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Determination of the sum of malachite green and leucomalachite green in salmon muscle by liquid chromatography–atmospheric pressure chemical ionisation-mass spectrometry

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Abstract

A sensitive method for the determination and confirmation of the sum of malachite green (MG) and leucomalachite green (LMG) in salmon muscle has been developed. It is based on the use of an oxidative pre-column reaction which converts LMG into MG previous to liquid chromatography–atmospheric pressure chemical ionisation–mass spectrometry (LC–APCI–MS) analysis. The determination of both compounds together constitutes a good screening method to confirm the presence of this kind of residue, taking into account that the combined signals will provide a gain of sensitivity. The detection limit, determined for spiked salmon samples using the confirmatory ion *m*/*z* 313, was 0.15 μg/kg. The recoveries determined at a spiking level of 2 μg/kg were 85 and 70% for LMG and MG, respectively, with respective relative standard deviations of 1.3 and 3.1%.

Keywords: Malachite green; Leucomalachite green; Salmon

1. Introduction

Malachite green (MG) is a triphenylmethane dye used in the fish industry as an antifungal agent. Leucomalachite green (LMG) is formed by the metabolic reduction of malachite green and persists in the tissues of exposed fish (Fig. 1). The use of malachite green has been banned in different parts in the world, including Chile, because it has been demonstrated that these dyes are linked to an increased risk of cancer [1].

In view of the fact that the presence of MG and LMG in fish has been banned, any related enforcement is being focused on the detection of the lowest concentrations for both compounds. Liquid chromatography (LC) with visible detection has traditionally been used for their determination, providing detection limits around 2 μg/kg for each substance [2–4]. The current trend related to the analytical determination of these dyes is not only to decrease the detection limits but also to increase the confirmatory character of the analysis in order to provide a more confident regulation enforcement. As can be observed in the literature, the way to satisfy such a demand is the use of tandem MS systems [2] or the use of MS detection with isotopic dilution [5], which unfortunately are very expensive methodologies.

On the other hand, it has been observed that the leuco form of malachite green is the major component present in incurred catfish tissue [3] and the relative amounts of the two compounds depend on the metabolic elimination time. Additionally, it is important to point out that during sample analysis there is a redox conversion between MG and LMG [6,7]. In this context, the joint determination of both compounds constitutes a good screening method for these residues, the combined signals will provide a gain of sensitiv-

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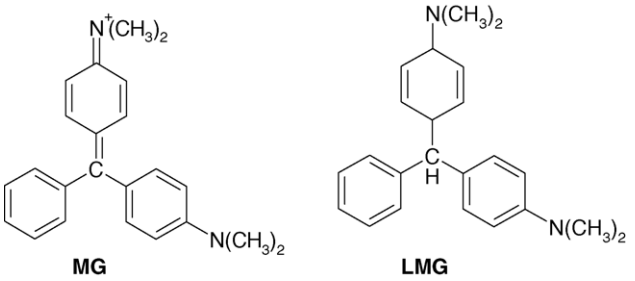

Fig. 1. Chemical structures of malachite green (MG) and leucomalachite green (LMG).

ity, allowing to use more simple equipment such as a single-quadrupole liquid chromatography–atmospheric pressure chemical ionisation–mass spectrometry (LC–APCI–MS) system.

The aim of this study was to develop a method for the quantification and confirmation of trace amounts of MG plus LMG in fish, based on the use of an oxidative pre-column reactor which converts LMG into MG, previous to LC–APCI–MS. The analytical signal corresponds to a single chromatographic peak obtained in the single ion recording mode.

2. Experimental

2.1. Reagents

All solvents were of HPLC grade and other chemicals were of analytical-reagent grade unless otherwise stated. Malachite green and leucomalachite green were obtained from Aldrich (Milwaukee, WI, USA). Standard solutions (1 mg/ml) were prepared in acetonitrile and were stable for at least 1 month at 4 °C. Intermediate standard solutions of 10 μg/ml were prepared daily by dilution with acetonitrile. Spiking solutions were prepared by dilution of the intermediate standards with acetonitrile. Calibration standards were prepared by dilution of the intermediate standard with 0.05 M ammonium acetate–acetonitrile (40:60, v/v).

Citric acid, sodium hydrogen phosphate, acetonitrile, ammonia, methanol, dichloromethane, ascorbic acid and acetic acid were obtained from Merck (Darmstadt, Germany).

1-Pentanesulfonic acid (sodium salt) (PSA) was delivered by Aldrich. A 1 mol/l PSA solution was prepared in water.

N,*N*,*N*′,*N*′-Tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD) was obtained from Aldrich. A 1 mg/ml TMPD solution was prepared in methanol.

McIlvaine solution pH 3 was prepared by mixing 18.9 ml 0.2 M sodium hydrogen phosphate and 81.1 ml 0.1 M citric acid. McIlvaine solution pH 6 was prepared by mixing 62.5 ml 0.2 M sodium hydrogen phosphate and 37.5 ml 0.1 M citric acid.

Aromatic sulphonic acid-bonded SPE columns (3 ml; 500 mg) were from J.T. Baker (Phillipsburg, NJ, USA). The solid phase extraction eluent was prepared just before use by mixing 2.5 ml 25% (*m*/*v*) ammonia, 2.5 ml 1.0 mg/ml methanolic ascorbic acid and 45 ml methanol.

The LC mobile phase was prepared by mixing 40 ml 0.05 M ammonium acetate pH 4.4 and 60 ml pure acetonitrile.

Celite (3.74 μm average size) and lead (IV) oxide (99.999%, 15 μm average size) were obtained from Aldrich.

2.2. Instruments and apparatus

The oxidative pre-column (4.6 mm × 20 mm) for the conversion of LMG into MG was hand packed with a mixture of 10% lead (IV) oxide and celite.

LC separation was carried out using an HPLC pump HP 1050 and a column (5 μm, 4.6 mm × 250 mm, Symmetry, Waters, MA,USA) at a flow rate of 0.7 ml/min with an isocratic mobile phase containing 60% (v/v) acetonitrile in aqueous ammonium acetate (50 mM, pH 4.4).

A Platform (Fisons Instruments, Altrincham, UK) single quadrupole mass spectrometer equipped with an APCI interface and heated nebulizer probe (500 °C) was used with an ion source temperature (150 °C). Positive ions were acquired in full scan (*m*/*z* 100–400) or selected ion monitoring (SIM, dwell time of 0.3 s, a span of 0.3 Da and an interchannel delay time of 0.01 s. A sample cone potential of 50 V was used in order to maximize formation of the specie derived from MG (*m*/*z* 329–313). No corona discharge potential was used.

2.3. General analytical procedure

Portions of about 4 g of previously homogenized salmon muscle were accurately weighed into 100 ml tubes. Fortified samples (1.0 μg/kg) were prepared by addition of appropriate amounts of LMG spiking solution to blank salmon samples. Extraction was based on the procedure described by Bergwerff and Scherpenisse [2], consisting of the following steps: McIlvaine buffer (pH 3, 4 ml), PSA solution (200 μl), TMPD solution (100 μl), and acetonitrile (25 ml) were added and the mixture was homogenized for 1 min using an ultra-turrax at 10,000 rpm. The suspensions were centrifuged for 5 min at 3400 rpm and the supernatant was decanted in a separatory funnel containing 12 ml of dichloromethane (DCM). The procedure was repeated, but now using McIlvaine buffer (pH 6, 4 ml) and acetonitrile (25 ml). The supernatant was decanted in the same separatory funnel. The funnel was shaken for approximately 1 min. After the layers were separated, the aqueous phase was removed and the organic layer centrifuged in order to eliminate water. The DCM extract was then passed through an SPE cationic cartridge. The cartridge was washed sequentially with 5 ml acetonitrile/DCM (2:1, v/v) and 5 ml methanol, and dried in a stream of nitrogen for 10 min. Analyte elution was carried out using 10 ml of eluent solution. The eluate was dried in a rotaevaporator at 50 °C. The residue was redissolved in 2 ml of mobile phase by vortex-mixing for 10 s and ultrasonication for 1 min and then transferred into

2-ml vials using a filter syringe (0.45 μm). Then a volume of 200 μl was subjected to LC. Under the selected conditions, MG plus LMG eluted together as MG at ca. 7.0 min. Just after elution of the analytes, the column was washed with pure acetonitrile for 15 min, in order to elute any non-polar traces present in the sample.

3. Results and discussion

An isocratic LC system (Fig. 2) was optimized to implement the method. The compounds MG and LMG present in the extract are injected and carried to the oxidative pre-column reactor, which converts LMG into MG. This latter compound is subsequently separated from the other components of the matrix in the LC column. Fig. 3 shows the signals obtained for both analytes by LC–APCI–MS.

The use of the oxidative reactor before the LC run allows both compounds, MG and LMG, to enter the LC column as MG. In this way, it is not possible to distinguish these analytes from each other in the sample. However, the convenience to merge both compounds into one signal is in the increase in the signal-to-noise ratio in order to detect smaller concentrations of banned fungicides.

Injections of LMG standards provides a signal at the same retention time as the signal observed for MG standards. The sensitivity of both signals were equivalent, which indicates that the oxidation in the reactor is quantitative.

3.1. Elution of MG and LMG from the SPE columns

A survey of the literature [2–4,8–10] shows that, previous to the LC run, analytical methods for MG and LMG involve a clean-up based on SPE (cation exchange resin) which basically allows to separate the analytes from low-polarity compounds in the matrix. The procedures use from 1 to 15 ml of eluting agent. However, none of these studies report on the elution pattern of both dyes from the columns. Fig. 4 shows the elution pattern of both compounds under the conditions described in this study. As can be seen, the required elution volume of MG and LMG should be over 6 ml. Taking this into account, a volume of 10 ml was selected for further studies.

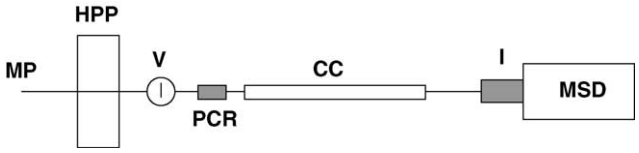

Fig. 2. Manifold for implementation of the method. MP, mobile phase; HPP, high pressure pump; V, injection valve; PCR, oxidative pre-column reactor; CC, chromatographic column; I, APCI interface; MSD, mass detector.

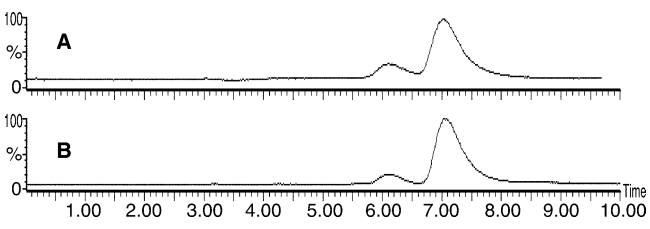

Fig. 3. Chromatographic response for MG (A) and LMG (B), both at 1.5 μg/kg.

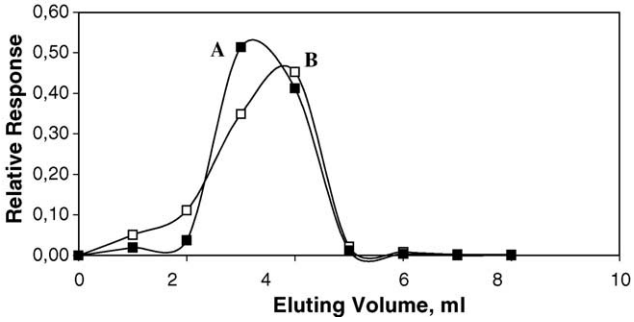

Fig. 4. Elution pattern of MG (A) and LMG (B) from the cation exchange cartridge used in the method. Both analytes were at 25 μg/kg concentration level.

3.2. Oxidative pre-column reactor

By using the manifold proposed in this study, in which the oxidative column reactor was located before the LC column, it was possible to detect that, in addition to the transformation of LMG into MG, the reactor produces the decomposition (oxidation) of MG to a fixed extent which depends on the amount of the PbO_2 in the reactor and on the residence time of the sample inside of the reactor. The detection of this decomposition product would not have been possible when the reactor was located after the LC column. The decomposition product of MG was detected as a peak which appeared about 1 min previous to the MG signal (Fig. 3) and was identified by LC–MS as desmethyl-MG (*m*/*z* 315), which can be used additionally as a confirmatory signal for the determination of MG or LMG. In a reactor containing celite/PbO_2 (50/50) the signal area ratio MG/decomposition product was 3, which increased to 30 when the PbO_2 mixed in the column decreased to 10%.

As can be seen in Fig. 3, the sensitivity of the signal is the same for both compounds, which means that conversion of LMG into MG in the pre-column reactor is quantitative. Regardless of whether the oxidation reaction of LMG occurs pre- or post-column, the sensitivity of the signal for this compound should be lower than or at most the same as that for MG, which is consistent with the signals observed in Fig. 3 (pre-column) and in the calibration equations (post-column) determined by Roybal et al. [8]. However, an opposite situation has also been reported [9] in which response for LMG is 1.5 times larger than that observed for MG.

The performance of the PbO_2 column in maintaining the oxidation of LMG was evaluated. Just as determined by Roybal et al. [8], after 60 injections, no loss of sensitivity was noted. In any case, it is recommended that the performance

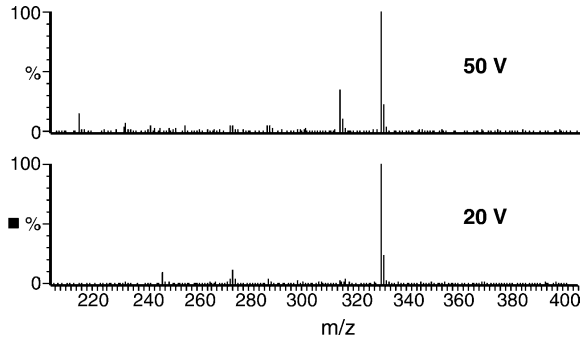

Fig. 5. Mass spectra for MG showing the effect of the cone potential as indicated.

of the system as a whole be periodically checked by injecting standards of both compounds every 20–30 injections. If the LMG response is <80% of the equivalent MG peak area response, the PbO_2 column should be refilled.

3.3. Robustness

The flow rate of the mobile phase was varied from 0.5 to 1.5 ml/min. The selected value of 0.7 ml/min allowed the quantitative conversion of LMG into MG, giving rise to equivalent signals, and a good resolution of MG from matrix signals. Higher flow rates decreased the sensitivity of the LMG signal and interferences with the matrix constituents became significant.

Injection volume was studied in the 50–200 μl range. As expected, the sensitivity of the signal increased with increasing injection volume. A large volume of 200 μl was selected taking into account both sensitivity and only a little degradation in performance.

As also observed by Doerge et al. [5], at cone potentials lower than 30 V the mass spectrum of MG consisted predominantly of the molecular species. When the potential was increased, there was increasing dissociation (Fig. 5). For confirmatory analyses using the SIM acquisition method, a sample cone potential of 50 V was selected because in this condition sensitivity of the target and qualifier ions are sufficiently high and comparable. The ion intensity ratio for ions 329 (target) and 313 (qualifier) in the standard was 3.0 ± 0.1. Consequently, as confirmatory criteria not only the retention time was used. In addition, the ratio between both ions in real samples should be in the 3.0 ± 0.3 interval. Additional confirmation by using more than the selected two ions was not practicable in the present study because the application of cone potentials higher than 50 V produces not only additional fragments but also a considerable decrease in the sensitivity of both target and confirmatory ions.

3.4. Mobile phase

When using the APCI interface, the intensity of the signal obtained for MG was found to diminished drastically when the mobile phase is enriched in acetonitrile, probably due to the lower activity of hydrogen ions required for appropriate chemical ionization [11]. The signal for MG eluted under the proposed conditions is approximately 20 times higher than that obtained for MG, coming from LMG after a post-column oxidation in a mobile phase containing 0.05 M ammonium acetate/acetonitrile (5/95). In the latter case, in addition to the lower extent of chemical ionization, the higher amount of acetonitrile would affect oxidation of LMG to MG in the column.

In the article Doerge et al. [5] it is possible to observe a similar effect to the one described here. These authors propose an LC–APCI–MS method which separately detects MG and LMG, the sensitivity observed for MG is 20 times higher than that for LMG, however the detection limit reached for the latter compound was 0.5 ng/ml by using isotopic dilution. Of course, this alternative is considerably more expensive than the method herein proposed.

Since LMG is not present during the LC run under the selected experimental conditions, cleaning of the LC column between samples is carried out by quickly increasing the acetonitrile proportion in the elution mixture. This results in considerable reduction in LC run time.

If the analytical problem necessarily involves the separate determination of MG and LMG, the present method can also be used. In this instance it would be necessary to carry out two injections, one with, and one without the reactor. The difference of both signals will give the LMG concentration.

3.5. Analytical features and application

Calibration curves were obtained by triplicate injection of different concentrations of MG and LMG. The following results were obtained:

$$\text{MG} \quad Y = 2.94X + 0.18; \quad \text{linear range} : 0 - 4.0\,\text{ng/ml}; \quad r = 0.9972$$

$$\text{LMG} \quad Y = 2.89X + 0.07; \quad \text{linear range} : 0 - 4.0\,\text{ng/ml}; \quad r = 0.9998,$$

where Y is the peak area and X the concentration in ng/ml.

As was stated above, the closely similar slopes confirm the quantitative performance of the oxidative pre-column reactor.

By using spiked salmon samples extracted under optimal conditions, the detection limits for MG and LMG were found to be 0.15 μg/kg, defined at a signal-to-noise ratio of 3 for the confirmatory ion m/z 313. The precision of the method was studied for samples spiked with 2.0 μg/kg of MG and LMG. The relative standard deviations of six determinations were 3.1 and 1.3% for MG and LMG, respectively.

The recovery of the method was calculated by spiking known negative salmon portions with 1.5 μg/kg of each compound. As already observed by Bergwerff and Scherpenisse [2], the best recoveries were observed with J.T. Baker Bond Cartridges. In this instance, the recoveries were 85 and 70 % ($n = 10$) for LMG and MG, respectively.

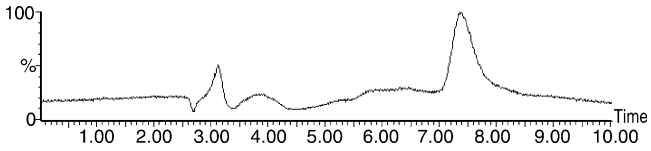

Fig. 6. Chromatographic response of a real sample containing a ca. 2 μg/kg concentration of MG plus LMG.

Table 1
Determination of MG plus LMG in real fish samples

Sample	Confirmation ratio (*m*/*z* 329/313)	Sum of MG and LMG (μg/kg)
1	3.2	1.5 ± 0.1
2	2.9	2.0 ± 0.5
3	2.8	2.5 ± 0.1
4	2.9	0.4 ± 0.1
5	2.9	1.1 ± 0.2
6	3.1	<0.15
7	3.1	7.0 ± 0.5
Standard	3.0 ± 0.1	

Samples were analysed in duplicate.

The method was applied to the determination of MG plus LMG in muscle from salmon collected in the Pacific Ocean, X Region, Chile. Fig. 6 shows the chromatogram of a sample containing a concentration of ca. 2.0 μg/kg of MG plus LMG. These samples had been classified as doubtful when previously analyzed by LC with visible detection. Table 1 shows the results obtained. As can be seen, the present method provides a confirmative character when the concentration level of the analytes is near the limit of detection of LC–vis.

4. Conclusions

A sensitive method for the quantification and confirmation of the sum of malachite green and leucomalachite green in salmon muscle has been developed. It is based on the use of an oxidative pre-column reaction which converts LMG into MG previous to LC–APCI–MS analysis. Although it is, with this screening system not possible to distinguish the analytes from each other, analyte detectability is improved. The detection limit of the procedure, using the confirmatory ion *m*/*z* 313, was 0.15 μg/kg.

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