Analysis of Pesticides by Supercritical Fluid Chromatography/ Mass Spectrometry

Optimizing the Agilent 1260 Infinity Analytical SFC System in Combination with the Agilent 6460 Triple Quadrupole LC/MS

Application Note

Food Testing & Agriculture

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Abstract

This Application Note describes the development of a method for the Agilent 1260 Infinity Analytical SFC Solution for the separation of a multi-pesticide sample in a short run time, which is faster than typical HPLC methods. Connecting the SFC to the Agilent 6460 Triple Quadrupole LC/MS System and the optimization of the necessary makeup flow is discussed, as well as MRM and MS source parameters. For the final method, important performance parameters such as limits of detection (LOD), limits of quantification (LOQ), retention time, and area RSD are determined.

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Introduction

The new generation of supercritical fluid chromatography (SFC) instruments offers robustness and performance that compare well to HPLC instruments. SFC's mobile CO_2 phase has a lower viscosity, increased diffusion, and better mass transfer capabilities compared to liquid phases, enabling higher separation speed while maintaining excellent separation and good peak shape. The sensitivity of measurement by SFC is comparable to sensitivities achievable by HPLC. Modern SFC instruments are easily connected to mass spectrometers such as triple quadrupole LC/MS instruments for quantification, which allows the use of SFC separation for applications that typically benefit from MS detection.

This Application Note describes the development of an SFC method for the separation of a 17-pesticide mix in 5 minutes by means of the Agilent method-scouting wizard. In addition, a robust instrument configuration to connect the Agilent 1260 Infinity Analytical SFC to modern triple quadrupole MS instruments for the measurement of multi-pesticide samples is shown. The optimization of the makeup flow necessary for the connection of the SFC to the MS is described. On the MS side, the automated optimization of MRM and source parameters are also shown. Data on the LOQs, LODs, linearity, retention time, and area RSD for the individual compounds are presented. The advantage of SFC compared to HPLC is the good separation of a larger number of compounds in a faster run time.

Experimental

The Agilent 1260 Infinity Analytical SFC Solution (G4309A) comprises:

- 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 1290 Infinity Thermostatted Column Compartment with valve drive
- Agilent 1260 Infinity DAD with high pressure SFC flow cell

For MS analysis and connection the following instruments are required:

- Splitter kit (p/n G4309-68715)
- Agilent 1260 Infinity Isocratic Pump (G1310B)
- Agilent 6460 Triple Quadrupole LC/MS System (G6460A)

Also required to run the SFC system for automated method development:

- Agilent 1290 Infinity Thermostatted Column Compartments (G1316C) with valve drive
- Two Agilent 1200 Infinity Series Quick-Change 8-position/9-port valves (G4230A)
- Agilent 1290 Infinity Valve Drive (G1170) with Agilent 1200 Infinity Series Quick-Change 12-position/13-port valve (G4235A)
- Capillary kit for method development (p/n 5067-1595)

Instrument setup

The recommended configuration of the Agilent 1260 Infinity Analytical SFC Solution with the Agilent 6460 Triple Quadrupole LC/MS is shown in Figure 1. The column is directly connected to a splitter assembly, which contains two combined splitters (and an additional check valve to prevent backflush of CO_2 into the makeup pump, and a solvent filter). At the first splitter, the makeup flow coming from an isocratic pump is introduced into the flow path. This splitter is connected to the second one by a short 0.12-mm id capillary. Here, the flow is split in two, one part going to the MS and the other to the backpressure regulator (BPR) of the SFC module. The connection to the MS is made by an innovative 50-µm id stainless steel capillary 1 m long, which is included in the splitter kit.

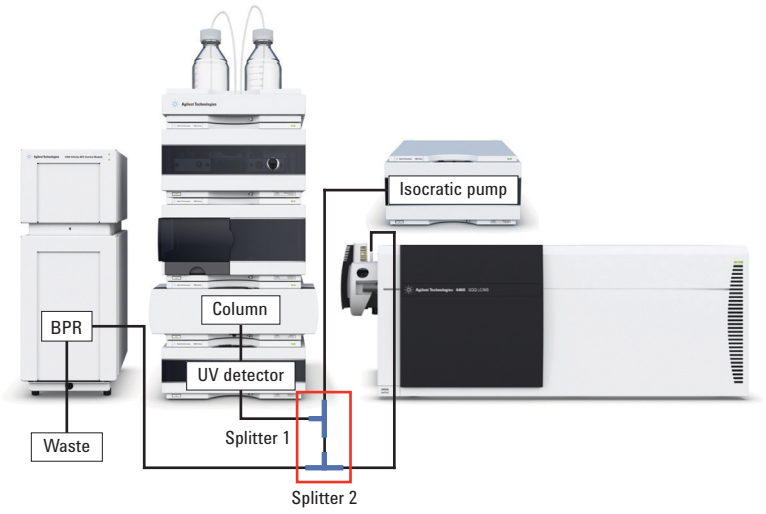


Figure 1. Configuration of the Agilent 1260 Infinity Analytical SFC Solution with the Agilent 6460 Triple Quadrupole LC/MS System. The column is directly connected to Splitter 1 in the splitter assembly (BPR = backpressure regulator, UV detector not used).

The split ratio depends on the backpressure generated by this restriction capillary and the pressure set by the BPR. As a general rule, 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. Since electrospray MS is concentration-dependent this has no influence on signal intensity.

Columns

- Agilent ZORBAX Rx-SIL, 4.6 × 150 mm, 5 µm (p/n 883975-901)
- Agilent ZORBAX SB-CN, 4.6 × 150 mm, 5 µm (p/n 883975-905)
- Agilent ZORBAX NH2, 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 MRM and Source Optimizer Software, Version 06.00
- Agilent MassHunter Qualitative Software, Version 06.00
- Agilent MassHunter Quantitative Software, Version 07.00
- Agilent OpenLAB CDS ChemStation Edition for LC & LC/MS Systems, Rev. C.01.06 with Agilent ChemStation Method Scouting Wizard, Version A.02.04, (G2196AA).

Method parameters

SFC method (final conditions in bold)	
SFC flow	**3 mL/min**
SFC gradient 1	0 minutes - 2 % B to 15, 10, 5 minutes – 50 % B Stop time 15, 10, 5 minutes Post time 2 minutes
SFC gradient 2	0 minutes - 2 % B to 5 minutes – 15 % B to 6 minutes – 15 % B Stop time 6 minutes Post time 2 minutes
SFC gradient 3	**0 minutes - 2 % B to 5 minutes – 20 % B** **Stop time 5 minutes** **Post time 2 minutes**
Modifier	**Methanol**, ethanol, isopropanol
BPR temperature	**60 °C**
BPR pressure	**120 bar**
Column temperature	**40 °C**
Injection volume	**5 µL, 3x loop overfill**
UV detection	220 nm, bandwidth 8 nm, ref. 360 nm, bandwidth 100 nm, data rate 10 Hz (not used in the final SFC/MS method)
Connecting SFC and MS by splitting and makeup flow (final conditions in bold)	
Makeup composition	**Acetonitrile + 0.2 % formic acid**
Makeup flow	0.1–1.0 mL/min, step 0.1 mL/min, **0.5 mL/min**
Flow gradient	**0 minutes – 0.5 mL/min to 5 minutes – 0.3 mL/min**
MS method (final conditions in bold)	
Ionization mode	**Positive**
Capillary voltage	2,000–4,500 V; step 500 V, **2,500 V**
Nozzle voltage	0–2,000 V; step 200 V, **2,000 V**
Gas flow	5–13 L/min; step 1 L/min, **8 L/min**
Gas temperature	160–340 °C; step 20 °C, **220 °C**
Sheath gas flow	8–12 L/min; step 1 L/min, **12 L/min**
Sheath gas temperature	200–400 °C; step 20 °C, **380 °C**
Nebulizer pressure	20–60 psi; step 5 psi, **25 psi**
MRM conditions (See Table 1)	

Standards

A standard mixture containing 10 ng/µL each of 17 pesticides in acetonitrile solution was obtained from LGC Standards GmbH (Pesticide Mix 44, p/n 18000044), Wesel, Germany.

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).

Results and Discussion

Optimizing the SFC separation

In the first step of the optimization of the SFC/triple quadrupole MS method, the SFC component was optimized by DAD detection using higher concentration pesticide samples (10 ng/µL of each compound in the mixture). The setup of different gradients for the automated screening was done using the Agilent ChemStation Method Scouting Wizard. For the scouting experiments, three different types of column (amino, silica, and cyano) and three solvents of increasing polarity (isopropanol, ethanol, and methanol) were used. To ensure that all pesticides eluted from the column at 50 % organic modifier at the latest, three gradients of increasing steepness up to 50 % modifier were used (Figure 2). It can be seen that the last compound eluted around 4.5 minutes for the long shallow gradient, and at about 2.8 minutes for the short steep gradient from the Agilent ZORBAX NH2 column using methanol as modifier (Figure 2A). The same experiment was done with ethanol as modifier with the result that compounds typically eluted later and showed some differences in resolution (Figure 2B). Due to very broad peaks and poor resolution, isopropanol was not a suitable modifier for this separation (not shown).

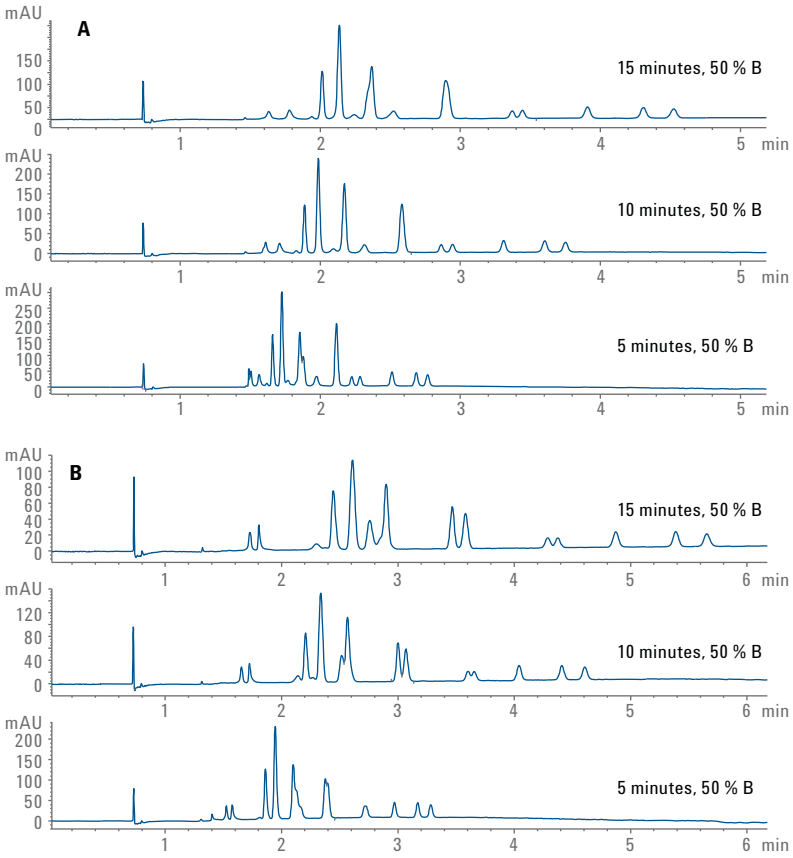


Figure 2. Separation of 17 pesticides on an Agilent ZORBAX NH2 column with three gradients of different steepness and methanol (A) or ethanol (B) as modifiers.

An identical screening was done on the silica and cyano columns. The results obtained with methanol are shown in Figure 3; other combinations provided no usable results. Compared to the amino column, the silica column delivered less retention and resolution (Figure 3A). On the cyano column, retention and resolution were even lower (Figure 3B). The screening of one column type against one solvent using the three different gradients took about 45 minutes. The complete screening of three columns against three solvents and three gradients was run in about 7 hours over night.

Because the resolution was sufficient even for the fast and steep gradients on the amino and silica columns with methanol as modifier, they were further optimized by lowering the final concentration of modifier (Figure 4). With a final concentration of 15 % methanol, the last peak eluted at about 5 minutes. The elution profile obtained from the amino column (Figure 4A) showed better resolution compared to the profile obtained from the silica column (Figure 4B). The final method went up to 20 % methanol in 5 minutes on the amino column.

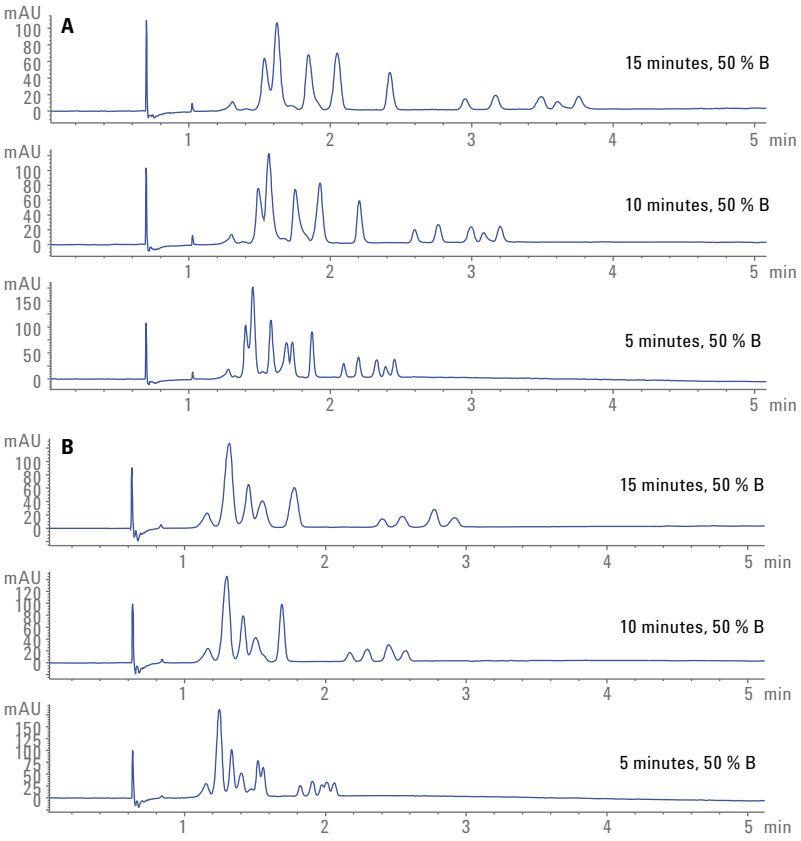


Figure 3. Separation of 17 pesticide compounds with three gradients of different steepness, and methanol as modifier, on Agilent ZORBAX RX-SIL (A) and Agilent ZORBAX SB-CN (B) columns.

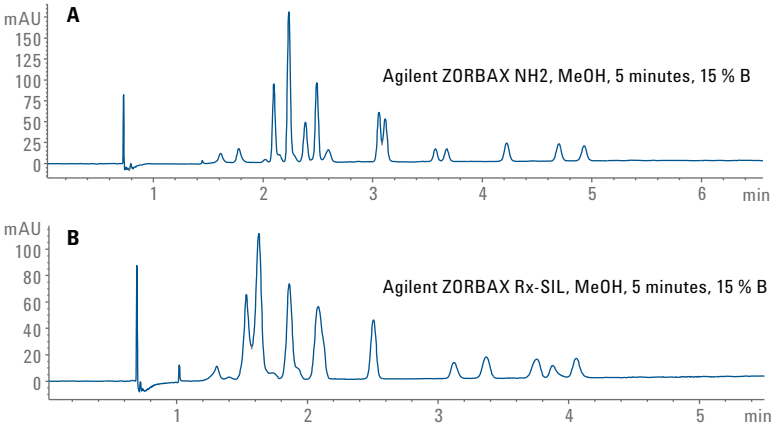


Figure 4. Separation of 17 pesticide compounds with methanol as modifier and a 5 minute gradient up to 15 % B on Agilent ZORBAX NH2 (A) and Agilent ZORBAX Rx-SIL (B) columns.

Optimizing the MRM settings

For the determination of the MRM settings of the triple quadrupole MS, using MassHunter Optimizer software, the makeup flow rate was adjusted to 0.3 mL/min, a value typically well suited for electrospray ionization. For the Agilent Jet Stream technology, typical source parameters used for LC/MS couplings were applied as a first approximation. The makeup flow rate and the source parameters were optimized later (see below). For the optimization, the complete suite of 17 pesticides was used and listed in the optimizer project. The pesticides were separated by the previously developed chromatographic SFC separation, and their fragmentor voltage, qualifier and quantifier ion, and collision energies were identified and optimized. This optimization took about 30 minutes per compound due to the chromatographic run time of 5 minutes per cycle.

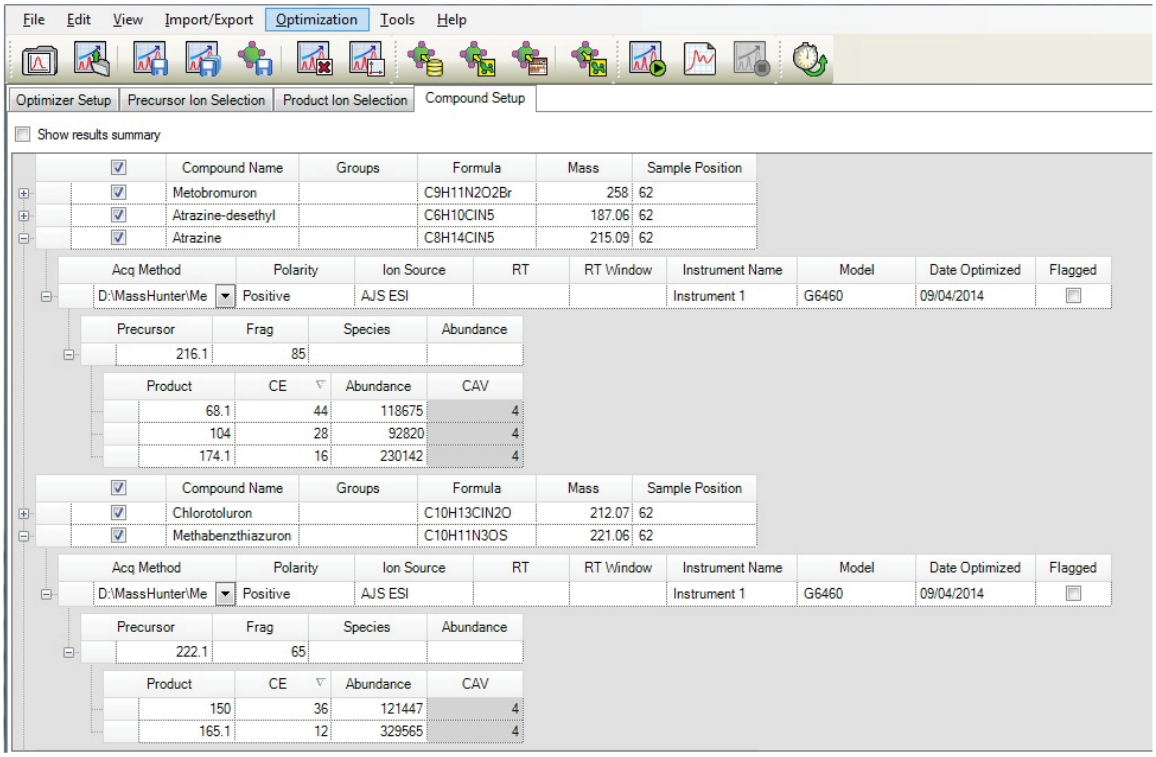


Figure 5. Compound setup screenshot of the Agilent MassHunter MRM Optimizer showing results from the optimization of precursor ions, fragmentor voltage, quantifier and qualifier ions, and collision energies. The image shows detailed optimization results for atrazine and methabenzthiazuron

As an example, the software screenshot with optimized values for atrazine and methabenzthiazuron is shown in Figure 5. The MRM values calculated for all compounds are listed in Table 1.

Table 1. MRM conditions for pesticides in the mixture obtained from Agilent MassHunter MRM Optimizer Software (dwell time, 10 ms; cell acceleration voltage, 5 V).

	Precursor ion (*m/z*)	Fragentor (V)	Product ion 1 (*m/z*)	Collsion energy (eV)	Product ion 2 (*m/z*)	Collsion 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
Atrazine-desethyl	188.1	90	146.0	16	104.0	24

Optimizing the splitting connection from SFC to MS

For the splitting approach, the flow going to the ion source depends on the backpressure applied on the SFC side and the resistance of the connection capillary (50 µm × 100 mm) from the splitter assembly to the MS ion source. In the setup described, an SFC backpressure of 120 bar diverts about 0.45 mL/min of the SFC flow to the ion source, and 200 bar backpressure would divert about 0.6 mL/min. The amount of split solvent depends only on the SFC backpressure and the capillary, and not on the flow rate coming from the SFC. Since electrospray LC/MS is a concentration-dependent detector, this has only a minor influence on the signal area. The makeup flow can be used to deliver the ionization reagent, which might not be part of the SFC modifier, and can stabilize the spray by keeping the organic solvent composition at a constant level. For optimization of the makeup, flow rates between 0.1 and 1.0 mL/min (acetonitrile, including 0.2 % formic acid for ionization) were applied to the split SFC effluent obtained from the optimized chromatographic separation. On the MS side, the optimized MRM transitions and default LC/MS settings for Agilent Jet Stream were applied. At a makeup flow rate of 0.1 mL/min, no ionization was obtained for the early-eluting compound metolachlor (Figure 6). Metazachlor showed some weak ionization due to the increase of organic coming from the SFC side by the progressing gradient. With an increase in makeup flow, the ionization of the early-eluting peaks increased up to a maximum at about 0.4 to 0.6 mL/min. Further increase in makeup flow rate decreased the peak heights again. Methabenzthiazuron, eluting in the middle of the run, was almost unaffected. The sensitivity of the late-eluting pesticide hexazinone declined slightly due to dilution effects by an increase of organic content from makeup and high organic content at the end of the run. To compensate for this effect, and to provide constant organic composition over the complete run, a flow gradient from 0.5 mL/min at the beginning of the gradient to 0.3 mL/min at the end was introduced in the final method.

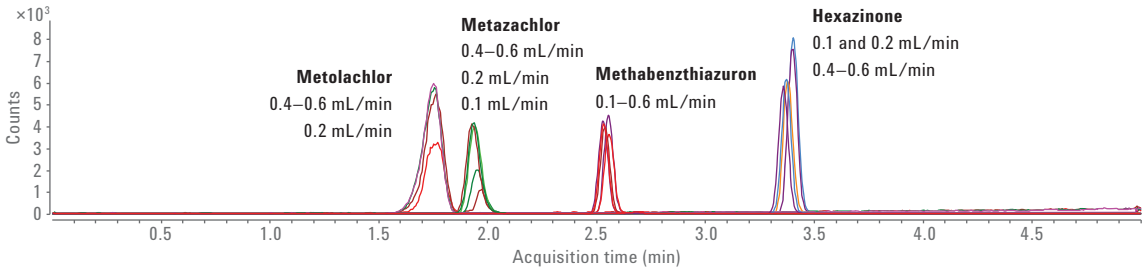


Figure 6. Optimization of makeup flow rate for early eluting metolachlor and metazachlor, medium eluting methabenzthiazuron, and late eluting hexazinone. Quantifier MRMs are shown as overlays for 0.1, 0.2, 0.4, 0.5, and 0.6 mL/min makeup flow.

Optimizing Agilent Jet Stream

Agilent MassHunter Source Optimizer was used to optimize the ion source conditions for the typical SFC effluent conditions. This specialized software allows the lowest and highest values and an incremental step size to be set for all source parameters, to cover the optimization range of each parameter (Figure 7). All necessary methods were automatically created and sent to the MassHunter Acquisition Software as a sequence of runs. In this case, the complete sequence for the optimization of all relevant source parameters under the final chromatographic conditions took approximately 22 hours.

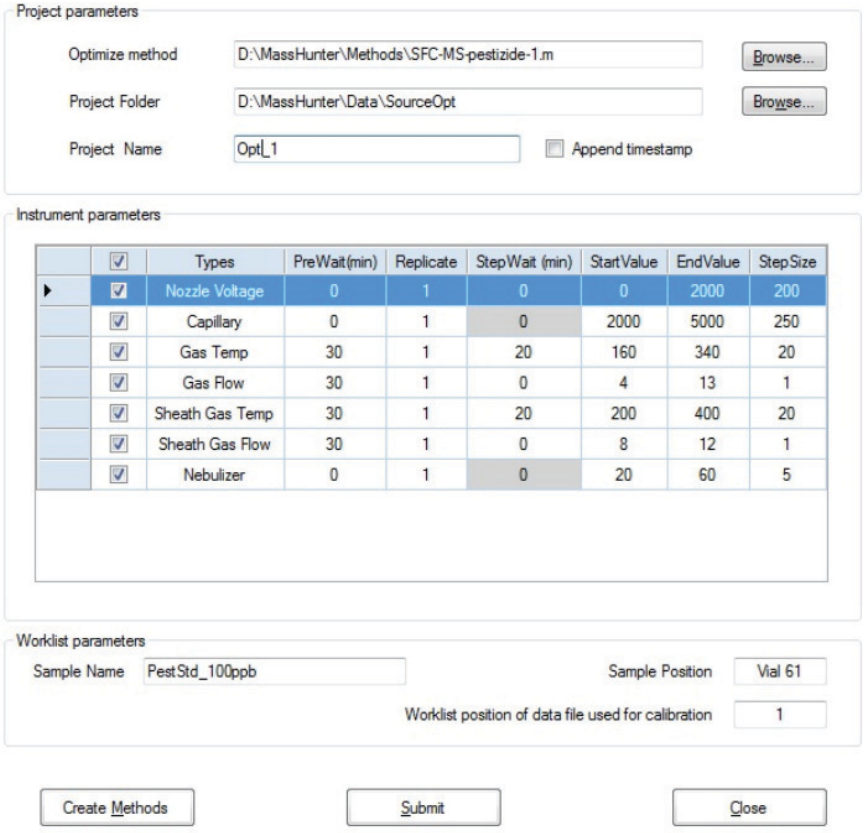
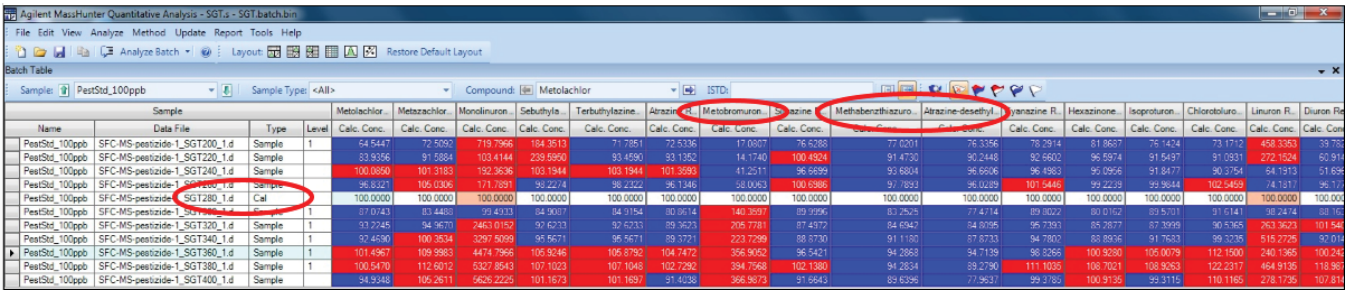


Figure 7. Software screenshot of Agilent MassHunter Source Optimizer Software, showing the different source parameters to optimize, their start and end values, and the size of the incremental step. The created methods are submitted to the MassHunter Acquisition Software and are run as a sequence.

Analysis of the rather large amount of data was fast and straightforward using MassHunter Quantitative Software. For this purpose, one of the data points was defined as 100 % in a single-point calibration and all other data points were compared to it, easily reviewable in a color-coded table (Figure 8). A sheath gas temperature of 280 °C was taken as the reference value for the Agilent Jet Stream (Figure 8A). It is clearly evident that metobromuron, for example, would provide higher intensities at higher temperatures, while the peak areas of methabenzthiazuron and atrazine desethyl have an optimum sensitivity at 280 °C. An increase of the sheath gas temperature up to 380 °C reveals an intensity optimum for most of the pesticides, such as metobromuron (Figure 8B), with an acceptable loss in intensity of up to 10 % for methabenzthiazuron and atrazine desethyl.

With this approach, each source parameter was adjusted to the optimum for the majority of compounds or the lower-responding compounds.

A



B

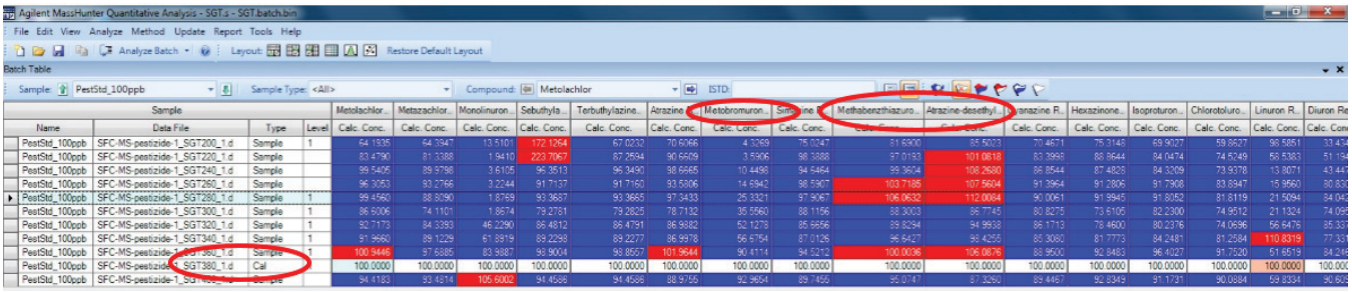


Figure 8. Analysis of the data created by the methods obtained from Agilent MassHunter Source Optimizer for the optimization of the Agilent Jet Stream sheath gas temperature from 200 to 400 °C, with a step size of 20 °C. A) Sheath gas temperature of 280 °C as the reference (100%) for a single-point calibration and compared to the values obtained for other temperatures. B) Same data with a sheath gas temperature of 380 °C as reference value. Lower values in blue and higher values in red.

Performance of the optimized SFC/triple quadrupole mass spectrometer method

With the optimized SFC method, nearly all compounds were clearly separated. In only two cases, two compounds eluted at the same time, namely terbuthylazine and atrazine, at 2.28 minutes, and simazine and methabenzthiazuron at 2.53 minutes (Figure 6). However, these compounds could be clearly distinguished by their MRM transitions. In the case of sebuthylazine and terbuthylazine, which have the same MRM fragmentation pattern, it was possible to distinguish them by their complete separation and retention at 2.15 and 2.28 minutes, respectively. The complete suite of compounds covered one order of magnitude in response between lowest and highest-responding compounds (Figure 9A and 9B).

To evaluate the performance of the method, a calibration over three orders of magnitude from 1,000 ng/mL down to 1 ng/mL was created. In most cases, the linearity was > 0.9990 for the high- as well as for the low-responding compounds (Figure 10).

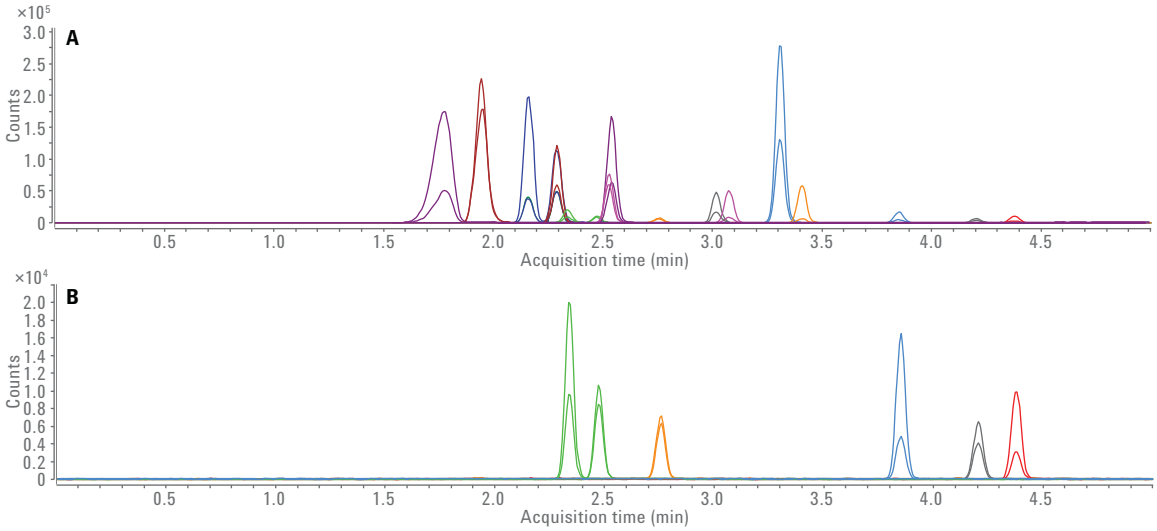


Figure 9. SFC separation of a mixture of 17 pesticides and detection by triple quadrupole MRM mass spectrometry at 100 ng/mL. A) MRM quantifier and qualifier of all 17 pesticides; B) MRM quantifier and qualifier of the six lower-abundant pesticides.

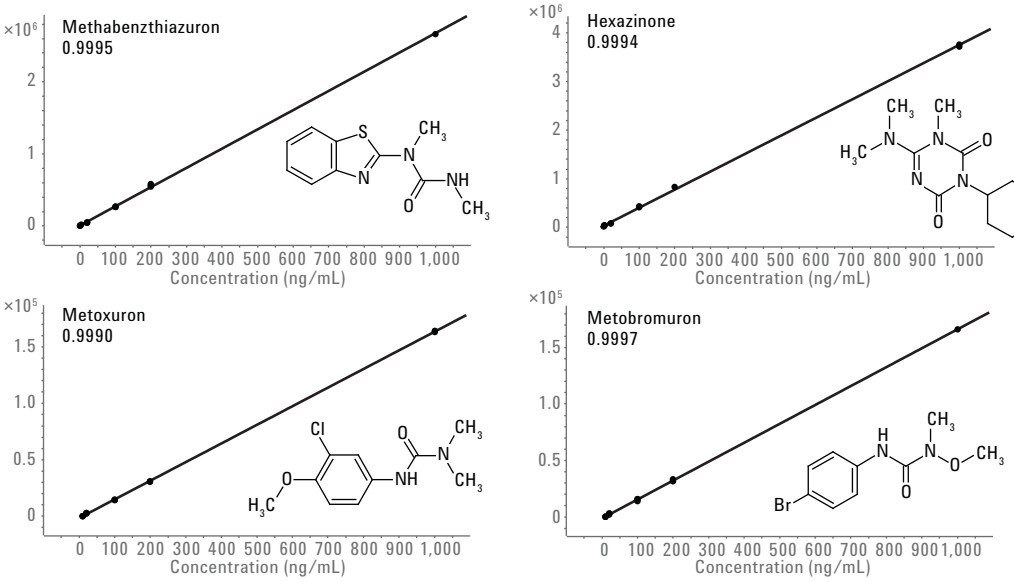


Figure 10. Calibration curves for the high-abundant compounds methabenzthiazuron and hexazinone and low-abundant compounds metoxuron and metobromuron.

The LOQs were typically below 2.9 ppb, and the LODs below 1.6 ppb. For a statistical evaluation, 10 replicates of the 100 ng/mL dilution were measured. The calculated retention time RSD values were typically below 0.4 %, and the area RSDs below 4 % (Table 2).

Conclusions

This investigation developed a workflow to optimize an Agilent 1260 Infinity Analytical SFC triple quadruple mass spectrometer method. Optimizing the SFC gradient was demonstrated for complete elution and separation of a large number of compounds in a sample by means of the method development capabilities of the SFC system. The optimization of the MRM settings was done using Agilent MassHunter Optimizer Software. The flow rate of the makeup solvent was optimized manually, and Agilent Jet Stream settings were optimized by Agilent MassHunter Source Optimizer. The settings achieved, especially for makeup flow and Agilent Jet Stream parameters, could be used as a good compromise for other SFC/MS methods. Finally, the performance of the system and method was evaluated for a pesticide mix, with LOQs typically below 2.9 ppb, retention time RSDs below 0.4 %, and area RSDs below 4 %. The advantage, compared to HPLC methods, was the good separation of a larger number of compounds with a faster run time at comparable performance.

Table 2. Performance results of the measurement of a sample comprising 17 pesticides (calibration from 1 to 1,000 ng/mL, LOD, LOQ, linearity, and statistical evaluation for retention time and area RSD % (n = 10)).

	RT	RT RSD	Area RSD	LOD	LOQ	R^2
Metolachlor	1.784	0.54	3.75	2.7	9.1	0.9996
Metazachlor	1.947	0.53	2.46	1.6	5.2	0.9992
Sebuthylazine	2.158	0.42	0.42	0.2	0.7	0.9998
Terbuthylazine	2.282	0.45	0.45	0.3	1.0	0.9992
Atrazine	2.283	0.38	4.56	0.1	0.4	0.9998
Monolinuron	2.338	0.38	3.07	0.3	1.0	0.9990
Metobromuron	2.465	0.37	4.22	1.4	4.5	0.9997
Simazine	2.525	0.31	2.73	0.2	0.7	0.9990
Methabenzthiazuron	2.538	0.34	2.02	0.2	0.7	0.9995
Linuron	2.743	0.27	2.91	3.7	12.5	0.9998
Atrazine-desethyl	3.011	0.23	2.54	0.9	2.9	0.9997
Cyanazine	3.068	0.23	2.39	0.4	1.2	0.9999
Hexazinone	3.307	0.12	2.38	0.1	0.3	0.9994
Isoproturon	3.404	0.11	3.31	0.4	1.2	0.9999
Chlorotoluron	3.852	0.11	2.69	3.7	12.5	0.9998
Diuron	4.212	0.05	2.89	3.7	12.5	0.9997
Metoxuron	4.375	0.06	3.11	2.2	7.4	0.9990

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