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アプリケーションノート

A Confirmatory Method for the Determination of Chloramphenicol, Thiamphenicol, and Florfenicol in Honey

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Abstract

In this application note, a rapid and sensitive method is described for the determination and confirmation of chloramphenicol, thiamphenicol and florfenicol in honey using a Symmetry C_8 Column, Oasis HLB Solid Phase Extraction (SPE) Cartridges and the Waters Micromass Quattro micro API Tandem Quadrupole Mass spectrometer.

Benefits

- A simple extraction method using Oasis HLB SPE Cartridges provides good recovery
- The Symmetry C_8 Column provides good retention, peak shape, and resolution for all three components

Introduction

Chloramphenicol

Chloramphenicol (CAP) is an inexpensive, potent, broad spectrum antibiotic, often used in food producing animals for the prevention and treatment of diseases. CAP can be used to treat bees infected with bacterial diseases such as the American foulbrood. However, residues of CAP may be found in honey if bees are treated during the harvesting season.[1] In the past few years, reports have surfaced stating that CAP has been found in several foodstuffs from Asia, including honey.[2-4]

Unfortunately, CAP is a cause of the fatal blood condition idiosyncratic aplastic anemia. Approximately one in thirty thousand individuals have a hypersensitivity to CAP that may cause this condition irrespective of exposure level.[5] For this reason, no Acceptable Daily Intake (ADI) can be determined, resulting in this drug being listed in Annex IV of European Union (EU) Council Regulation 2377/90/EEC. This means that CAP is prohibited for use in animal derived foods destined for human consumption. CAP is also banned in many other countries, including the USA, Canada, and Australia.

In the EU, there is no Maximum Residue Limit (MRL) set for CAP in animal derived food but zero tolerance is enforced. This is legislated in EU Decision 2003/181/EC, as a Minimum Required Performance Limit (MRPL) of 0.3 ppb for CAP.

The MRPL is the target for laboratories with the least sensitive analytical techniques, whereas the laboratories able to achieve the best detection limits will go lower. Therefore, the detection and confirmation of CAP at any concentration will lead to the condemnation of the produce. In order to effectively monitor the occurrence of residues of CAP, the most specific and sensitive methods are required.

Thiamphenicol and Florfenicol

Thiamphenicol (TAP) and Florfenicol (FP) are chloramphenicol related compounds. These two compounds do not have MRPLs like CAP, but antibiotics should not be present in any foodstuff as they could impact the effectiveness of antibiotics used in human medicine. No EU legislation exists for thiamphenicol and florfenicol in honey; however, TAP has an MRL of 50 µg/kg in milk and FP has an MRL in all foods of 100 µg/kg.

In this application note, a rapid and sensitive method is described for the determination and confirmation of chloramphenicol, thiamphenicol, and florfenicol in honey using a Symmetry C_8 Column, Oasis HLB Solid Phase Extraction (SPE) Cartridges and the Waters Micromass Quattro micro API Tandem Quadrupole Mass Spectrometer.

Experimental

Extraction Procedure

The procedure was developed from published methods by the Canadian Food Inspection Agency[6] and the US FDA[7], and existing Waters methodology.[8-9]

- 5 g of honey was spiked with D5-CAP and dissolved in water (5 mL)
- This solution was extracted with ethyl acetate (15 mL) and centrifuged
- The supernatant was transferred to a clean tube and evaporated to dryness under nitrogen at 50 °C
- The residue was reconstituted in methanol (1 mL) and diluted with water (20 mL)
- 5 mL of conditioning solvent (methanol) was passed through Oasis HLB 200 mg 6 cc Cartridge (WAT106202 < https://www.waters.com/nextgen/us/en/shop/sample-preparation--filtration/wat106202-oasis-hlb-6-cc-vac-cartridge-200-mg-sorbent-per-cartridge-30--m-.html>) followed by 5 mL of rinse solvent (water)
- The honey solution was loaded at approximately 2 drops followed by 5 mL of wash solvent (water)
- The phenicols were eluted with 2 x 2.5 mL of elution solvent (methanol)
- This solution was evaporated to dryness under nitrogen at 50 °C
- The residue was reconstituted in 9:1 water/methanol (500 µL) producing a matrix equivalent of 10 g/mL
- The extract was filtered through a syringe filter prior to injection (0.45 µm)

HPLC Method

LC:	Waters Alliance HPLC System
Mobile phase A:	Water
Mobile phase B:	Methanol
Column:	Symmetry C_8, 2.1 x 50 mm, 3.5 µm at 30 °C
Guard column:	Sentry Symmetry C8, 2.1 x 10 mm, 3.5 µm
Flow rate:	0.3 mL/min
Injection volume:	20 µL

Gradient

Time(min)	%A	%B
0	90	10
8	10	90
10	10	90
10.1	90	10
15	90	10

MS Method

MS:	Waters Micromass Quattro micro API Electrospray mode with negative polarity
Capillary voltage:	0.8 kV
Extractor:	3 V
RF lens:	0 V
Source temp.:	120 °C
Desolvation temp.:	450 °C
Cone gas flow:	100 L/hr
Desolvation gas flow:	1000 L/hr
Collision gas pressure:	Argon at $4.0e^{-3}$ mBar

Multiplier:	650 V

The phenicols were tuned so that the precursor and product ions were resolved with a peak width at half height of <0.7 Da. The Multiple Reaction Monitoring (MRM) transitions, along with the collision energies and dwell times for the method are listed in Table 1. Seven MRM transitions were monitored, a quantification and a confirmation transition for each component, and a transition for the internal standard.

	MRM Transition	Dwell Time (s)	Cone Voltage (V)	Collision Energy (eV)
Chloramphenicol	321→152	0.1	25	18
	321→257	0.1	25	12
Thiamphenicol	354→185	0.1	30	23
	354→290	0.1	30	14
Florfenicol	356→336	0.1	25	11
	356→185	0.1	25	22
Internal Std D_5-CAP	327→157	0.1	25	18

Table 1. MRM method parameters.

A series of matrix-matched calibration standards, matrix blanks, and recovery samples were analyzed to determine method accuracy, linearity, precision, repeatability and recovery. The Limit of Determination (LOD) was also estimated from the lowest concentration matrix-matched standard. The internal standard, D5-CAP, was spiked at 20 pg/µL in all samples. Matrix-matched calibration standards were made up at 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 µg/kg. Recovery samples were spiked at 0.3 µg/kg prior to extraction.

Results and Discussion

Overlaid chromatograms of chloramphenicol, thiamphenicol and florfenicol for a honey extract spiked at 2 µg/kg are illustrated in Figure 1. In accordance with EU guidelines on confirmation for compounds with an MRPL, two MRM transitions are acquired for each, and the ion ratio between these are monitored across a batch.[10] The Waters Symmetry C_8 Column provides good retention, peak shape and resolution for the three components.

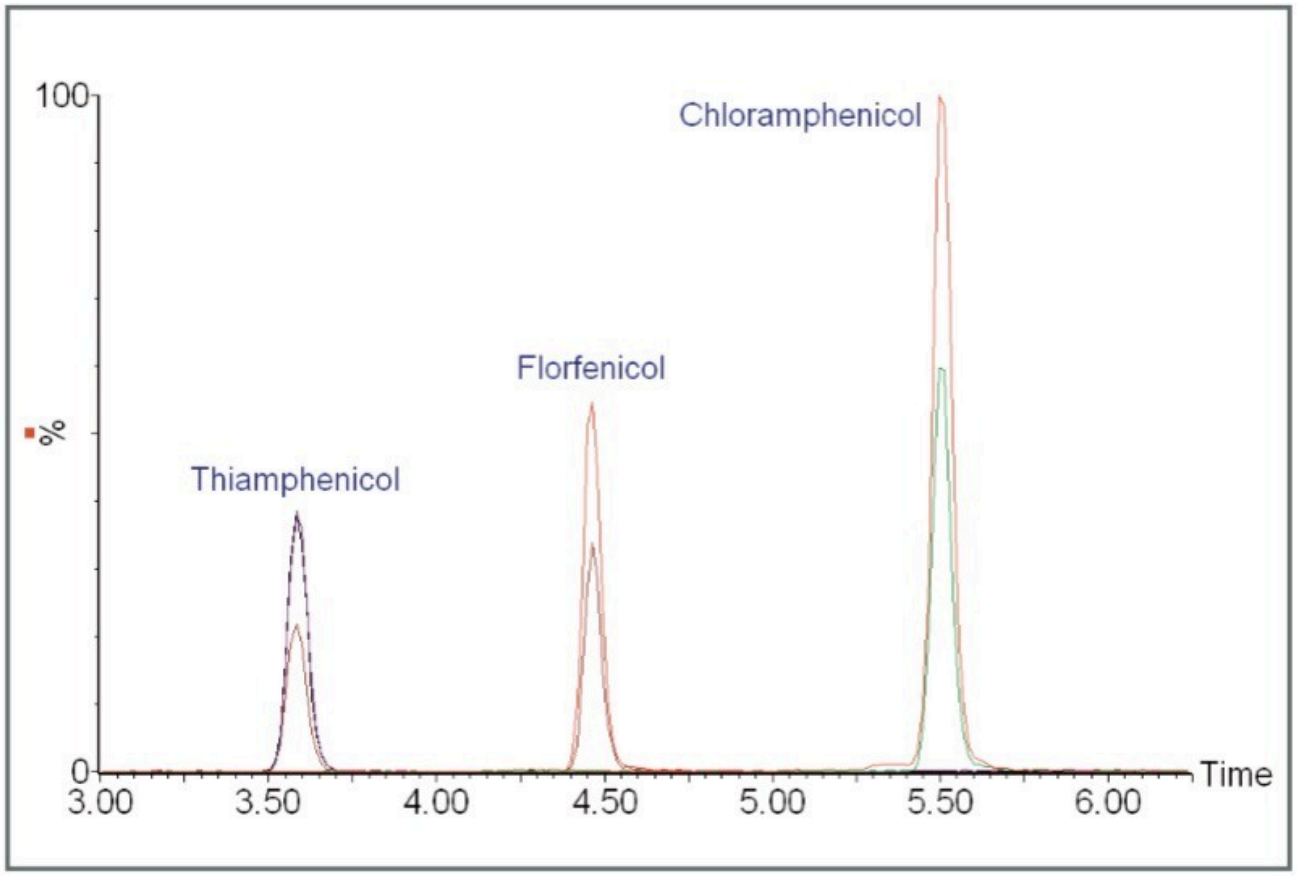

Figure 1. Overlaid chromatograms of CAP, TAP, and FP at 2 µg/kg in honey.

Figure 2 shows the difference in selectivity between MRM and Selected Ion Recording (SIR) when analyzing chloramphenicol at a spiked concentration of 1 µg/kg in honey. In this example, MRM improves the selectivity significantly compared to SIR, which can be seen by observing the signal-tonoise (S/N), background, ratio. The concentration of chloramphenicol in this instance is three to four times greater than the MRPL legislated by the EU. In this SIR experiment, only one mass was monitored for chloramphenicol. For confirmation purposes, four masses would need to be monitored, therefore reducing the overall sensitivity that can be seen here - this is the best case scenario. In the MRM experiment, two MRM transitions are already being monitored (satisfying confirmatory criteria); much lower concentrations could obviously be detected and quantified.

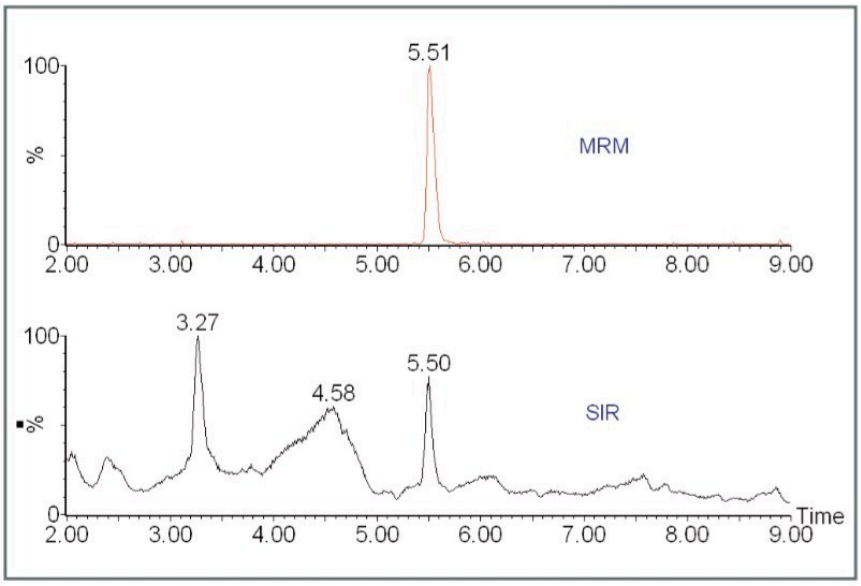

Figure 2. MRM versus SIR for chloramphenicol at 1 µg/kg in honey.

To test the extraction method described, five recovery experiments were performed for the phenicols in honey spiked at 0.3 µg/kg, the MRPL of chloramphenicol in the EU. Each sample was analyzed in duplicate and compared to a calibration curve of matrix-matched standards. The mean recoveries and relative standard deviations of each compound are listed in Table 2.

	Chloramphenicol	Thiamphenicol	Florfenicol
Mean Recovery	91.1%	91.9%	104.6%
% RSD (n = 5)	2.2	5.9	1.7

Table 2. Recovery data for CAP, TAP, and FP at 0.3 µg/kg.

The quantification transitions for a honey extract spiked with chloramphenicol, thiamphenicol, and florfenicol at 0.05 µg/kg are illustrated in Figure 3. For a 20 µL injection, the confirmation LODs (based on S/N >3:1 for both transitions) are estimated to be 0.01 µg/kg for all three components. For chloramphenicol, this confirmation LOD is thirty times lower than the MRPL legislated in the EU. Other analyses have used larger injection volumes,

e.g. 50 µL, to obtain lower LODs. Although this approach was not tested, injecting a larger volume in this instance would undoubtedly lower these limits further.

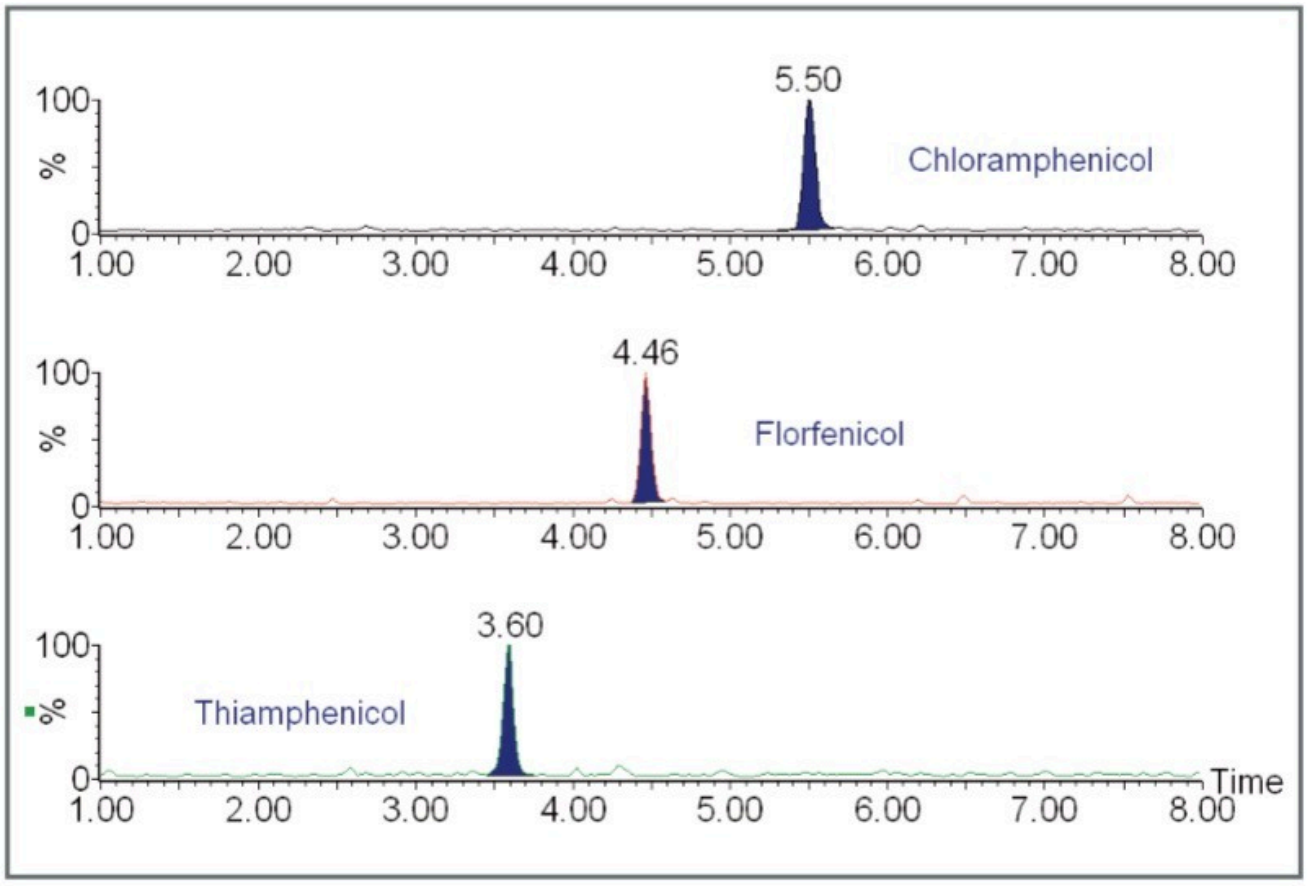

Figure 3. MRM transitions for CAP, TAP, and FP at 0.05 µg/kg in honey.

Matrix-matched standards were generated at the 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 µg/kg levels in honey. These standards were each injected five times in a typical batch analysis. The data was then processed using Waters TargetLynx Application Manager. The calibration curves were overlaid and a representative curve for chloramphenicol with a correlation coefficient of $r^2 = 0.9988$ is illustrated in Figure 4.

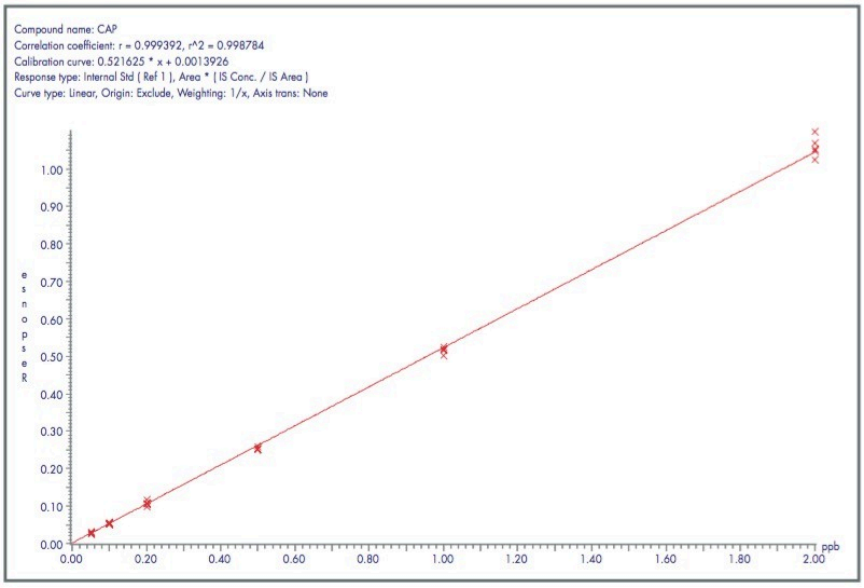

Figure 4. Overlaid calibration curves for CAP in honey.

The method accuracy and precision are listed in Table 3. Five injections were performed on day one, two injections on day two and three injections on day three, at each concentration level. These matrix spikes formed part of three batch analyses totalling 80 matrix injections. Good instrumental accuracy (mean) and precision (%RSD) were obtained at a range of concentrations around the MRPL for chloramphenicol, thiamphenicol, and florfenicol.

Concentration ug/kg	Chloramphenicol		Thiamphenicol		Florfenicol	
	Mean	% RSD	Mean	% RSD	Mean	% RSD
0.05	0.05	8.4	0.05	9.1	0.05	10.7
0.1	0.10	4.8	0.10	15.3	0.10	7.7
0.2	0.20	6.2	0.20	8.8	0.20	6.1
0.5	0.48	3.0	0.49	11.3	0.48	4.0
1.0	0.97	2.7	1.00	11.0	0.97	2.2
2.0	2.04	2.7	2.00	9.3	2.05	2.3

Table 3. Method accuracy and precision over three days.

The method repeatability of chloramphenicol for three batches of matrix-matched calibration standards is illustrated in Figure 5. This graph shows response factor (peak area/concentration) against injection number. Thirty injections were performed on day one, twelve injections on day two and eighteen injections on day three. No instrument maintenance was performed between each day. The response factor will remain constant if the response is linear and the source robustness is good. The graph indicates the repeatability of the method with a good relative standard deviation of 5.9% across all sixty injections.

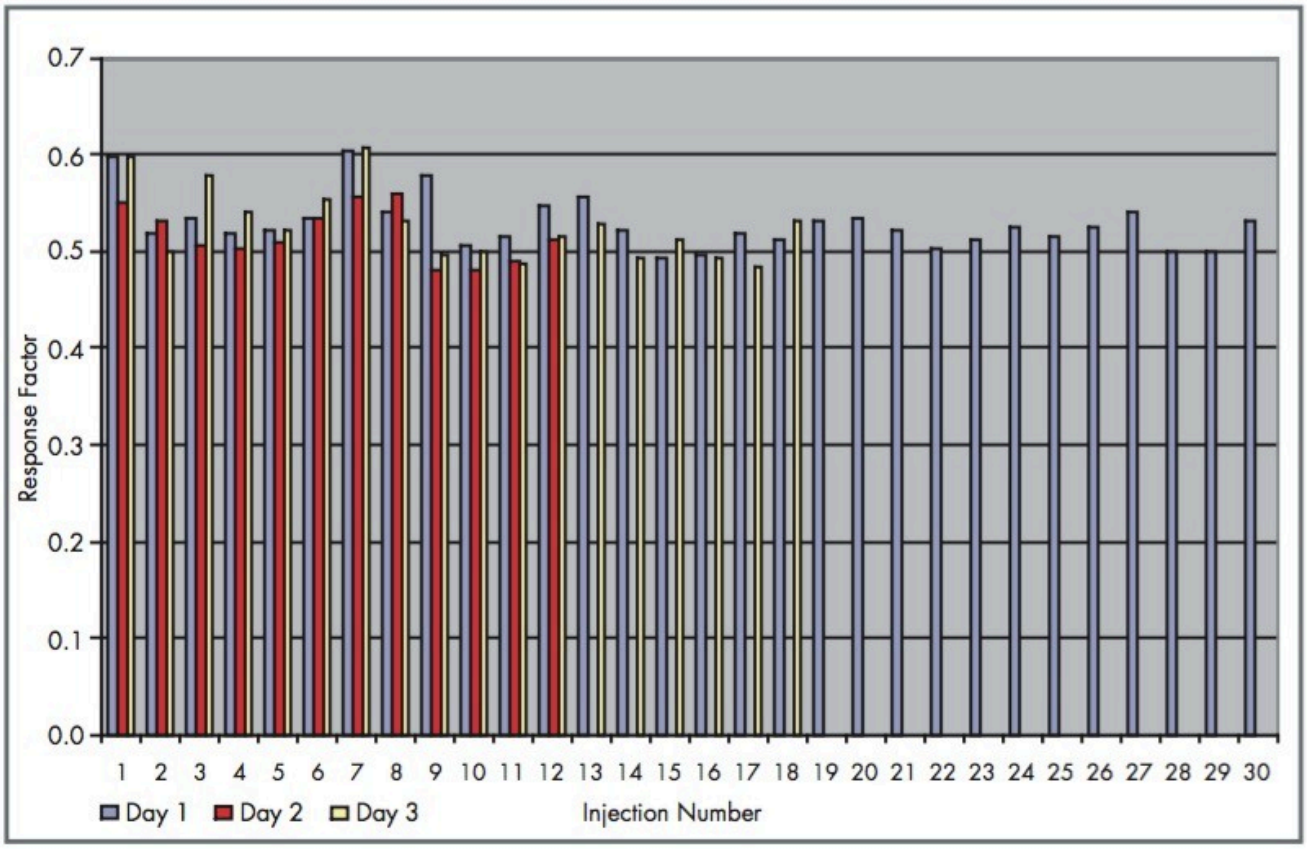

Figure 5. Response factor versus injection number over three days for CAP.

Ion ratios between the quantification transition and the confirmation transition are important as they provide the basis of confirmation. The ion ratio statistics are listed in Table 4 for sixty matrix injections over a three day period in three separate batch analyses. The relative standard deviation indicates good repeatability of the confirmation ion ratios with a number significantly less than the EU regulation[10] for MRM transitions with ion ratios of 1.78, 1.06, and 1.67 (56, 94 and 60%, respectively).

	Chloramphenicol	Thiamphenicol	Florfenicol
Mean Ratio	1.78	1.06	1.67
Std. Deviation	0.18	0.10	0.13
% RSD	9.9	9.3	8.0
EU Regulation 2002/657/EC	± 20%		

Table 4. Confirmation ion ratio repeatability over three days.

Conclusion

A rapid and sensitive method has been described for the determination and confirmation of chloramphenicol, thiamphenicol, and florfenicol in honey. The Symmetry C_8 Column provides good retention, peak shape, and resolution for all three components. A simple extraction method using Oasis HLB SPE Cartridges provides good recovery. The Waters Micromass Quattro micro API Tandem Quadrupole Mass Spectrometer provides sensitivity, selectivity, and reproducibility, and allows confirmation in a single injection. The limits of determination achieved are well below that required by legislation for any country in the European Union.

References

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