Determination of Chloramphenicol, Florfenicol, and Thiamphenicol in Honey Using Agilent SampliQ OPT Solid-Phase Extraction Cartridges and Liquid Chromatography-Tandem Mass Spectrometry

Application Note

Food Safety

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Abstract

A method for the simultaneous determination of three antibiotic residues of chloramphenicol (CAP), florfenicol (FF), and thiamphenicol (TAP) in honey has been developed and validated. The analytes are purified by liquid/liquid extraction and solid-phase extraction (SPE) and are quantified by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) operating in negative ion multiple reaction monitoring (MRM) mode. Chloramphenicol-D_5 is used as the internal standard. The method is validated by achieving reproducible, satisfactory, quantitative results. The method provides a sub-ng/g to ng/g level of limit of quantitation (LOQ) for all three antibiotics in honey. The overall recoveries range from 74.9 to 107% with RSD values between 0.5 and 9.7%. The dynamic calibration ranges for chloramphenicol and florfenicol are obtained over 0.1 to 20.0 ng/g and 1.0 to 20.0 ng/g for thiamphenicol. The method is demonstrated to be fast, simple, and efficient for monitoring chloramphenicol, florfenicol, and thiamphenicol residues in honey.

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Introduction

Chloramphenicol (CAP) is a broad-spectrum bacteriostatic antibiotic, obtained originally from the bacterium *Streptomyces venezuelae*. Due to potential side effects in humans, the drug is not recommended for the treatment of minor diseases, but is reserved for the treatment of serious infections. In veterinary medicine, CAP has been shown to be a highly effective, well-tolerated antibiotic; the potential side effects observed in humans have not been reported in animals. However, because of its toxicity in humans, the use of CAP in animal-derived foods, including honey from honeybees, has been strictly regulated. The European Union (EU) has defined a maximum residue limit (MRL) for CAP in food of animal origin at a level of 0.3 µg/kg [1], while China has an MRL level of 0.5 µg/kg [2]. Thiam-phenicol (TAP) and florfenicol (FF) are the analogue compounds of CAP. They can be used as a replacement veterinary antibiotic for CAP in many countries. The MRLs have been set for TAP (50 ng/g) and FF (100 ng/g) in food to date [3]. Table 1 shows the chemical structure and properties of these three compounds. This application note describes a method for the simultaneous determination of three phenicols in honey, and the results of validation.

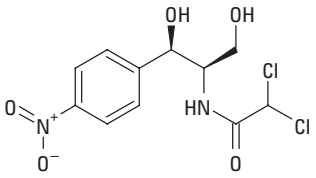
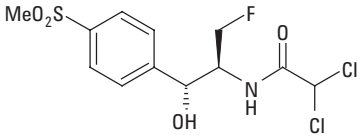
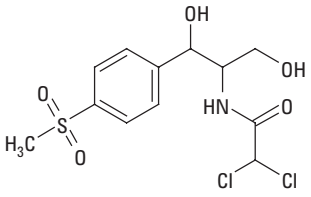
Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Acetonitrile and methanol were from Honeywell, Burdick & Jackson (Muskegon, MI); ethyl acetate was from J.T.Baker (Phillipsburg, NJ). Dimethyl sulfoxide was from Sigma-Aldrich (St. Louis, MO). The standards and other chemicals were purchased from Sigma-Aldrich.

Water (pH 8.5) was prepared by pH adjustment of Milli-Q water with 0.05% NH_4OH in water solution monitored by a pH meter. A solution of 20:80 methanol/ethyl acetate was prepared by combining 40 mL of methanol and 160 mL of ethyl acetate and mixing well. A solution of 20:80 acetonitrile/H_2O was prepared by adding 40 mL of acetonitrile into 160 mL of Milli-Q water.

Table 1. Chemical Structure and Properties of Target Analytes

Compounds	Log P	pKa	Structure
Chloramphenicol	1.02	9.61	
Florfenicol	–0.12	9.03	
Thiamphenicol	–0.27	9.76	

Standard stock solutions (1.0 mg/mL) were made in dimethyl sulfoxide (DMSO) individually, and stored in the refrigerator at 4 ºC. A combined working solution (2,500 ng/mL) was made weekly in 20:80 ACN/H_2O, and also stored at 4 ºC. The spiking solutions were then made daily by appropriate dilution of the combined working solution in Milli-Q water or 20:80 ACN/H_2O.

Internal standard (IS) stock solution (0.1 mg/mL) was made in DMSO and stored in the refrigerator at 4 ºC. An IS spiking solution (50 ng/mL) was made weekly by appropriate dilution of stock solution into Milli-Q water, and stored at 4 ºC.

Equipment and Materials

Agilent 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA)

Agilent 6410 Triple Quadrupole LC/MS/MS system with electrospray ionization source (Agilent Technologies Inc., Santa Clara, CA, USA)

Agilent SampliQ OPT solid-phase extraction cartridges, 50 × 3 mL tubes, 60 mg (p/n 5982-3036) (Agilent Technologies Inc., Wilmington, DE, USA)

CentraCL3R centrifuge (Thermo IEC, Needham Heights, MA, USA)

N_2 dryer (Glas-Col, Terre Haute, IN, USA)

Sample Preparation

Liquid-Liquid Extraction

5g of honey (± 0.05 g) was weighed into a 50 mL capped polypropylene tube. 0.5 mL of IS spiking solution (50 ng/mL) was added to the tube and vortexed until mixed. This was followed by the addition of 5 mL of Milli-Q water and vortexing for 3 minutes to mix the sample thoroughly. 5 mL of ethyl acetate was then added, capped tightly, and the tubes shaken for 5 minutes. The tubes were then centrifuged at 3,200 rpm for 5 minutes, before the upper organic layer was carefully transferred to another tube. Ethyl acetate addition, shaking, centrifuging, and organic layer transfer was repeated two more times with all supernatants combined. Samples were evaporated to dryness with a controlled N_2 flow drier at 50 ºC before being reconstituted into 5 mL of Milli-Q water, vortexed, and sonicated to completely dissolve the residue. The sample was then ready for SPE purification. Figure 1 shows the extraction procedure flowchart.

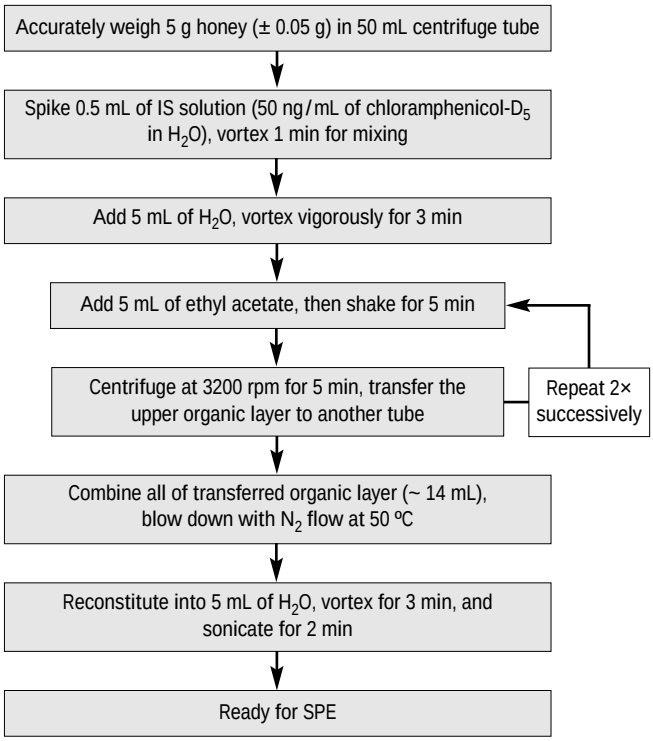


Figure 1. Sample preparation – liquid liquid extraction of phenicols in honey.

Solid-Phase Extraction

The procedure for SPE extraction is shown in Figure 2. Agilent SampliQ OPT cartridges were preconditioned with 3 mL of MeOH, and then equilibrated with 5 mL of water. The 5 mL sample extract was then loaded onto a cartridge and passed through the cartridge slowly by gravity (0.5 mL/min). The tubes were rinsed with 5 mL of Milli-Q water twice. Repeat the above wash procedure once. The entire effluent was discarded. Apply full vacuum to the cartridge for 3 minutes to completely dry the resin. Finally, the compounds were eluted with 5 mL of 20:80 MeOH/ethyl acetate (2.5 mL × 2) at a rate of 1 mL/min. The eluent was collected into clean tubes and dried under N_2 flow at 50 ºC. The residue was reconstituted in 0.5 mL of 20:80 AcN/H_2O. The sample was vortexed and sonicated to completely dissolve the residue in the tubes. The sample was transferred to a centrifuge tube and centrifuged at 3,200 rpm for 2 minutes. The samples were then transferred to 2 mL autosampler vials for analysis.

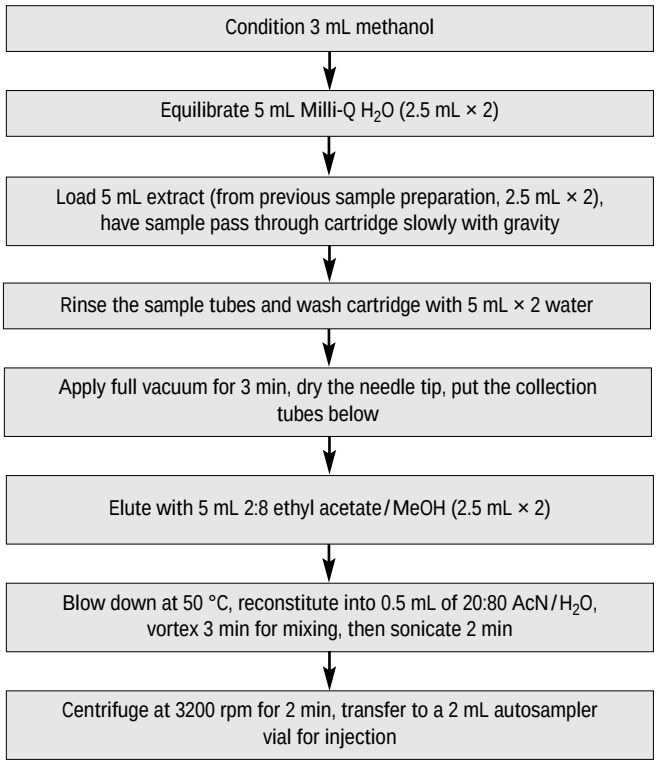


Figure 2. Sample clean-up – Agilent SampliQ solid-phase extraction.

Instrument Conditions

HPLC Conditions

Column:		Agilent ZORBAX Eclipse Plus 150 mm × 2.1 mm, 5 µm (PN: 959701-906)	
Flow rate:		0.3 mL/min	
Column temperature:		30 ºC	
Injection volume:		20 µL	
Mobile phase:		pH 8.5 H_2O (A), Acetonitrile (B)	
Gradient:	Time	% Acetonitrile	Flow rate (mL/min)
	0	20	0.3
	0.5	20	0.3
	6.0	80	0.3
	6.01	100	0.5
	6.50	100	0.5
	6.51	20	0.3
	7.00	STOP	

MS Conditions

The three compounds were monitored in the negative ionization mode. The multiple reaction monitoring channels are shown in Table 2.

Table 2. Masses Monitored in the Multiple Reaction Monitoring (MRM) Experiment

Analyte	MRM (*m/z* → *m/z*)	Dwell time (ms)
Thiamphenicol	354.0 → 184.9 (quantifier)	50
	354.0 → 290.0 (qualifier)	25
Florfenicol	355.8 → 185.0 (quantifier)	50
	355.8 → 336.0 (qualifier)	25
Chloramphenicol	320.9 → 152.0 (quantifier)	50
	320.9 → 176.0 (qualifier)	25
Chloramphenicol-D_5 (IS)	325.9 → 156.8	25

Results and Discussion

Linearity, Limit of Detection

The extracted ion chromatograms of fortified honey at a concentration of 0.2 ng/g are shown in Figure 3. The extracted honey blank was clean and free from any analytes, indicating that the cleaned-up honey extract does not contribute any interference with the target analysis.

The concentration ranges studied here are significantly below the limit of quantitation (LOQ) defined by the MRL for TAP (50 ng/g) [3]. In this study the limit of quantitation (LOQ) found for TAP is 1.0 ng/g, and the linear calibration range used for TAP is 1.0 to 20.0 ng/g. The linear calibration range for CAP (LOQ 0.1 ng/g, MRL 0.3 ng/g) and FF (LOQ 0.1 ng/g, MRL 100 ng/g) was 0.1 to 20.0 ng/g.

Calibration curves spiked in matrix blanks were made at levels of 0.1, 0.2, 1.0, 5.0, 10.0, 15.0, and 20.0 ng/g for CAP and FF. While for TAP they were spiked at a level of 1.0, 5.0, 10.0, 15.0, and 20.0 ng/g. The chloramphenicol-D_5 was used as internal standard at 5 ng/g level. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). The limit of detection (LOD) was determined with a signal-to-noise ratio between 4 and 5. Table 3 shows the linearity equation, correlation coefficient (R^2) and LOD. The calibration curve for chloramphenicol is shown in Figure 4.

Table 3. Linearity and LODs of Phenicols

Analytes	Regression equation	R^2	LOD (ng/g)
Chloramphenicol	Y = 0.5643X – 0.0001	0.9957	0.02
Florfenicol	Y = 0.8790X + 0.0006	0.9932	0.02
Thiamphenicol	Y = 0.1510X – 0.0018	0.9953	0.20

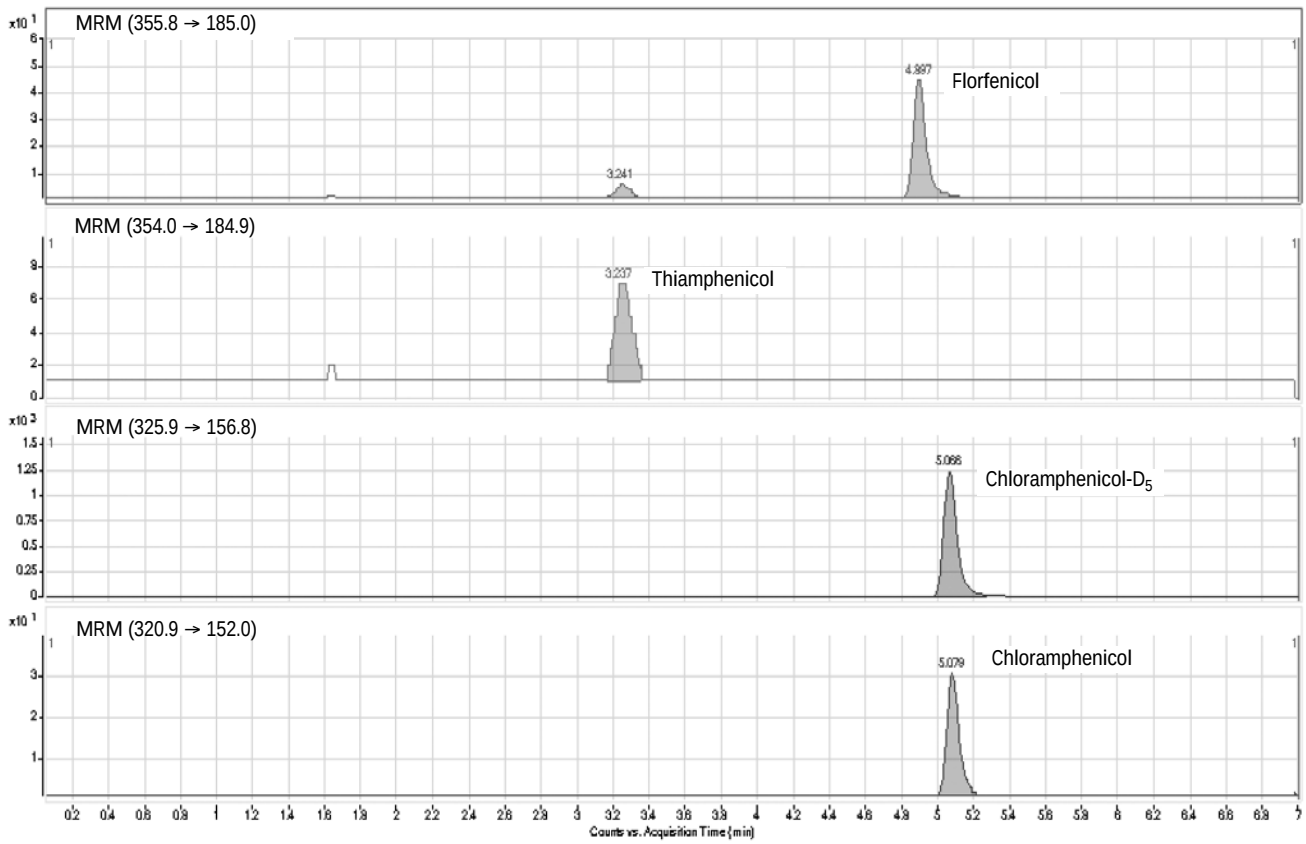


Figure 3. Chromatograms of 0.2 ng/g fortified honey extract.

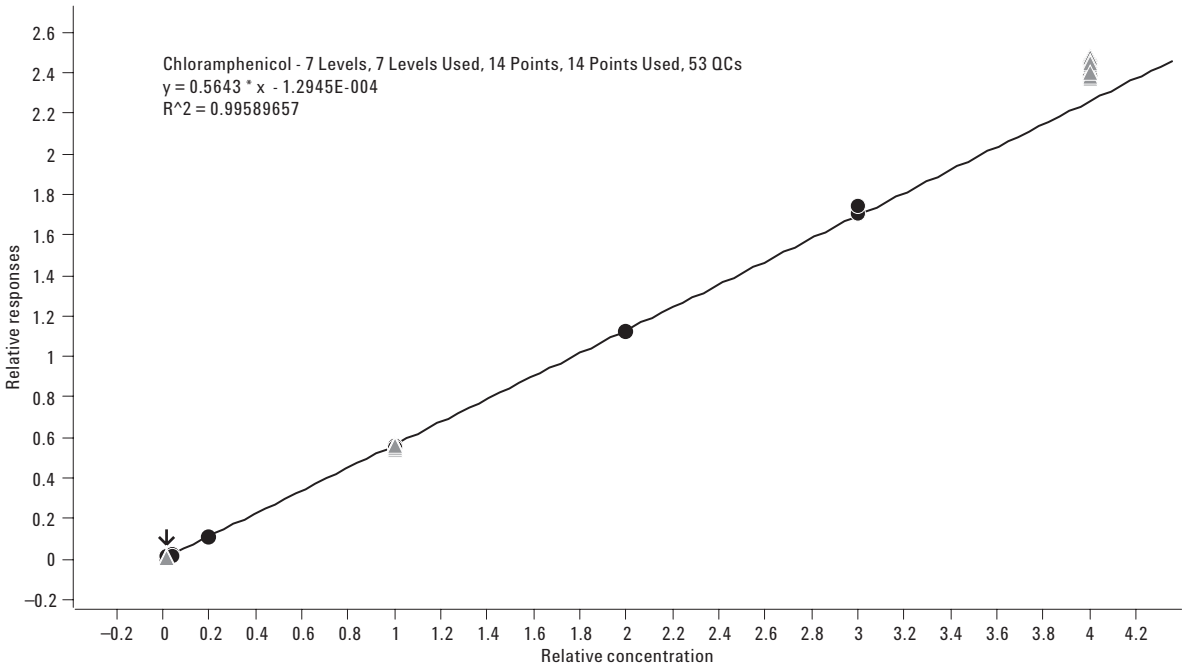


Figure 4. Calibration curve of chloramphenicol (0.1 to 20.0 ng/g). Dots (•) indicate sample results of calibration curve points, and trianges (Δ) indicate sample results of quality controls.

Recovery and Reproducibility

The recovery and reproducibility were evaluated by spiking phenicol standards in honey at levels of 0.1 ng/g (1.0 ng/g for TAP), 5.0 ng/g and 20.0 ng/g as quality control samples (QCs), and quantifying those QCs against the matrix spiked calibration curve. The analysis was performed in replicates of six at each level, except four replicates for TAP low level. The recovery and reproducibility (shown as %RSD) data are shown in Table 4. CAP and FF show excellent recovery and reproducibility at all QC levels. The recovery of TAP is adequate at all concentrations and the reproducibility is excellent.

Table 4. Recoveries and Reproducibility of Phenicols in Fortified Honey

Analytes	Spiking Level (ng/g honey)	Recovery (%)	RSD (%) n = 6
Chloramphenicol	0.10	96.94	3.51
	5.00	98.88	0.87
	20.00	107.32	0.46
Florfenicol	0.10	100.67	9.77
	5.00	100.28	2.84
	20.00	107.49	2.55
Thiamphenicol	1.00	76.00	4.39*
	5.00	74.89	2.34
	20.00	89.81	3.83

* The experiment was done in replicates of four.

Conclusions

Agilent SampliQ OPT SPE cartridges provide a simple and effective method for the purification and enrichment of chloramphenicol, florfenicol, and thiamphenicol in honey. The recovery and reproducibility results based on matrix spiked standards are acceptable for chloramphenicol residue determination in honey under EU or Chinese regulations. The impurities and matrix effect from honey are minimal and do not interfere with the quantitation of any target compound. The LOQs of the three phenicols are significantly lower than the MRLs.

References

1. GB/T 18932.19-2003, "Determination of Chloramphenicol Residues in Honey -LC/MS/MS Method."
2. "Commission Decision" 2003/181/ED of 13 March 2003, Off. J. Eur. Commun. L71/17 (2003).
3. "Handbook of Food Analysis: Residue and Other Food Component Analysis," CRC Press, 2004, pgs 937, 940.

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Printed in the USA
February 16, 2009
5990-3615EN

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