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Simultaneous Determination of 20 Pharmacologically Active Substances in Cow's Milk, Goat's Milk, and Human Breast Milk by Gas Chromatography−Mass Spectrometry

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ABSTRACT: This paper reports a systematic approach to the development of a method that combines continuous solid-phase extraction and gas chromatography−mass spectrometry for the simultaneous determination of 20 pharmacologically active substances including antibacterials (chloramphenicol, florfenicol, pyrimethamine, thiamphenicol), nonsteroideal anti-inflammatories (diclofenac, flunixin, ibuprofen, ketoprofen, naproxen, mefenamic acid, niflumic acid, phenylbutazone), antiseptic (triclosan), antiepileptic (carbamazepine), lipid regulator (clofibric acid), β-blockers (metoprolol, propranolol), and hormones (17α-ethinylestradiol, estrone, 17β-estradiol) in milk samples. The sample preparation procedure involves deproteination of the milk, followed by sample enrichment and cleanup by continuous solid-phase extraction. The proposed method provides a linear response over the range of 0.6−5000 ng/kg and features limits of detection from 0.2 to 1.2 ng/kg depending on the particular analyte. The method was successfully applied to the determination of pharmacologically active substance residues in food samples including whole, raw, half-skim, skim, and powdered milk from different sources (cow, goat, and human breast).

KEYWORDS: pharmacologically active substances, milk, continuous solid-phase extraction, gas chromatography−mass spectrometry

■ INTRODUCTION

Antibacterials, nonsteroidal anti-inflammatory drugs, β-blockers, lipid regulators, hormones, antiepileptics, and antiseptics are the most widely used medical and veterinary drugs at present. As a result, it is very common for milk to contain residues of pharmacologically active substances (PAS), which have undesirable effects on the quality and technological properties of dairy products and also, more importantly, on human health. In fact, contaminated milk can cause allergic reactions or indirect problems through bacterial resistance to clinical treatments. Human milk is also frequently contaminated with PAS as the result of many women using some personal care products or requiring medication while breast feeding. Accumulation of some drugs in milk can pose a risk to infants exceeding the benefits of breast feeding.[1] To protect consumer health and ensure a high product quality, the Codex Alimentarius Commission of the Food and Agriculture Organization (FAO), the World Health Organization (WHO),[2] and the European Community[3] have set maximum residue limits (MRLs) for some drugs in milk, primarily cow's milk and, occasionally, goat's milk. Carefully controlling the presence of PAS residues is especially important in milk and dairy products owing to their ubiquitous presence in human nutrition.

Detecting trace levels of PAS in such a complex matrix as milk requires sample preparation and cleanup. The sample preparation procedures usually employed for this purpose include homogenization, extraction/cleanup, enrichment and, when needed, derivatization of the analytes. Protein precipitation is also required to prevent emulsification during extraction. Sample preparation procedures such as liquid−liquid extraction,[4−6] solid-phase microextraction,[7,8] and solid-phase extraction (SPE) are common choices for simultaneous extraction and cleanup. The last has gained increasing popularity over liquid−liquid extraction by virtue of its ease of operation

and environmental friendliness. Sorbents including C_{18}, C_{30}, SCX, and ion-exchange resins have been used in the analysis of PAS residues in various matrices such as milk,[9−14] animal tissues, plasma, and urine.[14−17] Other polymeric sorbents such as Evolute ABN,[18] Amberlite XAD-2,[19] and Strata-X[20] have been used for antibiotics and nonsteroidal anti-inflammatory drugs in cow's milk, other food samples, and urine. Recently, polymeric SPE adsorbents with a dual nature (e.g., hydrophilic−lipophilic balance in Oasis-HLB) have enabled simultaneous sample cleanup and analyte enrichment in biological, environmental, and food matrices.[21] Oasis-HLB SPE cartridges have been successfully used to determine chloramphenicol, thiamphenicol, and florfenicol in poultry and porcine muscle and liver and in seafood[22,23] as well as antibacterial, nonsteroidal anti-inflammatories, antiepileptics, β-blockers, and hormones in milk, plasma, urine, hair, and water samples.[21,24−27]

A variety of analytical techniques have been used to determine PAS residues in foods. The most common choices for this purpose are gas chromatography (GC) and liquid chromatography.[28] Thus, liquid chromatography−mass spectrometry[5,8−11,13−16,18,22,26,27] and high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry[20,29] have been used to determine various types of PAS residues in some types of food. The prevalent analytical methods for determining these residues in milk, muscle tissue, urine, and water, based on gas chromatography−mass spectrometry (GC-MS), provide high sensitivity, specificity, and good chromatographic resolution.[12,14,17,19,21,23−25,30] However, the low volatility of some

Table 1. Analytical Figures of Merit of the Proposed Solid-Phase Extraction Method for the Determination of Pharmacologically Active Substances in Milk

compound	retention time (min)	linear range (ng/kg)	r^a	detection limit (ng/kg)	sensitivity [signal/(ng/kg)]	precision (RSD, %)[b] within-day	between-day	$M^{+\bullet}$ (m/z)	$[M-15]^+$ (m/z)	additional ions[c] (m/z)
clofibric acid	11.3	1.8−5000	0.995	0.6	1.075	5.1	5.5	286	271	128, **143**
ibuprofen	11.9	0.6−5000	0.994	0.2	3.185	4.9	5.3	278	263	**160**, 234
niflumic acid	18.8	0.6−5000	0.997	0.2	3.190	4.6	4.8	354	339	**236**, 263
metoprolol	19.1	1.9−5000	0.996	0.6	1.095	6.2	6.6	339	324	72, **223**
naproxen	19.3	1.2−5000	0.994	0.4	1.590	6.7	7.0	302	287	**185**, 243
flunixin	19.8	0.6−5000	0.995	0.2	3.065	4.5	5.7	368	353	251, **263**
triclosan	19.9	1.9−5000	0.998	0.6	1.030	6.7	7.4	362	347[c]	200, 310
propranolol	20.6	1.8−5000	0.999	0.6	1.075	6.1	6.5	331	316	72, **215**
mefenamic acid	20.9	0.7−5000	0.996	0.2	3.220	5.8	6.5	313	298	208, **223**
ketoprofen	21.0	1.2−5000	0.997	0.4	1.610	7.1	7.5	326	311	73, **282**
pyrimethamine	21.3	3.1−5000	0.997	1.0	0.615	6.9	7.0	392	377[c]	171, 281
carbamazepine	22.0	0.6−5000	0.998	0.2	3.230	5.8	6.4	308	293	**193**, 250
diclofenac	22.3	0.7−5000	0.994	0.2	3.180	5.9	6.2	367	352	**214**, 242
phenylbutazone	24.0	3.2−5000	0.996	1.0	0.610	7.6	7.8	380	365	**183**, 252, 308
chloramphenicol	24.5	0.6−5000	0.995	0.2	3.245	5.1	5.5	466	451	208, **225**, 242
florfenicol	26.2	0.6−5000	0.999	0.2	3.195	4.5	5.0	429	414	**257**, 360
estrone	26.9	3.2−5000	0.998	1.0	0.625	7.0	7.5	342[c]	327	218, 257
17β-estradiol	27.3	4.0−5000	0.997	1.2	0.515	5.3	5.7	416[c]	401	285, 326
thiamphenicol	27.9	0.6−5000	0.997	0.2	3.185	7.2	7.6	499	484	242, 257, **330**
17α-ethinylestradiol	28.7	3.4−5000	0.995	1.1	0.570	5.9	6.4	440	425[c]	232, 300

[a] r, correlation coefficient. [b] Relative standard deviation ($n = 11$) for 10 ng/kg. [c] The peaks used for quantitation are boldfaced. m/z for IS (triphenylphosphate): 77, 170, 325, **326**.

PAS and the presence of various polar groups in others require the use of an appropriate derivatization procedure to obtain more volatile products.[21]

Continuous flow systems are useful tools for the simplification and automation of analytical processes with a view to enabling the implementation of reliable separation techniques with increased sensitivity and selectivity. Thus, continuous solid-phase extraction has been successfully applied to the determination of pharmaceuticals in water,[21] amines in water and beer samples,[31,32] and organophosphorus pesticides in water.[33] The advantages of continuous solid-phase extraction over traditional SPE include (i) reduced sample, sorbent, and eluent consumption; (ii) increased precision; (iii) improved preconcentration by effect of the analytes being efficiently eluted with small volumes (100−300 μL) of organic solvent; and (iv) a high throughput derived from the ability to operate in the array mode.

The aim of this work was to develop a reliable multiresidue method for the simultaneous quantitative determination of antibacterials, nonsteroid anti-inflammatory drugs, hormones, and other PAS in milk by using a continuous SPE system to preconcentrate PAS residues and gas chromatography−mass spectrometry for their determination. The proposed method improves on an earlier one developed by our group to determine pharmaceuticals and hormones in water samples;[21] the new method affords application to a more complex matrix such as milk and spans a much broader range of analytes potentially present in animal and human samples. Milk proteins, which can interfere with the determination of PAS, are precipitated by the addition of acetonitrile and centrifugation prior to SPE. Also, PAS are converted into their silyl derivatives with a mixture of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1% trimethylchlorosilane (TMCS) to improve volatility and thus sensitivity in the subsequent GC-MS analysis.

■ MATERIALS AND METHODS

Gas Chromatography−Mass Spectrometry. GC-MS analyses were performed on a Focus GC instrument (Thermo Electron SA, Madrid, Spain) interfaced to a DSQ II mass spectrometer (single quadrupole) controlled via a computer running XCalibur software. The GC instrument was equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm) coated with 5% phenylmethyl-polysiloxane (Supelco, Madrid, Spain). The carrier gas was helium (purity = 6.0) and circulated at a flow rate of 1 mL/min. The oven temperature was held at 70 °C for 1 min following injection and then raised from 70 to 150 °C at 14 °C/min. After the first transition, the temperature was raised from 150 to 290 °C at 6 °C/min. The injection port and transfer line temperatures were maintained at 270 and 280 °C, respectively. A 10 μL syringe was washed three times with methanol before and after each injection and rinsed with 8 μL of sample solution before 1 μL of sample was injected in the split mode (1:20).

The mass spectrometer was used under the following conditions: ion source temperature, 200 °C; transfer line temperature, 280 °C; electron impact ionization mode at 70 eV. The time for solvent delay was set at 4 min. Samples were analyzed in the selected ion monitoring (SIM) mode. Quantitation was based on the peak areas of the analytes relative to the internal standard (IS). Scans spanned the m/z range from 60 to 500. Specific ions were selected for each compound, the most abundant being used as quantitation ion and three others as qualifier ions (Table 1). As prescribed by Commission Decision 2002/657/EC33, which was adhered to in its entirety, the two most abundant fragments were used as diagnostic ions.[34] The Commission criteria include the specification that the signal-to-noise ratio for each diagnostic ion should be ≥3. The relative retention times for the analytes differed by <0.5% from that for the calibration standard. The relative intensities of the detected ions, expressed as a percentage of the intensity of the most abundant ion, corresponded to those of samples fortified at comparable concentrations within the tolerances specified by the Commission Decision.[34]

Chemicals and Reagents. All reagents used were of analytical grade or better. The pharmacologically active substances used were

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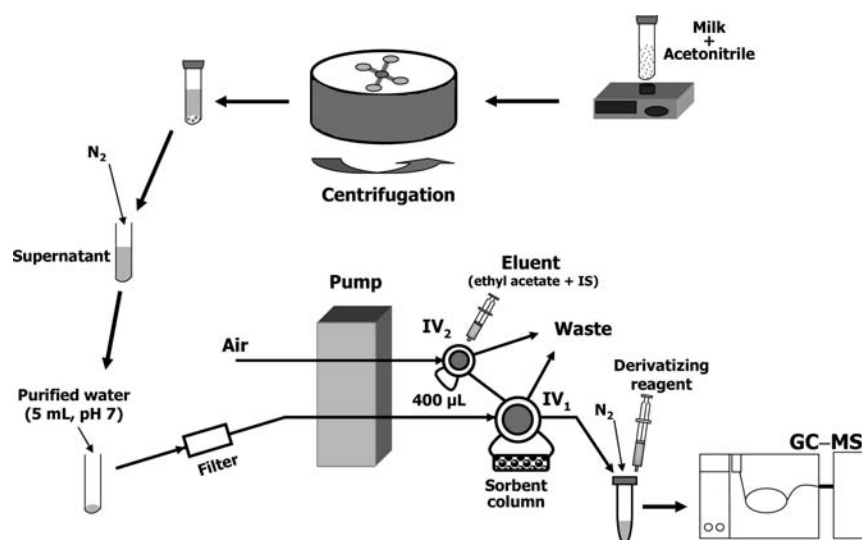dx.doi.org/10.1021/jf200364w |*J. Agric. Food Chem.* 2011, 59, 5125–5132

Figure 1. Experimental setup for the removal of protein from milk and the continuous preconcentration of pharmacologically active substances in milk samples. Abbreviations: IV, injection valve; IS, internal standard; GC-MS, gas chromatograph—mass spectrometer.

purchased from Sigma-Aldrich (Madrid, Spain). Triphenylphosphate (IS) and the derivatization agents (BSTFA and TMCS) were purchased from Fluka (Madrid, Spain). All solvents (methanol, ethyl acetate, acetonitrile, acetone, ethanol, n-hexane, dichloromethane, and 2-propanol) were supplied by Merck (Madrid, Spain). Oasis-HLB in particle size 50−65 μm was obtained from Waters (Madrid, Spain). Millex-LG filter units (hydrophilic, PTFE, pore size = 0.20 μm, diameter = 25 mm, filtration area = 3.9 cm^2) were obtained from Millipore Ibérica. Water was purified by passage through a Milli-Q system (Millipore Ibérica, Madrid, Spain).

Standards. Stock standard solutions of the individual antibacterials, nonsteroid anti-inflammatories, hormones, and other PAS at a 1 mg/mL concentration each were prepared in methanol and stored at 4 °C in the dark. Working-standard solutions were prepared on a daily basis by dilution of the individual stock standard solutions in purified water and adjustment to pH 7 with dilute NaOH as required. Freshly made solutions of ethyl acetate containing a 500 μg/L concentration of triphenylphosphate and prepared on a daily basis were used as eluents for continuous SPE.

Milk Samples. Cow's and goat's milk samples (whole, half-skim, skim, and powdered) were purchased at local markets in Spain or Morocco. Raw cow's milk samples were obtained from various farms using standard commercial breeding protocols. Human milk from lactating women who volunteered for the study with permission of the bioethics committee was also used. All samples were frozen at −20 °C in the dark until analysis. The powdered infant milk sample studied (half-skim cow's milk) was purchased a local chemist's shop and stored in a dry, cool place.

Sample Pretreatment. About 5 g of milk sample was weighed into a 50 mL round polypropylene centrifuge tube and mixed with 5 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 3500 rpm and 4 °C for 10 min. Then, the supernatant, which had previously been separated from the precipitate material, was evaporated under a stream of ultrahigh-purity N$_2$ to a final volume of approximately 100 μL and diluted to 5 mL with purified water at pH 7 adjusted with dilute NaOH as required. For powdered milk, an amount of 1 g was dissolved in 5 mL of purified water as per the manufacturer's instructions and the solution processed like all other samples. The pretreated sample was thus made ready for continuous solid-phase extraction.

The continuous solid-phase extraction manifold used was assembled from a Gilson Minipuls-3 peristaltic pump (Villiers-le-Bel, France) fitted with poly(vinyl chloride) pumping tubes, two Rheodyne 5041 injection valves (Cotati, CA), and a laboratory-made PTFE sorption column containing 60 mg of Oasis-HLB sorbent (5 cm × 3 mm i.d.). The sorbent column was conditioned with 1 mL of ethyl acetate and 1 mL of purified water. Under these conditions, the column remained effective for at least 1−2 months with no change in its properties. A laboratory-made polytetrafluoroethylene (PTFE) filter furnished with a paper disk (4 cm^2 filtration area) was also employed.

The continuous solid-phase extraction unit employed for the preconcentration of pharmaceutically actives substances is depicted in Figure 1. In the preconcentration step, 5 mL of pretreated sample was filtered to prevent suspended particles from reaching the continuous unit and passed at 4 mL/min through the sorbent column, located in the loop of injection valve IV$_1$. Retention of PAS residues was instantaneous, and the sample matrix was immediately sent to waste. In the drying step, IV$_1$ was switched to the injected position and the sorbent column dried for 2 min with an air stream at 4 mL/min inserted via the carrier line of the second valve (IV$_2$); simultaneously, the loop of IV$_2$ was filled with eluent (ethyl acetate containing 500 μg/L triphenylphosphate as IS) by means of a syringe. In the elution step, IV$_2$ was switched to have the loop contents (400 μL of eluent) injected into the same air stream used in the drying step to elute PAS residues 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 analyzed by GC-MS in the SIM mode.

■ RESULTS AND DISCUSSION

In previous work, we developed a continuous system consisting of a solid-phase extraction module for the preconcentration of pharmaceuticals and hormones from environmental water samples and the determination of derivatized (silylated) analytes by GC-MS.[21] We studied various SPE sorbents including Oasis-HLB, LiChrolut EN, XAD-2, XAD-4, RP-C18, Florisil, silica gel, and Isolute NH$_2$ and found Oasis-HLB to exhibit the highest

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Table 2. Variables Influencing the Milk Sample Pretreatment, Sorption/Elution Process, and Derivatization of Pharmacologically Active Substances

variable	studied range	optimum range (selected value)
Protein Precipitation and Centrifugation[a]		
volume of acetonitrile (mL)	1–10	4–10 (5)
centrifugation rate (rpm)	1500–5000	2500–5000 (3500)
centrifugation time (min)	1–30	5–30 (10)
centrifugation temperature (°C)	0–30	0–10 (4)
Continuous Solid-Phase Extraction		
sample pH	1–12	6.5–7.5 (7)
amount of Oasis-HLB (mg)	20–100	55–65 (60)
volume of ethyl acetate (μL)	50–500	350–450 (400)
flow rate of sample (mL/min)	1–5	3.5–4.5 (4)
breakthrough volume (mL)	1–500	1–200 (50)
flow rate of air (mL/min)	1–5	3.5–4.5 (4)
Derivatization (Silylation)		
percentage of TMCS (catalyst) in BSTFA[b]	0.25–15	0.75–15 (1)
reaction time	1–30	15–25 (20)
temperature	20–90	65–75 (70)

[a] To 5 g of sample. [b] BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane.

efficiency in retaining the analytes (94–100%). A study of the influence of pH on the SPE efficiency revealed that the best extraction results for all analytes were obtained in the neutral pH region (6.5–7.5), so pH 7 was adopted. We also examined the effects of other factors influencing analyte derivatization, retention, and elution. The results are shown in Table 2. In this work, we expanded the number of analytes. This entailed checking that the operational conditions chosen for the previous determination[21] fell within the optimum ranges for the new analytes as well. To this end, we used the above-described system to preconcentrate the 20 analytes from milk samples. The new analytes were found to respond similarly to the previous ones to the optimum operating conditions. The optimum amount of Oasis-HLB sorbent to be used was optimized by using columns containing between 20 and 100 mg of sorbent. To this end, a calibration test was conducted for each analyte and column by passing 5 mL of aqueous standard solutions containing a 0.1–8 μg/L concentration of each analyte and then eluting the column with 400 μL of ethyl acetate. The resulting analytical signals increased with increasing amount of sorbent up to 60 mg and then decreased above 65 mg of Oasis-HLB owing to the need for a higher volume of eluent to ensure the complete elution. Quantitative retention and the absence of carry-over were confirmed by subjecting an aqueous solution containing a 2000 ng/L concentration of each pharmacologically active substance to SPE as described under Materials and Methods. This elution was followed by a second one (IV$_2$ in Figure 1) with the same eluent (400 μL of ethyl acetate). The absence of carry-over was confirmed by the presence of none of the analytes in the second eluate, which indicates that the eluent volume used ensured complete elution. However, because the milk samples contained potentially interfering proteins, we examined the effects of the matrix in the pretreated samples to be passed through the SPE system. Below is described the influence of the variables affecting protein removal from the milk by precipitation and centrifugation.

Optimization of Variables Affecting Sample Pretreatment. One immediate requirement for the target multicompound analysis was to develop a generic sample preparation
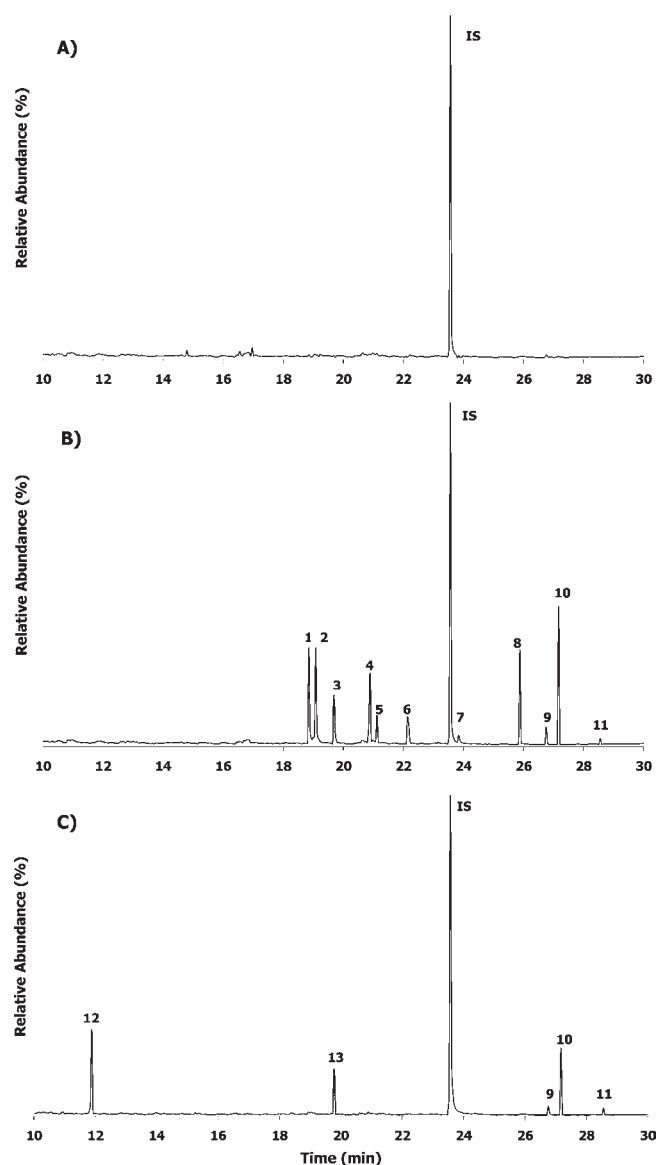

Figure 2. GC-MS chromatograms in the SIM mode for whole cow's milk samples 1 (A) and 3 (B) and human breast milk sample 2 (C) (see Tables 4 and 5). Peaks: (1) niflumic acid; (2) naproxen; (3) flunixin; (4) ketoprofen; (5) pyrimethamine; (6) diclofenac; (7) phenylbutazone; (8) florfenicol; (9) estrone; (10) 17β-estradiol; (11) 17α-ethinylestradiol; (12) ibuprofen; (13) triclosan; (IS) internal standard.

method suitable for extracting pharmaceuticals from various types of matrices of animal and human origin. Milk is a complex biological fluid containing a number of macromolecules such as proteins and lipids, the presence of which can damage an SPE column and suppress ionization in the mass spectrometer. As a result, sample preparation procedures for the determination of pharmaceutical residues in milk often involve protein precipitation with an appropriate solvent, centrifugation of the mixture, and filtration of the resulting supernatant.[7–9,11–14,18,20] In this work, we studied the efficiency of various solvents (acetonitrile, methanol, ethanol, acetone) and trichloroacetic acid in precipitating milk proteins. For this purpose, an amount of ca. 5 g of milk was mixed with a volume of 5 mL of each organic solvent or a aqueous solution of 30% m/v trichloroacetic acid and centrifuged

Table 3. Percent Recovery (± SD, $n = 3$) of Pharmacologically Active Substances Added to Milk Samples

	cow								goat (ng/kg)		powdered (ng/kg)		human (ng/kg)	
	whole (ng/kg)		half-skim (ng/kg)		skim (ng/kg)		raw (ng/kg)							
compound	10	50	10	50	10	50	10	50	10	50	10	50	10	50
clofibric acid	104 (6)	103 (5)	104 (5)	99 (5)	101 (6)	91 (4)	98 (4)	91 (4)	101 (5)	99 (4)	98 (5)	104 (6)	100 (5)	104 (5)
ibuprofen	103 (5)	96 (4)	101 (5)	104 (6)	98 (4)	94 (4)	103 (5)	96 (5)	103 (4)	95 (4)	104 (5)	98 (5)	103 (5)	98 (5)
niflumic acid	99 (5)	100 (4)	92 (4)	95 (4)	99 (5)	98 (5)	104 (5)	99 (4)	102 (5)	101 (5)	98 (4)	103 (5)	98 (5)	104 (5)
metoprolol	101 (7)	99 (6)	97 (7)	101 (6)	104 (6)	103 (5)	95 (5)	101 (6)	91 (5)	104 (7)	103 (6)	100 (6)	93 (5)	100 (6)
naproxen	98 (6)	102 (7)	104 (7)	98 (6)	101 (7)	99 (6)	97 (6)	103 (6)	92 (6)	93 (6)	96 (7)	104 (7)	101 (6)	103 (6)
flunixin	102 (5)	95 (5)	99 (5)	104 (6)	98 (5)	104 (5)	101 (5)	104 (5)	96 (4)	94 (4)	97 (4)	103 (6)	96 (5)	104 (5)
triclosan	96 (6)	103 (7)	102 (6)	99 (7)	95 (5)	104 (6)	100 (7)	92 (6)	102 (6)	92 (6)	98 (7)	97 (5)	98 (6)	101 (7)
propranolol	102 (6)	101 (6)	95 (6)	98 (6)	102 (5)	101 (7)	99 (6)	102 (6)	99 (5)	103 (7)	102 (6)	92 (5)	103 (5)	92 (5)
mefenamic acid	104 (6)	95 (5)	104 (6)	103 (6)	97 (4)	92 (4)	102 (6)	99 (6)	97 (5)	94 (5)	103 (5)	101 (6)	103 (6)	99 (6)
ketoprofen	104 (7)	97 (7)	102 (6)	94 (6)	93 (6)	102 (7)	98 (7)	101 (7)	101 (7)	93 (6)	102 (7)	99 (7)	92 (6)	103 (7)
pyrimethamine	96 (6)	98 (6)	101 (7)	102 (7)	99 (7)	95 (6)	103 (7)	96 (7)	100 (6)	92 (6)	102 (7)	98 (6)	104 (7)	98 (7)
carbamazepine	94 (5)	104 (6)	104 (6)	101 (6)	98 (5)	101 (6)	98 (6)	91 (5)	94 (5)	102 (5)	93 (6)	103 (6)	93 (5)	101 (6)
diclofenac	104 (6)	100 (6)	93 (5)	92 (5)	101 (6)	102 (6)	101 (6)	104 (6)	92 (5)	99 (6)	97 (6)	96 (6)	101 (7)	96 (6)
phenylbutazone	97 (7)	99 (7)	101 (6)	104 (8)	103 (8)	97 (7)	100 (6)	92 (6)	91 (6)	103 (7)	104 (8)	100 (7)	101 (7)	100 (7)
chloramphenicol	94 (5)	103 (6)	99 (5)	100 (5)	92 (4)	103 (5)	103 (5)	101 (6)	99 (5)	103 (6)	91 (4)	99 (5)	93 (4)	101 (5)
florfenicol	103 (5)	101 (5)	94 (4)	92 (4)	101 (4)	95 (4)	102 (5)	94 (4)	102 (5)	92 (4)	98 (4)	94 (5)	98 (4)	104 (5)
estrone	91 (6)	94 (6)	101 (7)	102 (7)	100 (7)	93 (6)	99 (7)	97 (7)	92 (6)	104 (8)	98 (6)	102 (7)	98 (6)	102 (7)
17β-estradiol	103 (6)	104 (5)	96 (5)	100 (6)	94 (5)	104 (5)	96 (5)	104 (6)	93 (4)	100 (5)	101 (6)	93 (5)	103 (6)	93 (5)
thiamphenicol	98 (7)	101 (7)	92 (7)	94 (7)	103 (7)	95 (6)	95 (7)	103 (7)	102 (7)	97 (7)	93 (7)	101 (7)	99 (7)	100 (7)
17α-ethinylestradiol	91 (5)	94 (6)	104 (6)	97 (6)	101 (6)	93 (5)	104 (6)	100 (6)	104 (7)	99 (6)	95 (6)	104 (6)	102 (6)	98 (6)

at 3500 rpm and 4 °C for 10 min. Acetonitrile, which caused >95% of the protein content to precipitate, proved to be the most efficient solvent; also, it facilitated separation of the precipitate by filtration, which is consistent with previous results.[11−13,18] The optimum volume of acetonitrile for addition to 5 g of milk was determined by changing it over the range of 1−10 mL; protein precipitation was found to peak at 4.5 mL of acetonitrile, so a solvent volume of 5 mL was adopted as optimal. We also examined the effects of centrifugation-related variables (rate, temperature, and time) over the respective ranges shown in Table 2. Centrifugation at 3500 rpm and 4 °C for 10 min resulted in optimal separation of precipitated milk proteins from the target species. Also, most of the fat initially present in the milk samples was removed by the effect of the low centrifugation temperature used in the protein precipitation step (4 °C).[35]

Once the precipitation and centrifugation conditions were optimized, the filtered supernatant matrix was checked to be compatible with the continuous SPE system. To this end, we examined the effect of the presence of acetonitrile on retention of the 20 studied PAS by the SPE system. This involved preparing aqueous solutions (50 mL) containing a 10 ng/L concentration of each PAS at pH 7 with dilute NaOH and a variable proportion of acetonitrile from 0 to 50%. The analytes present in the extract after elution were derivatized as described under Analytical Method and determined by GC-MS. The results were compared with those obtained in the absence of acetonitrile. The solvent had no effect on the retention of PAS in proportions up to 15%. This can be ascribed to the peculiar sorption mechanism, which involves the partitioning of moderately polar organic compounds from a polar phase (water) into a polymeric sorbent (Oasis-HLB) via a polar interaction such as hydrogen bonding between the hydroxyl group in the PAS and the underlying sorbent surface. When the aqueous sample contains a high concentration of acetonitrile, the solvent breaks the bonds and effectively solubilizes PAS, thereby dramatically reducing sorption of analytes. Therefore, the supernatant was evaporated under a stream of ultrahigh-purity N_2 to a final volume of 100 μL and diluted with 5 mL of purified water (pH 7) and introduced into a continuous SPE system.

Method Validation. All PAS studied exhibited a good gas chromatographic behavior. Control samples from milk previously checked to contain no residual PAS were prepared as described above. One control sample per calibration standard level was used. Calibration curves for PAS standards were obtained by processing an amount of 5 g of uncontaminated cow's milk sample (whole 1 in Table 4; see Figure 2A) that was aliquoted into 50 mL polypropylene tubes containing variable concentrations of the analytes over the range of 0.6−5000 ng/kg. After spiking, the samples were vortexed and allowed to stand for 15 min before pretreatment and SPE as described under Materials and Methods. Curves were constructed by plotting analyte-to-IS peak area ratios against analyte concentrations. The linear range, limit of detection (LOD), and precision for the determination of the 20 PAS studied by GC-MS in the SIM mode, along with the m/z values used for GC-MS confirmation, are shown in Table 1. Correlation coefficients were >0.994 in all instances (12 points per calibration). Limits of detection were calculated as 3 times the standard deviation (SD) of background noise divided by the slope of each calibration graph. The precision of the proposed method, as relative standard deviation (RSD), was calculated by measuring 11 uncontaminated milk samples spiked with a 10, 200, or 2000 ng/kg concentration of each PAS. A comparative study of within-day and between-day precision was conducted—the latter over 7 days—at three analyte concentration levels (10, 200, or 2000 ng/kg); the former parameter was found to range from 3.5 to 7.2% and the latter from 4.3 to 7.8%.

Because no certified reference material for milk containing the studied analytes was available, the proposed method was validated by analyzing various types of milk (raw, whole, half-skim, skim, and powdered) from different sources (cow, goat, and human breast) that were spiked with a 10, 50, or 2000 ng/kg concentration of a standard mixture of the analytes before pretreatment. Each sample was analyzed in triplicate ($n = 3$) to calculate a standard deviation. Some samples contained only a few PAS, which allowed recoveries to be calculated by

Table 4. Pharmacologically Active Substances Detected in the Bovine Milk Samples (± SD, n = 3)

milk sample[a]	pharmacologically active substance	concentration found (μg/kg)
raw 1	ketoprofen	0.15 ± 0.01
	estrone	0.075 ± 0.005
	17β-estradiol	3.1 ± 0.2
raw 2	pyrimethamine	0.15 ± 0.01
	phenylbutazone	0.020 ± 0.001
	estrone	0.080 ± 0.006
	17β-estradiol	3.2 ± 0.2
raw 3	phenylbutazone	0.13 ± 0.01
	estrone	0.025 ± 0.001
	17β-estradiol	0.085 ± 0.005
whole 1	none	
whole 2	niflumic acid	0.87 ± 0.04
	mefenamic acid	0.14 ± 0.01
	ketoprofen	0.11 ± 0.01
	diclofenac	0.070 ± 0.004
	phenylbutazone	0.075 ± 0.005
	florfenicol	1.2 ± 0.1
	estrone	0.035 ± 0.002
	17β-estradiol	1.1 ± 0.1
	17α-ethinylestradiol	0.020 ± 0.001
whole 3	niflumic acid	0.18 ± 0.01
	naproxen	0.35 ± 0.02
	flunixin	0.080 ± 0.004
	ketoprofen	1.1 ± 0.1
	pyrimethamine	0.21 ± 0.01
	diclofenac	0.045 ± 0.003
	phenylbutazone	0.060 ± 0.004
	florfenicol	0.10 ± 0.01
	estrone	0.13 ± 0.01
	17β-estradiol	1.2 ± 0.1
	17α-ethinylestradiol	0.035 ± 0.002
whole 4[b]	none	
whole 5[b]	17β-estradiol	5.4 ± 0.3
	17α-ethinylestradiol	0.11 ± 0.01
whole 6[b]	estrone	3.5 ± 0.2
semiskim 1	none	
semiskim 2	niflumic acid	0.075 ± 0.003
	triclosan	0.35 ± 0.02
	phenylbutazone	0.21 ± 0.01
	estrone	1.2 ± 0.1
	17β-estradiol	2.3 ± 0.1
skim 1	niflumic acid	0.015 ± 0.001
	naproxen	0.14 ± 0.01
	flunixin	0.065 ± 0.003
	ketoprofen	0.15 ± 0.01
	pyrimethamine	0.070 ± 0.005
	diclofenac	0.025 ± 0.001
	phenylbutazone	0.065 ± 0.005
	estrone	0.045 ± 0.003
	17β-estradiol	1.1 ± 0.1

Table 4. Continued

milk sample[a]	pharmacologically active substance	concentration found (μg/kg)
skim 2	niflumic acid	0.085 ± 0.004
	naproxen	0.35 ± 0.02
	ketoprofen	0.060 ± 0.004
	pyrimethamine	0.17 ± 0.01
	diclofenac	0.090 ± 0.005
	florfenicol	0.025 ± 0.001
	estrone	0.060 ± 0.004
	17β-estradiol	1.2 ± 0.1
	17α-ethinylestradiol	0.025 ± 0.001

[a] From Spain. [b] From Morocco.

subtracting the previously quantified endogenous compounds from the total contents. As can be seen in Table 3, recoveries ranged from 91 to 104% for 10 and 50 ng/kg of analytes added. When a high concentration of analytes (2000 ng/kg) was spiked to milk samples, recoveries ranged from 95 to 102%, which testifies to the applicability of the proposed method to any type of milk sample and also that matrix interferences are reduced or completely suppressed by the sample pretreatment and cleanup step in the SPE module.

Analysis of Milk Samples. The proposed method was applied to the determination of 20 PAS in 8 types of milk (whole, semiskim, skim, and raw cow's milk; whole and half-skim goat's milk; human milk; and powdered milk) from Spain and Morocco. Samples were analyzed in triplicate by using the analytical procedure described under Materials and Methods. If the concentration of any analyte lay outside the linear range (Table 1), then the sample concerned was diluted with purified water to bring it within the range. Previously, the effect of diluting the milk samples with water prior to analysis was examined by using an amount of 500 g of uncontaminated (whole) cow's milk that was supplied with a 4500 ng/kg concentration of each PAS and split into 50 g portions, which were diluted with 25, 50, 75, or 100 mL of purified water for analysis with the proposed method. The analyte concentrations thus found were similar to those in the undiluted sample, taking into account the dilution of each sample.

Preliminary freeze—thaw stability tests were conducted with a view to assessing the stability of the analytes in milk at a storage temperature of −20 °C, which is similar to those used by other authors.[8,13,18,36] An amount of 1 kg of whole cow milk spiked with a 100 ng/kg concentration of each analyte was split into 50 g portions and frozen at −20 °C; by exception, one portion was analyzed in triplicate as described under Materials and Methods on the same day. All other portions were subjected to the same analytical procedure in triplicate every 2 days for 1 month following thawing 1 h before preparation. Freezing the samples under these conditions was found to suppress any adverse effect of the matrix on analyte stability; in fact, the results were always similar, within the error range for the method (RSD < 8%), to those for the unfrozen sample. Similar results were obtained with whole cow's milk spiked with a 2000 ng/kg concentration of each analyte.

The results for the three raw cow's milk samples (Table 4) indicate the presence of the two natural hormones studied (estrone and 17β-estradiol) in amounts from 0.02 and 3.2 μg/kg. These samples additionally contained other PAS (ketoprofen, pyrimethamine, and phenylbutazone) at levels below 0.15 μg/kg. Their contents in ketoprofen were lower than those reported by Daeseleire et al.[11] One analyte frequently found in raw cow's milk,[11,16] flunixin, was not detected in our samples. All whole milk samples except samples 1 (Spain) and 4 (Morocco) contained several PAS. Worthy of special note are samples 2 and 3, which contained up to 12 analytes

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Table 5. Pharmacologically Active Substances in Human, Goat's, and Powdered Milk Samples ($\pm$ SD, n = 3)

milk sample	pharmacologically active substance	concentration found (μg/kg)
human breast 1	triclosan	0.16 ± 0.01
	17α-ethinylestradiol	0.035 ± 0.002
human breast 2	ibuprofen	0.37 ± 0.02
	triclosan	0.25 ± 0.02
	estrone	0.055 ± 0.004
	17β-estradiol	0.54 ± 0.03
	17α-ethinylestradiol	0.045 ± 0.003
human breast 3	naproxen	1.9 ± 0.1
	estrone	0.17 ± 0.01
	17β-estradiol	0.49 ± 0.03
goat (whole)	none	
goat (half-skim)	niflumic acid	0.085 ± 0.005
	flunixin	0.095 ± 0.006
powdered 1−2	none	

including nonsteroidal anti-inflammatories (niflumic, naproxen, flunixin, mefanamic acid, ketoprofen, diclofenac, and phenylbutazone), hormones (estrone, 17β-estradiol, and 17α-ethinylestradiol), and antibacterials (pyrimethamine and florfenicol). Figure 2B shows the chromatogram for sample 3, which contained 11 PAS. On the other hand, the whole milk samples from Morocco contained only hormones among the studied PAS. The half-skim and skim milk samples contained much lower levels of PAS than the whole milk samples; by exception, hormones were detected at similar levels in the three types of milk, which is consistent with the previous results of Courant et al.[37] Only diclofenac among the analytes present in the cow's milk samples has a maximum residue limit (MRL) for milk;[3] in any case, its content was always below such an MRL (0.1 μg/kg). Some samples contained florfenicol, use of which on animals producing milk for human consumption is banned by EU regulations.[3] One other legally restricted compound is thiamphenicol (MRL = 50 μg/kg), which, however, was found in none of the samples.

The goat's milk samples (whole and semiskim) were found to contain niflumic acid and flunixin at concentrations of 0.085 and 0.095 μg/kg, respectively (Table 5). On the other hand, neither sample of powdered milk for infant feeding contained any of the PAS studied.

As stated above, the samples included human milk from lactating women who volunteered for the study. As can be seen from Table 5, the antiseptic triclosan, which is widely used in a variety of personal care products, was detected in two samples; its concentrations (0.16 and 0.25 μg/kg) are within the range reported by other authors for this type of milk (<0.018−0.95 μg/kg).[36] Ibuprofen and naproxen were also detected in two samples (2 and 3); these analytes are among the most commonly used medical anti-inflammatories. Naturally occurring hormones, dietary, and synthetic hormones were found in the three human milk samples. The 17β-estradiol concentration in them was lower than that reported by Choi et al. (7.9−18.5 μg/kg) for human milk.[24] By way of example, Figure 2C shows the chromatogram obtained in the analysis of human milk sample 2 with the proposed method. As can be seen, it exhibited no peaks for the matrix, but only for the five PAS and the internal standard.

In summary, the proposed SPE-GC-MS method allows the simultaneous determination of different types of pharmacologically active substances (antibacterials, nonsteroidal anti-inflammatory,

hormones, antiseptics, lipid reguladors, β-blokers, and antiepileptics) in milk samples. The method features good linearity, accuracy, and precision. Its limits of detection (0.2−1.2 ng/kg) are better than those provided by existing methods for the determination of PAS in milk samples (LOD, 0.59−2.69 μg/L,[12] 0.46−2.86 μg/L,[18] 0.5−50 μg/L[29]). Also, recoveries with the proposed method ranged from 91 to 104%, which indicates that matrix interferences were reduced or completely suppressed during the precipitation of milk proteins and cleanup in the SPE module and also that derivatization of the PAS with the BSTFA + 1% TMCS mixture was quantitative. Therefore, our method is highly suitable for monitoring PAS residues in milk from different sources (cow, goat, lactating women).

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Notes
Safety. All products were handled with care, using efficiently ventilated hoods, wearing latex gloves, and avoiding inhalation or skin contact because pharmaceuticals are usually toxic.

■ ABBREVIATIONS USED

BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; GC-MS, gas chromatography−mass spectrometry; IS, internal standard; IV, injection valve; MRLs, maximum residue limits; PAS, pharmacologically active substances; RSD, relative standard deviation; SD, standard deviation; SIM, selected ion monitoring; SPE, solid-phase extraction; TMCS, trimethylchlorosilane.

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