Application of a hybrid ordered mesoporous silica as sorbent for solid-phase multi-residue extraction of veterinary drugs in meat by ultra-high-performance liquid chromatography coupled to ion-trap tandem mass spectrometry

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ABSTRACT

A quick, sensitive and selective analytical reversed-phase multi-residue method using ultra-high performance liquid chromatography coupled to an ion-trap mass spectrometry detector (UHPLC-IT-MS/MS) operating in both positive and negative ion mode was developed for the simultaneous determination of 23 veterinary drug residues (β-blockers, β-agonists and Non-Steroidal Anti-inflammatory Drugs (NSAIDs)) in meat samples. The sample treatment involved a liquid-solid extraction followed by a solid-phase extraction (SPE) procedure. SBA-15 type mesoporous silica was synthetized and modified with octadecylsilane, and the resulting hybrid material (denoted as SBA-15-C18) was applied and evaluated as SPE sorbent in the purification of samples. The materials were comprehensively characterized, and they showed a high surface area, high pore volume and a homogeneous distribution of the pores. Chromatographic conditions and extraction procedure were optimized, and the method was validated according to the Commission Decision 2002/657/EC. The method detection limits (MDLs) and the method quantification limits (MQLs) were determined for all the analytes in meat samples and found to range between 0.01-18.75 μg/Kg and 0.02-62.50 μg/Kg, respectively. Recoveries for 15 of the target analytes ranged from 71-98%. In addition, for comparative purpose SBA-15-C18 was evaluated towards commercial C18 amorphous silica. Results revealed that SBA-15-C18 was clearly more successful in the multi-residue extraction of the 23 mentioned analytes with higher recovery values. The method was successfully tested to analyze prepacked preparations of mince bovine meat. Traces of propranolol, ketoprofen and diclofenac were detected in some samples.

Keywords: Solid-phase extraction, multi-residue method, Veterinary drugs, meat, ultra-high performance liquid chromatography, SBA-15 hybrid mesoporous silica

1. Introduction

Nowadays, the presence of veterinary drugs and their residues in food is one of the main concerns of food safety. Veterinary drugs are generally used with a therapeutic purpose in animal production; however, sometimes they can be used fraudulently. β-blockers have a sedation effect, thus they are usually administered to prevent stress in animals during their transport to the slaughterhouse, because such stress can cause sudden death what results in an undesirable loss of meat quality [1, 2]. β-agonists can be used therapeutically as bronchodilatory and tocolytic agents, but the use of these drugs has been forbidden in many countries because of their well-known ability to act as growth promoters, reduce body fat and produce muscle growth enhancement [2, 3]. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly administered to food-producing animals because of their anti-inflammatory, analgesic and antipyretic properties. NSAIDs are used in the treatment of infectious diseases, musculoskeletal disorders, coliform mastitis, pulmonary diseases and enteritis in several animal species [4-6]. Nevertheless, sometimes NSAIDs are also administered to improve the quality of the final product, such as production of pale meats and reduction of edible fat [7, 8]. The incorrect and widespread use of these drugs in veterinary practice may lead to the presence of drug residues in edible tissue, what constitutes a potential health risk for consumers, since the long-term exposure to these drugs can cause different side effects, such as allergic reactions, toxic or microbiological effects, and carcinogenic or teratogenic effects [2, 5, 9]. For this reason, monitoring of drugs in animal tissues has become important to ensure food safety; therefore there is a need for the development of quick, sensitive and selective analytical methods for quantification and confirmation of these trace residues in edible tissues in order to avoid frauds and toxicological risks, and also to monitor their compliance with legislation.

The European Union (EU) has set maximum residues limits (MRLs) for many of these substances (Commission Decision 2010/37/EU [10]). For substances for which no MRL has been established, recommended concentrations (RC) have also been set and are listed in the CRL Guidance Paper [11]. β-agonists are classified into Group A (substances having anabolic effect and unauthorized substances) of Annex I of the Council Directive 96/23/EC [12], while β-blockers and NSAIDs are included in Group B (veterinary drugs and contaminants). Therefore, for Group A substances "zero tolerance" is established by EU, except for clenbuterol and ractopamine, which MRLs have been set for animal tissues and milk. On the other hand, many of the Group B substances have MRLs established by the EU.

Because of the low levels (μg/Kg to ng/Kg) of drug residues in animal tissues and the complexity of the matrix, the determination of veterinary drugs is a challenging task. In recent years, the cost-effectiveness of analytical procedures is becoming an important issue for all laboratories involved in residue analysis. A way to achieve this is to maximize the number of analytes determined by a single procedure through the development of multi-residue methods. However, most published veterinary drug multi-residue methods are focused on one drug class [1, 3, 4, 13-15]. There are only few reported procedures describing methods which cover several classes of veterinary drugs in meat samples [2, 9, 16-18]. As a result of the requirement of analytical criteria on confirmatory methods, mass spectrometry is becoming the most effective and widely used technique to identify trace levels of residues and contaminants in food matrices. Although gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) provides good sensitivity, specificity and resolution, it requires a derivation process which is time-consuming, tedious, laborious and expensive. Thus, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is frequently used in the determination of drug residues in

foodstuffs of animal origin, because of its high selectivity, specificity and sensitivity. However, owing to the high number of compounds to be analyzed, the run times could be relatively long. Hence, the interest in the ultra-high performance liquid chromatography (UHPLC) has recently increased due to the demand of rapid and precise analysis. The UHPLC technique provides low-dead-volume, high pressure (1000 bar), and strategies to improve resolution while maintaining or even shortening run times. An essential aspect of the UHPLC concept is the use of columns packed with sub-2 μm particles, what produces significant improvements in terms of efficiency and shorter chromatographic runs when compared with conventional columns packed with 3 or 5 μm particles.

Solid-phase extraction (SPE) has been commonly used to extract and purify veterinary drug residues from meat solvent extracts [2, 3, 14-18]. Current trends in sample treatment are focused on the synthesis of new materials and their application as sorbents in SPE [19, 20]. In this sense, mesoporous silicas could be used as excellent sorbents due to their textural properties. They present controllable and narrowly distributed pore sizes, ordered pore arrangement, good thermal and chemical stability, high surface area and large pore volume. In addition, high flexibility in functionalization enables the introduction of hydrophilic, hydrophobic, polar as well as charged functional moieties on their surface. For all these reasons, hybrid mesoporous silicas functionalized with different ligand types may be a good alternative to classical sorbents, such as amorphous silica and polymeric materials, and can allow an efficient extraction of the compounds of interest since they have successfully been applied for the extraction and pre-concentration of hormones and heavy metals [20-22]. Nevertheless, hybrid mesoporous silicas remain scarcely used due to their unknown potential for extracting many emerging contaminants, especially from food matrices. In this context, to best of our knowledge this is the first time that a hybrid

ordered mesoporous silica is applied for the simultaneous multi-residue extraction of different veterinary drug residues in meat samples in a single run analysis, taking advantage of the hydrophilic-lipophilic balance that the residual non-modified silanol groups of the silica surface provide which improves the retention of analytes in comparison with a conventional amorphous silica.

Therefore, the aim of this work was to synthetize a SBA-15 type mesoporous silica and functionalize it with C18 groups (denoted as SBA-15-C18) for its evaluation as SPE sorbent for the extraction of twenty three veterinary drugs in meat samples. The results were compared with those obtained with C18 commercial cartridges. Moreover, a quick and sensitive analytical reversed-phase multi-residue method using UHPLC-MS/MS coupled to an ion-trap mass spectrometry detector was developed and validated (according to the EU Commission Decision 2002/657/EC [23]) for the simultaneous determination of veterinary drug residues (β-blockers, β-agonists and NSAIDs) in bovine muscle.

2. Experimental

2.1. Chemicals, reagents and standard solutions

Tetraethylorthosilicate (TEOS) 98% (M = 208.33 g/mol, d = 0.934 g/mL), poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (EO20PO70EO20, Pluronic 123, M_{av} = 5800 g/mol, d = 1.019 g/mL), Chloro(dimethyl)octadecil silane (M = 347,09 g/mol), toluene, and diethylic ether were purchased from Sigma - Aldrich (St. Louis, MO, USA). Hydrochloric acid 35% (M = 36.45 g/mol, d = 1.19 g/mL) was obtained from Scharlau (Barcelona, Spain). Acetonitrile (ACN) and methanol (MeOH) LC-MS grade were purchased from Scharlab (Barcelona, Spain). Formic acid and ammonium acetate LC-MS grade were from Fluka (Busch, Switzerland). Sodium acetate trihydrate and ethanol were from Panreac Química (Castellar del Vallès, Bacerlona, Spain). Water (resistance 18.2 MΩ cm) was obtained from a Millipore Milli-Q-System (Billerica, MA, USA). Comercial C18 SPE cartridges (500 mg, 6 mL) were purchased from Análisis Vínicos (Tomelloso, Spain). All pharmaceuticals standards used were of high-purity grade ≥ 98%. Cimaterol, terbutaline acetate salt hemihydrates, clenproperol, ractopamine hydrochloride, mabuterol hydrochloride, carazolol, naproxen, diclofenac sodium salt, flunixin, tolfenamic acid, carprofen, and vedaprofen were purchased from Fluka (Busch, Switzerland). Salbutamol, atenolol, metoprolol tartrate salt, clenbuterol hydrochloride, brombuterol hydrochloride, tulobuterol hydrochloride, labetalol hydrochloride, propranolol hydrochloride, ketoprofen, meloxicam, and ibuprofen were supplied by Sigma -Aldrich (St. Louis, MO, USA).

Stock standard solutions (1000 mg/L) were prepared by diluting in MeOH adequate amounts of each compound and stored at -20 ºC. Working solutions (40 μg/L – 20 mg/L)

were prepared by appropriate dilution of the stock solutions with MeOH and were stored at 2-10 ºC. Mixed standard solutions were prepared daily by dilution of suitable volumes of working standard solutions with MeOH for the analytical method development and its validation (0.1 – 250 µg/L).

2.2. Synthesis and characterization of SBA-15-C18 mesoporous silica

SBA-15-C18 was synthesized according to the method described by Pérez-Fernández et al [22]. The resulting material was characterized by X-ray diffraction (XRD), transmission electron microscopy (TEM), scanning electron microscopy (SEM), nitrogen adsorption-desorption isotherms, cross-polarization magic-angle spinning ^{13}C and ^{29}Si solid-state nuclear magnetic resonance spectroscopy (CP-MAS-NMR), Fourier transform infrared spectroscopy (FT-IR), elemental analysis and thermogravimetric analysis (see Appendix A in supplementary material for equipment details).

2.3. Meat samples

Prepacked preparations of minced bovine meat with a 10-20 % fat (w/w) according to their labels were purchased from randomly chosen supermarkets in Madrid. They were stored at -20 ºC until analysis.

2.4. Solvent extraction procedure and SPE protocol

Different solvents and ratios were tested in order to optimize the extraction procedure and achieve good recoveries of analytes. The optimized extraction procedure was as follows: 2 g of minced meat were weighed into a 50 mL polypropylene centrifuge tube, 10 mL of acetate buffer 0.2 M (pH 5.2) were added and the mixture was vortexed for 1 min, then centrifuged at 3500 rpm for 10 min. The supernatant was transferred to a new tube, and the residue was extracted again with 5 mL of acetate buffer 0.2 M (pH 5.2) and

5 mL of MeOH, vortexed for 1 min and centrifuged at 5000 rpm for 10 min. The resultant supernatant was collected with the previous one, and then filtered under vacuum. This extract was purified by SPE. To prepare the SPE cartridges, 100 mg of SBA-15-C18 were packed into a 6 mL syringe type cartridge (65 mm length, 11 mm diameter) plugged with polyethylene frits at both ends. To prevent the material lost during sample loading, a 0.45 µm pore size nylon filter membrane was also inserted at the bottom of the mesoporous silica bed. In order to assess the best conditions for the SPE, some preliminary experiments were run to test critical factors affecting the extraction efficiency of the procedure, including the solvent and volume used for elution and the sample volume. In all instances conditioning of the cartridges was accomplished by passing 2 x 2 mL MeOH and 2 x 2 mL Milli-Q water at a flow rate of 1 mL/min. After sample extract loading, cartridges were dried with a Supelco Visiprep ^{TM} DL solid phase extraction vacuum manifold 12 port model (Sigma Aldrich, St. Louis, MO, USA) connected to a vacuum pump at 7.6 psi. Once the entire extract was loaded, cartridges were washed with 1 x 5 mL Milli-Q water to remove interferences. Finally, elution was performed by passing 2 x 2 mL MeOH. The eluate was evaporated to dryness and re-dissolved with 500 µL of MeOH for subsequent analysis in the UHPLC-IT-MS/MS system.

2.5. Chromatographic analysis

Chromatographic separation was performed on an UHPLC system (Dionex UltiMate 3000, Thermo Scientific, MA, USA) using an ACE Excel 2 C18 column (100 mm x 2.1 mm, 2 µm particle size, ACE, UK). The column oven temperature was set at 30 ºC, the flow rate was 0.3 mL/min, and the injection volume was 10 µL. The mobile phase consisted of ACN (A) and H_2O (B), both containing 0.1% formic acid and 4 mM ammonium acetate. The initial composition was 20% A and 80% B. A gradient elution was carried out where phase A increased linearly to 100% in first 6 min, and then returned

to initial conditions in 2 min. The column was then equilibrated for 2 min before the next injection. The total run-time of the method was 10 min. Table 1 shows the retention time for each analyte with the optimized gradient elution described above.

The UHPLC system was connected to an ion trap mass spectrometer detector (Bruker) equipped with an electrospray interface (ESI) operating in both positive and negative ion mode. The capillary voltage was held at -4500V, and the end plate offset at -500V. The nebulizer was set at 20 psi, the dry gas at 10 L/min, and the dry temperature at 200 ºC. Multiple reaction monitoring (MRM) mode was used for all analytes. The ESI source parameters were initially optimized by direct infusion of standard solutions of each compound (10 µg/mL) at a flow rate of 4 µL/min. The different parameters were manually varied to obtain the maximum total ion current signal (TIC) both in positive and negative operation mode within the mass range of 100-350 m/z. Table 1 lists the fragmentation amplitude and the product ions (daughter ions and granddaughter ions) selected for each compound during MRM acquisition.

2.6.Method validation

The developed method was validated in accordance with the Commission Decision 2002/657/EC [23] for a quantitative screening method in terms of identification, selectivity, linearity, decision limit, detection capability, accuracy and precision. The validation study of veterinary drugs in bovine muscle tissue was carried out at three concentration levels chosen around a validation level (VL). The three concentration levels (VL x 0.5, VL x 1.0, and VL x 1.5) used in this validation study are described in Table 2. To set the VL, the analytes were divided in two groups:

- For substances with an established MRL, the MRL was chosen as VL (Table 2).

- For unauthorized substances, permitted substances, and unregulated substances with and without RC, the VL was defined as a "specific level of interest" based on RC levels or based on the drug characteristics and its detection in the method (Table 2).

Although a MRL has been established for carprofen, it was validated at a concentration level below its MRL to prevent the amount of compound detected being outside the range for which the ion trap is linear and to prevent overloading of the analytical column.

Identification

According to the Commission Decision 2002/657/EC [23] substances listed in group A (β-agonist) of Annex I of Directive 96/23/EC [12] require minimum four identification points (IPs) for their confirmation, while substances listed in group B (β-blockers and NSAIDs) require minimum three IPs. To meet these requirements a precursor ion and two product ions (daughter ions or granddaughter ions) were selected to achieve the four IPs, since the 2002/657/EC document [23] establishes that a precursor ion is equivalent to 1 IP and each product ion is equivalent to 1.5 IPs. Confirmation was also checked with the relative standard deviation of the retention time for all analytes.

Selectivity

Twenty blank samples were analyzed and the chromatograms were monitored for intrusive peaks that can potentially interfere with the analytes of interest.

Linearity

Matrix-matched calibration curves were constructed for each analyte on each validation day. Linearity was determined for a concentration range of 0.0, 0.5, 1.0, 1.5 and 2.5 times the VL. Calibration curves were constructed by linear regression analysis, plotting peak area versus analyte concentration. Regression coefficients (R^2) were calculated, and criterion for good linearity was $R^2 > 0.99$.

Decision limit (CCα) and detection capability (CCβ)

According to the Commission Decision 2002/657/EC [23] the decision limit (CCα) can be defined as the limit above which it can be concluded with an error probability of α that a sample contains the analyte. For substances with a fixed MRL, CCα was determined by analyzing twenty blank samples spiked at the MRL level and using the standard deviation of the within-laboratory reproducibility. It was calculated using the following equation:

$$CC\alpha = MRL + 1.64\ SD_{MRL}$$

For substances without a fixed MRL, CCα was calculated according to Jedziniak et al [5] by analyzing twenty blank samples spiked at the first spiking level (VL x 0.5) and using the standard deviation of the within-laboratory reproducibility at the first spiking level with the following equation:

$$CC\alpha = 1^{st}\ \text{spiking level} + 1.64\ SD_{1^{st}\ \text{spiking level}}$$

The 2002/657/EC document [23] define the detection capability (CCβ) as the smallest content of analyte that may be detected, identified and/or quantified in a sample, with a

certainty of 1-β, where β=5%. For both groups of compounds, CCβ was determined by analyzing twenty blank samples spiked at the CCα level, using the following equation:

$$CC\beta = CC\alpha + 1.64\ SD_{CC\alpha}$$

Accuracy and precision

Blank samples were spiked at 0.5, 1.0 and 1.5 times the VL. Recoveries values were calculated by comparison of the areas of spiked meat samples with the areas of simulated samples (meat samples spiked at the end of the sample treatment process). The method precision was determined in terms of repeatability expressed as relative standard deviation percentage (% RSD) by the analysis of six replicates of each fortification level on 1 day, and in terms of within-laboratory reproducibility expressed as % RSD by repeating the procedure on three consecutive days. Guidelines for acceptable values of these parameters at different analyte concentrations were adopted from the 2002/657/EC document [23].

3. Results and discussion

3.1. Characterization of SBA-15-C18 mesoporous silica

XRD pattern of the synthesized SBA-15-C18 showed that this material display well-resolved pattern at low 2θ values, with a very sharp (100) diffraction peak around 0.92 Å and two well-resolved weak diffraction peaks (110) at 1.55 Å and (200) at 1.81 Å, that indicates a 2D ordered hexagonal mesostructure. TEM micrograph of SBA-15-C18 demonstrated a clear arrangement of hexagonal pores with uniform size and SEM micrograph showed a uniform particle size for this material with cylindrical shape. N_2 adsorption-desorption isotherms for SBA-15-C18 (Fig. S1a, supplementary data) were of type IV, according to the I.U.P.A.C. classification, with an H1 hysteresis loop that is representative of materials with pores of constant cross-section. Results obtained indicated a BET specific surface area of 654 m^2/g and a pore volume of 0.71 cm^3/g, with a narrow pore size distribution centred to 49 Å (Fig. S1b, supplementary data). The ^{29}Si MAS-NMR spectrum in the solid state for SBA-15-C18 (Fig. S2, supplementary data) confirmed the covalent bond formed between the C18 ligand and the silanol groups dispersed on the SBA-15 surface. The spectrum showed three main peaks at -111, -103 and -94(*sh*) ppm assigned to Q^4 ***Si***$(OSi)_4$, Q^3 (OH)***Si***$(OSi)_3$ and Q^2 $(OH)_2$***Si***$(OSi)_2$ silanol sites, respectively, being the Q^4 site the most abundant. This spectrum also showed a signal at 10 ppm due to M site, corresponding to the silicon atoms of the dimethyloctadecylsilane. ^{13}C MAS-NMR spectrum in the solid state of SBA-15-C18 clearly display peaks at 29, 22 and 16 ppm, corresponding to the carbon atoms on the C18 ligand chain (-$(CH_2)_{16}$-, -CH_3 and –CH_2-, respectively). Carbon atoms of the dimethyl groups in the organic ligand appeared at - 2 ppm. On the other hand, FT-IR spectrum of this material (Fig. S3, supplementary data) exhibited two peaks at 2924 cm^{-1} (asymmetrical stretching, CH_2) and 2856 cm^{-1} (symmetrical stretching, CH_3), confirming

the existence of C_{18} groups in the silica surface. The percentage of C in the material, calculated by elemental analysis, indicated that the quantity of C18 groups attached to the functionalized mesoporous silica (L_o) was 0.23 mmol/g. Finally, TGA of the SBA-15-C18 showed that an exothermic degradation process occurred between 250-600ºC with a weight loss around 6.5%, that demonstrated the good thermal stability of the material and confirmed the L_o estimated by elemental analysis.

3.2. Optimization of the MS detection and UHPLC conditions

After characterization of the SBA-15-C18 mesoporous silica, the work was focused on the development of a chromatographic separation method by UHPLC with an ion trap mass spectrometer detector for the simultaneous separation of the twenty three selected veterinary drugs.

First, the fragmentation patterns of all analytes were determined by direct infusion. The predominant ion was used as the precursor ion to obtain the product ion spectra (MS^2), as it can be seen in Fig. 1. The product ions for all precursor ions of the analytes were used to set up a MRM method to enhance detection specificity and sensitivity. The two most intense product ions were monitored for each compound leading to earn four IPs which are enough for confirmatory purpose according to the Commission Decision 657/2002/EC [23]. For those analytes (ketoprofen, naproxen, carprofen, diclofenac and tolfenamic acid) for which it was not possible to find two correct and sensitive product ions in MS^2 detection (Fig. 1), MS^3 detection was performed. Table 1 shows the precursor ion, product ions selected (daughter and granddaughter ions), and optimal analytical conditions (polarity mode and fragmentation amplitude) for each compound.

Secondly, to achieve the maximum ionization sensitivity of the ESI source and to optimize the chromatographic separation, different compositions of mobile phase

solvents (MeOH and ACN) and additives (ammonium acetate, ammonium formiate, and formic acid) were studied. The first step was to test MeOH and ACN as organic solvents. The mobile phase gradient elution started with a high aqueous content (100 % of water) and gradually the organic solvent content was increased to end with a 100% of MeOH or ACN. With this gradient elution it was observed good resolution in all peaks, but the first analytes eluted approximately at 5 min and the total run-time of the method was 14 min. The gradient elution was optimized in order to reduce the run-time analysis and bring forward the elution of the most polar compounds. In this sense, the initial organic composition of the mobile phase was increased, bearing in mind to avoid selectivity problems of the method caused by peak overlap in those analytes which have similar precursor ions (such as atenolol and metoprolol, terbutaline and tulobuterol, propranolol and tolfenamic acid, or flunixin and diclofenac). Thus, the final gradient elution started with a 20% of organic solvent content which increased linearly to 100% in the first 6 min, and then returned to initial conditions in 2 min. It was more desirable to use ACN:H_2O as mobile phase rather than MeOH:H_2O, because it provided better chromatographic efficiency and the run-time analysis was shorter. Thus, with a ACN:H_2O mobile phase, the first compounds eluted at 1.4 min and the total run-time of the method was 10 min, as it is shown in Table 1. Fig. 1 shows the extracted ion chromatograms using the precursor ions of each analyte with the optimized chromatographic conditions. Afterwards, the presence of different additives in the mobile phase was optimized. Formic acid is usually used in positive ESI mode, while ammonium acetate and ammonium formiate are additives mostly used in negative mode. It was observed that the signal of analytes detected in negative mode was more intense with ammonium acetate than with ammonium formiate. Therefore, since dual polarity detection was carried out, both formic acid and ammonium acetate were added to the mobile phases. The effect of different

concentrations of formic acid (0.05, 0.1 and 0.3%) and ammonium acetate (0.5, 2 and 4 mM) on the sensitivity was investigated. The signal of all analytes was higher when adding a concentration of 0.1 % formic acid, even for those detected in negative mode. Regarding the addition of ammonium acetate, it was observed that the concentration of 4 mM increased the signal of β-blocker and β-agonists. However, NSAIDs detected in positive mode had a higher signal when ammonium acetate 2 mM was added, while signal of NSAIDs detected in negative mode increased with a concentration of 4 mM. To reach a compromise, the concentration of ammonium acetate 4 mM was chosen because it improved the signal of the majority of compounds and it did not affect adversely the signal of NSAIDs registered in positive mode. Finally, it was conclude that the mixture of ACN with formic acid 0.1% and ammonium acetate 4 mM as organic phase and water with formic acid 0.1% and ammonium acetate 4 mM as aqueous phase, was the most suitable because it provided good results in all cases.

When comparing our developed chromatographic method with other reported multi-residue methods in meat samples, we observed that Sai et al [2] developed a multi-residue method for the separation of β-blockers and β-agonists in meat samples. However, they used high-performance liquid chromatography coupled with linear ion trap mass spectrometry (HPLC-LIT-MS) instead of UHPLC. Thus, their total chromatographic run-time was 45 min, what is a lot considering the run-time of our developed method (10 min). This underlines the advantages of the UHPLC in contrast to the HPLC technique. In addition, they used MeOH as organic solvent of the mobile phase, which is more pollutant than ACN. If we compare our developed methodology with those works of the literature that performed multi-residue methods in meat samples using UHPLC [9, 16, 17] we observed that all of them used ACN in the mobile phase and despite the use of higher flow rates (0.4 and 0.5 mL/min) their total analysis run-times are still longer than

ours (12-15 min). Robert et al [9] determined β-blockers, β-agonists and NSAIDs besides other compounds operating in both positive and negative ion mode but not simultaneously and changing the composition of the mobile phase. Therefore, we conclude that our developed method is more efficient and practical since it allows registering simultaneously in both positive and negative ion mode in a single run and without switching the mobile phase composition.

3.3. Optimization of the sample treatment

In order to optimize the sample treatment and to evaluate the SBA-15-C18 silica as a sorbent for the SPE, the extraction procedure was optimized evaluating the absolute recoveries of analytes. Four different cartridges were prepared following the procedure explained in Section 2.4. In each set of experiments, three cartridges were for meat samples spiked with the twenty three analytes at the VL, and the other one was a simulated sample prepared in the same way, but spiked with the analytes at the end of the sample treatment process. The recoveries obtained in each experiment were calculated by comparison of the areas of the meat samples with the areas of the simulated sample.

A first necessity for a multi-residue analysis is the development of a generic sample pre-treatment step. Enzymatic hydrolysis and acid hydrolysis are the commonly used techniques in the extraction of β-blockers, β-agonists and NSAIDs [2, 3, 5, 7, 13-15, 24, 25]. However, enzymatic hydrolysis requires a longer and tedious procedure and since some authors demonstrated that there is no need of enzymatic hydrolysis during animal tissues analysis [2, 14, 15, 24, 26], this step was avoided in the extraction protocol developed in this work. On the other hand, some preparation/extraction methods for multi-residue determination of veterinary drugs in meat have been described in the literature [9, 17, 18, 27, 28] by using different organic solvents. For example, Mol et al

[27] tested different solvents and their combinations, and the mixture water/ACN was chosen as the most suitable. Peters et al [17] also tried the combination water/ACN in different ratios, and in the end a 40:60 water/ACN ratio was selected as the most appropriate for their purpose. In many cases authors prefer ACN over other organic solvents such as MeOH or ethyl acetate, because it affords protein precipitation which can be considered a first clean-up step in matrices of animal origin. However, protein precipitation may lead to analyte co-precipitation and loss. To overcome this problem, the analyte-protein interactions should be disrupt before the protein precipitation, and this could be achieve by alteration of pH or by addition of small volume of organic solvents. Considering all this, to ensure the accuracy of results we decided to test two different extraction methodologies. In one of them, we studied the mixture water/ACN as extraction solvent, while in the other; the combination of buffers (at different pH) and organic solvents was investigated.

In the first extraction procedure, the meat sample was mixed with 10 mL of water/ACN (40:60 v/v), shaken for 30 min and then centrifuged for 15 min (3500 rpm). Afterwards, the extract was purified by SPE. The SPE was performed as it has been explained in Section 2.4. The eluate was evaporated to dryness and re-dissolved for its analysis in the UHPLC-IT-MS/MS. Acceptable recoveries were found for some β-agonists and β-blockers, but in general low recoveries were found with this extraction procedure, especially in the case of NSAIDs (Fig. 2).

In the other extraction procedure tested, a first extraction step was carried out adding 10 mL of acetate buffer to the meat sample, the mixture was vortexed for 1 min and then centrifuged at 3500 rpm for 10 min. The supernatant was collected in a new tube while a second extraction step was performed on the residue using a combination of acetate buffer and organic solvent (50:50 v/v). It was vortexed for 1 min, then centrifuged at 5000 rpm

for 10 min, and the resultant supernatant was collected with the previous one. The extract was purified by SPE which was performed according to what it has been described in Section 2.4. The eluate was evaporated to dryness and re-dissolved for subsequent analysis on the UHPLC-IT-MS/MS.

Regarding the second extraction step of this extraction procedure, it was first tested the combination of ACN and acetate buffer 0.2M (50:50 v/v) at pH 5.2. It was observed (Fig. 2) that this extraction procedure especially improved the recovery of NSAIDs in comparison with the first extraction methodology previously described (water/ACN 40:60), but low recoveries were found for those analytes which elute in the first two minutes of the chromatogram. Afterwards, in order to determine which organic solvent was more efficient for extraction of the target compounds, the combination of MeOH with acetate buffer 0.2M (50:50 v/v) at pH 5.2 was studied. Results showed that recoveries were higher when MeOH was used as organic solvent instead of ACN (Fig. 2). Moreover, since it is known that the use of different organic solvents may affect the ionization of analytes in the ESI source, the signal suppression was also studied comparing the matrix effect (ME) of both organic solvents. The evaluation of ME was carried out according to the strategy developed by Matuszewski et al [29] by using the following equation:

$$ME\ (\%) = (B/A) \times 100$$

where A is the mean peak area of the analyte in the standard solution and B is the mean peak area of the analyte in meat sample spiked after extraction. A value of >100% indicates ionization enhancement, while a value of <100% indicates ionization suppression [29]. To estimate the ME, firstly six replicates of a standard solution at VL concentration were injected into de UHPLC-IT-MS/MS system. Then, six replicates of

blank meat samples were processed using the optimized solvent extraction procedure; the extracts obtained were spiked at the VL concentration with the standard solution, evaporated to dryness, reconstituted in MeOH and injected into the UHPLC-IT-MS/MS.

As it can be seen in Fig. 3, ion suppression was similar for the majority of analytes using either of the organic solvents tested. In some cases ion suppression was higher when MeOH was used as extraction solvent, while in other compounds ion suppression increased with ACN. In the end, since almost all compounds showed higher recoveries with MeOH than with ACN (Fig. 2), MeOH was chosen as best organic extraction solvent. Finally, in this point, it was compared the combination of MeOH and acetate buffer 0.2M (50:50 v/v) at different pH (pH 7 and pH 5.2). Results obtained demonstrated that pH 5.2 was more desirable for all compounds, with exception of terbutaline and diclofenac (Fig. 2). Therefore, the combination of acetate buffer 0.2M (pH 5.2) and MeOH (50:50 v/v) was found to give the most satisfactory results.

The next step to optimize the sample treatment process was to choose the elution solvent in the SPE procedure, since this solvent should have enough elution ability to desorb the analytes. MeOH and ACN were tested for this purpose, setting an elution volume of 4 mL (2 x 2 mL). Best results (Fig. 4) were obtained using MeOH as elution solvent, obtaining good recoveries especially for β-agonists and β-blockers. ACN as elution solvent did not give satisfactory results since low recoveries were found for β-agonists and β-blockers; however it was observed that it was more effective for the recovery of the NSAIDs, since they are less polar compounds. A combination of both solvents (50:50 v/v) was also investigated. The recoveries values for β-agonists and β-blockers were higher than the ones obtained eluting only with ACN, but not as high as

with only MeOH. Thus, in the end MeOH (2 x 2 mL) was selected for the SPE elution step.

Finally, once the extraction procedure was optimized, the advantages of SBA-15-C18 towards commercial C18 amorphous silica were investigated. Experiments were performed under the same conditions described above, but employing 100 mg of commercial C18 amorphous silica (with similar L_o = 0.23 mmol/g) instead of SBA-15-C18. When comparing the results (Fig. 5), SBA-15-C18 was clearly more effective in the extraction of analytes than the commercial C18 cartridges, since recoveries achieved were higher for all compounds. These results could be attributed to the fact that hybrid SBA-15 material contains more accessible functional groups, which are homogeneously distributed in the silica surface without pore blocking, in comparison with C18 amorphous silica. In addition, in the SBA-15-C18 mesoporous silica the analytes experience a reversed-phase sorption with the C18 groups (by hydrophobic interactions) and polar secondary interactions (by hydrogen bonding interactions), as a result of the high number of residual non-modified silanol groups in the SBA-15-C18 surface. This fact has a pronounced effect on the retention of the most polar analytes in comparison with the commercial silica, thus it allows achieving better recoveries for these compounds in the SPE step. The majority of published works that performed multi-residue methods for determination of veterinary drugs in food matrix use HLB cartridges in the SPE step [4, 14-16, 18] instead of commercial C18 cartridges, because they have a hydrophilic-lipophilic balance that allows extracting a wider range of compounds of different nature and, in general, provide better recoveries. Therefore, since SBA-15-C18 has shown a clear improvement in the recoveries achieved for all analytes when compared with commercial C18, it could be an alternative sorbent to commercial C18 or HLB cartridges in the multi-residue extraction of veterinary drugs in food matrices.

3.4. Method Validation

The method validation was performed in accordance with the Commission Decision 2002/657/EC [23]. A summary of the validation parameters is reported in Table 2.

For the assessment of method selectivity twenty blank samples were analyzed and the chromatograms were monitored for intrusive peaks that can potentially interfere with the analytes of interest. Results obtained confirmed no interferences in the retention time of the target analytes. For linearity, matrix-matched calibration curves were obtained by plotting peak areas versus concentrations of analytes in spiked meat extracts obtained under the optimized extraction conditions (concentrations ranging from 0-2.5 x VL) according to what it has been explained in Section 2.6. All 23 compounds showed good linear regression and R^2 values were > 0.990 (Table 2). Moreover, to determine the existence of ion suppression as a result of the matrix effect, standard solution calibration curves were performed in order to compare the slope with the slope obtained for the matrix-matched calibration curves. Standard solution calibration curves were constructed by plotting peak areas versus concentrations of analytes in working standard solutions (concentrations from 1 to 500 μg/L). All showed R^2 values > 0.991 (Fig. 1), indicating a good correlation for each target compound. When comparing the slope of the standard solution calibration curves with the slope of the matrix-matched calibration curves, it was observed that slope values of the matrix-matched curves were lower than those of the standard solution curves. Therefore, it was concluded that analytes showed ion suppression due to the adverse influence of matrix effect, what is in accordance to that previously reported in Fig. 3. Thus, matrix-matched calibration curves should be used to accurately quantify the target analytes in real meat samples.

CCα and CCβ values were calculated as indicated in Section 2.6. and are presented in Table 2. CCα values obtained for naproxen and terbutaline were lower than their RC. For compounds with a VL set between 0.2 – 50 µg/Kg, the CCβ ranged from 0.23 – 78 µg/Kg. CCβ values of clenproperol, metoprolol, brombuterol, labetalol, mabuterol, propranolol, ketoprofen and naproxen were lower than the VL set for them, thus, it was concluded that their VL could be reduced to assess the validation method. For ibuprofen the VL was 100 µg/Kg and its CCβ was 142.6 µg/Kg. In addition, the method detection limit (MDL; defined as the concentration that yields a signal-to-noise ratio (S/N) of 3 measured in relation with the S/N obtained for the lowest concentration of the matrix-matched calibration curve (0.5 x VL) under the optimized conditions of the developed method) and the method quantification limit (MQL; defined as the concentration that yields a S/N of 10 for the chromatographic response measured in relation with the S/N obtained for the lowest concentration of the matrix-matched calibration curve (0.5 x VL) under the optimized conditions of the developed method) were also investigated for each compound. MDL and MQL values are shown in Table 2. The obtained values proved good sensitivity of the method, except for ibuprofen which presented the highest values. Moreover, the MQL of 14 compounds was even lower than the MRL or RC established for them.

The accuracy and precision of the method were evaluated at different concentration levels (0.5 x VL, 1.0 x VL, and 1.5 x VL). The results obtained at the three different levels are almost in the same range (Table 2). The Commission Decision 2002/657/EC [23] establishes that when analytes are determined in a mass fraction < 1 µg/Kg recovery values are only acceptable when they range between 50 - 120%, in this sense clenbuterol and tulobuterol showed acceptable accuracy values. For analytes determined in a mass fraction > 1 to 10 µg/Kg recovery values should range within 70 - 110% to be acceptable.

Terbutaline, salbutamol, ractopamine and naproxen showed recovery values < 70%. Finally, when analytes are determined in a mass fraction > 10 μg/Kg recovery values should range between 80 - 110%. Values < 80% were found for carprofen, ibuprofen, tolfenamic acid and vedaprofen. Therefore, according to legislation 2002/657/EC [23] we concluded that 15 tested compounds showed acceptable accuracy values within the permitted range in all levels. Precision values of this method are also summarized in Table 2 and are represented by the % RSD at each fortification level for each compound. All tested analytes showed repeatability values < 20 %, except terbutaline on the lowest spiking level. The 2002/657/EC [23] document states that the precision of a quantitative method should be as low as possible for mass fractions less than 100 μg/Kg, therefore according to legislation, satisfactory results were accomplished for analytes with a VL lower than 100 μg/Kg. Nevertheless, low precision was found in the case of terbutaline and tolfenamic acid since they showed reproducibility values > 30%. For mass fractions of 100 μg/Kg legislation establishes that precision values should not exceed RSD > 23 %, in this sense no satisfactory precision results were found for ibuprofen since it presented a reproducibility value > 30% at the VL. Taking all this into account, we have to conclude that according to the 2002/657/EC [23] document three β-agonists (terbutaline, salbutamol, and ractopamine) and five NSAIDs (naproxen, carprofen, ibuprofen, tolfenamic acid and vedaprofen) cannot be accurately quantified with the developed method. For these compounds accurate quantification is only possible by applying a standard addition procedure.

3.5. Method application to real samples

Ten different samples of prepacked preparations of minced bovine meat (with a 10-20 % fat according to their labels) commercially available from randomly chosen local supermarkets in Madrid were analyzed using the developed method. Each sample was analyzed in triplicate and injected 5 times in the UHPLC-IT-MS/MS.

None of the studied analytes was found at a concentration level higher than its CCα and CCβ in the meat samples analyzed. However, propranolol was found in one meat sample at 0.14 μg/Kg, since it showed a concentration level above its MQL. The presence of this analyte could be consequence of a non-observance of the withdrawing times before slaughter. Other sample was suspected for propranolol, presence of trace residues of naproxen and ketoprofen were detected in all meat samples analyzed, and diclofenac was detected in three samples since relative retention times and ion ratios fulfilled the EU analytical criteria. Nevertheless, these analytes showed concentration levels between their MDL and MQL (samples suspected positive but without a statistical certainty), therefore it was not possible to quantified them accurately. The other analytes selected for this study resulted negative in all the ten meat samples analyzed.

4. Conclusion

In this work, a quick, sensitive and selective analytical reversed-phase multi-residue method has been developed for the determination of 23 veterinary drug residues (5 β-blockers, 9 β-agonists and 9 NSAIDs) in meat samples by UHPLC-IT-MS/MS operating simultaneously in both polarities and allowing the separation of analytes in less than 10 min. Moreover, a SBA-15 type mesoporous silica modified with octadecylsilane (C18 groups) has been evaluated as SPE sorbent. Results showed it has a big extraction potential, and was clearly more successful in the multi-residue extraction of the 23 target analytes in comparison with commercial C18 amorphous silica.

Finally, the method was validated based on the EU criteria of the Commission Decision 2002/657/EC, and it was successfully tested to analyze meat samples. None of the studied analytes was found at a concentration level higher than its CCα and CCβ in the meat samples analyzed, but traces of propranolol, ketoprofen and diclofenac were detected in some samples.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material associated with this article can be found in the online version at

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Fig. 1 Extracted ion chromatograms and mass spectra (MS^2) of the target analytes in a standard solution at the validation level; Insert in chromatograms standard solution calibration curves values and regression coefficients (R^2) are shown.

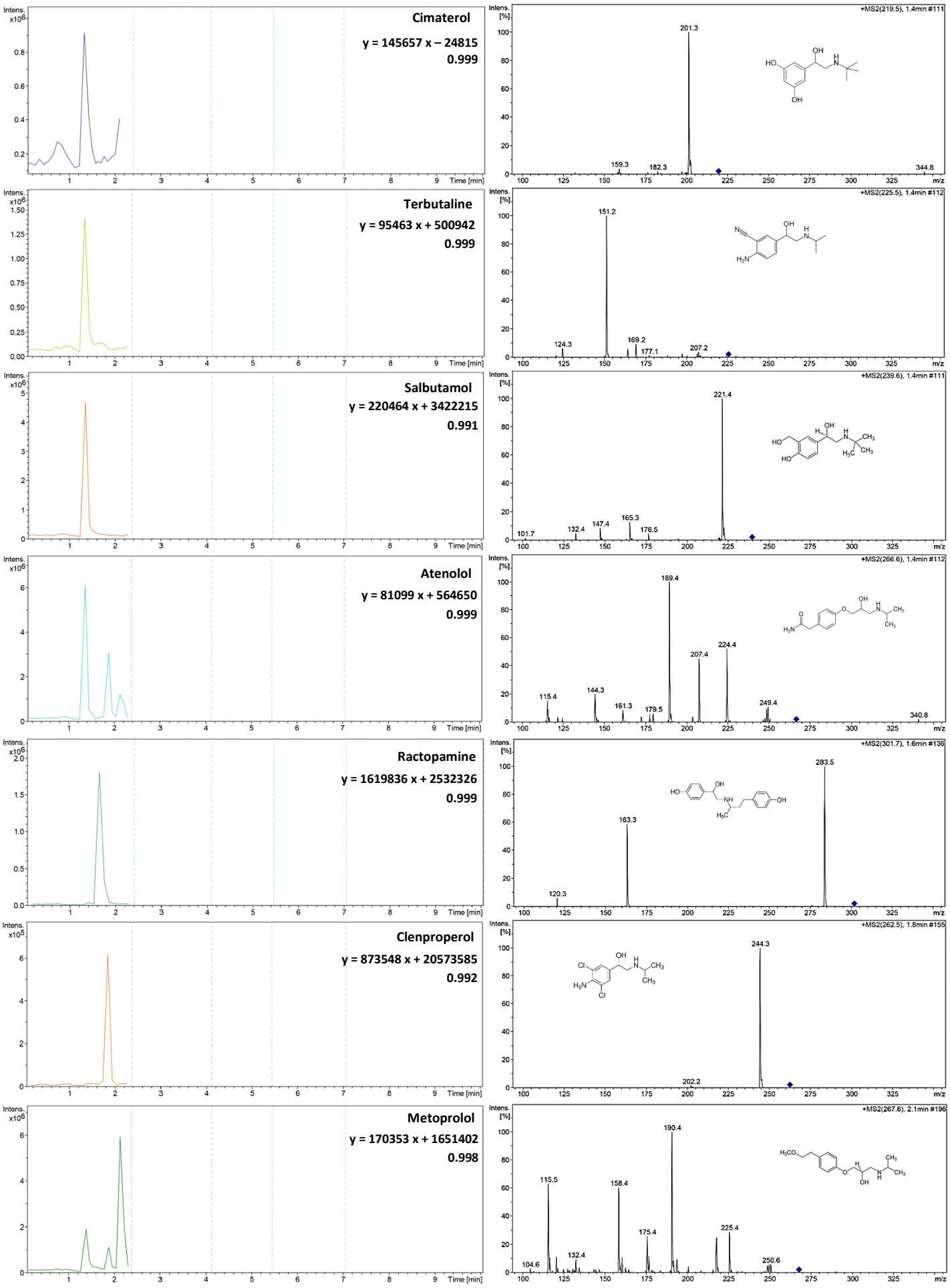
Fig. 2 Effect of different extraction procedures on the extraction efficiency of the target compounds in meat samples spiked at the validation level with SPE cartridges packed with mesoporous silica SBA-15-C18. Error bars represent the standard deviation of samples replicates (n=6).

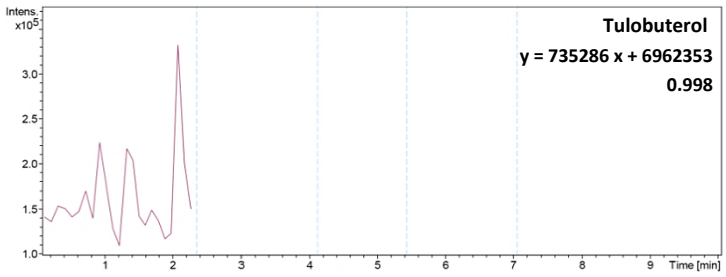
Fig. 3 Matrix effect of the tested organic solvents used in the extraction of meat samples spiked at the validation level with SPE cartridges packed with mesoporous silica SBA-15-C18. Error bars represent the standard deviation of samples replicates (n=6).

Fig. 4 Effect of different elution solvents on the SPE step in meat samples spiked at the validation level with SPE cartridges packed with mesoporous silica SBA-15-C18. Error bars represent the standard deviation of samples replicates (n=6).

Fig. 5 Comparison of the recovery percentages obtained from the analysis of meat samples spiked at the validation level extracted with SPE cartridges with 100 mg of SBA-15-C18 and 100 mg of commercial C18. Error bars represent the standard deviation of samples replicates (n=6).

Figure 1.

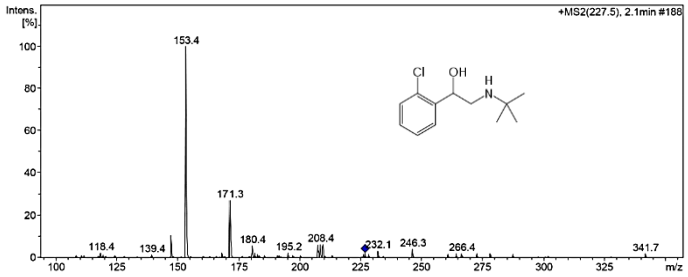

Cimaterol
y = 145657 x – 24815
0.999
+MS2(219.5), 1.4min #111
201.3
159.3
182.3
344.8
Terbutaline
y = 95463 x + 500942
0.999
+MS2(225.5), 1.4min #112
151.2
124.3
169.2
177.1
207.2
Salbutamol
y = 220464 x + 3422215
0.991
+MS2(239.6), 1.4min #111
221.4
101.7
132.4
147.4
165.3
176.5
Atenolol
y = 81099 x + 564650
0.999
+MS2(266.6), 1.4min #112
189.4
115.4
144.3
161.3
179.5
207.4
224.4
249.4
340.8
Ractopamine
y = 1619836 x + 2532326
0.999
+MS2(301.7), 1.6min #136
283.5
163.3
120.3
Clenproperol
y = 873548 x + 20573585
0.992
+MS2(262.5), 1.8min #155
244.3
202.2
Metoprolol
y = 170353 x + 1651402
0.998
+MS2(267.6), 2.1min #196
190.4
115.5
158.4
175.4
225.4
132.4
104.6
250.6
Time [min]
m/z
Intens.

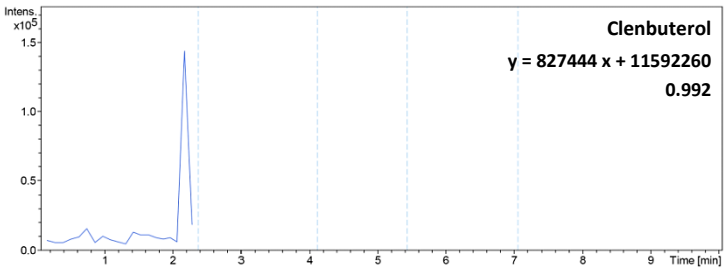


Tulobuterol

y = 735286 x + 6962353

0.998

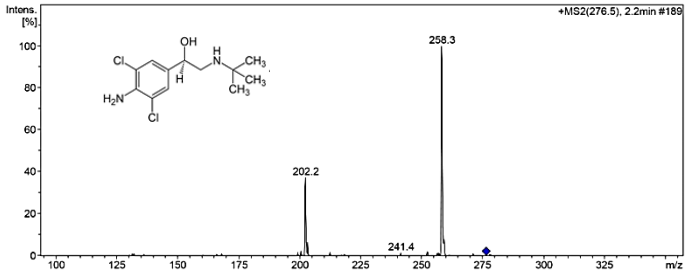


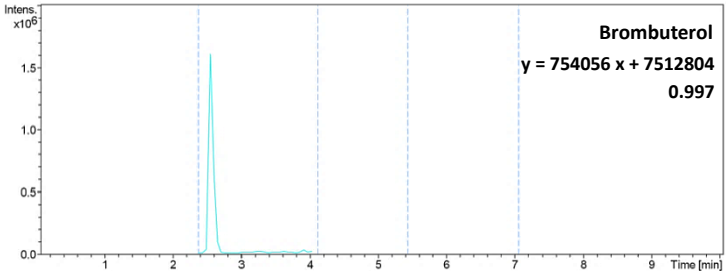


Clenbuterol

y = 827444 x + 11592260

0.992

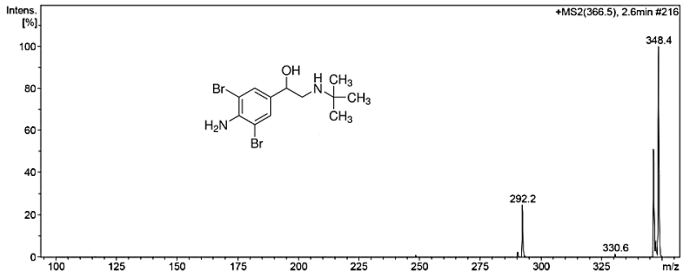


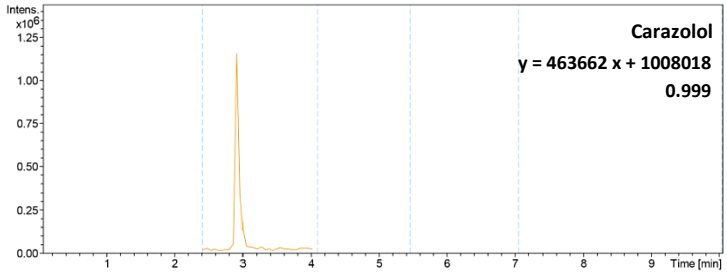


Brombuterol

y = 754056 x + 7512804

0.997

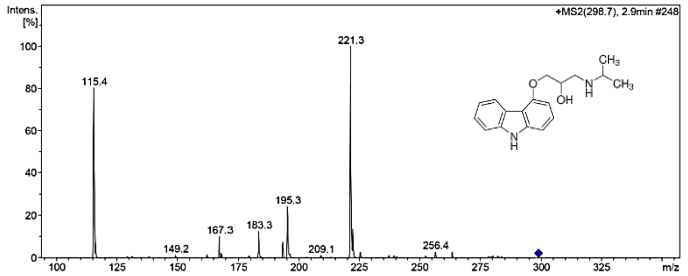


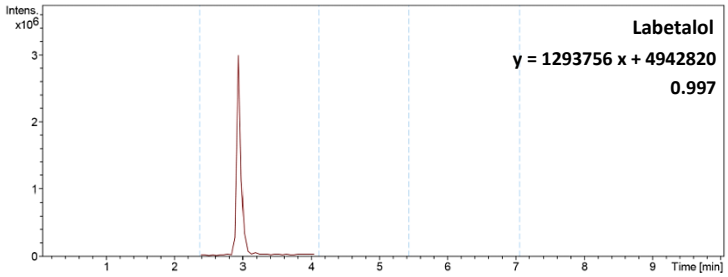


Carazolol

y = 463662 x + 1008018

0.999

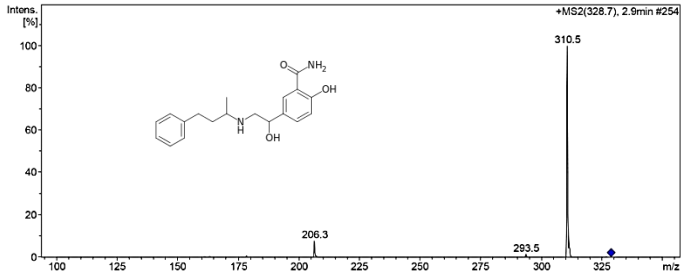


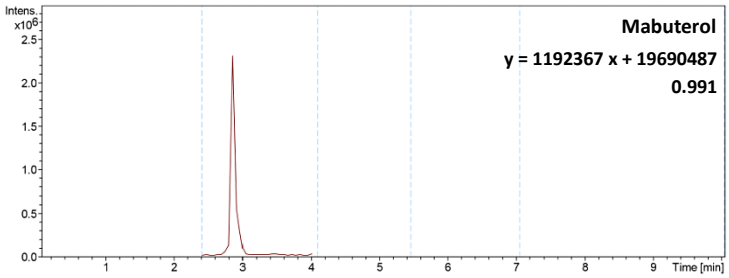


Labetalol

y = 1293756 x + 4942820

0.997

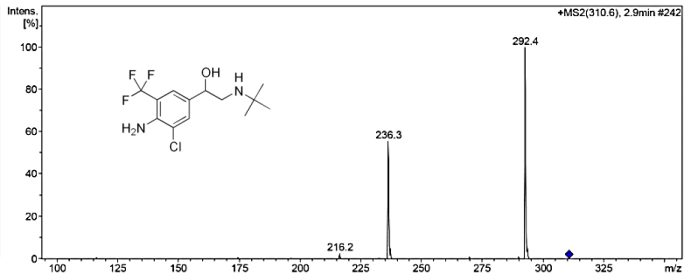


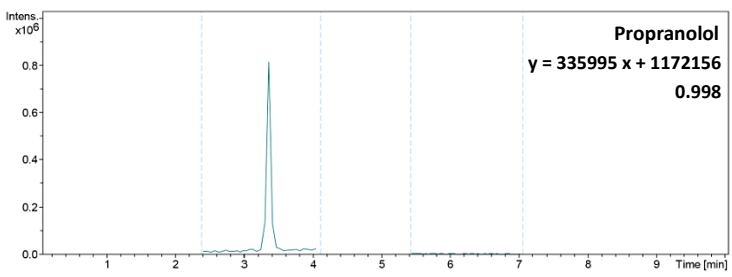


Mabuterol

y = 1192367 x + 19690487

0.991

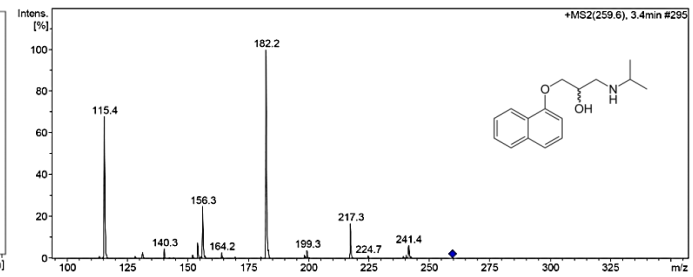


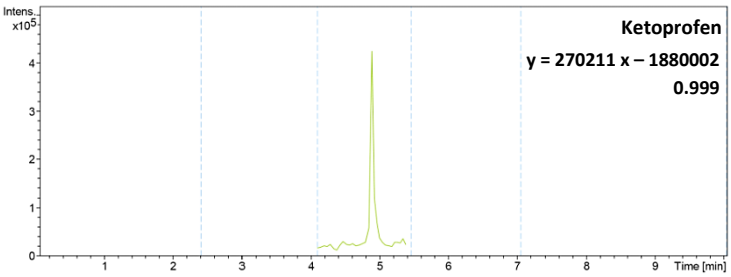


Propranolol

y = 335995 x + 1172156

0.998

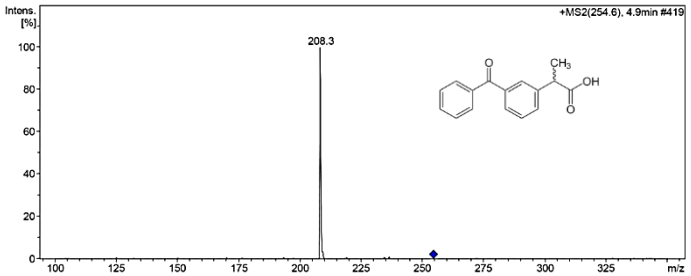


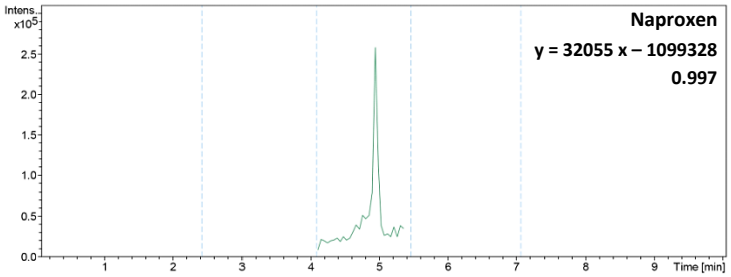


Ketoprofen

y = 270211 x – 1880002

0.999

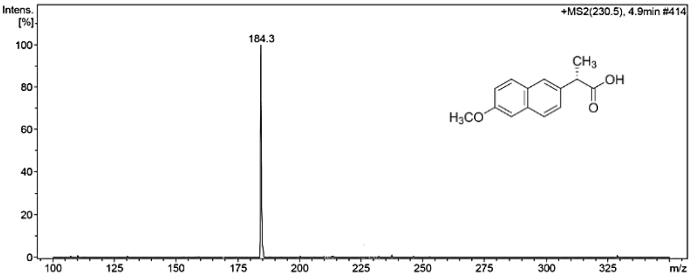


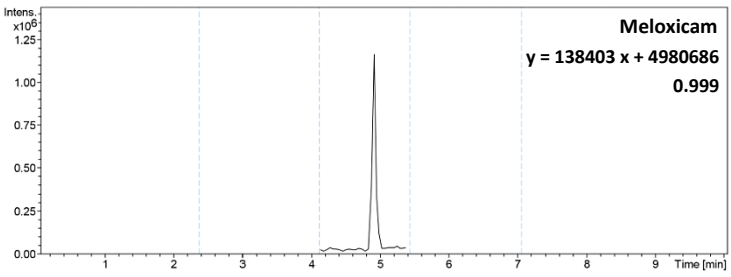


Naproxen

y = 32055 x – 1099328

0.997

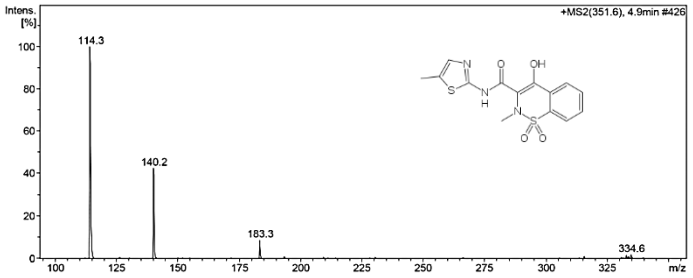


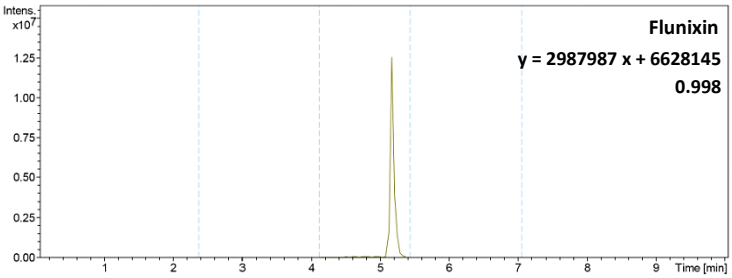


Meloxicam

y = 138403 x + 4980686

0.999

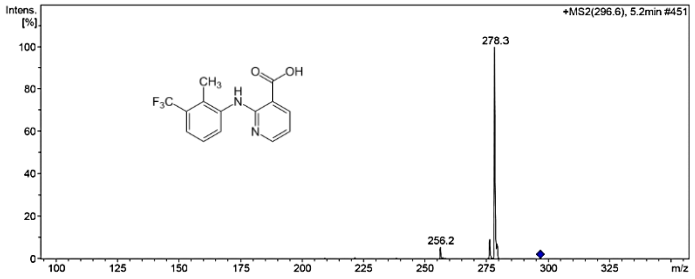


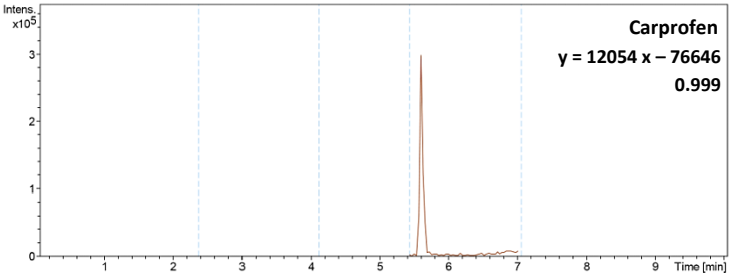


Flunixin

y = 2987987 x + 6628145

0.998

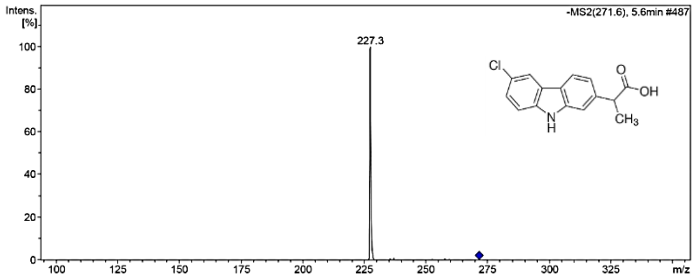


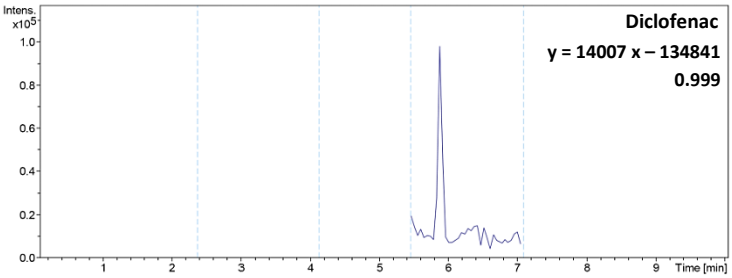


Carprofen

y = 12054 x – 76646

0.999

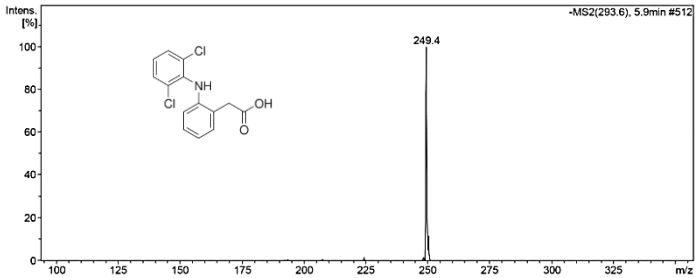


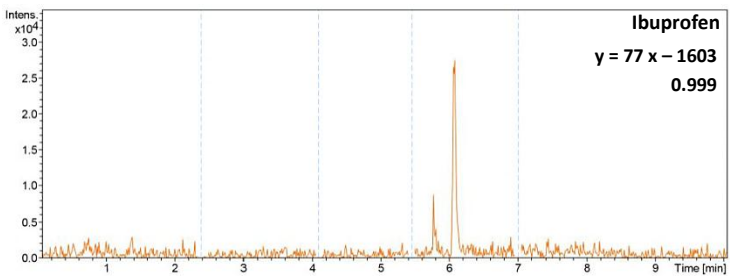


Diclofenac

y = 14007 x – 134841

0.999

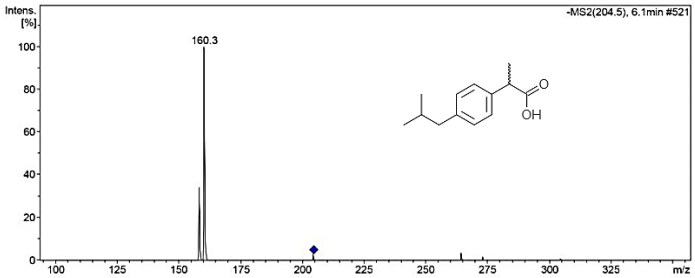


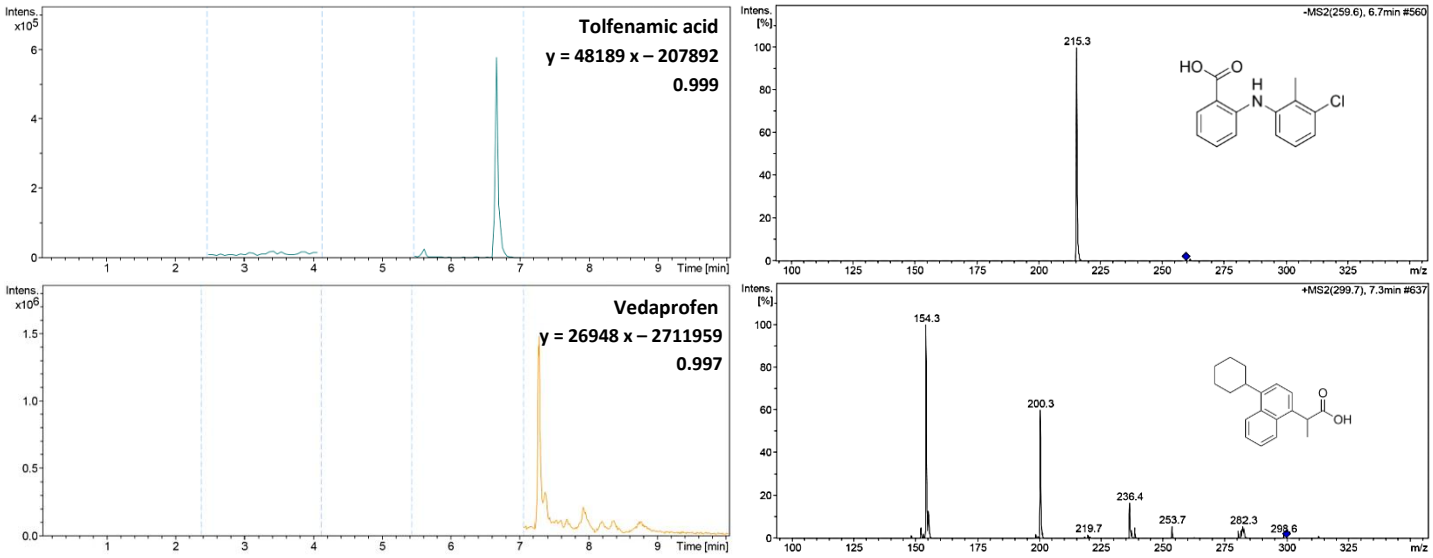


Ibuprofen

y = 77 x – 1603

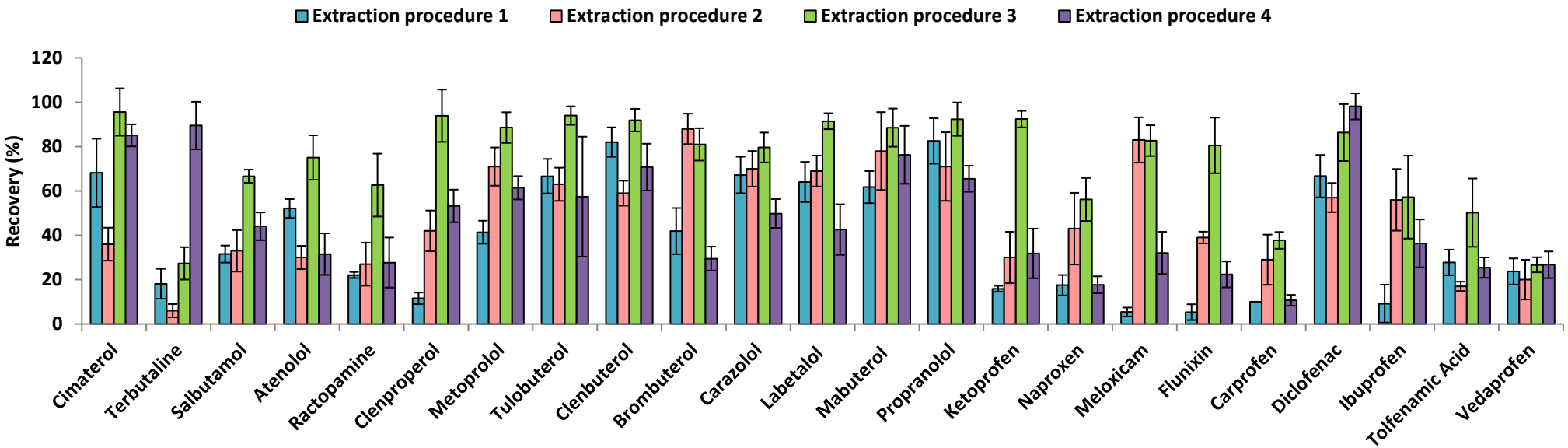
0.999





Chromatographic conditions described in Table 1.

Figure 2.



Extraction procedure 1: 10 mL of water/ACN (40:60 v/v)

Extraction procedure 2: 10 mL acetate buffer 0.2M (pH 5.2) + 10 mL acetate buffer 0.2M (pH 5.2):ACN (50:50 v/v)

Extraction procedure 3: 10 mL acetate buffer 0.2M (pH 5.2) + 10 mL acetate buffer 0.2M (pH 5.2):MeOH (50:50 v/v)

Extraction procedure 4: 10 mL acetate buffer 0.2M (pH 7) + 10 mL acetate buffer 0.2M (pH 7):MeOH (50:50 v/v)

Figure 3.

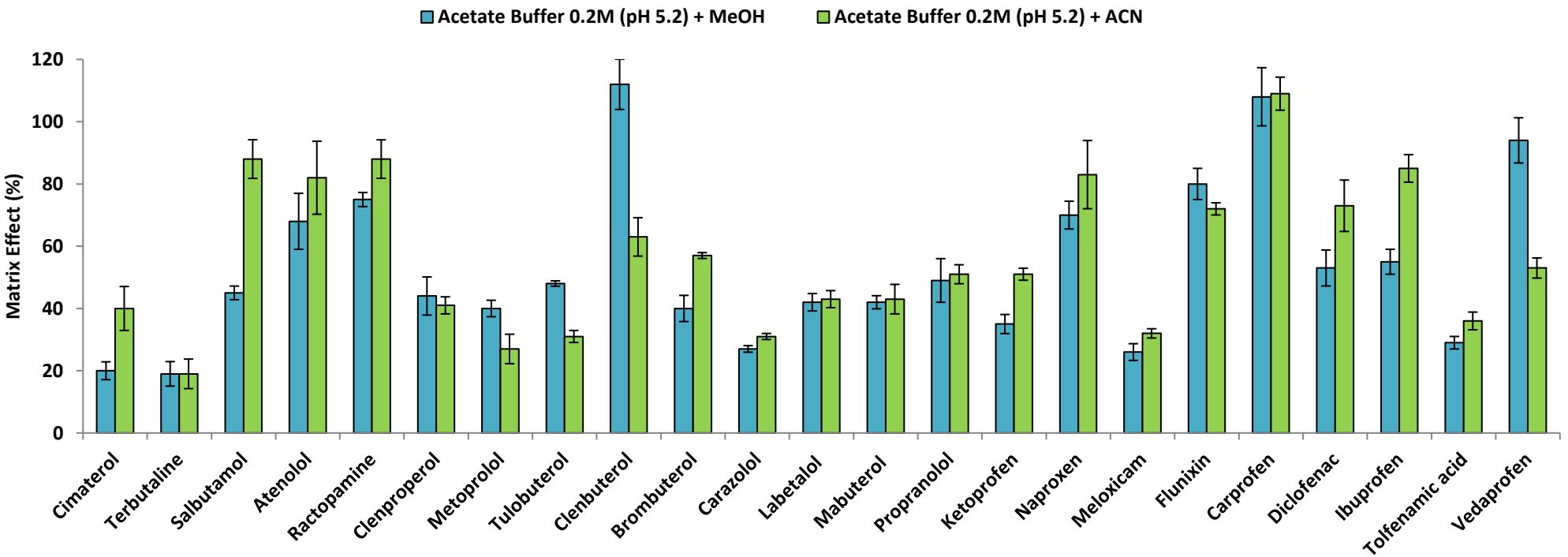

Acetate Buffer 0.2M (pH 5.2) + MeOH
Acetate Buffer 0.2M (pH 5.2) + ACN
Matrix Effect (%)
120
100
80
60
40
20
0
Cimaterol
Terbutaline
Salbutamol
Atenolol
Ractopamine
Clenproperol
Metoprolol
Tulobuterol
Clenbuterol
Brombuterol
Carazolol
Labetalol
Mabuterol
Propranolol
Ketoprofen
Naproxen
Meloxicam
Flunixin
Carprofen
Diclofenac
Ibuprofen
Tolfenamic acid
Vedaprofen

Figure 4.

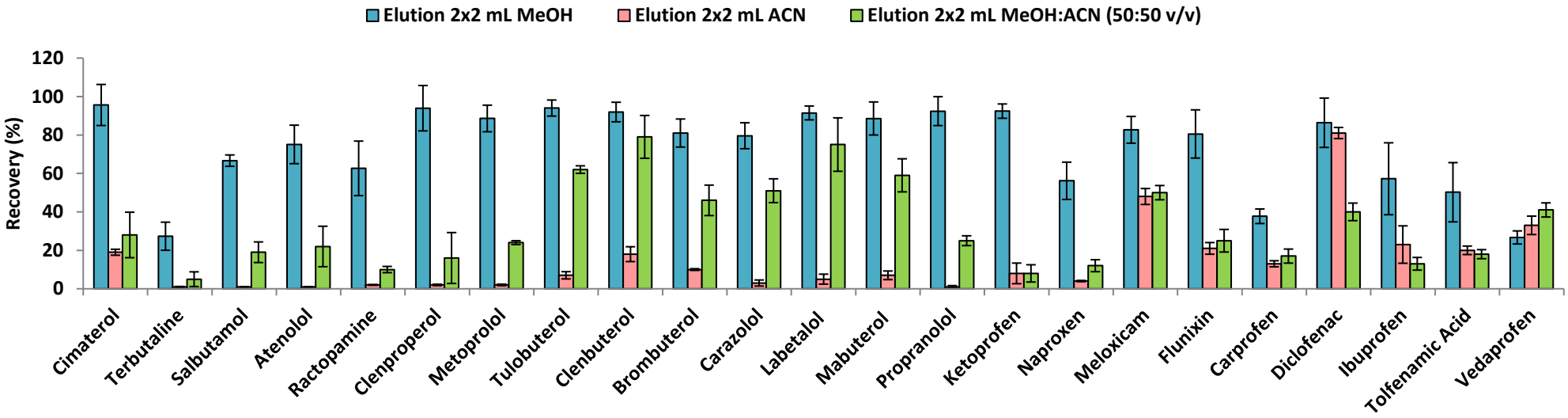

Elution 2x2 mL MeOH
Elution 2x2 mL ACN
Elution 2x2 mL MeOH:ACN (50:50 v/v)
Recovery (%)
120
100
80
60
40
20
0
Cimaterol
Terbutaline
Salbutamol
Atenolol
Ractopamine
Clenproperol
Metoprolol
Tulobuterol
Clenbuterol
Brombuterol
Carazolol
Labetalol
Mabuterol
Propranolol
Ketoprofen
Naproxen
Meloxicam
Flunixin
Carprofen
Diclofenac
Ibuprofen
Tolfenamic Acid
Vedaprofen

Figure 5.

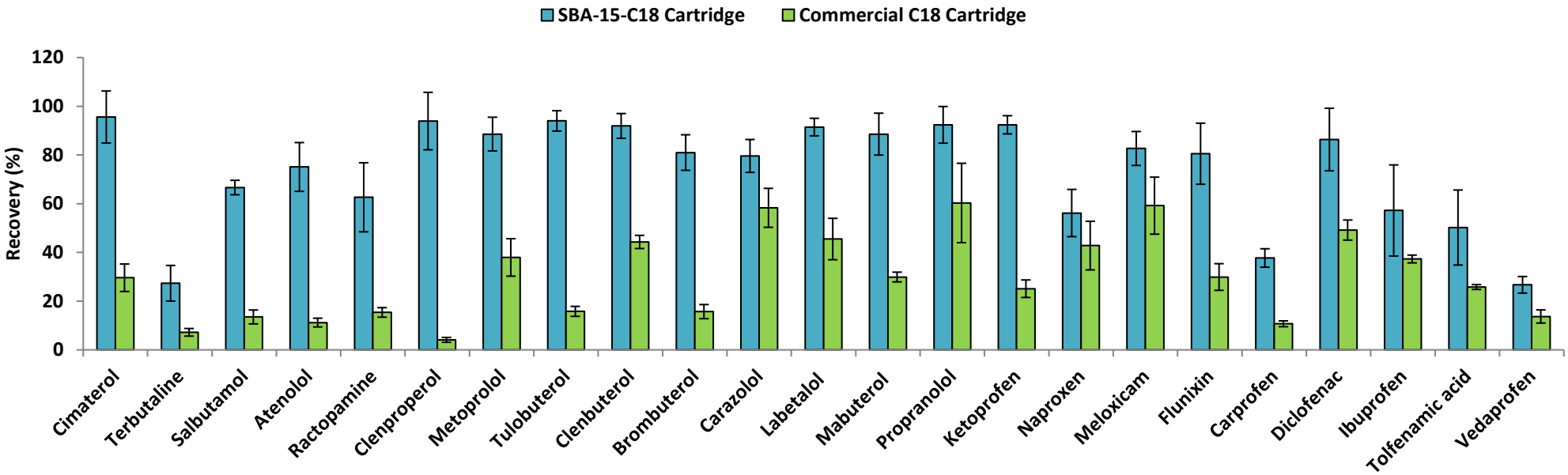

SBA-15-C18 Cartridge
Commercial C18 Cartridge
Recovery (%)
120
100
80
60
40
20
0
Cimaterol
Terbutaline
Salbutamol
Atenolol
Ractopamine
Clenproperol
Metoprolol
Tulobuterol
Clenbuterol
Brombuterol
Carazolol
Labetalol
Mabuterol
Propranolol
Ketoprofen
Naproxen
Meloxicam
Flunixin
Carprofen
Diclofenac
Ibuprofen
Tolfenamic acid
Vedaprofen

Table 1. Mass spectrum parameters and retention time for β-blockers, β-agonists and NSAIDs using the developed UHPLC-IT-MS/MS method.

Analyte	Ionization mode	Retention time (min)	Precursor ion (*m/z*)	Fragmentation amplitude	MS^2. Daughter ions [a] (*m/z*)	Fragmentation amplitude	MS^3. Granddaughter ions (*m/z*)
Cimaterol	ESI (+)	1.4	220	0.60	202*, 160		
Terbutaline	ESI (+)	1.4	226	0.70	170, 152*		
Salbutamol	ESI (+)	1.4	240	0.60	222*, 166		
Atenolol	ESI (+)	1.4	267	0.50	225, 190*		
Ractopamine	ESI (+)	1.6	302	0.50	284*, 164		
Clenproperol	ESI (+)	1.8	263	0.70	245*, 203		
Acebutolol	ESI (+)	1.8	337	0.60	319*, 260		
Metoprolol	ESI (+)	2.1	268	0.60	191*, 116		
Tulobuterol	ESI (+)	2.1	228	0.70	172, 154*		
Clenbuterol	ESI (+)	2.2	277	0.60	259*, 203		
Brombuterol	ESI (+)	2.6	367	0.70	349*, 293		
Carazolol	ESI (+)	2.9	299	0.70	222*, 116		
Labetalol	ESI (+)	2.9	329	0.50	311*, 207		
Mabuterol	ESI (+)	2.9	311	0.70	293*, 237		
Propranolol	ESI (+)	3.4	260	0.50	183*, 116		
Betaxolol	ESI (+)	3.5	308	0.70	177, 116*		
α-Zearalanol	ESI (-)	4.3	321	0.70	303, 277*		
Ketoprofen	ESI (+)	4.9	255	0.50	209*	0.70	194,131, 105
Naproxen	ESI (+)	4.9	231	0.80	185*	0.70	170
Meloxicam	ESI (+)	4.9	352	0.60	141, 115*		
Flunixin	ESI (+)	5.2	297	0.70	279*, 257		
Carprofen	ESI (-)	5.6	272	0.60	228*	0.40	226
Diclofenac	ESI (-)	5.8	294	0.70	250*	0.70	214, 178
Ibuprofen	ESI (-)	6.1	205	1.00	161*, 159		
Tolfenamic acid	ESI (-)	6.7	260	0.50	216*	0.60	180
Vedaprofen	ESI (+)	7.3	300	1.00	201, 155*		

[a] Predominant product ions.
* Ions used for quantitation.
Isolation width (*m/z)* is 4.
Chromatographic conditions with the optimized gradient elution: t = 0 min 20%A – 80%B, t = 6 min 100%A, t = 8 min 20%A – 80%B (2 min) (ACN as mobile phase A and water as mobile phase B, both containing 0.1% formic acid and 4 mM ammonium acetate). The flow rate was 0.3 mL/min.

Table 2. Validation results of the multi-residue method for the determination of the target compounds in meat samples.

Analyte	Linearity, R^2 Matrix-matched calibration	Spiked level (µg/Kg)			Recovery (%)			Repeatability, %RSD			Within-laboratory reproducibility, %RSD			CCα (µg/Kg)	CCβ (µg/Kg)	MDL (µg/Kg)	MQL (µg/Kg)
		VLx 0.5	VL x 1.0	VL x 1.5	VL x 0.5	VL x 1.0	VL x 1.5	VL x 0.5	VL x 1.0	VL x 1.5	VL x 0.5	VL x 1.0	VL x 1.5				
Cimaterol	y = 5478 x + 30083 0.994	2.5	5[a]	7.5	89	96	91	9.8	8.3	7.0	11.6	11.2	18.1	4.50	7.98	1.13	3.75
Terbutaline	y = 9346 x – 5748 0.995	5	10[b]	15	28	27	24	28.0	14.7	15.6	33.7	26.7	21.4	9.35	11.38	1.05	3.50
Salbutamol	y = 14145 x + 84984 0.994	2.5	5[b]	7.5	53	67	62	19.0	4.4	6.5	21.9	19.7	11.4	5.60	9.38	0.45	1.50
Atenolol	y = 17820 x + 90737 0.999	2.5	5[a]	7.5	76	75	85	1.6	11.7	5.0	13.8	13.3	16.1	3.90	7.38	0.23	0.75
Ractopamine	y = 227146 x + 7840049 0.999	5	10[c]	15	52	63	56	5.0	12.4	7.8	19.1	22.6	18.0	12.70	15.40	0.09	0.29
Clenproperol	y = 440695 x + 785440 0.997	2.5	5[a]	7.5	90	94	90	3.3	11.1	10.9	12.9	12.6	13.4	2.95	3.40	0.05	0.17
Metoprolol	y = 77229 x + 158382 0.993	2.5	5[a]	7.5	87	89	84	7.3	7.8	2.9	10.1	12.2	14.7	3.05	3.63	0.40	1.33
Tulobuterol	y = 294400 x + 254739 0.999	0.1	0.2[a]	0.3	93	94	93	8.5	4.5	12.5	9.8	15.7	13.9	0.18	0.23	0.01	0.02
Clenbuterol	y = 1387792 x + 116532 0.999	0.1	0.2[c]	0.3	87	92	89	7.1	5.5	10.9	8.4	10.1	13.2	0.25	0.30	0.01	0.03
Brombuterol	y = 304034 x + 875192 0.996	2.5	5[a]	7.5	78	81	77	3.2	9.0	9.2	8.6	14.2	9.2	3.08	3.68	0.35	1.17
Carazolol	y = 169871 x + 384335 0.992	2.5	5[c]	7.5	75	80	76	4.9	8.5	9.7	6.1	10.3	13.8	5.75	6.50	0.08	0.25
Labetalol	y= 914708 x + 3758070 0.990	2.5	5[a]	7.5	98	91	91	3.0	3.9	8.5	6.5	10.1	10.2	2.90	3.28	0.13	0.42
Mabuterol	y = 605146 x + 1619625 0.993	2.5	5[a]	7.5	91	89	93	1.1	7.1	4.8	7.3	9.7	11.4	2.93	3.35	0.45	1.50
Propranolol	y = 124978 x + 270652 0.995	2.5	5[a]	7.5	91	92	98	9.6	8.1	5.9	12.3	13.5	8.5	2.90	3.30	0.03	0.11
Ketoprofen	y = 52033 x – 31886 0.997	2.5	5[a]	7.5	97	92	98	3.2	4.0	8.3	7.7	11.8	10.5	3.25	4.00	0.48	1.58
Naproxen	y = 17204 x – 222345 0.995	5	10[b]	15	57	56	46	11.0	13.9	13.0	14.9	17.3	20.2	5.73	6.43	1.18	3.92

Meloxicam	y = 55260 x + 224613 0.996	10	20[c]	30	77	83	85	6.7	8.4	12.0	9.4	13.8	13.2	24.50	29.03	0.73	2.42
Flunixin	y = 720977 x + 33540254 0.999	10	20[c]	30	71	81	85	4.6	11.7	7.3	7.4	15.6	10.7	25.73	31.48	0.13	0.42
Carprofen	y = 9224 x + 26448 0.999	25	50[a]	75	34	38	38	8.0	10.0	10.9	13.3	11.4	23.5	63.90	77.80	0.30	1.00
Diclofenac	y = 7386 x + 32410 0.996	2.5	5[c]	7.5	85	86	79	14.2	9.0	4.8	14.6	14.9	15.3	6.13	7.25	0.25	0.83
Ibuprofen	y = 12 x + 2752 0.998	50	100[a]	150	56	57	57	16.2	14.8	8.6	22.5	32.7	13.5	121.30	142.58	18.75	62.50
Tolfenamic acid	y = 20741 x + 403169 0.990	25	50[c]	75	40	50	41	19.7	18.0	13.9	25.4	30.7	35.0	56.18	62.35	0.20	0.67
Vedaprofen	y = 13812 x – 44111 0.990	25	50[c]	75	29	27	24	17.6	12.7	12.6	28.5	15.3	16.2	62.80	75.58	0.63	2.08

[a] Specific level of interest based on the drug characteristics and its detection in the ion trap mass spectrometer and in the analytical method.
[b] Recommended concentration (EURL requirements).
[c] Maximum Residue limit (MRL).

Appendix A. Supplementary material

X-ray diffraction (XRD) pattern of the silicas were obtained on a Philips Difractometer model PW3040/00 X'Pert MPD/MRD at 45 KV and 40 mA, using a wavelength Cu Kα (λ = 1.5418 Å). Scanning electron micrographs and morphological analysis were carried out on a XL30 ESEM Philips with an energy-dispersive spectrometry system (EDS). The samples were treated with a sputtering method with the following parameters: sputter time 100 s, sputter current 30 mA, and film thickness 20 nm using sputter coater BAL-TEC SCD 005. Conventional transmission electron microscopy (TEM) was carried out on a TECNAI 20 Philips microscope operating at 200 kV, with a resolution of 0.27 nm and ± 70º of sample inclination, using a BeO sample holder. N_2 gas adsorption-desorption isotherms were performed using a Micromeritics ASAP 2020 analyzer. Cross-polarization magic-angle spinning (CP-MAS) ^{13}C and ^{29}Si solid-state NMR spectra were recorded on a Varian-Infinity Plus Spectrometer at 400 MHz operating at 100.52 MHz proton frequency (4 ms 908 pulse, 4000 transients, spinning speed of 6 MHz, contact time 3 ms, pulse delay 1.5 s). Infrared spectra were recorded on a Thermo Nicolet 380 FT-IR spectrophotometer in the region 4000 to 400 cm^{-1} by using spectra quality KBr powder. Elemental analysis (% H, % C, % N and % S) were performed using a microanalyser model LECO CHNS-932. The thermal stability of the modified nanostructured silicas was studied using a Setsys 18 A (Setaram) thermogravimetric analyzer with a 100 mL platinum crucible. A synthetic air atmosphere was used and the temperature increased from 25 ºC to 800 ºC at a speed of 10 ºC per minute.

Figure S1. a) N_2 adsorption-desorption isotherms and b) pore size distribution of SBA-15-C18.

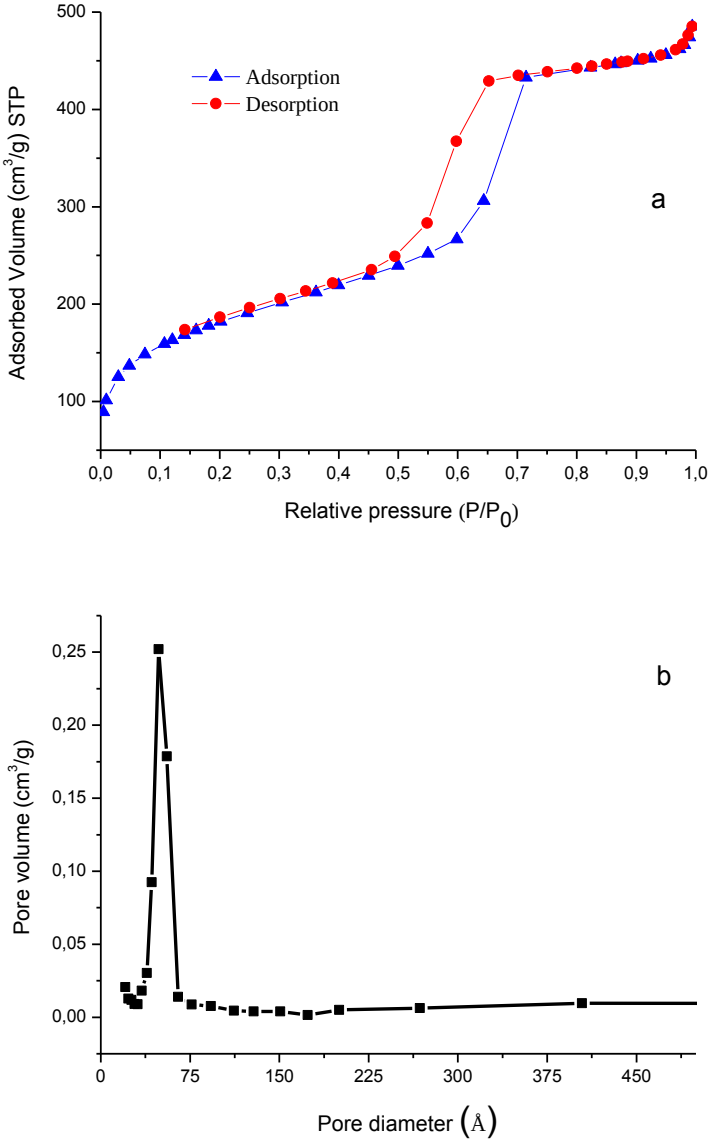


Figure S2. ^{29}Si MAS-NMR spectrum of SBA-15-C18.

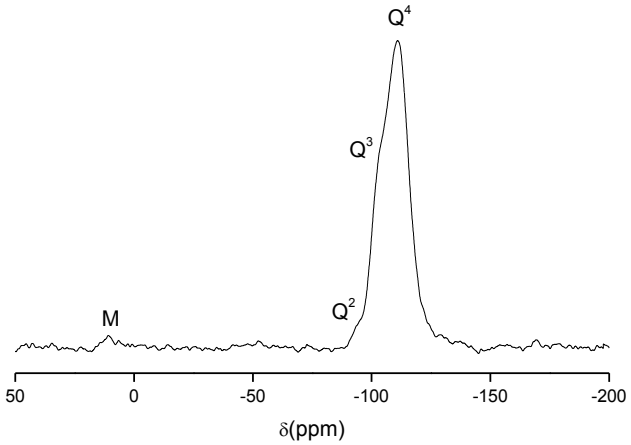


Figure S3. FT-IR spectrum of SBA-15-C18.