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Determination of Emamectin Benzoate in Eel, Halibut, and Shrimp Using QuEChERS-EDTA and LC-MRM

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Abstract : As a part of efforts to establish the positive list system (PLS) in South Korea, a method to determine residual emamectin benzoate (EB) in various aquatic products using QuEChERS-EDTA and LC-MRM was developed. The developed method was validated in the aspects of specificity, linearity (correlation coefficient of at least 0.996), sensitivity (the limit of detection and the lower limit of quantitation ≤ 5 ng/g), recovery (the recovery range of 87.4 and 96.2), and precision (the relative standard deviation of recovery between 0.9 and 13.5). Additionally, the validated method was successfully applied for monitoring EB contamination in eel, halibut, and shrimp collected from local food markets. To our knowledge, the present method is the first one to determine residual EB in various aquatic products at the level satisfying the PLS and could contribute to the establishment of the PLS in South Korea.

Keywords : Emamectin, Aquatic products, QuEChERS, EDTA, MRM, PLS

Introduction

Emamectin (EMA) is a pesticide which belongs to chloride channel activators.[1] It is widely used for controlling lepidopterous pests in agriculture[2] as well as sea lice in fish farming.[3] EMA is a semi-synthetic derivative of abamectin produced by the fermentation of *Streptomyces avermitilis* and it is a mixture between two homologue compounds EMA B1a and EMA B1b (Figure 1).[4] In general, the composition of EMA B1a in EMA is at least 90%[4]. In market, the salt of EMA with benzoic acid, emamectin benzoate (EB) is available, and many studies to determine residual EB in food commodities have been carried out.[5-8] For efficient extraction and purification of EB marker residues in various food commodities including milk, meat, and tea, QuEChERS has been widely used.[5-8] In the case of instrumental analyses of the extracted and purified marker residues, liquid chromatography and multiple reaction monitoring (LC-MRM) with electrospray ionization (ESI) in positive ion mode have been employed.[5-8]

While studies to determine residual EB in food commodities of various origin have been actively carried out, reports for those in aquatic products are relatively rare. For example, Hernando et al. determined four avermectins including EB (EMA B1a) in salmon using solid-liquid extraction and LC-MRM.[9] Also, Lopes *et al.* reported a method to analyze 32 veterinary drugs including EMA (EMA B1a) in gilthead sea bream using QuEChERS and LC-MRM.[10] However, these methods have limited lower limit of quantitation (LLOQ) values (for example, 15 ppb for Hernando et al.'s method and 10 ppb for Lopes et al.'s method) and were not validated for applications to the broad spectrum of aquatic products.[9,10] Thus, it is impossible to employ them for the positive list system (PLS), which sets the maximum residue limit (MRL) of 10 ppb (LLOQ of 5 ppb) as the default value for food-residual chemicals without specific MRL values.[11,12] Thus, here, as a part of efforts to establish the PLS in South Korea, a novel method to determine EB in various aquatic products was developed. The present method employs QuEChERS-EDTA and MRM assay for the purposes of extraction/purification and instrumental analysis, respectively. The method was successfully validated and applied to monitor EB in eel, halibut, and shrimp collected from local food markets.

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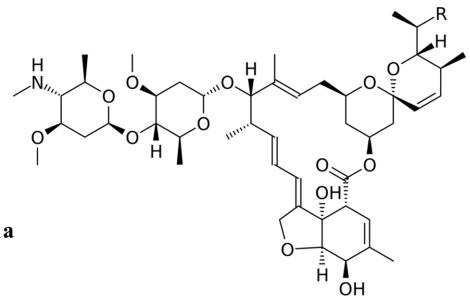

Emamectin B1a
R = CH_2CH_3

Emamectin B1b
R = CH_3

Figure 1. Chemical structures of emamectin B1a (EMA B1a) and emamectin B1b (EMA B1b).

Materials and Methods

Reagents and materials

Acetonitrile, methanol, and water (HPLC grade) were purchased from J. T. Baker (Phillipsburg, NJ, USA). Formic acid, ammonium acetate, ethylenediaminetetraacetic acid (EDTA), and sodium chloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). In the case of EB, its standard solution (94 μg/mL of EMA B1a in methanol) was supplied by AccuStandard (New Haven, CT, USA) and stored at -80°C until its analyses. All solvents and reagents for LC-MS/MS were of analytical grade and used without additional purification. Materials for QuEChERS were purchased from Phenomenex (Torrance, CA, USA).

Sample preparation

Eel, halibut, and shrimp samples were purchased from local food markets. Each sample was homogenously grounded in a blender, and 5 g of each sample was transferred to a 50-mL polypropylene conical tube. The grounded sample was stored at -20°C and thawed just before its preparation. In this study, three extraction methods based on QuEChERS were compared. They are the Standard QuEChERS Method EN 15662 using acetonitrile and citrate salts (Q1),[13] Cho et al.'s protocol that requires EDTA before extraction (Q2),[14] and the modified Q2 that requires less amounts of a sample as well as the dispersive solid phase extraction (dSPE) mixture than those for Q2 (Q3). In the cases of Q1 and Q2, individual QuEChERS procedures in original literatures were just followed for experiments.[13,14] For Q3, the ammonium acetate buffer solution (a 50 mmol/L ammonium acetate aqueous solution whose pH was adjusted to 4.0) was prepared first. Then, 14.6 g of EDTA was dissolved in 500 mL of the ammonium acetate buffer solution to prepare extraction solution. As a next step, 4 mL of the extraction solution was transferred into the tube containing 5 g of a thawed sample, and the tube was vortexed for a minute. Then, 12 mL of acetonitrile and 2 g of sodium chloride were added into the tube, and it was vortexed for 10 minutes. After the vortexing steps, the tube was centrifuged at 4°C and 2,700 × g for 10 minutes, and the whole above layer (organic layer) was transferred to a 15-mL polypropylene conical tube containing 37.5 mg of PSA, 225 mg of magnesium sulfate, and 37.5 mg of C18 for dSPE. The mixture was vortexed for a minute and centrifuged at 4°C and 2,700 × g for 15 minutes. Then, the resulting supernatant was completely taken and dried at 50°C under nitrogen gas stream. The dried residue was reconstituted in 1000 μL of a 50% aqueous methanol solution with 0.1% formic acid and the solution was sonicated for five minutes. Finally, a portion of the supernatant obtained from the centrifugation of the reconstituted solution (at 4°C and 2,700 × g for 3 minutes) was transferred into an autosampler vial for its LC-MS/MS analyses. A matrix-matched standard (MMS) and a standard-spiked sample (SSS) were prepared by spiking the standard solution into the final Q3 extract from a blank matrix sample and into a blank matrix sample before Q3 procedures, respectively.

LC-MS/MS

For LC-MS/MS analysis, a Shimadzu LC-20 Prominence system (Tokyo, Japan) and a SCIEX API 2000 triple quadrupole mass spectrometer (Poster City, CA, USA) with an electrospray ionization (ESI) source were used. The column oven and the autosampler were kept at 40°C and 4°C, respectively. Separation was performed with Phenomenex Luna C18 column (2.0 × 150 mm, 5 μm particle size). Two different kinds of mobile phases, 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B), were used with the isocratic mobile phase of 75% B and the flow rate was 0.25 mL/min. The sample injection volume was 3 μL and the total analysis time per sample was 15 minutes. For highly sensitive and specific mass analyses, multiple reaction monitoring (MRM) in positive ion mode was used and operating parameters were ionspray voltage of 5500 V, source temperature of 400°C, curtain gas of 16 psi, collision gas of 6 psi, ion source gas 1 of 30 psi, and ion source gas 2 of 40 psi. For the purpose of EMA B1a quantitation, an MRM transition (screening transition), 887.0 *m/z* (precursor ion)/158.0 *m/z* (product ion)/51 V (collision energy), was used, and another MRM transition (confirmatory transition), 887.0 *m/z*/126.0 *m/z*/ 65 V, was employed for the purpose of confirming the identity of peaks representing EMA B1a. The percentage ratio of the peak area value of the confirmatory transition to that of the screening transition was calculated for the additional confirmation of the identity of peaks representing EMA B1a. Finally, the data processing was performed with the Analyst software version 1.5.2 (SCIEX), and quantitation of emamectin B1a was based on the peak area of its screening transition.

Validation

The present method (Q3) was validated in terms of specificity, linearity, sensitivity, recovery, and precision. First, the specificity of the present method was tested by comparing final extracts from a blank sample and a 5 ppb SSS through the Q3 method. Its linearity was evaluated by calibration curves constructed from the analyses of 6 SSSs whose concentration values were between 5 and 50 ppb (n = 3). Limit of detection (LOD) and LLOQ were determined by confirming the concentration of EMA B1a in a SSS where MRM transition peaks from its analysis show the signal to noise ratio (S/N) at least 3 (all transitions) and 10 (10 for the screening transition but 3 for the confirmatory transitions), respectively. The recovery values were expressed by the ratio of a peak area from a SSS to that from a MMS at the same concentration (5 ng/mL, 10 ng/mL, and 20 ng/mL, n = 3). Intra-day precision and inter-day precision were evaluated by calculating the relative standard deviation of recovery values obtained in the same day and three consecutive days, respectively.

Results and discussion

Since the purpose of the present study is to develop a method to determine residual EB in various aquatic products for the PLS in South Korea, its sample matrices and the EB marker residue were cautiously selected. First, fat is considered as a major interfering substance in food residual analysis.[14] Thus, eel, halibut, and shrimp, whose fat content values are high (17.1%), medium (3.3%), and low (0.7%), respectively, were selected as sample matrices of this study for evaluating the applicability of the present method to the broad spectrum of aquatic products.[15] Also, EMA B1a was chosen as the marker residue of EB. In the present method, the MRL of 10 ppb and the LLOQ of 5 ppb are required for its future use in the PLS of South Korea.[11,12] Since its required sensitivity is high, only EMA B1a, the major component of EB, whose composition is at least 90%, was selected as its marker residue for its successful determination. In the present method, any internal standard (IS) was not employed. It was due to the fact that an analytical method to determine multiple residues simultaneously does not use any IS generally. While the present method targets only EMA B1a, there is a high possibility to be merged with other methods as a part of the PLS in the near future. Additionally, good performances of the present method even without IS was successfully confirmed in validation experiments followed.

For LC-MS/MS, the combination of a low grade triple quadrupole mass spectrometer and a traditional LC system was employed in the present study and it is for the wide applications of the present method in South Korea. LC-MS/MS conditions were optimized by the comparisons of its various parameters to obtain best peak shape, best separation, and best sensitivity. In the case the MRM assay, all experiments were carried out in positive ion mode and the $[M+H]^+$ ion (887.0 *m/z*) of EMA B1a was chosen as the precursor ion of all MRM transitions (Table 1). Additionally, the ions with 158.1 and 126.1 *m/z* values, the strongest and the second strongest fragment ions at the product ion scan of the $[M+H]^+$ ion of EMA B1a, respectively, were selected as the product ions for MRM transitions (data not shown). Thus, as shown in Table 1, the 887.0/158.1 transition with the highest sensitivity was used for quantitation (the screening transition), and another transition, 887.0/126.1 was used for the confirmatory purposes (the confirmatory transitions). Also, all transitions were observed at 4.0 minutes, and the ion ratio,

Table 1. Properties of emamectin B1a (EMA B1a)

Compound	Molar mass (Da)	Retention time (minutes)	MRM transitions			[c]Ion ratio (%)
			Precursor ion (*m/z*)	[a]Product ion (*m/z*)	[b]CE (V)	
EMA B1a	886.1	4.0	887.0	158.0	51	3.9
				126.0	65	

[a] The product ion of a screening transition; the product ion of a confirmatory transition
[b] Collision energy; the CE of a screening transition; the CE of a confirmatory transition
[c] The percentage ratio of the peak area value of the confirmatory transition to that of the screening transition

Table 2. Recovery of emamectin B1a (EMA B1a) at 5 ppb from different QuEChERS methods. Q1: the Standard Method EN 15662 [13]; Q2: the QuEChERS-EDTA method from [14]; Q3: the Q2 method with modification

Matrices	EMA B1a recovery values at 5 ppb from different methods (mean±standard deviation, %, n=3)		
	Q1	Q2	Q3
Eel	51.3±6.5	62.4±6.8	89.7±3.2
Halibut	28.4±2.2	62.3±5.8	95.5±9.2
Shrimp	58.5±11.1	61.6±26.8	94.1±0.9

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the percentage ratio of the peak area value of the confirmatory transition to that of the screening transition, was confirmed to be 3.9% (Table 1).

For the extraction and the purification of the marker residue from a sample, QuEChERS is used in this method. From the preliminary experiments, the Standard QuEChERS Method EN 15662 (the Q1 method) showed poor recovery in all matrices, and QuEChERS-EDTA method (the Q2 method) showed higher recovery values than those from the Q1 method (Table 2). Thus, the Q2 method was further optimized for its best performance and the resulting extraction and purification steps (the Q3 method) with high recovery and high precision in all matrices are expressed in Table 2 and Figure 2. However, as shown in Table 3, all matrices produced substantial matrix effect (35.6, 38.0, and 16.8% for eel, halibut, and shrimp, respectively) from the comparison between peak areas from MMS analyses (A_{MMS}) and standard solution analyses (A_{STD}) at 20 ppb.[14]

$$\text{Matrix effect (\%)} = [(A_{STD} - A_{MMS})/A_{STD}] \times 100$$

This observation was induced by the suppression of the EMA B1a signal by the co-existence of interfering compounds during ESI. Since it implies that all sample matrices can deteriorate quantitative characteristics of the present method, calibration curves built using the results from analyses of SSSs were applied to downstream for the acquisition of more accurate quantitation data.[16]

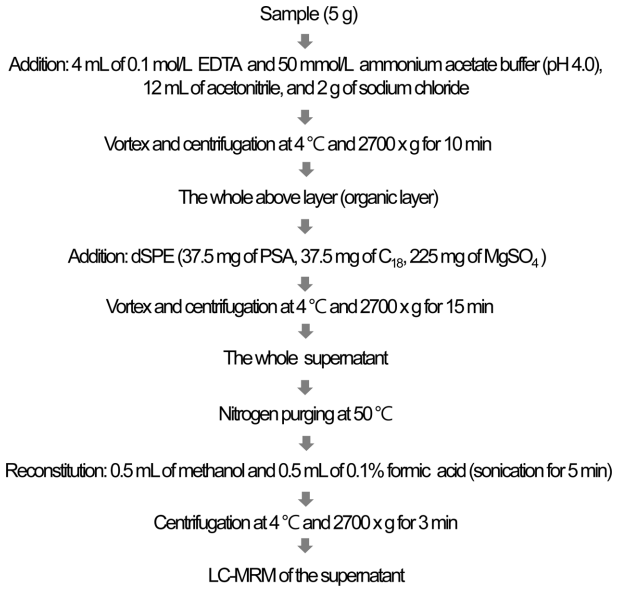

Figure 2. Schematic diagram of the present method using QuEChERS-EDTA (the Q3 method) and LC-MRM

Table 3. Matrix effect of emamectin B1a (EMA B1a) on eel, halibut, and shrimp samples

Compound	Matrix effect at 20 ppb on different matrices (mean±standard deviation, %, n=3)		
	Eel	Halibut	Shrimp
EMA B1a	35.6±6.0	38.0±6.2	16.8±4.3

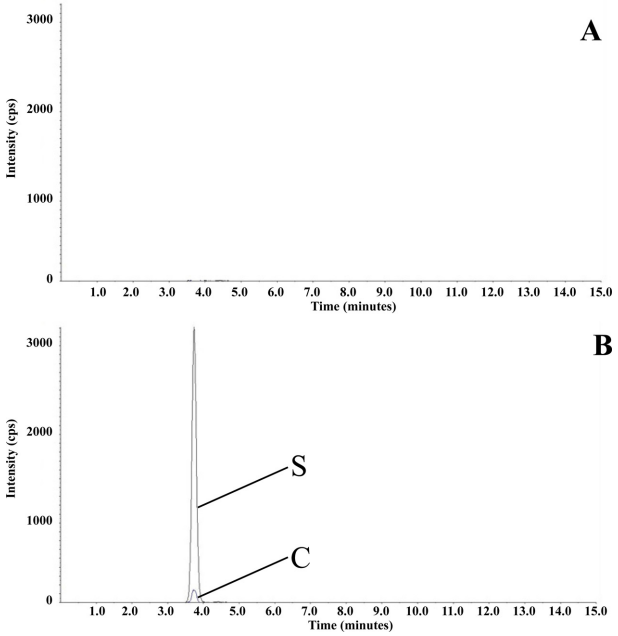

Figure 3. MRM chromatograms from blank eel (A) and standard (5 ppb)-spiked eel (B) analyses. S and C stand for the screening transition peak and the confirmatory transition peak, respectively.

Table 4. Method validation information

Matrices	[a]r	Intra-day [b]RSD (%)			Inter-day [b]RSD (%)			Recovery (mean±standard deviation, %)		
		5 ppb	10 ppb	20 ppb	5 ppb	10 ppb	20 ppb	5 ppb	10 ppb	20 ppb
Eel	0.996	3.5	2.6	2.2	4.3	6.2	5.4	89.7±3.2	96.2±5.5	91.7±3.8
Halibut	0.996	1.1	2.6	2.6	7.4	7.4	13.5	95.5±9.2	87.4±4.2	92.6±14.5
Shrimp	0.996	0.9	1.7	2.3	8.9	3.9	5.8	94.1±0.9	98.2±5.0	94.1±4.9

[a] Correlation coefficient
[b] Relative standard deviation

The present method was validated in the aspects of specificity, linearity, sensitivity, recovery, and precision (Table 4). First, its specificity was observed by the absence of MRM transition peaks for EMA B1a from blank (negative control) results (Figure 3). Also, its linearity was confirmed by correlation coefficient higher than 0.996. Third, since S/N values of all MRM transition peaks were higher than 3 (for confirmatory transitions) and 10 (for quantitative transitions) from the analyses of 5 ppb SSS extracts, the present method satisfies LOD and LLOQ criteria of the PLS (at least 5 ppb).[11,12] Fourth, its recovery values were found to be between 87.4% and 96.2% with intra-day RSD less than 3.5 and inter-day RSD less than 13.5. Thus, all validation results satisfy CODEX guidelines.[17]

The validated method was applied to monitor residual EB in eel, halibut, and shrimp (six samples per species) purchased from local food markets and each sample was prepared and analyzed in triplicates. During its monitoring experiments, there wasn't any positive determination of EB and it shows that no contamination of EB in all samples.

Conclusions

As a part of efforts to establish the PLS in South Korea, a method to determine residual EB in various aquatic products using QuEChERS-EDTA and LC-MRM was developed and validated. To our knowledge, this is the first method to determine residual EB in various aquatic products at the level satisfying the PLS. Also, its use of a low grade triple quadrupole mass spectrometer and a traditional LC system supports its wide applicability as a part of PLS in South Korea. Finally, the present method could contribute to construct the more efficient food safety system in South Korea.

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