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Application News

No. C200

Liquid Chromatography Mass Spectrometry

Analysis of Diarrhetic Shellfish Toxins (Okadaic Acid Group) Using Triple Quadrupole LC/MS/MS

In regard to the handling of shellfish containing diarrhetic shellfish toxins, an instrumental analysis method is introduced based on "Handling of Shellfish Contaminated with Paralytic Shellfish Toxins, etc.", (Notice 0306 No. 2, dated March 6, 2015, issued by the Food Safety Manager, Pharmaceutical and Food Safety Bureau, MHLW). A regulatory value of 0.16 mg OA equivalent/kg has been set for the okadaic acid (abbreviated as OA) group, and selling shellfish that exceed the regulatory value is prohibited under the provisions of Chapter 6, Article 2 of the Food Sanitation Act.

Since April 2016, it has been possible to reliably obtain certified reference materials produced domestically in Japan. Accordingly, the mouse toxicity test in Notice No. 37 "Testing for Diarrhetic Shellfish Toxins (Okadaic Acid Group)" dated May 19, 1981 was superseded as of April 1, 2017 by Notice 0308 No. 2 and Notice 0308 No. 9 "Partial Revision of <Testing for Diarrhetic Shellfish Toxins (Okadaic Acid Group)>" dated March 8, 2017. In this revision, a regulatory value for the OA group, which is recognized as toxic to humans, was introduced and this group has become targeted in an instrumental analysis method. On the other hand, the PTX and YTX groups, which do not cause diarrhea, are not covered by the instrumental analysis method. In addition to OA, which is a toxin produced by phytoplankton, the OA group includes the dinophysistoxin group (DTX1, DTX2 and DTX3) as similar compounds. Because each of these compounds has a different strength of toxicity, the toxicity of each compound is calculated by converting it into an equivalent toxicity in terms of OA. For this purpose, a toxicity equivalence coefficient (TEF) has been defined, and with OA set as 1, DTX1 is 1 and DTX2 is 0.5. OA, DTX1, and DTX2 quantitative results are converted to OA equivalent values by multiplying them by their respective TEF values, then the sum is calculated. DTX3 is an esterified compound with a fatty acid compound, which is a metabolite of scallops, and no TEF value is set for it because it is converted to OA, DTX1 or DTX2 by the hydrolysis process in the pretreatment operation.

In this paper, we introduce an instrumental analysis method (LC/MS/MS) for the OA group.

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Analysis of Standards

For the OA and DTX1 standards, certified reference materials from the National Metrology Institute of Japan / National Institute of Advanced Industrial Science and Technology (NMIJ/AIST), which is a national metrology body, were used. For DTX2, CRM-DTX2 from the National Research Council Canada was used.

Fig. 1 shows the chromatogram when 5 µL of a three-compound mixed standard solution (1 ppb each) was injected, and Table 1 shows the repeatability of retention times and area values for each compound over five repetitions. OAs can be detected using the electrospray ionization (ESI) method in the negative ion mode. This analysis complies with the method specified in the Notice, and the details are shown in Table 2.

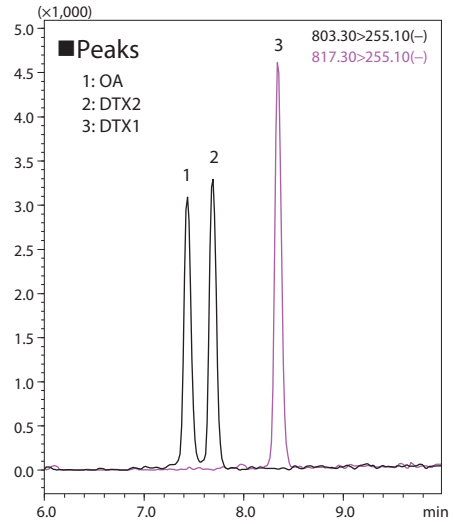

Fig. 1 MRM Chromatogram of the Standard Solution (1 ppb Each)

Table 1 Repeatability (1 ppb, n=5)

	R.T. %RSD	Area %RSD
OA	0.0419	2.03
DTX2	0.0401	2.98
DTX1	0.0385	2.08

Table 2 Analysis Conditions

Column	:	Shim-pack Scepter™ C18 (100 mm × 2.0 mm I.D., 1.9 µm)
Mobile Phases	:	A 2 mmol/L ammonium formate water with 50 mmol/L formic acid B Acetonitrile / Water: 95 / 5 (v/v) including 2 mmol/L ammonium formate with 50 mmol/L formic acid
Time Program	:	B conc. 40% (0 - 2.5 min) → 100% (7.5 - 12.5 min) → 40% (12.51 - 17.5 min) (Using the front cut valve, introduced into the MS only for 6 - 10 min)
Flow Rate	:	0.2 mL/min
Column Temperature	:	40 °C
Injection Volume	:	5 µL (2 µL when analyzing a scallop midgut gland certified reference material)
Probe Voltage	:	–3.0 kV (ESI-negative mode)
IF/DL/BH Temperature	:	350 / 150 / 450 °C
NG/HG/DG Flow	:	3 / 5 / 15 L/min
ESI probe position	:	+2 mm
MRM Transition	:	OA 803.30>255.10, 803.30>113.10 DTX2 803.30>255.10, 803.30>113.10 DTX1 817.30>255.10, 817.30>113.10

Linearity of Calibration Curve

Fig. 2 shows the calibration curves for each of the three compounds. When the calibration curve was created in the 0.1 to 10 ppb concentration range for each compound, favorable linearity was obtained with a coefficient of determination (r^2) of 0.999 or higher.

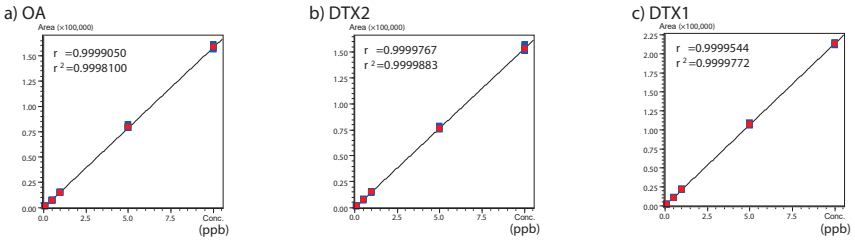

Fig. 2 Calibration Curve Linearity (0.1 to 10 ppb)

Analysis of Scallop Midgut Gland Certified Reference Material

Using a scallop midgut gland certified reference material, NMIJ CRM 7520-a*1, extraction, hydrolysis, and purification were implemented in accordance with the method specified in the Notice (Fig. 3). 300 µL (250 µL according to the Notice) of 2.5 mol/L HCl was added for neutralization after hydrolysis. A reverse-phase polymer solid phase extraction column (200 mg, 6 cc) was used for the purification.

1. Extraction

Midgut gland 2 g
↓ 9 mL methanol
Centrifugation 2000 rpm, 10 min
Collect supernatant in 20 mL measuring flask
↓ 9 mL 90% methanol
Centrifugation 2000 rpm, 10 min
Collect supernatant in 20 mL measuring flask
↓ Fixed 20 mL volume with 90% methanol
20 mL extract solution

2. Hydrolysis

2 mL extract solution
↓ 250 µL 2.5 mol/L NaOH
Hydrolysis at 76 °C for 40 min
↓
Cooling
↓ 300 µL 2.5 mol/L HCl
Hexane purification, 2 times
↓
2.5 mL water added
All volume of extract solution is treated in the next SPE purification step

3. Purification

SPE (6 cc, 200 mg)
↓ Conditioning
Methanol 5 mL
Water 5 mL
Load extract solution after hydrolysis
↓ Discard liquid
Wash flask with 2 mL of 40% methanol
Repeat twice
↓ Discard liquid
SPE washing with 3 mL of water
↓ Discard liquid
SPE washing with 3 mL of 40% methanol
↓ Discard liquid
Elution with 3 mL of 90% methanol
↓
Collect liquid in a flask
Concentrate (40 °C)
↓
Redissolution in 2 mL of methanol
↓
LC/MS/MS analysis

Fig. 3 Preparation

Table 3 Quantitative Value and Recovery Rate

Compounds	Certified Reference Materials		Extracted Samples				
	Certified Value (mg/Kg)	Expanded Uncertainty Mass Fraction (mg/Kg)	Quantitative Value (mg/Kg)	Recovery Rate (%)	Area Value Average (n=6)	Standard Deviation	Area Value %RSD (n=6)
OA	0.205	0.061	0.192	93	105424	2414.48761	2.29
DTX1	0.450	0.110	0.385	85	253677	1439.89408	0.57

It is generally known that the matrix effect of contaminants originating from midgut gland of scallops is large in LC/MS/MS analysis. Although it is possible to eliminate their influence by sufficiently diluting the sample, this time we introduce the standard addition method, which can be applied to various kinds of samples. Since the amount of OA standards purchased was small, standards were added to achieve concentrations at LC/MS/MS analysis of 10, 20, and 50 ppb for the extract before hydrolysis, and created a calibration point. Fig. 4 shows the chromatogram of the midgut gland extract after SPE purification (standard not added), Fig. 5 shows the calibration curves, Table 3 shows the quantitative value, recovery rate, and the area value repeatability of the certified reference material. The area repeatability %RSD of each peak, which is said to have an extensive matrix effect, is favorable with OA being 2.29 and DTX1 0.57 (n=6), the recovery rates of OA and DTX1 are 93% and 85%. It was shown that it is possible to quantify diarrhetic shellfish toxins according to the method specified in the Notice using LCMS™-8060.

*1 National Metrology Institute of Japan / National Institute of Advanced Industrial Science and Technology
Scallop midgut gland certified reference material, NMIJ CRM 7520-a No. 009 (for diarrhetic shellfish toxin analysis)
The uncertainty of certified values is the expanded uncertainty determined from the combined standard uncertainty and the coverage factor k = 2, representing half the width of the interval estimated to have a confidence level of approximately 95%.

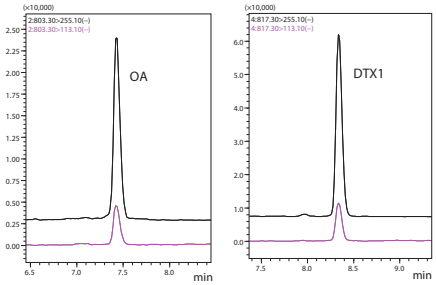

Fig. 4 Chromatograms of Certified Reference Materials

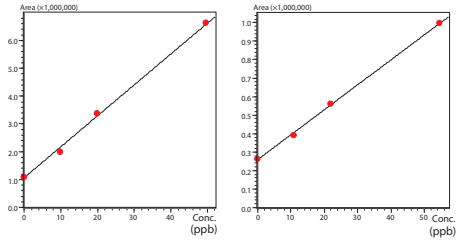

Fig. 5 Calibration Curves by Standard Addition Method

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