Development of a multi-class method to determine nitroimidazoles, nitrofurans, pharmacologically active dyes and chloramphenicol in aquaculture products by liquid chromatography-tandem mass spectrometry

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ABSTRACT

LC-MS/MS method was developed for the efficient identification and quantification of 21 banned substances including various nitroimidazoles, nitrofurans, pharmacologically-active dyes and chloramphenicol, respectively in aquaculture products. The sample preparation was started by acid-treatment with 2-nitrobenzaldehyde (NBA) to liberate matrix-bound residues of nitrofurans. A modified QuEChERS method was optimized for the extraction and clean-up of the target analytes. The metabolites of the four conventional nitrofurans (nitrofurantoin, furazolidone, nitrofurazone and furaltadone) and of three other nitrofurans (nifursol, nifuroxazide, and nitrovin), and an underivatizable nitrofuran (nifurpirinol) were simultaneously detected. Furthermore, 21 banned substances were quantified by LC-MS/MS with ESI using one single injection. To evaluate and validate the performance of the method the criteria of the Decision (EC) no 2002/657 were applied. Decision limit (CCα) of target analytes ranged 0.067-1.655 μg/kg in aquaculture products. The recovery ranged 77.2%-125.6%, and the relative standard deviations of inter-day analyses (RSD) were less than 25%.

KEY WORDS: Nitroimidazoles; Nitrofurans; Dyes; Chloramphenicol; Residues; LC-MS/MS; Aquaculture products

1. Introduction

Veterinary medicinal products (VMPs), such as nitrofurans (NFs), chloramphenicol (CAP), nitroimidazoles (NIIMs), and also the non-veterinary product pharmacologically-active were used extensively in aquaculture because of their low costs and high effectiveness. According to European Union legislation, the use of these compounds has been banned for food producing animals. They have been classed in table 2 of Commission regulation, except for dye compounds which do not enter this legislation because they never have been recorded as medicinal products (European Commission, 2009). Nevertheless no residues of these substances shall be found in food products. NFs are active broad-spectrum antibacterial drugs and in the past have been widely used in veterinary medicine. The most often used NFs compounds are furazolidone, furaltadone, nitrofurazone and nitrofurantoin. As mentioned by Hoogenboom et al. (Hoogenboom, Berghmans, Polman, Parker, & Shaw, 1992), McCracken & Kennedy (1997), and Zuidema et al. (2004), they can be rapidly metabolized into semicarbazide (SEM), 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), and 1-aminohydantoin (AHD), respectively. Recently, some other NFs including nifursol, nitrovin, nifuroxazide, and nifurpirinol (NPIR) have been notified due to having the similar structure. They are metabolized into 3,5-dinitrosalicylic acid hydrazine (DNSH), aminoguanidin hydrochloride (AMG), and salicylic acid hydrazine (PSH), respectively, and excluding NPIR undertaking no metabolisation. The chemical structures of parent compounds of NFs and their metabolites are shown in Fig. 1. The most frequently used NIIMs including metronidazole (MNZ), dimetridazole (DMZ), ronidazole (RNZ), and ipronidazole (IPZ) help combatting anaerobic bacterial and parasitic infections. The analytical method should cover as

well the three metabolites of these NIIMs including hydroxy-metronidazole (MNZOH), 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI), and hydroxy-ipronidazole (IPZOH), respectively. Triphenylmethane dyes have been used as antiparasites and antifungus infection agents in aquaculture, including malachite green (MG), crystal violet (CV) and brilliant green (BG). MG and CV can be easily metabolized into leucomalachite green (LMG) and leucocrystal violet (LCV), respectively. CAP is a broad-spectrum antibacterial drug which was also widely used in veterinary treatments.

However, these VMPs and Dyes are prohibited globally due to their carcinogenic and mutagenic potency or to the additional risk of causing aplastic anemia in the case of CAP. These compounds are currently regulated at the target level of 1 μg/kg for NFs, 3 μg/kg for NIIMs, 2 μg/kg for Dyes, and 0.3 μg/kg for CAP in the EU in accordance with the respective Minimum Required Performance Limits (MRPL) set for CAP, NFs and MG (Dyes) (European Commission, 2003; European Commission, 2004) and later Reference Points for Action (RPA) set for the same CAP, NFs and MG (Dyes) (EFSA, 2014; EFSA, 2015; EFSA, 2016) and in accordance with the EU-RLs Recommended Limits set for NIIMs (CRL Guidance, 2007). In order to reduce the number of implemented analytical methods for the control of banned antimicrobial and dye residues in food from animal origin and especially in aquaculture products, it is of interest to combine them all when possible but with keeping high standard level of reliability of the official control.

The recent mostly used methods for the determination of NFs, NIIMs, Dyes and CAP were LC-MS/MS due to the unambiguous identification and accurate quantification possibilities. For NFs, most currently published analytical methods mainly focused on AOZ,

AMOZ, SEM, and AHD in animal derived food (Zhang et al., 2017; Kaufmann, Butcher, Maden, Walker, & Widmer, 2015; El-Demerdash, Song, Reel, Hillegas, & Smith, 2015; Verdon, Couedor, & Sanders, 2007; Shendy, Al-Ghobashy, Alla, & Lotfy, 2016). There are very few methods for determination of the four other above-mentioned NFs. Kaufmann et al. (2015) have developed a method to determine PSH, DNSH and AMG. Verdon et al. (2007) reported a method to analyze DNSH in poultry muscle. There were many methods applied to analyze NIIMs in animal-derived food (Zhang et al., 2017; Granja et al., 2013; Tölgyesi, Sharma, Fekete, Fekete, Simon, & Farkas, 2012; Cronly et al., 2009; Hurtaud-Pessel, Delepine, & Laurentie, 2000). There also have been many methods to determine Dyes in aquaculture products (Hurtaud-Pessel, Couëdor, Verdon, & Dowell, 2013; Schneider, & Andersen, 2015; Kaplan, Olgun, & Karaoglu, 2014; Ascari, Dracz, Santos, Lima, Diniz, & Vargas, 2012). For CAP, many analytical methods have also been reported over the 20 past years.

However, very few multi-class methods were applied for the simultaneous analysis of these four groups of target analytes in animal-derived food due to their different physicochemical characteristics. Zhang et al. (2017) have reported to analyze four NFs (AOZ, AMOZ, SEM, and AHD), 7 NIIMs and CAP in chicken muscle and eggs. Shendy et al. (2016) described a method to simultaneously determine NFs and NIIMs including AOZ, AHD, AMOZ, SEM, RNZ, and DMZ in honey. There are a few methods to determine NFs and CAP in animal food product (Kaufmann et al., 2015; El-Demerdash et al., 2015; An et al. 2015; Veach, Baker, Kibbey, Fong, Broadaway, & Drake, 2015). Up to now, there are no methods for analysis of all the four groups of target analytes simultaneously in animal-derived food.

The sample preparation was a crucial factor for analysis of target analytes. NFs metabolites are strongly bound to proteins, which need to be released through mild acid hydrolysis before analysis. The derivatization of NFs metabolites has been recognized to be essential, since the strong polar (poor retention on RP columns) of underivatized metabolites show poor ionization properties in the electrospray interface of a mass spectrometer. Most methods for NFs analysis applied HCl for hydrolysis and 2-NBA for metabolite derivatization at 37 ◦C for 14 h followed by either liquid-liquid extraction or solid-phase extraction, which is time-consuming. For NIIM, Dyes and CAP, the mostly used methods extracted these substances either with ethyl acetate or acetonitrile or with a buffer solution, and followed by a solid-phase extraction (SPE) or a liquid-liquid extraction clean-up. For all the four groups of target analytes, the sample preparation became a subtle procedure to elaborate for achieving acceptable extraction and/or efficient clean-up at the same time for all groups.

The aim of our project is to develop a multi-residue method based on LC-MS/MS capable of reaching low Reference Point for Action (RPA) levels for the control for chloramphenicol, nitrofurans and their metabolites, and possible dyes or nitroimidazoles in the aquaculture products. In this article, LC-MS/MS in both positive and negative ESI modes is used to detect multi-banned substances. The sample preparation procedure was initiated with hydrolysis and derivatization followed by modified QuEChERS, which simplified the extraction method and reduced the extraction time. Internal standards are used to reach more accurate quantification. The performance of the method was evaluated and validated according to the criteria of the Decision (EC) no 2002/657.

2. Materials and methods

2.1 Chemicals and reagents

Analytes: 1-amino-hydantoin (AHD), 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), semicarbazide (SEM), aminoguanidin hydrochloride (AMG), 3,5-dinitrosalicylic acid hydrazine (DNSH), nifurpirinol (NPIR), salicylic acid hydrazine (PSH), dimetridazole (DMZ), metronidazole (MNZ), ronidazole (RNZ), ipronidazole (IPZ), hydroxy-metronidazole (MNZOH), 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI), hydroxy-ipronidazole (IPZOH), malachite green (MG), leuco-malachite green (LMG), crystal violet (CV), leuco-crystal violet (LCV), brilliant green (BG), and Chloramphenicol (CAP).

Internal standards: NP-AHD-$^{13}C_3$, NP-AOZ-D_4, NP-SEM-^{13}C,$^{15}N_2$, NP-AMOZ-D_5, DMZ-D_3, HMMNI-D_3, IPZ-D_3, MNZ-$^{13}C_2$,$^{15}N_2$, IPZOH-D_3, MNZOH-D_2, RNZ-D_3, MG-D_5, LMG-D_5, CV-D_6, LCV-D_6, and CAP-D_5.

These analytes and internal standards were obtained from Sigma-Aldrich (St-Quentin Fallavier, France), Witega (Berlin, Germany), LGC standards (Molsheim, France), Ehrenstorfer GmbH (Augsburg, Germany), and CIL Cluzeau (Ste Foy-La-Grande, France).

HPLC-MS grade methanol, acetonitrile, ethyl acetate, and iso-hexane were purchased from Fisher Scientific (Illkirch, France). HPLC grade formic acid and ammonium formate were from Fisher Scientific (Illkirch, France). Nitrobenzaldehyde (2-NBA), hydrochloric acid, and sodium chloride were obtained from Sigma-Aldrich (St-Quentin Fallavier, France). Di-potassium hydrogen phosphate was bought from VWR (Fontenay sous Bois, France).

2.2 LC–MS/MS analysis

HPLC system was composed of HPLC pump Ultimate 3000, Autosampler Ultimate 3000, and Column oven 3000 (Dionex, Villebon sur Yvette, France). Symmetry C_{18} analytical column (5 µm, 100 x 2.1 mm) from Waters Co. (Guyancourt, France) was used to separation the analytes. The mobile phase was 20 mM ammonium formate+0.02% formic acid (A) and methanol (B) with a gradient elution at 1.0 ml/min flow rate as follows (t in min): $t_{0'}$, A = 90%; $t_{6'}$, A = 10%; $t_{9'}$, A = 10%; $t_{9.1'}$, A =90%; $t_{12'}$, A = 90%.

Mass spectrometry was TSQ Vantage (Thermo scientific, Villebon sur Yvette, France) with electrospray interface (ESI). The source parameters were as follows: Spray voltage: 3000 V; Vaporizer temperature: 300 °C; Sheath gas pressure: 30 psi; Aux gas pressure: 30 psi; Capillary temperature: 300 °C; Cycle time: 0.5 s. The mass spectrometer was operated in a selective reaction monitoring (SRM) mode selecting one precursor ion and two product ions per each target compound, except for internal standard for which only one SRM transition was monitored for each The SRM acquisition parameters, ie. m/z precursor ion, m/z product ion, and collision energy are shown in Table S1.

2.3 Samples

Five hundred grams of aquaculture products, including salmon, trout, and shrimp, were purchased from a local supermarket. After being homogenized in a high-speed food blender, the samples were stored below -20 °C prior to using for the method developed . The samples taken for the validation and the way the sampling was operated. One gram portion of samples was taken to enter the extraction-purification process. The goal was to demonstrate the capacity of the method to deliver accurate data on different fish/aquaproduct species.

2.4 Sample preparation

One gram portion of sampled flesh was added 5 mL of hydrochloric acid (0.1 mol/L) and 0.2 mL of 50 mmol/L of nitrobenzaldehyde solution freshly prepared in methanol. After derivatization for 2 h at 60 ºC with moderate shaking, 3 mL of 0.5 mol/L di-potassium hydrogen phosphate, 5 mL of iso-hexane, and 2 g NaCl were added and mixed for 5 min. After centrifugation for 5 min at 6000 g at 4 °C, the (upper) iso-hexane phase was discarded. Five milliliters of ethyl acetate were added to the remaining extraction solution, and vortexed for 30 s, and then mixed for 10 min. After centrifugation for 10 min at 6000 g at 4 °C, 5 mL of the (upper) organic phase was transferred to a 10 mL polypropylene tube. Five milliliters of acetonitrile were added to the samples again, and vortexed 30 s and mixed for 10 min. After centrifugation for 10 min at 6000 g at 4 ° C, 5 mL of the (upper) organic phase was transferred to a 10 mL polypropylene tube. Then, all the extraction solution was collected and then evaporated under gentle nitrogen flow at 50 °C after adding of 2 g $MgSO_4$ in order to obtain an oily residual phase. The residues were then dissolved with 0.5 mL of acetonitrile and filtered through a 0.22 μm PVDF filter.

2.5 Method validation according to Decision No. (EC) 2002/657

2.5.1 Identification parameters

The performance of the method was assessed through its qualitative parameters: analyte specificity, molecular identification in terms of retention time (RT), and of transition ion ratios. The specificity of the assay was demonstrated by analyzing 20 representative blank tissue samples and checking interfering peaks at the retention time of target analytes. According to European Union Commission Decision No. 2002/657 (European Commission, 2002) with a minimum total score of 3 for Group B (authorized substances) or 4 for Group A

192 (non-authorized substances) identification points, one precursor ion and two product ions
193 were sufficiently monitored to fulfil this requirement. The analytes were additionally
194 identified by matching retention times of peaks with the values of the corresponding standard
195 analyzed under the same experimental conditions. The analyte in the sample should be eluted
196 at the retention time corresponding to the analyte in spiked samples (within a range of relative
197 retention time of ± 2.5%). Each analyte ion ratio was effectively measured on each
198 chromatogram, corresponding to the less intense SRM transition signal against the most
199 intense SRM transition ion one.

200 2.5.2 Quantitative parameters

201 The performance of the method was assessed through its 3 main quantitative parameters:
202 – 1) accuracy demonstrated in terms of trueness – 2) precision, the precision being expressed
203 as the intra- and inter-day/series repeatabilities, and – 3) confirmatory analytical limits (limit
204 of decision CCα and capacity of detection CCβ). The validation was performed with a set of
205 three series of analyses including salmon, trout, and shrimp. For each of the series, the
206 experiment comprised 24 samples: 6 calibration standard (CS) samples over the range of
207 calibration claimed for each group of substances, and 18 validating standard (VS) samples set
208 at three concentrations and for each concentration being repeated 6 times. One of the series
209 was analysed each day. The CS and VS samples are matrix-spiked samples, ie they are
210 prepared by addition of standards to blank matrix prior to extraction. CCα and CCβ were
211 recommended in Commission Decision No. (EC) 2002/657. In this article, CCα and CCβ
212 were calculated as described by Verdon et al. (2007).

213 This calibration curve was built from the CS samples using linear regression model
214 AX+B using specific internal standard for each substance. This calibration curve was then
215 used to measure the back-calculated concentration of each compound in the validating

samples (VS). The calibration range of the CS samples for each substance was made of 6 levels including the negative control: for Nitrofurans, at 0.0, 0.5, 1.0, 1.5, 2.0, and 3.0 µg/kg; for Dyes, at 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 µg/kg; for Nitroimidazoles, at 0.0, 1.5, 3.0, 4.5, 6.0, and 7.5 µg/kg; and for CAP, at 0.0, 3.0, 4.5, 6.0, 7.5, and 9.0 µg/kg. Internal standard concentration used for Nitrofurans, Dyes, Nitroimidazoles, and CAP were 2.0, 2.0, 5.0, and 5.0 µg/kg, respectively.

The percentage of trueness for the estimated concentration of the analytes back-calculated from the validating standards (VS) was expressed as the bias measured from the actually spiked concentration and estimated for each analyte at three levels (0.5, 1.0, and 2.0 µg/kg for Nitrofurans; 1.0, 2.0, and 4.0 µg/kg for Dyes; 1.5, 3.0, and 6.0 µg/kg for Nitroimidazoles, and 3.0, 4.5, and 6.0 µg/kg for CAP). Each concentration was repeated 6 times within a day for the intra-day precision test. The precision in terms of repeatability and reproducibility was evaluated by calculating the relative standard deviation (RSD) for each analyte at each level of concentration.

3. Results and discussion

3.1 Optimization of LC-MS/MS

In order to obtain nice resolution and high sensitivity, mobile phase and analytical column should be chosen based on the evaluation of ionization efficiency. Almost all references covering the topic of our project claimed LC separations performed on reversed-phase (RP) materials by applying either methanol/water or acetonitrile/water mixtures as mobile phase operated in the gradient as well as isocratic mode. In some cases, acetic acid, formic acid, and ammonium salts are added to support analyte ionization and improve the chromatographic separation efficiency. So, methanol, acetonitrile, formic acid, and different ratio of ammonium acetate (aqueous ammonia) were tested. Better optimized

241 sensitivity was observed when using a mixture of methanol and of 20 mmol/l ammonium
242 formate with 0.02% formic acid.
243 Four different columns were tested to separate the target compounds, including Acquity
244 BEH C_{18} (50 X 2.1 mm, 1.7 μm), Accucore Phenyl Hexyl (100 X 2.1 mm, 4.6 μm), Symmetry
245 C_{18} (150 X 3.9 mm, 5 μm), Colone Hypersil Gold (50 X 2.1 mm, 1.9 μm). The results showed
246 that Symmetry C_{18} (3.9 X 150 mm, 5 μm) gave both good peak shapes and higher ion
247 response.

248

249 ***3.2 Optimization of sample preparation***

250 3.2.1 Optimization of acid hydrolyzed and derivatized conditions
251 Since its easy combination with proteins *in vivo,* Nitrofuran metabolites need to be
252 released from tissues by means of acid hydrolysis, and simultaneously derivatized with
253 2-NBA after its release from the tissue proteins. The commonly used derivatization procedure
254 was 14 h at 37 ºC (Kaufmann et al., 2015) or 4 h at 55 ºC (Verdon et al., 2007). The
255 derivatization temperature and time have been investigated to improve the extraction efficient
256 and shorten the derivatization time. First, we compared the derivatization temperature and
257 time according to published articles (14 h at 37 ºC, and 4 h at 55 ºC). We also tested another
258 third condition (2 h at 60 ºC) to try shortening the derivatization time. The results showed that
259 derivatization for 2 h at 60 ºC delivered slightly higher recovery for most of the nitrofuran
260 analytes (Fig.1).
261 Moreover, different derivatization time (2, 3, and 4 h) were performed at 60 ºC to
262 evaluate the extraction efficiency. When increasing the derivatization time, the recovery of
263 nitrofuran metabolites did not significantly improve. So, 2 h was finally selected as extraction
264 time (Fig. 1).
265 In this study, we also confirmed that the acid hydrolysis and derivatization steps both

may affect the recovery of NIIMs, Dyes and CAP. When the derivatization time was increased up to 4 h, the recovery showed a start of decrease for Dyes and did not significantly change for NIIMs and CAP.

Moreover, the pH value of derivatization was also tested. When adding the hydrochloric acid and the nitrobenzaldehyde solution, the pH value was adjusted to 2.0，2.5, and 3.0, separately. The results showed that the derivatization efficiency for most of the analytes was decreased with increasing pH values from 2.0 to 3.0.

3.2.2 Optimization of the extraction procedure

To extract the analytes from animal food matrix with as little interference and as much high recovery as possible, is the most difficult and critical process. Originally, the QuEChERS method involved a single extraction step, i.e. a sample clean up via dispersive solid phase extraction using primary secondary amines (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003). In this study, a modified QuEChERS extraction protocol without sample clean up followed by evaporation was optimized and employed.

The most important impacting factors were the extraction solvent and the extraction volume. Some articles have reported to extract nitrofurans using ethyl acetate at pH 7 condition (Kaufmann et al., 2015; Verdon et al., 2007; Kim, Kim, Seok-Won, Lee, & Kim, 2015), some using acetonitrile (Shendy et al., 2016; An et al., 2015). For nitroimidazoles, most published papers used ethyl acetate (Granja et al., 2013; Boison, Asea, & Matus, 2012) and acetonitrile (Tölgyesi et al., 2012; Cronly et al., 2009) as the extraction solvent. Most articles developed a method to determine Dyes using acetonitrile as the extraction solvent (Hurtaud-Pessel et al., 2013; Schneider et al., 2015; Kaplan et al., 2014; Ascari at al., 2012; Qin et al., 2016; Dowling, Mulder, Duffy, Regan, & Smyth, 2007). For chloramphenicol, ethyl

289 acetate was used most of the time as the extraction solvent.

290 According to the properties of the four-family analytes, ethyl acetate, acetonitrile, and
291 their mixture, at different pH values, were chosen as extraction solvents and compared. The
292 tested matrix was trout. From the results (Fig. 2), we observed the mixture of ethyl acetate
293 and acetonitrile exhibited the best extraction efficiency especially for MG, CV, and BG,
294 which was about more than 50% higher than those exhibited by other solvents. Therefore, the
295 mixture of ethyl acetate and acetonitrile was chosen as the extraction solvent. When
296 comparing extraction volume of ethyl acetate (5 mL and 10 mL), the extraction efficiency was
297 not significantly different. So, 5 mL ethyl acetate was considered sufficient to extract the
298 target VMP residues from fish matrix.

299 After extraction with acetonitrile, several salts were investigated as salting-out agents in
300 order to increase the ratio of the analytes into the acetonitrile layer and decrease water mixing
301 into the acetonitrile layer. After optimization of the parameters, salting out and complete
302 phase separation was achieved via addition of 2 g $MgSO_4$ and 2 g NaCl for having a higher
303 efficiency of extraction, especially for Dyes.

304 When developing the method using trout as tested matrix, there was no fat-soluble
305 interference. However, when using salmon as matrix, fat-soluble interference was observed.
306 So, before extracting, iso-hexane was added to decrease/remove the fat-soluble interference.
307 In order to determine the targeted compounds in all matrices using the same sample
308 preparation method, iso-hexane was used to remove fat-soluble interference before extraction
309 in the three species: i.e. trout, salmon, and shrimp.

310 After derivatization, the main step described by published articles was to neutralize the

311 solution by adding 5 mL of 0.1M di-potassium hydrogenophosphate followed by adding 1M
312 NaOH to adjust the pH to 7.0±0.5 (controlled by pH strips). In order to simplify the
313 procedure, we tested different volumes and different concentrations of di-potassium
314 hydrogenophosphate to adjust pH to 7.0±0.5. After optimization, 3 mL of 0.5 mol/L
315 di-potassium hydrogen phosphate was finally kept to adjust pH to 7.0±0.5.
316 3.2.3 Optimization of reconstituting solvent recovery after evaporation step
317 The composition of the sample solvent medium before injection directly affects both the
318 separation behavior of analytes in the HPLC column and their sensitivity during LC–MS/MS
319 detection. For nitroimidazoles, nitrofurans, and chloramphenicol, most published articles
320 report using the mobile phase to dissolve/reconstitute the residues after the evaporation step.
321 However, for dyes, the residue was usually reconstituted with ammonium acetate-acetonitrile
322 (1:1) (Ascari et al., 2012), acetonitrile-water (60/40) (Huang, Zhao, Dai, Hou, Zhao, & Liang,
323 2016), or acetonitrile (Schneider et al., 2015). To select the optimal solvent medium, different
324 ratios of methanol-water, methanol-formic acid, methanol-ammonium formate solution, and
325 acetonitrile-water, acetonitrile-formic acid, and acetonitrile-ammonium formate solution were
326 compared in this study. The result showed that when using the mixture of methanol-water or
327 acetonitrile-water as reconstituting solvent, the extracting solvent recovery for dyes was very
328 low due to insufficient power of miscibility. When using pure methanol or pure acetonitrile to
329 dissolve the residues, a higher recovery was obtained (Fig. 3). Therefore, acetonitrile was
330 chosen as the dissolving/reconstituting solvent prior to injection. The injection volume for
331 LC-MS/MS mostly used in the published articles was 10 μL. However, when injecting 10 μL
332 to LC-MS/MS, the shape of nitroimidazole peaks were very wide. So, different volumes of

injection were tested, including 2, 5, 10 μL. The results showed that 2 μL as injection volume led to a satisfactory peak shape for all the analytes.

3.3 Method validation

The matrix-spiked calibrations of nitroimidazoles, nitrofurans, dyes, and chloramphenicol for salmon, trout, and shrimp were established, respectively. Matrix-spiked calibration regression' coefficient of correlation R were in the range from 0.9916 to 0.9997 for nitrofurans over the concentration range of 0-3.0 μg/kg, from 0.9980 to 1.000 for nitroimidazoles over the concentration range of 0-7.5 μg/kg, from 0.9909 to 0.9999 for dyes over the concentration range of 0-5.0 μg/kg, and from 0.9962 to 0.9976 for dyes over the concentration range of 0-9.0 μg/kg in aquaculture products.

The CCαs of confirmation for nitroimidazoles, nitrofurans, dyes and chloramphenicol in aquaculture products varied from 0.067 to 0.512 μg/kg for salmon, 0.077 to 0.848 μg/kg for trout, 0.083 to 1.655 μg/kg for shrimp, respectively.

The recoveries of nitrofurans, nitroimidazoles, dyes and chloramphenicol estimated at three concentrations in aquaculture products ranged 89.8-112.0%, 77.2-104.4%, 83.3-107.6%, and 93.0-125.6%, respectively, except for DNSH at 2.0 μg/kg and 3.0 μg/kg in shrimp slightly exceeding the criteria of performance recommended into the Decision 2002/657 to be ranging [-50% - +20%]. The RSD were less than 24.7%, 13.7%, 12.8%, and 16.1% in aquaculture products. The trueness of nitrofurans, nitroimidazoles, dyes and chloramphenicol, expressed as bias, were from -8.8% to +8.9% except for DNSH at 1.0 μg/kg and 2.0 μg/kg with a trueness in the range of 16%-18%. All the data of accuracy and precision are showed in Table

355 1.

356 The specificity of the assay was demonstrated by checking interfering peaks at the
357 retention time of target analytes. The results showed that there were no interference peaks
358 co-eluting with target analytes (Fig. 4). All the chromatograms obtained throughout the
359 validation study showed a very good stability of the retention times for all the analytes with
360 relative deviations always remaining below ±2.5%. According to European Union
361 Commission Decision 657/2002 (European Commission 2002) with a minimum total score,
362 one precursor ion and two product ions were monitored. This requirement is fulfilled for the
363 method (Table S1). Each analyte ion ratio was effectively measured on each chromatogram,
364 corresponding to the less intense signal against the most intense one. During the validation,
365 the ion ratios measured on the spiked samples were compared with those obtained from the
366 matrix matched calibration curve standards (Table 2). The calculated ion ratio results from
367 this work were in compliance with the ion ratio tolerance laid down in EU Commission
368 Decision No. (EC) 2002/657.

369 ***3.4 Further method improvement***

370 In this study, nitroimidazoles, nitrofurans, dyes and chloramphenicol in aquaculture
371 products were simultaneously determined by LC-MS/MS. However, some further method
372 development should still be carried out to improve the method's performance. In fact, the
373 analytical limits expressed as CCα were satisfactory according to the current RPAs but
374 excluding for CAP, for which the RPA level of 0.3 µg/kg is not reached. This method should
375 be further optimized to increase the sensitivity of the detection for CAP. Furthermore, the
376 matrices used to validate the method only included trout, salmon, and shrimp tissues. This

method can be more extensively developed to determine residues in other aquaculture products. Finally the pre-treatment of samples is still a crucial step before LC-MS/MS detection. We believe that fast and friendly-environmental techniques will be highly concerned in the future.

4. Conclusions

A new LC-MS/MS confirmatory method was tentatively developed to match with the relevant challenge of identifying and quantifying simultaneously the major prohibited veterinary substances in aquaculture products, ie. nitroimidazoles, nitrofurans, dyes, and chloramphenicol. The sample preparation procedure included acid hydrolysis and derivatization steps as to favor the detection of the nitrofuran metabolites. After optimization, the derivatization time was decreased compared to those of published articles dedicated to NFs. The extraction procedure was also simplified by using di-potassium hydrogen phosphate to adjust the pH to 7.0±0.5. It was the first time that a modified QuEChERS method was used to extract these four family compounds in aquaculture products before analysis. The results indicated that the sample preparation method was able to extract and clean up the residues of nitroimidazoles, nitrofurans, dyes, and chloramphenicol in aquaculture products in a quick and cheap way.

The simultaneous detection and quantification of 21 target compounds, ie. 8 nitrofurans, 7 nitroimidazoles, 5 dyes, and chloramphenicol were performed by a reversed-phase liquid chromatography coupled with LC-MS/MS. To the authors' knowledge, it is the first time to separate these four family compounds using one single liquid phase

399 separative system. The method demonstrates satisfactory validation characteristics with
400 respect to specificity, trueness, precision, and sensitivity for all of the target compounds
401 excepting a lightly reduced performance in trueness for DNSH and the fact that CAP cannot
402 be detected down to the EU reference point for action "RPA" level currently set at 0.3 μg/kg.
403 It will potentially be a useful tool for accurately monitoring the residues of nitroimidazoles,
404 nitrofurans, dyes, and chloramphenicol in aquaculture products and protecting consumer
405 health.
406
407 **Conflict of interest**
408 The authors declare that they have no known competing financial interests or personal
409 relationships that could have appeared to influence the work reported in this paper.
410
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Figure legends

Fig. 1 Comparative results for different derivatized temperatures (A) and time (B).

Fig. 2 The effect of extraction solvent on recoveries of analytes fortified in trout samples.

Fig. 3 The effect of extraction solvent recovery on injection in LC-MS/MS

Fig. 4 LC-MS/MS chromatograms of multi-banned substances in salmon samples.

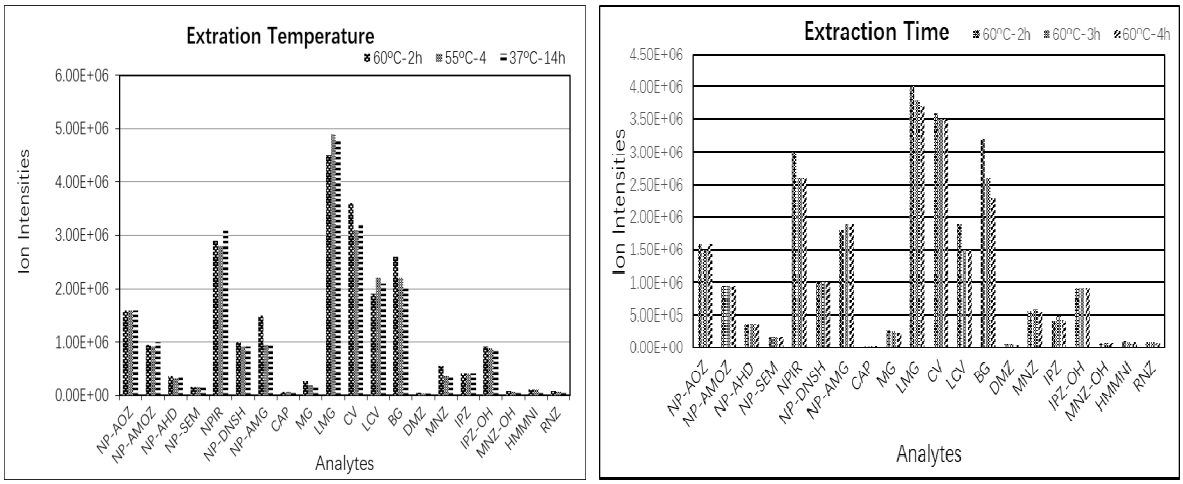
(0.5µg/kg for Nitrofurans; 1.0µg/kg for Dyes; 1.5µg/kg for Nitroimidazoles, and 3.0 µg/kg for CAP).

Tables

Table 1 The accuracy, expressed as recovery for each salmon, trout and shrimp matrix and expressed globally in terms of trueness (bias %) and intra-lab inter-series inter-day reproducibility

Table 2 Relative ion abundancy of analytes in matrix-matched standard solutions and in matrix-spiked salmon.

548 **Figures**



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550 Fig. 1 Comparative results for different derivatized temperatures (A) and time (B).

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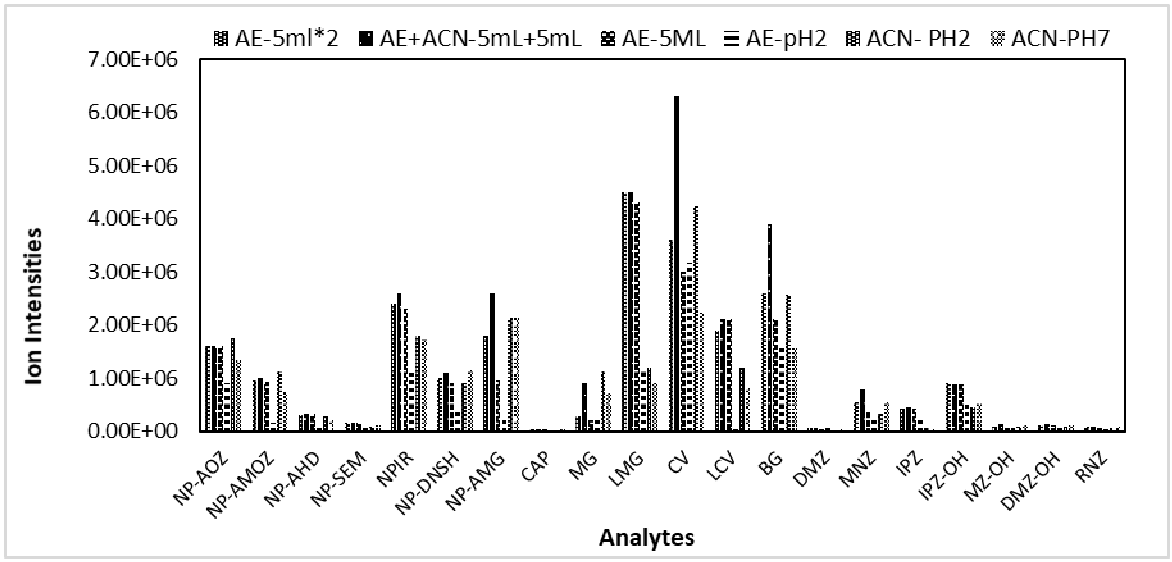

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553 Fig. 2 The effect of extraction solvent on recoveries of analytes fortified in trout samples.

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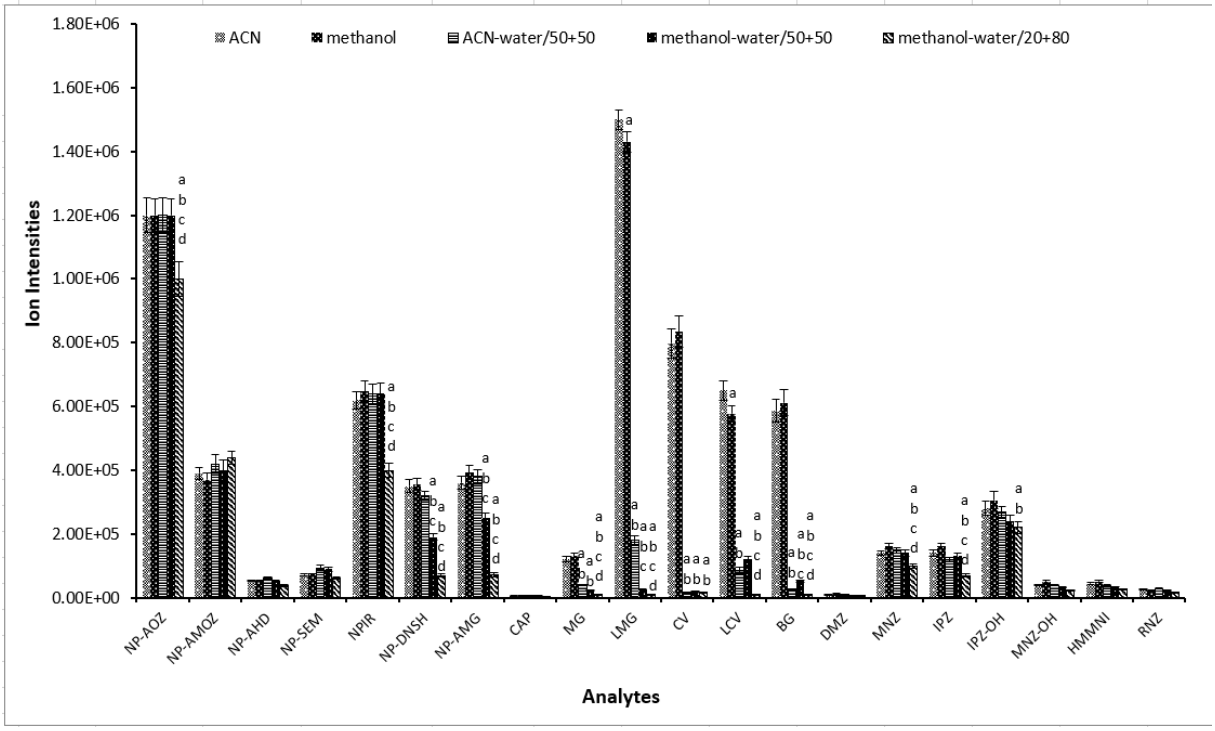


556

557 a Statistical significances compared with ACN are $p < 0.05$.

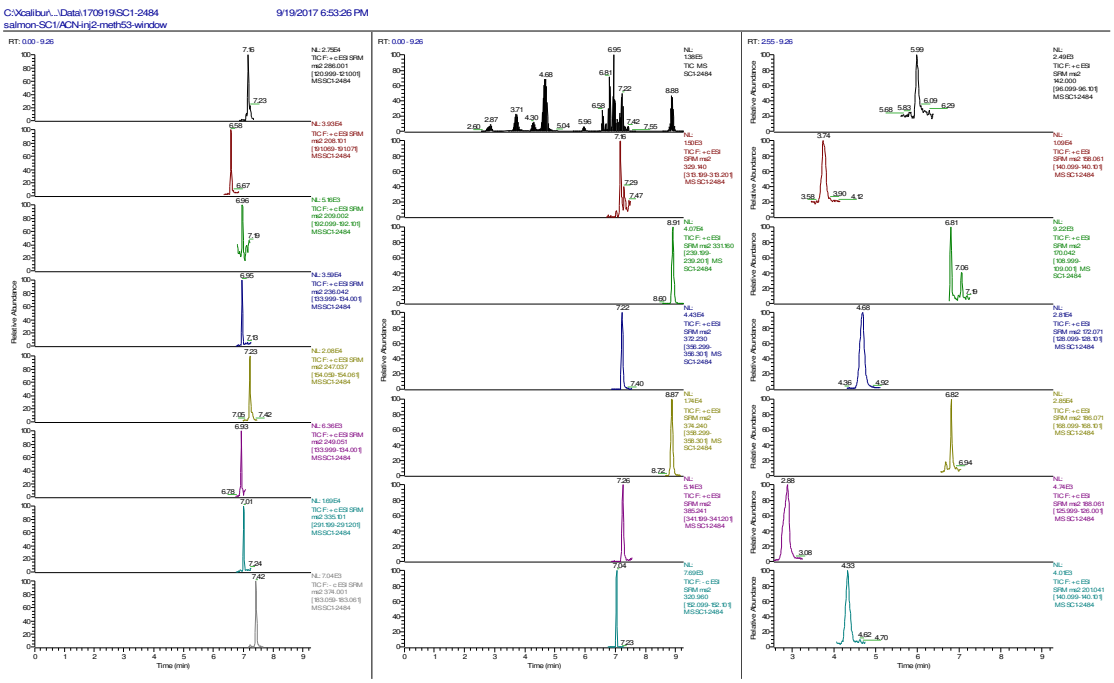
558 b Statistical significances compared with methanol are $p < 0.05$.

559 c Statistical significances compared with ACN-water/50+50 are $p < 0.05$.

560 d Statistical significances compared with methanol-water/50+50 are $p < 0.05$.

561 Fig. 3 The effect of extraction solvent recovery on injection in LC-MS/MS

562



563

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Tables

Table 1 The accuracy, expressed as recovery for each salmon, trout and shrimp matrix and expressed globally in terms of trueness (bias %) and intra-lab inter-series inter-day reproducibility

Compound	Spiked Concentration (µg/kg)	Salmon		Trout		Shrimp		Trueness (%) (n=18)	Reproducibility inter-series (%) (n=18)
		Estimated Concentration ±SD (µg/kg) (n=6)	Recovery (%)	Estimated Concentration ±SD (µg/kg) (n=6)	Recovery (%)	Estimated Concentration ±SD (µg/kg) (n=6)	Recovery (%)		
NP-AHD	0.5	0.562±0.023	112.4	0.485±0.060	97.0	0.494±0.038	98.8	2.8	10.5
	1.0	1.041±0.099	104.1	1.109±0.088	110.9	1.014±0.133	101.4	5.5	10.4
	2.0	1.920±0.115	96.0	1.903±0.088	95.2	1.929±0.154	96.5	4.1	6.0
NP-AOZ	0.5	0.484±0.008	96.8	0.487±0.013	97.4	0.474±0.030	94.8	0.8	6.5
	1.0	0.985±0.036	98.5	1.003±0.061	100.3	0.861±.0690	86.1	-2.9	5.2
	2.0	1.905±0.097	95.3	1.951±0.054	97.6	2.050±0.069	102.5	2.4	5.8
NP-SEM	0.5	0.467±0.051	93.4	0.466±0.074	93.2	0.498±0.058	99.6	-4.5	12.6
	1.0	1.043±0.088	104.3	0.898±0.084	89.8	0.935±0.145	93.5	-4.1	9.9
	2.0	1.977±0.109	98.9	1.966±0.181	98.3	2.014±0.134	100.7	0.7	6.9
NP-AMOZ	0.5	0.511±0.157	102.2	0.501±0.574	100.2	0.500±0.006	100.0	0.8	6.5
	1.0	0.976±0.038	97.6	0.985±0.058	98.5	0.950±0.054	95.0	-2.9	5.2
	2.0	1.931±0.089	96.6	2.051±0.113	102.6	1.877±0.066	93.9	-2.4	5.8
NP-AMG	0.5	0.516±0.008	103.2	0.484±0.037	96.8	0.496±0.022	99.2	-0.3	5.3
	1.0	1.015±0.064	101.5	0.946±0.072	94.6	1.061±0.087	106.1	2.2	8.5
	2.0	2.020±0.094	101.0	1.859±0.128	93.0	1.991±0.146	99.6	0.7	7.0
NP-DNSH	0.5	0.465±0.036	93.0	0.532±0.043	106.4	0.560±0.115	112.0	3.8	15.6
	1.0	1.094±0.043	109.4	1.000±0.081	100.0	1.409±0.280	140.9	16.7	20.7
	2.0	2.002±0.24	100.1	1.920±0.067	96.0	2.865±0.482	143.3	18.1	24.7
NP-PSH	0.5	0.471±0.041	94.2	0.571±0.015	114.2	0.454±0.216	90.8	-0.3	11.9
	1.0	1.081±0.092	108.1	0.904±0.072	90.4	1.032±0.089	103.2	0.5	11.0

	2.0	1.958±0.167	97.9	1.885±0.122	94.3	2.095±0.109	104.8	1.0	7.9
NPIR	0.5	0.500±0.038	100.0	0.527±0.023	105.4	0.452±0.029	90.4	-1.4	8.8
	1.0	1.036±0.042	103.6	1.050±0.079	105.0	0.946±0.102	94.6	1.1	8.7
	2.0	1.933±0.038	96.7	2.103±0.023	105.2	2.112±0.029	105.6	2.5	7.6
DMZ	1.5	1.457±0.044	97.1	1.490±0.054	99.3	1.158±0.185	77.2	-8.8	13.7
	3.0	2.948±0.109	98.3	2.906±0.158	96.9	2.843±0.225	94.8	-3.4	5.7
	6.0	5.71±0.243	95.2	6.026±0.213	100.4	5.929±0.247	98.8	1.9	4.4
HMMNI	1.5	1.473±0.049	98.2	1.533±0.072	102.2	1.502±0.072	100.1	0.2	4.4
	3.0	2.910±0.049	97.0	2.895±0.072	96.5	2.889±0.098	96.3	-3.4	2.5
	6.0	5.503±0.136	91.7	5.848±0.169	97.5	6.053±0.118	100.9	-3.3	4.6
IPZ	1.5	1.191±0.098	79.4	1.518±0.083	101.2	1.421±0.180	94.7	-1.5	8.6
	3.0	3.035±0.123	101.2	2.909±0.147	97.0	3.033±0.219	101.1	-0.3	5.7
	6.0	5.980±0.116	99.7	5.736±0.359	95.6	6.126±0.204	102.1	-0.9	4.8
MNZ	1.5	1.491±0.024	99.4	1.507±0.029	100.5	1.499±0.028	99.9	-0.1	1.8
	3.0	2.904±0.072	96.8	2.911±0.056	97.0	2.879±0.039	96.0	-3.4	1.9
	6.0	5.693±0.078	94.9	5.855±0.081	97.6	5.854±0.068	97.6	-3.3	1.8
IPZOH	1.5	1.547±0.083	103.1	1.314±0.036	87.6	1.475±0.122	98.3	-3.6	9.0
	3.0	2.955±0.079	98.5	2.937±0.109	97.9	3.042±0.192	101.4	-0.7	4.6
	6.0	5.824±0.334	97.1	5.871±0.109	97.9	6.021±0.198	100.4	-1.6	4.0
MNZOH	1.5	1.515±0.034	101.0	1.551±0.065	103.4	1.479±0.024	98.6	1.0	3.4
	3.0	2.874±0.088	95.8	2.987±0.105	99.6	2.911±0.064	97.0	-2.5	3.3
	6.0	5.832±0.073	97.2	5.787±0.146	96.5	5.793±0.082	96.6	-2.3	1.8
RNZ	1.5	1.508±0.088	100.5	1.566±0.047	104.4	1.555±0.037	103.7	2.9	4.1
	3.0	2.891±0.078	96.4	3.041±0.114	101.4	2.984±0.167	99.5	-0.9	4.5
	6.0	5.702±0.121	95.0	6.092±0.129	101.5	5.995±0.376	99.9	-1.2	4.8
MG	1.0	1.004±0.099	100.4	0.921±0.091	92.1	1.023±0.172	102.3	-1.7	13.0
	2.0	1.897±0.083	94.9	1.934±0.168	96.7	2.067±0.205	103.4	-1.7	8.6
	4.0	3.977±0.229	99.4	3.961±0.158	99.0	4.169±0.235	104.2	-0.9	5.5
LMG	1.0	1.029±0.040	102.9	1.021±0.031	102.1	0.993±0.029	99.3	1.4	3.5

	2.0	2.042±0.044	102.1	1.935±0.073	96.8	2.023±0.018	101.2	0	3.4
	4.0	3.692±0.049	92.3	3.746±0.075	93.7	4.024±0.086	100.6	4.5	4.3
CV	1.0	1.011±0.034	101.1	0.873±0.030	87.3	0.967±0.029	96.7	1.3	6.8
	2.0	1.928±0.035	96.4	1.872±0.065	93.6	1.881±0.043	94.1	-5.3	2.8
	4.0	3.815±0.086	95.4	3.896±0.087	97.4	3.840±0.031	96.0	-3.7	2.0
LCV	1.0	1.012±0.038	101.2	1.049±0.040	104.9	0.958±0.057	95.8	0.6	5.8
	2.0	2.046±0.049	102.3	1.895±0.074	94.8	2.032±0.041	101.6	-0.4	4.4
	4.0	3.984±0.109	99.6	3.804±0.104	95.1	4.034±0.120	100.9	1.5	3.7
BG	1.0	0.993±0.082	99.3	1.048±0.080	104.8	1.070±0.114	107.0	3.7	9.1
	2.0	1.939±0.218	97.0	2.011±0.035	100.6	2.152±0.104	107.6	1.7	7.1
	4.0	3.332±0.455	83.3	4.113±0.215	102.8	4.078±0.298	102.0	-3.8	12.8
CAP	3.0	2.790±0.368	93.0	3.243±0.221	108.1	3.769±0.421	125.6	8.9	16.1
	4.5	4.652±0.503	103.4	4.511±0.225	100.2	4.895±0.292	108.8	4.1	8.0
	6.0	6.027±0.284	100.5	5.981±0.312	99.7	6.456±0.626	107.6	2.6	7.6

570 Table 2 Relative ion abundancy of analytes in matrix-matched standard solutions and in
571 matrix-spiked salmon.

Toxin	Average of ion ratios of standard solution (%)	Maximum permitted tolerances	Average of ion ratios of matrix-spiked salmon (%)
NP-AHD	45.7	34.2%-57.1% (±25%)	45.4
NP-AOZ	60.5	48.4%-72.6% (±20%)	60.7
NP-SEM	70.6	56.5%-84.7% (±20%)	68.3
NP-AMOZ	34.7	26.0%-43.3% (±25%)	34.8
NP-AMG	30.3	22.7%-37.9% (±25%)	29.7
NP-DNSH	64.3	51.4%-71.1% (±20%)	63.8
NP-PSH	25.6	19.2%-32.0% (±25%)	23.1
NPIR	91.3	73.0%-109.6 % (±20%)	97.2
DMZ	77.5	62.0%-93.0% (±20%)	78.7
HMMNI	22.4	16.8%-28.0% (±25%)	22.6
IPZ	49.8	37.3%-62.2% (±25%)	52.1
MNZ	43.7	32.8%-54.6% (±25%)	43.0
IPZOH	53.0	42.4%-63.6% (±20%)	53.8
MNZOH	110.5	88.4%-132.6% (±20%)	110.2
RNZ	15.5	10.8%-21.1% (±30%)	16.0
MG	25.8	19.3%-32.2% (±25%)	27.1
LMG	37.6	28.2%-47.0% (±25%)	37.7
CV	40.8	30.6%-51.0% (±25%)	41.1
LCV	82.3	65.8%-98.8% (±20%)	83.4
BG	39.8	29.8%-49.8% (±25%)	39.2
CAP	91.2	72.9%-109.4% (±20%)	96.1

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