Accelerating Modifier-Free SFC Separations with the Agilent 1260 Infinity II SFC System

Focusing Late-Eluting Compounds Using Backpressure and Flow Gradients

Suitable for Agilent
1260 Infinity III LC

Technical Overview

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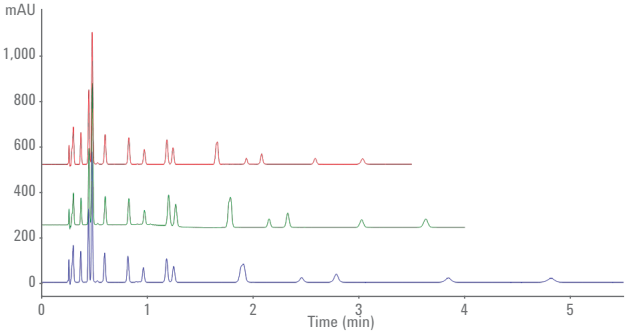
Abstract

Supercritical fluid chromatography (SFC) covers a wide range of analyte polarity and application range. For example, SFC offers modifier-free separations of highly hydrophobic compounds using exclusively CO_2, which makes SFC a truly green separation technique. Conversely, it is a common phenomenon that isocratic separations may lead to peak broadening of late-eluting compounds due to various band-broadening processes. This Technical Overview describes focusing late-eluting compounds by flow gradients and backpressure gradients in separations with pure CO_2 while maintaining separation power for the early eluting analytes. A dramatic reduction in run time, and a significantly improved peak shape could be achieved using the full pressure range of the Agilent 1260 Infinity II SFC System.



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Introduction

SFC is a highly versatile chromatographic technique with proven application examples covering a wide range of analyte polarities and application spaces. The applications can be as diverse as separating saturated and aromatic hydrocarbons with pure CO_2[1], or separating small peptides with strong-eluting modifiers[2], sometimes even containing low percentages of water. A full overview of SFC applications can be found in the Agilent SFC Primer[3].

The possibility of separating hydrophobic compounds with modifier-free methods, avoiding the use of organic solvents, makes SFC a truly green separation technology, especially since CO_2 meets the definition of green solvents previously proposed by Capello[4]. A problem of separations in pure CO_2 is their isocratic nature, which often leads to peak broadening of late-eluting compounds due to various band-broadening processes. In SFC, backpressure and flow gradients may be applied that influence system pressure and the pressure drop over the SFC column, and increase the elution strength of the mobile phase, thus significantly reducing run time especially for late-eluting compounds. System pressure may have an effect on retention and even selectivity in reversed-phase LC separations[5]. Flow and pressure have an even larger influence on key chromatographic parameters in SFC since they also vary the viscosity and density of the supercritical CO_2[6]. The interplay of these chromatographic parameters in SFC is not fully understood. Pressure and flow gradients can also be applied in gradient runs with a modifier to finely tune retention and selectivity. Since increasing the backpressure leads to a proportional increase in system pressure, fast separations with backpressure or flow gradients benefit from increased system pressure ranges of up to 600 bar at up to 5 mL/min.

To investigate the influence of backpressure and flow gradients, a mixture of 16 polycyclic aromatic hydrocarbons (PAHs) was used. The mixture was separated previously by means of SFC using methanol containing a certain percentage of water as a modifier[7]. PAHs are found in coal and crude oil, or are formed during incomplete combustion of carboniferous materials, causing environmental exposure in considerable amounts. Consequently, PAHs were declared by the US Environmental Protection Agency (US EPA) as priority pollutants.

Experimental

Instrumentation

The Agilent 1260 Infinity II SFC System comprised the following modules:

- 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 Diode Array Detector (G7115A) with high-pressure SFC flow cell
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with four-column selection valve (G4237A)

Instrumental setup

The setup used was the recommended configuration of the Agilent 1260 Infinity II SFC System with a four-column selection valve installed. This Technical Overview did not use any special modifications or modules. Backpressure gradients are also available for legacy control modules after a firmware upgrade (A/B/C/D 07.13 or higher) and a driver update (A.02.16 or higher).

Software

- Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, Rev. C.01.07 SR3
- Agilent LC Driver package A.02.16
- Agilent 1260 Infinity II SFC Control Module firmware version 07.20

Column

Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 150 mm, 2.7 µm (p/n 693975-302(T))

Isocratic separation method

Parameter	Value
SFC flow	2.5 mL/min
Mobile phase	100 % CO_2
Column temperature	40 °C
BPR temperature	60 °C
BPR pressure	150 bar
Total run time	5.5 minutes; no post time
Injection	1.0 µL
Feed speed	1,000 µL/min
Overfeed volume	4.0 µL
Feed solvent	MTBE
Needle wash	3 seconds MTBE
Diode array detection	Full spectra, 20 Hz data rate, 8 nm slit width, wavelength 223 ±4 nm, Reference 360 ±100 nm

SFC separation method with a backpressure gradient

- Initial isocratic separation method
- BPR pressure:
  0.0–1.0 minutes: 150 bar
  1.0–1.5 minutes: 150–300 bar
  1.5–4.0 minutes: 300 bar

SFC separation method with a flow gradient

- Initial isocratic separation method
- SFC Flow:
  0.0–1.0 minutes: 2.5 mL/min
  1.0–1.5 minutes: 2.5–4.0 mL/min
  1.5–3.5 minutes: 4.0 mL/min

Chemicals and samples

Methanol was purchased from Merck KGaA, Darmstadt, Germany. MTBE was bought from Sigma-Aldrich, Steinheim, Germany.

Sample

An Agilent 16-compound PAH mixture (p/n 8500-6035) was used, containing acenaphthene, acenaphthylene, anthracene, benzo[a]anthracene, benzo[b] fluoranthrene, benzo[ghi]perylene, benzo[a]pyrene, chrysene, dibenzo[a,h] anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, and pyrene at a nominal concentration of 500 µg/mL each. The original solution was diluted to 1/10 of the concentration with MTBE, resulting in a nominal concentration of 50 µg/mL. The dilute solution was used for the experiment.

Results and Discussion

Initial method development

Previous screening of stationary phases identified the Agilent InfinityLab Poroshell 120 EC-C18 column as the column of choice since it guaranteed elution of all compounds within a moderate time window and with low backpressure. An initial flow rate of 2.5 mL/min was a good compromise between overall run time and resolution, especially for the early-eluting compounds.

The isocratic nature of separations in pure CO_2 and the poor retention of the first two compounds made it necessary to optimize the feed injection parameters for this application, that is, the feed speed was increased to 1,000 µL/min (the default value is 400 µL/min). To keep feed solvent and sample solvent identical, MTBE was used as solvent. Chromatographic effects of overfeed volume and feed speed were described earlier[8]. The standard overfeed volume of 4 µL in combination with maximum feed speed of 1,000 µL/min did not show any influence on the chromatography, and was applied during all separations. From the spectral information obtained for all compounds, a wavelength of 223 ±4 nm with a reference wavelength of 360 ±100 nm was identified to give good detector response for all PAHs. Figure 1 shows the chromatogram of the developed isocratic base method.

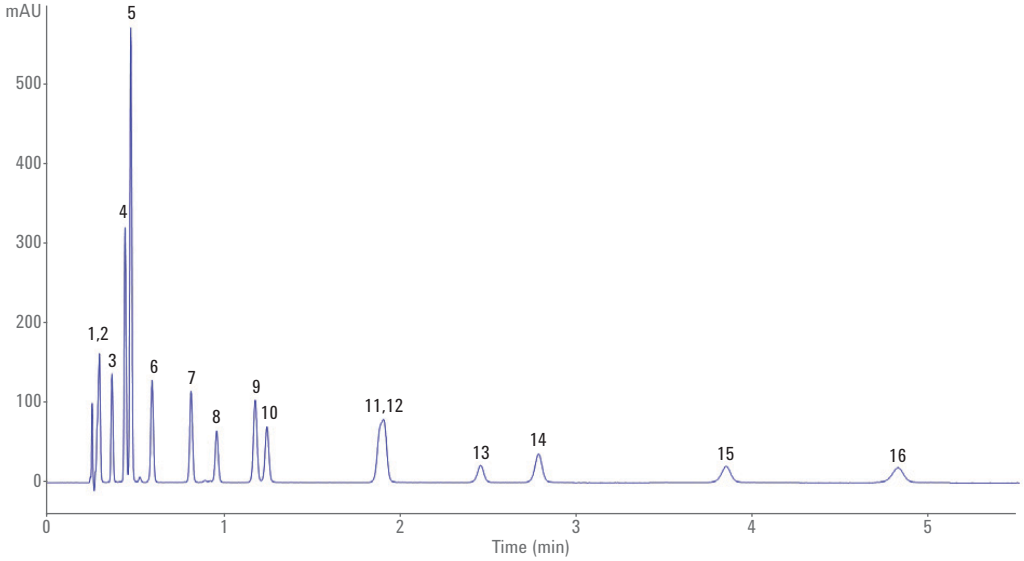

Figure 1. Initial separation method for the PAH standard. The method successfully separated 10 of 16 compounds, while compounds 4 and 5 were only partially separated. Compounds 1 and 2, and 11 and 12 coeluted. Mobile phase: 100 % CO_2, isocratic, no modifier used.

Full separation was achieved for 10 of 16 compounds, while two PAHs were partially separated (R_s = 1.54) and four PAHs coeluted (1 and 2, 11 and 12). To further speed up the analysis, the role of flow and backpressure gradients was investigated. To not compromise the separation of the early-eluting compounds, especially of the critical peak pairs 1 and 2, and 4 and 5, the flow and backpressure gradients were started after 1 minute.

Table 1 shows the key characteristics of the separation. Typical retention time RSD values were approximately 0.1 %, while area RSD values were typically below 1 % for a 1 µL injection. Only for late-eluting compounds, the area RSD increased significantly, most probably due to peak broadening and reduced signal-to-noise ratio.

Focusing by backpressure gradient

An increase in backpressure leads to higher CO_2 density and viscosity, which increases the elution strength of the mobile phase. To focus late-eluting compounds between 1 to 1.5 minutes, a backpressure gradient from 150 to 300 bar was applied, equaling a backpressure change of 5 bar/s, leading to a final system pressure of approximately 515 bar.

Focusing the late-eluting compounds significantly reduced the area RSD to values below 1 %, which was lower than under isocratic conditions. The overall run time was shortened by nearly 25 % to 3.62 minutes, while effectively improving peak shape (Figure 2).

The separation was highly reproducible in terms of retention time and area precision (Table 2). It was crucial to define a reference wavelength to compensate for the effects of varied backpressure to minimize detector noise. In addition, it was not surprising that the change in backpressure resulted in a decreasing detector response of up to 10 mAU, most probably due to changed CO_2 density. As a consequence, it reduced comparability of peak areas within one run.

Focusing by flow gradient

The faster elution of highly retained compounds by flow gradients was achieved due to a higher linear speed of the solvent and an increased pressure drop across the column, which led to an increased density and elution strength of the mobile phase.

The flow gradient was programmed between 1 and 1.5 minutes, with a flow rate increasing from 2.5 to 4 mL/min. This led to an overall system pressure of approximately 550 bar. In contrast to a backpressure gradient, flow gradients only increase the system pressure in front of the column, while the backpressure is maintained by the BPR module. This reduces the influence on viscosity and density of the mobile phase in the detector cell, and keeps the baseline more stable. The overall run time was shortened by 37 %, to 3.03 minutes, and offered the best peak shape for late-eluting compounds (Figure 2). In contrast to backpressure gradients, flow gradients led to higher retention time and area RSDs (Table 2).

Table 1. Key parameters for the separation of the PAH standard under isocratic conditions. Retention time RSD values were typically around 0.1 %, while area RSD values were typically below 1 % for a 1 µL injection. Area RSDs for coeluting peaks 1 and 2 as well as 8 (coelution with impurities) were not determined. Values were calculated as an average of 10 injections.

No.	RT (min)	Area (mAU*s)	Height (mAU)	Symmetry	Width (min)	Plates	Resolution	RT RSD (%)	Area RSD (%)
1,2	0.303	197.50	168.69	1.62	0.03	1,500	1.87	0.10	Coelution
3	0.374	100.94	137.68	0.95	0.02	5,634	2.77	0.12	0.52
4	0.448	246.53	317.98	0.86	0.02	7,576	3.65	0.11	0.61
5	0.480	447.47	565.48	0.87	0.02	8,415	1.54	0.10	0.63
6	0.600	133.03	127.83	0.95	0.03	7,832	3.04	0.07	0.65
7	0.820	128.34	114.17	0.93	0.03	12,400	7.79	0.07	0.92
8	0.965	82.12	63.85	1.07	0.04	14,504	4.72	0.08	Coelution with impurities
9	1.185	150.44	103.24	0.97	0.04	15,427	6.24	0.11	0.63
10	1.251	106.18	69.37	0.96	0.04	15,966	1.71	0.10	0.93
11,12	1.910	263.05	78.30	1.69	0.09	8,995	10.96	0.07	Coelution
13	2.459	56.28	21.32	1.04	0.08	21,899	7.47	0.05	1.81
14	2.787	105.17	35.39	1.02	0.09	21,847	4.62	0.06	1.85
15	3.846	77.25	19.61	1.10	0.11	24,303	12.16	0.06	1.56
16	4.816	90.59	18.50	1.12	0.14	25,676	8.86	0.06	3.36

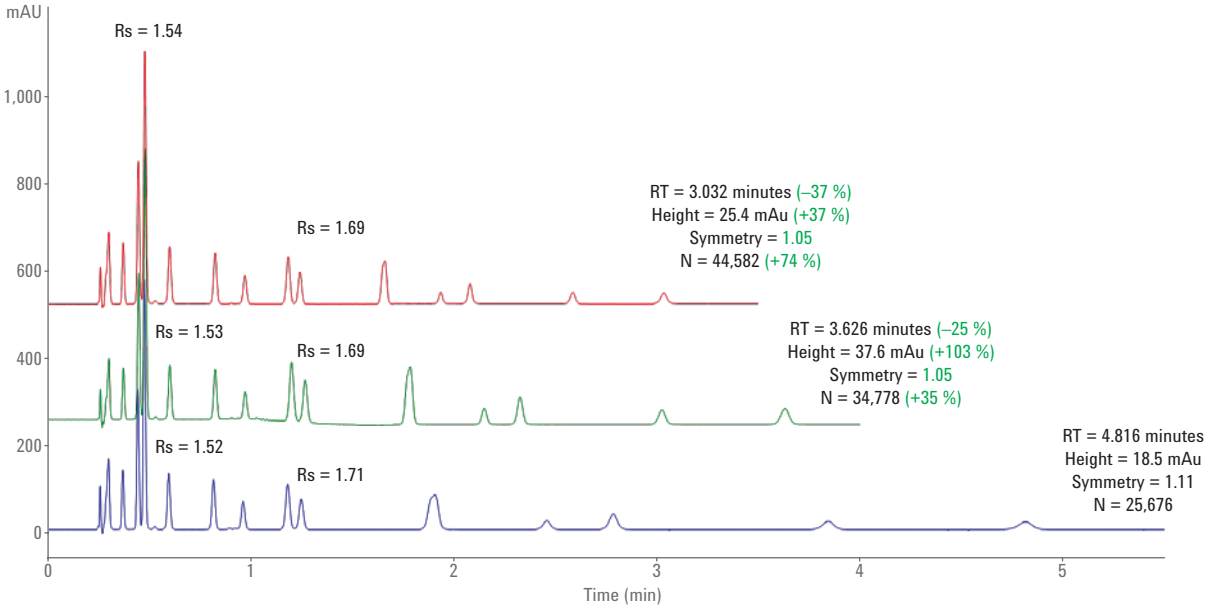

Figure 2. Comparison of the runs under isocratic conditions using pure CO_2: The last peak eluted after 4.816 minutes with no focusing (blue), after 3.626 minutes when a backpressure gradient was applied (green), and 3.032 minutes when a flow gradient was used (red). In total, the run time could be reduced by 25 % with a backpressure gradient, and 37 % with a flow gradient.

Table 2. Retention time and area RSDs for isocratic as well as gradient separations. Area RSDs for coeluting peaks 1 and 2 as well as 8 (coelution with impurities) were not determined. Values were calculated as an average of 10 injections.

	Isocratic run		Flow gradient		BPR gradient	
No.	RT RSD (%)	Area RSD (%)	RT RSD (%)	Area RSD (%)	RT RSD (%)	Area RSD (%)
1,2	0.10	Coelution	0.21	Coelution	0.15	Coelution
3	0.12	0.52	0.21	0.82	0.11	0.46
4	0.11	0.61	0.26	2.09	0.15	0.60
5	0.10	0.63	0.24	0.68	0.14	0.62
6	0.07	0.65	0.27	0.88	0.17	0.98
7	0.07	0.92	0.25	0.94	0.12	0.75
8	0.08	Coelution with impurities	0.25	Coelution with impurities	0.11	Coelution with impurities
9	0.11	0.63	0.22	0.85	0.09	0.80
10	0.10	0.93	0.20	1.04	0.09	1.03
11,12	0.07	Coelution	0.13	Coelution	0.07	Coelution
13	0.05	1.81	0.15	1.08	0.08	0.88
14	0.06	1.85	0.16	1.21	0.07	0.74
15	0.06	1.56	0.17	2.07	0.07	0.72
16	0.06	3.36	0.18	1.59	0.07	0.80

Comparison of both techniques

Table 2 presents a comparison of key characteristics for all separable compounds such as retention time RSDs, area RSDs. Both techniques showed advantages as well as disadvantages.

While the backpressure gradient led to better retention time and area RSDs, it was causing a decrease in the baseline, and a nonlinear increase of peak height and area due to density changes in the mobile phase. This reduced the comparability of peak areas within a run. However, considering the steep backpressure gradient of 5 bar/s, the reproducibility of the analysis is outstanding. The flow gradient could reduce analysis time and deliver better peak shape while creating higher retention time RSDs. Comparability of peak areas within a run was not compromised.

Conclusion

This Technical Overview describes the possibilities of modifier-free SFC separations as a green alternative to classic LC or SFC, using all possibilities of modern, state-of-the-art SFC instrumentation and columns. Focusing late-eluting compounds was successful both with flow and backpressure gradients, while both techniques showed advantages and disadvantages. Typical retention time RSDs were approximately 0.1 %, while area RSDs typically were found to be below 1 %.

Clearly, the possibility to change the backpressure during an analysis provides an additional degree of freedom in SFC method development. Both backpressure and flow gradients require a larger system pressure range. The Agilent 1260 Infinity II SFC System offers a pressure range of up to 600 bar at a flow of up to 5 mL/min, providing the necessary flexibility for both techniques.

The separation was performed on an Agilent InfinityLab Poroshell 120 EC-C18 column. InfinityLab Poroshell columns are a valuable enrichment when used in SFC due to their outstanding performance and efficiency while producing only moderate backpressures. The column enabled high-speed analyses in a pressure range up to 600 bar, making the 1260 Infinity II SFC System a true all-in-one solution.

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