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Gas chromatography–mass spectrometry determination of pharmacologically active substances in urine and blood samples by use of a continuous solid-phase extraction system and microwave-assisted derivatization

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ABSTRACT

A sensitive method based on gas chromatography–mass spectrometry was used to determine 22 pharmacologically active substances (frequently used in the treatment of human and animal's diseases) including analgesics, antibacterials, anti-epileptics, antiseptics, β-blockers, hormones, lipid regulators and non-steroidal anti-inflammatories in blood and urine samples. Samples were subjected to continuous solid-phase extraction in a sorbent column (Oasis HLB), and then the target analytes were eluted with ethyl acetate and derivatized in a household microwave oven at 350 W for 3 min. Finally, these products were determined in a gas chromatograph–mass spectrometer equipped with a DB-5 fused silica capillary column. The analyte detection limits thus obtained ranged from 0.2 to 1.3 ng L^{-1} for urine samples and 0.8–5.6 ng L^{-1} for blood samples. Recoveries from both blood and urine ranged from 85 to 102%, and within-day and between-day relative standard deviations were all less than 7.5%. The proposed method offers advantages in reduction of the exposure danger to toxic solvents used in conventional sample pretreatment, simplicity of the extraction processes, rapidity, and sensitivity enhancement. The method was successfully used to quantify pharmacologically active substances in human and animal (lamb, veal and pig) blood and urine. The hormones estrone and 17β-estradiol were detected in virtually all samples, and so were other analytes such as acetylsalicylic acid, ibuprofen, ketoprofen and triclosan in human samples, and florfenicol, pyrimethamine and phenylbutazone in animal samples.

1. Introduction

Controlling residual amounts of pharmacologically active substances (PAS) in biological fluids is extremely important with a view to assessing the effectiveness of medical therapies or the risks of consuming of food or water contaminated with such substances [1]. Non-opioid analgesics and non-steroidal anti-inflamamatories, which are used mainly for the treatment of osteoarthritis, rheumatoid arthritis and other painful musculoskeletal illnesses [2,3], are among the most widely used PAS in over-the-counter preparations. The most common side-effect of some of PAS is gastric or intestinal ulceration, which is occasionally accompanied by anemia due to the resulting blood loss. In addition, these substances can cause disturbances in platelet function, prolong pregnancy or spontaneous labor, and alter renal function [1]. Chloramphenicol, florfenicol and thiamphenicol are three broad-spectrum antibacterials commonly used as chemotherapy agents to control some diseases in veterinary and aquacultural practice [4]. Chloramphenicol is highly effective on animals because it inhibits a variety of aerobic and anaerobic microorganisms [5]; by contrast, it is toxic to human bone marrow and has been associated with blood disorders such as aplastic anemia [1]. β-Blockers are mainly used to treat hypertension, congestive heart failure and abnormal heart rhythms, as well as to relieve angina and prevent cardiac infarctions (heart attacks) in humans [1]. However, β-blockers have been misused by some athletes to relieve performance anxiety by controlling hand tremor, lowering heart rate and reducing blood pressure [6]. This has led to their banning by the World Anti-Doping Agency (WADA) in some sports such as archery and shooting [7]. Some hormones such as 17α-ethinylestradiol, a semi-synthetic estrogen used as a contraceptive, may have a positive impact on public health [8]. Other PAS such as triclosan, used in personal care products, may be a potentially toxic environmental contaminant since *in vitro* studies on rat and human material have shown low concentrations of this substance to disturb metabolic systems and hormone homeostasis [9].

Quantifying trace amounts of small molecules (<500 amu) in biological samples is rather challenging. Most biological matrices contain a wide variety of abundant species that can interfere with the determination of the target analytes. Determining PAS,

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Table 1
Analytical figures of merit of the proposed method for the determination of pharmacologically active substances in urine[a] and blood[b] samples.

Compounds	Linear range ($ng\,L^{-1}$)		Sensitivity [signal/($ng\,L^{-1}$)]		LOD ($ng\,L^{-1}$)		RSD (%) Within-day		RSD (%) Between-day		m/z[c]		
	Urine	Blood	Urine	Blood	Urine	Blood	Urine	Blood	Urine	Blood	$M^{+\bullet}$	$[M-15]^+$	Additional ions
Acetylsalicylic acid	2.0–5000	9.9–15,000	1.075	0.230	0.6	3.0	5.4	5.7	5.7	6.3	252	237	120, **195**, 210
Carbamazepine	0.6–5000	2.8–15,000	3.230	0.625	0.2	0.8	4.7	5.6	5.8	5.8	308	293	**193**, 250
Chloramphenicol	0.6–5000	2.9–15,000	3.245	0.660	0.2	0.9	4.1	4.8	4.6	5.2	466	451	208, **225**, 242
Clofibric acid	1.7–5000	8.8–15,000	1.080	0.230	0.6	2.6	4.5	4.7	5.0	4.9	286	271	128, **143**
Diclofenac	0.7–5000	3.4–15,000	3.195	0.645	0.2	1.1	4.8	4.3	5.0	5.3	367	352	**214**, 242
17α-ethinylestradiol	3.5–5000	17.0–15,000	0.540	0.110	1.2	5.2	5.5	6.0	5.9	6.5	440	**425**	232, 300
17β-estradiol	3.8–5000	19.3–15,000	0.515	0.100	1.3	5.6	4.5	4.8	5.5	5.3	**416**	401	285, 326
Estrone	3.3–5000	16.5–15,000	0.570	0.125	1.1	5.1	5.2	5.8	5.7	6.5	**342**	327	218, 257
Florfenicol	0.7–5000	3.4–15,000	3.190	0.655	0.2	1.1	6.2	6.4	6.8	6.6	429	414	**257**, 360
Flunixin	0.6–5000	3.3–15,000	3.050	0.605	0.2	1.0	4.7	4.2	5.0	4.7	368	**353**	251, 263
Ibuprofen	0.6–5000	3.3–15,000	3.095	0.630	0.2	1.0	3.9	4.3	4.5	5.5	278	263	**160**, 234
Ketoprofen	1.1–5000	6.0–15,000	1.625	0.340	0.4	1.9	4.3	4.5	5.2	5.7	326	311	73, **282**
Mefenamic acid	0.6–5000	3.1–15,000	3.210	0.665	0.2	0.9	5.4	4.6	6.0	5.6	313	298	208, **223**
Metoprolol	1.8–5000	9.1–15,000	1.115	0.215	0.6	2.9	4.8	4.3	5.5	5.6	339	324	72, **223**
Naproxen	1.2–5000	6.6–15,000	1.580	0.325	0.4	2.0	5.3	4.9	6.2	6.1	302	287	**185**, 243
Niflumic acid	0.6–5000	3.1–15,000	3.150	0.645	0.2	1.0	4.6	4.2	4.9	5.0	354	339	**236**, 263
Paracetamol	0.6–5000	3.3–15,000	3.215	0.660	0.2	1.0	5.3	5.6	5.9	6.0	295	**280**	116, 206
Phenylbutazone	3.1–5000	16.1–15,000	0.605	0.130	1.1	4.7	4.9	5.3	5.4	5.6	308[d]	–[d]	77, **183**, 252
Propranolol	1.7–5000	8.8–15,000	1.100	0.225	0.6	2.8	4.0	4.9	4.6	5.7	331	316	72, **215**
Pyrimethamine	2.9–5000	14.6–15,000	0.655	0.140	0.9	4.2	6.5	5.9	7.0	7.3	392	**377**	171, 281
Thiamphenicol	0.6–5000	3.0–15,000	3.205	0.635	0.2	0.9	6.0	6.8	6.6	7.4	499	484	242, 257, **330**
Triclosan	1.9–5000	9.3–15,000	1.050	0.205	0.6	2.9	4.4	4.6	5.1	5.5	362	**347**	200, 310

[a] Volume of sample: 5 mL.
[b] Volume of sample: 1 mL.
[c] The peaks used for quantification are boldfaced; m/z for IS (triphenylphosphate): 77, 170, 325, **326**.
[d] The phenylbutazone is determined as non-derivatized.

especially in biological fluids (urine and whole blood), is difficult owing to their low concentrations and those of their metabolites relative to the typically high levels of endogenous compounds in the matrix. Therefore, detecting trace levels of these substances requires sample preparation and cleanup. A number of authors have used extraction, sample clean-up and derivatization procedures to facilitate the determination of PAS in various types of biological samples. Such procedures are based on solid-phase extraction (SPE) [4,10–19], solid-phase microextraction [20,21], liquid–liquid extraction [13,22–25], stir bar sorptive extraction [26,27] and supercritical fluid extraction in combination with solid phase extraction [10,28]. Solid-phase extraction is probably the most popular technique in this context by virtue of its expeditiousness, reproducibility and low cost. This technique is usually performed using a small column or cartridge containing an appropriate packing. During the last years a series of different polymer-based materials for the SPE of either acidic, neutral and basic compounds out of different sample matrices have been developed [29]. Oasis-HLB is seemingly the most commonly used sorbent for the solid-phase extraction of PAS from biological samples [11,13,14,19,30]. Oasis HLB is a macroporous copolymer consisting of two monomer components, the lipophilic divinylbenzene and the hydrophilic *n*-vinylpyrrolidone.

Determinations of PAS in biological fluids have used various detection techniques (especially gas chromatography and liquid chromatography). In fact, liquid chromatography (LC) has been used in combination with mass spectrometry [12–16,18,20,24,25], UV–visible and diode array detectors [3,13,15,22,26,27] to determine PAS in different types of biological fluids, and so has gas chromatography, mostly in conjunction with mass spectrometers detector (GC–MS) [4,11,17,19,23,28].

The aim of this work was to develop an accurate, sensitive method for the simultaneous determination of different types of PAS (antibacterials, analgesics, non-steroidal anti-inflammatories, anti-epileptics, antiseptics, β-blockers, lipid regulators, and hormones) in human and animal biological samples (urine and whole blood) by using a continuous SPE system for preconcentration/clean-up previously employed by our group to determine PAS in water and food samples [30,31]. As a major novelty, a microwave oven was used here to expedite derivatization of the target analytes prior to their GC–MS determination.

2. Experimental

2.1. Instruments and apparatus

All analyzes were carried out on a Focus gas chromatograph coupled to a DSQ II mass spectrometer equipped with an AI/AS 3000 autosampler (Thermo Electron SA, Madrid, Spain) and controlled by a computer running XCalibur software (Thermo Electron SA, Madrid, Spain). The transfer line was kept at 280 °C. The mass spectrometer worked in the electron impact mode (70 eV) by scanning from 60 to 500 amu to obtain full spectra of the target analytes or by selected ion-monitoring (SIM) for the quantification of the analytes. For each silyl derivative, $M^{+\bullet}$, $[M-15]^+$, and other additional ions were monitored which are included in Table 1, where $M^{+\bullet}$ is the molecular mass and $[M-15]^+$ is the molecular mass minor 15 corresponding to the loss of a CH_3 of the $Si(CH_3)_3$ group (Figs. S1–S5 of Supplementary Information). 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 1 mL min^{-1} was employed as the carrier gas. The injection port was maintained at 280 °C, and all injections were done in the split mode (1:20 ratio). The time for solvent delay was set at 4 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^{-1}. After the first transition, the temperature was raised from 150 to 290 °C at 6 °C min^{-1}. The total GC run time was ~30 min.

The continuous SPE system was assembled from a Gilson Minipuls-3 peristaltic pump (Villiers-le-Bel, France) fitted with poly(vinylchloride) tubes and two Rheodyne (Cotati, CA) 5041 injection valves. A PTFE laboratory-made sorbent column packed with 60 mg of Oasis-HLB as described elsewhere was also employed

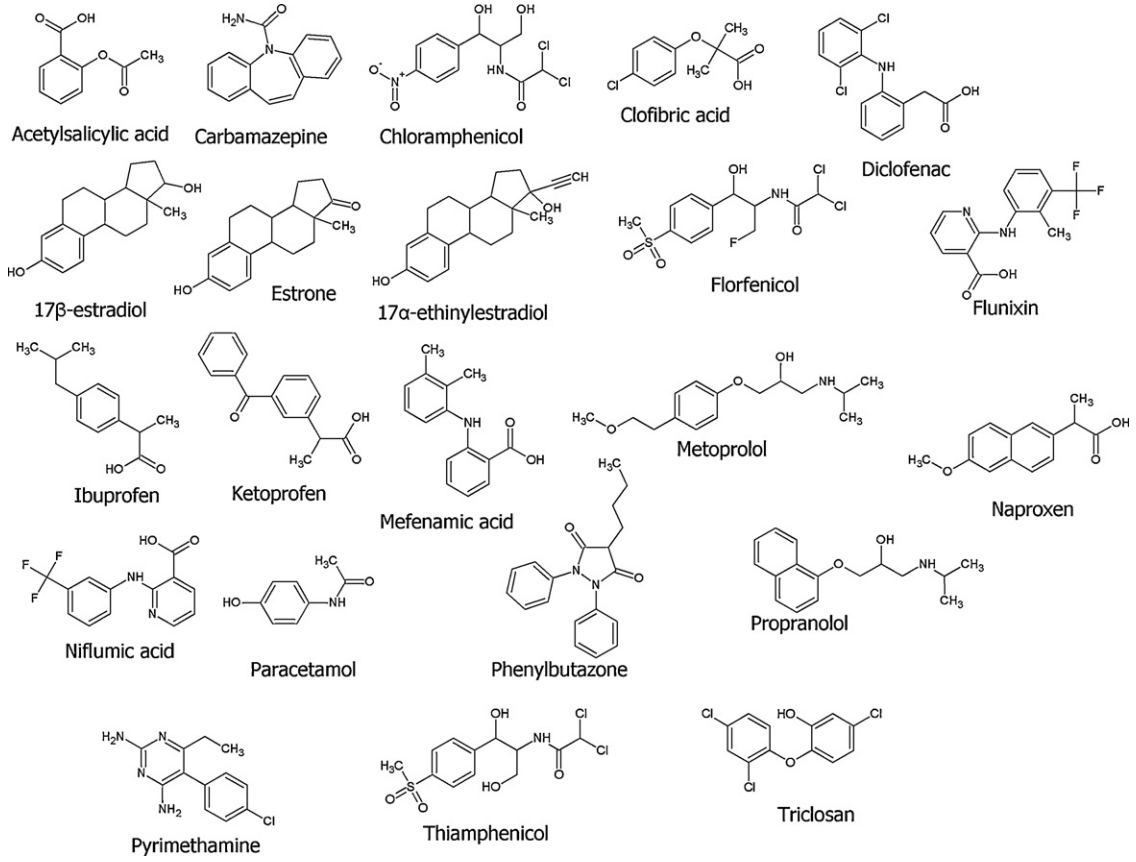

Fig. 1. Chemical structures of pharmacologically active substances.

[30]. The sorbent column was conditioned with 1 mL of ethyl acetate and 1 mL of purified water, which rendered it serviceable for at least 2 month.

2.2. Chemicals and materials

Pharmacologically active substances (Fig. 1) were all purchased from Sigma–Aldrich (Madrid, Spain) in the highest available purity. Triphenylphosphate and the derivatizing reagents [*N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS)] were obtained from Fluka (Madrid, Spain). All solvents (acetonitrile, methanol, dichloromethane and ethyl acetate), sodium hydroxide and inert poly-tetrafluoroethylene white beads (pore diameter 4 Å) were obtained from Merck (Darmstadt, Germany). 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 S.A. (Madrid, Spain). Ultrapure water was obtained using a Milli-Q purification system from Millipore Ibérica S.A.

Stock standard solutions of the individual PAS at 1 g L^{-1} 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^{-1} concentration of triphenylphosphate (internal standard, IS) and prepared on a daily basis were used as eluents for continuous SPE.

2.3. Biological samples

All biological samples were obtained in accordance with the guidelines of the bioethics committee. Human urine samples from healthy volunteers were collected in sterilized polyethylene bottles. Cow, pig and lamb urine samples were supplied by local stockbreeders not practicing intensive breeding, using sterilized polyethylene bottles to ensure the absence of contamination. When the time between sample collection and analysis was to exceeded 8 h, the samples were stored at −20 °C for up to 60 days to avoid degradation. Frozen samples were allowed to thaw at room temperature prior to analysis. Urine samples were gently mixed and directly transferred into vials for analysis in triplicate (n = 3).

Human whole blood samples were collected from healthy volunteers at various hospitals. Animal (veal, lamp and pig) blood samples were obtained by jugular venipuncture. All blood samples were collected in a blood-pack unit including sodium citrate (3.8%) as an anticoagulant, in a 1:16 ratio to whole blood [13]. Also, all were immediately frozen and stored at −20 °C until use.

2.4. Sample pretreatment

Previous study on 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 [30,31]. Therefore, urine samples were simply adjusted to pH 7 with dilute NaOH (0.5 M) and passed through a 0.20 μm Millex-LG filter. For blood samples, a volume of 1 mL of each was placed in a 15 mL round polypropylene centrifuge tube and mixed with 2 mL of acetonitirile in a REAX Control vortex mixer from Heidolph (Kelheim, Germany) for 2 min. Then, the mixture was centrifuged on a Centrofriger BL-II apparatus from JP Selecta (Barcelona, Spain) at 4000 rpm and 4 °C for 10 min, the supernatant being passed through a 0.20 μm Millex-LG filter and carefully evaporated to dryness under a stream of ultra-pure N_2 to a final volume of 200 μL and redissolved to 5 mL with purified water at pH 7.

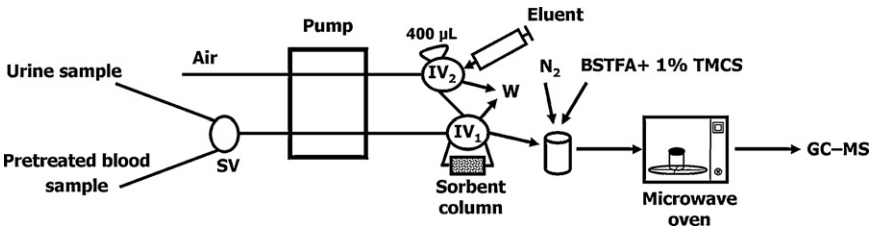

Fig. 2. Continuous solid-phase extraction system for the cleanup/preconcentration of pharmacologically active substances in urine and blood samples. IV, injection valve; SV, selection valve; W, waste; GC–MS, gas chromatograph with mass spectrometric detector; BSTFA-TMCS, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane (derivatizing reagents).

2.5. Continuous solid-phase extraction and microwave-assisted derivatization

The continuous SPE unit used for the cleanup/preconcentration of pharmacologically active substances from pretreated samples is depicted in Fig. 2. In the preconcentration step, a volume of 5 mL of pretreated sample was continuously passed through the sorbent column, located in the loop of injection valve IV_1, at 4 mL min^{-1}. PAS were immediately retained, and the sample matrix was sent to waste. Next, IV_1 was switched and the sorbent column dried for 2 min with an air stream at 4 mL min^{-1}; simultaneously, the loop of IV_2 (400 μL) was filled with eluent (ethyl acetate containing 500 ng L^{-1} triphenylphosphate as internal standard) by means of a syringe. In the elution step, IV_2 was switched to pass 400 μL of eluent, carried through the column by the air stream in the opposite direction of sample aspiration. The organic extract was collected in a 0.5 mL air-tight conical glass insert and evaporated to a volume of ~35 μL under a gentle stream of ultrapure N_2. Potential errors in measuring the final extract volume were avoided by using the internal standard. Next, 70 μL of mixture BSTFA + 1%TMCS (derivatizing agent) were added to sample extract of 35 μL. After that the vial was tightly sealed and the analytes were derivatized using a household microwave oven out for 3 min at 350 W. Finally, 1 μL aliquot of silylated derivatives was analyzed by GC–MS in the SIM mode.

3. Results and discussion

3.1. Variables affecting sample pretreatment

Appropriate sample preparation is an important prerequisite for chromatography of biosamples. The sample pretreatment to be used depends on the particular type of sample. Thus, whole blood and tissue (homogenates) require deproteination and filtration/centrifugation prior to SPE, whereas urine usually requires no more than dilution and/or centrifugation—and some SPE procedures allow urine to be used untreated [15,32]. We checked whether direct introduction of the urine samples into our previously developed continuous SPE system for determining PAS in water and milk samples [30,31] would pose any problem. To this end, a volume of 5 mL of uncontaminated urine sample was supplied with a 500 ng L^{-1} concentration of each PAS, adjusted to pH 7, passed through a 0.20 μm Millex-LG filter and analyzed as described in Section 2.5. An identical procedure was applied to 5 mL of a solution of purified water at pH 7 that was also supplied with each PAS at a 500 ng L^{-1} concentration. The results for both types of samples were very similar, so we chose not to pretreat the urine samples and simply filter them in order to prevent any solid particles from reaching the continuous SPE system.

Blood proteins are no doubt the components most likely to affect the solid-phase extraction of pharmacologically active substances at concentrations of 60–80 g L^{-1} from blood by effect of their blocking the sorbent column. This entails their prior removal by precipitation with a solvent such as acetone, acetonitrile [22], a mixture of methanol and acetonitrile [18] or dichloromethane [20], or their denaturation with a strong acid (trichloroacetic, hydrochloric or sulfuric acids) [9,16]. In this work, we assessed the efficiency of various solvents including 1% trichloroacetic acid, methanol, acetonitrile and dichoromethane, and of solvent mixtures (water–acetonitrile and methanol–acetonitrile), in precipitating proteins from whole blood. For this purpose, 1 mL of uncontaminated blood sample spiked with a 500 ng L^{-1} of each analyte was mixed with 2 mL of solvent and centrifuged at 5000 rpm for 10 min, after which the supernatant was filtered and evaporated to dryness under an N_2 stream to a final volume of 200 μL and redissolved to 5 mL with purified water at pH 7. Finally, the pretreated sample was introduced into the continuous SPE system [30,31] and processed as described in Section 2.5. The highest extraction efficiency for all PAS (~95%) was obtained with acetonitrile, which facilitated precipitation of proteins and their subsequent separation by centrifugation. We thus chose it to precipitate proteins from blood. The optimum volume of acetonitrile to be added to 1 mL of blood was determined by changing it over the range 1–10 mL; protein precipitation was found to peak at 1.5 mL acetonitrile, so a solvent volume of 2 mL was adopted as optimal. We also examined the effects of centrifugation-related variables such as rate, time and temperature over the ranges 1500–5000 rpm, 1–15 min and 0–25 °C, respectively. Centrifugation at 4000 rpm at 4 °C for 10 min resulted in optimal separation of precipitated blood proteins from the target species.

As previously shown by our group with the SPE of PAS in milk, the presence of acetonitrile at concentrations above 15% in the aqueous solution seriously impairs retention of the analytes on the sorbent column [30]. This led us to evaporate the supernatant from the centrifugation of the blood samples under a stream of ultra-pure N_2 to a final volume of 200 μL and dilute it with 5 mL of purified water at pH 7 prior to introduction into the continuous SPE system.

3.2. Optimization of microwave-assisted derivatization

Pharmacologically active substances have three polar groups: amino, hydroxyl and carboxyl (Fig. 1). Pharmacologically active substances require derivatization prior to GC–MS analysis and silylation is the most frequent choice for this purpose [4,11,17,19,23,30,31]. As shown in several studies, a mixture of BSTFA and TMCS can simultaneously silylate amino, alcohol and carboxyl groups in PAS in a single step [23,30,31,33]. However, the derivatization reaction takes a long time (more than 20 min) at 60–70 °C [23,30,31,33]. Combining a silylating reagent with microwave-assisted derivatization (MAD) can efficiently reduce analysis times. Microwave heating avoids the time for energy transfer required in traditional heating, thus resulting in a rapid rise in temperature and hence in fast completion of reactions. In this work, we optimized the microwave-assisted derivatization of PAS by examining the influence of four major variables (reaction

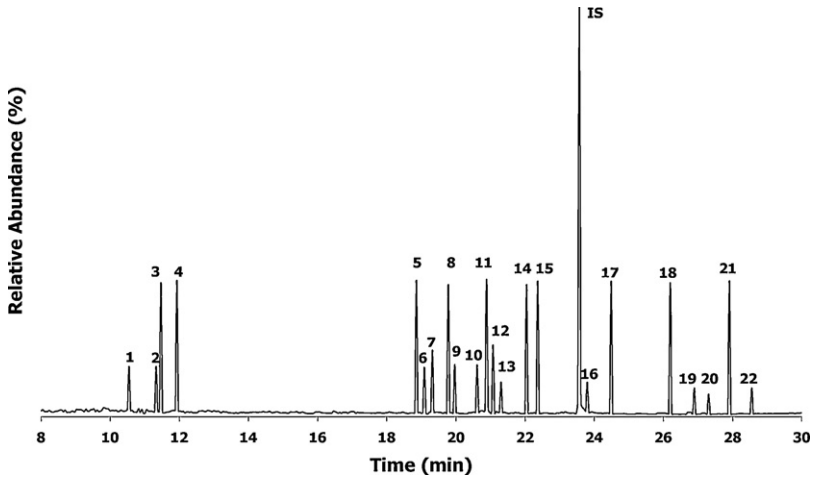

Fig. 3. Typical chromatogram (SIM mode) of pharmacologically active substances as N-trimethylsilyl derivatives (1 μg L^{-1}). 1 – acetylsalicylic acid; 2 – clofibric acid; 3 – paracetamol; 4 – ibuprofen; 5 – niflumic acid; 6 – metoprolol; 7 – naproxen: 8 – flunixin; 9 – triclosan; 10 – propranolol; 11 – mefenamic acid; 12 – ketoprofen; 13 – pyrimethamine; 14 – carbamazepine; 15 – diclofenac; 16 – phenylbutazone; 17 – chloramphenicol; 18 – florfenicol; 19 – estrone; 20 – 17β-estradiol; 21 – thiamphenicol; 22 – 17α-ethinylestradiol; IS – triphenylphosphate (internal standard).

solvent, derivatizing reagent, microwave power output and irradiation time). To this end, volumes of 100 μL of individual solutions of the derivatizing reagents [*N*,*O*-bis-(trimethylsilyl)acetamide, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide and trimethylcholorosilane, both individually and in mixtures] were added to 50 μL of a solution containing a 1 μg L^{-1} concentration of each analyte in ethyl acetate. The derivatization reaction was conducted in an air-tight 0.5 mL conical glass insert that was placed in the microwave oven at 250 W for 5 min. The derivatized PAS thus obtained were determined by GC–MS. The best results in this respect were obtained with a 99:1 mixture of BSTFA and TMCS.

Tests were conducted with ethyl acetate and acetonitrile, two solvents commonly used for the silylation of PAS [34]. A volume of 50 μL of a solution containing a 1 μg L^{-1} concentration of each PAS in each solvent was supplied with 100 μL of 99:1 BSTFA–TMCS mixture and placed in an air-tight conical glass insert of 0.5 mL for derivatization in a microwave oven at 250 W for 5 min. Although the two solvents provided similar results, ethyl acetate was selected because it surpassed acetonitrile in its ability to elute the sorbent column in the continuous SPE system [30]. Detection limits were found to depend on the volume of solvent used. In this work, we examined the effect of low volumes of solvents and derivatizing reagent (10–150 μL) in order to achieve detection limits relevant to environmental concentrations. The best results were obtained with a final volume of sample extract of 35 μL and one of derivatizing (BSTFA + 1% TMCS) of 70 μL.

The MAD conditions were also optimized, *via* the microwave power output and irradiation time. Thus, a volume of 5 mL of an aqueous sample containing a 500 μg L^{-1} concentration of each PAS was introduced into the continuous system as described in Section 2.5, the organic extract being collected in an air-tight 0.5 mL conical glass insert, evaporated to a volume of ~35 μL under a gentle stream of ultrapure N_2 and supplied with 70 μL of 99:1 BSTFA–TMCS mixture [34]. The vial containing the analytes was then tightly sealed and placed in a household microwave oven at variable power (70–500 W) for an also variable time (1–10 min). The highest derivatization efficiency was obtained by irradiation at 350 W for 3–4 min. It was observed that when applied a power under 350 W, low yields in the derivatization were achieved; for values greater than 400 W, the signal of the analytes decreases possibly due to the degradation of these compounds, as occurs with a power of 350 W for times higher of 4 min. Therefore, a power of irradiation of 350 W for 3 min was selected for subsequent work.

By way of example, Fig. 3 shows the typical chromatogram of standard as N-trimethylsily derivatives (1 μg L^{-1}). As can be seen, the 22 PAS studied were effectively separated with no difficulty in a single chromatographic run in about 30 min.

3.3. Analytical performance

Linear range, analyte detectability, and precision of the proposed method were studied under optimal experimental conditions (see Table 1). Calibration curves were constructed by using uncontaminated urine samples (5 mL, pooled human urine sample) and uncontaminated whole blood (1 mL, pooled sample of human blood) spiked with 0.6–5000 or 2.8–15,000 ng L^{-1} of each analytes, respectively, and processed as described in Sections 2.4 and 2.5. The equations for the standard curves were obtained by plotting the analyte to internal standard peak area ratios against the amount of PAS. Regression coefficients were over 0.995 in all cases. Limits of detection (LODs) were determined as the analyte concentration that provides a chromatographic peak equal to 3 times the regression standard deviation, S*y*/*x*, divided by the slope of each calibration graph, ranged between 0.2 and 1.3 ng L^{-1} or between 0.8 and 5.6 ng L^{-1} for urine or blood samples, respectively. As can be observed in Table 1, analytes with lower LODs are carbamazepine, chloramphenicol, diclofenac, florfenicol, flunixin, ibuprofen, mefanamic acid, niflumic acid, paracetamol and thiamphenicol. In contrast, PAS with higher LODs are the three hormones and phenylbutazone. The latter compound is determined as non-derivatized as demonstrated in the mass spectrum included in Fig. S6 of Supplementary Information. In the fragmentation pattern of phenylbutazone is observed that the peak *m*/*z* 308 corresponds to the atomic mass of this compound without derivatization.

The precision of the proposed method, as relative standard deviation (RSD), was calculated by measuring 11 uncontaminated urine samples spiked with 10, 100 and 1000 ng L^{-1}, or 11 uncontaminated blood samples spiked with 50, 500, and 5000 ng L^{-1} of each target compounds. A comparative study of within-day and between-day precision was conducted [35]. For the study of between-day precision, three pooled samples of urine (120 mL) or blood (25 mL) were taken to which were added the 22 PAS at the three concentration levels listed above. The pooled samples were split into portions of 15 mL (urine) or 3 mL (blood), and a portion was analyzed on the first day in triplicate. The others portions were frozen at −20 °C, and were subjected to the same analytical

Table 2
Percent recovery (± SD, $n = 3$) of pharmacologically active substances added to urine and blood samples.

Compounds	Urine (ng L^{-1})			Blood (ng L^{-1})		
	10	100	1000	50	500	5000
Acetylsalicylic acid	87 ± 4	94 ± 5	101 ± 6	92 ± 5	98 ± 6	99 ± 6
Carbamazepine	101 ± 5	97 ± 5	99 ± 6	97 ± 6	98 ± 6	97 ± 5
Chloramphenicol	101 ± 5	102 ± 5	96 ± 4	86 ± 5	100 ± 5	95 ± 5
Clofibric acid	90 ± 5	91 ± 5	99 ± 4	94 ± 5	96 ± 5	101 ± 5
Diclofenac	99 ± 5	90 ± 6	96 ± 5	100 ± 5	94 ± 5	101 ± 5
17α-Ethinylestradiol	99 ± 6	101 ± 5	97 ± 6	102 ± 6	92 ± 5	100 ± 6
17β-Estradiol	97 ± 5	100 ± 6	94 ± 5	101 ± 5	96 ± 5	93 ± 4
Estrone	99 ± 5	102 ± 5	101 ± 6	95 ± 6	94 ± 5	101 ± 6
Florfenicol	88 ± 5	92 ± 6	102 ± 6	98 ± 6	91 ± 5	92 ± 5
Flunixin	99 ± 5	96 ± 4	101 ± 5	93 ± 4	98 ± 5	95 ± 4
Ibuprofen	89 ± 4	97 ± 5	102 ± 4	95 ± 4	94 ± 4	98 ± 4
Ketoprofen	100 ± 5	93 ± 4	99 ± 5	91 ± 4	94 ± 5	101 ± 5
Mefenamic acid	97 ± 5	95 ± 5	102 ± 6	99 ± 5	97 ± 5	100 ± 5
Metoprolol	91 ± 5	100 ± 5	97 ± 4	88 ± 4	95 ± 4	100 ± 5
Naproxen	96 ± 5	89 ± 5	97 ± 6	94 ± 5	100 ± 5	99 ± 5
Niflumic acid	97 ± 4	101 ± 5	100 ± 5	86 ± 5	101 ± 4	98 ± 5
Paracetamol	95 ± 6	95 ± 5	98 ± 6	88 ± 5	90 ± 5	102 ± 6
Phenylbutazone	100 ± 5	89 ± 4	95 ± 5	88 ± 5	97 ± 6	92 ± 5
Propranolol	91 ± 4	100 ± 5	96 ± 4	100 ± 5	101 ± 5	97 ± 4
Pyrimethamine	96 ± 5	102 ± 6	98 ± 6	89 ± 5	93 ± 5	98 ± 6
Thiamphenicol	102 ± 6	94 ± 6	93 ± 5	99 ± 7	90 ± 6	91 ± 6
Triclosan	95 ± 5	91 ± 4	96 ± 4	85 ± 4	101 ± 5	98 ± 5

procedure in triplicate every day for 6 days following thawing 1 h before preparation. The within-day precision was found to range from 3.9 to 6.8% and the between-day from 4.5 to 7.4%. Also, freeze–thaw stability test were conducted with a view to assessing the stability of the analytes in urine and blood samples at a storage temperature of −20 °C. For this purpose, three pooled samples of urine (120 mL) or blood (25 mL) were spiked with all analytes at the three concentration levels listed above. The pooled samples was split into 15 mL portions and frozen at −20 °C; by exception, one portion was analyzed in triplicate as described under Section 2 on the same day. All other portions were subjected to the same analytical procedure in triplicate every 5 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 < 7.5%), to those for the unfrozen sample.

Because no certified reference material for urine or blood containing the studied analytes was available, the proposed method was validated by analyzing uncontaminated urine samples spiked

Table 3
Determination of pharmacologically active substances in blood and urine samples by proposed method.[a]

Sample[b]	Compound	Concentration found (μg L^{-1})	Sample[c]	Compound	Concentration found (μg L^{-1})
Human blood 1	Acetylsalicylic acid	1.2 ± 0.1	Human urine 1	17β-estradiol Estrone	2.8 ± 0.2
	17β-estradiol	1.6 ± 0.1		Ketoprofen	1.6 ± 0.1
	Estrone	2.8 ± 0.2		Triclosan	0.51 ± 0.03
	Triclosan	0.25 ± 0.01			0.41 ± 0.02
Human blood 2	Estrone	2.0 ± 0.1	Human urine 2	Acetylsalicylic acid	0.78 ± 0.04
	Ibuprofen	0.69 ± 0.03		17β-estradiol Estrone	0.87 ± 0.05
	Ketoprofen	0.34 ± 0.02			1.5 ± 0.1
	Triclosan	0.89 ± 0.05			
Human blood 3	–	–	Human urine 3	Estrone Ketoprofen	2.6 ± 0.1
				Triclosan	0.8 ± 0.1
					0.24 ± 0.01
Lamb blood 1	17β-estradiol Florfenicol	5.9 ± 0.3	Human urine 4	Acetylsalicylic acid	0.30 ± 0.01
		0.51 ± 0.03		Estrone	2.9 ± 0.2
				Ibuprofen	0.41 ± 0.02
				Triclosan	0.16 ± 0.01
Lamb blood 2	17β-estradiol	1.9 ± 0.1	Human urine 5	–	–
	Estrone	2.8 ± 0.2			
Cow blood 1	17β-estradiol	4.5 ± 0.3	Lamb urine 1	17β-estradiol	3.3 ± 0.2
	Estrone	4.8 ± 0.3		Estrone	0.78 ± 0.04
	Pyrimethamine	3.8 ± 0.2			
Cow blood 2	17β-estradiol Phenylbutazone	1.3 ± 0.1	Lamb urine 2	17β-estradiol Florfenicol	0.42 ± 0.02
		3.3 ± 0.2			0.16 ± 0.01
Pig blood 1	17β-estradiol Florfenicol	0.79 ± 0.04	Cow urine	17β-estradiol Estrone	0.52 ± 0.03
		1.9 ± 0.2		Pyrimethamine	1.7 ± 0.1
					1.8 ± 0.1
Pig blood 2	17β-estradiol	0.67 ± 0.04	Pig urine	17β-estradiol	0.47 ± 0.03
	Estrone	1.9 ± 0.1		Estrone	0.23 ± 0.01
	Pyrimethamine	2.7 ± 0.2			

[a] To 1 mL of blood samples or 5 mL of urine samples (± SD, $n = 3$).
[b] Human blood: 1, woman (25 years); 2, man (45 years); 3, man (30 years).
[c] Human urine: 1, woman (25 years); 2, woman (40 years); 3, man (26 years); 4, woman (50 years); 5, man (20 years).

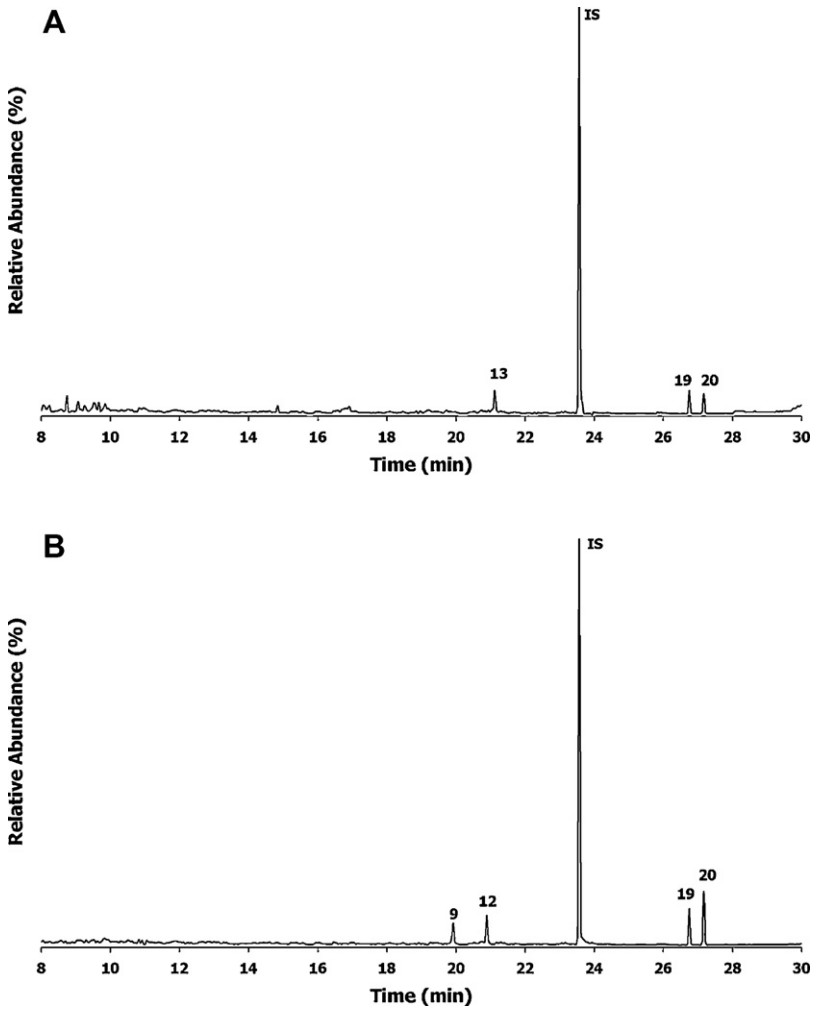

Fig. 4. Chromatograms in SIM mode for pharmaceutical active substances in the analysis of 1 mL of cow blood 1 (A) and 5 mL of human urine 1 (B) (see Table 3). For peak identification, see Fig. 3.

with 10, 100 and 1000 ng L^{-1}, and uncontaminated blood samples spiked with 50, 500, and 5000 ng L^{-1} of each target compounds and analyzed in triplicate. The average recoveries ranged from 85 to 102% (Table 2), which testifies to the applicability of the proposed method in these complex matrices.

3.4. Analysis of urine and blood real samples

The proposed method was applied to the determination of 22 pharmacologically active substances including analgesics, antibacterials, anti-epileptics, antiseptics, β-blockers, hormones, lipid regulators and non-steroidal anti-inflammatories in real urine and blood samples from humans and animals (lamb, cow and pig). Samples were analyzed in triplicate, following the pretreatment and analytical procedures described under Section 2.

As can be seen from Table 3, the hormones estrone and/or 17β-estradiol were detected in eight blood samples, at concentrations from 1.9 to 4.8 μg L^{-1} and 0.67 to 5.9 μg L^{-1}, respectively. These 17β-estradiol levels are higher than those previously reported by other authors for human blood [11]. The human blood samples contained PAS commonly used as analgesics (acetylsalicylic acid), non-steroidal anti-inflammatories (ibuprofen and ketoprofen) and antiseptics (triclosan). The antibacterials florfenicol and pyrimethamine were additionally found in some animal blood samples. Phenylbutazone was detected in a cow blood sample, at a concentration similar to those reported by Cárdenas et al. [32] for race horses.

Like the blood samples, most urine samples contained the hormones estrone and 17β-estradiol, albeit at lower concentrations (0.42–3.3 μg L^{-1}). This was also the case with the other PAS, which were found at concentrations of 0.16–1.8 μg L^{-1} in urine and 0.25–3.8 μg L^{-1} in blood. By way of example, Fig. 4 shows the SIM mode chromatograms for samples of cow blood and human urine processed with the proposed method.

4. Conclusions

The objectives of this work were acceptably fulfilled. In fact, the proposed method is rapid, sensitive and selective in the determination of PAS in blood and urine samples. The method has several salient advantages, namely: (*a*) urine samples require no treatment other than filtering and pH adjustment prior to introduction into the SPE system; (*b*) the SPE step is highly efficient, which significantly increases the sensitivity and selectivity of the determination, with limits of detection of 0.2–1.3 ng L^{-1} in urine and 0.8–5.6 ng L^{-1} in blood; (*c*) using a household microwave oven allows PAS to be silylated in 3 min as opposed to more than 20 min with conventional methods such as heating at 60–70 °C [23,30,31,34]; "(d) the proposed method affords the determination of a wide range of PAS belonging to a number of therapeutic classes, while most of the methodologies that had been developed so far focused on one or

two types of therapeutic classes, and (e) the method allows PAS to be determined in both animal and human biological fluids, while the methods found in the literature are concerned with the determination of PAS in these samples from human or animal samples separately.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.02.013.

References

[1] K.C. Osterhoudt, T.M. Penning, in: L.L. Brunton, B.C. Chabner, B.C. Knollmann (Eds.), Goodman and Gilman's The Pharmacological Basis of Therapeutics, vol. 1, 12 ed., McGraw-Hill Medical, New York, 2010.
[2] F.A. Aly, S.A. Al-Tamimi, A.A. Alwarthan, Anal. Chim. Acta 416 (2000) 87.
[3] P. Gowik, B. Jülicher, S. Uhlig, J. Chromatogr. B 716 (1998) 221.
[4] T. Nagata, H. Oka, J. Agric. Food Chem. 44 (1996) 1280.
[5] Z.Y. Huang, M.Y. Sun, S. Li, G.L. Huang, Aquacult. Res. 37 (2006) 1540.
[6] M. Lu, L. Zhang, B. Qiu, Q. Feng, S. Xia, G. Chen, J. Chromatogr. A 1193 (2008) 156.
[7] The World Anti-Doping Agency International Standard, WADA, Lausanne, 2006.
[8] E.M. Ghoneim, H.S. El-Desoky, M.M. Ghoneim, J. Pharm. Biomed. Anal. 40 (2006) 255.
[9] M. Allmyr, M.S. McLachlan, G. Sandborgh-Englund, M. Adolfsson-Eric, Anal. Chem. 78 (2006) 6542.
[10] K. Hartonen, M.L. Riekkola, J. Chromatogr. B 676 (1996) 45.
[11] M.H. Choi, K.R. Kim, J.K. Hong, S.J. Park, B.C. Chung, Rapid Commun. Mass Spectrom. 16 (2002) 2221.
[12] H.H. Maurer, O. Tenberken, C. Kratzsch, A.A. Weber, F.T. Peters, J. Chromatogr. A 1058 (2004) 169.
[13] M. Sultan, G. Stecher, W.M. Stöggl, R. Bakry, P. Zaborski, C.W. Huck, N.M. El Kousy, G.K. Bonn, Curr. Med. Chem. 12 (2005) 573.
[14] K. Suenami, L.W. Lim, T. Takeuchi, Y. Sasajima, K. Sato, Y. Takekoshi, S. Kanno, Anal. Bioanal. Chem. 384 (2006) 1501.
[15] J. Tuerk, M. Reinders, D. Dreyer, T.K. Kiffmeyer, K.G. Schmidt, H.M. Kuss, J. Chromatogr. B 831 (2006) 72.
[16] F. Vinci, S. Fabbrocino, M. Fiori, L. Serpe, P. Gallo, Rapid Commun. Mass Spectrom. 20 (2006) 3412.
[17] H. Budzinski, M.H. Devier, P. Labadie, A. Togola, Anal. Bioanal. Chem. 386 (2006) 1429.
[18] L. Kristoffersen, E.L. Øiestad, M.S. Opdal, M. Krogh, E. Lundanes, A.S. Christophersen, J. Chromatogr. B 850 (2007) 147.
[19] S. Magiera, C. Uhlschmied, M. Rainer, Ch.W. Huck, I. Baranowska, G.K. Bonn, J. Pharm. Biomed. Anal. 56 (2011) 93.
[20] X. Hu, J. Pan, Y. Hu, G. Li, J. Chromatogr. A 1216 (2009) 190.
[21] A. Aresta, D. Bianchi, C.D. Calvano, C.G. Zambonin, J. Pharm. Biomed. Anal. 53 (2010) 440.
[22] V.V. Pavan Kumar, M.C.A. Vinu, A.V. Ramani, R. Mullangi, N.R. Srinivas, Biomed. Chromatogr. 20 (2006) 125.
[23] B. Yilmaz, S. Arslan, V. Akba, Talanta 80 (2009) 346.
[24] H.P. Nguyen, L. Li, J.W. Gatson, D. Maass, J.G. Wigginton, J.W. Simpkins, K.A. Schug, J. Pharm. Biomed. Anal. 54 (2011) 830.
[25] L.K. Sørensen, Forensic Sci. Int. 206 (2011) 119.
[26] C. Almeida, J.M.F. Noguerira, J. Pharm. Biomed. Anal. 41 (2006) 1303.
[27] R.H. Costa Queiroz, C. Bertucci, W.R. Malfará, S.A. Carvalho Dreossi, A. Rodrigues Chaves, D.A. Rodrigues Valério, M.E. Costa Queiroz, J. Pharm. Biomed. Anal. 48 (2008) 428.
[28] G. Meissner, K. Hartonen, M.L. Riekkola, Fresenius J. Anal. Chem. 360 (1998) 618.
[29] C.W. Huch, G.K. Bonn, J. Chromatogr. A 885 (2000) 51.
[30] A. Azzouz, B. Jurado-Sánchez, B. Souhail, E. Ballesteros, J. Agric. Food Chem. 59 (2011) 5125.
[31] A. Azzouz, B. Souhail, E. Ballesteros, J. Chromatogr. A 1217 (2010) 2956.
[32] S. Cárdenas, M. Gallego, M. Valcárcel, R. Ventura, J. Segura, Anal. Chem. 68 (1996) 118.
[33] A. Lajeunesse, C. Gagnon, Int. J. Environ. Anal. Chem. 87 (2007) 565.
[34] L.W. Chung, K.L. Lin, T.C.C. Yang, M.R. Lee, J. Chromatogr. A 1216 (2009) 4083.
[35] Validation of Analytical Procedures: Text and Methodology, Q2 (R1), International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human USE, November 2005, http://www.ich.org.