Vol. 12, No. 1, 2021
ISSN 2233-4203/ e-ISSN 2093-8950
ARTICLE www.msletters.org | Mass Spectrometry Letters

Determination of Nitarsone in Pork, Egg, Milk, Halibut, Shrimp, and Eel Using QuEChERS and LC-MRM

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Received February 12, 2021; Revised March 9, 2021; Accepted March 9, 2021
First published on the web March 31, 2021; DOI: 10.5478/MSL.2021.12.1.11

Abstract : Nitarsone is an organoarsenic antiprotozoal drug widely used to treat blackhead disease in turkeys and chickens. However, since its biological conversion into inorganic arsenic, a carcinogen was known, its residue in foods should be regulated. Thus, here, a novel method to determine residual nitarsone in various food commodities (pork, milk, egg, halibut, eel, and shrimp) using QuEChERS and LC-MRM was developed. The developed method was successfully validated through specificity, linearity (coefficient of determination, at least 0.991), recovery (R, 63.6 - 85.6%), precision (the relative standard deviation of R, 0.5 - 10.6%), and sensitivity (the lower limit of quantitation, 5 ppb) by following the Ministry of food and drug safety (MFDS) guidelines. The present method is the first mean to quantitate nitarsone using LC-MRM, and it was designed to be conveniently merged into a new method to quantitate multiple veterinary drugs for the positive list system (PLS). Therefore, the present method could contribute to fortify the food safety system in South Korea.

Keywords : nitarsone, food, QuEChERS, MRM, PLS

Introduction

Nitarsone (4-nitrophenylarsonic acid, Figure 1), an organoarsenic compound with antiprotozoal activity has been widely used to treat blackhead disease in turkeys and chickens.[1,2] In 2015, the US Food and Drug Administration (FDA) withdrew the approval for its applications in animal feed due to its biological conversion into inorganic arsenic, a carcinogen.[3] However, since it is still used in other countries, its regulation in various food commodities is needed.[4] Organoarsenic compounds including nitarsone are analyzed by inductively coupled plasma mass spectrometry (ICP-MS).[5,6] Additionally, HPLC-ultraviolet oxidation hydride generation-atomic fluorescence spectrometry (HPLC-UV-HG-AFS), HPLC-ultraviolet detector (HPLC-UV), and gas chromatography-mass spectrometry (GC–MS) were reported to be used to analyze nitarsone.[7-9] Among these techniques, ICP-MS is considered as the gold standard for nitarsone analysis due to its high sensitivity, but there are some drawbacks. First, ICP-MS is relatively less common in laboratories due to its cost.[10] Also, since ICP-MS analyzes targets in elemental ion forms, majority of veterinary drugs without rare element cannot be determined using ICP-MS.[11] It means that ICP-MS-based methods to determine nitarsone cannot be merged into a new method to analyze various kinds of veterinary drugs simultaneously.

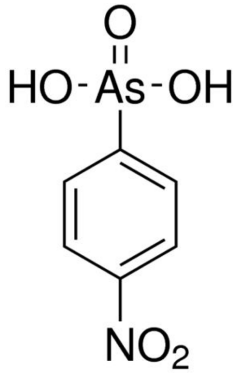

Figure 1. Chemical structure of nitarsone.

Thus, here, a novel method to determine residual nitarsone in various food commodities (pork, egg, milk, halibut, shrimp, and eel) using QuEChERS and LC-MRM was developed and validated. The present method is the first LC-MRM method to analyze nitarsone and could contribute to fortify the food safety system in South Korea.

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Experimental

Chemicals and reagents

Nitarsone (analytical standard grade) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Acetonitrile, methanol, and water were obtained from J. T. Baker (Phillipsburg, NJ, USA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents mentioned above were at least HPLC grade and used without further purification. All QuEChERS-related reagents were purchased from Phenomenex (Torrance, CA, USA).

Sample preparation

Samples (pork, egg, milk, halibut, shrimp, and eel) were obtained from local food markets and individual samples were homogenized (in the case of egg, blended without shell). A portion (2 g or 2 mL) of a homogenized sample was transferred to a 50-mL polypropylene (PP) conical tube and tubes were stored at -20°C until extraction and purification (E/P) processes. As the first step of E/P procedures, a frozen sample was thawed at room temperature. Then, the thawed sample was mixed with 0.2 mL of formic acid, 1 g of magnesium sulfate ($MgSO_4$), 0.25 g of sodium chloride (NaCl), 0.125 g of sodium citrate dibasic sesquihydrate (SCDS), 0.25 g of sodium citrate tribasic dihydrate (SCTD), and 10 mL of acetonitrile. The mixture was vortexed (for 10 min) and centrifuged (at 4°C and 2700 ×g for 10 min), and the whole top layer (organic layer) was transferred to a 15-mL PP conical tube containing 25 mg of primary secondary amine (PSA), 150 mg of $MgSO_4$, and 25 mg of C_{18}. The resulting mixture was vortexed (for 10 min) and centrifuged (at 4°C and 2700 ×g for 10 min), and the supernatant was completely taken for enrichment. After dried under nitrogen stream at 40°C, the residue was dissolved in 400 μL of a 50% aqueous methanol solution. Finally, the reconstituted solution was vortexed (for three min) and centrifuged (at 4°C and 2700 ×g for three min), and a portion of the supernatant was analyzed through liquid chromatography and tandem mass spectrometry (LC-MS/MS) (Figure 2). A matrix-matched standard (MMS) and a standard-spiked sample (SSS) were prepared by spiking an appropriate volume of a nitarsone standard solution into the final P/E extract from a blank matrix and into a blank matrix prior to P/E procedures, respectively.

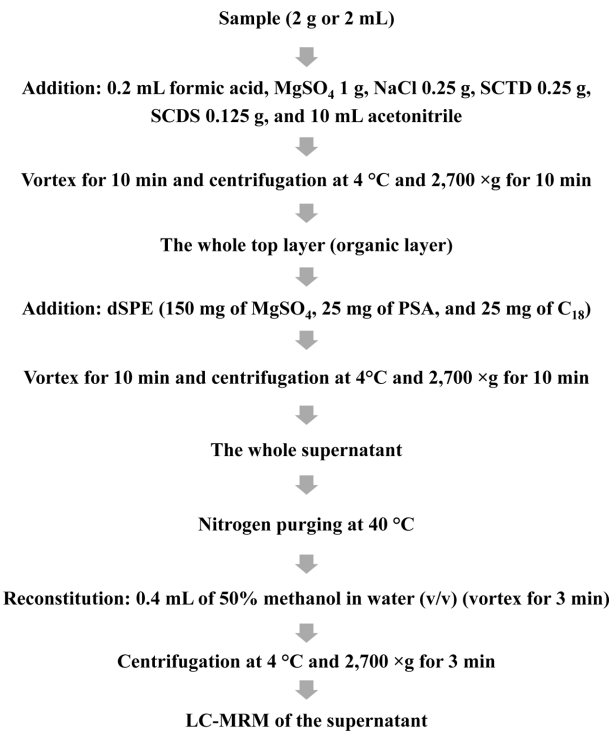

Figure 2. Schematic diagram of the present method using QuEChERS and LC-MRM.

LC-MS/MS

For separation and analysis of the P/E extract, a LC-MS/MS system composed of a Shimadzu Nexera UPLC system (Tokyo, Japan) and a Shimadzu LCMS 8050 triple quadrupole mass spectrometer were used. Additionally, electrospray ionization (ESI) with negative ion mode and a Phenomenex Luna C18 column (2.0 × 150 mm, 5 μm, Torrance, CA, USA) were employed. For separation, gradient mobile phase (MP) program between 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in methanol at 0.25 mL/min for 14 minutes were used and the column was kept at 40°C (Table 1). In the case of the sample injector, its temperature and injection volume were 4°C and 10 μL, respectively. The sensitive determination of nitarsone through mass spectrometry was achieved by multiple reaction monitoring (MRM), a selective as well as sensitive MS/MS scan method. As shown in Table 2, three MRM transitions for nitarsone were prepared: the screening transition of 245.9 *m/z* (precursor ion) / 137.9 *m/z* (product ion) / -15 V (collision energy); the confirmatory transition 1 of 245.9 *m/z* / 107.8 *m/z* / -24 V; the confirmatory transition 2 of 245.9 *m/z* / 122.7 *m/z* / -27 V. Additional parameters for the mass spectrometer were as follows: nebulizing gas flow at 3 L/min, heating gas flow at 10 L/min, drying gas flow at 10 L/min, interface temperature at

Table 1. Gradient mobile phase program.

Time (minutes)	0.1% (*v*/*v*) Formic acid in water (%, *v*/*v*)	0.1% (*v*/*v*) Formic acid in methanol (%, *v*/*v*)
0.0	100.0	0.0
1.0	100.0	0.0
6.0	10.0	90.0
8.5	10.0	90.0
8.6	100.0	0.0
14.0	100.0	0.0

Table 2. Properties of nitarsone.

Compound	Molar mass (Da)	Retention time (minutes)	MRM transitions		
			Precusor ion (*m/z*)	[a]Product ion (*m/z*)	[b]CE (V)
Nitarsone	247.0	5.6	245.9 $[M-H]^-$	137.9	15
				107.8	24
				122.7	27

[a]The product ion of the screening transition; the product ion of a confirmatory transition
[b]Collision energy; the CE of the screening transition; the CE of a confirmatory transition

300°C, DL temperature at 250°C, and heating block temperature at 400°C. All data were acquired and analyzed using Lab Solutions (version 5.93, Shimadzu). For quantitation, peak area values of the screening transition from sample analyses were compared to calibration curves built using those from MMS analyses. However, a couple of preconditions were tested prior to quantitation. First, three transitions peaks should have the same retention time (the identity confirmation). Also, the signal-to-noise ratio (S/N) values of the screening transition peak and the confirmatory transition peaks should be at least 10 and at least 3, respectively (the sensitivity test).

Validation

The present method was validated in the aspects of specificity, linearity, recovery, precision, and sensitivity following guidelines of the ministry of food and drug safety, South Korea (MFDS).[12] First, the specificity was tested by comparisons between blank matrices and their conjugate SSSs (5 ppb). Also, linearity (the coefficient of determination, r^2) was evaluated by individual calibration curves built from analyses of 6 MMSs (5, 10, 20, 30, 40, and 50 ppb, $n = 3$). Third, a recovery (R) value was calculated by the division of the screening transition peak area of a SSS by that of its counter MMS. In each matrix, recovery values at three levels (5, 10, and 20 ppb) for three consecutive days were studied and computed ($n = 5$). In the case of precision, it was expressed by the relative standard deviation (RSD) of R values. Finally, the lower limit of quantitation (LLOQ), a parameter representing sensitivity was determined to the lowest concentration which satisfies R criteria of MFDS guidelines within the linear dynamic range.

Results and discussion

To develop a highly sensitive method to determine nitarsone in diverse kinds of food commodities, food matrices with broad spectrum of fat content (0.7, 0.9, 3.3, 7.4, 16.4 and 17.1% in shrimp, milk, halibut, egg, pork and eel, respectively), considered as a major interfering factor in food residual analyses, were cautiously selected as sample matrices.[13-15]

Since the merge of the present method to a novel PLS method to determine various veterinary drugs in foods in a near future was considered, there are a couple of unique points in the present method. First, no internal standard (IS) was employed in the present method, because IS is not used in multiresidual analysis methods. Also, MRM was carried out in negative ion mode, but the pH of MPs was set to 2.8. While there must have been a disadvantage in the aspect of sensitivity due to less deprotonation of nitarsone, LC conditions of present method became compatible to LC conditions of most residual veterinary drug analyses in foods. Consequently, regardless of these unique points, the present method was found to be good enough for quantitation with LLOQ of 5 ppb, the requirement for PLS.[16]

For MRM transitions, the $[M-H]^-$ ion (245.9 *m/z*) of nitarsone was selected as the precursor ion. Also, the ions with 137.9, 107.8 and 122.7 *m/z* values, the strongest, the second, and the third strongest fragment ions from the product ion scan of the $[M-H]^-$ ion of nitarsone, respectively, were decided as product ions (data not shown). Thus, the most sensitive 245.9/137.9 transition was used for quantitation (the screening transition), and other transitions (245.9/107.8 and 245.9/122.7 transitions) were used as confirmatory transitions to confirm the identities of ions detected (Table 2).

E/P of nitarsone in matrices were performed using QuEChERS in the present method. To obtain the best recovery, major steps (the amount of a sample, the composition of the extraction solvent, the volume of the extraction solvent, the composition of the dSPE adsorbent, and the amount of the dSPE adsorbent) of our previous QuEChERS-EDTA method were changed and their resulting recovery values were compared (data not shown).[14] As a result, a novel QuEChERS method optimized for E/P of nitarsone in various food commodities was confirmed (Figure 2).

The present method was validated in the aspects of specificity, linearity, R, precision, and sensitivity (Table 3). First, specificity was confirmed by the absence of the nitarsone screening transition peak at the retention time of nitarsone from a blank matrix (negative control) results (Figure 3). Second, since all calibration curves built by using MMSs (5-50 ppb) of individual matrices showed r^2 values of at least 0.991, its linearity satisfied MFDS

Table 3. Method validation results.

Matrices	Linearity ([a]r^2, 5-50 ppb)	Fortified concentration (ppb)	Intraday (n = 5)		Interday (n = 5, 3 days)		[c]LLOQ (ppb)
			Recovery (%)	[b]RSD (%)	Recovery (%)	[b]RSD (%)	
Pork	0.993	5	80.57	0.46	83.12	2.93	5
		10	79.97	4.50	80.56	3.11	
		20	85.56	1.57	83.35	2.88	
Egg	0.997	5	67.50	7.58	64.02	6.12	
		10	73.12	8.39	67.73	9.12	
		20	76.71	5.24	73.27	4.56	
Milk	0.991	5	69.74	4.23	63.59	10.56	
		10	71.70	8.85	75.35	5.93	
		20	75.90	8.42	73.54	5.27	
Halibut	0.999	5	81.20	1.65	81.58	4.43	
		10	79.05	3.37	78.62	2.72	
		20	83.17	2.54	78.29	5.75	
Shrimp	0.995	5	68.27	2.84	70.25	4.34	
		10	65.88	3.95	68.02	6.08	
		20	76.99	2.97	73.95	3.53	
Eel	0.991	5	71.66	5.33	72.39	7.34	
		10	73.70	1.52	73.62	3.35	
		20	73.95	3.27	74.41	4.77	

[a]Coefficient of determination
[b]Relative standard deviation of recovery
[c]Lower limit of quantitation

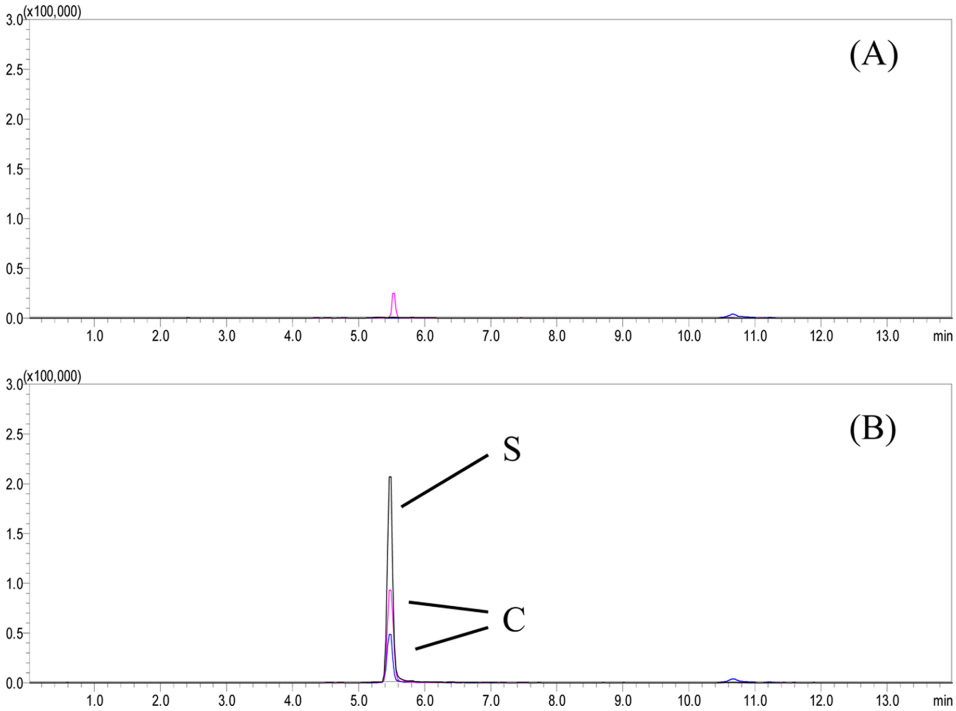

Figure 3. MRM chromatograms from blank halibut (A) and standard (5 ppb)-spiked halibut (B) analyses. S and C stand for the screening transition peak and the confirmatory transition peaks, respectively.

guidelines (at least 0.98).[12] Third, R values evaluated between 5 and 20 ppb were 63.6-85.6% with intra-day RSD less than 8.9% and inter-day RSD less than 10.6% and they are good enough to pass the criteria of MFDS guidelines.[12] Finally, the S/N values of all nitarsone MRM peaks observed over validation studies were found to be higher than 10 (for screening transition) and 3 (for confirmatory transitions) (data not shown). Thus, the good quantitative performance (including LLOQ of 5 ppb) of the present method was proved and it is good enough to be used for the PLS which requires LLOQ of 5 ppb.[16]

The validated method was applied to determine residual nitarsone in pork, milk, egg, halibut, eel, and shrimp (three samples per commodity) purchased from local food markets. Each sample extract was prepared and analyzed in triplicates and there was no sign of nitarsone residue in all samples (data not shown).

Conclusions

As a part of efforts to establish the PLS in South Korea, a novel method to determine residual nitarsone in various food commodities (pork, milk, egg, halibut, eel, and shrimp) using QuEChERS and LC-MRM was developed and validated. This is the first method to quantitate nitarsone using LC-MRM. Also, the present method was designed to be conveniently merged into a new method to quantitate multiple veterinary drugs for PLS. Therefore, the present method could contribute to fortify the food safety system in South Korea.

Acknowledgements

This research was supported by a grant (16162MFDS582) from Ministry of Food and Drug Safety, Republic of Korea in 2016-2018. Also, this work was funded by the Research-Focused Department Promotion Project as a part of the University Innovation Support Program 2020 to Dankook University. The authors would like to thank Dr. Sang Beom Han (Chung-Ang University), and Dr. Ho-Chul Shin (Konkuk University) for their technical support.

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