Determining Malachite Green and Leucomalachite Green in Food by LC/MS/MS

Application

Food Safety

Authors

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Abstract

This application note demonstrates a complete method to rapidly and precisely determine residue levels of malachite green and leucomalachite green in fish with the new Agilent 6410 LC/MS triple quadrupole system. Using positive mode electrospray ionization (ESI+) and multiple reaction monitoring (MRM), qualification and quantification were accomplished without the traditional tedious PbO_2 oxidation process. The LC/MS/MS method's LOQ is 0.01 µg/Kg, which easily meets the import requirement of 2 µg/Kg set by Japan and the EU.

Introduction

Malachite green (MG) is a metallic-looking crystal. It dissolves in water easily as a blue-green solution. It is a toxic chemical primarily used as a dye and has been found very effective in treating parasites, fungal infections, and bacterial infections in fish and fish eggs.[1] On uptake, MG is rapidly reduced into leucomalachite green (LMG) and deposited in the fatty tissue of the fish with little MG remaining.

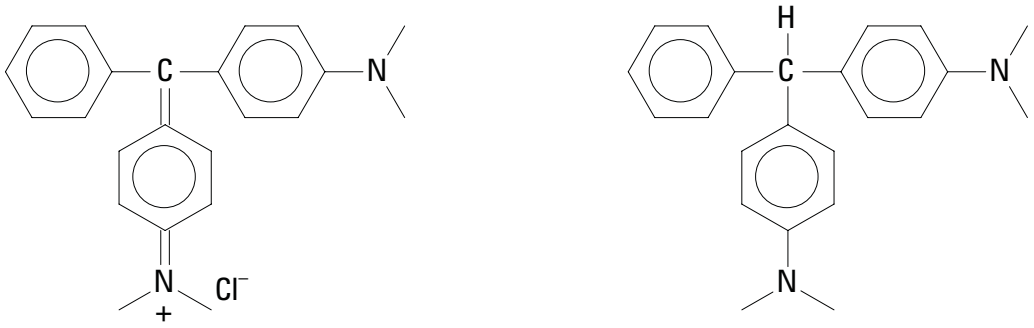
MG can cause significant health risk for humans who eat contaminated fish. For example, it can cause liver tumor formation and is suspected of carcinogenesis.[1] The United States, Japan, China, the European Union, and many other countries have already banned MG in fishery. Due to its low cost and antifungal effectiveness, MG is still being used illegally as indicated in the European Rapid Alert System for Food and Feed.[2]

HPLC with UV detection has been used to analyze MG and LMG. Figure 1 shows the structure of the two compounds. Loss of conjugation by reduction changes the chromaphore of LGM significantly. To obtain the sum of both, the method employs post-column oxidation with PbO_2 to convert LMG to MG, thus providing a sum of both comounds.[3] Most recently, LC/MS has been used to both meet the EU confirmation criteria and provide quantitative results for both compounds without the need for post-column oxidation. In this application, a simple and sensitive method for simultaneously determining MG and LMG is presented.[4, 5] The LC/MS/MS method's LOQ is 0.01 µg/Kg, which easily meets the import requirement set by Japan or the EU.[6]

Experimental

Reagents

MG	Sigma-Aldrich, CAS 569-64-2, USA
LMG	Dr. Ehreastorfer's lab, D-86199, 99% pure, Augsburg, Germany
Acetonitrile	CAS 75-05-8; Burdick & Jackson; Morristown, New Jersey, USA
Acetic acid	Merck, Germany
Ammonium acetate	CAS 631-61-8, Acros Organics, Morris Plains, New Jersey, USA



Malachite green Leucomalachite green

Figure 1. Molecular structure of malachite green and leucomalachite green.

Calibration Solutions

A stock standard solution of MG and LMG in acetonitrile was prepared at 100 µg/mL and stored at −18 °C, avoiding light. The stock solution was diluted in 50:50 acetonitrile:water to make the calibration solutions—10, 50, 100, 500, 1000, 5000, and 10,000 fg/µL.

Sample Preparation

To 5 g tilapia tissue was added 1 mL (0.25 mg/mL) hydroxylamine, 2 mL 1 M toluene sulfonic acid, 2 mL of 0.1 M ammonium acetate buffer (pH 4.5), and 40 mL acetonitrile. The mixture was then homogenized for 2 min. The supernatant was decanted, and to the precipitate was added 20 mL acetonitrile. This was filtered and added to the supernatant. To the combined acetonitrile extracts, 35 mL water and 30 mL methylene chloride were added. The solution was shaken and the methylene chloride layer collected. A second extract of 20 mL methylene chloride was made, and this layer added to the first extract. The methylene chloride was taken to dryness with a gentle stream of nitrogen and the extract reconstituted in 100 µL of acetonitrile

Instrumentation

LC	1100 LC	
Column	C18, 2.1 x 150 mm, 5 µm	
Column temp.	40 °C	
Mobile phase	A − 10 mmol/L ammonium acetate (adjust to pH 4.5 with acetic acid)	
	B − acetonitrile	
Column flow	0.3 mL/min	
Gradient	Time	%B
	0	30
	1	50
	2	95
	8	95
	8.01	30
	13	30
Injection vol.	10 µL	

MS	Agilent 6410 LC/MS Triple Quadrupole
Ionization	ESI(+)
Capillary	4000 V
Nebulizer P.	35 psi
Drying gas	11 L/min
Gas temp.	350 °C
Skimmer	15 V
OctDc1 (Skim2)	45 V
Oct RF	500 V
Q1 resolution	Unit
Q3 resolution	Unit
Collision gas	Nitrogen

The MRM parameters are listed in Table 1.

Table 1. MRM Method Parameters

Time	Compound	Precursor	Product	Dwell (ms)	Fragmentor (V)	Collision Energy (V)
0	MG	329.3	313.3	40	100	40
		329.3	208.2	40	100	40
7	LMG	331.3	316.3	40	100	30
		331.3	239.2	40	100	30

Results and Discussion

To obtain the most sensitive results, optimization of certain fragmentor voltages is important. Figure 2 shows the EICs of both target compounds at fragmentor values of 70 V, 90 V, and 100 V. The results show that the three different fragmentor values have little effect on the intensity of $[M+H]^+$ ions. Thus, 100 V was chosen for this study.

In addition, an optimal collision energy for the MS/MS must be set. Figure 3 shows the MS/MS spectra from three different collisional voltages, (a) 20 V, (b) 30 V, and (c) 40 V. Due to their structural differences, the voltage required for optimum fragmentation of each compound is different. For MG, the optimum fragmentation was observed at 40 V. The ion *m/z* 313 was due to the neutral loss of methane. The ion at *m/z* 208 was due to the neutral loss of N,N-dimethylaniline. For LMG, the optimum fragmentation was observed at 30 V. The ion at *m/z* 316 was due to the loss of a methyl radical. The ion at *m/z* 239 resulted from a subsequent loss of a benzene radical or, more likely, the rearrangement and neutral loss of toluene.

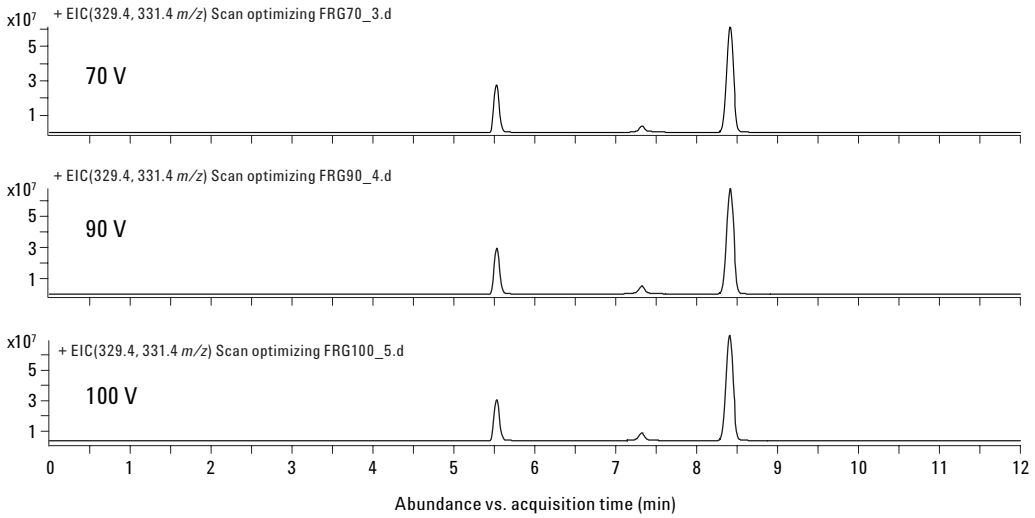


Figure 2. EICs of malachite green and leucomalachite green at fragmentor values of 70 V, 90 V, and 100 V.

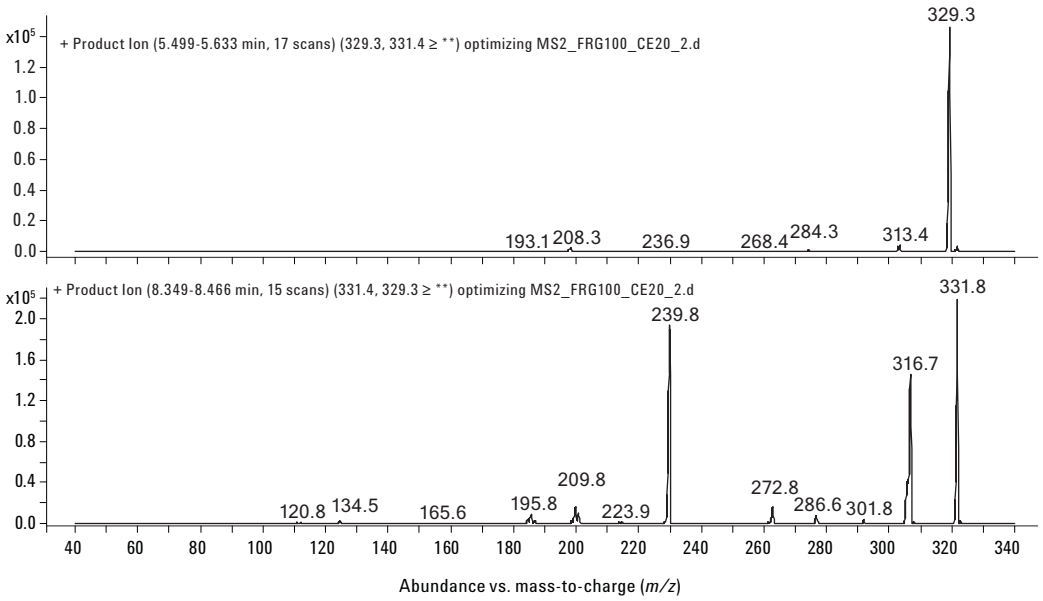


Figure 3a. MS/MS spectra of MG and LMG at collisional voltage of 20 V.

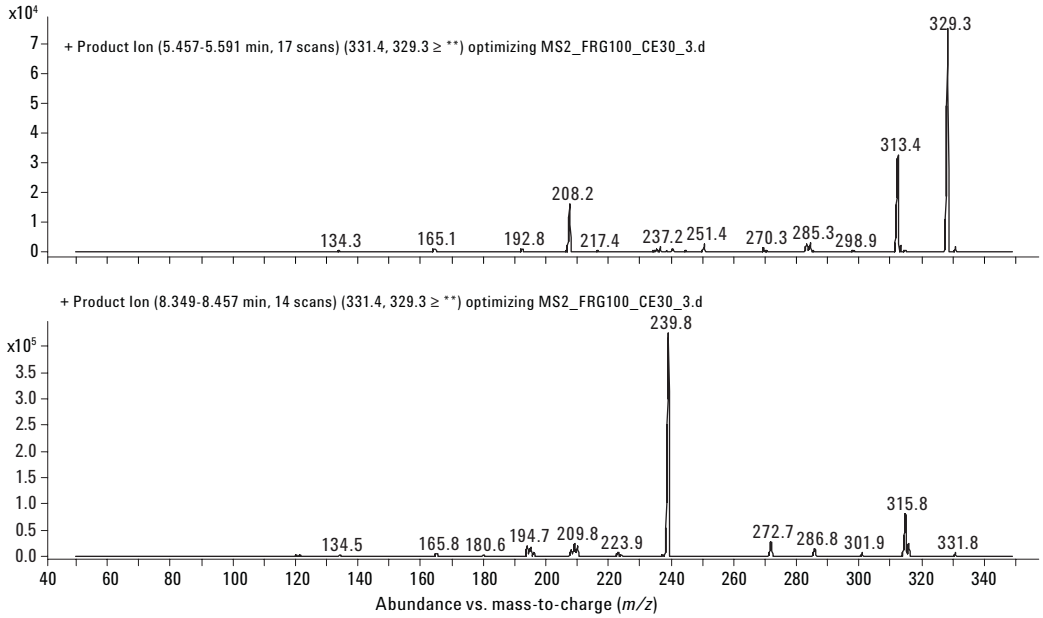


Figure 3b. MS/MS spectra of MG and LMG at collisional voltage of 30 V.

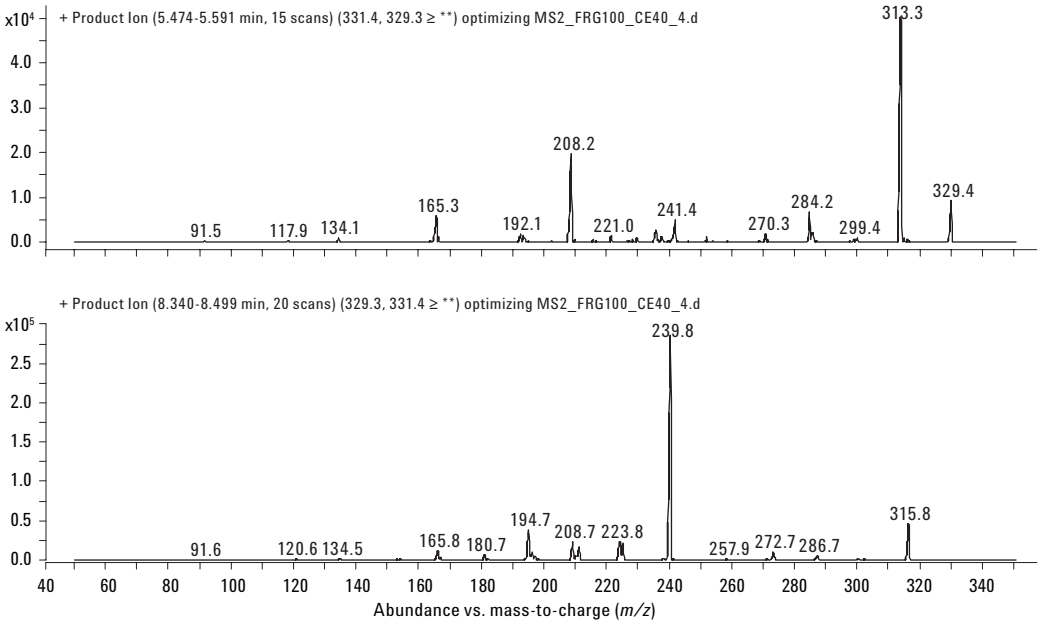


Figure 3c MS/MS spectra of MG and LMG at collisional voltage of 40 V.

Figure 4 shows the calibration curves for both MG (4a) and LMG (4b). Calibration solution concentrations were from 10 to 10,000 fg/µL. The linear calibration range is 100 to 100,000 fg on column for both compounds. The R^2 for both compounds was > 0.999 (origin ignored and no weighting). To demonstrate the sensitivity of the instrument, Figure 5 shows MS/MS spectra of a blank sample extract (5a) and sample extract spiked with 10 ppt of each compound (5b). A sample of tilapia spiked at 100 ppt MG and LMG before extraction was made to demonstrate method performance. The MRM results after extraction and cleanup are shown in Figure 6. The recover-

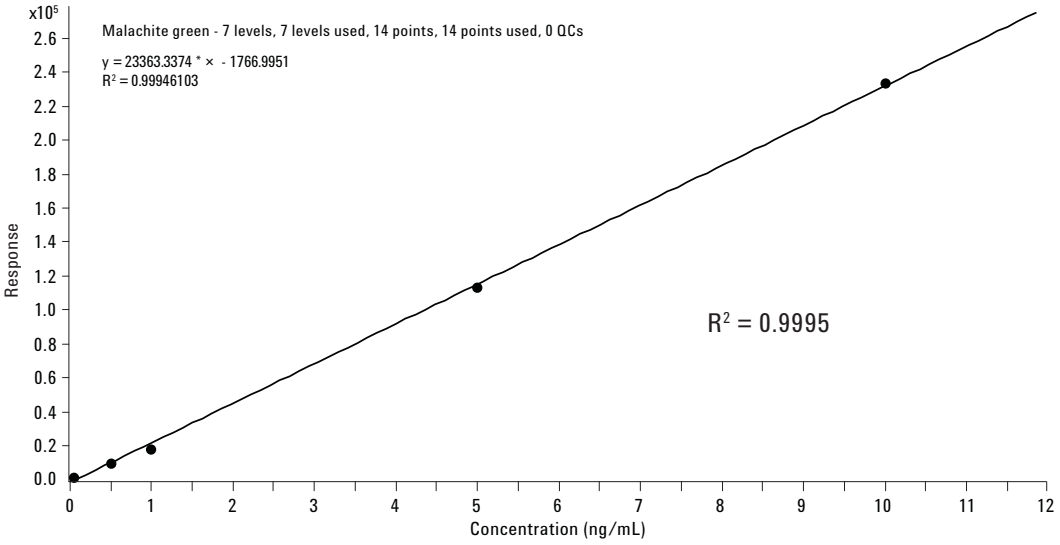


Figure 4a. Calibration curve of malachite green, linear range: 10 ppt to 10 ppb.

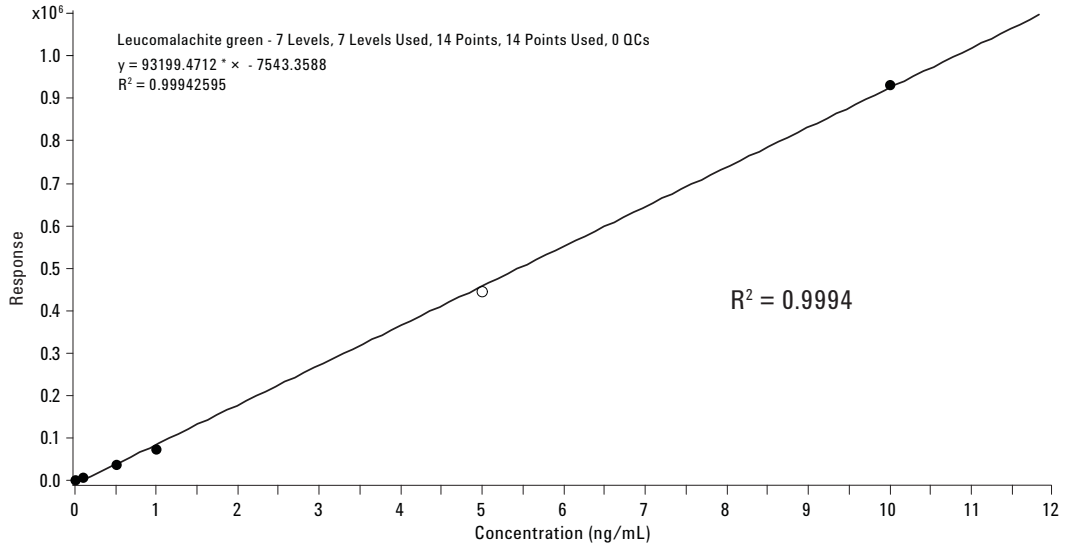


Figure 4b. Calibration curve of leucomalachite green, linear range: 10 ppt to 10 ppb.

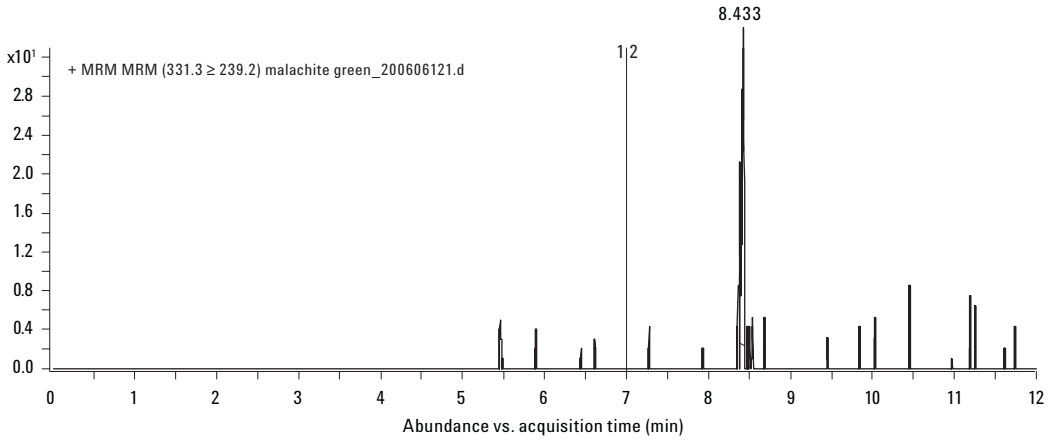
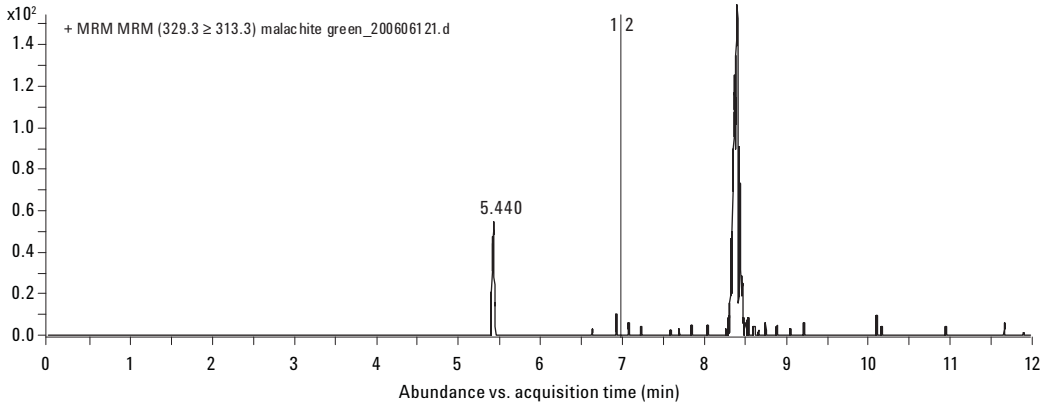


Figure 5a. MG and LMG MRM of a blank sample.



ppt spiked sample.

Figure 5b. MG and LMG MRM of a 10-ppt spiked sample.

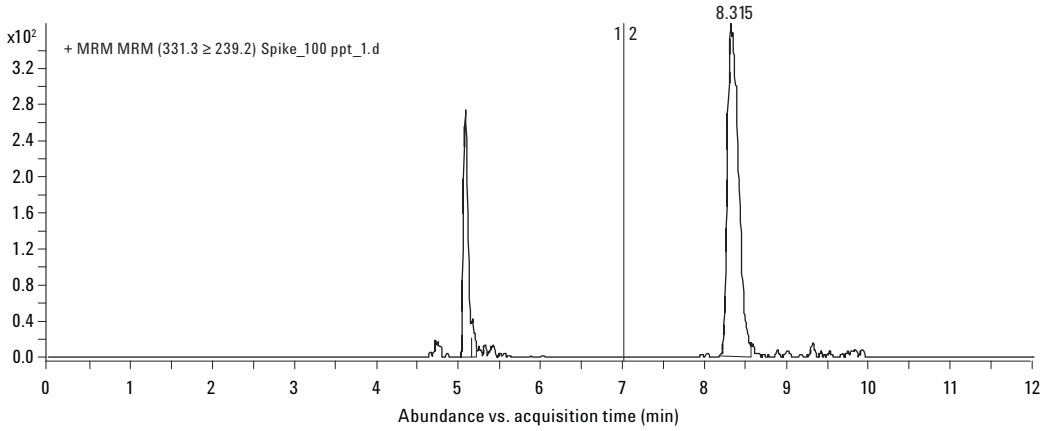


Figure 6. MRM result of talapia extract spiked with 100-ppt MG and LMG.

ies for MG were 48% and 23% for LMG. A mixture of MG and LMG at 100 fg/µL in 50:50 acetonitrile: ammonium acetate was used for the repeatability study for instrument performance. The RSD from eight injections for MG was 3.52% (S/N > 20). The RSD from eight injections for LMG was 2.25% (S/N > 40).

Conclusions

This application note demonstrates a complete method to rapidly and precisely determine residue levels of malachite green and leuco-malachite green in fish. Using positive mode electrospray ionization (ESI+) and multiple reaction monitoring (MRM) technique, the LC/MS/MS method shows detection limit of 10 ppt, which easily meets the import requirement set by Japan or EU.

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