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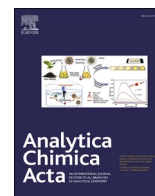
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Expanding the chemical coverage of polar compounds in water analysis by coupling supercritical fluid with hydrophilic interaction chromatography high-resolution mass spectrometry

A. Cerrato^{a,*}, T. Holmark^{b,c}, E. Emke^d, E.D. Amato^d, A.F.G. Gargano^{b,c,**}

^a Department of Chemistry, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185, Rome, Italy

^b Van't Hoff Institute for Molecular Sciences (HIMS), University of Amsterdam, Science Park 904, 1098 XH, Amsterdam, the Netherlands

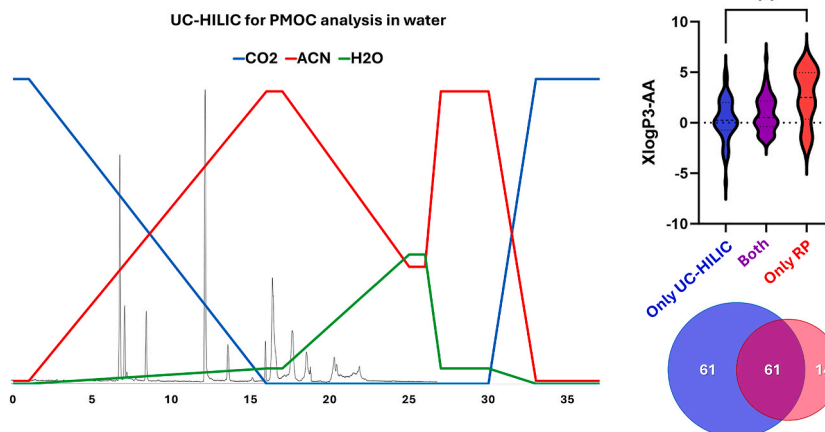
^c Centre for Analytical Sciences Amsterdam, Science Park 904, 1098 XH, Amsterdam, the Netherlands

^d KWR Water Research Institute, P.O. Box 1072, 3430 BB, Nieuwegein, the Netherlands

HIGHLIGHTS

- UC-HILIC-HRMS was proposed for the first time in environmental analysis.
- 122 compounds were annotated following untargeted UC-HILIC water analysis.
- The HILIC gradient helped eluting zwitterionic and positively charged compounds.
- Polarity was the key factor in differentiating UC-HILIC and RP-LC results.
- UC-HILIC was less affected than RP-LC by ion suppression and mask effects.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Persistent and mobile organic compounds (PMOC) are of great concern for water quality and human health. The recent improvement and availability of high-resolution mass spectrometry in combination with liquid chromatography have widely expanded the potential of analytical workflows for their detection and quantitation in water. Given their high polarity, the detection of some PMOC requires alternative techniques to reversed-phase chromatography, such as hydrophilic interaction liquid chromatography (HILIC) and supercritical fluid chromatography (SFC). Unified chromatography (UC), an SFC gradient in which the state of the mobile phase changes continuously from supercritical to liquid at 100 % polar co-solvent, has shown potential for the analysis of compounds in a broad range of polarity, including very polar compounds.

* Corresponding author.

** Corresponding author. Van't Hoff Institute for Molecular Sciences (HIMS), University of Amsterdam, Science Park 904, 1098 XH, Amsterdam, the Netherlands.

E-mail addresses: andrea.cerrato@uniroma1.it (A. Cerrato), a.gargano@uva.nl (A.F.G. Gargano).

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Results: In the present study, for the first time, a UC-HILIC method coupled with high-resolution mass spectrometry was set up for PMOC analysis in water. SFC and HILIC gradients were run sequentially on the same bare-silica column, with the first separation running to 100 % modifier (UC) followed by a HILIC gradient transitioning to water. The UC and UC-HILIC gradients were previously optimized on a mix of 18 representative PMOC to assess solvent and mobile phase composition and for the instrumental system setup. The final method was employed for the analysis of water samples in comparison with a traditional reversed-phase separation, resulting in a significant increase in the number of annotated polar PMOC, including compounds listed in the Candidate List of substances of very high concern for Authorisation by the European Chemicals Agency.

Significance: The proposed approach represents a robust alternative to traditional methods for broadening the chemical space of separation and mass spectrometric detection. The introduction of the HILIC section of the gradient was necessary for the elution of strongly retained compounds on the silica phase, thus also reducing the amount of compounds that would be permanently bound onto the phase of the column, resulting in possible irreproducibility, pressure increase, and loss of efficiency in the compound separation.

1. Introduction

Persistent and mobile organic compounds (PMOC) have recently gained substantial regulatory interest for their environmental stability and mobility in water [1,2]. The list of anthropogenic PMOC substances from industrial, domestic, personal care, and pharmaceutical applications that reach water environments is virtually endless, and many of these are not removed in wastewater treatment plants (WWTP) or are not subjected to treatment processes [2]. For these reasons, PMOC substances are of great concern regarding the quality of drinking water [3]. The European Commission has recently proposed to consider the mobility of substances as a critical property in risk assessment, emphasizing the need for further investigations of PMOC substances in aquatic environments [4].

The recent improvement and availability of high-resolution mass spectrometry (HRMS), especially in combination with gas- and liquid chromatography (GC-HRMS and LC-HRMS) have widely expanded the potential of the employed analytical workflows for the detection and quantitation of PMOC in water [5,6]. Theoretically, HRMS allows for the detection of several thousand compounds from a single injection, as long as they are ionizable, thus allowing simultaneous acquisition of expected, unexpected, and unknown compounds [7,8]. Notwithstanding, it is difficult in practice to set up experimental conditions that are free from several biases, given that all steps of analytical procedures, i.e., sampling, pretreatment, chromatographic separation, and type and conditions of the ion source, limit the nature of chemicals that can effectively be detected [1].

Reversed-phase liquid chromatography (RP-LC), which has numerous applications in environmental analysis [9–11], is mostly viable for non-polar and moderately polar compounds (with logD commonly between -1 and 4), whereas highly polar chemicals are usually not retained (logD < -1). Given that the high mobility of PMOC substances is intrinsically tied to their high polarity, several alternatives have been proposed to narrow the gap left by RP-LC, such as hydrophilic interaction liquid chromatography (HILIC). This technique extends the applicability of LC-HRMS to very polar compounds, but suffers from limited versatility and high dependence on the type of the stationary phase; as a result HILIC shows a better performance than RP-LC only for selected classes of compounds [12].

Recently, supercritical fluid chromatography (SFC) has emerged as a robust and versatile alternative to HILIC, with several applications in combination with HRMS that range from lipidomics [13] to environmental analyses [14]. Under supercritical conditions, molecular diffusion coefficients are often higher, and the mobile phase has lower viscosity compared to typical LC separations. This generally allows to reduce analysis time [15,16]. Furthermore, SFC was also proven to enhance compound desolvation and ionization by electrospray (ESI) [17]. However, SFC using only CO₂ is seldom used and is limited to the study of apolar substances. Typical SFC analysis methods use co-solvents such as methanol and additives (e.g. acids, bases, or buffers), to elute

more polar substances.

SFC-HRMS has shown to be a valid alternative to RP-LC and HILIC in water analysis [18,19]; Schulze et al. compared SFC and RP-LC for the analysis of PMOC in surface, ground-, and drinking water samples, demonstrating that SFC was suitable for highly polar substances, while RP-LC was characterized by poor retention of and peak shapes of these compounds [20]. Recently, Seiwert et al. compared the results of RP-LC and SFC for the non-targeted screening of transformation products of PMOC after ozonation treatment [21]. Their results suggested that, despite allowing the detection of a similar number of transformation products, SFC detected more compounds in the m/z 50–200 range, whereas RP-LC measured more compounds in the m/z 200–500 range, thus suggesting a preference for SFC for smaller and more polar compounds.

Unified chromatography (UC), where the state of the mobile phases change continuously from supercritical to subcritical and finally to liquid when 100 % polar co-solvent is reached, has been described as a bridge between SFC and LC and was proven promising in the simultaneous analysis of hydrophobic and hydrophilic compounds [22–24]. The use of liquified gases in addition to liquid mobile phases has long been used to enhance retention of polar analytes in HILIC, in an approach known as enhanced-fluidity liquid chromatography (EFLC) [25]. Yet, in some cases, even when running a gradient to 100 % of co-solvents, polar and charged compounds may not be eluted from the column [26,27]. One solution could be to use water-based mobile phases with additives as co-solvents to facilitate the elution of charged and highly polar compounds. However, in SFC, the amount of water that can be added to the mobile phases is typically kept within 5 to a maximum of 10 % depending on the amount of organic modifier in the mobile phase [28–30]. An interesting potential solution to this problem, applied to untargeted metabolomics, has been recently proposed by Si-Hung et al. [31], where authors combined a first separation running from CO₂ to ACN with a HILIC-like gradient to further extend the chemical space of the analysis and combine the best of both worlds. Due to the similar columns used, SFC and HILIC can be run sequentially on the same column, with the first SFC separation running to 100 % modifier (UC) followed by a HILIC gradient transitioning to water. Such an approach is known as UC-HILIC and has proved to expand the polarity range of compounds that can be retained and measured [31].

In the present study, for the first time, a UC-HILIC-HRMS approach was set up and employed to analyze PMOC in surface and wastewater samples in untargeted data acquisition mode. The method was optimized using a mixture of standards consisting of PMOC and more traditional compounds and compared to RP-LC to comprehensively evaluate the potential of UC-HILIC-HRMS for water quality monitoring.

2. Materials and methods

2.1. Chemical and reagents

Methanol absolute (MeOH) ULC/MS – CC/SFC grade and acetonitrile (ACN) LC-MS grade were purchased from Biosolve (Valkenswaard, The Netherlands) and milliQ water (H₂O) was filtered using Sartorius Arium 611UV; resistivity 18.2 M cm (Göttingen, Germany). Carbon dioxide (CO₂, purity N4.6) was purchased from Nippon Gases (Vlaardingen, The Netherlands). Ammonium formate (AmF), formic acid (FA), and sodium hydroxide (NaOH) were purchased from Sigma Aldrich (Darmstadt, Germany). Oxypurinol and maleic hydrazide were obtained from LGC Dr. Ehrenstorfer (Augsburg, Germany). 1,5-naphthalenedisulfonic acid tetrahydrate, pyrazole, maleimide, melamine, cotinine, valsartan, valsartan acid, and paraquat dichloride hydrate were obtained from Merck Life Science (Darmstadt, Germany). 5-fluorouracil was obtained from TCI Europe (Zwijndrecht, Belgium). Cyanuric acid was obtained from Acros Organics (Geel, Belgium). Desphenyl chloridazon (TRC, Toronto Canada), (aminomethyl)phosphonic acid (AMPA) (Acros Organics, Geel, Belgium), diglyme (TCI, Portland, United States), tetraglyme (TCI, Portland, United States) glyphosate (Sigma-Aldrich, Missouri, United States) and sucralose (Alfa Aesar, Karlsruhe, Germany) were all obtained in-house from the KWR water research institute (Nieuwegein, The Netherlands).

2.2. Model mixture of reference analytes

A model mixture of 18 reference analytes was employed for setting up the method (Table S1). Stock solutions were prepared at 1 mg mL⁻¹ in pure MeOH for all analytes except for paraquat, AMPA, and glyphosate, which were prepared in H₂O, and oxypurinol, which was prepared in H₂O 0.1 M NaOH, respectively. A working solution mix of the 18 analytes was prepared at 1 µg mL⁻¹ by appropriate dilution in ACN/H₂O 50:50 (v/v) and H₂O/ACN 99:1 (v/v) for UC-HILIC and RP-LC separation, respectively. These compounds were selected due to (i) frequent detection in surface water, (ii) challenging separation in RP-LC, and (iii) a wide range of polarities (with a focus on polar and very polar compounds). This model mixture was used for method optimization. In particular, we focused on (i) the type of organic modifier to use in the UC separation, (ii) the percentage and volume of water that could be injected, and (iii) the hardware configuration to realize the combination between UC and HILIC.

2.3. Water sample preparation

Grab samples were collected at the secondary clarifier of a municipal wastewater treatment plant (65,000 inhabitants) and from the river where the effluent is discharged. All samples (three distinct samples per site) were collected in high density polyethylene (HDPE) bottles and directly frozen upon sampling. Following the protocol of Labad with some modifications [32], samples (50 mL) were dried under vacuum, reconstituted in 1 mL ACN/H₂O 50:50 (v/v) and H₂O/ACN 99:1 (v/v) for UC-HILIC and RP-LC separation, respectively, and filtered onto Nano Spin Filter, 400 µL capacity, 0.2 µm pore size, polyvinylidene difluoride (PVDF) (BGB Analytik, Harderwijk, The Netherlands) in a Eppendorf® Centrifuge 5810 G at 2000×g for 15 min at room temperature.

2.4. UC-HILIC-HRMS untargeted analysis

The UC-HILIC separation on an Acquity Viridis® BEH column (100 × 3.0 mm i.d.), with particle size of 3.5 µm (Waters Corporation) at 40 °C consisted of two subsequent gradients and three mobile phases, i. e., CO₂ (phase A), ACN/H₂O/FA 94.5:5.0:0.5 (v/v/v) 1 mM AmF (phase B), and H₂O/FA 99.5:0.5 (v/v) 5 mM AmF (phase C) (Table 1). Moreover, for the MS coupling, a make-up flow consisting of pure MeOH was employed in combination with the CO₂ flow. Phases A and B were

Table 1

Details on the UC-HILIC gradient using the two connected instruments, i.e., Acquity UPC² and Acquity Binary Pump. The flow gradient scheme is reported in the Supplementary Material.

Time (min)	SFC pump			UHPLC pump		
	Flow (A-B) (µL min ⁻¹)	Phase A (%)	Phase B (%)	Flow (C-D) (µL min ⁻¹)	Phase C (%)	Phase D (MS make-up flow) (%)
1	500	99	1	490	0	100
16	500	0	100	0	0	100
17	500	0	100	0	100	0
25	200	0	100	200	100	0
26	200	0	100	200	100	0
27	500	0	100	0	100	0
30	500	0	100	0	0	100
33	500	99	1	490	0	100
37	500	99	1	490	0	100

pumped by an Acquity UPC² pump (Waters Corporation), whereas phase C and the make-up flow were pumped by an Acquity HPLC binary pump (Waters Corporation). Two mixers of 50 µL and 250 µL from Waters were used, allowing to combine the flow rate of two flows into one. The first mixer (50 µL) was used to mix channel A from the UHPLC pump (phase C) and channel B of the UPC² pump (phase B). The outlet of the mixer was then coupled via the second mixer (250 µL) mixing in the eluent with channel A of the UPLC² pump (CO₂, phase A) and then coupled to the column. The auto backpressure regulator (ABPR) was held constant at 120 bars for the whole gradient. For the UC section of the gradient, the CO₂ flow decreased from 99 % to 0 % in 16 min, whereas phase B increased from 1 % to 100 % in the same period. At the same time, the make-up flow decreased from 490 to 0 µL min⁻¹. After 1 min at 100 % phase B, a flow gradient was run for the HILIC section. The phase B flow decreased from 500 to 200 µL min⁻¹ in 8 min while the flow of phase C increased from 0 to 200 µL min⁻¹. An overall decrease in the flow was needed to comply with the APBR requirements. Subsequently, the flow of phase B returned to 500 µL min⁻¹ in 1 min and kept constant for 3 min to remove the water before reintroducing the CO₂ to the system. Later, phase B decreased from 100 to 1 % in 3 more minutes while phase A returned to 99 % and the make-up flow returned to 490 µL min⁻¹. A re-equilibration step of 4 min was finally added to the method. The injection volume was 5 µL. Table 1 and Fig. 1B summarize the overall gradient.

The UC-HILIC system was coupled with a hybrid quadrupole-Orbitrap Q Exactive plus mass spectrometer via a heated electrospray (HESI) source. Samples were analyzed in the positive (ESI+) and negative (ESI-) ion modes with the following HESI parameters: capillary temperature at 380 °C (ESI+) and 320 °C (ESI-), spray voltage at 4000 V (ESI+) and 3300 V (ESI-), sheath gas at 60 (arbitrary units), auxiliary gas at 20 (arbitrary units), sweep gas at 0 (arbitrary units), and S-Lens RF level was 50 (%). Full-scan MS data were acquired in the range of *m/z* 50–500 with a resolution (full width at half-maximum, FWHM at *m/z* 200) of 35,000. The automatic gain control (AGC) target value was 1,000,000, and the maximum ion injection time was 100 ms. The isolation window width was 2. The top 5 data-dependent acquisition (DDA) mode was performed at a resolution of 17,500 (FWHM, *m/z* 200), the AGC target at 100,000, maximum injection time at 50 ms, isolation window at *m/z* 1.0, and normalized collision energy at 30. The overall UC-HILIC-HRMS system is shown in Fig. 1. Raw MS and MS/MS data were acquired by Xcalibur software (version 3.1, Thermo Fisher Scientific).

2.5. RP-UHPLC-HRMS untargeted analysis

For RP-UHPLC-HRMS analysis, the standard mix and water samples were separated onto a Kinetex XB core-shell C18 (150 × 2.1 mm i.d.),

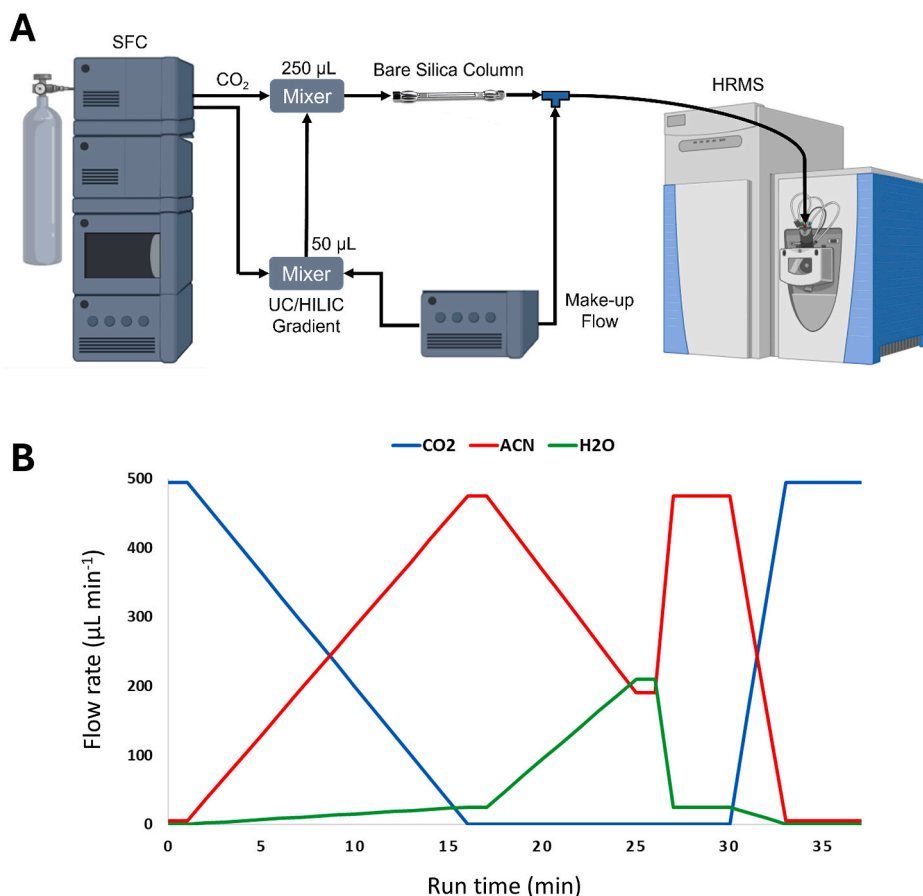


Fig. 1. (A) Graphical representation of the UC-HILIC system that was set up by connecting a Waters Acquity UPC² to Waters Acquity Binary pump to pump phases A, B, and C onto the silica column. In the figure, the connection between SFC and HPLC pump to the column is realized via mixers. A T-piece was used to add to the make-up flow. (B) Representation of the programmed gradient expressed in % of each solvent. The UC separation was completed at 500 $\mu\text{L min}^{-1}$ and HILIC separation at 400 $\mu\text{L min}^{-1}$ (see Table 1 for details). The mobile phase in the graph correspond to CO₂ (phase A, CO₂), ACN/H₂O/FA 94.5:5.0:0.5 (v/v/v) 1 mM AmF (phase B, ACN), and H₂O/FA 99.5:0.5 (v/v) 5 mM AmF (phase C, H₂O). Parts of the figure were created with BioRender.com.

with a particle size of 2.6 μm (Phenomenex, Torrance, CA, USA) at 40 °C and with a flow-rate of 300 $\mu\text{L min}^{-1}$ by an Ultimate 3000 quaternary pump (Thermo Fisher Scientific). The mobile phases consisted of H₂O/FA 99.9:0.1 (v/v, phase A) and ACN/FA 99.9:0.1 (v/v, phase B). The gradient was as follows: 5 % phase B for 1 min, 5–100 % phase B in 25 min, 100 % phase B for 3 min (washing step), and 5 % phase B for 4 min (re-equilibration step). The UHPLC system was hyphenated with the Q Exactive plus mass spectrometer using the same parameters described in section 2.3.

2.6. Data preprocessing and compound identification

Raw data files were processed by Compound Discoverer software (v. 3.1, Thermo Fisher Scientific) using the default data processing workflow dedicated to environmental analysis. A process blank sample, obtained by running a solvent sample through the whole analytical procedure, was analyzed together with the field samples to remove the contaminants deriving from sample preparation and data acquisition. Feature alignment was obtained by the adaptive curve regression model; whenever the adaptive curve model failed, the linear model was automatically selected instead. After the alignment, adducts were detected and grouped, and the list of features was filtered to remove the ones whose areas in the process blank were more than 10 % of the average peak areas in the samples employing the tools “Fill Gaps” and “Mark Background Compounds”. Filtered features were annotated by matching the experimental tandem mass spectra with the mzCloud database. The data presented are available at the following Zenodo Repository link:

<https://zenodo.org/records/13866560>.

2.7. Statistical analysis

Statistical analysis was performed by GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). T-test analyses were performed, and data were reported as bar charts with error bars and statistical significance: ns (non-significant, $p > 0.1$), * ($p < 0.1$), ** ($p < 0.01$), *** ($p < 0.001$), or **** ($p < 0.0001$).

3. Results and discussion

3.1. Preliminary UC experiments

The performance of an analytical method for the separation and MS-based identification of PMOC is closely related to the ability of the method to retain and separate polar and very polar chemicals. However, an ideal chromatographic system should be able to retain and separate a wide range of chemicals, including non-polar, polar, and very-polar compounds in a single run. This can be achieved by combining different separation techniques such as SFC and HILIC. RP-LC is the most commonly used method, however, this method provides limited retention for very polar compounds. In contrast, SFC allows the separation of non-polar and moderately polar lipids, whereas the former are not retained under common HILIC conditions [33]. To start our investigation, we selected 18 chemicals of interest in water analysis ranging from moderately polar to very polar compounds (Table S1).

3.1.1. Modifier

To evaluate the modifier in the UC separation, the standard mix was analyzed using a gradient from CO₂ with 1 % B to 100 % B, with B being ACN/H₂O/FA 94.5:5.0:0.5 (v/v/v) with 1 mM AmF, or the same composition using MeOH instead of ACN. Out of the 18 analytes, 14 were detected using both compositions (i.e., MeOH and ACN), whereas maleimide was only detected using ACN, and paraquat, glyphosate, and AMPA were not detected. Compared to MeOH, ACN has a lower elution strength, and thus, longer retention times were observed when using this solvent. For both ESI+ and ESI-, ACN allowed improved separation and peak shapes. For example, pyrazole and tetraglyme were efficiently separated using ACN (retention time, RT, 6.5 vs 7.8 min, respectively), while they coeluted using MeOH (RT 4.7) (Figs. S1–2). Moreover, the asymmetry factors (T) were generally lower with ACN, as in the case of desphenyl chloridazon (1.3 vs. 2.6) and tetraglyme (1.4 vs. 2.0) Unlike all other analytes, the elution of moderately non-polar valsartan and valsartan acid (XlogP3-AA 4.4 and 2.) was not delayed using ACN, implying a slightly different interaction mechanism. Based on these results, the ACN-based mobile phase was selected as the elution solvent for the UC gradient.

3.1.2. Solvent composition

The injection phase composition is crucial in SFC (and HILIC) methods. The presence of water or MeOH can be detrimental to SFC peak shapes, as these solvents both present elevated viscosity and polarity, contrasting with the initial CO₂-rich mobile phase [34,35]. ACN is a good polar sample diluent due to its aprotic feature and relatively low viscosity [36,37]. Different solvent compositions from 10 % to 50 % H₂O in ACN were tested, and the retention and peak shapes obtained were compared. For most chemicals, up to 50 % of the water content had minor effects on peak shapes and RT (Fig. S3). Peak widths were mostly unaffected, with cotinine representing an exception, whereas an expected increase of the peak asymmetry was measured for the compounds that eluted earlier (e.g., pyrazole). Therefore, 50 % H₂O was kept for further testing as it avoided excessive dilution of the water samples.

3.2. UC-HILIC hyphenation

To enable the hyphenation of UC with HILIC, two distinct and connected pumps were needed and the pressure during the overall gradient had to be within the limit ABPR. In our final setup, the mobile phase B was pumped from the SFC/UC pump, and the mobile phase C from the binary HPLC pump, respectively. The mixer was removed from the binary pump, allowing to use the two channels of the pump independently. This allowed the second pump of the binary pump to deliver a “makeup flow” (phase D) to facilitate post-column solvent addition and ESI ionization. To realize this combination, two mixers were used. One was installed between the two channels from the SFC/UC pump (phases A and B), and one was installed after the first mixer enabling to mixing the eluent from the SFC/UC pump with one of the pumps of the binary pump (phase C). This was then connected to the column, enabling the pumping of 3 mobile phases. The eluate from the column was finally connected to the makeup flow pump via a T piece which brought the phases to the ESI source of the MS system. The makeup flow was needed when a high percentage of CO₂ was pumped to ensure a liquid phase during ESI ionization. As described in the Materials and Methods section, the UC section of the gradient was entirely governed by the SFC pump, while the binary pump pumped the makeup flow that mirrored that of CO₂. Once 100 % phase B was reached (and kept for 1 min to remove possible residual CO₂ in the column), the binary pump switched from the makeup flow to phase C to start the HILIC section of the gradient. The HILIC part of the gradient had to take into account that the increase in the water flow is followed by higher pressure. Therefore, a flow gradient was run between the SFC pump system that pumped phase B and the binary pump that pumped phase C up to 50 % phase C. However, the overall flow was decreased from 500 to 400 $\mu\text{L min}^{-1}$ to

keep the pressure constant and stay within the APBR pressure limits. Later, a faster flow gradient was employed to go back to 100 % phase B (ACN-based) and kept for 3 min to remove most of the water and stabilize the pressure before returning to 99 % phase A, with the binary pump switching back from phase C to the makeup flow (Table 1). Once the gradient was set up, the stock solution was analyzed (Fig. 2). The system we report in this study is similar to the one used by Si-Hung [31] in the only previously reported UC-HILIC hyphenation. The main differences concern the pumping configuration, as the system employed by Si-Hung employs an SFC pump and a quaternary pump, whereas our configuration has two binary pumps. Based on the experimental conditions, the combination of dwell volume and column dead volume results in a 1.5-min delay; therefore, based on the gradient reported in Table 1, all compounds eluted after 18.5 min are eluting during the HILIC part of the gradient. In our experiments, a high % of water is used for elution up to 50 %, suggesting a limited contribution of hydrophilic partitioning to the HILIC retention mechanism. The retention under these conditions is, therefore, most probably driven by hydrogen bonding and ionic interactions rather than partition.

3.3. Analysis of PMOC in stock solutions

The UC-HILIC gradient did not have any effect on the RT and peak shapes of the compounds eluted in the UC part of the gradient and allowed the elution of the highly polar and charged paraquat. Noteworthy, glyphosate and AMPA were eluted as broad low-intensity peaks,

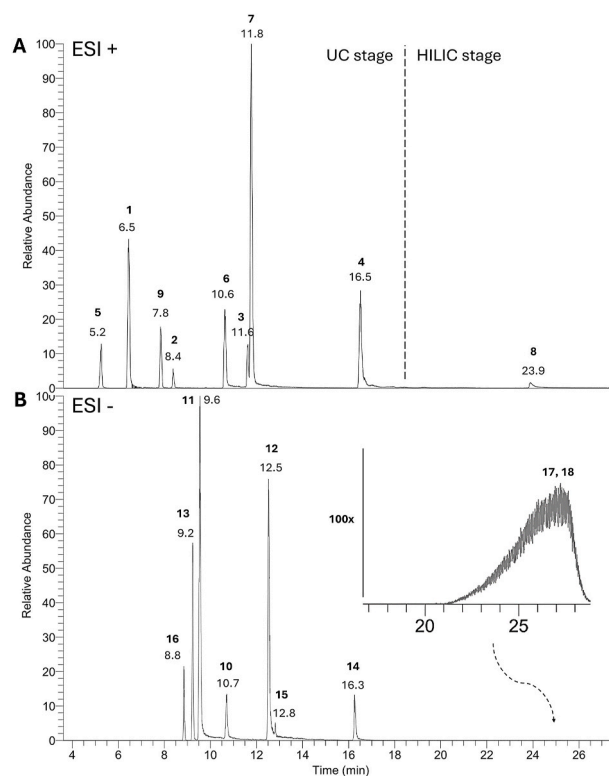


Fig. 2. Extracted ion chromatogram (EIC, 10 ppm for mass error toleration) in positive ion mode (ESI+, A) and negative ion mode (ESI-, B) of the mix of analytical standard PMOC following UC-HILIC-HRMS analysis. 1: pyrazole (m/z 69.0447); 2: maleimide (m/z 98.0237); 3: maleic hydrazide (m/z 113.0346); 4: melamine (m/z 127.0727); 5: diglyme (m/z 135.1016); 6: desphenyl chloridazon (m/z 146.0116); 7: cotinine (m/z 177.1022); 8: paraquat (m/z 186.1157); 9: tetraglyme (m/z 223.1540); 10: cyanuric acid (m/z 128.0102); 11: 5-fluorouracil (m/z 129.0106); 12: oxypurinol (m/z 151.0262); 13: valsartan (m/z 265.0731); 14: 1,5-naphthalenedisulfonic acid (m/z 286.9690); 15: sucralose (m/z 395.0073); 16: valsartan (m/z 434.2198); 17: AMPA (m/z 110.0007); 18: glyphosate (m/z 168.0061).

possibly due to their strong interactions with the silica phase. The proposed methodology allowed good separation of PMOC chemicals in a wide range of polarity ($-4.2 < \text{XLogP3-AA} < 4.4$). To assess the potential and limitations of the UC-HILIC workflow, we analyzed the standard mix using a reference RP-LC (C18 column) method.

The RP-LC separation of the PMOC chemical standard mix (Fig. S4) allowed the detection of 15 out of the 18 compounds, with glyphosate, AMPA, and 1,5-naphthalenedisulfonic acid being undetected. Moreover, except for 5 well-retained compounds, i.e., diglyme, tetraglyme, sucralose, valsartan, and valsartan acid, most analyzed compounds eluted in the first 2 min of the run, indicating poor or no retention on the C18 phase. The peak areas of the 15 compounds that were eluted from both approaches were evaluated by measuring the ratio of the average peak areas from three replicates in UC-HILIC and the corresponding areas of the peaks eluted by RP-LC. Among the analyzed compounds, 9 displayed higher peak area in UC-HILIC (UC-HILIC/RP > 1 , i.e., pyrazole, diglyme, desphenyl chloridazon, cotinine, paraquat, cyanuric acid, 5-fluorouracil, oxypurinol, and sucralose), 5 had higher peak area in RP-LC (UC-HILIC/RP < 1 , i.e., maleimide, maleic hydrazide, melamine, valsartan acid, and valsartan), and one (tetraglyme) did not show significant differences. The UC-HILIC/RP ratio, however, was in most cases between 0.6 and 1.5, thus implying a limited effect of the mobile phases on the ionization of the analyzed PMOC with five exceptions, i.e., desphenyl chloridazon (UC-HILIC/RP = 2.4), cotinine (2.0), paraquat (3.2), 5-fluorouracil (2.7), and oxypurinol (1.9), which were all eluted in the crowded first 1.5 min of the RP-LC gradient and would possibly suffer from ion suppression. The ionization properties of the analyzed PMOC were also evaluated to compare the adduct generated by the two separation strategies. Most of the analyzed chemicals generated the main deprotonated/protonated adduct with an abundance higher than 99 % and were not affected by the nature of the phase composition. Paraquat was ionized as singly and doubly charged adducts with a 1:2 ratio independent of the mobile phase composition (Fig. S5). Diglyme and tetraglyme were the only analyzed PMOC that were affected by the separation strategy, with UC-HILIC generating a significantly higher abundance of the sodiated adducts (Fig. S5), possibly due to higher sodium impurities in the employed mobile phase buffer. In terms of the reproducibility of the RT, the RSD were calculated from three replicates for UC-HILIC and RP-LC. The 16 analytes separated by UC-HILIC showed RT with RSD in the range of 0.01–0.03, except for 1,5-naphthalenedisulfonic acid (0.05), whereas the 15 chemicals separated by RP-LC had RSD of the RT in the range 0.00–0.02. In general, despite the much more complex hardware configuration of the UC-HILIC settings, the proposed methodology provided satisfying reproducibility of the RT. Further experiments were conducted using a bare silica HILIC column on the same LC-HRMS system used for RP-LC separation. The EIC shown in Fig. S6 displays the detection of only 12 out of the 18 standard analytes, with pyrazole, maleimide, paraquat, sucralose, glyphosate, and AMPA not detected. Whereas the behavior of glyphosate and AMPA was expected in analogy with the UC-HILIC results, the absence of the other four compounds was attributed to their extremely poor solubility in 95 % ACN, the solvent needed for compatibility with the HILIC gradient. Another key aspect is the lower separation efficiency of the HILIC gradient compared to the UC gradient in separating some analytes, such as desphenyl chloridazon and tetraglyme, maleic hydrazide and cotinine, and 5-fluorouracil and oxypurinol.

3.4. Untargeted annotation of chemicals from water samples

The analysis of water samples (i.e., wastewater and surface water) was carried out to evaluate the performance of the UC-HILIC method, especially in terms of the reproducibility of the RT, which is a crucial aspect of the untargeted annotation of several samples. As shown in Fig. S7, in which the aligned EIC of four exemplary annotated PMOC are displayed, the satisfactory results obtained from the PMOC mix were confirmed when complex samples were analyzed. After careful spectral

annotation, in which the experimental MS/MS data and the recorded spectra from mzCloud were manually checked, 122 compounds were tentatively identified from the UC-HILIC datasets, belonging to a wide array of classes of compounds, including several PMOC. The list of the annotated compounds is reported in Table S3, alongside their Xlog3P-AA, which is a computationally calculated measure of the water/octanol partition coefficients [38], pKA (when available on PubChem), and identification confidence level according to Schymanski et al. [39], and primary origin or use. Among the annotated compounds, four chemicals (1-vinylimidazole, N-methylpyrrolidone, tetraglyme, and melamine) are listed in the Candidate List of substances of very high concern for Authorisation by the European Chemicals Agency (ECHA, <https://echa.europa.eu/candidate-list-table>) under REACH agreement for being toxic for reproduction. Melamine, in particular, is increasingly being used in a diverse array of industrial and consumer products, e.g., building materials, reusable and single-use plastic tableware, and personal care products, and has been highlighted for having probable serious effects on both human health and the environment [40,41]. Furthermore, five other PMOC, i.e., metformin, guanyleurea, sulfamethoxazole, O-desmethylvenlafaxine, and trimethoprim, were listed in the Surfacewater WatchOut List of the EU [42]. The largest group of chemicals was prescription drugs, with 35 annotated compounds, including analgesics, antibiotics, anticonvulsants, antidepressants, antiepileptics, blood pressure medications, diabetes medications, and anxiety treatment drugs. Interestingly, among the annotated pharmaceuticals, eight were effectively drug metabolites, which not only are of great concern since they are often neglected in water analysis but can also help evaluate the origin of the contamination [43,44]. Two common metabolites of carbamazepine, carbamazepine 10,11-epoxide and 10,11-dihydroxy carbamazepine and 10,11-dihydro-10,11-dihydroxy carbamazepine, the latter being as active as its parent drug, were annotated at higher peak areas than the parent substance. Carbamazepine and its metabolites are also indicators of fecal contamination of water bodies since they are typically employed exclusively in human therapy [45]. Similarly, guanyleurea, the primary metabolite of metformin, was detected at a significantly higher peak area than its precursor in the wastewater effluent samples. Despite the parent substance metamizole not being detected, two of its major metabolites were annotated, i.e., 4-formylaminoantipyrine and 4-acetamidoantipyrine. The anthropogenic origin of the annotated chemicals is confirmed by the several caffeine metabolites and tobacco-related chemicals that were annotated, as well as 3,4-Methylenedioxyamphetamine (MDMA), which was previously found at environmentally damaging levels in local aquatic ecosystems in relation to large human gatherings such as music festivals [46]. Several personal care products were also annotated, including cosmetics, moisturizing agents, and UV filters, including small oligomers of PEG and PPG, which are employed as humectants or emulsifiers in several personal care cream-based products [47]. Other compounds closely related to anthropogenic activities were food additives, including acesulfame, an artificial sweetener that has been previously investigated as an indicator of domestic wastewater contamination in surface water [48]. Another artificial sweetener, saccharin, whose use is widespread for its low cost and sweetness 300 times greater than sugar, is an emerging contaminant with potential ecotoxicity risks to aqueous organisms [49]. Chemicals employed for industrial applications comprised a large group of annotations, including the compounds listed as candidates for being labeled substances of very concern under the REACH agreement. Quite large subsets include chemicals employed in the synthesis of polymers, including 1-vinylimidazole, δ -valerolactam, caprolactam, lauro lactam, N-vinyl-2-pyrrolidone, and terephthalic acid, and chemical employed as corrosion inhibitors, including benzotriazole, benzothiazole, and their related compounds. Benzothiazoles have gathered significant attention since they have been reported to be dermal sensitizers, respiratory tract irritants, endocrine disruptors, carcinogens, and genotoxicants [50]. Other annotated chemicals were pesticides (e.g., pyrazole), herbicides (paraquat), insect repellent (DEET), surfactants (e.g., dodecyl sulfate),

and tire additives (N,N'-dicyclohexylurea). Finally, several additional compounds of endogenous origin were tentatively identified, including amino acids, vitamins, nitrogen compounds, and small aromatics. In Table 2, 20 selected compounds that were exclusively annotated by UC-HILIC-HRMS are reported.

3.5. UC-HILIC vs RP-LC comparison

To evaluate the analytical performances of the UC-HILIC method, the water samples were also separated by RP-LC and analyzed with the same rationale. The manual spectra annotation against the mzCloud database resulted in 75 annotated compounds. Among the identified chemicals, 61 and 14 were only annotated from the UC-HILIC and RP-LC datasets, respectively, whereas 61 were common to both datasets. The annotated compounds (only UC-HILIC, only RP-LC, and both) were compared based on their pKa and XlogP3-AA. As shown in Fig. 3B, the hydrophobicity of the compound was a statistically significant parameter in distinguishing the compounds annotated only following UC-HILIC separation from those annotated after RP-LC ($p < 0.01$). On the other hand, the pKa of the compounds did not play a significant role (Fig. 3A), even though the parameter is somehow altered by the fact that for several compounds with amino groups, the reported pKa was effectively that of the conjugated acid. Based on the significance of the hydrophobicity, the correlation between the RT and the XlogP3-AA was evaluated for both UC-HILIC and RP-LC. As expected, the hydrophobicity was very highly correlated to the RT ($R^2 = 0.7291$, Fig. 3D) of the compounds eluted by RP-LC. Conversely, despite showing its orthogonality compared to RP-LC, the RT of the compound eluted by UC-HILIC was way less correlated to the predicted logP ($R^2 = 0.2097$, Fig. 3C), thus suggesting that the retention and elution of compounds separated by UC-HILIC are influenced by several distinct factors as previously pointed out Parr et al. [51]. The compounds eluted during the HILIC section of the gradient, and therefore strongly retained on the silica phase under the UC gradient conditions, were strongly alkaline (e.g., diethanolamine and triethanolamine), zwitterionic (e.g., amino acids, choline, betaine), and compounds with a permanent positive charge (e.g., paraquat), which are all positively charged under acidic conditions, thus implying a significant role of the charge status in the retention and elution of compounds under the UC-HILIC gradient.

Compounds separated by UC-HILIC were evenly distributed in the interval, whereas the elution of the annotated compounds by RP-LC was uneven, with several compounds clustered in the first 2 min of the

gradient due to their poor or non-retention of the C18 phase (Fig. 3C–D). The EIC of 10 exemplary compounds that were annotated by both UC-HILIC and RP-LC are shown in Fig. 4, displaying the coelution of several peaks near the dead volume. Moreover, the elution order of the 10 peaks is not exactly reversed, in agreement with the previous findings regarding the retention mechanisms of UC-HILIC compared to RP-LC.

The coelution of numerous peaks in a small section of the chromatogram can cause mask effects when DDA MS acquisition is employed. As such, since a limited number of ions (generally 5–10) are sequentially isolated by the quadrupole following each full scan, the higher the number of the compounds that coelute, the higher the chance that some of them are never isolated from the quadrupole for MS/MS spectra recording, resulting in several peaks that only exist as MS spectra but that cannot be identified since they are not associated with an MS/MS spectra. The m/z of the 61 chemicals that were annotated only following UC-HILIC were manually searched in the RP-LC raw data files. For 11 of them, including MDMA, tapentadol, and lamotrigine, there were peaks with a good peak shape and intensity that would be generally sufficient for selection, isolation, and MS/MS spectra recording, but that due to the mask effects were never fragmented in any of the replicates. Considering the PMOC that were employed for the setup of the UC-HILIC method, three were tentatively identified from both datasets, i.e., pyrazole, melamine, and paraquat, whereas three could only be annotated from the UC-HILIC datasets, i.e., cotinine, tetraglyme, and valsartan. It is worth noting that the latter were all detected from the standard mixture (Fig. S4), but not identified in the water samples, thus reinforcing the idea that RP-LC separation suffers not only from the poor separation of the most polar compounds but also from matrix effects and mask effects, that play a crucial role in limiting the annotation of PMOC.

4. Conclusions

In the present study, for the first time, a UC-HILIC approach was set up for the analysis of highly polar PMOC in water samples to expand the chemical space that could be covered in a single chromatographic run. Compared to widespread RP-LC-HRMS, our approach allowed a significant increase in the number of identified compounds (122 vs 75). Moreover, the UC-HILIC gradient allowed much better retention and separation of the polar compounds, thus reducing ion suppression and mask effects connected to DDA MS acquisition. The introduction of the HILIC section of the gradient was necessary for the elution of strongly retained compounds on the silica phase, including strongly alkaline,

Table 2

Annotation data of 20 selected PMOC of anthropic origin that were exclusively annotated from UC-HILIC-HRMS analysis in water samples.

Name	RT	Formula	Molecular Weight	Adduct	Type of chemical
Benzothiazole	4.5	C ₇ H ₅ NS	135.0140	[M+H] ⁺	Rubber production
N, N'-Dicyclohexylurea	6.7	C ₁₃ H ₂₄ N ₂ O	224.1883	[M+H] ⁺	Tire additive
Temazepam	6.9	C ₁₆ H ₁₃ ClN ₂ O ₂	300.0668	[M+H] ⁺	Pharmaceutical (benzodiazepine)
Sulfamethoxazole	7.8	C ₁₀ H ₁₁ N ₃ O ₃ S	253.0514	[M+H] ⁺	Pharmaceutical (antibiotic)
Tetraglyme ^a	7.9	C ₁₀ H ₂₂ O ₅	222.1461	[M+H] ⁺	Industrial chemical (solvent)
Valsartan	8.8	C ₂₄ H ₂₉ N ₅ O ₃	435.2263	[M+H] ⁺	Pharmaceutical (antihypertensive)
Saccharin	9.5	C ₇ H ₅ NO ₃ S	183.9985	[M - H] ⁻	Food additive
Acesulfame	11.6	C ₄ H ₅ NO ₄ S	162.9931	[M - H] ⁻	Food additive
Urea	12.0	CH ₄ N ₂ O	60.0331	[M+H] ⁺	Fertilizer; pharmaceutical (skin rehydration)
Carbamazepine 10,11-epoxide	12.2	C ₁₅ H ₁₂ N ₂ O ₂	252.0891	[M+H] ⁺	Pharmaceutical (anticonvulsant, metabolite)
10,11-Dihydro-10,11-dihydroxycarbamazepine	12.3	C ₁₅ H ₁₄ N ₂ O ₃	270.0997	[M+H] ⁺	Pharmaceutical (anticonvulsant, metabolite)
4-formylaminoantipyrine	12.5	C ₁₂ H ₁₃ N ₃ O ₂	231.1002	[M+H] ⁺	Pharmaceutical (analgesic, metabolite)
5-fluorocytosine	13.0	C ₄ H ₄ FN ₃ O	129.0337	[M+H] ⁺	Pharmaceutical (antifungal)
Amitriptyline	13.9	C ₂₀ H ₂₃ N	277.1834	[M+H] ⁺	Pharmaceutical (antidepressant)
1-Vinylimidazole ^a	14.9	C ₅ H ₆ N ₂	94.0525	[M+H] ⁺	Industrial chemical (synthesis of polymers)
N, N-Diisopropylethylamine (DIPEA)	15.0	C ₈ H ₁₉ N	129.1515	[M+H] ⁺	Industrial chemical
MDMA	15.2	C ₁₁ H ₁₅ NO ₂	193.1100	[M+H] ⁺	Drug of abuse
O-Desmethylvenlafaxine ^b	16.0	C ₁₆ H ₂₅ NO ₂	263.1878	[M+H] ⁺	Pharmaceutical (antidepressant, metabolite)
Atenolol	19.9	C ₁₄ H ₂₂ N ₂ O ₃	266.1623	[M+H] ⁺	Pharmaceutical (antihypertensive)
Miglitol	22.2	C ₈ H ₁₇ NO ₅	207.1102	[M+H] ⁺	Pharmaceutical (diabetes medication)

^a Compounds included in the Candidate List of substances of very high concern for Authorisation by the European Chemicals Agency (ECHA).

^b Compound included in the Surfacewater WatchOut List of the EU.

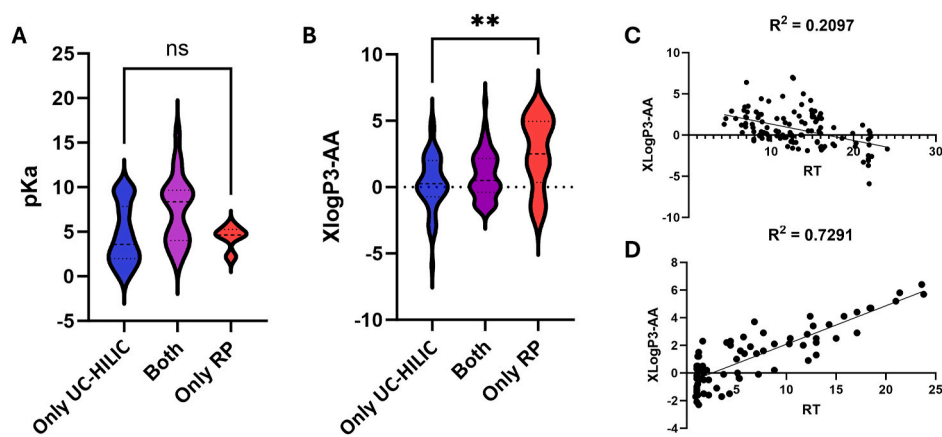


Fig. 3. Violin plots of the distribution of the compounds annotated by UC-HILIC only, RP-LC only, and by both based on their pKa (A) and XlogP3-AA (B). T-test statistical analysis was performed (ns: $p > 0.1$, **: $p < 0.01$). Linear regressions of the RT vs XlogP3-AA for UC-HILIC (C) and RP-LC (D).

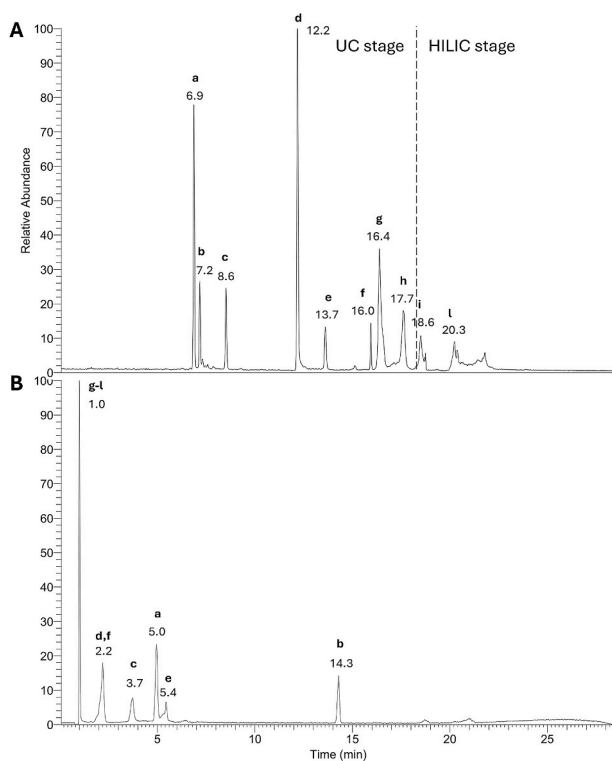


Fig. 4. Extracted ion chromatogram (EIC, 10 ppm) in UC-HILIC (A) and RP-LC (B) of 10 exemplary compounds annotated from water samples in ESI+. a: benzotriazole (m/z 120.0555); b: tapentadol (m/z 222.1847); c: caprolactam (m/z 114.0913); d: PEG n4 (m/z 195.1221); e: N,N'-diphenylguanidine (m/z 212.1178); f: sotalol (m/z 273.1259); g: melamine (m/z 127.0725); h: metformin (m/z 130.1085); i: choline (m/z 104.1071); l: triethanolamine (m/z 150.1120).

zwitterionic, or permanently charged compounds, thus not only expanding the chemical space of the analysis, but also reducing the amount of compounds that would be permanently bound onto the phase of the column, resulting in possible irreproducibility, pressure increase, and loss of efficiency in the compound separation.

Further research is needed to investigate the role of the column phase in the retention and the elution of extremely polar compounds such as glyphosate and AMPA, that were too strongly retained on the bare silica column employed in this study. Moreover, in terms of coverage of low abundance compounds, MS data recorded by data-independent

acquisition (DIA) could represent a powerful alternative to DDA, keeping in mind the need for tailored data processing approaches for data treatment, spectral deconvolution, and compound annotation.

CRediT authorship contribution statement

A. Cerrato: Writing – original draft, Methodology, Investigation, Formal analysis. **T. Holmark:** Visualization, Investigation. **E. Emke:** Writing – review & editing, Supervision, Resources. **E.D. Amato:** Writing – review & editing, Supervision, Resources. **A.F.G. Gargano:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aca.2025.343672>.

Data availability

There is a link in the text to the repository with the data

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