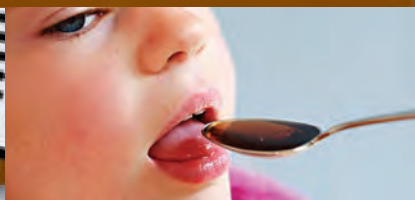
Infinity Series/Agilent LC Columns Pharmaceutical Compendium

OPTIMIZE UHPLC AND METHOD TRANSFER PRODUCTIVITY

The Measure of Confidence





Agilent Technologies

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Strengthen every link in your workflow chain

Achieve fast, flexible UHPLC and method transfer between instruments... or labs

Meeting the unrelenting productivity demands of drug discovery, development, manufacturing, and quality assurance requires you to get the most from *every* instrument in your lab.

UHPLC offers significant advantages in performance, speed, sensitivity, and resolution; however, today's labs must also collaborate with global partners that use different instruments. As a result, flexibility in method development and transfer have become essential for success in the pharmaceutical business.

Agilent's 1290 Infinity LC System with ISET combines maximum UHPLC productivity with flexible method development and transfer

The Agilent 1290 Infinity LC delivers outstanding sensitivity – up to 1200 bar. What's more, our Intelligent System Emulation Technology (ISET) lets you emulate *any* HPLC or UHPLC instrument in your worldwide network for fast, reliable method transfer between LC systems – *regardless of brand*.

Ensure stable, high-definition separations with ZORBAX Rapid Resolution High Definition (RRHD) columns

ZORBAX RRHD columns take sub-2 µm efficiency to new heights. They are stable up to 1200 bar, giving you more solvent options and allowing you to increase sensitivity by harnessing all the capabilities of our 1290 Infinity LC – or *any* UHPLC instrument.

You can also gain the advantages of scalability and method transfer to any HPLC or UHPLC by leveraging the full range of ZORBAX and Poroshell 120 chemistries. With more than 16 phases, Agilent LC columns give you the most choices for superior peak shape and method transfer, including:

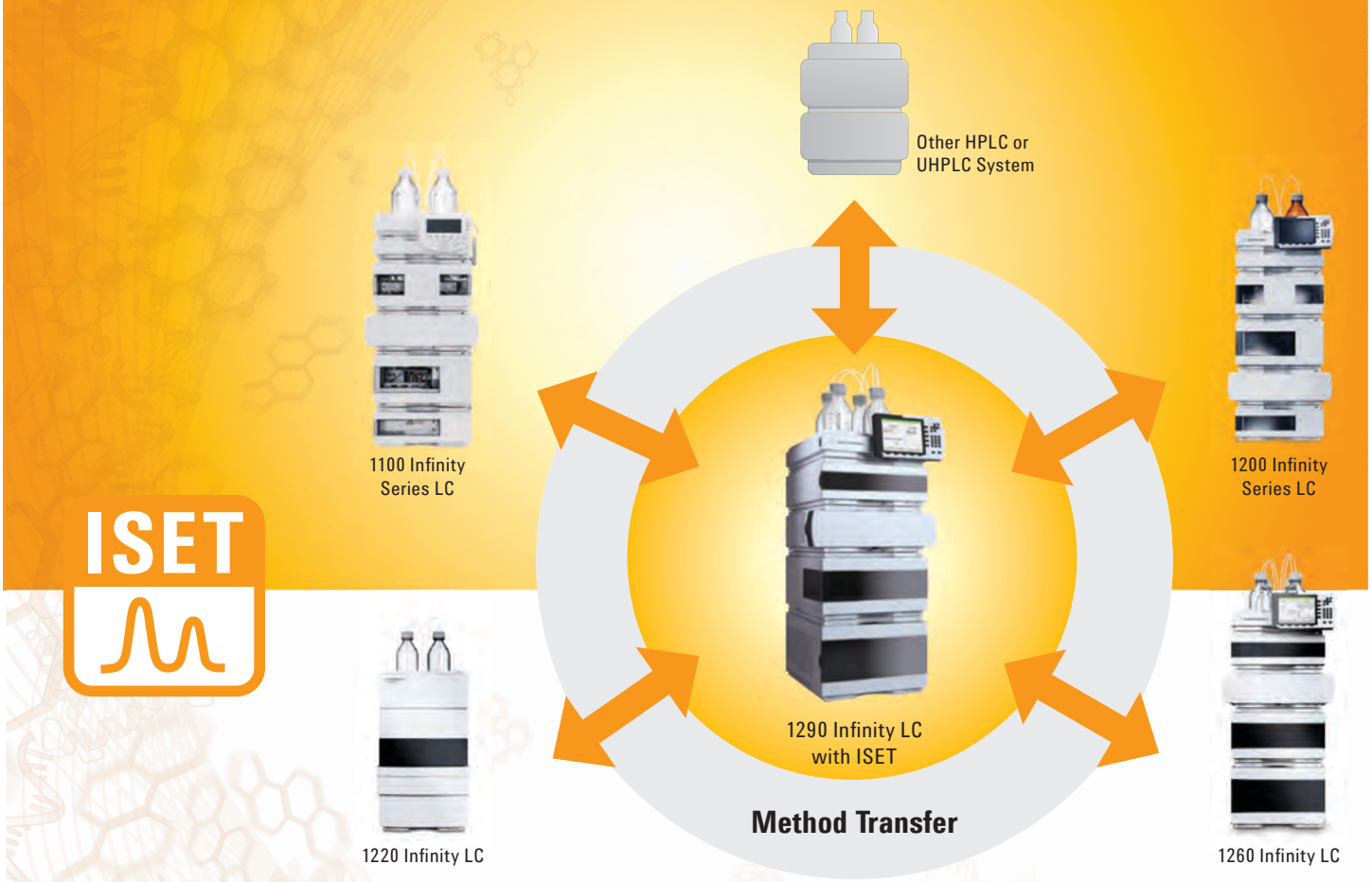
- Standard C18 and C8 phases
- A wide range of alternative selectivity options that enable you to perfect your most challenging separations

And of course, Agilent's worldwide technical support staff is always available to help resolve any problems you might encounter.

Method reproducibility from lab to lab... or around the world

Together, Agilent's 1290 Infinity LC system, sample prep products, and ZORBAX columns give you the tools you need to reduce analysis time – for **all** the instruments in your lab – without compromising reproducibility.

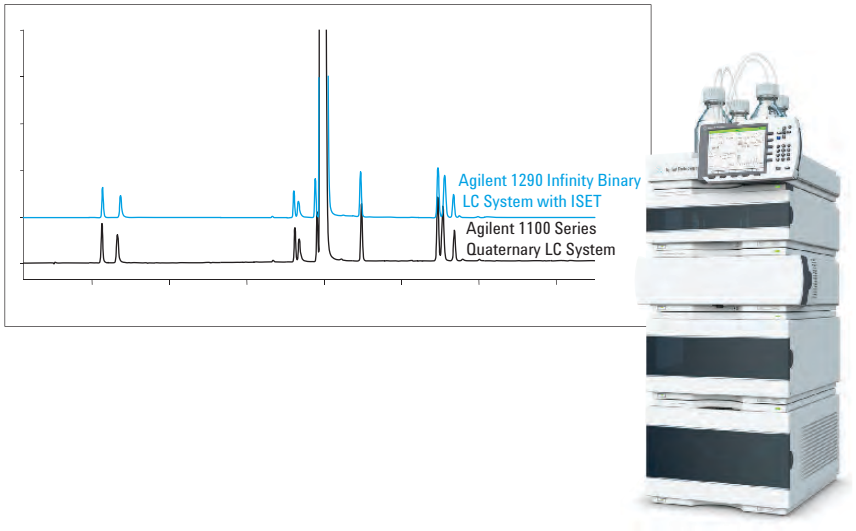
Intelligent System Emulation Technology



Transferring methods to the Agilent 1290 Infinity LC using Intelligent System Emulation Technology (ISET)

Analysis of metoclopramide hydrochloride and its impurities

Technical Overview



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Abstract

Agilent Intelligent System Emulation Technology (ISET) is a function within the Agilent 1290 Infinity LC System that offers seamless transfer of methods from conventional LC systems such as the Agilent 1100 Series Quaternary LC System, which has a higher delay volume and different mixing behavior, to the 1290 Infinity LC System. When the ISET function is enabled on the 1290 Infinity LC System, almost the same retention times and resolution can be obtained. This Technical Overview describes the transfer of a conventional HPLC method from a 1100 Series Quaternary LC System to the 1290 Infinity LC System. Metoclopramide and its impurities were analyzed on both systems. Measurements were done using the 1290 Infinity LC System both with and without ISET enabled. To further improve agreement of retention times and resolution, the fine-tuning option of ISET was deployed. Retention times and resolution of the different experiments were evaluated and compared.

Agilent Technologies

Introduction

The transfer of a method from a conventional LC to a UHPLC system such as the Agilent 1290 Infinity LC System will always result in significant differences in retention times and resolution of the analyzed peaks. To avoid the need for revalidation of legacy methods, an isocratic hold at the beginning of the run is often deployed or additional delay volume is installed. The disadvantage of these approaches is that only the delay volume is adjusted. The different mixing behavior of the two pumps cannot be compensated by these solutions. Using ISET, available in the 1290 Infinity LC, the difference in delay volume and in mixing behavior is compensated. As a result, retention times and resolution are typically very similar to the original data.

The experiments described in this Technical Overview show how the 1290 Infinity LC with ISET was used to emulate the behavior of an Agilent 1100 Series Quaternary LC. A method used to analyze metoclopramide and its impurities was transferred to a 1290 Infinity LC System. Experiments were done with and without using ISET functionality. Further, the fine tuning option of ISET was used to demonstrate how method transfer can be further optimized. Retention times and resolution for all experiments were evaluated and compared.

Experimental

Instrumentation and software

The Agilent 1290 Infinity LC System consisted of the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler with Thermostat (G4226A, G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector (G4212A)

The Agilent 1100 Series LC consisted of the following modules:

- Agilent 1100 Series Quaternary Pump (G1311A)
- Agilent 1100 Series Autosampler (G1313A)
- Agilent 1100 Series Thermostatted Column Compartment (G1316A)
- Agilent 1100 Series Diode Array Detector (G1315B)*

*The detector cell of the 1100 Series DAD had a volume of 13 µL. Note that ISET (revision 1.0) does not compensate for after-column dispersion volume.

Software: Agilent ChemStation revision C.01.03 and ISET revision 1.0. All LC modules had firmware revisions A.06.32, B.06.32, or B.06.41 or higher, and RC.Net drivers.

Sample

The following mixture of compounds was used for the experiments:

Main:	Metoclopramide hydrochloride
X:	Bromated metoclopramide
Impurity 1:	4-Amino-5-chloro-2-methoxybenzoic acid (EP C)
Impurity 2:	4-(Acetylamino)-2-hydroxybenzoic acid (EP H)
Impurity 3:	4-Amino-5-chloro-N-2-(diethylaminoethyl)-2-methoxybenzamide N-oxide (EP G)
Impurity 4:	4-Amino-5-chloro-N-2-(diethylaminoethyl)-2-hydroxybenzamide (EP F)
Impurity 5:	4-(Acetylamino)-5-chloro-N-2-(diethylaminoethyl)-2-methoxybenzamide (EP A)
Impurity 6:	Methyl 4-(acetylamino)- 2-methoxybenzoate (EP D)
Impurity 7:	Methyl 4-(acetylamino)-2-hydroxybenzoate
Impurity 8:	Methyl 4-(acetylamino)-5-chloro-2-methoxybenzoate (EP B)
Impurity 9:	Methyl 4-amino-2-methoxybenzoate

Chromatographic conditions

Column:	Agilent ZORBAX Eclipse Plus C18, 150 × 3.0 mm, 3.5 µm (959963-302)
Mobile phase:	Water + NH_4Ac (2.5 g/L), pH 6.99/Acetonitrile
Flow rate:	0.6 mL/min
Gradient:	5% ACN at 0 min to 57.5% ACN at 15 min
Stop time:	15 min
Post-time:	5 min
Injection volume:	1 µL (with needle wash for 3 s (for the Agilent 1290 Infinity Autosampler only)
Column temp.:	37 °C
Detection:	275/4 nm, Ref. 400/60 nm, 5 Hz, slit 4 nm

Results and discussion

Metoclopramide and its impurities are compounds that react extremely sensitively to changes in mobile phase composition and temperature. Therefore, transferring a method for these compounds is a demanding task.[2]

Metoclopramide and its impurities were analyzed using a conventional method with a 150 × 3.0 mm column packed with 3.5 µm particles. First, the method was transferred to the 1290 Infinity LC System without using ISET. In the next step, the method was transferred using ISET, see the resulting chromatograms in Figure 1, in which the original chromatogram from the 1100 Series LC System was overlaid with the chromatograms measured using the 1290 Infinity LC System with and without ISET.

Without deploying ISET, all peaks obviously shifted to lower retention times. In contrast, when ISET was used, all retention times correlated to the original chromatogram to a great extent. Using the 1290 Infinity LC System with or without ISET gave better resolution in both cases especially for peak 2, the main peak and peak 8. This was mainly due to the lower after-column dispersion volume of the 1290 Infinity LC System. The retention times for all three chromatograms were evaluated and the differences to the original chromatogram were calculated as percentages, (Figure 2).

Without ISET, the retention times shifted between 5 and 23% compared to the original retention times. With ISET, the retention times shifted only by 1.5% to later retention times except for the second peak, which shifted by 4% to a later retention time.

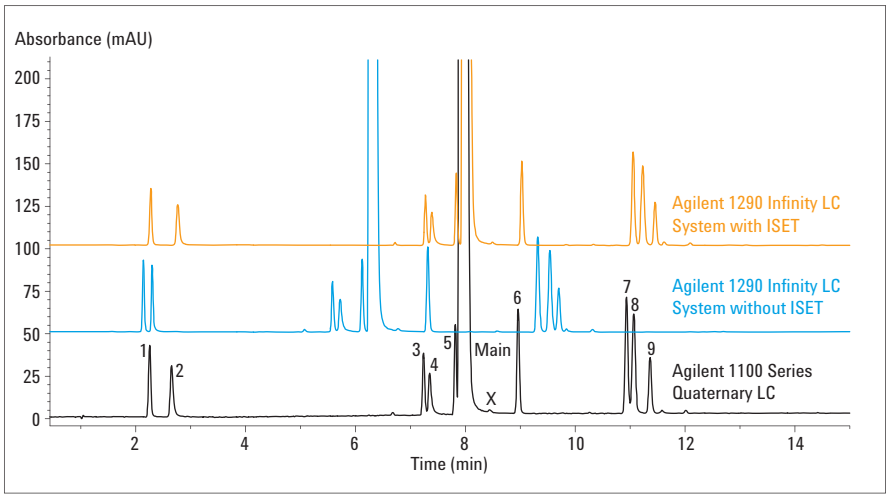

Figure 1
Overlay of original Agilent 1100 Series LC System chromatogram with the Agilent 1290 Infinity LC System chromatograms with and without ISET.

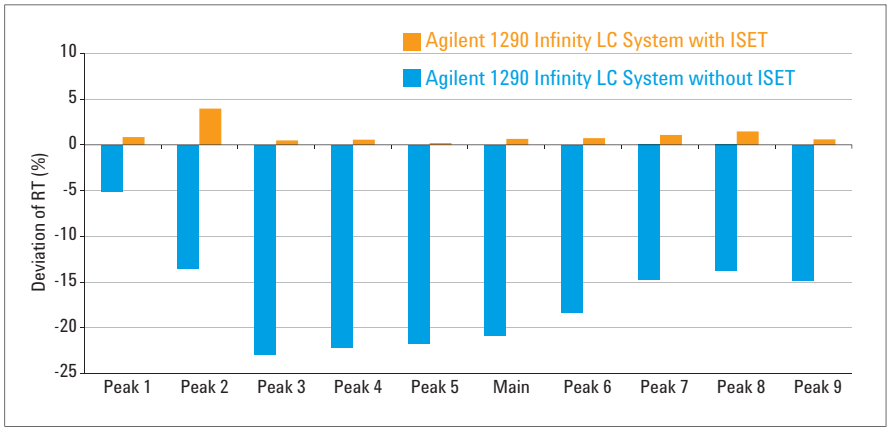

Figure 2
Deviation of retention times from original Agilent 1100 Series LC System data using the Agilent 1290 Infinity LC System with and without ISET.

To further improve the agreement between retention times, the *Enable Manual Fine Tuning* option within ISET was activated, (Figure 3).The retention times shifted to slightly earlier retention times by adding a 20-µL *Delay Volume Offset*. The maximum backpressure on the Agilent 1100 Series Quaternary LC was about 120 bar for the analysis of metoclopramide. This value was added to the *Typical Operating Pressure* field within the software. During the formation of gradients the system pressure increases and changes the damper volume of the 1100 Series LCs. The *Typical Operating pressure* function can be used to compensate for this additional volume.

Applying these new conditions, the agreement between the original 1100 Series data and 1290 Infinity chromatograms with ISET, and with fine-tuning, was further improved, (Figure 4).

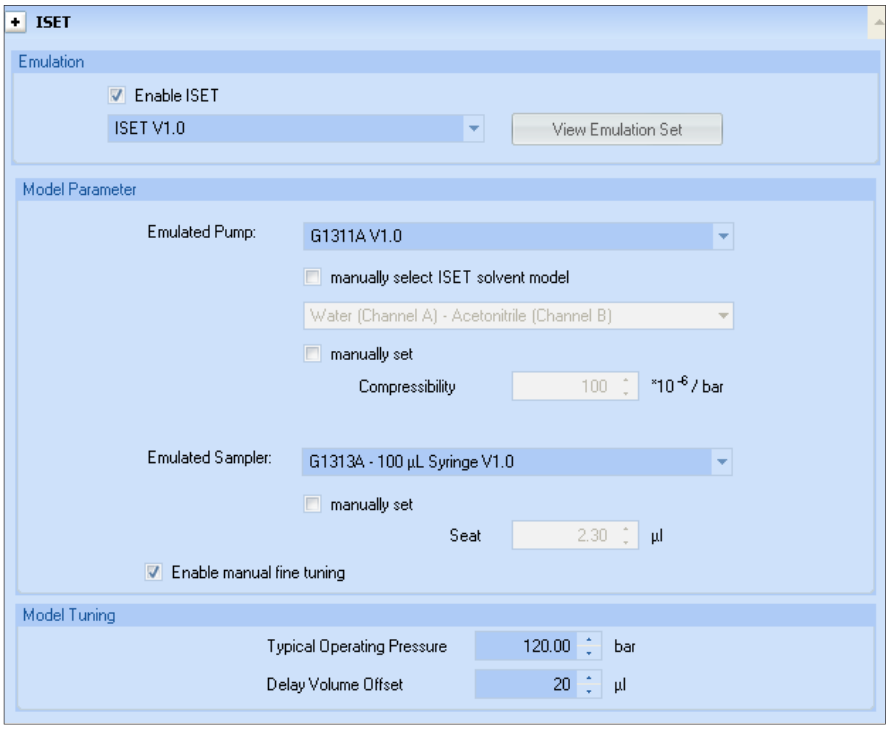

Figure 3
ISET software menu for enabling manual fine tuning.

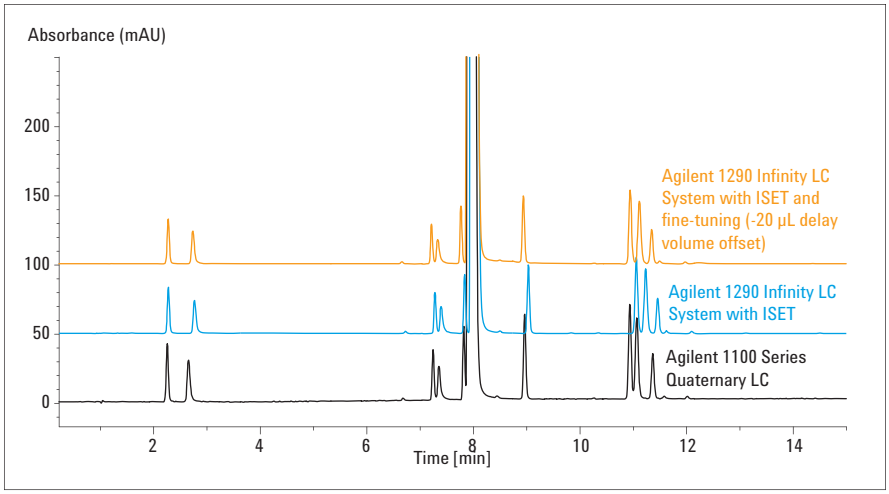

Figure 4
Overlay of the original Agilent 1100 Series LC System chromatogram, the chromatogram of Agilent 1290 Infinity LC System with ISET, and the chromatogram of 1290 Infinity LC System with ISET and with fine-tuning.

The retention times of the original chromatogram and of the chromatogram with the fine-tuning step showed nearly 100% agreement. The results for the three chromatograms with respect to deviation of retention times are summarized in Figure 5.

With ISET and additional fine-tuning, the deviation of retention times was less than 3% for the second peak and less than 0.8% for the remaining peaks. The resolution was typically better on the 1290 Infinity LC System. The absolute values for the resolution are combined in Figure 6. The agreement for all peaks was very good. The differences are due to the after-column dispersion volumes.

Conclusion

The Agilent Intelligent System Emulation Technology (ISET) facilitates seamless transfer of conventional methods to the 1290 Infinity LC System and thereby achieving excellent agreement of retention times and resolution. A conventional method for the analysis of metoclopramide and its impurities was transferred from the 1100 Series Quaternary LC System to the 1290 Infinity LC System. Enabling the ISET function for the 1290 Infinity LC resulted in deviations for the retention times smaller than 1.5% for all but one peak. Using in addition the fine-tuning option of ISET the deviation of retention times could be reduced to less than 0.8%. The resolution after method transfer to the 1290 Infinity LC System was typically slightly better than for the 1100 Series LC.

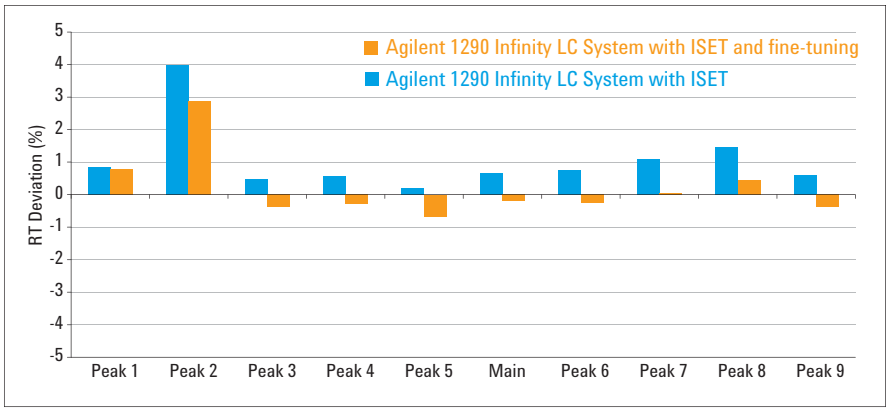

Figure 5
Deviation of retention time from the original Agilent 1100 Series LC System data using Agilent 1290 Infinity LC System with ISET and the Agilent 1290 Infinity LC System with ISET and fine-tuning.

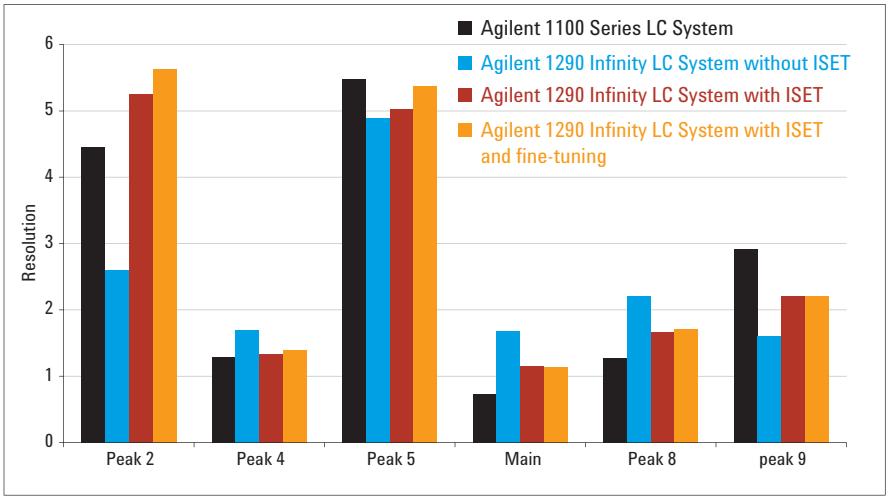

Figure 6
Differences of absolute data for the resolution of Agilent 1100 Series LC System, Agilent 1290 Infinity LC System with and without ISET, and Agilent 1290 Infinity LC System with ISET and additional fine-tuning.

References

1.
"Agilent 1290 Infinity LC with Intelligent System Emulation Technology", Agilent Brochure, Agilent Technologies publication number 5990-8670EN, **2011**

2.
Gerd Vanhoenacker, Frank David, Pat Sandra, Bernd Glatz, Edgar Naegele, *"Increasing productivity in the analysis of metoclopramide hydrochloride formulations using the Agilent 1290 Infinity LC system"*, Agilent Application Note, publication number 5990-3981EN, May **2009**

www.agilent.com/chem/iset

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5990-9692EN

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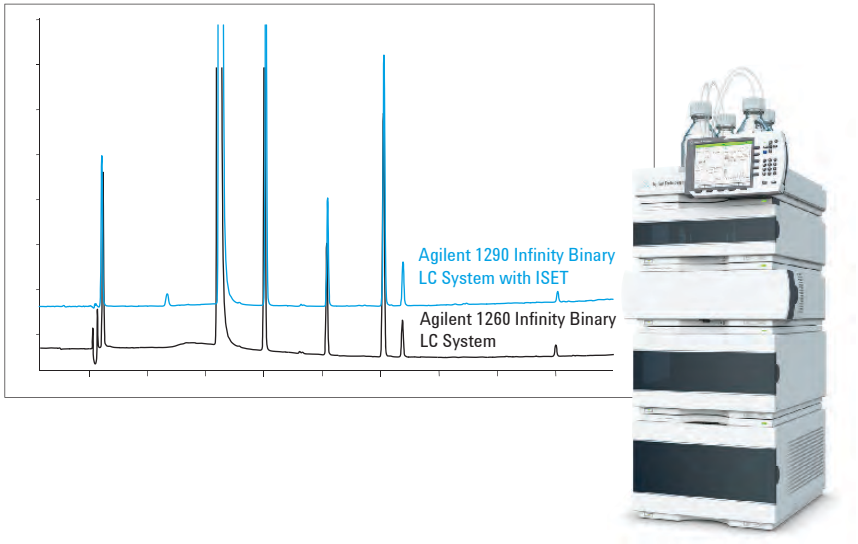
Transferring methods to the Agilent 1290 Infinity LC System using Intelligent System Emulation Technology (ISET)

Analysis of paracetamol and its impurities

Technical Overview



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Abstract

Agilent's Intelligent System Emulation Technology (ISET) offers seamless transfer of methods from conventional LC systems such as the Agilent 1100 Series Quaternary LC System, which has a higher delay volume and different mixing behavior, to the Agilent 1290 Infinity LC System. When the ISET function is enabled on the 1290 Infinity LC System, almost the same retention times and resolution can be obtained. This Technical Overview describes the transfer of a conventional HPLC method from an Agilent 1260 Infinity Binary LC System to the 1290 Infinity LC System. Paracetamol and its impurities were analyzed using the 1290 Infinity LC System both with and without ISET enabled. Retention times and resolution of the different experiments were evaluated and compared with the original data obtained using the 1260 Infinity Binary LC System.

Agilent Technologies

Introduction

Instrument-to-instrument method transfer is often problematic, especially in highly regulated environments, because critical parameters such as retention times and resolution might change. Agilent provides seamless method transfer from the Agilent 1100/1200 Series LC systems, as well as from the Agilent 1220/1260 Infinity LC systems to the Agilent 1290 Infinity LC System using the Agilent Intelligent System Emulation Technology (ISET)[1]. ISET is implemented in the method screen of the pump. In this screen, ISET can be simply switched on to enable legacy methods to be run unchanged, or switched off to run fast or high-resolution UHPLC methods. ISET compensates not only for the different system delay volumes, but also for different mixing behavior of, for example, the Agilent 1260 Infinity Binary LC Pump and the Agilent 1290 Infinity Binary Pump. Using ISET, nearly the same retention times and resolution can be achieved when a method is transferred to the 1290 Infinity LC System.

In this Technical Overview a conventional method was transferred from the 1260 Infinity Binary LC System to the 1290 Infinity LC System. Paracetamol and six impurities were analyzed. The application was transferred to the 1290 Infinity LC System with and without ISET. Retention times and resolution were evaluated and compared with the 1260 Infinity Binary LC System data.

Experimental

Instrumentation and software

The Agilent 1290 Infinity LC System used for the experiments consisted of the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler with Thermostat (G4226A, G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector (G4212A)

The Agilent 1260 Infinity Binary LC System was used for the experiments consisting of the following modules:

- Agilent 1260 Infinity Binary Pump (G1312A)
- Agilent 1260 Infinity Autosampler with Thermostat (G1329B, G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector (G4212B)

Agilent ChemStation revision C.01.03 and ISET revision 1.0 were used for the experiments. All LC modules had firmware revisions A.06.32 or B.06.32 or B.06.41 or higher, and all modules had RC.Net drivers.

Sample

The following mixture of compounds was used for the experiments:

Main:	Paracetamol
Impurity A:	2-Acetamidophenol
Impurity B:	N-(4-Hydroxyphenyl) propamide
Impurity F:	Nitrophenol
Impurity H:	4-(Acetylamino) phenyl Acetate (N,O-Diacetyl-4-aminophenol)
Impurity J:	4-Chloroacetanilide
Impurity K:	4-Aminophenol

Chromatographic conditions

Column:	Agilent ZORBAX Eclipse Plus C18, 100 × 4.6 mm, 3.5 µm
Mobile phase:	Water + 0.1% TFA, Acetonitrile + 0.09% TFA
Flow rate:	1.2 mL/min
Gradient:	5% ACN at 0 min, 5% ACN at 0.5 min, 90% ACN at 10 min
Stop time:	10 min
Post-time:	5 min
Injection volume:	5 µL (with needle wash for 6 s)
Column temp.:	30 °C
Detection:	220, 254, 270, 310/10 nm, Ref. 400/60 nm, 10 Hz, slit 4 nm

Results and discussion

The chromatographic method for paracetamol and its impurities was developed using the 1260 Infinity Binary LC System by deploying a conventional method with a 10 minute run time. The analysis of paracetamol and six impurities needs different wavelength settings to be able to quantify all peaks at maximum absorbance, (Figure 1). An isocratic step at low organic percentage at the beginning is needed to delay the elution of impurity K. To be able to elute impurity A in a reasonable time, the percentage of the organic phase was increased up to 90% within 10 minutes.

Compound	Detection wavelength
Impurity K	270 nm
Paracetamol	270 nm (linear)
Impurity B	254 nm
Impurity H	254 nm
Impurity F	310 nm
Impurity J	254 nm
Impurity A	220 nm

The original method was transferred to the 1290 Infinity LC without changing any parameters. As expected, the complete chromatogram shifted to lower retention times on the 1290 Infinity LC System due to the lower delay volume and different mixing behavior, (Figure 2).

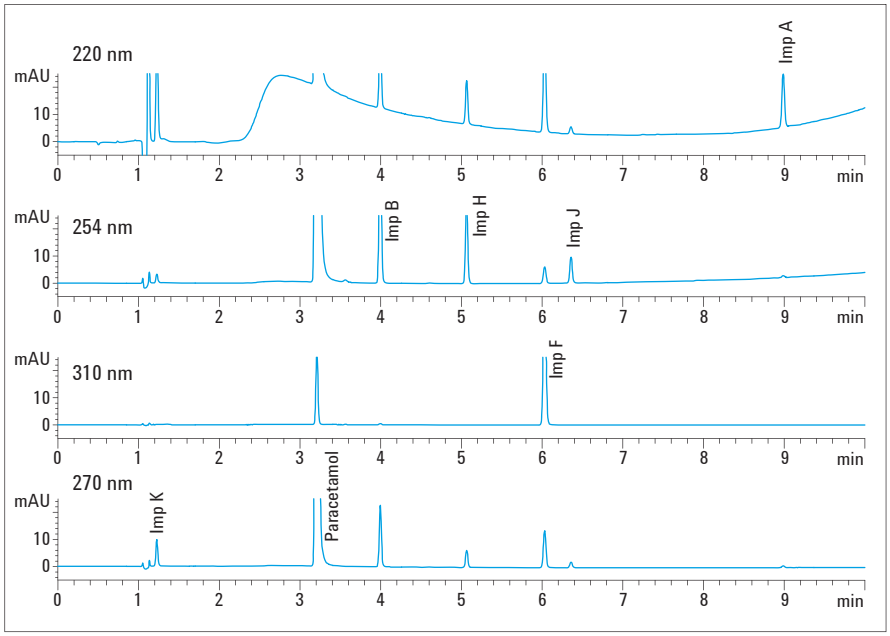


Figure 1
Analysis of paracetamol and six impurities at different wavelengths.

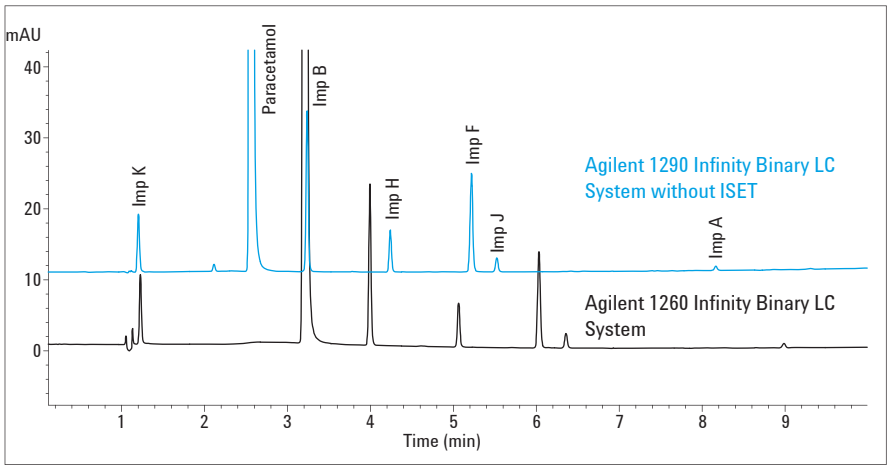


Figure 2
Overlay of chromatograms measured at 270 nm using the Agilent 1260 Infinity Binary LC System and the Agilent 1290 Infinity LC System without ISET enabled.

In the next experiment, the ISET function was enabled through the pump method screen of the software, (Figure 3). Here the originally used Agilent 1260 Infinity Pump and Autosampler had to be specified under *Module Parameter*. In our example, the original configuration of the 1260 Infinity Binary LC System included the pump (G1312B) and the autosampler (G1329B). All other parameters for detector, column thermostat, and autosampler remained the same. The new method was then saved under a new name and used in sequences or single runs.

The new method was transfered to the 1290 Infinity LC System and the resulting retention times and resolution evaluated. Figure 4 shows the original chromatogram was overlaid with a chromatogram obtained, and using the 1290 Infinity LC System with ISET.

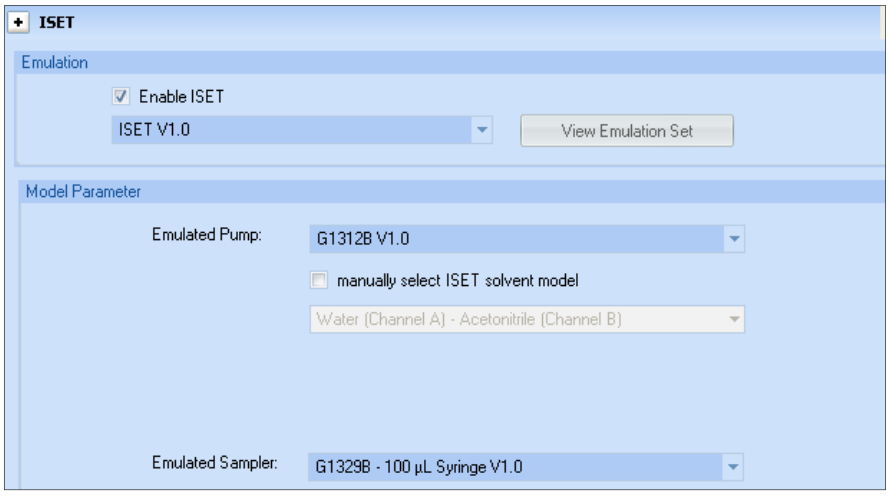


Figure 3
Software screen for enabling the ISET function.

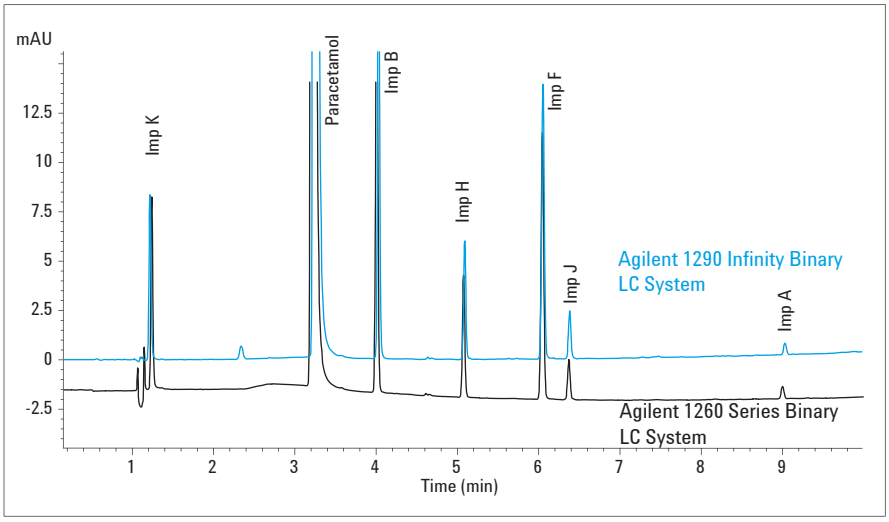


Figure 4
Overlay of chromatograms measured at 270 nm using the Agilent 1260 Infinity Binary LC System and the Agilent 1290 Infinity LC System with ISET enabled.

With ISET the retention times and the resolution showed a significantly better agreement with the original chromatogram. Figure 5 combines the results for the retention times with and without ISET.

The retention times without ISET differed up to -20%. The deviation of retention times on the 1290 Infinity LC with ISET was less than 1.3%. Figure 6 combines the results of the resolution data. The resolution without ISET differed up to -65%. The deviation of the resolution on the 1290 Infinity LC System with ISET was less than 2.3%.

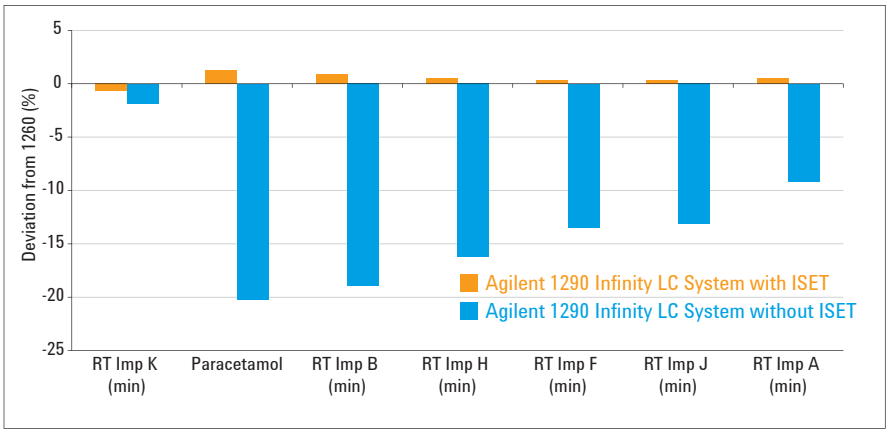


Figure 5
Deviation of retention times using the Agilent 1290 Infinity LC System with and without ISET.

Conclusion

The Agilent Intelligent System Emulation Technology (ISET) facilitates seamless transfer of conventional methods from an LC or another UHPLC system to the Agilent 1290 Infinity LC System. By specifying the pump and autosampler originally used, the 1290 Infinity LC System behaves like the original system and provides the same retention times and resolution. In the described experiments, a conventional method for the analysis of paracetamol and six impurities was transferred from the Agilent 1260 Infinty Binary LC System to the 1290 Infinity LC System with ISET enabled. Using ISET, the retention times did not differ more than 1.3% from the original data. The resolution did not differ more than 2.3%.

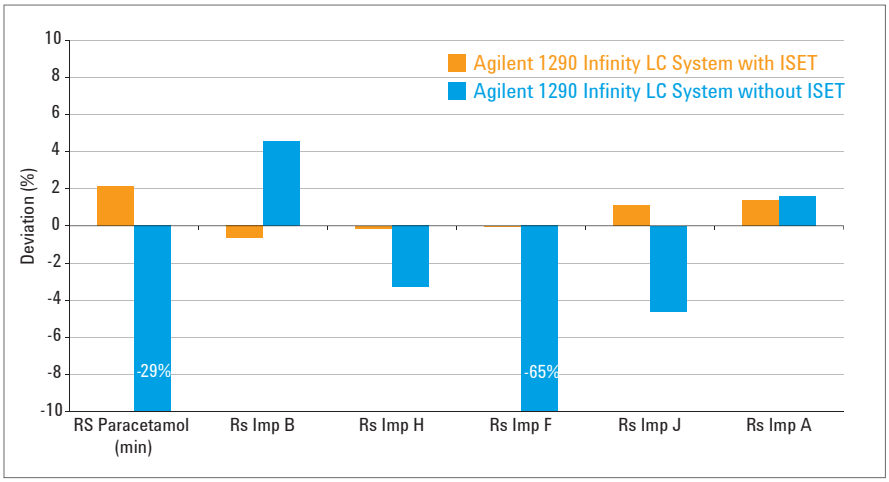


Figure 6
Deviation of resolution using the Agilent 1290 Infinity LC System with and without ISET.

Reference

1.
"Agilent 1290 Infinity LC with Intelligent System Emulation Technology", Agilent Brochure, publication number 5990-8670EN, **2011**

www.agilent.com/chem/iset

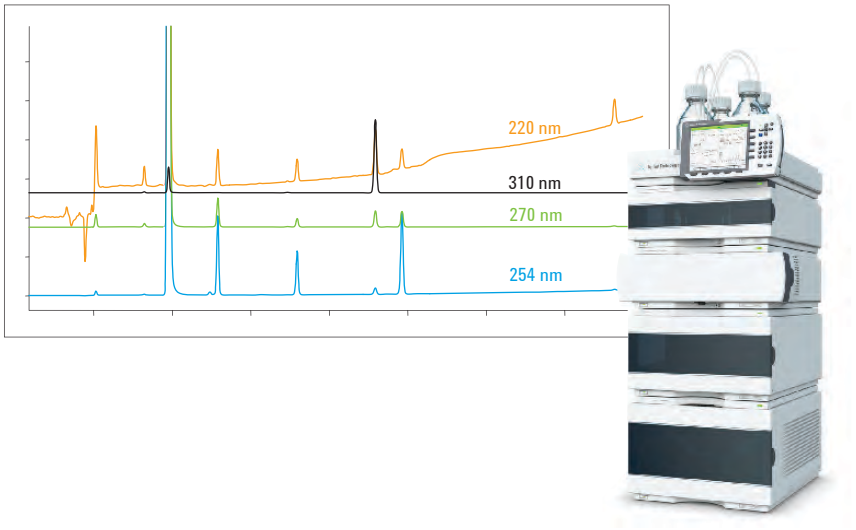
Published in the USA, January 1, 2012
5990-9703EN

Agilent Technologies

Method development on the Agilent 1290 Infinity LC using Intelligent System Emulation Technology (ISET) with subsequent transfer to an Agilent 1100 Series LC

Analysis of an analgesic drug

Technical Overview



Author

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Abstract

Agilent's Intelligent System Emulation Technology (ISET) offers seamless transfer of methods in both directions from conventional LC systems, which have higher delay volumes and different mixing behaviors, to the Agilent 1290 Infinity LC System. A method developed on the 1290 Infinity LC System can be transferred to a different LC system by first emulating the target LC on the 1290 Infinity LC System by using ISET. This gives valid information whether the method developed on the 1290 Infinity LC System will work using the target LC system. This Technical Overview shows the development of a chromatographic method for the analysis of paracetamol and its impurities using 1290 Infinity LC System with ISET. Having developed the method it was transferred to an Agilent 1100 Series Quaternary LC System. Retention times and resolution of the different experiments were evaluated and compared with the data obtained on the 1290 Infinity LC System with ISET.

Agilent Technologies

Introduction

In the pharmaceutical industry, method development for QA/QC is done in the R&D departments. The instrumentation used in R&D is often not the same as deployed routinely in the QA/QC departments. This can lead to problems because the developed and validated method might not fulfill the requirements when transferred to a different LC system. The 1290 Infinity LC System with ISET offers the possibility to emulate the target LC and to find out whether a method will run on a different LC system without problems and delivering the same results for retention times and resolution.

This Technical Overview shows the development of a method for the analysis of paracetamol and its impurities using the 1290 Infinity LC System. Having finalized the method, the 1290 Infinity LC System with ISET emulated the target LC, an 1100 Series Quaternary LC System, to determine whether the developed method was suitable for the 1100 Series LC System. Later on, the method was transferred to the 1100 Series Quaternary LC System and the results were compared to data obtained on the 1290 Infinity LC System with ISET.

Experimental

Instrumentation and software

The Agilent 1290 Infinity LC System used for the experiments consisted of the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler with Thermostat (G4226A, G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector (G4212A)

The Agilent 1100 Series LC was used for the experiments consisting of the following modules:

- Agilent 1100 Series Quaternary Pump (G1311A)
- Agilent 1100 Series Autosampler (G1313A)
- Agilent 1100 Series Thermostatted Column Compartment (G1316A)
- Agilent 1100 Series Diode Array Detector (G1315B)

Agilent ChemStation revision C.01.03 and ISET revision 1.0 were used for the experiments. All LC modules had firmware revisions A.06.32, B.06.32 or B.06.41 or higher, and all modules had RC.Net drivers.

Sample

The following mixture of compounds was used for the experiments:

Main:	Paracetamol
Impurity A:	2-Acetamidophenol
Impurity B:	N-(4-Hydroxyphenyl) propamide
Impurity F:	Nitrophenol
Impurity H:	4-(Acetylamino) phenyl Acetate (N,O-Diacetyl-4-aminophenol)
Impurity J:	4-Chloroacetanilide
Impurity K:	4-Aminophenol

Chromatographic conditions

Column:	Agilent ZORBAX SB C18, 150 × 4.6 mm, 5 µm (883975-902)
Mobile phase:	Water + 0.1% TFA, Acetonitrile + 0.09% TFA
Flow rate:	1.0 mL/min
Gradient:	5% ACN at 0 min, 90% ACN at 20 min
Stop time:	20 min
Post-time:	5 min
Injection volume:	5 µL
Column temp.:	30 °C
Detection:	220, 254, 270, 310/10 nm, Ref. 400/ 60 nm, 5 Hz, slit 4 nm

Results and discussion

Method development on the 1290 Infinity LC System

The separation was done using conventional chromatographic conditions. The column dimensions were 150 × 4.6 mm for length and internal diameter and the particle size was 5 µm. To increase the retention time of impurity K, the linear gradient started at low organic concentration. After 20 minutes, the mobile phase composition contained 90% organic to elute impurity A within a reasonable time, (Figure 1). Different wavelengths were necessary to be able to quantity all compounds at their absorbance maxima with high selectivity.

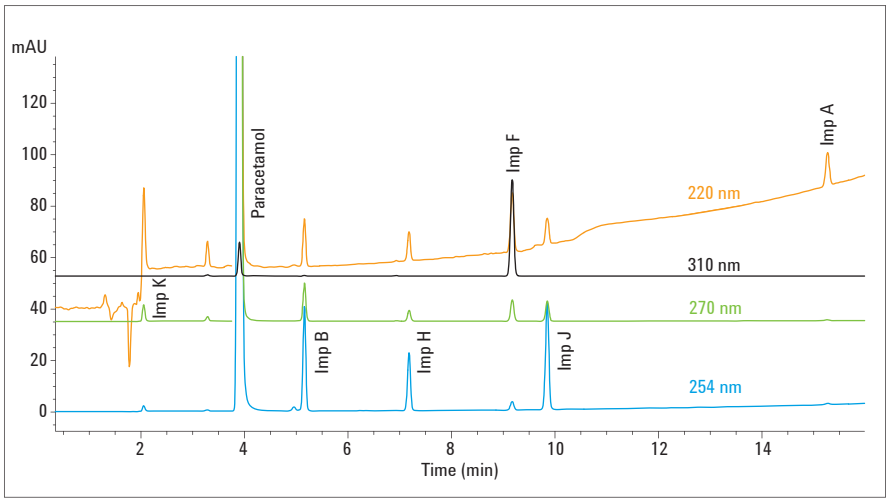


Figure 1
Overlay of chromatograms at different wavelengths.

The UV spectra, (Figure 2), delivered the information to select the optimum wavelength, (Table 1).

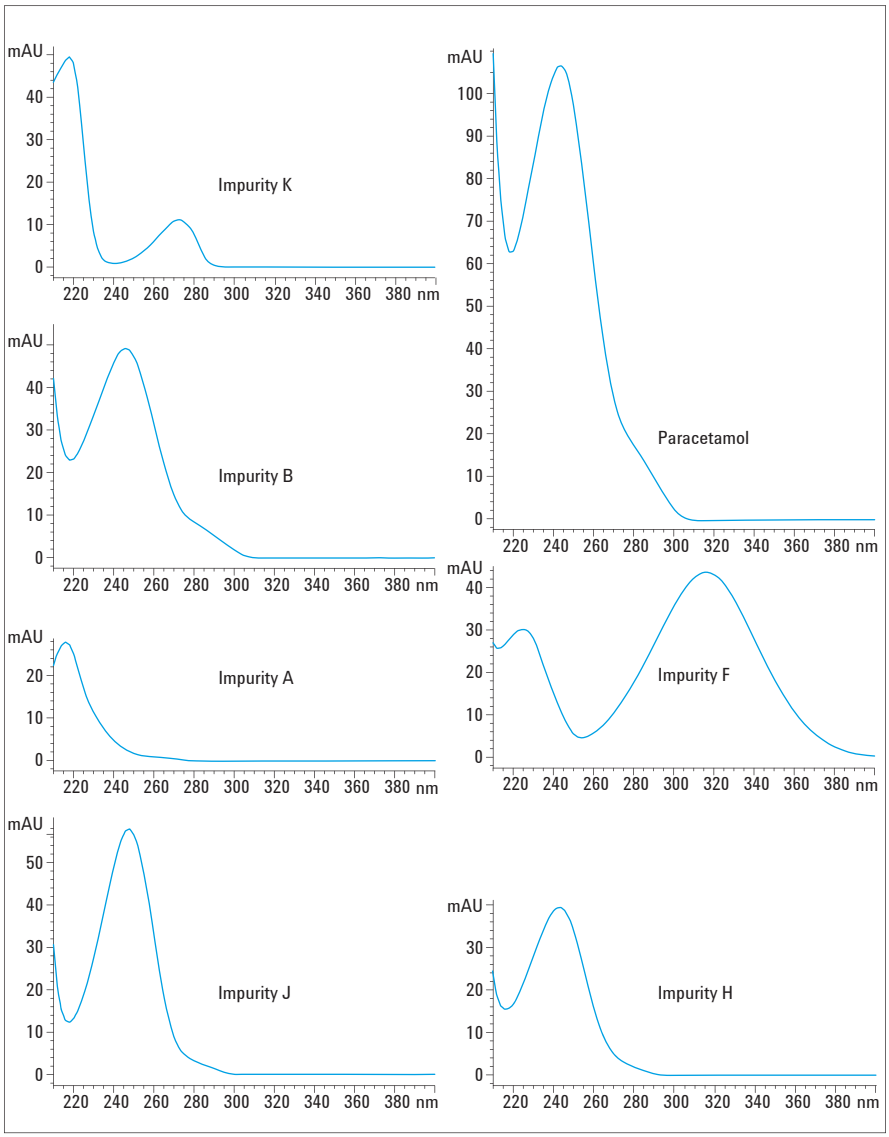


Figure 2
UV spectra of paracetamol and its impurities.

Compound	Detector wavelength
Impurity K	270 nm (better selectivity)
Paracetamol	270 nm (within linear range)
Impurity B	254 nm (absorbance maximum)
Impurity H	254 nm(absorbance maximum)
Impurity F	310 nm (absorbance maximum)
Impurity J	254 nm (absorbance maximum)
Impurity A	220 nm (absorbance maximum)

Table 1
Optimum wavelength for paracetamol and its impurities.

Emulation of the 1100 Series Quaternary LC System on the 1290 Infinity LC System using ISET and method transfer to the 1100 Series Quaternary LC System

The ISET tool on the 1290 Infinity LC System was enabled to emulate the 1100 Series Quaternary LC System. The finalized method was applied to the 1290 Infinity LC System using ISET to get an overview whether the method will work on an 1100 Series LC System. The method was then transferred to an 1100 Series Quaternary LC System. The 3 chromatograms obtained on the 1290 Infinity LC System with and without ISET and obtained on the 1100 Series quaternary LC System were compared, (Figure 3).

The retention times and the resolution using the 1290 Infinity LC System with ISET showed excellent agreement with the results of the 1100 Series Quaternary LC System. The deviation of retention times from the 1100 Series LC System data are combined in Figure 4.

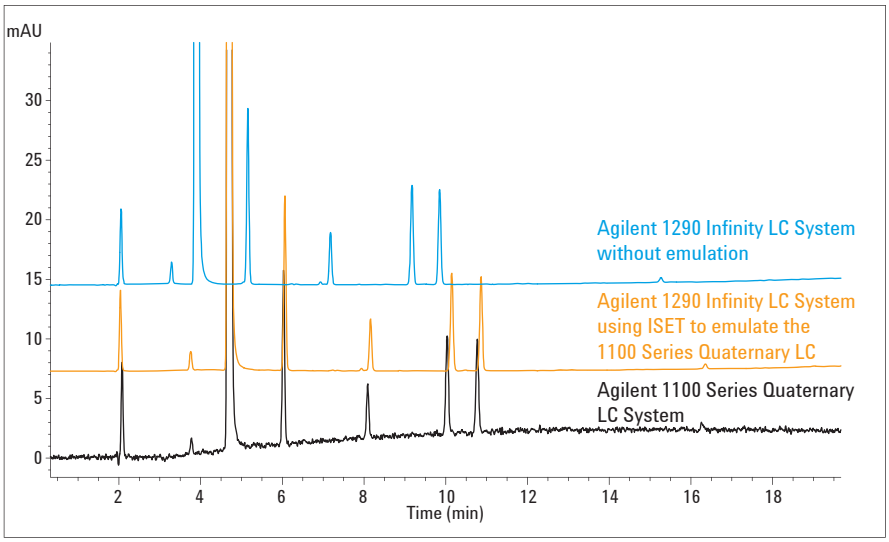


Figure 3
Overlay of chromatograms at 270 nm obtained on the Agilent 1290 Infinity LC System, on the Agilent 1290 Infinity LC System with ISET and on the Agilent 1100 Series Quaternary LC System.

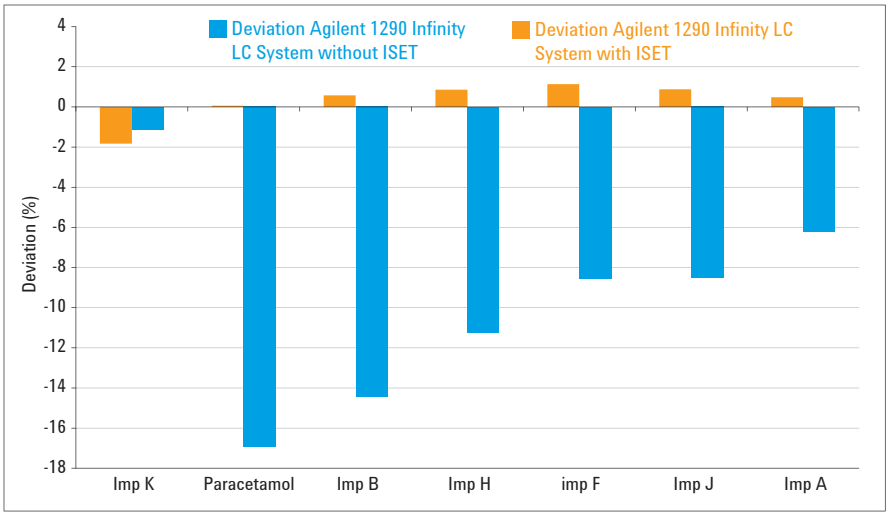


Figure 4
Deviation of retention times for the Agilent 1290 Infinity LC System with and without ISET in comparison with Agilent 1100 Series Quaternary LC System data.

The retention time deviation for the 1290 Infinity LC System with ISET was between 0.5 and 1.9%. The retention time deviation for the 1290 Infinity without ISET was as high as 17%. The results reading the resolution data are combined in Figure 5.

The deviation of the resolution on the 1290 Infinity LC System with ISET was between 0.11 and 2.4%. The deviation of the resolution on the 1290 Infinity LC System without ISET was up to 31%.

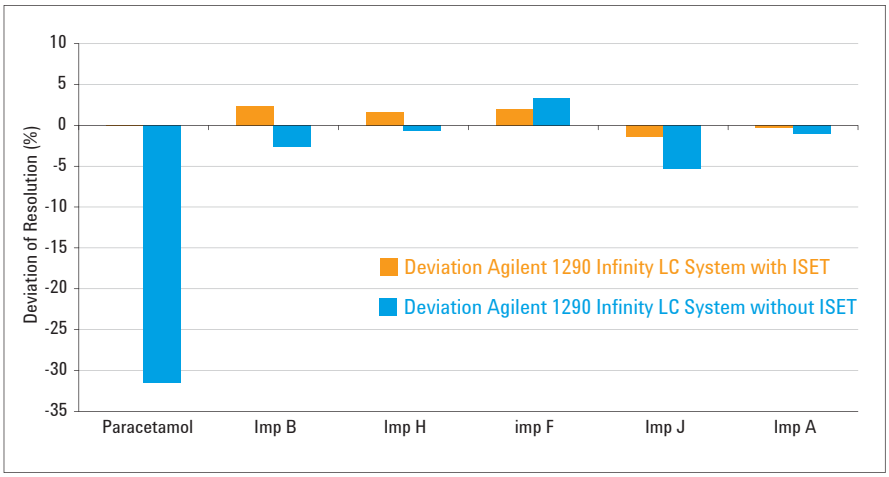


Figure 5
Deviation of resolution for the Agilent 1290 Infinity LC System with and without ISET compared with data obtained on the Agilent 1100 Series Quaternary LC System.

Conclusion

A method developed on an Agilent 1290 Infinity LC System for the analysis of paracetamol and its impurities was seamlessly transferred to an Agilent 1100 Series Quaternary LC System. To facilitate transfer the ISET function of the 1290 Infinity LC System was activated to emulate the 1100 Series LC System. This provided information whether the developed method worked on the target 1100 Series LC System. The results for retention times and resolution obtained on the 1100 Series LC System and the 1290 Infinity LC System using ISET were compared. The retention times differed less than 1.9% and the resolution less than 2.4%.

Reference

1.
"Agilent 1290 Infinity LC with Intelligent System Emulation Technology", Agilent Technologies Brochure, publication number 5990-8670EN, **2011**.

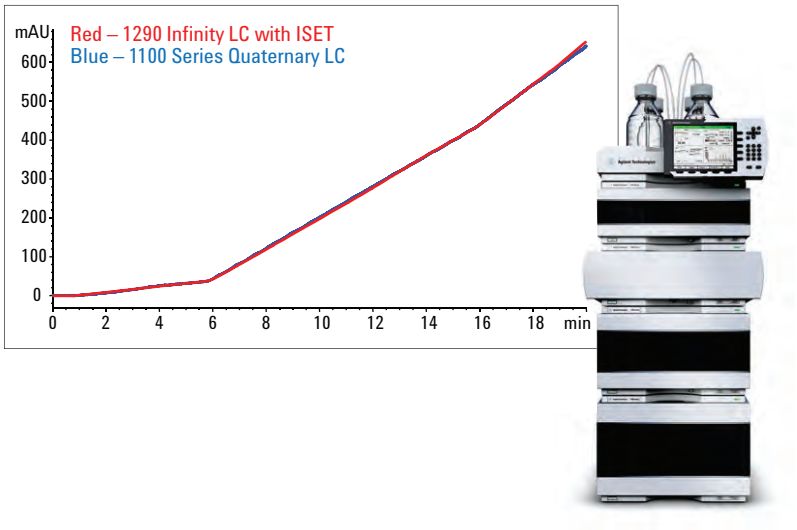
www.agilent.com/chem/iset

Published in the USA, January 1, 2012
5990-9715EN

Agilent Technologies

Seamless transfer of elution gradients from Agilent 1100/1200 Series LCs to an Agilent 1290 Infinity LC using ISET

Technical Overview



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Abstract

Agilent Intelligent System Emulation Technology (ISET) is a function within the Agilent 1290 Infinity LC System that allows to emulate other systems for seamless transfer of methods between LC instruments. Using ISET, the same chromatographic results can be achieved without any modifications to the instrument or changes to the original method.

In this Technical Overview, we demonstrate that gradients run on an Agilent 1100 Series LC System, including typical linear gradients as well as gradients with different slopes, steps, and isocratic parts, correlate almost 100% to gradient profiles created on the Agilent 1290 Infinity LC System with ISET.

Agilent Technologies

Introduction

Instrument-to-instrument method transfer is often problematic, especially in highly regulated environments, because critical parameters such as retention times and resolution might change due to differences in system delay volume and gradient mixing behavior. Agilent provides seamless method transfer, for example, from Agilent 1100/1200 Series LCs and Agilent 1220/1260 Infinity LCs to the Agilent 1290 Infinity LC with ISET[1]. Because ISET is a feature that can be simply switched on and off, you can run legacy methods unchanged.

Of special interest is whether typical gradient profiles obtained from a conventional LC can be transferred to an Agilent 1290 Infinity LC System with ISET with high correlation. High correlation implies that retention times and resolution will correlate to a high extent, even for peaks that react sensitively to small composition changes.

In this Technical Overview, tracer experiments were selected to demonstrate that ISET provides optimum results for a wide range of gradient applications. Tracer experiments give more detailed and complete results than injection of arbitrarily selected compounds.

In this Technical Overview, we demonstrate the transfer of several typical gradient profiles from an Agilent 1100 Series Quaternary LC to an Agilent 1290 Infinity LC System with ISET using tracer experiments.

Experimental

Instrumentation

Table 1 shows the configurations of the instrumentation used for the tracer experiments.

Software

Agilent Chemstation revision C 01.03 and ISET revision 1.0.

Chromatographic conditions

The following chromatographic conditions were used for all gradients:

Compound:	Uracil as tracer, 10 mg/L in methanol
Column:	Restriction capillary
Mobile phases:	Water (A)/methanol with tracer (B)
Column temperature:	30 °C
Detection with DAD:	254/4 nm, Ref. 360/100 nm, 10 Hz

	Agilent 1100 Series LC	Agilent 1290 Infinity LC with ISET
Module	**Product number**	**Product number**
Pump	G1311A manufactured in 2003	G4220A
Autosampler	G1313A	G4226A
Thermostat	none	G1330B
Column Compartment	G1316A	G1316C
Diode Array Detector	G1315B	G4212A

Table 1
Instrumentation used for the experiments.

Results and discussion

Method transfer from HPLC to UHPLC systems can be generally problematic due to the significant lower delay and different mixing behavior of UHPLC systems as shown in Figure 1. As a result, the gradient, the retention times, and the resolution of peaks will change.

There are two typical ways to overcome this problem: either an isocratic hold is added at the beginning of the gradient, or the delay volume is increased with additional tubing between the pump and autosampler. Both methods can only compensate for the smaller delay volume but not for the overall gradient behavior (transition volume, mixing performance) over the complete gradient and run time.

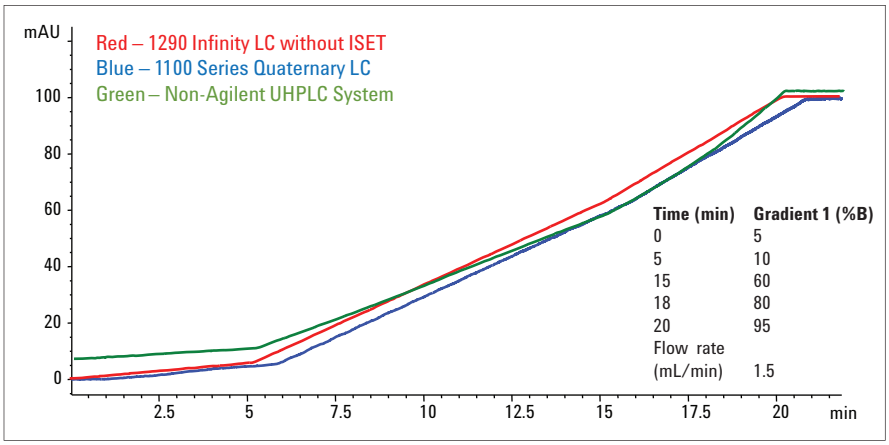

Figure 1
Overlay of gradient 1, run on an Agilent 1100 Series Quaternary LC, an Agilent 1290 Infinity LC without ISET, and another vendors' UHPLC instrument.

Transfer of gradients from an Agilent 1100 Series Quaternary LC System to an Agilent 1290 Infinity LC with ISET

Manifold separation problems may cause HPLC gradients to contain different lengths, slopes, steps and isocratic parts in their profile. Therefore, six typical gradient profiles were run using an Agilent 1100 Series Quaternary LC and an Agilent 1290 Infinity LC with ISET. Three gradient profiles contained different slopes and isocratic steps. The other three gradient profiles were linear, but with different run times. The agreement of both curves was calculated using correlation factors that were converted into percentage values.

Gradient 1 contained different slopes and the complete run time was 20 minutes. Figures 2 and 3 show overlays of the gradient profile from the Agilent 1100 Series Quaternary LC and from the resulting Agilent 1290 Infinity LC with ISET. In Figure 3, the view is enhanced to show the curve behavior after a slope change.

The emulated method of the Agilent 1290 Infinity LC with ISET provides perfect agreement of both curves.

Notice how the ISET function is able to follow the original gradient profile after a significant composition change such as the slope change at 15 minutes. Figure 3 shows that the original curve and the ISET curve correlate to nearly 100%, even after a slope change.

Due to the high correlation, similar retention times and resolution can be expected even for more demanding gradients.

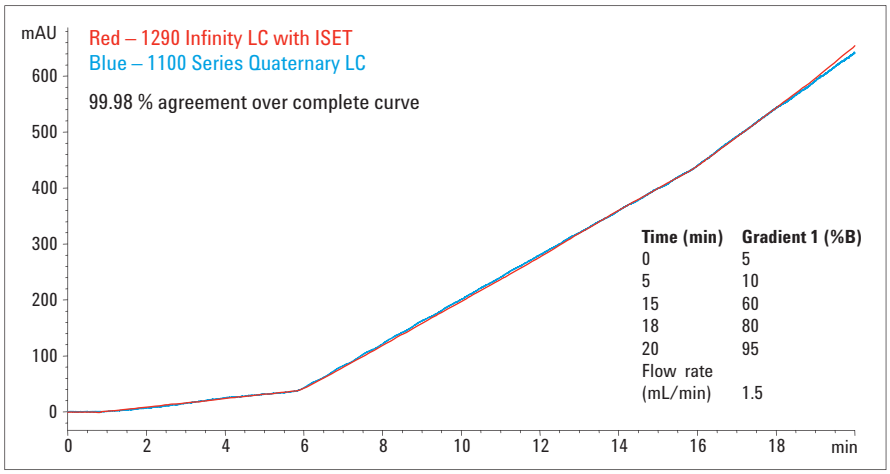

Figure 2
Overlay of gradient 1 using an Agilent 1100 Series Quaternary LC and an Agilent 1290 Infinity LC with ISET.

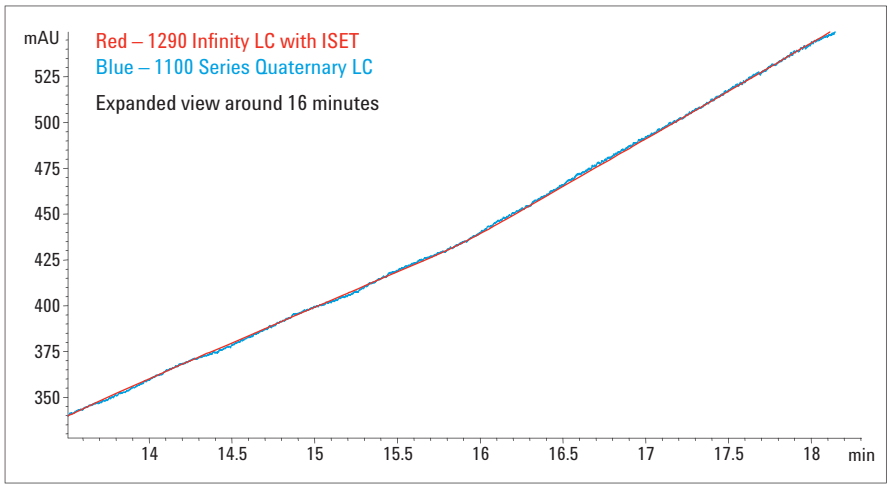

Figure 3
Expanded view around 16 minutes after change of slope at 15 minutes.

Gradient 2, with a run time of 10 minutes, contained different slopes and one isocratic section in the middle of the chromatogram. These gradients are frequently used if the resolution for closely eluting peaks should be improved. Figures 4 and 5 show overlays of the gradient profiles from an Agilent 1100 Series Quaternary LC System and an Agilent 1290 Infinity LC System with ISET.

In Figure 5, the view is enhanced to show curve behavior after slope changes. The agreement of both curves is close to 100%. The gradient contained an isocratic step from 3 to 5 minutes. In Figure 5, the chromatogram is expanded at 3 to 7 minutes, to show that both gradient profiles correlate well, even at the changeovers from slope 1 to the isocratic step, and from the isocratic step, to the next gradient slope.

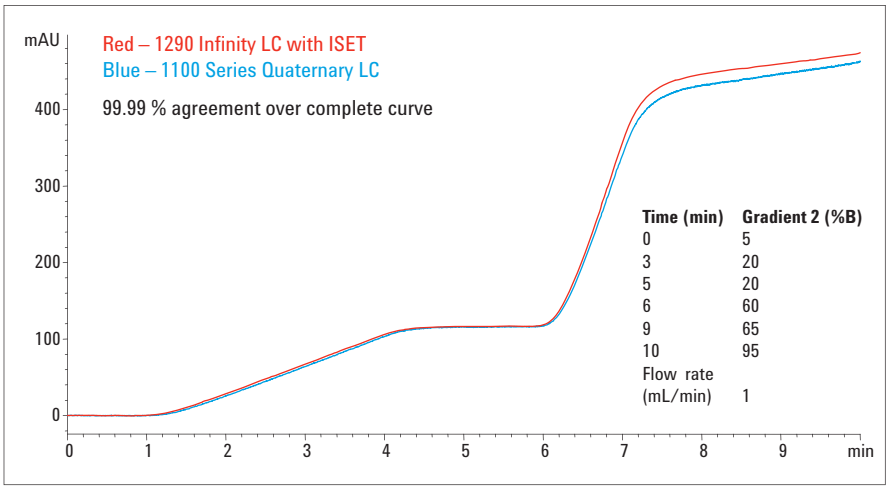

Figure 4
Overlay of gradient 2 using an Agilent 1100 Series Quaternary LC and an Agilent 1290 Infinity LC with ISET.

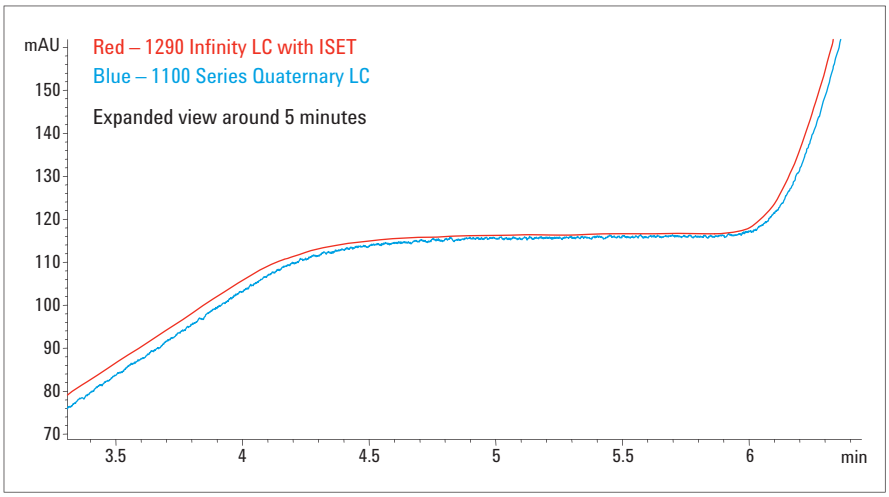

Figure 5
Expanded view at approximately 5 minutes at two critical composition changeovers.

Gradient 3, with a run time of 25 minutes, contained an isocratic step at the beginning. This type of gradient is usually applied when hydrophilic and hydrophobic compounds must be analyzed in one run. At the beginning of the run, a low organic percentage is used and after the elution of the hydrophilic compounds the organic percentage is increased significantly to elute the hydrophobic compounds in a reasonable time frame (Figure 6). Both curves show an agreement of close to 100%.

In Figure 7 the chromatogram is enhanced at 3 to 9 minutes, to show that even at the steep increase from 5% to 50% organic in 0.1 minutes, the correlation is very good.

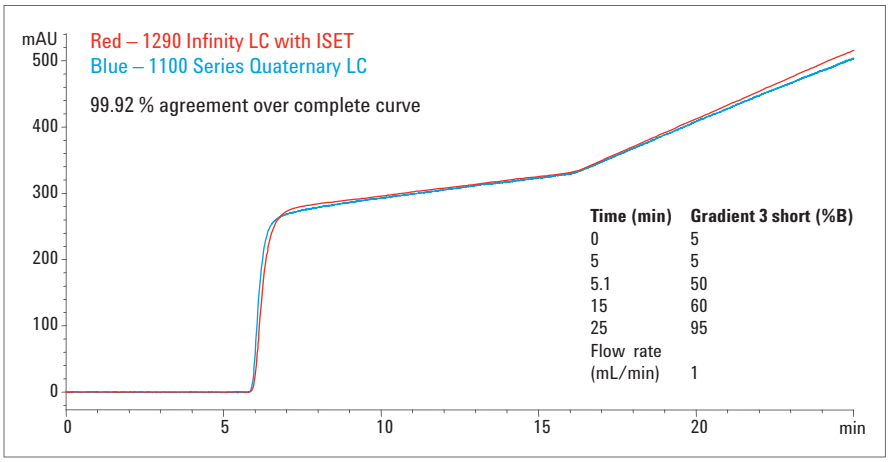

Figure 6
Overlay of gradient 3 using an Agilent 1100 Series Quaternary LC and an Agilent 1290 Infinity LC System with ISET.

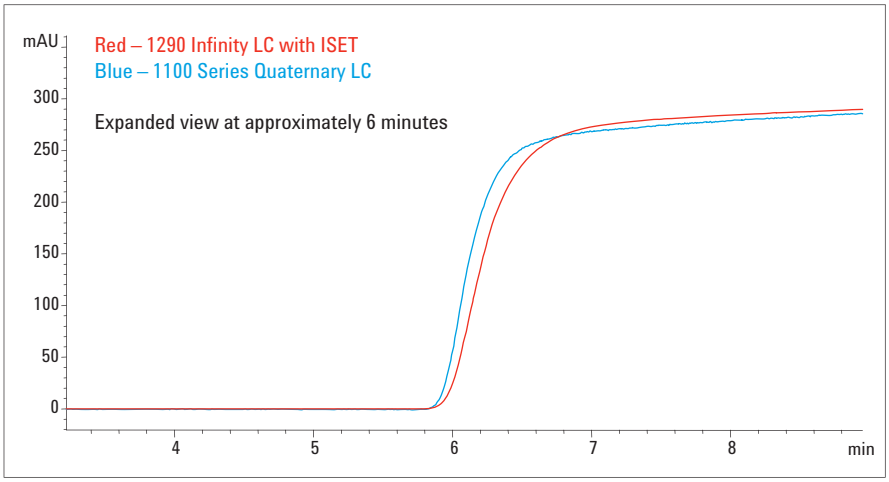

Figure 7
Expanded view at approximately 6 minutes at critical ultrafast composition changes.

In the following, linear gradients of different lengths were applied to both instruments.

Gradient 4, 5, and 6 were linear gradients from 5 to 95% organic with a run time of 3, 10, and 20 minutes. Figure 8 shows the linear gradient with a 20-minute run time as an example.

All linear curves show an agreement close to 100%. The agreement is close to 100% even for the gradient of 3 minutes, which is unusual for an Agilent 1100 Series Quaternary LC (Figure 9).

The performance results of the six gradients are summarized in Figure 9. Correlation factors were evaluated and transferred into percentage values. The agreement between the original gradient obtained from the Agilent 1100 Series Quaternary LC and the Agilent 1290 Infinity LC with ISET in all cases is close to 100%.

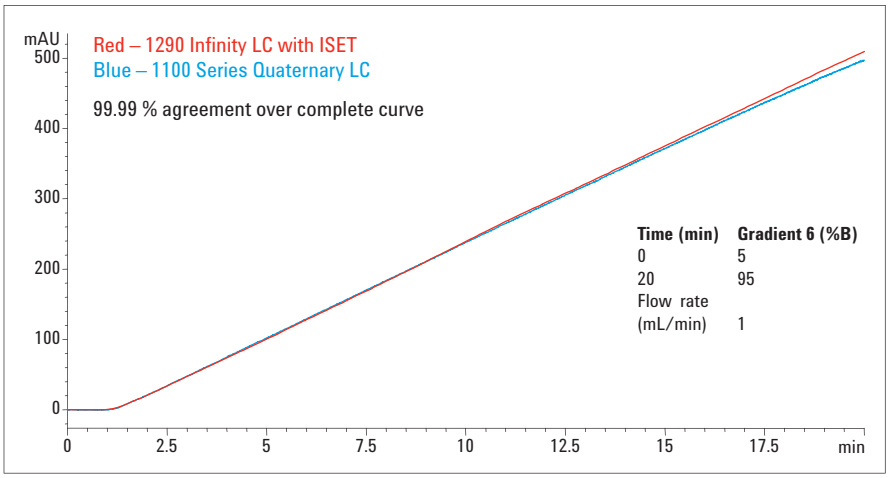

Figure 8
Overlay of gradient 6 applied on an Agilent 1100 Quaternary Series LC and an Agilent 1290 Infinity LC with ISET.

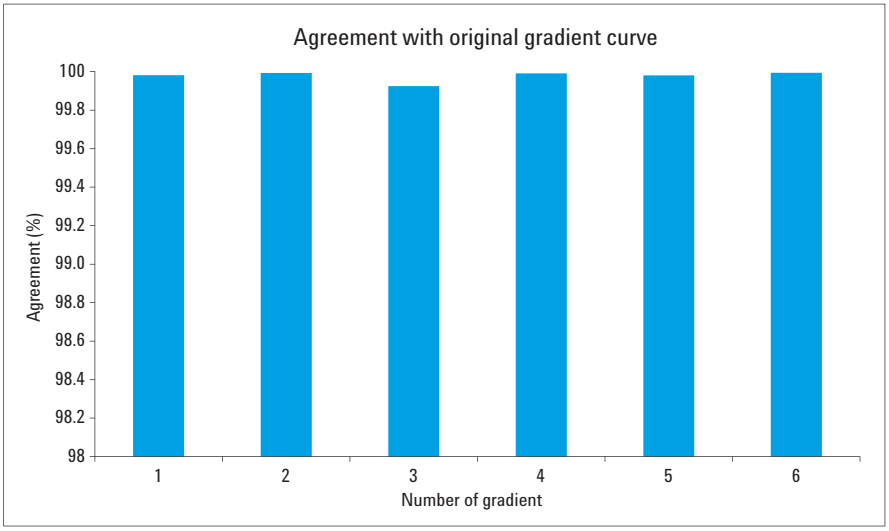

Figure 9
Agreement of gradients used and transferred from an Agilent 1100 Series Quaternary LC to an Agilent 1290 Infinity LC with ISET.

Conclusion

Design differences between HPLC and UHPLC instruments such as power range, delay volume, and mixing behavior affect the ability to transfer a method from one system to another. Therefore, identical methods used on different LC instrumentation could result in different retention times and chromatographic resolution.

The Agilent 1290 Infinity LC with ISET allows users to emulate methods from conventional instruments with a simple mouse click.

Six different gradients were applied to the Agilent 1100 Series Quaternary LC and to the Agilent 1290 Infinity LC with ISET to prove that there is high correlation between the transferred methods using the ISET function. The resulting gradients from each instrument were monitored using a tracer experiment. An overlay of the obtained curves and the calculation of correlation factors show excellent correlation. These data demonstrate that retention times and resolution are similar to a large extent.

Reference

1.
"Agilent 1290 Infinity LC with Intelligent System Emulation Technology", Agilent publication, Publication number 5990-8670EN, **2011**

www.agilent.com/chem/iset

Published in USA, January 1, 2012
5990-9545EN

Agilent Technologies

Seamless instrument-to-instrument method transfer from an Agilent 1100/1200 Series LC to an Agilent 1290 Infinity LC using Intelligent System Emulation Technology (ISET)

Technical Overview

Author

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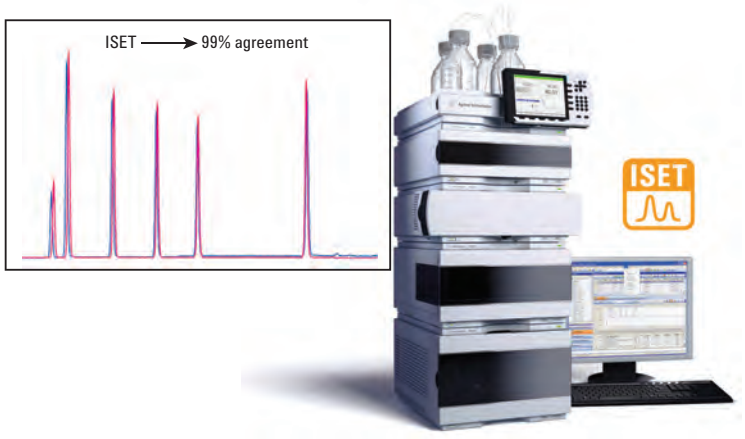

Abstract

The Intelligent System Emulation Technology (ISET) harnesses the wide power range and the superior accuracy and performance of the Agilent 1290 Infinity LC to emulate other systems for seamless transfer of methods between LCs, regardless of brand. It makes the Agilent 1290 Infinity LC the world's first truly universal LC system as it can execute other HPLC and UHPLC methods and deliver the same chromatographic results without any change of the instrument or the original method.

In this Technical Overview, we demonstrate that methods from an Agilent 1100 Series Quaternary LC instrument can easily be transferred to an Agilent 1290 Infinity LC without the need to change the original method, and that the same retention times with the same resolution are achieved by simply enabling the ISET function.

Agilent Technologies

Introduction

Instrument-to-instrument method transfer is often problematic, especially in highly regulated environments, because any modification of the original method should be avoided. Agilent Technologies provides seamless method transfer, for instance, between an Agilent 1100 Series, Agilent 1200 Series, and an Agilent 1220/1260 Infinity LC to the Agilent 1290 Infinity LC. The Agilent 1290 Infinity LC with ISET enables seamless LC method transfer without changing the original method[1] by adding isocratic steps, for example. Legacy methods can run unchanged and the user can still take full advantage of the UHPLC speed, resolution and sensitivity of an Agilent 1290 Infinity LC if new validated methods are transferred to any other department. Method development labs are able to speed up their method development with UHPLC performance and then fine-tune the new method by emulating the target system, and be confident that the method will run as intended.

In this Technical Overview we demonstrate:

- How to set up ISET
- Method transfer from an Agilent 1100 Quaternary LC system to an Agilent 1290 Infinity system with ISET
- Performance results, for example, agreement of retention times, resolution and precision

Experimental

The instruments used are listed in Table 1.

Chromatographic conditions

Compounds:	Uracil, phenol, methyl-, ethyl-, propyl-, butyl- and heptylparaben
Sigma sample:	HPLC Gradient System Diagnostic Mix, (Order No. 48271)
Column:	Agilent ZORBAX SB C18, 4.6 × 150 mm, 5 µm, (p/n 7995218-595)
Mobile phases:	Water/acetonitrile
Gradient:	20% to 95% in 10 min
Flow rate:	1 mL/min
Stop time:	12 min
Post time	5 min
Column temperature:	30 °C
Injection volume:	5 µL
DAD:	250/10 nm Ref. 360/100 nm, 10 Hz

Results and Discussion

Parameter screen for ISET

In Figure 1, the method screen of an Agilent 1290 Infinity Pump with ISET is shown. To initiate ISET, select *Enable ISET*. In this screen, it is mandatory to fill in the correct product number of the pump and autosampler that were originally used, and that should be emulated by the Agilent 1290 Infinity LC. Then, all other method parameters from the original method, such as flow and the gradient time table, are filled in. The agreement between the original

Module	Agilent 1100 LC product number	Agilent 1290 LC with ISET product number
Pump	G1311A, manufactured in 2003	G4220A
Autosampler	G1313A	G4226A
Thermostat for autosampler		G1330B
Column compartment	G1316A	G1316C
Detector	G1315B	G4212A
Chemstation	Version C 01.03 (26) and ISET version 1.0 are prerequisites	

Table 1
Instrumentation

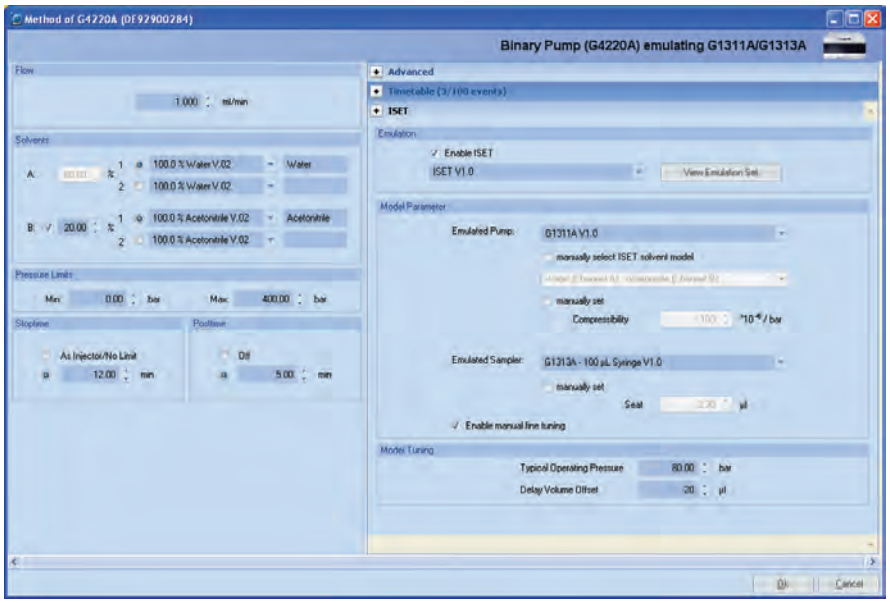

Figure 1
Pump method screen with ISET enabled.

chromatogram and the chromatogram obtained from an Agilent 1290 Infinity LC with ISET can be further optimized using the fine tuning option.

The method transfer from conventional instrumentation to UHPLC systems is generally problematic, due to the significant lower delay and transition volumes of the UHPLC systems, see Figure 2.

To overcome this problem, two solutions are typically applied. Either an isocratic step is used at the beginning of the gradient, or the delay volume is increased by adding additional tubing. Both methods can only compensate the smaller delay volume but not the overall gradient behavior (transition volume, mixing performance) over the complete gradient and run time. Without any compensation, the shift of retention time is > 9% and for the resolution of methylparaben the agreement is < 70% for both UHPLC systems, see Figure 3.

Method transfer from an Agilent 1100 Quaternary LC system

The Agilent 1100 Series Quaternary LC was built in 2003 and the method applied included a conventional 4.6 × 150 mm column packed with 5 µm particles. The gradient started with 20% organic going to 95% organic in 10 minutes. The total run time was 12 minutes and the post time was 5 minutes. This conventional method was applied to an Agilent 1100 Series Quaternary LC, to an Agilent 1290 Infinity LC without ISET, to an Agilent 1290 Infinity LC with ISET, and to an Agilent 1290 Infinity LC with ISET using additional fine tuning parameters, see Figure 4.

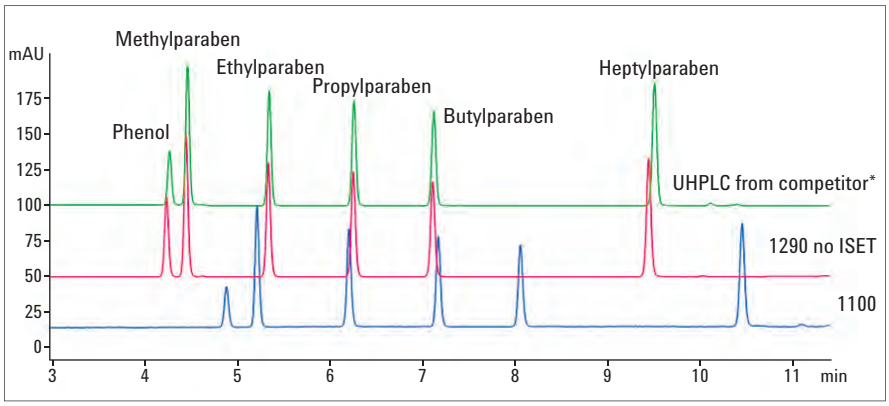

Figure 2
Transfer of a conventional method to two different UHPLC systems.
***Conventional column did not fit into small column compartment, weak wash and strong wash had to be selected carefully.**

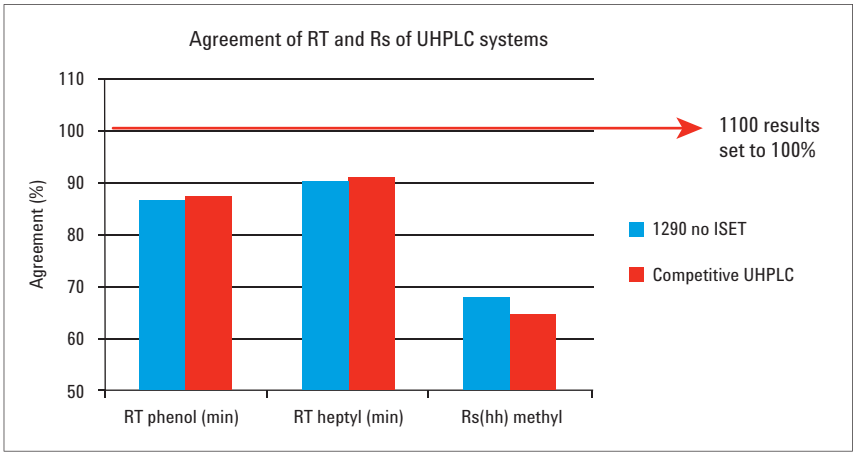

Figure 3
Agreement of retention time and resolution for UHPLC systems.

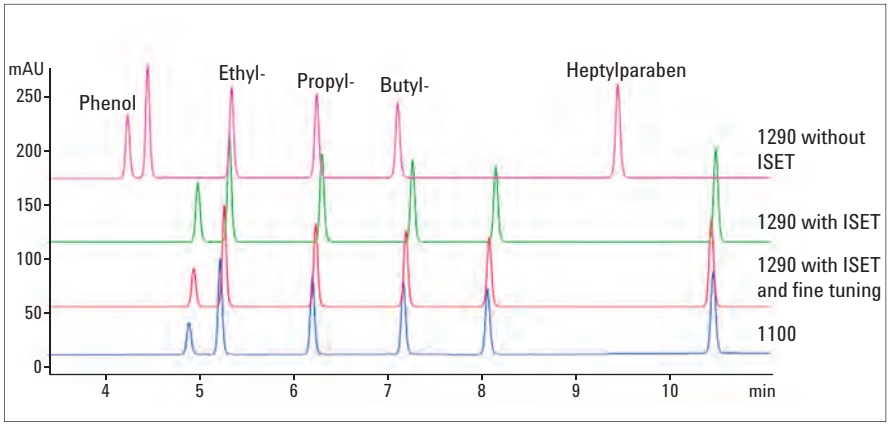

Figure 4
Overlay of chromatograms of a conventional method applied to an Agilent 1100 Series Quaternary LC, to an Agilent 1290 Infinity LC without ISET, to an Agilent 1290 Infinity LC with ISET and to an Agilent 1290 Infinity LC with ISET and fine tuning.

Without ISET, all peaks are shifted to lower retention times and also the resolution has changed. Using ISET the agreement for retention times fit already very well to the original chromatogram. A possibile way to further improve the agreement is to use the *Enable manual fine tuning* option. Filling in the average pressure from the Agilent 1100 Series LC analysis and a reduced delay volume of –20 µL, results in optimum agreement with the original chromatogram. In Figure 5, the results comparing the Agreement for retention times and resolution from an Agilent 1100 Series LC results are summarized. The results obtained from an Agilent 1100 Series LC were set to 100%. With the fine tuning options the RT shift is < 1.1% for phenol (first peak), RT shift is < 0.3% for heptylparabene (last peak) and resolution shift is < 0.26% for methylparaben (second peak).

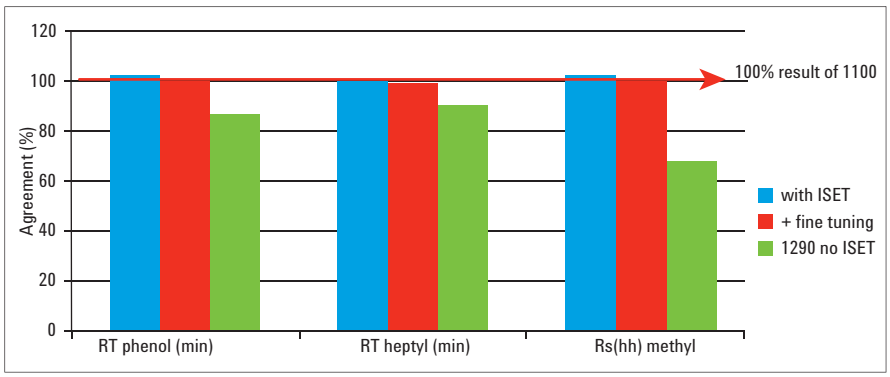

Figure 5
Agreement of retention times and resolution compared to Agilent 1100 Series LC results with and without ISET and with ISET plus fine tuning.

Parameter	Agilent 1100 Series LC	Agilent 1290 Infinity LC with ISET
RSD RT (%)	<0.027	0.009
RSD area (%)	<0.65	0.27

Table 2
Precision data.

The precision of retention times and areas obtained on an Agilent 1290 Infinity LC system with ISET is significantly better compared to those from an Agilent 1100 Series Quaternary LC system, see Table 2.

Conclusion

An Agilent 1290 Infinity LC with Intelligent System Emulation Technology (ISET) allows users to:

- Emulate other (U)HPLC instruments by a simple mouse click
- Run existing (U)HPLC methods without modifying method or system
- Deliver same retention times and peak resolution for "infinitely" better method transfer

A conventional LC method developed on an Agilent 1100 Series Quaternary LC was transferred to an Agilent 1290 Infinity LC using the ISET function. The resulting chromatograms agreed close to 100%. In addition, the precision of retention times and areas was significantly improved.

References

1.
"Agilent 1290 Infinity LC with Intelligent System Emulation Technology",
Agilent publication, Publication number 5990-8670EN, 2011

www.agilent.com/chem/iset

Published in USA, November 1, 2011
Publication Number 5990-9113EN

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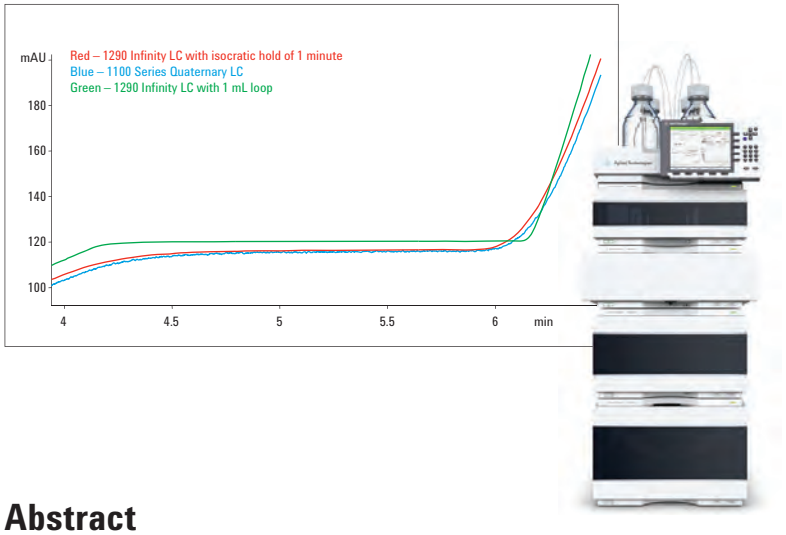
Comparing gradient transfer of isocratic hold and delay volume addition using the Agilent 1290 Infinity LC with ISET

Technical Overview



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Abstract

Agilent Intelligent System Emulation Technology (ISET) is a function within the Agilent 1290 Infinity LC System that allows to emulate other systems for seamless transfer of methods between LC instruments. Using ISET, retention times and resolution obtained on the Agilent 1290 Infinity LC are the same as obtained on the Agilent 1100 Series Quaternary LC system.

In this Technical Overview, we demonstrate that a gradient run on an Agilent 1100 Series LC correlates nearly 100% with the gradient profile of an Agilent 1290 Infinity LC System with ISET. Neither the addition of an isocratic step, nor the installation of an additional delay volume can deliver the same good correlation results.

Agilent Technologies

Introduction

Instrument-to-instrument method transfer is often problematic, especially in highly regulated environments, because any modifications of the original method should be avoided. Agilent Intelligent System Emulation Technology (ISET) provides seamless method transfer, for example, between earlier Agilent 1100/1200 Series LCs and Agilent 1220/1260 Infinity LCs to the Agilent 1290 Infinity LC System.[1] Legacy methods can run unchanged, retention times and resolution with the same as obtained from the emulated LC.

An important criterion for a seamless method transfer is whether gradient profiles obtained from a conventional LC can be transferred to the Agilent 1290 Infinity LC with ISET with high correlation. High correlation ensures that retention times and resolution will correlate to a high extent.

Another issue of interest is whether the addition of an isocratic step or the addition of an additional delay volume can compete.

Tracer experiments were chosen because they can deliver more complete correlation information than the injection of arbitrary selected peaks.

This Technical Overview details the following experiments:

- Transfer of a gradient from an Agilent 1100 Series Quaternary LC to an Agilent 1290 Infinity LC without ISET
- Applying the same gradient on an Agilent 1290 Infinity LC with an isocratic hold at the beginning of the run
- Applying the same gradient on the Agilent 1290 Infinity LC with an additional delay volume installed
- Applying the same gradient to the Agilent 1290 Infinity LC with ISET

All gradient curves were compared and the advantage of the ISET function is shown.

Experimental

Instrumentation

Table 1 shows the configurations of the instrumentation used for the tracer experiments.

Software

Agilent Chemstation revision C 01.03 and ISET revision 1.0.

Chromatographic conditions

The chromatographic conditions used for all gradients were as follows:

Compound:	Uracil as tracer, 10 mg/L in methanol
Column:	Restriction capillary
Mobile phases:	Water (A)/methanol with tracer (B)
Flow rate:	1 mL/min
Column temperature:	30 °C
Diode Array Detector:	254/4 nm, Ref. 360/100 nm, 10 Hz
Gradients:	Table 2

	Agilent 1100 Series Quaternary LC	Agilent 1290 Infinity LC with ISET
Module	**Product number**	**Product number**
Pump	G1311A manufactured in 2003	G4220A
Autosampler	G1313A	G4226A
Thermostat	none	G1330B
Column Compartment	G1316A	G1316C
Detector	G1315B	G4212A

Table 1
Instrumentation used for the experiments.

Tracer (%)	Time (min) *	Additional 1 mL loop on an Agilent Infinity LC	Isocratic hold 1 min on an Agilent Infinity LC Time (min)	Isocratic hold 0.95 min on an Agilent Infinity LC Time (min)
5	0	0	0	0
5	n/a	n/a	1	0.95
20	3	3	4	3.95
20	5	5	6	5.95
60	6	6	7	5.95
65	9	9	10	9.95
95	10	10	11	10.95

*** applied on 1100, 1290 Infinity LC with and without ISET.**

Table 2
Applied gradient on an Agilent 1100 Series LC and on an Agilent 1290 Infinity LC with and without ISET. Also applied on an Agilent 1290 Infinty LC with additional delay volume and two different isocratic holds.

Results and discussion

Typically, the transfer of a gradient from a conventional LC instrument to an UHPLC system will not deliver a good correlation (Figure 1).

Due to the significantly lower delay volume of the Agilent 1290 Infinity LC System, the gradient is shifted to shorter elution times. In addition, the mobile phase changeovers before and after the isocratic section show a sharper profile, which is characteristic of the Agilent 1290 Infinity LC with a delay volume of about 140 µL.

To overcome the transfer problems shown in Figure 1, two solutions are typically used. Either an isocratic hold is added at the beginning of the gradient, or the delay volume is increased by adding physical delay volume. Both methods can only compensate the smaller delay volume but not the overall gradient behavior (transition volume, mixing performance) over the complete gradient and run time. To compensate the different delay volumes, a 1-minute isocratic hold (simulates 1 mL of additional delay volume) was added to the method. In a second experiment, a 1-mL loop was installed between the pump and the autosampler on the Agilent 1290 Infinity LC. The resulting gradient curves from those two experiments were compared with the original gradient curve from an Agilent 1100 Series Quaternary LC (Figure 2).

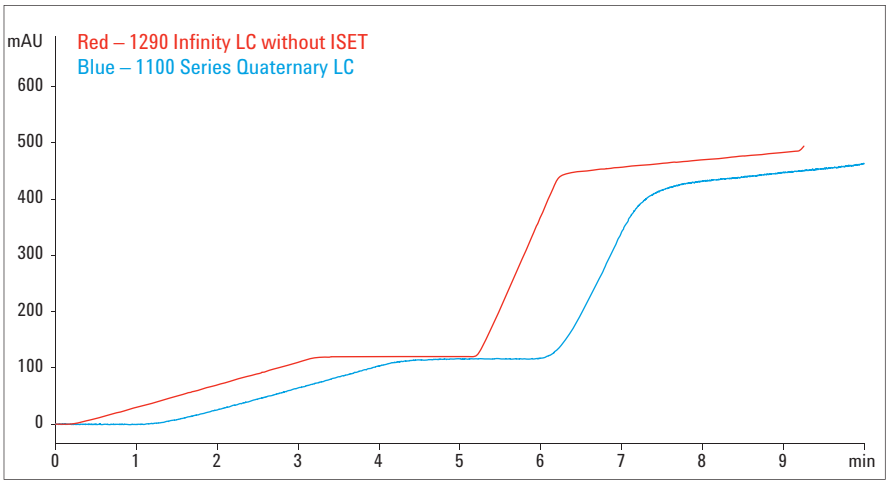


Figure 1
Transfer of gradient from the Agilent 1100 Series Quaternary LC to the Agilent 1290 Infinity LC without ISET.

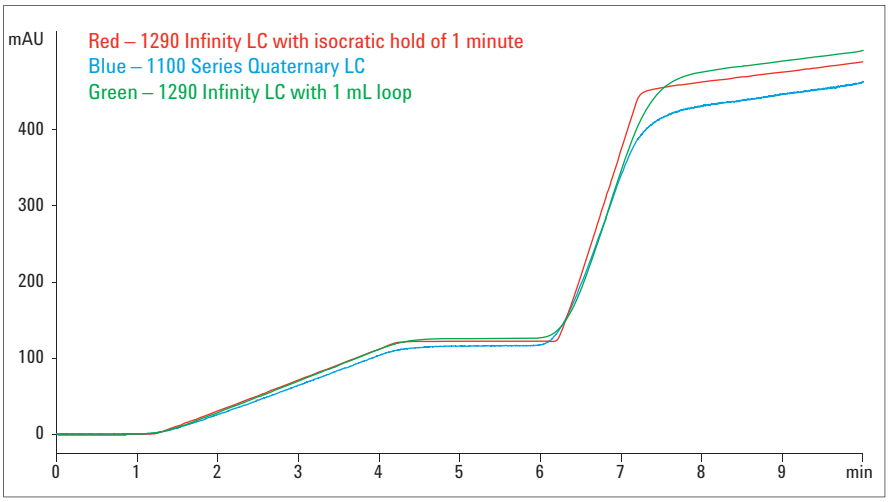


Figure 2
Overlay of the original curve with the curve obtained on the Agilent 1290 Infinity LC with isocratic hold of 1 minute and the addition of a 1-mL loop.

The most critical parts of a gradient are significant mobile phase changes. Figure 3 shows a more detailed view of before and after the isocratic range of the applied gradient, between 4 and 6.5 minutes.

Neither curve follows the original curve satisfactorily. However, using an isocratic hold allows the user to optimize the length of the isocratic hold at the beginning of the chromatogram. Figure 4 shows a better correlation by optimizing the isocratic hold and changing it to 0.95 minutes.

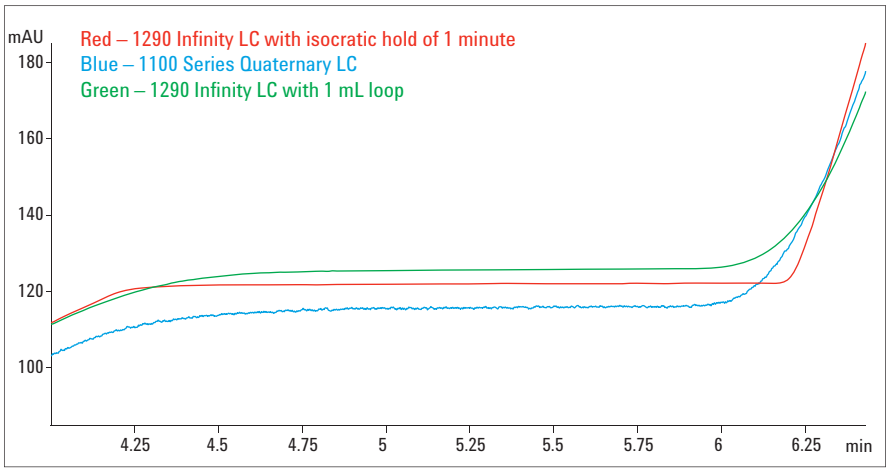


Figure 3
Enhanced part around the isocratic range of the gradient.

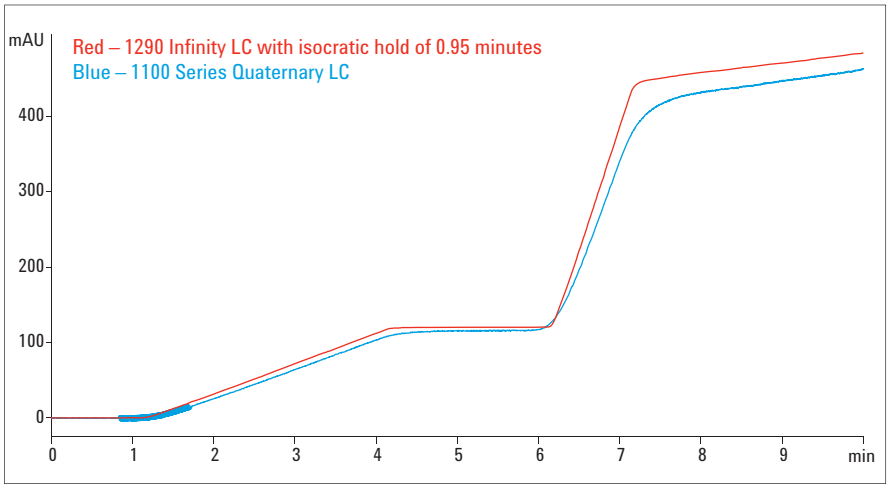


Figure 4
Overlay of gradient curve obtained on the Agilent 1100 Series Quaternary LC and an Agilent 1290 Infinity LC with an isocratic hold of 0.95 minutes.

Correlation of the gradients from an Agilent 1100 Series Quaternary LC and an Agilent 1290 Infinity LC with a 0.95-minute isocratic hold is better than a 1-minute isocratic hold, but still not as optimal as the one shown in the enlarged view in Figure 5.

The gradients on the Agilent 1290 Infinity LC with the isocratic hold are still different, in particular the changes to and from the isocratic and the gradient slopes.

In the last experiment, the ISET function of the Agilent 1290 Infinity LC was used to emulate the gradient of the Agilent 1100 Series Quaternary LC. Figure 6 shows the complete gradient of the 1100 Series LC (blue trace) the 1290 Infinity LC with ISET (red trace) and the 1290 Infinity LC with an isocratic hold of 0.95 minutes (green trace).

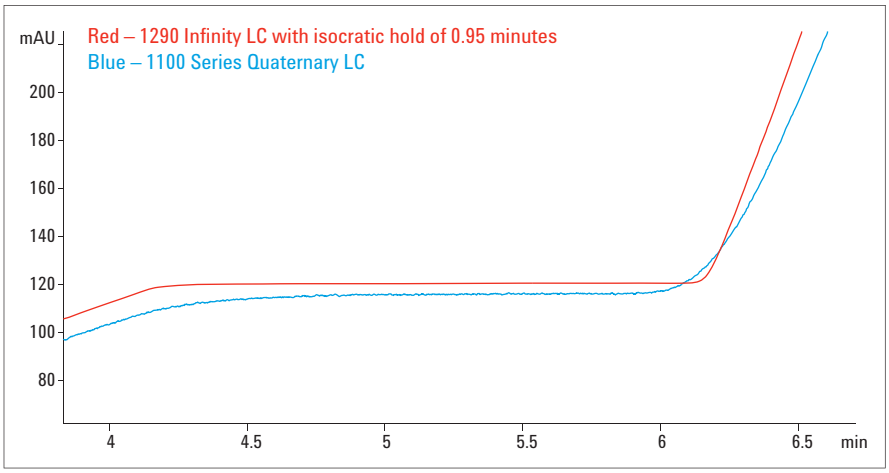


Figure 5
Overlay of an Agilent 1100 Series Quaternary LC gradient with curve obtained on an Agilent 1290 Infinity LC with an isocratic hold of 0.95 minutes (zoomed view around 5 minutes).

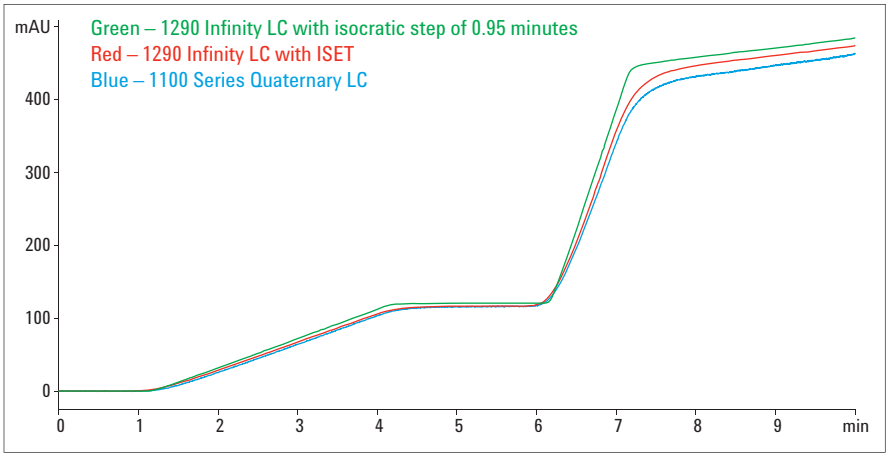


Figure 6
Overlay of gradients 1 applied on an Agilent 1100 Series Quaternary LC and an Agilent 1290 Infinity LC with ISET.

The gradient of the Agilent 1290 Infinity LC System with ISET follows the original gradient of the Agilent 1100 Series LC better than any of the gradients generated with additional delay volume or with an isocratic hold. This is even more obvious if the range around 5 minutes is expanded (Figure 7).

The red trace representing the gradient from the Agilent 1290 Infinity LC with ISET follows the gradient from the 1100 Series LC exactly, even at the changes to and from the isocratic step beween 4 and 6 minutes. The green curve representing the isocratic hold shows significantly less correlation.

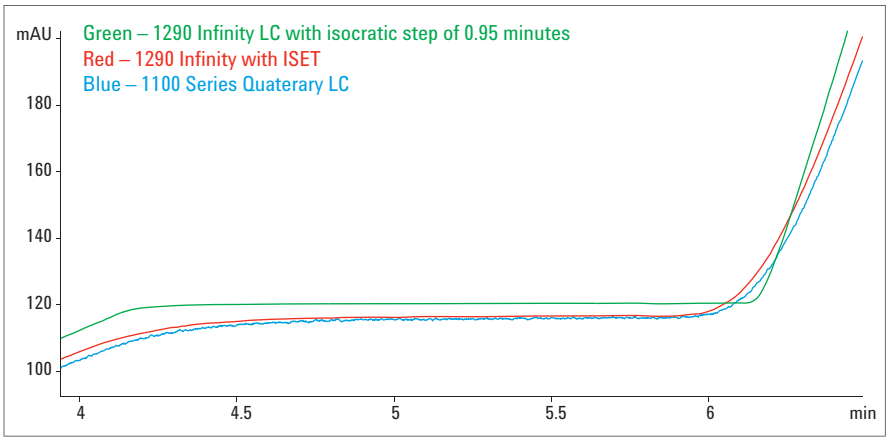


Figure 7
Overlay of gradient applied to the Agilent 1100 Series Quaternary LC System and the Agilent 1290 Infinity LC System with ISET (zoomed around 5 minutes).

Conclusion

One gradient was applied to the Agilent 1100 Series Quaternary LC using a tracer experiment. The same gradient was transferred to the Agilent 1290 Infinity LC with and without ISET, to the Agilent 1290 Infinity LC with additionally installed delay volume and to the Agilent 1290 Infinity LC with two different isocratic holds at the beginning of the run. The optimum correlation of the original curve was obtained using the Agilent 1290 Infinity LC with ISET. This ensures that retention times and resolution will both correlate to a high extent. Isocratic holds and the addition of delay volume did not deliver the same excellent performance.

Reference

1.
"Agilent 1290 Infinity LC with Intelligent System Emulation Technology", Agilent publication, Publication number 5990-8670EN, **2011**

www.agilent.com/chem/iset

Published in the USA, January 1, 2012
5990-9546EN

Agilent Technologies

Optimize UHPLC

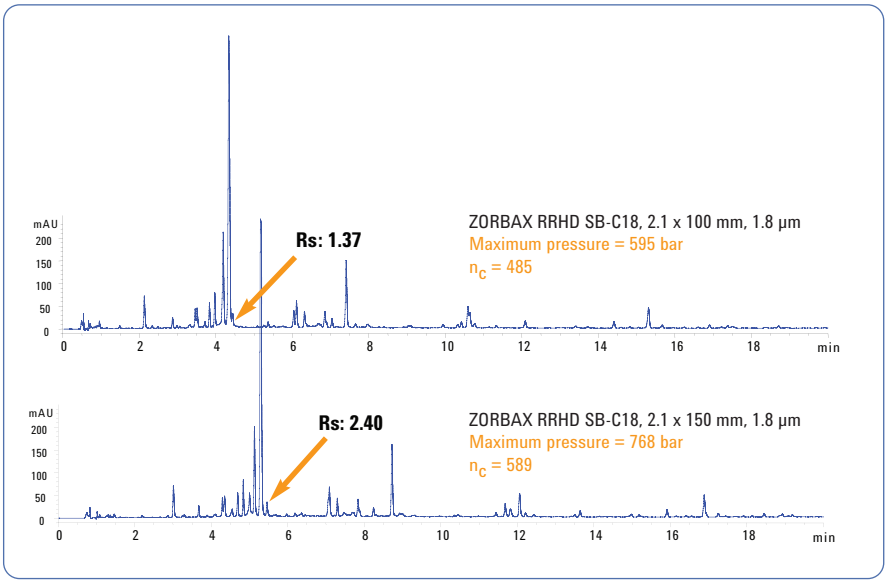
Ultra-Low Dispersion Max-Light Cartridge Flow Cell

Agilent Low Dispersion Kit

See "Optimizing Performance of an Agilent ZORBAX RRHD Eclipse Plus C18 Column by Enhancing an Agilent 1290 Infinity LC System for Ultra-Low Dispersion" (App Note 5990-9502EN)

Increasing Resolution and Speed by Operating UHPLC Columns up to 1200 Bar

Technical Note



Abstract

LC columns with sub-2-µm particles have gained popularity because they deliver high productivity and resolving power. In the past, some of the efficiency and throughput benefits of these columns could not be realized because LCs and columns were limited to backpressures of 400 to 600 bar. That changed when Agilent ZORBAX Rapid Resolution High Definition (RRHD) columns with 1.8 µm particles were designed to take advantage of the unique 1200-bar pressure limit of the Agilent 1290 Infinity LC System. Operation in this high-pressure domain allows chromatographers to achieve even more speed and resolution for complex separations.

Introduction

The productivity and resolution benefits of ultra high performance liquid chromatography (UHPLC) and columns with sub-2-µm particles are well recognized in the scientific community. Shorter LC columns with 1.8 µm particles allow analysts to achieve faster runtimes while maintaining column efficiency and resolution. Longer columns with these small particles enable additional resolution for complex mixtures. However, smaller particles produce higher backpressure. When the backpressure limit of the column or the LC is reached, it is impossible to further increase the flow rate for a faster analysis, or to use a longer column to achieve greater resolution.

The Agilent 1290 Infinity LC System uniquely addresses the need for a more flexible LC that operates under higher backpressures up to 1200 bar. (See Figure 1.) Agilent ZORBAX Rapid Resolution High Definition (RRHD) columns with 1.8 µm particles are designed for optimal performance over the entire operating range of this LC, and are the only commercially available columns that can be used up to the 1200-bar limit. This combination of the 1290 Infinity and ZORBAX RRHD (1.8 µm) columns forms a total solution that provides new levels of LC performance and flexibility.

The ZORBAX RRHD (1.8 µm) columns exceed the capabilities of the Agilent ZORBAX Rapid Resolution High Throughput (RRHT) columns with 1.8 µm particles, which have a 600-bar limit. New hardware and an improved packing process extend the stability of the ZORBAX RRHD (1.8 µm) columns to higher pressures. The ZORBAX RRHD (1.8 µm) columns are available in short and long lengths, and can be used up to 1200 bar to achieve the chromatographic definition needed to separate complex mixtures completely and reliably.

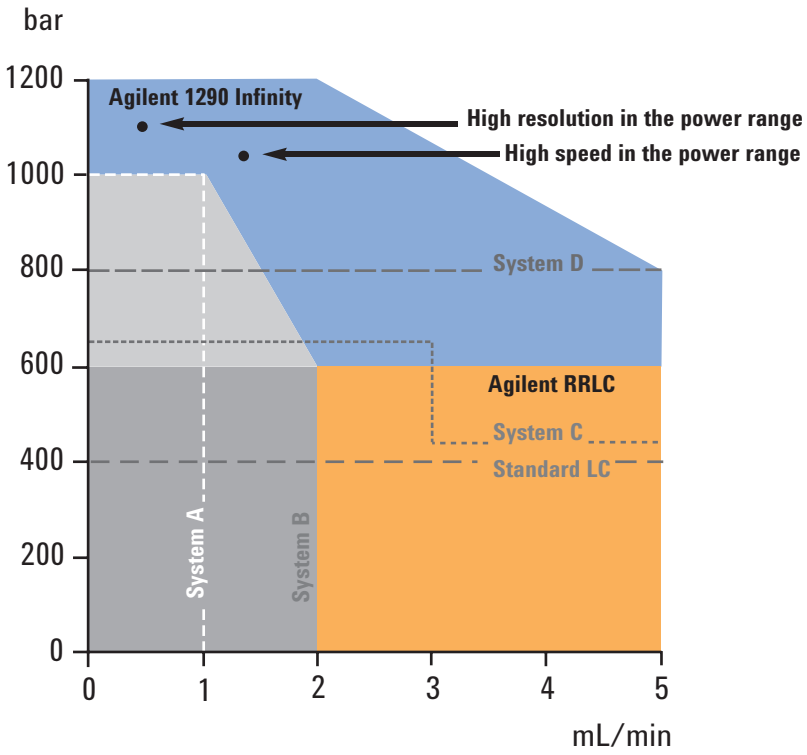


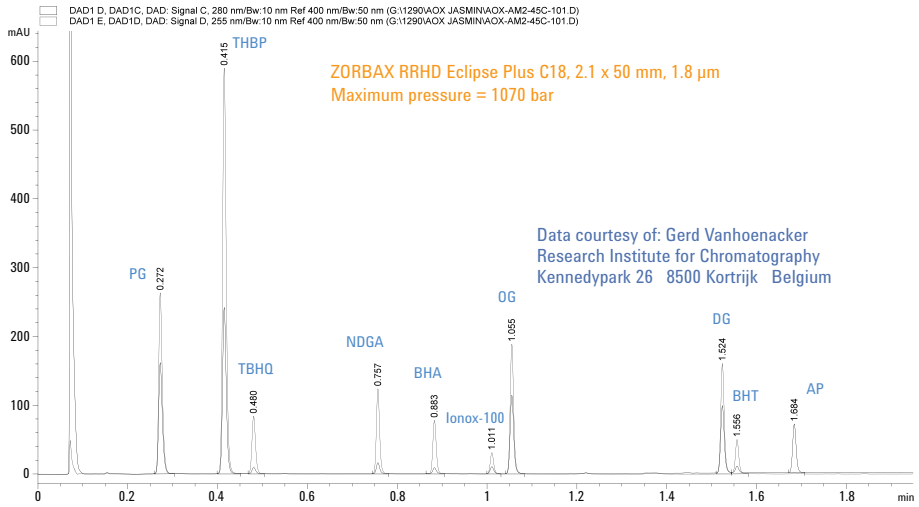
Figure 1: ZORBAX RRHD (1.8 µm) columns use the full pressure and flow range of the 1290 Infinity LC, for separations with higher speed and definition.

Results and discussion

As the following examples show, the combination of these columns with the 1290 Infinity LC System makes it possible to increase analysis speed for complex samples, or to achieve greater resolution in a limited amount of time.

Example 1: maximize speed with higher flow rates

Many labs need to decrease LC runtimes to increase sample throughput. In the example shown in Figure 2, the goal was to achieve maximum speed in a gradient separation of a 10-component mixture of antioxidants. This was a complex separation, but an analysis time of less than two minutes was achieved by using a fast flow rate of 1.6 mL/min with an Agilent ZORBAX RRHD Eclipse Plus C18 column, 2.1 x 50 mm, 1.8 µm. Note that a gradient can produce a pressure change of more than 300 bar during an analysis, especially if it covers a very wide range of organic mobile phase. The gradient program at this 1.6 mL/min flow rate generated a maximum pressure of 1070 bar – exceeding the limit on other UHPLC systems and columns. The extended pressure range of the 1290 Infinity LC with the ZORBAX RRHD (1.8 µm) columns enabled this fast separation.



Chromatographic conditions for Figure 2

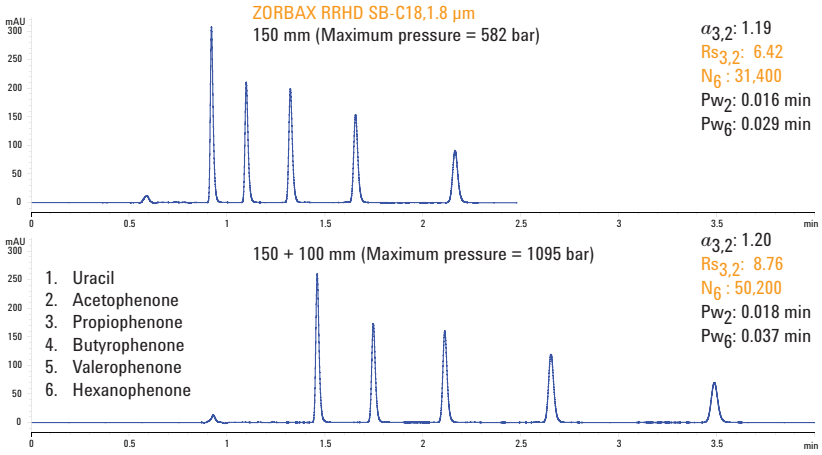
Column	ZORBAX RRHD Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm
Mobile phase	A = 0.02% H_3PO_4, B = acetonitrile:methanol 3:1
Flow rate	1.6 mL/min
Gradient	0 – 1.85 min 30 – 100% B
Injection volume	1 µL
Detector	Sig = 280/10 nm, Ref = 400/50, 80 Hz Sig = 255/10 nm, Ref = 400/50, 80 Hz
Temperature	45 °C
Sample	Antioxidant mixture, ~ 100 ppm

Figure 2. By operating at greater than 1000 bar, the ZORBAX RRHD (1.8 µm) column produced this gradient separation of 10 antioxidants in less than two minutes.

Agilent 1290 Infinity LC – *technology for more resolution, speed and sensitivity*

Example 2: maximize resolution with tandem columns

Longer LC columns deliver more resolving power for mixtures with a large number of components. While columns are available in standard lengths of 50, 100, and 150 mm, analysts may need additional options to increase resolution. As this example shows, they can combine columns in series and achieve even better separations. Figure 3 compares the separation of a group of alkylphenones on a 150 mm ZORBAX RRHD (1.8 µm) column versus two RRHD columns in series. In this example, tandem columns with a total length of 250 mm increased resolution by 36% and efficiency by the expected 60%. These columns in series generated very high resolution, but required close to 1100 bar pressure for this gradient separation. This high pressure was well within the limit for the Agilent LC and column.



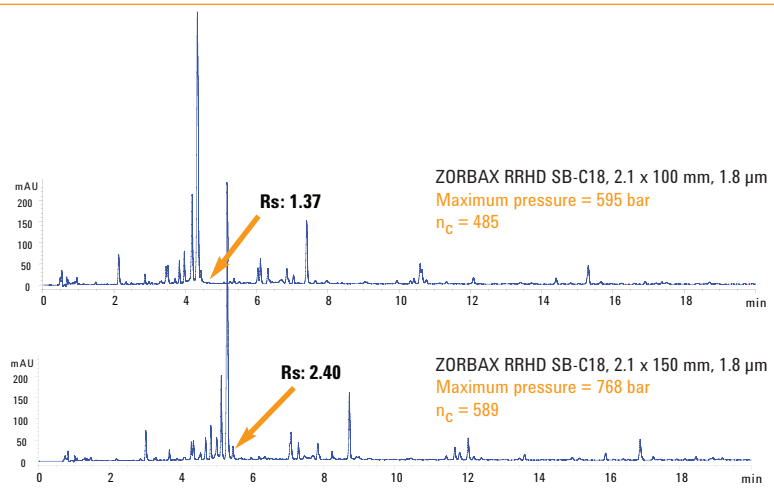
Chromatographic conditions for Figure 3

Columns	ZORBAX RRHD SB-C18, 1.8 µm
Mobile phase	25:75 water:acetonitrile
Flow rate	0.5 mL/min
Temperature	Ambient
Sample	Alkylphenones
Abbreviations	α = separation factor
	Rs = resolution
	N = number of theoretical plates
	Pw = peak width

Figure 3. Because they operate at pressures up to 1200 bar, ZORBAX RRHD (1.8 µm) columns can be used in tandem to maximize efficiency and resolution.

Example 3: increase peak capacity with longer columns

A third example (Figure 4) shows a separation of a complex licorice root extract. As the column length increased, the peak capacity increased from 486 on the 100 mm column to 589 on the 150 mm column, and some minor components were completely resolved. This gradient separation used a maximum pressure of almost 800 bar, representing a more typical UHPLC separation, and the sample was well-resolved with the ZORBAX RRHD (1.8 µm) column.



Chromatographic conditions for Figure 4

Method

Columns	ZORBAX RRHD SB-C18, 2.1 mm, 1.8 µm
Mobile phase	A = 0.1% formic acid
	B = acetonitrile with 0.1% formic acid
Gradient	10 – 100% B in 30 min
Flow rate	0.4 mL/min
Detector	UV, 280 nm
Temperature	Ambient
Sample	Licorice root extract
Abbreviation	Rs = resolution
	n_c = peak capacity

Figure 4. A longer 150 mm ZORBAX RRHD (1.8 µm) column enabled greater peak capacity and a better separation for this sample of licorice root.

Conclusion

Agilent ZORBAX Rapid Resolution High Definition (RRHD) columns with 1.8 µm particles are designed and manufactured for reliable operation at 1200 bar – the highest pressure in the industry. High-pressure operation enables use of longer columns, tandem columns, and/or higher flow rates. Chromatographers can use these columns over the full operating range of the Agilent 1290 Infinity LC System, providing greater flexibility to achieve maximum speed and resolution for complex samples.

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Agilent Technologies

New Dynamic MRM Mode Improves Data Quality and Triple Quad Quantification in Complex Analyses

Technical Overview

Authors

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Abstract

Multiple Reaction Monitoring (MRM) mode has become the preferred method for the quantitative analysis of known or target compounds using triple quadrupole mass spectrometry. The current solution for MRM analysis uses time segmentation, where a method is divided into a series of time segments and predefined sets of MRM transitions are monitored for each segment. As sample complexity increases (e.g. quantifying very low levels of hundreds of pesticide residues in a wide variety of food matrices), very real practical limitations in the time-segmentation methodology become apparent. A better solution is required.

New dynamic MRM methods on the Agilent 6400 Series triple quad instruments create new capability to tackle large multi-analyte assays and to accurately quantify exceedingly narrow peaks from fast Agilent 1200 Series RRLC and 1290 Infinity UHPLC separations. Examples of pesticide analysis and rapid screening of drugs of abuse are highlighted. Dynamic MRM methods yield equivalent, or better, quality data and results as compared to traditional time segment based methods – plus easier method development and modification.

Agilent Technologies

Introduction

Utilizing multiple reaction monitoring (MRM) with a triple quadrupole tandem mass spectrometer enables extraordinary sensitivity for multi-analyte quantitative assays. The first quadrupole (Q1) selects and transmits a precursor ion with a specific *m/z*. This ion is then fragmented in the second quadrupole (Q2 collision cell), and a specific product ion with a defined *m/z* is selected and transmitted in the third quadrupole (Q3). See **Figure 1**. The combination of a specific precursor mass and a unique product ion is generally an unambiguous and sensitive method to selectively monitor and quantify a compound of interest. Since two stages of mass selection are utilized, MRM assays are particularly useful for the specific analysis of target compounds in complex mixtures and matrices. MRM mode has become the preferred method for the quantitative analysis of known or target compounds.

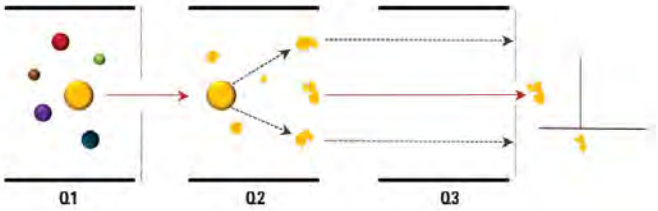

Figure 1: *A schematic diagram of MRM mode on a triple quadrupole instrument. The precursor ion is selected in Q1, fragmentation occurs in Q2, and the product ion is selected by Q3. Since two stages of mass selectivity are utilized, there is very little interference from background matrix resulting in excellent sensitivity.*

The Limitations of Time Segment Methods

The current solution to complex sample analysis is time segmentation. A method is developed with multiple predefined time segments and the triple quad MS is programmed to perform MRM assays for only those analytes that elute during each segment. **Figure 2** shows an example of a method with four time segments. One set of MRM transitions is analyzed during segment 1, another set during segment 2, etc. The benefit of such a method is that, rather than performing MRM scans for all analytes during the entire method, during any given segment the triple quad only monitors MRM transitions for the analytes that elute in that segment. The result is that there are fewer MRM transitions during each MS scan, allowing the mass spec method to use a longer dwell time and/or to reduce the overall cycle time for each MRM scan so that there are more data points per peak.

However, there are some limits to what can be accomplished with time segment methods. As the number of analytes in a method increases, so too will the number of concurrent MRM transitions in each segment. It will be necessary to either reduce the dwell times for these transitions or to increase the cycle time for each MS scan. Reducing dwell times (the amount of time required for the triple quad to analyze a single MRM transition) can compromise MS data integrity by introducing collision cell cross-talk (insufficient clearing of the collision cell between individual MRM experiments such that some product ions from a previous MRM may be detected in the subsequent MRM). Maintaining the same dwell time but increasing the overall MS cycle time may mean that not enough data points are collected during the elution of a very narrow LC peak to allow for reliable quantitation. Both of these factors can lead to compromises in data quality.

There is an additional challenge using time segments. In order not to compromise any data, the change from one segment to the next must occur during a time when no peaks are eluting from the LC column. In complex analyses such as pesticide analysis, where many co-eluting peaks are monitored at almost every time point during the chromatogram, this can be a formidable challenge as is highlighted in **Figure 2**. Furthermore, there is always the risk that adding analytes to a method may require complete redevelopment of a method to introduce these chromatographically quiet zones where segment changes can occur.

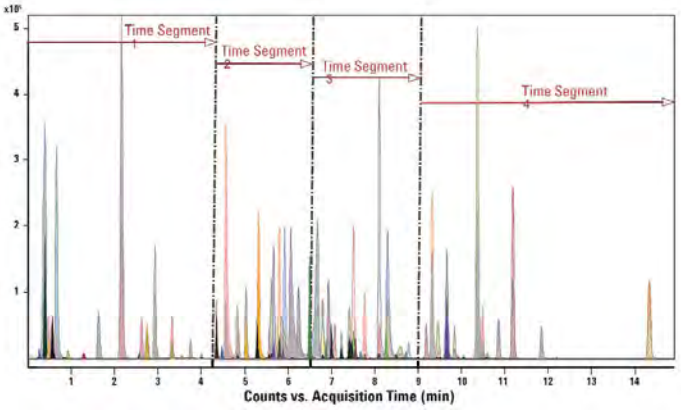

Figure 2: *Dividing the chromatogram into time segments. Detection of a complex pesticide mixture demonstrates the advantages and some of the limitations of time segment based MRM quantitation.*

Introducing Dynamic MRM Mode

Agilent's new and unique analytical method approach is now available on all 6400 Series Triple Quadrupole LC/MS systems. MassHunter acquisiton software allows the user to choose conventional MRM or dynamic MRM mode. Ion transitions and a retention time window for each analyte are stored in a method. MRM transition lists are then built dynamically throughout an LC/MS run, based on the retention time window for each analyte. In this way, analytes are only monitored while they are eluting from the LC and valuable MS duty cycle is not wasted by monitoring them when they are not expected. An added benefit of this approach is that MassHunter MS Optimizer software can readily determine and store optimal transition ions for each target analyte, greatly simplifying dynamic MRM method set up.

This approach addresses the limitations of the time segment methods for a large batch of compounds by replacing the group segmentation with individual time windows for every analyte transition and by dramatically reducing, on average, the number of individual MRM transitions that are monitored during each MS scan. This approach is demonstrated in **Figures 3-5**.

Dynamic MRM removes the requirement to resolve compounds to baseline and to create well-defined segments in the chromatogram where no compounds elute. This reduces the potential method impact of adding analytes and of retention time shifts. The inevitability of multiple co-eluting peaks is of lesser concern with dynamic MRM as long as the individual ion transitions are unique. **Figure 5** shows an expanded region of the analysis in **Figure 3**, with 22 compounds eluting between 5.58 and 6.51 min with substantially overlapped peaks. All analytes can be accurately quantified because their ion transitions are mutually exclusive, allowing total exclusion of background and interferences.

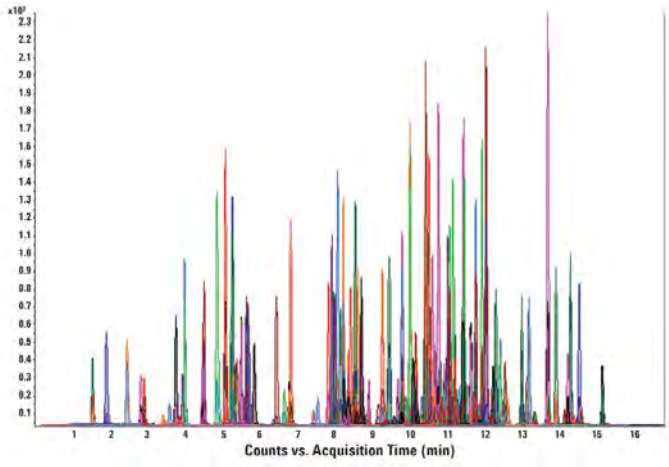

Figure 3: *Dynamic MRM method does not require time segments. Extracted ion chromatogram of a 250 pesticide mix spiked into tap water (500 total transitions, 2.5 pg on-column) using a dynamic MRM method run on a 1290 Infinity LC and a 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream technology.*

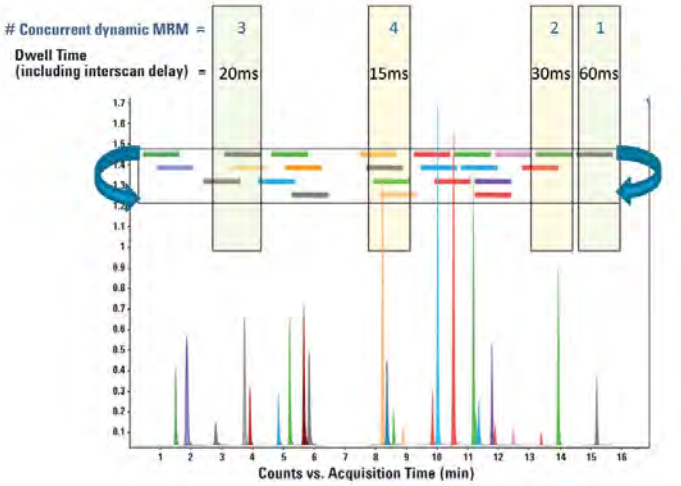

Figure 4: Dynamic MRM methods are based on individual retention time windows for each MRM transition. 24 pesticide transitions from the analysis in Figure 3 are highlighted and their retention time windows are shown. *Note that, on average, the number of transitions which are monitored at any point in the chromatogram is dramatically reduced relative to time segment methods, allowing much faster MS scan cycle times. Also note that this MS cycle time is held constant (60 ms in this case) in order to assure the highest possible data quality and quantitative result.*

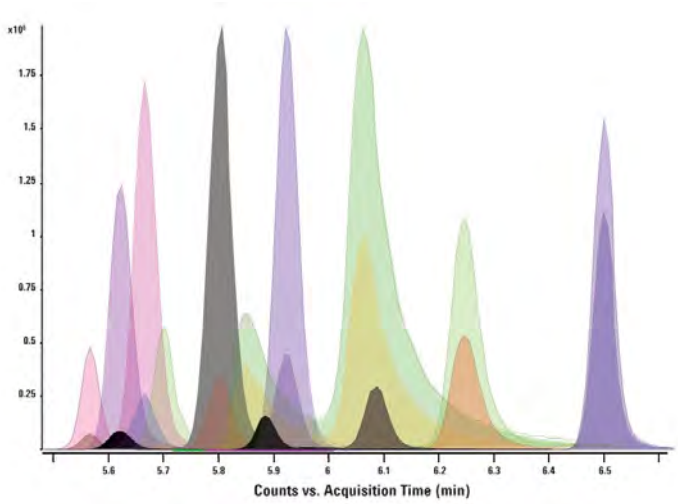

Figure 5: *Extracted ion chromatogram of 11 pesticides and 11 qualifier ions. In spite of significant co-elution, well-chosen MRM transitions allow for accurate quantification of all sample components.*

The Importance of Constant MS Scan Cycle Time in Dynamic MRM Methods

Agilent's dynamic MRM approach uses a constant sampling time across chromatographic peaks. Even data point spacing with adequate sampling across the peak provides the best and most precise representation of the peak. To maintain a constant cycle time, the individual MRM dwell time is also adjusted to keep a constant sampling rate across all peaks, even though the number of ion transitions being monitored will change dynamically and may vary cycle to cycle, dependent on elution time and the number of concurrent analytes. Because dynamic MRM yields generally fewer concurrent ion transitions per unit time than traditional time segments, MS cycle times can be reduced and individual transition dwell times are typically longer than traditional time segmented methods. While the Agilent 6460 and 6430 Triple Quadrupole LC/MS systems are capable of 1 ms dwell times, this is typically only required in the most extreme assays. Note: with the proprietary axial acceleration technology present on all Agilent triple quadrupole and Accurate Mass Q-TOF collision cells, all product ions are cleared from the collision cell in less than 600 µs so that there is no MRM cross-talk with the shortest dwell times (1).

Furthermore, by maintaining a constant dynamic MRM cycle time, MS methods can be matched to analyte peak widths to ensure that a statistically adequate number of data points is acquired for each analyte to yield excellent analytical accuracy and precision. This approach yields uniform data points across any given analyte peak and results in good peak symmetry — a distinct improvement over constant dwell time approaches.

Dynamic MRM Easily Accommodates Fast UHPLC

HPLC or UHPLC separations with the Agilent 1200 RRLC or 1290 Infinity LC systems can reduce method times dramatically without sacrificing peak capacity or chromatographic resolution. Individual peak widths may be reduced to just a few seconds. In the extreme, peak widths may be less than one second wide. Dynamic MRM methods require on average, fewer ion transitions to be monitored concurrently in a chromatogram. MS cycle times are much faster than with time segment methods and allow collection of many data points across narrow peaks, as is shown in **Figure 6**, for excellent quantitative results.

Linearity with dynamic MRM methods is at least as good as traditional time segment approaches. Typically, linear correlation coefficients are excellent and assay linearity exceeds three orders of dynamic range. **Figure 7** shows a calibration curve for the pesticide compound oxamyl, with excellent sensitivity, linearity, and dynamic range. Triplicate injections of a 25 pg sample on-column yielded a peak area %RSD of only 1.08.

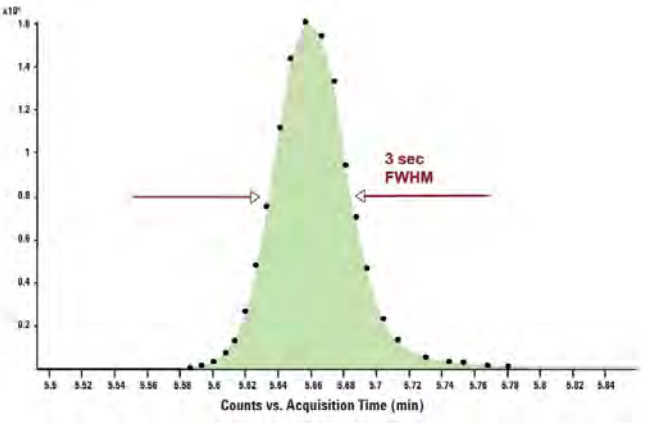

Figure 6: Dynamic MRM allows accurate quantification of narrow LC peaks. *A pesticide analysis gave this 6-sec wide peak for atrazine (5pg on-column). A dynamic MRM method allowed for collection of sufficient data points to assure an excellent quantitative result. The MS scan cycle time was 350 ms and remained constant across the peak. Quantitative precision showed a peak area %RSD < 3.5 for this compound.*

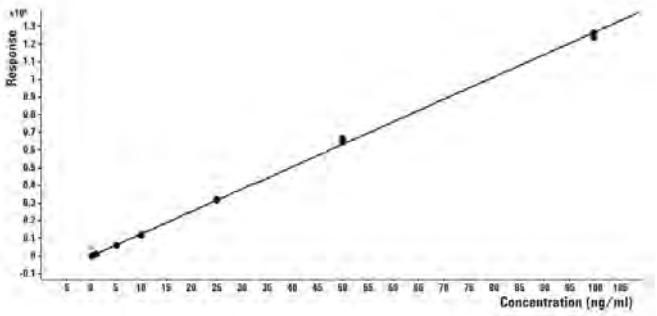

Figure 7: *Dynamic MRM methods provide excellent quantitative data. Linearity of oxamyl from 0.1 pg to 100 pg on-column, R2 = 0.9992.*

Application of dynamic MRM

Pesticide Screening

A challenging real world application for dynamic MRM is the quantitative analysis of a very complex sample run at ultra high pressure using a high resolution column and a fast gradient. 300 pesticides with internal standards were run on a sub-two micron column with a 15 min gradient at pressures exceeding 800 bar, or 11600 psi. A dynamic MRM method with 600 transitions was created using retention time windows of only 0.5 min. Results of this analysis are shown in **Figure 8**.

Comparison of dynamic MRM with conventional time segment methods reveals an excellent correlation. Eight pesticides were injected in 20 replicates at the 10 pg level and both average area and relative standard deviation were calculated. As shown in **Figure 9**, the correlation in peak areas derived with both the dynamic and time-based MRM methods was outstanding with $R^2 = 0.99992$. The peak area relative standard deviations for the time segment based method was less than 6% and less than 4% for the dynamic MRM method.

Rapid Screening for Drugs of Abuse

A further example of dynamic MRM capability is the fast screening for 100 drugs of abuse in oral fluids over a 5 minute gradient — a typical work-place drug test.

This is a particularly challenging analysis given the timescale of the assay relative to the number of analytes in the target screen. Further, since qualifier ions and quantifier ions were necessary for confirmatory purposes, a total of 200 dynamic MRM transitions were employed during the analysis, covering classes of analytes such as opiates, amphetamines, cannabinoids and benzodiazepines, among others.

In this example, peak widths were approximately 2 sec. A dynamic MRM method using retention time windows of only 12 sec was used. The maximum number of concurrent dynamic MRM transitions was never more than 52. The assay showed excellent sensitivity, (LOD = 23 fg on-column) and linearity. External calibration linearity: ($R^2 = 0.9987$) for one of the spiked analytes (Prazepam) is described in **Figures 11** and **12**, respectively.

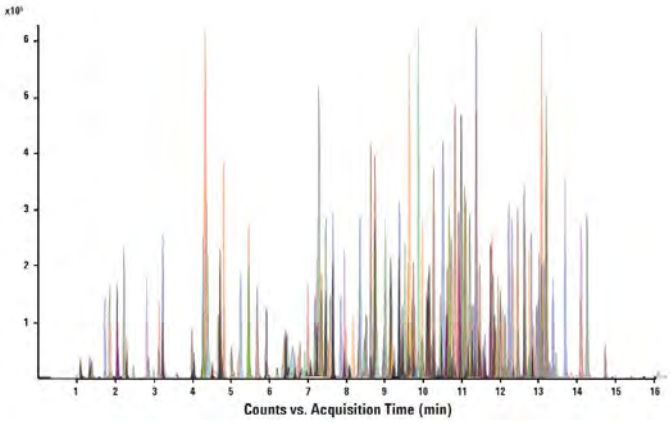

Figure 8: *Dynamic MRM analysis allows quantification of 300 pesticides using internal standards in a 15 min method. Data was generated with an Agilent 1200 Infinity LC and 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream technology.*

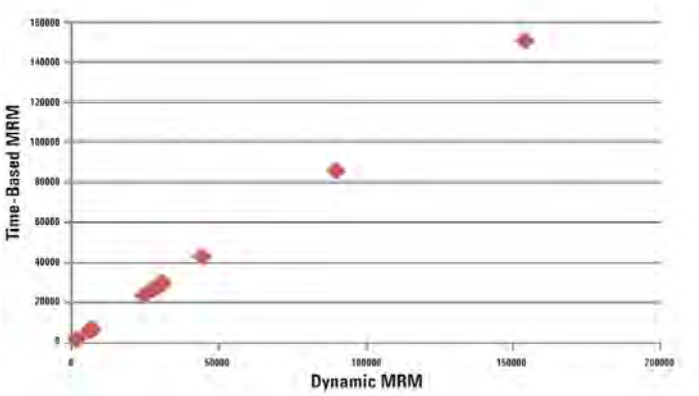

Figure 9: *Comparing pesticide peak areas with dynamic MRM and time segment based methods.*

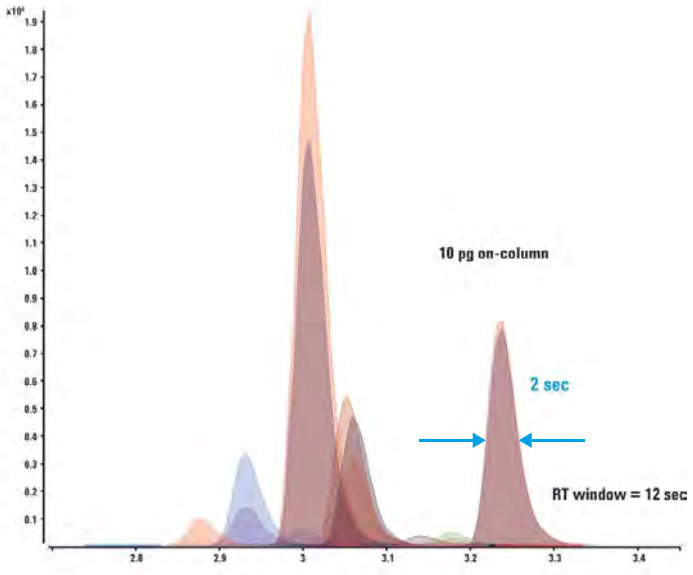

Figure 10. Detection of 10 spiked benzodiazepine drugs in an oral fluid extract using a dynamic MRM method with >200 MRM transitions and 12 sec retention time windows. *This study was performed on an Agilent 1290 Infinity LC and 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream technology.*

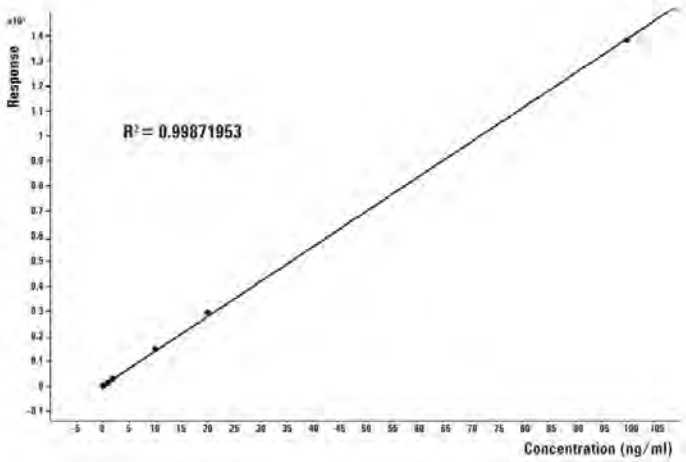

Figure 12. *Linearity of Prazepam from 23 fg to 100 pg on-column, R^2 = 0.9987.*

Compound	LOD (fg on-column)
Clobazam	126
Clonazepam	91.5
Flunitrazepam	47.5
Flurazepam	43.0
Lorazepam	186
Lormetazepam	45.9
Midazolam	1.2 pg
Oxazepam	145
Oxazolam	235
Prazepam	23.7
Temazepam	156

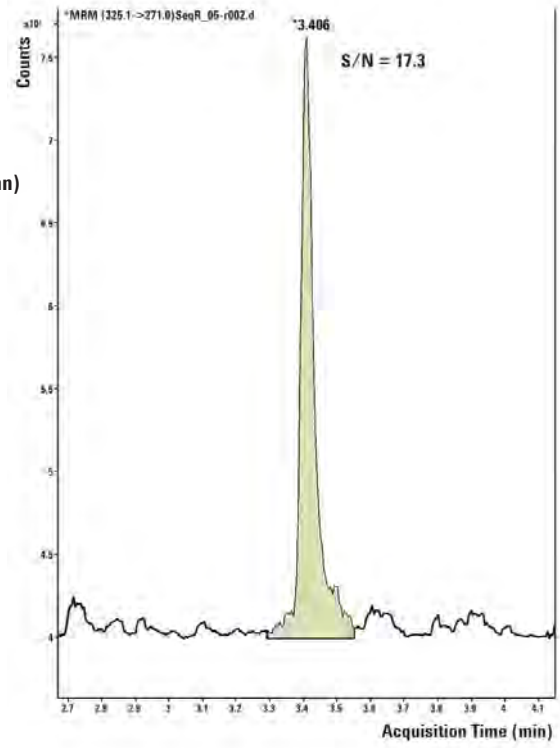

Figure 11. *Limit of detection (LOD) of Prazepam is 23.7 fg with a S/N of 17.3.*

Summary

New dynamic MRM methods on the Agilent 6400 Series triple quad instruments create new capability to tackle large multi-analyte assays and to accurately quantify exceedingly narrow peaks from fast Agilent 1200 Series RRLC and 1290 Infinity UHPLC separations. The number of MRM transitions is adjusted dynamically throughout the LC run, selecting only transitions with relevant retention time windows. This means that, on average, many fewer MRM transitions are monitored during a typical MS scan than would be the case with a time segment based method – with the added benefit that dynamic MRM methods are less demanding to develop and adapt.

Fewer transitions allow methods with shorter MS scan cycle times (more scans/second) and the ability to provide excellent quantification of very narrow (even sub-second) RRLC and UHPLC peaks. Importantly, this dramatically shortened MS scan cycle time is kept constant so optimized sampling and consistent accurate quantitation is ensured (the same cannot be said for methods that vary MS scan cycle time).

Practically, dynamic MRM methods can be used to accurately quantify hundreds of individual analytes, plus their internal standards and qualifier ions, in a relatively short LC run. Compared to benchmark time segment methods, dynamic MRM methods achieve similar sensitivity, linear dynamic range, and quantitative accuracy, with better precision.

Key Points

- Key enabling technology for fast, accurate LC/MS quantitation of complex samples
- Matches performance of Agilent 6400 series triple quad with separation power of 1200 Series RRLC and UHPLC with 1290 Infinity LC
- Many data points collected across very narrow peaks for accurate LC/MS quantitation
- Constant MS scan cycle time ensures accurate quantitation
- Equivalent and better quality data and results than traditional time segment based methods – plus easier method development and modification with MassHunter Optimizer software
- Up to 4,000 ion transitions per LC run
- Diverse applications: pesticide analysis, drug screening, targeted protein quantitation

References:
(1) Agilent publication 5989-7408EN: Ion optics innovations for increased sensitivity in hybrid MS systems

www.agilent.com/chem/qqq

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Agilent Technologies

Scalability of Agilent Columns Across HPLC and UHPLC Instruments

Application Note

Pharmaceutical

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Abstract

LC method transfer across several instrument types, using Agilent and non-Agilent systems, demonstrates the scalability of Agilent columns with different configurations and stationary phases. This ability to easily scale a method from one system to another is particularly useful in the pharmaceutical industry where samples may have to be analyzed by R&D, QC, or other laboratories where identical instrument setups may not be possible or where analytical needs may vary.

Introduction

Instrumentation and column technology for liquid chromatography continually improve, to deliver higher throughput, higher resolution, and higher sensitivity. Transferring an LC method from one instrument to another should be straightforward. However, columns specifically designed for one instrument are often not recommended, or necessary for another instrument. Newer, sub-2-µm columns are designed to withstand higher pressures than traditional HPLC systems, as a result, the smaller particles cannot be fully used within 400 bar. In addition, differences in system delay volume and extra-column volume could affect column performance from one instrument to another, most notably with small internal diameter columns. Therefore, scalability between column dimensions, especially with respect to particle size, is paramount to ensure straightforward method transfer. The flexibility of Agilent ZORBAX columns in several configurations and stationary phases is demonstrated by transferring an LC method across several instrument types, using Agilent and non-Agilent systems.

Agilent Technologies

Experimental

LC Method Parameters

An Agilent 1200 Series RRLC, a 1290 Infinity LC, a 1290 Infinity LC/6410 Triple Quadrupole MS system, and a non-Agilent UHPLC are used in this experiment.

Mobile phase	A: 0.2% formic acid in water; B: acetonitrile
Gradient	15% to 95% B, gradient time (t_g) varies according to column dimensions and flow rate, see Equations 1 and 2
DAD	Sig = 260, 4 nm; Ref = Off
MS Source	350 °C, 12 L/min, 50 psi, 3500 V
MS Scan	Positive ESI, Delta EMV 200, Fragmentor 135 V, Scan 100–400, 5 ms scan time, 0.2 amu step, 28.36 cycles/s, 35.3 ms/cycle
Analytes in elution order with identifying mass	acetaminophen (109), caffeine (194), 2-acetamidophenol (109), acetanilide (135), acetylsalicylic acid (120), phenacetin (179), salicylic acid (120), sulindac (356), piroxicam (332), tolmetin (257), ketoprofen (254), diflunisal (332), diclofenac (295), celecoxib (381), ibuprofen (160)
Sample	0.01 mg/mL (UV) and 1 µg/mL (MS) each in water

The following Agilent LC columns were used:

Description		P/N
Agilent ZORBAX Eclipse Plus C18	4.6 mm × 250 mm, 5-µm	959990-902
Agilent ZORBAX Eclipse Plus C18	4.6 mm × 150 mm, 5-µm	959993-902
Agilent ZORBAX Eclipse Plus C18	3.0 mm × 100 mm, 3.5-µm	959961-302
Agilent ZORBAX RRHD Eclipse Plus C18	3.0 mm × 100 mm, 1.8-µm	959758-302
Agilent ZORBAX RRHD Eclipse Plus C18	3.0 mm × 50 mm, 1.8-µm	959757-302
Agilent Poroshell 120 EC-C18	3.0 mm × 100 mm, 2.7-µm	695975-302
Agilent Poroshell 120 EC-C18	3.0 mm × 50 mm, 2.7-µm	699975-302

Gradient Scaling

Once a gradient separation has been optimized (selectivity and retention index), it is possible to further improve the chromatography by varying column length, particle size and flow rate. However, the k* value (Equation 1) must be maintained while varying these column conditions, so as not to lose selectivity while scaling the gradient.

Equation 1

$$k^* = (t_g F)/(d/2)^2 L(\Delta\%B)$$

where: t_g = gradient time
F = flow rate
d = column internal diameter
L = column length
$\Delta\%B$ = change in organic content across gradient segment

Assuming a constant k*, Equation 1 can be simplified to Equation 2 below:

Equation 2

$$t_{g2} = (t_{g1} d_2^2 L_2 F_1)/(d_1^2 L_1 F_2)$$

where: t_{g1} and t_{g2} = original and new gradient times
F_1 and F_2 = original and new flow rates
d_1 and d_2 = original and new column internal diameters
L_1 and L_2 = original and new column lengths

Additionally, in Equation 2, v_1 and v_2 can be substituted for t_{g1} and t_{g2} respectively to accurately scale a method's injection volume according to a new column's dimensions.

Instrument ⇔ Column Compatibility Considerations

While almost any column can be installed and run on any instrument, pressure limitations by the LC and pressure generated by the column can lead to the inability to optimally utilize any column on any LC. For example, small particles generate substantial back pressure, especially when packed into long columns, this particular column configuration is not best suited for a conventional 400 bar HPLC, as that pressure limit will easily be exceeded as flow rates increase. Table 1 lists the specifications of several Agilent LC systems that are critical to good performance, while Table 2 shows compatibility between instruments and columns for UHPLC and HPLC.

Table 1. Critical Parameters of Agilent LC Systems

	Agilent 1100/1200 Series Binary HPLC	Agilent 1200 Series RRLC (std)	Agilent 1260 Infinity Binary LC	Agilent 1290 Infinity Binary LC
Pressure limit (bar)	400	600	600	1200
Max flow rate (mL/min)	5	5	5	5
Pump Delay volume (µL)	600–900	600–800	600–800 (120*)	45/75
Capillary id (mm)	0.17	0.17	0.17 (0.12*)	0.12
Dispersion volume w/o cell (µL)	15	15	15 (7.5*)	7.5
Injection principle	Variable loop	Variable loop	Variable loop	Variable loop
Injection volume (std/ext) (µL)	100/1500	100/1500	100/1500	20/40 (100 up to 600 bar)
Area RSD (%)	< 0.25	< 0.25	< 0.25	< 0.25

*optimized for 2.1 mm id

Table 2. Instrument and Column Compatibility in Agilent UHPLC and HPLC Systems (Green [Compatible] to Red [Incompatible])

	UHPLC 1.8 µm particles						UHPLC superficially porous particles		HPLC 3.5–5 µm particles
Column length (mm)	**Short, 30–50**			**Long, 100–150**			**30–150**		**50–300**
Column id (mm)	2.1	3	4.6	2.1	3	4.6	2.1	3–4.6	3–4.6
Max pressure (bar)	1200	600	600	1200	600	600	600	600	400
Agilent column	RRHD*	RRHT^	RRHT	RRHD	RRHT	RRHT	Poroshell	Poroshell	Various
Agilent 1290 Infinity (1200 bar)									
Agilent 1260 Infinity/1200 RRLC (600 bar)									
Agilent 1100/1200 Series (400 bar)									

*rapid resolution high definition
^rapid resolution high throughput

Results and Discussion

Maintaining Selectivity with Different Column Dimensions

As shown in Figure 1, selectivity is maintained when a 5-µm column is shortened from 250 mm to 150 mm, and when transferred to a 3 mm × 100 mm, 3.5-µm column. Some resolution, however, is lost with the shorter columns, most notably with ibuprofen, the last peak to elute.

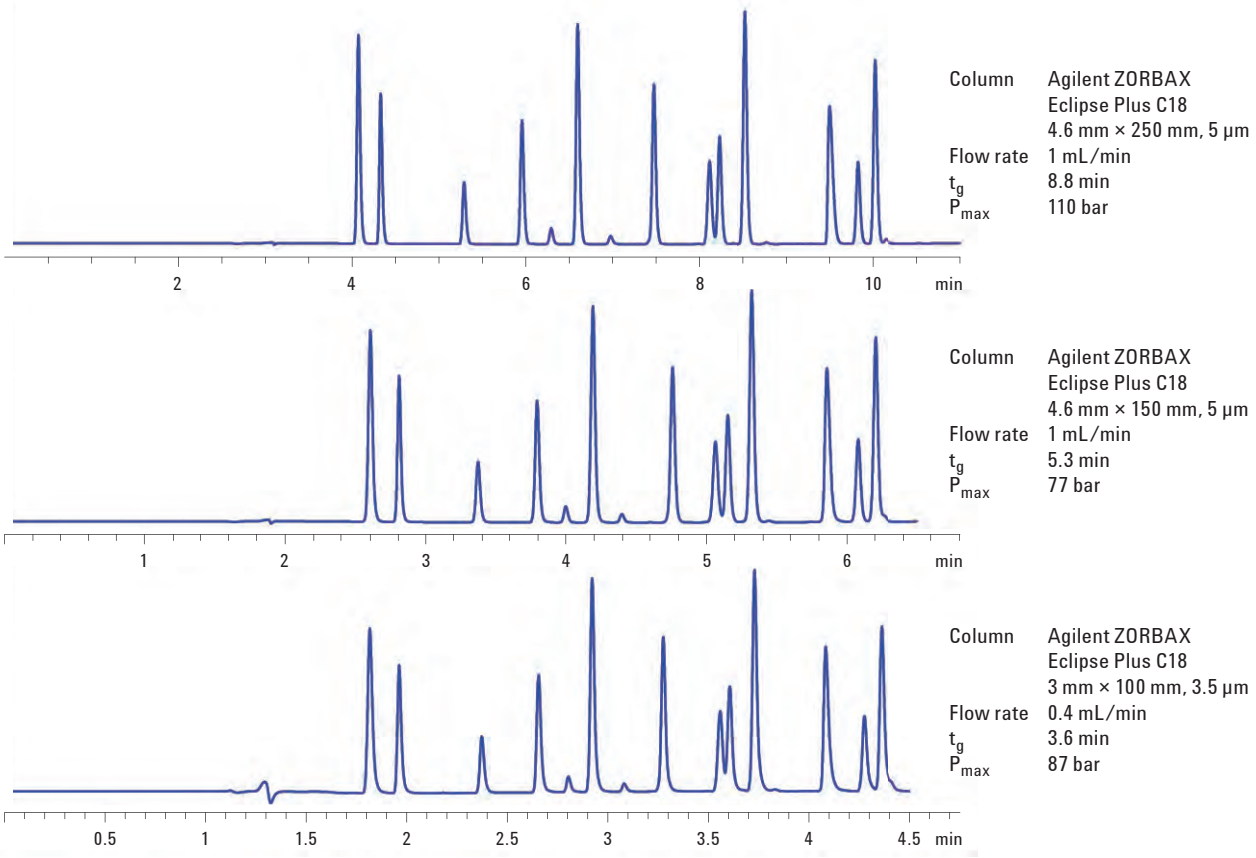

Figure 1. Maintaining selectivity on Agilent ZORBAX Eclipse Plus C18 columns with different dimensions and particle sizes using an Agilent 1200 Series RRLC.

Effect of System Delay Volume

A manual change in system delay volume is the cause of a pronounced difference in the early eluting peaks (Figure 2). The relatively large delay volume of an Agilent 1200 Series RRLC system if set-up in a standard delay volume and not in low delay volume configuration with a smaller dimension column (in this case 3 mm × 100 mm, 3.5 µm) causes delayed elution of all peaks. In order to make the chromatography more similar to the larger dimension columns shown in Figure 1, the automatic system delay volume reduction feature of the Agilent 1200 Series autosampler can be used, as shown in the bottom chromatogram in Figure 2.

Method Transfer across Agilent Instruments

Transferring the method from a 1200 Series RRLC to an Agilent 1290 Infinity LC yields very similar results with the 3.5-µm column, as shown in Figure 3, when the automatic delay volume reduction feature of the 1200 Series autosampler is used. As shown in Table 1, the delay volume of a 1290 Infinity LC system is substantially lower than the 1100/1200 Series or 1260 Infinity LC systems. The same is true for other vendors UHPLC systems optimized for lowest delay volumes. Also, typically the mixing behavior, that is, the slope of a step gradient, is much steeper for such instruments.

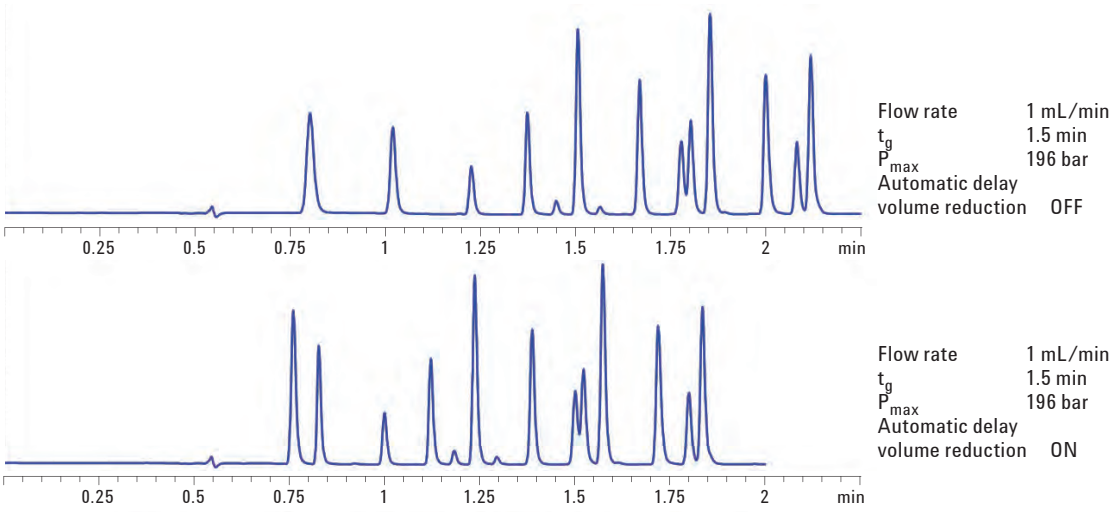

Figure 2. Effect of system delay volume on an Agilent ZORBAX Eclipse Plus C18 3.0 mm × 100 mm, 3.5-µm column using an Agilent 1200 Series RRLC without (top) and with (bottom) the delay volume reduction function on the autosampler.

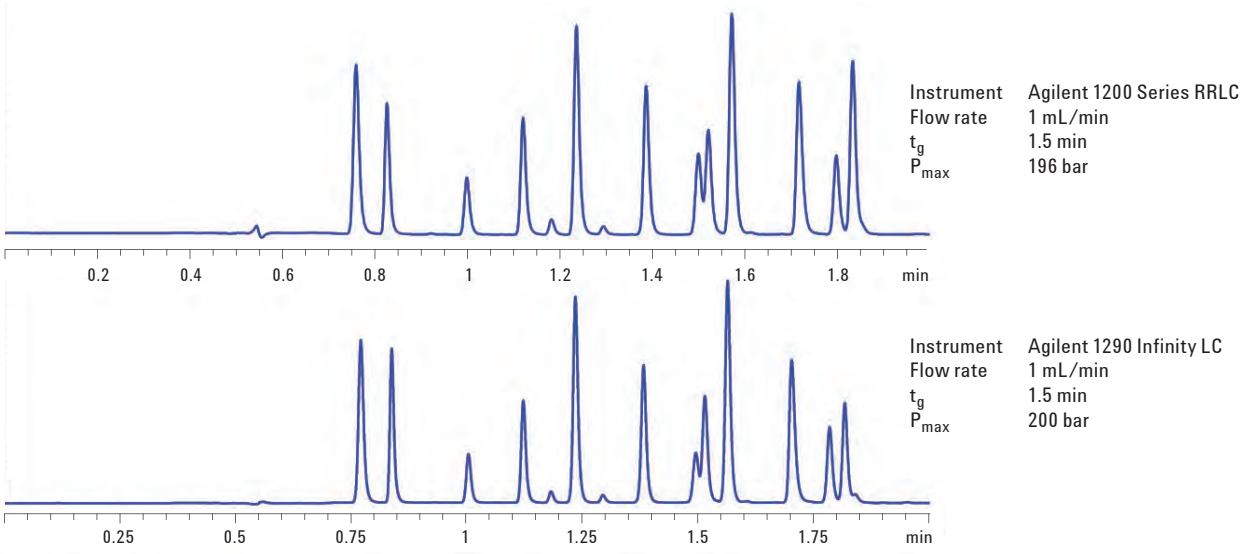

Figure 3. Comparing separations on Agilent 1200 Series RRLC and Agilent 1290 Infinity LC instruments, using an Agilent ZORBAX Eclipse Plus C18 3.0 mm × 100 mm, 3.5-µm column.

Conversely, if a method originally developed on a 1100/1200 Series or 1260 Infinity LC needs to be transferred to a 1290 Infinity LC system, a larger delay volume would be needed and, ideally, also an adoption of the mixing curve. For this, an isocratic hold can be added manually to the method, or physically the volume can be added by additional capillary tubing. Both need up-front determination of the required volume. The use of the new Intelligent System Emulation Technology (ISET), available only for 1290 Infinity LC systems, is a new technology that makes this process easier. ISET emulates the different delay volumes and mixing behaviors of Agilent's or other LC systems on a 1290 Infinity LC and delivers the same results without manual changes of the method or hardware. For more information see Agilent Pub. No. 5990-8670EN.

Method Transfer across Agilent Columns

Totally porous 1.8-µm and 3.5-µm Agilent ZORBAX columns yield the same selectivity, while superficially porous 2.7-µm Poroshell 120 delivers very similar selectivity as a result of similar bonding chemistry. Both 1.8-µm Agilent ZORBAX RRHD and 2.7-µm Agilent Poroshell 120 provide better resolution than the 3.5-µm ZORBAX column of the same dimensions, as can be seen in Figure 4. Referring to Table 2, the scalability illustrated in Figure 4 indicates that there is an Agilent LC column to meet any instrument need. In this case, the same separation can be performed on a 400 bar instrument with a 3.5-µm column, on a 600 bar instrument with a superficially porous 2.7-µm column, or on a 1200 bar instrument with a 1.8-µm column.

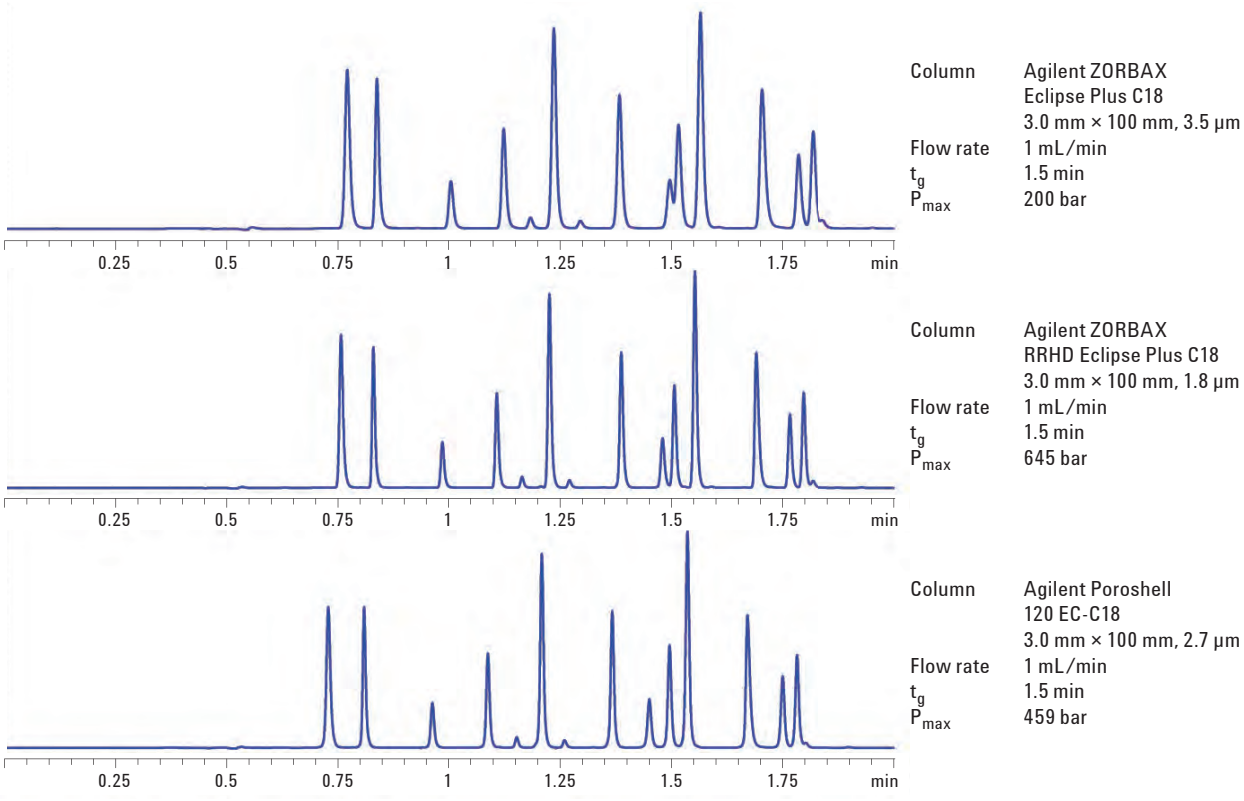

Figure 4. Comparing 1.8 and 3.5-µm Agilent ZORBAX Eclipse Plus C18 and 2.7-µm Agilent Poroshell 120 EC-C18 columns using an Agilent 1290 Infinity LC.

Method Transfer with Different Agilent Detectors

Selectivity is maintained when transferring this method from an Agilent 1290 Infinity LC with UV detection to a 1290 Infinity LC with MS detection, shown in Figure 5. Some peak broadening occurs with the MS due to more extra column volume in the detector, as compared to the DAD. Additionally, extra transfer tubing connecting the LC and MS accounts for the increase in system pressure and the slightly later elution time of all peaks.

Increasing Analysis Speed

Due to the highly selective nature of MS detection analysis speed can be increased to take full advantage of the high pressure limit of the RRHD column and the low back pressure generated by the Agilent Poroshell 120 column. With a 50 mm RRHD column, analysis time is increased significantly, resulting in a 0.4 minute run time for the 15 compounds, as seen in Figure 6.

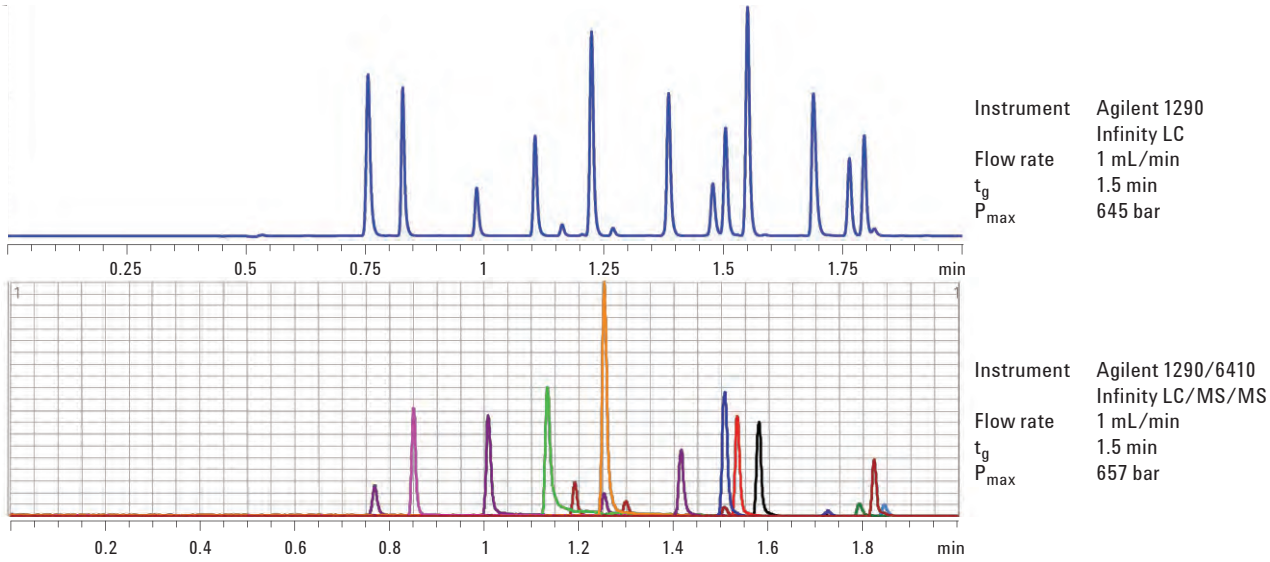

Figure 5. Comparing Agilent 1290 Infinity LC with UV detection with the Agilent 1290/6410 Infinity LC/MS/MS, using an Agilent ZORBAX RRHD Eclipse Plus C18 3.0 mm × 100 mm, 1.8-µm column.

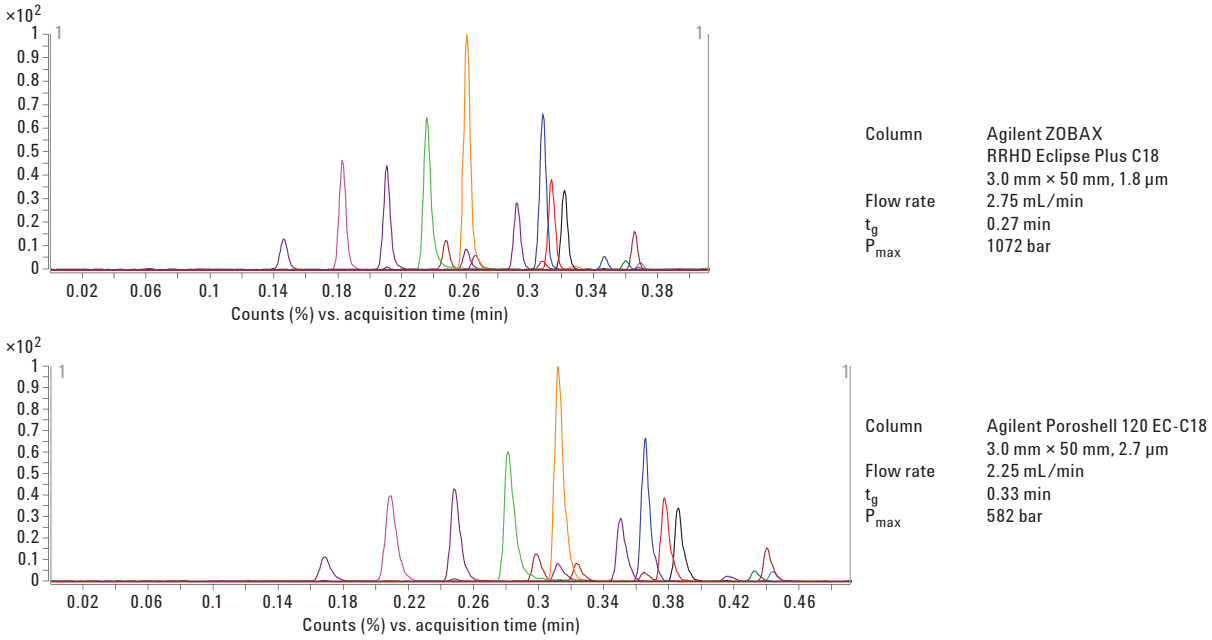

Figure 6. High-throughput methods with an Agilent 1290/6410 Infinity LC/MS/MS. (Note that these analyses were performed for demonstration purposes only, as it is not recommended to use such high flow rates with MS detection.)

Agilent Columns on a non-Agilent Instrument

Agilent's RRHD columns can be run not only with the Agilent 1290 Infinity LC, but also with a non-Agilent UHPLC system. The overall analysis is similar, but some slight modification would help resolve the last peak, ibuprofen. Smaller id capillary tubing in the non-Agilent UHPLC is probably the cause of increased system pressure and reduced delay volume, causing all peaks to elute earlier, as seen in Figure 7. The separation on the two instruments could be made more similar by adding either an isocratic hold to the beginning of the method on the non-Agilent LC or by adding additional capillary tubing to the non-Agilent LC to delay the gradient.

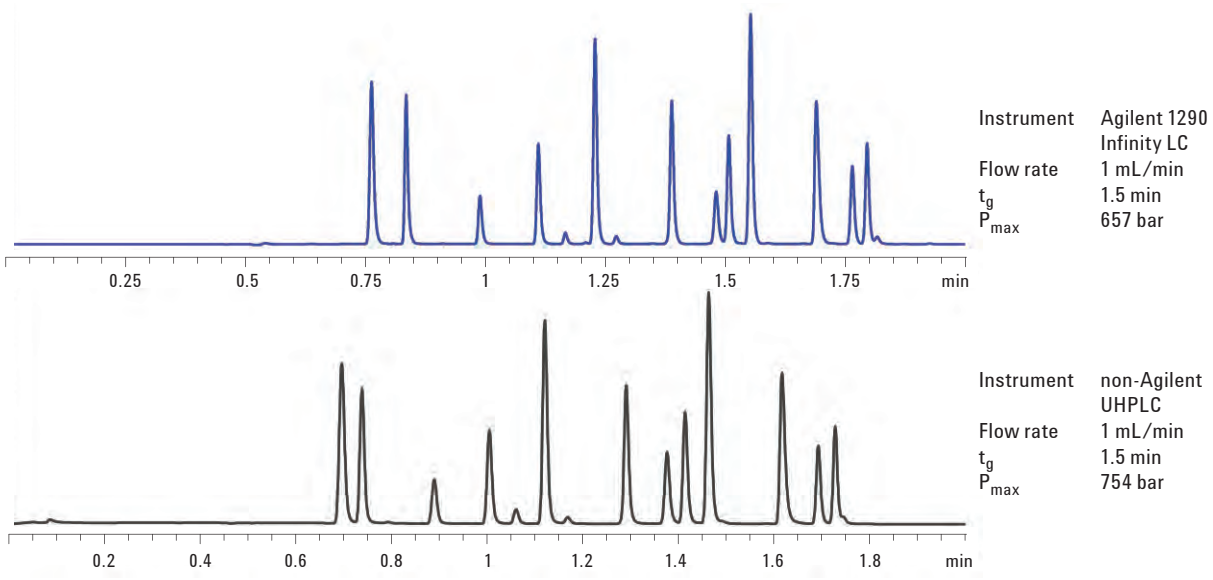

Figure 7. Using an Agilent ZORBAX RRHD Eclipse Plus C18 3.0 mm × 100 mm, 1.8-µm column to compare separation performance on an Agilent 1290 Infinity LC and a non-Agilent UHPLC.

Conclusions

Agilent ZORBAX columns offer the same selectivity across multiple particle sizes, including 5, 3.5 and 1.8 µm. The Agilent Poroshell 120 column has similar selectivity to the Agilent ZORBAX columns, with efficiency close to that provided by 1.8-µm particles, while generating substantially lower pressure due to its larger 2.7-µm particles. Scaling gradient methods according to column volume preserves selectivity when transferring methods, and methods can be easily transferred from Agilent 1200 Series RRLC systems to Agilent 1290 Infinity LC systems. Investigate ISET for more information on new technology from Agilent that makes this process easier.

Transferring methods to MS is easy, as it has no significant effect on the chromatography, other than a small increase in extra-column volume. Using MS detection delivers fast analyses because of its more selective nature when detecting co-eluting peaks.

Agilent ZORBAX RRHD columns, with their 1200 bar pressure limits, are easy to run on a non-Agilent UHPLC without significant method modification.

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

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Agilent Technologies

Agilent ZORBAX 300SB-C18 1.8 µm Rapid Resolution High Definition Columns for Proteins

Technical Overview

Introduction

Agilent ZORBAX RRHD 300SB-C18 1.8 µm is a new reverse phase media for UHPLC of proteins and peptides. The use of 1.8 µm particles in a column designed for UHPLC systems significantly reduces analysis time in HPLC, critical for increasing the efficiency of QC for protein primary structure analysis.

The eluents routinely employed for reverse phase analysis are acidic, containing trifluoroacetic acid or formic acid, which can limit the lifetime of many HPLC columns. However, by using StableBond technology it is possible to produce a 300Å pore-size media that is stable under acidic conditions, to provide the robust reproducible separations required for protein analysis.

Intact protein analysis

Short 50 mm columns are used to separate and resolve intact proteins. In these examples, different flow rates, from 0.5 mL/min to 1.0 mL/min, and temperatures, from 60 °C to 50 °C, are used to demonstrate the effect of flow rate on efficiency. As expected, higher flow rates improve efficiency.

The effect of three different flow rates is shown in Figures 1, 3, and 5. Figure 7 shows the separation at 50 °C, with a slight improvement in separation at this temperature which is below the boiling point of the solvent. Base line separations are given in Figures 2, 4, 6 and 8.

Conditions

Column	Agilent ZORBAX RRHD 300SB-C18, 2.1 x 50 mm, 1.8 µm (p/n 857750-902)
Sample	Sigma Protein Standards (ribonuclease A, cytochrome C, transferrin, myoglobin)
Sample conc	1 mg/mL
Inj vol	5 µL
Eluent	A, 0.1% TFA in water; B, 0.085% TFA in ACN
Gradient	20% B 0.5 min, 20-60% B 2 min, 60-90% B 0.5 min, 90% B 1 min, 90-20% B 0.1 min, 20% B 0.9 min
Temp	as indicated
Flow rate	as indicated
Pressure	as indicated
System	Agilent 1290 Infinity LC

Agilent Technologies

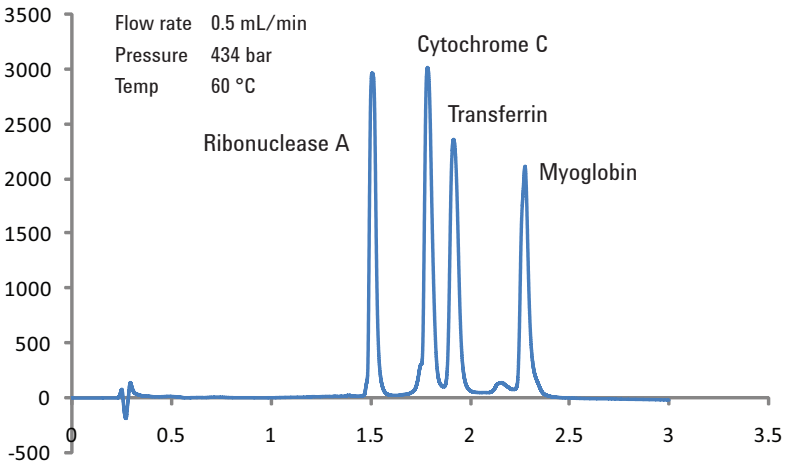

Figure 1. Protein standards on an Agilent ZORBAX RRHD 300SB-C18, 2.1 x 50 mm, 1.8 µm column at 0.5 mL/min.

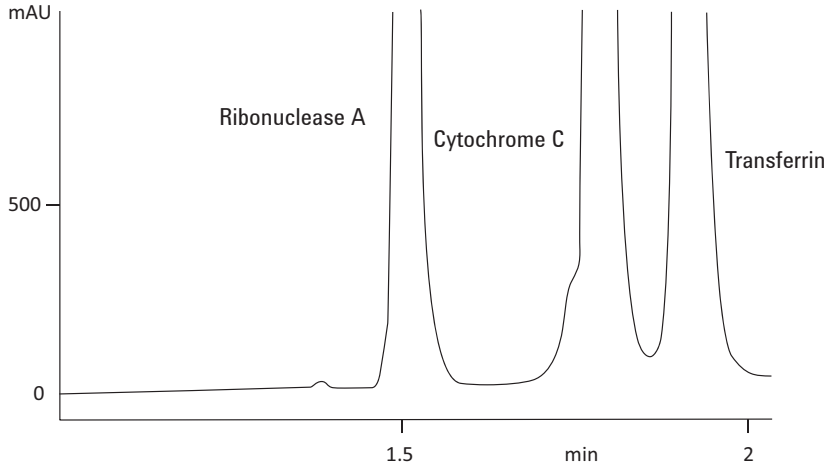

Figure 2. Base line expansion of Figure 1.

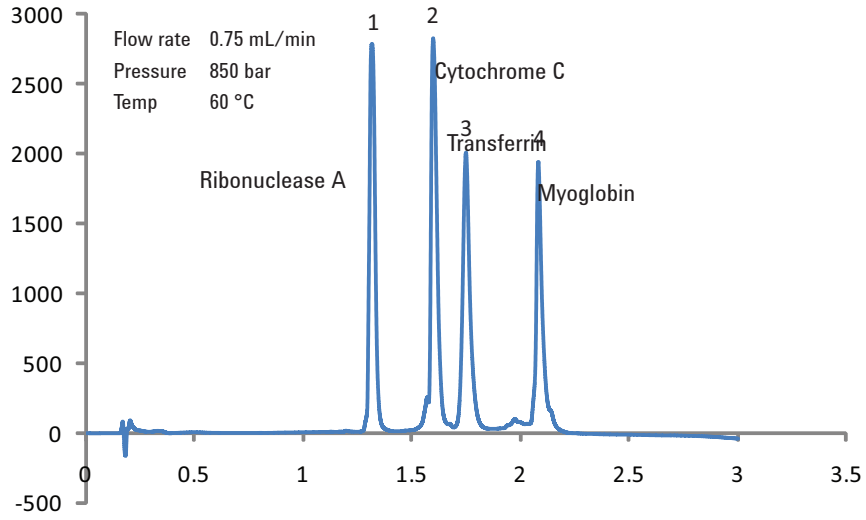

Figure 3. Protein standards on Agilent ZORBAX RRHD 300SB-C18, 2.1 x 50 mm, 1.8 µm at 0.75 mL/min.

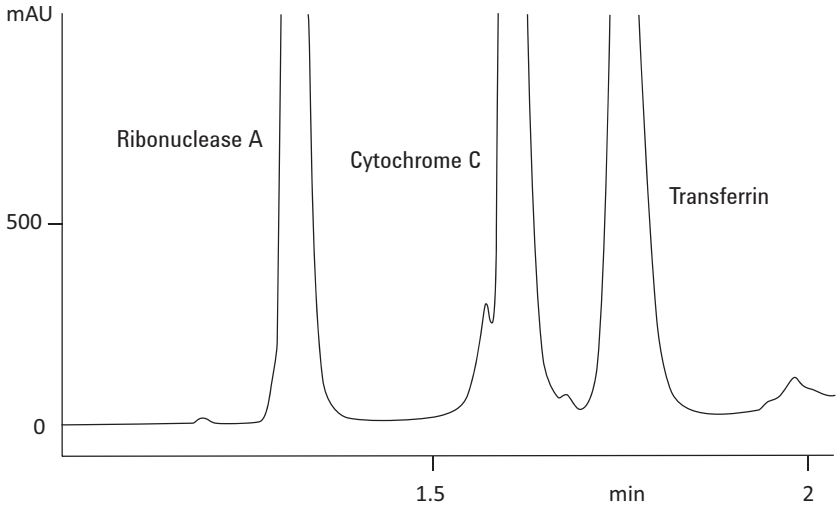

Figure 4. Base line expansion of Figure 3.

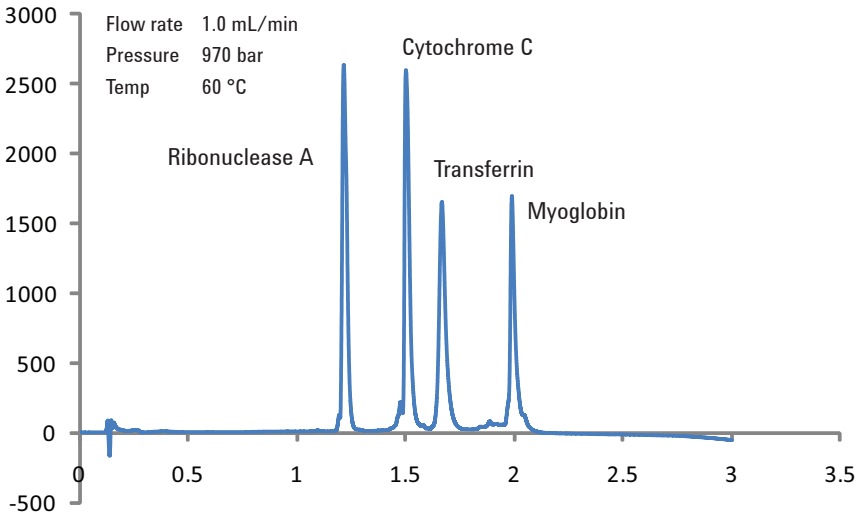

Figure 5. Protein standards on an Agilent ZORBAX RRHD 300SB-C18, 2.1 x 50 mm, 1.8 µm column at 1.0 mL/min.

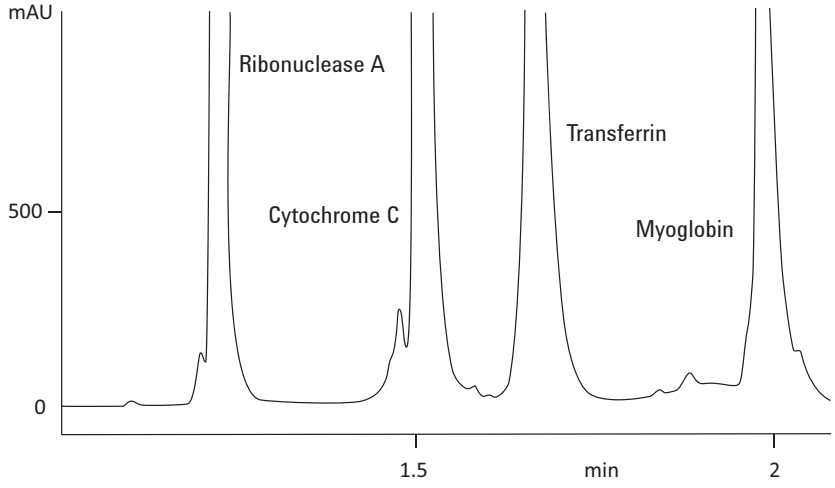

Figure 6. Base line expansion of Figure 5.

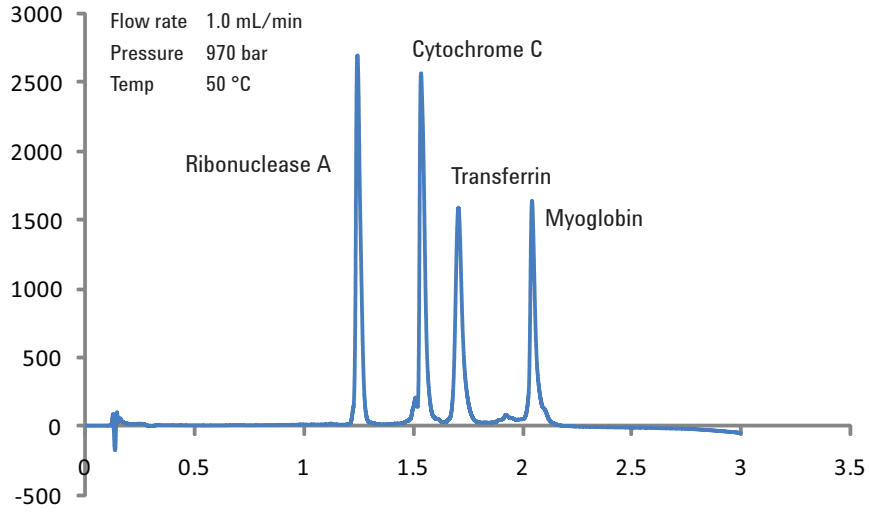

Figure 7. Protein standards at reduced temperature on an Agilent ZORBAX RRHD 300SB-C18, 2.1 x 50 mm,1.8 µm column.

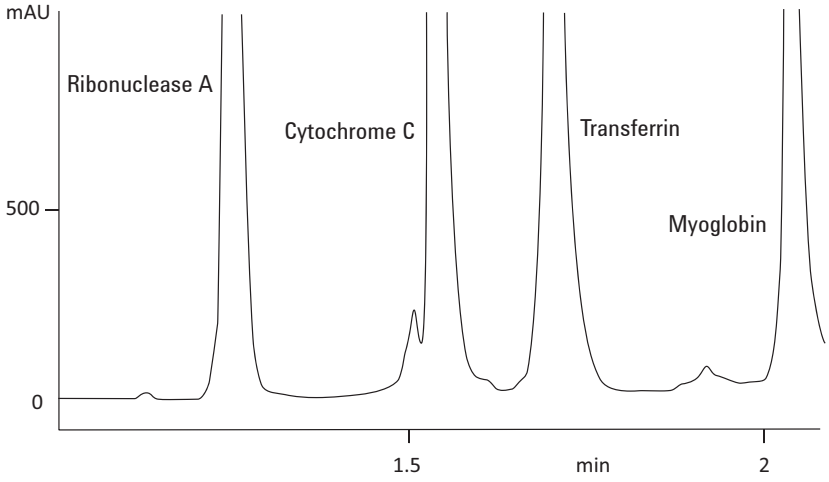

Figure 8. Base line expansion of Figure 7

Protein digest analysis

The same ZORBAX packing is used in longer 100 mm columns, for the analysis of peptide components and enzymatically digested proteins to identify changes in the primary amino acid sequence and amino acid modifications (Figure 9). Reproducibility of the column after 30 runs is shown in Figure 10.

Conditions

Column	Agilent ZORBAX RRHD 300SB-C18, 2.1 x 100 mm,1.8 µm (p/n 858750-902)
Sample	Protein digest
Sample conc	1 mg/mL
Inj vol	5 µL
Eluent	A, 0.1% TFA in water; B, 0.085% TFA in ACN
Gradient	20% B 1 min, 2-45% B 8.8 min, 45-95% B 0.2 min, 95% B 2 min, 98-2% B 0.2 min, 210% B 1.8 min
Temp	50 °C
Flow rate	0.5 mL/min
Pressure	~640 bar
System	Agilent 1290 Infinity LC

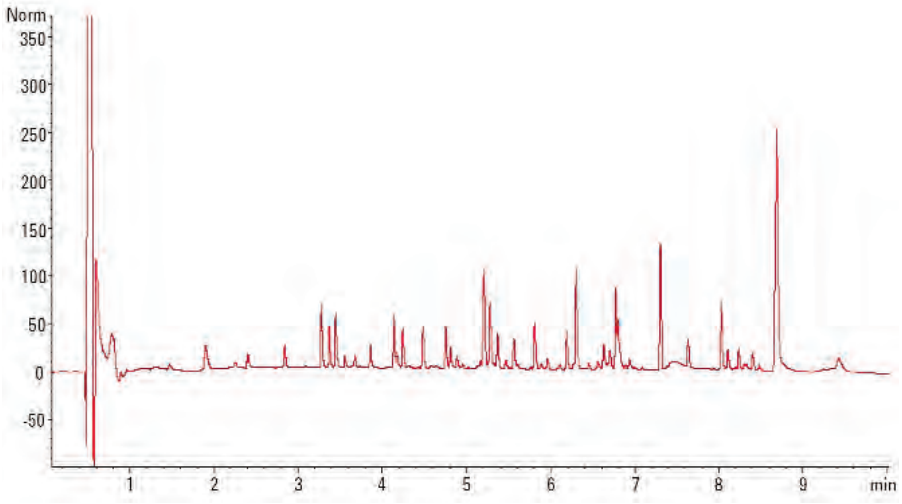

Figure 9. Peptide digest separation on an Agilent ZORBAX RRHD 300SB-C18, 2.1 x 100 mm, 1.8 µm column.

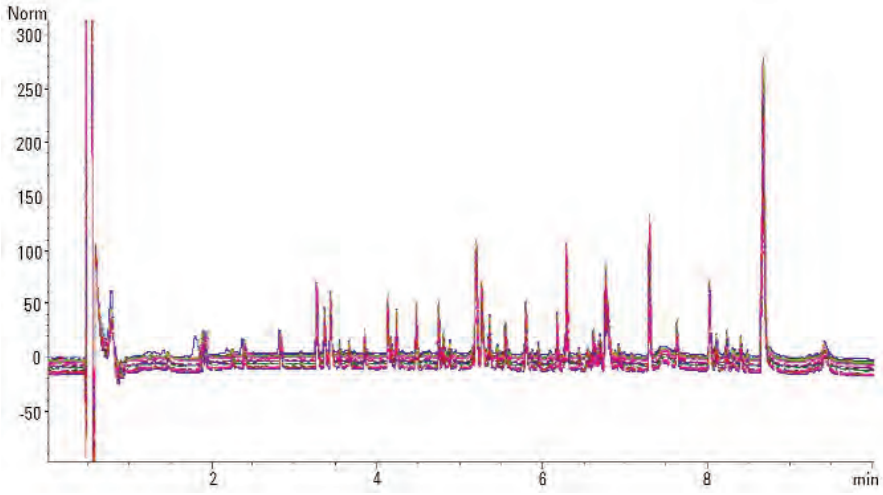

Figure 10. Overlaid chromatograms of 30 runs of a protein digest on an Agilent ZORBAX RRHD 300SB-C18, 2.1 x 100 mm, 1.8 µm column.

Agilent ZORBAX columns for proteins

Analyzing intact biotherapeutic proteins and peptide aliquots is fast and straightforward with Agilent ZORBAX RRHD 300SB-C18 1.8 µm columns. The column's rapid resolution high definition technology permits high pressure UHPLC, while the StableBond 300Å pore-sized particles are robust when analysis requires acidic conditions. Reproducibility is excellent, with good resolution, asymmetry and efficiency. The columns are ideal for protein primary sequence analysis.

Look at the Agilent Literature Library on www.agilent.com for a comprehensive range of application notes and technical overviews to help you get the best from your Agilent HPLC and UHPLC columns and instruments.

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Agilent Technologies

Transfer of Methods between Poroshell 120 EC-C18 and ZORBAX Eclipse Plus C18 Columns

Technical Overview

Introduction

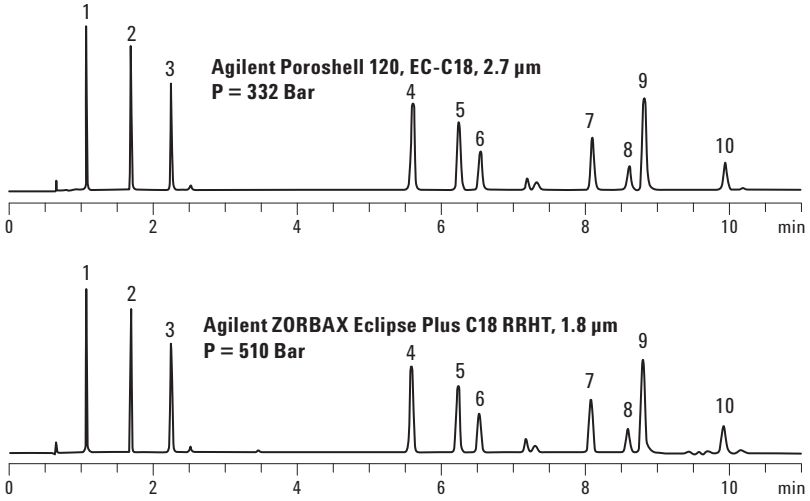
The developement of superficially porous particles has led to the possibility of method transfer from larger 5-µm totally porous particles, as well as from sub-2-µm totally porous particles. One of the benefits of transferring from larger particle columns is significant time savings, as the superficially porous particles are optimally run at a faster flow rate achieving similar resolution with a much shorter column length [1–4]. The high efficiency of superficially porous particles is similar to sub-2-µm totally porous particles because of the short mass transfer distance and substantially narrower particle size distribution. Transferring methods from totally porous sub-2-µm columns may also be desirable. Many development laboratories have chosen to use sub-2-µm columns. However, in some cases the higher operating pressure required of sub-2-µm methods may not be transferable to all HPLC systems. In many cases methods using sub-2-µm columns can be directly transferred to superficially porous particle columns, without adjustment. This is particularly true when columns like the Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18 are manufactured to have similar bonding chemistries and use similar retention mechanisms. Additionally, superficially porous particle columns can perform the same analysis as sub-2-µm columns, while generating less backpressure. This allows analysts to increase flow rates for higher throughput, or to increase column length to enhance resolution without exceeding the system pressure limits.

One asset of the Agilent ZORBAX family of HPLC columns is the scalability of methods between particle sizes. This allows a quick and reliable transfer of methods from method development to preparative lab and high throughput analysis.

Agilent Technologies

Several recent comparisons of Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18 have shown very similar chromatography. Poroshell 120 was designed to deliver 90 % of the efficiency of sub two micron columns such as Eclipse Plus C18 at approximately 60 % of the pressure. Superficially porous particles found in Poroshell 120 have the low pressure benefits of larger particles while achieving the performance of sub two micron particles.

Examples of this chromatographic similarity are shown using environmental phenols in Figure 1 with 0.1 % Formic acid and in Figure 2 in the analysis of soft drink additives using 10 mM ammonium acetate pH 4.8. In both cases, the retention order of the compounds are the same. The similarity of these two examples leads to the larger question, how similar are Poroshell 120 EC-C18 and Eclipse Plus C18, in terms of selectivity over a wider range of operating conditions and with a larger set of compounds including acids bases and neutral materials.

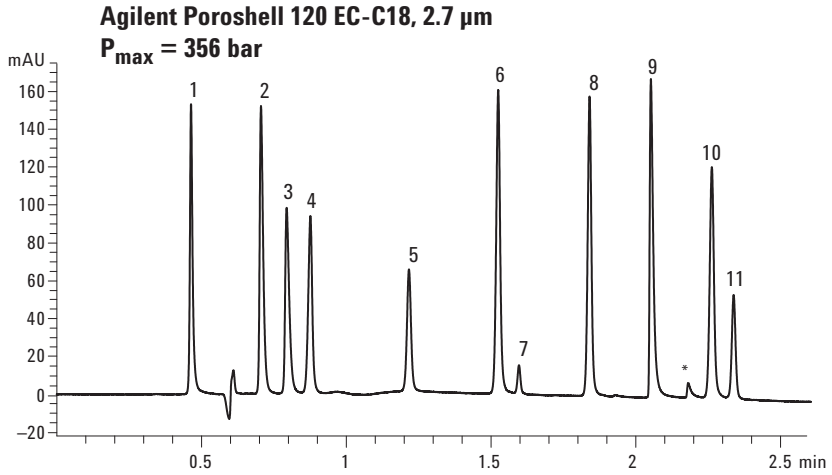


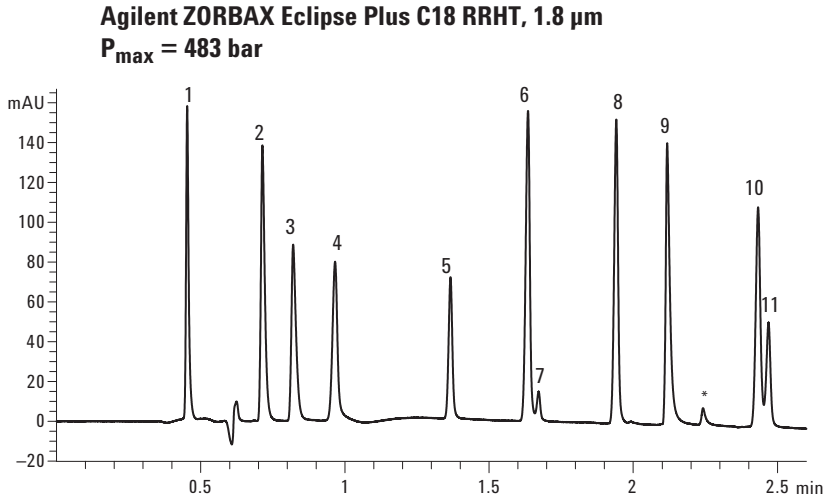
Conditions

Columns	Agilent Poroshell 120 EC-C18, 4.6 mm × 100 mm, 2.7 µm Agilent p/n 689975-902 Agilent ZORBAX Eclipse Plus RRHT C18, 4.6 mm × 100 mm, 1.8 µm Agilent p/n 959964-902
Mobile phase	A: 0.1% Formic acid B: MeCN + 0.1% Formic acid
Temperature	40 °C
Detection	275 nm
Injection volume	10 µL
Flow	2 mL/min
Initial	8% B, 10 min 30% B

1. Hydroquinone	6. o-cresol
2. Resorcinol	7. 2-Nitrophenol
3. Catechol	8. 2,3 Dimethyl phenol
4. 4-Nitrophenol	9. 2,5 Dimethyl phenol
5. p-cresol	10. 1-Naphtol

Figure 1. Comparison of Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18 using acetonitrile and formic acid mobile phase for the analysis of environmental phenols.





Conditions

Columns	Agilent Poroshell 120 EC-C18, 3.0 mm × 100 mm, 2.7 µm Agilent p/n 695975-302 Agilent ZORBAX Eclipse Plus C18 RRHT, 3.0 mm × 100 mm, 1.8 µm Agilent p/n 959964-302
Mobile phase	A: 20 mM Ammonium acetate, pH 4.80 B: Acetonitrile
Gradient	14% B at t_0, ramp to 52% B in 2.1 min
Flow rate	0.851 mL/min
Temperature	30 °C

1. Ascorbic Acid
2. Acesulfame K
3. Saccharin
4. p-Hydroxybenzoic Acid
5. Caffeine
6. Benzoic Acid
7. Aspartame
8. Sorbic Acid
9. Quinine
10. Dehydroacetic Acid
11. Methylparaben

* Quinine Impurity

Figure 2. Comparison of Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18 using acetonitrile and ammonium acetate mobile phase for the analysis of soft drink addities.

Experimental

Method development is often based upon the use of a generic gradient. Using a short Agilent Poroshell 120 EC-C18, 4.6 x 50 mm column, several different mobile phases can be quickly evaluated. The generic gradient used in this work is run at 2.0 mL/min, starts at 5% organic and increases to 95% organic over 2 min and holds at this concentration for 1 min. Mass spectrometer compatible mobile phases consisting of volatile buffers such as ammonium formate buffer and ammonium acetate buffer are used. These buffers were prepared by dissolving sufficient ammonium formate or ammonium acetate in water to produce 10 mM solutions and titrating the solutions to the desired pH with the appropriate concentrated acid. The pH of these buffers covers a range between 3 and 6.5.

An Agilent 1200 Method Development Solution LC system was used for this work:

- G1312B Binary Pump SL
- G1367D Automatic Liquid Sampler (ALS) SL
- Two G1316C Thermostatted Column Compartments (TCC) SL
- G1315C Diode Array Detector (DAD) SL, using a G1315-60024 micro flow cell (3-mm path, 2-µL volume)
- ChemStation version B.04.01 was used to control the HPLC and to process the data.

Correlation data was calculated and plotted using Microsoft Excel 7.0.

Four Agilent Poroshell 120 EC-C18 columns were used in this work:

- Agilent Poroshell 120 EC-C18, 4.6 mm × 50 mm, 2.7 µm p/n 699975-902
- Agilent Poroshell 120 EC-C18, 3 mm × 100 mm, 2.7 µm p/n 695975-302
- Agilent ZORBAX Eclipse Plus C18, 4.6 mm × 50 mm, 1.8 µm p/n 959943-902
- Agilent ZORBAX Eclipse Plus C18, 3 mm × 100 mm, 1.8 µm p/n 959964-302

Table 1 summarizes the list of compounds studied for this work. These compounds were prepared in water or 50/50 water/acetonitrile and injected individually.

Table 1. Sixty-six Compounds Including Acids, Bases and Neutrals Prepared in 50/50 MeCN/Water and Injected onto 4.6 x 50 mm Columns Individually

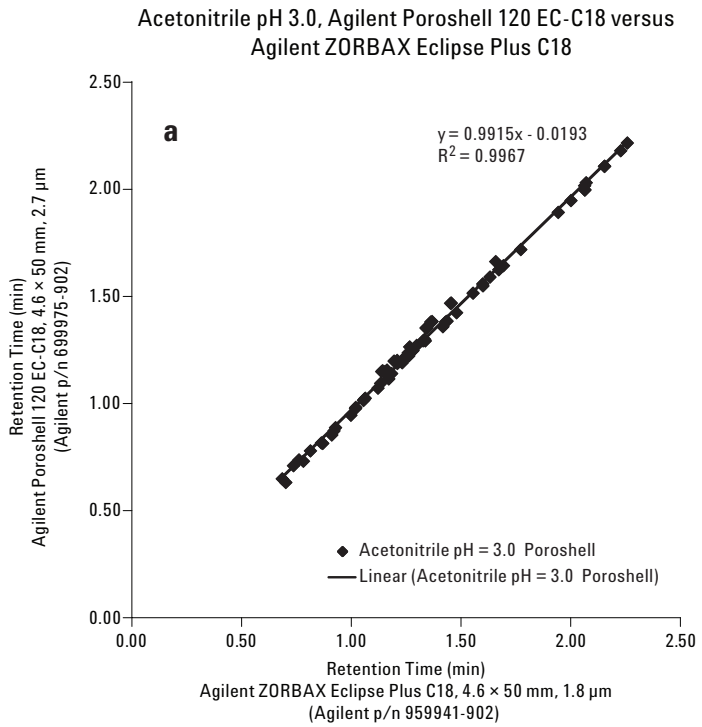
List of tested compounds	
furazolidone	phenacetin
chloramphenicol	acetanilide
impramithue	phenol
norethindrail	resorcial
cortisone acetate	hydroquinone
chloramphenicol	4 nitro phenol
busirone hydrochloride	o cresol
benzocaine	1 napthol
pyrimethamine	imipramine hydrochloride
sulfaquinoxaline	3 4 dihydroxy l phenyl alanine
sulfamonomethoxine	dl phenyalanine
nimopidin	ephedrine hydrochloride
sulfadimethoxine	loperamide
sulfamethoxazole	dibenzofuran
sulfachloropyridazine	procaine hydrochloride
sulfamethoxypyridazine	exonazole nitrate
sulfamethizole	gembigrozil
sulfamerazine	beta estradiol
sulfathiazole	metoprolol
sulfadiazine	protriptyline
benzaldehyde	hydroxy sophthalic
phenanthrene	flufenamic acid
biphenyl	pramoxine hydrochloride
acenaphthene	naproxen
methoxy naphthalene	diphenhydramine
dimethoxy benzene	diflunisal
alpha hydroxyprogesterone	nisoldipin
progesterone	diclofenac
prednisolone	hydrocortisone
deoxycorticosterone	procainamide hydrochloride
chlorphenamine	lidocaine
berberine	terfenaine
chlortetracycline hydrochloride	chlorpheniramine maleate

Discussion

Differences in column performance have been studied by many including Wilson, Nelson, Gilroy, Dolan, Snyder and Carr [5,6]. The United States Pharmacopeia lists many columns [7] and a tool to determine how interchangeable columns may be. Characteristics such as silica chemistry and bonding can change selectivity. Silanol activity affects peak shape dramatically through secondary interactions. It also can affect selectivity through H-bonding or ion-exchange. These effects become more pronounced at higher pH than at lower pH [8]. Both Agilent ZORBAX Eclipse Plus C18 and Agilent Poroshell 120 EC-C18 Columns are made from silica produced by Agilent at the same facility that makes the final columns. Both are intended to be highly inert columns and have been designed to yield excellent peak shape with basic compounds. In addition to the effect of pH, silanol activity can also be affected by differences in solvent. Methanol is an H-bonding solvent that has weaker elution strength than aprotic acetonitrile [10]. By choosing a wide range of conditions, it is more likely that differences in selectivity will be revealed.

Figure 3 shows similar retention of 66 compounds on Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18 columns using a generic gradient analysis with a variety of compounds from different chemical classifications. The high correlation coefficient (R^2) indicates a high degree of similarity between the interactions involved in the separation on the two Agilent C18 columns, while a slope of approximately 1 implies similar interaction strengths [9,10].

Generic Gradients using Acetonitrile, Buffered with 10 mM Ammonium Formate or Ammonium Acetate between pH 3 and 6.5



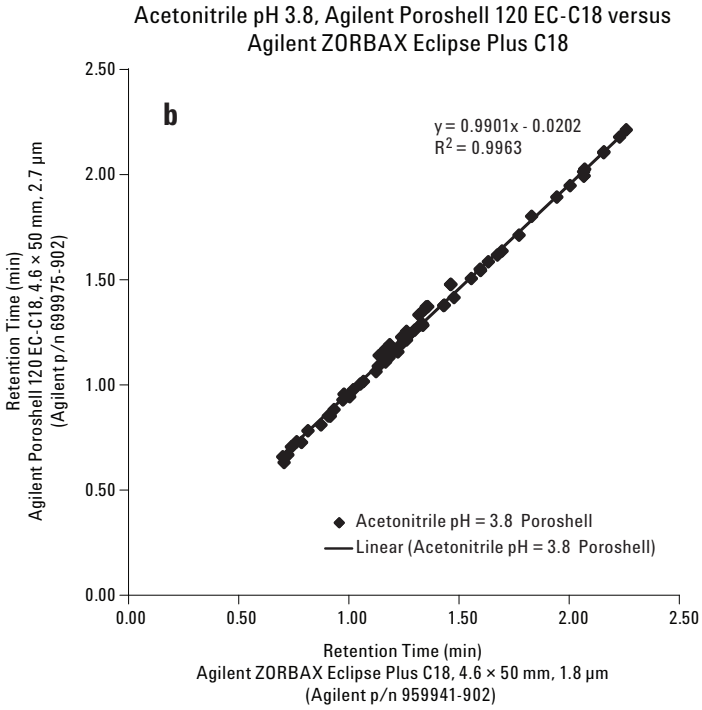
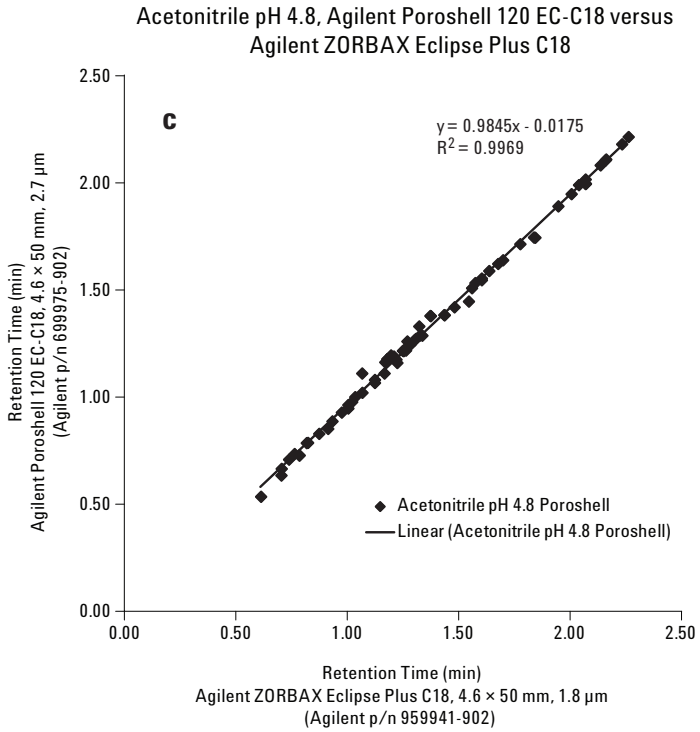
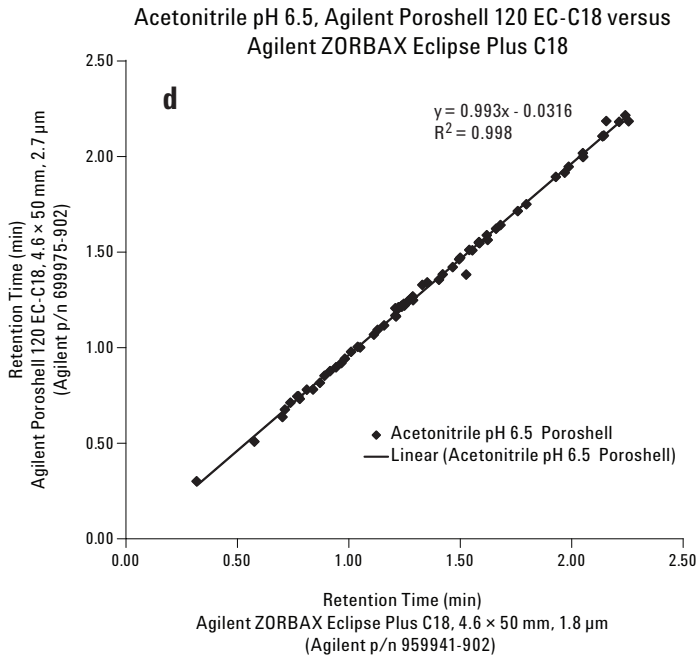


Figure 3. Scatter plot of retention time of 66 compounds on Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm versus Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 1.8 µm. (continued)





Conditions

Mobile phase	A: 10 mM Buffer B: Organic (ACN)
Gradient	5% B at t_0, ramp to 95% B in 2 min, hold 95% B for 1 min
Flow rate	2 mL/min
Sample	1 µL of 1 mg/mL standard in H_2O or H_2O/ACN

Figure 3. Scatter plot of retention time of 66 compounds on Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm versus Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 1.8 µm.

Figure 4 shows scatter plots of the retention times of 66 compounds on Agilent Poroshell 120 EC-C18 versus Agilent ZORBAX Eclipse Plus C18 columns at different pH values between 3 and 6.5 in acetonitrile. Figure 2 shows scatter plots at different pH values between 3 and 6.5 in methanol. The slope and R^2 values for these combinations are summarized in Table 2. As illustrated, the correlation between the two plots is quite good. While retention times sometimes change with the ionic compounds, the changes are proportional on both columns. A slight difference in the slopes of the correlation curves may indicate some difference in H bonding interaction between Agilent ZORBAX Eclipse Plus C18 and Agilent Poroshell 120 EC-C18 when comparing the acetonitrile and methanol data (slope of 0.99 and slope of 1.01), but this is not likely to cause any problems in method transfer and is only measureable given the large number of experiments and compounds studied.

Generic Gradients using Methanol, Buffered with 10 mM Ammonium Formate or Ammonium Acetate between pH 3 and 6.5

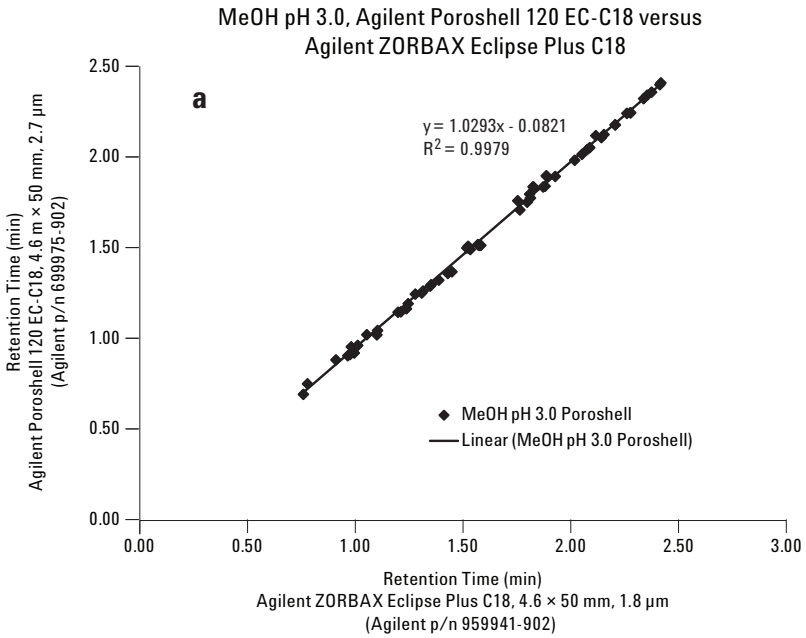
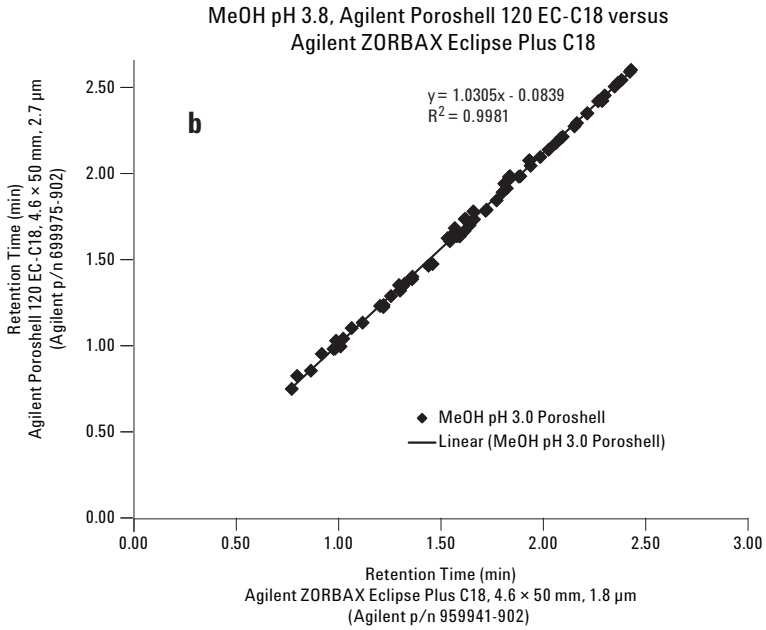


Figure 4. Scatter plot of retention time of 66 compounds on Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm versus Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 1.8 µm. (continued)



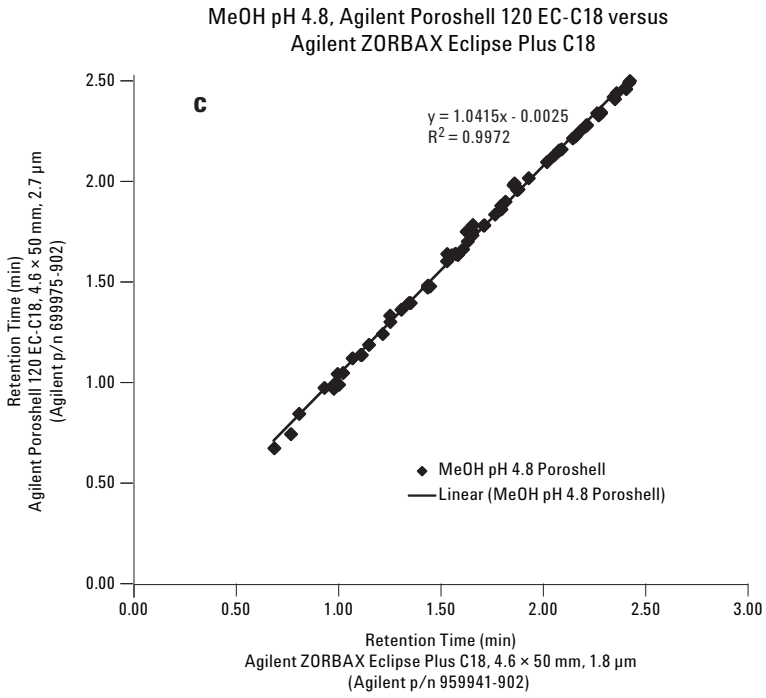
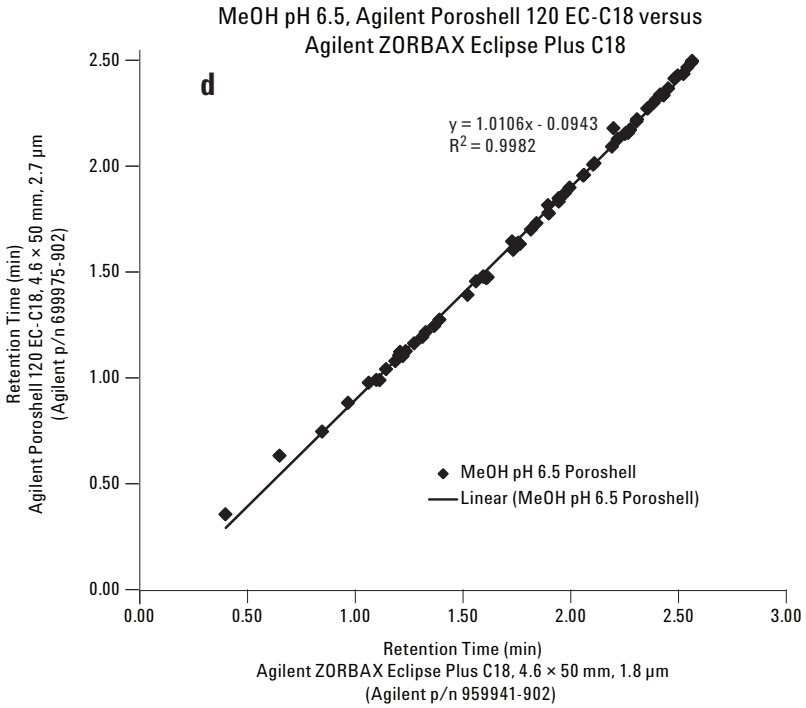


Figure 4. Scatter plot of retention time of 66 compounds on Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm versus Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 1.8 µm. (continued)



Conditions

Mobile phase:	A: 10 mM Buffer
	B: Organic (MeOH)
Gradient:	5% B at t_0, ramp to 95% B in 2 min, hold 95% B for 1 min
Flow rate:	2 mL/min
Sample:	1 µL of 1 mg/mL standard in H_2O or H_2O/ACN

Figure 4. Scatter plot of retention time of 66 compounds on Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm versus Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 1.8 µm.

Table 2. Summary of Correlation Data

Acetonitrile	Methanol
a. pH =3.0 y = 0.9915x -0.0193 R² = 0.9967	a. pH =3.0 y = 1.0293x -0.0821 R² = 0.9979
b. pH =3.8 y = 0.9901x -0.0202 R² = 0.9963	b. pH =3.8 y = 1.0305x -0.0839 R² = 0.9981
c. pH =4.8 y = 0.9845x -0.0175 R² = 0.9969	c. pH =4.8 y = 1.0415x -0.002 R² = 0.9972
d. pH =6.5 y = 0.993x -0.0316 R² = 0.998	d. pH =6.5 y = 1.0106x -0.0943 R² = 0.9982

Another benefit of the Agilent Poroshell 120 columns over sub-2-µm columns is lower operating pressure. The pressure is related to the particle size of the column; larger particles naturally yield lower pressure than smaller particles. In addition to the particle size, the pressure generated inside a column is dependent upon several other factors including solvent linear velocity, and solvent viscosity at a given composition and temperature. While this is a gradient study, the most viscous solvent composition in this study occurs between 40/60 and 50/50 methanol/water. At 25 °C the viscosity of this solvent is 1.62 cP. The most viscous acetonitrile composition is 10/90 acetonitrile/water. At 25 °C the viscosity of this solvent is 1.01 cP [11]. As indicated in the references the viscosity of the solutions is inversely dependent on the temperature. The pressure verses linear velocity graphs for Agilent Poroshell 120 EC-C18 columns and Agilent ZORBAX Eclipse Plus C18 1.8 µm columns are shown for both solvent pairs as Figures 5 and 6. In this case 100 mm columns are used. As stated earlier, this benefit can allow the use of longer columns achieving the same pressure (and larger injection volumes), or higher flow rates.

Differences in selectivity are more likely to occur in cases where the pore size difference becomes more important, typically for compounds between 1500 and 2500 mw. Compounds such as PAHs that involve shape selectivity may also be problematic.

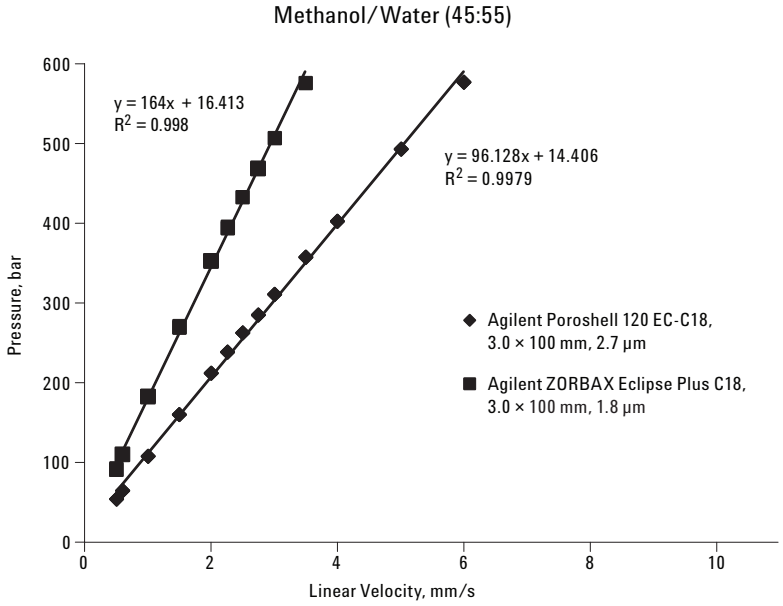


Figure 5. Pressure measured at varied linear velocities indicates lower operating pressure for Agilent Poroshell 120 than an a 1.8 µm column of similar length.

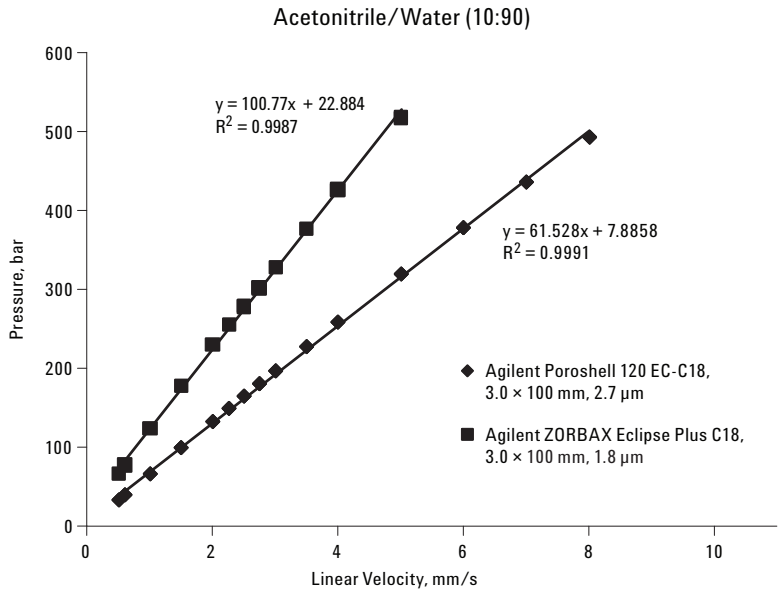


Figure 6. Pressure measured at varied linear velocities indicates lower operating pressure for Agilent Poroshell 120 than an a 1.8 µm column of similar length.

Conclusions

This work has demonstrated the equivalence of selectivity between Agilent ZORBAX Eclipse Plus C18 and Agilent Poroshell 120 EC-C18 columns across a wide range of pH and mobile phase conditions. Both column chemistries are manufactured using similar materials with similar proprietary bonding chemistries. Both columns were designed to achieve excellent peak shapes for bases without sacrificing low pH peak shape and performance for other compounds. The benefit of using Agilent Poroshell 120 EC-C18 columns is high efficiency at a lower backpressure. Based on this work, it is expected that if the need arises methods developed on Agilent ZORBAX Eclipse Plus C18 columns can be reliably transferred to Agilent Poroshell 120 EC-C18 columns and conversely with low risk.

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5990-6588EN

Agilent Technologies

Maximizing efficiency using Agilent Poroshell 120 columns

100000 plates in less than 5 min using coupled column technology

Application Note

Food, Environmental, Chemical, Pharmaceutical

Author

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Edgar Naegele

Agilent Technologies

Waldbronn, Germany

Abstract

Columns based on superficially porous technologies are an alternative to sub-2-µm particle based columns. The combination of these columns with the Agilent 1290 Infinity LC system produces high efficiency separations. Agilent Poroshell 120 columns offer:

- Lower back pressure
- Highest efficiency
- Comparable volume capacity

Introduction

Recently, sub-2-µm particle columns have gained a lot of interest, due to their high efficiency. They can be used at higher flow rates than those evaluated by the van Deemter equation. The loss in efficiency at higher flow rates is minor in comparison to the efficiency at the optimum flow rate. Run times and cycle times can be shortened and results obtained faster.

The drawback of these columns is that significantly higher back pressures are obtained, due to the small particle sizes. In many cases, especially for long sub-2-µm columns, the LC instrumentation must allow back pressures of >400 bar.

The superficially porous particle technology offers an alternative for very high resolution analyses[1], because these columns show significantly less back pressure. The efficiency of these columns, compared to that of sub-2-µm particle columns is slightly lower. It is possible to obtain very high plate counts by coupling columns, due to less back pressure.

Agilent Technologies

This Application Note demonstrates that the coupling of three long Agilent Poroshell 120 columns results in extremely high efficiencies. It is also demonstrated that the back pressure can be kept below 400 bar, unless special LC equipment is available. In that case higher flow rates are possible to save analysis and equilibration time. Finally, a comparison was made between one 2.7 µm porous shell column and one sub-2-µm particle size column.

Experimental

Equipment

An Agilent 1290 Infinity LC system equipped with a binary pump, autosampler, thermostatted column compartment and diode-array detector with a 10-mm path length cell was used for the experiments.

An Agilent ZORBAX Rapid Resolution HT 4.6 mm × 150 mm, 1.8 µm column and an Agilent Poroshell 120, 4.6 mm × 150 mm,
2.7 µm column were used. These columns can be used up to 600 bar.

The ChemStation software revision B.04.02 was used.

Results and discussion

Potential benefits of superficially porous columns

Superficially porous column technology is based on particles with a solid core and a superficially porous shell. These particles consist of a 1.7-µm solid core with a 0.5-µm porous silica shell. In total, the particle size is about 2.7 µm. The 2.7 µm superficially porous particles provide 40–50% lower back pressure and 80–90% of the efficiency of a sub-2-µm totally porous particle. The superficially porous particles have a narrower particle size distribution than a totally porous particle. This results in a more homogeneous column and reduces diffusion in the column. At the same time the small particle and the porous shell allow for lower resistance to mass transfer. The result is higher flow rates without efficiency loss.[1,2]

Configuring the system

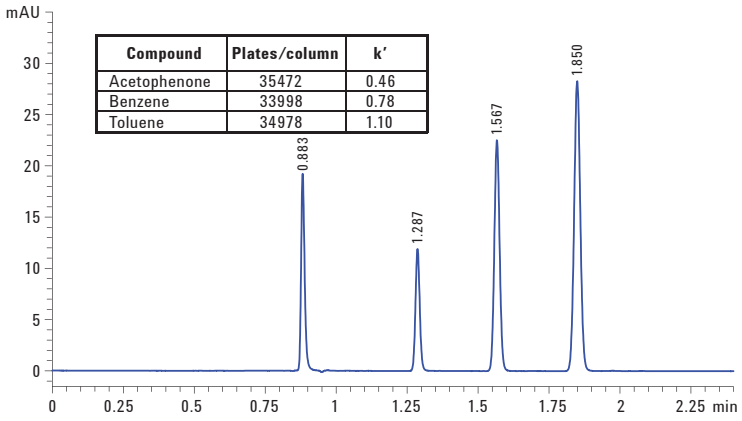
The following experiments evaluated the performance of the Agilent Poroshell 120 columns. The internal diameter was 4.6 mm and the column length 150 mm for all columns used.

- Evaluation of the plate number of a single column at 1.5 mL/min
- Evaluation of the plate number for three coupled columns at 1.5 mL/min
- Evaluation of the plate number for three coupled columns at higher flow rates
- Precision of retention times using isocratic and gradient conditions
- Comparison of a porous shell versus a sub-2-µm particle column

Column efficiency (plate number) is typically measured using isocratic conditions. For a symmetrical peak use the following equation to calculate the plate number (N):

$$N = 5.54\,(RT/W)^2$$

where RT is the retention time and W the peak width at half height.



Compound	Plates/column	k'
Acetophenone	35472	0.46
Benzene	33998	0.78
Toluene	34978	1.10

Chromatographic conditions:

Column:	Agilent Poroshell 120 SB-C18 150 mm × 4.6 mm, 2.7 µm
Sample:	Thiourea, Acetophenone, Benzene, Toluene
Mobile phase:	Water: ACN = 30:70
Flow rate:	1.5 mL/min
Injection volume:	1 µL
Column temperature:	50 °C
Detector:	DAD 254 nm/10, Ref 360/100 nm, 20 Hz, standard cell

Figure 1
Chromatogram to evaluate N for the Agilent Poroshell 120 150 mm × 4.6 mm column.

Evaluation of plate numbers for single column

The following compounds were used to evaluate the plate number for a single column: uracil, acetophenone, benzene and toluene.

The resulting chromatogram and evaluated plate numbers are shown in Figure 1.

The result was approximately 35000 plates/column for toluene under the chromatographic conditions specified.

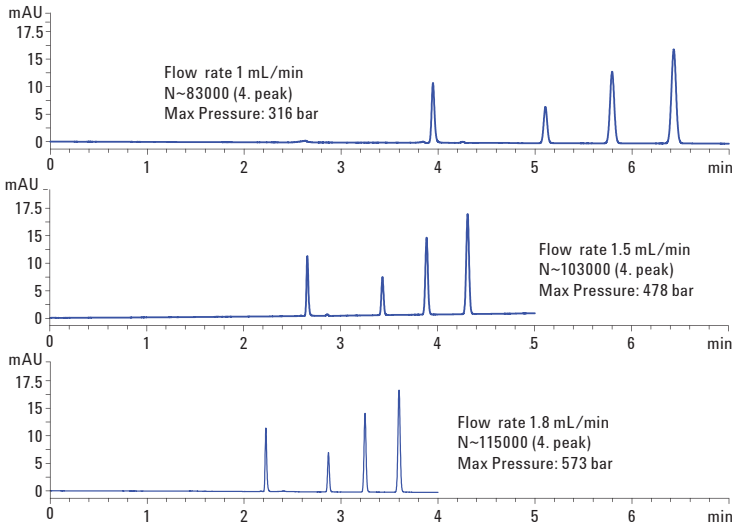
Evaluation of plate numbers for three coupled columns

The plate number for one column is approximately 35000 plates. The expectation is that three columns deliver a plate number of 105000 plates. Column coupling was done using stainless steel capillaries, 90 mm × 0.12 mm. Plate numbers were evaluated for different flow rates.

The resulting chromatograms are shown in Figure 2. If a 400-bar LC system is used, about 80000 plates can be obtained at 1 mL/min flow rate. However, higher flow rates and efficiencies can be obtained with this LC system, which allows pressures up to 1200 bars.

At 1.5 mL/min flow rate the obtained plate number of approximately 103000 plates is close to the expected value.

The best result for toluene with approximately 115000 plates was obtained at 1.8 mL/min with a retention time < 5 min (Table 1).



Chromatographic conditions:

Sample:	Thiourea, Acetophenone, Benzene, Toluene
Column:	Three coupled Agilent Poroshell 120 SB-C18, 150 mm × 4.6 mm, 2.7 µm columns
Mobile phase:	Water: ACN = 20:80
Flow rate:	1, 1.5, 1.8 mL/min
Injection volume:	1 µL
Column temperature:	60 °C
Detector:	DAD 254 nm/10, Ref 360/100 nm, 20 Hz, standard cell

Figure 2
Two chromatograms to evaluate N for three coupled Agilent Poroshell 120 150 mm × 4.6 mm columns at different flow rates.

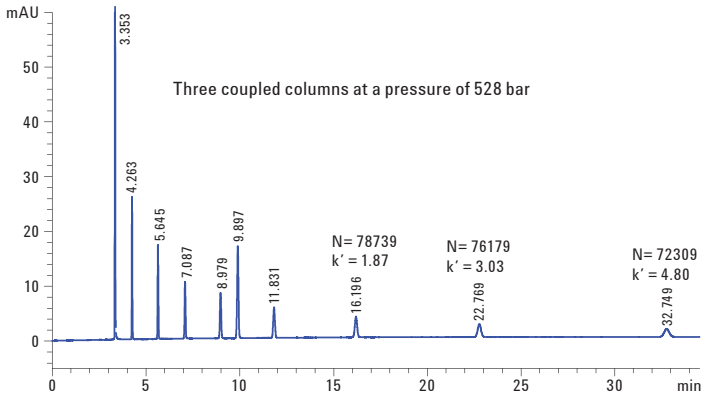
Compound	Plates	k'
Acetophenone	114120	0.29
Benzene	109931	0.46
Toluene	114800	0.62

Table 1
Plate numbers at 1.8 mL/min flow rate.

For higher k′ values good results are obtained using three coupled columns. A flow rate of 1.2 mL/min was used. (Figure 3)

Precision of retention times using isocratic conditions

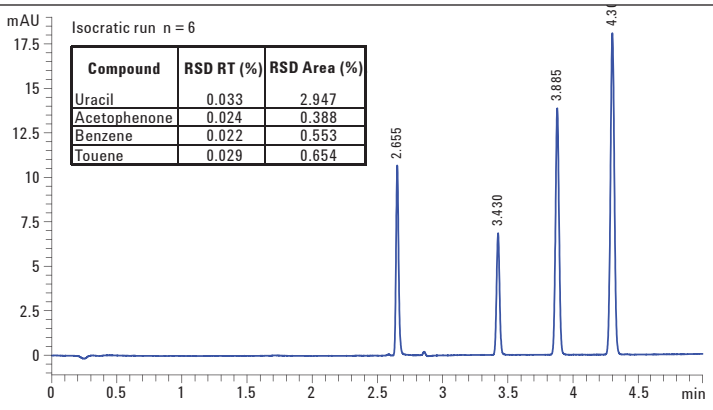
Precision for isocratic conditions at 1.5 mL/min was evaluated and results are shown in Figure 4 together with an overlay of six consecutive runs. The precision of retention times is < 0.034% RSD, and the precision for areas is < 0.66% RSD, except for uracil.



Chromatographic conditions:

Sample:	Thiourea+Test sample: Set of nine compounds, 100 ng/uL each, dissolved in water/ACN (65/35) 1. Acetanilide, 2. Acetophenone, 3. Propiophenone, 4. Butyrophenone (200 ng/µL), 5. Benzophenone, 6. Valerophenone, 7. Hexanophenone, 8. Heptanophenone, 9. Octanophenone
Column:	Three coupled Agilent Poroshell 120 SB-C18, 150 mm × 4.6 mm, 2.7 µm columns
Mobile phase:	ACN/Water 60/40
Column temperature:	60 °C
Flow rate:	1.2 mL/min
Detector:	DAD 254 nm/10 nm, ref 360/100 nm, 20 Hz, Standard cell

Figure 3
Plate numbers at higher k′ values for three coupled columns at 528 bar and 1.2 mL/min flow rate.



Chromatographic conditions:

Sample:	Uracil, Acetophenone, Benzene, Toluene
Column:	Three coupled Agilent Poroshell 120 SB-C18 150 mm × 4.6 mm, 2.7 µm columns
Mobile phase:	Water: ACN = 20:80
Flow rate:	1.5, mL/min
Injection volume:	1 µL
Column temperature:	60 °C
Detector:	DAD 254 nm/10, Ref 360/100 nm, 20 Hz, standard cell

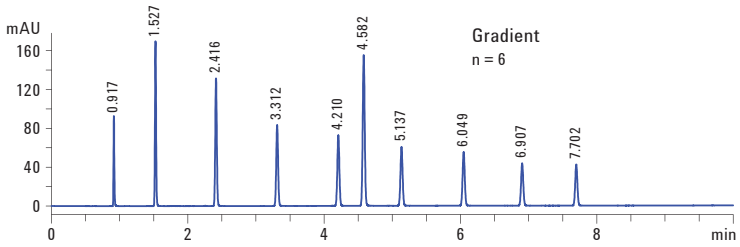
Figure 4
Overlay of six consecutive runs using isocratic conditions and precision data for retention times and areas.

Precision for retention times and areas using gradient conditions

The precision for gradient analysis was evaluated using a gradient from 35 to 95% in 10 min. The results and the overlay of six consecutive runs are shown in Figure 5.

Excellent precision was achieved for retention times of all compounds (RSD < 0.04%), except for Thiourea (Figure 5).

The RSDs for the areas of all compound peaks were less than 0.38% for a 1-µL injection.



Peak	RSD Retention time (%)	RSD Area (%)
Thiourea	0.092	0.372
1	0.020	0.238
2	0.038	0.255
3	0.033	0.211
4	0.029	0.186
5	0.027	0.227
6	0.023	0.194
7	0.018	0.183
8	0.017	0.251
9	0.017	0.167

Chromatographic conditions:

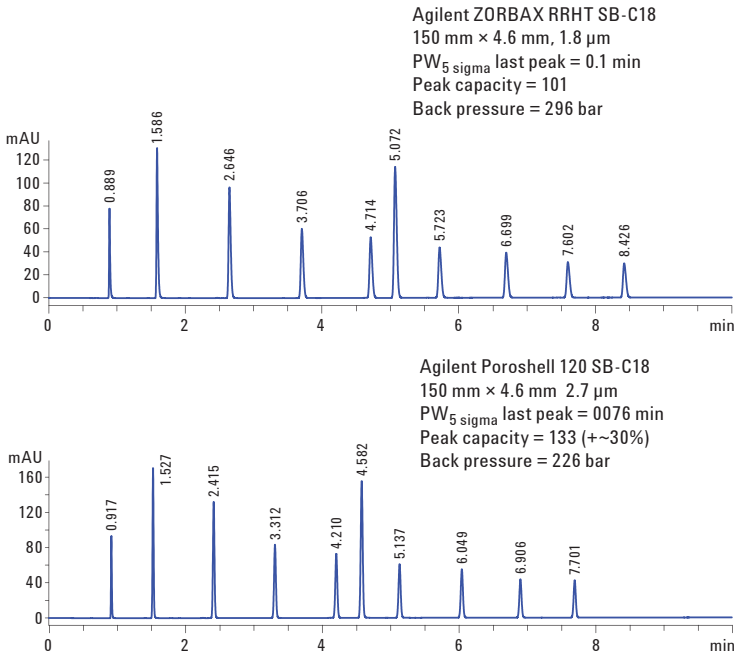
Sample:	Thiourea + Test sample: Set of nine compounds, 100 ng/µL each, dissolved in water/ACN (65/35) 1. Acetanilide, 2. Acetophenone, 3. Propiophenone, 4. Butyrophenone (200 ng/µL), Benzophenone, 6. Valerophenone, 7. Hexanophenone, 8. Heptanophenone, 9. Octanophenone
Column:	Agilent Poroshell 120 SB-C18, 150 mm × 4.6 mm, 2.7 µm
Mobile phase:	Water and ACN
Gradient:	At 0 min 35% ACN, at 10 min 95% ACN
Flow rate:	1.5 mL/min
Injection volume:	1 µL
Column temperature:	60 °C
Detector:	DAD 245/10 nm, Ref 400/100 nm, 20 Hz, standard cell

Figure 5
Overlay of 10 consecutive gradient runs and precision data for retention times and areas.

Comparison of the peak capacity of a porous shell column versus a sub-2-µm particle column

To illustrate the difference between porous shell and sub-2-µm columns, two 150 mm × 4.6 mm id columns were compared analyzing a set of 10 compounds (Figure 6).

The Agilent Poroshell 120 column shows shorter elution times, and smaller peak width, which results in a higher peak capacity for the porous shell column. The Agilent Poroshell 120 column shows 133 peaks with a higher peak capacity than the sub-2-µm column with a peak capacity of 101 peaks. This shows 30% higher efficiency for the Agilent Poroshell 120 column compared to the sub-2-µm column for the conditions used.



Chromatographic conditions:

Sample:	Thiourea + Test sample: Set of nine compounds, 100 ng/µL each, dissolved in water/ACN (65/35) 1. Acetanilide, 2. Acetophenone, 3. Propiophenone, 4. Butyrophenone (200ng/µL), Benzophenone, 6. Valerophenone, 7. Hexanophenone, 8. Heptanophenone, 9. Octanophenone
Column:	Agilent ZORBAX RRHT SB-C18, 150 mm × 4.6 mm, 1.8 µm, Agilent Poroshell 120 SB-C18, 150 mm × 4.6 mm, 2.7 µm
Mobile phase:	Water and ACN
Gradient:	0 min 35% ACN, 10 min 95% ACN
Flow rate:	1.5 mL/min
Injection volume:	1 µL
Column temperature:	60 °C
Detector:	DAD 245/10 nm, Ref 400/100 nm, 20 Hz, standard cell

Figure 6
Chromatograms of a "Phenone" mix analyzed on porous shell and sub-2-µm particle columns.

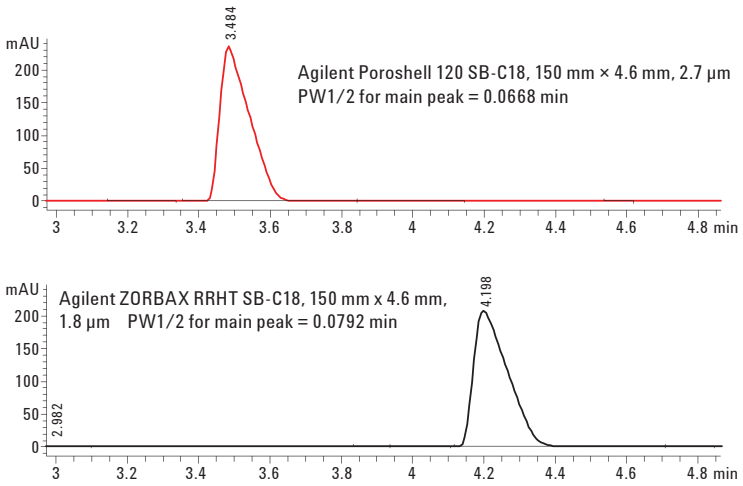
Comparison of volume capacity

To test whether porous shell columns have the same or lower volume capacity than column packed with 1.8 µm particles, a highly concentrated sample was injected. The injection volume was 10 µL and the concentration was approximately 20 µg in 10 µL (Figure 7).

No significant differences were observed for the main peak using the selected conditions. The peak width for the Poroshell 120 column was somewhat lower because in this case the peak eluted earlier. The peak width is typically smaller.

Comparison of signal-to-noise

Impurities in a pharmaceutical drug were analyzed to evaluate the signal-to-noise ratio. The impurities were present in a 0.02–0.03 percentage range. The chromatographic conditions are listed in Figure 7.



Chromatographic conditions:

Test Sample:	Tramadol 2,022 mL/mL containing impurities,
Column:	Agilent Poroshell 120 SB-C18, 150 mm × 4.6 mm, 2.7 µm Agilent ZORBAX RRHT SB-C18, 150 mm × 4.6 mm, 1.8 µm
Pump:	
Solvent A:	Water + 0.2 % TFA and Solvent B: ACN + 0.16% TFA
Gradient:	17 to 45% B in 5 min, Stop Time 7 min, Post time 3 min
Flow rate:	1.5 mL/min
Autosampler	
Injection volume:	10 µL
Wash time:	10 s
Thermostatted Column Compartment	
Temperature:	30 °C
DAD:	1290 270/10 nm, Ref. 360/100 nm, 20 Hz, Standard flow cell of 10 mm path length

Figure 7
Capacity comparison of porous shell and sub-2-µm columns; Injection volume 10 µL = 20 µg.

Figure 8 shows an overlay of a section of the complete chromatograms. The red trace represents the Poroshell 120 chromatogram and the black trace represents the sub-2-µm chromatogram.

In Table 2, the signal-to-noise calculations for both columns are combined. Impurity 1 and 2 were analyzed on the Poroshell 120 column and on the sub-2-µm column.

Conclusion

Porous shell columns represent a real alternative to sub-2-µm columns. The lower back pressure allows flow rates of 1 mL/min for a 4.6 mm x 150 mm, 2.7 µm column without exceeding the 400 bar limit. In this case, 35000 plates are achievable or more than 235000 plates/meter.

Column coupling of three 4.6 mm × 150 mm columns result in a plate number of 100000 plates in under 5 min without exceeding the 600 bar limit.

Agilent Poroshell 120 columns show excellent precision data for isocratic and gradient analysis.

Typically for Agilent Poroshell 120 columns shorter elution times than that of the similar sub 2-µm banded phase columns can be expected if the same chromatographic conditions are applied. The shorter elution times result in smaller peak widths and consequently higher peak capacities.

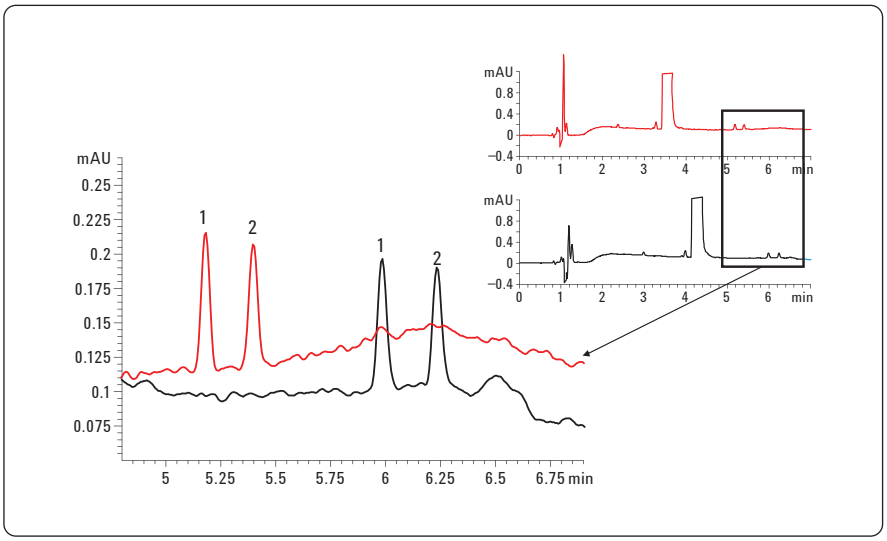


Figure 8
Comparison signal-to-noise ratio, red represents the porous shell column and black trace represents the 1.8 µm particle column. Modifier TFA was used.

Peak	Poroshell 120 S/N	1.8 µm S/N
1	14	13.6
2	12.8	12

Table 2
Comparison of signal-to-noise ratios for porous shell and 1.8 µm particle columns.

References

1. J. M. Cunliffe, T. D. Maloney, "Fused-core particle technology as an alternative to sub-2-µm particles to achieve high separation efficiency with low back pressure", *J. Sep. Sci.* 2007, 30, 3104-3109

2. F. Griiti, A. Cavazzini, N. Marchetti, G. Guiochon," Comparison between the efficiencies of columns packed with fully and partially porous C18-bonded silica materials", *Journal of Chromatography A*, 1157, 289-303, 2007

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Agilent Technologies

Superficially Porous HPLC Columns and Column Overload

Technical Overview

Introduction

New superficially porous particle HPLC columns are great analytical tools for achieving high efficiency, high resolution and fast analysis – all at HPLC pressures. Agilent Poroshell 120 columns have a 2.7-µm superficially porous particle and can deliver 80% to 90% of the efficiency of a sub-2-µm particle at nearly 50% lower backpressure – making these columns compatible with all HPLC's and UHPLC's.

The efficiency and performance of these superficially porous particle columns are excellent for many small molecule analytical separations. The column must also have the capability to be used with typical sample loads, in order to be considered a great analytical tool for method development and routine use. This ensures that adequate sensitivity is achieved for the separations done on this column. Many industries are interested in sample loading because of the number of studies required to determine impurities in samples. In many cases, a larger injection size is used to allow adequate detection of small quantities of impurity peaks. In these cases, it is important for the major component peak to be sharp enough to avoid interference with the quantitation of the smaller peaks due to band broadening, shifting or tailing.

Superficially porous particle columns have a solid core and a porous shell. The Poroshell 120 particle, for example, has a 1.7 µm solid core and a 0.5 µm porous shell as shown in Figure 1.

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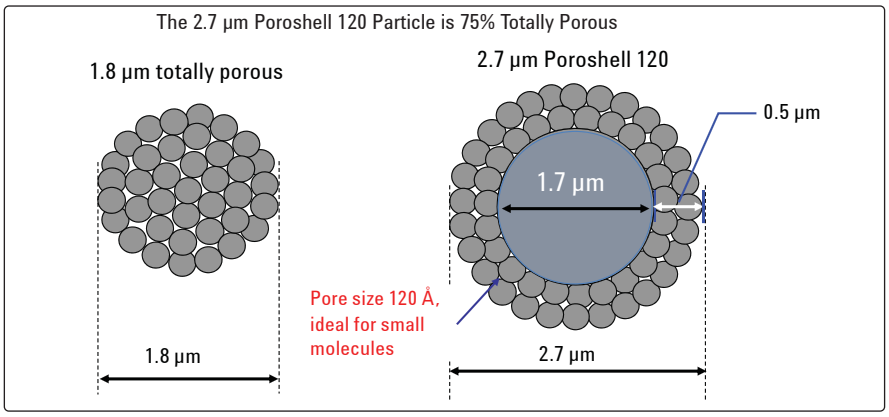

Figure 1. Diagram comparing a superficially porous Poroshell 120 particle to a totally porous 1.8-µm particle.

The total particle size is 2.7 µm. Based on this structure the 2.7 µm particle is 75% totally porous and only 25% of it is the solid core. With so much of the particle totally porous, sample loading on the column falls into a standard analytical range. And the sample loading compares well to that achieved on a totally porous sub 2-µm column.

The following illustrates calculations of Volume Total, and Volume Core:

$$V = \frac{4}{3} pi^{*} r^{3}$$

r_{core} = radius of the core = 0.85 µm

r_{total} = radius of the whole particle = 1.35 µm

Volume total = 10.31 μm^3 Volume Core = 2.57 μm^3

Volume % core = (2.57 μm^3/10.31 μm^3) × 100% = 25%

Volume Porous Shell = 100% – (volume % of core) = 100% – 25% = 75% totally porous

A series of loading experiments was performed to compare the loading capacity of the Poroshell 120 superficially porous particle column to another superficially porous particle column, as well as to both 1.8 µm and 3.5 µm totally porous particle columns (both Agilent ZORBAX Eclipse Plus C18's).

The experiments were designed to evaluate mass loading, which is the amount of material on the column with each injection. In mass overloading, the peak shape will start to change as the loading of the column increases. When the column is overloaded the peak shape will no longer be symmetrical. One typical change is for the peak to broaden with the increasing mass load and have a sharp peak front shifted earlier along with a sloping tail. This change in peak shape can be seen visually in a chromatogram, but that level of distortion clearly indicates sample overloading.

At a more analytical level we can measure the peak width at half-height, and when the peak width doubles we recognize the peak as being overloaded. Peak width is not the only parameter that can be measured, but it is one good choice. Efficiency loss is another parameter that can easily be measured.

The actual loading of acidic and basic compounds can vary based on the nature of these ionizable compounds. Therefore, the experiments included both acidic and basic compounds. A neutral compound was also analyzed, but in general neutral compounds have better sample loading and are of less interest to most chromatographers. The experiments were designed to start at low, analytical sample loads and increase the sample loading until the columns were clearly overloaded.

Peak width at half-height served as a primary measure of the sample overloading and the parameter used for comparison to the loading on the other columns. The first compound evaluated was an acidic compound, benzoic acid. The mobile phase was 25 mM NaH_2PO_4 at a pH of 3, which should typically result in good peak shape for benzoic acid, with pKa = 4.2. The sample loading started out at 0.082 µg on column and ended at 123 µg on column for 3 mm × 100 mm columns. For each injection a number of parameters were measured, including peak width at half-height. A plot of the peak width vs. sample load of benzoic acid is shown in Figure 2.

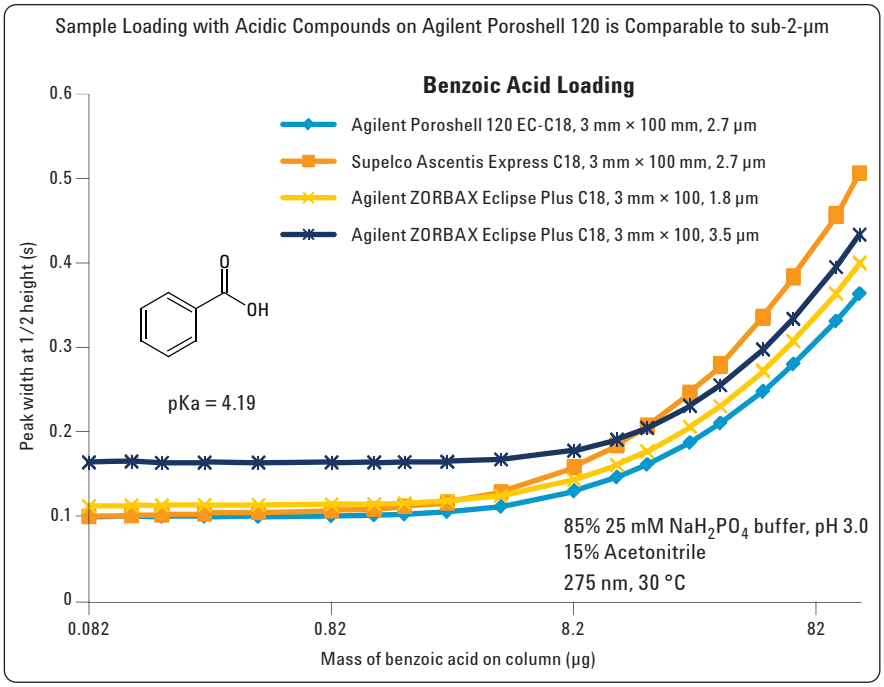

Figure 2. Benzoic acid loading on 3 mm × 100 mm columns.

A number of observations can be made from this plot. The plot shows that the superficially porous Poroshell 120 and 1.8 µm totally porous Eclipse Plus columns have roughly the same loading ability. Through a range of reasonable sample loads, each column maintains peak width at a given value. The flat region of the curves shows that each of the superficially porous and the 1.8 µm totally porous columns yields similar peak width for benzoic acid at pH 3. The only outlier in this plot is the 3.5 µm totally porous Eclipse Plus column, which produces notably wider peaks at low sample loads. This could compromise the resolution of small impurities. The chromatograms are shown in Figure 3.

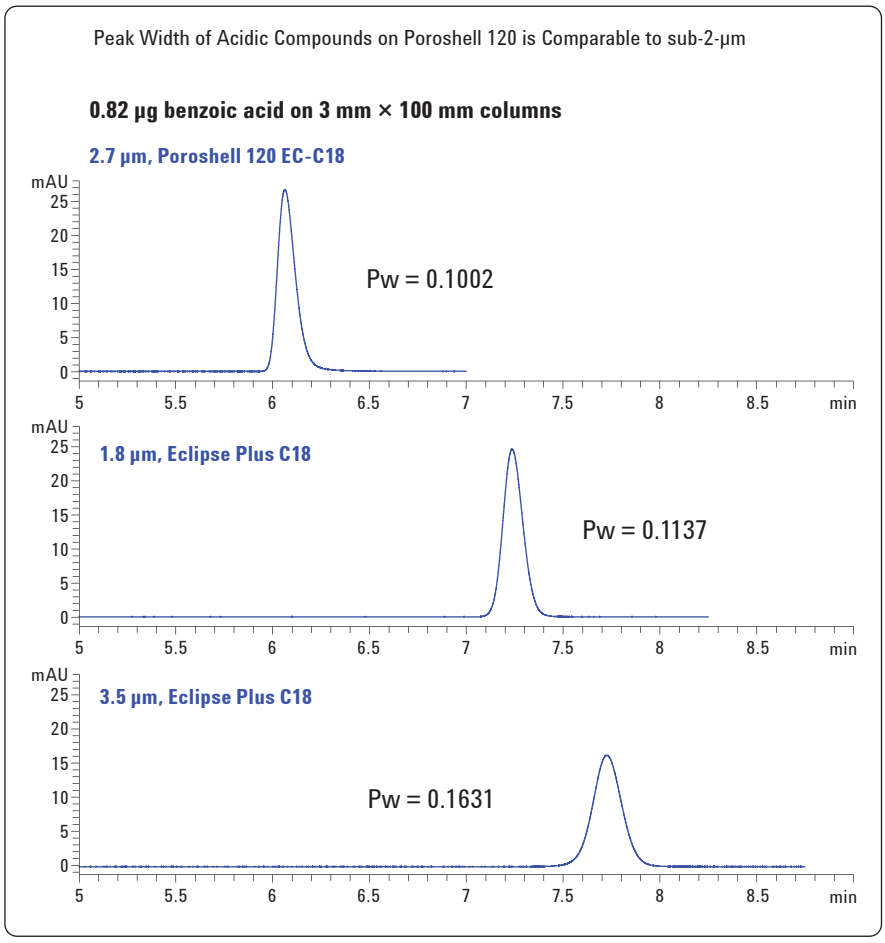

Figure 3. Normal peak width comparison of 2.7, 1.8 and 3.5 µm particles with benzoic acid on 3 mm × 100 mm columns.

Overloading of the sample is depicted when each curve in Figure 2 begins to trend upward. This can be seen at roughly the same amount of benzoic acid on-column for each of the superficially porous and sub-2-µm columns. Again, the 3.5 µm column stands out, because it starts with a much higher peak width, and the peak width doubles at approximately twice the concentration of the other columns. While this appears to be an advantage, the broader peak widths on the 3.5 µm column will limit resolution.

Overloading is noted by a change in peak width, peak shape and retention time on all these columns, as can be seen in Figure 4. For both Poroshell 120 and 1.8-µm Eclipse Plus columns, low sample load peak widths are roughly the same. They begin to increase as overloading occurs; for the two columns in Figure 4, overload occurs at about the same sample load of benzoic acid. In this experiment, overloading is determined when peak width at half-height doubles. Also notable is the inward shift in retention time and increase in tailing.

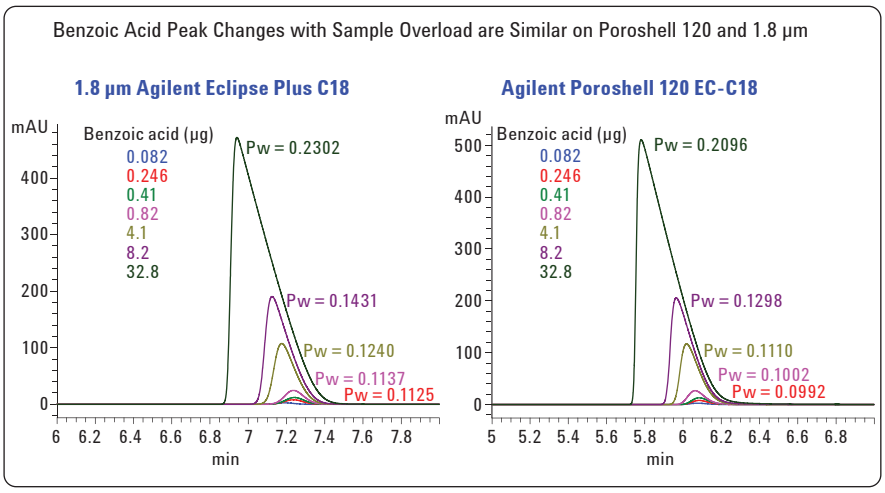

Figure 4. Overlay showing benzoic acid peak shape change with sample overload on 3 mm × 100 mm columns.

Figure 5 shows another loading curve with a basic compound, nortriptyline. This series of data was also collected with a 25 mM NaH_2PO_4 pH 3 mobile phase, which should produce good peak shape for nortriptyline with pKa=9.7. Sample loading ranged from 0.008 µg to 4 µg on 3 mm × 100 mm columns. For each injection, peak width at half-height was monitored and plotted in Figure 5 to determine the point of overload.

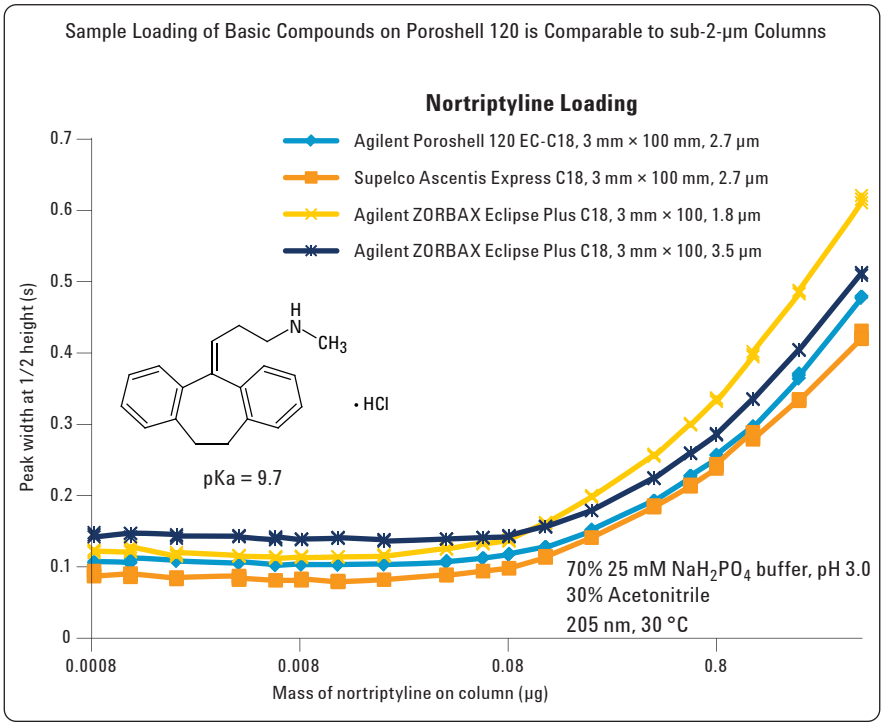

Figure 5. Nortriptyline loading on 3 mm × 100 mm columns.

As in the acidic compound plot, this nortriptyline plot shows similar loading for superficially porous particle columns and sub-2 µm totally porous columns. The most different column, as would be expected, is the 3.5-µm totally porous column because of the much broader peak width on this larger particle size. For the 3.5-µm Eclipse Plus column, the peak width doubles at a higher load as compared to the superficially porous and totally porous sub-2 µm columns because of the broader starting peak width. Each of the superficially porous columns and the 1.8 µm totally porous column start with similar peak width measurements, and display characteristics indicative of overload at nearly the same mass load on-column. Normal peak widths for 2.7 µm superficially porous, 1.8 and 3.5 µm totally porous columns are shown in Figure 6.

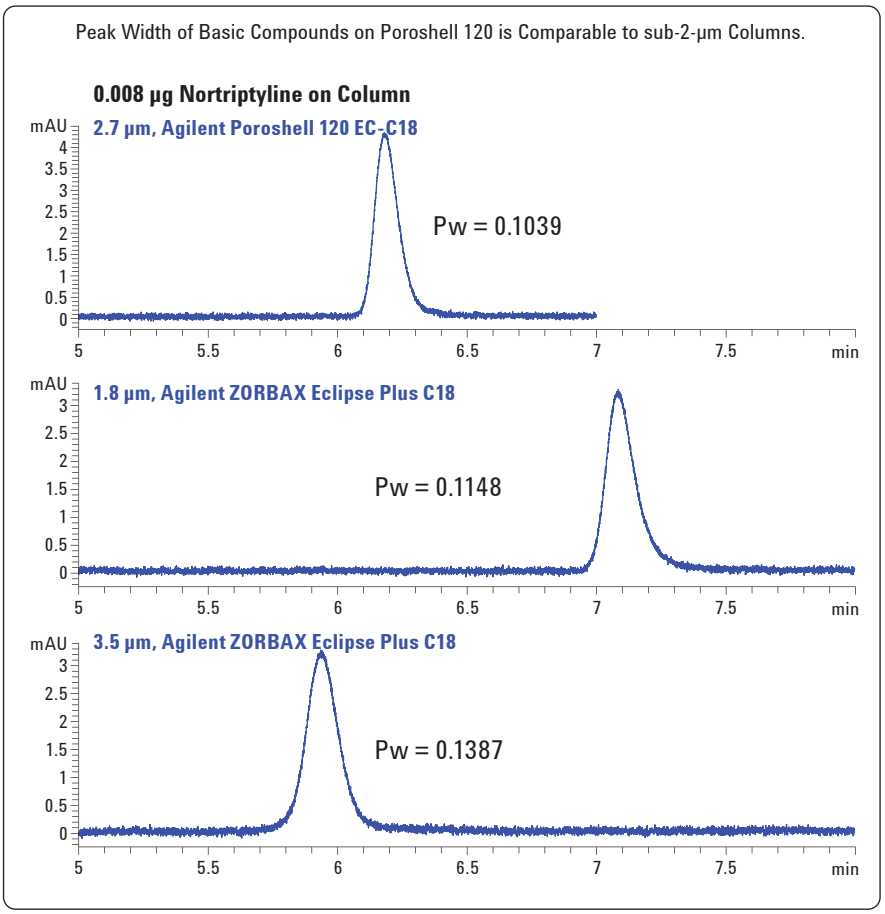

Figure 6. Normal peak width comparison of 2.7, 1.8 and 3.5 um particles with nortriptyline on 3 mm × 100 mm columns.

The trend in peak width, shape and retention time as nortriptyline load increases is similar to that of benzoic acid. Peaks shift inward, tail more and grow wider as sample is overloaded onto the column for both Poroshell 120 and 1.8-µm Eclipse Plus columns, shown in Figure 7. Most notable, again, is the similar mass load of nortriptyline for which peak width doubles on the superficially porous and totally porous sub-2-µm columns. This indicates very similar sample loading capabilities for these two columns.

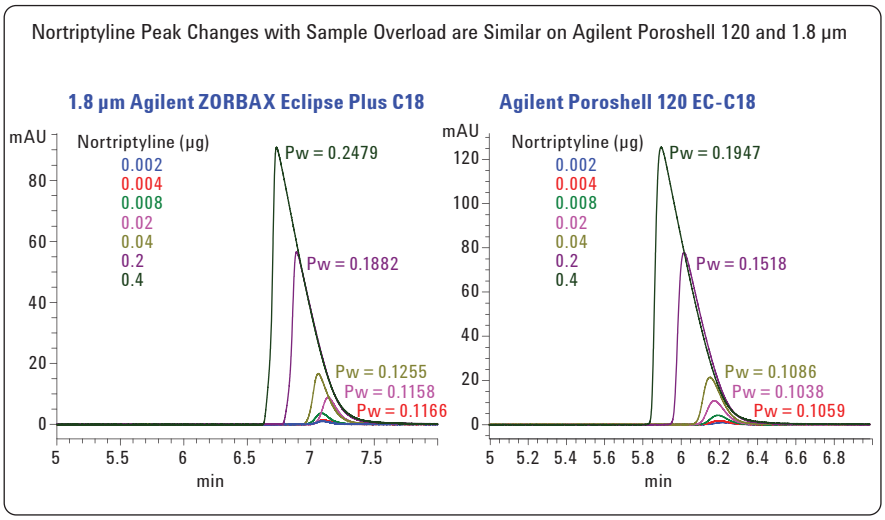

Figure 7. Overlay showing nortriptyline peak shape change with sample overload on 3 mm × 100 mm columns.

Valerophenone was used as the probe for a neutral compound loading study. Data was collected using a neutral mobile phase of water/acetonitrile, producing sharp, narrow peaks for valerophenone at low sample mass loading. Masses ranging from 0.2 to 300 µg of valerophenone were loaded onto 3.0 mm × 100 mm columns. Peak width at half-height was measured and plotted against sample load, as shown in Figure 8. Trends are similar to those found in both the acidic and basic compound loading studies.

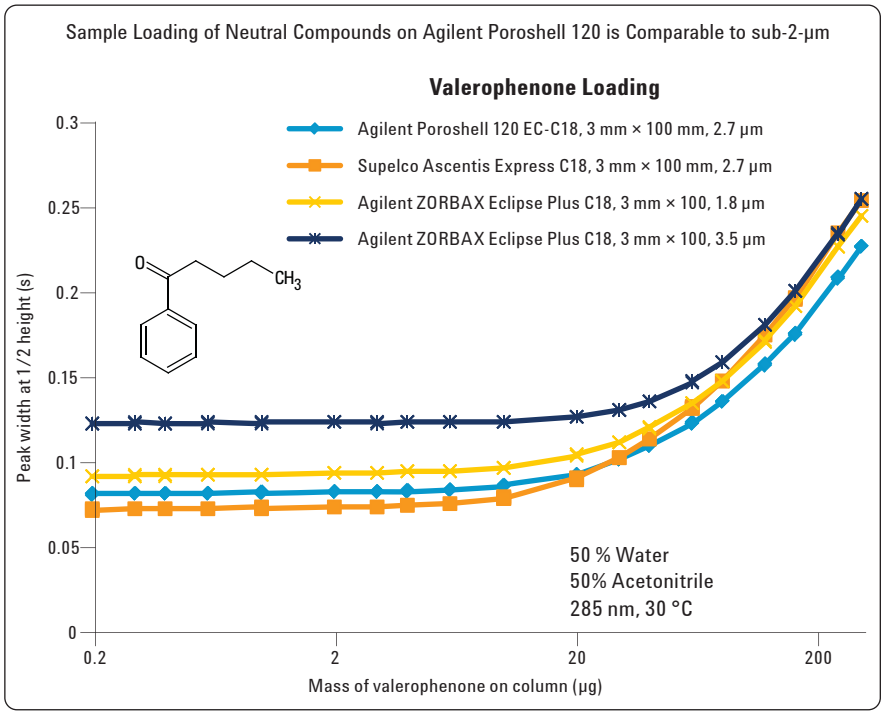

Figure 8. Valerophenone loading on 3 mm × 100 mm columns.

The totally porous 1.8 µm and superficially porous columns perform similarly with valerophenone loading, while the 3.5 µm totally porous column produces wider peaks (Figure 9). This allows for nearly twice the sample load on-column before the peak width at half-height doubles, but potentially compromises resolution more, due to the broader peaks.

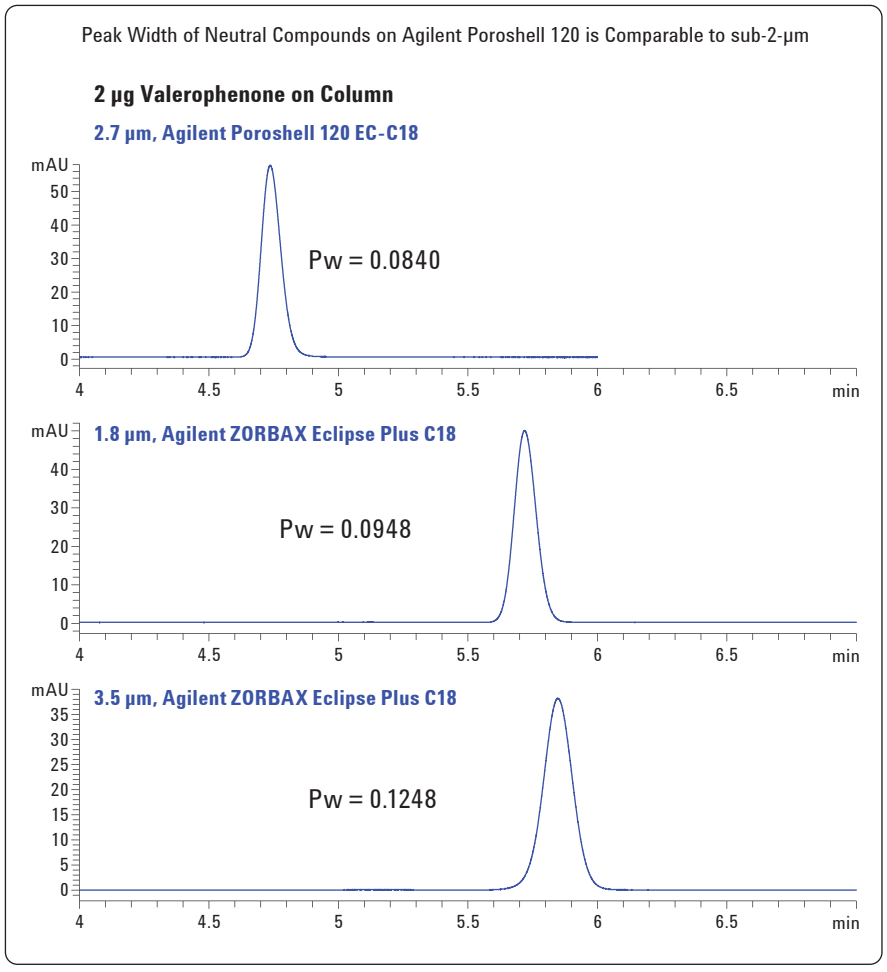

Figure 9. Normal peak width comparison of 2.7, 1.8 and 3.5 µm particles with valerophenone on 3 mm × 100 mm columns.

The chromatographic indications of sample overload for the neutral compound, valerophenone, are similar to the benzoic acid and nortriptyline examples provided earlier. Peak width increases as the sample mass approaches overload, while retention time shifts inward and tailing increases. Figure 10 shows the chromatographic changes that are evident of overload, highlighting the similarities between the superficially porous Poroshell 120 and totally porous 1.8-µm Eclipse Plus loading capacities.

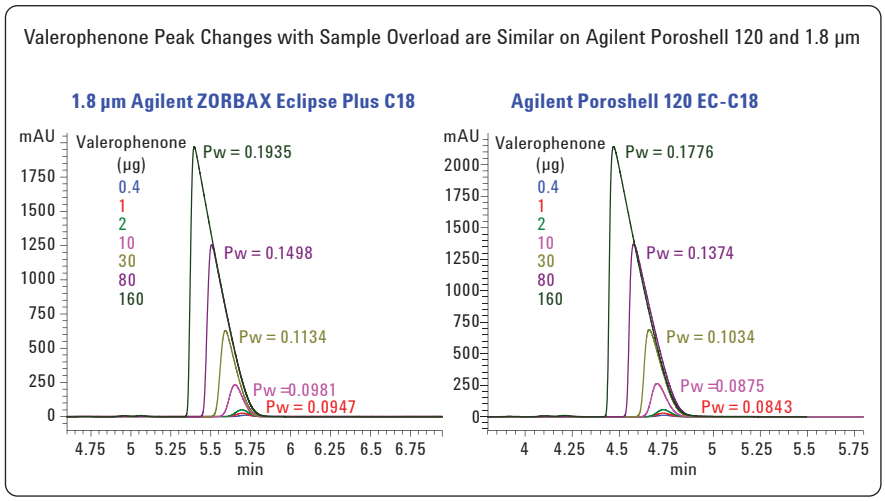

Figure 10. Overlay showing valerophenone peak shape change with sample overload on 3 mm × 100 mm columns.

Based on the loading curves for each of the compounds on each the columns tested, sample loads that produce peaks with twice the peak width at half-height were calculated. The results are shown in Table 1. The data shows that the superficially porous Poroshell 120 and totally porous 1.8-µm Eclipse Plus have similar loading abilities for all compounds tested, while the 3.5-µm Eclipse Plus can load about twice the sample mass in all cases. The superficially porous Ascentis Express was also tested, showing similar initial peak shape (Figure 11), but lower loading capacities with each compound, when compared to the Poroshell 120. Poroshell 120 was found to load about double the sample mass of a similar dimension Ascentis Express column before overloading occurred.

Table 1. Benzoic Acid, Nortriptyline and Valerophenone Loading Studies w/Calculated Mass on Column at Overload

	Benzoic Acid Loading			**Nortriptyline Loading**			**Valerophenone Loading**		
	Average Peak Width at 1/2 Height (s)	*Peak Width at Overload (2 x Ave PW) (s)*	*Mass Load on Column at Overload (µg)*	*Average Peak Width at 1/2 Height (s)*	*Peak Width at Overload (2 x Ave PW) (s)*	*Mass Load on Column at Overload (µg)*	*Average Peak Width at 1/2 Height (s)*	*Peak Width at Overload (2 x Ave PW) (s)*	*Mass Load on Column at Overload (µg)*
Agilent Poroshell 120 EC-C18, 3 mm × 100 mm, 2.7 µm	0.100	0.199	29.0	0.106	0.211	0.515	0.083	0.166	137
Supelco Ascentis Express C18, 3 mm × 100 mm, 2.7 µm	0.103	0.205	16.2	0.085	0.170	0.334	0.074	0.148	79.2
Agilent ZORBAX Eclipse Plus C18, 3 mm × 100, 1.8 µm	0.113	0.225	31.2	0.118	0.235	0.327	0.094	0.188	149
Agilent ZORBAX Eclipse Plus C18, 3 mm × 100, 3.5 µm	0.164	0.327	62.6	0.142	0.285	0.799	0.124	0.249	280

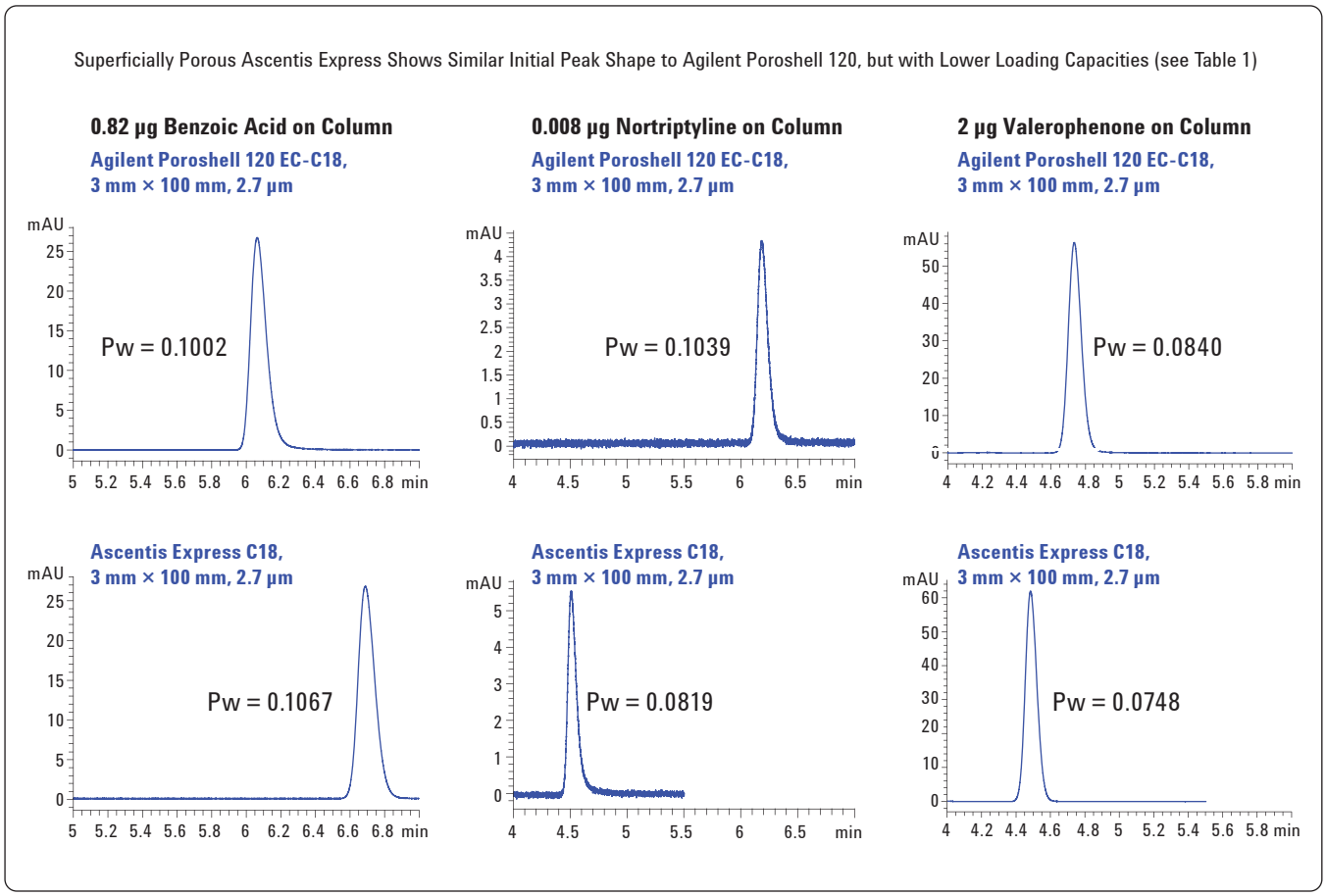

Figure 11. Agilent Poroshell 120 and Ascentis Express comparison under normal loading conditions with acidic, basic and neutral compounds.

An interesting observation from these loading studies is illustrated in Table 2. Overloading on a small particle column is not necessarily a reason to switch to a larger particle column to avoid overload. Table 2 shows the doubled peak width at which each column is determined to be overloaded, along with the calculated sample mass on-column needed to generate that peak width at overload. The third column for each of the respective loading studies gives the calculated peak width of the 3.5 µm totally porous column at the same mass load needed to double the peak width on the smaller particle columns. In nearly all cases, the 3.5 µm totally porous column produced wider peaks at the given sample load than both the overloaded superficially porous and sub-2-µm totally porous columns. This would suggest that even when high sample loading is required and sample overloading is unavoidable with small particle columns, superficially porous and 1.8-µm totally porous columns can still provide narrower peaks and potentially more resolution than a non-overloaded 3.5 µm totally porous column.

Table 2. Comparing Superficially Porous and sub-2-um Overloaded Peak Widths to Non-Overloaded 3.5-µm Peak Widths at Same Sample Mass Load

	Benzoic Acid Loading			**Nortriptyline Loading**			**Valerophenone Loading**		
	Peak Width at Overload (2 x Ave PW) (s)	*Mass Load on Column at Overload (µg)*	*3.5 µm Peak Width at Mass Load (s)*	*Peak Width at Overload (2 x Ave PW) (s)*	*Mass Load on Column at Overload (µg)*	*3.5 µm Peak Width at Mass Load (s)*	*Peak Width at Overload (2 x Ave PW) (s)*	*Mass Load on Column at Overload (µg)*	*3.5 µm Peak Width at Mass Load (s)*
Agilent Poroshell 120 EC-C18, 3 mm × 100 mm, 2.7 µm	0.199	29.0	0.244	0.211	0.515	0.245	0.166	137	0.190
Supelco Ascentis Express C18, 3 mm × 100 mm, 2.7 µm	0.205	16.2	0.203	0.170	0.334	0.210	0.148	79.2	0.160
Agilent ZORBAX Eclipse Plus C18, 3 mm × 100, 1.8 µm	0.225	31.2	0.250	0.235	0.327	0.208	0.188	149	0.197

The acidic, basic and neutral compounds used in this loading study show similar loading capacities for superficially porous and sub-2-µm totally porous columns. In all cases the symptoms of sample overload were similar:—retention time shifts inward, tailing increases, and peak width increases. However, despite the negative effects of sample overload on peak shape, superficially porous and sub-2-µm totally porous columns can still yield narrower peaks than larger 3.5 µm totally porous columns at the same sample mass load.

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Agilent Technologies

Optimizing Performance of an Agilent ZORBAX RRHD Eclipse Plus C18 Column by Enhancing an Agilent 1290 Infinity LC System for Ultra-Low Dispersion

Application Note

General Analysis

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Abstract

An Agilent 1290 Infinity LC System is optimized for the lowest possible extra-column volume, to ensure minimal band broadening and optimal results from a small dimension, Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm column. Agilent Technologies Ultra-Low Dispersion Tubing, Ultra-Low Dispersion Max-Light Cartridge Flow Cell (V(σ) = 0.6 µL) and LC System Rack decrease the 1290's extra-column volume by 60%. The effects on column performance are shown. Improvements to isocratic and gradient analyses are evident, with the early eluting peaks from the isocratic analysis showing the greatest improvement of up to a 58% increase in efficiency (k' = 1.6). Gradient analyses are improved by 24 to 32% with respect to conditional peak capacity (k' = 1.3 to 5.3). Additionally, there is no strong correlation between flow rate and the effect of extra-column volume, with respect to column performance for isocratic and gradient analyses.

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Introduction

Small dimension LC columns packed with small particles deliver increased productivity with faster analyses or more resolution, reduced solvent usage and better LC/MS and ELSD compatibility, as compared to larger bore columns with 4.6 or 3 mm internal diameters that require faster flow rates for equivalent linear velocities. Simply swapping a larger id column for a smaller one can yield these benefits. However, to take full advantage of small dimension columns, the LC system extra-column volume must be minimized; this can include connecting capillaries, needle seats, heat exchangers, and detector flow cells. Peak broadening occurs as soon as the sample is introduced into the LC system, as it travels through the autosampler, to the column, then to the detector, and finally through the detector flow cell. Minimizing this volume is especially critical for small dimension columns, as it will account for a higher percentage of the system's extra-column volume, compared to a larger volume column.

Previous work [1] shows the effect of extra-column volume on a variety of column dimensions and particle sizes. Extra-column volume is simplified in this experiment because the only variables are the diameter and length of the connecting capillary between the autosampler and column. We show that the effect of extra-column volume is dependent on column dimension, but is not dependent on particle size. For a 2.1 × 50 mm, 1.8 µm column, efficiency begins to decrease with as little as 2 µL of additional volume. In addition, we determined that larger 4.6 mm id columns are not significantly affected by extra volume ranging from 1.2 to 9.1 µL. Further work shows that a 5 µm column exhibits similar decreases in efficiency as compared to a same-dimension 1.8 µm column, when data is normalized to account for percent efficiency decrease as a function of additional system volume [1].

In this experiment, optimal column performance is obtained with an Agilent 1290 Infinity LC System, which is enhanced for minimal extra-column volume using Agilent Ultra-Low Dispersion Capillaries (0.08 mm id), an Agilent Ultra-Low Dispersion Max-Light Cartridge Flow Cell (V(σ) = 0.6 µL) and an Agilent LC System Rack. Isocratic and gradient performance improvements are demonstrated using a set of alkylphenone compounds in the Agilent RRLC Checkout Sample. The column is an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm.

Experimental

Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm (p/n 959757-902)
Mobile phase A	H_2O
Mobile phase B	CH_3CN
Flow rate	0.4 mL/min, unless specified otherwise
Gradient	Isocratic (60% B) or gradient (25 to 95% B in 1.2 min, unless specified otherwise)
Sample	A 1-µL injection of Agilent RRLC Checkout Sample (p/n 5188-6529) spiked with 50 µL 2 mg/mL thiourea in water/acetonitrile (65:35). See Table 1 for compound names, elution order, and retention factors for isocratic and gradient analyses
Thermostatted Column Compartment (TCC)	26 °C
Diode Array Detector (DAD)	Sig = 254, 4 nm; Ref = Off
System	Agilent 1290 Infinity LC Agilent Ultra-Low Dispersion Capillary Kit (p/n 5067-5189) Agilent Ultra-Low Dispersion Max-Light Cartridge Flow Cell, V(σ) = 0.6 µL (p/n G4212-60038) Agilent LC System Rack (p/n 5001-3726) MassHunter versions B.03.01, B.02.00 and B.03.01 were used for data acquisition, qualitative, and quantitative analyses respectively

Table 1. Retention Factors for all Alkylphenone Compounds for both Isocratic and Gradient Analyses

Compound	Isocratic k'	Gradient k'
Thiourea	0.0	0.0
Acetanilide	0.3	1.3
Acetophenone	0.9	2.4
Propiophenone	1.6	3.1
Benzophenone	2.6	3.6
Butyrophenone	3.0	3.8
Valerophenone	4.2	4.1
Hexanophenone	6.8	4.5
Heptanophenone	11.1	4.9
Octanophenone	18.2	5.3

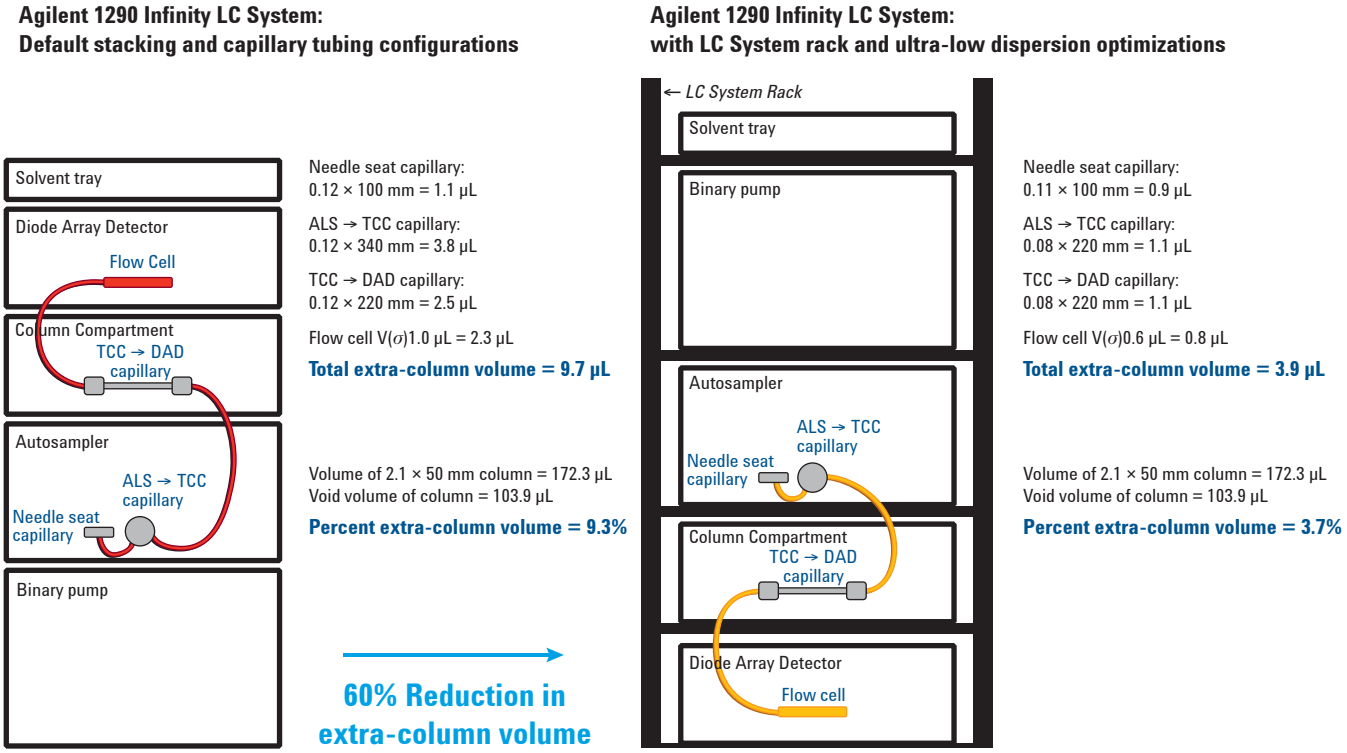


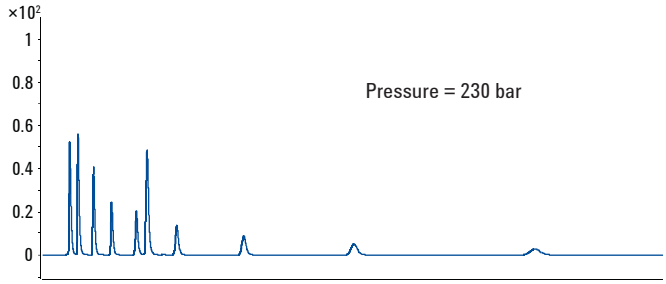
Figure 1. Comparison of extra-column volume on an Agilent 1290 Infinity LC System without (left) and with (right) ultra-low dispersion optimizations.

Results and Discussion

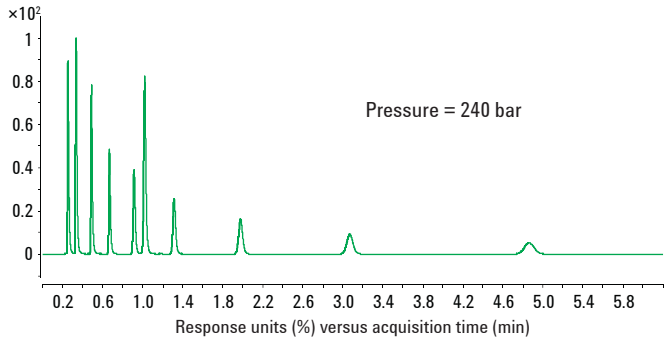
Narrow 0.08 mm id capillaries from the Agilent Ultra-Low Dispersion Capillary Kit replace the standard 0.12 mm id capillaries on the Agilent 1290 Infinity LC System (ALS→TCC capillary, TCC→DAD capillary and needle seat capillary [0.11 mm id]). Additionally, the V(σ) = 1.0 µL flow cell is replaced with the V(σ) = 0.6 µL (Ultra-Low Dispersion Max-Light cartridge) flow cell. The LC is further optimized by rearranging the modules with the LC System Rack. While the 1290 Infinity LC System is traditionally stacked with the binary pump on the bottom due to its weight, using an Agilent LC System Rack allows the pump to be safely located at the top of the stack (from top to bottom: solvent tray, binary pump, autosampler, column compartment, diode array detector); this permits use of the shortest possible capillaries. A shorter 220-mm length capillary connects the autosampler valve to the column inlet (as compared to 340 mm in the default configuration), and the same length capillary, 220 mm, is used to connect the column outlet to the detector flow cell. The result is a 60% reduction in extra-column volume from the default 1290 configuration (9.7 µL) to the optimized configuration (3.9 µL). See Figure 1 for a detailed illustrative and volumetric comparison of the extra-column volume in a default setup versus an optimized setup for the 1290 Infinity LC System.

The isocratic analyses in Figure 2 show a significant 3 to 65% improvement in efficiency with a 60% reduction in extra-column volume. Resolution is improved by 3 to 39% on the optimized Agilent 1290 Infinity LC System, with the critical pair, benzophenone (k′ = 2.6) and butyrophenone (k′ = 3.0), improved by 18%. Early eluting peaks are more affected than later eluting peaks. The bar chart below the chromatograms shows the percent improvement in peak width at half height, efficiency, and resolution for each peak, when comparing performance from the optimized LC to the default LC. Efficiency and resolution are functions of peak width, and so the trends for each of these three values is the same across the chart. These improvements in column performance for an isocratic analysis are possible with just a 5.8-µL (60%) decrease in extra-column volume.

Default Agilent 1290 Infinity LC System, 9.7 µL extra-column volume



Optimized Agilent 1290 Infinity LC System, 3.9 µL extra-column volume



Mobile phase A	H_2O
Mobile phase B	CH_3CN
Flow rate	0.4 mL/min
Isocratic	60% B
Sample	A 1-µL injection of RRLC checkout sample (p/n 5188-6529) spiked with 50 µL 2 mg/mL thiourea in water/acetonitrile
TCC	26 °C
DAD	Sig = 254, 4 nm; Ref = Off
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm
Analytes	1. Thiourea (v_0 marker) 2. Acetanilide 3. Acetophenone 4. Propiophenone 5. Butyrophenone 6. Benzophenone 7. Valerophenone 8. Hexanophenone 9. Heptanophenone 10. Octanophenone

% Improvement from default to optimized Agilent 1290 Infinity LC System with an isocratic analysis

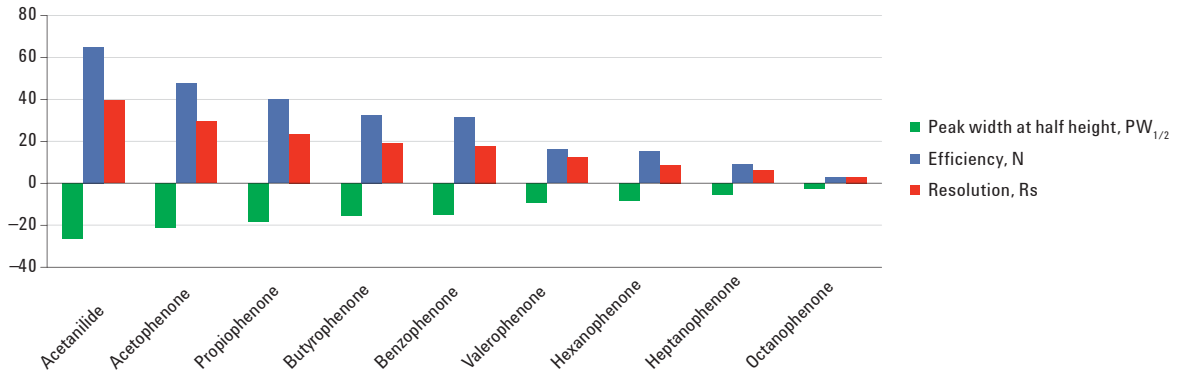


Figure 2. Effect of a 60% reduction in LC system extra-column volume on an isocratic analysis of alkylphenones.

% Improvement in efficiency with a 60% reduction in LC system extra-column volume with an isocratic analysis

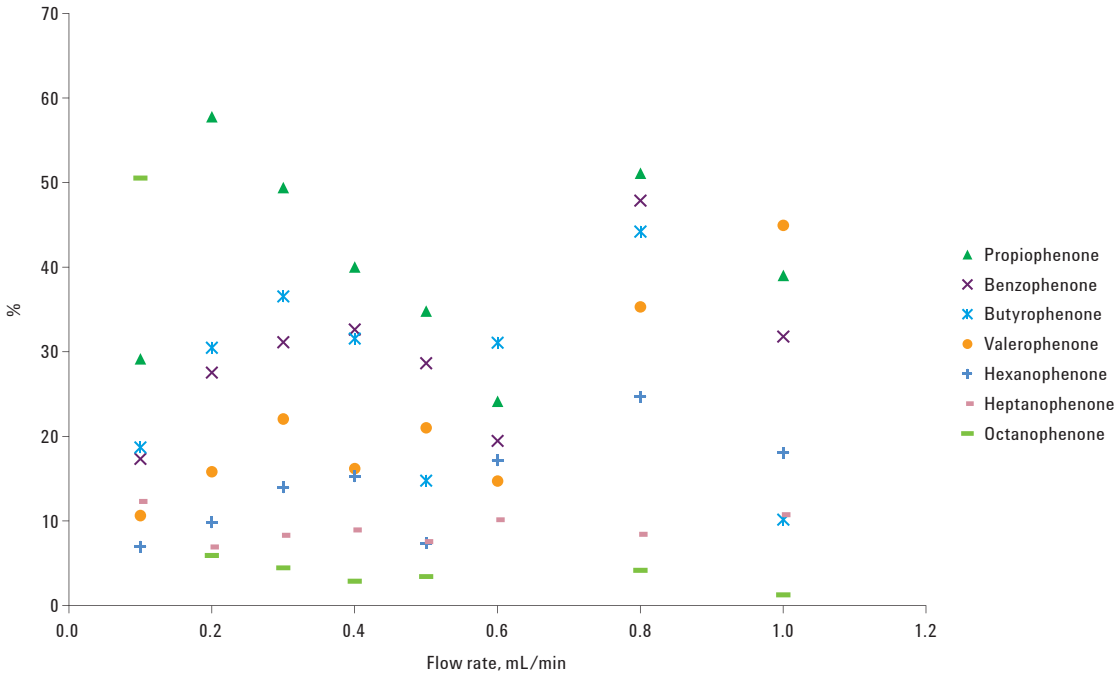


Figure 3. Scatter plot illustrating the effect of extra-column volume on efficiency with various flow rates for an isocratic analysis of alkylphenones.

Figure 2 shows example chromatograms and data from analyses run at 0.4 mL/min. The scatter plot in Figure 3 shows the percent improvement in efficiency for analyses ranging from 0.1 to 1 mL/min. The first two peaks, acetanilide and acetophenone, are poorly retained with k'<1. As a result, these data are not representative of good chromatography practice and are intentionally absent from this figure. Overall efficiency improvements range greatly from 1 to 58% (not including acetanilide and acetophenone). The flow rate of an isocratic analysis has little to no impact on the effects of extra-column volume with respect to column performance.

Gradient analyses for the same 10-component mixture are shown in Figure 4. Similar to Figure 2, the bar chart below the chromatograms depicts the percent improvement when comparing column performance on the optimized Agilent 1290 Infinity LC System to the default setup. Peak width at half height and resolution show the same trend across the chart, because resolution is dependent on peak width. Because efficiency is not a measure typically used to evaluate column performance of a gradient analysis, a line for the percent improvement in conditional peak capacity is displayed on the chart. Conditional peak capacity is the number of peaks that can be theoretically separated over a gradient time, and therefore is also dependent on peak width. See Equation 1.

Equation 1. Conditional peak capacity.

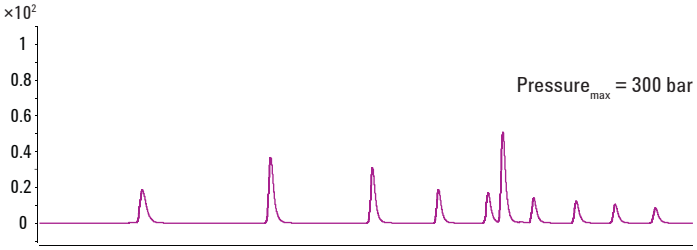
$$\text{Conditional peak capacity} = n_c = \frac{t_{R,n} - t_{R,1}}{W}$$

$t_{R,n}$ and $t_{R,1}$: Retention times of the last and first eluting peaks

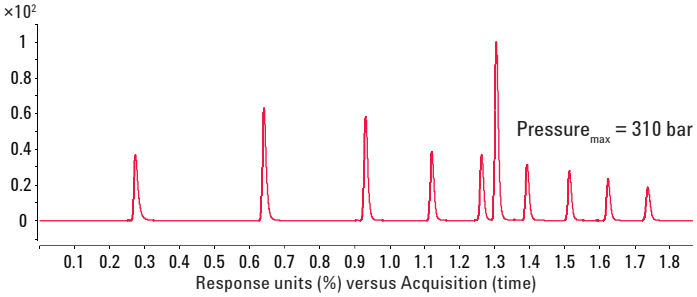
$$W: \frac{\overline{W_{1/2}}}{2.35} \times 4 \text{ (Average } 4\sigma \text{ peak width)}$$

$W_{1/2}$ is the average peak width at half height.

Default Agilent 1290 Infinity LC System, 9.7 µL extra-column volume



Optimized Agilent 1290 Infinity LC System, 3.9 µL extra-column volume



Mobile phase A	H_2O
Mobile phase B	CH_3CN
Flow rate	0.4 mL/min
Gradient	t (min) 0 1.2 %B 25 95
Sample	A 1-µL injection of RRLC checkout sample (p/n 5188-6529) spiked with 50 µL 2 mg/mL thiourea in water/acetonitrile
TCC	26 °C
DAD	Sig = 254, 4 nm; Ref = Off
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm
Analytes	1. Thiourea (v_o marker) 2. Acetanilide 3. Acetophenone 4. Propiophenone 5. Butyrophenone 6. Benzophenone 7. Valerophenone 8. Hexanophenone 9. Heptanophenone 10. Octanophenone

% Improvement from default to optimized Agilent 1290 Infinity LC System with a gradient analysis

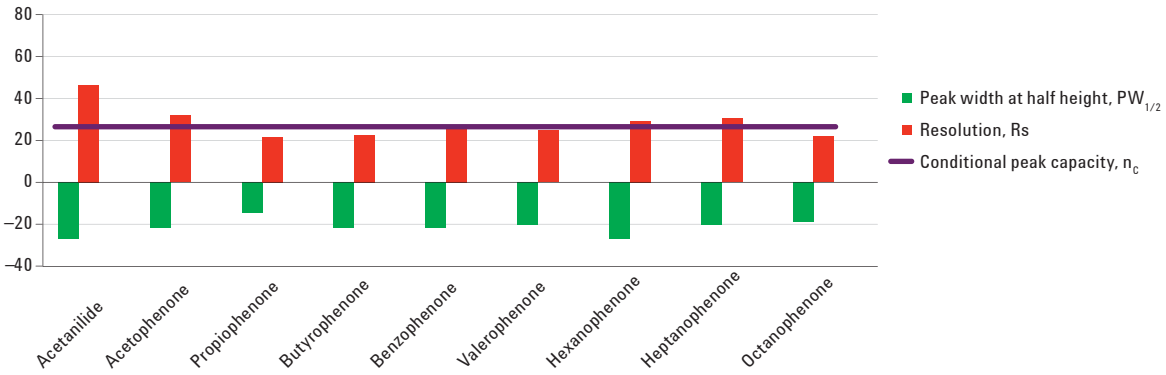


Figure 4. Effect of a 60% reduction in LC system extra-column volume on a gradient analysis of alkylphenones.

Reducing the extra-column volume of the LC system by 60% (5.8 µL) yields a 27% increase in conditional peak capacity of this gradient analysis. Furthermore, the narrower peaks improve the resolution of all peaks by >20%, including the critical pair (benzophenone and butyrophenone) by 27%.

Similar to Figure 3, Figure 5 is a scatter plot showing the percent improvement in resolution and conditional peak capacity for gradient analyses ranging from 0.1 to1 mL/min (gradient time is scaled according to column volume to maintain k' for all compounds). Once again, the first peak, acetanilide, elutes early and yields unusual values as compared to the other eight compounds and is therefore considered an outlier that is purposefully absent from this chart. Conditional peak capacity improves between 24 and 32%, while resolution improvements vary more and range from 18 to 37% (not including acetanilide). In general, it is evident that the flow rate of this fast gradient analysis has little to no impact on the effects of extra-column volume with respect to column performance.

When using the ultra-low dispersion 0.08 mm id capillaries with the Agilent 1290 Infinity LC System, system pressure increases, as shown in Figures 2 and 4. In this experiment, the observed pressure difference is 10 bar. However, if the Agilent LC System Rack was not used and longer 0.08 mm id capillaries (same length as the default setup) were required, the difference in system pressure would be greater than 10 bar. Because these capillaries are easily removable, and standard 0.12 mm id capillaries can be reinstalled, the Agilent 1290 Infinity LC System has significant flexibility. The 1200-bar pressure limit of the instrument can be used to accommodate very small 0.08-mm id capillaries for minimal sample band broadening, or the 0.12-mm id capillaries can be reinstalled and flow rates can be increased for higher sample throughput, whichever the method requirement.

% Improvement in resolution and peak capacity with a 60% reduction in LC System extra-column volume with a gradient analysis

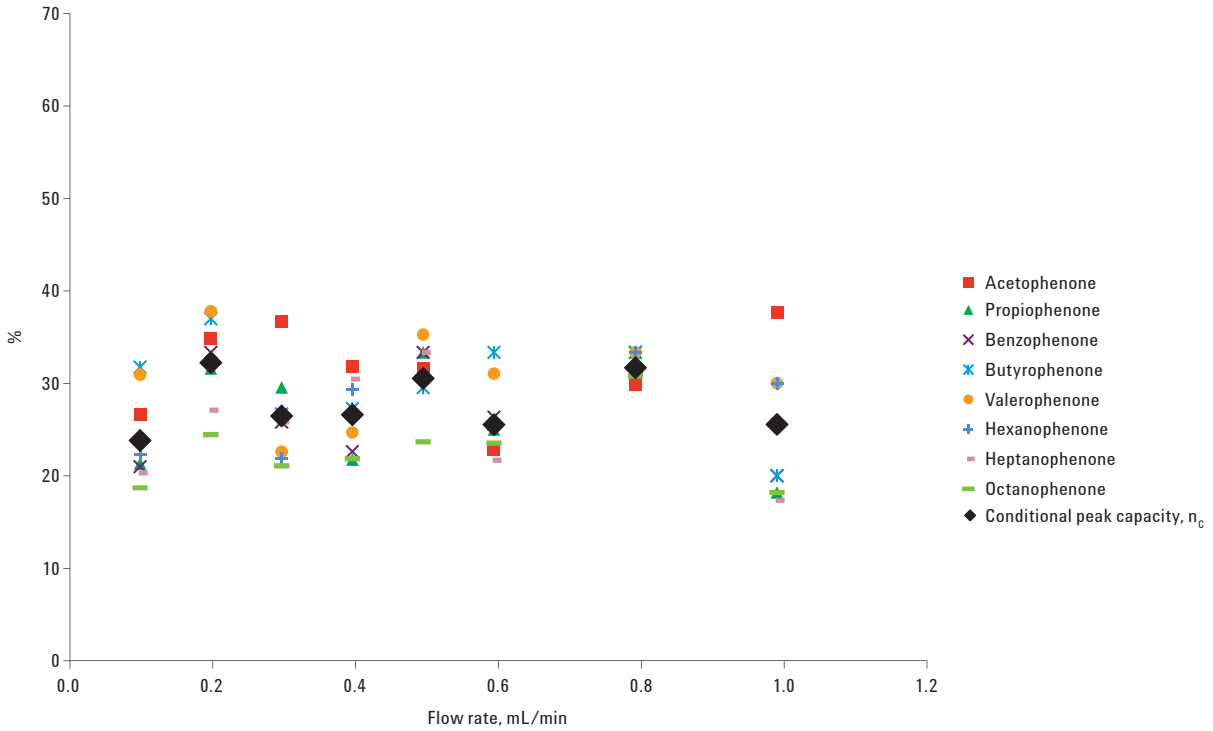


Figure 5. Scatter plot illustrating the effect of extra-column volume on resolution and conditional peak capacity over various flow rates for a gradient analysis of alkylphenones.

Conclusions

The performance of an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm column is improved for isocratic and gradient analyses by using the new Agilent Ultra-Low Dispersion Capillaries, Agilent Ultra-Low Dispersion Max-Light Cartridge Flow Cell (V(σ) = 0.6 µL), and Agilent LC System Rack to reduce extra-column volume in the Agilent 1290 Infinity LC System by 60% (5.8 µL). Conditional peak capacity in the gradient analysis increases by >24%. The efficiency of the isocratic analysis is more affected and increases by up to 58% for a compound with retention factor k' = 1.6. Additionally, the effects of extra-column volume are not further exacerbated by either increasing or decreasing flow rates.

Reference

1. W. Long and A. Mack. Reduce Tubing Volume to Optimize Column Performance. Agilent Publication 5990-4964EN. December 11, 2009. http://www.chem.agilent.com/Library/applications/5990-4964EN.pdf

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Agilent Technologies

Pharmaceutical

Fast Separation of Seven Biocides Using an Agilent ZORBAX Rapid Resolution High Definition Eclipse Plus C18 1.8 µm Column

Application note

Environmental

Authors

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Abstract

An Agilent ZORBAX Rapid Resolution High Definition (RRHD) Eclipse Plus C18 1.8 µm column separated seven different biocides rapidly with high resolution. The data presented illustrate the capability of the short 50 mm, 1.8 µm column to maintain high resolution of complex samples at very fast flow rates and high pressures, up to 1020 bar. The high quality separation was maintained when the method run was reduced from 3 minutes to 0.7 minutes by increasing the flow rate from 1.0 mL/min to 1.7 mL/min. The Agilent 1290 Infinity LC System was used because the column pressure just exceeded 1000 bar at this high flow rate. Methylparaben, listed as an echinacea supplement ingredient, was easily identified and a hand sanitizer containing 2-phenoxyethanol and methylparaben were also easily and rapidly identified using the method.

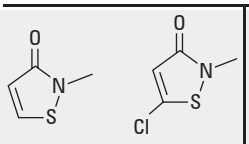
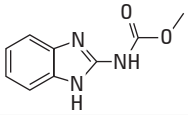
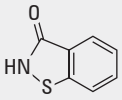
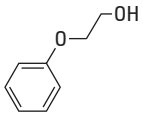
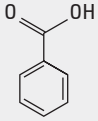
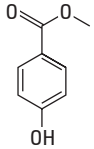
Agilent Technologies

Introduction

Biocides are used to control harmful organisms such as bacteria, fungi and rodents and to help protect health, improve product performance and prevent spoilage. They are found in many areas including medicine, agriculture, forestry, and industry. Due to their potential health risk to humans and the environment, they are highly regulated. In the US, biocides are listed on the national inventory of chemical substances called TSCA. There are also regulations governing the use of biocides on both the federal (EPA) and state level. In Europe, the regulations governing the use of biocides are on a countrywide level. The biocides industry in the European Union (EU) is undergoing a dramatic transformation because the European Commission recently presented draft legislation to achieve a higher level of protection of health and environment to take effect in 2013.

The demand for biocides will continue to grow as public awareness of the benefits of continued hygiene improvements becomes ever more apparent. It is vital therefore to ensure that only biocides safe for use are placed on the market. Because many biocides are synthetic, this work includes the fast separation of seven different synthetic biocides used in different applications. Obtaining results in the shortest time possible without compromising the quality of the results is an area of intense interest for lab analysts, because of the cost and efficiency advantages associated. Table 1 illustrates the biocides investigated in this study, along with their uses.

Table 1. Synthetic Biocides Included in Study and their Uses

Biocide Structure	Name	Uses
	Kathon CG/ICP 1. 2-methyl-4-isothiazonlin-3-one 2. 5-chloro-2-methyl-4-isothiazolin-3-one	Preservative with a wide variety of household and industrial uses.
	3. Carbendazim	Fungicide
	4. 1, 2-Benzisothiazol-3(2H)-one	Used as a preservative in emulsion paints, varnishes, adhesives, washing agents, fuels, and in the paper making process.
	5. 2-Phenoxyethanol	Preservative and bactericide used in vaccines and in dermatological products such as skin creams and sunscreen.
	6. Benzoic Acid	Used as a food preservative and an antimicrobial agent in toothpastes, mouthwashes, cosmetics, and deodorants.
	7. Methylparaben	Antifungal widely used as a preservative for food, drugs, and cosmetics.

Experimental

The biocides were purchased from Sigma Aldrich and include Kathon CG/ICP [containing 0.4% of 2-methyl-4-isothiazolin-3-one (1), 1.2% of 5-chloro-2-methyl-4-isothiazolin-3-one (2)], carbendazim (3), 1,2-benzisothiazol-3(2H)-one (4), 2-phenoxyethanol (5), benzoic acid (6), and methylparaben (7). Kathon was diluted with distilled, deionized water to make a 200-ppm solution of (1) and a 600-ppm solution of (2). Compounds (3-7) were dissolved in acetonitrile (ACN) to make a 200-ppm solution mix. Equal volumes of the Kathon solution and the solution mix were combined to give a 100-ppm stock solution (except for compound (2) that had a concentration of 300 ppm). Six levels of calibration standards were prepared in the range of 2.5 to 20 ppm by dilution of the 100-ppm stock solution in 5% ACN for compounds (1) and (3-7). A three-level calibration was made for compound (2) in the range 7.5 to 23 ppm by dilution of the stock solution with 5% ACN. A standard mix [50 ppm for (1) and (3-7), 150 ppm for (2)] was made by dilution of the stock solution [(100 ppm (1), (3-7) and 300 ppm (2)] with 5% ACN.

Three methods were used to analyze the seven biocides using an ACN/trifluoroacetic acid (TFA) mobile phase adjusting gradient conditions and flow rate. The ACN (CHROMASOLV for HPLC gradient grade, ≥99%) and TFA (≥99%) were purchased from Sigma-Aldrich. The experimental conditions for the three methods are listed in Table 2. Formic acid (98-100%) was from Riedel-de Haën. Formic acid (0.1%) can be substituted for TFA to assist in mass spectral detection.

Table 2. Experimental Conditions for Three, One and 0.7 Minute Methods

All Methods		
Column:	Agilent ZORBAX Rapid Resolution High Definition (RRHD) Eclipse Plus C18 2.1 × 50mm, 1.8-µm, p/n 959757-902	
Mobile Phase:	A: Water (0.05 v% Trifluoroacetic acid (TFA) B: Acetonitrile (0.04 v% TFA)	
Column Temp:	30 °C	
Injection:	1 µL	
UHPLC:	Agilent 1290 Infinity LC System	
Column Wash:	Column washed with 100% B at end of each run.	
Equilibration:	Column equilibrated for two minutes with 5% B before injection.	
Three Minute Method – Fast Analysis		
Gradient:	Time (min) 0: Time 2.9:	95/5 A/B 85/15 A/B
Flow Rate:	1.0 mL/min	
Detection:	Diode array (DAD) with programmed wavelength switching 275 nm (0 min) 225 nm (1.6 min) 255 nm (2.8 min)	
One Minute Method – Faster Analysis		
Gradient:	Time (min) 0: Time 0.1: Time 0.25: Time 1.0: Time 1.1:	95/5 A/B 95/5 A/B 90/10 A/B 65/35 A/B 0/100 A/B
Flow Rate:	1.0 mL/min	
Detection:	DAD with programmed wavelength switching 275 nm (0 min) 225 nm (0.8 min) 255 nm (1.08 min)	
0.7 Minute Method – Ultra Fast Analysis		
Gradient:	Time (min) 0: Time 1.0:	95/5 A/B 55/45 A/B
Flow Rate:	1.7 mL/min	
Detection:	DAD with programmed wavelength switching 275 nm (0 min) 225 nm (0.46 min) 255 nm (0.67 min)	

Results and Discussion

An Agilent ZORBAX Rapid Resolution High Definition (RRHD) Eclipse Plus C18 2.1 × 50 mm, 1.8 µm column separated seven different biocides rapidly with high resolution. Three different methods are presented in Figure 1, which show that a high quality separation can be achieved in 3 minutes, and in as short a time as 0.7 minutes. Using the ultrafast, 0.7 minute method, all compounds were baseline-resolved with a minimum resolution factor of 1.8 illustrating the power of sub 2-micron columns to maintain high resolution at fast flow rates. The very fast linear gradient and high flow rate of 1.7 mL/min used with the 0.7 minute method on the 50 mm RRHD column produced pressure slightly over 1000 bar. The RRHD columns are designed for UHPLC systems such as the Agilent 1290 Infinity operating at pressures up to 1200 bar. The Agilent 1290 Infinity LC System was equipped with a diode array detector and the method included programmed wavelength switching to maximize detection response and improve sensitivity.

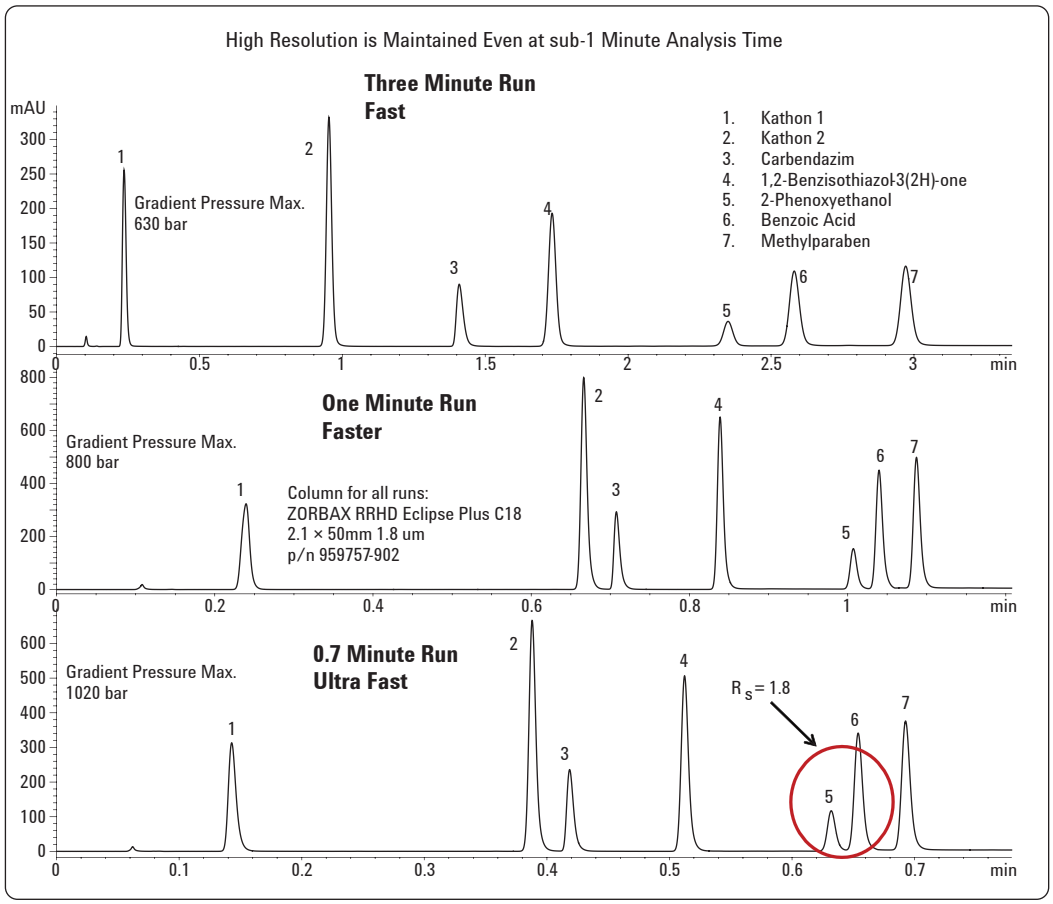


Figure 1. Seven biocides rapidly separated with high resolution using Agilent ZORBAX RRHD Eclipse Plus C18 with three different methods.

Ten consecutive injections of the biocide mix (10 ppm for biocides (1), and (3 to 7) and 30 ppm for biocide (2)) were made using the 1 minute method as shown in Figure 2. The retention time and peak area of each component is listed in Tables 3 and 4 respectively for each of the ten replicate injections. Statistical analysis of the data shows excellent % RSD results that are noteworthy because of the very fast analysis time.

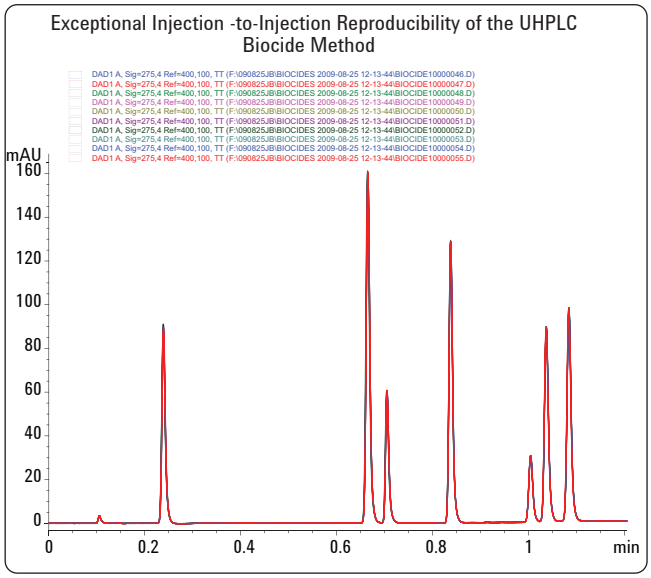


Figure 2. Ten consecutive injections of biocide mix (10 ppm) separated in 1.1 min using a short ZORBAX RRHD Eclipse Plus C18 column.

Table 3. Retention Time Precision Data for Ten Injections of Seven Biocides

	Retention Time (min)						
Injection	**Peak 1**	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**	**Peak 6**	**Peak 7**
1	0.239	0.665	0.705	0.838	1.004	1.037	1.084
2	0.239	0.665	0.705	0.838	1.004	1.037	1.084
3	0.239	0.664	0.704	0.838	1.004	1.037	1.084
4	0.24	0.665	0.705	0.838	1.005	1.038	1.085
5	0.239	0.665	0.705	0.838	1.004	1.037	1.084
6	0.239	0.665	0.705	0.838	1.004	1.037	1.084
7	0.239	0.665	0.705	0.838	1.004	1.037	1.084
8	0.24	0.665	0.705	0.838	1.005	1.037	1.084
9	0.239	0.665	0.705	0.838	1.005	1.037	1.084
10	0.239	0.665	0.705	0.838	1.004	1.037	1.084
AVG	0.2392	0.6649	0.7049	0.838	1.0043	1.0371	1.0841
STD	0.00042	0.00032	0.00032	0.00000	0.00048	0.00032	0.00032
%RSD	**0.18**	**0.05**	**0.04**	**0.00**	**0.05**	**0.03**	**0.03**

Table 4. Peak Area Precision Data for Ten Injections of Seven Biocides

	Peak Area						
Injection	**Peak 1**	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**	**Peak 6**	**Peak 7**
1	46.116	88.355	30.133	70.249	18.179	53.633	59.922
2	45.971	87.978	30.062	70.054	18.05	53.384	59.666
3	46.266	88.439	30.254	70.467	18.184	53.764	60.053
4	46.115	88.142	30.129	70.311	18.096	53.517	59.879
5	46.164	88.303	30.168	70.544	18.194	53.612	59.934
6	46.286	88.375	30.175	70.655	18.157	53.77	60.046
7	46.19	88.212	30.233	70.292	18.111	53.592	59.85
8	46.178	88.127	30.23	70.416	18.105	53.565	59.932
9	46.158	88.219	30.16	70.441	18.128	53.692	60.054
10	46.165	88.248	30.178	70.342	18.134	53.627	59.949
AVG	46.1609	88.2398	30.1722	70.3771	18.1338	53.6156	59.9285
STD	0.08696	0.13635	0.05724	0.16747	0.04541	0.11481	0.11694
%RSD	**0.19**	**0.15**	**0.19**	**0.24**	**0.25**	**0.21**	**0.20**

Calibration plots of the biocides were used to assess the linearity of the UHPLC method using the 1 minute method. Figure 3 shows an overlay of the seven calibration plots generated by plotting peak area vs. concentration in ppm. Excellent linearity was achieved in the 2.5 to 23 ppm range. Six level calibration plots were done for biocides (1) and (3 to 7) in a concentration range of 2.5 to 20 ppm. A three level calibration plot was done for biocide (2) in a range of 7.5 – 23 ppm.

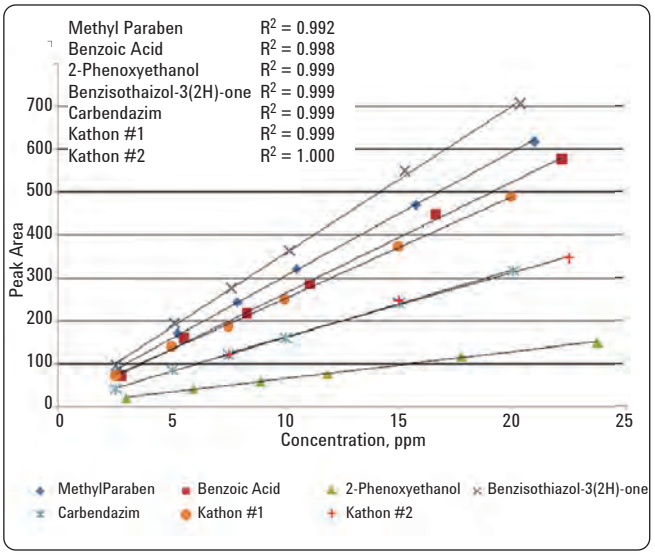


Figure 3. Calibration curves for seven biocides.

A commercial hand sanitizer was analyzed using the ultra fast 0.7 minute method with excellent results. The hand sanitizer was injected neat with no detrimental effects to the column and Figure 4 shows that 2-phenoxyethanol and methylparaben were both easily identified. Methylparaben and 2-phenoxyethanol are common preservatives used in personal care products and were expected to be in this hand sanitizer sample. A number of other trace components were found in the sample, as would be expected from this matrix and none of these peaks interfered with the detection of the biocides.

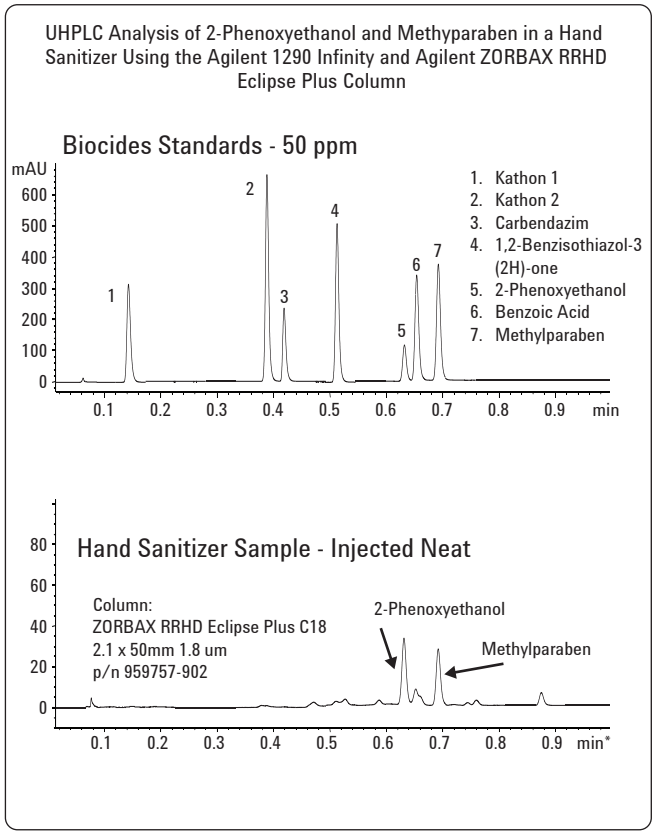


Figure 4. Overlay of biocide standard mix and a hand sanitizer using the sub-1 minute method.

Formic acid (0.1%) can be substituted for TFA for this method. Formic acid is often used in place of TFA when a mass spectrometer (MS) detector is used, to enhance ionization and assist in MS detection. Formic acid was used in the mobile phase to analyze an echinacea supplement using the 1 minute method. The echinacea supplement was diluted 1:5 in 70% methanol and 0.5 µL was analyzed. Methylparaben was easily identified in the sample as shown in Figure 5.

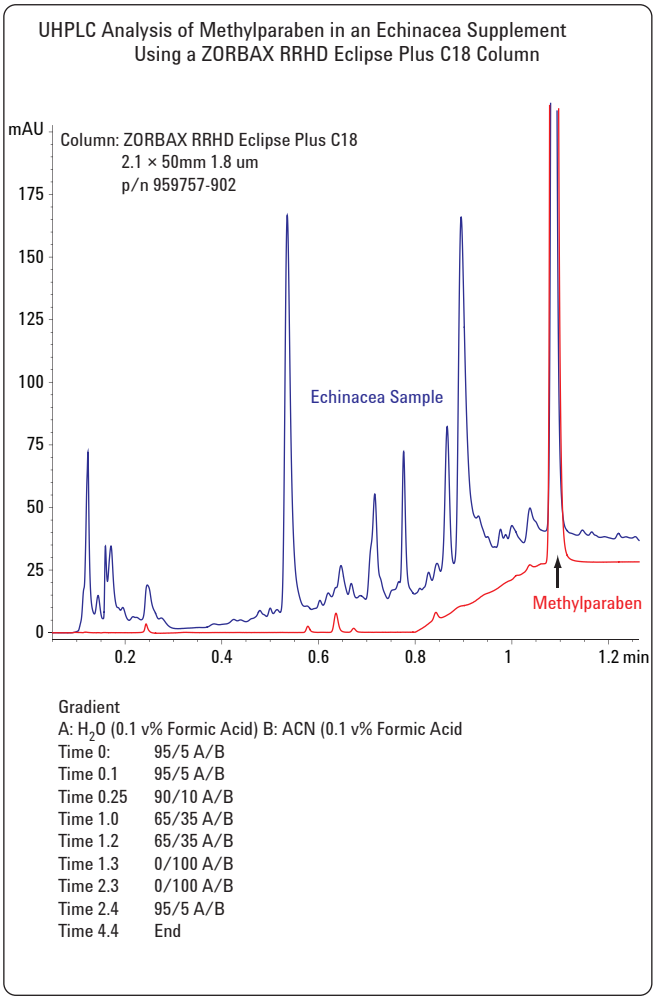


Figure 5. Overlay of methylparaben standard and an echinacea supplement using the 1 minute method with formic acid in the mobile phase.

Conclusion

The Agilent ZORBAX RRHD Eclipse Plus C18 1.8 um column was used in conjunction with the Agilent 1290 Infinity LC System for fast (3 min) to ultrafast (sub-minute), high resolution separation of biocides. The rapid analysis method was successfully applied to the separation and identification of biocides in complex samples such as a hand sanitizer and an echinacea supplement. Benefits include speed with high resolution for improved productivity and reproducibility.

References

1. Derek J. Knight and Mel Cooke, "The Biocide Business: Regulation, Safety and Applications," Wiley-VCH 2002.

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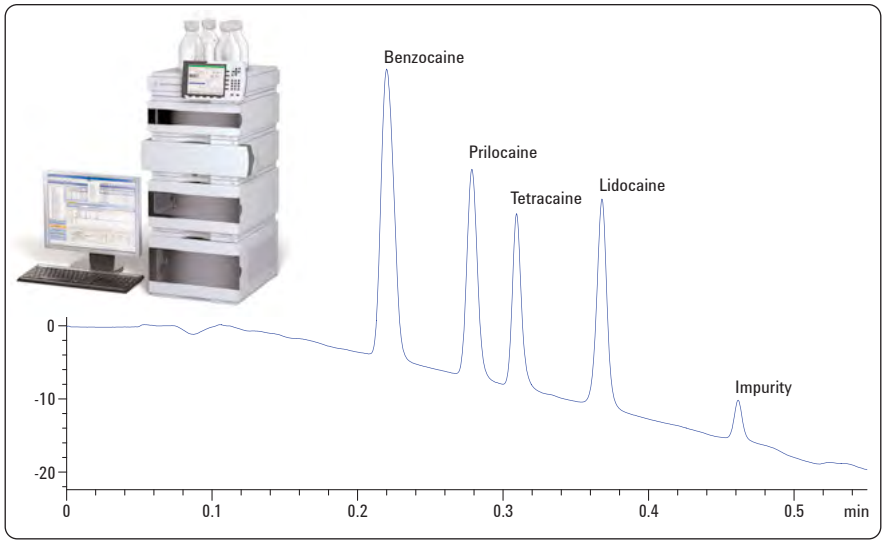
Agilent Technologies

High speed separation of anesthetics on the Agilent 1290 Infinity LC system with different columns

Application Note



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Abstract

The limits of resolution, peak capacity and pressure can be explicitly reduced when analyzing with the Agilent 1290 Infinity LC system. The power and flow design of the Agilent 1290 Infinity Binary Pump allows the use of various eluent compositions with any column type, and provides the high sensitivity of the new UV detection system.

The need to convert existing methods to fast or high resolution methods, causes difficulties such as the adaptation of delay volumes of the former HPLC system or the back pressure of the required column type to the new setup.

This Application Note shows the separation of local anesthetics with different column types. It demonstrates the transfer of parameters from a 5 µm column to columns with particles < 2 µm. The results show high resolution even under high throughput conditions. The best separation results (0.4 min) were achieved with the Agilent ZORBAX RRHD Eclipse Plus C18 HD 50 mm × 2.1 mm, 1.8 µm column with an overall runtime of 1 min, including regeneration. The results for the determination of the precision of areas and retention times (< 0.5 %) show that all criteria for qualified instruments are fulfilled. The correlation coefficients for linearity for all components are better than 0.999. No carryover was detected.

Agilent Technologies

Introduction

The development of the Agilent 1290 Infinity LC system resolved many issues around ultra-high performance, ultra-high pressure liquid chromatography. In addition, it has extended the limits of resolution, peak capacity, and pressure.

The power and flow design of the pump with reduced delay volumes, the elimination of an extra mechanical pulsation damper, and the new Jet Weaver for gradient mixing allows the use of any eluent composition, and any column type while still producing the highest sensitivity.

Many other HPLC systems need to be optimized to special column types, (such as columns with 4.6 mm diameter) because of their flow design. The Agilent 1290 Infinity LC system uses a small system volume, which has very little influence on dispersion and peak width. This allows the use of any column, with any diameter, or length, filled with any particle size packing, and still provides good results. This is especially true with 2.1 mm columns.

The recent trend to improve resolution, save time, and reduce solvent costs was to transfer methods from 4.6 mm columns with 5 µm particles to columns with smaller diameters and smaller particles. This also lowered the cost per analysis by shortening the analysis time. The transfer of methods by calculation to fast or high resolution methods provides the challenges of adaptating delay volumes of the former HPLC system, and adjusting the back pressure of the required column type to the new setup.

This Application Note describes the separation of four local anesthetics using different column types from different vendors.[1] It will also describe the transfer of parameters from a 5-µm column to columns with particles < 2 µm from different vendors. The results show high resolution even under the high throughput conditions. The best results were achieved with the Agilent ZORBAX RRHD Eclipse Plus C18 HD 50 mm × 2.1 mm, 1.8 µm column. The criteria for precision of retention times and areas are fulfilled, and demonstrate the versatility of high speed applications.

Experimental

Instrumentation

An Agilent 1290 Infinity LC system with the following configuration was used:

G4220A	1290 Infinity Binary pump with integrated vacuum degasser and 35 µL Jet Weaver as mixing device
G4226A	1290 Infinity Autosampler
G1316C	1290 Infinity Thermostatted Column Compartment
G4212A	1290 Infinity Diode Array Detector
Software:	ChemStation B.04.02

Configuration of the Agilent 1290 Infinity LC system

Preparation of samples

Reference samples

The stock solution of each anesthetic was prepared by dissolving 10 mg of each compound in water in a 100 mL volumetric flask yielding a concentration of 100 µg/mL (Figure 1). Samples were prepared by mixing aliquots of each component to yield the final concentration. The reference sample used to check the separation was prepared by mixing 2.5 mL of each stock solution in a 10-mL flusk to yield a ready-to-use solution. As an example for the calibration samples: the solution used for calibration of the 10 µg/ml point was prepared by mixing 1 mL of each stock solution in a 10-mL volumetric flask and diluting it to the final volume with water. Calibration points used to evaluate the correlation were: 1, 2.5, 10, 25, 50, 100 µg/mL with the Agilent ZORBAX RRHD Eclipse Plus C18 50 mm × 2.1 mm, 1.8 µm column at 1.9 mL/min.

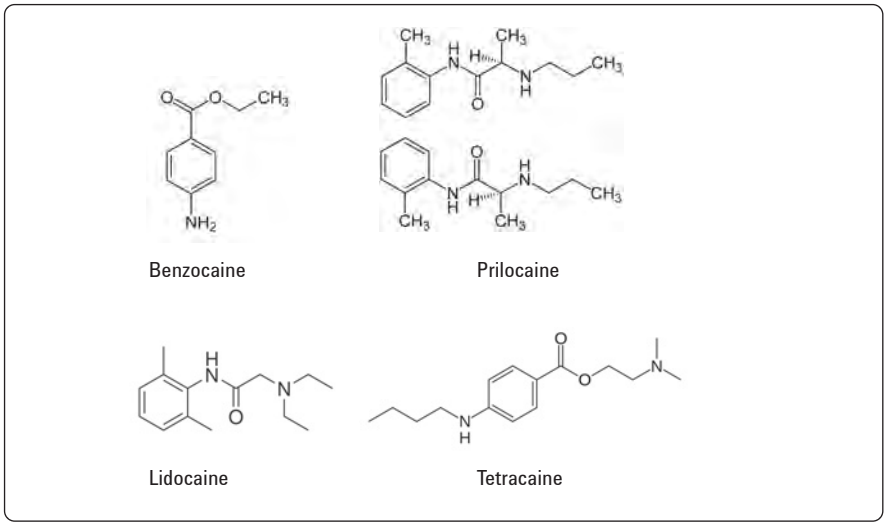

Figure 1
Chemical structures.

Chromatographic conditions

Columns	Agilent ZORBAX Eclipse Plus C18, 150 × 2.1 mm, 5 µm
	Agilent ZORBAX Eclipse Plus C18, 50 × 2.1mm, 3.5 µm
	Agilent ZORBAX RRHD Eclipse Plus C18, 50 × 2.1 mm, 1.8 µm
	Waters BEH C18, 50 × 2.1 mm, 1.7 µm
Mobile Phase	A: 50 mM Ammonium formate, pH=8.2
	B: Acetonitrile

Detailed chromatographic conditions are listed in Table 1.

Setup for testing

With the following setup for the reference sample the transferred methods can be checked:

- Establishment of a chromatographic separation to compare the performance of different column types (Resolution > 2)
- Precision of areas must be < 1 % RSD.
- Precision of retention times must be < 0.5 % RSD.
- Linearity should be given at least with $R^2 > 0.999$
- With these limits and settings for testing the following samples were prepared and analyzed (Table 2).

	Agilent ZORBAX Eclipse Plus C18, 150 × 2.1 mm, 5 µm	Agilent ZORBAX Eclipse Plus C18, 50 × 2.1 mm, 3.5 µm	Agilent ZORBAX RRHD Eclipse Plus C18, 50 × 2.1 mm, 1.8 µm	Waters BEH C18, 50 × 2.1 mm, 1.7 µm
Flow rate	0.8 ml/min	0.5 ml/min	1.9 ml/min	1.5 ml/min
Gradient	0-1 min 0-28% B 1-7 min 28-70% B	0-4 min 0-70% B	0-0.45 min 0-70% B	0-0.45 min 0-70% B
Temperature	40 °C	40 °C	40 °C	40 °C
Injection volume	5 µl	5 µl	1 µl	1 µl
Detection	DAD, Signal 225/4, Reference 400/80, standard Cell (1 µl, 10 mm)			
Data rate	2 Hz	10 Hz	80 Hz	80 Hz
Maximum pressure	98 bar	65 bar	945 bar	865 bar

Table 1
Instrument conditions.

Sample	Purpose	Number of injections
Blanc solution	Verify baseline stability and identify artifacts	3
Reference sample	Verify precision of areas and retention times for reference solution	10
Calibration	Verify linearity	3 for each level
Highest concentration and Blanc solution	Verify carryover	3 of each sample

Table 2
Sample setup for testing.

Results and discussion

Due to the varied pharmacological properties of local anesthetics, they are used in many different anesthesia applications.

The chromatographic properties result from the chemical structure; many of them are aminoesters or aminoamides. These primary or secondary amines (Figure 1) tend to tail on RP-columns at low pH-values. Separations in the mid or high pH-range (pH=8-10) are preferred to avoid asymmetric peaks. Therefore, RP materials with high stability such as the ZORBAX Eclipse Plus C18 are needed. A typical chromatogram for a separation of four local anesthetics, with the impurity originated from tetracaine, at pH = 8.2 is shown in Figure 2. The instrument conditions are listed in Table 1. A simple mixture for the eluents without attention to the baseline was chosen.

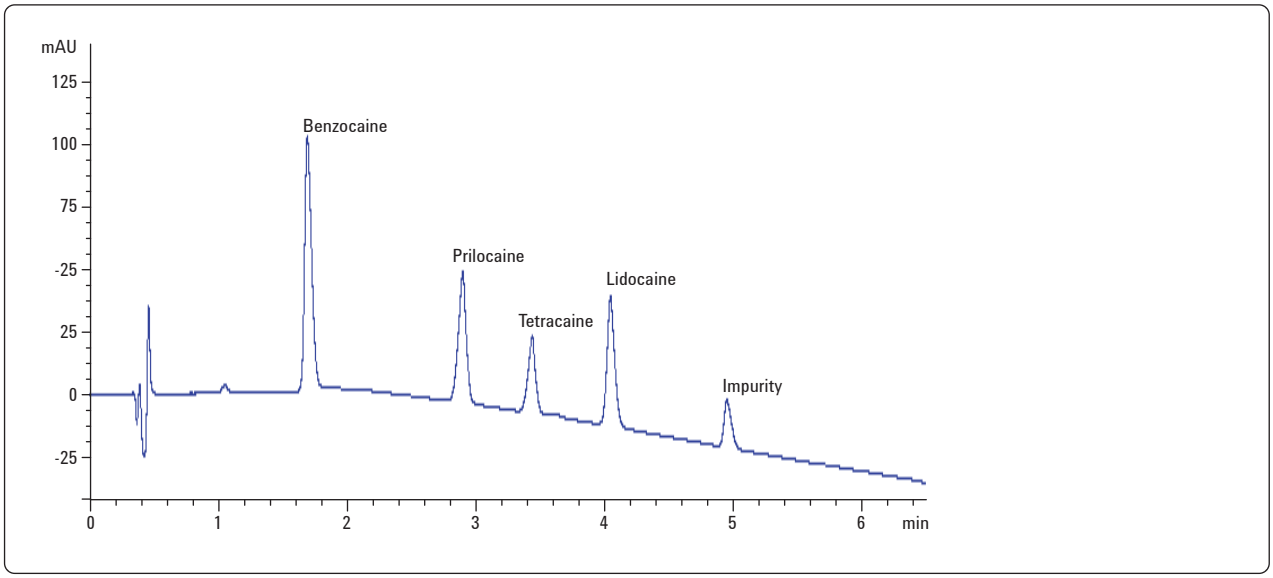

Figure 2
Separation of local anesthetics on Agilent ZORBAX Eclipse Plus C18, 150 × 2.1 mm, 5 µm.

When using 3.5 µm material to shorten analysis time, the parameters of the separation with the ZORBAX Eclipse Plus C18, 150 × 2.1 mm column with 5 µm-material can be used. With the Method Translator the new parameters can easily be calculated (Figures 3 and 4).

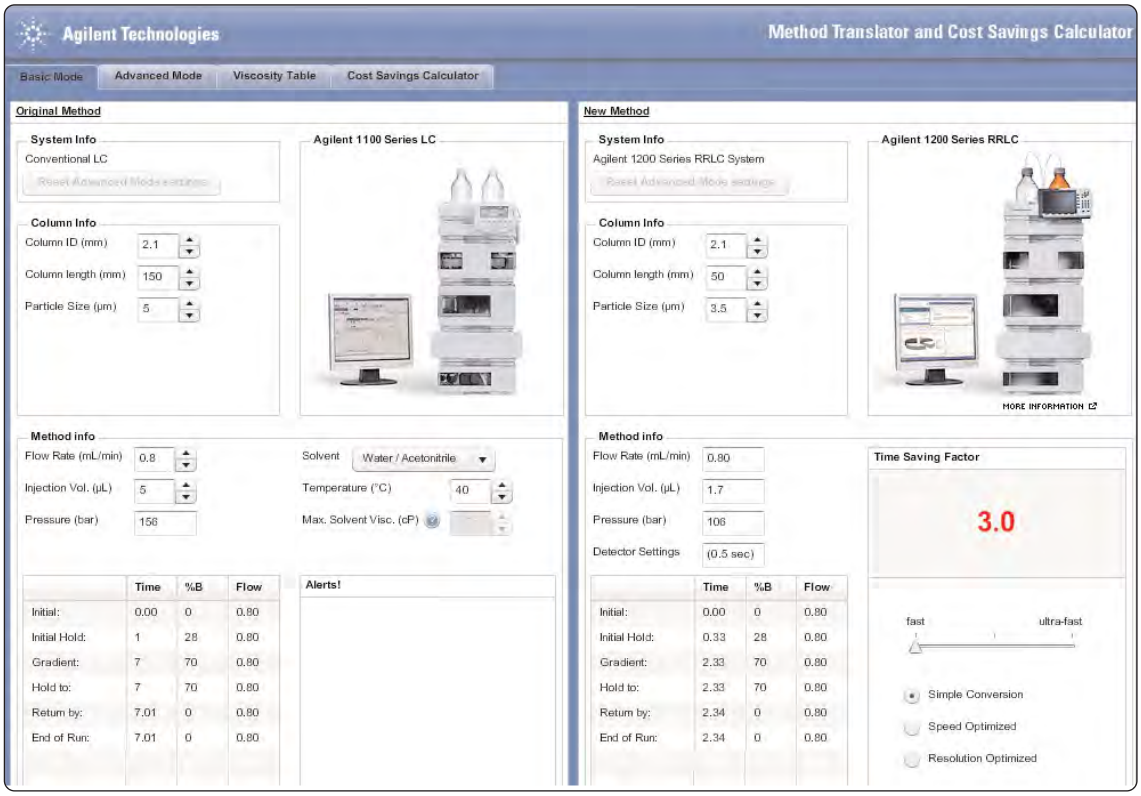

Figure 3
Calculating the new parameters for the 3.5 µm column with the Method Translator Software.

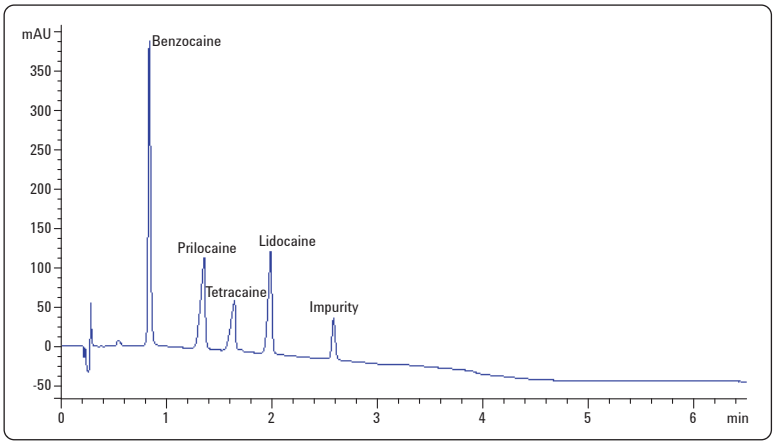

Figure 4
Separation of local anesthetics on Agilent ZORBAX Eclipse Plus C18, 50 × 2.1 mm, 3.5 µm.

To further reduce the analysis time the parameters can be transferred to columns with particles < 2 µm. Leaving the column dimension constant (50 mm × 2.1 mm) will improve the separation power because of the increased number of plates. When the system is independent of back pressure like the 1290 Infinity LC system the flow and the gradient shape can be increased, which dramatically decreases the run time. The results can be seen with separations in Figures 5 and 6. Both the Waters BEH C18 and the Agilent ZORBAX RRHD Eclipse Plus columns with particles < 2 µm provide a full separation of all peaks.

Table 3 lists the results of resolution calculations for all anesthetics separated with the different columns. For all peaks the resolution is greater than 2.5, even at highest flows and highest back pressures. With the BEH column the back pressure is remarkably higher resulting in lower flow rates and the peak shape shows some tailing, which is probably reduced at higher pH values. With the ZORBAX RRHD Eclipse Plus column no peak tailing at pH = 8.2 is seen as a result of good endcapping. An overall run time of 1.00 min is achieved with a flow of 1.9 mL/min. This is because reequilibration is done in 30 s, due to the small system and delay volume of the column.

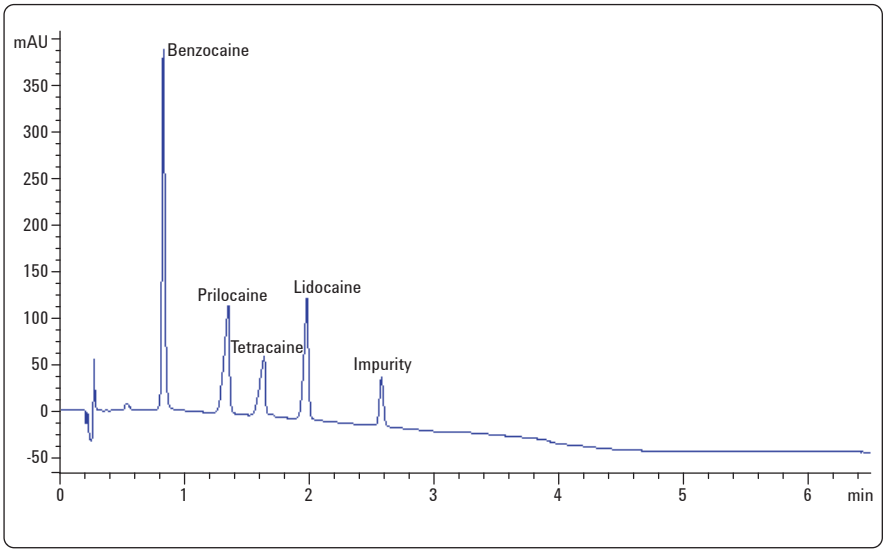

Figure 5
Separation of local anesthetics on Waters BEH C18, 50 × 2.1 mm, 1.7 µm, Flow: 1.5 ml/min.

Compound	Agilent ZORBAX Eclipse Plus, 150 × 2.1 mm, 5 µm, 0.8 ml/min	Agilent ZORBAX Eclipse Plus, 50 × 2.1 mm, 3.5 µm, 0.5 ml/min	Agilent ZORBAX RRHD Eclipse Plus, 50 × 2.1 mm, 1.8 µm, 1.9 ml/min	Waters BEH C18, 50 × 2.1 mm, 1.7 µm, 1.3 ml/min
Benzocaine	-	-	-	-
Prilocaine	11.57	8.52	3.91	4.23
Tetracaine	5.15	3.57	2.56	2.64
Lidocaine	5.94	4.65	5.13	3.88
Stop time	6 min	3 min	0.5 min	0.6 min

Table 3
Resolution of the anesthetics depending on column types (see Figures 2, 4-6).

Table 4 shows the data for the precision of the method applied to the separation with the Agilent ZORBAX RRHD Eclipse Plus C18, 50 mm × 2.1 mm column, and the high flow rate of 1.9 mL/min (Figure 6).

The data for precision of the retention times prove the high precision and stability of the flow, even at high pressure and high flow rates. The data also reflect the high efficiency of the new low volume jet weaver as a gradient mixing tool. The data for precision of areas show the good performance of the Autosampler. This is also illustrated by correlation coefficients for all components greater than 0.999 (Figure 7) with lidocaine as a reference.

	Retention times		Areas		Linearity
	Mean	RSD	Mean	RSD	R^2
Benzocaine	0.214	0.214	2,885,499.30	0.485	0.9998
Prilocaine	0.286	0.289	1,930,676.50	0.366	0.9999
Tetracaine	0.318	0.207	1,424,720.20	0.451	0.9998
Lidocaine	0.373	0.144	1,882,887.60	0.371	1.0000

Table 4
Determination of the precision of areas and retention times for the reference sample (chromatogram see Figure 6), linearity for 1-100 µg/ml calibration.

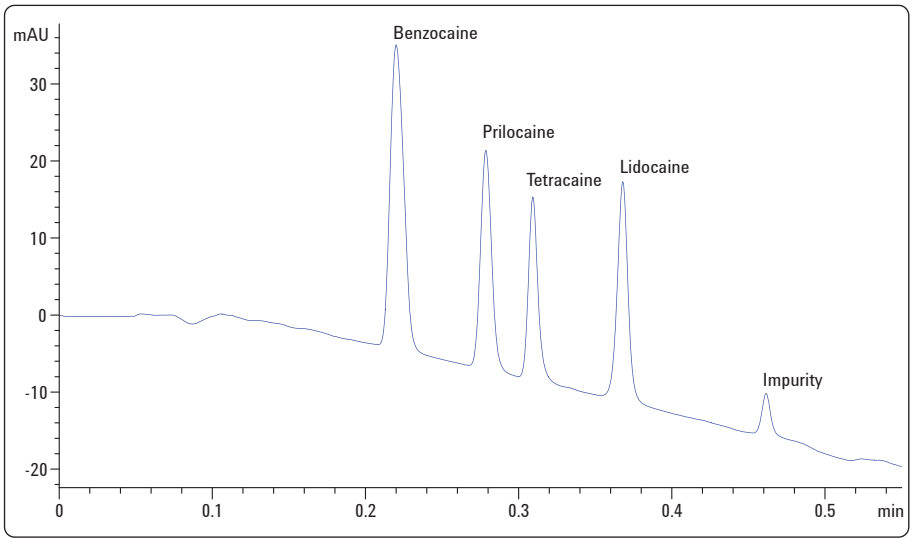

Figure 6
Separation of local anesthetics on Agilent ZORBAX Eclipse Plus RRHD C18, 50 × 2.1 mm, 1.8 µm, Flow: 1.9 ml/min.

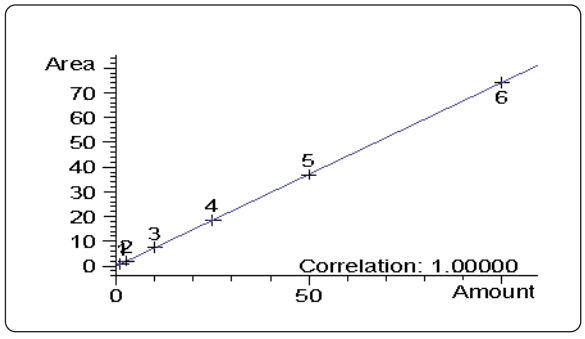

Figure 7
Calibration curve for lidocaine as example for all anesthetics.

A further test to evaluate the sampler performance is the determination of carryover. Figure 8 shows the chromatogram after an injection of the mixture. No carryover can be seen.

Conclusion

The new Agilent 1290 Infinity LC is designed to provide the highest speed, resolution and sensitivity. A new power range allows you to operate with columns filled with any particle type, any column dimensions, or any mobile and stationary phase. The 1290 Infinity LC is the first system to allow method transfer from any Agilent HPLC System to a new system.

The example separation of four local anesthetics has also shown that applications with conventional columns will run with high performance. The Method Translator is a helpful tool to make these methods faster. The good results of method transfer show that the selectivity and performance of the Agilent ZORBAX Eclipse Plus C18 material is independent of the particle size. The overall run time of the final method of 1.00 min, including reequilibration shows the infinite number of opportunities for establishing high resolution and ultrafast liquid chromatography.

With the new low volume jet weaver, effective gradient mixing provides high precision of gradient times.

The results shown in Tables 3 and 4 illustrate that all criteria for the precision of determination: areas, retention times, and resolution are fulfilled. Also the coefficients for linearity for all components are better than 0.999. All results explicitly show the applicability of the 1290 Infinity LC system for quality control testing as well as for high resolution and ultrafast liquid chromatography.

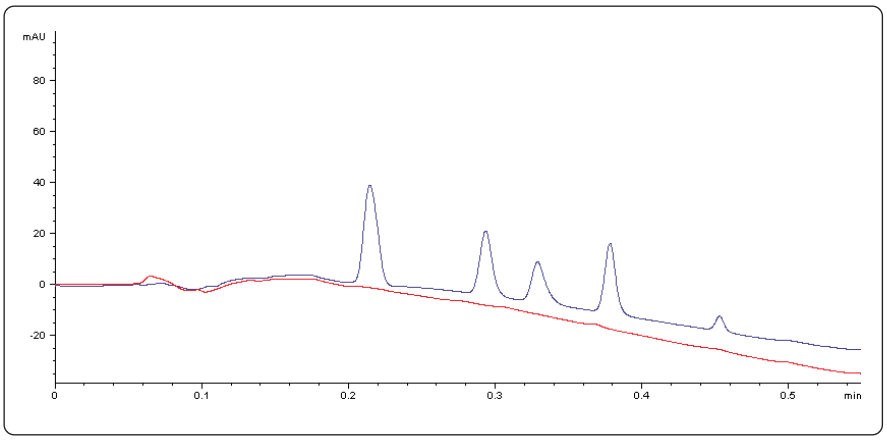

Figure 8
Blank injection to detect any carryover (blue-mixture, red-blank).

The infinite possibilities of the system are best shown by the overall run time including reequilibration of 1.00 min. The good flow design of the 1290 Infinity LC system assures the user that no band broadening or peak distortion will occur to hinder the separation power. In addition the "system pressure" will not limit the possible high operating flow rates.

In summary, the data obtained in this Application Note demonstrates the versatility and reliability of the Agilent 1290 Infinity LC system. It shows fast method transfer from or to any column and particle size and therefore, is applicable for any desired application. The Agilent 1290 Infinity LC system meets highest requirements for every LC function.

References

1. R. Ricker, "Bonded-phase selectivity, separation of local anesthetics", Agilent Publication 5988-6424EN, 2002

www.agilent.com/chem/1290

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Agilent Technologies

Several ZORBAX RRHD 1.8 µm Selectivities Facilitate Method Development

Application Note

Pharmaceuticals

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Abstract

Agilent Rapid Resolution High Definition columns offer new levels of productivity for HPLC because they are made with 1.8 µm particles, they are stable to 1200 bar and they are available in a variety of bonded phases for large potential selectivity differences. This application note describes the ways in which RRHD columns allow for selectivity refinement of methods for selected endocannabinoids.

Introduction

Rapid Resolution High Definition (RRHD) columns, used on UHPLC instruments, provide significant productivity enhancements because they are stable to 1200 bar and can withstand higher flow rates. The Eclipse Plus phase features a double end-capped process and unique bonding that delivers exceptional peak shapes across a broad range of analytes. This makes it an exceptional column for method development. Many users of UHPLC are working with analyses that require the highest level of sensitivity and resolution. Sometimes, alternate C18 phases are desirable because of the additional selectivity refinements they allow.

Endogenous cannabinoids are neurotransmitters that naturally occur in animal organs, especially the brain, and have a role as intercellular messengers similar to the well-known acetylcholine, gamma aminobutyric acid (GABA), or dopamine. They are quite different, however, because endocannabinoids are lipophilic and found in cell membranes, whereas acetylcholine, GABA, and dopamine are highly water soluble and are found in the vesicles inside cells.

Anandamide or arachidonoylethanolamide (AEA) was the first endocannabinoid discovered in 1992. Since then, research has shown that these neurotransmitters play significant roles in many life functions, including memory, sleeping and eating patterns, and even implantation of the blastocyst (embryonic stage) in the uterus. Structures of anandamide and other endocannabinoids analyzed in this application are represented in Figure 1.

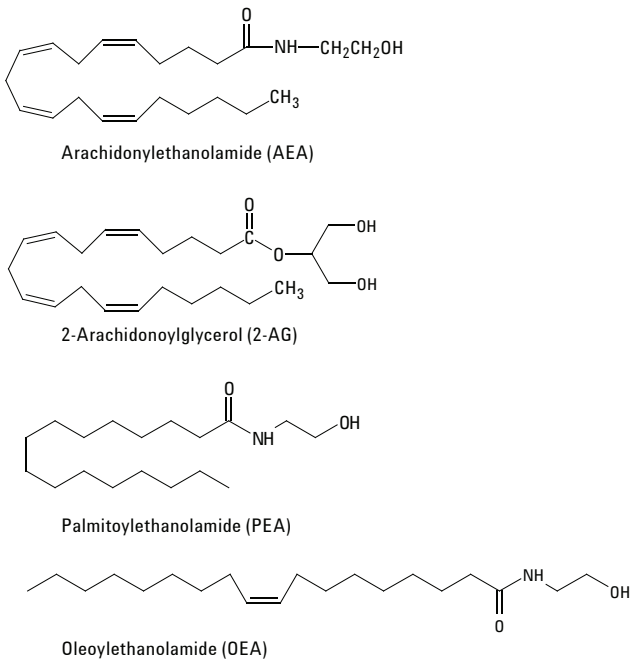


Figure 1. Encocannabinoid-related compounds.

Experimental

Four endocannabinoid fatty amides were obtained from Sigma-Aldrich (Bellefonte, PA, USA):

- Arachidonoylethanolamide (AEA)
- 2-Arachidonoylglycerol (2-AG)
- Palmitoylethanolamide (PEA)
- Oleoylethanolamide (OEA)

These were diluted in methanol to a concentration of about 1 to 5 mg/mL each component, then diluted 1:100 in 50% methanol/water for a final sample concentration of 0.01 to 0.05 mg/mL. The 2.1-mm ID columns are ideal for electrospray ionization-mass spectrometry (ESI-MS) because low flow rates (<1 mL/min) allow for optimal electrospray ionization and introduction to the high-vacuum mass spectrometer.

Long, 100-mm RRHD columns were used for this analysis to further improve efficiency and resolution. The longer RRHD columns coupled with Agilent's 1290 Infinity LC system and high linear velocity flow rates exploit the system and column's UHPLC pressure limits (1200 bar).

The following Agilent ZORBAX RRHD columns were used:

- Agilent ZORBAX Rapid Resolution High Definition (RRHD) Eclipse Plus C18, 2.1 mm × 100 mm, 1.8 µm
  Agilent Part Number: 959758-902
- Agilent ZORBAX Rapid Resolution High Definition (RRHD) Eclipse XDB-C18, 2.1 mm × 100 mm, 1.8 µm
  Agilent Part Number: 981758-902
- Agilent ZORBAX Rapid Resolution High Definition (RRHD) StableBond SB-C18, 2.1 mm × 100 mm, 1.8 µm
  Agilent Part Number: 858700-902
- Agilent ZORBAX Rapid Resolution High Definition (RRHD) Extend C18, 2.1 mm × 100 mm, 1.8 µm
  Agilent Part Number: 758700-902

The HPLC system was an Agilent 1290 Infinity LC with an Agilent 6410 Triple Quadrupole Mass Spectrometer.

– G4220A Binary Pump, with mobile phase A: H_2O and B: CH_3CN, each with 0.1% HCOOH. Analysis was isocratic at 1 mL/min, with varying amounts of CH_3CN.

– G4226A Automatic Liquid Sampler (ALS), with injection volume set to 1 µL.

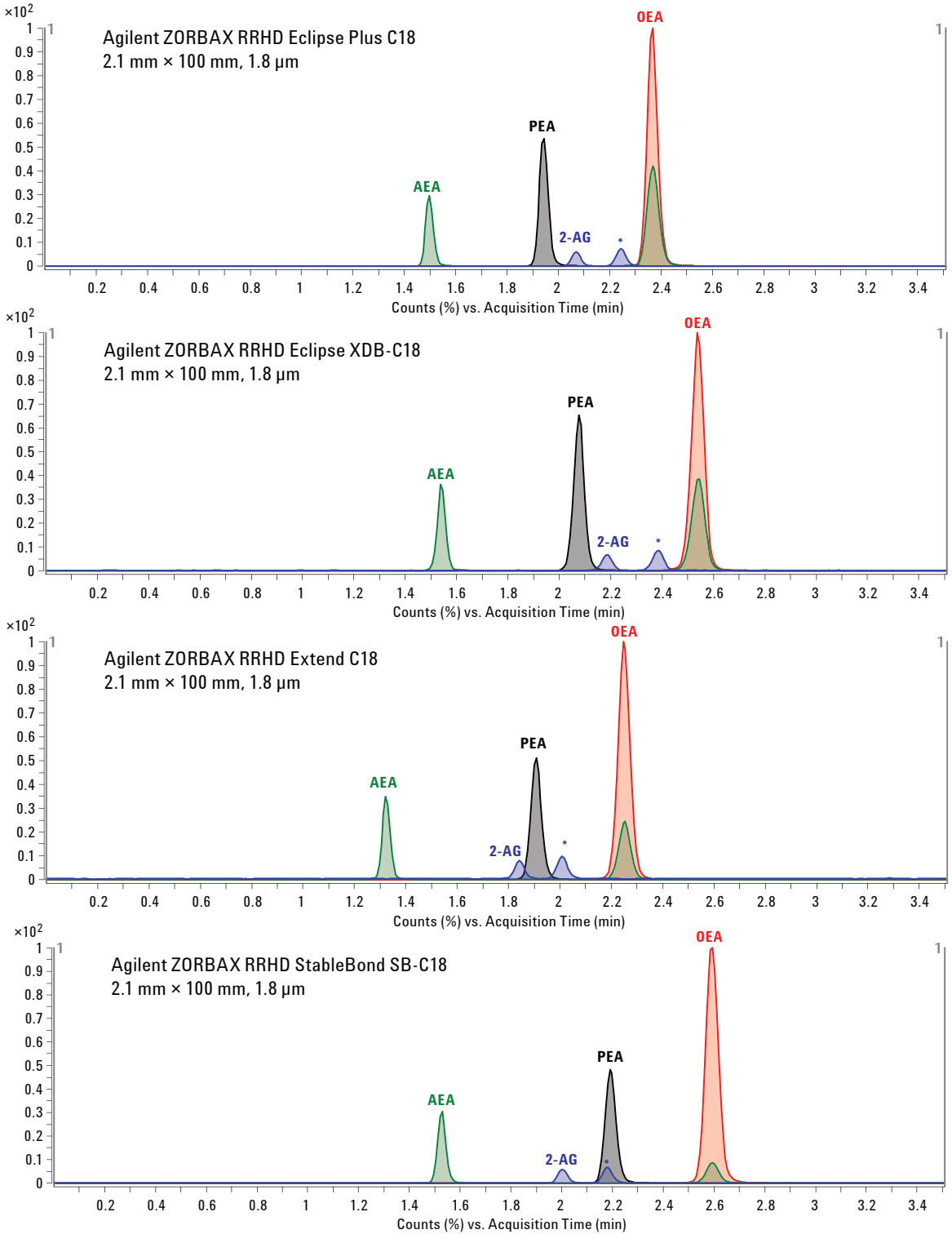
– G1316C Thermostatted Column Compartment (TCC), with temperature set to 30 °C.

– G6410A Triple Quadrupole Mass Spectrometer (QQQ), with MS source: electrospray AP-ESI; drying gas temperature and flow: 325 °C, 12 L/min; nebulizer gas pressure: 35 psi; capillary voltage: 3000 V; in MS2Scan mode from 290 to 390. Individual components were monitored at 348, 300, 379 and 326 for AEA, PEA, 2-AG and OEA respectively.

Discussion of Results

Examining Selectivity (α) for Best Resolution

The variety of stationary phases available on ZORBAX columns makes them useful for method development, especially for changing selectivity. Having a variety of bonded phases (columns) available to sequentially try in method development analyses demonstrates the different selectivity easily gained from the columns. Figure 2 is an overlay of four different C18 bonded phases available on ZORBAX RRHD 1.8 µm particles. All have a symmetrical peak shape and similar retention. Notice however, that although only four compounds comprise the endocannabinoid sample, a fifth peak is detected. This impurity has a mass of 379, and is believed to be 1,3-arachidonolyglycerol, a rearrangement of 2-AG. This is based on extracted ion MSD data.



*The second blue peak is an impurity, believed to be 1,3-arachidonolyglycerol, a rearrangement of 2-AG

Figure 2. The selectivtiy of four Agilent ZORBAX RRHD C18 columns is compared using a method for endocannabinoids (see Experimental section for detailed method parameters).

The differences in selectivity between the four columns are due to the subtle, yet important differences in bonding, such as the type of bonding, the endcapping, or the amount and type of silanols on the silica. Other factors that influence selectivity including mobile phase composition, temperature, and pH are identical.

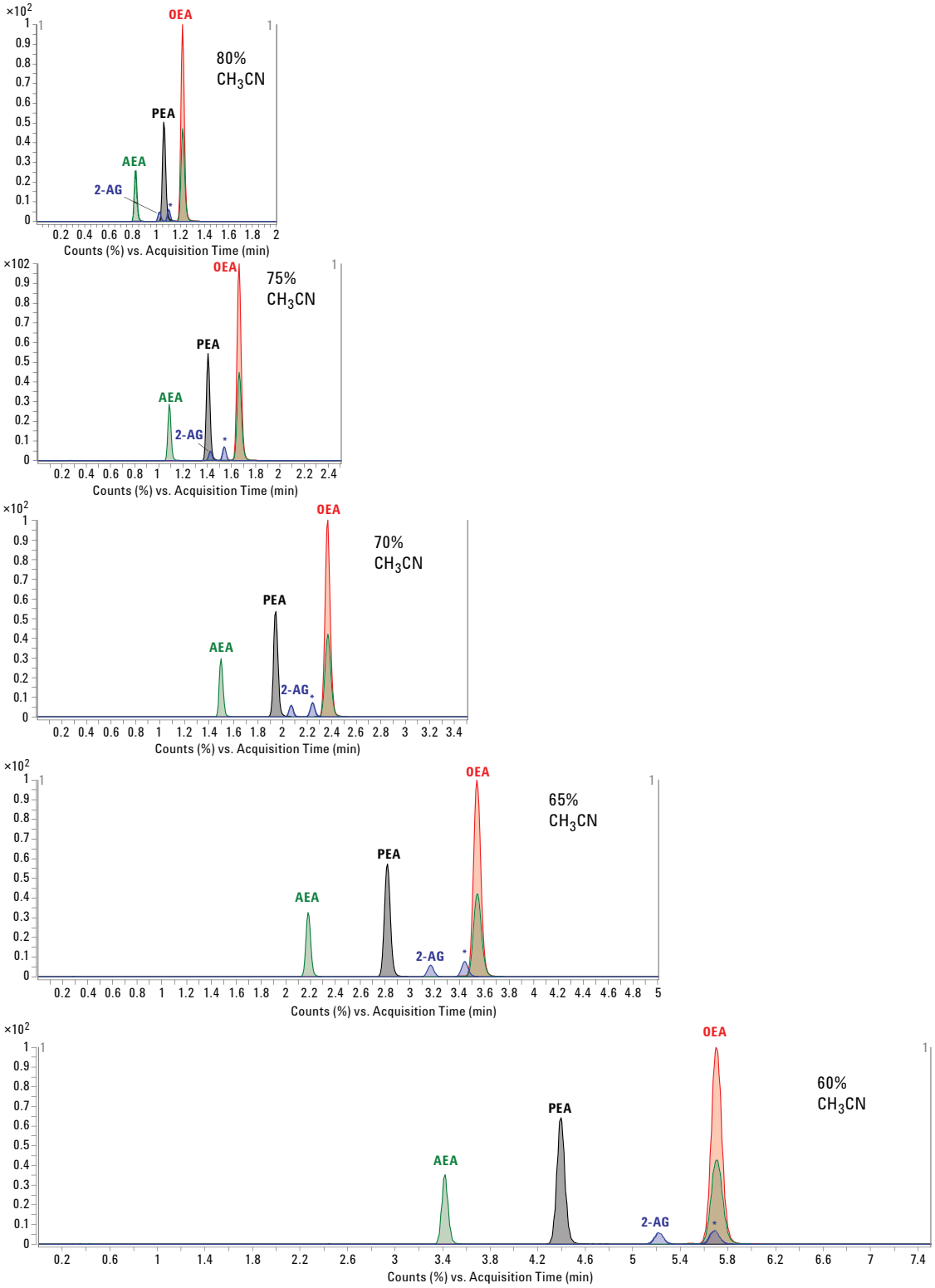
These four C18 bonded phases differ slightly, all based on 1.8 µm ZORBAX Rx-SIL silica, though the silica is modified to improve peak shape with the Eclipse Plus C18 column. Each column has its strengths. The Eclipse Plus is the most inert of the four. The Eclipse XDB and the Extend have slightly more silanols and the StableBond has the most exposed silanols. Silanols are often thought to be deleterious but can be used to provide selectivity.

Another way to alter selectivity is to change the mobile phase. In MSD applications, volatile mobile phases (MS friendly) are preferred. Here, a popular aqueous acetonitrile and formic acid mixture was used. However making different mobile phases for experimentation followed by flushing/equilibrating the HPLC system consumes more time than simply substituting columns. The goal here was rapid method development.

Examining Retention (k) for Best Resolution

Because Eclipse Plus C18 offers the most promising selectivity for the endocannabinoids, it will be used to address the retention factor of the resolution equation. The mobile phase strength (percent organic) was changed in 5% increments to alter the retention factor, and hopefully improve resolution. The entire sequence of runs to determine the best organic strength lasted less than two hours, including each run in triplicate with a 10-min equilibration between mobile phase compositions. This comparison used 100-mm RRHD columns; if 50-mm columns were used, the screening time could have been halved.

Figure 3 shows the effect of changing the organic solvent concentration on resolution. Typically, in reversed phase chromatography, k increases as the organic strength weakens. The 2-AG and 1,3-AG peaks are retained at a different rate (move faster than the other peaks). At 75% CH_3CN, the 2-AG coelutes with the PEA, and the 1,3-AG is resolved. At 60% CH_3CN, the two peaks have shifted so the 2-AG is completely resolved, but the 1,3-AG coelutes with the OEA peak. With this 100-mm RRHD Eclipse Plus C18 column, all five peaks are completely resolved with 70% CH_3CN.



*The second blue peak is an impurity, believed to be 1,3-arachidonolyglycerol, a rearrangement of 2-AG

Figure 3. The strength of the mobile phase is adjusted with this endocannabinoid method on Agilent ZORBAX Eclipse Plus to show the influence of k on resolution (see Experimental section for detailed method parameters).

Conclusions

The variety of bonded phases (selectivities) available on Agilent ZORBAX 1.8 µm packing adds to the strength of the column's 1200 bar stability. In addition to the four C18 phases described in this work, a variety of other phases are available, or in development, for RRHD columns, making them a flexible, scalable option for pharmaceutical labs that need to ensure method compatibility throughout the development cycle.

Reference

1. Henderson, J., Long W. "Exploiting RRHT Columns with Different C18 Selectivities to Quickly Develop Methods for Endocannabinoids," 2007, Agilent Technologies publication 5989-6128EN.

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Agilent Technologies

Speed up the LC Analysis of Notoginsenoside R1 and Ginsenosides Rg1, Re, and Rb1 in Compound TCM Using the Agilent 1290 Infinity LC system and ZORBAX RRHD 1.8 µm Column

Application Note

Pharmaceuticals

Introduction

Ginsenosides belong to a class of steroid glycosides found exclusively in the plant genus Panax (ginseng). Ginsenosides have been the target of research, because they are viewed as the active compounds behind the claims of ginseng's efficacy. As quality indicators, determination of those compounds is required for quality control methods of most ginsenosides containing TCM and neutraceutical products. Traditionally, it takes almost two hours to analyze these compounds with the China Pharmacopeia method using conventional HPLC. This Application Note describes a fast quality control method for the analysis of notoginsenoside R1 and Ginsenosides Rg1, Re and Rb1 using the Agilent 1290 Infinity LC system and an Agilent ZORBAX RRHD 1.8 µm column. Compared to conventional methods, the rapid method is much faster, and maintains the same performance and quality of separation. In addition, solvent consumption is also dramatically reduced.

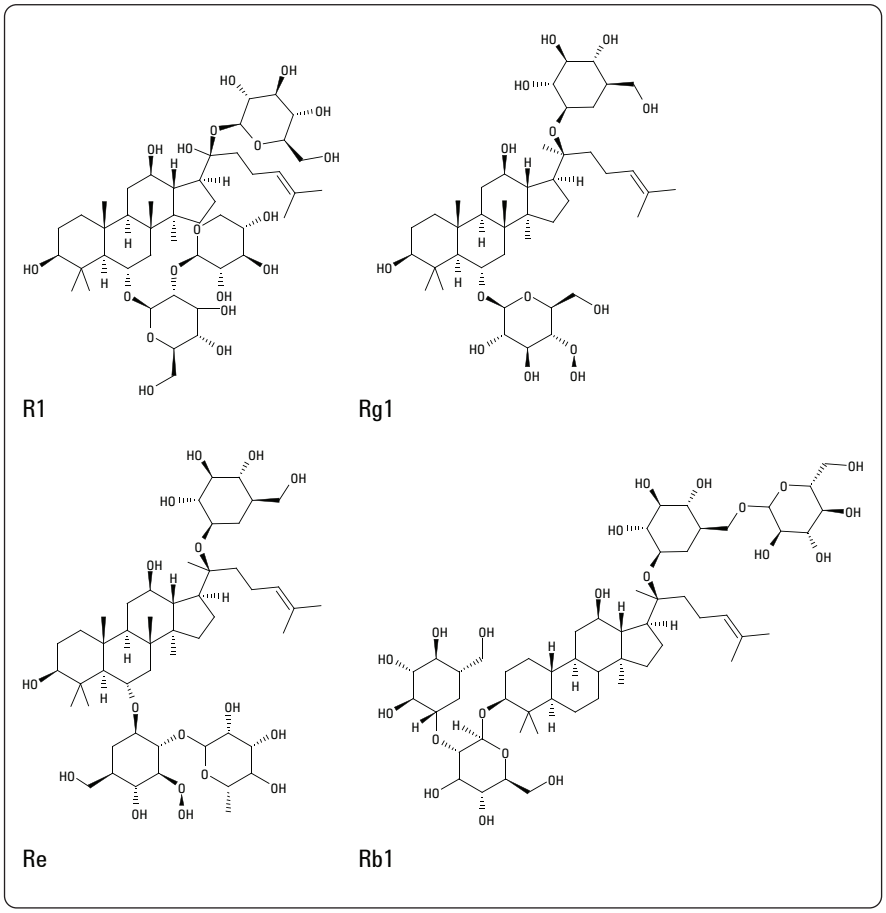

Figure 1
Structure of notoginsenoside R1 and Ginsenosides Rg1, Re and Rb1.

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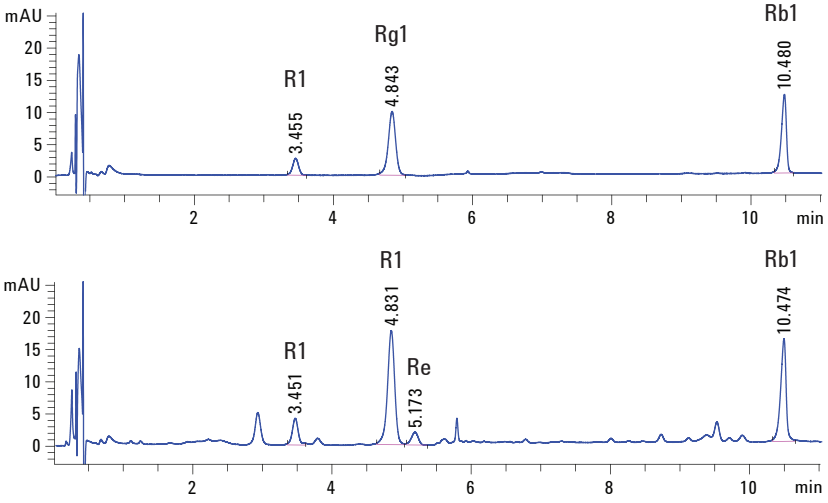
Results and Discussion

Compound TCM products normally consist of multiple active components that come from different plants. Because the matrix of those kinds of products is very complex, separation often requires a gradient method with a long period of time. In this Application Note, an extract of Fufang Dansen dripping pill, which is a compound TCM product, was analyzed. The original LC method used a 4.6 mm × 150 mm, 5 µm column, and took almost 120 mins to separate R1, Rg1, Re and Rb1, and recondition the column to initial gradient conditions. By using the Agilent 1290 Infinity LC system and a 2.1 mm × 50 mm RRHD column, method transfer and optimization were completed quickly. The analysis was completed in 12.6 min, while maintaining the same or even better resolution for the critical peak pair Rg1 and Re. Since the column chemistry did not change from 5 µm to 1.8 µm (both used Stablebond C18 chemistry), the separation achieved the same selectivity. In addition, solvent consumption was reduced from 120 mL to 5 mL.

Configuration

The Agilent 1290 Infinity LC system consisted of:

- Agilent 1290 Infinity Binary Pump with Integrated Vacuum Degasser (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1200 Diode Array Detector (G1315C)



Upper:	Chromatogram for standards; Bottom: chromatogram for sample
Condition:	Sample: extract of Fufang Danshen dripping pills, 0.4 mL/min,
Gradient:	0 min-19% ACN, 81% H_2O, 4.5 min-20% ACN, 5 min-25%ACN, 10 min-30% ACN, 10.50 min-30% ACN, 10.60 min-90% ACN,
Stoptime:	11.60 min.
Posttime:	1.00 min. 203 nm,
Column temperature:	20.00 °C
Column:	Agilent ZORBAX Stablebond C18 RRHD 2.1 mm × 50 mm, 1.8 µm

Figure 2
Separation of R1, Rg1, Re and Rb1 achieved with an Agilent 1290 Infinity LC system and Agilent ZORBAX StableBond C18, 1.8 µm RRHD column.

Conclusion

The shorter STM column reduces the separation time. The low delay volume of the Agilent 1290 Infinity LC system allows faster re-equilibration after gradient, especially when using a narrow bore column at a lower flow rate. The combination of those two factors allows fast analysis of the complex components of the TCM product, with much lower solvent consumption.

www.agilent.com/chem/1290

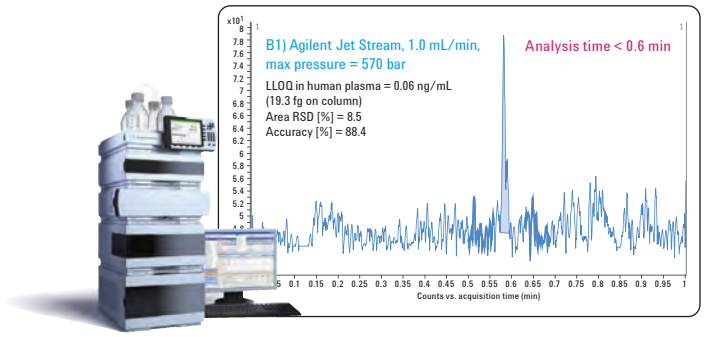
June 1, 2010
Publication Number 5990-5861EN

Agilent Technologies

High-throughput bioanalytical method development using UHPLC/triple quadrupole mass spectrometry

Application Note

Drug Discovery, Drug Development



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Abstract

Several factors cause fast LC/MS/MS method development in the bioanalytical area to be an arduous task. In order to maintain sensitivity while speeding up analysis time, target analytes should not elute in the chromatographic region affected by ion suppression. The scan speed of the mass spectrometer must be fast enough to acquire an adequate number of data points to define the narrow peaks generated using sub-2 µm columns. At typical fast LC conditions, current HPLC systems (pressure limit ≤ 400 bar (5800 psi)) would yield back pressures close to, or greater than the threshold limit. In this application example, we utilized the Agilent 1290 Infinity LC system coupled to an Agilent 6460 Triple Quadrupole mass spectrometer comprising thermal gradient focusing ESI (Agilent Jet Stream technology, AJS) to streamline high-throughput bioanalytical method development using alprazolam spiked in human plasma (concentration range: 2 nM to 5000 nM, corresponding to 0.06 ng/mL to 1544 ng/mL). A 100-µL sample of spiked plasma was precipitated with three parts of ACN and centrifuged. A 200-µL amount of the supernatant was diluted with three parts of H_2O containing 0.1% formic acid (FA). Each sample was injected three times. AJS technology was compared to conventional orthogonal ESI using generic source values.

The Agilent 1290 Infinity LC Triple Quadrupole MS/MS system, which allows flow rates up to 2 mL/min, pressures up to 1200 bar, and dwell times as low as 1-2 ms achieved an analysis time of less than 0.5 min without sacrificing quantitative data quality. The greater column efficiency of the Agilent rapid resolution high definition columns (RRHD) resulted in narrow peaks, increased analyte peak height, excellent resolution from matrix components, and improved analyte response (sensitivity).

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Instrument Conditions

Agilent 1290 Infinity LC MS/MS system: Agilent 1290 Infinity LC System comprising binary pump with integrated degasser, high performance autosampler with thermostat and thermostatted column compartment, Agilent 6460A Triple Quadrupole LC/MS with AJS Technology or with conventional orthogonal ESI.

Conditions	
Column:	RRHD ZORBAX Eclipse Plus C18, 2.1 mm × 50 mm, 1.8 µm
Mobile phase:	A= 0.1% FA in H_2O, B= 0.1% FA in ACN
Injection volume:	5 µL
Method 1	
Column temperature:	50 °C
Flow rate:	1.0 mL/min
Gradient:	0 min 25% B, 0.8 min 90% B, 0.81 min 25% B, stop time 1.5 min
Method 2	
Column temperature:	50 °C
Flow rate:	1.2 mL/min
Gradient:	0 min 25% B, 0.67 min 90% B, 0.68 min 25% B, stop time 1.37 min.
MS Scan type:	MRM
Polarity:	Positive
Parameters	
Drying gas temperature:	350 °C (ESI and ESI + AJS)
Drying gas flow:	10 L/min (ESI + AJS), 13 L/min (ESI)
Sheath gas temperature:	400 °C (ESI + AJS)
Sheath gas flow:	12 L/min (ESI + AJS)
Nebulizer pressure:	35 psig (ESI + AJS), 60 psig (ESI)
Nozzle:	0 V (ESI + AJS)
Capillary:	3500 V (ESI and ESI + AJS)
Transition:	309.2→281.1
Fragmentor:	145 V, CE: 24 V
Dwell time:	50 ms

Chromatograms

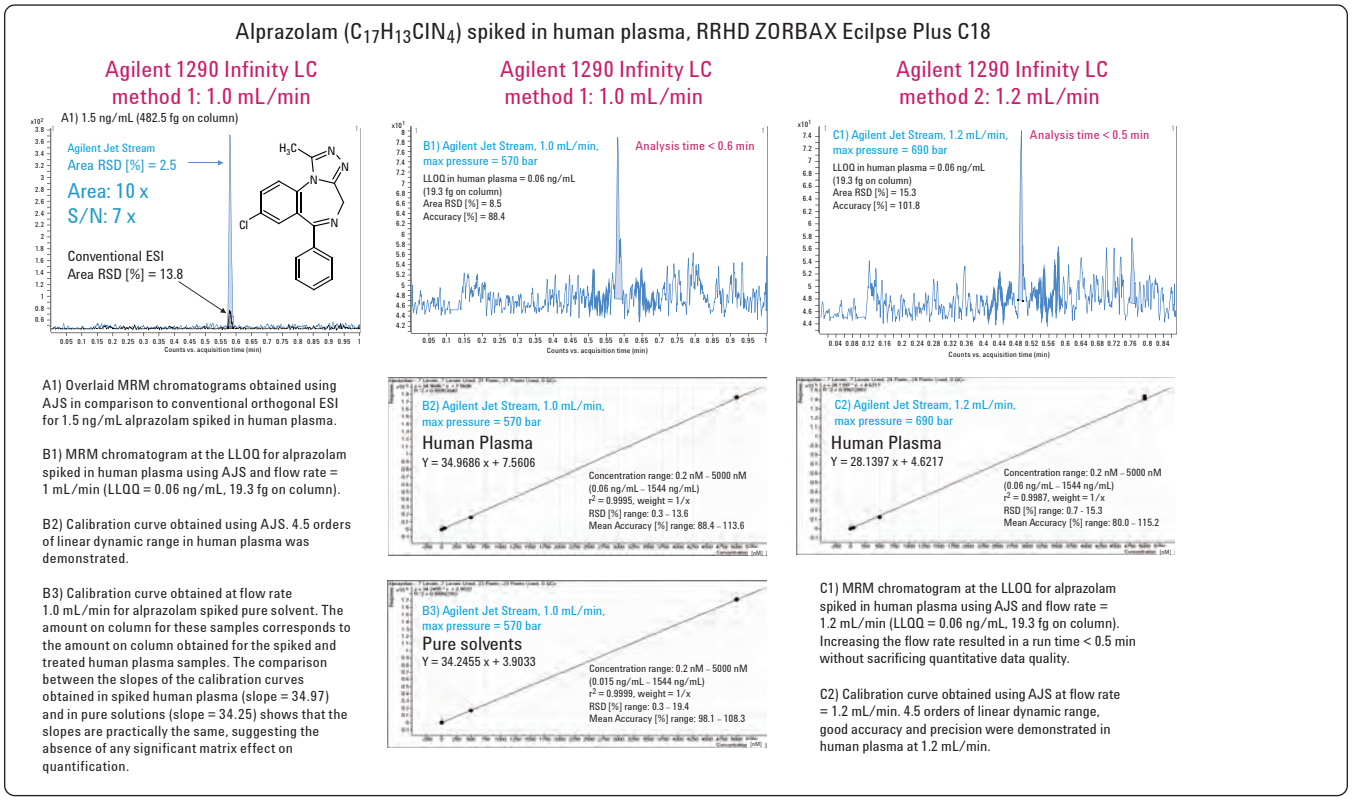


Figure 1.

A1) Overlaid MRM chromatograms obtained using AJS in comparison to conventional orthogonal ESI. B1) and C1) MRM chromatograms at the LLOQ (0.06 ng/mL, 19.3 fg on column) using AJS and flow rates = 1.0 and 1.2 mL/min, respectively. B2) and C2): Calibration curves obtained using AJS at 1.0 mL/min and 1.2 mL/min, respectively. B3) Calibration curve of alprazolam in pure solvents obtained using AJS at 1.0 mL/min shows practically the same slope in comparison to human plasma indicating the absence of any significant matrix effect.

www.agilent.com/chem

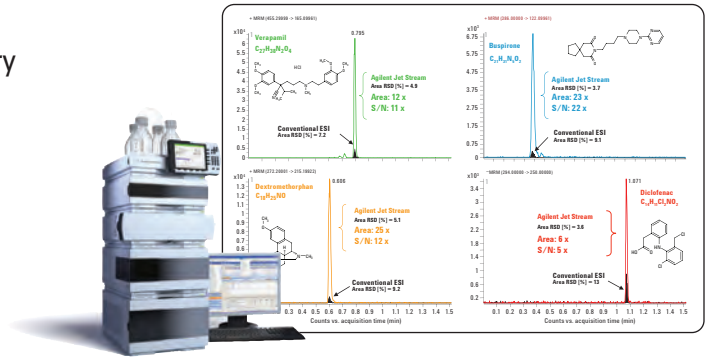
December 1, 2009
Publication Number 5990-4933EN

Agilent Technologies

Extended ionization capability of thermal gradient focusing ESI in high-throughput in-vitro ADME assays

Application Note

Drug Discovery



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Abstract

Several factors cause fast LC/MS/MS method development in the drug discovery area to be an arduous task. Combination of ESI/APCI sources offers broad response with multiple ionization modes, but optimization can be difficult and some sources limit flow rates to 1 mL/min, while others compromise chromatographic performance. The scan speed of the mass spectrometer needs to be fast enough to acquire an adequate number of data points across the narrow peaks generated using sub-2 µm columns. At typical fast LC conditions, current HPLC systems (pressure limit ~ 400 bar) would yield back pressures greater than the threshold limit. In this application example, we utilized the Agilent 1290 Infinity UHPLC system coupled with an Agilent 6460 Triple Quadrupole mass spectrometer comprising thermal gradient focusing ESI (Agilent Jet Stream technology, AJS) to streamline high-throughput bioanalytical method development using in-vitro metabolic stability samples. Incubations of the substrates buspirone, verapamil, dextromethorphan or diclofenac were carried out separately. An aliquot was taken at increasing time points from each incubate and then pooled together for analysis. AJS technology was compared to conventional orthogonal ESI using generic source values. The Agilent 1290 Infinity LC Triple Quadrupole MS/MS system, which allows flow rates up to 2 mL/min, pressures up to 1200 bar, dwell times as low as 1-2 ms, and polarity switching time of 30 ms, achieved an analysis time of less than 1.1 min without sacrificing quantitative data quality. Due to the high data acquisition rate provided by the Agilent 6460A Triple Quadrupole mass spectrometer, compounds ionizing in positive and negative modes were analyzed in a single run. An adequate number of data points (>10) could be collected across the extremely narrow peaks (Average full width half maximum (FWHM) < 1.3 sec) generated by the Agilent 1290 Infinity LC system. AJS showed enhanced area response and signal-to-noise in comparison to conventional orthogonal ESI.

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Instrument Conditions

Agilent 1290 Infinity LC MS/MS system: Agilent 1290 Infinity UHPLC System comprising binary pump/integrated degasser, high performance autosampler with thermostat and thermostatted column compartment, Agilent 6460A Triple Quadrupole LC/MS with AJS or with conventional orthogonal ESI.

Conditions	
Column:	RRHD ZORBAX SB-C18, 2.1 mm × 50 mm, 1.8 µm
Mobile phase:	A= 0.1% FA in H_2O, B= 0.1% FA in ACN
Injection volume:	1 µL
Method:	
Column temperature:	25 °C
Flow rate:	1.0 mL/min
Gradient:	25% to 80% B in 1 min, 1.25 min 80% B, 1.26 min 25% B, stop 1.8 min
Scan type:	MRM
Polarity:	Pos/Neg

Parameters	
Drying gas temperature:	350 °C (ESI / ESI + AJS)
Drying gas flow:	10 L/min (ESI + AJS), 13 L/min (ESI)
Sheath gas:	400 °C and 12 L/min (ESI + AJS)
Nebulizer:	35 psig (ESI + AJS), 60 psig (ESI)
Nozzle:	0 (+) 1500 V (–) (ESI + AJS)
Capillary:	3500 V (±) (ESI / ESI + AJS)
Dwell time:	5 ms

Chromatograms

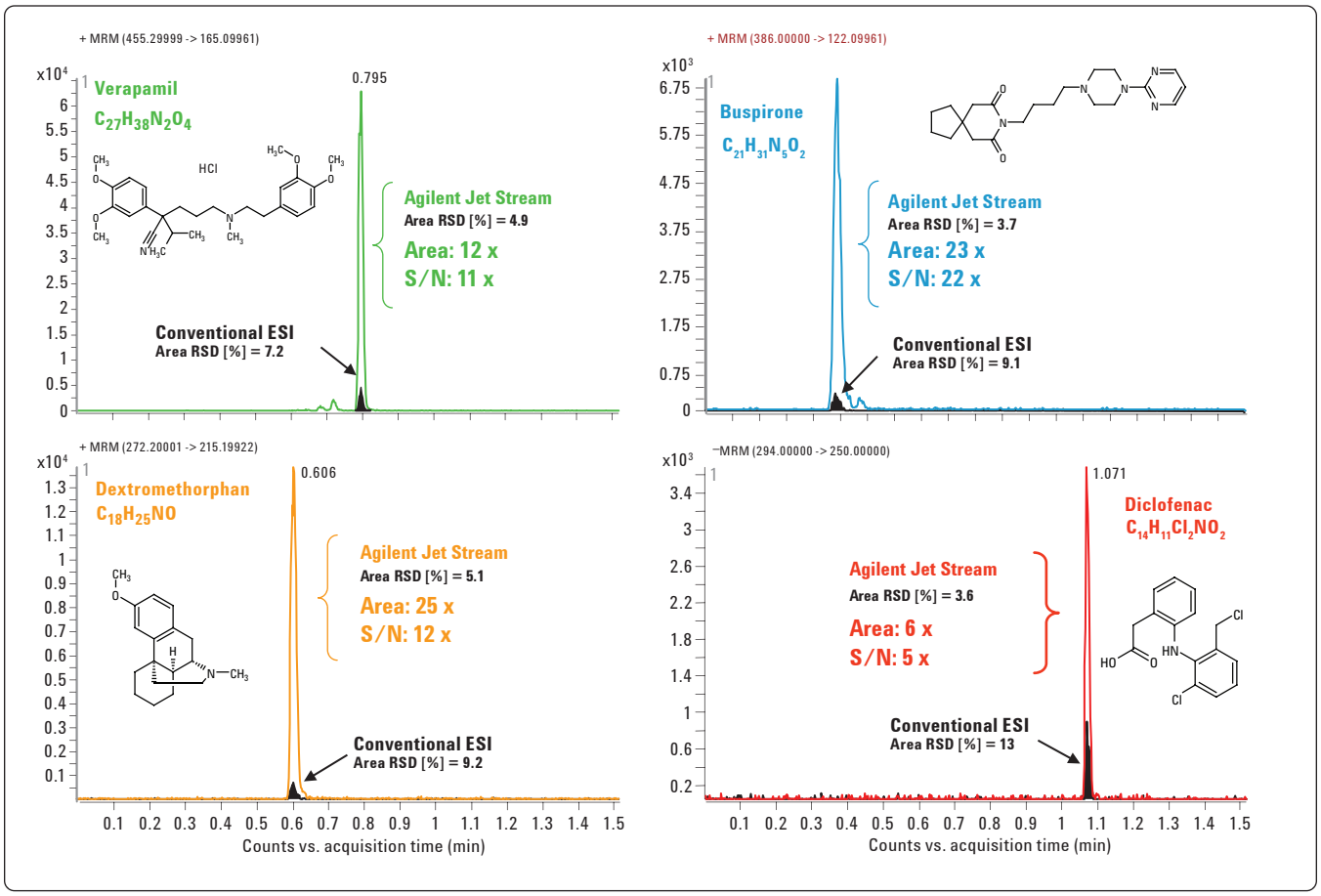

Figure 1.
Overlaid MRM chromatograms obtained using AJS in comparison to conventional orthogonal ESI for the metabolic stability substrates after 35 minutes of incubation with rat liver S9 fraction.

www.agilent.com/chem

Deceember 1, 2009
Publication Number 5990-4932EN

Agilent Technologies

Fast analysis of fat soluble vitamins using the Agilent 1290 Infinity LC and ZORBAX RRHT and RRHD 1.8 µm columns

Application Note

Food Analysis

Author

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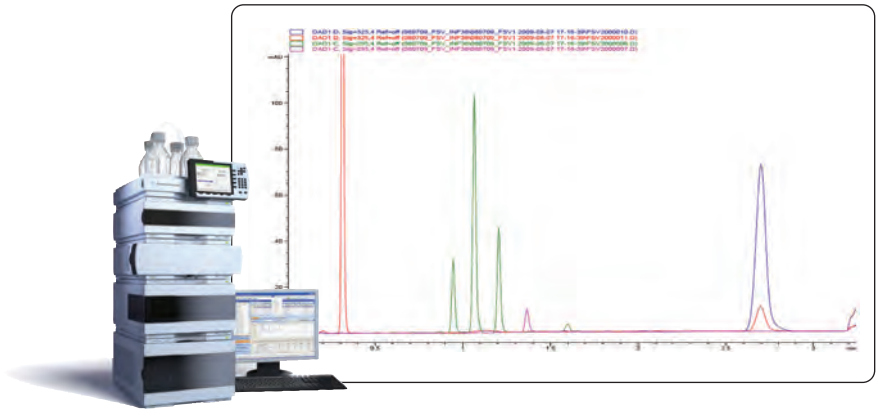

Abstract

The Agilent 1290 Infinity LC has significant capabilities for a wide range of HPLC and UHPLC applications. With a broader power range (that is, the combination of pressure and flow capabilities) than any other commercially available system, and the flexibility to operate a wide range of column dimensions and particle sizes, it is extremely useful for method transfer from any HPLC or UHPLC to the 1290 Infinity LC system. It allows the user to access capabilities not otherwise available.

Introduction

The speed and high resolution are demonstrated by a separation of fat-soluble vitamin isomers and esters, at a high pressure and flow rate. At 2 mL/min, utilizing a simple 1-min gradient and a 3.0 x 50 mm, 1.8 µm column, the analysis time is only 3 min including the late eluting retinyl palmitate component. The separation of the main components is shown in Figure 1.

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The speed, resolution and flexibility of the system are further demonstrated by a separation of vitamins D2 and D3. At 2 mL/min, utilizing a simple isocratic condition and a 3.0 mm × 150 mm, 1.8 µm column, the analysis time is only 3 min. The separation of the main components, at three column temperatures including sub-ambient, is shown in Figure 2. Sub-ambient column temperature control, a standard feature of the Agilent Thermostatted Column Compartment, has significant advantages for many difficult isomer separations, including enantiomeric separations, and for shape-selective separations such as polycyclic aromatic hydrocarbons.

Configuration

- G4220A 1290 Infinity Binary Pump with Integrated Vacuum Degasser
- G4226A 1290 Infinity Autosampler
- G1316C 1290 Infinity Thermostatted Column Compartment
- G4212A 1290 Infinity Diode Array Detector

Conclusion

Taking advantage of the combined high flow and high pressure capability of the system allows one to use high efficiency 3 mm id columns (having up to 40% higher efficiency than comparable 2.1 mm id columns) to produce rapid separations with remarkable resolution while conserving solvent over the use of 4.6 mm id columns.

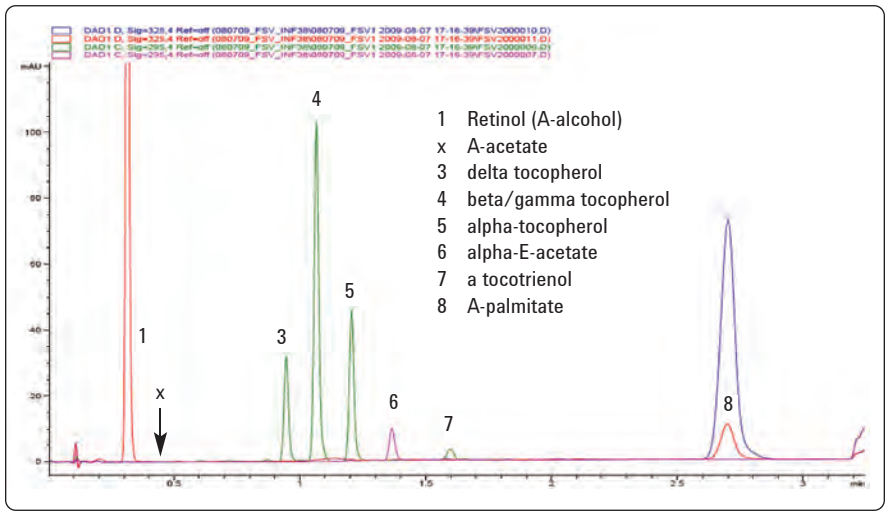

Figure 1
Analysis of important vitamins A and E components on the 1290 Infinity LC. Sample: solution of alcohols and esters of retinol and tocopherol. Conditions: 2.0 mL/min, 90% to 100% ACN at 1 min, hold to 3, run 4 min, ZORBAX RRHT StableBond C18, 3 mm × 50 mm, 1.8 µm, 45 °C.

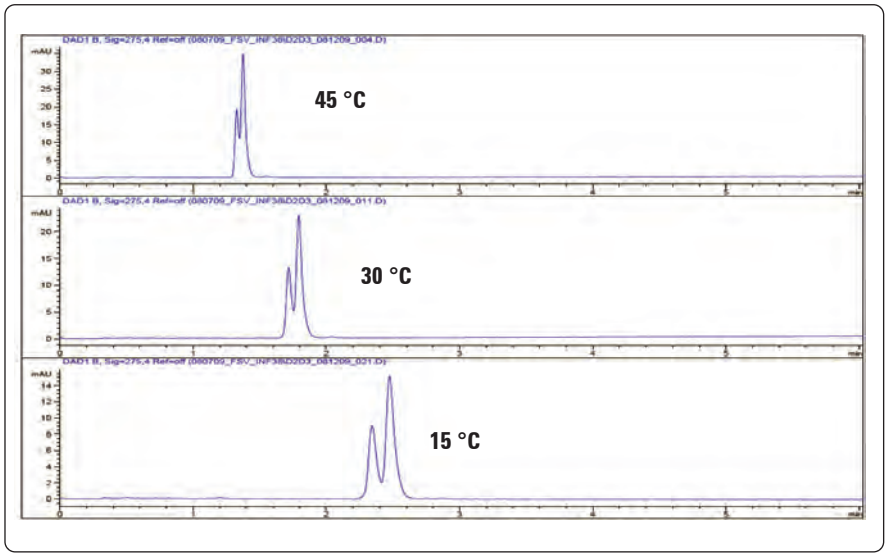

Figure 2
Analysis of vitamins D2 and D3 (order of elution) on the 1290 Infinity LC. Sample: standard mix (Sigma-Aldrich). Conditions: 2.0 mL/min, 75/25 ACN/MeOH isocratic, 280 nm UV ZORBAX RRHD StableBond C18, 3 mm × 150 mm, 1.8 µm, 45 °C, 30 °C and 15 °C.

www.agilent.com/chem/1290

October 1, 2009
Publication Number 5990-4882EN

Agilent Technologies

High resolution of complex lipids (triglycerides) using the Agilent 1290 Infinity LC and ZORBAX RRHT and RRHD 1.8 µm columns

Application Note

Lipid Analysis

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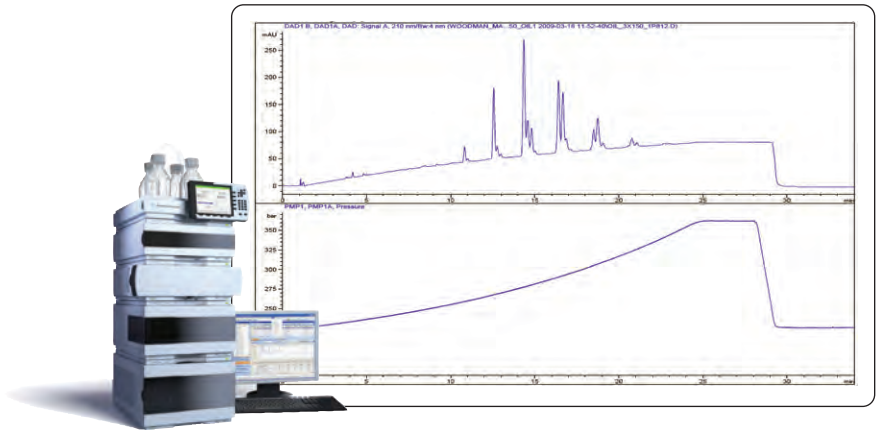

Abstract

The Agilent 1290 Infinity LC has significant capabilities for a wide range of HPLC and UHPLC applications. With a broader power range (that is, the combination of pressure and flow capabilities) than any other commercially available system, and the flexibility to operate a wide range of column dimensions and particle sizes, it is extremely useful for method transfer from any HPLC or UHPLC to the 1290 Infinity system. It allows the user to access capabilities not otherwise available.

Introduction

The typical HPLC resolution is shown by a separation of complex triglycerides in vegetable oil. Using a 24-min gradient and a 3.0 mm × 150 mm, 1.8 µm column, the analysis time of 35 min is typical; however, resolution is insufficient for good compositional investigation of the mixture. The separation of the main components is shown in Figure 1.

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The high resolution of the system is further demonstrated by separation on a much longer column, using more of the power range of the system. At 0.29 mL/min, incorporating a shallow gradient condition and an RRHD, 2.1 mm × 400 mm, 1.8 µm column, the separation is dramatically improved. The separation of the main components is shown in Figure 2. Subambient column temperature control, a standard feature of the Agilent Thermostatted Column Compartment, has significant advantages for many difficult isomer separations, including enantiomeric separations, and for shape-selective separations such as polycyclic aromatic hydrocarbons.

Configuration

- G4220A 1290 Infinity Binary Pump with Integrated Vacuum Degasser
- G4226A 1290 Infinity Autosampler
- G1316C 1290 Infinity Thermostatted Column Compartment
- G4212A 1290 Infinity Diode Array Detector

Conclusion

The high resolution and pressure capability of the system allows one to use high efficiency 2.1 mm id columns, generating approximately 97,000 theoretical plates and having approximately 400% lower solvent consumption compared to 4.6 mm id columns. With nearly 3 times higher efficiency, run time was increased by only about 80%. The low flow rate and high resolution facilitate the interfacing of the separation to high resolution TOF and QTOF mass spectrometers to produce high confidence peak identification and compositional information.

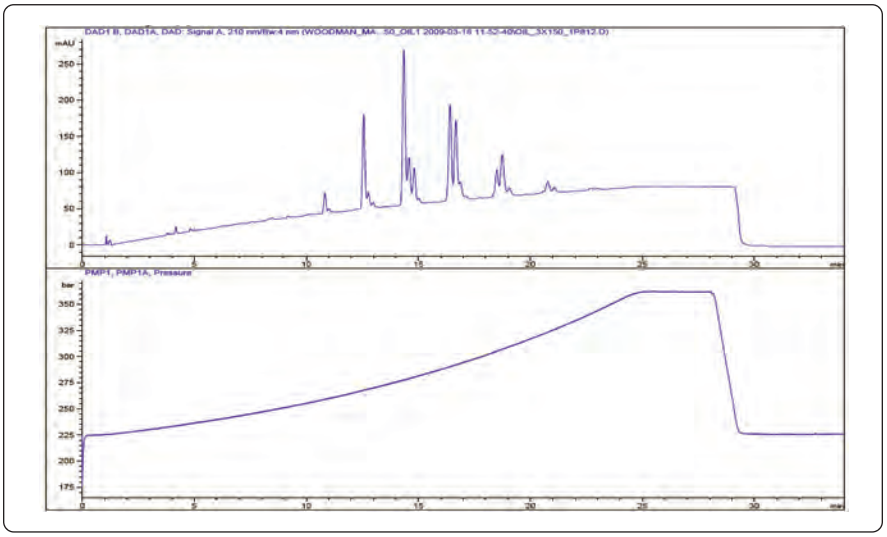


Figure 1
Analysis of vegetable oil components on the 1290 Infinity LC. Sample: soybean oil, 10 mg/mL, 10 µg on column. Conditions: 0.6 mL/min, 20% to 60% IPA vs. ACN at 24 min, hold to 30, run 35 min, ZORBAX RRHT StableBond C18, 3 mm × 150 mm 1.8, µm, 30 °C. Maximum operating pressure, 370 bar.

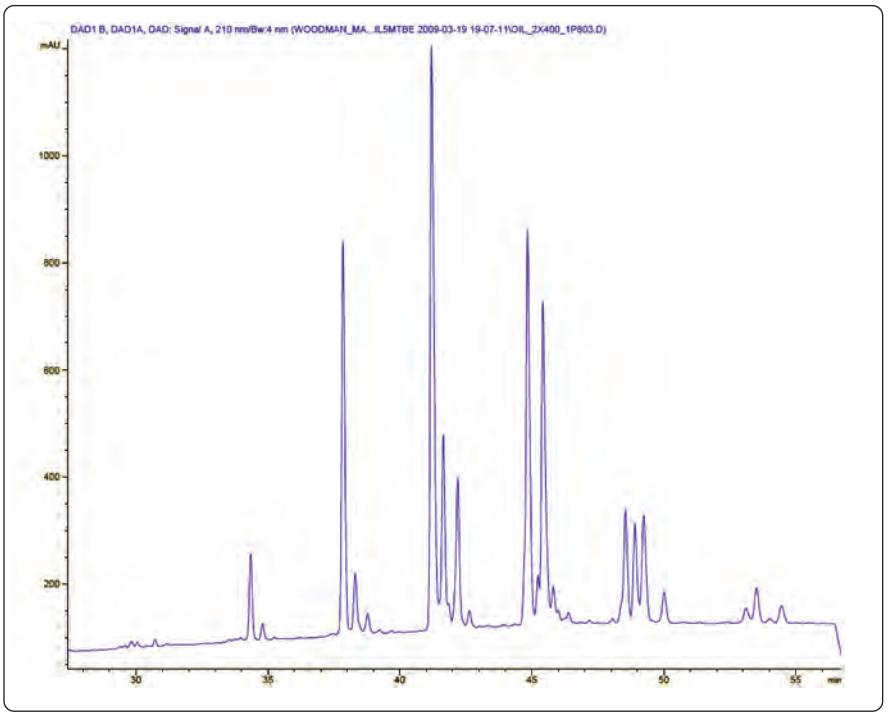


Figure 2
Analysis of soybean triglycerides on the 1290 Infinity LC. Sample: soybean oil, 10 mg/mL, 30 µg on column. Conditions: 0.29 mL/min, 10% to 40% MTBE vs. ACN at 42 minutes, hold to 55 minutes, run 60 minutes, 210 nm UV. ZORBAX RRHD StableBond C18, 2.1 mm × 400 mm (2–150 and 1–100 mm length in series), 1.8 µm, 20 °C. Operating pressure 730 bar.

www.agilent.com/chem/1290

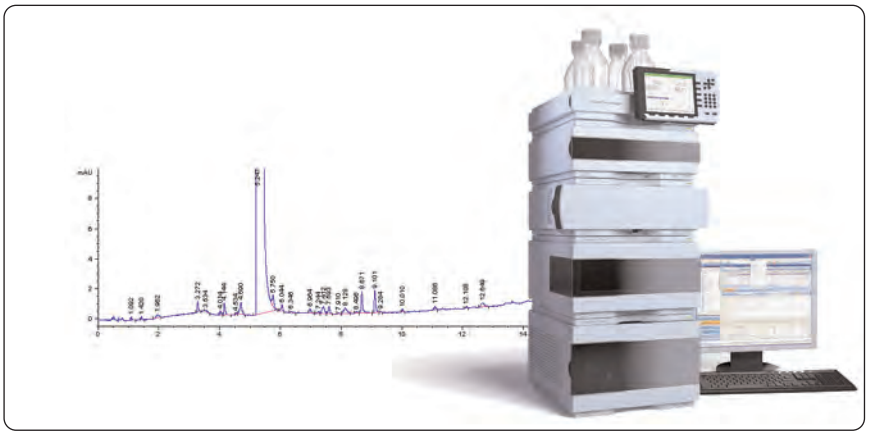
October 1, 2009
Publication Number 5990-4881EN

Agilent Technologies

Analytical instrument qualification and system validation according to USP Chapter <1058> for the Agilent 1290 Infinity LC system

Application Note

Pharmaceutical QA/QC



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Abstract

According to USP Chapter <1058> analytical instruments must be qualified before use. This Application Note will show a setup for testing the Agilent 1290 Infinity LC System. Metoclopramide was chosen as example substance to acquire data for testing accuracy, precision, and linearity and the results are presented.

The results of independent gradient testing with the Jet Weaver show steep gradient shapes and high plateau accuracy. The setup for different columns to achieve comparable separations starting with 5 µm material (250 mm × 4.6 mm), 3.5 µm material and columns with sub-2 µm materials of different vendors are shown. This versatility makes the system applicable for standard LC methodology as well as where highest separation power is needed.

The results for the determination of the precision of areas (<2% required), and retention times (<0.05% required) show that all criteria for qualified instruments are fulfilled. The coefficients for linearity for all components are better than 0.999. No carryover was detected.

Agilent Technologies

Introduction

USP Chapter <1058>[1] describes the relevant guidelines for analytical instrument qualification. These guidelines are not mandatory and allow different approaches. If analytical instruments are used in FDA regulated environments, related procedures are recommended. USP guidelines are mandatory only if any USP monographs require qualified instruments for a specific analysis, or if any regulated testing is applied.

The USP Chapter <1058> divides laboratory tools and instruments into three categories (A, B, C). Group A includes tools such as magnetic stirrers, Group B lists balances and pH-meters and Group C contains complex instruments like HPLCs or mass spectrometers. Depending on the complexity of the instrument and its application, the effort for qualification increases. The 4Q-model (design qualification, installation qualification, operational qualification, performance qualification) supports the guidelines of the instrument qualification for Group C instruments. Since it has been in use for 10 years, many users are familiar with the model.[2]

This model is applicable to all types of instruments because it is flexible and allows all laboratories to define test procedures and acceptance criteria .

The Agilent 1290 Infinity LC System belongs to category C, where testing according USP Chapter <1058> is necessary. This Application Note will show a setup for testing the instrument. Metoclopramide was chosen as an example substance to acquire data for testing of accuracy, precision and linearity. For setup of the method, the results of previous method developments were used.[3]

Experimental

Instrumentation

Table 1 shows the configuration of the Agilent 1290 Infinity LC system that was used. Several columns were used to show the performance.

Part No	Module
G4220A	1290 Infinity Binary pump with integrated vacuum degasser and different solvent mixers
G4226A	1290 Infinity Autosampler
G1316C	1290 Infinity Column Compartment
G4212A	1290 Infinity Diode Array Detector
Software:	Chemstation B.04.02

Table 1
Configuration of the Agilent 1290 Infinity LC system

Preparation of samples

Reference samples

The stock solution was prepared by mixing two different liquid formulations of metoclopramide hydrochloride (each 5 mg/mL, each 1 mL). One millilitre of the mixture was diluted with 4 mL of methanol to yield a concentration of 1 mg/mL for the main component. The resulting solution was diluted to the 0.01% concentration of the impurities.[3] Figure 1 shows the structure of metoclopramide.

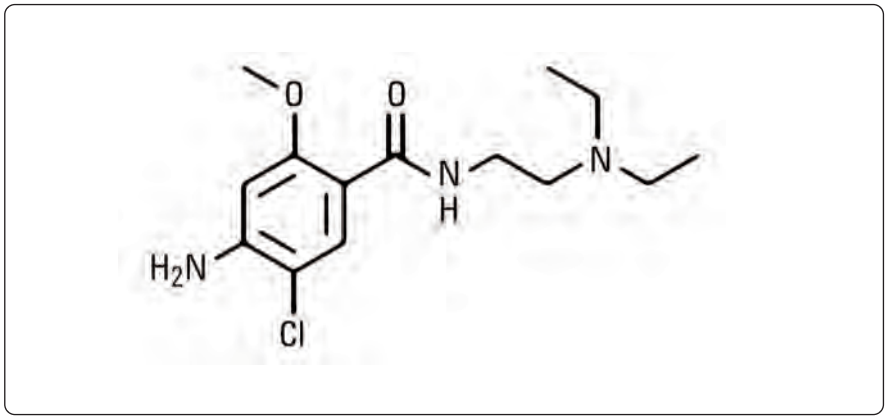

Figure 1
Structure of metoclopramide.

Chromatographic conditions

Columns:

- Agilent TC C18(2), 250 mm × 4.6 mm, 5 µm
- ZORBAX Eclipse Plus C18, 150 mm × 2.1 mm, 3.5 µm
- ZORBAX RRHD Eclipse Plus C18, 150 mm × 2.1 mm, 1.8 µm
- Waters BEH C18, 150 mm × 2.1 mm, 1.7 µm

Mobile phases: Gradient testing

- Mobile phase A: water
- Mobile phase B: water + 0.5% acetone (v/v)

Time	% A	% B
0.00	100.00	0.00
3.00	100.00	0.00
3.01	90.00	10.00
6.00	90.00	10.00
6.01	52.00	48.00
9.00	52.00	48.00
9.01	48.00	52.00
12.00	48.00	52.00
12.01	10.00	90.00
15.00	10.00	90.00
15.01	0.00	100.00
18.00	0.00	100.00
18.01	48.00	52.00
21.00	48.00	52.00
21.01	52.00	48.00
24.00	52.00	48.00
24.01	100.00	0.00
27.00	100.00	0.00

Table 2
Gradient.

Gradient for separation of metoclopramide and impurities

- Mobile phase A : 0.25% w/w ammonium acetate in water
- Mobile phase B : acetonitrile

The setup for all columns was found by using the parameters determined in Agilent publication number 5990-3981EN for the BEH column for starting conditions, converting them with the Method Translator Software[4]. (Instrument conditions are shown in Table 3.)

Setup for testing

USP Chapter <1058> defines tests and limits for the evaluation of HPLC systems. Typically, tests are used to evaluate pump performance, autosampler performance, the stability of temperatures, or the accuracy of optical detectors.

The chromatographic performance of the pump is shown by plotting and evaluating gradient mixing capabilities with a tracer and retention time precision. The autosampler can be validated by calculating the area precision of equal injection volumes, the correlation of calibration curves or the determination of the carryover. These results are only valid, if the detection system is stable enough to deliver reproducible data with sufficient sensitivity and high signal-to-noise values.

The following setup is the selection from a typical assortment of tests for system suitability with a reference sample :

- Determination of pump performance depending on dwell volumes by gradient tests
- Establishment of a chromatographic separation to achieve data for long time evaluations
- Similar peak pattern and resolution according to selected column with respect to particle size and column dimension and adapted to gradient shape and flow
- Precision of areas must be < 2 % RSD.
- Precision of retention times must be < 0.5 % RSD.
- Linearity should be $R^2 > 0.999$

	Agilent TC C18(2), 250×4.6mm, 5µm,	Eclipse Plus C18, 150×2.1mm, 3.5µm	RRHD Eclipse Plus C18, 150×2,1mm, 1.8µm	Waters BEH C18, 150×2.1mm, 1.7µm
Flow rate	1.058 mL/min	1.058 mL/min	0.221 mL/min	0.221 mL/min
Gradient	0-25 min 5-57% B	0-15 min 5-57% B	0-15 min 5-57% B	0-15 min 5-57% B
Temperature	37 °C	37 °C	37 °C	37 °C
Injection volume	8 µl	2 µl	1 µl	1 µl
Detection	DAD, Signal	275/4, Reference	400/60	standard cell (10 mm path length)
Data rate	2 Hz	2 Hz	40 Hz	40 Hz
Maximum pressure	138 bar	145 bar	310 bar	435 bar

Table 3
Instrument conditions.

There are more tests available to evaluate the accuracy of the optical unit of the detector. These tests can be established in addition to those mentioned, but are often part of a special setup available during diagnosis, and are not evaluated with a chromatographic test.

The samples in Table 4 were prepared and analyzed with these limits and settings for testing.

Results and discussion

USP Chapter <1058> defines tests and limits for the evaluation of HPLC modules and HPLC systems. The results shown here illustrate this process.

The accuracy of gradient mixing, as well as flow accuracy, are the main tests for evaluating a gradient pump. Gradient mixing becomes more and more influenced by the dwell volume. The testing setup must be variable to eliminate the effects of the mixer used.

The Agilent Jet Weavers for high efficiency mixing allow the use of different mixing volumes. The delay and dwell volume of the pumps have an influence on the separation for narrow bore applications combined with fast gradients. Enlarging the dwell volumes, thereby increasing retention times, could affect the resolution, and the gradient shape because of dispersion effects and different flush-out behavior. Chromatograms can appear different due to different mixers and mixing volumes. Figure 2 shows the different but steep gradient shapes, resulting in very short response times depending on the mixer. It also shows high accuracy of the gradient steps independent of the mixer used.

Sample	Purpose	Number of injections
Blanc solution	Verify baseline stability and identify artifacts	3
Suitability sample	Verify precision of areas and retention times for reference solution	10
Calibration	Verify linearity	3 for each level
Highest concentration and Blanc solution	Verify carryover	3 of each sample

Table 4
Setup for testing.

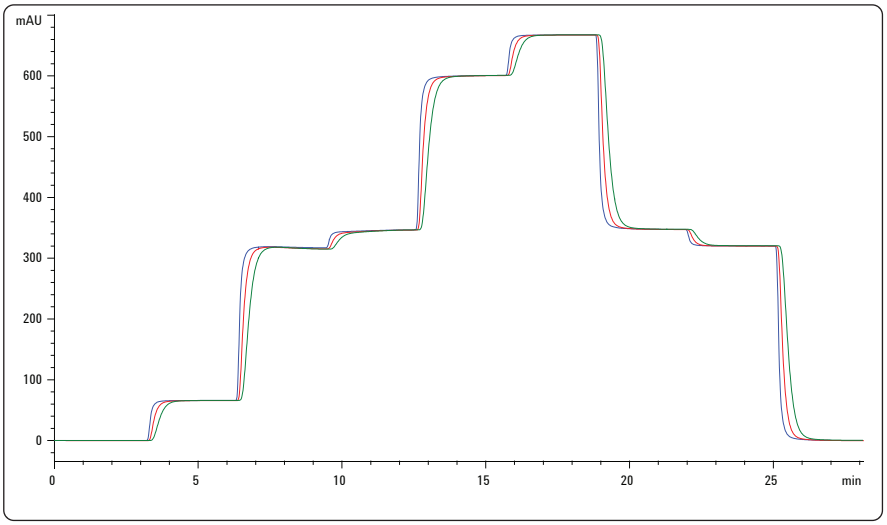

Figure 2
Gradient test depending on the volume of the mixers (blue-35 µL, red-135 µL, green-300 µL).

The next test evaulates the functionality of the system with different columns. For that purpose, four different columns with different particle sizes were tested (Figure 3).

In addition, the flow precision was evaluated using the retention time stability of some selected components in the mixture. With the same setup, the precision of the autosampler can be calculated, if the test sample is injected a minimum of 10 times and the relative standard deviation (RSD) of the areas is calculated. The data in Table 5 show high stability of retention times and high precision of areas even at low levels of impurities.

The accuracy of retention times is not only influenced by the flow precision but also by the temperature. The remarkable high stability is also a demonstration of excellent temperature stability in the column compartment.

The test for linearity shows correlation coefficients for all components greater than 0.999 which prove the high performance of the autosampler.

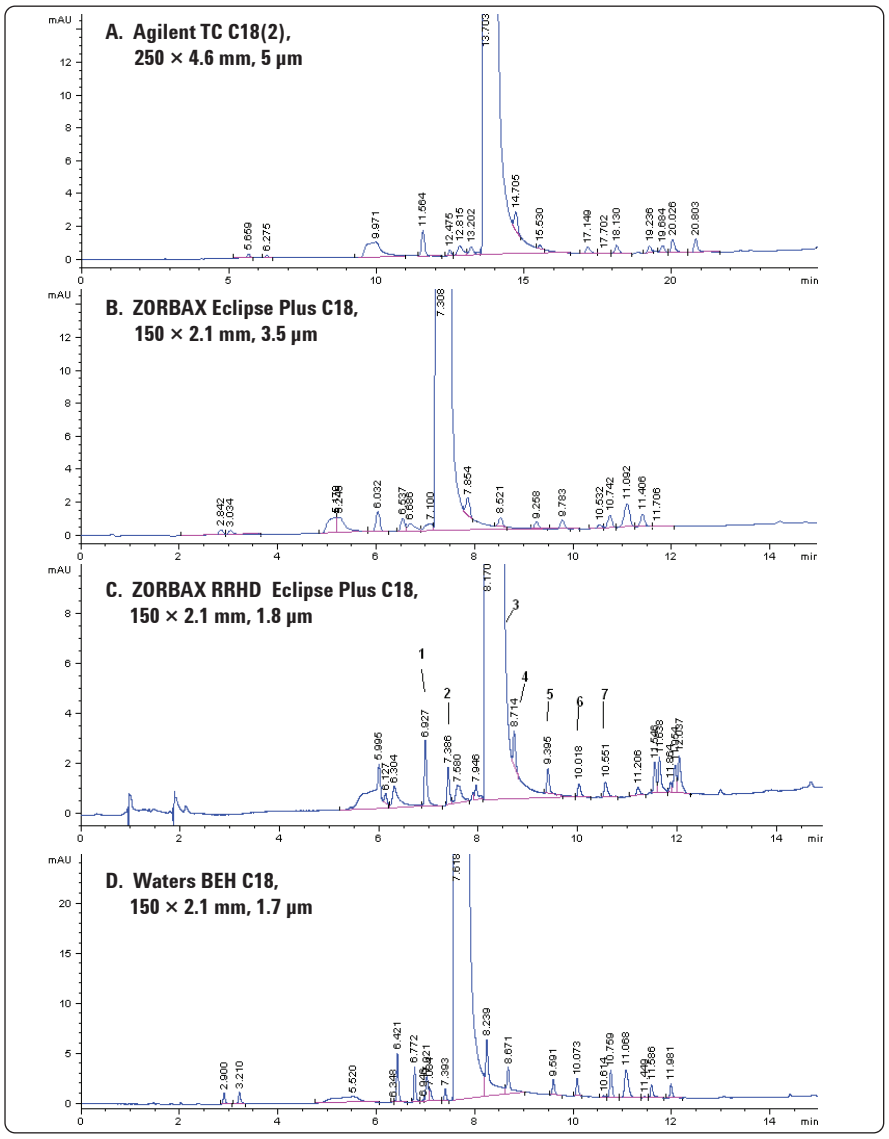

Figure 3
Separation of metoclopramide and its impurities.

	Retention times		Areas		Linearity
	Mean	RSD	Mean	RSD	R^2
Impurity 1	6.927	0.030	3.765	1.36	0.9994
Impurity 2	7.386	0.025	2.170	1.49	0.9997
Metoclopramide 3	8.170	0.039	10190.182	0.58	0.9998
Impurity 4	8.714	0.035	1.707	1.48	0.9993
Impurity 5	9.395	0.013	1.496	1.58	0.9993
Impurity 6	10.018	0.039	1.023	1.55	0.9990
Impurity 7	10.551	0.039	1.971	1.46	0.9993

Table 5
Determination of the precision of areas and retention times in Figure 3C.

A further test to evaluate sampler performance is the determination of carryover. Figure 4A shows the chromatogram after an injection of the highest concentration of metoclopramide. No carryover can be seen (Figure 4B).

The chromatogram in Figure 5 shows further method optimization, where the analysis time was shortened to improve the capacity of the system. It can be seen that all peaks are eluted within 10 minutes.

Conclusion

The Agilent 1290 Infinity LC system is designed to provide highest speed, resolution and sensitivity. A new power range allows operation with any particle type, any column dimension, or any mobile and stationary phase. The 1290 Infinity LC is the first system that allows method transfer from any Agilent HPLC System.

To use this system for quality control testing and development, as well as in an FDA regulated environment, it is necessary to meet the criteria of the new USP Chapter <1058>. These new regulations enforce procedures for testing and evaluating the applicability and versatility of the equipment before use.

This Application Note shows a selection of tests that can be established to evaluate an LC system.

Figure 2 shows the results of gradient testing. Independent of the Jet Weavers used and mixing volumes, the gradient shapes are steep and show high accuracy of each plateau.

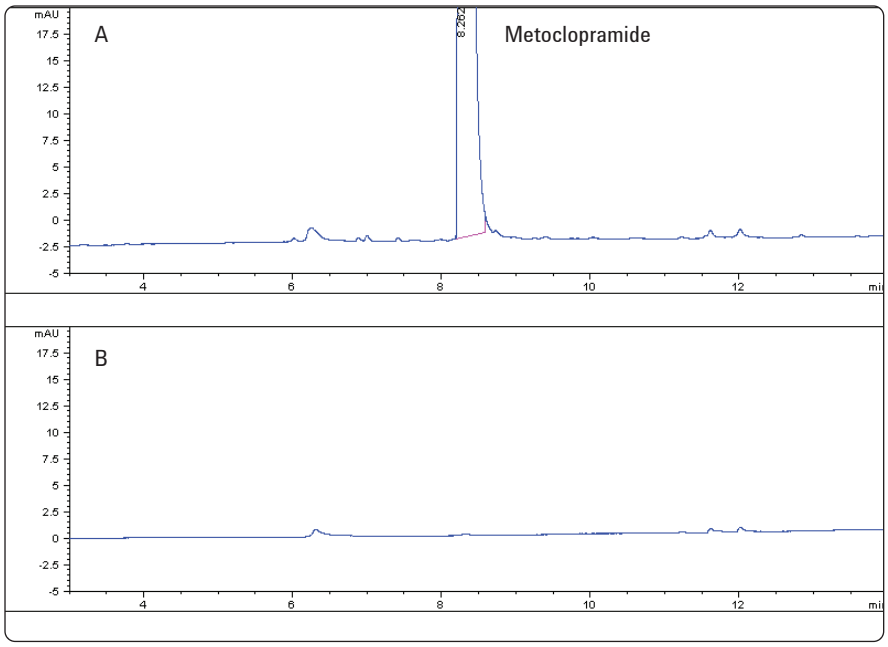

Figure 4
Determination of carryover. A. Injection of sample with highest concentration of metoclopramide. B. Injection of a blank solvent sample.

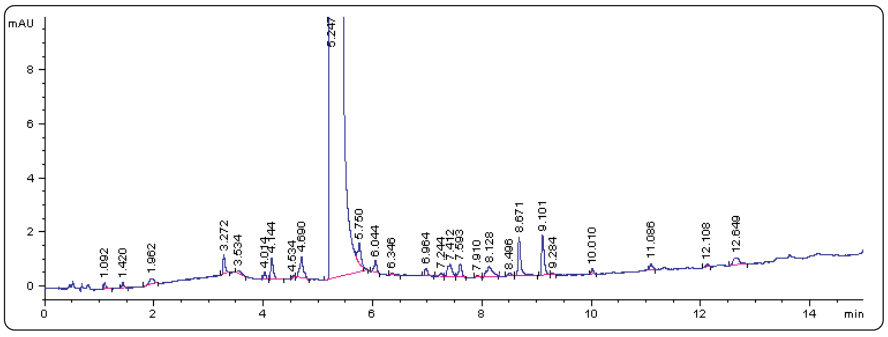

Figure 5
Separation of metoclopramide and its impurities on a ZORBAX RRHD Eclipse Plus C18, 150 × 2.1 mm, 1.8 µm, speed optimized (flow: 0.44 ml/min).

One of the great benefits of the system is the use of any column dimension or particle size. This was proven by the comparison of the separation power of a developed method with different column sizes. Figure 3 shows very similar chromatograms achieved from a column with 5 µm material (250 mm × 4.6 mm), a column with 3.5 µm material, and columns with sub-2 µm materials from different vendors. This versatility allows the use of the system for standard LC methodology as well as methods in which the highest separation power is needed.

The results shown in Table 5 show that all criteria for the precision of the determination (areas, retention times) are fulfilled. The coefficients of linearity for all components are better than 0.999. This is not only proven for the main component but also for the impurities at the 0.01%-level. No carryover was detected (Figure 4).

All results explicitly show the applicability of the 1290 Infinity LC system for quality control testing and development as well as in an FDA regulated environment.

In addition, the speed optimization test confirms that the system provides excellent separation possibilities (Figure 5).

The results of method transfer show that the selectivity and performance of the Agilent ZORBAX Eclipse Plus C18 material is independent of the particle size. The data also show the excellent flow design of the Agilent 1290 Infinity LC system, assuring the user that no band broadening or peak distortion will occur, hindering the separation power.

In summary, the data presented in this Application Note has illustrated the versatility and reliability of the Agilent 1290 Infinity LC system. This system allows fast method transfer to and from any column or particle size, allowing its use for almost any applications.

The Agilent 1290 Infinity LC system is qualified for the criteria of USP Chapter <1058> and will meet the highest requirements for every LC application.

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December 7, 2009
Publication Number 5990-4781EN

Agilent Technologies

High-Resolution Analysis of Intact Triglycerides by Reversed Phase HPLC Using the Agilent 1290 Infinity LC UHPLC System

Application Note

Food, Hydrocarbon Processing

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Abstract

The Agilent 1290 Infinity LC System with ultraviolet/visible (UV/VIS) Diode Array detection (DAD) is used to analyze triglycerides in soybean oil under non-aqueous reversed phase gradient conditions. The Agilent 1290 Infinity LC System was used for the chromatographic separation of the sample on 3.0 and 2.1 mm id C18 columns, of various lengths, with 1.8-µm packing materials prepared in 600 bar (9000 psi) or special 1200 bar (18,000 psi) configurations. The ability of the Agilent 1290 Infinity LC System to operate with long, high resolution columns is demonstrated with isopropanol (IPA) or methyl tert butyl ether (MTBE) as the strong solvent and acetonitrile as the weak component of the mobile phase mixture.

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Introduction

The analysis of intact triglycerides from animal or vegetable sources has many practical uses including understanding the chemical composition of the triglyceride, assessing fuel potential, and understanding lipid metabolism and behavior in living systems. The general conditions for successful analysis of these components by high-performance liquid chromatograph (HPLC) include gradient elution and low-wavelength monitoring of the overall separation. Because triglycerides have relatively few chromophores it is also beneficial to use evaporative light scattering detectors (ELSD) or mass spectrometers to facilitate other views of the separation.

During the development of this application, we analyzed a number of vegetable oils from various sources including soy, corn, rice bran, safflower, grape seed, olive, and palm oil. Because of the wide abundance of soybean oil in the United States and its growing significance in the production of biofuels, most of this work was standardized on maximizing the resolution of soybean oil triglycerides. These general conditions, however, are also suitable for a wide variety of samples including samples from animal lipid sources.

Intact triglycerides generally have very low water solubility and as such are commonly separated by normal phase chromatography, which separates species largely based on differences in polar functional groups, or by reversed phase chromatography operating in a non-aqueous mode of separation, which has more selectivity for small differences on carbon character such as chain length or unsaturation.

According to information published by Perkins [1] the predominant fatty acids, which are the building blocks of triglycerides on a glycerol backbone, found in soybean oil are myristic (14:0), palmitic (16:0), oleic (18:1), linoleic (18:2) and linolenic (18:3). Many other minor fatty acids are also present and because all of the fatty acids are randomly constructed into triglycerides, an extensive permutation of fatty acid substructure is obviously possible. Because the predominant difference between fatty acids consists of carbon chain length and number of double bonds, most of the diversity in triglycerides is found in the rather non-polar organic structural features. As a result, reversed phase chromatography is most useful for this application. Triglycerides have extremely poor solubility in water so one normally chooses either a high organic starting position, with respect to the aqueous content or, as in this work, a completely non-aqueous separation environment.

The typical structure of a triglyceride is shown in Figure 1. [2] In this example, from top to bottom, palmitic acid (C16:0), oleic acid (C18:1), alpha-linolenic acid (C18:3) are shown with respect to chain length and degree of unsaturation. The chemical formula is $C_{55}H_{98}O_6$.

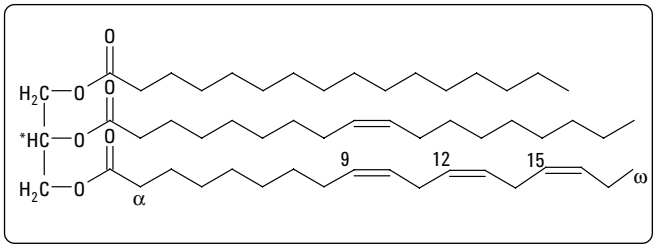

Figure 1. Typical triglyceride structure.

Experimental

Sample Preparation

The primary solution was prepared at a concentration of 10 mg/mL, in 2-propanol or 2:1 volume to volume MeOH/MTBE, and subsequently diluted to lower concentrations as needed. Injection volumes of 0.2-2 µL were made into the LC/DAD system.

LC Method Details

LC Conditions

Agilent 1290 Infinity LC System binary pump G4220A,
Agilent 1290 Infinity LC System autosampler G4226A
Agilent Thermostatted Column Compartment G1316C with switching valve
Agilent 1290 Infinity LC System diode array UV/VIS detector G4212A with 10 mm path fiber optic flow cell

Columns:	(See individual figures for specific usage) Agilent ZORBAX SB-C18 RRHT, 3 mm × 150 mm, 1.8 µm 600 bar, p/n 829975-302 Agilent ZORBAX SB-C18 RRHD, 2.1 mm × 100 mm, 1.8 µm 1200 bar, p/n 858700-902 Agilent ZORBAX SB-C18 RRHD, 2.1 mm × 150 mm, 1.8 µm 1200 bar, p/n 859700-902 In some cases, columns were coupled to extend the length and resolution.
Column temp:	20 °C or 30 °C
Mobile phase:	A = acetonitrile B = isopropanol (IPA) or tert butyl methyl ether (MTBE) (See individual figures)
Flow rate:	See individual figures
Gradient:	The gradient conditions were either 20% to 60% IPA or 10% to 40% MTBE, based on the strong eluting strength of MTBE when compared to IPA. The gradient slope was maintained at 2.6% organic phase increase per column volume for IPA gradients and 2.0% with MTBE, altering gradient time and flow rate accordingly. This was determined by calculations using a modification of the Agilent Method Translator. [3]

UV Conditions

Monitoring 210, 220 and 230 nm, bandwidth 4 nm, reference wavelength off

Results and Discussion

A typical gradient separation of triglycerides using acetonitrile IPA gradient is shown in Figure 2.

Some general comments are appropriate about the conditions and chromatographic profile shown in Figure 2. While it would be ideal to consider less expensive methanol as the weak eluent, introduction of methanol or denatured ethanol containing methanol has consistently shown a dramatic reduction in the overall resolution of the triglycerides. The significant increase in operating pressure, when running the gradient from acetonitrile to IPA, is clearly limiting and undesirable. Increasing the operating temperature of the column as a means of reducing solvent viscosity has proven to be undesirable because the chromatographic resolution tends to collapse as temperature increases.

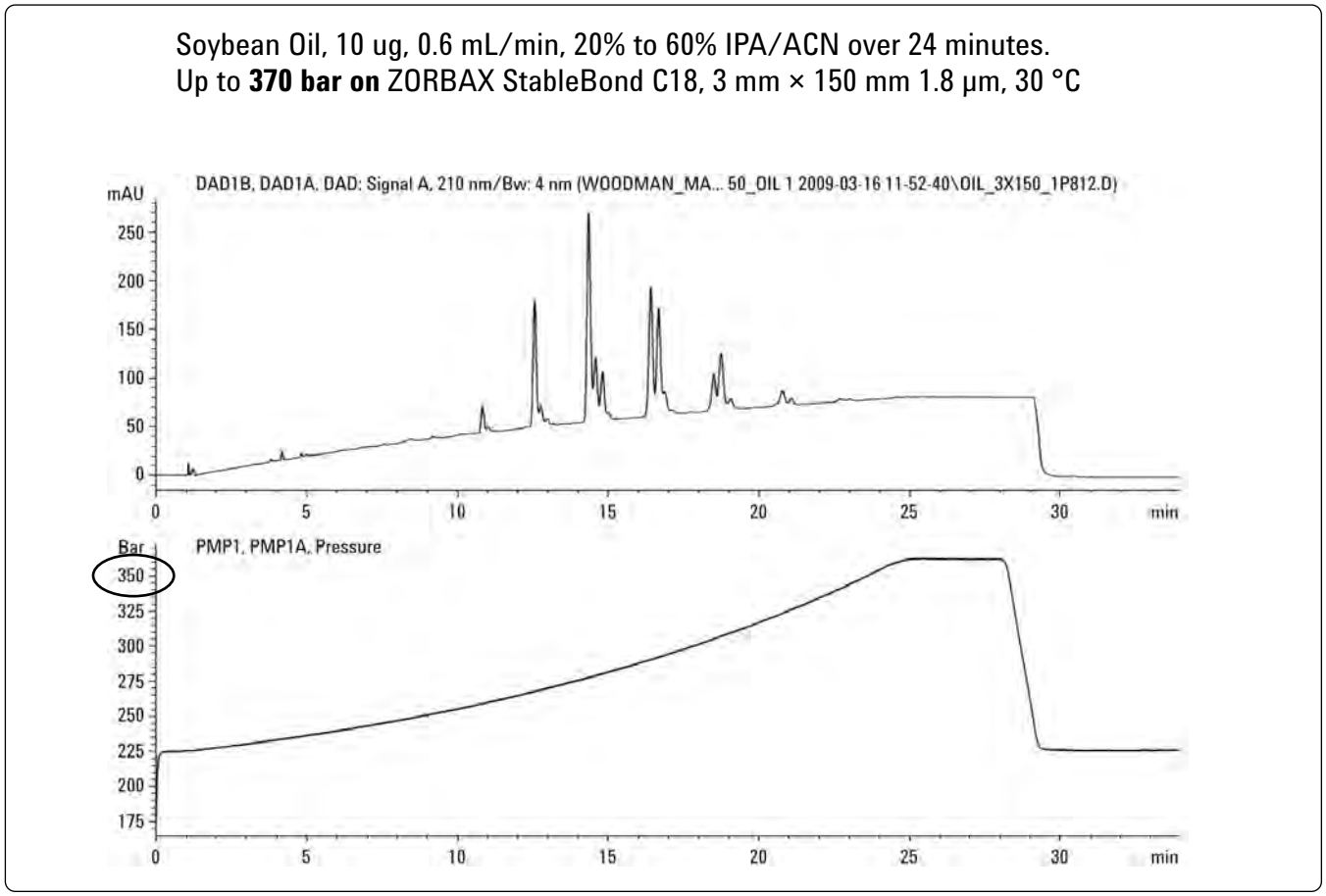

Figure 2. A 210-nm UV chromatogram of soybean oil sample on a 3 mm × 150 mm ZORBAX Rapid Resolution High Throughput (RRHT), 1.8 µm column, upper panel. System pressure trace showing the general progress of the gradient elution, lower panel. Flow rate 0.6 mL/min, gradient time 24 min. Strong solvent, isopropanol. The chromatogram demonstrates the typical difficulty encountered with this type of separation, which is small clusters of chromatographically similar triglycerides. These clusters are not positional isomers of the same carbon number and degree of unsaturation, rather a mixture of various chain lengths and number of double bonds as shown by mass spectrometric evaluation.

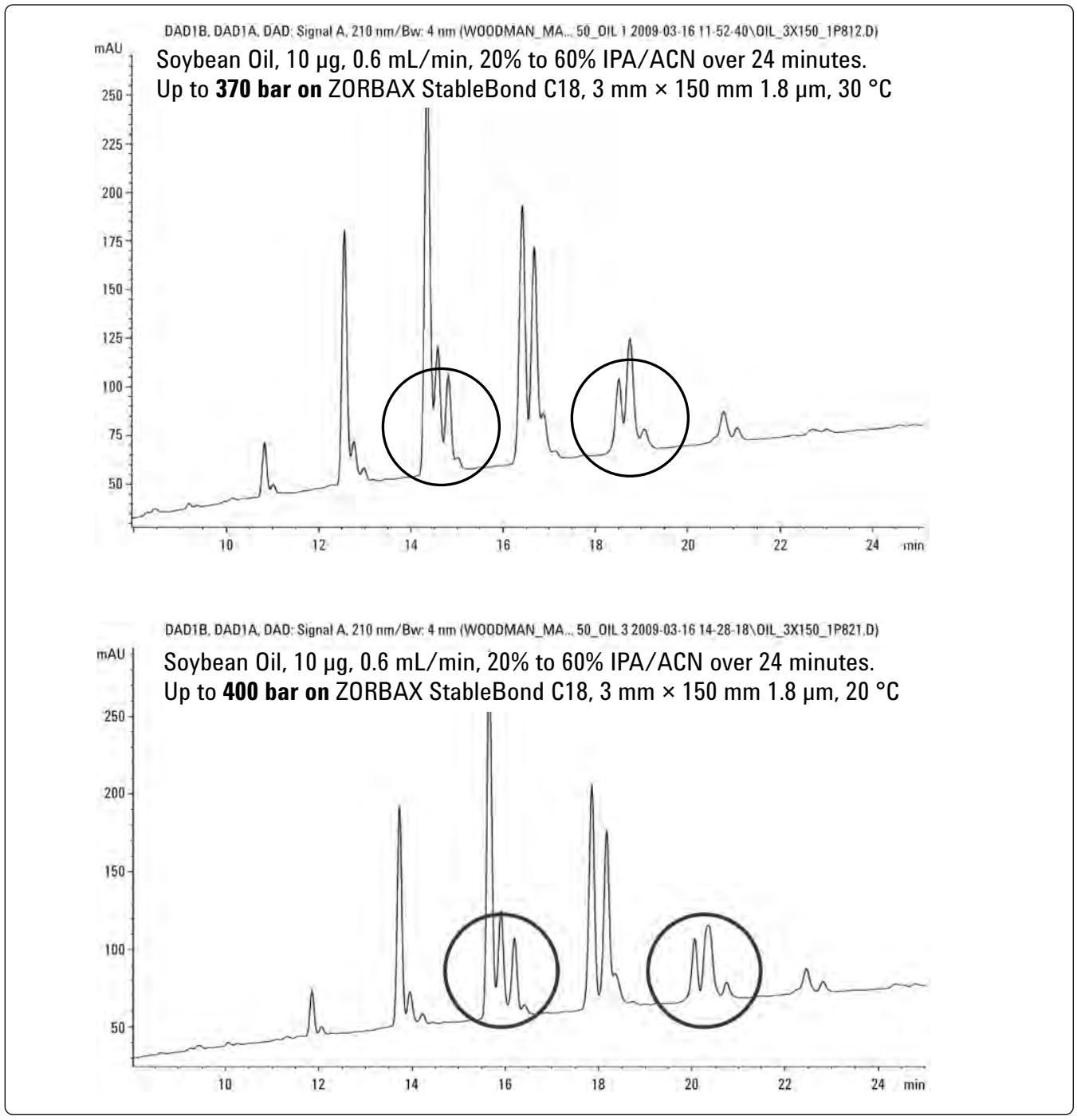

Figure 3. An expanded presentation of the chromatogram shown in Figure 2 at 30 °C, upper panel, compared with the same conditions in Figure 2 operating the column at 20 °C.

In Figure 3 we see the improvement achieved by operating the separation at 20 °C rather than 30 °C. The operating pressure increase is approximately 10% at the lower temperature. While many of our separations have been performed at 30 °C as a compromise between separation and operating pressure, the availability of the Agilent 1290 Infinity LC System with increased operating pressure capability has allowed us to reduce the temperature to 20 °C and demonstrate a usable improvement in separation.

Soybean Oil, 10 µg, 0.29 mL/min, 20% to 60% IPA/ACN over 27 minutes.
Up to **760 bar on** ZORBAX StableBond C18, 2.1 mm × 250 mm 1.8 µm, 20 °C

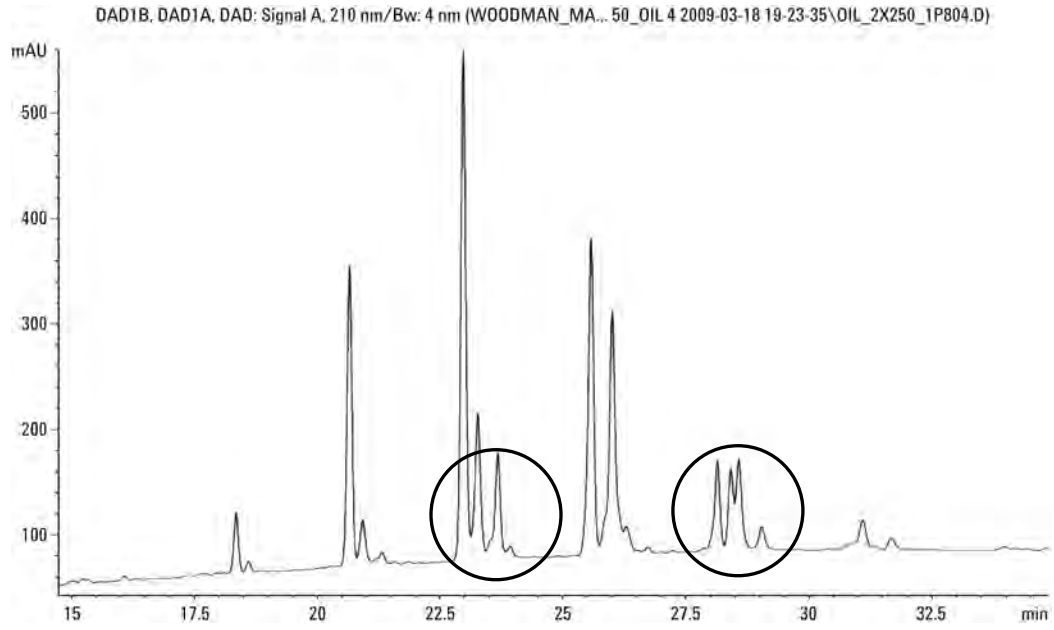

Figure 4. Analysis of the soybean oil sample on an Agilent ZORBAX StableBond C18 column, 2.1 mm × 250 mm, 1.8 µm, (150 mm in series with 100 mm) prepared for operation at 1200 bar pressure limit. Flow rate 0.29 mL/min, gradient time 27 min. Maximum observed pressure 760 bar.

In Figure 4, we see that increasing the length of the column has resulted in a significant increase in the resolution of some of the observed components. To further increase resolution, it would be practical to explore longer columns or explore alternative mobile phase or column chemistries. As with most very high performance separations, rate-limiting features tend to include operating pressure, operating temperature, and maximum flow rate. The triglyceride separations evaluated thus far have not been receptive to operation at higher column temperatures or higher flow rates, presumably because of their relatively high molecular weight and flexible organic structure. Even when gradient slope translations are carefully made to ensure organic strength consistency from method to method, operating at higher flow rates has consistently shown degradation of the overall separation. Because the isopropanol has significantly high viscosity and high pressure, it seemed appropriate to consider other non-polar solvents that are miscible with acetonitrile and friendly to low UV detection, as a substitute for isopropanol.

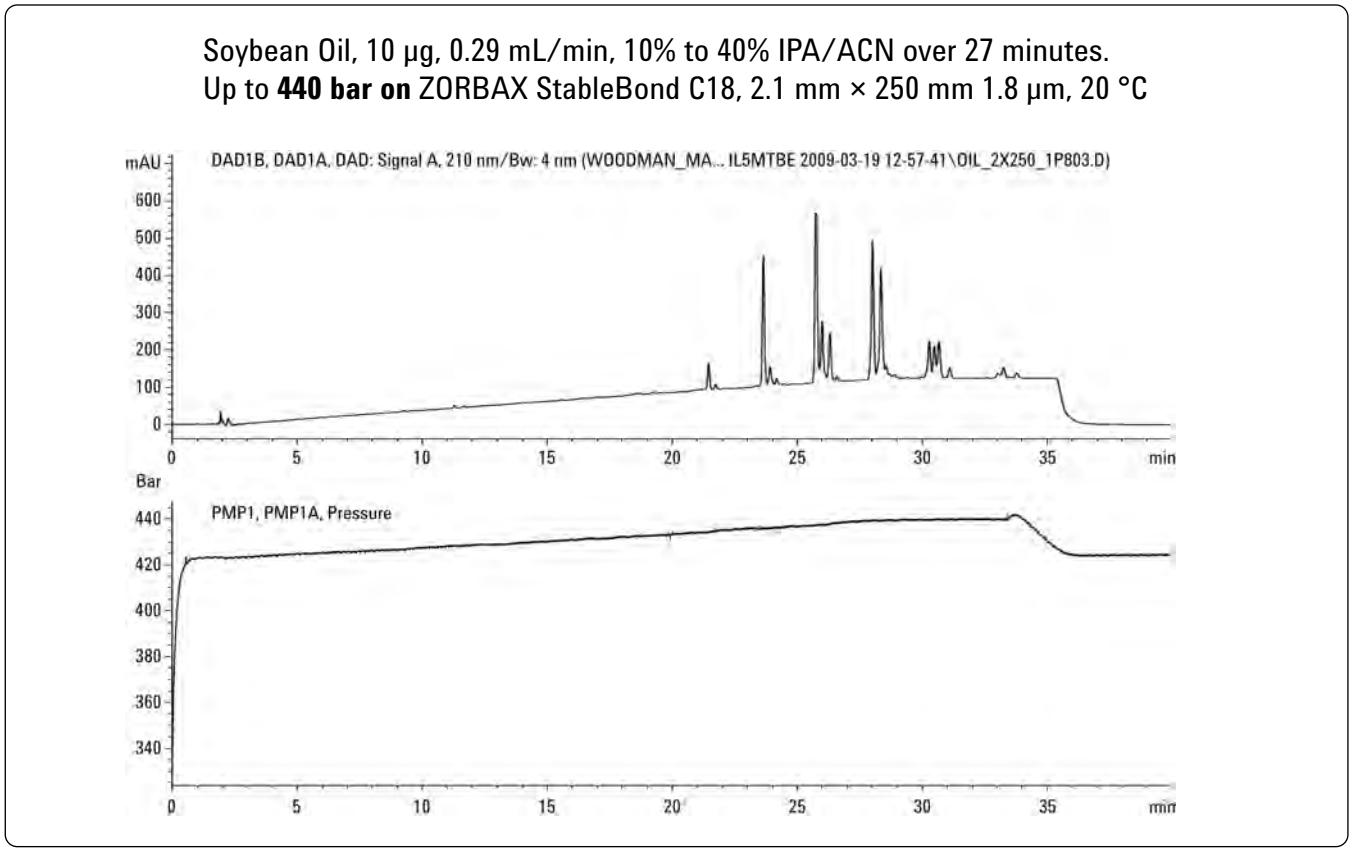

Figure 5. By substituting MTBE for isopropanol with otherwise the same conditions as Figure 4, and then re-optimizing the gradient for the significant increase in eluting strength of MTBE, we arrive at a new set of operating conditions where there is only a small difference in operating pressure over the gradient run. Flow rate 0.29 mL/min, gradient 27 min for 10% to 40% MTBE, maximum observed pressure 440 bar.

In Figure 5, the change to MTBE and subsequent readjustment of the gradient resulted in a separation that was very comparable to the original isopropanol separation, however at a much lower maximum operating pressure. In view of the prior evidence and comments regarding increased temperature or flow rate resulting in degraded separation, it seemed that the most appropriate way to take advantage of the new operating pressure capability of the Agilent 1290 Infinity LC System was to continue to increase the column length. The Agilent 1290 Infinity LC System and associated ZORBAX chemistries are capable of operating pressures up to 1200 bar, or approximately 18,000 psi. To ensure robust and rugged system operation many users typically specify the upper pressure limit for a method at a value less than 80% of the rated operating pressure.

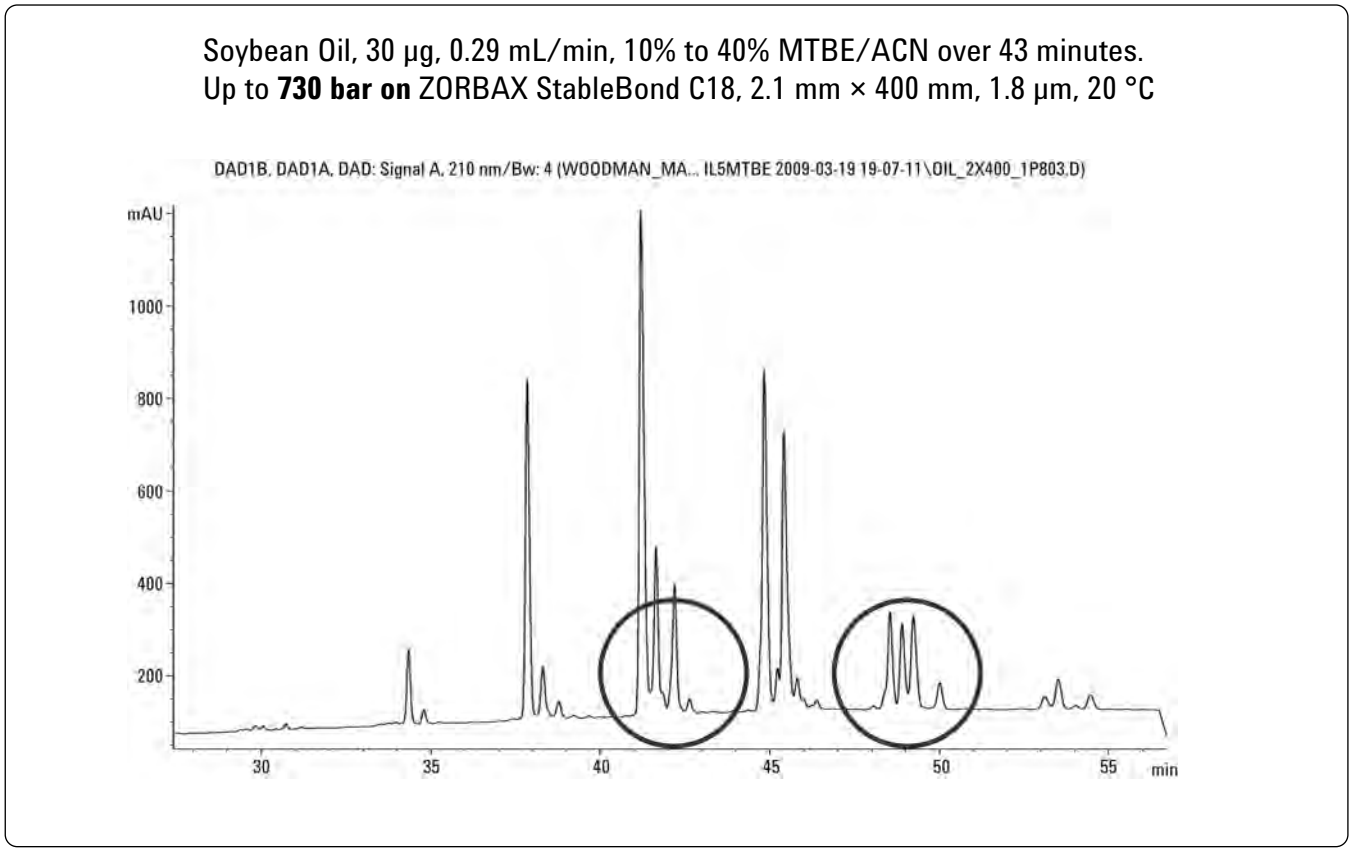

Figure 6. Separation of the soybean oil sample on a 2.1 mm × 400 mm ZORBAX StableBond C18, 1.8 µm 1200 bar columns (150 mm + 150 mm + 100 mm in series). Flow rate 0.29 mL/min gradient time 43 min, for a gradient of 10% to 40% MTBE. Maximum operating pressure 730 bar at 20 °C.

As shown in Figure 6, having previously optimized the column temperature, operating flow rate and gradient slope for the best possible balance between resolution and analysis time, and after investigating a variety of solvents as candidates for both the weak solvent and strong solvent choice, we are left with an ultimate opportunity to operate on a very long column set of 1.8 µm particle size columns under conditions ideal for the separation of this group of triglycerides. With an operating pressure of only 730 bar, which is about 60% of the rated capability of the Agilent 1290 Infinity LC System, it is clearly possible to consider even longer column lengths or a further reduction in the operating temperature as both of these seem promising in terms of delivering even higher resolution out of the mixture.

The separation with MTBE or isopropanol can be adapted for use with a mass spectrometer as one of the detectors. In previous studies (see www.Agilent.com/chem ASMS 2009 for a poster on this subject) we have been able to demonstrate the capability of quickly and confidently identifying the composition of many of the triglycerides found in this and other samples. For optimum electrospray performance in the non-aqueous, non-buffered environment it was useful to do post UV detector addition of a mixture of methanol and water with ammonium formate buffer to enhance ionization and to ensure a consistent ability to preserve the molecular ion into the mass spectrometer. It has been shown by McIntyre [4] that the presence of ammonium formate in the mobile phase significantly improves the probability that a molecular ion will be formed and preserved in the mass analyzer portion of a mass spectrometer.

Conclusions

Using the Agilent 1290 Infinity LC System, we were able to easily demonstrate UHPLC capabilities well within the operating range of the instrument. The significantly enhanced resolution afforded by long sub-2 micron particle size columns in the sub-ambient column compartment environment will contribute significantly to our understanding of the major and minor composition of this sample and other similar materials. This should significantly enhance the contribution of liquid chromatography to the understanding of seed oil composition, the role of triglycerides in metabolism, and the area of lipidomics where great interest has been directed on the LC separation coupled to time-of-flight high-resolution mass spectrometry (LC/TOF).

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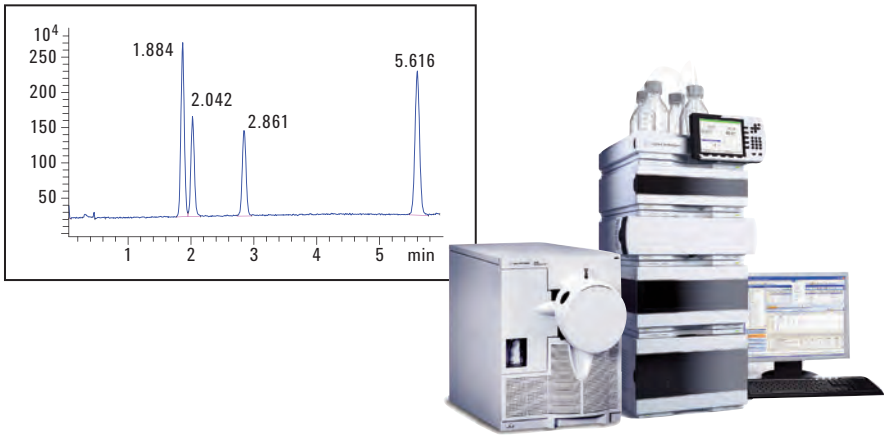
Influence of delay volume and system dispersion on resolution and sensitivity

Application Note

Pharmaceutical and Chemical



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Abstract

This Application Note demonstrates the influence of system delay volume and system dispersion on the resulting resolution and sensitivity achieved for LC/MS experiments. The advantages of using a low delay volume LC system and optimized connection of the LC to the MS are shown. The study illustrates that the Agilent 1290 Infinity LC system is an ideal partner for LC/MS instruments.

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Introduction

In LC/MS measurements, the achieved resolution and sensitivity of separated compounds heavily depend on the delay volume of the HPLC system and the connection between the LC system and the mass spectrometer.

The delay volume is responsible for the delay time, which is the time it takes a change in gradient to reach the column at a given flow rate. The delay volume also has an influence on the separation because it can cause isocratic elution and band broadening, especially for early eluting compounds.

The band broadening caused by the connection of the column outlet to the source of the mass spectrometer follows the Aris-Taylor equation, which gives the peak dispersion in cylindrical tubing:

$$\sigma^2_{v,ext} = \frac{\pi\, d^4\, L_{cap}\, u_{cap}}{96 D_m}$$

$\sigma^2_{v,ext}$	is the volume variance
d	is the tubing diameter
L	is the tubing length
u	is the linear velocity of the liquid
D_m	is the molecular diffusion coefficient

This equation clearly shows that the dispersion is proportional to the length of the tubing but more important, proportional to the fourth power of the inner diameter of the tubing.

This Application Note demonstrates the influence of system delay volume and system dispersion on the resulting resolution and sensitivity achieved for LC/MS experiments. The advantages of using a low delay volume LC system and optimized connection of the LC to the MS are shown. It is also shown that the Agilent 1290 Infinity LC system is the ideal partner for LC/MS instruments.

Experimental

Equipment:

Agilent 1290 Infinity LC system containing an Agilent 1290 Infinity Binary Pump, Agilent 1290 Infinity High Performance Autosampler, Agilent 1290 Infinity Thermostatted Column Compartment, Agilent 1290 Infinity DAD and Agilent 6140 Single Quadrupole Mass Spectrometer.

Column:	Agilent ZORBAX SB C18, 50 × 2.1 mm, 1.8 µm
Software for data acquisition and data analysis:	ChemStation B04.03

HPLC Method:

Solvent A:	Water + 0.1% formic acid Solvent B: Acetonitrile + 0.1% formic acid
Flow rate:	0.5 mL/min
Gradient A 0 min 12% B 5 min 20% B 5.01 min 95% B 6 min 95% B	Gradient B 0 min 10% B 5 min 20% B 5.01 min 95% B 6 min 95% B
Stop time:	6 min
Post time:	3 min
Injection volume:	1 µL
Needle wash:	6 s in MeOH
Column temperature:	40 °C
Diode array detector:	10 mm standard cell, wavelength 260/4 nm, ref. 360/16 nm, slit 4 nm, data rate 80Hz

MS Method:

Source:	Gas temperature: 350 °C, nebulizer pressure: 45 psi, gas flow: 11 L/min, positive polarity
Scan:	100 – 1000 *m/z*
SIM:	271.0 *m/z*, 279.0 m/z, 285.5 *m/z*, 311.0 m/z
Sample:	Solution of Sulfamethizole (first peak, *m/z* 271.0), Sulfamethazine (second peak, *m/z* 279.0), Sulfachloropyridazine (third peak, *m/z* 285.0), Sulfadimethoxine (fourth peak, *m/z* 311.0) each at a concentration of 100 ng/µL.

Results and discussion

The influence of the LC system delay volume

The delay volume of an HPLC system influences the separation performance which is determined with the detector directly connected to the LC column outlet. Typically, this is a diode array detector (DAD) for the detection of compounds absorbing UV light. This influence on the separation performance continues to an associated detector, typically a mass spectrometer. The first experiment was done with an Agilent 1290 Infinity LC system with a delay volume of about 125 µL. For the simulation of a system with a higher delay volume, two mixing devices with a combined volume of about 700 µL were connected in series to the Agilent 1290 Infinity LC system between the Jet Weaver mixer inherent in the Agilent 1290 Infinity Pump and the Agilent 1290 Infinity Autosampler. This produced an overall delay volume of about 1000 µL, which is similar to a standard HPLC system for 3 or 4.6 mm id columns.

The measurement of the separation of four compounds with the unchanged Agilent 1290 Infinity LC system is shown in Figure 1A. There are two peaks very early in the gradient separated with a resolution of about 2; one in the middle and the last one at the end of the gradient time. The retention

times, peak widths, areas and heights are summarized in Table 1. Figure 1Aa shows the peaks in the DAD chromatogram as an inherent detector, Figure 1Ab shows the chromatogram in MS scan mode and Figure 1Ac shows the chromatogram in MS SIM mode as and associated detector. In comparison, the peaks are delayed by a short time of about 0.015 minutes (0.9 s) between detection with DAD and MS due to the transfer tubing. The same is true for the small differences in peak width of about 0.012 minutes (0.7 s).

Figure 1B shows the same separation but on a system with a larger delay volume of about 800 µL. In the comparison of the UV and MS chromatograms for the last peak, a shift in the retention time of 1.45 minutes is seen. This is nearly the theoretically expected retention time shift caused by the added delay volume for the given flow rate of 0.5 mL/min (Figure 1Ba). There are additional effects such as additional peak broadening of about 0.9 seconds and a loss in peak height of about 20% which compromises the sensitivity. The same effects can be found in the associated MS detector for the last peak (Figures 1Bb and 1Bc). More impressive are the effects on the first and second peak, here the delay time is only about 0.1 minutes (6 s). This cannot be explained by the retention time shift due to the added delay volume but it can be explained by the fact that those early eluting peaks start to elute under isocratic conditions caused by the delay volume before the gradient hits the column. For these peaks, a reduction in peak height of about 20% can be seen with the DAD and about 10% by MS TIC detection, therefore a reduction in sensitivity. A similar behavior can be seen for the MS SIM data but on a higher level of sensitivity.

These results demonstrate the advantage of a system with the lowest delay volume for the detection of separated compounds with an inherent associated detector. There are two advantages: shorter run time and higher sensitivity.

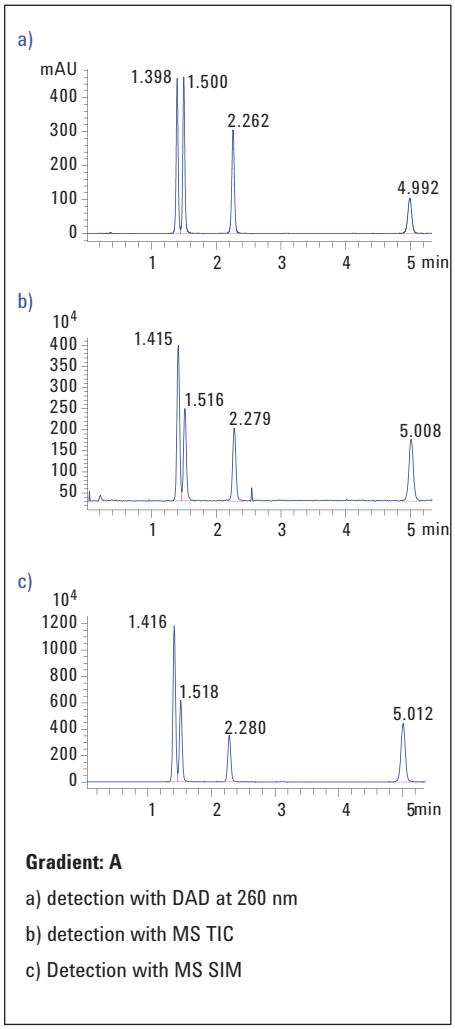


Figure 1A
Separation with lowest delay volume.

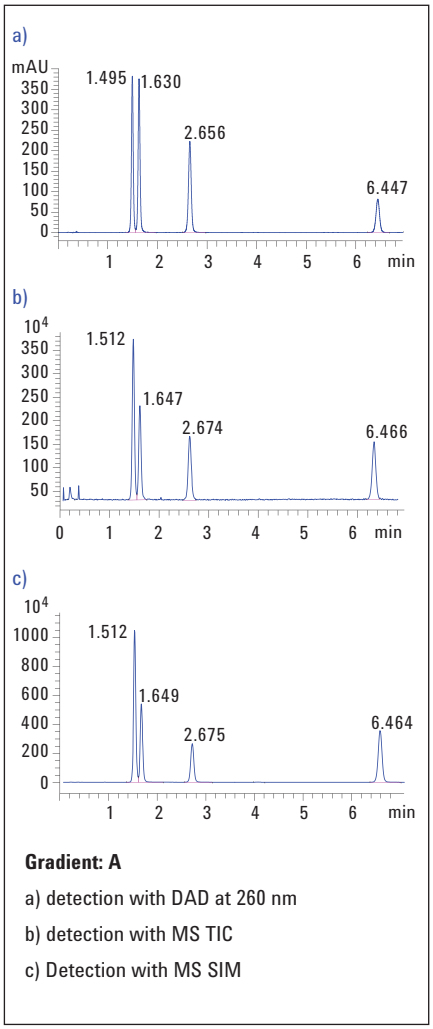


Figure 1B
Separation with high delay volume.

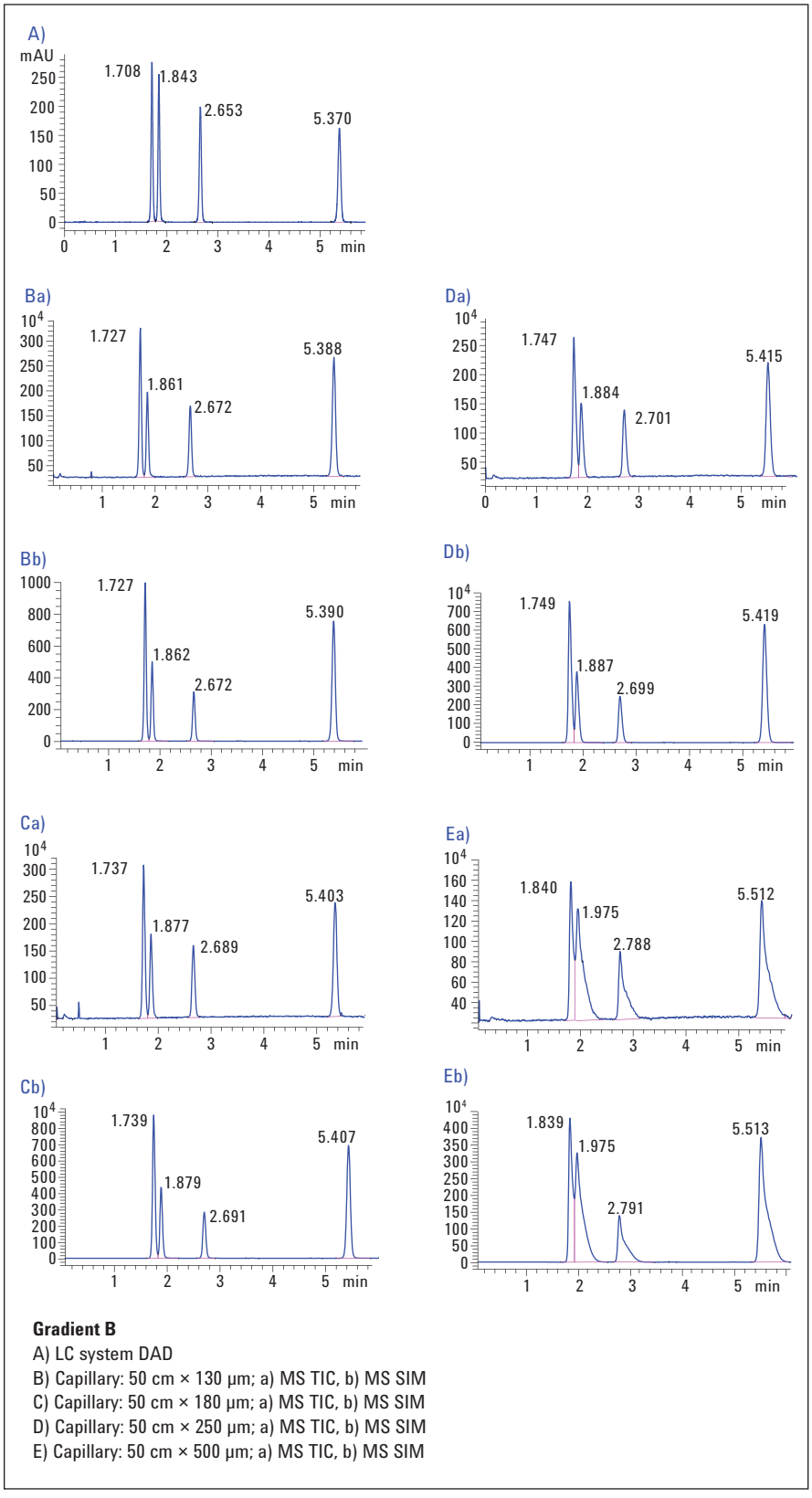
Peak	RT (min)	Width (min)	Height	Area
Standard delay volume - DAD				
1	1.398	0.0317	447.95	929.50
2	1.500	0.0327	451.27	956.05
3	2.262	0.0438	303.54	865.68
4	4.992	0.0622	103.46	445.63
Standard delay volume - MS TIC				
1	1.415	0.0442	3.59E+06	9.85E+06
2	1.516	0.0447	2.05E+06	5.70E+06
3	2.279	0.0551	1.72E+06	6.02E+06
4	5.008	0.0743	1.46E+06	6.99E+06
Standard delay volume - MS SIM				
1	1.416	0.0413	1.15E+07	3.06E+07
2	1.518	0.0431	5.71E+06	1.51E+07
3	2.280	0.0519	3.55E+06	1.12E+07
4	5.012	0.0763	4.45E+06	2.13E+07

Peak	RT (min)	Width (min)	Height	Area
Extended delay volume - DAD				
1	1.495	0.0382	377.67	946.35
2	1.630	0.0405	370.11	982.07
3	2.656	0.0608	221.65	879.79
4	6.447	0.0792	81.55	417.56
Extended delay volume - MS TIC				
1	1.512	0.0488	3.34E+06	1.05E+07
2	1.647	0.0510	1.91E+06	6.37E+06
3	2.674	0.0733	1.35E+06	6.38E+06
4	6.466	0.0749	1.21E+06	6.28E+06
Extended delay volume - MS SIM				
1	1.512	0.0486	1.03E+07	3.22E+07
2	1.649	0.0493	5.22E+06	1.66E+07
3	2.675	0.0695	2.66E+06	1.21E+07
4	6.464	0.0836	3.56E+06	1.99E+07

Table 1
Comparison of retention time, peak width, peak height and peak area for the low volume 1290 Infinity LC system with standard delay volume and extended delay volume. Data for the inherent DAD and the associated MS are shown. DAD height is given in mAU and DAD area is given in mAUs.

The influence of the connection to an LC-associated detector

Even when the delay volume in the front end LC system is optimized, the connection to the mass spectrometer has to be optimized to get the best performance for sensitivity and peak resolution. Both depend on peak broadening effects due to capillary length and inner diameter. The inner diameter of a capillary has a large impact on peak broadening. Hence, the behavior of separated peaks was determined with capillaries of the same length but different inner diameter (Figure 2). As a basis for this comparison, the results obtained with the LC inherent DAD were used (a gradient (B) slightly different from the first experiment was applied, because it gave better baseline separation of the first two peaks, Figure 2 A). For this initial experiment, a connection capillary of 50 cm × 130 µm from the LC to the MS was used (Figures 2 Ba and 2 Bb). The observed shift in retention time (about 2 s) and peak width (about 1 s) between DAD and MS are in the same order as described in the experiment above for the low delay volume LC system configuration (Table 2). When the capillary was exchanged the situation was different. In the following experiment, the first capillary had the dimensions 50 cm × 180 µm. In this case, the retention time was shifted about 2 seconds and the peak width increased by about 300 ms. Both peak 1 and peak 2 are still well separated (Figures 2 Ca and Cb). The effect of changing the transfer tubing to this size is still small. By changing the connection to a capillary dimension of 50 cm × 250 µm, the retention time shift is about two seconds and the peak width at half height increases by about one second. Looking on the chromatograms (Figures 2 Da and Db), it can be seen how dramatic this effect falls on the separation especially for peak 1 and peak 2, they are now not baseline separated. Finally, by exchanging the connection capillary to the dimension 50 cm × 500 µm, the chromatographic resolution becomes completely destroyed (Figures 2 Ea and Eb).



Gradient B

A) LC system DAD
B) Capillary: 50 cm × 130 µm; a) MS TIC, b) MS SIM
C) Capillary: 50 cm × 180 µm; a) MS TIC, b) MS SIM
D) Capillary: 50 cm × 250 µm; a) MS TIC, b) MS SIM
E) Capillary: 50 cm × 500 µm; a) MS TIC, b) MS SIM

Figure 2
Influence of the connection from the LC system to the mass spectrometer.

1290 Infinity LC - DAD				
Peak	RT (min)	Width (min)	Height (mAU)	Area (mAU*s)
1	1.708	0.036	273.770	635.490
2	1.843	0.036	252.440	594.630
3	2.654	0.046	197.960	586.420
4	5.370	0.060	161.770	631.420

MS connection: 50 cm, 0.13 mm id - MS TIC				
Peak	RT (min)	Width (min)	Height (× 10^6)	Area (× 10^6)
1	1.727	0.046	2.984	8.691
2	1.861	0.046	1.694	4.843
3	2.672	0.055	1.422	5.007
4	5.388	0.069	2.389	10.571

MS connection: 50 cm, 0.18 mm id - MS TIC				
Peak	RT (min)	Width (min)	Height (× 10^6)	Area (× 10^6)
1	1.737	0.048	2.805	8.963
2	1.877	0.051	1.507	4.947
3	2.689	0.059	1.329	5.144
4	5.403	0.074	2.097	10.070

MS connection: 50 cm, 0.25 mm id - MS TIC				
Peak	RT (min)	Width (min)	Height (× 10^6)	Area (× 10^6)
1	1.747	0.051	2.310	8.539
2	1.884	0.061	1.112	4.407
3	2.701	0.068	1.149	5.295
4	5.415	0.082	1.936	10.570

MS connection: 50 cm, 0.13 mm id - MS SIM				
Peak	RT (min)	Width (min)	Height (× 10^6)	Area (× 10^6)
1	1.727	0.044	9.858	28.345
2	1.862	0.043	4.875	13.851
3	2.672	0.055	3.124	10.954
4	5.390	0.071	7.563	34.166

MS connection: 50 cm, 0.18 mm id - MS SIM				
Peak	RT (min)	Width (min)	Height (× 10^6)	Area (× 10^6)
1	1.737	0.051	8.659	28.700
2	1.879	0.050	4.210	13.740
3	2.691	0.060	2.830	11.070
4	5.407	0.075	6.925	33.910

MS connection: 50 cm, 0.25 mm id - MS SIM				
Peak	RT (min)	Width (min)	Height (× 10^6)	Area (× 10^6)
1	1.747	0.057	7.222	27.570
2	1.887	0.058	3.281	12.430
3	2.699	0.070	2.475	11.470
4	5.419	0.087	6.303	34.700

Table 2
Comparison of retention time, peak width, peak height and peak area for the different conecting capillaries from the Agilent 1290 Infinity LC system to the mass spectometer. DAD height is given in mAU and DAD area is given in mAUs.

A summary of the behavior of peak widths is given in Figure 3. It can be seen that the peak widths increase for all used capillaries compared to the DAD peak width and that peak widths increase with the inner diameter of the capillaries. From the data in Table 2 it can be seen that there is not only an influence on the peak width but also in the peak height and on the sensitivity.

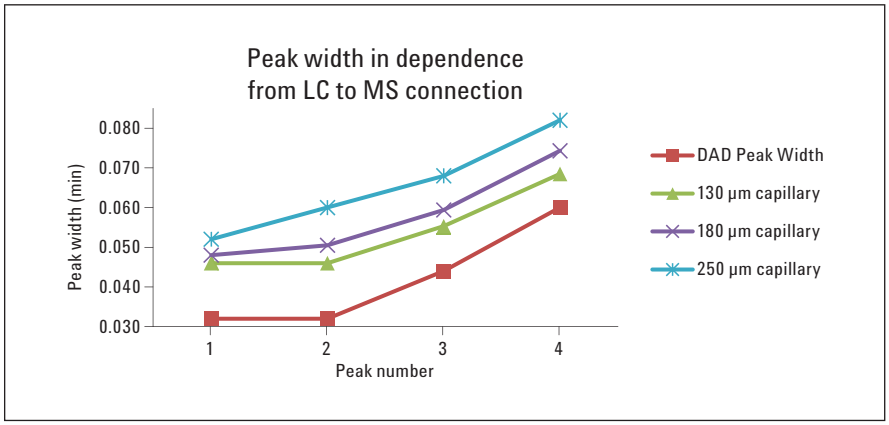


Figure 3
Peak width in relation to the inner diameter of the capillary connecting the LC to the MS.

A summary of the behavior of the peak height is given in Figure 4. It shows that the peak height decreases with the increase in the inner diameter of the connecting capillary and produces an accompanying loss in sensitivity. A similar behavior can be seen for the MS SIM data but on a higher level of sensitivity.

In a terminal experiment, the influence of the capillary length was determined. For this experiment, a capillary 500 cm × 130 µm was used. From the chromatograms, it can be seen that the peaks are well shaped and the early eluting peaks are well separated (Figure 5). The retention time is shifted by about 0.2 minutes compared to the DAD signals (Table 3 and Table 2). In theory, the peak width is most influenced by increased capillary inner diameter. In this case, the peak width is only increased by about 600 ms (Table 3 and Table 2). In comparison to Figures 3 and 4, the peak width performance for such a long capillary of 500 cm × 130 µm is in between the peak width performance of the 50 cm × 180 µm and the 50 cm × 250 µm capillary. The peak height performance is still between the 50 cm × 130 µm and the 50 cm × 180 µm capillary. A similar behavior can be seen for the MS SIM data but on a higher level of sensitivity. This demonstrates that the inner diameter is the much more critical parameter for the connection capillary from the LC system to the mass spectrometer than the length of the optimized capillary.

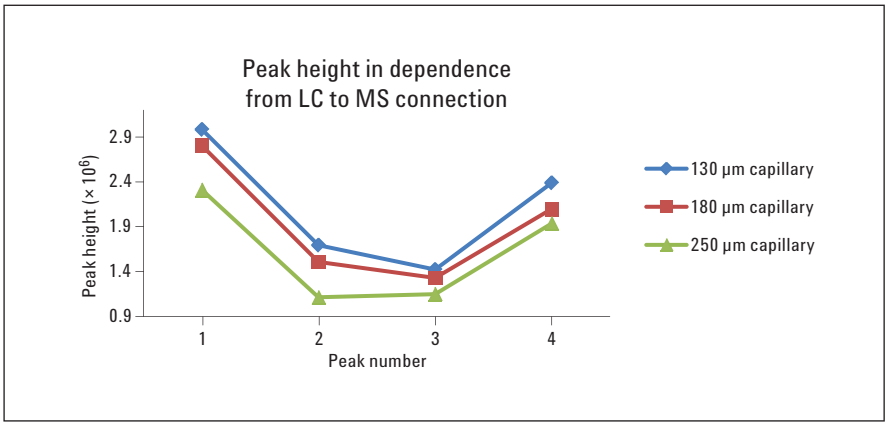
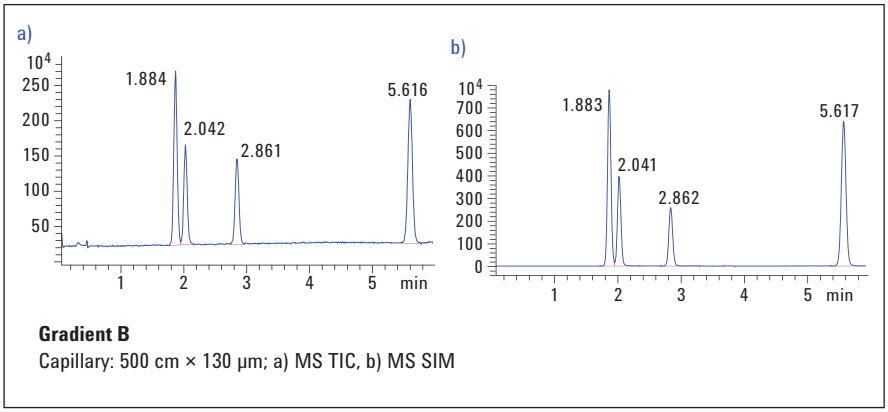


Figure 4
Peak height in relation to the inner diameter of the capillary connecting the LC to the MS.

MS connection: 500 cm, 0.13 mm id - MS TIC				
Peak	RT (min)	Width (min)	Height (× 10⁶)	Area (× 10⁶)
1	1.881	0.054	2.434	8.993
2	2.042	0.055	1.383	5.092
3	2.861	0.068	1.203	5.115
4	5.616	0.074	2.044	10.400
MS connection: 500 cm, 0.13 mm id - MS SIM				
Peak	RT (min)	Width (min)	Height (× 10⁶)	Area (× 10⁶)
1	1.883	0.061	7.671	29.210
2	2.041	0.060	3.857	14.290
3	2.862	0.064	2.594	11.120
4	5.617	0.082	6.398	33.590

Table 3
Retention time, peak width, peak height and peak area measured by MS TIC and MS SIM for a long 500 cm x 130 µm LC toMS connection capillary.



Gradient B
Capillary: 500 cm × 130 µm; a) MS TIC, b) MS SIM

Figure 5
Influence of the connection from the LC system to the mass spectrometer.

Conclusion

This Application Note demonstrates the value of the Agilent 1290 Infinity LC system as a partner for mass spectrometry. It shows that the low delay volume of the Agilent 1290 Infinity LC system is beneficial for the separation by shortening elution times, degreasing peak width, and therefore increasing the peak height. In addition, the detection sensitivity is improved with the inherent DAD as well as the associated MS detectors. It is also shown that the connection from the LC system to the associated detector has to be optimized to achieve the highest performance in separation for peak width and peak height by selecting the connection capillary with the lowest possible inner diameter and length.

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Agilent Technologies

Agilent 1290 Infinity LC
The ideal partner for MS – Part 2

Complementary, orthogonal detection by simultaneous UV and MS

Application Note

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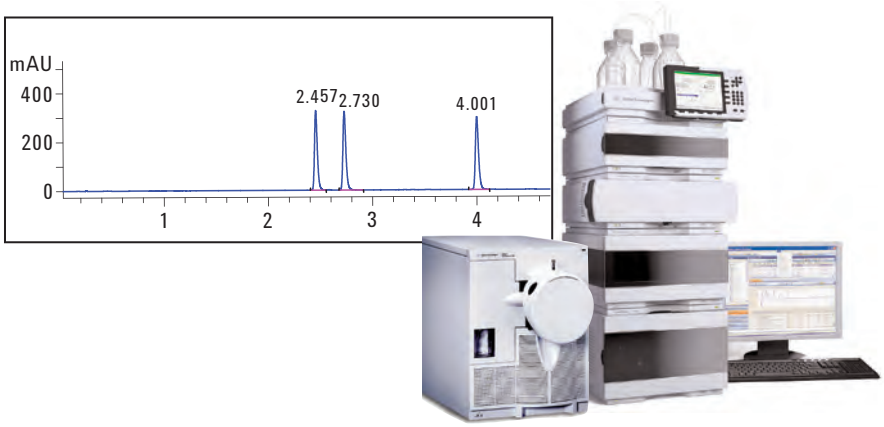

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Abstract

This Application Note demonstrates the use of DAD-UV detection and ESI-MS detection of compounds with different spectroscopic and ionization behavior in screening experiments. The compatibility of UV and MS detection is shown with example compounds of different spectroscopic and ionization properties. In addition, the Agilent 1290 Infinity LC Diode Array Detector is compared to the Agilent 1200 Series Diode Array Detector SL.

Agilent Technologies

Introduction

The screening of samples for their content is a widely used application including UV detection and mass spectrometric detection. This approach is typically used in quality control screening of pharmaceutical libraries, quality control screening of natural products, screening for pollutants in environmental samples, food control samples, and analytical walk-up solutions.

The detection by UV and mass spectrometry interact as complementary techniques in common screening approaches. It is not unusual that compounds which have UV activity do not ionize in electrospray mass spectrometry (ESI-MS) and vice versa. Compounds which undergo ionization in ESI-MS are often only detectable at one polarity, positive or negative. Therefore, it is desirable that a broad range of UV wavelengths is monitored and measured, a typical approach for a diode array detector (DAD). The mass spectrometer must be able to switch between positive and negative electrospray ionization polarity in a way, which is fast enough to follow the chromatographic separation.

This Application Note demonstrates the use of DAD-UV detection and ESI-MS detection of compounds with different spectroscopic and ionization behavior in screening experiments. The compatibility of UV and MS detection is shown with example compounds of different spectroscopic and ionization properties. Additionally, a comparison of the Agilent 1290 Infinity DAD (G4212A) to the Agilent 1200 Series DAD SL (G1315C) is shown.

Results and discussion

In special types of experiments such as screenings and walk-up solutions, LC/MS combines UV detection with the mass spectrometric detection of electrospray ionization giving comparable or complementary results. The separation of compounds in such a sample, and detection by UV and ESI-MS is shown in Figure 1. The detection by UV which was performed with the Agilent 1290 Infinity DAD, set on the wavelength 260 nm, is shown in Figure 1A. In this UV trace, it is seen that the sample contains five major compounds and one minor compound, which are detectable at 260 nm (other peaks are due to minor impurities in the compounds used to generate the sample). This does not show the full capabilities of the Agilent 1290 Infinity DAD, which is able to detect compounds at different wavelengths in parallel showing the complete spectral information comprised in the sample. For that purpose, all spectra where acquired and can be shown in a 3-dimensional plot over time, wavelength

Experimental

Equipment:
Agilent 1290 Infinity LC system containing an Agilent 1290 Infinity Binary Pump, Agilent 1290 Infinity High Performance Autosampler, 1290 Infinity Thermostatted Column Compartment, Agilent 1290 Infinity DAD and Agilent 6140 Single Quadrupole LC/MS system with ESI source.

Column:	Agilent ZORBAX SB C18, 50 × 2.1 mm, 1.8 µm
Software for data acquisition and data analysis:	ChemStation B.04.03

HPLC Method:

Solvent A:	Water + 0.1% formic acid
Solvent B:	Acetonitrile + 0.1% formic acid
Flow rate:	0.5 mL/min
Gradient:	0 min 5% B; 10 min 95% B
Stop time:	10 min
Post time:	3 min
Injection volume:	1 µL
Needle wash:	6 s in MeOH
Column temperature:	35 °C
Diode array detector:	10 mm standard cell, wave length 260/4 nm, Ref. 360/16 nm, slit 4 nm, data rate 40Hz

MS Method:

Source:	Gas temperature: 350 °C, nebulizer pressure: 45 psi, gas flow: 11 L/min, positive polarity
Signal 1:	Positive polarity
Signal 2:	Negative polarity
Scan:	100–1000 *m/z*
Sample:	Solution of: 1) Sulfamethazine (MW 278.0), 2) Sulfachloropyridazine (MW 284.0), 3) Hexanesulfonic acid (MW 166.0), 4) Sulfadimethoxine (MW 310.0), 5) Carbazole (MW 167.0), Crystall violet (MW 407.2), 7) 9-Phenanthrol (MW 194.0), each at a concentration of 100 ng/µL

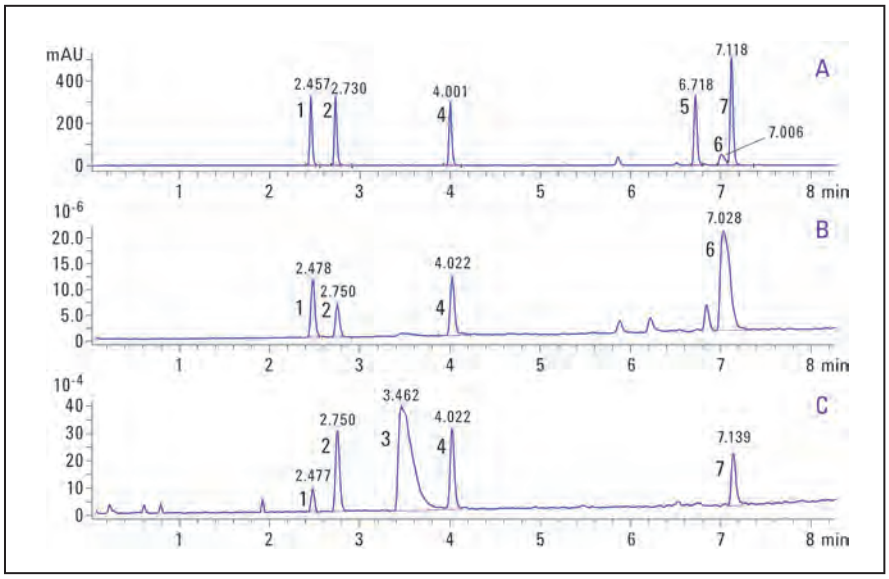

Figure 1
Separation and detection of sample compounds by UV and ESI-MS.
A) Detection by DAD UV at 260 nm. B) Detection by positive scan ESI-MS. C) Detection by negative scan ESI-MS.

and intensity (Figure 2). This plot presents the spectra of the five major compounds and shows that there is no other compound which has UV activity at another wavelength as the used one and the used wavelength is around the maximum absorbance wavelength for all compounds. It would be possible to measure other compounds with a maximum intensity at a different wavelength, because the DAD can acquire eight separate channels at different wavelenghts at the same time.

Comparable and complementary data can be acquired by adding a single quadrupole mass spectrometer such as the Agilent 6140 Single Quadrupole LC/MS system to the LC. This ionizes the compounds by electrospray ionization and allows measurement in positive and negative ion mode (Figures 1B and 1C). The total ion chromatogram from the positive electrospray ionization shows four major compounds and gives the [M+H]+ ion (Compound 1: *m/z* 279.0, Compound 2: *m/z* 285.0, Compound 4: *m/z* 311.0, Compound 6: *m/z* 372.0 (MW – Cl–)). Compounds 1, 2 and 4 can be detected by UV and under positive electrospray ionization conditions. Whereas Compound 6 has little UV activity but good ionization under positive electrospray conditions. The total ion chromatogram from the negative electrospray ionization shows five major compounds and gives the [M–H]– mass information (Compound 1: *m/z* 277.0, Compound 2: *m/z* 283.0, Compound 3: *m/z* 165.0, Compound 4: 309.0, Compound 7: *m/z* 193.0). Compound 3 shows only response in negative electrospray ionization, Compound 7 has UV activity and response in negative electrospray ionization.

Detection of Compounds 1, 2 and 4 is easiest because they have UV activity. Also, the compounds respond in positive and negative electrospray ionization (Figure 3). Compounds 6 and 7 show only UV activity and a positive or negative mass spectrum, respectively, but this is still sufficient for proper identification. Whereas, identification of Compounds 3 and 5 is somewhat more difficult because they show only negative mass spectrum or a UV spectrum, respectively. In Compound 5, the UV spectrum is very characteristic and could be identified by comparison to a spectral library (Figure 4). The result from this experiment clearly shows the value of adding an Agilent 1290 Infinity LC System with Agilent 1290 Infinity DAD to a mass spectrometer to gain comparative and complementary data. With the Agilent 6140 single quadrupole MS, it is possible to scan 10,000 amu/s in the ultrafast mode and to switch between positive and negative polarity in 20 msec for high speed separations.

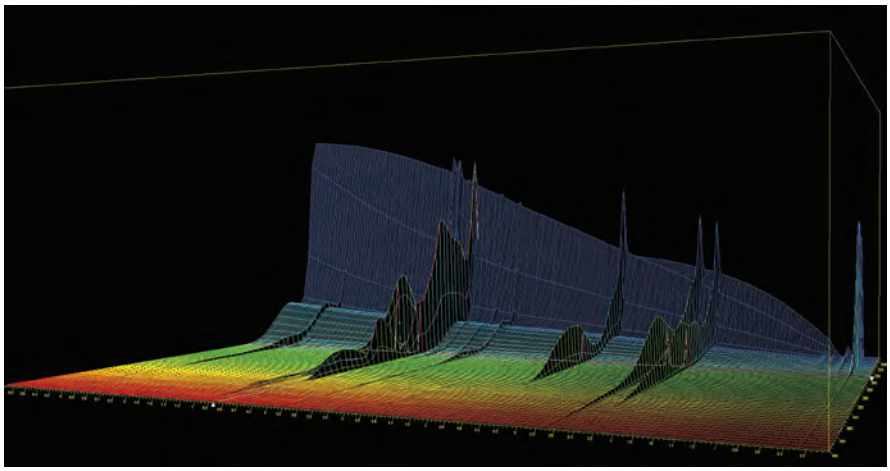

Figure 2
The 3-dimensional plot of wavelength over time and intensity, acquired with the Agilent 1290 Infinity DAD to show the complete spectral information comprised in the sample (X-axis: 0 min on the right side to 10 minutes, Y-axis: intensity in mAU, Z-axis: 190 nm to 400 nm on the front side).

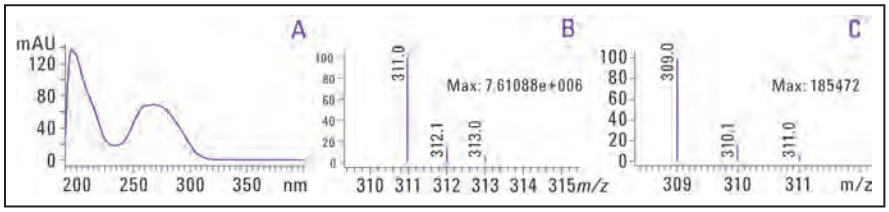

Figure 3
Complete spectral information of compound 4. A) UV spectrum with maximum 255 – 280 nm. B) Positive mass spectrum, *m/z* 311.0. C) Negative mass spectrum, *m/z* 309.0.

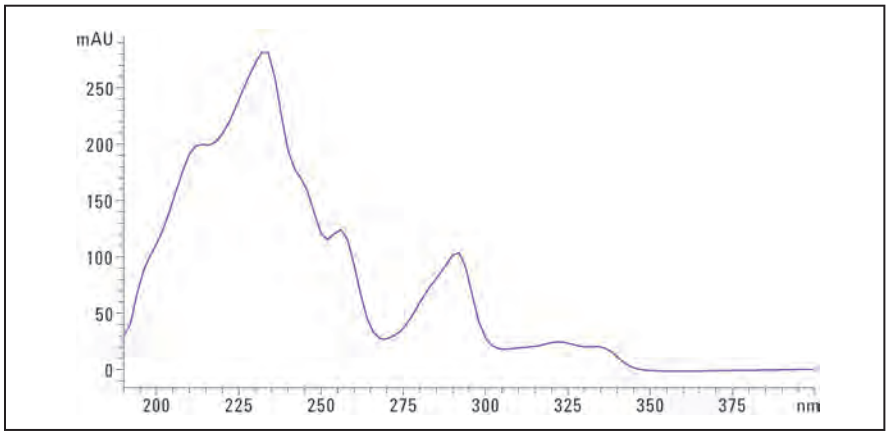

Figure 4
UV spectrum of Compound 5.

In the context of this work, an additional comparing evaluation of the Agilent 1290 Infinity DAD (G4212A) to the Agilent 1200 Series DAD SL (G1315C) was done to compare their performance. For this performance comparison, the peaks of Compounds 1, 2, and 4 where measured with the Agilent 1290 Infinity DAD equipped with the 10 mm Max-Light standard cell (G4212-60008) and with the Agilent 1200 Series DAD SL equipped with the 10 mm cell (G1315- 60022), the 6 mm cell (G1315-60025) and the 3 mm cell (G1315-60024). The obtained data are compared to show the influence on peak width, peak area and peak height (Table 1).

Comparing the Agilent 1290 Infinity DAD with 10 mm Max-Light cell to the Agilent 1200 Series DAD SL with 10 mm cell, it can be seen that the peaks are about 0.006 min (about 0.4 sec.) broader at half height and about 15% less in height, but the area remains unchanged. The loss in peak height and gain in peak broadness due to the larger volume of the flow cell results in less sensitivity for the Agilent 1200 Series DAD SL.

Comparing the results of the Agilent 1200 Series DAD SL with 10 mm cell to the 6 mm cell and the 3 mm cell, a decrease in peak width at half height can be seen, which is due to the shorter cell flow path length and lower flow cell volume. The peak width obtained with the 3 mm cell is comparable to the result obtained for Agilent 1290 Infinity DAD with the 10 mm cell. However, peak area and peak height declined due to the shorter flow path. The peak area and the peak height declined about 40% when exchanging the 10 mm cell with the 6 mm cell in the Agilent 1200 Series DAD SL. With the 3 mm cell the peak area and peak height declined down to one third compared to the 10 mm cell. The retention times are about 0.1 min higher in all cases compared to the Agilent 1290 Infinity DAD due to longer connecting cell capillaries. This result demonstrated the higher sensitivity of the Agilent 1290 Infinity DAD compared to the Agilent 1200 Series DAD SL even with the cell of the same path length. This is due to the improved cell design of the Max-Light cell. Furthermore the Max-Light cell leads to much less peak broadening due to its low dispersion volume. The valuable Max-Light cell can be protected from damages from overpressure due to MS sprayer blockage by an additional pressure relief valve between DAD and MS (Agilent in-line pressure relief valve kit, G4212-68001). This valve does not affect the performance of the separation by influence on the peak width.

1290 DAD G4212A, 10 mm cell (G4212-60008)				
Peak	**RT (min)**	**Width (min)**	**Area (mAU*s)**	**Height (mAU)**
1	2.457	0.0294	620.36	323.79
2	2.730	0.0306	646.19	319.40
4	4.001	0.0325	631.61	295.17
1200 DAD G1315C, 10 mm cell (G1315-600022)				
Peak	**RT (min)**	**Width (min)**	**Area (mAU*s)**	**Height (mAU)**
1	2.533	0.0350	621.95	273.74
2	2.842	0.0368	642.18	269.67
4	4.127	0.0395	631.03	245.46
1200 DAD G1315C, 6 mm cell (G1315-6025)				
Peak	**RT (min)**	**Width (min)**	**Area (mAU*s)**	**Height (mAU)**
1	2.530	0.0334	370.22	170.04
2	2.844	0.0353	381.69	166.04
4	4.126	0.0384	374.02	148.53
1200 DAD G1315C, 3 mm cell (G1315-60024)				
Peak	**RT (min)**	**Width (min)**	**Area (mAU*s)**	**Height (mAU)**
1	2.514	0.0295	193.22	100.20
2	2.824	0.0313	199.43	97.83
4	4.107	0.0334	195.17	89.64

Table 1
Data obtained from Agilent 1290 DAD and Agilent 1200 Series DAD SL to compare the influence of different flow cells on peak width, peak area, and peak height.

Conclusion

The results from these experiments clearly show the value of adding an Agilent 1290 Infinity LC system with an Agilent 1290 Infinity DAD to a mass spectrometer to gain comparative and complementary data. The use of DAD-UV detection and ESI-MS detection of compounds with different spectroscopic and ionization behavior is demonstrated and the compatibility of UV and MS detection is shown through example compounds of different spectroscopic and ionization properties. A comparison of the Agilent 1290 Infinity DAD to the Agilent 1200 DAD SL demonstrates the superior detection capabilities regarding UV sensitivity and lower dispersion volume of the Agilent 1290 Infinity DAD.

www.agilent.com/chem/lc

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Agilent Technologies

Agilent 1290 Infinity LC the ideal partner for MS – Part 3

Complimentary sensitivity of UV and MS detection

Application Note

Pharmaceutical and Chemical

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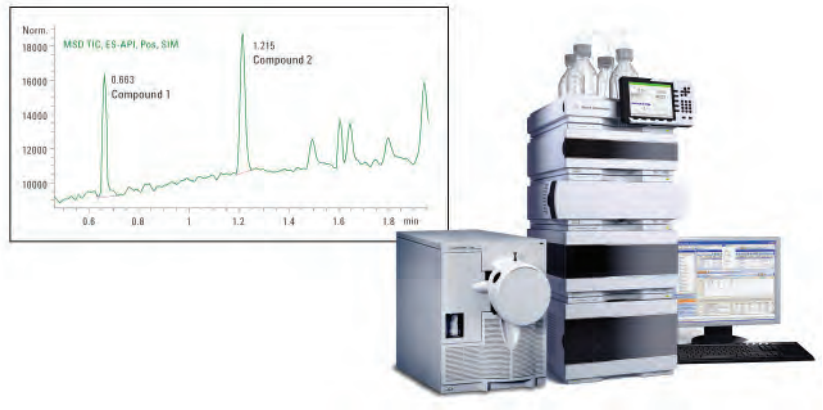

Abstract

This Application Note shows a comparison of sensitivities that can be achieved in typical fast LC/MS analysis with a single quadrupole mass spectrometer and diode array detectors for UV detection in different configurations. The limits of quantification are compared and the precision of peak areas as well as the range for detection by UV and MS together is determined for different DAD configurations.

Agilent Technologies

Introduction

Typical single quadrupole LC/MS systems contain a diode array detector (DAD) for additional UV detection. To achieve the best sensitivity for compound quantification, the MS is operated in selected ion monitoring (SIM) mode which is the most sensitive detection in such a system. On the DAD side, improvements were made to achieve higher sensitive detection, which enables better comparability to the MS SIM detection and quantification. The Agilent 1290 Infinity DAD is best suited for use in such a single quadrupole UHPLC/MS system for fast LC/MS analysis because of its high detection sensitivity. This can be achieved using different DAD flow cells, in particular with the Max-Light flow cell.

This Application Note compares the sensitivities that can be achieved in typical fast UHPLC/MS analysis between a single quadrupole mass spectrometer in SIM mode and a diode array detector (DAD) for UV detection using different flow cells. The limits of quantification are compared, and the overlapping range is determined for different DAD configurations. In addition, a comparison to an Agilent 1200 Series DAD SL detector is included.

Experimental

Equipment:

Agilent 1290 Infinity LC system containing an Agilent 1290 Infinity Binary Pump, Agilent 1290 Infinity High Performance Autosampler, Agilent 1290 Infinity Thermostatted Column Compartment, Agilent 1290 Infinity DAD and Agilent 6140 Single Quadrupole Mass Spectrometer.

Column:	Agilent ZORBAX SB C18, 50 × 2.1 mm, 1.8 µm
Software for data acquisition and data analysis:	ChemStation B.04.03

HPLC Method:

Solvent A:	Water + 0.1% formic acid
Solvent B:	Acetonitrile + 0.1% formic acid
Flow rate:	1.0 mL/min
Gradient:	0 min 10% B – 2.0 min 50% B – 2.01 min 95% B – 2.5 min 95% B
Stop time:	2.5 min
Post time:	1 min
Injection volume:	1 µL
Needle wash:	6 s in MeOH
Column temperature:	40 °C
Diode array detector:	10 mm standard Max-Light cartridge cell, 60 mm high sensitivity Max-Light cartridge cell, wave length 280/4 nm, Ref. 360/16 nm, slit 8 nm, data rate 20 Hz.

MS Method:

Source:	Gas temperature: 350 °C, nebulizer pressure: 60 psi, gas flow: 12 L/min, capillary: 4000 V, positive polarity		
Signal 1:	SIM, peak width: 0.04 min, cycle time: 0.24 s, gain: 1.0		
	Ion (m/z)	Fragmentor (V)	Dwell time (msec.)
	279.10	120	114
	311.10	120	114
Sample:	Stock solution of: 1) Sulfamethazine (MW 278.0), 2) Sulfachloropyridazine (MW 310.0) each at a concentration of 10 ng/µL. Dilution: (pg/µL): 10,000; 5,000; 2,000; 1,000; 500; 200; 100; 50; 20; 10; 5; 2; 1; 0.5; 0.2; 0.1.		

Results and discussion

In a quantitative HPLC/single quadrupole MS analysis, typically the mass spectrometer has the higher sensitivity compared to the diode array detector and therefore delivers the lower limits of detection (LOD) and lower limits of quantification (LOQ).

This work shows calibration curves acquired with an Agilent 1290 Infinity LC single quadrupole LC/MS system for quantification and discusses the comparison of achieved limits of quantification from a single quadruple MS and a DAD in different cell configurations and models. As a definition for the limit of detection (LOD), a signal-to-noise ratio ≥ 3 was used, and for the limit of quantification (LOQ) a signal-to-noise ratio of ≥ 10 was used.

With the Agilent 6140 Single Quadrupole Mass Spectrometer a LOQ of 1 pg (on column) for both tested compounds was found with a S/N ≥ 10. The calibration was continued up to 10,000 pg (on column) with good correlation factors (Figure 1).

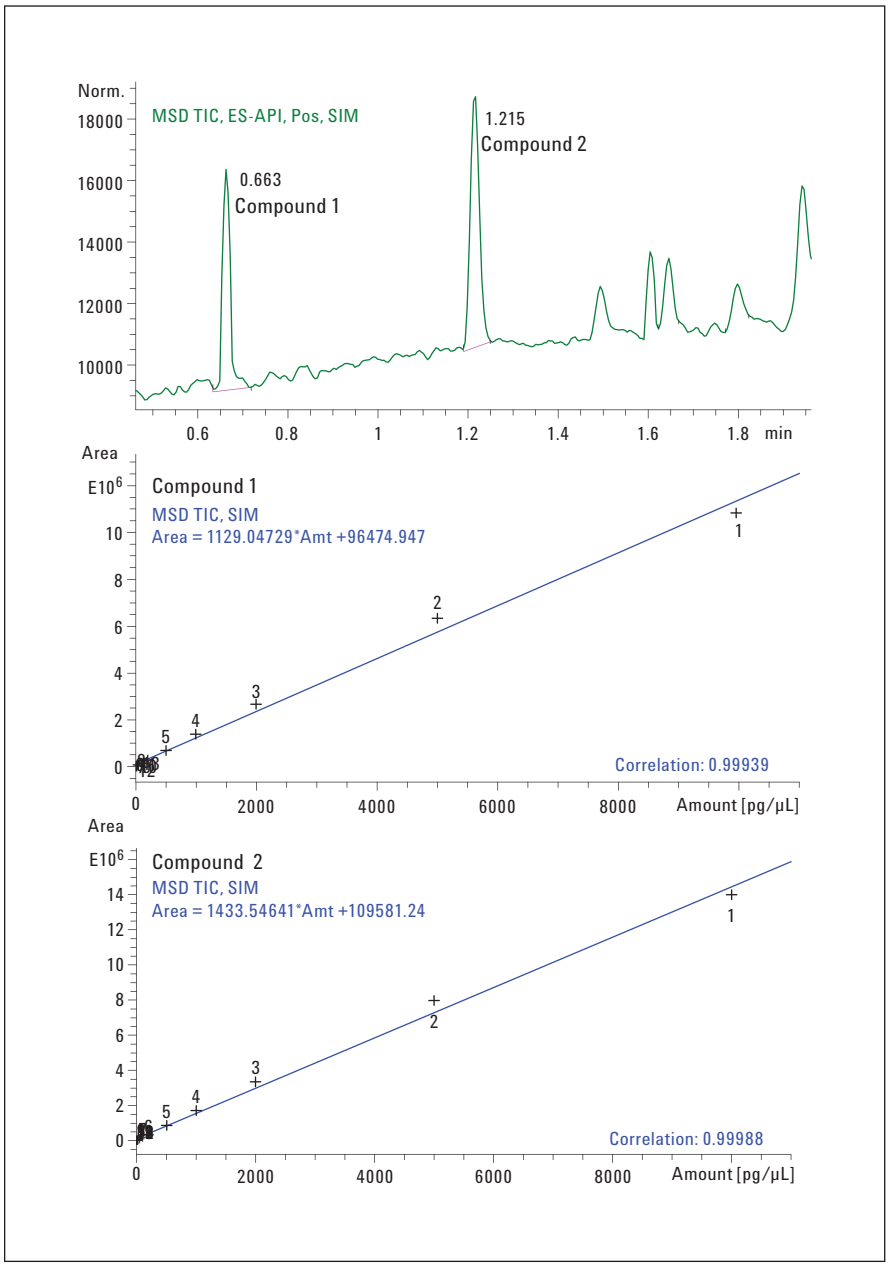

Figure 1
Calibration of compounds 1 and 2 with an Agilent 6140 Single Quadrupole Mass Spectrometer in SIM mode.
Compound 1: LOQ 1 pg/µL (S/N=10), Correlation: 0.99939.
Compound 2: LOQ 1 pg/µL (S/N=13), Correlation: 0.99988.

For the Agilent 1290 Infinity DAD equipped with the 10-mm Max-Light cell, the experiment delivered a LOQ of 100 pg (on column) with good correlation factors for a calibration up to 10,000 pg (on column) (Figure 2) which is a factor of 100 above the LOQ of the mass spectrometric detection. For DAD detection with higher sensitivity the 60-mm Max-Light cell is available for the Agilent 1290 Infinity DAD.

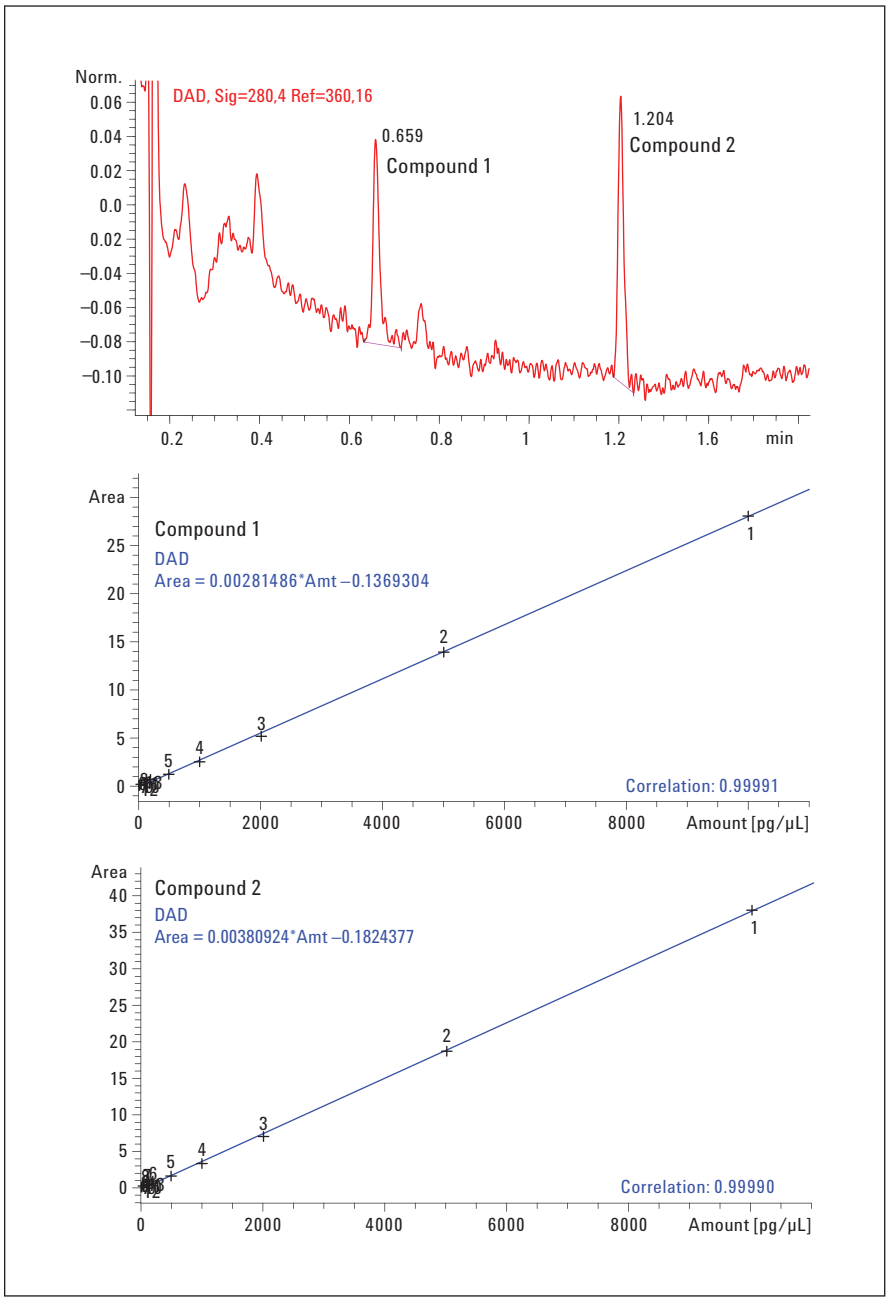

Figure 2
Calibration of compounds 1 and 2 with an Agilent 1290 Infinity DAD with 10 mm Max-Light cell.
Compound 1: LOQ 100 pg/µL (S/N=10), Correlation: 0.99991.
Compound 2: LOQ 100 pg/µL (S/N=11), Correlation: 0.99990.

This DAD configuration provides a LOQ of 20 pg (on column) for both compounds which reduces the gap between mass spectrometric detection and UV detection by a factor of 5 (Figure 3).

The used configuration comprising a 2.1-mm id column together with the 60-mm DAD flow cell works well under the chosen conditions but achieves best performance together with typically 3.0-mm id and 4.6-mm id columns at higher flow rates.

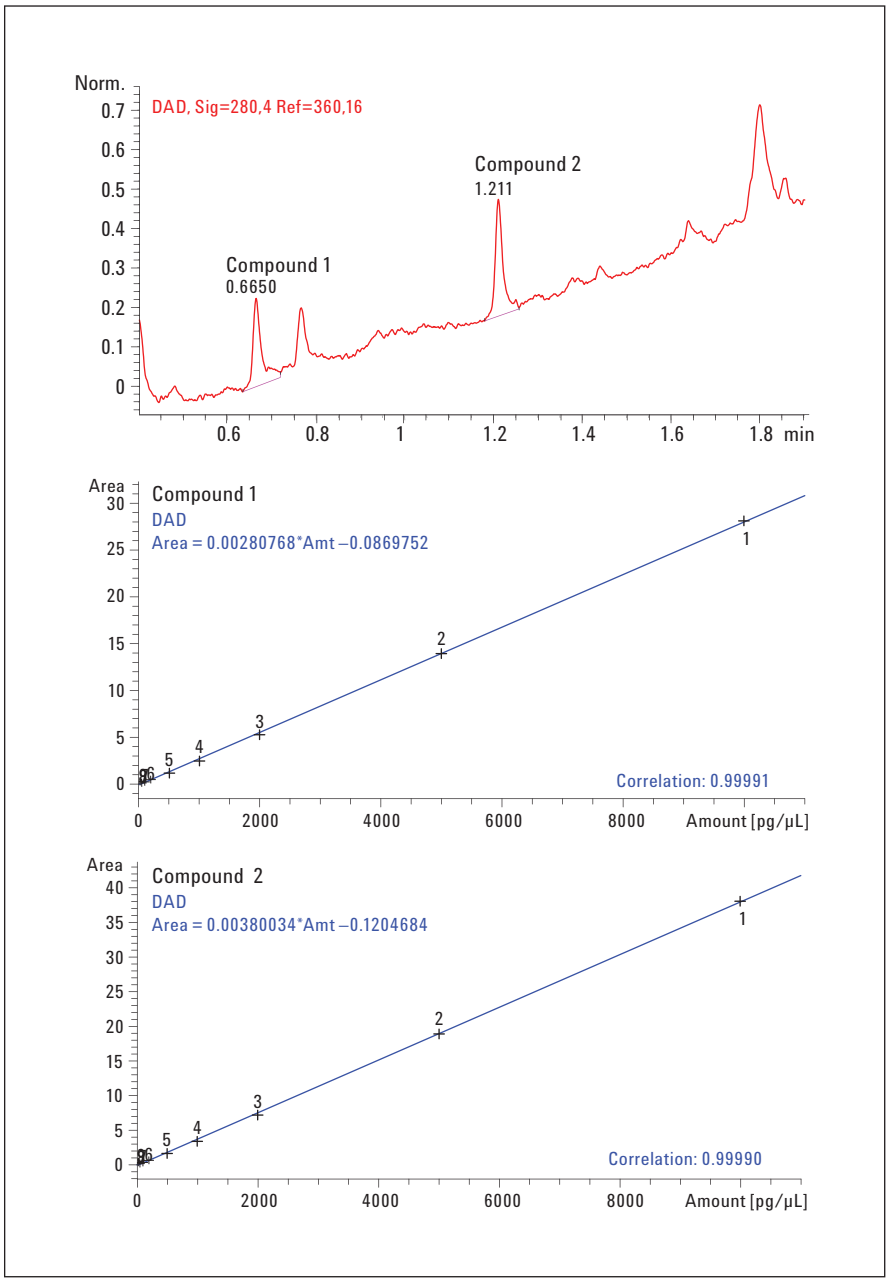

Figure 3
Calibration of compounds 1 and 2 with an Agilent 1290 Infinity DAD with 60 mm Max-Light cell.
Compound 1: LOQ 20 pg/µL (S/N=10), correlation 0.99991.
Compound 2: LOQ 20 pg/µL (S/N=12), correlation 0.99990.

Finally, a comparison to the Agilent 1200 Series DAD SL with a 10-mm cell was done, showing a LOQ of 200 pg (on column) which is far off from the mass spectrometric sensitivity (Figure 4).

The results for the achieved limits of detection are summarized in Table 1. This clearly shows the best overlap between mass spectrometric detection and UV detection is achieved with the Agilent 1290 Infinity DAD equipped with the 60-mm Max-Light cartridge cell. The Agilent 1290 Infinity DAD with the 60-mm cell provides a 5 times higher sensitivity compared to the 10-mm Max-Light cartridge cell and a 10 times higher sensitivity compared to the Agilent 1200 Series DAD SL with a 10-mm cell.

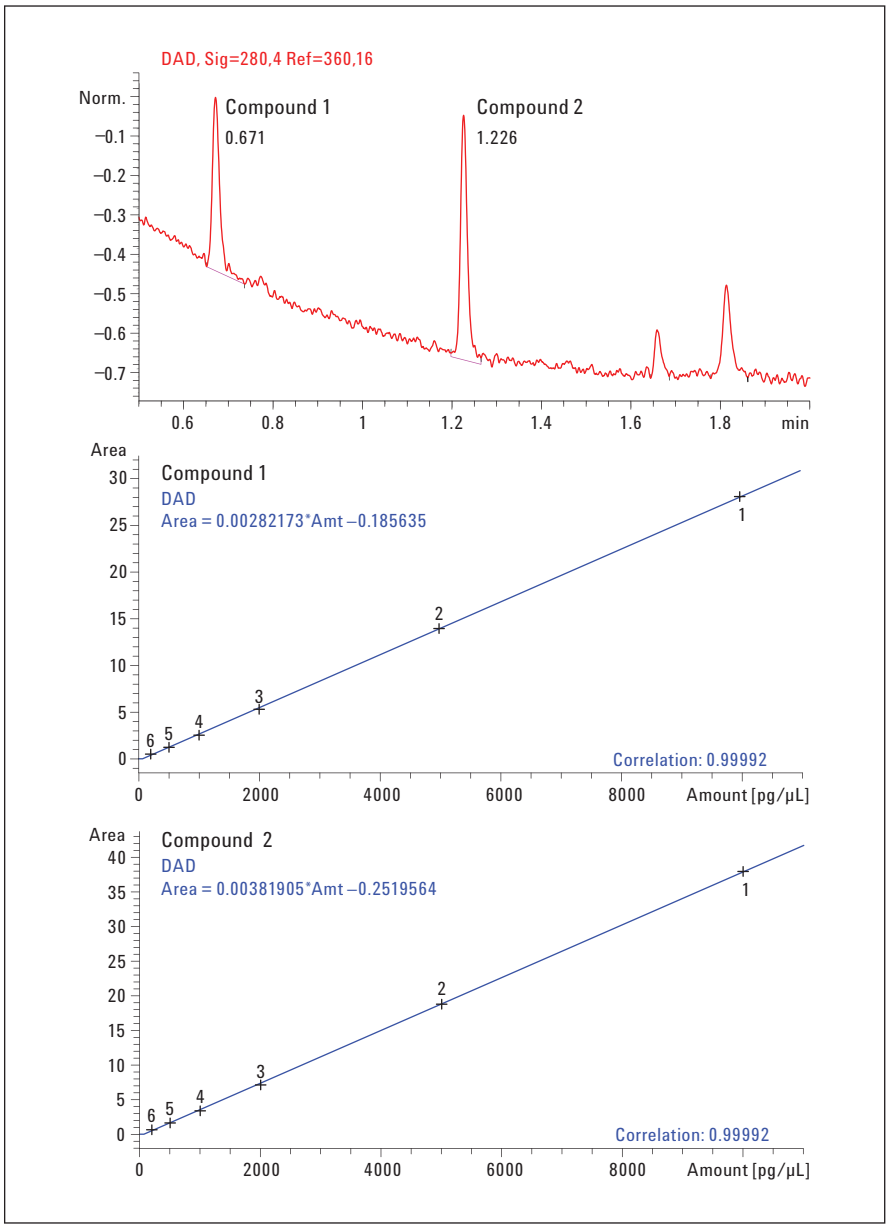

Figure 4
Calibration of compounds 1 and 2 with an Agilent 1200 Series DAD SL with 10 mm cell.
Compound 1: LOQ 200 pg/µL (S/N=10), correlation: 0.99992.
Compound 2: LOQ 200 pg/µL (S/N=15), correlation: 0.99992.

	Compound 1 [pg]		Compound 2 [pg]	
	LOD	LOQ	LOD	LOQ
Agilent 6140 MS SIM	0.2	1.0	0.2	1.0
Agilent 1290 Infinity DAD 60 mm Max-Light cell	10	20	10	20
Agilent 1290 Infinity DAD 10 mm Max-Light cell	20	100	20	100
Agilent 1200 Series DAD SL 10 mm cell	100	200	100	200

Table 1
LOD and LOQ for compound 1 and 2 measured with MS and DAD in different configurations.
LOD: S/N ≥ 3, LOQ: S/N ≥ 10.

In addition to LOQ, another important parameter for the quality of quantification is the precision of peak areas, which can be compared by the calculated relative standard deviations (RSD [%]). Therefore, all points used in the shown calibrations were done in five replicates and the RSD [%] was calculated from average area and standard deviation. For Compound 1, the area RSD [%] versus the concentration for mass spectrometric detection and detection with different DAD configurations and DAD models is shown in Figure 5. For the mass spectrometric detection, the relative standard deviation for the peak area at 5 pg (on column), is 5.70% (Figure 5 and Table 2). Towards the LOQ the RSD for the mass spectrometric detection goes up to about 10%. For higher concentrations above 20 pg (on column), the RSD for mass spectrometric detection is always between 1% and 2%. For the detection of Compound 1 with a DAD at the LOQ, the individual RSDs are between 4% and 5%. For higher concentrations, the RSDs for the detection with a DAD went down to about 0.2% for all DAD configurations and models. The comparison of the DAD detection to the mass spectroscopic detection for higher concentrations always shows a lower RSD value for the DAD detection and herewith higher precession. Comparison of the different DAD configurations and DAD models shows a lower RSD and herewith higher precession for the one with the higher sensitivity (lower LOQ) compared to the one with lower sensitivity (higher LOQ) at its LOQ. Similar results were found for Compound 2 (data not shown).

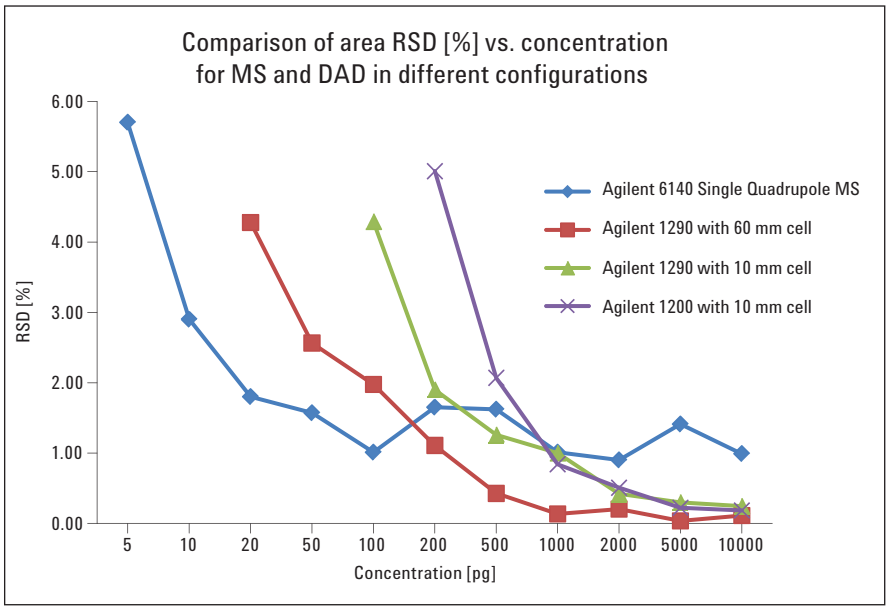

Figure 5
Area RSD [%] vs. concentration for mass spectrometric detection and detection with different DAD configurations and DAD models for Compound 1.

Compound 1											
Concentration [pg/µL]	5	10	20	50	100	200	500	1000	2000	5000	10000
Agilent 6140 SQ MS SIM	5.70	2.90	1.80	1.57	1.01	1.65	1.62	1.01	0.90	1.41	0.99
Agilent 1290 with 60 mm cell			4.28	2.56	1.97	1.11	0.43	0.14	0.20	0.04	0.11
Agilent 1290 with 10 mm cell					4.29	1.90	1.26	1.00	0.42	0.30	0.25
Agilent 1200 with 10 mm cell						5.01	2.07	0.84	0.51	0.22	0.19

Table 2
Area RSD [%] vs. concentration for mass spectrometric detection and detection with different DAD configurations and DAD models for Compound 1.

Conclusion

This Application Note shows a comparison of sensitivities that can be achieved in typical fast LC/MS analysis with a single quadrupole mass spectrometer and diode array detectors for UV detection in different configurations. The limits of detection and limits of quantification are determined with the result that the Agilent 1290 Infinity DAD equipped with a 60-mm Max-Light cartridge cell has the closest sensitivity to the mass spectrometric detection in an UHPLC system comprising an Agilent 1290 Infinity LC system and an Agilent 6140 Single Quadrupole Mass Spectrometer. Data are provided which show that the Agilent 1290 Infinity DAD configuration with the 60-mm Max-Light cartridge cell achieves a five times higher sensitivity than the configuration with the 10-mm Max-Light cartridge cell and a 10 times higher sensitivity than the Agilent 1200 Series DAD SL with 10-mm flow cell.

The displayed data and conclusion drawn from the discussion give verification that an LC/MS system with a DAD providing the necessary sensitivity and a single quadrupole mass spectrometer nicely fit together.

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Agilent Technologies

Agilent 1290 Infinity LC
The ideal partner for MS – Part 4

Increased sensitivity by enhanced separation between analyte and matrix with the Agilent 1290 Infinity LC

Application Note

Chemical and Pharmaceutical Analysis, Food Safety

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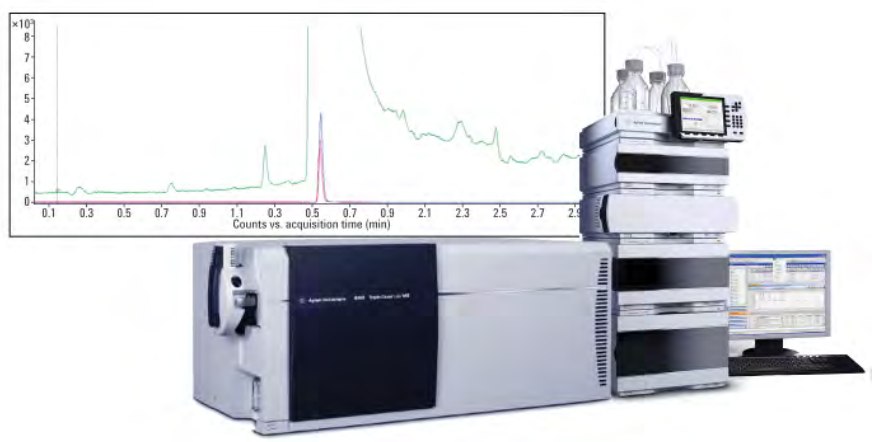

Abstract

This Application Note demonstrates the advantage of using an Agilent 1290 Infinity LC system with 1.8 µm columns as the front-end of an Agilent 6460 Triple Quadrupole MS system. This allows for better separation of the analyte compound from matrix compounds compared to conventional HPLC separation on a 5 µm column. The presented data shows an increase in peak height and sensitivity by decreased peak width and decreased matrix suppression caused by improved separation on an Agilent 1290 Infinity LC used in combination with 1.8 µm particle size columns.

Agilent Technologies

Introduction

Today, the analytical chemist is faced with the challenge to detect and quantify compounds in very low trace amounts buried in high amounts of complex matrixes. This is especially important for the determination of pesticides in different food matrixes in modern LC/MS analysis. A typical problem is the prevention of matrix suppression of the analytes. Matrix suppression occurs during the ionization of an analyte in the ion source of a mass spectrometer when other compounds are present in large excess at the same time, resulting from coelution out of the LC column. Modern UHPLC systems, which have the capability to work with 1.8 µm particle columns, can help solve this problem by minimizing matrix suppression effects due to improved separation of the analyte compounds from matrix components and producing sharper peaks.

This Application Note shows the advantage of using an Agilent 1290 Infinity LC system with 1.8 µm columns as the front end of a triple quadrupole MS instrument to achieve better separation of analyte compound from matrix compounds compared to conventional HPLC separation on a 5 µm column. As a matter of principle, the effect of matrix suppression is shown with a two compound mixture and by an example with plasma matrix. Finally, the effect is examined with a complex food matrix in a multi pesticide method. The presented data shows an increase in peak height due to an improved separation, sharper peaks and less ion suppression achieved with the Agilent 1290 Infinity LC running 1.8 µm columns.

Experimental

Equipment:
An Agilent 1290 Infinity LC system consisting of the following modules was used:
Agilent 1290 Infinity Binary Pump
Agilent 1290 Infinity High Performance Autosampler
Agilent 1290 Infinity Thermostatted Column Compartment
Agilent 6460 Triple Quadrupole Mass Spectrometer

Columns:
1) Agilent ZORBAX Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm
2) Agilent ZORBAX Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm
3) Agilent ZORBAX Eclipse Plus C18, 2.1 × 150 mm, 5 µm

Software for data acquisition and data analysis:
MassHunter Data Acquisition software
MassHunter Optimizer software
MassHunter Qualitative Data Analysis software

HPLC Method:

Solvent A:	Water + 0.1% formic acid
Solvent B:	Acetonitrile + 0.1% formic acid
Flow rate:	0.8 mL/min.
Gradient 1 (50 mm column):	0 min 5% B; 2.0 min 95% B; 2.5 min 95% B
Stop time:	2.5 min
Post time:	2 min
Gradient 2 (150 mm column):	0 min 5% B; 15.0 min 95% B; 16.0 min 95% B
Stop time:	16 min
Post time:	3 min
Injection volume:	1 µL Needle wash: 6 sec in MeOH
Column temperature:	35 °C

MS Method:

Source:	Sheath gas temperature: 350 °C, sheath gas flow: 11 L/min
Capillary gas temperature:	300 °C
Capillary gas flow:	5 L/min
Nebulizer pressure:	50 psi
Capillary:	4,000 V
Nozzle voltage:	500 V
Polarity:	positive
MRM settings:	see table 1

Samples:
Stock solutions (100 µg/mL) of: Sulfamethazine (MW 278.0), Sulfamethizole (MW 270.0), Verapamil (MW 454.1). Ehrenstorfer Pesticide Mix 44, content see Table 1, concentration 10 ng/mL each.

Compound name	Ion (m/z)	MRM transition	Fragmentor (V)	Collision energy (V)
Sulfamethizole	271.02	271.03 → 156.0	110	24
Sulfamethazine	279.09	SIM mode only	130	
Verapamil	455.29	455.29 → 165.1	190	24
Content of Ehrenstorfer Pesticide Mix 44				
Atrazinedesethyl	188.07	188.07 → 146.0	115	12
Atrazine	216.10	216.10 → 174.0	130	4
Chlorotoluron	213.08	213.08 → 72.1	105	20
Methabenzthiazuron	222.07	222.07 → 96.0	100	56
Metobromuron	259.01	259.01 → 91.0	100	32
Metolachlor	284.14	284.14 → 176.1	95	24
Cyanazine	241.10	241.10 → 214.1	120	12
Diuron	233.03	233.03 → 72.1	90	20
Hexazinone	253.17	253.17 → 171.1	100	12
Metoxuron	229.08	229.08 → 72.1	115	24
Monolinuron	215.06	215.06 → 99.0	70	35
Sebuthylazine	230.12	230.12 → 174.0	130	12
Isoproturon	207.15	207.15 → 165.1	100	8
Linuron	249.02	249.02 → 182.0	110	12
Metazachlor	278.11	278.11 → 105.0	75	48
Simazine	202.09	202.09 → 124.1	120	12
Terbuthylazine	230.12	230.12 → 104.0	125	32

Table 1
MRM transitions of used compounds. Fragmentor voltages and collision energies determined with MassHunter Optimizer. Dwell time: 10 ms.

Results and Discussion

In an atmospheric pressure ionization (API) source of a mass spectrometer, the effluent from the liquid chromatography is pneumatically sprayed into an electrical field countercurrent to a heated gas stream (electrospray ionization, ESI). Under these conditions, the formed spray droplets evaporate and ions are formed on the surface of the droplets. The droplets shrink to a critical size where the Coulomb forces become too strong and the droplets explode. The free ions are then drawn into the mass spectrometer by the electrical field. If compounds are coeluting from the liquid chromatography column, they compete in the formation of ions. If one compound is in a large excess, this compound can suppress the ionization of the minor compound and decrease its MS signal.

This effect was demonstrated in principle by coeluting sulfamethazine (100 ng/µL) and sulfamethizole (100 pg/mL) from the LC column, where sulfamethazine was present in a 1,000-fold excess (Figure 1). The comparison of the intensity of the MRM signal of sulfamethizole coeluting with a large excess of sulfamethazine shows about 25% less signal intensity compared to the signal in the absence of the large excess of sulfamethazine. This decrease in signal intensity by ion suppression is commonly called matrix effect.

Precipitated blood plasma is a matrix often encountered in clinical and forensic samples. To generate the sample, blood plasma is diluted with about a 3-fold excess of acetonitrile which precipitates the proteins comprised in the plasma. After removal of the precipitant by centrifugation, the supernatant is directly used for LC/MS injection. The advantage is the fast sample preparation, but the disadvantage is that some matrix components remain in the solution, especially glycerophospholipides and lysophospholipides. Common to all phospholipids is a phosphatidylcholine moiety. This part of the molecule can be cleaved off by collision induced dissociation (CID) and detected in a precursor ion scan experiment at *m/z* 184 (Figure 2A).

The molecular weight of glycerophospholipides and lysophospholipides is typically between 400 *m/z* and 800 *m/z*. This means all matrix poshoplipids can be detected and their position in the gradient form LC separation can be determined to avoid any suppression by coelution overlap with the analyte. There are still other components in the matrix which cannot be detected in such an experiment. For their localization, a suppression profile was acquired (Figure 2B). An example drug, verapamil, was taken and the triple quadrupole was run in MRM mode optimized for this compound. First, a blank was acquired with these settings.

In a second experiment, a blank matrix sample was injected and verapamil (10 pg/µL) was infused into the column effluent between column outlet and MS sprayer by a syringe with a T-piece (250 µL/h). The suppression profile was generated by subtraction of the MRM trace of the second experiment from the blank. It is shown, that the highest suppression occurs at the end of the run after 1.9 minutes, exactly where the phospholipids elute

(Figures 2A and 2B). Other early eluting polar compounds cause suppression at the beginning of the run between 0.2 and 0.3 minutes. In the middle of the run, where typical pharmaceutical compounds elute, there is no significant suppression, and separation is not a challenging task. In this example, a plasma sample spiked with verapamil was compared to a standard of verapamil at the same concentration (10 pg/µL) to determine the suppression (Figure 2C). The signal is suppressed by about 30% at the retention time in this gradient (gradient 1, column 1).

The influence of separation becomes more critical when matrix complexity increases which is often the case in food matrixes. In such a case, separation performance becomes important and the advantage of an UHPLC which is able to produce the necessary back pressure to work with 1.8 µm columns compared to an HPLC instrument which can only work with the classical 5 µm columns becomes obvious. A matrix from ginger, which is rich in background compounds, was taken as an example. This matrix was separated on a 2.1 × 150 mm, 5 µm column (column 3) and on a 2.1 × 150 mm, 1.8 µm column (column 2). The difference could be seen immediately by the achieved separation with better resolution in the chromatogram of the 1.8 µm column (Figures 3A and 4A). The back pressure of the 1.8 µm column at the flow rate of 0.8 mL/min is about 940 bar and the back pressure of the 5 µm column is approximately 160 bar at starting conditions. The separation performance with higher resolution and narrower peaks achieved with the 1.8 µm column can separate the compounds of interest much better from the matrix and proceed to a better detection performance.

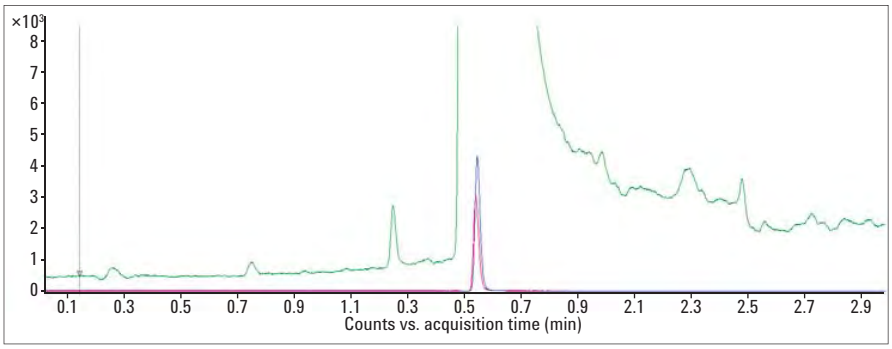

Figure 1
Principal effect of matrix ion suppression. A compound in large excess (sulfamethazine, 100 ng/µL, green, SIM trace) is coeluting from the LC together with a minor compound (sulfamethizole, 100 pg/µL, red, MRM trace) of interest and suppresses their signal by ionization competition compared to the standard solution of the same concentration without presence of matrix (blue, MRM trace).

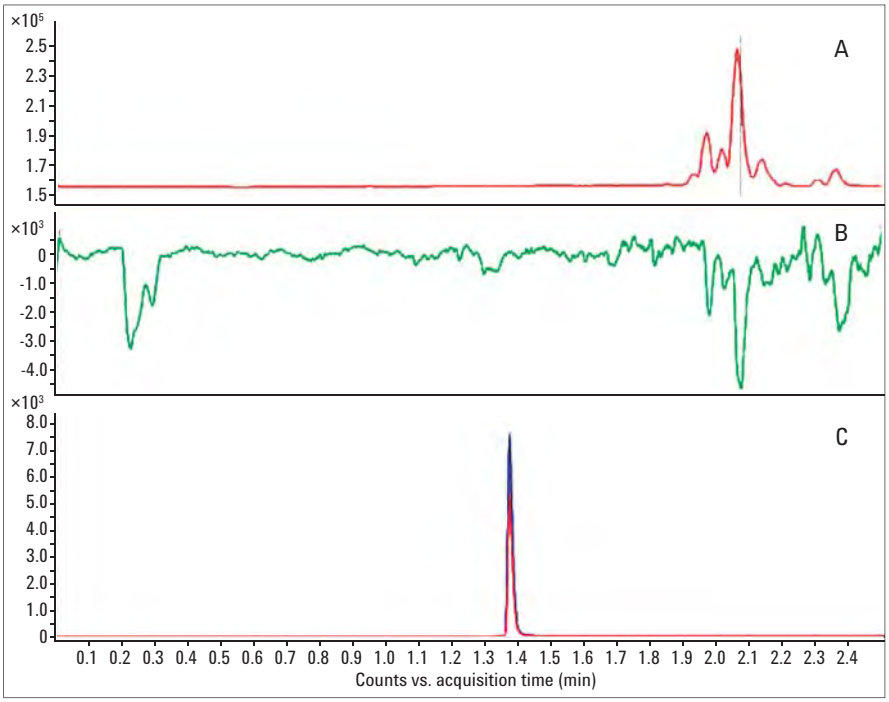

Figure 2
A) Precursor ion scan experiment with a precipitated plasma sample, 400 - 800 *m/z* → 184 *m/z*, for the detection of phospholipids.
B) Determination of the matrix effect for verapamil in plasma in MRM mode optimized for verpamil.
C) Verapamil in plasma (red) compared to a verapamil standard (blue) at the same concentration.

To demonstrate the influence of the matrix with suppression effects, the following experiment was done. A MRM method was developed for a defined mixture of pesticides (Table 1). The pesticide mixture (100 pg/µL, each component) was infused into the column effluent between the end of the column and the MS ESI sprayer by a syringe (250 µL/h). After the acquisition of a blank, the ginger sample was injected. To visualize the suppression effects, the sample separation was subtracted from the blank (Figures 3B and 4B). The resolution of the matrix compounds is much better on the 1.8 µm column than on the 5 µm column, peaks are much sharper and as a direct consequence matrix suppression occurs at more defined retention times (Figures 3B and 4B). With this higher resolution, it is expected that matrix effects are minimized for the measurement of pesticides in such a sample by using 1.8 µm columns on an Agilent 1290 Infinity LC system capable of delivering the necessary back pressure as front end for mass spectrometric analysis.

To compare the performance of both separations for a multi pesticide analysis, the mixture was spiked into ginger matrix to a final concentration of 100 pg/µL for each pesticide. The sample was measured with a 5 µm column and with a 1.8 µm column with the same method and the final result was compared to a measurement of the standard mixture (Figure 5). For the three most intense peaks, an ion suppression between measurement of the standard and measurement in matrix of about 10–15% could be seen with the 1.8 µm column (Figures 5A and 5B). The comparison between measurement of the pesticides in matrix on the 1.8 µm column and on the 5 µm column shows up to 50% less intense peaks for the separation on the 5 µm column (Figures 5B and 5C). The comparison of the measurement of the standard on

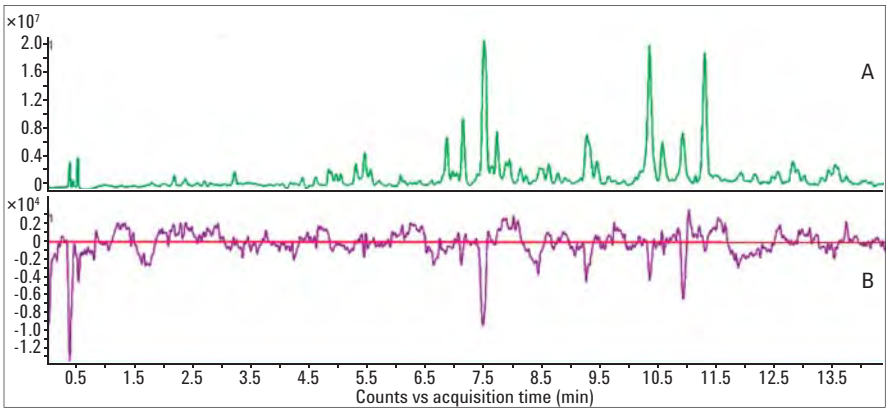

Figure 3
A) Separation of compounds comprised in a matrix of ginger on a 2.1 × 150 mm, 5 µm column (column 3) at 0.8 mL/min, applying gradient 2 at about 160 bar at starting conditions (ESI TIC).
B) Matrix suppression profile TIC MRM for a mixture of 17 pesticides. Pesticide concentration 100 pg/µL each, infused after the column by syringe at 250 µL/hour.

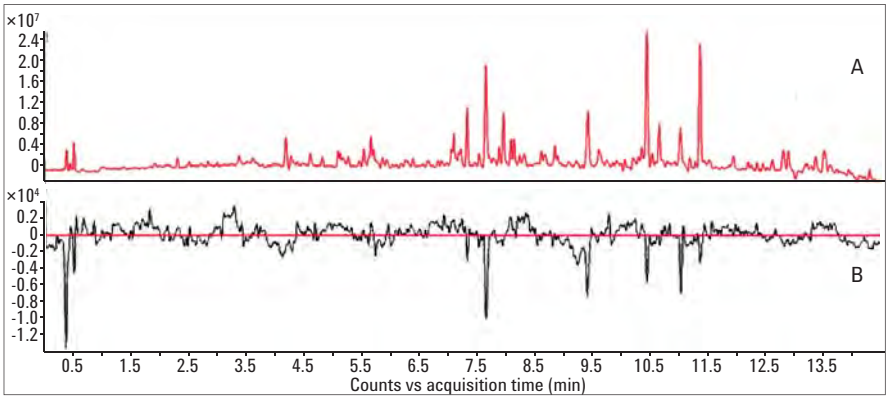

Figure 4
A) Separation of compounds comprised in a matrix of ginger on a 2.1 × 150 mm, 1.8 µm column (column 2) at 0.8 mL/min, applying gradient 2 at about 940 bar at starting conditions (ESI TIC).
B) Matrix suppression profile TIC MRM for a mixture of 17 pesticides. Pesticide concentration 100 pg/µL each, infused after the column by syringe at 250 µL/hour.

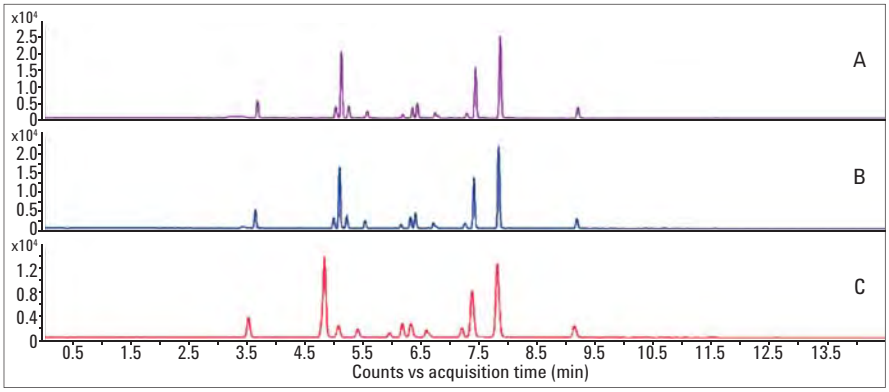

Figure 5
Measurement of ion suppression effects in a multi-pesticide method in ginger matrix (TIC MRM).
A) Standard of the pesticide mixture containing 17 compounds (some are coeluting) at 100 pg/µL each.
B) Measurement of the 17 pesticides in ginger matrix with a 1.8 µm column (940 bar at starting conditions).
C) Measurement of the 17 pesticides in ginger matrix with a 5 µm column (160 bar at starting conditions).

the 1.8 µm column and in matrix with a 5 µm column gives signal intensities even lower than 50% due to unresolved coelution of matrix compounds and peak broadening (Figures 5A and 5C).

This could be seen more accurately if the peaks were extracted individually and compared. As an example the peak of Monolinuron was extracted (Figure 6). Compared to the measurement in standard, an ion suppression of 17.5% could be seen for the measurement with a 1.8 µm column (Figures 6A and 6B) and an additional 25% decrease compared to the 5 µm column (Figures 6B and 6C).

Conclusion

This Application Note demonstrates the influence of ion suppression on peak height starting from principal examples up to examples of highest complexity. For an example of a multi-pesticide analysis in a complex sample, it is shown that the ion suppression critically depends on the quality of the separation. Superior results were achieved by using 1.8 µm columns compared to 5 µm columns. Due to the higher back pressure of the 1.8 µm columns the best separation performance with the minimized ion suppression and therefore most intense peaks even in complex matrixes were achieved with an Agilent 1290 infinity LC system as the front end for pesticide analysis by triple quadrupole mass spectrometry.

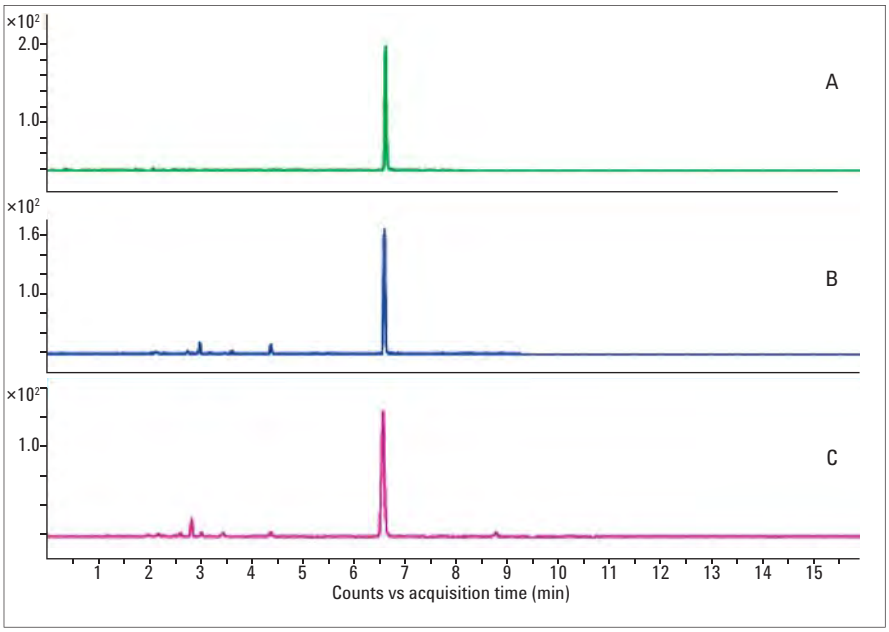

Figure 6
Ion suppression for the pesticide compound monolinuron, eluting at 6.6 minutes, measured by MRM.
A) Monolinuron MRM from measurement in the standard pesticide mixture at 100 pg/µL.
B) Measurement of monolinuron in ginger matrix with a 1.8 µm column.
C) Measurement of monolinuron in ginger matrix with a 5 µm column.

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Agilent 1290 Infinity LC
The ideal partner for MS – Part 5

Improved mass accuracy by enhanced separation of compounds of isobaric mass using Agilent 1290 Infinity LC technology

Application Note

Pharmaceutical and Chemical

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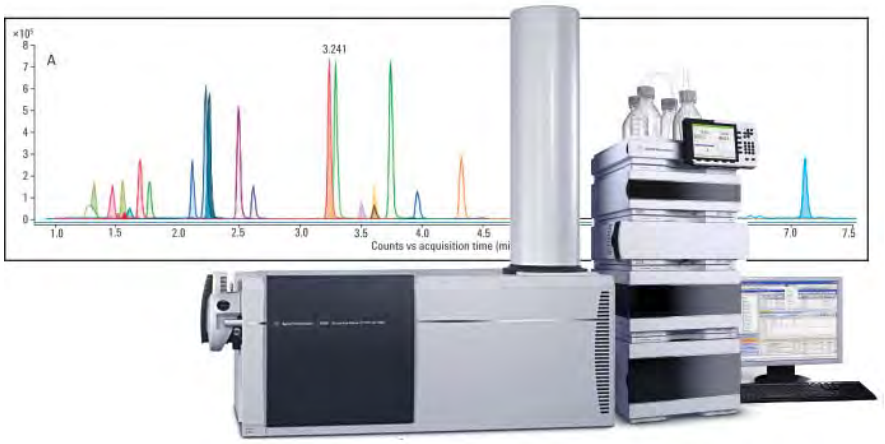

Abstract

This Application Note demonstrates the advantage of using an Agilent 1290 Infinity LC with 1.8 µm columns as the front-end of an Agilent 6500 Series Accurate Mass Quadrupole Time-of-Flight (Q TOF) mass spectrometer to achieve better separation of the analyte compound from other compounds compared to conventional HPLC separation on a 5 µm column. Coelution can compromise the identification of compounds by accurate mass measurement if coeluting compounds have isobaric mass. The presented data show that there is a lower probability for coeluting compounds by improved separation on an Agilent 1290 Infinity LC used with 1.8 µm particle size columns.

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Introduction

For an analysis, target compounds can be classified in three categories:

1) **Known knowns**, which could be present in the sample and therefore will be quantified with a triple quadrupole approach by previous adjustment of the mass spectrometer to the particular compounds.

2) **Known unknowns**, which are chemically characterized and inherent in a database but possibly not expected in the sample.

3) **Unknown unknowns**, which are compounds inherent in the sample but are chemically not characterized and not identified so far.

The first approach is called targeted analysis, and the second, a non-targeted screening.

A typical application for accurate mass measurement instruments like qadrupole time-of-flight (QTOF) mass spectrometers is the screening of samples for "known unknowns". Typical samples for screening are toxicological samples for drugs of abuse or food samples for pesticides. For the final search of the compounds in the acquired data accurate mass retention time (AMRT) data bases are available.

This is a clear contrast to the use of triple quadrupole (QQQ) mass spectrometers which are used for quantification of "known knowns" in a sample where unit mass resolution and accuracy is sufficient. For the application of QTOF instruments in compound screening, high resolution and high mass accuracy is required. A problem for the non-targeted analysis occurs when compounds of isobaric mass are coeluting where the mass difference between the compounds cannot be resolved by the mass spectrometer.

This problem can only be partially resolved by increasing the resolution of the instrument. Compounds with the same integer mass and containing only C-, H-, N-, and O-atoms are always in the same mass defect range as their natural matrix compounds and could be isobaric. It is possible that even mass spectrometric resolution above 100,000 could not be enough. This is where chromatography comes into play. The Agilent 1290 Infinity LC system with its capability to run high resolving 1.8 µm particle size columns can help to resolve these compounds chromatographically, to avoid coelution.

This Application Note demonstrates the advantage of using an Agilent 1290 Infinity LC system with 1.8 µm columns as the front end of an Agilent 6500 Series Accurate Mass Quadrupole Time-of-Flight (Q TOF) mass spectrometer to achieve better separation of the analyte compound from other compounds, compared to conventional HPLC separation on a 5 µm column. Coelution can compromise the identification of compounds by accurate mass measurement if coeluting compounds have isobaric mass. The presented data show that there is a lower probability for coeluting compounds by improved separation on an Agilent 1290 Infinity LC used with 1.8 µm particle size columns.

Experimental

Equipment:

- Agilent 1290 Infinity LC system including:
- Agilent 1290 Infinity Binary Pump,
- Agilent 1290 Infinity High Performance Autosampler,
- Agilent 1290 Infinity Thermostatted Column Compartment
- Agilent 6530 Accurate Mass Quadrupole Time-of-Flight (Q TOF) Mass Spectrometer

Columns:	• Agilent ZORBAX Eclipse Plus, RRHD, C18, 2.1 ×150 mm, 1.8 µm • Agilent ZORBAX Eclipse Plus C18, 2.1 ×150 mm, 5 µm
Software for data acquisition and data analysis:	• MassHunter data acquisition software for QTOF • MassHunter qualitative data analysis software • MassHunter Personal Compound Database Manager and Database (ForensicsTox_Testmix_AM_PCDL.cdb)

HPLC method:

Solvent A:	Water + 0.1% formic acid
Solvent B:	Acetonitrile + 0.1% formic acid
Flow rate:	0.8 mL/min
Gradient:	0 min 5% B – 7.0 min 95% B – 8.0 min 95% B
Stop time:	8.0 min.
Post time:	3 min
Injection volume:	1 µL
Needle wash:	6 s in MeOH
Column temperature:	40 °C

MS method:

Source:	Sheath gas temperature:	350 °C
	Sheath gas flow:	11 L/min
	Capillary gas temperature:	300 °C
	Capillary gas flow:	5 L/min
	Nebulizer pressure:	50 psi
	Capillary:	4,000 V
	Nozzle Voltage:	500 V
	Polarity:	positive
TOF:	MS only, 100-1,700 *m/z*, 2 scan/sec., 2 GHz data rate	
Samples:	Stock solutions (100 µg/mL)of: Mianserin ($C_{18}H_{20}N_2$, MW: 264.1626 ,$[M+H]^+$=265.1699), Tetracaine ($C_{15}H_{24}N_2O_2$, MW: 264.1838 ,$[M+H]^+$=265.1911) Agilent LC/MS Toxicology Test Mixture, (p/n 5190-0470), Table 1	

Results and Discussion

The mass spectrometric resolution is defined as the measure of separation between two peaks expressed as m/Δm. The resolving power of a TOF instrument is defined as the quotient of *m/z* and the peak width at half height. The resolving power defines a threshold for accurate mass measurement. If two compounds of isobaric mass below that threshold are approaching the detector at the same time, they cannot be resolved, and the measured accurate mass will be wrong. This will lead to false calculation results for the chemical formula[1].

Typically, this problem is avoided in most cases by chromatographic separation in the front end HPLC system. However, there are a few sets of isobaric compounds which undergo colelution under standard HPLC conditions and cause problems for their identification.

As an example for a set of coeluting, isobaric compounds tetracaine and mianserin were chosen. They would require a mass resolution of about 12,500 to be resolved while the used time-of-flight mass spectrometer delivers a resolution of about 7,500 at *m/z* 322. For the chromatographic separation, a conventional Agilent ZORBAX Eclipse Plus 2.1 × 150 mm, 5 µm column was used. Under the given conditions, both compounds could not be separated (Figure 1) and the accurate mass measured was off by a mass error of 34 ppm and 45 ppm from the masses of the test compounds tetracaine and mianserin, respectively. This will prevent the correct identification of both compounds and finally lead to a wrong formula calculation.

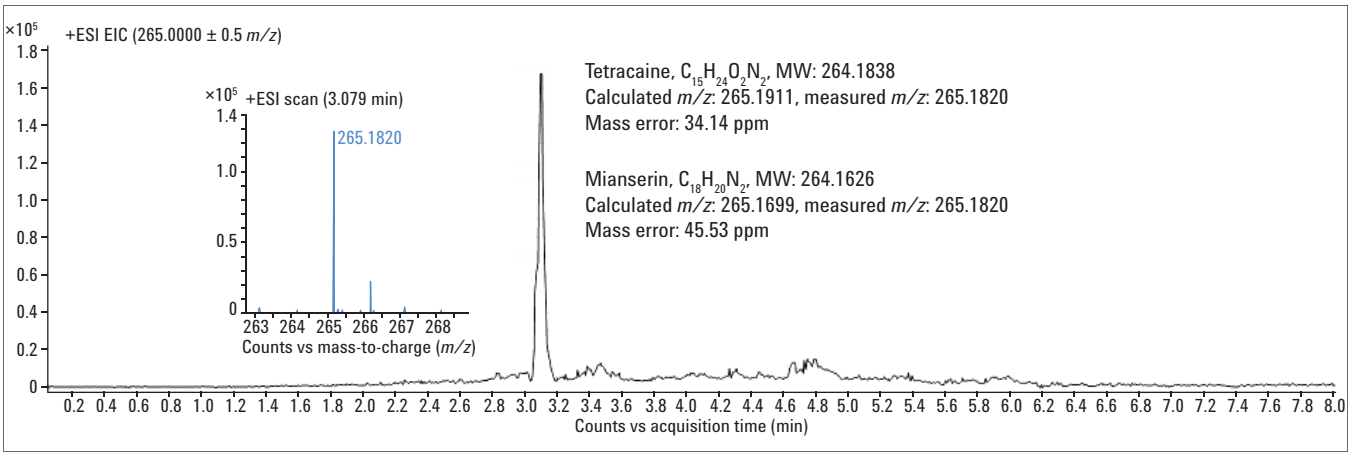

Figure 1

Coeluting compounds Tetracaine and Mianserin of isobaric mass at insufficient mass resolution of about 7,500 while a mass resolution of 12,500 would be required. The chromatographic separation was done on an Eclipse Plus 2.1 × 150 mm, 5 µm column.

The situation completely changes if the column is changed to a 1.8 µm particle size high resolving column run with an Agilent 1290 Infinity LC system as front end. Now, both compounds are chromatographically resolved peaks and can be identified as separated compounds (Figure 2).

Tetracaine and mianserin are identified with low mass errors of 2.81 ppm and 1.98 ppm, respectively. In a window of 10 ppm and a chemical space of $C_{3\text{-}50}H_{2\text{-}100}O_{0\text{-}10}N_{0\text{-}5}$, their formulae were calculated uniquely, which gives a reliable identification.

The high impact of chromatographic resolution for the identification of compounds is demonstrated in the following example on the identification of toxicologically important compounds. The mixture of compounds includes the toxicological test mixture (Table 1) and at the same concentration both the local anesthetic tetracaine and the tetracyclic antidepressant mianserin.

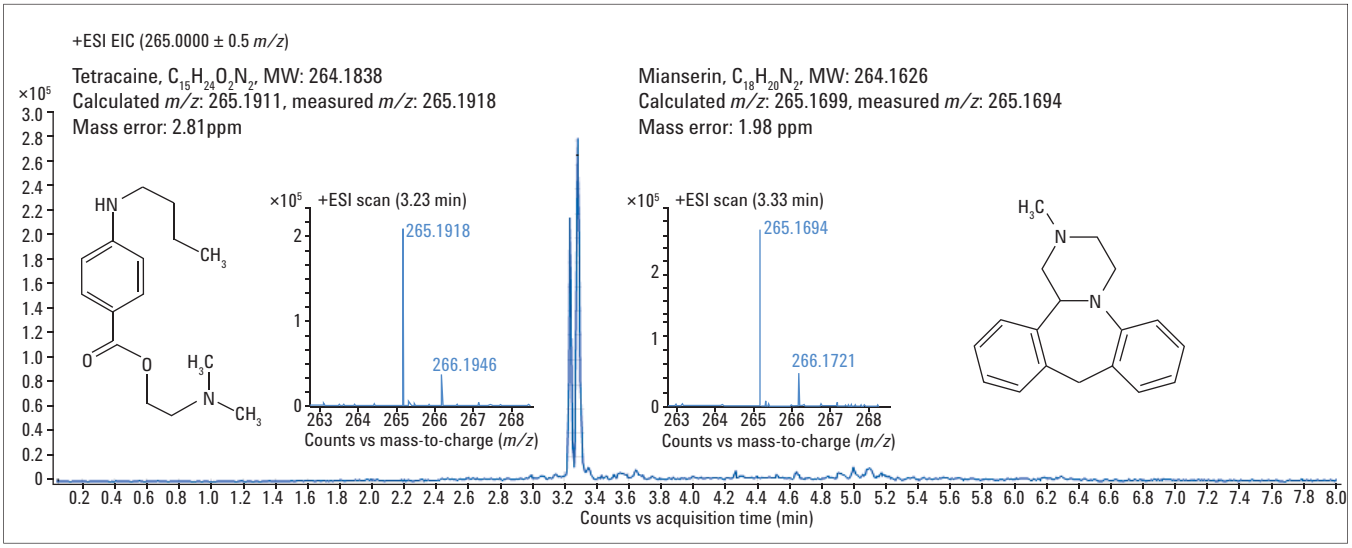

Figure 2

Chromatographically resolved isobaric compounds Tetracaine and Mianserin, column Eclipse Plus, RRHD, 2.1 × 150 mm, 1.8 µm both formulas were uniquely identified in $C_{3\text{-}50}H_{2\text{-}100}O_{0\text{-}10}N_{0\text{-}5}$ in a 10 ppm window.

Compound Name	Formula	Mass	RT (min)	CAS	IUPAC name
Amphetamine	$C_9H_{13}N$	135.10480	1.430	300-62-9	1-Phenyl-2-propanamine
Methamphetamine	$C_{10}H_15N$	149.12045	1.550	537-46-2	(2S)-N-Methyl-1-phenyl-2-propanamine
Phentermine	$C_{10}H_15N$	149.12045	1.660	122-09-8	2-Methyl-1-phenyl-2-propanamine
3,4-Methylendioxyamphetamine (MDA)	$C_{10}H_{13}NO_2$	179.09463	1.500	4764-17-4	1-(1,3-Benzodioxol-5-yl)-2-propanamine
Methylendioxymethamphetamine (MDMA)	$C_{11}H_{15}NO_2$	193.11028	1.600	69610-10-2	1-(1,3-Benzodioxol-5-yl)-N-methyl-2-propanamine
3,4-Methylenedioxyethamphetamine (MDEA)	$C_{12}H_{17}NO_2$	207.12593	1.770	14089-52-2	1-(1,3-Benzodioxol-5-yl)-N-ethyl-2-propanamine
Phencyclidine (PCP)	$C_{17}H_{25}N$	243.19870	2.630	77-10-1	1-(1-Phenylcyclohexyl)piperidine
Meperidine (Pethidine)	$C_{15}H_{21}NO_2$	247.15723	2.260	57-42-1	Ethyl 1-methyl-4-phenyl-4-piperidinecarboxylate
Nitrazepam	$C_{15}H_{11}N_3O_3$	281.08004	3.358	146-22-5	7-Nitro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Diazepam	$C_{16}H_{13}ClN_2O$	284.07164	4.382	439-14-5	7-Chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Oxazepam	$C_{15}H_{11}ClN_2O_2$	286.05091	3.591	604-75-1	7-Chloro-3-hydroxy-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Morphin-D3	$C_{17}H_{16}D_3NO_3$	288.15532	5.594		
Codeine	$C_{18}H_{21}NO_3$	299.15214	1.579	76-57-3	(5alpha,6alpha)-3-Methoxy-17-methyl-7,8-didehydro-4,5-epoxymorphinan-6-ol
Hydrocodone	$C_{18}H_{21}NO_3$	299.15214	1.340	125-29-1	(5alpha)-3-Methoxy-17-methyl-4,5-epoxymorphinan-6-one
Temazepam	$C_{16}H_{13}ClN_2O_2$	300.06656	4.040	846-50-4	7-Chloro-3-hydroxy-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Cocaine	$C_{17}H_{21}NO_4$	303.14706	2.240	50-36-2	Methyl (1R,2R,3S,5S)-3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate
Alprazolam	$C_{17}H_{13}ClN_4$	308.08287	3.730	28981-97-7	8-Chloro-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine
Methadone	$C_{21}H_{27}NO$	309.20926	3.300	76-99-3	6-(Dimethylamino)-4,4-diphenyl-3-heptanone
Delta9-tetrahydrocannabinol (THC)	$C_{21}H_{30}O_2$	314.22458	7.060	8/3/1972	(6aR,10aR)-6,6,9-Trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H-benzo[c]chromen-1-ol
Clonazepam	$C_{15}H_{10}ClN_3O_3$	315.04107	3.740	1622-61-3	5-(2-Chlorophenyl)-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Oxycodone	$C_{18}H_{21}NO_4$	315.14706	1.489	76-42-6	(5alpha)-14-Hydroxy-3-methoxy-17-methyl-4,5-epoxymorphinan-6-one
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	320.01193	3.698	846-49-1	7-Chloro-5-(2-chlorophenyl)-3-hydroxy-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Strychnine	$C_{21}H_{22}N_2O_2$	334.16813	1.730	57-24-9	Strychnidin-10-one
Proadifen	$C_{23}H_{31}NO_2$	353.23548	3.747	302-33-0	2-(Diethylamino)ethyl 2,2-diphenylpentanoate
Heroin	$C_{21}H_{23}NO_5$	369.15762	2.140	561-27-3	(5alpha,6alpha)-17-Methyl-7,8-didehydro-4,5-epoxymorphinan-3,6-diyl diacetate
Trazodone	$C_{19}H_{22}ClN_5O$	371.15129	2.520	19794-93-5	2-{3-[4-(3-Chlorophenyl)-1-piperazinyl]propyl}[1,2,4]triazolo[4,3-a]pyridin-3(2H)-one
Verapamil	$C_{27}H_{38}N_2O_4$	454.28316	3.270	52-53-9	2-(3,4-Dimethoxyphenyl)-5-{[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino}-2-isopropylpentanenitrile

Table 1
Content of the Agilent LC/MS Toxicology Test Mixture (p/n: 5190-0470), 1 µg/mL each, retention times refer to Figure 3.

In the first experiment, the mixture was separated under conventional HPLC conditions on a 2.1 × 150 mm, 5 µm column (Figure 3A). For the identification of the toxicological compounds in the mixture, a database search was done with the identification criteria settings: mass accuracy ± 10 ppm, retention time ± 0.2 min, EIC window ± 0.5 min. With this data base search, 27 compounds were identified by accurate mass and retention time. A large number of compounds coeluted between one and three minutes with differences in intensities by more than an order of magnitude. They were easily identified by their accurate mass and retention time. The compounds tetracaine and mianserin coeluting under this conditions were not identified because the mass resolution is not sufficient and the resulting mass is far of the settings window of the database search.

Changing to a 2.1 × 150 mm, 1.8 µm column, which can be operated by the Agilent 1290 Infinity LC system, changes the result especially for the compounds of isobaric mass tetracaine and mianserin (Figure 3B). Here, both compounds, coleuting under standard HPLC conditions are separated. This separation of the isobaric compounds removes the disturbing influence on their accurate mass measurement and enables their identification by the database search. Tertracaine was identified at a retention time of 2.749 minutes with a mass error of 2.34 ppm and mianserin was identified at a retention time of 2.807 minutes with a mass error of 4.05 ppm.

These measurements were done with an Agilent 6530 Accurate Mass QTOF in Dynamic Range Mode where a mass resolution of about 7,500 at 322 *m/z* can be achieved. The same instrument is able to deliver a resolution of about 13,500 at 322 *m/z* in high Resolution Mode. The Agilent 6540 Ultra High Definition Accurate Mass QTOF can even deliver a resolution of > 25,000 at 322 *m/z*. This means that the example here could be resolved by increasing the mass resolution in

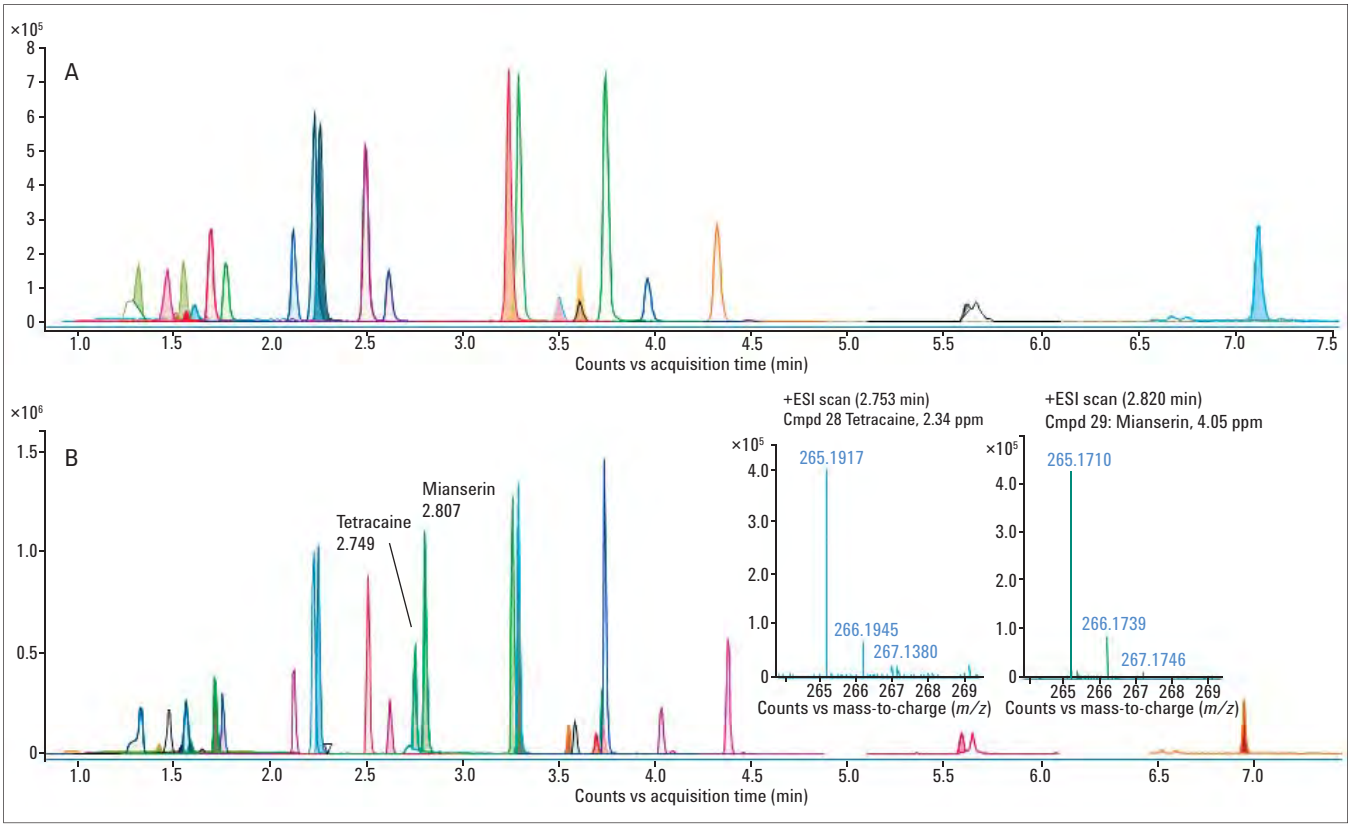

Figure 3

Identification of compounds in a Toxicology Test Mix of 29 compounds (see experimental, Table 1 + tetracaine and mianserin, 100 pg/mL).

A) Resolved on an Agilent ZORBAX Eclipse Plus 2.1 × 150 mm, 5.0 µm, 27 compounds identified.

B) Resolved on an Agilent ZORBAX Eclipse Plus, RRHD, 2.1 × 150 mm, 1.8 µm, 29 compounds identified. Chromatographic conditions: Gradient 5-95% AcN in 7 min. Search in Personal Compound Database: Threshold 10 ppm, retention time +/- 0.2 min, EIC extraction +/- 0.50 min.

case of coelution. This does not mean that chromatographic performance can be neglected. There may always be another coeluting compound of interest, a metabolite coeluting with the compound of interest, a coeluting standard or, most probably, a coeluting isobaric matrix compound where the achieved mass spectrometric resolution is not sufficient[1].

Conclusion

This Application Note demonstrates the use of the Agilent 1290 Infinity LC system as a valuable front end for high end mass spectrometers such as the Agilent 6500 QTOF series mass spectrometers. An example is shown where the mass spectrometric resolution of the accurate mass measurement instrument or the chosen data acquisition mode is not sufficient to resolve compounds of isobaric accurate mass coeltuing under conventional HPLC conditions. The Agilent 1290 Infinity LC system with its capability to run long 1.8 µm columns for high chromatographic resolution is able to resolve those compounds which enable the mass spectrometer to deliver the correct accurate mass for reliable formula calculation, database search and herewith compound identification.

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1.
Pleander A., Decker P., Baessmann C., Ojanperä I., „Evaluation of a high resolving power time-of-flight mass spectrometer for drug analysis in terms of resolving power and acquisition rate.“ *J. Am. Mass Spectrom.* 22 (2011) 379-385.

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Highly Sensitive UV Analysis with the Agilent 1290 Infinity LC System for Fast and Reliable Cleaning Validation – Part 1

Measurement of calibration curves, determination of LOD and LOQ and method validation using a DAD equipped with standard or high sensitivity flow cell

Application Note

Pharmaceutical and Chemical Industry

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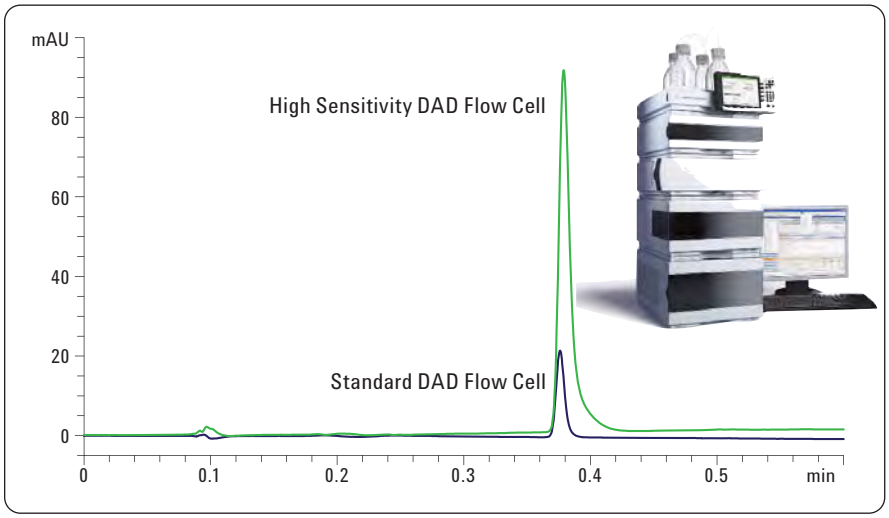

Abstract

This Application Note demonstrates high sensitivity measurement of pharmaceutical compounds with the Agilent 1290 Infinity LC. It also demonstrates a performance comparison of different flow cells with the Agilent 1290 Infinity LC Diode Array Detector (DAD) for highly sensitive UV measurement including calibration, validation, and determination of LOD and LOQ.

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Introduction

Cleaning validation is a process of providing documented evidence that cleaning methods employed within a facility consistently limit potential carryover of products to a level that is below predetermined levels[1]. A validation of cleaning procedures can be initiated by a change of customer requirements, regulatory requirements, or internal control and compliance. In an active pharmaceutical product (APP), different types of contaminants can be found, such as by-products, previous products, solvents, cleaning agents or micro-organisms.

Cleaning validation includes a number of steps. Acceptance criteria must first be established, then a cleaning procedure, an analytical method and sampling procedures must be defined. This is followed by validation, the generation of a protocol and the final report.

One approach for setting acceptance criteria for contamination of an APP with another APP is based on the pharmacological dose. The amount of contaminant must not be higher than 1/1000 of the normal dose of an APP present (APP1) per typical dose of the subsequent product (APP2). Another option is to define a general limit for any contaminant that could be present in the subsequent product (10 ppm up to 0.1%). A typical cleaning procedure for production equipment can be a swabbing or a rinsing process, while monitoring the contaminants in the extraction solvent of the swab or rinse solution.

A series of three Application Notes describes a complete quality control workflow including cleaning validation and final product quality control. This Application Note, which is Part 1 of the series, describes the measurement of calibration curves for APP1, method validation, and determination of LOD and LOQ with the Agilent 1290 Infinity LC and DAD with standard or high sensitivity flow cell.

Part 2 simulates the cleaning process of a reaction vessel (Figure 1). The difference in detection limits between different flow cells is also discussed. As a result, the high sensitivity DAD cell can detect compounds in low concentrations. Therefore cleaning procedures can be monitored with higher reliability and safety[2].

Part 3 describes the determination of contamination of APP2 with remains of APP1 (Figure 1). It is demonstrated that detection of very low level amounts of contaminant with the 60 mm cell shows five times higher sensitivity than with the standard cell[3].

Experimental

In this study two pharmaceutical compounds (APP1 and APP2) of the same class of active pharmaceutical products with equal therapeutic daily dosage were used. A method for compound separation was developed (Table 1) and a calibration curve for quantitation was obtained in the first step. The method was validated and the LOD and LOQ were determined. This was performed for both DAD configurations (standard

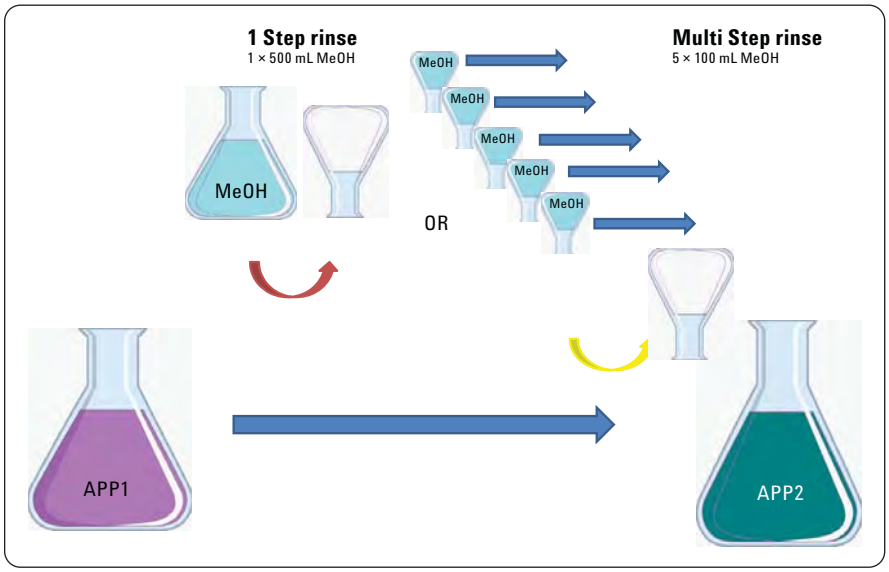

Figure 1
Schematic of the cleaning process during batch exchange in production of APPs.

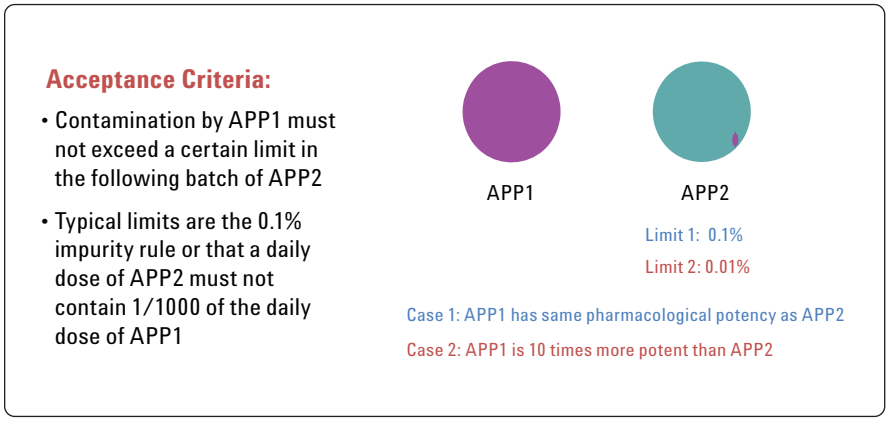

Figure 2
Schematic of the required detection limits according to the pharmacological daily doses of APP1 and APP2.

10 mm or high sensitivity 60 mm cell). Both calibrations and validations were compared and discussed.

Results and discussion

A calibration curve of APP1 was obtained for the two DAD flow cells, 10 mm and 60 mm (Figure 3).

Figure 3a shows the calibration curve of the 10 mm DAD cell. An injection of 1 µL of 5 ng/µL of APP1 produced an intensity of 22 mAU. The calibration curve had a range from 0.04 to 1000 ng/µL (shown: 0.04 to 10 ng/µL). And the low concentration section of the calibration curve was calculated separately from the respective LOQ_{10} (0.1 ng/µL) up to 50 times the LOQ_{10} (5 ng/µL) with five levels for a more precise quantitation of lower concentrations falling in this range (Figure 3a: A–C).

Figure 3b shows the calibration curve of the 60 mm DAD cell. An injection of 1 µL of 5 ng/µL of APP1 produced an intensity of 93 mAU. The calibration curve had a range from 0.005 to 1000 ng/µL (shown: 0.005 to 5 ng/µL). And the low concentration section of the calibration curve was calculated separately from the limit of quantification (LOQ_{60}, 0.02 ng/µL) up to 25 times the LOQ (0.5 ng/µL) with five levels for a more precise quantitation of lower concentrations (Figure 3b).

Agilent 1290 Infinity LC System	Product Number	Parameter	
Agilent 1290 Infinity Binary Pump	G4220	Mobile phase gradient	A: Water + 0.1% TFA. B: ACN + 0.08% TFA 0 min – 10% B, 1 min – 95% B
		Flow rate	1.5 mL/min
Agilent 1290 Infinity Autosampler	G4226	Injection volume	1 µL
		Needle wash	20 s Flush Port: MeOH/Water 50/50
Column		ZORBAX SB C18 Rapid Resolution HD	2.1 mm × 50 mm, 1.8 µm
Agilent 1290 Infinity Column Compartment	G1316C	Column temperature	25 °C
Agilent 1290 Infinity DAD	G4212A	Wavelength	270/4 nm; Ref: 380/40 nm Slit: 4 nm
		Flow cells	10 mm and 60 mm path length
		Peak width	40 Hz data rate
ChemStation Software	G2170BA	For data acquisition	Rev. B. 04.02 SP1 and data analysis
Sample		APP1	APP2 (subsequent compound)

Table 1
Equipment and chromatographic method.

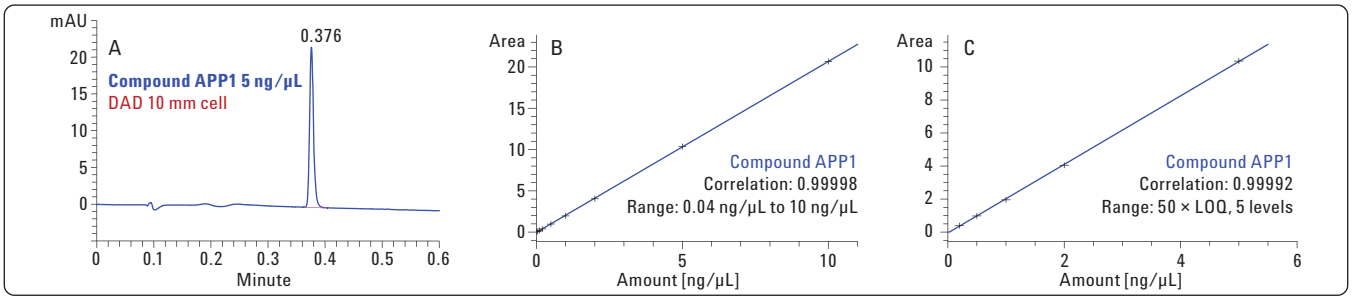

Figure 3a
Calibration curve for the compound APP1 with 10 mm DAD cell. A) Signal from 1 µL injection of 5 ng/µL with 22 mAU. B) Calibration curve 0.04 to 10 ng/µL. C) Calibration curve from LOQ (0.1 ng/µL) up to 50 x LOQ with five levels.

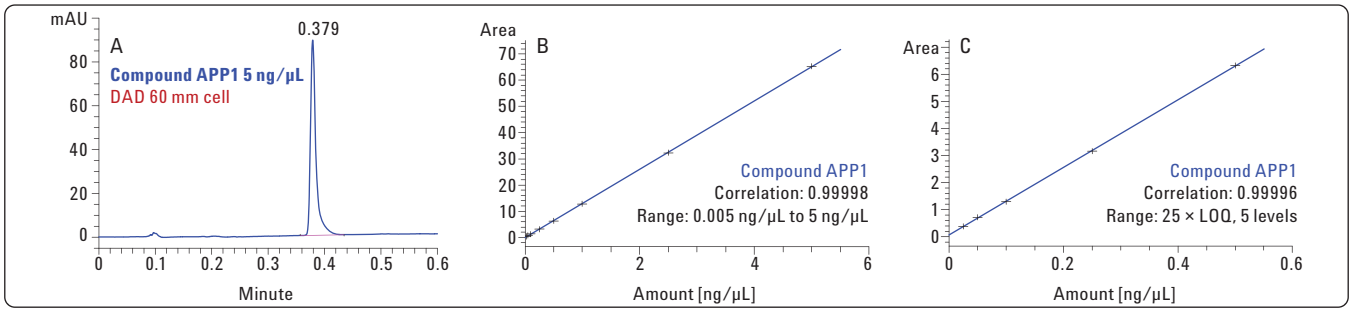

Figure 3b
Calibration curve for compound APP1 with 60 mm DAD cell. A) Signal from 1 µL injection of 5 ng/µL with 95 mAU B) Calibration curve 0.005 to 5 ng/µL C) Calibration curve from LOQ (0.02 ng/µL) up to 25 times the LOQ with five levels.

The analytical method for quantitation of APP1 was validated. Relevant validation parameters, such as retention time stability and area precision from replicate injections (n=10) of 5 ng/µL are shown in Table 2. Relative Standard

	Compound APP1			
	10 mm cell		60 mm cell	
n=10	RT	area	RT	area
Mean	0.377	10.679	0.379	61.253
SD	0.001	0.065	0	0.359
RSD	0.150	0.610	0	0.586

Table 2
Method validation for compound APP1 with 10 mm DAD cell and 60 mm DAD cell.

Deviation (RSD) of retention times for both cells was less than 0.15%, whereas area precision was less than 0.61%. Typical expected values are < 0.25 %RSD for the retention time and < 2 %RSD for the area precision. The linearity coefficient was between 0.99992 and 0.99998. LOD_{10} at a signal-to-noise ratio (S/N) of 3 was 0.04 ng/µL, and LOD_{60} was 0.005 ng/µL. LOQ_{10} (S/N=10) was 0.1 and LOQ_{60} was 0.02 ng/µL (Table 3).

	Compound A	
ng/µL	10 mm cell	60 mm cell
LOD	0.04	0.005
LOQ	0.1	0.02

Table 3
Limit of detection (LOD) at signal-to-noise ratio of 3 (S/N=3) and limit of quantification (LOQ) at signal-to-noise ratio of 10 (S/N=10) for compound APP1 measured with the 10 mm DAD cell and with the 60 mm DAD cell.

A comparison of the signals of APP1 at a concentration of 5 ng/µL showed a signal increase of about 4.5 times when changing from the 10 mm cell to the 60 mm cell (Figure 4). Due to higher signal detection achieved with the 60 mm DAD cell, the LOQ_{60} is five times lower than the 10 mm standard cell values.

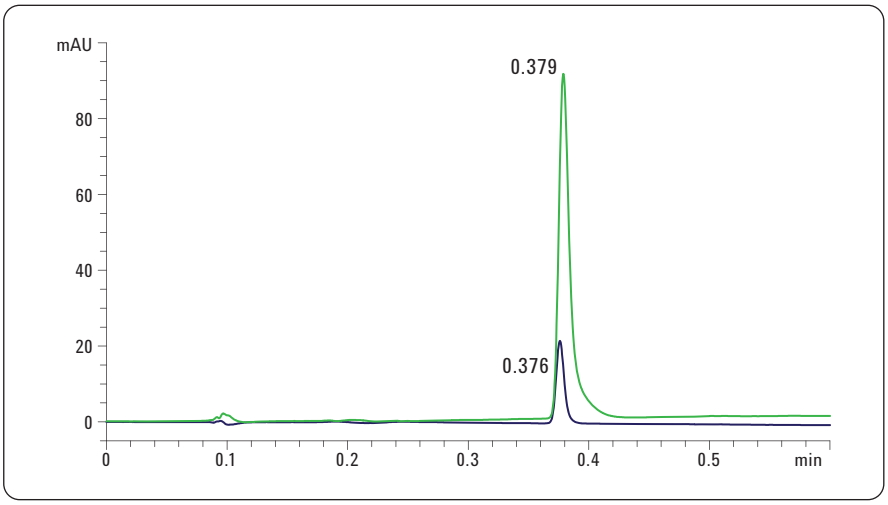

Figure 4
Comparison of the signal of compound APP1 at a concentration of 5 ng/µL measured with the Agilent 1290 Infinity DAD with the 10 mm standard cell (blue) and the 60 mm high sensitivity cell (green).

Conclusion

This Application Note demonstrates the use of the Agilent 1290 Infinity LC for calibration, determination of LOD and LOQ, as well as method validation. These data can be used for cleaning validation and determination of residual active pharmaceutical products in other active pharmaceutical products (see Part 2[2] and Part 3[3]). The 1290 Infinity DAD equipped with either a 10 mm standard or 60 mm high sensitivity cell was used. The gain in performance sensitivity and improvement in LOQ and LOD are shown for calibration and method validation.

References

1.
Cleaning validation in active pharmaceutical ingredient manufacturing plants, APIC Active Pharmaceutical Ingredients Committee, September 1999, http://apic.cefic.org/pub/4CleaningVal9909.pdf.

2.
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3.
"Highly sensitive UV Analysis with the Agilent 1290 Infinity LC System for Fast and Reliable Cleaning Validations – Part 3. Determination of residual active pharmaceutical impurities from a previous production batch using a DAD with standard or high sensitivity flow cell," Agilent Technologies publication 5990-6931EN.

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Highly Sensitive UV Analysis with the Agilent 1290 Infinity LC for Fast and Reliable Cleaning Validation – Part 2

Monitoring a cleaning validation procedure using a DAD equipped with standard or high sensitivity flow cell

Application Note

Pharmaceutical and Chemical Industry

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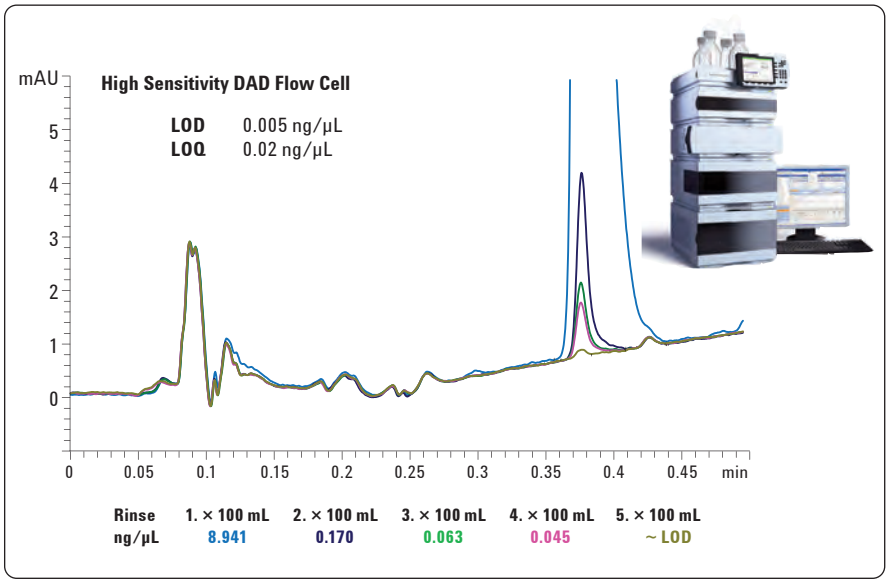

Abstract

This Application Note demonstrates the use of the Agilent 1290 Infinity LC in cleaning validation studies for high sensitivity monitoring of active pharmaceutical product residues in cleaning solutions. It shows that fast analysis is highly useful for cleaning validation, providing fast results and lower production equipment downtime.

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Introduction

Cleaning validation is a process of providing documented evidence that cleaning methods employed within a facility consistently limit potential carryover of products to a level that is below predetermined levels[1]. Validation of cleaning procedures can be initiated by a change of customer requirements, regulatory requirements or internal control and compliance. In active pharmaceutical products (APP), different types of contaminants can be found, such as byproducts, previous products, solvents, cleaning agents or micro-organisms.

Cleaning validation includes a number of steps. Acceptance criteria must first be established, then a cleaning procedure, an analytical method, and sampling procedures must be defined. This is followed by validation, the generation of a protocol and the final report.

One approach for setting acceptance criteria for contamination of an APP with another APP is based on the pharmacological dose. The amount of contaminant must not be higher than 1/1000 of the normal dose of an APP present (APP1) per typical dose of the subsequent product (APP2). Another option is to define a general limit for any contaminant that can be present in the subsequent product (10 ppm up to 0.1%). A typical cleaning procedure of production equipment can be a swabbing or a rinsing process, while monitoring the contaminants in the extraction solvent of the swab or rinse solution.

A series of three Application Notes describes a complete quality control workflow including cleaning validation and final product quality control. This Application Note, which is Part 2 of the series, describes detection and quantitation of a contaminant in the rinsing solvent during a cleaning procedure by the Agilent 1290 Infinity LC equipped with a DAD with a standard (10 mm) or high sensitivity (60 mm) flow cell. As a result, the 60 mm cell exhibits five times higher sensitivity than the standard cell.

Part 1 of the series describes the measurement of calibration curves for APP1, method validation and determination of LOD and LOQ with the Agilent 1290 Infinity LC and DAD with a standard or high sensitivity flow cell[2].

Part 3 describes the determination of contamination of APP2 with remains of APP1. It is demonstrated that detection of very low level amounts of contaminant with the 60 mm cell shows five times higher sensitivity than with the standard cell[3].

Experimental

One-step rinse

An experimental simulation of a cleaning procedure for a reaction vessel was performed twice; as a one-step rinse and as a multistep rinse. Each individual experiment was run in duplicate to provide fresh samples for measurement with individual DAD configurations. The distribution of residual APP1 in the cleaning solutions was measured with individual DAD configurations with the 10 mm standard cell and the 60 mm high sensitivity cell. The initial APP1 concentration in the reaction vessel was 1.0 g/L.

In the first experiment, the solution was poured out from the reaction vessel. The vessel was then cleaned with 500 mL methanol and the concentration of APP1 in the rinse solvent was measured in repeated experiments with both DAD configurations (Figure 1).

Multistep rinse

The disadvantage of using only one rinse to clean a reaction vessel is that the remaining amount is unknown and can contaminate the following reaction. Therefore, multiple rinse steps should be applied and the decreasing amount of APP1 should be monitored during the process in all rinse fractions.

In the multiple rinse the reaction vessel was rinsed five times with 100 mL and the experiment was performed twice (Figure 1).

Chromatographic analysis was done with an Agilent 1290 Infinity LC and DAD equipped with a standard 10 mm flow cell or high sensitivity 60 mm flow cell, respectively (for details of the

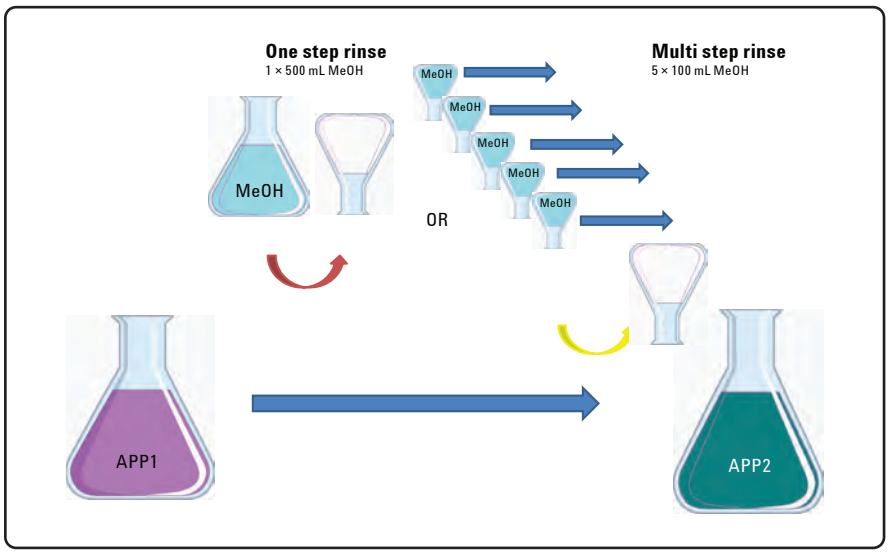

Figure 1
Schematic of the cleaning process during batch exchange in production of APPs.

chromatographic method see Part 1[2]) and the quantitative results from both configurations were compared.

Results and discussion

One-step rinse

In the one-step rinse experiment, the APP1-containing solution was poured out of the reaction vessel. Then the vessel was cleaned with 500 mL methanol. The remaining concentration of APP1 in the rinse solvent was measured with both DAD configurations (Figure 3). A concentration of 1.58 ng/µL (1.58 mg/L) was determined with the 10 mm DAD cell, and 1.52 ng/µL (1.52 mg/L) was determined with the 60 mm DAD cell. The results for both replicate experiments and subsequent measurements were similar, but the signal intensity of the 60 mm cell was about five times higher than for the 10 mm cell.

Multistep rinse

In a multistep rinse, the reaction vessel was rinsed five times with 100 mL and the experiment was performed twice.

In the first experiment, the residual amount of APP1 was measured in each 100 mL rinse fraction with the 10 mm cell (Figure 4). The amount of APP1 in the third fraction was 0.092 ng/µL (92 µg/L), which is below LOQ_{10} and therefore is at a level of uncertainty. The measured concentration in the fourth fractions cannot be confirmed. In accordance with an LOD_{10} of 0.04 ng/µL (40 µg/L) there was no signal detected for APP1 in the fifth fraction.

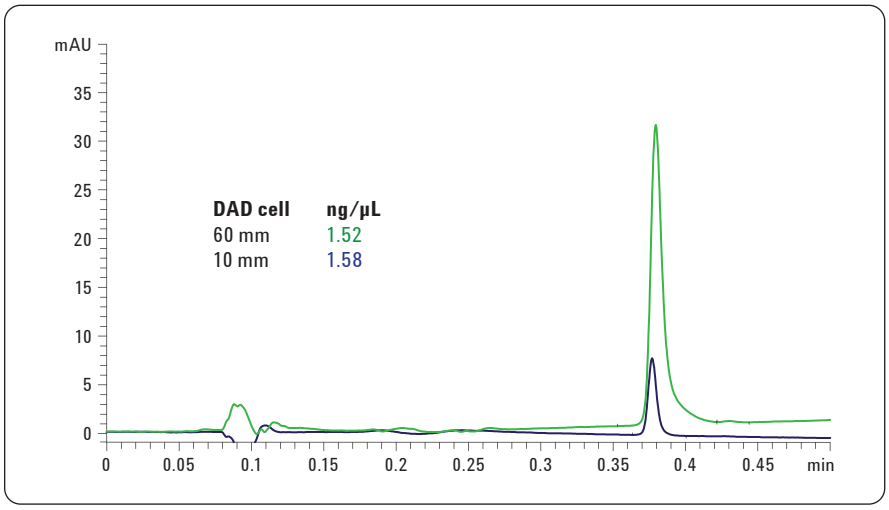

Figure 3
Residual active pharmaceutical product 1 in equipment rinse solution after one application of 500 mL MeOH.

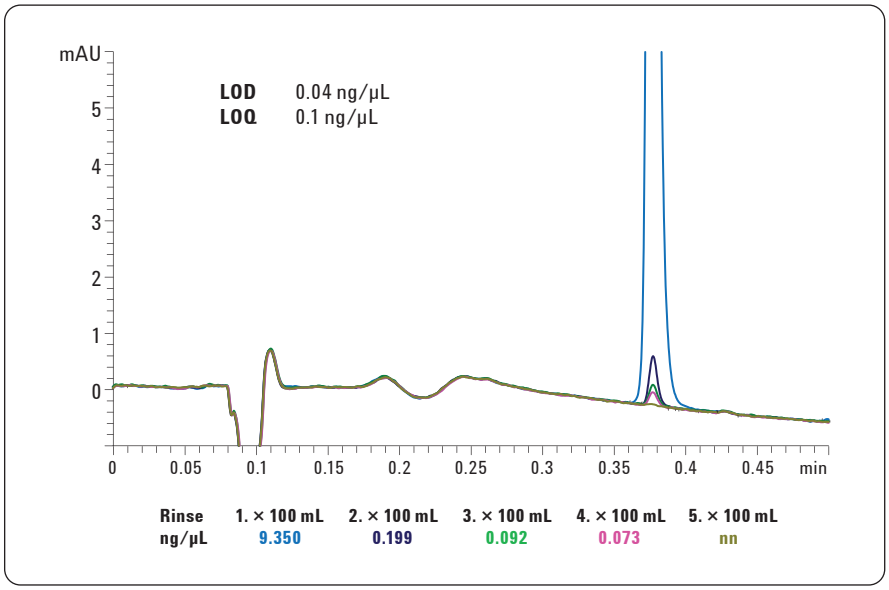

Figure 4
Residual active pharmaceutical product 1 in equipment rinse solution after five applications of 100 mL MeOH measured with the Agilent 1290 Infinity DAD with 10 mm cell.

The 60 mm cell was used for the second experiment (Figure 5). Here, the amount of APP1 in the fourth fraction was determined to be 0.045 ng/µL (45 µg/L), which is twice the LOQ_{60}. For the fifth fraction it was possible to detect a trace of APP1 at the LOD_{60} (0.005 ng/µL or 5 µg/L).

In comparison to the detection with the high sensitivity cell, it is possible to detect down to the LOQ_{10} at about 0.1 ng/µL (100 µg/L) of APP1 in the third fraction with the 10 mm cell. Using the 60 mm cell it was possible to detect APP1 reliably in one more fraction (fraction four) down to an LOQ_{60} of 0.02 ng/µL (20 µg/L) and even to detect a trace at LOD_{60} of 5 µg/L in fraction five (Figure 5). The 60 mm cell is five times more sensitive than the 10 mm cell offering more reliability and safety for the detection of residues in cleaning validation.

Conclusion

The Agilent 1290 Infinity LC with DAD and standard flow cell is an excellent instrument to determine residual amounts of APPs during a cleaning validation. If it is necessary to determine trace levels of contaminants, the high sensitivity 60 mm flow cell gives better certainty of results. It exhibits higher sensitivity and therefore helps to monitor cleaning procedures with higher reliability and safety.

In addition to the increased sensitivity for detection of residuals, the analysis time per sample was only one minute. The entire analysis took less than 30 minutes, including all replicates, quality controls, and system suitability samples. This allows faster decisions about production equipment use. Finally, it decreases downtime of equipment leading to higher productivity and reduced costs.

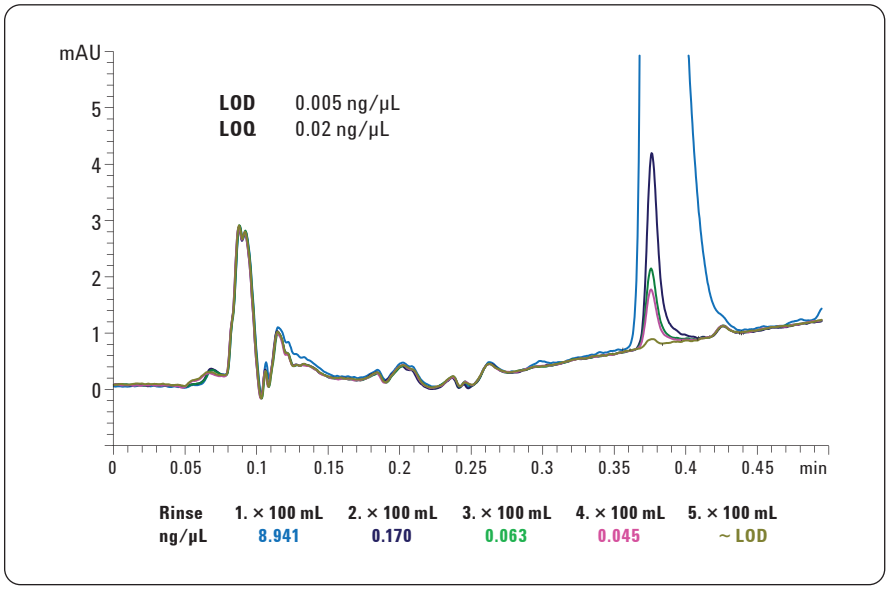

Figure 5
Residual active pharmaceutical product 1 in equipment rinse solution after five applications of 100 mL MeOH measured with the Agilent 1290 Infinity DAD with 60 mm cell.

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Cleaning validation in active pharmaceutical ingredient manufacturing plants, APIC Active Pharmaceutical Ingredients Committee, September 1999, http://apic.cefic.org/pub/4CleaningVal9909.pdf.

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"Highly Sensitive UV Analysis with the Agilent 1290 Infinity LC System for Fast and Reliable Cleaning Validation – Part 1 Measurement of calibration curves, determination of LOD and LOQ and method validation using a DAD equipped with standard or high sensitivity flow cell," Agilent Technologies publication 5990-6929EN.

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"Highly Sensitive UV Analysis with the Agilent 1290 Infinity LC System for Fast and Reliable Cleaning Validations – Part 3. Determination of residual active pharmaceutical impurities from a previous production batch using a DAD with standard or high sensitivity flow cell," Agilent Technologies publication 5990-6931EN.

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Highly Sensitive UV Analysis with the Agilent 1290 Infinity LC System for Fast and Reliable Cleaning Validation – Part 3

Determination of residual active pharmaceutical impurities from a previous production batch using a DAD with standard or high sensitivity flow cell

Application Note

Pharmaceutical and Chemical Industry

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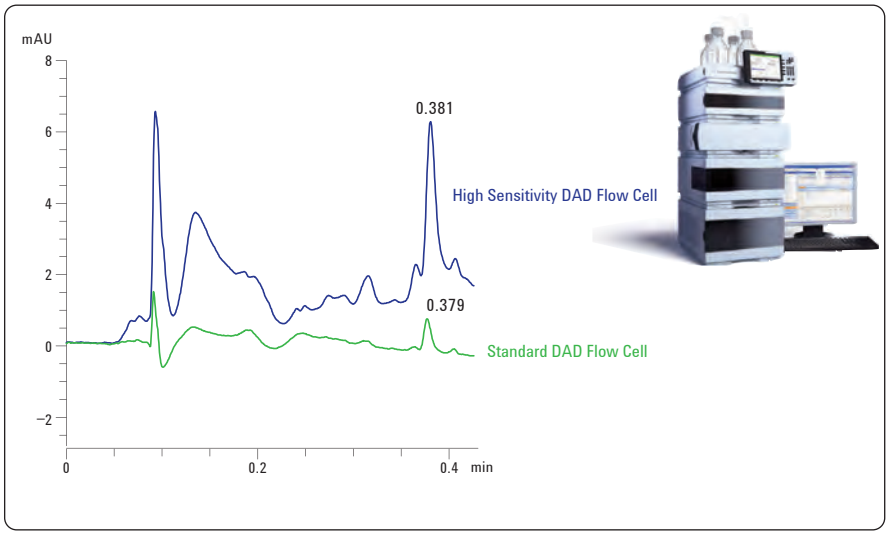

Abstract

This Application Note studies detection of the contamination of an active pharmaceutical product (APP) with the remains of another APP. It demonstrates that high sensitivity detection of very low level amounts of contaminant with the Agilent 1290 Infinity LC DAD equipped with a high sensitivity 60 mm flow achieves about five times higher sensitivity than the standard cell.

Agilent Technologies

Introduction

Cleaning validation is the process of providing documented evidence that cleaning methods used within a facility consistently limit potential carryover of products to a level that is below predetermined limits[1]. Validation of cleaning procedures can be initiated by a change in customer requirements, regulatory requirements, or internal control and compliance. In an active pharmaceutical product (APP), different types of contaminants can be found such as byproducts, previous products, solvents, cleaning agents, or microorganisms.

Cleaning validation includes a number of steps. Acceptance criteria must first be established, then a cleaning procedure, analytical method, and sampling procedures must be defined. This is followed by validation, the generation of a protocol, and the final report.

One approach for setting acceptance criteria for contamination of an APP with another APP is based on the pharmacological dose. The amount of contaminant must not be higher than 1/1000 of the normal dose of an APP present (APP1) per typical dose of the subsequent product (APP2) (Figure 1). Another option is to define a general limit for any contaminant that could be present in the subsequent product (10 ppm up to 0.1%). A typical cleaning procedure for production equipment can be a swabbing or a rinsing process with monitoring of the contaminants in the extraction solvent of the swab or rinse solution.

A series of three Application Notes describes a complete quality control workflow including cleaning validation and final product quality control. This Application Note, which is Part 3[2] of the series, describes the determination of APP2 contamination with remains of APP1. It is demonstrated that high sensitivity detection of very low level amounts of contaminant with the Agilent 1290 Infinity LC DAD equipped with a 60 mm cell shows five times higher sensitivity than the standard cell.

Part 1 of the series describes the measurement of calibration curves for APP1, method validation, and determination of LOD and LOQ with the Agilent 1290 Infinity LC and DAD with a standard or high sensitivity flow cell.[2]

Part 2 simulates the cleaning process of a reaction vessel. The difference in detection limits between different flow cells is also discussed. As a result, the high sensitivity DAD cell can detect compounds in the lowest concentrations. Therefore cleaning procedures can be monitored with higher reliability and safety.[3]

Experimental

In this study two pharmaceutical compounds (APP1 and APP2) of the same class of active pharmaceutical products with equal therapeutic daily dosage were used. The residual amount of APP1 was determined in the subsequent pharmaceutical product APP2 (Figure 1). Chromatographic analysis was conducted with an Agilent 1290 Infinity LC and DAD equipped with a standard 10 mm flow cell or high sensitivity 60 mm flow cell, respectively (the chromatographic method is given in part 1[2]). Quantitative results from both configurations were compared.

Results and discussion

After a change in the production of an active pharmaceutical product from APP1 to APP2, it is important that the contamination by APP1 does not exceed a certain limit in the following batch of APP2. Contaminations can occur due to improper cleaning of the production equipment used. Typical limits are the 0.1% impurity rule or that a daily dose of APP2 must not contain 1/1000 of the daily dose of APP1.

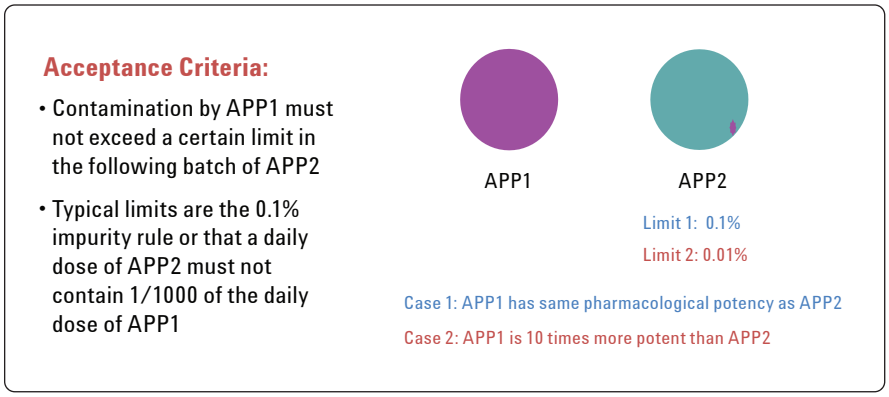

Figure 1
Schematic of the required detection limits according to the pharmacological daily doses of APP1 and APP2.

In the sample, as described in the experimental part, a 0.1% contamination of APP2 by APP1 was determined with a signal-to-noise ratio of 101 by measurement with the standard 10 mm DAD cell (Figure 2). This would be acceptable if the daily dose of APP1 and APP2 were the same amount. It would also meet the requirement of 1/1000 of the daily dose of APP2 (1 ng/µL APP1 in 1000 ng/µL APP2 or 1 µg APP1 in 1 mg APP2, dissolved in 1 mL). In the case, 0.01% of APP1 is contained in APP2, the signal-to-noise ratio is 13 for APP1. This is the limit of quantitation, where quantitation is no longer reliable or safe. This is especially important if the daily dose of APP1 is only 1/10 of the daily dose of APP2. In this case, contamination can pose a significant health risk, because the pharmaceutical potency of APP1 would be 10 times higher than that of APP2.

The experiment was repeated with the DAD configuration containing the 60 mm high sensitivity cell to demonstrate reliable detection of small amounts of contaminant by highly active APPs in APPs applied in a higher daily dose (Figure 3). In this experiment, the 0.1% contamination of APP2 by APP1 was detected with a signal-to-noise ratio of 500. This is a 1 to 1000 ratio for compounds with equal daily doses and there is additional dynamic range for quantitation in case the daily doses differ. In the case, APP1 has 10 times more pharmaceutical potency than APP2 and the daily dose is 1/10 of APP2. A contamination of 0.01% APP1 in APP2 must be detected, which is easily achieved with the 60 mm high sensitivity cell. The detection of the contamination of 0.01% APP1 is possible with a signal-to-noise ratio (S/N) of about 80 (Figure 3, inserts).

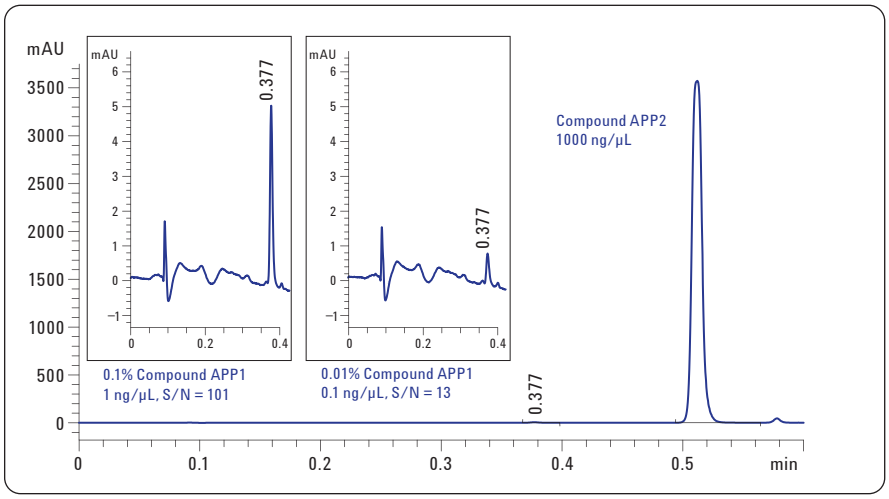

Figure 2
Residual active pharmaceutical product 1 in product 2 at 0.1% and 0.01% measured with the Agilent 1290 Infinity DAD with 10 mm standard flow cell.

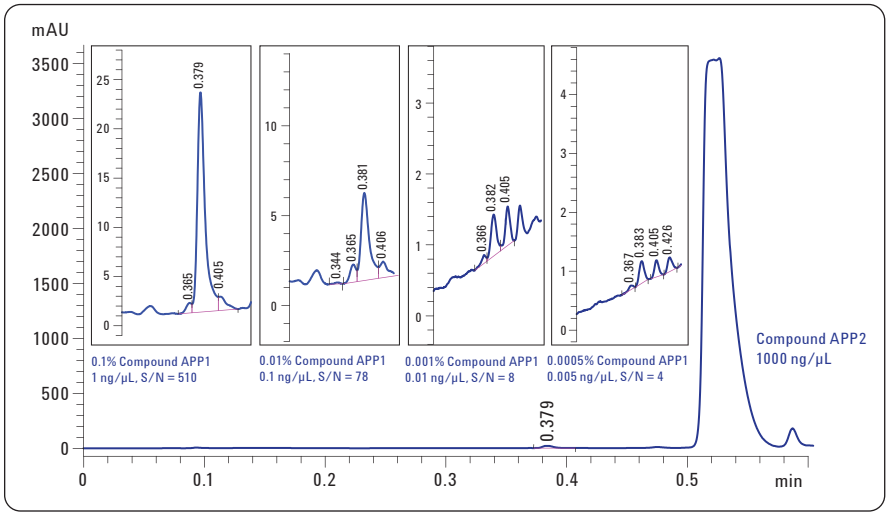

Figure 3
Residual active pharmaceutical product 1 in product 2 at 0.1% to 0.0005% measured with the Agilent 1290 Infinity DAD with 60 mm high sensitivity flow cell.

Quantitatively, it is 0.1 ng/µL or equivalent to 0.1 µg per 1 mg APP2, which is 1/10.000 of the daily dose of APP2 and therefore meets the pharmacological requirements. The LOQ_{60} is reached for a contamination at 0.001%, which is equivalent to 10 ng APP1/1 mg APP 2 (10 ppm). Even a contamination at the level of 0.0005% (5 ppm) can be seen at LOD_{60}. This shows that high sensitivity detection provides very reliable results for trace contaminations of APP2 by other APPs, even when their daily doses and pharmaceutical potency differ by a factor of 100.

Comparison of signal intensity of the 0.01% level contamination of APP2 by APP1 shows a S/N of 13 for the 10 mm standard cell and S/N of 78 for the 60 mm high sensitivity cell. This is an improvement of a factor of 6 for measurements at this low level (Figure 4).

Conclusion

This Application Note discusses measurement of contamination of an APP by a previous active pharmaceutical product. A significant sensitivity improvement of the 60 mm high sensitivity cell allows detection of highly active pharmaceutical compounds in another APP at very low levels. This is important when very low detection levels of APP1 are required, such as defined by an acceptance criteria of 1/1000 of daily dose in subsequent APP 2. The Agilent 1290 Infinity LC equipped with a 60 mm high sensitivity flow cell is an instrument suitably designed for this purpose. The 60 mm high sensitivity cell exhibits about six times higher sensitivity compared to the 10 mm standard DAD flow cell.

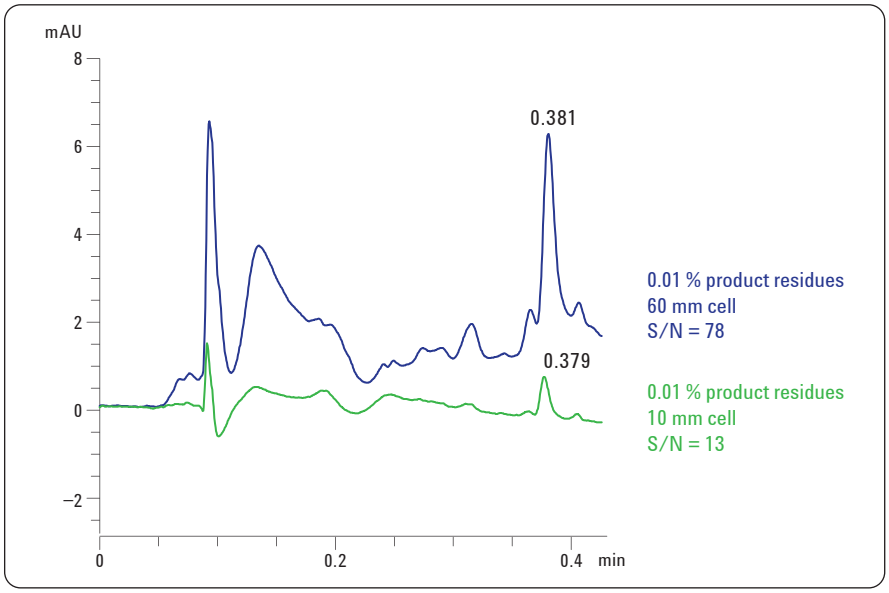

Figure 4
Comparison of the signal intensity measured with the DAD configuration using a 60 mm cell (blue) and a 10 mm cell (green) for a 0.01% impurity of APP1 in active pharmaceutical product 2.

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1.
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2.
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3.
"Highly Sensitive UV Analysis with the Agilent 1290 Infinity LC System for Fast and Reliable Cleaning Validation – Part 2. Monitoring of a cleaning validation procedure using a DAD equipped with standard or high-sensitivity flow cells," Agilent Publication 5990-6930EN.

www.agilent.com/chem/lc

December 1, 2010
Publication Number 5990-6931EN

Agilent Technologies

Comparing HILIC and RPLC of Morphine Using Agilent ZORBAX RRHD Columns with UHPLC/MS

Application Note

Forensics and Drug Testing

Author

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Abstract

UHPLC/MS analyses of morphine and 3 of its metabolites (normorphine, morphine-3-β-D-glucuronide [M3G], and morphine-6-β-D-glucuronide [M6G]) are used to compare mass spectrometer sensitivity with reversed-phase liquid chromatography (RPLC) and hydrophilic interaction chromatography (HILIC). Two Agilent ZORBAX Rapid Resolution High Definition (RRHD) columns are used in this comparison, an Agilent ZORBAX RRHD Eclipse Plus C18 for the RPLC method and an Agilent ZORBAX RRHD HILIC Plus for the HILIC method. Each is used with an Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer. Both methods use isocratic elution with an acetonitrile/ammonium acetate buffer mobile phase. Signal-to-noise calculations for the least sensitive M6G peak show HILIC mode allows for more sensitive MS detection due to more efficient spraying and desolvation in the ESI-MS source, as a result of the volatile acetonitrile rich mobile phase used.

Introduction

Hydrophilic interaction chromatography (HILIC) is gaining popularity in liquid chromatography, particularly for its ability to retain and separate small polar analytes - an area where common reversed-phase liquid chromatography (RPLC) methodology often fails. This novel mode of chromatography results in unique retention mechanisms, because water is used as the strong eluting solvent. HILIC can have distinct advantages over traditional RPLC in LC/MS sensitivity, due to the use of highly organic mobile phases. The highly organic mobile phases have higher volatility than traditional RPLC mobile phases, making HILIC well suited for applications with mass spectrometers.

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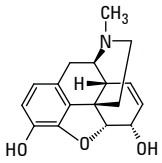
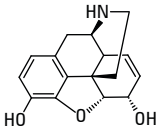
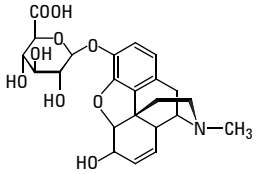
HILIC is used extensively to analyze polar molecules. In this work, morphine and 3 metabolites are analyzed by LC/MS to demonstrate the effectiveness of HILIC with regard to MS sensitivity, compared to RPLC. The compounds of interest are morphine, normorphine, morphine-3-β-D-glucuronide (M3G), and morphine-6-β-D-glucuronide (M6G) (Figure 1). Morphine is a powerful opiate analgesic. Normorphine is a major metabolite of morphine; it is a demethylated derivative that can be used to form both opioid agonists and antagonists. M3G is a non-active metabolite of morphine, while M6G is the major active metabolite. It is believed that M6G acts as an agonist at the opioid receptors, causing much of the pain relieving analgesic effects of morphine [1,2,3].



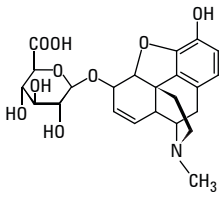
Morphine



Normorphine



Morphine-3-β-D-glucuronide (M3G)



Morphine-6-β-D-glucuronide (M6G)

Figure 1. Compounds of interest.

Experimental

An Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer was used in this experiment. The setup was optimized for lowest possible extra-column volume with short 0.075 mm id capillaries found in the Agilent Ultra-Low Dispersion Kit (p/n 5067-5189) and with an Agilent LC System Rack (p/n 5001-3726) [4].

All 4 analytes were purchased in methanol solutions from Cerilliant, and diluted to desired concentrations in either acetonitrile or water. Acetonitrile was purchased from Honeywell. Ammonium formate and formic acid were purchased from Sigma Aldrich. Water used was 18 MΩ Milli-Q water.

Conditions

Columns:	Agilent ZORBAX RRHD HILIC Plus, 2.1 × 100 mm, 1.8 µm (p/n 959758-901) Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm (p/n 959758-902) Agilent ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 3.5 µm (p/n 959793-902) Agilent ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 5 µm (p/n custom)
Mobile phase:	A: 10 mM NH_4HCO_2 pH 3.2 B: CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1) HILIC 70% B isocratic; RPLC 10% B isocratic
Flow rate:	0.4 or 1.0 mL/min
Temperature:	25 °C
Sample:	2 µL injection of 1 µg/mL each of morphine, normorphine, morphine-3-β-D-glucuronide, and morphine-6-β-D-glucuronide; HILIC sample was prepared in CH_3CN; RPLC sample was prepared in H_2O
MS source:	Positive ESI, capillary 4000 V, drying gas temperature, flow rate and nebulizer pressure vary with mobile phase flow rate and are specified in Table 1
MS acquisition:	Selected ion mode (SIM), delta EMV 200 V, MS dwell time varies with mobile phase flow rate and is specified in Table 1, compound specific parameters are detailed in Table 2
Software:	Agilent MassHunter versions B.03.01, B.02.00 and B.03.01 were used for data acquisition, qualitative, and quantitative analyses, respectively

Table 1. Mass spectrometer parameters for morphine analyses at various flow rates.

	0.4 mL/min	1.0 mL/min
Source	ESI+	ESI+
Delta EMV	200 V	200 V
MS dwell time	20 ms	10 ms
Drying gas temperature	250 °C	325 °C
Drying gas flow rate	11 L/min	12 L/min
Nebulizer pressure	30 psi	55 psi
Capillary voltage	4000 V	4000 V

Table 2. Mass spectrometer SIM parameters for morphine analyses.

	M+H	Fragmentor voltage
Morphine	286	170
Normorphine	272	170
Morphine-3-β-D-glucuronide	462	170
Morphine-6-β-D-glucuronide	462	170

Results and Discussion

Improvements in RPLC/MS Sensitivity with UHPLC Columns

Comparing traditional 5 µm columns to newer 1.8 µm UHPLC columns shows a substantial improvement in chromatographic performance, specifically regarding peak width, as shown in Figure 2. This RPLC analysis of morphine and its metabolites shows significantly narrower peaks generated by the highly efficient sub-2 µm UHPLC column as compared to the traditional 5 µm column. Method parameters for each of these analyses are identical, including the stationary phase chemistry; the only variable is particle size. This example shows overlays of the individual extracted ion chromatograms (EICs) for each compound in their full scale. Selectivity is maintained across all particles sizes with these ZORBAX Eclipse Plus C18 columns, which could allow for easy method scalability and transferability should the need arise.

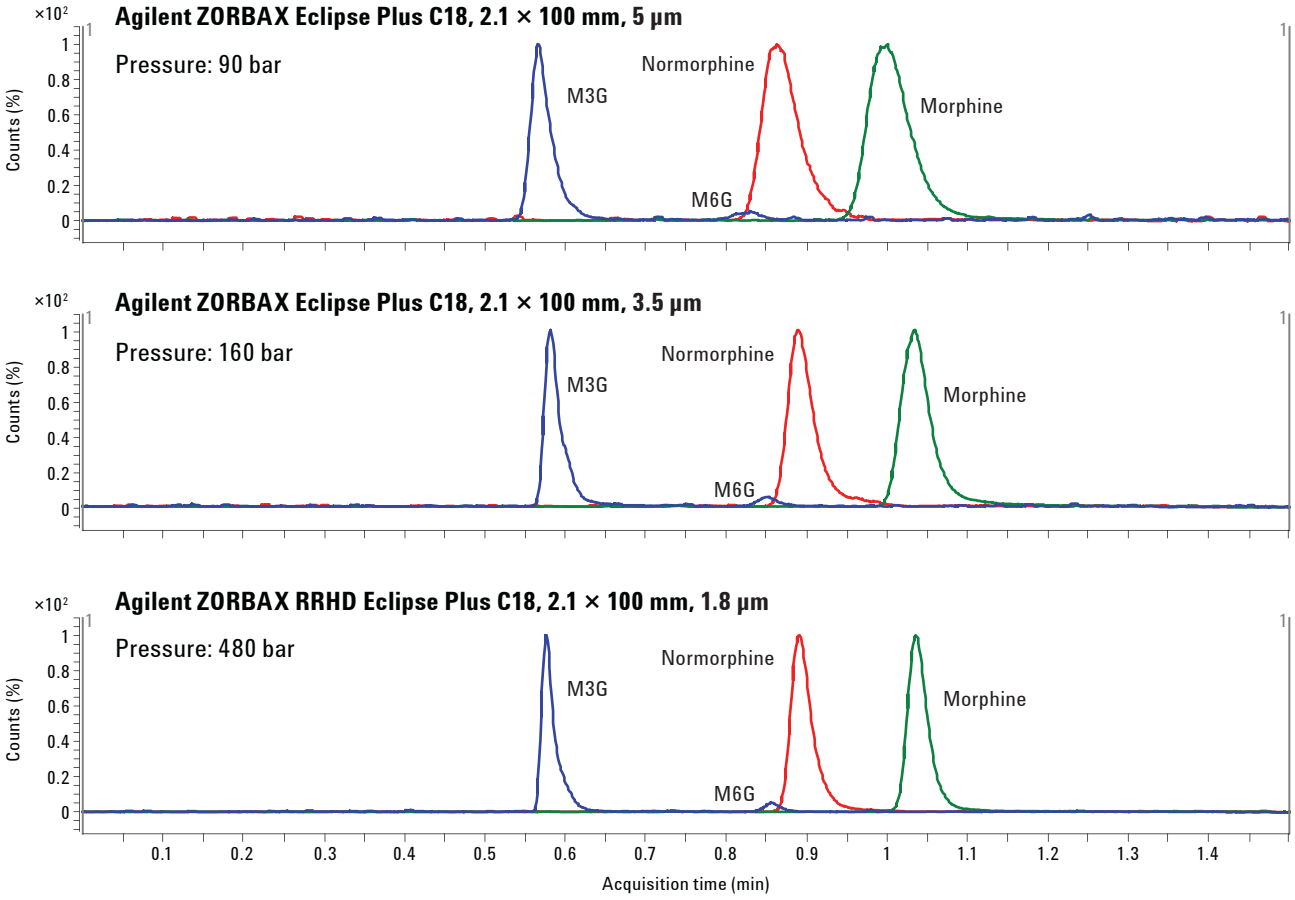


Figure 2. Peak width is improved with high efficiency 1.8 µm UHPLC columns compared to traditional 5 µm columns, while selectivity is maintained; LC/MS method parameters are detailed in the Experimental section.

M3G and M6G are isobaric and, consequently, are detected by the same mass at *m/z* 462. When these EICs are fit to the same scale (Figure 3), not only is the peak width improvement apparent with the smaller particle column, but also the difference in peak height. Comparing the signal-to-noise of the least sensitive M6G peak, shows that the highly efficient sub-2 µm UHPLC column is capable of improving the MS sensitivity by a factor of 5, when compared to a traditional 5 µm column.

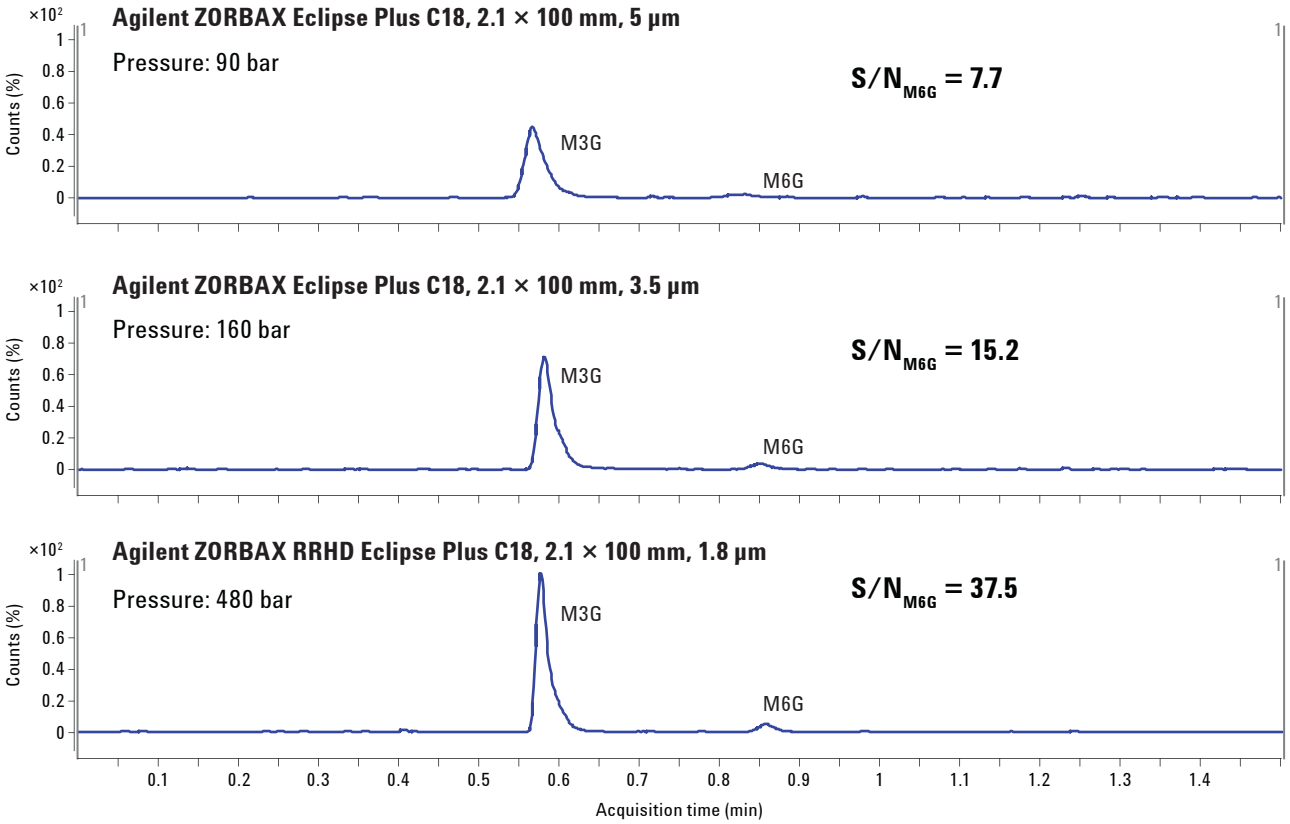


Figure 3. MS sensitivity (signal-to-noise) is improved by a factor of 5 with efficient 1.8 µm UHPLC columns compared to traditional 5 µm columns, while selectivity is maintained; LC/MS method parameters are detailed in the Experimental section.

Better LC/MS Sensitivity with HILIC Mode

Knowing the improvements possible with UHPLC columns compared to traditional LC columns, the impact of HILIC mode on MS sensitivity is explored. Figure 4 shows side-by-side chromatograms for similar analyses with RPLC and HILIC. Method parameters were kept as consistent as possible to eliminate unfair comparisons between the 2 analyses. The RPLC analysis uses 10% acetonitrile, while the HILIC uses 70% acetonitrile. Sample solvents matched the weak solvent in each case; the HILIC sample solvent was acetonitrile and the RPLC sample solvent was water to eliminate peak shape distortion that could occur if the strong solvents were injected in either mode. The ammonium formate buffer concentration is 10 mM in both the aqueous and organic portions of the mobile phase to eliminate differences in MS detection due to salt concentration. Each analysis is isocratic with similar retention factors to eliminate differences due to gradient focusing or column efficiency. These are overlays of EICs each in their full scale to show the selectivity differences between the C18 and HILIC columns. The HILIC selectivity is complementary to the C18, and almost opposite. An interesting observation is that the HILIC Plus column retains all compounds more than the C18, even though the C18 is run at the minimal suggested organic/aqueous ratio. Using less organic with the C18 column could cause phase collapse or column dewetting, leading to irreproducible or poor chromatography.

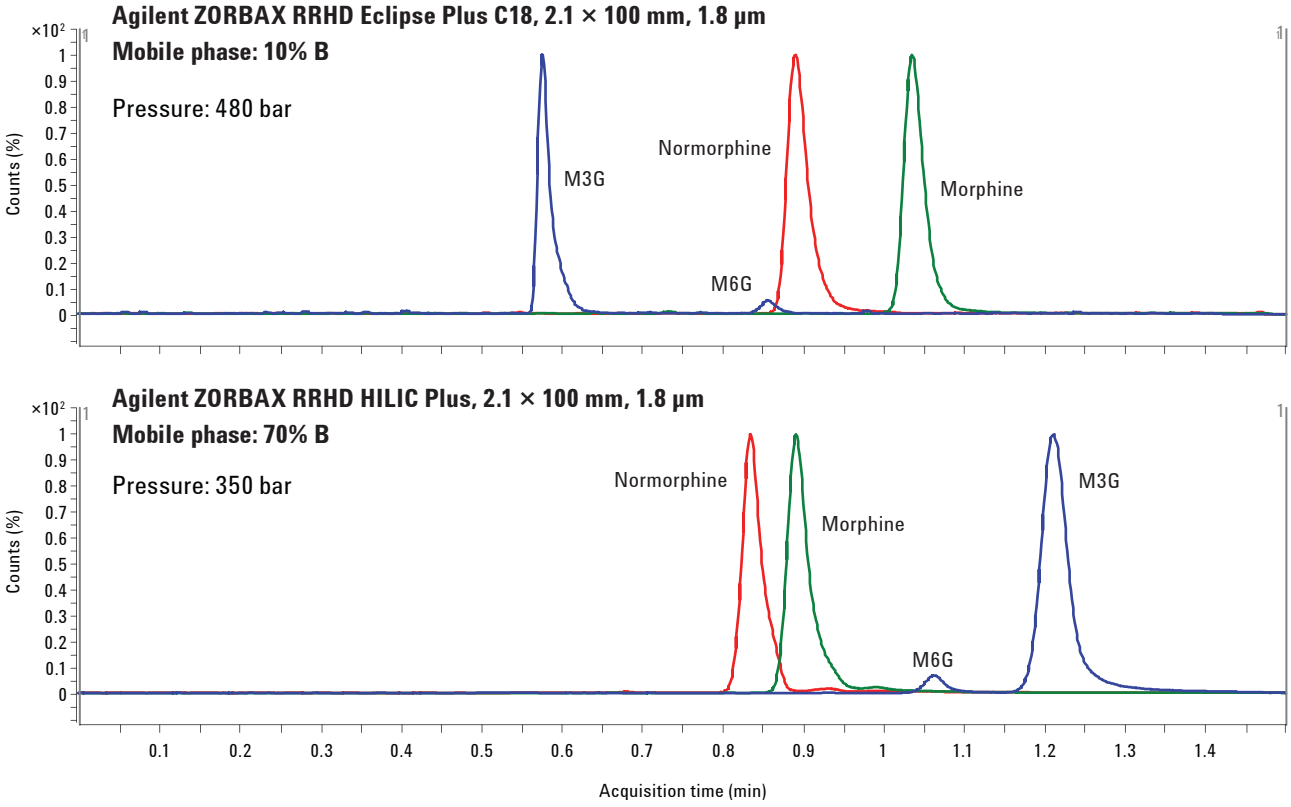


Figure 4. The Agilent ZORBAX RRHD HILIC Plus column offers complimentary selectivity to an Agilent ZORBAX RRHD Eclipse Plus C18 column, almost reversing elution order; LC/MS method parameters are detailed in the Experimental section.

Scaling the glucuronide metabolites to the same scale shows not only a selectivity difference between the C18 column and the HILIC, but also a more intense MS signal in HILIC mode, as shown in Figure 5. The HILIC Plus column uses 70% acetonitrile, compared to the 10% with the C18 column, which is more volatile and allows for more efficient spraying and desolvation in the ESI-MS source, thereby generating a more intense MS signal.

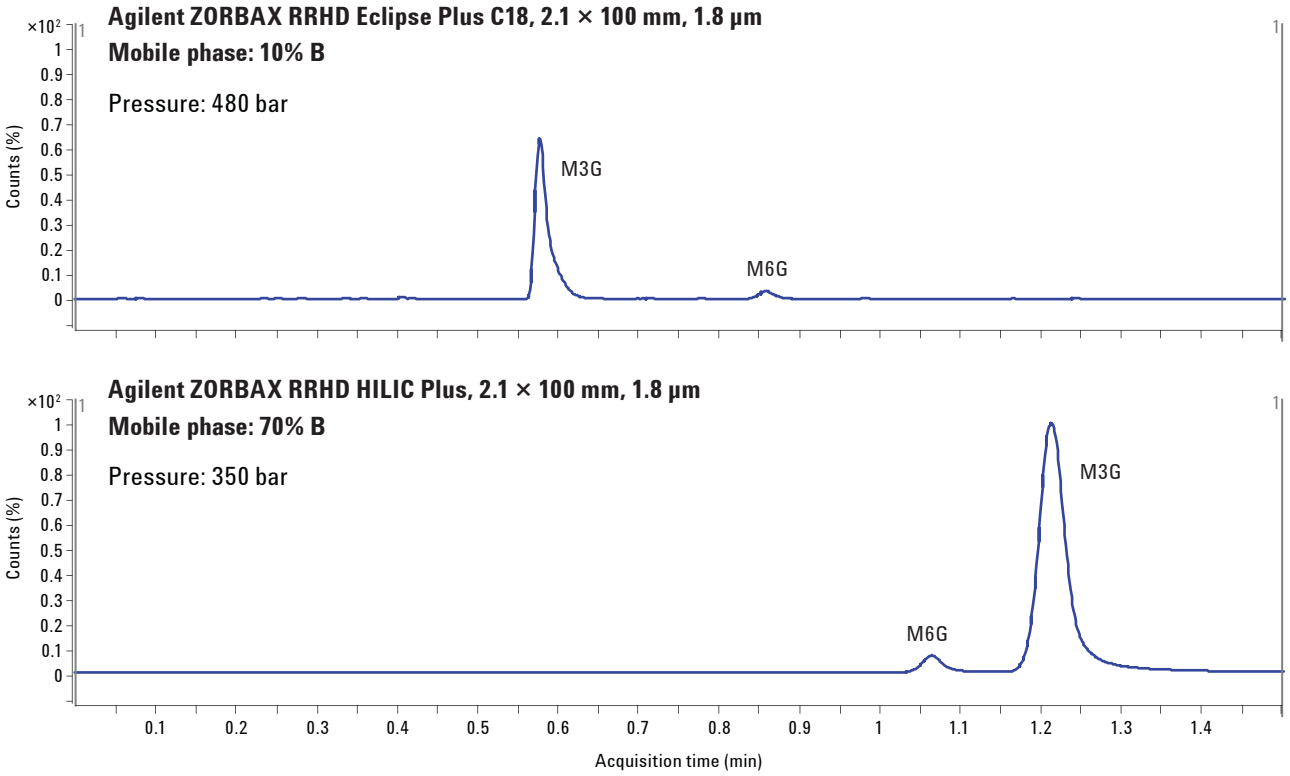


Figure 5. HILIC mode allows for more efficient spraying and desolvation in the ESI-MS source compared to RPLC, generating a more intense MS signal; LC/MS method parameters are detailed in the Experimental section.

A closer look at the M6G peak shows that HILIC mode not only improves signal intensity, but also reduces baseline noise. Figure 6 shows a fourfold improvement in signal-to-noise for M6G in HILIC mode compared to a similar UHPLC C18 method.

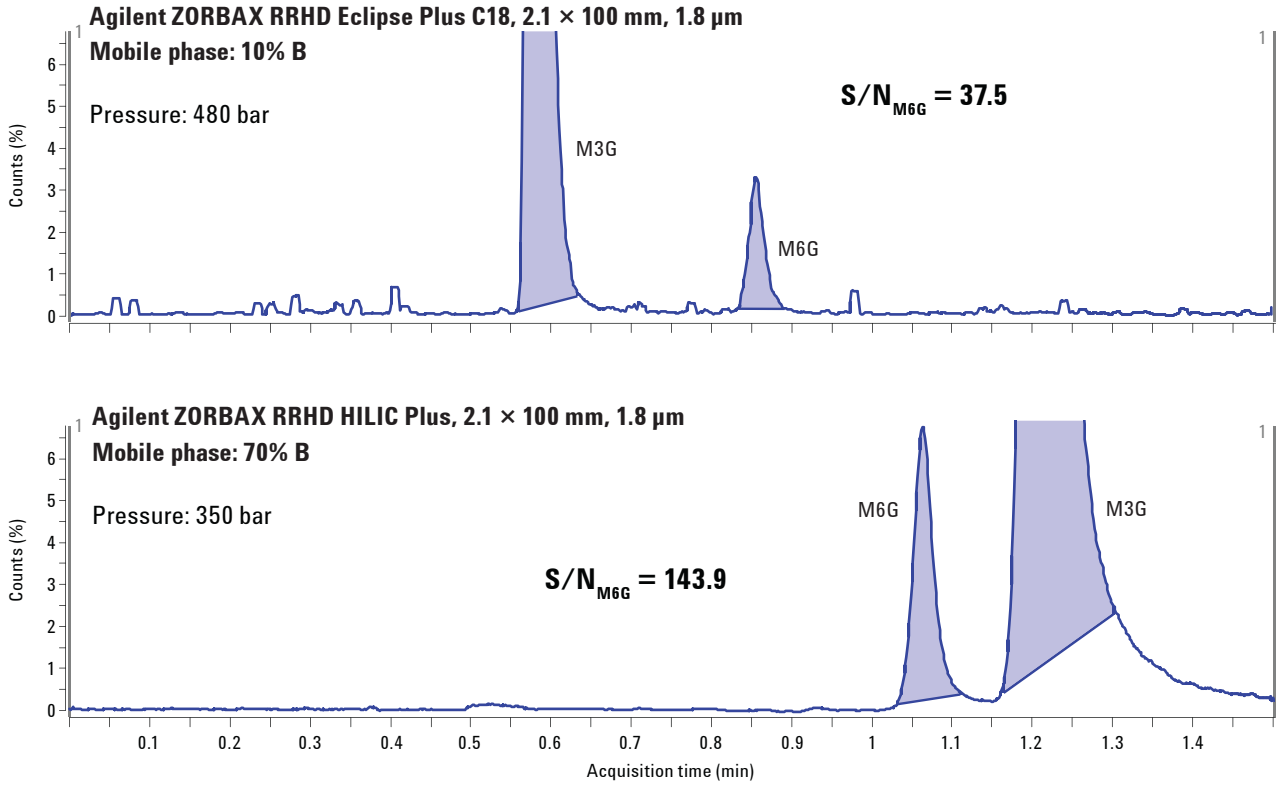


Figure 6. HILIC mode allows for more efficient spraying and desolvation in the ESI-MS source compared to RPLC, generating less baseline noise and a more intense MS signal resulting in a 4× improvement in sensitivity; LC/MS method parameters are detailed in the Experimental section.

To see the combined effects of using HILIC with UHPLC/MS the 1.8 µm HILIC Plus column can be compared to the earlier 5 µm column in RPLC mode. Again, the RPLC chromatogram shows more baseline noise, and when combined with the less efficient 5 µm particle column, the result is a 20x improvement in signal-to-noise for the active morphine metabolite M6G, as seen in Figure 7.

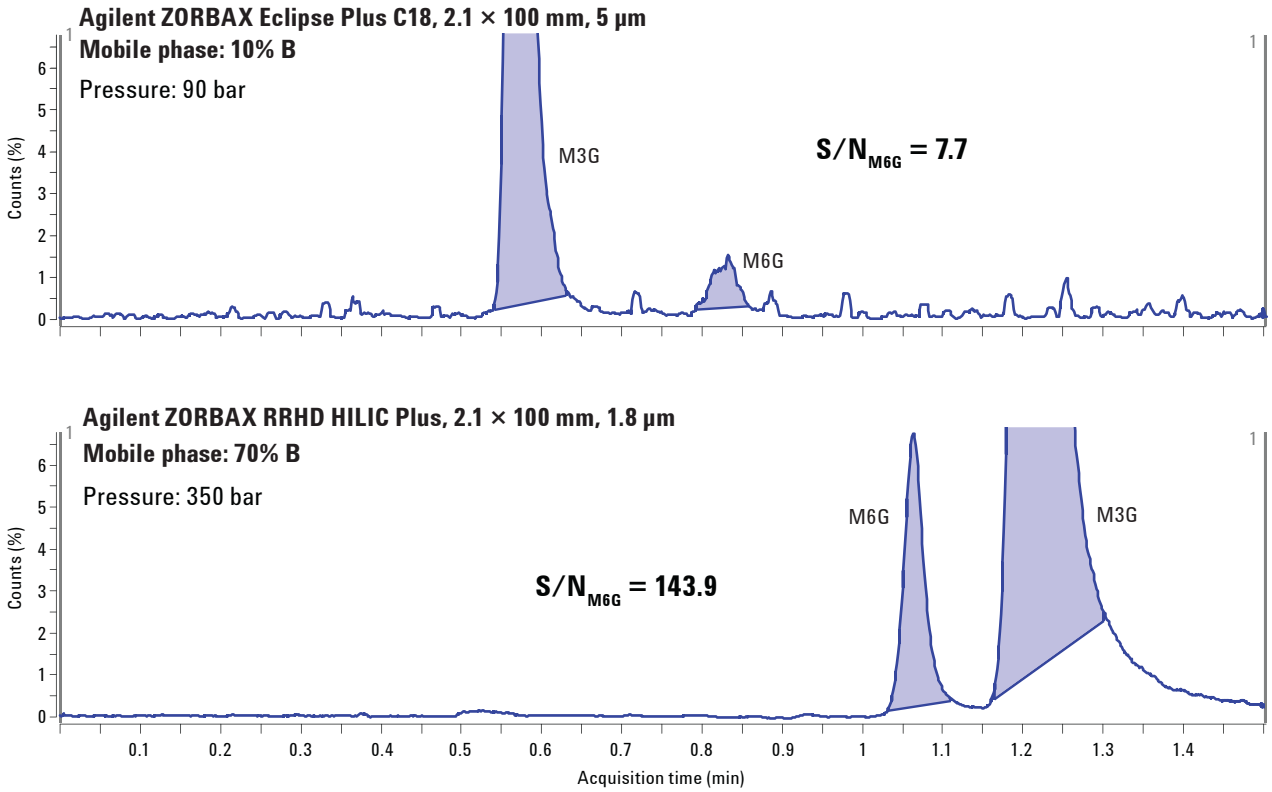


Figure 7. HILIC mode with UHPLC columns improves MS sensitivity by a factor of 20, compared to traditional LC columns in RPLC mode with MS detection; LC/MS method parameters are detailed in the Experimental section.

The high 1200 bar pressure limits of both the UHPLC column and LC system can be exploited by increasing the mobile phase flow rate. This shows that not only is the HILIC method more sensitive than a comparable RPLC method with traditional columns, but it can be accomplished in less than half the time while still substantially improving sensitivity by more than a factor of 10 (Figure 8). The sensitivity of this UHPLC analysis in HILIC mode is slightly less than the previous HILIC analysis, because the flow rate is increased to 1 mL/min from the optimal 0.4 mL/min, and so some column efficiency is likely to be lost.

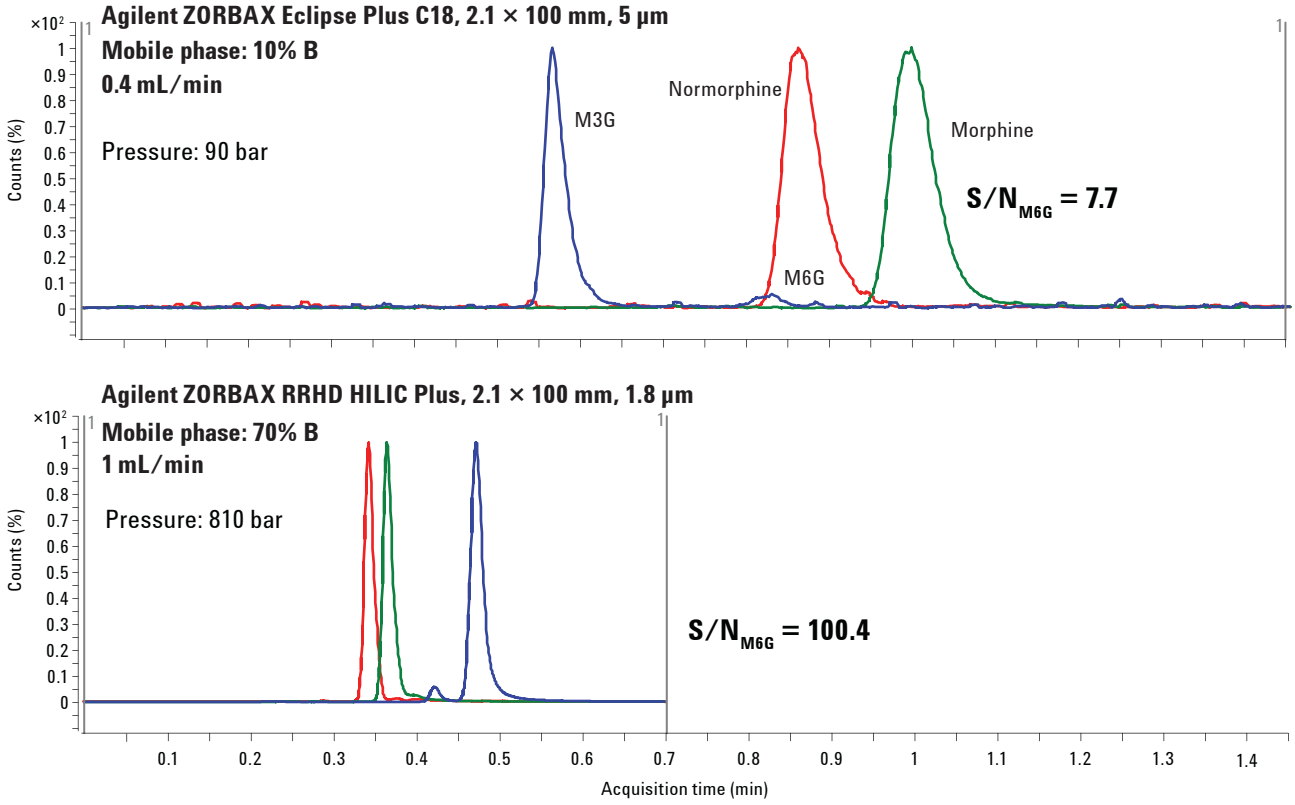


Figure 8. HILIC mode with UHPLC columns cuts analysis time in half, while improving sensitivity by more than a factor of 10, compared to traditional LC columns in RPLC mode with MS detection; LC/MS method parameters are detailed in the Experimental section.

Conclusions

Using these analyses for morphine and its metabolites, it is demonstrated that high efficiency sub-2 µm UHPLC columns can improve sensitivity by 5x when compared to traditional 5 µm columns. Also, HILIC mode can improve ESI-MS efficiency due to its more volatile highly organic mobile phase by 4x, compared to a similar UHPLC analysis in RPLC mode. Combining the improvements possible with UHPLC columns and HILIC mode with MS detection can yield as much as a 20x improvement in sensitivity, in contrast to a traditional 5 µm column in RPLC mode. Furthermore, the UHPLC column and LC system can be exploited with high flow rates to accomplish the analysis in less time, while still improving sensitivity, in this case by more than 10x.

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Agilent Technologies

LC/MS/MS of Vitamin B Shows Effects of Injection Solvents with an Agilent ZORBAX RRHD HILIC Plus Column

Application Note

Food Testing & Agriculture

Author

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Agilent Technologies, Inc.

Abstract

A rapid LC/MS/MS analysis of vitamin B related compounds (4-aminobenzoic acid, nicotinamide, riboflavin, and nicotinic acid) is optimized on an Agilent ZORBAX Rapid Resolution High Definition HILIC Plus column using an Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer. The method uses isocratic elution with an acetonitrile rich mobile phase and an ammonium acetate buffer. All analytes have good peak shape with this hydrophilic interaction chromatography (HILIC) application. The method is then used to demonstrate the importance of choosing an appropriate injection solvent when performing LC analyses in HILIC mode. Various injection solvents are investigated, including water, acetonitrile, methanol, and combinations. Of the solvents explored, it was determined that pure acetonitrile yields the best retention and peak shape for all compounds, with water being the worst solvent for analytical performance and peak shape.

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Introduction

HILIC is gaining popularity in liquid chromatography, particularly for its ability to retain and separate small polar analytes – an area where common reversed-phase liquid chromatography (RPLC) methods often fail. This novel mode of chromatography results in unique retention mechanisms, because water is used as the strong eluting solvent and can have distinct advantages over traditional RPLC in both sample preparation and LC/MS sensitivity, due to the use of highly organic mobile phases. The highly organic mobile phases do not require samples to be dried prior to injection, and their higher volatility than traditional RPLC mobile phases makes this technique well suited for applications with mass spectrometers [1].

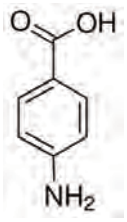
HILIC retention on a silica-based column is believed to involve a combination of mechanisms. First, a water layer must be adsorbed onto the polar silica surface, creating a liquid/liquid extraction system. The polar analytes can then partition into and out of this adsorbed water layer, with more polar analytes having a stronger interaction with this immobilized water layer. Charged polar analytes can also undergo ion exchange with the charged silica. Elution is typically from least to most polar, the opposite of RPLC. For HILIC method development, it is important to remember that the solvent strengths are different than in RPLC. For HILIC mode, solvent strength is tetrahydrofuran < acetone < acetonitrile < isopropanol < ethanol < methanol < water, with water being the strongest solvent [2,3,4].

HILIC is used extensively to analyze polar molecules. In this application note, an analysis of 4 vitamin B related compounds is optimized by LC/MS/MS using an Agilent ZORBAX Rapid Resolution High Definition HILIC column. The compounds of interest are 4-aminobenzoic acid, nicotinamide, riboflavin, and nicotinic acid, and they are shown in Figure 1.

Additionally, a major consideration for HILIC performance is its sensitivity to injection solvent strength. This is evaluated using the 4 vitamin B related compounds and a variety of injection solvents, ranging in strength from 100% acetonitrile to 100% water. Solvent effects on peak shape and retention are discussed.

Experimental

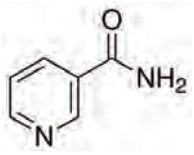
An Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer was used. The setup was optimized for lowest possible extra-column volume with short 0.075 mm id capillaries found in the Agilent Ultra Low Dispersion Kit (p/n 5067-5189) and with an Agilent LC System Rack (p/n 5001-3726) [5].



4-Aminobenzoic acid

$C_7H_7NO_2$

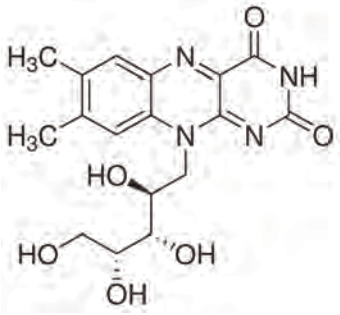
MW = 137.14



Nicotinamide

$C_6H_6N_2O$

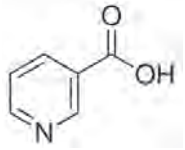
MW = 122.12



Riboflavin

$C_{17}H_{20}N_4O_6$

MW = 376.36



Nicotinic acid

$C_6H_5NO_2$

MW = 123.11

Figure 1. Vitamin B related compounds of interest.

Conditions

Columns:	Agilent ZORBAX Rapid Resolution High Definition (RRHD) HILIC Plus, 2.1 × 50 mm 1.8 µm (p/n 959757-901) and 2.1 × 100 mm, 1.8 µm (p/n 959758-901)
Mobile phase:	CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1)
Flow rate:	0.4, 0.7 or 1.0 mL/min, isocratic
Temperature:	25 °C
Sample:	For method optimization: 0.1 µL injection of 12.5 µg/mL each of 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid in acetonitrile (minimal amount of water, <5%)
	For injection solvent comparison: 1 µL injection of 5.7 µg/mL each of 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid in various solvents (including H_2O, CH_3CN, CH_3OH and combinations thereof); analytes are illustrated in Figure 1
MS source:	Positive ESI, capillary, 4000 V, drying gas temperature, flow rate and nebulizer pressure vary with mobile phase flow rate and are specified in Table 1
MS acquisition:	For method optimization: dynamic MRM (dMRM), delta EMV 200 V, MS cycle time varies with mobile phase flow rate and is specified in Table 1, compound MRM transitions are detailed in Table 2
	For injection solvent comparison: selected ion mode (MS2SIM), delta EMV 200 V, dwell time 15 ms, compounds were identified by their precursor ions listed in Table 2, and were generated using their respective fragmentor voltages in Table 2
Software:	Agilent MassHunter versions B.03.01, B.02.00, and B.03.01 were used for data acquisition, qualitative, and quantitative analyses, respectively.

All 4 analytes were purchased as powders from Sigma Aldrich and prepared to desired concentrations in various solvent systems. Acetonitrile and methanol were purchased from Honeywell. Ammonium formate and formic acid were purchased from Sigma Aldrich. Water used was 18 MΩ Milli-Q water.

Table 1. Mass spectrometer parameters for optimized vitamin analyses at various flow rates.

	0.4 mL/min	0.7 mL/min	1.0 mL/min
Source	ESI+	ESI+	ESI+
Delta EMV	200 V	200 V	200 V
MS Dwell Time	70 ms	50 ms	30 ms
Drying Gas Temperature	200 °C	200 °C	300 °C
Drying Gas Flow Rate	10 L/min	11 L/min	11 L/min
Nebulizer Pressure	30 psi	35 psi	55 psi
Capillary Voltage	4000 V	4000 V	4000 V

Table 2. Mass spectrometer MRM transitions for optimized vitamin analyses.

	Precursor ion	Fragmentor voltage	Product ion	Collision energy
4-Aminobenzoic acid	138	110	120	15
	138	110	94	15
Nicotinamide	123	130	80	25
	123	130	53	35
Riboflavin	377	160	243	30
	377	160	172	40
Nicotinic acid	124	130	80	20
	124	130	53	40

Results and Discussion

Optimized LC/MS/MS analyses of 4-aminobenzoic acid, nicotinamide, riboflavin, and nicotinic acid are shown in Figure 2 at various flow rates. The ZORBAX RRHD HILIC Plus column produces good peak shape and efficiency for all compounds regardless of the mobile phase flow rate.

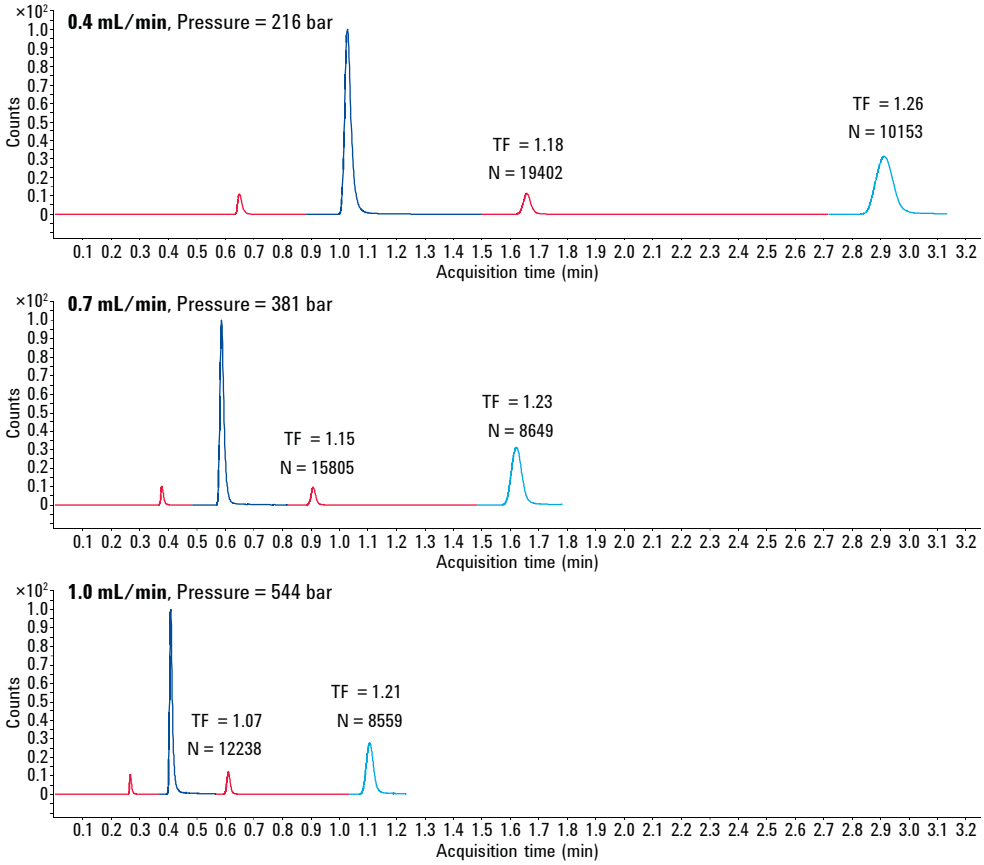


Figure 2. Optimized analyses of vitamin B related compounds at various flow rates by LC/MS/MS using a 2.1 x 100 mm, 1.8 µm Agilent ZORBAX RRHD HILIC Plus column and an Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer.

System:	Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer
Column:	Agilent ZORBAX RRHD HILIC Plus, 2.1 × 100 mm, 1.8 µm (p/n 959758-901)
Mobile phase:	CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1)
Isocratic:	0.4, 0.7, or 1.0 mL/min
Injection:	0.1 µL of 12.5 µg/mL each of: 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid in CH_3CN
Thermostatted column compartment:	25 °C
MS source:	ESI+, Capillary: 4000 V; Drying gas temperatures, Flow rates, Nebulizer pressures, and Dwell times are found in Table 1
MS acquisition:	dMRM; Compound transitions are found in Table 2

Figures 3A and 3B show the effects of injecting strong solvents in HILIC mode. Each sample was prepared to the same concentration in various solvents. A stock solution of the vitamin B related compounds in acetonitrile was diluted 1:10 with the different solvent combinations. Injecting water, the strongest solvent, severely distorts peak shape, particularly for nicotinamide and riboflavin. Because nicotinic acid is more retained, it is less affected than the earlier eluting peaks, however, there is still a shift in retention time. While methanol is not as strong a solvent as water in HILIC mode, it still has an effect on chromatography. Injecting between 100% and 50% methanol produces broader peaks than injecting the sample in pure acetonitrile. There is also a loss of retention for riboflavin and nicotinic acid when methanol is injected with HILIC mode. From these comparisons of injection solvents, it is apparent that the sample solvent in HILIC mode should be as weak as possible, which for HILIC means as high of a percentage of acetonitrile as possible (depending on analyte solubility).

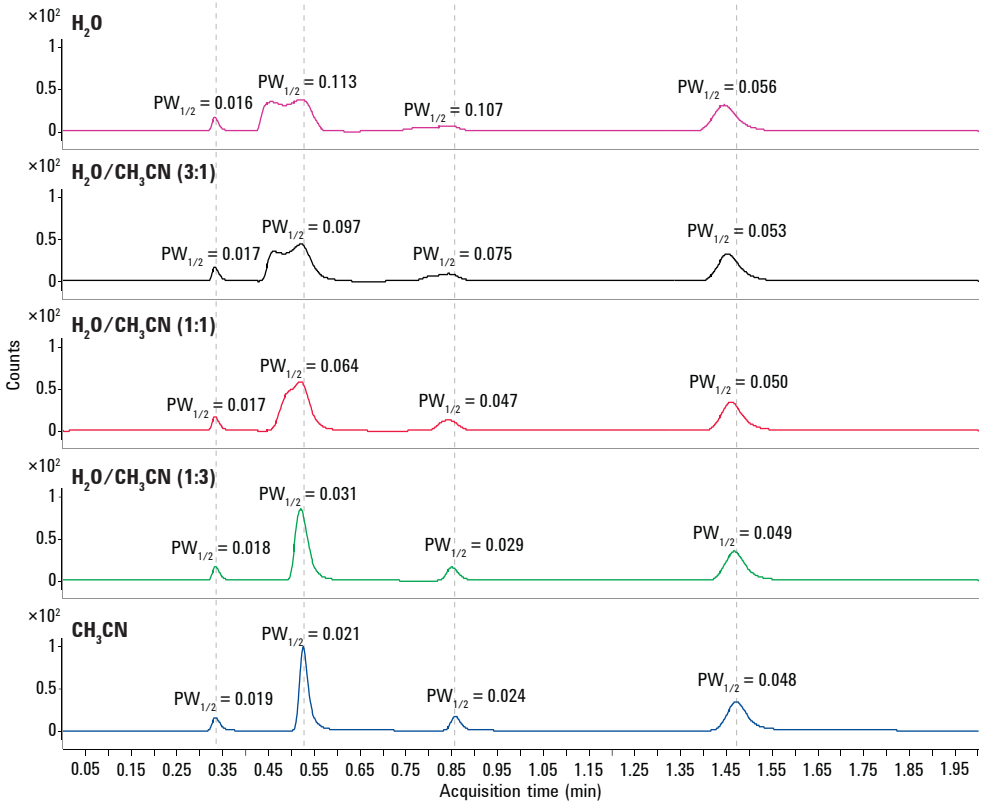


Figure 3A. The impact of injection solvent on HILIC/LC/MS performance using a 2.1 × 50 mm, 1.8 µm Agilent ZORBAX RRHD HILIC Plus column and an Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer. Water + acetonitrile.

System:	Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer
Column:	Agilent ZORBAX RRHD HILIC Plus, 2.1 × 50 mm, 1.8 µm (p/n 959757-901)
Mobile phase:	CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1)
Isocratic:	0.4 mL/min, Pressure = 135 bar
Injection:	1 µL of 5.7 µg/mL each of: 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid in various solvents
Thermostatted column compartment:	25 °C
MS source:	ESI+, Capillary: 4000 V, Drying gas: 200 °C, 10 L/min, Nebulizer: 30 psi, Dwell: 15 ms
MS acquisition:	MS2SIM: *m/z* 138 (Frag 110 V), *m/z* 123 (Frag 130 V), *m/z* 377 (Frag 160 V), *m/z* 124 (Frag 130 V)
Sample preparation:	100 µL stock solution in CH_3CN was diluted into 1 mL of the solvents labeled on the chromatograms to the left; final concentration was 5.7 µg/mL of each compound

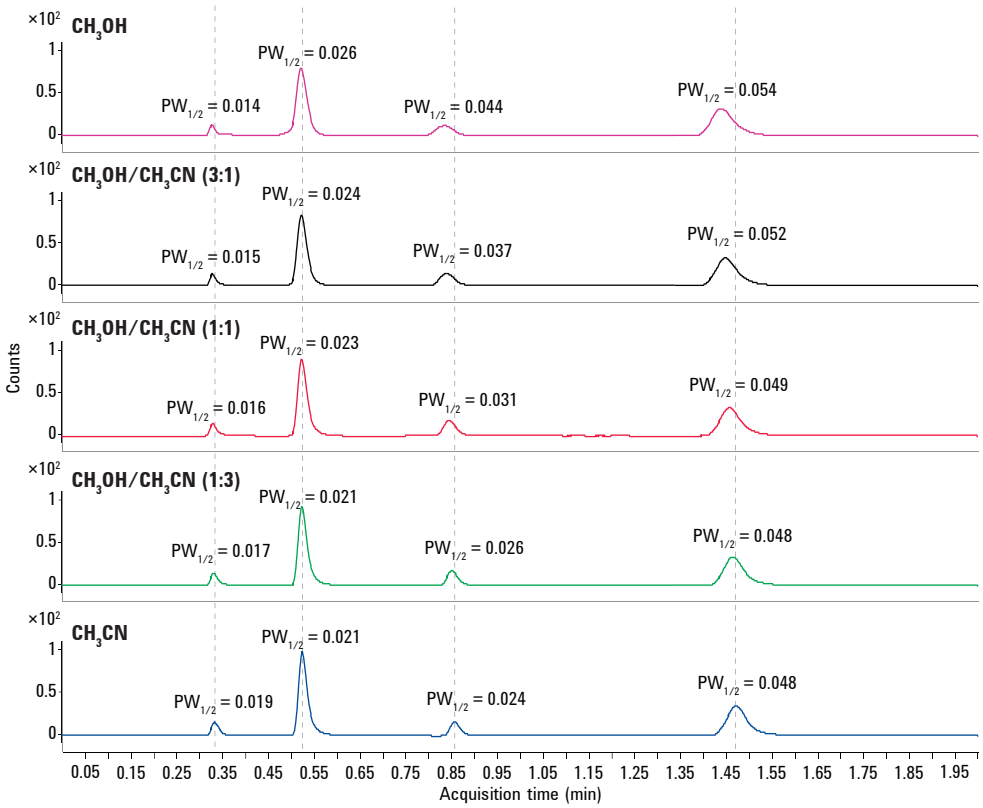


Figure 3B. The impact of injection solvent on HILIC/LC/MS performance using a 2.1 × 50 mm, 1.8 µm Agilent ZORBAX RRHD HILIC Plus column and an Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer. Methanol + acetonitrile.

System:	Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer
Column:	Agilent ZORBAX RRHD HILIC Plus, 2.1 × 50 mm, 1.8 µm (p/n 959757-901)
Mobile phase:	CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1)
Isocratic:	0.4 mL/min, Pressure = 135 bar
Injection:	1 µL of 5.7 µg/mL each of: 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid in various solvents
Thermostatted column compartment:	25 °C
MS source:	ESI+, Capillary: 4000 V, Drying gas: 200 °C, 10 L/min, Nebulizer: 30 psi, Dwell: 15 ms
MS acquisition:	MS2SIM: *m/z* 138 (Frag 110 V), *m/z* 123 (Frag 130 V), *m/z* 377 (Frag 160 V), *m/z* 124 (Frag 130 V)
Sample preparation:	100 µL stock solution in CH_3CN was diluted into 1 mL of the solvents labeled on the chromatograms to the left; final concentration was 5.7 µg/mL of each compound

Conclusions

Four vitamin B related compounds are successfully analyzed by LC/MS/MS in HILIC mode. The Agilent ZORBAX HILIC Plus column delivers good peak shape and efficiency for these polar analytes. Additionally, it is shown that water, the strongest solvent in HILIC mode, should never be used as the sample solvent, as it results in significant peak distortion for early eluting peaks. Methanol is also a strong solvent in HILIC mode, though not as strong as water, and it also affects peak shape and retention, though not as drastically as water.

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Agilent Technologies

Separation of enantiomers and conformers of Tofisopamon

Using Daicel immobilized polysaccharide-derived chiral columns using the Agilent 1260 Infinity Analytical SFC System

Application Note

Pharmaceuticals

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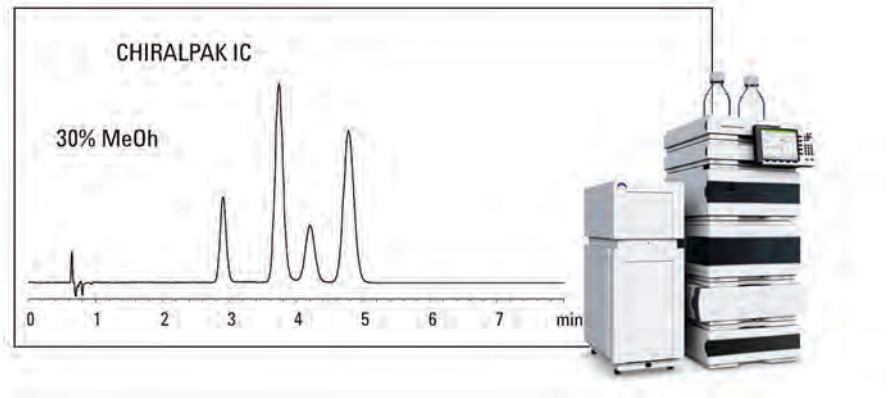

Abstract

Due to different physiological activities, the chromatographic separation of enantiomers is an important task in drug discovery and development. The determination of the enantiomeric excess (ee) is often done with supercritical fluid chromatography (SFC) in combination with chiral stationary phases. Since finding the right stationary phase that provides separation of the enantiomers is difficult to predict, automated screening of different stationary and mobile phases is advantageous. In this Application Note, the screening of different mobile and stationary phases for the separation of the enantiomers and conformers of Tofisopam is shown using the Agilent 1260 Infinity Analytical SFC and Daicel immobilized polysaccharide-derived chiral columns.

Agilent Technologies

Introduction

Tofisopam [1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine] is a member of the 2,3-benzodiazepine compound family. It has been applied as a pharmaceutical in the treatment of anxiety and alcohol withdrawal[1,2].

Due to its stereogenic center at C(5)-atom, Tofisopam exists as two enantiomers (R(+) and S(–)). Upon dissolution, its diazepine ring system will exist in two boat conformations, leading to two conformers for each enantiomer (Figure 1). The driving force for conformer transition is attributed to the steric repulsion effect between C(4)-methyl and C(5)-ethyl groups.

As a result of the pharmacological interest in Tofisopam[3], it is essential to have an easy and robust method to separate the four isomers in order to individually evaluate their different biological activities, to understand the kinetics and thermodynamics, and to control the quality of the drug.

In this Application Note, we have evaluated the separation of all enantiomers and conformers of Tofisopam with a single, robust, and fast chromatographic method using columns packed with the immobilized polysaccharide-derived chiral stationary phases (CSPs) in combination with the advanced SFC technology of the Agilent 1260 Infinity Analytical SFC System.

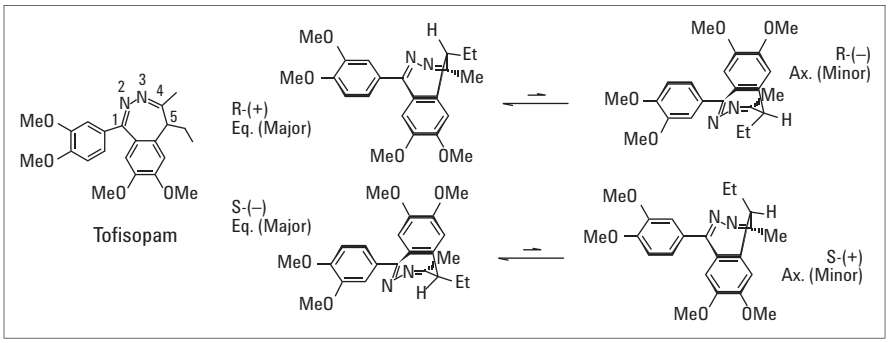

Figure 1
Conformational equilibria of Tofisopam enantiomers.

Experimental

Chemicals

The chiral columns CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC, and CHIRALPAK ID used in this study are manufactured by Daicel Chemical Industries, Ltd. They are sized 4.6 × 150 mm id and packed with 5-µm particles of immobilized amylose–or cellulose-derived CSPs.

As the main mobile phase component, supercritical CO_2 (industrial quality 4.8) was used in this study. Different mobile phase modifiers such as methanol (MeOH), 2-propanol (2-PrOH), and acetonitrile (ACN) were screened in different concentrations. All solvents used were HPLC quality. Due to the basic nature of the Tofisopam molecule, diethylamine (DEA) was added to all mobile phases (0.1% v/v).

Instruments

All SFC experiments were carried out on an Agilent 1260 Infinity Analytical SFC System. The system contains the A5 fusion module for CO_2 pre- and post-conditioning and a modified Agilent 1260 Infinity Binary LC System for accurate and constant metering of the mobile phase. The system (G4309A) consisted of the following modules:

- SFC Fusion A5 module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity Standard Degasser
- Agilent 1260 Infinity Standard Autosampler
- Agilent 1260 Infinity Diode Array Detector with high pressure SFC flow cell

In addition, an Agilent SFC Method Development kit was used consisting of:

- Two Agilent 1260 Infinity Thermostatted Columns Compartments with built-in valve drives
- Agilent ZORBAX Method Development Valve Kit, 600 bar

Chromatographic conditions

Throughout the experimental work, the flow rate was set at 3.0 mL/min, the temperature of the column compartments at 35 °C and the back pressure of CO_2 supercritical fluid at 150 bar.

Results and Discussion

Due to their remarkable enantioselectivity and versatility, CSPs based on polysaccharide derivatives have been widely used for separation of enantiomers or stereoisomers by LC and SFC. Since 2004, the advanced immobilized version of the polysaccharide-based CSPs has been successfully integrated into the tool box for chiral separation. These new columns have the advantages of universal solvent compatibility, creation of new selectivity profiles, and material robustness[4-6]. In addition, SFC is considered as an advantageous technique over LC due to its improved diffusion properties, more favorable mass transfer characteristics, and low mobile phase viscosity which result in faster separation. The simultaneous separation of the enantiomers and conformers of Tofisopam has been evaluated in our laboratories with the combination of SFC and the four immobilized chiral columns: CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC and CHIRALPAK ID. Various mobile phase modifiers were examined in a systematic way, including MeOH, 2-PrOH, and ACN.

As shown in Figure 1, R(+)- and S(–)-Tofisopam are the dominating conformers where the ethyl group attached to the C(5)-atom has a quasi-equatorial orientation (Eq.). Depending on the solvent in which the product is dissolved, the conformational equilibriums will move in favor of the formation of R(–)- or S(+)- conformers where the ethyl group attached to the C(5)-atom will change to the axial orientation (Ax.). Our study on conformer transition of Tofisopam in various sample media (MeOH, EtOH, 2-PrOH, Methyl tert-butyl ether/MeOH 90/10 and ACN) indicate that MeOH induces the fastest formation in the highest proportion (up to 26%) of the R(–)- or S(+)- conformers, while ACN leads to the slowest kinetics and in the lowest proportion (up to 16.5% only) of the same molecules[7]. In no case, does the presence of the R(–)- and S(+)- conformers overtake that of the R(+)- and S(–)- enantiomers in the sample mixture. As a consequence, the R(+)- and S(–)- enantiomers (Eq.) are always chromatographically eluted as major peaks and the R(–)- or S(+)- conformers (Ax.) are always eluted as minor peaks. The relative elution order between the R(+)- and S(–)- and between the R(–)- or S(+)- isomers were not determined in our study due to the lack of the reference standards. To facilitate the discussion, we denote here the minor peaks as $P_{(Ax.)}$ and the major peaks as $P_{(Eq.)}$ without assigning the absolute configuration of the molecules in each pair of enantiomer-conformers.

MeOH appears to be the most versatile co-solvent in resolution of Tofisopam isomers. As indicated in Table 1, the best resolution of the four peaks was achieved with CHIRALPAK IA and CHIRALPAK ID. Analysis times can be as short as 3 minutes (Figure 2 (a)) or 5 minutes (Figure 2 (b)), respectively. Interestingly, the apparent elution order is the same on CHIRALPAK IA, CHIRALPAK IC, and CHIRALPAK ID, that is, $P_{(Ax.)}$-$P_{(Eq.)}$-$P_{(Ax.)}$-$P_{(Eq.)}$. Co-elution of two $P_{(Ax.)}$ and one $P_{(Eq.)}$ is observed on CHIRALPAK IB.

	CHIRALPAK IA	CHIRALPAK IB	CHIRALPAK IC	CHIRALPAK ID
$tr_{1\ (min)}$	1.26	2.55	2.90	1.56
$tr_{2\ (min)}$	1.48	2.55	3.74	1.90
$tr_{3\ (min)}$	2.03	2.55	4.20	2.50
$tr_{4\ (min)}$	2.45	2.72	4.77	3.91
$Rs_{(12)}$	2.72		4.21	3.33
$Rs_{(23)}$	4.44		1.90	4.19
$Rs_{(34)}$	2.57	1.25	2.04	5.90

Table 1
Resolution of Tofisopam enantiomers and conformers with MeOH/CO_2.
Co-solvent: 20% (MeOH/DEA 100/0.5 v/v) for CHIRALPAK IA and IC; 10% (MeOH/DEA 100/1.0 v/v) for CHIRALPAK IB; 40% (MeOH/DEA 100/0.25 v/v) for CHIRALPAK ID

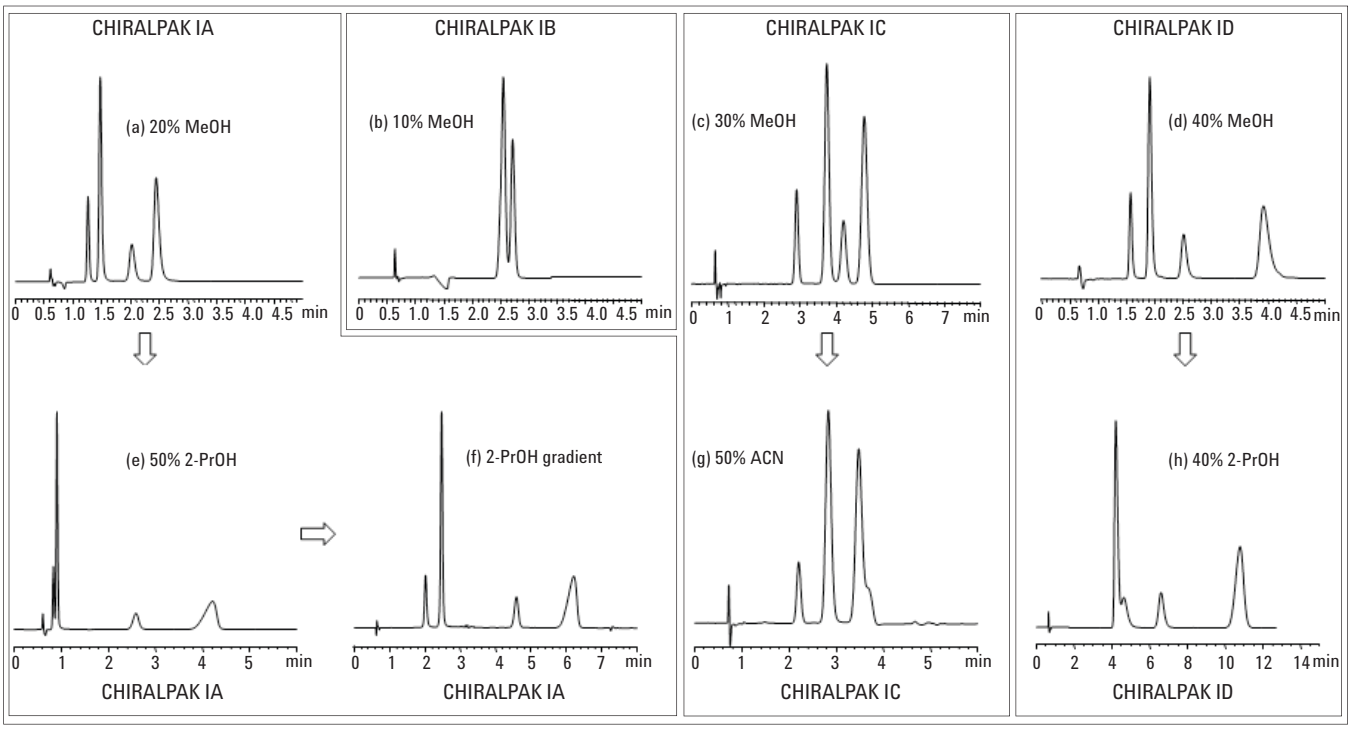

Figure 2
Separation examples. Modifier (solvent channel B, by volume):
20% (MeOH/DEA 100/0.5 v/v) for (a); 10% (MeOH/DEA 100/1.0 v/v) for (b); 30% (MeOH/DEA 100/0.33 v/v) for (c); 40% (MeOH/DEA 100/0.25 v/v) for (d); 50% (2-PrOH/DEA 100/0.2 v/v) for (e); Gradient* with (2-PrOH/DEA 100/0.2 v/v) for (f); 50% (ACN/DEA 100/0.2 v/v) for (g); 40% (2-PrOH/DEA 100/0.25 v/v) for (h)
***Gradient: 0-1.5 min 20% B; 1.5-2.5 min 20%-50% B; 2.5-6.5 min 50% B; 6.5-8.5 min 20% B**

The nature of the modifier in SFC can greatly impact the resolution of the four peaks. For instance, the complete resolution on CHIRALPAK IA between the two first eluting peaks is compromised when changing from MeOH (Figure 2 (a)) to 2-PrOH (Figure 2 (e)). In order to achieve full resolution of the first two eluting peaks with this modifier, a gradient run proved to be a good approach (Figure 2, $Rs_{(12)}$ = 1.27 in (e); $Rs_{(12)}$ = 4.18 in (f)).

Modifiers also have an effect on elution profile of Tofisopam peaks. This is demonstrated by the separation examples on CHIRALPAK IC and CHIRALPAK ID. On CHIRALPAK IC, the apparent elution order is $P_{(Ax.)}$-$P_{(Eq.)}$-$P_{(Ax.)}$-$P_{(Eq.)}$ with 20% MeOH (Figure 2 (c)), but becomes $P_{(Ax.)}$-$P_{(Eq.)}$-$P_{(Eq.)}$-$P_{(Ax.)}$ with 50% ACN (Figure 2 (g)). On CHIRALPAK ID, the four species are eluted in the order of $P_{(Ax.)}$-$P_{(Eq.)}$-$P_{(Ax.)}$-$P_{(Eq.)}$ with 40% MeOH (Figure 2 (d)), but changes to $P_{(Eq.)}$-$P_{(Ax.)}$- $P_{(Ax.)}$-$P_{(Eq.)}$ while switching to 40% 2-PrOH (Figure 2 (h)).

The separation of Tofisopam isomers by SFC is perfectly reproducible, as demonstrated by 120 injections of Tofisopam over 20 hours on CHIRALPAK IC under the given chromatographic conditions (Figure 3). This can be attributed to the high performance of the Agilent 1260 Infinity SFC system providing superior CO_2 pre- and post-conditioning in combination with precise mobile phase metering. Such a system performance is depicted in Figure 4 for the CO_2 back (or outlet) pressure (a) and the pressure of the Agilent 1260 Infinity SFC Binary Pump (b).

Conclusion

The Daicel columns packed with immobilized polysaccharide-derived chiral stationary phases are highly robust for multiple separation solutions for enantiomers and conformers of Tofisopam.

The complete resolution of Tofisopam isomers in a single chromatographic run can be achieved in SFC and HPLC on the same set of chiral columns[7]. SFC is the preferred choice for faster separation of Tofisopam. The high instrument performance of the Agilent 1260 Infinity Analytical SFC System renders the analytical separation of Tofisopam robust and reproducible.

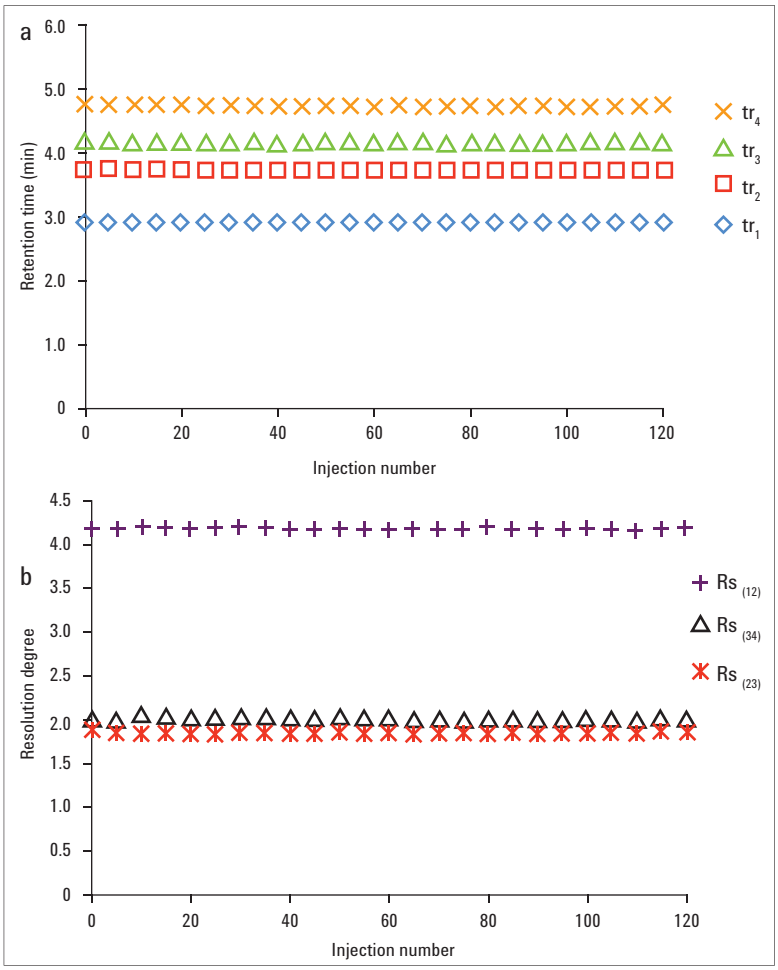

Figure 3
Reproducibility of the separation on CHIRALPAK IC
Modifier: 30% (MeOH/DEA 100/0.33 v/v), Flow rate: 3 mL/min, Temperature: 35 °C
(a) Retention time; (b) Resolution degree

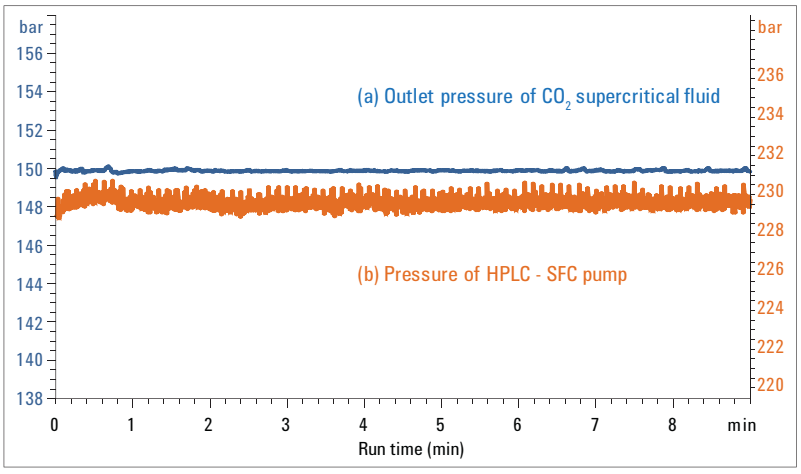

Figure 4
Pressure stability of the Agilent 1260 Infinity Analytical SFC System
Modifier: 30% (MeOH/DEA 100/0.33 v/v), Flow rate: 3 mL/min, Temperature: 35 °C

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Agilent Technologies

Enantiomer separation of non-steroidal anti-inflammatory drugs

Using Daicel immobilized polysaccharide-derived chiral columns and the Agilent 1260 Infinity Analytical SFC System

Application Note

Pharmaceuticals

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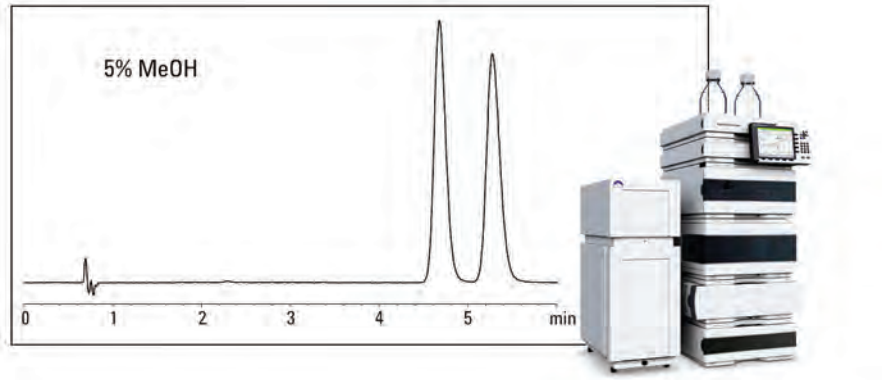

Abstract

The enantiomer separation by supercritical fluid chromatography (SFC) of six profen drugs was investigated on the new generation of chiral columns from Daicel Corporation, that are packed with the immobilized polysaccharide-derived chiral stationary phases CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC, and CHIRALPAK ID. Methanol, ethanol, 2-propanol and acetonitrile were used in combination with trifluoroacetic acid as the mobile phase modifier in the supercritical CO_2. Using the Agilent 1260 Infinity Analytical SFC System, systematic studies were carried out in terms of the mobile phase additive, the modifier nature and its percentage to achieve complete resolution of these profen drugs on the given column set.

Agilent Technologies

Introduction

Profens (2-arylmethylpropionic acids), such as Flurbiprofen, Ibuprofen, and Ketoprofen, have been widely used as non-steroidal anti-inflammatory drugs (NSAIDs). The enantiomers of these chiral drugs are reported to have different pharmacological and pharmacokinetic effects and undergo dissimilar metabolic processes[1-2]. As a consequence, the enantiomer resolution of profens and method optimization for diverse purposes have been the subject of intensive investigations by chromatography[3].

The columns packed with polysaccharide-based chiral stationary phases (CSPs) have proved suitable for enantiomer resolution of a number of profen drugs[4-8]. The advanced immobilized version of the polysaccharide-based CSPs offers the possibility of enhancing the application scope and enantioselective performance. Specifically, these new generation columns have the advantages of robustness, universal solvent compatibility and creation of new selectivity profiles[9]. They have now been successfully integrated into the tool box for chiral separation by LC and SFC.

The objective of the current study is to investigate the analytical separation feasibility of the typical profen enantiomers using a set of columns packed with the immobilized polysaccharide-derived CSPs in combination with advanced technology of the Agilent 1260 Infinity Analytical SFC System. The structures of the profen molecules under investigation are shown in Figure 1.

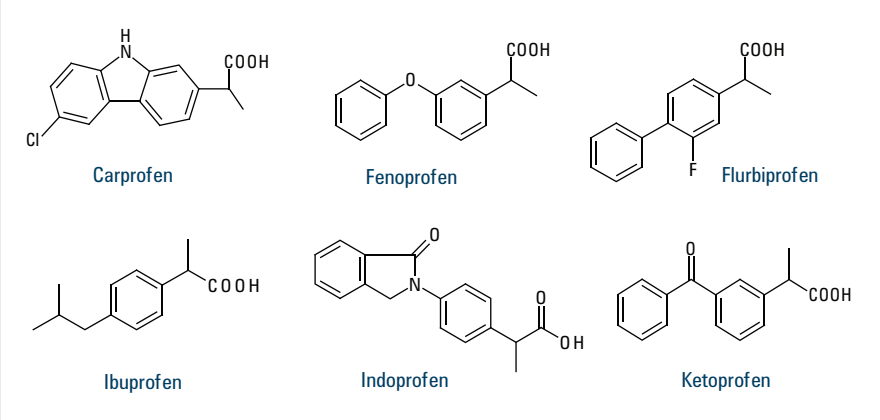

Figure 1
Structures of the profen molecules under investigation.

Experimental

Chemicals

The chiral columns CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC, and CHIRALPAK ID used in this study were manufactured by Daicel Corporation, Tokyo, Japan. Columns with dimensions of 4.6 × 150 mm and packed with 5-µm particles of immobilized amylose- or cellulose-derived CSPs were used.

The liquid carbon dioxide (industrial quality 4.8) in a cylinder of B50 was used for CO_2 supply. Different mobile phase modifiers such as methanol (MeOH), ethanol (EtOH), 2-propanol (2-PrOH), and acetonitrile (ACN) were employed in different proportions. All solvents used were HPLC quality. Trifluoroacetic acid (TFA) was used as the acidic additive and added to the modifier solvents equivalent to 0.06% by volume in the mobile phase.

Instrumentation

All SFC experiments were carried out on an Agilent 1260 Infinity Analytical SFC System. The system (G4309A) consists of the following modules:

- Aurora SFC Fusion A5 module for CO_2 pre- and post-conditioning
- Agilent 1260 Infinity SFC Binary Pump for accurate and constant metering of the mobile phase
- Agilent 1260 Infinity Degasser
- Agilent 1260 Infinity Autosampler
- Agilent 1260 Infinity Diode Array Detector with high pressure SFC flow cell

In addition, the Agilent SFC Method Development Kit was integrated into the system consisting of two Agilent 1260 Infinity Thermostatted Column Compartments with built-in valve drive and the Method Development Valve Kit (600 bar).

Chromatographic conditions

Throughout the experimental work, the flow rate was set at 3.0 mL/min, the temperature of the column compartments at 35 °C, and the back pressure of supercritical fluid carbon dioxide ($SF\text{-}CO_2$) at 150 bar.

Results and Discussion

The first screening of the profen molecules was undertaken by running a gradient (Figure 2). This allowed the determination of the modifier percentage to be used in isocratic mode so that the compounds were eluted in a reasonable time window (for example, 2 to 10 minutes). With this approach, isocratic runs delivering no enantiomer separation were avoided. The guideline for method transfer from gradient to isocratic is given in Table 1. In certain cases, fine-tuning regarding the modifier concentration may be necessary to optimize the separation.

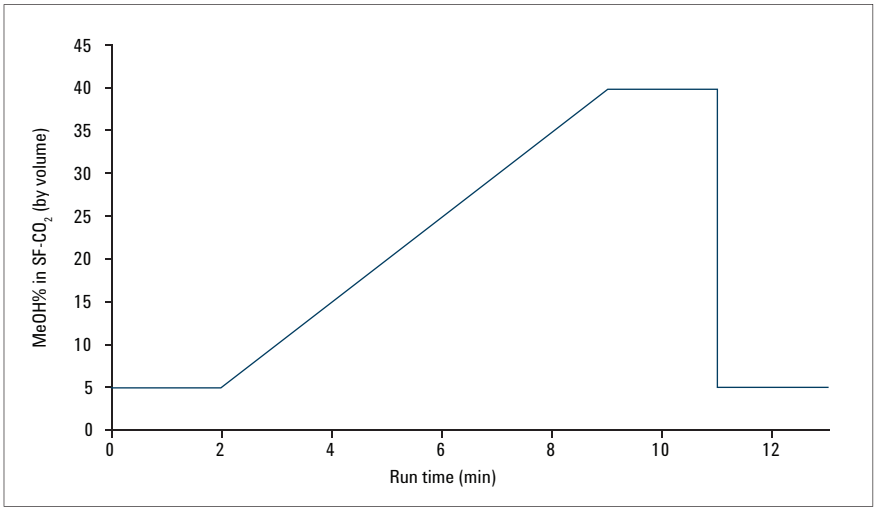

Figure 2
MeOH gradient program.

*t grad. (min)	Modifier%
>11	40 - 50
8 - 11	30
5.5 - 8	20
4 - 5.5	10
< 4	2 - 5

Table 1
Guideline from gradient to isocratic.
(* t grad. = (tr1+tr2)/2)

Effect of acidic additive

Due to its acidic character, SF-CO_2 has been used as the major mobile phase component for enantiomer resolution of acidic compounds with no additional acidic additives[10].

Using this approach, the racemic profens were first injected on the columns using MeOH as modifier without any additives. However, some comparative trials in the presence of TFA at trace level (0.06%) led to improved separation of the enantiomers. As indicated by the data in Table 2 and Table 3, the addition of 0.06% TFA in the mobile phase systematically resulted in:

- Shorter retention times (tr)
- Slightly higher enantioselectivity (α)
- Increased plate counts (N)
- Better peak symmetry (S)

These four benefits led to increased resolution (Rs) of the enantiomers.

	MeOH%	TFA %	tr_1	tr_2	α	Rs
Carprofen	40	-	1.72	1.93	1.19	1.86
	40	**0.06**	**1.67**	**1.88**	**1.20**	**2.21**
Flurbiprofen	10	-	2.05	2.34	1.20	2.55
	10	**0.06**	**1.84**	**2.14**	**1.25**	**3.40**
Ibuprofen	5	-	5.62	5.90	1.08	0.85
	5	**0.06**	**5.22**	**5.51**	**1.09**	**0.96**
Indoprofen	30	-	3.10	3.23	1.05	0.64
	30	**0.06**	**2.94**	**3.08**	**1.06**	**0.84**

Table 2
Chromatographic results with and without TFA in the mobile phases.
Column: CHIRALPAK IA

	MeOH%	TFA %	N_1	N_2	S_1	S_2
Carprofen	40	-	4478	4418	0.75	0.77
	40	**0.06**	**5643**	**5511**	**0.89**	**0.92**
Flurbiprofen	10	-	6250	6643	0.67	0.73
	10	**0.06**	**7797**	**8199**	**0.95**	**0.97**

Table 3
Effect of TFA on plate counts (N) and peak symmetry factors (S).
Column: CHIRALPAK IA

The effect of TFA on enantiomer separation of Carprofen and Flurbiprofen is demonstrated in Figure 3. The presence of TFA in the mobile phase, even at very low concentration, can more efficiently suppress the undesirable achiral interaction between the acidic molecules and the silica matrix therefore enhancing the enantiomeric resolution of acidic compounds. As a consequence, 0.06% TFA was added to the mobile phases throughout the following experiments.

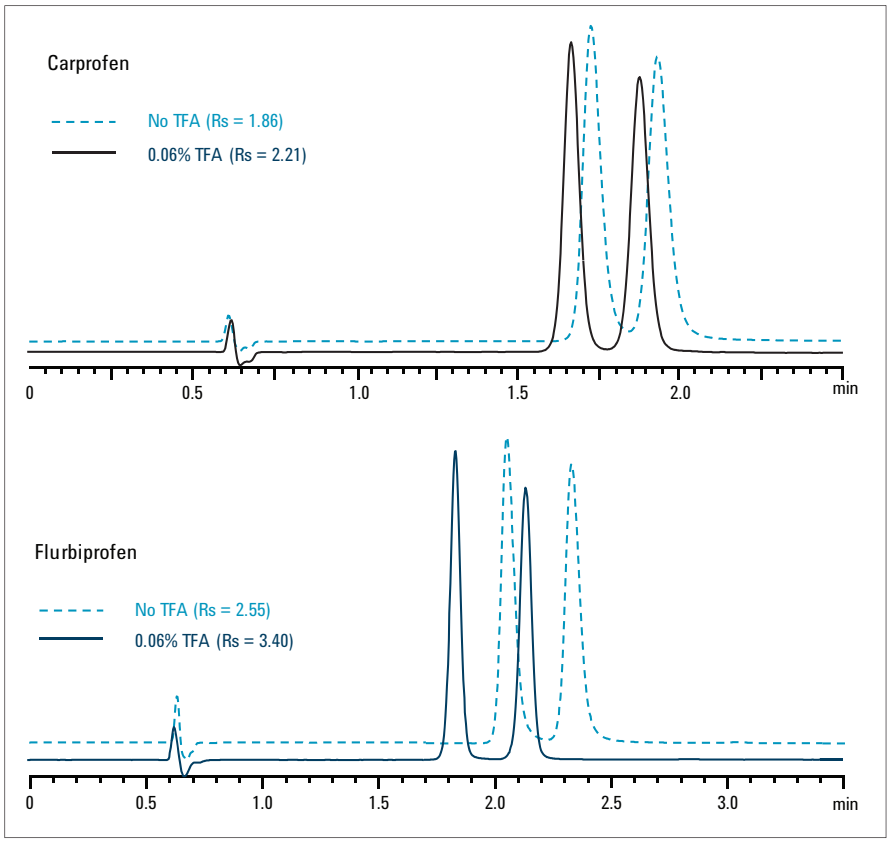

Figure 3
Improved enantiomer resolution of profens with 0.06% TFA.
Column: CHIRALPAK IA

Effect of modifier

It has been reported that the organic modifier may have an important effect in chiral SFC with packed columns of polysaccharide-derived CSPs[11].

This was also observed with the profen molecules. As summarized in Table 4, the most suitable modifier for the separation varied depending on the molecule structure. For instance, the highest resolution of Carprofen enantiomers was obtained with 2-PrOH (Figure 4a) while no separation was observed when ACN was used as modifier (Figure 4b). In the case of Flurbiprofen, MeOH resulted in the highest enantio-selectivity and the largest resolution. For the separation of Ketoprofen enantiomers, however, ACN delivered the best results. Considering these findings, the automated screening of modifiers is the most effective approach for SFC method development.

It should be noted that, in comparison with the alcoholic modifiers, ACN appeared to be a relatively poor modifier. It has considerably weaker eluting strength (needing higher percentage) and sometimes induces broader peak shape associated with compromised peak symmetry, even in the presence of TFA.

	CHIRALPAK	Modifier and its %		tr_1	tr_2	α	Rs
Carprofen	IA	MeOH	30	3.44	3.96	1.19	2.88
		EtOH	30	2.67	2.79	1.06	0.67
		2-PrOH	**30**	**3.53**	**5.23**	**1.59**	**7.38**
		ACN	50	3.16	3.16	1.00	0.00
Flurbiprofen	ID	**MeOH**	**5**	**2.06**	**2.75**	**1.50**	**4.33**
		EtOH	5	1.97	2.31	1.27	2.01
		2-PrOH	5	2.35	2.65	1.18	1.36
		ACN	20	2.21	2.88	1.45	3.66
Ketoprofen	ID	MeOH	10	2.08	2.25	1.12	1.44
		EtOH	10	2.16	2.26	1.07	0.74
		2-PrOH	10	2.98	2.98	1.00	0.00
		ACN	**30**	**2.10**	**2.68**	**1.42**	**2.73**

Table 4
Chromatographic results with various modifiers.

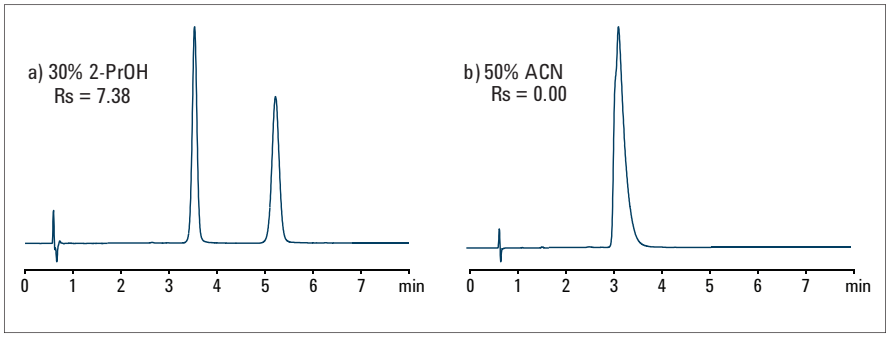

Figure 4
Separation of Carprofen enantiomers with different modifiers.
Column: CHIRALPAK IA

Effect of modifier percentage

In SFC, the eluting strength of the mobile phase increases with the concentration of the polar organic modifiers. As a general rule, the retention times decrease with the increase in percentage of a given modifier.

In SFC, the decrease in modifier percentage to a reasonable extent may be favorable for the enantiomer separation. The experimental results with variation of the modifier content in SF-CO_2 are presented in Table 5. It can be observed that the lower percentage of the modifier resulted in increased enantiomer resolution,. However, such variations did not significantly impact the enantio-selectivity. The separation examples in Figure 5 show that the incomplete separation of Ketoprofen enantiomers was transformed into full separation by simply reducing MeOH concentration from 10% to 5%. However, the gain in resolution in this case was at the expense of a significantly longer analysis time.

Conclusions

The complete enantiomer separations of the six racemic profen drugs are readily achieved by SFC using the columns packed with Daicel immobilized polysaccharide-derived CSPs. The best separations of the profen enantiomers are obtained alternately on CHIRALPAK IA, CHIRALPAK IC, and CHIRALPAK ID (Table 6) depending on the individual compound. CHIRALPAK IB certainly merits its involvement in the general chiral screening by offering some specific separations, although it does not deliver the best separation results with the current series of compounds.

Applying automated method development and solvent selection with the Agilent 1260 Infinity Analytical SFC System is fast, straightforward and highly successful for the selected profen drug examples.

	CHIRALPAK	Modifier and its %		tr_1	tr_2	α	Rs
Carprofen	IA	MeOH	40	1.67	1.88	1.20	2.21
			30	3.44	3.96	1.19	2.88
			20	**6.58**	**7.74**	**1.19**	**3.34**
Ibuprofen	IA	2-PrOH	5%	2.78	2.82	1.02	0.19
			2%	**9.49**	**10.15**	**1.08**	**0.80**
Indoprofen	IC	MeOH	30%	2.51	2.67	1.08	1.12
			20%	**6.22**	**6.70**	**1.09**	**1.61**
Ketoprofen	ID	MeOH	10%	2.08	2.25	1.12	1.44
			5%	**4.67**	**5.27**	**1.15**	**2.29**

Table 5
Chromatographic results of different modifier percentages.

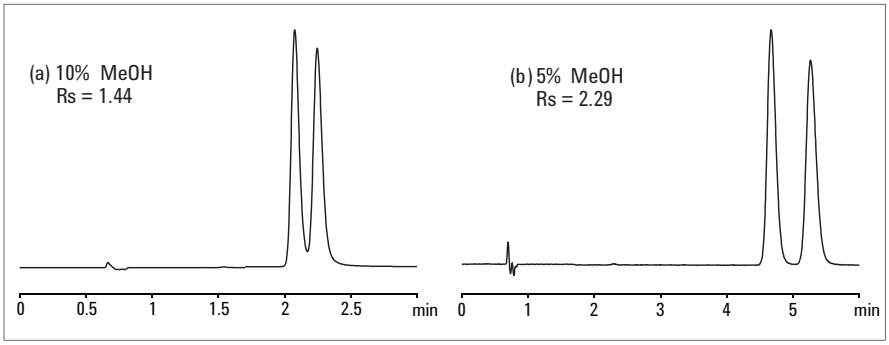

Figure 5
Separation of Ketoprofen enantiomers with different modifier percentage.
Column: CHIRALPAK ID

	CHIRALPAK	Modifier and its %		tr_1	tr_2	α	Rs
Carprofen	IA	2-PrOH	30	3.53	5.23	1.59	**7.38**
Fenoprofen	IC	ACN	20	1.89	2.12	1.19	**2.15**
Flurbiprofen	ID	MeOH	5	2.06	2.75	1.50	**4.33**
Ibuprofen	IC	ACN	15	2.05	2.33	1.21	**2.46**
Indoprofen	ID	MeOH	30	2.66	3.19	1.27	**2.95**
Ketoprofen	ID	MeOH	5	4.67	5.27	1.15	**2.29**

Table 6
Optimized SFC separations of the profen enantiomers.

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Agilent Technologies

Enantiomer separation of acidic compounds

Using Daicel CHIRALPAK QN-AX and QD-AX columns and the Agilent 1260 Infinity Analytical SFC System

Application Note

Pharmaceuticals

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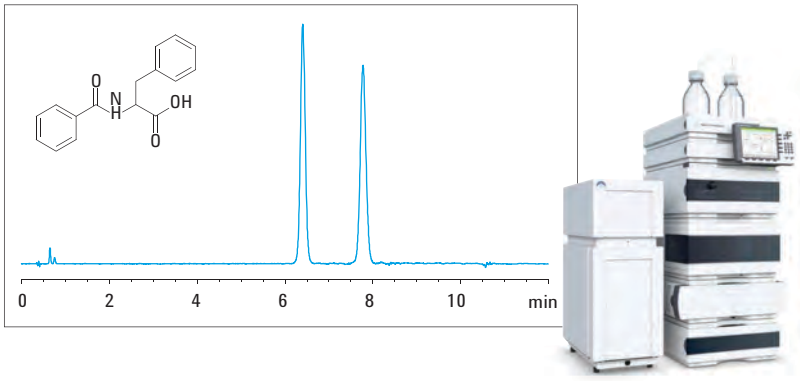

Abstract

CHIRALPAK QN-AX and QD-AX are anion exchanger chiral stationary phases providing specific enantioselectivity for acidic compounds. In this Application Note, we demonstrate that the application of these columns can be extended to super-critical fluid chromatography (SFC) for enantiomer separation of acidic compounds. Using the advantages of the advanced technology of the Agilent 1260 Infinity Analytical SFC system, we were able to assess the influence of a series of para-meters such as the mobile phase additives and the flow rate to gain insight into the general approaches for method development and optimization.

CHIRAL TECHNOLOGIES EUROPE
DAICEL GROUP

Agilent Technologies

Introduction

CHIRALPAK QN-AX and CHIRALPAK QD-AX (Figure 1) are anion exchanger chiral stationary phases providing specific enantioselectivity for acidic compounds. The chiral selectors are O-9-tert-butylcarbamate of Quinine (QN) and Quinidine (QD) respectively and immobilized on 5 µm spherical silica gel. The enantiomer recognition mechanism is based on the ionic exchange between the protonated tertiary nitrogen of the quinuclidine moiety of the chiral selector and the anionic analytes. Such an ion-pairing is accompanied by additional intermolecular interactions including hydrogen bonding, dipole-dipole, π-π, hydrophobic as well as steric interactions[1-2].

These chiral columns have been exhaustively investigated in HPLC with aqueous- and non-aqueous polar organic mobile phases and show remarkable performance in enantiomer resolution of a wide variety of acidic compounds[2-8]. The investigation of these columns for enantiomer separation by SFC is a current area of research[9].

This Application Note demonstrates that the application of these columns can be extended to supercritical fluid chromatography (SFC) for enantiomer separation of acidic compounds. Using the advantages of the advanced technology of the Agilent 1260 Infinity Analytical SFC system, we were able to assess the influence of a series of parameters such as the mobile phase additives and the flow rate to gain insight into the general approaches for method development and optimization on CHIRALPAK QN-AX and CHIRALPAK QD-AX by SFC.

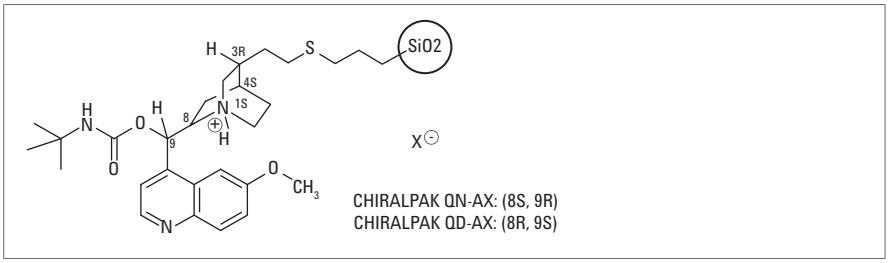

Figure 1
The chiral stationary phases.

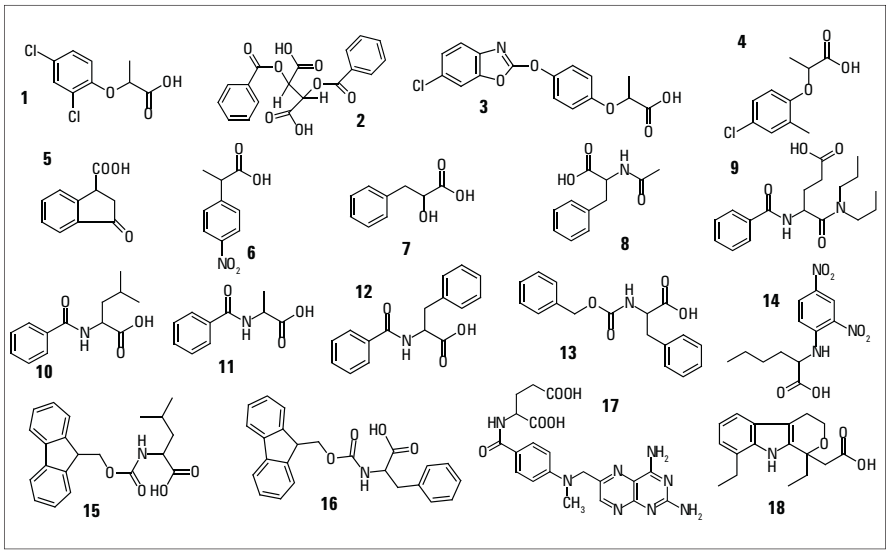

Figure 2
Structures of the acidic analytes.

Experimental

Chemicals

The chiral columns CHIRALPAK QN-AX and CHIRALPAK QD-AX used in this study are products of Daicel Group, Tokyo, Japan. They are manufactured at Chiral Technologies Europe, Strasbourg, France. Columns with dimensions of 4.6 × 100 mm and packed with 5 µm particles of the CSPs were used.

The CO_2 supply was from a cylinder of B50 containing the liquid carbon dioxide of industrial quality 4.8. Methanol (MeOH) of HPLC quality was used as the bulk modifier of the supercritical fluid carbon dioxide (SF-CO_2). Formic acid (FA) or acetic acid (HOAc) was employed as the acidic additive. They were matched respectively by ammonium formate (NH_4OOCH) and ammonium acetate (NH_4OAc) to balance the analyte retention times. The acidic compounds from Sigma-Aldrich (Figure 2) were dissolved in methanol for 5 µL injections.

Instrumentation

All SFC experiments were carried out on an Agilent 1260 Infinity Analytical SFC System (G4309A) consisting of the following modules:

- Aurora SFC Fusion A5 module for CO_2 pre- and post-conditioning
- Agilent 1260 Infinity SFC Binary Pump for accurate and constant metering of the mobile phase
- Agilent 1260 Infinity Degasser
- Agilent 1260 Infinity Autosampler
- Agilent 1260 Infinity Diode Array Detector with high pressure SFC flow cell

In addition, the Agilent SFC Method Development Kit was integrated into the system consisting of two Agilent 1290 Infinity Thermostatted Column Compartments with built-in valve drives and the Method Development Valve Kit.

Chromatographic conditions

Unless specifically indicated, the flow rate was conventionally set at 3.0 mL/min, the temperature of the column compartments at 40 °C and the back pressure of SF-CO_2 at 150 bar.

Results and discussion

In HPLC, methanol is proved to be a versatile mobile phase on CHIRALPAK QN-AX and CHIRALPAK QD-AX columns. Owing to its pronounced protic properties, methanol provides efficient solvation of all the ionized species involved in the ion-exchange equilibriums. Used as a single solvent in HPLC, the eluotropic strength of methanol can be adjusted by the concentration of the counter-ion (acidic additives) and by the ionic strength (acidic and salt additives).

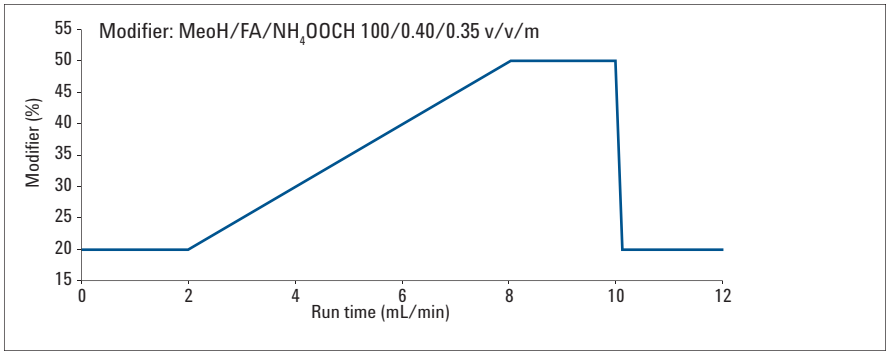

Figure 3
The gradient program.

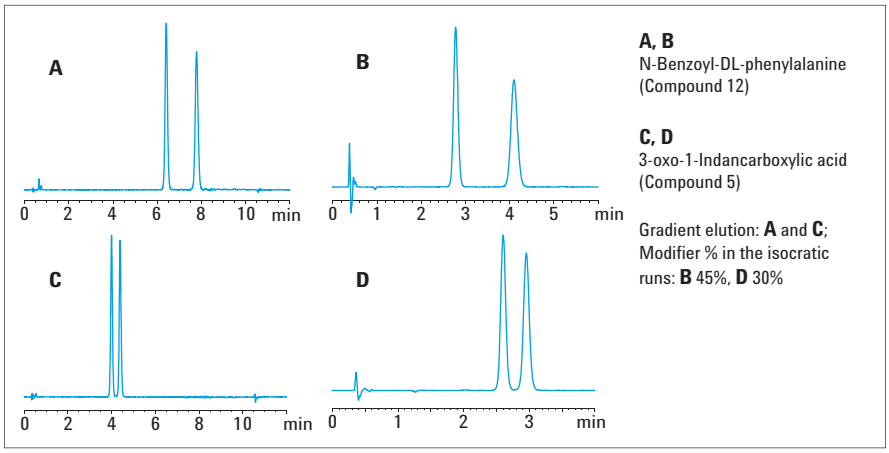

Figure 4
Straightforward method transfer from gradient to isocratic.

The same principles can be applied in SFC regarding the choice of the bulk modifier and the acid-salt additives. Throughout the current study, FA (up to 0.6% by volume) and NH_4OOCH (up to 0.50% by mass) were added into methanol and the resulted mixtures were used as the modifier of SF-CO_2.

Gradient to isocratic transfer

The first screening of the acidic compounds was carried out by running a gradient program (Figure 3) on the single column CHIRALPAK QN-AX using a mixture of MeOH/FA/NH_4OOCH 100/0.40/0.35 v/v/m as the modifier. The results from the gradient runs allowed targeting the modifier percentage to be used in isocratic mode (Table 1) so that the compounds were eluted in a reasonable time window. The chromatograms in Figure 4 demonstrate the direct transfer from the gradient to the isocratic method. The optimization steps, if needed, were then carried out in isocratic mode.

$^*t_{grad.}$ (min)	Modifier %
2–3	20
3–4	25
4–5	30
5–6	35
6–7	40
7–8	45
8–10	50

$^*t_{grad.} = (t_{r1}+t_{r2})/2$

Table 1
Determination of modifier % for isocratic run from the gradient results.

Effect of the acidic additive

In HPLC, the typical working conditions on CHIRALPAK QN-AX and CHIRALPAK QD-AX are with weakly acidic mobile phases (pH 5–7)[4]. Under such conditions, the chiral selector is protonated at the quinuclidine ring (Figure 1) and the acidic analytes are dissociated. An ionic exchange mechanism is thus activated between the positively charged chiral selector and the negatively charged analyte molecules.

It seems that the ionic exchange mechanism in SFC can be established owing to the mild acidity of SF-CO_2 itself. As shown in Table 2, the chiral recognition happened with no need of external addition of the acidic additive ([FA] =0%). The enantioselectivity was mostly unaffected by the absence or presence of the acidic additive and its concentration (Figure 5B). Except in the case of Compound 9 (proglumide), the addition of FA did not induce notable loss in resolution (Table 2, Figure 5B). Especially when exceeding the level of 0.20%, the presence of FA in the mobile phase provided the benefits of reducing the analysis times to a significant extent (Figure 5A). It can be presumed that FA played the role of the counter-ion, induced competitive effect and favored the mass transfer kinetics of the chromatographic process.

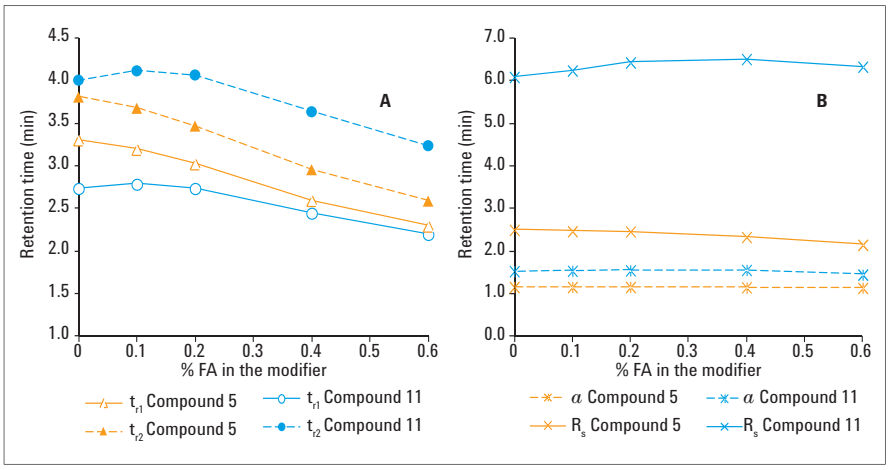

Figure 5
Dependence of chromatographic results on percentage of FA.

Compound	Modifier (%)	[FA]=0% t_{r1}	t_{r2}	α	Rs	[FA]=0.4% t_{r1}	t_{r2}	α	Rs
5	30	3.31	3.81	1.17	2.51	2.60	2.96	1.16	2.34
6	20	3.97	4.29	1.09	1.60	2.87	3.07	1.08	1.46
9	10	3.25	3.69	1.16	2.51	2.56	2.84	1.13	1.29
11	35	2.74	4.01	1.54	6.10	2.45	3.64	1.57	6.52
15	35	3.26	4.92	1.57	6.36	2.48	3.68	1.56	6.27
20	20	4.55	5.10	1.13	2.13	3.20	3.57	1.13	2.18
Average		**3.51**	**4.30**	**1.28**	**3.54**	**2.69**	**3.29**	**1.27**	**3.34**

Table 2
Chromatographic results with and without FA in the modifier.
Column: CHIRALPAK QN-AX, Modifier: MeOH/NH_4OOCH 100/0.35 v/m + FA.

Effect of the salt and its concentration

The ion-exchange chromatographic process is not only dependent on the ionization state of the chiral selector and the analyte molecules but also on the ionic strength of the mobile phase. Adding a certain amount of a suitable salt into the mobile phase can efficiently modulate the ionic strength of the mobile phase and regulate the absorption-desorption process between the analyte and the chiral selector. In practice, it is preferable to choose a salt of high solubility in the modifier or the mobile phase, high volatility for the potential LC/MS hyphenation and high UV transparency to ensure good UV detection of the analytes. NH_4OOCH meets all these requirements.

NH_4OOCH was added into the methanolic modifier at two different concentrations: 0.35% and 0.20%. As shown in Table 3, the enantioselectivity was unaffected by the concentration of NH_4OOCH. The higher concentration of NH_4OOCH led to reduced retention times at about 10% with minor decrease in resolution of the enantiomers. A concentration of 0.35% NH_4OOCH in MeOH seems to be a good compromise for generic method development.

Because of the unknown solubility of the salt in SF-CO_2, caution would be needed if significantly higher salt concentrations are to be used.

It is worth noting that the binary mixture of methanol/FA with no salt is not a stable system due to the rapid esterification reaction. It would lead to longer and longer sample retention over time for a given analysis. The presence of the salt NH_4OOCH in the modifier plays the role of the "stabilizer" of the system and is essential for reproducible chromatographic results.

Compound	Modifier (%)	[NH_4OOCH] = 0.35% t_{r1}	t_{r2}	α	Rs	[NH_4OOCH] = 0.20% t_{r1}	t_{r2}	α	Rs
3	40	2.70	2.85	1.07	0.76	3.30	3.50	1.07	0.91
4	35	2.33	2.58	1.12	1.74	2.71	3.00	1.13	1.85
5	30	2.60	2.96	1.16	2.34	2.85	3.25	1.16	2.39
6	20	2.87	3.07	1.08	1.46	3.03	3.25	1.08	1.48
9	10	2.56	2.84	1.13	1.29	2.59	2.88	1.13	1.34
11	35	2.45	3.64	1.57	6.52	2.80	4.21	1.58	6.72
13	35	3.54	3.90	1.11	1.57	4.04	4.47	1.12	1.65
15	35	2.48	3.68	1.56	6.27	2.69	4.02	1.57	6.42
18	20	3.20	3.57	1.13	2.18	3.33	3.72	1.13	2.19
Average		**2.75**	**3.23**	**1.22**	**2.68**	**3.04**	**3.59**	**1.22**	**2.77**

Table 3
Effect of the salt concentration.
Column: CHIRALPAK QN-AX; Modifier: MeOH/FA 100/0.40 v/v + NH_4OOCH.

Elution of strongly retained compounds

From the experimental data, the mixture of MeOH/FA/NH_4OOCH at the proportion of 100/0.40/0.35 v/v/m represents a good compromise in terms of elutropic strength and achievable resolution and can be used as a starting point for SFC method development. However, a few of the acidic compounds under investigation (such as Amethopterin (Compound 17), 2,3-Dibenzoyl-DL-tartaric acid (Compound 2) and N-DNP-DL-norleucine (Compound 14)) have strong retention on the columns and could not be (completely) eluted with such a methanolic modifier in the gradient as described above.

Compound	Modifier (%)	Flow rate (mL/min)	t_{r1}	t_{r2}	α	Rs
2	60	5	16.39	21.17	1.30	2.43
7	50	3	2.05	2.37	1.19	2.13
14	50	3	4.20	5.74	1.40	4.68
16	60	5	1.16	1.47	1.38	2.52
17	60	5	3.17	5.95	1.99	5.01

Table 4
Enantiomer separation employing strong eluting conditions.
Column: CHIRALPAK QN-AX; Modifier: MeOH/FA/NH_4OOCH 100/0.60/0.50 v/v/m.

In this scenario, efficient elution could be achieved by employing a high percentage of the modifier (50–60%) containing slightly higher concentrations of the acid and salt additives and, if necessary, increasing the flow rate. Some chromatographic results obtained under the enhanced eluting conditions are presented in Table 4.

Effect of flow rate

As previously mentioned, the enantiomer separation on CHIRALPAK QN-AX and CHIRALPAK QD-AX involves multiple specific interactions of high affinity, particularly the long range electrostatic interaction. These combined forces ensure the enantioselectivity for acidic compounds of various molecular structures. However, these effects may be too strong for the dissociation or desorption process even in the copresence of the counterion (the acidic additive) and the salt.

This will unavoidably result in slow mass transfer kinetics as confirmed by a large C-term in the Van Deemter plot (Figure 6A) and the drastic variation of the resultant resolution degree according to the flow rate (Figure 6B).

This disadvantage can actually be converted into effective means to improve the enantiomer separation by reducing the flow rate. As depicted in Figure 7, the just base-line resolution of Compound 1 (Dichlorprop) was significantly increased by reducing the flow rate from 5.0 mL/min to 1.0 mL/min.

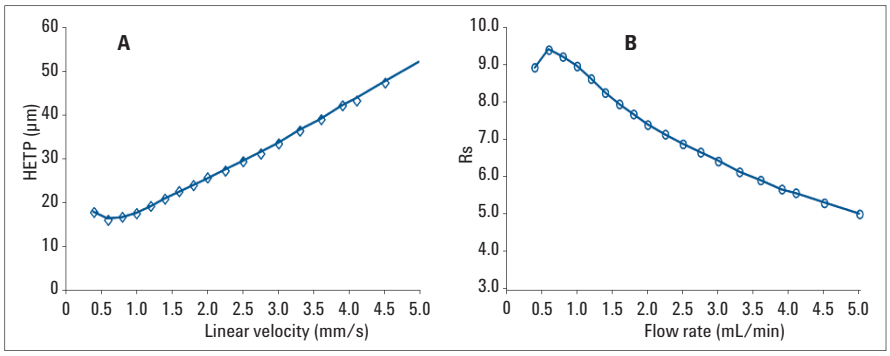

Figure 6
Dependence of peak efficiency and resolution degree on flow rate.
Compound 11 (N-benzoyl-DL-alanine; (A) peak-2
Column: CHIRALPAK QN-AX; Modifier: 35% (MeOH/FA/NH_4OOCH 100/0.40/0.35 v/v/m).

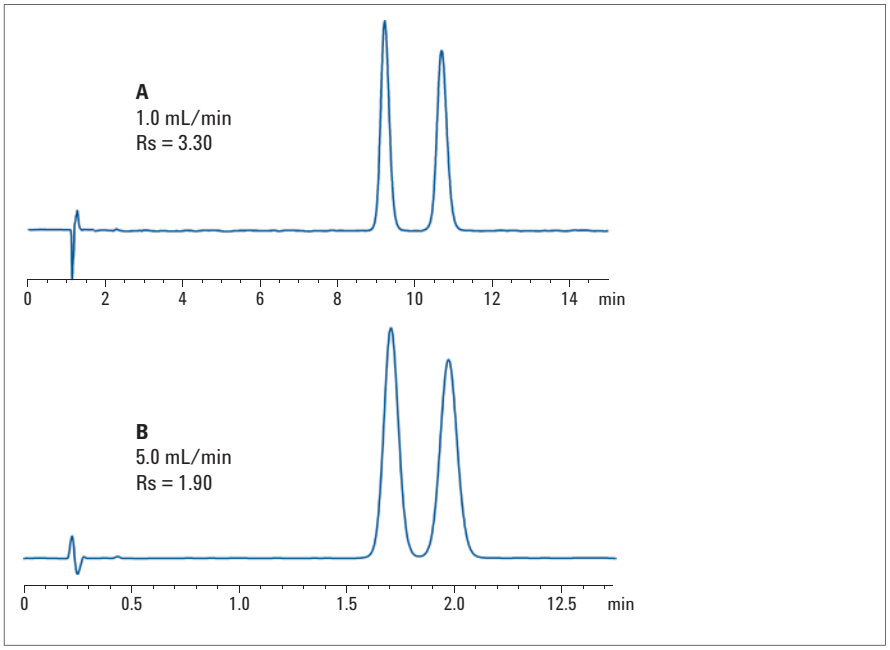

Figure 7
Enantiomer resolution of Dichlorprop (Compound 1) at different flow rates.
Column: CHIRALPAK QN-AX; Modifier: 45% (MeOH/FA/NH_4OOCH 100/0.40/0.35 v/v/m).

Elution order and complementarity

As shown in Figure 1, QN and QD are diastereomers. Chromatographically, the two CSPs behave as "pseudo-enantiomers" due to the fact that the stereoselectivity is under the control of C8 and C9, which have the opposite configurations[1,6]. Consequently, the elution order (EO) of the enantiomers is reversed on these two columns. In SFC, such a phenomenon was monitored by injecting one of the pure enantiomers, which were available in our laboratory (Table 5). Coincidently, all the four D-enantiomers examined were first eluted on CHIRALPAK QN-AX and secondly eluted on CHIRALPAK QD-AX. The feasibility to choose the column in terms of EO is undoubtedly of value especially for applications in trace analysis of a given enantiomer.

As indicated by the averaged values in Table 5, CHIRALPAK QN-AX and CHIRALPAK QD-AX afforded the same enantioselectivity, but with a stronger retentivity and a slightly improved resolution degree on CHIRALPAK QD-AX. If the individual cases are examined, a complementarity in enantiomer resolution between the two columns can be observed. As exemplified in Figure 8 A-B, CHIRALPAK QN-AX offered the full separation of enantiomers for 3-oxo-1-indancarboxylic acid (Compound 5) whilst a compromised resolution was found on CHIRALPAK QD-AX for the same compound. The trend is inversed with the Fenoxaprop enantiomers (Compound 3) as shown in Figure 8 C-D. This type of complementarity is highly desirable for compounds being difficult to be resolved into enantiomers.

Compound	Modifier (%)	CHIRALPAK QN-AX					CHIRALPAK QD-AX				
		t_{r1}	t_{r2}	α	Rs	EO	t_{r1}	t_{r2}	α	Rs	EO
1	50	2.65	3.05	1.18	2.24		2.66	3.29	1.27	3.35	
3	40	2.70	2.85	1.07	0.76		2.74	3.18	1.18	2.27	
4	35	2.33	2.58	1.12	1.74		2.50	2.81	1.14	2.03	
5	30	2.60	2.96	1.16	2.34		2.72	2.87	1.07	1.02	
8	30	2.64	3.40	1.34	4.30	D/L	2.57	3.46	1.40	5.01	L/D
9	10	2.56	2.84	1.13	1.29		3.05	3.05	1.00	0.00	
10	30	2.24	4.19	2.04	10.21		2.13	3.73	1.90	9.38	
13	35	3.54	3.90	1.11	1.57	D/L	3.71	4.35	1.19	2.68	L/D
15	35	2.48	3.68	1.56	6.27	D/L	2.56	3.61	1.48	5.72	L/D
16	50	3.37	4.35	1.33	3.64	D/L	3.59	4.75	1.36	4.10	L/D
18	20	3.20	3.57	1.13	2.18		3.70	4.36	1.20	3.31	
Average		**2.76**	**3.40**	**1.29**	**3.32**		**2.90**	**3.59**	**1.29**	**3.53**	

Table 5
Comparison between CHIRALPAK QN-AX and CHIRALPAK QD-AX.
Modifier: MeOH/FA/NH_4OOCH (100/0.40/0.35 v/v/m).

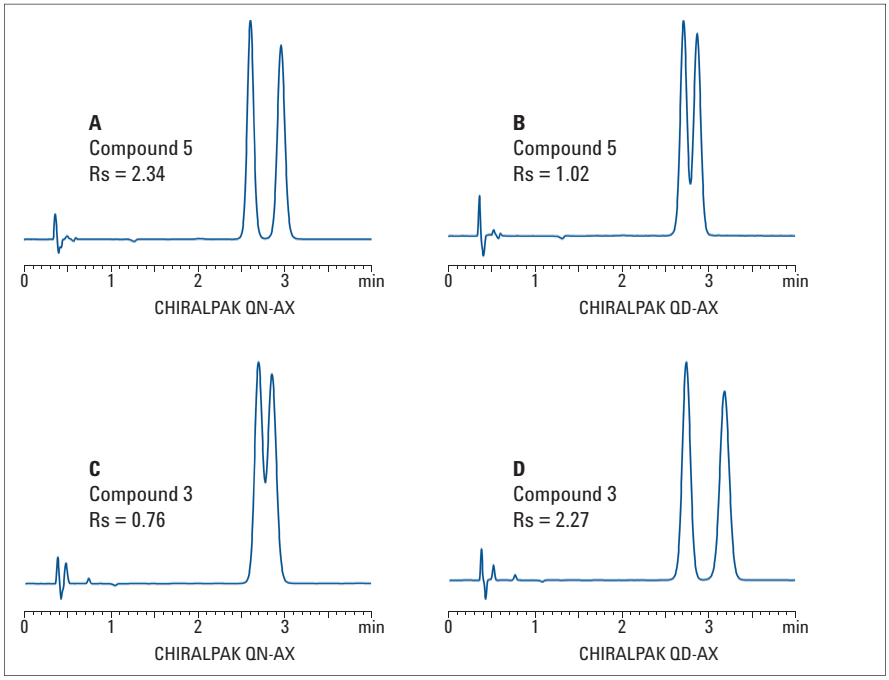

Figure 8
Examples of enantiomer separation on CHIRALPAK QN-AX and CHIRALPAK QD-AX.
Modifier: MeOH/FA/NH_4OOCH 100/0.40/0.35 v/v/m, 30% for Compound 5; 40% for Compound 3.

Conclusions

CHIRALPAK QN-AX and CHIRALPAK QD-AX are versatile in enantiomer resolution of acidic compounds by SFC using methanolic modifiers. The major factors influencing the chiral separation include the acidic additive, the salt concentration and the flow rate. The mixture of MeOH/FA/NH_4OOCH 100/0.40/0.35 v/v/m is a suitable modifier for the first trials of method development.

Some other parameters such as the temperature, the variation of the modifier percentage and different pair of acid-salt additives (for example, HOAc-NH_4OAc) were investigated as well. It was observed that the enantiomer separations by SFC are mostly unaffected by temperatures in the range between 20 °C and 40 °C. Lower modifier percentage usually lead to longer retention times but has no major effect on enantioselectivity and resolution degree. The additive pair of FA-NH_4OOCH would be the preferred choice over the pair of HOAc-NH_4OAc in terms of efficiency of the enantiomer resolution.

The effect of other polar organic modifiers (such as ethanol, 2-propanol and acetonitrile) instead of methanol was not investigated because of their poor solubility to the salt additives and the resultant potential inconvenience in the balance of eluotropic strength of the mobile phase.

Most of the enantiomer separations on the CHIRALPAK QN-AX and CHIRALPAK QD-AX columns can be obtained within 5 minutes. The advanced technology of the Agilent 1260 Infinity Analytical SFC System makes the method development easy, fast and reliable.

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Agilent Technologies

Analysis of phosphate compounds with the Agilent 1260 Infinity Bio-inert Quaternary LC System

Application Note

Biopharmaceuticals

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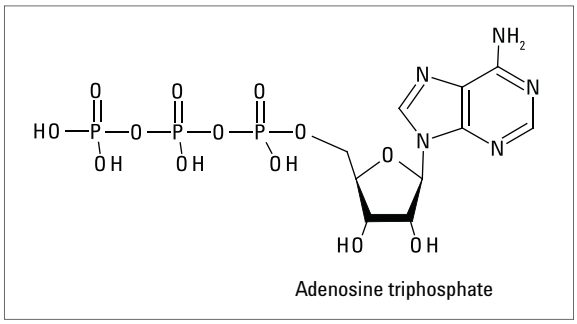

Adenosine triphosphate

Abstract

This Application Note shows that unwanted retention and peak tailing of adenosine triphosphate can be completely prevented when using the Agilent 1260 Infinity Bio-inert Quaternary LC System. Due to the iron and steel-free design of the Agilent 1260 Infinity Bio-inert Quaternary LC System, phosphate compounds can be analyzed without any issues regarding the formation of phosphate-iron complexes found with stainless steel systems. Even in the presence of high organic mobile phases, good peak shapes were observed without peak tailing or area reduction.

Agilent Technologies

Introduction

Severe peak tailing of phosphate compounds is a well described issue in HPLC analysis[1, 2]. It has been reported for a variety of adenosine and guanidine mono-, di- and triphosphate nucleotides and other phosphate compounds in different HPLC separation techniques[3, 4]. Interaction between stainless steel and phosphate groups were described by Liu *et al.* leading to the formation of phosphopeptide-Fe(III) complexes[5]. With the use of PEEK tubing instead of stainless steel tubing, the peak tailing can be reduced[1].

PEEK has a limited backpressure tolerance, therefore, UHPLC is not possible with PEEK-only capillaries. In addition, standard systems also have stainless steel injector parts and detector flow cells, making them not fully bio-inert. The 1260 Infinity Bio-inert Quaternary LC System has capillaries that consist of PEEK inside and stainless steel outside, enabling UHPLC with a metal-free sample flow path[6]. With this system, a user can analyze a variety of phosphate compounds without the emersion of peak tailing or other unwanted sample retention in the system due to phosphate-iron complexes.

The analysis of adenosine triphosphate (ATP) has been employed as a measure of microbial biomass[7], to determine the energy status in plant tissues[8] and in various other metabolic analytical experiments.

Experimental

The Agilent 1260 Infinity Quaternary LC System used for the experiments consisted of the following modules:

- Agilent 1260 Infinity Quaternary Pump (G1311B)
- Agilent 1260 Infinity Standard Autosampler (G1329B)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector SL (G1315C), equipped with standard flow cell, 10 mm

The Agilent 1260 Infinity Bio-inert Quaternary LC System used for the experiments consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High Performance Bio-inert Autosampler (G5667A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector VL (G1315D), equipped with bio-inert standard flow cell, 10 mm

A PEEK restriction capillary was used instead of a stainless steel column.

Software
Agilent OpenLAB CDS, ChemStation Edition for LC & LC MS Systems, Rev. C.01.02 [14]

Solvents and Samples

Buffer A: 10 mM ammonium acetate

Buffer B: 10 mM ammonium acetate + 10% methanol

Buffer C: 10 mM ammonium acetate + 50% methanol

Buffer D: 10 mM ammonium acetate + 70% methanol

Buffer E: 10 mM ammonium acetate + 90% methanol

Sample
Adenosine triphosphate (ATP), solved in H_2O_{dd} (5 mg/mL)

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). Ammonium acetate and ATP were purchased from Sigma-Aldrich, St.Louis, USA.

Chromatographic conditions

Flow rate:	0.5 mL/min
Isocratic run with buffer A, B, C, D or E	
Stop time:	5 minutes
Injection volume:	0.2 µL
Temperature TCC:	40 °C
Diode array detector:	254 nm, Reference 360 nm
Peak width:	> 0.1 minutes (2.5 Hz)

Results and discussion

Significant peak tailing could be observed for ATP analysis in a stainless steel based system, the 1260 Infinity Quaternary LC System. With increasing amount of organic mobile phase, the retention of the phosphate compound was increasing to a huge extent, also resulting in relevant area reduction. With the use of 90% MeOH in the mobile phase, complete retention of the ATP was observed, see Figures 1, 3, and 4.

With the 1260 Infinity Bio-inert Quaternary LC System, the unwanted retention of the used phosphate sample could be completely prevented, resulting in good peak shapes without substantial peak tailing or area reduction, see Figures 2, 3, and 4.

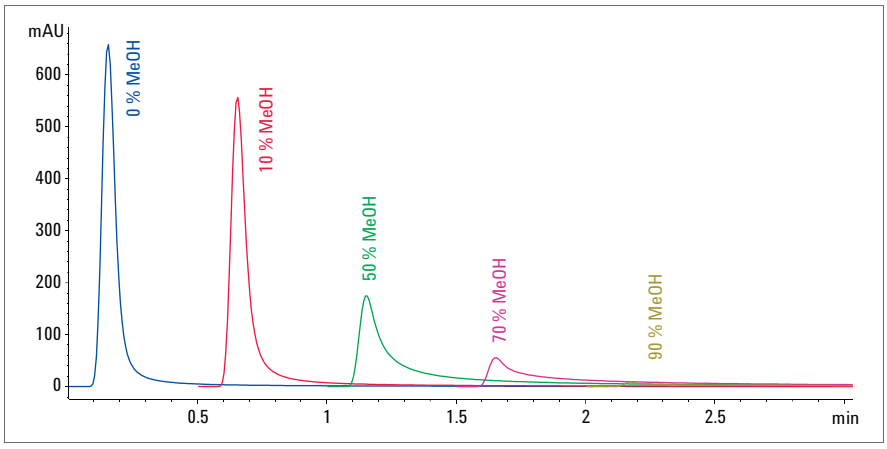

Figure 1
ATP analysis on the Agilent 1260 Infinity Quaternary System (stainless steel system).

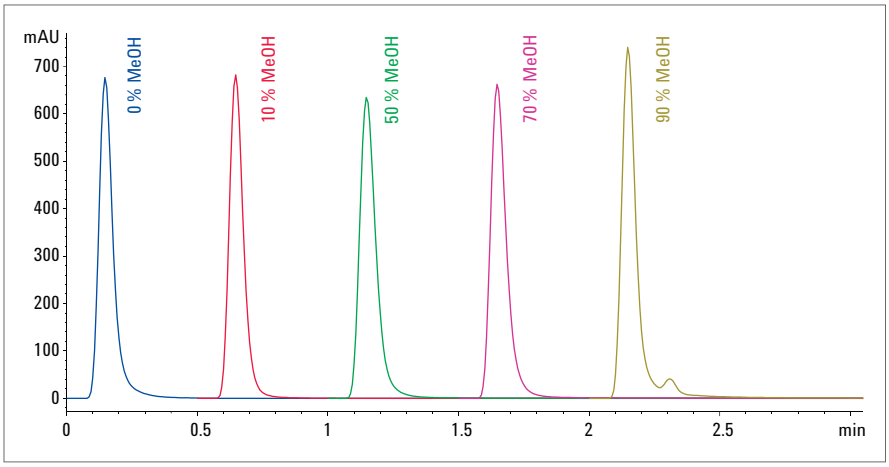

Figure 2
ATP analysis on the Agilent 1260 Infinity Bio-inert Quaternary System.

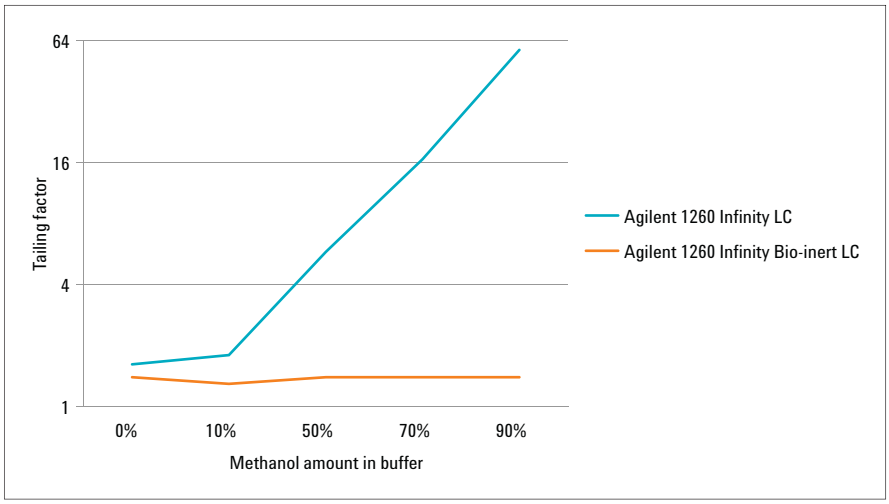

Figure 3
Tailing factors of ATP relating to the MeOH amount in the used buffers.

Conclusions

Unwanted retention and peak tailing of adenosine triphosphate could be completely prevented when using the Agilent 1260 Infinity Bio-inert Quaternary LC System. In contrast to the use of a stainless steel system, good peak shapes were observed. Peaks without substantial peak tailing or area reduction due to extensive sample retention in the flow path were found even with increasing organic content in the used mobile phases.

Due to the iron and steel-free design of the 1260 Infinity Bio-inert Quaternary LC system, phosphate compounds can be analyzed without any issues regarding the formation of phosphate-iron complexes as found with stainless steel systems.

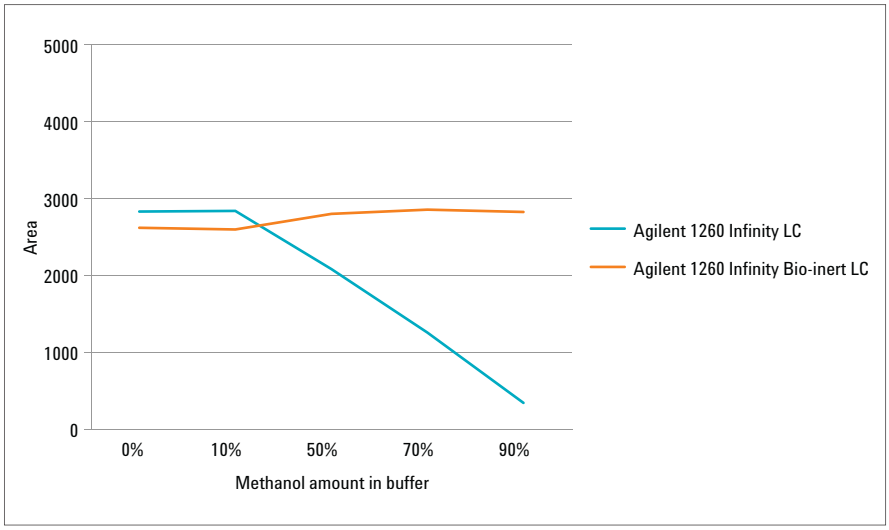

Figure 4
Area of ATP relating to the MeOH amount in the used buffers.

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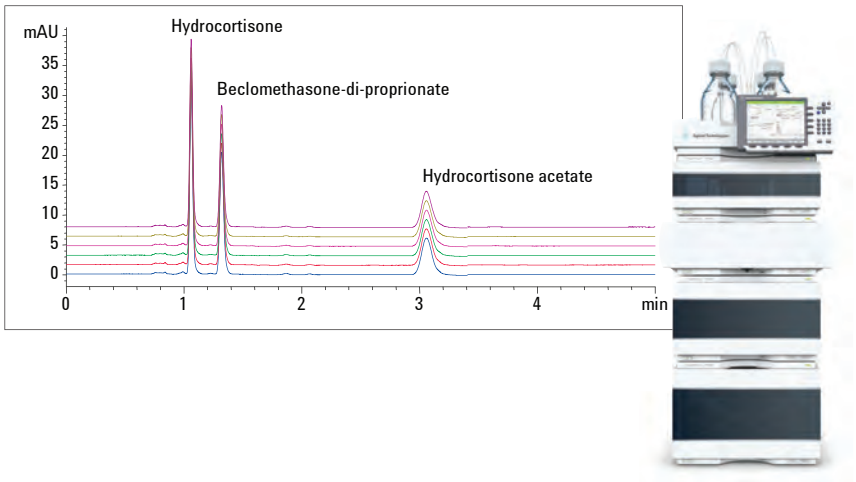
Agilent Technologies

Agilent 1290 Infinity Quaternary Pump: Comparing premixed isocratic conditions with dynamically-mixed conditions

Analysis of glucocorticoid drugs

Technical Overview



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Abstract

The most important performance parameter for an ultrahigh performance liquid chromatography (UHPLC) pump is precision of retention times. Highest precision is required to enable identification of compounds by retention times and subsequently to be able to accurately quantify the analyzed sample compounds. To achieve highest precision of retention times, many applications are performed using isocratic conditions by premixing the mobile phase and then pumping the mixture through a single pump channel. This Technical Overview demonstrates that the Agilent 1290 Infinity Quaternary Pump eliminates the need to premix the mobile phase.

Agilent Technologies

Introduction

Recently in the pharmaceutical industry, quality control analyses of drug preparations are frequently done using isocratic UHPLC conditions. To ensure highest precision for the retention times, premixed phases are often preferred. To ensure that the premixed phases always have the same compositions, the mixing procedures have to be very reproducible. Slight composition changes could occur between preparations and also between different users. For compounds that react to even small composition changes, non-reproducible mixing will result in lower precision of retention times.

This Technical Overview demonstrates that the Agilent 1290 Infinity Quaternary Pump is able to provide highest precision for retention times using dynamically-mixed mobile phases over prolonged periods, and that the retention time precision is comparable to results obtained from different charges of premixed mobile phases. Three glucocorticoids were chosen as analytes, which show significant retention time shifts even for slight composition changes, see Figure 1.

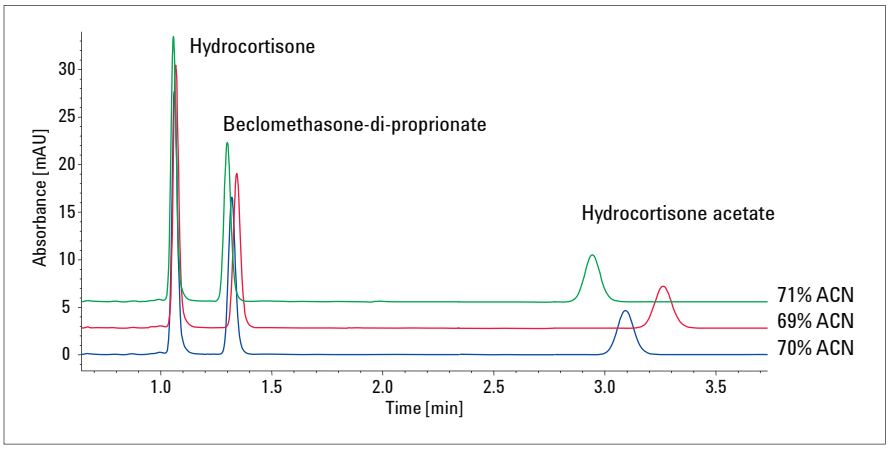


Figure 1
Influence of acetonitrile percentage on elution.

Experimental

Instrumentation and software

The Agilent 1290 Infinity LC used for the experiments consisted of the following modules:

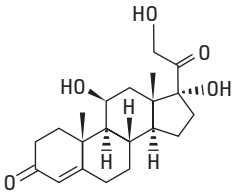
- Agilent 1290 Infinity Quaternary Pump (G4204A)
- Agilent 1290 Infinity Autosampler with Thermostat (G4226A, G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector (G4212A)

Software

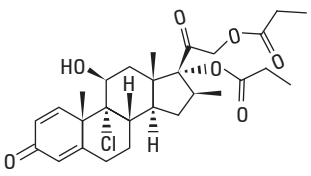
Agilent ChemStation revision C.01.03. All Agilent LC modules had RC.Net drivers with appropriate firmware revision.

Compounds analyzed

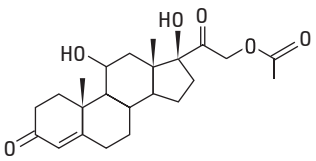
Hydrocortisone



Beclomethasone-di-proprionate



Hydrocortisone acetate



Chromatographic conditions

Column:	Agilent ZORBAX Eclipse Plus C18, 100 × 4.6 mm, 5 µm
Mobile phase:	Water:Acetonitrile / 30:70
Flow rate:	1 mL/min
Stop time:	5 min
Injection volume:	3 µL with needle wash for 6 s
Column temperature:	30 °C
Detection:	254/10 nm, Ref 400/80, 20 Hz, slit 4 nm

Results and discussion

All experiments were performed using an 1290 Infinity LC equipped with the 1290 Infinity Quaternary Pump. For all experiments using premixed mobile phases, the C channel of the pump was deployed; for all experiments with dynamically-mixed mobile phases, channels A and B were used. Figure 2 shows six example chromatograms based on dynamically-mixed phases.

The following experiments were performed and the relative standard deviation (RSD) of retention times (RT) was evaluated:

1. RSD of RT for six consecutive runs using a dynamically-mixed mobile phase
2. RSD of RT for six consecutive runs using a premixed mobile phase
3. RSD of RT of the 10th run of six sequences using dynamically-mixed mobile phases over several days
4. RSD of RT of the 10th run of six sequences using six premixed mobile phases prepared by one user
5. RSD of RT for three runs using three premixed mobile phase prepared by three different users

Figure 3 shows the results for experiments 1 and 2. The precision of the retention times for the experiments with premixed mobile phases is typically slightly better than the precision for experiments with dynamically-mixed mobile phases, as expected. Nevertheless, the precision of the retention times using premixed phases is only approximately two times better, in worst case, but still very good overall.

In experiments 3 and 4, six sequences were applied each containing 10 runs with dynamically-mixed mobile phases and 10 runs with premixed mobile phases. For each sequence, the premixed phase was freshly prepared by one user. The mobile phases for the dynamic mixing were just refilled. The 10th run from each sequence was taken and the RSD of the six retention times were evaluated for the dynamically and the premixed experiments. In Figure 4, the chromatograms for the six sequences (dynamically and premixed) are overlaid. The precision over several sequences using dynamically-mixed mobile phases was significantly better than the precision for data obtained

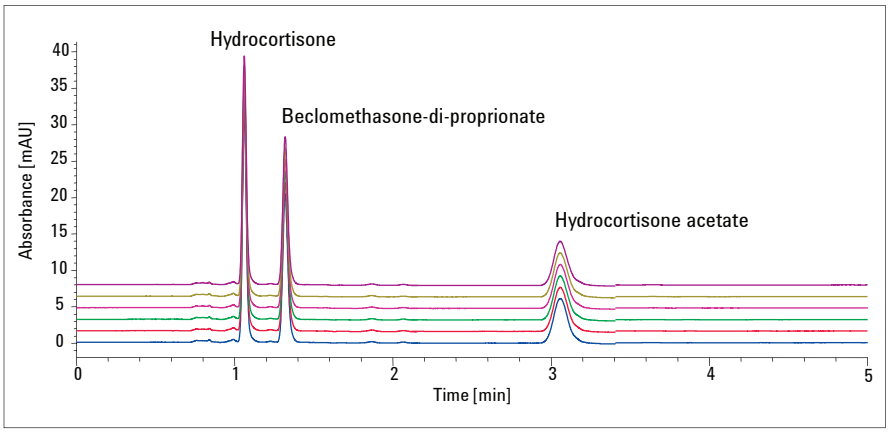


Figure 2
Analysis of glucocorticoids using a dynamically-mixed mobile phases.

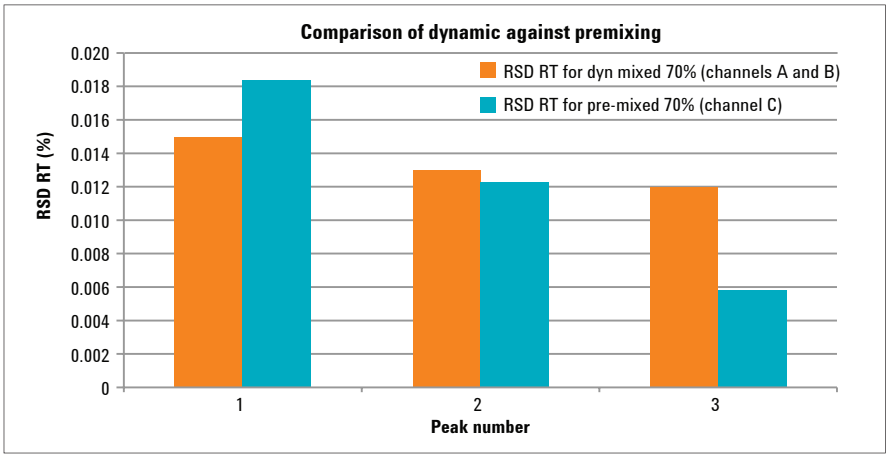


Figure 3
RSD of retention times using dynamically and premixed mobile phases.

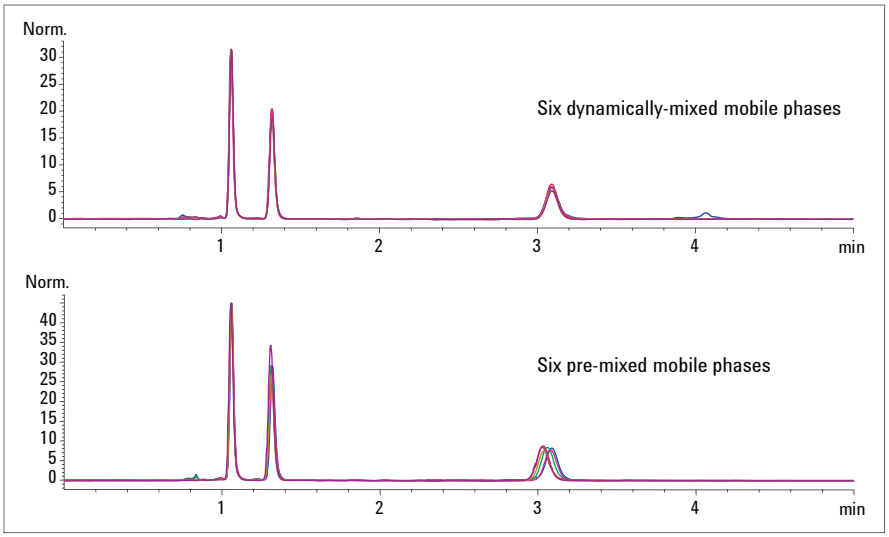


Figure 4
Comparison of chromatograms from six sequences using dynamically and six premixed mobile phases.

using premixed mobile phases, prepared by the same user. The relative standard deviation over six sequences with dynamically-mixed mobile phases was for the last peak 0.16% RSD. The relative standard deviation over six sequences with freshly prepared premixed mobile phases for the last peak was 0.83% RSD. As expected, dynamically mixed mobile phases showed better precision over several sequences even when performed on different days.

Similar RSD values were obtained when different users prepared the premixed phases, see Figure 5.

The relative standard deviation for the three runs is 0.982% RSD. The results from experiments 3 to 5 are combined in Figure 6.

Conclusions

Within a sequence, a premixed phase out of one channel provided slightly better precision than a dynamically-mixed mobile phase out of two channels. In contrast, comparing several sequences over several days, the dynamically-mixed mobile phase provided better precision. This was mainly due to the individual error that occurred when one user prepared fresh mobile phase every day. The day-to-day precision of retention times for dynamically-mixed phase was approximately 0.16% RSD. The day-to-day precision for retention times using premixed mobile phases was approximately 0.89% RSD. Similar results were obtained when different users prepared the premixed mobile phases. In conclusion, using the Agilent Infinity 1290 Quaternary Pump eliminates the need to premix mobile phase. Day-to-day reproducibility of retention times is typically better than achieved with premixed mobile phases.

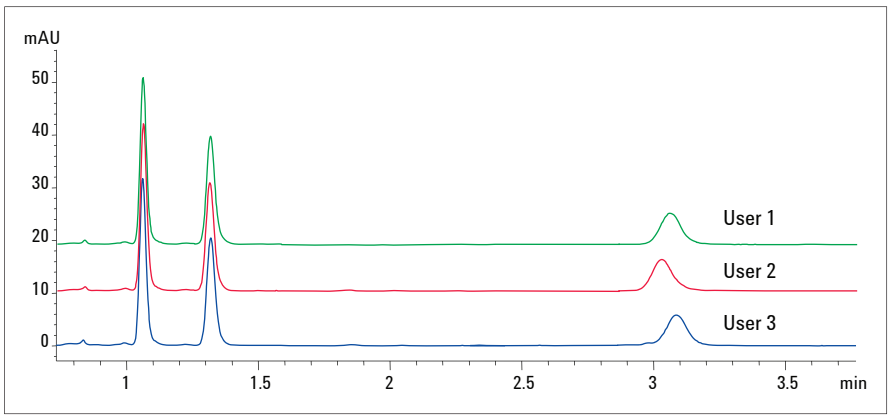


Figure 5
Comparison of chromatograms obtained by premixed phases prepared by three different users.

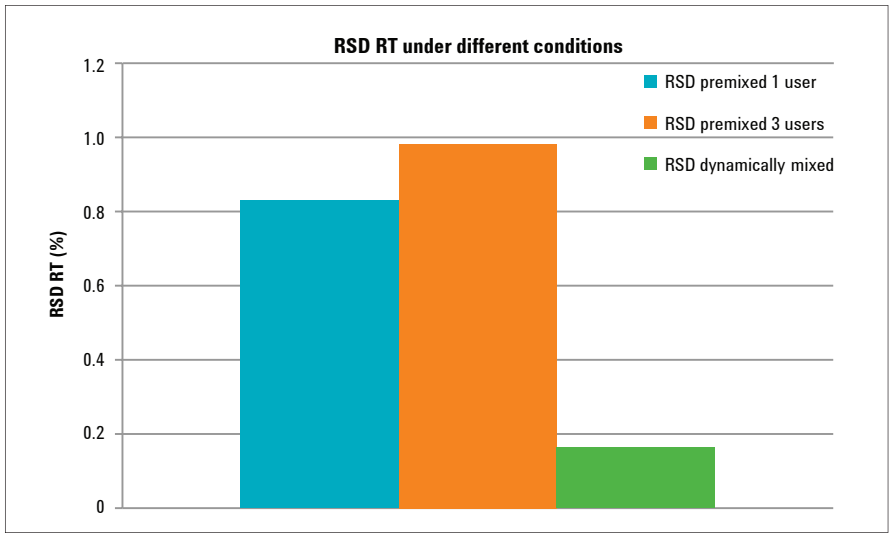


Figure 6
Precision of retention times for differently mixed mobile phases.

www.agilent.com/chem/1290QuatPump

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Agilent Technologies

Single-run assay and impurity testing of fixed-dose combination drugs using the Agilent 1200 Infinity Series High Dynamic Range Diode Array Detector Solution

Technical Overview

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Abstract

The Agilent 1200 Infinity Series High Dynamic Range Diode Array Detection Solution provides a 30× wider linear dynamic UV-range. By combining the signals from two diode array detectors with different path length Max-Light flow cells, the high dynamic range DAD solution detects and quantifies main and trace compounds in a single run without exceeding the linear UV-range.

Using a conventional DAD, typically two injections with different injection volumes are needed to determine the low dose and high dose as well as the trace compounds. At the high injection volumes that are required to determine the low dose and trace compounds, the high dose compounds exceed the linear range of a conventional detector.

This Technical Overview demonstrates that one single injection is adequate to reliably quantify low dose, high dose, and trace compounds of a fixed-dose combination drug using the high dynamic range detection solution from Agilent. The results are compared to those from a conventional DAD. In addition, the precision of areas and limit of detection for both detection solutions are evaluated and compared.

Agilent Technologies

Introduction

Fixed-dose combination drugs are used in the medication of various disease patterns. The composition percentage of the active ingredients can vary depending on the desired physiological effect. If high dose and low dose ingredients are combined, the analysis using conventional HPLC and UHPLC diode array detectors may need at least two injections. This ensures that all compounds are quantified within the linear range of the detector with reliable integration and quantification of trace compounds.

This Technical Overview shows that with the Agilent 1290 Infinity Series High Dynamic Range (HDR) DAD Solution, high dose and low dose compounds as well as their impurities can be determined in a single run with high sensitivity and excellent area precision without exceeding the linear UV-range.

Experimental

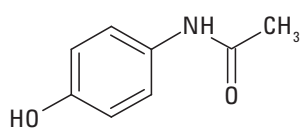
Instrumentation

An Agilent 1290 Infinity LC System consisting of the following modules was used;

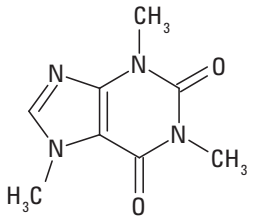
- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Autosampler Thermostat (G1330B)
- Agilent 1290 Infinity Column Compartment (G1316C)
- Two Agilent 1290 Infinity Diode Array Detectors (G4212A)
- Detector cells: 60-mm path length cell (G4212-60007)
  3.7-mm path length cell (G4212-60032)
- Agilent Dide Array 1200 Series Diode Array Detector (G1315C) with 10-mm path length cell

Compounds

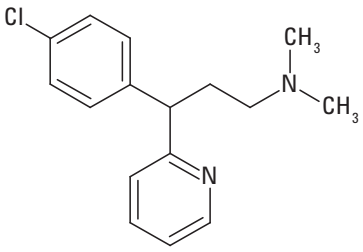
A fixed-dose combination drug was used as sample with the following ingredients: Paracetamol and chlorphenamine with a ratio 1:80, other compounds were vitamin C and caffeine and further small unknown impurities.



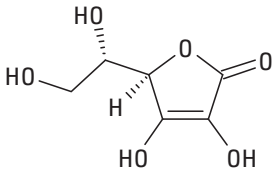
Paracetamol



Caffeine



Chlorphenamine



Vitamin C

Sample preparation

1. Two capsules of a cold medication were opened and dissolved in 20 mL distilled water
2. Extraction with ultrasonic bath for 5 minutes
3. Filtering with Satorious Minisart 0.8-µm filter
4. Clear liquid was filled and stored in 1.5-mL LC vials containing:
   - 250 ng/µL chlorphenamine
   - 20,000 ng/µL paracetamol
   - 2,500 ng/µL caffeine
   - 15,000 ng/µL vitamin C
5. Dilution 1: 20 with water containing:
   - 12.5 ng/µL chlorphenamine
   - 1000 ng/µL paracetamol
   - 125 ng/µL caffeine
   - 750 ng/µL vitamin C
6. This solution was injected

Chromatographic conditions

Column:	Agilent ZORBAX Eclipse Plus C18, 4.6 × 100 mm, 5 µm (p/n 959996-902)
Mobile phase:	Water+ 0.1% TFA/Acetonitrile + 0.09% TFA = 95/5
Gradient:	5% B at 0 minutes, 5% B at 0.5 minutes, 40% B at 6.1 minutes, 95% B at 6.5 minutes, 5% B at 8 minutes
Stop time:	10 minutes
Post time:	2 minutes
Injection volume:	1 µL and 5 µL for conventional DAD 5 µL for high dynamic range DAD solution
Column temperature:	40 °C
Detection:	254/20 nm, Ref 380/80, 10 Hz

Software and firmware

Agilent OpenLAB CDS ChemStation revision B.04.03 SP1, C.01.03 and C.01.04 can be used.

All Agilent LC modules had RC.Net drivers with appropriate firmware revision.

Principle of the Agilent 1200 Infinity Series High Dynamic Range DAD Solution

The Agilent 1200 Infinity Series High Dynamic Range DAD Solution provides a 30× wider linear dynamic UV-range. By combining the signals from two diode array detectors with different path length Max-Light flow cells, the high dynamic range detection solution detects and quantifies components with significantly different concentrations in a single run. Two Agilent 1260 or 1290 Infinity DADs are clustered, (Figure 1). One DAD is equipped with a 60-mm path length cell to analyze highly concentrated compounds, and the second DAD is equipped with a 3.7-mm path length cell to analyze low concentrated compounds. The 60-mm cell must be the first in the flow path followed by the 3.7-mm cell. The output signal is a combined signal, normalized to 10-mm path length. The linear UV-range of the 1200 Infinity Series High Dynamic Range DAD Solution is typically as wide as 0.6×10^{-6} to 6.7 AU/cm. In comparison, the earlier Agilent 1200 Series DAD has a maximum linear range of 7×10^{-6} to 2 AU/cm.

The high dynamic range (HDR) tool can be activated through the ChemStation by selecting the appropriate field, (Figure 2). For adjustment of the retention time shift between the detector cells, the delay volume must be entered. This is the volume of the connection capillary between the 60-mm and 3.7-mm path length cells. A capillary with a volume of 0.0125 µL is supplied with the 3.7-mm path length cell.

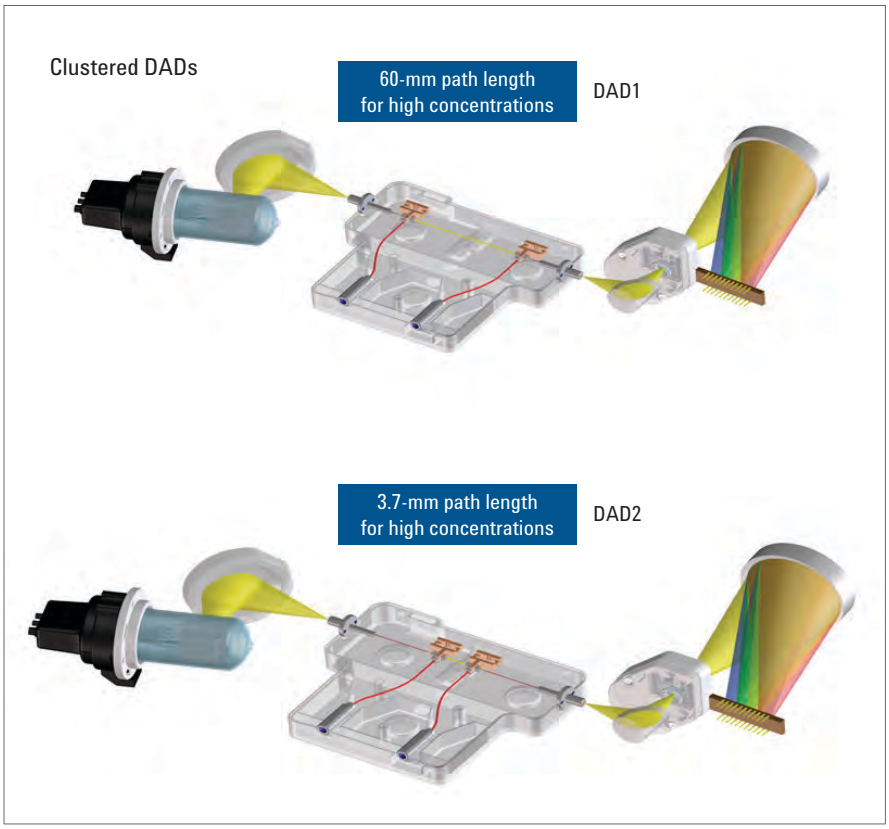


Figure 1
Clustered 1200 Infinity Series DADs.

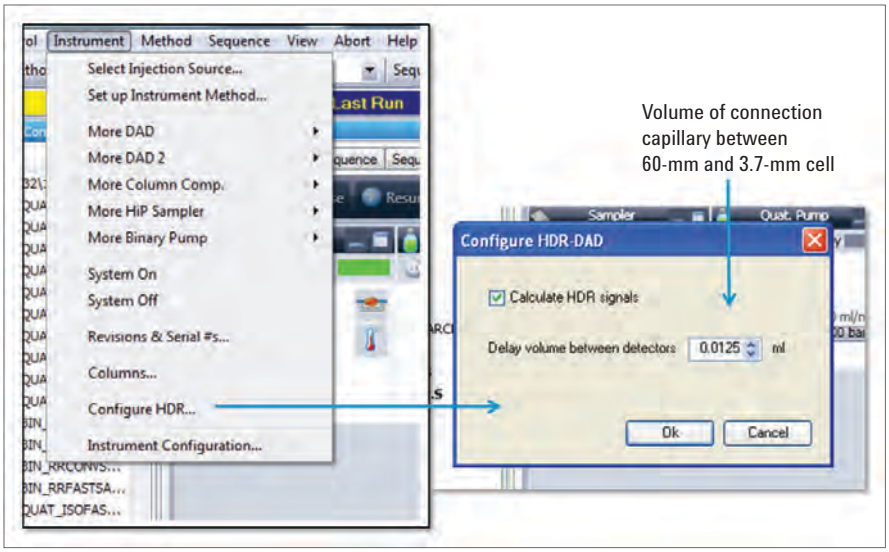


Figure 2
Activating the HDR tool in the ChemStation software.

Results and discussion

For all experiments the same Agilent 1290 Infinity LC System configuration was used with the exception of the detectors. To evaluate the performance differences between the conventional DAD and the Agilent high dynamic range solution, the following experiments were performed:

- Analysis of a fixed-dose combination drug on a 1290 Infinity LC System with conventional DAD using two injections of 1 and 5 µL volumes to determine high and low-dose drugs and further trace compounds.
- Analysis of a fixed-dose combination drug on a 1290 Infinity LC System with the HDR solution using one single injection of 5 µL volume to determine low-dose and high-dose drugs, and further trace compounds within the linear UV-range of typically 6.7 AU.

For the first experiment, the conventional DAD was used. Two injections were necessary to determine all compounds and trace impurities within the linear UV-range, (Figures 3, 4). Vitamin C, paracetamol, and caffeine were determined by injecting 1 µL. The maximum peak height was approximately 1,500 mAU, which is within the linear UV-range of the conventional DAD. The other compounds were determined by injecting 5 µL. Vitamin C and paracetamol were now at a maximum peak height of 3,800 mAU out of the UV-linear range.

Figure 4 shows enlarged the 1- and 5-µL injections using the conventional DAD. Only with the 5-µL injection it was possible to reliably identify and determine chlorphenamine and further trace compounds. For chlorphenamine the peak height for the 1-µL injection was only as low as 3.7 mAU, whereas for the 5-µL injection approxiamtely 22 mAU were achieved. This ensured easier integration and subsequently more reliable quantification. The impurities 3 and 4 (impurities 1 and 2 elute earlier in the chromatogram, Figure 6) could only be analyzed by injecting 5 µL.

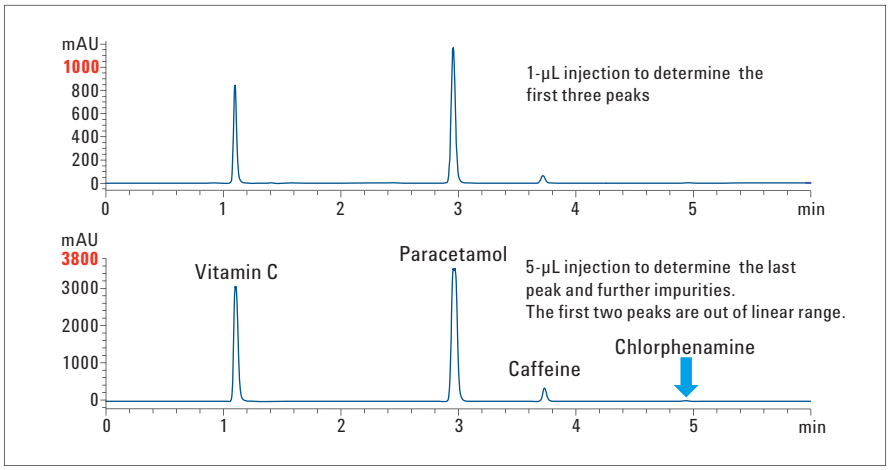


Figure 3
Chromatograms of the drug using two injections with different injection volumes.

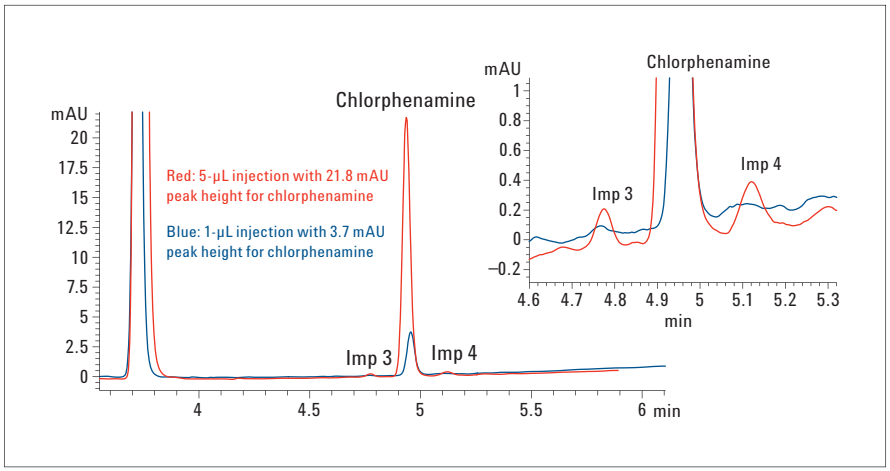


Figure 4
Enlargement of the 1-µL and 5-µL injections for the conventional DAD.

In a second experiment, 5 µL of the fixed-dose combination drug was injected using the 1290 Infinity HDR DAD Solution. Figure 5 shows an overlay of the 5-µL injection of the conventional DAD signal and the HDR DAD signal. Using the HDR DAD signal, all peaks were within the linear range and could be precisely quantified. The HDR DAD signal is close to 4,000 mAU, which is within the linear UV-range for the HDR DAD of approximately 6,000 mAU. Consequently vitamin C, paracetamol and caffeine could be quantified without problems by saturated signals.

Figure 6 shows enlargement of the 5-µL injection for the conventional and the HDR DAD signal.

As expected, the HDR DAD signal allowed the evaluation of chlorphenamine and impurities 1-4 simultaneously with the determination of the high-dose compounds as shown in Figure 5

Performance evaluation

The limit of detection with S/N = 3 of low-dose compound chlorphenamine was evaluated for the conventional DAD and the HDR DAD solution, (Table 1). The limit of detection for the HDR DAD signal was 10 times lower than for the conventional DAD signal. This is mainly due to the excellent noise performance of the HDR DAD signal, (Figure 7). For the limit of detection calculation the noise range between 4.2 and 4.6 minutes was chosen.

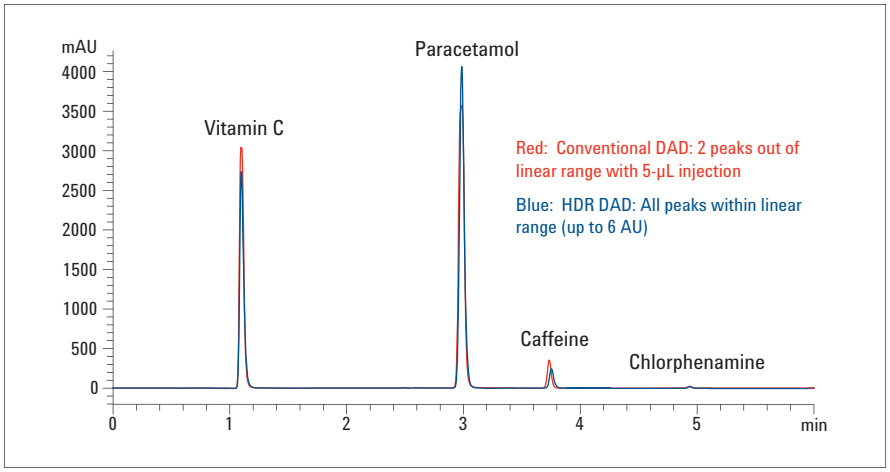


Figure 5
Overlay of 5 µL injection of conventional DAD signal and HDR DAD signals.

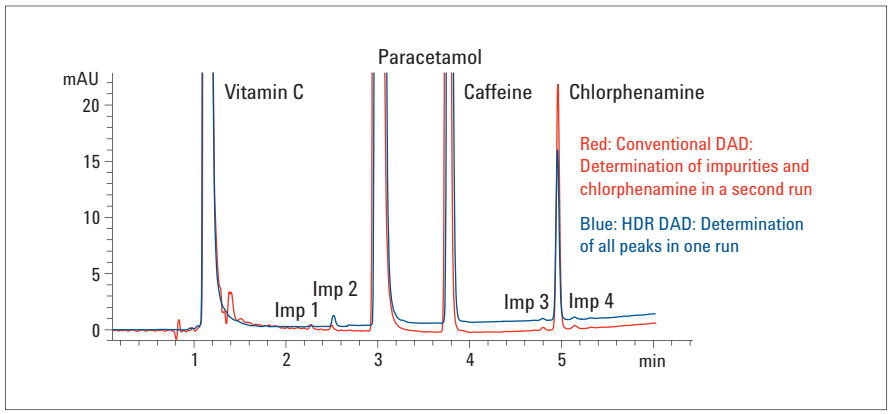


Figure 6
Enlargement of 5-µL injection of the conventional and the HDR DAD signals.

	LOD with S/N=3 for conventional DAD	LOD with S/N=3 for HDR DAD
Chlorphenamine	~1 ng	~ 0.1 ng

Table 1
Limit of detection chlorphenamine for the conventional DAD and the HDR DAD signals.

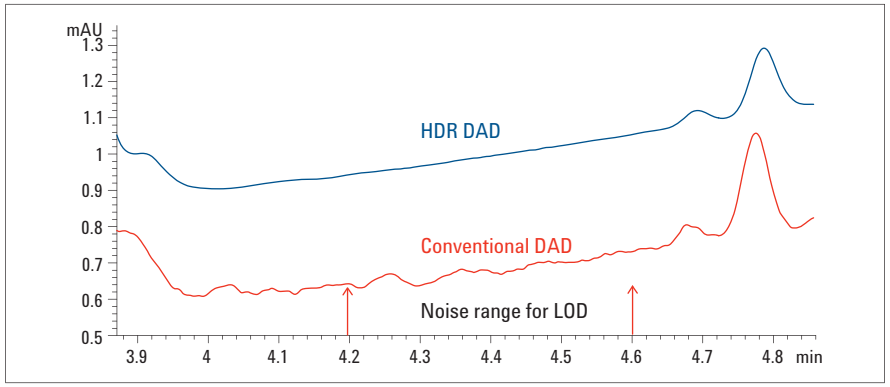


Figure 7
Baseline noise of conventional DAD and HDR DAD signals.

The area precision was evaluated for all main and trace compounds, (Figure 8). The blue bars represent the HDR DAD signal. The red bars represent the 1-µL injection of the conventional DAD that was used to determine vitamin C, paracetamol, and caffeine. The green bars represent the 5-µL injection of the conventional DAD that was used to determine chlorphenamine and impurities 1 to 4.

The HDR DAD signal provided significantly better precision for all compounds. For vitamin C, paracetamol, caffeine, and chlorphenamine the area precision was < 0.1% RSD. The area precision for the impurities was between 1 and 3% RSD.

For the conventional DAD, the area precision for vitamin C, paracetamol and caffeine and chlorphenamine was < 2% RSD and for the impurities between 5 and 15% RSD.

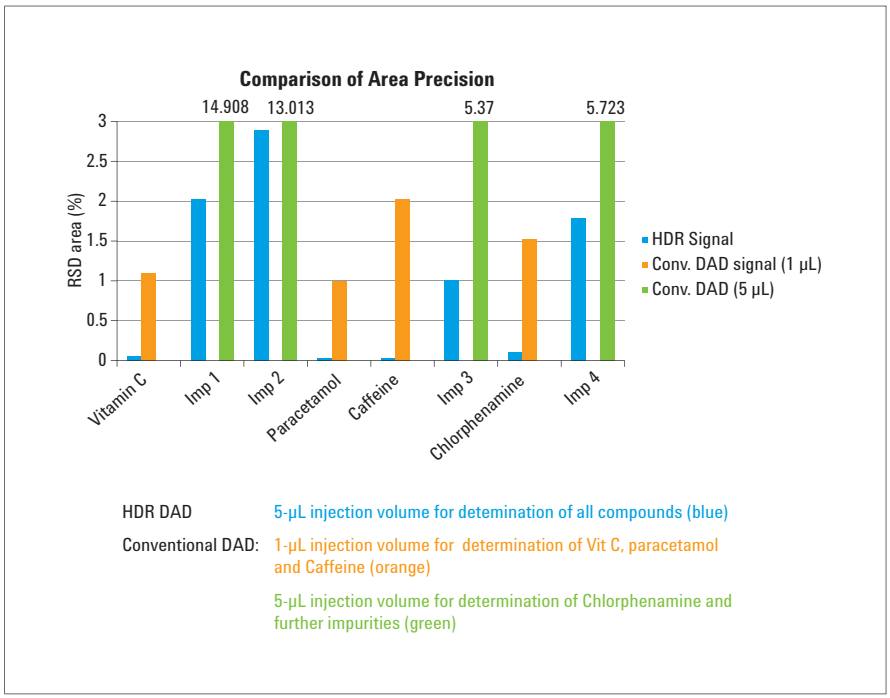


Figure 8
RSD of areas for conventional DAD and HDR DAD signals.

Conclusion

With the Agilent 1290 Infinity HDR DAD Solution, determination of low and high-dose drugs and additional impurities present in the analyzed fixed-dose combination drug was possible in one run. The complete determination of all compounds using the conventional DAD required two injections with different injection volumes to avoid saturated signals for the high-dose drugs.

Further, significantly improved precision and lower detection limits were achieved with the 1290 Infinity HDR DAD Solution compared to the conventional DAD. The limit of detection for chlorphenamine was 10 times better using the HDR DAD solution. The area precision for the additional unknown impurities was three to seven times better. For the high dosed ingredient paracetamol, the area precision was 30 times better for the HDR DAD signal.

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Agilent Technologies

Analysis of Cholesterol Lowering Drugs (Statins) Using Dried Matrix Spots Technology

Application Note

Pharmaceutical

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Introduction

Dried blood spot (DBS) technology combined with the analytical capability of modern mass spectrometers (LC-MS/MS) has recently emerged as an important method for the quantitative bioanalysis of small molecules. It is increasingly being looked at as a microsampling approach for preclinical and clinical pharmacokinetic/toxicokinetic (PK/TK) studies [1]. The primary advantage of DBS is the significant reduction in blood volume requirements, leading to cost and ethical benefits (3Rs implications - reduction, refinement, and replacement) for animal use, facilitating pediatric studies, and offering simplified sample collection [2]. It also facilitates reduction in processing, sample shipping, and storage costs under ambient conditions. An unexpected benefit of this technology is the on-card metabolite stability, specifically those metabolites known to be very labile and susceptible to degradation.

Statins (or HMG-CoA reductase inhibitors) are a class of drugs used to lower cholesterol levels. They act by inhibiting the enzyme HMG-CoA reductase, which plays a central role in the production of cholesterol in the liver. Increased cholesterol levels have been associated with cardiovascular diseases (CVD), therefore statins are used in the prevention of these diseases.

Four statins, atorvastatin, simvastatin, pravastatin, and lovastatin were analyzed on novel non-cellulose based Dried Matrix Spotting (DMS) cards. Except atorvastatin, these compounds, being acidic in nature, are challenging in terms of achieving lower detection limits.

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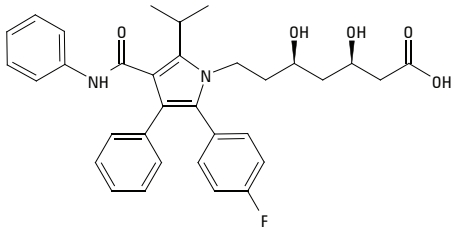
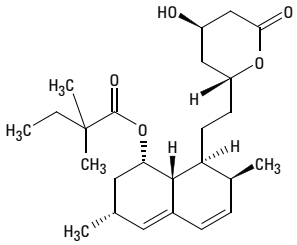
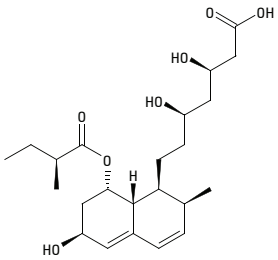
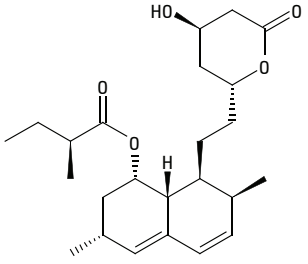
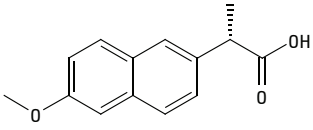
Experimental

The structures, log P, and pKa information of the four statins along with naproxen (internal standard) screened on DMS cards is given in Table 1.

Table 1. Statins Investigated – Structures, and General Information

Compound	Structure	Log P	pKa
Atorvastatin		6.36	6.36
Simvastatin		4.68	N/A
Pravastatin		2.18	4.70
Lovastatin		4.26	N/A
Naproxen (IS)		3.18	4.15

DMS Procedure

Fresh human whole blood (from Biochemed) was spiked with a mix of four statins at a concentration range of 20–2000 ng/mL for generating calibration curves. Specifically, 990 µL human blood was spiked with 10 µL of each 100x concentrated working standard to create a calibration curve of 20, 50, 100, 200, 500 and 2,000 ng/mL. After vortexing, 15 µL of each concentration of spiked blood was spotted on Agilent Bond Elut DMS cards (p/n A400150), which are non-cellulose in nature. For accuracy and precision, three replicates of blood concentrations at 20 ng/mL, and 500 ng/mL were also prepared. Accuracy and precision studies were also extended to competitive cellulose-based cards. Cards, once spotted, were left overnight for drying. 3 mm disks were punched and placed in 2 mL vials. Each spot was dissolved in 300 µL desorption solvent (60% methanol with 1% ammonium hydroxide containing 0.5 ng/mL naproxen as an internal standard), and vortexed. Spots were left to soak in desorption solvent for ~ 2 hours, samples were then removed and put in conical vials, followed by evaporation to dryness. Samples were reconstituted in 100 µL of mobile phase (70% 5 mM Ammonium formate: 30% CH_3CN), vortexed and subjected to LC-MS/MS analysis.

Results and Discussion

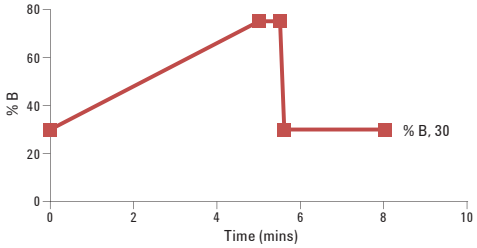
Figure 1 is an example of 50 ng/mL spiked blood after work-up with DMS cards. The column used for the analysis is an Agilent Poroshell 120 EC-C8 2.7 µm column based on a superficially porous microparticulate column packing. This new particle technology is designed to generate high efficiency separations at lower back-pressures. Back-pressures of 394 bar for this separation on a 150 mm × 2.1 mm column format on an ultra high pressure system such as an Agilent 1290 Infinity LC system are impressive. Poroshell 120 EC-C8 is an endcapped bonded phase which helps in providing excellent peak shapes of all analyte and being a C8, is less retentive for non-polar analytes (all in the current mix except pravastatin). Amongst all the compounds examined, atorvastatin was the most sensitive. It could be detected easily at 1 ng/mL with a SNR of 797 (Figure 2), while others could barely be seen at 20 ng/mL.

Linearity was observed in the calibration curves for 6 levels for pravastatin and atorvastatin using linear regression with correlation coefficients better than 0.998. Lovastatin and simvastatin curves were non-linear at the highest concentration and yielded quadratic regression with correlation coefficients better than 0.999.

LC/MS conditions

Column	Agilent Poroshell 120 EC-C8, 150 mm × 2.1 mm, 2.7 µm (p/n: 693775-906)
Mobile phase	A: 5 mM Ammonium formate, B: CH_3CN
Flow rate	200 µL/ min
Gradient	t_0 A: 70%, B: 30% $t_{5.0}$ A: 25%, B: 75% $t_{5.5}$ A: 25%, B: 75% $t_{5.6}$ A: 70%, B: 30% $t_{8.0}$ A: 70%, B: 30%

Mobile phase gradient



Column temp	30 °C
Back pressure	394 bar
Run time	8:00 min
Instrument	Agilent Infinity LC/6460 QQQ
Gas temp	275 °C
Gas flow	10 L/min
Nebulizer	10 psi
Sheath gas temp	250 °C
Sheath gas flow	7 L/min
Polarity	Negative

Table 2. MS/MS Transition Parameters of Statins Screened

Compound	Parent ion	Daughter ion	Collision energy (V)
Atorvastatin	557.2	397.1	27
Simvastatin	435.3	318.9	11
Pravastatin	423.2	321.1	7
Lovastatin	421.3	318.9	11
Naproxen (IS)	229.1	169	27

The data presented is generated by the Agilent Masshunter software.

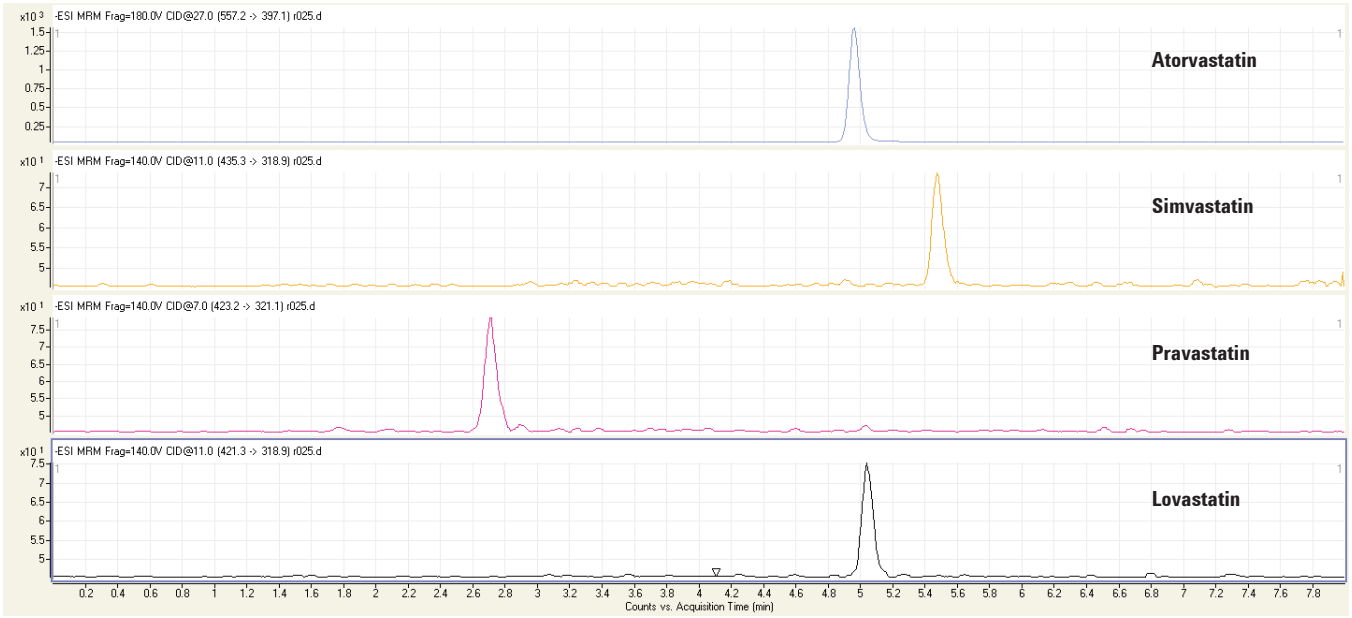


Figure 1. LC-MS/MS chromatogram of 50 ng/mL blood spiked with statins after DMS work-up.

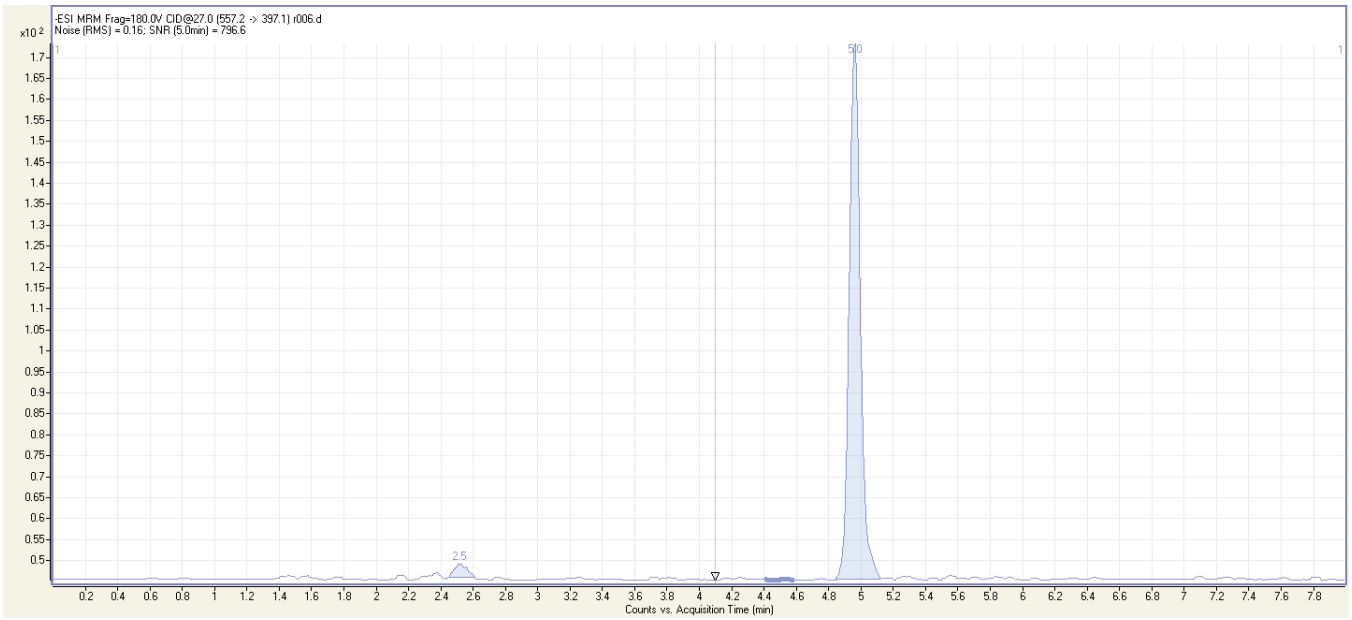


Figure 2. LC-MS/MS chromatogram of atorvastatin at 1 ng/mL spiked blood.

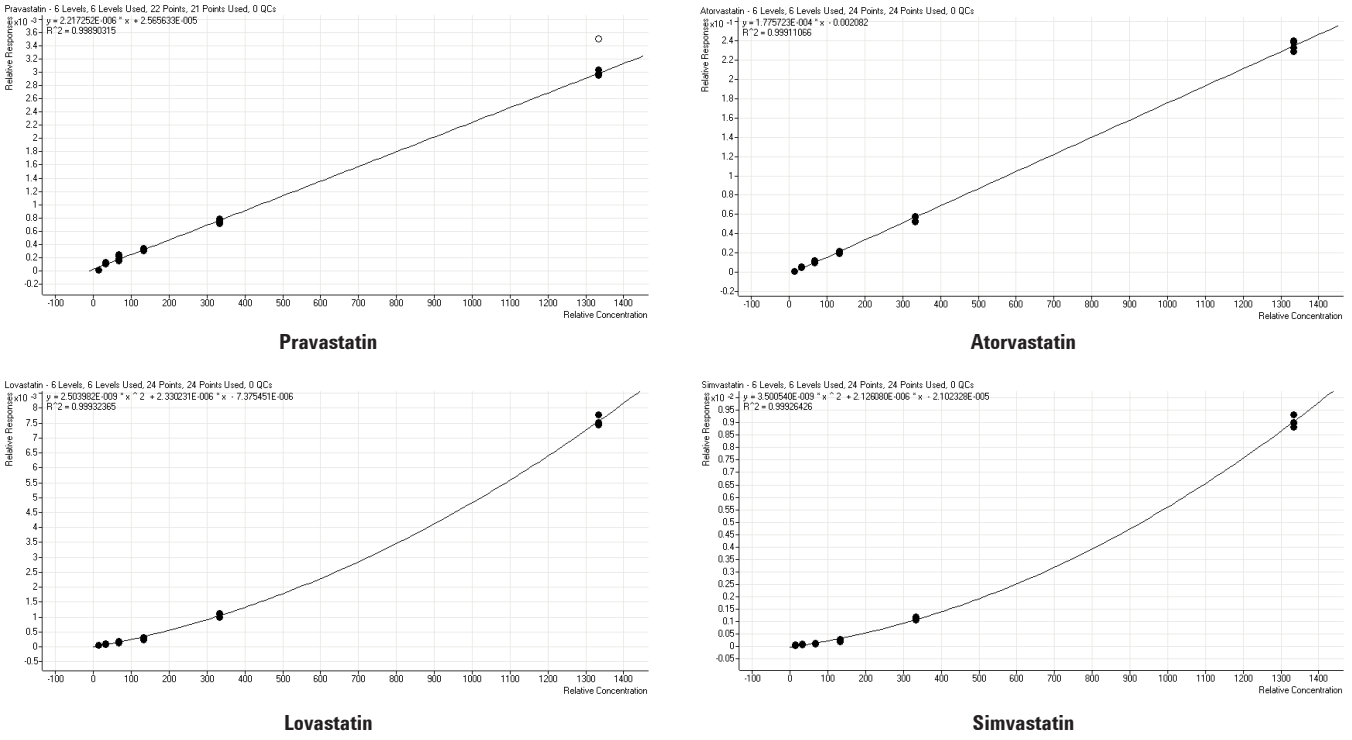


Figure 3. Calibration curves of statins in spiked blood.

Table 3 lists relative recoveries of statins from blood after DMS desorption from Agilent Bond Elut DMS cards and competitive cards. Pravastatin could not be detected at 20 ng/mL on either card. Even at 500 ng/mL, recoveries were artificially high. Being the most polar statin amongst the four investigated, it may be suffering from ion-enhancement. Further method development efforts could involve looking at additional sample cleanliness via LLE, PPT, SPE, or other sample clean-up technique. For the rest of the series, recoveries on Agilent Bond Elut DMS cards are within 16% of the true value, and those on the cellulose-based cards are within 65% of the true value. RSD values on the non-cellulose Agilent Bond Elut cards are within 17%, while those on the cellulose based cards are within 9%. Even though the RSDs on the non-cellulose based cards are somewhat higher for simvastatin at 20 ng/mL and some others marginally, the recoveries are unrealistic on the competitive product (165% vs. 111% on Agilent Bond Elut DMS). The same is true for lovastatin at 500 ng/mL (162% vs. 116% on Agilent Bond Elut DMS) This indicates that there is ion-enhancement occurring with blood constituents when the cellulose product is used, resulting in artificially high recoveries. This data supports that the non-cellulose product exhibits better quality data for desorption compared to traditional cellulose cards.

Table 3. Recoveries of Statins from Bond Elut DMS Cards and Competitive Cards (n = 3)

		Bond Elut DMS cards (non-cellulose based)		Competitive cards (cellulose based)	
	Concentration (ng/mL)	**% Recovery**	**% RSD**	**% Recovery**	**% RSD**
Atorvastatin	20.0	103	3	105	1
	500.0	98	1	103	1
Simvastatin	20.0	111	17	**165**	9
	500.0	98	5	119	6
Pravastatin	500.0	**150**	4	**198**	11
Lovastatin	20.0	104	11	112	8
	500.0	116	5	**162**	2

Conclusions

A simple and rapid method has been developed for the analysis of an acidic group of compounds such as statins in human DMS samples by LC-MS/MS. The method was demonstrated to be accurate, precise, and robust. Linearity was demonstrated for pravastatin and atorvastatin using linear regression with correlation coefficients better than 0.998. Atorvastatin displayed much lower sensitivity compared to the other statins screened and had good signal-to-noise ratios at 1 ng/mL. Relative recoveries on the non-cellulose based Agilent Bond Elut DMS cards were within 16% of the true value and RSDs within 17%, however, atorvastatin delivered RSDs of up to 5%. These cards offered better desorption properties compared to the cellulose-based competitive product. An Agilent Poroshell 120 EC-C8 column yielded good peak shapes and decreased back pressures for all the analyses. Dried blood spots should be considered as a sample collection technique when developing and validating quantitative bioanalytical methods for the analysis of drugs in pre-clinical and clinical studies.

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For More Information

Bond Elut DMS cards are intended for use in DMPK/ADME research applications only. They should not be used in diagnostic procedures. For more information on our products and services, visit our Web site at www.agilent.com/chem.

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Agilent Technologies

Investigation of Varying Blood Hematocrit Level in Dried Blood Spotting

Application Note

Pharmaceuticals

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Introduction

Dried blood spotting or dried matrix spotting has been gaining popularity in recent years. Ease of sample handling and the ability to use small sample sizes has been very attractive to the pharmaceutical industry. An important issue with dried blood spotting is the effect of blood hematocrit (HCT) on analytical results. Human HCT levels vary in adult females from 36–48 and adult males from 40–52. Higher hematocrit levels cause increased blood viscosity, altering the diffusion characteristics of blood and leading to variability in spot area. More viscous blood does not diffuse as well through a cellulose paper, but recently a new non-cellulose media has been introduced. This new material exhibits better diffusion properties with less variability than cellulose. Various HCT levels (20, 30, 45, 65, and 80) were investigated.

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Experimental

Hematocrit levels were adjusted by adding or removing plasma from whole blood. The cell volume of the original blood sample was measured at 45. Samples were diluted or concentrated to HCT levels of 20, 30, 45, 65, 80. Two analytes were chosen, paroxetine and nortriptyline. The deuterated equivalents were utilized as the internal standard for each analyte.

Results and Discussion

A 15 µL of a 20 ng/mL blood sample containing these standards were spotted onto an Agilent Bond Elut DMS card (p/n A400150).

A 3 mm disk was punched from each dried spot and placed into a 96 well collection plate.

A 300 µL of 0.1% formic acid in 80% methanol (with 0.066 ng/mL of deuterated internal standard mix) was added to each well and vortexed. Samples were allowed to soak for ~2 hours before being transferred to a conical autosampler vial.

The samples were evaporated to dryness and reconstituted in 100 µL of mobile phase.

The variation from HCT 20 to HCT 80 in a non-cellulose membrane was ~11% in spot area. Cellulose membranes on average deviated 31% over the same HCT range (see Table 1).

Column	Agilent Poroshell 120 EC-C18, 50 mm × 4.6 mm 2.7 µm (p/n 699975-902)
Mobile phase	A: 0.1% Aqueous Formic Acid B: MeOH
Pump program	Flow rate 400 µL/min
t_0	A: 40%, B: 60%
$t_{2\text{-}2.1}$	A: 20%, B: 80%
$t_{2.01\text{-}3}$	A: 40%, B: 80%
Run time =	3:00 minutes
Gas temp	350 °C
Gas flow	10 L/min
Nebulizing	20 psi
Pol	Pos

Compound	Q1 ion	Product ion	CE (V)
Paroxetine	330.2	192.1	19
Paroxetine-D6	336.2	198.1	19
Nortriptyline	264.2	233.1	11
Nortriptyline-D3	267.2	233.1	11

Table 1. Spot Areas for Cellulose and Non-Cellulose Media with Varying HCT

Non-cellulose paper			Cellulose paper		
HCT 20	HCT 45	HCT 80	HCT 20	HCT 45	HCT 80
0.0369 in^2	0.0378 in^2	0.0409 in^2	0.0750 in^2	0.0653 in^2	0.0550 in^2
CV = –3%		CV = 8%	CV = 15%		CV = –16%

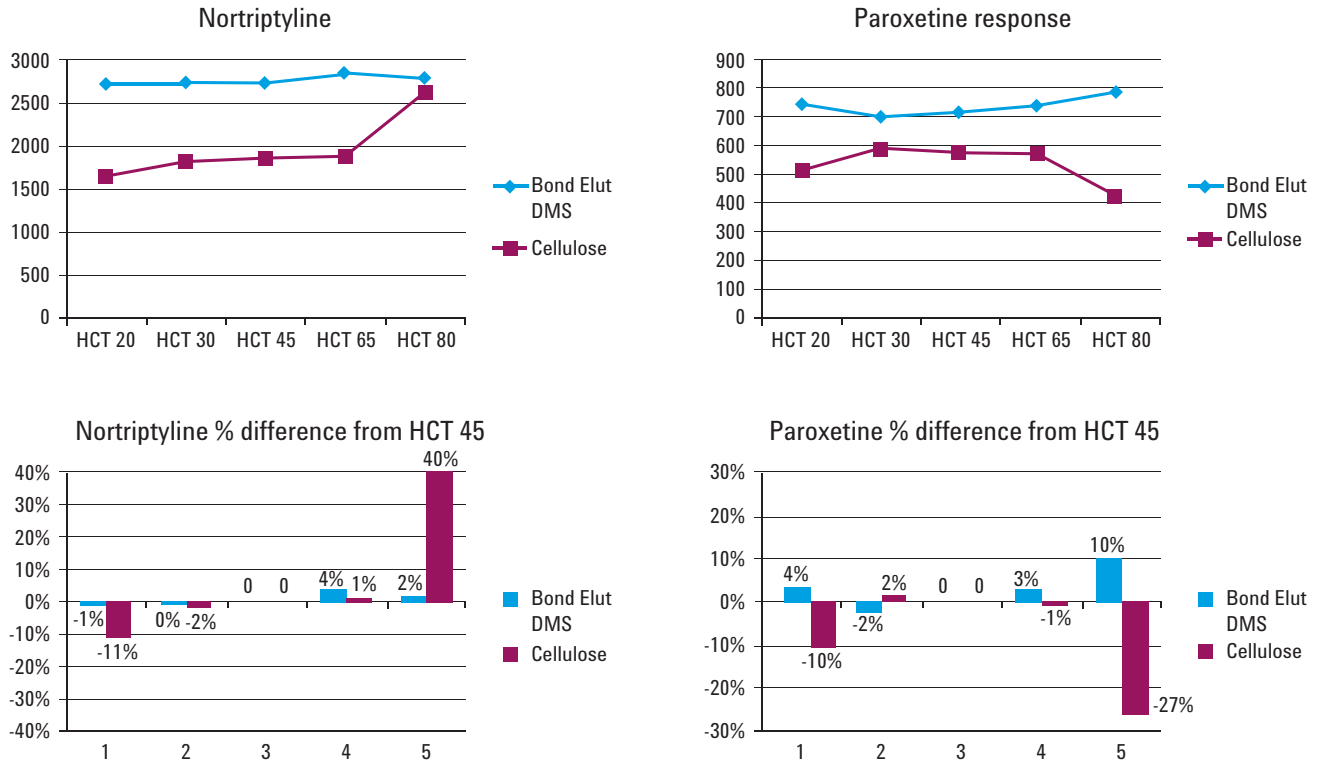

Figure 1. Comparison of Analyte response and % difference versus different HCT values on non-cellulose and cellulose-based papers. Analyte response for 20 ng/mL of analytes in whole blood. No internal area ratio was used. The non-cellulose paper shows better and more consistent response for both nortriptyline and paroxetine compared to the cellulose base paper.

Conclusions

The non-cellulose media shows significant advantage over its cellulose counterpart for dried blood spotting. There was a decreased variability in spot area and therefore less variability in the corresponding analytical result. Spot size increased only 11% from HCT 20 to 80 which is counter to the cellulose spot area migration. The difference of spot size could be correlated to the analytical data as well. Recoveries on the non-cellulose media remained consistent from HCT 20 to 80; sample response %CVs were 10% or less across the entire range. Cellulose demonstrated a much greater variability, up to 50% for nortriptyline from HCT 20 to 80. Paroxetine exhibited a decrease in response, which is counter to the spot being smaller in size. A potential cause for the variability with the cellulose paper is that chromatography could be taking place on the paper altering the diffusion rate of the analyte compared to that of the blood. The effect of hematocrit variability on compound response in dried blood spotting has been shown to vary depending on the substrate used which plays an important role in method validation. The Agilent Bond Elut DMS card with a non-cellulose media removes the effects of sample variability easing method validation and overall analysis.

Bond Elut DMS cards are for use in DMPK/ADME bioanalytical applications only. They should not be used in clinical diagnostic procedures.

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Agilent Technologies

Improving Sensitivity of Basic Drugs in Dried Blood Spotting through Optimized Desorption

Application Note

Pharmaceutical

Authors

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Agilent Technologies, Inc.

Introduction

Dried matrix spotting (DMS) or dried blood spotting (DBS) technology, combined with the analytical capability of modern mass spectrometers (LC/MS/MS), has recently emerged as an important method in the quantitative bioanalysis of small molecules. The great interest in DBS lies in the small volume of sample required, ease of collection, reduced sample shipping costs, and versatile storage conditions [1, 2, 3, 4].

As a relatively new technique in bioanalysis, investigating the impact of variables that may affect its overall efficiency is essential. Agilent Bond Elut Dried Matrix Spotting cards use a novel, noncellulose-based substrate for dried matrix and dried blood spotting. These were used to evaluate method development options available for basic analytes to reach optimal desorption conditions, and the resulting impact on mass spectrometric sensitivity.

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Experimental

Materials and Methods

- Agilent Bond Elut Dried Matrix Spotting (DMS) cards, (p/n A400150)
- Agilent Poroshell 120 EC-C18, 30 × 2.1 mm, 2.7 µm column (p/n 691775-902)
- Human whole blood (pooled, mixed gender) was purchased from Biochemed Services
- Chemicals: Atenolol, pindolol, metoprolol, and propranolol were purchased from Sigma Chemicals
- Water and methanol (LC/MS grade) were purchased from VWR

Fresh human whole blood, pooled (in Heparin) was spiked with a mix of four basic pharmaceuticals, comprising β-blockers such as atenolol, pindolol, metoprolol, and propranolol, at a concentration of 20 ng/mL. The log P and pKa values of the four drugs screened are listed in Table 1. After vortexing, 15 µL of blood was aliquoted per spot on Agilent Bond Elut Dried Matrix Spotting cards followed by overnight drying. Circular punches of 3 mm diameter were taken from the DMS cards and used for the desorption studies.

Table 1. Basic drugs screened - general information.

Compound	Log P	pKa	Therapeutic use
Atenolol	0.5	9.6	β-blocker, antihypertensive
Pindolol	1.9	8.8	β-blocker
Metoprolol	1.6	9.7	β-blocker, antihypertensive
Propranolol	3.0	9.5	β-blocker, antihypertensive

Desorption methods

Different techniques were tested to evaluate the best way to desorb the analytes from the membrane. Each test was compared to a standard and protein precipitated sample. All blood samples were evaporated and reconstituted in 100 µL mobile phase.

a) **Standard:** By determining the ratio of the area of the punch to the area of the spot, the volume taken from a 3 mm punch can be determined. The actual blood volume sampled is 4 µL and is consistent regardless of the amount of volume spotted on the card [5]. 4 µL of 20 ng/mL standard was desorbed with 300 µL of 0.1% formic acid 80:20 MeOH:H_2O, centrifuged at 15,000 rpm for 15 minutes, evaporated to dryness, and diluted to 100 µL with mobile phase.

b) **Protein precipitation:** A 4 µL sample of blood was diluted to 100 µL with H_2O. Then, 300 µL of 0.1% formic acid in MeOH was used as a crash solvent (1:3 aqueous:organic crash) to precipitate the proteins. This was done to compare DMS extracts with the traditionally used protein crash technique.

c) **Centrifugation (15 minutes):** A 3 mm punched DMS blood spot was desorbed using 300 µL of 0.1% formic acid 80:20 MeOH:H_2O. The sample was centrifuged at 15,000 rpm for 15 minutes.

d) **Soak (1 hour):** A 3 mm DMS blood spot was desorbed using 300 µL of 0.1% formic acid 80:20 MeOH:H_2O. The sample was soaked for 1 hour before evaporation.

Centrifugation time

The centrifugation time was increased in 15 minute increments to test if recoveries/responses could be improved with higher centrifuge times. Spots of 3 mm diameter were taken from different 20 ng/mL spots and put into 2 mL centrifuge tubes. Then, 300 µL of 0.1% formic acid in 80:20 MeOH:H_2O was added to each spot and centrifuged for 15, 30, 45, and 60 minutes. Each sample was then evaporated and reconstituted in 100 µL of mobile phase.

Desorption solvents

Both MeOH and ACN were tested at various concentrations with 0.1% formic acid (FA), that is, 100%, 80%, 60%, and 40% organic. A concentration study of FA was also carried out with 80% MeOH and ACN: 0%, 0.1%, 0.5%, and 1% FA. Spots of 3 mm diameter were taken from different 20 ng/mL spots and put into 2 mL centrifuge tubes. They were then desorbed with 300 µL of each of the desorption solvents, centrifuged at 15,000 rpm for 15 minutes, evaporated, and reconstituted in 100 µL of mobile phase.

LC/MS conditions

Column:	Agilent Poroshell 120 EC-C18, 2.1 × 30, 2.7 µm (p/n 691775-902)
Mobile phase:	A: 0.1% Formic acid in H_2O, B: MeOH
Flow rate:	200 µL/min
Gradient:	t_0 A: 80%, B: 20%
	$t_{1.0-2.0}$ A: 20%, B: 80%
	$t_{2.01-3.0}$ A: 80%, B: 20%
Run time:	3 min
Gas temperature:	350 °C
Gas flow:	10 L/min
Nebulizer:	15 psi
Sheath gas temperature:	250 °C
Sheath gas flow:	7 L/min
Polarity:	Positive
Capillary:	4000 V
Instrument:	Agilent 1290 Infinity LC System, Agilent 6460 Series Triple Quadrupole LC/MS System

LC/MS transitions

Compound	Parent ion	Product ion	CE (V)	Dwell time (ms)	Fragmentor (V)
Atenolol	267.2	145.1	22	200	140
Pindolol	249.2	116.1	14	200	100
Metoprolol	268.2	116.2	14	200	140
Propranolol	260.2	116.1	14	200	100

Results and Discussion

Figure 1 is an example of 20 ng/mL spiked blood after work-up with DMS cards. The column used for the analysis is an Agilent Poroshell 120 EC-C18 2.7 µm column based on a superficially porous microparticulate column packing. This new particle technology is designed to give all the performance advantages of sub 2-µm particles with backpressures that are comparable to sub 3-µm particles. All four analytes separate with base-line resolution and good peak shapes on a short 30-mm column.

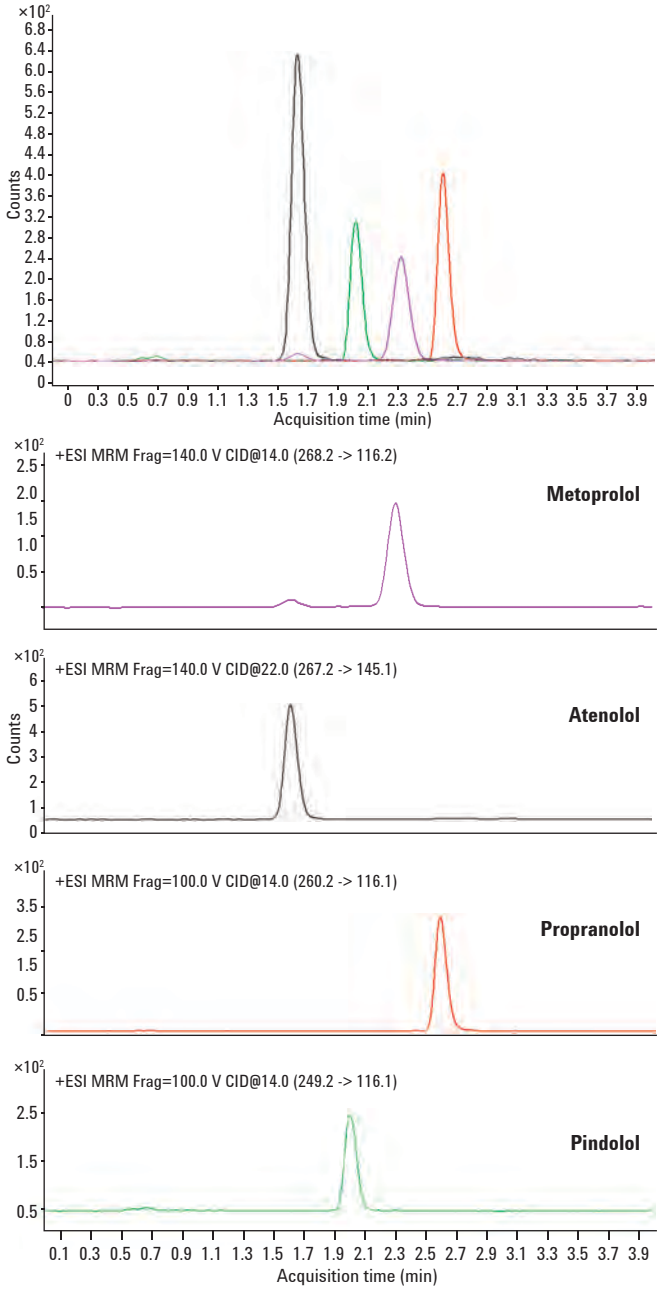

Figure 1. LC-MS/MS chromatogram of 20 ng/mL blood spiked with β-blockers after DMS work-up.

Agilent Bond Elut DMS cards use an innovative, noncellulose technology that delivers significantly improved analytical sensitivity, reproducibility, and ease-of-use. The improved MS signal results from a cellulose-free format that has reduced non-specific binding with no impregnated chemical reagents. This feature makes the noncellulose product exhibit better quality data for desorption compared to traditional cellulose cards [6, 7].

The cards display excellent spot homogeneity with reproducible extractions, at higher recoveries, across a range of hematocrit levels [8, 7, 9, 10]. The effect of hematocrit on assay bias was examined across a wide range of hematocrit levels, the cards generated a narrow assay bias in all the three critical parameters affecting overall performance, namely, spot area, ion suppression, and analyte recovery [11]. Bond Elut DMS is amenable to a broad range of biological matrices including plasma [12].

Analyte desorption was measured in terms of MS response for each analyte. The effect of different desorption methods, centrifugation times, and desorption solvents on analyte sensitivity is presented to reflect the overall efficiency of the technique.

Figure 2 illustrates that centrifuging the sample for 15 minutes gave the best overall results, especially for the more hydrophobic, higher Log P analyte, propranolol. Atenolol, which is the most polar, had the poorest recovery. In the protein precipitated sample, propranolol yields recoveries higher than the standards, most likely due to co-extraction of hydrophobic endogenous interferences, because the technique does not provide any sample clean-up. It is evident that centrifugation for 15 minutes yields higher responses than soak for 60 minutes for three out of the four drugs screened. This implies significant reduction in sample processing time once the spots are punched, leading to increased throughput.

Figure 3 compares response in 15 minute increments when the blood spots were desorbed and centrifuged at 15,000 rpm for 15-60 minutes. Centrifugation for 15 minutes appeared to be sufficient. Propranolol response decreased as centrifugation time increased, probably due to more interferences being desorbed with time.

Desorption methods

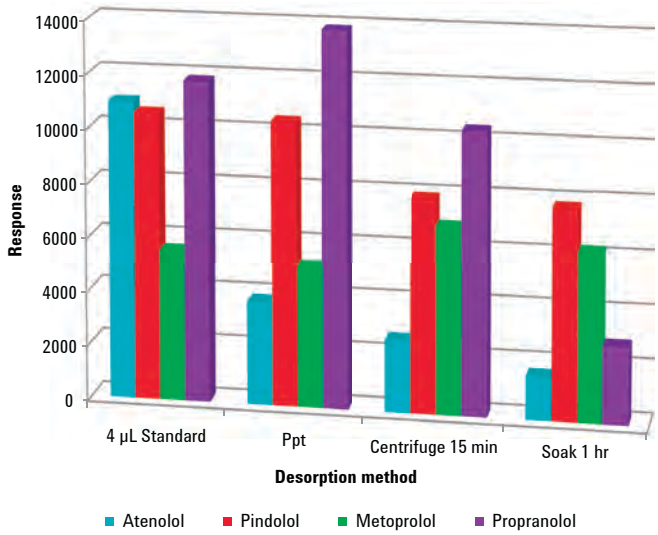

Figure 2. Effect of desorption methods on the response of β-blockers (n = 4).

Centrifugation time

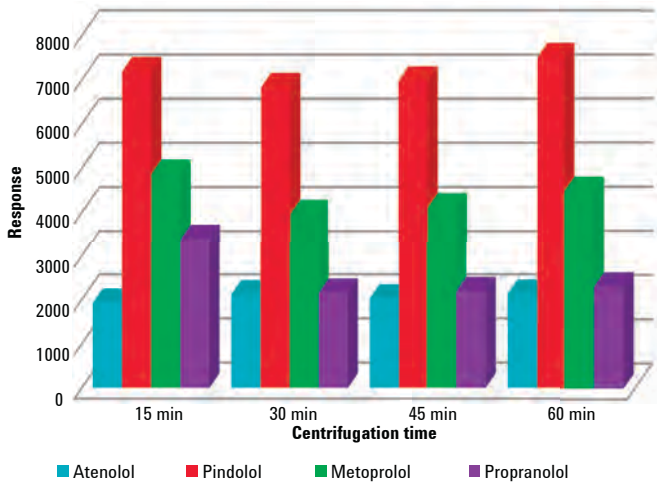

Figure 3. Effect of centrifugation times on the response of β-blockers (n = 4).

Desorption solvents

Figure 4 reflects that atenolol yields the best response at 60% ACN:0.1% FA or 40% MeOH:0.1% FA. Previous published work with basic analytes and dried blood spot technology cites the use of 80% MeOH with 0.1% FA as a generic desorption solvent resulting in high and reproducible recoveries [5, 7-12]. Thus, even though 80% organic did not generate the highest response when the organic composition was varied, it was still chosen as the composition in which FA concentrations were varied and the corresponding responses examined. In the FA concentration study experiments, the best response was obtained with 0.5% FA in 80% ACN, followed closely by 0% FA in 80% MeOH.

For pindolol, 60% ACN:0.1% FA generated the best response. No FA in 80% ACN or MeOH worked best in the FA concentration studies (Figure 5).

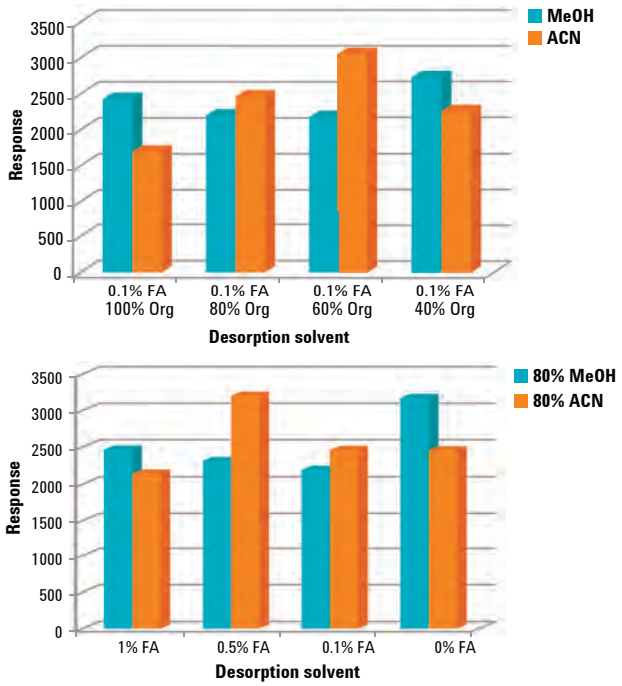

Figure 4. Effect of methanol and acetonitrile-based desorption solvents on the sensitivity of atenolol (n = 4).

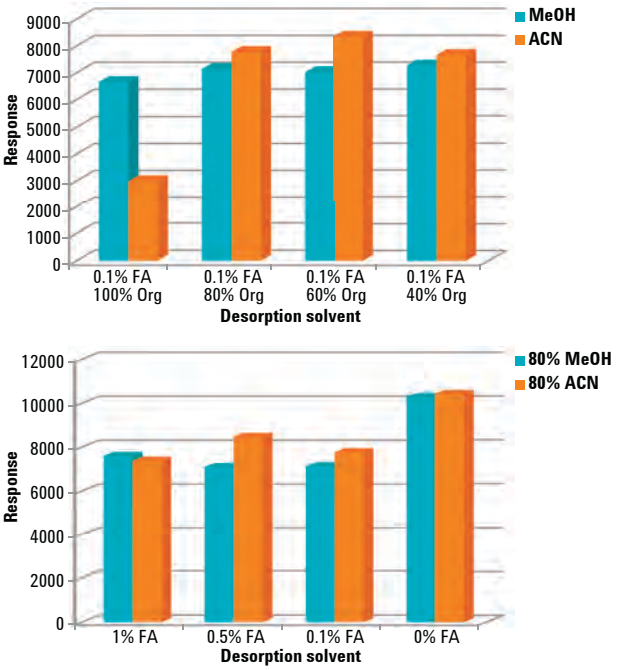

Figure 5. Effect of methanol and acetonitrile-based desorption solvents on the sensitivity of pindolol (n = 4).

In the case of metoprolol, 100% ACN:0.1% FA yielded the best response. In general, ACN worked better than MeOH for every organic % tried. In the FA concentration study, the best response resulted from using 0.5% FA in 80% ACN (Figure 6).

For the more hydrophobic propranolol, MeOH worked better than ACN in general, with 80% MeOH:0.1% FA yielding the highest response. In the FA concentration study, 0.1% FA in 80% MeOH was far above the other concentrations investigated (Figure 7).

When selecting desorption solvents for basic compounds, such as β-blockers, there was no generic method for all compounds. Polar compounds, such as atenolol, pindolol, and metoprolol, worked better with acetonitrile, while nonpolar analytes like propranolol yielded better responses with methanol. Pindolol worked best with no FA, but propranolol response was very poor.

Conclusions

Analyte desorption and its resulting impact on sensitivity was improved when centrifugation was used, in comparison to soak for 1 hour, for basic analytes such as β-blockers. A 15 minute centrifugation time was adequate, and longer centrifugation times did not offer increased sensitivity. By replacing a soak step of 60 minutes with a short 15 minute centrifugation, there is a significant reduction in sample processing time, leading to overall increased throughput. Among the variety of solvents tried, the best overall desorption solvent that resulted in improving sensitivity used 80% MeOH with 0.1% FA. This was the best solvent for propranolol, and it provided sufficient response for the other compounds.

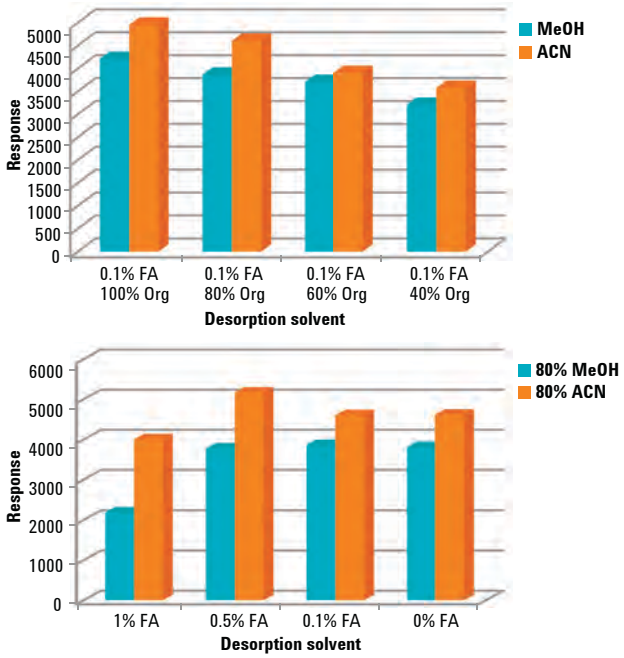

Figure 6. Effect of methanol and acetonitrile-based desorption solvents on the sensitivity of metoprolol (n = 4).

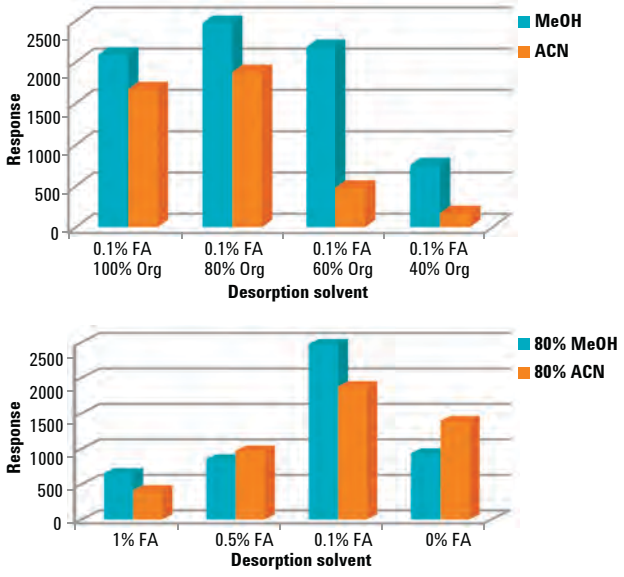

Figure 7. Effect of methanol and acetonitrile-based desorption solvents on the sensitivity of propranolol (n = 4).

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Agilent Technologies

Analysis of Danshen (Salvia Miltrorrhiza) and Compound Danshen Dropping Pills using Poroshell 120 Superficially Porous LC Columns

Application

Pharmaceuticals

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Abstract

A popular traditional Chinese medicine, Danshen (Salvia Miltrorrhiza), and its preparation Compound Danshen Dropping Pills were analyzed by HPLC with Agilent Poroshell 120 LC columns. The columns are made from superficially porous particles that have a solid core (1.7 µm) and porous outer layer with a 0.5-µm diffusion path. The advantages of the new column type include high performance similar to that of sub-2-µm particles but with 40-50% lower back pressure, and high peak capacity. The method on the Poroshell 120 column was compared to that generated on a UPLC with a Waters HSS T3 column. The Poroshell 120 column provided excellent peak shape and was operated at a pressure of less than 287 bar. The method can be run on any HPLC.

Introduction

Danshen (Salvia miltiorrhizae) is widely used as a traditional Chinese medicine (TCM), often in combination with other herbs. Remedies containing Danshen are used traditionally to treat a variety of ailments, particularly cardiac (heart) and vascular (blood vessel) disorders such as atherosclerosis ("hardening" of the arteries with cholesterol plaques) or blood clotting abnormalities. Danshen Dropping Pills are a compound preparation consisting of Danshen, notoginseng, borneol, and other compounds and are used to treat for coronary heart disease and angina.[1]

Because there are so many compounds in the matrix, quality control for TCM's and other natural products is very difficult. HPLC methods can be used for quantitive analysis of only one or several compounds in the TCM, which may not represent the real quality of the TCM. The current trend is to find a unique chromatogram to be used like a fingerprint for a respective material. The more peaks found, the more information is presented for the sample. So the peak capacity and resolution are important for the TCM fingerprint to control its quality.

Regulations have included the Waters UPLC method for TCM fingerprint analysis [2]. In this note, we developed methods with an Agilent Poroshell 120 that can be run on any LC instrument.

Experimental

Sample preparation

Pill preparation: Dissolve 10 pills in 10 mL of water. Filter with a 0.45 µm Regenerated Cellulose Membrane filter (p/n: 5064-8221) before injecting into the HPLC for analysis.

Danshen preparation: Weigh 2.0 g of Danshen powder, extract with water at 80 °C for 1 hour, filter and bring the aqueous volume to 50 mL. Filter with a 0.45 µm Regenerated Cellulose Membrane filter (p/n 5064-8221) before injecting into the HPLC for analysis.

HPLC conditions

The HPLC analysis was performed with an Agilent 1200 Series Rapid Resolution LC (RRLC) system including a G1312B Binary Pump SL, G1376C Automatic Liquid Sampler SL (ALS), G1316B Thermostatted Column Compartment SL (TCC), G1316C Diode Array Detector SL (DAD).

Table 1. Conditions for Danshen Dropping Pills

Mobile Phase:	A, 0.02% H_3PO_4; B, 0.02% H_3PO_4 in ACN/water (80/20)					
Gradient:						
Time (min)	0	1.6	1.8	8.0	8.4	10
%B	9	22	26	39	9	9
Column	Waters HSS T3, 2.1 × 100 mm, 1.8 µm Agilent Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm					
Column temp	40 °C					
Flow Rate	0.4 ml/min for 2.1 mm ID, 0.8 mL/min for 3.0 mm ID, 2mL/min for 4.6 mm ID					
Wavelength	280 nm					
Injection Volume	2 µL on 2.1 and 3.0 mm ID columns, 4.0 µL on 4.6 mm ID column					

Table 2. Conditions for Danshen Water Extraction

Mobile Phase	A, 0.02% H_3PO_4 :B, ACN					
Gradient	Flow rate=1 ml/min					
Time(min)	0	6	14	18	22	28
%B	10	20	25	30	90	90
Gradient	Flow rate = 2 ml/min					
Time(min)	0	3	7	9	11	14
B%	10	20	25	30	90	90
Gradient	Flow rate = 3 ml/min					
Time(min)	0	2	4.67	6	7.33	9.33
B%	10	20	25	30	90	90
Column	Agilent Poroshell 120 EC-C18, 4.6 × 100mm, 2.7 µm; Agilent ZORBAX RRHT Eclipse Plus C18, 4.6 × 100 mm, 1.8 µm					
Column temp	30 °C					
Wavelength	280 nm					
Injection Volume	4 µL					

Results and Discussion

The Compound Danshen Dropping Pill was analyzed on both a Poroshell 120 EC-18 and a Poroshell 120 SB-C18 column, as shown in Figure 1. All eight target compounds were separated on both columns. The Poroshell 120 EC-C18 column provided a little more retention than the Poroshell 120 SB-C18, and the selectivity was different between the two columns. The selectivity difference indicates that the Poroshell 120 EC-C18 is the better choice for this sample because a small impurity peak was separated from peak 6 on the Poroshell 120 EC-C18 but not on the Poroshell 120 SB-C18.

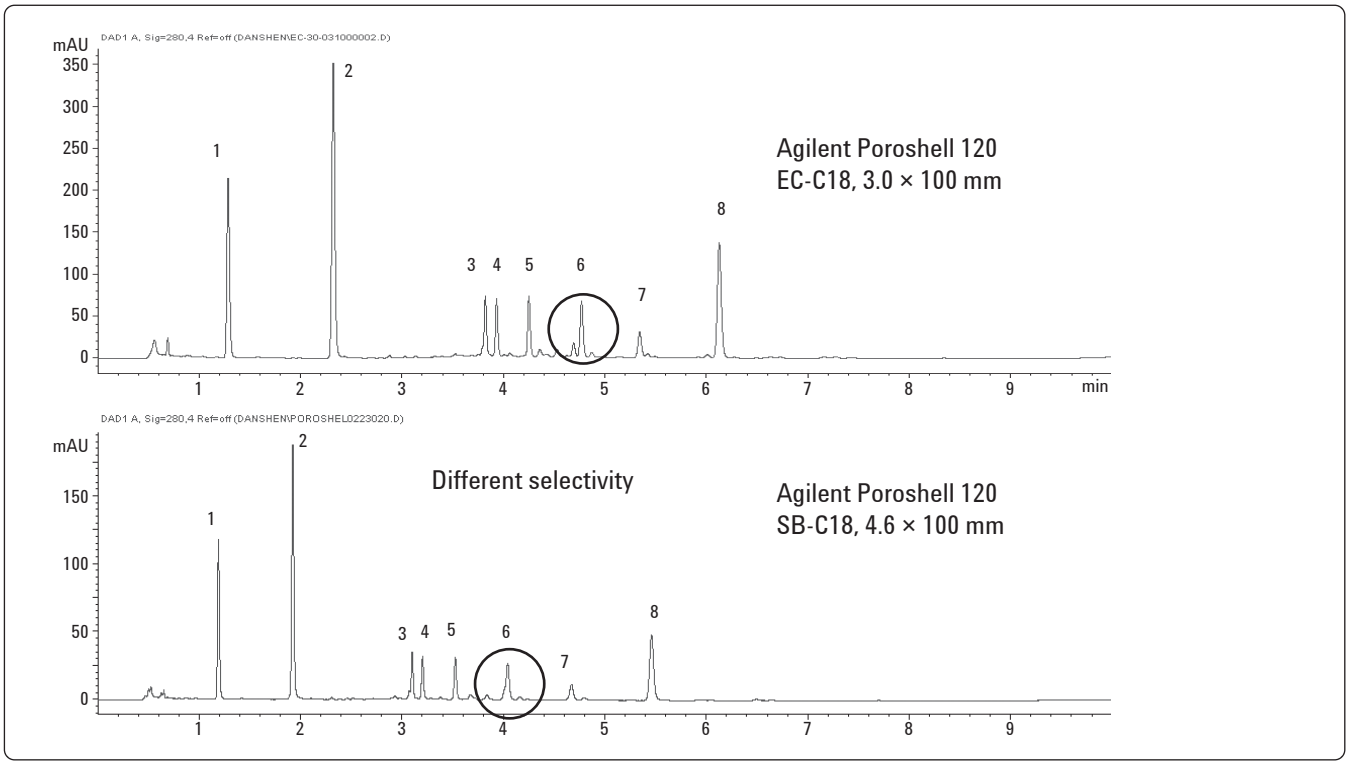

Figure 1. Danshen Dropping Pills on Agilent Poroshell 120 EC-C18 and SB-C18 columns. Both columns have a particle size of 2.7 µm.

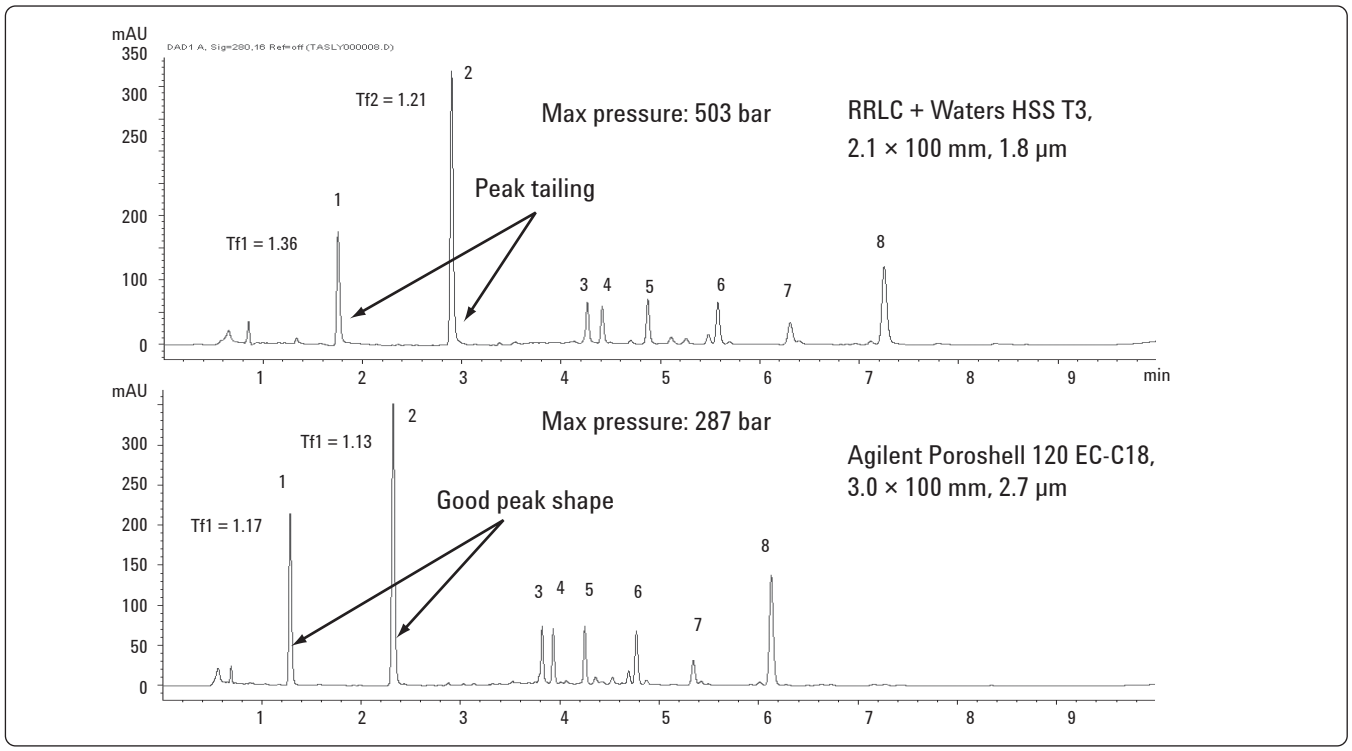

Figure 2. Comparison of the Agilent Poroshell 120 EC-C18 and competitor column shows the better peak shape and shorter analysis time on the Poroshell 120 column.

The original method was developed on a Waters HSS T3, 1.8 µm column and was repeated on an Agilent LC with the results shown in Figure 2. A comparison of the results on the Poroshell 120 EC-C18 column and the Waters HSS T3 showed that the Poroshell 120 EC-C18 column had several advantages for this separation. The analysis time was shorter and the peak shape was better than with the Waters HSS T3 column and the resolution was very similar for the eight target compounds. The most important advantage for chromatographers without a UHPLC was that the maximum pressure of the Poroshell 120 EC-C18 column was only 57% of the Waters HSS TS, 1.8 µm column. The method with the Poroshell 120 EC-C18 column can be run on any LC but a high pressure LC is needed to run the same method with the Waters HSS T3 column.

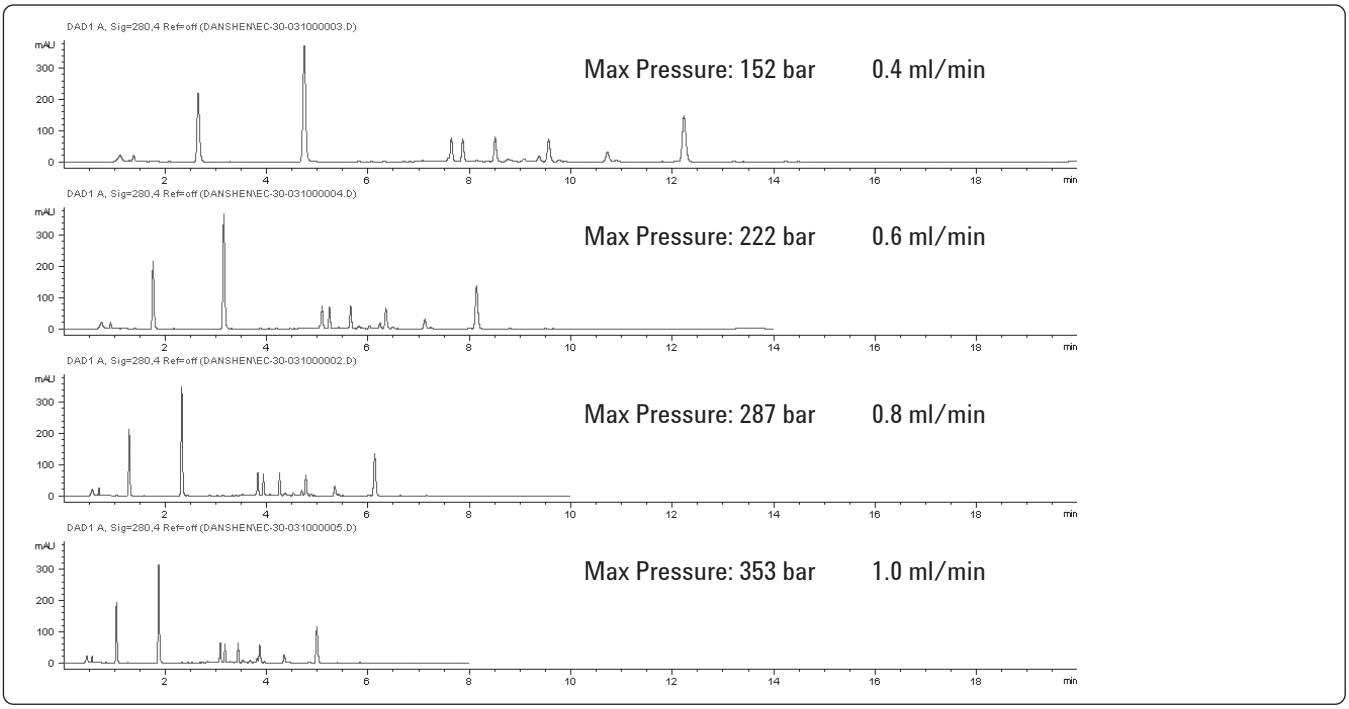

Figure 3. Chromatograms at different flow rates on the Agilent Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm column.

Figure 3 shows chromatograms at different flow rates. The separation can perform faster at higher flow rates with almost no loss of resolution, while maintaining pressures under 400 bar.

Water extractions of Danshen were analyzed on the Agilent Poroshell 120 EC-C18 (2.7 µm) and the Agilent ZORBAX Rapid Resolution HT Eclipse Plus C18 (1.8 µm) columns as shown in Figure 4. The Poroshell 120 EC-C18 column with superficially porous particles provided a little less retention and better resolution than the column with sub-2-µm totally porous particles. The small, superficially porous particles generate high efficiency, similar to the efficiency of sub-2-µm columns. The different bonded phases produce results with higher performance and resolution. A small peak was separated from the main peak on the Poroshell 120 EC-C18 column due to these selectivity differences. In addition, the separation can be performed faster at a higher flow rate without resolution loss with this column due to the lower back pressure of the superficially porous column. Columns with superficially porous particles generate higher efficiency and resolution compared to those with totally porous particles due to faster mass transfer in the porous shell particles and the 20% narrower particle size distribution.

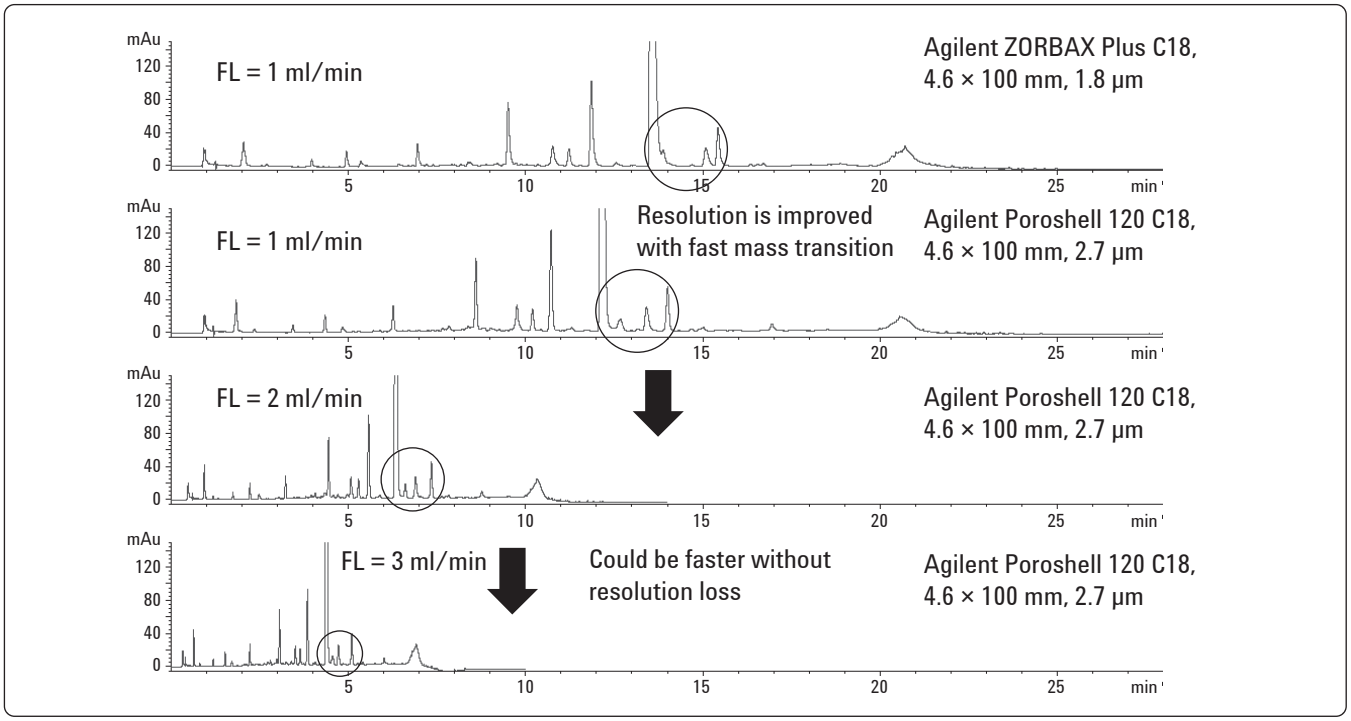

Figure 4. Water extractions of Danshen on the Agilent Poroshell 120 EC-C18 (2.7 µm) and the Agilent ZORBAX Rapid Resolution HT Eclipse Plus C18 (1.8 µm) column.

Conclusion

The superficially porous particles in the Agilent Poroshell 120 columns provide high performance and fast analyses at HPLC pressures. These columns are suitable for the complex sample analysis of a TCM, such as the Danshen extract, because of the high resolution achieved. The pressure on the Agilent Poroshell 120 column is about 40-50% less than that with a column with totally porous sub-2-µm particles. This makes the Agilent Poroshell 120 columns suitable for any LC instrument, especially a standard one with a maximum pressure of 400 bar. Users with standard LC instruments can achieve fast analyses using Poroshell 120 columns.

Reference

1. http://www.nlm.nih.gov/medlineplus/druginfo/natural/patient-danshen.html
2. Compound Danshen Dropping Pills, China Pharmacopoeia 2010: 906

www.agilent.com/chem

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Agilent Technologies

Fast method for Ginseng Analyses using Agilent Poroshell 120 Columns Scaled from a Traditional Method

Application Note

Pharmaceutical

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Abstract

The traditional method for analyzing ginseng was scaled from a 4.6 × 150 mm, 5 µm column to an Agilent Poroshell 120 EC-C18, 3.0 mm × 100 mm, 2.7 µm column. The gradient time required decreased from over 100 min to about 70 min with the same linear velocity, and could be decreased further to below 25 min by increasing the flow rate. In this instance, the pressure exceeded 400 bar at high flow rates, therefore a 600-bar instrument should be used. The time can be further reduced using an Agilent Poroshell 120 EC-C18 or Poroshell 120 SB-C18 3.0 mm × 50 mm column. The fastest method has an 11-min gradient with resolution of 1.57 for the critical pair of compounds, ginsenosides Rg1 and Re. Finally, the new method does not need changes in sample preparation because the column uses 2 µm frits. The pressure is still below 400 bar and can be run on any HPLC instrument.

Agilent Technologies

Introduction

Asian ginseng is native to China and Korea and is used in various systems of medicine for many centuries. Treatment claims for Asian ginseng are numerous and the herb is used to support overall health and boost the immune system. Traditional and modern uses of ginseng include: improving the health of people recovering from illness; increasing a sense of well-being and stamina; improving both mental and physical performance; treating erectile dysfunction, hepatitis C, and symptoms related to menopause; lowering blood glucose; and controlling blood pressure. The root of Asian ginseng contains active chemical components called ginsenosides that are thought to be responsible for the herb's medicinal properties [1].

In the Chinese Pharmacopeia, an HPLC method is listed to measure amounts of the three main ginsenosides Rg1, Re and Rb1 in ginseng [2]. The original method uses a traditional 4.6 mm × 150 mm, 5 µm column and applies a long gradient of 100 minutes. A rapid analysis method is necessary to increase the work throughput and reduce lab costs.

In this study, the original method was scaled to both Agilent Poroshell 120 EC-C18 and Poroshell 120 SB-C18 columns. The Agilent Poroshell 120 2.7 µm columns are packed with superficially porous materials, which make the separation fast and achieves performance similar to sub-2-µm totally porous materials. The method was scaled for column length, column id, and sample size. The linear velocity was maintained on the smaller column.

Experimental

The 1200 Series SL LC system includes a binary pump, a thermostatted column compartment (TCC), a high performance autosampler and a diode array detector (DAD).

The columns used in the application are:

- Agilent ZORBAX StableBond C18, 4.6 mm × 150 mm, 5 µm (p/n: 883975-902)
- Agilent ZORBAX Eclipse Plus C18, 4.6 mm × 150 mm, 5 µm (p/n: 959993-902)
- Agilent Poroshell 120 EC-C18, 3.0 mm × 100 mm, 2.7 µm (p/n: 695975-302)
- Agilent Poroshell 120 EC-C18, 3.0 mm × 50 mm, 2.7 µm (p/n: 699975-302)
- Agilent Poroshell 120 SB-C18, 3.0 mm × 50 mm, 2.7 µm (p/n: 689975-302)

The ginseng sample was made using following steps:

1. Weigh 1.0 g of the dried powder.
2. Degrease with Soxhlet extractor using 50 mL ethyl ether for 3 h.
3. Filter after cooling down, discard the liquid phase, then dry the residues.
4. Put the residues in a 25-mL flask with filter paper. Add 20 mL of water-saturated n-butanol and extract for 30 min with a supersonic extractor.
5. Filter the extraction and collect 10 mL of the liquid of n-butanol.
6. Evaporate the n-butanol using a water bath and add 2 mL of methanol to the residue to dissolve.
7. Filter the final sample with a 0.45-µm regenerated cellulose membrane filter (p/n: 5064-8221) before injecting into HPLC for analysis.

Results and Discussion

The original ginsenosides separation method separating Rg1, Re and Rb1 ginseng was repeated on an Agilent ZORBAX Eclipse Plus C18, 4.6 mm × 150 mm, 5 µm and an Agilent ZORBAX SB-C18, 4.6 mm × 150 mm, 5 µm column. Theoretical plates of ginsenosides Rg1 on both columns were more than 9000 and the resolution was around 2.0, which is sufficient for the determination of three ginsenosides. The time for a single run was more than 100 min as shown in Figure 1.

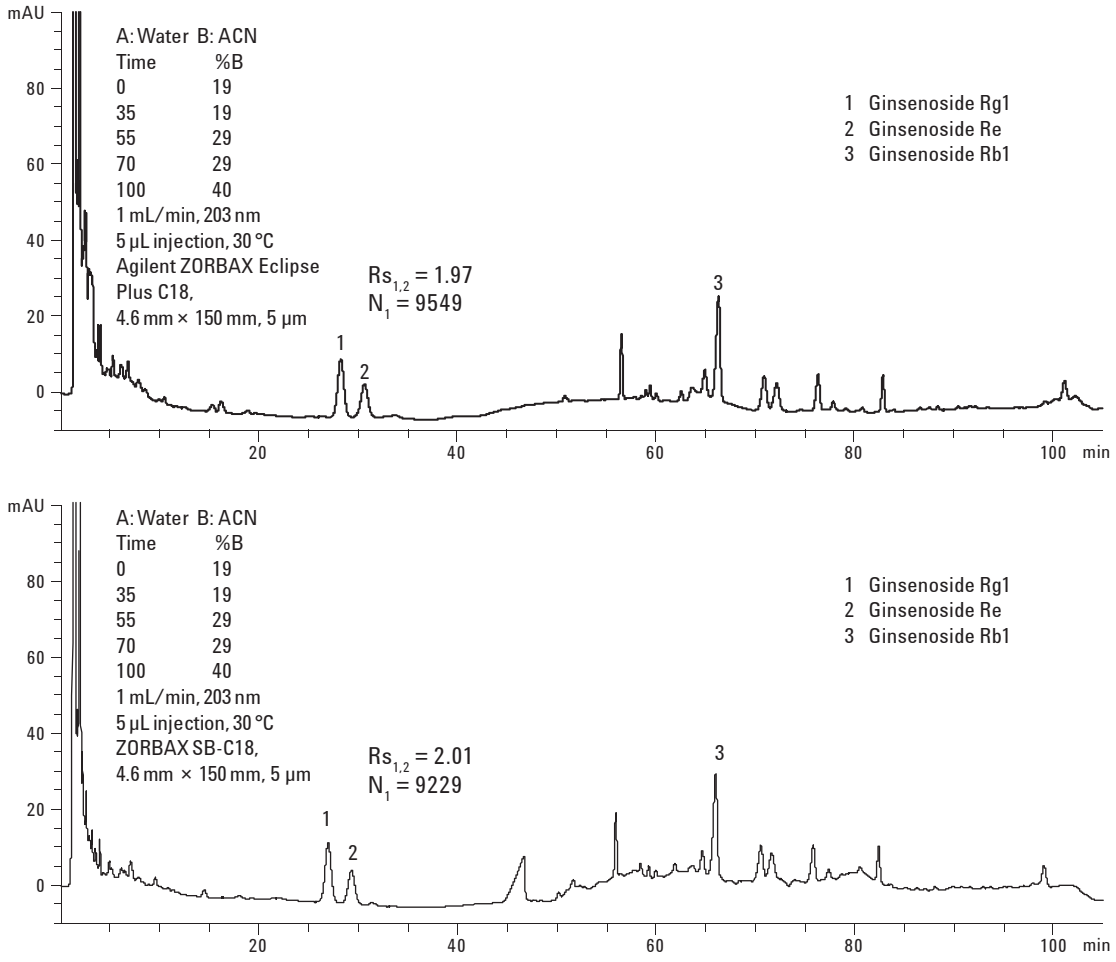

Figure 1. Original method for analyzing ginseng on an Agilent ZORBAX Eclipse Plus C18, 4.6 mm × 150 mm, 5 µm column and an Agilent ZORBAX SB-C18, 4.6 mm × 150 mm, 5 µm column.

The original method was then transferred to an Agilent Poroshell 120 EC-C18, 3.0 mm × 100 mm, 2.7 µm column. The flow rate was changed according to the below equation to maintain the same linear velocity.

Equation 1: $F_1/(r_1)^2 = F_2/(r_2)^2$

where

F_1 is the flow rate of original column
F_2 is the flow rate of new column
r_1 is the radius of original column
r_2 is the radius of new column

The gradient time is proportional to the column length while maintaining the original separation. Since the original column is 4.6 mm × 150 mm, using a 3.0 mm × 100 mm column at the same linear velocity shortens the gradient time by 100/150. The injection volume is decreased properly to avoid sample overload. The analysis time, therefore, decreased from 100 min to 66.7 min as shown in Figure 2. The backpressure of 174 bar is acceptable for a 400-bar HPLC. The chromatogram shows better resolution and theoretical plates than the original method.

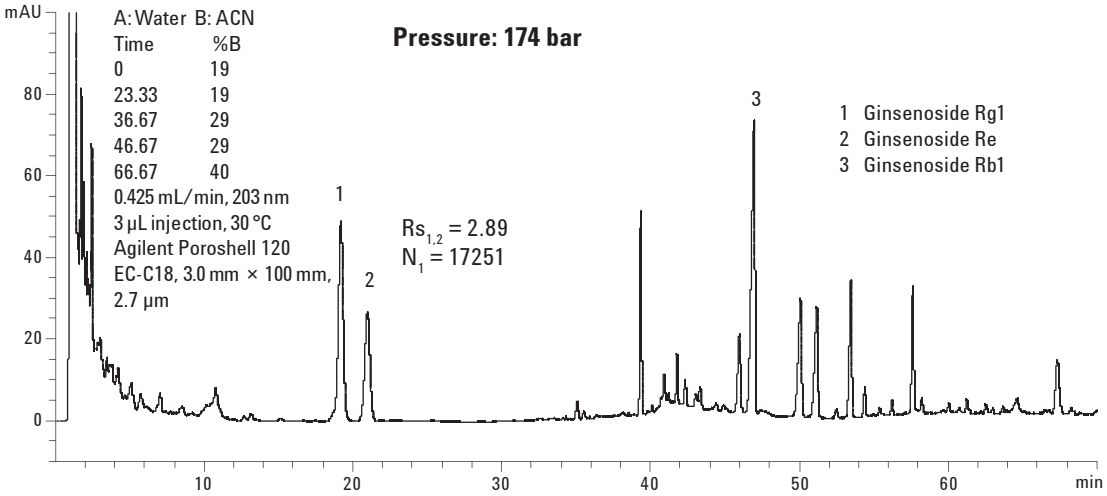

Figure 2. Chromatogram using an Agilent Poroshell 120 EC-C18, 3.0 mm × 100 mm, 2.7 µm column at normal flow rate.

The new method saves one-third of the original analysis time, and is a substantial improvement. The analysis time can be further shortened by increasing the flow rate and decreasing the gradient time. The gradient time is proportional to the flow rate using the same column. Therefore the analysis time reduces to half that with the new method when using twice the original flow rate (increasing from 0.425 mL/min to 0.85 mL/min). The cycle time is further reduced by using three times the original flow rate (Figure 3) with some loss in resolution and theoretical plates, which are still better than those on traditional columns. This is possible because the Van Deemter curve of the superficially porous Poroshell 120 2.7 µm particles is similar to columns with 1.8 µm particles. The efficiency performance of the Poroshell column does not decrease at high flow rate, compared to that of columns with 1.8 µm packing. At three times the original flow rate the pressure is about 3 times higher than the original 174 bar and is about 485 bar. This pressure is fine for the column, which can be used up to 600 bar, but does require an HPLC or UHPLC with a pressure limit of at least 600 bar, such as the Agilent 1260 Infinity LC.

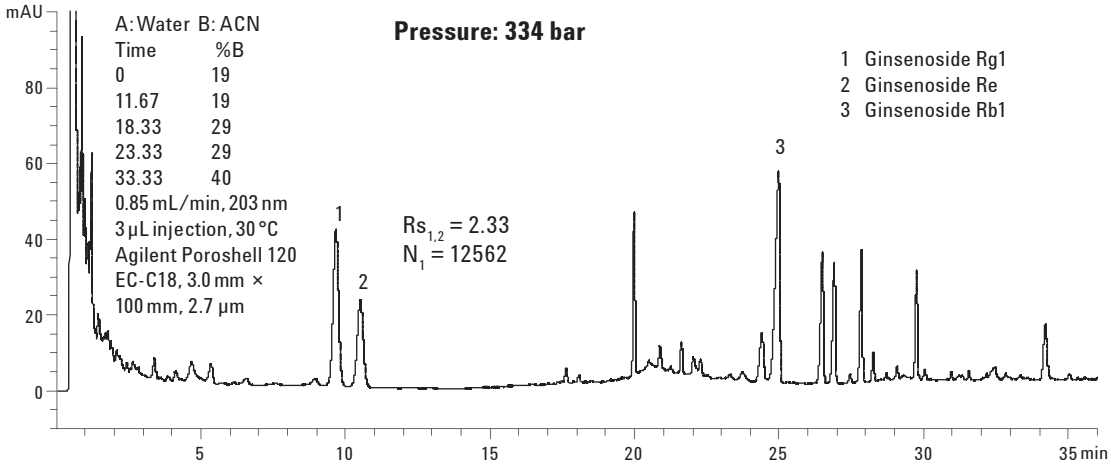

Figure 3. Chromatograms at higher flow rate using an Agilent Poroshell 120 EC-C18, 3.0 mm × 100 mm, 2.7 µm column. (Continued)

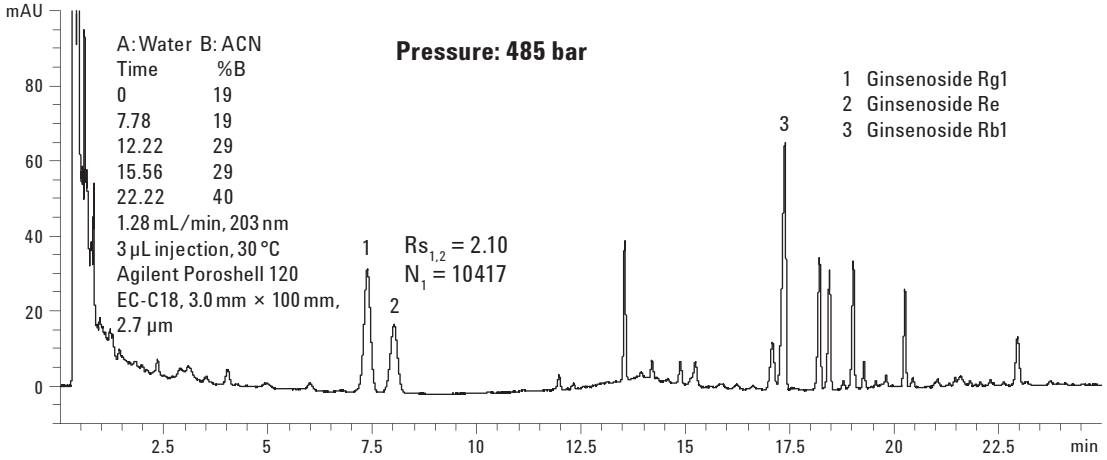

Figure 3. Chromatograms at higher flow rate using an Agilent Poroshell 120 EC-C18, 3.0 mm × 100 mm, 2.7 µm column.

Significant time savings is found in the overlaid chromatogram (Figure 4) using an Agilent Poroshell 120 EC-C18, 3.0 x 100 mm, 2.7 µm column with different flow rates.

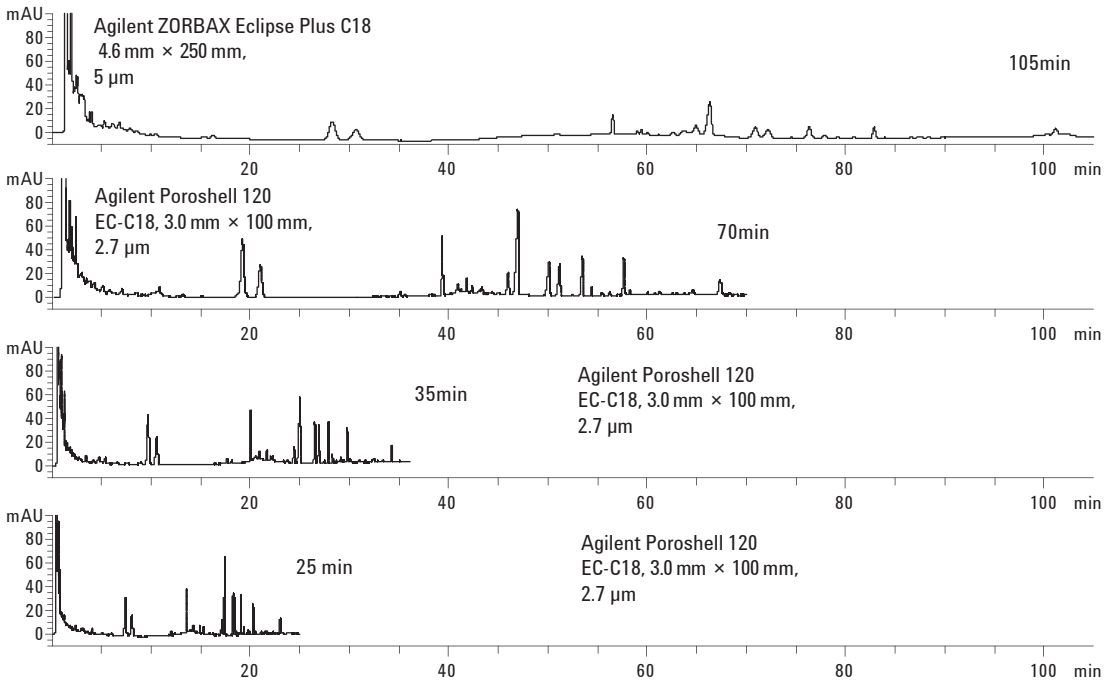

Figure 4. Overlaid chromatograms using an Agilent ZORBAX Eclipse Plus C18, 4.6 mm x 150 mm, 5 µm column and an Agilent Poroshell 120 EC-C18, 3.0 mm × 100 mm, 2.7 µm column at different flow rates.

The method developed on an Agilent Poroshell 120, 3.0 mm × 100 mm column saved three-fourths (75%) of the original analysis time, however the separation time can be further shortened using a shorter column. The method was then transferred to an Agilent Poroshell 120, 3.0 mm × 50 mm column. The separation requires 23 min with the Rs of ginsenosides Rg1, Re close to 2.0. The separation could be complete in only 11 min (about one-tenth of original time) when the flow rate was doubled with some compromise of resolution and performance, which is still acceptable for quantitative measurement.

It is important to note that the pressure is only around 200 bar, which is far below the limit of a 400-bar instrument. Theoretically, this fast separation could be run on a 400-bar instrument, but for the delay volume and post column volume, the instrument should be optimized and HPLC condition adjusted to achieve the ideal separation.

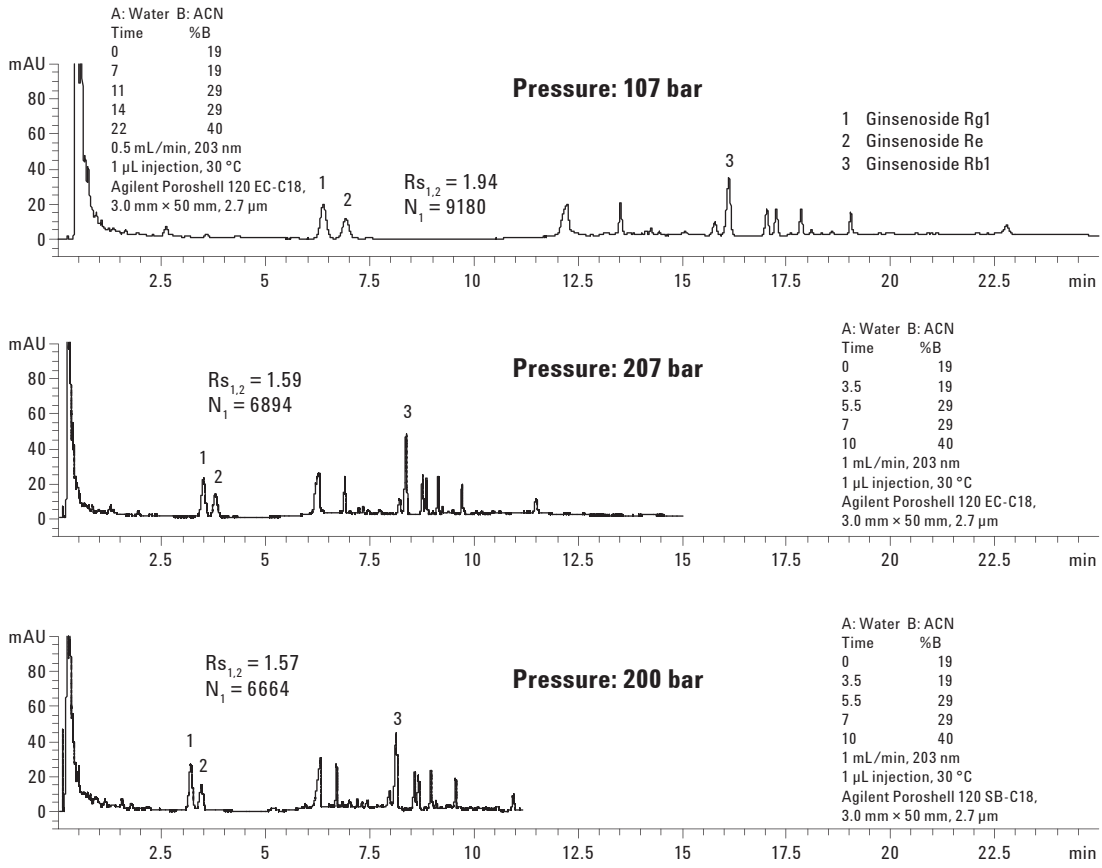

Figure 5. Chromatograms of ginseng using an Agilent Poroshell 120 EC-C18, 3.0 mm × 50 mm, 2.7 µm and an Agilent Poroshell 120 SB-C18, 3.0 mm × 50 mm, 2.7 µm columns

Conclusion

The method for the analysis of ginsenosides in ginseng was successfully transferred from a traditional 4.6 × 150 mm, 5 µm column to a Poroshell 120 column with substantial time savings and no compromise in resolution. The best method choice depends on what time is desired and what HPLC's are available for use. The superficially porous 2.7 µm particle columns provide similar performance to that of the totally porous sub-2-µm columns but with lower pressure. Due to the low pressure, a 400-bar instrument can run this method. A higher flow rate allows faster separations on a UHPLC, up to the 600-bar pressure limit of the column.

Reference

1. http://nccam.nih.gov/health/asianginseng/
2. Ginseng, China Pharmacopoeia, edition 2010

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Analysis of Naproxen Using Poroshell 120 EC-C18: Headache Free Method Adjustment

Application Note

Pharmaceuticals

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Abstract

The USP method for Naproxen is demonstrated using Eclipse Plus C18 and Poroshell 120 EC-C18. When a 4.6 x 50 mm Poroshell 120 EC-C18 column is used, the modified method reduces analys time to 22% of the original method without revalidation.

Agilent Technologies

Introduction

The costs associated with pharmaceutical testing are considerable and many lab managers are seeking ways to reduce costs by reducing solvent usage and improving productivity while still using the LC instruments in their lab.

Compendia methods from the USP (United States Pharmacopeia) are widely used in pharmaceutical drug product and raw materials testing. However, not all methods in the USP use modern technologies and may be more time consuming than desired. These methods can be updated by making adjustments following the recommendations in USP chapter <621>. The ranges for adjustments that were used in this method are: column length, column material, particle size, and injection volume. While other parameters can be adjusted according to the USP, none of those were needed to improve the throughput of this method. Modifications outside these ranges are considered changes and require revalidation of the method.

Naproxen is classified as a non-steroidal anti-inflammatory drug or NSAID and is available as generic tablets. The USP contains a method for the analysis of Naproxen tablets, which uses an L1 (C18), 5 µm column. The structure of Naproxen is shown in Figure 1.

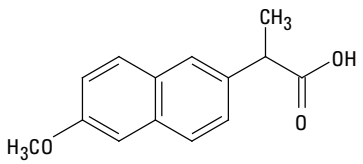

Figure 1. Structure of Naproxen.

Agilent Poroshell 120 columns are an LC column choice that can provide improved performance on a typical LC instrument. These columns have a 2.7 µm superficially porous particle that can provide faster analysis and high resolution in shorter columns for testing more samples in less time on existing LC instruments. The columns are available in a C18 bonded phase, a typical L1 material. In Figure 1, a 4.6 mm × 150 mm, 5 µm L1 or C18 column is used as the starting point for the method. The conditions are unchanged and both an Agilent Poroshell 120 EC-C18 4.6 mm × 100 mm, 2.7 µm and an Agilent Poroshell 4.6 mm × 50 mm, 2.7 µm column are included for comparison.

Experimental

- Agilent 1200 Series Binary Pump SL, Mobile phase Channel A: Acetonitrile: Water: Glacial Acetic Acid (500:490:10); Flow rate was 1.2 mL/min, in some work the flow rate is increased up to 2.2 mL/min (G1312B)
- Agilent 1200 Series Automatic Liquid Sampler SL (ALS), injection volume was 20 µL for the 150 mm column, 13.34 µL for the 100 mm column, 6.67 µL for the 50 mm column (G1376C)
- Agilent 1200 Series Thermostatted Column Compartment SL(TCC), Temperature was 25 °C (G1316B)
- Agilent 1200 Series Diode Array Detector SL (DAD), wavelength used was 254, 4 nm, with a G1315-60024 micro flow cell (5-mm path, 6 µL volume) (G1316C)

Agilent ZORBAX Columns:

- Agilent Eclipse Plus C18, 4.6 mm × 150 mm, 5 µm p/n 959993-902
- Agilent Eclipse Plus C18, 4.6 mm × 100 mm, 3.5 µm p/n 959961-902
- Agilent Poroshell 120 EC-C18, 4.6 mm × 100 mm, 2.7 µm p/n 695975-902
- Agilent Poroshell 120 EC-C18 4.6 mm × 50 mm, 2.7 µm p/n 699975-902

Acetonitrile used was Burdick and Jackson ACS/HPLC Certified solvent, purchased from Honeywell. Glacial Acetic Acid used was ACS/USP Grade purchased from VWR. Water used was produced on site using a Millipore Milli-Q system,18 MΩ filtered to 0.2 µm. USP Naproxen was purchased from United State Pharmacopeia. Butyrophenone was purchased from Sigma-Aldrich. Sample and mobile phase preparation are from the USP method. [1]

Mobile Phase Preparation

The mobile phase is prepared by mixing acetonitrile, water, and glacial acetic acid (500 mL: 490 mL: 10 mL). [1]

Sample Preparation

Samples and internal standards are prepared in a mixture of acetonitrile and water (90:10). The internal standard is prepared by diluting 5 mL of butyrophenone with acetonitrile to make 100 mL. 1 mL of the resulting solution is diluted with acetonitrile to make 100 mL. Each mL of this solution contains about 0.5 µL of butyrophenone.

The USP Resolution Standard or USP Naproxen RS is prepared by dissolving an accurately weighed quantity of in Solvent mixture to obtain a solution having a known concentration of about 2.5 mg per mL. 1.0 mL of the resulting solution and 2.0 mL of Internal standard solution is transferred to a 100-mL volumetric flask, diluted with Mobile phase to volume, and mixed. This solution contains about 25 µg of USP Naproxen RS per mL. [1]

The chromatographic and performance requirements of the method are listed in the USP method. These are summarized below. [1]

- 4.6 mm × 150 mm column, L1 column (C18)
- N of the analyte not less than 4000 plates
- Resolution between the analyte and internal standard peaks is not less than 11.5

As can be seen in Figure 2, the efficiency and other chromatographic performance requirements of the USP method are easily met. During the course of a day using the USP method as written, an analysis can be performed every 9 minutes. This leads to a throughput of six to seven analyses per hour, or approximately 160 injections that can be made per day at 9 minutes per injection. Over the course of a week, 1120 injections can be performed using a 150 mm, 5-µm column. In many applications this throughput is sufficient. An increased throughput can be achieved by adjusting the method. The USP updated chapter <621> presents recommendations on how much a method can be modified such that the changes are considered an adjustment. [2]

- Column length ± 70%
- Column internal diameter ± 25%
- Column material particle size: Reduction of up to 50%, no increase
- Flow rate ± 50%
- Injection volume Changes are allowed as long as system suitability testing (SST) criteria are met
- Column temperature ± 10%
- pH of mobile phase ± 0.2
- UV wavelength: no change outside manufacturer specifications
- Concentration of salts in buffer ± 10%

Modifications outside these ranges are considered changes and require re-validation. If the analyst chooses to use a shorter column, such as a 4.6 mm × 100 mm, 3.5-µm column as shown in Figure 2, the same analysis could be accomplished in 67% of the time (approximately 6 minutes per sample). The method could also be easily applied to an Agilent Poroshell EC-C18 4.6 mm × 100 mm, 2.7 µm or an Agilent Poroshell EC-C18 4.6 mm × 50 mm, 2.7 µm. The 50 mm column is still within the allowed adjustment window and easily allows reduction of analysis time to 33% of the initial method time.

In order to achieve the best performance with Poroshell 120 or other small volume columns, it is necessary to optimize detector speed and minimize extra column volume. Typically, a data collection rate of 40 Hz is used. [3] To avoid column overloading, the injection volume is scaled geometrically as the column volume is reduced. This means a 150 mm column with a 20 µL injection is scaled to a 100 mm column with an injection volume equal to 20 × (100/150) or 13.67 µL and a 50 mm column is scaled to use an injection volume of 20 × (50/150) or 6.67 µL.

The performance requirements of the method are exceeded when changing from the 5 µm L1 columns to either of the superficially porous 2.7 µm C18 columns. The analysis on the 100 mm column is 2 × faster than the original method, and on the 50 mm long column the method is 4.5 × faster than the original method. Either column choice improves productivity.

One of the allowed adjustments is a change in flow rate by ± 50%. This would allow an increase of up to 1.8 mL/min under current rules. A suggested change is currently being discussed that would allow the linear velocity of the column and particle to remain constant, allowing a flow rate increase of almost 100% (up to 2.4 mL/min) without revalidation of the method. [4] Implementing these changes would increase through put even more.

Figure 3 shows the effect of increasing flow rate on an Agilent Poroshell 120 EC-C18 4.6 mm × 100 mm column. In this case, the flow is increased from 1.2 mL/min to 2 mL/min, while exceeding the efficiency and resolution requirements and remaining under 400 bar pressure. At 2.2 mL/min, the efficiency and resolution requirements are still met but we are now slightly above the 400 bar threshold.

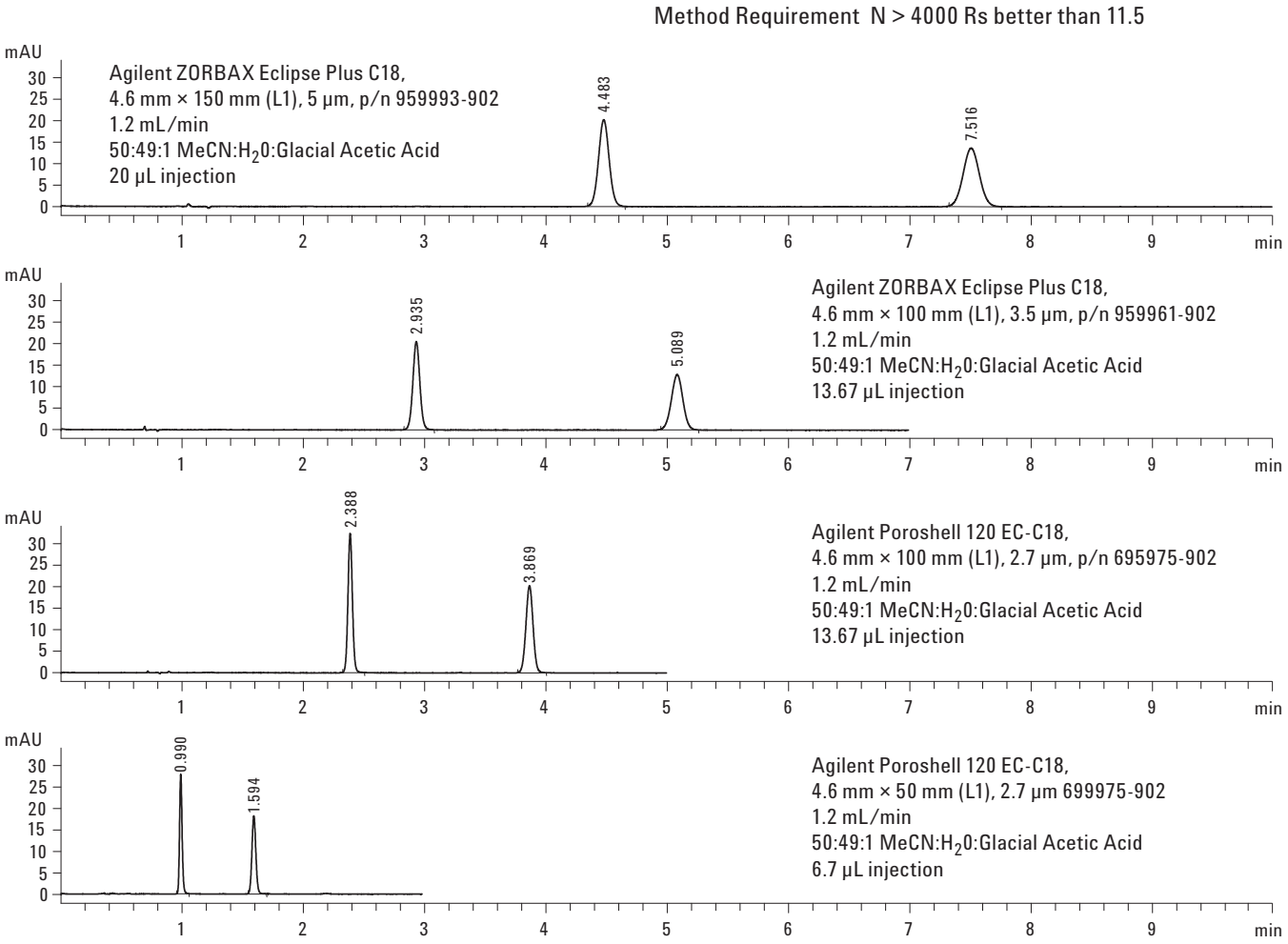

Figure 2. USP Naproxen Method Demonstrated on Varied Totally Porous and Superficially Porous Columns.

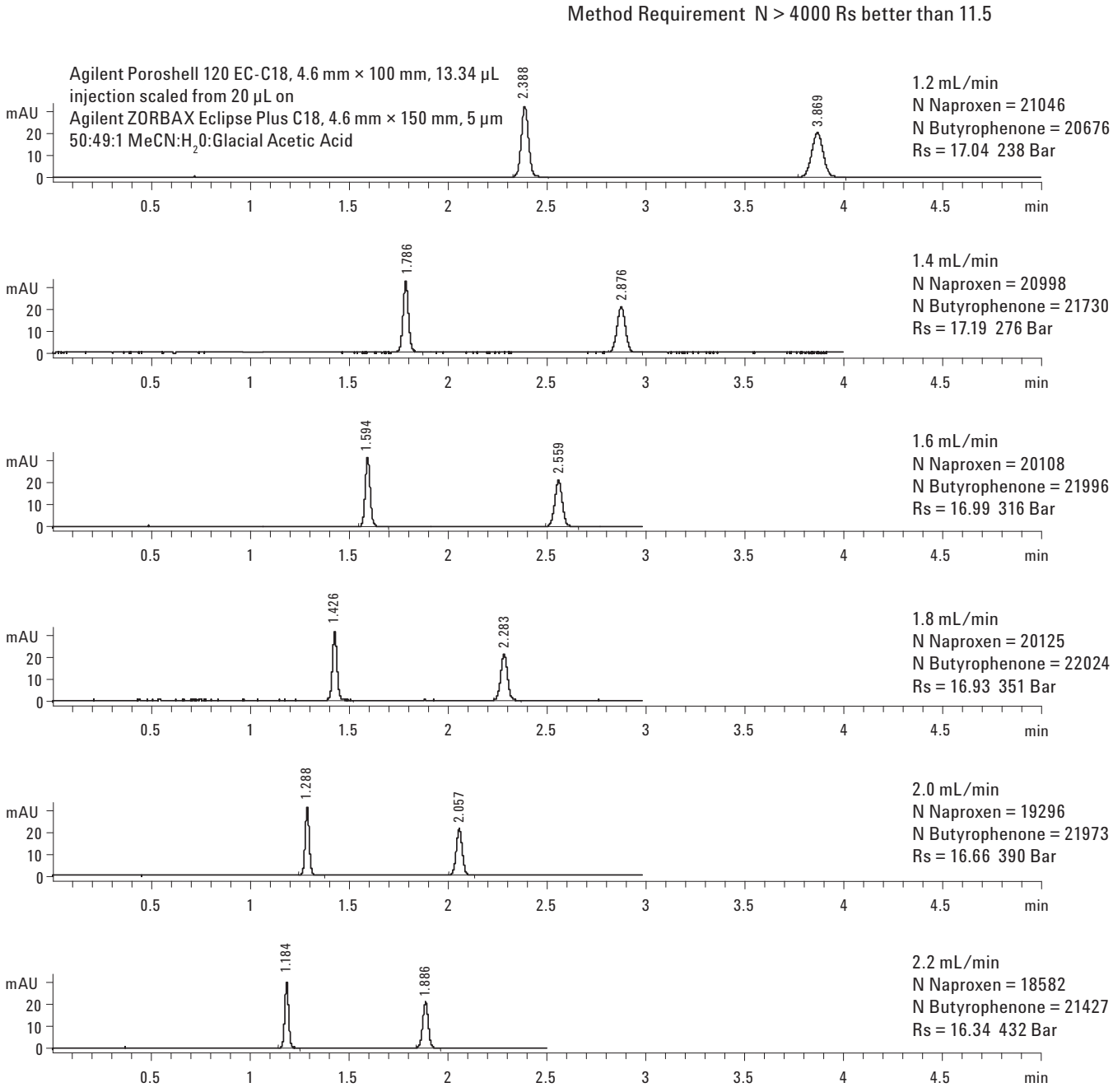

Figure 3. USP Naproxen Method Demonstrated on an Agilent Poroshell 120 EC-C18, 4.6 mm × 100 mm at varied flow rates.

Figure 4 shows the effect of increasing flow rate on an Agilent Poroshell 120 EC-C18, 4.6 mm × 50 mm column. In this case, the flow is increased from 1.2 mL/min to 2.4 mL/min, while exceeding the efficiency and resolution requirements and remaining under 300 bar pressure.

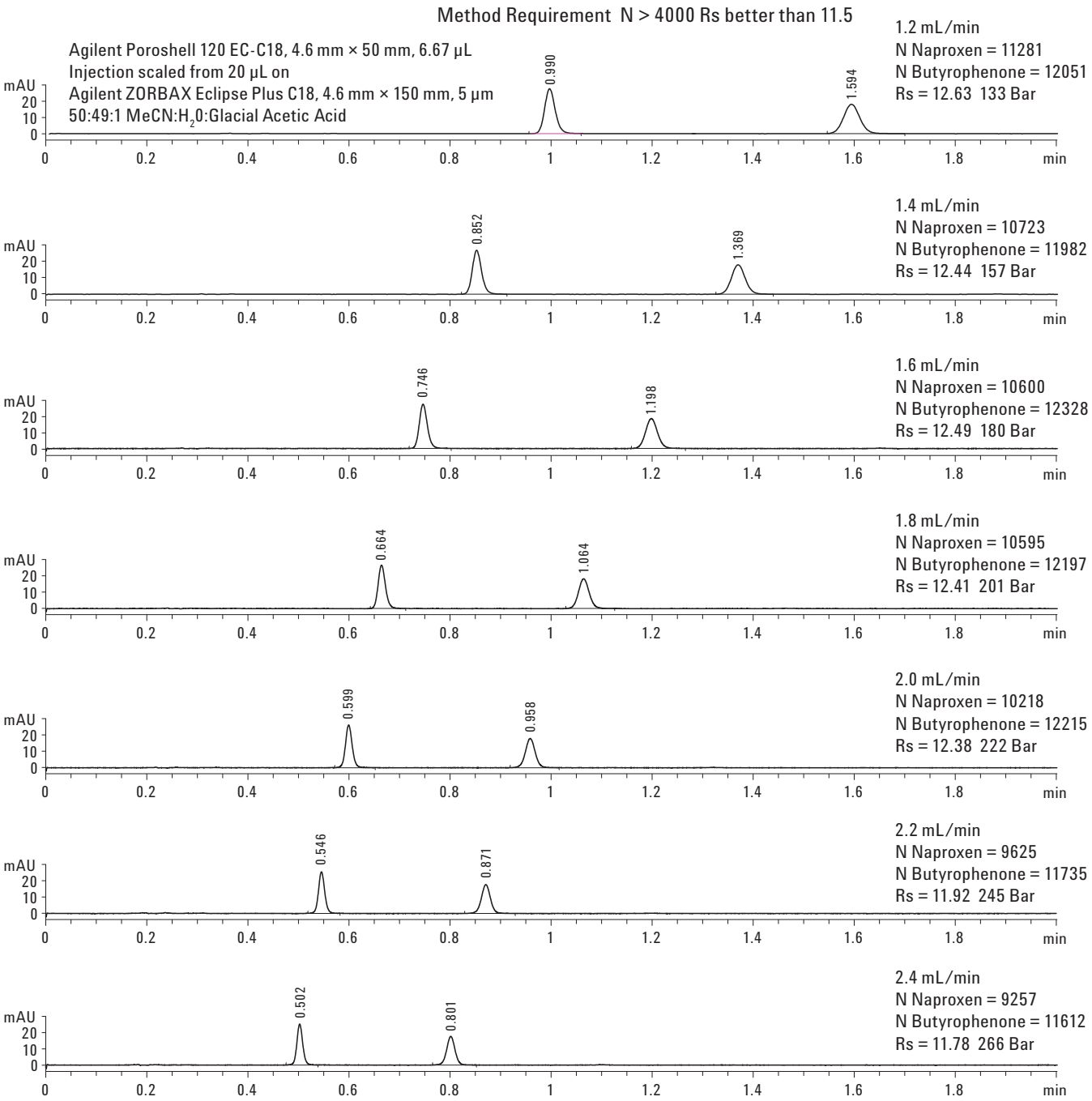

Figure 4. USP Naproxen Method Demonstrated on an Agilent Poroshell 120 EC-C18, 4.6 mm × 50 mm at varied flow rates.

Conclusions

Laboratories performing compendia analysis with fully-porous LC columns can benefit from the increased speed, resolution, and sensitivity that superficially porous, Agilent Poroshell 120 columns provide without having to replace existing instrumentation. Faster analysis times resulting in higher throughput and greater productivity can be achieved with Agilent Poroshell 120 columns. Method adjustments to these compendia methods with shorter length columns and the smaller 2.7 µm particle size provide these improved results.

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Fast Analysis of Cefepime and Related Impurities on Poroshell 120 EC-C18

Application Note

Pharmaceuticals

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Introduction

Cephalosporins are the most frequently prescribed class of antibiotics. They are structurally and pharmacologically related to the penicillins. Like the penicillins, cephalosporins have a beta-lactam ring structure that interferes with synthesis of the bacterial cell wall which means that they kill bacteria. Cephalosporin compounds were first isolated from cultures of Cephalosporium acremonium in 1948 by Italian scientist Giuseppe Brotzu. The first commercial product, Cephalothin was launched by Eli Lily in 1964.

Cephalosporins are bactericidal agents and have the same mode of action as other beta-lactam antibiotics (such as penicillins). All bacterial cells have a cell wall that protects them. Cephalosporins disrupt the synthesis of the peptidoglycan layer of bacterial cell walls, which causes the walls to break down and eventually the bacteria die. Cephalosporins are beta-lactam compounds in which the beta-lactam ring is fused to a 6-membered dihydrothiazine ring, thus forming the cephem nucleus. Modifications to the side chain modifications of the ring structure can improve antibacterial and pharmacokinetic activity. Based on their spectrum of activity, cephalosporins can be broadly categorized into four generations.

First generation cephalosporins are predominantly active against gram-positive bacteria, and successive generations have increased activity against gram-negative bacteria (often with reduced activity against gram-positive organisms). Gram-negative bacteria have a unique outer membrane that prevents many drugs from penetrating them, making gram-negative bacteria generally more resistant to antibiotics than are gram-positive bacteria [1].

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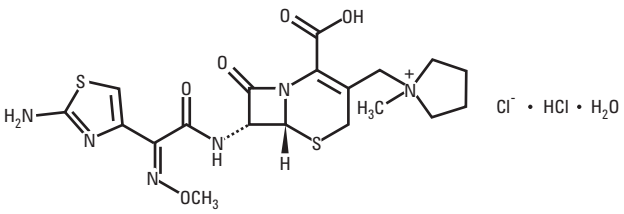

Figure 1. Structure of Cefepime.

Cefepime, fourth generation cephalosporin, is a broad spectrum antibiotic with improved activity against Gram-negative bacteria over other commercially available cephalosporin drugs. The structure of Cefepime is shown in Figure 1.

Despite extensive research on this class of drugs, quantitative analysis and purity assays remain problematic [2]. The EP and USP have published methods determine cefepime and the related compounds [3,4]. These methods use a phosphate/acetonitrile eluent at 1.0 mL/min on a 4.6 × 250 mm, 5 µm column. In this work, an Agilent Poroshell 120 EC-C18 column, 4.6 × 75 mm, 5 µm will be used to quickly analyze the Cefepime related compounds while meeting the requirements for both USP and EP methods. The shorter column rate used with this column saves time solvent due to less frequent eluent preparation, and produces less waste compared to the original EP and USP methods. Moreover, using a shorter column allows quick evaluation of allowable adjustments to methods resulting in better performance in less time.

Experimental

An Agilent 1200 Series Rapid Resolution LC (RRLC) system with an Agilent:

- G1312B Binary Pump SL Gradient times vary depending on column dimensions and flow rate, (Table 1).
- G1367C Automatic Liquid Sampler (ALS) SL, injection volumes are dependent upon specific method parameters, see Table 1.
- G1316B Thermostated Column Compartment (TCC) SL with temperature controlled at 25 °C.
- G1367C Diode Array Detector, set to 254 nm as described in the method.

Two Agilent columns were used in this work:

- Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm, p/n 959990-902
- Agilent Poroshell 120 EC-C18, 4.6 × 75 mm, 2.7 µm, p/n 697975-902

In addition, two 4.6 × 250 mm, 5 µm C18 competitive columns were also examined, and are designated C1 and C2.

Acetonitrile used was Burdick and Jackson ACS/HPLC Certified solvent, purchased from Honeywell. Monobasic Potassium Phosphate ACS/USP Grade purchased from VWR. Water used was produced on site using a Millipore Milli-Q system,18 M filtered to 0.2 µm. 0.45 µm Regenerated Cellulose Filter media (Agilent Technologies) was used for buffer filtration. USP Cefepime Hydrochloride and USP Cefepime Hydrochloride System Suitability RS was purchased from United State Pharmacopeia. Sample and mobile phase preparation are made following directions from the USP and EP. [3,4].

Mobile Phase Preparation

This method uses a gradient composed of a monobasic potassium phosphate buffer mixed with an amount of acetonitrile. The buffer is prepared by dissolving 0.68 g of monobasic potassium phosphate in 1000 mL of water. This buffer is adjusted with potassium hydroxide or phosphoric acid to a pH of 5.0, filtered through 0.45 µm filter media (regenerated cellulose was used in our lab) and degassed ultrasonically. The initial USP method uses a 9:1 ratio of buffer to acetonitrile for Mobile Phase A. All samples are subsequently prepared from this mobile phase. In the course of method adjustments, varied ratio's of "Mobile Phase A" are prepared. Mobile Phase B is prepared from the monobasic potassium phosphate buffer mixed with a 1:1 ratio v/v acetonitrile. The European Pharmacopeia (EP) method is similar but specifies the concentration of Potassium Hydroxide or Phosphoric acid used to adjust the solution pH, (0.05 M) and specifies that the mobile phase pH is adjusted before the addition of acetonitrile.

Assay Preparation

About 70 mg of Cefepime Hydrochloride, should be accurately weighed and transferred, to a 50-mL volumetric flask, dissolved in and diluted with Mobile Phase A to volume. This solution should be sonicated for approximately 30 minutes. The system suitablility sample is prepared at 7 mg/5 mL in Mobile Phase A. Sonication is important as impurity B is reluctantly soluble. NOTE: These solutions should be used immediately, or stored in a refrigerator and injected within 12 hours.

Results and Discussion

Chromatographic conditions as described in the USP and EP

were followed. In both cases an L1 (C18 column) is 4.6 × 250 mm, 5 µm is specified. The gradient program shown in Table 2. The liquid chromatograph uses an initial isocratic hold for 10 min of 100% mobile phase A increasing to 50% over the next 20 min. An isocratic hold at 50% A is maintained for 5 min, after which the solvent re-equilibrates to the initial 100% composition A. The total run time is 36 min.

In the original USP and EP methods a 4.6 × 250 mm, 5 µm L1 column is specified. Three different columns are shown in Figure 2. The method specifies a gradient and with recovery time approximately 45 min are required for each sample. Speeding up this method through adjustments presents a good opportunity for improving the method.

The chromatographic and performance requirements of the method are listed in the USP method. These are summarized below [1].

- 4.6 mm × 250 mm column, L1 column (C18)
- N of the analyte not less than 4000 plates.
- The resolution, R, between cefepime and cefepime related compound A is not less than five.
- The resolution, R between cefepime and cefepime related compound B is not less than 10.
- The capacity factor, k' of cefepime, is more than 0.6.
- Column efficiency is not less than 4000 theoretical plates.
- The tailing factor is not more than 1.5.

For the purpose of identification, the relative retention times are about 1.0 for cefepime, 2.7 for cefepime related compound A, and about 4.3 for cefepime related compound B.

The USP updated chapter <621> presents recommendations on how much a method can be modified such that the changes are considered an adjustment. [5] Table 1 summarizes these modifications. In previous work, modification of particle size and column dimensions have been demonstrated. [6,7]. In addition, new changes to these compendial methods are proposed that could allow linear velocity to remain constant as particle size decreases, thus increasing the flow rate beyond the present ± 50% [8]. In this case, we also look at the advantages of modifying the mobile phase composition on a substantially smaller column.

Table 1. Allowable Method Modifications under USP Chapter 621

Column length	± 70%
Column internal diameter	± 25%
Column material particle size	Reduction of up to 50%, no increase
Flow rate	± 50%
Injection volume	Changes are allowed as long as system suitability testing (SST) criteria are met
Column temperature	± 10%
pH of mobile phase	± 0.2
UV wavelength	No change outside manufacturer specifications
Concentration of salts in buffer	± 10%
Composition of mobile phase (adjustment of the minor component is allowed ± 30% or ± 10% absolute whichever is smaller (as discussed in the USP)	

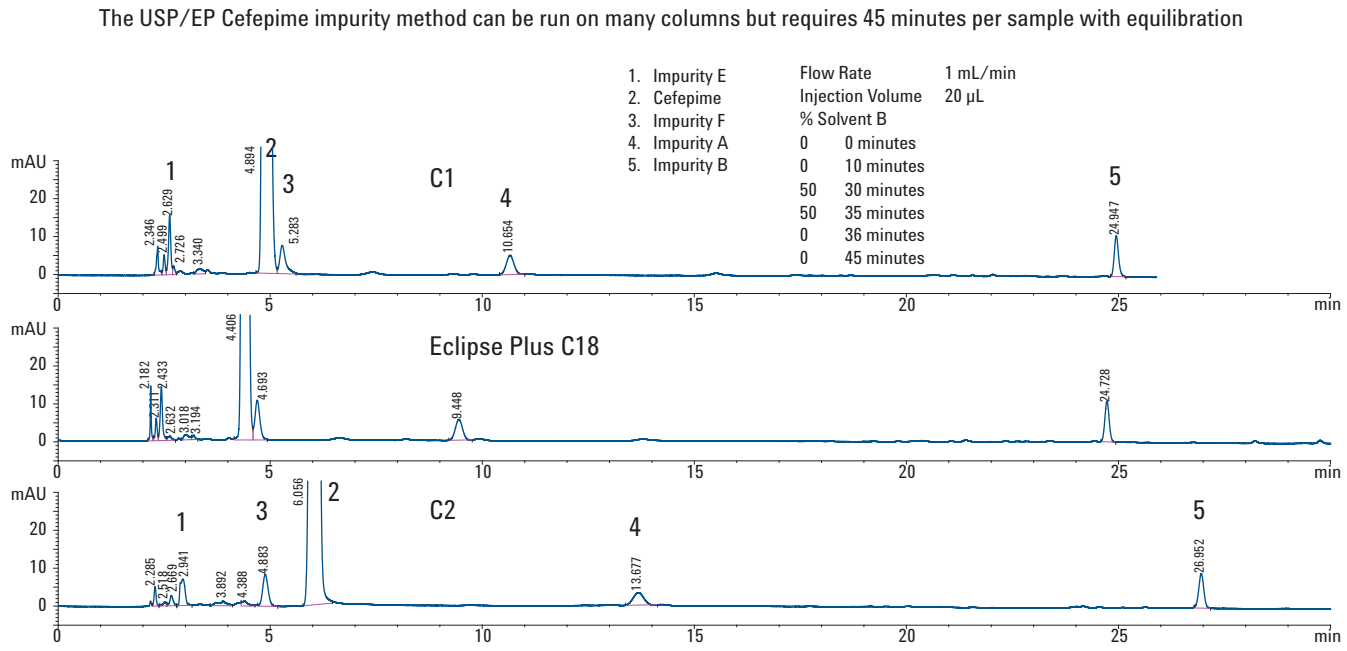

Figure 2. Original Method on 3 different columns, approximately 45 minutes per analysis.

Gradient Conditions were scaled according to the formula:

When a constant k* value is to be maintained, the equation above can be reduced into the following equation to calculate new gradient times:

$t_{g2} = (t_{g1} d_2^2 L_2 F_1)/(d_1^2 L_1 F_2)$

t_{g1} and t_{g2} are the original and new gradient times

d_1 and d_2 are the original and new column id's

L_1 and L_2 are the original and new column lengths

F_1 and F_2 are the original and new flow rates

For this work several allowed modifications were made. First the particle size is changed from 5 µm to 2.7 µm. This change yields an increase in efficiency as well as an increase in pressure. Because the column is 70% shorter the increase in pressure is minimized. An advantage of an Agilent Poroshell 120 is the narrow particle size distribution because of this, a 2 µm frit can be used, the same size used on 5 and 3.5 µm columns. This means that no additional care must be made in preparing samples than was used in the original method. Columns with particle sizes of 3, 2.5, and of course sub 2 µm use smaller size frit to retain the packing material in the column and as such are more apt to clogging [9].

The second modification made is a change from 250 mm length to 75 mm. This 70% reduction in length is allowed and can easily lead to higher throughput of samples if the performance of the column allows such a change.

Table 2. Table of Gradients

Inj. Volume % Solvent B	4.6 × 250 mm 5 µm, 1 mL/min, 20 µL	4.6 × 75 mm 2.7 µm, 1 mL/min, 6 µL	4.6 × 75 mm 2.7 µm, 1.5 mL/min, 6 µL	4.6 × 75 mm 2.7 µm, 2 mL/min, 6 µL
0	0 min	0.0 min	0.0 min	0.0 min
0	10 min	3.0 min	2.0 min	1.5 min
50	30 min	10.0 min	6.67 min	5.0 min
50	35 min	10.5 min	7.0 min	5.25 min
0	36 min	10.8 min	7.2 min	5.4 min
0	45 min	13.5 min	10 min	6.75 min

The minor component in the mobile phase is the Acetonitrile at 10%. Under USP modification rules an allowed change of ± 30% or ± 10% absolute whichever is smaller means that the acetonitrile can be reduced to 7% or increased to 13%. Under EP rules this change is still 7 to 13%. In this work, several concentrations of acetonitrile can be quickly calculated using this dramatically shorter column. It is important to note that the chromatographic solvents are varied in this method but

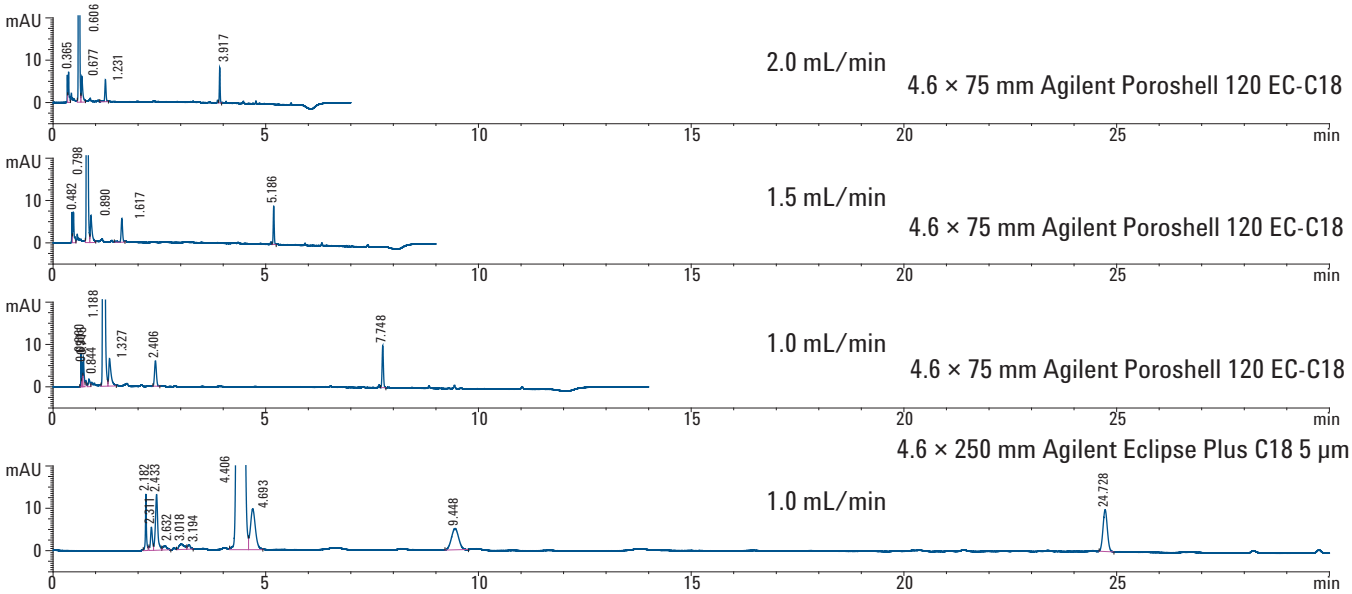

Figure 3. Totally Porous Agilent Eclipse Plus C18 column and Agilent Poroshell 120 EC-C18 column.

the sample preparation is left intact. It was noticed during the course of this work that Impurity B is less soluble than other components, and without sufficient ultra-sonication as specified in the method, this compound is not dissolved.

In Figure 4, the flow rate is increased to 2 mL/min to allow even faster method evaluation.

As can be seen, the initial acetonitrile is varied from 11% to 8%, within compendial guidelines for adjustment. At 10% the resolution of the cefepime and impurity B is 2, but by decreasing the initial organic modifier content to 8% the resolution between these peaks is increased to 4.9. It becomes evident that decreasing the initial concentration beyond 7% could potentially lead to additional peak resolution, but modifications outside these ranges are considered changes and require re-validation.

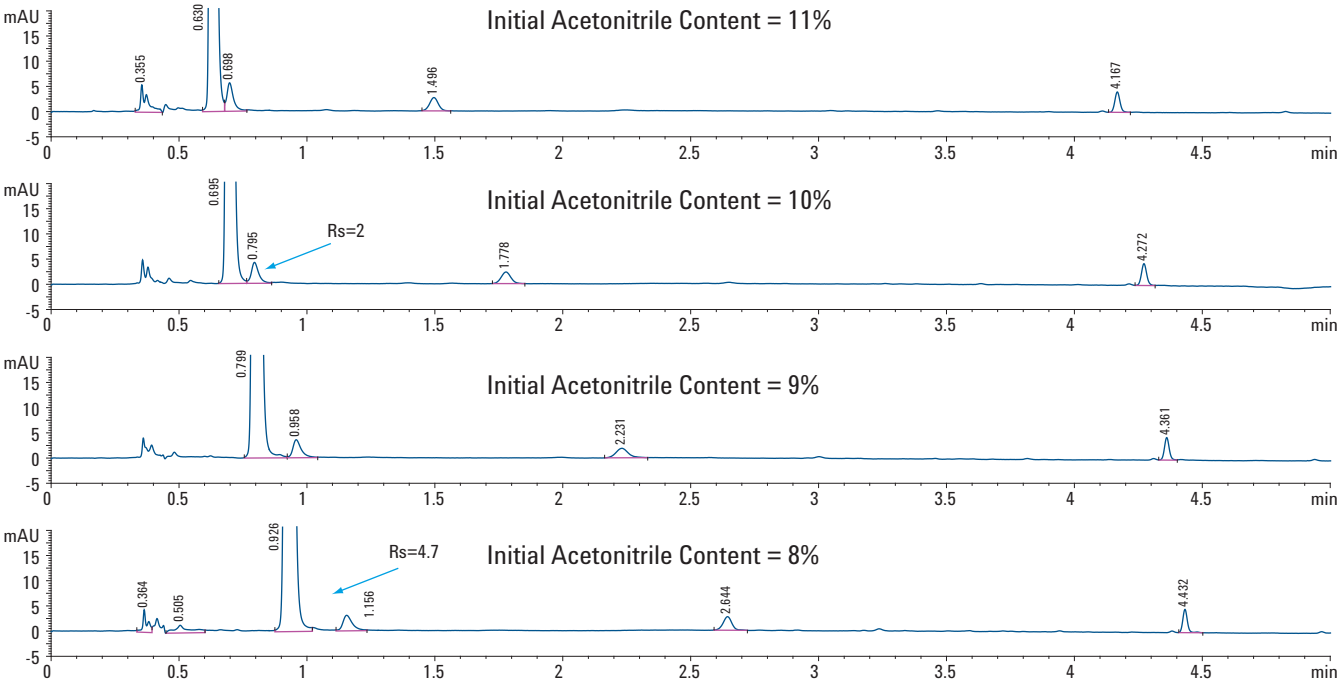

Figure 4. Modification of organic content to improve resolution. Fast method development with Agilent Poroshell 120 EC-C18.

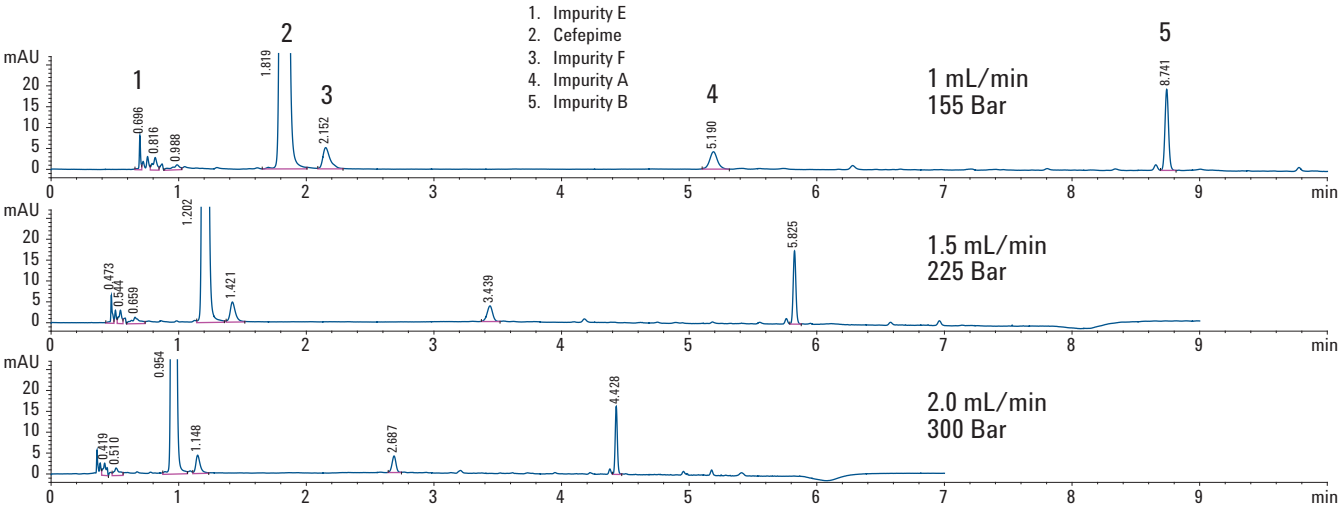

Figure 5. Final optimization of USP cefepime impurity Method, 8% initial acetonitrile at varied flow rates, using Agilent Poroshell 120 EC-C18 4.6 × 75 mm column.

Table 3.
Requirement: Agilent Poroshell 120 EC-C18 4.6×75 mm, 2.7 µm

	1 mL/min 156 bar	1.5 mL/min 221 bar	2 mL/min 300 bar
Tf<1.5	1.28	1.28	1.30
N>4000	18288	11993	7250
k'>0.6	1.4	1.4	1.4
Rs C/a	5.9	5.9	5.9
Rs C/b	10.7	10.7	10.7

Modifications outside these ranges are considered changes and require re-validation. If the analyst chooses to use a shorter column, such as a 4.6 mm × 50 mm, the same analysis could be accomplished in potentially 20% of the time. Further only 20% of the solvent would be used. However, this would require a complete revalidation. In cases such as assay methods, it might be easier to justify revalidation of a method, but impurity methods, are run less frequently. Tables 2 and 3 indicate that this analysis could easily be reduced in time from 36 minutes to 7.2 minutes without any need for new equipment, with an 80% reduction in analysis time. This would allow a lab to assay an incoming raw material within two hours of receipt instead of within 24 hours of receipt.

Conclusion

Laboratories performing compendia analysis with 250 mm fully-porous LC columns can benefit from the increased speed, resolution, and sensitivity that superficially porous, Poroshell 120 columns provide without having to replace existing instrumentation. The 75 mm column length is within the allowed modification range of USP and EP guidelines. Faster analysis times resulting in higher throughput and greater productivity can be achieved with Poroshell 120 columns. Method adjustments to these compendia methods with shorter length columns and the smaller 2.7 µm particle size provide these improved results.

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Converting a CHP Method for Insulin to Agilent Poroshell 120 Columns

Application Note

Biopharm

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Abstract

A regulatory method in the China Pharmacopeia (CHP) for insulin analysis calls for a traditional C18 LC column of either 4.6 × 150 mm or 4.6 × 250 mm, 5 µm. The traditional 5 µm particle size columns provide low efficiency performance for the insulin peak in either column length. In this application note, the traditional column was replaced with an Agilent Poroshell 120 EC-C18 or Agilent Poroshell 120 SB-C18 column. The adjusted methods using Poroshell 120 columns achieved significant improvements in efficiency performance and resolution while still meeting the requirements of the CHP or United States Pharmacopia (USP) regulatory methods.

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Introduction

Insulin is a peptide hormone composed of 51 amino acids and has a molecular weight of 5808 Da. It is produced in the islets of Langerhans in the pancreas. Its structure varies slightly between species of animals. Insulin "strength" from animals differs from humans because of variations in carbohydrate metabolism control effects. Bovine insulin differs from human in only three amino acid residues, and porcine insulin is close to human insulin. Insulin has been widely used for the treatment of both type 1 and some cases of type 2 diabetes. Regulatory methods in the CHP [1] and USP [2] specify a long isocratic elution for the insulin assay and a long gradient for the analysis of related compounds. Both methods share the same HPLC conditions for the assay but have slightly different gradient methods for the analysis of related compounds.

In this application note, the HPLC methods for the assay and related compounds in the CHP were first run on an Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm or an Agilent ZORBAX Eclipse Plus C18, 4.6 × 150, 5 µm column. The methods were then transferred to a column with superficially porous particles, the Agilent Poroshell 120 column, which delivers similar performance to columns with sub-2 micron particles for fast separations.

Materials and Methods

The CHP HPLC conditions for related compounds and assay of porcine insulin

Columns	Octadecyl silane (C18) chemically bonded to porous silica
Flow rate	1.0 mL/min
Injection volume	20 µL
Column temp	40 °C
Wavelength	214 nm

Mobile Phase for related compounds:

Mobile phase A	0.2 mol/L sulfate (Dissolve 28.4 g anhydrous sodium sulfate in 1000 mL of water, pipet 2.7 mL of phosphoric acid the solution and adjust with ethanolamine to a pH of 2.3, and mix) -acetonitrile (82:18)
Mobile phase B	Acetonitrile:water (50:50)

Referring to the gradient as follows, adjust the mobile phase composition and the duration of the isocratic elution to obtain a retention time of about 25 minutes for insulin, with the A-21 desamido insulin eluting just prior to the start of the gradient elution phase.

Time (min)	%B
0	22
35	22
61	67
67	67

Mobile phase for assay

Mobile phase A	0.2 mol/L sulfate (Dissolve 28.4 g anhydrous sodium sulfate in 1000 mL of water, pipet 2.7 mL of phosphoric acid solution and adjust with ethanolamine to a pH of 2.3, and mix)
Mobile phase B	Acetonitrile (74:26)

Materials used for the note

Sample	Porcine insulin (Provided by NIFDC China)
Columns	Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm (p/n 883975-902)
	Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm (p/n 959993-902)
	Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm (p/n 685975-902)
	Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7 µm (p/n 695975-302)
System	The Agilent 1200 SL LC system includes a binary pump, a thermostatted column compartment (TCC), a high performance autosampler and a diode array detector (DAD).

Results and Discussion

Traditional columns

The CHP requires a column with octadecyl silane (C18) chemically bonded to porous silica as the packing material which is also within USP L1 materials. Traditional 5 µm columns are commonly used for CHP methods, but smaller particle sizes are allowed if the results meet the requirements. Therefore the method was first run on an Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm column. The mobile phase composition was modified according to the requirements of CHP method to obtain a retention time of about 25 minutes for insulin. The chromatogram from the analysis of related compounds is shown in Figure 1 and the assay chromatograms are shown in Figure 2.

The system suitability of insulin analysis requires the resolution between insulin and A-21 desamido insulin not less than 1.8 and the tailing factor for the insulin peak not more than 1.8. As you can see in Figure 1 and Figure 2, both traditional columns meet the suitability requirements in the CHP. The Agilent ZORBAX Eclipse Plus C18 column provides more symmetrical peaks and higher efficiency than the SB-C18 column, which may be due to two differences between the columns. The first difference is the complete endcapping of the Eclipse Plus C18 column versus no endcapping on the SB-C18 column. Endcapped columns typically have better peak shape for basic compounds. The second difference is the larger pore size (95 Å for Eclipse Plus C18, and 80Å for SB-C18). The larger pore size of the Eclipse Plus C18 column improves the efficiency and peak shape with improved diffusion in the larger pore.

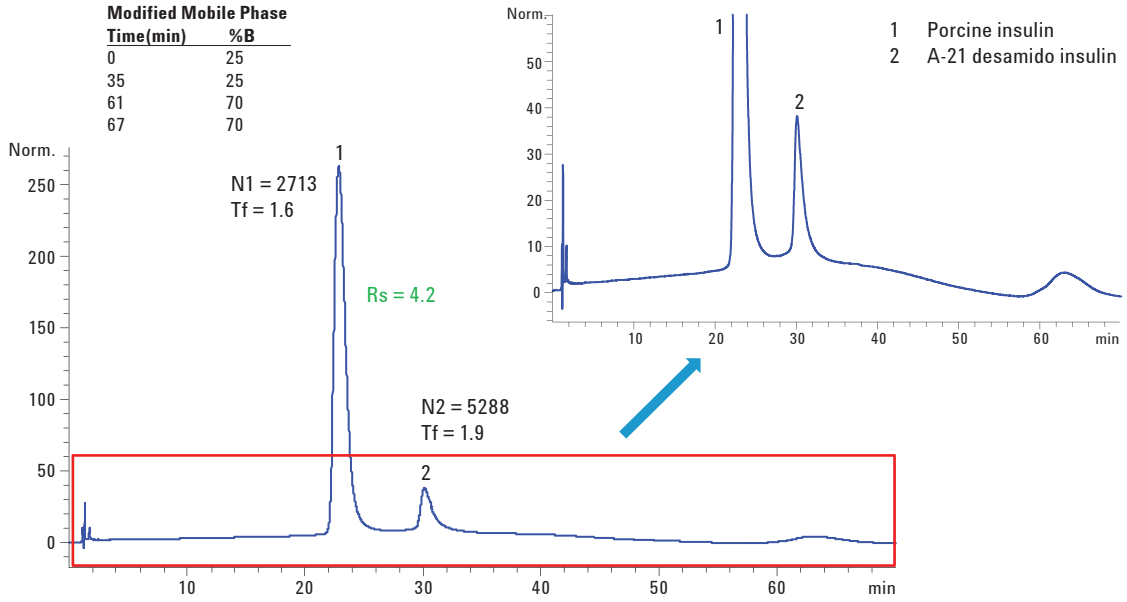

Figure 1. Chromatogram of related compounds analysis on a traditional Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm column.

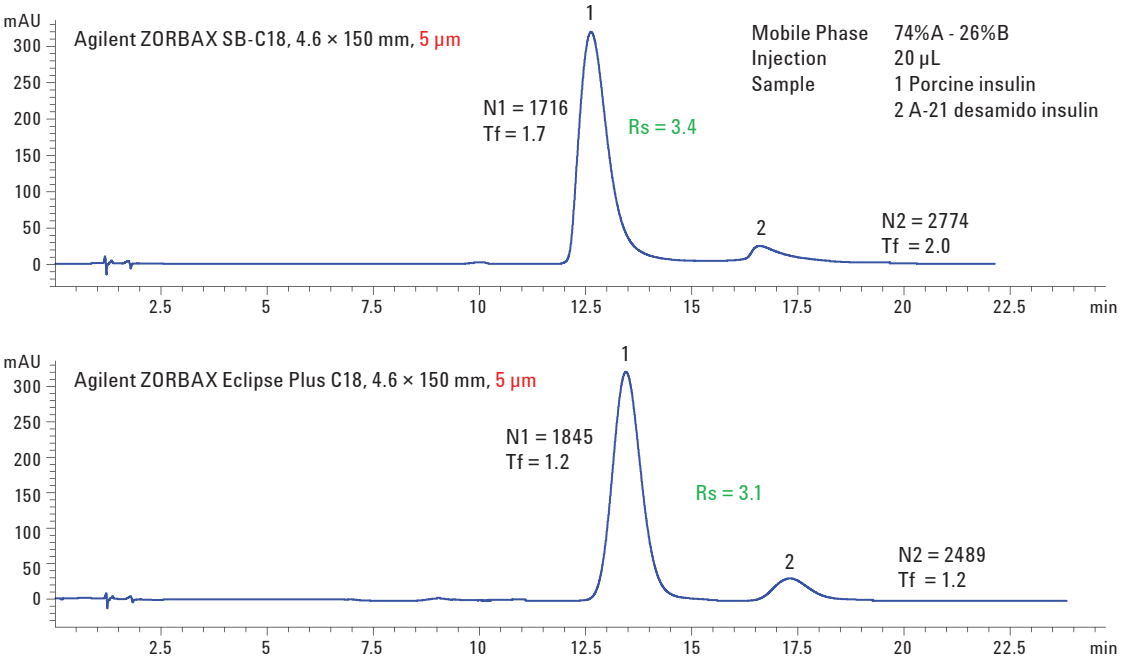

Figure 2. Chromatograms for assay analysis on traditional Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm and Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm columns.

Agilent Poroshell 120 Columns

The related compounds analysis method was transferred to an Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm column. A slight change in the mobile phase composition was made to fit the requirements of the CHP. The gradient time and injection volume need to be recalculated when scaling the original method to a new one.

To maintain the resolution and overall separation the retention index K* in Equation 1 should be kept constant.

Equation 1: $K^* = (t_G F)/(S\ \Delta\Phi\ Vm)$

Where:
t_G is the gradient time
F is the flow rate
S is constant
Vm is volume of column, $(Vm = \Pi\ (d/2)^2(L)(0.6)$, L is the column length, d is the column diameter)
$\Delta\Phi$ is the change in organic percentage across the gradient segment

According to Equation 1, the flow rate and gradient time should be changed with column diameter and length.

To keep almost the same response of the peaks, the injection volume should be changed proportional to the volume of the column (Equation 2).

Equation 2: $(d_1/2)^2 L_1 = (d_2/2)^2 L_2$

Where:
d_1, d_2 are the column diameter for column 1 and column 2 separately
L_1, L_2 are the column length for column 1 and column 2 separately

The new gradient method for related compounds was run on an Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm column. The chromatogram and data is shown in Figure 3. The assay method was run on a Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm column and a Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7 µm column. The Poroshell 120 columns show many improvements when compared to the traditional SB-C18 column (Figure 1). These improvements include peak shape, efficiency, and resolution. The performance on Poroshell 120 columns is 4-6 times higher than on traditional 5 µm columns. Some minor impurities were found in the chromatogram using the Poroshell 120 columns (Figure 3) due to the improved peak shape, increased efficiency, greater sensitivity, and resolution of the superficially porous columns. While these impurities may have been present in the separation on the traditional column the broader, less efficient peaks reduced the resolution such that they were not detected on the traditional 5 µm column.

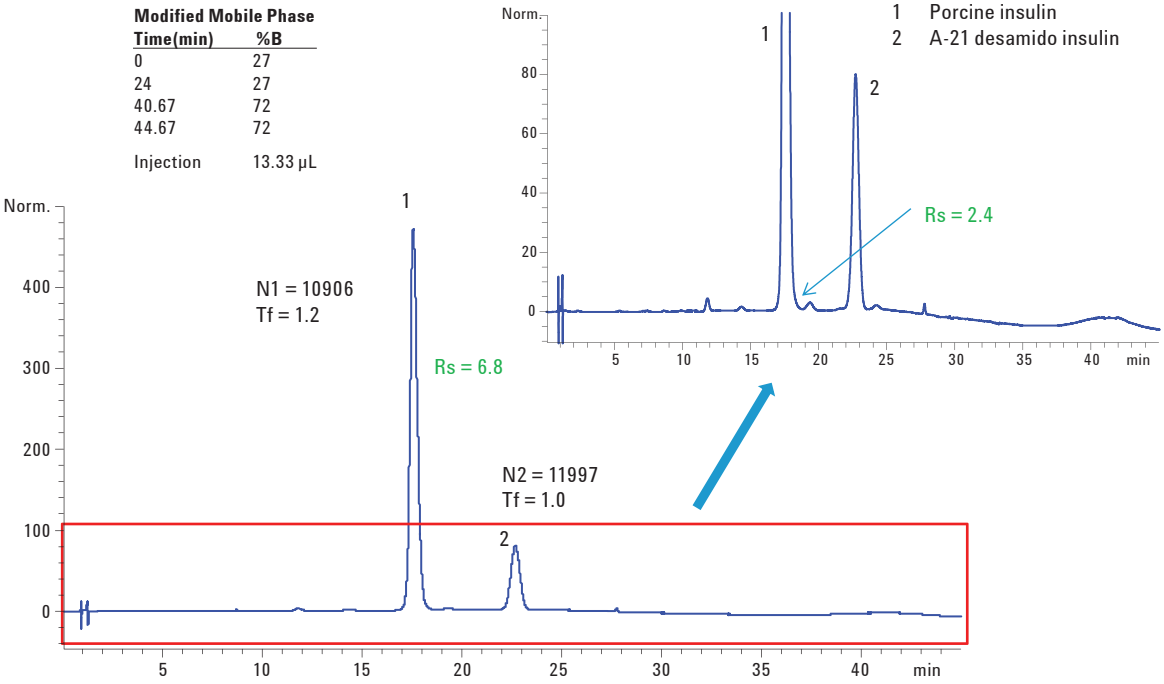

Figure 3. Chromatogram of related compounds analysis on a traditional Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm column.

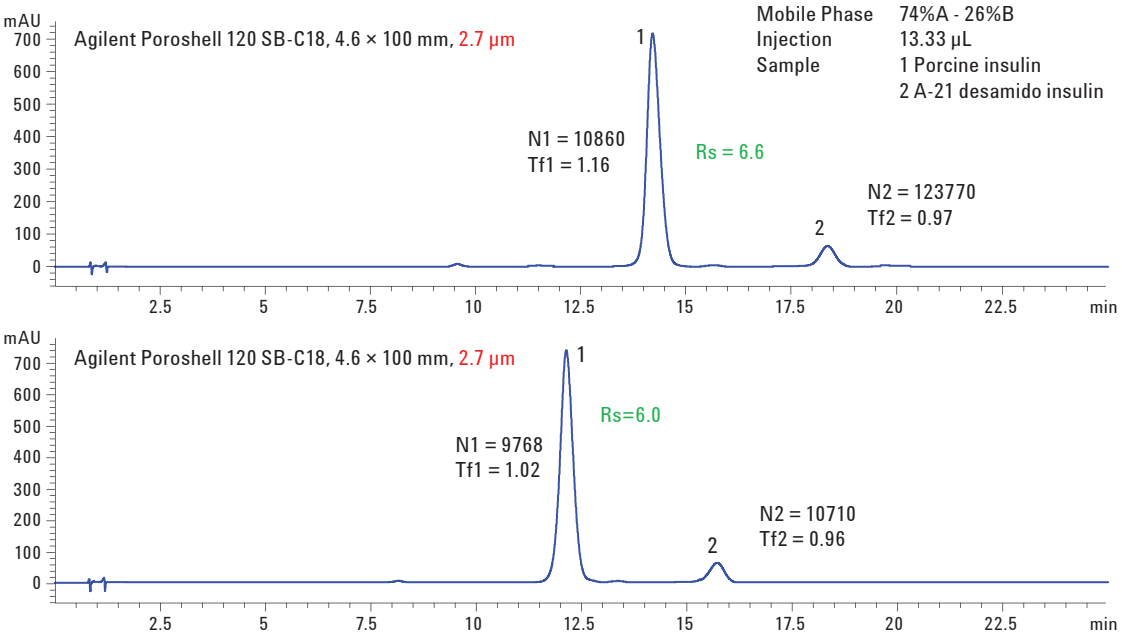

Figure 4. Chromatograms for assay analysis on Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm and Poroshell EC-C18, 4.6 × 100 mm, 2.7 µm columns.

Agilent Poroshell 120 SB-C18 and EC-C18 columns provide good performance for insulin analysis (Figure 4). They easily meet the system suitability requirements. The dramatic increase in performance is due to the smaller particles (2.7 µm) and the larger pore size (120 Å) of the superficially porous Poroshell 120 columns. For more information on the relationship between pore size, particle size, and molecular weight, consult publication number 5990-9028EN.

Reproducibility from injection to injection is important for reliable results. The CHP requirement is a Relative Standard Deviation (RSD) for five replicate injections of not more than 2%. This is a typical requirement for many LC methods. The RSDs of peak area from five replicate injections using the Poroshell 120 column (Figure 5) were 0.2% for porcine insulin and 0.4% for A-21 desamido insulin, easily meeting the requirements.

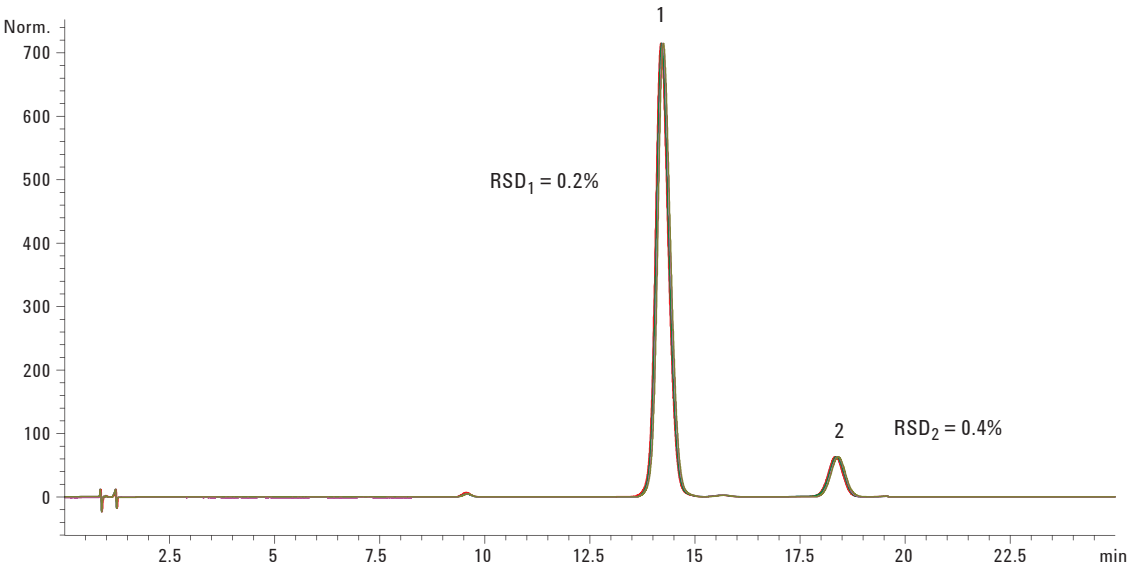

Figure 5. Overlay chromatograms of five injections on an Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm column.

Conclusion

The method for the analysis of insulin was successfully converted from a traditional 5 µm column to superficially porous Agilent Poroshell 120 columns with significant improvements in performance. A Poroshell column with a particle size of 2.7 µm, and pore size of 120 Å is suitable for the highly efficient analysis of small proteins, such as insulin, and can be used to meet the system suitability requirements of the CHP for insulin. The new method using the Poroshell 120 column is well suited for quality control testing of manufactured insulin.

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Analysis of Echinacoside and Verbascoside in *Cistanche deserticola* Chinese Medicine Using an Agilent Poroshell 120 EC-C18

Application Note

Traditional Chinese Medicine

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Introduction

Due to the complex components in traditional Chinese medicines (TCM), a gradient method with a long time period is often required to separate the target compounds from the complex matrix. HPLC has been an effective method for quality control of TCM applied in the China Pharmacopoeia (CHP).

Cistanche deserticola Y. C. Ma (1960), a commonly used TCM included in the CHP, is prescribed to reinforce the vital function of the kidney and to influence fertility, leukorrhea, and metrorrhagia in women. Phenylethanoid glycosides in *C. deserticola* are the most important active compounds, which have functions of antioxidation, protecting liver and nerves [1]. In the CHP, the amounts of the two main phenylethanoid glycosides of echinacoside and verbascoside (Figure 1) in *C. deserticola* extracts are regulated using HPLC for quality control.

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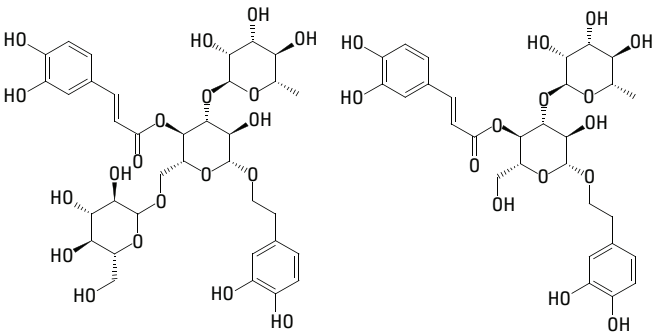

echinacoside verbascoside

Figure 1. Figure 1. Structures of echinacoside and verbascoside

Traditionally, it takes about 35 minutes to analyze the two compounds with the CHP method using a conventional 5 µm particle column. This application note describes a fast quality control method for the analysis of echinacoside and verbascoside using the Agilent 1290 Infinity LC System and an Agilent Poroshell 120 EC-C18, 2.7 µm column. Compared to conventional methods, the rapid method is much faster, and maintains the same performance and quality of separation. In addition, solvent consumption is dramatically reduced.

HPLC conditions

The analysis was performed with the 1290 Infinity LC System including a G4220A Infinity binary pump, G4226A Infinity sampler (ALS), G1316C Infinity Thermostatted Column Compartment (TCC), and G4212A Diode Array Detector SL (DAD).

Conditions

Sample	Extract of Cistanche deserticola
Mobile phase	A, 0.1% (v/v) formic acid; B, methanol
UV	330 nm
TCC temp	30 °C

Conditions for Figure 2

Column	Agilent ZORBAX Eclipse Plus C18 4.6 × 150 mm, 5 µm (p/n 959993-902)	
Gradient	time (min)	%B
	0	26.5
	17	26.5
	20	29.5
	32	29.5
	33	80
Stop time	35 min	
Flow rate	1 mL/min	
Injection volume	10 µL	

Conditions for Figures 3 and 4

Column	Agilent Poroshell 120, EC-C18, 3.0 × 50 mm, 2.7 µm (p/n 699975-902)	
Gradient	time (min)	%B
	0	26.5
	5.67	26.5
	6.67	29.5
	10.67	29.5
	11	80
Stop time	11.67 min	
Flow rate (Figure 3)	0.425 mL/min	
Flow rate (Figure 4)	0.425, 0.85, and 1.7 mL/min	
Injection volume	1.4 µL	

Results and discussion

The original LC method for the analysis of *C. deserticola* used an Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm column. Analysis took approximately 35 minutes to separate echinacoside and verbascoside and recondition the column to initial gradient conditions (Figure 2).

By using the 1290 Infinity LC system and a Poroshell 120 EC-C18, 3.0 × 50 mm column, method transfer and optimization were completed quickly and easily. Analysis was accomplished in 12 minutes, while maintaining the same or even better performance for the two target compounds. Since both Agilent columns have similar chemistry, the separation achieved almost the same selectivity (Figure 3).

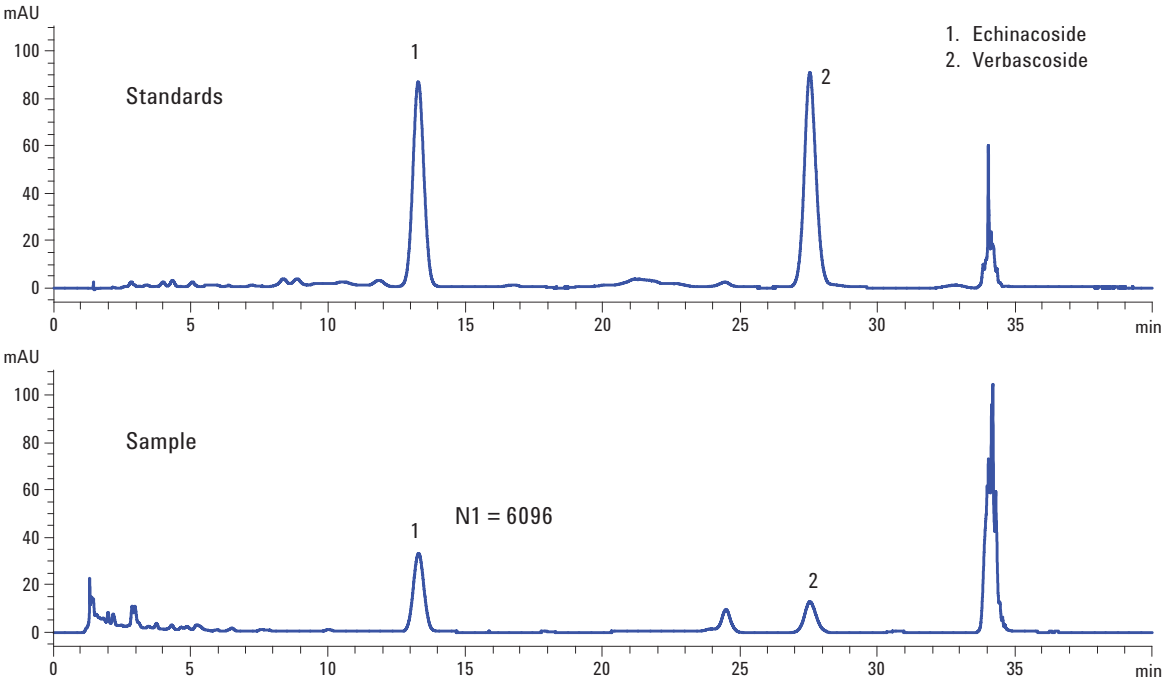

Figure 2. *Figure 2. Echinoside and verbascoside standards, and extract from Cistanche deserticola, analyzed on an Agilent ZORBAX Eclipse Plus C18 4.6 × 150 mm, 5 µm column.*

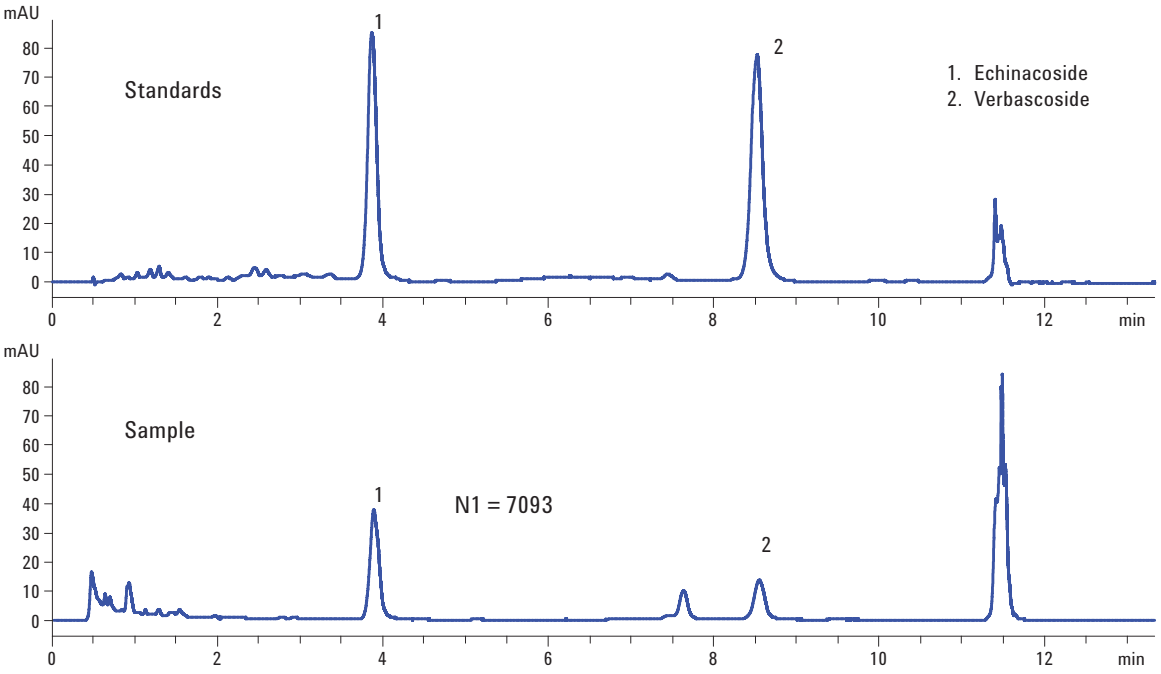

Figure 3. *Echinoside and verbascoside standards, and extract from Cistanche deserticola, analyzed on an Agilent Poroshell 120, EC-C18, 3.0 x 50 mm, 2.7 µm column.*

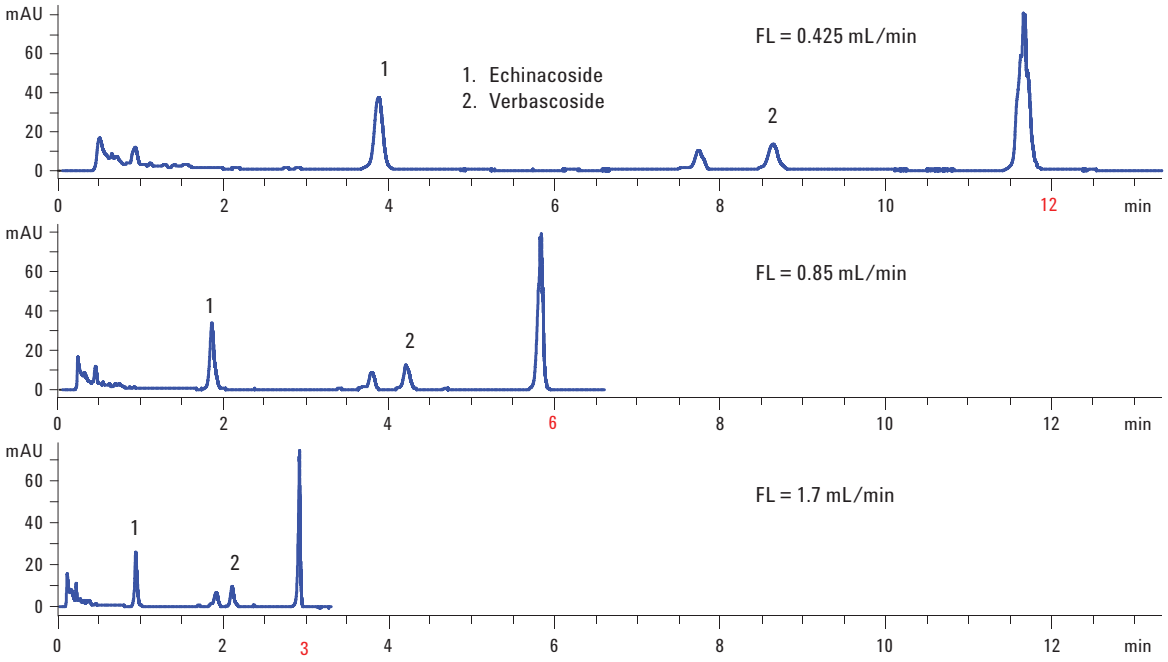

Figure 4. Echinoside and verbascoside standards, and extract from Cistanche deserticola, analyzed on an Agilent Poroshell 120, EC-C18, 3.0 x 50 mm, 2.7 µm column at different flow rates

In addition, solvent consumption was reduced from 35 mL to 5 mL. To take full advantage of the small-particle column, a higher flow rate could be used to further increase the speed of analysis, as shown in Figure 4.

Conclusion

The shorter Poroshell 120 column with 2.7 µm superficially porous particles dramatically reduce the separation time of *C. deserticola* extracts while maintaining a separation similar to that obtained with conventional 5 µm columns. Therefore, quality control of this traditional Chinese medicine is easy and fast when using Agilent Poroshell 120 columns, and time and solvent could be saved for complex analysis of other such medicines.

Reference

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Faster LC Analysis of Notoginseng Total Saponins Using an Agilent Poroshell 120 EC-18

Application Note

Traditional Chinese Medicine

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Abstract

The traditional method for analyzing notoginseng total saponins was transferred from an Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm column to an Agilent Poroshell 120 EC-C18, 4.6 × 75 mm, 2.7 µm column. Gradient time decreased from 60 minutes to 18 minutes. The transferred method was fast, with higher efficiency and a 2.11 resolution for the critical pair of compounds, ginsenosides Rg1 and Re. In addition, backpressure was below 270 bar and so the analysis could be run on a traditional HPLC instrument.

Introduction

Panax notoginseng, also known as San Qi, is a plant of the Araliaceae family. It is a traditional Chinese herb well-known for its therapeutic abilities to stop hemorrhage [1], to influence blood circulation, and to act as a tonic. *P. notoginseng* contains about 8–12% by weight of saponins. Total saponins of *P. notoginseng*, the major bioactive components, are used to treat coronary heart disease, cardiac angina, apoplexy, and atherosclerosis [2, 3]. However, notoginseng total saponins contain several kinds of active components such as notoginsenoside R1, ginsenoside Rg1, Rb1, Re, and Rd. The analysis of saponins is important for evaluating the quality of notoginseng and its Chinese medicine preparations.

Traditionally, the HPLC run time is greater than 60 minutes for the analysis of notoginseng total saponins with the China Pharmacopeia method using a conventional LC column [4]. Agilent Poroshell 120 EC-C18, 2.7 µm columns are packed with superficially porous materials, which deliver fast separation and achieve performance similar to sub-2 µm totally porous materials, but with lower pressure. This application note describes a fast quality control method for the analysis of notoginsenoside R1 and Ginsenosides Rg1, Re, Rb1, and Rd using the Agilent 1290 Infinity LC System and a Poroshell 120 EC-C18 column. Compared to conventional methods, the rapid method is much faster, with better performance, and quality of separation. In addition, solvent consumption is dramatically reduced.

Experimental

Analyses were performed on an Agilent 1290 Infinity LC System consisting of a binary pump (G4220A), a thermostatted column compartment (TCC, G1316C), an autosampler (G4226A), and a diode array detector (DAD, G4212A).

Columns

Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm (p/n 959990-902)
Agilent Poroshell 120 EC-C18, 4.6 × 75 mm, 2.7 µm (p/n 697975-902)

Compounds

Compounds of interest are shown in Figure 1, with their respective structures. They were dissolved in 70% methanol aqueous solution at 2.5 mg/mL. Notoginseng total saponins were purchased from a local TCM store. Twenty five mg of the sample powder were transferred to a 10 mL volumetric flask and a 70% methanol aqueous solution was added to dissolve and dilute to volume. This solution was then filtered through a 0.45 µm regenerated cellulose membrane filter (p/n 5064-8221) and injected directly into the HPLC system.

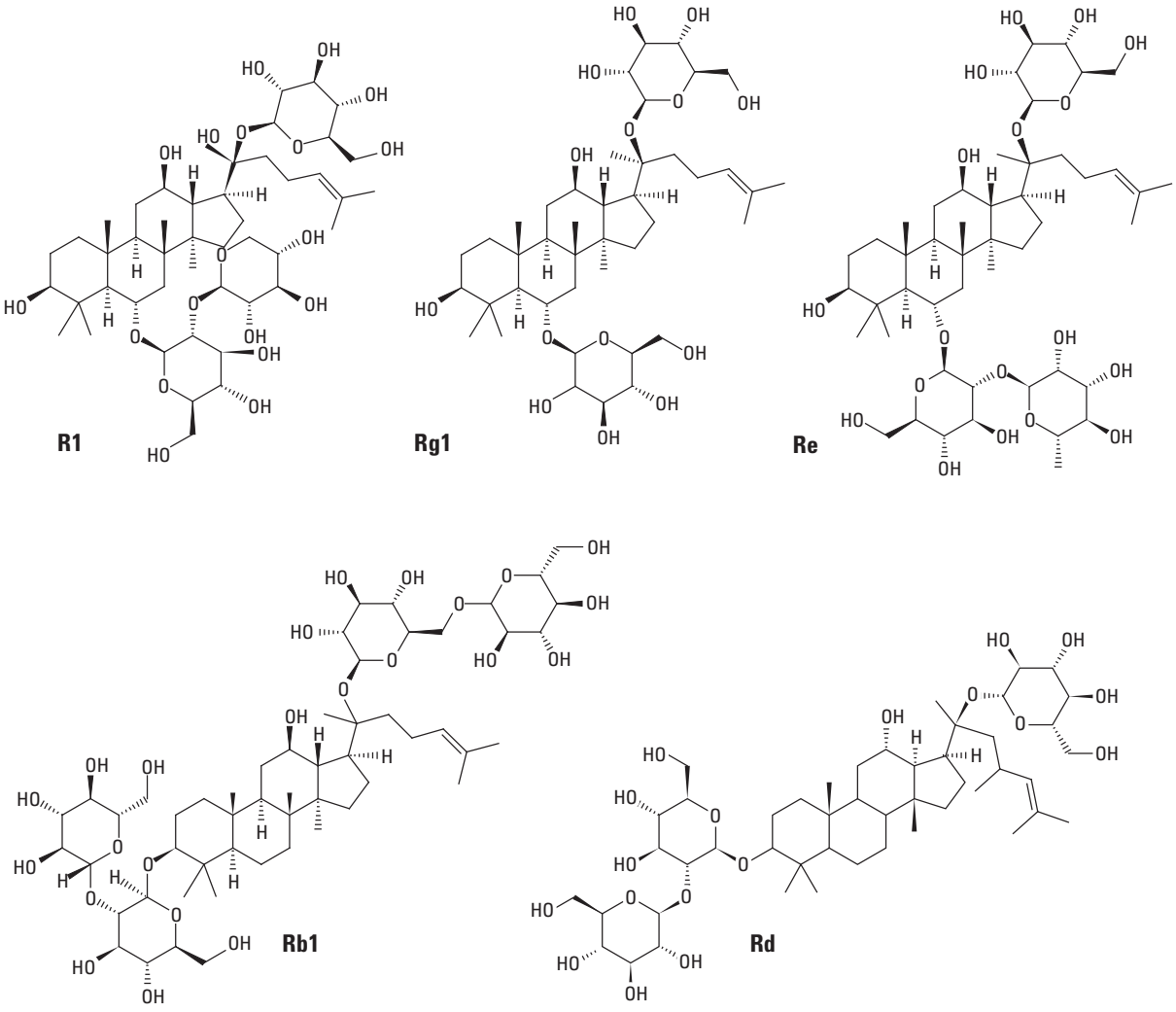

Figure 1. Structure of notoginsenoside R1 and ginsenosides Rg1, Re, Rb1 and Rd.

Results and Discussion

The original separation method of notoginseng total saponins published in the 2010 Chinese Pharmacopoeia was repeated on an Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm column. It took approximately 60 minutes to separate notoginsenoside R1 and ginsenosides Rg1, Re, Rb1, and Rd. Compounds of interest were baseline separated with excellent peak shape. The Agilent Poroshell 2.7 µm particle columns provided similar performance to that of totally porous sub-2 µm columns, but with lower pressure. By using an Agilent Poroshell 120 EC-C18 4.6 × 75 mm, 2.7 µm column, method transfer and optimization were completed quickly. As shown in Figure 2, the analysis time decreased from 60 minutes to 18 minutes, while achieving better resolution for the critical pair Rg1 and Re, and better theoretical plates for Rg1, which exceeded the requirement of the 2010 Chinese Pharmacopoeia (N>6000). The pressure was less than 270 bar, which is quite acceptable for a 400-bar HPLC when using an Agilent Poroshell 120 EC-C18, 4.6 × 75 mm, 2.7 µm column. In addition, solvent consumption can be significantly decreased, thereby lowering costs.

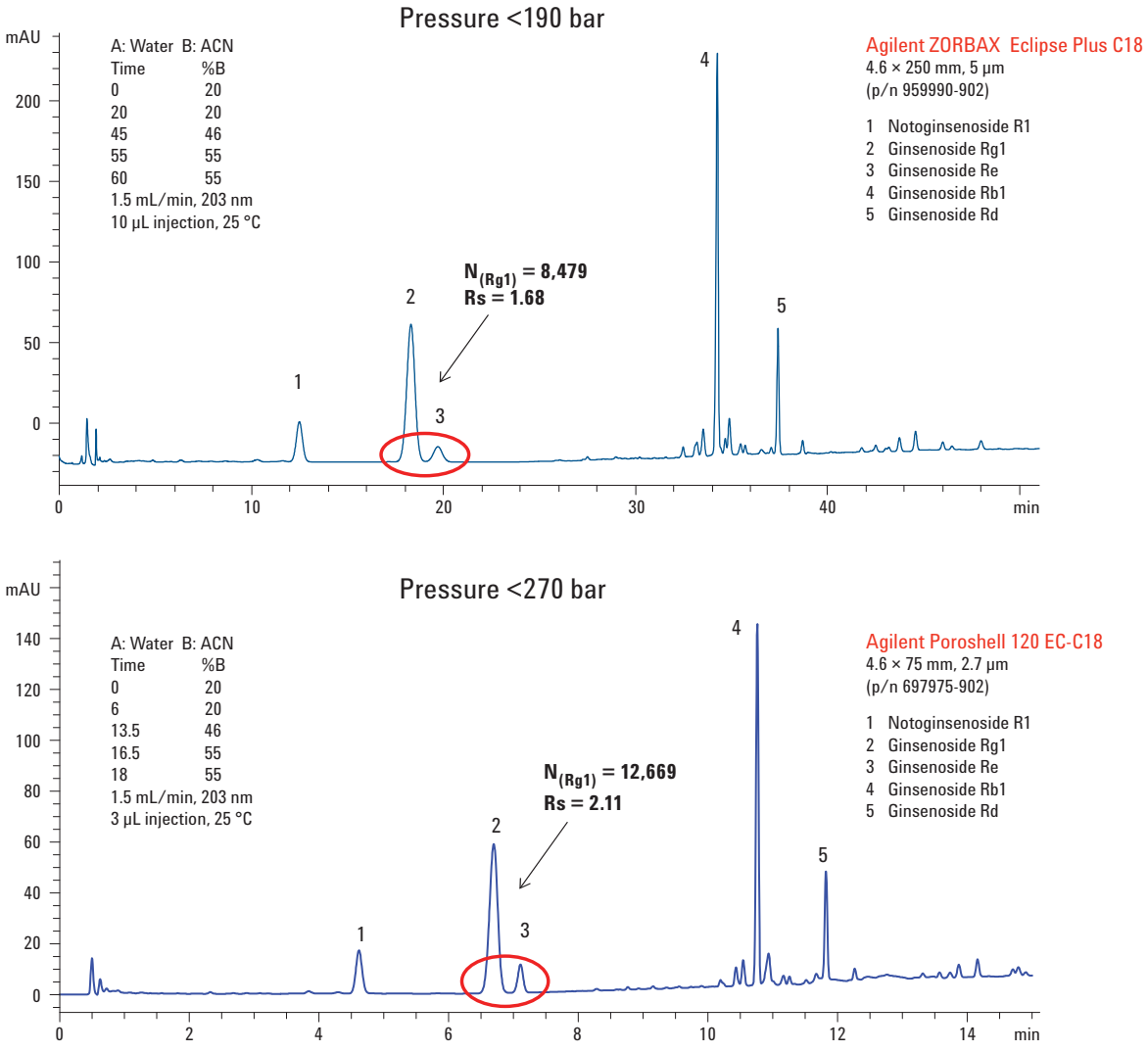

Figure 2. Overlaid chromatograms of notoginseng total saponins using an Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm and an Agilent Poroshell 120 EC-C18, 4.6 × 75 mm, 2.7 µm.

Conclusions

The traditional method for analyzing notoginseng total saponins was reproduced successfully on an Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm column. The shorter Agilent Poroshell 120 EC-C18 column can greatly reduce the analysis time and provide better separation and peak shape, and thereby substantial time and cost savings. The Poroshell 120 column can exceed the requirements of the 2010 Chinese Pharmacopoeia for notoginseng total saponins analysis. It is well suited for evaluating the quality of notoginseng and its Chinese medicine preparations.

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Analysis of Water Soluble Vitamins in Multivitamin Tablets Using Poroshell 120 EC-C18

Application Note

Pharmaceutical

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Abstract

A method separating nine multivitamin compounds originally developed on Agilent ZORBAX Eclipse Plus C18 columns of various sizes is transferred to an Agilent Poroshell 120 EC-C18 4.6 mm × 75 mm, 2.7 µm column using an Agilent 1260 Rapid Resolution LC. The gradient and flow rate are scaled, maintaining retention index with the objective of determining the optimal flow rate for each column evaluated. The new separation keeps the analysis time constant at 5 minutes and allows the use of a longer column with a higher flow rate and still maintains the pressure under 400 bar. Sample preparation used is dissolution of a tablet in water followed by either filtration through a 0.45 micron syringe filter or centrifugation. Over 800 injections were made without substantially increasing pressure.

Introduction

A compound is called a vitamin when it cannot be synthesized in sufficient quantities by an organism, and must be obtained through diet. Vitamins have diverse biochemical functions. Many vitamins function as precursors for enzyme cofactors, that help enzymes in their work as catalysts in metabolism. Vitamins may also be less tightly bound to enzyme catalysts as coenzymes, detachable molecules that function to carry chemical groups or electrons between molecules.

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The two types of vitamins are classified by the materials in which they will dissolve. Fat-soluble vitamins, such as vitamins D, or E, dissolve in fat before they are absorbed in the blood stream to carry out their functions. Excesses of these vitamins are stored in the liver. Because they are stored, they are not needed every day in the diet.

By contrast, water-soluble vitamins dissolve in water and are not stored; they are eliminated in urine. We need a continuous supply of them in our diets. The water-soluble vitamins are the B-complex group and vitamin C.

The B-complex group: thiamin, riboflavin, niacin, pyridoxyl phosphate, folic acid, cobalamin, pantothenic acid and, in addition, ascorbic acid, or vitamin C, are widely distributed in foods. Riboflavin, for example, can be obtained from liver, milk, dark green vegetables, whole and enriched grain products, eggs. Their influence is felt in many parts of the body. They function as coenzymes that help the body obtain energy from food. They also are important for normal appetite, good vision, healthy skin, healthy nervous system and red blood cell formation.

Until the mid-1930s, when the first commercial yeast-extract and semi-synthetic vitamin C supplement tablets were sold, vitamins were obtained solely through food intake, and changes in diet (following a bad harvest) can alter the types and amounts of vitamins ingested. Vitamins have been produced as commodity chemicals and made widely available as inexpensive synthetic-source multivitamin dietary supplements, since the middle of the 20th century [1].

In addition to containing vitamins of interest, other excipient materials such as cellulose, maltodextrin, dextrin, gelatin, dextrose, soy lecithin are formulated into the tablets. Given the large size of the tablet and relatively small quantities of many of the vitamins, clogging problems have plagued this analysis when it has been attempted on small particle size columns.

Reverse Phase HPLC is well suited for vitamin analysis. Qualitative and Quantitative analysis of vitamins is important for clinical, food and pharmaceutical applications. Using a previously described method developed on an Agilent ZORBAX Eclipse Plus C18, 1.8 µm column, a fast separation method is converted to be used on an Agilent Poroshell 120 EC-C18 column [2]. The new method remains fast, but is less prone to clogging by excipients found in multivitamins.

In this work,a method using an Agilent Eclipse Plus C18 4.6 mm × 50 mm, 1.8 µm column is converted to use an Agilent Poroshell 120 EC-C18 4.6 mm × 75 mm column. Injection volume is increased and the risk of clogging is reduced.

Experimental

An Agilent 1260 Rapid Resolution LC (RRLC) system was used for this work:

- G1312B Binary Pump SL with mobile phase A: 25 mM Sodium Phosphate pH 2.5 in Water and B: Methanol. The gradient started at 1% B, held at that concentration, then ramped to 12% B and finally 30% B, held at that concentration, and then re-equilibrated to the initial condition. The system is configured with the pulse damper and standard mixer installed.
- G1367E Automatic Liquid Sampler (ALS) SL. Injection volume of 5 µL was used.
- G1316B Thermostatted Column Compartment (TCC) SL with temperature set to 35 °C.
- G4212C Diode Array Detector (DAD) SL with the signal set to 230, 4 nm and reference not used, using a G4212-60008 micro flow cell (10-mm path, 1-µL variance).
- ChemStation version B.04.02 was used to control the HPLC and process the data.
- Agilent Poroshell 120 EC-C18, 4.6 mm × 75 mm, 2.7 µm, p/n 697975-902

The compounds of interest are shown in Reference 2, with their respective structures. Compounds were dissolved in water at 1 mg/mL and used for identification. The following compounds were purchased from Sigma Aldrich: thiamin (vitamin B_1), riboflavin (vitamin B_2), niacin, vitamin B_6, folic acid, vitamin B_{12}, biotin, pantothenic acid, and ascorbic acid. Additionally purchased from Sigma Aldrich (Bellefonte, PA) was the Sodium Monophosphate and Phosphoric Acid. Methanol was purchased from Honeywell, Burdick and Jackson High Purity, (Muskegon, MI). Water used was 18 MΩ WMilli-Q water (Bedford, MA). A multivitamin tablet was purchased at a local pharmacy. (One a Day Women's Active Metabolism, Bayer HealthCare, Morristown NJ.).

Tablets were dissolved by grinding them individually using a mortar and pestle and transferring the entire amount (about 1.6766 g/tablet) with 100 mL water to a 150 mL plastic coated bottle. The bottle is then sealed and shaken vigorously for 5 min. A cloudy solution is produced which is clarified by either filtration (using a 0.45 µm, 30 mm regenerated cellulose filter (p/n 5061-3364) and a 10 mL syringe or by centrifugation in a polypropylene tube (6000 rpm for 5 min). The resulting clear solution is then transferred to an Agilent MS Analyzed Write-On Vial (p/n 5190-2278).

Results and Discussion

In transferring this method from the original method, a longer column was chosen and used at a proportionally higher flow rate. Previous work has shown that higher peak capacity for an Agilent Poroshell 120 EC-C18 (75 mm instead of the previously used 50 mm column) can be obtained at higher linear velocity (1.5 mL/min instead of 1 m/min) [3]. In addition, a larger injection volume, which should be proportional to column volume, would be possible on the larger Poroshell column. The selectivities of an Agilent ZORBAX Eclipse Plus C18 (75 mm instead of the previously used 50 mm column) and Poroshell 120 EC-C18, have been shown to be very similar in previous work [4,5]. In addition, a larger column would be less affected by extra column effects such as additional tubing [6] required if a cooled autosampler was used, as recommended in a previous note on analysis of water soluble vitamins by Huber [7]. In this work, a slight change in elution order is noted at the beginning of the gradient. This could be the result of the change in column porosity (from fully porous to superficially porous), the dwell volume of the instrument, or the equilibration time between runs. Figure 1 shows a representative sample chromatogram, scaled to the largest peak. In the upper left corner, an expanded chromatogram is shown that reveals the lower intensity peaks. The chromatographic conditions, as well as the list of components in their elution order, are found on the right.

Over 800 injections of a vitamin tablet extract were made on this column without an increase in pressure. This is due in part to the 2 µm column inlet frit, which is less likely to clog than the smaller porosity frits used on sub 2-micron and totally porous 3 µm columns [8]. At least 100 injections were made using the centrifuge sample clarification method without any change in system pressure. In this case, filtration was found to be a faster method, requiring fewer steps in producing a final sample. The 0.45 µm regenerated cellulose filter presents less resistance than a 0.2 µm filter required for use with a sub 2-micron column. A plot describing pressure changes per injection is shown in Figure 2. An increase in pressure from 177 to 179 bar is noted over 3 days and 6 L of phosphate buffer.

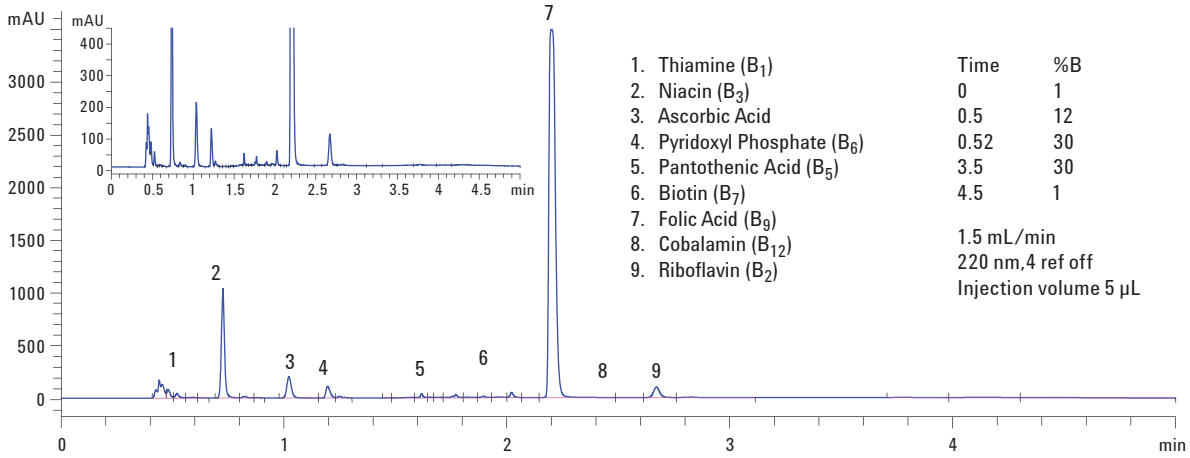

Figure 1. Elution order water soluble vitamins.

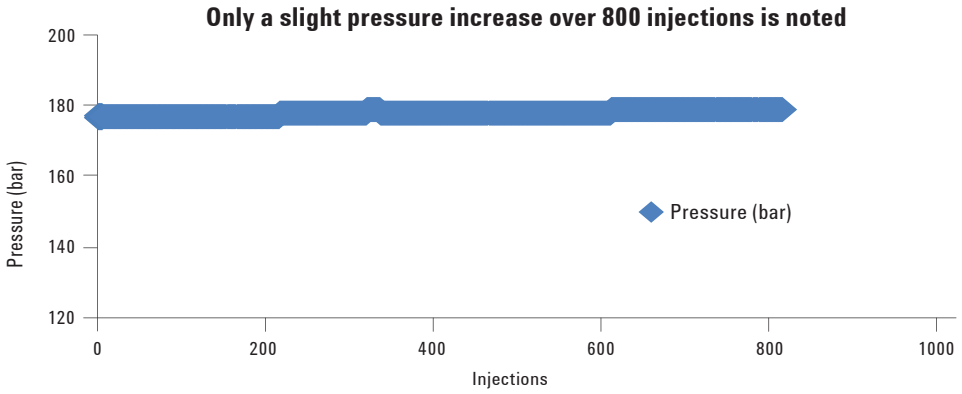

Figure 2. Agilent Poroshell 120 EC-C18 pressure remains nearly constant after 800 injections water soluble vitamin tablet.

Other wavelengths could also be used for this analysis, as several of these compounds form yellow or even red solutions in water, indicating absorbance in the visible region of the spectra [9]. Care should be taken when using a reference wavelength as an improper choice could lead to higher reference absorbance than in the analytical wavelength and to negative peaks.

Conclusion

HPLC columns packed with superficially porous particles offer many advantages over columns packed with conventional, fully porous particles. The superficially porous 2.7-µm Agilent Poroshell 120 EC-C18 offers similar efficiency and selectivity to the 1.8 µm Agilent ZORBAX Eclipse Plus C18 column, without the high back pressure. The 2 µm frit has demonstrated a resistance to clogging through the analysis of over 800 samples.

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Analysis of Anthocyanins in Common Foods Using an Agilent Poroshell 120 SB-C18

Application Note

Food/Pharmaceutical

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Abstract

Methods separating anthocyanins compounds originally developed on an Agilent StableBond SB-C18 4.6 × 250 mm 5 µm column or a 4.6 mm × 150 mm, 3.5 µm column are transferred to an Agilent Poroshell 120 SB-C18, 4.6 mm × 75 mm, 2.7 µm column using an Agilent 1260 Rapid Resolution Liquid Chromatograph. The gradient, injection volume, and flow rate are scaled, maintaining retention index for each column evaluated. One Method reduces the time per analysis from 100 minutes to 20 minutes and reduces solvent consumption by 70%. The second transfer reduces time from 67 minutes to 40 minutes and reduces solvent consumption by 40%.

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Introduction

Anthocyanins are water soluble plant pigments responsible for red, blue and purple colors found in many fruits, flowers and plants. The analysis of anthocyanins by HPLC and HPLC MS has been shown to be useful in identifying the fingerprint of different varieties of fruits and or assist in determination of authenticy of fruit juices.

Interest in anthocyanin pigments has intensified because of their possible health benefits as dietary antioxidants. Over 300 structurally distinct anthocyanins have been identified in nature. Anthocyanins are one class of flavonoid compounds, which are widely distributed plant polyphenols. Flavonols, flavan-3-ols, flavones, flavanones, and flavanonols are additional classes of flavonoids that differ in their oxidation state from the anthocyanins.

Qualitative and quantitative analysis of anthocyanins can be used to distinguish between different cultivars of blueberry plants and determine their quality. Therefore, the chromatographic separation of anthocyanins is of increasing importance to the agricultural and wine industries. Recent interest in medicinal use of anthocyanins, as antioxidants/anticancer agents, has also stimulated interest in their chromatographic separation [1].

Traditionally, a low-pH mobile phase (containing formic acid) in these types of separations has caused degradation of the column and change in the separation [2]. Agilent ZORBAX StableBond SB-C18 columns provide the chromatographer with long-term stability for reverse-phase separations requiring very low pH. Many published methods use 50 mL/L (5%) formic acid or 30 mL/L (3%) phosphoric acid. In this work, the methods using phosphoric acid and formic acid are scaled for use with an Agilent Poroshell 120 SB-C18. Several fruit or juice samples are assayed with this new method including blueberries, blackberries, cranberries, strawberries and pomegranate juice.

Experimental

- G1312B Binary Pump SL with mobile phase A: 3% phosphoric acid or 5% Formic Acid in Water and B: Methanol.
- G1367E Automatic Liquid Sampler (ALS) SL.
- G1316B Thermostatted Column Compartment (TCC) SL with temperature set to 30 °C.
- G4212C Diode Array Detector (DAD) SL with the signal set to 525, 16 nm and reference not used, using a G4212-60008 micro flow cell (10-mm path, 1-µL variance).
- ChemStation version B.04.02 was used to control the HPLC and process the data.
- Agilent Poroshell 120 SB-C18, 4.6 mm × 75 mm, 2.7 µm, (p/n 689775-902)
- Agilent ZORBAX SB-C18, 4.6 mm × 250 mm, 5 µm (p/n 880975-902)
- Agilent ZORBAX SB-C18, 4.6 mm × 150 mm, 3.5 µm, (p/n 863953-902)

The formic acid, and phosphoric acid were purchased from Sigma Aldrich (Bellefonte, PA). Methanol was purchased from Honeywell, Burdick and Jackson High Purity, (Muskegon, MI). Water used was 18 M-Ω Milli-Q water (Millipore, Bedford, MA). Fresh blackberries, blueberries, strawberries, cranberries as well as pomegranate juice were purchased from a local grocery store.

Method of Preparing Fruit Extracts

Begin by mixing: 10 g blueberries (or other fruit), 10 mL solvent (70:28:2, MeOH:H_2O, Formic acid), Blend for 10 minutes on dry ice allow ice to sublime. Filter through glass wool in a 10 mL syringe. Allow filtrate to sit for 1 hour. Filter through a 0.2 µm filter. Inject 50 µL immediately for HPLC analysis. (4.6 mm × 250 mm columns) [3,4]. The resulting clear solution is then transferred to an Agilent MS Analyzed write on vial (p/n 5190-2278).

Equation 1: $$k^* = (t_G F)/(d/2)^2 L(\Delta\%B)$$

Where:

t_G is the gradient time,
F is the flow rate
L is the column length
d is the column diameter
Δ%B is the change in organic content across the gradient segment

Figure 1 shows the separation of blueberry extract using totally porous 5 and 3.5 µm SB-C18 columns as well as with a shorter, superficially porous Agilent Poroshell 120 SB-C18 2.7 µm column. Injection volumes are scaled to column volume. The selectivity on both columns should be very similar as they both have the same bonded phase [5,6,7]. Agilent ZORBAX StableBond SB-C18 is particularly well suited to very low pH methods. Previous work has shown that Poroshell 120 achieves approximately 90% of the peak capacity of 1.8 µm totally porous columns at roughly half the pressure. In addition, the efficiency of Poroshell 120 was shown to be 2 × of a 3.5 µm column[6,7]. In this work, the logical progression from long 5 µm columns to shorter 3.5 µm columns to shorter still Poroshell 120 columns is demonstrated. By scaling the gradients to the column length (diameter is kept constant), retention index (k') is kept constant using Equation 1.

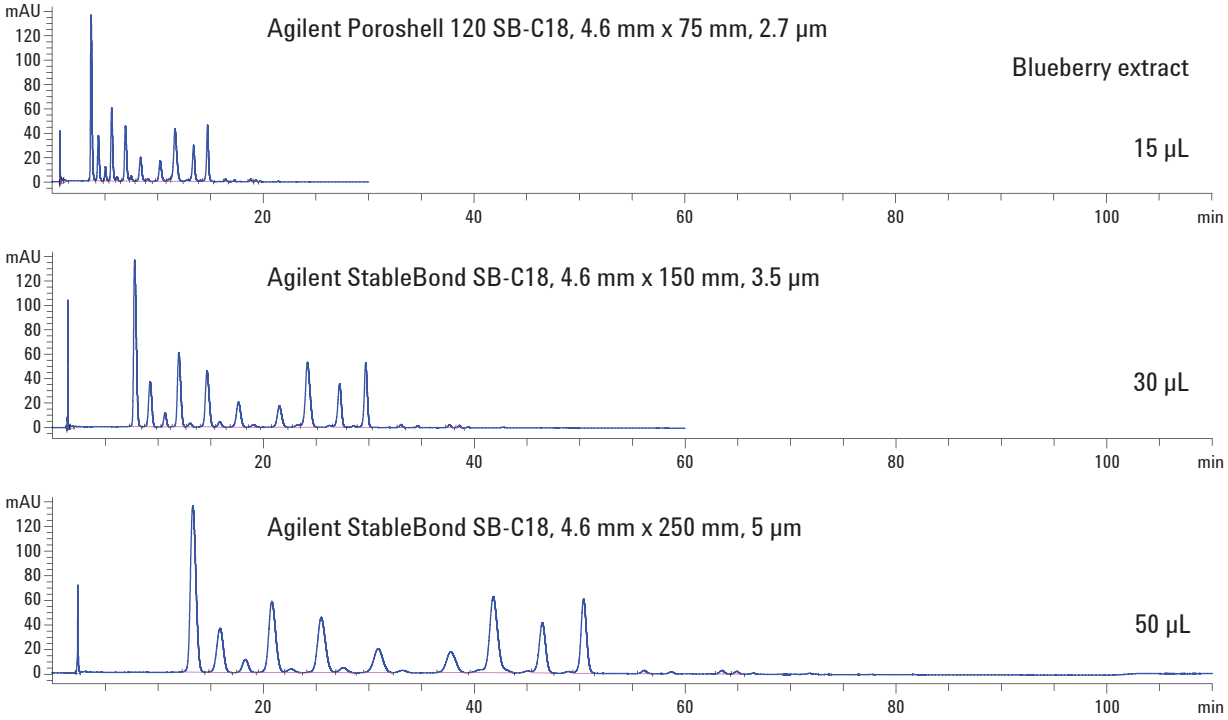

Figure 1. Blueberry anthocyanin analysis on totally porous and superficially porous stableBond C18 columns. Overlay of anthocyanin method with 250 mm 5 µm, 150 mm 3.5 µm, and 75 mm 2.7 µm at 1 mL/min.

Even in the complex example of blueberry extract, all peaks are separated with identical (albeit faster) separations saving both time and solvent. Table 1 shows the gradients used, and the reduction of time from 97 minutes to 29 minutes. No additional sample preparation was performed as all columns evaluated use the same 2 µm column frits. These frits have been shown to be more resistant to clogging than those used on totally porous 3 µm columns [8].

Table 1. Phosphoric Acid Gradients Used in Figures 1,2, and 3a Scaled from Reference 3

Length	**4.6 mm × 250 mm**	**4.6 mm × 150 mm**	**4.6 mm × 75 mm**	**4.6 mm × 75 mm**	**4.6 mm × 75 mm**
Particle	5	3.5	2.7	2.7	2.7
Part number	880975-902	863953-902	689775-902	689775-902	689775-902
Flow rate	1	1	1	1.5	2
Max pressure	183 bar	236 bar	236 bar	349 bar	448 bar
Injection volume	50	30	15	15	15
% B	**Time**	**Time**	**Time**	**Time**	**Time**
23	0	0	0	0	0
26	35	21	10.5	7.5	5.25
60	97	58	29.1	20	10

In Figure 2, flow rate or linear velocity is increased. Previous work has shown that higher peak capacity for Agilent Poroshell 120 EC-C18 can be obtained at higher linear velocity between 1.5 and 2.5 mL/min on a 4.6 mm column [9]. By employing equation 1 and keeping k' constant the separation is maintained. In this case pressure maxima of 448 bar is reached at 2 mL/min at 50% Methanol content as show in the chromatograms. However at 1.5 mL/min the maximum pressure is under 400 bar. Gradients used in this work are listed in Table 1.

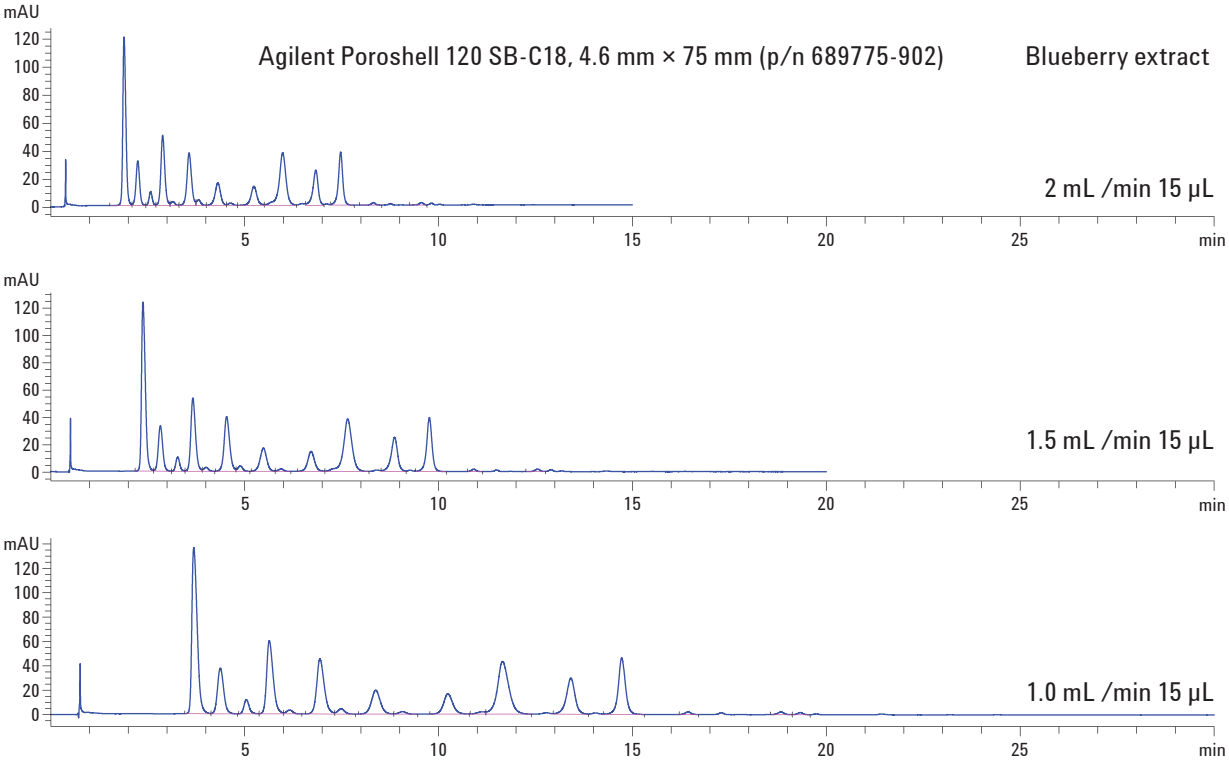

Figure 2. Overlay of blueberry extract on Anthocyanin Method using an Agilent Poroshell 120 SB-C18 4.6 mm × 75 mm 2.7 µm at varied flow rates.

Figure 3a shows real samples as analyzed using the 1.5 mL/min method as described in Table 1. Samples of blueberry, blackberry, cranberry and strawberry were prepared using acidified methanol [3,4]. Pomegranate juice was injected without further preparation. As shown in Figure 1, the complex blueberry chromatogram shows approximately 20 peaks (16 major, 4 minor). Work by Kalt has shown that the more wild and stressed blueberry varieties have more Anthocyanin peaks [4]. Blackberry, a cultivated variety shows few peaks, but is consistent with previously reported data [10]. Cranberry shows the distinctive (cyd-3-gal, cyd-3-glu, cyd-3-arab, pnd-3-gal, pnd-3-glu, pnd-3-arab) depicted in reference [11], and also shown in references [10, 12, 13]. Strawberry also appears consistent with previously reported data. Figure 3b shows similar results using 5% formic acid on a slightly different gradient. The original gradient run was 1.5 mL/min and no attempt was made to increase the linear velocity of the mobile phase. The original and resulting scaled gradient used is listed in Table 2. The use of formic acid instead of phosphoric acid allows the use of mass spectrometry for identification, but the chromatography using either mobile phase modifier leads to similar fingerprinting results.

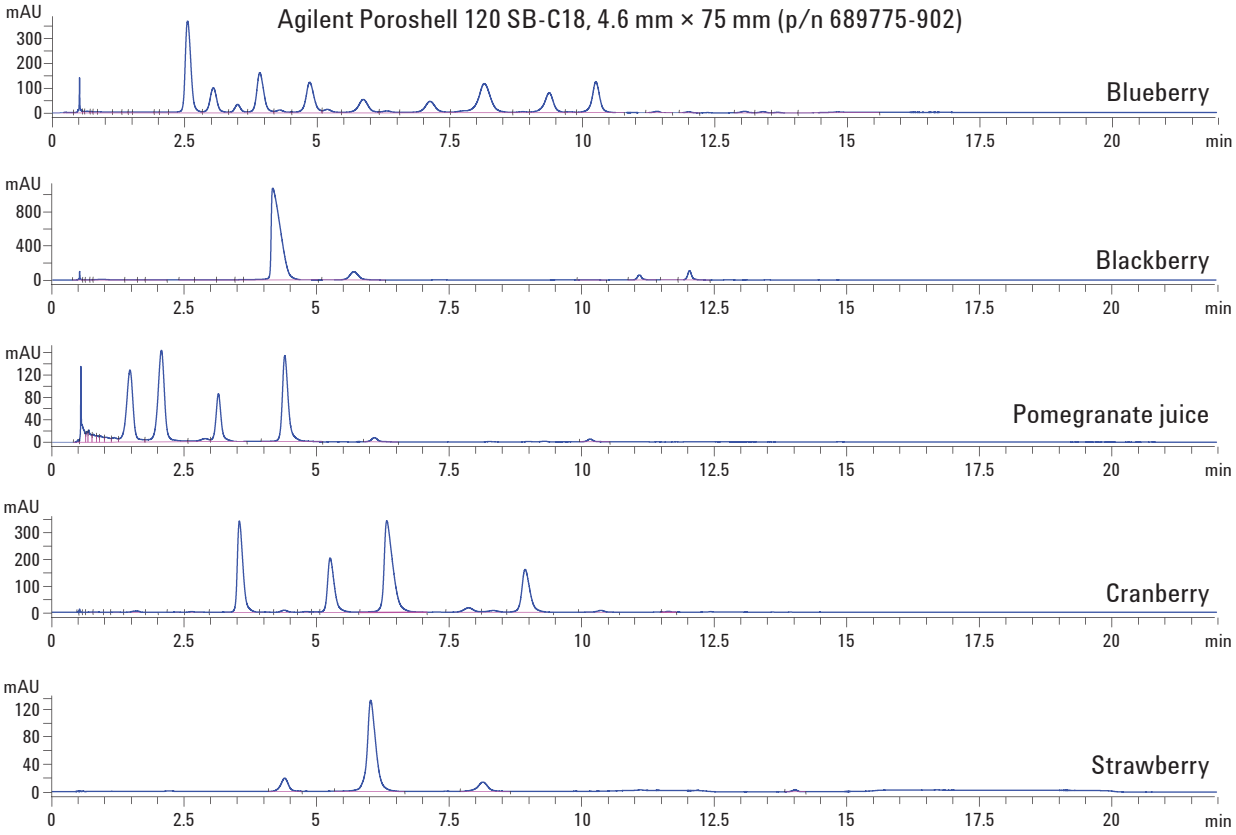

Figure 3a. Overlay of Anthocyanin Method using an Agilent Poroshell 120 SB-C18 of varied samples using a H_3PO_4 gradient.

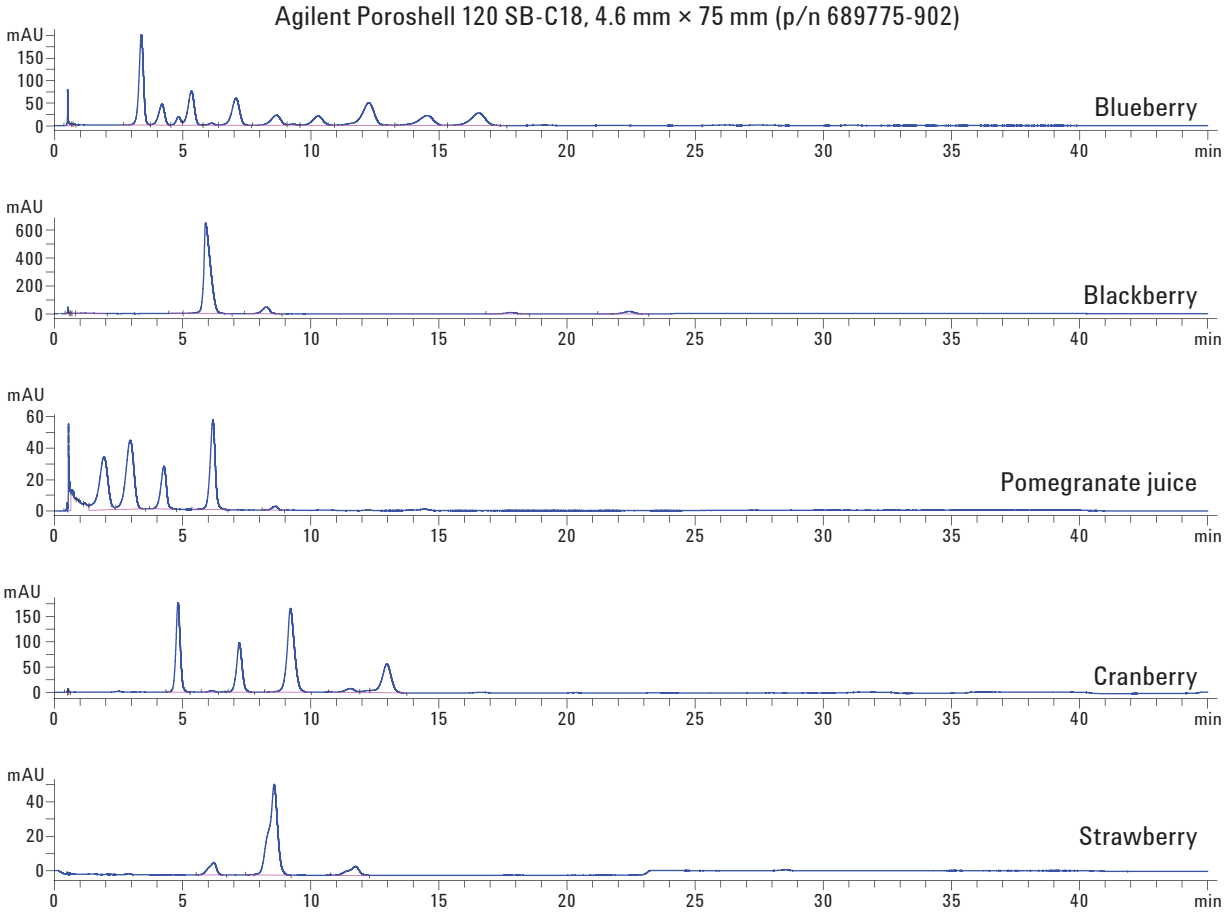

Figure 3b. Overlay of Anthocyanin Method using an Agilent Poroshell 120 SB-C18 of varied samples using a HCO_2H gradient.

Table 2. Formic Acid Gradients Used in Figure 3b Scaled From Reference 4

Length	4.6 x 250	4.6 x 75
Particle	5	
Part number	880975-902	68775-902
Flow rate	1.5	1.5
Max pressure	274 bar	349 bar
Injection volume	50	15
%B	**Time**	**Time**
14	0	0
17	10	6
23	35	21
47	65	39
14	67	40.2

Conclusion

HPLC columns packed with superficially porous particles offer many advantages over columns packed with conventional, fully porous particles. The superficially porous Agilent Poroshell 120 SB-C18, 2.7-µm offers similar selectivity to Agilent ZORBAX StableBond SB-C18 columns. The use of Poroshell 120 SB-C18 for the analysis of Anthocyanins has been shown to allow faster analysis with lower solvent use per sample. The importance of this faster and less solvent expensive change can be easily seen when applying the analysis to differentiate plant species [4] or assay varieties of foods [10, 12] where hundreds of samples have been analyzed requiring more than an hour to separate each sample. Substantial amounts of time could have been saved or more samples assayed allowing further differentiation of plant species. Using formic acid as a mobile phase additive would allow further identification using mass spectrometry.

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Agilent Technologies

Analysis of Amitriptyline, Fluoxetine, Quetiapine, and Sertraline Using Dried Plasma Spots

Application Note

Pharmaceutical

Author

William Hudson

Ben Yong

Paul Boguszewski

Introduction

Dried blood spotting (DBS) has been gaining popularity with pharmaceutical laboratories in recent years. There has been recent interest in pre-clinical and clinical trials for new chemical entities within the pharmaceutical industry, leading to pharmaceutical companies use of DBS for sample storage and blood sample analysis for new analytes. Traditional bioanalysis has always been performed with plasma and there is concern over whether blood or plasma is the optimal choice for drug analysis in human patients.

While dried blood spotting cards are typically used for blood, other biological fluids may also be spotted onto non-cellulose cards and analyzed by LC-MS/MS. Dried plasma spots offer advantages over cold plasma storage in that greater space is required for storage, and that sample stability is improved over cold storage. 15 µL of plasma is spotted onto the paper and allowed to dry for at least 2 hours. A 2–4 mm core is punched from the spot, followed by analyte desorption using solvent and analyzed by LC-MS/MS.

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Experimental

Four analytes were chosen: Quetiapine, Amitriptyline, Sertraline, and Fluoxetine. Fluoxetine-D6 was readily used as an internal standard, and was used for all compounds. Precision and accuracy recoveries were calculated based on linear regression of the calibration standards. A mid-level (5.0 ng/mL) and high level (500 ng/mL) sample were chosen.

A 3 mm disk was punched and placed into a 96-well collection plate.

300 µL of 0.1% formic acid in 80% methanol (with 0.66 ng/mL of deuterated internal standard mix) was added to each well and vortexed.

The samples were evaporated to dryness and reconstituted in 100 uL of mobile phase.

LC/MS conditions

Column	Agilent Poroshell 120 EC C18, 3.0 mm x 50 mm, 2.7 µm
Mobile phase	A: 0.1% Aqueous formic acid
	B: ACN
Pump program	Flow rate 200 µL/min
t_0	A: 50%, B: 50%
$t_{1.5–2.0}$	A: 10%, B: 90%
$t_{2.01–3.00}$	A: 50%, B: 50%
Run time	3:00 minutes
Gas temp	350 °C
Gas flow	10 L/min
Nebulizing	20 psi

Compound	Q1 ion	Product ion	CE
Quetiapine	384.0	253.1	18 V
Amitriptyline	278.2	105.1	22V
Sertraline	306.1	159.01	26 V
Fluoxetine	310.1	163.0	26 V
Fluoxetine-D6	316.1	154.1	2 V

Results and Discussion

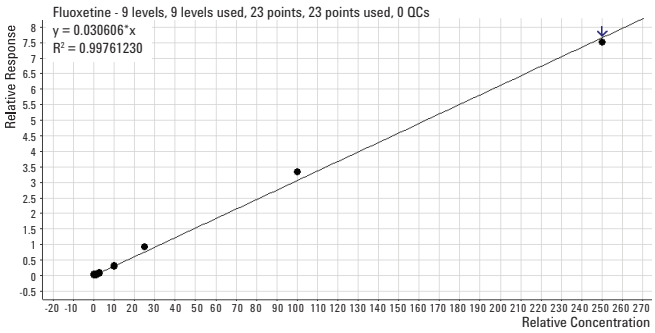
Spot Area

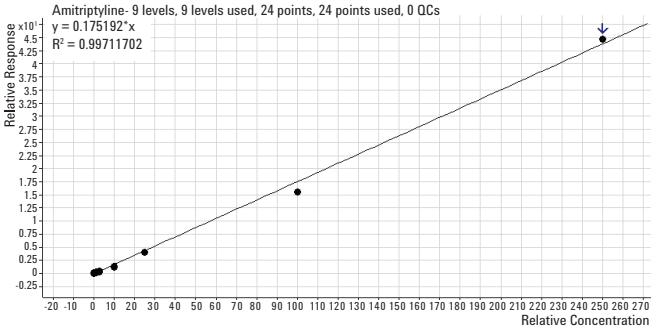
Spot area is a concern for dried blood spotting. In cellulose based cards, spot size can vary up to 40% due to hematocrit levels of 20 to 80. Plasma spot areas were 11% smaller than the Hematocrit 45 spots and only 19% smaller than the HCT 80 spots.

Calibration Curves

1 mL human blood was spiked with 10 µL of each working standard to create a calibration curve of 0.5, 1.0, 2.0, 5.0, 20, 50, 200, and 500 ng/mL. 15 uL of each of these standards were spotted onto DMS paper.

1st order regression was used and correlation coefficients were better than 0.995, which demonstrated good linearity.





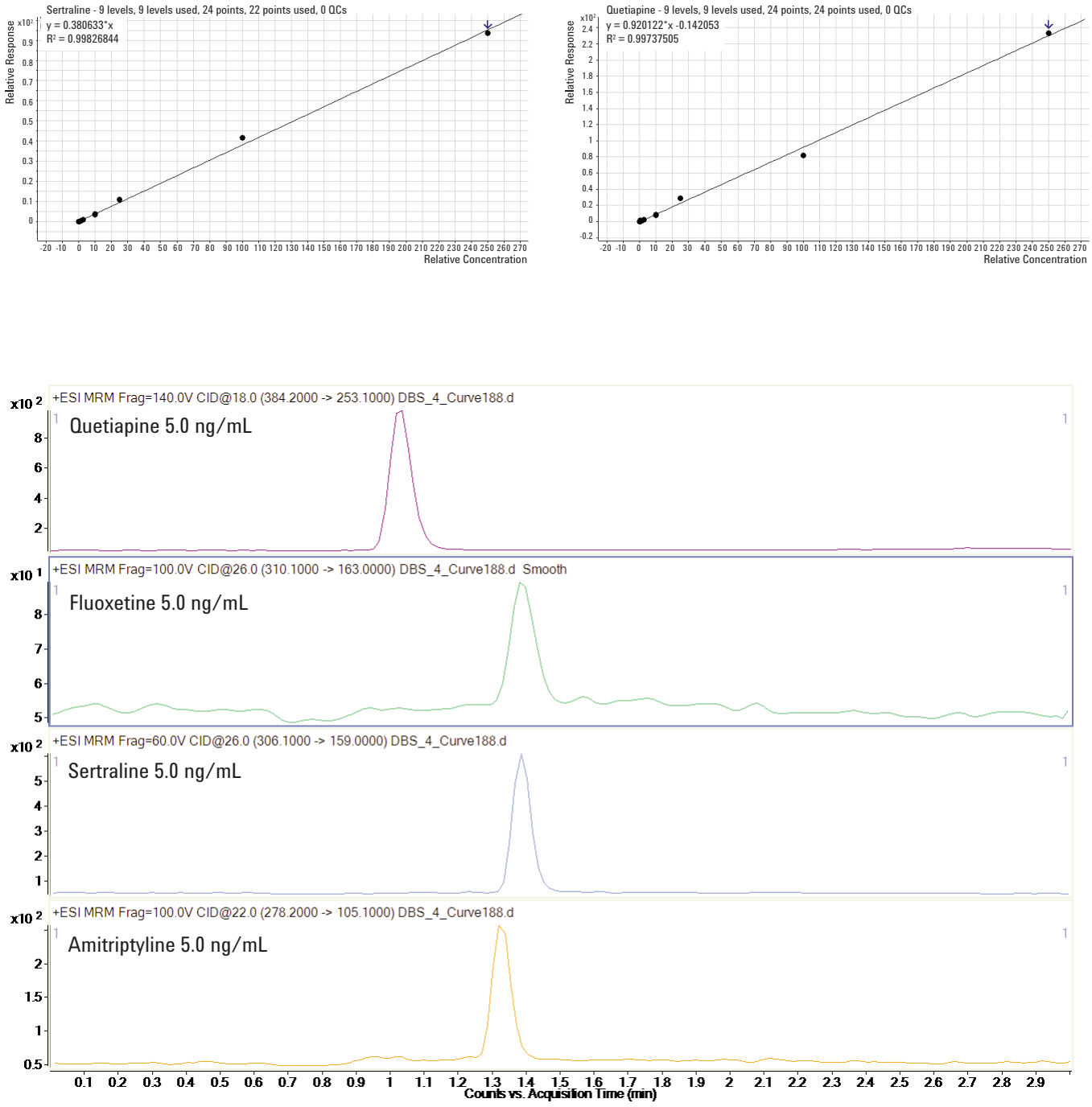


Figure 1. A mid-level extracted blood sample chromatogram.

Analyte recoveries were calculated based on the 1st order regression calibration curves. RSDs were based on eight replicates.

Analyte Recoveries (n=6)

	5.0 ng/mL		500 ng/mL	
Compound	**% Rec**	**RSD**	**% Rec**	**RSD**
Quetiapine	98%	7%	94%	4%
Amitriptyline	103%	8%	94%	7%
Fluoxetine	97%	7%	99%	2%
Sertraline	106%	5%	98%	2%

Conclusions

Four compounds in plasma were successfully desorbed and analyzed by LC-MS/MS. Good detection levels were achieved using an Agilent 1290 LC system and an Agilent 6460 mass spectrometer. Linearity was demonstrated using a 1st order regression and correlation coefficients were better than 0.995. Relative recoveries were within 10% of the true value and RSDs were less than 10%. DBS proved to be an effective means of storing plasma samples as well as crashing out any unwanted proteins for a simple approach to sample preparation prior to LC-MS/MS.

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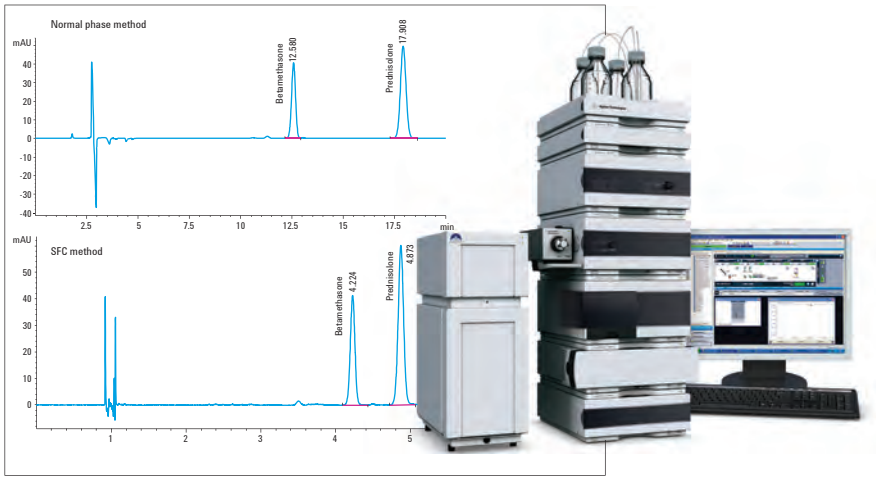
Agilent Application Solution

Transfer of a USP method for prednisolone from normal phase HPLC to SFC using the Agilent 1260 Infinity Hybrid SFC/UHPLC System

Saving time and costs

Application Note

Pharmaceutical QA/QC



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Abstract

Normal phase liquid chromatography (LC) methods often have long run times and involve environmentally toxic/costly solvents. Supercritical fluid chromatography (SFC) methods on the other hand are faster, inexpensive, and eco-friendly. SFC involves the use of low viscosity supercritical carbon dioxide that can be operated at flow rates up to 3x higher than LC without losing separation efficiency and thereby leading to faster analysis. In this Application Note, we describe a method to transfer a United States Pharmacopeia (USP) prednisolone assay normal phase HPLC method to SFC. The Agilent 1260 Infinity Hybrid SFC/UHPLC System was used to perform both normal phase as well as the SFC methods. The results show that the SFC method meets the system suitability criteria, is 4x faster, and results in 17x lower solvent expenses. Robustness tests on the SFC method demonstrate excellent robustness for routine analysis.

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Introduction

Prednisolone is a synthetic adrenal corticosteroid. Corticosteroids have potent anti-inflammatory properties. They are used in a wide variety of inflammatory conditions such as arthritis, asthma, bronchitis, and others. The USP assay method for prednisolone uses a normal phase method that includes chloroform as a sample diluent while 1-chlorobutane (butyl chloride) is used as the mobile phase. Chloroform is a known carcinogen, potentially toxic to analysts, and expensive to dispose. SFC is considered a green technology, because of the use of carbon dioxide (CO_2) as a major component of the mobile phase. In the recent decade, SFC has shown the capability to replace many achiral LC methods. Especially, compared to normal phase methods, SFC methods offer faster separation without losing efficiency, and faster column re-equilibration[1]. In this Application Note we show the development of a SFC method to replace a normal phase prednisolone assay in which betamethasone is used as an internal standard (Figure 1). This method uses methanol as sample diluent instead of chloroform. Linearity, limit of detection (LOD), limit of quantification (LOQ) and robustness[2] of the method was demonstrated.

The Agilent 1260 Infinity SFC/UHPLC Hybrid system[3] was used to perform both the normal phase as well as the SFC method on a single instrument. With this unique hybrid solution, the need to invest in two individual systems is eliminated. This eliminates the need for system-to-system variability and saves significant cost and a laboratory space.

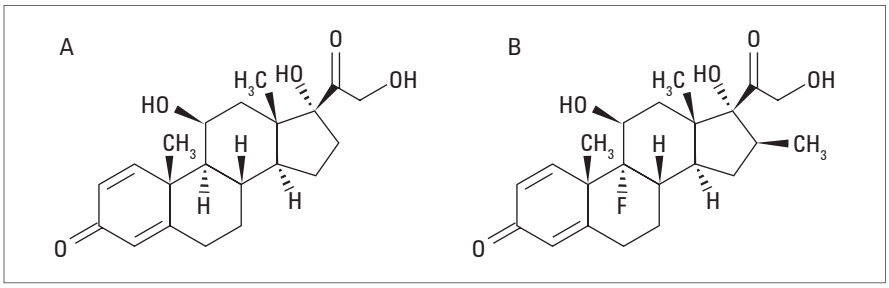

Figure 1
Molecular structures of Prednisolone (A) and Betamethasone (B).

Experimental

Instruments

An Agilent 1260 Infinity Hybrid SFC/UHPLC system (G4309A) consisting of the following modules was used:

- Aurora SFC Fusion A5 module
- Agilent 1260 Infinity Degasser
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity SFC Autosampler
- Agilent 1260 Infinity Thermostatted Column Compartment
- Agilent 1260 Infinity Diode array Detector

Additional components were needed:

- Agilent 1260 Infinity Binary Pump (G1312B)
- Agilent 1290 Infinity Universal Valve Drive (G1170A)
- Agilent 2-position/10-port valve kit – 600 bar (G4232A)
- Agilent 1260 SFC/UHPLC Hybrid Capillary Kit (G4306A)

Software

- Agilent ChemStation B.04.03

Reagents and materials

All solvents used were HPLC grade. Purified water was used from a Milli-Q water purification system (Millipore, USA). Methanol super gradient were purchased from Lab-Scan. HPLC grade, butyl chloride, tetrahydrofuran, glacial acetic acid, and chloroform were purchased from Sigma-Aldrich (India). Prednisolone (Vetranal, analytical reagent >99%), and betamethasone (USP grade) were also purchased from Sigma-Aldrich (India). For the testing of assay method, another prednisolone standard with a different part number was purchased from Sigma-Aldrich (India).

Chromatographic parameters

The chromatographic parameters for SFC chromatography with the 1260 Infinity Hybrid SFC/UHPLC System are shown in Table 1. The SFC flow rate and back pressure regulator (BPR) was maintained at a low value of 1 mL/min and 90 bar respectively, to keep the system under pressure while switching to SFC after normal phase runs.

Preparation of standards

Preparation of water-saturated chloroform: To 500 mL of chloroform, 300 mL of water was added in a separatory funnel and mixed. After phase separation, the bottom layer (chloroform) was collected.

Normal phase internal standard solution preparation: Betamethasone was accurately weighed out, to which tetrahydrofuran was added to obtain a concentration of 5 mg/mL. This solution was then diluted to 0.5 mg/mL using water saturated chloroform.

Normal phase standard solution: 1 mg prednisolone (USP grade) was added to a 10-mL volumetric flask, followed by 0.5 mL of methanol to dissolve. To this flask, 2 mL of internal standard solution was added, followed by dilution to the 10-mL mark using water saturated chloroform.

SFC internal standard solution: Betamethasone, which was accurately weighed out, was dissolved in 100% methanol to obtain 0.5 mg/mL.

SFC standard solution: First, 1 mg of USP prednisolone was dissolved in 0.5 mL of 100% methanol and then, 2 mL of SFC internal standard solution was added. The solution was filled to the 10-mL mark with 100% methanol.

Linearity and robustness sample preparation: The SFC solution described above was used for linearity and robustness studies (100 ppm of prednisolone and betamethasone).

Sample preparation

Normal phase/SFC assay test solution: Approximately 1 mg of prednisolone (test standard) was transferred to a 10-mL volumetric flask, followed by 0.5 mL methanol to dissolve. 2 mL of normal phase internal standard solution was added, followed by water saturated choloroform to the 10-mL mark.

Parameters	Normal phase method	SFC method
Column	Agilent ZORBAX Rx-SIL 4.6 × 250 mm, 5 µm (p/n 880975-901)	Agilent ZORBAX Rx-SIL 4.6 × 250 mm, 5 µm (p/n 880975-901)
Thermostatted column compartment solvent preheating	25 °C	40 °C
Thermostatted column compartment solvent post conditioning	not controlled	37.5 °C
Detection	254/16 nm (Ref 360/100 nm) 40 Hz acquisition rate	254/16 nm (Ref 360/100 nm) 40 Hz acquisition rate
Flow cell	10 mm path length, 13 µL volume high pressure flow cell	10 mm path length, 13 µL volume high pressure flow cell
Injection volume	5 µL*	5 µL
Injector program	Yes	Yes
BPR	90 bar	150 bar
SFC flow rate	1 mL/min	2.9 mL/min
Normal phase flow rate	1 mL/min	0 mL/min
SFC run	-	15% B isocratic
Normal phase run	100% A isocratic	-
Run time	20 minutes	5.5 minutes
Mobile phase	Mixture of butyl chloride, water-saturated butyl chloride, tetrahydrofuran, methanol, and glacial acetic acid (95:95:14:7:6)	85% supercritical fluid CO_2, 15% methanol

*The injector volume was decreased from 10 µL to 5 µL to fit the 5 µL fixed loop.

Table 1
Chromatographic parameters used in the Agilent 1260 Infinity Hybrid SFC/UHPLC System.

Procedure

The normal phase pump seal (p/n 0905-1420) was used in the 1260 Infinity Binary Pump of the hybrid system. The pump was equilibrated with isopropyl alcohol prior to use normal phase solvents. The 1260 Infinity SFC/UHPLC Hybrid System was operated in normal phase mode by switching the 2-position/10-port valve. The normal phase runs were performed using the "normal phase standard solution" to determine the USP system suitability parameters. The 2-position/10-port valve was then switched to SFC mode to perform the SFC runs to determine the system suitability parameters. In the SFC mode, linearity and robustness studies were also performed.

A solution of 100% methanol (super gradient) was injected as blank, followed by 11 linearity levels in replicate injections. The average of six area and retention time (RT) information for each level was used to calculate the relative standard deviation (RSD) values. The average area of each linearity level in the linearity range was plotted against the concentration to obtain a calibration curve. The LOD and LOQ for prednisolone and betamethasone was established from the lower linearity level injections based on signal-to-noise ratio. The dilutions for the linearity levels were prepared as per Table 2.

Calibration levels	Prednisolone (µg/mL)	Betamethasone (µg/mL)
1	7.2	6.0
2	24.0	20.0
3	50.4	42.0
4	100.8	84.0
5	144.0	120.0
6	192.0	160.0
7	240.0	200.0
8	288.0	240.0
9	360.0	300.0
10	408.0	340.0
11	480.0	400.0

Table 2
Dilution table for prednisolone and betamethasone.

To evaluate the robustness of the method, five method parameters were evaluated:

- Flow rate ± 2%
- Column temperature ± 2.5%
- Injector volume ± 3%
- Absorption wavelength ± 1 nm
- Modifier concentration ± 1%

For each robustness parameter, a SFC standard preparation of 100 ppm solution of prednisolone and betamethasone were injected, six replicates were used to calculate area, RT and resolution of prednisolone compared to betamethasone. The original method was also performed similarly. The percentage deviation (% accuracy) of area/retention time was calculated from the original method.

To determine the amount of prednisolone in the test sample, the normal phase/SFC assay test solution was used. The same sample was run on the normal phase method as well as on the SFC method. The prednisolone peak area was compared against the prednisolone peak area obtained from the "normal phase standard." The quantity of prednisolone was determined in mg using the formula specified in the USP assay method.

Results and discussion

Separation and detection

The system suitability mixture was used to optimize the separation conditions. The separation was initially performed at initial SFC conditions (TCC temperature of 35 °C, back pressure regulator at 150 bar, Agilent ZORBAX Rx-Sil column and flow rate of 3.0 mL/min). The methanol percentage was varied from 20% B (methanol) isocratic by decreasing it systematically to 5% isocratic in different runs. The ideal separation was found to be at 15% B isocratic. Following the mobile phase optimization, flow rate optimization was carried out. The flow rate was changed from 1.5 mL/min to 3.5 mL/min in increments of 0.2 mL/min where area/RT of the peaks were recorded. The ideal flow rate was determined to be 2.9 mL/min. The TCC temperature was also varied from 25 °C to 45 °C where the ideal temperature was found to be at 40 °C.

Figure 2 shows the chromatogram of the SFC method performed at the final optimal condition overlaid with the USP normal phase method. The detector was set at 254 nm as suggested in the USP method. Figure 2, in the SFC method, shows some additional peaks around the column void time (~0.9 minute). These peaks originate from the "super gradient" methanol used to dilute the sample.

The system suitability test was performed using both methods. The SFC method provided acceptable relative retention time values and resolution (Table 3). The area precision for four replicate injections showed better results in the SFC method as compared to the USP normal phase method. The added benefit of SFC is to be able to run the sample at a faster flow rate. It also used methanol as the only modifier. The advantage of the SFC method compared to normal phase method in regards to analysis time and solvent cost (US $) per 100 sample analysis is displayed in Table 4. A 4-fold decrease in analysis time and a 17-fold decrease in cost was achieved with the SFC method for every analytical run. Assuming analysis time to be US $ 80/hr, the overhead cost would decrease to US $ 20/hr.

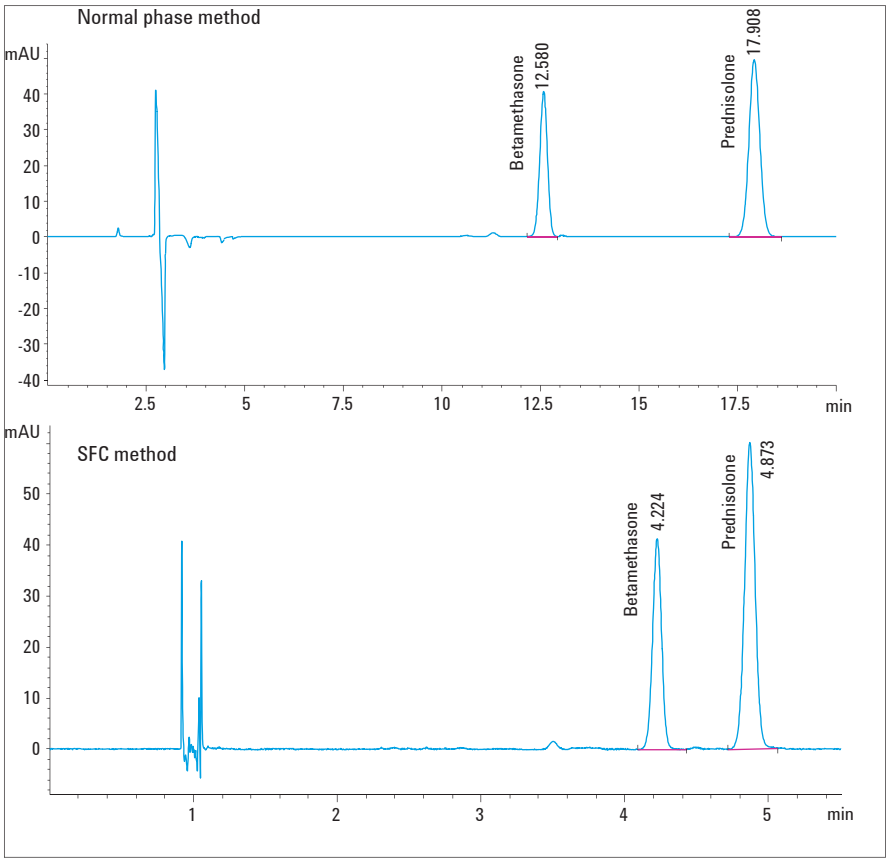

Figure 2
Separation of 100 ppm solution of prednisolone and betamethasone using an Agilent ZORBAX Rx- SIL 4.6 × 250 5 µm column.

Parameter		USP method	USP normal phase method	SFC method
RRT	Prednisolone	1.0	1.0	1.0
	Betamethasone	0.7	0.7	0.9
Resolution		NLT 3.5	11.6	4.1
Std injection (n=4) (Prednisolone)		RSD Area NMT 2.0%	0.5%	0.1%

Table 3
USP prednisolone system suitability acceptable limits compared with USP normal phase method and SFC method. NLT = Not less than, NMT = Not more than.

	USP normal phase method	SFC method	Savings
Analysis time per sample (min)	20	5.5	3.6×
Solvent cost per 100 analysis (US $)	292	17.2	17×

Table 4
Savings in analysis time and solvent cost per 100 sample analysis when using the SFC method compared with the normal phase method.

LOD, LOQ, and linearity using the SFC method

The analyte concentration that provides a signal-to-noise ratio (S/N) was considered as LOD, while the analyte concentration with S/N ratio > 10 was considered as LOQ. LOD, LOQ, and linearity was performed for SFC method only. Table 5 shows that the LOD for prednisolone was found to be at 2.4 µg/mL while the LOQ was found to be at 7.0 µg/mL.

The linearity levels were determined using the SFC method starting from the LOQ level of prednisolone and betamethasone. Figure 3 shows calibration curves for these two compounds. Both calibration curves were found to be linear having correlation coefficient (R^2) values of >0.999. The results show the excellent performance of SFC as a replacement method for normal phase method.

		LOD		LOQ		Linearity range		Number
Sl no	Name	µg/mL	S/N	µg/mL	S/N	µg/mL	R^2 value	of levels
1	Prednisolone	2.4	5.0	7.2	13.2	7.2–480	0.9993	11
2	Betamethasone	2.0	3.9	6.0	10.0	6.0–400	0.9992	11

Table 5
LOD, LOQ, and linearity of prednisolone and betamethasone.

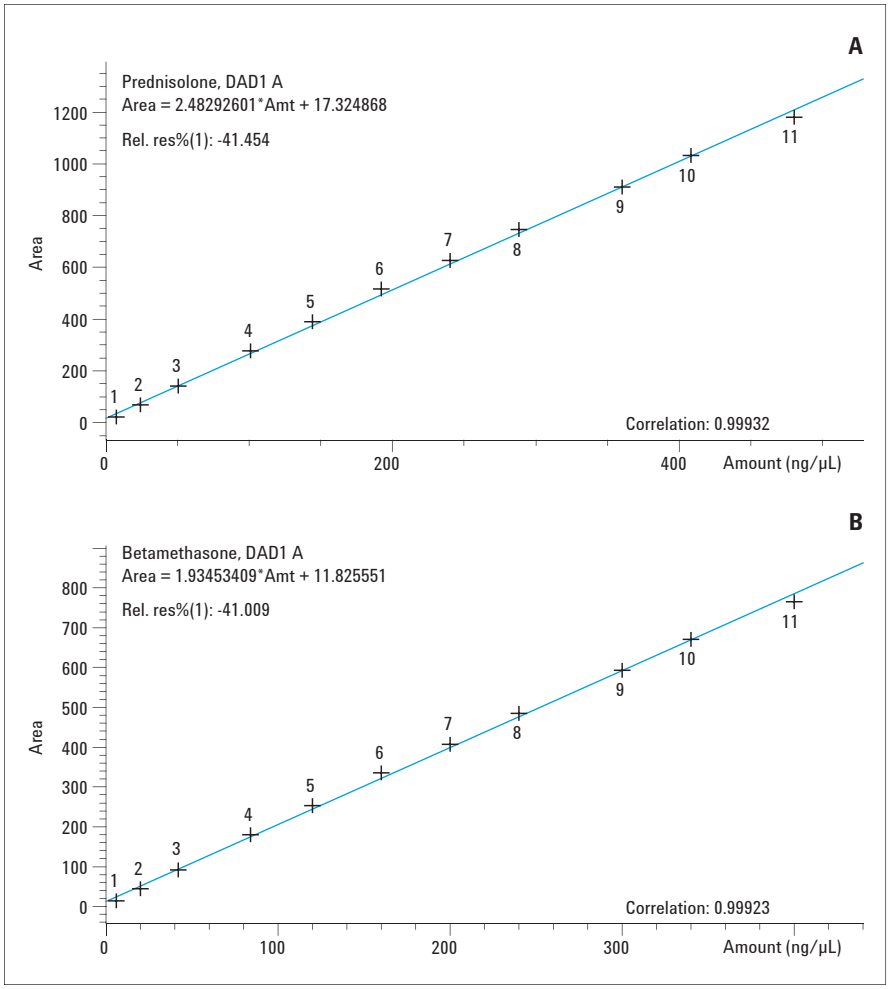

Figure 3
Linearity curves of prednisolone (A) and betamethasone (B).

Precision of retention time and area

The area precision was measured as RSD (%) across the linearity levels with the SFC method. The maximum RSD value of 2.2% and 2.1% for level 1 (L1) were obtained for prednisolone and betamethasone respectively. Similarly, RT precision calculations obtained a maximum RSD value <0.2% for both prednisolone and betamethasone. Graphical representation of area RSD values are displayed in Figure 4.

Robustness

To test the robustness of the method, a standard solution containing 100 ppm of prednisolone and betamethasone was used. Five critical method parameters (flow rate, column temperature, injector volume, absorption wavelength, and modifier concentration) were varied individually. The peak areas from the six replicate injections were compared. The allowed deviation for the area and retention time was set to ± 5% and ± 3% respectively. The results of the robustness tests are summarized in Table 6. The red numbers indicate where the result exceeded the allowed deviation. A change in flow rate and TCC temperature does not vary the method. The injection volume of 15 µL is taken in order to fill 3x the fixed injection loop of 5 µL. A deviation in injection volume of ±3% from 15 µL also does not affect the method. It is recommended to use 15 µL to overfill the 5 µL injection loop. The modifier concentration rise by 1%, changes the RT of the compound but not the area. The lowering of the modifier concentration however does not change the RT. The deviation in area due to wavelength of 254 nm is also very sensitive because of the position of 254 nm in the absorption spectra of both prednisolone and betamethasone.

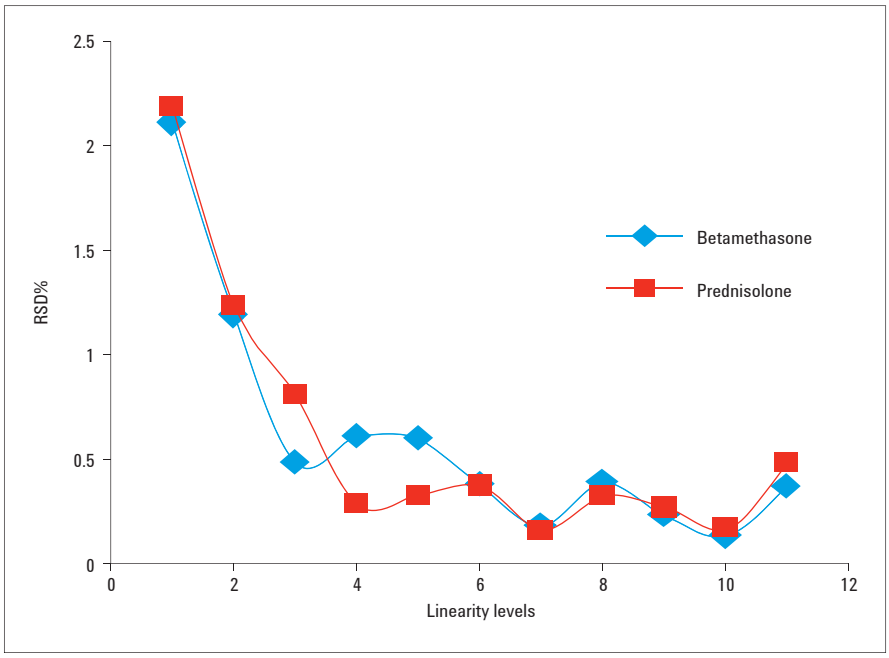

Figure 4
Area precision measured as RSD(%) for six replicates at each concentration level for prednisolone and betamethasone.

		Prednisolone			Betamethasone	
Parameters	**Variations**	**% area**	**% RT**	**Resolution**	**% area**	**% RT**
Flow: 2.9 mL/min ± 2%	High: 2.96 mL/min	-3.5	-2.9	4.1	2.2	-2.8
	Low: 2.84 mL/min	1.9	1.7	4.1	-3.4	1.8
TCC: 40 °C ± 2.5%	High: 41°C	0.1	0.8	4.0	0.1	0.9
	Low: 39°C	-0.4	-1.4	4.0	-0.4	-1.3
Injector: 15 µL ± 3%	High: 15.5 µL	-0.1	-0.6	4.0	-0.2	-0.3
	Low: 14.5 µL	-0.3	-0.7	4.0	-0.2	-0.5
Wavelength: 254 ± 1 nm	255 nm	-4.8	-0.8	4.0	-6.0	-0.6
	253 nm	2.5	-0.8	4.0	3.6	-0.5
Modifier concentration: 15% B ± 1%	High: 15.2 %B	0.2	-3.7	3.9	0.2	-3.3
	Low: 14.8%B	0.0	1.3	4.0	0.0	1.5

Table 6
Results of the robustness test methods compared to the standard method at concentration of 100 ppm. The red values in the table indicate that the deviations exceeding the allowed limits of 5% for area and 3% for retention time.

The absorption wavelength needs to be constant as well as unchanging during the analysis. Alternatively, a different UV region such as 240 nm can be chosen for further studies. The resolution of prednisolone was not found to be changing in any of the robustness testing methods. Robustness results indicate that the method is reliable for normal usage, where, to a great extent, the performance remains unaffected by deliberate changes of the method parameters. However, some parameters, such as the wavelength and percentage modifier concentration are critical, which must be carefully controlled.

Assay results

To test the accuracy of both the methods, the normal phase/SFC assay test solution (see page 3) was tested with both the SFC method and the normal phase method. The analysis was performed according to the USP assay method, which involves comparing the area with that of the system suitability mix. The results from Table 7 show that for the same test sample approximately 1.3 mg of prednisolone was detected in a 10-mL solution, for both of the methods, confirming similar performance.

Method	Assay results (mg)
SFC method	1.278
Normal phase method	1.272

Table 7
Assay results obtained from SFC method and the USP normal phase method.

Conclusion

The Agilent 1260 Infinity Hybrid SFC/UHPLC System was used to develop a novel SFC prednisolone assay method and this method was compared to the original USP normal phase method. While meeting the system suitability requirements, the new SFC method was 4x faster and 17x less expensive than the normal phase method. Additionally, the amount of prednisolone from a test sample delivered similar results with both methods. The linearity and robustness test results were excellent for the SFC method with a LOD value of about 2 ppm for both prednisolone and betamethasone. The SFC method does not require purchase and disposal of expensive environmentally hazardous chemicals. Hence, the newly developed SFC method provides a fast, cost effective, and safe solution.

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Agilent Technologies

Fast Screening Methods for Steroids by HPLC with Agilent Poroshell 120 Columns

Application Note

Pharma, BioPharma, and Clinical

Author

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Introduction

Steroids are a type of lipid derived from cholesterol. The main feature of steroids is the ring system of 3 cyclohexanes and 1 cyclopentane in a fused ring system, as shown in Figure 1. There are a variety of functional groups that may be attached. The main feature, as in all lipids, is the large number of carbon-hydrogens, which makes steroids non-polar [1]. Steroids include such well known compounds as gonadal steroids, birth control pills, cortisone, and anabolic steroids.

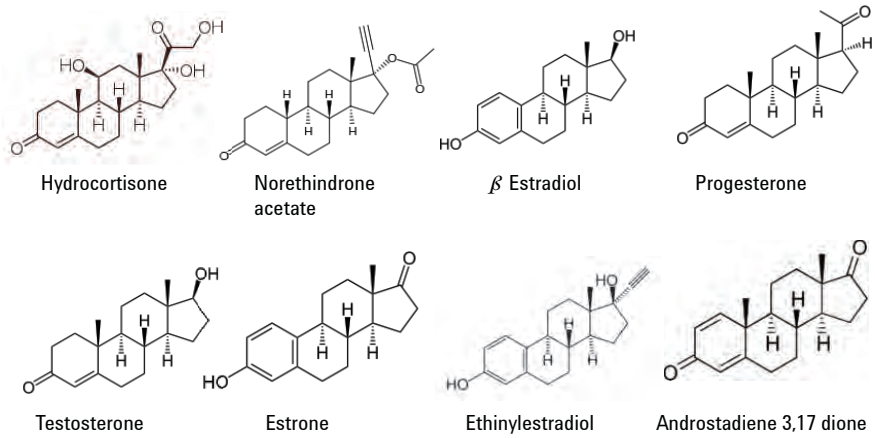

Figure 1. Structures of selected steroids.

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Gonadal steroids are steroid hormones that interact with vertebrate androgen or estrogen receptors. Gonadal steroids, also known as sex steroids, are a subset of the sex hormones that produce sex differences or support reproduction. There are also many synthetic sex steroids.

The 2 main classes of sex steroids are androgens and estrogens, of which the most important human derivatives are testosterone and estradiol. A third class of sex steroids are progestagens. Progesterone is the most important and only naturally occurring human progestagen. In general, androgens such as anabolic steroids (androstenedione, dehydroepiandrosterone, dihydrotestosterone, and testosterone) are considered male sex hormones, since they have masculinizing effects. Estrogens such as estradiol, estriol, estrone, and progestagens, which are primarily used to maintain pregnancy, are thought of as female sex hormones. Hydrocortisone, cortisone, corticosterone, 17-alpha hydroxyprogesterone, and progesterone belong to the progestagen class of steroid hormones, although all types are present in each sex, albeit at different levels [2,3].

Using Selectivity to Enhance Separation of Steroids

When considering the best way to increase chromatographic resolution, it can be useful to consider the resolution equation, which relates efficiency, selectivity, and retention faction.

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{1 + k'_B}{k'_B}\right)$$

To obtain high resolution, the 3 terms must be maximized. An increase in N, the number of theoretical plates, by lengthening the column, leads to an increase in retention time and increased band broadening. This may not be desirable. Instead, to increase the number of plates, the height equivalent to a theoretical plate can be reduced by reducing the particle size of the stationary phase particles. Superficially porous particles, such as Agilent Poroshell 120, achieve 90% of the efficiency of 1.8 µm materials with considerably lower pressure.

The selectivity factor, α, can also be manipulated to improve separations. Changing selectivity is the variable that can have the largest impact on any separation. Selectivity can be increased by:

- Changing mobile phase composition
- Changing column temperature
- Changing composition of stationary phase

Selectivity is the most powerful tool to optimize separations in HPLC. This parameter is changed by using different bonded phases, including C18, C8, polar embedded, and phenyl bonded phases, or by changing the mobile phase. In this work, Poroshell 120 columns and the Agilent 1200 SL Method Development Solution were used to quickly evaluate method development choices for the analysis of steroids. The short column length and high efficiency provided short analysis times and rapid equilibration leading to fast investigations of selectivity.

Experimental

The Agilent 1200 Infinity Series LC Multi-Method Solution was used. This system consisted of:

- 1260 Infinity Binary Pump (G1312B)
- 1290 Infinity Thermostatted Column Compartment (G1316C)
- 1260 Infinity High Performance Autosampler (G1367E)
- 1290 Infinity Diode-Array Detector (G4212A), equipped with 10 mm MaxiLight cartridge flow cell
- G6140 Single Quadrupole Mass Spectrometer.

The Agilent 1200 Infinity Series LC Multi-Method Solution is a highly flexible system that can be used for up to 4 (100 mm) columns. In addition, the Agilent ChemStation Method Scouting Wizard automates the setup of methods and sequences to screen the available combinations of columns, solvents, predefined gradients, and temperatures. In this work, 4 Agilent Poroshell 120 columns were used:

- Agilent Poroshell 120 StableBond SB-C18, 2.1 × 100 mm, 2.7 µm (p/n 685775-902)
- Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-902)
- Agilent Poroshell 120 Bonus-RP, 2.1 × 100 mm, 2.7 µm (p/n 685775-901)
- Agilent Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 2.7 µm (p/n 695775-912)

The TCC was fitted with a 6 position/6 port selection valve. This is a new Quick Change Valve mounted on a slide-out rail to make plumbing and maintenance more convenient. Port 1 was connected to a StableBond C18 column, and port 2 was connected to an EC-C18 column. Port 3 was connected to a Bonus-RP column, port 4 to a Phenyl-Hexyl column, and port 6 to a bypass connecting capillary.

The solvent passing into each column was heated using 1 of 4 individual low-dispersion heat exchangers. A G1160 12 solvent selection valve was connected to valve position A1 on the G1312B. Together with the internal solvent selection valve of the Binary SL Pump, up to 15 solvents could be screened using this system. The mobile phase was methanol or acetonitrile with 0.1% formic acid and water with 0.1% formic acid. An acetonitrile/water mixture (50%/50% v/v) was used to rinse the modifiers from the columns and allow proper column storage. Agilent ChemStation version B.04.02 was used to control the instrument and process the data.

The compounds examined included hydrocortisone, norethindrone acetate, estradiol, progesterone, testosterone, estrone, ethinylestradiol, and boldione, which were all purchased from Sigma Aldrich. Structures and details are shown in Figure 1 and Table 1. All samples were prepared at 10 mg/mL in acetonitrile and were diluted in water to a final concentration of 0.1 mg/mL.

Column choice to enhance selectivity

The columns were chosen to improve selectivity in the separation. They included a highly end capped column recommended as a first choice in method development (Poroshell 120 EC- C18), and a non end capped C18 (Poroshell 120 StableBond SB-C18) that could have interaction with silanol groups to provide an alternative C18 selectivity using neutral to low pH mobile phases. A polar-embedded amine column (Poroshell 120 Bonus-RP) and a phenyl-hexyl column (Poroshell 120 Phenyl-Hexyl) were also used. Phenyl bonded phases are known for their improved selectivity for aromatic compounds.

Table 1. Steroid nomenclature and molecular characteristics.

Common name	IUPC name	Molecular formula	Molecular weight
Hydrocortisone	Cortisol	$C_{21}H_{30}O_5$	362.460
Norethindrone acetate	(17α)-17-ethynyl-3-oxoestr-4-en-17-yl acetate	$C_{22}H_{28}O_3$	340.456
β Estradiol	(17β)-estra-1,3,5(10)-triene-3,17-diol	$C_{18}H_{24}O_2$	272.38
Progesterone	Pregn-4-ene-3,20-dione	$C_{21}H_{30}O_2$	314.46
Testosterone	(8R,9S,10R,13S,14S,17S)- 17-hydroxy-10,13-dimethyl- 1,2,6,7,8,9,11,12,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one	$C_{19}H_{28}O_2$	288.42
Ethinylestradiol	19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol	$C_{20}H_{24}O_2$	296.403
Androstadiene 3,17 dione (boldione)	(8R,9S,10R,13S,14S)-10,13-dimethyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-dione	$C_{19}H_{24}O_2$	284.39
Estrone	3-hydroxy-13-methyl- 6,7,8,9,11,12,13,14,15,16-decahydrocyclopenta[a]phenanthren-17- one	$C_{18}H_{22}O_2$	270.366

A polar-embedded group inserted into the hydrophobic C14 alkyl chain allows the Bonus-RP phase on totally porous Poroshell 120 to minimize interaction of polar samples with silanols, providing symmetrical peaks for a wide variety of applications. This phase is especially useful at neutral pH where amines can interact strongly with ionized silanols. The polar-embedded group also helps to wet the hydrophobic chains and prevents phase collapse in highly aqueous mobile phases.

Poroshell 120 Bonus-RP can be used for many of the same separations as a C18 column while avoiding some of the disadvantages of C18, such as poor wettability in high aqueous mobile phases. In addition, it is much more retentive for those molecules that can interact by hydrophobic interactions and also by H-bonding with the amide group. Compared to alkyl only phases, Bonus-RP has enhanced retention and selectivity for phenols, organic acids, and other polar solutes due to strong H-bonding between polar group (H-bond acceptor) and H-bond donors, like phenols and acids. Bonus-RP gives retention slightly less than a C18 allows, for easy column comparison without the need to change mobile phase conditions. The Bonus-RP phase gives different selectivity than C18 for polar compounds. It is also compatible with 100% water.

The Phenyl-Hexyl phase has unique reversed-phase selectivity, especially for polar aromatics and heterocyclic compounds, derived from analyte interaction with the aromatic ring of the bonded phase and its delocalized electrons. Poroshell 120 Phenyl-Hexyl can be orthogonal to both C18 and Bonus-RP phases. More retention and selectivity will often be observed for solutes with aromatic electron-withdrawing groups such as fluorine or nitro groups [4,5,6].

Poroshell 120 Phenyl-Hexyl columns deliver unique selectivity for compounds with aromatic groups, providing superior resolution for these samples. Poroshell 120 Phenyl-Hexyl can also provide optimum separations of moderately polar compounds where typical alkyl phases (C18 and C8) do not provide adequate resolution. Acetonitrile tends to decrease the π–π interactions between aromatic and polarizable analytes and the phenyl-hexyl stationary phases, but methanol enhances those same interactions, giving both increased retention and changes in selectivity [7]. This does not mean that acetonitrile should not be used with a phenyl bonded phase or that it might not provide an acceptable separation, but methanol is more likely to deliver the additional selectivity that is desired from a phenyl phase.

Results and Discussion

As can be seen in Figure 2, the separation of all 8 compounds was attempted on all columns surveyed. The Poroshell 120 EC-C18 and Poroshell 120 Phenyl Hexyl columns showed very similar profiles, although the elution on the Phenyl Hexyl column was faster. This could indicate that the π–π interactions on the Phenyl Hexyl column were being reduced by the acetonitrile. The overlap of estradiol and androstadiene was less severe on the Phenyl Hexyl column. The Poroshell 120 SB-C18 column delivered a very different separation, resolving estradiol but loosing resolution on ethylestradione and estrone. This could be due to the exposed silanols on the SB-C18 phase or to some additional shape selectivity derived from the di-isobutyl side chains on the SB-C18 phase. Some additional work is needed to determine this. The Poroshell 120 Bonus RP phase almost separates all 8 compounds, and when using acetonitrile, it would provide the best method development option for further development.

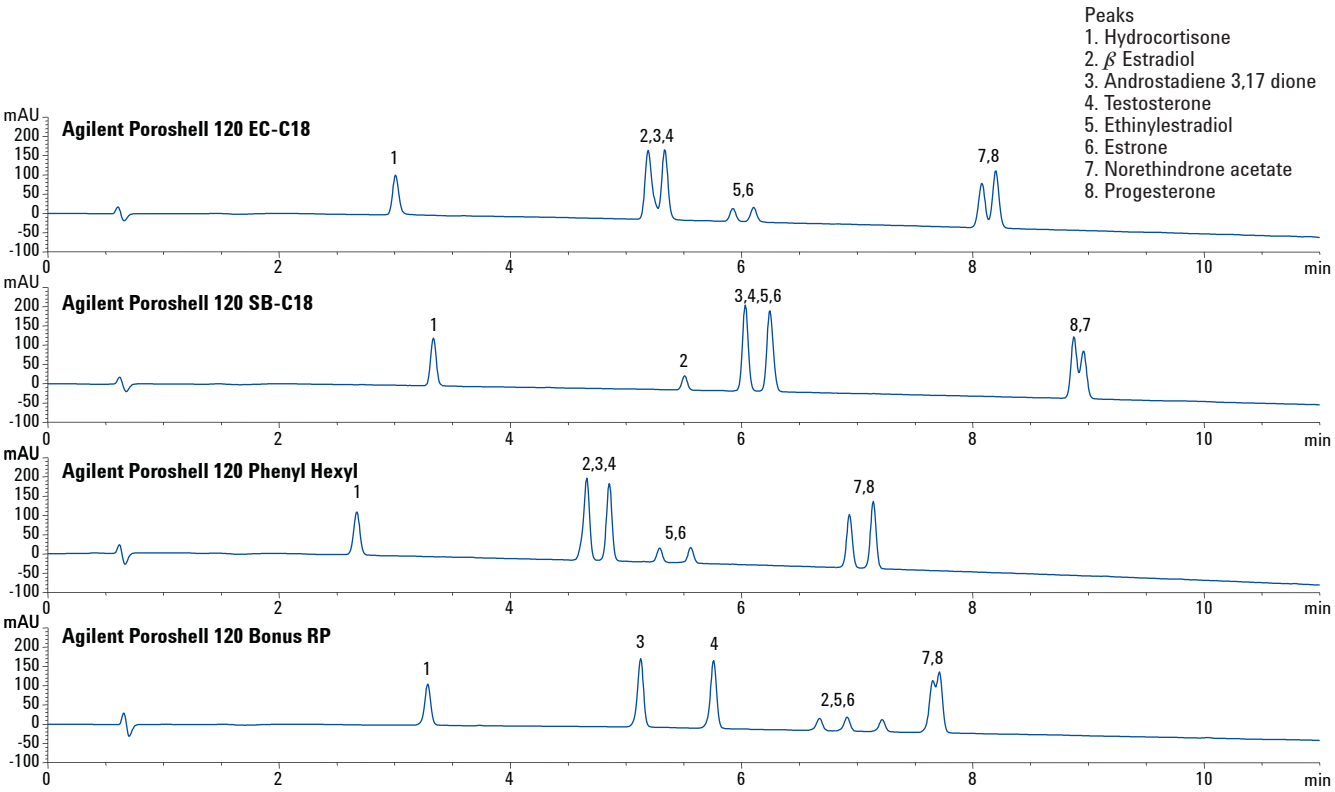

Figure 2. Separation of steroids using Agilent Poroshell 120 columns with acetonitrile.

Conditions

Columns:	Agilent Poroshell 120, 2.1 × 100 mm
Flow rate:	0.4 mL/min
Gradient:	40-80% MeOH/14 min (0.1% formic acid in water and MeOH)
Temperature:	40 °C
Detection:	DAD 260, 80 ref = off

In Figure 3, the separation was carried out using methanol at slightly elevated temperature (40 °C). In this case, the 2 C18 phases (Poroshell 120 EC-C18 and Poroshell 120 SB-C18) yielded nearly identical chromatographic profiles. Some additional retention was seen on the SB-C18 phase due to some silanol interaction. The Poroshell 120 Bonus-RP chromatogram had 3 overlapping peak pairs, which would likely make further method development difficult in methanol. However, the Poroshell 120 Phenyl Hexyl phase resolved 8 compounds at better than baseline resolution.

Peaks
1. Hydrocortisone
2. β Estradiol
3. Androstadiene 3,17 dione
4. Testosterone
5. Ethinylestradiol
6. Estrone
7. Norethindrone acetate
8. Progesterone

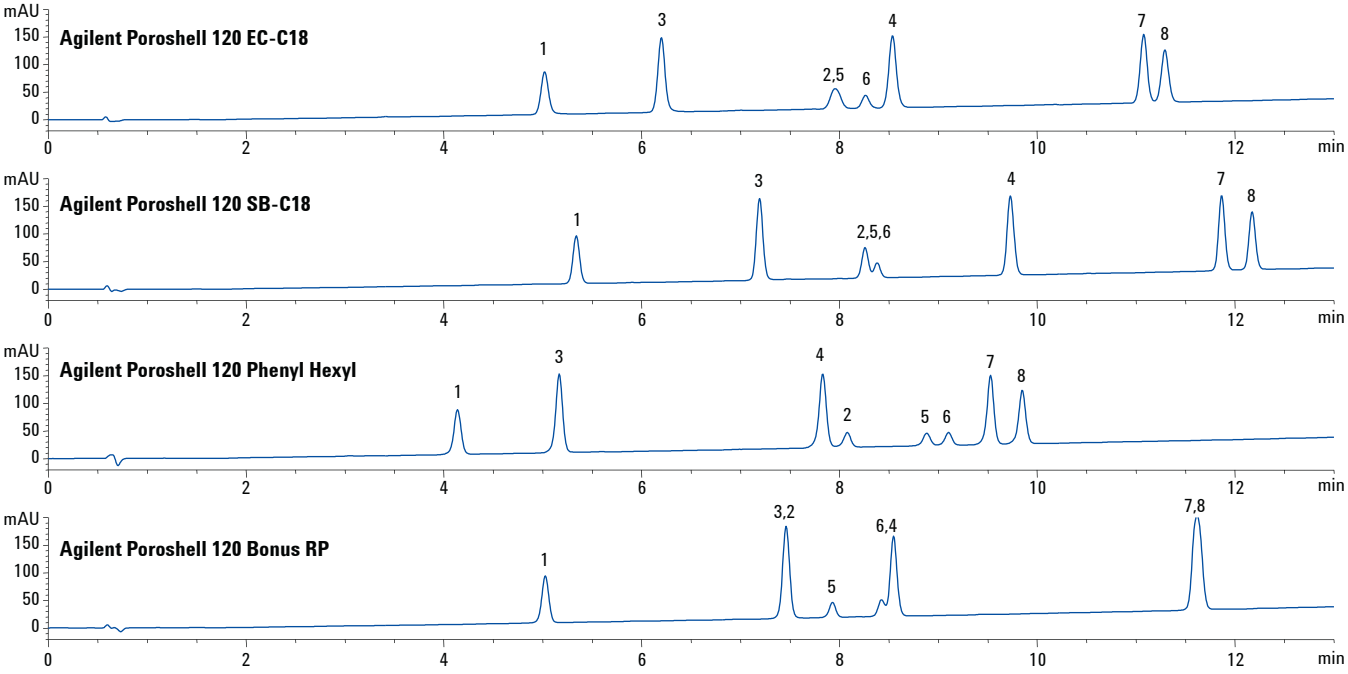

Figure 3. Separation of steroids using Agilent Poroshell 120 columns with methanol.

Conditions

Columns:	Agilent Poroshell 120, 2.1 × 100 mm
Flow rate:	0.4 mL/min
Gradient:	25-80% MeCN/10 min (0.1% formic acid in water and MeCN)
Temperature:	25 °C
Detection:	DAD 260,80 ref = off

Conclusions

Analysis problems can be quickly resolved by including survey methods with generic gradients as part of the method development scheme. This work used steroids as an example, and showed how phases and organic modifiers, such as acetonitrile and methanol, could develop different selectivity that could be used to optimize the separation. In this case, the widely used C18 phases, as found on Poroshell 120 EC-C18 and SB-C18 columns, did not provide adequate separation. Using an alternative selectivity column such as Poroshell 120 Bonus-RP in acetonitrile or Poroshell 120 Phenyl Hexyl yielded better results, and could be used for several thousand samples.

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Agilent Technologies

Fast Screening Methods for Beta Blockers by HPLC with Agilent Poroshell 120 Columns

Application Note

Pharma, Biopharma, and Clinical

Author

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Agilent Technologies, Inc.

Introduction

Beta blockers, or beta-adrenergic blocking agents, are a class of drugs used to treat hypertension and to manage cardiac arrhythmias after a heart attack. As beta adrenergic receptor antagonists, they diminish the effects of epinephrine (adrenaline) and other stress hormones. The first beta blocker was synthesized in 1958 by Eli Lilly Laboratories, but in 1962, the first clinically significant beta blockers, propranolol and pronethalol, were developed and used for the treatment of angina pectoris.

Beta blockers block the action of epinephrine (adrenaline) and norepinephrine (noradrenaline), in particular, on β-adrenergic receptors, part of the sympathetic nervous system that mediates the fight-or-flight response. Three types of beta receptors are known, designated β_1, β_2, and β_3 receptors. β_1-Adrenergic receptors are located mainly in the heart and in the kidney, β2-adrenergic receptors are mainly in the lungs, gastrointestinal tract, liver, uterus, vascular smooth muscle, and skeletal muscle. β_3-Adrenergic receptors are found in fat cells [1,2].

Beta blockers can be abused in sports involving little physical activity, such as archery, to reduce cardiac contraction, heart rate, and coronary blood flow. They have, therefore, been included in the list of forbidden substances by the International Olympic Committee [3].

Selectivity is the most powerful tool to optimize separations in HPLC. This parameter is changed by using different bonded phases, including C18, C8, polar embedded, and phenyl bonded phases, or by changing the mobile phase. In this work, Agilent Poroshell 120 columns and the Agilent 1260 Infinity Method Development Solutions were used to quickly evaluate method development choices for the analysis of beta blockers. The short column length and high efficiency provided short analysis times and rapid equilibration leading to fast investigations of selectivity [4].

Agilent Technologies

Experimental

The Agilent 1200 Infinity Series LC Multi-Method Solution was used. This system consisted of:

- 1260 Infinity Binary Pump (G1312B)
- 1290 Infinity Thermostatted Column Compartment (G1316C)
- 1260 Infinity High Performance Autosampler (G1367E)
- 1290 Infinity Diode-Array Detector (G4212A), equipped with 10 mm MaxiLight cartridge flow cell
- G6140 Single Quadrupole Mass Spectrometer.

The Agilent 1200 Infinity Series LC Multi-Method Solution is a highly flexible system that can be used for up to 4 (100 mm) columns. In addition, the Agilent ChemStation Method Scouting Wizard automates the setup of methods and sequences to screen the available combinations of columns, solvents, predefined gradients, and temperatures. In this work, 4 Agilent Poroshell 120 columns were used:

- Agilent Poroshell 120 StableBond SB-C18, 2.1 × 100 mm, 2.7 µm (p/n 685775-902)
- Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-902)
- Agilent Poroshell 120 Bonus-RP, 2.1 × 100 mm, 2.7 µm (p/n 685775-901)
- Agilent Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 2.7 µm (p/n 695775-912)

The TCC was fitted with a 6 position/4 port selection valve. This is a new Quick Change Valve mounted on a slide-out rail to make plumbing and maintenance more convenient. Port 1 was connected to a StableBond C18 column, and port 2 was connected to an EC-C18 column. Port 3 was connected to a Bonus-RP column, port 4 to a Phenyl-Hexyl column, and port 6 to a bypass connecting capillary.

The solvent passing into each column was heated using 1 of 4 individual low-dispersion heat exchangers. An additional 12-solvent selection valve was connected to valve position A1 on the 1260 Infinity Binary pump. Together with the internal solvent selection valve of the 1260 Infinity Binary pump, up to 15 solvents could be screened using this system. The mobile phase was methanol and 10 mM ammonium formate titrated to pH 3.8 with formic acid. Water was used as a final weak solvent and to rinse the modifiers from the columns and allow proper column storage. Ammonium formate and formic acid were purchased from Sigma. Milli-Q 18 MΩ water was used.

Methanol was used throughout as a strong solvent and was obtained from Honeywell. Temperature was controlled at 25 °C, and flow rate was set at 0.4 mL/min. Agilent ChemStation version B.04.02 was used to control the instrument and process the data.

The compounds examined included nadolol, atenolol, alprenolol, acebutalol, pindolol, propranolol, metoprolol, and labetalol, which were all were purchased from Sigma Aldrich. Structures are shown in Figure 1. The pKa of these basic compounds ranged from 8.8 to 9.7. All samples were prepared at 10 mg/mL in DMSO and were diluted in water to a final concentration of 0.1 mg/mL.

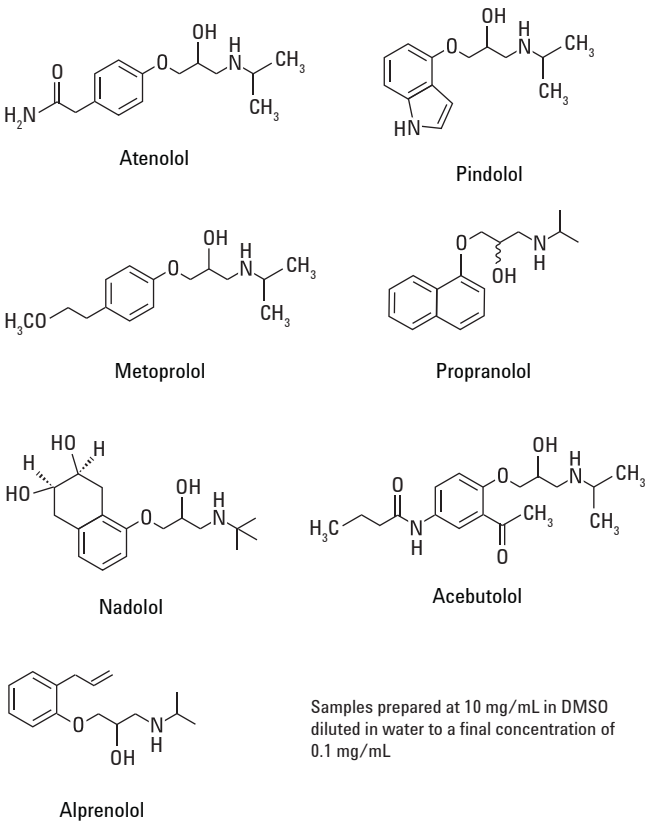

Figure 1. Structures of some beta blockers.

Column Choice to Enhance Selectivity

The columns were chosen to improve selectivity in the separation. They included a highly end capped column recommended as a first choice in method development (Poroshell 120 EC- C18); a non end capped C18 (Poroshell 120 StableBond SB-C18) that could have interaction with silanol groups, providing an alternative C18 selectivity using neutral to low pH mobile phases; a polar embedded amine column (Poroshell 120 Bonus-RP), and a phenyl-hexyl column (Poroshell 120 Phenyl-Hexyl). Phenyl bonded phases are known for their improved selectivity for aromatic compounds.

A polar-embedded group inserted into the hydrophobic C14 alkyl chain allows the Bonus-RP phase on totally porous Poroshell 120 to minimize interaction of polar samples with silanols, providing symmetrical peaks for a wide variety of applications. This phase is especially useful at neutral pH where amines can interact strongly with ionized silanols. The polar-embedded group also helps to wet the hydrophobic chains and prevents phase collapse in highly aqueous mobile phases.

Poroshell 120 Bonus-RP can be used for many of the same separations as a C18 column while avoiding some of the disadvantages of C18, such as poor wettability in high aqueous mobile phases. In addition, it is much more retentive for those molecules that can interact by hydrophobic interactions and also by H-bonding with the amide group. Compared to alkyl only phases, Bonus-RP has enhanced retention and selectivity for phenols, organic acids, and other polar solutes due to strong H-bonding between polar group (H-bond acceptor) and H-bond donors, like phenols and acids. Bonus-RP gives retention slightly less than a C18 allows for easy column comparison without the need to change mobile phase conditions. The Bonus-RP phase gives different selectivity than C18 for polar compounds. It is also compatible with 100% water.

The Phenyl-Hexyl phase has unique reversed-phase selectivity, especially for polar aromatics and heterocyclic compounds, derived from analyte interaction with the aromatic ring of the bonded phase and its delocalized electrons. Poroshell 120 Phenyl-Hexyl can be orthogonal to both C18 and Bonus-RP phases. More retention and selectivity will often be observed for solutes with aromatic electron-withdrawing groups such as fluorine or nitro groups [5,6].

Poroshell 120 Phenyl-Hexyl columns deliver unique selectivity for compounds with aromatic groups providing superior resolution for these samples. Poroshell 120 Phenyl-Hexyl can also provide optimum separations of moderately polar compounds where typical alkyl phases (C18 and C8) do not provide adequate resolution Acetonitrile tends to decrease the π–π interactions between aromatic and polarizable analytes and the phenyl-hexyl stationary phases, but methanol enhances those same interactions, giving both increased retention and changes in selectivity [7]. This does not mean that acetonitrile should not be used with a phenyl bonded phase or that it might not provide an acceptable separation, but methanol is more likely to deliver the additional selectivity that is desired from a phenyl phase.

Results and Discussion

As can be seen in Figure 2, the separation of all 7 compounds was accomplished on all columns surveyed. The Poroshell 120 EC-C18 column showed very close elution of acebutanol and propranolol and a double peak with the same molecular ion for naldolol. The double peak for nadolol was attributed to a diastereomer. The Poroshell 120 SB-C18 column delivered almost the same separation. This was not found with the Poroshell 120 Bonus-RP column, as it tended to minimize secondary interactions. The Poroshell 120 Bonus-RP column also reversed peaks 6 and 7 (propranolol and alprenolol), compared to the C18 columns. The Poroshell 120 Phenyl-Hexyl column shared this 6, 7 peak reversal and additionally reversed peaks 4 and 5 (metoprolol and acebutolol). The separation was similar to that shown by the other C18 columns but was not as retentive.

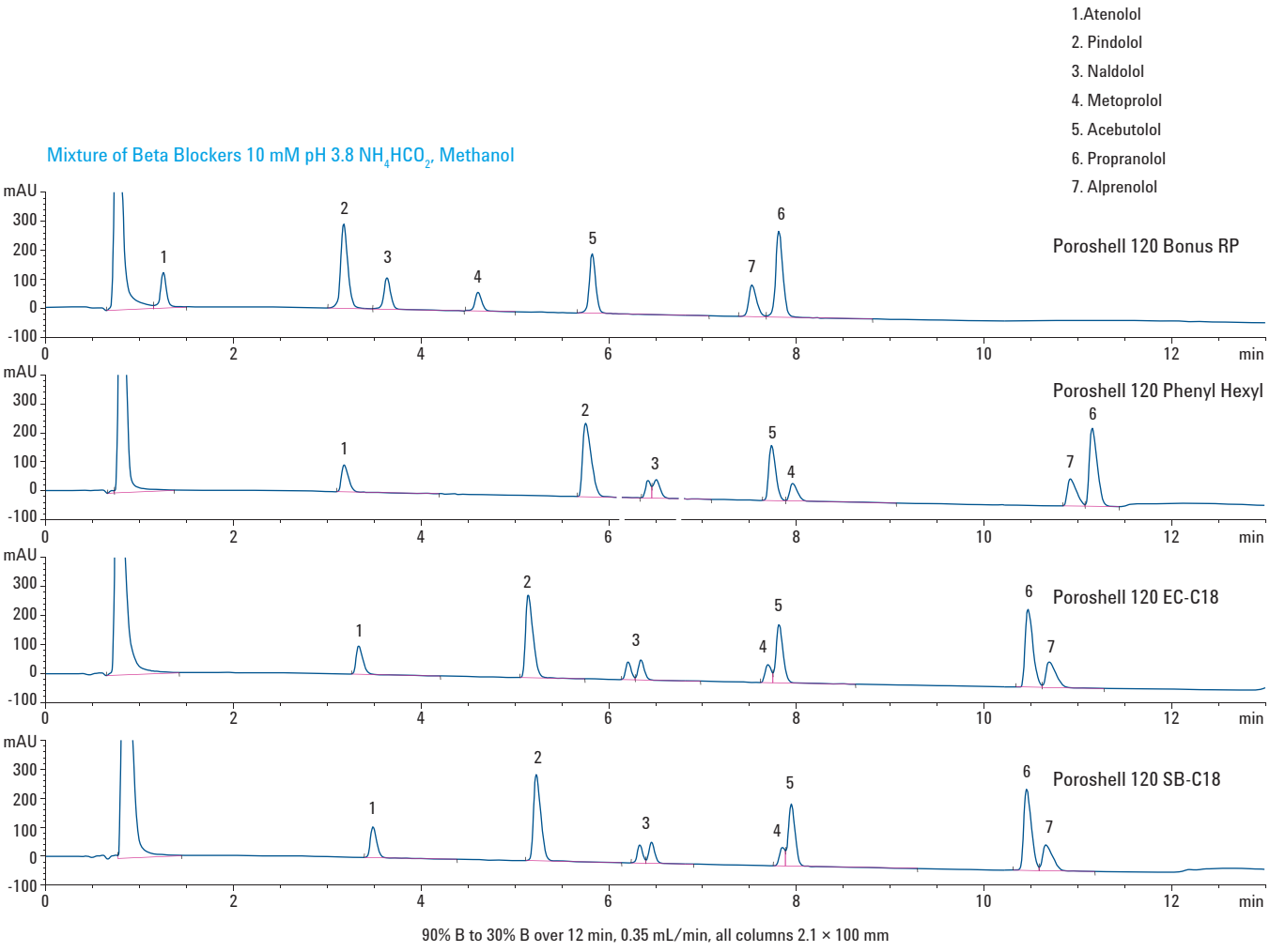

Figure 2. Separation of beta blockers using Agilent Poroshell 120 columns.

Conclusions

Analysis problems can be quickly resolved by including survey methods with generic gradients as part of the method development scheme. This work uses beta blockers as an example, and shows how phases with different selectivity can be used to optimize the separation. While the Poroshell 120 EC-C18 and SB-C18 columns provide adequate separation, using an alternative selectivity column, such as Poroshell 120 Bonus-RP, yields even better results and can be used for several thousand samples. Automatic setup of methods and sequences for the Poroshell 120 columns was straightforward using the Agilent 1200 Infinity Series LC Multi-Method Solution.

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Agilent Technologies

Analysis of Paracetamol and Aspirin in pain relievers on the Agilent 1220 Infinity Isocratic LC System with manual injector

Excellent chromatographic results at lowest costs

Application Note

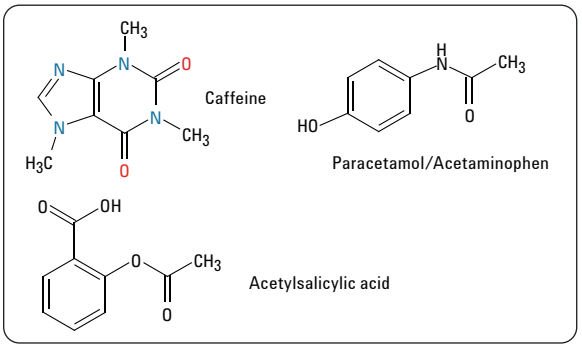
Pharmaceuticals

Author

A.G.Huesgen

Agilent Technologies

Waldbronn, Germany



Abstract

The Agilent 1220 Infinity LC System is an integrated LC system consisting of an isocratic pump, manual injector and variable wavelength detector. This Application Note describes the analysis of paracetamol and aspirin to demonstrate that typical acceptance criteria for a USP method can be fulfilled. These criteria are:

- Precision of areas < 2% RSD
- Precision of retention times < 0.5% RSD
- Resolution > 1.4
- Tailing factor < 1.2

The data from this analysis show that the Agilent 1220 Infinity LC System provides excellent performance at low costs.

Agilent Technologies

Introduction

The Agilent 1220 Infinity LC System is a liquid chromatography (LC) system for routine standard analysis. Due to its extraordinary pressure range up to 600 bar, the system can perform UHPLC applications. It is an easy to use, integrated LC system consisting of an isocratic pump, manual injector and variable wavelength detector (VWD).

The isocratic pump has a flow range of 0.2 to 10 mL/min (5 mL at 600 bar, 10 mL at 200 bar) and an integrated degasser. The VWD detector features 80 Hz data acquisition rate and a wavelength range from 190 nm to 600 nm.

The system can be upgraded according to growing needs with:

- Oven upgrade kit – adds a click-in oven to your Agilent 1220 Infinity LC System
- Isocratic to gradient pump upgrade kit – adds gradient capabilities to your isocratic Agilent 1220 Infinity LC System
- Manual injector to autosampler upgrade kit – exchanges your manual injector with an autosampler

Paracetamol and aspirin were chosen as examples in this analysis to demonstrate that typical acceptance criteria for a USP method can be fulfilled (Fgure 1). The isocratic USP method with UV detection according to USP/NF 23 was applied for analysis.

Experimental

Instrumentation

For the analysis of paracetamol, aspirin and caffeine, an Agilent 1220 Infinity LC System with the following configuration was used:

- Agilent 1220 Infinity LC System (G4286B) consisting of an isocratic pump, manual injector and VWD
- 20 µL loop (p/n 0100-1922) installed

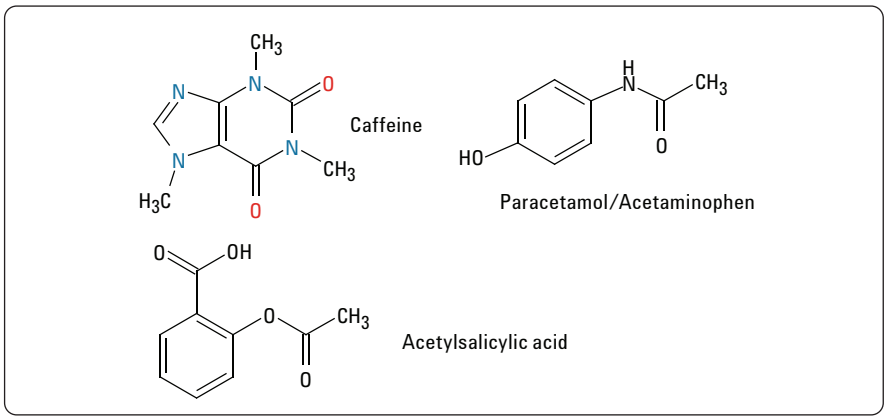


Figure 1
Structures of acetaminophen (paracetamol), aspirin and caffeine.

Chromatographic conditions according to USP method	
Column:	Agilent ZORBAX Eclipse Plus C18 Column, 3 mm × 100 mm, 3.5 µm (internal diameter 35% less than original method, particle size 30% less than original method)
Mobile phase:	Water/methanol/acetic acid = 69/28/3
Pump settings:	No gradient (in accordance with EP regulations)
Stop time:	6 min
Flow rate:	1 mL/min, isocratic (50% less than original method)
Injection volume:	20 µL
Column temp:	Ambient (Laboratory temperature between 24 °C and 25 °C)
Detector:	Agilent 1220 Infinity LC System with 10 mm path length flow cell Peak width 0.05 min (10 Hz) Signal 275 nm

The original method was changed according to the typically allowed changes for chromatographic parameters (Table 1).

Chromatographic parameter	Typically allowed changes
Mobile phase pH	± 0.2 units
Concentration of salts in buffers	± 10%
Ratio of mobile phase percentages	± 30% of the minor component, or 0.2% absolute of that component, whichever is greater. However a change in any component cannot exeed ± 10% absolute, nor can the final concentration be reduced to zero
Wavelength of UV detector	No change permitted
Column length	± 70%
Internal diameter of column	± 50%
Particle size of column packing material	Can be reduced by 50%
Flow rate	± 50%
Injection volume	Increased up to twice the volume specified, provided no adverse effects. Must be within stated linearity range of the method
Column compartment temperature	± 10°C

Table 1
Typically accepted changes for USP methods.

Preparation of samples

The reference solution was prepared according to the concentrations listed in Table 2.

Results and discussion

System suitability testing was performed to verify that the LC system fulfills the acceptance criteria typical for USP methods. The following acceptance criteria had to be fulfilled:

- Precision of areas must be < 2% RSD
- Precision of retention times must be < 0.5% RSD
- Resolution must be > 1.4 for benzoic acid
- Tailing factor < 1.2

An overlay of six consecutive chromatograms is shown in Figure 2.

Table 3 combines the results to show that the acceptance criteria are fulfilled.

Conclusion

The Agilent 1220 Infinity LC system is a liquid chromatography (LC) system for routine standard analysis. Due to its extraordinary pressure range up to 600 bar, the system can also perform UHPLC applications. It is an integrated LC system consisting of an isocratic pump, manual injector and variable wavelength detector (VWD). The application example of the analysis of pain relievers shows that typical USP acceptance criteria are fulfilled on the Agilent 1220 Infinity LC system, showing excellent performance at lowest costs.

	Stock solution in mobile phase	1:5 diluted in water
Acetaminophen	5.5 mg/10 mL	1.1 µg/10 µL
Caffeine	1.3 mg/10 mL	0.26 µg/10 µL
Aspirin	3.9 mg/10 mL	0.78 µg/10 µL
Benzoic acid	4 mg/10 mL	0.8 µg/10 µL
Salicylic acid	4 mg/10 mL	0.8 µg/10 µL

Table 2
Sample concentration.

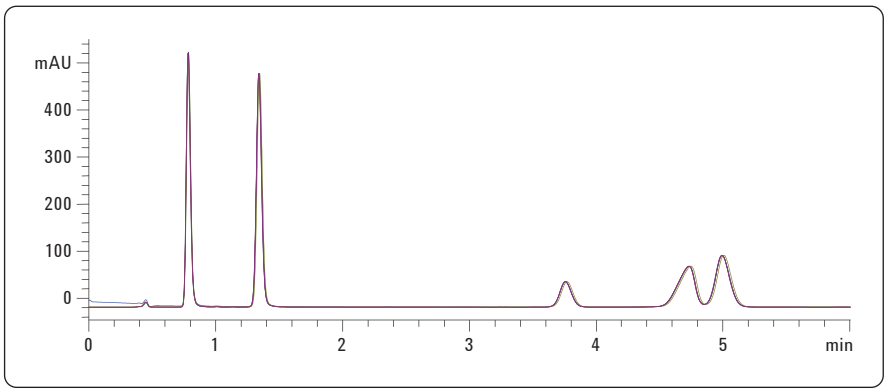


Figure 2
Overlay of chromatograms, six consecutive runs injected with manual loop injector.

Compound	Retention time (min) Average	RSD RT (%)	RSD area (%)	Resolution (hH)	Peak tailing
Acetaminophen	0.784	0.208	0.371		1.179
Caffeine	1.341	0.209	0.341	8.117	1.105
Aspirin	3.756	0.147	0.100	19.557	1.041
Benzoic acid	4.730	0.129	0.195	4.600	0.800
Salicylic acid	4.991	0.117	0.191	1.139	1.005

Table 3
Results for retention time and area precision, resolution data and peak tailing.

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March 1, 2012
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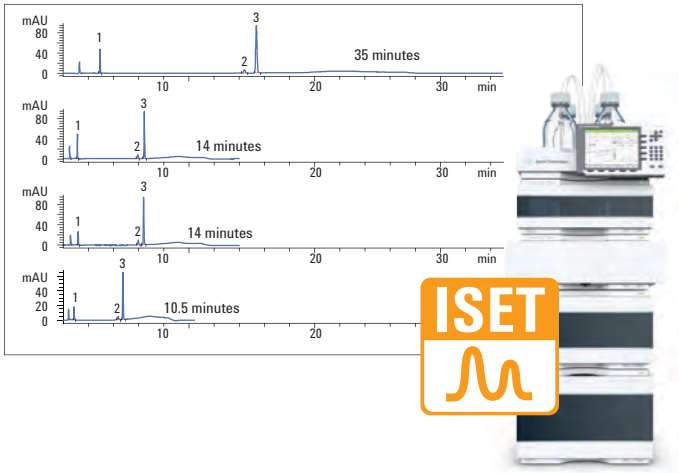
Agilent Technologies

Developing faster methods for generic drugs within USP <621> allowed limits

Higher throughput and cost reduction for purity analysis of Olanzapine tablets using the Agilent 1290 Infinity LC System with ISET

Application Note

Pharmaceutical QA/QC



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Abstract

This Application Note describes an approach to reduce cost per analysis by reducing analysis time and solvent consumption through varying column dimensions. The modifications are made according to the United States Pharmacopeia (USP) guidelines on allowed deviations for column dimensions and thus eliminate the need for method revalidation. The USP method of organic impurities for olanzapine tablets was used to demonstrate the approach. The USP method was efficiently converted in to three shorter gradients using Agilent Poroshell 120 EC C8 columns of various dimensions within the allowed USP limit. The Agilent 1290 Infinity LC System with Intelligent System Emulation Technology (ISET) was used for the experiments to emulate other HPLC system configurations depending on the column dimensions used. The results proved that smart selection of column dimensions can save more than 90% solvent and more than 70% time compared to the original Pharmacopeia method.

Agilent Technologies

Introduction

For every liquid chromatography method listed in the pharmacopeia, recommendations for column dimensions and packing materials are given in addition to other method parameters. There are general guidelines to chromatographers which clearly state what deviations are permitted for these column parameters. If the deviations are within the allowed limit, no method revalidation is required, but system suitability criteria should be met.[1]

olanzapine is one of the best selling block buster drugs on the global pharma market. The USP method of analysis for organic impurities in olanzapine tablets takes approximately 35 minutes, using a 250 × 4.6 mm column with 5-µm L7 packing[2]. The USP <621> guideline on permitted column dimension deviations for LC methods is given in Table 1.

The system suitability test for the olanzapine tablet includes:

a) Resolution of the active pharmaceutical ingredient (API) peak with one of the impurities

b) Tailing factor and relative standard deviation (RSD) of retention time (RT) of the API peak

c) Signal-to-noise for a diluted sample of the API

Three different smaller column dimensions are selected by varying column length, diameter, and particle size within the allowed deviation on column dimension and system suitability testing. Total cost savings in gradient time and solvent consumption was calculated while meeting the system suitability results. The use of a smaller column length and diameter reduced analysis time and solvent consumption. Smaller particle sizes promised uncompromised resolution of peaks. Different instrument models with different characteristics either from the same or different vendors offer different delay volumes and may result in a compromise on critical separations while performing Pharmacopeia methods. This issue was eliminated by using ISET with the Agilent 1290 Infinity LC System. The ISET emulation algorithm delivers identical gradient mixing conditions as selected other instruments and gives matching retention time and chromatographic resolution. ISET ensures the uncompromised performance of a 1290 Infinity LC System as a universal LC system.

Column parameter	USP limit for deviation
Length	± 70%
Internal diameter	No limit, but keep constant linear velocity
Particle size	– 50%

Table 1
Allowed column deviations as per USP <621> recommendation.

Experimental

Instruments, software and columns

The Agilent 1290 Infinity LC System consisted of the following modules:

- Agilent 1290 Infinity Binary Pump with integrated vacuum degasser (G4220 A) and 35 µL Jet Weaver mixer.
- Agilent 1290 Infinity High Performance Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A) with Max-Light flow cell (1.0 µL dispersion volume, 10-mm path length) (G4212-60008)
- Software: Agilent ChemStation C.01.03

In addition to the USP recommended method, three more methods were performed with modified column dimensions (within the allowed USP deviation limit) to evaluate the time and solvent savings in comparison to the original pharmacopeia method. The details of columns dimensions with observed deviations for all four experiments are tabulated in Table 2.

Column parameter	USP recommendation	Experiment 1 (Original USP method)		Experiment 2		Experiment 3		Experiment 4	
		Actual	% Deviation	Actual	% Deviation	Actual	% Deviation	Actual	% Deviation
Length	250 mm	250 mm	0	100 mm	–60	100 mm	–60	70 mm	–70
Diameter	4.6 mm	4.6 mm	0	4.6 mm	0	2.1 mm	–54	2.1 mm	–54
Particle size	5 µm	5 µm	0	2.7 µm	–46	2.7 µm	–46	2.7 µm	–46

Table 2
Various column dimensions percentage deviations used for the experiments.

Reagents and materials

USP reference standards for olanzapine and corresponding related compounds B and C were purchased from USP-India Private Limited (Hyperbad, India). Acetonitrile was of 'super gradient grade' and was purchased from Lab-Scan (Bangkok, Thailand). Highly purified water from a Milli Q water purification system (Millipore Elix 10 model, USA) was used for the experiment. Chemicals for making buffers, phosphoric acid, sodium hydroxide, sodium dodecyl sulfate, and edetate disodium were purchased from Aldrich (India).

Chromatographic parameters

The buffers and mobile phases were prepared as per the USP method. The details of the buffers, mobile phases and diluent preparation used for this experiment are given in Table 3. The column temperature was maintained at 35 °C and the detection was done at 220 nm. The detailed chromatographic method parameters used for each experiment are tabulated in Table 4.

Procedure

The system suitability, standard, and sensitivity solutions were prepared as per USP method for the olanzapine tablet described in USP 34–NF 29.

System suitability solution: 20 µg/mL of USP olanzapine, and 2 µg/mL each of USP olanzapine related compound B and C in diluent.

Standard solution: 2 µg/mL of USP olanzapine RS in diluent

Sensitivity solution: 0.4 µg/mL of USP olanzapine in diluent, from the standard solution.

Method transfer for all the experiments were carried out using Agilent Method Translator (v:2) in *Simple conversion* mode. System suitability testing was performed using all the four experiment conditions.

Buffer	Details
Buffer 1	3.3 mL/L of phosphoric acid. Adjust with 50% NaOH to a pH of 2.5.
Buffer 2	8.7 g/L of sodium dodecyl sulfate in Buffer 1
Buffer 3	18.6 mg/L of edetate disodium (EDTA) in Buffer 2
Mobile phase A	Acetonitrile and Buffer 2 (12:13)
Mobile phase B	Acetonitrile and Buffer 2 (7:3)
Diluent	Acetonitrile and Buffer 3 (2:3)

Table 3
Buffers, mobile phases and diluent as per USP method.

	Agilent 1290 Infinity Binary LC System with ISET			
Parameter	Experiment 1: Emulated as Agilent 1100 Series LC	Experiment 2: Emulated as Agilent 1260 Infinity LC	Experiment 3: Without ISET	Experiment 4: Without ISET
Injection volume	20 µL	8 µL	1.7 µL	1.3 µL
Column	Agilent ZORBAX Eclipse Plus C8, 4.6 × 250 mm, 5 µm	Agilent Poroshell 120 EC-C8, 4.6 × 100 mm, 2.7 µm	Agilent Poroshell 120 EC-C8, 2.1 × 100 mm, 2.7 µm	Agilent Poroshell 120 EC-C8, 2.1 × 75 mm, 2.7 µm
Flow rate	1.5 mL/min	1.5 mL/min	0.31 mL/min	0.31 mL/min
Gradient	At 0 min: 0% B At 10 min: 0% B At 20 min: 100% B At 25 min: 100% B At 27 min: 0% B At 35 min: 0% B	At 0 min: 0% B At 4 min: 0% B At 8 min: 100% B At 10 min: 100% B At 10.8 min: 0% B At 14 min: 0% B	At 0 min: 0% B At 4 min: 0% B At 8 min: 100% B At 10 min: 100% B At 10.8 min: 0% B At 14 min: 0% B	At 0 min: 0% B At 3 min: 0% B At 6 min: 100% B At 7.5 min: 100% B At 8.1 min: 0% B At 10.5 min: 0% B
Acquisition rate	5 Hz	10 Hz	10 Hz	10 Hz

Table 4
Detailed chromatographic parameters for all the four experiments.

Results and discussion

Separation and detection

The USP recommended column dimension is 4.6 × 250 mm, 5-µm packing L7 column which is a typical column dimension for LC system configurations with a pressure limit of 400 bar. The analysis using this USP recommended column dimension (experiment 1) was carried out using an Agilent 1290 Infinity Binary LC System with ISET emulating to an Agilent 1100 Series Binary Pump. An Agilent ZORBAX Eclipse Plus C8 column was used here. For experiment 2, an Agilent Poroshell 120 EC-C8 100 × 4.6 mm, 2.7-µm column (length about 1/3 of original length) was selected. Analysis using smaller particle sized columns may require LC systems which have higher pressure withstanding capabilities. To address this, experiment 2 was performed using an Agilent 1290 Infinity LC System with ISET emulating an Agilent 1260 Infinity Binary Pump which has a pressure limit of 600 bar. For experiment 3, an Agilent Poroshell 120 EC-C8 100 × 2.1 mm, 2.7-µm column (same length as experiment 2, but with a 2.1 mm id) was selected and for experiment 4, an Agilent Poroshell EC-C8 75 × 2.1 mm, 2.7-µm column (minimum length according to USP limits, and 2.1 mm id) was selected. Using narrow bore columns may demand Ultra High Pressure LC (UHPLC) systems with low delay and dispersion volumes. Experiments 3 and 4 were performed using an Agilent 1290 Infinity LC System without ISET. The peaks are well separated in all four experiments, and Figure 1 shows the observed chromatograms. The system suitability results obtained from all the four experiments are tabulated in Table 5, and the results are found to be within the acceptance criteria.

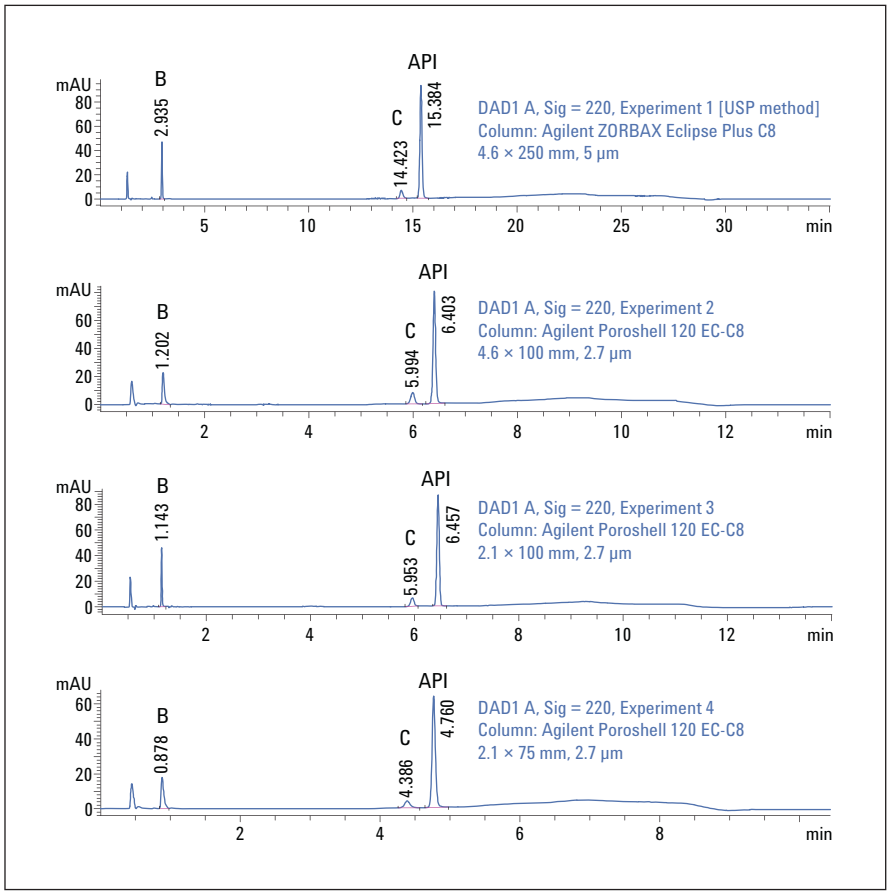

Figure 1
Separation of Olanzapine system suitability mix in USP method and newly developed cost effective methods.

No	Parameter	Limit	Results			
			Exp 1	Exp 2	Exp 3	Exp 4
1	Resolution between Olanzapine and related compound C (system suitability solution)	NLT* 3.0	4.31	4.79	3.63	3.33
2	USP Tailing factor for Olanzapine (system suitability solution)	NMT** 1.5	1.082	0.984	1.034	1.077
3	RSD RT of Olanzapine peak (Standard solution)	NMT** 2.0%	0.07	0.07	0.02	0.10
4	RSD Area of Olanzapine peak (Standard solution)	NMT** 2.0%	0.33	0.33	0.31	0.26
5	Signal-to-noise ratio for Olanzapine peak (Sensitivity solution)	NLT* 10	>13	>13	~50	>38

*NLT: Not less than,
**NMT: Not more than

Table 5
System suitability results for all four experiments.

The system suitability results were found to be within the acceptance criteria even with a 75-mm Poroshell 120 EC C8 column. The resolution between olanzapine and Impurity C was one of the critical parameters to be monitored for the system suitability testing. The system suitability limit for resolution between the olanzapine peak and the Impurity C peak was greater than 3 for a 250-mm column, and the results with modified column dimensions met this requirement. The USP tailing factor for the API peak also passed in all trials. The small values for area and RT RSD confirmed the precision and accuracy of the UHPLC system. Using a shorter column reduced the analysis time and increased the throughput. A reduced flow rate from 1.5 mL/min to 0.31 mL/min with a 2.1-mm id column reduced the total solvent consumption 1/10 fold. Using the experiment 3 and 4 conditions, a 3-fold increment in signal-to-noise was observed and this confirmed the gain in sensitivity with narrow bore columns with smaller particle size. As a result of the savings in time and solvent consumption, a total cost saving factor was calculated for each experiment (Figure 2). In experiments 2, 3, and 4, approximately 60%, 91.7% and 93.8% of acetonitrile could be saved respectively. Experiments 2 and 3 were approximately 60% and experiment 4 was approximately 70% faster than the original Pharmacopeia method. The cost of acetonitrile was calculated with US $60/L, as well as a cost factor for the disposal of solvent waste. The running cost of the HPLC system was calculated as US $80/hour. The method developed in this Application Note (experiment 4) can potentially save a total of US $34 per injection compared to the original USP method.

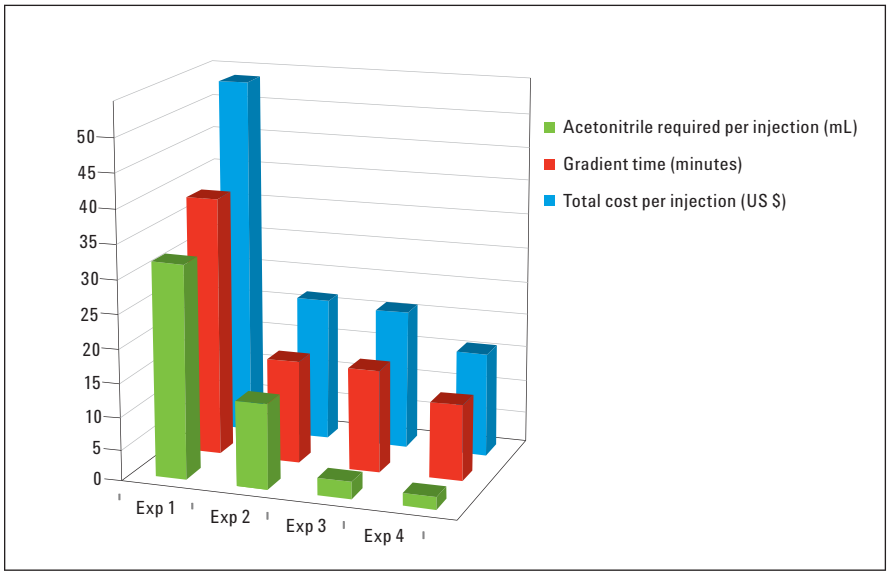

Figure 2
Solvent, time and total cost calculation for all experiments.

Analysis of the olanzapine tablet

The effective usage of the developed, cost effective method with shorter gradients for high throughput was demonstrated by analysis of the olanzapine tablet samples. The API was extracted from olanzapine tablets as per the USP protocol and analyzed using the Pharmacopeia and the three developed methods. The label claim on the tablet as per the manufacturer was 2.5 mg of API and the calculated amount from all the four chromatographic assay methods was about 2.4 mg. The presence of 0.1–0.2% of Impurity C (confirmed by retention time) was observed in the chromatographic separation. The calculated percentage area of API and Impurity C were found to be similar. The chromatograms obtained from the olanzapine tablet analysis using all four methods are shown in Figure 3. Area distribution of API and impurity peaks with calculated API percentage is given in Table 6.

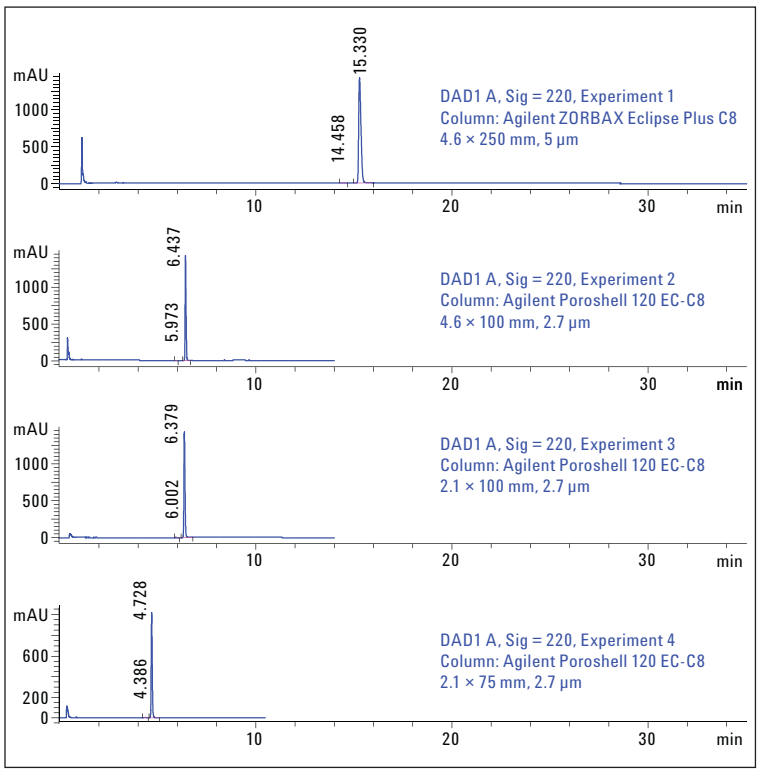

Figure 3
Chromatograms of extracted Olanzapine samples from tables using USP and newly developed methods.

Experiment	Peak	Area	Area%	Calculated API content (mg)
1	Impurity C	13.9	0.119	2.38
	API	11674.9	99.881	
2	Impurity C	5.6	0.118	2.34
	API	4731.8	99.882	
3	Impurity C	8.4	0.174	2.35
	API	4832.2	99.826	
4	Impurity C	6.2	0.168	2.36
	API	3676	99.832	

Table 6
Area percentage of API and impurity C along with calculated amount of API observed from Olanzapine tablet analysis.

Conclusion

- The original column dimension for the USP organic impurity analysis for the olanzapine tablet was adapted to shorter dimensions as allowed by the USP guidelines for column deviations.
- Revalidation is not required as the modifications incorporated are within the USP limits.
- Deriving new gradient parameters for each modified column dimension was performed with the Agilent Method Translator.
- An Agilent 1290 Infinity LC System with Intelligent System Emulation Technology (ISET) was efficiently used to emulate to other different instrument configurations.
- System suitability testing was performed with modified column dimensions using the new methods and the results were within the acceptance criteria.
- The usage of smaller column dimensions reduced the analysis time and solvent consumption and thus reduced the cost per analysis.
- Smart selection of column dimensions within the Pharmacopeia allowed limit reduces the total cost per analysis by up to 70% and increased the high throughput.

References

1.
"Validation of analytical methods" Agilent Publication Number 5990-5140EN, **2011**

2.
USP method for olanzapine tablet, USP34–NF29

www.agilent.com/chem/ISET

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5991-0278EN

Agilent Technologies

Biopharmaceutical

Automated Sample Preparation by Protein Precipitation for High Throughput Bioanalysis

Application Note

BioPharma

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Abstract

Protein removal is an essential step in sample preparation for LC/MS/MS analysis of compounds in biological matrices. Protein precipitation followed by centrifugation is one of the most popular sample preparation techniques for removing proteins. However, it is not well-suited for high throughput environments due to the multiple manual steps involved. Fully automatable in-well precipitation in a 96-well plate format would reduce the time, cost, and manpower required for sample preparation in high throughput labs.

Agilent introduces a new protein precipitation filtration plate, Captiva ND, with non-drip technology for simple operation. Specially designed filtration materials effectively hold organic solvents used for precipitation with no dripping. This prevents sample loss and enables trouble-free automated methods. Simply add organic solvent to precipitate followed by biological samples, mix them in the well, and apply vacuum to filter out precipitated proteins. The result is particulate-free, protein-free samples in just a few minutes–five times faster than centrifugation methods.

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Materials and Methods

Reagents and solutions

Add 10 µL formic acid to 10 mL ACN to produce a 0.1% formic acid in ACN crash solvent.

Sample preparation using Captiva ND

SPE: Agilent Captiva ND 96-well plate, 0.45 µm, 10 mg (p/n A5969045)

Figure 1 shows the sample preparation method using Captiva ND.

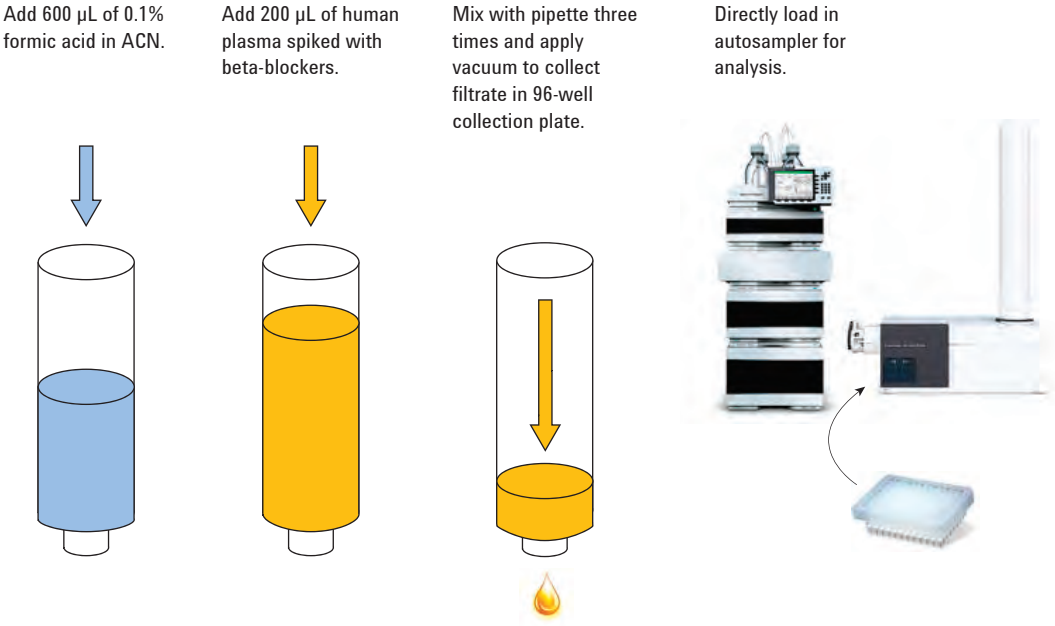

Figure 1. Schematic of sample preparation method for Captiva ND.

Sample preparation by centrifugation of protein precipitation

Centrifuge: Eppendorf centrifuge 5424 with 24 centrifuge tube holders.

1. Add 600 µL of 0.1% formic acid in ACN and 200 µL of human plasma spiked with beta-blockers.
2. Centrifuge at 10,000 RPM for 10 min or more.
3. Filter supernatant if needed.
4. Carefully transfer (filtered) supernatant to injection vials manually for analysis.

LC conditions

Column	Agilent ZORBAX Eclipse Plus RRHD C18, 2.1 × 5.0 mm, 1.8 µm (p/n 959757-902)
LC/MS/MS	Agilent 1290 Infinity UHPLC coupled with a generic MS system
Eluent A	0.1% formic acid in H_2O
Eluent B	0.1% formic acid in ACN
Flow rate	0.5 mL/min
Injection volume	1 µL
Gradient	Time (min) %B 0 30 1.0 90 1.1 30 1.5 30
Temperature	Ambient
Ion-source	ESI+
Drying gas temperature	300 °C
Drying gas pressure	18 psi
Nebulizer	55 psi
Vortex gas temperature	300 °C
Vortex gas pressure	25 psi
Needle voltage	4,000 V
CID gas pressure	1.5 mTorr

Table 1. Samples

	pKa	log P	MS/MS transition	Collision energy	Capillary
Nadolol	9.67	0.81	310.4 → 254.0	15.5	120
Propranolol	9.42	3.48	260.3 → 115.9	15.0	120
Pindolol	9.25	1.75	249.3 → 115.9	17.5	120
Metoprolol	9.70	1.90	268.4 → 115.9	18.0	120

Results and Discussion

A longevity experiment with continuous injections over an extended period of time is the easiest and most effective way to demonstrate the cleanliness of biological samples. Samples prepared by Captiva ND were tested in a 5,000-injection longevity experiment while monitoring retention times, MS area counts, and backpressure (see Figures 2 to 4).

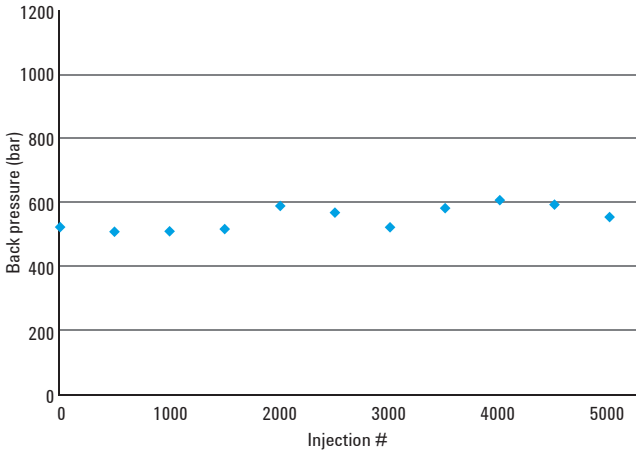

Figure 2. System backpressure data for longevity experiment using Agilent Captiva ND.

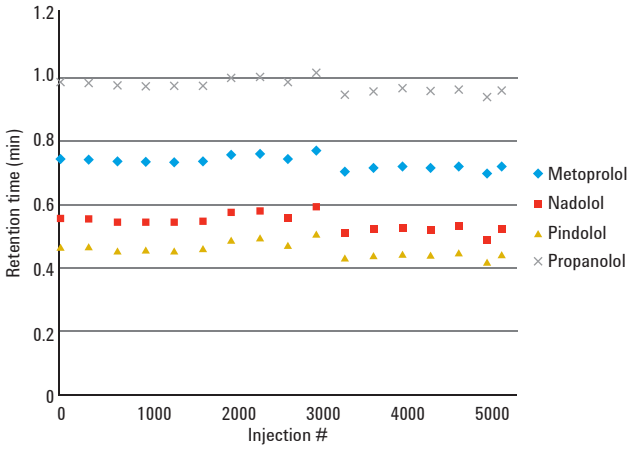

Figure 3. Retention time data for 5,000 continuous injections of Agilent Captiva ND samples.

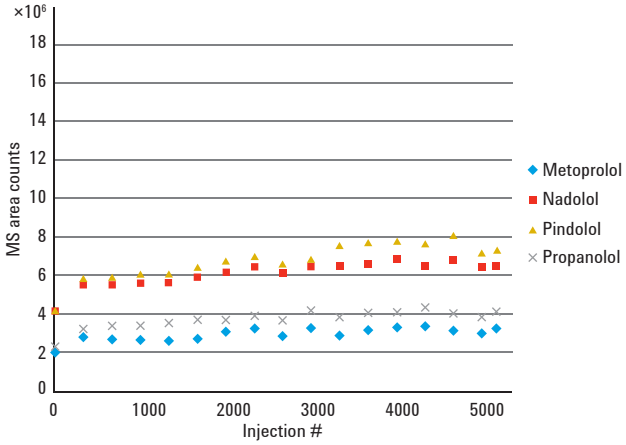

Figure 4. MS area count data for plasma samples spiked beta-blockers prepared by Agilent Captiva ND.

As implied by stable monitoring parameters in the longevity experiment such as backpressure, retention times, and MS area counts (see Figures 2 to 4), Captiva ND showed superb capability in protein removal, and delivered ultra cleanliness in biological samples. The longevity experiment, using over 5,000 injections, proved Captiva ND's ideal applicability in a high throughput environment especially using sub-2-µm columns and UHPLC systems. Comparison of high throughput applicability between Captiva ND and centrifugation protein precipitation is summarized in Table 2.

Table 2. Sample Preparation Time Comparison Between Agilent Captiva ND and Centrifugation Protein Precipitation Methods

Centrifugation protein precipitation	Time (min)	Captiva ND*	Time (min)
Add 0.2 mL of spiked plasma sample and 0.6 mL of ACN + 0.1% formic acid to centrifugation tubes or an empty 96-well plate.	5	Add 0.2 mL of spiked plasma sample and 0.6 mL of ACN + 0.1% formic acid to Captiva ND 96-well plate.	5
Centrifuge at 10,000 RPM for 10 min.	11	Mix each well with a pipette 5 times and apply vacuum.	
Transfer supernatant to 2 mL injection vials (if tubes were used) or a new empty 96-well plate for analysis (if plate format was used).	10	Directly transfer injection plate for analysis.	0
Total time required for sample preparation.	26	Total time required for sample preparation.	5

* Based on automation with robotic system such as Tomtec or Hamilton.

Approximately 80% reduction in cycle time is anticipated by switching from centrifugation protein precipitation to Captiva ND. Switching from centrifugation protein precipitation to Captiva ND saves time, money, and training.

Conclusion

Captiva ND plate is an optimal solution for the high throughput analytical industry. It enables the production of a large volume of samples in just a few minutes, with an extremely easy to use method. The three major monitoring parameters in the longevity experiment, backpressure, retention times, and MS signals, were all stable assuring the performance of Captiva ND.

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

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Agilent Technologies

Analysis of Oxidized Insulin Chains using Reversed Phase Agilent ZORBAX RRHD 300SB-C18

Application Note

BioPharma

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Abstract

A new reverse phase media, Agilent ZORBAX RRHD 300SB-C18 1.8 µm, was used for the separation of a typical protein biopharmaceutical, insulin. The value of sub 2-µm particles for protein separations was assessed under denaturing conditions. The advantages of these particles in separating small molecules were also realized in protein separations.

The use of a 1.8 µm column designed for UHPLC systems significantly reduced analysis time, critical for increasing the efficiency of QC for protein primary structure analysis. The separations also demonstrated how this technology achieved resolution of various insulin isoforms.

The eluents routinely used for reverse phase analysis are acidic, containing trifluoroacetic acid or formic acid, which can limit the lifetime of many HPLC columns. Using StableBond technology, it was possible to produce a 300Å pore-size media that was stable under acidic conditions, to provide the robust reproducible separations required for protein QC.

Agilent Technologies

Introduction

Due to the heterogeneity of a protein biopharmaceutical, it is necessary to use a number of chromatographic techniques to fully characterize the API. Methods include size exclusion chromatography for the quantitation of dimers and aggregates and ion exchange for the identification of charge variants. Both of these techniques use aqueous eluents and non-denaturing conditions. As part of the full characterization of a protein it is also necessary to look at the primary amino acid sequence and any post translational modifications to the sequence that may have occurred during the purification or formulation steps of manufacture. To perform this type of analysis, denaturing conditions are required, and so reversed phase HPLC is normally the technique of choice. In this example, we use Agilent ZORBAX Rapid Resolution High Definition (RRHD) columns, which benefit from improved packing processes to achieve stability up to 1200 bar for use with the Agilent 1290 Infinity LC.

Materials and Methods

The conditions in Table 1 were used throughout the investigation, with variations as noted in the relevant chromatograms.

Table 1. Standardized Chromatographic Conditions

Parameter	Item
Column	Agilent ZORBAX 300SB-C18 1.8 µm, 2.1 × 50 mm (p/n 857750-902) (Agilent Technologies, Wilmington DE)
Sample	Insulin, oxidized insulin chain A and chain B from bovine pancreas (Sigma Aldrich, St. Louis, MO.)
Sample concentration	1 mg/mL
Injection volume	3 µL
Flow rate	1.0 mL/min
Pressure	~ 650 bar
Mobile phase A	0.1% TFA in distilled water
Mobile phase B	80% ACN + 0.01% TFA in distilled water
Detector	UV, 280 nm
System	Agilent 1290 Infinity HPLC

Results

Speed

The system separated the test mixture very quickly, distinguishing insulin, a small molecule, from its impurities in less than five minutes (Figure 1). Using multiple gradients achieves the same fast analysis time (Figure 2). Rapid equilibration is evident even with the screening gradient, which starts from a highly aqueous eluent, demonstrating that the column is suitable for use with a wide range of organic content.

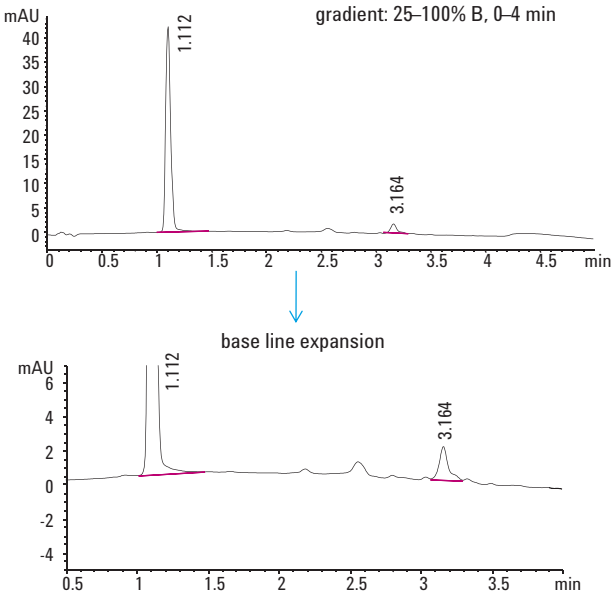

Figure 1. Fast resolution of insulin and some impurities on an Agilent ZORBAX RRHD 300SB-C18 1.8 µm column.

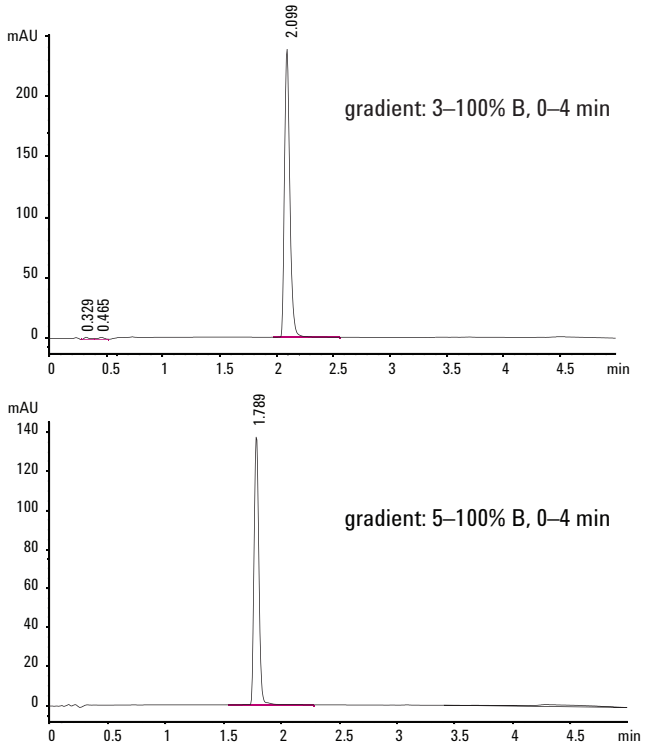

Figure 2. Multiple gradient systems can be selected to separate insulin in less than 5 minutes using the Agilent ZORBAX RRHD 300SB-C18 1.8 µm column, including eluents with high water content.

Flow rate can also be manipulated to provide a fast separation (Figure 3). Peak asymmetry and efficiency remain unchanged (Table 2), a feature of sub 2 µm particles that facilitates rapid separations.

Table 2. Effect of Flow Rate on Retention Time, Asymmetry, and Efficiency in the Analysis of Insulin

Flow rate (mL)	Pressure (bar)	Retention time (min)	Asymmetry	Plate count
0.3	230–150	2.39	0.80	8815
0.5	350–250	2.04	0.82	8390
1.0	680–520	1.78	0.88	8034
1.5	890–670	1.72	0.88	8060

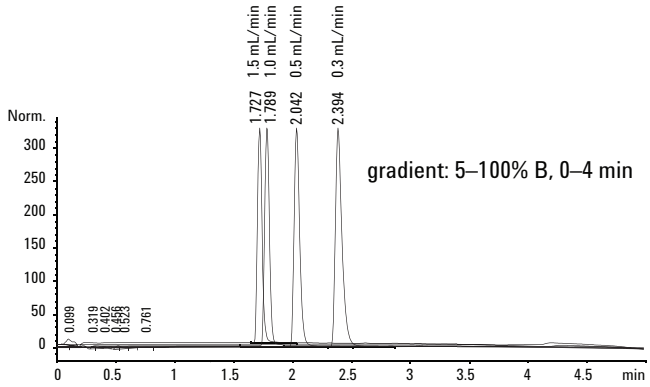

Figure 3. Different flow rates can be selected to separate insulin on the Agilent ZORBAX RRHD 300SB-C18 1.8 µm column.

Reproducibility

Two hundred consecutive injections were done to examine the column's reproducibility. The results show that the integrity of peak shape, asymmetry, retention time and efficiency remained the same after 200 injections of insulin without cleaning the column (Table 3 and Figure 4).

Table 3. Two Hundred Injections of Insulin Demonstrates the Reproducibility of Agilent ZORBAX RRHD 300SB-C18 1.8 µm

Run no	Pressure (bar)	Retention time (min)	Asymmetry	Plate count
1	680–520	1.789	0.86	9758
50	680–520	1.790	0.91	9752
100	680–520	1.788	0.88	9758
200	680–520	1.789	0.87	9741

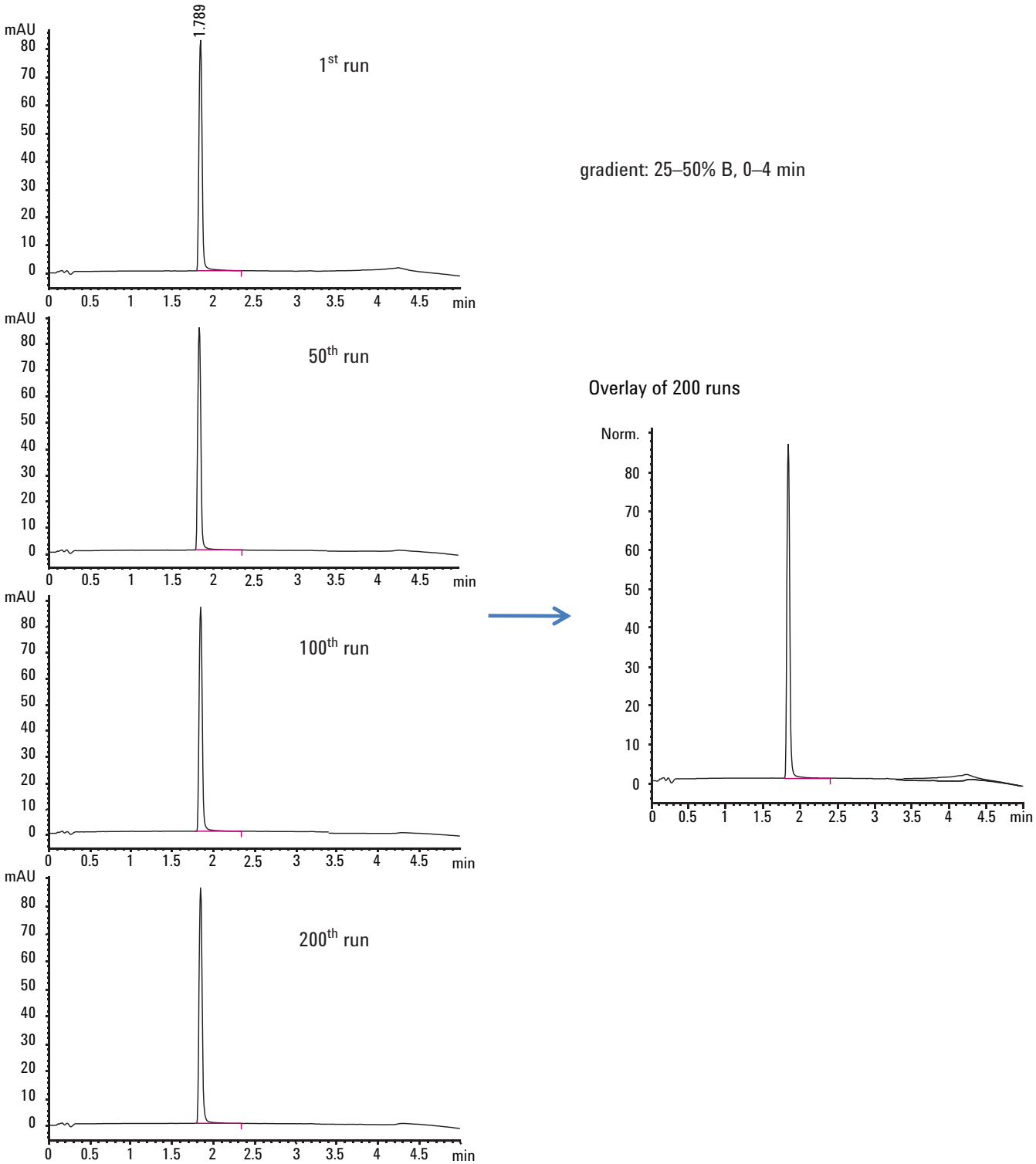

Figure 4. Two hundred injections reveal the reproducibility of the Agilent ZORBAX RRHD 300SB-C18 1.8 µm column.

Separation of Heat-Degraded Insulin

A forced degradation study can be performed by heating APIs, and HPLC used to monitor the degradation products. Heat treating insulin produces degradation products that can be quickly resolved by the column from the monomer insulin (Figure 5).

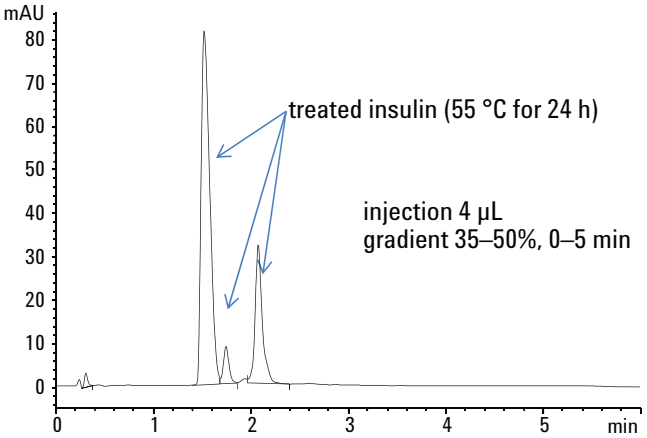

Figure 5. Heat-treated insulin quickly resolved on an Agilent ZORBAX RRHD 300SB-C18 1.8 µm column.

Separation of Insulin Isoforms

Figures 6 and 7 showed that the column can also separate isoforms of insulin; in this case, oxidized insulin chain A (Figure 6) and the mixture of insulin and oxidized insulin chain A (Figure 7). Once again, different gradient systems can be selected with very similar results.

For fast analysis of insulin, high gradient systems are usually used. However, these conditions force oxidized insulin chain A to be eluted rather quickly. To analyze oxidized insulin chain A from its subspecies, a shallow gradient system is required to ensure that the molecule can retain longer on the column.

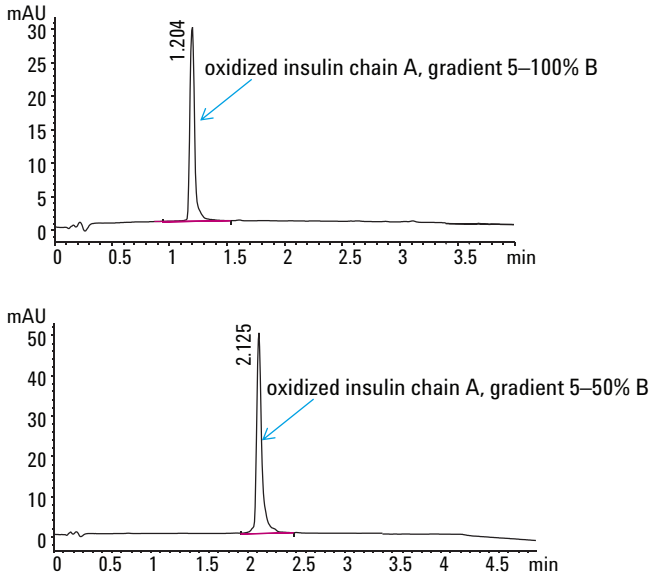

Figure 6. Analysis of oxidized insulin chain A with different gradients.

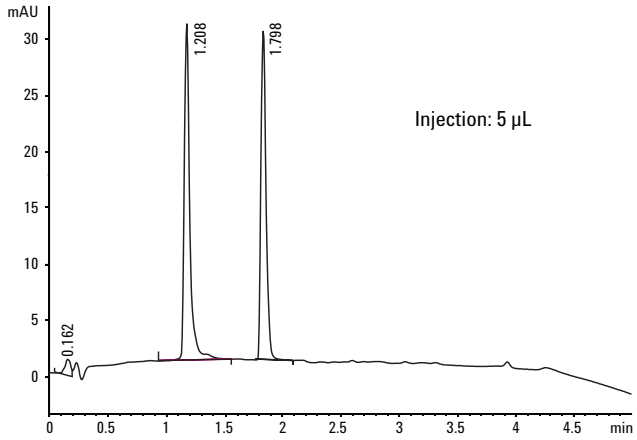

Figure 7. Analysis of insulin and oxidized insulin chain A on Agilent ZORBAX RRHD 300SB-C18 1.8 µm.

Similarly, Figures 8 and 9 demonstrate that the column separates oxidized insulin chain B (Figure 8). Again, different gradient systems can be selected with very similar results. The column also discriminates degradation products of oxidized insulin chain B (Figure 9).

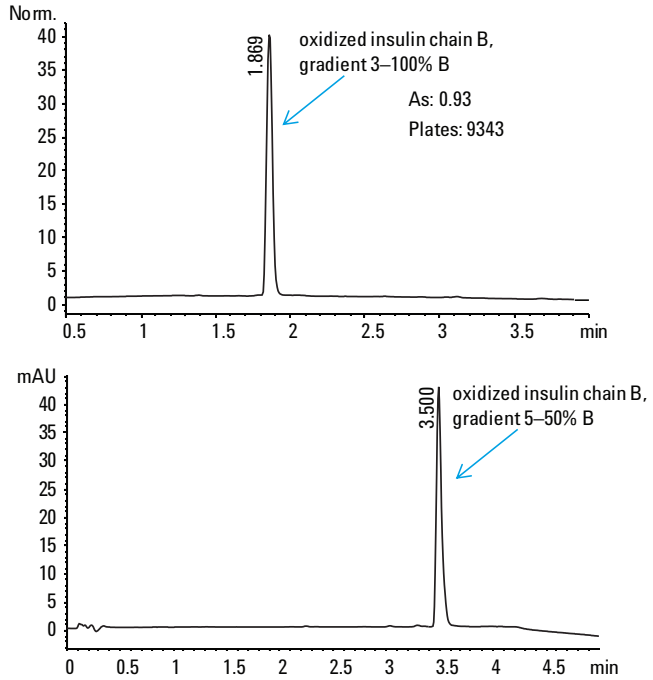

Figure 8. Optimized conditions for fast analysis of oxidized insulin chain B on the Agilent ZORBAX RRHD 300SB-C18 1.8 µm column.

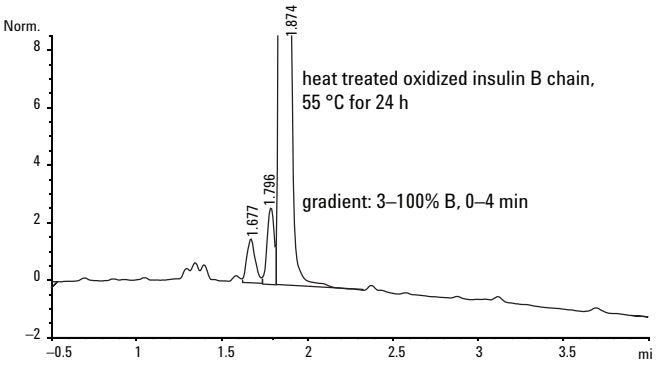

Figure 9. Oxidized insulin chain B and some degradation and impurities are well resolved on Agilent ZORBAX RRHD 300SB-C18 1.8 µm.

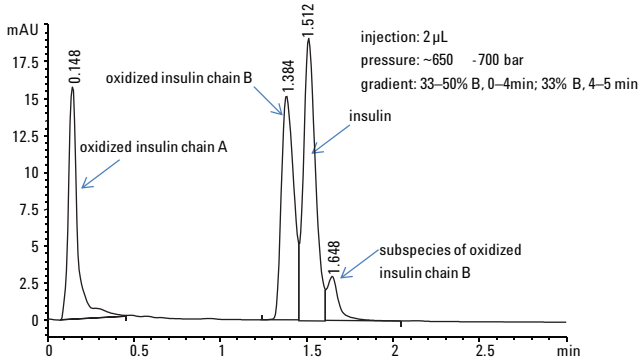

Figure 10. Insulin and oxidized insulin A and B chains are resolved quickly but insulin and oxidized chain B often co-elute.

Conclusions

Analyzing small molecule, protein biotherapeutic insulin, together with its isoforms and breakdown products, is fast and simple with the Agilent ZORBAX RRHD 300SB-C18 1.8 µm column. The column's rapid resolution high definition technology permits high pressure UHPLC, while the StableBond 300Å pore-sized particles are robust when analysis requires acidic conditions. Reproducibility is excellent, with good resolution, asymmetry and efficiency. The column is well suited to the needs of QC when assessing the structure of primary proteins.

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Agilent Technologies

Analyze MAb and BSA digests by UHPLC with UV detection and Agilent ZORBAX RRHD 300SB-C18

Application Note

BioPharma

Author

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Abstract

A new reversed-phase media, Agilent ZORBAX RRHD 300SB-C18 1.8 µm, is used for the analysis of trypsin-digested monoclonal antibody and bovine serum albumen. The robustness of the media is demonstrated using different acidic eluents, temperatures and flow rates. Good reproducibility is also evident.

Agilent Technologies

Introduction

Agilent ZORBAX RRHD 300SB-C18 1.8 µm is a new reversed-phase media designed for UHPLC of proteins and peptides. The use of 1.8 µm particles in a column designed for UHPLC systems significantly reduces analysis time in HPLC. Protein digest, enzymatic cleavage of the protein, into peptide fragments is used to confirm the identity of a protein through database matching of the fragments and in the qualitation or identification of amino acid modifications. To analyze trypsin digests, denaturing conditions are required, and so reversed-phase HPLC is normally employed. In this example, we use ZORBAX Rapid Resolution High Definition (RRHD) columns, which benefit from improved packing processes to achieve stability up to 1200 bar for use with the Agilent 1290 Infinity LC.

Analysis of trypsin-digested MAb at different digestion times

Monoclonal antibody was obtained from Reactive Biolabs, and BSA, Trypsin Gold (digestion grade), TFA and formic acid from Sigma Aldrich. An Agilent 1290 Infinity LC system was used for all analyses.

Protocol for trypsin digestion

Add Trypsin Gold to a final protease:protein ratio of 1:20 (w/w) in tris-HCl buffer pH 8.0 - it is desirable that the protein concentration is 1 mg/mL. Incubate at 50 °C, for at least 4 h to overnight (16 h) (method adapted from *Trypsin Digestion of Proteins in Solution*, Promega, Madison WI, USA).

Depending on the nature of the investigation, protein digestion times and analysis temperatures may vary. Notwithstanding, monoclonal antibody and its digested fragments are well resolved with ZORBAX RRHD 300SB-C18 1.8 µm, as shown in Figure 1.

Conditions

Column:	Agilent ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Humanized monoclonal antibody (MAb) (1 mg/mL) (Reactive Biolabs)
Flow rate:	0.6 mL/min (~ 680 bar)
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA in ACN
Gradient:	1 to 100% B in 20 min
Injection:	5 µL
Temp:	25 °C or 50 °C
Digest time:	As indicated
Detection:	280 nm

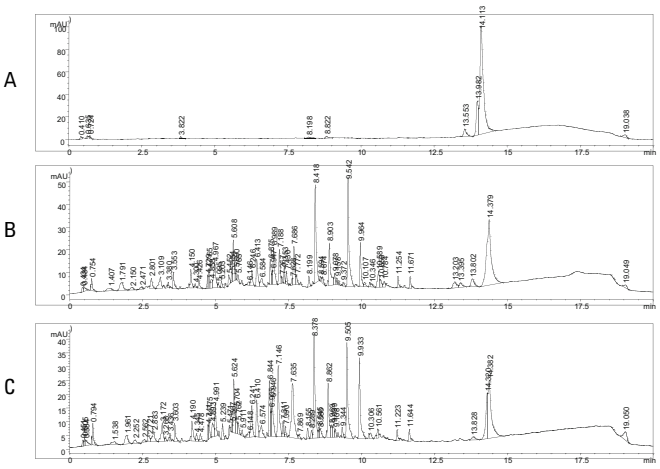

Figure 1. *Resolution of monoclonal antibody and its digested fragments at different temperatures and digestion times using Agilent ZORBAX RRHD 300SB-C18 1.8 µm. A, humanized monoclonal antibody at 25 °C; B, 10 h trypsin-digested MAb at 50 °C; C, 16 h trypsin-digested MAb at 50 °C*

Separation of trypsin-digested BSA fragments

Conditions

Column:	ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Trypsin-digested BSA (1 mg/mL) (Sigma Aldrich)
Flow rate:	0.6 mL/min (~ 680 bar)
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA in ACN
Gradient:	1 to 100% B in 20 min
Injection:	5 µL
Temp:	25 °C
Digestion time:	16 h
Detection:	280 nm

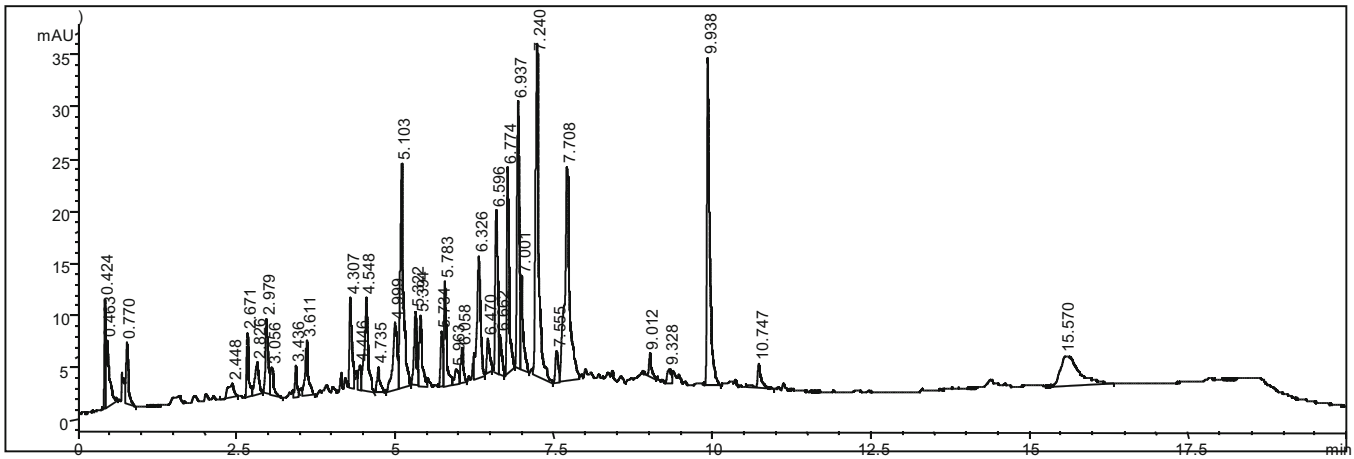

Figure 2. *Fragments from digested BSA are well separated using Agilent ZORBAX RRHD 300SB-C18 1.8 µm*

Analysis of trypsin-digested MAb with formic acid

TFA reduces the signal and trace sensitivity of the method when using HPLC-MS. This can limit the utility of the method, making it more difficult to identify minor peptides in the digest when complete coverage for database matching is needed. Formic acid can be used as an alternative to TFA for protein digest analysis using UHPLC-MS. The analysis of monoclonal antibody digest is shown in Figure 3.

Conditions

Column:	ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Trypsin-digested MAb (1 mg/mL)
Flow rate:	0.6 mL/min (~ 720 bar)
Mobile phase A:	0.1% formic acid
Mobile phase B:	0.01% formic acid in ACN
Gradient:	0.5 to 100% B in 20 min
Injection:	5 µL
Temp:	25 °C
Digestion time:	16 h
Detection:	280 nm

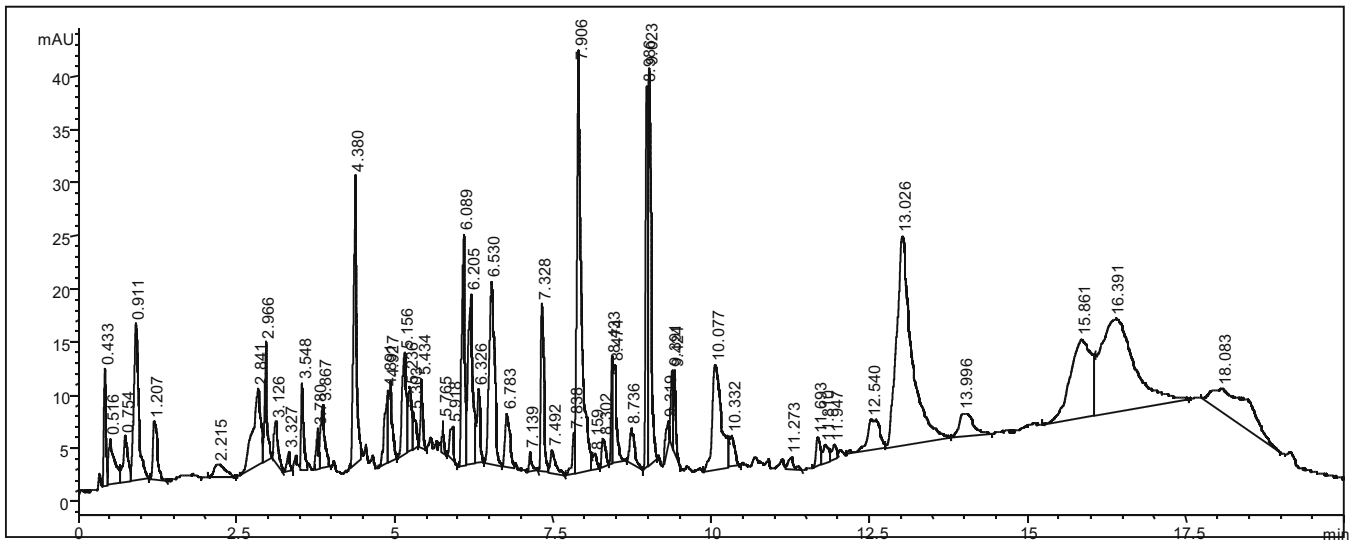

Figure 3. *Agilent ZORBAX RRHD 300SB-C18 1.8 µm is used with formic acid to analyze trypsin-digested MAb*

High pressure separation of trypsin-digested MAb and BSA

If fast analysis times are required, the ZORBAX RRHD 300SB-C18 1.8 µm handles the increased pressure required to separate digested MAb and BSA in 17 minutes (Figure 4).

Conditions

Column:	ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Trypsin-digested BSA and MAb (1 mg/mL)
Flow rate:	1 mL/min (~ 1010 bar)
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA in ACN
Gradient:	1 to 100% B in 20 min
Injection:	5 µL
Temp:	25 °C
Digestion time:	16 h
Detection:	280 nm

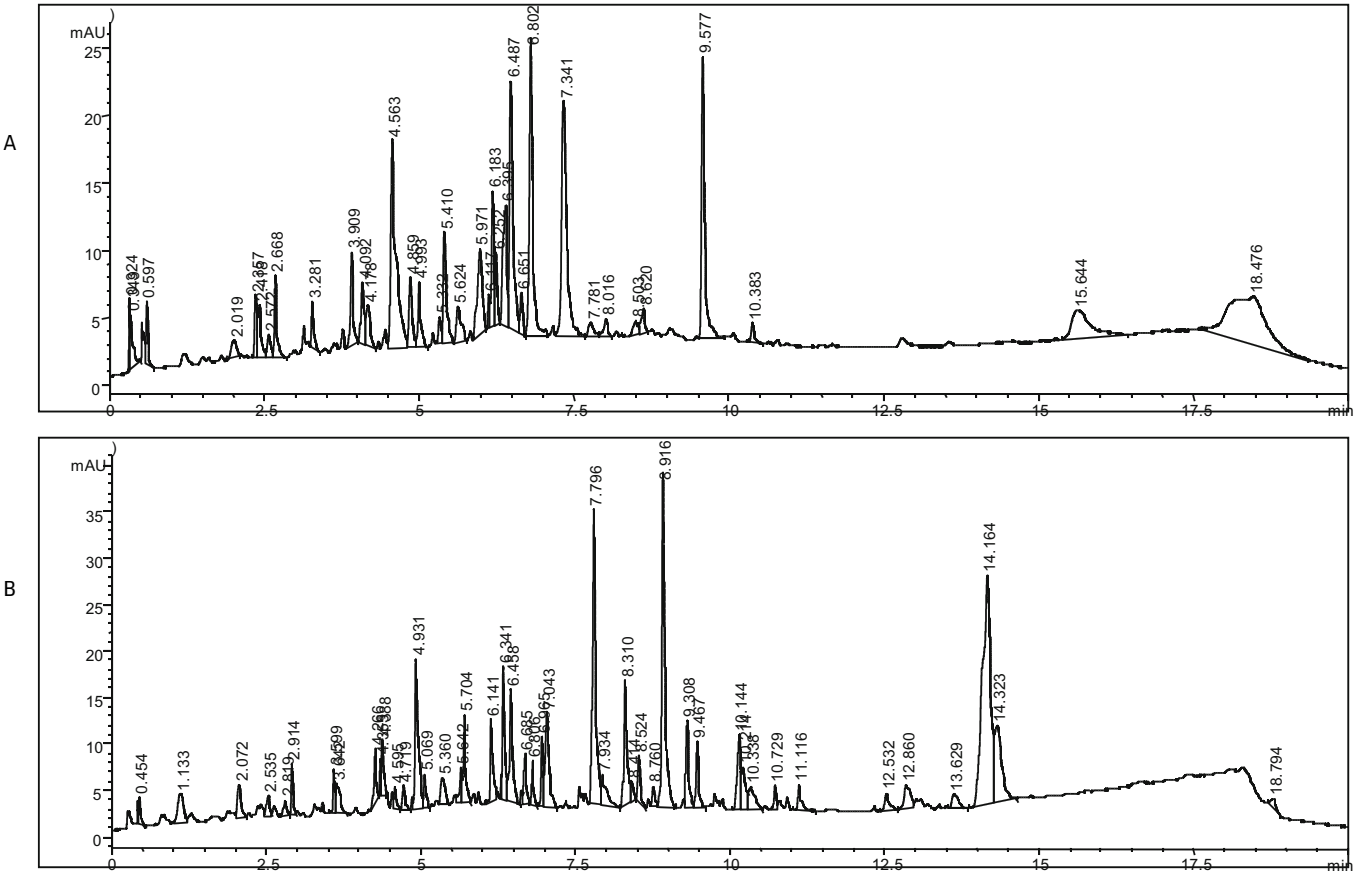

Figure 4. *High speed and high pressure deliver fast analysis of peptides using Agilent ZORBAX RRHD 300SB-C18 1.8 µm. A, trypsin-digested BSA; B, trypsin-digested MAb*

High temperature separations

Elevated temperatures may be needed to analyze highly bound peptides and proteins, to improve peak shape and recovery. However, this must be accomplished without damaging the integrity of the column. The ZORBAX RRHD 300SB-C18 column is stable to 90 oC. Figure 5 shows such an analysis using TFA. Similarly, Figure 6 indicates a high temperature separation achieved using formic acid.

Conditions

Column:	ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Trypsin-digested BSA and MAb (1 mg/mL)
Flow rate:	0.6 mL/min (~ 1010 bar)
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA in ACN
Gradient:	1 to 100% B in 20 min
Injection:	5 µL
Temp:	50 °C
Digestion time:	16 h
Detection:	280 nm

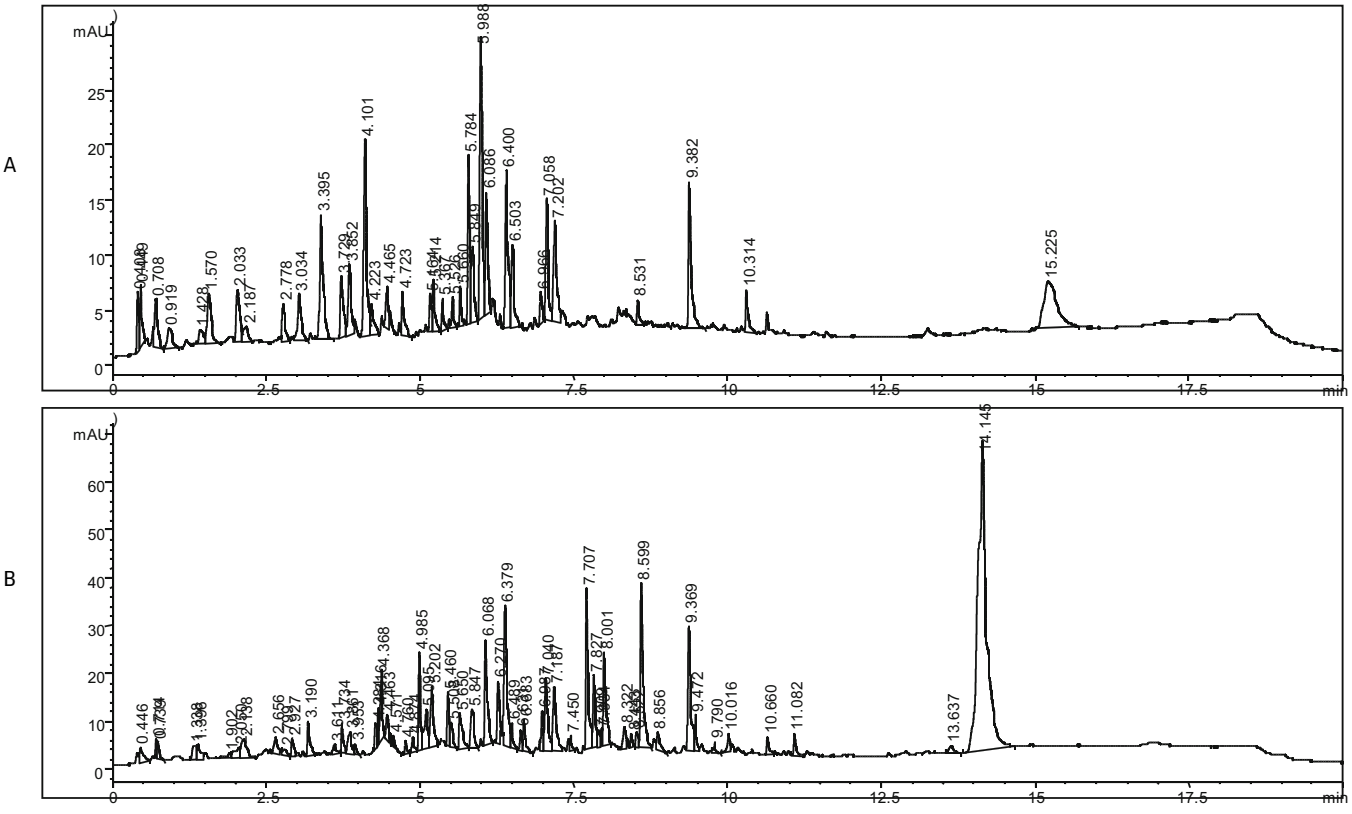

Figure 5. *Agilent ZORBAX RRHD 300SB-C18 with TFA eluent can be used at high temperature to efficiently analyze highly bound peptides and proteins without damaging the column's integrity. A, trypsin-digested BSA; B, trypsin-digested MAb*

Conditions

Column:	ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Trypsin-digested MAb (1 mg/mL)
Flow rate:	0.6 mL/min (~ 720 bar)
Mobile phase A:	0.1% formic acid
Mobile phase B:	0.01% formic acid in ACN
Gradient:	0.5 to 100% B in 20 min
Injection:	5 µL
Temp:	50 °C
Digestion time:	16 h
Detection:	280 nm

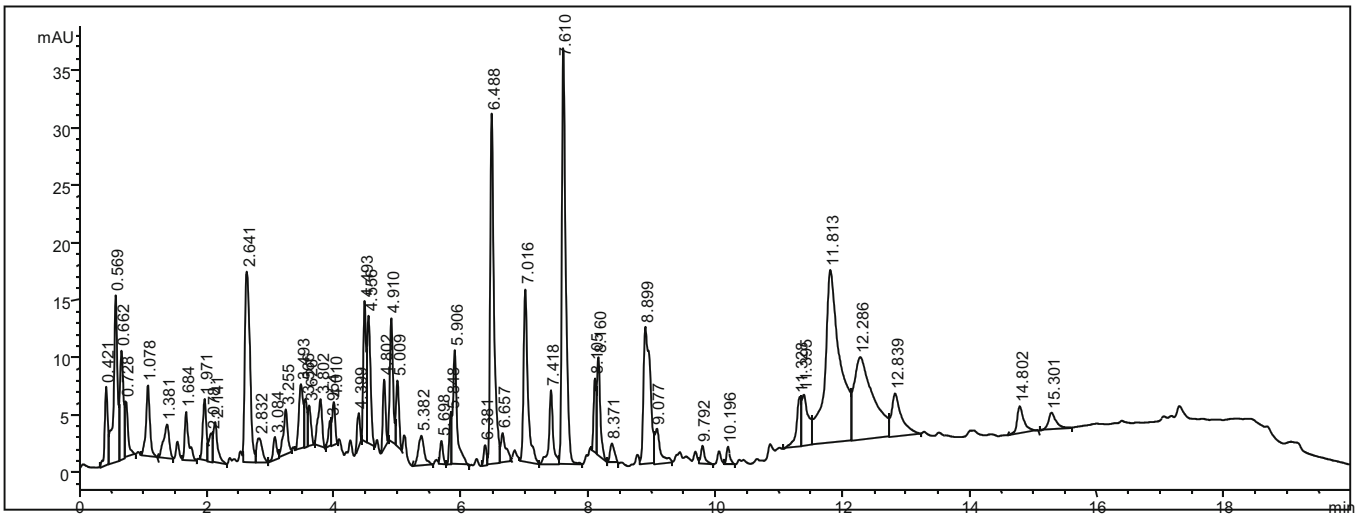

Figure 6. *High temperature separation of trypsin-digested MAb using an Agilent ZORBAX RRHD 300SB-C18 with formic acid eluent*

Conclusions

Rapid analysis of trypsin digests of monoclonal antibody and bovine serum albumen is accomplished using an ZORBAX RRHD 300SB-C18 1.8 µm column. The analysis time is reduced significantly from the 3 to 5 hours previously achieved with conventional HPLC. The efficiency of the column is evident over the range of temperature and flow rate elucidated in this study. In addition, the eluents routinely employed for reversed-phase analysis are acidic, containing trifluoroacetic acid or formic acid, which can limit the lifetime of many HPLC columns. With formic acid, preferred for UHPLC-MS, there is no deterioration in peak shape or efficiency. However, by using Agilent's StableBond technology it is possible to produce a 300Å pore-size media that is stable under acidic conditions, to provide the robust reproducible separations required for protein digest analysis.

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Reversed-phase Separation of Intact Monoclonal Antibodies Using Agilent ZORBAX Rapid Resolution High Definition 300SB-C8 1.8 µm Column

Application Note

Biopharmaceuticals

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Abstract

Analysis and separation of monoclonal antibodies (mAb) by reversed-phase chromatography presents many challenges for maximizing resolution, recovery and run-to-run reproducibility. Additionally, adapting UHPLC methods to these separations for increasing analysis times and throughput, requires robust column technologies and specific bonded phase selection for complete optimization. The Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8 1.8 µm column is designed to address complex protein challenges, such as mAbs, by providing increased sensitivity in reduced analysis times, enhanced resolution and long column lifetimes with high reproducibility. The stable bonded short chain C8 proprietary phase offers an alternate selectivity to C18 and is an ideal choice for resolving large hydrophobic proteins, such as monoclonal antibodies. The 300SB-C8 coating technology also provides exceptional thermal and low pH stability allowing mobile phase eluents with trifluoroacetic acid or formic acid to be routinely used at temperatures up to 80 °C. This application note highlights these beneficial column characteristics in the analysis of a derived intact monoclonal antibody produced from Chinese hamster ovary.

Agilent Technologies

Introduction

Monoclonal antibodies (MAbs) currently represent the largest class of therapeutic drugs made by the biotechnology industry and will play a significant role in the future of pharmacological interventions of disease. Purification, characterization, and monitoring of mAbs are all critically important to drug development, with a variety of analysis techniques routinely used. Due to the heterogeneity in hydrophobic structure of mAbs, reversed-phase separation is thus becoming an option for monitoring purity and stability during manufacturing, formulation and storage. However, too many reversed-phase methods fall short in providing robust separation performance, with fast analysis times, to consider this technique mainstream for mAb impurity characterization. Additionally, there are limited column choices which can provide reproducible, high resolution separations.

We used an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8 1.8 µm column for intact monoclonal antibody separations to demonstrate utility for fast analysis during mAb screening and optimization of critical separation parameters. The ZORBAX StableBond C8 coating technology, in combination with an optimized packing process, enabled high resolution mAb separations during faster run times. The columns displayed exceptional tolerance to back pressure increases beyond 1,000 bar and ensured reproducible column operation under acidic conditions and elevated temperatures. What's more, the RRHD 300SB-C8 1.8 µm column achieved greater sensitivities with enhanced peak shapes and greater resolution when compared to ZORBAX 300SB-C8 3.5 µm columns.

Materials and Methods

The Chinese hamster ovary (CHO)-cell derived monoclonal antibody was purchased from Creative Biolab, Pennsylvania. Triflouroacetic acid was purchased from Sigma-Adrich, St. Louis, MO, and iso-propanol and acetonitrile were supplied from Honeywell-Burdick & Jackson, Muskegon, MI

Conditions

Instrument	Agilent 1290 LC Infinity system with auto injector (ALS), binary pump and thermostatted oven and diode array detector (DAD)
Column	Agilent ZORBAX Rapid Resolution High Definition 300SB-C8, 2.1 × 50 mm, 1.8 µm (p/n 857750-906)
Mobile Phase	A. H_20:IPA (98:2) + 0.1% TFA (v/v) B. IPA:ACN:H_20 (70:20:10) + 0.1% TFA (v/v)
Injection	1 µL (2 mg/mL)
Flow rates	Between 0.5 mL/min and 1.0 mL/min
Gradient	Multi-segmented and linear elution
Temperature	80 °C
Detection	UV, 225 nm

For consecutive chromatographic runs, a one-minute post run was added to re-equilibrate the column.

Results and Discussion

Elevating column temperature to enhance peak shape performance and decrease retention

Solvent viscosity, protein diffusivity and mobile phase polarity depend strongly on temperature. The manipulation of column temperature is a crucial variable in the separation of hydrophobic peptides and proteins [1]. The expanded chromatographic overlays in Figure 1 show the effect of column temperature on a mAb separation as temperature is increased from 23 °C to 80 °C. Employing a gradient of 25% to 40% B (1.5% B/min), the mAb shoulder peak (highlighted in yellow) shows improved resolution from the base peak, while the pressure and retention time of both peaks decreases, thus making the 80 C separation more amendable for a fast and highly efficient analysis.

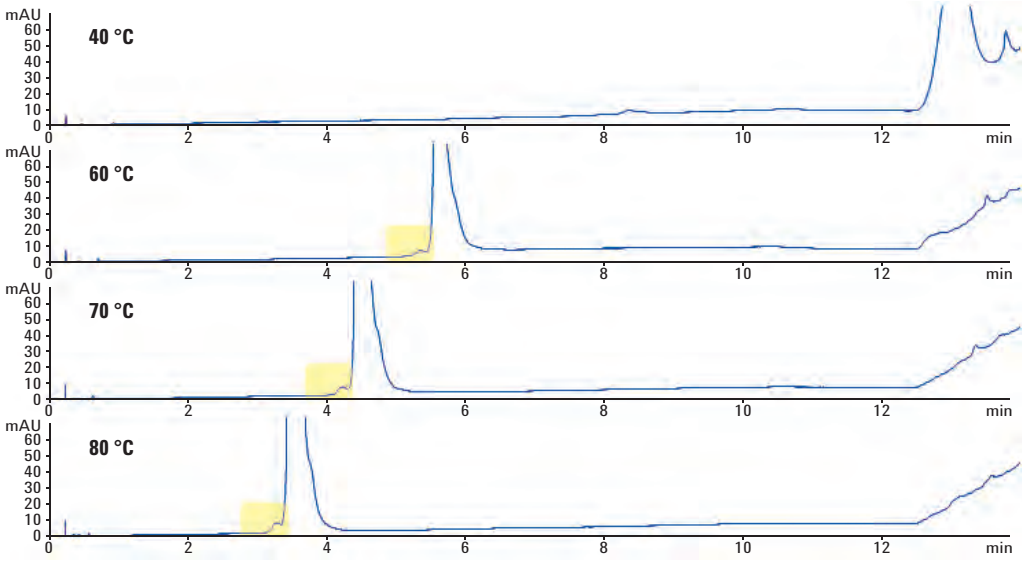

Figure 1. Temperature-dependant performance of intact monoclonal antibody separations on an Agilent ZORBAX Rapid Resolution High Definition 300SB-C8 1.8 µm column. During each chromatographic run temperature was increased (top to bottom) while flow rate remained constant at 0.5 mL/min. The shoulder peak, contrasted in yellow, details the resolution improvement and decrease in retention as temperature is increased.

Gradient flow rate optimization for improving sub-2 µm resolution

Changes in flow rate for optimizing protein separations, particularly with mAb's, can have dramatic outcomes in terms of resolution, efficiency and peak shapes due to mass transfer constraints with large protein diffusion. Protein dynamic size, hydrophobicity, polarity and eluent environment can all create separation difficulties in obtaining adequate peak shapes at various flow rates. Thus flow rate determinations for larger proteins, such as mAbs, become critical to the gradient optimization. Ideally, fast gradient run times are desired for 2.1 × 50 mm columns; however, this requires careful evaluation and optimization of the gradient slope at different flows. Flow rates of 0.5 mL/min, 0.75 mL/min, and 1.0 mL/min were evaluated under steep gradient conditions (Table 1, gradient B). As shown in Figure 2, a fast flow rate of 1.0 mL/min (relative to a 2.1 mm column id) produced enhanced mAb peak resolution in a shorter retention window. The intact mAb peak shape displayed better resolution of the intact peak at faster flow, while the increase in subsequent back pressures was well tolerated.

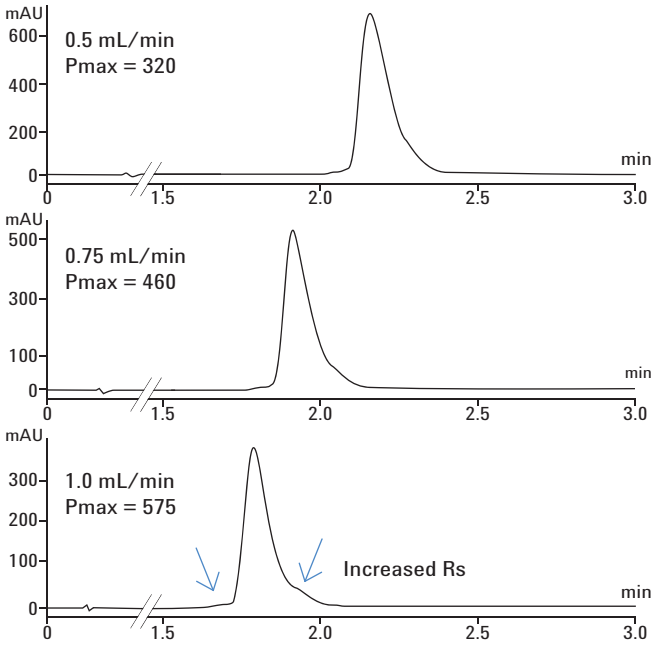

Figure 2. Three chromatographic overlays comparing increasing flow rates using fast gradient analysis conditions at 80 °C on an Agilent ZORBAX Rapid Resolution High Definition 300SB-C8 1.8 µm column. Increasing flow rate from 0.5 mL/min (top), 0.75 mL/min (middle) to 1.0 mL/min (bottom) improved intact mAb resolution and decreased retention.

Optimizing gradient conditions for high resolution and fast intact mAb analysis

Systematic gradient optimizations were performed under various column flow velocities to evaluate separation speed and the subsequent resolution effects towards intact mAb separation. We identified two gradients to highlight C8 separation efficiency for very fast mAb monitoring or for achieving ultra high resolution in a longer run time. To obtain a highly resolved mAb separation, a shallow gradient was identified and optimized at 0.5 mL/min (Table 1–Gradient A). The top chromatogram in Figure 3 details the resolution and highlights the base peak shoulder profile obtained when employing Gradient A. To obtain higher sensitivity and less band broadening in a much shorter run time, useful for mAb screening, a steeper gradient slope was used at a faster flow rate of 1.0 mL/min (Table 1–Gradient B). The bottom chromatogram and inset in Figure 3 displays a faster mAb separation and details an increase in sensitivity and earlier elution time compared to the top chromotagram, while still enabling adequate resolution for peak profiling.

Table 1. Optimized Gradient A and B Conditions for Figure 3

Gradient A, 0.5 mL/min	% solvent B	Time (min)
	25	0
	35	10
	35	12
	90	14
	25	18

Gradient B, 1.0 mL/min	% solvent B	Time (min)
	25	0
	35	3
	90	4
	25	5

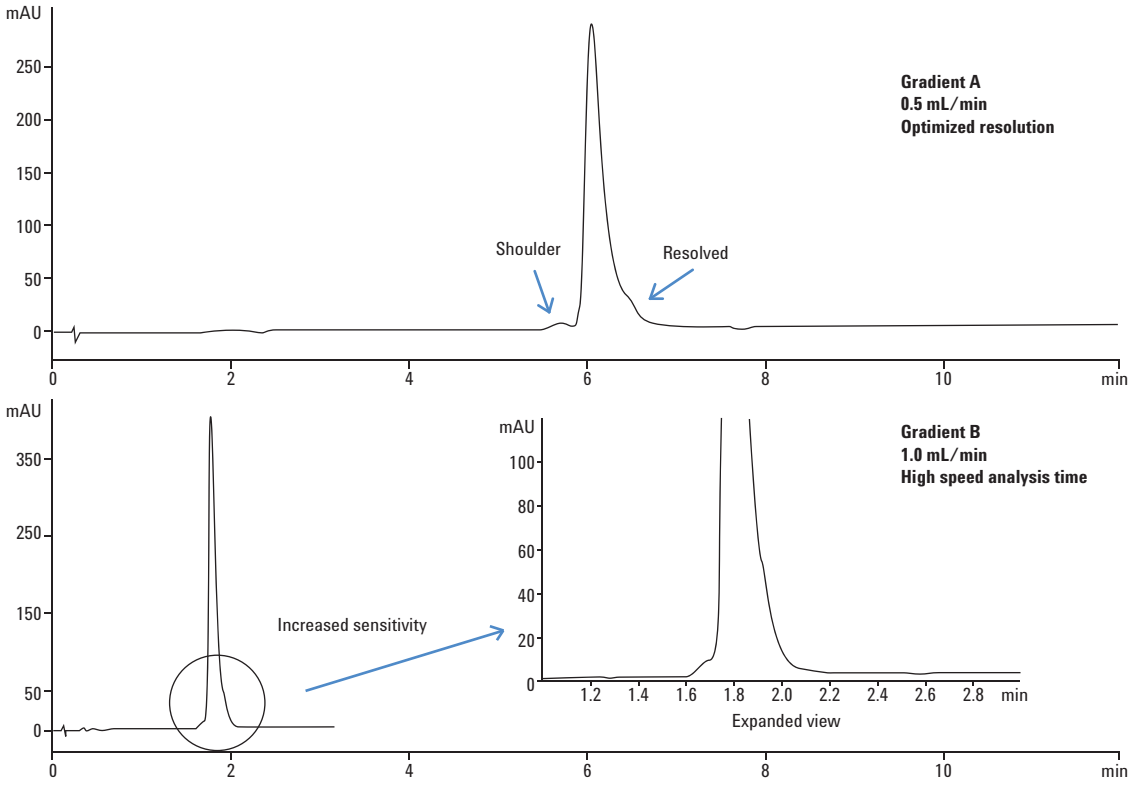

Figure 3. Two chromatographic comparisons showing optimized gradient separations of intact mAb's on an Agilent Rapid Resolution High Definition ZORBAX 300SB-C8 1.8 µm column. The top chromatogram details ultra high resolution obtained during a longer run time and slower flow rate, while the bottom chromatogram ,with expanded view, shows increased sensitivity with adequate resolution in a very fast analysis time, useful for mAb screening.

Performance comparison of Agilent ZORBAX RRHD 300SB-C8 1.8 µm and 300SB-C8 3.5 µm columns

Using optimized gradients A and B in Table 1, a ZORBAX RRHD 300SB-C8 1.8 µm column was compared directly to a ZORBAX 300SB-C8 3.5 µm column. The results of these comparisons are shown in Figure 4a and Figure 4b. Figure 4a is a comparison using the longer gradient time for obtaining higher resolution of the intact mAb. Under these gradient conditions (gradient A), the 1.8 µm 300SB-C8 outperforms the 3.5 µm 300SB-C8 column, delivering better resolution and higher sensitivity with a slightly shorter retention factor. In this comparison, the 3.5 µm front shoulder peak has been reduced and the subsequent base peak resolution has been diminished. Alternatively, in Figure 4b, employing the steeper B gradient with faster flow rate, the 1.8 µm 300SB-C8 shows greater resolution, enhanced peak shape and almost 2× higher sensitivity.

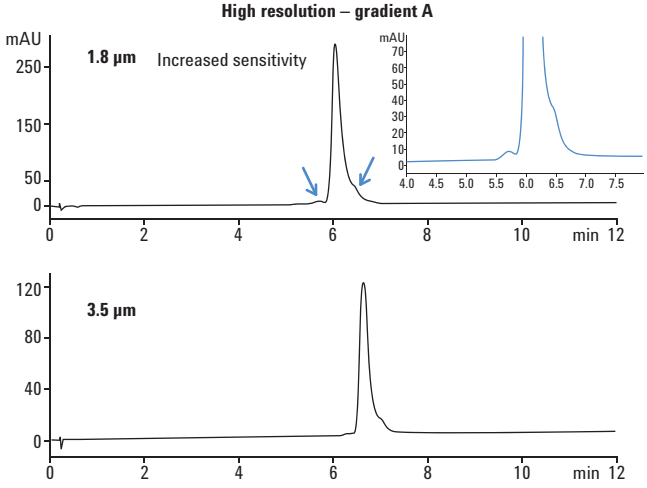

Figure 4a. Gradient A (Table 1) comparison of 1.8 µm (top) and 3.5 µm (bottom) Agilent ZORBAX Rapid Resolution High Definition 300SB-C8, 2.1 × 50 mm columns at 80 °C. The top 1.8 µm chromatogram inset details the improved resolution.

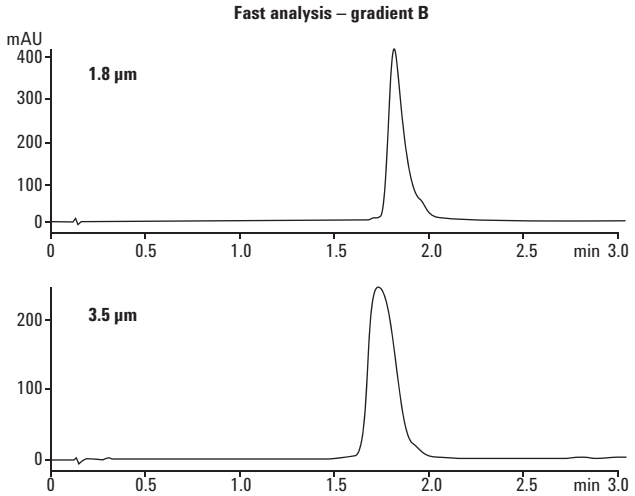

Figure 4b. Gradient B (Table 1) comparison of 1.8 µm (top) and 3.5 µm (bottom) Agilent ZORBAX Rapid Resolution High Definition 300SB-C8, 2.1 × 50 mm at 80 °C. The top 1.8 µm chromatogram shows improved peak shape, resolution and sensitivity.

Determining column performance for run-to-run reproducibility and mAb recovery

Column reproducibility and recovery were investigated at 80 °C using Gradient A conditions (Table 1). A faster separation time, requiring a quicker equilibration time, is the preferred method to fully evaluate the column for run-to-run reproducibility and total protein recovery. High protein recovery is a critical attribute for intact mAb analysis [2]. Although specific post run wash regimes can be employed for column cleanup between injections, it is more desirable to develop in-run conditions that allow high mass balance transfer and the absence of peak ghosting from run-to-run. To evaluate column reproducibility and recovery of the ZORBAX 300SB-C8 1.8 µm, 150 consecutive runs were performed. The repeated intact mAb separations gave no indications of retention time shifts, peak broadening or changes in symmetry (Table 2). The bottom chromatogram in Figure 5 displays the pre- and post-150 injection blank runs (0 µL injected) and gradient pressure curves. The UV trace at 225 nm shows no apparent peak ghosting or baseline disturbance after 150 injections, and the pressure remained stable indicating no fouling of the inlet frit or potential shifts in bed stability.

Table 2. Retention Time, Peak Width, and Symmetry Calculated During Reproducibility Runs at Every 50th Run Beginning From Run #1 to Run #150

Reproducibility injection #	Ret. time (min)	Peak width (W)	Symmetry	Reproducibility injection #	Ret. time (min)	Peak width (W)	Symmetry
1	1.975	0.0938	0.518	100	1.990	0.0999	0.539
50	1.980	0.0969	0.540	150	1.977	0.0828	0.546

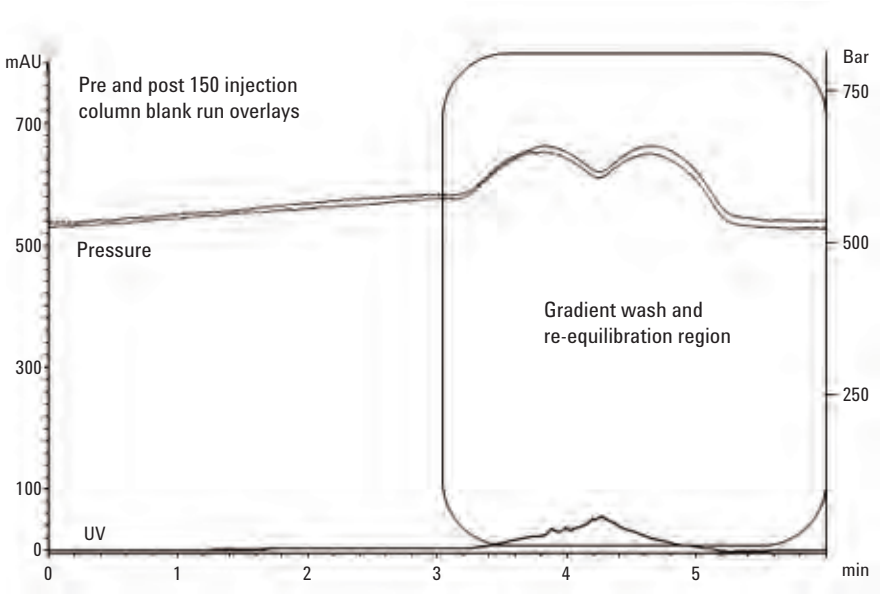

Figure 5. Chromatographic pressure and UV trace overlays for the 1st and 150th injection of the intact mAb on an Agilent ZORBAX Rapid Resolution High Definition 300SB-C8 1.8 µm column. All injections from 1 to 150 were consecutive with total run-to-run injection times at six-minute intervals.

Conclusions

The Agilent ZORBAX RRHD 300SB-C8 1.8 µm column was investigated under gradient optimized conditions at 80 °C for separation of an intact monoclonal antibody. Employing the use of two preferred gradients, separations were optimized to efficiently resolve an intact mAb for very fast mAb screening or ultra high resolution of the mAb and its constituents. In addition, temperature, recovery and flow rate optimizations were demonstrated and established, and compared to a 3.5 µm ZORBAX RRHD 300SB-C8 column. The 1.8 µm ZORBAX RRHD 300SB-C8 delivered fast-highly efficient separations for intact mAb analysis and performed reliably at elevated temperatures and low pH. In combination with the optimized gradient conditions and elevated temperature, the RRHD 300SB-C8 column delivered reproducible separations, during very fast run times, for 150 consecutive injections.

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2. C. T. Mant, L. H. Kondejewski, P. J. Cachia, O. D. Monera, R. S. Hodges, Methods Enzymol., 1997, 289, 426-429.

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Fast Separation of Recombinant Human Erythropoietin Using Reversed Phased Agilent ZORBAX RRHD 300SB-C18, 1.8 µm

Application Note

BioPharma

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Abstract

Fast separation of proteins with increased sensitivity, reduced separation time and run-to-run reproducibility is in great demand by biotechnology and pharmaceutical companies. Highly sensitive analytical techniques are necessary for monitoring the purity and stability of proteins during manufacturing, purification, formulation, and storage. The Agilent ZORBAX 300SB-C18 1.8 µm Rapid Resolution High Definition (RRHD) column is designed to address these challenges. The C18 StableBond coating technology provides increased sensitivity, exceptional pH, and thermal stability. The use of a 1.8-µm column designed for UHPLC systems significantly reduces analysis time, critical for increasing the efficiency of QC for protein primary structure analysis. The data presented here focuses on the fast separation of various recombinant human erythropoietin isoforms. Like many other biomolecules, recombinant EPO protein exhibits heterogeneity due to modifications that occur during manufacturing. Methods are optimized for flow rates, gradient and reproducibility under acidic conditions that contain trifluoroacetic acid.

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Introduction

Erythropoietin protein (EPO) is a glycoprotein hormone found in plasma. It is a cytokine for erythrocyte (red blood cell) precursors in the bone marrow. EPO controls red blood cell production. It also has neuroprotective activity against a variety of potential brain injuries and antiapoptotic functions in several tissue types. Recombinant human EPO protein (rEPO) is produced by Chinese hamster ovary (CHO) cells using recombinant DNA technology. The rEPO single polypeptide chain contains 165 amino acids and has a predicted molecular mass of 24,000 Dalton, and apparent glycosylated molecular mass of 30,400 Dalton. Recombinant human EPO protein is one of the most widely produced by many bio-and pharmaceutical companies throughout the world for therapeutic agents. The HPLC separation of EPO protein from its impurities can be achieved by using a variety of chemistries, including reversed-phase chromatography. The data presented here focuses on the use of analytical reversed phase for separation of varying hydrophobicities of a CHO derived-EPO protein. Methods were developed on the Agilent ZORBAX 300SB-C18 1.8-µm column using the Agilent 1290 Infinity LC system.

Material and Methods

HPLC system

The Agilent 1290 Infinity LC system operates at a maximum pressure of 1,200 bar. The Agilent 1290 Infinity LC is designed to provide the highest speed, resolution, and ultra-sensitivity. A new power range lets you deploy any particle type, any column dimensions, or any mobile, and stationary phases. Innovative technology components offer the next level of performance for UHPLC, RRLC, and HPLC applications. With revolutionary Intelligent System Emulation Technology, the 1290 Infinity is the world's first universal LC system, as it can execute other HPLC and UHPLC methods and deliver the same chromatographic results without any change of the instrument or the original method.

HPLC column

The column used was an Agilent ZORBAX 300 SB-C18 RRHD, 2.1 × 50 mm, 1.8 µm (p/n 857750-902). These columns are used for protein primary structure analysis to confirm protein identity, quantify post translational modifications and provide impurity profiles for biotherapeutic protein discovery and development and production applications. This new wide-pore (300 Å) RRHD column brings UHPLC performance to the reversed-phase separation of intact proteins and protein digests. The ZORBAX StableBond stationary phase provides high stability in low pH that allows mobile phase eluents with triflouroacetic acid (TFA) or formic acid (FA) to be used routinely without compromising the columns' life time. The columns are stable up to 90 °C.

Chemicals and reagents

CHO-cell derived, humanized EPO protein from Creative BioLabs, Shirley, NY
Triflouroacetic acid from Sigma-Aldrich, St. Louis, MO
Acetonitrile from Honeywell-Burdick & Jackson, Muskegon, MI

LC methods

The conditions in Table 1 were used throughout the investigation with variations as noted in the relevant chromatograms.

Table 1. Standardized Chromatographic Conditions

Parameter	Item
Column	Agilent ZORBAX RRHD 300 SB-C18, 2.1 × 50 mm, 1.8 µm
Sample	Recombinant human EPO protein (rEPO)
Sample concentration	1.0 mg/mL
Injection volume	3 µ
Flow rate	1.0 mL/min
Pressure	650 bar
Mobile phase A	0.1% TFA in deonized water
Mobile phase B	0.01% TFA in 100% ACN
Detector	UV, 280 nm
System	Agilent 1290 Infinity HPLC

Results and Discussion

Optimization of flow rate and gradient for rapid separation and increased resolution

Three flow rates were selected, 0.5, 1.0, and 1.5 mL/min, with gradient started at 5.0% mobile phase B and ended at 100% mobile phase B from 0 to 2.5 min, respectively. The column was then equilibrated at 5.0% mobile phase B for another 2.5 min. The column separated the test mixture very quickly, distinguishing rEPO protein from its impurities in less than five minutes. The integrity of peak shape and asymmetry of 1.0 and 1.5 mL/min indicated that fast flow rates had a major advantage when using ZORBAX 300 SB-C18, 1.8 µm for protein separation (Figure 1, Table 2), a feature of 1.8-µm particles that facilitates rapid separation.

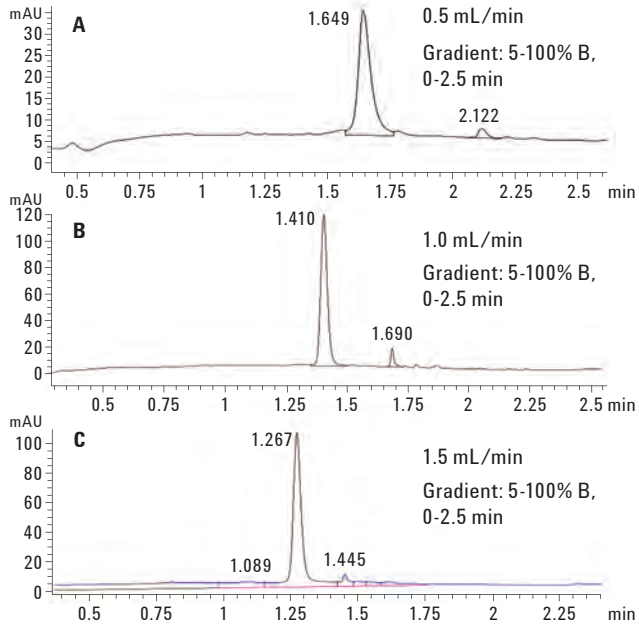

Figure 1. Different flow rates can be selected to separate rEPO protein on the Agilent ZORBAX RRHD 300 SB-C18, 2.1 × 50 mm, 1.8 µm column.

Table 2. Effect of Flow Rate on Retention Time, Asymmetry, and Peak Width in the Separation of rEPO Protein

Flow rate (mL)	Pressure (bar)	Retention time (min)	Asymmetry	Peak width
0.5	350	1.64	0.6	0.047
1.0	650	1.41	0.85	0.030
1.5	890	1.26	0.84	0.030

Different gradient systems were also selected to achieve the same fast analysis time. Figure 2 shows the separation of rEPO protein at 1.0 mL/min with gradient set at 10 to 50 to 70% mobile phase B from 0, 2.5 and 3.0 min, respectively. The column was then equilibrated for another 2 min at 10% mobile phase B before the next injection. Figure 2 indicates that the column was able to separate EPO protein from its impurities in a similar manner to the gradient shown in Figure 1, panel B. The same number of peaks was obtained, just with different retention times. In addition, rapid equilibration was evident, demonstrating that the column was suitable for use with a wide range of organic content.

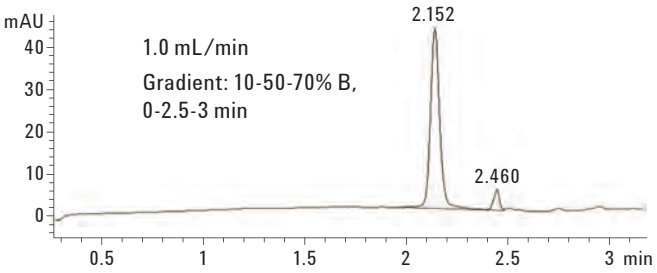

Figure 2. Different gradients can be selected to separate EPO protein from its impurities in less than 5 min using the Agilent ZORAX RRHD 300 SB-C18, 2.1 × 50 mm, 1.8 µm column.

Separation of heat-degraded recombinant human EPO protein

Recombinant human EPO protein was heated at 60 °C overnight (16 hours) at neutral pH (pH 7.0) and acidic pH (pH 4.0). Due to its nature, rEPO protein will be degraded or form other isoforms when heated at high temperatures such as 60 °C at different pH. At neutral pH, rEPO forms limited isoforms, but when acidic pH conditions are used, the structure of rEPO protein will be altered significantly. The lower the pH, the more changes[1]. Figure 3 shows both conditions of heating. Panel A shows data from a sample was heated at neutral pH. The column resolved the main peak of rEPO from its degraded products or isoforms very well. Note that the conformation of rEPO protein was not much changed. The retention of the main peak remained at 1.4 min, as seen in Figure, panel A (unheated sample). However, the impurity peak was substantially increased and well separated by the column (compared to Figure 1, panel B). Figure 3, panel B shows the separation of heat-treated rEPO at pH 6.0. The separation conditions were the same with neutral pH but the chromatogram was drastically changed. As indicated earlier, heating with acidic conditions can highly alter and degrade the conformation of the rEPO protein. The retention time of the major peak changed from 1.4 min to 1.68 min and the peak also contained a shoulder peak on the right. In addition, more impurity peaks also were observed. This clearly indicated that the ZORBAX RRHD 300 SB-C18, 1.8 µm could also very well resolve rEPO impurities and degradation products.

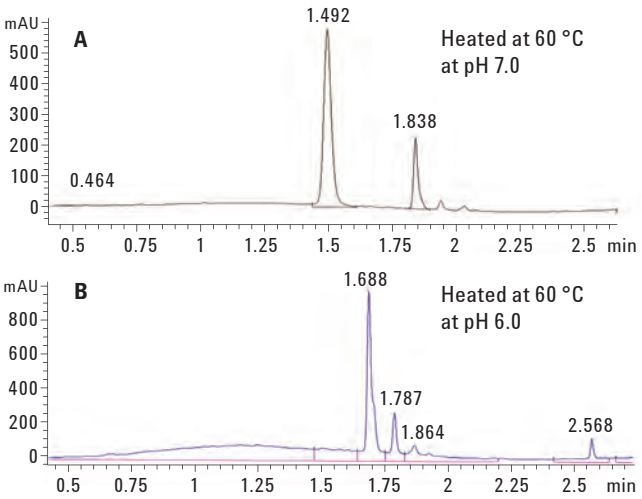

Figure 3. Heat-treated rEPO protein well resolved by the Agilent ZORBAX RRHD 300 SB-C18, 2.1 × 50 mm, 1.8 µm column. The column separated these heat-treated rEPO proteins at 1.0 mL/min, 5 to 100% B solvent from 0 to 2.5 min.

Reference

1. Yoshiyuki Endo, *et al.*, *J. Biochem* (1992) 112 (5): 700-706.

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Rapid UHPLC Analysis of Reduced Monoclonal Antibodies using an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8 Column

Application Note

BioPharma

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Abstract

An Agilent ZORBAX sub-2 µm Rapid Resolution High Definition (RRHD) 300SB-C8 reversed-phase column was used under optimized chromatographic conditions for delivering highly resolved ultra fast separations of reduced and alkylated monoclonal antibodies. The StableBond C8 phase and RRHD column technology, in combination with robust gradient elution conditions enabled efficient, fast, and effective separation of light chain and two heavy chain monoclonal antibody variants in under 4 minutes. Additionally, alternate mobile phase compositions were employed, making separations flexible for ultra fast LC/MS analysis. At elevated operating pressures and temperature, the stable C8 phase and rugged column packing technology delivered highly reproducible separations and delivered excellent chromatographic run-to-run results.

Agilent Technologies

Introduction

Biotherapeutic drug development is rapidly growing among the pharmaceutical industry and antibody drug characterization is an important challenge to this development pipeline. Although antibodies can be characterized by many separation techniques, separation by reversed-phase chromatography has been limited due to inefficient protocols, poor resolution, and loses in total protein recovery. Structurally, monoclonal antibody is a glycoprotein comprised of two identical copies of heavy chains (50 kDa) and two identical copies of light chains (25 kDa) attached through disulfide bridges. Fast and efficient characterization of these chains and isoforms are becoming increasingly desired and critical, for high throughput monitoring of purity and stability during manufacturing, formulation and storage.

We have used an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8, 1.8 µm column in combination with optimized chromatographic conditions for reduced monoclonal antibody analysis to demonstrate utility for ultra fast mAb characterization and screening. The ZORBAX StableBond C8 phase, elevated temperature (75 °C), and optimized gradient conditions enabled high resolution separation of the light and heavy chain mAbs in an extremely short elution time that to our knowledge, has not previously been demonstrated. The separations were performed under various mobile phase compositions with mass spectrometer (MS) friendly ion pairing additives to provide flexibility for different LC/MS user preferences. The columns displayed exceptional tolerance to high backpressure increases beyond 900 bar and ensured reproducible column operation under acidic mobile phase conditions and elevated temperatures. Additionally, protein recovery was repeatedly evaluated during 250 runs and gave no indications of peak ghosting or changes in retention behavior.

Experimental

Materials

The human monoclonal antibody used in this study was produced using CDH media at Agilent (p/n 010774) and stored at pH 7.2 in 10 mM phosphate buffer and 0.09% (w/v) sodium azide at 4 °C. Antibody concentration was 10 mg/mL. Triflouroacetic acid was purchased from Sigma-Adrich, St. Louis, MO, and 1-propanol and acetonitrile were supplied from Honeywell-Burdick & Jackson, Muskegon, MI. The dialysis cassettes had a 3,500 MWCO and were purchased from Thermo Scientific (p/n 66330).

Reduction and Alkylation

Reduction and alkylation were performed under denaturing conditions using guanidine hydrochloride to produce the free light and heavy chains for reversed-phase analysis. A 0.5 mL (1.5 mg/mL) aliquot of antibody was dialyzed against water for preservative removal. Once dialyzed, the 0.5 mL aliquot was diluted to a final concentration of 0.75 mg/mL with 100 mM TRIS-HCl and 4M guanidine hydrochloride (Mallinckrodt, Phillipsburg, NJ, USA). The solution pH was adjusted to pH 8.0. A 10 µL aliquot of 0.5 M dithiothreitol (DTT, Sigma) stock solution was added to obtain a final concentration of 5 mM. The mixture was placed in a 37 °C water bath and incubated for 30 minutes. The antibody solution was then briefly cooled to room temperature and a 26 µL aliquot of a 0.5 M iodoacetamide (IAM, Sigma) stock solution was added for a final concentration of 13 mM. The antibody solution was placed in the absence of light at room temperature for 45 minutes. Once removed, the solution was quenched with 20 µL of 0.5 M DTT for a final concentration of 10 mM. The 1.0 mL of reduced and alkyklated antibody was then desalted through a 4 mL, 3.5 K MWCO concentrator (p/n 5185-5991) at 3800 RPM for 30 minutes using water (0.1% TFA). The concentrating process was repeated two times for a final volume of 0.5 mL (1.5 mg/mL).

UHPLC Conditions

Instrument	Agilent 1290 LC Infinity system with auto injector (ALS), binary pump, thermostatted oven, and diode array detector (DAD)
Column	Agilent ZORBAX Rapid Resolution High Definition 300SB-C8, 2.1 × 100 mm, 1.8 µm (p/n 858750-906)
Mobile Phase	(Various) A. H_20 + 0.1% TFA (v/v) B. n-propanol:ACN:H_20 (80:10:10) + 0.1% TFA (v/v) A. H_20 + 0.05% TFA/0.5% acetic acid (v/v) B. n-propanol:ACN:H_20 (80:10:10) + 0.05% TFA/0.5% acetic acid (v/v) A. H_20 + 0.05% TFA/0.05% formic acid (v/v) B. ACN + 0.05% TFA/0.05% formic acid (v/v)
Injection	1-3 µL
Flow rate	0.5 mL/min
Gradient	multi-segmented
Temperature	75 °C
Detection	UV, 225 nm

For consecutive chromatographic runs, a 2-minute post run was added to re-equilibrate the column.

Results and Discussion

Separation optimization for ultra fast analysis of reduced and alkylated monoclonal antibody

The chromatograms in Figures 1A and 1B detail two optimized chromatographic profiles for ultra fast separation of reduced and alkylated monoclonal antibody. The Agilent ZORBAX RRHD 300SB-C8, 1.8 µm column, elevated temperature of 75 °C and optimized mobile phase compositions enabled two well resolved, high speed separations. The faster separation profile displayed in the top chromatogram (1A) details a reduced antibody separation completing in less than 4 minutes. The separation exhibits narrow light and heavy chain bands with high efficiency. In contrast, the chromatogram shown in Figure 2B also delivers a fast separation, but during a longer run time. In this separation, the light and heavy chain bands are slightly broadened, however the two heavy chain peaks 1 and 2 are now fully resolved. For each separation, mobile phase A contained water (0.1%TFA) and mobile phase B contained an 80/10/10 solvent mixture of 1-propanol, acetonitrile and water (0.1% TFA). Alternate eluent compositions of mobile phase B were tried, including 100% acetonitrile, however they were unable to deliver satisfactory peak shapes as those displayed in Figures 1 and 2. The optimized segmented gradient conditions (Table 1A) used in the top panel separation were critical to resolve the light and heavy chains in under 4 minutes, while the shallower gradient conditions (Table 1B) were optimized to enable baseline resolution of the two heavy chain variants. For both separations, each gradient completed with a fast column washing step that facilitated faster column equilibration for high throughput analysis and continued run-to-run testing.

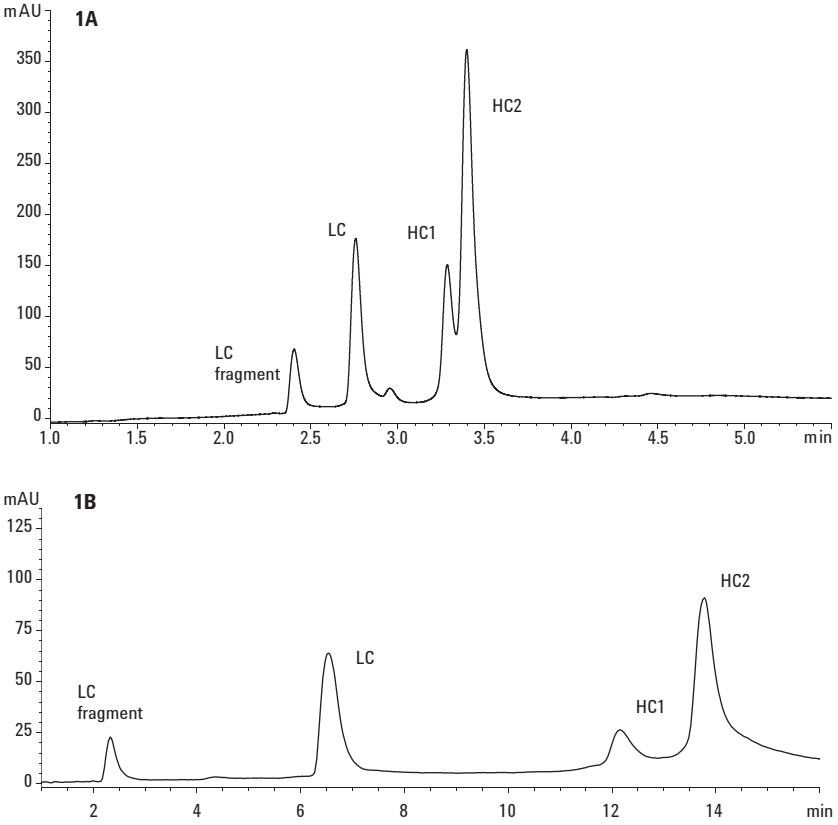


Figure 1. Comparison of two optimized gradients for the ultra fast separation of reduced and alkyklated monoclonal antibodies on an Agilent ZORBAX Rapid Resolution High Definition 300SB-C8, 1.8 µm column, 2.1 × 100 mm. The top panel details a rapid separation of the light and heavy chain variants in a shortened run time of less than 4 minutes. The bottom panel displays complete baseline resolution of the two heavy chain variants during a longer runtime using a shallower gradient profile. Both separations were performed at 75 °C and completed with a fast 90% 1-propanol wash step (UV not shown).

Table 1A. Optimized for Speed

Gradient	% Solvent B	Time (min)
	20	0
	35	3
	40	4
	40	5
	90	5.1
	90	5.5
	25	6

Table 1B. Optimized for Resolution

Gradient	% Solvent B	Time (min)
	25	0
	32	15
	32	16
	90	17
	90	17.5
	25	18

Mobile phase compositions for fast enhanced LC/MS analysis

MS electrospray ionization in positive ion mode requires reduced quantities of trifluoroacetic acid (TFA), which can greatly affect signal suppression. Additionally, MS analysis benefits from the addition of volatile mobile phase additives that increase signal intensity. Small organic acids such as formic acid (FA) or acetic acid (AcOH) are therefore the preferred ion pairing reagents for reversed-phase chromatography coupled with MS detection. We have evaluated several combinations of organic mobile phase B with various ion pair additives for providing higher signal intensity alternatives to conventional ACN/TFA only systems for LC/MS analysis. The concentration amounts of the ion pair reagents were adjusted between 0.5% and 5% while the B mobile phases included ACN, 1-propanol and iso-propanol or combinations thereof. Figures 2A and 2B below display the top two separation results from this study. Under the defined gradient conditions (Tables 2A and 2B), separation of light and two heavy chain variants were fully optimized for fast analysis with each separation completing in under 5 minutes. The top panel separation has been optimized using the same mobile phase compositions used in Figures 1A and 1B, however the TFA has been reduced from 1.0% to 0.05%, while 0.5% AcOH has been added. The gradient for this separation is shown in Table 2A. The bottom panel (Figure 2B) displays the separation from a more conventional water/ACN gradient but with TFA reduced to 0.05% and 0.05% FA added. In this separation, the two heavy chain variants show different selectivity compared to the 2A separation, which used n-propanol, where HC1 and HC2 have switched retention positions. Additionally, it was observed that using the same gradient slope and mobile phase compositions for 2B, but eliminating FA, did not provide sufficient resolution between HC1 and HC2. Conditions for each separation, using reduced amounts of TFA with FA or AcOH, are very amendable for ultra fast analysis of mAb and suggest profiling alternatives for LC/MS analysis.

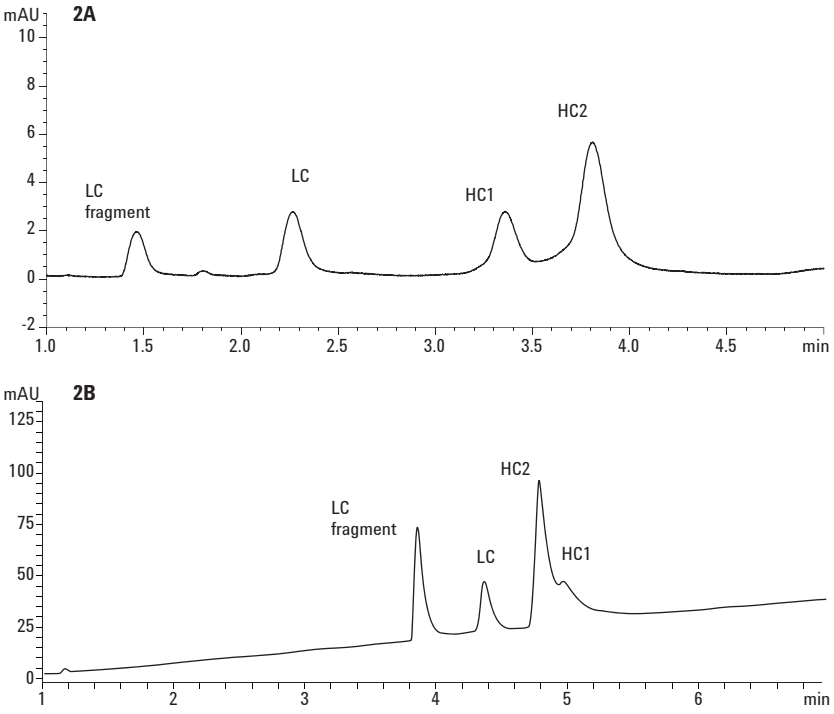


Figure 2. MS friendly mobile phase compositions for ultra fast LC/MS characterization of reduced and alkylated antibodies using an Agilent ZORBAX RRHD 300SB-C8, 2.1 × 100 mm column. The top panel (2A) separation uses the same solvent composition as noted in Figures 1A and 1B, however with the addition of acetic acid for enhancing MS signal intensity. The bottom panel (2B) was performed with a more common water/acetonitrile mobile phase, but with a reduced amount of TFA and addition of formic acid to aid in less signal suppression.

Table 2A. A: H_2O + 0.5% AcOH/0.05% TFA (v/v)
B: n-propanol:ACN:H_2O (80:10:10) + 0.5% AcOH/0.05% TFA (v/v)

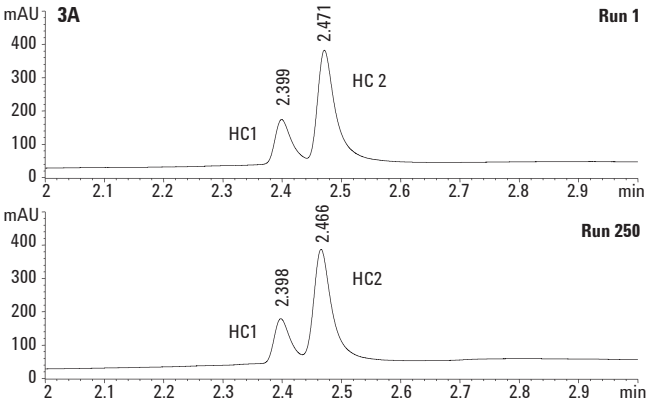
Gradient	% Solvent B	Time (min)
	25	0
	35	10
	35	12
	90	14
	25	18

Table 2B. A: H_2O + 0.05% FA/0.05% TFA (v/v)
B: n-propanol:ACN:H_2O (80:10:10) + 0.05% FA/0.05% TFA (v/v)

Gradient	% Solvent B	Time (min)
	25	0
	50	7
	90	8
	25	9

Reproducibility and Recovery during repeated mAB analysis

Using a new ZORBAX RRHD SB C8, 2.1 × 100 mm, 1.8 µm column, run-to-run reproducibility and recovery of reduced monoclonal antibodies were investigated under the gradient composition and conditions described in Table 3. To evaluate column reproducibility and recovery, 250 consecutive column runs were performed, while post run blanks and column back-pressures were evaluated every 20th injection. During the lifetime sequence, the column was exposed to repeated high pressure operation (>700 bar) and 75 °C temperature. As detailed in Figure 3A, repeated separations of heavy chain variants 1 and 2 were highly reproducible with no changes in retention time or peak shapes. The blank run UV overlap traces shown in the bottom of chromatogram of Figure 3B indicate consecutive runs do not exhibit carryover of residual mAb, while the top pressure overlays in 3B demonstrate rugged column longevity for repeated analysis and thus provided high tolerance to internal column fouling and frit blinding.



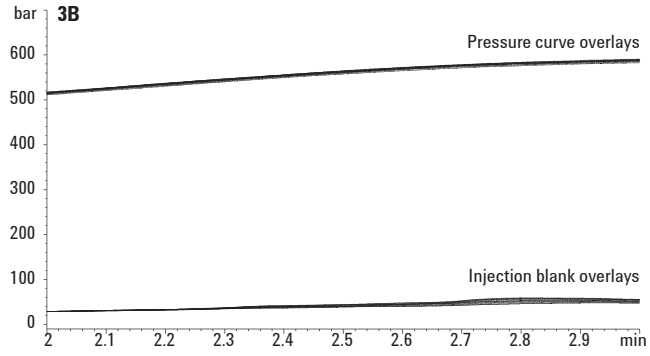


Figure 3A. Run 1 (top) and run 250 (bottom) comparison of heavy chain 1 and heavy chain 2 peak shape and retention repeatability.

Figure 3B. Thirteen overlay comparisons of blank injection UV and pressure traces during 250 repeated injections. Blank traces were collected after every 20 injection during the lifetime sequence.

Table 3. Mobile Phase and Gradient Used During Repeated Analysis of mAb

Mobile phase	A. H_2O + 0.1% TFA (v/v)	
	B. n-propanol:ACN:H_2O (80:10:10) + 0.1% TFA (v/v)	
Gradient	% solvent B	Time (min)
	25	0
	35	10
	35	12
	90	14
	25	18

Conclusions

The Agilent ZORBAX RRHD 300SB-C8, 1.8 µm column provided ultra fast and efficient separation of reduced and alkylated monoclonal antibodies. Optimized gradient conditions and elevated operating temperature (75 °C), in combination with the rugged ZORBAX C8 coating chemistry, enabled high resolution separations of antibody light chain and two heavy chain variants in under 4 minutes. Gradient conditions and compositions were systematically optimized to provide complete separation, washing and re-equilibration of antibody in a greatly reduced analysis time. Additionally, separations were optimized in ultra fast run times with use of alternate mobile phase additives (for example, formic acid and acetic acid), to provide different separation options for obtaining better MS sensitivity.

Excellent reproducibility and recovery results were obtained after 250 consecutive runs of reduced monoclonal antibody separations. At elevated pressures greater than 700 bar, repeated separations maintained peak shape and retention time, and gave no indications of early column failure due to frit plugging or a packed bed instability. The 1.8 µm ZORBAX RRHD 300SB-C8 column displayed excellent recovery as indicated by the absence of peak carryover and demonstrates robust column operation for the repeated analysis of reduced and alkylated antibodies. All separations provided high resolution and ultra fast separation of the reduced light and heavy chains and thus demonstrated utility for high throughput mAB screening.

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Agilent Technologies

Reversed-Phase Optimization for Ultra Fast Profiling of Intact and Reduced Monoclonal Antibodies using Agilent ZORBAX Rapid Resolution High Definition 300SB-C3 Column

Application Note

Biopharma

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Abstract

Rapid reversed-phase separations of intact and reduced monoclonal antibodies (mAbs) were optimized using Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C3 columns. The StableBond C3 phase and robust sub-2 µm RRHD column technology enabled high resolution separation of monoclonal antibody structure and has demonstrated utility for high throughput mAb characterizations. Monoclonal antibodies expressed by different cell lines were evaluated and optimized for high resolution separations during rapid run times. The ZORBAX 300SB-C3 columns were evaluated for lifetime stability and reproducibility during continuous run sequences and demonstrated high tolerance to elevated temperature and pressure while delivering robust separation performance.

Agilent Technologies

Introduction

Drug development of biotherapeutics is rapidly growing among the pharmaceutical industry and reliable antibody drug characterization is an important challenge to this development pipeline. Separation of antibodies can be achieved by various chromatographic techniques and requires multiple modes of separation to detect and measure them. One approach to antibody characterization is by reversed-phase (RP) methods, due to its attractiveness with LC/MS detection. However, separation of large molecules such as monoclonal antibodies (mAbs) by RP, has traditionally suffered from broad, diffuse and poorly resolved peaks and has thus limited its use for analysis. Now with the advent of UHPLC and smaller particle columns, these separations can be achieved during faster run times with increased resolution. Also, with the introduction of newer phase chemistries, RP separations can provide alternate selectivity's with greater sensitivity towards proteins. These alternatives to RP analysis for mAbs are now showing great promise for facilitating biotherapeutic analyses.

In this work, we have achieved ultra high resolution separations of both intact and reduced antibodies during a rapid run time using ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C3 columns. Specifically, we systematically optimized gradient conditions at 75C to deliver ultra fast- high efficiency separations of both intact and reduced light and heavy chain mAbs. We have also evaluated the ZORBAX 300SB-C3 for column lifetime and reproducibility at elevated temperature, high operating pressures and low pH to monitor column changes in retention behavior, peak shape and efficiency. All the work herein demonstrates utility for fast mAb profiling while delivering reproducible high resolution separations.

Experimental

Materials

Two humanized monoclonal antibody lines were used in this study. One line was expressed at Agilent using CDH media (p/n 010774) and the other was expressed from a Chinese hamster ovary (CHO)-cell derived monoclonal antibody purchased from Creative Biolab, Pennsylvania. Triflouroacetic acid was purchased from Sigma-Adrich, St. Louis, MO, and iso-propanol, n-propanol, and acetonitrile were supplied from Honeywell-Burdick & Jackson, Muskegon, MI. The 1-propanol was purchased from VWR (p/n BJ322-4). The dialysis cassettes had a 3,500 MWCO and were purchased from Thermo Scientific (p/n 66330).

Reduction and Alkylation

Reduction and alkylation was performed under denaturing conditions using guanidine hydrochloride (GuHCl) to produce the free light and heavy chains. 0.5 mL (1.5 mg/mL) of antibody was dialyzed against water for preservative removal. Once dialyzed, a 0.5 mL aliquot was diluted to a final concentration of 0.75 mg/mL with 100 mM TRIS-HCl and 4 M GuHCl (Mallinckrodt, Phillipsburg, NJ, USA). The pH was adjusted to 8.0 and a 10-µL of 0.5 M dithiothreitol (DTT, Sigma) stock solution was added for a final concentration of 5 mM. The mixture was placed in a 37 °C water bath and incubated for 30 minutes. The antibody was briefly cooled to room temp and a 26-µL aliquot of 0.5 M iodoacetamide (IAM, Sigma) stock solution was added for a final concentration of 13 mM. The alkylated antibody solution was placed in the absence of light at room temperature for 45 minutes. Once removed, the mixture was quenched with 20 µL of 0.5 M DTT for a final concentration of 10 mM. A 1.0 mL amount of reduced and alkylated antibody was desalted through a 4 mL 3.5 K MWCO concentrator (p/n 5185-5991) at 3800 RPM for 30 minutes using water (0.1% TFA). The concentrating process was repeated two times to a final volume of 0.5 mL (1.5 mg/mL)

UHPLC Conditions

Instrument	Agilent 1290 LC Infinity system with auto injector (ALS), binary pump, thermosttated oven (TLC) and and diode array detector (DAD)
Column	Agilent ZORBAX RRHD 300 SB-C3, 1.8 µm 2.1 × 100 mm (p/n 858750-909) 2.1 × 50 mm (p/n 857750-909)
Mobile phase (intact mAb)	A. 98/2 water/n-propanol (0.1% TFA) B. 70/20/10 iso-propanol/ACN/water (0.1% TFA)
Mobile phase (reduced mAb)	A. H_20 + 0.1% TFA (v/v) B. 80/10/10 n-propanol/ACN/water (0.1% TFA)
Injection	2 µL
Flow rates	0.5 mL/min 1.0 mL/min (intact) 1.25 mL/min (lifetime testing)
Gradient	multisegmented
Temperature	75 °C
Detection	UV, 280 nm

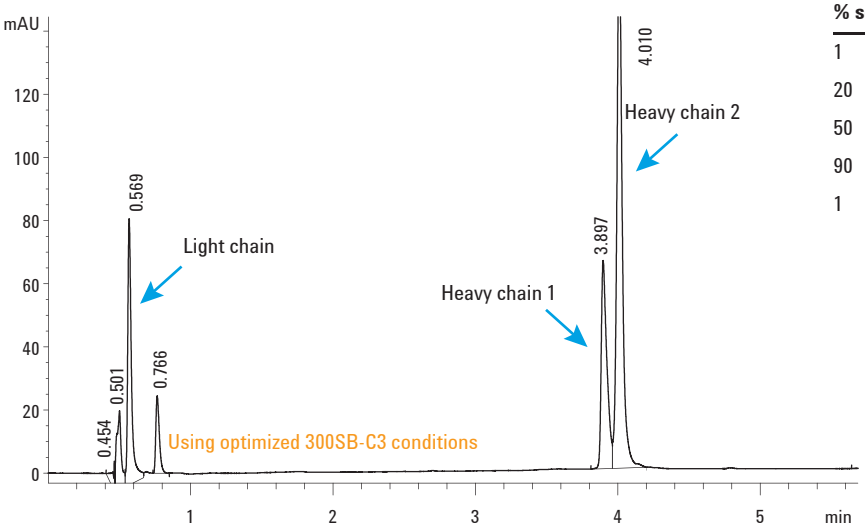
For consecutive chromatographic runs, a 2-minute post run was added to re-equilibrate the column.

Results

Gradient Optimizations for Ultra Fast Analysis of Reduced and Alkylated Monoclonal Antibody

The separation shown in Figure 1 details a high speed separation of reduced and alkylated CHO cell derived mAb. The Agilent ZORBAX RRHD 300SB-C3 2.1 × 100 mm column, 75 °C temperature, and optimized gradient enabled a highly resolved separation of the light chain and two heavy chain variants in under 4.5 minutes. The separation of the two heavy chain peaks, 1 and 2, display narrow band widths and are well resolved. The light chain peak at 0.6 minutes displays excellent separation from the surrounding light chain fragment peaks at 0.5 and 0.7 minutes. The optimized gradient for this separation is shown in Table 1.

Fast separation of reduced monoclonal antibody using an Agilent ZORBAX RRHD 300SB-C3 2.1 × 100 mm, 1.8 µm column



Columns	Agilent ZORBAX RRHD 300SB-C3, 2.1 × 100 mm, 1.8 µm
Sample	Reduced mAb (IgG1) (1.0 mg/ml)-BioCreative IgG1
Sample injection	2 µL
Mobile phase A	0.1% TFA in water
Mobile phase B	80% n-propyl alcohol, 10% ACN, 9.9% water and 0.1% TFA
Temperature	74 °C
Flow rate	0.5 mL/min
Detection	UV 280

Table 1. Agilent ZORBAX RRHD 300 SB-C3 Gradient Conditions

% solvent B	Time (min)
1	0
20	2
50	5
90	6
1	6.1

Figure 1. Rapid reduced monoclonal mAb separation achieved on an Agilent ZORBAX Rapid Resolution High Definition 300SB-C3, 2.1 × 100 mm, 1.8 µm at 75 °C, 0.5 mL/min and monitored at UV 280. Gradient conditions are defined in Table 1.

Optimizing Conditions for High Resolution and Fast Analysis of Intact mAbs from Different Cell Lines

Monoclonal antibodies derived from different cell lines were separated and optimized on an Agilent ZORBAX RRHD 300SB-C3 2.1 x 50 mm, 1.8-µm to demonstrate C3 selectivity towards different mAb expression. Under systematic gradient methods, we identified gradients for each mAb to deliver high speed separations with optimum resolution. The separation displayed in the top panel of Figure 2, was optimized for the Agilent standard antibody expressed from CDH media and using gradient conditions shown in Table 2A. The separation was completed in under 4 minutes and exhibits excellent resolution of the intact peak with a very narrow band width. In comparison, the bottom chromatogram shown in Figure 2 was optimized for the humanized mAb expressed from a CHO cell line using the gradient conditions shown in Table 2B. In this separation, the gradient was optimized with a shallower gradient curve that resolved a front shoulder from the mAb base peak. Both separations in Figure 2 were developed to facilitate high throughput run to run mAb characterization with high efficiency. Each separation finishes with a fast 90% isopropanol wash and rapid re-equilibration for enabling repeated injection sequences.

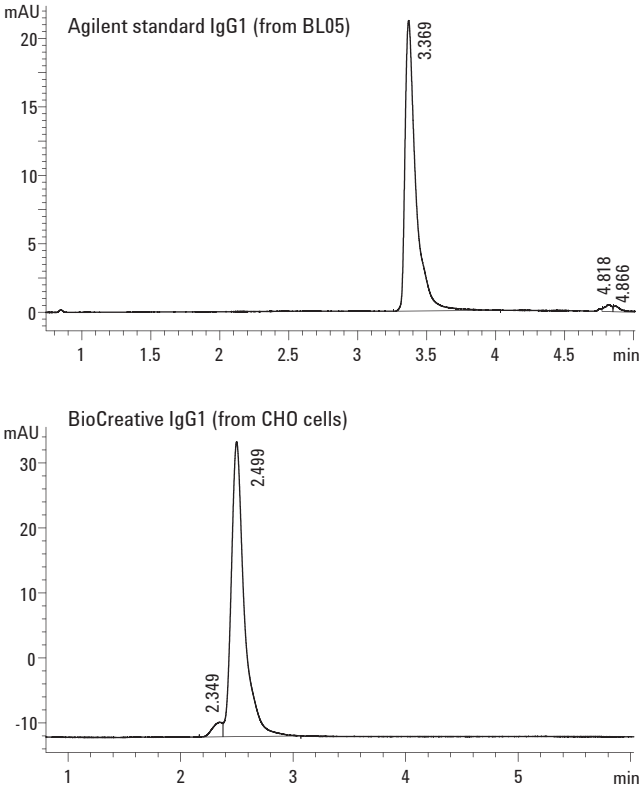
Table 2A Gradient for Agilent Std IgG1

% solvent B	Time (min)
10	0
25	2.5
35	4.5
90	4.56
90	5.0
10	6

Table 2B Gradient for BioCreative IgG1

% solvent B	Time (min)
5	0
25	5
25	7
90	8
5	9

Fast separation Intact IgG1 and their degradation products using an Agilent ZORBAX RRHD 300SB-C3, 2.1 × 50 mm, 1.8 µm column



Columns	Agilent ZORBAX RRHD 300SB-C3, 2.1 × 100 mm, 1.8 µm
Sample	monoclonal antibody (IgG1) 1.0 mg/mL Agilent Standard IgG1 (top) BioCreative IgG1(bottom)
Sample injection	2 µL
Mobile phase A	0.1% TFA in water
Mobile phase B	70% iso-propyl alcohol, 20% ACN, 10% water and 0.1% TFA
Temperature	74 °C
Flow rate	0.5 mL/min
Detection	UV 280

Figure 2. UHPLC separations optimized for two monoclonal antibodies on an Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm column. The separations were performed at 1.0 mL/min and 75 °C with iso-propanol/acetonitrile/water. The top panel in Figure 2 was optimized for an mAb expressed from CDH media, while the bottom chromatogram was optimized for a humanized mAb expressed by a CHO cell line. Each separation was followed with a fast 2-minute equilibration post run time.

Column Lifetime

An Agilent ZORBAX RRHD 300SB-C3 column was evaluated for lifetime at low pH during repeated injection sequences at 900 bar. Column packed bed stability, phase stabilization, and inlet frit performance are all critical for continued operation at elevated temperatures and pressures during repeated mAb analysis. To evaluate this performance, 1,000 repeated injections of Ribonuclease A, Cytochrome C, and Lysozyme were performed on a 2.1 x 50 mm column at a flow of 1.25 mL/min. A chromatographic view of this separation can be seen in Figure 3. During each lifetime run, the gradient peak width of the proteins was recorded, while backpressure was closely monitored. As shown in Figure 4A, 10 runs of peak width performance was plotted every 100th injection interval. The peak width performance remained stable during the 1,000 run sequence and column backpressure (Figure 4B) remained unchanged at 900 bar. Maintaining reproducible peak widths and efficiency, while limiting column backpressure increases, are indicators of an optimally packed column bed with excellent resilience to high flow, low pH and repeated high pressure operation. Additionally, maintaining excellent flow dynamics during the lifetime analysis ensured high tolerance to inlet frit plugging from protein or system micro-particulates.

Fast separation standard proteins and their degradation products using Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm, 1.8 µm column

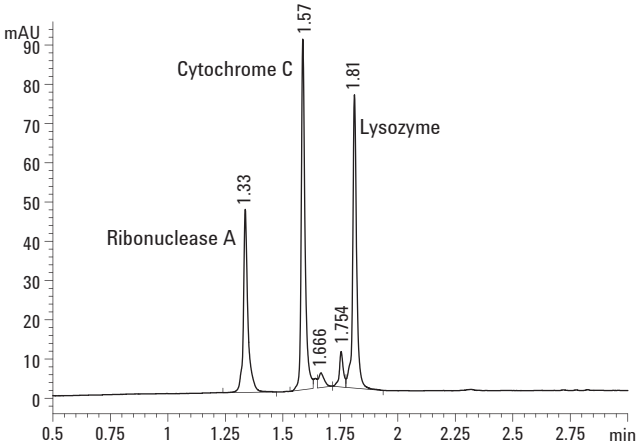


Figure 3. High speed separation of Ribonuclease A, Cytochrome C and Lysozyme for lifetime stability monitoring using an Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm. The chromatographic conditions are described in Table 2.

Column	Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm, 1.8 µm (p/n 857750-909)	
Sample	Ribonuclease A, Cytochrome C and Lysozyme (3 mg/mL)	
Sample injection	1 µL	
HPLC instrument	Agilent 1290 Infinity Series	
Detection	UV 280	
Mobile phase A	H_20 + 0.1% TFA (v/v)	
Mobile phase B	ACN + 0.1% TFA (v/v)	
Flow	1.25 mL/min	
Injection	1 µL (1 mg/mL)	
Temperature	ambient	
Gradient	% Solvent B	Time (min)
	10	0
	70	2.5
	90	2.6
	90	3.0
	10	5.0

Life test of Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm, 1.8 µm

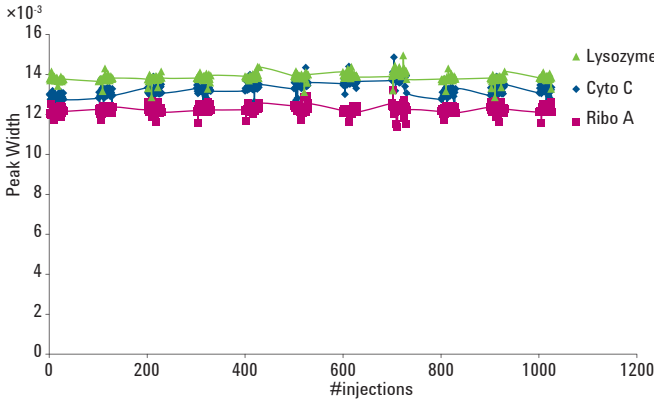


Figure 4A. Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm lifetime plot at 900 bar backpressure and high flow (1.25 mL/min). The graph displays a continuous series of protein peak width recordings of Ribonuclease A, Cytochrome C, and Lysozyme plotted during every 100th run interval over the course of 1,000 injections.

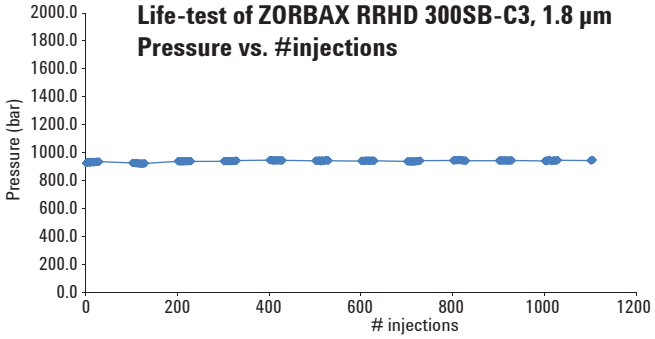
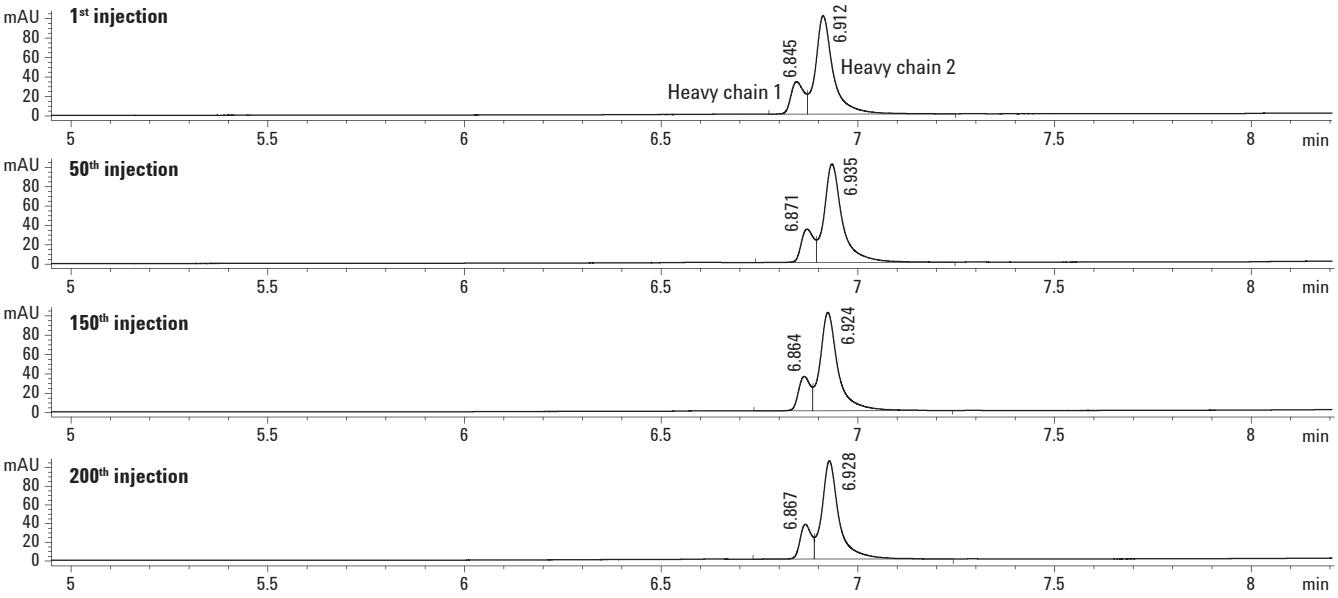


Figure 4B. Agilent ZORBAX RRHD 300SB-C3, 2.1 × 50mm, pressure plot. Consecutive column backpressure readings were recorded and plotted at every 100-injection interval.

Column Reproducibility

An Agilent ZORBAX RRHD 300SB-C3 column reproducibility was examined during 200 repeated injection of a reduced mAb sample. Using a 2.1 x 50 mm column two reduced heavy chain variants were separated, and evaluated for retention time and resolution. As shown in Figure 5, the results of these separations detail excellent run-to-run column reproducibility during continued exposure to 75 °C and low pH. The mAb heavy chain 1 and 2 peaks shown at the 1 st, 50 th, 150 th and 200 th run, maintained retention and peak shape delivering consistent separation performance without any indications of peak deterioration or efficiency loss.

Column reproducibility - 200 injections of reduced monoclonal antibody using Agilent ZORBAX RRHD 300SB-C3, 2.1 × 100 mm, 1.8 µm column



Columns	Agilent ZORBAX RRHD 300SB-C3, 2.1 × 100 mm, 1.8 µm
Sample	Reduced monoclonal antibody (IgG1) (1.0 mg/ml)- Agilent BL05 IgG1
Sample injection	2 µL
Mobile phase A	0.1% TFA in water
Mobile phase B	80% n-propyl alcohol, 10% ACN, 9.9% water and 0.1% TFA
Gradient	0 min–1% B, 2 min–20% B, 5 min–50% B, 7 min–50% B, 8.0 min–90% B, 8.3 min–1% B hold for 2 min
Temperature	75 °C
Flow rate	0.4 mL/min
Detection	UV 280

Figure 5. Reduced and alkylated mAb profiling during 200 repeated injections using an Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm column at 75 °C. Sequence runs 1, 50, 150, and 200 are shown. Mobile phase: A: water (0.1% TFA), B: 80/10/10 n-propanol/ACN/ water (0.1% TFA). Gradient: 0 min–1% B, 2 min–20% B, 5 min–50% B, 7 min–50% B, 8.0 min–90% B, 8.3 min–1% B hold for 2 min.

Conclusions

The Agilent ZORBAX RRHD 300SB-C3, 1.8 µm column provided ultra fast and efficient separation of monoclonal antibodies. Optimized chromatographic conditions in combination with the ZORBAX RRHD 300 column technology, enabled rapid and high resolution separation of both intact and reduced mAb's expressed from different sources. The gradient conditions and compositions were systematically optimized to provide complete separation, washing and re-equilibration in a reduced analysis time and demonstrated utility for high throughput mAb characterizations.

ZORBAX RRHD 300SB-C3 column stability (lifetime) and reproducibility were also demonstrated during repeated injection analyses. Throughout 1,000 injections at 900 bar and low pH, the column efficiency performance remained stable and showed excellent tolerance to backpressure increases from bed instability or frit plugging. Additionally, during 200 consecutive runs of two heavy chain mAb variants, the separations displayed consistent peak shape and resolution while they maintained retention position.

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Ultra High Speed and High Resolution Separations of Reduced and Intact Monoclonal Antibodies with Agilent ZORBAX RRHD Sub-2 µm 300 Diphenyl UHPLC Column

Application Note

Biopharma

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Abstract

Rapid separations of intact and reduced monoclonal antibodies (mAbs) were achieved with the use of an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300 diphenyl reversed-phase column and optimized chromatographic conditions. The unique diphenyl phase and robust rapid resolution high definition column technology, in combination with optimized gradient conditions, provided ultra fast separations and delivered excellent peak shapes demonstrating utility for high throughput mAb characterizations. Monoclonal antibodies expressed by both Chinese Hamster Ovary and CDH media cell lines were evaluated and compared with the goal of obtaining high resolution and high efficiency separations during rapid run times. The ZORBAX RRHD 300 diphenyl was also evaluated at elevated operating pressures and temperature, and exhibited high operational tolerance during continuous investigations of reproducibility and lifetime.

Agilent Technologies

Introduction

Drug development of biotherapeutics is rapidly growing among the pharmaceutical industry and reliable antibody drug characterization is an important challenge to this development pipeline. Although antibodies can be characterized by many separation techniques, separation by reversed-phase chromatography has been rapidly growing due to the introduction of more efficient and desirable chromatographic materials such as those offered by sub 2 µm columns and the newer phase chemistries.

In this work, we have achieved ultra high resolution separation of intact and reduced monoclonal antibodies (mAbs) during a rapid run time, and demonstrate different selectivity beyond traditional C18, C8, and C3 for mAb characterization. Specifically, we systematically optimized the gradient conditions at elevated temperatures to deliver rapid separation of intact mAb's and reduced, light, and heavy chain mAb variants. The goal of these investigations focused on ultra fast and efficient run-to-run method optimizations that eliminated long equilibration times or, extensive post run washings. We have also evaluated the ZORBAX RRHD 300 diphenyl column for longevity and reproducibility. To evaluate longevity, the columns were tested at elevated operating pressures (> 900 bar) and temperature (75 °C), low pH and high flow for 1,000 injections with a protein standard mix. Run-to-run reproducibility and retention behavior was examined during 200 runs using an mAb standard.

Experimental

Materials

Two humanized monoclonal antibody lines were used in this study. One line was expressed at Agilent using CDH media (p/n 010774) and the other was expressed from a Chinese hamster ovary (CHO)-cell derived monoclonal antibody purchased from Creative Biolab, Pennsylvania. Triflouroacetic acid was purchased from Sigma-Adrich, St. Louis, MO, and iso-propanol, n-propanol, and acetonitrile were supplied from Honeywell-Burdick & Jackson, Muskegon, MI. The 1-propanol was purchased from VWR (p/n BJ322-4) The dialysis cassettes had a 3,500 MWCO and were purchased from Thermo Scientific (p/n 66330).

Reduction and Alkylation

Reduction and alkylation was performed under denaturing conditions using guanidine hydrochloride (GuHCl) to produce the free light and heavy chains. Antibody in the amount of 0.5 mL (1.5 mg/mL) was dialyzed with water for preservative removal. Once dialyzed, a 0.5-mL aliquot was diluted to a final concentration of 0.75 mg/mL with 100 mM TRIS-HCl, and 4 M GuHCl (Mallinckrodt, Phillipsburg, NJ, USA). The pH was adjusted to 8.0 and 10 µL of 0.5 M dithiothreitol (DTT, Sigma) stock solution was added for a final concentration of 5 mM. The mixture was placed in a 37 °C water bath and incubated for 30 minutes. The antibody was briefly cooled to room temp and a 26-µL aliquot of a 0.5 M iodoacetamide (IAM, Sigma) stock solution was added for a final concentration of 13 mM. The alkylated antibody solution was placed in the absence of light at room temperature for 45 minutes. Once removed, the mixture was quenched with 20 µL of 0.5 M DTT for a final concentration of 10 mM. Then 1.0 mL of reduced and alkylated antibody was desalted through a 4 mL 3.5 K MWCO concentrator (p/n 5185-5991) at 3,800 RPM for 30 minutes using water (0.1% TFA). The concentrating process was repeated two times to a final volume of 0.5 mL (1.5 mg/mL).

UHPLC Conditions

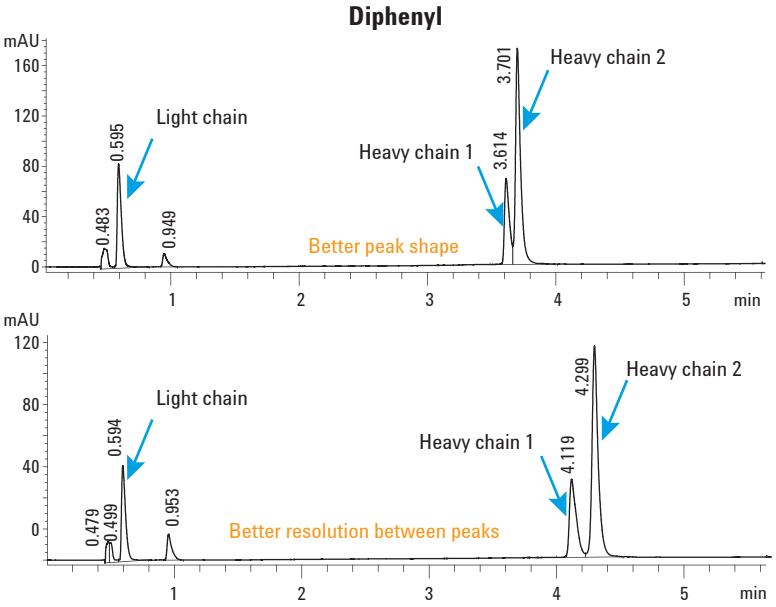
Instrument	Agilent 1290 LC Infinity system with auto injector (ALS), binary pump, thermostatted oven (TLC), and diode array detector (DAD)
Column	Agilent ZORBAX Rapid Resolution High Definition 300 diphenyl, 1.8 µm 2.1 × 100 mm, (p/n 858750-944) 2.1 × 50 mm, (p/n 857750-944)
Mobile phase	(intact mAb) A. 98/2 water/iso-propanol (0.1% TFA) B. 70/20/10 iso-propanol/ACN/water (0.1% TFA)
Mobile phase	(reduced mAb) A. H_20 + 0.1% TFA (v/v) B. 80/10/10 n-propanol/ACN/water (0.1% TFA)
Injection	1–3 µL
Flow rates	0.5 mL/min (reduced) 1.0 mL/min (intact) 1.25 mL/min (lifetime testing)
Gradient	multisegmented
Temperature	75 °C
Detection	UV, 280 nm

For consecutive chromatographic runs, a 2-minute post run was added to re-equilibrate the column.

Results

Gradient Optimizations for ultra fast Analysis of Reduced Monoclonal Antibody

The chromatographic comparisons in Figure 1 show two optimized high speed separations of reduced and alkylated monoclonal antibody. The ZORBAX RRHD 300 diphenyl, 2.1 × 100 mm, column and chromatographic conditions enabled well resolved separations of the reduced mAb light chain and two heavy chain variants. The top panel chromatogram in Figure 1 detail a separation with narrow bands and high resolution of the heavy chains achieved using the gradient conditions shown in Table 1A. In comparison, the separation displayed in the bottom panel of Figure 1 has been optimized for obtaining better resolution of the heavy chains, but with a slight increase in peak width. In this separation, the two heavy chains display near baseline resolution. The optimized conditions for this separation are shown in Table 1B. In contrast between the two separations, the diphenyl phase enabled enhanced separation control for resolving the two heavy chain peaks with minor changes to the gradient slopes. Additionaly, we have observed less dramatic effects towards improving this resolution when the same dimension C3 and C8 columns were used under these identical conditions, suggesting the diphenyl offers a unique selectivity advantage towards this particular antibody beyond the traditional short chain phase chemistries.



Columns	Agilent ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm
Sample	Reduced monoclonal antibody (IgG1) (1.0 mg/ml)-BioCreative IgG1
Sample injection	2 µL
Mobile phase A	0.1% TFA in water
Mobile phase B	80% n-propyl alcohol, 10% ACN, 9.9% water and 0.1% TFA
Gradient	1st condition: 0 min–1% B, 2 min–20% B, 5 min–70% B
	2nd condition: 0 min–1% B, 2 min–20% B, 5 min–50% B
Flow rate	0.5 mL/min
Temperature	74 °C
Detection	UV 280

Figure 1. Comparison of two ultra-fast separations of reduced monoclonal antibodies achieved on a Agilent ZORBAX Rapid Resolution High Definition 300 diphenyl (2.1 × 100 mm) under different optimized gradient conditions. The top panel separation delivered narrow peak widths with shorter retention times. The bottom panel separation displays higher resolution between the two heavy chain peaks, but with less efficiency.

Table 1A Gradient A

% Solvent B	Time (min)
1	0
20	2
70	5
90	5.1
1	7

Table 1B Gradient B

% Solvent B	Time (min)
1	0
20	2
50	5
90	5.1
1	7

Optimizing Conditions for Ultra High Resolution and Fast Analysis of Intact mAb's

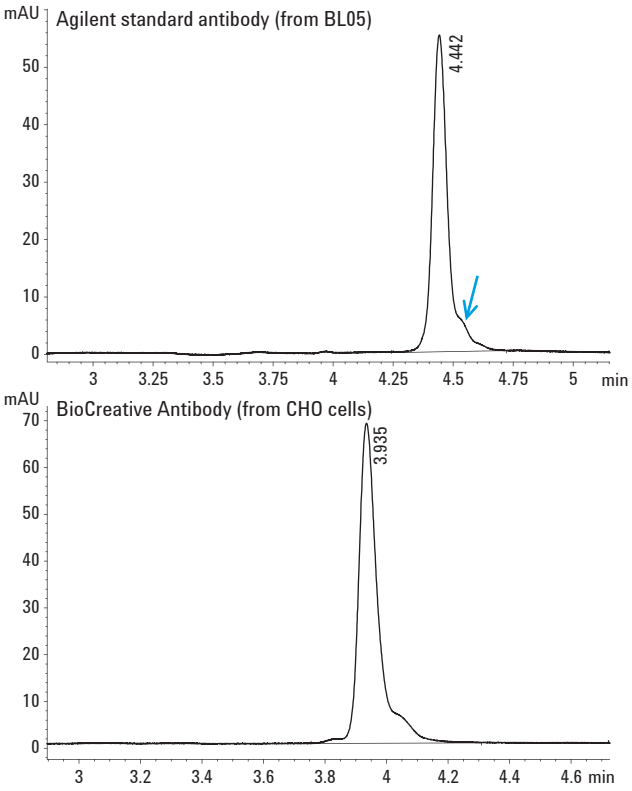
To evaluate separation performance of an intact monoclonal antibody on the ZORBAX 300 diphenyl (2.1 × 50 mm), two mAbs were selected and individually optimized for speed and resolution. Under systematic gradient investigations we identified optimized gradients for each mAb to highlight diphenyl resolving power towards fast intact fast mAb separations. The separation displayed in the top panel of Figure 2, was optimized for the Agilent standard antibody which was expressed from CDH media using gradient conditions shown in Table 2A. The separation was completed in less than 5 minutes and exhibits excellent resolution of the intact peak and shoulder peak identified at 4.5 minutes (arrow). In comparison, the bottom chromatogram shown in Figure 2 was optimized for the mAb expressed from a CHO cell line using gradient conditions shown in Table 2B. In this separation, the gradient was optimized using a slightly modified gradient curve to deliver enhanced resolution at the front and back of the intact mAb peak. In addition to obtaining high resolution-high speed run times, both separations were developed to facilitate run-to-run mAb profiling. Each separation completes with a fast 90% iso-propanol wash and rapid re-equilibration for repeated high throughput injection sequences.

Table 2A Gradient

% Solvent B	Time (min)
15	0
25	2.5
35	4.5
35	4.9
90	5.0
90	5.5
15	6.0

Table 2B Gradient

% Solvent B	Time (min)
10	0
25	2.5
35	4.5
90	4.56
90	5.0
10	6.0

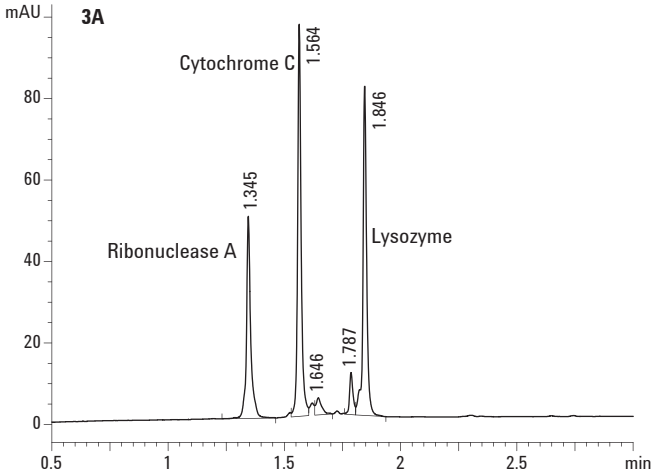


Columns	Agilent ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm
Sample	monoclonal antibody (1.0 mg/mL)- BioCreative and Agilent Standard IgG1
Sample injection	2 µL
Mobile phase A	98/2 water/iso-propyl (0.1% TFA)
Mobile phase B	70/20/10 iso-propyl/ACN/water (0.1% TFA)
Flow rate	1.0 mL/min
Temperature	74 °C
Detection	UV 280

Figure 2. UHPLC separations optimized for two sources of monoclonal antibodies on a 2.1 × 50 mm Agilent ZORBAX RRHD 300SB-diphenyl column. The separations were performed at 1.0 mL/min and 75 °C with %B compositions of 70/20/10 iso-propanol/acetonitrile/water (0.1% TFA). The top panel in Figure 2 was optimized for an mAb expressed from CDH media, while the bottom chromatogram was optimized for a humanized mAb expressed from a CHO cell line. Each separation was completed with a fast 2-minute equilibration post run time.

Column Lifetime

Column packed bed stability and inlet frit performance is critical for continued operation at elevated temperatures and pressures during repeated mAb analysis. ZORBAX RRHD 300 diphenyl column lifetime was evaluated for ruggedness at low pH during repeated injection sequences at 900 bar under the conditions displayed in Table 3. Repeated injections totaling 1,000 of a protein standard mix comprising of Ribonuclease A, Cytochrome C and Lysozyme were performed on a 2.1 × 50 mm column at a flow of 1.25 mL/min. A chromatographic view of this separation can be seen in Figure 3A and details a fast run time under 2 minutes to facilitate the lifetime analysis. During each run, the gradient peak width of each protein was recorded while backpressure was closely monitored. As shown in Figure 3B, 10 runs of peak width performance was plotted at every 60th injection interval. The peak width performance remained stable during the 1,000 injection sequence, while the column backpressure remained unchanged at 900 bar. Steady peak width efficiency and a stable pressure curve indicate an optimally packed column bed with excellent resilience to high flow (relative to 2.1 mm column id), low pH and continuous operation at high pressure. Additionally, after extensive protein injection the ZORBAX RRHD 300 diphenyl column maintained excellent frit flow with high tolerance to inlet plugging from protein or system microparticulates.



Samples	Diphenyl RT (min)	Diphenyl "plates"
Ribonuclease A	1.35	50816
Cytochrome C	1.56	63533
Lysozyme	1.85	92272

Figure 3A. High speed separation of Ribonuclease A, Cytochrome C and Lysozyme for Lifetime stability monitoring using a 2.1 × 50 mm ZORBAX RRHD 300-diphenyl. The separation conditions are described in Table 3.

Table 3

Column	Agilent ZORBAX RRHD 300 2.1 × 50 mm, 1.8 µm	
Sample	Ribonuclease A, Cytochrome C and Lysozyme (3 mg/mL)	
Mobile phase A	H_20 + 0.1% TFA (v/v)	
Mobile phase B	ACN + 0.1% TFA (v/v)	
Flow	1.25 mL/min	
Injection	1 µL (1 mg/mL)	
Temp	ambient	
Gradient	% Solvent B	Time (min)
	10	0
	70	2.5
	90	2.6
	90	3.0
	10	5.0
HPLC instrument	Agilent 1290 Infinity Series	
Detection	UV 280	

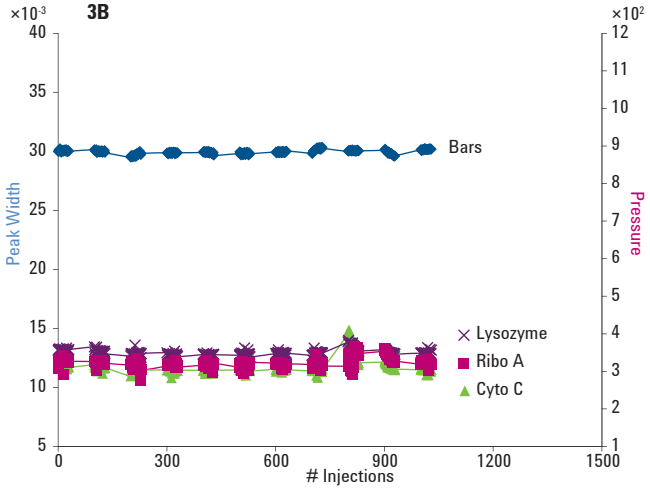
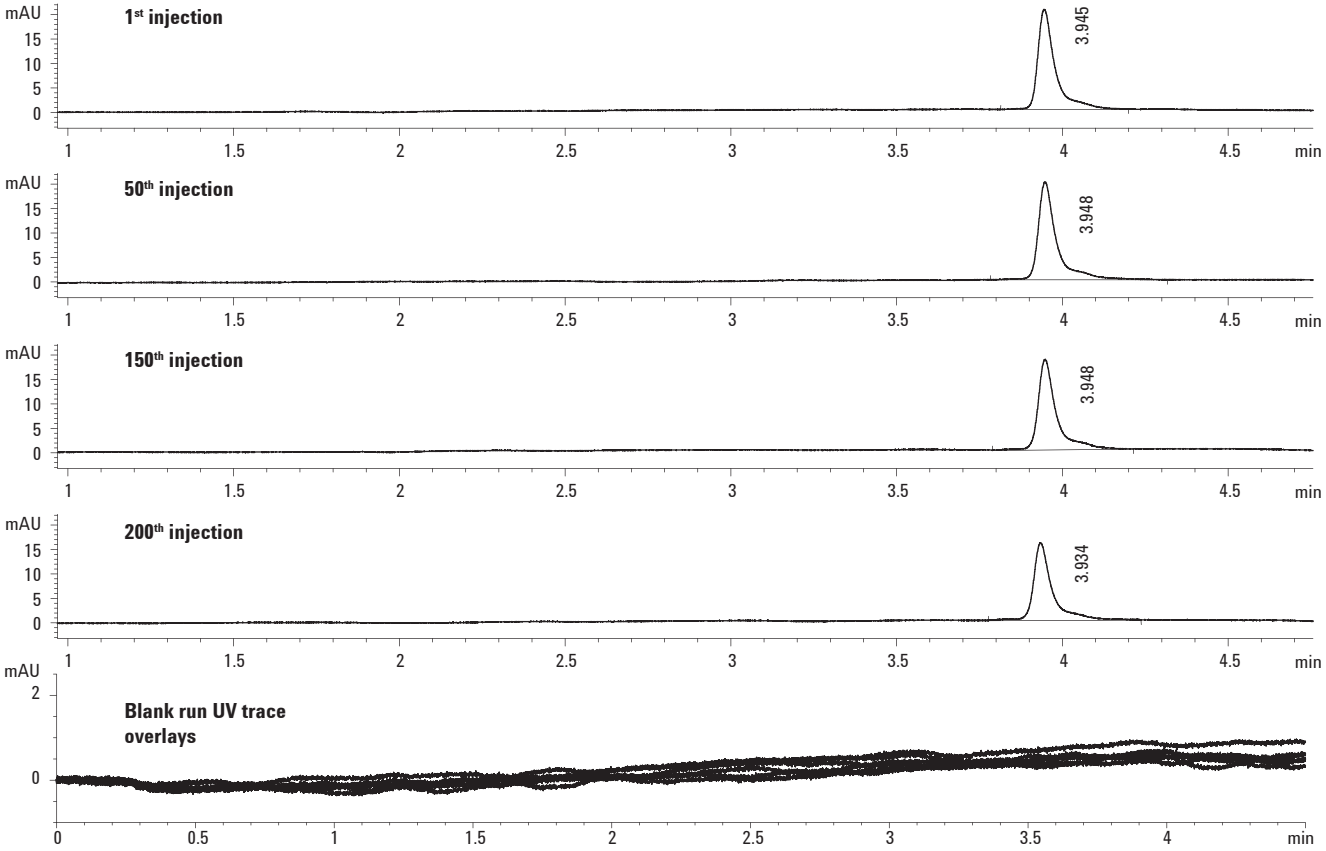


Figure 3B. Agilent ZORBAX RRHD 300-diphenyl 2.1 × 50 mm lifetime plot detailing column lifetime stability curves at elevated pressure and high flow (1.25 mL/min). Ten consecutive protein peak widths of Ribonuclease A, Cytochrome C, and Lysozyme were plotted every 60th run interval during 1,000 injections. Column backpressure was recorded during the intervals and plotted versus the number of injections as shown.

Column Reproducibility

Column reproducibility was examined during repeated injection of an intact humanized mAb during 200 runs. Using a new 2.1 × 50 mm ZORBAX RRHD 300-diphenyl column, run-to-run reproducibility was performed under the gradient compositions and conditions described in Table 2A. To evaluate column reproducibility, and post run recovery, 200 consecutive injections were performed with column blanks collected during every 20th run to monitor the carryover effects from multiple protein injections. The defined separation conditions resulted in repeated high pressure column operation (>700 bar), continued exposure to 75 °C operating temperature and low pH. As detailed in Figure 4, repeated separations of the intact mAb were highly reproducible maintaining retention time and peak shape. Additionally, the post run blanks gave no evidence of run to run mAb carryover (Figure 4 bottom panel).



Columns	Agilent ZORBAX RRHD 300 diphenyl, 2.1 × 100 mm, 1.8 µm
Sample	monoclonal antibody (IgG1) (1.0 mg/mL)– BioCreative IgG1 and Agilent Standard IgG1
Sample injection	1 µL
Mobile phase A	0.1% TFA in water
Mobile phase B	80% n-propyl alcohol, 10% ACN, 9.9% water and 0.1% TFA
Flow rate	1.0 mL/min
Temperature	74 °C
Detection	UV 280

Figure 4. Details intact mAb profiling during 200 repeated injections using an Agilent ZORBAX RRHD 2.1 × 50 mm 300-diphenyl column at 75 °C. Intact mAb separations shown were collected at 1, 50, 150, and 200th run intervals. The bottom panel displays 5 UV blank run trace overlays collected every 20th run during the column evaluation (note: overlay traces are scaled to 2 mAu). Gradient conditions for the separations are provided in Table 2B.

Conclusions

The Agilent ZORBAX RRHD 300 diphenyl, 1.8 µm, column provided ultra fast and efficient separation of intact and reduced monoclonal antibodies (mAbs) and demonstrated utility for high throughput mAb screening. Through systematic optimization of gradient conditions, tailored to antibody type, high resolution separations of intact and reduced mAb isoforms were achieved in under 5 minutes. The unique diphenyl phase and RRHD column technology enabled robust and repeatable separations while delivering excellent peak shapes, and displayed a unique selectivity advantage during gradient separations than those observed with traditional C3 and C8 phases.

ZORBAX RRHD 300 diphenyl column stability (lifetime) and reproducibility were also demonstrated during repeated injection analyses. During 1,000 injections at 900 bar and low pH, column efficiency performance remained stable and showed high tolerances to backpressure increases from bed instability or frit plugging. Additionally, during 200 consecutive runs at 700 bar, the separations maintained peak shape, resolution and retention position while column blank runs gave no indications of peak carryover or UV trace ghosting.

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Agilent Technologies

Proof of Performance

Determination of low-metal release from the Agilent 1260 Infinity Bio-inert Quaternary LC system using ICP-MS

Technical Overview

Authors

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Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

Proteins are chelating agents and especially iron is often found in protein-metal complexes. The Agilent 1260 Infinity Bio-inert Quaternary LC system is based on the stainless-steel Agilent 1260 Infinity Quaternary LC system, but comprises new metal-free components in the sample flow-path to ascertain the integrity of bio-molecules. The bio-inertness of the Agilent 1260 Infinity Bio-inert Quaternary LC system was investigated by determining the metal content of different, system passing eluents using inductively-coupled-plasma mass-spectrometry (ICP-MS). The results were compared with the Agilent 1260 Infinity LC system and with a bio-inert system from another vendor. The Agilent 1260 Infinity Bio-inert Quaternary LC system releases less metals using acidic, basic, and salt containing buffers compared to the Agilent 1260 Infinity LC system and to the bio-inert LC system from the other vendor.

Agilent Technologies

Introduction

Bio-chromatographers are faced with two challenges when using a traditional stainless-steel HPLC system:

- First, biological samples such as proteins might interact with the sample flow path and, therefore, lead to a lower protein yield.
- Second, the liquid chromatography system must be able to withstand harsh cleaning procedures (such as cleaning in place with HCl) which are applied to remove potential contaminations in Bio-analysis.

As some proteins are chelating agents, bio-inertness of the system is essential for the accuracy of, for example, protein recovery. Especially the metal ions Fe^{2+} and Fe^{3+} are often found in protein-metal complexes such as in iron-containing oxygen-transporting Hemoglobin in the red blood cells of all vertebrates.

The Agilent 1260 Infinity Bio-inert Quaternary LC system is based on the stainless-steel Agilent 1260 Infinity Quaternary LC system, but it comprises new metal-free components in the sample flow-path to ascertain the integrity of bio-molecules. The absence of iron and steel in solvent delivery minimizes unwanted surface interactions and increases column lifetime. All capillaries and fittings throughout the autosampler, column compartment, and detectors are completely metal-free so that the bio-molecules come in contact only with ceramics or PEEK. In front of the sample path, the Agilent 1260 Infinity Bio-inert Quaternary Pump is assembled using only bio-inert metals (titanium, gold, and platinum-iridium).

Due to the iron and steel-free design, the system has a higher salt tolerance and a wider pH range (1–13, short term 14) than a standard system. Based on the proven technology of the Agilent 1200 Infinity Series liquid chromatography platform, the 1260 Infinity Bio-inert Quaternary LC has the same performance specifications as the standard 1260 Infinity LC system – resulting in compatibility with standard methods[1].

To verify low-metal release from the 1260 Infinity Bio-inert Quaternary LC, different eluents were run through the system and the metal content of the eluents was measured by ICP-MS after the system passage. In parallel, the same procedure was conducted on a 1260 Infinity LC stainless steel system and a bio-inert LC system from another vendor.

Experimental

Systems

1. Agilent 1260 Infinity LC system consisting of:
- Agilent 1260 Infinity Binary Pump (G1312B)
- Agilent 1260 Infinity High performance Autosampler (G1392B)
- Agilent 1260 Infinity DAD (G4212B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316A)

2. Agilent 1260 Infinity Bio-inert Quaternary LC system consisting of:
- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High performance Autosampler (G5667A)
- Agilent 1260 Infinity DAD (G4212B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)

3. Bio-inert LC system from other vendor

4. Agilent 7700x ICP MS system

Software

Agilent MassHunter ICP-MS, B 1.02

Eluents

- Double-distilled water (ddH_2O)
- Acetonitrile/ddH_2O → 50:50
- 0.1% Trifluoroacetic acid (TFA) in ddH_2O
- 0.1% Formic acid (FA) in ddH_2O
- 100 mM Sodium hydroxide (NaOH)
- Phosphate buffer (150 mM sodium phosphate, 150 mM sodium chloride (NaCl))

The same bottle of each eluent was used for all systems, and blanks of all eluents were analyzed before the system was flushed with the respective eluent. All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). Acetonitrile (LiChrosolv) and NaCl were purchased from Merck KGaA, Darmstadt, Germany. TFA (Reagent Plus 99%), FA (98% to 100% puriss. p.a.), NaOH (Standard Solution, 1M), and sodium mono- and diphosphate (99%) were purchased from Sigma-Aldrich, St.Louis, USA.

The HPLC system was flushed with each eluent for 10 minutes at a flow rate of 1 mL/min. Then the mobile phase was collected from 10–20 minutes, 20–30 minutes, and from 30–40 minutes, resulting in three samples per eluent, per system, plus blank.

With ICP-MS, the following metals were determined in the different eluents:

- Titanium
- Chromium
- Manganese
- Iron
- Cobalt
- Nickel
- Copper
- Zinc
- Zirconium
- Molybdenum

Results and Discussion

Table 1 shows the amount of metals in the eluents before (→ blanks) and after flowing through the three different LC systems used. The blank amounts are already subtracted in the results.

Summing up the results of the six different solvents used, ddH_2O and 50% ACN were the two solvents with the smallest impact concerning metal leaching out of the LC system. Small amounts of iron and manganese were detected in the eluents, which had been running through an Agilent 1260 Infinity LC system. Chromium, copper, and nickel were found in the bio-inert system from the other vendor.

< 1 µg/L < 10 µg/L > 10 µg/L

	Blanks	Agilent 1260 Infinity LC	Bio-inert system from other vendor	Agilent 1260 Infinity Bio-inert LC
H_2O_{dd}	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo
ACN/ H_2O_{dd}	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo
0.1 % FA	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo
0.1 % TFA	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo
100 mM NaOH	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo
Buffer (150 mM NaPO4, 150 mM NaCl)	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo	Ti Cr Mn Fe Co Ni Cu Zn Zr Mo

Table 1
Metal content of different eluents, run through three different LC systems (Agilent 1260 Infinity LC, Agilent 1260 Infinity Bio-inert LC, and a bio-inert system from another vendor). The blank amounts are already subtracted in the results.

Regarding acidic buffers, 0.1% TFA had a greater impact on the metals eluting from the systems. More and higher amounts of metals could be detected in these eluents. Iron is, in marginal amounts (0.3%), part of the titanium alloy used in the Agilent 1260 Infinity Bio-inert Quaternary LC system. Due to the highly corrosive abilities of TFA, small amounts of iron were found in the eluents passing the 1260 Infinity Bio-inert Quaternary LC system and also in the bio-inert system from the other vendor. However, the amount of iron, found in the Agilent 1260 Infinity LC system, was 10 times higher. Titanium was found in both bio-inert systems in higher amounts after acidic, basic, and salt-containing eluents, due to the high content of titanium, used in the pump modules. Though, titanium is considered a bio-inert material with high corrosion resistance and excellent biocompatibility.[2]

When chemicals, for example, salts, are part of the eluents, the blanks revealed already certain amounts of metals, particularly iron. Especially the use of sodium phosphate and sodium chloride made ICP-MS measurements difficult due to the high metal content of the salts.

Conclusion

The Agilent 1260 Infinity Bio-inert Quaternary LC system releases an almost negligible amount of metals when flushed with acidic, basic, and salt containing buffers. It has significantly lower release compared to the Agilent 1260 Infinity LC stainless-steel system and to the bio-inert LC system from the other vendor. We conclude that it is, therefore, highly recommended for bio-inert UHPLC applications.

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www.agilent.com/chem/bio-inert

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Agilent Technologies

Proof of Performance

Analysis of compounds in mobile phases with high pH

Feasibility of the Agilent 1260 Infinity Bio-inert Quaternary LC system for generic high pH applications

Application Note

Chemical and Pharmaceutical Analysis

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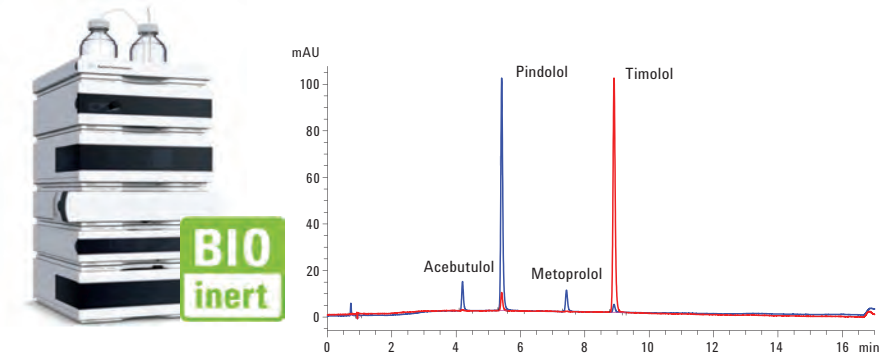

Abstract

In this Application Note, the feasibility of the Agilent 1260 Infinity Bio-inert Quaternary LC system for generic high pH applications is demonstrated. The iron and steel-free design enables the user to work in a wider pH range (1–13, and up to 14 for short periods) compared to the stainless-steel based Agilent 1260 Infinity LC system. Retention time and resolution stability over a time period of 20 hours are demonstrated for the analysis of four ß-blockers, serving as examples for small molecules. A tryptic BSA digest served as an example for biomolecules and showed retention time stability over a time period of 37.5 hours. This confirms that high pH mobile phases (pH 10 to 11) have no adverse effects on the stability of retention time or resolution for the analyzed samples over extended periods.

Agilent Technologies

Introduction

Performing high pH liquid chromatography in pH ranges above 10 puts stainless-steel systems at high risk of damage after long-term use due to corrosion effects. It is, therefore, highly recommended to use an iron-free system that is not deteriorated by aggressive agents used in high pH applications.

The Agilent 1260 Infinity Bio-inert Quaternary LC consists of metal-free components in the sample flow path. All capillaries and fittings throughout the autosampler, column compartment, and detectors are completely metal-free so that bio-molecules interact only with ceramics or PEEK. This assembly type (iron and steel-free design) enables the user to deploy not only buffers containing high amounts of salt but also to work in a wider pH range (1–13, and up to 14 for short periods) compared to the Agilent 1260 Infinity LC system. Further, it has been already proven that the 1260 Infinity Bio-inert Quaternary LC system releases less metal ions in presence of high pH eluents (for example 100 mM NaOH) compared to the 1260 Infinity LC system[1].

In this Application Note, the feasibility of the 1260 Infinity Bio-inert Quaternary LC system for generic high pH applications is demonstrated. ß-Blockers, as examples for small molecules and a peptide mix (tryptic BSA digest) as an example for bio-molecules were used as samples, analyzed at high pH[2,3]. The influence of high pH eluents on the stability of retention time and resolution was investigated.

Experimental

The Agilent 1260 Infinity Bio-inert Quaternary LC system consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High Performance Bio-inert Autosampler (G5667A)
- Agilent 1260 Infinity DAD VL (G1315D with bio-inert standard flow cell, 10 mm)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)

Column

Agilent ZORBAX Extend-C18, 4.6 × 100 mm, 1.8 µm, 600 bar

Software

Agilent OpenLAB CDS, ChemStation Edition for LC & LC MS Systems, Rev. C.01.02 [14]

Eluents and samples

1. 50 mM triethylamine (TEA, **pH 11**) and methanol, for the analysis of four ß-blockers:
   - Acebutulol
   - Pindolol
   - Metoprolol
   - Timolol
2. 15 mM ammonium hydroxide (NH_4OH, **pH 10**) and acetonitrile for peptide analysis

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). TEA, NH_4OH and the ß-blockers were purchased from Sigma-Aldrich, St.Louis, USA.

Chromatographic method

	ß-Blockers	Peptide mix (Tryptic BSA digest)
Solvents:	A: 50 mM TEA, pH 11 B: Methanol	A: 15 mM NH_4OH, pH 10 B: 90% Acetonitrile, 15 mM NH_4OH, pH 10
Gradient:	0 min – 45% B 15 min – 80% B 15.01 min – 95% B	0 min 5% B 25 min – 30% B 30 min – 100%
Stop time:	17 min	35 min
Post time:	3 min	10 min
Temperature:	35 °C	35 °C
Flow rate:	1 mL/min	1 mL/min
Injection volume:	3 µL	3 µL
DAD:	260 nm, reference 360 nm 300 nm, reference 400 nm	214 nm, reference 400 nm
Peak width:	0.025 min (0.5 s response time) (10 Hz)	<0.013 min (0.13 s response time) (20 Hz)

Results and Discussion

High pH Analysis of Small Molecules

Four different ß-blockers were analyzed using TEA as basic aqueous buffer (pH 11) and methanol as organic mobile phase. Figure 1 shows the chromatogram after detection by two different wavelengths. The first three ß-blockers (Acebutulol, Pindolol and Metoprolol) were detected at 260 nm and Timolol at 300 nm. RSD of retention time was < 0.2% for all four peaks.

Retention time stability was monitored over a time period of 20 hours and remained stable from 0 to 1,200 minutes, see Figure 2.

Resolution stability for Pindolol, Metoprolol and Timolol was monitored over a time period of 20 hours and remained stable from 0 to 1,200 minutes, see Figure 3.

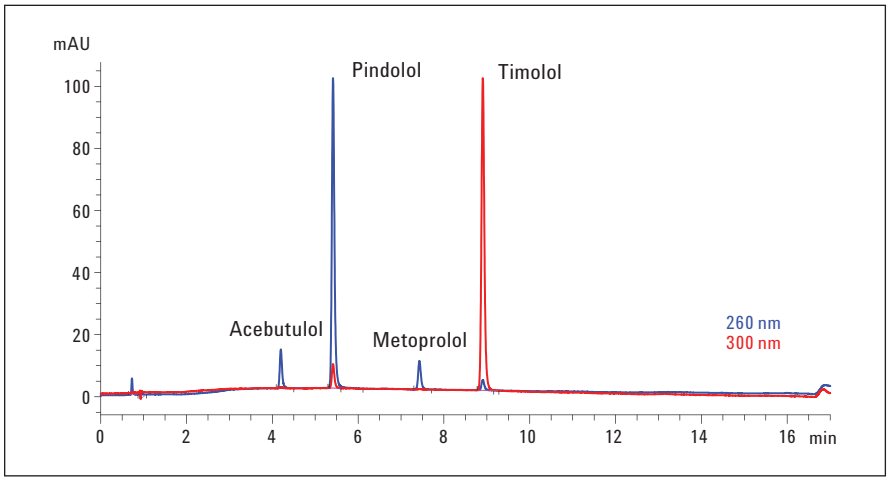

Figure 1
LC chromatogram of the four ß-blockers, analyzed with basic mobile phase at pH 11.

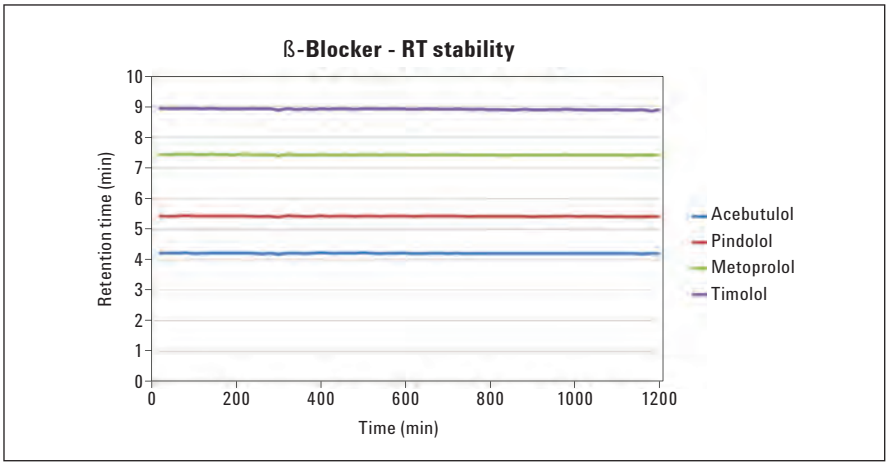

Figure 2
Retention time stability of four ß-blockers, analyzed at high pH.

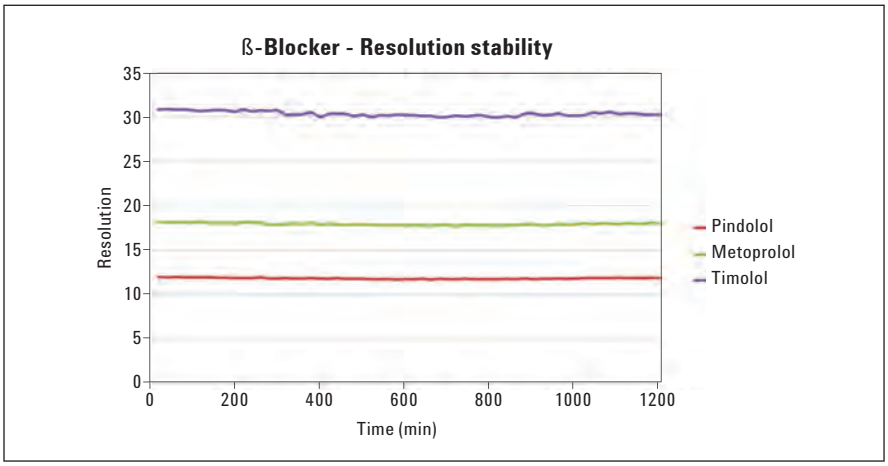

Figure 3
Resolution stability of four ß-blockers, analyzed at high pH.

High pH Analysis of Peptides

A tryptic BSA digest was analyzed using 15 mM NH_4OH as basic aqueous mobile phase (pH 10) and 90% ACN with 15 mM NH_4OH as organic mobile phase. Figure 4 shows the chromatogram of the BSA digest measured at 214 nm. Four peptides were randomly picked for the monitoring of retention time stability.

Retention times stability was monitored over a time period of 37 hours and remained stable from 0 to 2,250 minutes, see Figure 5.

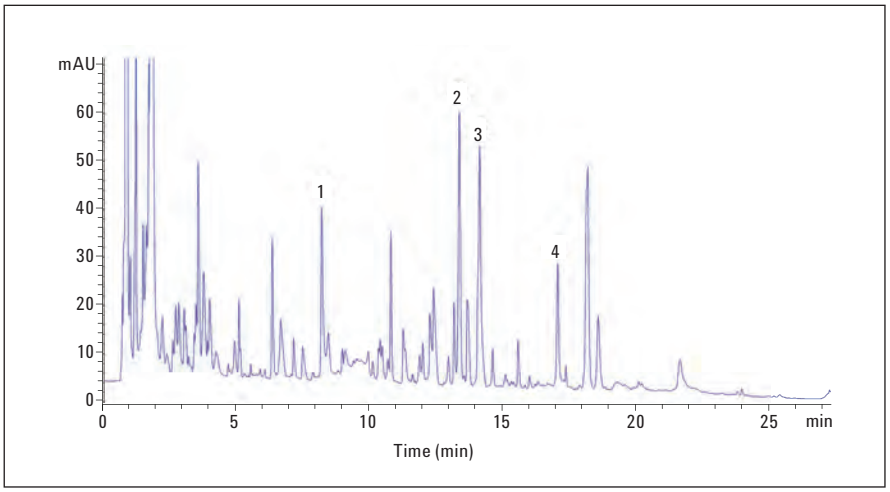

Figure 4
LC chromatogram of a tryptic BSA digest.

Conclusion

This Application Note demonstrates the feasibility of the Agilent 1260 Infinity Bio-inert Quaternary LC system for generic high pH applications. Retention time and resolution stability over a time period of 20 hours was confirmed for the analysis of ß-blockers representing small molecules. A tryptic BSA digest, as an example for biomolecules, showed retention time stability for a longer time range of 37.5 hours.

In summary, high pH mobile phases (pH 10 to 11) had no adverse effects on the stability of RT or resolution over multiple hours.

The 1260 Infinity Bio-inert Quaternary LC system as well as the Agilent ZORBAX Extend-C18 column show high stability concerning RT and resolution for high pH applications.

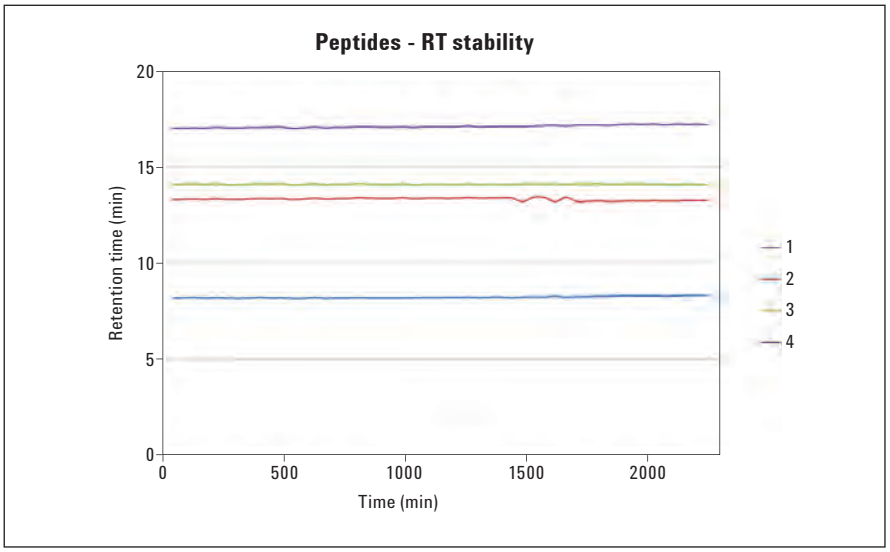

Figure 5
Retention time stability of four peptides out of a tryptic BSA digest, analyzed at high pH.

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Agilent Technologies

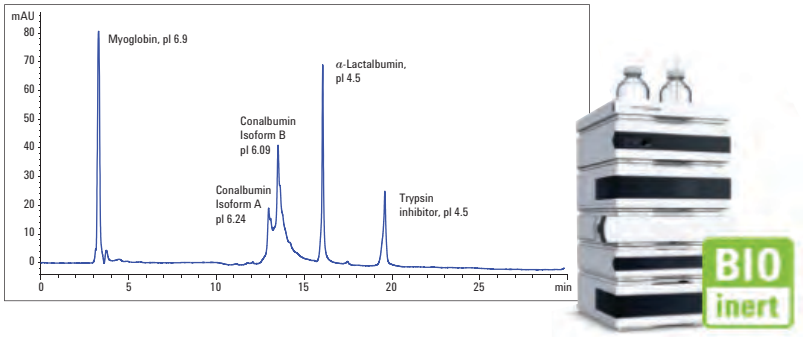
Proof of Performance

Analysis of proteins by anion exchange chromatography

Feasibility of the Agilent 1260 Infinity Bio-inert Quaternary LC System for applications using high-salt buffers

Application Note

Chemical and Pharmaceutical Analysis



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Abstract

In this Application Note, we show the separation of four proteins by anion exchange chromatography using four different high salt-containing elution buffers (up to 2 M) for linear and step gradients. Due to its iron/steel-free design, the Agilent 1260 Infinity Bio-inert Quaternary LC System was able to withstand the harsh conditions used in bio-analytic and bio-purification applications and thereby maintain its UHPLC performance. High precision of retention time and area was demonstrated using linear gradients for four salt types: sodium chloride (2 M), potassium chloride (1 M), sodium acetate (1 M) and tetramethylammonium chloride (1 M). In addition, the stability of retention time and resolution over 48 hours was proven using 2 M sodium chloride as eluting buffer.

Under these conditions, stainless steel-based LC-systems face problems such as salt-based corrosion and therefore require special care and tedious cleaning procedures. These procedures are no longer necessary with the Agilent 1260 Infinity Bio-inert Quaternary LC System thus increasing throughput and efficiency of bio-analytical or bio-purification application.

Agilent Technologies

Introduction

High salt-containing mobile phases, such as those used in ion exchange (IEX) or size exclusion chromatography (SEC), can be problematic for stainless steel-based LC systems. Due to corrosion effects after long-term usage of salt-containing buffers, these LC systems are at risk of being damaged. Tedious cleaning procedures are the consequences. It is therefore highly recommended to use an 'iron-free' system that is not affected by high salt concentrations.

The Agilent 1260 Infinity Bio-inert Quaternary LC System consists of metal-free components in the sample flow-path. All capillaries and fittings throughout the autosampler, column compartment and detectors are completely metal-free so that biomolecules interact only with ceramics or PEEK. This allows the user to deploy buffers containing high amounts of salt. Additionally, the system is stable in a wider pH range (1–13, short term 14) compared to the Agilent 1260 Infinity LC System[1].

In this Application Note, the feasibility of the Agilent 1260 Infinity Bio-inert Quaternary LC System for anion exchange chromatography (AEX), as an example for a high salt application, is demonstrated. A mix of four proteins was separated by anion exchange chromatography (AEX) by using four different eluting salts: sodium chloride (2 M NaCl), potassium chloride (1 M KCl), sodium acetate (1 M CH3COONa) and tetramethylammonium chloride (1 M [(CH3)4N]Cl). Precision of retention time and area was analyzed for linear and step gradients. In addition, long-time stability (48 h) of retention time and resolution using 2 M NaCl elution buffer was investigated.

Experimental

The Agilent 1260 Infinity Bio-inert Quaternary LC System consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High Performance Bio-inert Autosampler (G5667A)
- Agilent 1260 Infinity DAD VL (G1315D) with bio-inert standard flow cell, 10 mm
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)

Column

Agilent Bio WAX, NP5, 4.6 × 250 mm, PK

Software

Agilent OpenLAB CDS, ChemStation Edition for LC & LC MS Systems, Rev. C.01.02 [14]

Solvents

Buffer A: 20 mM Tris, pH 7.6

Buffer B: 20 mM Tris, pH 7.6 +

- 2 M NaCl
- 1 M KCl
- 1 M CH_3COONa
- 1 M $[(CH_3)_4N]Cl$

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). Tris was purchased from Fluka (Sigma-Aldrich, St. Louis, USA). NaCl was purchased from VWR, Radnor, PA, USA. $[(CH_3)_4N]Cl$ and KCl were purchased from Merck KGaA, Darmstadt, Germany. CH_3COONa was purchased from J.T. Baker (VWR, Radnor, PA, USA).

Chromatographic conditions

	Linear gradients	Step gradients
Gradient 1 M	5 min – 100% A	5 min – 100% A
	20 min – 70% B	5.01 min – 20% B
	25 min – 100% B	10 min – 20% B
		10.01 min – 40% B
		15 min – 40% B
		15.01 min – 60%B
		20 min – 60 %B
		20.01 min – 100%B
Gradient 2 M	5 min – 100% A	5 min – 100% A
	20 min – 35% B	5.01 min – 10% B
	25 min – 50% B	10 min – 10% B
	25.01 min – 100%B	10.01 min – 20% B
		15 min – 20% B
		15.01 min – 30%B
		20 min – 30 %B
		20.01 min – 100%B
Stop time	30 min	25 min
Post time	20 min	20 min
Temperature	25 °C	
Flow rate	0.5 mL/min	
Injection volume	5 µL	
DAD	280 nm	
Peak width	0.025 min (0.5 s response time) (10 Hz)	

Piston seal wash

- 100% ultrapure water
- Active for 0.3 min every 1.5 min

Proteins

- Myoglobin from equine skeletal muscle, 17,053 Da, pI 6.9 (1 mg/mL)
- Conalbumin from Chicken Egg White, 76,000 Da, pI 6.24 and 6.092 (2 mg/mL)
- α-Lactalbumin from bovine milk, 14,175 Da, pI 4.5 (1 mg/mL)
- Trypsin Inhibitor from Glycine max (soybean), 20,100 Da, pI 4.5 (1 mg/mL)

All proteins were purchased from Sigma-Aldrich, St. Louis, USA.

Results and discussion

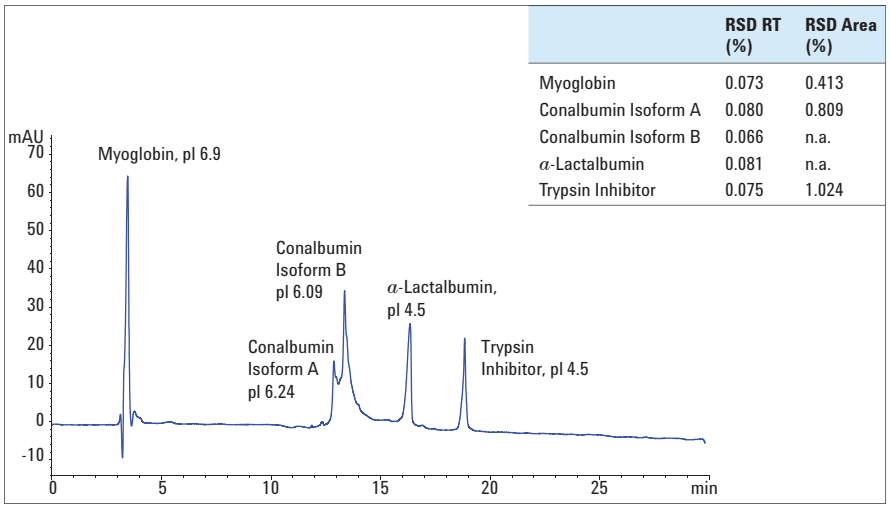
Linear gradients

Anion exchange chromatography using 2 M NaCl

A protein mixture of four proteins was separated via AEX with linear gradients using 2 M NaCl as eluting salt in buffer B. Figure 1 shows the chromatogram of the protein separation by a linear gradient. The proteins are mostly eluting according to their isoelectric point (pI). The separation of α-lactalbumin and trypsin inhibitor is possible due to charge changes on the surface of the protein, which are not necessarily identical with the total net charge. Precision was determined over seven runs as relative standard deviation of retention time and area. Conalbumin exists in different isoforms, depending on the amount of bound iron[2]. Therefore, five peaks were used for the evaluation of retention time precision. In contrast, the conalbumin peaks were not implemented into the calculation of area RSD due to the non-baseline separation of the isoforms. RSD of retention time was <0.09 % for all five peaks. RSD of area was <1.1 % for the evaluated proteins.

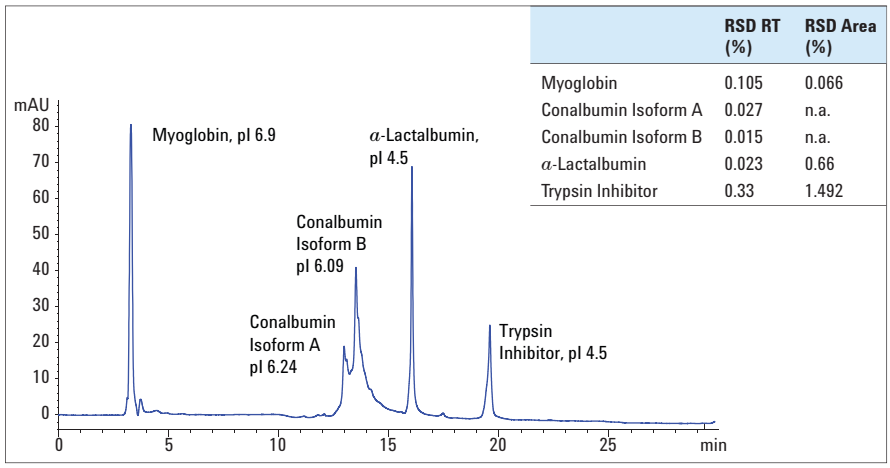
Anion exchange chromatography using different salt types

Resolution and selectivity can be influenced by a changing of the eluting ions, respectively salt types[3]. Depending on the protein to be analyzed, the salts might have different chromatographic effects on the elution of the protein. To find the most appropriate eluting ion for the individual application, various salts can be tested. If more than three salt types are to be tested in one sequence, a solvent selection valve is a good option for method development. In this Application Note, four salts were evaluated in the elution buffer. Figures 2, 3 and 4 show the protein separation using linear gradients with 1 M KCl (Figure 2), 1 M CH_3COONa (Figure 3) and 1 M $[(CH_3)_4N]Cl$ (Figure 4) as eluting salts. RSD of retention time and area was calculated with n = 7.



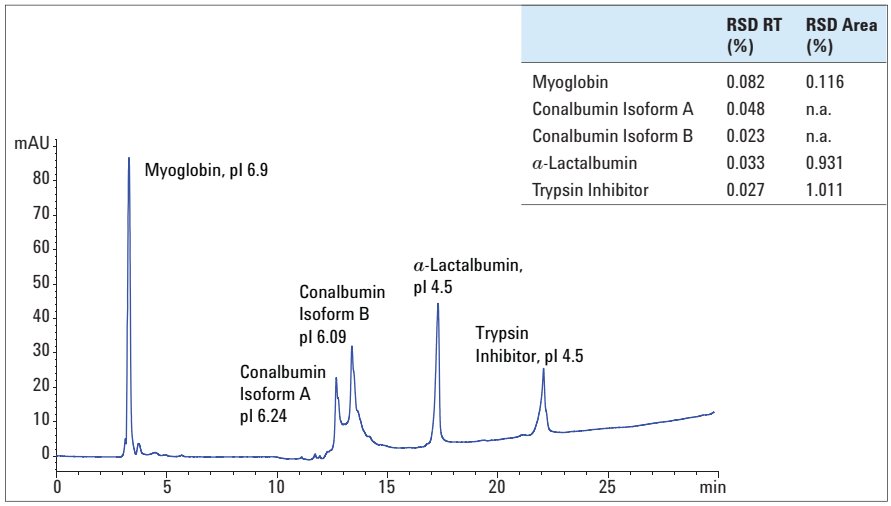
	RSD RT (%)	RSD Area (%)
Myoglobin	0.073	0.413
Conalbumin Isoform A	0.080	0.809
Conalbumin Isoform B	0.066	n.a.
α-Lactalbumin	0.081	n.a.
Trypsin Inhibitor	0.075	1.024

Figure 1
Protein separation by AEX by a linear gradient using 2 M NaCl as eluting salt.



	RSD RT (%)	RSD Area (%)
Myoglobin	0.105	0.066
Conalbumin Isoform A	0.027	n.a.
Conalbumin Isoform B	0.015	n.a.
α-Lactalbumin	0.023	0.66
Trypsin Inhibitor	0.33	1.492

Figure 2
Protein separation by AEX by a linear gradient using 1 M KCl as eluting salt.



	RSD RT (%)	RSD Area (%)
Myoglobin	0.082	0.116
Conalbumin Isoform A	0.048	n.a.
Conalbumin Isoform B	0.023	n.a.
α-Lactalbumin	0.033	0.931
Trypsin Inhibitor	0.027	1.011

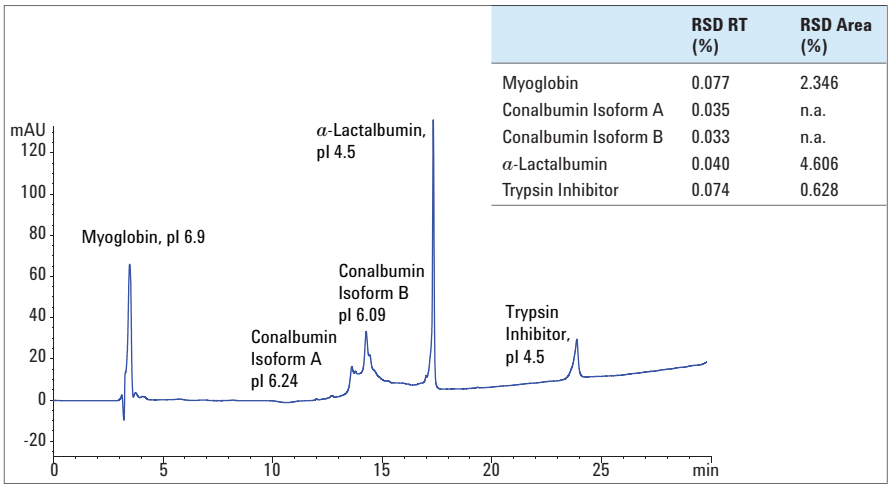
Figure 3
Protein separation by AEX by a linear gradient using 1 M CH_3COONa as eluting salt.

The experiments demonstrate changes in retention time, resolution and in addition changes in peak shape and intensity, depending on the eluting salt used. Especially, the resolution of conalbumin A and B differs between the salt types, whereas 1 M CH_3COONa showed the best resolution. Considering all factors mentioned above (best resolution, best peak shape and highest intensity) together with a flat baseline 1 M KCl was the optimal salt for the separation of the four proteins used.

Stepg radients

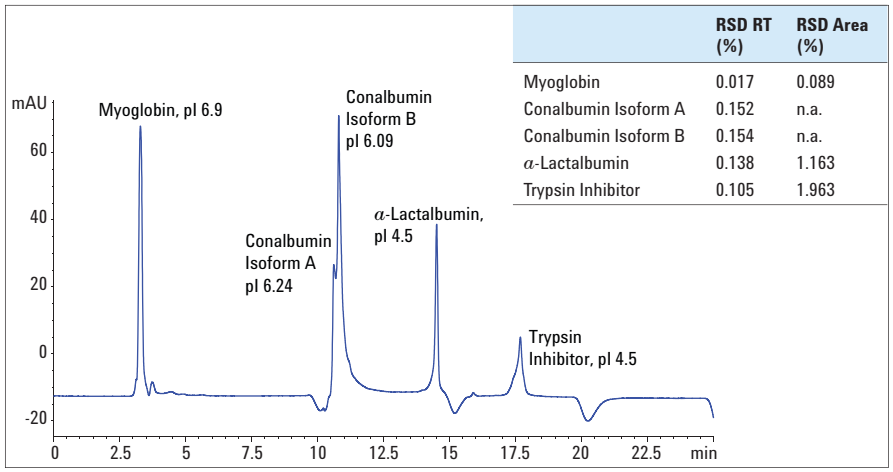
With the use of step gradients, especially if only one protein is to be separated, it is possible to accelerate separation time and thus reduce buffer consumption. Figures 5 and 6 show protein separations by step gradients using 1 M KCl (Figure 5) and 1 M CH_3COONa (Figure 6). RSD of retention time and area is calculated with n = 7.

Due to long equilibration times of the AEX column, precision of retention time and area is slightly inferior compared to the linear gradient. In addition, negative peaks could be observed at the time point of the gradients steps, which is likewise based on missing equilibration time.



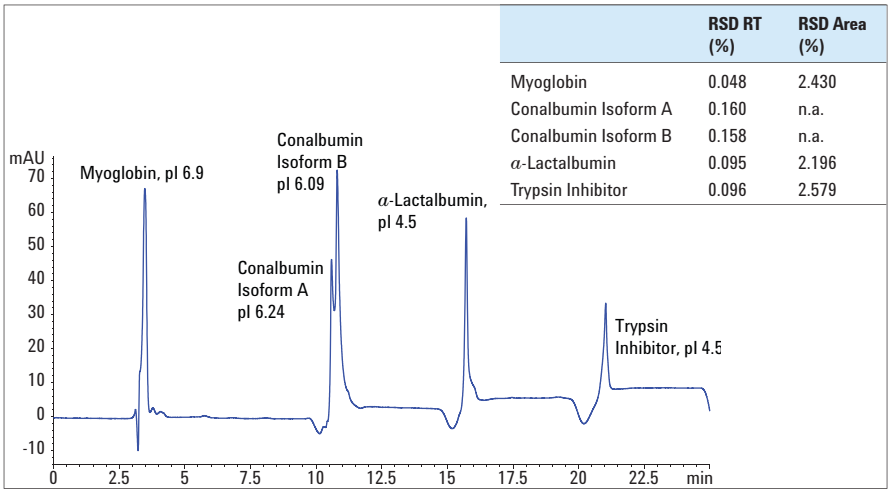
	RSD RT (%)	RSD Area (%)
Myoglobin	0.077	2.346
Conalbumin Isoform A	0.035	n.a.
Conalbumin Isoform B	0.033	n.a.
α-Lactalbumin	0.040	4.606
Trypsin Inhibitor	0.074	0.628

Figure 4
Protein separation by AEX by a linear gradient using 1 M $[(CH_3)_4N]Cl$) as eluting salt.



	RSD RT (%)	RSD Area (%)
Myoglobin	0.017	0.089
Conalbumin Isoform A	0.152	n.a.
Conalbumin Isoform B	0.154	n.a.
α-Lactalbumin	0.138	1.163
Trypsin Inhibitor	0.105	1.963

Figure 5
Protein separation by AEX by a step gradient using 1 M KCl as eluting salt.



	RSD RT (%)	RSD Area (%)
Myoglobin	0.048	2.430
Conalbumin Isoform A	0.160	n.a.
Conalbumin Isoform B	0.158	n.a.
α-Lactalbumin	0.095	2.196
Trypsin Inhibitor	0.096	2.579

Figure 6
Protein separation by AEX by a step gradient using 1 M CH_3COONa as eluting salt.

To prove the long-term stability of retention time and resolution with high salt containing buffers, protein separation (linear gradient) using 2 M NaCl as eluting salt was monitored over 48 h, see Figure 7 and 8. The stability of retention time and resolution could be demonstrated over the whole time period. During all measurements, the piston seal wash (containing 100% water) was active in regular intervals for 0.3 min every 1.5 min to remove salt from the pump pistons.

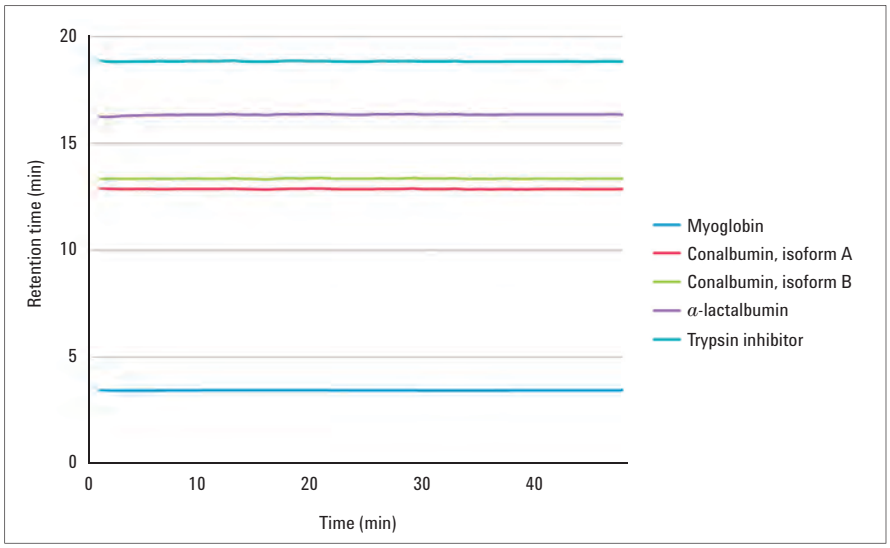


Figure 7
Stability of retention time over 48 h with 2 M NaCl.

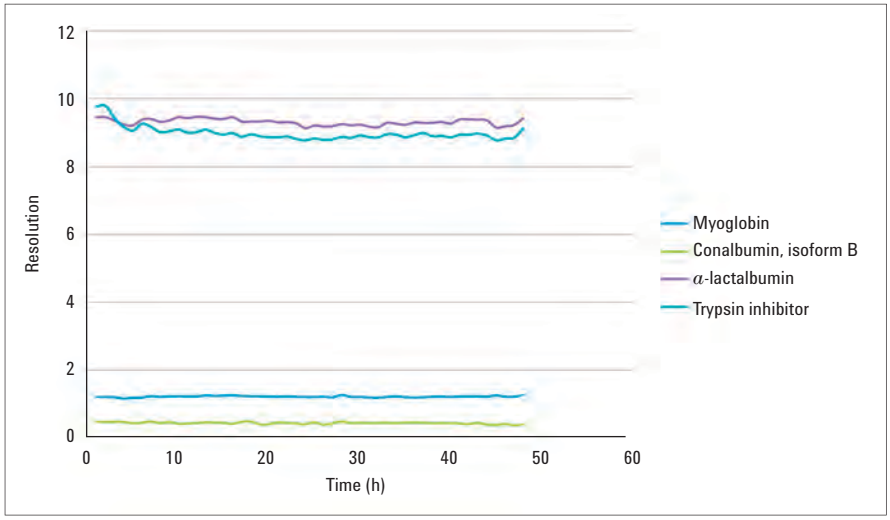


Figure 8
Stability of resolution over 48 h with 2 M NaCl.

Conclusions

This Application Note demonstrated that the Agilent 1260 Infinity Bio-inert Quaternary LC System is an ideal system for high salt applications. By employing anion exchange chromatography for the separation of four different proteins, high precision was achieved for linear gradients with four different eluting ions/salt types. Depending on the salt type used, the chromatograms varied with regards to retention time, resolution, peak shape and intensity. Hence, it is recommended to test different salt types to find the most adequate one for optimal sample separation. If more than three salt types are to be tested, a solvent selection valve is a good option for method development.

Retention time and resolution stability over a time period of 48 hours was confirmed for the analysis of the protein mix with 2 M NaCl as eluting salt.

With the use of step gradients, it is possible to speed up separation time and buffer consumption. However, long equilibration times of the columns have to be considered. Therefore, it is highly recommended to consider enough equilibration time between the single runs, both in step and linear gradients. It is also very important to activate the seal wash (containing 100% water) in regular intervals (for example, 0.3 min every 1.5 min) to remove salt from the pump pistons.

The Agilent 1260 Infinity Bio-inert Quaternary LC System as well as the Agilent Bio WAX, NP5, 4.6 x 250 mm, PK column show high stability concerning RT and resolution for high salt applications.

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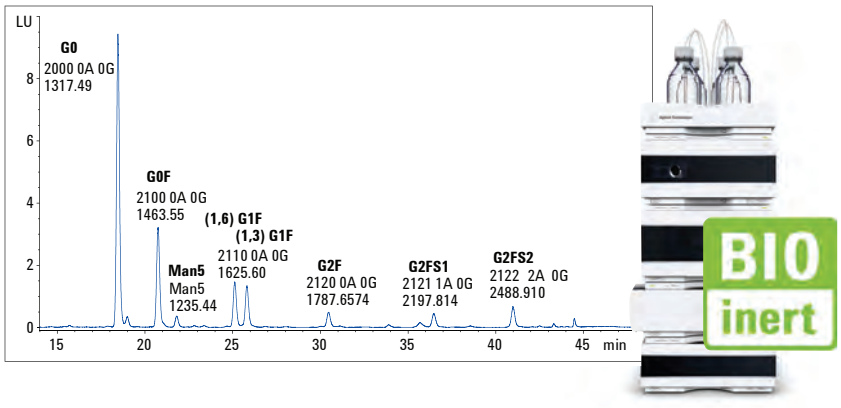
Agilent Technologies

N-Glycan analysis of monoclonal antibodies and other glycoproteins using UHPLC with fluorescence detection

Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent 1260 Infinity Fluorescence Detector

Application Note

Biopharmaceuticals



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Abstract

This Application Note shows the analysis of N-linked glycans with hydrophilic interaction chromatography (HILIC) separation using the Agilent 1260 Infinity Bio-inert Quaternary LC System together with the Agilent 1260 Infinity Fluorescence Detector. Enzymatic glycan release with PNGase F followed by derivatization by 2-aminobenzamide (2-AB) was conducted on monoclonal antibodies (mAbs) and two other glycoproteins from avian egg white: ovalbumin and conalbumin. The glycan pattern of the monoclonal antibody showed very good resolution and all major N-glycans occurring in mAbs could be detected with good signal-to-noise ratios. In addition, the complex glycan patterns of the two major types of avian egg glycoproteins were well resolved, resulting in over 30 to 35 detected peaks. The 1260 Infinity Bio-inert Quaternary LC System together with the 1260 Infinity Fluorescence Detector is an ideal solution for sensitive and high resolution analysis of 2-AB derivatized glycans released from mAbs and other glycoproteins.

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Introduction

Protein glycosylation is one of the most frequently observed post translational modifications. Mammalian glycoproteins contain three major types of glycans: N-linked, O-linked, and glycosylphosphatidyinositol (GPI) lipid anchors, which consist of one or more monosaccharide units. A single glycosylation site can generate considerable heterogeneity regarding mass and charge of glycoproteins. These oligosaccharides are involved in many biological regulation and recognition processes, for example, protein sorting, immune and receptor recognition, inflammation, pathogenicity, metastasis, and other cellular processes. Therefore, certain glycosylation patterns can be associated to the diseased or healthy state of a patient[1, 2]. In addition, properties like safety, efficacy and the serum half-life of therapeutic proteins can be affected by their glycosylation pattern.

Recombinant monoclonal antibody therapeutics (mAbs) represent the largest group of therapeutic proteins as a major new class of drug. The efficacy of these therapeutics is highly dependent on the correct glycosylation patterns of the mAbs and so far, all licensed therapeutic mAbs are immunoglobulines G (IgGs)[3]. Human IgG has a single conserved N-linked glycosylation site located on the Fc region of each heavy chain at Asn-297[4] (Figure 1). This fact results in two sugar moieties per IgG, which are highly heterogeneous and contain up to 30 different glycan types[5]. The combination of glycans at each of the two glycosylation sites on the Fc region leads to large numbers of different glycoforms in each batch of mAb production.

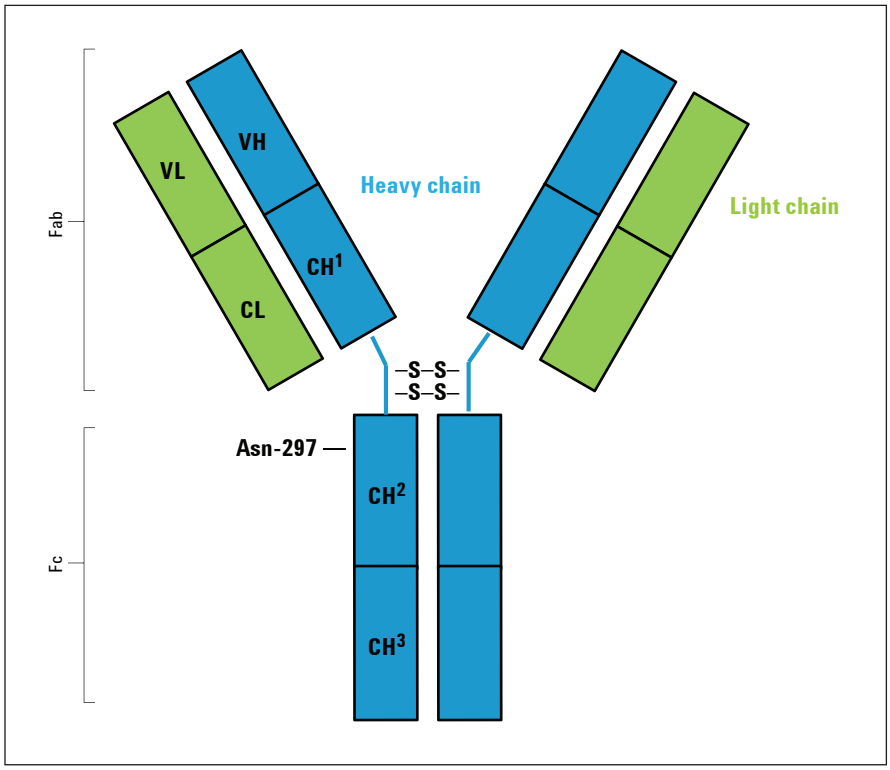


Figure 1
IgG antibody structure.

The glycan structure on this glycosylation site plays a critical role in complement activation and receptor affinity[6], which affects the efficacy of the therapeutic mAbs. Moreover, nonhuman glycans are a safety issue due to induced immune responses.

Therefore, analysis of the glycan pattern is an important part of characterization of therapeutic glycoproteins, especially mAbs. Figure 2a shows the general nomenclature used to describe sugar residues of different glycan structures on proteins. Figure 2b shows the predominant glycan structures present on the Asn-297 site in IgG. In general, N-glycans have a core structure, containing two β-D-N-acetylglucosamine (GlcNac) and three mannose (Man) units. IgG Fc N-glycans are predominantly biantennary complex-type structures, partially core-fucosylated (for example, G0F).

Different strategies for the analysis of glycans have been described. A large number of methods are based on protein-released and subsequently derivatized glycans[7] due to the lack of chromophores needed for optical detection methods, for example, UV detection. We have presented a combination of enzymatic release of N-glycans using PNGase F with subsequent derivatization with 2-aminobenzamide (2-AB) for fluorescence detection.

2-AB is a neutral and stable bonded label, numerously used in glycan analysis[7, 8, 10]. Figure 3 shows the 2-AB-labeling by reductive amination (Schiff's base intermediate not shown). The 2-AB labeling of the glycans results in the mass of the glycans +119 Da. Due to protonation, the resulting mass shift in the MS is 120 Da (Tables 1, 2, 3).

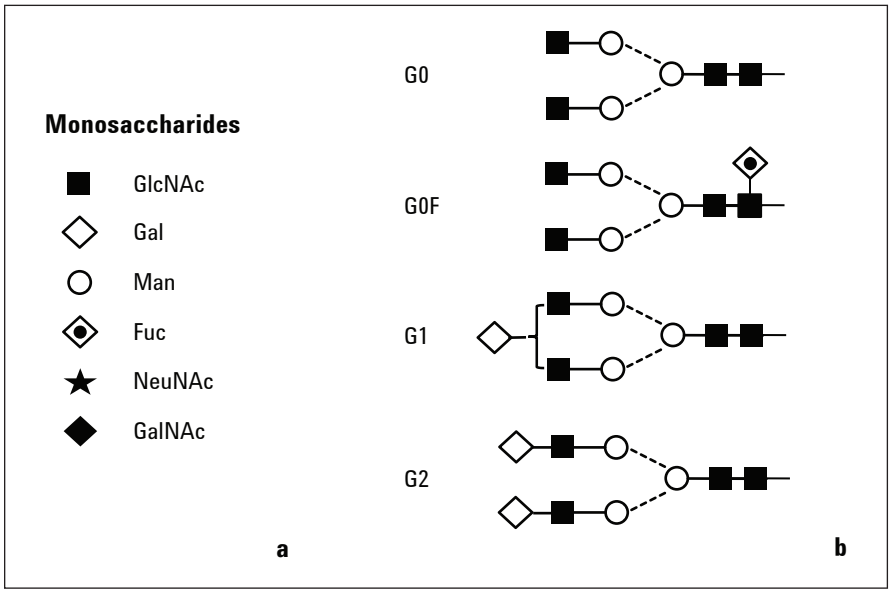


Figure 2
Glycan structure and isoforms. a) General nomenclature for glycans b) Predominant glycan structures of IgGs. G = Galactose units, F = Fucose units. Modified after Arnold *et al*[6].

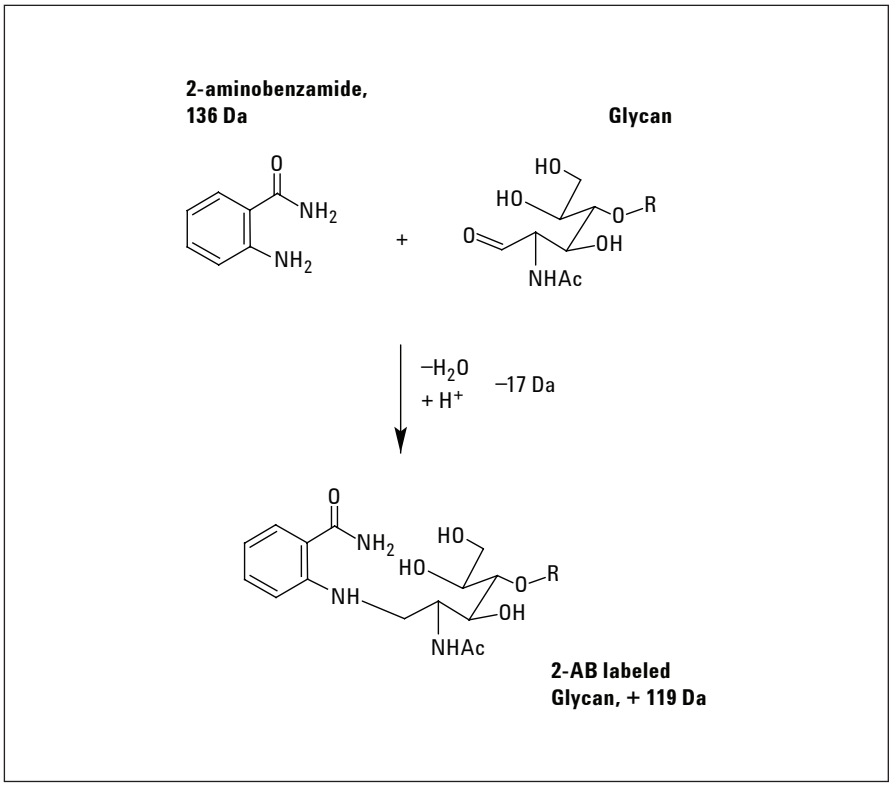


Figure 3
Labeling of a glycan with 2-aminobenzamide (2-AB).

Due to the hydrophilic properties of glycans, subsequent purification using Hydrophilic Interaction Chromatography – Solid Phase Extraction (HILIC-SPE) is added for removal of excess label[8].

Separation using HILIC with fluorescence detection is a robust method for glycan analysis[9]. Pauline Rudd and coworkers established a database (http://glycobase.nibrt.ie/) based on HIILIC retention properties, a frequently used technology for the analysis of protein glycosylation[8, 10]. To identify the monosaccharide composition of the glycans within a chromatographic peak, HILIC-LC can be coupled to ESI-QToF-MS for mass and structure information.

Experimental

The Agilent 1260 Infinity Bio-inert Quaternary LC System consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High Performance Bio-inert Autosampler (G5667A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector VL (G1315D), with bio-inert standard flow cell, 10 mm
- Agilent 1200 Series Fluorescence Detector (G1321A), with standard flow cell

MS system
Agilent 6530 Accurate Mass QTOF LC/MS system

Column
HILIC Glycan Amide column, 2.1 × 150 mm, < 2 µm

Software
Agilent OpenLAB CDS, ChemStation Edition for LC & LC/MS Systems, Rev. C.01.02 [14]

Agilent MassHunter Workstation Software, Version B.04.00, Build 4.0.479.0

Sample preparation

Deglycosylation procedure
Deglycosylation of the monoclonal antibody and the glycoproteins was performed using PNGase F to release asparagine-linked oligosaccharides (N-glycans) from the glycoproteins. PNGase cleaves asparagine-linked high mannose as well as hybrid and complex oligosaccharides from the glycoproteins and leaves the glycans intact. The average glycosylation of proteins is 2–5 % with 1,000 Da as average molecular weight. Therefore, to release approximately 20 µg glycans 400 µg of glycoproteins were used. Ovalbumin and conalbumin have only one glycosylation site, whereas the mAb contains two glycosylation sites. The amount of PNGase F was adjusted to the amount of glycans. The proteins were deglycosylated according to instructions from Sigma-Aldrich for 3 hours at 37 °C. The reaction was then stopped and the sample was vacuum dried for further processing.

AB-labeling for fluorescence detection and sample cleanup
The dried glycan samples were labeled with the fluorophore 2-AB (2-aminobenzamide) using the GlycoProfil 2-AB Labeling Kit according to the preparing protocol from Sigma-Aldrich for 3 hours at 65 °C. After the labeling procedure, the samples were purified using the GlycoProfil Glycan Cleanup Cartridges from Sigma-Aldrich according to the instructions manual. After the HILIC cleanup procedure, the samples were vacuum dried and reconstituted in 15 µL ultrapure water for LC analysis.

Chromatographic conditions

	Gradient antibody standard	Gradient glycoproteins
Starting flow rate:	0.5 mL/min	0.5 mL/min
Gradient:	0 minutes – 85% B	0–6 minutes – 85% B
	5 minutes – 75% B	10 minutes – 80%B
	35 minutes – 64%	60 minutes – 64% B
	40 minutes – 50%	65 minutes – 50% B
	42 minutes - Flow 0.25 mL/min	67 – Flow 0.25 mL/min
	43 minutes – 0% B	68 minutes – 0% B
	48 minutes – 0% B	73 – 0% B
	50 minutes – 85% B	75 minutes – 85% B
	55 minutes – Flow 0.5 mL/min	80 minutes – Flow 0.5 mL/min
Stop time:	55.01 minutes	80.01 minutes
Post time:	20 minutes	20 minutes
Injection volume:	10 µL	1 µL
Column temperature:	60 °C	
FLD:	Ex. 260 nm Em. 430 nm	
Peak width:	> 0.025 minutes (18.52 Hz)	

Solvents and samples

Buffer A: 100 mM ammonium formate, pH 4.5

Buffer B: acetonitrile

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). The monoclonal antibody standard, that was used, is part of the mAb-Glyco Chip Kit (p/n G4240-64026). Ammonium formate, ovalbumin and conalbumin, PNGase F from Elizabethkingia miricola, GlycoProfil 2-AB Labeling Kit and GlycoProfil Glycan Cleanup Cartridges were purchased from Sigma-Aldrich, St.Louis, USA.

Results and discussion

Analysis of N-glycans from monoclonal antibodies

The glycans from the monoclonal antibody standard (included in Agilent mAb-Glyco Chip Kit) were AB-labeled and analyzed using HILIC-UHPLC. Figure 4 shows the separation of the mAb glycans. The resulting HILIC glycan pattern was compared to a mAb glycan pattern generated by Melmer *et al*,[10] and the single peaks were assigned to the corresponding glycan structures. For peak labeling, both the nomenclature used in various publications[4, 5, 10] and the nomenclature of the Agilent mAb-Glyco Chip manual was used. The mAb glycan pattern was optimally resolved, allowing separation of all major N-glycans occurring in mAbs: G0, G0F, Man5, (1,6)G1F and (1,3)G1F. In addition, two more peaks could be detected, representing two sialylated glycans (G2FS1, G2FS2) containing N-acetylneuramic acid (NANA).

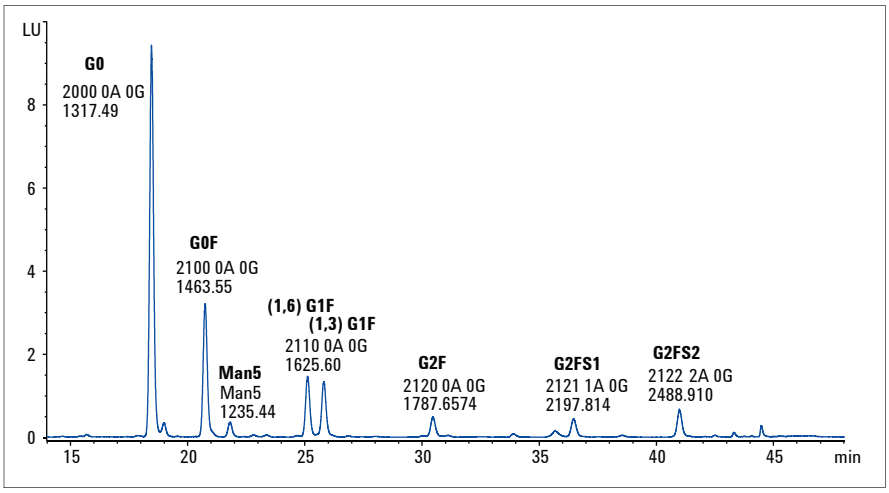


Figure 4
Separation of mAB N-glycans.

Name	Calculated mass	Mass + AB	Monosaccharide composition	Structure
G0	1317.5	1437.6	$GlcNAc_2Man_3GlcNAc_2$	
G0F	1463.6	1583.6	$GlcNAc_2Man_3GlcNAc_2Fuc$	
Man5	1235.4	1355.5	$Man_5GlcNAc_2$	
(1,6)G1F	1625.6	1745.7	$GalGlcNAc_2Man_3GlcNAc_2Fuc$	
(1,3)G1F	1625.6	1745.7	$GalGlcNAc_2Man_3GlcNAc_2Fuc$	
G2F	1787.6	1907.7	$GalGlcNAc_2Man_3GlcNAc_2Fuc$	
G2FS1	2077.8	2197.8	$NeuNAcGal_2GlcNAc_2Man_3GlcNAc_2Fuc$	
G2FS2	2368.9	2488.9	$NeuNAc_2Gal_2GlcNAc_2Man_3GlcNAc_2Fuc$	

Table 1
This table gives an overview of the detected glycan structures of the monoclonal antibody.

The glycan pattern of the mAb standard (Figure 3) showed a high amount of glycan G0, which is added as internal standard for system checkout. Table 1 shows an overview of the detected glycan structures.

High intensity of the detected labeled glycans was achieved by setting the optimal wavelengths for glycan detection on the 1260 Infinity Fluorescence Detector, using 260 nm as excitation wavelength and 430 nm as emission wavelength. Usually, an excitation wavelength of 330 nm is preferred for the analysis of 2-AB labeled glycans[11]. We used the lower 260 nm, due to higher intensity, resulting in better signal-to-noise ratios, as described by Melmer *et al*[10].

Two additional glycoproteins (ovalbumin and conalbumin) were deglycosylated, the glycans derivatized and analyzed using HILIC-UHPLC. Figure 5 shows the separation of ovalbumin glycans. Ovalbumin is N-glycosylated only at one site (Asn-292), but a complex glycosylation pattern can be associated to this site[11]. Due to the complexity of the glycan pattern, the gradient had to be adjusted to achieve higher resolution. Six glycans were identified with ESI-QTOF detection and mass correlation to the glycan library published by Harvey *et al* 2000[12], see Table 2 for detailed glycan information. Over 35 peaks could be resolved in the glycan sample, released from ovalbumin.

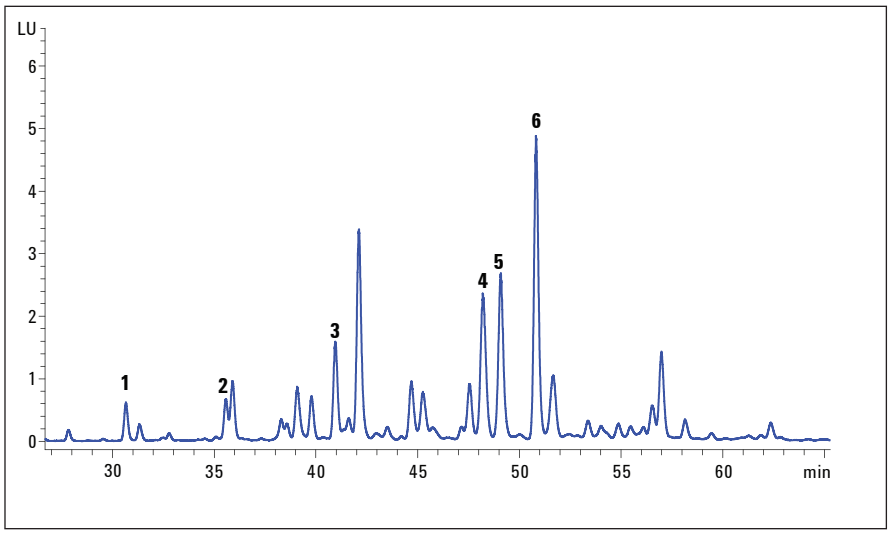


Figure 5
Separation of N-glycans, released from ovalbumin.

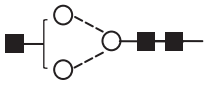
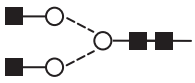
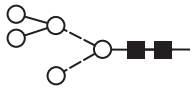
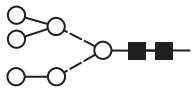
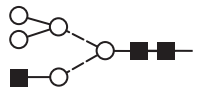
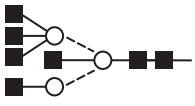
Peak	Retention time	Calculated mass	Mass + AB	Name	Structure
1	30.66	1114.5	1234.5	$GlcNAcMan_3GlcNAc_2$	
2	35.59	1317.6	1437.6	$GlcNAc_2Man_3GlcNAc_2$	
3	40.95	1235.5	1355.5	$Man_5GlcNAc_2$	
4	48.21	1397.6	1517.6	$Man_6GlcNAc_2$	
5	49.09	1438.6	1558.6	$GlcNAcMan_5GlcNAc_2$	
6	50.81	1927.8	2047.8	$GlcNAc_5Man_3GlcNAc_2$	

Table 2
Detailed information identified N-glycans ovalbumin. For more and detailed information, see 5990-9774EN.

Figure 6 shows the glycosylation pattern of conalbumin (synonym: ovotransferrin). Like ovalbumin, conalbumin has only one N-linked glycosylation site per mol protein[13]. Despite the complexity of the glycan pattern, high resolution resulting in successful glycan separation with good peak shape was achieved. Three glycans were identified with ESI-QTOF detection, see Table 3 for detailed glycan information. Overall, more than 30 peaks could be detected with good signal-to-noise ratio in the glycan sample from conalbumin.

Compared to the relative simple glycan pattern of the mAb, the two egg white glycoproteins have a higher variety of glycan structures, although they are only assigned to a single glycosylation site on both proteins. No fucosylated glycans were detected in ovalbumin and conalbumin in contrast to the mAb glycans, due to the fact that all avian egg glycoproteins are not fucosylated[14].

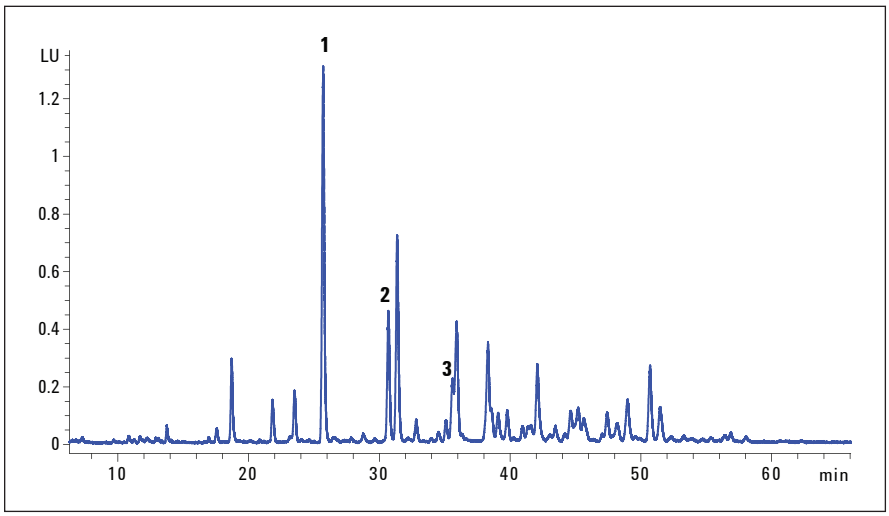


Figure 6
Separation of N-glycans released from conalbumin.

Peak	Retention time	Calculated Mass	Mass + AB	Name	Structure
1	25.6	911.4	1031.4	$Man_3GlcNAc_2$	
2	30.66	1114.5	1234.5	$GlcNAcMan_3GlcNAc_2$	
3	35.59	1317.6	1437.6	$GlcNAc_2Man_3GlcNAc_2$	

Table 3
Retention time, masses, monosaccharide composition and structure of the conalbumin glycans identified.

Conclusion

This Application Note demonstrates, that the Agilent 1260 Infinity Bio-inert Quaternary LC System together with the Agilent 1260 Infinity Fluorescence Detector is an ideal solution for the analysis of protein-released glycans, derivatized with 2-aminobenzamide (2-AB). Sample preparation using PNGase F for the release of N-linked glycans following 2-AB derivatization with subsequent HILIC sample cleanup was shown for the monoclonal antibody standard and two glycoproteins from avian egg white.

The glycan pattern of a monoclonal antibody standard was optimally resolved, allowing separation of all major N-glycans occurring in mAbs: G0, G0F, Man5, (1,6)G1F, (1,3)G1F, G2F, even G2FS1 and G2FS2 could be detected with good signal-to-noise ratios.

With fluorescence wavelengths optimized for our system, higher intensities and, therefore, better signal-to-noise ratios were achieved using the 1260 Infinity Fluorescence Detector. Instead of an excitation wavelength of 330 nm (as recommended by Anumula 2005[11]) the lower wavelength 260 nm was used.

In addition, the complex glycan patterns of two avian egg glycoproteins, ovalbumin and conalbumin, were well resolved, resulting in over 30 to 35 detected peaks with good peak shape and signal-to-noise ratio. Subsequent ESI-QTOF analysis enabled the identification of different glycan masses and related monosaccharide composition.

The 1260 Infinity Bio-inert Quaternary LC System together with fluorescence detection provides an optimal system for sensitive and high resolving analysis of 2-AB derivatized glycans released from mAbs and other glycoproteins.

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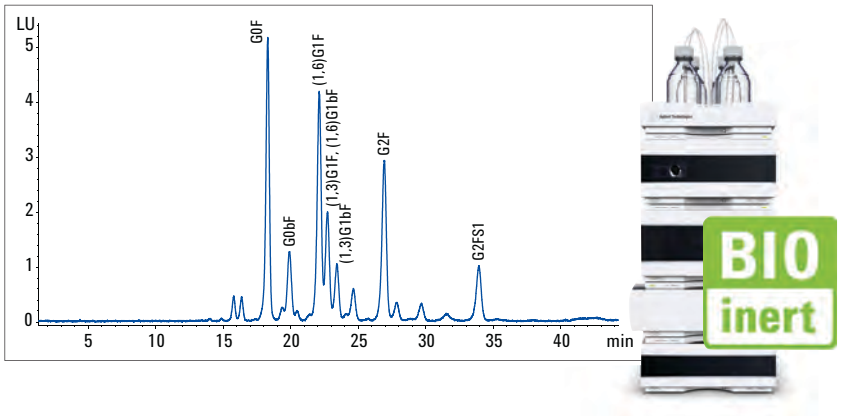
Sensitive and reproducible glycan analysis of human immunoglobulin G

Agilent 1260 Infinity Bio-inert Quaternary LC system with fluorescence detection

Application Note

Biopharmaceuticals



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Abstract

In this Application Note, sensitive and reproducible HPLC analysis of N-linked glycans of human immunoglobulin G is demonstrated using the Agilent 1260 Infinity Bio-inert Quaternary LC System together with fluorescence detection. Precision of retention times and areas was determined for analysis of a N-linked human IgG glycan library. In addition, good linearity in a range from 0.008 to 1 pmol was determined for a mix of five glycan standards (Man5, G2, G2S1, G2FS1, and G2S2) with low limits of detection and quantification.

The Agilent 1260 Infinity Bio-inert Quaternary LC System together with fluorescence detection is an optimal system for sensitive and reproducible analysis of 2-AB derivatized N-glycans released from human immunoglobulin G.

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Introduction

Antibodies represent the largest group of recombinantly produced therapeutic proteins as a major new class of drug. The efficacy of these therapeutics is highly dependent on the correct glycosylation pattern and, so far, all licensed therapeutic mAbs are immunoglobulines G (IgGs)[1]. The analysis of their glycosylation pattern is an important part of the QA/QC process. In addition, the glycosylation pattern of plasma derived human IgGs can reflect the healthy or diseased status of a patient[2].

Human IgG has a single conserved N-linked glycosylation site located on the Fc region of each heavy chain at Asn-297[3]. This fact results in two sugar moieties per IgG, which are highly heterogeneous and can result in a mixture of at least 30 glycoforms[1]. Plasma-derived human IgG N-linked glycans are predominantly biantennary complex-type structures, mostly core-fucosylated with none to two galactose residues and one to two sialic acids. In addition, human IgGs carry a small amount of bisecting N-acetylglycosamine (GlcNAc) residues. In general, recombinantly produced monoclonal antibodies (mAbs) reveal a marginally less complex pattern without nonfucosylated or bisecting GlcNAc glycan structures[4]. Other more immature structures are also found, for example high-mannose structures like mannose-5, although primarily in small amounts[5].

The analysis of protein glycosylation pattern can be challenging, especially due to the fact that the glycan analytes are often of low abundance in complex biological samples. The separation of AB-labeled glycan structures using Hydrophilic Interaction Chromatography (HILIC)-HPLC with fluorescence detection is a robust and sensitive method for glycan analysis[6] and was used in this Application Note for the determination of precision, linearity, and sensitivity.

Figure 1 shows the glycan structures, isoforms and nomenclature used in this Application Note, which have already been used in a previous publication[7]. The glycans linked to IgG are classified according to the number of terminal galactose residues attached, see Figure 1b. G0, G1, and G2 contain none, one and two terminal galactose residues.

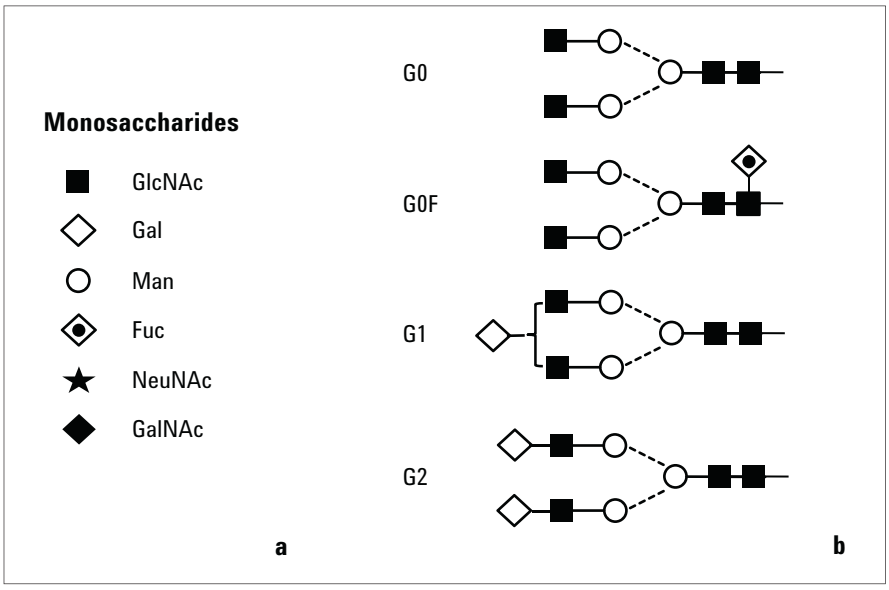


Figure 1
Glycan structure and isoforms. a) General nomenclature for glycans, b) Predominant glycan structures of IgGs. G = Galactose units, F = Fucose units. Modified after Arnold *et al.*[1].

Experimental

The Agilent 1260 Infinity Bio-inert Quaternary LC System used for the experiments consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High Performance Bio-inert Autosampler (G5667A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector VL (G1315D), equipped with bio-inert standard flow cell, 10 mm
- Agilent 1260 Infinity Fluorescence Detector (G1321B), equipped with bio-inert FLD flow cell

Column
TOSOH TSK-GEL Amide-80, 2 × 150 mm, 3 µm

Software
Agilent OpenLAB CDS, ChemStation Edition for LC & LC MS Systems, Rev. C.01.02 [14]

Solvents and samples
Buffer A: 100 mM ammonium formate, pH 4.5
Buffer B: acetonitrile

Glycan standards
- GLYKO 2-AB-(HUMAN IgG N-LINKED GLYCAN LIBRARY), 200 pmol
- GLYKO 2-AB-(MAN-5) → *Man5*, 100 pmol
- GLYKO 2-AB-(NA2) → *G2*, 100 pmol
- GLYKO 2-AB(A1) → *G2S1*, 100 pmol
- GLYKO 2-AB-(A1F) → *G2FS1*, 100 pmol
- GLYKO 2-AB-(A2) → *G2S2*, 100 pmol

All glycan standards were dissolved in 50 µL 100 mM ammonium formate, pH 4.5.

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). Ammonium formate was purchased from Sigma-Aldrich, St.Louis, USA. AB-labeled glycan standards were purchased from Prozyme, Hayward, USA.

Chromatographic conditions

Gradient	Minutes	% B	Flow rate [mL/min]
	0	85	0.65
	5	75	
	10	72	
	30	67	
	40	64	
	40.01		0.65
	42	50	
	42.01		0.4
	45	100	
	50	100	
	55	85	
	55.01		0.4
	60		0.65
Stop time:	60.01		
Post time:	20 minutes		
Injection volume:	2.5 µL		
Column temperature:	50 °C		
FLD:	Ex. 260 nm Em. 430 nm		
Peak width:	Peak width: > 0.05 minutes (9.26 Hz)		
PMT:	PMT Gain: 14		

Results and discussion

Reproducible analysis of N-glycans from AB-labeled human IgG standard

Figure 2 shows the separation of the AB-labeled human IgG N-linked glycan library. All major glycan structures found in human IgGs were well resolved. For peak labeling, the nomenclature employed in various publications [3, 8, 9] was used. All dominant glycans are core fucosylated with none, one or two galactosylated structures, see Table 1. Except for the mono-sialylated G2FS1 (last peak), all assigned glycans have neutral structures.

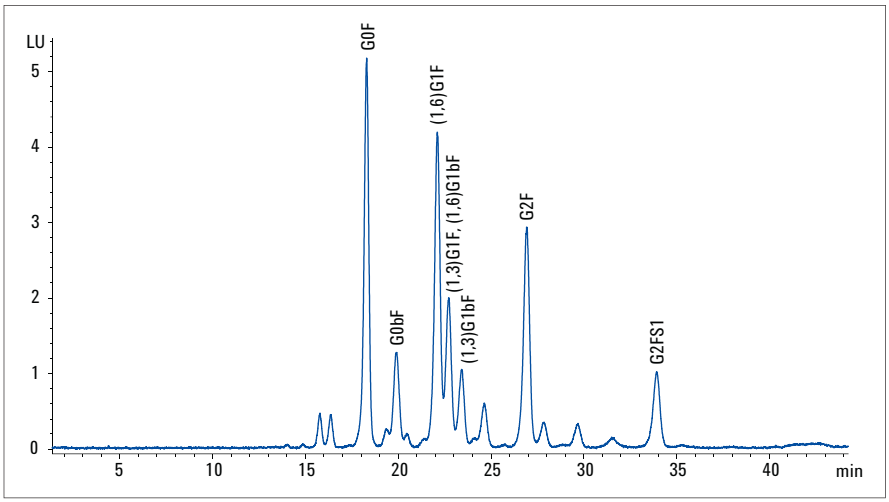


Figure 2
Separation of AB-labeled human IgG N-linked glycan library. Assignments were made based on Omtvedt *et al.* 2006.

Name	Monosaccharide composition	Structure
G0F	$GlcNAc_2Man_3GlcNAc_2Fuc$	
G0bF	$GlcNAc_3Man_3GlcNAc_2Fuc$	
(1,6)G1F	$GalGlcNAc_2Man_3GlcNAc_2Fuc$	
(1,3)G1F	$GalGlcNAc_2Man_3GlcNAc_2Fuc$	
(1,6)G1bF	$GalGlcNAc_3Man_3GlcNAc_2Fuc$	
(1,3)G1bF	$GalGlcNAc_3Man_3GlcNAc_2Fuc$	
G2F	$Gal_2GlcNAc_2Man_3GlcNAc_2Fuc$	
G2FS1	$NeuNAcGal_2GlcNAc_2Man_3GlcNAc_2Fuc$	

Table 1
Names, monosaccharide composition and structure of the assigned N-glycans in the human IgG glycan library.

Precision of retention time and area was determined for five consecutive injections of the human IgG N-linked glycan library. For all glycan structures, the retention time precision was below 0.16% and area precision between 0.66% and 4.11%, see Table 2. All measured RSD values for RT and area were within the commonly reported range[10].

Glycan	Precision of RT [%]	Precision of area [%]
G0F	0.135	0.74
G0bF	0.146	4.11
G1F	0.142	0.66
G1bF	0.153	1.03
G2	0.149	1.92
G2F	0.155	2.44
G2FS1	0.151	1.82

Table 2
Precision of retention time and area for five consecutive injections of human N-linked IgG glycans.

Linearity

Linearity was determined with a mix of five glycan standards (Man5, G2, G2S1, G2FS1, and G2S2) diluted in series 1:3 in a range from 0.008 to 1 pmol. Figure 3 shows a chromatogram of the five N-glycan mix at a sample amount of 1 pmol on column.

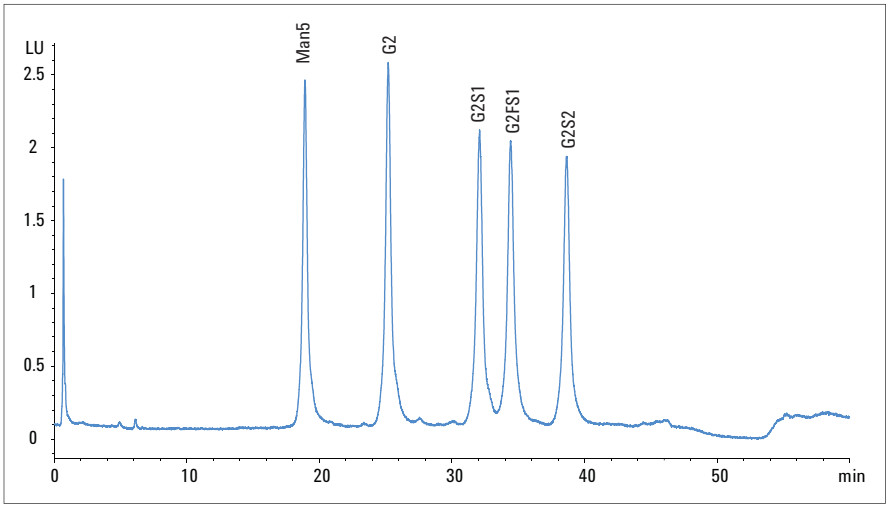


Figure 3
Mix of five AB-labeled N-linked glycan standards.

Limit of detections (LOD) were found between 10 and 15 fmol for S/N = 3. Limits of quantifications (LOQ) were found between 34 and 51 fmol for S/N = 10. All correlation curves showed good linearity with correlation coefficients over 0.999, see Table 3.

The PMT (photomultiplier tube) gain of the FLD detector was set to 14 for maximal sensitivity. In addition, using the Agilent 1260 Infinity Fluorescence Detector for the analysis of up to four different excitation and emission wavelengths, an optimal method development was possible, resulting in an excitation wavelength of 260 nm and an emission wavelength of 430 nm. This wavelength combination showed much higher intensity values compared to the wavelength normally used for glycan analysis (as, for example, recommended by Anumula 2005[11])

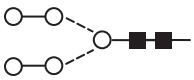
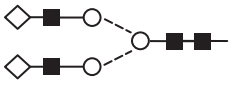
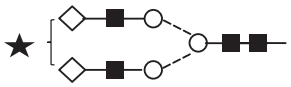
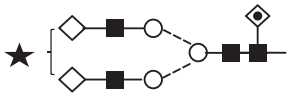
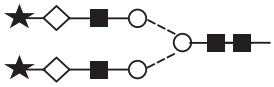
Glycan standard	Structure	LOD [fmol]	LOQ [fmol]	Correlation coefficient
Man5		10	34	0.9994
G2 (NA2)		11	37	0.9997
G2S1 (A1)		13	45	0.9998
G2FS1 (A1F)		14	47	0.9997
G2S2 (A2)		15	51	0.9993

Table 3
Linearity, LOD and LOQ for five AB-labeled N-linked glycan standards.

Conclusions

This Application Note demonstrates that the Agilent 1260 Infinity Bio-inert Quaternary LC System together with fluorescence detection is an ideal system for the sensitive and reproducible HPLC analysis of AB-labeled N-linked glycans derived from human IgG.

The analysis of a N-linked glycan library from human IgG showed good resolution for all major glycan structures. Precision of retention time was found to be below 0.16% respectively between 0.66% and 4.11% for area precision. All measured RSD values for RT and area were within the commonly reported range[10]. With regard to structure assignment software, as presented by Campbell *et al.*[12], high precision of retention time is extremely important for correct assignment of the glycan structures.

Linearity was determined with a mix of five glycan standards (Man5, G2, G2S1, G2FS1, and G2S2) diluted in series 1:3 in a range from 0.008 to 1 pmol. All correlation curves showed good linearity with correlation coefficients over 0.999. Limit of detections (LOD) were found between 10 and 15 and limits of quantifications (LOQ) were found between 34 and 51 fmol.

With optimized fluorescence wavelengths from the Agilent 1260 Infinity Fluorescence Detector, higher intensities and, therefore, better signal to noise ratios were achieved. Instead of an excitation wavelength of 330 nm (as recommended by Anumula 2005[12]) the lower wavelength 260 nm was used.

The Agilent 1260 Infinity Bio-inert Quaternary LC System combined with fluorescence detection provides an optimal system for sensitive and reproducible HPLC analysis of 2-AB derivatized N-linked glycans.

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Agilent Technologies

Reduced Ion Suppression and Improved LC/MS Sensitivity with Agilent Bond Elut Plexa

Application Note

BioPharma

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Abstract

In a comparison of SPE products, Agilent Bond Elut Plexa polymeric SPE reduces ion suppression for better sensitivity and low LOD and LOQ. Beta blockers are extracted with excellent correlation coefficients, recoveries, and % RSD.

Introduction

Ion suppression is often an issue in bioanalysis by LC/MS, resulting in poor recovery and inaccuracy, as well as increased instrument maintenance cost and time. Ion suppression cannot be avoided completely when biological samples are handled. However, it should be avoided wherever possible.

Agilent Bond Elut Plexa has a hydroxylated surface whereas other competitor's products have amide residues on the surface of the sorbent. The presence of amide residue can cause increased interaction between the SPE sorbent and the endogenous materials in biological samples, which can be directly responsible for ion suppression during bioanalysis. Due to hydroxylation of the sorbent's surface, Bond Elut Plexa reduces the interaction between the sorbent and endogenous materials in biological matrices to improve sensitivity. In this example, we present clear evidence of ion-suppression reduction and improved sensitivity with Bond Elut Plexa mono-dispersed polymeric SPE. The sample was human plasma spiked with beta blockers.

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Materials and Methods

SPE reagents and solutions

2% aqueous ammonia	Add 20 µL diluted NH_4OH to 1 mL H_2O
MeOH	Reagent grade or better
5% MeOH	Add 50 µL MeOH to 1 mL H_2O
50:50 MeOH:ACN	Add 1 mL MeOH to 1 mL ACN

SPE method

All samples were processed by the same SPE method.

SPE products	Agilent Bond Elut Plexa 96-well plate (10 mg) (p/n A4969010)
	Competitor W 96-well plate (10 mg)
	Competitor P 96-well plate (10 mg)
Sample	100 µL human plasma[1]
Pretreatment	Dilute with 300 µL 2% aqueous ammonia
Condition	1. 500 µL MeOH
	2. 500 µL H_2O
Load	400 µL diluted sample from pretreatment (actual plasma 100 µL)
Wash	500 µL 5% MeOH
Elute	Twice with 250 µL 50:50 ACN:MeOH

Experiment set up

For ion-suppression comparison, a drug compound mixture (50 ng/mL) was continuously infused by syringe pump at 20 µL/min. The blank plasma sample was injected. Blank plasma samples were prepared by Agilent Bond Elut Plexa and two other competitor's products based on the SPE methods specified above. MS transition 184 → 184 *m/z* was selected for lipid content monitoring during the analysis. Figure 1 shows the experimental set up.

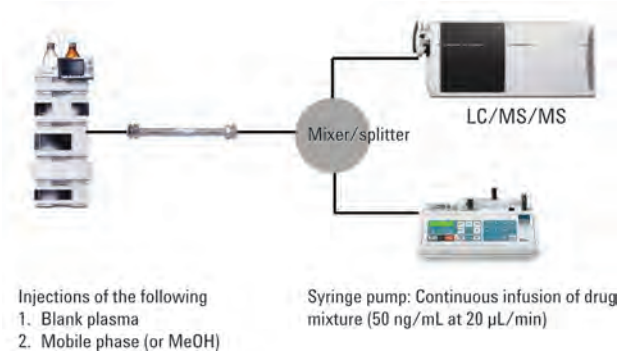

Figure 1. Schematic of experiment setup for ion-suppression comparison.

LC Conditions

Column	Agilent Poroshell 120 EC-C18, 2.1 mm × 5.0 mm, 2.7 µm (p/n 699775-902)
Instrument	Agilent 1260 Infinity LC/MS
Mobile phase A	0.1% formic acid in H_2O
Mobile phase B	0.1% formic acid in MeOH
Flow rate	0.4 mL/min
Injection volume	10 µL
Gradient	Time (min) %B 0 10 4.0 90 4.1 10 6.5 10
Temperature	sample (25 °C), column (ambient)
Ion-source	ESI+ with JetStream
Gas temperature	350 °C
Gas flow	10 L/min
Nebulizer	35 psi
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
Capillary	4000 V

Samples

Beta blocker	pKa	log P	MS/MS transition	Collision energy	Fragmentor
Acebutolol	9.40	1.71	337.2 → 116.1	20	128
Nadolol	9.67	0.81	310.2 → 254.1	12	92
Atenolol	9.60	0.16	267.2 → 190.1	12	92
Propranolol	9.42	3.48	260.2 → 116.2	16	92
Pindolol	9.25	1.75	249.2 → 116.1	12	92
Metoprolol (ISTD)	9.70	1.90	268.2 → 116.2	16	92

Results and Discussion

Good separation and retention between all analytes were obtained, as shown in Figure 2. Reduced ion-suppression delivered higher sensitivity resulted, as demonstrated in Figure 3. The figure shows an overlay of extracted MS chromatograms for nadolol from all three SPE products when blank plasma sample was injected and continuous infusion of drug mixture were executed simultaneously. The signal from Bond Elut Plexa was higher than from the other products during most of the analysis. Figure 4 shows an overlay of extracted MS/MS chromatogram 184 → 184 *m/z* for all three SPE products. The data show clearly that Bond Elut Plexa has reduced ion-suppression when compared to the others.

[1]For calibration and recovery, plasma was spiked with drug compounds of corresponding concentrations. For ion-suppression comparison, blank plasma samples were processed with SPE.

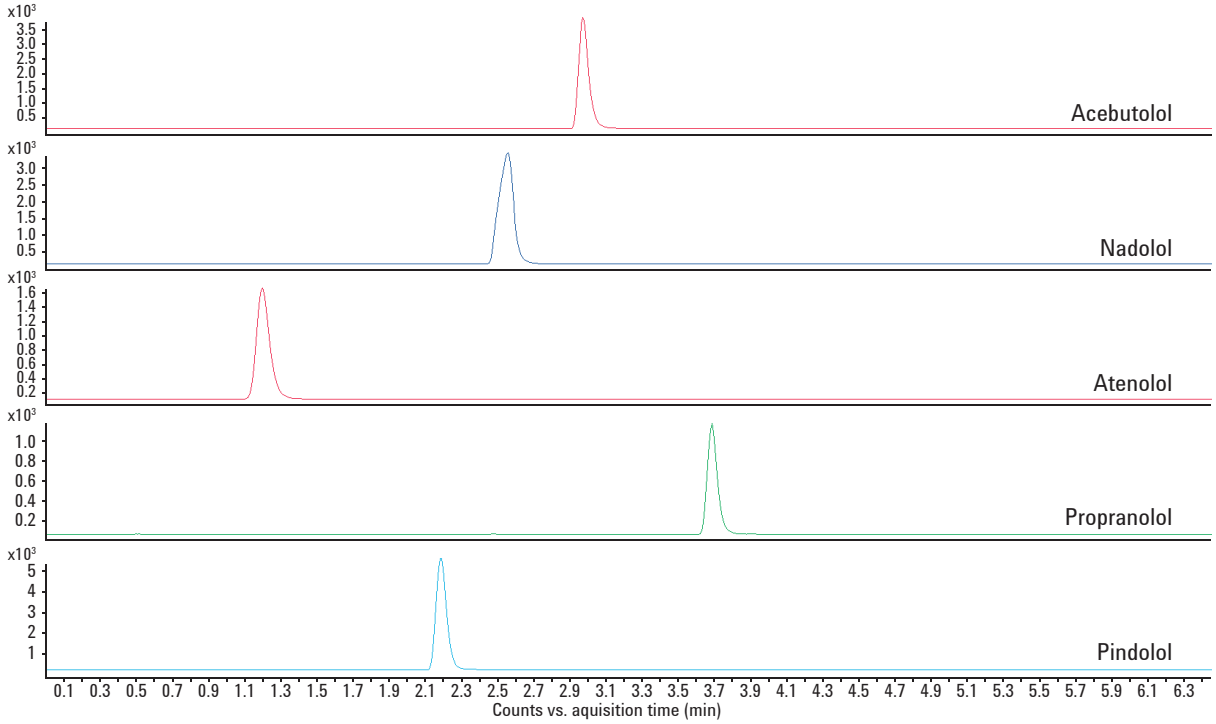

Figure 2. MS chromatogram of spiked plasma sample processed by Agilent Bond Elut Plexa (5 ng/mL of each analyte).

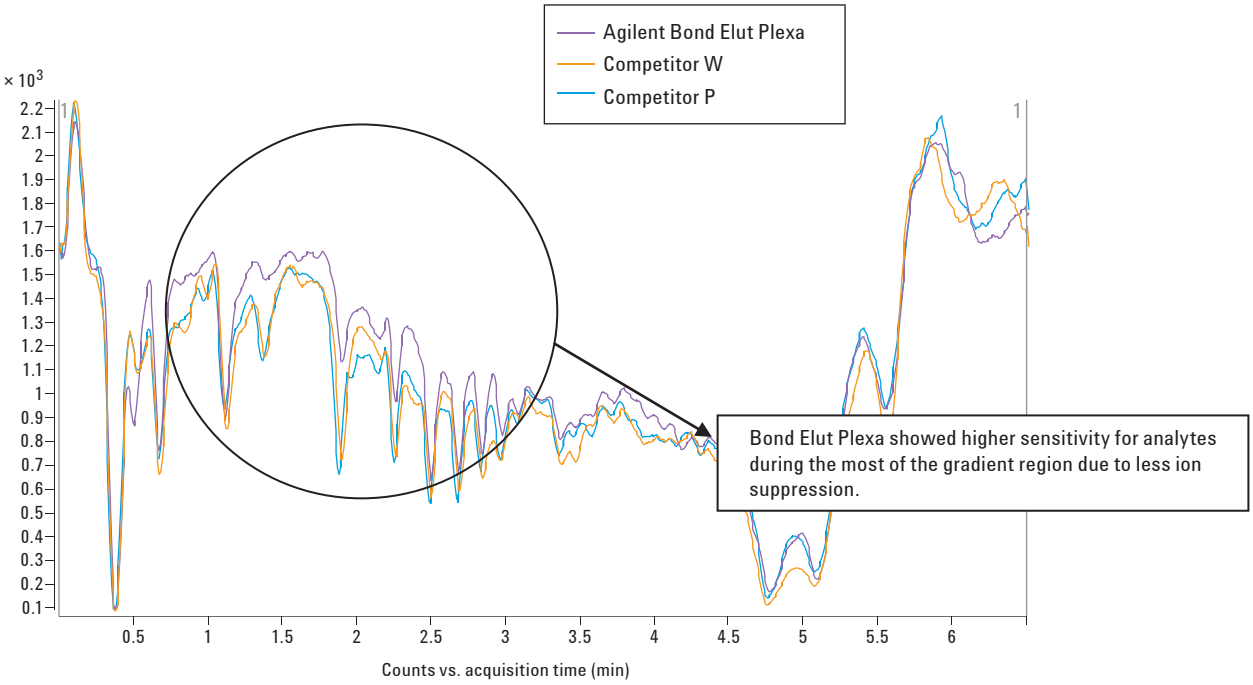

Figure 3. Overlay of the nadolol signal from simultaneous injection of blank plasma sample and continuous infusion of drug mixture, showing the superiority of Agilent Bond Elut Plexa.

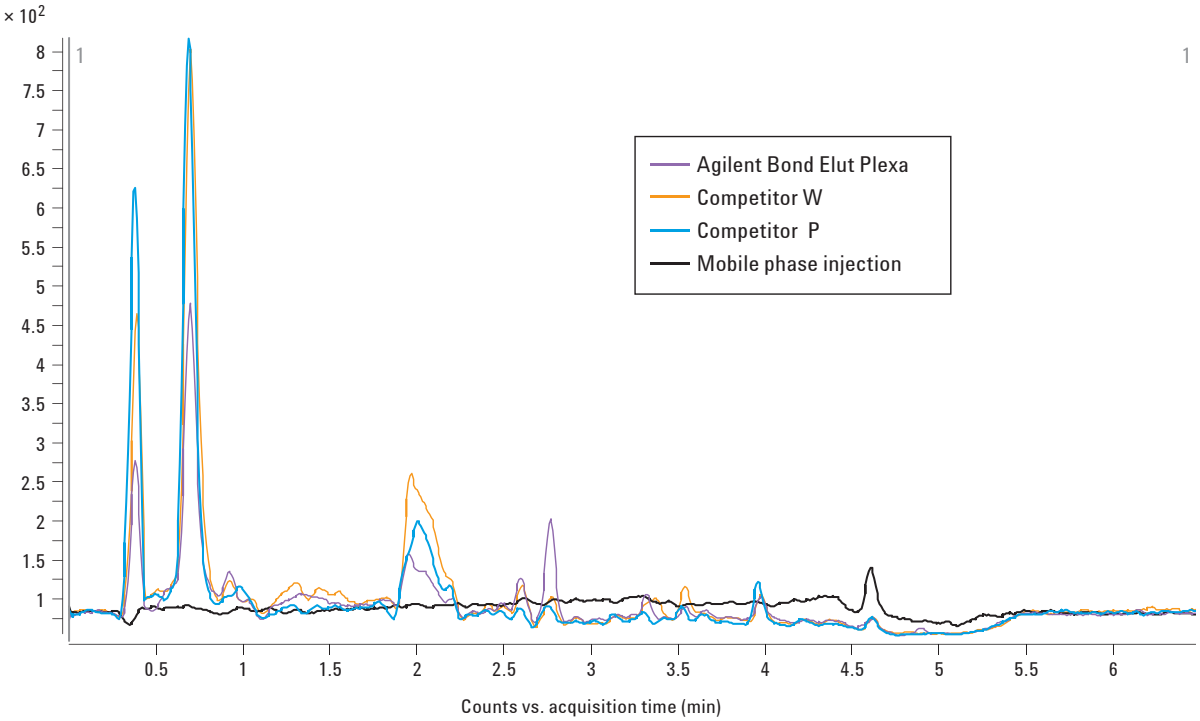

Figure 4. Lipid content monitoring of blank plasma sample injection by 184 → 184 m/z transition, with reduced ion suppression using Agilent Bond Elut Plexa.

Bond Elut Plexa achieved excellent limit of detection (LOD ≤ 0.05 ng/mL) and limit of quantitation (LOQ ≤ 0.5). A recovery experiment was performed at three different concentration levels (low, mid, and high, n = 6) and the data are summarized in Table 1, with excellent recovery and % RSD. Calibration curves were created with nine concentration levels (0.01 – 100 ng/mL) and all compounds showed good linearity with correlation coefficients $R^2 \geq 0.995$ (Figure 5).

Table 1. Results of a Recovery Experiment using AgilentBond Elut Plexa

					5 ng/mL		50 ng/mL		100 ng/mL		
	pKa	log P	LOD (ng/mL)	LOQ (ng/mL)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Correlation coefficient, R^2
Acebutolol	9.40	1.71	0.01	0.05	79.3	0.5	84.9	0.7	97.0	0.4	0.996
Nadolol	9.67	0.81	0.01	0.05	98.5	0.8	94.7	1.4	108.1	0.8	0.997
Atenolol	9.60	0.16	0.05	0.5	119.7	2.9	104.0	2.5	109.0	4.5	1.000
Propranolol	9.42	3.48	0.05	0.5	106.2	3.7	109.9	7.3	126.9	9.7	0.995
Pindolol	9.25	1.75	0.01	0.05	111.6	1.3	106.0	3.0	115.1	2.8	0.998

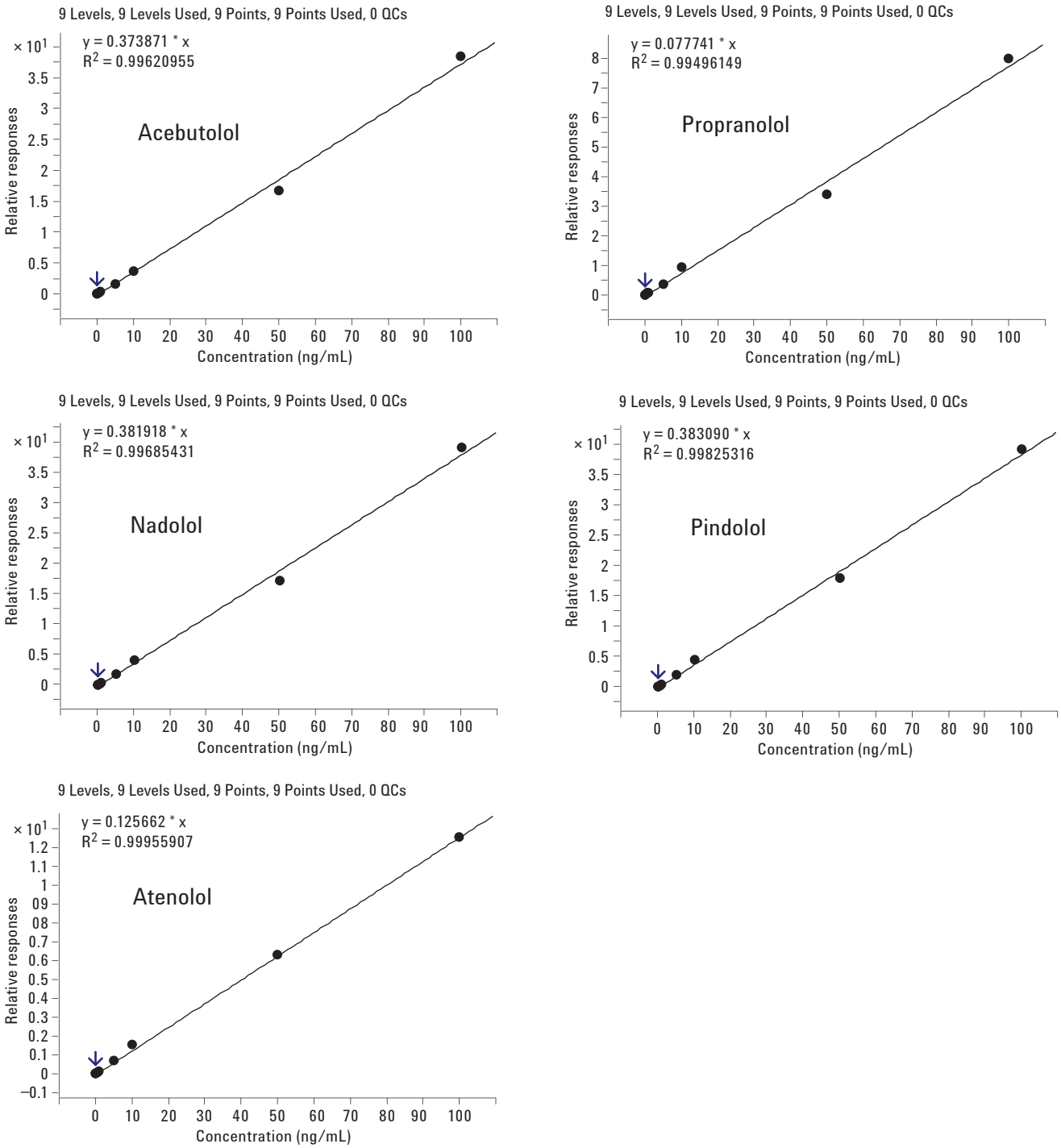

Figure 5. Calibration curves of five beta blockers at nine concentration levels (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 ng/mL).

Conclusion

Agilent Bond Elut Plexa reduced ion suppression when compared to other SPE products, resulting in better sensitivity. The improved sensitivity delivered low LOD (0.01 – 0.05 ng/mL) and LOQ (0.05 – 0.5 ng/mL). In addition, excellent correlation coefficients ($R^2 \geq 0.995$) and good recovery data were obtained with very good % RSD.

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Agilent Bond Elut Plexa PCX – Cation Exchange SPE

A Destination to a Better Sensitivity in LC/MS Bioanalysis Resulting from Minimized Ion-Suppression

Application Note

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Introduction

Throughout the drug development process in pharmaceutical industry, it is of essence to develop and validate fast methods for bioanalysis without losing sensitivity. Ion-suppression often can be the most commonly encountered issue in achieving that goal, which causes low recovery, inaccuracy, as well as increased instrument maintenance cost and time. While ion-suppression cannot be fully avoided when biological samples are handled, it should be avoided as much as possible.

The nature of hydroxylated surface on Agilent Bond Elut Plexa PCX makes it stand out among other cation exchange SPE products with amide residue on the surface of the sorbent. The presence of amide residue causes increased interaction between the SPE sorbent and the endogenous material in biological sample, which can be directly responsible for ion-suppression during bioanalysis. Due to hydroxylation of the sorbent's surface, Bond Elut Plexa PCX reduces the interaction between the sorbent and the endogenous material in the biological matrices, hence, they achieve improved sensitivity. The following experiment shows clear evidence of ion-suppression reduction and improved sensitivity with Bond Elut Plexa PCX, mono-dispersed polymeric SPE.

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Materials and Methods

SPE reagents and solutions

2% H_3PO_4	Add 20 µL H_3PO_4 to 1 mL H_2O
2% formic acid	Add 20 µL formic acid to 1 mL H_2O
MeOH	Reagent grade or better
50:50 MeOH:ACN	Add 1 mL MeOH to 1 mL ACN
5% ammonia in 50:50 MeOH:ACN	Add 50 µL diluted NH_4OH to 1 mL 50:50 MeOH:ACN

SPE Method

All samples were processed by the same SPE method.

SPE products	Agilent Bond Elut Plexa PCX 96-well plate (10 mg) (p/n A4968010)
	Competitor W 96-well plate (10 mg)
	Competitor P 96-well plate (10 mg)
Sample	100 µL human plasma[1]
Pretreatment	Dilute with 300 µL 2% H_3PO_4
Conditions	1. 500 µL MeOH
	2. 500 µL H_2O
Load	400 µL diluted sample from pretreatment (actual plasma amount 100 µL)
Wash	1. 500 µL 2% formic acid
	2. 500 µL 50:50 ACN:MeOH
Elute	2 × 250 µL 5% ammonia in 50:50 ACN:MeOH

Experiment Design

For ion-suppression comparison, drug compound mixture (50 ng/mL) was continuously infused by a syringe pump at 20 µL/min while a blank plasma sample was injected. Blank plasma samples were prepared by Agilent Bond Elut Plexa PCX and two competitor's products based on the SPE methods specified in the previous section. MS transition 184 → 184 was selected for lipid contents monitoring during the analysis.

LC Conditions

Column	Agilent Poroshell 120 EC-C18, 2.1 × 5.0 mm, 2.7 µm (p/n 699775-902)	
LC/MS	Agilent 1260 Infinity LC/MS	
A	0.1% formic acid in H_2O	
B	0.1% formic acid in MeOH	
Flow rate	0.4 mL/min	
Injection volume	10 µL	
Gradient	Time (min)	%B
	0	10
	4.0	90
	4.1	10
	6.5	10
Temperature	sample (25 °C), column (ambient)	
Ion-source	ESI+ with JetStream	
Gas temperatue	350 °C	
Gas flow	10 L/min	
Nebulizer	35 psi	
Sheath gas temperature	400 °C	
Sheath gas flow	12 L/min	
Capillary	4000 V	

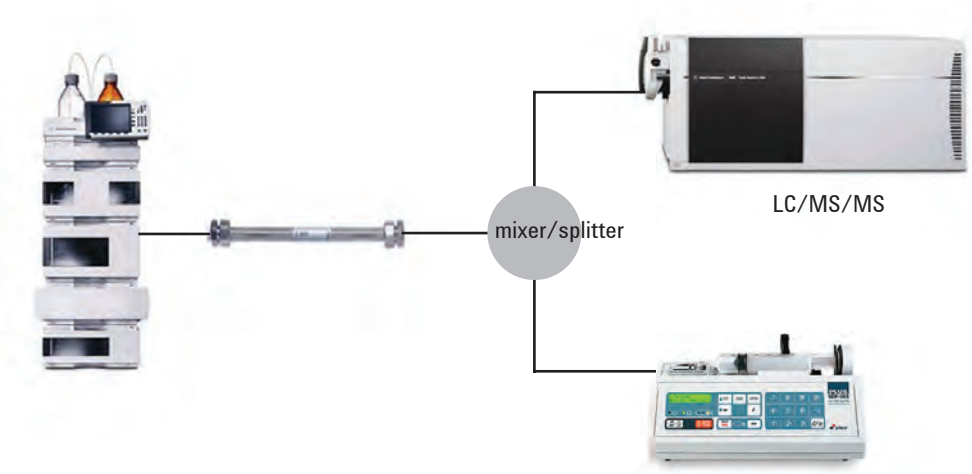


Figure 1. Schematic of ion-suppression comparison experiment setup.

1. For calibration and recovery, plasma was spiked with drug compounds of corresponding concentrations. For ion-suppression comparison, blank plasma samples were processed with SPE.

Table 1. Samples

	pKa	log P	MS/MS transition	Collision energy	Fragmentor
Acebutolol	9.40	1.71	337.2 → 116.1	20	128
Ranitidine	8.20	0.27	315.2 → 176.1	12	92
Nadolol	9.67	0.81	310.2 → 254.1	12	92
Atenolol	9.60	0.16	267.2 → 190.1	12	92
Propranolol	9.42	3.48	260.2 → 116.2	16	92
Procainamide	9.32	0.88	236.2 → 120.1	16	92
Metoprolol (ISTD)	9.70	1.90	268.2 → 116.2	16	92

Results and Discussion

Good separation and retention among all analytes were achieved and shown in Figure 2. Chromatograms shown in Figure 3 were obtained during continuous infusion of drug mixture with blank plasma sample injections processed by each SPE product. The data show clearly that Agilent Bond Elut Plexa PCX has reduced ion-suppression when compared to its competitive SPE products.

Excellent limit of detection (LOD) and limit of quantitation (LOQ) were achieved with Bond Elut Plexa PCX. A recovery experiment was performed at three different concentration levels (low, mid, and high, n = 6) and the data are shown in Table 1 with excellent recovery and % RSD. All compounds showed good linearity with correlation coefficients $R^2 \geq 0.995$ (Figure 4).

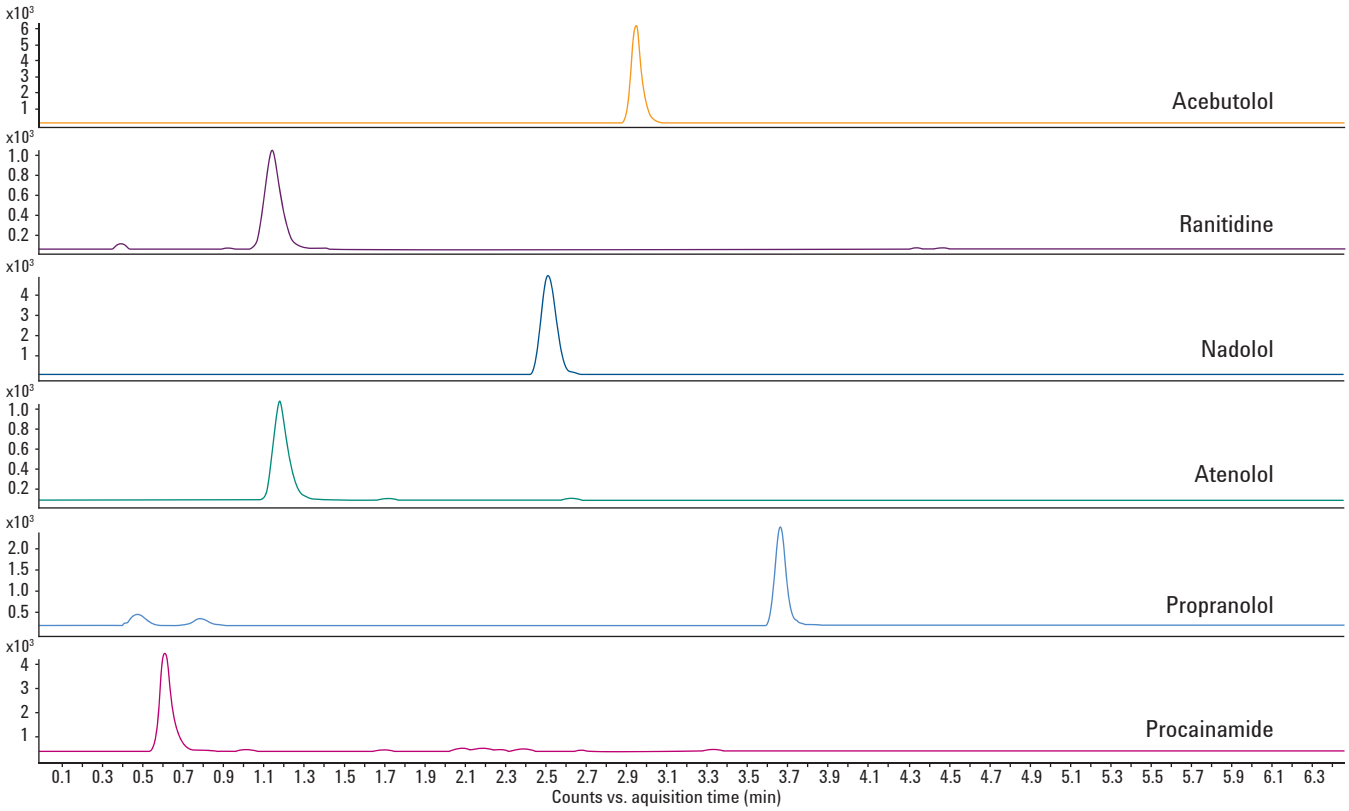


Figure 2. MS chromatogram of spiked plasma sample processed by Agilent Bond Elut Plexa PCX (5 ng/mL each).

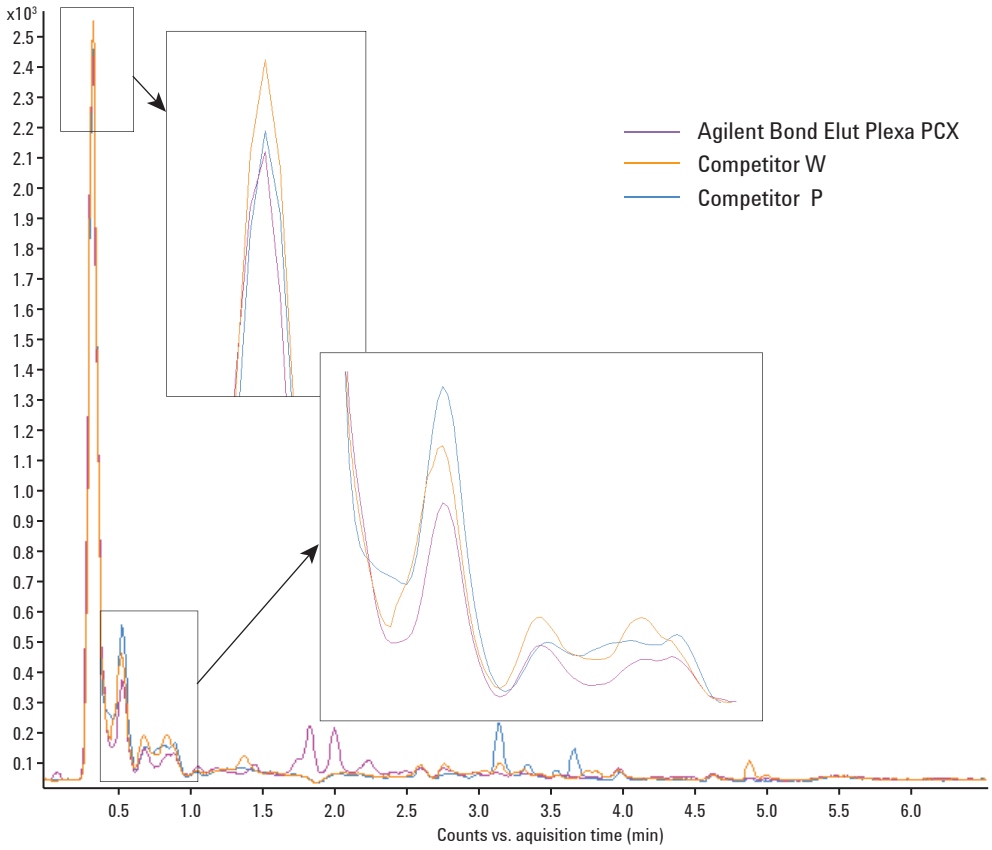
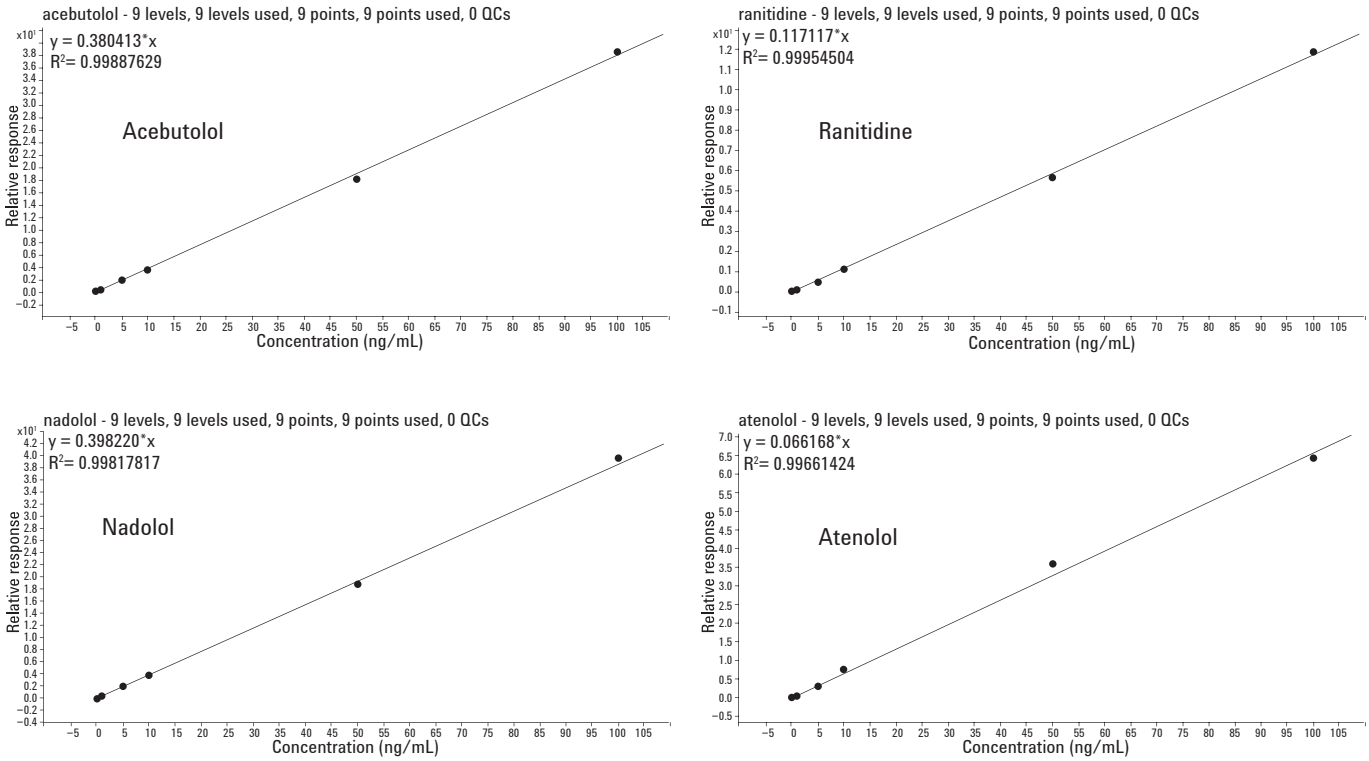


Figure 3. Lipid contents monitoring of blank plasma sample injection by 184 → 184 m/z transition.



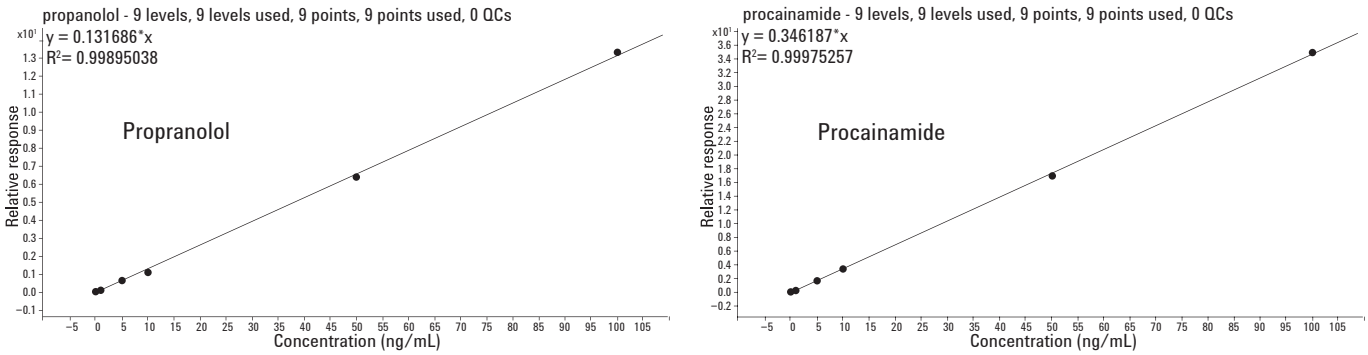


Figure 4. Calibration curves of six beta blockers at nine concentration levels (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 ng/mL).

Table 2. Agilent Bond Elut Plexa PCX Data Summary

					5 ng/mL		50 ng/mL		100 ng/mL		
	pKa	log P	LOD (ng/mL)	LOQ (ng/mL)	Recovery	% RSD	Recovery	% RSD	Recovery	% RSD	Correlation coefficient, R^2
Atenolol	9.60	0.16	0.05	0.1	109.0	1.2	95.6	2.3	95.5	3.3	0.997
Nadolol	9.67	0.81	0.01	0.05	110.8	1.4	120.7	1.5	95.4	1.6	0.998
Acebutolol	9.40	1.71	0.01	0.1	113.9	0.9	108.6	2.0	98.7	2.4	0.999
Propranolol	9.42	3.48	0.05	0.1	120.2	1.1	103.5	2.7	93.6	2.5	0.999
Procainamide	9.32	0.88	0.05	0.1	93.0	2.1	104.5	1.8	96.9	3.9	1
Ranitidine	8.20	0.27	0.05	0.1	90.7	1.9	96.4	2.7	91.1	3.9	1

Conclusion

Agilent Bond Elut Plexa PCX showed reduced ion-suppression when compared to their competitive SPE products. Low LOD (0.01 – 0.05 ng/mL) and LOQ (0.05 – 0.5 ng/mL) were obtained resulted from minimized ion-suppression. Excellent correlation coefficients ($R^2 \geq 0.995$) and good recovery data were obtained with very good % RSD as well.

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The Influence of Silica Pore Size and Particle Size on Insulin – A Small Protein Molecule Separation

Application Note

Biopharm

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Abstract

Insulin, a small protein molecule, was separated on columns with different pore size and particle sizes. The efficiency and resolution was compared among columns with different pore sizes including 80Å, 95Å, 120Å, 170Å, and 300Å and different particle sizes including 1.8 µm, 2.7 µm, 3.5 µm, and 5 µm.

The comparison shows that a larger pore size gives higher efficiency for insulin analysis. A pore size larger than 100Å is enough for the efficient separation of insulin and a macro pore size of 300Å is not necessary for such a moderate molecular weight molecule. A small particle size gives higher efficiency for insulin analysis. This is demonstrated by changing the particle size from 5 µm to 3.5 µm then to 1.8 µm.

The Agilent Poroshell 120 columns combination of pore size (120Å) and superficialy porus particle size (2.7 µm), makes it the best choice for insulin analysis.

Agilent Technologies

Introduction

Particle size and pore size of silica are very important parameters of a reversed phase column when chosen for HPLC analysis. Small particle size columns, those with particles less than 3 µm including sub-2 µm particles, have been widely used for achieving high performance and fast separations. Additionally, the pore size of silica particles should be considered when a column is selected. For molecules with molecular weights less than 5000 Da, a column with a small pores size (60-120Å) is typically selected, a pore size of 200-300Å is typically used for low molecular weight proteins (5 kDA – 50 kDa), and larger pore sizes (1000Å-4000Å) are used for very high molecular weight proteins and vaccines.

A small protein, such as insulin with a molecular weight of about 5800 Da, could be analyzed on columns with small pores, but larger pore size should be evaluated. The proper pore size choice maximizes efficiency. Too small a pore size and the molecule experiences restricted diffusion in and out of the pores. With an appropriate pore size, higher efficiency is seen. To maximize efficiency, smaller particle sizes with an optimum pore size should be selected.

A regulatory method (USP) for insulin with an isocratic mobile phase was used in this study of optimum pore and particle size. The performance, including efficiency and resolution, is compared among columns with different pore sizes including 80Å, 95Å, 120Å, 170Å, and 300Å and different particle sizes including 1.8 µm, 2.7 µm, 3.5 µm, and 5 µm. These choices also include different particle types, including both superficially porous and totally porous particles. The end result of this application note is recommendations for achieving the highest efficiency and resolution while still meeting the requirements of the regulatory methods of the China Pharmacopeia [1] and USP [2].

Materials and Methods

HPLC conditions

Columns	All columns were C18 columns, meeting pharmacopeia definitions for octadecyl silane (C18) chemically bonded to porous silica
Flow rate	1.0 mL/min
Injection volume	20 µL
Column temp	40 °C
Wavelength	214 nm
Mobile phase	74% A:26% B, where A: 0.2 mol/L sulfate (Dissolve 28.4 g anhydrous sodium sulfate in 1000 mL of water, pipet 2.7 mL of phosphoric acid into the solution, and adjust with ethanolamine to a pH of 2.3, and mix) B: acetonitrile

Materials

Sample

Porcine insulin (Provided by NIFDC China)

Columns

Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm (p/n 883975-902)
Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm (p/n 959993-902)
Agilent ZORBAX 300SB-C18, 4.6 × 150 mm, 5 µm (p/n 883995-902)
Agilent TC-C18(2), 4.6 × 150 mm, 5 µm (p/n 588935-902)
Agilent ZORBAX SB-C18, 4.6 × 100 mm, 3.5 µm (p/n 861953-902)
Agilent ZORBAX Eclipse Plus C18, 4.6 × 100 mm, 3.5 µm (p/n 959961-902)
Agilent ZORBAX SB-C18, 4.6 × 100 mm, 1.8 µm (p/n 828975-902)
Agilent ZORBAX Eclipse Plus C18, 4.6 × 100 mm, 1.8 µm (p/n 959964-902)
Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm (p/n 685975-902)
Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7 µm (p/n 695975-902)

System

The Agilent 1200 SL LC system includes a binary pump, a thermostatted column compartment (TCC), a high performance autosampler and a diode array detector (DAD).

Results and Discussion

Influence of silica pore size on efficiency and resolution

To find out how the pore size of silica influences the efficiency, resolution, and tailing factor of the insulin separation, the same method for insulin was run on four traditional columns with a 5 µm particle size (Figure 1), but with four different pore sizes. Insulin has the poorest performance both on the SB-C18, 5 µm and the Eclipse Plus C18, 5 µm columns with the smallest pore sizes of 80Å and 95Å. A slight increase of the pore size from 80Å to 95Å provided a minimal increase in the efficiency of insulin.

The Agilent TC-C18(2) and Agilent ZORBAX 300SB-C18 columns both have a larger pore size and gave higher efficiency for insulin. Columns with a 300Å are often used for a molecular weight of more than 5000 Da. As seen in Figure 1, the efficiency of insulin more than doubled when comparing the results from the columns with <100Å pore sizes to a column with a 300Å pore size. The peak shape also improved dramatically between the smallest pore size column, (Agilent ZORBAX SB-C18) and the largest pore size column, (300SB-C18). These two columns differ only in pore size, the type of bonding and particle size of the columns are the same. This provides the most exact comparison and shows clearly that a pore size larger than 80Å is needed for the most efficient separation of insulin.

The fourth column compared, the Agilent TC-C18(2) has a 170Å pore size that is intermediate between the 80Å, 95Å, and 300Å pore sizes. The efficiency on this column was also more than double the efficiency of the smaller pore size columns, indicating that a 300Å pore size is not necessary, but a pore size >100Å is needed for insulin molecule at 5800 Da to fully access the pores for an efficient separation.

When we compare the four 5 µm columns in more detail, we see some differences in retention. These four columns have different surface area and types of bonding. The 300SB-C18 column has the lowest surface area at about 45 m^2/g, followed by the Agilent ZORBAX Eclipse Plus C18 at 160 m^2/g, then SB-C18 at 180 m^2/g, and finally the TC-C18(2) at 290 m^2/g. The retention is most similar on the two columns with the smallest pore sizes, the Eclipse Plus C18 and the SB-C18, however these are not the lowest surface area. Without being able to fully access the pores and the bonded phase, the retention is limited. The C18 bonding on the Eclipse Plus C18 and the SB-C18 is very different. The SB-C18 has a low carbon load and no endcapping. The Eclipse Plus C18 has a higher carbon load, with denser bonding and thorough endcapping. While this change in the bonding helps improve peak shape it does not change retention because the insulin is not interacting much with the bonded phase in the pores.

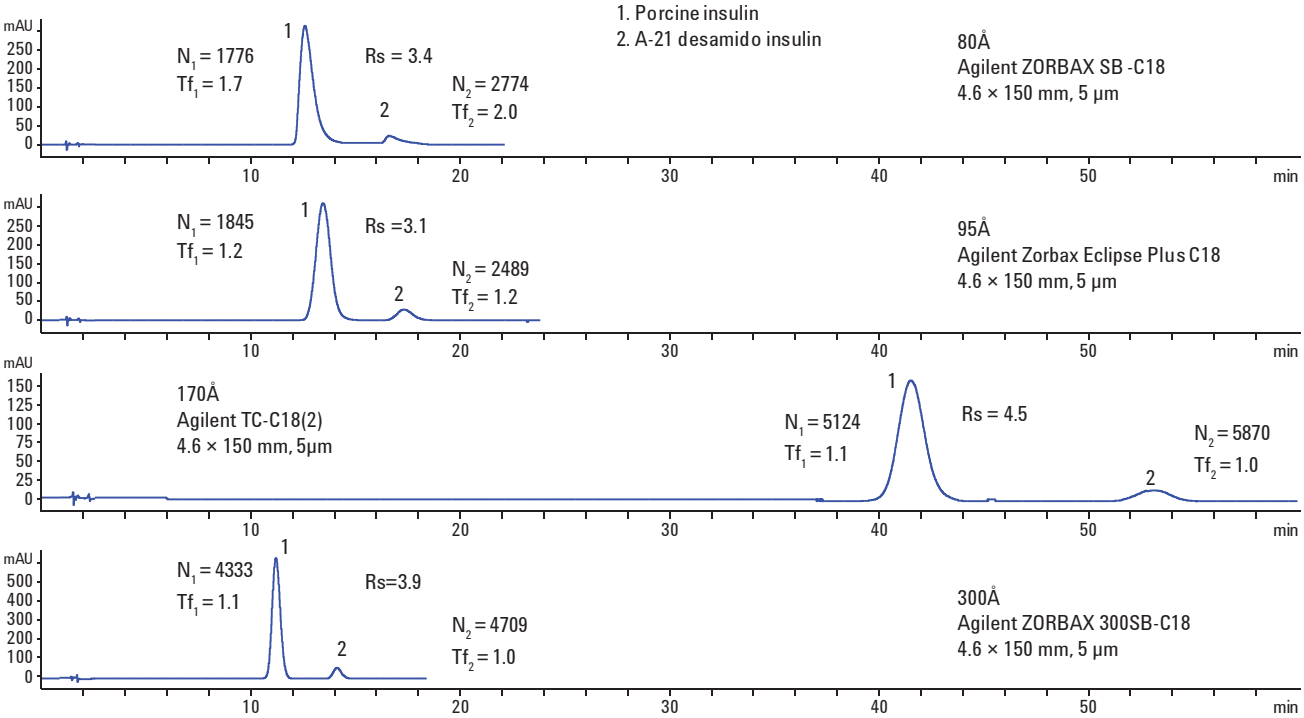

Figure 1. Chromatograms on 4.6 × 150 mm, 5 µm columns with different pore size.

The Agilent ZORBAX 300SB-C18 column has the lowest surface area, but the retention is only slightly less than the small pore size columns. Once the insulin can access the pores and interact with all of the bonded phase in the pores it is better retained. This more efficient access is also seen in the improved peak shape, even on a non-endcapped bonded phase. The Agilent TC-C18(2) column has the highest surface area and the strongest retention, most likely due to accessing all the pores and retention increasing with this higher surface area of silica particles (290 m^2/g).

The retention of the Agilent TC-C18(2) column is very long, at over 40 minutes, and not ideal. Therefore the method on the TC-C18(2) column could be modified with a higher organic component in the mobile phase to substantially reduce the retention time, while still maintaining good peak shape and efficiency (Figure 2). The retention is reduced from 44 minutes to 15 minutes by increasing the organic in the mobile phase by only 1.5%. When organic content in the mobile phase is changed, large molecules (such as proteins) show a much greater change in retention than small molecules, so it is easy to adjust the retention time substantially. The resolution is slightly reduced, but similar to the other 5 µm columns once the retention matches the other columns.

The result of comparing these four 5 µm columns is that the 170Å pore size is large enough to provide access to the pores and results in an efficient separation. Pore sizes >100Å are too small, and for a mid molecular weight compound a pore size of 300Å is not necessary.

Table 1. Theoretical Plates per M on 5 µm Columns with Different Pore Sizes

N/m	80Å	95Å	170Å	300Å
Peak 1	11840	12300	34160	28887
Peak 2	18493	16593	39133	31393

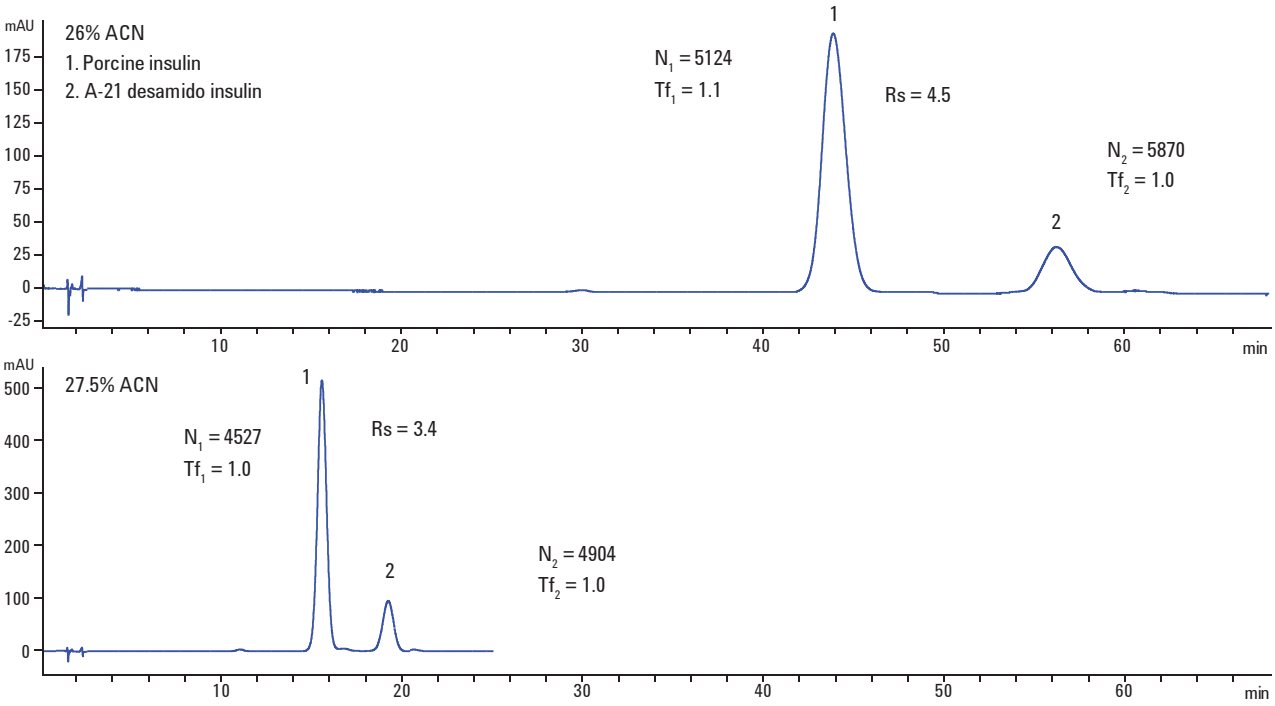

Figure 2. Organic phase modification on an Agilent TC-C18(2), 4.6 ×150 mm, 5 µm column.

Influence of silica particle size

For method development, the standard particle size for HPLC columns was 5 µm until the mid-1990s, when 3.5 µm increased in popularity. More recently, as higher speed and higher resolution is required, chromatographers often select packings with particle sizes less than 3 µm or 2 µm, such as 1.8 µm particles.

The insulin method was run on 5 µm, 3.5 µm, and 1.8 µm Agilent ZORBAX SB-C18 columns and Agilent ZORBAX Eclipse Plus C18 columns. To make sure the data are comparable with different length of columns, the theoretical plate counts were all divided by the length of column to represent the efficiency in plates/meter (N/m). The data in Tables 2 and 3 shows improved efficiency using the smaller 1.8 µm particle columns. While these columns are not the ideal pore size columns, efficiency should still be expected to improve with smaller particle sizes, as it does here with insulin.

Table 2. Theoretical Plates per M on an Agilent ZORBAX SB-C18 with Different Particle Sizes

N/m	SB-C18 5 µm	SB-C18 3.5 µm	SB-C18 1.8 µm
Peak 1	11840	18060	52150
Peak 2	18493	21720	66910

Table 3. Theoretical Plates per M on an Agilent ZORBAX Eclipse Plus C18 Columns with Three Different Particle Sizes

N/m	Plus 5 µm	Plus 3.5 µm	Plus 1.8 µm
Peak 1	12300	21570	78540
Peak 2	14580	26040	94340

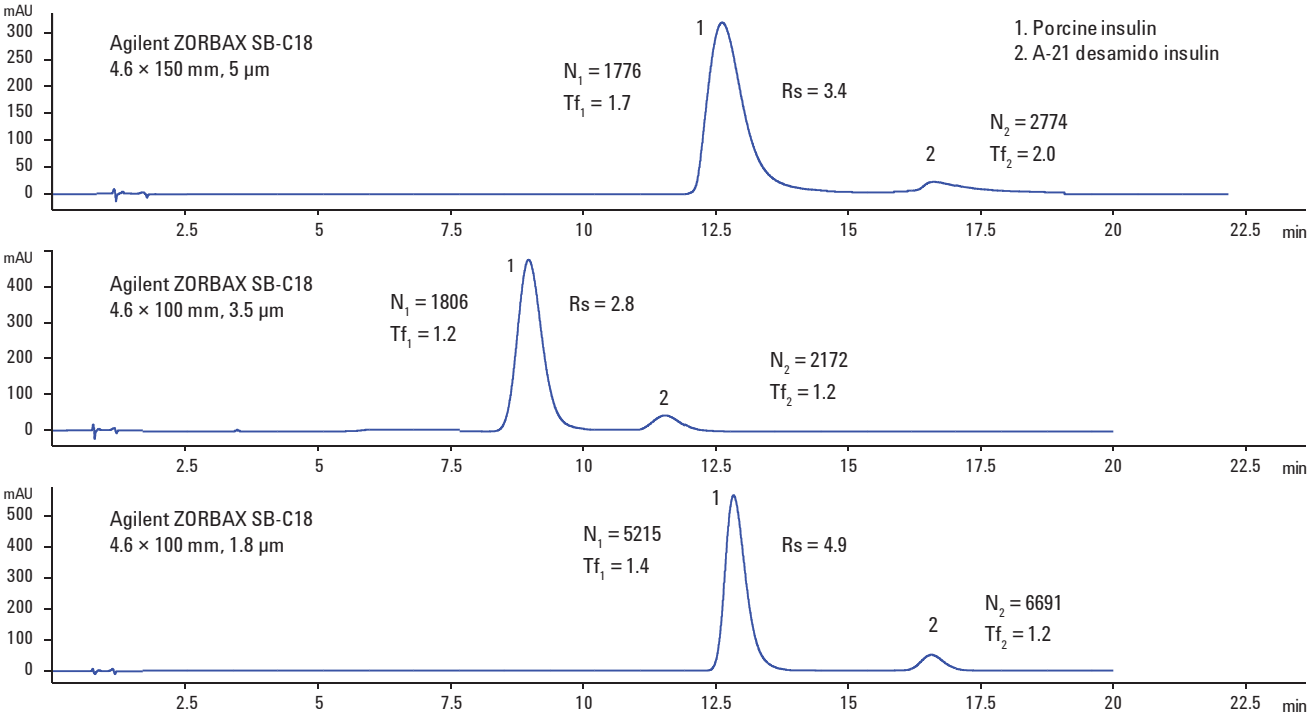

Figure 3. Chromatograms on Agilent ZORBAX SB-C18 columns with different particle sizes.

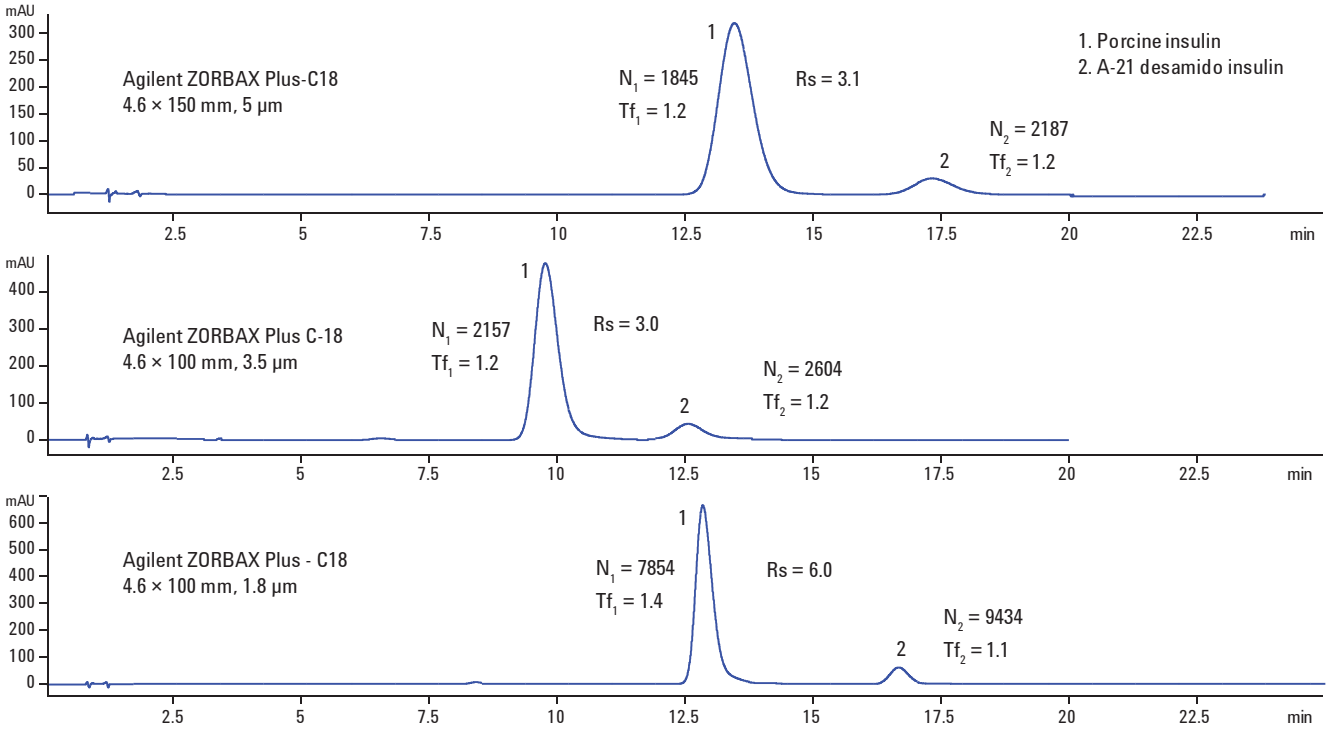

Figure 4. Chromatograms on Agilent ZORBAX Eclipse Plus C18 columns with three different particle sizes.

Comparison between superficially porous Agilent Poroshell 120 2.7 µm and totally porous sub-2 micron columns

The columns compared thus far have all been totally porous columns, most available in a range of pore and particle sizes. The Agilent Poroshell 120, 2.7 µm superficially porous particle column, has a 120Å pore size and a sub 3 µm particle size. The 120Å pore size is >100Å but smaller than the 170Å pore size previously compared. In addition, the 2.7 µm particle size is known to provide similar performance to the sub-2 micron particle columns for small molecules (less than 2000 Da). For molecules around 5000 molecular weight, like insulin, a comparison is needed.

Figure 5 shows the direct comparison of two Poroshell 120 columns with different bonded phases to the closest matching 1.8 µm particle size columns, the Agilent ZORBAX SB-C18 and Agilent ZORBAX Eclipse Plus C18. In these chromatograms, the Poroshell 120 SB-C18 120Å column provides double the efficiency of the SB-C18 80Å. This is due to the larger pore size and more rapid diffusion in the 120Å pores. Switching from the Agilent ZORBAX Eclipse Plus C18 to Agilent Poroshell 120 EC-C18 also provided an increase in efficiency with the change from 95Å to 120Å pore size. In addition, the peak shape of insulin on both Poroshell 120 columns was improved with greater access to the pores (Figure 5). The data in Table 4 provides the theoretical plates per meter (N/m) of the 2.7 µm and 1.8 µm particle sized and shows the increase when using a Poroshell 120 column.

The pressure of the 1.8 µm columns is around 250 bar. The pressure of the Poroshell 120 columns is below 200 bar. Both kinds of column can be used on standard instruments, but Porshell 120 columns are more suitable for standard instruments because they have about 30% lower pressure than the 1.8 µm columns.

Figure 6 shows the Agilent Poroshell 120 SB-C18 column provides higher efficiency than the 5 µm Agilent TC-C18(2). The chromatogram shown for the TC-C18(2) column was run with 27.5% organic in the mobile phase so that the retention times could be more closely matched. The improvement in the efficiency when comparing these two columns is due to the benefit of the smaller particle size with a larger pore size.

Table 4. Theoretical Plates per M on an Agilent Poroshell 120 and Sub-2 µm Columns

N/m	SB-C18, 1.8 µm	Poroshell 120, SB-C18, 2.7 µm	Plus C18, 1.8 µm	Poroshell 120, EC-C18, 2.7 µm
Peak 1	52150	108600	78540	97680
Peak 2	66910	123770	94340	107100

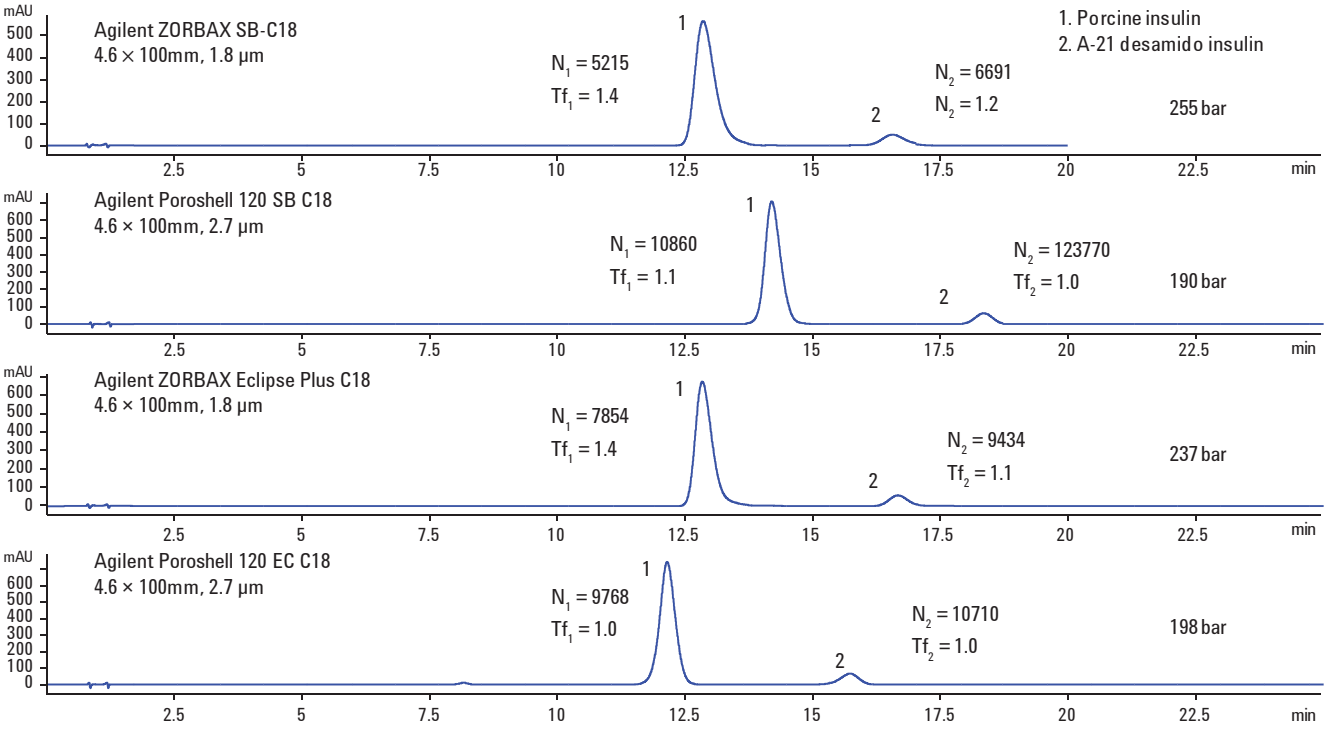

Figure 5. Chromatograms for comparison between Agilent Poroshell 120 and sub-2 micron columns.

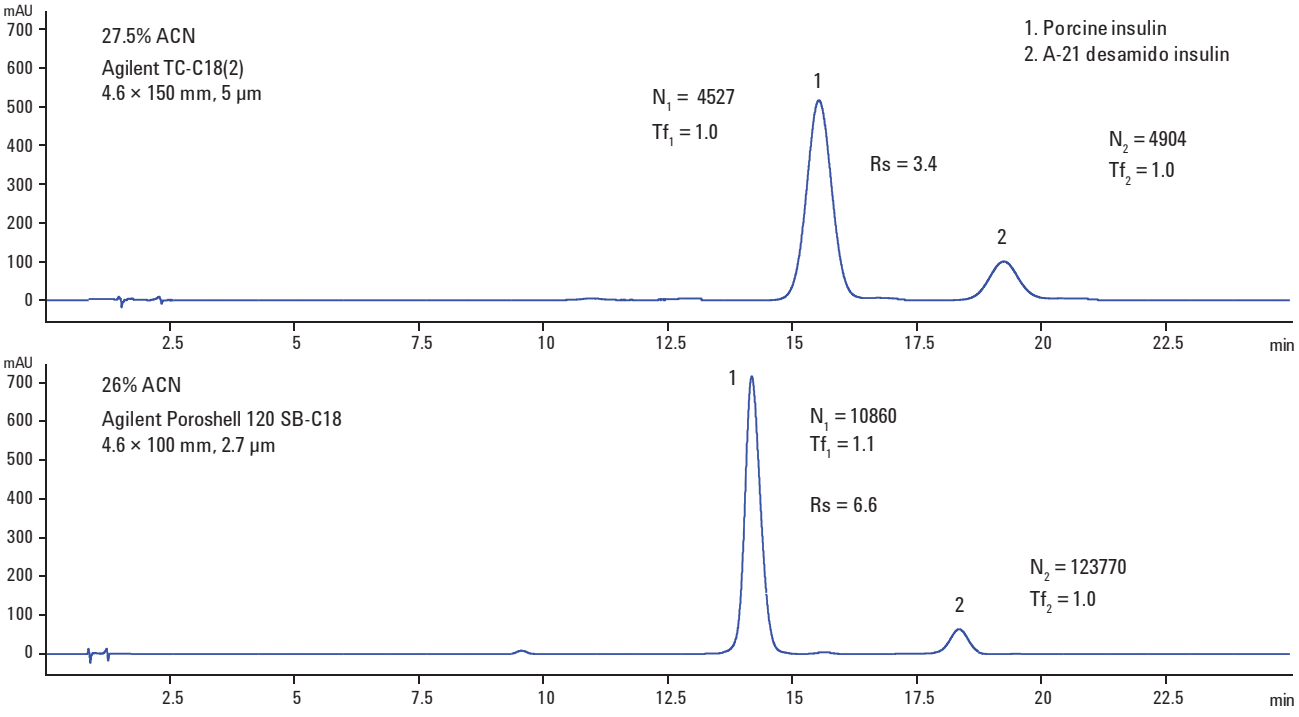

Figure 6. Chromatograms for comparison between Agilent Poroshell 120 SB-C18 and Agilent TC-C18(2) columns.

The remaining obvious column comparison would be between the sub-2 µm particles with 300 Å pore size in the 300SB-C18 material and the 80Å SB-C18. This is something that will be done in the future with the mobile phase called for in this method, but under different conditions, insulin has been shown to have good performance on this column [3].

Conclusion

The comparisons shown here were done to determine what type of C18 columns could effectively be used for the analysis of insulin according to pharmacopeia methods. Columns with too small a pore size, <100Å did not provide the best results. The efficiency on these columns was low and the tailing factors were a little high due to the restricted access to the bonded phase in the pores of these columns. Columns with a larger pore size, >100Å, such as the Agilent Poroshell 120, Agilent TC-C18(2), and Agilent ZORBAX 300SB-C18 provided much higher efficiency and lower tailing factors. A pore size as large as 300Å was not needed for an efficient separation of insulin. The intermediate pore size columns, Poroshell 120 and TC-C18(2), were suitable and would be a good choice for separations of other small proteins or peptide mapping.

Particle size was evaluated to compare efficiency and resolution. Smaller particle sizes provided the highest efficiencies. The Agilent Poroshell 120 columns delivered the most efficiency. These columns had a 2.7 µm particle size with a pore size of 120Å and are suitable for the highly efficient analysis of insulin.

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2. The United States Pharmacopoeia USP 31 (vol 2) Insulin, 2403–2404.
3. Phu T Duong, Analysis of Oxidized Insulin chains using Reversed Phase Agilent ZORBAX RRHD 300 SB-C18, Agilent application note, 5990-7988EN.

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Optimum Pore Size for Characterizing Biomolecules with Agilent Bio SEC Columns

Application Note

BioPharma

Author

Keeley Mapp

Agilent Technologies, Inc.

Introduction

Size exclusion chromatography (SEC) is a technique for separating proteins, oligonucleotides, and other complex biopolymers in their native forms by size using aqueous eluents. For molecules of discreet molecular weight, such as proteins, SEC can be used to detect and quantitate monomers, dimers, aggregates and fragments.

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Pore size choice

The choice of media pore size will influence the resolution in SEC. As the separation is based on differences in molecular size in solution, the sample must be able to permeate the porous structure of the particles. If the pore size is too small the samples will be excluded from the pores and elute in the void volume of the column, and if too large, all will be able to fully permeate the particles and there will be very little separation. This application note demonstrates the effect of pore size on separations of proteins and antibody using Agilent Bio SEC-3 HPLC columns. These columns are available in 100Å, 150Å, and 300Å pore sizes to accommodate most peptide and protein size exclusion separations.

Protein separation

For this experiment, we used a standard protein mix that covers the typical range of characteristics of recombinant biopharmaceuticals.

Conditions

Columns	Agilent BioSEC-3 100Å, 4.6 x 300 mm, 3 µm (p/n 5190-2503) Agilent BioSEC-3 150Å, 4.6 x 300 mm, 3 µm (p/n 5190-2508) Agilent BioSEC-3 300Å, 4.6 x 300 mm, 3 µm (p/n 5190-2513)
Sample	Bio-Rad Protein Standards Mix
Eluent	100 mM Sodium phosphate buffer + 0.15 M NaCl, pH 6.8
Flow rate	0.35 mL/min
Detector	UV, 220 nm
System	Agilent 1260 Bio-inert Quaternary LC

Table 1 lists the components of the protein test mix. Figure 1 shows the resulting separation achieved for each of the three different pore size columns. The 100Å column excludes the larger globular proteins (thyroglobulin, its aggregates, and γ-globulin), and so is only suitable for proteins with molecular weights less than 100,000.

Table 1. Peak Identification and Molecular Weight of a Standard Protein Mix

Peak number	Name	Molecular weight
1	Thyroglobulin aggregates	
2	Thyroglobulin	670,000
3	IgA	320,000
4	IgG (γ-globulin)	158,000
5	Ovalbumin	44,000
6	Myoglobin	17,000
7	Vitamin B12	1,355

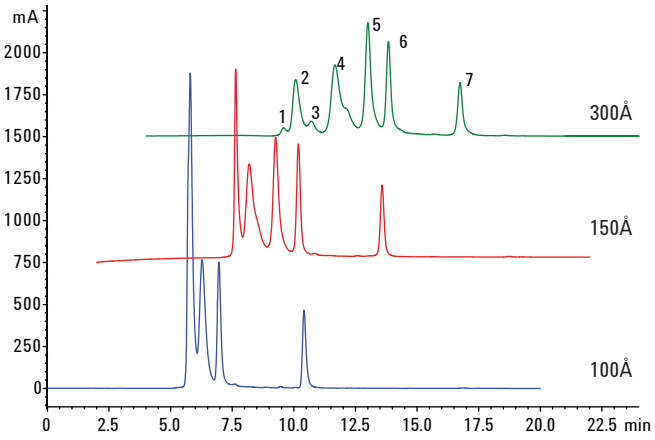

Figure 1. Separation of the standard protein mix on Agilent BioSEC-3 columns with three different pore sizes. The 100Å and 150Å columns demonstrate exclusion of the larger proteins. Note how the 300Å pore size gives definition between the thyroglobulin and its aggregates, also the resolution between the IgA and IgG peaks.

The 150Å column shows very good resolution between the ovalbumin and myoglobin (Table 2) and the only protein excluded is the high molecular weight thyroglobulin. The 300Å column is able to resolve proteins across the whole range.

Table 2 shows the resolution factors between the proteins for each pore size. From this, it is possible to make an informed decision on the most suitable column to choose for your application.

Table 2. Resolution Factors Achieved with the Separation of the Protein Mix

Media	Rs Factor Thyro/Aggs	Rs IgA/Thyro	Rs IgG/IgA	Rs Ovalb/IgG	Rs Myo/Ovalb	Rs Vit B12/Myo
100Å	*	*	*	*1.17*	1.87	12.94
150Å	*	*	*1.28*	2.02	2.59	11.66
300Å	0.93	1.06	1.38	2.32	2.19	8.24

* no resolution, excluded
Numbers in italics are achieved using the retention time of the excluded peaks

Antibody separation

In this trial, we used a mouse IgG sample to evaluate the suitability of the pore sizes for the analysis of the monomer and its dimer components. The size, type, and content of aggregates present in protein biopharmaceuticals can affect both efficacy and formulation, or worse, induce an immunogenic response. Aggregation formations occur through a variety of mechanisms, including disulfide bond formation and non-covalent interactions.

Conditions

Columns	Agilent BioSEC-3 100Å, 4.6 x 300 mm, 3 µm (p/n 5190-2503) Agilent BioSEC-3 150Å, 4.6 x 300 mm, 3 µm (p/n 5190-2508) Agilent BioSEC-3 300Å, 4.6 x 300 mm, 3 µm (p/n 5190-2513)
Sample	Mouse IgG
Eluent	100 mM Sodium phosphate buffer + 0.15 M NaCl, pH 6.8
Flow rate	0.35 mL/min
Detector	UV, 220 nm
System	Agilent 1260 Bio-inert Quaternary LC

Figure 2 shows the separation using Agilent BioSEC 3 µm columns with different pore sizes, demonstrating that the 100Å pore size column excludes the IgG and so no definition is achieved. However, both the 150Å and 300Å columns are able to resolve the monomer and dimer. Figure 3 shows the separation achieved on the 300Å column magnified and shows the detail seen by the 300Å column, making it a suitable choice for analysis of monoclonal antibodies and their aggregates.

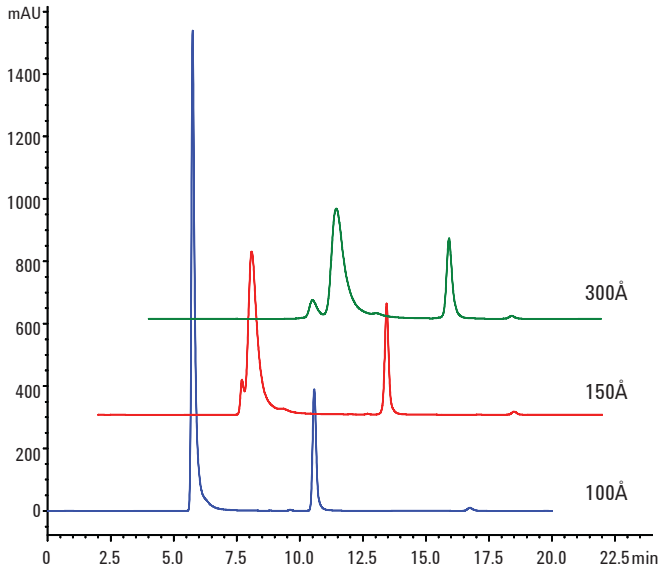

Figure 2. Separation of mouse IgG on Agilent BioSEC-3 columns with three different pore sizes. Again, resolution increases with pore size.

Conclusions

Because the size of protein aggregates, including dimers, is sufficiently different from the protein monomer, it is possible to separate the various forms using SEC. In fact, SEC with UV or light scattering is a standard technique for quantifying protein aggregation and molecular weight. However, because the choice of media pore size influences the resolution obtained when using SEC, it is worthwhile testing a range of pore sizes to match the pore size to the analyte. This approach will identify the best choice before conducting in-depth investigations, and reduce the risk of missing valuable information through the choice of media with an inappropriate pore size.

Agilent Bio SEC-3 HPLC columns are available in 100Å, 150Å, and 300Å pore sizes to accommodate most peptide and protein size exclusion separations.

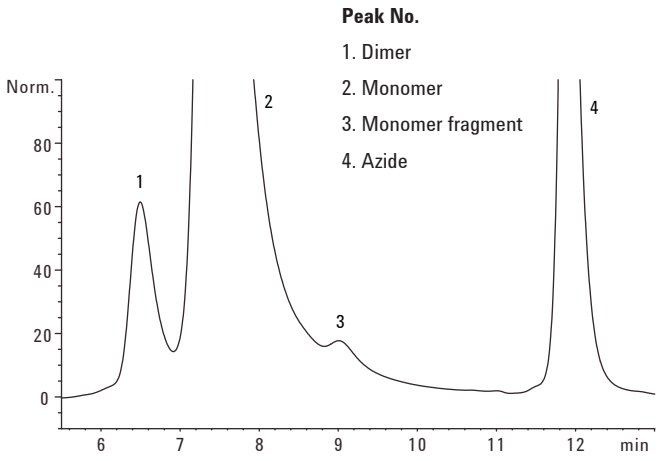

Figure 3. Magnification of the separation of mouse IgG using an Agilent BioSEC-3 300Å column – the best choice for analyte.

For More Information

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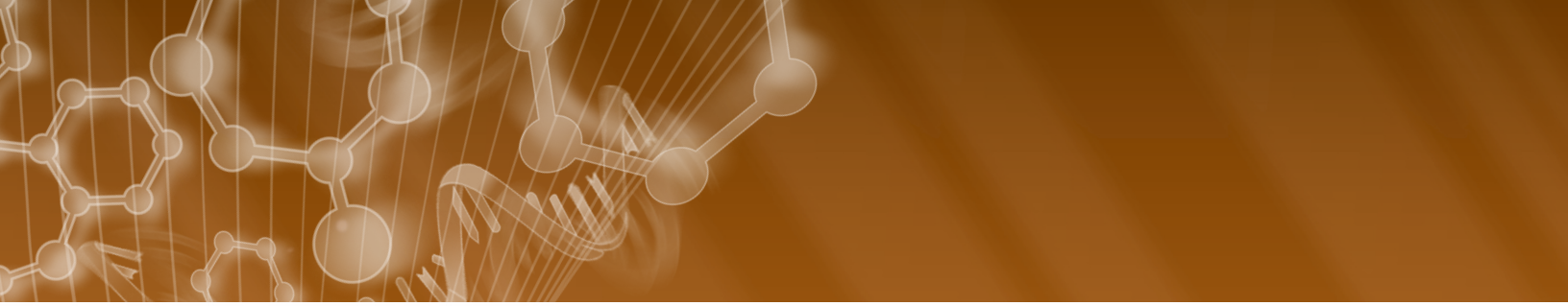

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