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Multiclass detection and quantitation of antibiotics and veterinary drugs in shrimps by fast liquid chromatography time-of-flight mass spectrometry

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ABSTRACT

A fast liquid chromatography time-of-flight mass spectrometry (LC–TOFMS) method has been developed for simultaneous quantitative multiclass determination of residues of selected antibiotics and other veterinary drugs (benzalkonium chloride, ethoxyquin, leucomalachite green (LMG), malachite green (MG), mebendazole, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethizole, sulfanilamide, sulfapyridine, sulfathiazole and trimethoprim) in shrimps. Different sample treatment methodologies were tested for the extraction of the targeted species based on either liquid partitioning with different solvents, solid-phase extraction or and matrix solid-phase dispersion. The final selected extraction method consisted of solid–liquid extraction protocol using acetonitrile as solvent followed by a clean-up step with primary secondary amine (QuEChERS). Recovery rates for the extraction of the selected multiclass chemicals were in the range 58–133%. Subsequent identification, confirmation and quantitation were carried out by LC–TOFMS analysis using a reverse-phase C_{18} column with 1.8 μm particle size. The confirmation of the target species was based on accurate mass measurements of the protonated molecules ($[M+H]^+$) and their fragment ions, obtaining routine accuracy errors lower than 2 ppm in most cases. The optimized LC–TOFMS method displayed excellent sensitivity for the studied analytes, with limits of detection (LODs) in the range 0.06–7 μg kg^{-1}. Finally, the proposed method was successfully applied to the analysis of 12 shrimp samples collected from different supermarkets, showing the potential applicability of the method for ultratrace detection of these chemicals in such complex matrix.

1. Introduction

The use of veterinary drugs in food production has been an issue of increasing concern for consumers during the last years. For instance, the intensive use of chemotherapeutic agents to treat infectious diseases in aquaculture has led to a frequent occurrence of drug-resistant microorganisms (including multiple-antibiotic resistant bacteria). In fact, in Europe there are currently in force some regulations establishing maximum residue levels (MRLs) for various antibiotics in fish [1,2], including, among others, sulfonamides, tetracyclines and emamectin. Other veterinary drugs (e.g. malachite green (MG) and its main metabolite, leucomalachite green (LMG)) are not authorized for use in food producing animals in the EU, USA and Canada. For these banned substances, the European Commission have established minimum required performance limits (MRPLs) in food, defined as "*minimum content of an analyte in a sample, which at least has to be detected and confirmed*" [3–5]. Thus, in order to monitor properly these products in aquatic animals, sensitive, fast and accurate analytical methods have to be developed.

Multi-residue analysis of veterinary drugs at trace levels in seafood matrices, such as shrimps, is a challenging task taking into account the inherent complexity of the matrix. The simplification of sample treatment steps (typically previous to mass spectrometry-based measurements) is of great relevance in such applications. The desirable features of alternative methodologies are low organic solvent consumption, reduced labor time and the use of cheaper and disposable material. A variety of sample treatment methodologies have been tested in the last years for the analysis of antibacterial residues in foods [6]. A wide array of the proposed procedures for analyzing veterinary drug residues in food-producing animal tissues employs liquid-phase extraction [7]. Solid-phase extraction (SPE) is the most widely used technique for the clean-up of the extracts. The combination of liquid-phase extraction followed by SPE clean-up step has been satisfactorily applied, for example, to the analysis of several antibacterials in salmon [8] and chicken [9] tissues. Matrix solid-phase dispersion (MSPD) extraction has been also used for the determination of different antibacterials in a

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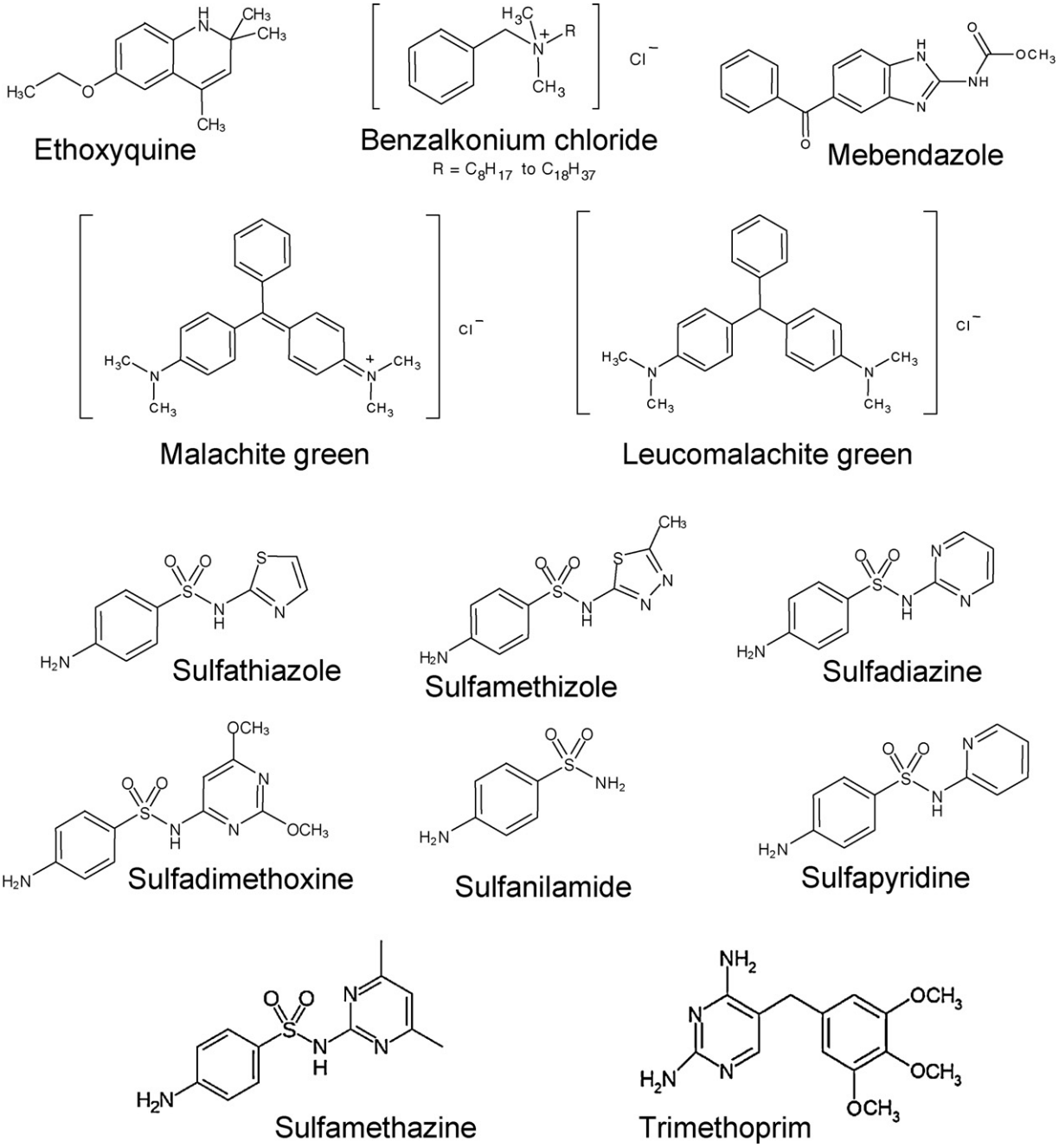

Fig. 1. Chemical structures of the selected multi-class antibiotics.

great variety of foodstuffs [6]. Other methodologies such as pressurized liquid extraction (PLE), microwave assisted extraction (MAE) or solid-phase microextraction (SPME) have also been employed [6].

With regards to the determination step, liquid chromatography has been extensively used for the analysis of veterinary drugs in seafood and fish. Several LC methods have been reported using UV–vis detection and confirmation by mass spectrometry for the determination of MG and LMG in fish and shrimps [10,11], tetracyclines in shrimps [12], or sulfonamides in salmon [13]. Because of the increasingly demanding requirements of sensitivity, liquid chromatography–tandem mass spectrometry (LC–MS/MS), using triple quadrupole MS, is currently the preferred technique for residue analysis of antimicrobials in food [14–18], although accurate mass full scan MS techniques as time-of-flight mass spectrometry (LC–TOFMS) has also been recently applied [8,19–24].

The aim of this work is to develop an analytical method for the sensitive determination of 13 multiclass antibiotics and veterinary drugs in shrimps using fast liquid chromatography electrospray time-of-flight mass spectrometry. Different sample treatment methodologies based on solid–liquid extraction, solid-phase extraction or matrix solid-phase dispersion have been evaluated for the extraction of the selected chemicals from seafood. Method performance in terms of sensitivity, LODs, linearity and matrix effects has been evaluated in detail. Finally, the proposed method was successfully tested with different samples of different origin purchased in local markets.

Table 1
In-source CID fragmentation of the studied multiclass antibiotics and veterinary drugs using three different fragmentor voltages.

Compound	Ion	*m/z*	Elemental composition	Relative abundance (%)		
				160 V	190 V	230 V
Benzalkonium chloride-C12	$[M+H]^+$	304.2999	$C_{21}H_{38}N$	100	100	100
	Frag 1	161.1328	$C_{12}H_{17}$	4	6	–
Benzalkonium chloride-C14	$[M+H]^+$	332.3318	$C_{23}H_{41}N$	100	100	100
	$[M+H]^+$	218.1539	$C_{14}H_{20}NO$	100	100	100
	Frag 1	202.1226	$C_{13}H_{16}NO$		37	23
	Frag 2	190.1223	$C_{12}H_{16}NO$			12
Ethoxyquin	Frag 3	174.0918	$C_{11}H_{12}NO$			15
	Frag 4	162.0912	$C_{10}H_{12}NO$			7
	Frag 5	148.0751	$C_9H_{10}NO$			4
	Frag 6	134.0601	C_8H_8NO			3
Leucomalachite green	$[M+H]^+$	331.2168	$C_{23}H_{27}N_2$	100	100	100
	Frag 1	196.1120	$C_{14}H_{14}N$		71	73
Malachite green	$[M+H]^+$	329.2012	$C_{23}H_{25}N_2$	100	100	100
Mebendazole	$[M+H]^+$	296.1029	$C_{16}H_{14}N_3O_3$	100	100	100
	Frag 1	264.0774	$C_{15}H_{10}N_3O_2$		15	63
Sulfadiazine	$[M+H]^+$	251.0597	$C_{10}H_{11}N_4O_2S$	100	100	100
	Frag 1	185.0824	$C_{10}H_9N_4$		2	35
	$[M+H]^+$	311.0808	$C_{12}H_{15}N_4O_4S$	100	100	100
	Frag 1	245.1038	$C_{12}H_{13}N_4O_2$		–	3
Sulfadimethoxine	Frag 2	156.0768	$C_6H_{10}N_3O_2$		4	29
	Frag 3	108.0445	C_6H_6NO		1	8
	Frag 4	92.0500	C_6H_6N		–	4
Sulfamethazine	$[M+H]^+$Frag 1	279.0911	$C_{12}H_{15}N_4O_2SC_{12}H_{13}N_4$	100	100	100
		213.1136			–	5
Sulfamethizole	$[M+H]^+$	271.0317	$C_9H_{11}N_4O_2S_2$	100	100	100
	$[M+H]^+$	173.0379	$C_6H_9N_2O_2S$	100	100	100
Sulfanilamide	Frag 1	108.0447	C_6H_6NO		7	36
	Frag 2	92.0498	C_6H_6N		29	25
	Frag 3	80.0498	C_5H_6N		22	21
	$[M+H]^+$	250.0644	$C_{11}H_{12}N_3O_2S$	100	100	100
Sulfapyridine	Frag 1	184.0872	$C_{11}H_{10}N_3$		5	60
	Frag 2	157.0091	$C_{10}H_5S$		–	12
Sulfathiazole	$[M+H]^+$	256.0208	$C_9H_{10}N_3O_2S_2$	100	100	100
	$[M+H]^+$	291.1451	$C_{14}H_{19}N_4O_3$	100	100	100
	Frag 1	276.1141	$C_{13}H_{15}N_4O_3$		1	10
	Frag 2	261.0987	$C_{12}H_{13}N_4O_3$		2	11
Trimethoprim	Frag 3	247.1175	$C_{12}H_{15}N_4O_2$		–	1
	Frag 4	233.1034	$C_{11}H_{13}N_4O_2$		0	2
	Frag 5	232.0959	$C_{13}H_{14}NO_3$		–	0
	Frag 6	230.1156	$C_{14}H_{16}NO_2$		–	1

2. Experimental

2.1. Standards and reagents

Benzalkonium chloride standard was obtained from Aldrich, and its expanded structure was $[C_6H_5CH_2N(CH_3)_2R]Cl$, where R is predominantly n-$C_{12}H_{25}$, but the commercial product also contained C_{14} and C_{16} homologues. For the rest of compounds, individual analytical standards were purchased from Fluka Chemie (Zwijndrecht, The Netherlands) and Sigma–Aldrich (Zwijndrecht, The Netherlands). Individual analyte stock solutions (500 $\mu g\,mL^{-1}$) were prepared in methanol and stored at −20 °C (Fig. 1).

HPLC-grade acetonitrile, methanol and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany). Formic acid, anhydrous magnesium sulfate, sulfuric acid, sodium hydroxide, ethanol, hydrochloric acid, acetic acid, metaphosphoric acid, and tungstic acid were obtained from Fluka (Buchs, Switzerland). Sodium chloride was obtained from J.T. Baker (Phillipsburg, NJ, USA). Primary–secondary amine (PSA) was purchased from Supelco (Bellefonte, PA, USA). A Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA, USA) was used throughout the study to obtain the HPLC-grade water used during the analyses. Florisil cartridges and C_{18} sorbent were from Análisis Vínicos (Tomelloso, Castilla-La Mancha, Spain). Bond Elut NH_2 sorbent was obtained from Varian Inc. (Palo Alto, CA, USA).

2.2. Liquid chromatography time-of-flight mass spectrometry

2.2.1. Chromatography

The chromatographic separation was carried out using an HPLC system (consisting of vacuum degasser, autosampler and a binary pump) (Agilent Series 1200, Agilent Technologies, Santa Clara, CA) equipped with a reversed phase rapid resolution C_{18} analytical column of 50 mm × 4.6 mm and 1.8 μm particle size (RR Zorbax Eclipse XDB-C_{18}). 20 μL of extract was injected in each study. Mobile phases A and B were water with 0.1% formic acid and acetonitrile respectively. Initial mobile phase composition (10% B) was held constant for 1 min, followed by a linear gradient to 100% B at 11 min. Then, 100% B was pumped for 5 min. The flow-rate used was 0.5 mL min^{-1}.

2.2.2. Electrospray time-of-flight mass spectrometry

The HPLC system was connected to a time-of-flight mass spectrometer Agilent 6220 accurate mass TOF (Agilent Technologies, Santa Clara, CA) equipped with an electrospray interface operating in the positive ionization mode, using the following operation parameters: capillary voltage, 4000 V; nebulizer pressure, 40 psig; drying gas flow rate, 9.0 L min^{-1}; gas temperature, 325 °C; skimmer voltage, 65 V; octapole 1 rf, 250 V; fragmentor voltage (in-source CID fragmentation), 160, 190, and 230 V. LC–MS accurate mass spectra were recorded across the *m/z* range

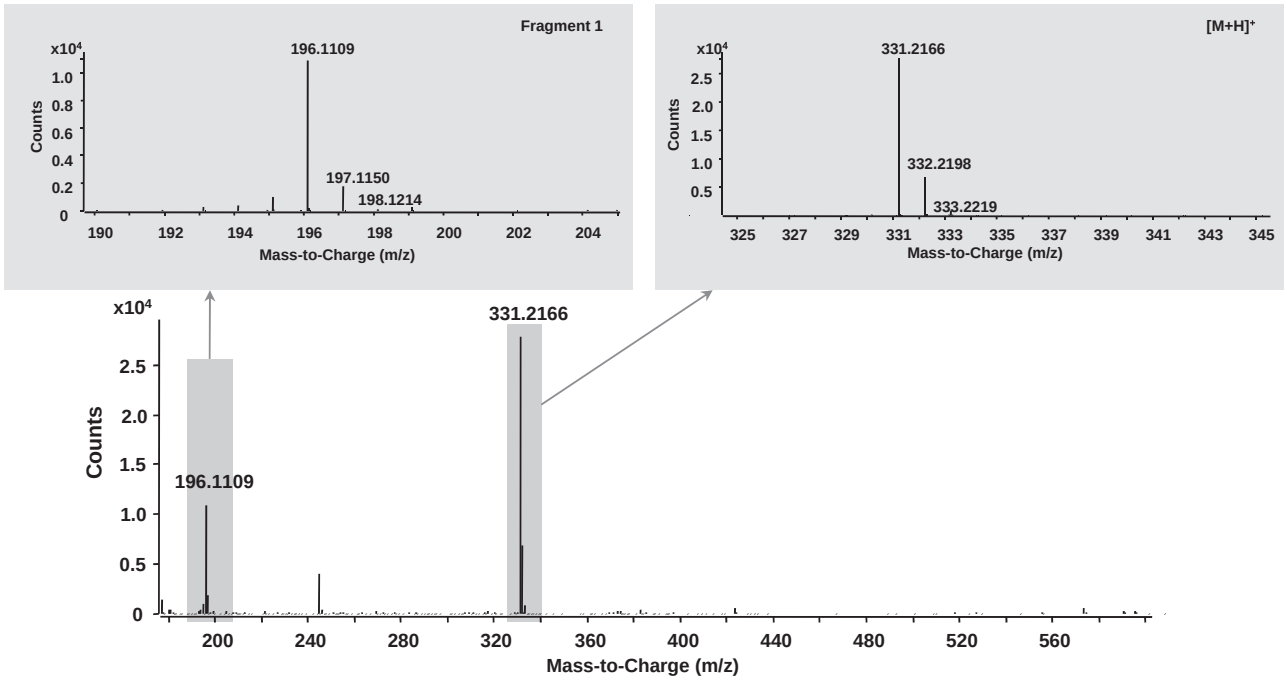

Fig. 2. LC–ESI-TOFMS(+) accurate mass spectrum of leucomalachite green at a concentration level of 10 μg kg^{-1}.

of 50–1000. The instrument performed the internal mass calibration automatically, using a dual-nebulizer electrospray source with an automated calibrant delivery system, which introduced the flow from the outlet of the analytical column together with a low flow (approximately 40 μL min^{-1}) of a calibrating solution which contained the internal reference masses purine ($C_5H_4N_4$, at *m*/*z* 121.050873, in positive ion mode) and HP-0921 (Hexakis-(1H,1H,3H-tetrafluoropropoxy)phosphazine, $C_{18}H_{18}O_6N_3P_3F_{24}$, at *m*/*z* 922.009798 in positive ion mode). The instrument provided a typical resolution higher than 10,000 at *m*/*z* 118 and higher than 18,000 at *m*/*z* 1522. The full scan data were recorded with Agilent Mass Hunter Data Acquisition software (version B.02.00, Patch 3) and processed with Agilent Mass Hunter Qualitative Analysis software (version B.02.00, Patch 3) and Agilent Mass Hunter Quantitative Analysis software (version B.01.04, Patch 2).

2.3. Sample treatment

Five different sample treatment methodologies were tested.

2.3.1. Acetonitrile extraction followed by clean-up by dispersive solid-phase extraction

The employed method also known as QuEChERS [25] comprises the following steps: a representative 10 g portion of previously homogenized crushed shrimps sample was weighed into a 50 mL PTFE centrifuge tube. Then 10 mL of acetonitrile containing 1% acetic acid were added, and the tube was vigorously shaken for 1 min. After this time, 4 g of anhydrous magnesium sulfate and 1.75 g of sodium chloride were added, and inmediately the shaking process was repeated for 1 min to prevent coagulation of anhydrous magnesium sulfate. The extract was then centrifuged (3700 rpm) for 3 min. 5 mL of the supernatant (acetonitrile phase) was then taken with a pipette and transferred to a 15 mL graduated centrifuge tube containing 250 mg of PSA and 750 mg of anhydrous magnesium sulfate that was then energetically shaken for 20 s. The extract was then centrifuged again (3700 rpm) for 3 min. Finally, 2 mL of this extract were evaporated to near dryness, and reconstituted with 20% (v/v) methanol in water up to a final volume of 2 mL. Prior to LC–MS analysis the extract was filtered through a 0.45 μm PTFE filter (Millex FG, Millipore, Milford, MA, USA) and transferred into a vial.

2.3.2. Extraction with trichloroacetic acid

The employed treatment was adapted from Furusawa [26]. 1 g of crushed shrimp homogenate was placed in a 15 mL graduated centrifuge tube with 6 mL of TCA (20%, w/v) solution and were homogenized with the aid of an ultrasonic bath for 30 s. Then the mixture was centrifuged for 5 min (3700 rpm). 3 mL were taken up from the supernatant and evaporated until near dryness, being then dissolved in 20% (v/v) methanol in water up to a final volume of 0.5 mL so that the analyzed extract contained the equivalent of 1 g of sample per mL. The final extract was filtered again through a 0.45 μm PTFE filter (Millex FG, Millipore, Milford, MA, USA) prior to LC–MS analysis.

2.3.3. Matrix solid-phase dispersion (MSPD) procedure

1 g of crushed shrimp homogenate was transferred to a glass mortar, where it was gently blended and homogenized together

Table 2
Recovery study at a concentration level of 100 μg kg^{-1}, using the selected extraction method (QuEChERS procedure).

Compound	Average recovery (%) (n = 6)	RSD (%) (n = 6)
Benzalkonium chloride-C12	53	11.2
Ethoxyquin	53	12.5
Leucomalachite green	90	8.1
Malachite green	118	9.3
Mebendazole	118	5.7
Sulfadiazine	82	6.8
Sulfadimethoxin	85	7.2
Sulfamethazine	114	5.2
Sulfamethizole	33	14.9
Sulfanilamide	115	4.7
Sulfapyridine	109	5.9
Sulfathiazole	81	6.3
Trimethoprim	87	9.1

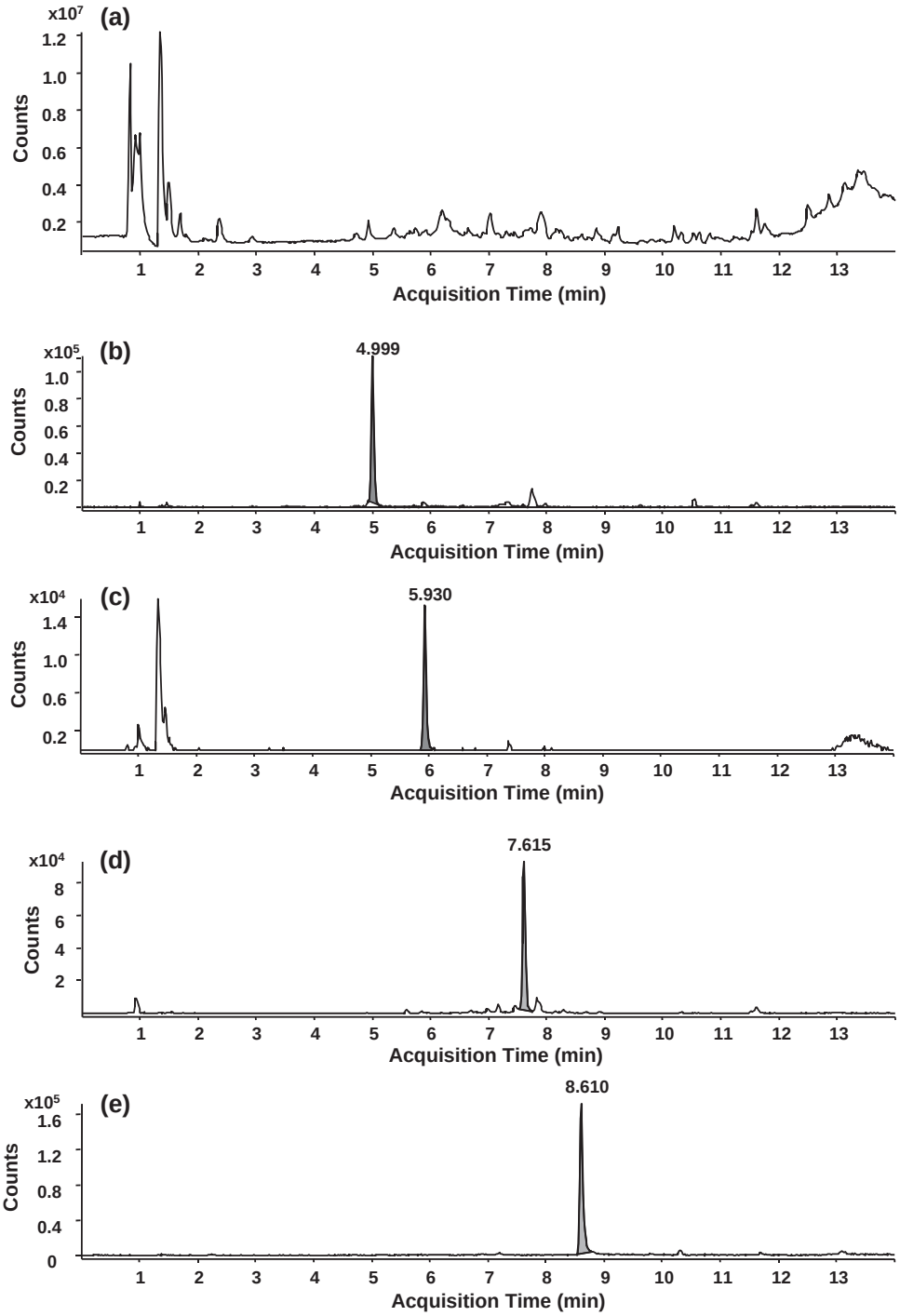

Fig. 3. (a) TIC corresponding to a matrix-matched standard spiked with 10 μg kg^{-1} of the target analytes. Extracted ion chromatograms (EICs) corresponding to (b) trimethoprim, (c) sulfamethizole, (d) sulfadimethoxine and (e) malachite green.

with 2 g of aminopropyl (Bondesil-NH_2) until a fine powder was obtained. This mixture was then transferred to a commercially available 12 mL SPE cartridge containing 2 g of Florisil and connected to a vacuum system. The elution step was carried out with 2× 5 mL of acetonitrile. The first aliquot of the eluting solvent was used to backwash both the mortar and the pestle. The final extract was evaporated until near dryness, then reconstituted with 20% (v/v) methanol in water, up to a final volume of 1 mL. The resulting extract contained the equivalent of 1 g of sample per mL and was filtered through a 0.45 μm PTFE filter (Millex FG, Millipore, Milford, MA, USA) and transferred into a vial, prior to LC–MS analysis.

2.3.4. SPE-based method I

This procedure adapted from Ramos et al. [27], can be divided in three stages:

(1) *Protein precipitation*: 1 g of crushed shrimp homogenate was weighed into a 10 mL graduated centrifuge tube, where 3 mL of sulfuric acid 0.17 M, 0.158 g of sodium tungstate and 12 mL of acetonitrile were added. The mixture was shaken and then centrifuged until phases partitioned. Then the supernatant of 10 mL was filtered through a 0.45 μm PTFE filter (Millex FG, Millipore, Milford, MA, USA).

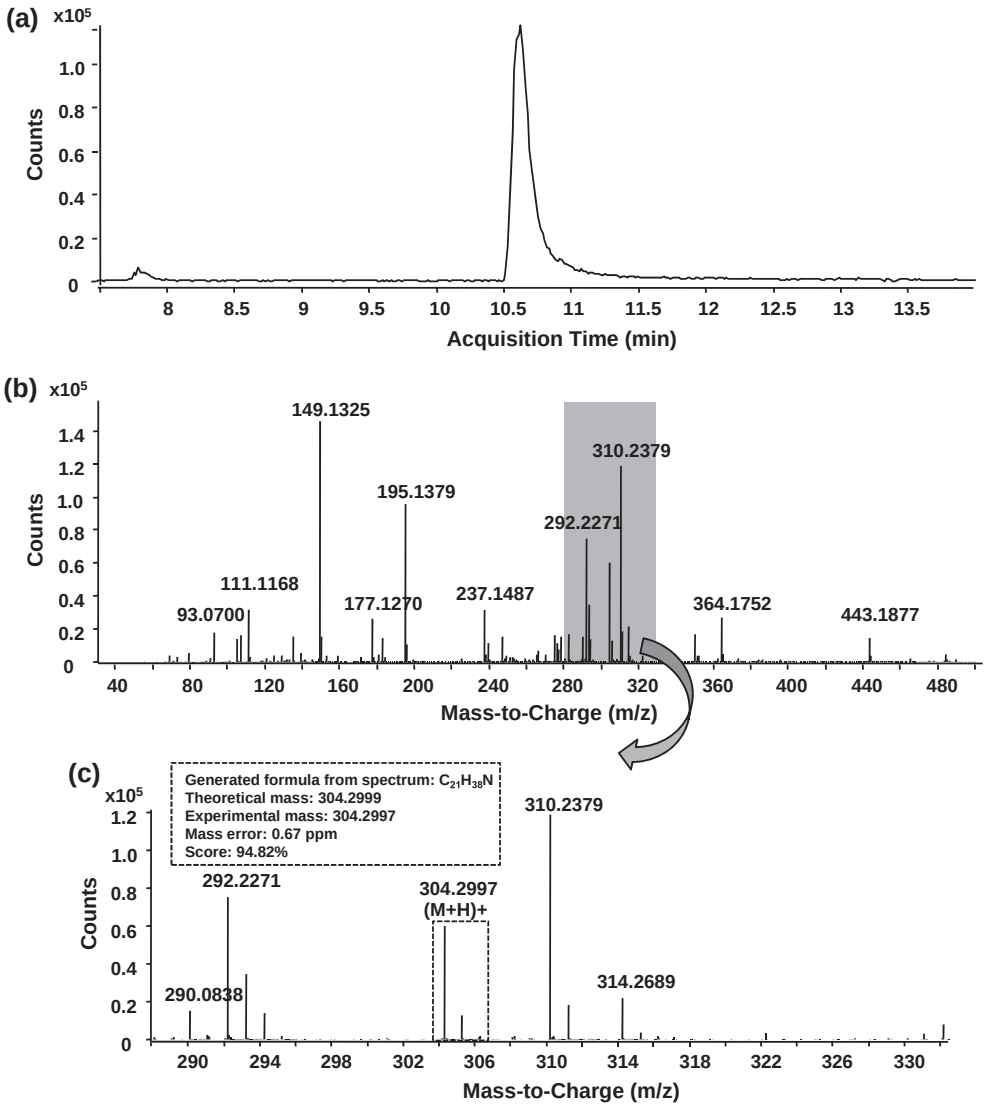

Fig. 4. (a) Extracted ion chromatogram (EIC) corresponding to the positive finding of benzalkonium chloride in a sample of prawns. (b) ESI-TOF-MS (+) mass spectrum corresponding to the detected peak. (c) Mass spectrum corresponding to the highlighted grey section of spectrum (b); benzalkonium chloride-C_{12} profile is showed in the dotted box.

(2) *Solid-phase extraction*: A 3 mL-aliquot was transferred to a C_{18} cartridge (previously conditioned with 5 mL of methanol and 5 mL of water). The following step was washing the cartridge with 500 μL of water and 500 μL of acetonitrile/water (5:95). The elution step was carried out with 1 mL of acetonitrile/water (30:70).
(3) *Liquid–liquid extraction*: The SPE eluate was transferred to a 20 mL graduated centrifuge tube and 2 mL of ethyl acetate were added. After shaking for 30 s, the organic phase was separated and the extraction was repeated with another 2 mL of ethyl acetate. Both organic extracts were combined and evaporated near dryness, being then reconstituted with 20% (v/v) methanol in water to give a final volume of 1 mL. The resulting extract contained the equivalent of 1 g of sample per mL and was filtered through a 0.45 μm PTFE filter (Millex FG, Millipore, Milford, MA, USA) and transferred into a vial, prior to LC–MS analysis.

2.3.5. SPE-based method II

This procedure has been adapted from Horiea and Nakazawab [28]. The method started with protein precipitation by weighting 5 g of crushed shrimp homogenate and mixing with 100 mL of 0.2% of metaphosphoric acid in acetonitrile. The mix was filtered through 0.45 μm filter and subsequently evaporated under a gentle N_2 stream until 30 mL of extract remained. Then the extract was loaded onto a Oasis™ HLB cartridge (6 mL, 200 mg), which was then washed with 5 mL of acetonitrile:water (20:80) and eluted with 5 mL of acetonitrile. The eluate was finally evaporated to near dryness and reconstituted with 20% (v/v) methanol in water, to give a final volume of 1 mL. The resulting extract contained the equivalent of 1 g of sample per mL, and was filtered through a 0.45 μm PTFE filter and transferred into a vial, prior to LC–MS analysis.

2.4. Spiking procedure

For recovery studies, the samples were spiked with the studied chemicals before the corresponding extraction procedure. A representative 100 g portion of a crushed shrimp homogenate was weighed and fortified with an appropriate volume of mixed standards solution to provide 100 μg kg^{-1} of each of the studied compounds. The mixture was then gently blended in the mortar for an hour in order to produce a homogeneous sample. Then the

Table 3
Analytical parameters of the proposed LC–TOFMS method for the determination of multiclass antibiotics and veterinary drugs in shrimps. Comparison with EU MRLs established in food producing species.

Compound	RT (min)	Linearity (r^2)	LOD ($\mu g\,kg^{-1}$)	EU MRL[a] ($\mu g\,kg^{-1}$)
Benzalkonium chloride-C12	10.3	0.9983	0.60	–
Benzalkonium chloride-C14	11.3	–	–	–
Ethoxyquin	8.9	0.9833	7.10	–
Leucomalachite green	10.0	0.9985	0.60	Non authorized
Malachite green	8.6	0.9844	0.06	Non authorized
Mebendazole	7.7	0.9992	0.10	–
Sulfadiazine	3.7	0.9976	4.50	100[b]
Sulfadimethoxine	7.6	0.9999	0.30	100[b]
Sulfamethazine	5.7	0.9994	0.10	100[b]
Sulfamethizole	5.9	0.9944	0.80	100[b]
Sulfanilamide	1.7	0.9996	3.50	100[b]
Sulfapyridine	4.8	0.9999	0.50	100[b]
Sulfathiazole	5.0	0.9995	2.90	100[b]
Trimethoprim	5.0	0.9996	0.70	50

[a] Commission Regulation (EU) No. 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.
[b] The combined total residues of all substances within the sulfonamides group should not exceed the 100 $\mu g\,kg^{-1}$.

sample was allowed to stand at room temperature for three hours, before it was kept at −18 °C, before analysis.

3. Results and discussion

3.1. Identification and confirmation of selected antibiotics and veterinary drugs by LC–TOFMS

The main instrumental parameters (drying and nitrogen flow rates, vaporizer and drying temperatures and capillary voltage) were optimized to provide the best possible sensitivity. The most significant compound-dependent parameter is the fragmentor voltage, which has a significant effect on in-source CID fragmentation which may affect the sensitivity of the method. Actually, in-source CID fragmentation on LC–TOFMS instruments provides highly valuable structural information for confirmatory purposes based on the use of accurate mass measurements of each characteristic fragment ion together with its elemental composition, which can be used with the accurate mass of the protonated target molecule. For this reason, fragmentor voltage was studied in the range from 160 to 230 V under optimized source conditions. Values of 230 V or higher led to extensive fragmentation in most cases, while values of 160 V provided no significant fragmentation in most of the studied chemicals. A compromise value between sensitivity for quantitation (using the protonated molecule) and fragmentation information for confirmation purposes, a value of 190 V was chosen for further experiments.

Accurate mass measurements of the protonated molecules were used for identification purposes in all cases. Typical mass errors obtained in real matrices were less than 2 ppm in most cases. The mass measurement accuracy along with the characteristic retention time usually provides unambiguous confirmation of the targeted species. In addition, accurate mass measurements performed on selected fragment ions (Table 1) may provide additional information for unequivocal identification. Fig. 2 shows the accurate mass spectrum of leucomalachite green. Quantitation was performed using the extracted ion chromatograms of the m/z values corresponding to protonated molecules with ±10 mDa accurate mass window.

3.2. Selection of the extraction method

From all studied sample treatment procedures, QuEChERS (liquid partitioning with acetonitrile followed by clean-up with PSA) was found to be the most appropriate method for the determination of the studied veterinary drugs in shrimps. The obtained matrix extracts from shrimps using the QuEChERS method were suitable in terms of cleanliness for LC–MS analysis. The rest of the studied methods were not completely satisfactory. In the case of the extraction with trichloroacetic acid (Section 2.3.2), a turbid extract was obtained, and it was found not clear enough to be analyzed by LC–MS. Regarding to SPE-based methods, SPE method I (Section 2.3.4) was a laborious process and the extracts obtained were not as clean as those obtained using the QuEChERS procedure. In contrast, SPE method II (Section 2.3.5) was quicker than method I, but the resulting extracts were similar in cleanliness to extracts obtained by method II. With respect to MSPD procedure, cleanliness of the obtained extracts was satisfactory, but the recoveries of the studied analytes were not suitable for

Table 4
Matrix effects evaluation: comparison between matrix and solvent calibration curves slopes.

Compound	Solvent-based calibration curve	Matrix-matched calibration curve	Matrix effects (%)
Benzalkonium chloride-C12	$y = 1.02 \times 10^5 x + 3.67 \times 10^5$	$y = 8.54 \times 10^4 x + 2.12 \times 10^5$	−16%
Ethoxyquin	$y = 8.28 \times 10^4 x + 5.60 \times 10^5$	$y = 3.06 \times 10^4 x - 1.89 \times 10^6$	−63%
Leucomalachite green	$y = 3.03 \times 10^4 x + 1.11 \times 10^5$	$y = 1.71 \times 10^4 x + 1.33 \times 10^5$	−43%
Malachite green	$y = 7.12 \times 10^4 x + 5.04 \times 10^5$	$y = 7.11 \times 10^4 x + 4.11 \times 10^5$	0%
Mebendazole	$y = 4.60 \times 10^4 x - 7.35 \times 10^4$	$y = 4.49 \times 10^4 x - 5.00 \times 10^4$	−3%
Sulfadiazine	$y = 3.84 \times 10^3 x - 8.51 \times 10^2$	$y = 3.19 \times 10^3 x + 2.34 \times 10^4$	−17%
Sulfadimethoxin	$y = 2.90 \times 10^4 x + 5.18 \times 10^4$	$y = 3.18 \times 10^4 x - 3.15 \times 10^4$	10%
Sulfamethazine	$y = 2.45 \times 10^4 x + 8.02 \times 10^3$	$y = 2.26 \times 10^4 x + 1.99 \times 10^4$	−8%
Sulfamethizole	$y = 6.24 \times 10^3 x - 4.28 \times 10^4$	$y = 4.67 \times 10^3 x + 3.38 \times 10^4$	−25%
Sulfanilamide	$y = 4.53 \times 10^3 x - 4.92 \times 10^3$	$y = 3.09 \times 10^3 x - 8.81 \times 10^3$	−32%
Sulfapyridine	$y = 1.63 \times 10^4 x + 3.34 \times 10^4$	$y = 1.49 \times 10^4 x - 3.28 \times 10^3$	−8%
Sulfathiazole	$y = 5.61 \times 10^3 x - 4.71 \times 10^4$	$y = 4.43 \times 10^3 x + 7.42 \times 10^3$	−21%
Trimethoprim	$y = 4.99 \times 10^4 x + 1.49 \times 10^5$	$y = 4.40 \times 10^4 x - 7.72 \times 10^3$	−12%

the target analysis. Actually, most of the tested species yielded recovery rates below 40% in most cases when using the MSPD procedure.

For these reasons, QuEChERS procedure was selected as more suitable for multiclass detection of antibiotics in shrimps. To evaluate the efficiency of the proposed extraction procedure, recovery studies were carried out at a concentration level of 100 $\mu g\,kg^{-1}$ ($n = 6$). The results obtained from recovery studies performed using QuEChERS sample treatment are shown in Table 2. The recovery rates obtained were satisfactory for most tested analytes, exceeding 80% for the majority of the analytes investigated. Ethoxyquin yielded an average recovery of 53%, while sulfamethizole yielded de lowest value, with an average recovery of 33%. Benzalkonium chloride C_{12} alkyl derivative yielded estimated recoveries of about 50%.

3.3. Analytical performance

The linearity of the method was studied by preparing calibration curves using matrix-matched standards in matrix spiked at five concentration levels from 5 to 500 $\mu g\,kg^{-1}$. Results are shown in Table 3, the correlation coefficients being higher than 0.994, except in three cases (ethoxyquin, malachite green and leucomalachite green). The limit of detection (LOD) was defined as the lowest concentration showing a signal-to-noise ratio of 3, and was empirically determined by fortifying extracts at decreasing analyte concentrations. Results obtained are shown in Table 3. LODs were in the range 0.06–7 $\mu g\,kg^{-1}$. These results demonstrate enough sensitivity for the proposed method to be applied to the quantitative analysis of trace residues of multiclass antibiotics and veterinary drugs in shrimps. As an example, LC–TOFMS analysis of a shrimp extract spiked with selected antibiotics (at a concentration level of 10 $\mu g\,kg^{-1}$) is shown in Fig. 3, together with the extracted ion chromatograms (EICs) of four of the studied analytes (trimethoprim, sulfamethizole, sulfadimethoxine and malachite green).

Matrix effects in LC–MS are well known and this phenomenon may play an important role for quantification of analytes, particularly when electrospray ionization is used. An eventual signal suppression/enhancement of *ca.* 20–25% or higher can be considered relevant for quantitation purposes [29,30]. Matrix effects depend on the ionization source (ESI in positive mode suffers more signal suppression than negative mode), on each analyte, on each matrix and also depends on the sample treatment procedure selected. To evaluate matrix effects we studied the relationship between solvent and matrix-matched calibration curves slopes. According to Table 4, eight of the target analytes showed no significant matrix effect (<20%), whereas five of them displayed signal suppression because of matrix coextractives. Therefore, matrix effects are not excessive, but the use of matrix-matched standards is necessary in order to minimize errors during analyte quantitation in real samples.

Finally, the proposed method was applied to twelve shrimp samples of different origin (Huelva (Spain), Algeria, Turkey, Argentina and Senegal). Each sample was analyzed in duplicate. Positive results on benzalkonium chloride were found in the two replicates of a prawn sample from Huelva, at an estimated concentration level of 2 $\mu g\,kg^{-1}$. Fig. 4 shows the EIC corresponding to benzalkonium chloride-C_{12} and its accurate mass spectrum, matching the isotopic pattern of theoretical elemental composition (94.82% score) with a mass error of 0.74 ppm mass error which provides unambiguous confirmation of the positive. Benzalkonium chloride is authorized in the EU for use as additive at concentrations up to 0.05% [2], and there are no MRL or MRPL established for this compound in food.

4. Conclusions

In this work, a liquid chromatography time-of-flight mass spectrometry (LC–TOFMS) method has been developed for simultaneous quantitative multiclass determination of residues of selected antibiotics and other veterinary drugs in shrimps. Different sample treatment methodologies were tested for the extraction of the targeted species based on either liquid partitioning with different solvents, solid-phase extraction or and matrix solid-phase dispersion. From four sample treatment procedures tested, a QuEChERS procedure was selected as the most appropriate for the analysis of veterinary drugs in shrimps in terms of recovery yields and matrix effects. The developed method allows the screening of 13 analytes representative of families of compounds commonly used as chemotherapeutic drugs in fish farms. The high sensitivity attained by the proposed method (with low detection limits as low as 0.06 $\mu g\,kg^{-1}$ for malachite green) is in compliance with the stringent EU regulations. Since most of the studied analytes are representative of family classes of compounds with similar physicochemical properties, the methodology presented here could be suitable for the extraction and analysis of a large number of veterinary drugs in this type of matrix.

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