

# Orthogonal Chromatographic Separations using the Agilent 1260 Infinity II SFC/UHPLC Hybrid System

Suitable for Agilent  
1260 Infinity III LC

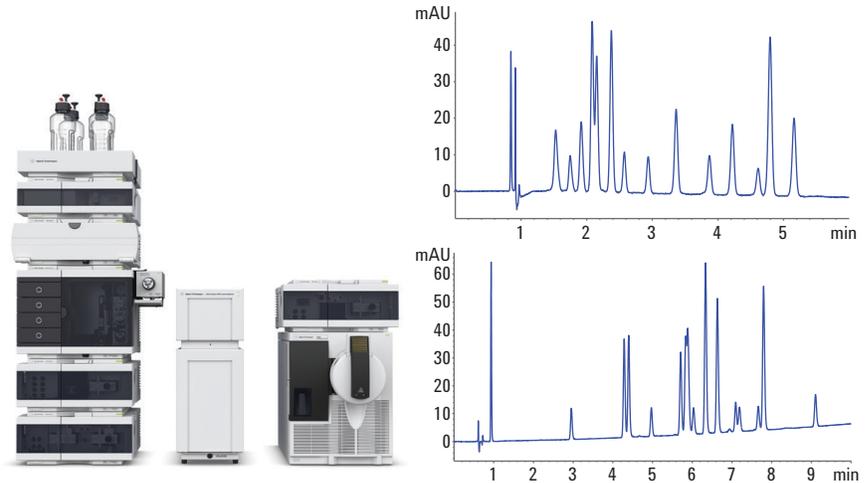
## Technical Overview

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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<sub>2</sub>/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 <sub>2</sub>
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-position/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-position/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,

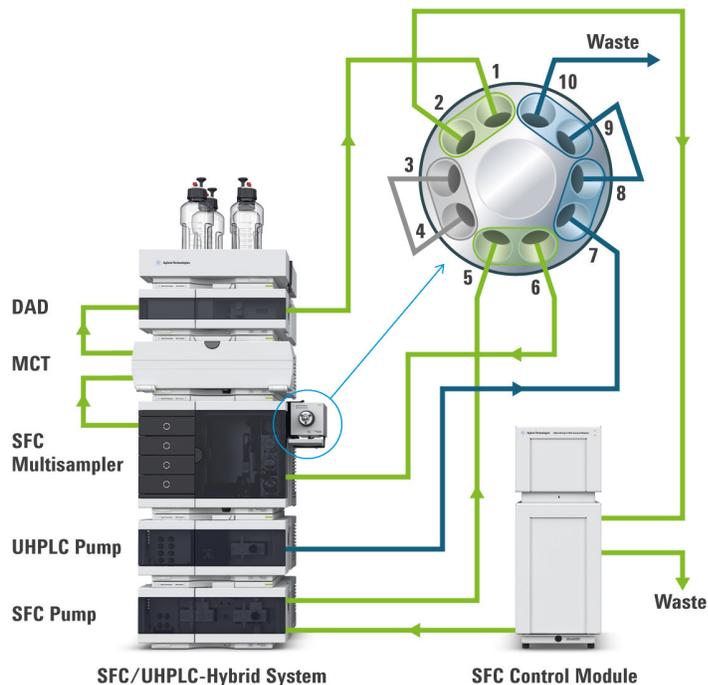


Figure 1. Agilent 1260 Infinity II SFC/UHPLC Hybrid System in SFC Mode. The SFC Control Module is connected to the SFC pump and the shared modules (green tubing). The UHPLC pump is connected to the waste position (blue tubing).

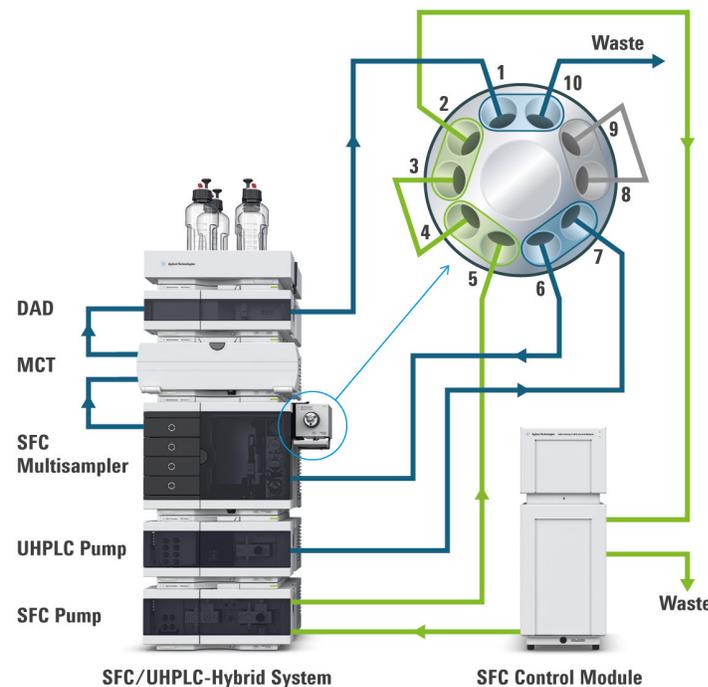


Figure 2. Agilent 1260 Infinity II SFC/UHPLC Hybrid System in UHPLC Mode. The UHPLC pump is connected to the shared modules (blue tubing). The SFC pump is directly connected to the SFC Control Module in a loop (green tubing).

and the detector, the CO<sub>2</sub> 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-position/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-position/10-port valve to include the SFC make-up flow 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<sub>2</sub> 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.

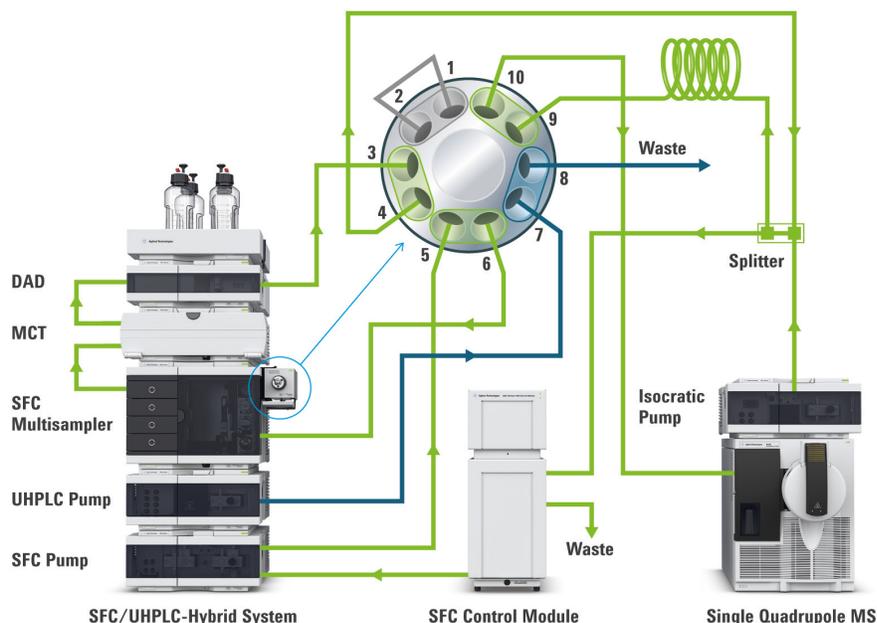


Figure 3. Agilent 1260 Infinity II SFC/UHPLC/MS Hybrid System in SFC/MS mode. The SFC Control module is connected to the SFC pump and the shared modules (green tubing). The UHPLC pump is connected to the waste position (blue tubing). The CO<sub>2</sub> stream is connected to the first splitter for the 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).

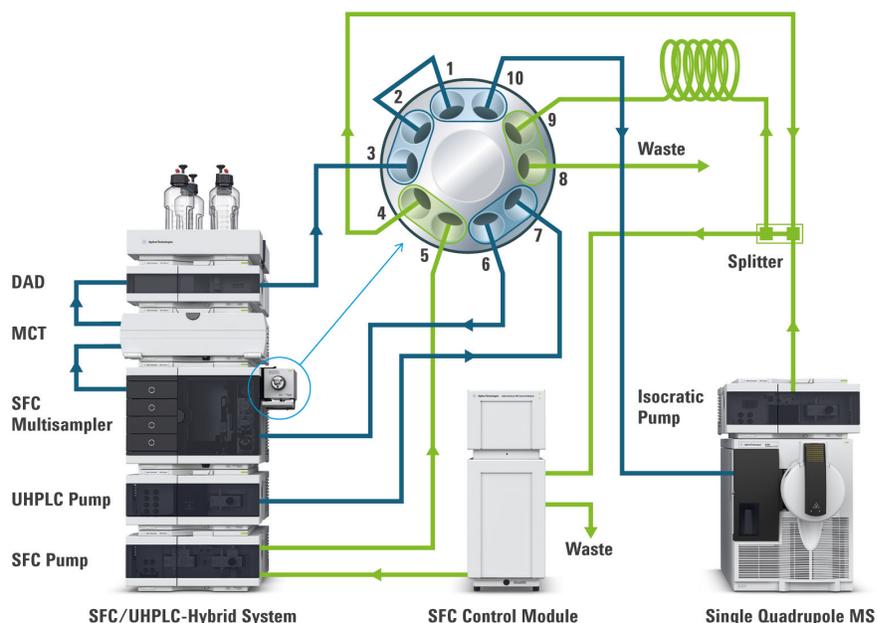


Figure 4. Agilent 1260 Infinity II SFC/UHPLC/MS Hybrid System in UHPLC/MS Mode. The UHPLC pump is connected to the shared modules (blue tubing) and in direct flow to the MS. The SFC pump is directly connected to the SFC control module in a loop (green tubing) including the splitter (coiled green capillary: 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<sub>2</sub>, 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 <i>m/z</i>
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	<i>m/z</i> [M+H] <sup>+</sup>	SFC RT (min)	LC RT (min)
1	Prometryn	C <sub>10</sub> H <sub>19</sub> N <sub>5</sub> S	242.10	1.528	5.880
2	Sebuthylazine	C <sub>9</sub> H <sub>16</sub> ClN <sub>5</sub>	230.11	1.748	7.190
3	Terbuthylazine	C <sub>9</sub> H <sub>16</sub> ClN <sub>5</sub>	230.11	1.917	7.664
4	Atrazine	C <sub>8</sub> H <sub>14</sub> ClN <sub>5</sub>	216.10	2.084	6.035
5	Metobromuron	C <sub>8</sub> H <sub>11</sub> BrN <sub>2</sub> O <sub>2</sub>	259.00	2.153	6.632
6	Methabenzthiazuron	C <sub>10</sub> H <sub>11</sub> N <sub>3</sub> O <sub>5</sub>	222.06	2.153	5.710
7	Linuron	C <sub>9</sub> H <sub>10</sub> Cl <sub>2</sub> N <sub>2</sub> O <sub>2</sub>	249.02	2.375	7.798
8	Terbuthylazine-desethyl	C <sub>7</sub> H <sub>12</sub> ClN <sub>5</sub>	202.08	2.572	4.971
9	Atrazine-desethyl	C <sub>6</sub> H <sub>10</sub> ClN <sub>5</sub>	188.06	2.939	2.954
10	Hexazinone	C <sub>12</sub> H <sub>20</sub> N <sub>4</sub> O <sub>2</sub>	253.16	3.362	4.400
11	Nimodipine	C <sub>21</sub> H <sub>26</sub> N <sub>2</sub> O <sub>7</sub>	419.18	3.870	9.107
12	Chlorotoluron	C <sub>10</sub> H <sub>13</sub> ClN <sub>2</sub> O	213.08	4.220	5.838
13	Nifedipine	C <sub>17</sub> H <sub>18</sub> N <sub>2</sub> O <sub>6</sub>	347.10	4.613	7.095
14	Diuron	C <sub>9</sub> H <sub>10</sub> Cl <sub>2</sub> N <sub>2</sub> O	233.02	4.795	6.333
15	Metoxuron	C <sub>10</sub> H <sub>13</sub> ClN <sub>2</sub> O <sub>2</sub>	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<sub>2</sub> 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<sup>2</sup> = 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<sup>1</sup>. 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<sup>2</sup>. 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<sup>1</sup>.

### 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.

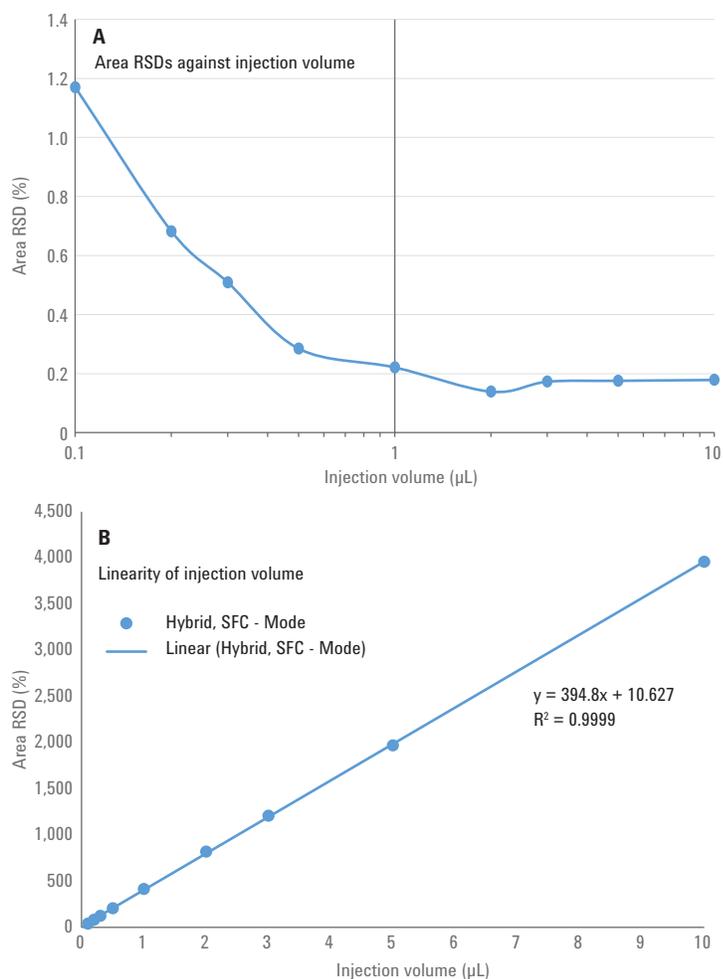


Figure 5. Performance in SFC mode. A) Determination of peak area RSDs in dependence of the injection volume (0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0, 5, and 10 µL). Area RSDs are typically below 0.3 % for all injection volume above 0.5 µL. B) Peak area linearity of injection volumes between 0.1 and 10 µL,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

The analytical range from 0.1 to 10  $\mu\text{L}$  injection volume showed an RSD performance of 0.2 % and lower for all injection volumes above 0.5  $\mu\text{L}$  (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  $\mu\text{L}$ . 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<sup>3</sup>.

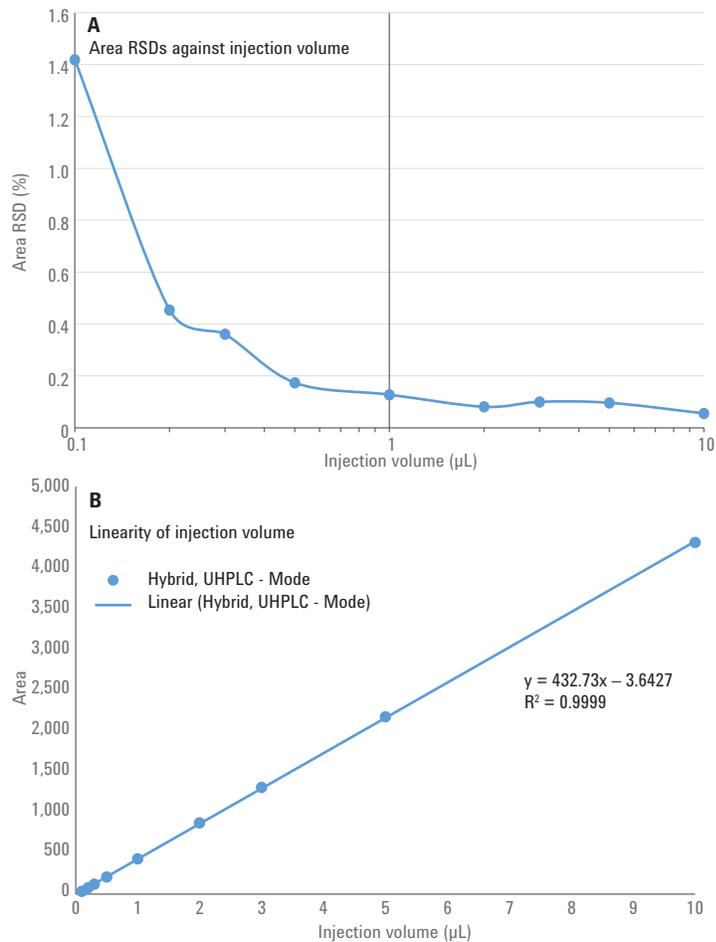


Figure 6. Performance in UHPLC mode. A) Determination of peak area RSDs in dependence of the injection volume (0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0, 5, and 10  $\mu\text{L}$ ). Area RSDs are typically below 0.2 % for all injection volume above 0.5  $\mu\text{L}$ . B) Peak area linearity of injection volumes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

## 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-position/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  $\mu\text{L}$  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.

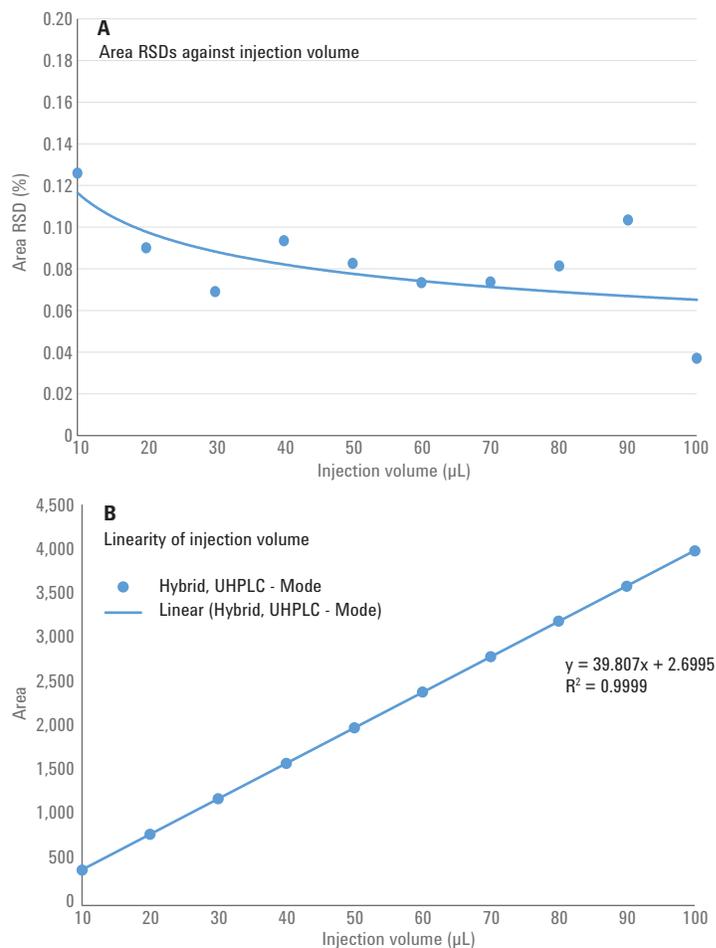


Figure 7. Performance in UHPLC mode for large injection volumes. A) Determination of peak area RSDs in dependence of the injection volume (10 to 100  $\mu\text{L}$ , step 10  $\mu\text{L}$ ). Area RSDs are typically below 0.12 %. B) Peak area linearity of injection volumes between 10 and 100  $\mu\text{L}$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

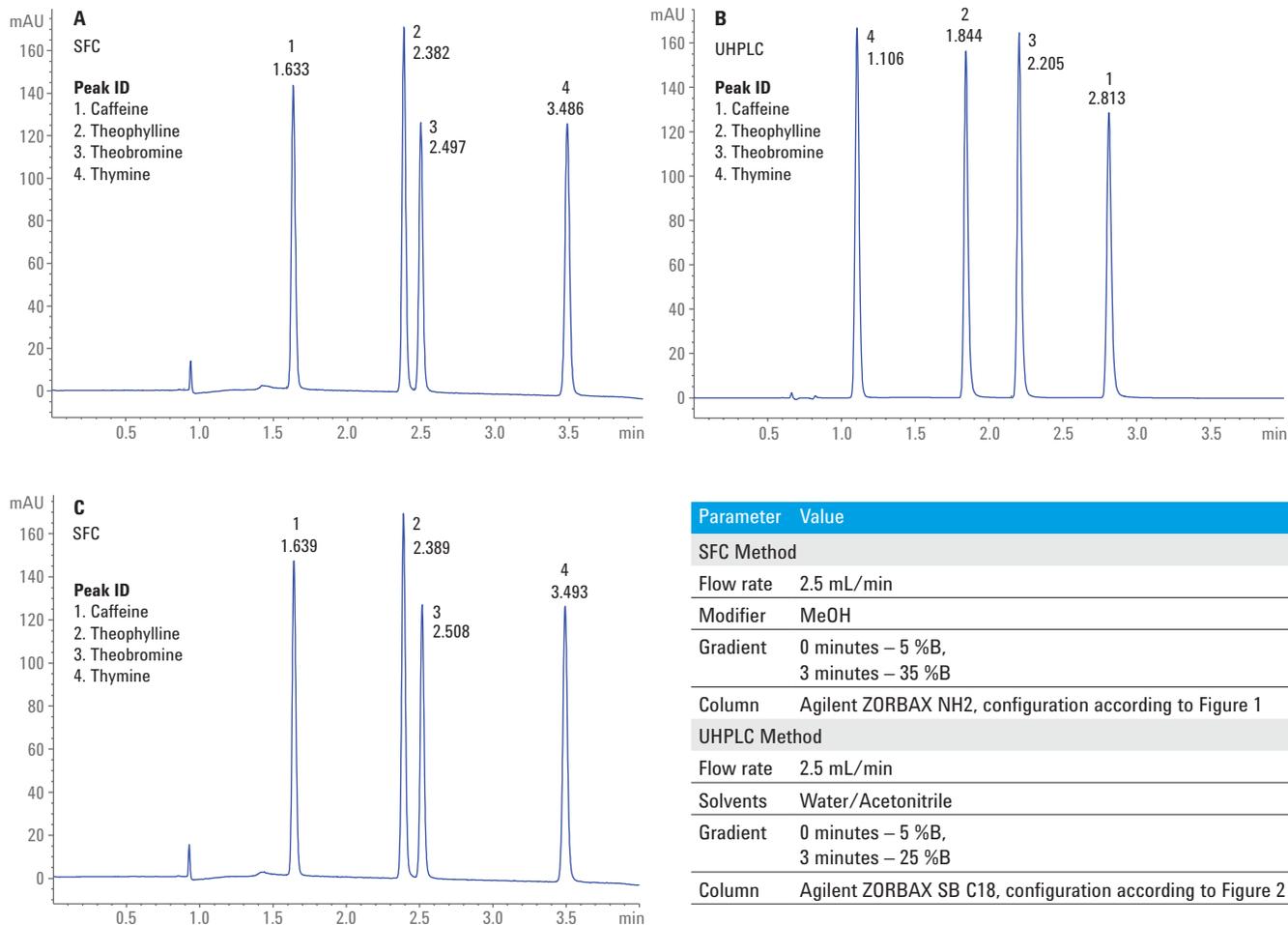


Figure 8. Sequence of multiple SFC and UHPLC runs on the SFC/UHPLC Hybrid System. The sequence started with replicate SFC runs (A) and continued with replicate UHPLC runs (B) after switching to a method with the described UHPLC method settings. Finally, multiple SFC runs were done at the end of the sequence (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.

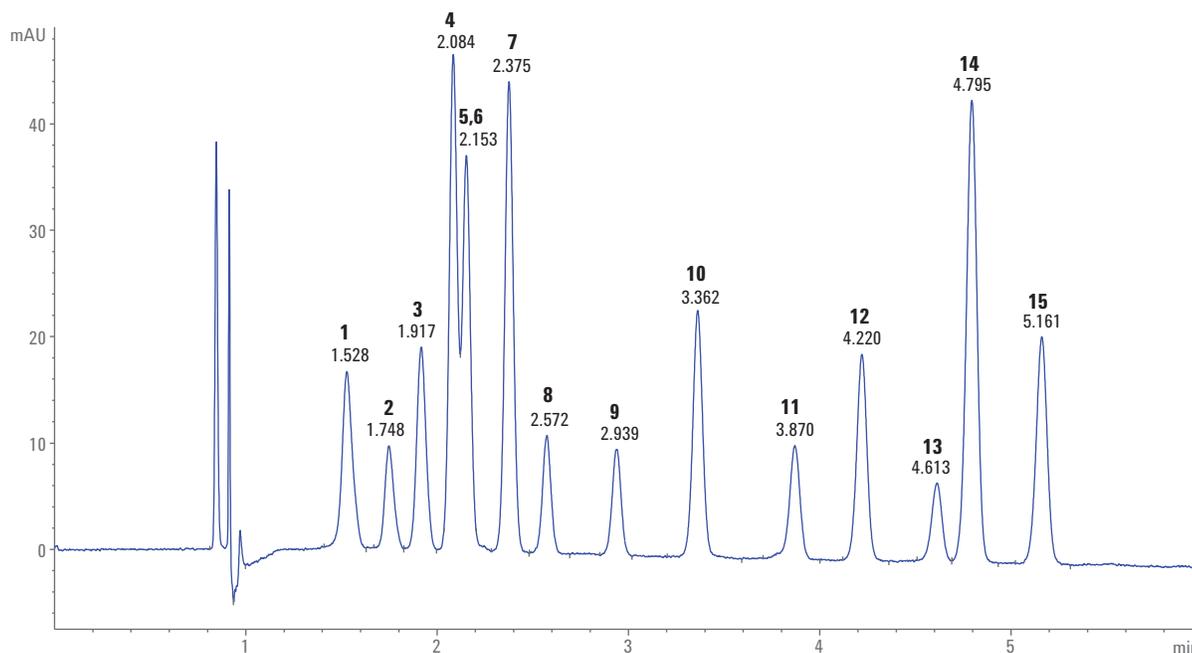


Figure 9. Separation of a complex 15 pesticide sample in SFC mode on an amino phase column with methanol as modifier (see the Experimental section).

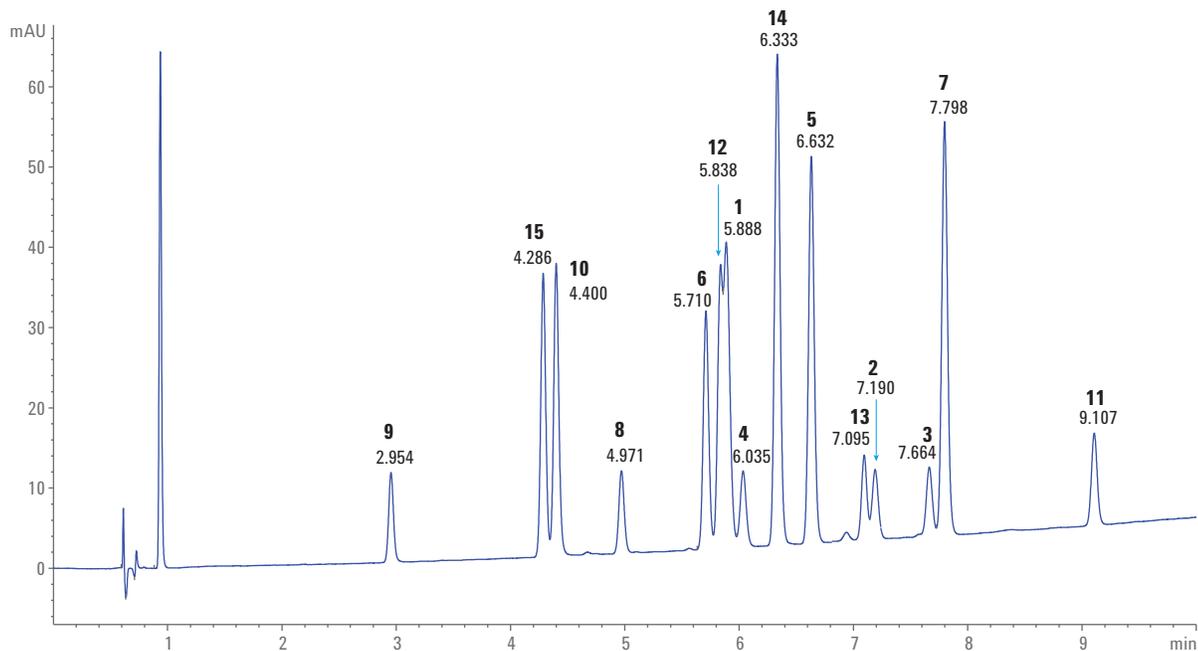


Figure 10. Separation of a complex 15 pesticide sample in UHPLC mode on a C18 phase column with water/acetonitrile as solvents (see the Experimental section).

## 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

1. Naegele, E. 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, *Agilent Technologies Technical Overview*, publication number 5991-7623EN, **2017**.
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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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