# Matrix solid-phase dispersion followed by liquid chromatography tandem mass spectrometry for the determination of COXIBs in sewage sludge samples

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## Abstract

A straightforward single-step extraction method based on matrix solid-phase dispersion (MSPD), followed by high-performance liquid chromatography with hybrid quadrupole time of flight mass spectrometry (LC-QTOF-MS), was developed and optimized to determine five non-steroidal anti-inflammatory drugs (Valdecoxib, Etoricoxib, Parecoxib, Celecoxib and 2,5-Dimethylcelecoxib) in sewage sludge samples. The influence of different operational parameters on the extraction efficiency a well as in the matrix effects of the produced extracts was evaluated in detail. Under final working conditions, freeze dried samples (0.2 g) were first soaked with 100 µL of aqueous potassium hydroxide solution (60%, w/v), mixed with 1 g of anhydrous sodium sulfate and dispersed with 1 g of Florisil. This blend was transferred to the top of a polypropylene column cartridge containing 3 g of silica. Analytes were recovered using 15 mL of hexane/acetone (1:2, v/v) mixture. The extracts were concentrated by evaporation and reconstituted with 1mL of methanol/water (1:1, v/v), filtered and injected in the LC system. Quantification limits from 0.005 and 0.05 ng $g^{-1}$ and absolute recoveries

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between 86 and 105% were achieved. Results indicated the presence of two of the targeted COXIBs in real samples of sewage sludge, the highest average concentration (22 ng $g^{-1}$) corresponding to celecoxib. Moreover, the screening capabilities of the LC-QTOF-MS system demonstrated that the developed MSPD extraction procedure might be useful for the selective extraction of some other pharmaceuticals (e.g. amiodarone and their metabolite N-desethylamiodarone, miconazole, clotrimazole and ketoprofen) from sludge samples.

**Keywords**

COX-2 specific inhibitors; NSAID; matrix solid-phase dispersion; liquid chromatography-quadrupole time-of-flight-mass spectrometry; sludge analysis

## *1. Introduction*

Active pharmaceutical ingredients (APIs) are a very large and diverse group of compounds used in considerable quantities through the world designed to prevent, cure and treat diseases and improve health. Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most consumed groups [1,2]. As with many other API residues and metabolites, one of the most important routes into the environment is sewage treatment plants (STPs) and some studies have reported the occurrence of NSAIDs in treated wastewater effluents, indicating that some of these compounds are not efficiently removed in STPs [3,4,5]. On the other hand, when STPs appear efficient in removing pharmaceutical residues as judged by the absence in treated aqueous effluents, these residues frequently

may remain intact accumulated in sludge. In contrast to the many studies of pharmaceutical residues in the aquatic environment, the occurrence and fate of pharmaceuticals in solid matrices, such as sludge, soil and sediments have been rarely studied [6], possibly because the matrix complexity, especially in the case of sludge. This means that several NSAIDs drugs (including the COXIBs), especially the more hydrophobic, low biodegradable compounds are likely re-entering into the environment through the sludge [6,7].

The amount of sewage sludge produced per year in the UE is estimated over 10 million tones [8, 9]. In particular, Spain produces around 1.13 million tons per year and, 81% are employed in agriculture, 7% are eliminated in landfill, another 7% is incinerated and 5% of tons go to other uses [10]. Consequently, it is a real technological challenge the elimination of these compounds as well as the analytical control of its levels in these complex matrices.

Pharmaceutical residues in soils, sediments and sludge have been extracted by ultrasonic solvent extraction (USE) [4], microwave assisted extraction (MAE) [11] and pressurized liquid extraction (PLE, ASE) [4,12-14]. In most cases, the extracts need further clean-up using solid phase extraction (SPE) and concentration to provide analytical extracts allowing the reliable quantification of analytes. An alternative strategy for the extraction of organic environmental pollutants is matrix solid-phase extraction (MSPD), developed by Baker et al. [15] that has been applied for the extraction of a large variety of analytes from solid, semi-solid, viscous and biological matrices [16]. This technique involves a process allowing simultaneous extraction and clean-up of analytes from solid or semi-solid samples with significant reduction in solvent consumption not requiring particularly expensive instrumentation [6].

In this study, five COXIBs were selected on the basis of their recent use as a convenient alternative to the traditional non-steroidal anti-inflammatory drugs (t-NSAIDs) [17]. The aim was to assess the suitability of the matrix solid-phase dispersion technique (MSPD) for the one-step extraction of COXIBs from sludge samples. While the necessary selectivity in the determination is provided by LC-ESI-Q/TOF, the objective was to develop a simple process allowing the quantitative extraction of the analytes while providing clean extracts with a minimum of sample preparation operations. As far as we know this is the first time MSPD has been applied to process sludge samples for the analysis of COXIBs. Different important parameters, such as solid sorbent types, eluting solvents or the amount of additives were studied and optimized. The complete procedure was evaluated for linearity, sensitivity, matrix effects, repeatability and reproducibility demonstrating satisfactory performance. Additionally, using the information gathered by the LC-QTOF-MS instrument, other non-target pharmaceutical residues were screened in the LC–MS chromatograms of samples which extends the practical applicability of the developed sample preparation procedure.

## ***2. Experimental***

### *2.1. Reagents, standards and materials*

Acetonitrile (ACN), methanol (MeOH) (gradient-grade, Lichrosolv), n-hexane, acetone, ethyl acetate (EtOAc) and dichloromethane (DCM) (Suprasolv) were purchase from Merck (Darmstadt, Germany). Ultrapure water was produced by means of a Milli-Q gradient A-10 system (Millipore, Billerica, MA, USA). The commercial selective COXIBs standards (*Valdecoxib (VDC)* (4-(5-methyl-3-

phenyl-4-isoxazolyl)benzenesulfonamide), *Parecoxib (PRC)* (N-{[4-(5-Methyl-3-phenyl-1,2-oxazol-4-yl)phenyl]sulfonyl}propanamide), *Etoricoxib (ETC)* (5-Chloro-6'-methyl-3-[4-(methylsulfonyl)phenyl]-2,3'-bipyridine), *Celecoxib (CLC)* (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide) and *2,5-Dimethylcelecoxib (2,5-DMCLC)* (4-[5-(2,5-dimethylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]-benzenesulfonamide)),*Amiodarone* ((2-butyl-1-benzofuran-3-yl)(4-{[2-(diethylamino)ethyl]oxy}-3,5-diiodophenyl)methanone) and *N-desethylamiodarone* ((2-Butyl-1-benzofuran-3-yl){4-[2-(ethylamino)ethoxy]-3,5-diiodophenyl}methanone), *clotrimazole* (1-[(2-Chlorophenyl)(diphenyl)methyl]-1H-imidazole), *micomazole* nitrate salt (1-{2-[(2,4-Dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl}-1H-imidazole nitrate (1:1) ) and *ketoprofen (*2-(3-Benzoylphenyl) propanoic acid) standards were obtained from Sigma-Aldrich (Madrid, Spain). Potassium hydroxide (Pellets, 85%+, AC) was also purchased to Sigma-Aldrich, and sodium sulfate anhydrous was obtained from Panreac (Barcelona, Spain). Florisil (60-100 mesh) and silica bonded to ethylenediamine-N-propyl groups (PSA sorbent) were purchased from Supelco (Bellefonte, PA, USA). Diatomaceous earth was provided by Sigma-Aldrich and silica bonded to $C_{18}$ ($C_{18}$ sorbent) was purchased to Agilent Technologies (Santa Clara, CA, USA). Silica gel 60 (0.040-0.063 mm) was obtained from Merck. For some experiments, Florisil and silica gel were activated at 120 ºC for at least 12 h and then allowed to cool at room temperature in a desiccator before use.

MSPD empty polypropylene syringes (15 mL capacity) and 20 µm polyethylene frits were acquired from International Sorbent Technology (Mid Glamorgan, UK).

*2.2. Samples and sample preparation*

123 Stabilized and non-stabilized, spiked and non-spiked, sewage sludge samples
124 were used in this study. The sludge samples were obtained from two STPs
125 located in the Northwest of Spain.

126 MSPD conditions were optimized with a pool of non-stabilized sludge fortified with
127 the target analytes at 500 ng $g^{-1}$ level. Spiked samples were prepared by mixing
128 an accurately weighed amount of sludge with a standard solution of COXIBs in
129 acetone. The slurry was manually blended and left in the hood for 2 days
130 (protected from direct exposure to sun light) to allow acetone removal. This
131 operation was carried out one month before sample analysis. Spiked and non-
132 spiked sewage sludge samples were freeze dried and stored in amber glass
133 bottles at 4ºC. Recoveries of the extraction procedure were evaluated with a pool
134 of primary sludge samples, spiked at different concentration levels (100, 250 and
135 500 ng $g^{-1}$). Other sludge samples of different origin, spiked at the lower
136 concentration level (100 ng $g^{-1}$) were used to verify the absence of matrix effects.

137 The influence of different operational parameters of the MSPD method such as
138 the type of dispersant and amount/type of additives, clean-up co-sorbents and
139 extractant solvent were systematically tested considering extraction efficiencies
140 and matrix effects. Under final working conditions, freeze dried sludge samples
141 (0.2 g) were first soaked with 100 µL of aqueous potassium hydroxide solution
142 (60%, w/v) and mixed with 1 g of anhydrous sodium sulfate in a glass mortar with
143 a pestle. Then, 1 g of Florisil was added and the mixture was blended and
144 dispersed during 3 min. The dispersed sample was transferred into a
145 polypropylene column fitted with a single bottom frit containing a layer of 3 g of
146 silica as clean-up sorbent, and the whole solid phase is covered with another 20-
147 µm frit. Analytes were recovered passing 15 mL of hexane/acetone (1:2, v/v)

mixture through the packed cartridge. The extracts were concentrated by evaporation under a stream of nitrogen (e.g. using a Turbo Vap), and finally reconstituted with 500 µL of MeOH diluting to 1 mL with ultrapure water. Extracts were filtered through 0.2 µm GHP Acrodisc 13 mm syringe filters and 15 µL were injected into the LC-QTOF-MS system.

### *2.3. Chromatographic separation and determination*

Compounds were determined using an Agilent LC-ESI-QTOF-MS system (Wilmington, DE, USA). The LC instrument was a 1200 Series consisting of a vacuum degasser unit, a binary high pressure gradient pump, a chromatographic oven and an auto sampler. The Q-TOF mass spectrometer was a 6520 model, equipped with a Dual-Spray ESI source and a hexapole collision cell controlled by the Mass Hunter software (version B.05.01). Compounds were separated in an Ascentis Express C8 fused core column (Supelco) of 50 mm×2.1 mm and 2.7 µm particle size. The mobile phase consisted of ultrapure water containing 0.1% acetic acid (eluent A) and MeOH/ACN (80:20, v/v) (eluent B). Elution conditions were taken from a previous study [5]. In short, the gradient started with 10% solvent B, which was maintained for 2 min and then increased to 80% solvent B over 5 min, and then hold for 5 min. The gradient decreased back to the initial conditions (10% solvent B) in 5 min and 13 min of column re-equilibration was allowed. Flow rate was set at 0.2 mL $min^{-1}$ and the oven temperature was maintained at 40ºC. Injection volume was 15 µL.

### *2.4. Matrix effects, MSPD extraction efficiency and samples quantification*

Potential matrix effects (ME) occurring in the ESI source and MSPD extraction efficiency were studied. In our work, the quantitative evaluation of matrix effects follows the strategy suggested by Matuszewski et al. [18]. Matrix effects during ESI were tested for each analyte spiking an aliquot of the final MSPD extract using a non-spiked aliquot from each sample as contrast. So, the difference in responses (peak area) of the spiked ($R_{se}$) and non-spiked ($R_{nse}$) extracts, was compared to the response of a standard solution prepared in MeOH/$H_2O$ (1:1, v/v) ($R_s$) containing the adopted spiking concentration of the analyte. Matrix effects percentage was calculated as: $\%ME = [(R_{se}-R_{nse})/R_s] \times 100$. Thus, a ME value of 100% indicates the absence of significant effects in the ionization yields both for standard solutions and sludge samples extracts.

The efficiency of the MSPD extraction was evaluated as the ratio between the concentration measured in the extract from the spiked sample and the concentration added to the sample, multiplied by a factor of 100.

The overall recoveries (R) of the procedure were calculated as follows: $\%R = [(C_s-C_b)/C_t] \times 100$, where $C_s$ is the concentration measured in the extract from the spiked sample, $C_b$ is the concentration in the extract from the non-spiked fraction of the same sample and $C_t$ is the concentration added to the sample. $C_s$ and $C_b$ were determined using calibration curves obtained from standard solutions prepared in MeOH/$H_2O$ (1:1, v/v).

## *3. Results and discussion*

### *3.1 Preliminary experiments and MSPD extraction optimization.*

Several preliminary extraction assays were conducted in order to explore the main parameters affecting MSPD process, such as the type of sorbent and the

195 solvent polarity, as well as the clean-up sorbents and the sample additives. These
196 experiments were conducted using multilevel factorial experimental designs as
197 well as some one factor at a time trials to fix the levels of some of the factors
198 considered. An important aspect to consider in the optimization of the conditions
199 for MSPD is the intentional ionization or suppression of ionization of analytes and
200 matrix components. This operation can be carried out by adding acids, bases,
201 salts, antioxidants, etc., during sample blending and/or as additives to the eluting
202 solvents. Starting conditions in these preliminary experiments were taken from
203 published reports [19, 20] dealing with MSPD extraction of hydrophobic
204 compounds from sludge samples. These reports clearly show the convenience
205 of soaking the freeze dried samples with KOH before proceeding with the
206 dispersing stage. The concentration of KOH as well as the use of methanolic or
207 aqueous solutions of KOH was investigated. Also a drying step with anhydrous
208 sodium sulfate (0.5 g) was introduced before the dispersion. This action is
209 necessary to help the sorbent dispersants (e.g. activated Florisil and PSA),
210 extracting the hydrophobic fraction of samples. Considering the detection limits
211 of the QTOF-MS detector a fixed amount of 0.2 g of sludge sample was fixed in
212 all experiments. Additionally, were evaluated factors such as the nature and
213 proportions of the solvent mixtures (hexane/acetone (1:1 and 1:2 v/v), AcOEt,
214 ACN/AcOEt (1:1, and 2:1, v/v), DCM/MeOH (90:10, v/v), ACN/acetone (1:1 and
215 2:1, v/v)) used to elute analytes from cartridges, as well as the needed volumes
216 to fully recover the analytes. A very important parameter in MSPD is the use of
217 clean-up layers in the cartridges helping in obtaining extracts amenable to
218 chromatographic separations. Florisil, silica, PSA and C18 in different amounts
219 and combinations were considered in these preliminary experiments.

Except for etoricoxib, the extraction efficiencies obtained with Florisil as dispersant were higher than those obtained with PSA. Some other sorbents (silica and diatomaceous earth) were assayed as dispersants but produced worse extraction efficiencies than Florisil.

The analysis of experimental design results (not shown) indicated that the proportions of silica and Florisil used in the clean-up layer were the most significant factor for the majority of analytes. Best extractions efficiencies (between 42 and 118%) were obtained with the combination of Florisil as dispersant and 1 g of Florisil and 2 g of silica as clean-up layers. Also the amount of KOH played a significant effect for some analytes (e.g. negative for VDC) as well as the nature of the solvent used to elute the cartridges. The mixture DCM/MeOH (90:10, v/v) produced visually dirty extracts that were not injected. Mixtures containing ACN played a clear negative effect in the extraction of ETC, but positive for CLC and 2,5DMCLC). The mixtures hexane/acetone (1:1 and 1:2) appears providing a compromise solution for most analytes, with extraction efficiencies between 90 and 119%, when KOH was used in aqueous solution because the interaction of the nature of the solvent mixtures and the solvent of KOH appear as significant. Thus, mixtures hexane/ acetone were retained for further experiments.

The effects of different additives (no additive, water, $KOH_{(MeOH)}$ (35%, w/v), $KOH_{(aqueous)}$(120%, w/v)) in the efficiency of the MSPD extraction were compared. Fig. 1 shows that 0.1 mL $KOH_{(aqueous)}$ (120%, w/v) provided the higher extraction efficiencies (between 90% and 106%). Therefore, subsequent experiments were performed using $KOH_{(aqueous)}$ (120% w/v) and several mixtures of silica and Florisil (3 g of total amount) as the clean-up layer were developed. From the

245 results obtained (Fig. 2) it is apparent, especially for PRC, that the efficiencies of
246 the MSPD extraction were better using a single co-column formed by 3 g of silica.

247 The influence of the amount of sodium sulfate during the drying step on the
248 efficiency of the MSPD extraction was also evaluated (Fig. 3). For the majority
249 of analytes, recoveries increased when the amount of sulfate increases from 0.5
250 to 1 g (which corresponds to [g sulfate / mL $KOH_{(aqueous)}$] ratios from 5 to 10), but
251 decreases when the amount of sulfate increases from 1 to 2 g (ratios from 10 to
252 20).

253 Another stage in the optimization of the MSPD procedure consisted on the
254 evaluation of the effect of the amount of aqueous KOH added during the initial
255 blending of samples. Preliminary assays indicated that this treatment was
256 statistically significant to maximize COXIB's recoveries and also that this factor
257 and the composition of the solvent mixture used to elute the analytes are not
258 independent. Thus, a series of experiments were carried out varying the amounts
259 of added alkali, using hexane/acetone (1:1 and 1:2, v/v) as extraction solvents.
260 When using hexane/acetone 1:1, only the efficiency for PRC extraction was
261 slightly increased (about 10%) on increasing the percentage of KOH, but
262 extraction efficiencies were on average 36–63% lower for all analytes (results not
263 shown) when larger (c.a. 200 µL) amounts 120%, w/v aqueous KOH are added.
264 Using hexane/acetone (1:2, v/v) as solvent, the higher efficiencies of extraction
265 were obtained with 100 µL of 60% aqueous KOH (Figure 4), being similar in value
266 to those obtained with 100 µL of 120 % aqueous KOH using hexane/acetone (1:1,
267 v/v). Thus, similar extraction efficiencies can be obtained by adjusting the relative
268 composition of the sample soaking agent and the final eluting solvents mixture.
269 However, as the Figure 4 shows, the elution profiles produced by hexane/acetone

mixtures are also dependent of the amount of aqueous KOH used in the soaking stage. Figure 4 displays the elution profiles of the target compounds from the MSPD packed syringe, using 10 mL fractions of hexane/acetone 1:2 v/v. It is clear in that figure that not only the recoveries for the analytes are better when using 60% aqueous KOH. If lower percentages of KOH are used, only the first fraction contains the analytes which cannot be detected in further fractions. A quite similar behavior is apparent for higher concentrations of KOH (120%) although in that case, small amounts of analytes are also detected in the second and third fractions. If 60% KOH is used, all analytes are eluted over 80% in the first fraction and elution is nearly quantitative in the second fraction. Some additional experiments demonstrated that 15 mL of mixture hexane/acetone (1:2, v/v) were sufficient to elute the analytes from the MSPD column. In all, conditions described in section 2.2 were adopted as optimal for a fixed amount of 0.2 g of freeze dried sludge sample. These conditions provide a really simple and relatively quick procedure for sample preparation of sludge in the analysis of COXIBs residues.

*3.2. Performance of the method*

Table 1 summarizes some features of the optimized method, including chromatographic and MS determination parameters.

Nine-point calibration curves were constructed using linear regression analysis by injecting standard solutions in the range of 0.005-200 ng $mL^{-1}$. Recoveries of the overall sample preparation process were calculated against external standards.. Recoveries during the procedure optimization process were evaluated with a pool of primary sludge spiked at different concentration levels (Table 1). Later, verified with some stabilized and non-stabilized sludge samples

of different origins and sampling dates, spiked with target compounds at 100 ng $g^{-1}$ (Table 2). Recoveries ranged from 86 to 105%, with standard deviations below 4%. Most recovery data shown in Tables 1 and 2 correspond to repeatability conditions (n=3, same day). Data for samples fortified at 250 ng $g^{-1}$ correspond to extractions performed in three consecutive days (9 replicates). ME values ranging from 91 to 105 % (Table 1) indicates the absence of significant changes between ionization yields for standard solutions and sludge extracts, thus confirming that the developed extraction procedure provides quite clean extracts which avoids the need of time consuming standard additions calibration.

Instrumental limits of quantification (LOQs) were calculated using signal to noise ratio (*S/N*) as 10×*S/N*. Procedural blanks did not exhibit detectable traces of COXIBs, thus the attained LOQs were controlled by sensitivity of the LC-QTOF-MS. Consequently, LOQs of the reported method were calculated from instrumental LOQs multiplied by the final extract volume (1 mL) and divided by the sample intake (0.2 g). The attained LOQs (referred to freeze dried sludge material) ranged from 0.005 (for ETC) to 0.05 ng $g^{-1}$ (for VDC, CLC and 2,5-DMCLC) .

*3.3 Sludge sample analysis*

The optimized method was applied to determine COXIBs in several samples of non-stabilized and stabilized sludge, collected during different months between November 2014 and December 2015 from two STPs serving cities of about 100000 inhabitants. ETC was detected in all samples, with average concentrations ranging to 1.8 to 14.7 ng $g^{-1}$ (Table 2). CLC could be detected in three samples (collected on 2015), with average concentrations ranging to 6.5 to

21.6 ng $g^{-1}$ (Table 2). Although it is clear that results obtained for sludge samples are insufficient to evaluate the efficiency of STPs, the comparison of concentrations found in non-stabilized and stabilized sludge samples (samples collected in November 2014 and December 2015, Table 2), and particularly the presence of some investigated COXIBs in stabilized sludges, suggest low efficacy of the waste water treatments done. Fig. 5 shows the extracted LC-MS chromatogram for a non-stabilized sludge. The overall score for the peaks was between 95.6 and 99.7 over 100, and the mass error remained below 1 ppm, as shown in the figure (Fig 5 A). The accurate ion products scan MS/MS spectrum of the peaks of each investigated compound in the samples provided an unambiguous confirmation of its identity. Absolute differences between calculated and experimental masses of the most intense ion in MS/MS spectra were between 2.5 to 1.4 ppm (Fig. 5 B). It should be pointed out that stabilized sludge is used frequently as fertilizer in agriculture, and these practices may contribute to the potential bioaccumulation in terrestrial organisms, and/or to the transferring of the target compounds to the water cycle and eventually into the food chain.

*3.4 Applicability of the method to the Screening of other pharmaceuticals in sludge.*

The information existing in LC-MS files of the analyzed sludge samples was used to investigate the presence of other non-target pharmaceutical residues. The mechanisms that control pharmaceuticals sorption onto sludge are complex not depending only on the lipophilicity of the compounds. Other factors, including solubility, vapor pressure, and the environmental conditions (temperature, air

345 disturbance, or soil organic-matter content), are also important [21]. To develop
346 a post-target screening in the processed sludge samples some frequently
347 reported [22, 23] pharmaceuticals with relatively high values of log $K_{OW}$ were
348 considered (Table S1).
349 The approach used in post-target studies has been described in several articles
350 [24, 25]. In short, the Mass Hunter software was used to search for their
351 characteristic ions (normally $[M-H]^+$) in the LC-MS chromatograms of samples,
352 within a mass window of 20 ppm around their theoretical values. This software
353 extracts the accurate LC-MS chromatograms and compares the experimental MS
354 spectra of detected peaks with the theoretical (calculated) ones. Then, a
355 normalized score (0-100), which combines mass accuracy, isotopic pattern and
356 spacing among ions in the cluster for the characteristic ion, is calculated. A score
357 of 100 represents a perfect match between the empirical and the theoretical
358 spectrum. Tentative identifications obtained from this post-target strategy
359 requires additional confirmation, using product ion scan MS/MS spectra (a
360 second injection was made considering different collision energies).
361 Two anti-mycotic drugs, miconazole and clotrimazole were detected in all the
362 samples studied. Amiodarone, a drug prescribed for the treatment of chronic and
363 severe cardiac diseases and its N-desethyl metabolite were also detected in all
364 samples except for a pool of samples collected on May 2015. Ketoprofen, an anti-
365 inflammatory drug (t-NSAIDs), was detected in five samples (both in stabilized
366 and non-stabilized sludge) of the seven studied.
367 Fig. 6 shows the LC–MS chromatogram for the characteristic ions of the
368 ketoprofen (255.1009-Da, retention time 12.15 min) corresponding to the extract
369 from a non-stabilized sludge sample and confirmation MS spectrum. Similar

figures for Miconazole (414.9936–Da, retention time 12.71 min), Clotrimazole (277.0788 Da, retention time 12.10 min), amiodarone (646.0310 Da, retention time 13.21 min), and N-desethylamiodarone (617.9997-Da, retention time 13.10 min), have been included in Figure S1 of the supplementary material. The superposed boxes represent the theoretical spectra of the peak. The calculated scores stayed between 91.34 and 98.49 over 100, and the mass error remained below 4 ppm (Fig S1 A). The MS/MS spectra for these ions displayed a fragmentation pattern coherent with de chemical structures of the above drugs (Fig. S1 B). Some of these findings (e.g. for amiodarone) have been reported previously [26]. Furthermore, the presence of the compounds in the samples was confirmed by spiking with pure standards verifying retention times coincidence.

## *4 Conclusions*

This study has demonstrated for the first time the suitability of MSPD as a single-step extraction method for the quantitative determination of the most relevant COXIB non-steroidal anti-inflammatory drugs in sewage sludge samples. The proposed method improved the selectivity of COXIBs extraction, providing clean extracts with no significant matrix effects during ESI ionization. The developed MSPD method followed by LC-QTOF-MS determination provided LOQs low enough for selective and unambiguous determination of target compounds in sludge samples. Data obtained for real samples confirmed the systematic presence of CLC and also a high frequency of ETC in sludge samples from urban STPs. Finally, the post-target capabilities of the QTOF instrument were used for the post-target identification of additional pharmaceuticals in the samples. These results show clearly that a simple MSPD extraction method could be extended to

the extraction of several other drug residues (e.g. the basic drugs clotrimazole, miconazole, amiodarone and their metabolite N-desethylamiodarone, and acid drugs as ketoprofen) from complex sludge samples.

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524 Figure legends

525

526 Figure 1. Efficiency of MSPD extraction as a function of the additive. (Sample:
527 0.2 g ; Elution solvent: 30 mL hexane/acetone (1:1, v/v)), n=3.

528

529 Figure 2. Efficiency of MSPD extraction as a function of the co-column. (Sample:
530 0.2 g ; Elution solvent: 30 mL hexane/acetone (1:1, v/v)), n=3.

531

532 Figure 3. Efficiency of MSPD extraction as a function of the amount of sodium
533 sulfate added as drying agent. (Sample: 0.2 g; Elution solvent: 30 mL
534 hexane/acetone (1:1, v/v); n=3)

535

536 Figure 4. Influence of the amount of aqueous KOH on the efficiency of the MSPD
537 extraction and elution profiles of COXIBs from MSPD cartridge using fractions
538 (10 mL each) of hexane/acetone (1:2, v/v). (Volume of aqueous KOH: 100 µL in
539 all experiments).

540

541 Figure 5. A) LC-QTOF-MS chromatograms (20 ppm mass extraction window) for
542 the $[M+H]^+$ ions for CLC and ETC, with their MS spectra, in a non-stabilized
543 sludge sample. Boxes in red correspond to theoretical MS spectra for both
544 species. B) Experimental MS/MS spectra for above compounds.

545

546 Figure 6. A) LC-MS chromatograms for ketoprofen $[M+H]^+$ ion, and B) MS/MS
547 confirmation spectrum.

Table 1 Summary of chromatographic and QTOF–MS determination parameters, linearity data, Global recoveries (R%, Average±SD) and limits of quantification of the method (ng $g^{-1}$)

| Compound | Ret. Time (min) | [a]Precursor $[M+H]^+$ | Linearity | | Global recovery± SD | | | %ME±SD | LOQs (ng g-1) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Calibration range, 9 levels (ng $mL^{-1}$) | $R^2$ | [b]100 ng g-1 (n=3) | [b,c]250 ng g-1 (n=9) | [b]500 ng g-1 (n=3) | [d]100 ng g-1 (n=3) | |
| VDC | 12.1 | 315.0798 | 0.01-200 | 0.9996 | 97.1±3.4 | 103.5±1.3 | 98.5±0.5 | 95.8±4.7 | 0.05 |
| ETC | 11.1 | 359.0616 | 0.005-200 | 0.9995 | 104.2±0.9 | 100.3±2.1 | 100.9±1.6 | 99.3±0.2 | 0.005 |
| PRC | 12.6 | 371.1060 | 0.01-200 | 0.9994 | 98.0±1.8 | 97.6±1.7 | 96.5±0.2 | 91.4±2.4 | 0.015 |
| CLC | 13.2 | 382.0832 | 0.01-200 | 0.9996 | 99.8±1.4 | 97±1.7 | 98.9±0.7 | 103.3±1.7 | 0.05 |
| 2,5-DMCLC | 13.5 | 396.0988 | 0.01-200 | 0.9998 | 101.2±1.8 | 104.5±1.0 | 100.9±0.4 | 105.8±3.9 | 0.05 |

[a]Used as quantification ion

[b] Concentrations added to sludge samples

[c] Intermediate precision conditions

[d] Concentrations added to sample extracts

**Table 2.** Concentration (ng $g^{-1}$, Mean±SD) of COXIBs in environmental sludge samples and recoveries (%, [a]Average ±SD) of the optimized method, n=3 replicates

| Sample Sludge type | Non-treated (non-stabilized) | | | | | | Treated (stabilized) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Concentration Mean± SD | | | | %R± SD | | Concentration Mean± SD | | %R± SD | |
| Sample origin code | **1** | **2** | **2** | **2** | **2** | **2** | **2** | **2** | **2** | **2** |
| Date | May 2014 | November 2014 | May 2015 | December 2015 | November 2014 | December 2015 | November 2014 | December 2015 | November 2014 | December 2015 |
| VDC | n.d. | n.d. | n.d | n.d. | 86.4±2.0 | 102.1±1.4 | n.d. | n.d. | 84.9±2.0 | 89.1±1.3 |
| ETC | 5.4±0.6 | 14.7±0.5 | 1.9±0.2 | 11.4±0.5 | 100.6±1.6 | 103.2±1.0 | 14.1±0.5 | 1.8±0.1 | 97.6±0.3 | 105.7±1.7 |
| PRC | n.d. | n.d. | n.d. | n.d. | 85.7±1.7 | 105.3±1.6 | n.d. | n.d. | 87.1±0.6 | 88.1±1.1 |
| CLC | n.d. | n.d. | 21.6±1.6 | 12.5±0.9 | 94.8±1.6 | 96.2±2.3 | n.d. | 6.5±0.25 | 94.5±1.6 | 96.5±2.1 |
| 2,5-DMCLC | n.d | n.d | n.d | n.d | 90.5±2. | 87.1±2.0 | n.d | n.d | 89.3±2.2 | 89.9±2.0 |

[a] Concentrations added to sludge samples:100 ng $g^{-1}$

n.d., not detected

**Table** S0 Studied COXIBs and physicochemical properties.

| Compound | Abbreviation | Formula | Precursor $[M+H]^+$ | p*Ka** | log $K_{ow}$ |
|---|---|---|---|---|---|
| Etoricoxib | ETC | $C_{18}H_{15}ClN_2O_2S$ | 359.0616 | 3.68±0.12 (Most Basic) | 2.455±0.420 |
| Valdecoxib | VDC | $C_{16}H_{14}N_2O_3S$ | 315.0798 | 9.83±0.10 (Most acidic)<br>-3.25±0.10 (Most Basic) | 3.565±0.624 |
| Parecoxib | PRC | $C_{19}H_{18}N_2O_4S$ | 371.1060 | 5.08±0. 10 (Most acidic)<br>-3.45±0.10 (Most Basic) | 4.330±0.602 |
| Celecoxib | CLC | $C_{17}H_{14}F_3N_3O_2S$ | 382.0832 | 9.68±0.10 (Most acidic)<br>-3.81±0.10 (Most Basic) | 2.593±0.696 |
| 2,5-Dimethylcelecoxib | 2,5DMCLC | $C_{18}H_{16}F_3N_3O_7S$ | 396.0988 | 9.67±0.10 (Most acidic)<br>-4.02±0.10 (Most Basic) | 3.120±0.696 |

*Calculated using Advanced Chemistry Development (ACD/Labs) Software Solaris V 11.02 (©1994-2015), Chemical Abstracts Service (CAS), American Chemical Society, DC, 2015.

Table S1.- Summary of pharmaceuticals included in the screening study

| | CAS number | Formula | $[M+H]^+$ mass | pKa | $logK_{ow}$ | Class |
|---|---|---|---|---|---|---|
| Amiodarone | 1951-25-3 | $C_{25}H_{29}I_2NO_3$ | 646.0310 | 9.37 | 7.81 | Antiarrhythmic |
| Amitryptiline | 50-48-6 | $C_{20}H_{23}N$ | 278.1909 | 9.18 | 4.41 | Antidepressant |
| Atorvastatin | 134523-00-5 | $C_{33}H_{35}FN_2O_5$ | 559.2603 | 0.38 ; 4.29 | 3.85 | Antidepressant |
| Azelastine | 58581-89-8 | $C_{22}H_{24}ClN_3O$ | 382.1681 | 9.16 | 3.47 | Antiasthmatic |
| Azithromycin | 83905-01-5 | $C_{38}H_{72}N_2O_{12}$ | 749.5151 | 8.59;13.28 | 2.58 | Antibiotic |
| Bromocriptine | 25614-03-3 | $C_{32}H_{40}BrN_5O_5$ | 654.2286 | 6.44; 9.60 | 8.60 | Antiparkinson |
| Carbamazepine | 298-46-4 | $C_{15}H_{12}N_2O$ | 237.1022 | 13.94 | 2.67 | Antiepileptic<br>Analgesic<br>Anticonvulsant |
| Chlorpromazine | 50-53-3 | $C_{17}H_{19}ClN_2S$ | 319.1030 | 9.41 | 5.20 | Antipsychotics |
| Chlorprothixene | 113-59-7 | $C_{18}H_{18}ClNS$ | 316.0921 | 9.05 | 5.21 | Antipsychotic |
| Climbazole | 38083-17-9 | $C_{15}H_{17}ClN_2O_2$ | 293.1051 | 5.66 | 3.50 | Antimycotic |
| Clomipramine | 303-49-1 | $C_{19}H_{23}ClN_2$ | 315.1623 | 9.46 | 4.94 | Antidepressants |
| Clotrimazole | 23593-75-1 | $C_{22}H_{17}ClN_2$ | 277.0788<br>$[M-C_3H_3N_2]^+$ | 6.12 | 4.10 | Antimycotic |
| Cimetidine | 51481-61-9 | $C_{10}H_{16}N_6S$ | 253.1230 | 7.07;14.13 | -0.07 | Antiulcer |
| Ciprofloxacine | 85721-33-1 | $C_{17}H_{18}FN_3O_3$ | 332.1405 | 6.40 ; 8.70 | 1.60 | Antibiotic |
| Clemastine | 15686-518 | $C_{21}H_{26}ClNO$ | 344.1776 | 10.23 | 5.30 | Dermatologic drug |
| Codeine | 76-57-3 | $C_{18}H_{21}NO_3$ | 300.1594 | 8.23; 13.40 | 1.39 | Antitussive |
| Cyproheptadine | 129-03-3 | $C_{21}H_{21}N$ | 288.1747 | 8.95 | 5.80 | Dermatologic |
| Dihydroergotamine | 511-12-6 | $C_{33}H_{37}N_5O_5$ | 584.2867 | 7.22 ;9.64 | 5.70 | Analgesic |

| | | | | | | |
|---|---|---|---|---|---|---|
| Diphenhydramine | 88637-37-0 | $C_{17}H_{21}NO$ | 256.1696 | 8.76 | 3.00 | Antiparkinson |
| Domperidone | 57808-66-9 | $C_{22}H_{24}ClN_5O_2$ | 426.1691 | 9.0; 11.11 | 4.05 | Antiemetic |
| Doxepine | 1668-19-5 | $C_{19}H_{21}NO$ | 280.1696 | 9.40 | 3.84 | Antidepressant |
| Doxycycline | 564-25-0 | $C_{22}H_{24}N_2O_8$ | 445.1605 | 4.50; 10.84 | 1.78 | Antibiotic |
| Duloxetine | 116539-59-4 | $C_{18}H_{19}NOS$ | 298.1260 | 10.0 | 4.81 | Antidepressant |
| Econazole | 27220-47-9 | $C_{18}H_{15}Cl_3N_2O$ | 383.0229 | 6.68 | 5.50 | Antimycotic |
| Ezetimide | 163222-33-1 | $C_{24}H_{21}F_2NO_3$ | 408.1397 | 9.72 | 3.96 | Antilipidemic |
| Flucomazole | 86386-73-4 | $C_{13}H_{12}F_2N_6O$ | 307.1113 | 2.64;11.01 | 0.45 | Antimycotic |
| Escitalopram | 128196-01-0 | $C_{20}H_{21}FN_2O$ | 325.1711 | 9.57 | 3.47 | Antidepressant |
| Etaconazole | 60207-93-4 | $C_{14}H_{15}Cl_2N_3O_2$ | 328.0614 | 2.90 | 3.60 | Antimycotic |
| Fluoxetine | 54910-89-3 | $C_{17}H_{18}F_3NO$ | 310.1413 | 10.05 | 3.93 | Antidepressant |
| Fluphenazine | 69-23-8 | $C_{22}H_{26}F_3N_3OS$ | 438.1821 | 7.39; 14.96 | 3.92 | Antipsychotic |
| Haloperidol | 52-86-8 | $C_{21}H_{23}ClFNO_2$ | 376.1474 | 8.04; 13.86 | 3.76 | Antipsychotic |
| Ketoconazole | 65277-42-1 | $C_{26}H_{28}Cl_2N_4O_4$ | 531.156 | 6.88 | 4.30 | Antimycotic |
| Ketoprofen | 22071-15-4 | $C_{16}H_{14}O_3$ | 255.1009 | 4.23 | 2.91 | Anti-inflammatory |
| Levomepromazine | 60-99-1 | $C_{19}H_{24}N_2OS$ | 329.1682 | 9.32 | 4.94 | Analgesic; |
| Lidocaine | 137-58-6 | $C_{14}H_{22}N_2O$ | 235.1805 | 7.96 ;14.23 | 2.20 | Local Anesthetic |
| Meclizine | 569-65-3 | $C_{25}H_{27}ClN_2$ | 391.1936 | 6.73 | 5.28 | Antiemetic |
| Miconazole | 22916-47-8 | $C_{18}H_{14}Cl_4N_2O$ | 414.9936 | 6.64 | 6.10 | Antimycotic |
| N-Desethylamiodorone | 83409-32-9 | $C_{23}H_{25}I_2NO_3$ | 617.9997 | 9.01 | 7.32 | Metabolite Amiodarone |
| Norsertraline | 87857-41-8 | $C_{16}H_{15}Cl_2N$ | 275.0385 $[M-NH_3]^+$ | 9.13 | 4.88 | Metabolite Sertraline |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ofloxacine | 82419-36-1 | $C_{18}H_{20}FN_3O_4$ | 362.1511 | 5.19;7.37 | 1.85 | Antibiotic |
| Paroxetine | 61869-08-7 | $C_{19}H_{20}FNO_3$ | 330.1500 | 9.68 | 3.70 | Antidepressant |
| Perphenazine | 58-39-9 | $C_{21}H_{26}ClN_3OS$ | 404.1558 | 7.39;14.96 | 3.94 | Antipsychotic |
| Pizotifen | 15574-96-6 | $C_{19}H_{21}NS$ | 296.1467 | 9.04bp | 2.71 | Antidepressant |
| Promethazine | 60-87-7 | $C_{17}H_{20}N_2S$ | 285.1420 | 8.98bp | 4.89 | Antienemic |
| Quinacrine | 83-89-6 | $C_{23}H_{30}ClN_3O$ | 400.2150 | 10.47 | 5.59 | Antimalarial, antiplatyhelmintic |
| Roxithromycin | 80214-83-1 | $C_{41}H_{76}N_2O_{15}$ | 837.5318 | 8.16; 13 | 2.84 | Antibiotic |
| Sertraline | 79617-96-2 | $C_{17}H_{17}Cl_2N$ | 306.0811 | 9.47 | 5.08 | Antidepressant |
| Simvastatin | 79902-63-9 | $C_{25}H_{38}O_5$ | 419.2792 | 13.49 | 4.72 | Antilipemic |
| Sulfamethoxazole | 723-46-6 | $C_{10}H_{11}N_3O3S$ | 254.0594 | 1.39; 5.81 | 0.70 | Antibiotic |
| Sulfapyridine | 144-83-2 | $C_{11}H_{11}N_3O_2S$ | 250.0645 | 2.13;8.54 | 0.47 | Antibiotic |
| Tamoxifen | 10540-29-1 | $C_{26}H_{29}NO$ | 372.2322 | 8.69 | 5.13 | Anti-estrogen |
| Terbinafine | 91161-71-6 | $C_{21}H_{25}N$ | 292.206 | 7.1 | 5.60 | Antimycotic |
| Tetracycline | 60-54-8 | $C_{22}H_{24}N_2O_8$ | 445.1605 | 4.50; 11.02 | 0.62 | Antibiotic |
| Tramadol | 27203-92-5 | $C_{16}H_{25}NO_2$ | 264.1958 | 9.61; 14.47 | 2.32 | Analgesic |
| Verapamil | 52-53-9 | $C_{27}H_{38}N_2O_4$ | 455.2904 | 8.97 | 4.02 | Vasodilator agent |
| Zolpidem | 82626-48-0 | $C_{19}H_{21}N_3O$ | 308.1757 | 6.77 | 3.089 | Sedative |

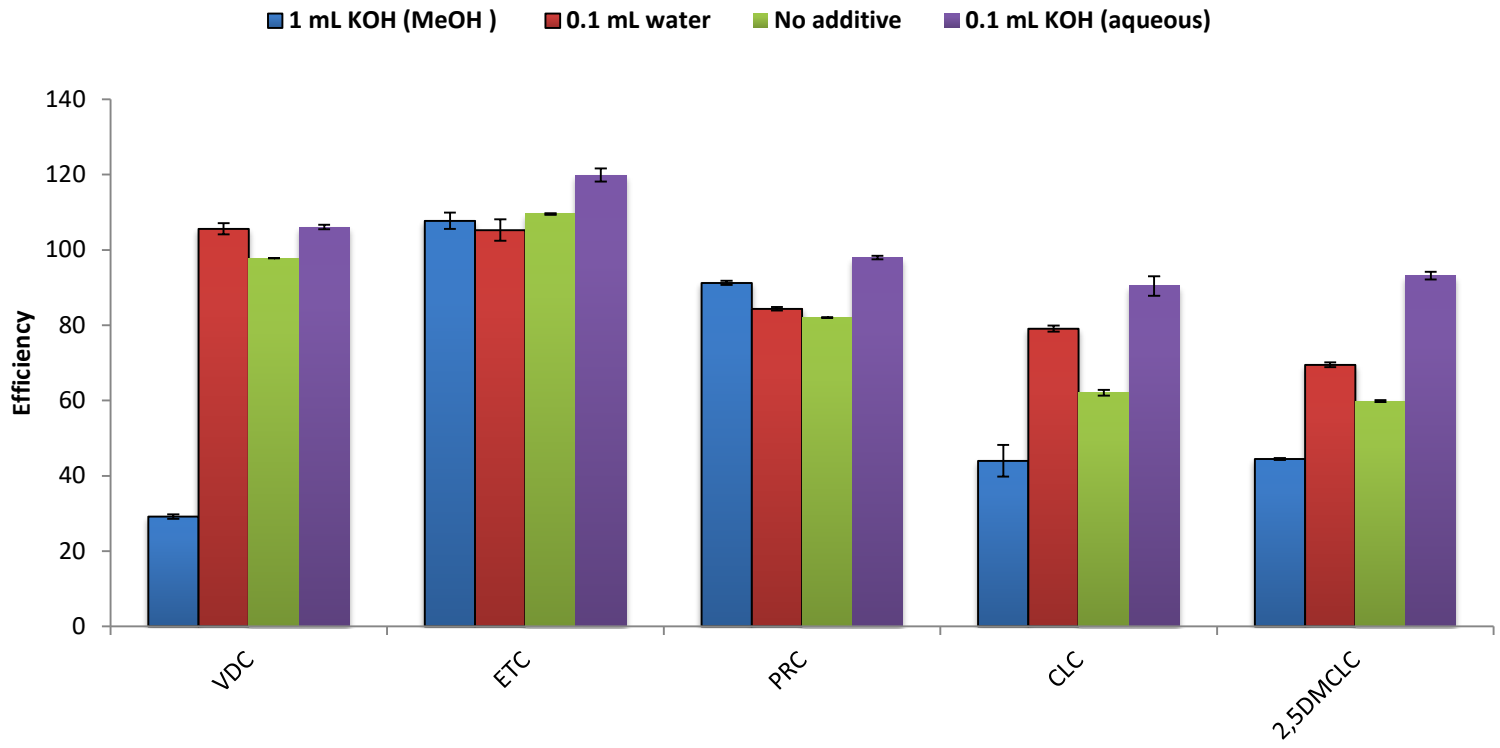



Figure 1.-Efficiency of MSPD extraction as a function of the additive. Sample: 0.2 g .Elution solvent: 30 mL hexane/acetone (1:1, v/v), n=3.

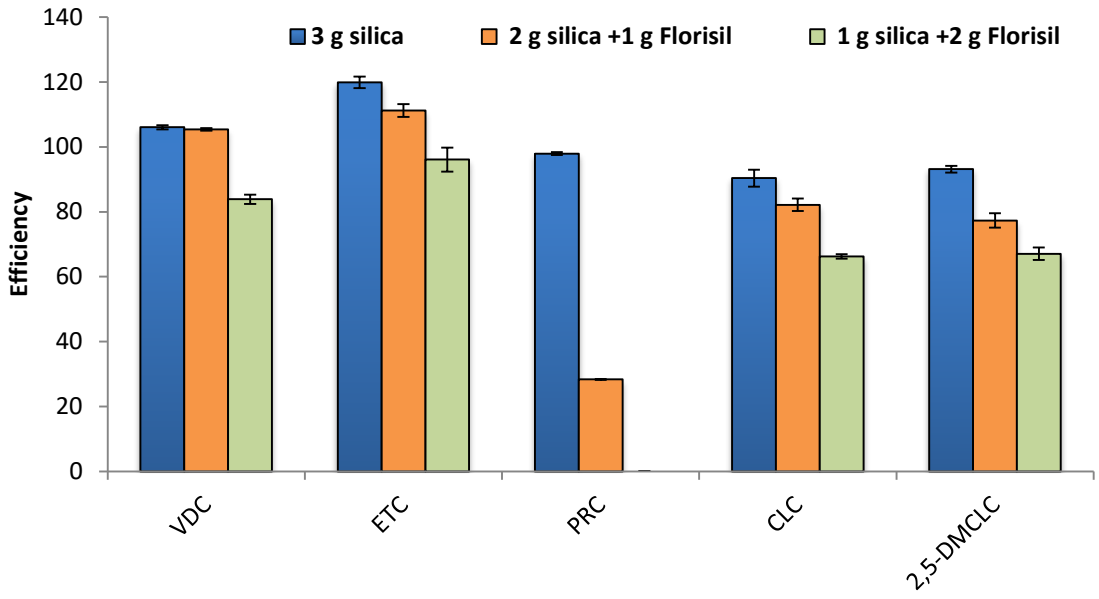



Figure 2.- Efficiency of MSPD extraction as a function of the co-column. Sample: 0.2 g .Elution solvent: 30 mL hexane/acetone (1:1, v/v), n=3

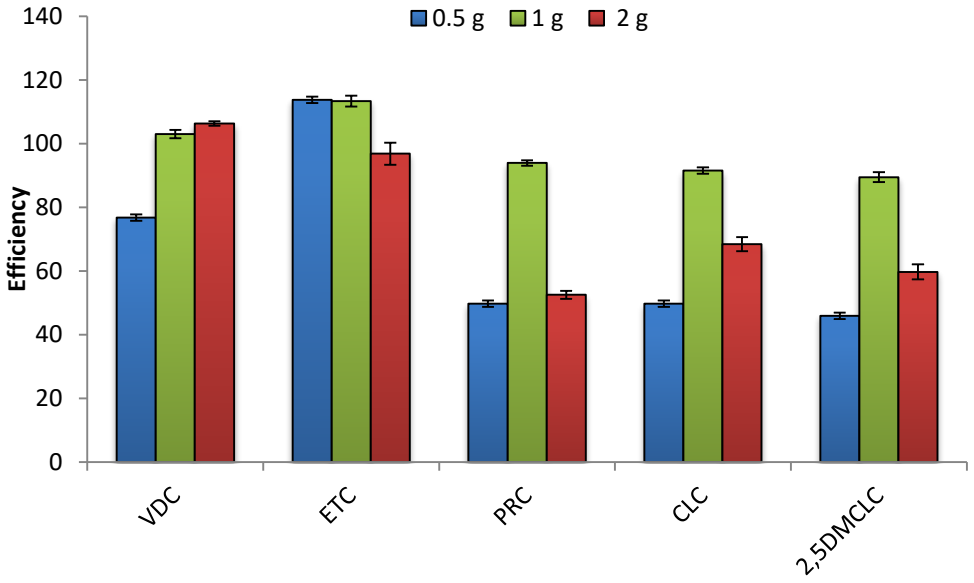



Figure 3. Efficiency of MSPD extraction as a function of the amount of sodium sulfate added as drying agent. Sample: 0.2 g .Elution solvent: 30 mL hexane/acetone (1:1, v/v),n=3

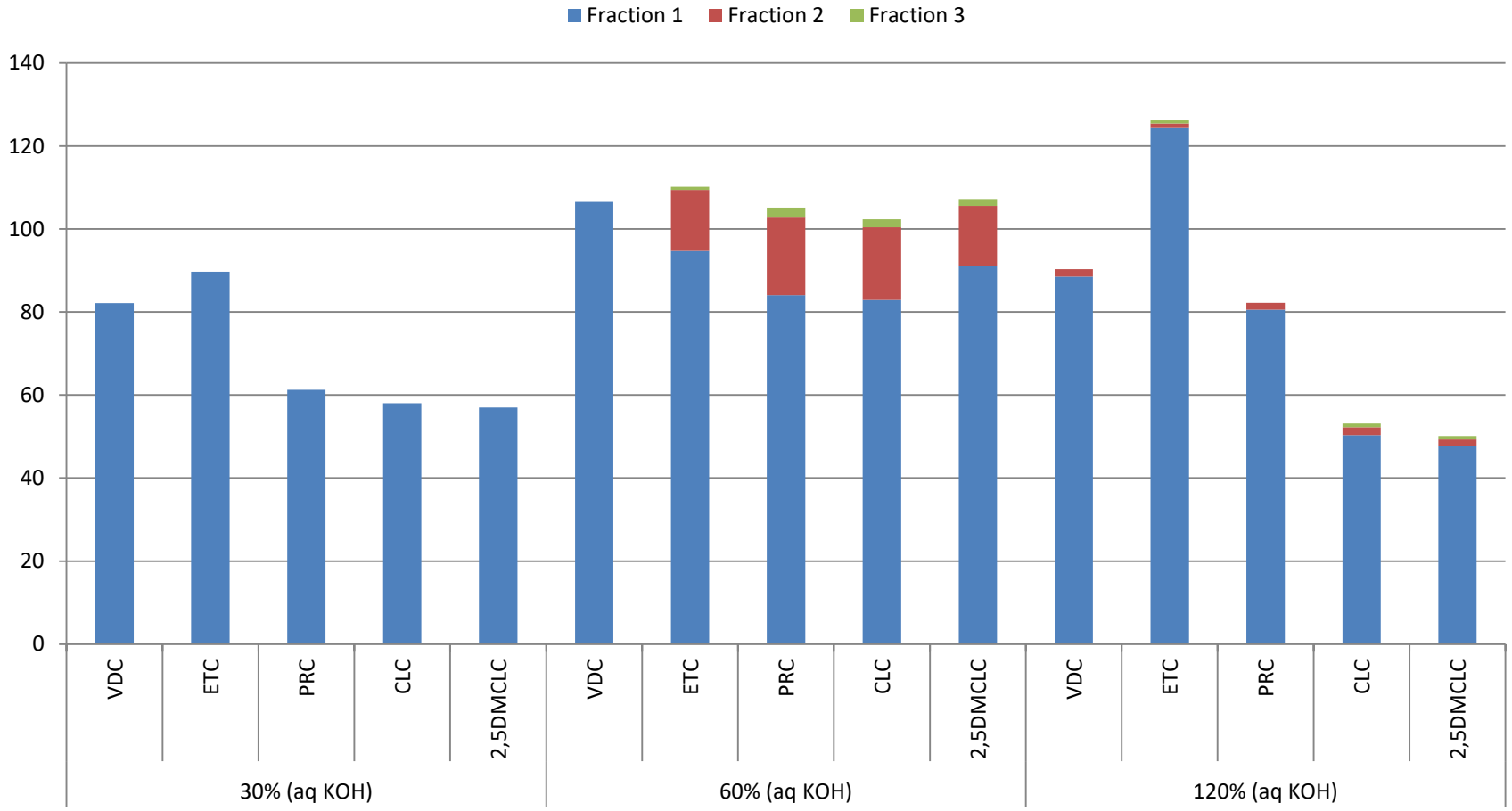
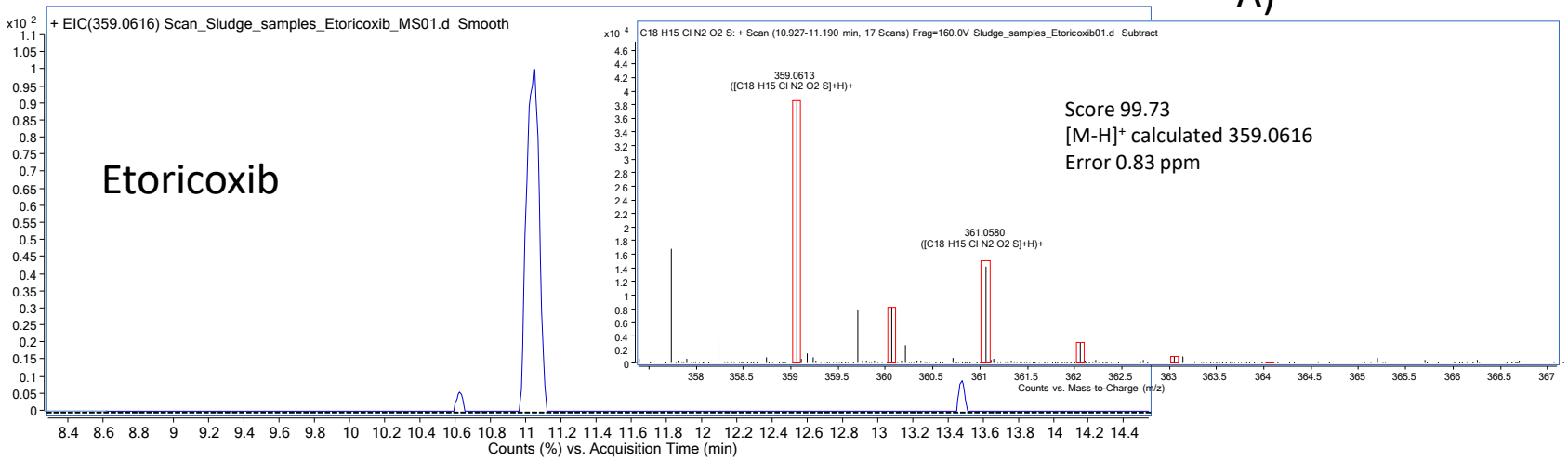
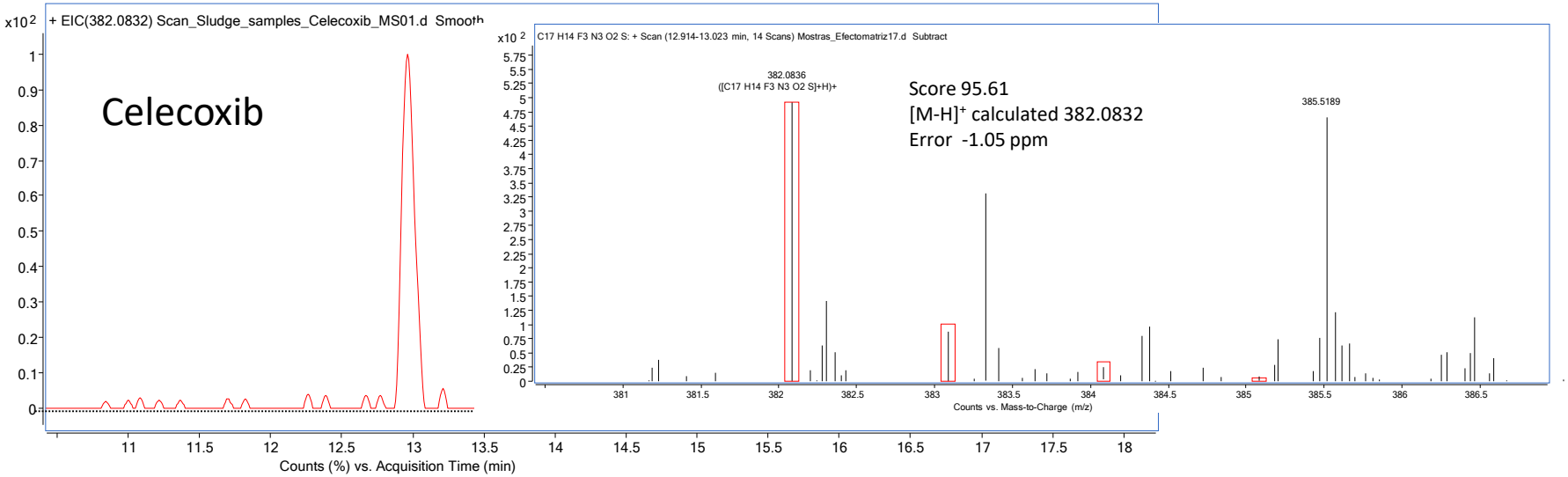



Figure 4. Influence of the amount of aqueous KOH on the efficiency of the MSPD extraction and elution profiles of COXIBs from MSPD cartridge using fractions (10 mL each) of hexane/acetone (1:2, v/v). Volume of aqueous KOH: 100 µL in all experiments.

A)


+ EIC(359.0616) Scan_Sludge_samples_Etoricoxib_MS01.d Smooth
Etoricoxib
C18 H15 Cl N2 O2 S: + Scan (10.927-11.190 min, 17 Scans) Frag=160.0V Sludge_samples_Etoricoxib01.d Subtract
359.0613
([C18 H15 Cl N2 O2 S]+H)+
361.0580
([C18 H15 Cl N2 O2 S]+H)+
Score 99.73
[M-H]+ calculated 359.0616
Error 0.83 ppm
Counts vs. Mass-to-Charge (m/z)
Counts (%) vs. Acquisition Time (min)


+ EIC(382.0832) Scan_Sludge_samples_Celecoxib_MS01.d Smooth
Celecoxib
C17 H14 F3 N3 O2 S: + Scan (12.914-13.023 min, 14 Scans) Mostras_Efectomatriz17.d Subtract
382.0836
([C17 H14 F3 N3 O2 S]+H)+
385.5189
Score 95.61
[M-H]+ calculated 382.0832
Error -1.05 ppm
Counts vs. Mass-to-Charge (m/z)
Counts (%) vs. Acquisition Time (min)

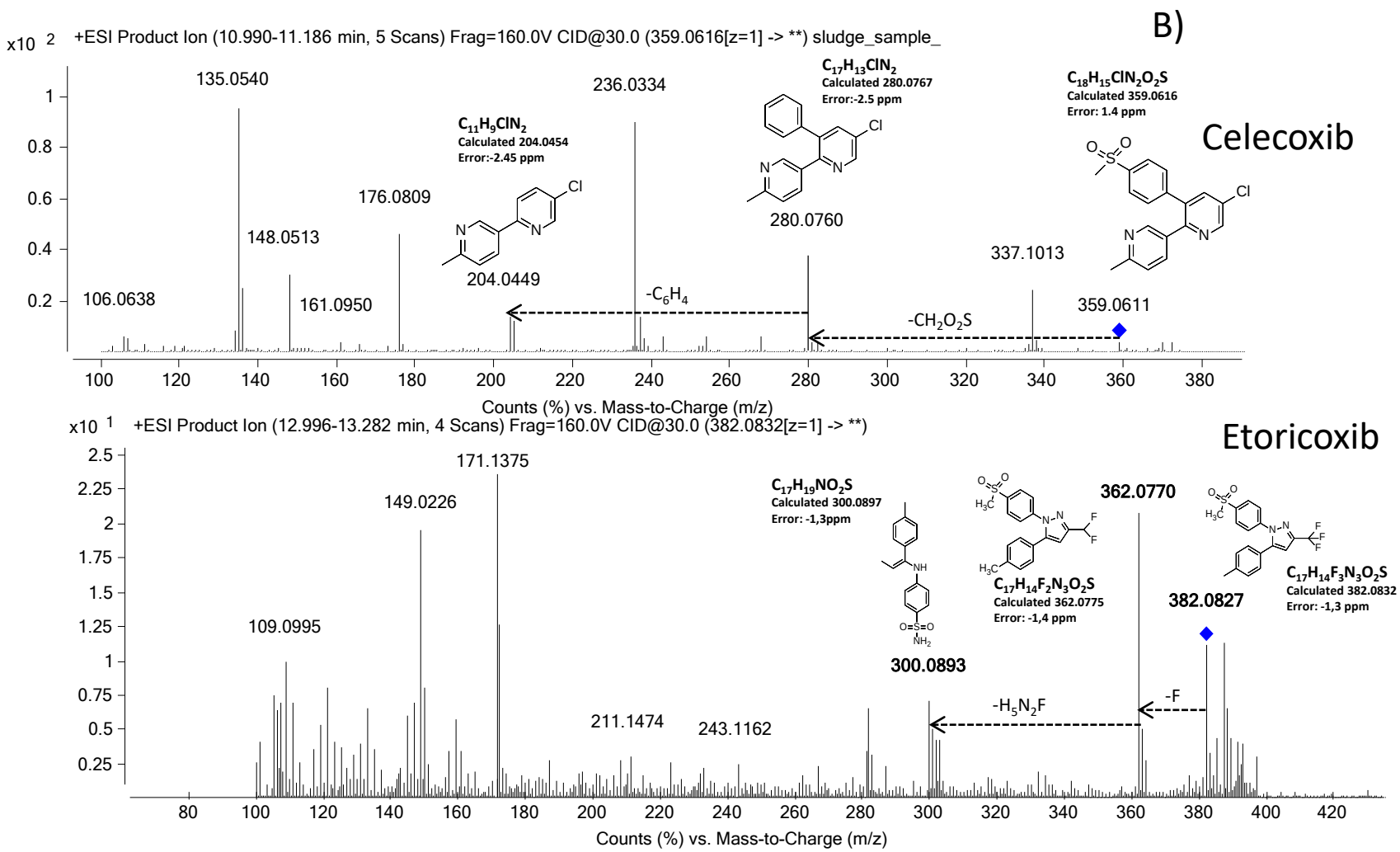



Figure 5. A) LC-QTOF-MS chromatograms (20 ppm mass extraction window) for the $[M+H]^+$ ions for CLC and ETC, with their MS spectra, in a non-stabilized sludge sample. Boxes in red correspond to theoretical MS spectra for both species. B)Experimental MS/MS spectra for above compounds.

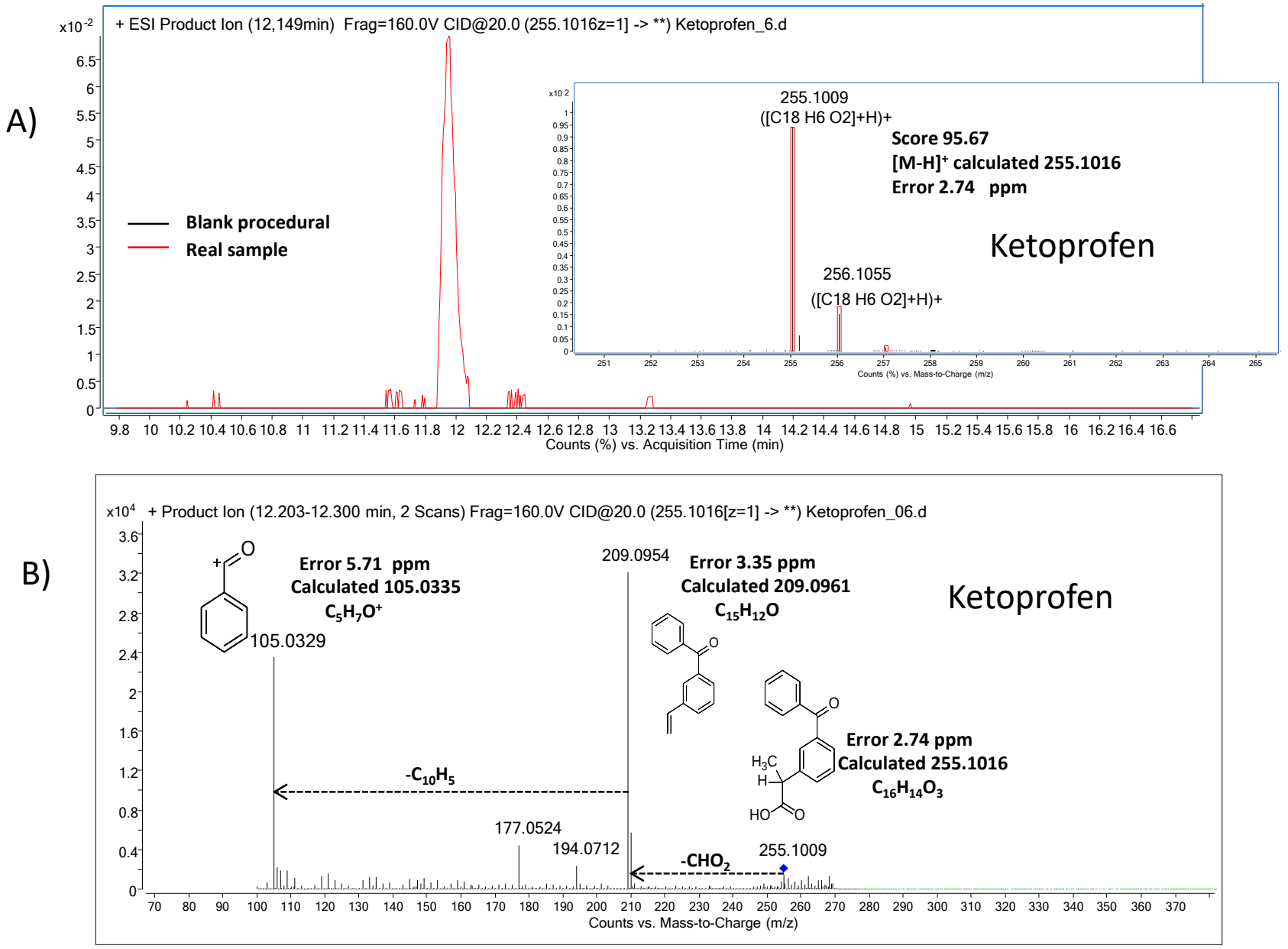
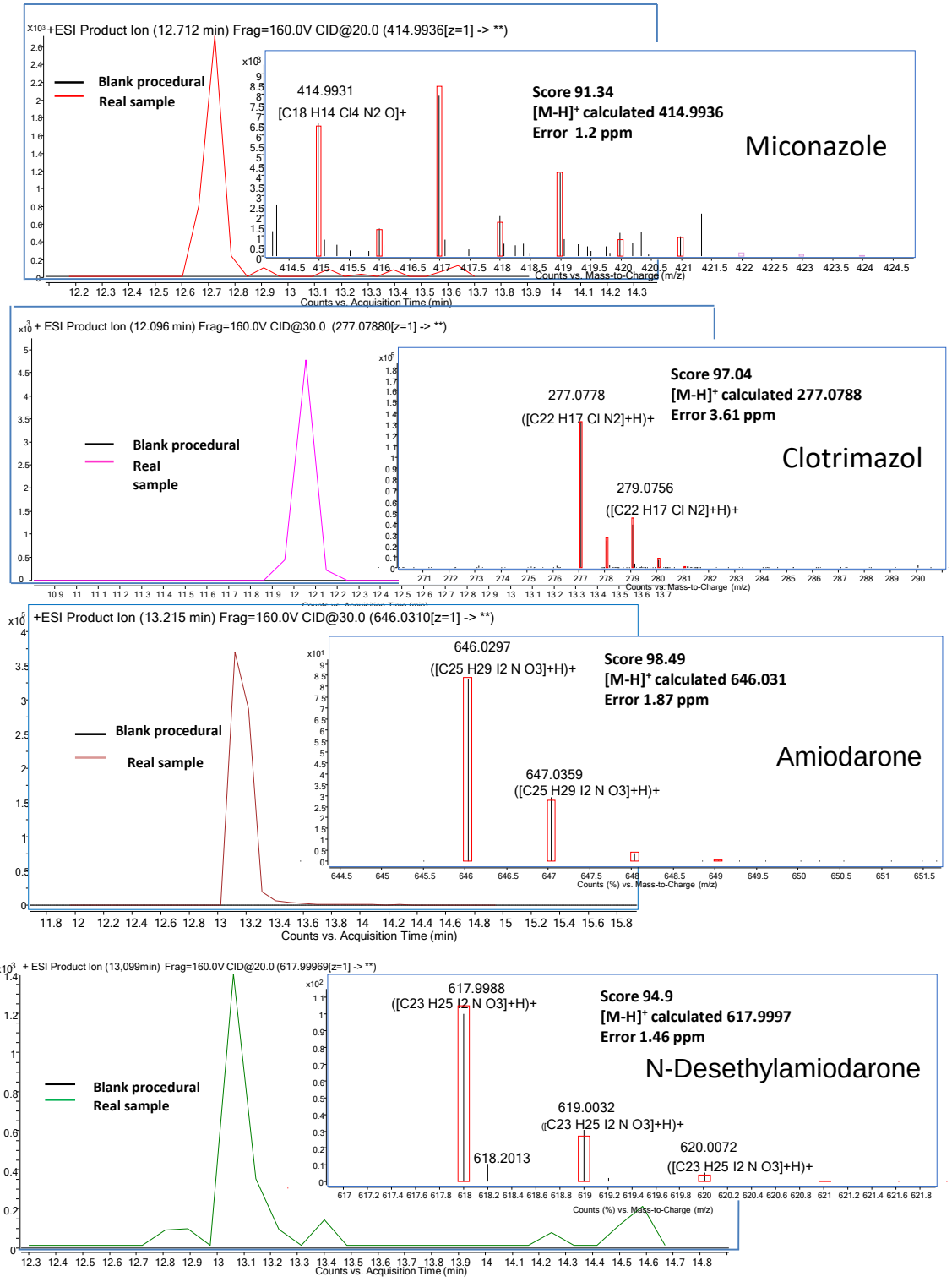



Figure 6. A) LC-MS chromatograms for ketoprofen $[M+H]^+$ ion, and B) MS/MS confirmation spectrum .

A)


+ESI Product Ion (12.712 min) Frag=160.0V CID@20.0 (414.9936[z=1] -> **)
Blank procedural
Real sample
414.9931
[C18 H14 Cl4 N2 O]+
Score 91.34
[M-H]+ calculated 414.9936
Error 1.2 ppm
Miconazole
Counts vs. Mass-to-Charge (m/z)
Counts vs. Acquisition Time (min)
+ ESI Product Ion (12.096 min) Frag=160.0V CID@30.0 (277.07880[z=1] -> **)
Blank procedural
Real sample
277.0778
([C22 H17 Cl N2]+H)+
Score 97.04
[M-H]+ calculated 277.0788
Error 3.61 ppm
Clotrimazol
279.0756
([C22 H17 Cl N2]+H)+
Counts vs. Mass-to-Charge (m/z)
+ESI Product Ion (13.215 min) Frag=160.0V CID@30.0 (646.0310[z=1] -> **)
Blank procedural
Real sample
646.0297
([C25 H29 I2 N O3]+H)+
Score 98.49
[M-H]+ calculated 646.031
Error 1.87 ppm
Amiodarone
647.0359
([C25 H29 I2 N O3]+H)+
Counts (%) vs. Mass-to-Charge (m/z)
Counts vs. Acquisition Time (min)
+ ESI Product Ion (13,099min) Frag=160.0V CID@20.0 (617.99969[z=1] -> **)
Blank procedural
Real sample
617.9988
([C23 H25 I2 N O3]+H)+
Score 94.9
[M-H]+ calculated 617.9997
Error 1.46 ppm
N-Desethylamiodarone
619.0032
([C23 H25 I2 N O3]+H)+
618.2013
620.0072
([C23 H25 I2 N O3]+H)+
Counts (%) vs. Mass-to-Charge (m/z)
Counts vs. Acquisition Time (min)

B)

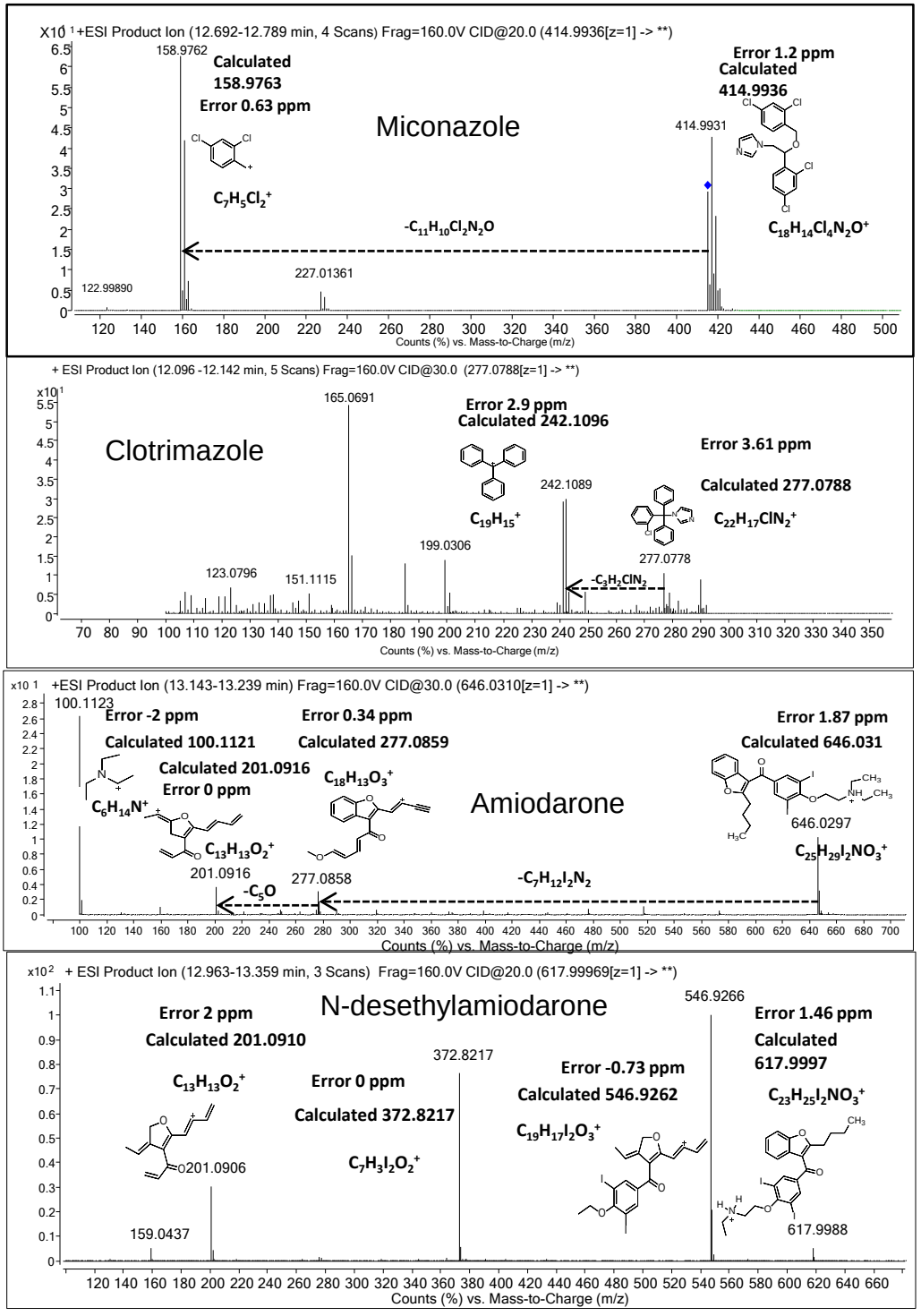



Figure S1. A) LC-MS chromatograms ($[M+H]^+$ ions) for miconazole, amiodarone and N-desethylamiodarone, and $[M-C_3H_3N_2]^+$ for clotrimazole. B) MS/MS spectra interpretation .