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Article

Simultaneous Determination of Malachite Green, Crystal Violet, Methylene blue and the Metabolite Residues in Aquatic Products by Ultra-Performance Liquid Chromatography with Electrospray Ionization Tandem Mass Spectrometry

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This work describes solid-phase extraction–ultra-performance liquid chromatography with electrospray ionization tandem spectrometry for determination of malachite green and metabolite leucomalachite green, crystal violet and metabolite leucocrystal violet, methylene blue and metabolites including azure A, azure B and azure C in aquatic products. Samples were extracted with acetonitrile and ammonium acetate buffer and purified by liquid extraction with dichloromethane, and then on MCAX solid-phase extraction cartridges. Then the extract was evaporated at 45°C by nitrogen blow. The residue was dissolved and separated by an Acquity BEH C18 column. The mobile phase was acetonitrile (A) and 5 mmol/L of ammonium acetate containing 0.1% formic acid (B). Analytes were confirmed and quantified using a tandem mass spectrometry system in multiple reaction mode with triple quadrupole analyzer using positive polarity mode. The limits of detection of malachite green, leucomalachite green, crystal violet and leucocrystal violet were 0.15 μg/kg, the limits of quantification were 0.50 μg/kg, and the average recoveries were more than 75% with spiked residues from 0.5 to 10 μg/kg. The relative standard deviations were less than 13%. The limits of detection of methylene blue, azure A, azure B and azure C were 0.3 μg/kg, the limits of quantification were 1.0 μg/kg, the average recoveries were more than 70% with spiked residues from 1.0 to 10 μg/kg and the relative standard deviations were less than 15%. The method has the merits of simplicity, sensitivity and rapidity, and can be used for simultaneous determination of the analytes in aquatic products.

Introduction

Malachite green (MG) and crystal violet (CV) are triphenylmethane dyes that were originally used in the textile process (1). Because of their disinfection and sterilization properties, they are widely used in aquatic products throughout the world (2, 3). It has been found that dyes of this family (like rosaniline) can induce hepatic and renal tumors in mice and reproductive abnormalities in fish, and the dyes have been linked to increased risk of human bladder cancer (4, 5). MG is highly cytotoxic to mammalian cells and also acts as a liver tumor-enhancing agent (6). CV is also known to be effective in the treatment of fungal infections. It has found widespread use as a feed additive to inhibit mold and fungal growth in poultry feed (7). Methylene blue (MB) is a thiazine dye. MB can also be helpful in fields such as surgery, microbiology and diagnosis. If presented at low concentrations, it can be used as toxinicide of nitrite and chlorate. Currently, MB is used as a replacement of other anti-fungal dyes in aquaculture for preventing and curing saprolegniasis, red mouth disease and ichthyophthiriasis. It is indicated that MB can be poisonous and mutagenic to the animal body (8).

MG, CV and MB are readily absorbed by fish and reduced to the corresponding metabolites, MG to leuco form LMG, CV to leuco form LCV, MB to metabolites azure A (AZ-A), azure B (AZ-B) and azure C (AZ-C), which are the majority of prevalent residues present in fish tissues (9), as shown in Figure 1. For this reason, the European Commission requires methods that can determine MG and LMG residues in the meat of aquaculture products. In addition, the Commission has established a minimum required performance limit (MRPL) of 2 μg/kg for the sum of MG and LMG (10). The US Food and Drug Administration explicitly banned the use of MG in fish farming in 1991 due to its suspected carcinogenic properties. However, no safe levels for the presence of MG, LMG, CV, LCV, MB, AZ-A, AZ-B or AZ-C in aquatic products for human consumption have been established, and therefore, determination of these compounds at sub μg/kg levels is required.

Amid concerns about the health risks associated with the abuse of the drugs, an increasing number of methods have been developed for their determination in recent years. Mitrowska *et al.* extracted MG and LMG from carp sample with acetonitrile–acetate buffer mixture followed by portioning with dichloromethane, cleanup on a SCX solid-phase extraction (SPE) cartridge and detection by tandem visible absorbance and fluorescence detectors connected inline without any post-column procedure (11). Lee *et al.* reported that 16 mL of acetonitrile containing 250 mg ascorbic acid and 0.8% perchloric acid were used for extraction from edible goldfish muscle, followed by partitioning with dichloromethane and cleaniup with a Strata-x 33 μm polymeric cartridge and detection with ion trap mass spectrometry. These two methods were both based on solvent extraction using acetonitrile with aqueous buffer. In the cleanup process, both methods used liquid–liquid partitioning and SPE. This method has been adopted by many other groups (12). Dowling *et al.* modified the two preceding methods without liquid–liquid partitioning and purified on a Bakerbond strong cation exchange SPE cartridge (13).

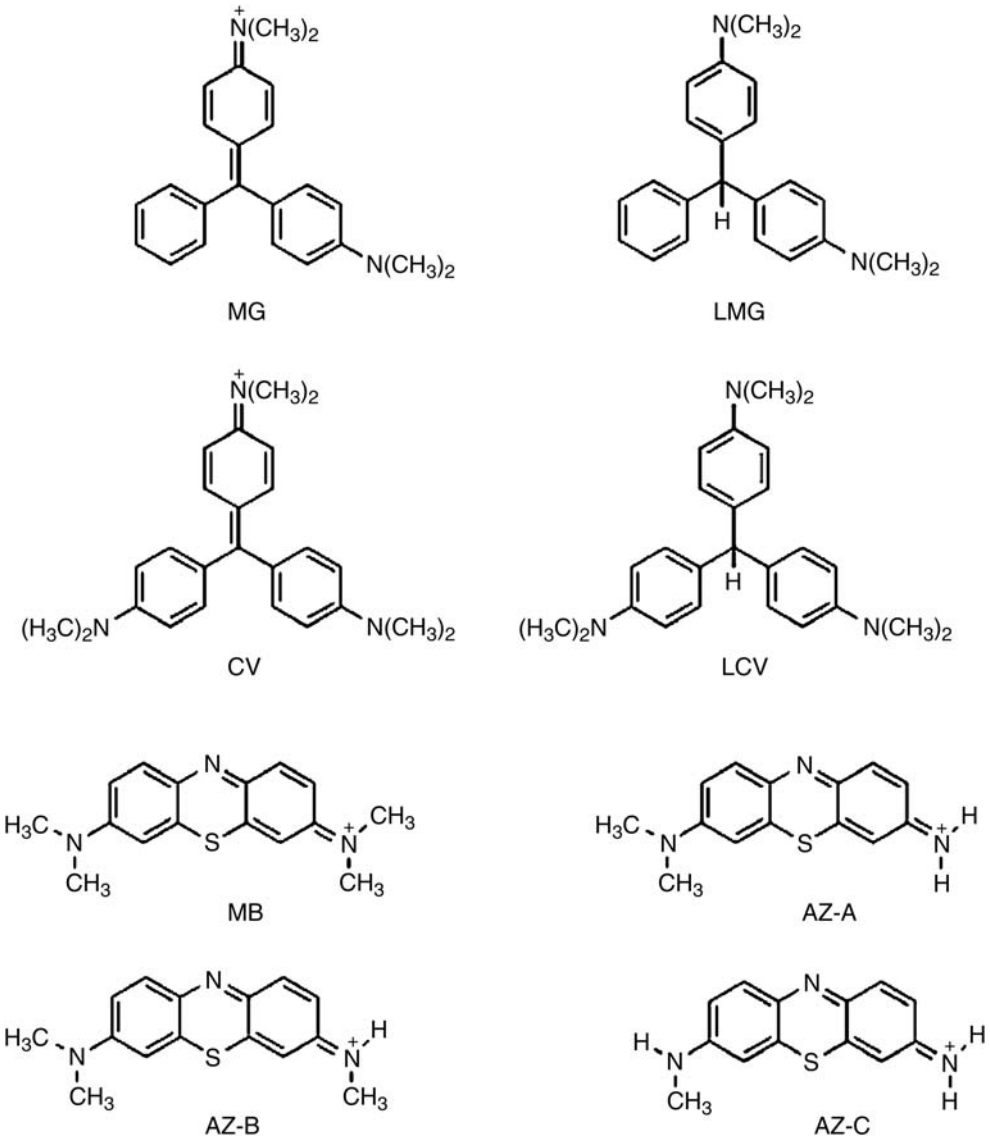

Figure 1. Structures of MG, LMG, CV, LCV, MB, AZ-A, AZ-B and AZ-C.

Yang *et al.* extracted MB from homogenized aquatic product tissues with acetonitrile–dichloromethane based on ion-pair formation with *p*-toluenesulfonic acid using SPE cartridge without any alternative cleanup before liquid chromatography–tandem mass spectrometry (LC–MS-MS) analysis (14).

Other analytical methods have been reported for determination of the residues. Some methods are based on LC, with visible detection, single fluorescence detection or confirmation by MS and tandem MS detection (15, 16, 17). Much attention has been devoted to the analysis of MG and CV and their lecuo forms, but less has been shown to MB and its metabolites (18, 19). Our method was adapted to include MB, AZ-A, AZ-B and AZ-C. Ultra–performance liquid chromatography–tandem mass spectrometry (UPLC–MS-MS) has the virtue of efficient separation and accurate characterization, and has become the primary method of determining residues in recent years. In this study, a method for simultaneously determining eight analytes in aquatic products has been established. The result is satisfactory after systematized optimization of sample pretreatment methods and instrumental analysis conditions.

Experimental

Materials and reagents

MG and LMG were purchased from Dr. Ehrenstorfer (Germany). CV, LCV and MB were all obtained from Sigma (St. Louis, MO). AZ-A, AZ-B and AZ-C were obtained from Fluka (Buchs, Switzerland). Methanol, acetonitrile, dichloromethane and formic acid were all of LC grade. Ammonium acetate and ammonia were of guaranteed reagent grade. Water was deionized ultrapure water. All other reagents used in the experiment were of analytical grade. MCAX SPC cartridges (3 mL, 500 mg) were obtained from Supelco (Bellefonte, PA). 20% Hydroxylamine hydrochloride was prepared by dissolving 20.0 g of hydroxylamine hydrochloride in a 100-mL volumetric

flask. Ammonium acetate (0.1 mol/L) was prepared by dissolving 3.85 g of ammonium acetate in a 500-mL volumetric flask. P-toluenesulfonic acid (1.0 mol/L) was prepared by dissolving 19.0 g of p-toluenesulfonic acid in a 100-mL volumetric flask.

Preparation of stock and standard solutions

Individual stock solutions of 100 μg/mL for MG, LMG, CV and LCV were prepared in LC-grade acetonitrile, and MB, AZ-A, AZ-B and AZ-C in LC-grade methanol. All were kept at −20°C (stable for three months). Standard solutions containing all compounds were mixed and diluted with methanol, and working solutions of all compounds and calibration concentrations were prepared by appropriate dilution of the stock solutions on the day of analysis. All standards were stored at 4°C (stable for one month).

Preparation and purification of samples

Silver carp obtained from a local supermarket was homogenized with bones in a blender and stored at −20°C. Laboratory samples of silver carp tissue were analyzed and those found to contain no detectable residues of the analytes were used as negative controls.

Silver carp samples (5 g) were weighed into 50-mL polypropylene tubes. Nineteen milliliters of extraction solution (containing 1.5 mL of 20% hydroxylamine hydrochloride, 2.5 mL of 1.0 mol/L p-toluenesulfonic acid, 5.0 mL of 0.1 mol/L ammonium acetate and 10.0 mL of acetonitrile) was added and the samples were homogenized for 30 s. Samples were shaken 5 min and ultrasonicated 20 min at room temperature. Then the homogenates were centrifuged at 7,000 rpm for 10 min and the supernatant was transferred to a clean 100-mL polypropylene tubes. The samples were re-extracted with 4.0 mL of extraction solution as before. The supernatants were combined and the sample extracts were further purified.

15 mL of dichloromethane, 4.0 mL of water and 2.5 mL of diethylene glycol were added into the sample extracts and shaken for 1 min,then centrifuged at 7,000 rpm for 10 min. The subnatant was transferred to a clean 150-mL round flask and to the supernatant was added 5.0 mL of acetonitrile and 15 mL of dichloromethane, then it was shaken for 1 min and centrifuged at 7,000 rpm for 10 min. The subnatant was combined and evaporated to 0.5 mL at 45°C and the residue was dissolved in 5.0 mL of acetonitrile, ready for loading.

The SPE process with MCAX cartridges can be summarized as follows: (i) activated with 5 mL of methanol and 5 mL of water (both steps at 3 mL/min); (ii) 5 mL sample loaded at 1.2 mL/min; (iii) rinsed with 10 mL of water and 10 mL of methanol and dried under vacuum for 5 min; (iv) first elution with 5 mL of dichloromethane, methanol and ammonia (50:50:5, v/v/v), then the second with 3 mL of dichloromethane, methanol and formic acid (50:50:1, v/v/v); (v) both eluates evaporated to near dryness under a gentle stream of nitrogen at 40°C and residues redissolved with a volume of 0.5 mL of acetonitrile and 0.5 mL of 5 mmol ammonium acetate, acetonitrile and formic acid, respectively (80:20:0.1, v/v/v); the first for determining MG, LMG, CV and LCV; the second for MB, AZ-A, AZ-B, and AZ-C. Then 0.3 mL of each was taken for mixture and syringe filtered using a 0.22-μm filter into an autosampler vial. An aliquot (10 μL) was injected onto the LC column for analysis.

UPLC–MS-MS conditions

The UPLC–MS-MS system comprised an Acquity UPLC system connected online with a Quattro Premier tandem mass spectrometer (Waters, Milford, MA). The column used in the experiment was an Acquity BEH C18 (2.1 × 100 mm, 1.7 μm particle size) maintained at 40°C. Mobile phase was acetonitrile (A) and 5 mmol/L of ammonium acetate containing 0.1% formic acid (B). After sample injection (10 μL), a linear gradient was programmed for 0.5 min from 10:90 A–B to obtain 85:15 A–B composition, then the composition was held for 2 min. Finally, A was directly decreased to 10% and held for 0.5 min. The total analysis time was 4.5 min, while 1.5 min was required for re-establishing and equilibrating the initial conditions. The flow rate was set at 0.25 mL/min during the chromatographic process. The entire eluate was electrosprayed, ionized and monitored by MS-MS detection in the multiple reaction mode using positive electrospray ionization. The flow rate and temperature of the drying gas (N_2) were 700 L/h and 350°C, respectively. The collision gas (Ar) flow was 0.12 mL/min and the capillary voltage was 2,500 V. The dwell time was set at 100 ms.

Validation procedures

Standard calibration curve and quality control (QC) samples were analyzed in three consecutive days. Linearity of calibration curves based on the analyte area as function of the nominal concentration were assessed by weighted ($1/C^2$) least square regression. Linearity correlation coefficients were calculated in our experiment, as shown in Table I.

Results and Discussion

Extraction optimization

The eight analytes are the dyes that are tightly associated with the tissues, and have the insertion function and coulombic forces with the tissues. It is necessary to choose the suitable extraction and method for determining them. MG, CV, MB, AZ-A, AZ-B and AZ-C are cationic compounds and a single organic solvent is very difficult to get good extraction recovery. In the experiment, p-toluenesulfonic acid used as ion pair

Table I
Linear Equations, Linearity Range, Correlation Coefficient, Limits of Detection and Limits of Quantification for the Eight Analytes

Compound	Linear equations	Linearity range (ng/mL)	Correlation coefficient	LOD (μg/kg)	LOQ (μg/kg)
MG	$Y = 0.706x + 0.0303$	2.5 ~ 100	0.9995	0.15	0.5
LMG	$Y = 0.0372x + 0.0058$	2.5 ~ 100	0.9992	0.15	0.5
CV	$Y = 7593.3x + 520.98$	2.5 ~ 100	0.9987	0.15	0.5
LCV	$Y = 0.00694x + 0.000636$	2.5 ~ 100	0.9954	0.15	0.5
MB	$Y = 1302.51x + 365.35$	5.0 ~ 200	0.9937	0.30	1.0
AZ-A	$Y = 991.824x + 220.27$	5.0 ~ 200	0.9974	0.30	1.0
AZ-B	$Y = 2752.51x + 594.03$	5.0 ~ 200	0.9928	0.30	1.0
AZ-C	$Y = 267.59x + 95.85$	5.0 ~ 200	0.9953	0.30	1.0

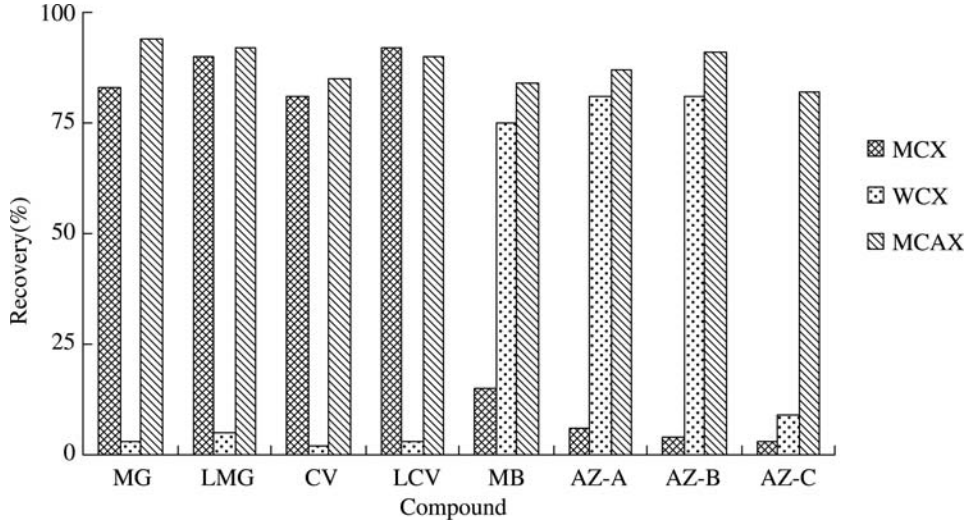

Figure 2. Recoveries of eight analytes by different SPE cartridges.

reagent can form ion pairs with the analytes, which were easily extracted by organic solvent. Hydroxylamine hydrochloride can protect LMG and LCV from oxidation. Both can enhance the extraction efficiency and improve the analyte recoveries. In liquid–liquid partitioning, water can prevent delamination in disorder. Diethylene glycol used as emulsifying agent can improve partitioning and protect from boiling in the later evaporation process. Acetonitrile in the second step of partitioning can not only prevent from delamination in disorder, but also separate protein for certain purification. It was also found that evaporation to dryness could lead to a relatively lower recovery, so evaporating the analytes to 0.5 mL before the SPE process and to near dryness after the SPE process could make better recoveries.

SPE optimization

According to the properties of the targets in the study, MCX (strong cation exchange cartridge), WCX (weak cation exchange cartridge) and MCAX (C8 and cation exchange compound cartridge) were selected for further purification. It was found that MCX was too strong adsorption for MB, AZ-A, AZ-B and AZ-C to elute and WCX had such a weak adsorption for MG and LMG that it led to a lower recovery for them in the elution process. However, MCAX gave easy elution and better adsorption for all the targets and, causing a better recovery, so we chose MCAX in the experiment. The recoveries of eight analytes by different SPE cartridges are shown in Figure 2.

Elution optimization

In the elution process, formic acid can sharpen elution of all targets, but can cause MG, LMG, CV and LCV to be easily decomposed in the nitrogen blowing process. It was found that dichloromethane, methanol and ammonia (50:50:5, v/v/v) can only elute MG, LMG, CV and LCV. Dichloromethane, methanol and formic acid (50:50:1, v/v/v) can be used for elution of MB, AZ-A, AZ-B and AZ-C, so dichloromethane, methanol and ammonia (50:50:5, v/v/v) were used for elution of MG, LMG, CV and LCV and dichloromethane, methanol and formic acid (50:50:1, v/v/v) were later used for MB, AZ-A, AZ-B and AZ-C.

Table II
UPLC–MS-MS Parameters for Eight Analytes in Positive Electrospray Ionization Mode*

Compound	Parent ion (**m/z**)	Daughter ion (**m/z**)	Cone Voltage/V	Collision Energy/eV
MG	329.2	**313.2**	30	34
		208.1	30	36
LMG	331.0	**239.1**	30	30
		316.2	30	20
CV	372.2	**356.2**	30	40
		340.2	30	50
LCV	374.3	**238.2**	30	26
		358.4	30	26
MB	284.2	**268.1**	50	40
		252.2	50	50
AZ-A	255.8	**213.7**	45	30
		198.8	45	40
AZ-B	269.8	**253.7**	40	28
		227.8	40	30
AZ-C	241.9	**199.8**	38	33
		226.8	38	26

*Numbers in bold are quantification ions.

Redissolved solution optimization

MG and CV are weak cationic compounds, and methanol used as redissolving solution can make them decompose easily, but acetonitrile had no effect on their stability. Therefore, acetonitrile was used as redissolved solution for MG, LMG, CV and LCV and ammonium acetate, acetonitrile and formic acid (80:20:0.1, v/v/v) were used for MB, AZ-A, AZ-B and AZ-C.

Optimization of LC–MS-MS

Each tuning solution was introduced into the electrospray source by direct infusion (10 μL/min). The main ions produced in MS and MS-MS were identified in positive ionization modes. The diagnostic fragment ions were selected and all

mass spectrometry parameters were optimized to increase sensitivity. Table II shows the parent and daughter ions for each compound as well as the optimum values of MS-MS parameters: voltage of the first quadrupole for isolation of the parent ion and collision energy for efficient fragmentation. In the study, two daughter ions were routinely monitored. This fulfils the recommendations of the European Union concerning identification, because two multiple reaction mode transitions from the ionized molecule of the target compound gave four points in the scale—a value regarded as sufficient for unequivocal identification. The commonly used mobile phase compositions such as acetonitrile–water and methanol–water were also optimized. In terms of ion response, acetonitrile–water was a better choice.

Matrix effect

Matrix effect is a special phenomenon associated with LC–MS-MS determination of analytes from biological samples such as aquatic products. Components extracted along with analytes from aquatic products may suppress or enhance ionization of the analytes in electrospray source if they co-elute with the analytes from the LC column. Matrix effect may impair accuracy and reproducibility. For this reason, matrix effect was evaluated under the experimental conditions. It was evaluated by comparing the peak area of analytes dissolved in the reconstituted solution of blank sample with dissolved mobile phase. If the ratio was to be $<85\%$ or $>115\%$, standard solution diluted by the matrix blank that was operated under the same process as the samples was adopted to calculate the concentration of the drugs. If it was of $85 \sim 115\%$, the matrix effect was absent.

Limit of quantification and linearity

The limits of detection (LODs) were calculated and defined as signal-to-noise ratio of 3:1 and they were obtained from blank samples spiked with 0.75 mL of 1.0 ng/mL of MG, LMG, CV and LCV and 150 μL of 10 ng/mL MB, AZ-A, AZ-B and AZ-C. The limits of quantification (LOQs) were also calculated and defined as signal-to-noise ratio of 10:1, which were the lowest concentration of the analytes that could be quantificationally determined with accuracy. LOQs were obtained from blank samples spiked with 250 μL of 10 ng/mL of MG, LMG, CV and LCV and 0.50 mL of 10 ng/mL MB, AZ-A, AZ-B and AZ-C. The calibration curves obtained were suitable for the quantification of all the analytes in the sample during the intra-day and inter-day validations and stability tests, and were suitable on the day of analysis. According to the concentration factor in the process of preparation and purification of samples, we achieved the LODs and LOQs, as shown in Table II.

Accuracy, precision and recovery

Confirmation of the analytes in aquatic products was performed using criteria of two ion transitions and LC retention time of each compound. Specifically, the relative intensities of two major daughter ions of given analytes were unique and did not appreciably change over the concentration range tested; therefore, they were used for confirmation of the presence of

Table III
Results for Repeatability and Within-Laboratory Reproducibility of the Eight Analytes in Silver Carp

Analyte	Spiked level(μg/kg)	Accuracy							
		Assay 1 Repeatability		Assay 2 Repeatability		Assay 3 Repeatability		Within-laboratory reproducibility	
		Mean (%)	RSD (%)*	Mean (%)	RSD (%)*	Mean (%)	RSD (%)*	Mean (%)	RSD (%)†
MG	0.5	90.2	7.69	86.5	6.41	87.6	6.78	88.1	6.82
	1.0	83.4	8.50	88.3	7.37	86.8	4.69	86.1	7.03
	10.0	85.2	12.1	81.7	7.83	84.1	6.18	83.6	8.73
LMG	0.5	89.0	7.93	86.5	7.11	86.1	1.73	87.2	6.09
	1.0	87.8	7.67	85.4	5.22	87.6	5.38	86.9	5.98
	10.0	88.2	5.67	82.5	3.03	86.2	1.84	85.6	4.64
CV	0.5	77.0	9.81	78.4	4.33	83.8	10.4	79.7	9.00
	1.0	75.5	4.49	79.2	10.7	82.6	10.1	79.1	9.28
	10.0	76.3	6.71	80.0	9.66	81.9	6.91	79.4	8.12
LCV	0.5	76.5	7.39	78.0	8.18	80.9	10.2	78.5	8.55
	1.0	78.5	6.84	78.5	9.44	82.4	6.49	79.8	7.59
	10.0	79.0	6.39	79.7	10.6	85.9	5.38	81.5	8.22
MB	1.0	84.7	8.62	85.3	8.37	80.8	4.99	83.6	7.55
	5.0	83.5	6.18	83.3	6.11	87.8	5.20	84.9	6.00
	10.0	78.8	5.44	75.7	5.66	75.4	10.0	76.6	7.17
AZ-A	1.0	79.2	8.10	83.8	9.65	84.6	4.36	82.5	7.80
	5.0	79.4	8.36	80.7	6.13	76.2	3.41	78.8	6.45
	10.0	77.2	6.60	79.8	7.71	81.2	6.98	79.4	7.04
AZ-B	1.0	81.0	6.10	85.3	7.75	77.9	7.68	81.4	7.80
	5.0	74.5	8.79	75.7	10.2	79.6	5.84	76.6	8.40
	10.0	75.7	5.14	77.0	8.16	79.6	5.84	77.4	6.49
AZ-C	1.0	75.8	10.3	74.8	10.2	78.1	14.8	76.3	11.5
	5.0	71.2	7.39	72.3	8.82	74.1	9.97	72.5	8.46
	10.0	72.5	5.84	71.5	9.05	74.8	7.35	72.9	7.31

*RSD represented as repeatability ($n = 6$).
†RSD represented as within-laboratory reproducibility ($n = 18$).

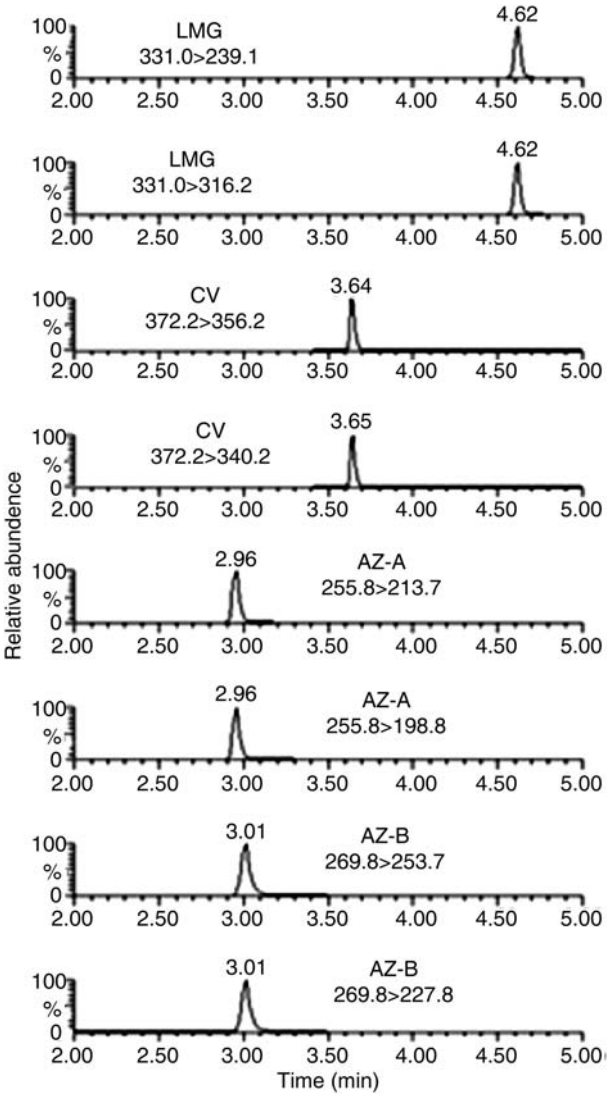

Figure 3. Reconstituted ion chromatograms of the analytes in samples. Ion peak areas of each analytes as follows: LMG (331.0 > 239.1) 275,798; LMG (331.0 > 316.2) 104,532; CV (372.2 > 356.2) 195,438; CV (372.2 > 340.2) 59,869; AZ-A (255.8 > 213.7) 8,328; AZ-A (255.8 > 198.8) 6,376; AZ-B (269.8 > 253.7) 40,073; AZ-B (269.8 > 227.8) 12,007.

the analytes in the samples. Variation of the relative ion intensities within 20% is usually acceptable for confirmation purposes. Intra-day and inter-day accuracy and precision were obtained from three analytical runs on separate days. Each contained six replicates of each QC concentration. The intra-day assay precision (relative standard deviation; RSD) values for QC samples were between 1.73 and 14.8%, and inter-day values were between 4.64 and 11.5%. The average recovery of the method was within 15%. The data obtained from silver carp samples for the eight analytes were within acceptable limits stated for bioanalytical method validation, as shown in Table III.

Stability

Preliminary studies showed that the eight analytes were stable under the conditions used in this work. Freeze–thaw, reinjection and long-term stability were evaluated and the results were satisfactory. The analytes were stable in samples at room temperature for 4 h, three freeze-thaw cycles in 48 h and in samples after freezing at −20°C for 15 days. The stability of processed samples was determined at 10°C in an autosampler for 16 h. The analytes did not degrade at these conditions, showing recovery between 70.3 and 87.2%. The analytes were considered stable in the matrix when an average of 80% of the initial concentrations was found.

Application

The method was successfully applied to determining the eight analytes in aquatic products, for example, silver carp. The results were calculated as a graph of peak area versus analyte concentration, and the recoveries for the eight analytes were between 70 and 85%. We also detected the eight analytes from five fish (including carp, crucian, tilapia, mandarinfish and bream), *penaeus vannamei*, *penaeus chinensis*, sea cucumber and seashell, which were obtained from a local market. No compounds were detected in carp, crucian, *penaeus vannamei*, *penaeus chinensis*, sea cucumber or seashell. We detected 16.3 μg/kg of LMG in bream, 7.15 μg/kg of CV in tilapia, and 1.62 μg/kg of AZ-A and 3.27 μg/kg of AZ-B in mandarinfish. The reconstituted ion chromatograms of the analytes in these samples are shown in Figure 3. Interfering peaks were observed at the retention time for AZ-C transitions, but upon quantification were so low as to be of little significance.

Conclusions

A method has been proposed allowing identification and quantification of MG, CV MB and their corresponding metabolite residues LMG, LCV, AZ-A, AZ-B and AZ-C in aquatic products at low μg/kg levels. Compared to the traditional LC, UPLC has a very short single run time of 4.5 min per sample, which makes it an attractive procedure for analysis of the residues, and no method has yet been published for the simultaneous determination of the eight analytes. According to the properties of each analyte, we adopted the same extraction method, but changed the elution solution in the SPE process and redissolved solution, and introduced it into the LC column for simultaneous determination in the end. The method is economical with time, reagent and energy. It meets not only the required sensitivity of the MRPL of 2 μg/kg for the sum of MG and LMG, but is also a useful method for determination of CV, LCV, MB, AZ-A, AZ-B and AZ-C. In short, the method shows good sensitivity, linearity, precision and accuracy and it is also very useful for the determination of MG, LMG, CV, LCV, MB, AZ-A, AZ-B and AZ-C residues in aquatic products.

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