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Performance Assessment and Comparability of a Commercial Enzyme-Linked Immunosorbent Assay Kit with Liquid Chromatography–Tandem Mass Spectrometry for Chloramphenicol Residues in Crab and Shrimp

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ABSTRACT

Monitoring for chloramphenicol (CAP) in aquaculture products is primarily performed by liquid chromatography–tandem mass spectrometry (LC-MS/MS), which requires expensive equipment and specialized training. Many laboratories prefer to screen samples with facile and high-throughput enzyme-linked immunosorbent assay (ELISA) kits for CAP residues before submitting samples for LC-MS/MS quantification and confirmation. We evaluated the performance of a Ridascreen (R-Biopharm) ELISA kit for CAP in spiked and incurred crab and shrimp muscle at levels bracketing the minimum required performance level for analysis (0.3 ng/g). The Ridascreen ELISA kit incorporates antibody directed against CAP. Incurred CAP levels in crab and shrimp muscle were verified using LC-MS/MS. We found good repeatability (relative standard deviation) of the ELISA in spiked and incurred crab and shrimp muscle samples, with values ranging from 6.8 to 21.7%. Recoveries of CAP from tissues spiked at 0.15 to 0.60 ng/g ranged from 102 to 107%. Minimal cross-reactivity with blank crab and shrimp muscle matrix components was observed. ELISA data were highly correlated with those of LC-MS/MS for CAP in incurred muscle tissue. We believe this study to be the first evaluation of the performance and comparability of a CAP ELISA kit and LC-MS/MS for determination of CAP residues, as well as their elimination, in crab muscle. Our findings support the use of this ELISA kit for screening purposes and, when used in conjunction with validated instrumental methods, for regulatory monitoring of CAP in these species.

Chloramphenicol (CAP) was first isolated from *Streptomyces venezuelae* in 1947. It is a highly effective, broad-spectrum drug, with a history of use in treating bacterial infections in food animals, including aquaculture species. However, due to safety issues, CAP is banned from food animal use in the United States *(13–15)* and many other countries. In humans, this drug has been associated with aplastic anemia and bone marrow suppression *(7)*. With no tolerance or safe level established for CAP, residues are not permissible in food animal products. For analytical methods, a minimum required performance level (MRPL) of 0.3 ng/g has been adopted by the United States *(14)*.

CAP is banned from use in the United States for aquaculture production. However, it continues to be used for disease treatment in other countries *(11)*. Sapkota et al. *(11)* reported that 69% of the 13 top aquaculture-producing countries used CAP between 1990 and 2007. CAP residues in aquaculture species could result from direct or indirect exposure. In a study by Lu et al. *(8),* CAP was present in water, sediment, and fish from a freshwater aquaculture pond in China even though CAP had not been used. The authors suggest that CAP may have entered the pond from a nearby pig farm or may have been residual from previous usage at the pond. Hassan et al. *(5)* reported CAP in cultured shrimp and prawn, and their feeds, from the southwest coastal region of Bangladesh. Recently, a study of trout in Iran by Mahmoudi et al. *(9)* found CAP present in seven of 100 fish purchased from markets. A study by Raffi and Suresh *(10)* of cultured and wild shrimp from India reported the presence of CAP. The presence of illegal drug residues in aquaculture products is a major concern for the United States, particularly with increases in imports.

There are several methods for determining the presence of CAP in food products. Shen and Jiang *(12)* provided a comparison of available methods for CAP detection, including enzyme-linked immunosorbent assay (ELISA), liquid chromatography (LC)–UV, gas chromatography (GC)–electron capture detection, and GC–mass spectrometry (MS). In addition, there are LC–tandem MS (LC-MS/MS) techniques, which are the regulatory method of choice in the United States. However, these instrumental methods are expensive and often require highly trained individuals to perform the analysis. There is also a need for facile, rapid,

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and inexpensive methods, such as ELISA, to screen for CAP in seafood products. Impens et al. *(6)* described a tiered approach for screening for CAP in shrimp: ELISA followed by GC-MS/MS and LC-MS/MS confirmation.

The R-Biopharm ELISA kit has been used to test for the presence of CAP in aquaculture products, such as trout *(9)* and shrimp *(10)*. Although the kit has been used for screening purposes, performance comparisons to that of LC-MS/MS in laboratory-incurred tissue have not been described. In the present study, we evaluated the performance of this kit in crab and shrimp. To assess selectivity, we compared ELISA results with those of LC-MS/MS. We believe that this is the first assessment of the kit for incurred residues in crab muscle.

MATERIALS AND METHODS

Chemicals and reagents. CAP and ethyl acetate (Chromasolv) was purchased from Sigma-Aldrich (St. Louis, MO). High-pressure LC (HPLC)-grade water was purchased from Burdick & Jackson (Muskegon, MI). HPLC-grade *n*-hexane (95%) was purchased from J.T. Baker (Phillipsburg, NJ). CAP spiking solutions (50 ng/ml aqueous solution) were purchased from R-Biopharm (Darmstadt, Germany).

Crab and shrimp samples. Live crabs and fresh shrimp were purchased locally for all investigations. Crab and shrimp tissue samples were separately homogenized in a blender. Spiked samples were prepared at 0.15, 0.30, and 0.60 ng/g by adding 9, 18, and 36 μl, respectively, of spiking solution (50 ng/ml of CAP) to aliquots of homogenized control tissues (3 g). Incurred crab and shrimp was generated by waterborne exposure to CAP, with sampling for up to 2 weeks following dosing. Incurred tissue samples selected for the present study provided a range of CAP levels appropriate to evaluate the ELISA kit. Homogenates were stored at –80°C until analysis.

Dosing of crab and shrimp. Waterborne exposures were conducted by dissolving 1, 10, or 20 g of CAP into 100 liters of seawater in aerated stainless steel tanks, representing 10, 100, and 200 mg/liter concentrations, respectively. Crabs were removed from tanks following either a 4- or 24-h exposure period. Shrimp were removed after a 24-h exposure period at a 200 mg/liter concentration. Animals were held in flow-through tanks after dosing. Muscle tissue (whole body) was dissected, pooled, and homogenized from five crabs per sampling time-point.

ELISA kit. ELISA kits for CAP (R1505) were purchased from R-Biopharm and contained a single 96-well microtiter plate coated with capture antibodies against CAP, standard solutions, and all reagents for colorimetric detection of drug residue in samples. The spiking solutions were purchased separately from R-Biopharm.

ELISA standard series. CAP standard solutions were provided in the ELISA kit at concentrations of 0, 0.025, 0.05, 0.1, 0.25, and 0.75 ng/ml. Standards were directly analyzed with the ELISA kit, and the reaction product was read on a BioTek ELx800 plate reader (Winooski, VT). Relative absorbance was plotted on a linear scale against concentration on a log scale. The data were best fit within the working range to a sigmoidal Hill three-parameter fit model using SigmaPlot 11 software (Systat Software, Inc., San Jose, CA).

ELISA sample extraction. Extractions of CAP from tissue samples were performed according to ELISA kit directions (shrimp, meat, and fish meal), with minor modifications. Briefly, homogenates were vortexed with HPLC-grade water. Ethyl acetate was added and was mixed on a vortex mixer. Sample tubes were placed (horizontally) on a New Brunswick Scientific orbital shaker (Edison, NJ) and were mixed at 350 rpm for 10 min. Solutions were centrifuged at 3,000 × *g* for 10 min at 20°C in a Beckman Coulter Allegra 6R centrifuge (Palo Alto, CA). A portion (4 ml) of the upper ethyl acetate layer was transferred to a clean glass tube (13 by 100 mm) and was evaporated under a nitrogen gas stream at 60°C in a TurboVap LV evaporator (Zymark, Hopkinton, MA). The dried residue was suspended in 1 ml of *n*-hexane and was mixed with 1 ml of sample buffer on a vortex mixer. Solutions were centrifuged at 3,000 × *g* for 20 min at 20°C. The lower aqueous phase was transferred to a clean LC vial for assay.

ELISA sample analysis. ELISA was performed according to kit instructions. Standards and samples were evaluated in duplicate. Standard or sample solutions were added to the wells, followed by CAP enzyme conjugate. The plate was manually mixed and was incubated at room temperature for 1 h. The liquid was removed from the plate, and wells were washed with buffer. Chromagen solution was added, and the plate was incubated for 15 min at room temperature in the dark. Stop solution was added to wells, and the absorbance of solutions was read at 450 nm.

LC-MS/MS. Incurred CAP levels in crab and shrimp muscle were determined and confirmed using LC-MS/MS. The extraction methods commonly used for LC-MS/MS analysis of CAP in seafood involve either ethyl acetate or basic ethyl acetate *(2, 4)*. The ethyl acetate extraction method used for ELISA was also used for LC-MS/MS to provide direct comparison of results and to eliminate extraction bias. Standards and samples were analyzed with selected reaction monitoring, as described below.

Preparation of LC-MS/MS standards. A stock standard solution (1,000 μg/ml) was prepared by weighing 100 mg of CAP into a 100-ml volumetric flask and bringing to volume with methanol. An intermediate standard solution (10 μg/ml) was prepared by dilution of the stock solution with methanol. Working standard solutions (1 and 0.01 μg/ml) were prepared by diluting the intermediate solution with methanol. Solutions were stable for 3 months when stored at ≤10°C. Neat standards were prepared by diluting the appropriate CAP solutions to the range 0.1 to 30 ng/ml in methanol.

LC-MS/MS sample extraction. Samples were extracted with ethyl acetate, as performed for ELISA CAP analysis, with the slight modification of diluting the residue in 80:20 methanol:water and defatting twice with *n*-hexane. Samples were filtered by centrifugation (8,000 × *g*) with a Millipore PVDF 0.22-μm filter unit (Billerica, MA) and were transferred to LC vials prior to analysis (tissue equivalent concentration is 2 g/ml).

LC-MS/MS sample analysis. Samples were analyzed by LC-MS/MS using a Waters Acquity Ultra Performance LC coupled to a Xevo TQ MS (Waters, Milford, MA). LC separations were performed on a Phenomenex Kinetex C18, 2.6-μm, 100Å column (100 by 2.1 mm), with a Phenomenex KrudKatcher Ultra HPLC in-line filter (0.5-μm depth by 0.004-in. inside diameter; Phenomenex, Macclesfield, UK), at a flow rate of 0.6 ml/min. Column temperature was 40°C. Mobile phase consisted of (A) water and (B) 95:5 acetonitrile-water, with 0.01% formic acid

TABLE 1. *Interday repeatability of ELISA kits for CAP at* I_{25}, I_{50}, *and* I_{75} *of the calibration curves*[a]

Run	CAP (ng/ml)		
	I_{25}	I_{50}	I_{75}
1	0.235	0.075	0.027
2	0.373	0.101	0.036
3	0.308	0.073	0.027
4	0.301	0.073	0.027
5	0.433	0.097	0.028
6	0.341	0.085	0.030
7	0.402	0.101	—
8	0.447	0.093	0.032
9	0.264	0.076	—
10	0.233	0.073	—
11	0.243	0.077	0.028
12	0.249	0.083	0.034
13	0.251	0.076	0.027
14	0.229	0.069	—
15	0.225	0.071	—
16	0.308	0.103	0.041
17	0.261	0.082	0.033
18	0.256	0.087	0.033
19	0.283	0.091	0.034
20	0.222	0.080	0.030
21	0.248	0.077	—
22	0.227	0.067	0.025
n	22	22	16
Avg	0.288	0.082	0.031
SD	0.069	0.011	0.004
RSD (%)	24	13	14

[a] I_{25}, I_{50}, and I_{75} are calculated concentrations derived from calibration curves at relative absorbance at 25, 50, and 75% responses, respectively.

added as a modifier. The column gradient was 8% B for 1 min, linear gradient to 30% B at 3.7 min, then to 98% B at 4.7 min, hold for 0.7 min, return gradient to 8% B at 5.5 min, and hold for 1 min before next injection. The mass spectrometer was operated in negative ESI mode. The *m*/*z* 321 $[M\text{-}H]^-$ deprotonated molecule of CAP was selected as the precursor ion for collision-induced dissociation. The CAP transitions used for selected reaction monitoring were *m*/*z* 321→152, 194, 257. The transition *m*/*z* 321→152 was used for quantitation, along with the transitions *m*/*z* 321→194, 257 for confirmation. Tune settings were capillary voltage, 1.5 kV; desolvation temperature, 600°C; source temperature, 150°C; cone voltage, 30 V; desolvation gas flow rate, 900 liters/h; and cone gas flow rate, 100 liters/h.

The performance of the LC-MS/MS method in the analysis of CAP in crab and shrimp muscle was verified. Recovery and repeatability were determined using control crab and shrimp muscle (3 g) spiked at 0.15, 0.30, and 0.60 ng/g ($n = 6$ per level), bracketing the MRPL. Muscle samples were spiked ($n = 3$ per level) and were analyzed on separate days.

Statistical analysis. Repeatability or precision was calculated as relative standard deviation (RSD). RSD (%) was calculated as $SD/\bar{x} \times 100$, where SD is the standard deviation and $\bar{x}$ is the sample mean. Recovery (%) of CAP was calculated as $C_m/C_s \times 100$, where C_m and C_s are the measured and spiked concentrations, respectively. The average method detection limits (MDLs) of the ELISA kit were calculated by the equation MDL $= B_0 - 3SD$, where B_0 is the absorbance with blank matrix and SD is the standard deviation on three blank matrix replicates over 4 days.

RESULTS AND DISCUSSION

LC-MS/MS method performance. Performance testing established that the method was suitable for analysis of CAP in crab and shrimp muscle. RSDs (%) for neat standards ($n = 7$ per concentration) at 0.125, 0.25, 0.5, and 1 ng/ml were 10, 15, 10, and 7, respectively. Recovery (%) of spikes was determined directly against a neat standard curve. Interday recoveries (and RSDs) at spiking concentrations of 0.15, 0.30, and 0.60 ng/g were 91 (7), 91 (6), and 94 (6), respectively, in crab muscle, and 96 (3), 94 (8), and 94 (4), respectively, in shrimp muscle. The limit of quantitation and limit of detection were 0.05 and 0.0015 ng/g, respectively.

Sample preparation for ELISA. The ELISA sample extraction is fast and repeatable, with minimal solvent waste generated. The final step of the kit method was modified by suspending the dried extract residues of muscle with 1 ml of sample buffer to facilitate phase separation and to perform sample dilutions. According to ELISA kit instructions, 10 samples can be prepared for analysis in parallel in 1.5 to 2 h. The assay itself takes approximately 1.25 h. We have realized throughput of up to 42 samples in parallel in this time frame.

Repeatability of ELISA kit calibration curves. Calibration curves were generated with the standards (six levels) included in the kit. The assay is a competitive enzyme immunoassay. Maximum absorbance occurs with the 0 ng/ml standard, and minimum absorbance with the highest CAP standard concentration (0.75 ng/ml). Absorbance values are plotted relative to the zero standard (B/B_0%), with the overall working standard range falling between 20 and 80%. Interday repeatability values ($n = 22$) for concentrations calculated at 25, 50, and 75% response (I_{25}, I_{50}, and I_{75}) from each calibration curve are presented in Table 1. Values were calculated using SigmaPlot 11 software with a sigmoidal Hill three-parameter fit model. Mean CAP values for I_{25}, I_{50}, and I_{75} were 0.288, 0.082, and 0.031 ng/ml, respectively. Values for I_{75} could not be calculated from the standard curve fit in six instances of the 22 assay determinations, being close to the lower limits of the assay using the standards provided. Interday variability (RSD) at these points ranged from 13 to 24%, with four different kit lots demonstrating good standardization between kits.

ELISA kit specificity. Buffer blanks, solvent blanks, and blank tissue samples were assayed on a single plate to determine the repeatability of absorbance readings (Table 2). Kit directions recommend setting the cutoff value at 0.0125 ng/g for shrimp, meat, and fish meal, to avoid matrix effects. The kit manufacturer also recommends running a solvent blank (ethyl acetate) for background correction, because

TABLE 2. *Repeatability of buffer, reagent, and crab muscle blanks assayed in triplicate on a single plate*

Sample	Avg absorbance (450 nm) ($n = 3$)	RSD (%)	CAP (ng/g)
Buffer 1	1.180	16	ND[a]
Buffer 2	1.213	19	ND
Buffer 3	1.145	13	ND
Buffer 4	1.301	8	ND
Reagent 1	1.238	12	ND
Reagent 2	1.171	8	ND
Reagent 3	1.095	7	ND
Reagent 4	1.220	16	ND
Reagent 5	1.324	15	ND
Crab muscle 1	1.110	10	ND
Crab muscle 2	1.130	2	ND
Crab muscle 3	1.173	5	ND
Crab muscle 4	1.017	10	ND
Crab muscle 5	1.185	17	ND

[a] ND, absorbance below that of lowest standard in the working range.

absorbance readings can be found above that of the 0.025 ng/ml standard. We did not observe matrix or solvent effects with the blank crab muscle or ethyl acetate used (Table 2). Specificity was evaluated with three homogenates of blank crab and shrimp muscle obtained at different times. Blank matrices typically did not exhibit cross-reactivity. However, on some plates, blank crab and shrimp matrices yielded ELISA readings ranging from 0.01 to 0.04 ng/g equivalent (data not shown). Due to the possibility of cross-reactivity in blank tissue, the presence of CAP residues should be confirmed by LC-MS/MS. The calculated MDLs for crab and shrimp muscle were 0.039 and 0.065 ng/g, respectively. The R-Biopharm kit directions do not provide a detection limit for crab. This study may be the first to determine an MDL for crab with this kit, because none could be found in the literature. Our calculated MDL is higher than the value stated by the kit for shrimp, partly due to the increased dilution factor when resolubilizing the final residue in 1 ml of buffer instead of the recommended volume of 0.5 ml. This increases the method dilution factor twofold. Our calculated MDL values are all well below the MRPL for CAP of 0.3 ng/g.

Recovery of CAP from shrimp and crab. For recovery, blank shrimp and crab muscle were spiked with CAP (R-Biopharm spiking solution) at concentrations of 0.15, 0.30, and 0.60 ng/g ($n = 9$ per level), bracketing the MRPL (0.3 ng/g). Recoveries of CAP in spiked tissues ranged from 102 to 107% (Table 3).

TABLE 3. *Recovery of CAP by ELISA kits in fortified crab and shrimp muscle*

Matrix	n	CAP spike level (ng/g)	ELISA level (ng/g)	Recovery (%)
Crab muscle	9	0.15	0.161	107
	9	0.30	0.322	107
	9	0.60	0.618	103
Shrimp muscle	9	0.15	0.153	102
	9	0.30	0.319	106
	9	0.60	0.625	104

TABLE 4. *Interassay repeatability of ELISA for CAP levels in incurred and fortified crab and shrimp muscle*

Matrix	n	Incurred level (ng/g)	Spike level (ng/g)	RSD (%)
Crab muscle	9		0.15	17
	9		0.30	11
	9		0.60	12
	9	0.13		10
	9	0.35		9
Shrimp muscle	9		0.15	18
	9		0.30	13
	9		0.60	7
	9	11.5		8
	9	1.4		14

Repeatability and precision. ELISA kit repeatability and precision were evaluated in CAP-spiked shrimp and crab muscle and also in incurred shrimp and crab muscle. Interday repeatability (RSD) for spiked (0.15 to 0.60 ng/g) crab and shrimp muscle ranged from 11 to 17% and 7 to 18%, respectively (Table 4). Incurred CAP residue levels in crab muscle used in ELISA repeatability experiments were previously determined by LC-MS/MS as 0.109 and 0.312 ng CAP/g. Levels were chosen to evaluate assay performance at and below 0.3 ng CAP/g. The average interday RSD for incurred crab muscle by ELISA was 10%, and that for incurred shrimp muscle was 11% (Table 4).

TABLE 5. *Comparability of ELISA and LC-MS/MS values for CAP in incurred crab samples*

Exposure				
Concn (mg/liter)	Duration (h)	Elimination time (day)	ELISA CAP (ng/g)	LC-MS/MS CAP (ng/g)
200	24	0	2,194	2,987
		0.5	3,287	3,404
		1	1,213	721
		2	48.1	56.3
		3	1.78	1.91
		6	0.85	1.08
		7	0.32	0.31
		10	0.21	0.18
		13	0.12	0.11
100	24	1	27.0	60.4
		2	6.67	9.56
		5	0.21	0.31
		7	0.53	0.81
		14	ND[a]	0.02
10	4	1	0.40	0.94
		2	0.14	0.20

[a] ND, absorbance below that of lowest standard in the working range.

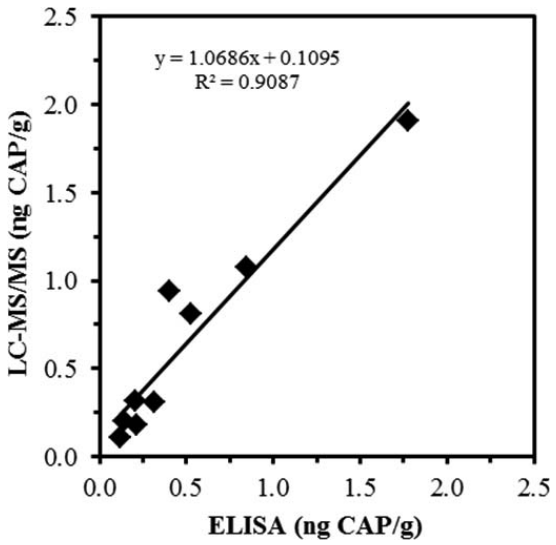

FIGURE 1. *Correlation between ELISA and LC-MS/MS values for incurred CAP residues in crab muscle.*

Comparability of ELISA and LC-MS/MS values. Incurred residues of CAP in crab muscle samples were analyzed by ELISA and LC-MS/MS (Table 5), with good agreement. ELISA and LC-MS/MS values were highly correlated ($R^2 = 0.91$) for incurred crab muscle samples in the range of concentrations relevant to the intended application of the method, bracketing the MRPL (0.1 to 1.8 ng/g) (Fig. 1). The slope of the generated linear regression equation (1.069) indicates that the ELISA and LC-MS/MS values are numerically similar. For shrimp, two incurred CAP levels were generated; the concentrations determined by LC-MS/MS were 0.8 and 4.0 ng/g, and those of ELISA for the same samples were 1.4 and 11.5 ng/g. The higher values found with ELISA, relative to LC-MS/MS, are thought to be related to cross-reactivity with shrimp matrix and/or CAP metabolites.

Elimination of CAP residues in crab. CAP was rapidly eliminated following waterborne exposures. At the highest level of exposure (200 mg/liter), levels of CAP in crab tissue declined from >3,000 to 0.1 ng/g within 2 weeks after dosing. Following the 100 and 200 mg/liter waterborne exposures, CAP levels were below the MRPL (0.3 ng/g) by 5 and 7 days, respectively. CAP was not detectable by ELISA in crab muscle at 14 days following the 100 mg/liter exposure, although a trace concentration (0.02 ng/g) was detected by LC-MS/MS. In other aquatic animals, CAP was also found to be rapidly eliminated, including trout after oral gavage *(1, 3)* and shrimp following ingestion of medicated feed *(16)*.

In conclusion, the performance of the R-Biopharm CAP ELISA was evaluated to determine its usefulness as a screening assay for CAP residues in crab muscle. Incurred shrimp samples ($n = 2$) were not sufficient for meaningful correlation of ELISA values with those of LC-MS/MS but were useful in extending the performance evaluation of the CAP ELISA kit. Blank crab and shrimp muscle had low cross-reactivity in the ELISA, and our calculated MDLs were well below the MRPL of 0.3 ng/g. CAP recovery at spike levels of 0.15 to 0.60 ng/g in crab and shrimp muscle ranged from 102 to 107%. Overall, the average interassay RSD of CAP values for spiked and incurred crab and shrimp muscle was 12%. ELISA and LC-MS/MS values for CAP were highly correlated in crab muscle. CAP is quickly eliminated in crab following dosing. CAP levels were below the MRPL in crab muscle at 5 to 7 days following waterborne exposure. We believe this study to be the first evaluation of the kit in crab muscle. Based on performance and LC-MS/MS comparability data, the kit demonstrates utility as a screening method for regulatory monitoring of CAP residues in crab and shrimp tissues. When coupled with LC-MS/MS for determinative and confirmatory analyses, these complementary techniques offer a powerful approach to regulatory monitoring.

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