A simple and rapid method for the identification and quantification of malachite green and its metabolite in hake by HPLC-MS/MS

Carolina. Nebot, Alejandra Iglesias, Rocio Barreiro, Jose Manuel Miranda, Beatriz Vázquez, Carlos Manuel Franco and Alberto Cepeda

Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Veterinary Medicine, University of Santiago de Compostela, 27002, Lugo, Spain. Tel.: +34 982 285900 Fax: +34 982 254592. *Corresponding author: carolina.nebot@usc.es

Corresponding author:

Carolina. Nebot

Email address: carolina.nebot@usc.es

Corresponding address: Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Veterinary Medicine, University of Santiago de Compostela, 27002, Lugo, Spain.

Telephone number +34 982 285900

Fax number: +34 982 254592. *

ABSTRACT

Aquaculture is the fastest growing animal food-producing sector in the world and similarly to any other type of food production systems, the use of veterinary drugs is unavoidable and common, but it needs to be controlled. Special attention should be paid to the use of illegal substances such as malachite green. This substance was found in the last three control monitoring plans conducted in Europe. Methods for the detection of malachite green in commonly farmed species, including salmon, trout, carp, shrimp and panga can be easily found but not emerging species such as hake. Hake is becoming an emerging species and nowadays, farmed hake can be found in some markets.

This study presents an HPLC-MS/MS method for the simultaneous determination of malachite green and its metabolite, leucomalachite green, in hake muscle. The recoveries, decision limit, and limit of quantification of malachite and leucomalachite green were greater than 70%, below 2 µg kg^{-1}, between 0.5 and 1 µg kg^{-1}, respectively.

Keywords: Residues; Malachite green; Hake, LC-MS/MS; 2002/657/EC

1. Introduction

While wild capture fisheries have been stabilised at approximately 90 million metric tons since the late 1980s, world aquaculture has grown tremendously over the past years, from 12 million metric tons in 1985 to 48.1 in 2005. It is likely that aquaculture will continue to grow at significant rates through 2025 [Subasinghe et al., 2009; Diana, 2009], becoming the fastest growing food-producing sector in the world [Subasinghe et al., 2009]. Currently, more than 220 species of finfish and shellfish are farmed, with the most common species being sea bass, carp, eel, salmon, trout and catfish, and this number will continue to grow as a result of wild captures decreased and increased consumer demand. Each year, the domestication of new species, such as cod, haddock, halibut and sole, is reported [Fotedar, 2011; Suquet, Divanach, Hussenot, Coves & Fauvel, 2009; Moksness, Kjorsvik & Olsen, 2004]. Hake is also one of the species that has great potential for aquaculture, because of its fast growth, its high quality when it is fresh and its high demand in Europe [Groison, Suquet, Cosson, Mayer, Severe, Bouquet, Geffen, Utne-Palm & Kjesbu, 2010]. In Norway, for example, nine fish farms are already producing hake [Paisley, Ariel, Lyngstad, Jónsson, Vennerström, Hellström & ØStergaard, 2010].

One of the problems of aquaculture is animal diseases that involve significant cost and management [Subasinghe 2009]. Veterinary drugs are legally and regularly employed to prevent, control and treat animal diseases. Although the use of illegal substances is not a common practice because it is not considered a good management practice and has legal implications [Subasinghe 2009; Love, Rodman, Neff & Nachman, 2011], some illegal substances, such as malachite green (MG), are still be found in national residue monitoring plans conducted in the EU and the United States [EU, The European

61 parliament and the Council of the European Union 2005; FDA, US Food and Drug
62 Administration USA 2007].

63 Residues of MG are not permitted in food of animal origin in Canada, the United States
64 and Europe, and its use is completely prohibited in food-production. MG is a
65 triphenylmethane dye originally used as a dyeing agent in the textile and paper
66 industries. It was introduced as an ecto-parasiticide, fungicide and antiseptic in
67 aquaculture in 1933 because of its broad fungicidal and anti-parasitical spectrum
68 [Bergwerff and Scherpenisse, 2003]. The effectiveness of MG against proliferative
69 kidney disease has rapidly expanded its illegal use in the culture of fish eggs, fingerlings
70 and adult fish. MG and its metabolite (Figure 1), leucomalachite green (LMG), are
71 multi-organ toxic to mammals [Srivastava, Sinha, Roy, 2004] and have negative effects
72 on the immune and reproductive systems and genotoxic and carcinogenic properties
73 [Srivastava et al. 2004; Li, Shen, Liang, He & He, 2011; Oplatowska, Donnelly,
74 Majithiya, Glenn Kennedy & Elliott, 2011]. LMG is accumulated in tissues, and its
75 elimination is strongly dependent on the fat content [Lee, Lee, Lee & Kim, 2006]. To
76 protect consumer health, therefore, Commission Decision 2004/25/EC stated that
77 analytical methods for the determination of MG residues in meat of aquaculture
78 products must meet a minimum required performance limit (MRPL) of 2 $\mu g\ kg^{-1}$
79 [Commission Decision 2004/25/EC].

80 Most of the methods reported for the analysis of MG and LMG describe protocols that
81 consisted in two steps. The first step involves extraction of the analytes with
82 acetonitrile, combined with different reagents. The second consists of purification of the
83 extract on solid phase extraction (SPE) cartridges. Determination has generally being
84 conducted by high performance liquid chromatography (HPLC) using a

chromatographic separation column followed by a derivatisation column with a lead oxide post-column reactor for LMG detection. The detectors employed were photodiode array (PDA), fluorescence and tandem mass spectrometers (MS/MS). Most extraction techniques are developed independently of the method employed and validated for one species of fish, usually species that are commonly produced in fish farms (e.g., salmon or trout). However, to the best of our knowledge, there are no protocols available for emerging fish farm species, such as hake.

The aim of this article is to present an HPLC-MS/MS method for the identification and quantification of MG and LMG in a potential fish farm species, hake. The method was also investigated for application in other fish species, including sole, trout, salmon, sea bream, sea bass, and gilthead. The method was validated for hake muscle samples following all of the criteria established in Commission Decision 2002/657/EC.

2. Materials and Methods

2.1. Chemicals, reagents and stock solutions

MG oxalate (MG), LMG, and malachite green-d_5 picrate, sulfathiazole were of analytical grade (purity > 95%) and obtained from Sigma–Aldrich (St. Louis, USA). While, malachite green-d_5 picrate was employed as the internal standard (IS-MG) for the quantification of MG and LMG, sulfathiazole was used for to investigate the selectivity/specificity of the method. All the solvents employed during this work (methanol, acetonitrile, hexane, dichloromethane, chloroform and petroleum ether) were analytical grade and obtained from Scharlau Chemie (Barcelona, Spain). Formic acid (purity > 99% for analysis) was purchased from Acros Organics (Geel, Belgium).

Nitrogen gas was generated using an in-house nitrogen generator from Peak Scientific Instruments, Ltd. (Chicago, IL). Purified water was prepared in-house with a Milli-RO water system from Millipore (Bedford, MA).

Acidified methanol, dichloromethane and Milli-RO water were prepared by diluting one millilitre of formic acid in 999 mL of each solvent (methanol, dichloromethane or Milli-RO water). The stock solutions of individual compounds were prepared at 0.6 g L^{-1} by dissolving 30 mg of each compound into 50 mL of methanol. These solutions were then mixed and diluted with acidified methanol to obtain a standard solution mixture of MG and LMG at 1 µg L^{-1}. The stock solution of the IS-MG was prepared by diluting 10 mg of malachite green-d_5 picrate in 10 mL of methanol. This solution was further diluted to 1 µg L^{-1} with acidified methanol. All of the standard solutions were stored in amber glass bottles at -18 °C for a maximum of three months.

2.2. Extraction and analysis of MG and LMG

Homogenised fresh hake muscle (2 g) was transferred into a clean 10 mL glass centrifuged tube, 10 µL of the IS-MG stock solution (10 µg mL^{-1}) and 3 mL of acidified dichloromethane were added to the tube. The mixture was vortex-mixed, sonicated (10 min) and centrifuges at 93,000 g (10 min) on a H-103N series Kokusan centrifuge (Tokyo, Japan). The supernatant (lower layer) was collected with a glass Pasteur pipette and transferred into a clean 10 mL Pyrex® conical centrifuge tube which contained 3 mL of acidified methanol. The resultant extract was vortex-mixed and and evaporated to approximately 3 mL at 35 ºC on a turbo evaporator, model Turbo Vap® II from Zyrmark (Hopkinton, MA, USA). The remaining extract was vortex-mixed and evaporated to

130 approximately 100 µL at 45 ºC, the remaining extract was dissolved to 0.2 mL with
131 acidified methanol.

132 Matrix-matched calibration curves were prepared for control and quantification
133 purposes. These samples were prepared with fresh muscle sample spiked at five
134 concentrations (0 ×, 0.5 ×, 1 ×, 1.5 ×, 2 × and 5 × MRPL) with MG and LMG and they
135 were processed as described above.

136 The HPLC-MS/MS system consisted of a HPLC model 1100 from Agilent
137 Technologies (Waldbronn, Germany) equipped with a quaternary pump, a degasser, and
138 an auto-sampler. The mass spectrometer (MS), model API 4000™ from Applied
139 Biosystems MSD Sciex (Toronto, Canada), integrated a TurboIonSpray® probe for
140 molecule ionisation. The whole system HPLC-MS/MS was controlled by the software
141 Analyst 1.4.1, also from Applied Biosystems, MSD Sciex (Toronto, Canada).

142 Before conducting the analysis, the MS settings were optimised for the detection of
143 MG, LMG, IS-MG and sulfathiazole by infusing standard solutions of each analyte at 1
144 µg L^{-1}. Table 1 details MS parameters required for their detection, as well as the
145 precursor and product ions selected for their identification. The determination of the
146 analytes was performed with the electrospray ionisation (ESI) source operating on
147 positive-ion mode (ESI^+) and obtaining an optimum signal response for each analyte
148 with the source temperature set up at 520 ºC and a vacuum gauge of 2.4 x 10^5 Pa.

149 For the analysis of MG and LMG 20 µL of extract was injected into a Synergi 4 µm
150 Polar-RP 80A (50 mm × 2.00 mm) HPLC analytical column used in conjunction with a
151 Polar-RP (4.0 mm × 2.0 mm) SecurityGuard cartridge, both from Phenomenex
152 (Macclesfield, UK). The elution of the analytes was performed with a binary mobile

153 phase with consisted of acidified Milli-RO water (phase A) and acidified methanol
154 (phase B). The mobile phase was pumped at 0.2 mL min^{-1} and the gradient profile was
155 as follows: 0-4 min, 90% A; 4-9 min, 50% A; 9-17 min, 25% A; 17-24 min, 0% A; 24-
156 26 min, 0% A; 26-30 min, 75% A; 30-32 min, 90% A; 32-40 min, 90% A.

157 **2.3. Validation of the HPLC-MS/MS for the detection of MG and LMG in hake**

158 Validation was conducted on four different days, on day 1, 2 and 3 three batches of
159 matrix-matched samples were employed for the validation; each batch consisted of 21
160 samples enriched with MG and LMG at 0 ×, 0.5 ×, 1 ×, 1.5 ×, 2 × and 5 × MRPLs.
161 While, six replicates were need for levels 0.5 ×, 1 ×, 1.5 × MRPLs, only one was
162 employed for levels 0, 2 ×, 2 × and 5 × MRPLs. For each batch of samples (n=21) it
163 was calculated the recoveries and the precision under repeatability conditions (RDS_r),
164 expressed as the relative standard deviation (RSD) of the recovery. Since the three
165 batches were analysed on different days and employing different batches of solvents,
166 vials, pipettes and laboratory conditions the combination of these results were employed
167 to evaluate the precision of the method under reproducibility conditions (RSD_R),
168 expressed as RSD of the recovery obtained for the three days. Additionally, the linearity
169 was evaluated with calibration curves constructed with batch of sample each sample,
170 they were built by representing concentrations of MG or LMG against the
171 corresponding ratio analyte area/IS-MG peak area.

172 On day 4 it was investigated the specificity/selectivity of the method, 10 fresh hake
173 muscle samples obtained from different specimens of hake were analysed. The samples
174 were divided into two batches of ten samples; the first one was processed following the
175 protocol described for routine analysis, without spiking the sample, and samples of the

second one were spiked with MG, LMG and sulfathiazole, a common drug employed in fish farms [Commission Decision 2002/657/EC].

Each day of the validation (day 1, 2, 3 and 4) standard solutions containing a mixture of MG and LMG at 0, 10, 20, 40, 80 and 100 μg L^{-1} and the IS-MG were analysed prior to the analysis of the sample batches. The decision limit (CCα) and the detection capability (CCβ) were calculated as indicated in Commission Decision 2002/657/EC for substances with no established permitted limit and as described by Wu et al. (2007). To calculate CCα and CCβ, the matrix-matched calibration curves analysed on day 1, 2 and 3 were employed.

2.4. Fish samples

Fish samples, especially hake, were purchased from local supermarkets. Once in the laboratory, all of the fish were accuracy weighed, and their head, gills, skin, and thorns were removed. The muscle was separated, homogenised and stored at -18 °C until analysis. Furthermore, the applicability of the method was investigated in different specimens of hake and sole, trout, salmon, sea bream, sea bass and gilthead.

2.5. Applicability of the method in other species of fish

The applicability of the method was also investigated in sole, trout, salmon, sea bream, sea bass and gilthead muscle, fish with different percentages of fat. The study was conducted with ten replicates of fish muscle (sole, trout, salmon, hake, sea bream, sea bass, and gilthead) spiked with MG and LMG at two concentrations (5 and 25 μg kg^{-1}). After homogenisation, the samples were shaken for 10 min and kept in the dark. The same experiment was repeated with lyophilised muscles samples prepared in a lyophiliser model LyoQuest from Telstar (Terrasa, Spain). The water content was taken

199 into account and sample size of the lyophilised sample were: 0.5 g of sole, 0.5 g of
200 trout, 0.8 g of salmon, 0.5 g hake, 0.4 g sea bream, 0.6 g of sea bass, and 0.6 g of
201 gilthead. Fresh and lyophilized muscle samples were extracted as described earlier and
202 analysed by HPLC-MS/MS for each type of fish the following parameters were
203 calculated: recoveries, RSD_r, CCα and CCβ.

204 **2.6. Analysis of the crude lipid content by soxhlet**

205 For the measurement of crude lipid content 4.0 g of fish muscle was transferred to a
206 cellulose extraction thimble (26 x 60 mm) from Filtros Anoia (Barcelona, Spain). The
207 extraction thimbles were then inserted into a soxhlet sample vessel, which was
208 previously dried at 100 °C for 15 min, cooled in desiccators and weighed. Extraction of
209 the lipid content was performed on a Soxtec system, Model 1046, from Tecator
210 (Hoganes, Sweden) employing petroleum ether at a temperature of 110 ºC for 1 hour
211 and 54 min. After the extraction, samples vessel were dried for 15 min at 105 °C, cooled
212 to room temperature in a desiccator and weighed. Each sample was analysed in
213 triplicate.

214

215 **3. Results and discussion**

216 **3.1. Optimisation of the LC-MS/MS method**

217 Even though atmospheric pressure chemical ionisation (APCI) mass spectrometry has
218 previously been employed for the detection of MG [Doerge, Churchwell, Gehring, Pu,
219 Plakas, 1998;Valle, Diaz, Zanocco, Richtera, 2005), ESI is the technique most
220 frequently utilised [Lee, Wu & Cai, 2006; Martínez-Bueno, Herrera, Uclés, Agüera,
221 Hernando, Shimelis, Rudolfsson, Fernández-Alba, 2010; Halme, Lindfors & Peltonen,

222 2007] and the one selected for this investigation. MG, LMG and the IS-MG were
223 detected using positive ESI^+, as reported previously [Halme et al. 2007; Arroyo, Ortiz,
224 Sarabia, Palacios, 2009].

225 The combination of several mobile phase components, such as ammonium acetate,
226 formic acid, methanol, acetonitrile and water, was comprehensively investigated to
227 achieve an optimum chromatographic separation and high MS signal response for the
228 analytes. The highest resolution between peaks and the most symmetric peak shapes
229 were achieved with the combination of acidified methanol and acidified water mixed in
230 a gradient mode. These mobile phase components were also employed by Martínez et
231 al. (2010) who analysed MG and other triphenylmethane dyes by HPLC-MS/MS. The
232 use of formic acid helped to prevent peak tailing and ion formation. The MS was
233 operating on a selected reaction monitoring (SRM) mode because it permits more
234 selectivity and sensitivity. Each analyte was identified by its t_R and two SRM
235 transitions. Variability of the t_R was not observed (RSD < 0.1 %) for any of the analytes
236 when standard solutions containing all of the analytes, including the IS-MG, at 25, 50,
237 100 and 250 ng L^{-1} were injected ten times. One of the advantage of this method was
238 the fact that post-column derivatisation was not necessary. Although some authors have
239 reported its use for the detection of LMG by HPLC-MS/MS [Scherpenisse and
240 Bergwerff, 2005], most HPLC-MS/MS methods did not include the implementation of
241 post-column derivatisation based on the characteristics of this technique [Halme et al.
242 2007; Hurtaud-Pessel, Couedor, Verdon, 2011].

243 The objective of this investigation was to develop a simple, low cost and rapid
244 extraction procedure for the analysis of MG and its metabolite in hake. Because the
245 amount of reagent required for the extraction depends on the sample size, the use of

246 different sample weights of fresh hake muscles were tested (1, 1.5, 2, 2.5 and 3 g). The
247 recoveries of MG and LMG with different organic solvents (acidified and non-acidified
248 solvents and combinations of solvents) were compared (data not shown). The highest
249 recoveries were achieved with acidified dichloromethane combined with acidified
250 methanol. Recoveries obtained with methanol combined with dichloromethane were
251 approximately 60% higher than with dichloromethane alone, most likely because the
252 dichloromethane combined with methanol evaporated less quickly, preventing the loss
253 of analytes during the evaporation step.

254 Two additional factors have been shown to have significant effects on the MG and
255 LMG recoveries: light and oxygen. It is well known that MG is sensitive to light
256 [Hurtaud-Pessel et al. 2011; Mitrowska, Posyniak & Zmudzki, 2005], and this effect
257 was clearly observed even within the two hours during the extraction process.
258 Therefore, to avoid MG degradation, all of the material employed was stored in amber
259 vials or wrapped with aluminium foil. It was also noticed that recoveries decreased
260 more than 70% when air, instead of nitrogen, was employed to evaporate the extracts.

261 Different procedures can be found in the literature for the extraction of MG and LMG
262 from fish samples. Most techniques employ an initial extraction with acetonitrile
263 combined with ammonium acetate [Arroyo, Ortiz, Sarabia & Palacios, 2009], McIlvaine
264 buffer [Bergwerff and Scherpenisse, 2003; Valle et al. 2005; Dowling, Mulder, Duffy,
265 Regan & Smyth, 2007] hydroxylamide [Arroyo et al. 2009] or N,N,N,N′-tetramethyl-
266 1,4-phenylenediamine dihydrochloride [Bergwerff and Scherpenisse, 2003;
267 Scherpenisse and Bergwerff, 2005]. Purification of the resultant extract is then
268 conducted with SPE cartridges, such as Bakerbond SCX, Strata™-X, or aromatic
269 sulphonic acid-bonded [Lee et al. 2006; Valle et al. 2005; Dowling et al. 2007] or

molecularly imprinted polymers [Martínez-Bueno et al. 2010; Long, Mai, Yang, Zhu, Xu, Lu & Zou, 2009]. Therefore, compared with previously published methods, the method presented in this article is shorted and less time consuming.

3.2. Method validation for fresh hake muscle samples

Validation of the method was conducted as described in the Decision 2002/657/EC employing hake muscle samples spiked with the analytes at various concentrations because no certified reference materials were available for MG and LMG in hake muscle. The linear response of the instrument to analytes concentration was determined using a linear regression model. Standard solution and matrix-matched calibration curves were built each day of the calibration. The linearity of the calibration curves for MG and LMG was satisfactory with determination coefficients (R^2) above 0,978 for concentration of MG and LMG between 0 and 10 μg kg^{-1}.

The recoveries obtained for 1, 2 and 3 μg kg^{-1} of MG and LMG over the three days of the validation fulfilled the criteria established in 2002/657/EC (Table 2). The mean recoveries achieved for MG at 1 μg kg^{-1} (71%) were lower than those reported by Martínez Bueno et al. (2010) for salmon (118%) and than those reported by Long et al. (2009) for grass carp (89%), shrimp (91%) and shellfish (94%) but higher than those reported by Halme et al. (2007) for trout (58%). On the other hand, the recoveries achieved for LMG in this work were higher than those reported previously for other fish species [Halme et al. 2007; Long et al. 2009].

The precision of the method, determined as relative standard deviation of the concentration (RSD), under repeatable (RSD_r) and reproducible (RSD_R) within-laboratory conditions was obtained from the matrix-matched samples analysed each day

293 of the validation. The RSD obtained fulfilled the criteria established in 2002/657/EC,
294 the RSD_R were < 38% and greater than the RSD_r (Table 2). These values were greater
295 than those reported by Long et al. (2009), the only other work which reported method
296 precision under repeatability and reproducibility conditions. This method employs
297 molecularly imprinted polymers for the extraction of malachite green, leucomalachite
298 green and other substances (leucogentian violet and gentian violet) this certainly could
299 results in a cleaner extract which will gave lower RSD.

300 Although some methods only reported CCα and CCβ values for MG and LMG, for the
301 method presented in this work in addition to CCα and CCβ, LOQ was also calculated
302 due to the toxicity of the analytes which impact human health [Srivastava et al. 2004;
303 Lee et al. 2006; Hurtaud-Pessel et al. 2011]. CCα, CCβ and LOQ were determined with
304 matrix-matched hake samples spiked with the analytes at concentrations of 0,25, 0,5, 1,
305 2 and 3 μg kg^{-1}. Mean CCα values for MG and LMG were below 2 μg kg^{-1}. The LOQ of
306 the method, established by measuring the S/N at different concentrations, were 0.5 μg
307 kg^{-1} for MG and 1 μg kg^{-1} for LMG (Table 3). Figure 2 shows SRM chromatograms of
308 malachite green and leucomalachite green and the internal standard (IS-MG) in hake
309 muscle samples spiked with MG and LMG at 2 μg kg^{-1}.

310 **3.3. Application of the method in other fish species**

311 In the literature it can be found methods for the analysis of residues of MG and LMG in
312 different species of fish, such as salmon, trout [Martínez-Bueno et al. 2010; Hurtaud-
313 Pessel et al. 2011; Long et al. 2009; Lee et al. 2008]. In these works, even though
314 validation has been conducted with only one type of fish, authors report their
315 applicability in other fish species. Based on the requirement of the Decision

316 2002/657/EC which specifies that a method can only be applied to a particular species
317 of fish when it has been validated for it, this requirement is not fulfil by many authors.
318 Even though, it has to be highlighted that the work reported by Long et al. (2010) is the
319 only method that conducted the validation on different species of fish (grass carp,
320 shrimp and shellfish). Based on the 2002/657/EC, the method developed for hake
321 muscle was also evaluated in muscle of salmon, trout, sole, sea bass, gilthead bream and
322 sea bream. As a first test, instead of conducting the whole validation protocol for each
323 fish, only recoveries and CCα and CCβ values were calculated to investigate the
324 applicability of the method. Recoveries were calculated for MG and LMG at two
325 concentrations (5 and 25 μg kg^{-1}), and the CCα and CCβ values were estimated by
326 employing matrix-matched samples spiked at 0, 5 and 25 μg kg^{-1}. Even if, the Decision
327 indicates that recoveries, CCα and CCβ should be calculated for 0.5, 1 and 1.5 x MRPL,
328 the aim of this part of the work was only to investigate the potential of the method in
329 other fish species.

330 Table 4 summarises R^2, CCα, CCβ, recoveries and RSDr of MG and LMG in each type
331 of fish species. Recoveries of MG were better than those achieved for LMG, as they
332 were above 70% for all of the fish species. Only recoveries of LMG in hake and sole
333 were above 70%. Recoveries of sea bream, sea bass and gilthead were below 40%. No
334 relation between the percent of LMG recovery and fat content in each type of fish was
335 found (Table 4 and 5). CCα values achieved for MG were below 2 μg kg^{-1} for hake and
336 salmon and for LMG only in hake. Even if, the results indicated that the method could
337 only be employed for the control of residues of MG and LMG in hake, according to the
338 European Decision, it should be highlighted that the method could be successfully
339 employed with sample muscles of sole, trout, salmon, sea bream, sea bass and gilthead

when it is not necessary to follow the required of the Decision. These results also indicated that a complete validation process should be conducted for each type of matrix for which the method will be applied and that only recovery data are insufficient to confirm the applicability of the method.

To improve recoveries of MG and LMG in salmon, trout, sole, sea bass, gilthead, sea bream and sea bream the fresh muscle samples of each species of fish was lyophilized before implement the extraction procedure developed for fresh muscle of hake. Because recoveries, CCα and CCβ obtained with lyophilized muscle samples were unsatisfactory and have not being included. The authors believe that lyophilised fish led to more interference in the extraction because of the absence of water.

3.4. Application of the method in real samples

The presence of MG and LMG residues was investigated in fish samples purchased from local supermarkets. None of the samples tested contained residues of MG or LMG at measurable levels.

4. Conclusion

The aim of this research study was to develop an HPLC-MS/MS method for the simultaneous extraction and analysis of MG and LMG in hake muscle samples and to validate this method following the requirements established by European legislation [2002/657/EC]. The method was also evaluated in other species of fish (sole, trout, salmon, sea bream, sea bass and gilthead) and even if the recoveries more o less satisfactory, the CCα and CCβ were greater than 2 $\mu g\ kg^{-1}$.

5. Acknowledgments

The authors wish to thank the Spanish Ministry of Science and Innovation through for funding this study through project AGL-2009-14707 and the staff of the Cactus of the University of Santiago de Compostela for their help in the analysis of fish muscle samples.

6. References

Arroyo et al., 2009. D. Arroyo, M.C. Ortiz, L.A. Sarabia, F. Palacios, Determination and identification, according to European Union Decision 2002/657/EC, of malachite green and its metabolite in fish by liquid chromatography-tandem mass spectrometry using an optimized extraction procedure and three-way calibration. *Journal of Chromatography A,*1216 (2009), pp 5472-5482.

Bergwerff and Scherpenisse, 2003. A. Bergwerff and P. Scherpenisse, Determination of residues of malachite green in aquatic animals. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **788** (2003), pp 351-359.

Doerge et al., 1998. D.R. Doerge, M.I. Churchwell, T.A. Gehring, Y.M. Pu, S.M. Plakas, Analysis of malachite green and metabolites in fish using liquid chromatography atmospheric pressure chemical ionization mass spectrometry. *Rapid Communication in Mass Spectrometry* **12** (1998), pp 1625-1634.

Dowling et al., 2007. G. Dowling, P.P. Mulder, C. Duffy, L. Regan, M.R. Smyth, Confirmatory analysis of malachite green, leucomalachite green, crystal violet and leucocrystal violet in salmon by liquid chromatography-tandem mass spectrometry. *Analytical Chimica Acta* **586** (2007), pp 411-419.

EU, The European parliament and the Council of the European Union (2005) Report for 2004 on the Results of Residue Monitoring in Food of Animal Origin in the Member States. .

Commission Decision 2004/25/EC. EU, The European Parliament and the Council of the European Union (2004) of 22 December 2003 amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. *Official Journal of the European Union* **L 6**.

Diana, 2009, Aquaculture Production and Biodiversity Conservation. BioScience **59** (2009), pp 27-38.

FDA, US Food and Drug Administration USA Import Alert (2007), pp 16-131.

393 Fotedar, 2001. R.K. Fotedar, Recent advances and new species in aquaculture (2011),
394 pp 397.

395 Groison et al., 2010. A. Groison, M. Suquet, J. Cosson, I. Mayer, I. Severe, J. Bouquet,
396 A.J. Geffen, A.C. Utne-Palm, O.S. Kjesbu, Sperm motility in European hake,
397 *Merluccius merluccius*, and characterization of its spermatozoa concentration and
398 volume, spermatocrit, osmolality and pH. *Aquaculture* **301** (2010), pp 31-36.

399 Halme et al., 2007. K. Halme, E. Lindfors, K. Peltonen, A confirmatory analysis of
400 malachite green residues in rainbow trout with liquid chromatography-electrospray
401 tandem mass spectrometry. *Journal of Chromatography B Analytical Technologies*
402 *Biomedical Life Sciences* **845** (2007), pp 74-79.

403 Hurtaud-Pessel et al., 2011. D. Hurtaud-Pessel, P. Couedor, E. Verdon, Liquid
404 chromatography-tandem mass spectrometry method for the determination of dye
405 residues in aquaculture products: development and validation. *Journal of*
406 *Chromatography A* **1218** (2011), pp 1632-1645.

407 Lee et al., 2006. K. Lee, J. Wu, Z. Cai, Determination of malachite green and
408 leucomalachite green in edible goldfish muscle by liquid chromatography–ion trap mass
409 spectrometry. *Journal of Chromatography B Analytical Technologies Biomedical Life*
410 *Sciences* **843** (2006), pp 247-251.

411 Lee et al., 2008. Y. Lee, S. Lee, DS. Lee, Kim, Risk assessment of human antibiotics in
412 Korean aquatic environment. *Environmental Toxicology and Pharmacology* ***26*** (2008),
413 pp 216-221.

414 Li et al., 2011. G. Li, D. Shen, X. Liang, Y. He and S He, Effects of malachite green on
415 the mRNA expression of detoxification-related genes in Nile tilapia (Oreochromis
416 niloticus) and other major Chinese freshwater fishes. Environmental Toxicology (2011)

417 Long et al., 2009. C. Long, Z. Mai, Y. Yang, B. Zhu, X. Xu, L. Lu and X. Zou,
418 Determination of multi-residue for malachite green, gentian violet and their metabolites
419 in aquatic products by high-performance liquid chromatography coupled with
420 molecularly imprinted solid-phase extraction. *Journal of Chromatography A* **1216**
421 (2009), pp 2275-2281.

422 Love et al., 2009. D.C. Love, S. Rodman, R.A. Neff and K.E. Nachman, Veterinary
423 drug residues in seafood inspected by the European Union, United States, Canada, and
424 Japan from 2000 to 2009. *Environmental Science & Technology* **45** (2011), pp 7232-
425 7240.

426 Martínez-Bueno et al., 2010. M.J. Martínez-Bueno, S. Herrera, A. Uclés, A. Agüera,
427 M.D. Hernando, O. Shimelis, M. Rudolfsson, A.R. Fernández-Alba. Determination of
428 malachite green residues in fish using molecularly imprinted solid-phase extraction
429 followed by liquid chromatography–linear ion trap mass spectrometry. *Analytical*
430 *Chimica Acta* **665** (2010) 47-54.

Mitrowska et al., 2005. K. Mitrowska, A. Posyniak, J. Zmudzki, Determination of malachite green and leucomalachite green in carp muscle by liquid chromatography with visible and fluorescence detection. *Journal of Chromatography A* **1089** (2005), pp 187-192.

Moksness et al., 2004. E. Moksness, E. Kjorsvik, Y. Olsen, Culture of cold-water marine fish (2004), pp 528.

Oplatowska et al., 2011. M. Oplatowska, R.F. Donnelly, R.J. Majithiya, D. Glenn Kennedy, C.T. Elliott, The potential for human exposure, direct and indirect, to the suspected carcinogenic triphenylmethane dye Brilliant Green from green paper towels. *Food and Chemical Toxicology* **49** (2011) 1870-1876.

Paisley et al., 2010. L.G. Paisley, E. Ariel, T. Lyngstad, G. Jónsson, P. Vennerström, A. Hellström, P. ØStergaard, An Overview of Aquaculture in the Nordic Countries. *Journal of the World Aquaculture Society* **41** (2010), pp 1-17.

Scherpenisse and Bergwerff, 2005. P. Scherpenisse and A.A. Bergwerff, Determination of residues of malachite green in finfish by liquid chromatography tandem mass spectrometry. *Analytical Chimica Acta* **529** (2005), pp 173-177.

Srivastava et al., 2004. S. Srivastava, R. Sinha, D. Roy, Toxicological effects of malachite green. *Aquatic Toxicology* **66** (2004) 319-329.

Subasinghe, 2009. R. Subasinghe, Disease control in aquaculture and the responsible use of veterinary drugs and vaccines: The issues, prospects and challenges. *Options Méditerranéennes* (2009), pp 86.

Subasinghe et al., 2009. R. Subasinghe, D. Soto, J. Jia, Global aquaculture and its role in sustainable development. *Reviews in Aquaculture* **1** (2009), pp 2-9.

M. Suquet, P. Divanach, J. Hussenot, D. Coves, C. Fauvel, Pisciculture marine de nouvelles espèces d'élevage pour l'Europe; Marine fish culture of "new species" farmed in Europe. *Cahiers Agricultures* **18** (2009), pp 148-156.

Wu et al., 2007. X. Wu, G. Zhang, Y. Wu, X. Hou, y Z. Yuan, Simultaneous determination of malachite green, gentian violet and their leuco-metabolites in aquatic products by high-performance liquid chromatography–linear ion trap mass spectrometry. *Journal of Chromatography* **1172** (2007), pp 121-126

Valle et al., 2005. L. Valle, C. Diaz, A.L. Zanocco, P. Richtera, Determination of the sum of malachite green and leucomalachite green in salmon muscle by liquid chromatography-atmospheric pressure chemical ionisation-mass spectrometry. *Journal of Chromatography A* **1067** (2005), pp 101-105.

Table Captions

Table 1. Retention times (t_R), precursor ions, product ions, declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE) and cell exit potential (CXP) employed to identify malachite green and leucomalachite green.

Table 2. Accuracy and relative standard deviation of malachite green and leucomalachite green under repeatability conditions (RSD_r) and within-laboratory reproducibility conditions (RSD_R).

Table 3. Decision limit (CCα), detection capability (CCβ) and limit of quantification (LOQ) achieved with the method presented for malachite green and leucomalachite green.

Table 4. a, b, Regression coefficients (R^2) and recoveries (relative standard deviation under repeatability conditions (RSD_r)) of malachite green and leucomalachite green in fresh muscle samples.

Table 5. Crude lipid content of each species of fish investigated

482 **Figure Captions**

483 **Figure 1. Structures of malachite green and leucomalachite green.**

484 **Figure 2. SRM chromatograms of malachite green and leucomalachite green and**
485 **the internal standard (IS-MG) in hake muscle samples spiked with MG and LMG**
486 **at 2 µg kg^{-1}.**

487