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Optimization of pressurized liquid extraction using a multivariate chemometric approach for the determination of anticancer drugs in sludge by ultra high performance liquid chromatography–tandem mass spectrometry

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A B S T R A C T

The present paper describes an analytical method for the determination of 2 widely administered anticancer drugs, ifosfamide and cyclophosphamide, contained in sewage sludge. The method relies on the extraction from the solid matrix by pressurized liquid extraction, sample purification by solid-phase extraction and analysis by ultra high performance liquid chromatography coupled with tandem mass spectrometry. The different parameters affecting the extraction efficiency were optimized using an experimental design. Solvent nature was the most decisive factor for the extraction but interactions between some parameters also appeared very influent. The method was applied to seven different types of sludge for validation. The performances of the analytical method displayed high variability between sludges with limits of detection spanning more than one order of magnitude and confirming the relevance of multi-sample validation. Matrix effect has been determined as the most limiting analytical step for quantification with different extent depending on analyte and sludge nature. For each analyte, the use of deuterated standard spiked at the very beginning ensured the complete compensation of losses regardless of the sample nature. The suitability of the method between freshly spiked and aged samples has also been verified. The optimized method was applied to different sludge samples to determine the environmental levels of anticancer drugs. The compounds were detected in some samples reaching 42.5 $\mu\text{g}/\text{kg}_{\text{DM}}$ in ifosfamide for the most contaminated sample.

Keywords:

Anticancer drugs
Sludge
Experimental design
Pressurized liquid extraction
Ultra high performance liquid chromatography
Tandem mass spectrometry

1. Introduction

Pharmaceutical residues in the environment and their possible biological or side effects on non-target organisms are an emerging research in environmental sciences [1]. The interest about their occurrence, their fate and their toxicity in the environment really took off at the end of 1990s and the number of publications has been constantly increasing since then [2].

After administration, large fractions of pharmaceuticals are not completely assimilated or metabolized in the body and then excreted as parent compounds or metabolites via urine and feces [3]. These compounds are collected and mixed in wastewaters, in which their concentrations can reach some $\mu\text{g}/\text{L}$ [4]. Pharmaceutical compounds suffer from partial removal during activated sludge treatment, the most common wastewater treatment plant (WWTP). Consequently, WWTP effluents are recognized as the primary spreading source of pharmaceutical pollution in the environment.

During activated sludge treatment, trace pollutants can mainly be affected by three mechanisms: volatilization, biodegradation or sorption onto sludge, depending on both compound and sludge physico-chemical properties. Therefore, volatilization is usually neglected for pharmaceuticals because of low Henry's constant [5]. While biodegradation has sometimes the signification of complete elimination, sorption onto sludge can be considered as a

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displacement of the pollution from the aqueous to the solid phase. Monitoring trace pollutants in solid part could be of crucial importance because of (1) possible influence toward bioavailability (i.e. biodegradation) to microorganisms and (2) stabilized-sludge landfill applications which can introduce sludge-born trace pollutants in the environment, increasing potential exposure risks. Consequently, investigating occurrence of trace compounds in biosolids could be a key factor for (1) upgrading WWTPs and trace pollutants removal and (2) the establishment of new regulations which are only focused on heavy metals, polychlorobiphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) for trace pollutants in sludge-amended soil applications.

Among the broad spectrum of available pharmaceutical products, there is still one class that paid little attention in spite of an environmentally devastating potential: the anticancer drugs. Including antineoplastic and endocrine-therapy drugs, these compounds are designed to prevent or disrupt cellular proliferation in cancer treatment schemes [6]. Unlike some other therapeutic classes, anticancer drugs exhibit very different physico-chemical properties. To name a few, some examples are log K_{ow} ranging from -2.46 to 6.3 and pK_a ranging from 1.45 to 9.8 [2]. Most of the anticancer drugs possess a strong carcinogenic, mutagenic and teratogenic potential and are thought as one of the most hazardous contaminants in water cycle [7]. Due to their mode of action, it is assumed that almost all eukaryotic organisms are vulnerable to genetic damages at very low concentrations [8]. As highlighted by the literature, their consumption is increasing and trends, including type of consumed drugs and practices of consumption, are diversifying [6].

The monitoring of anticancer drugs in the environment has encountered a tremendous interest for the last 3 years. Comprehensive overviews including analytical methods for their analysis [9], data about their environmental occurrence and fate [2] and assessment of environmental exposure [6] have been published under this period. These states-of-art revealed that environmental occurrence of anticancer drugs in water samples are few documented but data about their occurrence in solid samples are definitively scarce. Although analytical development for their determination in liquid samples is still of concern but fairly common, there is a great need of accurate analytical method focused on their detection in more challenging matrices such as solid part of sludge.

Performing extraction of trace pollutants from solid matrices is not easy to handle. A variety of procedures has been defined in the literature and can be divided in two distinct groups: classical and recent extraction techniques [10]. Classical techniques include mechanical stirring, Soxhlet and Soxtec, and ultrasound extraction (USE), the later has been used one time for the extraction of anticancer drugs in sludge samples [11]. Most of them are labor-intensive, time-consuming and require large amounts of solvents. Their application to solids is noticeably dropped and replaced with more time-saving and eco-friendly processes. Recent extraction techniques include micro-wave assisted extraction (MWE) and pressurized liquid (including hot water) extraction (PLE) among many examples. A comprehensive overview about the extraction of trace pollutants from sludge according different extraction techniques is available [10]. Due to the increasing number of published papers, PLE and its derivatives appear as the most promising technique for efficient extraction [12,13]. Up to now, only one application of PLE has been reported for the extraction of anticancer drugs in sludge samples [14].

Depending on the extractive conditions applied, the recovery of variable amounts of co-interfering compounds during PLE is possible [15]. To address this well-known drawback, a clean-up extract is often required. In most of cases, this step is performed by solid-phase extraction (SPE). With the emergence of mixed-mode SPE implying polar, non-polar and ionic interactions with the sorbent,

selective purification is allowed. Thus, mixed-mode SPE could be promising for recovering analytes with different physico-chemical properties and enhancing method specificity. Mixtures containing anticancer drugs are usually separated by liquid chromatography [2,9]. The trace level occurrence of these drugs in environmental samples justifies the use of sophisticated systems such as mass spectrometry (MS) detection. Thus, ultra high pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) appears as the most powerful and adequate tool for fast separation and very selective and sensitive detection in complex matrices [10].

In light of these concerns, one of our objectives was to develop and validate an analytical method for determining the occurrence of anticancer drugs in the solid part of sludge. The drugs of interest are the alkylating cytotoxics cyclophosphamide (CP) and ifosfamide (IFO) and the antiestrogen hormonally active tamoxifen (TAM). Some of their relevant physico-chemical properties are given in Fig. 1. Among all the anticancer drugs, the investigation of their fate has recently been defined as preferential due to their consumption data, their behavior in WWTP and related predicted environmental concentrations (PEC) in the literature [6]. The method is based on the extraction from the solid matrix using semi-exhaustive PLE, extract clean-up using tandem Oasis MAX/MCX selective SPE and analysis by UHPLC-MS/MS. To highlight the influence of experimental conditions, optimization of PLE parameters was realized according to an experimental design. The method was validated for seven sludge samples representative of the French WWTPs profile. Some efforts have been carried out to identify which analytical step was detrimental in the determination of anticancer drugs. The use of deuterated standards has also been applied to check for possible complete compensation of losses through the analytical procedure. To our knowledge, this is the first time that anticancer drugs have been investigated in sludge originated from French WWTPs.

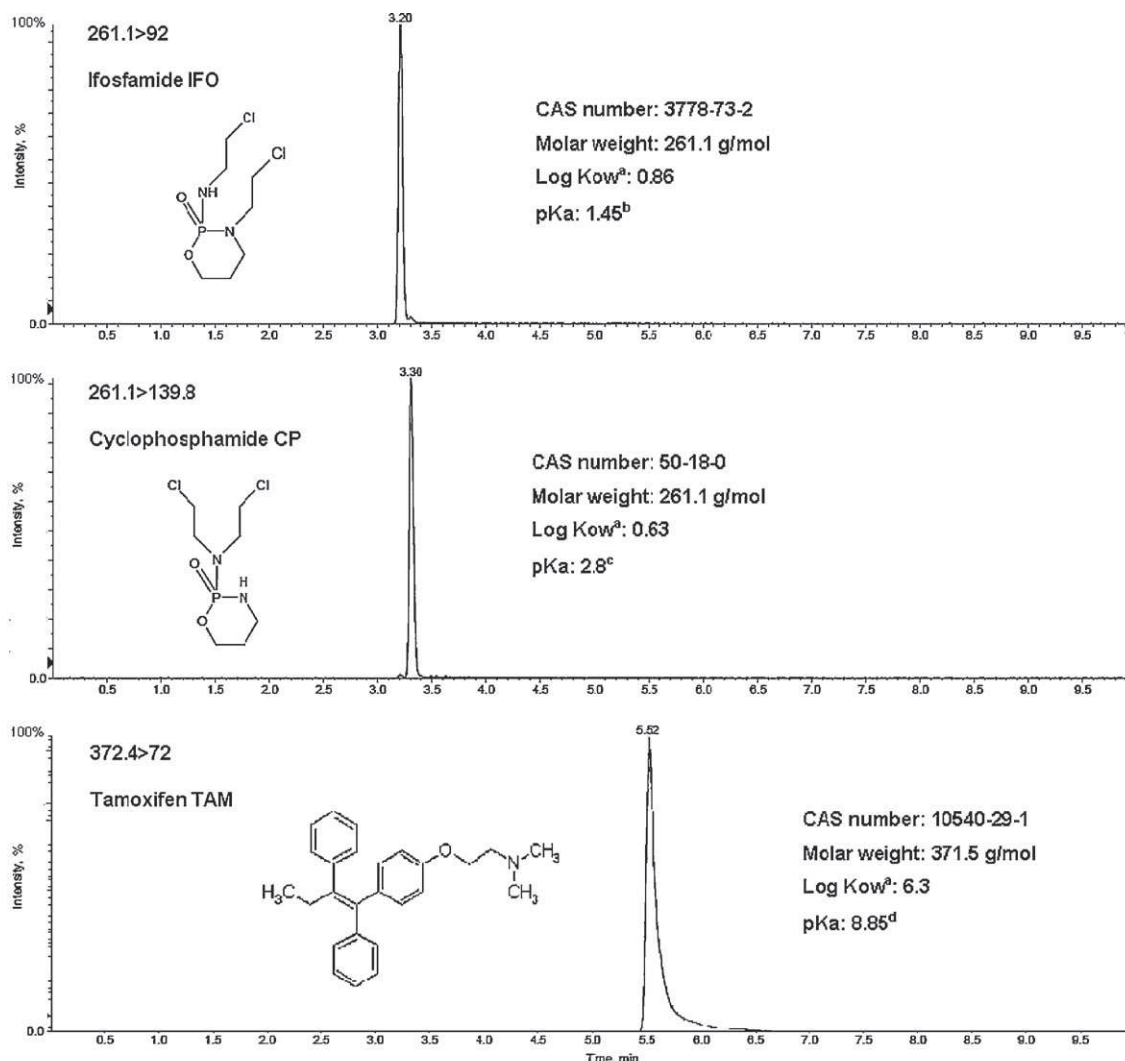
2. Experimental

2.1. Analytical standards and chemicals

Analytical standards cyclophosphamide monohydrate (CP), ifosfamide (IFO), tamoxifen (TAM) were purchased from Sigma-Aldrich (Saint-Quentin de Fallavier, France) and deuterated cyclophosphamide-d₄ (CP-d₄), ifosfamide-d₄ (IFO-d₄), tamoxifen-d₅ (TAM-d₅) were purchased from Toronto Research Chemicals (North York, Ontario, Canada) as chemical powders.

Methanol (MeOH), acetonitrile (ACN) and acetone were HPLC grade and purchased from Scharlau (Spain). Hydrochloric acid (HCl) 37% and formic acid (HCOOH) 99% were purchased from VWR Prolabo (Fontenay-sous-Bois, France). Ammonium hydroxide (NH₄OH) 35% was purchased from Fischer Chemical (Loughborough, Leicestershire, UK). Ammonium acetate (NH₄CH₃COO) 98% was purchased from Merck (Damstadt, Germany). Na₂EDTA was purchased from ICN BioMedicals (Aurora, OH, USA). The ultra pure water used for laboratory purposes as well as LC mobile phase was produced from demineralized water by a MilliPore system (Molsheim, France).

Stock solution (~1000 mg/L) of each individual standard was prepared every 4 months by dissolving the appropriate amount in MeOH. Before any experiment, working solutions (i.e. dilution of the stock solution) were prepared in MeOH to the required concentration. Two distinct mixtures of standards CP (~2 mg/L), IFO (~2 mg/L), TAM (~0.5 mg/L) and deuterated CP-d₄ (2 mg/L), IFO-d₄ (2 mg/L), TAM-d₅ (0.3 mg/L) were prepared in this way. For convenience, the terms MIX Standards and MIX Deuterated will now be used throughout this document. To minimize degradation of standards, stock and working standards solutions were wrapped in aluminum and stored at -20 °C after preparation.



Spiking concentrations: IFO = 300 ng/g_{DM}; CP = 300 ng/g_{DM}; TAM = 100 ng/g_{DM}

^a from [33]

^b from [32]

^c from [34]

^d monographs.iarc.fr/ENG/Monographs/vol66/mono66-15.pdf

Fig. 1. MRM chromatogram of spiked FS.IMBR sludge sample.

2.2. Analytical procedure

Determining the anticancer compounds in sludge was carried out according to a procedure of several determinative steps including sample pre-treatment, extraction, purification and analysis (Fig. 2).

2.2.1. Sample collection and pre-treatment

Sludge samples used in this study were originated from different full-scale or pilot-scale WWTPs in Midi-Pyrenees and Languedoc-Roussillon regions (France). Samples were collected during grab sampling campaigns between March 2009 and November 2011. For each sampling campaign, a sufficient amount of sludge (>5 L) was retrieved and transferred to polypropylene cans. Samples were originated from three conventional activated sludge (CAS), one full-scale (FS) internal membrane bioreactor (IMBR), two

pilot-scale (PS) IMBR and external MBR (EMBR) and one thickened primary–secondary (TPS) sludge. Details about WWTPs and some related features such as sludge acronyms used throughout this document are given in Table 1. All the above mentioned samples were characterized and distinguished according to volatile suspended solid (VSS) measurement. VSS was obtained after calcination of total suspended solid (TSS) at 525 °C during 2 h in a furnace. TSS measurement was determined by filtration of a known volume of sludge according to AFNOR regulation NF EN 872 [16].

Briefly after transport to the laboratory, each sludge sample was allowed to settle and supernatant was discarded. The remaining sludge was then centrifuged to ensure a complete separation between particular and aqueous phases. A great amount of settled sludge (1 L for each run) was centrifuged during 20 min at 5000 × g with a Megafuge 40 R centrifuge from Fischer Scientific (Illkirch, France) operated at sludge temperature. All the pellets

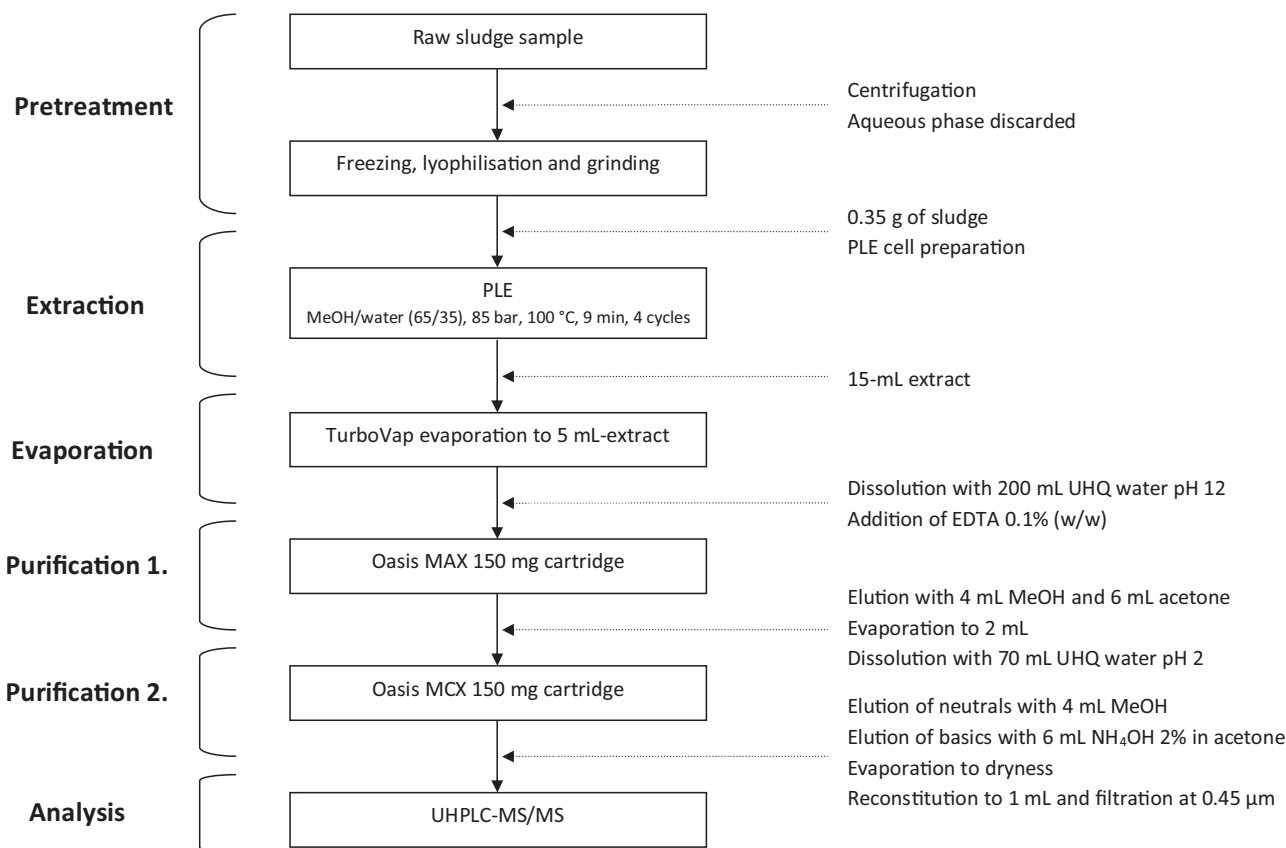


Fig. 2. Methodology applied for the determination of anticancer drugs in sludge.

were combined and frozen at -20°C . Iced sludge pellets were then freeze-dried at obscurity and -60°C under 0.045 bar vacuum (Christ Alpha 1-2 LD, Bioblock Scientific, Illkirch, France), ground to thin particles ($<0.5\text{ mm}$) using a mortar and pestle and stored at -20°C prior to use.

2.2.2. Sample extraction

A Dionex accelerated solvent extraction (ASE) 200 device (Dionex, Sunnyvale, USA), which is the trade name for PLE, was used for the extraction of anticancer drugs from sludge.

At the bottom of each extraction cell, one glass-fiber filter (Dionex, Voisins-le-Bretonneux, France) was placed to ensure the filtration of semi-aqueous extracts. A thin sand layer (Fisher Scientific, Loughborough, UK) was then applied for pre-filtration. The dried biosolid sample was weighted (0.35 g), spiked with 100 μL of MIX Deuterated and mixed thoroughly with sand as dispersing agent to prevent aggregation during extraction process and reduce clumping and channeling. The ratio between sample and sand

weight was about 0.04. The mixture was then placed in the extraction cell and covered with an additional layer of sand. The cell was not completely filled with sand. A dead space about 0.25 cm was left to keep threads and sealing surfaces safe. To allow more representative adsorption of spiked analytes in sludge, built cells were left at room temperature for a minimum of 24 h before extraction.

The extraction solvent and operating conditions were optimized according to a multivariate experimental design shortly detailed in this paper. MeOH/ultra pure water mixture (65/35, v/v) was used as extraction solvent. The operating conditions were as follows: extraction pressure, 85 bar; extraction temperature, 100°C ; no pre-heat period; static extraction time, 9 min; number of static cycles, 4; flush volume, 60% of the cell; purge time, 120 s. This procedure led to a final extract volume of $15 \pm 2\text{ mL}$ for all the samples.

2.2.3. Extract clean-up

Extracts were transferred to rocket-shaped bottles (200 mL) and evaporated to around 5 mL with a TurboVap II concentration

Table 1
Features of sludges used in this study.

Sludge	Scale	Person equivalent	Organic load	Technology	pH	VSS (%)
FS.VLCAS	Full	300 000	Very low	CAS	8.3	79
FS.LCAS	Full	2000	Low	CAS	-	83
FS.MCAS	Full	800 000	Medium	CAS	7.25	91
FS.IMBR	Full	9000	Very low	Internal MBR	7.55	75
LS.EMBR	Lab (20 L)	-	Low ^a	External MBR	7.7	84
LS.IMBR	Lab (15 L)	-	Low ^b	Internal MBR	7.5	89
TPS	Full	>30 000	-	Thickener	7.8	71

CAS: conventional activated sludge; MBR: membrane bioreactor; VSS: volatile suspended solids; FS.VLCAS: FullScale.VeryLowCAS; FS.LCAS: FullScale.LowCAS; FS.MCAS: FullScale.MediumCAS; FS.IMBR: FullScale.InternalMBR; LS.EMBR: LabScale.ExternalMBR; LS.IMBR: LasScale.InternalMBR; TPS: thickened primary secondary.

^a Semi-synthetic influent using same wastewater as FS.MCAS.

^b Fed with the same wastewater as FS.MCAS.

workstation (Caliper Life Sciences, Hopkinton, USA) operating at 30 °C under a nitrogen N₂ pressure of 1 bar. The evaporation lasted 2 h. The clean-up procedure has already been submitted for aqueous samples [17] and was adapted to our purposes. Clean-up has been carried out using selective SPE tandem approach Oasis MAX/MCX cartridges from Waters (Saint-Quentin-en-Yvelines, France). The solvent mixtures used for SPE were prepared every week.

The 5-mL extract was dissolved in 150 mL of ultra pure water. ASE vial collection was also rinsed with 50 mL (5 × 10 mL) of ultra pure water and transferred to the mixture for a final volume about 200 mL. Sample pH was adjusted to 12 with NH₄OH 35% and mixed thoroughly with EDTA 5% (0.01% in the sample, w/w). A MAX cartridge (6 cm³, 150 mg) was initially conditioned with 4 mL of MeOH, 4 mL of acetone and 4 mL of NH₄OH 0.5%. A 70-mL SPE propylene sample reservoir from Macherey-Nagel (Hoerd, France) was stacked on the cartridge before loading the sample at a flow rate of 1 mL/min. A wash solution of 4 mL NH₄OH 0.5% in MeOH/ultra pure water mixture (5/95, v/v) was applied and followed by the elution of targeted analytes with 4 mL of MeOH and 6 mL of acetone collected in a same fraction. The volume of the fraction was concentrated down to 2 mL and dissolved in 70 mL of ultra pure water corrected at pH 2 with HCl 37%. A MCX cartridge (6 cm³, 150 mg) was then conditioned with 4 mL of MeOH, 4 mL of acetone and 4 mL of ultra pure water at pH 2. The sample was loaded (1 mL/min) on a 70-mL SPE propylene adaptor. The cartridge was rinsed with 4 mL of MeOH/ultra pure water (pH 2) mixture (5/95, v/v). The excess water present in the cartridge was removed with a strong vacuum during 15 min and the sorbent was completely dried under N₂ stream during 20 min. The elution of neutrals IFO and CP was performed with 4 mL of MeOH followed by the elution of basic TAM with 6 mL of NH₄OH 2% in acetone in two distinct fractions. Details of SPE procedure, retention mechanisms and interest of cartridges combination are given elsewhere [17]. The volume of the extracts was reduced down to 1 mL and transferred to vials from Agilent Technologies (Massy, France). The extracts were then evaporated to dryness and redissolved in 1 mL of (A)/(B) mobile phase mixture (75/25, v/v) (see Table 2 for composition) using a vortex apparatus from Fischer Scientific (Illkirch, France). A filtration on a Spartan RC 0.45 µm syringe filter from VWR (Fontenay-sous-Bois, France) was performed for each extract. The extracts were finally stored at 4 °C and obscurity during a maximum duration of 7 days prior to analysis.

2.2.4. UHPLC–MS/MS analysis

LC separation was carried out using an Ultimate 3000 UHPLC System from Dionex (France). The column used for separation was an ACQUITY UPLC[®] BEH C₁₈ (50 mm × 2.1 mm) with a 1.7 µm particle size diameter (Waters, Saint-Quentin en Yvelines, France). All details about LC conditions such as injection volume, flow rate, auto sampler and column temperatures, elution gradient are given in Table 2.

Table 3

MS and MRM conditions used to identify and quantify pharmaceuticals.

Pharmaceutical	Detection	Transitions (m/z)	DP ^a (V)	EP ^b (V)	CE ^c (V)	CXP ^d (V)
IFO	Positive	261.1 > 92.0 (Q)	65	10	30	12
		261.1 > 153.8 (q)	65	10	24	12
CP	Positive	261.1 > 139.8 (Q)	65	10	27	12
		261.1 > 105.9 (q)	65	10	22	12
TAM	Positive	372.4 > 72.0 (Q)	65	10	40	15
		372.4 > 128.9 (q)	65	10	35	15

Q: quantification transition; q: confirmatory transition.

^a Declustering potential.

^b Entrance potential.

^c Collision energy.

^d Collision cell exit potential.

Table 2

Liquid chromatography conditions.

Parameter	Applied condition	
Injection volume	10 µL	
Flow rate	400 µL/min	
Auto sampler temperature	15 °C	
Column oven temperature	25 °C	
Mobile phase	Eluent A	Eluent B
	Ultra pure water/ACN (90/10, v/v) NH ₄ CH ₃ COO 1 mM HCOOH 0.3%	Pure ACN
LC gradient	% Eluent A	% Eluent B
Time (min)		
0	100	0
0.5	100	0
2	78	22
3.5	77	23
4	0	100
6	0	100
8	100	0
10	100	0

Detection was achieved with an Applied Biosystems Sciex QTRAP[®] hybrid linear ion-trap triple quadrupole mass spectrometer (Foster City, USA) equipped with a Turbolon-Spray Interface. The instrument was operated in ElectroSpray (ESI) positive (+) in Multiple Reaction Monitoring (MRM) mode (dwell time, 80 ms). The operating parameters were: capillary voltage, 5000 V; source temperature, 450 °C; gas N₂; curtain gas, 20; Ion source gas 1, 20; ion source gas 2, 70. Before any experiment, a soft cleaning of the cone entrance was performed to maintain top instrumental performance. For each compound, cone voltage and collision energies of the main transitions were optimized. MS and MRM conditions are summarized in Table 3. For MS spectra and chromatogram acquisition and exploitation, Analyst 1.6.1 software from Applied Biosystems Sciex (Foster City, USA) was used.

A minimum of 3 identification points were applied to unambiguously identify the analytes in environmental samples. Each compound was characterized according to (1) its retention time *t_R* in comparison with the corresponding standard for each batch process with a tolerance of ±5%, (2) the monitoring of two transitions per analyte and (3) its presence in one of the 2 SPE extracts. A typical chromatogram of targeted analytes in real sample is given in Fig. 1.

For quantification, MRM transitions were used. Six-point calibration curves were generated. From working solutions, identical amounts of deuterated analytes were added to the calibration standards, which contained related analytes in concentration spanning about 2 orders of magnitude. The calibration standards were evaporated to dryness, redissolved in 1 mL of (A)/(B) mobile phase mixture (75/25, v/v) and filtered at 0.45 µm. Calibration curves

were performed at the beginning of each batch process. Curves were built by calculating the ratios between the peak area of each analyte and the peak area of corresponding deuterated standard using weighted $1/x$ model for linear regression. Along the sequence, quality control (QC) samples were also analyzed to confirm their validity. QC samples were a high- and low-concentration level of the curves (1 order of magnitude). No significant (<12%) deviation has been observed. As sludge extracts may contain many interfering compounds, blank samples (mobile phase mixture without analytes) were included every 5 injections. No cross-contamination has been observed. At the end of each sequence, chromatographic column was washed thoroughly with acidified water (pH 3) and pure ACN.

Instrumental detection limits (IDL) and instrumental quantification limits (IQL) were determined by serial dilution of each standard down to 2 pg injected. The IDL and IQL were set as a signal-to-noise (S/N) ratio of 3 and 10 of the chromatographic response respectively.

2.3. Method performances

The performances of the analytical procedure were evaluated for each analyte through the estimation of method efficiency, repeatability and reproducibility, sensitivity and matrix effect. Estimation of the linearity was also considered as part of the validation.

2.3.1. Validation procedure

To demonstrate the robustness of the analytical procedure, the seven sludge samples defined in Table 1 were submitted to the validation process. For each freeze-dried biosolid, 4 samples were spiked with 100 μL of both MIX Standards and Deuterated and 1 sample was spiked with 100 μL of MIX Deuterated for native analyte concentration. All the samples were then submitted to the previously described protocol. This experimental set-up allows for the determination of the efficiency of the entire procedure (i.e. method efficiency MEff) and not for each analytical step. The determination of the MEff was calculated following Eq. (1):

$$\text{method efficiency MEff (\%)} = \frac{Q_{\text{preextract}} - Q_{\text{back}}}{Q_{\text{spike}}} \times 100 \quad (1)$$

where $Q_{\text{preextract}}$ is the amount in the extract after complete procedure (ng), Q_{back} is the amount present in the native sample (background quantity) (ng) and Q_{spike} is the quantity of the spike (ng).

For three freeze-dried sludges (FS.LCAS, FS.MCAS, FS.IMBR), MEff was also determined over a range of 4 concentrations. For each sludge candidate, 4 samples were spiked with 100 μL of MIX Deuterated and different volumes of MIX Standards (10, 50, 100, 200 μL) to achieve concentrations in the samples of 60, 300, 600, 1200 $\mu\text{g}/\text{kg}$ of dry matter (DM) and then submitted to the entire protocol. Measured analyte concentrations were plotted as a function of their related spiked concentrations and the corresponding slope was determined ($Slope_{\text{plotted}}$). Four-concentration MEff was determined for each analyte according to Eq. (2):

$$\text{four-concentration method efficiency MEff (\%)} = \frac{Slope_{\text{plotted}}}{Slope_{\text{calibration}}} \times 100 \quad (2)$$

where $Slope_{\text{plotted}}$ is the slope previously defined, $Slope_{\text{calibration}}$ is the slope of the calibration curve. In both experiments, absolute and relative MEff were calculated. For relative MEff, all the values were corrected relative to the deuterated analogues.

Repeatability (intra-day precision) was expressed as the relative standard deviation (RSD, %) obtained from the MEff experiment at a single concentration and extracted, purified and analyzed in the

same batch. Reproducibility (inter-day precision) was defined and conducted in the same conditions but on different batches and was determined only for three freeze-dried sludges (FS.LCAS, FS.MCAS, FS.IMBR).

The sensitivity of the analytical method was determined according to the definitions of method detection limits (MDL) and method quantification limits (MQL). MDL and MQL were calculated using Eq. (3):

$$\text{method limits ML (\mu g/kg}_{\text{DM}}) = \frac{IL \times V_{\text{extract}}}{MEff_{\text{abs}} \times m} \quad (3)$$

where IL is the considered instrumental limit ($\mu\text{g}/\text{L}$), V_{extract} is the volume of the final extract (=1 mL), $MEff_{\text{abs}}$ is the absolute method efficiency calculated for a single concentration ($0 < MEff_{\text{abs}} < 1$), m is the dried sample weight (=0.35 g).

2.3.2. Analytical limitation

To evaluate the performances of each analytical step, freeze-dried samples and subsequent extracts were spiked at different steps of the procedure with 100 μL of both MIX Standards and Deuterated. The experimental scheme was inspired from the literature [18] and conducted in triplicate for FS.MCAS and FS.IMBR. Spikes were applied:

- Before freeze-drying on rehydrated freeze-dried samples to assess *true* MEff;
- Before extraction to evaluate the combined recovery of extraction, purification and analysis (MEff defined in Section 2.3.1);
- Before purification on Oasis MAX to evaluate the recovery of both purification and analysis;
- Before purification on Oasis MCX to evaluate the recovery of second purification and analysis;
- Before analysis to evaluate the recovery of the analysis.

Absolute and relative recoveries were determined in the same way as for MEff estimation. The following Eq. (4) was used for calculation:

$$\text{recovery (\%)} = \frac{Q_{\text{step}} - Q_{\text{back}}}{Q_{\text{spike}}} \times 100 \quad (4)$$

where Q_{step} is the amount in the final extract after spike to the corresponding analytical step (ng). For relative recovery, all the values were corrected relative to the deuterated analogues.

The spiking procedure applied in (e) also allows for the determination of matrix effect (ME), according to Eq. (5):

$$\text{matrix effect ME (\%)} = \left(\frac{A_{\text{postextract}} - A_{\text{back}}}{A_{\text{spike}}} - 1 \right) \times 100 \quad (5)$$

where $A_{\text{postextract}}$ is the area in the extract spiked just before the analysis, A_{back} is the area in the extract of native unspiked sample (background area) and A_{spike} is the area of the corresponding spike. Absolute ME calculation was based on the area of analyte without correction while relative ME was calculated related to the deuterated analogue area.

The accurate determination of the recoveries for each analytical step was possible. The efficiency of each detailed step was determined according to Eq. (6):

$$\text{analytical step } n \text{ efficiency (\%)} = \frac{R_n}{R_{n+1}} \times 100 \quad (6)$$

where R is the absolute or relative recovery (%) at a given spiking step, n is a value ranging from 1 to 4 and describing a specific analytical step. Thus, the corresponding steps are:

($n = 1$) pretreatment (i.e. freeze-drying) by comparing experiments (a) and (b)

($n = 2$) extraction by comparing experiments (b) and (c)

($n = 3$) purification I (i.e. Oasis MAX) by comparing experiments (c) and (d)

($n = 4$) purification II (i.e. Oasis MCX) by comparing experiments (d) and (e)

3. Results and discussion

3.1. Optimization of PLE

3.1.1. Selection of extraction solvent

The solvent must be able to solubilize the targeted analytes from the matrix with few interfering compounds as far as possible. Since the analytes vary in physico-chemical properties, the choice of solvent mixtures was crucial but also limited. Our strategy for selecting mixtures relies on (1) solvents previously applied with success in the literature and (2) close polarity matching between analytes and solvent mixtures. Different pure and binary solvents were tested. Pure solvents were acetone, MeOH, ACN, water (pH 7) and binary mixtures were acetone/ACN (1:1), MeOH/ACN (1:1), acetone/water (1:1), MeOH/water (1:1) and ACN/water (1:1). As no detectable concentration of targeted anticancer drugs was measured, FS.LCAS sludge was selected, spiked with 100 μ L of both MIXs and submitted to the whole analytical process. All the experiments were performed in duplicate. Initial PLE conditions were applied from the literature: extraction pressure, 138 bar; extraction temperature, 100 °C; no pre-heat period; static cycle extraction time, 5 min; number of static cycles, 3; flush volume, 60% of the cell; purge time, 120 s [14]. The solvent mixture efficiency was investigated by comparing the mean areas of targeted analytes for each tested condition (data not shown). Areas of deuterated analogues were also compared. In the same time, extraction cells filled with dispersing agent were spiked and extracted in the same conditions to investigate the thermal degradation of analytes. No significant losses occurred under chosen parameters, thus confirming the stability.

For the tested solvents, all targeted analytes were recovered in different amounts. Extracts exhibiting different aspects were also obtained. Pure and mixed organic solvents led to highly colored and clear extracts while water led to brown and very turbid ones. Semi-organic mixtures gave intermediate profiles. Turbid aqueous samples were responsible for the clogging of the cartridge during the purification. Consequently, water (pH 7) was not selected as extraction solvent in our experimental scheme. Higher areas were obtained for IFO and CP using MeOH/water (1:1) and for TAM using pure MeOH. No discrepancies were observed for deuterated analogues areas. ACN and derived mixtures gave the worst results for each compound. The lower efficiency of ACN for extracting pharmaceuticals from solid samples has already been reported [13,18,19]. Unsurprisingly, water mixtures were efficient to extract polar analytes IFO and CP while pure organic solvents were efficient to extract apolar TAM. As TAM analysis was more sensitive than for IFO and CP, MeOH/water as extraction solvent was found to be a good compromise. From the literature and our findings, the superior capability of MeOH/water mixture to extract pharmaceuticals from solid samples has been found [13,18–23].

3.1.2. Optimization using experimental design

The number of parameters affecting PLE is very high so the one variable at a time (OVAT) strategy was not to consider here. Finding the best operating conditions for maximizing recoveries with few experiments was achieved using a central composite design (CCD). According to the literature, the parameters of interest were the solvent (MeOH/water) ratio (variable A), the extraction temperature

(variable B), the extraction pressure (variable C), the static cycle duration (variable D) and the number of cycles (variable E) [24]. The CCD consisted in a fractional factorial design including the five variables at two levels (2^{5-1}), each augmented by ten star points and 6 center points. The total number of extractions was 32. The low and high levels (domain boundaries) for each parameter were common PLE values determined from the literature [10,24]. These values were 10–90% (MeOH/water ratio), 70–110 °C (temperature), 70–130 bar (pressure), 4–16 min (cycle duration) and 1–5 (number of cycles). The complete definition of the experimental design applied is given in Supplementary Content 1. FS.LCAS sludge was chosen for optimization as no targeted analytes have been detected. To evaluate the efficiency of extraction, 100 μ L of MIX Standards were spiked prior to extraction and 100 μ L of MIX Deuterated were spiked into the corresponding extract.

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.chroma.2013.01.114>.

The recoveries obtained for each analyte and experiment are given in Supplementary Content 2. Some yields were superior to 100% which could be attributed to method errors, sludge sample inhomogeneity [25] or signal ion enhancement during analysis. In the defined experimental domain, TAM displayed strong variability with values ranging from 0 to 205%. Moreover, the variability was remarkably high for the 6 center points (experiments 27–32). It suggested that TAM extraction was affected by an unconsidered parameter or any other unknown process. A simple experiment was conducted by washing thoroughly with organic solvent the laboratory vessel and analyzing the solvent. Quantifiable amounts of TAM have been measured, confirming adsorption phenomena. Determination of TAM was therefore not possible. For the other analytes, the variability at the 6 center points has been determined (Supplementary Content 2). IFO exhibited less variability than CP with a relative standard deviation (RSD) of 6% versus 13%.

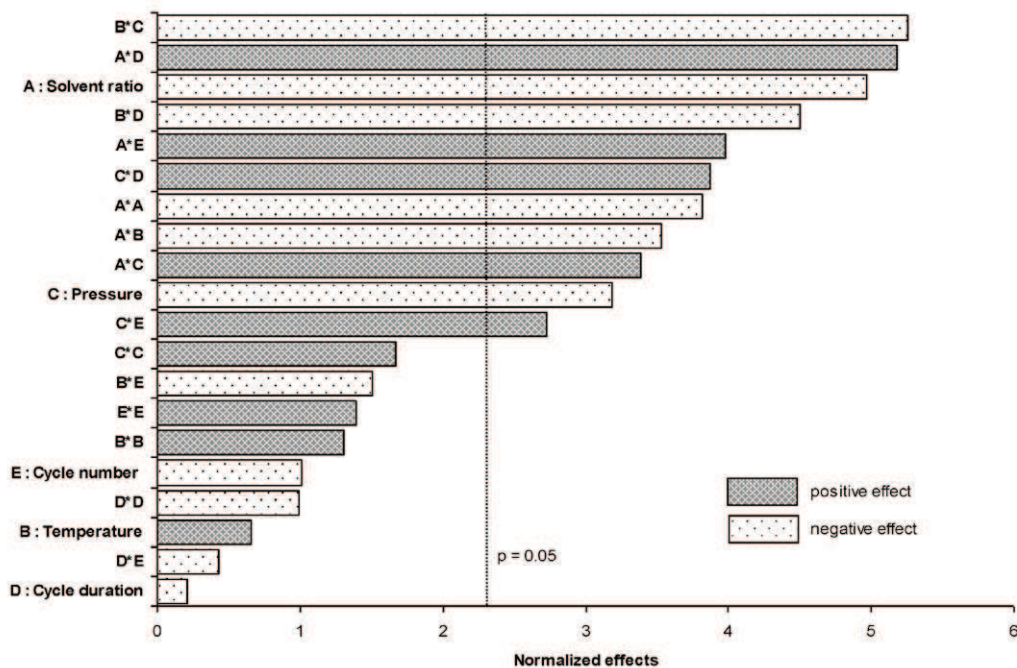
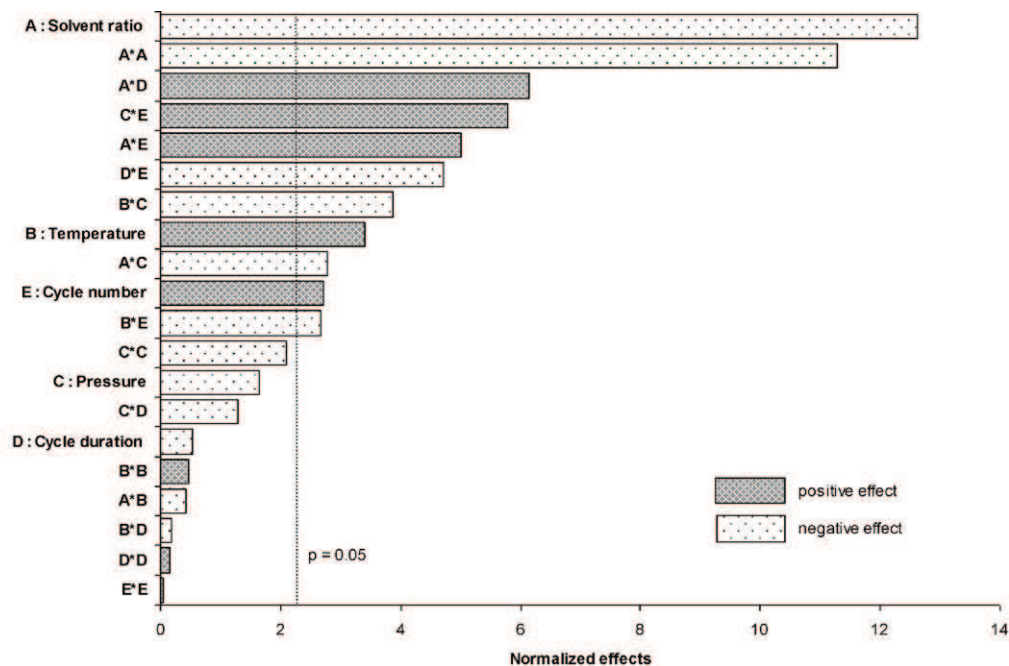
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The Minitab® software was used for the statistical study. Owing to the CCD, the coefficients of a second order polynomial model describing the effects of the 5 variables on IFO and CP recovery have been estimated. The two models adequately represented the data as lack-of-fit p -values were superior to 0.05 (0.21 for IFO and 0.26 for CP). The correlation between predicted and observed recoveries was up to 99% for IFO and 98% for CP.

In order to see which variables (i.e. parameters) were the most influent on the response, standardized Pareto charts were constructed and are given in Fig. 3. Trends between IFO and CP were rather similar. In both cases, the solvent ratio was probably the most determining factor for extraction efficiency. However, its influence was difficult to assess, as this parameter was implied in several significant interactions sometimes of opposite trends. Indeed, it appeared that some interactions between parameters, such as $A * D$ and $A * E$ for example, were strongly influent. It means that variations in extraction recovery were not strictly assigned to a single parameter but could also be due to synergistic effects of two or more variables. These results justify the use of experimental design rather than OVAT strategy.

Our objective was to determine the best values of the five parameters that allow a recovery of around 100% with a 5% tolerance. Due to the second order of the models, an infinite combination of the factors allows to reach this goal. So response surface methodology was used to determine the area where the criterion is fulfilled. The values of the five parameters were chosen in these areas, taking into account the following experimental considerations.

First, aqueous or highly aqueous extracts were not recommended in our experimental scheme due to possible cartridge clogging. Moreover, the more polar the solvent mixture, the less selective extraction is [15]. Consequently, semi-organic content



The vertical straight line is the limit of significance

Fig. 3. Standardized Pareto chart highlighting the effect of PLE parameters in applied experimental design for IFO (up) and CP (down). The vertical straight line is the limit of significance.

was preferential (*middle of domain*). Then, the application of high temperature in PLE decreases the viscosity of the solvent, thus allowing its better penetration into sample matrix and increasing its capacity to solubilize the analytes [20]. Faster extraction rates are also expected with high temperatures [15]. Nevertheless, high temperature could also lead to loss in method selectivity due to the extraction of more co-extractable compounds [20]. Relatively high temperature was thus preferential (*upper part of domain*). Next, pressure seemed to be the less significant parameter, which is a common finding in the literature for PLE [13,25,26]. Its role is to

maintain the solvent in the liquid state at extraction temperature. Low pressure was sufficient (*lower part of domain*). Finally, the duration and number of cycles were determined simultaneously. Long cycle time could lead to a better diffusion of analytes but the multiplication of short cycle could be favorable to recovery [20]. Indeed, the introduction of fresh solvent at each cycle could allow new equilibrium between analytes and solvent, which could be interesting for strongly entrapped analytes. Consequently, low cycle duration (*lower part of the domain*) and many cycles (*upper part of the domain*) were preferential. Taking account of these reasons,

surface responses were plotted and displayed in Supplementary Contents 3 and 4. The chosen experimental conditions were the following: MeOH/water 65/35 (v/v), extraction temperature 100 °C, extraction pressure 85 bar, static cycle duration 9 min and 4 cycles.

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.chroma.2013.01.114>.

3.2. Extract clean-up

Extract clean-up was required to concentrate the analytes and to remove the interfering components. As sludge was expected to contain much more interferents than wastewater samples, high sorbent weights (150, 500 and 1000 mg) were applied. Briefly, three types of sorbents were selected: reversed phase, hydrophilic–lipophilic balance (HLB) and mixed-mode anionic–cationic exchange. FS.LCAS sludge PLE extracts were generated and spiked prior to purification. Reversed phase sorbent yielded very low recoveries for IFO and CP and were rejected. HLB yielded better recoveries but the major part of interfering compounds were concentrated in the final extract, which could introduce analytical troubles (i.e. strong matrix effect) with more complex sludge samples. Therefore, HLB sorbents were rejected. In our previous study [17], mixed-mode anionic- and cationic-exchange SPE has proven value in the selective recover of targeted analytes in sludge aqueous samples with relatively low matrix effect. This procedure has been retained. As the sorbent weight for purification was two times and a half higher, the conditioning, washing and eluting volumes were multiplied by two. Lightly colored and clear extracts were obtained for most of the samples. Purification procedure was then considered satisfactory.

3.3. Performances of the analytical method

As no CRM was available for validation, in-house material was used. In-house material was freeze-dried sludge spiked with a known amount of targeted analytes. Seven different types of sludge were studied to demonstrate the complete suitability of the procedure.

The linearity of the internal calibration curves was satisfactory ($R^2 > 0.995$) for IFO and CP over the tested concentrations (1–500 µg/L) and validation period (2 months). Indirectly, method linearity was also studied during MEff estimation over four concentrations (see Section 2.3.1) for FS.LCAS, FS.MCAS and FS.IMBR. Linearity was observed ($R^2 \geq 0.990$) for each analyte and sludge tested (data not shown). Thus, the method showed good specificity for the analysis of targeted analytes.

Recoveries of selected drugs for different types of sludge are given in Table 4. Both absolute and relative method recoveries were distinguished as recommended in the literature [12]. Absolute MEff values were very different and dependent on the compound and sludge considered. Absolute MEff ranges were 1.5–33% for IFO and 2.2–47% for CP. For FS.LCAS, FS.MCAS and FS.IMBR, the agreement between MEff at a single and four concentrations validate the “single-point” procedure for each sludge. The absolute recoveries for IFO and CP were limited for all the samples (<50%) but not critical for their determination due to the high sensitivity of MS/MS detection. No significant correlation has been found between the recoveries and sludge features according to pH, VSS and the biological process (see Table 1). The very low method efficiency for TPS sludge impedes the quantitative determination of IFO and CP. Since VSS was the lowest, other characteristic might be more relevant to explain the very low method efficiency. As TPS sludge appeared partially digested, harsh chemical surroundings of TPS sludge could have been detrimental for IFO and CP recovery during the extraction or purification. Strong matrix effect occurring during analysis was also possible.

Table 4
Analytical method performances and validation data.

Sludge	IFO				CP			
	MEff ± SD ^a (%)		MEff ^b (%)		MEff ± SD ^a (%)		MEff ^b (%)	
	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
FS.VLCAS	29 ± 0	102 ± 3	15	98	38 ± 5	99 ± 11	3.0	7.5
FS.LCAS	16 ± 1	99 ± 7	15	108	27 ± 1	100 ± 3	4.2	10.4
FS.MCAS	14 ± 2	110 ± 13	15	95	24 ± 3	105 ± 12	4.7	11.8
FS.IMBR	29 ± 2	104 ± 10	28	95	40 ± 1	97 ± 6	2.8	7.1
PS.EMBR	33 ± 3	106 ± 4			47 ± 2	96 ± 4	2.5	6.1
PS.IMBR	20 ± 1	104 ± 3			35 ± 1	98 ± 1	3.2	8.1
TPS	1.5 ± 0.2	106 ± 4			2.2 ± 0.3	92 ± 5	51	128

MEff: method efficiency; SD: standard deviation; MDL: method detection limit; MQL: method quantification limit; DM: dry matter.

^a Calculated for a single concentration (n = 4).

^b Calculated over a four-concentration range.

For the different sludges, relative MEff values were considered excellent and ranged from 99 to 110% for IFO and from 92 to 105% for CP (see Table 4). Therefore, deuterated standards were completely suitable for IFO and CP determination in each case. Moreover, the use of only one surrogate standard along the entire protocol provided more accurate results in comparison with analytical methods using at least two surrogate standards, one for extraction and one for analysis, as encountered in the literature [20,21].

The repeatability of the method was calculated from the standard deviations given in Table 4 for each sludge. For IFO, RSD for absolute and relative MEff were in the range 0.8–15% and 2.4–12% respectively. For CP, RSD were in the range 2.4–14% and 1.0–11% respectively. These values have the significance of good overall repeatability (<15%) in each case. The reproducibility of the method has been calculated as the same manner and was below 14% and considered satisfactory (<15%) for FS.LCAS, FS.MCAS and FS.IMBR (data not shown). Therefore, the robustness of the procedure has been proven.

For IFO and CP, MDL ranged from 3.9 to 74 $\mu\text{g}/\text{kg}_{\text{DM}}$ and from 2.5 to 51 $\mu\text{g}/\text{kg}_{\text{DM}}$ respectively (Table 4). With the exception of TPS sludge, all the MDLs were lower than 10 $\mu\text{g}/\text{kg}_{\text{DM}}$ displaying good overall method sensitivity. The conclusions are the same for MQs lower than 20 $\mu\text{g}/\text{kg}_{\text{DM}}$ which are the best quantification limits reported in the literature for both compound [11]. The uncommonly low sample and purification sorbent weights applied in the experimental scheme were not limiting in the achievement of low method limits, reaching possible environmental requirements.

In the overall, our analytical strategy proved good sensitivity, selectivity and specificity due to the validation on seven sludge samples from different origins. However, it is important to note that recoveries obtained for spiked samples could overestimate the efficiency of the method for incurred native analyte [25]. Because of limitations in diffusion and kinetics of the sorption process, spiked analytes will always be less retained than the native ones [27]. To assess the representativeness of freshly spiked compared to incurred analytes, an additional experiment on PS.EMBR sludge has been carried out. PS.EMBR has been continuously contaminated with anticancer drugs during 80 days. This procedure allows analytes to penetrate much more into the volume of the matrix rather than on the surface. Sludge was sampled on days 10, 30 and 60 during campaign, which corresponds respectively to 0.5, 1.5 and 3 times the sludge age. Each sample was freeze-dried and split equally in two. The second aliquot received an additional spiking of 10 μL of MIX Standards. All the samples were then submitted to the whole analytical procedure. The measured concentration of the freshly spiked sample was corrected by subtracting the amount of the spike to assess the native concentration. The corrected value was compared to the concentration measured in the sample without additional spike. No significant differences were measured for IFO (RSD < 4%) and CP (RSD < 3%). It appears that the proposed analytical method is not specific to freshly spiked samples and can be applied to aged samples. This could be attributed to the numerous extraction cycles in PLE, allowing the exhaustion of the matrix from easily accessible compartments (spiked) to less accessible ones (incurred). The sorptive interactions of IFO and CP in freshly spiked and aged samples could also be comparable.

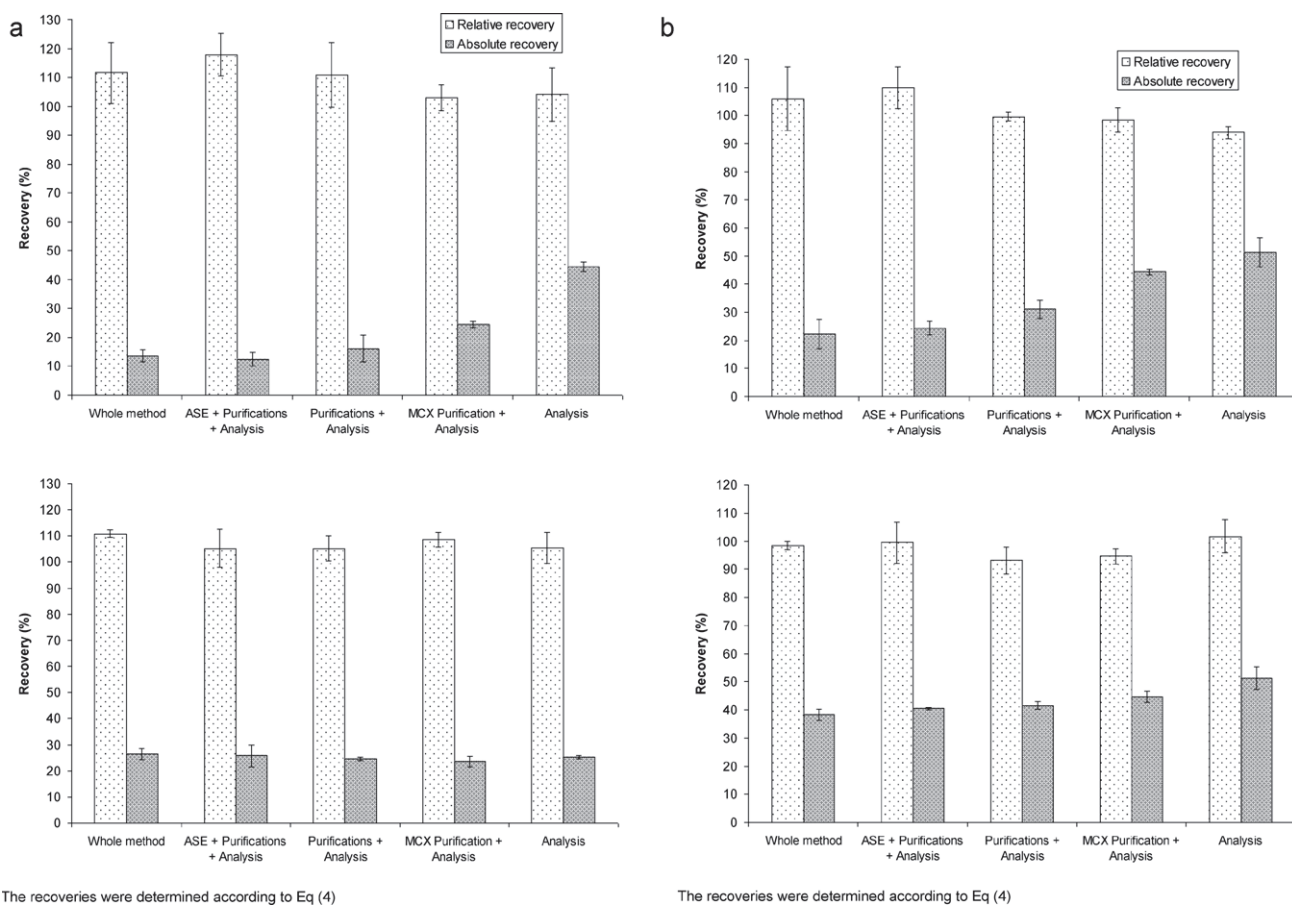


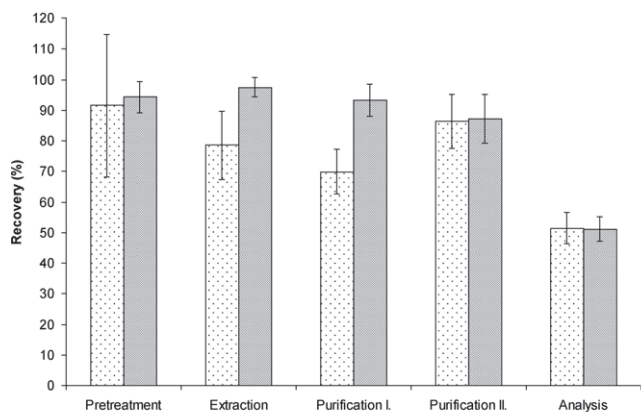
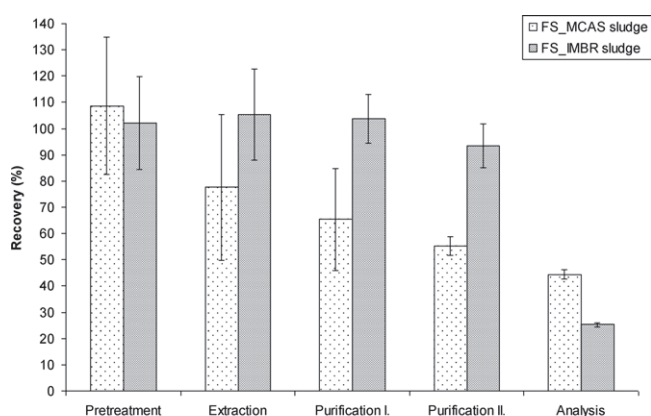
Fig. 4. (a) Mean recoveries \pm standard deviation for IFO in FS.MCAS sludge (up) and FS.IMBR sludge (down) for the different steps of the analytical procedure ($n=3$). (b) Mean recoveries \pm standard deviation for CP in FS.MCAS sludge (up) and FS.IMBR sludge (down) for the different steps of the analytical procedure ($n=3$). The recoveries were determined according to Eq. (4).

3.4. Which analytical step is the most limiting?

As sludge matrix components can strongly influence the efficiency of the sample treatment stage, the objective here was to determine whether the limited absolute recoveries were linked to a same analytical stage or if they were related to different stages depending on the sludge nature. To do so, two types of sludge with different organic content (i.e. VSS) have been selected and spiked at different analytical steps described in Section 2.3.2. FS_MCAS was selected for its high organic content (91%) and FS_IMBR for its relatively low organic one (75%). TPS sludge (71%) was rejected due to the analytical challenge previously described.

The profiles obtained for IFO and CP are displayed in Fig. 4a and b respectively. The absolute recoveries displayed the true efficiency of the spiking stages. Even if recoveries related to the analysis are comparable or somewhat higher than those related to the whole method, the quantification of IFO and CP is deeply disturbed by the matrix effect (ME) in each sample, possibly due to the use of semi-organic solvent during PLE. For IFO, recoveries associated with the couple "Whole method; Analysis" are 14%; 45% for FS_MCAS and 26%; 25% for FS_IMBR. For CP, recoveries are 22%; 51% and 38%; 51%. The use of (semi-) organic solvent during PLE could be responsible for the extraction of many interfering compounds as suggested in the literature [10,15,24] thus decreasing clean-up efficiency and resulting in relatively high ME.

The efficiency of each analytical step from the pretreatment until the analysis has been calculated following Eq. (6) given in Section 2.3.2. The results are displayed in Fig. 5. Only absolute recoveries were used for calculation.



The recoveries were determined according to Eq (6)

Fig. 5. Recovery profiles for IFO (up) and CP (down) in two types of sludge. The recoveries were determined according to Eq. (6).

The profiles are very different between sludges but not between analytes. For a given sludge sample, it suggests that IFO and CP are submitted to the same or close processes during each stage. The high variability observed for some analytical steps is fully explained by the addition of variances implied by Eq. (6) but not critical for trend explanation.

Pretreatment stage did not imply any significant losses for IFO and CP in each case. Freeze-drying is often required because wet samples can prevent from efficient PLE [15]. Grinding ensures shorter diffusion path-lengths during extraction and enhances solvent penetration [15]. Both steps can be responsible for losses but are usually neglected during method development. From our result it is demonstrated that non-volatile analytes, which is the case of pharmaceuticals, are not sensitive to freeze-drying and grinding. Therefore, the use of spiked freeze-dried samples during method validation was effectively sufficient. The extractive step led to satisfactory recoveries between 78 and 105% in each case. For sludge samples, the versatility of the optimized PLE method has been demonstrated. The purification stage efficiency was strongly dependent on the sludge nature. For both analytes, higher losses were observed for FS_MCAS sludge. It could be explained by the nature of interfering compounds present in the PLE extract, which may have competed for binding sites and lowering the clean-up efficiency. It is also important to note that evaporative steps along the procedure were not responsible for any analyte loss.

In the overall, the analysis was the most limiting factor in the quantification. CP suffered from ME up to 49% for both sludges while IFO suffered from ME of 55 and 75% for FS_MCAS and FS_IMBR sludges respectively. Additionally, it appeared that sludge organicity according to VSS measurement was not sufficient to explain ME as no correlation between VSS, analytes and ME was found. Even if VSS is an easy-to-handle and quick measurement, it seems that the characterization of the sludge matter and related extract could be more relevant in the understanding of ME origins.

3.5. Application to environmental samples

Optimized method was applied to the biosolid samples described in Table 1. Measured mean concentrations are given in Table 5.

Except for FS_LCAS, one or two of the targeted drugs were detected or quantified in our samples thus confirming the occurrence of anticancer drugs in solid part of sludge. Concentrations in solid phase for IFO ranged from 11.4 to 42.5 $\mu\text{g}/\text{kg}_{\text{DM}}$ while CP was quantified only in FS_MCAS at a concentration of 12.6 $\mu\text{g}/\text{kg}_{\text{DM}}$. This concentration is of the same order of magnitude than one reported in the literature for excess sludge [14]. From our data, contaminated sludges are mostly those of WWTPs treating each day large amounts of wastewater. It could be thought that the

Table 5
Anticancer drugs concentrations in collected biosolid samples.

Sludge	Pharmaceuticals ^a ($\mu\text{g}/\text{kg}_{\text{DM}}$)	
	IFO	CP
FS_VLCAS	11.4 \pm 2.1	<MQL
FS_LCAS	<MDL	<MDL
FS_MCAS	41 \pm 23	12.6 \pm 4.9
FS_IMBR	42.5 \pm 14.6	<MQL
PS_EMBR	<MQL	<MDL
PS_IMBR	<MQL	<MQL
TPS	<MQL	<MQL

MDL: method detection limit; MQL: method quantification limit.

^a $n=2$, medium concentration \pm standard deviation.

contamination is much more related to the treated person equivalent number than the sludge physico-chemical nature. The quantification of IFO in FS-IMBR could be attributed to a possible accumulation as sludge age is long (100 days) and biodegradation is not expected [28–32]. In the overall, very low levels of anticancer drugs were determined in our solid samples originated from different WWTPs. This is in good agreement with levels of concentration found or predicted in the literature [6,11,30]. It could be explained by the relatively low consumption and the possible low sorption affinity for sludge due to high polarity of IFO and CP. However, low concentrations in sludge may not have the significance of low toxicity for microorganisms and more. Some other field results are requested to confirm or not these first conclusions.

4. Conclusion

In this paper, an original analytical method was proposed to recover anticancer drugs from solid part of sludge. The experimental set-up consists of extraction from the solid matrix using PLE, clean-up by selective SPE and analysis by UHPLC–MS/MS. Some efforts focused on the extraction efficiency, the method validation and the analytical limitation. The use of an experimental design to optimize the extraction revealed the concomitant effect of some parameters during extraction, which helped to understand the true functioning of PLE. The validation of the method was applied to seven different sludge samples. Method validation requirements implying linearity, repeatability, and reproducibility were fulfilled. The analytical performances were very different between sludge samples with method efficiencies and MDLs spanning more than one order of magnitude. Thus, method validation should be systematically applied for each new sample and could be of great interest for monitoring programs. Matrix effect occurring during analysis was demonstrated as the most limiting factor for the quantification of each analyte. However, the use of deuterated standards spiked at the very beginning was efficient to overcome analytical troubles regardless of the matrix composition. Various sludge samples were analyzed, confirming the environmental occurrence of anticancer drugs in sludge. Up to now, the proposed method is only the third analytical procedure available in the literature for the extraction of anticancer drugs from environmental solid samples, each of them dealing with sludges. The developed method is also the most sensitive (up to low $\mu\text{g}/\text{kg}_{\text{DM}}$), detailed and versatile. The need of analytical methods and environmental data about anticancer drugs is still of concern to establish their occurrence in the water cycle at national and international scales.

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