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Sample preparation development and matrix effects evaluation for multianalyte determination in urine

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The development of a generic analytical method remains difficult when a high number of compounds has to be simultaneously considered. This study proposes an innovative strategy for the development of a solid phase extraction (SPE) procedure before liquid chromatography-mass spectrometry analysis of 34 diuretics and beta-blockers in urine samples. These compounds have been selected since they are often encountered in anti-doping control. The principle is based on the selection of representative analytes during SPE protocol optimization, allowing a drastic reduction of generated data and development time. To select the representative compounds, all substances were classified based on their SPE behavior with a generic method and groups were formed with the help of a chemometric tool, namely hierarchical cluster analysis (HCA). One representative analyte per group was selected and used for subsequent SPE method development. Once the SPE method was developed, compounds were analyzed by LC-MS and matrix effects were evaluated to determine the influence of the matrix on the SPE process and MS signal alteration due to endogenous compounds. As a result, matrix effects evaluation must be performed on all analytes; representative compounds previously selected for SPE development were unable to predict matrix effects.

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1. Introduction

The analysis of a large number of compounds in biological matrices (*e.g.* urine, blood) is required in various domains such as therapeutic drug monitoring (TDM), doping control, forensic sciences, and toxicology. From a practical point of view, the use of a reduced number of generic methods, allowing the simultaneous determination of different substances, is generally preferred in routine analyses. When handling biological samples for liquid chromatography (LC) which remains the method of choice for this kind of application, a sample preparation is mandatory to limit column clogging, presence of co-eluting substances as well as matrix effects frequently encountered with mass spectrometric detection (MS) [1–4]. In this regard, solid phase extraction (SPE) is often chosen, allowing good sample clean-up. The use of on-line SPE in the column-switching mode is particularly adapted to TDM [5], but requires a dedicated instrument. On the other hand, the off-line SPE on multi-well plates is qualified for multianalyte procedures. Since each sample is independently extracted, this format is compatible with different separation techniques working with various mechanisms (reversed or normal phase, ion exchange, *etc.*), reduces contamination risk, which presents a great advantage in analyses where a legal aspect has to be taken into account, and does not require a particular technical skill [6].

The analysis of a large variety of substances can induce a tedious and time-consuming method development. To the best of our knowledge, only a small number of studies have reported solutions for time reduction in the development of a generic sample preparation procedure. A solution was the use of an apparatus capable of extracting a large number of samples (*i.e.* multi-well plates, on-line extraction supports, *etc.*) [7]. In this approach, all analytes of interest are evaluated, inducing a tedious task and generating large amounts of data. Additionally, the use of a chemometric tool was proposed to determine the principal interaction effects of the extraction conditions, leading to a reduced number of experiments for method development [8]. The last approach mentioned in the literature was based on the reduction of used analytes, drastically lowering time and the number of procedures [9]. However, no rigorous compound selection has been proposed, leading to a sample preparation that is not necessarily focused on the full set of compounds.

Moreover, it is well known that the sample preparation must reduce matrix effects on subsequent LC-MS analysis to obtain repeatable and reliable results. A characterization and reduction of these effects must be operated as already described in several publications [10–19]. The method proposed by Matuszewski et al. [18], to identify matrix effects was considered in this study to determine

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their origin and overcome the problem by the use of an internal standard or a modification of the sample preparation prior to validation.

The aim of this paper is to propose a simple and low-cost method to reduce the time required for developing a sample preparation in the case of multianalyte analysis by an appropriate selection of representative analytes. Moreover, matrix effects after the optimized sample preparation are evaluated *via* a method analogous to the one previously mentioned [18], and a classification of the different types of matrix effects is proposed.

2. Experimental

2.1. Chemicals

Acebutolol, acetazolamide, atenolol, bendroflumethiazide, betamethasone, bumetanide, chlorothiazide, chlorthalidone, clopamide, dexamethasone, ethacrynic acid, furosemide, hydrochlorothiazide, indapamide, metolazone, metoprolol, nadolol, probenecid, sotalol, strychnine, formic acid and 37% hydrochloric acid solutions were purchased from Sigma–Aldrich (Buchs, Switzerland). Adrafinil, canrenone, celiprolol, dichlorphenamide, esmolol, gestrinone, piretanide, torasemide and xipamide were kindly provided by the Laboratoire d'Analyse du Dopage (Epalinges, Switzerland). Benzoylecgonine was purchased from Cerilliant (Austin, TX, USA), carteolol was extracted from Arteoptic® tablets (Novopharma, Cham, Switzerland) and finasteride was obtained from Propecia® tablets (MSD, Glattbrugg, Switzerland). Metipranolol was present in Betanol® eye-drops (Europhta, Monaco), and modafinil was extracted from Modasomil® 100 tablets (Cephalon, Martinsried, Germany). A list of pK_a, log D and log P values for each compound is reported in Table 1. Acetonitrile (ACN) and methanol (MeOH) were purchased from Panreac (Barcelona, Spain) and water was provided by a Milli-Q Gradient A10 water purifier system from Millipore (Bedford, MA, USA). The ammonia solution (25%) was purchased from Fluka (Buchs, Switzerland). All chemicals were of the highest purity grade commercially available, and all reagents used were of HPLC grade.

Stock solutions of all compounds at 10,000 μg mL^{-1} were individually prepared in a mixture of water–ACN (50:50, v/v). Ammonium buffer (50 mM) was prepared every 3 days by diluting 3.9 mL of ammonia solution (25%) in 500 mL of Milli-Q water. pH was adjusted to pH 10 with a Metrohm 691 pH-meter (Herisau, Switzerland) by adding formic acid drop-by-drop.

2.2. SPE

2.2.1. Generic protocol

All sample extractions were performed on a 10 mg 2 mL Waters Oasis Sorbent Selection Plate (comprising Oasis MCX, Oasis MAX, Oasis WCX and Oasis WAX sorbents [20]) and on an Oasis HLB 10 mg 2 mL plate using a Waters SPE manifold and a Gast DOA-P504-BN pump (Benton Harbor, MI, USA) kindly loaned by Waters. The generic Oasis 2 × 4 Method was applied to extract standard solutions with Oasis MCX, WAX, MAX, and WCX. Sorbents were conditioned with 500 μL of MeOH and equilibrated with 500 μL of water. One thousand and five hundred microliters of sample was loaded on each type of sorbent (MCX, MAX, WCX, and WAX). Washing was performed with 900 μL of 2% HCOOH for MCX and WAX and 900 μL of NH_4OH in water (5:95, v/v) for MAX and WCX. The first

Table 1
List of therapeutic classes, pK_a, log D and log P values for all compounds of the set. Values were calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (© 1994–2006 ACD/Labs).

Compound	Therapeutic class	pK_a acid	pK_a basic	log D			log P
				pH 1	pH 7	pH 10	
Acebutolol	β-Blocker	13.8	9.1	−0.5	0.5	2.5	2.6
Acetazolamide	Diuretic	7.4	–	−0.3	−0.4	−3.8	−0.2
Adrafinil	Stimulant	8.2	–	1.0	1.0	−0.8	1.0
Atenolol	β-Blocker	13.9	9.2	−3.0	−2.0	0.0	0.1
Bendroflumethiazide	Diuretic	8.6	–	2.0	2.0	−0.1	2.0
Benzoylecgonine	Narcotic	3.5	10.8	−0.4	0.2	0.2	2.7
Betamethasone	Corticosteroid	12.1	–	2.1	2.1	2.2	2.0
Bumetanide	Diuretic	3.2	4.5	0.5	−0.3	−1.5	2.7
Canrenone	Diuretic	–	–	3.0	3.0	3.0	2.9
Carteolol	β-Blocker	13.8	9.1	−1.4	−0.4	1.6	1.6
Celiprolol	β-Blocker	13.8	9.1	−0.3	0.8	2.8	2.8
Chlorothiazide	Diuretic	9.2	–	−0.2	−0.2	−1.1	−0.1
Chlorthalidone	Diuretic	9.6	–	−0.7	−0.7	−1.4	−0.7
Clopamide	Diuretic	9.4	4.1	−1.2	1.6	0.9	1.5
Dexamethasone	Corticosteroid	12.1	–	1.9	1.9	1.9	1.8
Dichlorphenamide	Diuretic	9.0	–	0.9	0.9	−0.9	0.9
Esmolol	β-Blocker	13.9	9.2	−1.2	−0.2	1.9	1.9
Ethacrynic acid	Diuretic	2.8	–	3.4	−0.5	−0.7	3.3
Finasteride	Diuretic	–	–	3.2	3.2	3.2	3.2
Furosemide	Diuretic	3.0	–	2.9	−0.8	−1.5	2.9
Gestrinone	Anabolic agent	–	–	3.3	3.3	3.3	3.3
Hydrochlorothiazide	Diuretic	9.0	–	−0.1	−0.1	−1.8	−0.1
Indapamide	Diuretic	9.4	–	2.1	2.1	1.2	2.1
Metipranolol	β-Blocker	13.9	9.2	−0.4	0.5	2.6	2.6
Metolazone	Diuretic	10.0	–	3.2	3.2	2.9	3.1
Metoprolol	β-Blocker	13.9	9.2	−1.3	−0.3	1.7	1.7
Modafinil	Stimulant	–	–	1.4	1.4	1.4	1.4
Nadolol	β-Blocker	13.9	9.2	−1.8	−0.8	1.2	1.2
Piretanide	Diuretic	10.2	4.3	−0.0	−1.2	−2.4	1.8
Probenecid	Diuretic	3.7	–	3.3	0.1	−0.8	3.3
Sotalol	β-Blocker	9.6	9.2	−2.8	−1.8	−0.3	0.3
Strychnine	Stimulant	–	8.3	−1.4	0.3	1.6	1.6
Torasemide	Diuretic	3.1	4.8	0.7	0.5	−0.9	3.1
Xipamide	Diuretic	5.1	–	4.0	2.1	0.5	4.0

Table 2
Recovery values of all fractions with the HLB sorbent in three loading conditions (pH 2.5, 7 and 12).

Compound	HLB pH 2.5			HLB pH 7			HLB pH 12		
	Load (%)	Wash (%)	Elute (%)	Load (%)	Wash (%)	Elute (%)	Load (%)	Wash (%)	Elute (%)
Acebutolol	12	27	61	3	2	95	0	0	99
Acetazolamide	0	0	62	0	0	86	51	3	0
Adrafinil	0	0	82	0	0	86	0	0	88
Atenolol	81	8	0	79	8	13	1	1	89
Bendroflumethiazide	0	0	90	0	0	100	35	4	61
Benzoylecgonine	14	8	71	0	0	93	0	2	4
Betamethasone	0	0	88	0	0	99	0	0	81
Bumetanide	0	0	93	0	0	100	3	3	93
Canrenone	0	0	85	0	0	100	0	0	100
Carteolol	45	24	26	1	0	94	0	0	90
Celiprolol	1	6	89	0	0	97	0	0	97
Chlorothiazide	2	0	79	0	0	100	0	0	45
Chlortalidone	0	0	100	0	0	100	66	6	20
Clopamide	4	0	94	0	0	97	33	11	56
Dexamethasone	0	0	100	0	0	99	0	0	62
Dichlorphenamide	0	0	86	0	0	100	93	0	0
Esmolol	37	2	61	0	0	98	0	0	1
Ethacrynic acid	0	0	100	0	0	100	0	14	33
Finasteride	0	0	98	0	0	100	2	0	98
Furosemide	0	0	100	0	0	93	0	0	100
Gestrinone	0	0	95	0	0	97	0	0	83
Hydrochlorothiazide	0	0	68	0	0	100	79	0	0
Indapamide	0	0	90	0	0	97	0	0	7
Metipranolol	20	6	62	0	0	100	0	0	10
Metolazone	0	0	93	0	0	83	0	0	77
Metoprolol	9	20	63	6	6	88	0	0	89
Modafinil	0	0	88	55	0	45	0	0	100
Nadolol	47	22	13	29	3	68	0	0	100
Piretanide	0	0	96	0	0	98	20	14	49
Probenecid	0	0	88	0	0	99	0	0	98
Sotalol	75	14	1	67	8	25	78	12	0
Strychnine	0	0	82	0	0	99	0	0	83
Torasemide	1	0	86	0	0	96	5	1	86
Xipamide	0	0	92	0	0	98	53	0	28

elution was assessed with 900 μL of MeOH, and the second elution was performed with 900 μL of NH_4OH in MeOH (5:95, v/v) for MCX and WAX and with 900 μL of HCOOH in MeOH (2:98, v/v) for MAX and WCX. Extraction on Oasis HLB was evaluated with loading in acidic or neutral media. When loading in acidic conditions, the sorbent was conditioned with 500 μL of MeOH and equilibrated with 500 μL of HCl (120 mM). One thousand and five hundred microliters of acidified sample (120 mM HCl) was loaded. Washing was performed with 900 μL of HCOOH in water (2:98, v/v) and elution with 900 μL of MeOH. When loading in neutral conditions, the sorbent was conditioned with 500 μL of MeOH and equilibrated with 500 μL of water. A volume of 1500 μL of sample was loaded. Washing was performed with 900 μL of water and elution with 900 μL of MeOH. All fractions were evaporated to dryness from the 96-well collection plate using a Univapo 150 ECH (UniEquip, Martinsried, Germany) vacuum concentrator centrifuges set at 40 °C. Samples were reconstituted in 200 μL of water. Collection plates were directly used as injection vials for the UPLC.

2.2.2. Optimized SPE protocol (Oasis MCX)

The sample (urine spiked with all tested substances at 100 ppb each) was first centrifuged at 10,000 × *g* for 10 min, and 750 μL of HCl (240 mM) was added to 750 μL of the collected supernatant. The sorbent was conditioned with 500 μL of MeOH and equilibrated with 500 μL of HCl (120 mM). A volume of 1000 μL of the acidified sample was loaded and washed with a mixture of HCl (120 mM)–MeOH (90:10, v/v). First elution was performed with 250 μL of MeOH and the second elution with the same volume of NH_4OH in MeOH (5:95, v/v). Both elutions were evaporated to dryness with the same apparatus as used in Section 2.2.1 and reconstituted in 50 μL of a mixture of water–MeOH (50:50, v/v).

2.3. Chromatographic separation

Chromatographic separation was optimized with Osiris (Datalys, Grenoble, France), a HPLC modeling software.

Analyses were performed on a Waters Acquity UPLC system (Milford, MA, USA) equipped with a binary solvent manager, an auto-sampler with a 2 μL injection loop and a stainless steel needle (allowing for injections directly from 96-well plates capped with a silicon sealing cap), and a UV–vis programmable detector, including a 500 nL flow cell. The Empower Software was used for instrument control, data acquisition and data handling.

For all separations, a volume of 1 μL was injected on an Acquity BEH Shield RP18, 2.1 mm × 50 mm, 1.7 μm column used with a gradient of acetonitrile in a ammonium buffer (50 mM, pH 10) from 11.3% to 51.2% in 2.2 min delivered at 900 μL min^{-1}. UV detection was operated at 254 nm with a 25 ms time constant and data sampling rate set at 80 Hz.

2.4. Matrix effect evaluation

All experiments for matrix effect evaluation were performed on an Agilent Series 1100 LC system (Agilent Technologies, Waldbronn, Germany) equipped with an auto-sampler and a binary pump. Five microliters of the sample was injected on a XBridge Shield 100 mm × 2.1 mm, 3.5 μm analytical column from Waters. The mobile phase (acetate buffer 20 mM pH 5/ACN 67/33 (v/v)) was delivered in the isocratic mode at 300 μL min^{-1}. The LC system was coupled to an Agilent Series 1100 MSD single quadrupole equipped

Table 3
Recovery values of all fractions with MCX, WAX, MAX and WCX sorbents.

Compound	MCX				WAX				MAX				WCX			
	Load (%)	Wash (%)	Elute 1 (%)	Elute 2 (%)	Load (%)	Wash (%)	Elute 1 (%)	Elute 2 (%)	Load (%)	Wash (%)	Elute 1 (%)	Elute 2 (%)	Load (%)	Wash (%)	Elute 1 (%)	Elute 2 (%)
Acebutolol	0	0	0	98	29	24	29	1	77	0	11	0	0	0	16	59
Acetazolamide	0	0	35	0	0	0	44	0	0	0	0	46	0	53	0	0
Adrafinil	0	0	99	1	0	0	13	87	0	9	0	91	14	61	24	1
Atenolol	0	0	31	69	76	0	8	0	100	0	0	0	0	0	64	13
Bendroflumethiazide	0	0	42	4	0	0	85	0	0	0	0	100	0	0	100	0
Benzoylecgonine	23	0	3	74	62	21	18	0	71	0	13	16	35	9	18	0
Betamethasone	0	0	100	0	0	0	100	0	0	0	100	0	0	0	98	2
Bumetanide	0	0	91	2	0	0	15	85	0	0	0	100	36	41	17	5
Canrenone	0	0	10	0	0	0	100	0	0	0	100	0	0	0	100	0
Carteolol	0	0	0	98	44	28	16	0	94	0	5	1	0	0	84	2
Celiprolol	0	1	0	99	36	24	33	0	78	0	14	0	0	0	93	1
Chlorothiazide	0	0	95	0	0	0	76	9	0	0	0	48	0	100	0	0
Chlortalidone	0	0	99	1	0	0	98	2	0	0	5	87	10	66	22	2
Clopamide	0	0	94	6	23	0	73	4	18	0	2	80	7	91	2	0
Dexamethasone	0	0	100	0	0	0	97	0	0	0	61	18	0	51	48	1
Dichlorphenamide	0	0	83	1	0	0	79	3	0	0	0	71	6	72	7	3
Esmolol	0	0	0	85	40	28	21	0	67	0	17	0	0	0	58	16
Ethacrynic acid	27	0	70	2	0	0	0	82	0	0	0	19	57	18	3	1
Finasteride	0	0	97	3	0	0	97	3	0	0	97	3	0	0	97	3
Furosemide	0	0	97	3	0	0	13	87	0	3	0	97	14	59	23	4
Gestrinone	0	0	80	2	0	0	75	0	0	0	80	2	0	92	8	0
Hydrochlorothiazide	0	0	90	2	0	0	92	2	10	0	0	90	27	71	2	0
Indapamide	0	0	98	2	0	0	90	3	0	0	0	75	0	24	45	1
Metipranolol	0	0	0	100	0	59	39	0	0	0	45	0	0	0	42	58
Metolazone	0	0	91	1	0	0	81	1	0	0	0	71	0	20	49	1
Metoprolol	0	0	0	100	49	21	28	2	84	0	15	1	0	0	1	99
Modafinil	4	0	70	1	14	0	13	1	10	0	85	1	11	0	82	2
Nadolol	0	0	0	100	52	0	0	0	100	0	0	0	0	98	0	2
Piretanide	0	0	75	0	0	0	9	81	0	0	0	84	51	44	3	1
Probenecid	0	0	88	2	0	0	8	82	0	0	0	78	0	23	25	6
Sotalol	0	0	0	100	62	28	10	0	94	0	0	5	0	95	1	4
Strychnine	0	0	1	93	35	27	18	0	59	0	5	0	0	54	26	8
Torasemide	0	0	0	100	0	0	80	11	0	4	0	87	0	41	52	4
Xipamide	0	0	97	3	0	0	6	94	0	0	0	100	10	68	14	8

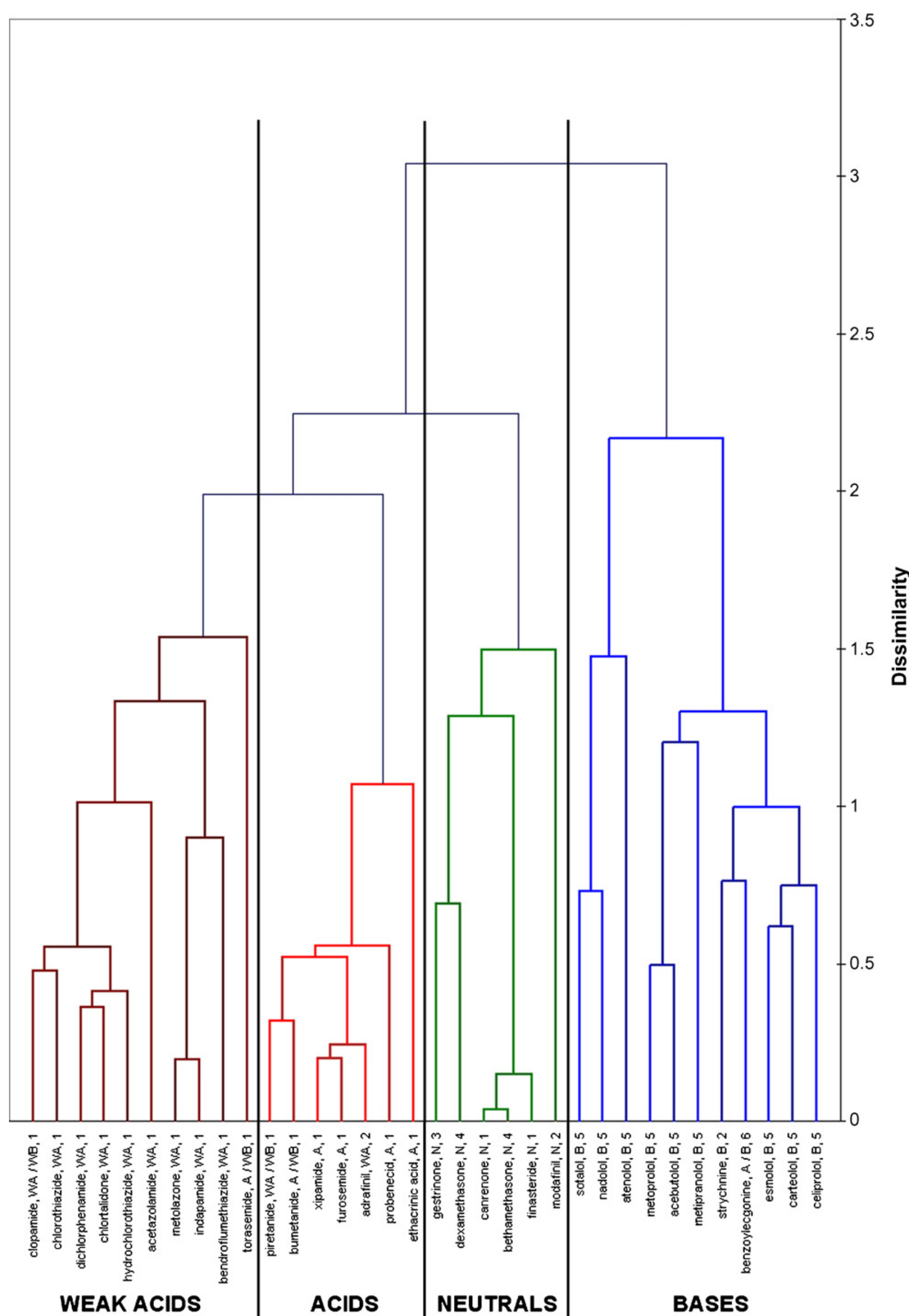

Fig. 1. Dissimilarity dendrogram for SPE behavior. A = acid, B = base, N = neutral, WA = weak acid, WB = weak base, 1 = diuretic, 2 = stimulant, 3 = anabolic agent, 4 = corticosteroid, 5 = β-blocker, 6 = narcotic.

with an orthogonal ESI source. Nitrogen was used as both nebulizing (5 L min^{-1}) and drying gas (250 °C). Vaporizer temperature was set at 250 °C, nebulizer pressure at 45 psig and capillary voltage at +2000 V. Detection of protonated analytes was always conducted in the selected ion monitoring (SIM) mode. The Chemstation A.10.03 software (Agilent Technologies) was used for instrument control, data acquisition and data handling.

2.4.1. Neat standards

Five microliters of each stock solution at 10,000 μg mL^{-1} was added to 1000 μL of water. Seven hundred and fifty microliters of HCl (240 mM) was added to 750 μL of the neat standard and vortex mixed. One thousand microliters was finally extracted with the optimized protocol described in Section 2.2.2.

2.4.2. Pre-extraction spiked urine

One thousand microliters of blank urine was spiked with 5 μL of each stock solution at 10,000 μg mL^{-1}. The sample was then centrifuged at 10,000 × g for 10 min. Seven hundred and fifty microliters of HCl (240 mM) was added to 750 μL of the collected supernatant and vortex mixed. One thousand microliters was finally extracted with the optimized protocol described in Section 2.2.2.

2.4.3. Post-extraction spiked urine

One thousand microliters of blank urine was centrifuged at 10,000 × *g* for 10 min. Seven hundred and fifty microliters of HCl (240 mM) was added to 750 μL of the collected supernatant and vortex mixed. One thousand microliters was extracted with the optimized protocol described in Section 2.2.2. Finally, the resulting elutions were spiked with 5 μL of a standard solution at 200 μg mL^{-1} of each corresponding analyte.

2.5. Data handling software

Data analysis, including principal component analysis (PCA) and hierarchical cluster analysis (HCA), was performed with the XLStat 7.5.3 (AddinSoft, France) software package. For HCA, a flexible linkage ($\beta = -0.1$) aggregation based on Euclidean distance was selected.

3. Results and discussion

3.1. Compound descriptions

Clinical, forensic, and anti-doping laboratories currently employ different screening methods for the analysis of drug substances in urine. In this paper, the determination of a mixture of 34 molecules was studied constituted by 18 diuretics (12 acids, 2 neutrals and 4 bases), 9 β-blockers (ampholytes), 2 corticosteroids (bases), 3 stimulants (1 acid, 1 neutral and 1 basic), 1 narcotic (ampholyte) and 1 anabolic agent (neutral). Thus, acidic compounds and ampholytes represent the majority of studied analytes (Table 1). In regards to polarity, 27 molecules show high $\log P$ values ($\log P \geq 1$) and 7 exhibit intermediate polarities ($-1.0 < \log P < 1.0$), whereas no highly polar molecules are present.

The study presented in this paper will focus on the compounds that must be extracted and analyzed with a generic confirmatory analysis when a positive result is evidenced during the screening step.

3.2. Sorbent selection

Since the physico-chemical properties of the analytes are quite diverse, a stationary phase able to provide hydrophobic and hydrophilic interactions was initially selected (HLB). Compounds were first extracted using a generic protocol provided by the manufacturer. Each of the three SPE solutions (loading, washing and elutions) was collected, analyzed, and recoveries (expressed in %) were calculated for each compound (Table 2). To evaluate the best retention conditions for the loading step, various pH conditions were tested (pH 2.5, 7, and 10). At acidic and neutral pH, basic compounds were inadequately retained. At basic pH, weak acids with low polarity were eluted during the loading step, as evidenced for chlorthalidone ($\log D = -1.4$) and hydrochlorothiazide ($\log D = -1.8$). Oasis HLB was therefore not suitable for quantitative extraction of this compound's set.

In the second step, strong and weak cation exchangers (MCX and WCX, respectively), and strong and weak anion exchangers (MAX and WAX, respectively) were tested. As expected, recoveries from the various fractions demonstrated that MAX and WAX sorbents were unable to retain basic compounds, whereas the weak cation exchanger (WCX) was unable to keep acidic compounds during the loading step. However, the Oasis MCX sorbent allowed good retention and elution of almost all compounds, and was therefore selected as the sorbent of choice for the remainder of the study (Table 3). This support probably presents the best compromise when a complex mixture of analytes is considered, as demonstrated elsewhere [21]. An optimization of the generic extraction protocol was achieved on the basis of this sorbent selection. In order to rapidly develop a generic method, a restricted number of compounds was selected as representative substances.

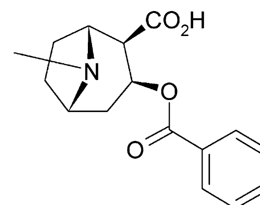

Fig. 2. Benzoylecgonine chemical structure.

3.3. Selection of representative compounds

For subsequent SPE optimization, representative compounds were selected. To identify groups of compounds presenting similar SPE behavior, it is possible to obtain from the literature physico-chemical data (*i.e.* pK_a, $\log D$ values) and cluster compounds with the help of chemometric tools as already reported [22]. However, two major restrictions were identified. First, missing values were generally observed and the reliability of data determined by mathematical algorithms could significantly differ from the actual value or be uncertain in cases of particular chemical structures (*e.g.* cyclization). In this paper, group clustering was achieved owing to the analysis of recoveries for all compounds in each fraction obtained during the sorbent selection step. Due to the large number of values (544), multivariate analysis was achieved on the entire data set with a hierarchical cluster analysis. Global variation is summarized on a dendrogram, where dissimilarity between scores is indicated *via* the distance between the branches of the tree. The recovery values on all tested sorbents were necessary to obtain better differentiation among compounds than that brought by Oasis MCX only. For the purpose of clarity, compound names as well as therapeutic and chemical families were identified (Fig. 1). Four main groups clearly emerged, corresponding to main chemical classes (7 acids, 10 weak acids, 6 neutrals and 11 bases) because these four chemical classes presented different SPE behaviors leading to clear clustering.

Acids and weak acids were evidenced by a different SPE behavior brought by extraction results from WAX and MAX sorbents. The observed clustering confirmed that evaluation of fundamental physico-chemical properties was the simplest method to determine classification in SPE. Therefore, screening analyses should be preferentially operated by considering the chemical class rather than the therapeutic one. Moreover, the presence of zwitterionic compounds, such as benzoylecgonine, emphasized the importance of clustering compounds by their practical SPE behavior rather than on values from the literature. Indeed, benzoylecgonine behaved like a base, whereas it presents both acidic and basic functions (Fig. 2). Finally, one compound per group was selected for protocol optimization by taking into account $\log P$ and $\log D$ values. Betamethasone, chlortalidone, ethacrynic acid, and metoprolol were chosen. For instance, the choice of ethacrynic acid was made to characterize the group of acids because it presents average $\log P$ (3.38) and $\log D$ values (2.9, −0.8, and −1.5, respectively at pH 1, 7, and 10, see Table 1). Betamethasone, chlorthalidone and metoprolol were chosen following the same criteria.

Time required for the LC separation of all four representatives was also taken into account. Indeed, optimizing a protocol is time-consuming and must be performed on a large number of compounds. Therefore, representative analytes were chosen to be separated in a short time in the simple isocratic mode.

Table 4
Recovery values in the loading step with four different urines at (A) pH 5.3, (B) pH 7.8, (C) pH 6.8, and (D) pH 6.3.

	Recovery (%)			Mean (%)	RSD (%)
(A)					
Betamethasone	3	5	6	5	0
Chlortalidone	4	5	7	5	0
Ethacrynic acid	5	9	8	7	0
Metoprolol	0	1	0	0	2
(B)					
Betamethasone	1	4	7	4	1
Chlortalidone	6	2	3	4	1
Ethacrynic acid	7	9	6	7	0
Metoprolol	0	0	0	0	0
(C)					
Betamethasone	2	7	4	4	1
Chlortalidone	4	6	3	4	0
Ethacrynic acid	8	5	6	6	0
Metoprolol	0	0	0	0	0
(D)					
Betamethasone	4	8	1	4	1
Chlortalidone	3	1	7	4	1
Ethacrynic acid	9	5	8	7	0
Metoprolol	0	0	0	0	0

3.4. Sample preparation optimization

3.4.1. SPE

Each step was optimized with the selected analytes taking into account pH, volume, nature, and proportion of the solvent mixture. Regarding loading, a strong adjustment had to be planned, as urine pH can vary from 4 to 9. Acidification was chosen in agreement with the retention principle of the sorbent and as the most reported pre-treatment for extraction on MCX [23,24]. The sample was therefore loaded after dilution (50:50, v/v) with HCl (240 mM) and several urines with various pH were investigated. At these conditions, no compound loss was observed in the loading step (Table 4). The washing step was also optimized regarding pH and organic solvent content, and 10% of methanol in washing solution was further used. Finally, both elutions were optimized regarding the volumes used of methanol and basified methanol with 5% ammonia. Increasing volumes of methanol (50 μL, 100 μL, 150 μL, 200 μL, 250 μL, 500 μL, 750 μL, and 1000 μL) were tested. It appeared that, for both elutions, a volume of 250 μL of solvent was the lower limit, allowing 100% recovery and this volume was therefore considered optimal.

3.4.2. Reconstitution

Both elutions required a high amount of methanol, which can induce further chromatographic issues. Therefore, the reduction of organic content in injection solvent was tested. As expected, dilution with water while maintaining a constant injection volume was found to be fast and easy, but caused sensitivity loss. Thus, evaporation to dryness was selected for sample concentration and a complete plate required about 2.3 h (mainly due to the above-mentioned evaporation step), corresponding to less than 2 min per sample. Reconstitution was evaluated with increasing volumes (25 μL, 50 μL, 100 μL, 150 μL, 200 μL, and 250 μL), and increasing proportions of MeOH (20%, 50% and 100%) and the best compromise was found to be 50 μL of a mixture of water–MeOH (50:50, v/v) (Table 5). Finally, the optimized method required sample centrifugation at 10,000 × g for 10 min. Seven hundred and fifty microliters of HCl (240 mM) was added to 750 μL of the collected supernatant. The sorbent was conditioned with 500 μL of MeOH and equilibrated with 500 μL of HCl (120 mM). One thousand microliters of the acidified sample was loaded and washed with a mixture of HCl (120 mM)–MeOH (90:10, v/v). First elution was operated with 250 μL of MeOH, and the second elution with the same volume of 5% NH_4OH in MeOH. Both elutions were evaporated to dryness with the same apparatus used in Section 2.1 and reconstituted in 50 μL of a mixture of water–MeOH (50:50, v/v).

Table 5
Reconstitution yield with 50 μL of water/MeOH 50:50.

	Recovery (%)			Mean (%)	RSD (%)
Betamethasone	105	96	99	100	5
Chlortalidone	102	95	98	98	4
Ethacrynic acid	98	100	99	99	1
Metoprolol	96	104	98	99	4

The optimized method was tested on the full set of compounds. Thus, a neat extraction standard of the 34 analytes was extracted in triplicate following the optimized protocol (illustration 1 in Fig. 3a). Recoveries were calculated in comparison to a neat standard solution (illustration 2 in Fig. 3a) and were comprised in the 80–110% range with repeatability RSD < 10% for all compounds, demonstrating a good method transfer from the four representatives to the full set of analytes.

3.5. Matrix effects

Matrix effects were evaluated and their origin determined *via* a method based on Matuszewski et al. [18]. First, the process efficiency was considered in urine samples. A urine sample spiked with all analytes was extracted in triplicate (pre-extraction spiked urine, illustration 3 in Fig. 3a) and compared to a neat standard solution (illustration 2 in Fig. 3a). Process efficiencies were determined by 3/2 ratios (Fig. 3b) and ranged from 10% to 97% with RSD values <27% (Table 6), indicating an effect of the matrix on the extraction process and/or on MS ionization. In order to determine the matrix effect

Table 6
Process efficiency expressed as matrix effect on MS and on SPE with repeatability values (RSD) for each evaluated compound.

Compound	Process efficiency (RSD) (%)	MS (RSD) (%)	SPE (RSD) (%)
Acebutolol	59 (2)	65 (3)	90 (4)
Acetazolamide	26 (10)	28 (6)	92 (10)
Adrafinil	54 (3)	55 (2)	98 (6)
Atenolol	48 (6)	53 (3)	91 (7)
Bendroflumethiazide	69 (6)	72 (2)	96 (5)
Benzoylecgonine	46 (5)	49 (8)	91 (1)
Betamethasone	77 (27)	72 (33)	106 (4)
Bumetanide	82 (1)	86 (5)	95 (7)
Canrenone	69 (11)	71 (10)	99 (5)
Carteolol	64 (4)	64 (9)	100 (7)
Celiprolol	71 (3)	79 (5)	90 (4)
Chlorothiazide	24 (8)	26 (5)	91 (4)
Chlorthalidone	75 (2)	65 (10)	114 (10)
Clopamide	35 (2)	38 (4)	91 (3)
Dexamethasone	79 (3)	80 (1)	98 (3)
Dichlorphenamide	86 (5)	78 (4)	109 (6)
Esmolol	10 (12)	9 (4)	109 (11)
Ethacrynic acid	52 (8)	56 (6)	93 (10)
Finasteride	73 (11)	75 (12)	94 (5)
Furosemide	83 (1)	98 (4)	85 (3)
Gestrinone	74 (10)	74 (15)	100 (5)
Hydrochlorothiazide	62 (2)	55 (6)	113 (4)
Indapamide	97 (16)	94 (13)	98 (7)
Metipranolol	56 (16)	61 (5)	93 (12)
Metolazone	33 (20)	26 (15)	111 (10)
Metoprolol	27 (7)	30 (3)	89 (7)
Modafinil	96 (11)	83 (10)	107 (15)
Nadolol	54 (10)	53 (2)	97 (10)
Piretanide	51 (25)	46 (8)	93 (9)
Probenecid	93 (3)	100 (3)	93 (10)
Sotalol	58 (15)	51 (6)	109 (9)
Strychnine	36 (6)	39 (15)	94 (9)
Torasemide	57 (8)	66 (7)	85 (5)
Xipamide	68 (5)	67 (2)	101 (7)

Table 7
Summary of matrix effect cases (negative effect (−), no effect (0), positive effect (+)).

	Case							
	1	2	3	4	5	6	7	8
Process efficiency	−1	−1	−1	−1	0	0	1	1
MS	−1	−1	0	1	0	1	1	1
SPE	−1	0	−1	−1	0	−1	−1	0

origin for each compound, a blank urine sample was extracted and spiked with the considered analytes (post-extraction spiked urine, illustration 4 in Fig. 3a). The influence of the matrix on the SPE process was determined by 3/4 ratios, since in this case, matrix effects on LC-MS were similar with both samples (all analytes were dissolved in extracted urine). In this study, urine was not considered to affect the extraction process because values of the 3/4 ratio were all comprised between 85% and 114% with repeatability RSD < 15%. Finally, matrix effects on LC-MS were estimated through 4/2 ratios, as they compared the analytical response given by a neat standard and the same solution added in extracted urine. The latter effects were found to contribute the most to the low process efficiency values (Table 6).

However, other situations emerged, such as the case of furosemide, where matrix effects were observed only during the SPE step. Metoprolol and torasemide presented matrix effects during both SPE and analysis steps, while indapamide, modafinil and probenecid were not subjected to any matrix effect. Since various combinations of matrix effects on SPE and on the analysis came out, a classification of all possibilities encountered when a sample preparation is performed prior to the analysis is proposed, still based on the protocol developed by Matuszewski et al. The effect of the matrix on SPE can only be negative (−1) or null (0), since analytes can compete for access to interaction sites. Matrix effect on ionization can be negative (−1), null (0), or positive (+), since signal suppression, no signal alteration, or signal enhancement can occur with MS detection. These different situations lead to eight possible cases, summarized in Table 7. A negative process efficiency can be due to the combination of a negative effect of the matrix on SPE and a signal suppression (case 1), only signal suppression (case 2),

Table 8
Matrix effect case reported for each analyte (bold: representative compound).

Compound	Case	Group
Acebutolol	1	B
Acetazolamide	2	WA
Adrafinil	2	A
Atenolol	2	B
Bendroflumethiazide	2	WA
Benzoylecgonine	2	B
Betamethasone	2	N
Bumetanide	2	A
Canrenone	2	N
Carteolol	2	B
Celiprolol	2	B
Chlorothiazide	2	WA
Chlortalidone	2	WA
Clopamide	2	WA
Dexamethasone	2	N
Dichlorphenamide	2	WA
Esmolol	2	B
Ethacrynic acid	2	A
Finasteride	2	N
Furosemide	3	A
Gestrinone	2	N
Hydrochlorothiazide	2	WA
Indapamide	5	WA
Metipranolol	2	B
Metolazone	2	WA
Metoprolol	1	B
Modafinil	5	N
Nadolol	2	B
Piretanide	2	A
Probenecid	5	A
Sotalol	2	B
Strychnine	2	B
Torasemide	1	WA
Xipamide	2	A

only negative effect of the matrix on SPE (case 3) or a combination of a negative effect of the matrix on SPE heavier than signal enhancement (case 4). By the thought process, a good process efficiency around 100% can reflect an absence of matrix effect (case 5), but may also be due to a contribution of a negative effect on

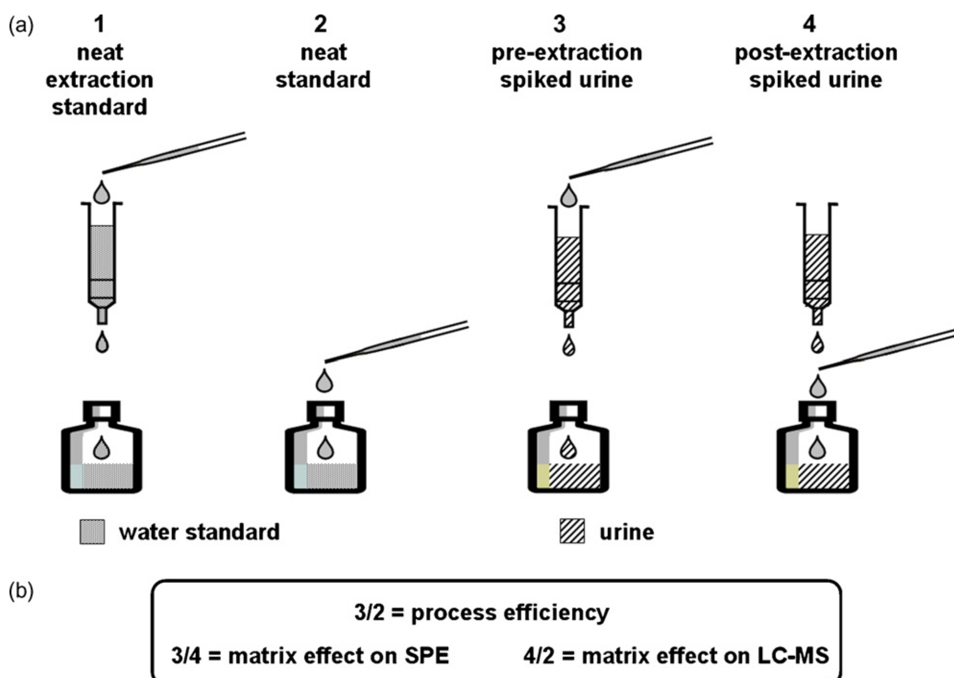

Fig. 3. Schematic illustration of experiments for matrix effects evaluation.

the SPE balanced by a signal enhancement (case 6). Finally, a positive process efficiency should be reasonably attributed to signal enhancement exceeding a negative effect on SPE (case 7) or to signal enhancement only (case 8). Thus, process efficiencies, matrix effects on the SPE step, and signal alterations were used to define the situation of each compound, summarized in Table 8. A reasonable limit was set at ±10% to determine if an effect was significant. It is revealed that there is no correlation between matrix effects and the groups of compounds determined in Section 3.2. Therefore, representative compounds cannot be used for matrix effect evaluation, the latter being specific to each compound and needing to be estimated individually. Finally, it must be emphasized that no compound presented signal enhancement, certainly due to the use of the ESI source, which is more susceptible to signal suppression than signal enhancement, explaining the absence of cases 4, 6, 7 and 8.

In conclusion, the extraction of urine samples was found to have only a low influence on the SPE process (4 cases over 34), but endogenous compounds were not completely removed and produced matrix effects during LC-MS analysis. Subsequent quantification would not be affected when deuterated internal standards are used, at least for compounds subject to matrix effects (all compounds except indapamide, modafinil and probenecid). If these standards are not available, quantification should absolutely be achieved within the matrix to prevent from the important matrix effect. Finally, it has to be noted that the optimization of extraction could be rapidly achieved by the help of representative analytes, whereas matrix effects must be evaluated for each analyte, as physico-chemical properties do not allow a good prediction.

4. Conclusion

The main objective of this work was to propose the use of representative compounds to perform easier and faster optimization of sample preparation in the case of multianalytes determination in a complex matrix such as urine. All compounds were first extracted onto different sorbents. From this data set, four main groups of compounds presenting similar SPE behaviors were brought out with the help of a chemometric tool. For each group of analytes, one compound was chosen as representative and used for subsequent protocol optimization. The optimized SPE protocol allows a complete preparation of 96 samples in less than 2 min per sample.

The optimized protocol was tested on a neat standard solution of the 34 compounds and provided excellent recovery and repeatability, proving good representation of the entire set by the four selected compounds. Matrix effects were carefully evaluated, and it was determined that most of the compounds were subject to signal suppression, indicating the difficulty in removing interferents when a complex mixture of compounds with various properties has to be extracted. A classification of probable matrix effects encountered during sample preparation prior to the analysis was then proposed, including eight different cases. It emphasized that matrix effects should be evaluated for each compound individually, since representative compounds were not adapted for matrix effect determination.

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References

[1] Y. Hsieh, M. Chintala, H. Mei, J. Agans, J.M. Brisson, K. Ng, W.A. Korfmacher, Rapid Commun. Mass Spectrom. 15 (2001) 2481–2487.
[2] I. Fu, E.J. Woolf, B.K. Matuszewski, J. Pharm. Biomed. Anal. 18 (1998) 347–357.
[3] C. Muller, P. Schafer, M. Stortzel, S. Vogt, W. Weinmann, J. Chromatogr. B 773 (2002) 47–52.
[4] C.G. Enke, Anal. Chem. 69 (1997) 4885–4893.
[5] S. Souverain, C. Eap, J.l. Veuthey, S. Rudaz, Clin. Chem. Lab. Med. 41 (2003) 1615–1621.
[6] D.A. Wells, High Throughput Bioanalytical Sample Preparation: Methods and Automation Strategies, Elsevier, New York, 2003.
[7] P. Lopez-Roldan, M.J. Lopez de Alda, D. Barcelo, Anal. Bioanal. Chem. 378 (2004) 599–609.
[8] A. Pena, F. Ruano, M.D. Mingorance, Anal. Bioanal. Chem. 385 (2006) 918–925.
[9] C. Lopez-Blanco, S. Gomez-Alvarez, M. Rey-Garrote, B. Cancho-Grande, J. Simal-Gandara, Anal. Bioanal. Chem. 384 (2006) 1002–1006.
[10] S. Ito, K. Tsukada, J. Chromatogr. A 943 (2001) 39–46.
[11] E.T. Gangl, M. Annan, N. Spooner, P. Vouros, Anal. Chem. 73 (2001) 5635–5644.
[12] R. Pascoe, J.P. Foley, A.I. Gusev, Anal. Chem. 73 (2001) 6014–6023.
[13] H. Mei, Y. Hsieh, C. Nardo, X. Xu, S. Wang, K. Ng, W.A. Korfmacher, Rapid Commun. Mass Spectrom. 17 (2003) 97–103.
[14] J. Zrostlikova, J. Hajslova, J. Poustka, P. Begany, J. Chromatogr. A 973 (2002) 13–26.
[15] B.K. Choi, A.I. Gusev, D.M. Hercules, Anal. Chem. 71 (1999) 4107–4110.
[16] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175–1185.
[17] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942–950.
[18] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019–3030.
[19] E. Chambers, D.M. Wagrowski-Diehl, Z. Lu, J.R. Mazzeo, J. Chromatogr. B 852 (2007) 22–34.
[20] D.W. Brousmiche, J.E. O'Gara, D.P. Walsh, P.J. Lee, P.C. Iraneta, B.C. Trammell, Y. Xu, C.R. Mallet, J. Chromatogr. A 1191 (2008) 108–117.
[21] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, Anal. Bioanal. Chem. 391 (2008) 1293–1308.
[22] A. Pagliara, E. Khamis, A. Trinh, P.A. Carrupt, R.S. Tsai, B. Testa, J. Liq. Chromatogr. 18 (1995) 1721–1745.
[23] R. Coles, E.D. Kharasch, J. Chromatogr, B: Anal. Technol. Biomed. Life Sci. 857 (2007) 67–75.
[24] D. Siluk, D.E. Mager, N. Gronich, D. Abernethy, I.W. Wainer, J. Chromatogr. B 859 (2007) 213–221.