Journal of Chromatography B, 913–914 (2013) 123–128

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of malachite green, crystal violet and their leuco-metabolites in fish by HPLC–VIS detection after immunoaffinity column clean-up

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ARTICLE INFO

Article history:
Received 31 August 2012
Accepted 4 December 2012
Available online 10 December 2012

Keywords:
Malachite green
Crystal violet
Leuco metabolites
Immunoaffinity column
High performance liquid chromatography

ABSTRACT

A high performance liquid chromatography method with visible detection (HPLC–VIS) for the determination of malachite green (MG), crystal violet (CV), leucomalachite green (LMG), and leucocrystal violet (LCV) in fish has been developed after clean-up through an immunoaffinity column (IAC). Residues were simultaneously extracted from fish muscle with acetonitrile and ammonium acetate buffer. The leuco-forms, LMG and LCV, were oxidized quantitatively to the chromic CV and MG by reaction with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. Extracts were then purified on an IAC which prepared by immobilizing the anti-MG–CV antibodies by the sol–gel method. Finally, the eluents were analyzed by HPLC–VIS. The limits of detection were 0.15, 0.1, 0.18 and 0.14 ng/g for MG, CV, LMG and LCV, respectively. The average recoveries in samples fortified with MG, CV, LMG and LCV over the range 0.5–10 ng/g were from 71.6% to 96.8% with RSDs of 5.1–12.3% ($n = 6$). This novel method was confirmed by liquid chromatography–tandem mass spectrometry with electrospray interface in positive mode using multiple reaction monitoring.

1. Introduction

Malachite green (MG) and crystal violet (CV, also known as gentian violet) are both triphenylmethane dyes. They are highly effective against protozoal and fungal infections as well as skin flukes and gill flukes, and therefore, extensively used as biocide in the aquaculture industry in the past [1,2]. When MG and CV are readily absorbed by fish, the main part of them is metabolically reduced to the lipophilic leucomalachite green (LMG) and leucocrystal violet (LCV) [3]. Because of potential hazards on human health and environment, MG, CV, and their leuco-metabolites, LMG and LCV, have been prohibited to be used in aquatic products for human consumption in many countries, including the United States, Canada, China and the European Union. It has prompted the development of a control programme that necessitated robust and reliable analytical method for the determination of MG, CV, LMG, and LCV residues in aquatic products.

Numerous methods have been reported for the detection of MG, CV, LMG and LCV residues in aquatic products, including enzyme-linked immunosorbent assay (ELISA) [4–6], spectrophotometer [7], gas chromatography–mass spectrometry (GC–MS) [8], high performance liquid chromatography (HPLC) [9–16] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [17–20]. ELISA can give quick and sensitive result, and a few successful attempts which used ELISA with polyclonal antibodies to detect triphenylmethanes [4–6] have been reported. However, ELISA often shows false positive result. And the GC or GC–MS analysis with derivatization is more tedious and time-consuming. Although the ability to detect MG, CV and their leuco-metabolites at regulated levels has been dramatically developed by the application of LC–MS/MS, the costly price of equipment blocks its universal use. MG and CV have strong visible absorption in the range of 580–620 nm. Moreover, HPLC–VIS has become the preferred method for the detection of MG and CV because of its high sensitivity, better quantitation ability, and less interferences. These are the reasons why HPLC method is still widely used.

Since the matrix of the aquatic samples is complicated, it is very important to develop effective pretreatment procedures to enrich analytes and minimize the matrix interference before trace analysis. The most common methods for the extraction of MG, CV, LMG, and LCV residues from aquatic samples have been performed using organic solvents (methanol or acetonitrile) with aqueous buffer [21–25]. For further clean-up of these sample extracts,

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http://dx.doi.org/10.1016/j.jchromb.2012.12.002

liquid–liquid partition (LLP) combined with solid phase extraction (SPE) [26–28] and a modified QuEChERS extraction [29,30] have been developed in this progress. Nevertheless, SPE techniques often provide nonspecific retention and have the pitfall of requiring a lot of organic solvent and elaborate sample pretreatment. In contrast, the immunoaffinity column (IAC), which is based on the highly specific interaction between antigen and antibody, is a good alternative method to clean up samples. Because of higher selectivity and specificity compared with other clean-up columns, IAC can bring generally cleaner extracts, smaller variability between samples, and make the chromatograms to be free from matrix interferences [31–36]. Moreover, IAC method is very efficient, simple and environmentally friendly as no toxic solvents are used. It has been applied in environmental monitoring [37], pharmaceutical and biomedical analysis [38,39], as well as food analysis [40]. To the best of our knowledge, the IAC clean-up has not been used for the determination of MG and CV with HPLC method.

The aim of this study was to develop a novel IAC–HPLC–VIS method with high selectivity, sensitivity, and low cost to detect MG, CV, LMG, and LCV residues in fish samples after pre-column oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and IAC cleanup procedure. Several important parameters affecting the IAC purification efficiency such as sample volume, sample flow rate, and eluent flow rate were studied. And this method was also confirmed by LC–MS/MS.

2. Experimental

2.1. Chemicals and materials

MG, CV, LMG and LCV were purchased from Sigma–Aldrich (St. Louis, MO). MG as the oxalate salt dimers (CAS 2437-29-8; FW = 929.0; 99.3% purity); CV as the chloride salt (CAS 548-62-9; FW = 407.98; >99.9% purity); LMG (CAS 129-73-7; FW = 330.48; >99.9% purity); LCV (CAS 603-48-5; FW = 373.53; 99.9% purity). Their stock solutions were prepared individually at a concentration of 100 mg/L in methanol, and stored in low-actinic glassware protected from light at −20 °C (stable for at least 3 months). All working solutions were prepared by serial dilution of the stock solutions with methanol every month and stored at 4 °C.

Acetonitrile and methanol were chromatographic grade obtained from J.T. Baker. Alumina B (basic alumina, pH 10, chromatographic grade, 80–200 mesh) was obtained from Sinopharm Chemical Reagent Co., Ltd. DDQ (98% purity), ammonium acetate buffer, acetonitrile, ammonia chloride, hydroxylamine hydrochloride (HAH), p-toluene sulfonic acid (p-TSA), acetic acid and dichloromethane were analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. Ultra pure grade water from a Millipore Milli-Q system was used throughout. A 1.0 mol/L of DDQ stock solution was prepared by dissolving 2.27 g of DDQ in 10 mL of acetonitrile and stored at 4 °C for up to 1 month. A 0.1 mol/L of DDQ working solution of DDQ in acetonitrile was stored at room temperature and prepared fresh each week. The IAC contains 1 mL of gel combined with MG–CV antibody was provided by Clover Technology Group. The samples of grass carp were purchased from a local market.

Table 1
LC gradient profile for determination of MG and CV.

Time (min)	Flow rate (ml/min)	Component A (%)	Component B (%)	Curve
0.0	0.500	20.0	80.0	
0.3	0.500	20.0	80.0	6
2.5	0.500	68.0	32.0	6
3.0	0.500	95.0	5.0	1
4.0	0.500	20.0	80.0	1

2.2. LC–VIS conditions

The HPLC system consisted of a Waters pump, a stainless-steel column (Cloversil-C_{18} 4.6 mm × 250 mm I.D, particle size 5 μm), and an ultraviolet detector. The mobile phase was acetonitrile: 0.1 mol/L ammonia chloride (7:3 v/v, pH 4.5). The column temperature was 35 °C. The flow rate was 0.8 mL/min and visible detector was set at a wavelength of 588 nm. The injection volume was 50 μL.

2.3. LC–MS/MS conditions

A gradient LC system (Table 1) using acetonitrile (mobile phase A) and 0.1% formic acid solution (mobile phase B) at a flow rate of 0.500 mL/min, was used to separate the analytes on a Waters BEH column (C_{18}, 2.1 mm × 50 mm, 1.7 μm particle size). The column temperature was 35 °C. The injection volume was 10 μL. The analysis was performed using positive-ion electrospray interface (ESI^+) with multiple reaction monitoring (MRM) mode. Interface conditions were as follows: capillary voltage was 3.5 kV; source temperature was 150 °C; desolvation temperature was 350 °C; the flow rates of cone and desolvation gas (nitrogen) were 100 L/h and 800 L/h, respectively; collision gas was argon; parameters were shown in Table 2.

2.4. Sample preparation

Fresh fish muscles were cut into 3–5 cm cubes, blended and stored at −20 to −30 °C. The samples with no detectable residues of the analytes confirmed by LC–MS/MS were used as negative controls. Then the negative samples spiked with MG, LMG, CV, and LCV standard solutions prior to extraction were used as positive controls.

2.5. Extraction

Into a 50-mL polypropylene capped centrifuge tube, were added 10.0 g of thawed fish sample, ammonium acetate buffer (5 mL, 0.1 mol/L, pH 4.5), HAH solution (1 mL, 0.25 g/mL), p-TSA solution (100 μL, 1 mol/L), and acetonitrile (15 mL) in turn. The mixture was homogenized for 1 min. Then, acetonitrile (10 mL) and alumina (10 g) were added and the mixture was shaken periodically for 30 min followed by centrifuging at 5000 rpm for 5 min at 0 °C. The supernatant was decanted into a 200-mL separatory funnel. A second portion of acetonitrile (25 mL) was added to the solids remaining in the centrifuge tube, this extraction step was repeated again. The supernatant was decanted into the separatory funnel containing the first extract. The resulted supernatant was liquid–liquid extracted twice, each time with dichloromethane

Table 2
MS/MS parameters for determination of MG and CV.

Compound	Precursor ions	Product ions	Capillary voltage (V)	Cone voltage (V)	Collision (eV)
MG	329.415	208.161 (strong)	3500	55	33
		165.078 (weak)	3500	55	30
CV	372.479	340.324 (strong)	3500	55	25
		235.269 (weak)	3500	55	16

(25 mL). The lower dichloromethane layer was collected into a 200-mL pear-shaped boiling flask. By heating in a water bath set at 50 °C, the contents of the boiling flask were rotoevaporated just to dryness under reduced pressure, resulting an oily residue.

2.6. Oxidation

Acetonitrile (1980 μL) was added to the above mentioned oily residue and swirled to dissolve. DDQ solution (20 μL, 0.1 mol/L) was added and swirled to mix. The oxidation reaction was allowed to proceed for 30 min with periodic sample agitation.

2.7. IAC clean-up

For sample clean-up, the oxidized solution was diluted to 10 mL with pH 7.4 phosphate buffer solution (PBS), then passed through an IAC with a flow rate of 1 mL/min to remove most of the interferences resulted from the fish matrixes. The column was washed with water (10 mL) and eluted by acetonitrile (700 μL). The obtained elution was mixed with ammonia chloride (300 μL, 0.1 mol/L, pH 4.5), which was finally subjected to HPLC–VIS determination, and LC–MS/MS confirmation.

3. Results and discussion

3.1. Optimization of extraction

Sample extraction is always a crucial step in residue analysis, because the matrix of the aquatic samples is very complicated. Most methods currently used are based on the solvent extraction of MG, CV, and their leuco-metabolites from aquatic samples using acetonitrile or methanol with aqueous buffer [11–16,21–28]. Andersen et al. [11] developed a method for the simultaneous extraction of MG ± LMG, CV ± LCV and BG ± LBG, in catfish samples, using acetonitrile: 0.1 mol/L ammonium acetate buffer (10:1, v/v) with recoveries 90.6% for LCV, 89% for LMG, 84.4% for CV, and 64% for MG. Andersen et al. also reported that real samples were found to have CV in the range of 0.4–0.8 ng/g. The extraction method used in this study is same as the procedure previously described by Andersen et al. [11–14]. Therefore, acetonitrile–ammonium acetate buffer was selected as the extraction solvent of extracting MG, CV, LMG, and LCV from the homogenized fish samples.

As previously reported [11,12,14], it was found that the alumina clean-up of matrix interferences and fats was especially important for the extraction step because LMG and LCV are lipophilic and can be stored in tissues. In this study, different amounts of alumina (5, 6, 7, 8, 9, 10, and 15 g) were used in the initial extraction. The results indicated that 10 g of alumina was the most sufficient amount for the extraction of MG, CV and their leuco-metabolites for 10.0 g of the grass carp sample.

3.2. Optimization of oxidation

This method requires the oxidation of the colorless LMG and LCV to MG and CV, which can be captured by IAC loaded with MG–CV antibodies. Batch experiments were carried out to find out the optimal amount of DDQ used in the oxidation step. The results showed that addition of 20 μL of DDQ (0.1 mol/L) to the fish extracts provided quantitative conversion of LMG and LCV to MG and CV. The ratio of conversion was higher than 99%. The DDQ oxidation, therefore, provided efficient leuco-form conversion, eliminating the need for post-column lead oxide oxidation.

Table 3
The optimized conditions for IAC of the MG and CV.

Optimum IAC conditions	
Loading buffer	pH 7.4 PBS–acetonitrile (80:20, 10 mL)
Loaded amount bound	250 ng
Flow rate	1 mL/min
Washing solution	Pure water (10 mL)
Eluted solution	700 μL of acetonitrile (add 300 μL pH 4.5 solution of ammonia chloride)

3.3. Optimization of the IAC clean-up

In the clean-up procedure for triphenylmethane dyes residue analysis, SPE is the most commonly used technique [10–14,20–27]. Methods reported cleaned up tissue extracts directly using Bakerbond™ strong cation exchange (SCX) [3], Bondesil-NH_2 [16], or C_{18} [24] SPE cartridges. Swarbrick et al. [15] also used activated charcoal to clean up extracts instead of C_{18} SPE. Compared with SPE, the IAC has higher selectivity and specificity [31–36], which is based on the highly specific interaction between antigen and antibody. Moreover, up to now, no paper has been published concerning the use of IAC clean-up for the determination of MG and CV in aquatic samples.

Several conditions which have a strong influence on the association and dissociation of antigen–antibody complex with the IAC were necessary to be optimized. The optimized conditions were shown in Table 3.

3.3.1. Capacity determination of IAC

The column capacity was determined by loading 1000 ng of MG and 1000 ng of CV mixed standard solution at a flow rate of 1.0 mL/min under optimized loading, washing, and elution conditions. As shown in Fig. 1, the maximum binding capacity of the IAC for MG–CV was found to be 250 ng of the sum of MG and CV. Above this level (250 ng) no increase of the response was observed, indicating the saturation of MG–CV binding sites. MG–CV recoveries from the column below the saturation level were higher than 90%.

The reusability of the IAC was evaluated by 10 cycles of use in 30 days. Although the column capacity gradually decreased as the cycles increased, the recoveries of MG, CV, LMG and LCV had no significant change. And, the capacity of the IAC was still greater than 205 ng/mL after being used for 10 cycles.

3.3.2. Loading conditions

In order to study whether the loading medium had an effect on the recoveries of the analytes, a set of PBS (pH 7.4) containing different ratios of acetonitrile (0:100, 5:95, 10:90, 15:85, 20:80, 25:75, 30:70, v/v) was investigated. The results (shown in Fig. 2) demonstrated that the recovery increased gradually when the ratio of acetonitrile was increased from 0 to 20%, and then decreased

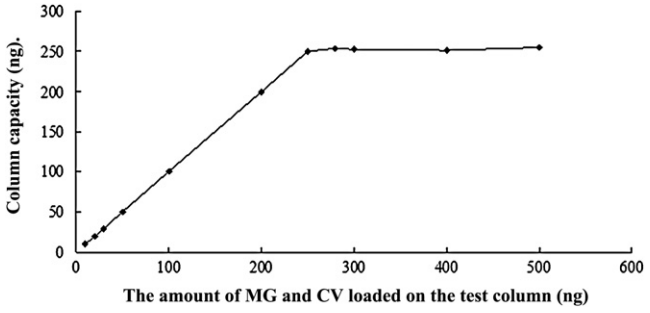

Fig. 1. Binding performance of antibodies used in the test immunoaffinity columns.

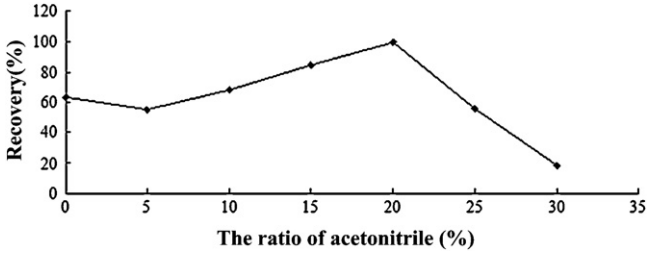

Fig. 2. The loading curve of MG and CV on an IAC with different ratios of pH 7.4 PBS–acetonitrile.

sharply as the ratio increased further. Thus pH 7.4 PBS containing 20% acetonitrile was selected as the loading medium.

The different volumes of loading solution (1, 2, 5, 10, 15, and 20 mL) on recovery were also investigated. Increasing in the volume of loading solution from 1 to 10 mL resulted in the increase of average recoveries from 51.6 to 94.0%. Further increase of the loading solution did not significantly improve the recoveries, thus 10 mL of loading solution was chosen as the optimum loading volume.

3.3.3. Flow rate conditions

In this study, a decrease in the loading flow rate caused an increase in the recoveries of the analytes. However, the recoveries at gravity-flow rate of 0.2–1.0 mL/min had no significant difference, thus the flow rate was set at 1.0 mL/min, at which satisfactory recoveries for the target analytes were obtained.

3.3.4. Washing conditions

The target analytes in samples could be selectively adsorbed by specific antibodies immobilized on the sol–gel IAC. However, the interferents in the matrix may also be retained on the sol–gel column because of nonspecific absorption, and could be largely removed by a washing procedure. Therefore, various washing buffers (water, PBS, and different ratios of PBS–acetonitrile) were tested in the washing step. The results indicated that there were no significant differences among these washing buffers, thus sufficient pure water was subsequently used as washing solution to remove the interference.

3.3.5. Elution conditions

In this study, the recoveries of MG and CV were found to be decreased dramatically from 97.5% to 66.1% when the eluent was evaporated to dryness at 50 °C, which may be due to the decomposition of MG and CV under such conditions. If 700 μL of acetonitrile was used to elute the analytes from the IAC and the obtained eluent was mixed with 300 μL of 0.1 mol/L ammonia chloride (pH 4.5) via vortex, then a portion of the mix solution was directly injected into the HPLC system for analyses, well-shaped chromatographic peaks (Fig. 3A) and good recoveries (Table 4) were obtained. Other eluent

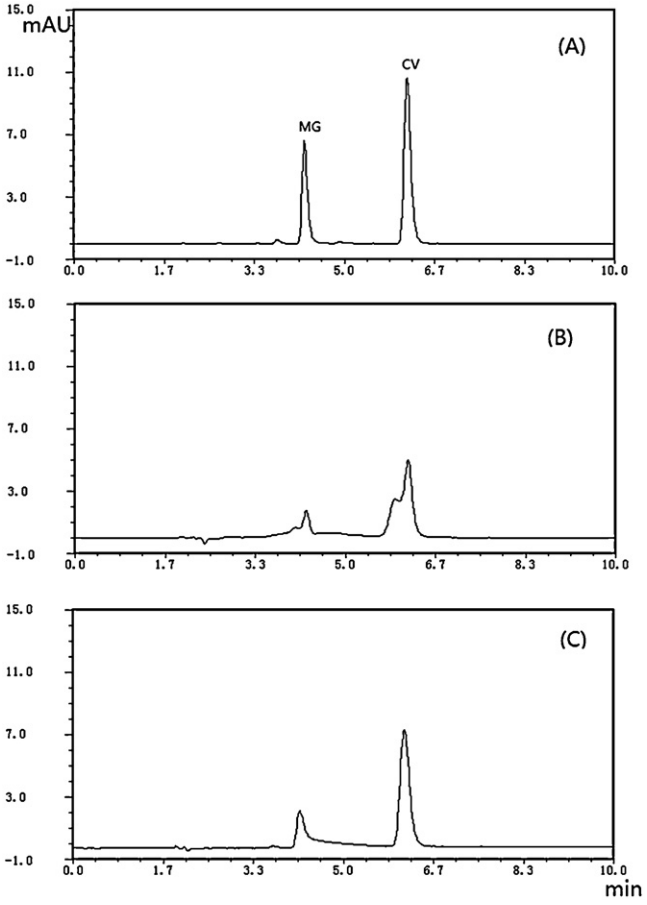

Fig. 3. The chromatograms of malachite green and crystal violet: (A) elute with 700 μL of acetonitrile and then mix with 300 μL of 0.1 mol/L ammonia chloride (pH 4.5); (B) elute with 1 mL of methanol; (C) elute with 1 mL of acetonitrile; the spiked concentration was 5 ng/g of MG and 5 ng/g of CV.

Table 4
Recoveries and precision (RSD) of the fish samples fortified with MG, CV, LMG, and CV ($n = 6$).

Analyte	Fortification level (ng/g)	Accuracy							
		Day 1 repeatability		Day 2 repeatability		Day 3 repeatability		Within-laboratory reproducibility	
		Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)
Control grass carp	0	ND	ND	ND	ND	ND	ND	ND	ND
MG	0.5	85.7	6.3	91.5	10.9	79.7	5.9	85.6	12.3
	1.0	78.0	7.5	85.3	5.1	95.7	7.2	86.3	8.1
	1.5	88.7	5.4	72.4	6.7	94.9	8.8	85.3	9.7
LMG	0.5	71.8	10.9	91.6	10.4	77.9	5.3	80.4	6.3
	1.0	83.4	8.9	89.5	6.2	82.5	8.4	85.1	7.7
	1.5	95.8	7.3	93.9	7.7	80.1	6.1	89.9	8.0
CV	0.5	77.8	10.5	90.6	7.9	81.9	5.2	83.4	11.5
	1.0	83.5	6.8	89.6	8.3	83.6	7.4	85.6	9.1
	1.5	94.6	7.2	71.6	6.7	79.8	9.5	82.0	10.2
LCV	0.5	85.8	11.1	79.9	8.4	93.1	12.1	86.3	6.3
	1.0	90.1	9.3	73.6	7.3	96.8	9.8	86.8	7.6
	1.5	87.3	5.7	80.9	9.5	94.9	6.2	87.7	5.3

ND: not detected.

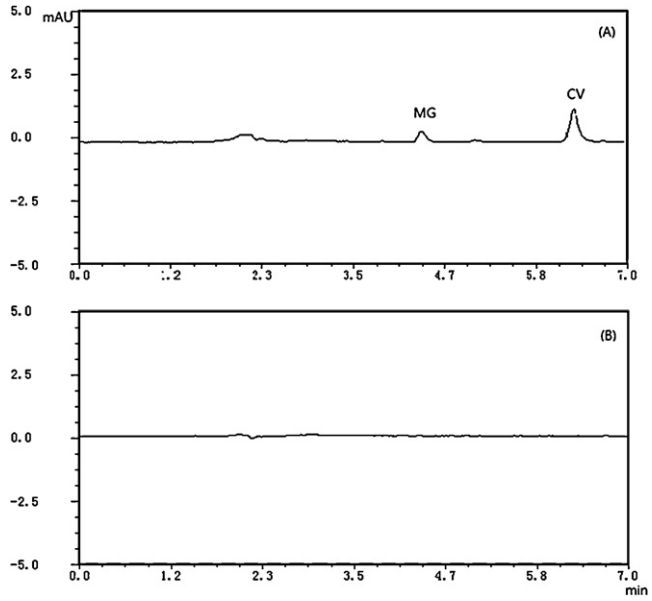

Fig. 4. Typical chromatograms of negative and spiked samples: (A) spiked samples were purified by IAC; (B) control samples were purified by IAC; the spiked concentration was 0.5 ng/g of LMG and 0.5 ng/g of LCV.

such as methanol or acetonitrile resulted poor chromatographic peaks (Fig. 3B and C).

3.4. Method validation

3.4.1. Linearity

The calibration curves for MG and CV were constructed by plotting the peak area (y) versus concentration (x) of each analyte which were expressed by the equation given as: $y = 277.87x - 268.17$ with a correlation coefficient (r^2) of 0.9998 and $y = 1077x - 1698.5$ with a correlation coefficient (r^2) of 0.9997 for MG and CV, respectively. The calibration curves were generated daily from the peak area responses of standards with concentrations ranging from 0.3 to 15.0 ng/mL.

3.4.2. Limit of detection (LOD) and limit of quantification (LOQ)

The negative samples were selected and spiked with the standard solution, then treated and analyzed following the method described above. The LODs and LOQs for MG and CV were obtained from the analysis of the negative samples spiking with MG–CV mixed standard solution. The LODs and LOQs for LMG and LCV were determined by spiking with LMG–LCV mixed standard solution. The LODs based on three times the signal to noise ratio were 0.15, 0.1, 0.18 and 0.14 ng/g and the LOQs based on 10 times the signal to noise ratio were 0.47, 0.31, 0.49 and 0.45 ng/g for MG, CV, LMG and LCV, respectively. Typical chromatograms are shown in Fig. 4 for grass carp fortified with 0.5 ng/g of LMG and 0.5 ng/g of LCV.

3.4.3. Accuracy and precision

Accuracy was expressed by the recovery. The mean recoveries of MG and CV residues in grass carp fortified with MG–CV mixed standard solution at the concentrations of 0.5, 1.0 and 1.5 ng/g were higher than 71.6%. The mean recoveries of LMG and LCV from grass carp fortified with 0.5, 1.0 and 1.5 ng/g of LMG–LCV mixed standard solution were in the range of 71.8–96.8%.

Precision was expressed as relative standard deviation (RSD). The results of intraday, interday and within-laboratory reproducibility for MG, CV, LMG and LCV were listed in Table 4. As shown in Table 4, the overall precision of the assay expressed as RSD was less than 12.3% in the fish samples.

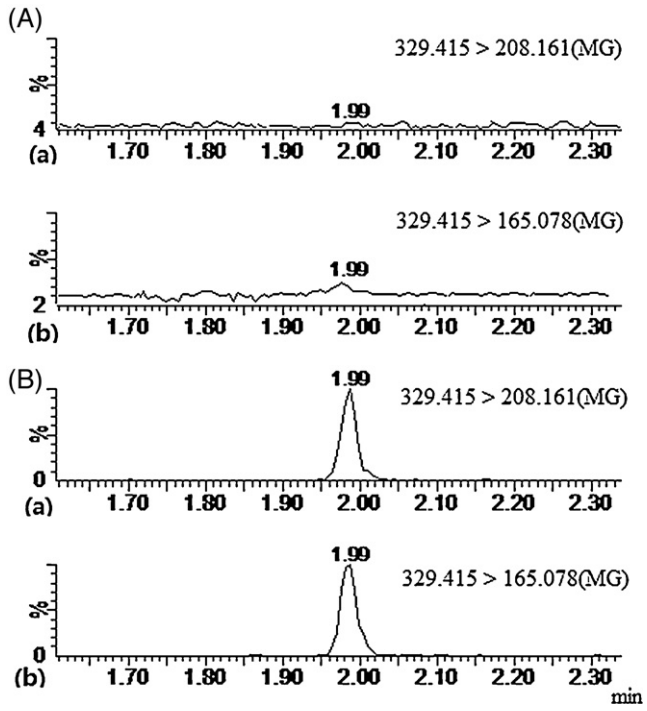

Fig. 5. LC–MS chromatograms and a spectrum of MG fortified grass carp samples. (A) Negative control fish (top); (B) negative control fish spiked with 0.5 ng/g of: (a) MG (strong transition) and (b) MG (weak transition).

3.5. Confirmation and application of the method

In current study, ESI^+ with MRM mode was used for the confirmation of the sample identification. The LC–MS/MS conditions were described in Section 2.3. The transitions m/z 329 → 208, 329 → 165 for MG, and m/z 372 → 340, 372 → 235 for CV, which were coincided with the results by Dowling et al. [3], were

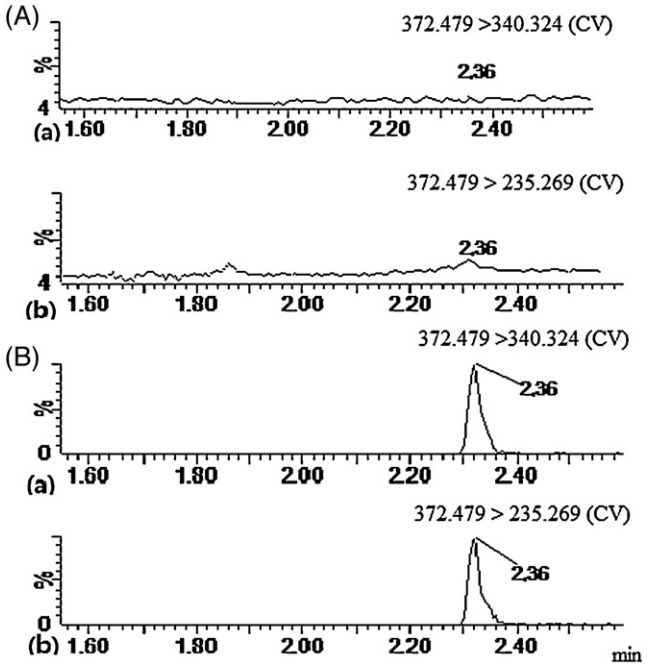

Fig. 6. LC–MS chromatograms and a spectrum of CV fortified grass carp samples. (A) Negative control fish (top); (B) negative control fish spiked with 0.5 ng/g of LCV: (a) CV (strong transition) and (b) CV (weak transition).

chosen for confirmation. The positive control sample fortified at 0.5 ng/g for MG, LMG, CV and LCV was confirmed by LC–MS/MS. And the MRM chromatograms of MG and CV were illustrated in Figs. 5 and 6. The developed method was applied to determine the residue of MG ± LMG and CV ± LCV in 20 grass carp samples purchased from the local supermarkets in Beijing (China). No target compounds were detected in these samples.

4. Conclusions

In this study, a sensitive, reproducible and accurate IAC–HPLC–VIS method was successfully developed for the first time for determination of MG, CV, and their leuco-metabolites in fish samples, which was also confirmed by LC–MS/MS. The leuco-metabolites, LMG and LCV, were converted to the chromic forms, MG and CV, by oxidation with DDQ. Then the sum of MG–LMG and CV–LCV were measured, and presented as the concentration of MG ± LMG and CV ± LCV, respectively. Compared with the clean-up procedure by traditional solid phase extraction, the IAC clean-up can give good purification effect and high sensitivity in HPLC–VIS analysis. Moreover, this method is more selective, environmentally friendly and simpler for the routine analysis of MG, CV and their leuco-metabolites in fish samples.

Acknowledgements

This study was supported financially by grants from the General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (201210086 and 201210016), and the Ministry of Science and Technology (2012BAD33B02). The authors would like to thank staff at Chinese Academy of Inspection and Quarantine and Clover Technology Group Limited for their practical assistance.

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