Talanta 84 (2011) 820–828

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Determination of residual pharmaceuticals in edible animal tissues by continuous solid-phase extraction and gas chromatography–mass spectrometry

Abdelmonaim Azzouz[a], Badredine Souhail[b], Evaristo Ballesteros[a,*]

[a] Department of Physical and Analytical Chemistry, E.P.S of Linares, University of Jaén, 23700 Linares, Jaén, Spain
[b] Department of Chemistry, Faculty of Science, University Abdelmalek Essaadi, B.P. 2121, M'Hannech II, 93002 Tétouan, Morocco

ARTICLE INFO

Article history:
Received 24 November 2010
Received in revised form 31 January 2011
Accepted 14 February 2011
Available online 19 February 2011

Keywords:
Pharmaceutical residues
Edible animal tissues
Continuous solid-phase extraction
Gas chromatography–mass spectrometry

ABSTRACT

A sensitive, reliable method using continuous solid-phase extraction and gas chromatography–mass spectrometry was developed for the simultaneous determination of twenty pharmaceuticals including antibacterials, anti-epileptics, antiseptics, β-blockers, lipid regulators, hormones and non-steroidal anti-inflammatories at trace levels in edible animal tissues. The procedure involves deproteination and delipidation of samples by precipitation/centrifugation/filtration, followed by sample enrichment and cleanup by continuous solid-phase extraction. The proposed method was validated with quite good analytical results including low limits of detections (0.4–2.7 ng kg^{-1} for 2 g of sample) and good linearity ($r^2 > 0.995$) throughout the studied concentration ranges. In addition, the method is quite accurate (recoveries ranged from 92 to 101%) and precise (within-day and between-day RSD values were less than 7%), which allows the determination of residual pharmaceuticals in tissues from agricultural farm and fish hatchery animals (pig, veal, lamb and chicken muscle, kidney and liver; and salmon, sea bass and sole flesh). The analytes most frequently found in the studied samples were the hormones estrone and 17β-estradiol, and the antibacterials florfenicol and pyrimethamine.

1. Introduction

The usually high density of animals grown in agricultural facilities and fish hatcheries can increase the potential for disease outbreak. This raises the need for medications to be given therapeutically to treat existing infections, or prophylactically, to minimize the impact of an outbreak spreading across an animal population. A portion of administered pharmaceuticals remains in the animal's body, but a significant fraction is discharged into the environment by excretion. The ensuing risks of pharmaceutical residues (PRs) reaching edible products and the potential health hazards associated to their consumption have become a public safety issue [1]. Veterinary and human pharmaceuticals can also reach animal foods by effect of animals eating or drinking contaminated feed or water. Residues may include the unaltered parent compound and its metabolites and/or conjugates, and may have direct toxic effects on consumers (e.g. allergic reactions in hypersensitive individuals or the development of resistant bacterial strains in response to some antibacterials) [2]. The Codex Alimentarius Commission of the Food and Agriculture Organization, the World Health Organization [3] and the European Community [4] have set maximum residue limits (MRLs) for a variety of pharmaceuticals in foodstuffs of animal origin [5].

The extremely low (parts-per-billion) levels at which pharmaceutical residues can be encountered in so highly complex biological matrices as milk, meat or eggs pose a major analytical challenge that can only be met by using effective procedures for the selective extraction of analytes, removal of coextractants, and sensitive, specific detection of the analytes. The sample preparation procedures typically used with pharmaceutical residues include homogenization and/or enzymatic digestion of the homogenate, extraction/cleanup, enrichment, and, where needed, derivatization of the analyte [1]. Liquid–liquid extraction [6,7], solid-phase extraction (SPE) [8,9] and solid-phase microextraction [10] are common choices for extraction and cleanup in this context. Emulsification during extraction of the sample is usually avoided by prior protein precipitation or delipidation. Proteins in meat, egg and milk samples can be precipitated and fat delipidated by adding methanol [11,12], acetonitrile [13,14], hydrochloric acid [15,16] or trichloroacetic acid [17], for example. In some cases, deproteination can be accomplished simply by heating the sample in the presence of a buffer [18], and delipidation by freezing-lipid filtration [11]. Finally, SPE of pharmaceutical residues usually relies on sorbent materials such as octadecyl bonded silica (RP-C_{18}) [7,12,19], Oasis-HLB (polystyrene–divinylbenzene–*N*-vinypyrrolidone terpolymer) [8,9,20,21], Oasis MCX (a strong cation-exchange mixed-mode polymer) [22] or amino-propyl-NH_2 [11,12,18,23].

* Corresponding author. Tel.: +34 953648560; fax: +34 953648560.
E-mail address: eballes@ujaen.es (E. Ballesteros).

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doi:10.1016/j.talanta.2011.02.016

Immunoassays are often used in control analyses of pharmaceutical residues in meat products. The analyte (antigen) binds highly specifically to the antibodies raised against it [24]. The high sensitivity of immunoassays facilitates the rapid screening of large numbers of samples for individual pharmaceuticals, which has led to its gradually superseding multi-residue techniques [1]. Pharmaceutical residues present in various animal meat products, and their derivatives, have been determined by liquid chromatography with fluorescence or electrochemical detection [25,26]. This methodology is simple and rapid, but has limited sensitivity. In combination with mass spectrometry, however, liquid chromatography allows PRs in various types of foods to be determined with good sensitivity and accuracy [6,10,14]. Recently, new approaches using the potential of liquid chromatography coupled with tandem mass spectrometry (MS–MS) [8,12,15,16,22,23] or time-of-flight mass spectrometry [13,14] have been developed for multiclass residue screening. The prevalent instrumental methods for determining PRs in meat samples, which are based on gas chromatography–mass spectrometry (GC–MS), provide increased sensitivity, specificity and chromatographic resolution, but require derivatization of the analytes prior to their determination [7,9,11,21,27,28]. Gas chromatography–tandem mass spectrometry has also been used for the determination of hormones in kidney and meat, and milk and egg [18,29]; and that of non-steroidal anti-inflammatories at the microgram-per-kilogram level in milk [30].

The aim of this work was to develop a reliable multi-residue method for the simultaneous determination of selected pharmaceuticals (antibacterials, non-steroidal anti-inflammatory, hormones, antiseptics, β-blockers, lipid regulators and anti-epileptics) in tissues from agricultural farm and fish hatchery animals (viz. pig, veal, lamb and chicken muscle, liver and kidney; and salmon, sea bass and sole flesh). The proposed method, which improves on an earlier one developed by our group to determine pharmaceuticals in water samples [21], involves the removal of protein and lipids – which can interfere with the determination of PRs – from the sample matrix by precipitation/centrifugation/filtration. Following cleanup of the resulting supernatant, the analytes are preconcentrated by continuous solid-phase extraction on an Oasis-HLB column for conversion into their silyl derivatives to improve sensitivity and resolution in their GC–MS measurements.

2. Experimental

2.1. Standards and reagents

All products were handled with care, using latex gloves, a respiratory protection device and fume hoods. Stock solutions of the 20 pharmaceuticals studied, which were supplied in the highest available purity by Sigma–Aldrich (Madrid, Spain), were prepared at a 1 g L^{-1} concentration in methanol and stored at 4 °C in the dark. Triphenylphosphate and the derivatizing reagents [*N,O-bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS)] were purchased from Fluka (Madrid, Spain). Oasis-HLB (particle size 50–65 μm) was obtained from Waters (Madrid, Spain). Solvents (acetonitrile, methanol and ethyl acetate) were obtained from Merck (Darmstadt, Germany). Millex-LG filter units (hydrophilic, PTFE, pore size 0.20 μm, diameter 25 mm, filtration area 3.9 cm^2) were supplied by Millipore Ibérica, S.A. (Madrid, Spain). The standard solutions used to prepare spiked samples were obtained by diluting the stocks in purified water from a Milli-Q System (Millipore, Bedford, MA, USA) and adjusted to pH 7.

2.2. Instruments and apparatus

GC–MS analyses were performed on a Focus GC instrument (Thermo Electron SA, Madrid, Spain) interfaced to a DSQ II mass spectrometer controlled via a computer running XCalibur software. The chromatograph was equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness) coated with 5% phenylmethylpolysiloxane (Supelco, Madrid, Spain). Helium (purity 6.0) at a flow rate of 1 mL min^{-1} was employed as the carrier gas. For pharmaceutical determinations, the column temperature was initially kept at 70 °C for 1 min, raised to 150 °C at 14 °C min^{-1}, and then to 290 °C at 6 °C min^{-1}. The injection port and transfer line temperatures were kept at 270 and 280 °C, respectively. The ion source temperature for the 70 eV electron impact ionization mode was 200 °C. The mass spectrometer was operated in the selected ion monitoring (SIM) mode. The MS instrument was set in full scan mode (70–500 amu) for identification, and the quantification fragments (m/z) for each pharmaceutical (SIM mode) were selected according to abundance (highest sensitivity) and specific criteria. The m/z values used for each analyte are listed in Table 1. The time for solvent delay was set to 8 min. In all analyses, a volume of 1 μL of the silylated derivatives was injected in the split mode (1:20 ratio) and the resulting peak area was used as analytical signal for quantification.

The flow system comprised a peristaltic pump (Gilson Minipuls-3, Villiers-le-Bel, France), two Rheodyne 5041 injection valves (Cotati, CA, US), poly(vinylchloride) pumping tubes, PTFE tubing of 0.5 mm ID and standard connectors. The sorbent column was prepared by packing a PTFE column with 60 mg of Oasis-HLB sorbent material as described elsewhere [21].

2.3. Sampling

Tissues from agricultural farm and fish hatchery animals (viz. pig, veal, lamb and chicken muscle, liver and kidney; and salmon, sea bass and sole flesh) were purchased at a local supermarket. Samples were all received in frozen form and stored at −20 °C until analysis.

2.4. Sample pretreatment

Fig. 1 depicts the procedure used to prepare the samples and determine the residual pharmaceuticals. Tissues were homogenized in an A320R1 grinder from Moulinex (Barcelona, Spain). A portion of about 2 g of tissue homogenate was then weighed into a 50 mL round polypropylene centrifuge tube and mixed with 4 mL of water and 6 mL of acetonitrile in a vortex mixer (REAX Control, Heidolph, Kelheim, Germany) for 30 s. This was followed by centrifugation on a Centrofriger BL-II apparatus (JP Selecta, Barcelona, Spain) at 4000 rpm for 10 min (4 °C). Next, the supernatant was passed through a 0.20 μm Millex-LG filter. The filtered supernatant was carefully evaporated under a stream of ultrahigh-purity N_2 to a final volume of 200 μL and redissolved to 5 mL with purified water a pH 7. The pretreated sample was thus made ready for continuous solid-phase extraction.

2.5. Cleanup and continuous solid-phase extraction

Fig. 2 shows the continuous system used for the solid-phase extraction of pharmaceutical residues from animal tissues. A volume of 5 mL of pretreated sample or standard aqueous solution containing a 1.3–10,000 ng kg^{-1} concentration of each analyte at pH 7 was continuously passed at 4 mL min^{-1} through the sorbent column placed in the loop of injection valve IV_1. All pharmaceuticals were sorbed and the sample matrix was sent to waste. Simultaneously, the loop of IV_2 was filled with eluent containing the IS

Table 1
Analytical figures of merit of the proposed method for determination of residual pharmaceuticals in edible animal tissues.

Therapeutic class	Compounds	Retention time (min)	LOD ($ng\,kg^{-1}$)	Linear range ($ng\,kg^{-1}$)	r^a	RSD (%) ($n=11$) within-day	RSD (%) ($n=11$) between-day	$m/z^{b,c}$
Non steroidal anti-inflammatories	Diclofenac	22.3	1.1	3.3–10,000	0.996	5.1	5.8	**214**, 242, 367
	Flunixin	19.7	0.5	1.6–10,000	0.996	5.0	5.6	251, 263, **353**
	Ibuprofen	11.9	0.5	1.5–10,000	0.995	4.8	5.3	**160**, 234, 263, 278
	Ketoprofen	21.0	0.5	1.5–10,000	0.999	5.8	6.7	73, **282**, 311
	Mefenamic acid	20.9	0.4	1.3–10,000	0.996	4.9	5.5	208, **223**, 313
	Naproxen	19.3	1.0	3.2–10,000	0.996	5.4	6.0	**185**, 243, 302
	Niflumic acid	18.6	0.6	1.8–10,000	0.996	4.5	5.1	**236**, 263, 353
	Phenylbutazone	24.0	2.3	7.4–10,000	0.997	4.9	5.4	**183**, 252, 308
Hormones	Estrone	26.9	2.4	7.9–10,000	0.998	5.4	6.3	218, 257, **342**
	17β-estradiol	27.3	2.6	8.3–10,000	0.998	5.6	6.4	285, **416**
	17α-ethinylestradiol	28.7	2.7	8.7–10,000	0.996	6.2	6.7	**425**, 440
Antibacterials	Chloramphenicol	24.5	0.4	1.3–10,000	0.996	5.0	5.5	208, **225**
	Florfenicol	26.2	0.5	1.6–10,000	0.998	5.8	6.5	**257**, 414
	Pyrimethamine	21.3	2.5	8.0–10,000	0.998	5.5	5.9	**377**, 392, 394
	Thiamphenicol	27.9	0.5	1.5–10,000	0.998	6.3	6.9	242, 257, **330**
Anti-epileptics and β-blokers	Carbamazepine	22.0	0.6	1.9–10,000	0.997	4.1	5.0	**193**, 236
	Metoprolol	19.0	1.4	4.5–10,000	0.996	5.3	6.2	72, **223**
	Propranolol	20.6	1.6	5.2–10,000	0.998	5.1	6.1	72, **215**
Antiseptic and lipid regulators	Clofibric acid	11.3	1.5	5.0–10,000	0.995	5.2	6.2	128, **143**, 286
	Triclosan	19.9	1.5	4.8–10,000	0.997	4.6	5.3	200, **347**

[a] r, correlation coefficient.
[b] The base peaks under for quantification are boldfaced.
[c] m/z for IS (triphenylphosphate): 77, 325, **326**.

(500 $\mu g\,L^{-1}$ triphenylphosphate in ethyl acetate) by means of a syringe. Any residual water remaining inside the column or connectors was flushed by passing an air stream at 4 mL min^{-1} through the carrier line for 2 min. Next, IV_2 was switched to have the loop contents (400 μL) injected into the same air stream used in the drying step in order to elute PRs – in the opposite direction of sample aspiration. The organic extract was collected in a conical glass insert of 0.5 mL and concentrated to a volume of 35 μL under a stream of ultrapure N_2. Potential errors in measuring the final extract volume were avoided by using an internal standard. Next, a volume of 70 μL of BSTFA + 1% TMCS was added and the vials were heated at 70 °C for 20 min. Finally, 1 μL aliquots of the silylated derivatives were

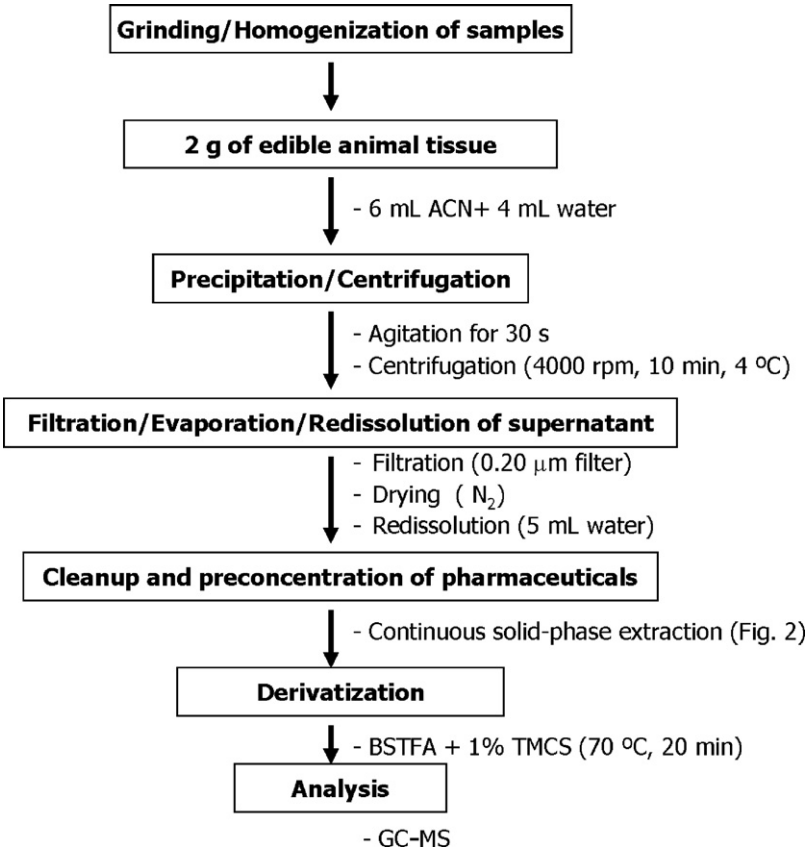

Fig. 1. Flow chart of the procedure for determining residual pharmaceuticals in edible animal tissues.

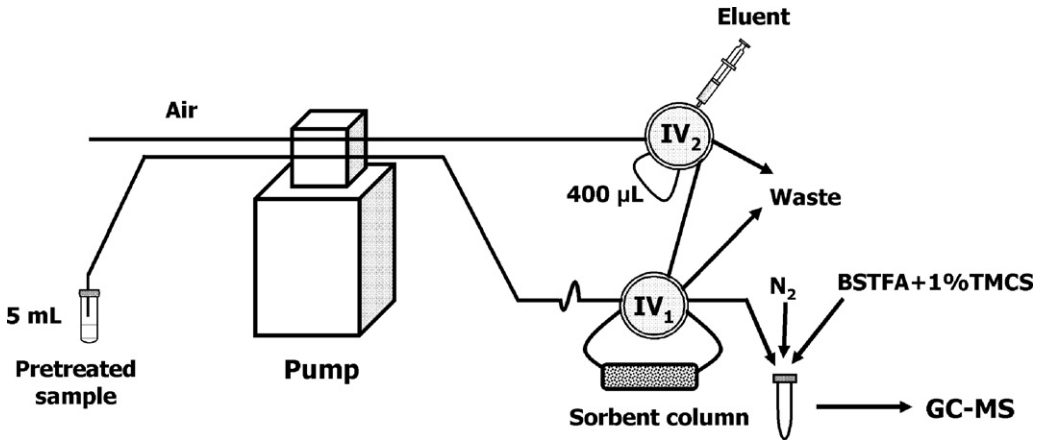

Fig. 2. Continuous flow system fort the cleanup and preconcentration of pharmaceutical residues in edible animal tissues. IV, injection valve.

analysed by GC–MS in the SIM mode. The sorbent column was conditioned with 1 mL of methanol and 5 mL of purified water between samples. Under these conditions, the column remained serviceable for about 2 months.

3. Results and discussion

In previous work, we developed a method for the determination of pharmaceuticals in environmental water samples [21]. The method was optimized by examining the performance of various sorbents (viz. Oasis-HLB, LiChrolut EN, XAD-2, XAD-4, RP-C18, Florisil, Silica Gel and Isolute NH_2) in the preconcentration and solid-phase extraction of the pharmaceuticals and Oasis-HLB selected on the grounds that it retained all analytes virtually completely. Also, various organic solvents including ethyl acetate, acetonitrile, acetone, methanol, ethanol, 2-propanol and dichloromethane were examined as eluents and ethyl acetate was found to be the most efficient in eluting the pharmaceuticals.

In this work, we analysed a greater number of compounds and examined the effect of all variables potentially influencing retention and elution of the 20 pharmaceuticals studied, in addition to those having some effect on their derivatization with a 99:1 BSTFA–TMCS mixture. To reach the best operational conditions, we used a univariate method, where one variable is varied by maintaining constant the rest. The optimum range and selected values for each variable are given in Table 2. The high complexity of the matrix of edible animal tissues required the removal of proteins and lipids in order to avoid their interference with the GC–MS determination of the pharmaceuticals [11]. Below are described the effects of the variables influencing the removal of proteins and lipids by precipitation and centrifugation.

3.1. Variables influencing sample pretreatment

One preliminary step to be taken towards the intended multicomponent analyses was the development of a generic sample preparation method suitable for extracting the analytes from various types of food matrices of animal origin. In fact, a large amount of lipids (phospholipids, triacylglycerolipids, phosphocholine lipids and cholesterol, mainly) and protein is co-extracted with the target compounds owing to their high solubility in the organic solvents used to extract pharmaceuticals from animal tissues. Also, lipids and proteins are easily adsorbed in different parts of a gas chromatograph such as the injection port and column, which detracts from chromatographic performance. In addition, pharmaceutical residues in meat and fish tissues may be present as conjugates (e.g. modified by glucuronide or acetyl groups) [2]. A number of preparation methods including the removal of lipids and proteins are available for the determination of pharmaceuticals in meat and fish [1,14,31,32]. Many authors prefer acetonitrile over methanol or ethyl acetate since the latter two extract too many matrix compounds and complicate further cleanup as a result. Mol et al. [33] tested a number of solvents and their combinations, and found the best choice to depend on the particular matrix; they selected the water/acetonitrile mixture. In this work, we assessed the efficiency of various solvents (water, acetonitrile, methanol, ethanol and ethyl acetate) and trichloroacetic acid in removing proteins and lipids from meat and fish tissues. For this purpose, an amount of ca. 2 g of animal tissue was mixed with a volume of 10 mL of each solvent or solvent mixture (water–methanol, water–acetonitrile and water–ethanol), or a solution of 30% m/v trichloroacetic acid, and centrifuged at 4 °C at 4000 rpm for 10 min. A 3:2 (v/v) acetonitrile–water mixture caused more than 95% of the protein and lipid content to be removed and proved the most efficient solvent; also, it facilitated separation of the precipitate, which is consistent with previous results [14]. The optimum volume of acetonitrile–water mixture for addition to meat and fish tissues was established by examining its effects over the range 1–20 mL; protein and lipid precipitation was found to peak at 9 mL, so a solvent volume of 10 mL (6 mL acetonitrile + 4 mL purified water) was

Table 2
Variables affecting the meat and fish tissues pretreatment, continuous solid-phase extraction and derivatization processes of pharmaceutical residues.

Variable	Optimum range (selected value)
Sample pretreatment (precipitation and centrifugation)[a]	
Volume of water–acetonitrile mixture (3:2, v/v, mL)	9–20 (10)
Centrifugation rate (rpm)	3500–5000 (4000)
Centrifugation time (min)	5–30 (10)
Centrifugation temperature (°C)	0–10 (4)
Continuous solid-phase extraction[b]	
Sample pH	6.5–7.5 (7)
Amount of sorbent (Oasis-HLB, mg)	55–65 (60)
Volume of eluent (ethyl acetate, µL)	350–450 (400)
Sample flow rate (mL min^{-1})	3.5–4.5 (4)
Air flow rate (mL min^{-1})	3.5–4.5 (4)
Breakthrough volume (mL)	1–200 (5)
Percentage of acetonitrile (%)	0–15
Derivatization (silylation of pharmaceuticals)	
Percentage of TMCS in BSTFA[c]	1–15 (1)
Reaction time (min)	15–25 (20)
Temperature of reaction (°C)	65–75 (70)

[a] To 2 g of samples.
[b] These variables have been previously optimized [21].
[c] TMS, trimethylchlorosilane; BSTFA, *N*,*O*-bis-(trimethylsilyl)trifluoacetamide.

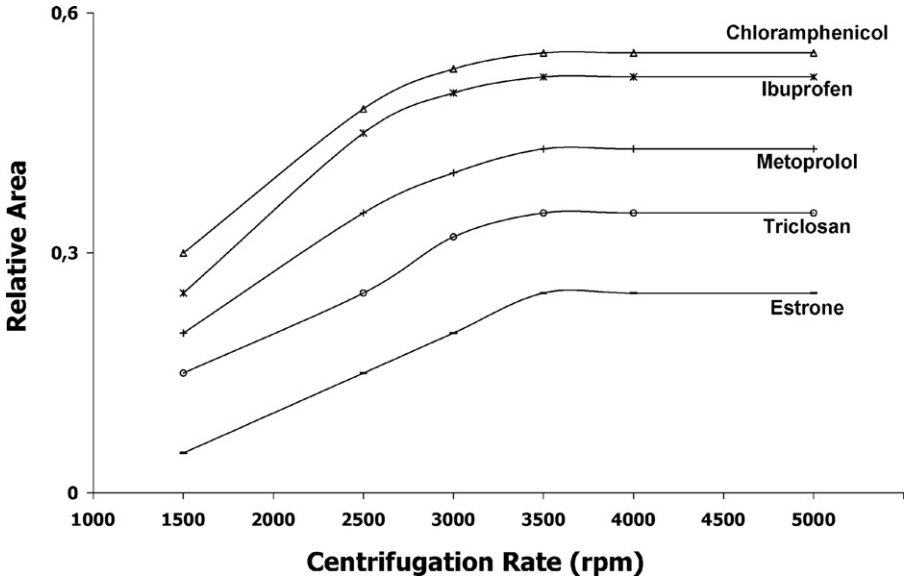

Fig. 3. Effect of centrifugation rate for separating proteins and lipids from the sample matrix.

adopted as optimal. We also examined the effects of the centrifugation rate, temperature and time used with the sample–solvent mixture to facilitate separation of the protein–lipid precipitate from the other components of the sample matrix. The effects of the centrifugation rate, temperature and time were examined over the ranges 1500–5000 rpm, 0–20 °C and 1–30 min, respectively. As can be in Fig. 3 for six representative analytes, the signals increase the up to 3500 rpm of centrifugation rate, after which it stays constant. For the other analytes the behaviour is similar. We therefore selected as centrifugation rate 4000 rpm. In the case of the temperature and time of centrifugation, 4 °C and 10, respectively, have been selected as intermediate values in their optimum ranges (Table 2).

Once the operating conditions for separating proteins and lipids from the sample matrix by precipitation and centrifugation were established, we checked that the filtered supernatant was compatible with the continuous solid-phase extraction of the target pharmaceuticals. To this end, we examined the effect of acetonitrile on retention of the 20 pharmaceuticals by the sorbent column in the continuous SPE system by preparing aqueous solutions containing a 50 ng L^{-1} concentration of each compound and a variable proportion of acetonitrile from 0 to 50% in a volume of 10 mL. The pharmaceuticals present in the eluate were derivatized as described under analytical procedure and determined by GC–MS for comparison with the results obtained in the absence of acetonitrile. Based on them, acetonitrile in proportions below 15% had no effect on retention. Higher proportions, however, resulted in diminished sorption of the analytes. This was the likely consequence of the special sorption mechanism involved, by which moderately polar organic compounds partitioned between a polar phase (water) and a polymeric sorbent (Oasis-HLB) via polar interactions such as hydrogen bonding between the hydroxyl group in the pharmaceuticals and the underlying sorbent surface. In the presence of a high proportion of acetonitrile in the aqueous sample, the solvent probably broke bonds and helped dissolve the pharmaceuticals, thereby dramatically reducing their adsorption. This entailed lowering the proportion of solvent in the supernatant remaining after the sample treatment, which contained about 40%

Table 3
Percent recovery (±SD, $n = 3$) of pharmaceuticals added to animal tissue samples.

Compounds	Chicken muscle (ng kg^{-1})		Veal liver (ng kg^{-1})		Pig kidney (ng kg^{-1})		Sole flesh (ng kg^{-1})	
	25	100	25	100	25	100	25	100
Diclofenac	101 ± 6	95 ± 5	101 ± 6	99 ± 6	94 ± 5	97 ± 6	99 ± 6	100 ± 6
Flunixin	100 ± 5	96 ± 5	100 ± 6	101 ± 6	96 ± 5	98 ± 5	92 ± 5	97 ± 6
Ibuprofen	96 ± 5	101 ± 5	96 ± 5	98 ± 5	100 ± 6	94 ± 5	95 ± 5	96 ± 5
Ketoprofen	99 ± 6	100 ± 7	101 ± 6	96 ± 6	93 ± 5	100 ± 6	97 ± 6	98 ± 6
Mefenamic acid	101 ± 6	95 ± 5	100 ± 5	98 ± 5	95 ± 5	94 ± 5	101 ± 6	99 ± 5
Naproxen	100 ± 6	99 ± 6	95 ± 5	98 ± 6	101 ± 6	93 ± 6	97 ± 6	92 ± 5
Niflumic acid	97 ± 5	101 ± 5	101 ± 5	99 ± 5	92 ± 5	98 ± 5	100 ± 5	99 ± 5
Phenylbutazone	96 ± 5	93 ± 5	92 ± 5	101 ± 5	101 ± 6	97 ± 5	95 ± 5	93 ± 5
Estrone	92 ± 5	98 ± 6	99 ± 6	101 ± 6	96 ± 6	95 ± 6	100 ± 6	97 ± 6
17β-estradiol	99 ± 7	100 ± 6	96 ± 6	94 ± 6	99 ± 6	101 ± 7	95 ± 6	98 ± 6
17α-ethinylestradiol	93 ± 6	97 ± 6	99 ± 7	100 ± 7	95 ± 6	92 ± 6	101 ± 7	100 ± 6
Chloramphenicol	100 ± 6	101 ± 6	97 ± 6	98 ± 6	100 ± 6	93 ± 5	95 ± 6	98 ± 5
Florfenicol	101 ± 6	97 ± 5	97 ± 7	92 ± 6	98 ± 6	100 ± 7	94 ± 6	94 ± 6
Pyrimethamine	98 ± 6	96 ± 6	99 ± 6	101 ± 6	95 ± 6	96 ± 6	101 ± 6	96 ± 6
Thiamphenicol	94 ± 6	97 ± 7	100 ± 7	92 ± 6	96 ± 6	92 ± 6	95 ± 6	101 ± 7
Carbamazepine	101 ± 5	95 ± 5	101 ± 5	99 ± 5	98 ± 5	97 ± 5	93 ± 5	92 ± 5
Metoprolol	100 ± 6	101 ± 6	94 ± 5	96 ± 5	101 ± 6	97 ± 6	97 ± 6	101 ± 6
Propranolol	97 ± 6	101 ± 6	95 ± 5	100 ± 6	97 ± 6	93 ± 6	101 ± 6	94 ± 5
Clofibric acid	98 ± 6	96 ± 5	101 ± 6	99 ± 6	101 ± 5	92 ± 6	98 ± 6	95 ± 5
Triclosan	99 ± 5	94 ± 5	101 ± 6	101 ± 5	98 ± 5	100 ± 5	97 ± 5	95 ± 5

Table 4
Pharmaceuticals detected in edible animal tissues (±SD, μg kg^{-1}, $n = 3$).

Compounds	Muscle[a]	Muscle[b]	Muscle[c]	Muscle[d]	Liver[a]	Liver[b]	Live[c]	Live[d]	Kidney[a]	Kidney[b]	Kidney[c]	Flesh[e]	Flesh[f]	Flesh[g]
Diclofenac	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Flunixin	–	1.5 ± 0.1	–	–	–	–	–	–	–	–	3.5 ± 0.2	–	–	–
Ibuprofen	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ketoprofen	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mefenamic acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Naproxen	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Niflumic acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Phenylbutazone	7.8 ± 0.4	2.8 ± 0.2	–	–	–	–	–	1.2 ± 0.1	–	–	–	–	–	–
Estrone	0.65 ± 0.04	0.99 ± 0.07	0.51 ± 0.03	–	–	1.9± 0.1	0.85 ± 0.05	0.64 ± 0.04	4.5 ± 0.3	–	–	1.3 ± 0.1	0.78 ± 0.05	0.52 ± 0.03
17β-estradiol	1.5 ± 0.1	1.3 ± 0.1	0.82 ± 0.05	–	1.2 ± 0.1	1.7 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	3.7 ± 0.2	6.7 ± 0.4	1.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	0.81 ± 0.05
17α-ethinylestradiol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Chloramphenicol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Florfenicol	28.2 ± 1.8	0.59 ± 0.04	18.2 ± 1.1	–	–	3.5 ± 0.2	–	1.5 ± 0.1	27.5 ± 1.8	–	–	3.4 ± 0.2	1.9 ± 0.1	0.60 ± 0.04
Pyrimethamine	14.0 ± 0.8	0.83 ± 0.05	–	–	5.3 ± 0.3	–	3.2 ± 0.2	–	–	1.4 ± 0.1	1.7 ± 0.1	–	–	–
Thiamphenicol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Carbamazepine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Metoprolol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Propranolol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Clofibric acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Triclosan	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Lamb.
[b] Veal.
[c] Pig.
[d] Chicken.
[e] Sea bass.
[f] Sole.
[g] Salmon.

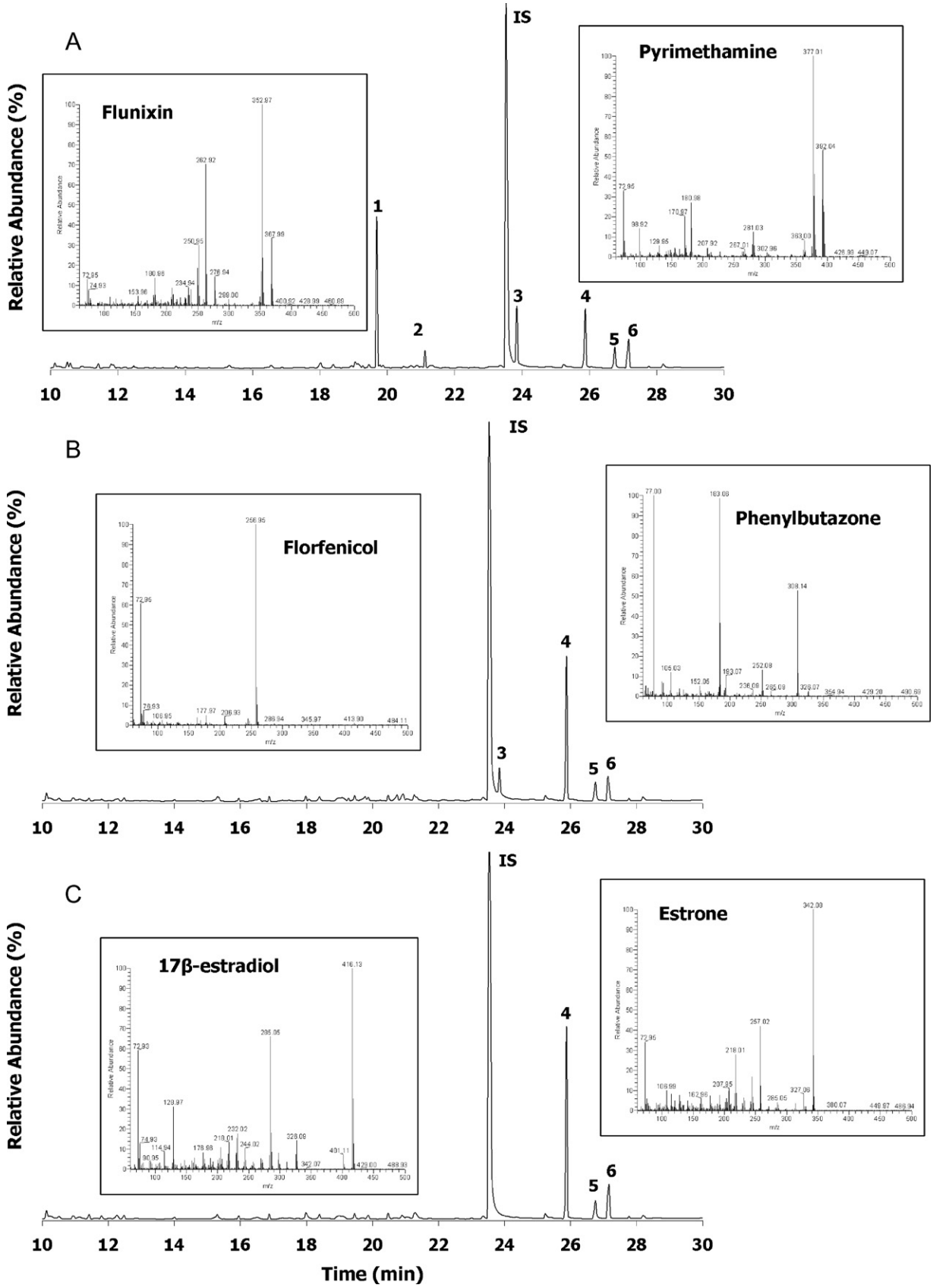

Fig. 4. GC–MS chromatograms obtained in the SIM mode in the analysis of 2 g of veal muscle (A), chicken liver (B) and sole flesh (C) (see Table 4). Peaks: 1, flunixin; 2, pyrimethamine; 3, phenylbutazone; 4, florfenicol; 5, estrone; 6, 17β-estradiol; IS, internal standard (triphenylphosphate).

of acetonitrile, by evaporation to a final volume of 200 μL under a stream of ultrapure N_2 and redissolution in 5 mL of purified water at pH 7 for introduction into the continuous SPE system.

3.2. Analytical performance

The performance and reliability of the proposed method were assessed by determining the regression equation, linear range, analyte detectability and precision for the 20 pharmaceuticals. For this purpose, an amount of 2 g of uncontaminated muscle (chicken) tissue was fortified with 50–200 μL of standard solutions containing all pharmaceuticals at concentrations over the range 1.3–10,000 ng kg^{-1}. The fortified samples were pretreated and extracted as described in Section 2 (Fig. 1). Chromatographic resolution and efficiency were assessed from plots of analyte-to-IS peak area ratio against analyte concentration. The figures of merit of the proposed method are listed in Table 1. As can be seen, the correlation coefficient was higher than 0.995 (12 points per calibration) in all instances. The limit of detection (LOD, defined as the analyte concentration giving a chromatographic peak equal to three times the corresponding regression standard deviation, Sy/x, divided by the slope of the calibration graph) ranged from 0.4 to 2.7 ng kg^{-1}. The precision, calculated as the relative standard deviation for 11 chicken muscle samples spiked with a 50 ng kg^{-1} concentration of each analyte ranged from 4.1 to 6.3% (within-day) and from 5.1 to 6.9% (between-day).

The proposed method for animal tissues was validated in terms of recovery. For this purpose, various types of tissues (muscle, liver, kidney and fish) spiked with a 25 or 100 ng kg^{-1} concentration of a standard mixture of the analytes (before sample pretreatment) were analysed in triplicate ($n = 3$). The fact that most of the samples contained some residual pharmaceutical allowed recoveries to be calculated by subtracting the previously quantified endogenous compounds from the total contents. The average recoveries thus obtained are listed in Table 3. As can be seen, all analytes were accurately identified; also, the average recoveries (92–101%) for all matrices were quite acceptable. Therefore, the precipitation/centrifugation step, or the cleanup step in the SPE system, efficiently reduced or even completely suppressed matrix interferences.

3.3. Analysis of edible animal tissues

The proposed method was successfully applied to the determination of 20 pharmaceuticals in tissues from agricultural farm and fish hatchery animals (viz. lamb, veal, pig and chicken muscle, liver and kidney; and salmon, sea bass and sole flesh). Samples were analysed in triplicate, following the analytical procedure described in Section 2. If the concentration of any analyte fell outside the linear range (Table 2), then the SPE extract from the sample concerned was diluted with eluent (500 μg L^{-1} triphenylphosphate in ethyl acetate) after derivatization of the analytes with BSTFA + 1% TMCS. The results of these tests are shown in Table 4. As can be seen, the hormones estrone and 17β-estradiol were present in all samples except chicken muscle, at concentrations from 0.51 to 6.7 μg kg^{-1}. These concentrations are consistent with others previously found in muscle, kidney and liver tissues [29]. As regards non-steroidal anti-inflammatories, flunixin was detected at concentrations from 1.5 to 3.5 μg kg^{-1} in veal muscle and pig kidney, and phenylbutazone was detected at concentration from 1.2 to 7.8 μg kg^{-1} in lamb and veal muscle and liver chicken. The antibacterials florfenicol and pyrimethamine were found in all samples except chicken muscle; their concentrations were relatively high (0.59–28.2 μg kg^{-1}), but always below their MRLs (from 100 μg kg^{-1} for poultry muscle to 3000 μg kg^{-1} for bovine, ovine and caprine liver) [4]. Other authors detected florfenicol a 260 μg kg^{-1} levels, and pyrimethamine at lower concentrations (0.6–14.0 μg kg^{-1}), in porcine muscle [20]. No other pharmaceuticals were detected. By way of example, Fig. 4 shows the SIM mode chromatograms for samples of veal muscle, chicken liver and sole flesh processed with the proposed SPE–GC–MS method. As can be seen, the chromatogram contained few significant peaks due to the sample matrix, which facilitated identification of the analytes and testifies to the efficiency of the cleanup treatment.

4. Conclusions

A method for the simultaneous determination of 20 pharmaceuticals (antibacterials, non-steroidal anti-inflammatories, antiseptics, anti-epileptics, lipid regulators, β-blockers and hormones) in agricultural farm and fish hatchery animals was developed. Precipitation/centrifugation/filtration of on a mixture of animal tissue with acetonitrile–water and subsequent continuous solid-phase extraction provide a reliable procedure for removing co-extracting interferences (proteins and lipids, mainly) from complex matrices and facilitate preconcentration of the pharmaceuticals. The method is quite sensitive, accurate and precise. Thus, its LODs (0.4–2.7 ng kg^{-1}) are better than those for other methods such as those for the determination of ten hormones in meat (0.1–0.4 μg kg^{-1}) [11], flunixin in bovine tissues (0.1–0.2 μg kg^{-1}) [16], and four antibacterials in farmed aquatic species (0.1–1.0 μg kg^{-1}) [6] or chicken muscle (0.1–1.0 μg kg^{-1}) [22]. Pharmaceutical recoveries from various samples of edible animal tissues ranged from 92 to 101%. By contrast, previously reported recoveries for some pharmaceuticals varied over wider ranges (e.g. 77.1–98.3% for flunixin in edible bovine tissues [16], and 80.9–105.5% for antibacterials in poultry and porcine muscle [20] or 72.4–107.4% in meat, kidney and liver tissues [23]). The proposed method is therefore applicable to a variety of animal tissues. Nearly all samples were found to contain the hormones estrone and 17β-estradiol, and the antibacterials florfenicol and pyrimethamine, all at concentrations below their maximum allowed levels.

Acknowledgements

This work was funded by Grant D/023185/09 from Agencia Española de Cooperación Internacional y Desarrollo (AECID). The authors would like to thank the DGI of the Spanish Ministry of Education for supporting this research in the framework of Project CTQ2010–17008. They are also indebted to the staff at the Technical Research Service of the University of Jaén for access to its gas chromatograph–mass spectrometer system.

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