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Dilute-and-shoot coupled to mixed mode liquid chromatography-tandem mass spectrometry for the analysis of persistent and mobile organic compounds in human urine

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ABSTRACT

In this work, a comprehensive method for the simultaneous determination of 33 diverse persistent and mobile organic compounds (PMOCs) in human urine was developed by dilute-and-shoot (DS) followed by mixed-mode liquid chromatography coupled with tandem mass spectrometry (MMLC-MS/MS). In the sample preparation step, DS was chosen since it allowed the quantification of all targets in comparison to lyophilization. For the chromatographic separation, Acclaim Trinity P1 and P2 trimodal columns provided greater capacity for retaining PMOCs than reverse phase and hydrophilic interaction liquid chromatography. Therefore, DS was validated at 5 and 50 ng/mL in urine with both mixed mode columns at pH = 3 and 7. Regarding figures of merit, linear calibration curves ($r^2 > 0.999$) built between instrumental quantification limits (mostly below 5 ng/mL) and 500 ng/mL were achieved. Despite only 60% of the targets were recovered at 5 ng/mL because of the dilution, all PMOCs were quantified at 50 ng/mL. Using surrogate correction, apparent recoveries in the 70–130% range were obtained for 91% of the targets. To analyse human urine samples, the Acclaim Trinity P1 column at pH = 3 and 7 was selected as a consensus between analytical coverage (i.e. 94% of the targets) and chromatographic runs. In a pooled urine sample, industrial chemicals (acrylamide and bisphenol S), biocides and their metabolites (2-methyl-4-isothiazolin-3-one, dimethyl phosphate, 6-chloropyridine-3-carboxylic acid, and ammonium glufosinate) and an artificial sweetener (aspartame) were determined at ng/mL levels. The outcomes of this work showed that humans are also exposed to PMOCs due to their persistence and mobility, and therefore, further human risk assessment is needed.

1. Introduction

In recent years, the impact of external factors in combination with the intrinsic genome on human health status has been demonstrated with increasing evidence [1]. As a consequence, the concept of "exposome" has emerged as a novel research paradigm aiming to understand and correlate both external exposures (e.g. lifestyle habits, chemical contaminants, socioeconomic context) and internal factors (e.g. metabolism, endocrine system, ageing process) with disease origin [2,3].

In the analytical chemistry field, efforts have been made in developing methods to analyse chemicals in human matrices, such as urine [4,5], plasma [6,7], or breast milk [8,9]. In many of those works, only semi-polar or non-polar chemicals are monitored when it comes to organic compounds, while very polar compounds are overlooked, as happens in most environmental studies [10].

The issue arises when very polar compounds, which are highly mobile in water, are also persistent. Therefore, they are not degraded or eliminated in wastewater treatment plants and, eventually, they reach drinking water [11]. Those chemicals have been named "persistent and mobile organic contaminants" (PMOCs) or even "(very) persistent, (very) mobile and toxic substances" (PMT/vPvMT) if they are known to be toxic [12]. Examples of such chemicals include quaternary ammonium compounds (e.g.

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diquat and chlormequat), short-chain perfluorinated substances (e.g. carboxylates and sulfonates), pesticides (e.g. acephate and glyphosate), complexing agents (e.g. oxalic acid and EDTA), industrial chemicals (e.g. melamine and acesulfame), and transformation products of the mentioned examples [13,14].

At present, an analytical gap exists for the analysis of PMOCs, which makes the study of their occurrence, fate, or potential adverse effects, and, therefore, their subsequent integration into regulatory lists challenging [10]. In that scenario, The German Environment Agency (UBA) proposed over several years criteria for identifying PMT or vPvMT substances in the regulatory context of the EU REACH (registration, evaluation, authorisation, and restriction of chemicals), for instance [15]. However, robust and reliable analytical methods to analyse PMOCs in aqueous samples are mandatory to continue with that work.

Regarding sample treatment, the extraction of PMOCs from aqueous samples is demanding and common extraction and/or clean-up techniques, such as solid phase extraction (SPE) or liquid-liquid extraction (LLE) usually fail to achieve the required objective [10]. In that context, lyophilization and reconstitution of the sample [16,17] or even "dilute-and-shoot" (DS) approaches with direct injection [18,19] have been tested for minimizing losses in sample preparation.

In the analysis step, PMOCs also pose a major challenge. Even though gas-chromatography (GC) and reverse-phase liquid chromatography (RPLC) cover a wide range of substances in terms of polarity, PMOCs are neither volatile nor retainable in RP columns [13]. Therefore, other LC-based approaches are compulsory to retain very polar compounds and allow their subsequent detection, which is carried out mostly by mass spectrometry (MS) due to its detection limits and identification power [20]. Particularly, hydrophilic interaction liquid chromatography (HILIC) [17,21], mixed-mode liquid chromatography (MMLC), combining either RP or HILIC with an ion-exchange mechanism [22,23], or even supercritical fluid chromatography (SFC) using normal phases [24] seem promising to fulfil that requirement.

While most works determining polar chemicals have been focused on water samples [17,22,25], the number of works involving human matrices is scarce and the real exposure of humans to PMOCs remains unknown [18,26,27]. In that context, the present work aimed to develop an analytical method for the simultaneous determination of different families of diverse PMOCs including acids, bases, neutrals and zwitterions in human urine. To that end, non-discriminatory sample preparation procedures (DS and lyophilization), several chromatographic columns (RP, HILIC, and trimodal MMLC) and elution conditions (mobile phase pH, eluotropic strength, buffer concentration, flow, and column temperature) were optimized.

2. Materials and methods

2.1. Reagents and solutions

In this work, a wide variety of PMOCs in terms of usage and physicochemical properties were selected. Precisely, 33 PMOCs were used including industrial chemicals, biocides and pharmaceuticals. Targets were divided into neutrals, acids, bases, cations, or zwitterions depending on their functional groups and respective acidity constants (pK_a). Moreover, 9 isotopically labelled internal standards (IS) were employed as surrogates for recovery correction. All the information about the PMOCs and surrogates is collected in Table S1 as Supplementary Information (SI).

UHPLC-MS quality acetonitrile (ACN, Scharlau, Spain) and HPLC quality ACN (ChromAR, Macron Fine Chemicals, Avantor, Poland), optima grade ammonia (NH_3, 20–22%, Fisher Chemical, Belgium), optima grade formic acid (HCOOH, Fisher Chemical, Belgium), methanol (MeOH, ChromAR HPLC quality, Macron Fine Chemicals, Avantor), Milli-Q water (H_2O, < 0.05 μS/cm, Millipore 185, Millipore, USA) and UHPLC H_2O (UHPLC-MS quality, Scharlau) were employed as solvents. Individual stock solutions of all targets and surrogates were prepared at 3000 µg/mL level in 1:1 H_2O:ACN (v/v) and from those concentrated solutions, working solutions containing all the analytes were prepared at appropriate concentration levels and solvents depending on the specific experimental requirements. All working solutions were stored at -20 °C in amber vials.

Regarding the solid reagents, ascorbic acid (99.0 – 100.5%, Avantor VWR Chemicals, Slovenia), citric acid (99.5–100.5%, Merck, Germany), creatinine (anhydrous, ≥ 98%, Sigma-Aldrich, Germany), KCl (99.5–100.5% Panreac, Spain), K_3PO_4 (> 98%, Sigma-Aldrich), sodium acetate (NaOAc trihydrate, ≥ 99.5%, Fluka, Germany), NaCl (≥ 99.5%, Merck), $NaHCO_3$ (≥ 99.5%, Merck), NaOH (≥ 98%, Honeywell, Germany), ammonium acetate (NH_4OAc, ≥ 98%, Sigma-Aldrich) and urea (> 98.0%, Sigma-Aldrich) were used. Nitrogen gas (N_2, 99.999%) was purchased from Air Liquid (Spain).

2.2. Sample preparation

DS and lyophilization were evaluated as non-discriminatory sample preparation procedures using synthetic urine. The preparation of the artificial matrix is described elsewhere [28]. In the lyophilization [16], 1 mL of urine was frozen at -20 °C overnight and freeze-dried at -60 °C and 0.05 mbar using a LyoMicron (Coolvacuum Technologies, Spain). Then, the freeze-dried extract was redissolved in 1 mL MeOH, evaporated to dryness using a gentle stream of N_2 in a Turbovap LC Evaporator (Zymark, Biotage, Sweden) at 35 °C, and redissolved in 200 µL H_2O:ACN (75:25, v/v). Regarding DS [29], 1 mL H_2O:ACN (1:1, v/v) was added to 1 mL urine and the mixture was vortexed for 1 min and centrifuged at 15,000 rpm for 5 min at 4 °C. Then, 250 µL of the supernatant were recovered and frozen at -20 °C overnight. All extracts were filtered with 0.22 µm polypropylene filters (Phenomenex, Torrance, USA) and stored at -20 °C in a freezer until analysis.

For each protocol, 3 procedural blanks, 3 samples spiked with all target PMOCs at 40 ng/mL before sample preparation and 3 samples spiked before the analysis (only for lyophilization) at the respective final concentration were processed. To compare both non-invasive sample preparation protocols, the number of detected PMOCs, absolute recoveries and matrix effects were considered. For recovery calculation, the chromatographic peak areas of analytes spiked before sample treatment were compared to the areas of standards of the same final theoretical concentration. In the case of matrix effects, the chromatographic peak areas of procedural blank samples spiked before the analysis were compared to the areas acquired for pure standards.

2.3. UHPLC-MS/MS analysis

The analysis of the target analytes was carried out by a UHPLC system (Agilent 1290 Infinity II) coupled to a triple quadrupole (QqQ) mass detector (Agilent Technologies 6430 Triple Quad). The system had a binary pump, a degasifying system, an automatic injector, an electrospray ionization (ESI) interface, and an ACE Ultra-Core 5 SuperC18 (2.1 mm x 30 mm, 5 µm, Avantor, Sympta) column placed between the loop and the mobile phase line to delay interfering compounds coming from the LC system.

For the separation of the analytes, several chromatographic columns were tested: (i) a Kinetex RP column (C18, 100×2.1 mm, 2.5 µm, Phenomenex), (ii) an Acquity HILIC column (amide, 100×2.1 mm, 1.7 µm, Waters), and two mixed-mode trimodal columns, (iii) Acclaim Trinity P1 (50×2.1 mm, 3.0 µm, Thermo Scientific) and (iv) Acclaim Trinity P2 (50×2.1 mm, 3.0 µm, Thermo

Table 1
Optimal gradient separations for reverse phase, HILIC and Acclaim Trinity P1 and P2 columns at pH = 3 and 7.

Column	Kinetex reverse phase			Acclaim Trinity P1			Acclaim Trinity P2		
Mobile phase	A line (pH = 3 or 7, 5 mM)			A line (pH = 3, 5 mM)			A line (pH = 3, 5 mM)		
	B line (pH = 3 or 7, 5 mM)			B line (pH = 3, 40 mM)			B line (pH = 3, 40 mM)		
Temperature (°C)	35			35			35		
Gradient elution	*Time* (min)	*B line (%)*	*Flow (mL/min)*	*Time* (min)	*B line (%)*	*Flow (mL/min)*	*Time* (min)	*B line (%)*	*Flow (mL/min)*
	0	5	0.3	0	2	0.3	0	2	0.3
	5	5	0.3	3	2	0.3	3	2	0.3
	15	100	0.3	6	50	0.3	6	50	0.3
	20	100	0.3	8	50	0.3	8	50	0.3
	25	5	0.3	12	50	0.6	12	50	0.6
	30	5	0.3	15	80	0.6	15	80	0.6
				25	80	0.6	35	80	0.6
				30	2	0.3	40	2	0.3
				35	2	0.3	45	2	0.3
Column	Acquity HILIC			Acclaim Trinity P1			Acclaim Trinity P2		
Mobile phase	A line (pH = 3, 5 mM)			A line (pH = 7, 5 mM)			A line (pH = 3, 5 mM)		
	B line (pH = 3, 5 mM)			B line (pH = 7, 20 mM)			B line (pH = 3, 20 mM)		
Temperature (°C)	35			35			35		
Gradient elution	*Time* (min)	*B line (%)*	*Flow (mL/min)*	*Time* (min)	*B line (%)*	*Flow (mL/min)*	*Time* (min)	*B line (%)*	*Flow (mL/min)*
	0	95	0.3	0	2	0.3	0	2	0.3
	5	95	0.3	3	2	0.3	3	2	0.3
	15	50	0.3	6	50	0.3	6	50	0.3
	20	50	0.3	10	50	0.3	12	50	0.3
	25	95	0.3	12	80	0.3	15	80	0.3
	35	95	0.3	20	80	0.3	25	80	0.3
				25	2	0.3	30	2	0.3
				30	2	0.3	35	2	0.3

Scientific). The P1 column combines RP with a weak anion exchanger (WAX) and a strong cation exchanger (SCX), while the P2 consists of a HILIC phase, a strong anion exchanger (SAX) and a weak cation exchanger (WCX). For each column, its respective pre-column from the same vendor was used. In all cases, 5 µL were injected.

For the mobile phases, UHPLC H_2O (A line) and ACN (B line) were used at pH = 3 (ammonium formate) and pH = 7 (ammonium acetate) and the chromatographic gradients employed for each column are described in Table 1. For comparing the retention of the PMOCs in each column, retention factor (k) values were calculated according to Equation 1, where RT is the retention time of the analyte and t_0 is the retention time of a non-retained compound.

$$\text{Retention factor}(k) = (RT - t_0)/t_0 \quad (1)$$

The quantification of the target compounds and surrogates was carried out using the Dynamic Multiple Reaction Monitoring (DMRM) acquisition mode at positive and negative ionization. The ESI parameters were defined according to the experience of the research group [30]. Briefly, the capillary voltage used was 3000 V, while a flow of 12 L/min of N_2 was required for the evaporation of the mobile phase. The temperature and nebulizing pressure were set at 350 °C and 30 psi, respectively. The DMRM methods' parameters were optimized using the MassHunter Optimizer by individual injection of the PMOCs without column and are summarized in Table S2. Finally, instrumental operation, data acquisition, and peak integration were performed with the MassHunter Workstation Software (Acquisition, Quantitative and Qualitative Analysis for QqQ, Version 10.0, Agilent Technologies).

2.4. Method validation and quality control/quality assurance (QC/QA)

The DS protocol was validated at two concentration levels (5 and 50 ng/mL in synthetic urine) using both trimodal columns (Acclaim Trinity P1 and P2) for LC separation, while surrogates were added at 25 ng/mL at both levels. Calibration curves for target PMOCs and surrogates were built between 0 and 500 ng/mL in 75:25 H_2O:can and the method was validated in terms of instrumental limits of quantification (iLOQ), linearity ranges, instrumental repeatability, procedural limits of quantification (pLOQ), trueness (absolute and apparent recoveries) and precision for all target compounds using both trimodal chromatographic columns.

Calibration solutions prepared at low concentrations (0.1–50 ng/mL) were injected in triplicate to calculate iLOQs, which were defined for each analyte as the lowest concentration level that fitted the calibration curve with a relative standard deviation (RSD%) and systematic error lower than 30% [31]. Linearity ranges were defined considering the determination coefficients (r^2) of the curves built between iLOQ and the upper limit. pLOQs were estimated from the corresponding iLOQs considering the dilution factor. Moreover, the RSD of the 50 ng/mL calibration point injected in triplicate was calculated to define the instrumental precision.

Absolute recoveries (%) were determined as the average ratio (n = 3) of the concentration obtained from the calibration curve and the nominal concentration used for spiking synthetic urine. In the case of apparent recoveries (%), they were estimated after correction of the absolute recoveries with the corresponding surrogate. Finally, the methodś repeatability was estimated by calculating the RSD for the replicates (n = 3) of spiked samples at each spiking level and only RSD values below 35% were considered for discussion. Procedural blanks were also processed (n = 3) and used for signal correction, while $a \pm 30$ s error was permitted in the RTs.

Regarding the analytical sequence, blank H_2O:ACN (75:25) samples were introduced every 6 samples to check for possible carryover or background contamination, while the 100 ng/mL calibration solution was also injected throughout the sequence every 10 samples to study retention time (RT) shifts and signal intensity.

2.5. Pooled urine analysis

A pool of real urine samples provided by 5 volunteers from the research group was analysed to test the applicability of the developed method, while the obtention of environmental or epidemiological results was out of the scope of this work. The urine samples

were handled following the indications of the Ethics Commission for Research and Teaching of the University of the Basque Country after ethical approval (CEISH-UPV/EHU, BOPV 32, 17/2/2014 M10 2021 124 and CEIAB-UPV/EHU, BOPV 32, 14/2/14, M30 2021 158) and informed consent of all individual participants who provided the samples was obtained before conducting the experiments.

Briefly, first-morning urine samples were collected in glass vials, anonymized and pooled using equal volumes. The pooled sample was stored at 4 °C and processed within 24 h. For that, 1 mL aliquots ($n = 3$) were tempered to room temperature, spiked with the surrogates at 25 ng/mL and the validated DS-MMLC-MS/MS method was applied. The QC/QA criteria mentioned in Section 2.4 (i.e. procedural blank correction, RT deviation and precision) were also considered for real samples.

3. Results and discussion

3.1. Optimization of the chromatographic separation

For the LC separation of PMOCs, four chromatographic columns were compared (Section 2.3). For RP and HILIC, scouting gradients detailed in Table 1 were employed, while several parameters that affect the mixed-mode retention were first studied to build the gradients for the Acclaim Trinity P1 and P2 columns. For that, the following model analytes covering a broad range of acid-base properties were selected: (i) 2-methyl-4-isothiazolin-3-one and acephate as neutrals; (ii) benzyltrimethylammonium as a cation; (iii) cytarabine as a weak base; (iv) trifluoromethanesulfonic acid as a strong acid; (v) bisphenol S as a weak acid; and (vi) aspartame and ammonium glufosinate as zwitterions.

Firstly, the retention of the model analytes at both mixed-mode columns was studied at isocratic ACN proportions (0–90%), pHs (3 and 7) and buffer concentrations (5, 20 and 40 mM) at a constant 0.3 mL/min flow and 35 °C. Figures S1 – S8 in the SI show k values (Equation 1) at the tested conditions and the qualitative observations are also compiled in Table 2. Considering the information gathered in Table 2 and Figures S1 – S8, increasing ACN content decreased retention in most cases although it was limited to 80% to avoid salt precipitation. For zwitterions, strong retention at the maximum ACN percentage was observed and, therefore, an isocratic composition of 50:50 H_2O:ACN was included in the gradients to favour the elution of zwitterions. The HILIC-like behaviour of the Acclaim Trinity P1 column at high organic modifier values has also been observed in the literature for very polar compounds [16]. However, despite the reported HILIC nature of the Acclaim Trinity P2 column, it did not provide better retentions at high ACN percentages overall. Since the details of the HILIC material are not provided, no further discussion is possible.

Regarding buffer concentration, 40 mM turned out to be compulsory to avoid high RT values that led to very broad peaks at pH = 3 for both columns, while 20 mM was enough at pH = 7. Therefore, binary gradients with a simultaneous increase of the organic modifier and buffer concentration were designed (gradients shown in Table 1 for mixed-mode columns at constant 0.3 mL/min flow and 35 °C).

To compare the separation of PMOCs at the four columns, all targets were included and the overlayed Extracted Ion Chromatograms (EICs) are shown in Figs. S9-S12 at the conditions compiled in Table 1. Considering the k values (Equation 1) shown in Fig. 1 as boxplots, both mixed-mode columns rendered wider ranges of k values than RP and HILIC. In fact, both RP and HILIC failed to retain all PMOCs resulting in poor peak shapes and severe co-elution that resulted in decreased sensitivity in the DMRM mode (Figures S9 and S10). Therefore, mixed-mode columns were selected and further optimized since some PMOCs, especially the ones positively charged at pH = 3, were still strongly retained, and broad peaks were obtained. Peak broadening was also observed in the literature for the Acclaim Trinity P1 column by Montes and co-workers, but no measures were taken to reduce retention, and therefore, cationic PMOCs were not further considered in their screening work [16]. In our case, other approaches were tested, such as increasing both the column temperature up to 50 °C and the mobile phase flow to 0.75 mL/min.

The column temperature did not significantly improve peak broadening and it was set to 35 °C (Figure S13). On the contrary, increasing the column flow, decreased retention time and consequently, narrower peaks were obtained (Figure S14). Since similar peak shapes and sensitivity were obtained at 0.6 and 0.75 mL/min, the flow was set at 0.6 mL/min as a consensus between sensitivity, peak shape, and avoidance of high pressures. Despite low flow rates render better ionization in the ESI, higher flow values have been reported in the literature for mixed-mode trimodal columns [32]. All in all, the gradients described in Table 1 (Section 2.1) were used for separating PMOCs in the trimodal columns. Neutral PMOCs, however, were not retained at any of the tested columns and other approaches, such as SFC seem to be necessary for retaining neutral and very polar compounds [33].

3.2. Sample preparation

The comparison of lyophilization and DS was carried out using the Acclaim Trinity P2 column at pH = 3 and pH = 7. Since the lyophilization protocol required a final evaporation and reconstitution step, the injection solvent was first assessed using a mix-solution containing 40 ng of all PMOCs. The mixture was evaporated and the analytes were redissolved in 200 µL of a H_2O:ACN mixture. The optimal solvent was chosen by consensus amongst re-dissolving power (i.e. recoveries) and chromatographic peak shape (i.e. full width at half maximum (FWHM)). Concerning re-dissolving strength (Fig. 2a), a minimum of 25% of ACN was required to properly dissolve all PMOCs. In the case of the peak shape, similar FWHM values were obtained regardless of the ACN percentage as can be observed in Fig. 2b. Consequently, 75:25 in H_2O:ACN (v/v) was selected as the optimum injection solvent.

Regarding sample preparation procedures, in the lyophilization experiments, the preconcentrated salts caused a strong signal suppression and matrix effect values ranged from - 39 to - 87%. Therefore, only 54% of the target PMOCs were successfully detected, with absolute recoveries between 13% and 39%. It could be concluded that despite being a promising non-discriminatory procedure for the determination of PMOCs in water samples [16,17], lyophilization is not suitable for salty aqueous samples such as urine. The DS protocol, however, provided much better results in terms of detected compounds (76%) and absolute recoveries (34–101%). It should be underlined that in the DS protocol, absolute recovery consists of matrix effect since only a dilution of the samples is carried out.

3.3. Method validation

The DS protocol was validated for a total of 33 compounds at 5 and 50 ng/mL in urine using both mixed-mode Acclaim Trinity columns to increase analytical coverage. Isotopically labelled standards were also employed for recovery correction. The results concerning all the figures of merits (Section 2.4) are shown in Tables S3 and S4 in the SI for the P1 and P2 columns, respectively.

3.3.1. Limits of quantification, linearity ranges and instrumental precision

Regarding iLOQs, pH = 3 turned out to be more sensitive than pH = 7 as can be seen in Fig. 3, specially for neutral PMOCs although they were not retained at any condition. Cationic com-

Table 2
Observations of the retention of model PMOCs in the Acclaim Trinity P1 and P2 trimodal columns at the tested mobile phase pH, organic modifier content and ionic strength conditions.

pH	Analyte	Class	Analyte charge	Acclaim Trinity P1			Acclaim Trinity P2		
				Column charge	Retention with increasing acetonitrile content	Ionic strength	Column charge	Retention with increasing acetonitrile content	Ionic strength
3	2-Methyl-4-isothiazolin-3-one	Neutral	Not charged	Negative and positive	Only at 0% and 90% of acetonitrile	No effect	Positive (could be partially negative)	Only at 0% of acetonitrile	No effect
	Acephate	Neutral	Not charged		Only at 0% and 90% of acetonitrile	No effect		Only at 0% and 90% of acetonitrile	No effect
	Benzyltrimethylammonium	Cation	Positive		Decreases	40 mM compulsory		Decreases	40 mM compulsory
	Cytarabine	Weak base	Partially positive		Decreases	20 mM recommended		Higher at 0% and 90% of acetonitrile	20 mM recommended
	Trifluoromethanesulfonic acid	Strong acid	Negative		Decreases	40 mM compulsory		Decreases	40 mM compulsory
	Bisphenol S	Weak acid	Not charged		Decreases	No effect		Decreases	No effect
	Aspartame	Zwitterion	Negative and positive		Only at 0% and 10% of acetonitrile	No significant effect		Only at 0% and 90% of acetonitrile	No effect
	Ammonium glufosinate	Zwitterion	2 negatives and 1 positive		Increases	No significant effect		Increases	Decreases
7	2-Methyl-4-isothiazolin-3-one	Neutral	Not charged	Negative (could be partially positive)	Only at 0% of acetonitrile	No effect	Negative and positive	Only at 0% of acetonitrile	No effect
	Acephate	Neutral	Not charged		Only at 0% and 90% of acetonitrile	No effect		Only at 0% and 90% of acetonitrile	No effect
	Benzyltrimethylammonium	Cation	Positive		Decreases	20 mM compulsory		Decreases	20 mM compulsory
	Cytarabine	Weak base	Not charged		No retention	No effect		No retention	No effect
	Trifluoromethanesulfonic acid	Strong acid	Negative		Decreases	No significant effect		Decreases	20 mM compulsory
	Bisphenol S	Weak acid	Negative		Decreases	No effect		Only at 0% of acetonitrile	20 mM recommended
	Aspartame	Zwitterion	Negative and positive		Only at 0% of acetonitrile	No effect		No retention	No effect
	Ammonium glufosinate	Zwitterion	2 negatives and 1 positive		Increases	No significant effect		Increases	No significant effect

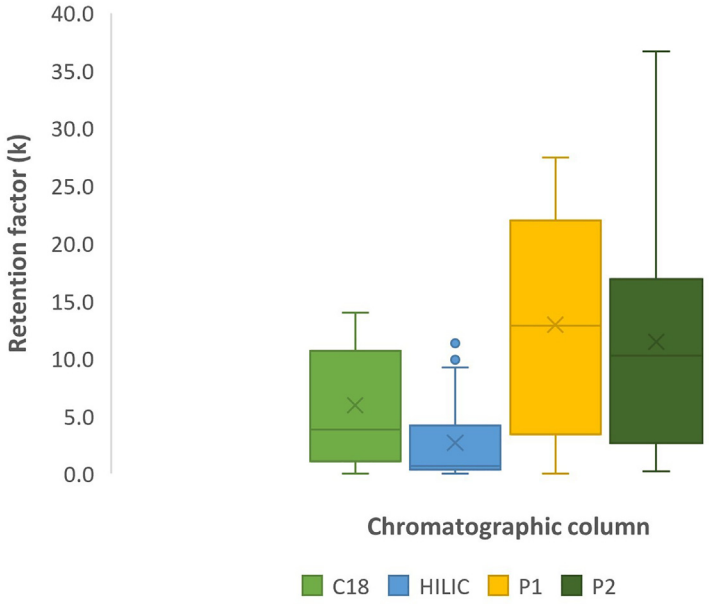

Fig. 1. Boxplots of the retention factor (k) values for the tested chromatographic columns. The "x" indicates the mean value while the dots outside the whiskers are outliers.

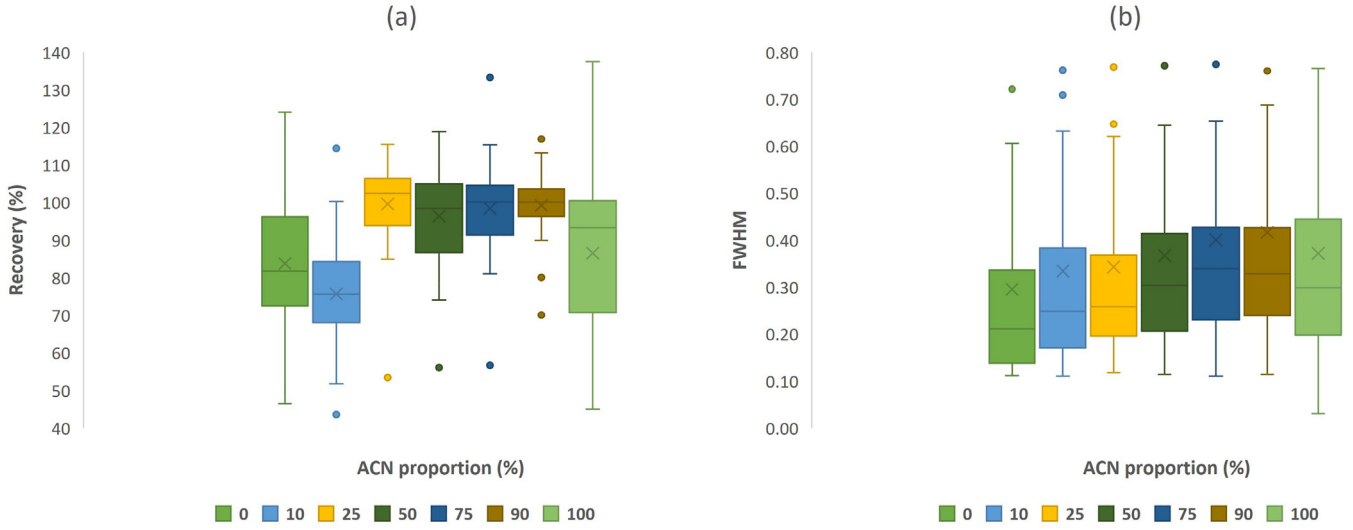

Fig. 2. Injection-solvent results: (a) re-dissolving recovery (%) and (b) FWHM. The "x" indicates the mean value while the dots outside the whiskers are outliers.

pounds rendered iLOQs below 0.5 ng/mL in all the cases except for tetrapropylammonium in the P2 column at pH = 7, which was 2.2 ng/mL. In the case of the weak bases, a similar pattern was observed since iLOQs around 2 ng/mL were obtained in all the cases except for the neutral pH in the P2 column (3–7 ng/mL). The partial neutralization at pH = 7 for bases resulted in a lower retention, and thus, poorer chromatographic peak shape. For strong acids, the higher iLOQs obtained at pH = 7 for P1 (2–15 ng/mL) are related to the non-retention of acids at neutral conditions where WAX is uncharged. For weak acids, instead, similar iLOQs around 5 ng/mL were achieved in all cases.

Lastly, different scenarios were observed for the two zwitterions. On the one hand, ammonium glufosinate was only detected at pH = 7, while aspartame provided the lowest iLOQ in the P1 column at acidic media (0.5 ng/mL). In the literature, mean iLOQ values of 54.3 ng/mL using a QTOF [16] and 1 ng/mL using a QqQ [22] were achieved although few works aim to analyse diverse PMOCs in a single run.

In addition, wider linear ranges were obtained at the P1 column at both pHs and the P2 column at pH = 3 since almost all PMOCs were calibrated up to 450 – 500 ng/mL with r^2 values above 0.998. On the contrary, in the case of the P2 column at neutral pH, linear ranges up to 350 – 400 ng/mL with r^2 values above 0.998 for 70% of the target compounds were obtained. Finally, regarding the instrumental precision, the RSD mean values were below 5% for both columns and pHs, showing good repeatability for the trifunctional columns without carry-over effect or retention-time shifts.

Regarding pLOQs, the mean values for more than 70% of the PMOCs at pH = 3 for P1 and P2 columns were around 5.5 ng/mL. The rest of the analytes provided values higher than 50 ng/mL since they were not detected at the high spiking level. At the neutral pH, however, slightly higher pLOQ values were achieved for both columns since 70% of the analytes rendered a mean pLOQ of around 7.5 ng/mL, while the rest provided pLOQs above 50 ng/mL.

To the best of our knowledge, this is the first work that aims for the quantification of a wide variety of PMOCs in urine sam-

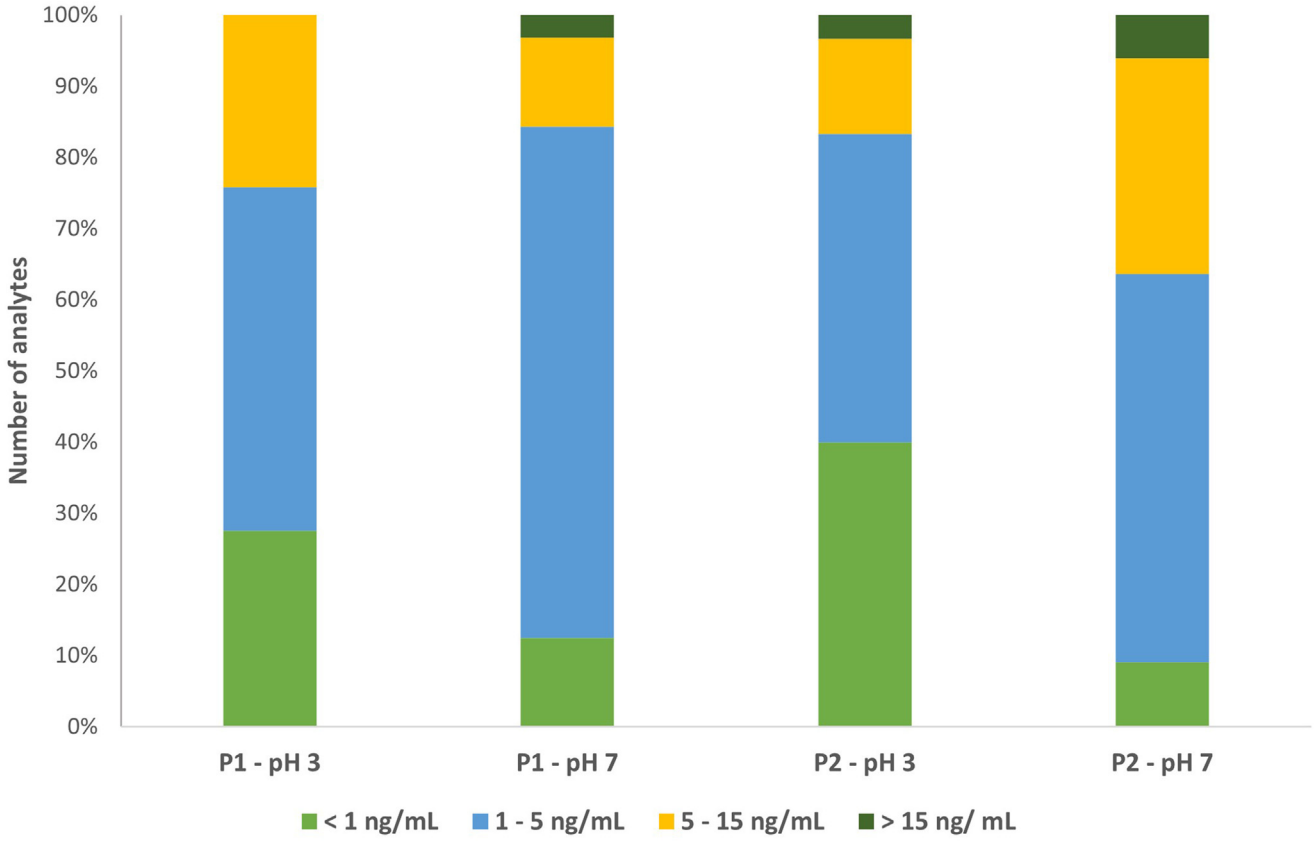

Fig. 3. Summary of the iLOQs for the PMOCs at both Acclaim Trinity columns (P1 and P2) at pH = 3 and 7.

Table 3
Absolute and apparent recovery (%) ranges, average values and detected PMOCs from the total 33 at 5 and 50 ng/mL spiking levels at both Acclaim Trinity P1 and P2 columns at pHs 3 and 7.

LEVEL	PARAMETER	COLUMN	P1		P2	
		pH	3	7	3	7
5 ng/mL	*Absolute recoveries (%)*	Range	19 - 68	41 - 96	23 - 103	81 - 112
		Average	44	69	64	95
		Detected (%)	39	30	42	24
	Apparent recoveries (%)	Range	96 - 122	79 - 102	107 - 128	97 - 120
		Average	107	88	118	109
		Corrected (%)	24	27	18	24
50 ng/mL	*Absolute recoveries (%)*	Range	12 - 105	16 - 82	12 - 99	21 - 103
		Average	66	54	55	71
		Detected (%)	79	70	61	61
	Apparent recoveries (%)	Range	93 - 120	70 - 101	88 - 113	93 - 116
		Average	106	87	97	104
		Corrected (%)	36	55	33	42

ples. In fact, there is only one work in the literature aiming to analyse a wide range of PMOCs in urine [18]. However, that work is focused on suspect screening for both PMOCs and PFAS and therefore, quantitative results were not provided. Consequently, no pLOQs of PMOCs are reported in the literature for urine samples.

3.3.2. Trueness and precision

The figures of merit regarding trueness (absolute and apparent recoveries) are summarized in Table 3 for both spiking levels at all analysis conditions. All the assays were performed in triplicate, and the absolute and apparent recoveries of compounds with an RSD below 35% were only computed as satisfactory results in terms of precision and considered for further discussion.

At 5 ng/mL, around 40% of PMOCs could be quantified at pH = 3 due to the lower iLOQs obtained (Section 3.3.1), with recoveries ranging between 19 and 103%. At pH = 7 instead, higher recoveries (41–112%) were obtained but less than 30% of targets were quantified. In both cases, recoveries for ~ 25% of the total analytes could be corrected by surrogate correction, obtaining apparent recoveries close to 100%. Complementary results were obtained with the four conditions and almost 60% of the target analytes were properly quantified at the low spiking level. Most phosphates could not be quantified due to their complicated nature and the complexity of the urine matrix (see Tables S3 and S4). In fact, very laborious sample preparation methods were used in the literature to extract and preconcentrate small organic phosphates from urine [34].

At 50 ng/mL instead, better recoveries (mean values of 54–71%) were obtained for a higher number of detected compounds (more than 60% in all cases) as can be seen in Table 3. Considering the

Table 4
Concentrations (ng/mL, $n = 3$, 95% confidence level, 2 s) of the target PMOCs measured in the pooled urine sample by DS-MMLC-QqQ at pH = 3 and/or pH = 7.

PMOCs	pH = 3 (ng/mL)	pH = 7 (ng/mL)
2-Acrylamido-2-methyl-1-propanesulfonic acid	< Limit Of Quantification	Not Detected
2-Methyl-4-isothiazolin-3-one	23 ± 6	36 ± 8
6-chloropyridine-3-carboxylic acid	Not Detected	251 ± 54
Acrylamide	671 ± 130	< Limit Of Quantification
Amicarbazone	Not Detected	Not Detected
Ammonium glufosinate	Not Detected	110 ± 12
Aspartame	558 ± 202	459 ± 108
Bisphenol S	21 ± 3	Not Detected
Dicyclohexyl sulfosuccinate	< Limit Of Quantification	< Limit Of Quantification
Diethyl phosphate	< Limit Of Quantification	< Limit Of Quantification
Dimethyl phosphate	Not Detected	99 ± 4
Melamine	< Limit Of Quantification	Not Detected
N,N-dimethylbenzylamine	< Limit Of Quantification	< Limit Of Quantification
N-desmethyl-acetamiprid	< Limit Of Quantification	< Limit Of Quantification

four analysis conditions, all the compounds were quantified and recoveries could be corrected for 91% of the targets (70- 130%). However, acephate was only recovered at P2 – pH = 7 with a low recovery (10%), while phosphates were mostly quantified at the P1 column (see Table S3 for specific recoveries).

To reduce the number of analyses for future monitorization experiments of PMOCs in urine samples, the complementation of the columns was carefully studied to limit the analysis to two conditions. The P1 column allowed the quantification of 94% of the PMOCs, while that value was reduced to 84% in the P2 column. Additionally, the combination between P1 at pH = 3 and P2 at pH = 7 allowed also the quantification of 94% of the total compounds. In the P1 column, acephate and diethanolamine were lost, while in the combination of P1 at pH = 3 and P2 at pH = 7 diethanolamine and toluene-4-sulfonamide could not be quantified. Based on all observations, the use of the P1 column at both pHs is the most straightforward analytical approach to determine PMOCs in terms of a consensus between fewer LC analyses (i.e. only two conditions) and comprehensive detection of PMOCs in urine by DS (i.e. 94% of the targets).

3.4. Pooled real urine sample analysis

In the pooled urine sample from 5 volunteers, 13 out of the 33 target PMOCs (39%) were detected, from which 8 were quantified using the Acclaim Trinity P1 column at pH = 3 and/or pH = 7. The concentrations at a 95% confidence level (2 s, where s is the standard deviation) are shown in Table 4. The Extracted Ion Chromatograms (EICs) of each target PMOC quantified are shown in Figures S15a and S16a for pH = 3 and pH = 7, respectively. Additionally, EICs of procedural blanks (i.e. non-spiked synthetic urine) are also shown in Figures S15b and S16b to ensure that the quantified PMOCs were not artifacts coming from elsewhere.

The PMOCs quantified at higher concentrations were acrylamide, aspartame and 6-chloropyridine-3-carboxylic acid, which were above 200 ng/mL in urine, as can be seen in Table 4. Acrylamide, apart from being an industrial chemical used in the synthesis of polymers, is also a by-product formed when carbohydrate-rich foods are heated [35]. In addition, it has also been found in tobacco smoke [36]. As a consequence, acrylamide and its metabolites have already been detected in urine samples in biomonitoring studies [37,38]. Regarding aspartame, it is a widely used and studied artificial sweetener [39], while 6-chloropyridine-3-carboxylic acid is a metabolite of neonicotinoid insecticides that was previously detected in urine samples at low ng/mL levels (0.01–33.5 ng/mL) using a target method by SPE-RPLC-MS/MS for pesticides and their metabolites [40].

Other two PMOCs related to biocides (i.e. ammonium glufosinate and dimethyl phosphate) were also quantified at 100 ng/mL level. The former is a widely-applied broad-spectrum herbicide that controls weeds in a huge variety of crops and has been detected in numerous foodstuffs and environmental waters [41,42], indicating that humans are in direct contact with that herbicide [43]. Although it has not been detected in urine samples, other similar aminophosphonic pesticides, such as glyphosate and its transformation product (aminomethyl)phosphonic acid have been previously reported [44]. Regarding dimethyl phosphate, it is a metabolite of dialkyl organophosphate pesticides and it has also been detected in human urine samples at low ng/mL levels [45,46]. It should be underlined that those works are specific target methods for organophosphorus pesticides and their transformation products.

At concentrations lower than 100 ng/mL, the antifouling agent 2-methyl-4-isothiazolin-3-one and the industrial chemical bisphenol S were also quantified. Regarding the antifouling agent, its urinary metabolite N-methylmalonamic acid has been followed to assess the exposure. For instance, the metabolite was quantified in urine samples from children and adolescents in Germany with a mean concentration of 6.25 ng/mL [47]. In the case of bisphenol S, it has been previously quantified in urine samples in several works since it has been reported as the alternative for bisphenol A (BPA) by the ECHA (European Chemicals Agency) [48]. In particular, in the work published by Sanchis and colleagues, BPS was quantified at the 0.5 – 8.5 ng/mL range by DS-RPLC-MS/MS [49].

A few more xenobiotics were detected in the pooled urine sample although their concentrations were below the pLOQs, including several industrial chemicals, such as 2-acrylamido-2-methyl-1-propanesulfonic acid, dicyclohexyl sulfosuccinate, melamine, and N,N-dimethylbenzylamine, as well as diethyl phosphate and N-desmethyl-acetamiprid, which are metabolites of the insecticides chlorpyrifos and acetamiprid, respectively.

4. Conclusions

A method for the simultaneous analysis of 33 PMOCs in human urine was developed and validated by means of DS-MMLC-MS/MS. DS was chosen over lyophilization for sample preparation due to the higher number of detected PMOCs (25% more of the targets) and less ion suppression. Besides, in the analysis by LC, after comparing several chromatographic columns (RP, HILIC and mixed mode), Acclaim Trinity P1 and P2 trimodal columns were selected due to their higher capacity of retaining a wider range of PMOCs. In both columns, 40 mM and the increment of the flow from 0.3 to 0.6 mL/min turned out to be compulsory for avoid-

ing peak broadening of strongly retained cationic PMOCs at pH = 3, while 20 mM and a constant flow of 0.3 mL/min were enough at pH = 7. In the validation of the method in both columns at 5 and 50 ng/mL, satisfactory figures of merit were obtained in comparison with other works in the literature. However, pLOQs should be lowered in the future testing sample preparation procedures involving pre-concentration steps. From the complementary results observed, using the column P1 at pH = 3 and pH = 7 seems the best approach for further monitoring works in urine since 31 out of 33 PMOCs were satisfactorily validated at only two analysis runs. At those conditions, a pooled urine sample was analysed and 8 diverse PMOCs in terms of polarity (log D value at pH = 7 ranging from −6.5 to 2.2) and acid-base properties were simultaneously quantified, while additional 5 compounds were detected under the pLOQs. Precisely, aspartame, acrylamide and 6-chloropyridine-3-carboxylic acid were quantified at the highest concentrations showing not only the comprehensiveness of the developed method, but also the exposure of humans to PMOCs. That exposure enhances the necessity of developing methods for PMOCs determination in human matrices and their consequent human health risk evaluation for legacy purposes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Mikel Musatadi: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Jon Zumalabe:** Software, Validation, Formal analysis, Investigation, Writing – original draft. **Leire Mijangos:** Conceptualization, Software, Investigation, Writing – review & editing. **Ailette Prieto:** Resources, Supervision, Project administration. **Maitane Olivares:** Methodology, Data curation, Writing – review & editing. **Olatz Zuloaga:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.464141.

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