Feed Speed and Overfeed Volume

New Parameters for Injection in Supercritical Fluid Chromatography

Suitable for Agilent
1260 Infinity III LC

Technical Overview

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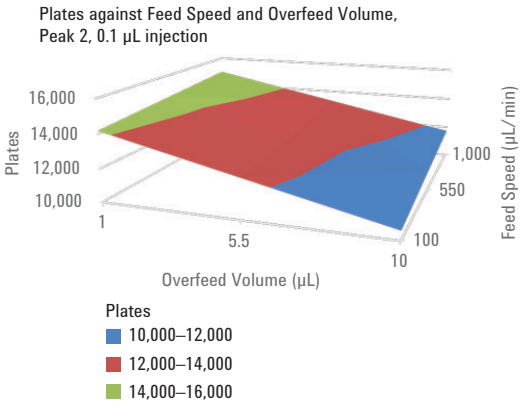
Abstract

This Technical Overview demonstrates and discusses the influence of the injection parameters Feed Speed and Overfeed Volume on chromatographic separation. These parameters were introduced with the Agilent 1260 Infinity II Supercritical Fluid Chromatography (SFC) Multisampler. The influence on the isocratic separation of closely eluting compounds, shown for different injection volumes commonly used in analytical SFC, and guidelines for optimization are given.



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Introduction

The Agilent 1260 Infinity II SFC Multisampler offers the injection of flexible sample volumes into the Agilent 1260 Infinity II SFC System[1]. The sample volume range, which is addressable by the 1260 Infinity II SFC Multisampler, starts at a possible injection volume of 0.1 µL. Because the 1260 Infinity II SFC Multisampler has a 100 µL sample loop, it is possible to inject larger volumes. With the injection principle of the 1260 Infinity II SFC Multisampler, two extra injection parameters, Feed Speed and Overfeed Volume, are applied. The Feed Speed is equal to the speed of sample introduction, and the Overfeed Volume is a flush-out volume added to the end of the sample plug.

This Technical Overview presents and discusses the chromatographic results of sample injections for different sample volumes at different Feed Speed and Overfeed Volume values. The resulting isocratic separations are shown, and performance parameters such as linearity and area precision are discussed. From the results, guidelines and default parameters are suggested.

Experimental

Instrumentation

The Agilent 1260 Infinity II SFC System comprises:

- Agilent 1260 Infinity II SFC Control Module (G4301A)
- Agilent 1260 Infinity II SFC Binary Pump (G4782A)
- Agilent 1260 Infinity II SFC Multisampler (G4767A)
- Agilent 1260 Infinity II DAD with high-pressure SFC flow cell (G7115A)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)

Instrumental setup

The 1260 Infinity II SFC Multisampler is directly connected to the SFC pump and the column. All necessary flushing and washing steps are done by the factory-installed plumbing. It is only necessary to connect two solvents, one for the flushing and feeding process, and one for the needle wash.

Column

Agilent ZORBAX RX-SIL, 4.6 × 150 mm, 5 µm (p/n 883975-901)

Software

Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, Rev. C.01.07 SR3

Sample

SFC Checkout Standard (p/n 5190–0584) containing theophylline, caffeine, thymine, and theobromine (250 µg/mL in methanol)

Chemicals

All solvents were purchased from Merck, Germany.

Results and Discussion

Feed Injection technology provides a prepressurized sample that is injected into the mobile phase stream before the column by a syringing process[1]. There are two instrument parameters controlling the injection, the Feed Speed and the Overfeed Volume. The Feed Speed could be described as the speed of the syringe injection of the sample into the mobile phase stream. The Overfeed Volume is a solvent plug that is injected after the sample, to flush out the sample completely. Both parameters can influence the chromatography.

SFC methods

Parameter	Description
Solvent A	CO_2
Modifier B	Methanol
SFC flow	2.5 mL/min
Isocratic elution	12 %B Stop time: 6 minutes
Backpressure regulator (BPR)	60 °C, 130 bar
Column temperature	40 °C
Injection volume	0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0, 5.0, 10.0 µL
Feed solvent	Methanol
Overfeed volume	4, 3, 2, 1, 0 µL
Feed speed	1,000, 400, 200, 100, 50 µL/min
Needle wash	3 seconds in methanol
Diode array detector	254 nm/4 nm; Ref. 360 nm/100 nm, data rate: 10 Hz, standard high-pressure SFC flow cell

The influence of Feed Speed on separations at different injection volumes

The influence of Feed Speed on the chromatography was tested. The range of injection volumes typically used for analytical work (0.1–10.0 µL), various Feed Speed values (1,000, 400, 200, 100, and 50 µL/min), and constant Overfeed Volume (4 µL) were used. The resulting separations with the isocratic separation method, as mentioned in Experimental, showed a clear separation of the four compounds in the test sample (Figures 1A–D).

The injections of 0.1, 1.0, 5.0 and 10.0 µL are shown as examples. The peaks are baseline separated up to the 5 µL injection (Figure 1C), and they started to elute with valleys at the highest injection volume of 10.0 µL (Figure 1D). The injection linearity was determined for the applied settings over the whole injection volume range (Figure 2) as a measure of the performance.

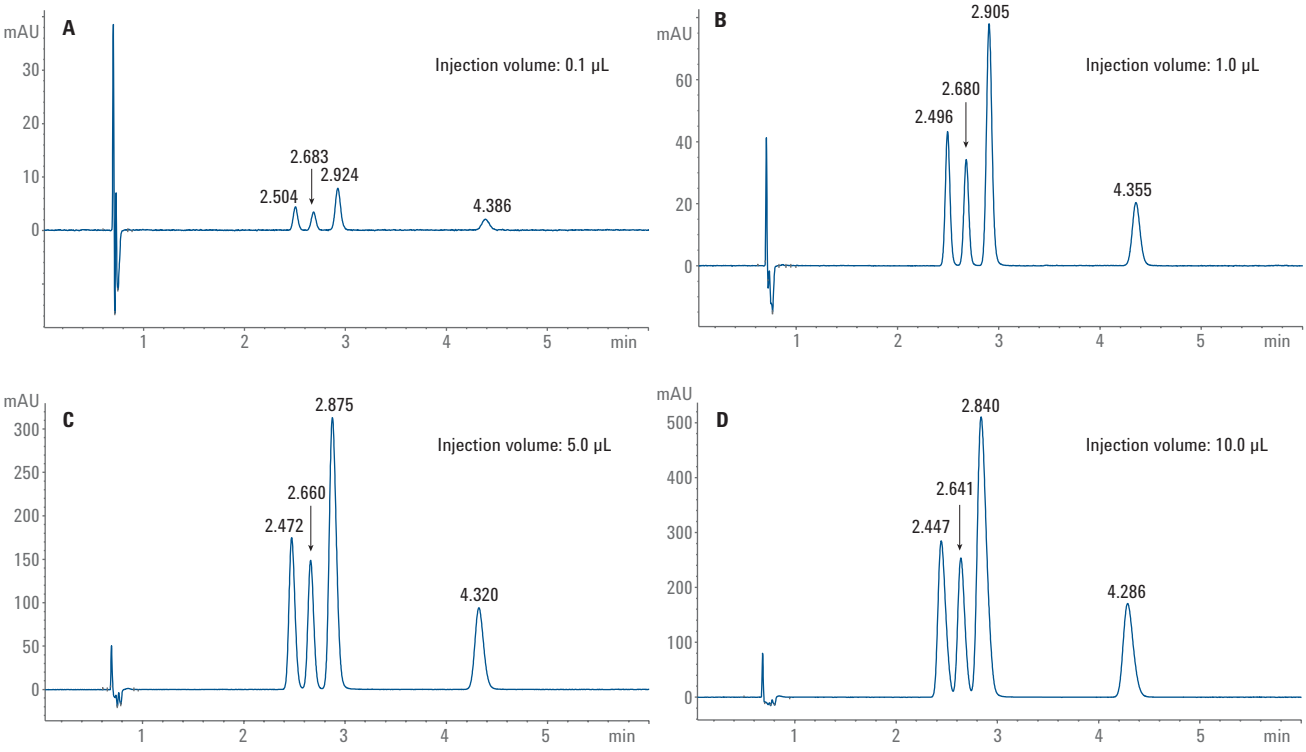

Figure 1. Different injection volumes at a Feed Speed of 400 µL/min, and an Overfeed Volume of 4 µL. A) 0.1 µL, B) 1.0 µL, C) 5.0 µL, D) 10.0 µL.

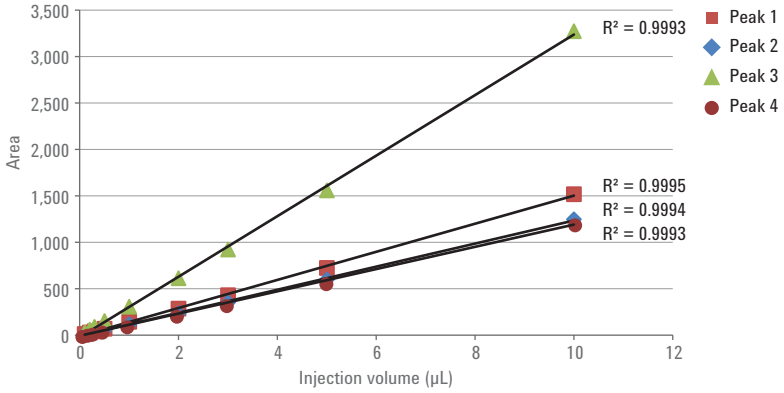

Figure 2. Injection volume linearity of compounds 1 to 4 at 400 µL/min Feed Speed, and 4 µL Overfeed Volume.

All peaks showed excellent linearity values over the whole injection volume range, typically better than R^2 >0.999. As another measure of performance, the relative standard deviation (RSD) of the peak area was determined by multiple injections of each injection volume. Typically, the area RSDs started, for the lowest volume injection (0.1 µL), at 3.0 to 3.5 %, and declined to 0.3 % or less for injection volumes above 0.5 µL. For all higher injection volumes, up to 10.0 µL, the RSD values remain at 0.3 % or less. For a detailed discussion, see Agilent Technical Overview[1].

In the further experiments evaluating the limits of Feed Speed in relation to the maximum injectable sample volume, the Feed Speed was decreased. Figure 3 displays the resulting chromatographic limitations, dependent on decreasing Feed Speed in relation to the injection volume under isocratic separation conditions. The first three peaks started to coelute for an injection volume of 10 µL at a Feed Speed of 200 µL/min. The peaks were still separated for the 5 µL injection and less (Figure 3, A1 and A2). At a Feed Speed of 100 µL/min, the coelution started at 5 µL injection volume. The resolution was retained for the 3 µL injection volume and less (Figure 3, B1 and B2). The lowest Feed Speed value of 50 µL/min in this comparison showed a coelution for the 3 µL injection volume, and good resolution for the 1 µL injection volume and less (Figure 3, C1 and C2).

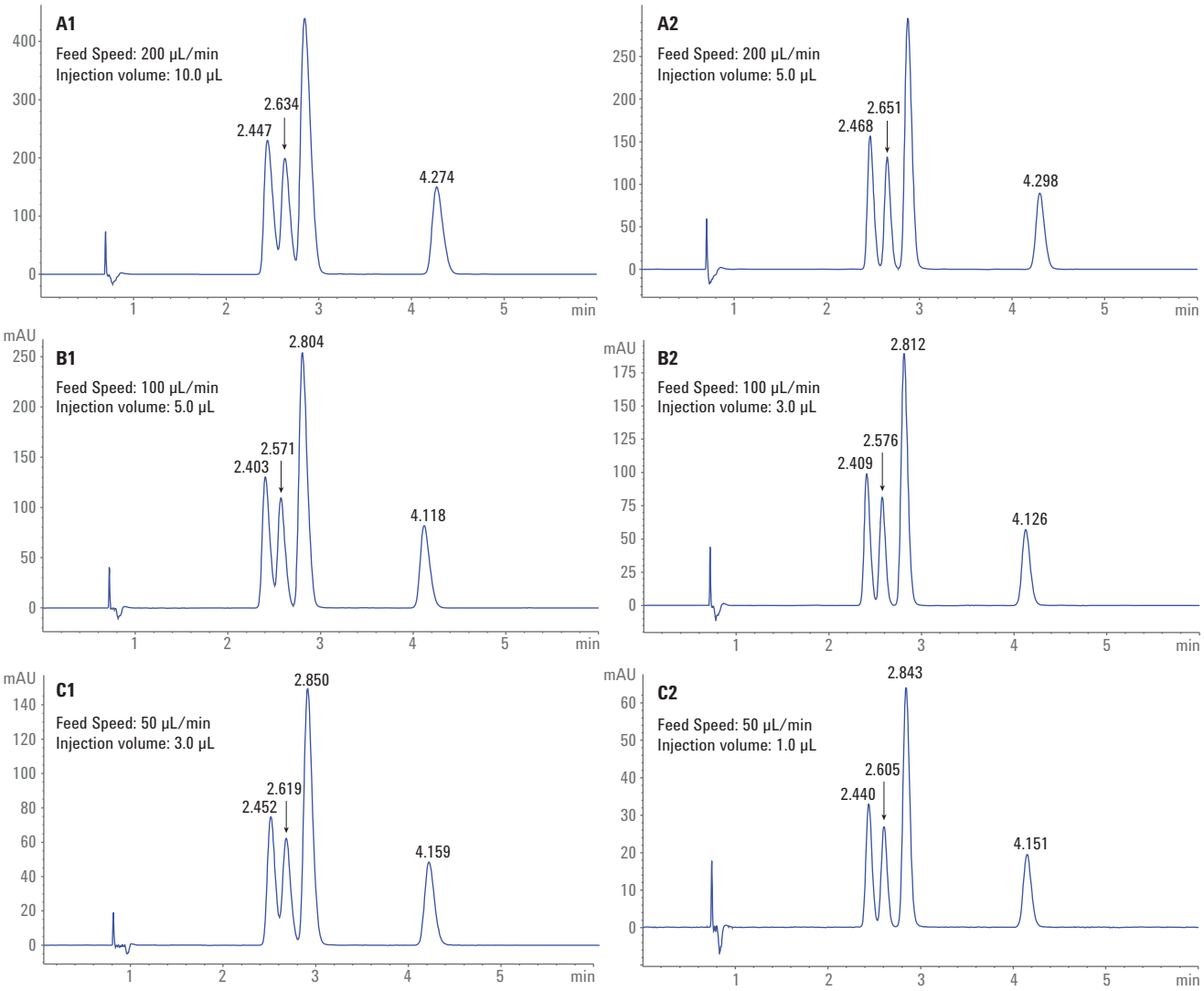

Figure 3. Chromatographic limitation depending on Feed Speed and injection volume under isocratic separation conditions. A) The first three peaks started to coelute for an injection volume of 10 µL at a Feed Speed of 200 µL/min (A1). The peaks were still separated for the 5 µL injection (A2). B) At a Feed Speed of 100 µL/min, the coelution started at 5 µL injection volume (B1) and resolution was retained until 3 µL injection volume (B2). C) The lowest Feed Speed value of 50 µL/min in this comparison showed coelution beginning at 3 µL injection volume (C1), and good resolution for 1 µL injection volume (C2).

The injection linearity was determined from 0.1 µL up to the maximum injection volume, according to the applied Feed Speed (Table 1) for all experimental Feed Speed conditions.

The obtained injection linearity was typically better than 0.9995. If higher injection volumes were included in the linearity calculation, a decrease of the R^2 values was observed, because chromatographic separation degraded, as shown in Figure 3.

Another important value for quantification is the peak area RSD. For experimental conditions applying higher Feed Speed values, the area RSDs decreased to 0.3 % or less for injection volumes above 0.5 µL. Figure 4 shows an example of a slow Feed Speed of 50 µL/min, starting with the low injection volume of 0.1 up to 2 µL.

In all described experiments, peak 4 was not affected by coelution effects with other compounds due to its late and distanced elution. Therefore, it could be used to examine the influence of the Feed Speed at different injection volumes on typical peak parameters. For instance, the peak height decreased dramatically with decreasing Feed Speed at fixed injection volumes, especially when larger volumes were injected (Figure 5).

For Feed Speed values of 50 and 100 µL/min, the peak height started to suffer for injection values above 3 and 5 µL, respectively. Conversely, there was no further improvement in peak height, which means less peak broadening, for Feed Speed values above 500 µL/min.

Table 1. Injection linearity depending on the applied Feed Speed from 0.1 µL up to the maximum injection volume.

Feed Speed (µL/min) and maximum injection volume (µL)	Compound 1	Compound 2	Compound 3	Compound 4
500/10	0.9995	0.9994	0.9993	0.9993
200/5	1.0000	0.9999	0.9998	0.9997
100/3	0.9999	0.9999	1.0000	1.0000
50/1	0.9998	0.9998	1.0000	1.0000

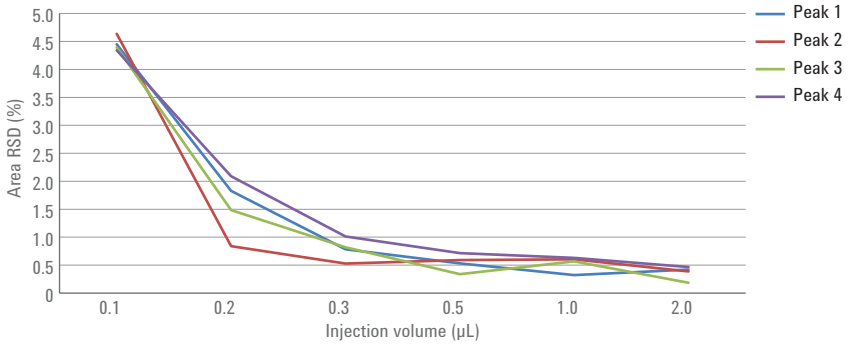

Figure 4. Area RSD values of injection volumes from 0.1 to 2 µL at 50 µL/min Feed Speed.

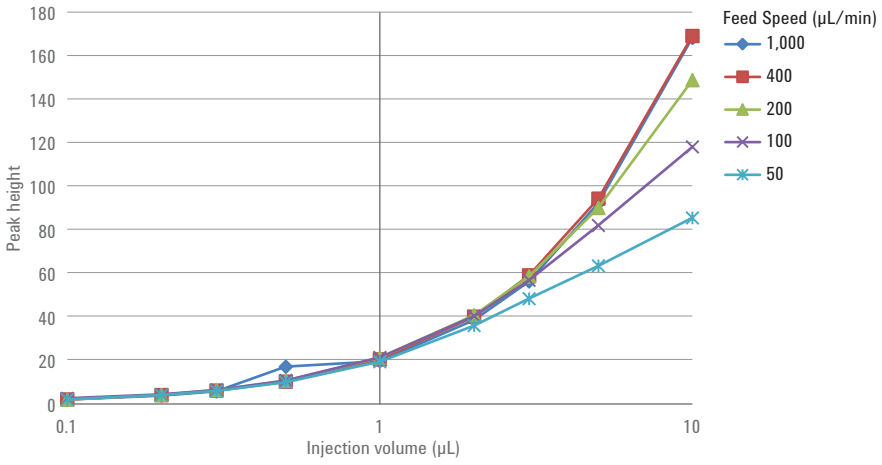

Figure 5. Peak height of peak 4 in comparison to the applied Feed Speed for different injection volumes.

Figure 6 presents a contrasting view in which peak width increased with increasing injection volume for different Feed Speed values. For lower injection volumes up to 0.5 µL, peak width constantly increased, almost independent from the Feed Speed. For higher injection volumes, the peak width increased dramatically, with lower Feed Speed values of 50 and 100 µL/min for injection volumes above 2 and 5 µL, respectively. The influence of the injection volume on the peak width was almost the same for the higher Feed Speed values of 400 and 1,000 µL/min.

The peak symmetry is discussed as a last peak parameter (Figure 7). For low volumes, the peak symmetry is typically approximately 0.9 for different Feed Speed values. The symmetry value starts to decline for the higher injection volumes, which means the peaks start to tail, especially if the lower Feed Speed values were applied.

The influence of Overfeed Volume on separations at different injection volumes

The second parameter, the Overfeed Volume, ensures that the complete sample volume is transferred from the loop capillary into the column. Having a sufficient Overfeed Volume is especially critical for the injection of small sample volumes to avoid any loss of sample (Figure 8).

For instance, an injection of 0.1 µL could be transferred completely to the column by the application of an Overfeed Volume of 4 µL. As an extreme example, a low amount of sample is possible without any Overfeed Volume, but with the partial loss. Figure 9 shows the results of the complete tested range of injection volumes and Overfeed Volumes.

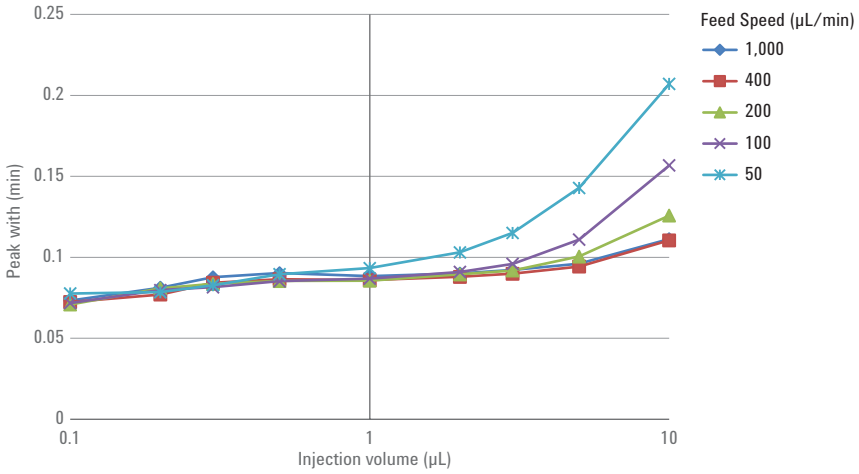

Figure 6. Peak width of peak 4 in comparison to the applied Feed Speed for different injection volumes.

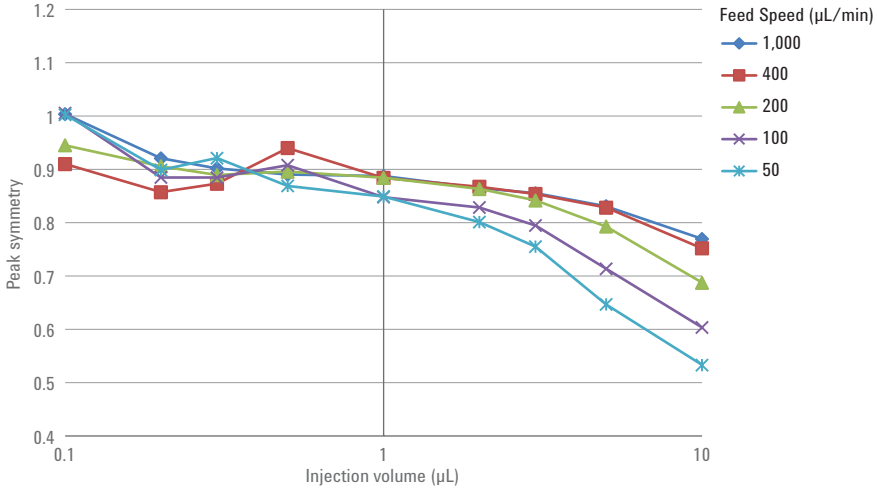

Figure 7. Peak symmetry of peak 4 in comparison to the applied Feed Speed for different injection volumes.

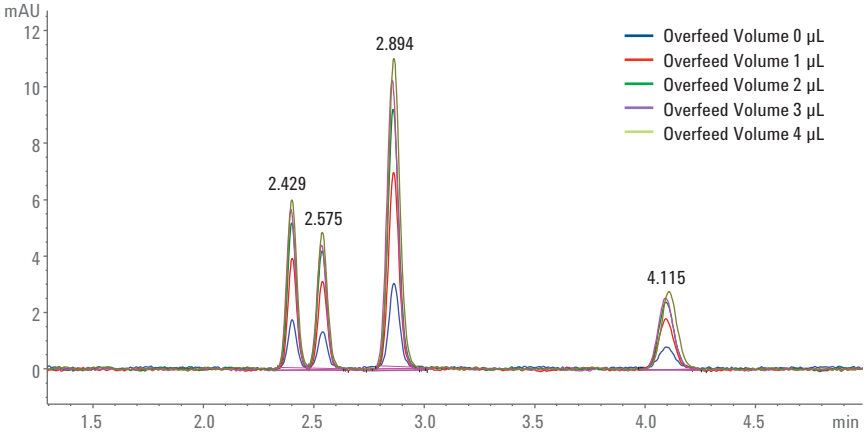

Figure 8. Influence of the Overfeed Volume on sample introduction for 0.1 µL injection volume.

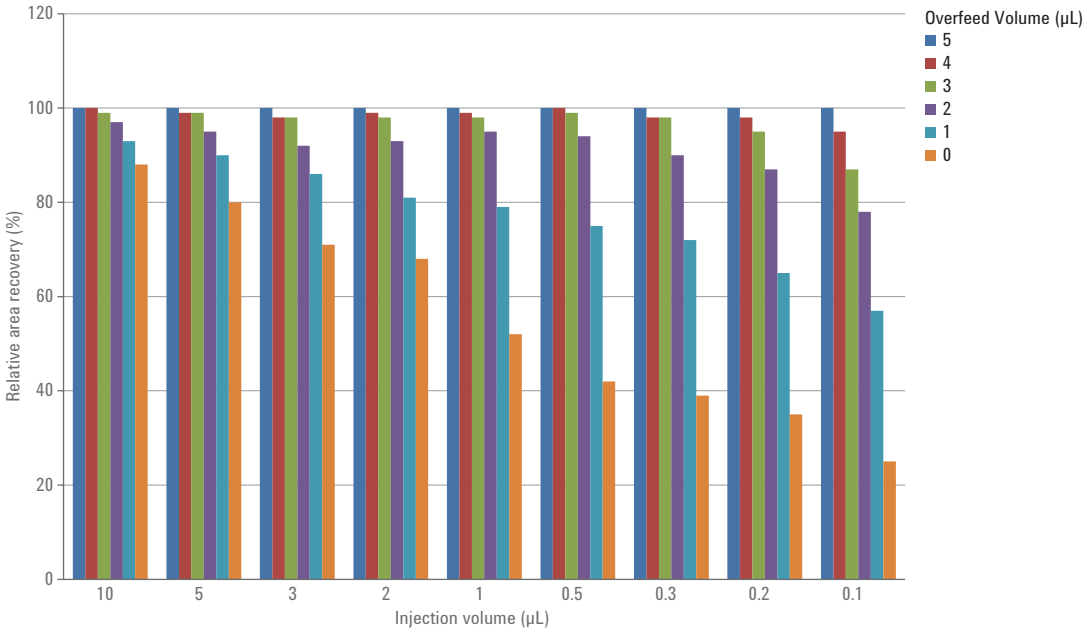

Figure 9. Comparison of the influence of the Overfeed Volumes on sample introduction for different injection volumes. The relative area recoveries of peak 4 are shown for different Overfeed Volumes. The areas obtained for the Overfeed Volume of 5 µL were used as a basis, and assumed to deliver a complete sample transfer into the eluent stream to the column.

It was described that there was only a limited peak area of approximately 20 % detection obtained for the lowest injection volume of 0.1 µL and no Overfeed Volume. The detected peak area increased up to approximately 90 % for a 10 µL sample injection without Overfeed Volume. The lower volumes could be transferred into the solvent stream by the application of a 4 µL Overfeed Volume. The application of 3 and 4 µL of Overfeed Volume ensured a good value of sample transfer over the whole range of injection volumes. An Overfeed Volume of 5 µL was used as the basis for this calculation. In comparison to the Overfeed Volume of 5 µL, it is described that 4 µL of Overfeed Volumes typically delivered >98 % recovery.

The combined influence of Feed Speed, Overfeed Volume, and injection volume on chromatographic performance expressed as chromatographic plates

A Design of Experiment (DOE) matrix was set up to demonstrate the combined influence of the Feed Speed, Overfeed Volume, and injection volume parameters on the chromatographic performance. Combined influence was expressed in the form of chromatographic plates. This DOE matrix was based on the results described in the earlier sections of this Technical Overview. The values of 100 and 1,000 µL/min were used as ranges of this DOE matrix for the Feed Speed. For Overfeed Volume, the values of 1 and 10 µL were used, and respectively combined with the Feed Speed values. In addition, a method for the center point at a Feed Speed 550 µL/min and an Overfeed Volume of 5.5 µL was created. The resulting five methods (other method parameters were used, as mentioned in Experimental) were combined with the injection volumes of 0.1, 5.05, and 10 µL.

The methods were combined in a sequence wherein typically all parameters were changed from one run to the next. The center point method was applied three times during the sequence, which led to 17 sequence lines, with each run being replicated 10 times. A mixture of caffeine and theobromine (250 mg/L in methanol) was used as a test sample. Caffeine eluted at approximately k' = 2 and theobromine at approximately k' = 4. Figure 10 summarizes the results as 3D-plots.

For example, in Figure 10-A1, the general influence of the different parameters can be seen at the lowest injection volume of 0.1 µL. The highest number of plates was achieved for the highest Feed Speed and the lowest Overfeed Volume. The plate count typically decreased with a decreasing Feed Speed and, in contrast, the plate count increased with a decreasing Overfeed Volume. The later-eluting peak showed the same behavior, but with a lower plate count (Figure 10-A2). For higher injection volumes, under identical Feed Speed and Overfeed Volume conditions, the achieved plate number was lower.

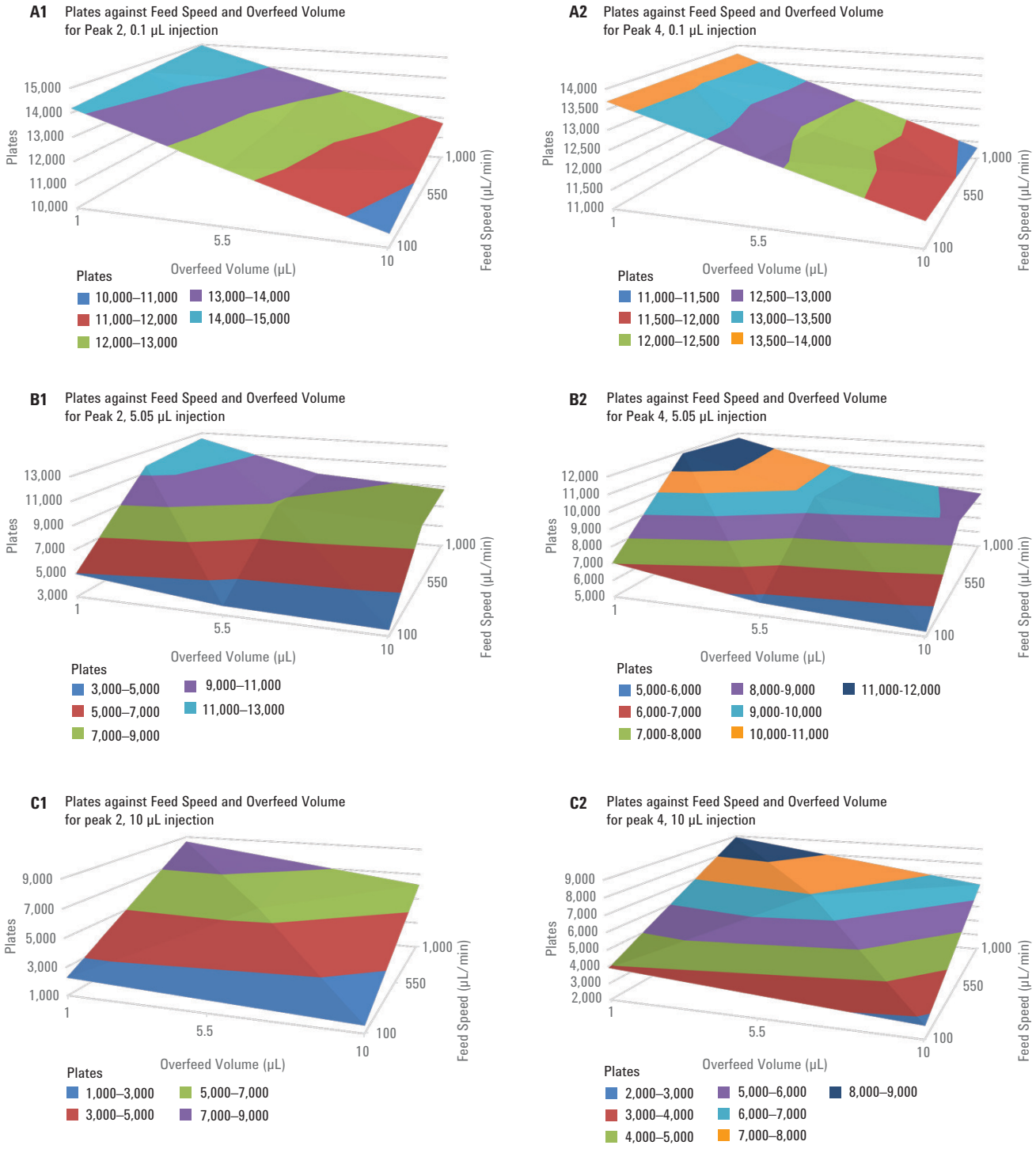

Figure 10. Comparison of the chromatographic performance expressed as chromatographic plates in a DOE space for Feed Speed, Overfeed Volume, and injection volume. Two compounds were used as examples, caffeine (peak 2, k′ = 2) and theobromine (peak 4, k′ = 4). Feed Speed range: 100–1,000 µL/min, Overfeed Volume range: 1 to 10 µL, Center point: 550 µL/min Feed Speed and 5.05 µL Overfeed Volume. A) Injection volume 0.1 µL. B) Injection volume 5.05 µL. C) Injection volume 10 µL (colors indicate areas of a common range of plate number as outlined).

This effect became even more dominant when the Feed Speed was reduced below 550 µL/min. (Figure 10, B1 and B2.) The injection volume of 10 µL showed the same behavior for both peaks, but with lower plate numbers (Figure 10, C1 and C2). From these experiments for optimized chromatographic performance, the following conclusion could be drawn: if the separation conditions are in the upper left part of the 3D-plots, a higher chromatographic performance can be achieved. That means the Feed Speed should typically be higher than 500 µL/min with a lower Overfeed Volume. As discussed earlier, the optimum Overfeed Volume should be 3 to 4 µL for an injection range between 0.1 and 10 µL. The chromatographic performance in terms of plates was higher for lower injection volumes up to 5 µL. With regards to the previously discussed area RSD, it should be between 0.5 and 5 µL.

Summary

The results obtained in the earlier discussed experiments were obtained for isocratic separations. The isocratic elution shows clearer effects than gradient elution. In gradient elution, the compounds are typically focused at the front of the column, and start to elute later in the gradient. Typically, isocratic elution plays an important role for a major application of SFC, the separation of enantiomers[2]. From the results, a Feed Speed value of 400 µL/min and an Overfeed Volume of 4 µL were suggested as starting values for these parameters. If the injection of large sample volumes with initial enrichment on the front of the column has to be done[1], lower values for the Feed Speed make sense.

If the sample comprises sticky compounds or highly contaminating matrices, higher values of Overfeed Volumes could be applied.

Conclusion

This Technical Overview describes the influence of the Feed Speed and Overfeed Volume on the chromatographic performance as applied with the Agilent 1260 Infinity II SFC Multisampler. The suggested default values for these parameters are explained as an outcome from a large set of experiments. Their application for optimum chromatographic performance of the Agilent 1260 Infinity II SFC Multisampler is demonstrated.

References

1. Naegele, E. Supercritical Fluid Chromatography with Flexible Injection Volumes at Highest Precision, *Agilent Technologies Technical Overview*, publication number 5991-7623EN, **2017**.

2. Naegele, E. Chiral Multicolumn Method Development on the Agilent 1260 Infinity II SFC System, *Agilent Technologies Application Note*, publication number 5991-7624EN, **2017.**

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