Detection, Confirmation, and Quantification of Chloramphenicol in Honey, Shrimp and Chicken Using the Agilent 6410 LC/MS Triple Quadrupole

Application

Food Safety

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Abstract

This method was developed using the Agilent G6410AA Triple Quadrupole Mass Spectrometer (QQQ) for chloramphenicol in honey, shrimp, and chicken. The sensitivity obtained exceeds the minimum required performance level (MRPL) established by the European Union regulation for food monitoring programs. Using a deuterated internal standard and one simple sample solid phase extraction (SPE) procedure can provide a limit of detection at 10 ppt in sample matrix. The analytical performance of the method was evaluated for three different matrixes and the results show little or no matrix effects. Linearity of response over 2 orders of magnitude was demonstrated (r > 0.99). In addition, good reproducibility of the two required product ion ratios was obtained to meet the EU identification points needed for confirmation. This study is a valuable indicator of the ability of the QQQ for routine quantitative trace analysis of chloramphenicol in honey, shrimp, and chicken.

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic. It was concluded that human exposure to CAP can cause aplastic anemia [1]. Chloramphenicol and other bacterial inhibitors have arguably been the biggest issue facing international seafood trade over the past year. Because chloramphenicol has displayed significant toxicological effects on humans, it has been banned from foods in the European community, Japan, and the United States at levels of 0.3 ppb.

LC/MS has been demonstrated for this analysis by the U.S. Food and Drug Administration[2-4] and others[5]. In this application, the Agilent G6410AA QQQ is used. This method employs negative ion mode with electrospray ionization. An internal standard (IS), CAP-d5, is added at the beginning of the extraction. The use of this IS self-corrects for any extraction variability from sample to sample and response variability caused by the matrix. With the use of this IS, 50 parts per trillion (ppt) CAP levels can be reliably quantified. A solid phase extraction (SPE) procedure is used along with a mobile phase of only methanol and water without salt buffers, which should help minimize MS maintenance.

Experimental

Reagents and Materials

Agilent AccuBond SPE ENV PS DVB Cartridges (P/N 188-3060)
Ethyl acetate from Burdick and Jackson (Morristown, NJ)
Methanol HPLC-Grade from Burdick and Jackson
Water (18 MΩ) from Milli-Q Synthesis System
Chloramphenicol (CAP) from Aldrich Chemical Co. (Milwaulkee, WI)
Deuterated (d5) CAP internal standard from Cambridge Isotope Laboratories (CIL, Andover, MA, U.S.)
Syringe filter (0.2 µm, PTFE) from Agilent (P/N 5185-5843)

Overview of Method

Internal Standard Preparation

1. A 100-µg/mL (100 ppm) stock standard CAP-d5 solution in methanol (MeOH) is purchased from Cambridge Isotope Laboratories, Inc. (Lot SCCE-005)
2. A 1:100 dilution in MeOH of the stock standard gives an intermediate standard concentration of 1 µg/mL (1 ppm) or 1000 ng/mL CAP-d5
3. A 1:100 dilution in MeOH gives a diluent solution (This diluent solution is used to prepare the samples) concentration of 10 ppb.
4. Every 1-g sample is fortified with 25 µL of CAP-d5 diluent solution for a 0.25 ppb IS (internal standard) concentration

Standard Solution Preparation

1. A 100-µg/mL stock standard CAP solution in methanol (MeOH) is prepared by weighing 5.0 mg CAP std into 50 mL methanol.
2. A 1:100 dilution with methanol of the stock standard gives an intermediate standard concentration of 1 µg/mL (1 ppm) or 1000 ng/mL CAP
3. Add 25 µL CAP-d5 diluent solution into each vial.
4. Prepare standard solutions in these vials: 1 ppb, 0.2 ppb, 0.1 ppb, 0.02 ppb, and 0.01 ppb, with IS at 0.25 ppb level.

Sample Preparation

All SPE cartridges are conditioned with 2 mL of water before use.

1. **Honey**, 1 g of sample is diluted to 5 mL with water and 25 µL 10 ppb IS is added. The solution is loaded onto the SPE cartridge and allowed to stand for 5 min. Elution is performed with 10 mL ethyl acetate. The eluate is collected and the solvent is evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min. The solution is filtered, using a syringe filter, before injection. No additional clean-up of the sample solution is performed.
2. **Shrimp**, 1 g of shrimp is defrosted and mixed in a blender. To the 1 g of the mixed shrimp, 3 mL of water and 25 µL 10 ppb IS is added. The portion is centrifuged for 5 min (8,000 rpm). The supernatant is loaded on the cartridge and allowed to stand for 5 min. Elution is performed with 5 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min; the solution is filtered before injection.
3. **Chicken**, 1 g of chicken is defrosted and mixed in a blender. To the 1 g of the mixed chicken, 3 mL of water and 25 µL 10 ppb IS is added. The portion is centrifuged for 5 min (8,000 rpm). The supernatant is loaded on the cartridge and allowed to stand for 5 min. Elution is performed with 5 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min.; the solution is filtered before injection.

LC/MS conditions

The LC system was the Agilent 1200-SL binary pump with the ALS-SL autosampler. The MS was an Agilent 6410 LC/MS triple quadrupole mass spectrometer. See Table 1 for conditions.

Table1. LC/MS Conditions

HPLC	
Column	ZORBAX SB-C18, 2.1 × 50 mm, 1.8 µm (p/n 827700-902)
Flow rate	0.4 mL/min
Mobile phase	A: water
	B: methanol
Gradient	0-5 min, 30~70% B
	5-6 min, 70~100% B
	8 min, 100% B
Post time	4 min
Temperature	45 °C
Injection	5 µL
MS Source Settings	
Source	ESI
Ion polarity	Negative
Drying gas temperature	350 °C
Drying gas flow rate	10 L/min
Nebulizer	45 psi
V_{cap}	3500 V
Fragmentor	100 V
Collision energy	10 V for *m/z* 257(qualifier ion)
	15 V for *m/z* 152 (quantitation ion)

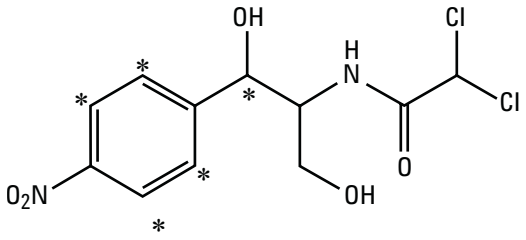
Results and Discussion

Spectral Quality and Sensitivity of Standard

Table 2 lists the structure of the CAP and the fragment ions used for quantitation and confirmation as described by the identification point system.[6] To obtain the most sensitivity, only two or three parameters need to be optimized on this instrument. They are the fragmentor, to provide highest transmission of the precursor ion, the collision energy, to maximize signal for the quantitation and qualifier ion, and possibly the V_{cap} (electrospray voltage), to maximize the number of ions generated.

Table 2. Structure and Fragment Ions of CAP and CAP-d5 (* indicates deuterated positions for the CAP-d5 IS)

Chloramphenicol structure



	m/z	
CAP	257	Qualifier ion
	152	Quantitiation ion
CAP-d5	262	
	157	

Optimization of MS Condition

Figure 1 shows the results of varying the V_{cap}. For this analyte there was little effect from varying this parameter. Only a slight increase in signal is observed at 3,500 V, and this voltage was used. The fragmentor was varied from 90 V to 160 V. Above 120 V, fragment ions are observed and the precursor ion signal drops significantly. At 160 V on the fragmentor almost no *m/z* 321 is observed. This results show that 100 V on the fragmentor provided the highest precursor ion signal. Finally, using a product ion scan of the precursor, m/z 321, the collision energy (CE) was varied from 2 V, 5 V, 8 V, 10 V, 15 V, 18 V to 40 V.

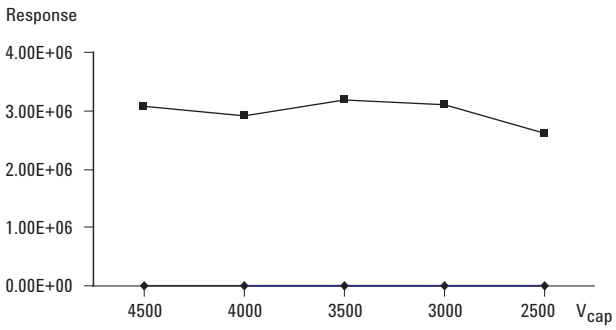


Figure 1. Plot of V_{cap} voltage vs. response of precursor ion at *m/z* 321.

Comparison of extracted ion chromatograms of the quantitation and qualifier ions showed that response maximized at 10 V for *m/z* 257 and at 15 V for *m/z* 152. The product ion spectra for these two collision energy experiments are shown in Figure 2 and Figure 3. As shown in Table 3, the same CE were used for the deuterated internal standard.

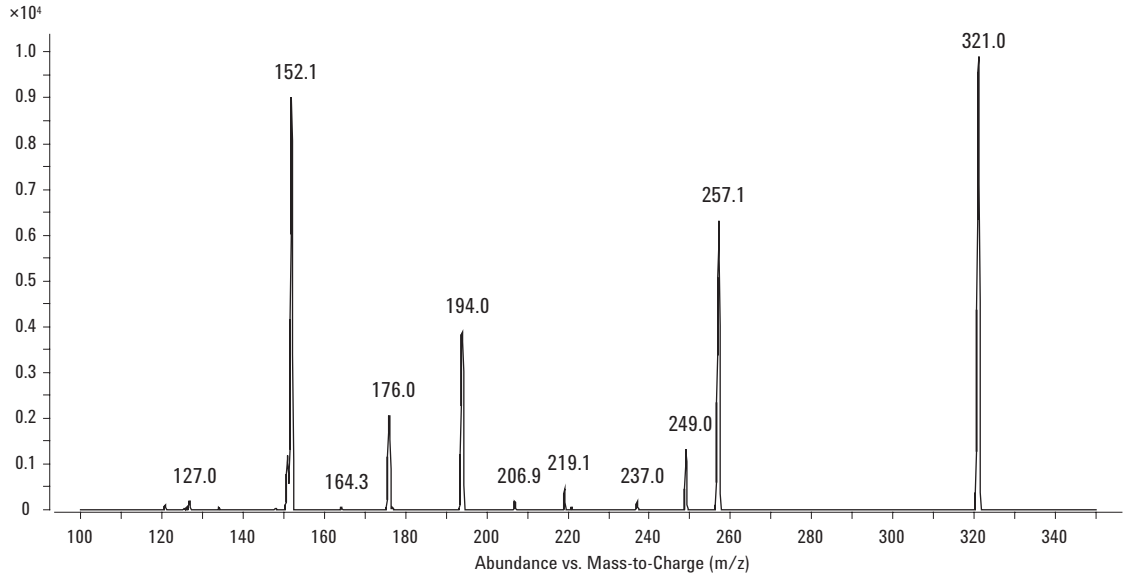


Figure 2. Product ion spectrum of *m/z* 321 at 10 V collision energy.

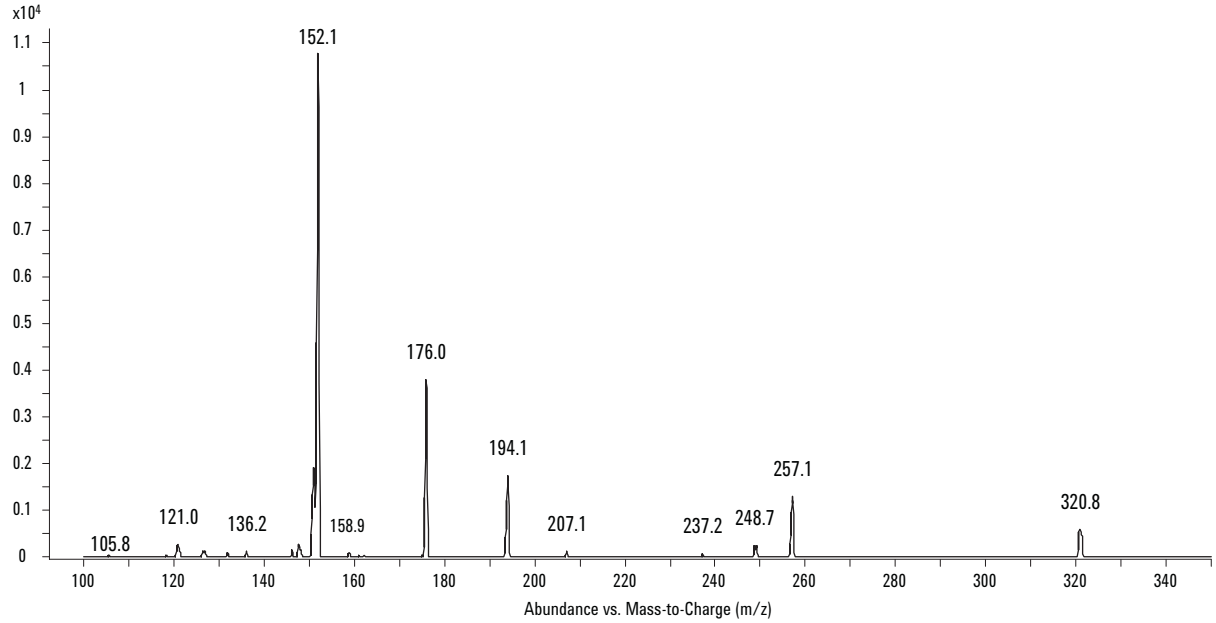


Figure 3. Product ion spectrum of *m/z* 321 at 15 V collision energy.

Table 3. MRM Mode Parameters

Compound	Transition	Dwell time (ms)	Fragmentor Voltage (V)	Collision) Energy (V)	MS2 resolution
CAP	321–257	200	100	10	Unit
	321–152	200	100	15	Unit
CAP-d5	326–262	200	100	10	Unit
	326–157	200	100	15	Unit

Repeatability

Using honey matrix spiked at 0.1 ppb level as an example, the repeatability was tested by running the extract 15 times. Table 4 shows the area of the qualifier and quantitation ions in both the analyte and the IS. On average the areas of each ion vary about 8% and the ratios 5%, well within the 20% required for ratios 50% and above. Masshunter quantitation software tabulates these results and gives a graphic representation as shown in Figure 4.

Table 4. Integrated Areas of the Quantitation Ion and Qualifier Ion and Their Associated Internal Standard Ion

	Chloramphenicol Quantitative ion (321–152)	Qualifier ion (321–257)	Ratio	d5-chloramphenicol Quantitative ion (326–157)	Qualifier ion (326–262)	Ratio
1	350	165	47.1	262	121	50.4
2	346	157	45.2	258	114	55.3
3	346	5	44.6	259	118	49.4
4	313	164	52.3	267	127	47.6
5	301	154	49.5	261	121	46.4
6	313	168	53.6	253	124	49.0
7	320	160	50.1	228	111	48.6
8	326	145	44.5	225	113	50.4
9	317	141	44.5	241	117	48.6
10	290	135	46.6	226	107	47.1
11	300	138	46.2	253	90	45.7
12	281	136	48.4	240	90	47.6
13	303	143	47.3	220	101	45.9
14	290	140	48.3	214	107	49.8
15	261	131	50.3	217	101	46.6
RSD	8.11%	8.30%	5.91%	7.67%	9.99%	4.83%

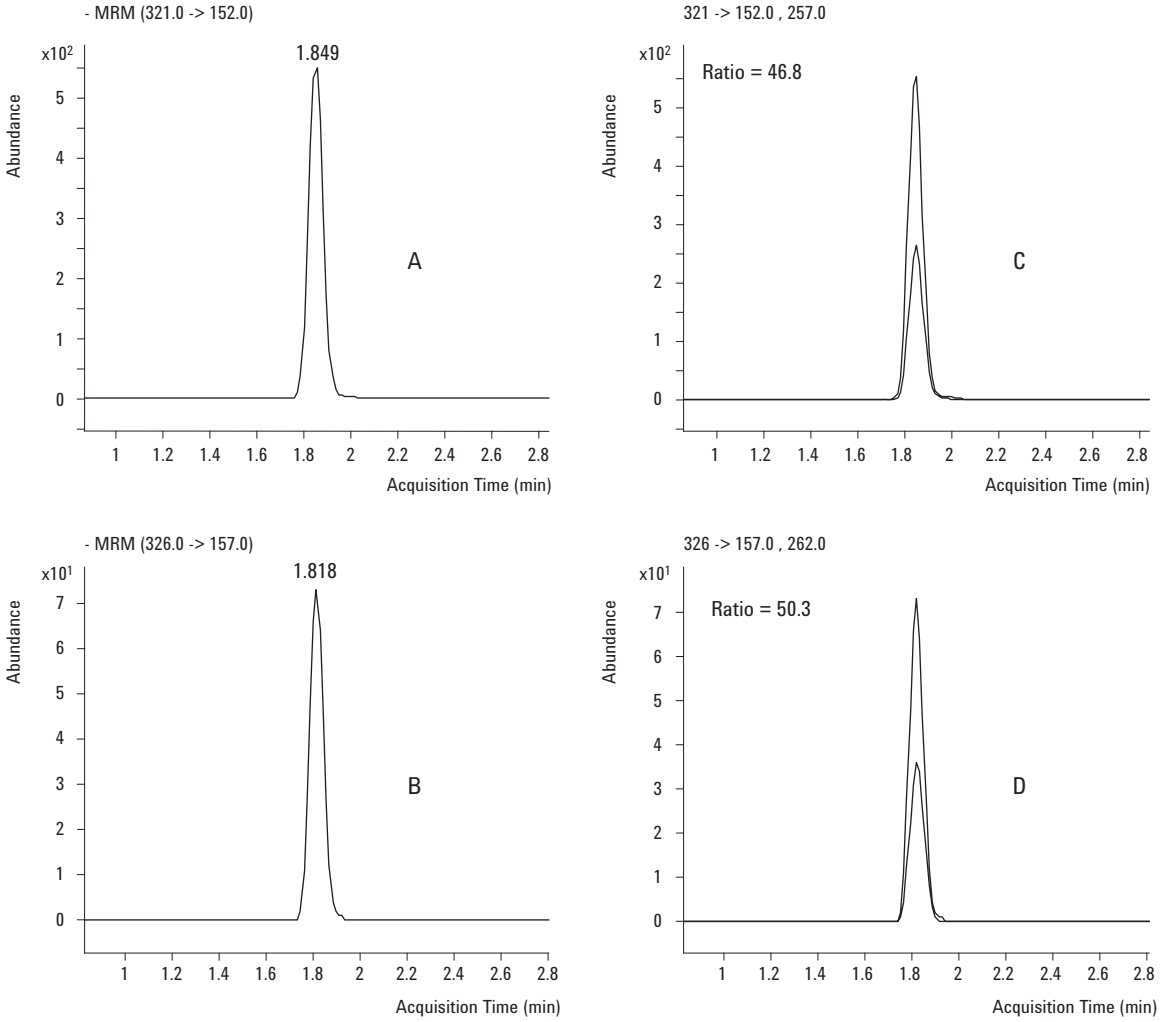


Figure 4. Panels A and B show the CAP and IS peak for the quantitation transition. Panels C and D are the graphic representation of quantitation ion and qualifier ion ratio as shown by MassHunter software.

Linearity

The linearity of the method was determined for CAP in solvent and each of the matrices. This was done from 10 ppt to 1 ppb, well below the minimum required performance level (MRPL) and above that concentration. Figures 5 through 8 show the graphic representation of those results. Each was well above an r^2 value of 0.99.

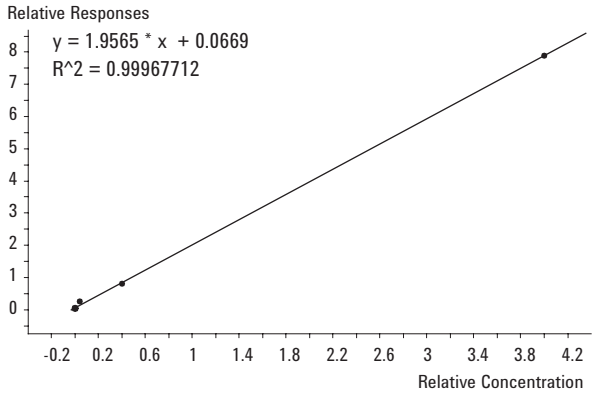


Figure 5. Linearity of CAP in solvent from 10 ppt to 1 ppb.

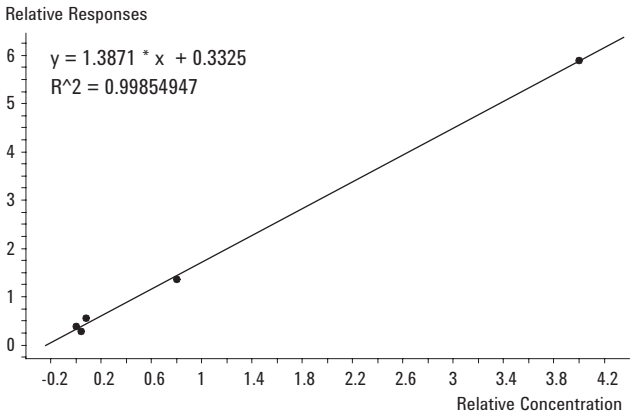


Figure 6. Linearity of CAP in honey from 10 ppt to 1 ppb.

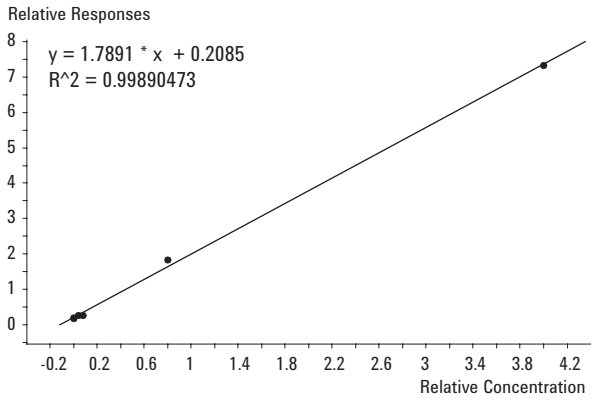


Figure 7. Linearity of CAP in shrimp from 10 ppt to 1 ppb.

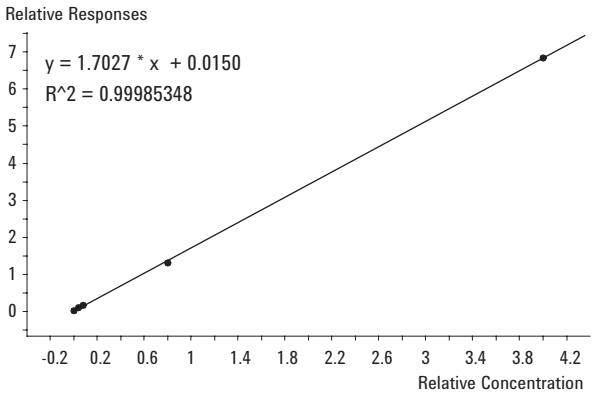


Figure 8. Linearity of CAP in chicken from 10 ppt to 1 ppb.

Sensitivity

The sensitivity of CAP standard in solvent is observed at 10 ppt with an injection volume of 5 µL. The MRM chromatogram is shown in Figure 9. Although this demonstrates the sensitivity of the instrument, it is also important to determine the sensitivity in real sample matrix. This is shown in Figure 10 with a spike concentration of CAP at 10 ppt with a 5-µL injection. Not only is the analyte detectable, but the ratio of the qualifier ion is within the specified tolerance so confirmation can be obtained.

Recovery

Recovery was determined by spiking CAP into three samples of matrix and extracting using the specified SPE. Table 5 shows both the repeatability of extraction and analysis and the mean recovery. Using the internal standard spiked before extraction, recovery is automatically compensated. Thus accuracy of the quantification is very good using this methodology. The recovery results show the overall effectiveness of the method.

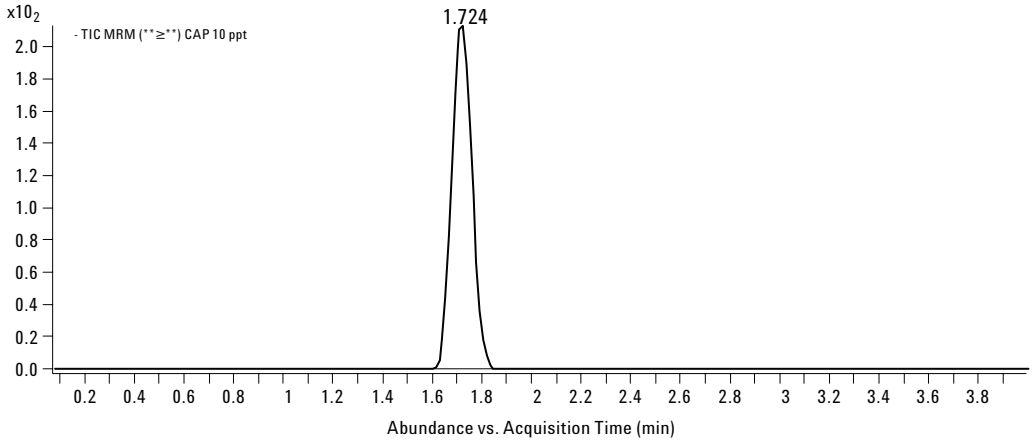


Figure 9. MRM chromatogram of 10 ppt CAP in solvent with injection volume of 5 µL.

Table 5. Recovery of CAP at 0.1 ppb Where Three Sample Aliquots of Each Matrix Were Spiked and Determined

	Honey (n=3)	Shrimp (n=3)	Chicken (n=3)
RSD (%)	6.29	3.93	3.29
Recovery (%)	89.5	85.4	86.4

Conclusions

The method described herein for the analysis of CAP in three important matrices has been shown to be highly effective and meet the criteria for quantitation and confirmation well below the required 0.3 ppb MRPL. Optimization of the method was simple, as few parameters in the mass spectrometer need adjustment. In addition, the requirements for a validated method have been shown. These include sensitivity, repeatability, linearity, and recovery. The Agilent 6410 LC/MS triple quadrupole instrument has been shown to be a highly effective instrument for the analysis of chloramphenicol.

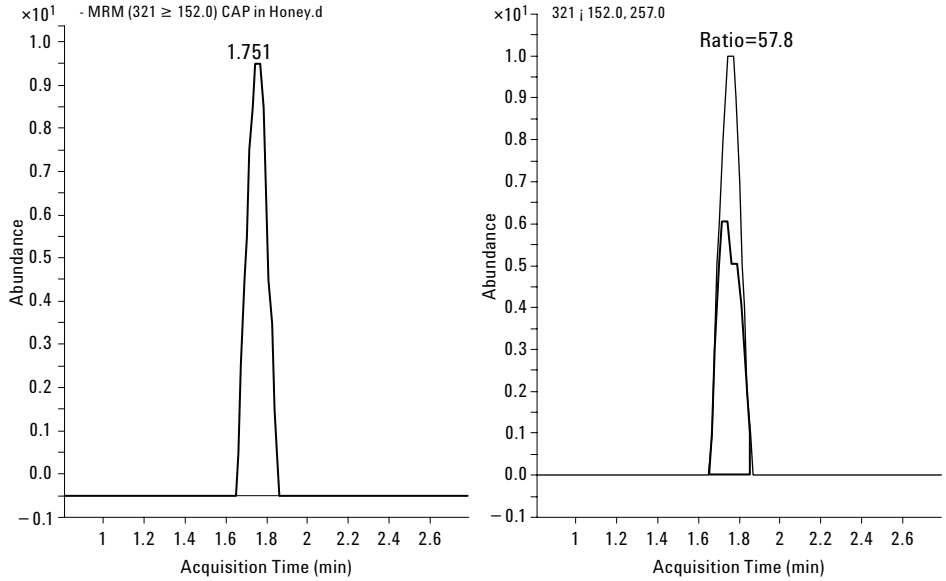


Figure 10. MRM chromatogram of quantitation ion and ratio of qualifier ion for 10 ppt CAP in honey.

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