Application Compendium

SUPERCRITICAL FLUID CHROMATOGRAPHY

Agilent Technologies

EXTEND YOUR APPLICATION REACH WITH STATE-OF-THE-ART SFC

With further improvements in technology, supercritical fluid chromatography (SFC) has gained increasing interest among chromatographers. The new Agilent InfinityLab SFC Solutions allow you to separate and quantify complex mixtures faster and more efficiently than ever before. Deploying SFC in your lab gives you a technique that is orthogonal to normal- or reversed-phase LC and enables you to reach beyond your current application spectrum.

Benefits of Agilent InfinityLab SFC Solutions at a glance

Maximize analytical efficiency

- Achieve analysis speeds up to 10-times faster than HPLC
- Eliminate sample solvent effects with feed injection
- Benefit from variable injection from 0.1 to 90 µL

Maximize instrument efficiency

- Exploit the full orthogonality of supercritical fluid chromatography
- Compare SFC and UHPLC results on one system

Maximize laboratory efficiency

- Reduce costs of solvent purchase and waste disposal by a factor of five
- Eliminate toxic solvents
- Make your lab leaner and greener

What's Inside?

This compendium demonstrates the power and application reach of SFC. An introduction to recent technology advancements of the new Agilent InfinityLab SFC Solutions is followed by examples from the application fields of pharma, biopharma, food, and forensics as well as from chemicals and energy.

All applications are available for download from the Agilent website. Simply search for the publication number given at the end of each application. Alternatively, you can search for SFC applications using the Agilent Application Finder at **www.agilent.com/chem/sfc-applications**

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For Forensic Use.

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Chapter 1

PERFORMANCE CHARACTERISTICS OF THE AGILENT INFINITYLAB SFC SOLUTIONS

Supercritical Fluid Chromatography with Flexible Injection Volumes at Highest Precision

Performance Evaluation of the Agilent 1260 Infinity II SFC Multisampler in the Agilent 1260 Infinity II SFC System

Technical Overview

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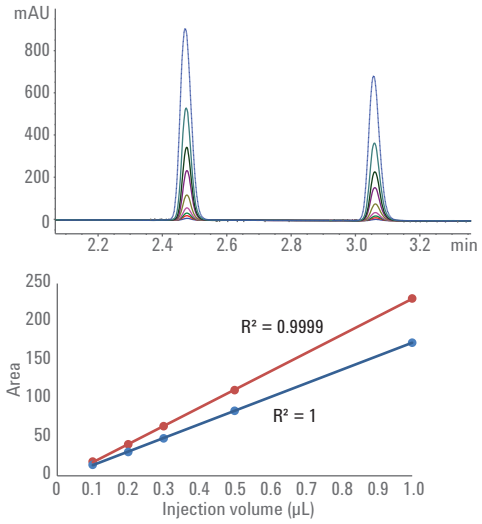
Abstract

This Technical Overview demonstrates the injection principle used in the Agilent 1260 Infinity II SFC Multisampler. In the Agilent 1260 Infinity II SFC Multisampler, the sample volume is drawn under atmospheric pressure conditions, pressurized to system pressure, and injected by an ultrafast syringing process. Data are presented that the 1260 Infinity II SFC Multisampler enables the injection of flexible sample volumes with highest precision, and excellent linearity over a broad volume range.



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Introduction

In contrast to the variable sample introduction of classical HPLC instruments, where the mobile phase filled sampling path can harmlessly experience atmospheric pressure, SFC instruments must avoid using mobile phase at ambient pressure in sampling paths to prevent evaporation of the dense CO_2. Evaporation of the CO_2 in the sampling path could lead to either a complete loss of the sample or an incomplete injection. Therefore, the fixed loop approach, where a previously filled loop was switched into the pressurized CO_2 stream, has been the method of choice for SFC. While this approach yields good peak area precision for full loop injections, it requires loop overfilling and hence a waste of sample. When used for partial loop filling, it requires complicated implementations, and compromises precision performance.

This Technical Overview demonstrates the injection principle used in the Agilent 1260 Infinity II SFC Multisampler. The 1260 Infinity II SFC Multisampler enables the injection of flexible sample volumes with the highest precision, in contrast to the widely used fixed-loop approach, which wastes sample by loop overfilling or suffers poor injection precision for partial loop injections. In the 1260 Infinity II SFC Multisampler, the sample volume is drawn under atmospheric pressure conditions, and pressurized to system pressure before it is injected into the analytical flow path by an ultrafast syringing process.

Experimental

Instrumentation

Agilent 1260 Infinity II SFC System comprised:

- 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 (G7115A) with high-pressure SFC flow cell
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)

Instrumental setup

The 1260 Infinity II SFC Multisampler is connected directly to the SFC pump and downstream to the column. All necessary flushing and washing steps are done through 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

Software

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

Samples

Solutions of caffeine and theobromine (250 mg/L each in methanol), caffeine (0.5 g/100 mL in methanol) and theobromine (250 mg/L in methanol).

Chemicals

All solvents were purchased from Merck, Germany. Chemicals were purchased from Sigma-Aldrich (Germany).

Results and Discussion

The fixed-loop approach is used for sample injection in SFC instruments as a state-of-the-art technique. This approach enables the injection of fixed volumes with high precision, but volumes that are injected by a partial loop fill suffer from compromised precision. The variable-loop concept that is widely used for sample injection in HPLC instruments cannot be used because liquid CO_2 cannot be subjected to atmospheric pressure. This would lead to partial or complete loss of the sample due to evaporation. To overcome this drawback, a flexible injection principle was introduced with the 1260 Infinity II SFC Multisampler for the Agilent 1260 Infinity II SFC System.

The Agilent feed-injection technology provides a pressurized sample, which is injected into the CO_2 stream prior to the column by a syringing process. Before the sample is drawn, the connected loop, needle, and seat are cleaned by purging with feed solvent, while the SFC pump is connected to the column. After drawing the flexible sample volume, it is pressurized in the loop to the pressure of the system. The pressurized loop containing the sample is connected to the CO_2 stream coming from the analytical SFC pump and leading to the column. In this position, the sample can be injected by a syringing process with variable injection speed (feed speed). To flush the complete sample out of the loop, an overfill volume (over-feed volume) can be defined.

SFC method

Parameter	Value
Solvent A	CO_2
Modifier B	Methanol
SFC flow	2.5 mL/min
Isocratic elution	12 %B
Stop time	6 minutes
Gradient elution	5 to 35 %B in 4 minutes
Stop time	6 minutes
Post time	2 minutes
Gradient for large volume injection	0 to 1 minute – 1 %B 1.1 minutes – 5 %B 4 minutes – 35 %B
Stop time	6 minutes
Post time	2 minutes
Back pressure regulator (BPR) temperature	60 °C
BPR pressure	130 bar
Column temperature	40 °C
Injection volume	0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0, 5.0, and 10.0 µL
Large volume injection	10, 20, 30, 40, 50, 60, 70, and 80 µL
Feed solvent	Methanol
Over-feed volume	4 µL
Feed speed	400 µL/min (up to 10 µL injection) and 100 µL/min for large volume injection (>10 µL)
Needle wash	3 seconds in methanol
Detection	272 nm/bandwidth 4 nm; reference 360 nm/bandwidth 100 nm; standard high-pressure SFC flow cell; data rate 10 Hz

There are two instrument parameters controlling the injection: the feed speed and the over-feed volume. For standard injections (0.1 to 10 µL), the feed speed should typically be higher than 100 µL/min (default 400 µL/min, up to 1,000 µL/min) to avoid peak broadening. A lower feed speed could be used for trapping injections into an initial isocratic step. The over-feed volume should not be below 2 µL (default 4 µL) due to possible sample loss. Higher values could be used to flush out sticky compounds or heavy matrix loaded samples. The influence of these parameters on chromatographic performance is discussed in more detail in an Agilent Technical Overview[1].

To determine the performance of the 1260 Infinity II SFC Multisampler, the injection linearity and peak area precision were determined under isocratic and gradient elution conditions for the injection volume range from 0.1 µL to 10.0 µL (Figures 1, 2, and 3). For both sets of experiments, the default conditions for over-feed volume and feed speed (see method) were applied. This ensured that the sample was fed into the CO_2 stream quickly, and made certain that the sample was flushed out completely from the sampling loop. Under isocratic conditions, both compounds were well separated, and peak shapes were excellent for all injection volumes from 0.1 to 10.0 µL (Figure 1A). The injection linearity was calculated for the different ranges of injection volumes: 0.1 to 10.0 µL, 0.1 to 1.0 µL, and 1.0 to 10.0 µL. In all cases, the injection linearity was excellent, with $R^2 > 0.9995$ (Figures 1B to 1D) for both compounds. The results for the gradient separation also showed excellent peak shapes for all tested injection volumes from 0.1 µL up to 10.0 µL (Figure 2A). The injection linearity for both compounds showed excellent values of $R^2 > 0.9999$ for all tested injection volume ranges at 0.1 to 10.0 µL, 0.1 to 1.0, and 1.0 to 10.0 µL (Figures 2B to 2D).

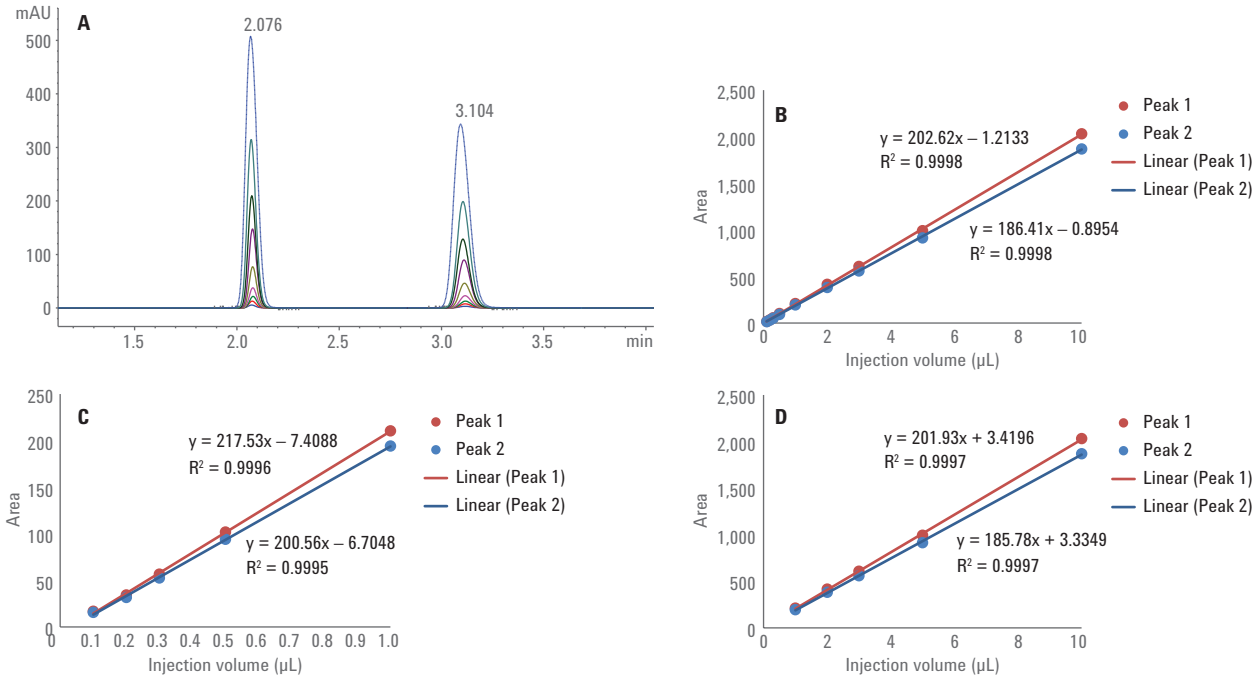


Figure 1. Injection linearity for peak 1 and peak 2 under isocratic elution conditions. A) Chromatogram of the isocratic separation. Peak 1: Caffeine, 2.076 minutes. Peak 2: Theobromine, 3.104 minutes. Injection volume: 0.1 to 10.0 µL. B) Linearity for 0.1 to 10 µL injection volume. C) Linearity for 0.1 to 1.0 µL injection volume. D) Linearity for 1.0 to 10 µL injection volume. R^2 is typically >0.9995.

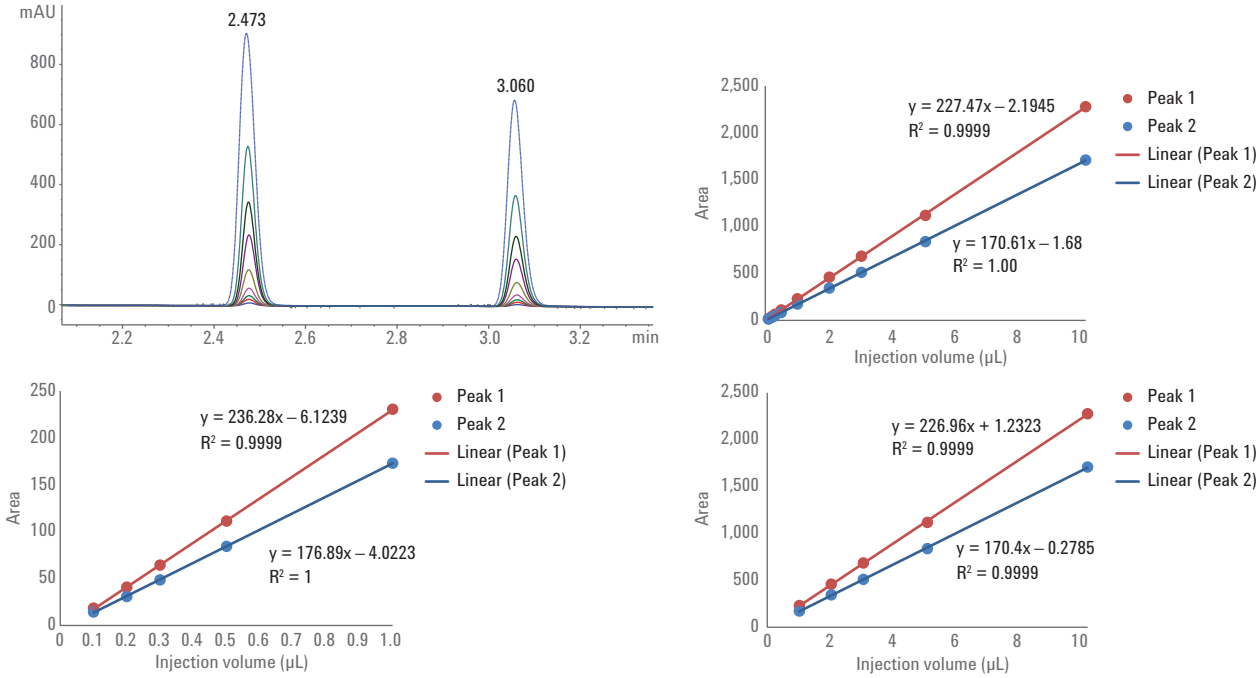


Figure 2. Injection linearity for peak 1 and peak 2 under gradient elution conditions. A) Chromatogram of the gradient separation. Peak 1: Caffeine, 2.473 minutes. Peak 2: Theobromine, 3.060 minutes. Injection volume 0.1 to 10.0 µL. B) Linearity for 0.1 to 10 µL injection volume. C) Linearity for 0.1 to 1.0 µL injection volume. D) Linearity for 1.0 to 10 µL injection volume. R^2 is typically >0.9999.

The area RSD values for all injection volumes were calculated from 10 replicates for the isocratic and gradient experiments (Figure 3). For both compounds, the area RSDs start for the lowest volume injection (0.1 µL) at 3.0 to 3.5 % for the isocratic separation (Figure 3A), and at 2.0–2.5 % for the gradient separation (Figure 3B). For both cases, the RSD values decline to 0.3 % and below for increasing injection volume above 0.5 µL. For all higher injection volumes, up to 10.0 µL, the RSD values remain below 0.3 %.

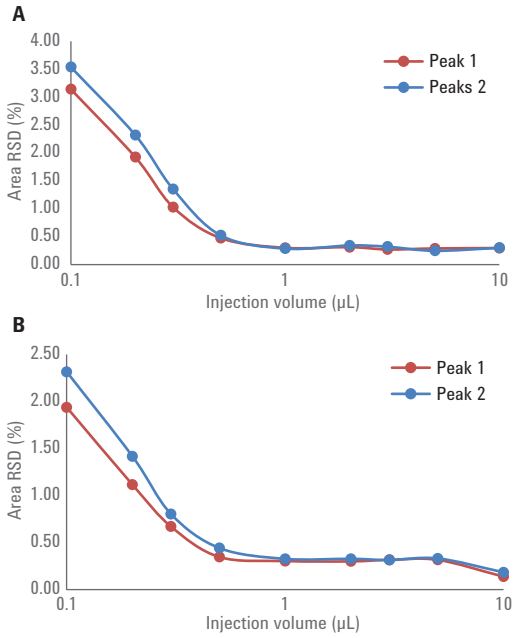
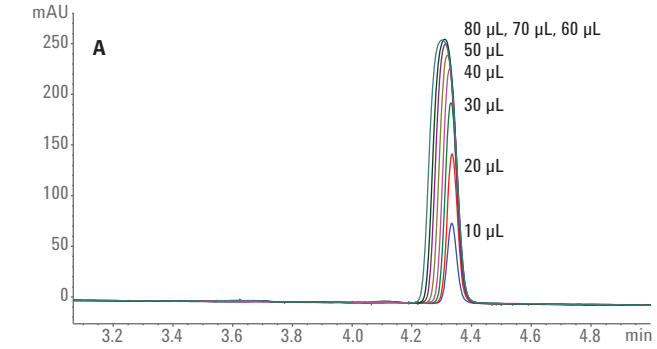


Figure 3. Area RSDs for injection volumes of 0.1 to 10 µL for peak 1 and 2 under A) isocratic and B) gradient separation conditions.

Beyond the range of up to 10 µL injections, the SFC multisampler is capable of injecting even larger volumes. For that purpose, it has a 100 µL sample loop installed. The injectable sample volume can be calculated by subtraction of the used over-feed volume from 100 µL loop volume. To demonstrate this capability, increasing volumes from 10 to 80 µL of a solution of theobromine were injected (250 mg/L in methanol). The large sample volume of methanol solution, which is a strong eluting solvent, was slowly fed into the CO_2 stream (100 µL/min). After the feeding process, the modifier concentration was increased from 1 % to 5 % in a fast step, and the eluting gradient was started. The theobromine peaks increased with the injection volume in peak height and peak width (Figure 4). For the higher injection volumes, the peak height does not increase much, but with the increase of the peak width, the peak area increases linearly (see the table in Figure 4). The correlation of the injection volume versus the peak area shows excellent linearity, with a correlation of 0.9999 (Figure 4B). The peak width at half height increased from 0.04 minutes at 10 µL injection volume to very acceptable 0.1 minutes for 80 µL injection volume, showing symmetrical peaks for all injection volumes (see table in Figure 4). From the peak areas of all injection volumes, the relative standard deviations were calculated, and showed excellent values typically at or below 0.3 % area RSD (Figure 4C).



Inj. volume (µL)	RT (min)	Area	Height	Width (min)	Symm.
10	4.337	187.27	79.89	0.036	0.875
20	4.336	375.48	148.32	0.039	0.876
30	4.331	566.40	197.70	0.045	0.872
40	4.326	760.59	231.65	0.053	0.881
50	4.320	938.66	246.14	0.062	0.891
60	4.314	1,127.29	255.96	0.073	0.905
70	4.309	1,315.80	261.58	0.084	0.933
80	4.302	1,494.58	261.62	0.096	0.947

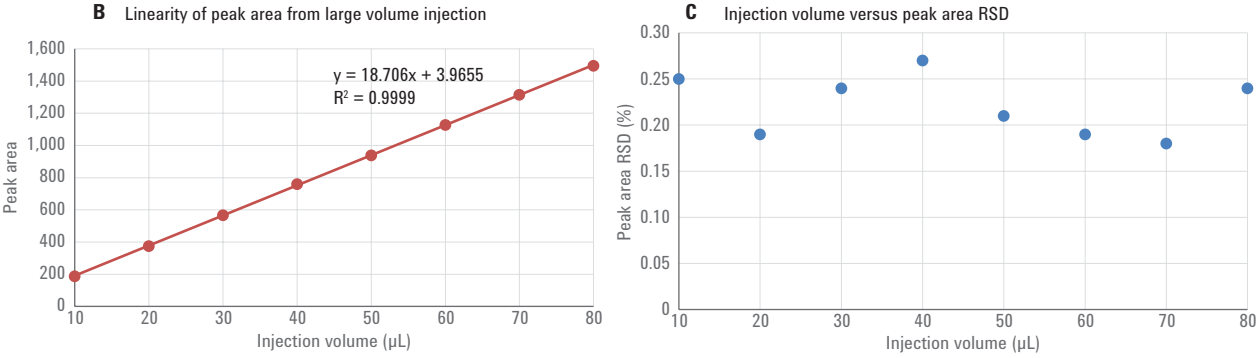


Figure 4. Results of large volume injections.
A) Overlay of the chromatograms obtained for injection volumes from 10 to 80 µL sample.
B) Area linearity obtained from large volume injection with R^2 0.9999.
C) Peak Area RSDs (%) calculated from 10 replicate injections of each large volume injection.
Table) Summary of all parameters measured for large volume injections.

Finally, the carryover behavior of the 1260 Infinity II SFC Multisampler caused by an injection of a highly concentrated caffeine sample (5 g/L in MeOH, injection volume 5 µL) was examined. The carryover was determined from the first blank injection after the injection of the high caffeine concentration. The carryover was calculated as area percentage in comparison to the peak of the high caffeine concentration to be 0.0014 % (14 ppm). No carryover was detected in the second blank injection after the injection of the high caffeine concentration (Figure 5).

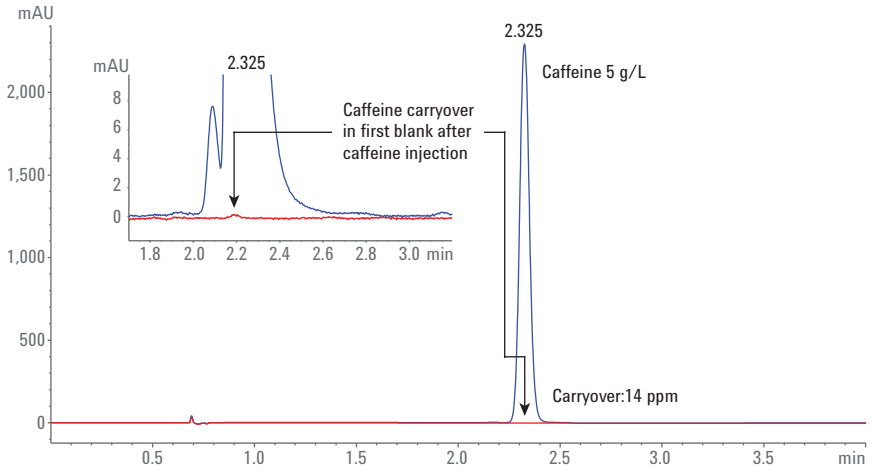


Figure 5. Determination of carryover of the Agilent 1260 Infinity II SFC Multisampler by an injection of a high concentrated caffeine sample (5 g/L in MeOH, injection volume 5 µL). Carryover was determined from the first blank injection after the injection of the high caffeine concentration. No carryover was detected in the second blank injection after the injection of the high caffeine concentration. Due to the fact that the injected amount of caffeine reaches the nonlinear range of the detector, the correct area for the 5 µL injection was calculated by extrapolation from lower volume injections.

Conclusion

This Technical Overview discusses the performance results obtained for the Agilent 1260 Infinity II SFC Multisampler, which enables variable injection volumes at highest precision. The area RSDs for injection volumes between 0.5 and 10 µL are typically below 0.3 %. Even lower injection volumes, down to 0.1 µL, showed area RSDs typically below 2.5 %. The demonstrated injection linearity is typically better than 0.9995. Even for injection volumes of up to 80 µL, excellent area RSD values below 0.3 % could be achieved. The linearity of the peak area for large injection values is also extremely good. The flexible sample introduction showed a negligible carryover of only 14 ppm. This offers performance that is comparable to the typically used fixed-loop autosamplers in full-loop mode, but with the high flexibility of a variable-loop autosampler.

Reference

1. Naegele, E. Feed Speed and Over-Feed Volume – New Parameters for SFC Injection, *Agilent Technologies Technical Overview*, publication number 5991-7626EN, **2017**.

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Agilent Technologies

Feed Speed and Overfeed Volume

New Parameters for Injection in Supercritical Fluid Chromatography

Technical Overview

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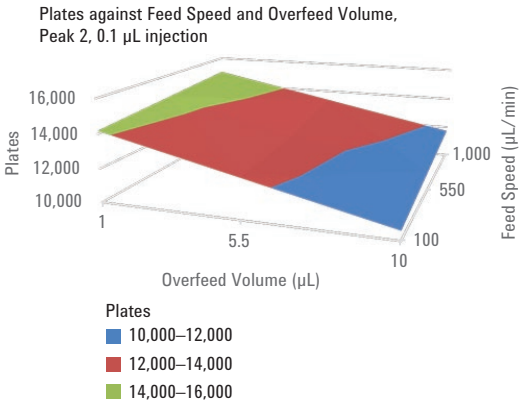
Abstract

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.

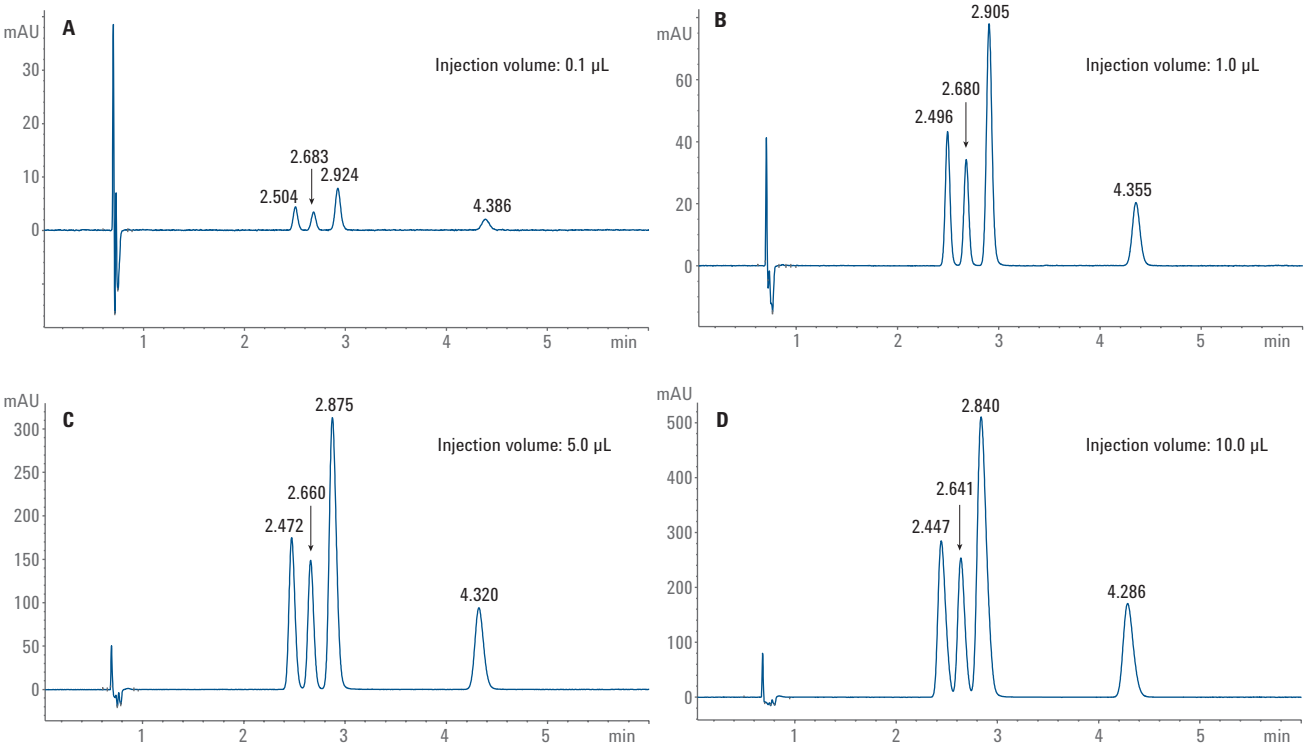

Figure 1. Different injection volumes at a Feed Speed of 400 µL/min, and an Overfeed Volume of 4 µL. A) 0.1 µL, B) 1.0 µL, C) 5.0 µL, D) 10.0 µL.

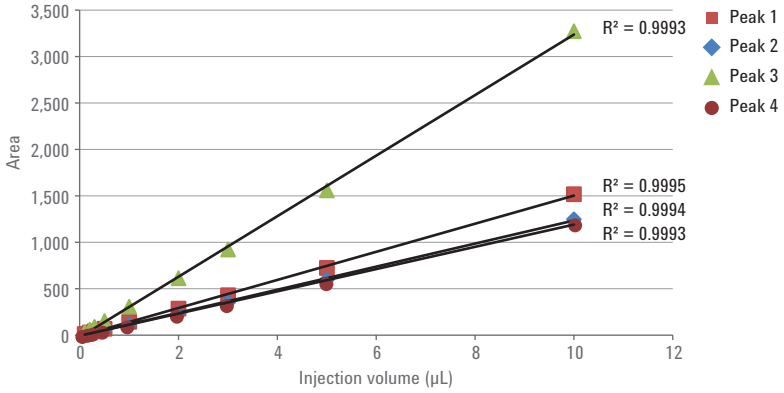

Figure 2. Injection volume linearity of compounds 1 to 4 at 400 µL/min Feed Speed, and 4 µL Overfeed Volume.

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).

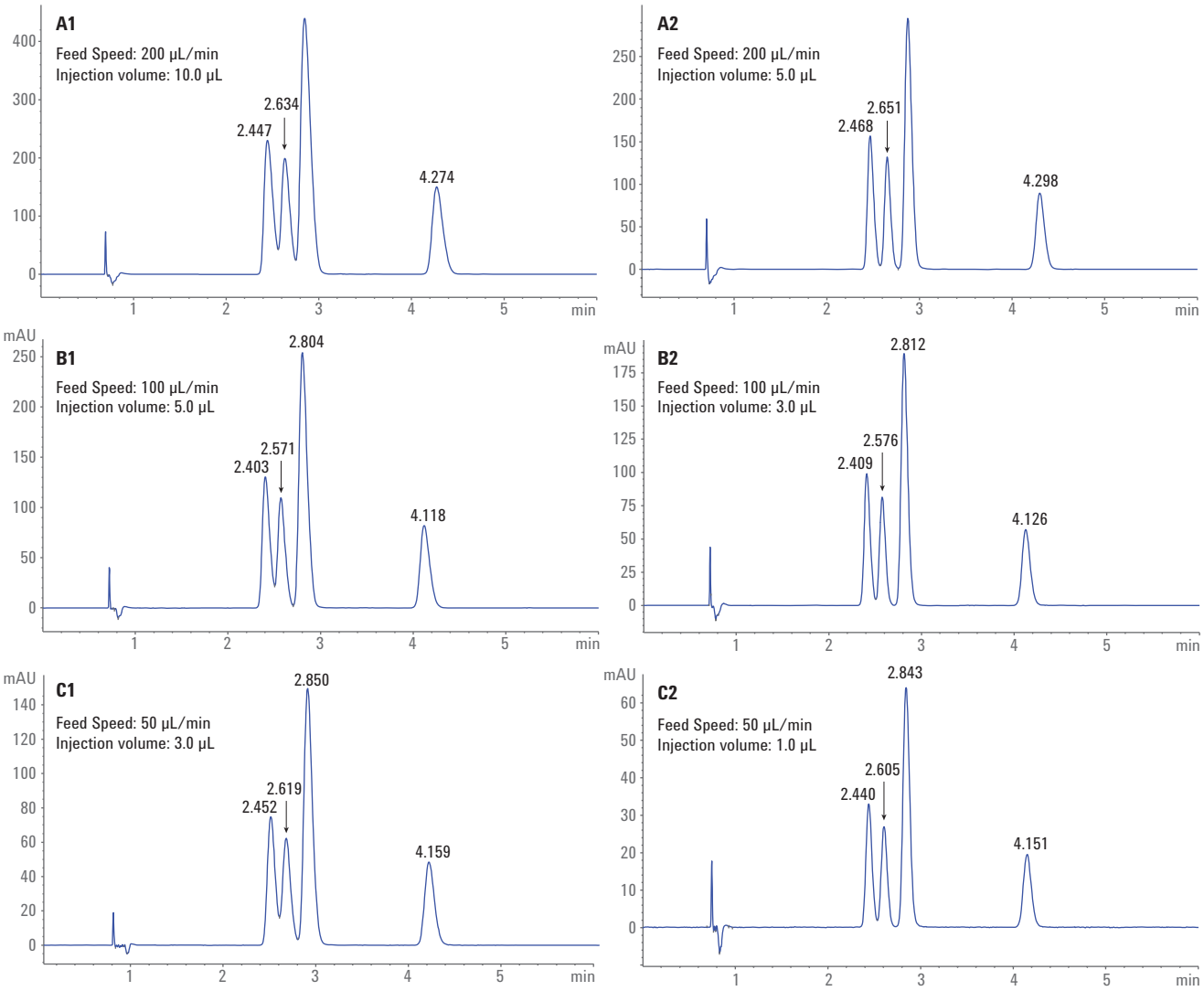

Figure 3. Chromatographic limitation depending on Feed Speed and injection volume under isocratic separation conditions. A) The first three peaks started to coelute for an injection volume of 10 µL at a Feed Speed of 200 µL/min (A1). The peaks were still separated for the 5 µL injection (A2). B) At a 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

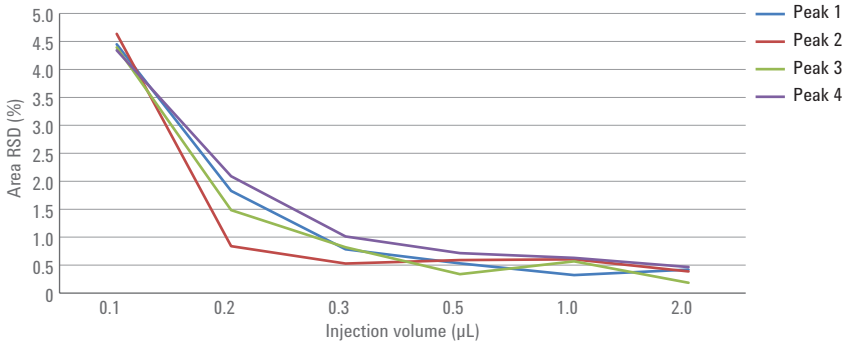

Figure 4. Area RSD values of injection volumes from 0.1 to 2 µL at 50 µL/min Feed Speed.

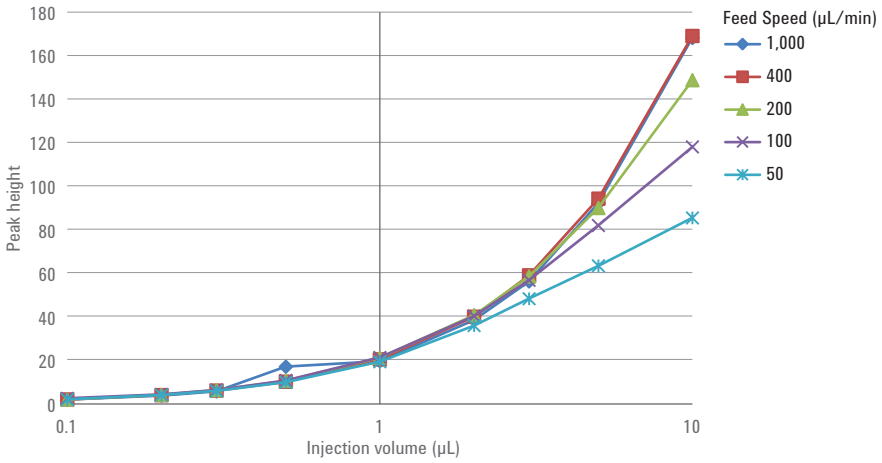

Figure 5. Peak height of peak 4 in comparison to the applied Feed Speed for different injection volumes.

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.

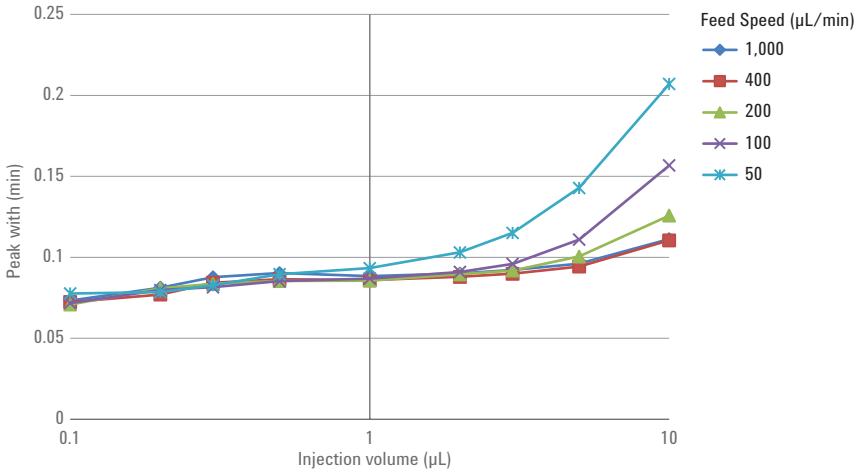

Figure 6. Peak width of peak 4 in comparison to the applied Feed Speed for different injection volumes.

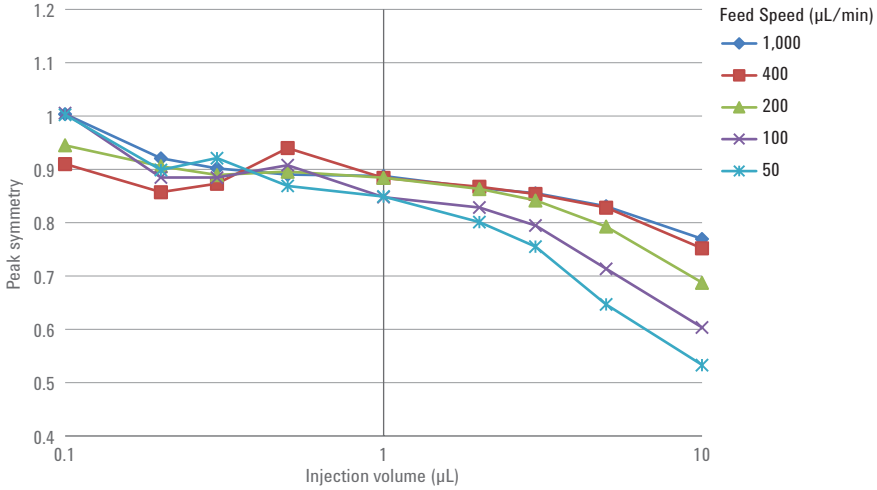

Figure 7. Peak symmetry of peak 4 in comparison to the applied Feed Speed for different injection volumes.

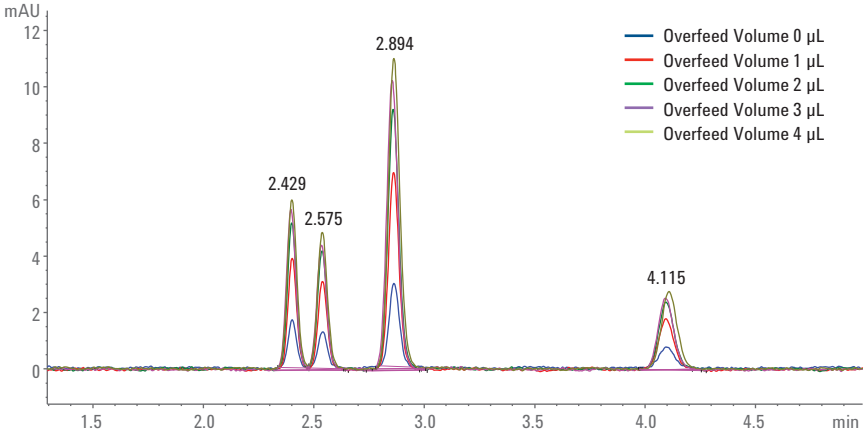

Figure 8. Influence of the Overfeed Volume on sample introduction for 0.1 µL injection volume.

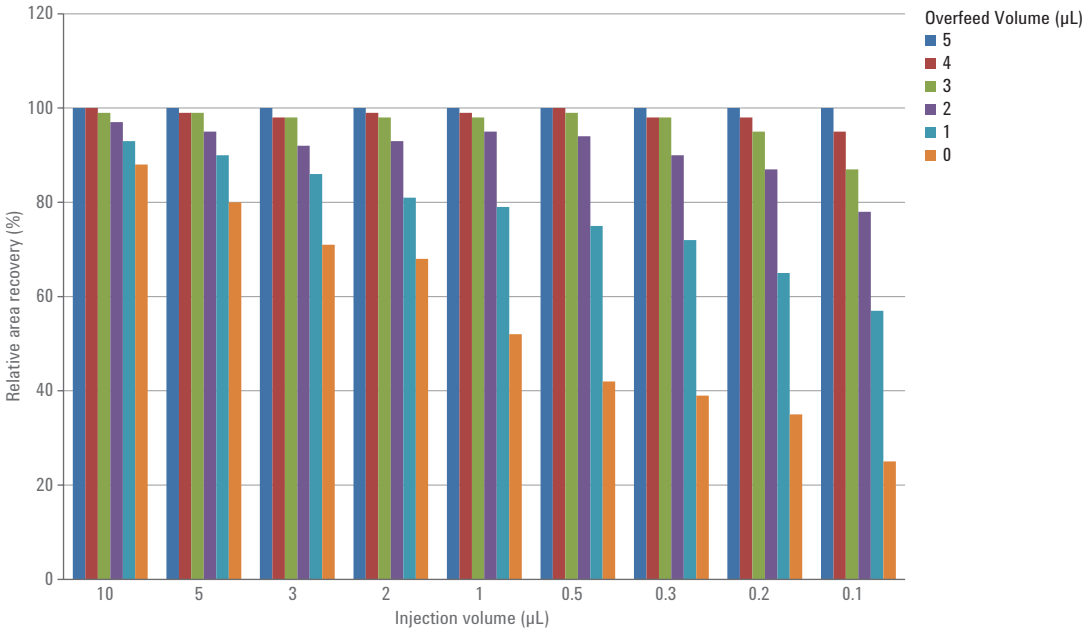

Figure 9. Comparison of the influence of the Overfeed Volumes on sample introduction for different injection volumes. The relative area recoveries of peak 4 are shown for different Overfeed Volumes. The areas obtained for the Overfeed Volume of 5 µL were used as a basis, and assumed to deliver a complete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

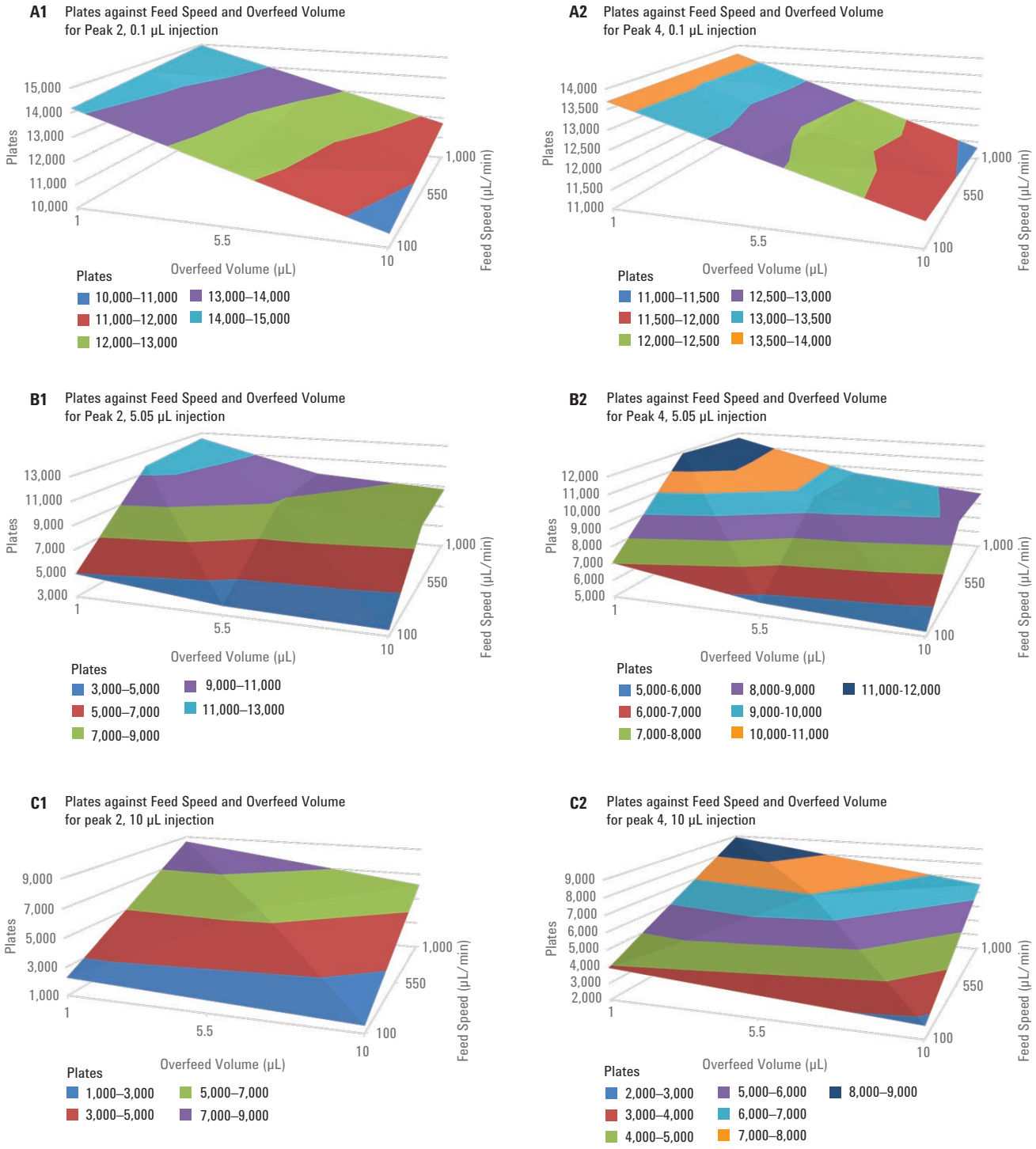

Figure 10. Comparison of the chromatographic performance expressed as chromatographic plates in a DOE space for Feed Speed, Overfeed Volume, and injection volume. Two compounds were used as examples, caffeine (peak 2, k' = 2) and theobromine (peak 4, k' = 4). Feed Speed range: 100–1,000 µL/min, Overfeed 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.

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

Focusing Late-Eluting Compounds Using Backpressure and Flow Gradients

Technical Overview

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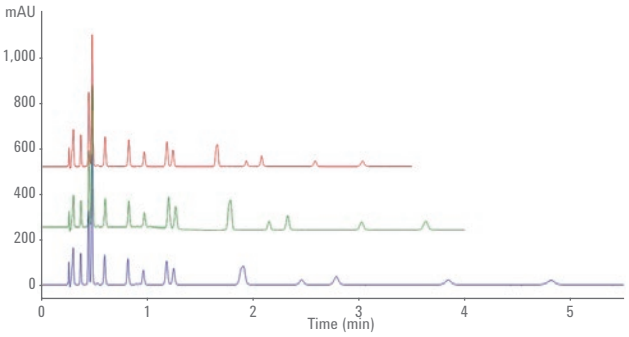
Abstract

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.

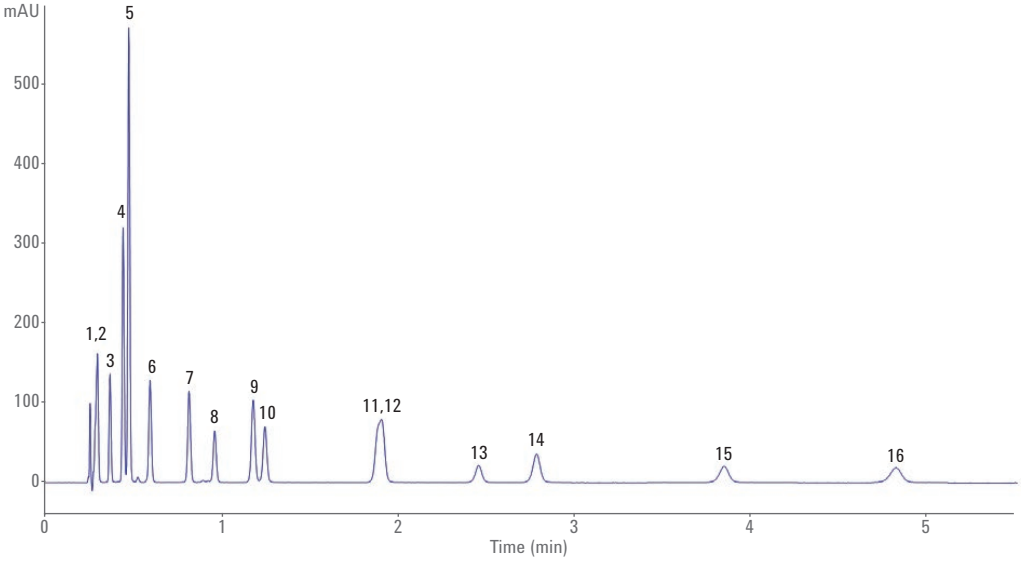

Figure 1. Initial separation method for the PAH standard. The method successfully separated 10 of 16 compounds, while compounds 4 and 5 were only partially separated. Compounds 1 and 2, and 11 and 12 coeluted. Mobile phase: 100 % CO_2, isocratic, no modifier used.

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

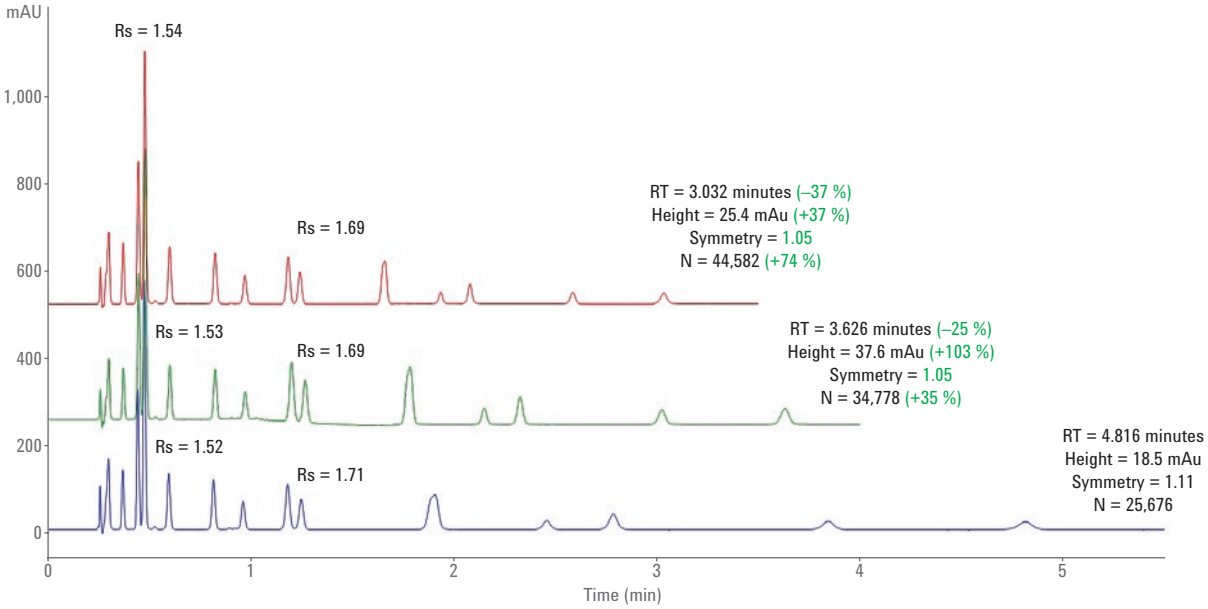

Figure 2. Comparison of the runs under isocratic conditions using pure CO_2: The last peak eluted after 4.816 minutes with no focusing (blue), after 3.626 minutes when a backpressure gradient was applied (green), and 3.032 minutes when a flow gradient was used (red). In total, the run time could be reduced 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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Orthogonal Chromatographic Separations using the Agilent 1260 Infinity II SFC/UHPLC Hybrid System

Technical Overview

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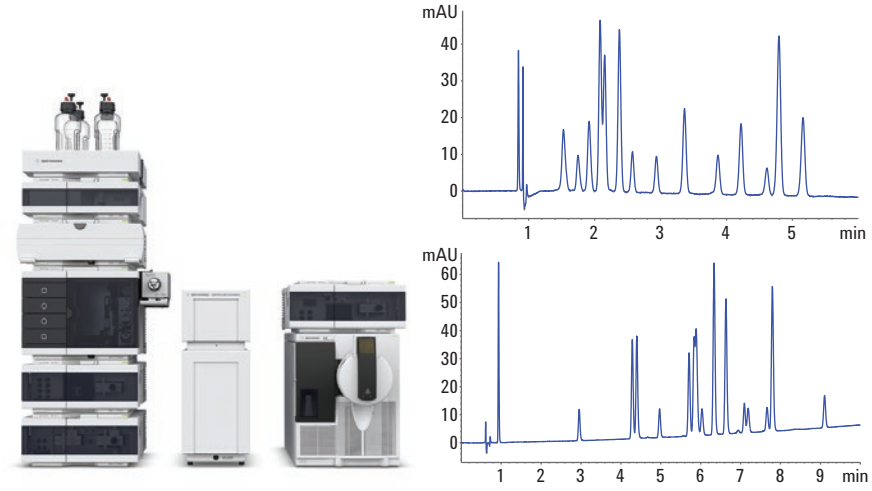
Abstract

This Technical Overview provides a detailed explanation of how to set up an Agilent 1260 Infinity II SFC/UHPLC Hybrid System with or without an MS system. Performance data will be shown and discussed for both modes of operation. As an application example, the separation of a mixture of pesticides in SFC mode and in UHPLC mode will be shown, and the orthogonality of the separation will be discussed.



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Introduction

Performing both an SFC-based separation and a UHPLC-based separation of a given complex sample delivers complementary information about the sample content. These separations are truly orthogonal due to their different separation mechanisms, which are based on the interaction of the analytes in completely different fluid media and stationary phases. Reversed-phase separation typically uses hydrophobic stationary phases with organic-aqueous liquid phases, whereas SFC typically uses polar stationary phases with CO_2/organic liquid phases similar to normal phase separations.

To avoid the burden of purchasing independent SFC and UHPLC instruments, the Agilent 1260 Infinity II SFC/UHPLC Hybrid System offers both SFC and UHPLC capability in a single instrument. The common modular parts such as autosampler, column compartment, and detector are shared between both techniques. The setup of such an instrument is an easy task, requiring just one single valve to switch between both techniques. This enables high reliability and robustness for both SFC and UHPLC. In addition, the quality of the acquired data can be improved by adding an MS system, which works with a make-up pump under split-flow conditions in SFC mode and with full flow in UHPLC mode.

This Technical Overview demonstrates the performance of the Agilent 1260 Infinity II SFC/UHPLC Hybrid System in SFC and LC modes by showing retention time, area precision, and linearity data. As an example of the orthogonality of both techniques, the separation of a complex mixture of pesticides on the 1260 Infinity II SFC/UHPLC Hybrid System by means of both separation techniques will be shown and discussed.

Experimental

SFC method for pesticide separation

Parameter	Value
Solvent A	CO_2
Modifier B	Methanol
SFC flow	2.5 mL/min
Gradient	5 %B to 25 %B in 6 minutes Stop time: 6 minutes Post time: 2 minutes
Backpressure regulator (BPR) temperature	60 °C
BPR pressure	140 bar
Column temperature	40 °C
Injection volume	1.0 µL
Feed solvent	Methanol
Over feed volume	4 µL
Feed speed	400 µL/min
Needle wash	3 seconds in methanol
DAD	254 nm/band width 4 nm; Ref. 360 nm/band width 100 nm Standard high-pressure SFC flow cell
Data rate	20 Hz

LC method for pesticide separation

Parameter	Value
Solvent A	Water + 0.1% FA
Solvent B	Acetonitrile + 0.1% FA
Flow rate	2.5 mL/min
Gradient	15 %B to 70 %B in 6 minutes Stop time: 6 minutes Post time: 3 minutes
Column temperature	40 °C
Injection volume	1.0 µL
Needle wash	3 seconds in methanol
DAD	254 nm/band width 4 nm; Ref. 360 nm/bandwidth 100 nm Standard high-pressure SFC flow cell
Data rate	20 Hz

Instrumentation

Agilent 1260 Infinity II SFC/UHPLC Hybrid 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 Diode Array Detector (G7115A) with high-pressure SFC flow cell
- Agilent 1260 Infinity II Multicolumn Thermostat (MCT) (G7116A) with Agilent InfinityLab Quick Change 4-position/10-port four-column selection valve (p/n 5067-4287)
- Agilent 1260 Infinity II Quaternary Pump (G7111B)
- Agilent 1290 Infinity valve drive (G1170A) with 2-position/10-port valve (G4232B)
- Agilent 1260 Infinity II Isocratic Pump (G7110B) and SFC/MS splitter kit (G4309-68715)
- Agilent 6150 Single Quadrupole LC/MS with Agilent Jet Stream technology

Instrumental setup

For the conversion of an SFC system to an SFC/UHPLC hybrid system, a quaternary or binary UHPLC pump is added and connected by a 2-positon/10-port valve, which allows direct switching between SFC and LC modes (Figures 1 and 2). The multicolumn thermostat (MCT) is equipped with a 4-position/10-port four-column selection valve for column switching, for example, from a typical SFC column to a typical analytical UHPLC column (not shown in Figures 1 or 2). A central point, which is outlined in Figures 1 and 2, is the plumbing at the 2-positon/10-port valve. The position of the valve shown in Figure 1 connects the SFC pump and the SFC control module to the shared modules of the instrument. After flowing through the autosampler, the column oven with the SFC column,

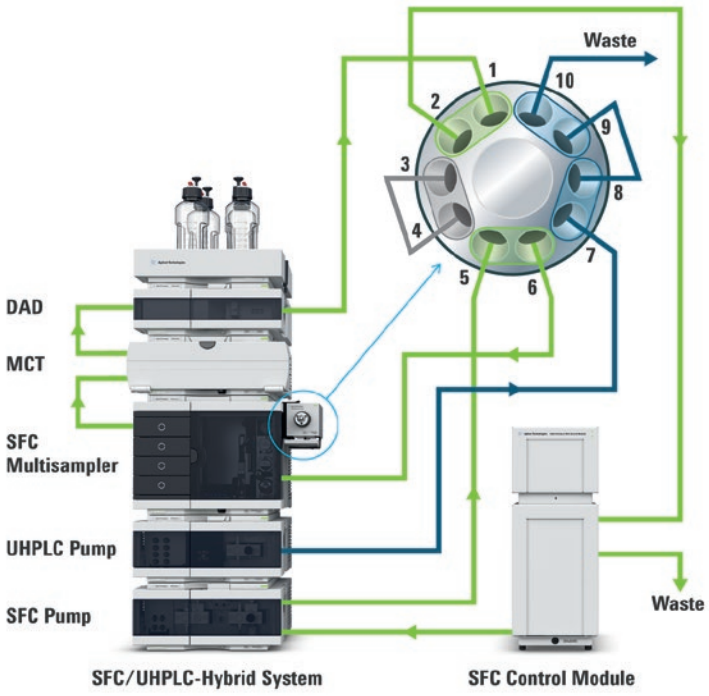


Figure 1. Agilent 1260 Infinity II SFC/UHPLC Hybrid System in SFC Mode. The SFC Control Module is connected to the SFC pump and the shared modules (green tubing). The UHPLC pump is connected to the waste position (blue tubing).

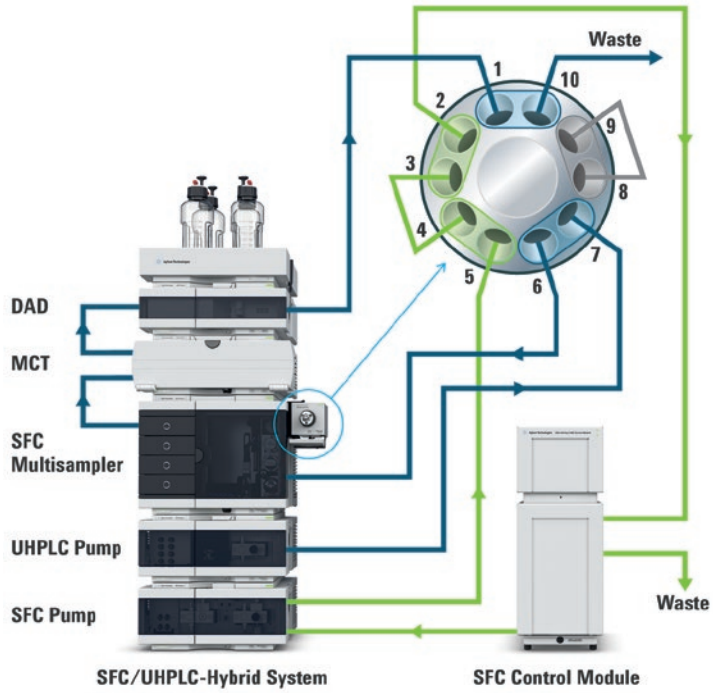


Figure 2. Agilent 1260 Infinity II SFC/UHPLC Hybrid System in UHPLC Mode. The UHPLC pump is connected to the shared modules (blue tubing). The SFC pump is directly connected to the SFC Control Module in a loop (green tubing).

and the detector, the CO_2 stream is connected back to the SFC control module for backpressure regulation. In this position, the quaternary UHPLC pump is connected to waste.

After switching the 2-positon/10-port valve to the UHPLC position, the quaternary pump is connected to the shared modules (Figure 2). The SFC pump is directly connected to the SFC control module to maintain backpressure.

The additional connection of the SFC/UHPLC hybrid instrument to a mass spectrometer does not increase complexity. It just needs a simple replumbing at the 2-postion/10-port valve to include the SFC make-up pump with flow splitting to the MS as well as full UHPLC flow to the MS (Figures 3 and 4). In this case, in SFC mode, the CO_2 stream is connected to the first splitter for the introduction of the make-up flow after passing the shared modules. The second splitter divides the flow between backpressure regulation at the SFC control module and the MS (Figure 3). In UHPLC mode, the quaternary pump is connected to the shared modules, and directly connected to the MS for full flow (Figure 4). To make use of a split-flow approach in UHPLC mode, the bridging capillary at ports 1–2 could be replaced by a splitter for the UHPLC side.

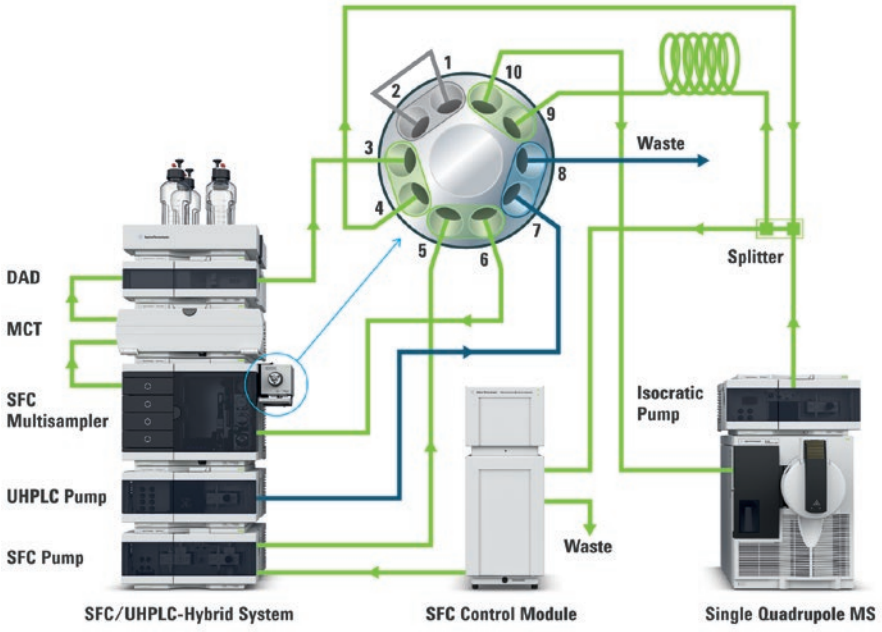


Figure 3. Agilent 1260 Infinity II SFC/UHPLC/MS Hybrid System in SFC/MS mode. The SFC Control module is connected to the SFC pump and the shared modules (green tubing). The UHPLC pump is connected to the waste position (blue tubing). The CO_2 stream is connected to the first splitter for the introduction of the make-up flow after passing the shared modules. The second splitter divides the flow between backpressure regulation at the SFC control module and the MS (coiled green capillary: 50 µm, 100 cm, restriction to prevent decompression).

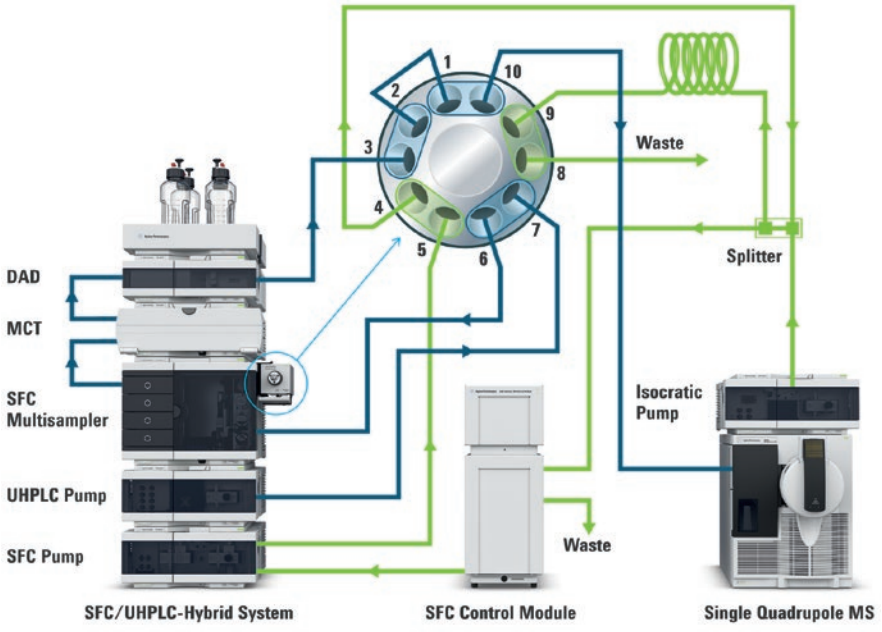


Figure 4. Agilent 1260 Infinity II SFC/UHPLC/MS Hybrid System in UHPLC/MS Mode. The UHPLC pump is connected to the shared modules (blue tubing) and in direct flow to the MS. The SFC pump is directly connected to the SFC control module in a loop (green tubing) including the splitter (coiled green capillary: 50 µm, 100 cm, restriction to prevent decompression).

Columns

- **SFC mode:**
  Agilent ZORBAX RX-SIL,
  4.6 × 150 mm, 5 µm
  (p/n 883975-901)
- **SFC mode:**
  Agilent ZORBAX NH_2, 4.6 × 150 mm,
  5 µm (p/n 883952-708)
- **LC mode:**
  Agilent ZORBAX SB C18,
  4.6 × 150 mm, 3.5 µm
  (p/n 883975-902)

Software

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

Samples

- Solution of caffeine (0.5 g/100 mL in methanol)
- SFC checkout mixture (theophylline, caffeine, thymine, theobromine; 250 mg/L each in MeOH)
- Solutions of pesticides, 1 mg/mL (MeOH) and mixture of equal volumes (Table 1)

Chemicals

All solvents were purchased from Merck, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22 µm membrane point-of-use cartridge (Millipak). Chemicals were purchased from Sigma-Aldrich (Germany).

MS Conditions

Parameter	Value
Electrospray Ionization with Agilent Jet Stream Ion Source	
Ionization mode	positive
Capillary voltage	2,500 V
Nozzle voltage	2,000 V
Gas flow	8 L/min
Gas temperature	220 °C
Sheath gas flow	12 L/min
Sheath gas temperature	380 °C
Nebulizer pressure	35 psi
Make up flow	0.3 mL/min, MeOH + 3 % water + 0.1 % formic acid
MS-Parameter for single quadrupole	
ESI Polarity	positive
Scan	180–450 *m/z*
Dwell time	200 ms
Fragmentor	70 V
Gain	1.0

Table 1. Fifteen pesticides used in the complex test sample demonstrate orthogonal separation in SFC mode and UHPLC mode. The individual retention times demonstrate the shifts of the compounds under the used conditions. The identification of the individual compounds was done using a single quadrupole MS (see the Experimental section and Figures 3 and 4)

No.	Name	Chemical formula	m/z $[M+H]^+$	SFC RT (min)	LC RT (min)
1	Prometryn	$C_{10}H_{19}N_5S$	242.10	1.528	5.880
2	Sebuthylazine	$C_9H_{16}ClN_5$	230.11	1.748	7.190
3	Terbuthylazine	$C_9H_{16}ClN_5$	230.11	1.917	7.664
4	Atrazine	$C_8H_{14}ClN_5$	216.10	2.084	6.035
5	Metobromuron	$C_9H_{11}BrN_2O_2$	259.00	2.153	6.632
6	Methabenzthiazuron	$C_{10}H_{11}N_3OS$	222.06	2.153	5.710
7	Linuron	$C_9H_{10}Cl_2N_2O_2$	249.02	2.375	7.798
8	Terbuthylazine-desethyl	$C_7H_{12}ClN_5$	202.08	2.572	4.971
9	Atrazine-desethyl	$C_6H_{10}ClN_5$	188.06	2.939	2.954
10	Hexazinone	$C_{12}H_{20}N_4O_2$	253.16	3.362	4.400
11	Nimodipine	$C_{21}H_{26}N_2O_7$	419.18	3.870	9.107
12	Chlorotoluron	$C_{10}H_{13}ClN_2O$	213.08	4.220	5.838
13	Nifedipine	$C_{17}H_{18}N_2O_6$	347.10	4.613	7.095
14	Diuron	$C_9H_{10}Cl_2N_2O$	233.02	4.795	6.333
15	Metoxuron	$C_{10}H_{13}ClN_2O_2$	229.07	5.161	4.286

Results and Discussion

In the SFC/UHPLC hybrid system, both separation principles are combined by dedicated and shared modules. For proof of concept, it is essential that the performance of the instrument is excellent in both modes and at the same level as for the stand-alone SFC and UHPLC instruments. To demonstrate this performance, the relative standard deviation of peak areas and retention times, and the injection linearity was measured.

Performance in SFC mode

For the measurement of peak area RSD, retention time RSD, and injection linearity data, the SFC/UHPLC instrument was run in SFC mode according to the configuration and valve position shown in Figure 1. A caffeine sample, as described in the experimental section, was used as the test standard, and the chromatography was done on an Agilent ZORBAX Rx-SIL column with methanol as a modifier for the CO_2 under isocratic conditions (12 % methanol, for other conditions, see the method parameters in the Experimental section). For the evaluation of the analytical range, injections from 0.1 to 10 µL were done (n = 10). The calculated peak area RSD for the injection volume of 0.1 µL was determined to be approximately 1.2 %, decreasing to below 0.3 % for injection volumes above 0.5 µL, and remaining around 0.2 % for all injection volumes up to 10 µL (Figure 5A). The peak area linearity for the complete range of injection volumes was excellent, with R^2 = 0.9999 (Figure 5B). The calculated average retention time RSD value was 0.08 %. These values are in accordance with the corresponding values measured for the Agilent 1260 Infinity II SFC System[1]. The possibility to inject the described variable injection volumes is enabled by the concept of Feed Injection performed by the Agilent 1260 Infinity II SFC Multisampler, which was introduced with this system. For the optimization of the Feed Injection process, two new injection parameters were introduced: Feed Speed and Overfeed Volume[2]. With the SFC Multisampler, it is also possible to inject larger volumes beyond 10 µL, up to >90 µL (100 µL minus Overfeed Volume) while preserving the same excellent performance[1].

Performance in UHPLC mode

For the experimental determination of peak area RSD, retention time RSD, and injection linearity under UHPLC conditions, the setup and valve position described in Figure 2 were used.

The chromatography of the caffeine sample, identical to the one applied under SFC conditions, was done on an Agilent ZORBAX SB-C18 column under isocratic conditions (14 % acetonitrile, for other conditions, see the method parameters in the Experimental section). In contrast, the 1260 Infinity II SFC Multisampler was switched to UHPLC mode and from Feed Injection mode to a standard flow-through injection mode.

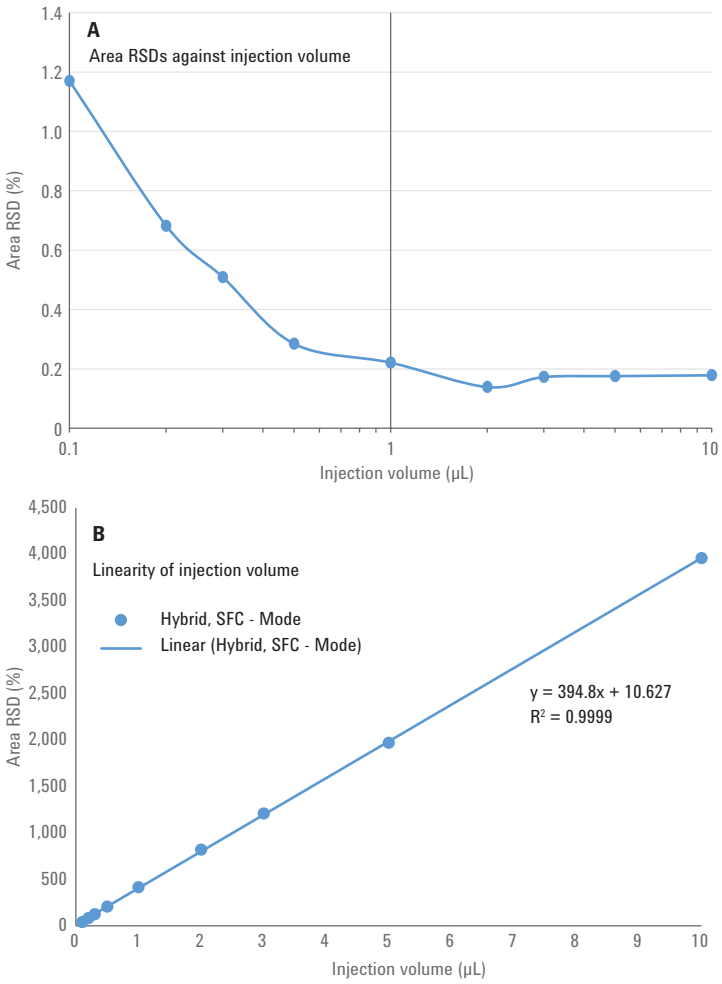


Figure 5. Performance in SFC mode. A) Determination of peak area RSDs in dependence of the injection volume (0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0, 5, and 10 µL). Area RSDs are typically below 0.3 % for all injection volume above 0.5 µL. B) Peak area linearity of injection volumes between 0.1 and 10 µL, R^2 = 0.9999.

The analytical range from 0.1 to 10 µL injection volume showed an RSD performance of 0.2 % and lower for all injection volumes above 0.5 µL (Figure 6A). The linearity of injection volumes in this range was typically R^2 >0.9999 (Figure 6B). For the evaluation of the large injection volumes under UHPLC conditions, the caffeine sample was diluted 1:10 with water and injected in the range of 10 to 100 µL. The determined peak area RSDs were typically below 0.12 %, and injection volume linearity was better than R^2 >0.9999 (Figure 7). The average retention time RSD values were typically 0.08 % for the injections in the analytical range and the same for the large volume injection range. These results are in accordance with the values already determined for pure UHPLC systems[3].

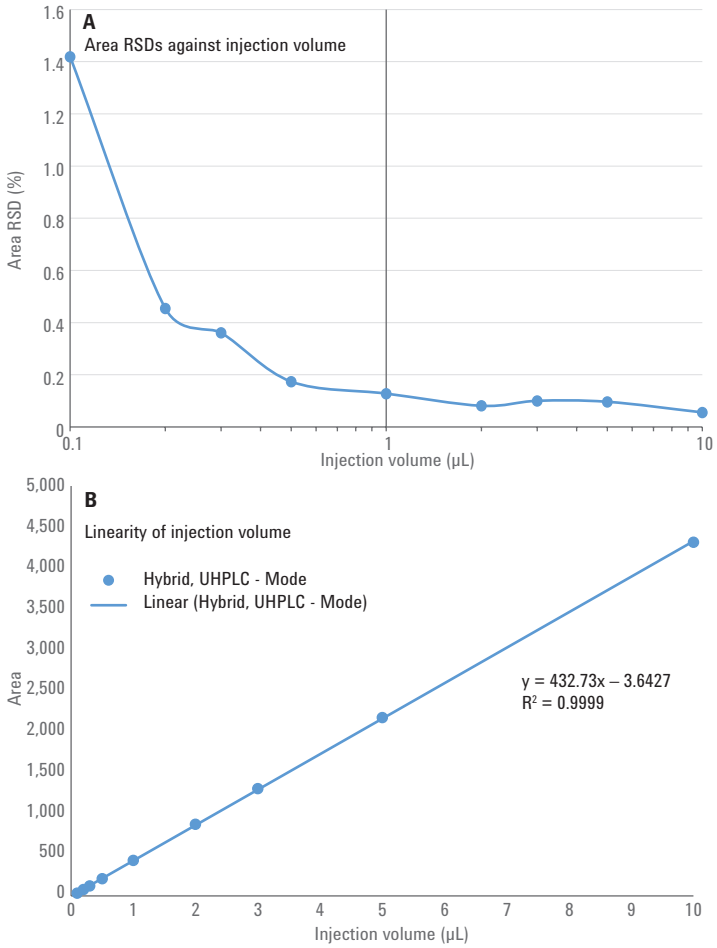


Figure 6. Performance in UHPLC mode. A) Determination of peak area RSDs in dependence of the injection volume (0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0, 5, and 10 µL). Area RSDs are typically below 0.2 % for all injection volume above 0.5 µL. B) Peak area linearity of injection volumes between 0.1 and 10 µL, R^2 = 0.9999.

Rapid switching between SFC and UHPLC modes

The SFC/UHPLC hybrid system offers the advantage to run samples in both modes by means of separate SFC and UHPLC methods, which comprise all necessary settings to switch the SFC control module, the 1260 Infinity II SFC Multisampler, the pumps, and the complete flow stream by the 2-postion/10-port valve, as shown in Figures 1 and 2.

The typical problem of clogging the system by icing during switching between UHPLC and SFC is caused by residual water plugs from reversed-phase HPLC use. This problem can be avoided by flushing the instrument with an organic solvent such as methanol before switching from UHPLC to SFC. With the 1260 Infinity II SFC Multisampler, this is done automatically by a special automated flushing procedure, if the mode given in the method settings changes from UHPLC to SFC. This avoids a separate flushing method and clears the commonly shared Multisampler of aqueous solvent, which could be a residue in the largest system dead volume, the 100 µL flow through sample loop. As a recommendation, separate dedicated columns should be used for both modes. They can be switched by a column selection valve located in the MCT with the chosen SFC or UHPLC method. To demonstrate the switching between SFC and UHPLC, replicates of SFC and UHPLC runs within a single sequence were set up (Figure 8). The complete sequence ran without any problems with highly reproducible peak areas and retention times, as shown in Figure 8.

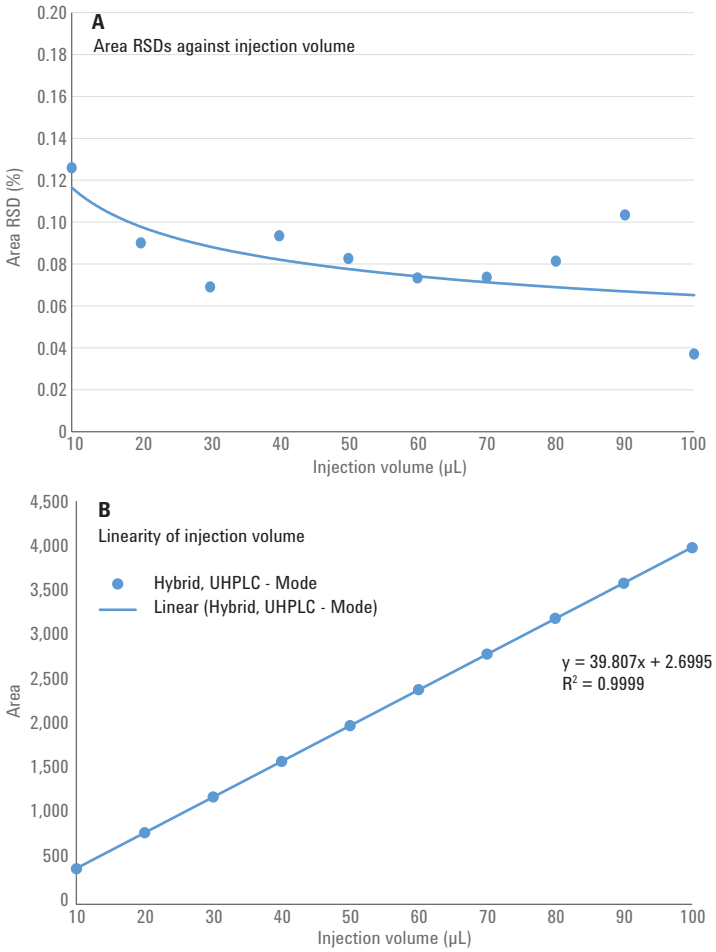
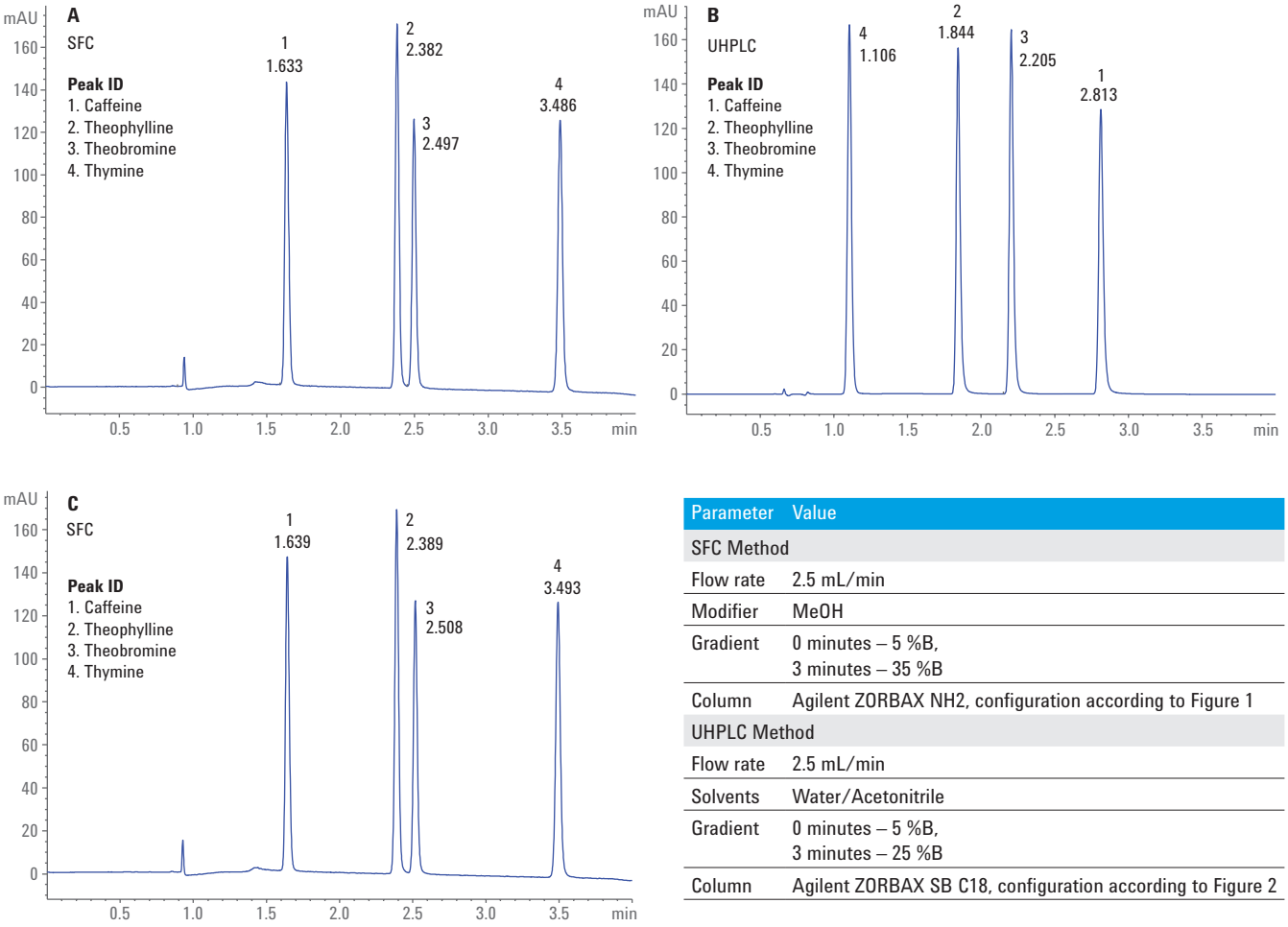


Figure 7. Performance in UHPLC mode for large injection volumes. A) Determination of peak area RSDs in dependence of the injection volume (10 to 100 µL, step 10 µL). Area RSDs are typically below 0.12 %. B) Peak area linearity of injection volumes between 10 and 100 µL, R^2 = 0.9999.



Parameter	Value
SFC Method	
Flow rate	2.5 mL/min
Modifier	MeOH
Gradient	0 minutes – 5 %B, 3 minutes – 35 %B
Column	Agilent ZORBAX NH2, configuration according to Figure 1
UHPLC Method	
Flow rate	2.5 mL/min
Solvents	Water/Acetonitrile
Gradient	0 minutes – 5 %B, 3 minutes – 25 %B
Column	Agilent ZORBAX SB C18, configuration according to Figure 2

Figure 8. Sequence of multiple SFC and UHPLC runs on the SFC/UHPLC Hybrid System. The sequence started with replicate SFC runs (A) and continued with replicate UHPLC runs (B) after switching to a method with the described UHPLC method settings. Finally, multiple SFC runs were done at the end of the sequence (C) after switching back to a SFC mode method. To equilibrate the respective column, a blank run was done at the beginning of each SFC mode or UHPLC mode part of the sequence. Other parameters are the same as in the methods given in the Experimental section. The columns were switched with the different methods by a 4-position/10-port four-column selection valve. The configuration of the SFC control module mode and the Multisampler mode was chosen with the SFC or UHPLC method.

Orthogonal separation in SFC and UHPLC mode

One advantage of combining SFC and UHPLC in one hybrid system is that the examination of a given sample by means of orthogonal separation techniques can be done in a cost-effective way, using one system. Due to the application of orthogonal separation techniques, compounds can be unraveled that would be hidden by coelution while applying a single chromatographic separation technique. As an example, a complex sample comprising 15 pesticides (Table 1) was examined under SFC and UHPLC conditions. This was done by means of an amino phase and methanol as modifier under SFC conditions, on a C18 phase, under reversed-phase conditions, applying water/acetonitrile in UHPLC mode (Figures 9 and 10). Under SFC conditions, the compounds could be separated in a 6-minute run (Figure 9). Under the chosen separation conditions, compounds 5 and 6 were completely coeluted, and also showed a partial coelution with compound 4. All other compounds were baseline-separated. The same sample was also separated under reversed-phase conditions, which showed a completely different selectivity (Figure 10). Here, compounds 4, 5, and 6, which coeluted under SFC conditions, were now separated. However, compounds 12 and 1 showed nearly complete coelution under these conditions. In addition, from the comparison of both chromatograms, it could be seen that the selectivity was completely different. For instance, compound 15, which eluted last under SFC conditions, eluted second under reversed-phase UHPLC conditions.

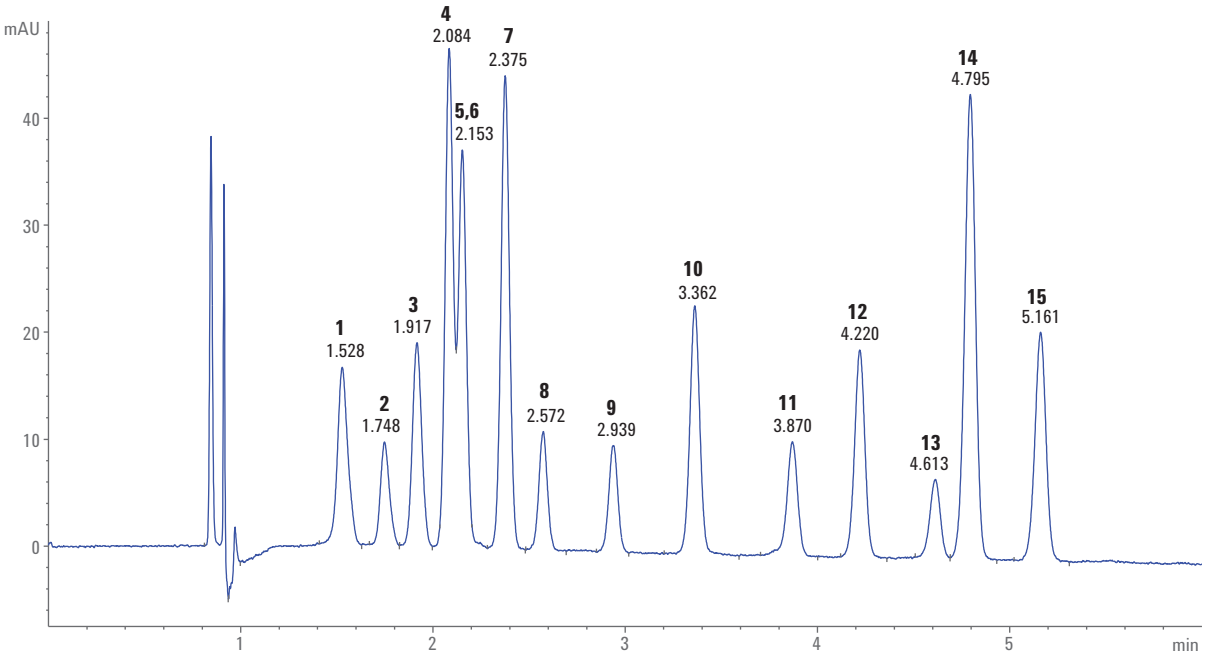


Figure 9. Separation of a complex 15 pesticide sample in SFC mode on an amino phase column with methanol as modifier (see the Experimental section).

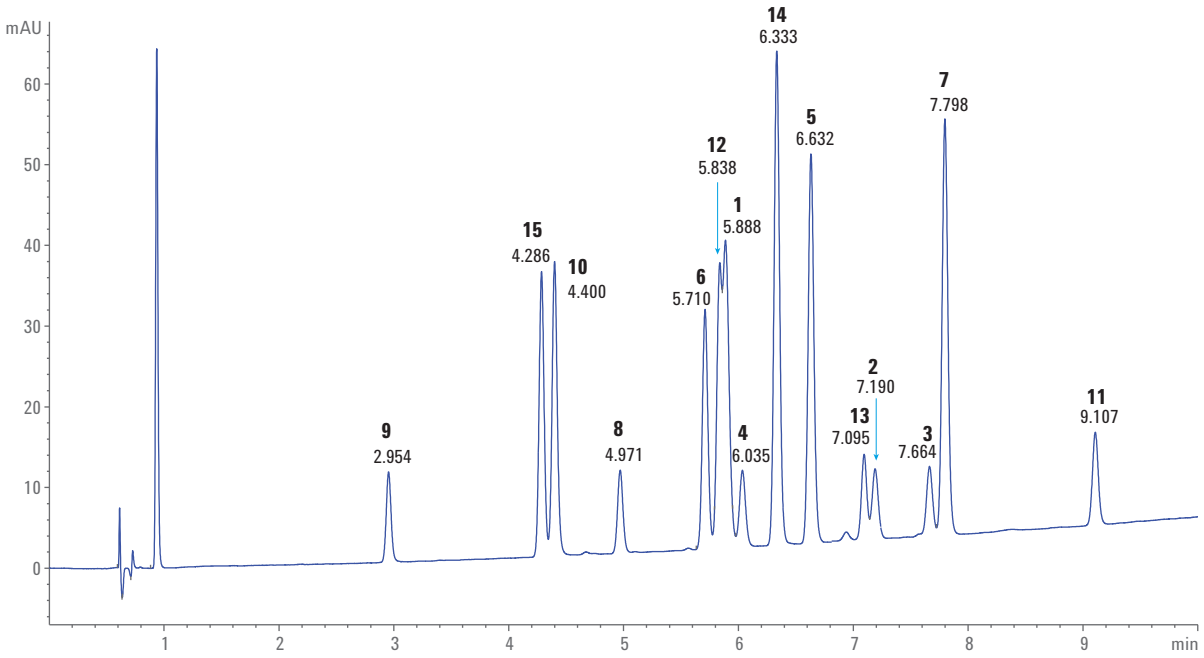


Figure 10. Separation of a complex 15 pesticide sample in UHPLC mode on an C18 phase column with water/acetonitrile as solvents (see the Experimental section).

Conclusion

This Technical Overview demonstrates that the SFC mode and the UHPLC mode of an Agilent 1260 Infinity II SFC/UHPLC Hybrid System delivers the same performance as the standalone SFC and UHPLC instruments. The values determined with the Agilent 1260 Infinity II SFC/UHPLC Hybrid System for peak area RSD, retention time RSD, and linearity are at the same levels as for the individual SFC and UHPLC instruments. The possibility of switching between SFC and UHPLC modes, even within a sequence, is demonstrated, and an example of the orthogonal separation of a complex sample is given.

References

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2. Naegele, E. Feed Speed and Overfeed Volume – New Parameters for SFC Injection, *Agilent Technologies Technical Overview*, publication number 5991-7626EN, **2017**.

3. Huesgen, A. G.; Schneider, S. Performance Characteristics of the Agilent 1290 Infinity II Multisampler, *Agilent Technologies Technical Overview*, publication number 5991-5348EN, **2014**.

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Agilent Technologies

High-Precision Temperature Control for Supercritical Fluid Chromatography Using the Agilent 1260 Infinity II Multicolumn Thermostat

Technical Overview

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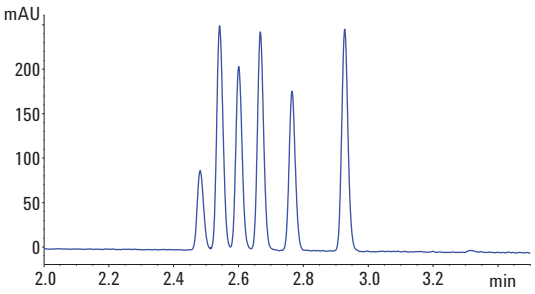
Abstract

This Technical Overview demonstrates the use of the Agilent 1260 Infinity II Multicolumn Thermostat in the Agilent InfinityLab Supercritical Fluid Chromatography (SFC) Solution. It describes how the post column temperature influences the noise at the diode array detector, and how this temperature could be optimized. With the obtained settings, the noise at different column temperatures was measured, and showed stability over a wide column temperature range. The high retention time stability of some compounds with a temperature-sensitive retention time is shown.



Agilent Technologies

Introduction

Due to the high sensitivity of modern diode array detectors used in LC and SFC systems, the minimization of detector noise is crucial to reach maximum performance. Beyond the electronic noise, typical detector noise in any liquid chromatography detection system may be caused by refractive index effects. These effects are primarily caused by incomplete mixing of the solvents used, and temperature differences between the column and detector cell. The latter can be compensated by adjusting the temperature of the column effluent to the temperature of the detector cell in SFC. Therefore, a highly accurate and stable temperature control is needed. In addition, a highly accurate and stable temperature is needed for the column itself. A stable column temperature is necessary because there are some compounds whose retention times shift considerably with small changes in column temperature. The Agilent 1260 Infinity II Multicolumn Thermostat provides control of both temperatures, the precolumn temperature that determines the column temperature, and the post column temperature that adjusts the column effluent to the detector cell temperature.

This Technical Overview demonstrates and discusses both effects. An optimization process for the minimization of detector noise is given, and the resulting noise over a broad range of column temperatures is displayed. The influence of the column temperature itself is demonstrated by using compounds whose retention is sensitive to temperature changes.

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 with high-pressure SFC flow cell (G7115A)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)

Instrumental setup

To minimize detector noise, it was necessary to optimize the post column temperature as close as possible to the detector cell temperature. For that purpose, the column effluent was guided through a heat exchanger (3 µL internal volume), which was set to the detector cell temperature. The precolumn temperature adjustment was done by another heat exchanger of the same internal volume. For that purpose, two independent heat-exchanging blocks of the Agilent 1260 Infinity II Multicolumn Thermostat were used (see Results and Discussion).

Columns

- Agilent ZORBAX RxSil, 4.6 × 150 mm, 5 µm (for noise measurements)
- Agilent ZORBAX RxSil, 3.0 × 100 mm, 1.8 µm (for temperature stability measurements)

Software

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

Sample

The sample was a mixture of six compounds: sulfadimethoxine, sulfachloropyrazine, sulfamethazine, sulfamethizole, sulfamerazine, and sulfadiazine (each 10 mg in 25 mL methanol with equivalent volumes in the mixture).

Chemicals

All solvents were bought from Merck, Germany. Chemicals were bought from Sigma-Aldrich (Germany).

SFC method for noise measurements

Parameter	Description
Solvent A	CO_2
Modifier B	Methanol
SFC flow	2.5 mL/min
Isocratic elution	20 % modifier
Stop time	60 minutes
Backpressure regulator (BPR)	60 °C, 140 bar
Column temperature	20 to 60 °C (step 5 degrees)
Precolumn heat exchanger	3 µL
Post column temperature	42 °C
Post column heat exchanger	3 µL
Injection volume	0.0 µL (blank run)
Feed solvent	Methanol
Overfeed volume	4 µL
Feed speed	400 µL/min
Diode array detector	254 nm/4 nm, Ref.: 360 nm/60 nm, 16 nm slit, data rate 5 Hz, standard high-pressure flow cell

SFC method for temperature stability measurements

Parameter	Description
Solvent A	CO_2
Modifier B	Methanol
SFC flow	1.5 mL/min
Gradient	0 minutes – 5 %B 4 minutes – 40 %B
Backpressure regulator (BPR)	60 °C, 140 bar
Column temperature	20 to 80 °C (step 10 degrees)
Precolumn heat exchanger	3 µL
Post column temperature	42 °C
Post column heat exchanger	3 µL
Injection volume	10.0 µL
Feed solvent	Methanol
Overfeed volume	4 µL
Feed speed	400 µL/min
Diode array detector	270 nm/4 nm, Ref.: 360 nm/60 nm, 16 nm slit, data rate 10 Hz, standard high-pressure flow cell

Results and Discussion

To identify the optimum temperature, the detector noise was measured over the post column temperature range of 35 to 45 °C (step size 1 degree), which is the typical range of the detector cell temperature. This was done with the SFC method described in the Experimental section (column temperature 40 °C). The line through the measured data points of detector noise showed a minimum at 60 µAU peak-to-peak noise between 38 and 44 °C for the post column temperature (Figure 1). The direct measurement of the temperature at the surface of the detector cell by a thermocouple confirmed 42 °C as cell temperature. The post column temperature setting at 42 °C was kept for all further experiments.

A set of nine runs with different column temperatures between 20 and 60 °C (step 5 degrees) was done. Each run for the determination of the detector noise was 1 hour, and was done in duplicate. The noise was determined every 10 minutes over the run time, and reported by Agilent ChemStation as peak-to-peak noise. Finally, all values of peak-to-peak noise obtained from one run were averaged and displayed in a chart showing column temperature against peak-to-peak noise (Figure 2). The chart shows that the peak-to-peak detector noise was constant over the entire column temperature range, and typically was approximately 65 µAU.

A minimum of detector noise is especially important when high sensitivity is required, for instance, when determining lower-level impurities at or below 0.1 %, which is required for impurity profiling. This is demonstrated by the separation of a main compound from its impurity at a level of 0.1 %, where the peak of the main compound was still in the linear range of the detector (Figure 3).

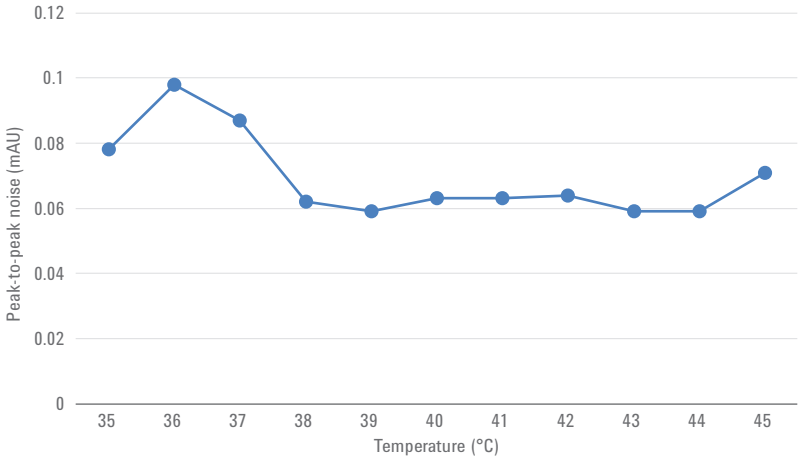


Figure 1. Determination of the post column temperature setting for minimization of detector noise (see *SFC method for noise measurement*).

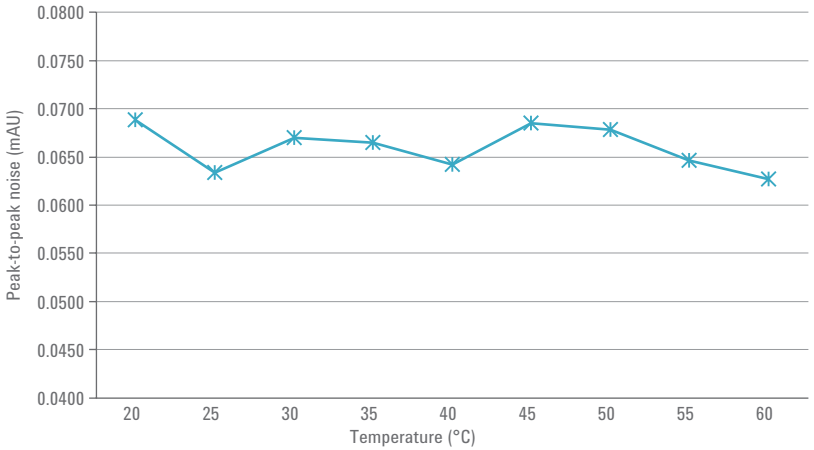


Figure 2. Dependence of peak-to-peak noise on column temperature, showing constant detector noise over a wide range of column temperatures (see *SFC method for noise measurement*).

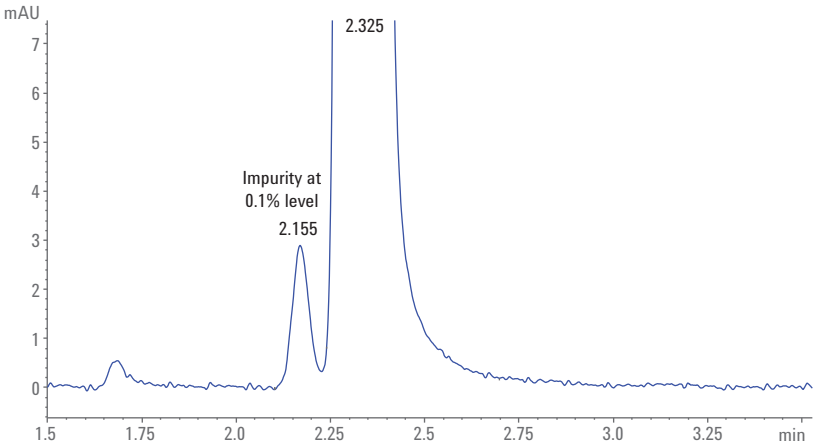


Figure 3. Detection of a low-level impurity at 0.1 % compared to the main peak on the SFC system with minimized noise.

For the evaluation of the stability of the column temperature and the precolumn temperature adjustment, a mixture of six compounds was used. The mixture included compounds with highly temperature-sensitive retention and temperature-dependent coelution. Under the given conditions, the six compounds were clearly separated at a column temperature of 30 °C (Figure 4).

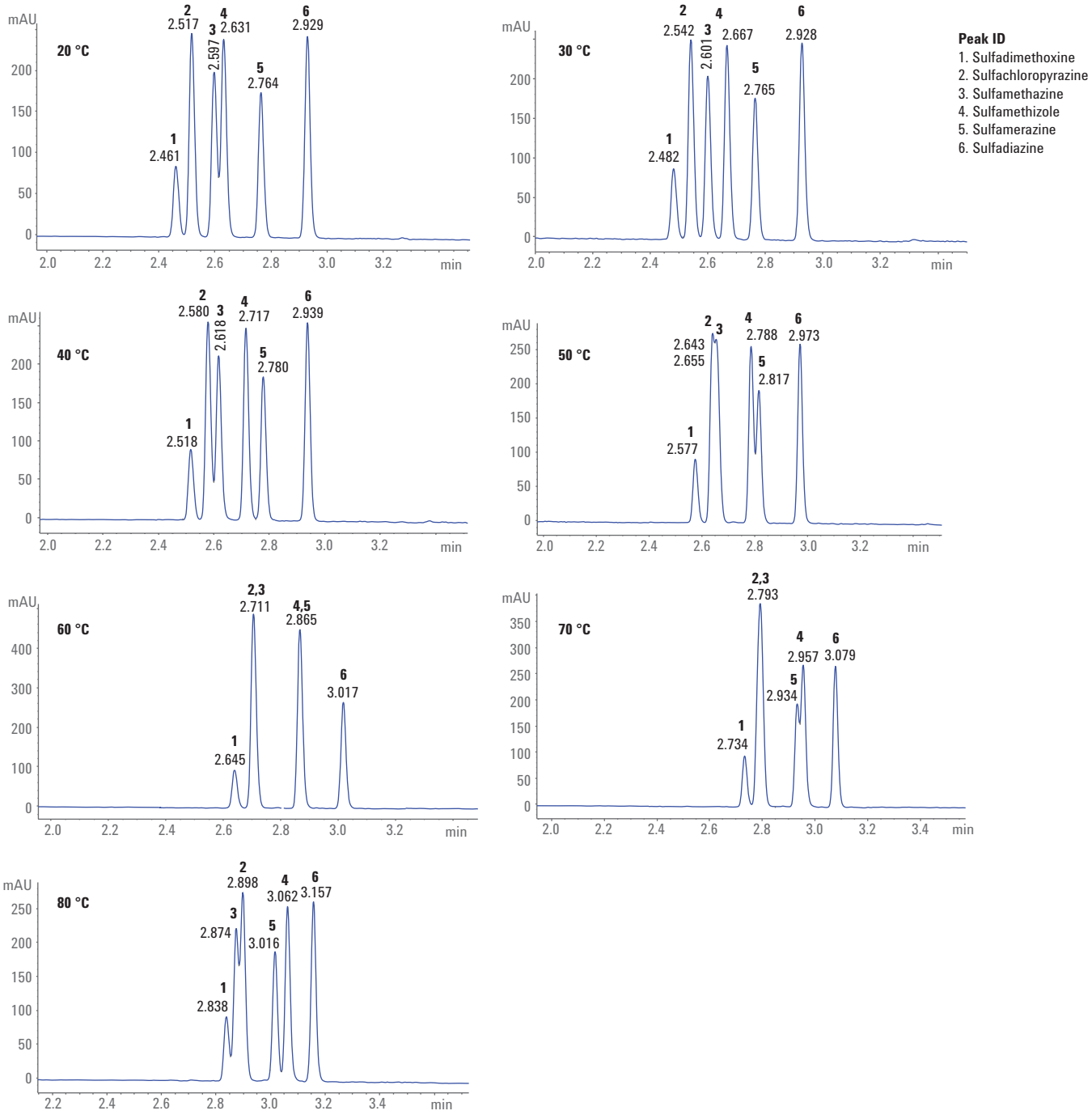


Figure 4. Temperature-dependent elution behavior of six compounds. The best separation was obtained at 30 °C. All other temperatures show temperature-dependent retention-time shifts up to complete coelution and reversal of the elution order (see *SFC method for temperature stability measurements*).

At a lower column temperature (20 °C), peak 4 moved to a shorter retention time, and started to coelute with peak 3. At a higher column temperature (40 °C), peaks 2 and 4 moved markedly to longer retention times. At a column temperature of 50 °C, peaks 2 and 3 coeluted completely. The same situation occurred at a column temperature of 60 °C for peaks 4 and 5. At higher column temperatures (70 °C), peaks 4 and 5 started to separate again but in reversed order. At the highest column temperature in the experiment (80 °C), peaks 4 and 5 again showed good separation. Peaks 2 and 3 also started to separate again at that temperature, but with reversed order and partial coelution with peak 1. To gain a better overview of the temperature-dependent movements, retention times were plotted against temperature (Figure 5). This chart shows clearly that peaks 4 and 5 overlapped completely at 60 °C with reversed retention above and below this temperature. Compounds 2 and 3 coeluted over a broader temperature range between 50 and 70 °C with reversed retention above and below this range. At 80 °C, the peaks of compounds 1, 2, and 3 eluted closely to each other, and were no longer separated.

The precision of the precolumn heating temperature and temperature stability are reflected in the retention time RSD values, which were calculated for all compounds at each applied column temperature for 10 runs. To get a comprehensive overview, all data points were plotted (Figure 6). The chart shows that the retention time RSDs for all six compounds were typically distributed within a small range of approximately 0.003 to 0.01 % for each column temperature. As an example for the frequently used column temperature of 40 °C, the RSDs were between 0.014 and 0.022 %. Table 1 summarizes the measured values for all retention times, and the calculated RSD values.

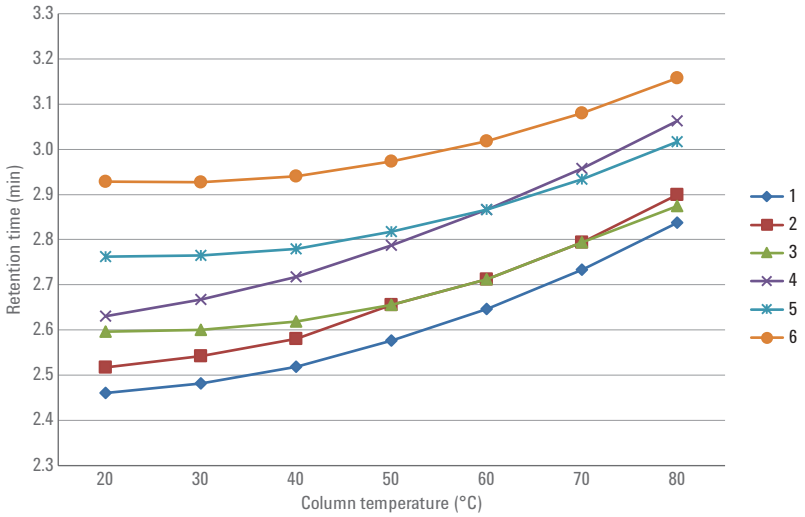


Figure 5. Dependence of retention time on column temperature, showing retention time changes of the six compounds in the mixture.

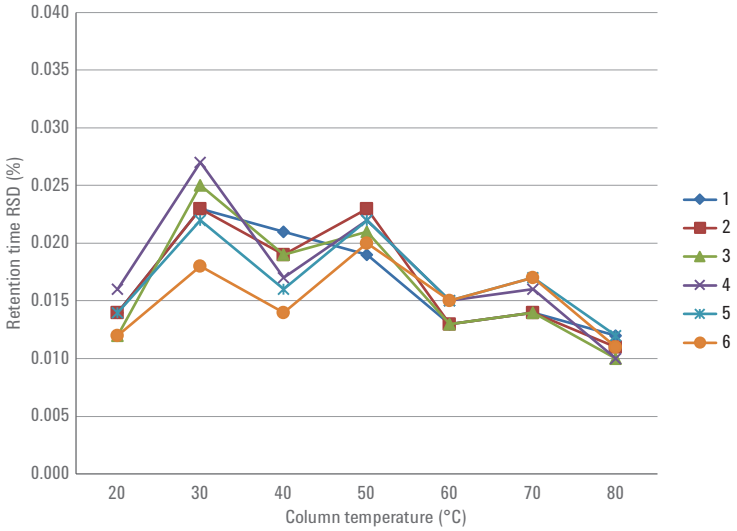


Figure 6. Retention time RSDs obtained for the set of the six compounds at different column temperatures (10 runs at each temperature level).

Table 1. Average retention time and retention time RSD values for all six compounds at all measured temperature levels (10 runs at each temperature level, see *SFC method for temperature stability measurements*).

	20 °C		30 °C		40 °C		50 °C		60 °C		70 °C		80 °C	
Compound	RT	RSD (%)	RT	RSD (%)	RT	RSD (%)	RT	RSD (%)	RT	RSD (%)	RT	RSD (%)	RT	RSD (%)
1	2.460	0.014	2.482	0.023	2.518	0.021	2.577	0.019	2.646	0.013	2.734	0.014	2.838	0.012
2	2.517	0.014	2.542	0.023	2.581	0.019	2.655	0.023	2.712	0.013	2.794	0.014	2.899	0.011
3	2.596	0.012	2.600	0.025	2.619	0.019	2.656	0.021	2.712	0.013	2.794	0.014	2.875	0.010
4	2.630	0.016	2.667	0.027	2.718	0.017	2.788	0.022	2.866	0.015	2.957	0.016	3.063	0.010
5	2.763	0.014	2.765	0.022	2.780	0.016	2.818	0.022	2.866	0.015	2.934	0.017	3.017	0.012
6	2.928	0.012	2.927	0.018	2.940	0.014	2.974	0.020	3.018	0.015	3.080	0.017	3.158	0.011

Conclusion

This Technical Overview describes a routine to determine the post column temperature adjustment to minimize the detector noise caused by temperature differences between column effluent and detector cell temperature. With the optimized post column temperature applied to the column effluent, the detector noise was determined over a broad temperature range from 20 to 60 °C as peak-to-peak noise of approximately 65 µAU. This low level of noise is excellent, and enables the sensitive measurements of low-level impurities at or even below 0.1 %. The stability of the column temperature, achieved by precolumn heating, was determined by the analysis of a set of compounds with high temperature sensitivity. The typical RSD values of the retention time over a range of 20 to 80 °C was between 0.010 to 0.025 %. These excellent peak-to-peak noise and retention time RSD values can be achieved using the Agilent 1260 Infinity II Multicolumn Thermostat for column heating and post column temperature adjustment.

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Agilent Technologies

Chapter 2

PHARMA AND BIOPHARMA APPLICATIONS

Chiral Multicolumn Method Development on the Agilent 1260 Infinity II SFC System

Application Note

Small Molecule Pharmaceuticals

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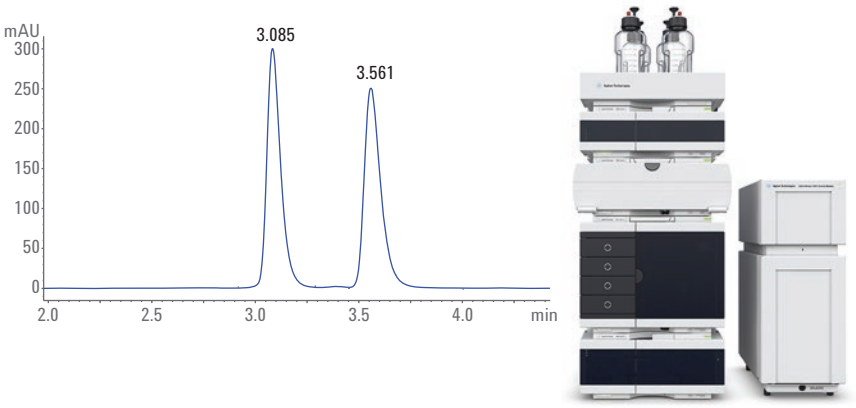
Abstract

This Application Note demonstrates the use of the Agilent ChemStation Method Scouting Wizard for the development of a chiral separation method on the Agilent 1260 Infinity II SFC System. The SFC system was equipped with a four-column selection valve and four different chiral columns for scouting runs against different isocratic separation conditions.



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Introduction

The separation of enantiomers is one of the main application areas for modern SFC instruments. On a SFC instrument, chiral separations are typically 10 to 20 times faster than their classical separation on normal phase HPLC. In addition, the solvents used for the SFC separation are less harmful, and waste disposal is less expensive than for normal phase solvents.

This Application Note demonstrates software-assisted method development for the separation of enantiomers of a chiral pharmaceutical compound. For this purpose, four chiral columns were used and screened against different isocratic solvent compositions. The necessary methods and all flushing and equilibration steps were created automatically with Agilent ChemStation Method Scouting Wizard.

Experimental

Instrumentation

The Agilent 1260 Infinity II SFC System comprised of 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)

In addition, the following parts were required to run the SFC system for automated method development:

- Agilent InfinityLab Quick Change 4-position/10-port four-column selection valve (p/n 5067-4287)
- Agilent 1290 Infinity Valve Drive (G1170A) with Agilent InfinityLab Quick Change 12-position/13-port valve (G4235A)
- Capillary kit for method development with four-column selection valve (p/n 5067-6596)

Instrumental setup

The Agilent SFC Binary Pump was clustered with an Agilent InfinityLab Quick Change 12-position/13-port valve for the selection of up to 12 different solvents in the Instrument Configuration dialog of the Agilent OpenLAB CDS ChemStation Edition software. The solvents were defined in the Pump Setup dialog. For the experiments described in Results and Discussion, only one of the solvents was used.

The Agilent 1260 Infinity II Multicolumn Thermostat was equipped with the Agilent InfinityLab Quick Change 4-position/10-port four-column selection valve, and the columns were set up in the Instrument Configuration dialog of the OpenLAB CDS ChemStation Edition software. With the method development capillary kit, up to four columns could be used. The columns were entered in the Columns Table, and assigned in the MCT dialog. All columns can be used with ID Tags (p/n 5067-5917) for automated recognition in ChemStation and assignement in the MCT dialog.

All methods necessary for column and gradient screening as well as instrument flushing and column equilibration were created with the Method Scouting Wizard (Figure 1).

Columns

- Chiral Technologies, Chiralpak IA, 4.6 × 250 mm, 5 µm
- Chiral Technologies, Chiralpak IB, 4.6 × 250 mm, 5 µm
- Chiral Technologies, Chiralpak IC, 4.6 × 250 mm, 5 µm
- Chiral Technologies, Chiralpak ID, 4.6 × 250 mm, 5 µm

Software

Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, version C.01.07 SR3, including LC and CE Drivers A.02.16 with Agilent ChemStation Method Scouting Wizard, version A.02.07

Sample

Propranolol, 1 mg/mL, in methanol.

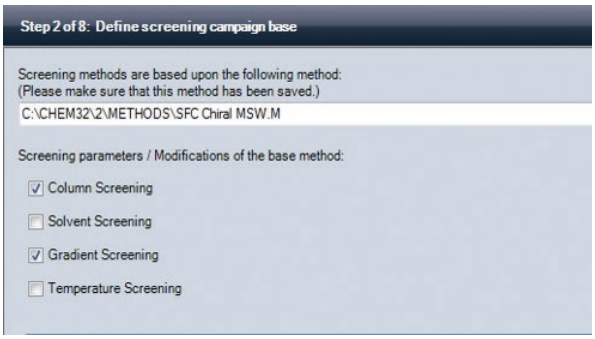
Chemicals

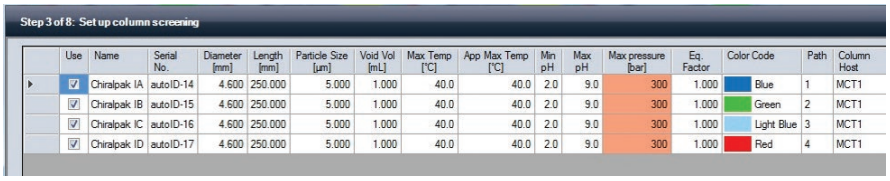
All solvents were purchased from Merck, Germany. Chemicals were purchased from Sigma-Aldrich (Germany).

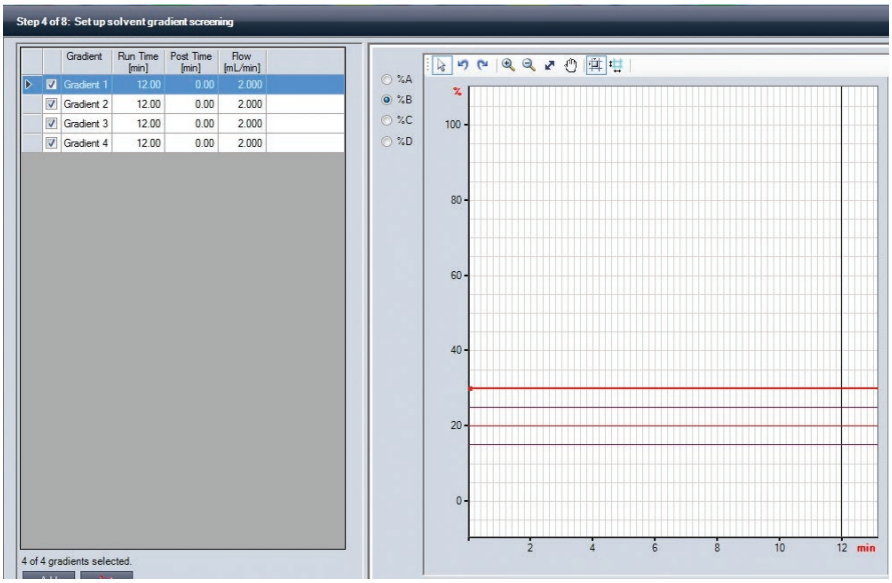
SFC method

Parameter	Value
Solvent A	CO_2
Modifier B	Methanol + 0.1 % diethyl amine
SFC flow	2.0 mL/min
Isocratic elution	15, 20, 25 and 30 % modifier
Stop time	12 minutes
Backpressure regulator (BPR) temperature	60 °C
BPR pressure	140 bar
Column temperature	30 °C
Injection volume	5 µL
Feed solvent	Methanol; feed speed 400 µL/min; over feed volume 4 µL
Needle wash	3 seconds with methanol
Diode array detection	230 nm/bandwidth 4 nm; reference 360 nm/bandwidth 100 nm; data rate 10 Hz

Method Scouting Wizard setup







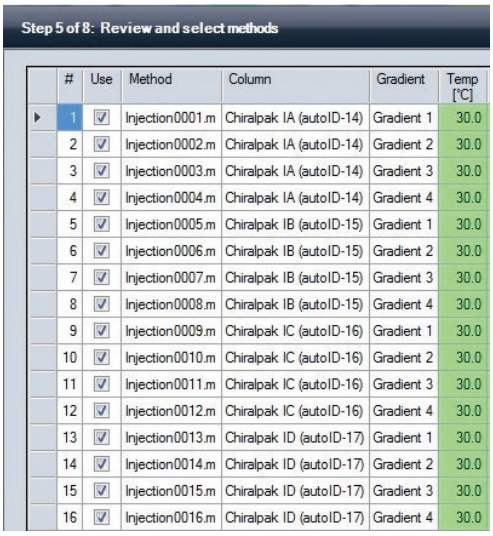


Figure 1. The Method Scouting Wizard enables the setup of the described chiral screening campaign in a 10-step procedure. This setup defines, for example, the different screening options in Step 2, here the column and gradient screening. All other parameters will be used as setup in the chosen main method. In Step 3, the columns will be selected for the column screening. Some parameters such as maximum pressure and maximum temperature will be defined here and later compared to the final method for the elimination of incompatibilities. Step 4 defines the applied gradients, here the choice of four isocratic conditions. Step 5 enables review of the methods that will be created. The other steps (not shown) will define the flushing and equilibration methods as well as sample positons.

Results and Discussion

In the described chiral screening campaign, four different chiral columns were used with the strong eluting solvent, methanol, as the modifier. The goal of the campaign was the identification of a fast method with a good baseline separation of the propranolol enantiomers within a maximum 6 minutes run time. Therefore, four isocratic compositions containing 15, 20, 25, and 30 % methanol were used. On chiral column IA, it is seen that the separation of the two enantiomers started already at 30 % methanol at a retention time of 3.25 and 3.41 minutes. However, a near baseline separation was not achieved until 15 % methanol, and at a retention time of 7.68 and 8.36 minutes. Unfortunately, this compromised the peak shape, the peak height decreased, and the absolute run time was relatively high (Figure 2).

The result of the screening showed good separation for the chiral column IB (Figure 3). The enantiomers were well separated at the baseline with 30 % methanol at 4.49 and 5.44 minutes. The retention time and the distance of both peaks increased with the decreasing content of the methanol modifier, but the peak shape was still acceptable.

By means of the chiral column IC with methanol as modifier, no separation of the propranolol enantiomers could be seen (Figure 4).

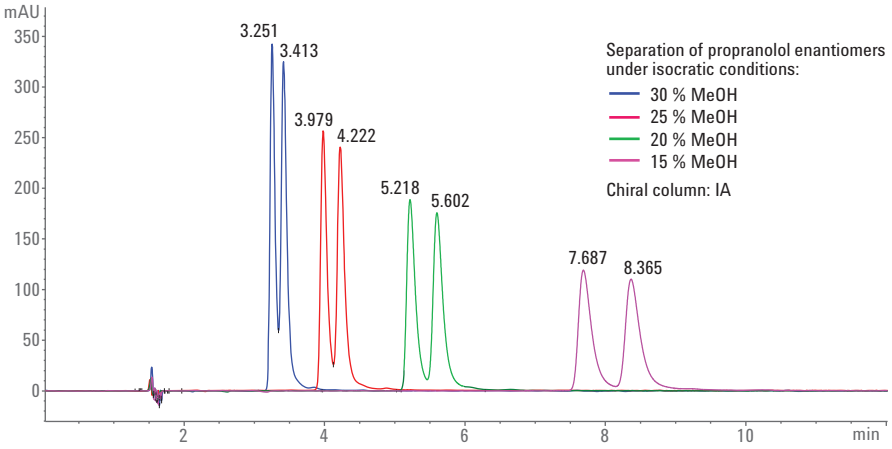


Figure 2. Separation of propranolol enantiomers on the chiral column IA with methanol as modifier.

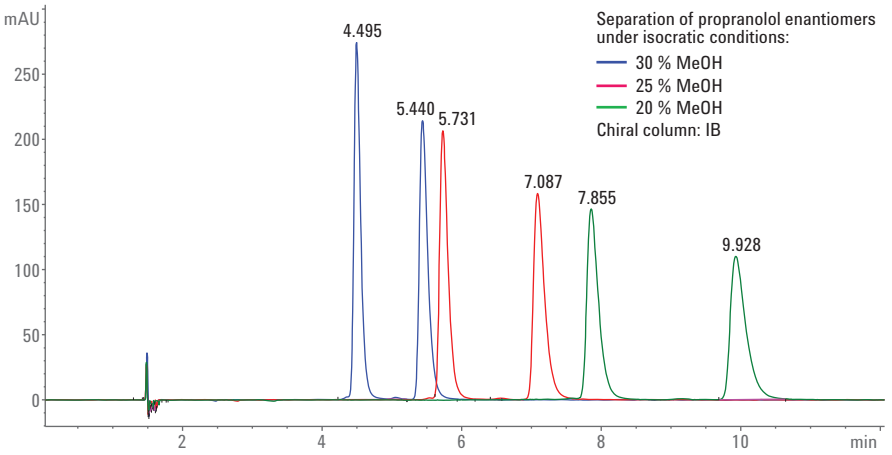


Figure 3. Separation of propranolol enantiomers on the chiral column IB with methanol as modifier.

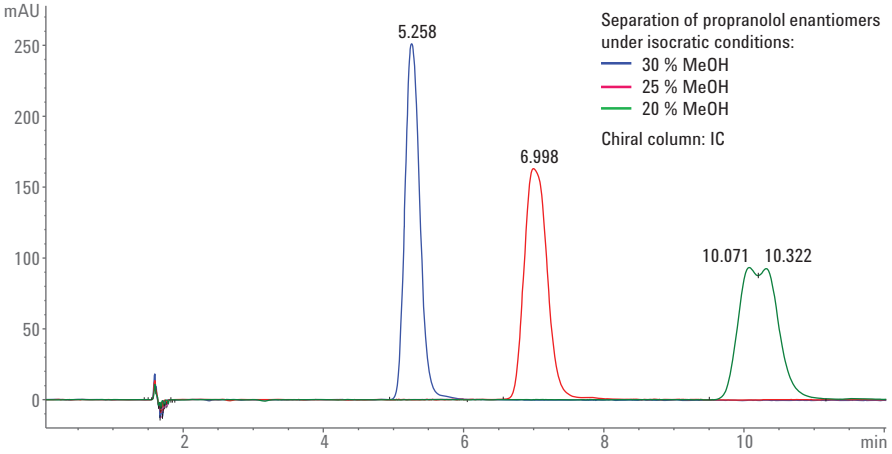


Figure 4. Separation of propranolol enantiomers on the chiral column IC with methanol as modifier.

The separation of the propranolol enantiomers started at 3.0 minutes on chiral column ID with 30 % methanol, and showed a valley at half the peak height for 25 % methanol (Figure 5). No real baseline separation could be achieved for the separation on this column.

For further optimization, chiral column IB was chosen, because there was enough distance between the peaks to increase the speed of this separation, even at high methanol content. After an increase of the flow rate from 2.0 to 2.5 mL/min, and an increase in column temperature from 30 to 40 °C, the retention times could be shifted from the range of 4.0 to 6.0 minutes down to 3.0 to 3.7 minutes (Figure 6). This yielded a short final run time of 4 minutes and the calculated retention time RSD values were 0.11 and 0.13 %, respectively.

The well separated enantiomers also offered the possibility to purify them easily on an analytical scale, and collect the enantiomers in an enantiomerically pure form in single flasks. To optimize the separation process for highest yield, a highly concentrated solution or a high injection volume was used. In this example, a high injection volume of 80 µL was used, which can be achieved by the SFC multisampler[1]. The goal of such an experiment is to find a method that overloads the column by the high concentration, but still has a sufficient separation for the collection of fractions (Figure 7). The identified method uses an isocratic composition with 25 % methanol as modifier, and separates the enantiomers on the highly overloaded column at 5.9 and 6.8 minutes with baseline separation for best fraction collection.

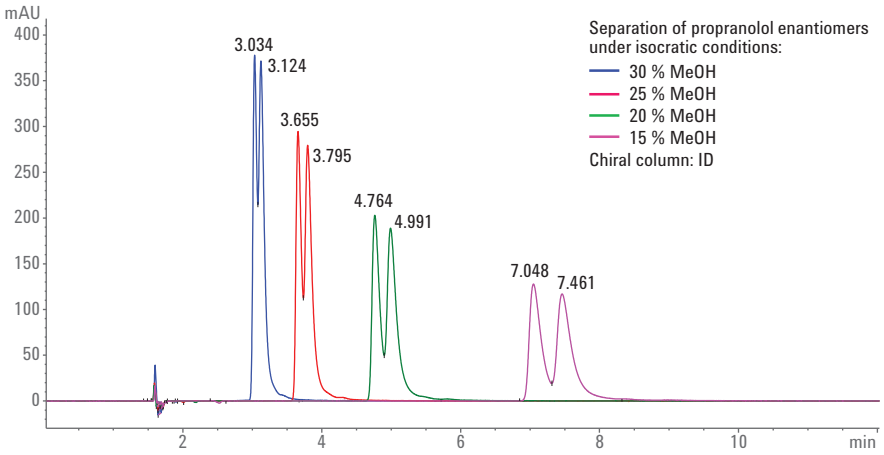


Figure 5. Separation of propranolol enantiomers on the chiral column ID with methanol as modifier.

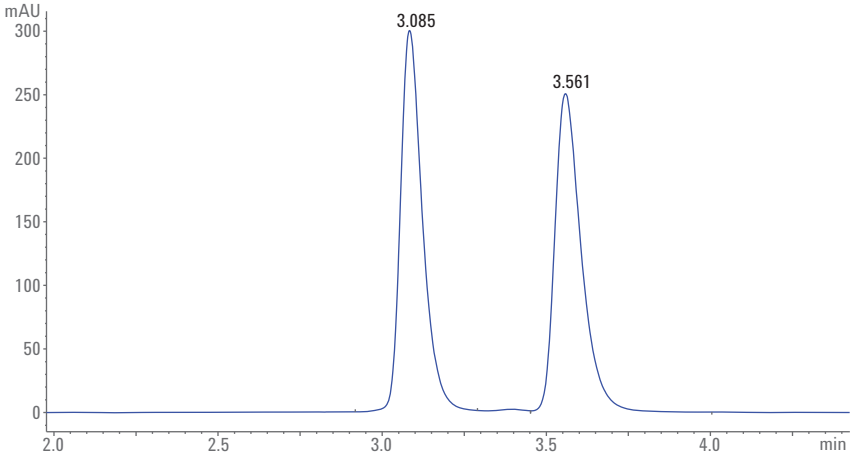


Figure 6. Final speed-optimized analytical separation method of propranolol enantiomers (flow rate: 2.5 mL/min, temperature: 40 °C, organic solvent: 30 % MeOH).

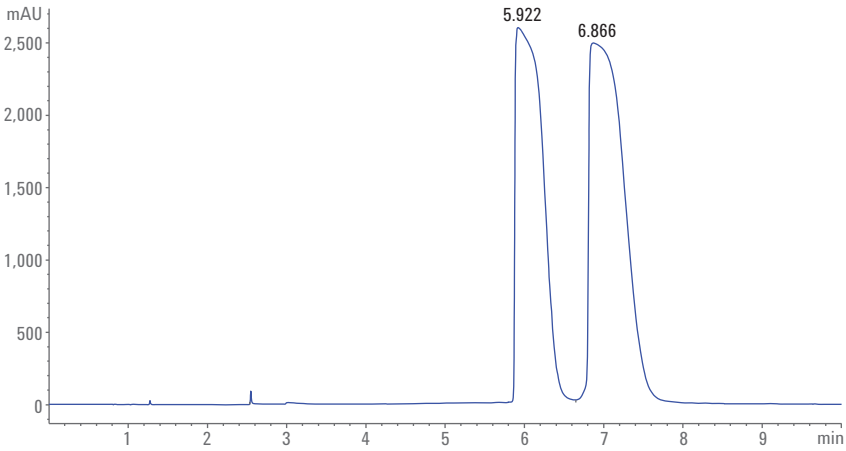


Figure 7. Column overloading experiment for the analytical preparative separation of the enantiomers of propranolol. The injected sample volume was 80 µL and the baseline separation occurred under isocratic conditions with 25 % methanol (feed speed: 100 µL/min, gradient: 5 %B at 0 minutes, 5 %B at 1.0 minutes, 25 %B at 1.1 minutes, stop time: 10 minutes).

Conclusion

This Application Note demonstrates the use of the Agilent 1260 Infinity II SFC System with the Agilent Method Scouting Wizard for software-aided method development. Four chiral columns were automatically screened under different isocratic conditions for the rapid identification of best separation conditions. After a quick optimization of the identified conditions, a separation of two enantiomers in under 4 minutes could be achieved. This is typically a factor of 10-times faster than the classical separation of enantiomers by normal phase chromatography. Finally, it is shown that the identified separation method could also be used for the analytical preparative separation of enantiomers.

Reference

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

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Separation of Large and Small Peptides by Supercritical Fluid Chromatography and Detection by Mass Spectrometry

Application Note

Biologics and Biosimilars

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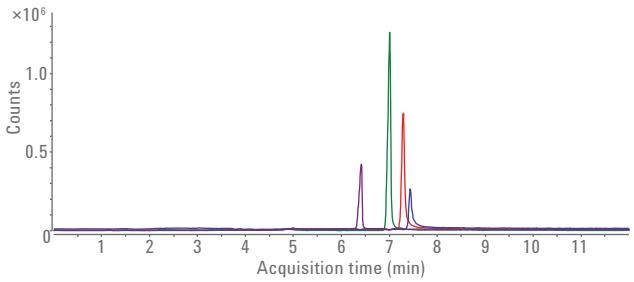
Abstract

This Application Note demonstrates the use of supercritical fluid chromatography (SFC) for the separation of di- and tri-amino acids and peptides. It shows that the separation of smaller and larger peptides is possible by SFC. For detection, a mass spectrometer was used. For larger peptides occurring in different charge states, Agilent MassHunter Bioconfirm software was used for deconvolution to determine the molecular weight of the peptides.



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Introduction

Today, supercritical fluid chromatography (SFC) could be used in different analytical fields such as the pharmaceutical industry. This is documented by a scientific review written by a large pharmaceutical company[1]. They demonstrated the successful use of SFC/MS for high-throughput SFC/APPI-MS, ultrafast SFC/MS, chiral SFC/MS, and SFC/MS for the analysis of peptides and proteins. The biologically important *trans*-membrane proteins comprise highly hydrophobic regions with helical structures of amino acids piercing through the cell membrane. They are typically insoluble in water and difficult to analyze by RP-HPLC. Here, SFC has been successfully used to separate highly hydrophobic peptides such as gramicidin and integral membrane proteins such as bacteriorhodopsin[1,2]. The analysis of peptides with various basic and acidic amino acids in their sequence are described up to a 40-mer[3].

This Application Note describes the separation of small peptides and hydrophilic peptides of similar amino acid sequences by SFC, and their detection by mass spectrometry. As an example for the separation of a large peptide, the determination of the molecular weight of the large peptide insulin is demonstrated.

Experimental

Instrumentation

The Agilent 1260 Infinity Analytical SFC System (G4309A) comprised the following modules:

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity High-Performance Degasser
- Agilent 1260 Infinity SFC Standard Autosampler
- Agilent 1260 Infinity Thermostatted Column Compartment
- Agilent 1260 Infinity DAD with high-pressure SFC flow cell
- Agilent 6460 Triple Quadrupole LC/MS System (G6460C) with Agilent Jet Stream
- Agilent 1260 Infinity Isocratic Pump (G1310B)
- Agilent splitter kit G4309-68715

Instrumental setup

Figure 1 shows the recommended configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS System.

Column

Princeton Chromatography Inc., 2-Ethylpyridine, 4.6 × 250 mm, 5 µm

Software

- Agilent MassHunter Data Acquisition software for triple quadrupole mass spectrometer, Version 07.01
- Agilent MassHunter Qualitative Software, Version 07.00
- Agilent MassHunter Quantitative Software, Version 07.00
- Agilent MassHunter Bioconfirm Software, Version 07.00
- Skyline Software, McCoss Lab Software, University Washington, Version 3.1, for peptide quantification and targeted proteomics[4]

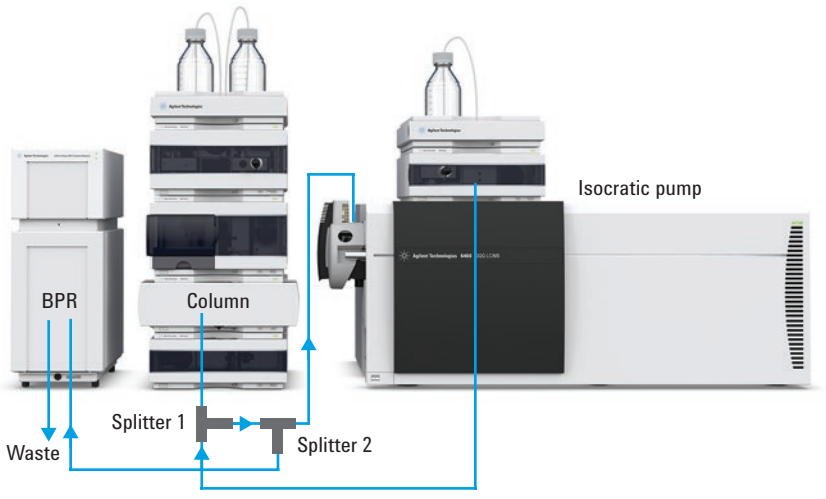

Figure 1. Configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS System. The column is directly connected to splitter 1 in the splitter assembly (BPR = backpressure regulator, UV detector not used, splitter kit p/n G4309-68715).

SFC method

Parameter	Value
SFC flow	3 mL/min
SFC Gradient 1	0 minutes, 5 %B; 10 minutes, 50 %B
SFC Gradient 2	0 minutes, 25 %B; 10 minutes, 75 %B
Stop time	10 minutes
Post time	2 minutes
Modifier	Methanol + 0.1 % TFA
BPR temperature	60 °C
BPR pressure	150 bar
Column temperature	40 °C
Injection volume	5 µL, three-times loop overfill

Connection of the SFC to the MS by splitting and make-up flow

Parameter	Value
Make-up composition	Methanol/Water (95/5) + 0.1 % TFA
Make-up flow	0.5 mL/min

MS method

Parameter	Value
Ionization mode	positive
Capillary voltage	3,500 V
Nozzle voltage	2,000 V
Gas flow	8 L/min
Gas temperature	220 °C
Sheath gas flow	12 L/min
Sheath gas temperature	380 °C
Nebulizer pressure	25 psi
Fragmentor	130 V
SIM mode used for peptides	See Table 1
MRM mode for Angiotensin II	Transition 1: 532.7 → 784.4, CE: 17 eV Transition 2: 532.7 → 647.3, CE: 17 eV
Scan mode, used for insulin	400–1,600 *m/z*

Table 1. Molecular weight and m/z for SIM detection of the analyzed peptides.

Peptide	MW	*m/z*
Gly-Tyr	238.2	239.2
Val-Tyr-Val	379.5	380.5
Leu-enkephalin	555.3	556.3
Met-enkephalin	573.2	574.2
Angiotensin I	1,296.5	433.2
Angiotensin II	1,046.2	524.1
Angiotensin III	931.1	466.5
Angiotensin IV	774.9	388.5

Standard solutions

All peptide samples were prepared at a concentration of 1 mg/mL in make-up solvent, and diluted or mixed to a final concentration of 100 µg/mL.

Chemicals

All chemicals were purchased from Sigma-Aldrich, Taufkirchen, Germany. All solvents were LC/MS grade. Methanol was purchased from J.T. Baker, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak).

Results and Discussion

First, the method for the separation of peptides by SFC was developed for di- and tri-amino acids and smaller peptides. As an example, a di- and tri-amino acid were separated by a gradient increasing from 5 to 50 % methanol in 10 minutes on a 2-ethylpyridine column (Figure 2A). Both small peptides were clearly baseline-separated, and eluted in sharp peaks. As examples for smaller peptides, Leu- and Met-enkephalin were used. The tyrosine residue is an analog to the 3-hydroxyl group of morphine with actions on the δ-opioid receptor. Both were separated with the same method that was used for the smaller peptides (Figure 2B). Leu-enkephalin elutes under the chosen conditions at 6.78 minutes, and Met-enkephalin at 7.11 minutes, both with sharp and baseline-separated peaks. All used di- and tri-amino acids, and the enkephalin penta-peptides were detected by SIM-MS as their singly charged ions (Table 1). From repeated injections, the retention time precision and the area precision were determined to be better than 0.05 and 1.75 %, respectively (Table 2).

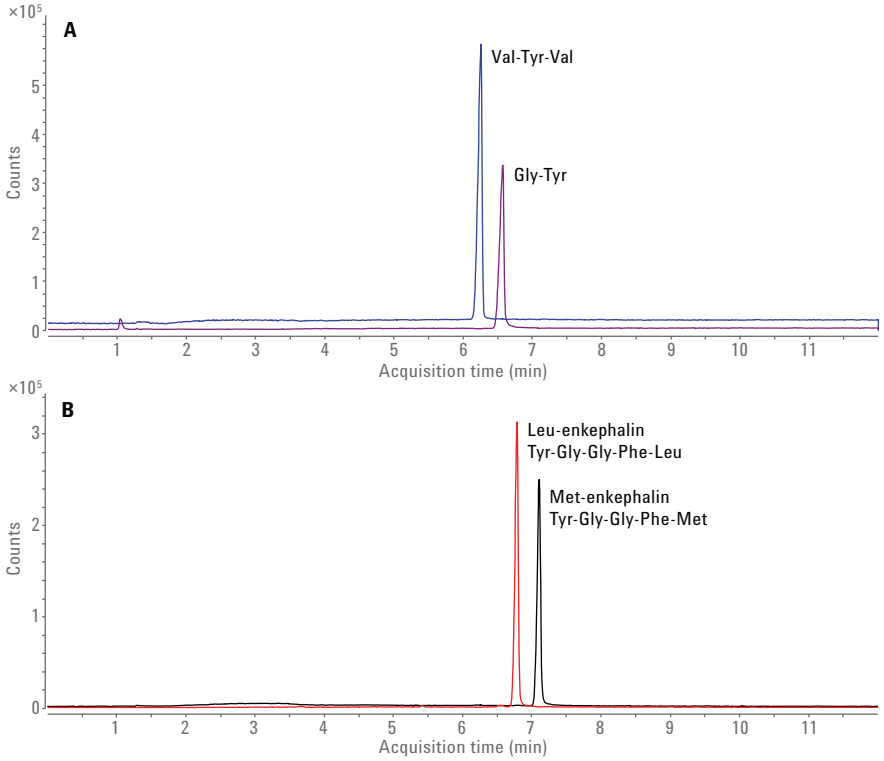

Figure 2. Separation of di- and tri-amino acids and peptides by SFC (gradient 1) and detection by SIM-MS. A) Separation of di- and tri-amino acids Gly-Tyr and Val-Tyr-Val (for abbreviations of amino acids, see Table 4). The di-amino acid Gly-Tyr elutes at 6.59 minutes and the tri-amino acid Val-Tyr-Val at 6.25 minutes with baseline separation. B) Separation of the penta-peptides Leu-enkephalin (6.78 minutes) and Met-enkephalin (7.11 minutes).

Table 2. Retention times, peak areas, and RSD values of di- and tri- amino acids and the penta-peptides Leu-and Met-enkephalin.

	Gly-Tyr		Val-Tyr-Val		Met-enkephalin		Leu-enkephalin	
	RT	Area	RT	Area	RT	Area	RT	Area
AV	6.596	1,495,481	6.254	2,338,841	7.107	892,900	6.782	1,094,260
RSD (%)	0.03	1.74	0.03	1.53	0.04	1.48	0.04	1.36

As a more complex example, the different angiotensin peptides were separated by SFC with the prior developed method. Angiotensin I is a deca-peptide that is released from the protein angiotensinogen, and has no activity itself. Further cleavage of amino acids from angiotensin I releases the most active angiotensin II and the less active angiotensin III and IV. In the separation of these angiotensin peptides by SFC, angiotensin IV elutes first at 6.39 minutes followed by angiotensin III at 6.98 minutes, angiotensin II at 7.27 minutes, and finally angiotensin I at 7.41 minutes. In this separation, angiotensin II, III, and IV were clearly baseline-separated, and only angiotensin I and II showed a partial coelution due to some tailing (Figure 3). Ten replicative injections of the sample showed that the retention time and area RSDs were lower than 0.2 % and 2 %, respectively (Table 3). The separated angiotensin peptides were detected by SIM-MS with their triply- and doubly-charged ions (Table 1).

The combination of SFC with a triple quadrupole mass spectrometer, as used in this study, enables quantification of peptides, if the transitions are set up according to the target peptide. As an example, the Dynamic MRM (DMRM) and MRM method was developed for the main active angiotensin II peptide by means of Skyline software. Possible fragmentations are created by the software according to the peptide fragmentation scheme of Roepstorff and Pohlman[4] (Figure 4). A triple quadrupole acquisition method comprising these fragment masses and different collision energies for optimization was created automatically. The acquired data delivered the most intense fragments and their optimized collision energies (Figure 5A). The peptide fragmentation, all replicates of the collision energy optimization, and the automatically generated final DMRM and MRM results (Figure 5B) can be displayed at-a-glance in the Skyline software.

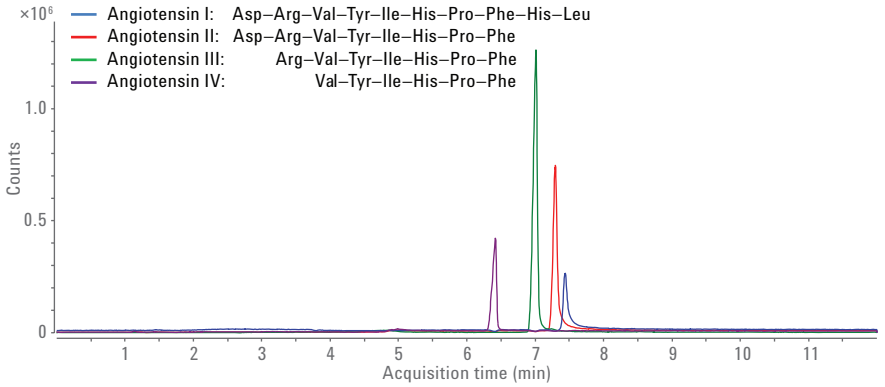

Figure 3. Separation of angiotensin I, II, III and IV by SFC (Gradient 1).

Table 3. Retention times, peak areas, and RSD values of angiotensin peptides.

	Angiotensin I		Angiotensin II		Angiotensin III		Angiotensin IV	
	RT	Area	RT	Area	RT	Area	RT	Area
av	7.415	1,544,293	7.270	3,327,340	6.988	5,476,613	6.395	1,802,408
RSD	0.15	1.91	0.16	1.83	0.19	1.43	0.19	1.10

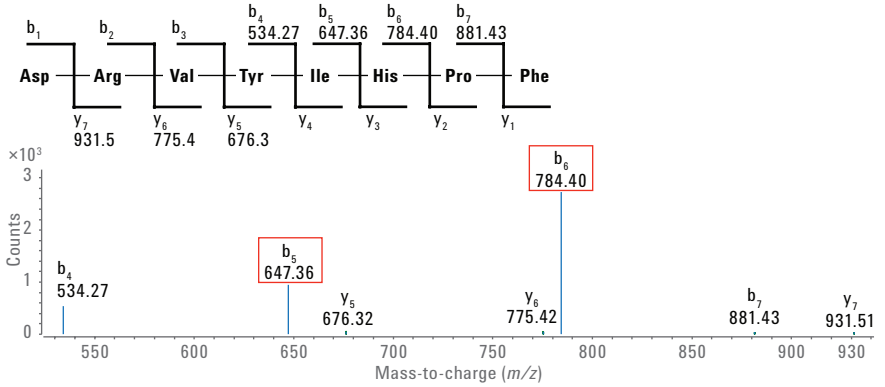

Figure 4. Fragmentation scheme of angiotensin II (MW = 1,046.2, $[M+2H]^{2+}$ = 532.7) and fragments identified from MS/MS spectra. The final DMRM and MRM method used the fragmentation 532.7 → 784.4 and 532.7 → 647.3.

Finally, as an example for a large peptide, insulin was analyzed by SFC/MS. Insulin is a peptide molecule with a molecular weight of 5,808, comprising 51 amino acids. It is built as a heterodimer of two peptide strands comprised of 21 and 30 amino acids. They are connected by two cysteine disulfide bridges (Cys—Cys). A solution of insulin in methanol/water/TFA was injected onto the 2-ethylpyridine column, and eluted as a single peak at 4.987 minutes in the middle of applied gradient 2 (Figure 6A). The extracted mass spectrum showed two charge states of $[M+4H]^{4+}$ and $[M+5H]^{5+}$ with a mass of 1,162.4 and 1,452.6, respectively (Figure 6B). After deconvolution of the charge state mass spectrum, the molecular weight of the double stranded peptide was determined to be 5,807.3 (Figure 6C).

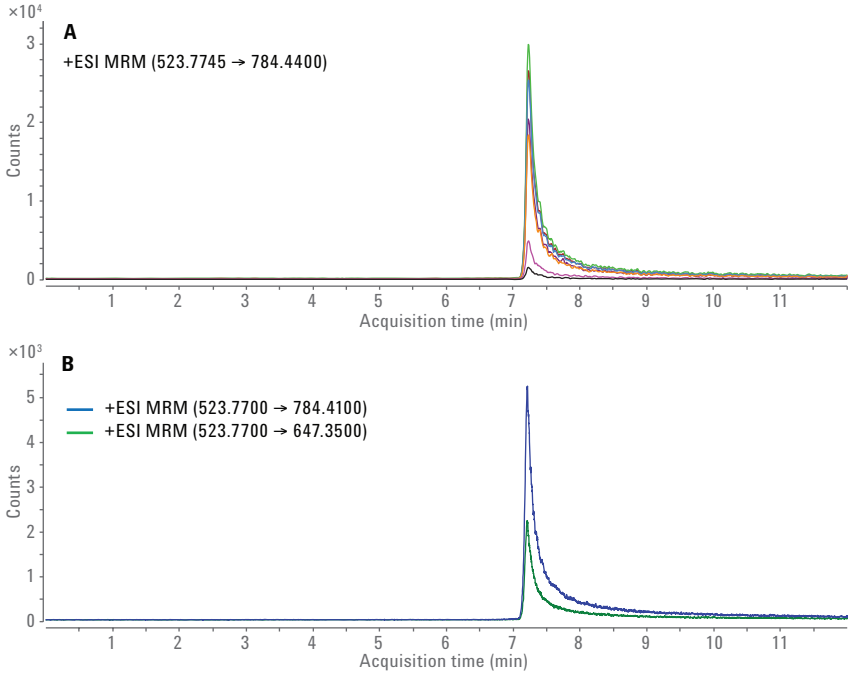

Figure 5. Collision energy optimization and final MRM measurement. A) Optimization of collision energy for the transition 523.77 → 784.44. B) Final MRM measurement with transitions 532.7 → 784.4 and 532.7 → 647.3.

Table 4. Proteinogenic amino acids with three-letter abbreviation and code.

Name	Abbreviation	Code
Alanine	Ala	A
Cysteine	Cys	C
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asp	N
Proline	Pro	P
Glutamine	Glu	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

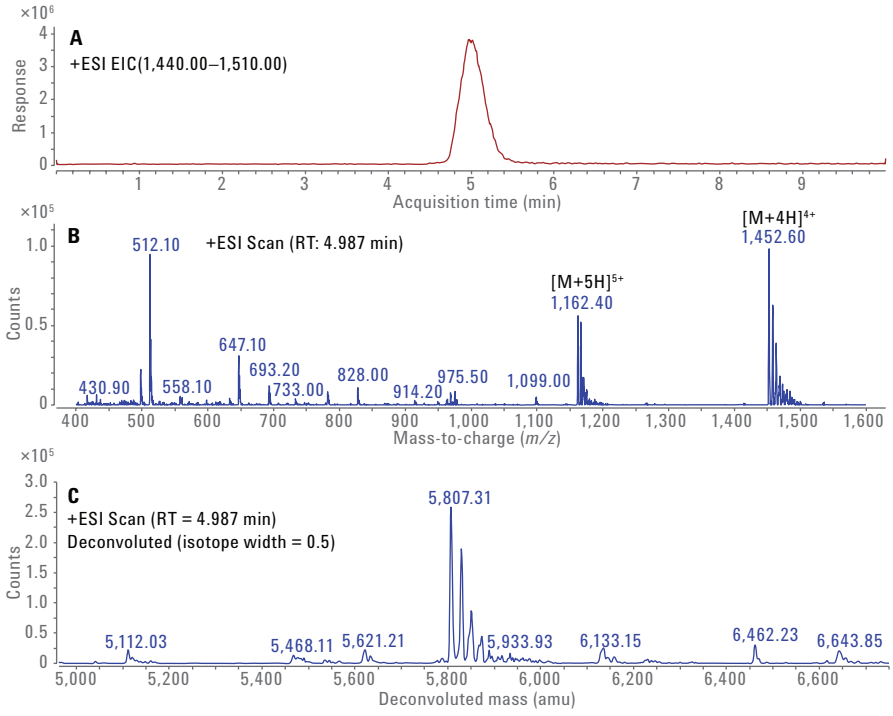

Figure 6) Analysis of insulin by SFC/MS. A) Elution of Insulin from a 2-ethylpyridine column under SFC conditions by gradient 2, extracted ion chromatogram. B) Mass spectrum of insulin at 4.987 minutes showing two charge states, $[M+4H]^{4+}$ and $[M+5H]^{5+}$. C) Deconvoluted mass spectrum of insulin, showing a molecular weight of 5,807.3.

Conclusion

This Application Note demonstrates the analysis of di- and tri-amino acids, small peptides, and large peptides by SFC/MS. The peptides are eluted in a gradient going typically up to 50 % modifier. The measured retention time RSDs are typically below 0.2 %, and the area RSDs are typically below 2 %. As an example, an MRM method was created from the octa-peptide angiotensin II with the aid of Skyline software. This demonstrates the capability of the SFC/Triple Quadrupole combination to analyze peptides. Finally, insulin, as a large peptide, was analyzed by SFC/MS and the molecular weight was determined by deconvolution of the measured charge states.

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Chapter 3

FOOD TESTING AND AGRICULTURE APPLICATIONS

Analysis of Pesticides in Vegetables Using the Agilent 1260 Infinity Analytical SFC System with Triple Quadrupole MS Detection

Application Note

Food Testing & Agriculture

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Abstract

This Application Note demonstrates the optimization of a separation method for the determination of pesticides in a complex food matrix by supercritical fluid chromatography (SFC) with triple quadrupole MS detection. Several gradients of different steepness are applied to the analysis of a vegetable matrix spiked with different concentrations of a multipesticide standard. The optimum separation conditions are determined by software-aided batch comparison to identify the gradient with the lowest matrix impact.

Agilent Technologies

Introduction

Pesticides are widely used in the production of all plant food products such as vegetables, fruits, corn, and grain to protect against various pests. Before plant-based food products enter the market, they have to be tested for possible pesticide residues, which must meet legal limits[1]. Therefore, samples of the complete plant food product have to be extracted and transferred into an analyzable form, typically a solution in organic solvent. This extraction is mostly done by the QuEChERS procedure[2]. The analysis of such samples by HPLC with triple quadrupole MS detection is state of the art. Unfortunately, during sample preparation, not only the pesticide residues are extracted, but also naturally inherent compounds, which make up the matrix. Pesticide and matrix compounds compete for ionization in the ion source of a mass spectrometer when they are eluted from the HPLC column at the same time. This hampers the accurate quantification of pesticides in complex food matrixes. If matrix compounds are present in a large excess, it is possible that they suppress the ionization of the pesticide completely.

Good separation of all compounds on the column can help to avoid this situation and have a strong influence on the mass spectrometric detection of the analytes. Careful optimization of the separation becomes as important as the adjustment of the MS parameters[3]. To compare several separation conditions, a batch analysis can be performed and the optimum conditions for best and broadest detection can be identified.

This Application Note demonstrates the detection of pesticides by supercritical fluid chromatography (SFC) with triple quadrupole mass spectrometry in a complex food matrix after optimization of the SFC separation and batch comparison of different separation conditions. The advantages of using SFC as a front end for the analysis of pesticides in plant food samples by means of mass spectrometry are separation speed, orthogonal selectivity, and tolerance of injections with organic solvents used during sample preparation.

Experimental

Instrumentation

All experiments were carried out on an Agilent 1260 Infinity Analytical SFC System (G4309A) comprising:

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity High Performance Degasser
- Agilent 1260 Infinity SFC Autosampler
- Agilent 1290 Infinity Thermostatted Column Compartment
- Agilent 1260 Infinity Diode Array Detector with high pressure SFC flow cell
- Agilent 6460 Triple Quadrupole LC/MS System (G6460C)
- Agilent 1260 Infinity Isocratic Pump (G1310B)
- Splitter kit (G4309-68715)

Instrumental setup

The recommended configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS System is shown in Figure 1. The column is directly connected to a splitter assembly, which contains two combined splitters, an additional check valve to prevent of CO_2 flowing back into the make-up pump, and a solvent filter. At the first splitter the make-up flow coming from the isocratic pump is introduced into the flow path. This splitter is connected to the second splitter by a short 0.12-mm id capillary. Here, the flow is split into one part going to the MS and the other part going to the backpressure regulator (BPR) of the SFC module. The connection to the MS is made by a newly developed 50-µm id stainless steel capillary of 1-meter length, which is included in the splitter kit. The split ratio depends on the backpressure generated by this restriction capillary and the pressure set by the BPR. As a rule of thumb, an SFC backpressure of 120 bar diverts about 0.45 mL/min of the SFC flow to the ion source and 200-bar backpressure diverts about 0.6 mL/min to the ion source. Since electrospray MS is concentration-dependent, this has no influence on signal intensity.

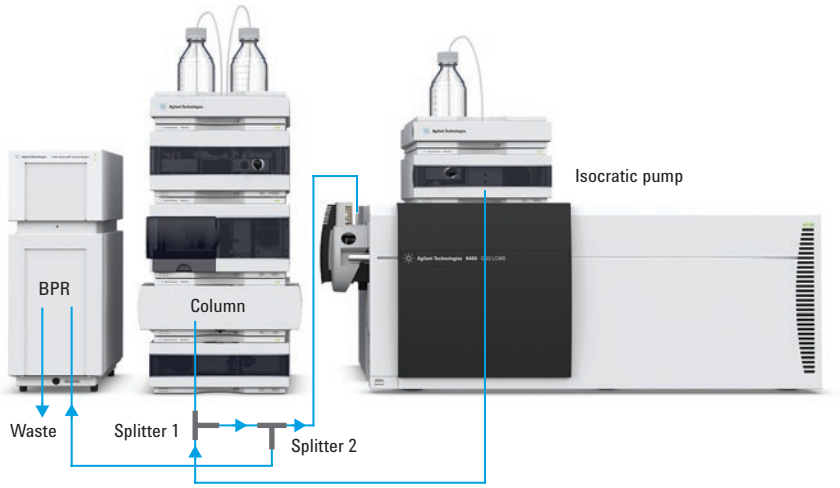

Figure 1. Configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS System. The column is connected directly to splitter 1 in the splitter assembly.

Column

Agilent ZORBAX NH2,
4.6 × 150 mm, 5 µm (p/n 883952-708)

Software

- Agilent MassHunter Data Scquisition Software for triple quadruple mass spectrometer, version 06.00. including SFC software add-on
- Agilent MassHunter Qualitative Software, version 06.00
- Agilent MassHunter Quantitative Software, version 07.00

Standards

A standard mixture containing 10 ng/µL of each of the 17 pesticides in acetonitrile solution was obtained from LGC Standards GmbH (Pesticide Mix 44, part no. 18000044) Mercatorstrasse 51, 46485 Wesel, Germany. The inherent pesticide degradation product atrazine desethyl was not investigated in this study because it is not relevant for vegetables and fruits – it is a degradation product from atrazine occurring in soil and water.

Chemicals

All solvents were LC/MS grade. Acetonitrile and methanol were purchased from J.T. Baker, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak).

SFC method

Parameter	Value
SFC flow	3 mL/min
SFC gradient 1	0 minutes, 2 % B; 5 minutes, 20 % B; 5.1 minutes, 50 % B Stop time 7 minutes Post time 2 minutes
SFC gradient 2	0 minutes, 2 % B; 10 minutes, 20 % B; 10.1 minutes, 50 % B Stop time 12 minutes Post time 2 minutes
SFC gradient 3	0 minutes, 2 % B; 15 minutes, 20 % B; 15.1 minutes, 50 % B Stop time 17 minutes Post time 2 minutes
SFC gradient 4	0 minutes, 2 % B; 8 minutes, 12 % B; 8.1 minutes, 50 % B Stop time 10 minutes Post time 2 minutes
Modifier	Methanol
BPR temperature	60 °C
BPR pressure	120 bar
Column temperature	40 °C
Injection volume	5 µL, 3 times loop overfill

Connection of SFC to MS by splitting and make-up flow

Parameter	Value
Make up composition	Acetonitrile + 0.2 % formic acid
Make-up flow	0.5 mL/min
Flow gradient	0 min, 0.5 mL/min to 5, 10, 15, or 8 minutes, 0.3 mL/min

MS method

Parameter	Value
Ionization mode	Positive
Capillary voltage	2,500 V
Nozzle voltage	2,000 V
Gas flow	8 L/min
Gas temperature	220 °C
Sheath gas flow	12 L/min
Sheath gas temperature	380 °C
Nebulizer pressure	25 psi
MRM conditions	See Table 1

Table 1. MRM conditions for pesticide compounds inherent in the used mixture obtained from MRM Optimizer (dwell time 10 ms, cell acceleration voltage 5 V).

	Precursor ion (*m/z*)	Fragmentor (V)	Product ion 1 (*m/z*)	Collision energy (eV)	Product ion 2 (*m/z*)	Collision energy (eV)
Metolachlor	284.1	90	252.1	12	176.1	24
Metazachlor	278.1	70	210.1	4	134.1	20
Metobromuron	259.0	85	170.0	16	148.1	12
Hexazinone	253.1	85	171.1	12	71.1	32
Linuron	249.0	85	181.1	12	159.9	16
Cyanazine	241.1	100	214.1	12	104.1	32
Diuron	233.1/235.1	95	72.1	20	72.1	20
Metoxuron	229.1/231.1	135	72.1	16	72.1	16
Terbuthylazine	230.1	55	174.1	12	104.1	32
Sebuthylazine	230.1	85	174.1	12	104.1	36
Methabenzthiazuron	222.1	65	165.1	12	150.0	36
Atrazine	216.1	85	174.0	16	104	28
Monolinuron	215.1	95	148.0	16	125.9	12
Chlorotoluron	213.1/215.1	65	72.1	20	72.1	20
Isoproturon	207.1	95	165.0	12	72.1	20
Simazine	202.1	105	132.1	16	124.1	16

Sample preparation

Rocket was obtained from a local greengrocer. Samples were extracted according to the official citrate buffered QuEChERS protocol using Agilent BondElut QuEChERS kits (p/n 5982-5650). 10 g homogenized rocket sample was weighed in a 50-mL polypropylene tube and extracted with 10 mL acetonitrile for 1 minute while shaking vigorously by hand. After the addition of an extraction salt packet containing 4 g of anhydrous $MgSO_4$, 1 g NaCl and 1.5 g buffering citrate salts, the mixture was again shaken for 1 minute, and then centrifuged at 3,000 rpm for 5 minutes.

After phase separation, a 6-mL aliquot of the upper acetonitrile phase was transferred to an Agilent BondElut QuEChERS EN dispersive SPE tube (p/n 5982-5256) containing 150 mg primary secondary amine (PSA) and 15 mg graphitized carbon black for sample cleanup and 900 mg anhydrous $MgSO_4$ to remove water. The tubes were closed and shaken for another minute. Afterwards, the tubes were centrifuged at 3,000 rpm for 5 minutes. A 4-mL amount of the final extract were transferred into a clean polypropylene vial. To improve the stability of the target pesticides, 40 µL formic acid was added to the final extract.

Results and Discussion

For evaluation of matrix effects, the final QuEChERS extract of the rocket sample was spiked with the pesticide solution to a concentration of 10, 20, and 100 ppb. The lowest spiking level of 10 ppb was chosen because a proper detection of all pesticides in a standard solution with 10 ppb each was possible with all applied gradients. The level of 10 ppb is also a typical performance requirement for the detection of pesticides in vegetables and fruits. The used rocket matrix is one of the more complex matrices occurring in the analysis of vegetables and fruits. Gradient 1 was used as the reference separation for this comparison. For comparison, two other gradients with lower steepness were generated by increasing the run time to 10 and 15 minutes while maintaining the final maximum content of organic modifier. In the shallowest gradient (gradient 3), the pesticide compound with the highest retention elutes at 7.5 minutes at approximately 10 % organic modifier. Therefore, in the optimized gradient 4, the organic content is increased to a final concentration of 12 % methanol in 8 minutes. A later increase to 50 % organic is used to clean the column from remaining matrix compounds. Figure 2 shows the separation of the 16 standard pesticide compounds under the conditions of gradient 4 in a standard mix at a concentration of 10 ppb.

For evaluation of matrix effects in the different gradients, a spiked rocket extract was compared to the separation of a calibration standard. In the MassHunter Quantitative Software, the standard solution with a concentration of 10 ppb was set to 100 %, and was used as a one-point calibration. In this way, changes in peak intensities for the spiked sample are flagged in the batch table for fast batch review. This table was transferred into the diagram shown in Figure 3, displaying the results at a glance. When comparing the pure standard solution analyzed by gradient 1 to a sample spiked in matrix analyzed by the same gradient, the intensity of the compounds typically decreases due to matrix suppression.

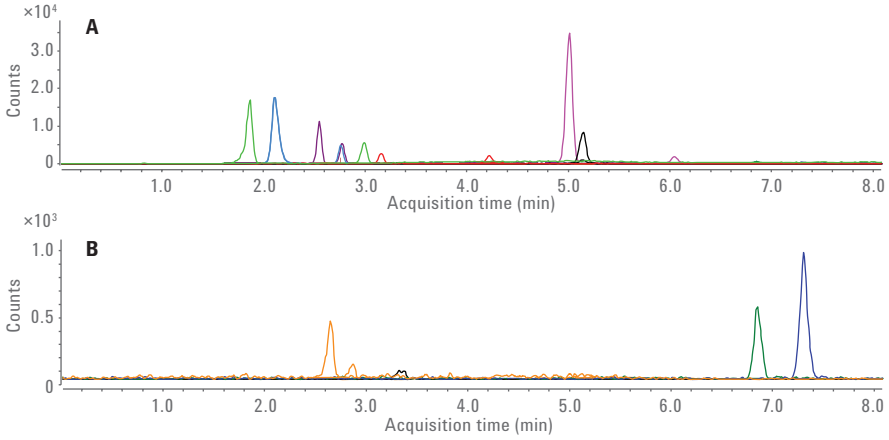

Figure 2. A) Separation of all 16 standard pesticide compounds at the 10 ppb level by means of gradient 4. B) Five pesticide compounds of lowest abundance at the 10 ppb level.

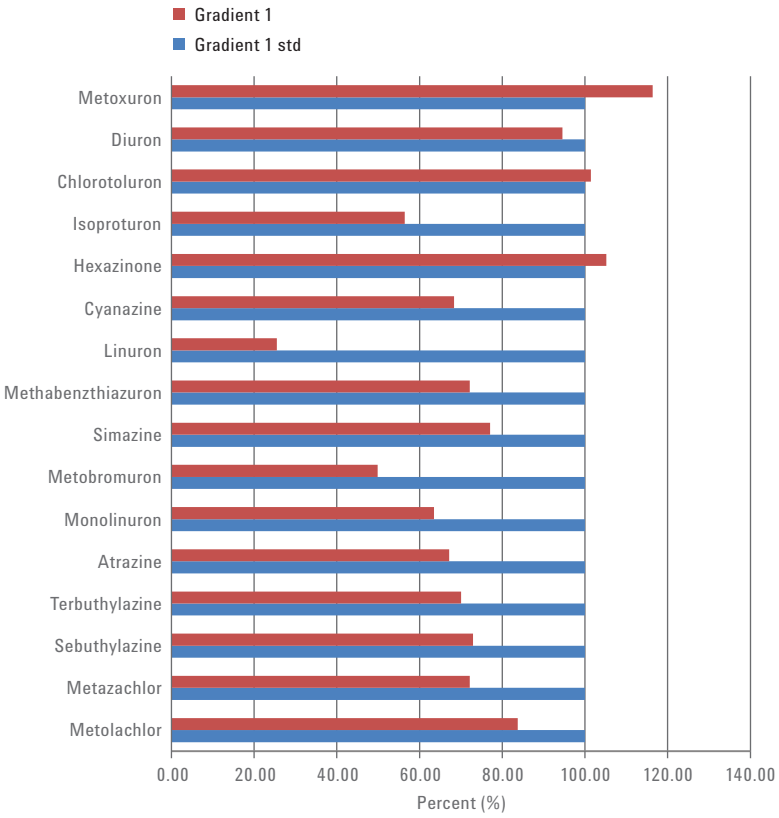

Figure 3. Comparison of matrix suppression of 16 pesticide compounds in rocket matrix (red) to a standard solution (blue).

A change in the gradient to a less steep increase in organic modifier possibly improves the separation of the compounds from the matrix compounds and results in higher signal intensities by less suppression due to high abundant matrix compounds.

To demonstrate this effect, gradients 2, 3, and 4 were also applied to the described spiked sample and the standard. The complete batch of samples and standards was compared by means of the MassHunter Qualitative Software. The area values obtained for the standards from each gradient were used as the basis in a one point calibration to compare to the spiked samples. The comparison of matrix effects is displayed in the graphical chart shown in Figure 4. It can be seen, that for most of the compounds the shallow gradients 2, 3, and 4 result in an improvement in signal intensity compared to the fastest gradient 1. Typically, the shallowest gradient 4 provides the highest signal intensities. For instance, the chromatograms of the pesticide compound isoproturon show an increase in response for the comparison of gradient 2 to 4 for the spiked matrix sample with the chromatogram of the initial gradient 1 (Figure 5). Presumably, the intensity increases due to the better separation from the matrix background and, thus, higher ionization yields. There are three exceptions, cyanazine, atrazine, and tertbuthylazine which gave higher intensities with gradient 1. However, due to the fact, that the majority of compounds produces higher intensities with gradient 4, and the majority of compounds have recoveries between 70 and 120% this one was used for the next experiments.

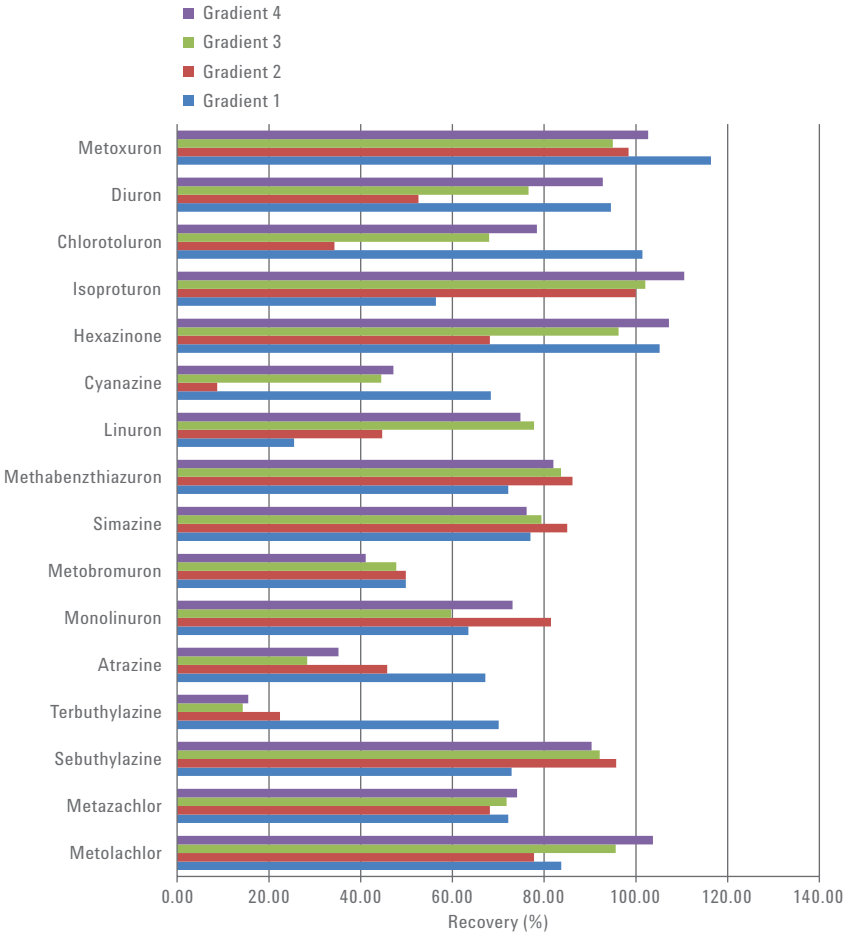

Figure 4. Comparison of matrix effects in different gradients of different steepness. The matrix effect is at its minimum for most of the compounds for gradient 4.

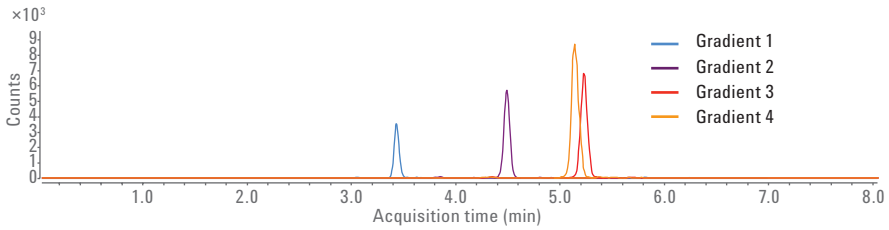

Figure 5. Signal intensities for isoproturon for the four applied gradients of different steepness.

Finally, quantification in rocket extract was done based on a solvent calibration using the 10, 20, and 100 ppb levels by means of gradient 4. The same concentration levels spiked in rocket matrix were used as samples. The comparison of the measured concentration shows the matrix effects in relative percentages (Figure 6). According to the SANCO guidelines SANCO/12571/2013, an apparent recovery of 70 to 120 % is acceptable[4]. The matrix effects per compound are very similar over the examined concentrating range (Figure 6). When compared to a solvent calibration, most of the tested compounds show recoveries within the acceptable range of 70 to 120 %. The two compounds atrazine and terbuthylazine were quantified with recoveries below 50 %. Dilution is an accepted way of minimizing matrix effects in complex samples. When diluting the QuEChERS extract spiked with pesticides to 100 ppb 1:10 with acetonitrile, recoveries for atrazine and terbuthylazine were 87 and 85 % respectively and, thus, within the acceptable range. The linearity of calibration for all compounds, calculated limits of quantitation (LOQ) and limits of detection (LOD) are summarized in Table 2.

Conclusion

This Application Note demonstrates the importance of optimizing the SFC separation on the influence of the sample matrix for the measurement of pesticides in vegetable and fruit samples by SFC with triple quadrupole MS. The optimization of the used gradient can improve the separation between analyte and high abundant matrix compounds and, thus, help to lower detection limits. For all tested pesticides, the required LOQ of 10 ppb could be met, and most of the compounds could be quantified in the required recovery range of 70 to 120 % based on a solvent calibration. In addition, the use of an SFC instrument brings the advantage of increased speed of the separation and the usability of samples dissolved in pure organic solvent directly from sample preparation by QuEChERS.

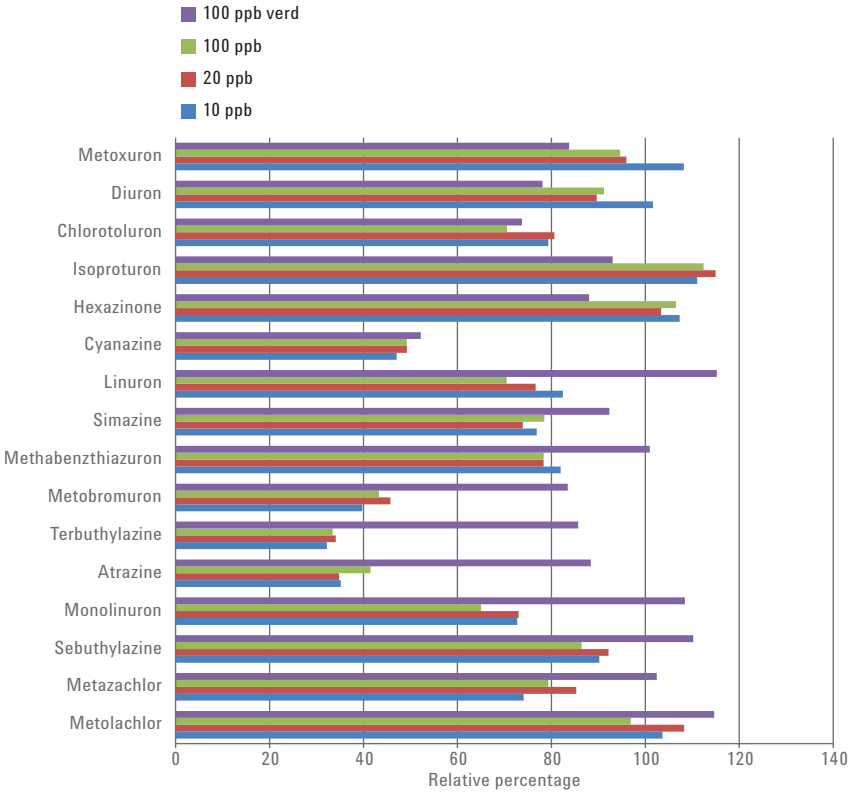

Figure 6. Comparison of spiked samples to a calibration in standard solution. Matrix effects are typically in a range of 70 to 120 %. Matrix effects could be additionally minimized by sample dilution.

Table 2. Summary of calibration, showing linearity of the individual compounds, LOQ, and LOD.

10 ppb	RT	LOD	LOQ	R^2
Metolachlor	1.869	0.08	0.25	0.9991
Metazachlor	2.117	0.12	0.40	0.9990
Sebuthylazine	2.554	0.55	1.83	0.9993
Monolinuron	2.647	1.77	5.90	0.9993
Atrazine	2.754	0.06	0.20	0.9993
Terbuthylazine	2.776	0.08	0.25	0.9995
Metobromuron	2.866	2.49	8.30	0.9991
Methabenzthiazuron	2.993	0.05	0.18	0.9994
Simazine	3.158	0.24	0.80	0.9995
Linuron	3.307	3.00	10.00	0.9990
Cyanazine	4.219	0.20	0.66	0.9992
Hexazinone	5.006	0.03	0.10	0.9995
Isoproturon	5.142	0.04	0.13	0.9998
Chlorotoluron	6.046	0.23	0.77	0.9991
Diuron	6.846	0.87	2.90	0.9992
Metoxuron	7.287	0.30	1.00	0.9992

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Development of a Method for Multipesticide Analysis Using the Agilent 1260 Infinity Analytical SFC System with Triple Quadrupole MS Detection

Application Note

Food Testing & Agriculture

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Abstract

This Application Note describes the development of a method for multipesticide analysis by supercritical fluid chromatography (SFC) using the Agilent 1260 Infinity Analytical SFC System in combination with an Agilent 6460 Triple Quadrupole Mass Spectrometer. The final multipesticide method was used for the determination of more than 200 pesticides in a single analysis. Different matrixes from fruits and vegetables were spiked with pesticides at several levels in a relevant concentration range and quantified. Individual calibration and performance data are presented and discussed.

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Introduction

Today, several hundreds of pesticide compounds are available on the market, and are in use on a worldwide basis for protection against various pests of plant food products such as vegetables, fruits, corn, and grain. Before plant-based food products enter the market, they have to be tested for possible pesticide residues, and they have to meet the legal limits[1]. The sheer number of possible pesticide-matrix combinations makes it necessary that methods used for the quantitative determination of pesticides in food products cover the widest possible range of compounds. This is typically done by HPLC methods in combination with mass spectrometry, where the compounds are separated by LC, and the selective detection is performed by triple quadrupole mass spectrometry in multiple reaction monitoring (MRM) mode. The optimization of supercritical fluid chromatography (SFC) separations for pesticides, the optimization of their mass spectrometric detection, and the influence of matrix compounds was shown previously[2,3].

Compared to HPLC, SFC offers the ability to use cheaper solvents such as carbon dioxide, less harmful solvents such as methanol or ethanol, lower costs for solvent waste disposal, and shorter run times. Samples of the complete plant food product have to be extracted and transferred into an analyzable form, typically a solution in organic solvent. This extraction is primarily done by the QuEChERS procedure[4], and the final extracts are analyzed by HPLC/triple quadrupole MS. While the extraction of samples in pure solvents such as acetonitrile in HPLC often compromises the peak shapes of the early eluting compounds, they are directly usable for injection in SFC.

This Application Note demonstrates the detection of more than 200 pesticide residues by SFC with triple quadrupole mass spectrometry in complex food matrixes after optimization of the SFC separation of a multiple-pesticide standard. The advantages of using an SFC as a front end for mass spectrometry for the analysis of pesticides in plant food samples are the separation speed, the orthogonal selectivity to LC, and the tolerance to injections with organic solvents as they are obtained from sample preparation. Data about the limits of detection (LODs), limits of quantitation (LOQs), linearity, retention time, and area RSDs of selected individual compounds are presented.

Experimental

Instrumentation

All experiments were carried out on an Agilent 1260 Infinity Analytical SFC System (G4309A) comprising:

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity High Performance Degasser
- Agilent 1260 Infinity SFC Autosampler
- Agilent 1290 Infinity Thermostatted Column Compartment
- Agilent 1260 Infinity Diode Array Detector with a high pressure SFC flow cell
- Agilent 6460 Triple Quadrupole LC/MS system (G6460C)
- Agilent 1260 Infinity Isocratic Pump (G1310B)
- Splitter Kit (G4309-68715)

Instrument setup

Figure 1 shows the recommended configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS System. The column is directly connected to a splitter assembly, which contains two combined splitters, an additional check valve to prevent CO_2 flowing back into the make-up pump, and a solvent filter. At the first splitter, the make-up flow coming from the isocratic pump is introduced into the flow path. This splitter is connected to the second splitter by a short 0.12-mm id capillary. Here, the flow is split with one part going to the MS and the other part going to the backpressure regulator (BPR) of the SFC module.
The connection to the MS is made by a special 50-µm id stainless steel capillary of 1-m length, which is included in the splitter kit. The split ratio depends on the backpressure generated by this restriction capillary and the pressure set by the BPR. Generally, an SFC backpressure of 120 bar diverts about 0.45 mL/min of the SFC flow to the ion source, and a 200-bar backpressure diverts about 0.6 mL/min to the ion source. Since electrospray MS is concentration-dependent, this has no influence on signal intensity.

Column

Agilent ZORBAX NH_2, 4.6 × 150 mm, 5 µm (p/n 883952-708)

Software

- Agilent MassHunter Data Acquisition Software for triple quadruple mass spectrometer, version 06.00. including SFC software add-on
- Agilent MassHunter Qualitative Software, version 07.00
- Agilent MassHunter Quantitative Software, version 07.00

Standards

The Agilent LC/MS Pesticides Comprehensive Test Mix (p/n 5190-0551) was used as standard mixture. This mix comprises eight submixtures, with a total of 254 pesticide compounds. The stock solutions contain the pesticides at a concentration of 100 ppm each. This stock solution was diluted to a working stock solution of 1 ppm in acetonitrile.

Chemicals

All solvents were LC/MS grade. Ethanol was purchased from J.T. Baker, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak).

Sample preparation

Fruits and vegetables were obtained from a local greengrocer. Samples were extracted according to the official citrate buffered QuEChERS protocol using Agilent BondElut QuEChERS kits (p/n 5982-5650). A 10-g amount of homogenized sample was weighed in a 50-mL polypropylene tube, and extracted with 10 mL acetonitrile for 1 minute while shaking vigorously by hand. After the addition of an extraction salt packet containing 4 g anhydrous $MgSO_4$, 1 g of NaCl, and 1.5 g buffering citrate salts, the mixture was again shaken for 1 minute, then centrifuged at 4,000 rpm for 5 minutes.

After phase separation, a 6-mL aliquot of the upper acetonitrile phase was transferred to an Agilent BondElut QuEChERS EN Dispersive SPE Tube (p/n 5982-5056) containing 150 mg of primary secondary amine (PSA) for sample cleanup, and 900 mg of anhydrous $MgSO_4$ to remove water. The tubes were closed and shaken for another minute. Afterwards, the tubes were centrifuged at 4,000 rpm for 5 minutes. A 4-mL aliquot of the final extract was transferred to a clean polypropylene vial. To improve the stability of the target pesticides, 40 µL of formic acid was added to the final extract.

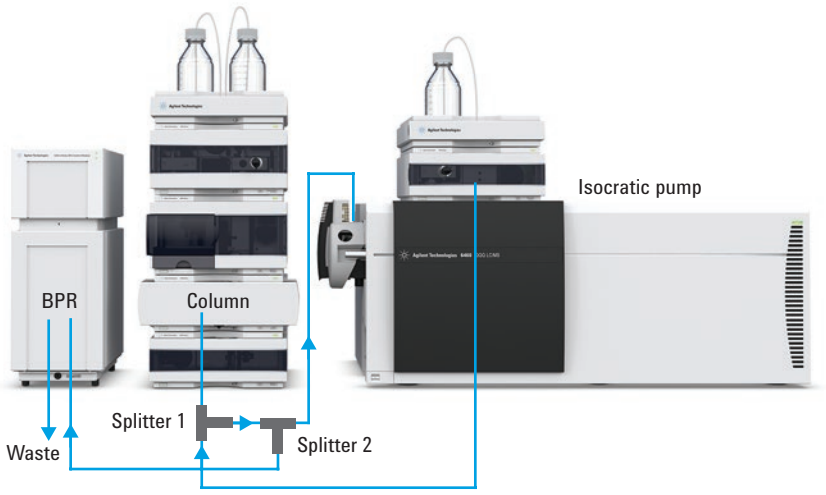

Figure 1. Configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS System. The column is directly connected to splitter 1 in the splitter assembly (BPR = backpressure regulator, UV detector not used, splitter Kit p/n G4309-68715).

SFC method

Parameter	Value
SFC flow	3 mL/min
SFC gradient	0 minutes, 2 %B 10 minutes, 10 %B 14 minutes, 26 %B 14.1 minutes, 50 %B Stop time 20 minutes Post time 2 minutes
Modifier	Methanol
BPR temperature	60 °C
BPR pressure	120 bar
Column temperature	40 °C
Injection volume	5 µL, 3-times loop overfill

Connection of SFC to MS by splitting and make-up flow

Parameter	Value
Make up composition	Methanol/water (95/5), 0.5 mM ammonium formate, + 0.2 % formic acid
Make-up flow	0.5 mL/min

MS method

Parameter	Value
Ionization mode	Positive
Capillary voltage	2,500 V
Nozzle voltage	2,000 V
Gas flow	8 L/min
Gas temperature	220 °C
Sheath gas flow	12 L/min
Sheath gas temperature	380 °C
Nebulizer pressure	25 psi
DMRM conditions	See Appendix Table 1, showing detailed retention time, retention time window, fragmentor, and collision energy details.

Results and Discussion

The Agilent LC/MS Pesticides Comprehensive Test Mix contains eight submixtures, each with approximately 33 compounds. These mixtures were used to develop and optimize the SFC separation method. The amino phase column was chosen due to experience based on an earlier method development work for a multipesticide sample. Ethanol was chosen as a modifier due to its lower elution strength compared to methanol, to enable a broader elution range[2].

In the first experiment, the pesticides from the different submixtures were eluted in a steep gradient, to 50 % modifier in 10 minutes, to see which pesticides could be eluted from the chosen combination of column phase and modifier. Because the elution behavior of most of the compounds under SFC conditions is susceptible to minor changes in the organic modifier even at low values, the submixtures were also tested in a gradient from 2 to 10 % in 10 minutes. Under these conditions, 195 compounds were eluted. An additional 28 compounds were eluted when the modifier was increased to 26 % in 14 minutes, then to 50 % at 14.01 minutes, then held there to 20 minutes. Overall, 223 compounds of the 254 compounds inherent in the mixtures were eluted and detected by MRM. In the remaining group of 31 compounds, some ionized only under negative ionization mode conditions, and others were not eluted with good peak shapes because they did not seem to fit well with the chosen combination of column phase and modifier. Several compounds of the group of sulfunyolurea herbicides were present in this group. To improve the sensitivity of the final method, the MRM method was transferred to a dynamic MRM (DMRM) mode method where each compound was measured at its retention time with a window of twice the peak width. Figure 2 shows the DMRM chromatogram of the separation of 223 compounds within 20 minutes. Figure 3 explains the distribution of the compounds over the complete runtime.

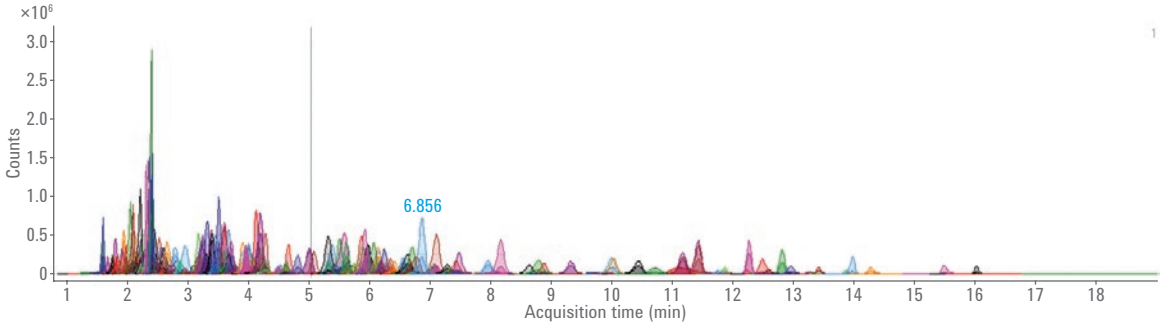

Figure 2. Measurement of 223 pesticides in the Agilent LC/MS Pesticides Comprehensive Test Mix by DMRM. There were 195 compounds eluted within 10 minutes from an amino phase column with 2 to 10 % ethanol as organic modifier, and 28 additional compounds eluted with up to 50 % organic modifier in 20 minutes.

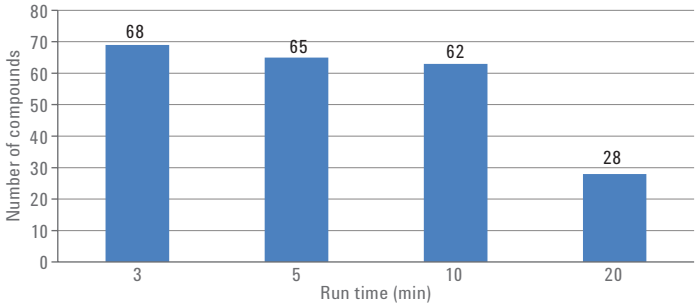

Figure 3. Distribution of pesticide elution over total runtime. The first compounds eluted at 1.5 minutes. There were 68 compounds eluted within the first 3 minutes, another 65 compounds between 3 and 5 minutes, and a further 62 compounds between 5 and 10 minutes. In total, 195 compounds eluted within 10 minutes with a gradient from 2 to 10 % ethanol. The elution was broadly distributed in the first 10 minutes.

For the complete set of 223 pesticides measured, a distribution of their LOQs is shown in Figure 4. A total of 102 pesticides had an LOQ of 0.5 ppb with a signal-to-noise (S/N) ratio greater than 10, and 167 had an LOQ of 1 ppb or lower. Only seven pesticides out of the 223 compounds had an LOQ below 10 ppb. Nevertheless, all had LODs below 10 ppb, and thereby met the requirement of the regulations[1]. The calibration curves for all compounds were created from their LOQ up to 100 ppb. All compounds showed a linearity of $R^2 = 0.999$ or better. Figure 5 shows the distribution of retention time precision. The majority of the 165 compounds had a retention time precision better than 1 % RSD. Figure 6 shows the distribution of the area precision. In total, 162 compounds had area RSDs below 5 %, and the majority of the compounds had RSDs between 2 and 5 %.

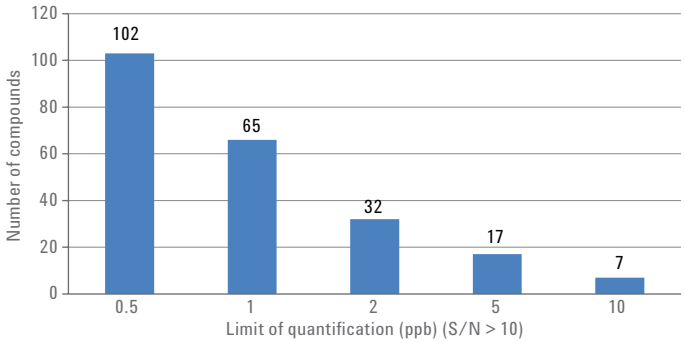

Figure 4. Distribution of LOQ for tested pesticides. There were 102 pesticides with a LOQ of 0.5 ppb, with an S/N > 10 and 167 had an LOQ of 1 ppb or lower. Only seven pesticides out of the 223 compounds had an LOQ of 10 ppb.

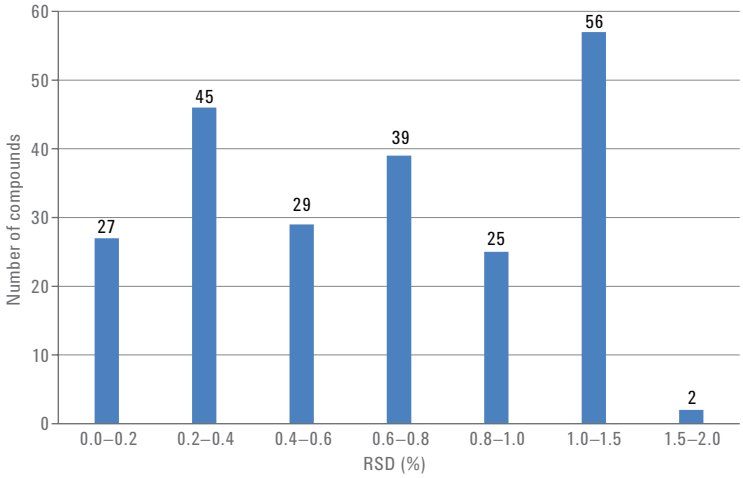

Figure 5. Distribution of retention time precision. There were 165 compounds with a retention time precision below 1 % RSD.

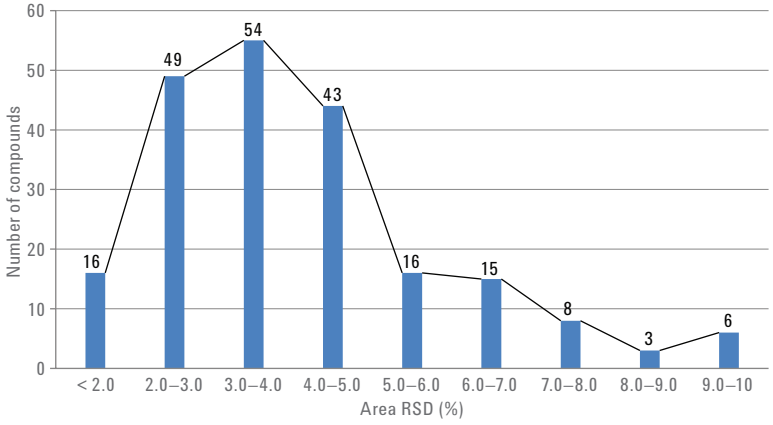

Figure 6. Distribution of area precision. There were 162 compounds with RSDs below 5 %, and the majority of the compounds had RSDs between 2 and 5 %.

As examples, the compounds displayed in Figure 7 are discussed in more detail. The first example is oxasulfuron, which belongs to the group of sulfonylurea herbicides, and displays good chromatographic behavior when using SFC. The lowest level of the calibration was 10 ppb, the calculated LOQ was 0.14 ppb, and the LOD was 0.04 ppb with a linearity of R^2 = 0.99993 (Figure 7A). The second example is methamidosphos, which is widely used for the protection of rice plants. It is a highly polar compound, and often peak broadening is observed when injecting pure QuEChERS extracts in reversed phase HPLC separations due to early elution. QuEChERS sample preparation results in a final extract of pure acetonitrile. In contrast to HPLC, this solution can be used in SFC directly, without compromising peak shape. Under the SFC conditions, it eluted at 7.055 minutes. The 10 ppb calibration level and the calibration curve are shown in Figure 7B. The calculated LOQ was 0.38 ppb, and the LOD was 0.13 ppb, with a linearity of R^2 = 0.99991.

As an example of real-life samples, strawberries, apples, and tomatoes were extracted according to the described QuEChERS procedure[4], and the obtained acetonitrile extract was injected directly. In this part of the experiment, all 223 pesticides were calibrated from 10 to 100 ppb, whereby the 10-ppb value is the highest legally accepted pesticide residue. From the measured 223 pesticides, only five were detected in minor amounts near the LOD: tebuconazole, triadimenol, chlorantraniliprol, trifloxystrobin, and boscalid.

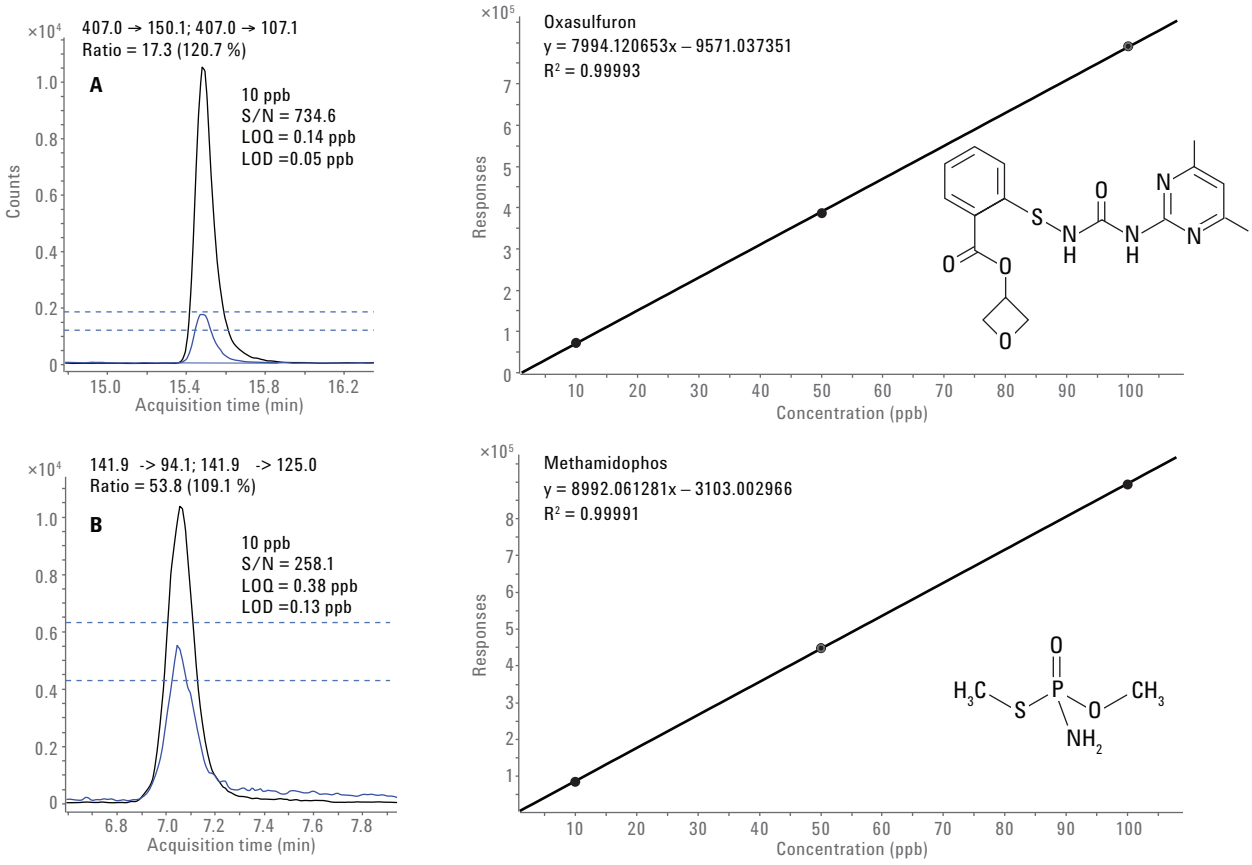

Figure 7. (A) Oxasulfuron, lowest level of the calibration at 10 ppb with an S/N = 734.6, LOQ = 0.14 ppb, LOD = 0.05 ppb, and linearity 0.99993. (B) Methamidophos, lowest level of the calibration at 10 ppb with an S/N = 258.1, LOQ = 0.38 ppb, LOD = 0.13 ppb, and linearity 0.99991.

Triadimenol is a systemic fungicide used predominantly against rust and powdery mildew, for example, on fruits, grapes, and tomatoes. Triadimenol is a metabolite of triadimefon, but is also used as an active ingredient itself. Often, it is used in combination with other fungicides such as tebuconazole. In the tomato sample, triademenol was detected at a low level (Figure 8). The lowest level of the calibration was 10 ppb with S/N = 971.2, LOQ = 0.1 ppb, and LOD = 0.03 ppb (Figure 8A). The triadimenol residue detected in tomatoes corresponded to a level of 1.36 ppb (Figure 8B). The calibration curve for triadimenol at levels of 10, 50, and 100 ppb showed a linearity of R^2 = 0.99929. Another example of a low level residue found in the strawberry sample is boscalid. It was detected at a concentration of 0.75 ppb and, thus, very close to the estimated LOD. Boscalid is widely used as a fungicide for the protection of fruits, vegetables, and wine grapes. According to the United States Environmental Protection Agency (EPA), boscalid has some carcinogenicity, but with minor potential on humans[5]. The maximum accepted daily dose is 0.04 mg/kg. However, the minimum reporting level (MRL) for triadimenol in tomatoes and boscalid in strawberries is significantly higher (1,000 and 500 ppb, respectively). These examples show the performance of the presented method for the analysis of trace level residues in complex food matrixes.

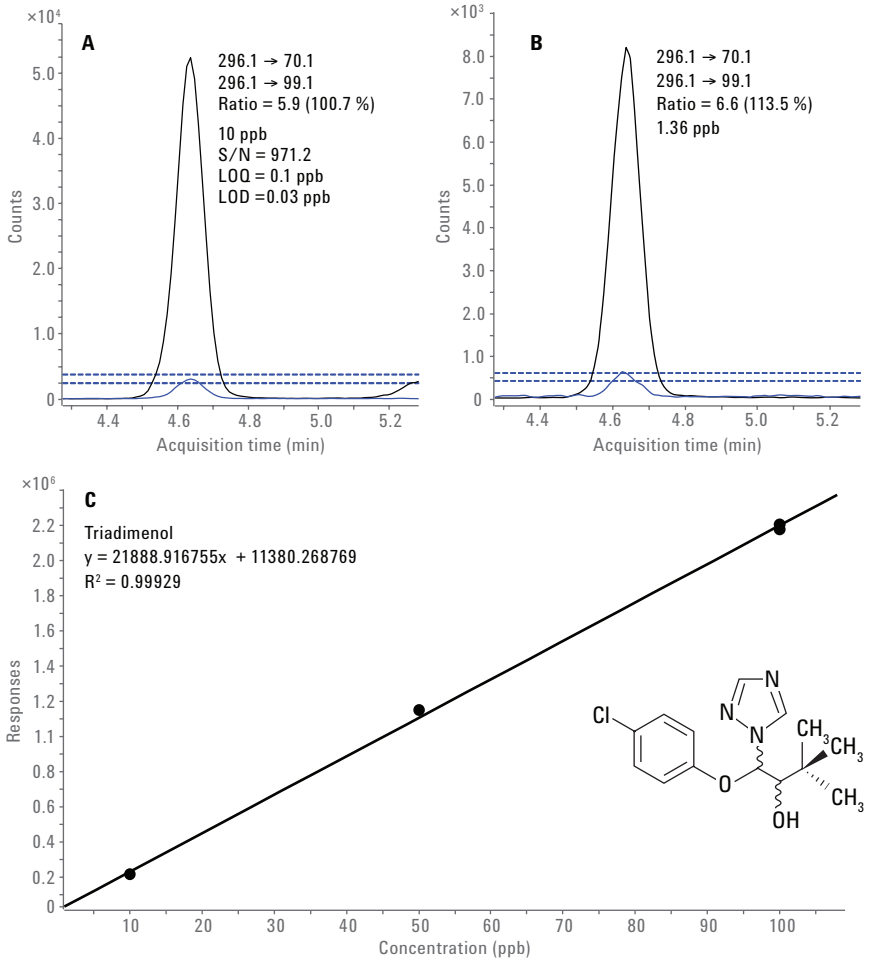

Figure 8. Triadimenol residue in tomatoes. A) Lowest level of the calibration at 10 ppb with an S/N = 971.2, LOQ = 0.1 ppb, and LOD = 0.03 ppb. B) Triadimenol residue detected in tomatoes at 1.36 ppb. C) Calibration curve for triadimenol at levels of 10, 50, and 100 ppb.

The influence of the respective matrix was examined by comparing spiked matrix samples and standards. The recovery for most compounds was in the range of 70 to 120 %, which is accepted by SANCO guidelines for method validation[6]. This was also shown in an earlier work[4]. For instance, for the strawberry matrix, at the 10-ppb level, 193 compounds out of the measured 223 fall in the recovery range of 70 to 120 % (Figure 9). Accounting for the matrix effect, a matrix calibration with compound addition could be done to further improve these results. In addition, standard addition can be used as a means to compensate for matrix effects.

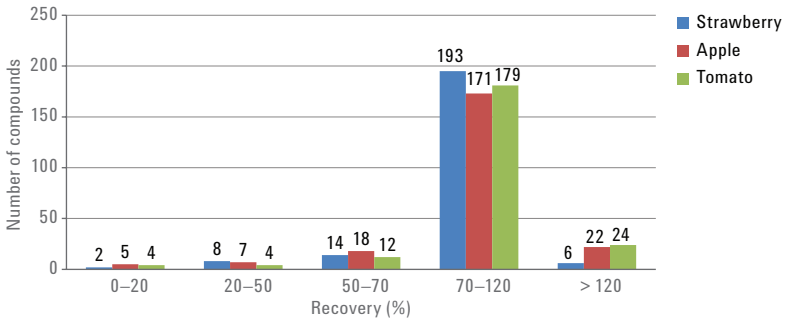

Figure 9. Distribution of pesticide recoveries. Most of the compounds have recoveries in the required range of –30 to +20 %.

Figure 10 shows the standard addition for trifloxystrobin in apple, calculated using the built-in function of the Agilent MassHunter Quantitative Software. The quadratic symbol in the calibration line corresponds to the sample, and the round symbols show the various spiking levels. While the external calibration resulted in a final concentration of 8.1 ppb trifloxystrobin, the standard addition resulted in 11.3 ppb. This shows how a matrix suppression of nearly 30 % can give a result that lies below the actual value. For trifloxystrobin in apples, the MRL is significantly higher (700 ppb) than the default MRL of 10 ppb and, therefore, no MRL exceeding has to be reported.

Conclusion

This Application Note describes the development of a multipesticide method for SFC coupled to triple quadrupole MS for the determination of 223 pesticide compounds. In this method, the majority of 195 pesticide compounds eluted within 10 minutes using a gradient from 2 to 10 % organic modifier. By focusing on these pesticides, this could shorten the method dramatically compared to typical HPLC methods for the measurement of the same number of compounds. The targeted pesticides were determined with typical LOQs at or below 1 ppb, and calibration linearity better than $R^2 = 0.999$. Polar pesticide compounds that are difficult to determine by standard reversed phase HPLC/MS are easily separated and determined by SFC/MS directly from the organic sample extract. Matrix effects are in the same range as reported before, and matrix calibration or the use of internal standards is recommended to compensate for strong matrix effects for specific compounds.

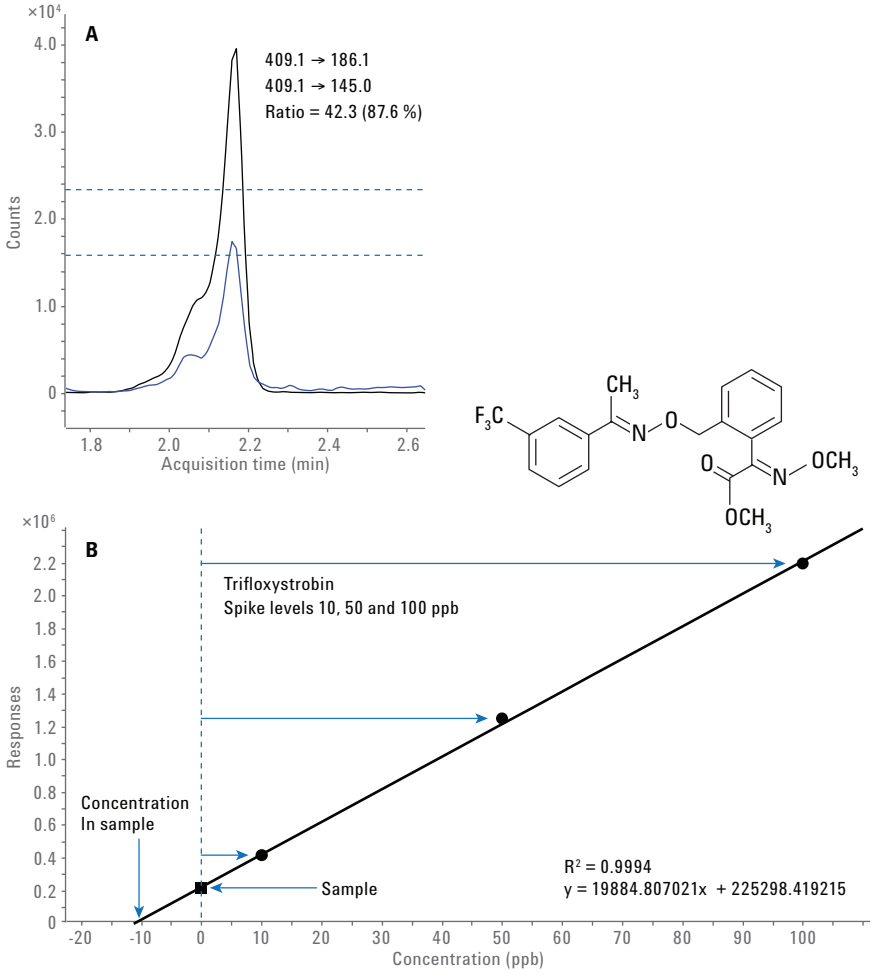

Figure 10. Trifloxystrobin residue in apples. A) Trifloxystrobin residue detected in tomatoes at 8.1 ppb by external calibration. B) Calibration curve for trifloxystobin including standard addition at levels of 10, 50, and 100 ppb. This approach resulted in 11.3 ppb.

References

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Appendix

Table 1. Dynamic MRM method information for the 223 measured pesticides, including retention times, molecular and fragment masses, and fragmentor, collision, and cell acceleration voltages.

No.	Compound name	Retention time (min)	Precursor ion (*m/z*)	Fragmentor (V)	Product ion 1 (*m/z*)	Collision energy (V)	Product ion 2 (*m/z*)	Collision energy (V)	Cell accel. (V)
1	Methacrifos	1.56	241	55	209.1	0	125.1	28	3
2	Carfentrazone-ethyl	1.61	412	150	366	15	346.1	20	3
3	Pendimethalin	1.61	282.1	85	212.1	5	194.1	15	3
4	Dichlorvos	1.62	220.9	100	109	12	79	24	4
5	Molinate	1.62	188.1	90	126	10	83.2	15	3
6	Diazinon	1.63	305.1	105	169	20	153.1	20	4
7	Malathion	1.65	331	80	126.9	5	99	10	3
8	Oxadiazon	1.65	345	90	303	10	220	15	3
9	Prosulfocarb	1.66	252.1	90	128.1	5	91.1	20	3
10	Pirimiphos-methyl	1.67	306	130	164.2	20	108.1	30	3
11	Phoxim	1.72	299.1	70	129.1	4	77.1	24	3
12	Tolclofos-methyl	1.76	300.9	115	269	10	125	15	3
13	Bifenthrin	1.78	440.2	100	181	5	–	–	4
13	Bifenthrin	1.78	442.2	100	–	–	181	5	4
14	Ethion	1.81	385	95	199	4	143	20	4
15	Mecarbam	1.85	330	70	227	0	97.1	45	3
16	Mevinphos	1.85	225	65	193.1	0	127	10	3
17	Ethoprophos	1.89	243	90	131	15	97	30	4
18	Quinalphos	1.89	299	90	163	20	147	20	7
19	Chlorpyriphos-methyl	1.90	322	110	290	10	125	25	4
20	Phenthoate	1.90	321	75	247	4	79.1	48	3
21	Propargit	1.93	368.1	80	231.2	5	175.1	10	3
22	Ethofumesat	1.97	287	80	259.1	0	121.1	10	3
23	Clomazone	1.98	240	70	125.1	15	89.1	45	3
24	Ethoxyquin	1.98	218	120	174	30	160	35	3

Table 1. Dynamic MRM method information for the 223 measured pesticides, including retention times, molecular and fragment masses, and fragmentor, collision, and cell acceleration voltages. (continued)

No.	Compound name	Retention time (min)	Precursor ion (*m/z*)	Fragmentor (V)	Product ion 1 (*m/z*)	Collision energy (V)	Product ion 2 (*m/z*)	Collision energy (V)	Cell Accel. (V)
25	Flufenacet	2.00	364	90	194.2	5	152.1	15	3
26	Proquinazid	2.03	372.9	85	331	12	289	24	3
27	Isoxaflutole	2.04	359.8	95	250.9	20	220	35	3
28	Propetamophos	2.05	282.1	125	156	10	138	15	3
29	Triadimefon	2.06	294.1	90	197.1	10	69.1	20	3
30	Metolachlor	2.13	284.1	100	252.2	10	176.1	20	3
31	Kresoxim-methyl	2.14	314.1	85	267.1	0	222.2	10	3
32	Profenofos	2.15	374.9	120	347	5	304.9	15	3
33	Trifloxystrobin	2.19	409.1	110	186.1	10	145	45	3
34	Malaoxon	2.20	315.1	85	127	4	99	20	3
35	Diflufenican	2.21	395	150	266	25	246	40	3
36	Methidathion	2.25	302.9	55	145	0	85.1	15	3
37	Dimethachlor	2.26	256	120	224	10	148	25	3
38	Etofenprox	2.27	394.2	100	177.2	10	107.1	45	3
39	Pyripoxyfen	2.28	322.1	110	185.1	20	96.1	10	3
40	Carbosulfan	2.30	381.1	105	160.1	8	118.1	16	3
41	Furathiocarb	2.30	383.1	110	252.1	5	195.1	15	3
42	Propham	2.33	180.1	60	138.1	4	120	12	3
43	Quinoxyfen	2.33	308	115	197	35	162	45	7
44	Tolylfluanide	2.37	346.9	70	238.1	0	137	25	3
45	Tebufenpyrad	2.39	334.1	145	145.1	25	117.1	40	3
46	Chlorfenvinphos	2.39	358.9	105	170	40	155.1	8	4
47	Metazachlor	2.41	278	70	210.1	0	134.1	15	3
48	Spirodiclofen	2.42	411.1	110	313	5	71.2	15	3
49	Picoxystrobin	2.44	368.1	70	205.1	0	145.1	20	3
50	Pirimicarb	2.45	239.1	100	182.2	10	72.1	20	3
51	Spiromesifen	2.47	388.2	110	273	10	255	25	3
52	Phosalone	2.49	368	70	182	10	111.1	45	3
53	Fenazaquin	2.50	307.2	105	161.1	10	57.1	25	3
54	Hexythiazox	2.50	353	90	228.1	10	168.1	25	3
55	Benfuracarb	2.51	411.1	95	252.1	10	195.1	20	3
56	Spiroxamine	2.55	298.2	125	144.2	15	100.2	35	3
57	Picolinafen	2.56	377.1	120	359	24	238	32	3
58	Fenpyroximat	2.59	422.1	135	366.1	15	135.1	30	3
59	Propaquizafop	2.60	444	125	371	10	100.2	15	3
60	Benalaxyl	2.62	326.1	90	294.2	5	148.1	15	4
61	Propiconazole	2.70	342	115	158.9	30	69.1	15	4
62	DEET	2.71	192.14	110	119	16	91.1	32	3
63	Metalaxyl	2.76	280.1	95	220.1	10	160.1	20	3
64	Indoxacarb	2.82	528	110	203	45	149.9	20	3
65	Cymoxanil	2.83	199	50	128	0	111.1	15	3
66	Buprofezin	2.85	306.1	105	201.2	5	116.1	10	3
67	Trietazin	2.95	230.1	105	202.1	15	99	25	3
68	Bupirimate	2.97	317.1	125	166.1	20	108.1	25	4
69	Phosmet	3.03	317.9	70	160	10	133	40	3
70	Silthiopham	3.03	268	135	252.1	5	139	15	3

Table 1. Dynamic MRM method information for the 223 measured pesticides, including retention times, molecular and fragment masses, and fragmentor, collision, and cell acceleration voltages. (continued)

No.	Compound name	Retention time (min)	Precursor ion (*m/z*)	Fragmentor (V)	Product ion 1 (*m/z*)	Collision energy (V)	Product ion 2 (*m/z*)	Collision energy (V)	Cell Accel. (V)
71	Pyrimethanil	3.05	200.1	120	107.1	20	82.1	25	3
72	Benzoximate	3.08	364.1	80	198.1	4	104.9	20	3
73	Aldicarb-fragment	3.16	116	70	89.1	4	70.1	4	3
74	Clofentezin	3.16	303	110	138	10	102.1	40	3
75	Flumioxazin	3.23	355.1	100	327.1	20	299	28	3
76	Diethofencarb	3.25	268.1	70	226	0	124	30	3
77	Azinphos-ethyl	3.28	346.05	70	132	8	97	32	3
78	Fluquinconazole	3.28	376	120	349.1	16	307	24	4
79	Fenoxycarb	3.29	302.1	90	116.1	5	88.1	15	3
80	Epoxyconazol	3.34	330	100	121.1	20	101.1	45	4
81	Tetraconazole	3.34	372	130	159	30	70.1	20	4
82	Butocarboxim	3.35	213	70	156.1	5	75	10	3
83	Beflubutamid	3.37	356	145	162.1	25	91	30	3
84	Metobromuron	3.37	259	120	170	15	148	10	3
85	Penconazole	3.4	284	70	159	30	70.1	15	3
86	Flusilazole	3.42	316	120	247.2	15	165.1	25	4
87	Promecarb	3.42	208.1	80	151	0	109.1	10	3
88	Cyprodinil	3.43	226.1	140	93.1	40	77.1	45	3
89	Azamethiphos	3.44	325	120	182.9	12	111.9	40	4
90	Phosphamidon	3.44	300.1	110	174.1	8	127	16	3
91	Azinphos-methyl	3.46	318.02	60	261	0	132	8	3
92	Coumaphos	3.46	363	120	307	16	226.9	28	4
93	Temephos	3.47	467	155	419	20	124.9	44	3
94	Triflumizol	3.48	346	85	278.1	5	73.1	10	3
95	Pyridaben	3.49	365.1	80	309.1	10	147.1	25	3
96	Isocarbophos	3.54	231	100	121	20	65	40	3
97	Fosthiazate	3.55	284	90	228.1	5	104.1	20	3
98	Propyzamid	3.59	256	105	190	10	173	20	3
99	Metrafenon	3.6	409	110	226.9	25	209.1	10	3
100	Cymiazol	3.61	219	95	171	25	144	35	3
101	Prometon	3.62	226.2	100	184	16	142.1	24	3
102	Isoprothiolane	3.63	291.1	80	231	8	188.8	20	3
103	Fenobucarb	3.70	208.1	65	152.1	5	95.1	10	3
104	Triazophos	3.70	314	110	162.1	15	119.1	35	3
105	Tralkoxydim	3.71	330.1	170	284.2	5	138.1	15	3
106	Furalaxyl	3.72	302.1	110	242.1	10	95	27	3
107	Iprovalicarb	3.74	321.1	80	203.1	0	119.1	20	3
108	Trimethacarb	3.77	194.1	80	137	4	122.1	28	3
109	Mexacarbate	3.82	223.1	110	166.1	12	151	24	3
110	Azaconazole	3.83	300	130	230.8	16	158.9	32	3
111	Propoxur	3.83	210.1	55	168.1	0	111.1	10	3
112	Mepanipyrim	3.88	224	140	209.1	16	106.1	25	3
113	Cyazofamid	3.89	325	90	261.1	5	108.1	10	3
114	Bromuconazole	3.98	377.9	115	159	35	70.1	20	4
115	Methoprotryne	4.10	272.2	140	198	24	169.9	28	3
116	Carbofuran	4.11	222.1	80	165.1	5	123.1	20	3

Table 1. Dynamic MRM method information for the 223 measured pesticides, including retention times, molecular and fragment masses, and fragmentor, collision, and cell acceleration voltages. (continued)

No.	Compound name	Retention time (min)	Precursor ion (*m/z*)	Fragmentor (V)	Product ion 1 (*m/z*)	Collision energy (V)	Product ion 2 (*m/z*)	Collision energy (V)	Cell Accel. (V)
117	Methabenzthiazuron	4.11	222	90	165	15	150	35	3
118	Linuron	4.14	249	100	182.1	10	160	15	3
119	Pyraclostrobin	4.18	388	95	194.1	5	163.1	20	3
120	Difenoconazole	4.20	406	120	337.1	15	251.1	25	3
121	Secbumeton	4.24	226.2	100	170.1	16	67.9	50	3
122	Aminocarb	4.34	209.1	105	152	12	137.2	24	3
123	Fenamiphos	4.39	304.1	120	217.1	20	202	35	3
124	Prochloraz	4.39	376	70	308	5	266	10	3
125	Methiocarb	4.40	226.1	70	169.1	0	121.1	15	3
126	Fenpropidin	4.43	274	120	147	30	86	25	3
127	Myclobutanil	4.50	289.1	110	125	35	70.1	15	3
128	Clethodim	4.67	360.1	100	268.2	10	164.1	15	3
129	Imazalil	4.69	297	115	201	15	159	20	4
130	Fluopicolide	4.72	382.9	110	172.9	25	144.9	45	3
131	Triadimenol	4.78	296.1	70	99.1	10	70.1	5	3
132	Rotenone	4.79	395	145	213.1	20	192.1	20	3
133	Cycluron	4.84	199.2	120	88.9	12	72.1	28	3
134	Dimethomorph	5.05	388	145	301.1	20	165.1	30	3
135	Dimoxystrobin	5.16	327.1	115	205.1	5	116	20	3
136	Hexaconazole	5.27	314	95	159	30	70.1	15	4
137	Triflumuron	5.34	359	90	156	10	139	35	3
138	Paclobutrazol	5.46	294.1	115	125	40	70.1	20	3
139	Aldicarb	5.49	208	70	116	0	89.1	10	3
140	Quinoclamin	5.49	208	125	88.9	44	76.9	44	3
141	Carboxin	5.51	236	105	143	10	93	40	3
142	Tebuconazole	5.69	308.1	100	125	40	70.1	20	4
143	Azoxystrobin	5.75	404	110	372.2	10	344	25	3
144	Fenbuconazol	5.78	337.1	145	125.1	35	70.1	15	4
145	Dioxacarb	5.81	224	80	167	10	123	10	3
146	Monocrotophos	5.89	224	65	193.1	0	127	10	3
147	Bitertanol	5.91	338.1	70	269.2	0	70.1	0	3
148	Fenarimol	5.99	331	130	268.1	20	81.1	30	4
149	Fenamidon	6.05	312.1	100	236.2	10	92.1	25	3
150	Flutriafol	6.05	302	90	123	30	70.1	15	3
151	Pyracarbolid	6.14	218.1	145	125	16	96.9	28	3
152	Tebuthiuron	6.18	229.1	105	172.1	12	116	24	3
153	Omethoat	6.19	214	80	125	20	109	25	3
154	Spinosyn A	6.19	732.4	155	142.1	30	98.1	45	3
155	Bifenazate	6.23	301.1	95	198.2	5	170.1	15	3
156	Lufenuron	6.23	510.9	138	158	20	141	45	3
157	Metconazole	6.25	320.1	130	125.1	40	70.1	20	4
158	Diniconazole	6.27	326	75	159	28	70.1	28	4
159	Spinosyn D	6.30	746.5	145	142.1	35	98	55	3
160	Novaluron	6.31	493.1	90	158.1	20	141.1	45	3
161	Tepraloxydim	6.33	342.1	130	250.2	10	166.1	20	3
162	Cyproconazole	6.36	292.1	100	125.1	35	70.1	15	3

Table 1. Dynamic MRM method information for the 223 measured pesticides, including retention times, molecular and fragment masses, and fragmentor, collision, and cell acceleration voltages. (continued)

No.	Compound name	Retention time (min)	Precursor ion (*m/z*)	Fragmentor (V)	Product ion 1 (*m/z*)	Collision energy (V)	Product ion 2 (*m/z*)	Collision energy (V)	Cell Accel. (V)
163	Uniconazole-P	6.36	292.1	135	125	36	70	24	4
164	Ipconazole	6.39	334.1	115	125	45	70	25	4
165	Dimethoate	6.41	230	70	199	0	125	20	3
166	Alanycarb	6.45	400.1	130	238	4	91	50	3
167	Mandipropamid	6.52	411.9	110	356.1	5	328.1	10	3
168	Carbaryl	6.54	202	65	145	0	127.1	25	3
169	Diflubenzuron	6.55	311	80	158	10	141	35	3
170	Flufenoxuron	6.68	489	100	158	15	141	45	3
171	Oxadixyl	6.88	279.1	70	219.1	5	132.1	35	3
172	Triticonazole	6.92	318.1	90	125.1	40	70.1	10	4
173	Fluoxastrobin	6.94	459	130	427.1	15	188.1	40	3
174	Spirotetramat	7.13	374.1	120	330.1	10	302.1	10	3
175	Vamidothion	7.17	288.1	95	146	8	146	8	4
176	Pencycuron	7.21	329.1	120	218.1	10	125	25	3
177	Methamidophos	7.26	141.9	85	125	10	94.1	10	3
178	Diuron	7.27	235	110	72.1	20	–	–	3
178	Diuron	7.27	233	110	–	–	72.1	20	3
179	Famoxadone	7.27	392.1	85	331.2	0	238.2	10	3
180	Fluometuron	7.27	233.1	105	72.1	15	46.2	15	3
181	Zoxamide	7.32	336	120	187	20	159	45	3
182	Carbendazim	7.50	192	105	160.1	15	132.1	30	3
183	Methomyl	7.58	162.9	50	106.1	5	88.1	0	3
184	Bosclid	7.68	343	145	307.1	12	271	28	3
185	Acephate	7.73	183.9	70	143	0	125	15	3
186	Flonicamid	7.98	230	110	203	15	174	15	3
187	Hexaflumuron	8.09	461	120	158	15	141	45	3
188	Tricyclazol	8.28	190	130	163	20	136	30	4
189	Isoxaben	8.30	333.2	100	165	16	150	48	3
190	Sulfentrazone	8.31	404	110	306.9	28	273	36	3
191	Chlorotoluron	8.85	213.1	120	140	20	72	20	3
192	Lenacil	8.93	235.2	85	153.1	15	136	35	3
193	Oxamyl	9.17	237	60	90.1	0	72.1	15	3
194	Metaflumizone	9.45	507	150	287.1	20	178.1	20	3
195	Tebufenozid	9.45	353	95	297.2	0	133.1	15	3
196	Moxidectin	10.16	640.4	148	622.2	12	528.2	4	3
197	Metamitron	10.18	203.1	100	175.1	15	104.1	20	3
198	Fenuron	10.25	165.1	180	76.9	32	72	16	3
199	Chloroxuron	10.27	291	130	164	10	72.1	20	3
200	Thiodicarb	10.28	355	82	108.1	10	88.1	10	3
201	Methoxyfenozide	10.56	369.2	85	313.2	0	149.1	10	3
202	Tribenuron-methyl	11.08	396	110	181.1	15	155.1	5	3
203	Thiabendazol	11.24	202	130	175.1	25	131.1	35	3
204	Desmedipham	11.47	318.1	80	182.2	5	136.1	25	3
205	Phenmedipham	11.47	318.1	90	168.1	4	136	20	3
206	Propamocarb	11.88	189.1	90	144	5	102.1	15	3
207	Ethidimuron	11.98	265.1	120	207.9	12	57	32	3

Table 1. Dynamic MRM method information for the 223 measured pesticides, including retention times, molecular and fragment masses, and fragmentor, collision, and cell acceleration voltages. (continued)

No.	Compound name	Retention time (min)	Precursor ion (*m/z*)	Fragmentor (V)	Product ion 1 (*m/z*)	Collision energy (V)	Product ion 2 (*m/z*)	Collision energy (V)	Cell Accel. (V)
208	Acetamiprid	12.06	223	80	126.1	2	90.1	35	3
209	Chlorantraniliprole	12.31	483.9	105	452.9	15	285.9	10	3
210	Fuberidazol	12.34	185.1	145	157.1	20	156.1	30	3
211	Fenhexamid	12.56	302	130	97.2	20	55.1	40	3
212	Pymetrozin	12.84	218	110	105.1	20	78.1	45	3
213	Ethirimol	12.87	210.1	145	140.1	20	98.1	25	3
214	Hydramethylnon	12.99	495.2	200	323	36	170.9	48	3
215	Imidacloprid	13.48	256	80	209.1	10	175.1	15	3
216	Thiamethoxam	13.69	292	85	211.1	5	181.1	20	3
217	Chloridazon	13.93	222	130	104.1	25	77.1	35	3
218	Thiacloprid	14.15	253	100	126	20	90.1	40	3
219	Nitenpyram	14.60	271.1	95	225.2	3	56.1	30	3
220	Oxasulfuron	15.57	407	120	150.1	15	107.1	45	3
221	Forchlorfenuron	16.02	248.1	110	129	16	92.9	40	3
222	Mesosulfuron-metyl	16.30	504.1	125	182.1	25	139.1	45	3
223	Triasulfuron	17.86	401.9	130	167.1	10	141	10	3

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Determination of Vitamin E in Olive Oil Using the Agilent 1260 Infinity Analytical SFC System

Rapid, high-resolution separation of all isomers of tocopherol and tocotrienol

Application Note

Food Testing and Agriculture

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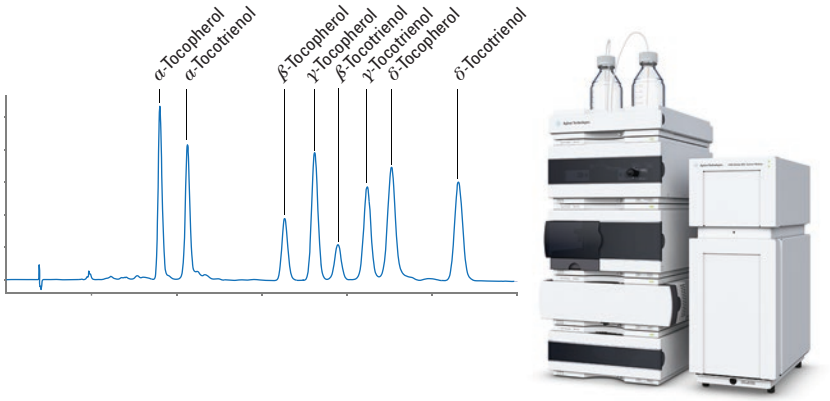
Abstract

This Application Note demonstrates the separation of eight tocopherol and tocotrienol compounds by supercritical fluid chromatography (SFC) using the Agilent 1260 Infinity Analytical SFC System. Calibration curves were generated for all compounds, and a real-life virgin olive oil sample was analyzed. The limits of detection and quantification (LOD and LOQ) were determined as well as relative standard deviations (RSDs) of retention times and areas.



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Introduction

Supercritical fluid chromatography (SFC) is a versatile tool to replace separations that to date have been preferably done on a normal-phase liquid chromatography (LC) system. The advantage of SFC is that separations are much faster and of higher precision compared to normal-phase LC. In addition, SFC does not use harmful solvents.

Fat-soluble vitamins are typical examples of compounds that can be separated by SFC. A group of such compounds is the vitamin E family. Tocopherols and their unsaturated relatives, tocotrienols, belong to this group in various isomeric forms known as α, β, γ, δ-tocopherols and -tocotrienols. These compounds have high bioactive and antioxidant potential, and are nutritionally beneficial for human health. One natural source of vitamin E, as well as other healthy substances, is virgin olive oil obtained from the fruit of the olive tree (*Olea europea* L.)[1,2]. Typically, about 95 % of the vitamin E in olive oil is α-tocopherol[2]. Since olive oil is a commodity of important economic value for the producing countries, various methods for the analysis of olive oil have been developed to ensure authenticity and quality[3].

This Application Note describes the analysis of vitamin E compounds in olive oil by SFC that has the advantages of faster run times and higher precision compared to widely used normal-phase LC.

Experimental

Instrumentation

All experiments were performed with an Agilent 1260 Infinity Analytical SFC System (G4309A) comprising the following modules:

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity High-Performance Degasser
- Agilent 1260 Infinity SFC Standard Autosampler
- Agilent 1260 Infinity Diode Array Detector (DAD) with high-pressure SFC flow cell
- Agilent 1290 Infinity Thermostatted Column Compartment (TCC)

Column

Agilent ZORBAX NH2,
4.6 × 150 mm, 5 µm (p/n 883952-708)

Software

Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, Rev. C.01.06

Standards

Tocopherol and tocotrienol mixed solution standards (in hexane) were purchased from LGC Standards, Teddington, UK.

Sample

A native olive oil was bought in a local supermarket. The olive oil was diluted in hexane 100 mg/mL, and directly used for analysis.

Chemicals

All solvents were purchased from Merck, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak).

SFC method

Parameter	Value
Solvent A	CO_2
Modifier B	Ethanol
SFC flow	4.5 mL/min
Gradient	3 % B at 0 minutes to 4.5 % B at 6 minutes
Stop time	6 minutes
Post time	2 minutes
BPR pressure	210 bar
BPR temperature	60 °C
Column temperature	50 °C
Injection volume	5 µL, 3 × loop overfill, needle wash in vial with hexane
DAD (UV/VIS)	Wavelength 295 nm, bandwidth 4 nm Reference 550 nm, bandwidth 100 nm Slit 8 nm Data rate 10 Hz

Results and Discussion

An amino phase column with a flow rate of 4.5 mL/min was used to separate the mixture of the four isomeric tocopherols and the four isomeric tocotrienol compounds. The organic composition of the gradient was close to isocratic behavior, increasing only from 3 to 4.5 % ethanol. This gradient separated the early eluting compounds, and decreased the retention of the later eluting compounds to produce sharper peaks. The optimized backpressure of 210 bar and the higher column temperature of 50 °C also helped to increase the resolution of the late eluting compounds. Figure 1 shows the chromatogram of the separation of tocopherol and tocotrienol compounds in the standard stock solution within a run time of 6 minutes.

To calibrate all compounds, the stock solution (level 1) was diluted using a 1:2 dilution pattern over six levels (Table 1).

The peaks obtained for the dilution at level 5 showed a signal-to-noise (S/N) ratio of about 10, which was used as the lowest level in the calibration curves and to calculate the limit of quantification (LOQ). The limit of detection (LOD) was calculated from the peaks measured at level 6 with a S/N ratio of 3. The LOQ was typically less than 23 µg/mL and the LOD was less than 7 µg/mL (Table 2).

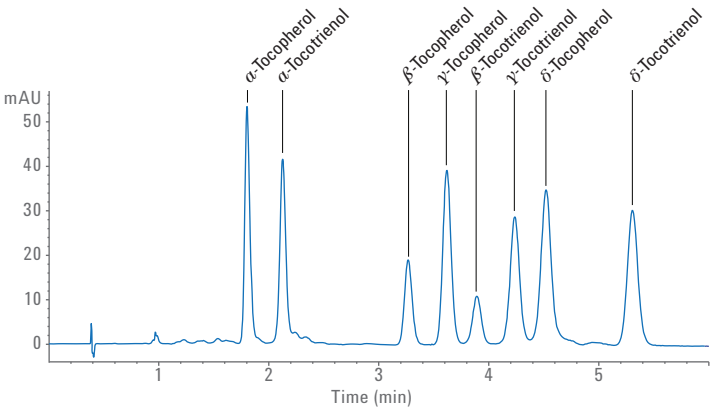

Figure 1. Separation of a mixture of α, β, γ, δ-tocopherol and α, β, γ, δ-tocotrienol by SFC.

Table 1. Concentrations of individual compounds and calibration levels using a 1:2 dilution pattern.

	Level 1 (µg/mL)	Level 2 (µg/mL)	Level 3 (µg/mL)	Level 4 (µg/mL)	Level 5 (µg/mL)	Level 6 (µg/mL)
α-Tocopherol	463.00	231.50	115.75	57.88	38.94	14.46
α-Tocotrienol	447.00	223.15	111.75	55.88	27.94	13.96
β-Tocopherol	203.00	101.50	50.75	25.34	12.69	4.23
γ-Tocopherol	430.00	215.00	107.50	53.75	26.88	13.44
β-Tocotrienol	135.00	67.50	22.50	11.25	5.65	2.81
γ-Tocotrienol	467.00	233.50	116.75	58.37	29.18	14.59
δ-Tocopherol	423.00	211.50	105.75	52.88	26.44	13.22
δ-Tocotrienol	414.00	207.00	103.50	51.75	25.88	12.94

Table 2. Relative standard deviations of retention times and areas, LOQ, LODs, and linearity of individual tocopherols and tocotrienols.

	Retention time	RSD of retention times	RSD of areas	LOQ (µg/mL)	LOD (µg/mL)	R^2
α-Tocopherol	1.799	0.24	5.75	18.11	5.43	0.9997
α-Tocotrienol	2.124	0.31	4.89	22.88	6.87	0.9991
β-Tocopherol	3.273	0.23	5.59	14.58	4.38	0.9999
γ-Tocopherol	3.624	0.27	6.45	22.03	6.62	0.9998
β-Tocotrienol	3.884	0.23	6.27	14.79	4.44	0.9992
γ-Tocotrienol	4.240	0.16	5.04	35.58	10.68	0.9991
δ-Tocopherol	4.517	0.14	6.62	27.54	8.27	0.9998
δ-Tocotrienol	5.302	0.14	5.58	27.53	8.27	0.9999

Figure 2 shows an overlay of the peaks of all compounds obtained from level 1 to level 5. The linearity for all calibrations was in a good range with R^2 better than 0.999. For statistical evaluation, a 1:10 dilution of the stock solution was injected 10 times, and the relative standard deviation (RSD) values of the retention times and areas were calculated. The RSD of retention times was typically better than 0.3 %, and the RSD of areas was better than 6 % (Table 2). The high RSD values for areas could be explained with the known fact that tocopherols and tocotrienols degrade or adsorb on steel capillaries in LC systems[4].

Finally, an extra virgin olive oil sample was measured to determine the content of vitamin E compounds. The olive oil was diluted in hexane and directly injected. The chromatogram showed only α-tocopherol at 1.79 minutes as the quantifiable main peak and a trace of γ-tocopherol at 3.6 minutes (Figure 3). The final concentration of α-tocopherol in the measured olive oil was 184.8 mg/kg.

Conclusion

This Application Note demonstrates the use of the Agilent 1260 Infinity Analytical SFC System for quantification of vitamin E compounds such as tocopherols and tocotrienols in olive oil. The separation of four isomeric tocopherols and four isomeric tocotrienols is shown in a run time of 6 minutes, which is about five times faster than the typically used normal-phase LC methods. Another aspect is that the organic solvents used in normal-phase separations are harmful. This is in contrast to the ethanol modifier used for the SFC separation. The obtained LOQs are typically less than 23 µg/mL, LODs are less than 7 µg/mL, and R^2 better than 0.999.

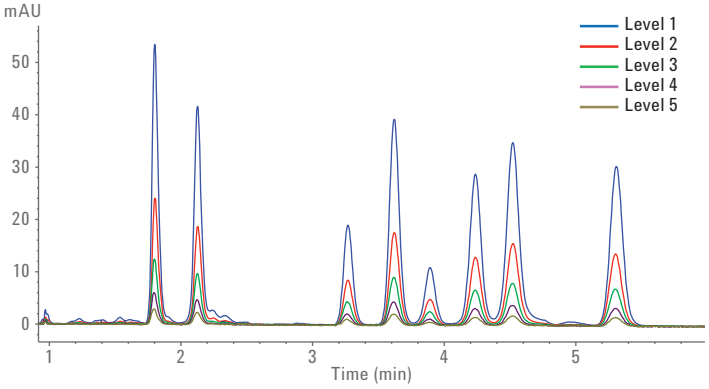

Figure 2. Overlay of calibration levels used to generate calibration curves for α, β, γ, δ-tocopherol and α, β, γ, δ-tocotrienol. (See Figure 1 for compound names, and Table 1 for the concentration of individual compounds and levels.)

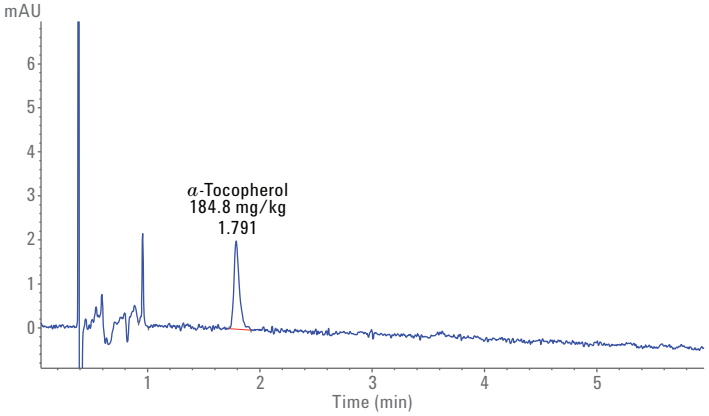

Figure 3. Measurement of an extra virgin olive oil sample and determination of the concentration of α-tocopherol.

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Analysis of Antioxidants in Vegetable Oils Using the Agilent 1260 Infinity Hybrid SFC/UHPLC System with MS Detection

Application Note

Food Analysis & Agriculture

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Abstract

This Application Note demonstrates that SFC and UHPLC are complementary for the analysis of antioxidants in vegetable oil samples. The Agilent 1260 Infinity Hybrid SFC/UHPLC System combined with single quadrupole mass spectrometry detection is capable of performing both supercritical fluid chromatography (SFC) and ultrahigh performance liquid chromatography (UHPLC) by switching automatically between the two techniques.

Good MS-peak area repeatability (RSD < 5.0%) and sensitivity were achieved, allowing the system to be used for qualitative as well as quantitative analysis. The figures of merit are shown using standard solutions and vegetable oils. Using a simple methanol extraction, good recovery was obtained for all antioxidants in the oil sample.

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Introduction

The Agilent 1260 Infinity Hybrid SFC/UHPLC-MS System represents state-of-art, packed-column SFC, providing HPLC-like sensitivity, 600-bar power range, and high instrument and method robustness, all achieved on a truly modular and flexible LC-based system[1].

SFC using packed columns is a valuable complementary technique to liquid chromatography. Especially for chiral and normal phase separations, SFC has demonstrated its potential. This Application Note describes the possibility to obtain complementary data on analyte mixtures in a single sequence of runs by switching between SFC and UHPLC mode. This eliminates the need to invest in two individual systems, excludes system-to-system variability, and saves significant cost and laboratory space[1].

Vitamin E plays an important role as antioxidant. Different stereo-isomers (vitamers) are prevalent in various vegetable oils exhibiting differences in vitamin activity. This Application Note describes the analysis of 14 antioxidants in vegetable oils using the 1260 Infinity Hybrid SFC/UHPLC/MS System in the SFC/MS and LC/MS mode. Since the biological activities and chemical properties of tocols (tocopherols and tocotrienols) differ from each other, it is important to be able to determine and quantify each vitamer separately. The complete resolution of the eight tocols is only possible by using the SFC-MS mode. In this case, the separation by SFC was significantly faster than with UHPLC.

Experimental

Solutions

Stock solutions of the individual antioxidants were prepared in methanol (1–5 mg/mL, depending on solubility). These stock solutions were then mixed to obtain a 14-compound test mixture. Most experiments were performed using a 100-ppm solution; however, a dilution series was also prepared from 0.1–100 ppm. Table 1 provides peak identification, chemical name, and formula weight. For the spiked samples, a stock solution of the antioxidants in the solvent was added prior to extraction.

Oil samples were purchased from a local supermarket. The extraction of the oil and the spiked oil sample was carried out by weighing 100 mg of oil and adding 1 mL of the solvent. This mixture was vortexed for 30 seconds, allowed to stand for 2 minutes, and vortexed once more for 30 seconds.

Peak id	Chemical name	CAS	MW (g/mol)	Supplier
1	Propyl Gallate (PG)	121-79-9	212.2	Sigma
2	Tert-butyl-hydroquinone (TBHQ)	1948-33-0	166.2	Sigma
3	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX)	53188-07-1	250.3	Sigma
4	Butylated hydroxyanisole (BHA)	25013-16-5	180.2	Sigma
5	Octyl Gallate (OG)	1034-01-1	282.3	Sigma
6	Butylated hydroxytoluene (BHT)	128-37-0	220.3	Sigma
7	Lauryl Gallate (LG)	1166-52-5	338.4	Sigma
8	δ-Tocotrienol (δ-TT)	25612-59-3	396.6	Cayman Chem.
9	γ-Tocotrienol (γ-TT)	14101-61-2	410.6	Cayman Chem.
10	α-Tocotrienol (α-TT)	58864-81-6	424.7	Cayman Chem.
11	δ-Tocopherol (δ-TP)	119-13-1	402.6	Cabiochem
12	γ-Tocopherol (γ-TP)	54-28-4	416.7	Calbiochem
13	β-Tocopherol (β-TP)	148-03-8	416.7	Calbiochem
14	α-Tocopherol (α-TP)	59-02-9	430.7	Calbiochem

Table 1
Analyzed antioxidants.

The sample was then centrifuged at 5,000 × g for 5 minutes and the supernatant was transferred into an autosampler vial for injection.

System Configuration

A 1260 Infinity Analytical SFC System (G4309A) can be converted into a hybrid SFC/UHPLC system by simple addition of a 2-position/10-port valve comprising universal valve drive (G1170A) with valve head (G4232B) and a second pump (G1311B). The system can be run in UHPLC mode (Figure 1a) or in SFC mode (Figure 1b). Alternating between modes is accomplished by switching the 2-position/10-port valve, which can be programmed as a method parameter at the beginning of the respective method[1]. The thermostatted column compartment is equipped with a 2-position/6-port column switching valve which enables the selection of the appropriate column for each mode.

Some modifications should be taken into account when coupling the SFC to a MS (or ELSD)[2]. A capillary heating device is installed just before the MS inlet. In SFC mode, the effluent, mainly consisting of carbon dioxide, is decompressed before entering to the MS source. The expanding CO_2 results in significant cooling which can cause freezing of the transfer line. Additionally, a make-up flow is added

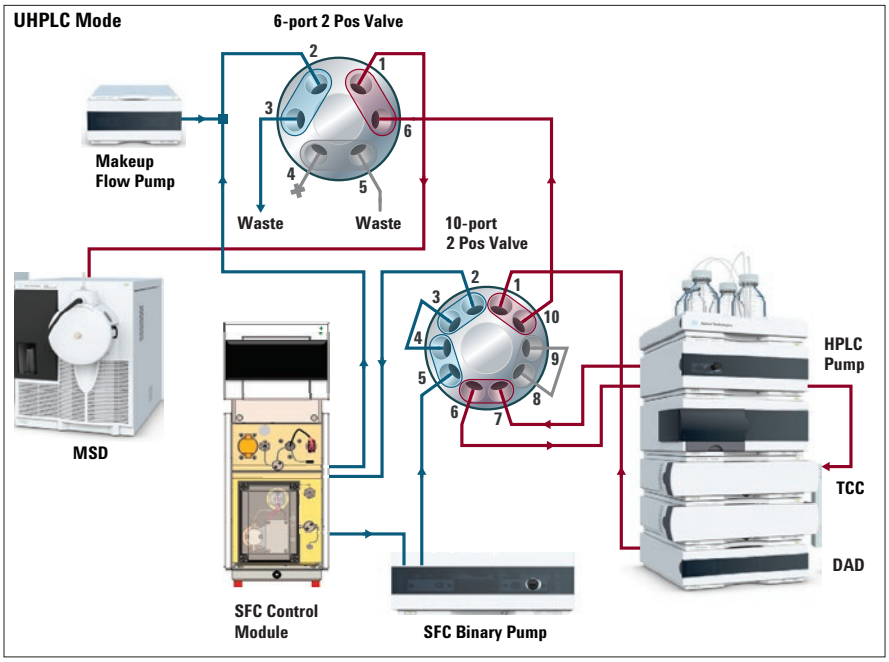

Figure 1a
Schematic of the hybrid system in UHPLC/MS mode.

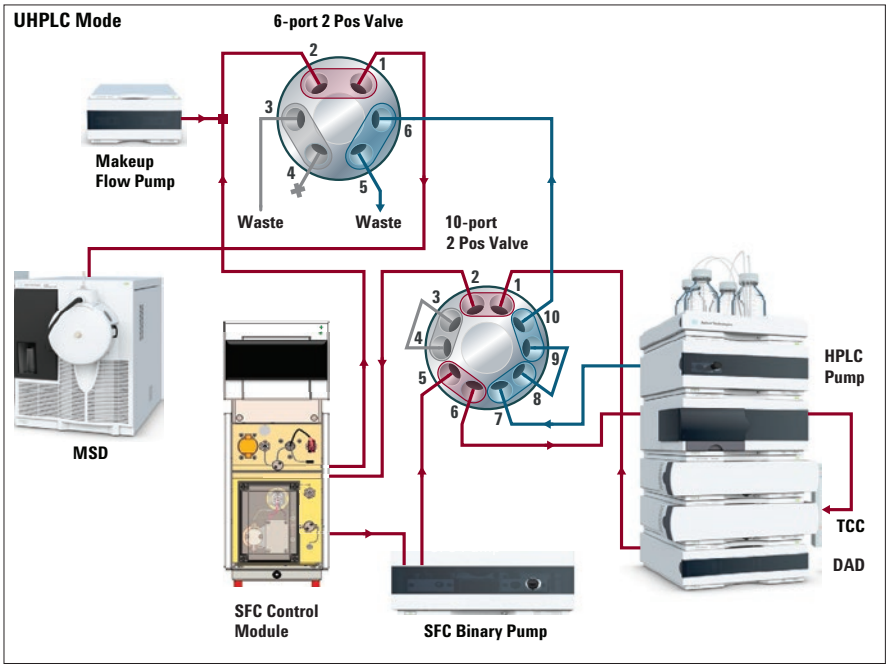

Figure 1b
Schematic of the hybrid system in SFC/MS mode

to the system between the UV detector and the BPR. This additional make-up flow is required in order to obtain the best retention time and peak area reproducibility.

From the UV detector, a 0.12 mm × 105 mm SS capillary (p/n 5021-1820) is connected to an Agilent zero dead volume T-piece (p/n 0100-0969). An Agilent G1311B Pump was used to supply the make-up flow and was connected to the T using a 0.25 mm × 800 mm SS capillary (p/n 5065-9930). A 0.12 mm x 400 mm SS capillary (p/n 5021-1823) was used to connect the T to the BPR. The Caloratherm preheater sleeve was placed over a 0.17 mm × 10 mm SS capillary (p/n 5061-3361) and the tubing containing the preheater device was connected directly to the inlet of the MS. In addition, a 2-position/6-port valve needs to be added as shown in Figures 1a and 1b to combine the flow paths from SFC and UHPLC prior to MS detection.

With the heating device and make-up flow present in the SFC configuration, freezing does not occur, and the MS reproducibility is significantly improved.

Experimental conditions

Table 3 shows the method parameters used in the separation of the 14-component antioxidant mixture and oil samples.

Agilent 1260 Infinity Hybrid SFC/UHPLC System		
G4309A	Agilent 1260 Infinity Analytical SFC System	
G1311B	Agilent 1260 Infinity Quaternary Pump (can be replaced by G1312B, G1310B, G4220A/B, and G4204A)	
G1170A	Agilent 1290 Infinity Valve Drive	
G4232B	2-position/10-port valve head – 600 bar	
G6130B	LC/MS Single Quad	
G4231A	2-position/6-port valve head -600 bar	
G1170A	Agilent 1290 Infinity Valve Drive (a second valve drive is necessary to support the 2-position/6-port valve head)	
AG1	Caloratherm[2]	Available through RIC[1]
AG004	Preheater[2]	Available through RIC[1]

[1]Contact info@richrom.com for more information.
[2]A Capillary heater can be replaced by the usage of a G1316A or G1316C heat exchanger

Table 2
System modules.

Conditions	UHPLC mode	SFC mode
Injection volume:	15 µL (5 µL on column)	15 µL (5 µL on column)
Column:	Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-902)	Agilent ZORBAX Rx-SIL, 4.6 × 250 mm, 5 µm (p/n 880975-901)
BPR:	90 bar*	120 bar
SFC flow rate:	-	2 mL/min
UHPLC flow rate:	0.4 mL/min	1 mL/min
Supercritical fluid:	-	CO_2
Modifier:	(A) Water 0.1% FA (B) Methanol 0.1% FA	MeOH
UHPLC gradient:	20–100% B in 15 minutes (total 25 minutes)	
SFC gradient:		3–12% B (0–25 minutes)
Column temperature:	30 °C	50 °C
Make-up flow:		MeOH 0.1% FA at 0.8 mL/min
Caloratherm:		60 °C
DAD:	292/10 nm, Ref. 400/50 nm	292/10 nm, Ref. 400/50 nm
APCI:	Capillary V ± 4,000 V Corona I = 4.0 µA (+), 20 µA (-) Drying gas = 6.0 L/min at 325 °C Nebulizer = 55 psig Vaporizer = 350 °C	Capillary V ± 4,000 V Corona I = 4.0 µA (+), 20 µA (-) Drying gas = 6.0 L/min at 325 °C Nebulizer = 60 psig Vaporizer = 350 °C

* Only to maintain functioning of BPR. The pressure is not applied on the UHPLC column.

Table 3
Experimental conditions of hybrid system.

Results and Discussion

A 14-component antioxidant mix was analyzed to demonstrate the ease and complementary use and performance of the 1260 Infinity Hybrid SFC/UHPLC/MS System. Both UV and MS data (APCI) were collected; MS data was used to confirm the identity. Figure 2 shows the separation of the antioxidant standard mixture (10 µg/mL) in the UHPLC mode.

Calibration curves were constructed and excellent linearity was obtained for both SFC and LC mode. Table 4 summarizes the UHPLC results.

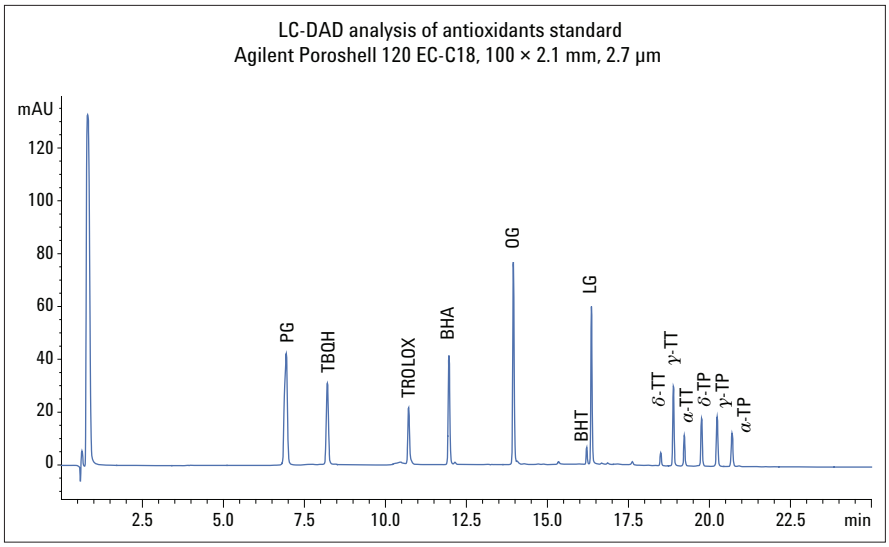

Figure 2
Analyses 14-compounds antioxidant mixture by LC-DAD (10 µg/mL).

	Linearity (R^2)[1]	Repeat. (% RSD)[2]	Repeat. (% RSD)[3]	Recovery 5 mg/kg (%)	Repeat. (% RSD)	Recovery 100 mg/kg (%)	Repeat. (% RSD)
PG	0.99977	3.7	4.11	102.8	2.0	103.3	0.7
TBHQ	0.99807	4.4	4.8	72.6	20	87.2	23
TROLOX	0.99969	5.0	4.3	94.9	17	92.6	6
BHA	0.99978	0.7	2.1	105.2	4.5	100.8	1.2
OG	0.99978	3.0	4.5	101.2	1.6	104.0	1.2
BHT	0.99981	4.9	1.7	104.7	5.6	99.4	2.0
LG	0.99974	0.8	1.4	99.97	7.2	104.4	0.9
δ-TT	0.99965	4.5	2.2				
γ-TT	0.99969	1.8	2.5				
α-TT	0.99953	2.1	2.5				
δ-TP	0.99972	1.8	2.3				
γ-TP and β-TP	0.99987	1.8	2.7				
α-TP	0.99943	1.3	2.6				

[1] 0.1, 0.5, 1, 5, 10, 25, 50, and 100 µg/mL standard solution, 1 injection/level (UV)
[2] 6 consecutive injections of 0.5 µg/mL
[3] 6 consecutive injections of 25 µg/mL
* Recoveries of tocopherols and tocotrienols were not calculated because they are already in the oil samples.

Table 4
LC mode method performance data.

The repeatability and linearity of the method were investigated using standard solutions of the antioxidant and spiked oil samples. The detection limit was equal to, or below 0.1 µg/mL for all antioxidants. This corresponds to approximately 1 mg/kg or lower in an oil or fat sample. Extracts of vegetable oil and spiked oils were analyzed to determine recovery and accuracy (Figure 3). The oil sample was spiked with 5 mg/kg and 100 mg/kg of each antioxidant, and the detected amounts in the extracts were compared to standard solutions at the same concentration.

Similar resolution and peak widths for the UV and MS results were obtained and linearity was good from 0.1–100 ppm. The MSD was approximately 10 times more sensitive than UV detection for all of the components of the test mixture. Thus, APCI MS was used to confirm the identification of the peaks. It is important to note that two compounds (TBHQ and trolox) in the spiked sample were slightly decomposed during the sequence analysis, which resulted in the low recovery for these compounds.

Although the performance of the LC method was good, not all tocopherols were resolved (co-elution of β-TP and γ-TP). Additionally some other co-elutions were observed (BHA and α-tocopherol (Figure 4). Complete resolution of the eight tocols was obtained by SFC-MS mode, enabling the analysis of the individual tocols in different oils extracts (deep frying oil, sunflower, rapeseed, and tocomix). Tocomix is a commercial mixture of tocols in sunflower oil (AOMS, S.A., Argentina).

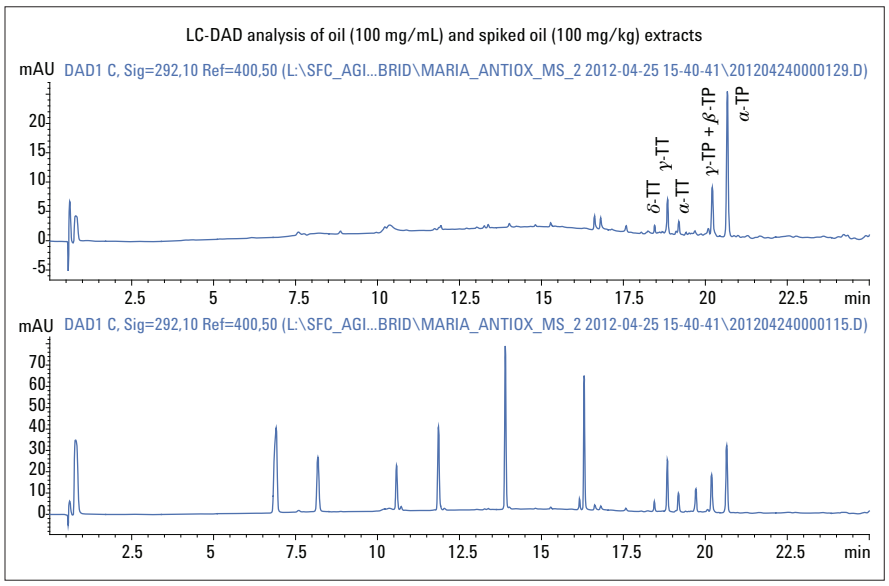

Figure 3
Analysis of oil (100 mg/mL) and spiked deep frying oil (100 mg/kg) extracts with the LC method.

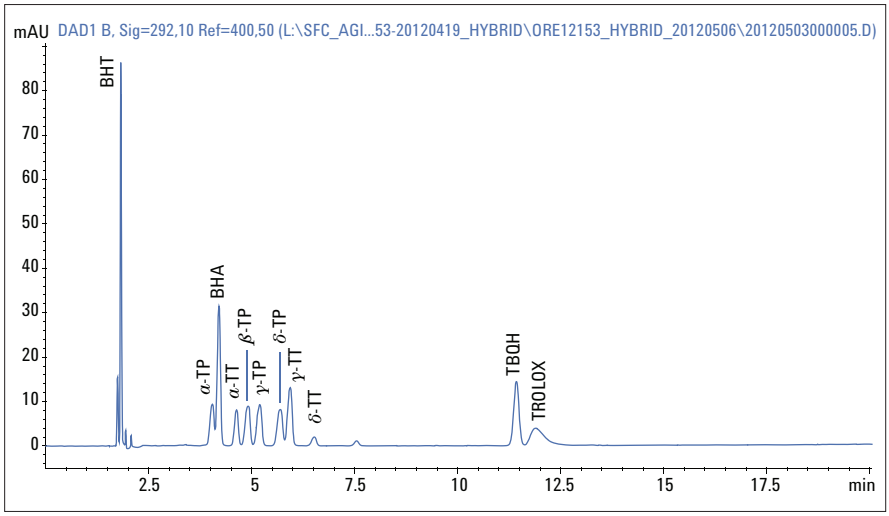

Figure 4
Analyses 14-compounds antioxidant mixture by SFC-DAD (10 µg/mL).

The polarity of the tocopherols and tocotrienols is mainly influenced by the number of methyl groups in the chromanol ring, and to a lesser extent by steric effects of the methyl groups and slightly increased polarity of the unsaturated side chains of tocotrienols compared to those of tocopherols. The most difficult compounds to be separated were the β- and γ- tocols (Figures 5 and 6), because they have three methyl groups in their ring structure. APCI mass to charge ratios (m/z) of [M-H]+ ions were 429, 415, 415, and 401 for α-, β-, γ-, and δ-tocopherols, and 423, 409, 409, and 396 for α-, β-, γ-, and δ-tocotrienols.

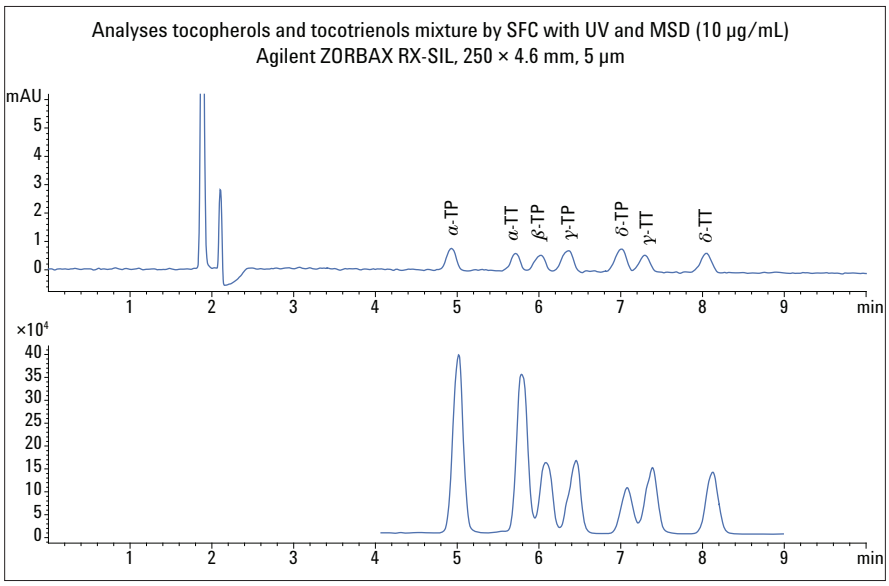

Figure 5
Analyses tocopherols and tocotrienols mixture by SFC with UV and MSD (10 µg/mL) (β-tocotrienol was not available as pure standard).

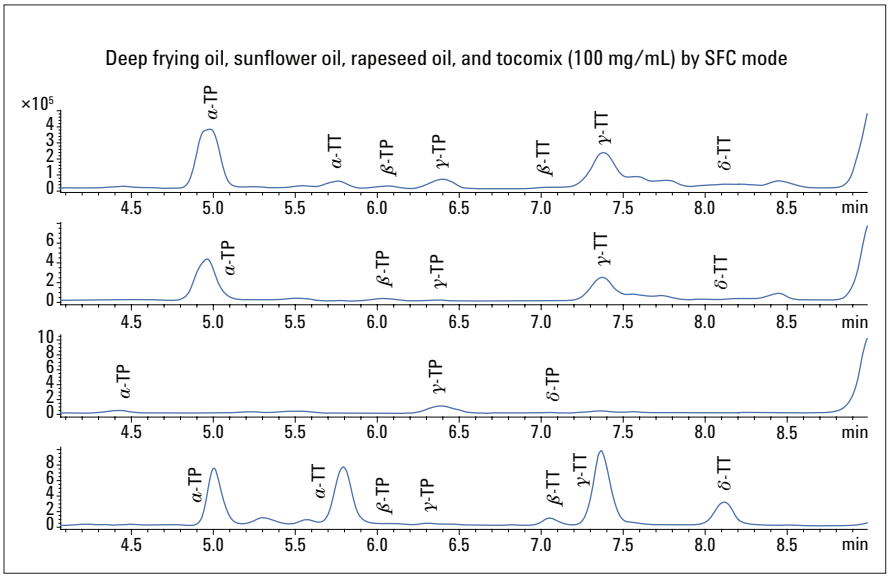

Figure 6
Deep frying oil, sunflower oil, rapeseed oil (100 mg/mL) and tocomix by SFC mode.

Figure 7 shows a Spectrum of α-tocopherol in deep frying oil sample by SFC-APCI analyses. Ion *m/z* 429 was selected for further analyses, which was attributed to [M-H]+ formed by initial protonation of α-tocopherol followed by dehydrogenation[4,5].

Comparable resolution in both UV and MSD were achieved when the separation was performed. Linearity was good with R^2 values of 0.99 from 0.1–50 ppm. Overall, the limits of detection (LODs) of LC/MS mode and SFC/MS mode were in the same order of magnitude. The results show that high separation power and good reproducibility were achieved with both techniques for a complex mixture of analytes. It is important to note that tocopherols and tocotrienols could only be completely resolved in the SFC-MS mode.

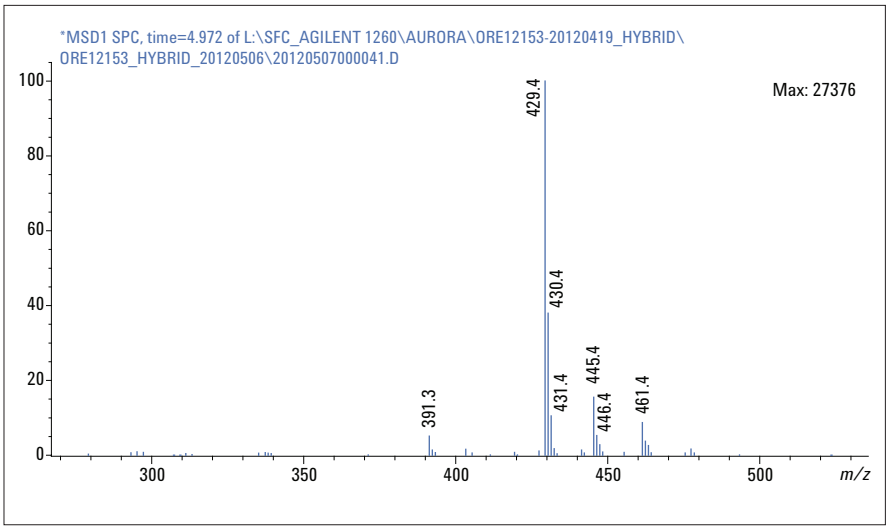

Figure 7
Spectra α-tocopherol in Deep frying oil by SFC mode.

				Deep frying[4]		Sunflower[4]		Rapeseed[4]		Tocomix	
	Linearity (R^2)[1]	Repeat. (% RSD)[2]	Repeat. (% RSD)[3]	Recov. (mg/kg)	RSD (%)	Recov. (mg/kg)	RSD (%)	Recov. (mg/kg)	RSD (%)	Recov. (ppm)	RSD (%)
δ-TT	0.99993	3.3	2.7	24	5.5	9	4.9	-	15.4	4.2	
β-TT*	NA	NA	NA	-	-	-	Detected				
γ-TT	0.99975	4.6	4.4	96	1.7	70	6.0	-	43.3	5.2	
α-TT	0.9994	3.9	2.9	19	4.3	-	-	20	4.6		
δ-TP	0.99942	3.8	4.4	-	-	0.5	5.8	-			
β-TP	0.99764	4.7	4.7	3	5.8	9	5.6	-	0.4	4.4	
γ-TP	0.99805	3.3	4.0	42	6.0	2.1	5.4	40	5.3	0.2	5.3
α-TP	0.99692	2.1	4.5	165	3.2	124	4.0	2.5	2.9	16.4	3.6

[1] 0.1, 0.5, 1, 5, 10, 25, 50 µg/mL standard solution, 1 injection/level (MS)
[2] 6 consecutive injections of 0.5 µg/mL
[3] 6 consecutive injections of 25 µg/mL
[4] 6 consecutive injections
* No quantitative data. No pure reference material available.

Table 5
SFC mode method performance data.

Conclusions

The Agilent 1260 Infinity Hybrid SFC/UHPLC/MS System provides an excellent tool to obtain complementary data from both SFC and UHPLC on a single instrument. Vegetable oil samples from different origins and spiked vegetable oil samples were extracted and the recoveries of the antioxidants were calculated. Good recovery was obtained for all antioxidants.

Phenolic antioxidants were analyzed by UHPLC. Using this mode, not all tocopherols were separated. Complete resolution of vitamers was achieved only when performing SFC-MS mode. Good sensitivity and high robustness led to the conclusion that hybrid SFC/UHPLC/MS is highly capable to separate and detect all antioxidant isomers for quantitative as well as for qualitative analyses.

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Chapter 4

FORENSIC TOXICOLOGY AND DOPING CONTROL APPLICATIONS

Development of a Method for the Chiral Separation of D/L-Amphetamine

A Quantitative Determination by SFC/MS in an Authentic Whole Blood Sample

Application Note

Forensic Toxicology, Criminalistics

Authors

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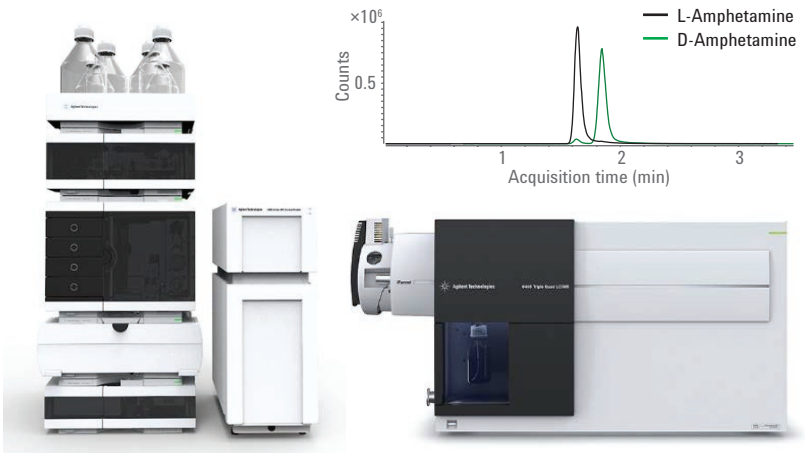
Agilent Technologies Inc.

Abstract

This Application Note demonstrates method development for the chiral separation of D/L-amphetamine using an Agilent 1260 Infinity II SFC System. The Agilent 6495 triple quadrupole MS demonstrates the quantification of these two chiral enantiomeric forms. The method development process is described, and the final analytical method was used for the determination of calibration curves and the limit of quantification using a triple quadrupole MS. The analysis of a processed authentic whole blood sample is shown.



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Introduction

The compound D/L-amphetamine occurs in two chiral enantiomeric forms (Figure 1). The D-amphetamine isomer is the more active, and pharmaceutically produced in enantiomeric pure form[1].

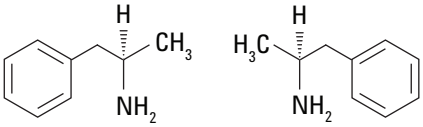

Figure 1. Formula of D-and L-amphetamine.

In forensic toxicology, amphetamine can be qualitatively and quantitatively determined in bodily fluids by chromatographic methods such as GC and HPLC coupled to mass spectrometry[2].

This Application Note demonstrates the development of a fast analytical SFC/MS method for the separation of D- and L-amphetamine and its quantitative determination using a triple quadrupole mass spectrometer. This analytical method can distinguish between the quantitative amount of D-amphetamine from medical use, and the amount of D/L-amphetamine from illegal sources. Finally, this method was verified for use in forensic toxicology by the analysis of an authentic extracted whole blood sample.

Experimental

Instruments

An Agilent 1260 Infinity II SFC/MS System comprising:

- 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 (MCT) (G7116B) with four-column selection valve
- Agilent 1260 Infinity II Isocratic Pump (G7110B) and SFC/MS Splitter kit (G4309-68715)
- Agilent 6495 Triple Quadrupole MSD with Agilent Jet Stream and iFunnel Technology

Instrumental setup

The recommended configuration of the Agilent 1260 Infinity II SFC System with Agilent LC/MS Systems was described earlier[3].

Software

- Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, Rev. C.01.07 SR3
- Agilent MassHunter Triple Quadrupole Acquisition Software, Version B.08.02
- Agilent MassHunter Optimizer Software, Version B.08.02
- Agilent MassHunter Source and iFunnel Optimizer Software, Version B.08.02
- Agilent MassHunter Quantitative Software, Version B.08.00
- Agilent MassHunter Qualitative Software, Version B.07.00 SP1

Columns

- Chiral Technologies, CHIRALPAK AD-H 150 × 4.6 mm, 5 µm
- Chiral Technologies, CHIRALPAK AD-H 250 × 4.6 mm, 5 µm
- Chiral Technologies, CHIRALPAK IA 250 × 4.6 mm, 5 µm
- Chiral Technologies, CHIRALPAK IC 250 × 4.6 mm, 5 µm
- Chiral Technologies, CHIRALPAK ID 250 × 4.6 mm, 5 µm

Chemicals

All solvents were purchased from Merck, Germany.

Samples

Solutions of D-amphetamine, L-amphetamine, and D/L-amphetamine were prepared in methanol according to the related concentrations of the described calibration curve from individual stock solutions (stock solution: 1 ppm in methanol).

A processed, authentic whole blood sample was provided (see Acknowledgments).

Sample preparation

The authentic whole blood sample was processed by protein precipitation with acetonitrile and diluted 1:1,000/1:100/1:10 with mobile phase B (ethanol + 0.1 % aq. NH_3) before analysis.

Final SFC method

Parameter	Value
SFC flow	4 mL/min
Modifier	Ethanol + 0.1 % NH_3(aq)
Isocratic	10 % modifier
Column temperature	20 °C
BPR temperature	60 °C
BPR pressure	200 bar
Total run time	3 minutes
Injection	5 µL
Feed speed	400 µL/min
Overfeed volume	4 µL
Needle wash	3 seconds methanol

MS triple quadrupole method

Parameter	Value
Make up composition	Methanol/water (95/5) + 0.2 % formic acid
Make up flow	0.4 mL/min
Electrospray Ionization with Agilent Jet Stream Ion Source	
Drying gas	170 °C, 16 L/min
Sheath gas	300 °C, 9 L/min
Nebulizer	60 psi
Capillary	2,500 V
Nozzle	500 V
iFunnel	High-pressure RF: 80, low-pressure RF: 60
MS parameters	
ESI polarity	Positive
Scan type	MRM
Transitions	2
Cycle time	502 ms
ΔEMV	+200 V

Compound name	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell (ms)	Fragmentor (V)	Collision energy (V)	Cell accelerator voltage (V)
D/L-Amphetamine	136.1	119.1	250	380	7	1
D/L-Amphetamine	136.1	91.1	250	380	17	1

Results and Discussion

A racemic amphetamine standard solution (100 ppb) was screened against four different chiral stationary phase columns and two organic modifiers (see Experimental, columns 2 to 5). Because amphetamine is a basic compound, a basic additive, 0.1 % aqueous ammonia, was added to methanol and ethanol, which were used as the CO_2 modifier. In the initial method development steps, different isocratic separations were carried out on all the columns. The experiments resulted in an initial separation on column 2 (Figure 2). The separation of both enantiomers became, in tendency, better with decreasing amount of modifier. Baseline separation under chosen conditions was not possible with methanol.

To achieve a better separation of both amphetamines, ethanol as a solvent of weaker elution strength, was tested with column 2 (Figure 3). A clear separation of both enantiomers was obtained for modifier concentrations below 10 %B. The enantiomers eluted between 3.5 and 4.5 minutes at 10 %B, and between 7 and 9 minutes for a modifier concentration of 6 %B.

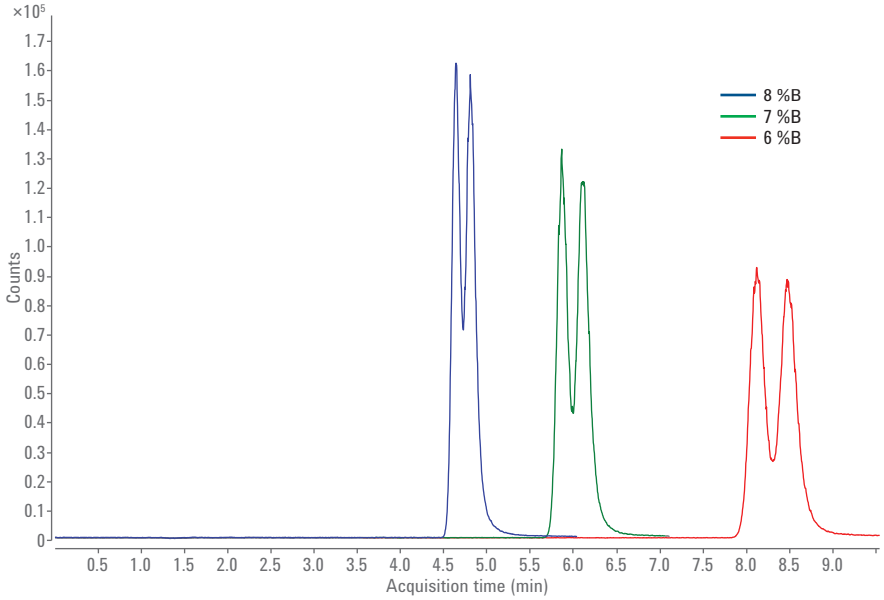

Figure 2. Chiral separation of D/L-amphetamine enantiomers (100 ppb) using different modifier content (modifier B: MeOH+ 0.1 % NH_3(aq), flow rate: 3 mL/min, column temperature: 20 °C, column: CHIRALPAK AD-H 4.6 × 250 mm; 5 µm).

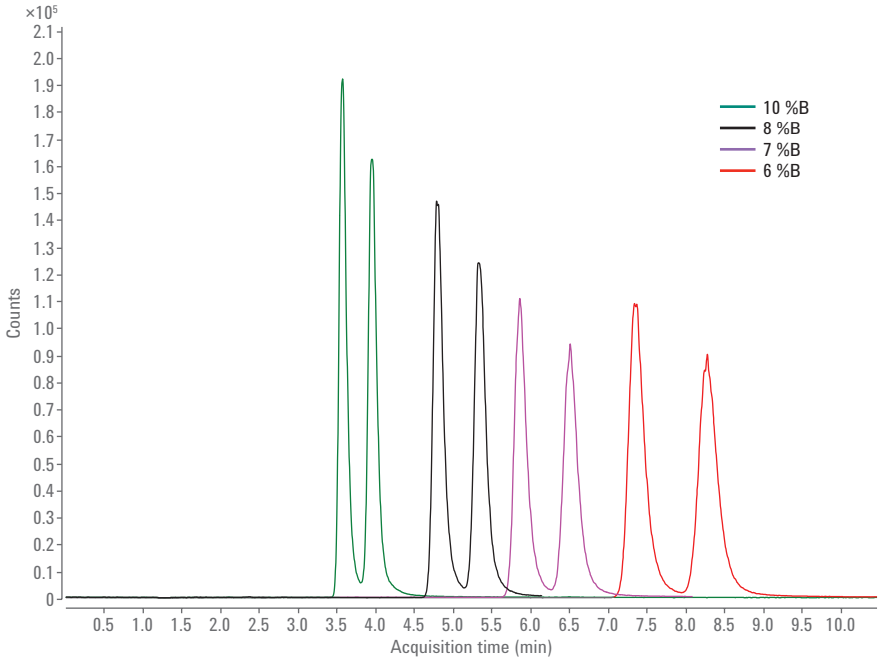

Figure 3. Chiral separation of D/L-amphetamine enantiomers (100 ppb) using different modifier content (modifier B: EtOH+ 0.1 % NH_3(aq), flow rate: 3 mL/min, column temperature: 20 °C, column: CHIRALPAK AD-H 4.6 × 250 mm; 5 µm).

In the final step, the method run time was optimized by increasing the flow rate from 3 to 4 mL/min. The increased flow rate resulted in elution of the enantiomers between 2.5 and 3.2 minutes (Figure 4). To further shorten the run time, a shorter column (column 1) consisting of the same stationary phase, inner diameter, and particle size was used (Figure 5). The change from a 250-mm column to a shorter 150-mm column led to an earlier elution of between 1.5 and 2.1 minutes for both enantiomers. Furthermore, different temperatures (40, 30, and 20 °C) were tested, and a column temperature of 20 °C achieved the highest resolution (data not shown).

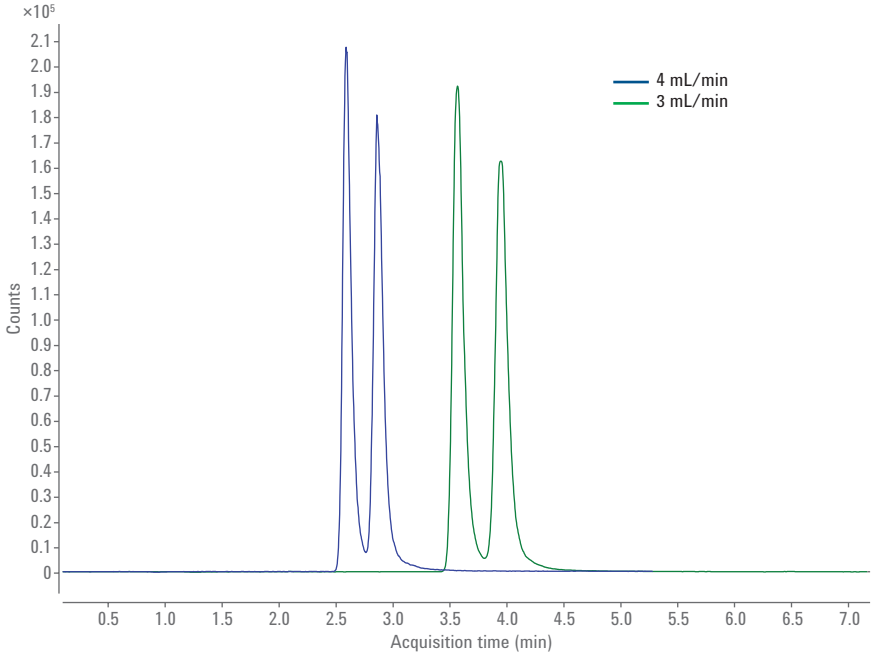

Figure 4. Chiral separation of D/L-amphetamine enantiomers (100 ppb) using different flow rates (modifier B: 10 % EtOH+ 0.1 % NH_3(aq), flow rate: 3 and 4 mL/min, column temperature: 20 °C, column: CHIRALPAK AD-H 4.6 × 250 mm; 5 µm).

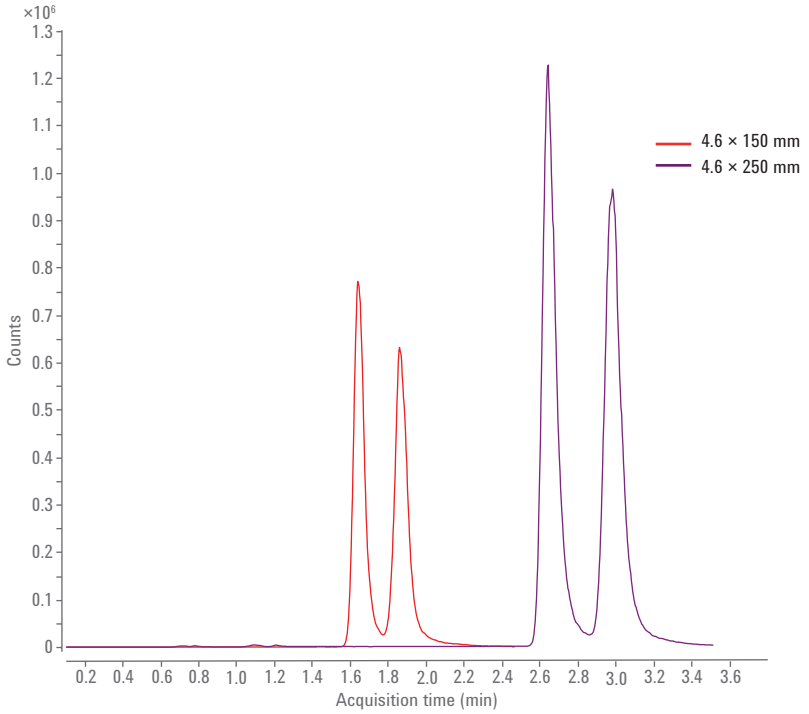

Figure 5. Chiral separation of D/L-amphetamine enantiomers (100 ppb) using different column sizes (modifier B: 10 % EtOH+ 0.1 % NH_3(aq), flow rate: 4 mL/min, column temperature: 20 °C, column: CHIRALPAK AD-H 4.6 × 250 mm and 4.6 × 150 mm, 5 µm).

Finally, the transfer to the source of the mass spectrometer includes the addition of make-up solvent for proper ionization, which was optimized to gain the maximum sensitivity. For that purpose, different backpressure settings were tested, because with the splitter setup the backpressure regulates the amount of column effluent that is transferred to the ionization source (Figure 6). For the final method, a backpressure of 200 bar was applied. The influence of the flow rate of the added make up solvent (methanol/water (95/5) + 0.2 % formic acid) was also examined, but since there was virtually no influence on sensitivity, it was kept at 0.4 mL/min. All source parameters of the MS were fully optimized to obtain the highest sensitivity (see Experimental).

For a final confirmation of the SFC/MS method, commercially available separate D- and L-amphetamine standards in enantiomeric pure form were analyzed. Single peaks resulted for the respective enantiomers: the L-amphetamine eluted at 1.632 minutes and the D-amphetamine at 1.860 minutes (Figure 7).

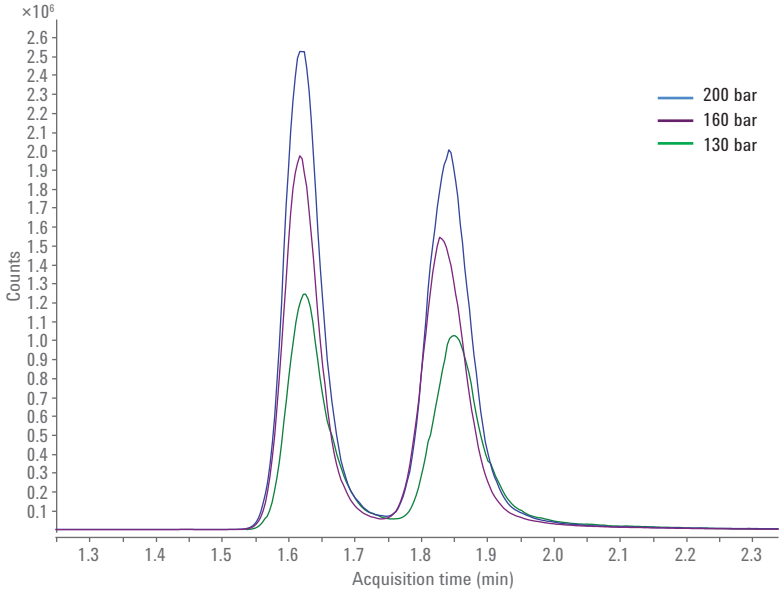

Figure 6. Optimization of SFC backpressure settings to maximize MS sensitivity.

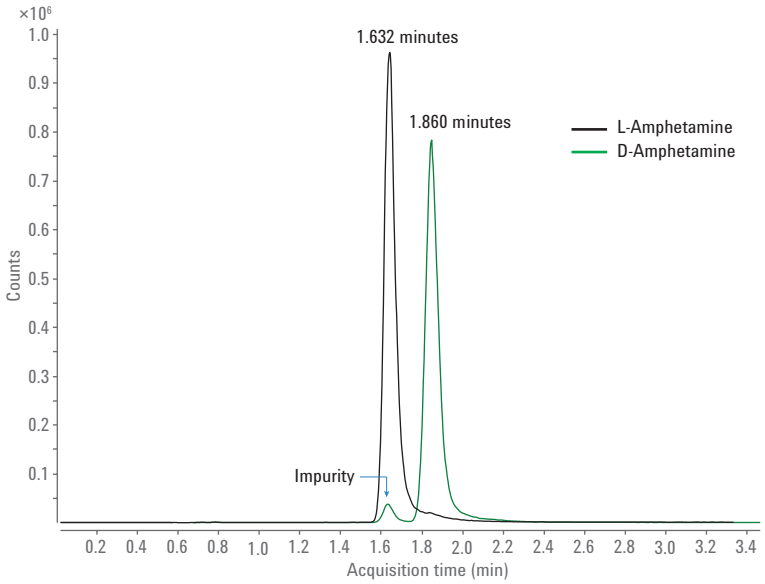

Figure 7. Chiral separation of commercially available enantiomerically pure standards of D-amphetamine and L-amphetamine (100 ppb) with the developed chiral method. The arrow indicates an impurity of the L-enantiomer in the standard of the D-amphetamine.

For the quantitative determination of D- and L-amphetamine, individual calibration curves were created between 100 ppt and 100 ppb, which showed excellent linearity (Figure 8). The limits of quantitation (LOQs) were determined to be at 100 ppt at a signal-to-noise ratio (S/N) of 10 and the limits of detection (LODs) were at 40 ppt (S/N = 3). The linearity coefficients were 0.9998 and 0.9996 for L- and D-amphetamine, respectively.

To demonstrate the capability of the developed SFC/MS method, a real whole blood sample, which was prepared as described in the Experimental section, was measured using the developed method. The blood sample was diluted 1/10, 1/100, and 1/1,000 with modifier B (Figure 9 and Table 1). Table 1 gives a detailed view of the measured areas and peak heights of D- and L-amphetamine in the sample, and demonstrates that it is present *in vivo* in its racemic form, which came originally from a nonpharmaceutical drug. Figure 9 shows the corresponding chromatograms of the 1:1,000 and the 1:10 dilutions.

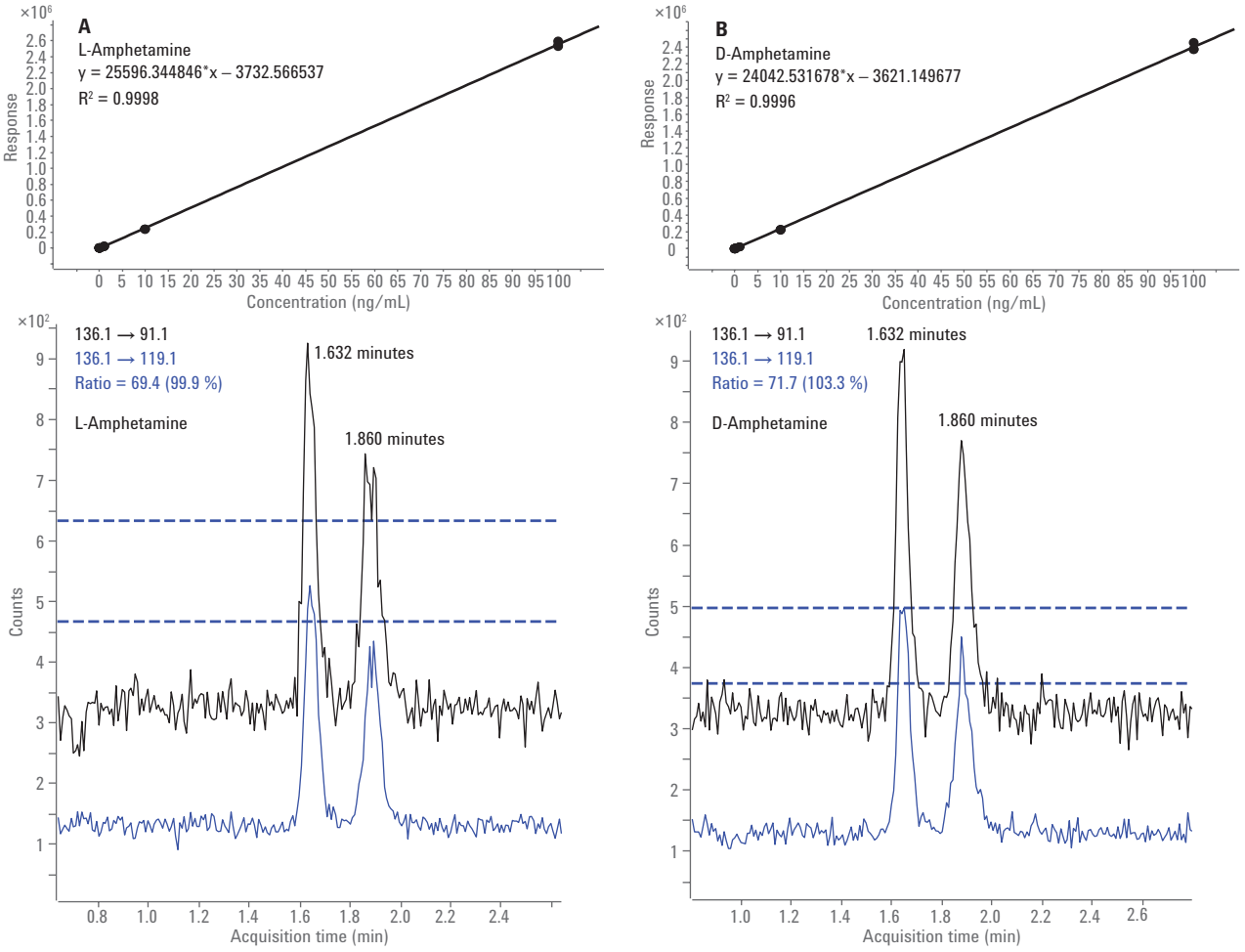

Figure 8. Calibration curves and qualifier/quantifier signal at 100 ppt for L-amphetamine, retention time 1.632 minutes (A) and D-amphetamine, retention time 1.860 minutes (B). The achieved resolution between D-and L-amphetamine was >1, and the total run time was 3 minutes.

Conclusion

This Application Note demonstrates the development of a method for the fast separation of the enantiomers of D/L-amphetamine using the Agilent 1260 Infinity II SFC and a highly sensitive quantitative determination using an Agilent 6495 triple quadrupole mass spectrometer. The SFC separation was possible within a total run time of 3 minutes, showing fast analysis time. The quantitative determination was performed with LOQs below 100 ppt. Finally, a reliable determination of amphetamine in a prepared whole blood sample was shown successfully.

Acknowledgements

Thanks to Martin Josefsson and Markus Roman from the National Board of Forensic Medicine, Linkoping, Sweden for providing the processed authentic whole blood sample.

References

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2. Kde Mariotti, C.; *et al.* Simultaneous analysis of amphetamine-type stimulants in plasma by solid-phase microextraction and gas chromatography-mass spectrometry. *J. Anal. Toxicology* **2014**, *38(7)*, 432–437.

3. Use of the SFC-MS Splitter Kit G4309-68715. *Agilent Technologies Technical Note*, publication number G4309-90130, **2015**.

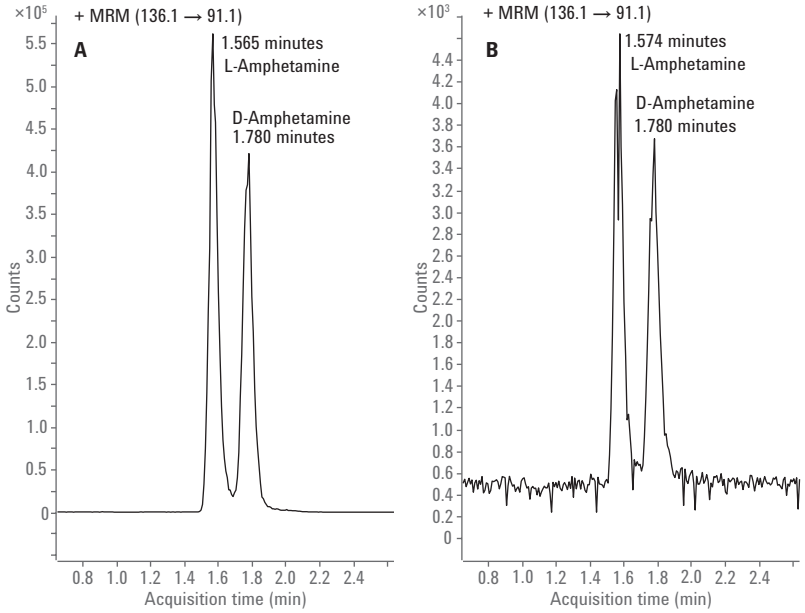

Figure 9. D/L-Amphetamine in a processed real whole blood sample in different dilutions: 1:10 (A) and 1:1,000 (B) with modifier.

Table 1. Detailed results of the measurement of the prepared real whole blood sample for D-and L-amphetamine diluted 1:10, 1:100, and 1:1,000 with the modifier.

	L-Amphetamine results			D-Amphetamine results		
Name	RT	Area	Height	RT	Area	Height
Case 2 Dil 1_1000	1.574	14,605.41	4,129.08	1.780	14,342.73	3,159.12
Case 2 Dil 1_100	1.574	178,409.64	48,475.84	1.780	165,744.26	34,952.28
Case 2 Dil 1_100	1.574	165,569.54	45,037.95	1.780	154,606.59	35,124.32
Case 2 Dil 1_100	1.565	156,671.29	44,736.99	1.780	139,086.04	32,365.24
Case 2 Dil 1_10	1.565	2,073,946.07	561,571.92	1.780	1,780,961.78	417,402.00

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Application Note

Forensic Toxicology - Criminalistics

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Separation of Enantiomers of Amphetamine-Related Drugs and Their Structural Isomers

Using the Agilent 1260 Infinity II SFC and Detection by Coupled Mass Spectrometry

Authors

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Herbert Godel, and
Edgar Naegele
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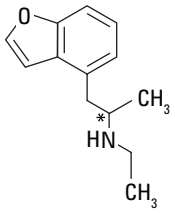
Abstract

This Application Note demonstrates the separation of enantiomers of amphetamine-related drugs and their positional isomers as well as enantiomers of the same compounds. This separation was performed using chiral phase columns with the Agilent 1260 Infinity II SFC System. The qualitative detection and quantitative determination of both the structural isomers and enantiomers was achieved using the Agilent 6495 triple quadrupole MS and the Agilent 6150 single quadrupole MSD.

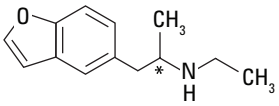
Introduction

The isomeric compounds 4-, 5-, and 6-EAPB are psychedelic drugs structurally related to amphetamine, and belong chemically to the class of benzofuran compounds (Figure 1)[1]. The three compounds are structural isomers comprising one chiral center; therefore, each exists in two enantiomeric forms.

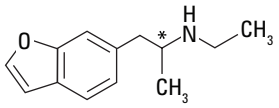
This Application Note demonstrates the separation of these structural isomers and their enantiomers by chiral chromatography. This separation was achieved using the Agilent 1260 Infinity II SFC System coupled to mass spectrometry for qualitative and quantitative determination.



1-(Benzofuran-4-yl)-N-ethylporpan-2-amine
(4-EAPB)



1-(Benzofuran-5-yl)-N-ethylporpan-2-amine
(5-EAPB)



1-(Benzofuran-6-yl)-N-ethylporpan-2-amine
(6-EAPB)

Figure 1. Formulae of 4-, 5-, and 6-EAPB. Each of the isomeric compounds also has a stereo center (asterisk), and thus exists in two enantiomeric forms.

Experimental

Instruments

Agilent 1260 Infinity II SFC/MS 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 (MCT) (G7116A) with
- Agilent InfinityLab Quick Change 4-position/10-port four-column selection valve (p/n 5067-4287)
- Agilent 1260 Infinity II Isocratic Pump (G7110B) and SFC/MS Splitter kit (G4309-68715)
- Agilent 6495 Triple Quadrupole MSD with Agilent Jet Stream and iFunnel Technology
- Agilent 6150 Single Quadrupole MSD with Agilent Jet Stream

SFC Method for the separation of all six isomers (enantiomers and structural isomers) on Column 1

Parameter	Value
SFC Flow	3 mL/min
Modifier	Methanol + 0.1 % NH_3 aq.
Isocratic	10 % modifier
Column temperature	20 °C
BPR Temperature	60 °C
BPR Pressure	200 bar
Total run time	7 minutes
Injection	5 µL
Feed speed	400 µL/min
Overfeed volume	4 µL
Needle wash	3 seconds methanol

SFC Method for the separation of the three structural isomers on Column 2

Parameter	Value
SFC Flow	2.5 mL/min
Modifier	Methanol + 0.1 % NH_3 aq.
Isocratic	11 % modifier
Column temperature	30 °C
BPR Temperature	60 °C
BPR Pressure	200 bar
Total run time	3.5 minutes
Injection	1 µL
Feed speed	400 µL/min
Overfeed volume	4 µL
Needle wash	3 seconds methanol

Instrumental setup

The recommended configuration of the Agilent 1260 Infinity II Analytical SFC System with Agilent LC/MS Systems was described previously[2].

Software

- Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, Rev. C.01.07 SR3
- MassHunter LC/TQ Acquisition Software, Version B.08.02
- MasHunter Optimizer Software, Version B.08.02
- MassHunter Source and iFunnel Optimizer Software, Version B.08.02
- MassHunter Quantitative Software, Version B.08.00
- MassHunter Qualitative Software, Version B.07.00 SP1

Columns

1. Chiral Technologies, CHIRALPAK AD-H, 250 × 4.6 mm, 5 µm
2. Chiral Technologies, CHIRALPAK AD-3, 150 × 4.6 mm, 3 µm

Chemicals

All solvents were purchased from Merck, Germany.

Samples

Separate stock solutions of 4-, 5-, and 6-EAPB (1 ppm in methanol) were used in dilution, as outlined in the text.

MS Method for triple quadrupole MS and single quadrupole MS

Parameter	Value
Make up composition	Methanol/water (95/5) + 0.2 % formic acid
Make up flow	0.4 mL/min
Electrospray Ionization with Agilent Jet Stream Ion Source	
Drying gas	150 °C, 11 L/min
Sheath gas	350 °C, 12 L/min
Nebulizer	45 psi
Capillary	2,500 V
Nozzle	0 V
iFunnel*	High-pressure RF: 90 Low-pressure RF: 70
Triple quadrupole parameters	
ESI Polarity	positive
Scan type	MRM
Transitions	2
Cycle time	502 ms
ΔEMV	+200 V
Single quadrupole parameters	
ESI Polarity	positive
Scan type	SIM (*m/z* 204.1)
Dwell time	590 ms
Fragmentor	70 V
Gain	1.0

* Only for triple quadrupole

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell (ms)	Fragmentor (V)	Collision energy (V)	Cell acc. voltage (V)
4/5/6-EAPB	204.1	159.1	250	380	12	1
4/5/6-EAPB	204.1	131.1	250	380	22	1

Results and Discussion

Separation of enantiomers, and quantitative determination by triple quadrupole MS

A method developed for the chiral separation of D- and L-amphetamine[3] was chosen as the starting point for developing a separation method for the six isomers of 4-, 5-, and 6-EAPB. This method immediately led to a promising separation of all six possible stereoisomers (Figure 2), with all six isomers being partially separated between 2.8 and 6.0 minutes. This separation was then further optimized due to the incomplete separation.

Method parameters for Figure 2

Parameter	Value
Column	Chiralpak AD-H, 4.6 × 250 mm, 5 µm
Column temperature	20 °C
Mobile phase	10 %B (EtOH + 0.1 % NH_3 aq.)
Flow rate	4 mL/min
Injection volume	5 µL
Sample	10 ppb each in MeOH

The ethanol content of modifier B was varied in the next step, which did not result in improved separation. Reducing the content of modifier B led to higher retention times with broader peaks and a lower resolution, especially of the later eluting peaks (data not shown). The effect of column temperature on the separation of the enantiomers was also examined (Figure 3).

Method parameters for Figure 3

Parameter	Value
Column	Chiralpak AD-H, 4.6 × 250 mm, 5 µm
Column temperature	20, 30, 40, and 45 °C
Mobile phase	10 %B (EtOH + 0.1 % NH_3 aq.)
Flow rate	4 mL/min
Injection volume	5 µL
Sample	10 ppb each in MeOH

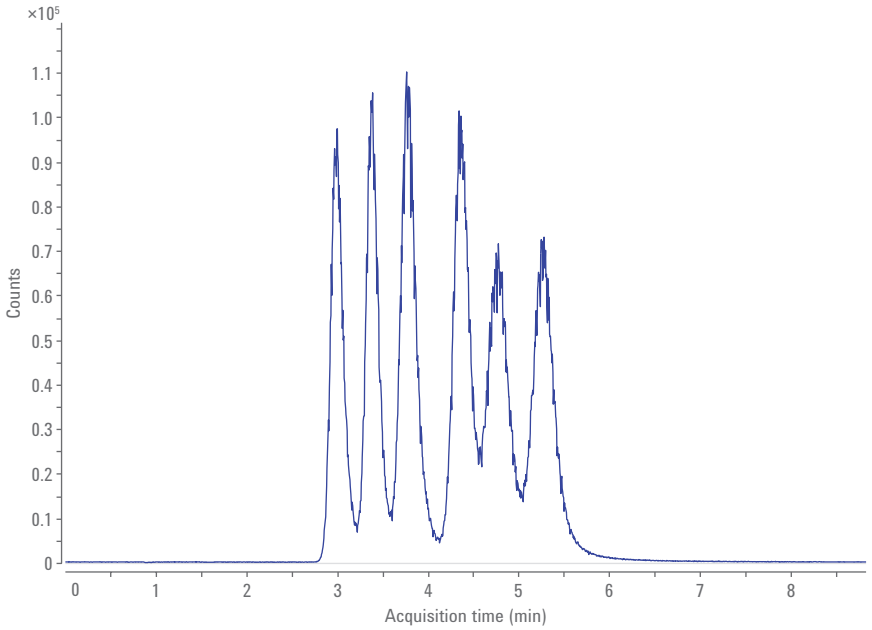


Figure 2. Initial method for the separation of all six isomers of 4-, 5- and 6-EAPB.

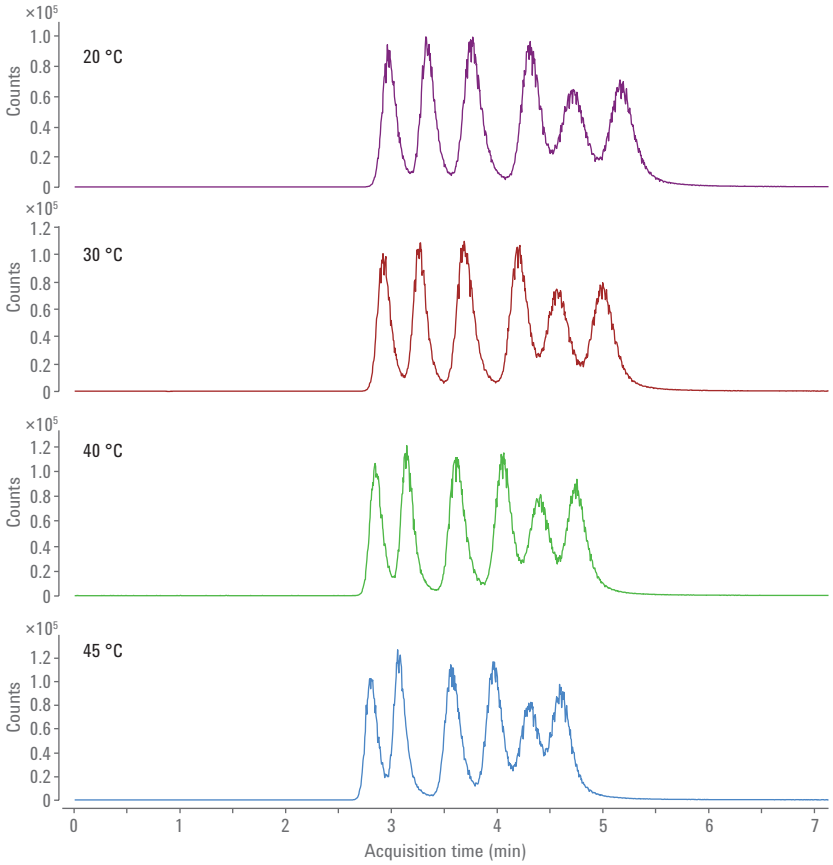


Figure 3. Separation of all six isomers of 4-, 5-, and 6-EAPB depending on the column temperature.

The highest resolutions were achieved at the lower temperatures of 20–30 °C, and the resolution decreased when using higher temperatures. Different solvents were also tested to improve the resolution between the six isomers. When methanol was used as a modifier instead of ethanol, the peaks eluted earlier, between 2.0 and 3.5 minutes, and with better peak shape. However, the peaks were still not completely resolved (Figure 4).

Method parameters for Figure 4

Parameter	Value
Column	Chiralpak AD-H, 4.6 × 250 mm, 5 µm
Column temperature	20 °C
Mobile phase	10 %B (MeOH + 0.1 % NH_3 aq.)
Flow rate	4 mL/min
Injection volume	5 µL
Sample	10 ppb each in MeOH

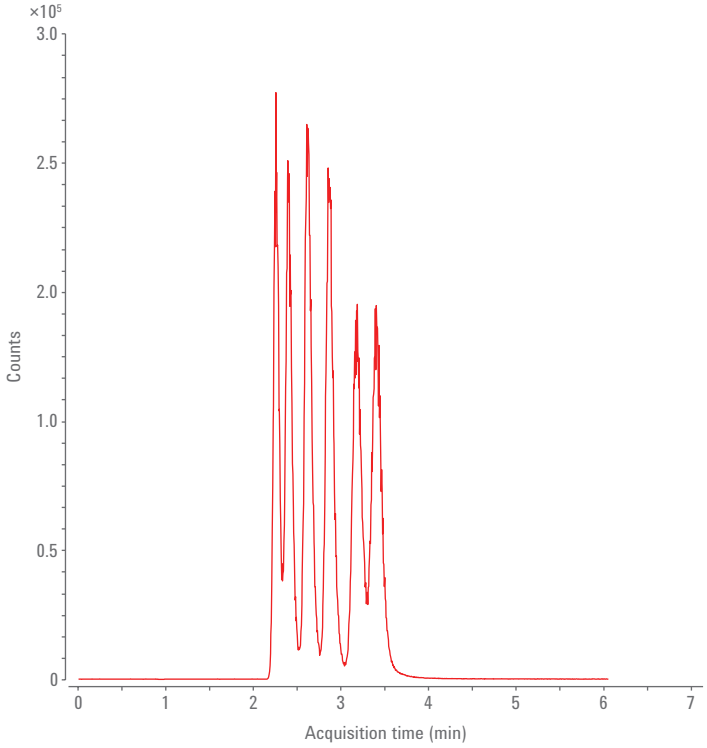


Figure 4. Method for the separation of all six isomers of 4-, 5-, and 6-EAPB with methanol as modifier.

Finally, it was found that 10 % methanol (with 0.1 % NH_3 aq.) as modifier gave the best separation at a flow rate of 3 mL/min (Figure 5).

Method parameters for Figure 5

Parameter	Value
Column	Chiralpak AD-H, 4.6 × 250 mm, 5 µm
Column temperature	20 °C
Mobile phase	10 %B (MeOH + 0.1 % NH_3 aq.)
Flow rate	3 mL/min
Injection volume	5 µL
Sample	10 ppb each in MeOH

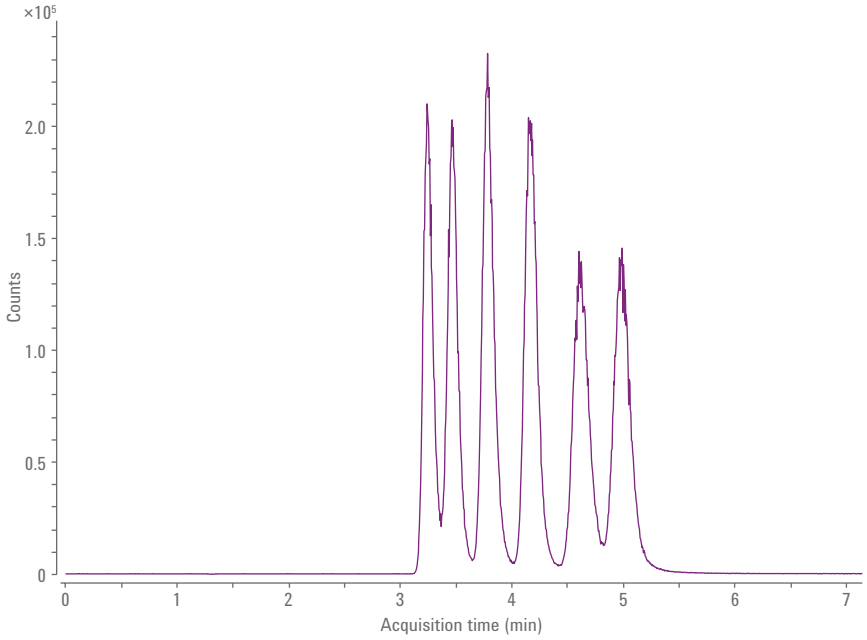


Figure 5. Optimized separation of all six isomers of 4-, 5-, and 6-EAPB.

The compounds eluted between 3.2 and 5.5 minutes. The compounds were identified by comparison to racemic single standards of 4-, 5-, and 6-EAPB (Figure 6). Under the optimized conditions:

- 4-EAPB elutes between 3.1 and 3.7 minutes
- 5-EAPB elutes between 3.6 and 4.6 minutes
- 6-EAPB elutes between 4.4 and 5.4 minutes

In particular, 5- and 6-EAPB were successfully separated by the developed method, which was the aim for the development of this method.

After optimization of the MS source and all related MS parameters, calibration curves were created for 4-, 5-, and 6-EAPB between 100 ppt and 100 ppb (Figure 7), with three replicates for each calibration level. The linearity, R^2, showed values of 0.9998, 0.9993, and 0.9990 for 4-, 5-, and 6-EAPB, respectively. The limit of quantification (LOQ) was determined at 100 ppt for a signal-to-noise ratio (S/N) of 10. The limit of detection (LOD) was determined at 30 ppt for an S/N of 3.

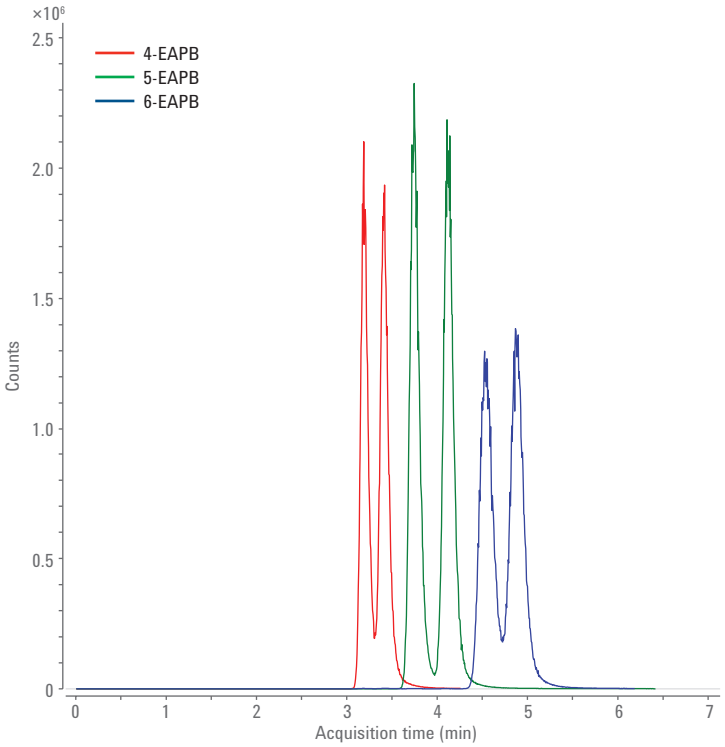


Figure 6. Identification of the enantiomeric compounds 4-, 5-, and 6-EAPB by injection of their racemic single standards.

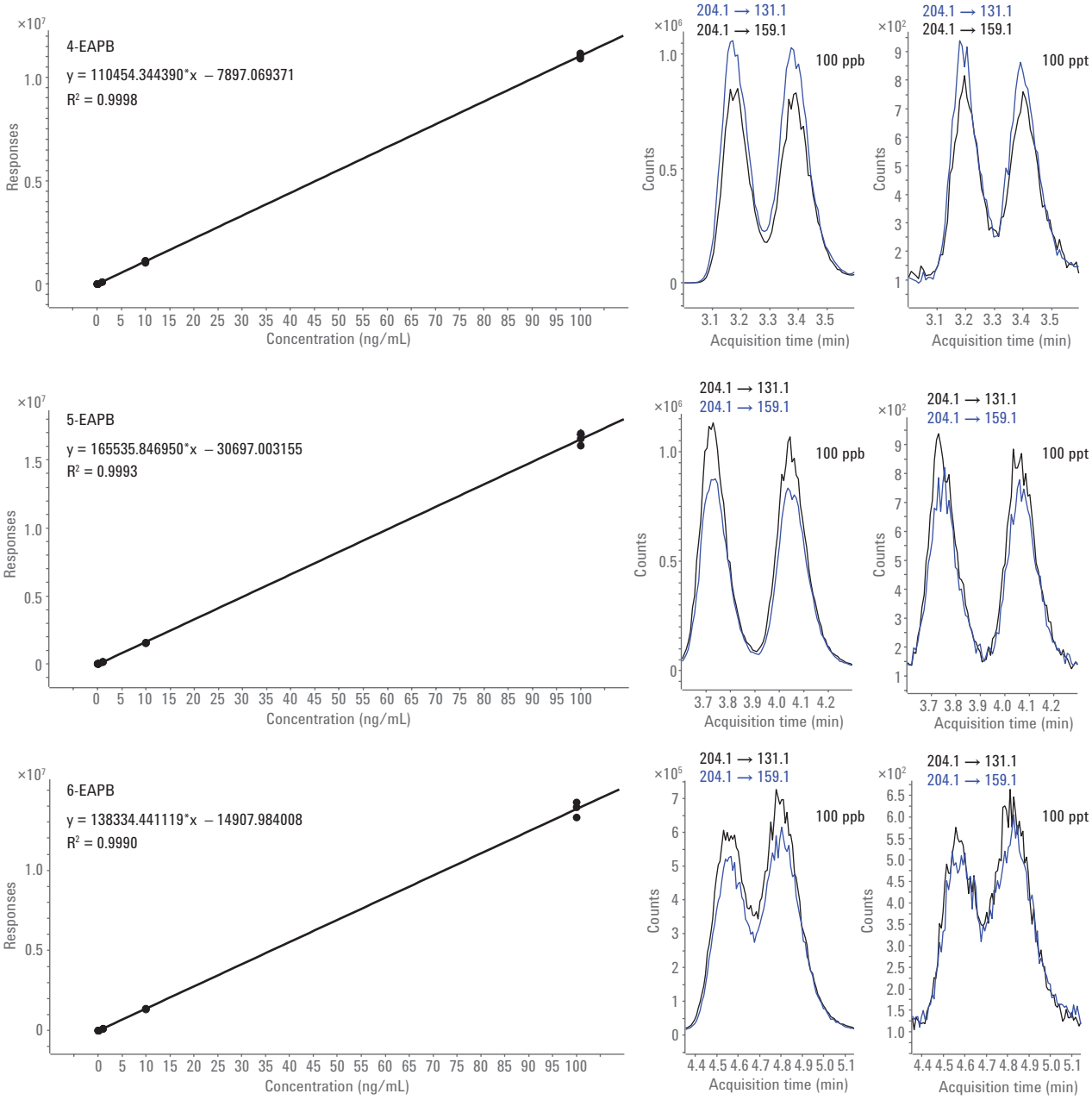


Figure 7. Calibration curves for 4-, 5-, and 6-EAPB between 100 ppt and 100 ppb. The quantifier and qualifier transition at 100 ppb and at 100 ppt (LOQ) are shown next to the individual calibration curves.

Separation of position isomers of 4-, 5-, and 6-EAPB and qualitative determination by single quadrupole MS

Another possibility for the determination of 4-, 5-, and 6-EAPB is the nonchiral separation of the structural isomers. Unfortunately, there was no existing separation method found that worked on an achiral stationary phase and separates all three structural isomers. Typically, the 4-EPAB is separated, but the other two isomers, 5- and 6-EPAB, coelute completely or elute with insufficient separation. To solve this problem, it has been attempted to separate only the isomers without separation of the enantiomers, but on a chiral stationary phase.

The initial experiment, which showed some separation of the three structural isomers without showing a separation of the enantiomers was performed on a CHIRALPAK AD-3 column with methanol as mobile phase (15 % with 0.1 % NH_3 aq.) (Figure 8). To improve the partial separation of the three isomers, the methanol content was decreased in steps of 1 %. A sufficient separation with acceptable peak width and run time for the three compounds was found for a modifier content of 11 % methanol (with 0.1 % NH_3 aq.) (Figure 9).

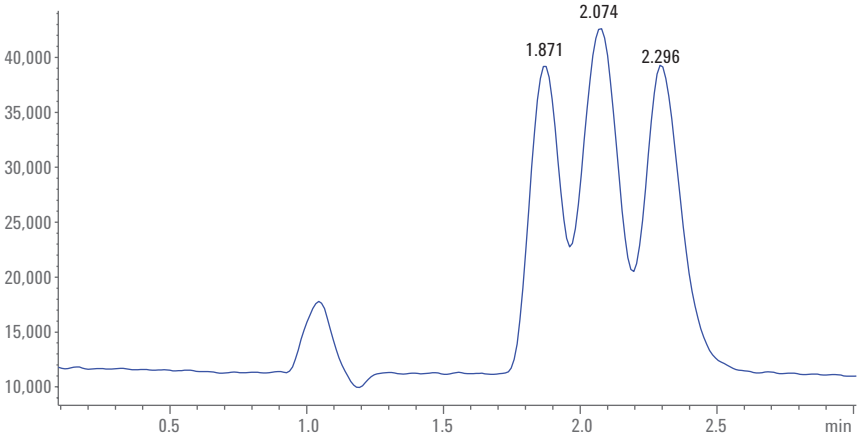


Figure 8. Separation of 4-, 5-, and 6-EAPB structural isomers with 15 % methanol as modifier.

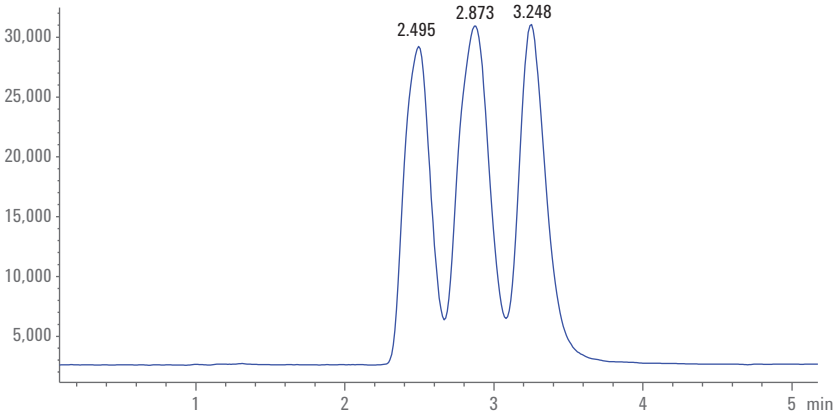


Figure 9. Separation of 4-, 5-, and 6-EAPB structural isomers with 11 % methanol as modifier.

Method parameters for Figure 8

Parameter	Value
Column	Chiralpak AD-3, 4.6 × 150 mm, 3 µm
Column temperature	30 °C
Mobile phase	15 %B (MeOH + 0.1 % NH_3 aq.)
Flow rate	2 mL/min
Injection volume	1 µL
Sample	100 ppb each in MeOH

Method parameters for Figure 9

Parameter	Value
Column	Chiralpak AD-3, 4.6 × 150 mm, 3 µm
Column temperature	30 °C
Mobile phase	11 %B (MeOH + 0.1 % NH_3 aq.)
Flow rate	2 mL/min
Injection volume	1 µL
Sample	100 ppb each in MeOH

The final method was achieved by increasing the flow rate to shorten the run time to 3.5 minutes (Figure 10). The identity of the compounds was confirmed by injection of the single standards of the three achiral isomers (Figure 11). The 4-EAPB elutes at 2.15 minutes, 5-EAPB at 2.50 minutes, and 6-EAPB at 2.83 minutes. This method could be used as quick qualitative detection of the EAPBs by a combination of SFC and a single quadrupole MS in SIM mode. For a proper quantification, the method shown for the combination of the SFC and a triple quadrupole with its additional selectivity can be used.

Method parameters for Figure 10

Parameter	Value
Column	Chiralpak AD-3, 4.6 × 150 mm, 3 µm
Column temperature	30 °C
Mobile Phase	11 %B (MeOH + 0.1 % NH_3 aq.)
Flow rate	2.5 mL/min
Injection volume	1 µL
Sample	100 ppb each in MeOH

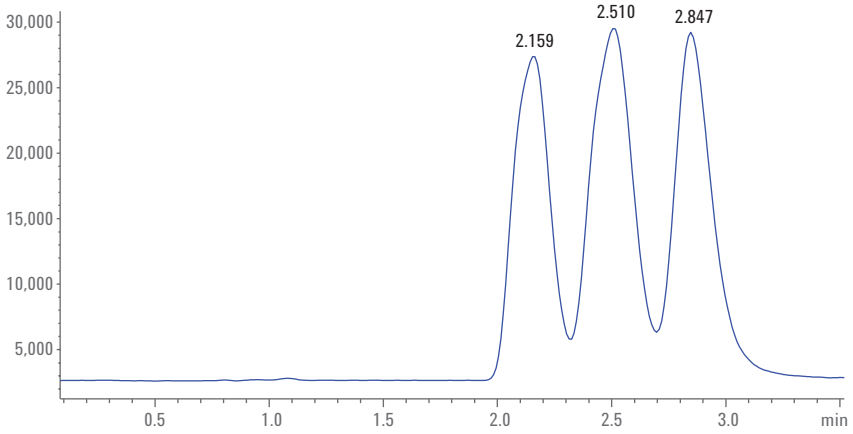


Figure 10. Final method for the separation of 4-, 5-, and 6-EAPB structural isomers.

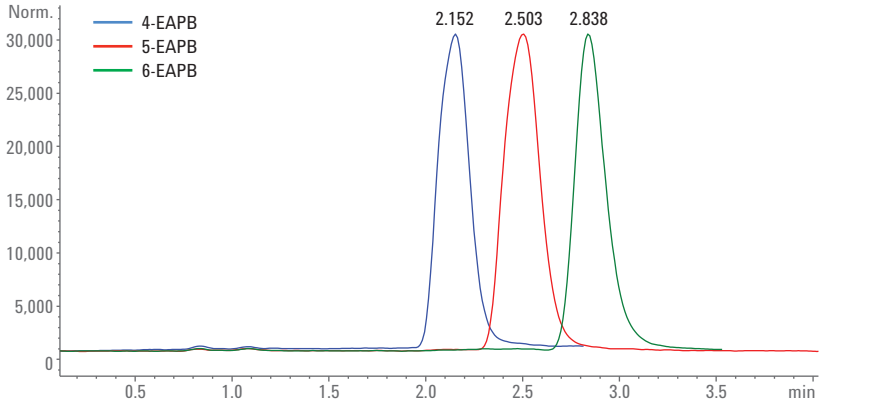


Figure 11. Overlay of the separation of individual samples of 4-, 5,- and 6-EAPB structural isomers with the final method.

Conclusion

This Application Note demonstrates the use of the Agilent 1260 Infinity II SFC for the separation of either all six possible isomers of 4-, 5-, and 6-EAPB, or the separation of only the three respective structural isomers. The detection and quantitative determination have been done by coupling the SFC either to a single quadrupole or a triple quadrupole MS. The calibration curves for quantitative analysis were performed on the triple quadruple MS and showed excellent linearity (R^2 >0.9990) and sensitivity. The LOQs were found at 100 ppt and the LODs at 30 ppt.

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Quantitative Determination of Drugs Using Supercritical Fluid Chromatography with Triple Quadrupole Mass Spectrometry

Application Note

Forensic Toxicology

Author

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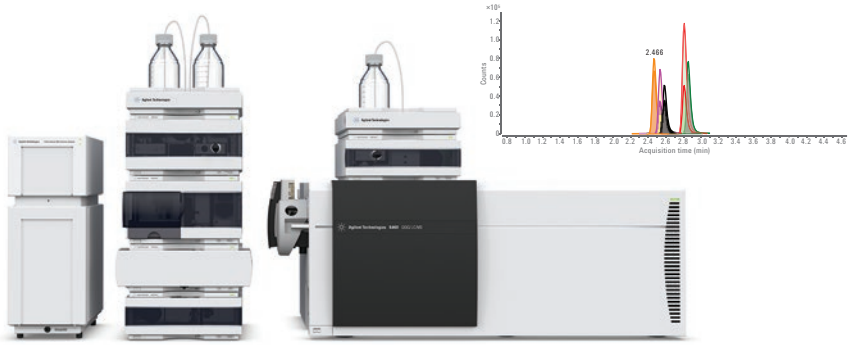
Waldbronn, Germany

Abstract

This Application Note demonstrates the use of the Agilent 1260 Infinity Analytical SFC System in combination with triple quadrupole mass spectrometry for the fast separation of 25 drugs, and quantitative determination down to a limit of detection of 30 pg/mL. For all compounds, calibration curves showed excellent linear correlation. The statistical evaluation of replicate measurements showed highest precision and accuracy for all 25 compounds. Finally, the determination of amphetamines in a urine sample is described.



Agilent Technologies

Introduction

A broad range of compounds of forensic interest are screened and quantified for several application areas in forensic toxicology. These fields range, for example, from doping control, postmortem forensic toxicology, drug testing, and even to the determination of explosive residues.

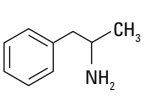
The group of drugs itself is also diverse regarding chemical properties, which are important for separation and detection. Chemical structures range from simple aromatic amines and polycyclic aromatic benzodiazepines to complex morphine-like structures, and even hydrophobic compounds such as tetrahydro cannabinol (THC). So far, the challenging separation for quantitative screening of all compound classes at-a-glance was done by reversed-phase HPLC/MS[1].

This Application Note demonstrates the separation of different classes of drugs in a single quantitative screening run by supercritical fluid chromatography (SFC). Quantitative screening by SFC can be done in a short run time of only a few minutes, and can achieve highest sensitivity when combined with triple quadrupole mass spectrometry. The test suite used for this Application Note comprised 25 compounds of amphetamines, benzodiazepines, morphines, morphine analogs, and THC. After the creation of calibration curves and a statistical evaluation, a spiked biological sample was analyzed with focus on the class of amphetamines. In this study, the following compounds of interest were screened:

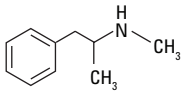
- **Amphetamine** – A central nervous system stimulant that is abused[2], for instance, by college students as a test-taking aid, due to its performance enhancing effects[3,4,5]. However, amphetamine in larger doses can have serious side effects, and may impair cognitive function and induce rapid muscle breakdown[6].
- **Methamphetamine** – Also a central nervous stimulant. The abuse, especially by smoking of the clear crystals (crystal meth) is associated with strong side effects such as psychosis, paranoia, hallucination, rhabdomyolysis, and cerebral hemorrhage[7].
- **Substituted amphetamines** – 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-methamphetamine (MDMA) and 3,4-methylenedioxy-N-ethyl-amphetamine (MDEA) belong to the class of substituted amphetamines. They are psychoactive drugs. Pharmacologically, they act as a serotonin-, norepinephrine-, and dopamine-releasing drug.

Figure 1 shows the chemical formulas. Related chemical and toxicological information are publicly available[8].

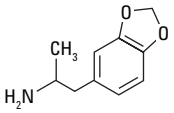
Experimental



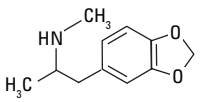
Amphetamine



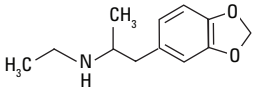
Methamphetamine



3,4-methylenedioxy-amphetamine (MDA)



3,4-methylenedioxy-methamphetamine (MDMA)



3,4-methylenedioxy-N-ethyl-amphetamine (MDEA)

Figure 1. Chemical structures of amphetamines used in this study.

Instrumentation

Agilent 1260 Infinity Analytical SFC System (G4309A):

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity High-Performance Degasser
- Agilent 1260 Infinity SFC Standard Autosampler
- Agilent 1260 Infinity Thermostatted Column Compartment
- Agilent 1260 Infinity Diode Array Detector with high-pressure SFC flow cell
- Agilent 6460 Triple Quadrupole LC/MS system (G6460C) with Agilent Jet Stream
- Agilent 1260 Infinity Isocratic Pump (G1310B)
- Agilent splitter kit (G4309-68715)

Instrumental setup

The recommended configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS system has been described in a previous study[9].

Column

Agilent ZORBAX SB-C8, 4.6 × 100 mm, 1.8 µm (p/n 828975-906)

Software

- Agilent MassHunter Data Acquisition Software for triple quadrupole mass spectrometer, version 07.01.
- Agilent MassHunter Qualitative Software, version 07.00
- Agilent MassHunter Quantitative Software, version 07.00
- Agilent MassHunter MRM and Source Optimizer Software, version 07.00

Connection of the SFC to the MS by splitting and make-up flow:

- Make up composition: Methanol/Water (95/5) + 0.2 % formic acid
- Make-up flow: 0.3 mL/min

Standards

The Agilent LC/MS Forensic Toxicology Test Mixture was used as a standard stock solution. This mixture comprises 25 compounds at a concentration of 1.00 µg/mL, each in methanol. A 1:10 dilution in methanol was used as stock solution for the generation of the calibration curve (100 ng/mL).

Chemicals

All chemicals were purchased from Sigma-Aldrich, Taufkirchen, Germany. All solvents were LC/MS grade. Methanol was purchased from J.T. Baker, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with an LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak).

Sample preparation

A urine sample was spiked with the complete suite of compounds inherent to the Agilent LC/MS Toxicology Test Mixture (100 ng/mL), diluted 1:5 with methanol, vortexed, then centrifuged at 14,000 g for 5 minutes. The supernatant was filtered; the filtrate was used directly for injection.

SFC method

Parameter	Description
SFC flow	2 mL/min
SFC gradient	0 minutes – 2 %B, 5 minutes – 25 %B
Stop time	5 minutes
Post time	2 minutes
Modifier	Methanol + 0.2 % formic acid (FA) + 10 mM ammonium formate
BPR temperature	60 °C
BPR pressure	200 bar
Column temperature	60 °C
Injection volume	1 µL, 3 times loop overfill

MS method

Parameter	Description
Ionization mode	Positive
Capillary voltage	3,000 V
Nozzle voltage	500 V
Gas flow	8 L/min
Gas temperature	220 °C
Sheath gas flow	12 L/min
Sheath gas temperature	380 °C
Nebulizer pressure	25 psi
MRM conditions	See Table 1, showing precursor ions, fragment ions, fragmentor voltage, and collision energy details. The system was used in dynamic MRM mode to ensure best sensitivity.

Results and Discussion

The chromatographic method for the separation of the 25 drugs was developed using the 100 ng/mL dilution. This solution was also used to optimize the conditions for make-up flow, Agilent Jet Stream, and MS conditions by means of the MRM optimizer software and the source optimizer software.

The final SFC method separated the 25 compounds in a run time of 5 minutes in a gradient from 2 to 25 % methanol comprising formic acid and ammonium formate (Figure 2). The first compound that eluted from the column was THC at 0.99 minutes, and the last eluting compound was strychnine at 4.05 minutes. The compound that showed the highest intensity was methadone, eluting at 2.95 minutes

The 100 ng/mL solution was used to create individual calibration curves for the inherent compounds by a dilution pattern of 1:5:2 with methanol. The dilution series was measured down to a concentration of 0.01 ng/mL for all compounds to identify the individual limit of quantification (LOQ) and limit of detection (LOD). The compounds were detected with highest sensitivity showing LOQs below 100 pg/mL, and LODs below 30 pg/mL, all at good linearity correlations (Table 2). For a statistical evaluation, the 10 ng/mL calibration solution was injected 15 times. The calculated relative standard deviation (RSD) of the retention times was typically below 0.3 %, and the area RSDs were in a good range, below 4 %. The calculated concentration precision was below 3.5 %, and the corresponding concentration accuracies were between 95 and 105 %.

Table 1. MRM conditions: Precursor ions, fragment ions, fragmentor voltage, and collision energy (sorted by retention time, see Table 2). The final DMRM method was created from the MRM method.

Compound	Precursor ion	Fragmentor (V)	Quantifier ion	CE	Qualifier ion	CE
THC	315.2	150	193.2	20	123.3	30
Temazepam	301.1	117	255.1	29	177	45
Clonazepam	316.1	110	270	24	214	40
Diazepam	285.1	169	193	45	154	25
Lorazepam	321	102	275	21	194	49
Nitrazepam	282.1	148	236.1	25	180	41
Proadifen	354.2	153	167	29	91.1	45
Oxazepam	287	150	269	12	241	20
Cocaine	304.2	138	182.1	17	77	61
Verapamil	455.3	158	165	37	150	45
Trazodone	372.2	159	176	25	148	37
Oxycodone	316.2	143	298.1	17	256.1	25
Meperidine	248.2	128	220.1	21	174.1	17
MDEA	208.1	107	163	9	105	25
Heroin	370.2	149	268.1	37	165	61
PCP	244.1	86	91	41	86.1	9
Amphetamine	136.1	66	119.1	5	91	17
MDA	180.1	61	163	5	105	21
Methamphetamine	150.1	92	119	5	91	17
MDMA	194.1	97	163	9	105	25
Methadone	310.2	112	265.1	9	105	29
Alprolazame	309.1	179	281	25	205	49
Codeine	300.2	158	165.1	45	58.1	29
Hydrocodone	300.2	159	199	29	128	65
Strychnine	335.2	195	184	41	156	53

Abbreviations: tertrahydro cannabinol (THC), 3,4-methylenedioxy-N-ethyl-amphetamine (MDEA), phencyclidine (PCP), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-ethyl-amphetamine (MDMA).

Table 2. Retention times of the 25 drugs, retention time and area RSDs, concentration precision, and accuracy of the 10 ng/mL concentration level. LOD and LOQ, linearity from individual calibration curves from 100 ng/mL down to the individual LOQ.

Compound	RT	RT RSD (%)	Area RSD (%)	LOD (pg/mL)	LOQ (pg/mL)	Linearity correlation R^2	Concentration precision (%)	Concentration accuracy (%)
THC	0.997	0.44	4.34	60	200	0.9994	3.78	101.7
Temazepam	1.498	0.44	2.59	40	130	0.9951	2.42	105.5
Clonazepam	1.642	0.39	2.66	100	300	0.9982	4.25	102.4
Diazepam	1.668	0.41	3.81	30	100	0.9997	3.79	101.2
Lorazepam	1.742	0.32	4.78	300	1000	0.9975	5.15	106.9
Nitrazepam	1.768	0.37	1.64	20	65	0.9993	3.91	110.9
Proadifen	1.771	0.27	2.43	15	40	0.9996	1.61	106.9
Oxazepam	1.862	0.23	2.04	150	500	0.9952	2.15	105.8
Cocaine	1.994	0.39	1.42	10	40	0.9998	1.27	98.5
Verapamil	2.147	0.29	3.09	<5	10	0.9998	1.99	105.6
Trazodone	2.370	0.25	4.04	<5	10	0.9993	3.61	112.1
Oxycodone	2.478	0.29	3.65	40	130	0.9951	5.34	105.8
Meperidine	2.494	0.26	4.53	6	20	0.9951	2.42	105.5
MDEA	2.506	0.18	3.48	<5	10	0.9956	3.31	104.1
Heroin	2.518	0.27	3.53	40	150	0.9983	3.18	106.3
PCP	2.550	0.22	2.73	15	55	0.9991	2.34	110.1
Amphetamine	2.592	0.17	3.34	20	70	0.9943	2.29	93.1
MDA	2.631	0.16	4.34	60	200	0.9995	2.86	95.2
Methamphetamine	2.839	0.15	4.67	<5	10	0.9983	4.24	105.5
MDMA	2.900	0.16	3.13	10	30	0.9991	2.69	105.6
Methadone	2.947	0.15	2.86	10	30	0.9998	2.43	102.4
Alprolazame	3.228	0.13	2.13	10	30	0.9995	2.89	105.8
Codeine	3.290	0.19	4.39	20	50	0.9931	3.83	111.8
Hydrocodone	3.631	0.21	2.91	25	80	0.9931	2.73	112.3
Strychnine	4.055	0.13	1.15	50	150	0.9992	1.28	100.3

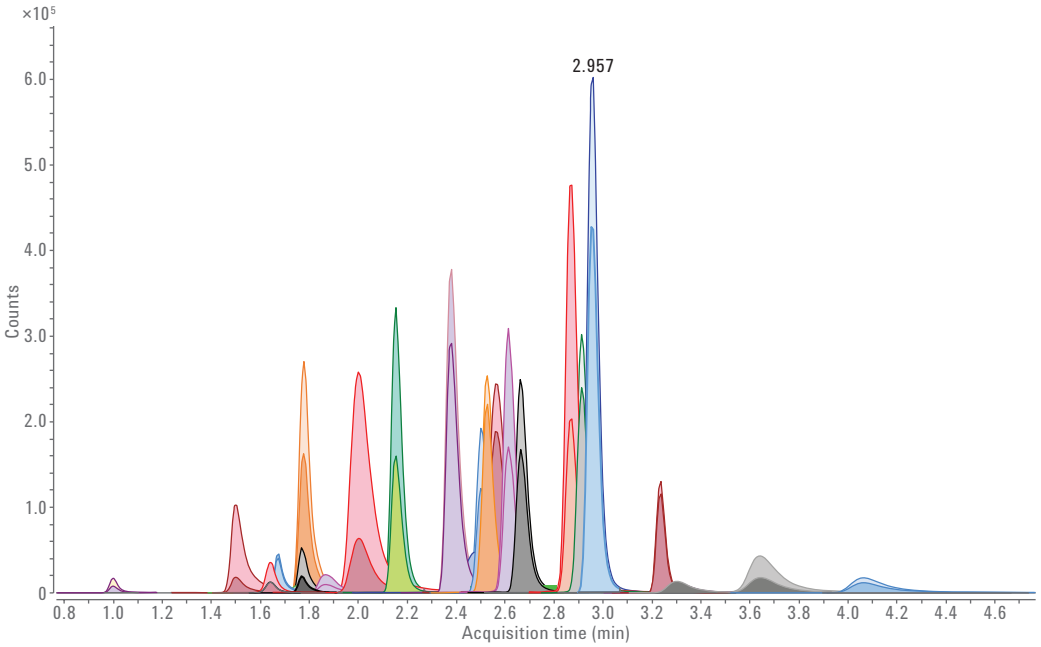


Figure 2. Separation of the mixture comprising 25 drugs by SFC separation in a run time of 5 minutes and detection by DMRM.

As an example, the compounds belonging to the class of amphetamines were examined more closely in a spiked urine sample. The sample was spiked at a level of 100 ng/mL, diluted 1:5 with methanol, yielding a final concentration of 20 ng/mL and used for injection as described in the experimental section. The five amphetamine compounds eluted between 2.4 and 3.1 minutes in the short gradient, ranging within 5 minutes from 2 to 25 % methanol (Figure 3). For a more precise evaluation, the sample was injected 10 times. The RSDs for retention time and concentration, calculated from the replicate injections, were below 0.4 % and below 3 %, respectively. The concentration accuracy was in the range of 82 % to 101 %, which is excellent for quantification (Table 3).

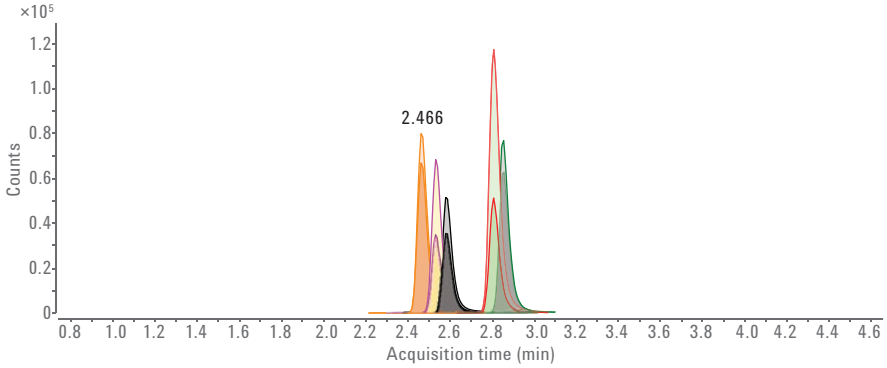


Figure 3. Sample of 20 ng/mL amphetamines (amphetamine, methamphetamine, MDA, MDMA, and MDEA) in spiked urine (100 ng/mL), diluted 1:5 with methanol.

Table 3. Results for the quantitative measurement of amphetamine compounds by SFC/triple quadrupole in a spiked and diluted urine sample.

Compound	RT (min)	RT RSD (%)	Measured concentration (ng/mL)	Concentration precision RSD (%)	Concentration accuracy (%)
MDEA	2.466	0.42	19.61	2.75	97.98
Amphetamine	2.554	0.42	16.41	3.03	82.05
MDA	2.595	0.37	20.19	1.37	100.95
Methamphetamine	2.813	0.21	17.27	1.75	86.35
MDMA	2.860	0.17	17.71	2.16	88.55

As an example, the calibration curve obtained for MDA, from 0.2 ng/mL up to 100 ng/mL, showed an excellent linearity coefficient of 0.9995. The quantifier and qualifier ions obtained from the measured sample at a concentration level of 20 ng/mL showed good peak shape, and their ratio was in the expected range (Figure 4).

Conclusion

This Application Note demonstrates the use of the Agilent 1260 Infinity Analytical SFC System for the fast separation of a large number of drugs. The combination of the SFC system with the Agilent 6460 triple quadrupole MS enabled rapid screening and quantification. All compounds were eluted and separated in a short 5-minute gradient with high retention time and area precision of 0.3 and 4 %, respectively. All calibration curves showed excellent linearity, and the LODs were below 30 pg/mL, which gives evidence of the high sensitivity achievable. The concentration precision was below 3.5 %, and the accuracy between 95 and 105 %. The analysis of a forensic toxicology sample was demonstrated by the quantification of amphetamines in a spiked urine sample. The concentration of spiked compounds was determined with excellent concentration precision and accuracy.

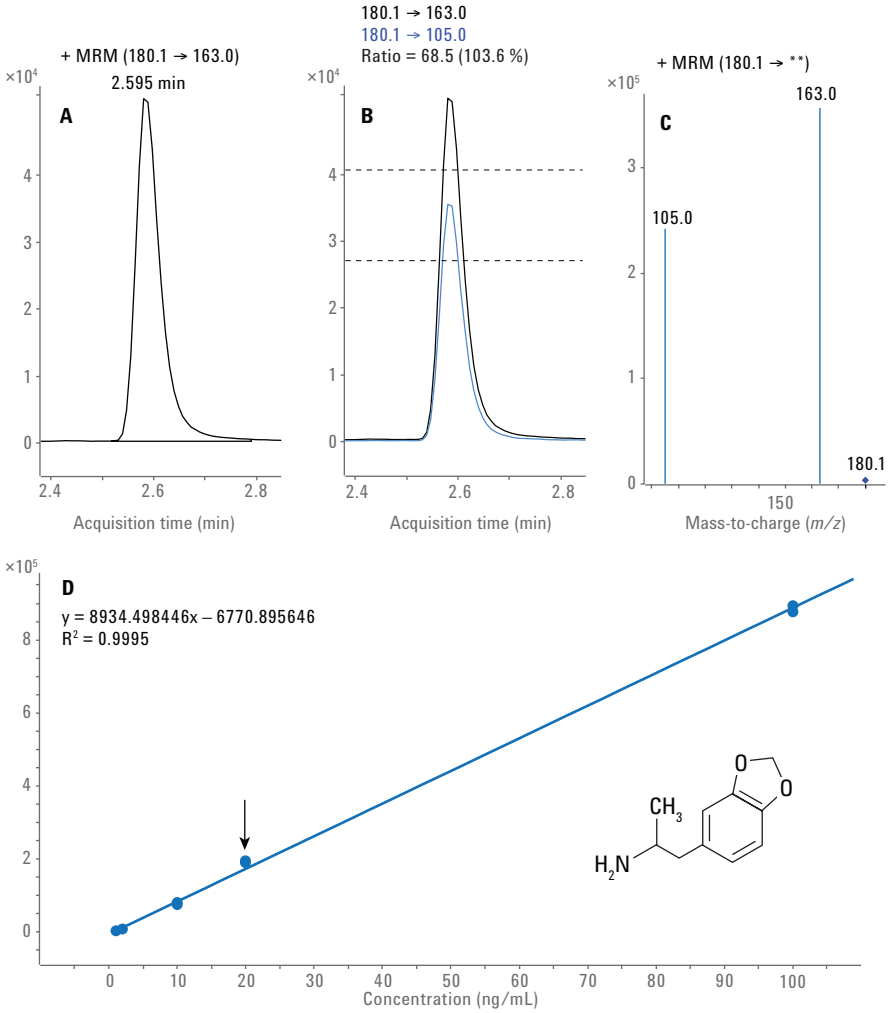


Figure 4. Qualitative measurement of MDA in a spiked urine sample. A) Quantifier ion of MDA at a concentration level of 20 ng/mL. B) Quantifier ion, qualifier ion and their ratio. C) MS/MS spectrum of MDA. D) Calibration curve of MDA between 0.2 ng/mL and 100 ng/mL with linearity correlation 0.9995. The measured concentration is indicated by the arrow.

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For Forensic Use.

Published in the USA, October 15, 2017
5991-6747EN

Agilent Technologies

Determination of Beta-Blockers in Urine Using Supercritical Fluid Chromatography and Mass Spectrometry

Application Note

Doping Control

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Abstract

This Application Note demonstrates the use of the Agilent 1260 Infinity Analytical SFC System for the separation of a library of polar pharmaceutical compounds. It is focused on the larger number of beta-blockers included in the used library. For all beta-blockers, performance data such as linearity, limit of detection (LOD) and limit of quantification (LOQ), and retention time and area RSDs are discussed. Finally, a urine sample spiked with a beta-blocker was measured.

Agilent Technologies

Introduction

A subsection of a larger library of polar compounds, listed as prohibited substances by the World Anti-Doping Agency (WADA)[1], has been analyzed under HILIC conditions[2].

In this study, this compound library was investigated by supercritical fluid chromatography (SFC) to evaluate the separation capabilities for such polar compounds and their detection by triple-quadrupole mass spectrometry. Due to the high sample load in doping control analysis, a fast method for the analysis of known doping compounds is required. Due to its fast separation capabilities, SFC can play a significant role to cope with the number of samples in this application area. For the evaluation, the 13 beta-blockers inherent in the used library were used to determine typical limit of detection (LOD), limit of quantification (LOQ), retention time and area RSDs, as well as precision and accuracy data from the quantification of a sample.

Experimental

Instrumentation

Agilent 1260 Infinity Analytical SFC System (G4309A):

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity High-Performance Degasser
- Agilent 1260 Infinity SFC Standard Autosampler
- Agilent 1260 Infinity Thermostatted Column Compartment
- Agilent 1260 Infinity Diode Array Detector with high-pressure SFC flow cell
- Agilent 6460 Triple Quadrupole LC/MS System (G6460C) with Agilent Jet Stream
- Agilent 1260 Infinity Isocratic Pump (G1310B)
- Agilent Splitter kit G4309-68715

Instrumental setup

Figure 1 shows the recommended configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS System.

Column

Agilent ZORBAX NH2,
4.6 × 150 mm, 5 µm (p/n 883952-708)

Software

- Agilent MassHunter Data Acquisition Software for triple quadrupole mass spectrometer, Version 07.01
- Agilent MassHunter Qualitative Software, Version 07.00
- Agilent MassHunter Quantitative Software, Version 07.00

Connection of the SFC to the MS by splitting and make-up flow

Make up composition

Methanol/Water (95/5), 0.5 mM Ammonium Formate, + 0.2 % formic acid

Make-up flow

0.5 mL/min

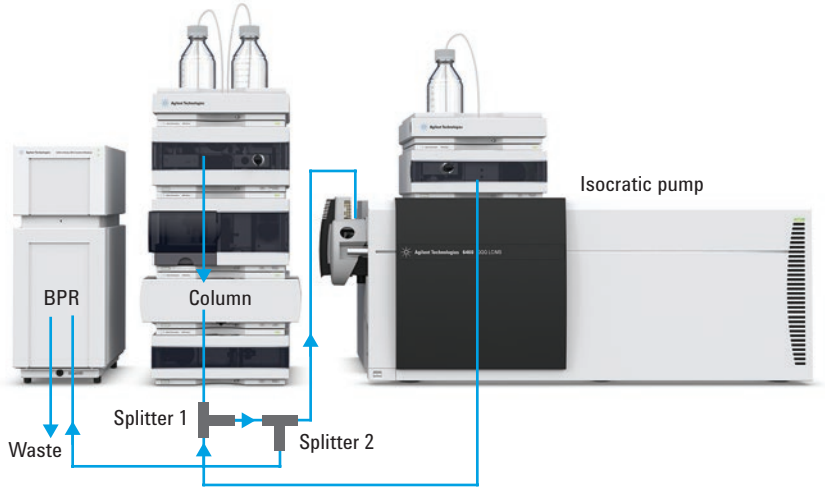


Figure 1. Configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS System. The column is directly connected to splitter 1 in the splitter assembly (BPR = backpressure regulator, UV detector not used, splitter kit p/n G4309-68715).

Standards

The compound library comprised polar pharmaceutical compounds including 13 beta-blockers at a concentration of 1 mg/mL each in acetonitrile. To prepare the stock solution for the described work, a 1:100 dilution in methanol was made.

Chemicals

All chemicals were purchased from Sigma-Aldrich, Taufkirchen, Germany. All solvents were LC/MS grade. Methanol was purchased from J.T. Baker, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22 µm membrane point-of-use cartridge (Millipak).

Sample preparation

A urine sample was spiked with penbutolol (250 ng/mL), diluted 1:5 with methanol, vortexed, and centrifuged at 14,000 g for 5 minutes. The supernatant was filtered, and the filtrate was used directly for injection.

SFC method

Parameter	Value
SFC flow	3 mL/min
SFC gradient	0 minutes, 2 %B; 10 minutes, 50 %B
Stop time	10 minutes
Post time	2 minutes
Modifier	Methanol + 0.1 % formic acid (FA)
BPR temperature	60 °C
BPR pressure	150 bar
Column temperature	40 °C
Injection volume	5 µL, three-times loop overfill

MS method

Parameter	Value
Ionization mode	positive
Capillary voltage	2,500 V
Nozzle voltage	2,000 V
Gas flow	8 L/min
Gas temperature	220 °C
Sheath gas flow	12 L/min
Sheath gas temperature	380 °C
Nebulizer pressure	25 psi
MRM conditions	See Table 1, showing detailed retention time, retention time window, and fragmentor and collision energies

Table 1. MRM conditions for the studied beta-blockers, listing precursor ion mass, fragment ion mass, and fragmentor and collision energies.

	Precursor ion (m/z)	Fragmentor (V)	Quantifier	CE (V)	Qualifier	CE (V)
Penbutolol	292	113	236	12	74	20
Alprenolol	250	11	116	16	56	28
Oxprenolol	266	113	116	12	72	16
Bisoprolol	326	141	116	16	74	28
Esmolol	296	121	145	24	56	32
Propranolol	260	95	116	16	56	28
Celiprolol	380	136	251	20	74	32
Acebutolol	337	145	116	20	56	28
Nebivolol	406	151	151	28	44	44
Pindolol	249	110	116	16	56	28
Sotalol	273	83	255	8	133	28
Atenolol	267	110	145	28	56	28
Nadolol	310	88	254	12	56	36

Results and Discussion

The separation of the 13 beta-blockers inherent in the 44-compound drug mix was performed on an amino column with a gradient of methanol (+0.1 %FA) as modifier, starting at 2 % and going up to 50 % in 10 minutes. With this gradient, all 44 compounds could be separated. In this example, only the 13 beta blockers were monitored, and are discussed in detail (Figure 2).

The beta-blockers eluted between 4.68 and 7.38 minutes with a minimum of coelution. The first compound of the whole drug mix eluted at a retention time of 2.27 minutes, with the last one eluting at 8.19 minutes, respectively.

For all beta-blockers, calibration curves were generated from 1,000 ng/mL down to 1 ng/mL (in modifier) to measure linearity, LOQ (S/N > 10), and LOD (S/N > 3) (Table 2). Typical LODs are below 1.5 ng/mL, and typical LOQs are below 5 ng/mL. Linear calibration was obtained for all compounds, with an R^2 better than 0.9990.

The 100 ng/mL calibration concentration was injected 10 times for a statistical evaluation (Table 2). The measured retention time RSDs are typically below 0.25 %, and the area RSDs are below 5 %.

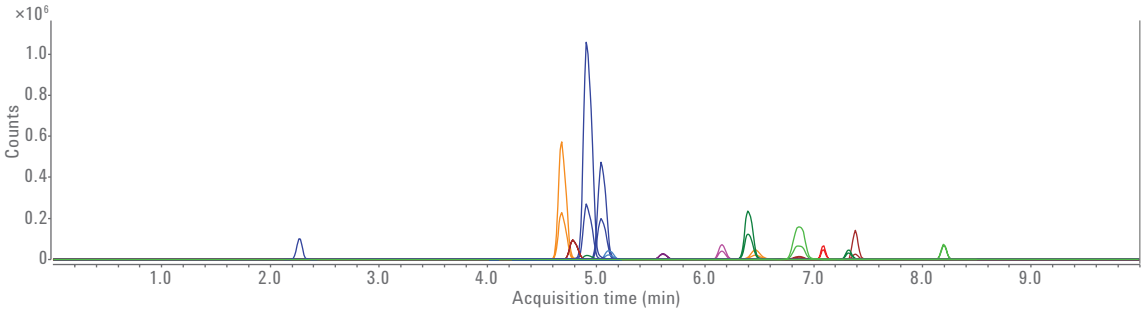
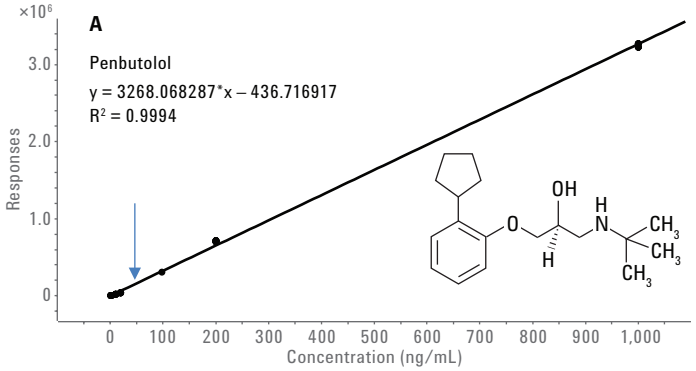


Figure 2. Separation of 13 beta-blockers from a library of 44 compounds. The applied gradient to separate all compounds went from 2 to 50 % methanol in 10 minutes. The earliest eluting compound has a retention time of 2.270 minutes, and the latest eluting compound has a retention time of 8.198 minutes. The beta-blockers eluted between 4.68 and 7.38 minutes.

Table 2. Retention time, RSD's of retention time and areas as well as LODs, LOQs, and linearity of the 13 beta-blockers. The LODs and LOQs are calculated from calibration curves created between 1 and 1,000 ng/mL. The RSDs are calculated from 10 injections of the 100 ng/mL calibration point.

Compound	RT (min)	RT RSD (%)	Area RSD (%)	LOD (ng/mL)	LOQ (ng/mL)	R^2
Penbutolol	4.680	0.18	3.54	0.20	1.60	0.9994
Alprenolol	4.784	0.16	4.42	1.50	5.00	0.9990
Oxprenolol	4.912	0.16	2.76	0.10	0.25	0.9992
Bisoprolol	5.045	0.15	3.00	0.19	0.60	0.9991
Esmolol	5.111	0.15	6.83	1.41	4.71	0.9992
Propranolol	5.611	0.18	8.05	1.71	5.70	0.9997
Celiprolol	6.158	0.26	3.29	0.37	1.25	0.9998
Acebutolol	6.397	0.26	1.68	0.66	2.19	0.9997
Nebivolol	6.463	0.24	5.31	2.85	9.52	0.9996
Pindolol	6.854	0.28	3.67	3.64	10.90	0.9994
Sotalol	7.088	0.24	6.29	5.51	18.38	0.9995
Atenolol	7.322	0.28	3.73	1.98	6.66	0.9995
Nadolol	7.383	0.29	2.53	0.41	1.38	0.9997

As an example of a real-life sample, penbutolol was spiked into urine at a concentration of 250 ng/mL, and prepared as described in the experimental section. Because pure aqueous samples should not be injected into the SFC, the sample was diluted 1:5 in methanol. For the quantitative determination of the penbutolol, a calibration curve from 1,000 ng/mL down to 2 ng/mL was created in solvent (Figure 3A). The measured average concentration of the diluted sample was 45.59 ng/mL, and the determined sample concentration 228.45 ng/mL. The compound eluted at 4.53 minutes with good signal intensity (Figure 3B) and the quantifier/quantifier ratio was in the expected range (Figure 3C). For a statistical evaluation, the sample was injected 10 times, and the retention time and area RSD were calculated to be 0.14 and 2.29 %, respectively. The concentration precision and the accuracy was calculated to be 2.28 and 91.38 %, respectively.



Penbutolol	
RT RSD (%)	0.14
Area RSD (%)	2.29
Average concentration (ng/mL)	45.69
Concentration precision (%)	2.28
Concentration accuracy (%)	91.38

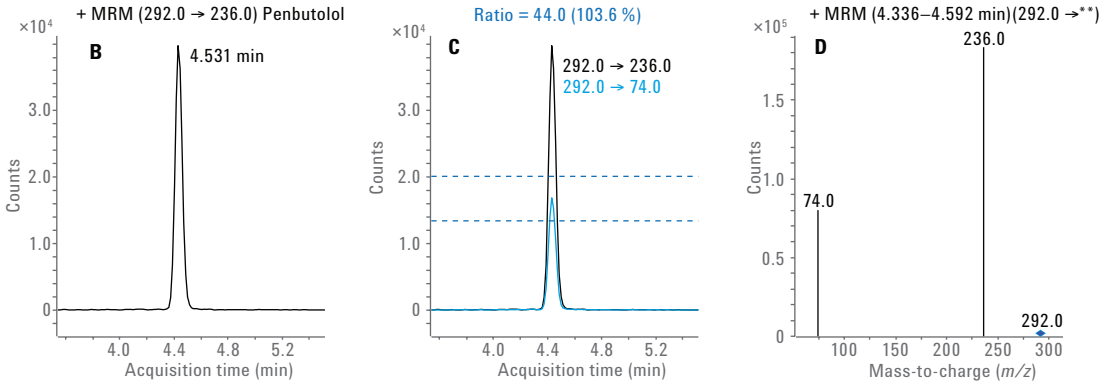


Figure 3. Determination of penbutolol in urine. A) Calibration curve of penbutolol between 2 and 1,000 ng/mL. The concentration measured in the diluted sample is indicated by the arrow. B) Quantifier transition of penbutolol from the diluted sample. C) Quantifier/qualifier ratio of the measured sample. D) MRM transitions measured for penbutolol in the sample. The table inset shows retention time RSD and area RSD as well as average concentration, concentration precision and concentration accuracy calculated from 10 injections of the sample.

Conclusion

This Application Note demonstrates the possibility to separate a large number of highly polar pharmaceutical compounds within a short run time by SFC. Their detection by connecting the SFC to a triple-quadruple mass spectrometer with limits of detection typically below 1.5 ng/mL has been shown. The retention time RSDs were below 0.25 %, and area RSDs were below 5 %.

It was demonstrated that pharmaceutical compounds could be measured in an aqueous real-life sample by dilution with organic solvents with sufficient sensitivity and concentration precision and accuracy.

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Chapter 5

ENERGY AND CHEMICALS APPLICATIONS

Quantification of Compounds in the E7 Liquid Crystal Mixture by Supercritical Fluid Chromatography with UV Detection

Application Note

Specialty Chemicals

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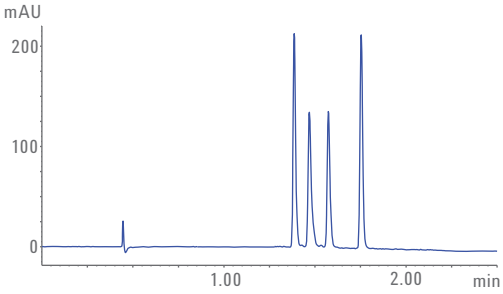
Abstract

This Application Note demonstrates the capability of the Agilent 1260 Infinity Analytical SFC System to perform quantitative analysis of liquid crystal compounds. It demonstrates that fast separation of commercial liquid crystal mixtures is possible within a few minutes. Detection was done by UV, and a relative quantification of the composition of the E7 mixture is described. The results are supported by a statistical evaluation.



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Introduction

Liquid crystal is a type of matter that has physical properties between the solid state and the liquid state[1,2]. The liquid crystalline state of some chemical and biological compounds was discovered in 1888 by the physiologist Friedrich Reinitzer on derivatives of cholesterol[3]. He observed that such compounds have two melting points. At the first one, the compounds melt into a cloudy liquid, and at the second one into a clear liquid. This work was continued by the physicist Otto Lehmann, who examined the behavior of liquid crystals under polarized light[4]. A large number of synthetic liquid crystals were produced by the chemist Daniel Vorländer at the beginning of the 20th century[5].

Liquid crystals can be obtained in thermotropic, lyotropic, and metallotripic phases. The thermotropic liquid crystals are, for example, rod-shaped organic molecules. They exhibit a phase transition to the liquid crystal phase with change of temperature. Typically, below a certain temperature range, thermotropic liquid crystals behave like a solid, and above like a liquid. Within the range of liquid crystallinity, they may show some nematic and smectic phases. In the nematic phases, rod-shaped molecules typically order along their axis and align to magnetic and electric fields. In smectic phases, the molecules are ordered in layers.

Typically, the nematic range is far above room temperature. But some molecules or mixtures exhibit a nematic temperature range within the temperature range required for the use of liquid crystal displays (LCD). An LCD consists of a layer of liquid crystals between two glass layers with electrodes and two polarization filters. Depending on the electrical field and the orientation of the liquid crystal, the LCD can be switched to a highly transparent ON state or to a light-scattering OFF state[6].

The compounds class of cyanobiphenyls and cyanoterphenols comprises liquid crystals that exhibit a nematic phase in the required temperature range. Their properties can be designed by synthesizing compounds that differ only in the length of the aliphatic moiety, or by mixing different liquid crystal compounds. The commonly used liquid crystal 4-cyano-4'-pentylbiphenyl (5CB) is about 20 Å long, and exhibits a phase transition at 18 °C to the nematic phase and to an isotropic state at 35 °C[7]. The widely used E7 liquid crystal mixture (Merck KGaA, Darmstadt, Germany) is such an example (Figure 1). It possess a high birefringence and positive dielectric anisotropy, which enables its broad use in polymer LCD[8]. The composition of the E7 mixture is critical to provide the physical properties and characteristics of the liquid crystal. Even small changes in the composition compromise, for example, the nematic to isotropic temperature (T_{NI}).

The liquid crystal compounds and mixtures are typically analyzed by reversed-phase, high-performance liquid chromatography (HPLC) with UV or mass spectrometric (MS) detection. A typical reversed-phase HPLC separation of a mixture of liquid crystals takes about a one hour run time[9,10].

This Application Note demonstrates the separation of a mixture of liquid crystal compounds in a short run time by means of the Agilent 1260 Analytical SFC System. The quantification of all compounds is demonstrated by creation of the respective calibration curves after UV detection. The relative quantification of the content of a commercially available liquid crystal mixture is shown.

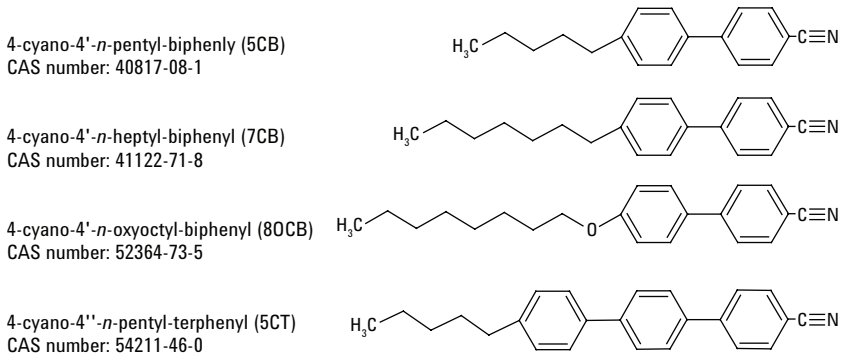


Figure 1. Chemical structures of cyanobiphenyls and cyanoterphenols used in this study and incorporated in the commercially available E7 liquid crystal mixture.

Experimental

Instrumentation

The Agilent 1260 Infinity Analytical SFC System (G4309A) comprised the following modules:

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity High-Performance Degasser
- Agilent 1260 Infinity SFC Standard Autosampler
- Agilent 1260 Infinity DAD with high-pressure standard SFC flow cell
- Agilent 1260 Infinity Thermostatted Column Compartment

Column

Agilent ZORBAX SB-C8, 4.6 × 100 mm, 1.8 µm (p/n 828975-906)

Software

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

Sample

All liquid crystal compounds (10 mg, each), as shown in Figure 1, were dissolved in 10 mL heptane/ethanol 90/10. The final mixed stock solution was at a concentration of 200 µg/mL each. The calibration curve was created using heptane as diluent with a 1:2 dilution pattern.

Chemicals

All solvents were purchased from Merck, Germany. Chemicals were purchased from Sigma-Aldrich (Germany).

SFC method

Parmeter	Value
Solvent A	CO_2
Modifier B	Acetonitrile
SFC flow	2.5 mL/min
Gradient	1 %B at 0 minutes, 13 %B at 1.5 minutes
Stop time	2.25 minutes
Post time	1 minute
BPR temperature	60 °C
BPR pressure	100 bar
Column temperature	60 °C
Injection volume	1 µL, nine-times loop over fill
Needle wash in vial and loop flush with heptane/ethanol 90/10	
Detection	280 nm/band width 4 nm; Ref. 360 nm/band width 100 nm; data rate: 20Hz

Results and Discussion

From the individual stock solutions of the liquid crystal compounds 5CB, 7CB, 8OCB, and 5CT, a mixed stock solution was generated. This solution was used for the development of the SFC separation method. The single wavelength at 280 nm for the detection of all compounds was determined from individual spectra, where the response of all compounds was sufficient. The developed method separated the four similar compounds between 1.38 minutes and 1.73 minutes (Figure 2). 5CB and 7CB, which differ only by the length of the alkyl chain by a $-CH_2CH_2-$ moiety, are baseline separated at 1.38 minutes and 1.46 minutes. The complete run time was 2.25 minutes, with a fast gradient of 1 to 13 % modifier in 1.50 minutes. In this separation on a SB-C8 column, acetonitrile was used as a modifier of medium polarity.

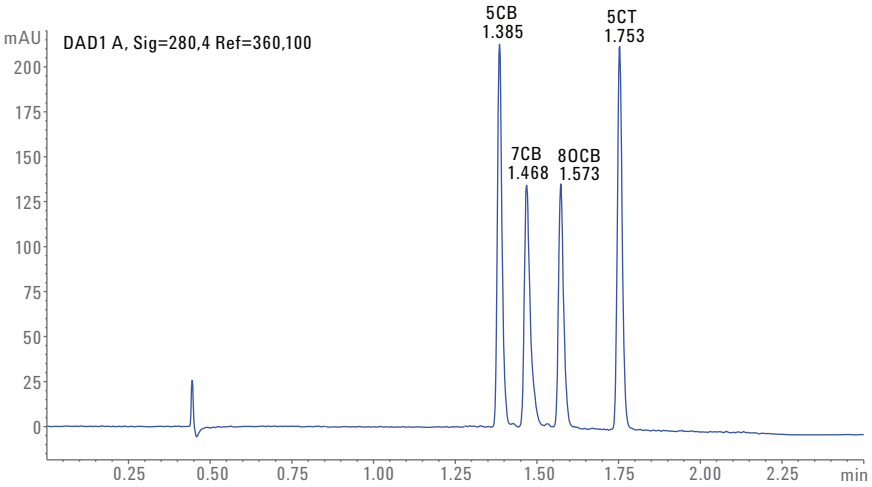


Figure 2. Separation of a mixture of liquid crystal compounds 5CB, 7CB, 8OCB, and 5CT using a fast gradient and within a short run time (50 µg/mL, each).

The mixed stock solution was also used to generate calibration curves for the individual compounds by a dilution in a 1:2 pattern down to a concentration of 6.25 µg/mL for each compound. All individual calibration curves showed a linearity better than 0.99990 (Figure 2). The calibration dilution of 50 µg/mL was injected 10 times for a statistical evaluation of the most important results (Table 1). The retention time RSDs were typically below 0.24 %, and confirm the high retention time precision even for these short retention times. The precision of the measured amount was between 1.75 and 1.97 %, and the calculated accuracies were approximately 98 %. The compounds eluted as sharp peaks with typical peak widths of about 0.015 minutes (0.9 seconds) at half height, and the resolution of all peaks was always above three.

Table 1. Results from a statistical evaluation of the liquid crystal mixture at 50 µg/mL, showing retention time RSDs, concentration precision, peak width, and peak resolution.

	5CB				7CB			
	RT (min)	Amount (mg/mL)	Peak width	Resolution	RT (min)	Amount (mg/mL)	Peak width	Resolution
Average	1.384	48.72	0.015		1.466	49.06	0.018	3.01
RSD (%)	0.25	1.97	1.09		0.24	1.95	1.69	1.29
	8OCB				5CT			
	RT (min)	Amount (mg/mL)	Peak width	Resolution	RT (min)	Amount (mg/mL)	Peak width	Resolution
Average	1.571	49.04	0.016	3.56	1.751	48.61	0.016	6.61
RSD (%)	0.24	1.75	1.09	1.31	0.21	1.76	1.01	1.01

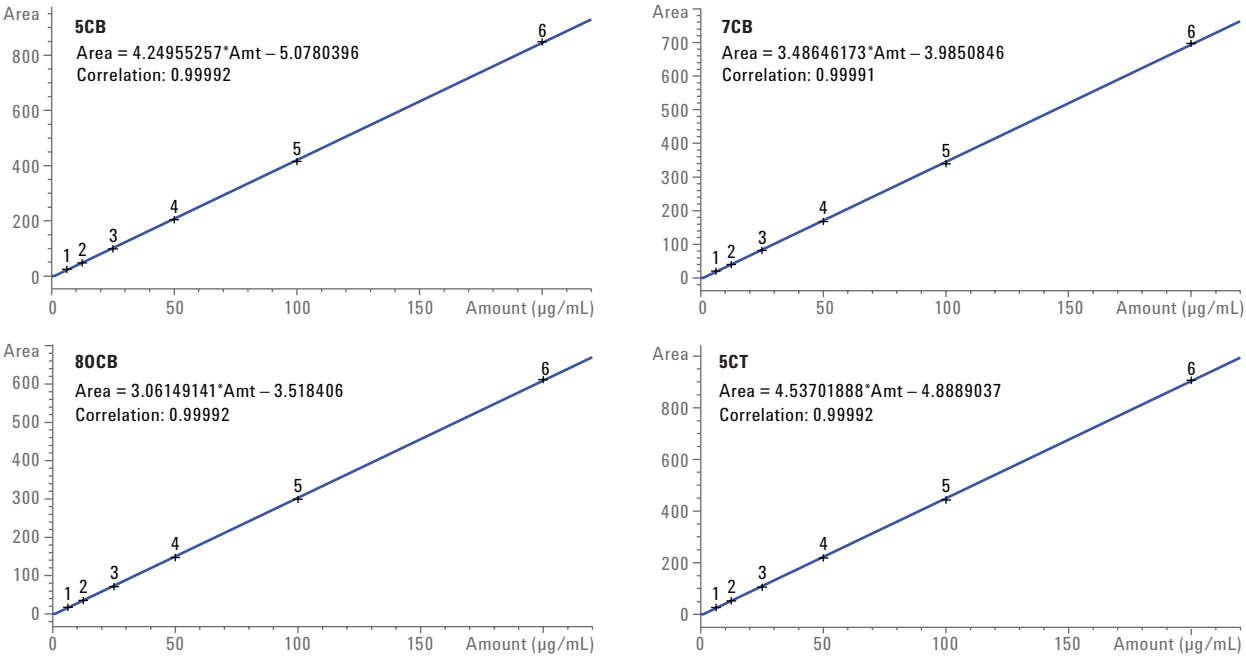
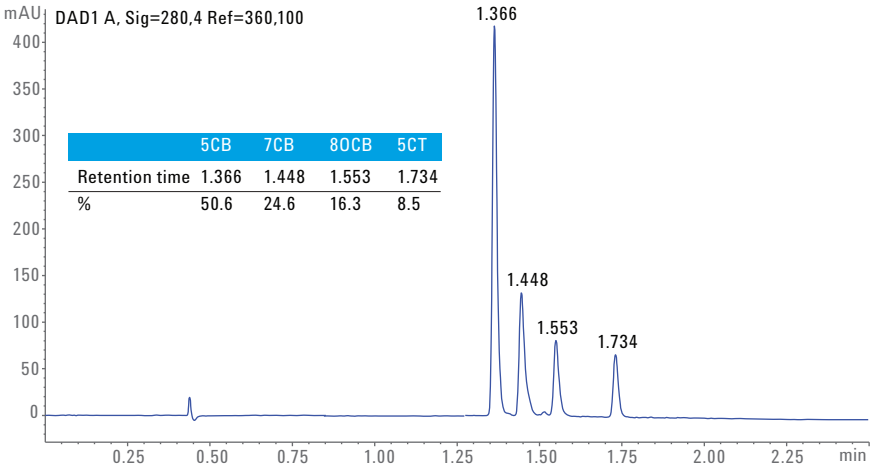


Figure 3. Calibration curves for liquid crystal compounds 5CB, 7CB, 8OCB, and 5CT in a mixture from 200 µg/mL down to 6.25 µg/mL with linearity better than 0.99990.

The compounds used are commercially available as the E7 liquid crystal mixture. This mixture has a composition of 51 weight percent (wt.%) 5CB, 25 wt.% 7CB, 16 wt.% 8OCB, and 8 wt.% CT. This is critical to ensure its required physical properties[8]. The developed method is able to be used in the quality control of the production of the E7 liquid crystal mixture, for example. For a fast decision whether the composition is within the tolerances, a report showing area percentages could be generated in ChemStation or customized by means of the Intelligent Reporter based on the above described calibration. Figure 4 shows a chromatogram of a typical E7 mixture.



	5CB	7CB	8OCB	5CT
Retention time	1.366	1.448	1.553	1.734
%	50.6	24.6	16.3	8.5

Figure 4. Chromatogram of an E7 liquid crystal mixture. The required composition is 51 wt.% 5CB, 25 wt.% 7CB, 16 wt.% 8OCB, and 8 wt.% CT.

Conclusion

This Application Note demonstrates the separation of a mixture of liquid crystal compounds using the Agilent 1260 Infinity Analytical SFC System. The baseline separation of a mixture of four liquid crystal compounds of technical importance is demonstrated in a fast, 2.25-minute run time. This fast run time is about a factor of 20-times faster than the typically used conventional RP-HPLC separations. The retention time RSDs are below 0.25 %, and the calibration curve linearity is better than 0.99990. The quantitative determination of the composition of a commercial E7 liquid crystal mixture is demonstrated.

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Agilent Technologies

Determination of Enantiomeric Excess of Metolachlor from Chiral Synthesis using the Agilent 1260 Infinity Analytical SFC System

Application Note

Specialty Chemicals

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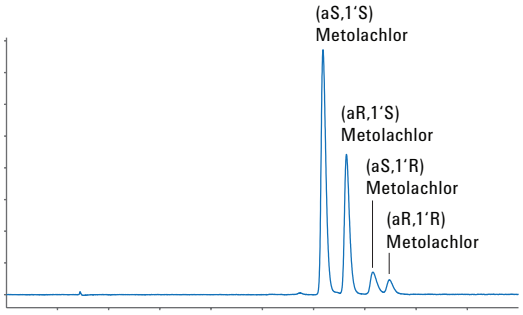
Abstract

This Application Note describes the development of a method for the separation of four stereoisomers, which were derived from a compound with two steric centers. The method was developed using an Agilent 1260 Infinity Analytical Supercritical Fluid Chromatography (SFC) System with Agilent ChemStation Method Scouting Wizard software. The developed method was used to compare the ratio of stereoisomers obtained from racemic and stereoselective syntheses.



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Introduction

The herbicide metolachlor is used worldwide in large amounts for the control of a variety of broad-leaved weeds in corn and other crops. Worldwide production exceeds 30,000 tons per year[1,2]. Metolachlor has one stereogenic center located at an asymmetrically-substituted carbon atom and an additional chiral axis. This structure means metolachlor exists as four stereoisomers (Figure 1).

The biological activity depends mainly on the configuration on the stereogenic carbon atom. About 95 % of the biological activity stems from both forms of the S-enantiomer, (aS,1′S) and (aR,1′S), which differ in spatial arrangement at the chiral axis[3]. Due to this fact, a metolchor formulation enriched with the S-enantiomers could lower the intake of the compound by the environment while maintaining the desired herbicidal effect. Large efforts were taken to replace the racemic syntheses by a stereoselective synthesis of the S-enantiomer. The final solution was found with a catalytic hydrogenation reaction driven by a chiral ferrocenyl catalyst (Figure 2)[4,5]. This synthesis enables production of metolachlor with an enantiomer excess of about 80 % in amounts greater than 50,000 tons per year. To determine the enantiomer excess and thereby the success of the chiral synthesis, all four stereoisomers must be resolved. This separation has been done by normal phase HPLC[6,7]. With the HPLC method, the four stereoisomers elute between 20 and 30 minutes from the column, and typical harmful normal phase solvents are used.

In this Application Note, we demonstrate how a method for the separation of all four stereoisomers of metolachlor can be developed using the Agilent 1260 Infinity Analytical SFC System. The developed method is able to separate the four stereoisomers within a much shorter time than typically needed for the separation using normal phase HPLC conditions. In addition, the SFC method avoids using harmful solvents.

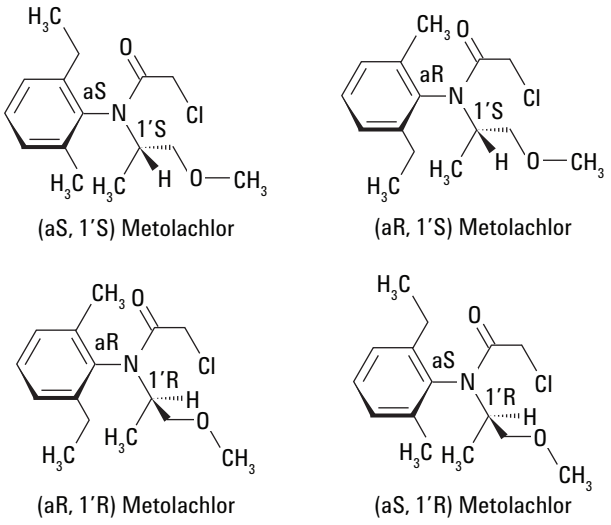

Figure 1 Stereoisomers of the pesticide metolachlor. The 1′S-enantiomers are biologically active, independent of the spatial arrangement at the chiral axis.

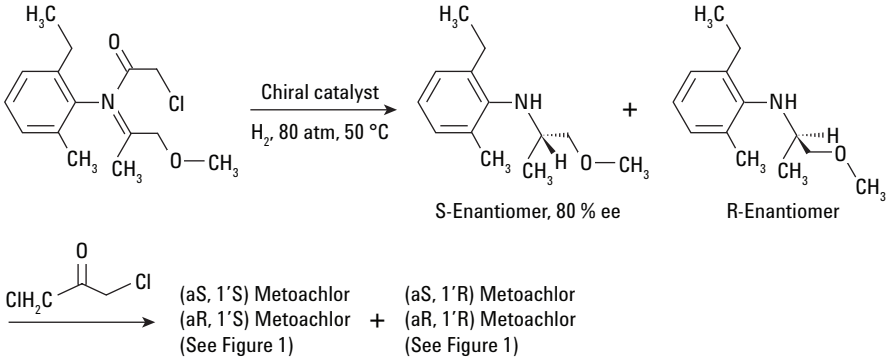

Figure 2 Stereoselective synthesis of S-metolachlor.

Experimental

Instrumentation

All experiments were carried out on an Agilent 1260 Infinity Analytical SFC System (G4309A) comprising:

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity High Performance Degasser
- Agilent 1260 Infinity SFC Autosampler
- Agilent 1290 Infinity Thermostatted Column Compartment with valve drive
- Agilent 1260 Infinity Diode Array Detector with high pressure SFC flow cell

The following additional equipment was required for automated method development with the SFC system:

- Agilent 1290 Infinity Thermostatted Column Compartments (G1316C) with valve drive
- Agilent 1200 Infinity Series 8-position/9-port Quick-Change Valves, 2x (G4230A)
- Agilent 1290 Infinity Valve Drive (G1170A) with Agilent 1200 Infinity Series 12-position/13-port Quick-Change Valve (G4235A)
- Capillary kit for method development (p/n 5067-1595)

Instrumental setup

For solvent selection, the instrument configuration menu in OpenLAB CDS was used to cluster the SFC binary pump with a 12-position/13-port valve. The solvents were defined in the pump setup menu of OpenLAB CDS. For column selection, the instrument configuration menu in OpenLAB CDS was also used to cluster the two thermostatted column compartments, each of which were equipped with an 8-position/9-port valve. The method development capillary kit enables using up to eight columns. Details of the columns were entered in the columns database of OpenLAB CDS and configured in the column compartment menu.

Software

Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, version C.01.06, with Agilent ChemStation Method Scouting Wizard, version A02.04 (G2196AA)

Columns

- Chiral Technologies, Chiralpak IA3, 4.6 × 250 mm, 3 µm
- Chiral Technologies, Chiralpak IB, 4.6 × 250 mm, 5 µm
- Chiral Technologies, Chiralpak IC, 4.6 × 250 mm, 5 µm
- Chiral Technologies, Chiralpak ID, 4.6 × 250 mm, 5 µm

Chemicals

- Metolachlor and S-Metolachlor were purchased from Sigma-Aldrich, Germany. A solution in 5 mg/mL isopropanol was used for the experiments.
- All solvents were purchased from Merck, Germany.
- Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak).

SFC methods

Conditions of the optimized final method are in shown in bold.

Parameter	Value
Solvent A	CO_2
Modifier B	Methanol, ethanol, isopropanol
SFC flow	3 mL/min
Isocratic elution	2.5 %, 5 %, 10 %, and 20 % modifier
Modifier	Methanol
BPR temperature	60 °C
BPR pressure	120 bar
Column temperature	35 °C
Injection volume	1 µL, fixed loop, 10-times overfill, needle wash in vial with isopropanol
Detection	220 nm/bandwidth 4 nm, reference 360 nm/bandwidth 100 nm, 10-Hz data rate

Results and Discussion

A racemic mixture of metolachlor comprising all four stereoisomers was used for a screening with four different chiral columns and three organic modifiers of increasing eluting strength; isopropanol, ethanol, and methanol. Three different isocratic conditions were used for the initial screening with 5, 10, and 20 % of the organic modifier. The screening of one column with three different solvents and three different isocratic compositions took about 90 minutes, including solvent exchange and equilibration. The most promising elution pattern obtained by the screening process was achieved on the Chiralpak IA3 column (Figure 3, other columns are not shown).

During screening on the Chiralpak IA3 column, the four seteroisomers were not separated using methanol, which has the highest eluting strength (Figure 3A). Using ethanol, which has a lower eluting strength, separation began at a ratio of 10 %. At a ratio of 5 %, the two pairs were separated, but the individual compounds were not completely separated (Figure 3B). The weakest eluting solvent, isopropanol, separated the first and last compounds almost at baseline, and the second and third compound with a valley at 5 % under isocratic conditions (Figure 3C). The final optimization was done using these starting conditions. The final method for the separation of the four stereoisomers applied isocratic conditions with 2.5 % isopropanol and separated the four compounds between 6.16 and 7.44 minutes (Figure 4).

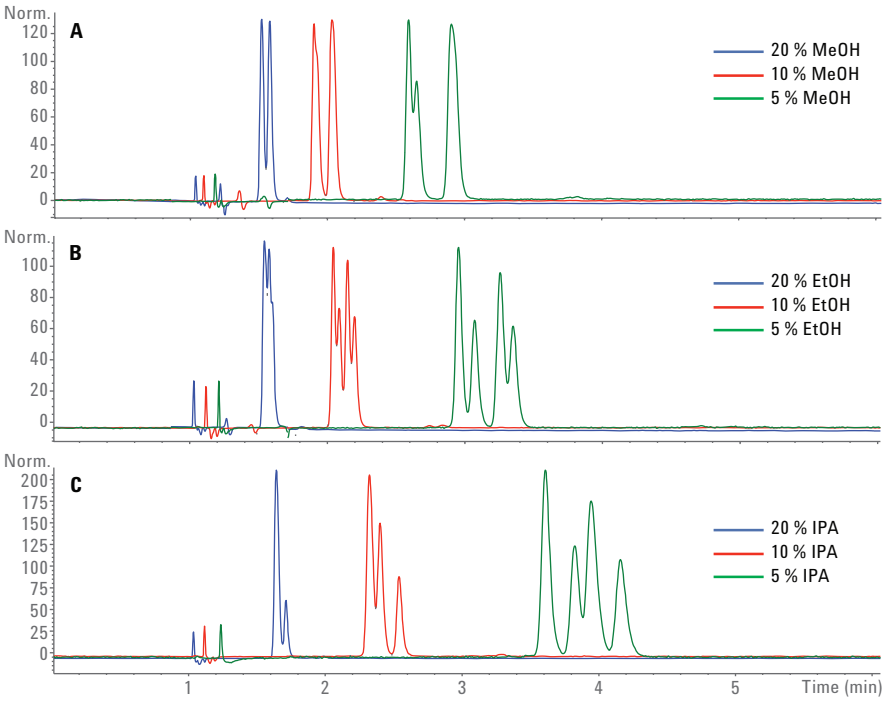

Figure 3. Separation of the four chiral metolachlor isomers with 5, 10, and 20 % of A) MeOH, B) EtOH, and C) isopropanol (IPA). As starting point for the final optimization of the separation method on column Chiralpak IA3, 5 % isocratic IPA was chosen.

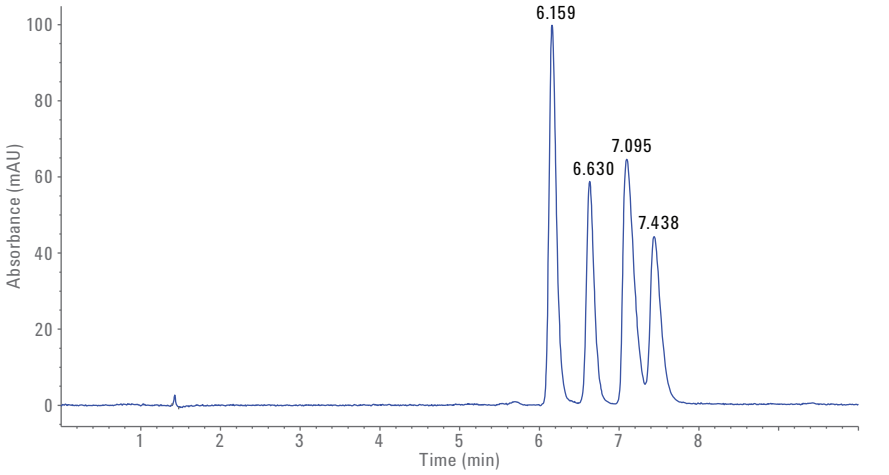

Figure 4. Separation of four stereoisomers of metolachlor with the final optimized method between 6.159 and 7.438 minutes on the Chiralpak IA3 column with 2.5 % isocratic isopropanol.

This optimized method was applied to analyze metolachlor obtained from a stereoselective synthesis, which had an enantiomeric excess of the S-enantiomers (Figure 5). The (aS,1'S) and (aR,1'S) enantiomers elute at 6.18 and 6.64 minutes as the main compounds in the mixture. The (aS,1'R) and (aR,1'R) enantiomers elute at 7.16 and 7.48 minutes as the minor components. The enantiomeric excess of the S-enantiomers was calculated from the peak areas to be about 78 % (Table 1). The peak areas obtained from the racemic metolachlor sample showed a 50:50-% ratio of the S- and R-enantiomers, whereas one of the stereoisomers of the chiral axis, aS or aR, was preferred (Table 1).

A statistical evaluation with 10 injections of the racemic and the S-selective mixture showed retention time RSDs typically below 0.2 % and area RSDs typically at 1 % (Table 1).

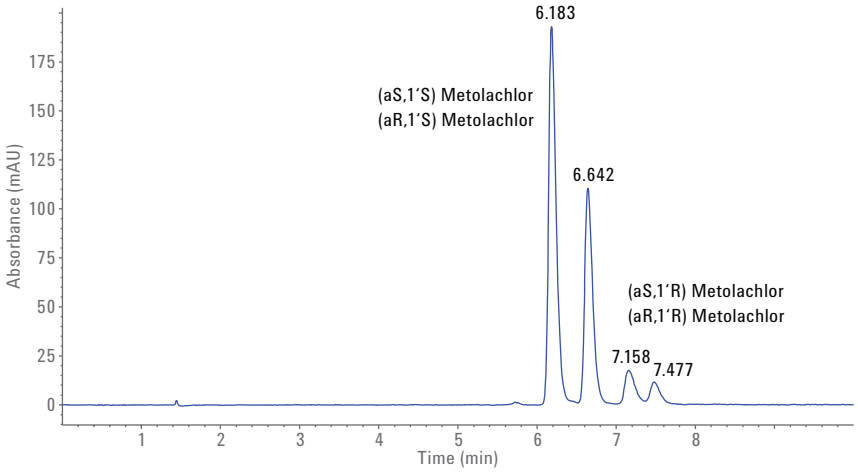

Figure 5. Separation of four stereoisomers of metolachlor synthesized by a steroslecitve synthesis. The major components, the S-enantiomers, elute at 6.183 and 6.642 minutes. The enantiomeric excess of the S-enantiomer is about 78 %.

Table 1. Statistical evaluation of the separation of four metolachlor stereoisomers from a racemic and S-selective synthesis, and relative determination of the enantiomeric composition.

		Racemic synthesis			S-selective synthesis		
	Retention time	Retention time RSD	Area RSD	Area %	Retention time RSD	Area RSD	Area %
Peak 1	6.183	0.25	1.06	30.77	0.20	0.90	55.35
Peak 2	6.642	0.22	1.05	19.24	0.19	0.97	33.66
Peak 3	7.158	0.16	1.06	30.08	0.18	1.93	6.70
Peak 4	7.477	0.17	1.04	19.91	0.16	1.34	4.29

Conclusion

This Application Note demonstrates the development of a method for the chiral separation of a compound, the pesticide metolachlor, with more than one chiral center and, hence, more than two enantiomers. The final method was used to determine the enantiomeric excess of the product from stereoselective synthesis. The developed method had a run time of 10 minutes. In contrast, the typical normal phase HPLC method takes at least 30 minutes. The SFC method is about three-times faster than a classically used normal phase method. In addition, compared to the normal phase method, the SFC does not use harmful solvents such as *n*-hexane. The obtained retention time RSD values were below 0.2 %, and area RSD values were about 1 %.

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Determination of Olefin Content in Denatured Ethanol According to ASTM D7347

Application Note

Energy & Chemicals

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Abstract

This Application Note demonstrates the determination of the olefin content in denatured ethanol using the Agilent 1260 Infinity Analytical SFC System together with the SIM Flame Ionization Detector (FID). Combining the 1260 Infinity Analytical SFC System with this FID meets all requirements of the ASTM D7347-07 method such as the required retention time precision for time-based column switching, good area precision, and calibration function.

SIM Scientific Instruments Manufacturer GmbH

Agilent Technologies

Introduction

Denatured ethanol is used as an oxygenate additive in spark ignition engine fuel. It is added to fuel at the terminals, and can contain olefinic species, which contribute to the total olefins content. Olefinic hydrocarbons have been demonstrated to contribute to photochemical reactions in the atmosphere. This can result in the formation of smog in susceptible urban areas. Therefore, the California Air Resources Board (CARB) has specified a maximum allowable limit of total olefins in spark ignition engine fuel[1,2].

An analytical method is necessary to determine the amount of olefins in denatured ethanol intended for spark ignition engine fuel, as described in ASTM method D7347-07. Regulators and producers must abide by this method[2]. An appropriate analytical test method for the determination of total olefins in gasoline is described in ASTM D6550[3,4].

SIM Scientific Instruments Manufacturer GmbH (Germany) has developed a flame ionization detector (FID) for the Agilent 1260 Infinity Analytical SFC System to enhance the range of applications, for example, to meet petrochemical requirements described in ASTM D5186 for the determination of aromatic compounds in diesel fuels[5,6].

This Application Note demonstrates the determination of the olefin content in denatured ethanol using the 1260 Infinity Analytical SFC System together with the SIM Flame Ionization Detector. It is shown that the SFC/FID system meets all requirements of ASTM D7347-07. The test method is automated, does not require any sample preparation, and has a relatively short analysis time of approximately 10 minutes. The application range is from 0.1 to 1.0 mass% total olefins. For the final method, the retention time (RT) and area precision, the accuracy of olefin quantification, and detector linearity is demonstrated. Finally, a denatured ethanol sample is measured.

Experimental

Instrument

An Agilent 1260 Infinity Analytical SFC System with the following configuration was used:

- Agilent 1260 Infinity Binary SFC Pump
- Two Agilent 1290 Infinity Thermostatted Column Compartments with valve drives and 2-position/6-port Agilent InfinityLab Quick Change valves
- Agilent 1260 Infinity SFC Control Module
- CTC Analytics LC-Injector HTC PAL (50 cm width) modified with 4-port valve (internal 1-µL loop) and DLW or Agilent 1260 Infinity Standard Autosampler (alternative)
- SIM Flame Ionization Detector
- Restrictor for hexane purging path: PEEKsil, 100 µm id, 20 cm

The complete solution can be ordered through SIM Scientific Instruments Manufacturer GmbH, Oberhausen, Germany.

Analytical column

- Agilent ChromSpher 5 Lipids (silver loaded), 4.6 × 30 mm (p/n G7601-85000)
- YMC-PACK-SIL-06, 4.6 × 250 mm, S-5 µm, 6 nm (p/n SL06S05-2546WT)
- YMC-Pack-PVA-Sil, 4.0 × 50 mm, S-5 µm, 12 nm (p/n PV12S05-0504QT)

Software

Agilent OpenLAB CDS ChemStation Edition, Rev. C.01.05

SFC/FID configuration

The outlet of the column is connected to the upper T-piece to the FID (Figure 1). For purging the backpressure regulator (BPR), hexane is permanently pumped from pump head B of the binary pump. To maintain constant backpressure and a continuously operating system, a restriction capillary is integrated between pump head B and the lower T-piece of the FID.

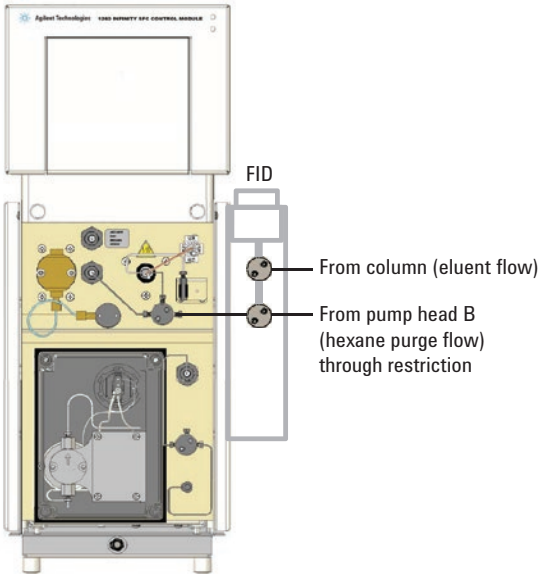

Figure 1. Connection of the SFC/FID module with the LC instrument.

Column and valve configuration

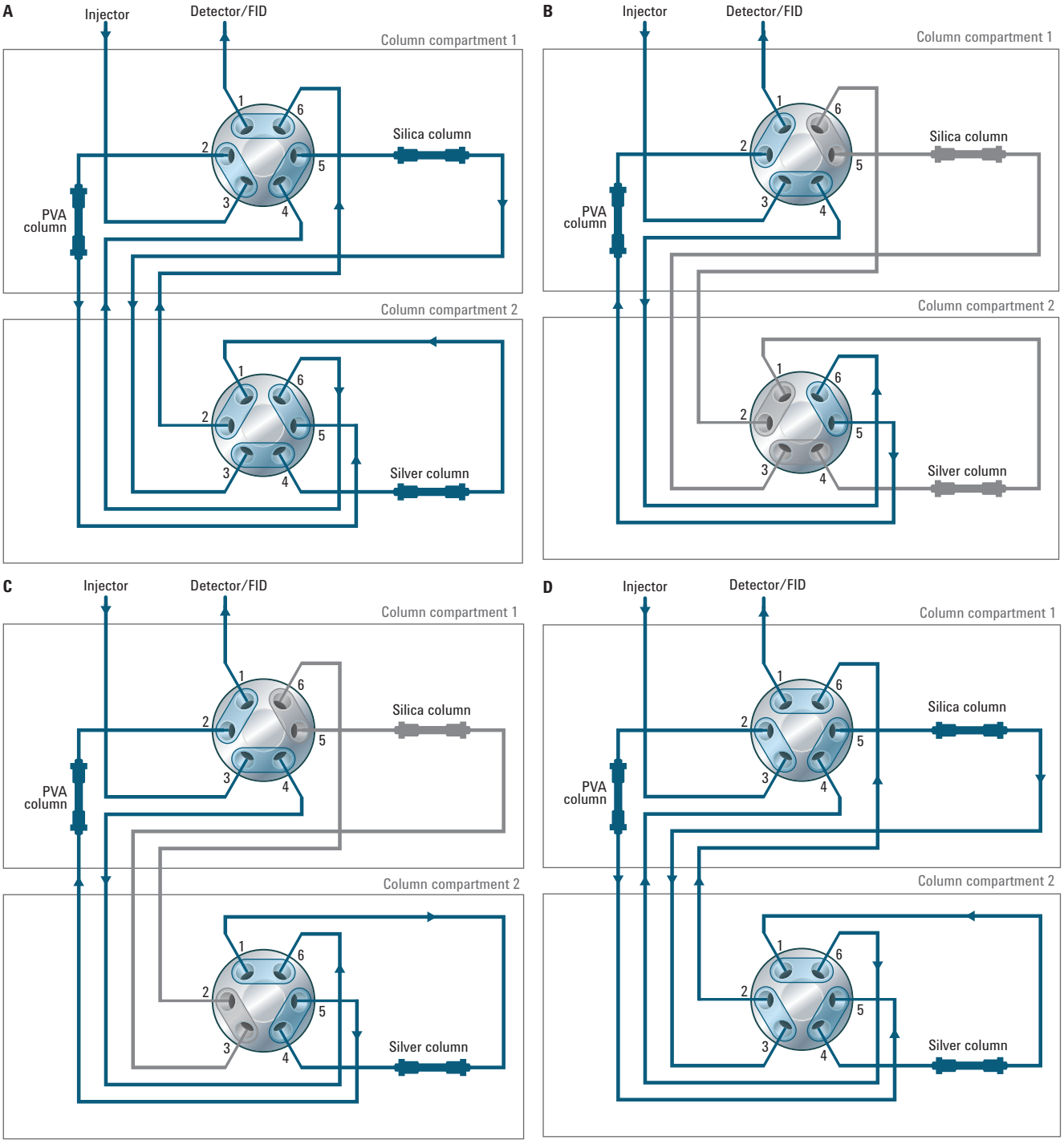

Figure 2. Column and valve configurations. Position A) PVA column, silica column, and silver-loaded column in forward-flush mode, connected in series. Injection of the sample onto the three columns retains the ethanol on the PVA column. After elution of the ethanol (position B), this position will be used to elute the saturates, load olefins onto the silver-loaded column, and retain the aromatics. Position B) Backflush of the ethanol from the PVA column. The silica and the silver-loaded column are not in the flow path. Position C) Backflush of the silver-loaded column to elute the olefins. The silica column is not in the flow path. Position D) The flow enters the PVA column first, then the silver-loaded column, and last the silica column in forward-flush mode to elute the aromatics off the silica column to the detector.

Determination of valve switching times (ASTM D7347, section 9)

The times for valve switching were determined by the experiments described below by means of the loading-time mixtures A to C. This procedure has to be done at initial system setup, or whenever something was changed such as columns, capillaries, and so on.

- For the determination of the sample loading time, t_L, the valves were switched to positions described in Figure 2B; loading-time mixture A was used. This valve position only allows use of the PVA column, while the silica column is protected from ethanol, and the silver-loaded column is protected from aromatics (Figure 3A, t_L = 0.75 minutes).
- Pure ethanol was used to determine the time to backflush the ethanol, t_E, from the PVA column after the loading time, t_L. The ethanol was injected with the valves in the positions shown in Figure 2A, and the valves were switched to the positions shown in Figure 2B at t_L = 0.75 minutes. The time t_E was determined when the ethanol peak returned to the baseline (Figure 4, t_E = 3.0 minutes).
- Loading-time mixture B was used for the determination of the loading time of the olefinic compounds, t_0, from the silicia column onto the silver-loaded column (Figure 5, t_0 = 5.15 minutes).
- Loading-time mixture C was used to determine the time period, t_{BO}, necessary for the complete elution of the olefins from the silver-loaded column in backflush mode (Figure 6, t_{BO} = 7.5 minutes).

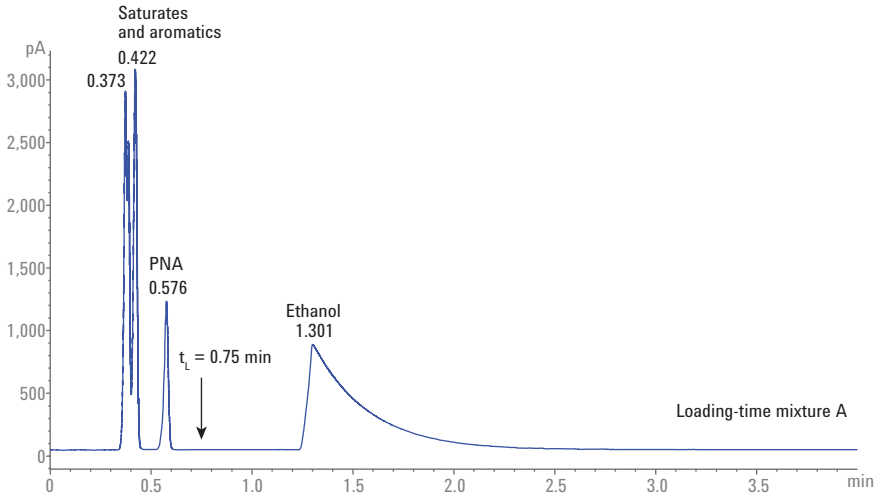

Figure 3. Determination of the sample loading time, t_L. Valves were switched to positions described in Figure 2B. Loading-time mixture A was used. The loading time t_L is determined when the aromatic and saturate compounds exit the PVA column, and the signal is back to baseline (t_L = 0.75 minutes).

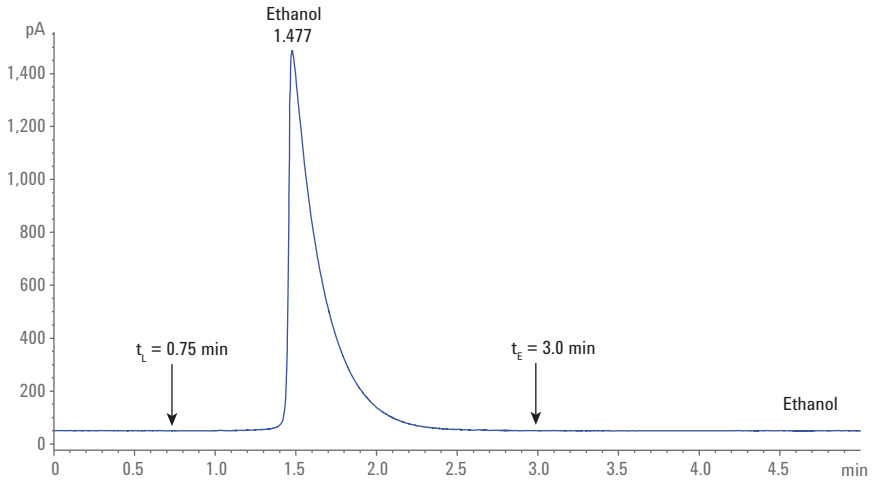

Figure 4. Determination of the backflush time for ethanol, t_E, after loading time, t_L. Ethanol was injected in valve position 2A, switching to position 2B (t_L = 0.75 minutes). The backflush time t_E is determined when the ethanol peak returns to the baseline (t_E = 3.0 minutes).

- Loading-time mixture B was used for the determination of the elution time of the aromatics, t_A. After the time period t_{BO}, the aromatics were eluted from the silica column by switching to the valve positions shown in Figure 2D. After elution of the mono-aromatics and the polynuclear aromatics (PNA), the run can be stopped (t_A = 9.5 minutes). Figure 7 shows the complete chromatogram.
- Loading-time mixtures D and E can be used to show whether there are saturates or aromatics eluted onto the silver-loaded column. They can be used to optimize the valve switching time point t_0 (chromatograms not shown).

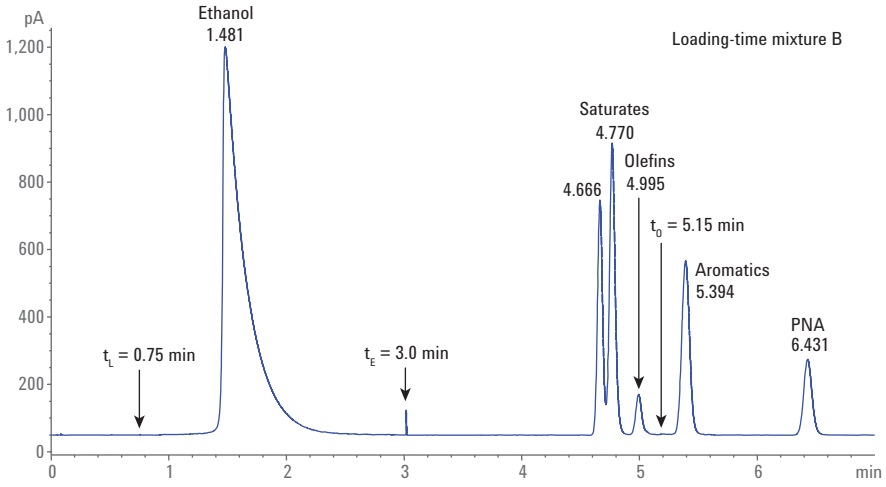

Figure 5 Determination of the loading time of the olefinic compounds, t_0, onto the silver-loaded column. Loading-time mixture B was used. To determine t_0, the loading-time mixture B was injected with the valve positions given in Figure 2A. After t_L, the valves were switched to positions shown in Figure 2B to backflush the ethanol. At t_E, the valves were switched to positions shown in Figure 2D. This configuration elutes saturates, olefins, and aromatics directly to the detector without contaminating the silver-loaded column (t_0 = 5.15 minutes).

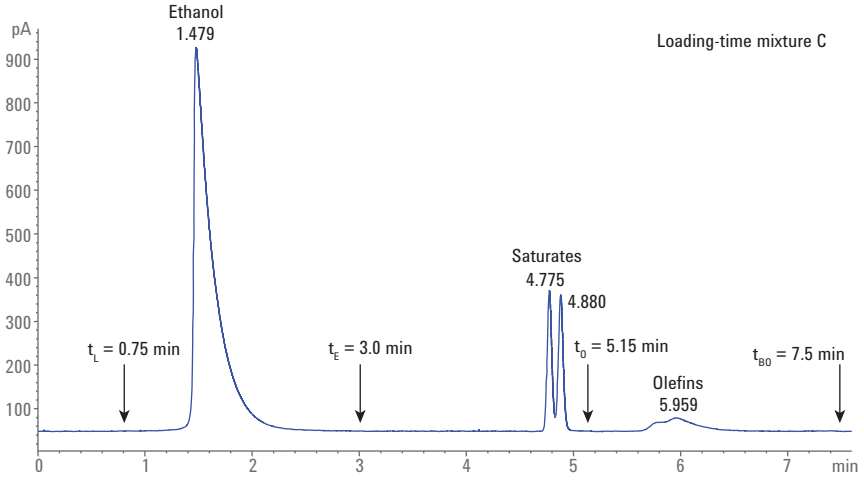

Figure 6 Determination of the time period t_{BO} necessary for the complete elution of the olefins from the silver-loaded column in backflush mode. Loading-time mixture C was used. This sample was injected by means of valve positions according to Figure 2A. At t_L, the valves were switched to the positions according to Figure 2B to backflush the ethanol. At t_E, the valves were switched back to the initial positions until saturates were eluted from the silica column through the silver-loaded column to the detector, until time t_0. The valves are now actuated to the position shown in Figure 2C, and the retained olefins are eluted from the silver-loaded column in backflush mode (t_{BO} = 7.5 minutes).

Chemicals and solutions

Samples and standards were prepared according to guidance published in ASTM D7347. All chemicals: benzene, toluene, naphthalene, hexane, cyclohexane, cyclohexene, 2-pentene, 1-hexene, and petrol (gasoline, puriss., bp 90 to 100 °C) were purchased from Sigma-Aldrich, Taufkirchen, Germany. Ethanol was HPLC grade, and was purchased from Carl Roth, Germany.

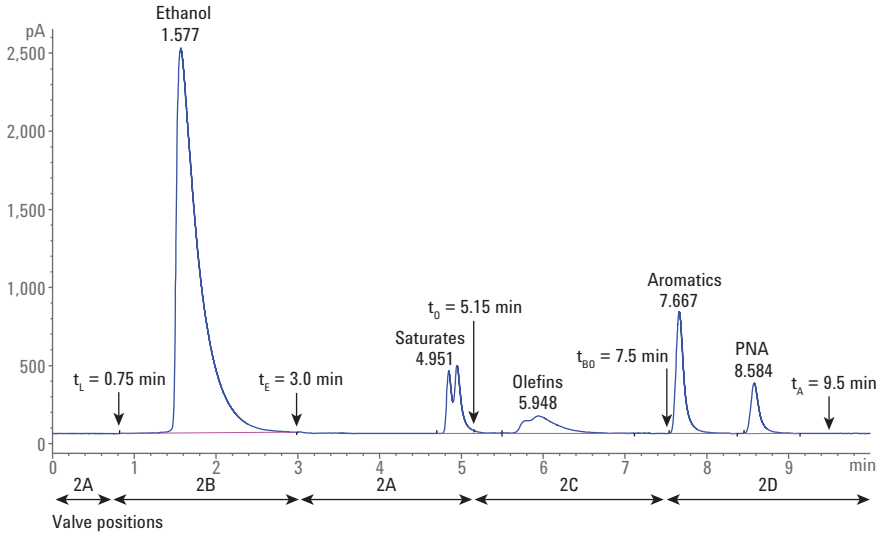

Figure 7. Performance test for the measurement of olefins in denatured ethanol. Valve switching time points and time ranges are indicated. The respective flow paths are shown in Figure 2.

Component	Composition
FID gases	Hydrogen, air, and nitrogen (as make-up gas).
Eluent	Carbon dioxide (purity > 99.998 %, pressurized in a cylinder without DIP tube according to the specifications of the SFC module).
Purge solution for backpressure regulator	Hexane (not used as modifier. Pumped with channel B of the binary pump).
Loading-time mixture A	10 % Alkanes (*n*-hexane and cyclohexane), 10 % aromatics (benzene, toluene, and naphthalene), and 80 % ethanol were used to determine the loading time of saturates and aromatics on the silica column while the silica column is protected from ethanol by the PVA column (ASTM D7347, section 7.5.1).
Loading-time mixture B	10 % Alkanes (*n*-hexane and cyclohexane), 7 % aromatics (benzene, toluene, and naphthalene), 3 % olefins (2-pentene, 1-hexene, and cyclohexene), and 80 % ethanol were used to determine the loading time of saturates and olefins on the silver-loaded column without aromatic contamination (ASTM D7347, section 7.5.2).
Loading-time mixture C	7 % Alkanes (*n*-hexane and cyclohexane), 3 % olefins (2-pentene, 1-hexene, and cyclohexene), and 90 % ethanol were used to determine the elution time of the olefins from the silver-loaded column (ASTM D7347, section 7.5.3).
Loading-time mixture D	10 % Alkanes (*n*-hexane and cyclohexane) and 90 % ethanol were used to check the absence of saturates on the silver-loaded column (ASTM D7347, section 7.5.4).
Loading-time mixture E	10 % Aromatics (benzene, toluene, and naphthalene) and 90 % ethanol were used to check the absence of aromatics on the silver-loaded column (ASTM D7347, section 7.5.5).
Performance Test Mixture (PTM)	Alkanes (*n*-hexane and cyclohexane), mono-aromatics (benzene and toluene), and polynuclear aromatic (naphthalene) at no more than 10 % by weight and mono-olefins (2-pentene, 1-hexene, and cyclohexene) at no more than 3 % by weight in ethanol (ASTM D7347, section 7.7).
Quality control mixture	Ethanol containing olefins at a known concentration to monitor the precision of the analytical SFC system (ASTM D7347, section 7.8).
Calibration mixture	10 % Alkanes (hexane and cyclohexane) by weight and 2 % olefins (2-pentene, 1-hexene, and cyclohexene) by weight with 88 % ethanol by weight (ASTM D7347, section 8.5). This stock solution was diluted 1:1 with ethanol containing 10 % alkanes (hexane and cyclohexane) by weight. The resulting 1 % olefin solution was diluted to calibration solutions with olefin content down to 0.1 % in steps of 0.1 %.
Spiked sample	Ethanol (denatured with no more than 10 % petrol) containing mono-olefins (2-pentene, 1-hexene, and cyclohexene) at no more than 1 %.

Results and Discussion

This ASTM D7347 test method for the determination of the total amount of olefins in denatured ethanol is based on a chromatographic SFC LC system with three columns and two six-port switching valves. Step by step, the olefins are separated from ethanol, aromatics, and saturates; an FID is used for quantification of the olefins. It is important to determine the valve switching times thoroughly to protect the column from ethanol, and the silver-loaded silica column from ethanol and aromatics, and to guarantee the absence of aromatics and saturates on the silver-loaded column at the time of the olefin elution.

At the beginning, the valve switching times were determined as described in the experimental section, then the Performance Test Mixture (PTM) was measured. This mixture comprised saturated hydrocarbons, olefins, mono-aromatics, and PNAs. This mixture was used to evaluate the accurateness of valve switching, and to ensure that all compounds were eluted in time from their respective columns to meet the required retention time (RT) (Figure 7).

SFC Method

Agilent 1260 Infinity Binary SFC Pump	
Solvent A	CO_2 (precompressed), 99.998 %
Solvent B	Hexane, 0.5 % (used for purging the backpressure regulator (BPR) only, not used as eluent). Depending on the composition of the sample, it might be necessary to purge the BPR with a higher amount of hexane (for example, setting in the software to 50 % eluent B) from time to time.
Flow rate	1.5 mL/min
BPR temperature	40 °C
BPR pressure	170 bar
Agilent 1290 Infinity Thermostatted Column Compartment	
Column temperature PVA and silica column	40 °C
Column temperature silver-loaded column	70 °C
Valve positions (Figure 2)	
Valve position A	At 0 minutes
Valve position B	At 0.75 minutes
Valve position A	At 3.0 minutes
Valve position C	At 5.15 minutes
Valve position D	At 7.5 minutes
CTC Analytics LC-Injector HTC PAL	
Injection volume	1.0 µL loop, 30-times overfill, wash in port with hexane
Preclean with solvent 1	1
Preclean with sample	1
Filling speed	5 µL/s
Filling strokes	3
Inject to	LCVIv1
Injection speed	10 µL/s
Pre-injection delay	500
Postinjection delay	500
Postclean solvent 1	2
Valve clean solvent 1	2
Agilent 1260 Infinity Injector (alternative)	
Injection volume	1.0 µL loop, 10-times overfill, wash in vial with hexane
FID	
Temperature	300 °C
Gases	Hydrogen (H_2) 50 mL/min Air 500 mL/min Make-up gas (N_2) 50 mL/min

In the first step, ethanol has to be removed from the hydrocarbons. While they have entered the silica column completely at t_L (0.75 minutes), ethanol is retained in the PVA column and eluted by backflush until t_E (3.0 minutes). The valve positions are assigned at the bottom of Figure 7. Figure 2 shows their respective flow paths. After elution of the saturates and olefins from the silica column, the olefins are retained on the silver-loaded column, and the saturates are guided through to the detector (4.95 minutes). At t_0 = 5.15 minutes, the silver-loaded column is switched to backflush for the elution of the olefins. The aromatics are still retained on the silica column, which is in bypass mode. The olefins elute from the backflushed silver-loaded column in a broader peak with the maximum at 5.95 minutes. After complete elution and flushing of the silver-loaded column, the aromatics are eluted from the silica column by switching it back into the flow path at 7.5 minutes. The mono-aromatics elute at 7.67 minutes, and the PNAs at 8.58 minutes. Figure 8 shows an overlay of 20 repeating measurements of PTM to show that the time-based valve switching is working well. The resolution between the olefins and the mono-aromatic peak is around 6 (required > 4).

For the measurement of real samples, it is necessary to generate a calibration curve with calibration points from 0.1 to 1.0 % olefins dissolved in denatured ethanol. The calibration showed good linearity with a correlation better than 0.99 (Figure 9). Figure 9 also displays an overlay of the 0.1 to 1.0 % calibration concentrations of the olefin peak.

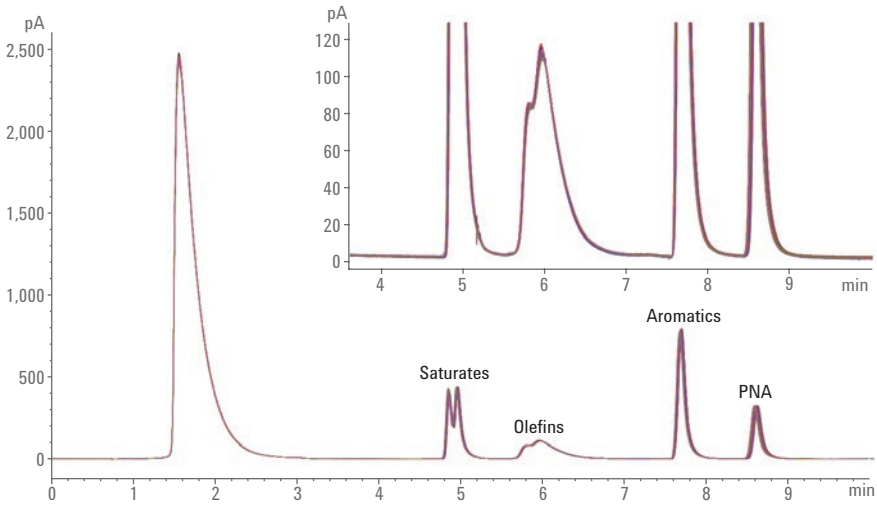

Figure 8. Performance test with PTM for the olefin measurement in denatured ethanol with zoom in (overlay; n = 20).

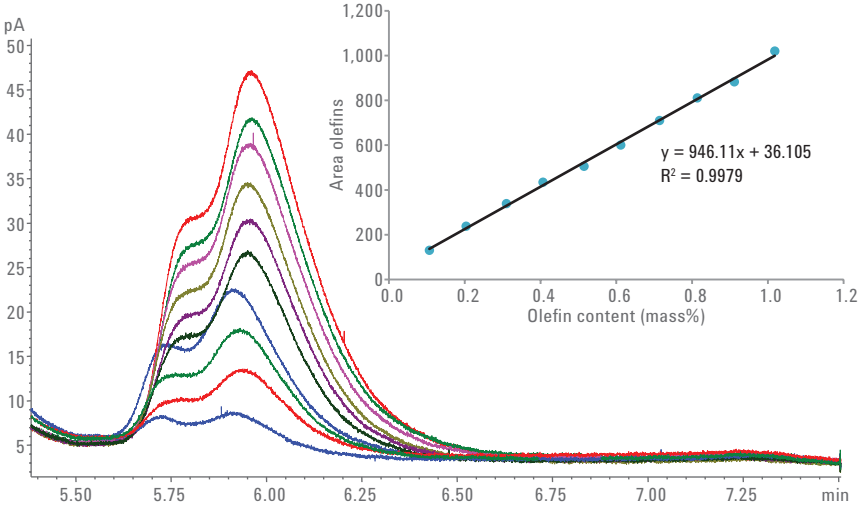

Figure 9. Overlay of 0.1 to 1.0 mass% content of olefin and calibration curve for the measurement of olefins in denatured ethanol with linearity correlation 0.9979.

Together with the calibration curve, a performance sample (PTM), a quality control (QC) sample, and a spiked sample of denatured ethanol (with 9.5 % petrol) were measured. Figure 10 shows a sample of denatured ethanol spiked with 0.5 % olefin. All samples were injected 20 times for a statistical evaluation of the olefin peak (Table 1). The relative standard deviation (RSD) of the RT is typically below 0.2 % (required < 0.5 %), and the peak area RSDs were typically below 2 %. The repeatability limit was calculated according to ASTM D7347-07, section 13.1.1. All samples met this criterion as the difference between successive results did not exceed the calculated limit.

Conclusions

This Application Note demonstrates the improved capabilities of the Agilent 1260 Infinity Analytical SFC System in combination with the SIM/FID for the determination of olefins in denatured ethanol according to ASTM D7347-07.

The olefin content of a denatured ethanol sample was separated from saturated and aromatic hydrocarbons as well as from the ethanol itself by means of three different columns, and a valve switching solution for separation in forward-flush and elution in backflush mode. The olefins were quantified between 0.1 and 1.0 mass%, with good linearity. RT precision, which is important for time-based column switching, was within the given limits. The difference between the successive results agree to the repeatability limits stated in section 13.1.1 of ASTM D7347-07.

Table 1. RT, area, concentration, and repeatability values for the PTM, QC, and spiked sample (n = 20) in accordance to ASTM 7347-07, section 13.1.1.

Sample	PTM 3.0 % olefin			Quality control 0.25 % olefin			Spiked sample 0.5 % olefin		
Olefin content by weight (mass%)	3.027			0.257			0.527		
	RT	Area	Amount	RT	Area	Amount	RT	Area	Amount
Average (mass%)	5.95	3,024.21	3.158	5.93	271.70	0.249	5.95	482.90	0.472
SD	0.01	26.57	0.03	0.01	4.80	0.01	0.01	5.19	0.01
RSD (%)	0.13	0.88	0.89	0.14	1.77	2.04	0.14	1.07	1.16
Repeatability*(Δ_{max})			0.107			0.022			0.023
Repeatability limit*			0.216			0.065			0.077

* calculated according to ASTM D7347-07, section 13.1.1

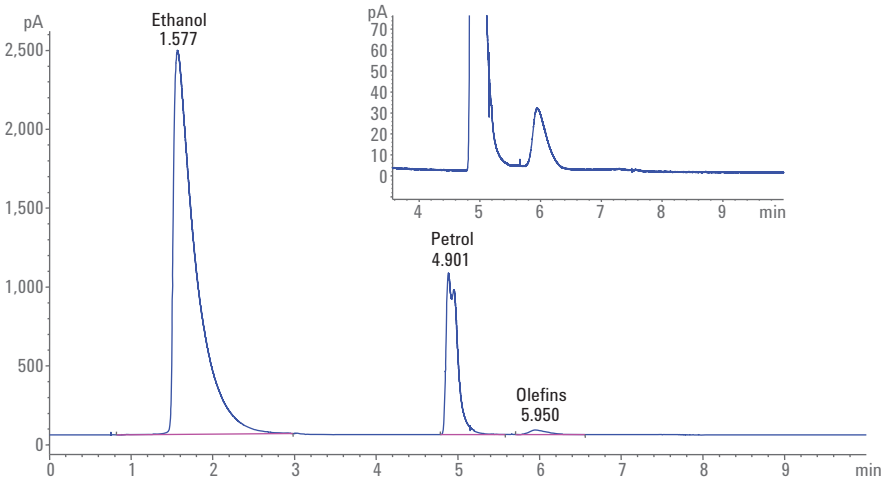

Figure 10. Sample of denatured ethanol (9.5 % petrol) spiked with 0.5 % olefins.

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Agilent Technologies

Determination of Aromatic Content in Diesel Fuel According to ASTM D5186

Enhancing the Agilent 1260 Infinity Analytical SFC System with a Flame Ionization Detector

Application Note

Energy & Chemicals

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Abstract

This Application Note describes the determination of the aromatic content of diesel fuel using the Agilent 1260 Infinity Analytical SFC system with the SIM flame ionization detector (FID). Combining the SFC system with this developed FID system meets all requirements of the ASTM D5186 method such as detector accuracy and linearity. This combination offers a cost-effective and fast alternative to the existing normal phase HPLC methods D1319 and D2425.

Agilent Technologies

Introduction

SIM Scientific Instruments Manufacturer GmbH, Oberhausen, Germany, has developed a flame ionization detector (FID) for use with the Agilent 1260 Infinity Analytical SFC system. This combination extends the range of applications, for example, to meet petrochemical requirements as described in the ASTM method D5186. Control of the FID is possible through Agilent OpenLAB CDS ChemStation Edition Software with the appropriate electronic components and the FID software module. The development of the FID was done by taking particular account of detector accuracy and linearity as well as integration in the 1260 Infinity Analytical SFC system. The starting point of the FID development was the determination of aromatic compounds in diesel fuels as published in the ASTM method D5186. This approach was taken so that system performance could be verified according to the requirements in this test method. This enables the separation of monoaromatics and polynuclear aromatics in fuel samples using an FID. An important challenge to be solved was the contamination of the backpressure regulator (BPR) with high-melting compounds such as naphthalene. This challenge was overcome by using Channel B of a binary pump to purge the BPR continuously with hexane.

Diesel and aviation turbine fuels contain nonaromatic, mono- and polyaromatic hydrocarbons. Best performance and maximum lifetime of an engine is achieved when the amount of aromatics is as low as possible. Since the aromatic hydrocarbon content can affect the cetane number of fuels and cause emissions due to incomplete burning, there are different regulations to protect the environment and public health. Examples of such regulations are those from the United States Environmental Protection Agency (USEPA) and the California Air Resources Board (CARB) as well as self-regulation of distilleries for process and quality control.

The American Society for Testing and Materials (ASTM) published the test method D5186 to determine the aromatic and polynuclear aromatic content of diesel fuels and aviation turbine fuels by supercritical fluid chromatography with flame ionization detection. This test method shows clear benefits compared with test methods D1319 and D2425 because it is:

- Applicable for an expanded application range
- Statistically more precise than or at least as precise as other methods
- Unaffected by fuel coloration
- Not as expensive and time-consuming to perform

The method is applicable to samples containing total aromatics in the range of 1 to 75 mass %, and polyaromatics in the range of 0.5 to 50 mass %[1-3].

Instrumentation

An Agilent 1260 Infinity Analytical SFC system with the following configuration was used:

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity Thermostatted Column Compartment
- CTC Analytics LC-Injector HTC PAL (50-cm width) modified with 4-port valve (internal 0.5-µL loop) and DLW
- SIM flame ionization detector

The complete solution can be ordered through SIM Scientific Instruments Manufacturer GmbH, Oberhausen, Germany.

Experimental

Chemicals and solutions

Samples and standards were prepared according to guidance published in the ASTM method.

FID gases	Hydrogen, air, and nitrogen (as make-up gas)
Eluent	Carbon dioxide (purity > 99.995 %, pressurized in a cylinder without DIP tube, according to the specifications of the SFC module)
Purge solution for back pressure regulator	Hexane (not used as modifier! Pumped with channel B of the binary pump)
Performance mixture	Quantitative mixture prepared according to section 7.6 of the ASTM method from fine chemicals (Sigma Aldrich) with the following composition (approximate values): 75 mass % hexadecane (*n*-C_{16}) 20 mass % toluene (T) 3 mass % tetralin (1,2,3,4-tetrahydronaphthalene, THN) 2 mass % naphthalene (N)
Diesel sample for linearity check	Diesel sample, aromatic content 21.8 % (according to manufacturer's analysis results)

Analytical column

YMC-PACK-SIL_06, 250 × 4.5 mm, S-6 nm, 5 µm (YMC America, Inc., Allentown, PA, USA), or
Agilent ZORBAX RX-SIL,
4.6 × 250 mm, 5 µm (p/n 880975-901)

Restrictor

PEEKsil, 100-µm inside diameter, 20 cm (for hexane purging path)

Software

Agilent OpenLab CDS ChemStation Edition, Rev. C.01.05

SFC/FID Configuration

The outlet of the column is connected to the upper T-piece of the FID (Figure 1). To purge the backpressure regulator (BPR), hexane is pumped continuously through Channel B of the binary pump. To maintain constant backpressure and a continuously operating system, a restriction capillary is integrated between pump head B and the lower T-piece of the FID (Figure 1).

LC Method

Agilent 1260 Infinity Binary SFC Pump		
Solvent A	CO_2 (precompressed), 99,995 %	
Solvent B	Hexane, 0.5 %, used for purging the backpressure regulator (BPR) only, not as eluent. Depending on the composition of the sample, it might be necessary to purge the BPR with a higher amount of hexane (for example, setting in the software to 50 % eluent B) from time to time.	
Flow rate	1.8 mL/min	
Agilent 1260 Infinity Thermostatted Column Compartment		
Column temperature	25 °C	
LC-Injector HTC PAL		
Injection volume	0.5 µL	
Injection cycle	Preclean with Solvent 1 (hexane)	1
	Preclean with Sample	1
	Filling speed (µL/s)	10
	Filling strokes	3
	Inject to	LC Vlv1
	Injection speed (µL/s)	5
	Pre-inject delay	500 ms
	Post-inject delay	500 ms
	Post clean with Solvent 1 (hexane)	2
	Valve clean with Solvent 1	1
	(all other parameters not listed here have the value zero)	
FID		
Temperature	300 °C	
Gases		
Hydrogen (H_2)	50 mL/min	
Air	500 mL/min	
Make-up gas (N_2)	50 mL/min	

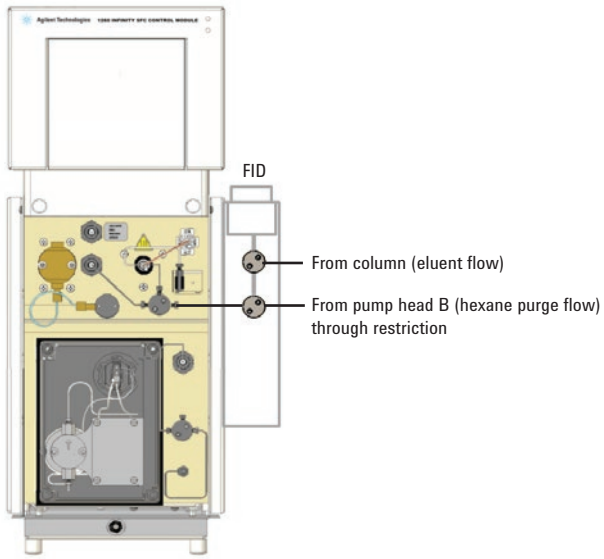

Figure 1. Connection of the SFC/FID module with the LC instrument.

Results and Discussion

In the following, the compliance with the requirements of the system performance listed in section 8.2 of the ASTM test method is shown. The areas in the chromatogram are integrated corresponding to a performance mixture of hexadecane, toluene, tetrahydronaphthalene (THN), and naphthalene (N). The mass % content of each of these groups in the fuel is calculated by area normalization.

FID sensitivity

The first step, according to the ASTM method, was to show that the FID sensitivity is sufficient to detect 0.1 mass % toluene in hexadecane (see ASTM section 6.1). Figure 2 illustrates that the FID exceeds this requirement.

The performance mixture (PFM), according to ASTM section 7.6, is used to determine most of the method acceptance criteria such as resolution, retention time reproducibility, and detector accuracy. Moreover, it is used to determine the integration marks for performing the grouping and integration of the complex fuel samples.

Resolution

Figure 3 shows the resolution between nonaromatics (hexadecane) and monoaromatics (toluene), referred to as R_{NM}. According to ASTM D5186 a R_{NM} value of at least 4 is required, but a value of 10 was determined for the actual experiment. Also, the resolution between mono-(tetralin) and polynuclear (naphthalene) aromatics, referred to as R_{MD}, is twice as high as required (see ASTM method, section 8.2.1).

Retention time reproducibility

Retention-time reproducibility is also determined with the PFM. It should not be higher than 0.5 % RSD for hexadecane and toluene peaks. This is important because the integration marks for the diesel sample are determined from the analysis of the PFM. Figure 4 shows an overlay of five chromatograms as well as the assignment of the areas to the corresponding diesel fractions.

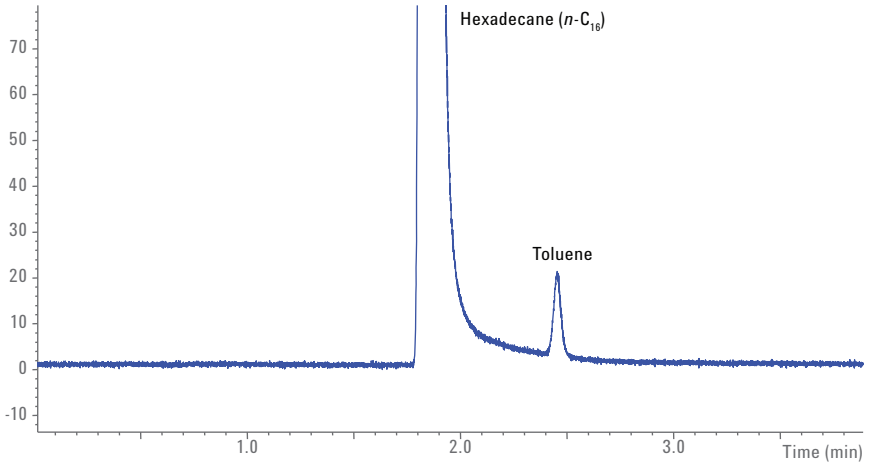

Figure 2. Chromatogram detail of a solution with 0.1 mass % toluene in hexadecane (n-C_{16}).

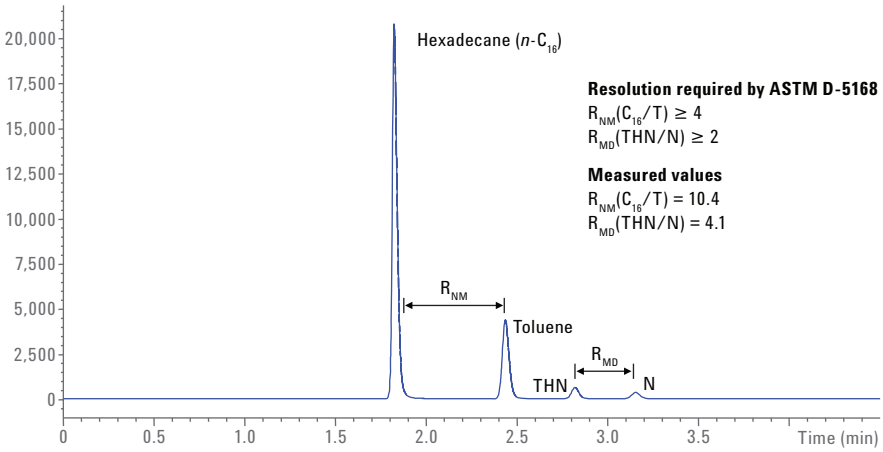

Figure 3. Determination of the resolution values for RNM (hexadecane to toluene) and RMD (THN to N) using the performance mixture.

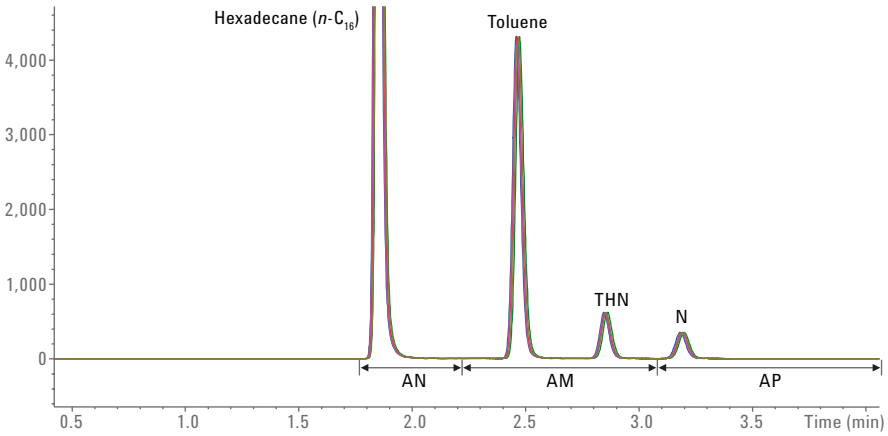

Figure 4. Overlay of five chromatograms of the PFM with the assignment to the fractions of a diesel sample (AN = Area of nonaromatics, AM = Area of monoaromatics, AP = Area of polyaromatics).

Table 1 lists the calculated values for all four substances of the PFM. The relative standard deviations (RSDs) for the retention time (RT) were all in the range of 0.3 %, so that all values fit the acceptance criterion.

Detector accuracy test

To check the assumption that the FID response approximates to the theoretical unit carbon response, the response factors relative to hexadecane (RRF) were calculated for each of the components in the performance mixture. The measured RRF for each component must be within ±10 % of the theoretical value when it is assumed that the FID response approximates to the theoretical unit carbon response. The calculated RRF values were within these limits, as summarized in Table 2.

Detector linearity check

A diesel sample was used for this check. Neat fuel and two dilutions (containing fuel and *n*-C_{16} in proportions of 1:1 and 1:3) were analyzed according to ASTM section 9. Determination of the mass % aromatics in the two dilutions, and comparison with their corresponding expected aromatics results was used to verify detector linearity. Figure 5 shows an overlay of these three diesel samples. Integration marks were set according to the chromatogram of the PFM (Figure 4) to determine the content of mono- and polyaromatics. The sum of both areas is the content of total aromatics in the diesel sample (given in mass %).

Table 3 shows the aromatic content of the diesel sample and its weighed dilutions. The difference between the measured and expected values agree to within the repeatability limits stated in 13.1.1 of the ASTM method, so that the FID linearity could be verified.

Table 1. Retention time reproducibility of PFM (average of 10 runs, *n*-C_{16} = Hexadecane, T= Toluene, THN = 1,2,3,4-tetrahydronaphthalene, N = Naphthalene)

	n-C_{16}	T	THN	N
RT average (n = 10)	1.83	2.44	2.82	3.16
RT SD	0.004	0.007	0.008	0.009
RT RSD %	0.24	0.28	0.29	0.30

Table 2. Determination of the relative response factors (RRF) of the performance mixture (average of 10 runs).

RRF value	Minimum	Maximum	Calculated*	Compliance to test method
RRF (toluene)	0.9675	1.1825	1.0753	Yes
RRF (tetrahydronaphthalene)	0.9630	1.1825	1.0420	Yes
RRF (naphthalene)	0.9936	1.2144	1.0840	Yes

* Average based on 10 injections, all within the given limits.

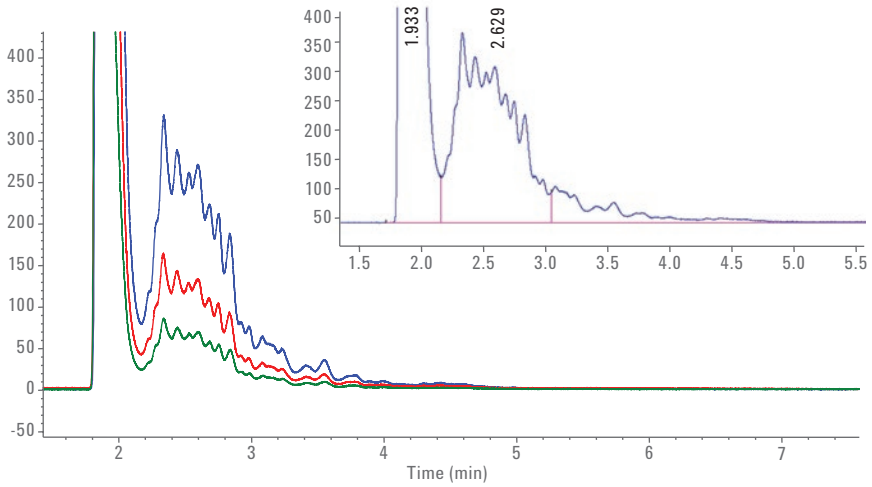

Figure 5. Chromatogram of the diesel sample (blue) and its 1:1 (red) and 1:3 (green) dilution; the inset on the right shows the setting of the integration marks corresponding to the PFM.

Table 3. Total aromatic content of a diesel fuel.

Dilution	Measured aromatics (mass %)*	Expected aromatics (mass %)	Aromatics deviation (mass %)
Neat	21.8		
1:1	11.1	10.8	0.3
1:3	5.9	5.7	0.2

*average based on 10 injections

Analysis of diesel samples

To see the repeatability of the diesel sample measurements, Figure 6 shows an overlay of 10 chromatograms. If fatty acid methyl esters (FAMEs) are present as a biodiesel additive, a longer run time is required to elute the FAMES from the column.

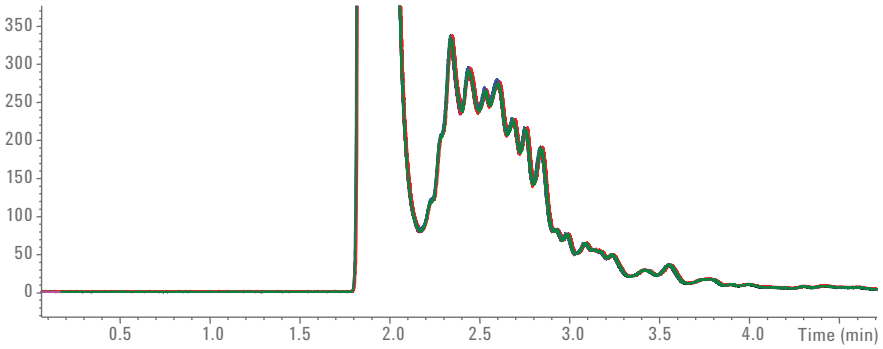

Figure 6. Overlay of 10 chromatograms of a diesel sample.

Conclusion

The performance of the Agilent 1260 Infinity Analytical SFC system with the SIM FID meets the requirements of ASTM D5186 for the determination of aromatics in diesel fuel. Specifically, the detector accuracy and linearity tests show the suitability of the SIM FID for the determination of aromatic substances with the 1260 Infinity Analytical SFC system. The system is equipped with continuous purging of the backpressure regulator to ensure trouble-free and reliable operation of the system.

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