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Research Article

Determination of methylene blue residues in aquatic products by liquid chromatography-tandem mass spectrometry

A method for the determination and confirmation of methylene blue (MB) in aquatic products was developed. Residues of MB were extracted from homogenized tissues with acetonitrile/sodium acetate buffer solution, and simply cleaned up with dichloromethane liquid/liquid extraction. After concentration and dissolution, the sample solutions were cleaned up by the neutral alumina and weak cation-exchange solid phase extraction (SPE) cartridge, prior to LC-MS/MS analysis. MB was determined at 1.0–20 μg/kg in eel, toasted eel and shrimp, with a limit of quantification of 0.5 μg/kg. Recovery for MB was between 73.0% and 108.3%. This method is fast, exact and sensitive. It can be applied to determine MB in aquatic products.

Keywords: Aquatic products / LC-MS/MS / Methylene blue / Residue
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1 Introduction

Methylene blue (MB) is a thiazine dye that, although not regulated for use with edible aquatic animals, may sometimes be used as a chemotherapeutic agent in the aquaculture industry [1]. Nowadays MB is widely used in lots of different areas, for example in microbiology, surgery and in the diagnostic field. It is a good alexipharmic of nitrite, chlorate, quinine if present at low concentration levels. However, in large doses, MB can oxidize haemoglobin to methaemoglobin [2]. In bodies, highly colored oxidized form of MB can be reduced to colorless leuco form leuco-methylene blue (LMB), as shown in Fig. 1. However, LMB is not available as a standard substance and has only been obtained by diluting MB-solutions with reducing reagent [3].

Because MB is used as a replacement for other antifungal dyes in aquaculture nowadays, Japan has established a maximum residue limit (MRL) of 10 μg/kg for aquatic products. It is important to have a practical method to monitor MB in edible aquatic product tissues. Numerous approaches have been reported for the determination of MB's concentration, such as HPLC, CE, spectrophotometry, chemiluminescence and so on [1, 3–8]. Anyway, few papers have been published for the determination of MB or its metabolites in fish or other aquatic animals by LC-MS/MS [9]. But in this method, just the fish was studied and the detection limit was 15 μg/kg. Most approaches are applicable to urine or blood [3, 10–12].

This study aimed at the development of a simple method for the determination and confirmation of residues of MB in aquatic products. Determination procedure involves only a liquid-liquid extraction and cleaning up by SPE, detection by LC-MS/MS. Several factors affecting the extraction efficiency were studied.

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Abbreviations: ALN-N, alumina-neutral; **LMB,** leuco-methylene blue; **MB,** methylene blue; **MCX,** mix-mode cationic exchanger; **MRL,** maximum residue limit; ***p*-TSA,** *p*-toluenesulfonic acid; **SCX,** strong cationic exchanger; **SRM,** selected reaction monitoring; **WCX,** weak cationic exchanger; **SPE,** solid phase extraction

2 Experimental

2.1 Reagents and chemicals

MB standard was purchased from Shanghai reagent No.3 factory (Shanghai, China). Purity was listed as 96%. Analytical grade sodium acetate, *p*-toluenesulfonic acid (*p*-TSA), neutral alumina were obtained from Nanjing Chemical Reagent No.1 factory (Nanjing, China). HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany), HPLC grade formic acid was bought from TEDIA (Fairfield, USA), dichloromethane and diglycol were obtained from Lingfeng (Shanghai, China). Water was of Milli-Q quality (Millipore, Molshelm, France). Weak cationic exchanger (WCX) SPE cartridges (3 mL, 60 mg) were obtained from Waters and alumina-

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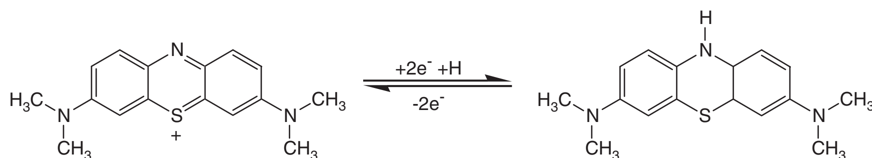

Figure 1. Conversion of methylene blue to leuco-methylene blue.

neutral (ALN-N) SPE columns (3 mL, 500 mg) were purchased from Supelco.

Buffers and solutions: (1) 0.1 M Sodium acetate buffer: 8.2 g sodium acetate was dissolved in 1000 mL water, pH was adjusted to 4.5 with glacial acetic acid. (2) 1 M *p*-Toluenesulfonic acid (*p*-TSA) solution: 44 g *p*-TSA was dissolved in 250 mL water. (3) 20% Diglycol solution: 200 mL diglycol was diluted with 800 mL water. (4) 3:7 (v/v) Methanol solution: 300 mL methanol was diluted with 700 mL water.

Standard preparation: (1) Stock solution (100 μg/mL): Approximately 10 mg MB was weighed and dissolved in 100 mL methanol solution. This solution was stable at 4°C for several months. (2) Fortification standard solution (1 μg/mL): Stock solution (100 μL) was pipetted into 10 mL methanol solution. The fortification standards were made up at least once a week. (3) Standard curve preparation: Dilute fortification standard solution to six concentrations of 100.0 ng/mL, 50.0 ng/mL, 20.0 ng/mL, 10.0 ng/mL, 5.0 ng/mL and 2.0 ng/mL.

2.2 Equipment and apparatus

The centrifuge used was the RJ-LD-IIB model of Ruijiang (Wuxi, China). Vortex mixers of WH-861 model were bought from science and educational equipment company of Taicang. Budhi Rotavapor R-200 evaporator (Flawil) was from Switzerland with a water bath. An Organomation Assoc. N_2 evaporator made in USA was used, with water temperature set to 50°C.

2.3 Tissue preparation and storage

Eel fillets with heated skins and edible parts of shrimp were blended until homogenous, and stored in clean bags. Samples were stored at –18°C.

2.4 Extraction and cleanup

Blended tissue (5.0 g) was weighed into a 100 mL centrifuge tube. 3.0 g neutral alumina, 1 mL 1 M *p*-TSA and 4 mL sodium acetate buffer were added into the tissue. The samples were vortex-mixed for 30 s. Then, 8 mL acetonitrile was added to the tissue and the samples were vortex-mixed for 2 min. The tubes were centrifuged for 5 min at 20°C and 2000 rpm. The supernatant was poured into another 100 mL centrifuge tube. Residual material was vortex-mixed with another 4 mL acetonitrile for 1 min followed by centrifugation for 5 min at 20°C and 2000 rpm. Combined supernatants were vortex-mixed with 10 mL dichloromethane, 8 mL 20% diglycol solution for 1 min followed by centrifugation for 5 min at 20°C and 2000 rpm. The lower organic layer was collected into a 50 mL round-bottom flask. Then the extract was evaporated to dryness while the flask was in a 45°C water bath.

A WCX cartridge was placed on a vacuum manifold, and a ALN-N column was placed on top of the WCX cartridges with adapters. The columns were conditioned with 5 mL acetonitrile. The residues were re-dissolved in flask with 2 mL acetonitrile three times. The tissue extract was added to the top ALN-N column with a Pasteur pipette and the flow rate was about 1 mL/min. The column was washed with 2 mL acetonitrile and vacuum was applied until the ALN-N column was dry. Then the ALN-N cartridge was discarded. Wash the WCX column with 3 mL water and 3 mL acetonitrile. Finally, MB was eluted from the WCX column with 4 mL 5% formic-methanol. The elution solvent was removed from the extract with an N_2 evaporator, and the residue was re-dissolved in 1 mL 3:7 (v/v) methanol solution, and filtered through a 0.45 μm Teflon filter, and transferred into LC vial for analysis.

In order to confirm the function of extraction solution, the acetonitrile, acetonitrile/acetate buffer and acetonitrile/*p*-TSA/acetate buffer were used to extraction the MB from the sample. When trying different solvents, the other steps were the same.

2.5 LC-MS/MS analysis

Aliquots of 25 μL were analyzed on a Surveyor HPLC module integrated with autosampler, solvent delivery system, MS pump and a triple-quadrupole mass spectrometer equipped with electrospray ionization probe (Thermo-Fisher Finnigan, USA). The interface of the LC-MS/MS was the Xcalibur 2.0 system. The separation was performed on a Waters Sunfire C_{18} column (150 × 2.1 mm, 3.5 μm), with A: methanol and B: 0.1% formic acid as the mobile phases. The gradient conditions were set as follows: 0 min, B = 90%; 6.0 min, B = 10%; 8.0 min, B = 10%; 8.1 min, B = 90%; 10.0 min, B = 90%. The flow rate was set at 250 μL/min and the column temperature was ambient.

The MS detector was operated in the positive ion mode. The capillary voltage was set at 3.8 kV, and the capillary temperature at 320°C. Nitrogen was used as sheath gas and

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Table 1. Precursor ions, product ions and corresponding collision energies for MB and LMB

Analyte	Precursor ion	Product ion	Collision energy (eV)
MB	284	239.9	32
		241.0	30
		267.9[a)]	30
LMB	286	228.0	40
		242.6	35
		255.8	34
		270.0	32

a) The daughter ion used for quantification is shown with asterisks.

auxiliary gas, with gas pressure at 30 arb and 4 arb, respectively. Argon was used as collision gas and its pressure was 1.5 mTorr. The collision energies were separately optimized for the selected ion transitions of both MB and LMB (Table 1).

2.6 Data evaluation

The content of residues of MB was calculated by interpolation of the standard curve which was determined by peak areas of standard solutions.

3 Result and discussion

3.1 Extraction

Because MB and malachite green both are dyes, ionic compounds and easy to dissolve into water, our sample processing method is based on the extraction method of malachite green [13]. In consideration of having a positive charge, adding *p*-TSA as ion-pairing reagent will improve the extraction of MB with acetonitrile. Sodium acetate buffer is added to control solution's pH.

The experiments were carried out to contrast the extraction efficiency of acetonitrile, acetonitrile/acetate buffer and acetonitrile/*p*-TSA/acetate buffer. The recovery of the first one was about 65%, and 80% for the second system. For the third system, the recovery reached to 100%. Moreover, the ion-pair form of MB and *p*-TSA also improved the extraction of MB in dichloromethane. The efficiency of dichloromethane extraction is nearly 100%.

3.2 Clean-up

3.2.1 Liquid-liquid extraction

There were many impurities left in the sample extraction solution after just extraction of acetonitrile. The leading area and tailing area of the chromatographic peaks were observed in this condition. If the acetonitrile solution was brought to the next SPE cleanup step right now, plentiful impurities would saturate the cartridges easily. Thus the capacity of cartridges must be increased, and the cost of the SPE also increases. In contrast, the liquid-liquid extraction of dichloromethane can remove most ion impurities. That is more convenient and cheaper. And after the extraction of dichloromethane, evaporation and SPE cleanup, the sample could be cleaned up well.

3.2.2 SPE clean-up

The ALN-N column was chosen to separate the fatty impurities based on the method of malachite green. And it did well. When the cationic exchanger column was used only in this step, the hold of MB was bad. About 40% of MB was lost.

Three kinds of cationic exchanger column were compared in this method. They are MCX (3 mL, 60 mg, Waters), WCX (3 mL, 60 mg, Waters) and SCX (3 mL, 200 mg, Supelco). Because MB has a positive charge, it can be held by them very well. Since MB's combination with cations on MCX and SCX column is very firm, it is difficult to be eluted completely. We tried 5% ammonia-methanol (v/v), 5% ammonia-acetone (v/v) and 5% ammonia-ethyl acetate to elute MB. The highest recovery is less than 60%. However, MB can be eluted thoroughly with just 3 mL 5% formic acid/methanol from WCX column.

So the ALN-N column and WCX SPE column were chosen and connected in tandem. ALN-N cartridge was discarded after sample application. And MB was eluted with 3 mL 5% formic acid/methanol from WCX column.

3.3 Influence of teflon filter

As a kind of dye, MB tends to be adsorbed on plastic polymer [4]. It was also found that after filtering from blue aqueous phase filter, about 60–70% of MB was lost. But white organic phase filter almost did not absorb MB. So the organic phase film filter was chosen to filter the granules before injecting the sample.

3.4 Stability of LMB

Like other dyes, MB also has a reduced colorless leuco form. Researchers studying MB metabolism in mammalian species indirectly measured LMB [10]. Because it tends to revert back to MB, there is no standard of it which could be purchased. LMB can be made by adding ascorbic acid to the solution of MB, finding that the blue solution turns to colorless. During the chromatogram process, once ascorbic acid and LMB were separated, LMB turned to MB quickly. That can be shown by observing color transformation in doing this with SPE cartridges. And a balance was found between MB and LMB. The ratios of their peak area were almost equal no matter adding LMB solution before sample processing or LC-MS/MS analysis. Therefore, it can be

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considered that any LMB present in the samples was oxidized to MB during analysis.

3.5 Optimization of LC and tandem mass spectrometry conditions

C8 and C18 chromatographic columns were compared in the separation, and found that the column efficiency of C18 is higher. After that, different brands of C18 columns were also compared. The results showed that the Waters Sunfire C18 column had good separation power, and hardly had tailing. So the Sunfire C18 column was used in this method.

Using positive ion electrospray ionization, triple quadrupole mass spectrometer parameters were optimized with MB standards. The LMB was not stable and easy to be oxidized to the MB, but the LMB could be observed during the MS scan. The product ions of the LMB were similar with the MB, and its *m/z* just increased 2 for the LMB than the MB. MS/MS-full scan spectra of MB and LMB are given in Fig. 2. Selected reaction monitoring (SRM) was used to measure the transitions from the precursor ions to product

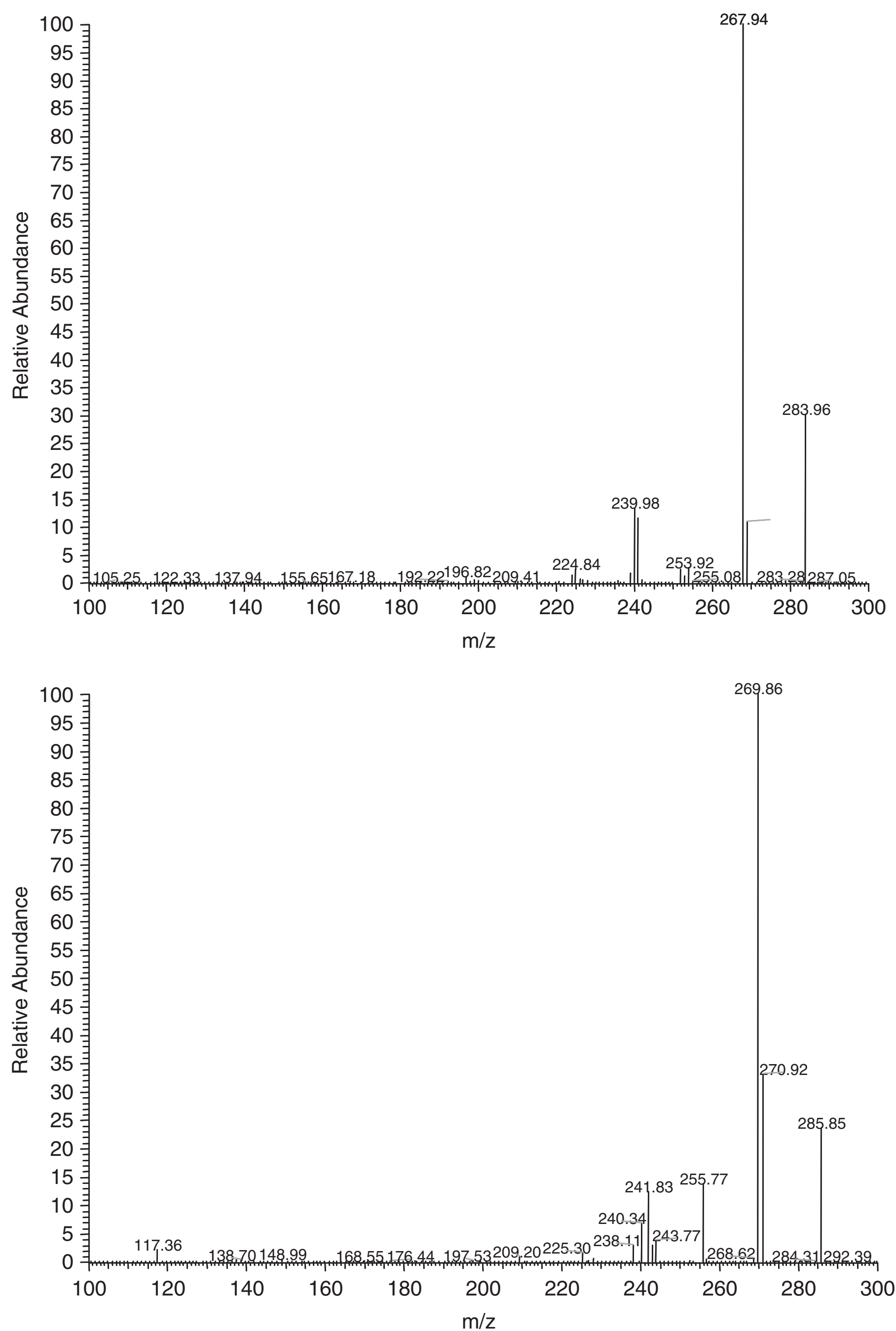

Figure 2. MS/MS full scan spectra of MB and LMB.

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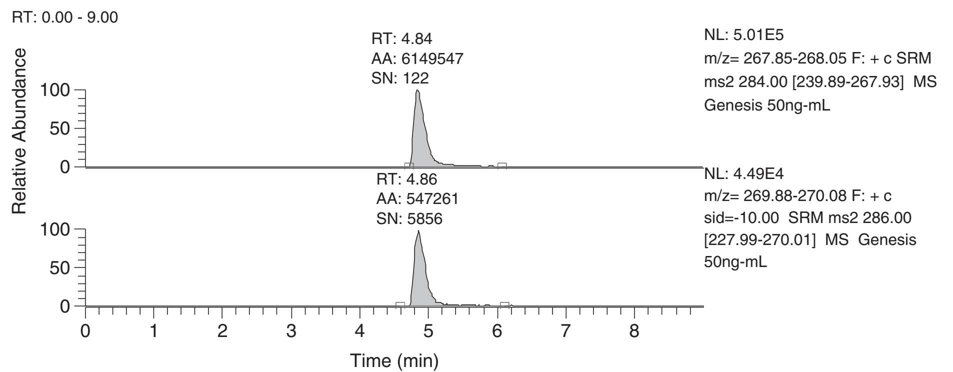


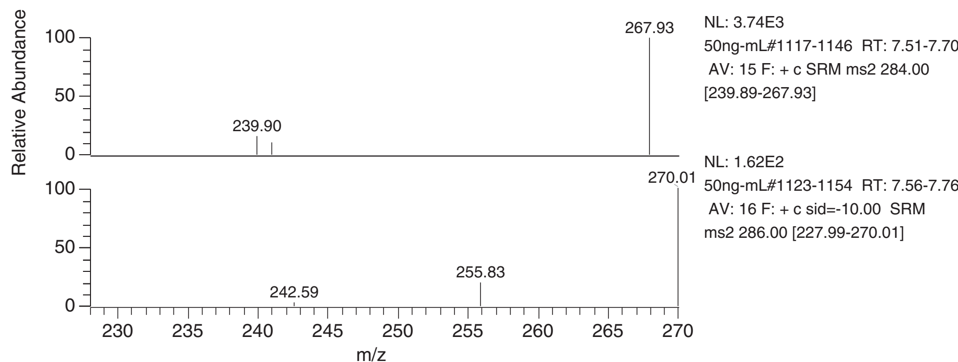
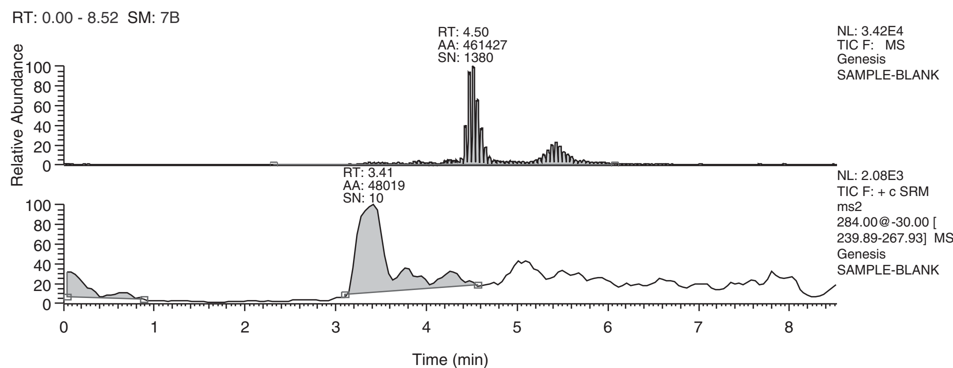


Figure 3. LC-MS/MS chromatograms of MB and LMB.



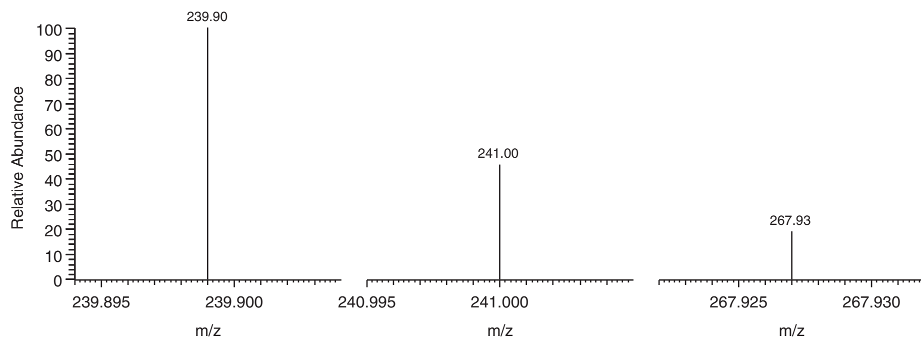


Figure 4. LC-MS/MS chromatograms of blank eel matrix.

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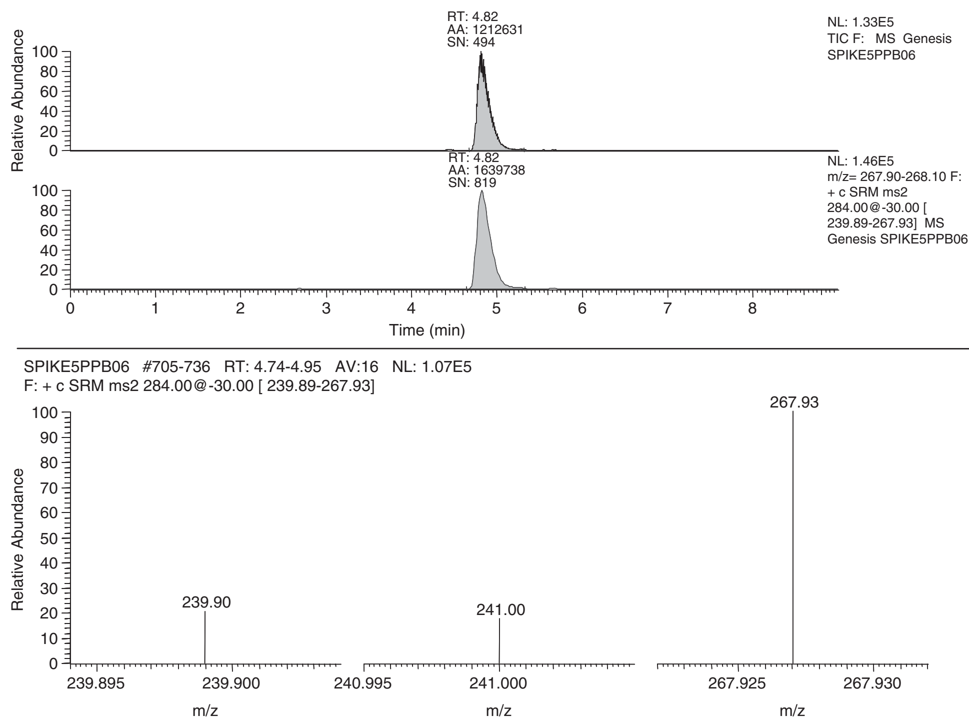

Figure 5. LC-MS/MS chromatograms of eel matrix spiked with 5 μg/kg.

Table 2. Recoveries of MB at different fortification levels in LC-MS/MS method

Spike level (μg/kg)	Eel			Toasted eel			Shrimp		
	1.0	5.0	10.0	1.0	5.0	10.0	1.0	5.0	10.0
1	0.875	3.490	9.751	0.979	3.994	9.952	0.832	5.533	8.591
2	0.954	3.961	7.266	0.898	4.719	9.559	0.864	4.673	7.416
3	0.859	3.719	8.530	0.820	4.179	9.472	0.774	5.042	7.617
4	0.972	3.596	9.638	0.950	3.666	8.703	0.967	3.858	8.688
5	1.026	3.782	6.266	0.976	4.056	12.001	0.963	5.503	7.579
6	0.921	3.648	8.356	0.955	3.628	8.379	0.745	5.109	7.534
Average (μg/kg)	0.935	3.699	8.301	0.930	4.040	9.678	0.857	4.953	7.904
RSD%	6.7	4.4	16.3	6.6	9.9	13.2	10.9	12.6	7.3
Recovery (%)	93.5	74.0	83.0	93.0	80.8	96.8	85.7	99.1	79.0

ions. Fig. 3 shows typical SRM chromatograms of MB and LMB in standard solution. The area of the LMB was about 10 percent of the MB in the standard. In the sample extraction solution, the area ratio of the LMB and MB was the same.

In real sample analysis, only the MB was detected and calibrated as the residue drugs. The matrix blank and spiked chromatograms of eel were shown in Figures 4 and 5. There are no disturbed peaks in the MB retention time. The results of the shrimp and toasted eel were similar.

3.6 Calibration and detection limits

Standard curves of the regressed concentration points gave squared correlation coefficient of 0.9938 in this method. The equation of calibration curve was $Y = -29976 + 1502610 * X$. The LOD was calculated by 4 times of the signal *vs.* noise, the LOQ was calculated by 10 times of the signal *vs.* noise. The LOD was 0.1 μg/kg, and the LOQ was 0.5 μg/kg.

3.7 Recovery and RSD

With the experimental method described above, MB was extracted from eel, toasted eel and shrimp separately at 1.0, 5.0 and 10.0 μg/kg levels, with recoveries of 74.0–99.1% in LC-MS/MS method and the RSD corresponding to every level. The results were listed in Table 2.

4 Concluding remarks

A LC-MS/MS method has been developed to determine MB residue in aquatic products. The liquid-liquid extraction and

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SPE columns were used to clean up the sample. The detection and quantification limit for this method were low enough to determine MB residues in aquatic products below the permissible MRLs established by Japan.

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The authors have declared no conflict of interest.

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