Improved Analytical Method for the LC/MS/MS Analysis of Chloramphenicol in Shrimp and Other Marine Food Products using Strata™-X Solid Phase Extraction Cartridges and Kinetex® 2.6 µm Core-Shell Columns

Philip J. Koerner, Matthew Trass, Liming Peng, and Jeff Layne
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

A method for the analysis of Chloramphenicol in shrimp has been developed with a limit of quantitation (LOQ) of 0.001 ng/g in shrimp (0.001 ppb) based on the calibration standards. This is three hundred times lower than the current United States Food and Drug Administration (USFDA) method. The method described uses Strata-X solid phase extraction (SPE) cartridges for sample clean-up and concentration, followed by ultra-fast LC/MS/MS analysis (<5 minutes) using a Kinetex core-shell column.

Introduction

Chloramphenicol (CAP) is a broad spectrum antibiotic that exhibits activity against both gram-positive and gram-negative bacteria.[1-3] CAP has been commonly used in aquaculture as a disinfectant to prevent diseases, or as a chemotherapeutic agent to control diseases. CAP has been implicated as a probable causative agent of aplastic anemia, a condition where bone marrow does not produce sufficient new cells to replenish blood cells, and as a possible carcinogen in humans. Therefore, its use in aquaculture and meat producing animals has been banned in the European Union (EU), Canada, and the United States (USA). Despite this ban, CAP is still used illegally to treat seafood products because of its broad spectrum activity, ready availability, and low cost.[4]

The detection of low levels of CAP in imported shrimp from China, Thailand and Vietnam has been reported by government agencies in the EU, Canada and USA. As a result, the USFDA and other government agencies throughout the world have increased their sampling of imported shrimp and other food products. The present methodology specified in the EU has defined a maximum residue limit (MRL) of CAP at 0.3 ppb (0.3 µg/kg)[5], while China has set an MRL of 0.5 ppb. The USFDA method for CAP in shrimp has a limit of detection (LOD) of 0.08 ppb with a limit of quantitation (LOQ) of 0.3 ppb.[6]

Until LC/MS became more readily accessible, the most common approach for the analysis of CAP in seafood tissues involved sample cleanup utilizing liquid-liquid extraction and SPE followed by derivatization to form volatile derivatives that are subsequently analyzed by GC/Electron Capture Detection (GC-ECD).[4,7] The current official USFDA method uses HPLC coupled with MS/MS detection, which eliminates the need for derivatization following liquid-liquid extraction and SPE.

We describe here an alternative approach for the analysis of chloramphenicol in shrimp which utilizes liquid-liquid extraction of the shrimp tissue followed by SPE for sample cleanup and concentration, and ultra-fast LC/MS/MS analysis using a Kinetex core-shell C18 HPLC column.

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. HPLC Grade Ethyl acetate was purchased from Fischer Scientific. HPLC Grade Methanol and Acetonitrile were purchased from Honeywell, Burdick & Jackson (Muskegon, MI), Milli-Q Water was used for SPE and sample preparation. HPLC Grade Water was purchased from Honeywell, Burdick & Jackson and used to prepare the LC mobile phase. Chloramphenicol was purchased from Sigma-Aldrich, and Chloramphenicol-d5 was purchased from Cambridge Isotope Laboratories.

The internal standard solution (IS) 50 ng/mL was prepared by diluting 10 µL of the 100 µg/mL stock solution of Chloramphenicol-d5 in 20 mL of Water. A 1 mg/mL Chloramphenicol stock solution was prepared in Water and diluted 100-fold in Water/Acetonitrile (80:20) to yield a 10 µg/mL Chloramphenicol standard solution, which was then used to prepare the 200 ng/mL calibration standard. Serial dilutions yielded the remaining calibration standards (50, 25, 5, 0.5 and 0.1 ng/mL).

The standard calibration curve was generated by spiking 50 µL of IS into 0.5 mL of each of the calibration standards. The extracted calibration curve was generated by spiking 5 grams of homogenized shrimp with 0.5 mL of each respective calibration solution plus 50 µL of IS, and the samples prepared for LC/MS/MS analysis using the SPE procedure.

Two QC samples were prepared in four replicates. For each sample, 5 grams of homogenized shrimp was spiked with 200 µL of 25 ng/mL CAP calibration solution plus 50 µL of IS, or 187.5 µL of 200 ng/mL standard plus 50 µL of IS to provide sample concentrations of 10 and 75 ng/mL, respectively. The samples were prepared for analysis using the SPE procedure. Each of the four replicates was then analyzed in duplicate (N = 8).

Equipment and Materials

Agilent® 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) was interfaced with API 4000™ MS/MS with ESI TurboIonSpray® (Applied Biosystems, Foster City, CA, USA) operated in negative ionization mode (ESI-).

Sample Preparation

The shrimp sample was prepared as follows:

1. Homogenize ~100 g of thawed shrimp using a blender or tissue homogenizer. **Note:** a small amount of water may need to be added to facilitate homogenization.
2. Weigh out 5 g of homogenized shrimp and transfer to a 15 mL polypropylene tube.
3. Add 50 µL of Chloramphenicol-d5 internal standard (ISTD) solution and vortex thoroughly to ensure adequate distribution of the ISTD throughout the homogenate.
4. Add 2 mL of Water and vortex well to mix.
5. Add 5 mL of Ethyl acetate. Transfer the tube(s) to a mechanical shaker and shake rigorously for 30 minutes.
6. Centrifuge at 7000 rpm for 15 minutes.

7. Transfer the supernatant to a new 15 mL polypropylene test tube, reserving the tissue pellet.
8. Add 5 mL of Ethyl acetate to the tissue pellet from **Step 7** and repeat the extraction process. Combine the resulting Ethyl acetate supernatant with the extraction previously obtained in **Step 7**.
9. Dry the combined supernatant extracts under Nitrogen gas at 55 ºC.
10. Reconstitute with 300 µL of Methanol and dilute to 10 mL with Water. At this point, the sample is ready for solid-phase extraction.

Solid Phase Extraction

The extracted shrimp tissue is further cleaned up and concentrated using SPE.

Cartridge: Strata-X
Part No.: 8B-S100-UBJ
Condition: 3 mL Methanol (1-2 mL/min)
Equilibrate: 3 mL Water (1-2 mL/min)*
Load: 10 mL extracted shrimp tissue sample (1 mL/min)
Wash: 3 x 1 mL Water
Dry: >10" Hg for 5-10 minutes to remove residual water
Elute: 3 x 1.0 mL Ethyl acetate (1 mL/min)
Dry down: Nitrogen gas at 55 ºC
Reconstitute: 500 µL of Acetonitrile/Water (20:80)

*Note: Do not let sorbent run dry.

MS/MS Conditions

Ionization: ESI
Polarity: Negative
Scan Type: MRM
Gas 1 (GS1): 45
Gas 2 (GS2): 50
Collision Gas (CAD): 5
Temperature (TEM): 450
Curtain Gas (CUR): 10
IS: -4500
DP: -50
Entrance Potential (EP): -10
CEM: 2000
DF: 350

Analyte	Ionization	Q1	Q3	Time	DP	CE	CXP
Chloramphenicol	Negative	**321.2**	**152.0**	**150**	**-50**	**-24**	**-25**
	Negative	321.2	257.2	150	-50	-16	-3
Chloramphenicol-d5	Negative	**326.0**	**157.0**	**150**	**-50**	**-26**	**-13**

Results and Discussion

The use of the Kinetex® core-shell technology allowed for the very fast elution of CAP at 2.15 minutes **(Figure 1)**. In ESI negative mode, CAP was detected by monitoring the 321.2/152 mass transition and the CAP-d5 internal standard by monitoring the 326.0/157.0 mass transition. The 321.2/152 mass transition was chosen for quantitation since m/z 152 yielded the most intense product ion. The 321.2/257.2 mass transition provided the second largest product ion and was used for confirmation.

An impurity peak, eluting at about 3.0 minutes, was observed in the extracted shrimp tissue samples but was easily resolved from the CAP peak **(Figure 2)**. The presence of a similar impurity peak had previously been reported by the USFDA[6] and found to co-elute with the CAP peak; however, with the Kinetex core-shell technology and the conditions used here, the impurity was well resolved and did not interfere with quantitation of CAP. An additional benefit compared with the USFDA method is the dramatically shorter LC cycle time – 4.5 minutes versus 20.5 minutes - allowing for significant increases in sample throughput and productivity.

A standard calibration curve was generated over the concentration range of 0.1 ng/mL to 200 ng/mL by plotting the relative response (peak area of CAP/peak area of IS) versus concentration. This concentration range corresponds to 0.01 ng/g – 20 ng/g (0.01 – 20 ppb) in shrimp tissue. The standard calibration curve was linear over the calibration range with an R^2 value of 0.9998 **(Figure 3)**. At the lowest level standard concentration (0.1 ng/mL) the signal-to-noise was 115.9 **(Figure 2)**, therefore the limit of quantitation (LOQ) was estimated to be ≤ 0.01 ng/mL; this corresponds to ≤ 0.001 ng/g in shrimp or ≤ 0.001 ppb.

The extracted calibration curve was generated over the concentration range of 0.1 to 200 ng/mL (0.01 to 20 ng/g shrimp) by spiking 5 g of homogenized shrimp with 0.5 mL of each respective calibration solution and 50 µL of IS and following the SPE protocol to prepare the samples for LC/MS/MS analysis. The extracted calibration curve was linear over this concentration range with an R^2 value of 0.9997 **(Figure 4)**. Two QC samples were prepared (N = 4) at 1.0 and 7.5 ng/g shrimp and analyzed in duplicate to assess reproducibility. The RSDs were 6.4 % and 2.6 %, respectively. The recovery for the QC samples (1.0 and 7.5 ng/g) was 106.5 % and 86.2 %, respectively.

The results obtained with this method compared very favorably with the currently accepted USFDA method[6] where the LOQ was reported to be 0.30 ng/g of shrimp (0.3 ppb) and the LOD was estimated at 0.08 ng/g (0.08 ppb). Based on the standard calibration curve, this method provides an LOQ of 0.001 ng/g shrimp (0.001 ppb) which is over 300 times lower than reported for the USFDA method. However, the practical LOQ and LOD are approximately the same as reported in the currently accepted USFDA method due to the background levels observed. The extension of this methodology to the analysis of other food products is being investigated.

Figure 1: Chloramphenicol analysis (5 ng/mL extracted standard)

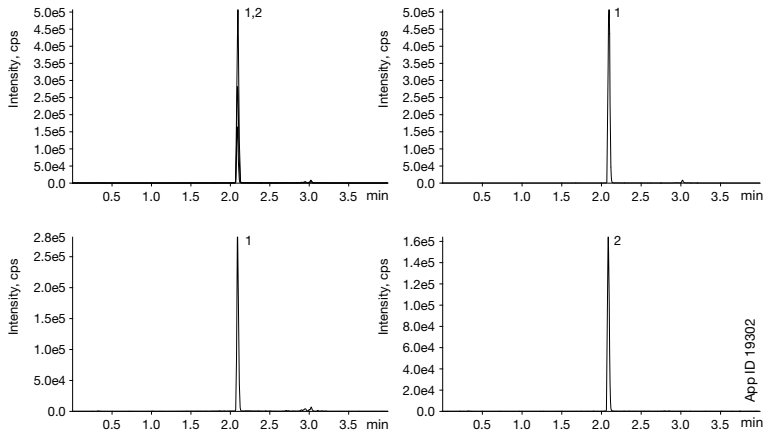
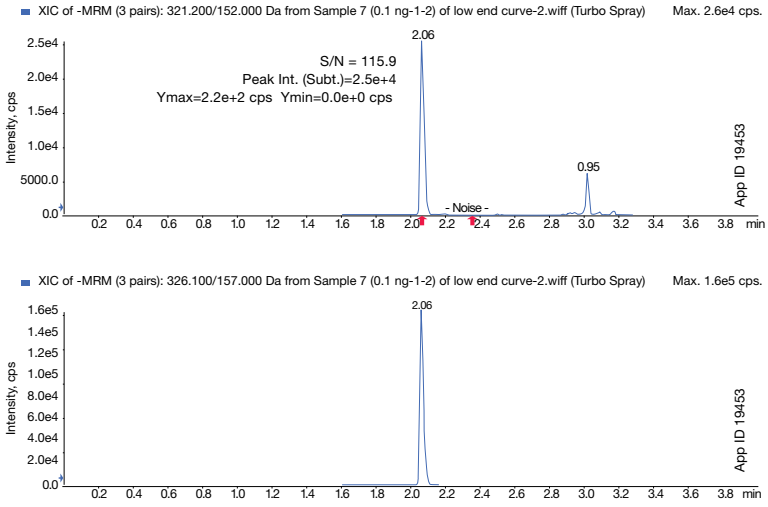


Figure 2: The lowest level (0.1 ng/mL or 0.01 ng/g of shrimp tissue) of extracted standard of Chloramphenicol (321.2/152) and internal standard (326/157). The impurity peak eluting at about 3.0 minutes is well-resolved from Chloramphenicol and does not affect quantitation. 0.1 ng/g Chloramphenicol in shrimp using Strata-X SPE tube and Kinetex C18 LC column.



LC/MS/MS

Same conditions for all columns

Column: Kinetex 2.6 µm C18
Dimensions: 50 x 2.1 mm
Part No.: 00B-4462-AN
Mobile Phase: A: 5 mM Ammonium bicarbonate
B: Acetonitrile

Gradient:

Time (min)	B (%)
0.00	5
2.00	5
2.01	95
4.50	95

Flow Rate: 0.4 mL/min
Injection Volume: 25 µL
Temperature: 25 °C
Detection: API 4000™ MS/MS, ESI negative (ESI-)
Sample: 1. Chloramphenicol
2. Chloramphenicol-d5

Figure 3: Chloramphenicol standard curve from 0.1 ng/mL to 200 ng/mL. The quantitation ion used was 321.2/152. The response was linear over the testing range (y = 0.2663x + 0.2169) with a R^2 value of 0.9998.

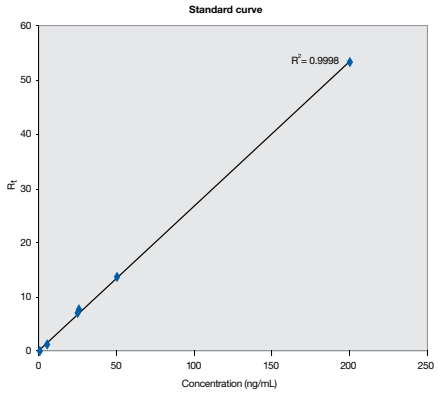
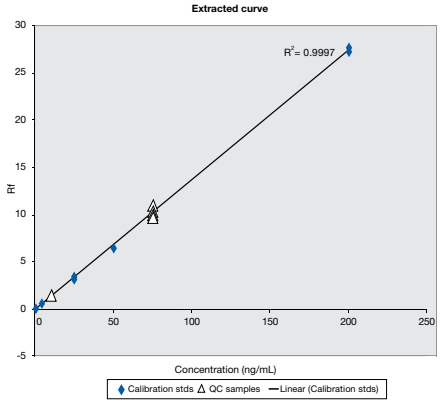


Figure 4: Extracted calibration curve for Chloramphenicol over a range of 0.1 ng/mL - 200 ng/mL (0.01 ng/g - 20 ng/g of shrimp tissue homogenate). The curve was linear (y = 0.1377x - 0.0875) with an R^2 value of 0.9997.



Conclusions

An improved method for the analysis of Chloramphenicol in marine food products has been developed and applied to the analysis of shrimp for residual levels of CAP. The application of the Kinetex core-shell column in this method results in a shorter chromatographic analysis time, providing a productivity benefit for the food safety testing laboratory with a dramatic increase in efficiency providing resolution from a known impurity peak. Sample preparation using Strata-X SPE following extraction concentrates the analyte and removes potential sample matrix interferences, which coupled with the high efficiency core-shell column provides for the low level detection and quantitation of Chloramphenicol in shrimp.

References

1. L.K. Sorensen, T.H. Elbek, H. Hansen; *J. Assoc. Off. Anal. Chem.*, **2003**, 86, 703–706.
2. I.S. Park, N. Kim; *Anal. Chim. Acta*, **2006**, 578, 19-24.
3. R.J. Shakila, S.A.P.R. Vyla, S. Kumar, G. Jeyasekaran, G.I. Jasmine; *Food Microbiol.*, **2006**, 23, 47–51.
4. A.P. Pfenning, J.E. Roybal, H.S. Rupp, S.B. Turnipseed, S.A. Gonzales, J.A. Hurlbut; *J. AOAC Int.* **2000**, 83, 26-30.
5. "Commission Decision" **2003**/181/ED of 13 March **2003**; *Off. J. Eur. Commun.* **2003**, L71/17.
6. B.K. Neuhaus, J.A. Hurlbut, W. Hammack; *U.S. FDA – Laboratory Information Bulletin No. 4290, Volume 18, No. 9, September* **2002**.
7. R.K. Munns, D.C. Holland, J.E. Roybal, J.M. Storey, A.R. Long, G.R. Stehly, S.M. Plakas; *J. AOAC Int.* **1994,** 77, 596-601.

APPLICATIONS

Australia
t: 02-9428-6444
f: 02-9428-6445
auinfo@phenomenex.com

Austria
t: 01-319-1301
f: 01-319-1300
anfrage@phenomenex.com

Belgium
t: +31 (0)30-2418700
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: (800) 543-3681
f: (310) 328-7768
info@phenomenex.com

Denmark
t: 4824 8048
f: +45 4810 6265
nordicinfo@phenomenex.com

Finland
t: (09)4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: 01 30 09 21 10
f: 01 30 09 21 11
franceinfo@phenomenex.com

Germany
t: 06021-58830-0
f: 06021-58830-11
anfrage@phenomenex.com

Ireland
t: 01 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: 051 6327511
f: 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: (55) 5018 3791
f: (310) 328-7768
tecnicomx@phenomenex.com

The Netherlands
t: 030-2418700
f: 030-2383749
nlinfo@phenomenex.com

New Zealand
t: 09-4780951
f: 09-4780952
nzinfo@phenomenex.com

Norway
t: 810 02 005
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: (800) 541-HPLC
f: (310) 328-7768
info@phenomenex.com

United Kingdom
t: 01625-501367
f: 01625-501796
ukinfo@phenomenex.com

All other countries:
Corporate Office USA
t: (310) 212-0555
f: (310) 328-7768
info@phenomenex.com

www.phenomenex.com
Phenomenex products are available worldwide. For the distributor in your country, contact Phenomenex USA, International Department at international@phenomenex.com

Ordering Information

Kinetex® 2.6 µm Analytical Columns (mm)

	30 x 4.6	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	SecurityGuard™ Ultra Cartridges /3pk
XB-C18	—	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	AJ0-8768
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	AJ0-8768
C8	—	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	AJ0-8770
PFP	00A-4477-E0	00B-4477-E0	00C-4477-E0	00D-4477-E0	00F-4477-E0	AJ0-8773
HILIC	—	00B-4461-E0	00C-4461-E0	00D-4461-E0	00F-4461-E0	AJ0-8772
						for 4.6 mm ID

SecurityGuard Ultra cartridges require holder, Part No.: AJ0-9000.
Check for availability in your country.

2.6 µm Minibore Columns (mm)

	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	KrudKatcher Ultra In-Line Filter /3pk
XB-C18	—	00B-4496-AN	00D-4496-AN	—	AF0-8497
C18	00A-4462-AN	00B-4462-AN	00D-4462-AN	00F-4462-AN	AF0-8497
C8	—	00B-4497-AN	00D-4497-AN	—	AF0-8497
PFP	00A-4477-AN	00B-4477-AN	00D-4477-AN	00F-4477-AN	AF0-8497
HILIC	—	00B-4461-AN	00D-4461-AN	00F-4461-AN	AF0-8497

KrudKatcher Ultra requires $^{5}/_{16}$ in. wrench. Wrench not provided.

Strata™-X

Sorbent Mass	Part No.	Unit
Tube		
30 mg	8B-S100-TAK	1 mL (100/box)
30 mg	8B-S100-TBJ	3 mL (50/box)
60 mg	8B-S100-UBJ	3 mL (50/box)
100 mg	8B-S100-EBJ	3 mL (50/box)
100 mg	8B-S100-ECH	6 mL (30/box)
200 mg	8B-S100-FBJ	3 mL (50/box)
200 mg	8B-S100-FCH	6 mL (30/box)
500 mg	8B-S100-HBJ	3 mL (50/box)
500 mg	8B-S100-HCH	6 mL (30/box)
Giga™ Tube		
500 mg	8B-S100-HDG	12 mL (20/box)
1 g	8B-S100-JDG	12 mL (20/box)
1g	8B-S100-JEG	20 mL (20/box)
2 g	8B-S100-KEG	20 mL (20/box)
5 g	8B-S100-LFF	60 mL (16/box)
96-Well Plate		
10 mg	8E-S100-AGB	2 Plates/Box
30 mg	8E-S100-TGB	2 Plates/Box
60 mg	8E-S100-UGB	2 Plates/Box

Additional sizes available. Contact your Phenomenex Sample Preparation Specialist for additional information.

guarantee

If Phenomenex products in this technical note do not provide at least an equivalent separation as compared to other products of the same phase and dimensions, return the product with comparative data within 45 days for a FULL REFUND.

Terms and Conditions
Subject to Phenomenex Standard Terms and Conditions, which may be viewed at www.phenomenex.com/TermsAndConditions

Trademarks
Kinetex is a registered trademark of Phenomenex, Inc. in the United States, EU, and other jurisdictions. Strata-X is a trademark of Phenomenex, Inc. Agilent is a registered trademark of Agilent, Inc. TurboIonSpray is a registered trademark, and API4000 is a trademark of Applied Biosystems/MDS Sciex, a joint venture between Applera Corporation and MDS, Inc.

Strata-X is patented by Phenomenex, Inc. U.S. Patent No. 7,119,145

TN82060810_L