CONFIRMATION OF CHLORAMPHENICOL RESIDUE IN CRAB BY ELECTROSPRAY LC/MS

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SCOPE

The analysis of crab tissues for chloramphenicol (CAP) is important for several reasons. Residues of 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 (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 other 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).

Recently we have developed confirmatory methods for phenicol residues in shrimp and honey (9,10) using an ion trap LC/MS with an electrospray interface. This LIB describes the application of that method to the confirmation of chloramphenicol in crab tissue. This data shows that this method is suitable for the confirmation of CAP in this matrix. Although the method was designed to also analyze for thiamphenicol and florfenicol, these two analytes were not confirmed at this time.

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PRINCIPLES

I. Extraction.

Ten grams of crab composite is extracted with 20 mL ethyl acetate/ammonium hydroxide homogenized and centrifuged. The extraction steps are repeated and the ethyl acetate/ammonium hydroxide 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 eluted 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.

II. Mass Spectral Analysis

Qualitative Confirmation

The qualitative confirmation of CAP in crab is based on unique mass spectral characteristics of these compounds as evaluated by established guidelines (11,12). One unique aspect of this compound is the fact that it contains two chlorine atoms, thus giving rise to a unique isotopic pattern. 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.

REAGENTS

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

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

OR Gelman PVDF Acrodisc LC13 filter 0.45 µm

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 acquisition program may need to be adjusted to account for shift in the retention time of CAP.

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, Pasteur pipettes

PROCEDURES

1. Standard Preparation

CAP was purchased from: Chloramphenicol (USP).

Fortification Standards. For fortification of crab, a stock solution of CAP at 1000 µg/mL (1000 ng/µL) was made up in ACN. An intermediate standard solution (10 ng/µL) was made by pipetting 1 mL of the stock solution into 100 mL volumetric flask and diluting to volume with ACN. Prepare fortification standards, as applicable: Pipet 0.2, 0.1, or 0.05 mL

standard solution into 10 mL volumetric and dilute to volume with acetonitrile for 2, 1, or 0.5 ppb fortification standards, respectively.

MS Standards For MS analysis, a stock solution of CAP at 100 µg/mL (100 ng/µL) was made up in methanol. An intermediate standard (1 ng/µL) was made by diluting 500 µL of the 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 crab (ppb)***
400	4600	0.08	2
200	4800	0.04	1
100	4900	0.02	0.5

* Assuming 10 g of crab 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 0.5 to 2 ppb.

Incurred Samples. Were not evaluated during method development.

Sample Compositing. The crab meat that is of regulatory interest is canned in liquid. Open and drain can of crab. Allow to continue to drain for about 3 to 4 minutes by pouring contents of can into a large glass funnel. Place crab meat in blender, and blend with dry ice with pulsed action until contents are uniform.

3. Sample Extraction.

Accurately weigh about 10.0 g of blended crab composite into a 50 mL P/P centrifuge tube. (If spiking control crab, 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 5 minutes at 4000 RPM at approximately 5 ºC. Pour into 100 mL P-S flask. Repeat extraction twice more, combining the extracts in the 100mL P-S flask. Ensure that there is no residual aroma of ethyl acetate or ammonium hydroxide. If necessary, add a few mLs of IPA, vortex and roto-evaporate to dryness. There will remain an oily residue. Add 30 mL H_2O, vortex, sonicate 4 min, adjust pH (<4.6) with approximately 0.4mL of 0.1% acetic acid. Filter aqueous through glass microfiber filter into a 50 mL P/P centrifuge tube. After the liquid has drained through the filter, rinse the filter with enough water to raise the level in the 50 mL P/P centrifuge tube to 30 mL. Discard the P-S flask. Do not rinse the flask with hexane. Add 10 mL of hexane to P/P centrifuge tube; shake vigorously for about 20 seconds. Centrifuge @ 4000 RPM at 5 ºC for 3 min, aspirate upper hexane layer and discard. 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 1 mL H_2O to columns and allow the PRS column to run dry into the C18 column. Discard PRS column. When the level is even with the top of the C18 column, add 1mL 2% MeOH in H_2O and allow to run dry (under strong vacuum for a min). Elute the C_{18} SPE with 4 mL MeOH into 15mL disposable P/P centrifuge tube. Evaporate MeOH eluate to dryness with the aid of acetonitrile 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). The spray voltage can be set at –4kV to –5kV. The lower setting (-4kV) will minimize arcing from the capillary to the spray needle without any apparent loss of sensitivity.

(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: Fixed MS^2 Acquisition

Isolation width was set to 2 amu for MS^2 transitions.

Time Segment 1: 10-15 minutes CAP

Scan Event 1: (-) MS scan from 300-350 amu

Scan Event 2: (-) MS^2 m/z 321.2 (CE 24%) scan from 100-300 amu

Scan Event 3: (-) MS^2 m/z 323.2 (CE 24%) scan from 100-300 amu

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

ten minutes. The MS was on from 10-15 minutes and the LC flow diverted again. 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

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) and a reagent blank should also be run along with any unknown sample extracts and must demonstrate the absence of CAP. The fortified matrix control samples must demonstrate the confirmation criteria in the Validation Section #2v. A solvent blank (mobile phase) may be run after an external standard to ensure that there was no carryover.

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, and 257 in the MS^2 spectra of 321, as well as, ions m/z 194 and the summation of ions 257 and 259 in the MS^2 spectra of 323 should be shown with the S/N calculated for each of these MS^2 transactions. 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 $\pm$ 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

Figure 1 shows chromatograms for a 1 ppb crab fortified extract.

2. Parameters Evaluated

(i) ***Recovery.*** Fortified matrix samples were analyzed at 0.5, 1 and 2 ppb. All levels demonstrated appropriate responses and met confirmation criteria. The recoveries ranged from 53% to 71%.

(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% (n=4).

(iii) ***Specificity.*** This method meets the specificity guidelines for confirmation methods outlined by Sphon[11] and recently elaborated in CVM's draft guidance[12]. During the course of this investigation, two lots of control crab were analyzed repeatedly and there were no significant interfering peaks in any of the control tissue samples analyzed using the mass filters as described.

(iv) ***Sensitivity*** The ion trap instrument was able to confirm approximately 300-500 pg of standards on-column and crab 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 qualitative confirmation:

For chloramphenicol:

1) **Spectral criteria**: 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, the ions corresponding to [M-H-(HCOCl)]$^-$ (m/z 257 and 259 in MS^2 spectra of 323) must be observed with an approximate relative abundance to the base peal m/z 194 as is observed in the external standards. Other peaks that may be observed in the MS^2 spectra, but are not required for confirmation, include m/z 249 [M-H-(2HCl)], m/z 176 [m/z 194 – (H_2O)]$^-$, and m/z 152. Any other predominant ions in the spectra should be able to be explained, i.e. present in reagent or matrix blank.
2) **Signal threshold (ST) criteria:** a) The signal for the peak at the correct retention time for CAP in the ion chromatogram of 194 from the MS^2 spectra should be equal to or greater than ten times the negative control (blank matrix). b) The signal for the ions corresponding to [M-H-(HCOCl)]$^-$ (m/z 257 from 321 and summation of m/z 257 and 259 from MS^2 of 323) should be equal to or greater than ten times the negative control (blank matrix).
3) **Retention time criteria**: The retention time should be +-5% of external standards run on that day.

*(vi)**Practicality, Sample Throughput, Solvents and Time Requirements.*** Extraction and LC/MS analysis of 6-8 individual samples can be accomplished in one day/overnight. For example, initial extraction can be performed in 5 hours. Each LC/MS run takes 28 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. Do not rinse the pear shaped flask with hexane. 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. It is important with this matrix to divert most of the LC effluent away from the MS (only sampling the 10-15 time interval) to prevent clogging of stainless steel capillary at the MS orifice and resulting instrument performance.

(iii) 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).

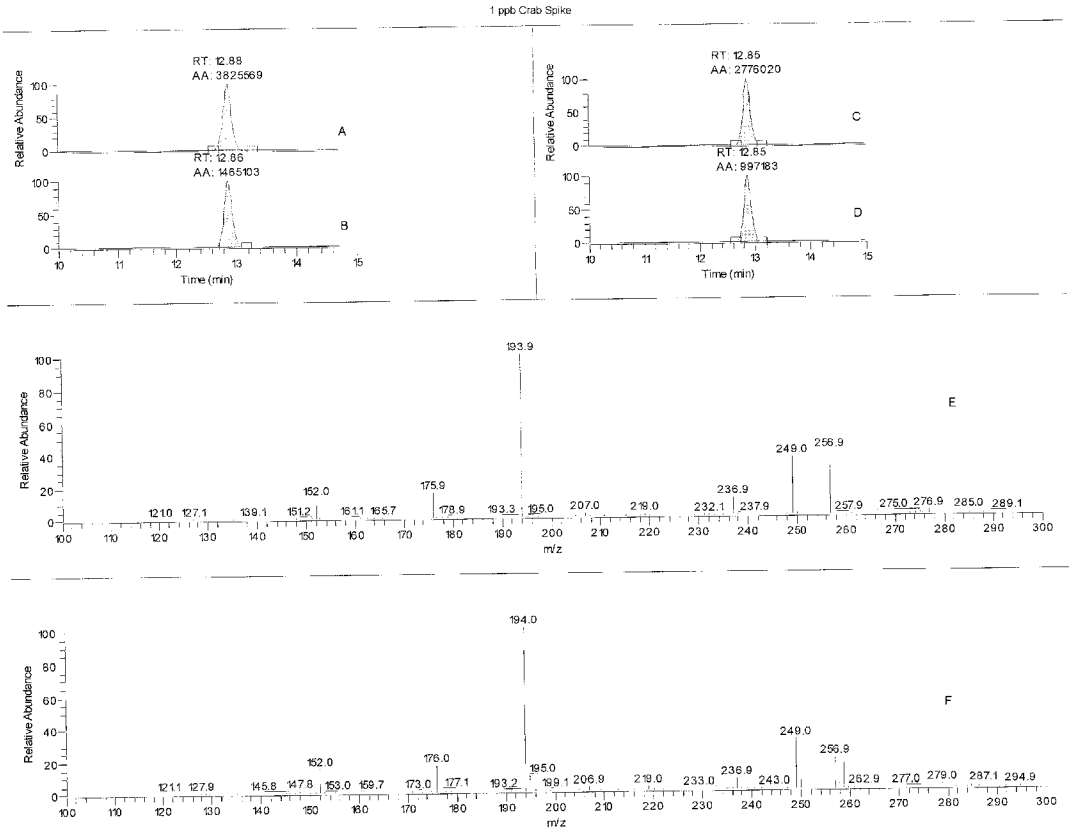
(3) Stability

Stability of residues in crab 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).

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



Extracted ion chromatograms for MS^2 (A) m/z 194 from m/z 321 (B) m/z 257 from m/z 321(C) m/z 194 from m/z 323 and (D) m/z 257 +259 from m/z 323. (E) MS^2 spectrum for m/z 321 (F) MS^2 spectrum for m/z 323.