CONFIRMATION OF MULTIPLE PHENICOL RESIDUES IN SHRIMP BY ELECTROSPRAY LC/MS

Al Pfenning, Sherri Turnipseed, Jose Roybal, Cathy Burns, Mark Madson,
Joseph Storey, Rebecca Lee
Animal Drugs Research Center and
Denver District Laboratory
Food and Drug Administration,
P.O. Box 25087, Denver CO 80225-0087

SCOPE

The analysis of shrimp for chloramphenicol and related compounds is important for several reasons. Residues of chloramphenicol (CAP) are of particular concern because this drug can cause serious acute reactions, including aplastic anemia, in susceptible individuals (1). Recently it has been reported that chloramphenicol has been found in several foodstuffs from Asia, including shrimp (2).

There are limited reports of the analysis of CAP and other phenicols in food from animal origin substances using electrospray LC/MS (3). Several others government (4,5) methods have also been reported, but are not published in the open literature. Our laboratory has been working with these compounds for many years. The traditional approach to the determination and confirmation of these compounds is isolation from tissue or fluids using liquid/liquid extraction, derivatization with silylating agents to form volatile derivatives, and analysis by GC/ECD and/or GC/MS with negative chemical ion detection (6-8).

The scope of this method is to describe a confirmatory (qualitative) method for chloramphenicol (CAP) and several related compounds (florfenicol [FF] and thiamphenicol [TAP]) in shrimp using negative ion electrospray with ion trap LC/MS^n analysis. Because the chromatographic and MS conditions were initially developed to look for the metabolite florfenicol amine as well as these other drugs, the method allows for detection of this compound in the first part of the chromatographic run, but at this time a confirmation limit for this drug has not been determined in shrimp.

Both fixed MS^2 scans and data dependent acquisition were used successfully to confirm these drugs in shrimp tissue. The fixed MS^2 program outlined in this SOP was chosen for the final method. Certain parameters, such as matrix effects, reproducibility of the instrument and extractions must be evaluated more thoroughly before this method would meet standards for quantitative analysis. Better performance for quantitation at low residue levels (<1 ppb) will most likely be obtained using a triple quadrupole instrument.

(1) Roybal, J.E. “Chloramphenicol and Related Drugs” in *Analytical Procedures for Drug Residues in Food of Animal Origin* (1998) ed, S.B. Turnipseed and A.R. Long, Science Technology System, W. Sacramento, CA pp. 227-260.

(2) http://www.fst.rdg.ac.uk/foodlaw/news/eu-02031.htm

(3) Hormazabal, Y J. Liq Chromatogr. & Related Technique

(4) Canadian Food Inspection Agency (CFIA), Dartmouth Laboratory Draft method: Analysis of Florfenicol, Florfenicol Amine, Thiamphenicol and Chloramphenicol in Fish, Shellfish and Crustaceans (2002)
(5) Florida Chemical Residue Laboratories, Florida Department of Agriculture and Consumer Services, Preparation and Analysis of Chloramphenicol in Shrimp. (2002)
(6) Pfenning, A.P., Roybal, J.E., Rupp, H.S., Turnipseed, S.B., Gonzales, S.A., Hurlbut, J.A. (2000) *J.AOAC Int*. 83, 26.

(7) Pfenning, A.P., Madson, M.R., Roybal, J.E, Turnipseed, S.B., Gonzales, S.A., Hurlbut, J.A., Salmon, G.D (1998) *J.AOAC Int*. 81, 714.
(8) Kijak, P.J (1994) *J.AOAC. Int*. 77, 34.

PRINCIPLES

I. Extraction.

Ten grams of shrimp composite is extracted with 20 mL basic ethyl acetate/acetonitrile, homogenized and centrifuged. The extraction steps are repeated and the ethyl acetate/acetonitrile layers are evaporated to dryness. Thirty mL water is added to the flask, sonicated and followed by hexane defatting steps. The aqueous phase is passed through a series of SPE columns. The analyte is extract off the final SPE with methanol. The methanol is evaporated to dryness. The extracts are reconstituted into a small volume of 0.1% formic acid and filtered into LC vials. In addition, only the parent phenicols (not the florfenicol amine) were confirmed by this method (only the C18 cartridge was eluted and analyzed).

II. Mass Spectral Analysis

A. Qualitative Confirmation

The qualitative confirmation of phenicols in shrimp is based on unique mass spectral characteristics of these compounds as evaluated by established guidelines (9,10). One unique aspect of these compounds is the fact that they contain two chlorine atoms, thus giving rise to unique isotopic patterns. In order to take advantage of this fact, the MS^2 spectra is obtained not only from the parent ion ([M-H]-, but also from the corresponding M+2 ($^{35}Cl^{37}Cl$) isotope peak. For example, in the MS^2 spectra of CAP ([M-H]- pair m/z 321/323) the predominant ion is m/z 194 which corresponds to $[M- H-(NH_2COCCl_2H)]^-$. Also present in this spectra are the ions m/z 176 $[m/z\ 194 – (H_2O)]^-$ (15%), 249 $[M-H-(2HCl)]^-$ (30%), and 257 $[M-H-(HCOCl)]^-$ (25%). These ions are also present in the MS^2 spectra of m/z 323, although the peak at 257 is split (into peaks of approximately equal abundance) between ions at m/z 257 and 259, indicating the loss of one chlorine atom (either ^{35}Cl or ^{37}Cl) from the $^{35}Cl^{37}Cl$ parent ion.

The florfenicol MS^2 spectra is dominated by the loss of HF from the parent ions. This is observed as m/z 335.8 when the $^{35}Cl^{35}Cl$ parent ion (m/z 356) is isolated or m/z 337.8 when the $^{35}Cl^{37}Cl$ ion is fragmented. To obtain additional confirmatory ions, MS^3 is performed on ion 335.8 to give a spectra which includes the ions 219 (usually 100%,), as well as m/z 119, 184, 264. Thiamphenicol [M-H]- equal to 354/356, fragments to give the following ions, m/z 227, 240, 270, and 290/292.

The florfenicol amine spectra is not as unique as the parent phenicols because it does not include the lipophilic chlorine containing moiety. This compound responds very well by positive ion electrospray to give $[MH]^+$ of m/z 248. The predominant ion in the MS^2 spectra is m/z 230, representing the loss of water. The dominant ion in the MS^3 is m/z 130. Because of the non-specific ions and losses associated with this compound, as well as, the fact that it elutes very early in the chromatographic run, the confirmation of the drug was complicated by the fact that low-level false positives were observed. Therefore, although the chromatographic program would allow for its detection, confirmation limits for the amine were not evaluated in shrimp at this time.

REAGENTS

Solvents: Distilled-in glass, pesticide-grade, hexane, ethyl acetate (EtOAC), acetonitrile (ACN), isopropanol (IPA), methanol (MeOH).

Formic acid used to prepare the mobile phase was purchased from Baker (88%).

Solid-phase extraction columns: C18: Varian Bond Elut 6 cc/500 mg

PRS: Varian Bond-Elut LRC-PRS 500mg

Syringe filters: 4 mm syringe filter 0.45 µm, PFTE. Phenomenex P/N AFO-0422

Ammonium hydroxide (assay ca. 30% as NH_3),

Glacial acetic acid, LC grade.

EQUIPMENT

1. Ion Trap LC/MS: The instrument used was a Finnegan LCQ DECA Ion Trap Mass Spectrometer coupled to a modular Spectrasystem LC system. The components of the LC system include a SCM1000 degasser, P4000 LC pump, AS3000 autosampler, and a UV6000LP UV/VIS detector. The software used was Xcaliber Version 1.2.

2. LC Column. The LC Column was an Xterra phenyl (2.1 x 100 mm, 3.5 µ, Waters Corp. P/N 186001180). Other phenyl columns would also be acceptable. In this laboratory an Inertsil phenyl (2 x 150 mm, 5 µ, Phenomenex Corp. P/N 0301-150X020) was also tested during method development. If other columns are used, the time segments in acquisition program need to be adjusted to account for shift in retention times.

3. Other.

Tissue disrupter --High speed shearing tool, i.e. tissuemizer, of a diameter < 20 mm.

Rotoevaporator: with ice trap and water bath set at 50 C

Nitrogen evaporator: 12-sample nitrogen evaporator, with 50 C water bath

Plasticware: 50 mL and 15 mL disposable, conical polypropylene with screw cap

Glassware: pear shape flask, Pastuer pipettes

PROCEDURES

1. Standard Preparation

The compounds were purchased or obtained from: Chloramphencol (USP), Thiamphenicol (Sigma), Florfenicol and Florfenicol Amine (Schering-Plough).

Fortification Standards. For fortification of shrimp, individual stock solutions of drug at 1000 µg/mL (1000 ng/µL) were made up in acetonitrile. A combined intermediate standard solution (10 ng/µL) was made by pipetting 1 mL of each individual stock solution into 100 mL volumetric flask and diluting to volume with acetonitrile. Prepare fortification standards, as applicable: Pipet 0.5, 0.2, or 0.1 mL combined standard solution into 10 mL volumetric and dilute to volume with acetonitrile for 5, 2, and 1 ppb fortification standards, respectively.

MS Standards For MS analysis, stock solutions of drug at 100 µg/mL (100 ng/µL) were made up in methanol. A mixed intermediate standard (1 ng/µL of each drug) was made by diluting 500 µL of each stock solution to 50 mL with 0.1% formic acid.

Working LC/MS Standards. As applicable, LC/MS standards were made as follows:

µL of intermediate standard	µL of 0.1% Formic Acid	[ng/µL]	equivalent in shrimp (ppb)*
1000	4000	0.2	5
400	4600	0.08	2
200	4800	0.04	1
100	4900	0.02	0.5

* Assuming 10 g of shrimp is processed and final extract volume is 250 µL.

Stability. Working LC/MS standard are stable for at least one week.

2. Sample Preparation

Control Samples. At least one control (matrix blank) sample should be run with every set of samples.

Fortified Samples. At least two fortified samples should be run with every set of incurred or unknown samples. The concentration of the fortified sample should be in the range of 1-5 ppb.

Incurred Samples. Were not evaluated during method development.

3. Sample Extraction.

Hold frozen shrimp at room temperature until they feel limber. Remove the heads, chitinous shell and body appendages from partially thawed shrimp. Place shrimp meat in blender, and blend with dry ice with pulsed action until contents are uniform. Accurately weigh about 10.0 g of blended shrimp composite into a 50 mL P/P centrifuge tube. (If spiking control shrimp, add 100 µL of the desired concentration of Standard Solution to completely thawed 10 g blank composite and allow to sit at room temperature for at least 20 minutes before proceeding.) Add 20 mL of extraction solution (EtOAC:NH_4OH, 98:2) homogenize with tissue disrupter until the entire mass is broken up (about 30 sec). Centrifuge for 7 min @ 4000 RPM, 5 ^oC; decant through medium retention filter paper into 100 mL P-S flask. Repeat extraction with another 20 mL of extraction solution, combining the extracts in the 100mL P-S flask. Repeat extraction a third time with 10 mL of extraction solution + 10 mL ACN combining the extracts in the 100 mL P-S flask. Add 5mL IPA, to prevent bumping and foaming and roto-evaporate at 50-55 ^oC to dryness. Add 30 mL H_2O, vortex, sonicate 2 min, adjust pH (<4.6) with approximately 0.4mL of 0.1% acetic acid and pour into a 50 mL P/P centrifuge tube. Add 5 mL of hexane to the 100 mL P-S flask; vortex, swirl to dissolve contents, and transfer contents to same tube as the acidified aqueous; repeat with another 5

mL aliquot of hexane. Shake tube well or vortex for about 30 sec, centrifuge @ 4000 RPM at room temperature for 3 min, aspirate upper hexane layer and discard. Repeat hexane defatting steps two more times with an additional 5 mL portion of hexane each time and discard the hexane each time. Condition each PRS and C_{18} SPE column with 3 mL MeOH followed by 3 mL H_2O. Transfer remaining aqueous from P/P tube to a (conditioned) SPE system consisting of a C_{18} SPE column on bottom, PRS SPE column on top of the C_{18}, with a 70 mL reservoir atop the PRS; all on a vacuum manifold (allow to flow through at about 1 drop/sec). When level just reaches the top of PRS column, add 2 mL H_2O to columns. Allow the columns to run dry, separate system, discarding reservoir, identify and place PRS column in 50mL centrifuge tube and store in freezer, if needed for florfenicol amine analysis. Elute the C_{18} SPE with 4 mL MeOH into 15mL disposable P/P centrifuge tube. Evaporate MeOH eluate to dryness in N-Evap with water-bath set at 50°C. The dried extracts are reconstituted into 250 µL of 0.1% formic acid, and filtered for injection into LC-MS system.

4. Instrument Operating Parameters.

Regardless of the instrument used, certain performance verification criteria should be incorporated into the operating parameters. These include mass calibration, tuning, and appropriate fragmentation patterns. Mass axis calibration should be performed according to the instrument manufacturers' specifications or according to internal laboratory MS standard operating procedures. Signal optimization (tuning) should be adjusted to maximize the abundance of ions of interest. Daily system suitability requirements (described in #7 of this section) should also be met. The following describes the specific operating procedures for the instrument used to validate this method in the developer's laboratory.

(i) Instrumental Configuration. LC/MS analysis is performed using a LCQ DECA mass spectrometer coupled to a TSP P4000 LC via an electrospray interface. The instrument is operated using positive and negative ion detection. The instrument was calibrated according to the manufacturer's instructions. The response for CAP was optimized by tuning on ion m/z 321. For tuning, CAP (1 ng/µL in mobile phase) was pumped through a syringe pump at 10 µL/min and then introduced into the LC flow (250 µL/min 80/20 0.1% formic acid/acetonitrile) via a T before entering the MS source. In the tune file the MS parameters were set to a prescan of 2 and a

maximum inject time of 100 ms. The MS^2 parameters were also optimized using the tune function of the instrument. For this mode the prescan was set to 1 with a maximum inject time of 500 ms . The collision energy was optimized for both total MS^2 ion current, as well as for specific ions (m/z 194, 249) with no significant differences (optimal collision energy was 24-26% in all cases).

(ii) Monitored Response. Using the ion trap, MS^2 was performed on the molecular ions for each of the analytes according to the following program:

Program 1: Fixed MS^2 Acquisition

Isolation width was set to 2 amu for all MS^2 transitions. Positive ion tune should be used for time segment 1 if used. Tune file developed for CAP (described above) should be used for other time segments.

Time Segment 1: 2-5 minutes FFA (CAN DELETE THIS SEGMENT)
Scan Event 1: (+) MS [m/z 180-350]
Scan Event 2: (+) MS^2 of m/z 248.1 (24% CE) [m/z 65-250]
Scan Event 3: (+)MS^3 of m/z 248.1 (24%CE) → m/z 230.1 (32% CE) [m/z 60-250]

Time Segment 2: 5-11 minutes TAP
Scan Event 1: (-) MS [m/z 320-375]
Scan Event 2: (-) MS^2 m/z 354.2, (CE 35%) [m/z 65-250]
Scan Event 3: (-)MS^2 356.2 (CE 35%)

Time Segment 3: 11-12.5 minutes FF
Scan Event 1: (-) MS m/z 320-375
Scan Event 2: (-) MS^2 m/z 356.2,(CE 24%)
Scan Event 3: (-)MS^2 m/z 358.2 (CE 24%)
Scan Event 4: (-) MS^3 of m/z 356.2 (24%CE) → m/z 335.8 (20% CE)

Time Segment 4: 12.5-18 minutes CAP
Scan Event 1: (-) MS m/z 300-350
Scan Event 2: (-) MS^2 m/z 321.2 (CE 24%)
Scan Event 3: (-)MS^2 m/z 323.2 (CE 24%)

A UV/Vis diode array detector was also utilized with a scan range of 190-800nm and channel A set to 270 nm (bandwidth 9 nm) and channel B set to 236 nm (bandwidth 9 nm).

(iii) Specific Operating Conditions. The electrospray interface was operated with a temperature of 275°C. The sheath gas was nitrogen at approximately 35 psi; the auxiliary gas was also nitrogen at approximately 6 psi (optimized for CAP signal). The mobile phase was at flow of 250 µL/min and a column oven was not used. Automated injections of 75 µL were made using

"push loop" type injection. The LC flow was diverted away from the mass spectrometer for the first minute. The MS was on from 1-18 minutes. The chromatographic gradient is as follows:

Time (minutes)	% Acetonitrile	% 0.1% Formic Acid
0-5 *	2	98
6-18	20	80
20-22	90	10
23-28	2	98

* note- if not interested in florfenicol amine, chromatographic program could begin at 20% acetonitrile. Time windows might need to be adjusted.

5. Procedures for Instrumental Analysis of Samples, Controls, and Standards

Standards are to be run with each set of samples (at the beginning and end of a set of samples, and in the middle of the sequence if many samples are being analyzed). At least two positive controls, i.e. fortified matrix should be run along with any unknown sample extracts. A blank matrix sample (negative control) should also be run along with any unknown sample extracts and must demonstrate the absence of CAP. At least one of the fortified matrix control samples must demonstrate the confirmation criteria in the Validation Section #2v. A solvent blank (mobile phase) should be run before each sample to ensure that there was no carryover from the previous sample or standard. Solvent blanks are not required between duplicates of the same test sample, or when a fortified sample of higher concentration than a previous fortified sample is analyzed.

6. Calculations

For qualitative analysis, the important factor is to obtain information to determine if the data meet the confirmation criteria described in the Validation Section #2v. Ion chromatograms from the full MS (m/z corresponding to $[M-H]^-$) and from MS^2 (m/z 194 corresponding to $[M- H-(NH_2COCCl_2H)]^-$ from both fragmentation of both m/z 321 and 323) can be shown along with the MS^2 spectra averaged across the chromatographic peaks. In addition, extracted ion chromatograms for several ions (m/z 194, 257/259, 249, and 176) in the MS^2 spectra of 321 and 323 can be shown.

As scan data are obtained, relative abundances of representative ions can be estimated from the appearance of the MS^2 spectra, or from tabulation data. Integration of ion chromatograms is not necessary.

7. System Suitability

The instrument should meet calibration and tuning criteria as described above.
In addition, for each day's analysis, a standard mixture should be analyzed initially to determine the performance qualifications, or system suitability of the instrument. The analytes need to elute at the correct retention time; within ± 5% of what was observed for standards previously (unless column or mobile phase have been changed) and within the time-dependent window if used. It may require one or two injections of standard for compounds to elute at correct retention time if instrument has not been used recently. In addition, the response for 75 µL injection of a 1 ppb standard for CAP should be > 200,000 counts for the 321-> 194 MS^2 transition.

VALIDATION INFORMATION

1. Validation Data

Validation data for ion trap MS confirmation of multi phenicol residues in shrimp are shown in Table 1. Figure 1 shows chromatograms for a 1 ppb shrimp fortified extract.

2. Parameters Evaluated

(i) ***Recovery.*** Fortified samples were analyzed at 1 and 2 ppb with recoveries of approximately 55 percent.

(ii) Reproducibility. A series of standard injections (75 µL injection size) were analyzed using the following standards: At 1 ppb (3 ng on-column) the reproducibility of standard injections as measured by the CAP 321 to 194 transition was 16% (n=6), at 0.25 ng (750 pg on-column), 19.9% (n=5) and at 0.1 ppb (300 pg on-column) it was 40.0% (n=4).

(iii) Specificity. This method meets the specificity guidelines for confirmation methods outlined by Sphon[9] and recently elaborated in CVM's draft guidance[10]. During the course of this investigation, several lots of control shrimp were analyzed and there were no significant interfering peaks in any of the control tissue samples analyzed using the mass filters as described.

(iv) Sensitivity. For CAP, the ion trap instrument was able to confirm approximately 300-500 pg of standards on-column and shrimp tissue fortified at 1.0 ppb was confirmed with a 75 uL injection volume (final extract volume of 250 μL).

(v) Accuracy, Proof of Recovery from Authentic Samples.

Using an ion trap instrument the following criteria must be met for positive qualitative confirmation:

For chloramphenicol: 1) The ion m/z 194 $[M- H-(NH_2COCCl_2H)]^-$ must be observed in the MS^2 spectra from both parent ions (m/z 321 and 323), and should be a predominant peak in the mass range m/z 100-270. 2) In addition, at least one of the other structurally significant lower abundance ions (m/z 257/259 $[M-H-(HCOCl)]^-$, m/z 249 [M-H-(2HCl)], or m/z 176 [m/z 194 – $(H_2O)]^-$) must also be present in at least one of the MS^2 spectra at an approximate relative abundance to the base peak m/z 194 as is observed in the external standards, and 3) the retention time should be +-5% of external standards run on that day.

The qualitative criteria for the other phenicols is similar. The florfenicol MS^2 spectra is dominated by the loss of HF from the parent ions to give only one ion (335.8 from m/z 356 or m/z 337.8 from m/z 358). To obtain additional confirmatory ions MS^3 is performed on m/z 337.8. For thiamphenicol $[M-H]^-$ (354/356) fragments to give several ions m/z 227, 240, 270, and 290/292. At least two of these should be observed in MS^2 spectra from each parent isotope peak. In addition, the retention times for these other residues must also be +- 5% of what is observed from external standards analyzed on the same day.

(vi) Practicality, Sample Throughput, Solvents and Time Requirements. Extraction and LC/MS analysis of 6-8 samples can be accomplished in one day/overnight. For example, initial extraction can be performed in 5 hours. Each LC/MS run takes 28

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[9] J.A. Sphon *J. Assoc. Off. Anal. Chem*. **61**, 1247 (1978)

[10] Center for Veterinary Medicine (2001) *Guidance for Industry: Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues*

minutes therefore 6 sample analyses (bracketed by analysis of standards, separated by solvent blanks) can be done in 8-12 hours.

QUALITY CONTROL POINTS

(1) Critical Points

(i) Extraction. When filtering, be careful that the syringe filter does not disengage.

(ii) Chromatography. A formic acid/acetonitrile mobile phase at 0.25 mL/min on a semi-micro phenyl column resulted in the best chromatographic performance and electrospray sensitivity. The migration of peaks, especially at the beginning of the chromatographic analysis, can be a problem and several injections of standard may be necessary to allow compounds to "settle" into reproducible retention time. Retention times are stable during continuous sequences, even as long as 40-50 samples.

(i) Mass spectral analysis. In addition to obtaining good agreement between samples and standards analyzed on the same day, a review of the data shows that the relative abundances of ions obtained different days is also very reproducible.

(2) Performance Specifications.

Performance Specifications are outlined above in Procedures section #4.ii (tuning of mass spectrometer), #7 (system suitability for standards) and the Validation section #2.v (criteria for confirmation).

(3) Stability

Stability of residues in shrimp stored for extended periods of time was not evaluated.

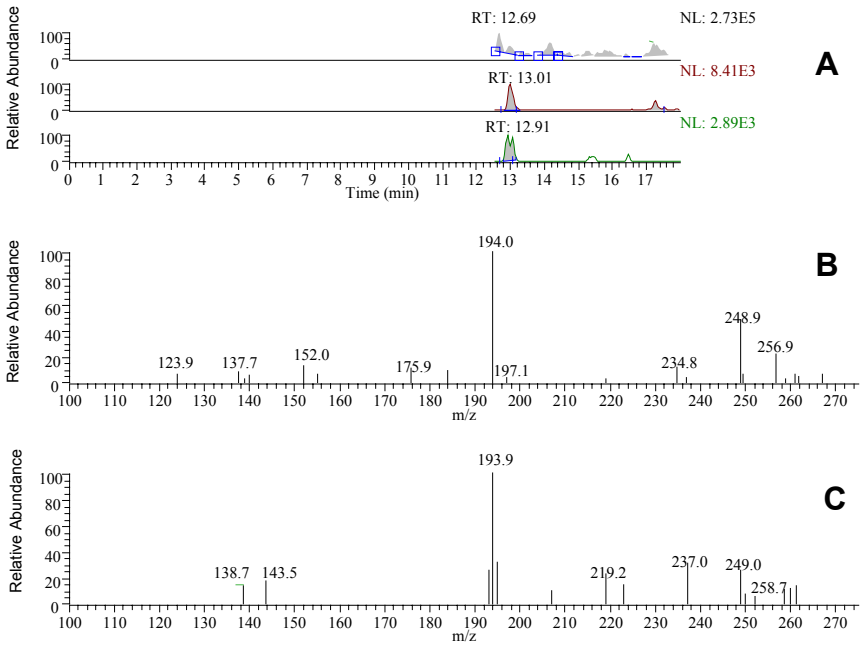
(4) Safety.

Standard laboratory safety practices (lab coats, eye protection) should be followed. In addition any safety precautions listed in the determinative SOP for preparation of reagents should be followed. Also follow instrument manufacturers guidelines for safe operation of electrospray LC/MS (particularly with respect to high voltages, high current, and high temperatures).

Table 1. Summary of Confirmation of Phenicols in Shrimp Using Ion Trap

Sample	Number Confirmed/Number Analyzed		
	CAP	**FF**	**TAP**
Control Tissue	0/7	1/6	0/6
Fortified 0.5 µg/kg	3/4	1/3	0/3
Fortified 1 µg/kg	7/7	3/4	4/4
Fortified 2 µg/kg	7/7	3/3	3/3
Fortified 5 µg/kg	7/7	6/6	6/6

Figure 1. Extract from shrimp fortified with 1 ppb CAP.



(A) Extracted ion chromatograms for full MS (m/z 321) and MS^2 (m/z 194) from m/z 321 and 323.

(B) MS^2 spectrum for m/z 321 (C) MS^2 spectrum for m/z 323