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Liquid chromatography with time-of-flight mass spectrometry for simultaneous determination of chemotherapeutant residues in salmon

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Abstract

Liquid chromatography with time-of-flight mass spectrometry (LC–TOF-MS) method has been developed for simultaneous confirmation by accurate mass measurement and quantitative determination of antibiotics (enrofloxacin, oxolinic acid, flumequine, erythromycin), fungicides (malachite green MG, leucomalachite green LMG) and parasiticide (emamectin benzoate) residues in edible portion of salmon. Confirmation of chemotherapeutant residues has been based on the system of identification points (IPs) established in the Commission Decision 2002/657/EC concerning the use of mass spectrometry (MS) techniques. A validation study on matrix is presented evaluating accuracy in terms of precision (λ_{ppm} 0.83–1.15) and trueness (0.22–0.70 Da). Limits of detection (LODs) and limits of quantification (LOQs) were in ranges of 1–3 and 3–9 μg/kg, below the maximum residue limits (MRLs) established in current EU legislation (100–200 μg/kg) for these chemotherapeutants. Considering the EU guidelines, decision limits (CCα) and detection capabilities (CCβ) were determined (ranges of 103–218 and 107–234 μg/kg, respectively) for authorised substances. For no authorised compounds (MG and LMG), LODs were 2 and 1 μg/kg, respectively, but exceed the MRPL (minimum required performance limit) established in the legislation which corresponds to the sum of MG and LMG (2 μg/kg). Acceptable intra-day and inter-day variability, in terms of relative standard deviation (R.S.D.) of the analytical method, were obtained (2–15%). Linearity was demonstrated from the LOQs of the analytes to 600 μg/kg ($r > 0.9991$). The method has involved an extraction procedure based on solid–liquid extraction (SLE) with recoveries higher than 80% for most target chemotherapeutants, with exception of enrofloxacin (40%).

Keywords: Chemotherapeutant residues; Salmon; Liquid chromatography; Time-of-flight mass spectrometry

1. Introduction

In becoming an important contributor to the markets for seafood, aquaculture is increasingly subject to safety mechanisms and analytical controls. Chemicals used within the aquaculture industry include those associated with structural materials or water treatment, but also chemotherapeutants for veterinary treatments which can remain in fish tissues as residues. To ensure safe food, analytical control in aquacultural products is a major issue for better fin fish product quality. Among the chemotherapeutants used in aquaculture, the widespread use of antibiotics in food production is of concern as there is evidence that this may lead to development of bacterial resistance in humans [1]. In Europe, maximum residue limits (MRLs) are established in regulations for various classes of antibiotics, among them, quinolones with MRLs from 30 to 300 μg/kg in fish muscle/skin [2].

For other therapeutants, such as malachite green (MG) and its derivative leuchomalaquite green (LMG), no authorisation exist in current European legislation. However, MG is one of the most traditional products used to treat and prevent fungal infections as well as other diseases [3]. It use is not authorised by Food and Drug Administration (FDA), due to its potential carcinogenic, genotoxic, mutagenic and teratogenic properties [4], but application of MG in worldwide aquaculture is probable to continue due to low cost, ready availability and high efficacy [5]. The non-polar leuco form of MG, LMG, is accumulated in fish fat tissues and the control of the use of MG in aquaculture fish is also by LMG as marker residue [6,7].

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The variety of chemotherapeutants used in aquaculture and the possibility of trace level residues has made it necessary to develop sensitive screening methods for confirmatory purposes. Liquid chromatography–mass spectrometry (LC–MS) [8–11] or LC–MS/MS methods [12–17] have been applied to the analysis of antibiotics in seafood because of its high sensitivity and ability to provide compound confirmation. The majority of LC methods for MG or LMG in seafood have been reported by using UV–vis detection [6,18–20] fluorescence detection [21], LC–MS [22–24] and LC–MS/MS systems [25,26]. So, different methodological approaches have been used for a variety of food matrices and site-specific equipments [27]. However, to the best of our knowledge, the analysis of chemotherapeutant residues in fish fin by LC–TOF-MS system has not previously been published. The ability to provide high quality data, by exact mass measurements can make it a very compelling instrument for use as confirmatory methods [28–30] under the EU legislative context (Commission Decision 2002/657/EEC) [31]. According to this regulation, confirmatory methods for organic residues in food shall provide information on the chemical structure of the analytes and spectrometry detection is considered as suitable in this context. The use of MS techniques of low and high resolution can fulfil the criteria to confirm residues based on the established system of identification points (IPs) that is related to fragment masses [8–11,25,26,32]. For MS techniques of low resolution, three or four ions depending on the commodity or the analyte are necessary for confirmation, while techniques of high resolution need two ions. With this system, a minimum of three IPs is required for authorised substances and four for banned compounds [31,32]. So, the use of TOF-MS system, could earn the required IPs by the sum of two ions, the protonated molecular ion and a fragment ion produced by in-source collision-induced dissociation (CID). In addition, similar to other MS techniques with high selectivity (i.e. LC–MS/MS) [12–17,25,26], the TOF-MS system can confirm the presence of residual chemotherapeutants and although sensitivity is lower than that achieved by LC–MS/MS [27], it can be sufficient to detect substances at lower levels than the MRLs established in the EU legislation.

The aim of this work has been to develop a multi-residue method using LC–TOF-MS system for the determination of chemotherapeutant residues in salmon, one of the major seafood producing species in aquaculture. Both antibiotics (enrofloxacin, oxolinic acid, flumequine, erythromycin), fungicides (malachite green, leucomalachite green) and parasiticide (emamectin benzoate) have been investigated. Analytical parameters such as accuracy, sensitivity, decision limits, detection capability, intra-day and inter-day variability, as well as linear range, are presented in a study on matrix.

2. Experimental

2.1. Reagents, chemicals and working solutions

Enrofloxacin, oxolinic acid, flumequine and emamectin benzoate were supplied by Dr. Ehrensdorfer (Augsburg, Germany) with analytical grade (purity >98%). MG, LMG and erythromycin with analytical grade (>90%, >95% and >98%, respectively) were supplied by Sigma–Aldrich (St. Louis, MO, USA). Fig. 1 shows the chemical structure of the chemotherapeutants.

HPLC grade methanol and acetonitrile were supplied by Merck (Darmstadt, Germany). Formic acid (purity, 98%) was purchased from Fluka (Buchs, Switzerland). Acetic acid (purity, 98%) was purchased from Merck.

Stock solutions containing 5 mg/ml of analytes were prepared in methanol and stored in screw-capped glass tubes in the dark at −20 °C. Spiking standard solutions were also prepared by dilution in methanol.

2.2. LC–TOF-MS analysis

LC–TOF-MS system (LC Agilent MSD TOF from Agilent Technologies, Palo Alto, CA, USA) was used to analyze chemotherapeutant residues in salmon. The LC equipment was an HPLC binary solvent delivery system (Agilent Series 1100). LC analysis was performed on a ZORBAX SB-C18 column (3 mm × 250 mm, 5 μm i.d.) from Agilent Technologies. Gradient elution was performed with acetonitrile as solvent A and 0.1% formic acid in water (pH 3.5) as solvent B, starting at 20% of A in B, and increasing to 100% of A in B, in 40 min at a flow rate of 0.4 ml/min. HPLC system was connected to a TOF-MS with an electrospray interface (ESI) and was operated in positive ionization mode. The conditions for the acquisition parameters were: capillary voltage 4 kV, nebulizer pressure 40 psi, desolvation temperature 350 °C, gas temperature 300 °C, skimmer voltage 60 V, octapole dc 1 37.5 and octapole RF 250 V. Nitrogen generated from pressurized air in a nitrogen generator (Peak Scientific, Inchinnan, UK) was used as nebulizing and drying gas. TOF-MS internal mass calibration was performed using a calibration solution (ES-TOF reference mass solution, Agilent) that provided *m/z* 121.0509 and 922.0098 in positive mode. TOF-MS resolution was approximately 9500 ± 500 (full peak width at half-maximum, FWHM). Different fragmentor values were explored for proper optimization and for obtaining of useful structural information to be interpreted according the EU concept of IPs (see Tables 1 and 2). The values were in the range of 100–190 V to provide a soft or mild in-source CID fragmentation and at high values (280 V) for further fragmentation. Spectra were acquired over the *m/z* 50–1000 range at a scan rate of 1 s per spectrum. Data were processed with Applied Biosystem/MDS-SCIEX Analyst QS software (Frankfurt, Germany), with accurate mass application-specific additions from Agilent MSD TOF software. Elemental composition program was set at the following parameters: C: 0–50, H: 0–100, N: 0–10, O: 0–20, and F: 0–2. Double-bond equivalent (DBE) parameter was set from −0.5 to 50. The software option of electron state "even" and number of charges "+1" was selected for pseudo-molecular ions and fragment ions. Possible elemental compositions for ions with a maximum deviation of 5 ppm were assigned.

2.3. Solid–liquid extraction (SLE) method

The salmon was kept at −20 °C not more than 2 days till ready for sample treatment and analysis. After thawing, the

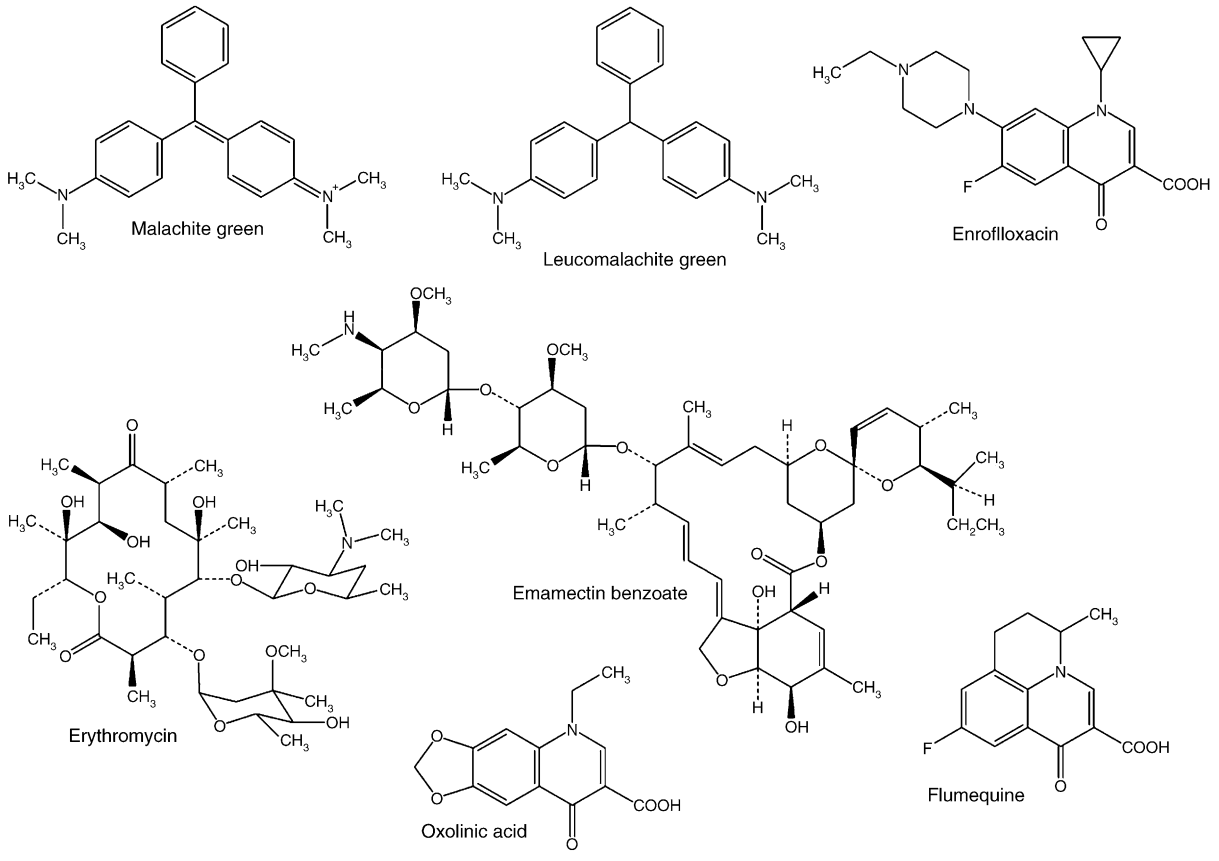

Fig. 1. Chemical structure of antibiotics (enrofloxacin, oxolinic acid, flumequine, erythromycin), fungicide (malachite green, leucomalachite green) and parasiticide (emamectin benzoate).

Table 1
Protonated molecular ions of chemotherapeutant residues in salmon muscle

Chemotherapeutants	$[M+H]^+$ (*m/z*)	Accuracy (20 μg/kg)		
		Trueness		Precision (λ_{ppm})
		Mean error (ppm, $n=11$)	Mean error (mDa, $n=11$)	
Enrofloxacin	360.17179	1.23	0.44	0.87
Erythromycin	734.46851	0.99	0.62	0.98
Oxolinic acid	262.07099	1.21	0.32	0.83
Flumequine	262.08739	1.76	0.46	1.11
Malachite green	329.20122	0.70	0.22	0.71
Leucomalachite green	331.21687	0.90	0.29	1.15
Emamectin benzoate	886.53111	0.79	0.70	0.96

Determination of accuracy in terms of precision and trueness.

Table 2
Fragment ions observed for target analytes in salmon muscle under different fragmentor values

Chemotherapeutants	Fragment ions (*m/z*)	Fragmentor (V)	Elemental composition	Error	
				mDa	ppm
Enrofloxacin	342.1612	280	$C_{19}H_{21}N_3O_2F$	0.7	2.0
	316.1819	280	$C_{18}H_{23}N_3OF$	0.3	1.2
Erythromycin	576.3742	280	$C_{29}H_{54}NO_{10}$	1.1	1.9
Oxolinic acid	244.0604	190	$C_{13}H_{10}NO_4$	0.5	2.0
Flumequine	244.0768	190	$C_{14}H_{11}NO_2F$	0.2	0.9
Malachite green	313.1699	280	$C_{22}H_{21}N_2$	0.8	0.1
Leucomalachite green	196.1120	190	$C_{14}H_{14}N$	0.024	0.1
Emamectin benzoate	700.4055	280	$C_{49}H_{58}NO_{10}$	1.5	2.0

Elemental composition and mass error.

fish muscle was separated from the skin and bones. For recovery studies, salmon samples (20 g) free of target residues were grinded and spiked at 20 μg/kg level, and after homogenisation, were incubated during 30 min at ambient temperature. The extraction method was based on solid–liquid extraction (SLE) procedure. The salmon muscle portions (2 g) were homogenised with 12 ml of acetonitrile (0.1% acetic acid) in 30 ml disposable screw-capped polypropylene tubes by a mixer (Polytron PT-MR 2100, Kinematica, Switzerland) for 3 min. NaCl (0.2 g) was added to facilitate the extraction process. The samples were centrifugated for 5 min at 3700 rpm and the supernatant was collected into a 15 ml disposable screw-capped polypropylene centrifuge tube. A 0.2 g of Bondesil-NH_2, 40 μm particle size (Varian, Middleburg, The Netherlands) was added for additional clean-up step. The tubes were shaken on a vortex mixer for 2 min (800 rpm) and centrifugated for 3 min at 3700 rpm. As last step, the supernatant was evaporated under a stream of nitrogen and 200 μl of the mobile phase were added in order to reconstitute the residue. Injection volumes of 10 μl of extracts in acetonitrile–0.1% formic acid in water (20:80, v/v) were used in LC analysis. Average recoveries ($n = 10$) were determined comparing the analytical response (peak area) of target analytes in salmon muscle spiked at 20 μg/kg before extraction and the analytical response in extract of salmon spiked at the same concentration level.

2.4. Validation study

A validation study is presented in terms of selectivity, sensitivity, accuracy, intra-day and inter-day variability, as well as linearity. Samples of salmon free of target residues were used in validation studies. Selectivity was investigated analyzing extracts of salmon spiked at 15 μg/l level. The suitability of the TOF-MS system for a confirmatory method of chemotherapeutant residues is discussed based on the application of IPs system. With the use of the TOF-MS system, the required IPs could be gained by the sum of the IPs corresponding to the protonated molecular ion and one fragment ion produced by in-source collision-induced dissociation (CID). In addition, the relative ion intensities (ion ratios) of the diagnostic ions are the criteria to be qualified as IPs. So, a minimum of at least one ion ratio shall be measured and must match the maximum allowable variation tolerances established under the EU concept of IPs [31].

For assessment of matrix-induced effects, signal suppression/enhancement was studied by analyzing a standard solution of the target analytes in solvent at a concentration of 20 μg/l and extract of salmon spiked with the same analytes and concentration level.

Accuracy has been determined in terms of precision and trueness. Precision of exact mass measurements was evaluated according to ISO 5725 by determination of λ_{ppm} [33] (calculated by 95% confidence limit, 1.36 × standard deviation, S.D., $n = 11$). Trueness was evaluated as the mean of the deviation in the measured mass of a theoretical exact mass from experimental mass, in other words, the closeness of the agreement between both values. For this purpose 11 analytical determinations of a salmon sample spiked at 20 μg/kg were performed for a period of 5 days (Table 1).

Intra-day and inter-day variability were determined by the repeated analysis ($n = 5$) of a salmon sample spiked with the 0.2 times the MRL, the MRL and 1.5 times the MRL concentration of the target compounds, from run-to-run during 1 day and 5 days, respectively. Table 3 shows intra-day and inter-day variability data that have been computed as relative standard deviation (R.S.D.). Decision limits (CCα, alpha error 5%) and detection capability (CCβ, beta error 5%) were calculated by calibration curves using the data generated during validation studies. For MRL compounds, CCα was determined as the corresponding concentration at the MRL plus 1.64 times S.D. and above which it can be decided with a 95% of statistical certainty that a sample is non-compliant. For banned compounds, CCα is calculated as the corresponding concentration at the *y*-intercept plus 2.33 times the S.D. of the intercept. CCβ for MRL compounds was calculated as the corresponding concentration at the decision limit plus 1.6 times the S.D. of the mean measured content at the decision limit. To calculate CCβ and CCα for no authorised compounds, samples were fortified at and below the minimum required performance level (MRPL) in equidistant steps. For these compounds so-called "zero tolerance" levels a progressive establishment of MRPLs is being carried out in order to come harmonization in the EU. Analytical limit of detection (LOD) was determined as the minimum concentration of analyte providing a spectrum in which exact mass measurement is feasible with a trueness value ≤2 ppm and which MS screening ion has a signal-to-noise (S/N) ratio of 3. Similarly, the limit of quantification (LOQ) was determined for a S/N ratio of 10. Linearity was tested assessing signal responses of target analytes from salmon samples spiked in a concentration ranging from the LOQs for each analyte to 600 μg/kg.

3. Results and discussion

3.1. LC–TOF-MS analysis

3.1.1. Selectivity

One of the main advantages of TOF-MS is improved selectivity due to the high-resolution power linked to the capability to provide exact mass chromatograms over nominal mass chromatograms (1 Da mass range). Thus, mass interferences with analytes having the same nominal mass and chromatographic retention time can be identified by LC–TOF-MS analyses. Fig. 2(a) shows overlapped extracted ion chromatograms (EIC) using a mass window of 0.01 Da around the masses of interest which correspond to an extract of salmon spiked at 15 μg/l. With this improved selectivity, compounds that are resolved at different retention time, but have the same nominal mass, such as oxolinic acid and flumequine (*m*/*z* 262) are discriminated as it is shown in Fig. 2(b) by exact mass 262.0709 and 262.0873, respectively. Fig. 2(c) and (d) shows an additional example corresponding to MG and matrix interference, where enhanced selectivity is demonstrated by the EIC reconstructed using a mass window of ±0.01 Da (Fig. 2(d)) over the EIC with ±0.2 Da (Fig. 2(c)) for *m*/*z* 329. Thus, MG and matrix inter-

Table 3
Validation parameters of LC–TOF-MS method for target residues in salmon muscle

Target residues	MRLs (μg/kg)	LODs (μg/kg)	LOQs (μg/kg)	Intra-day variability (R.S.D.%, $n=5$) (20 μg/kg)			Inter-day variability (R.S.D.%, $n=5$) (20 μg/kg)			CCα (μg/kg)	CCβ (μg/kg)	R^2 (LOQs-600 μg/kg)	% Ion suppression (20 μg/l)	Recoveries (R.S.D., %, $n=10$; 20 μg/kg) with clean-up Bondesil-NH_2	Recoveries (R.S.D., %, $n=3$; 20 μg/kg) without clean-up
				0.2 MRL*	MRL	1.5 MRL**	0.2 MRL*	MRL	1.5 MRL**						
Enrofloxacin	100[a]	2.5	7.5	2	2	1.5	15	12	10	103	107	0.9993	25	40 (6)	20 (2)
Erythromycin	200[b]	2	6	10	8	7	12	11	11	217	234	0.9993	1	80 (9)	56 (7)
Oxolinic acid	100[c]	3	9	6	6	5	14	12	9	109	118	0.9999	4	90 (12)	63 (2)
Flumequine	200[d]	3	9	5	4	2	13	12	7	208	218	0.9991	12	100 (10)	72 (6)
Malachite green	No authorised[e]	2	6	3	3	2	2	2	2	8	13	0.9992	2	100 (7)	55 (4)
Leucomalachite green	No authorised[e]	1	3	10	6	6	15	12	9	38	65	0.9996	8	100 (9)	60 (8)
Emamectin benzoote	100[f]	2	6	6	5	3	11	11	9	109	118	0.9998	0.1	90 (8)	62 (5)

LODs, LOQs, intra-day and inter-day variability (R.S.D.), decision limit (CCα) and detection capability (CCβ) and linearity. Assessment of signal ion suppression (expressed in %). Recovery data from salmon muscle spiked at 20 μg/kg. MRLs for authorised chemotherapeutants in fin fish according to EU legislation.

[a] MRL related to the sum of enrofloxacin and ciprofloxacin for fin fish in natural portions. Unofficial Consolidated version of the Anexes I to IV of Council Regulation no. 2377/90 update up to 22.12.2004. http://www.biavl.dk/varroa/eu-mrl.pdf.

[b] MRL for fin fish related to muscle and skin in natural portions. Unofficial Consolidated version of the Anexes I to IV of Council Regulation no. 2377/90 update up to 22.12.2004. http://www.biavl.dk/varroa/eu-mrl.pdf.

[c] MRL for fin fish related to muscle in natural portions. Unofficial Consolidated version of the Anexes I to IV of Council Regulation no. 2377/90 update up to 22.07.2003. http://www.pharmacos.eudra.org/F2/mrl/conspdf/MRL%20consol%202003-07-22%2OEN.pdf (previous legislation 807/2001/EC).

[d] MRL for fin fish related to muscle in natural portions. Unofficial Consolidated version of the Anexes I to IV of Council Regulation no. 2377/90 update up to 22.12.2004. http://www.biavl.dk/varroa/eu-mrl.pdf (previous legislation 2728/1999/EC).

[e] No authorisation for MG and its derivative LMG exist in European legislation in aquaculture (2377/90/EC Directive).

[f] MRL for salmonids related to muscle in natural portions. Unofficial Consolidated version of the Anexes I to IV of Council Regulation no. 2377/90 update up to 22.12.2004. http://www.biavl.dk/varroa/eu-mrl.pdf (previous legislation 1931/1999/EC).

* 0.2 MRL, 0.2 times the MRL concentration of the target residues.

** 1.5 MRL, 1.5 times the MRL concentration of the target residues.

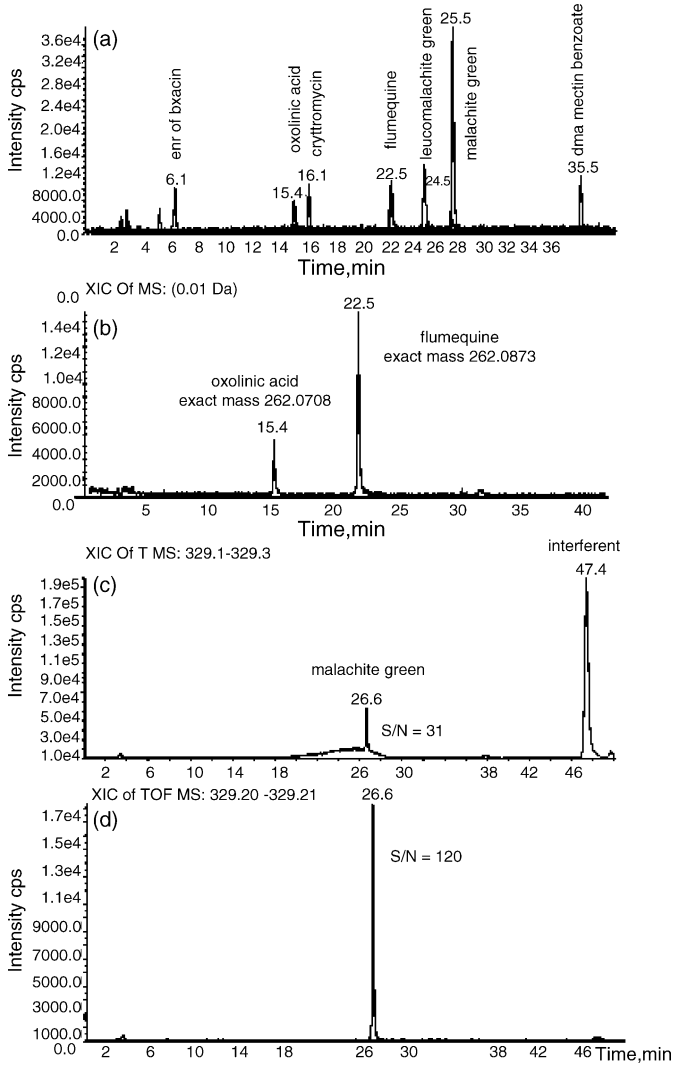

Fig. 2. (a) Extracted ion chromatogram (EIC) using a mass window of ±0.01 Da for target analytes in extract of salmon spiked at 15 μg/l; (b) detection of analytes with the same nominal mass (oxolinic acid, *m/z* 262.0709 and flumequine, *m/z* 262.0873) EIC using a mass window of ±0.01 Da; (c) detection of MG and matrix interference in EIC (±0.2 Da); (d) EIC of MG (±0.01 Da).

ference which are resolved with different retention times, are discriminated by exact mass measurement (Fig. 2(d)) using a mass window of 0.01 Da. On the other hand, the selectivity is related with an enhanced S/N ratio which is observed when narrow mass windows from ±0.2 to ±0.01 Da are applied and which is showed in Fig. 2(d) for MG. Exact masses used to identify target compounds and errors are presented in Table 1. In order to compute errors, an average mass spectrum was taken across the largest peak in the EIC, and masses for each analyte were compared against the theoretical mass.

CID fragments obtained in TOF-MS analysis can provide useful structural information for confirmation of residues. Since MS spectra, are recorded at high resolution, the use of TOF for confirmatory methods could fulfil the EU legislation criteria, based on the use of IPs. Although the use of exact mass as a confirmatory tool is not explicitly mentioned in the legislation, the number of IPs required can be reached by TOF analyzers, the EU criteria requires 2 and 2.5 IPs for high-resolution MS ions and product ions, respectively [31]. For this, different fragmentor values were assayed (100, 140, 190, 280 V). In the experiments performed with fragmentor values of 100 and 140 V, most compounds showed $[M+H]^+$ ions (see Tables 1 and 2) and at a medium value (190 V), a mild in-source CID fragmentation was observed. The MS spectra for oxolinic acid and flumequine showed one product ion and the spectral behaviour was analogous to previous reported work performed with IT-MS [14]. Those fragment ions at *m/z* 244.0604 and 244.0768, corresponds to the loss of water from protonated molecular ions. With this same fragmentor value (190 V), MS spectra of LMG also showed one fragment ion at *m/z* 196.1120 whose elemental composition was $C_{14}H_{14}N$ and with a trueness of 0.12 ppm. From the corresponding MS spectra was observed which the ion ratios obtained between the intensity of an individual ion over that of the base ion matched the maximum permitted tolerance (±20 for oxolinic acid and flumequine, ±25 for LMG). So, as two ions were obtained within of tolerable ion ratios, these compounds could be confirmed following the IP system. On the other hand, comparing the results obtained by TOF and the previous reported by IT-MS [14], the corresponding sum of ions should give 4 and 3.5 IPs, respectively. Therefore the confirmation of MRL compounds such as oxolinic acid and flumequine could be achieved by TOF. In the case of LMG which is metabolite of a no authorised compound (MG), the use of TOF-MS result also suitable, since 4 IP are earned. With this experiments, performed at 100, 140 and 190 V, the sensitivity observed in each case was similar, so the capability of obtaining useful structural information for confirmatory purposes using IP system, would not represent a limitation with the sensitivity achieved for determining these compounds. Table 2 shows elemental composition and mass error of fragment ions observed for the target residues in matrix.

To explore extensive fragmentation for the rest of compounds, a higher fragmentor value (280 V) was applied. MS spectra of enrofloxacin showed two fragment ions, at *m/z* 342.1612 which was attributed to the loss of water and at *m/z* 316.1819. For erythromycin, emamectin benzoate and MG, a soft fragmentation providing one fragment ion in addition to $[M+H]^+$ ions, was observed in MS spectra (see Table 2). In these cases, the relative ion intensities were within the tolerable range (±25%). The MS spectra of MG with one fragment ion at *m/z* 313.1699, was also analogous to previous publications, where MS–MS analysis were performed [25,34]. In both cases, 4 and more than 4 IPs could be obtained, so by MS–MS techniques, the confirmation of MG should be suitable and by TOF-MS it would be also possible. However, in spite of that for the rest of compounds (enrofloxacin, erythromycin and emamectin benzoate), the required IPs could be reached, the conditions of operation are limiting and at fragmentor values higher than 280 V, further extensive fragmentation could be obtained but also mass error. In Table 2, fragmentation values, fragment ions and mass error which were less or equal than 2 ppm for target residues are presented.

Selectivity was also assessed by studying matrix effects, since it is well known that in LC–MS analysis, interference from

residual matrix components may result in signal suppression-enhancement, depending to a great extent on the electrospray source ionization (ESI). This effect can adversely affect confirmation and detection of analytes. To address matrix effect assessment, MS response under exact mass conditions was monitored for each target analyte from standard solution in methanol and extract of salmon spiked at 20 μg/l level. Table 3 shows signal suppression observed for each analyte calculated as the percentage decrease in signal intensity in spiked salmon, versus a standard solution in methanol. Signal suppression was estimated for most of analytes between 1 and 10%, with the exception of enrofloxacin (20%) which is the first LC eluting compound. This effect is generally described in located areas of the chromatogram when complex matrices are analyzed and is usually attributed to the presence of moderately polar interferences [35].

3.1.2. Sensitivity

One limitation of the TOF-MS instrument is its low sensitivity in comparison to MS/MS techniques such as LC–MS^2, under selected reaction monitoring (SRM). But, improved selectivity of TOF-MS can also offer an improved signal-to-noise ratio. So, sensitivity can be affected depending of the selected mass window to reconstruct the EIC. Different mass intervals and S/N ratio have been evaluated. An example is shown in Fig. 3, where EIC are drawn using narrow mass windows from ±0.25 to ±0.001 Da for MG, flumequine and enrofloxacin. For MG, an enhancement of S/N ratio from 31 to 120 was observed when narrow windows from ±0.2 to ±0.01 Da were applied. Under these conditions, S/N ratio enhancement was also observed from 20–25 to 50–108, for enrofloxacin and flumequine, respectively. No improvement in S/N ratio was achieved, when mass window was smaller than ±0.01 Da. Thus, the optimal mass window was established at ±0.01 Da for all target analytes, considering a mass error ≤2 ppm.

The LODs and LOQs achieved for veterinary medicines, whose use in treatments is authorised according to EU legislation, were between 2–3 and 6–9 μg/kg, respectively (Table 3). For MG and LMG, whose use is not authorised, LODs and LOQs were in the range of 1–2 and 3–6 μg/kg, respectively. Subsequently, despite the fact that other MS techniques, in particular LC–MS^2 under SRM mode, can offer better sensitivity, the use of TOF-MS also allows the confirmation of residues in salmon at low ppb levels (μg/kg). Therefore, its application can be successful in the control of the authorised veterinary medicines, subject of this study, which MRLs are established in the range of 100–200 μg/kg. The method was also validated determining CCα and CCβ. For the compounds for which presence in food of animal origin, are tolerable, were in the range of 103–218 and 107–234 μg/kg, respectively. With these data it could conclude that a sample is non-compliant (with α and β error of 5%). For compounds whose use is not authorised (MG and LMG), CCα and CCβ were 8–38 and 13–65 μg/kg, respectively. In this case, according to EU legislation, as no permitted limit has been established, it would be necessary to meet the MRPL of the analytical method, established in 2 μg/kg for the sum of both. Therefore, considering this context, the results obtained with TOF-MS exceed this limit (see Table 3), so MG and LMG can be detected at 2 and 1 μg/kg, respectively.

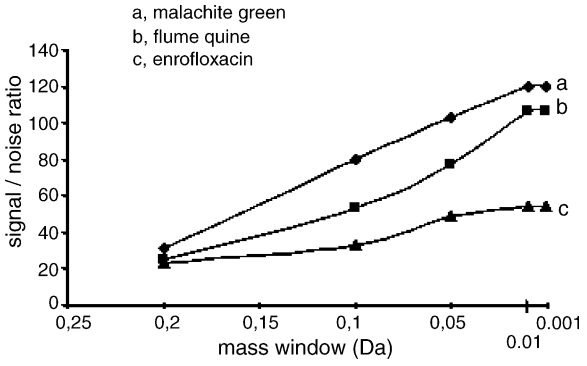

Fig. 3. Signal-to-noise ratio against mass windows applied to reconstruct EIC for MG, flumequine and enrofloxacin. Enhancement of S/N ratio is shown when narrow mass windows from ±0.25 to ±0.001 Da are applied.

3.1.3. Accuracy

For authorised veterinary medicines, in addition to EC criteria, confirmatory methods used need be evaluated for accuracy and precision to ensure whether or not a sample is violative if exceeding established MRLs, with sufficient statistical confidence. For banned veterinary medicinal products, confirmatory methods need be evaluated as screening methods in order to ensure with sufficient statistical confidence the presence/absence of the compound. Systematic and random mass errors, even when the instrument has been well calibrated and the operation procedure has been optimized, can be a source of error. Factors affecting mass accuracy have been described in literature [36–38].

To assess accuracy, the statistical approach established in ISO 5725, has been followed in terms of precision and trueness [33]. Precision (with a 95% confidence limit) has been calculated for 11 measurements under conditions described in Section 2. Since precision depends principally on ion statistics (number of ions sampled in making the measurement, S), the magnitude of the statistical component of the error will be different for each analyte [38,39]. In previous studies, the dependence of precision on S values was assessed using a non-weighted linear least-square analysis. This relationship was calculated by the formula $\lambda_{ppm} = 10^6/CRS^{1/2}$, where λ_{ppm} is an expression of statistical error, C is an instrument constant, which depends on the shape of spectral peak, the centroiding and mass correction algorithms employed, and R is the resolution of the mass analyzer [39,40]. Similarly, in this study, the precision of mass measurements has been calculated under conditions of operation described in Section 2, obtaining λ_{ppm} values ranging from 0.83 to 1.15 (95% confidence limit). Comparable values were obtained when trueness was determined for each analyte in error between 0.70 and 1.76 ppm. Results are summarized in Table 1. Taking into account that accuracy of mass measurements in TOF-MS system, is typically less than 5 ppm over the mass range m/z 150–900, the results of precision and trueness can be considered adequate for the use for which data is intended.

3.1.4. Inter-day, intra-day variability and linear range

Method validation was also performed by the determination of intra-day and inter-day variability. The repeated analysis ($n = 5$) of salmon muscle spiked at three levels of concentration (0.2 MRL, MRL, and 1.5 MRL) showed an acceptable variability for run-to-run in the same day and during 5 days. Intra-day and inter-day variability that were computed as R.S.D. were within 2–15% range (Table 3).

Narrow dynamic range has been a limiting factor in TOF-MS instruments for quantitative purposes. However, the last generation of TOF mass spectrometers provides the capability to perform a linear dynamic range over several orders of magnitude for exact mass measurements [41,42]. In this sense, the use of TOF-MS system has been tested for quantitative analysis of chemotherapeutant residues in salmon muscle. Standard calibration curves were constructed by plotting the corresponding peak area against concentration of analytes in salmon sample spiked in a range of concentration from the LOQs of the compounds to 600 μg/kg and computed by the least-square linear regression. Correlation coefficients ($r > 0.9991$) for the analytes are summarized in Table 3 and show an acceptable linearity of the analytical response across the range of concentration tested.

3.2. Extraction procedure: recovery studies

Extraction efficiency for the target analytes was calculated by the ratio of signal response obtained in salmon spiked at 20 μg/kg before extraction, and the corresponding response in salmon spiked after extraction at the same concentration level. Sample matrix clean-up procedure was also evaluated without and with the use of Bondesil-NH_2. From different experiments performed varying the added amount (0.1, 0.2, 0.5, 0.8 g, of Bondesil-NH_2), the clean-up was effective in removing matrix interferences using 0.2 g of Bondesil-NH_2. The mechanisms could be attributed to the fact that NH_2 functionality may exhibit polar interactions, including hydrogen bonding or dipole–dipole interactions with matrix interferences. This procedure to eliminate interfering species during sample preparation was efficient, but it does not always offer the complete removal. Results of extraction procedure are shown in Table 3 and correspond to averaging recovery data from repeated extraction procedure ($n = 10$). Recoveries exceed 80% for all the analytes investigated, with the exception of enrofloxacin (40%). The elimination of possible compounds that could interfere in the LC analysis is observed in a cleaner chromatogram. So, in the case of enrofloxacin, as it was mentioned above, the impact of matrix effect was observed as signal suppression (20%), while for the rest of compounds was not higher than 10%. The precision of the extraction procedure was acceptable with R.S.D. as less than 12% for the analytes.

4. Conclusions

Accurate mass measurements provided qualitative information which could be applied for confirmation of chemotherapeutant residues in fin fish analysis according to IP criteria established in EU legislation [31]. This would be the case of oxolinic acid, flumequine and LMG. The rest of compounds (MG, erythromycine, emamectin benzoate and enrofloxacin) could be confirmed following the EU criteria, if high fragmentor values for extensive fragmentation are applied. Accuracy determined in terms of precision (λ_{ppm} 0.87–1.15) and trueness (0.22–0.70 Da), is adequate for confirmatory purposes of target residues. LODs and LOQs (1–3 and 3–9 μg/kg, respectively) obtained were lower than the established MRLs (100–200 μg/kg) for the target veterinary medicines. The method was also validated following EU guidelines giving CCα and CCβ for MRL substances which were in the range of 103–217 and 107–234 μg/kg, respectively. For no authorised compounds (MG and LMG), CCα and CCβ were 8–38 and 13–65 μg/kg, respectively. The analytical limits (LODs) obtained for MG and LMG (2 and 1 μg/kg, respectively) exceeded the (MRPL) of the analytical method, established in 2 μg/kg for the sum of both. Calibration curves prepared at a range of concentration from LOQs to 600 μg/kg were linear with $r > 0.9991$ for the target analytes. Acceptable intra-day and inter-day variability computed as R.S.D. were obtained (2–15%). With the extraction method based on SLE, recoveries higher than 80% were obtained for most of the target analytes, with the exception of enrofloxacin (40%).

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