Determination of the cardiac drug amiodarone and its N-desethyl metabolite in sludge samples

R. Montes[a•b], I. Rodríguez[a•*], J. Casado[a], M.C. López-Sabater[b], R. Cela[a]

[a] Departamento de Química Analítica, Nutrición y Bromatología, Instituto de Investigación y Análisis Alimentario (IIAA), Universidad de Santiago de Compostela, Santiago de Compostela 15782, Spain
[b] Departamento de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Barcelona, Barcelona 08028, Spain

a r t i c l e i n f o

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a b s t r a c t

For the first time, a procedure for the simultaneous determination of the iodinated drug amiodarone and its major metabolite, N-desethylamiodarone, in sludge from urban sewage treatment plants (STPs) is proposed. Matrix solid-phase dispersion (MSPD) followed by on-line cationic exchange clean-up, in modular configuration, was used as sample preparation technique. Liquid chromatography with tandem mass spectrometry (LC–MS/MS), based on a hybrid quadrupole time-of-flight (QTOF) system, was employed for the selective determination of target compounds. The optimized procedure provided exhaustive recoveries with little effect of the sample matrix in the efficiency of electrospray ionization (ESI). The overall recoveries of the method ranged between 95 and 111%, for samples spiked at different concentration levels. The achieved limits of quantification (LOQs) remained below 10 ng g^{-1} for both compounds, and the linear response range extended up to 2500 ng g^{-1}. Amiodarone and N-desethylamiodarone were ubiquitous in sludge samples, from different STPs located in the Northwest of Spain, with maximum concentrations above 300 ng g^{-1} referred to the freeze-dried matrix. They were also present in stabilized sludge (mixed with lime and thermally dehydrated), which is mostly disposed in agriculture fields as fertilizer. Furthermore, mono-iodinated analogues of amiodarone and N-desethylamiodarone were also tentatively identified in some samples from their accurate MS and MS/MS spectra.

1. Introduction

Amiodarone is an iodinated drug prescribed for the treatment of chronic and severe cardiac diseases since the middle of the eighties. This pharmaceutical displays a high bioavailability, remaining in human body tissues for months after administration [1], with faeces representing the main excretion via for the active ingredient and its metabolites [2]. Despite the successful of amiodarone cardiac treatments, this compound is highly toxic to human beings due to side effects in pulmonary, hepatic and thyroid functions [3,4]. Environmental toxicity is also expected to be relevant on the basis of (1) the potential of amiodarone to decrease T4 levels in zebra fish larvae [5], and (2) quantitative structure–activity relationships (QSAR) estimations [6]; nevertheless, experimental ecotoxicity data are still limited.

Considering a combination of prescription data, lipophilicity and estimated biodegradability rates, Howard et al. [7] labelled amiodarone as a potential persistent and/or bioaccumulative micropollutant, not yet detected in environmental samples by year 2011. Escher et al. [6] performed a mass balance of amiodarone consumption in a hospital, located in Switzerland, and the excretion rates of patients who were treated with this drug. The predicting average concentrations were 0.8 ng mL^{-1} in the hospital sewer, and 0.013 ng mL^{-1} after dilution with urban sewage; however, the employed prediction model did not take into account the domiciliary consumption of amiodarone. Apart from above estimations, quantitative data related to the levels of this drug and its main metabolite, N-desethylamiodarone [8], in sewage water could not be found. Also, little information is available regarding the suitability of screening methods (solid-phase extraction, SPE, followed by liquid chromatography tandem mass spectrometry, LC–MS/MS) for amiodarone determination in sewage water. In fact, Grabic and co-workers [9] reported recoveries below 40% during SPE of amiodarone from spiked surface and sewage water samples, concluding that the developed multi-residue LC–MS/MS method was unsuitable for the screening of this pharmaceutical. Likely, the highly lipophilic character of amiodarone (log K_{ow} 7.82) is responsible for losses occurring during SPE of water samples. Such behaviour points out to the accumulation of this drug in sludge, as already predicted in the

* Corresponding author. Tel.: +34 881814387; fax: +34 881814468.
E-mail address: isaac.rodriguez@usc.es (I. Rodríguez).

literature [10], with negligible concentrations in the water phase at STPs.

To the best of our knowledge, Peysson et al. [11] reported the first quantitative data corresponding to amiodarone levels in sludge. This compound was included in a list of 119 pharmaceuticals screened in sludge from STPs located in France. Four out of seven samples did not contain detectable levels of this species. On the other hand, a maximum value of 1200 ng g^{-1} was found in an urban digested sludge. In a previous study from our group, dealing with antimycotic drugs analysis in sludge [12], we have tentatively identified the existence of amiodarone and N-desethylamiodarone residues in sludge using high resolution MS. However, as the performance of the sample preparation method had not been assessed for any of both compounds, quantitative results could not be reported. Thus, the aims of this research were (1) to validate a sample preparation procedure suitable for the quantitative extraction of amiodarone and N-desethylamiodarone from sludge, and (2) to provide an overview of their levels in samples obtained from STPs located in the Northwest of Spain, an area with an elevated incidence of cardiovascular diseases and, in some points, with an elderly population. Also, the stability of amiodarone and its N-desethyl metabolite during sludge stabilization by combining thermal dehydration with lime (CaO) and iron trichloride ($FeCl_3$) addition is discussed by processing pairs of samples of raw (non-stabilized) and stabilized sludge from the same STP. Finally, other potential amiodarone metabolites were screened in the LC–MS chromatograms of processed samples.

2. Experimental

2.1. Standards, solvents and sorbents

Amiodarone and N-desethylamiodarone standards were obtained from Sigma (Milwaukee, WI, USA). Amiodarone-d_4 (98%), used as internal surrogate (IS) through sample preparation steps, was also acquired from Sigma. Chemical structures and some relevant properties (pK_a and log K_{ow} values) of target compounds are provided as supplementary information, Fig. S1. Individual solutions of each compound and the IS were prepared in methanol. Further dilutions were made in the same solvent. Calibration standards were prepared in methanol with a 0.5% of NH_3.

Acetonitrile and methanol, HPLC-grade purity; ammonia (12% solution in methanol) and ammonium acetate (99%) were supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system by Millipore (Billerica, MA, USA).

Regarding the matrix solid-phase dispersion (MSPD) materials, diatomaceous earth was provided by Sigma, and silica bonded to C_{18} (C_{18} sorbent) was acquired from Agilent Technologies (Santa Clara, CA, USA). Florisil and silica bonded to ethylenediamine-N-propyl groups (PSA sorbent), as bulk materials, were purchased from Supelco (Bellefonte, PA, USA). Florisil was activated at 130 °C, for 24 h, before being employed as MSPD co-sorbent. The rest of materials were used as received.

Empty polypropylene syringes (15 mL capacity), used for MSPD extraction, and 20 μm polyethylene frits were acquired from International Sorbent Technology (Mid Glamorgan, UK). Bond Elut SCX (500 mg) cationic exchanger SPE cartridges were purchased from Agilent Technologies.

2.2. Samples and sample preparation

Grab samples of sludge were obtained from different STPs serving populations between 20,000 and 100,000 inhabitants in the Northwest of Spain. In addition, a reference material of sludge, acquired from the Resource Technology Corporation (Laramie, WY, USA), CRM CNS 312-04-050, and three pairs of non-stabilized and stabilized sludge samples from the same STP, obtained in three consecutive months, were also processed. Samples were received in glass vessels and stored at −20 °C before lyophilization.

Fractions of different sludge samples were combined, spiked with a methanolic solution of the target analytes and homogenized. After overnight solvent evaporation, the spiked sludge (addition level 2 μg g^{-1}) was stored at 4 °C for one week, and then used for optimization of sample preparation conditions. The carbon content (TC) of this pooled matrix was 32.9% of its mass. Recoveries of the optimized methodology were assessed with different sludge samples, spiked at lower concentration levels (100, 200 and 500 ng g^{-1}), and aged for one week before extraction.

Different sorbents and solvents were investigated during optimization of the MSPD extraction and the on-line SPE clean-up steps. The performance of each combination of tested parameters was characterized in terms of matrix effects and extraction efficiencies evaluated as discussed further.

Under final conditions, freeze-dried samples (0.5 g) were dispersed with 2 g of C_{18}, with the help of a pestle, in a glass mortar for 5 min. MSPD syringes were loaded with 1 g of a mixture of PSA and activated Florisil (1:1) over a frit. Then, the dispersed sample was poured on top of the primary clean-up layer. A second frit was placed on top and the packing was slightly pressed. The MSPD syringe was serially connected to a SCX (500 mg) cartridge. A scheme of this modular MSPD system is provided as supplementary information, Fig. S2. Then, 20 mL of methanol were passed through the previously described on-line system. The analytes were retained in the SCX sorbent through cationic exchange interactions, while neutral interferences were removed in the extraction solvent, and the most polar compounds stayed in the primary clean-up layer, within the MSPD cartridge. Thereafter, the modular sample preparation system was disassembled, discarding the MSPD syringe and rinsing the SCX cartridge with 5 mL of methanol. Finally, the SCX cartridge was dried using a gentle stream of nitrogen and analytes were recovered with 10 mL of methanol containing a 0.5% (v:v) of NH_3. This extract was concentrated down to 5 mL before LC–MS/MS analysis.

2.3. Determination conditions

Compounds were determined using a LC–ESI–QTOF–MS system acquired from Agilent Technologies (Wilmington, DE, USA). The LC instrument was an Agilent 1200 Series, comprised of a vacuum degasser unit, two isocratic high-pressure mixing pumps, an autosampler and a chromatographic oven. The QTOF mass spectrometer was an Agilent 6520 model, equipped with a Dual-Spray ESI source and a hexapole collision cell.

Chromatographic separations were developed in a Zorbax Eclipse XDB C_{18} column (100 mm × 2 mm, 3.5 μm), acquired from Agilent Technologies, under gradient programme and at a constant flow of 0.2 mL min^{-1}. The column, connected to the binary pump after a C_{18} (4 mm × 2 mm) guard cartridge from Phenomenex (Torrance, CA, USA), was maintained at 30 °C. The mobile phases consisted of water (A) and methanol (B), both containing 5 mM of ammonium acetate, and the gradient programme was as follows: 0–2 min, 5% B; 4 min, 50% B; 10–15 min, 100% B; 16–25 min, 5% B. The injection volume for standards and sample extracts was 10 μL.

The ESI source used nitrogen (99.999%) for nebulization (30 psi) and also as drying gas (350 °C, 11 L min^{-1}). Analytes were quantified in ESI(+), applying a capillary voltage of 4500 V. Regarding the QTOF hybrid analyzer, it worked in the 2 GHz Extended Dynamic Range resolution mode (mass resolution 11,000 at m/z value of 622.0290). A mass reference solution (Agilent calibration solution A) was infused in the source of the QTOF system through a second nebulizer, to guarantee the accuracy of m/z assignations.

This way, recalibration of the mass axis was continuously performed considering the ions 121.0509 and 922.0098. The Mass Hunter Workstation software (Agilent) was used to control the LC–ESI–QTOF–MS system and to process the recorded data.

Precursor $[M+H]^+$ ions corresponding to target compounds were formed at the source with the fragmentor voltage set at 150 V. Product ion scan spectra were acquired at a rate of 2.5 spectra s^{-1}, in a range of m/z values between 55 and 700 units, selecting a window of 3 min around the retention time of each analyte. MS scan spectra (m/z range from 100 to 1400 units) were simultaneously recorded at the same rate. Selective LC–MS and LC–MS/MS chromatograms were extracted using a window of 20 ppm around the m/z values of the precursor and the most intense product ions of each compound, respectively. The LC–MS/MS signal was used for quantitative purposes, whereas, additional amiodarone metabolites were investigated from their $[M+H]^+$ ions (extraction window 20 ppm) in LC–MS chromatograms from processed sludge samples [13].

2.4. Matrix effects, extraction efficiency and sample quantification

Matrix effects (ME) during ESI (+) ionization were evaluated as follows: $ME = [(A_{se} - A_{be})/A_s] \times 100$, following the criterion established by Matuszewski et al. [14]. A_{se} is the response for a target compound (peak area without IS correction) measured in the spiked extract from a sludge sample, A_{be} is the response for the same compound in an non-spiked extract of the same sample, and A_s is the response for a standard solution containing the spiked concentration of the analyte. Thus, a ME value of 100% indicates the absence of changes between ionization yields for standard solutions and sludge extracts [14].

The efficiency of the sample preparation process (EE) was calculated as the ratio between the responses (peak areas without IS correction) measured for spiked sludge samples and the extracts from the same sample, fortified after finishing sample preparation, multiplied by 100.

The overall recoveries (R) of the procedure were defined as $R = [(C_s - C_b)/C_t] \times 100$. Being C_s, the concentration measured in the extract from a spiked sludge sample; C_b, the concentration in the extract from a non-spiked fraction of the same sample; and C_t, the concentration added to the sample. C_s and C_b were determined against calibration curves obtained for standard solutions prepared in methanol (0.5% NH_3).

3. Results and discussion

3.1. Determination and sample preparation conditions

ESI(+) and collision induced dissociation (CID) parameters were optimized to enhance the detectability of amiodarone and N-desethylamiodarone in MS and MS/MS modes. As regards source parameters, the voltage of the capillary exerted a relevant influence on the intensities of their $[M+H]^+$ ions, with a steady increase between 2500 and 4500 V. Other variables, such as nebulizing and drying gases pressure and fragmentor voltage, played less significant effects in the responses measured for their $[M+H]^+$ ions. The MS/MS fragmentation path for the parent ion of N-desethylamiodarone ($C_{23}H_{25}I_2NO_3^+$) pointed out to a cleavage of the ether bond with the positive charge remaining in any of the resulting fragments: the amino moiety ($C_4H_{10}N^+$, calculated mass 72.0813 Da) or the substituted phenolic species ($C_{19}H_{17}I_2O_3^+$, calculated mass 546.9262 Da) (Fig. 1A). Further fragmentations led to product ions with empirical formulae $C_{13}H_{13}O_2^+$ and $C_7H_3I_2O_2^+$ (Fig. 1A). The product ion with the highest m/z ratio (546.9262 Da) was used for quantitative purposes. On the other hand, CID of the $[M+H]^+$ ion of amiodarone (calculated mass 646.0310) rendered mostly product ions with low m/z ratios, resulting again from the cleavage of the ether bond, but with the positive charge remaining attached to the amino moiety. The collision energy was fixed at 35 eV, since it maximized the response for the tertiary amine fragment (100.1121 Da, corresponding to $C_6H_{14}N^+$), despite a relevant percentage of the parent compound still remained un-fragmented (Fig. 1B). Other product ions (e.g. $C_3H_8N^+$) appeared at lower masses as a result of a further dealkylation of the $C_6H_{14}N^+$ cation (Fig. 1B). Obviously, the MS/MS fragmentation route of the IS (deuterated amiodarone) was analogue to that of the native drug (Fig. 1C). Table 1 summarizes retention times and exact values of precursor and product ions for both compounds and the IS.

MSPD was adopted as sample preparation (extraction and clean-up) technique considering its low cost, adjustable selectivity [15,16] and the fact that target compounds were already found in MSPD extracts corresponding to optimal extraction conditions for antimycotic drugs [12]. Thus, the setup of the MSPD extractions was adopted from our previous study. In brief, the MSPD cartridge contained the dispersed sample over a layer of a primary clean-up sorbent. The extract from the MSPD syringe flows through a SCX cartridge where amiodarone and N-desethylamiodarone are retained through electrostatic interactions with sulphonic groups existing in this sorbent. The investigated extraction conditions involved the use of different dispersant sorbents (C18 and diatomaceous earth), elution solvents (methanol and acetonitrile) and primary clean-up materials (PSA and Florisil). In all cases, analytes were removed from the SCX cartridges with 10 mL of methanol containing a 0.5% of NH_3. All assays were performed in triplicate, without addition of the IS to sludge samples and adjusting the final extract to a volume of 5 mL.

Table 2 compiles the list of the evaluated MSPD extraction conditions together with the obtained EE and ME values. As reported in Section 2.4, ME of 100% point out to identical ESI(+) efficiencies for sludge extracts and standard solutions. Thus, the aim of the optimization was to attain values as close as possible to 100% for both variables. Obviously, the level of attenuation in the ionization of target compounds during sludge samples analysis is defined as 100 minus the normalized ME values compiled in Table 2. Departure extraction conditions (Exp. 1, Table 2) were directly adopted from the extraction of antimycotic drugs [12]. Although the yield of the extraction was quantitative, ME values remained between 45 and 62%. Slightly lower MEs, that is higher signal attenuation effects, were noticed when replacing C_{18} by diatomaceous earth as dispersant. Acetonitrile was unsuitable as extraction solvent (EEs below 5%, Exp 3) and the role of the SCX cartridge to improve the selectivity of the process can be appreciated by comparing the ME values for Exp. 1 and Exp. 4, Table 2. Exp. 5–7 reflect the effect of the primary clean-up sorbent in the performance of the extraction. Florisil rendered cleaner extracts than PSA (higher MEs values) at the expense of lower EEs when using the same volume of methanol, Exp. 1, 5 and 6. Thus, under final conditions (Exp. 7) 0.5 g of both sorbents were mixed and included in the primary clean-up layer, within the MSPD syringe, and the volume of methanol employed in the elution step was set at 20 mL. Such combination of experimental parameters rendered EE above 95% with ME around 90% (Table 2). The configuration of the MSPD extraction is provided as supplementary information, Fig. S2. Total ionic current LC–MS chromatograms corresponding to waste (20 mL volume) and analytical fractions (5 mL final volume) are depicted in Fig. 2. The lower baseline observed for the 2nd fraction demonstrates the elimination of many

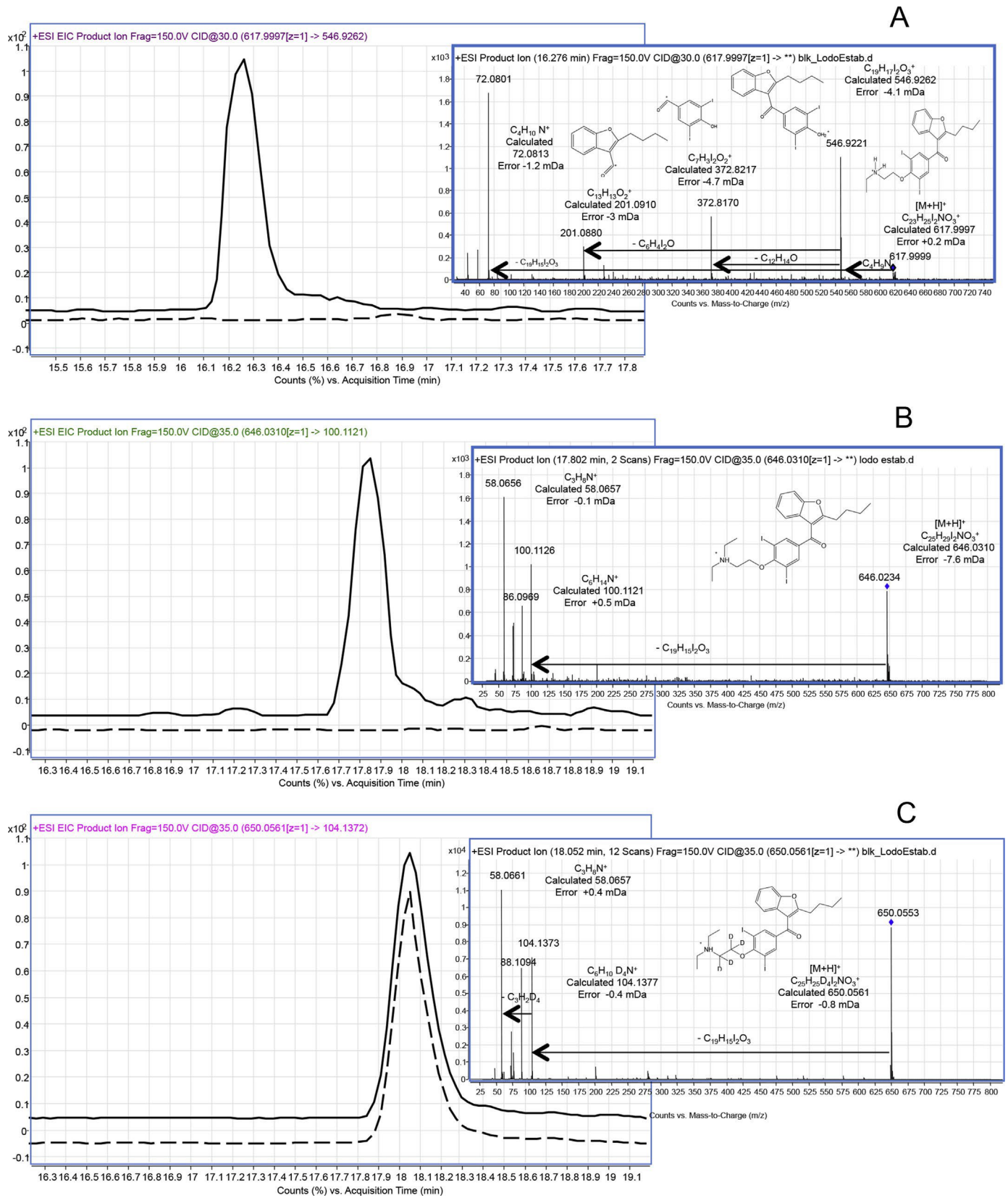

Fig. 1. LC–MS/MS chromatograms for a procedural blank (dotted line) and a non-spiked sludge (solid line) sample, code 12 in Table 5. Product ion scan spectra for each compound and the IS are also shown. (A) N-Desethylamiodarone; (B) Amiodarone; (C) IS.

Table 1
Retention times and quantification ions of target compounds.

Compound	Retention time (min)	[a][M+H]$^+$ ion	Collision energy (eV)	[a] Quantification product ion	[a]Other product ions
N-Desethylamiodarone	16.27	617.9997	30	546.9262	372.8217, 201.910, 72.0813
Amiodarone	17.85	646.0310	35	100.1121	86.0964, 58.0651
Amiodarone-d_4 (IS)	18.05	650.0561	35	104.1377	88.1090, 58.0651

[a] Theoretical mass values (Da) calculated for empirical formulae of product ions given in Fig. 1.

Table 2
Summary of MSPD extraction conditions with achieved extraction efficiencies (EE) and calculated matrix effects (ME), $n = 3$ replicates.

Exp.	Extraction conditions				EE (%) ± SD		ME (%) ± SD	
	Dispersant sorbent	MSPD clean-up sorbent	MSPD elution solvent	SCX clean-up	N-Desethylamiodarone	Amiodarone	N-Desethylamiodarone	Amiodarone
1	C18 (2 g)	PSA (1 g)	MeOH (10 mL)	Yes	96 ± 7	102 ± 3	45 ± 2	62 ± 2
2	Diatomaceous earth (2 g)	PSA (1 g)	MeOH (10 mL)	Yes	102 ± 2	92 ± 1	43 ± 1	53 ± 2
3	C18 (2 g)	PSA (1 g)	ACN (10 mL)	Yes	3.1 ± 0.1	4.2 ± 0.2	100 ± 7	102 ± 5
4	C18 (2 g)	PSA (1 g)	MeOH (10 mL)	No	90 ± 16	91 ± 14	5 ± 1	12 ± 2
5	C18 (2 g)	Florisil (1 g)	MeOH (10 mL)	Yes	50 ± 9	53 ± 6	82 ± 1	81 ± 4
6	C18 (2 g)	Florisil (1 g)	MeOH (20 mL)	Yes	93 ± 5	96 ± 7	80 ± 2	79 ± 6
7	C18 (2 g)	Florisil (0.5 g)/PSA (0.5 g)	MeOH (20 mL)	Yes	96 ± 5	98 ± 6	89 ± 6	92 ± 6

interferences in the rinsing (waste) fraction, which displayed a yellowish appearance versus the completely transparent analytical fraction eluted from the SCX cartridge.

3.2. Performance of the method

The figures of merit of the determination method were calculated alternating the LC–QTOF–MS instrument between MS and MS/MS acquisition modes in the same injection, and using signals corresponding to ions compiled in Table 1 (3rd and 5th columns) for quantification purposes. The IS was added to sludge samples before extraction and included in the calibration standards at 50 ng mL^{-1}.

Linearity was investigated with duplicate injections of standard solutions in the range between 1 and 250 ng mL^{-1} ($n = 7$ levels) with determination coefficients (R^2) values between 0.9987 and 0.9994 in both detection modes. After IS correction, R^2 values of 0.9999 were obtained (Table 3). The precision of the injection was slightly better in the MS mode and the instrumental limits of quantification (LOQs), calculated as the concentration of each compound providing a signal 10 times higher than the standard baseline deviation around the considered peak, were also lower in the single MS mode (Table 3). Such results can be understood since (1) the use of narrow mass extraction windows (20 ppm) guarantees a very low baseline noise level, independent of the detection mode, and

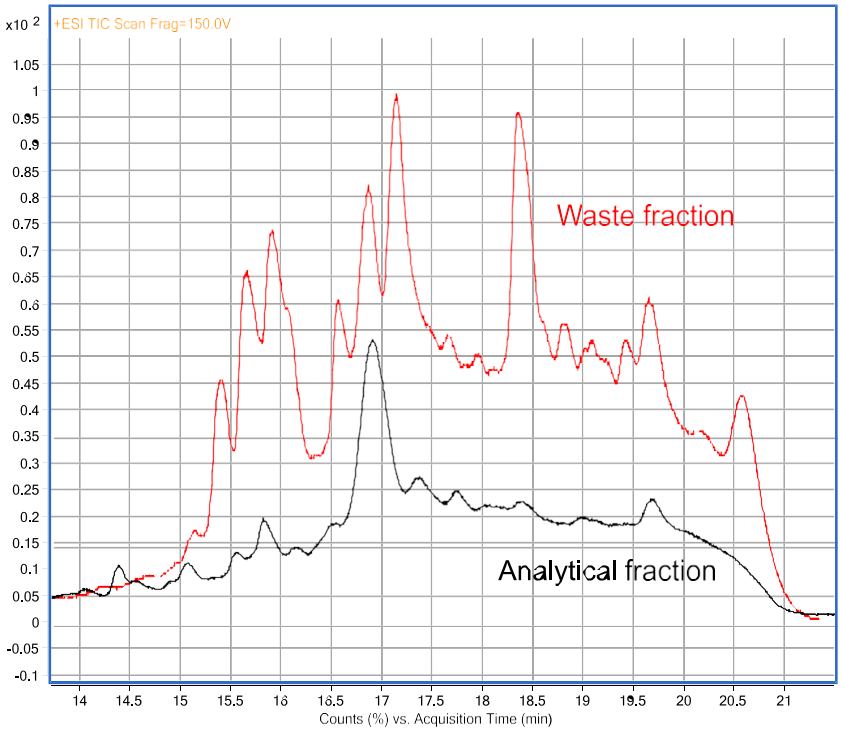

Fig. 2. Total ion current LC–MS chromatograms corresponding to the waste (washing) fraction (20 mL volume) and the analytical fraction (5 mL) obtained with the proposed sample preparation methodology for a sludge sample containing a 33% of total carbon.

Table 3
Performance of the LC–ESI(+)–MS/MS method, without the sample preparation step.

Detection mode	Compound		Linearity, R^2 (1–250 ng mL^{-1}, n = 7 levels)	Injection repeatability, % (n = 7)		LOQs (ng mL^{-1})
				[a]10 ng mL^{-1}	[a]100 ng mL^{-1}	
LC–MS	N-Desethylamiodarone	Without IS	0.9990	1.8	0.6	0.25
		With IS	0.9999	2.2	2.8	
	Amiodarone	Without IS	0.9987	3.0	1.9	0.20
		With IS	0.9999	3.9	4.0	
LC–MS/MS	N-Desethylamiodarone	Without IS	0.9994	6.7	3.3	0.5
		With IS	0.9999	6.7	4.1	
	Amiodarone	Without IS	0.9990	5.1	3.1	0.75
		With IS	0.9999	5.2	3.8	

[a] Concentration level.

Table 4
Recoveries of the overall procedure for samples with different addition levels, and limits of quantification of the method (LOQs, ng g^{-1}). Values corresponding to MS/MS detection.

Compound	Recovery (%) ± SD			LOQs (ng g^{-1})
	[a]100 ng g^{-1}	[b]200 ng g^{-1}	[a]500 ng g^{-1}	
N-Desethylamiodarone	111 ± 11	109 ± 11	106 ± 5	5
Amiodarone	101 ± 8	95 ± 7	104 ± 2	8

[a] n = 3 replicates.
[b] n = 9 replicates, three different days.

(2) intensities of product ions are lower than those corresponding to the respective $[M+H]^+$ precursors in MS spectra. The difference of LOQs between MS and MS/MS modes was most significant for amiodarone than in the case of *N*-desethylamiodarone due to a less efficient MS/MS fragmentation of the active pharmaceutical in comparison to its N-dealkylated metabolite (Fig. 1). However, considering that product ion scan spectra contain more qualitative information than scan MS spectra, the first mode was used for recoveries evaluation and to estimate the concentration of target compounds in real samples.

The absolute recoveries of the method are compiled in Table 4. Obtained data correspond to spiked fractions of sludge samples labelled with code 1 (500 ng g^{-1} addition level) and code 3 (100 and 200 ng g^{-1} addition level) in Table 5. Recoveries evaluated at 100 and 500 ng g^{-1} addition levels correspond to triplicate extractions (3 spiked and 3 non-spiked fractions of each sam-

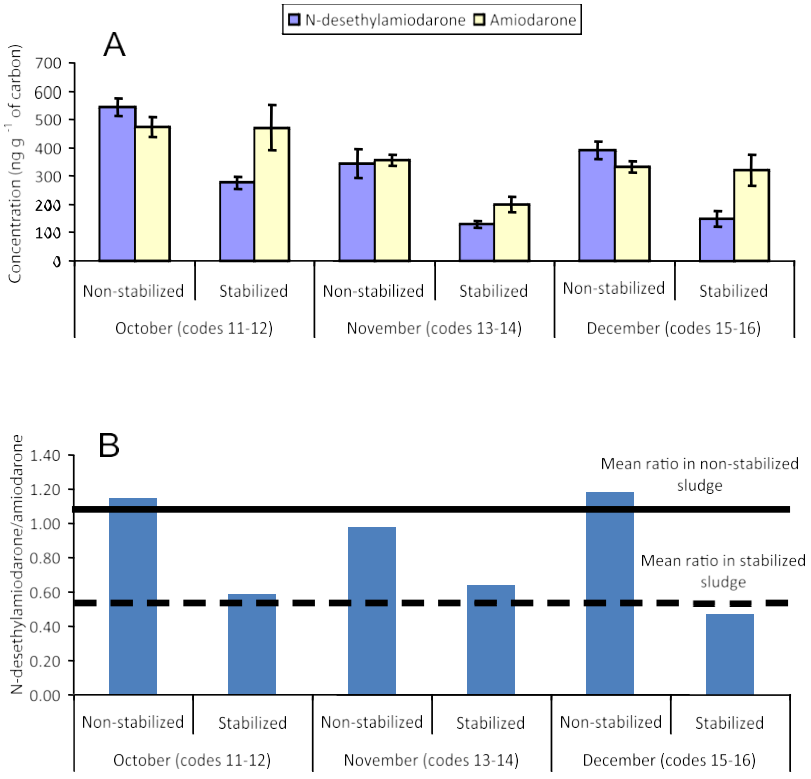

Fig. 3. (A) Concentrations measured in the pairs of samples of non-stabilized and stabilized sludge obtained from the same STP in three consecutive months. Data in ng g^{-1}, referred to the carbon content of each sample. (B) Ratios between N-desethylamiodarone and amiodarone concentrations in the above pairs of samples.

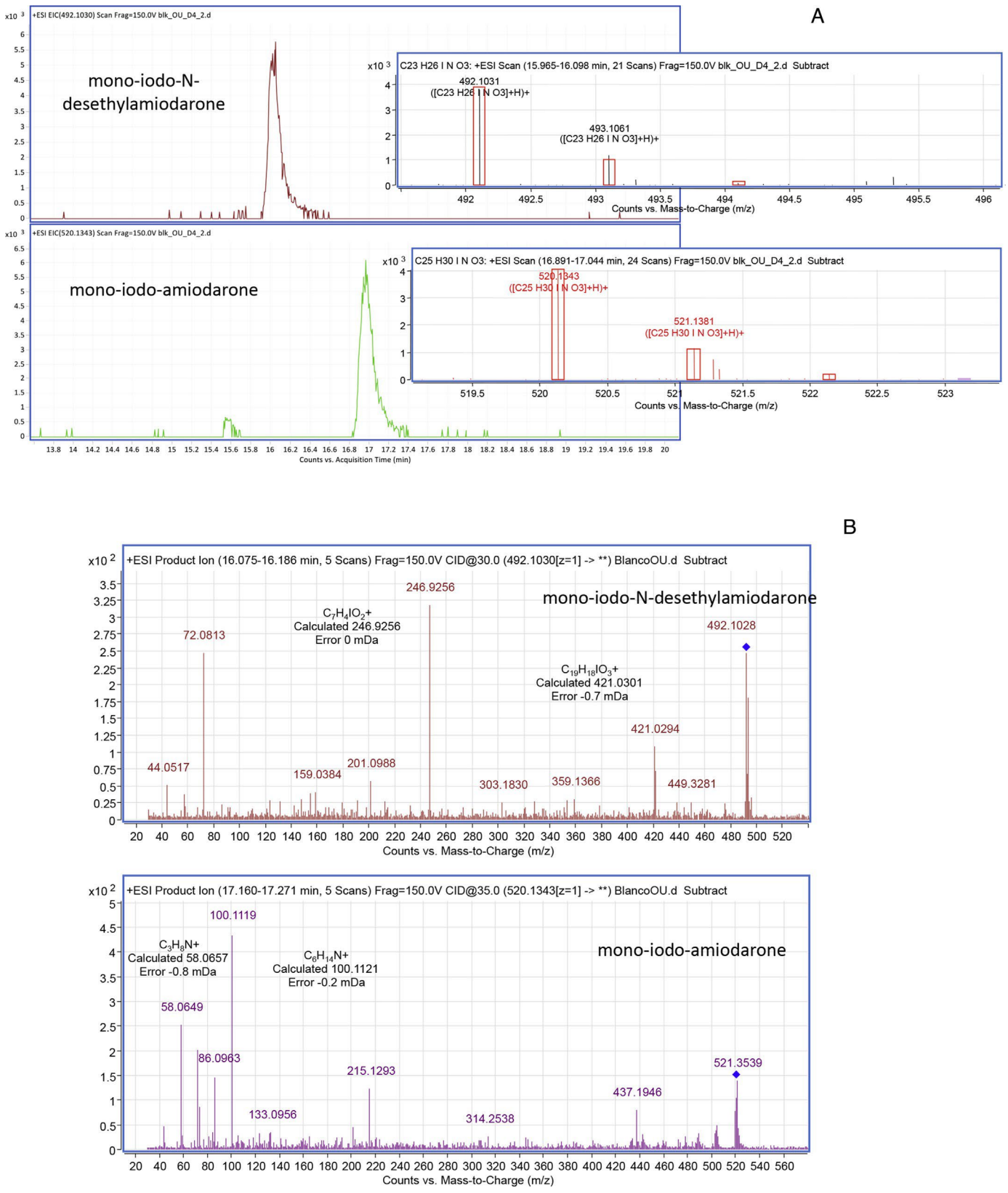

Fig. 4. (A) LC–MS chromatograms (20 ppm mass extraction window) for the $[M+H]^+$ ions of the mono-iodinated analogues of N-desethylamiodarone ($C_{23}H_{26}INO_3$) and amiodarone ($C_{25}H_{30}INO_3$), with their MS spectra, in sludge sample code 1, Table 5. Boxes in the red correspond to theoretical MS spectra for both species. (B) Experimental MS/MS spectra for above compounds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 5
Summary of concentrations (ng g^{-1} of freeze-dried sample) measured in sludge samples, n = 3 replicates.

Code	Concentration ± SD (ng g^{-1})	
	N-Desethylamiodarone	Amiodarone
1	285 ± 8	362 ± 4
2	154 ± 6	146 ± 2
3	95 ± 4	79 ± 1
4	97 ± 5	95 ± 5
5	190 ± 12	273 ± 17
6	96 ± 5	97 ± 6
7	356 ± 29	290 ± 20
8	52.2 ± 0.4	35.5 ± 0.1
9	336 ± 24	311 ± 30
10	28 ± 2	32 ± 2
11	212 ± 12	185 ± 14
12	50 ± 4	85 ± 15
13	137 ± 20	141 ± 8
14	23 ± 2	37 ± 5
15	163 ± 13	138 ± 8
16	22 ± 4	47 ± 8

ple) performed in the same day; whereas, those measured for the 200 ng g^{-1} level were obtained under reproducibility conditions (n = 9 spiked and n = 3 non-spiked aliquots, processed in three consecutive days). In summary, the recoveries of the method varied between 95 and 111%, with standard deviations below 11% (Table 4). The chromatograms for procedural blanks reflected the absence of contamination problems (Fig. 1). Thus, considering (1) the quantitative yield of the sample preparation process, (2) the absence of significant variations in the efficiency of the ESI(+) ionization between sample extracts and methanolic standards, and that (3) baseline noise remained unaltered between standards and sample extracts, the procedural LOQs can be estimated from instrumental values multiplied by the final extract volume (5 mL) and divided by the sample intake (0.5 g). The attained LOQs stayed below 10 ng g^{-1} referred to the freeze-dried sludge (Table 4). Using the same assumption, the linear response range of the method reached up to 2500 ng g^{-1} for both analytes.

3.3. Sludge samples analysis

Table 5 summarizes the concentrations of target pollutants in a total of 16 sludge samples. Codes 1–9 correspond to concentrated sludge (ca. 90% of water at samples reception in the laboratory), obtained during 2013 and 2014 from different STPs in the Northwest of Spain; code 10 corresponds to a reference material of sludge (CNS 312-04-050); and codes 11–16 are samples obtained from the same STP, which receives the wastewater from a 100,000 inhabitants city and a hospital serving a population of 500,000. This latter group of specimens (sample codes 11–16) was collected in three consecutive months (October–December 2014), with pairs 11–12, 13–14 and 15–16 taken before and after sludge stabilization with lime, $FeCl_3$ and thermal treatment, respectively. The above stabilization process is carried out at the STP before disposal of sludge as fertilizer in agriculture fields.

All samples, including the reference sludge sample and the stabilized sludge specimens, contained measurable concentrations of the active drug and the N-desethyl metabolite, with maximum values of 350 ng g^{-1} for both species (Table 5). Apparently, the comparison of concentrations found in the three pairs of non-stabilized and stabilized sludge specimens suggests a relevant elimination of both compounds during this process. However, a different conclusion is drawn if concentrations referred to the freeze-dried matrices (Table 5, codes 11–16) are normalized to their carbon contents (14–17% and 39–41% for stabilized and non-stabilized sludges, respectively). As graphically shown in Fig. 3A, for the pairs of samples collected in October (codes 11–12) and December (codes 15–16), the apparent removal of amiodarone, observed in Table 5, is just the result of sludge dilution with lime and $FeCl_3$. Obviously, the above data correspond to grab samples, which might not be fully representative of the total amount of sludge produced in the STP and; thus, the negligible removal of amiodarone in these two pairs of samples requires further confirmation.

In order to compensate for sampling variability, the ratios between N-desethylamiodarone and amiodarone in the above pairs of samples were calculated. Obtained values are shown in Fig. 3B. In case of non-stabilized samples the ratios remained around 1 (average value 1.1 ± 0.1), similar to the average value calculated for the rest of samples whose concentrations are provided in Table 4 (average ratio 1.0 ± 0.2). On the other hand, this ratio decrease to 0.57 ± 0.09 for stabilized sludge (Fig. 3B). Thus, it is clear that N-desethylamiodarone is removed in a higher extend than the precursor drug during sludge stabilization.

In addition to N-desethylamiodarone, several human metabolites of amiodarone have been reported. Some of them arise from hydroxylation, carboxylation and glucuronidation reactions [8]. Consequently, these species are more polar than amiodarone and are not expected to be sorbed on sludge. In agreement with this prediction, no peak could be observed when LC–ESI(+)–MS chromatograms were explored for the $[M+H]^+$ ion of 4^1-carboxylamiodarone ($C_{25}H_{27}I_2NO_5$ at 676.0060 Da), which is one of the major biliar metabolites of amiodarone. Another potential metabolization route of amiodarone consists of iodine replacement by hydrogen to produce the mono-iodinated amiodarone ($C_{25}H_{30}INO_3$) and the mono-iodinated N-desethylamiodarone ($C_{23}H_{26}INO_3$) compounds [8]. Both species retain the structures of their precursors and thus, they are expected to be amenable to the reported sample preparation method. In this case, low intensity peaks were noticed when LC–MS chromatograms of samples compiled in Table 5 were explored for the $[M+H]^+$ ions of these potential amiodarone transformation products (Fig. 4A). These peaks were noticed in a half of the analyzed samples. Mono-iodinated metabolites appeared at lower retention times than their precursors, which is coherent with the chromatographic behaviour reported by Deng et al. [8]. The calculated scores for their MS spectra (accounting for mass accuracy, isotopic profile and mass spacing [13]) stayed above 90% in a scale from 0 to 100%. In order to confirm the identities of these peaks, their MS/MS spectra were acquired using same collision energies as for their di-iodinated analogous. As observed in Fig. 4B, the spectrum assigned to mono-iodinated N-desethylamiodarone contained fragments whose empirical formulae ($C_7H_4IO_2^+$, calculated mass 246.9256 Da; and $C_{19}H_{18}IO_3^+$, calculated mass 421.0301 Da) differed in the substitution of iodine by hydrogen when compared to product ions in the spectrum of N-desethylamiodarone (372.8217 and 546.9262 Da for $C_7H_3I_2O_2^+$ and $C_{19}H_{17}I_2O_3^+$, respectively, Fig. 1A). Also, product ions in the spectrum of mono-iodinated amiodarone (100.1119, 86.0963 and 58.0549 Da, Fig. 4B) are common to those in the MS/MS spectrum of amiodarone (Fig. 1B).

4. Conclusions

An analytical method for the quantitative determination of the cardiac drug amiodarone and its N-desethyl metabolite in sludge from urban STPs has been developed. The sample preparation process provides quantitative recoveries and LOQ values below 10 ng g^{-1} for both compounds, with a moderate consumption of organic solvents (methanol). Accurate, high resolution product ion full scan detection guarantees the unambiguous determination of both species in complex sludge matrices. All the analyzed samples contained significant concentrations of amidorane and its N-desethyl metabolite (from 20 to 350 ng g^{-1}) confirming the

predicted sorption of this pharmaceutical in sludge and highlighting its ubiquity in this aquatic compartment. In addition, trace levels of the mono-iodinated derivatives of both species were also noticed in some sludge samples. Sludge stabilization, combining lime, iron trichloride and thermal dehydration, reduced the ratio between N-desethylamiodarone and amiodarone levels, suggesting a significant reduction in the concentrations of the first species; nevertheless, this matrix still contained detectable amounts of both iodinated pollutants. Thus, the potential bioaccumulation of amiodarone in terrestrial organisms (i.e. earth worms) merits to be evaluated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.1016/j.chroma.2015.03.024.

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