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Corresponding Author: Dr. Massimo Del Bubba, Ph.D.

Corresponding Author's Institution: University of Florence

First Author: Daniele Rossini

Order of Authors: Daniele Rossini; Lorenzo Ciofi; Claudia Ancillotti; Leonardo Checchini; Maria Concetta Bruzzoniti; Luca Rivoira; Donatella Fibbi; Serena Orlandini; Massimo Del Bubba, Ph.D.

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Suggested Reviewers: José Juan Santana-Rodriguez
Full Professor, Department of Chemistry, University of Las Palmas (Gran Canaria, Spain)
josejuan.santana@ulpgc.es
For his widely recognized expertise in extraction and clean-up techniques coupled with LC-MS/MS analysis.

Virginia Coman
Raluca Ripan Institute for Research in Chemistry, Babes-Bolyai University, Cluj-Napoca, Romania
coman_virginia@yahoo.com
She has worked in the last years on the determination of drugs in environmental matrices and very recently on innovative analytical methods for the analysis of NSAIDs in wastewater.

Bogusław Buszewski
Nicolaus Copernicus University, Torun, Poland
bbusz@chem.uni.torun.pl
For his widely recognized expertise in Analytical Chemistry, with particular regard to separation sciences.

Luigi Mondello
Full Professor of Analytical Chemistry, Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Italy

luigi.mondello@unime.it
For his widely recognized expertise in separation sciences.

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Dear Editor,

I send you the manuscript entitled "Innovative combination of QuEChERS extraction with on-line solid-phase extract purification and pre-concentration, followed by liquid chromatography-tandem mass spectrometry for the determination of non-steroidal anti-inflammatory drugs and their metabolites in sewage sludge" for the submission to Abalytica Chimica Acta.

Corresponding author and the submitter of the manuscript: Prof. Massimo Del Bubba, Department of Chemistry, University of Florence, Via della Lastruccia n.3, 50019 Sesto Fiorentino (Florence), Italy. E-mail address: delbubba@unifi.it

In this manuscript, for the first time, QuEChERS extraction of sewage sludge was innovatively combined with the automatic solid-phase pre-concentration and purification (SPPCP) of the extract and LC-MS/MS analysis, for the determination of 13 non-steroidal anti-inflammatory drugs and their metabolites. Various stationary phases have been tested for extract clean-up and chromatographic analysis. The proposed approach is characterized by a higher analytical throughput, compared to others previously published and allows for analysing target compounds with very high sensitivities (from tens of pg/g to ng/g) in 30 min per sample. After optimization, the proposed automatic preconcentration and purification strategy allowed for obtaining low matrix effects and also from this viewpoint it is very interesting compared to previously published articles. The method was applied to five sludge samples collected in different sewage facilities, highlighting the importance to include in the group of target analytes the metabolites.

Prof. Massimo Del Bubba
Via della Lastruccia, 3 – 50019 Sesto Fiorentino (Firenze)
phone +39 055 4573326 | fax +39 055 4573439 | e-mail: delbubba@unifi.it
P.IVA | Cod. Fis. 01279680480

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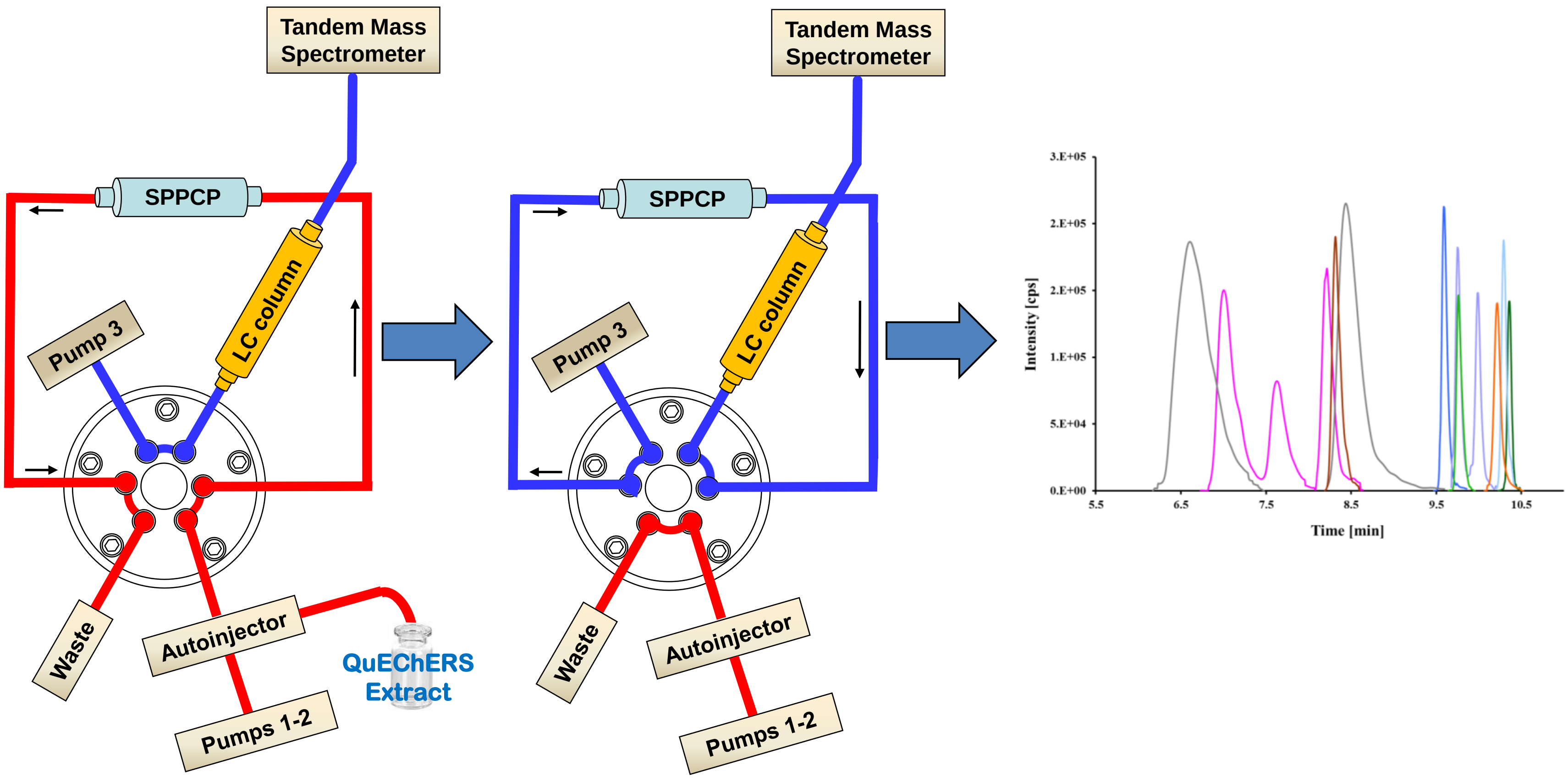
Massimo Del Bubba

***Graphical Abstract**

GRAPHICAL ABSTRACT



***Highlights**

Non-steroidal anti-inflammatory drugs and their metabolites are analysed in sludge

QuEChERS extract is automatically preconcentrated, purificated and analysed by LC-MS

In most cases matrix effect was ≤ 20% and recovery ≥ 50%

The determination of target analytes in sludge is achieved in 30 minutes

The method sensitivity is high, being it from tens of pg g^{-1} to ng g^{-1} of dry sludge

*Manuscript (including figures, tables, text graphics and associated captions)
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Innovative combination of QuEChERS extraction with on-line solid-phase extract purification and pre-concentration, followed by liquid chromatography-tandem mass spectrometry for the determination of non-steroidal anti-inflammatory drugs and their metabolites in sewage sludge

D. Rossini[a,b], L. Ciofi[a], C. Ancillotti[a], L. Checchini[a], M.C. Bruzzoniti[b], L. Rivoira[b], D. Fibbi[c], S. Orlandini[a], M Del Bubba[a,*]

[a] Department of Chemistry "Ugo Schiff", University of Florence, Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence, Italy)

[b] Department of Chemistry, University of Turin, Via Pietro Giuria 5, 10125 Turin (Italy)

[c] GIDA S.p.A., Via di Baciacavallo 36, 59100 Prato (Italy)

* Corresponding author: Massimo Del Bubba, Department of Chemistry, University of Florence, Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence, Italy). Phone number: +39-055-4573326. E-mail: delbubba@unifi.it

Abstract

For the first time QuEChERS extraction of sewage sludge was combined with the automatic solid-phase pre-concentration and purification (SPPCP) of the extract and LC-MS/MS analysis, for the determination of the non-steroidal anti-inflammatory drugs acetylsalicylic acid (ASA), diclofenac (DIC), fenbufen (FEN), flurbiprofen (FLU), ketoprofen (KET), ibuprofen (IBU) and naproxen (NAP), and their metabolites salicylic acid (SAL), 4'-hydroxydiclofenac (4'-HYIDIC), 1-hydroxyibuprofen (1-HYBU), 2-hydroxyibuprofen (2-HYBU), 3-hydroxyibuprofen (3-HYBU) and o-desmethylnaproxen (O-DMNAP). Various commercial pellicular stationary phases (i.e. silica gel silanized with octadecyl, biphenyl, phenylhexyl and pentafluorophenyl groups) were preliminarily investigated for the resolution of target analytes and different sorbent phases (i.e. octyl or octadecyl silanized silica gel and a polymeric phase functionalized with N-benzylpyrrolidone groups) were tested for the SPPCP phase. The optimized method involves the QuEChERS extraction of 1 g of freeze-dried sludge with 15 mL of water/acetonitrile 1/2 (*v/v*), the SPPCP of the extract with the N-benzylpyrrolidone polymeric phase and the water/acetonitrile gradient elution on the pentafluorophenyl stationary phase at room temperature. Matrix effect was always suppressive and in most cases low, being it ≤ 20% for ASA, DIC, FLU, KET, IBU, 1-HYBU, 2-HYBU, 3-HYBU, NAP and O-DMNAP, and included in the range of 35-47% for the other analytes. Recoveries were evaluated at three spiking levels, evidencing almost quantitative values for HYIBUs and O-DMNAP; for ASA, SAL KET the recoveries were included in between 50-76%, whereas for the other compounds they ranged from 36% to 55%. The proposed method is more performing than those so far published, being suitable for target compound determination in real samples from tens of pg g^{-1} to ng g^{-1} of freeze-dried sludge, with a total analysis time of 30 minutes per sample.

Keywords

QuEChERS; Solid-phase pre-concentration and purification; Liquid chromatography-tandem mass spectrometry; Sewage sludge; Non-steroidal anti-inflammatory drugs; Drug metabolites

1 Introduction

Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method is an extraction and clean-up technique originally developed for recovering pesticide residues from fruits and vegetables [1-3] and thereafter applied to the analysis of various organic micropollutants in different environmental matrices, mainly of solid nature, such as sediments and soil [4]. Briefly, the QuEChERS extraction method, in its original approach to fruits and vegetables, is based on the recovery of target analytes in acetonitrile, which is partitioned from the native water of the sample by the addition of proper amounts of sodium chloride and magnesium sulphate. Afterwards, the acetonitrile extract is treated again with magnesium sulphate and finally purified by dispersive solid-phase extraction (d-SPE) using "primary secondary amine" (PSA) as sorbent [1]. Improvements later highlighted as crucial for maximizing recovery from solid environmental matrices, are the controlled pH conditions [2, 3] and hydration [4] of the sample during extraction. The recovery from soil of selected drugs and herbicides, characterized by low values of the octanol-water partition coefficient (i.e. log K_{OW}=0.8-2.8), has been also demonstrated by the QuEChERS method [5], thus suggesting the suitability of this extraction technique also for a wide range of polar compounds, including pharmaceuticals and their metabolites.

The determination of organic micropollutants in sewage sludge is without doubts a topic of great interest from an environmental point of view. In fact, biological sludge may represent the final sink of organic micropollutants in wastewater treatment plants (WTPs), the determination of which can give useful information concerning the overall efficiency of the wastewater treatment process, as well as the potential soil contamination, when these bio solids are used for land applications [6, 7]. Among solid environmental matrices, biological sludge is much less investigated than sediments and especially soil by using the QuEChERS approach. To date, these studies focus on the determination of selected benzotriazole, benzothiazole and benzenesulfonamide derivatives [8], and a number of hormones, pharmaceuticals and personal care products [9-11]. In these works the above-described QuEChERS extraction procedure followed by the traditional d-SPE purification of the extract and liquid chromatographic (LC) analysis with tandem mass spectrometric (MS/MS) [8, 9, 11] or single time of flight mass detection [10], have been applied under both positive and negative electrospray ionization (ESI) modes.

Even though the QuEChERS technique can be considered as a high-throughput analytical approach, the d-SPE step doubles the analysis time and involves an extra sample manipulation, compared to the extraction alone. Moreover, large matrix effects (ME) have been often observed, especially when ESI-MS detection is employed, notwithstanding various d-SPE sorbents, besides PSA, were investigated to lower the matrix influence [10]. A remarkable decrease in total analysis time, together with a significant increase of the overall pre-concentration factor, would be achieved by treating the QuEChERS extract like a water sample, according to a protocol similar to the on-line SPE-LC-MS/MS approach, which has been extensively applied to the determination of various classes of organic micropollutants in environmental waters [12-14].

Based on the considerations reported above, the aim of this research was to investigate the combination of QuEChERS extraction with solid-phase pre-concentration and purification (SPPCP) of the extract, automatically coupled with LC-MS/MS (on-line SPPCP-LC-MS/MS), for the determination of selected pharmaceutical compounds in sewage sludge. More in detail, various commercially available sorbent phases (i.e. silica gel silanized with octyl or octadecyl groups and a polymeric phase functionalized with N-benzylpyrrolidone groups) were evaluated for replacing the d-SPE step traditionally included in the QuEChERS approach. Furthermore, some analytical stationary phases (i.e. silica gel silanized with octadecyl, biphenyl, phenylhexyl and pentafluorophenyl groups), characterized by different physicochemical properties, were tested.

Target compounds of this study (i.e. acetylsalicylic acid, diclofenac, fenbufen, flurbiprofen, ibuprofen, ketoprofen and naproxen) were chosen within the group of non-steroidal anti-inflammatory drugs (NSAIDs), which represent one of the most worldwide consumed class of pharmaceutical compounds [15-17], characterized by significant endocrine disruption properties

[18, 19] and previously found in biological sludge [5, 10, 20, 21]. Moreover, some NSAID metabolites (i.e. salicylic acid, 4'-hydroxydiclofenac, 1-hydroxyibuprofen, 2-hydroxyibuprofen, 3-hydroxyibuprofen and O-desmethylnaproxen), never investigated before in sewage sludge, were included in the study. Target analytes were characterized by a very wide range of polarity (log K_{OW} included in the range 1.4-4.5), thus representing a group of chemicals very interesting to be studied from an analytical point of view during the various partition steps involved in both the QuEChERS and the SPPCP phases.

2 Experimental

2.1 Chemicals and materials

LC–MS grade methanol, acetonitrile, water, formic acid, HPLC grade methanol and acetonitrile were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Sodium chloride and magnesium sulphate heptahydrate used for QuEChERS extraction were obtained from Sigma–Aldrich.

Acetylsalicylic acid (ASA, CAS: 50-78-2), acetylsalicylic acid D3 (ASA D3, CAS: 921943-73-9), salicylic acid (SAL, CAS: 69-72-7), diclofenac (DIC, CAS: 15307-79-6), diclofenac D4 (DIC D4, CAS: 153466-65-0), 4'-hydroxydiclofenac (4'-HYDIC, CAS: 64118-84-9), fenbufen (FEN, CAS: 36330-85-5), flurbiprofen (FLU, CAS: 5104-49-4), ketoprofen (KET, CAS: 22071-15-4), ketoprofen D3 (KET D3, CAS: 159490-55-8), ibuprofen (IBU, CAS: 15687-27-1), ibuprofen D3 (IBU D3, CAS: 121662-14-4), 1-hydroxyibuprofen (1-HYIBU, CAS: 53949-53-4), 2-hydroxyibuprofen (2-HYIBU, CAS: 51146-55-5), 3-hydroxyibuprofen (3-HYIBU, CAS: 53949-54-5), naproxen (NAP, CAS: 22204-53-1), o-desmethylnaproxen (O-DMNAP, CAS: 52079-10-4) were supplied by Sigma–Aldrich. 2-hydroxyibuprofen D6 (2-HYIBU D6, CAS: 50474-67-4) was obtained by Green-Pharma (Orléans, France).

The solid-phase cartridges employed in this study for the extraction of target analytes (see Table 1) were all from Phenomenex (Torrance, CA, USA): octadecyl-bonded silica (Strata C18-E), octyl-bonded silica (Strata C8) and surface-modified N-benzylpyrrolidone polymeric phase (Strata-X).

The following LC pellicular columns (100 mm×3 mm, 2.6 μm particle size), purchased from Phenomenex, were used: (i) octadecylsilane Kinetex XB-C18 (C18), (ii) biphenylsilane Kinetex Biphenyl (BP), (iii) phenyl-hexylsilane (PhH) Kinetex Phenyl-Hexyl and (iv) pentafluorophenylsilane Kinetex PFP (PFP).

The following syringe filters were used: Phenex-RC (cellulose membrane, pore size 0.2 μm, Phenomenex) and Minisart SPR-PTFE (polytetrafluoroethylene membrane, pore size 0.45 μm) (Sartorius-Stedim, Goettingen, Germany).

Acidic water employed for the preparation of standard solutions, the QuEChERS extraction, the on-line SPPCP of the extract and LC-MS/MS analysis was a 0.2% (*v/v*) solution of formic acid in Milli-Q or LC-MS grade water (pH=2.50±0.05).

2.2 Sampling sites and sludge samples

The samples were collected (i) in two different activated sludge WTPs (i.e. Baciacavallo and Calice facilities) devoted to the treatment of wastewater from the industrial textile district and the city of Prato (Tuscany, Italy), and (ii) in three activated sludge WTPs (i.e. Vernio, Vaiano and Cantagallo facilities) treating the domestic and industrial wastewater from the civil and textile areas of Bisenzio Valley (Tuscany, Italy). The sludge lines of WTPs consisted in a gravity thickening and a filter press and/or centrifugal dewatering.

Sewage sludge used for method development and application on real samples were collected in July 2015 and September 2015, respectively. After collection, the samples were immediately treated with liquid nitrogen and transported to the laboratory, where they were freeze-dried and finally stored in the dark at -20°C, until analysis.

For method development, an average representative sample of the different sludge collected in the five WTPs was prepared by mixing equal amounts of each freeze-dried sample (following identified as “sludge mix”).

2.3 QuEChERS extraction

One gram of freeze-dried sludge was weighed into a 50 mL centrifuge tube and 5 mL of acidic water were added. The mixture was hand-shaken for 15 seconds and vortex-mixed for 1 min, and 10 mL of CH_3CN were added. After a further step of hand-shaking and vortex mixing, 2 g of NaCl and 2 g of $MgSO_4$ were added, and the obtained mixture underwent to additional hand-shaking and vortex-mixing processes. The tube was centrifuged at 1200 x g for 4 min and 1 mL of the CH_3CN supernatant phase was made up to 10 mL with acidic water. The diluted extract was finally filtered with a 0.2 μm RC membrane and analysed by on-line SPPCP-LC-MS/MS. Accordingly, the QuEChERS extraction lasted about 9 min.

2.4 On-line SPPCP-LC analysis

The system used for the on-line SPPCP-LC analysis was home-made assembled as schematically illustrated in Fig. S1 of the "Supplementary Material". The single modular devices were purchased from Shimadzu (Kyoto, Japan) and consisted of two isocratic pumps LC-20AD XR (pumps 1 and 2), an autoinjector SIL-30AC equipped with a 2 mL loop, a low-pressure gradient quaternary pump Nexera X2 LC-30AD (pump 3), a thermostatted column compartment CTO/20AC, a degassing unit DGU-20A 5R, and a module controller CBM-20A. The Shimadzu LC system was coupled with a Vici (Schenkon, Switzerland) two-position six-port switching valve model HT. A sorbent cartridge and an analytical column were installed on the six-port valve, as illustrated in Fig. S1.

The automatic SPPCP of the extract consisted in a first step ("loading phase") in which 2 mL of the QuEChERS extract are loaded into the cartridge, using an appropriate carrier eluent, supplied by pump 1 (see Fig. S1-A of the "Supplementary Material"). Afterwards, the valve is switched so as to allow the mobile phase supplied by pump 3 to back-flush the cartridge and target analyte to be desorbed and transferred into the analytical column ("desorption and injection phase", see Fig. S1-B of the "Supplementary Material"), where they undergo the chromatographic separation. After the analyte injection in the analytical column, the valve switch in the previous position and the cartridge is fed by pump 2 in order to remove matrix constituents from the sorbent phase; finally the cartridge is re-equilibrated with the loading solvent supplied by pump 1. The entire chromatographic procedure is programmed and automatically controlled by the Analyst® software, version 1.6.2 (ABSciex, Ontario, Canada).

In the optimized conditions, the automatic pre-concentration and purification phases of the QuEChERS extract were carried out by loading 2 mL (sample drawing speed equal to 11 µL s^{-1}) of the diluted extract on the Strata-X cartridge, with a mixture of acidic water/CH_3OH 80/20 (*v/v*), supplied by pump 1 at a flow rate of 1.50 mL min^{-1} for 3.5 minutes ("loading phase"). Afterwards, the six-port valve switched to the "desorption and injection phase" and the target compounds were eluted from the cartridge to the analytical column, in the counter-flow mode, by the below-reported LC gradient, supplied by pump 3. After 2 min, the valve switched in the "loading phase" and the cartridge was flushed with 100% CH_3CN (pump 2) for 10 min in order to wash the sorbent and for further 6 min with acidic water/CH_3OH 80/20 (*v/v*) (pump 1) for cartridge re-equilibration ("cartridge washing and re-equilibration phase").

The LC analysis was carried out at 25°C, on the PFP column, using acidic LC-MS grade water (A) and CH_3CN (B), as eluents. Flow rate was 0.450 mL min^{-1} and the gradient elution was the following: 25% B for 1.5 min, from 25% to 95% in 5.6 min and a final isocratic for 4 min. A final re-equilibration step at 25% B lasted 7 min. Accordingly, total analysis time per sample, including loop filling, was 25 min.

2.5 Tandem mass spectrometry

The LC system was coupled with a 5500 QTrap™ mass spectrometer (ABSciex), by a Turbo V™ interface equipped with an ESI probe. Tandem mass analysis was carried out using the Multiple Reaction Monitoring (MRM) mode by negative ESI.

Source dependent parameters were optimized in flow injection analysis at optimal LC flow and mobile phase composition and were as follows: Curtain Gas (CUR) 40, Collision-Activated

Dissociation Gas (CAD) medium, Temperature (TEM) 550°C, Gas 1 (GS1) 50, Gas 2 (GS2) 50, and Ion Spray Voltage (IS) -4500 V.

Compound dependent parameters were optimized by direct infusion of properly diluted standard solution of each analyte (see Table 2).

2.6 Identification and quantification of target analytes

For each investigated compound, the most intense transition was used for quantification and the second most intense, when present, for confirming identification (Table 2). In order to confirm the identities of target analytes, criteria proposed by the Commission Decision 2002/657/CE were adopted [22]. The positive identification is achieved when: (i) LC chromatographic retention time agrees within ±2%; (ii) relative abundance of the two transitions, selected as precursor ion and product ion, fall within the permitted tolerances for relative ion intensities using the LC-MS technique.

For quantification of target analytes in real samples, the standard addition method was adopted; accordingly, sludge samples were fortified with four different concentration levels, each one replicated three times, and subjected to the whole analytical process, together with unfortified samples. The spiking procedure was performed by adding 500 µL of CH_3CN standard solution to 1 g of dried sludge, the sample was then vigorously vortex stirred and the solvent was evaporated at room temperature. Finally, the sludge was incubated for 24 h at 4°C prior analysis.

Peak attribution and quantitative determination were performed using MultiQuant software version 3.0.2 (ABSciex). All statistical analyses were performed using SPSS® software, version 22 (SPSS Inc., Chicago, IL, USA).

3 Results and discussion

Structure formula, log K_{OW} and pKa values of the investigated analytes are shown in Fig. S2 of the "Supplementary Material".

3.1 On-line SPPCP-LC-MS/MS approach

3.1.1 Chromatographic behaviour

In this paper the four different commercially available pellicular analytical columns listed in Section 2.1 were tested to study the chromatographic behaviour of target analytes. The choice of pellicular analytical columns allows to achieve the same peak capacity of fully porous stationary phases, using larger particle diameters, thus leading to lower backpressures, which are generally more advisable for lowering the mechanical stress of chromatographic systems and specifically more compatible with the use of on-line SPE cartridges [12].

The four stationary phases selected for this study (i.e. C18, BP, PhH and PFP) were characterized by very different functionalization of silica particles, thus covering a wide and interesting range of interactions between target analytes and stationary phases themselves. More in detail, C18 stationary phase, which has been extensively used for LC analysis of pharmaceutical compounds, including NSAIDs [23, 24] is characterized by hydrophobic interactions. PFP, which was employed for NSAID determination only in few cases [25, 26], is conversely distinguished by a much wider set of interactions, including π-π, hydrogen bonding, dipole-dipole and steric ones. A similar broad variety of interactions is also shown by BP and PhH columns, which have been herein investigated for LC analysis of NSAIDs for the first time.

As illustrated in Table 2, among target compounds of this study, FEN and KET are characterized by the same quantifier MRM transition; furthermore, 1-HYIBU, 2-HYIBU and 3-HYIBU have common quantifier and/or qualifier transitions, being them positional isomers (see Fig. S2 of the "Supplementary Material"). Hence, for the above-mentioned compounds the chromatographic separation is mandatory for their LC-MS/MS determination.

The chromatographic behaviour of target analytes on the four different stationary phases included in this study was first investigated using mixtures of 0.2% (*v/v*) aqueous solution of formic acidic and methanol or 0.2% (*v/v*) aqueous solution of formic acidic and acetonitrile, as eluents, according to a gradient elution from 10% to 90% of the organic solvent at a column temperature of 25°C. Separation of isobaric compounds was achieved with all stationary phases using CH_3CN as organic solvent, whereas when CH_3OH was adopted, 2-HYIBU and 3-HYIBU were not resolved on the C18 stationary phase, and 3-HYIBU and 1-HYIBU co-eluted on the PFP column. As expected, a general much higher retention was highlighted using CH_3OH instead of CH_3CN, irrespective of the stationary phase employed. More in detail, with the former eluent, PFP and BP columns were the most retentive. PFP stationary phase showed the highest retention with CH_3CN, as well, especially for the more polar analytes (i.e. SAL, ASA, 1-HYIBU, 2-HYIBU and 3-HYIBU, see log K_{OW} values reported in Fig. S2 of the "Supplementary Material"). In this regard, it should be remarked that a higher analyte retention is more advisable when a reversed-phase SPPCP step is planned to be combined with the analytical chromatography. In fact, in order to achieve a narrow band during the analyte desorption from the cartridge and a satisfactory peak focusing in the analytical column, an aqueous-organic mixture with proper eluting power must be used, so as to minimize the loss of resolution of the chromatographic system, especially for early eluting compounds. Thus, much higher is the analyte retention on the analytical column, less important is the influence of the initial organic percentage in the eluent employed for desorption from the cartridge on the chromatographic separation.

Based on the above-reported findings, BP and PFP columns were selected employing acidic water/CH_3OH and acidic water/CH_3CN eluent mixtures, respectively.

3.1.2 Optimization of the analyte desorption within the on-line SPPCP step

Among the few sorbents commercially available as on-line cartridges, those selected for this study were: (i) an octadecyl-bonded silica; (ii) an octyl silica and (iii) a styrene-N-benzylpyrrolidone co-polymeric phase, which provide different retention characteristics. Even though octyl- and octadecyl-bonded silica sorbents are more suitable for the recovery of hydrophobic species from aqueous solutions, they have been also successfully employed for SPE of medium- to high-polarity compounds, such as estrogens [12] and pharmaceuticals [27, 28]. Accordingly, they can be adopted for NSAIDs recovery under proper experimental conditions that essentially concern the use of low loading volumes [29], the use of solvent mixtures with low eluting strength during the SPPCP step and the pH correction of loaded sample and eluents, in order to prevent ionization of target analytes. The Strata-X cartridge belongs to the group of stationary phases that allows for establishing hydrophilic, π-π bonding, hydrogen bonding and dipole-dipole interactions, which are particularly important for the retention of molecules like drugs, which have multiple functional groups.

The three cartridges (i.e. Strata C18-E, Strata C8 and Strata-X) were preliminarily tested to evaluate the desorption profile of target compounds from the SPE sorbents, so as to define the optimal eluent composition to be used for analyte transfer to the analytical column. This latter aspect is very important in order to obtain a narrow chromatographic band during the desorption phase and, consequently, a satisfactory peak focusing in the analytical column.

Initially, standard water solutions of target compounds were loaded at room temperature into the SPE sorbents using an acidic water/CH_3OH 90/10 (*v/v*) mixture as loading carrier and acidic water/CH_3OH or acidic water/CH_3CN as cartridge backflush mixture, with organic solvent percentages included in the range of 20-50%.

The use of aqueous methanol mixtures for the desorption of target compounds was not able to provide a good mass transfer from Strata-X, not even by eluting with acidic water/CH_3OH 50/50 (*v/v*). The strong retention of the N-benzylpyrrolidone polymeric phase was mainly due to the π–π interactions between sorbent and target analytes. Conversely, when C8 and C18-E sorbents were used, a narrow detachment band (i.e. 30-60 sec, respectively) was achieved with methanol percentages of 50% (see Fig. S3 of the "Supplementary Material"). The higher eluting strength of CH_3CN allowed to obtain the desorption of investigated compounds from all the sorbents in a short time window (i.e. 1 min) using percentages of organic solvent of 25% (see Fig. S4 of the "Supplementary Material").

Based on the aforementioned considerations, the subsequent optimization steps have been performed on the following on-line sorbents/analytical column configurations: (a) Strata C8/PFP; (b) Strata C18-E/PFP; (c) Strata-X/PFP; (d) Strata C8/BP and (e) Strata C18-E/BP. According to the chromatographic behaviour observed for the PFP and BP analytical columns (see section 3.1.1), for configurations (a-c) and (d-e), acidic water/CH_3CN and acidic water/CH_3OH mixtures must respectively be used.

3.1.3 On-line SPPCP-LC-MS/MS chromatographic method

The chromatographic behaviour of target analytes was investigated for the five sorbents/analytical column configurations reported above and common elution gradients were respectively optimized for configurations (a-c) and (d-e), with the aim of identifying the best compromise between chromatographic resolution and analysis time. For this optimization the injection volume was 2000 µL (sample drawing speed equal to 11 µL s^{-1}) and loading solution was acidic water/CH_3OH 90/10 (*v/v*) at the flow rate of 1.50 mL min^{-1} for 3.5 min.

For the instrumental configurations (a-c) the separation was carried out at 25°C, with a flow rate of 450 µL min^{-1}, using acidic water (A) and CH_3CN (B) according to the following gradient elution: 25% B for 4.5 min, from 25% to 95% in 5.6 min and final isocratic for 4 min. The "two position six-port" switching valve (see Fig. S1A-B of the "Supplementary Material") was scheduled as follows: 0-3.5 min "loading phase", 3.5-5.5 min "desorption and injection phase", 5.5-21.6 min "cartridge washing and re-equilibration phase". The duration of the whole chromatographic method, including loop filling, sample loading and system re-equilibration, was 24.6 min. Representative chromatograms obtained under the above-mentioned experimental conditions with the Strata-X and Strata C8 coupled with the PFP analytical column are shown in Fig. 1A-B, as examples of the chromatographic behaviour with a-c configurations.

Analogously, for configurations (d-e) the column temperature was set at 20°C and the chromatographic analysis was performed at 300 µL min^{-1} using acidic water (A) and CH_3OH (B), eluting as follows: 50% B for 8 min, from 50% to 95% in 4.5 min and final isocratic for 4 min. The two position six-port switching valve was scheduled as follows: 0-3.5 min "loading phase", 3.5-4.5 min "desorption and injection phase", 4.5-22 min "cartridge washing and re-equilibration phase". Total analysis time per sample, including loop filling, sample loading and system re-equilibration, was 25 min. A representative chromatogram obtained under the above-mentioned experimental conditions with the Strata C18-E/BP configuration is shown in Fig. 1-C, as an example of the chromatographic behaviour with d-e configurations.

The chromatographic resolution of the MS/MS isobaric compounds (see Table 2) was achieved on each investigated configuration, even though different elution orders and chromatographic profiles were observed, depending on sorbents and analytical columns used. In any case, a very good peak shape was obtained for O-DMNAP, 4'-HYDIC, KET, FEN, NAP, FLU, IBU and DIC. Conversely, the peak shape of ASA, SAL and HYIBUs resulted to be affected by the different nature of the SPE cartridge. More in details, broader peaks were observed for the above-mentioned compounds when the Strata-X sorbent was used (see Fig. 1-A), due to the multiple interactions, typical of this phase. On the contrary, a better peak focusing was achieved by means of the octyl and octadecyl sorbent phases (Fig. 1-BC).

Since baseline separation of MS/MS isobaric compounds was obtained in all cases, each proposed configuration was further investigated for the following optimization steps.

3.1.4 Optimization of the dilution factor of QuEChERS extract

The raw QuEChERS extract is typically a CH_3CN solution that cannot be directly loaded into the commonly available sorbent cartridges, the retention mode of which is based on the reversed-phase mechanism. Thus, the raw organic extract must be diluted with water before the SPPCP procedure, and the dilution factor to be applied is a key–parameter in method development, since it affects the overall method performance.

In order to assess the minimum dilution factor to be applied to the raw QuEChERS extract, acidic water/CH_3CN mixtures at the relative percentages of 95/5, 90/10 and 80/20 (*v/v*) (corresponding to dilution factors of 20, 10 and 5, respectively) were properly spiked to final concentrations of 25 ng L^{-1} for SAL, DIC, 4'-HYDIC, FEN, KET and NAP, 100 ng L^{-1} for FLU, IBU and O-DMNAP and 250 ng L^{-1} for ASA and HYIBUs. The standard solutions were subjected to the on-line SPPCP-LC-MS/MS analysis using Strata C8, Strata C18-E and Strata-X cartridges coupled to the PFP analytical column. The spiked acidic water/CH_3CN solutions were loaded into the cartridges using an aqueous-methanolic solution containing the minimum organic solvent percentage (i.e. 5%), so as to enhance the influence on the sorbent retention of CH_3CN present in the diluted extract. For each compound, the mean peak areas (n=5) were compared to those obtained from five replicated analysis of a reference standard solution in acidic water (representing the "infinite dilution" of the raw organic extract), containing the aforementioned concentrations of target analytes. Fig. 2-AB illustrates the results obtained for Strata-X and Strata C18-E, the latter as an example of the retention observed for alkyl bonded silica sorbents, which behaved very similarly.

For the most lipophilic compounds the retention of alkyl bonded silica and Strata-X sorbents was high for all the acidic water/CH_3CN relative percentages, compared to acidic water 100%, whereas for compounds characterized by the lowest log K_{OW} values (i.e. ASA, SAL, HYIBUs and O-DMNAP, see Fig. S2 of the "Supplementary Material") a strong analyte loss was observed during the loading step, when the highest CH_3CN percentage (20%) was employed. Furthermore, for SAL and above all ASA, the drop of normalized peak area was evident also for CH_3CN percentages of 10% and 5%, evidencing that even very low percentages of organic solvent in the loading solution significantly hinder the retention of these molecules under the reversed-phase mode. More in detail, irrespective of the cartridge considered, the percent decrease of the chromatographic response with increasing CH_3CN content in the loading solution from 5% to 10% was in the worst case (e.g. SAL with Strata C18-E) less than 40%. Conversely, when CH_3CN percentage increased from 10% to 20% the signal drop was much more relevant, being it about 50%; moreover, using the Strata C18-

E, a 50% decrease of the chromatographic area was also observed for HYIBUs (Fig. 2B). In this regard, it should be underlined that signal losses ≥50% observed with the doubling of CH_3CN percentage, make negligible the signal increase due to the halving of the dilution factor and the corresponding doubling of the pre-concentration one.

Accordingly, an acidic water/CH_3CN 90/10 (*v/v*) ratio, equivalent to a 1:10 dilution factor of the raw QuEChERS extract, can be considered the best compromise that allows to obtain a high pre-concentration factor, together with satisfactory recoveries.

3.1.5 Influence of the methanol percentage in the loading solution on the recovery profile within the on-line SPPCP step

The recoveries of target analytes during the SPPCP phase were evaluated for the three investigated sorbents as a function of the eluting strength of the loading solution dispensed by Pump 1. An acidic water/CH_3CN mixture 90/10 (*v/v*), which simulates the composition of a raw QuEChERS extract after its 1:10 dilution with acidic water, was properly spiked to final concentrations of 25 ng L^{-1} for DIC, 4'-HYDIC, FEN, KET and NAP, 100 ng L^{-1} for FLU, IBU, O-DMNAP and SAL and 250 ng L^{-1} for HYIBUs. For ASA a spiking concentration of 250 or 1000 ng L^{-1} was adopted, depending on the sorbent used for the SPPCP phase.

The spiked solution was subjected to the on-line SPPCP-LC-MS/MS analysis using acidic water/CH_3OH mixtures with relative percentages of organic solvent in the range of 5-30%, as loading solution. The lowest CH_3OH percentage corresponded to the lowest organic solvent concentration necessary to avoid alkyl bonded phase collapse and subsequent retention loss of analytes.

This evaluation was performed using the PFP column, according to the elution gradient described in Section 3.1.3. For each eluent composition, five replicated on-line SPPCP-LC-MS/MS analysis were performed and the corresponding chromatographic areas were compared with those obtained by direct injections (n=5) of equivalent amounts of target analytes. Accordingly, recovery values for a given compound were calculated as the percent ratio of the mean peak area obtained in the on-line SPPCP configuration and the corresponding mean value obtained by direct injection.

Fig. 3 illustrates the mean recovery percentages and corresponding standard deviations obtained for each investigated compound, using Strata-X (Fig. 3A), Strata C18-E (Fig. 3B) and Strata C8 (Fig. 3C) cartridges coupled to the PFP column.

The Strata-X sorbent (Fig. 3A) exhibited satisfactory recoveries, ranging from 70% to 107%, for all the target analytes and under all the loading conditions tested, with the only exception of ASA (41%) using 30% CH_3OH in the loading solution. The use of CH_3OH percentages as high as 30% was not investigated on octadecyl (Fig. 3B) and octyl (Fig. 3C) silica sorbents since with a percentage of the organic solvent as high as 20% CH_3OH, ASA and SAL were washed out of the sorbents.

The acidic water/CH_3OH ratios 90/10 and 80/20 (v/v) showed similar recoveries for all target compounds. Accordingly, the latter relative percentage was chosen for the loading solution, being it the best compromise between satisfactory recovery and efficient clean-up of the matrix in the analysis of real samples.

Data reported in Fig. 3, together with those discussed in the previous sections, indicated the feasibility of using Strata-X sorbent for the on-line SPPCP analysis of QuEChERS extracts, after their 1:10 dilution, employing an acidic water/CH_3OH 80/20 (*v/v*) loading solution and performing the LC-MS/MS analysis on the PFP column under the optimized elution conditions reported in the Section 3.1.3.

3.1.6 Instrumental figure of merits of the SPPCP configuration

Before investigating real QuEChERS extracts, this instrumental configuration was preliminarily evaluated for limits of detection (LODs), limits of quantification (LOQs), linearity and precision by replicated injections of standard solutions in acidic water/CH_3CN 90/10 (see Table S1 of the "Supplementary Materials"). LODs and LOQs were taken as the minimum concentrations of target analytes that give rise to a signal to noise ratio (s/n) equal to 3 and 10, respectively. LODs were included in the range 0.33-36 ng L^{-1}, which represents sensitivities lower or comparable with those recently obtained for target analytes on environmental waters using on-line SPE-LC-MS/MS [30-32]. The linearity was investigated by replicated analyses (n=5) of standard solutions from four to ten calibration levels. Concentration ranges from LOQs to 5000-10000 ng L^{-1} were chosen, depending on the analyte, in order to cover a concentration linearity range of about three magnitude orders (Table S1). Determination coefficients ≥0.992 were obtained in all cases. Intra-day ($RSD\%_{intra}$) and inter-day ($RSD\%_{inter}$) precision were evaluated by ten replicated injections of standard solutions, at concentration levels twice higher than LOQs. $RSD\%_{intra}$ and $RSD\%_{inter}$ values were found in the ranges of 1.7-8.2% and 4.1-9.9%, respectively.

3.2 QuEChERS extraction

The QuEChERS approach mainly involves two steps: (i) a water/CH_3CN salting-out liquid/liquid partition of target analytes desorbed from the solid matrix and (ii) a d-SPE for the clean-up of the CH_3CN extract. For the first time, in this paper, d-SPE clean-up is replaced with the on-line SPPCP approach that allows the automated pre-concentration and purification of the raw QuEChERS extract (see Section 3.1), together with LC-MS/MS analysis.

The QuEChERS method is usually applied to solid matrixes with a high water content (e.g. fruit and vegetables) and, if dried samples are extracted, their rehydration before QuEChERS procedure is recommended for increasing analyte recovery; moreover, an excess of solvent compared with the sample is suggested for improving the extraction efficiency [4] and the use of solvent/sample ratios up to ten has been proposed for the analysis of organic micropollutants in sludge [8].

In our study a classical QuEChERS procedure based on CH_3CN as extractant and NaCl and $MgSO_4$ as salting-out agents, was adopted; more in detail, a sample/H_2O/CH_3CN ratio of 1/5/10 (*w/v/v*) and 2 g of each salt were used (see Section 2.3).

3.2.1 Extraction efficiency of the QuEChERS procedure

In order to evaluate the QuEChERS extraction efficiency, three 1 g-aliquots of the "sludge mix" (see Section 2.2 for further details) were fortified with mass labelled compounds to the following final concentrations: 5 ng g^{-1} for DIC D4 and KET D3, 10 ng g^{-1} for ASA D3 and NAP D3, 25 ng g^{-1} for IBU D3 and 2-HYIBU D6. It should be noted that these compounds cover the entire range of physicochemical properties of the investigated molecules (e.g. log K_{OW} and acid-base properties, see Fig. S2 of the "Supplementary Material") and are therefore representative of the whole set of target analytes.

The spiking procedure was performed by adding 500 µL of the CH_3CN standard solution (concentration range from 10 to 50 ng mL^{-1}, depending on the compound investigated) to 1 g of dried sludge, the sample was then vigorously vortex stirred and the solvent was evaporated at room temperature. Finally, the sludge was incubated for 24 h at 4°C. The spiked samples were subjected to the QuEChERS extraction, followed by the on-line SPPCP-LC-MS/MS analysis; the resulting mean areas (n=3) were compared to the mean areas (n=3) obtained by spiking the QuEChERS extract of a non-fortified representative sample with equivalent amounts of mass labelled compounds (i.e. 0.5 ng mL^{-1} for DIC D4 and KET D3, 1 ng mL^{-1} for ASA D3 and NAP D3, 2.5 ng mL^{-1} for IBU D3 and 2-HYIBU D6).

Filtration of QuEChERS extracts before on-line SPPCP-LC-MS/MS analysis was carried out on RC membranes, which guaranteed the absence of adsorption phenomena towards target analytes (see Fig. S5 of the "Supplementary Material").

The QuEChERS extraction efficiency of mass labelled analytes was found in the range of 80-94%. and resulted therefore suitable for the extraction of selected NSAIDs and their metabolites from sewage sludge.

3.3 *Method recovery evaluation*

3.3.1 *Overall analytical process efficiency*

The overall method performance for the analysis of real samples are expected to be affected by the presence of the co-extracted matrix components, which may: (i) interfere with the partitioning processes within the on-line SPPCP step, thus decreasing the overall analytical recovery (RE%) [33] and (ii) alter the efficiency of the ionization process in the MS source. The latter phenomenon, which affects method sensitivity and accuracy is commonly referred as “matrix effect” (ME%) [34]. The evaluation of these effects is of paramount importance for a reliable quantification of target compounds in real samples. Accordingly, in this study the combination of RE% and ME% has been initially evaluated in terms of overall analytical process efficiency (PE%) [33]. To this aim, three aliquots (1 g each) of the “sludge mix” were fortified to three different concentration levels: spike level 1: 5 ng g^{-1} for SAL, DIC, 4’-HYDIC, FEN and KET; 10 ng g^{-1} for ASA, NAP and O-DMNAP; 25 ng g^{-1} for FLU, IBU and HYIBUs; spike level 2: 25 ng g^{-1} for SAL, DIC, 4’-HYDIC, FEN and KET; 50 ng g^{-1} for ASA, NAP and O-DMNAP; 125 ng g^{-1} for FLU, IBU and HYIBUs; spike level 3: 250 ng g^{-1} for SAL, DIC, 4’-HYDIC, FEN and KET; 500 ng g^{-1} for ASA, NAP and O-DMNAP; 1250 ng g^{-1} for FLU, IBU and HYIBUs.

For each compound and spike level, PE% was defined as follows:

$$\mathrm{PE\%} = \frac{\mathrm{A_{spiked}} - \mathrm{A_{unspiked}}}{\mathrm{A_{standard}}} \cdot 100$$

where A_{spiked} is the mean chromatographic area of three replicated QuEChERS-on-line SPPCP-LC-MS/MS analysis of the fortified ”sludge mix”; $A_{unspiked}$ is the mean peak area of three replicated QuEChERS-on-line SPPCP-LC-MS/MS analysis of the unspiked ”sludge mix”; $A_{standard}$ is the mean chromatographic area (n=3) obtained by direct injection of an equivalent amount of the analyte in CH_3CN. The results, illustrated in Table 3, indicate different trends of PE% values as a function of the spike levels, depending on the analyte considered. For most analytes, no statistically significant differences were observed at the three fortification levels investigated. Conversely, for ASA, DIC, 4’-HYDIC and KET, PE% values found at the fortification level 1 were significantly higher than those determined at higher spiking concentrations. Finally, for FLU and NAP a slight increasing PE% trend was evidenced. Very good overall method performances were observed for HYIBUs and O-DMNAP, which showed PE% values in the range of 71-94%. Very low PE% values (≤ 30%) were conversely found for 4'-HYDIC and FEN, whereas intermediate performances (PE% = 31-67%) were found for the remaining compounds.

These results strongly differed from those previously obtained during the performance evaluation of the on-line SPPCP procedure (see Section 3.1.5), indicating that the sample matrix actually affects the SPPCP step and/or the analyte detection via tandem mass spectrometry.

3.3.2 Matrix effect and recovery evaluations of the QuEChERS-on-line SPPCP-LC-MS/MS method

The evaluation of the “matrix effect” occurring in MS source is performed by comparing the signal in solvent of a certain amount of a given analyte, with the one obtained from the injection of a sample or an extract containing the same amount of the analyte [34]. Accordingly, in our case, the sample fraction that should be injected into the analytical column after the SPPCP step (purified matrix) was collected and fortified with target analytes, as followed specified: 2 mL-aliquots of the QuEChERS diluted extract (obtained from the extraction of the “sludge mix”) were loaded onto the cartridge (“loading phase”, see Fig. S1-A of the “Supplementary Material”), treated according to the SPPCP procedure (see Fig. S1-B of the “Supplementary Material”) and finally collected without being introduced in the analytical column. More in detail, in accordance with the SPPCP procedure described in Section 3.1.3, about 900 µL-aliquots of the purified matrix were collected.

The matrix effect was evaluated through the standard additions method, by spiking the 900 µL purified matrix aliquots with the following different equally-spaced amounts of target analytes: 10-20-30-40 pg for SAL, DIC, 4’-HYDIC, and KET; 50-100-150-200 pg for ASA, FEN and NAP; 150-300-450-600 pg for FLU, IBU and O-DMNAP; 250-500-750-1000 pg for HYIBUs. The same amounts of target compounds were added to 900 µL-aliquots of a reference solution with a solvent composition equal to the purified matrix (i.e. acidic water/CH_3CN 75/25). Direct injections (n=3) of the whole 900 µL-aliquots of spiked purified matrix aliquots and reference solutions were performed, and the mean peak areas obtained were plotted as a function of the amount of added compound.

Matrix effect percentage (ME%) was defined as:

$$\mathrm{ME\%} = \frac{S_{\text{purified matrix}}}{S_{\text{solvent}}} \cdot 100 - 100$$

where $S_{\text{purified matrix}}$ is the slope of the calibration line in matrix, whereas S_{solvent} is the slope of the calibration line in solvent (i.e. acidic water/CH_3CN 75/25). ME% values higher or lower than 0 indicate the presence of signal enhancement or suppression in comparison with the instrumental response observed in solvent. However, ion suppression ≤ 20%, is considered by several authors to have a negligible influence on the analytical performance [35-37]. In our study, ME% was always found to be suppressive, being it for most compounds ≤ 20% (Fig. 4). A significant suppressive effect was found only for SAL, 4'-HYDIC, FEN and FLU, which showed ME% values included between -21% and -47%. These results are very satisfactory and indicate the high clean-up efficiency of the proposed SPPCP procedure, especially considering that biological sludge is an extremely complex matrix. Peysson et al. [10], who performed a multiresidual study on 136 pharmaceuticals and hormones in aerobic biological sludge using an optimized QuEChERS extraction followed by d-SPE with PSA and LC-ESI-TOF-MS analysis, reported strong matrix effects for the determination of IBU, KET, DIC and SAL (i.e. from -80% to +251%); moreover, ME found for NAP was so high to prevent its determination. High suppressive matrix effects were also observed by Jelic et al. (i.e. from -14% to -79%) and above all Radjenovic et al. (i.e. from -52% to -85%) for the LC-ESI-MS/MS analysis of DIC, NAP, IBU and KET in aerobic biological sludge from two Spanish WTPs, after pressurized liquid extraction (PLE) and extract clean-up on a styrene-N-vinylpyrrolidone co-polymeric phase [38, 39], which is very similar to the Strata-X sorbent herein selected for the SPPCP analytical step (see Section 3.1.5).

Matuszewski et al. (2003) [33] highlighted the dependency existing among PE%, ME% and RE% by the equation 2:

$$\mathrm{RE\%} = \frac{\mathrm{PE\%}}{\mathrm{ME\%} + 100}$$

that allows for estimating the overall method recovery when PE% and ME% are known.

Table 3 illustrates the RE% ranges of target analytes, corresponding to the PE% values obtained at the three spiking levels and reported in the same table. Recoveries higher than 80% were obtained for HYIBUs and O-DMNAP; moreover, for these analytes, the recovery ranges were quite narrow (difference between minimum and maximum RE% ≈ 10%). For ASA, SAL and KET, RE% values were lower, even though still satisfactory, being them in any case ≥ 50%. The lowest observed recoveries ranged approximately from 40% to 50% and concerned the most hydrophobic compounds. According to the RE% values discussed above, the most polar analytes (i.e. ASA, SAL, HYIBUs and O-DMNAP, log $K_{OW} \leq 2.25$) exhibited RE% values comparable with those observed in solvent (Fig. 3A). Conversely, for the most hydrophobic compounds, larger differences were found, thus evidencing a stronger competitive effect of matrix components on the partitioning process occurring during the SPPCP phase.

Our RE% values can be compared to the ones obtained in the studies mentioned above with regards to the matrix effect. Peysson et al. [10], who attempted the RE% calculation at three different spike levels (250, 1000 and 25000 ng g^{-1}), obtained results for SAL, DIC, KET and IBU only at the highest spiking concentration (RE% = 48-98%), due to a low method sensitivity; moreover, for NAP, the very strong matrix signal suppression did not allow any recovery evaluation. The recovery data herein obtained were comparable or higher than those achieved by Radjenovic and co-workers [38], for KET, IBU and NAP (33-49%), whereas for DIC the same authors reported a value as high as 122%. The same extraction and clean-up procedure performed on aerobic sludge collected in two Spanish WTPs, showed for these analytes a much higher recovery performance (from 81% to 125%) [39], highlighting that the analysis of similar matrixes can give rise to very different method performances.

3.3.3 Evaluation of the overall method sensitivity and precision

The QuEChERS-on-line SPPCP-LC-MS/MS method was evaluated for sensitivity, linearity and precision. Table 4 summarizes the results obtained for these performance parameters.

Method detection limits (MDLs) were established by replicated analysis (n=5) of 1 g-aliquots of the "sludge mix" sample spiked with decreasing concentrations of target compounds and were taken as the concentration that gave rise to a mean signal-to-noise ratio (s/n) equal to three. The MQLs were assessed by the same approach, but considering a s/n equal to ten.

Very good method sensitivities were achieved for target analytes in the optimized experimental conditions, being MDLs and MQLs included in the ranges of 0.065-6.7 and 0.22-22 ng g^{-1}, respectively (Table 4). These limits were found to be lower or comparable than others previously published regarding the LC-MS/MS analysis of NSAIDs in sludge samples processed with various sample preparation techniques, with the exception of the determination of IBU and NAP by Jelic and co-workers, who quantified these analytes at one-two magnitude orders lower (Table 5) [10, 38-40].

Linearity was evaluated in matrix, by spiking a "sludge mix" QuEChERS extract to concentration ranges included between MQLs and 500-1000 ng g^{-1}, depending on the analyte investigated. Hence, two-three magnitude orders were covered, obtaining in any case determination coefficients ≥0.995 (Table 4).

Finally, the method showed very good intra-day and inter-day precision, with $RSD\%_{intra}$ and $RSD\%_{inter}$ in the ranges of 3.1-9.6% and 5.1-12.8%, respectively, as estimated by means of triplicated QuEChERS-on-line SPPCP-LC-MS/MS analysis of a representative sludge sample spiked to the following final concentration: 5 ng g^{-1} for SAL; 10 ng g^{-1} for ASA, DIC, 4'-HYDIC, KET, NAP and FEN; 25 ng g^{-1} for O-DMNAP; 50 ng g^{-1} for FLU, IBU and HYIBUs.

3.4 Method application to real samples

The method was successfully applied to the identification and quantitative determination of selected NSAIDs and their metabolites in sewage sludge samples collected in the five WTPs described in the Section 2.2. Matrix matched calibration approach and sample spiking with surrogate standards (2.5 ng g^{-1} for DIC D4 and KET D3; 10 ng g^{-1} ASA D3 and NAP D3; 25 ng g^{-1} for IBU D3 and 2-HYIBU D6) were adopted for ME correction and PE evaluation.

Table 6 summarizes the mean concentrations of NSAIDs and their metabolites found in real sludge samples. For target compounds detected in real samples with s/n values in between 3 and 10 the MDL-MQL interval was reported.

The highest number of analytes (eight out of the thirteen target compounds) was detected in sample A, which refers to the sludge collected in the "Baciacavallo" WTP, the facility receiving by far the highest hydraulic loading (about 130000 m^3 d^{-1} of treated wastewater, compared to 2000-40000 m^3 d^{-1} of the other WTPs), with a large percentage of civil contribution (about 60%). Interestingly, a high number of NSAID metabolites was generally detected in the investigated samples, thus highlighting the importance to include these analytes in environmental studies regarding this drug class. SAL was detected and/or quantified in all samples, even when its precursor (i.e. ASA) was below MDL (see Table 6). However, for this compound an important natural contribution can be hypothesized, since it is synthesized by plants within the shikimate pathway [41].

4 Conclusions

The QuEChERS-on-line SPPCP-LC-MS/MS method proposed in this paper represents an innovation in terms of sample preparation and analysis of NSAIDs and their metabolites in sewage sludge, one of the more complex environmental matrices, from the analytical viewpoint. In fact, for the first time, the QuEChERS extraction of biological sludge was successfully coupled with a fully automatic pre-concentration and purification of the extract and the LC-MS/MS analysis. This analytical approach offers several advantages, such as the minimization of sample handling and the improvement of the overall analytical throughput, being the total analysis time (about 30 min per sample) the lowest reported in literature.

Both the QuEChERS extraction and the chromatographic analysis were optimized, providing satisfactory overall method recoveries and low matrix effects. Very low detection limits (from tens of pg g^{-1} to ng g^{-1} of freeze-dried sludge, depending on the compound considered) were also achieved.

Even though this study was not designed as an environmental monitoring of target compounds in sludge and included only a few samples collected in a brief period, the results showed that NSAIDs and, above all their metabolites, are present in the investigated matrix.

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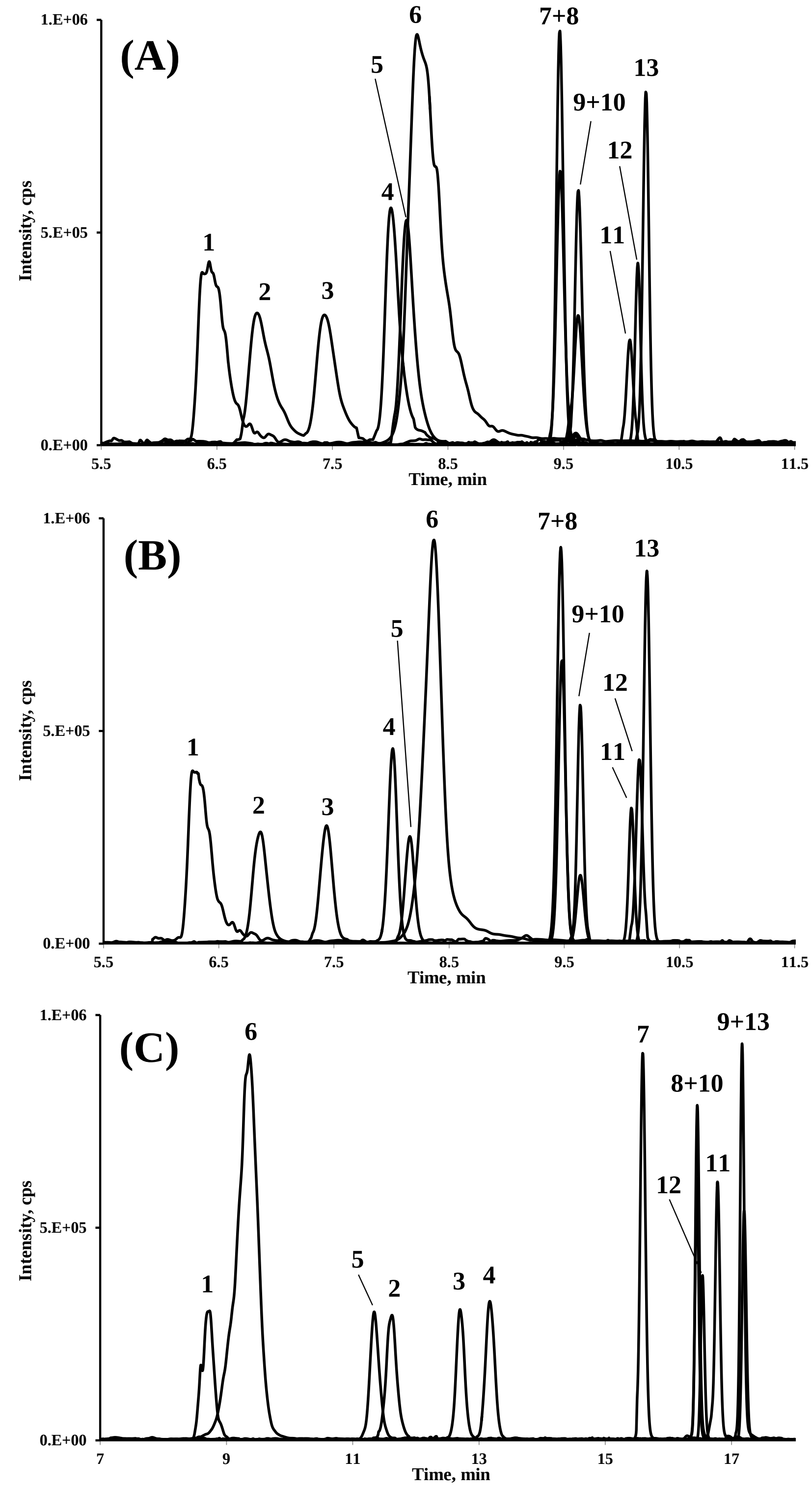

Fig. 1. Reconstructed MRM chromatograms based on the quantifier transitions illustrating the elution order on the resolution of target compounds on the investigated instrumental configurations. (A) Strata-X/PFP; (B) Strata C8/PFP; (C) Strata C18-E/BP (see paragraph 3.1.3). Peak number: (1) ASA; (2) 2-HYIBU; (3) 3-HYIBU; (4) 1-HYIBU; (5) O-DMNAP; (6) SAL; (7) 4’-HYDIC; (8) KET; (9) FEN (10) NAP; (11) FLU; (12) IBU; (13) DIC (see paragraph 2.1 for acronyms meaning).

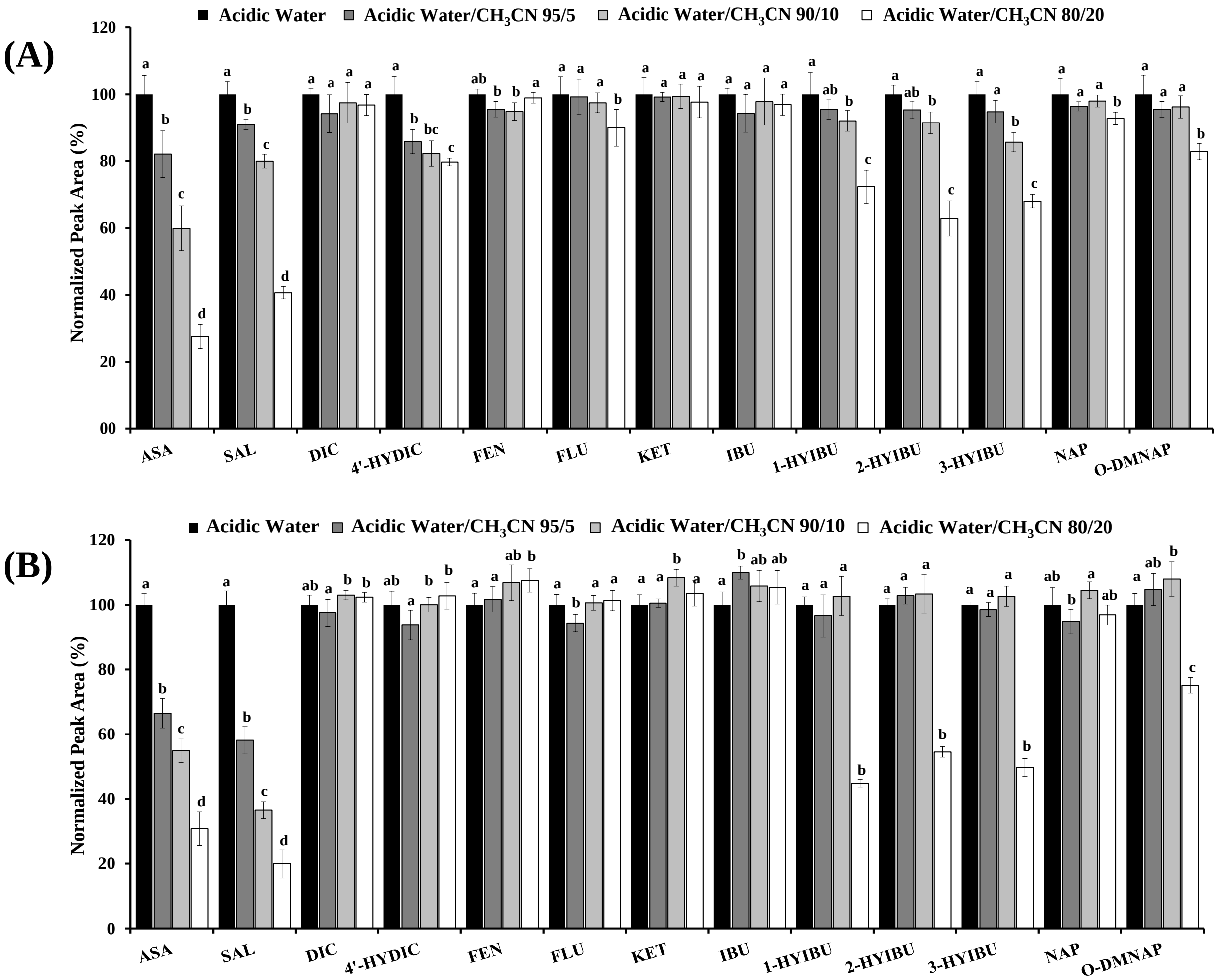

Fig. 2. Mean values (n=5) of normalized peak areas of target analytes obtained after the on-line SPPCP-LC-MS/MS analysis as a function of the dilution factor applied to a reference standard solution in CH_3CN (see paragraph 3.1.4) on following SPE cartridges: (A) Strata-X; (B) Strata C18-E. Error bars represent standard deviations. Values with the same letter are not statistically different at 5% significance level according to the Dunnett T3 nonparametric test. See paragraph 2.1 for acronyms meaning.

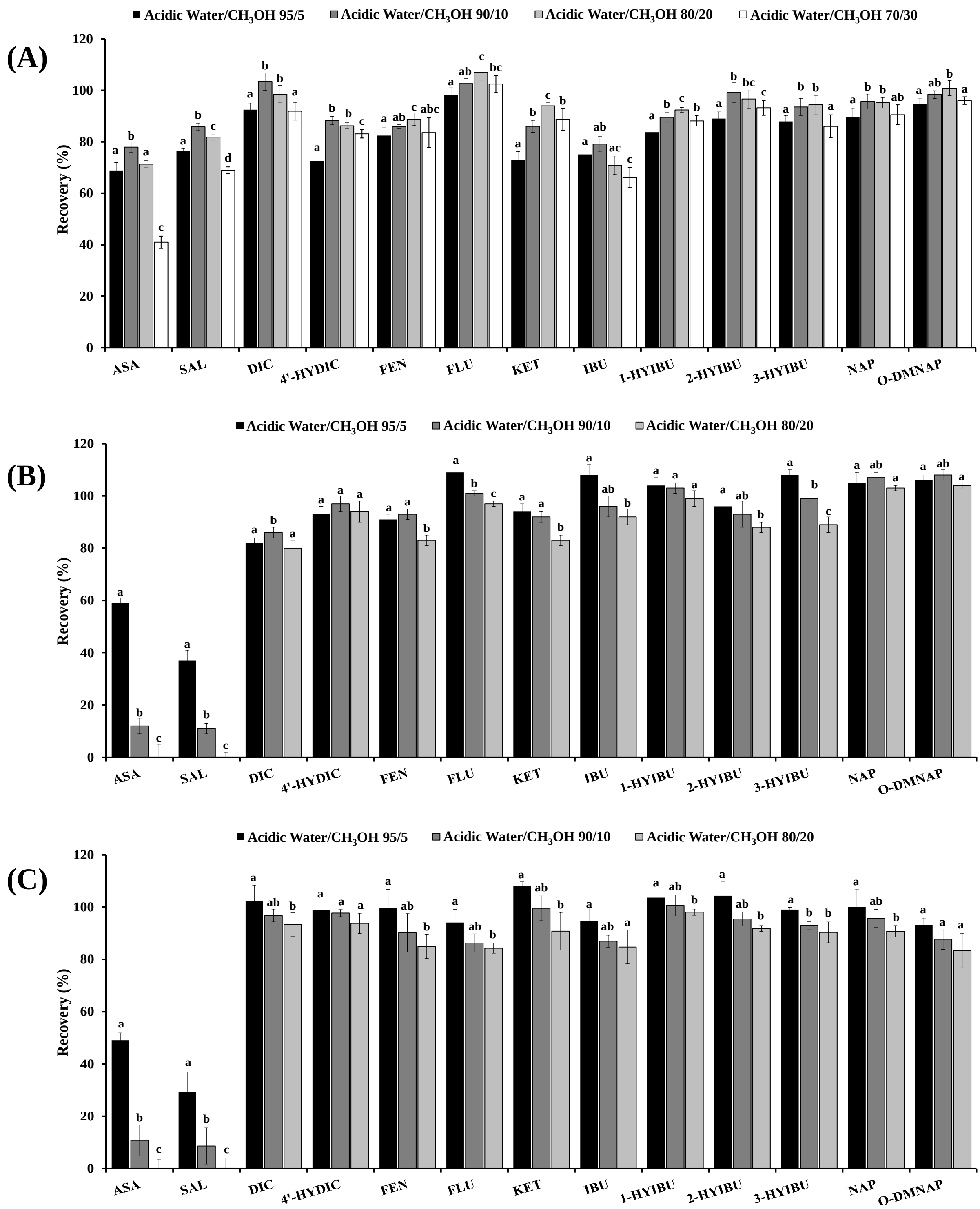

Fig. 3. Mean values (n=5) of recovery percentage of target analytes in acidic water/CH_3CN 90/10 solution as a function of the acidic water/methanol relative percentage in the eluent mixture employed during the "loading phase". (A) Strata-X; (B) Strata C18-E; (C) Strata C8. Error bars represent standard deviations. Values with the same letter are not statistically different at 5% significance level according to the Dunnett T3 nonparametric test. See paragraph 2.1 for acronyms meaning.

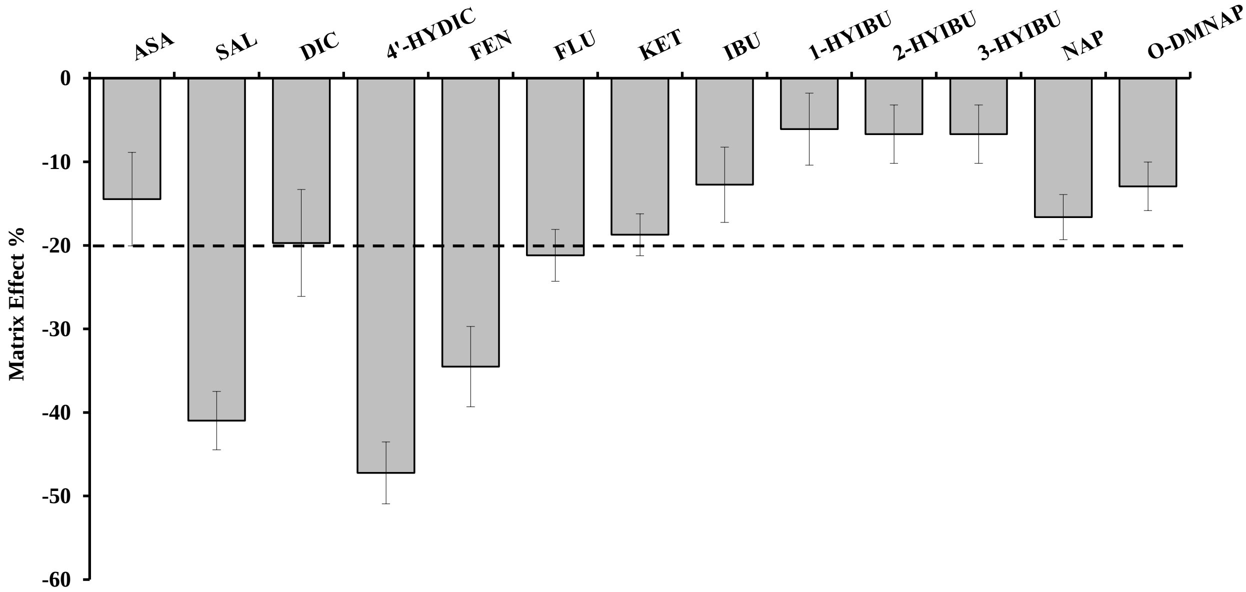

Fig. 4. Mean values (n=3) of matrix effect for target analytes obtained submitting a representative sludge sample to the QuEChERS-on-line SPPCP-LC-MS/MS analysis. Error bars represent standard deviations. See paragraph 2.1 for acronyms meaning.

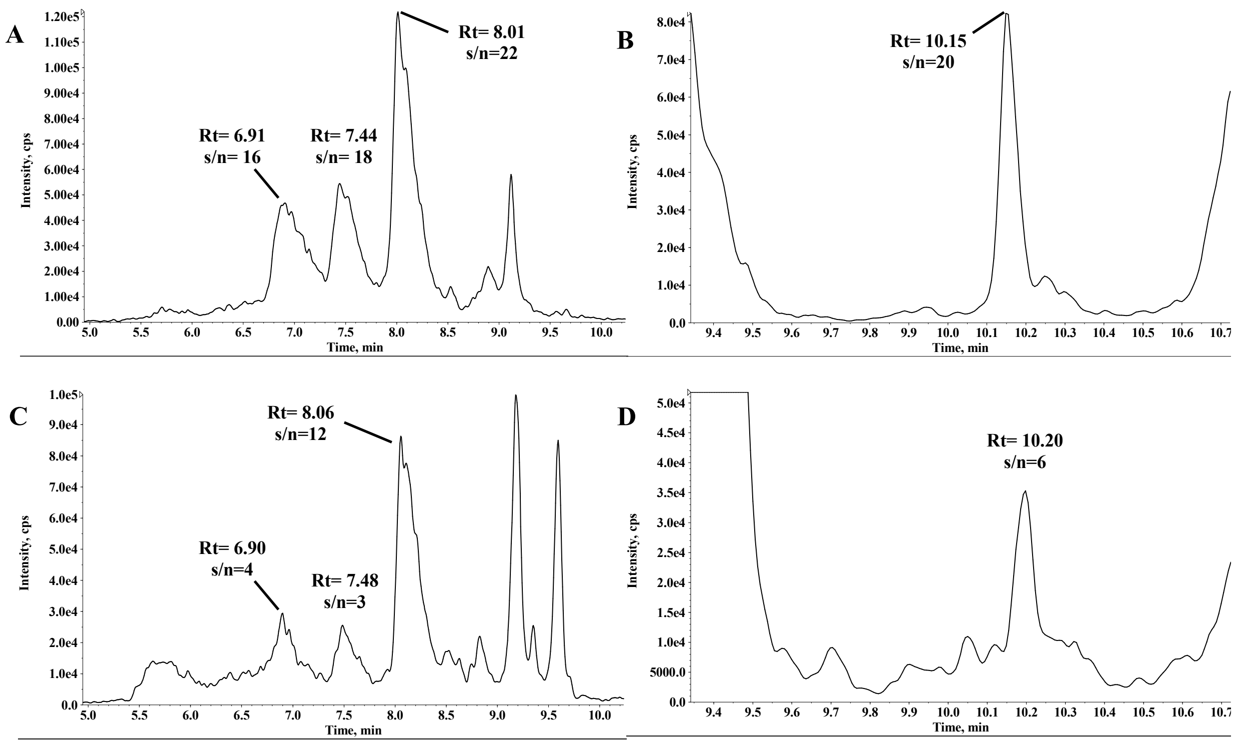

Fig. 5. MRM chromatogram, retention times (Rt) and signal-to-noise ratio (s/n) of selected compounds in the spiked "sludge mix" (first row) and in a sludge sample collected at the Baciacavallo WTP (second row). (A) 2-HYIBU (Rt=6.91, 30 ng g^{-1}), 3-HYIBU (Rt=7.44, 30 ng g^{-1}) and 1-HYIBU (Rt=8.01, 30 ng g^{-1}); (B) IBU (Rt=10.15, 40 ng g^{-1}); (C) 2-HYIBU (Rt=6.90, 5.6-18 ng g^{-1}), 3-HYIBU (Rt=7.48, 5.0-16 ng g^{-1}) and 1-HYIBU (Rt=8.06, 15.3 ng g^{-1}); (D) IBU (Rt=10.20, 6.7-22 ng g^{-1}). See paragraph 2.1 for acronyms meaning.

Table 1. Characteristics of the sorbent cartridges investigated in this study.

Support	Functionalization	Commercial name	Carbon load (%)	Surface area (m^2 g^{-1})	Particle size (µm)	Dimension (mm)
Silica	Octadecyl endcapped	Strata C18-E	18	500	20	20 x 2
Silica	Octyl	Strata C8	10.5	500	20	20 x 2
Polymer	Styrene-N-vinylpiperidinone	Strata-X	n.a.	800	25	20 x 2

n.a. = not available

Table 2
Retention time (Rt, obtained under the experimental conditions described in Section 2.4) and optimized MS/MS parameters of target analytes. (CE) collision energy (reported in bracket, together with the related product ion); (DP) declustering potential; (EP) entrance potential and (CXP) collision cell exit potential. See Section 2.1 for acronym meaning.

Compound	Rt (min)	Precursor Ion	Product Ions (CE)		DP	EP	CXP
			Quantifier Ion	Qualifier Ion			
ASA	6.56	179	137 (-15)	93 (-30)	-40	-9	-10
ASA D3	6.56	182	138 (-10)	94 (-30)	-40	-9	-10
SAL	8.23	137	93 (-25)	—	-60	-9	-10
DIC	10.18	294	250 (-25)	214 (-28)	-60	-5	-10
DIC D4	10.18	298	254 (-15)	217 (-30)	-60	-9	-10
4’-HYDIC	9.43	310	266 (-15)	230 (-15)	-60	-9	-10
FEN	9.59	253	209 (-15)	153 (-30)	-60	-9	-10
FLU	10.14	243	199 (-15)	—	-40	-9	-10
KET	9.47	253	209 (-10)	—	-60	-10	-15
KET D3	9.47	256	212 (-10)	—	-60	-10	-15
IBU	10.20	205	161 (-10)	—	-60	-9	-15
IBU D3	10.20	208	164 (-10)	—	-60	-5	-10
1-HYIBU	7.96	221	177 (-10)	—	-40	-9	-10
2-HYIBU	6.80	221	177 (-10)	—	-40	-9	-10
2-HYIBU D6	6.80	227	183 (-15)	—	-40	-8	-10
3-HYIBU	7.35	221	177 (-10)	—	-40	-10	-15
NAP	9.53	229	169 (-40)	185 (-10)	-50	-10	-10
NAP D3	9.53	232	169 (-40)	188 (-10)	-50	-9	-10
O-DMNAP	8.08	215	171 (-20)	169 (-40)	-80	-10	-20

Table 3

Mean values (n=3) and standard deviation of overall analytical process efficiency (PE%) and overall method recovery (RE%) ranges of target analytes evaluated on three aliquots (1 g each) of a representative sludge sample fortified with three concentration levels. Spike level 1: 5 ng g^{-1} for SAL, DIC, 4'-HYDIC, FEN and KET; 10 ng g^{-1} for ASA, NAP and O-DMNAP; 25 ng g^{-1} for FLU, IBU and HYIBUs; spike level 2: 25 ng g^{-1} for SAL, DIC, 4'-HYDIC, FEN and KET; 50 ng g^{-1} for ASA, NAP and O-DMNAP; 125 ng g^{-1} for FLU, IBU and HYIBUs; spike level 3: 250 ng g^{-1} for SAL, DIC, 4'-HYDIC, FEN and KET; 500 ng g^{-1} for ASA, NAP and O-DMNAP; 1250 ng g^{-1} for FLU, IBU and HYIBUs. PE% values with the same letters are not statistically different at 5% significance level, according to the Dunnett T3 nonparametric test. See Section 2.1 for acronym meaning.

Compound	**Spike level 1**	**Spike level 2**	**Spike level 3**	**RE% range**
	PE%	**PE%**	**PE%**	
ASA	67±7 (a)	46±2 (b)	50±4 (b)	52-76
SAL	48±12 (a)	45±8 (a)	38±4 (a)	58-74
DIC	44±3 (a)	31±1 (b)	30±1 (b)	37-55
4'-HYDIC	29±1 (a)	22±1 (b)	22±1 (b)	42-55
FEN	26±3 (a)	24±3 (a)	30±4 (a)	37-46
FLU	31±2 (a)	36±2 (ab)	37±1 (b)	39-47
KET	60±2 (a)	44±2 (b)	41±2 (b)	50-74
IBU	36±3 (a)	43±6 (a)	42±3 (a)	41-49
1-HYIBU	81±11 (a)	84±8 (a)	88±3 (a)	86-96
2-HYIBU	90±10 (a)	81±7 (a)	82±2 (a)	87-96
3-HYIBU	89±9 (a)	88±8 (a)	94±1 (a)	94-101
NAP	30±1 (a)	35±2 (b)	40±1 (c)	36-48
O-DMNAP	71±4 (a)	81±6 (a)	82±5 (a)	82-94

Table 4

Method Detection Limits (MDLs), Method Quantification Limit (MQLs), linearity range determination coefficient of linear regression, intra-day ($RSD\%_{intra}$) and inter-day ($RSD\%_{inter}$) precision of the QuEChERS-on-line SPPCP-LC-MS/MS method, evaluated in a representative mix of sludge from the five investigated WTPs (see paragraph 2.2). See paragraph 2.1 for acronyms meaning.

Compound	MDL (ng g^{-1})	Linearity range (ng g^{-1})[a]	R^2	$RSD\%_{intra}$	$RSD\%_{inter}$
ASA	0.78	2.6-1000	0.999	4.5	6.2
SAL	0.065	0.22-500	0.997	3.8	5.4
DIC	0.56	1.9-500	0.996	4.2	7.0
4'-HYDIC	1.0	3.3-500	0.995	3.8	6.8
FEN	1.5	5.0-1000	0.997	9.6	12.4
FLU	6.7	22-1000	0.999	3.1	5.6
KET	0.39	1.3-500	0.998	4.8	7.5
IBU	6.7	22-1000	0.998	3.5	5.1
1-HYIBU	4.1	13-1000	0.999	6.0	8.4
2-HYIBU	5.6	18-1000	0.996	7.5	9.6
3-HYIBU	5.0	16-1000	0.996	8.7	10.4
NAP	0.94	3.1-1000	0.999	9.6	12.8
O-DMNAP	2.2	7.4-1000	0.999	5.1	7.5

[a] The bottom limits of linearity range represent MQLs

Table 5
Main characteristics of the analytical method proposed herein, compared to the ones previously published and developed by using different extraction and clean-up procedures for the analysis of selected non-steroidal anti-inflammatory drugs in biological sludge. See paragraph 2.1 for acronym meanings.

Extraction	Enrichment/Clean-up	Analysis time (h)	MQLs (ng g^{-1})						[Reference]
			SAL	DIC	KET	IBU	2-HYIBU	NAP	
QuEChERS	on-line SPPCP [a]	0.5	0.22	1.9	1.3	22	18	3.1	This study
QuEChERS	n.p./d-SPE [b]	1.0 [e]	2500	50	83	3000	n.i.	n.d.	[10]
PLE	off-line SPE [c]	2.5 [e]	n.i.	69	26	89	n.i.	70	[38]
PLE	off-line SPE [c]	2.5 [e]	n.i.	3.1	1.9	0.3	n.i.	0.2	[39]
USE	off-line SPE [d]	1.5 [e]	n.i.	20	50	20	20	n.i.	[40]

[a] N-benzylpyrrolidone polymer
[b] Primary secondary amine
[c] Polystyrene-divinylbenzene-N-vinylpyrrolidone co-polymer
[d] Polystyrene-divinylbenzene-N-vinylpyrrolidone co-polymer functionalized with sulphonated groups
[e] Estimated from the information reported in the paper
n.p. = not performed
n.i. = not investigated
n.d. = not determined due to strong matrix effect

Table 6

Mean concentration (n=3) and standard deviation (in brackets) of target compounds in real samples. All results are expressed in ng g^{-1}. Sample A: Baciacavallo WTP; Sample B: Calice WTP; Sample C: Cantagallo WTP; Sample D: Vaiano WTP; Sample E: Vernio WTP. See paragraph 2.1 for acronyms meaning.

Compound	**Sample A**	**Sample B**	**Sample C**	**Sample D**	**Sample E**
ASA	<0.78 [a]	31.7 (1.4)	<0.78 [a]	<0.78 [a]	<0.78 [a]
SAL	44.5 (1.8)	11.7 (0.7)	32.1 (1.2)	57.1 (2.0)	16.6 (0.5)
DIC	<0.56 [a]	0.56 [a]-1.9 [b]	<0.56 [a]	<0.56 [a]	<0.56 [a]
4'-HYDIC	<1.0 [a]	1.8 (0.1)	2.1 (0.3)	1.0 [a]-3.3 [b]	<1.0 [a]
FEN	11.4 (2.5)	<1.5 [a]	5.9 (0.4)	1.5 [a]-5.0 [b]	10.3 (0.4)
FLU	24.8 (2.2)	<6.7 [a]	<6.7 [a]	<6.7 [a]	<6.7 [a]
KET	<0.39 [a]	11.7 (1.5)	<0.39 [a]	0.39 [a]-1.3 [b]	<0.39 [a]
IBU	6.7 [a]-22 [b]	43.0 (2.1)	<6.7 [a]	<6.7 [a]	<6.7 [a]
1-HYIBU	15.6 (2.8)	<4.1 [a]	4.1 [a]-13 [b]	<4.1 [a]	<4.1 [a]
2-HYIBU	5.6 [a]-18 [b]	<5.6 [a]	<5.6 [a]	<5.6 [a]	<5.6 [a]
3-HYIBU	5.0 [a]-16 [b]	<5.0 [a]	<5.0 [a]	<5.0 [a]	<5.0 [a]
NAP	<0.94 [a]	<0.94 [a]	<0.94 [a]	<0.94 [a]	<0.94 [a]
O-DMNAP	10.5 (0.2)	<2.2 [a]	2.2 [a]-7.4 [b]	2.2 [a]-7.4 [b]	2.2 [a]-7.4 [b]

[a] MDLs= method detection limits at signal-to-noise ratio of 3.
[b] MQLs= method quantification limits at signal-to-noise ratio of 10.

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