Combination of different chromatographic and sampling modes for high-resolution mass spectrometric screening of organic microcontaminants in water

Verónica Castro[1], José Benito Quintana[1,*], Inmaculada Carpinteiro[1], Julio Cobas[2], Nieves Carro[2], Rafael Cela[1], Rosario Rodil[1,*]

[1] Department of Analytical Chemistry, Institute of Research on Chemical and Biological Analysis (IAQBUS), Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

[2] INTECMAR - Technological Institute for the Monitoring of the Marine Environment of Galicia, Peirao de Vilaxoán S/N, 36611 Vilagarcía de Arousa, Spain

*Corresponding authors: Phone: +34881816035; E-mails: rosario.rodil@usc.es, jb.quintana@usc.es

Abstract

This study explores the combination of two sampling strategies (polar organic compounds integrative sampler (POCIS) Vs. spot sampling) and four chromatographic retention modes (reversed-phase liquid chromatography (RPLC), hydrophilic interaction liquid chromatography (HILIC), mixed-mode liquid chromatography (MMLC) and supercritical fluid chromatography (SFC)) for high-resolution mass spectrometry (HRMS) screening of organic pollutants in water samples. To this end, a suspect screening approach, using iterative data-dependent tandem mass spectrometry (MS/MS) driven by a library of 3227 chemicals (including pharmaceuticals, pesticides, drugs of abuse, human metabolites, industrial chemicals and other pollutants) was employed. Results show that POCIS can afford a larger number of positive identifications as compared to spot sampling. On the other hand, the best suited retention mechanisms, in terms of identified analytes are: SFC, followed by RPLC, MMLC and HILIC. However, the best combination (POCIS + SFC) would only allow the identification of 67% of the detected analytes. Thus, the combination of the two sampling strategies, spot and passive sampling, with two

orthogonal retention mechanism, RPLC and SFC, is proposed in order to maximize the number of analytes detected (89%). This strategy was applied to different surface water (river and estuary) samples from Galicia (NW Spain). A total of 155 compounds were detected at a confidence level 2a, from which the major class was pharmaceuticals (61%).

Keywords: liquid chromatography, supercritical fluid chromatography (SFC), quadrupole-time-of-flight (QTOF), passive sampling, contaminants of emerging concern (CECs), surface water

INTRODUCTION

The list of anthropogenic organic microcontaminants employed in industrial, domestic, personal care or pharmaceutical applications, as well as leachates from landfills or agricultural fields that may reach the aquatic environment is virtually endless, as many of them are not completely removed during treatment in wastewater treatment plants (WWTPs) or even not subjected to treatment processes (e.g. runoff from agriculture). Therefore, it is very important to deliver efficient analytical tools capable of detecting as many as possible different chemical pollutants. Such task has become possible by the improvements in high-resolution mass spectrometry (HRMS) instrumentation and data processing that popularized screening methods as the best approach for comprehensive environmental quality assessment, especially when liquid chromatography is combined with HRMS (LC-HRMS) [1-3]. HRMS makes theoretically possible to detect, in one injection, several thousand compounds without a preselection bias. Nevertheless, bias cannot be fully avoided, given the fact that sampling, pretreatment, chromatographic or ionization conditions limit the type of chemicals being detectable [4].

An ideal chromatographic system should deliver high retention and separation power of a wide range of compounds, from non-polar to very polar, in a single run, but this has not been achieved so far. Reversed-phase (RP) LC, which is the workhorse in LC-HRMS, works fine with nonpolar to moderately polar molecules (logD typically >0) [5], but may not be the mode of choice for extremely polar (log D < −2) compounds. In those cases, hydrophilic interaction liquid chromatography (HILIC), mixed-mode liquid chromatography (MMLC) and supercritical fluid chromatography (SFC) have been proven to be promising alternatives [5-11].

On the other hand, many of these micropollutants are present in the environment at ng L^{-1} concentrations, which also fluctuate along time and locations. Thus, spot/grab sampling provides merely a snapshot of contamination at a given time and place [5, 12-14]. Alternatively, passive sampling devices are a complementary sampling approach that combine sampling and

analyte's preconcentration in one step providing time weighted average concentrations. Among them, polar organic chemical integrative samplers (POCIS) are some the most popular passive sampling devices for the analysis of water samples [15-17], due to their easy preparation and capacity to capture and preconcentrate moderately polar chemicals in-situ, thus enabling their determination even at very low concentration [18]. Due to POCIS's accumulative character, the combination of passive sampling and LC–HRMS has already shown its ability to detect more organic compounds than when using grab sampling [19, 20]. Recently, other passive sampling devices have also been combined with HRMS for screening purposes [21-24].

The main objective of this study was to evaluate the performance of different chromatographic and sampling modes in data-dependant acquisition (DDA) suspect screening of water samples. To this end, four chromatographic modes (RPLC, HILIC, MMLC and SFC) were combined to HRMS. As mentioned, several of those chromatographic modes have already shown promising results for very polar analytes. However, in this work, we employ a suspect list of 3227 chemicals (including pharmaceuticals, pesticides, drugs of abuse, human metabolites, industrial chemicals and other pollutants) for which MS/MS spectra were available (Table S1) with rather wide variable physico-chemical properties. Moreover, we also compared the outcomes of POCIS passive sampling and traditional grab sampling. Finally, the best combination of two chromatographic modes plus the two sampling approaches was applied to the analysis of eight surface water samples, four river and four estuary samples, from Galicia (NW Spain).

MATERIALS AND METHODS

Chemicals and reagents

Formic acid (≥ 99.9%) for LC-MS and dichloromethane for pesticide residue analysis were provided by VWR Chemicals (Radnor, PA, USA). Acetonitrile hypergrade for LC-MS, ethyl acetate for gas chromatography-MS were provided by Merck (Darmstadt, Germany). Ammonia 7 N solution in methanol was provided by Sigma-Aldrich (Steinheim, Germany). Methanol optima

LC-MS grade was provided by Thermo Fisher Scientific (Waltham, MA, USA). Ultrapure water was obtained in the laboratory from a Milli-Q Gradient A-10 system (Merck-Millipore, Bedford, MA, USA).

Oasis HLB 6 cc (200 mg) cartridges were supplied by Waters (Milford, MA, USA). For POCIS preparation, polyethersulfone (PES) membrane Supor®-450 47 mm 0.45 µm was supplied by Pall Corporation (Willoughby, OH, USA) and Oasis HLB 12 cc (500 mg) cartridges by Waters. The Oasis HLB 500 mg cartridges were dismounted to attain the sorbent phase for POCIS preparation.

POCIS stainless steel holders were constructed by Nodosafer (Pontevedra, Spain) with 70 mm external diameter and 40 mm internal diameter. Before assembly, stainless steel rings were washed with soap, Milli-Q water and methanol. POCIS were assembled by enclosing 100 mg of HLB sorbent (previously washed with methanol) between two PES membranes (previously sonicated in methanol). These are sandwiched between two support rings held together with three thumbscrews. After that, the POCIS devices were placed in stainless-steel cage (canister) that let water run through and protect them against potential damages on the sampling points.

Sampling and deployment of POCIS

The treated wastewater (effluent) from a wastewater treatment plant (WWTP) that receives mostly domestic wastewater and serves a population of ca. 100,000 inhabitants in the NW of Spain was chosen for an initial comparison of chromatographic and sampling modes. A steel canister with the POCIS was deployed during 1 week in December 2018. Spot water samples (1 L) were also collected at the beginning and at the end of the week-long sampling in glass prewashed amber bottles.

Furthermore, eight surface water samples, four river (R1-R4) and four estuary (S1-S4) samples, from Galicia (NW of Spain) were sampled during the first semester of 2019 (Figure S1). The steel canisters with the POCIS were deployed for either one (river) or two (estuary) weeks [25-29]. The longer deployment time in estuarine water was employed ought to the lower

concentrations that were expected. Spot water samples were collected at the beginning and at the end of the POCIS deployment time at all locations.

POCIS procedural blanks were also prepared in order to check for any contamination during POCIS preparation or transport.

POCIS extraction procedure

At the end of each sampling period, the POCIS were collected and transported to the laboratory. After reception, they were rinsed with Milli-Q water, wrapped in aluminium foil and stored at −20 ◦C until extraction.

Each POCIS was disassembled and the sorbent material (HLB sorbent) was transferred into empty 10 mL SPE cartridges (syringe Discardit II, BD® Biosciences, San Jose, CA, USA) and packed between two polyethylene frits (ISOLUTE® Frits, 16 mm, 20 µm PE, Biotage, Uppsala, Sweden). Elution of the analytes from the sorbent was performed with 6 mL of methanol (noted as POCIS sorbent F1). After that, a second elution of each cartridge was carried out with 6 mL dichloromethane (noted as POCIS sorbent F2). PES membranes were also eluted with 10 mL of methanol (noted POCIS membranes F1) followed by 10 mL of ethyl acetate (noted POCIS membranes F2) using a vacuum filtration system. Eluent selection was based on the fact that methanol is by far the most frequently used eluent for POCIS, while some authors include a less polar solvent to increase the extractability of more hydrophobic chemicals [26, 30]. PES is not compatible with chlorinated solvents [31], thus, ethyl acetate was used in that case [32].

The eluates (F1 and F2 from POCIS sorbent and membranes) were concentrated approximately to ca. 0.5 mL using a Turbovap II concentrator (Zymark, Hopkinton, MA, USA), then to dryness under a gentle nitrogen stream and finally reconstituted in 500 µL of methanol and filtered with a 0.2 µm GHP® 13 mm Syringe filter (Pall Corporation).

Solid-Phase Extraction procedure

Spot water samples were transferred to the laboratory and processed on the same sampling day. Before SPE, the water samples were vacuum filtered through 0.7 µm glass microfiber filters GF/A (Whatman). Aliquots (200 mL) were extracted with Oasis HLB-200 mg cartridges, previously conditioned with 5 mL of methanol and 5 mL of Milli-Q water. After sample percolation, the cartridge was washed with 10 mL of Milli-Q water and dried under a purified nitrogen stream for approximately 30 min. Elution was carried out by gravity using 10 mL of methanol (noted as SPE F1) followed by 10 mL of dichloromethane (noted as SPE F2). These solvents are typically used with Oasis HLB [33] and were also selected for a better comparability to POCIS sorbents. The eluates were concentrated and reconstituted as POCIS eluates.

Chromatographic conditions

RPLC, HILIC, MMLC analysis were performed on an Agilent Technologies (Wilmington, DE, USA) 1290 Infinity II series. SFC was performed on an Agilent 1260 Infinity II system equipped with an Agilent 1260 Infinity II SFC Control Module. Chromatographic programs of 30 min were used in all chromatographic modes. The columns used where a ZORBAX Extend-C18 (Agilent) for RPLC, a Nucleodur HILIC (Macherey Nagel, Düren, Germany) zwitterionic HILIC column, an Acclaim Trinity P1 (Thermo Scientific, San Jose, CA, USA) with both anion- and cation-exchange functionalities for MMLC, and a Torus DIOL (Waters) for SFC. Details on gradients and other chromatographic conditions are provided in the Supporting Information (Text S1). A mixture containing 18 organophosphorus flame retardants [34] and 10 phthalate metabolites (phthalic acid monoester derivatives) [35] of 500 ng mL^{-1} was acquired every ca. 15 injections in order to verify absence of chromatographic (i.e. retention time stability and peak shape) and MS (mass accuracy and sensitivity) issues during the acquisition sequence.

The LC columns were directly connected to a quadrupole-time-of-flight (QTOF) mass spectrometer source using a PEEK tube. The mobile phase emerging from the SFC column was mixed with the make-up solution and then split in two streams. One of them is connected to the

158 back-pressure regulator (BPR), responsible to maintain CO_2 under supercritical conditions, and
159 the other one to the ESI source. The connection between the splitting point and the ESI source
160 was made using a 500 mm (length) x 0.05 mm (i.d.) silica tubing provided by Agilent.

161 **Mass spectrometry**

162 A quadrupole-time-of-flight (QTOF) mass spectrometer model Agilent 6550 iFunnel Q-TOF
163 LC/MS system was coupled to the SFC and LC systems. The QTOF was furnished with a Dual
164 Agilent Jet Stream electrospray (ESI) ion source. The ESI interface was operated either in positive
165 or negative modes (in different injections) and the needle voltage of the ESI was fixed at 3500
166 V. Nitrogen was used as nebulizing (30 psi) and drying gas (200 °C, 12 L min^{-1}) in the ESI source,
167 and also as collision gas in the MS/MS experiments.

168 Instrument control and data acquisition were performed with Agilent MassHunter Workstation
169 software B.10.00. Data acquisition was performed by an Auto MS/MS (DDA) method using three
170 consecutive injections per sample excluding the precursors selected for MS/MS fragmentation
171 in the previous injections (iterative acquisition). Iterative MS/MS parameters in the acquisition
172 method were set at 20 ppm of mass error tolerance and 0.2 min of retention time exclusion
173 tolerance.

174 Three collision energies (10, 20 and 40 V) were collected for each precursor ion, with a maximum
175 of 2 precursor ions per cycle and an isolation width set at medium (4 m/z units). Precursors
176 previously selected for fragmentation were excluded after 3 spectra and released after 0.5 min.
177 Precursor ion selection was solely based on abundance only by using the “common organic
178 molecules” isotopic model, with an absolute threshold of 1000 counts and a relative threshold
179 of 0.001%. The acquisition frequency and the scan range in single-stage MS and MS/MS were 3
180 and 6 spectra per second, and 60-1100 m/z and 30-1000 m/z, respectively. A reference
181 calibration solution, supplied by Agilent, was continuously sprayed in the source during the
182 chromatographic run in order to continuously recalibrate the m/z axis. The purine and HP-0921

183 (hexakis(1H, 1H, 3H-tetrafluoropropoxy)phosphazine) $[M+H]^+$ ions, 121.050873 m/z and
184 922.009798 m/z, respectively were employed in positive mode for this purpose. The
185 trifluoracetic acid $[M-H]^-$ ion (112.985587 m/z) and the formate (966.000725 m/z) and
186 trifluoroacetate (1033.988109 m/z) adducts of HP-0921, were used in negative mode.

187 **Data analysis**

188 The obtained data were processed using the Agilent MassHunter Qualitative Workflow B.08.00
189 software. Data analysis consisted of the use of the search algorithm Find by Auto MSMS, which
190 automatically extracts the MS and MS/MS information, aligns, sorts and display the spectra as a
191 single compound. The peak threshold was set to 1000 counts in both positive and negative
192 modes. MS and MS/MS spectra extraction was set to average all scans above a 20% of peak
193 height and exclude scans in case of detector saturation and automatically remove background
194 spectra by averaging spectra at peak start and peak end.

195 Analytes were considered as identified at a confidence level 2a as defined in [36, 37] when at
196 least one collision energy was collected for each precursor ion and the library search reverse
197 score was higher than 80% in MS/MS mode (threshold of MS/MS peaks used for library search
198 relative to the largest peak in the spectra: 5%), and also a minimum score at MS level of 70%
199 (please note that the MS score is a combination of mass error against theoretical mass, isotopic
200 profile match and spacing among those isotopes). A maximum mass error of 5 ppm was
201 permitted at MS level, while for MS/MS library comparison, this was set to 5 mDa. In addition,
202 MS/MS spectra matching was manually checked and only in those cases were at least two
203 product ions matched those in the library were considered as positively detected.

204 **Comparison strategy**

205 The comparison of chromatographic modes and sampling approach was performed with a
206 wastewater effluent. Such sample was selected for method evaluation because of the higher
207 probability to find a substantial number of compounds in this matrix and also, because it is a

major source of surface water contaminants. Furthermore, it contains several other natural chemicals and background ions, which may interfere in DDA, as such masses may be selected by the AutoMS/MS algorithm, preventing contaminants being subject to MS/MS experiments and being detectable. Thus, the comparison does not only account for the availability of different modes to retain chemicals, but also on the separation power respective to background chemicals/MS signals.

The accurate mass MS/MS spectral library of 3227 chemicals contains mainly pharmaceuticals (ca. 60% of the compounds), pesticides (ca. 24 %), drugs of abuse (ca. 10%), human metabolites (ca. 3%), industrial chemicals (ca. 1%) and other pollutants (ca. 2%) (Table S1). Such library was created combining four commercial libraries supplied by Agilent (ForTox PCDL, Water PCDL, Pesticides PCDL and VetDrugs PCDL) and an in-house empirical MS/MS library. Duplicates and compounds that did not contain MS/MS spectra from the commercial libraries were deleted. The average log D (at pH 7) values (calculated using the JChem add-on for Office; https://chemaxon.com/products/jchem-for-office) of the chemicals included in the library is 1.5 (median 1.6) being the lower quartile -0.1 and the upper quartile 3.2, being in this way quite broad in terms of polarity range. So, 25% of the investigated chemicals can be considered as nonpolar (log D (pH 7) > +3.2), 50% chemicals as polar (-0.1 < log D (pH 7)< +3.2) and 25% as very polar (log D (pH7) < -0.1), among which 223 compounds (7% of the total) would be extremely polar with logD (pH7) < -2.

As regards sample preparation, a POCIS conventional configuration with HLB sorbent and PES membrane was selected, while SPE was also performed with HLB sorbents for a better comparison. Thus, sampling/sample preparation is expected to represent the most conventional methods used in water analysis and not dedicated towards very polar or very hydrophobic analytes.

Besides, in order investigate the performance of the screening workflows, 45 chemicals (from those detected in river water) were spiked over a filtered surface water sample (from location R4) at two concentration levels (100 and 500 ng L^{-1}) and subjected to the SPE protocol above mentioned (n=5 at each concentration level). The extracts were then injected in RPLC and SFC modes and the screening approach applied. Also, apparent recoveries were calculated by integrating the protonated or deprotonated molecular ion (with a ±50 ppm extraction window) chromatograms and interpolation into an external standard calibration. Non-spiked samples (n=3) were also processed for recovery calculations.

RESULTS AND DISCUSSION

Comparison strategy

In total, 135 different compounds were identified at a confidence level 2a [36, 37] in the wastewater sample by using the different sampling strategies and chromatographic modes, as compiled in Table S2. Their physicochemical properties calculated using the JChem add-on for Office are summarized in Table S3. The average log D (pH 7) values of the identified chemicals is 0.95 (median 1.04) being the lower quartile -0.37 and the upper quartile 2.19.

Iterative acquisition

Conventional DDA HRMS screening is limited by the number of precursor ions that can be selected for fragmentation during a single analysis. Thus, in order to increase the number of detected compounds, an iterative acquisition mode was employed. In this acquisition mode, the sample is injected multiple times, the precursors selected for MS/MS fragmentation being automatically excluded on a rolling basis in the subsequent injections by the acquisition software.

In this study, three consecutive iterative injections (coded A, B and C were acquired for each sampling approach, chromatographic mode (RPLC, MMLC, SFC and HILIC) and polarity (negative and positive). The number of compounds found in injections A, B and C per retention mechanism and sampling mode in positive and negative polarity are compiled in Figure S2, where it can be observed that the compounds found in injection A represented between 49% and 69% in positive polarity (Figure S2a) of the total identified compounds and more than 60% in negative polarity (Figure S2b), between 14% and 40% in injection B, and between 0% and 21% in injection C. Hence, in general terms, if we compare iterative acquisition with a conventional single injection (which would mean injection A only), the number of detections is doubled in positive mode by the iterative approach. So, in order to maximize the number of compounds identified, the samples were analysed using three consecutive injections in interactive acquisition mode.

Spot Vs passive sampling

The two sampling strategies were compared considering the number of compounds identified in the passive sampler, either POCIS sorbent or POCIS membrane, and the spot water SPE extracts. In SPE extracts, compounds were considered as detected if they were found in at least one of the two spot samples that were collected at each sampling point (at the beginning or end of the passive sampler deployment time). All the compounds identified in the second fraction extracts (POCIS sorbent F2, POCIS membrane F2 and SPE F2) were also detected in the corresponding first fraction (Table S2). So, given the fact that those second fractions did not provide new compounds, only the first fraction results are discussed. The results obtained are shown in Figure 1 regardless of the chromatographic mode (RPLC, MMLC, SFC or HILIC) compounds were detected by. As presented in the Figure, the number of compounds identified in the POCIS sorbent (115 compounds, 85.2%) were slightly higher than in the two spot water samples together (104 compounds, 77.0%), while the number of compounds detectable in the POCIS membrane (23 compounds, 17.0%) was much lower. Thus, more organic compounds

were identified with POCIS than by analysing grab samples, similarly to the results of Soulier et al. [20] for groundwater.

When comparing the log D (pH 7) values of these chemicals found using the different sampling strategies, statistically significant differences were observed (Kruskal-Wallis test p-value 0.01166, data was not normally distributed). The differences analysed by the Bonferroni procedure showed that the log D of those compounds found into the POCIS membrane is statistically higher than those found in SPE and POCIS sorbent extracts (Figure S3). This is likely associated to the fact that the diffusion coefficient of hydrophobic compounds through the PES membrane is lower preventing them from reaching the sorbent in significant amounts [38]. On the other hand, there are no statistically significant differences between the log D (pH 7) values of the compounds found into the POCIS sorbent and SPE extracts, which may also be expectable since both SPE and POCIS materials where Oasis HLB. Most of the compounds (91 out of 135) were identified by both sampling strategies (Figure 1): 76 in SPE and POCIS sorbent extracts (e.g. amantadine, Figure S4a) and 14 compounds in SPE and POCIS sorbent and POCIS membrane (e.g. carbendazim, Figure S4b). If we consider those chemicals detected exclusively in in POCIS sorbent or SPE, even when there is no statistical difference (Student's t test p-value: 0.13), chemicals detected by SPE and not in POCIS sorbent tend to have a lower logD (SPE median: -0.4 Vs POCIS sorbent median: 1.4), due to the fact that very polar ionic at natural water pH (as nicotine, ritalinic acid or gabapentin) chemicals may have difficulties to get through the membrane. Anyway, as discussed also in the section below, samples were screened by a DDA approach, so that there is a competition between matrix constituents and organic micropollutants in triggering MS/MS acquisition (see next section), making it difficult to get a clear explanation on the sole basis of physico-chemical properties.

Moreover, the signal obtained in the POCIS sorbent extract is generally higher than in SPE or POCIS membrane (Figure S4), likely due to the POCIS sorbent accumulative character. A further

relevant output shown in Figure 1 is the number of compounds detected only in one of the POCIS materials or SPE. As shown, 23 out of 135 identified compounds were found only in the POCIS sorbent (e.g. climbazole, Figure S4c), 13 in the spot water samples (e.g. ritalinic acid, Figure S4d) and 6 in the POCIS membrane (e.g. fludioxonil, Figure S4e).

In view of these results, in the following steps of the study, only the POCIS sorbent F1 and SPE F1 extracts were considered, since such combination covers 95.6% (129) of the chemicals.

Chromatographic modes

Four different retention mechanisms (HILIC, SFC, RPLC and MMLC) were considered to maximize the coverage of organic micropollutants that could be detected. The chromatographic columns and experimental conditions (see Text S1) were selected based on the literature [5, 8, 39].

As depicted in Figure 2, SFC provided the best results in terms of number of chemicals that could be detected, with 103 compounds (79.8% of the 129 compounds detected), followed closely by RPLC with 86 compounds (66.7% of the 129 compounds detected) and then by MMLC with 70 compounds (54.3%). Finally, HILIC provided the worst results with only 37 compounds (28.7%). In addition, 20 compounds were detected using the four chromatographic modes, 38 compounds by 3 chromatographic modes and 38 compounds by 2 chromatographic modes. As a final consideration, 23 of these compounds were detected exclusively by SFC, 7 by RPLC, 7 by MMLC and only 2 by HILIC (denoted in Figure 2 as unique).

The relationship between the log D and the retention time (RT) of the compounds is presented in Figure S5, both for logD values calculated at pH 7 (i.e. more relevant from the environmental perspective) and at pH values closer to the mobile phases used in each chromatographic mode. A significant correlation of RT and compound log D/polarity could be verified for all the separations modes (Pearson p-values <0.05). SFC and HILIC separations (Pearson p-values <0.0001 and 0.0001-0.0003, respectively) can be considered comparable to normal-phase LC, consequently nonpolar compounds elute earlier than polar compounds. This finding agree with

those of Schulze et al. [5] but contrasts however with Bieber et al. [6] who could not verify any correlation of RT and compound log D/polarity in SFC. On the other hand, as expected, in RPLC (Pearson p-values <0.0001) RT increases with increasing log D values. Finally, the correlation between RT on MMLC and log D is weaker (Pearson p-value 0.0271-0.0430). The main reason could be the fact that MMLC combines three retention mechanisms (anionic-exchange, cationic-exchange and RPLC) altogether. No statistically significant differences (ANOVA p-value 0.7557) were observed among the chromatographic modes when the log D (pH 7) of the chemicals detected by each column are compared (Figure S6, overall log D (pH 7) ranged from −3.73 to +5.17), although it would be expected that MMLC and particularly HILIC and SFC (which show a normal-phase behaviour) would lead to an improved detectability of the most polar analytes [5]. This finding may be the consequence of several facts: 1) that neither sample preparation (nor the MS/MS library) were particularly focussed towards very polar analytes; 2) that the RPLC column employed is double endcapped to improve the retention of basic analytes, which may result into improved performance towards more polar chemicals; and 3) that DDA was employed, so that even when analytes are retained and have a good peak shape, they may remain undetected if they coelute with several matrix constituents. Therefore, in this comparison, driven by DDA, the ability of the column to separate chemicals from each other and from the matrix turns out to be a key issue.

Thus, it becomes difficult to interpret which chemicals are detected, only based on their polarity or physico-chemical properties. When looking at matrix effects, evaluated by comparing the signal of the reference calibration solution at m/z 121.0509 and 922.0098 in positive mode and m/z 119.0363 and 966.0007 (formate adduct) or 980.0164 (acetate adduct) in negative mode during the analysis of a methanol injection to that of either a POCIS sorbent or SPE extracts, it is evident that they were more important in positive mode than in negative mode (Figure S7). Also, in general, a stronger signal suppression is observed for HILIC (particularly for m/z 121.0509) and in MMLC modes (at both m/z), reaching ca. 80% of signal suppression. On the other hand,

matrix effects were negligible in SFC, which may explain the higher detectability of analytes with this chromatographic mode due to an improved separation of analytes from the matrix. Some former works have already pointed to stronger signal suppression in HILIC [40] and MMLC [39, 41]. Conversely, several publications devoted to bioanalysis [42, 43] and even including wastewater [44] have shown that SFC seems to be less prone to matrix effects than RPLC.

Given the above-mentioned results, only SFC, RPLC and MMLC were further considered as to select the optimal combination, see below. With these three columns the number of compounds detected would be 127.

Selection of the optimal combination

If only the best sampling strategy (POCIS sorbent) and the best retention mechanism (SFC) would be employed, this approach would allow detecting 67.4% of the compounds (91 compounds). So, in order to increase the number of detectable chemicals, different combinations of sampling and retention modes were investigated. These combinations were either one sampling strategy and two retention mechanisms, two sampling strategies and one retention mechanism, or even two sampling strategies and two retention mechanisms (Figure S8).

With only one sampling approach and two retention mechanisms, the best results would be provided by the combination of SFC and RPLC with the POCIS sorbent (108 compounds, 80% coverage). On the other hand, the use of two sampling strategies and one chromatographic mode (SFC) would provide slightly lower coverage (103 compounds, 76.3%). Finally, with two sampling strategies and two chromatographic mechanisms, both the combinations of SFC and RPLC and SFC and MMLC achieved the best results 120 compounds (88.9%), while using MMLC + RPLC 101 compounds (74.8%) would be identified.

Therefore, it was decided to select the combination of both spot sampling and passive sampling with both RPLC and SFC retention mechanism as the approach providing enhanced chances of capturing micropollutants. If analytical efforts (and cost) need to be further minimized, then the

recommendation would be performing passive sampling, followed by HRMS analysis on both RPLC and SFC, or SFC and MMLC.

Performance of the SFC and RPLC screening workflow

As explained in the Materials and Methods section, 45 chemicals from those detected in the river samples (see next section) were employed to investigate the performance of the screening workflow with both SFC and RPLC after the SPE of a real sample spiked at two concentration levels. The results of these assays (presented into Tables S4 and S5) show that the screening workflow was able to positively detect 60-74% and 75-83% of the analytes at the lower and higher concentration levels, with the detection rate being slightly more favourable for SFC. Some polar analytes that were more difficult to be detected by RPLC such as benzoylecgonine were detected by SFC. The SPE apparent recoveries were overall good (mean values in the 62-88% range), with metformin (a very polar chemical) being the main exception. Also, some analytes exhibiting good recoveries were not detectable in the screening workflow at lower concentrations (e.g. sulfadiazine and trazodone in RPLC), which was associated to the coelution with other matrix constituent not triggering DDA MS/MS experiments, as previously mentioned.

Screening of surface water samples

This dual sampling-chromatography strategy was applied to different surface water samples from Galicia (NW Spain): four river samples (R1, R2, R3 and R4) and four estuarine seawater samples (S1, S2, S3 and S4). A total of 155 compounds were detected (Table 1, further details in Table S6). As an example, Figure 3 presents some chromatograms and experimental spectra compared to the library of some of the identified chemicals.

By sample (Figure 4), 89 compounds were detected in R1, 97 in R2, 69 in R3, 51 in R4, 11 in S1, 98 in S2, 14 in S3 and 10 in S4. As regards river water, R1 and R2, which are the closest sampling points to the discharge of a WWTP, were the most polluted samples and the number of identified compounds decreases in the connected samples (R2-R4) with distance to the WWTP.

River water samples were, in general, more polluted than sea water samples, in terms of number of chemicals detected, except sample S2. The higher number of compounds identified in this sample could be related to the proximity of the sampling point to three marine outfalls, two from urban WWTPs (ca. 30,000 inh. in total) and one from a canned seafood industry.

Regarding sampling and chromatographic modes, again, the POCIS sorbent and SFC analysis provided the larger number of identified compounds (Figure 4). Attending to chromatographic mode, 53% of total compounds were detected exclusively by SFC, 13% by RPLC and 34% by both modes (Figure 4a). On the other hand, concerning sampling mode, 26% of total compounds were exclusively detected in the POCIS sorbent, 22% in the spot sample and 52% in both sampling strategies (Figure 4b).

The chemicals detected included 95 pharmaceuticals (61%), 20 human metabolites (13%), 14 natural products (9.0%), 8 pesticides (5.2%), 7 industrial chemicals (4.5%), 3 drugs of abuse (1.9%), 5 compounds from multiple sources (3.2%) and 3 from other classes (1.9%), Figure S10. This profile was also observed when looking individually at highly polluted samples (R1-R4 and S2). In the less polluted ones in terms of number of chemicals detected (S1, S3 and S4), a lower percentage of pharmaceuticals (ca. 30%) and no human metabolites were found. On the other hand, industrial chemicals, mainly plastic additives and flame retardants (25%) and compounds from multiple sources (i.e. 4-hydroxybenzoic acid, 4-nitrophenol, melamine and phenethylamine; 25%) became more important. This finding accounts for a lower contribution of WWTP effluents in such samples, while other diffuse pollution sources become more relevant.

By compound, 4-nitrophenol (Figure 3a) was found in all samples. This compound can have several sources and uses, mainly intermediate or precursor in different industrial processes as well as pesticide. Also, 4-nitrophenol is involved, as intermediate or final product, in the degradation of different organophosphorus pesticides such as fenitrothion and methylparathion [45]. Then, 3 industrial chemicals used as plastic additives, diethyl phthalate, tri-iso-butyl

phosphate and tri-n-butyl phosphate (Figure 3b) have been identified in 7 out of 8 samples, while another plastic additive, tris(2-butoxyethyl) phosphate, and 2 pharmaceuticals, benzododecinium and salicylic acid were detected in 6 samples. Benzododecinium is an antiseptic agent and disinfectant while salicylic acid has several uses as keratolytic, food preservative, bactericidal and antiseptic chemical, and also being the active metabolite of acetylsalicylic acid [46].

Moreover, other 23 compounds were identified in all the highly polluted samples (R1-R4 and S2, see details in Table S4): 19 pharmaceuticals (3 analgesics, 3 antibiotics, 3 antihypertensives, 2 antidepressants, 2 antiepileptics, 2 antipsychotics, 1 antiarrhythmic, 1 beta blocker, 1 stimulant and 1 antidiabetic drug), 3 human metabolites (benzoylecgonine, metabolite of the drug of abuse cocaine, O-desmethyltramadol, metabolite of the opioid painkiller tramadol and ritalinic acid, metabolite of methylphenidate, used to treat attention deficit hyperactivity disorder) and 1 drug of abuse (methylendioxymethamphetamine, MDMA), as mentioned related to wastewater discharges.

Conversely, two chemicals were found only in the low polluted samples (S1, S3 and S4) azelaic acid, which is a natural product found in cereal plants, an industrial chemical precursor to diverse industrial products and a component of a number of hair and skin conditioners, and the herbicide dinoterb.

CONCLUSIONS

The combination of different strategies widens the possibilities to separate and detect compounds which would not be captured using only one chromatographic mode or sampling approach. Hence, the proposed screening strategy, based on the combination of two sampling strategies (POCIS and spot sampling) and two chromatographic modes (SFC and RPLC), maximized the number of identified compounds (88.9% of the total identified compounds). If

only one sampling methodology and chromatography could be used (in order to save time and costs) passive sampling and SFC would lead to 67.4% identification rate, comparatively more than what would be obtained by the classical approach of combining SPE with RPLC (that would offer a 49.6% rate), even when two grab samples are collected and analysed. The application of the developed method to several surface water samples, provided the tentative identification (level 2a) of over 150 chemicals, but could indeed be expanded in the future to other screening approaches (e.g. data-independent acquisition, non-target analysis, use of public open libraries, etc.).

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Ethics declarations

The authors declare that they have no conflict of interest.

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623

FIGURE CAPTIONS

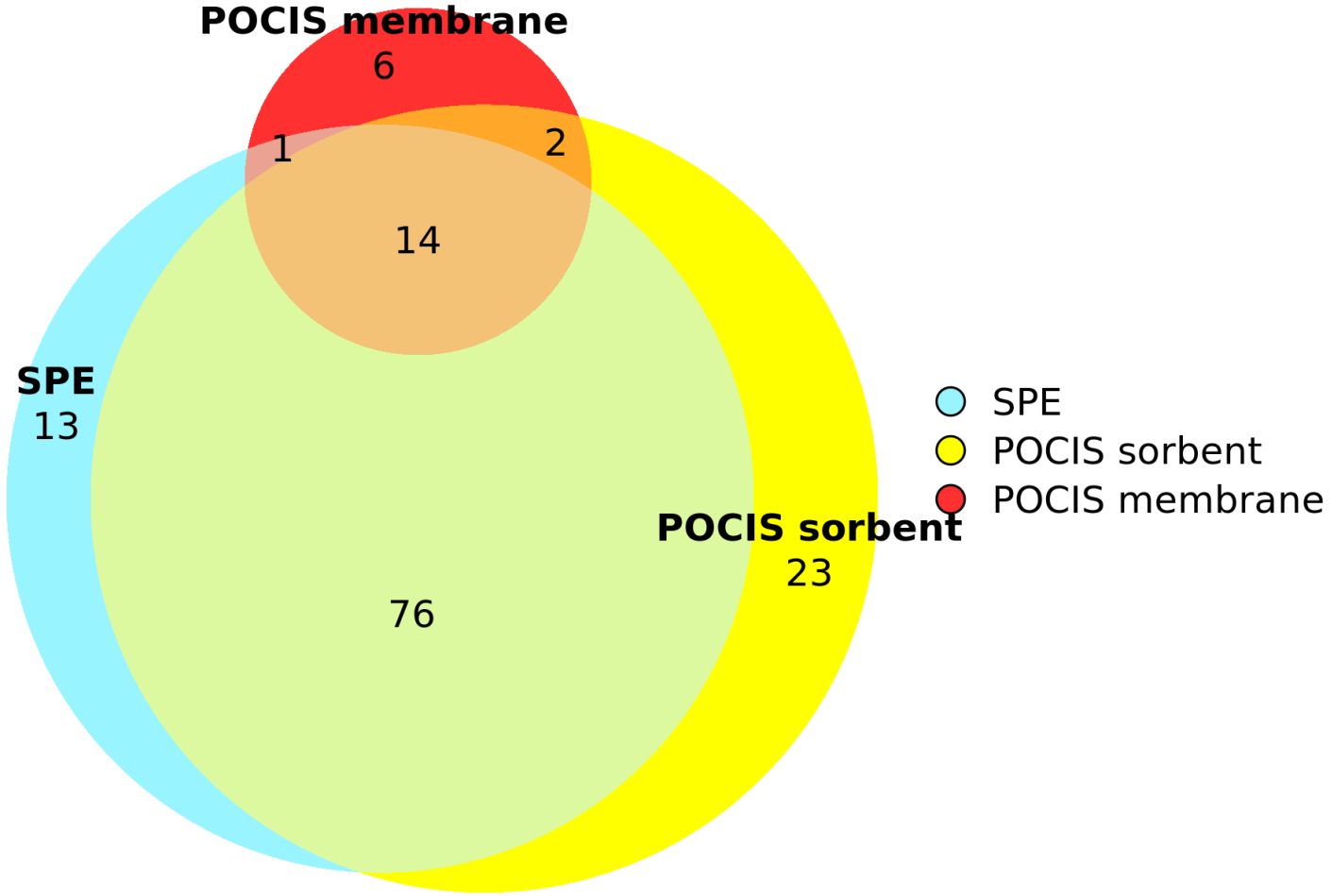
Figure 1: Area proportional Venn diagram of compounds identified in the wastewater effluent according to sampling mode (generated with Larsson J (2020). eulerr: Area-Proportional Euler and Venn Diagrams with Ellipses. R package version 6.1.0, https://cran.r-project.org/package=eulerr).

Figure 2: Number of compounds identified in the wastewater effluent according to chromatographic mode, either in spot samples (SPE) or POCIS sorbent. N.B.: “Unique” denotes those chemicals exclusively detected in the given chromatographic mode, while “Non-unique” means they were detected by several chromatographic approaches.

Figure 3: Chromatogram and comparison of experimental and library spectra of (a) 4-nitrophenol identified in sample S1 using POCIS sorbent and RPLC; (b) tri-n-butyl phosphate identified in sample S1 using SPE and RPLC and (c) azithromycin in sample R2 using SPE and SFC.

Figure 4: Number of compounds identified in each surface water sample (A) by chromatographic mode and (B) by sampling strategy.

Figure 1


POCIS membrane
6
1
2
14
SPE
13
POCIS sorbent
23
76
SPE
POCIS sorbent
POCIS membrane

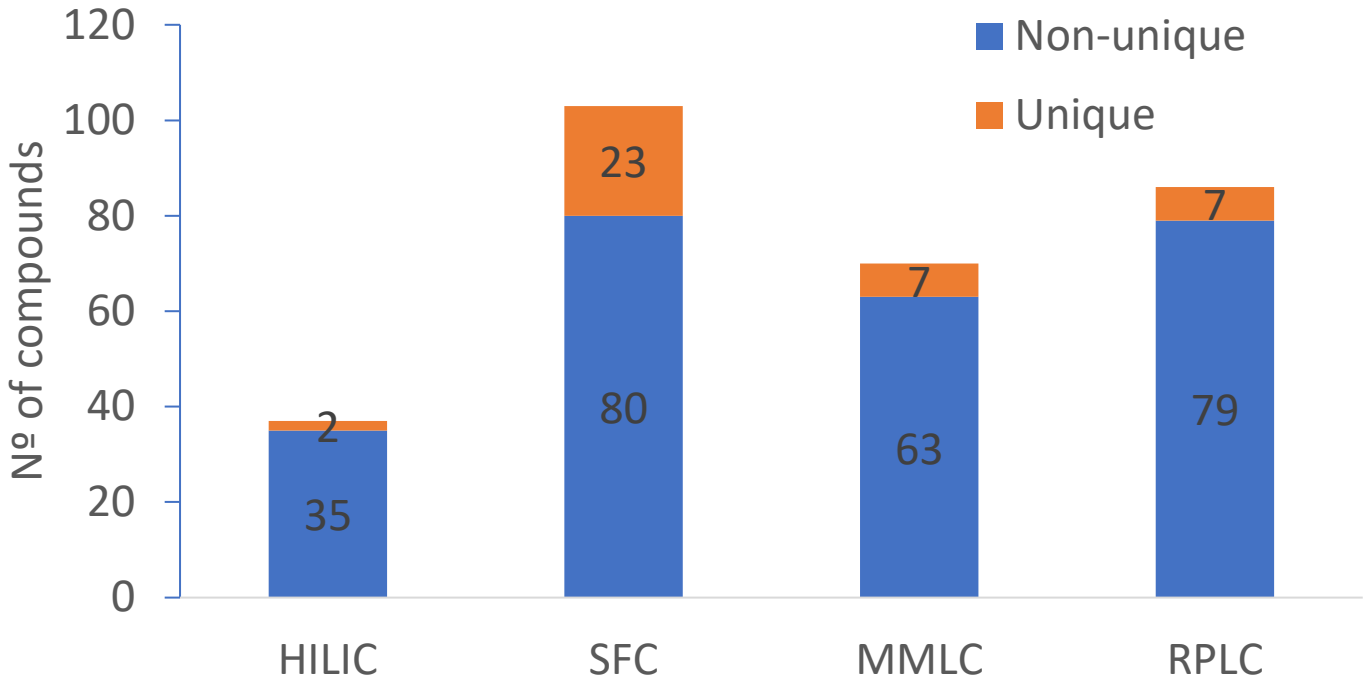

120
100
80
60
40
20
0
Nº of compounds
Non-unique
Unique
23
7
7
2
80
63
79
35
HILIC
SFC
MMLC
RPLC

Figure 2

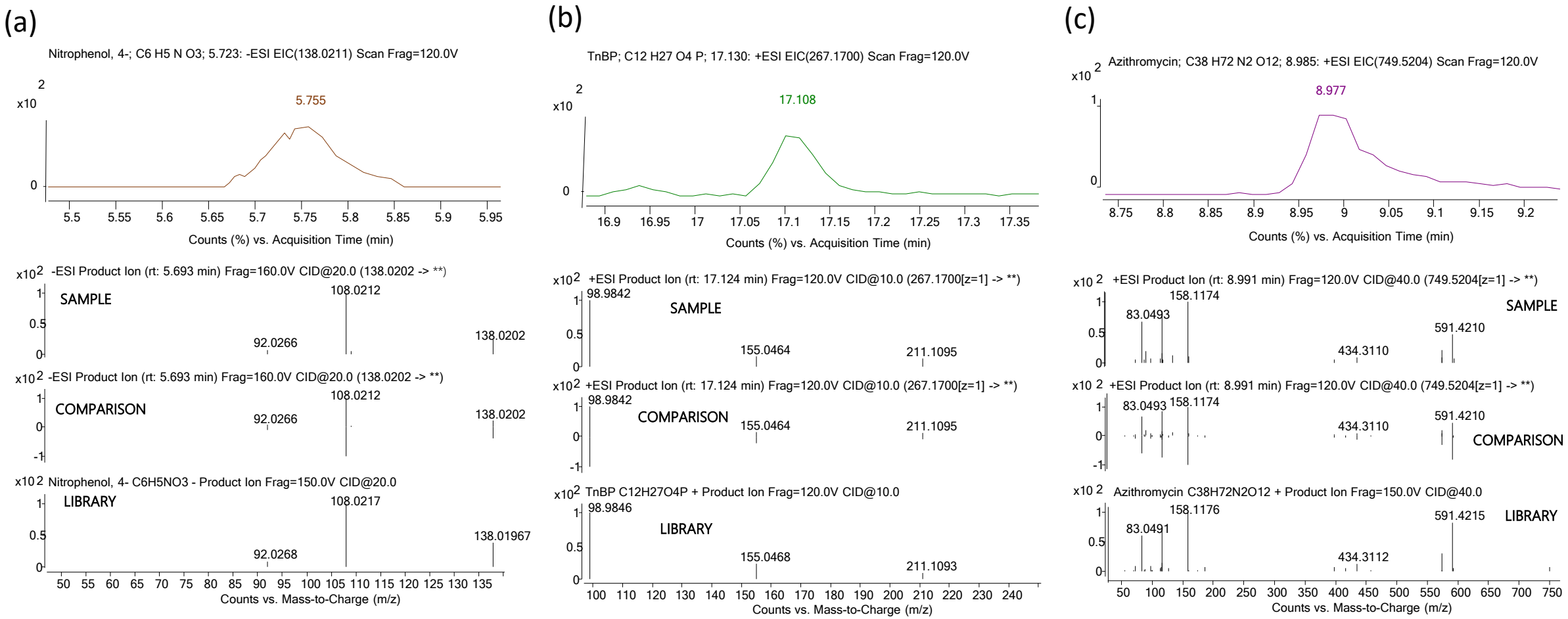

(a)
Nitrophenol, 4-; C6 H5 N O3; 5.723: -ESI EIC(138.0211) Scan Frag=120.0V
x10 2
5.755
Counts (%) vs. Acquisition Time (min)
-ESI Product Ion (rt: 5.693 min) Frag=160.0V CID@20.0 (138.0202 -> **)
SAMPLE
108.0212
92.0266
138.0202
-ESI Product Ion (rt: 5.693 min) Frag=160.0V CID@20.0 (138.0202 -> **)
COMPARISON
108.0212
92.0266
138.0202
Nitrophenol, 4- C6H5NO3 - Product Ion Frag=150.0V CID@20.0
LIBRARY
108.0217
92.0268
138.01967
Counts vs. Mass-to-Charge (m/z)
(b)
TnBP; C12 H27 O4 P; 17.130: +ESI EIC(267.1700) Scan Frag=120.0V
17.108
Counts (%) vs. Acquisition Time (min)
+ESI Product Ion (rt: 17.124 min) Frag=120.0V CID@10.0 (267.1700[z=1] -> **)
SAMPLE
98.9842
155.0464
211.1095
+ESI Product Ion (rt: 17.124 min) Frag=120.0V CID@10.0 (267.1700[z=1] -> **)
COMPARISON
98.9842
155.0464
211.1095
TnBP C12H27O4P + Product Ion Frag=120.0V CID@10.0
LIBRARY
98.9846
155.0468
211.1093
Counts vs. Mass-to-Charge (m/z)
(c)
Azithromycin; C38 H72 N2 O12; 8.985: +ESI EIC(749.5204) Scan Frag=120.0V
8.977
Counts (%) vs. Acquisition Time (min)
+ESI Product Ion (rt: 8.991 min) Frag=120.0V CID@40.0 (749.5204[z=1] -> **)
SAMPLE
158.1174
83.0493
591.4210
434.3110
+ESI Product Ion (rt: 8.991 min) Frag=120.0V CID@40.0 (749.5204[z=1] -> **)
COMPARISON
83.0493
158.1174
434.3110
591.4210
Azithromycin C38H72N2O12 + Product Ion Frag=150.0V CID@40.0
LIBRARY
158.1176
83.0491
591.4215
434.3112
Counts vs. Mass-to-Charge (m/z)

Figure 3

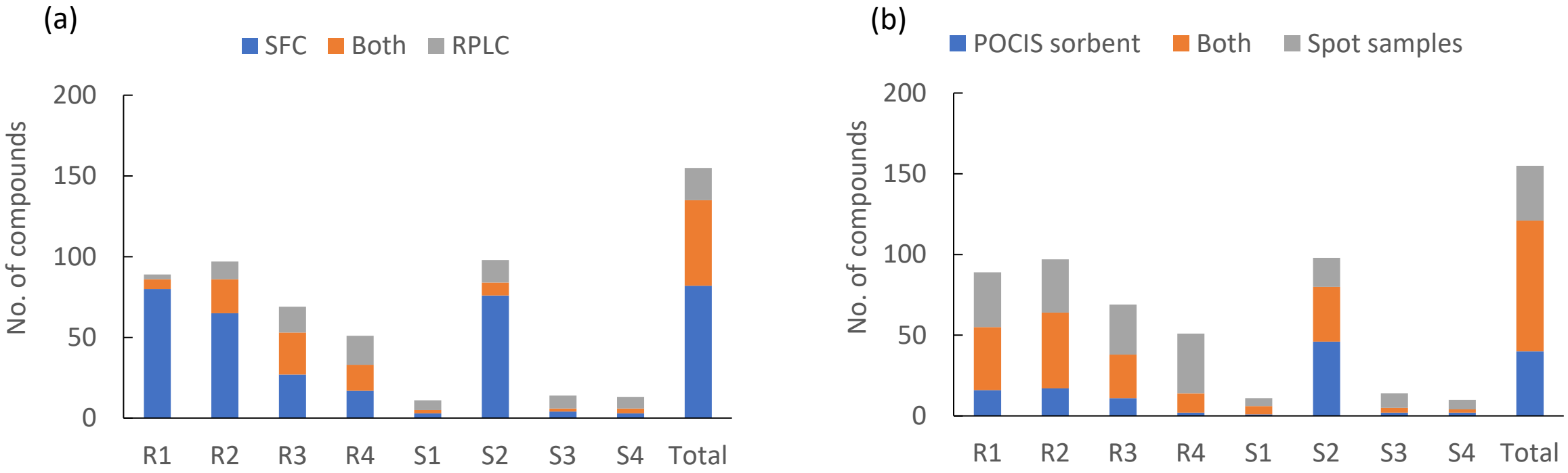

(a)
SFC
Both
RPLC
No. of compounds
200
150
100
50
0
R1
R2
R3
R4
S1
S2
S3
S4
Total
(b)
POCIS sorbent
Both
Spot samples
No. of compounds
200
150
100
50
0
R1
R2
R3
R4
S1
S2
S3
S4
Total

Figure 4

Table 1: List of compounds detected in surface water samples. n: number of samples where the compounds were identified. Further details are provided in Table S4.

Compound name	CAS	n	Compound name	CAS	n	Compound name	CAS	n	Compound name	CAS	n
10-Hydroxycarbazepine	29331-92-8	4	2,4-Dinitrophenol	51-28-5	1	3-Hydroxycotinine	34834-67-8	3	4-Hydroxybenzoic acid	99-96-7	3
4-Nitrophenol	100-02-7	8	5-Hydroxyomeprazole	92340-57-3	3	8-Hydroxyquinoline	148-24-3	1	Abacavir	136470-78-5	4
Acebutolol	37517-30-9	3	Acetaminophen	103-90-2	5	Adenosine	58-61-7	1	Amantadine	768-94-5	3
Amisulpride	71675-85-9	3	Amphetamine	300-62-9	2	Ampyrone	83-07-8	2	Atenolol	29122-68-7	5
Atorvastatin	134523-00-5	3	Atrazine	1912-24-9	1	Azelaic acid	123-99-9	3	Azithromycin	83905-01-5	5
Benzododecinium	10328-35-5	6	Benzoylecgonine	519-09-5	5	Benzydamine	642-72-8	2	Berberine	2086-83-1	4
Bezafibrate	41859-67-0	1	Bicalutamide	90357-06-5	2	Bisoprolol	66722-44-9	3	Butylbenzylphthalate	85-68-7	1
Butylscopolaminium	149-64-4	1	Caffeine	58-08-2	3	Candesartan	139481-59-7	1	Carbamazepine	298-46-4	5
Carbamazepine 10,11-epoxide	36507-30-9	4	Carbendazim	10605-21-7	3	Cetirizine	83881-51-0	3	Cetylpyridinium	7773-52-6	1
Chlorpheniramine	132-22-9	1	Citalopram	59729-33-8	1	Clarithromycin	81103-11-9	5	Clindamycin	18323-44-9	1
Cloperastine	3703-76-2	1	Clozapine	5786-21-0	2	Cocaethylene	529-38-4	1	Cocaine	50-36-2	4
Cotinine	486-56-6	3	Denatonium	47324-98-1	1	Dexpanthenol	81-13-0	4	Dextrorphan	125-73-5	2
Diclofenac	15307-86-5	4	Diethyl phthalate	84-66-2	7	Diethyltoluamide (DEET)	134-62-3	4	Diltiazem	42399-41-7	5
Dimethoate	60-51-5	1	Dinoterb	1420-07-1	3	Diuron	330-54-1	2	Doxylamine	469-21-6	2
Empenthrin	54406-48-3	1	Enalapril	75847-73-3	3	Ephedrine	299-42-3	5	Eprosartan	133040-01-4	2
Erythromycin	114-07-8	2	Estrone sulfate	481-97-0	1	Flecainide	54143-55-4	5	Fluconazole (II)	86386-73-4	4
Flucytosine	2022-85-7	3	Flufenamic acid	530-78-9	3	Furosemide	54-31-9	1	Gabapentin	60142-96-3	1
Haloperidol	52-86-8	1	Harmol	487-03-6	1	Hydrochlorothiazide	58-93-5	5	Hydroxybupropion	357399-43-0	1
Imidapril	89371-37-9	1	Iminostilbene	256-96-2	3	Iopromide	73334-07-3	3	Irbesartan	138402-11-6	4
Ketoprofen	22071-15-4	1	Labetalol	36894-69-6	1	Lamivudine	134678-17-4	1	Levamisole	14769-73-4	1
Levetiracetam	102767-28-2	2	Levomethadone	125-58-6	1	Levorphanol	77-07-6	1	Lidocaine	137-58-6	5
Loratadine	79794-75-5	5	Lorazepam	846-49-1	1	Losartan	114798-26-4	4	Mecetronium	3006-10-8	1
Melamine	108-78-1	2	Memantine	19982-08-2	1	Mepivacaine	22801-44-1	4	Metformin	657-24-9	5
Methadone	76-99-3	4	Methenamine	100-97-0	1	Methylendioxymethamphetamine	42542-10-09	5	Methylphenidate	113-45-1	2
Metoclopramide	364-62-5	1	Metoprolol	37350-58-6	1	Metronidazole	443-48-1	5	Minoxidil	38304-91-5	5

Compound name	CAS	n	Compound name	CAS	n	Compound name	CAS	n	Compound name	CAS	n
Mirtazapine	61337-67-5	2	Mycophenolic acid	24280-93-1	1	N-Desalkylverapamil	34245-14-2	2	Nicotine	54-11-5	4
Norcitalopram	62498-67-3	3	Norcocaine	18717-72-1	2	Norethindrone acetate	51-98-9	1	O-Desmethyltramadol	73986-53-5	5
Omeprazole	73590-58-6	1	Omeprazole sulfone	88546-55-8	4	Oxazepam	604-75-1	3	Oxcarbazepine	28721-07-5	5
Palmidrol	544-31-0	1	Paraxanthine	611-59-6	1	Pentoxifylline	6493-05-6	5	Phenethylamine	64-04-0	1
Piperine	94-62-2	3	Propranolol	525-66-6	2	Psilocine	520-53-6	2	Quetiapine	111974-69-7	4
Ranitidine	66357-35-5	2	Rhein (cassic acid)	478-43-3	1	Ritalinic acid	19395-41-6	5	Saccharin	81-07-2	4
Salicylic acid	69-72-7	6	Sertraline	79617-96-2	2	Sitagliptin	486460-32-6	2	Sotalol	3930-20-9	3
Sulfadiazine	68-35-9	2	Sulfamethoxazole	723-46-6	4	Sulfapyridine	144-83-2	1	Sulpiride	15676-16-1	5
Tapentadol	175591-23-8	5	Telmisartan	144701-48-4	4	Terbinafine	91161-71-6	1	Terbuthylazine-2-hydroxy	66753-07-9	2
Terbutryn	886-50-0	4	Theobromine	83-67-0	2	Theophylline	58-55-9	2	Thiabendazole	148-79-8	1
Tiapride	51012-32-9	5	Tramadol	27203-92-5	3	trans-10,11-Dihydroxy-10,11-dihydrocarbazepine	58955-93-4	4	Trazodone	19794-93-5	3
Triamterene	396-01-0	1	Tri-iso-butyl phosphate	126-71-6	7	Trimethoprim	738-70-5	3	Tri-n-butyl phosphate	126-73-8	7
Triphenyl phosphate	115-86-6	1	Tris(2-butoxyethyl) phosphate	78-51-3	6	Tris(2-chloroisopropyl) phosphate	13674-84-5	3	Umbelliferone	93-35-6	3
Usnic acid	125-46-2	1	Valsartan	137862-53-4	5	Venlafaxine	93413-69-5	5			