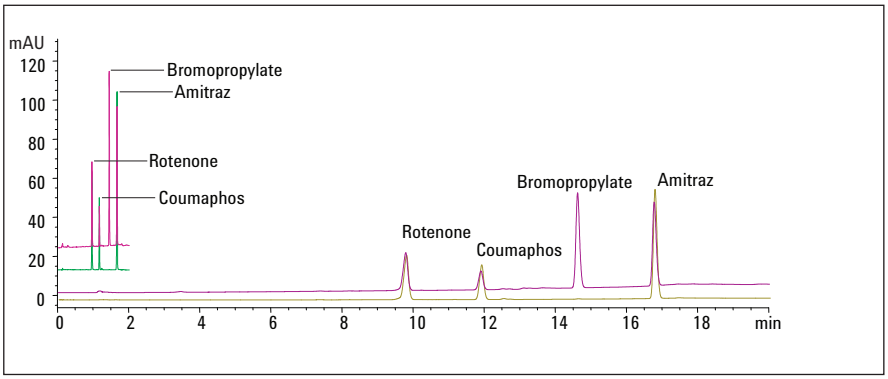
Agilent Application Solution

Analysis of acaricides in honey

Application Note

Food



Authors

A.G. Huesgen
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

Varroosis is a bee disease caused by the mite *Varroa Jacobsoni*, which endangers beekeeping all over the world. In order to prevent economic losses, beekeepers treat their colonies with acaricides. Using these acaricides inside beehives implies a risk of direct pollution of honey and other hive products, therefore maximum residue levels (MRLs) on honey have been fixed in many countries to protect consumers.

In this Application Note, an HPLC method was developed and validated for four acaricides, Rotenone, Coumaphos, Bromopropylate, and Amitraz using the Agilent 1260 Infinity LC system. In addition, an UHPLC method was developed using the Agilent 1290 Infinity LC system, which saves time and solvent consumption, and results in better sensitivity.

Agilent Technologies

Introduction

Acaricides are widely used to keep the possibility of infection by *Varroa Jacobsoni* under control. Rotenone, Coumaphos, Bromopropylate, and Amitraz are frequently used. These acaricides belong to four different chemical families, see Figure 1.

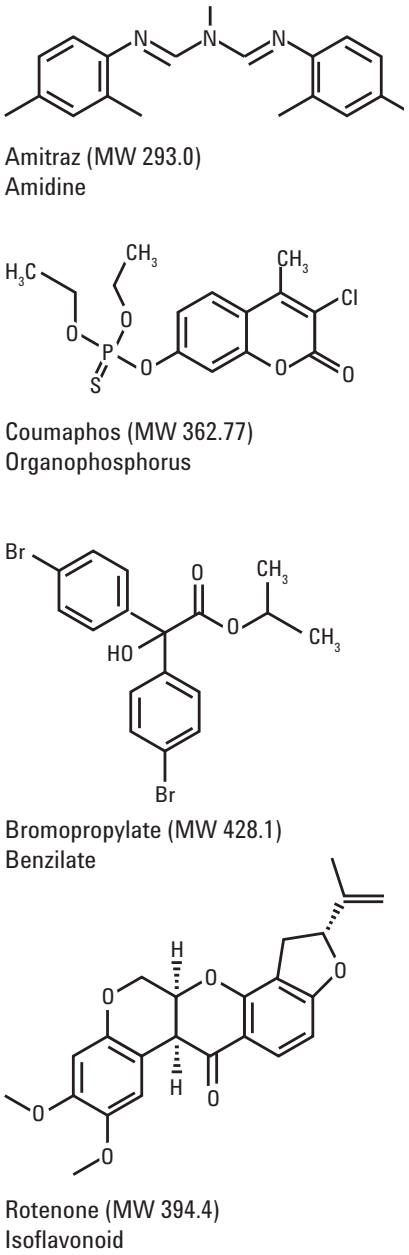


Figure 1
Chemical structure of acaricides.

The use of these pesticides implies the risk of contamination of consumer products like honey and other beehive products. Therefore, many countries have set maximum residue limits (MRL) to reduce the health risk for consumers.

Two norms were defined in ECC Regulation 2377/90 for Coumaphos and Amitraz : 0.1 and 0.2 mg/kg respectively.[1,2]

To determine acaricides in honey several LC methods have been used.[3-6] This method was developed based on these publications.

Experimental

Instrument and software

An Agilent 1260 Infinity Binary LC system consisting of the following modules was used:

- Agilent 1260 Infinity Binary Pump (G1312B)
- Agilent 1260 Infinity Autosampler and sample thermostat (G1367E, G 1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316A)
- Agilent 1260 Infinity Diode Array Detector (G4212B) with 10-mm path length flow cell

The UHPLC analysis was developed and performed using an Agilent 1290 Infinity LC system consisting of the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler and sample thermostat (G4226A and G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A) with 10-mm Max-Light flow cell

Columns:

- Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm (p/n 95993-902)
- Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm (p/n 959757-902)

Software:

- Agilent ChemStation B.04.02

Chromatographic parameters

Parameter	Conventional method	Fast UHPLC method
Column:	Agilent ZORBAX Eclipse Plus C18, 4.6 x 150 mm, 5 µm	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm
Mobile phase:	water (A), acetonitrile (B)	water (A), acetonitrile (B)
Gradient:	40 to 90% B in 15 min	40 to 90% ACN in 1.16 min
	At 20 min 90% B	At 1.557 min 90% B
	At 20.1 min 40 % B	At 1.56 min 40% B
	At 25 min 40 % B	At 1.93 min 40% B
Flow rate:	1 mL/min	0.9 mL/min
Column temp:	30 °C	30 °C
DAD:	0.5 sec, 10 Hz, 290 nm (rotenone), 290 nm (Amitraz), 230 nm (Bromopropylate), 290 nm (Coumaphos), Identification through UV spectra	0.13 sec, 40 Hz, 290 nm (rotenone), 290 nm (Amitraz), 230 nm (Bromopropylate), 290 nm (Coumaphos), Identification through UV spectra
Injection volume:	20 µL with 6 sec for exterior needle wash with methanol, sample compartment was cooled to 10 °C and kept dark	1.5 µL with 6 sec for exterior needle wash with methanol, sample compartment was cooled to 10 °C and kept dark

Reagents and materials

All chemicals and solvents used were HPLC grade, and highly purified water from a Milli Q water purification system was used. Acetonitrile gradient grade was purchased from Merck (Darmstadt, Germany). All standards were ordered from Sigma Aldrich, Germany

Preparation of standards

To perform the validation tests, a dilution series was set up, see Table 2. For the stock solution and all other dilutions, Acetonitrile was used as solvent. The stock solution contents are shown in Table 1.

Compound	Weighted sample (mg/10 mL)
Coumaphos	18.65
Amitraz	18.25
Bromopropylate	28.55
Rotenone	18.9

Table 1
Concentration of stock solution.

The stock solution was stored at 4 °C in the refrigerator and was stable for at least two months. Dilutions 2 to 4 are typical concentrations obtained after sample preparation of honey samples.

Precautions

Rotenone is very light sensitive and should be kept in the dark. The light of the autosampler should be switched off.

Amitraz should not be heated above 40 °C due to decomposition.

Sample preparation

Sample preparation was done in the following way:

- 10 g honey + 10 mL Acetonitrile
- Mechanical stirring for 30 minutes
- 8 mL of the supernatant liquid are transferred into 250 mL rotary vessel.
- Evaporate to dryness at 30 °C with vacuum rotary evaporator from Büchi.
- Reconstitute in 500 µL acetonitrile.
- Transfer to a LC vial and inject 20 µL.

Spiking procedure:

- 10 g spiked with 200 µL of dilution 2 (~ 0.1 mg/kg) or dilution 3 (~ 0.05 mg/kg)
- Mechanical stirring for 10 minutes

Procedure

The following steps were taken to develop and validate the method on the Agilent 1260 Infinity LC system:

- **Method development**: Standards were injected to elute all peaks in a reasonable time (~ 20 minutes) with an Agilent 1260 Infinity LC system using standard-bore 4.6 mm id columns.
- **Method validation**: Area and RT precision, LOD/LOQ, linearity (relevant range), recovery rates, and accuracy (from spiked matrix), robustness (column temperature, flow, gradient steepness, wavelength, injection volume) were evaluated.
- **Sample preparation**: One relevant matrix was chosen. Unspiked honey and two spiked honeys were prepared and analyzed.
- **Analysis**: Injection of spiked real-life sample with quantification and identification through UV spectra was performed.

Having developed and validated the conventional method, the analysis was transferred to the Agilent 1290 Infinity LC system to develop an UHPLC method.

- **Method transfer to UHPLC**: An UHPLC method was developed increasing speed and sensitivity using the Agilent 1290 Infinity LC system and short sub-2-µm columns.
- **Proof of UHPLC method performance**: Precision of area and RT, LOD/LOQ was tested.

Compound	1st dilution, 1:200 of stock solution (ng/20 µL)	2nd dilution, ng/20 µL	3rd dilution, ng/20 µL	4th dilution, ng/20 µL	5th dilution, ng/20 µL	6th dilution, ng/20 µL	7th dilution, ng/20 µL	8th dilution, ng/20 µL
Coumaphos	186.5	93.25	46.625	23.3125	11.65625	5.828125	2.914063	1.457031
Amitraz	182.5	91.25	45.625	22.8125	11.40625	5.703125	2.851563	1.425781
Bromopropylate	285.5	142.75	71.375	35.6875	17.84375	8.921875	4.460938	2.230469
Rotenone	189	94.5	47.25	23.625	11.8125	5.90625	2.953125	1.476563

Table 2
Dilution series.

Results and Discussion

Separation and detection

A conventional method was developed using an Agilent ZORBAX Eclipse Plus C-18, 4.6 × 150 mm, 5 µm particles column. A gradient from 40 to 90% organic was used and a flow rate of 1 mL/min. Figure 2 demonstrates the excellent separation. Only water and acetonitrile were used to achieve the separation the four compounds. Buffers or further modifiers were not needed.

Two wavelengths were needed to measure all compounds at their absorbance maximum. For Rotenone, Coumaphos, and Amitraz, a wavelength of 290 nm was used for calibration. For Bromopropylate, a wavelength of 230 nm was used. The characteristic spectra (Figure 3) of the four compounds were used to create a UV spectral library. This library, along with the retention times, helped identify the compounds in the honey sample.

Method validation

Precision of retention times and areas

Retention time RSD values for all four acaricides across the linearity levels were calculated.

The precision of retention times for six consecutive runs was typically < 0.05% RSD. The precision of the complete sequence was < 0.16 % RSD, over 70 runs within 29 hours.

The Precision of Areas was tested over the complete dilution series. The results are combined in Figure 4. Even though the peak height of dilution 8 is only ~ 0.1 to 0.2 mAU, the RSD value of < 6.5%, is very good. All other RSD values were < 2% over the complete series and < 1% RSD from dilution 5 on.

Limit of detection (LOD) and Limit of quantitation (LOQ)

The limit of detection and quantitation was evaluated using dilution 8. In Figure 5, the chromatogram for this concentration is shown.

The analyte concentration that provides a signal to noise ratio (S/N) of > 3 was considered as LOD and compound concentration with 10 * LOD was considered as LOQ, which corresponds to dilution 8.

Overall the LOD is < 0.2 ng/20 µL and LOQ (10*LOD) is < 1.8 ng/20 µL.

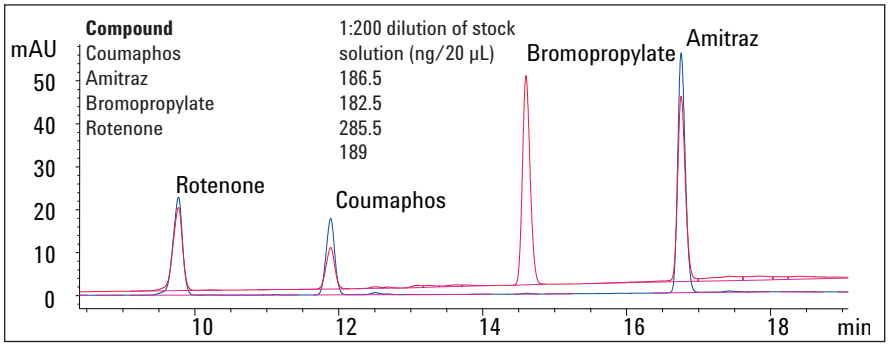


Figure 2
Analysis of the four Acaricides on Agilent ZORBAX Eclipse Plus C18, 4.6 x 150 mm, 5 µm column.

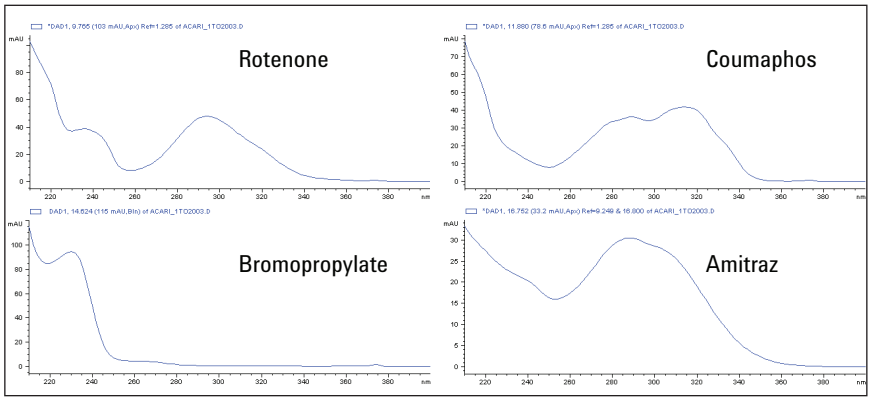


Figure 3
Spectra of acaricides.

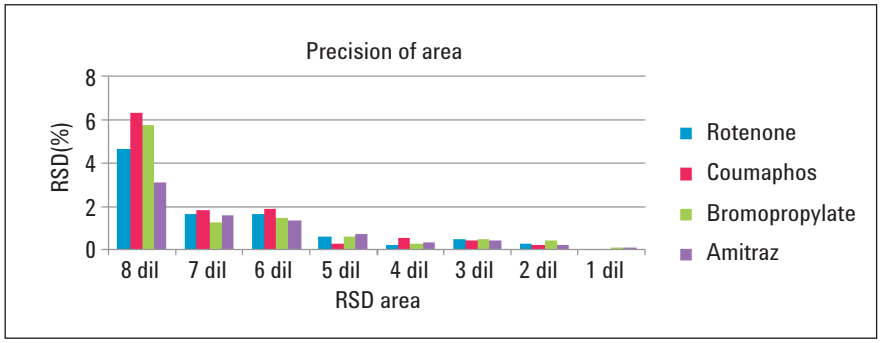


Figure 4
Precision of areas over the complete dilution series, over 6 consecutive runs for each concentration.

Linearity

To test the linearity dilution, 1 to 8 were used. Each linearity solution was injected six times and the area was used to construct the linearity curve. The linearity of all four compounds was > 0.9999 for the coefficient of correlation over the complete dilution series.

Carry over

To test the carry over behavior dilution 1 was injected followed by the injection of pure solvent. No carry over was observed for the concentration range used.

Robustness test

Five critical parameters were changed and data collected in 10 replicate injections. Values from the last six replicates were used for the analysis. Allowed deviation for retention time and area was set to ± 3.0% and ± 5% respectively. Robustness of the method was tested using dilution 1. The results showed that retention time shifts > 3% have to be expected for gradient slope differences of ± 10%, see Table 3.

Regarding robustness related to compound amounts, it is critical to change the injection volume and the flow rate.

Robustness results indicate that the method is reliable for normal usage and to a great extent the performance remains unaffected by deliberate change in parameters. However, some parameters are critical and must be carefully controlled.

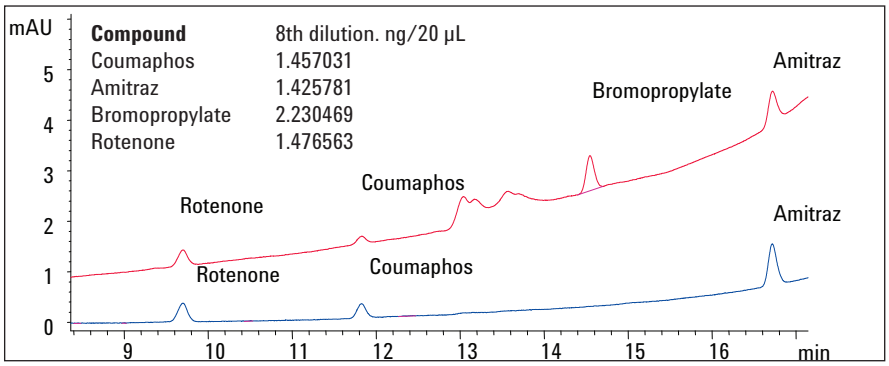


Figure 5
Analysis of dilution 8.

Parameters changed	Changes	% Deviation for RT Rotenone	% Deviation for RT Coumaphos	% Deviation for RT Bromopropylate	% Deviation for RT Amitraz	± % Deviation limits
Flow ± 2% Standard: 1 mL/min	High: 1.04 mL/min	– 2.23	– 1.87	– 1.59	– 1.47	3
	Low: 0.96 mL/min	+ 1.99	– 1.94	– 1.69	– 1.57	3
TCC ± 5% Standard: 30 °C	High: 31.5 °C	– 0.03	– 0.20	– 0.18	– 0.27	3
	Low: 28.5 °C	+ 0.79	+ 0.76	+ 0.67	+ 0.64	3
Inj ± 5% Standard: 20 µL	High: 21 µL	+ 0.26	+ 0.20	+ 0.19	0.14	3
	Low: 19 µL	– 0.32	– 0.24	– 0.23	– 0.19	3
Gradient slope ± 10%	55% in 15 min + 10%	– 3.91	– 4.75	– 5.3	– 5.64	3
	45% in 15 min – 10%	+ 4.66	+ 5.68	+ 6.41	+ 6.83	3
Wavelength ± 3 nm 290 nm 230 nm 254 nm	DAD 293 nm 233 nm 257 nm	0	– 0.02	– 0.01	– 0.01	3
	DAD 287 nm 227 nm 251 nm	– 0.02	0	0	– 0.02	3

Table 3
Robustness tests related to retention times.

Parameters changed	Changes	% Deviation for RT Rotenone	% Deviation for RT Coumaphos	% Deviation for RT Bromopropylate	% Deviation for RT Amitraz	± % Deviation limits
Flow ± 2% Standard: 1 mL/min	High: 1.04 mL/min	– 3.13	– 3.33	– 2	– 3.87	5
	Low: 0.96 mL/min	+ 5	+ 4.4	+ 6	+ 5.16	5
TCC ± 5% Standard: 30 °C	High: 31.5 °C	+ 3.13	+ 2.78	+ 3	+ 1.94	5
	Low: 28.5 °C	+ 2.5	+ 2.2	+ 2.5	– 0.65	5
Inj ± 5% Standard: 20 µL	High: 21 µL	+ 6.88	+ 6.67	+ 7	+ 5.81	5
	Low: 19 µL	+ 3.75	+ 3.9	+ 3.5	+ 4.52	5
Gradient slope ± 10%	55% in 15 min + 10%	+ 1.25	+ 0.56	+ 2	+ 0.65	5
	45% in 15 min – 10%	+ 1.25	+ 1.1	± 2.5	+ 5.16	5
Wavelength ± 3 nm 290 nm 230 nm 254 nm	DAD 293 nm 233 nm 257 nm	+ 2.5	– 2.2	– 4.5	+ 3.23	5
	DAD 287 nm 227 nm 251 nm	– 6.88	– 0.56	0	+ 3.23	5

Table 4
Robustness test related to compound amounts.

Recovery rates

The honey used was a fully liquid honey which contained none of the tested acaricides. The honey was spiked with the four compounds to obtain a resulting concentration of 0.1 and 0.05 mg/kg of the acaricides. In Figure 6, the chromatogram of honey spiked with ~ 0.05 mg/kg is shown after sample preparation. The recovery rate was for honey spiked with:

- 50 ng/g, 0.05 mg/kg = recovery 67–88%
- 100 ng/g, 0.1 mg/kg = recovery 84–105%

Amitraz shows the lowest recovery rate, this may be due to decomposition.

In addition, the UV spectral library was used to identify the compounds using spectral match.

Method transfer to UHPLC method

A UHPLC method was established for the separation of acaricides using the Agilent method translator. This tool enables one to easily convert methods from either binary or quaternary pump systems to optimized methods for the Agilent 1290 Infinity LC system.

The analysis time was decreased to 2 minutes using a short sub-2-µm column, see Figure 7.

The benefits of the fast analysis are:

- Time savings versus conventional of 92%
- Solvent savings versus conventional of 92.8%

In Figure 7, all peaks are baseline separated and show sufficient resolution and good peak shape.

Partial validation of ultra fast method

The following parameter were tested

- Limit of detection and quantitation using dilution 8
- RSD of retention times and areas for dilution 3, 4, and 5

The RSD for retention times over 38 runs was < 0.12%, which is comparable to results obtained for the conventional method. The RSD of areas was typically < 0.9% for amounts
< 6 to < 1 ng injected amount. Even though only 1.5 µL were injected, the LOD was 0.02–0.06 ng injected amount and the LOQ (10*LOD) < 0.6 injected amount. This is overall 3 times better than measured for the conventional method.

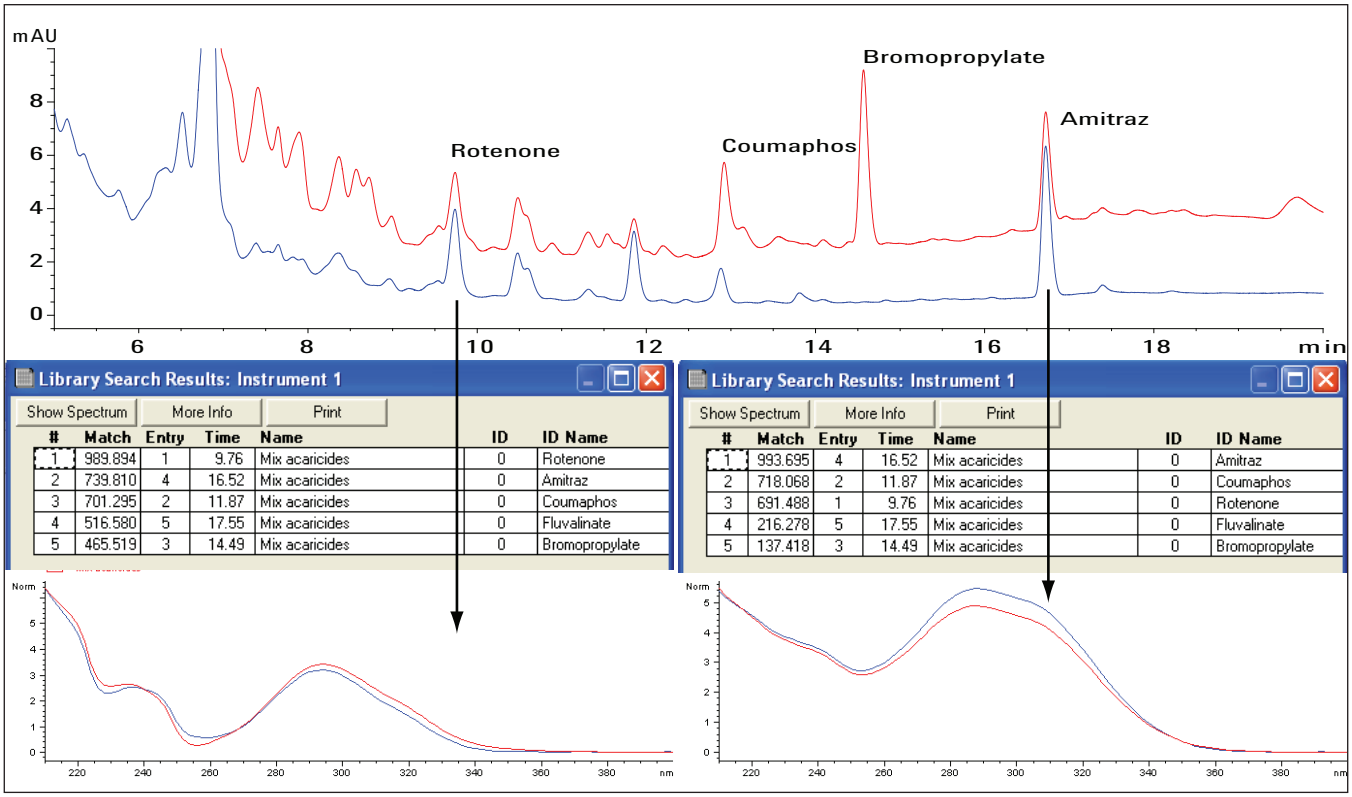


Figure 6
Analysis of pesticides in honey in a concentration range of 0.05 mg/kg and identification through UV spectral comparison.

Conclusion

A conventional method for the analysis of, Rotenone, Coumaphos, Bromopropylate, and Amitraz in honey was developed and validated using the Agilent 1260 Infinity Binary LC system. The method is robust and suitable for the quantitation of concentrations of acharicides < 0.05 mg/kg in honey. The extraction method is based on the extraction of the compounds with Acetonitrile and is fast and simple to perform. Recovery rates between 67 and 88% are typically achieved for the 0.05 mg/kg range. The LOD is typically < 0.2 ng injected amount. Faster results with significant decrease in solvent consumption and time can be achieved by applying an UHPLC method using the Agilent 1290 Infinity LC system. In addition, lower limits of detection can be achieved.

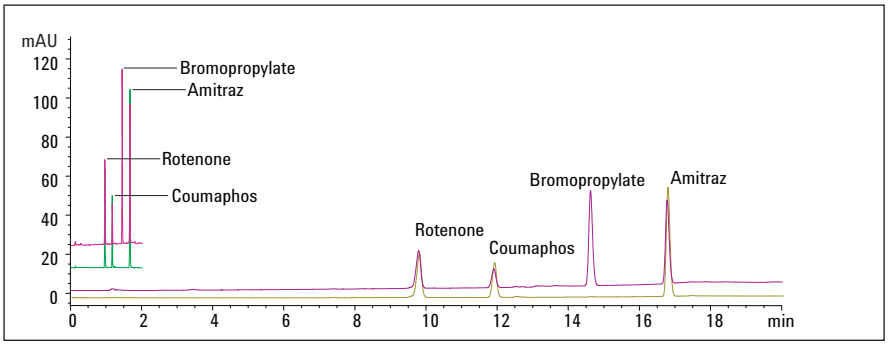


Figure 7
Comparison of conventional method versus ultra fast method.

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