

1 Screening for polar chemicals in water by trifunctional mixed- 2 mode liquid chromatography-high resolution mass 3 spectrometry

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11 Abstract

12 The presence of persistent and mobile organic contaminants (PMOC) in aquatic environments is
13 a matter of high concern due to their capability of crossing through natural and anthropogenic
14 barriers, even reaching the drinking water. Most analytical methods rely on reversed-phase liquid
15 chromatography (RPLC), which is quite limited for the detection of very polar chemicals. Thus,
16 many of these PMOCs may have not been recognized as water pollutants yet, due to the lack of
17 analytical methods capable to detect them. Mixed-mode LC (MMLC), providing the combination
18 of RP and ion-exchange functionalities is explored in this work with a trifunctional column,
19 combining RPLC, anion and cation exchange, which allows the simultaneous determination of
20 analytes with extremely different properties. A non-discriminant sample concentration step
21 followed by a MMLC-high resolution mass spectrometry method was developed for a group of 37
22 very polar model chemicals with different acid/base functionalities. The overall method
23 performance was satisfactory with a mean limit of detection of 50 ng/L, relative standard deviation
24 lower than 20% and overall recoveries (including matrix effects) higher than 60% for the 54% of
25 model compounds. Then, the method was applied to 15 real water samples, by a suspect
26 screening approach. For those detected PMOC with standard available, a preliminary estimation
27 of concentrations was also performed. Thus, 22 compounds were unequivocally identified in a
28 range of expected concentrations from 6 ng/L to 540 µg/L. Some of them are well-known PMOC,
29 such as acesulfame, perfluorobutanoic acid or metformin, but other novel pollutants were also
30 identified, as for example di-o-tolylguanidine or trifluoromethanesulfonic acid, which had not or
31 were scarcely studied in water so far.

32 Introduction

33 High resolution mass spectrometry (HRMS) instrumentation and software has greatly improved
34 during the last decade. Indeed, the increasing popularity of this technique has been a turning
35 point in screening studies, since it allows the identification of unknowns without the need of initially
36 having pure standards and post-targeted analysis approaches, because the full accurate
37 spectrum is registered for each injection.¹⁻³ Hence, the coupling of liquid chromatography (LC) to
38 HRMS has been applied in several water screening studies.⁴⁻⁸ However, most of these LC-HRMS
39 screening methods rely on reversed-phase LC (RPLC),^{5,9} which is limited for the detection of very
40 polar chemicals, which usually exhibit poor retention in traditional C18 columns.¹⁰ Thus, many
41 very polar organic chemicals may have not been recognized as water pollutants of concern yet,
42 because the analytical methods developed so far are unable to detect them.¹¹ Since these polar
43 pollutants are by definition highly mobile in water, many of them can spread in the water cycle
44 and even reach drinking water if they are persistent, i.e. “persistent and mobile organic pollutants”
45 (PMOC). Thus, it is necessary to explore new LC retention mechanisms which allow the
46 determination of PMOC in water.

47 The limitations of RPLC for polar analytes have been commonly avoided by the use of alternative
48 chromatographic modes (e.g. ion-exchange or ion-pairing), but they present well-known intrinsic
49 drawbacks in LC-MS (such as interface and spectrometer contamination).¹² Hydrophilic
50 interaction liquid chromatography (HILIC)¹³⁻¹⁴ and mixed-mode LC (MMLC) can be viable
51 alternatives in the determination of such polar compounds.¹⁵ The use of HILIC has been described
52 for the identification of polar compounds and isomers when retention problems using RPLC were
53 observed.¹⁶ However, the main advantage of MMLC is that it provides more than one type of
54 interaction between the stationary phase and the analytes, allowing the simultaneous
55 determination of compounds with different physico-chemical properties (e.g. ionic, basic, acid and
56 neutral chemicals, or hydrophilic and hydrophobic compounds) in one run.¹⁷⁻¹⁸ There are several
57 types of MMLC columns available nowadays¹⁹, but among the newest generations, recently,
58 trimodal RP/anion exchange/cation exchange (RP/AX/CX) columns based on embedded double
59 functionalized ligands on nanopolymeric silica hybrid technology have also been
60 commercialized.^{12, 17, 20-22} Several applications involving trimodal MMLC have been described in

61 the pharmaceutical field ^{17, 21, 23}, as for instance counterions and active ingredients can be
62 measured in one run, but in environmental analysis applications are still rare. ²⁴

63 Thus, the aim of this work was to develop a new analytical methodology for PMOC screening by
64 using trimodal MMLC combined to HRMS. To this end, a group of polar model chemicals with
65 different acid/base functionalities was employed for exploring their retention behavior and method
66 development, before final application in suspect screening studies of over 3000 chemicals aiming
67 to detect PMOC in a set of 15 water samples from different countries of Europe. To the best of
68 our knowledge, this is the first application of MMLC in LC-HRMS environmental screening studies,
69 despite its potential for effectively detecting chemicals of different physico-chemical properties.

70

71 **Experimental**

72 **Chemicals and reagents**

73 Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Acetic acid ($\geq 99\%$)
74 and ammonium hydroxide solution (25%) were purchased from Sigma-Aldrich (Steinheim,
75 Germany). For method development, a mixture of 45 model analytes, with logD values at pH 7.4
76 ranging from -6 to 1.8 (average -2.3), was used, including acidic, basic, neutral and amphoteric
77 substances. Detailed information regarding these model analytes, including physico-chemical
78 properties and suppliers is given in **Table S1**.

79 **Samples and sample preparation**

80 The 15 analyzed samples were obtained from different locations in Spain, Germany, France, The
81 Netherlands and Switzerland and included surface (5), ground (7), drinking (2) water and also
82 effluent wastewater (1). The sampling campaign was programmed within the project PROMOTE
83 - Protecting Water Resources from Mobile Trace Chemicals. ²⁵

84 These samples were submitted to a simple freeze-drying protocol that avoids discrimination of
85 PMOC that could be produced e.g. by solid-phase extraction (SPE). Under final working
86 conditions 100 mL of filtered and frozen water were completely freeze-dried and reconstituted
87 using 15 mL (3x5mL) of methanol. The organic extract was filtered through 0.22 μm polypropylene

88 (PP) filters and evaporated to dryness in a Büchi Syncore PolyVap (Flawil, Switzerland), following
89 the supplier recommended conditions for methanol evaporation. Two hundred microliters of
90 water:acetonitrile (9:1) were used to reconstitute samples, which were finally filtered through 0.22
91 µm PP filters and injected in the chromatographic system. Thus, the final concentration factor
92 reached was 500 times.

93 **Determination conditions**

94 The MMLC columns used were the Acclaim Trinity P1 (2.6 µm particle size; 3 mm internal
95 diameter, in both 50 and 100 mm length format), supplied by Dionex (Sunnyvale, CA, USA). A
96 Varian (Walnut Creek, CA, USA) LC-triple quadrupole-MS (LC-QQQ) system was used for
97 investigating the parameters affecting MMLC retention. The Varian instrument comprises a
98 ProStar 210 dual pump coupled to a Varian 320MS furnished with an ESI interface. Nitrogen was
99 used as nebulizing (50 psi) and drying gas (200 °C, 19 psi) and Argon was employed as collision
100 gas (2 mTorr). The ESI interface was operated both in the positive and negative modes and the
101 voltage of the ESI needle was fixed at 5,000 V. Compounds were recorded in the multiple reaction
102 monitoring (MRM) mode as detailed in **Table S1**.

103 For the screening studies, an Agilent (Wilmington, DE, USA) 1200 Series LC was used. The LC
104 system was interfaced to a quadrupole-time of flight (QTOF) HRMS model Agilent 6520 equipped
105 with a Dual electrospray (ESI) ion source. Nitrogen was used as nebulizing (40 psi) and drying
106 gas (300 °C, 5 L min⁻¹) in the dual ESI source and also as collision gas in the MS/MS experiments.
107 The QTOF instrument was operated in the high resolution 4 GHz mode. This mode provides a
108 full width at half maximum (FWHM) resolution of ca. 10,600 at m/z 118.0862 and ca. 16,900 at
109 m/z 922.0097. A reference calibration solution, supplied by Agilent, was continuously sprayed in
110 the source during the chromatographic run, providing the required accuracy of mass assignments.

111 Several chromatographic conditions, in isocratic mode, were tested in order to establish adequate
112 compromise elution conditions for the majority of model compounds, including mobile phase pH
113 (3.5 and 5.5), concentration of buffer (ammonium acetate, 10-80 mM) and percentage of organic
114 modifier (acetonitrile, 5-95%). The final LC method consisted of a simultaneous binary gradient
115 from low organic content (2% ACN) and buffer (5 mM ammonium acetate, pH 5.5) to high organic

116 (80% ACN) and buffer (20 mM ammonium acetate, pH 5.5) in 10 min, with a final isocratic time
117 of 15 min, with the 50 mm length column being used.

118 **Method performance evaluation**

119 The analytical parameters evaluated were limits of detection (LODs), repeatability and apparent
120 recovery with the LC-QTOF instrument for the model compounds. Linearity of the method was
121 not evaluated, since the quantification of samples was beyond the original goal of this
122 methodology. The instrumental LODs (iLODs) were determined by the injection of standards, for
123 a signal to noise ratio (S/N) of 3. The method LODs (mLODs) were calculated by spiking real river
124 water samples at the 200 ng L⁻¹ level, submitting them to the entire protocol and calculating the
125 S/N. Instrumental and full methodology repeatability was measured by the relative standard
126 deviation (RSD) of three consecutive injections of either standards or extracts, and expressed as
127 iRSD and mRSD, respectively. The apparent recovery was assessed by the addition of model
128 analytes to real surface water samples (200 ng L⁻¹), and comparing the obtained response against
129 pure standards. Blank samples were processed in order to evaluate the presence of these
130 compounds in the selected matrix.

131 **Workflow for MMLC-QTOF suspect screening**

132 The scheme of the whole screening workflow is presented in **Figure 1** (see **Figure S1** for more
133 detailed information). The accurate mass obtained results were processed using the Qualitative
134 Analysis of MassHunter Workstation software B.07.00. The strategy used to analyze the data
135 consisted on the use of the search algorithm "*Find by Formula*" (FBF), which analyzes each data
136 file searching for the specified compounds, i.e those from a pre-selected database. In this work,
137 two different databases were used: a) a database taken from Wode et al.²⁶ containing over 2000
138 typical water micropollutants and b) a lab-developed library prepared within the PROMOTE
139 consortium consisting of over 1000 polar compounds extracted from REACH (Registration,
140 Evaluation, Authorization and restriction of Chemicals) database and fulfilling the criteria of being
141 PMOCs and having a high emission score into the environment.²⁷⁻²⁸ Thus, FBF computes the
142 neutral mass and the theoretical isotopic pattern corresponding to each specified formula,
143 calculates the m/z of permitted ions (in this work set to protonation, Na⁺ K⁺ and NH₄⁺ adducts in
144 positive mode; deprotonation only in negative) and searches for chromatographic peaks for such

145 m/z values. Then, it extracts the spectrum and for each compound the software calculates a
146 normalized score parameter (0-100) combining mass deviation, isotope abundance and spacing
147 between ions. Thus a MS score of 100 implies an absolute match. As screening criteria, a
148 maximum mass error (± 5 ppm) and score > 80 were set as cut-off values. Finally, the software
149 provided a list of candidate compounds that were present in the databases and met the selected
150 data processing tolerances.

151 For those suspect compounds identified in the MS mode (scan range: 50-1100 m/z), MS/MS
152 experiments (scanning from 40 m/z to 10 m/z units above the precursor ion) at two different
153 collision energies (15 and 30 V) were carried out. Thus, the presence of some analytes was
154 confirmed by their fragmentation pattern. Finally, the tentatively identified compounds that were
155 commercially available were acquired and injected into the system in order to unequivocally
156 confirm their presence. Thus, to give a positive identification the retention and at least two product
157 ions in MS/MS spectrum should match ²⁹.

158 Finally, in order to obtain a tentative idea of the detected chemicals' concentrations, the 10-90%
159 percentile (P_{10-90}) was calculated from the recovery evaluation of model analytes (see above and
160 **Figure S2**). These percentile values were 15.4-96.3% apparent recoveries. Therefore, the final
161 estimated range of concentration of the analytes in the extracts was calculated by comparison
162 with the injection of standards and applying the P_{10-90} percentiles of recovery.

163 **Results and discussion**

164 **Development of the MMLC-MS methodology**

165 **Evaluation of chromatographic conditions: pH, ionic strength and organic modifier** 166 **percentage**

167 In the first steps of this work, the retention mechanisms of model analytes were studied under
168 isocratic conditions using a 100 mm Acclaim Trinity P1 column. The selected chromatographic
169 parameters were evaluated taking into account the column supplier specifications, where the use
170 of methanol as organic modifier and pH values outside the range 3-6 were not recommended.
171 Thus, pH 3.5 and 5.5, different percentages of acetonitrile as organic modifier (5-95%) and 10 to
172 80 mM of ammonium acetate buffer were evaluated.

173 **Figure 2** shows the retention behavior for some model compounds, belonging to different
174 chemical classes, i.e. acidics, neutrals, amphoteric, basics and cations. As shown, most of
175 compounds eluted earlier at high pH (dotted lines) and buffer concentrations (round markers).
176 Also, in general, the higher the acetonitrile percentage, the lower the retention time, as expectable
177 because of the RPLC functionality of the column. However, for some model analytes, such as
178 aspartame (**Figure 2**) retention is higher at either low or high acetonitrile content, i.e. there is
179 RPLC retention at low organic and HILIC-like retention when this is increased sufficiently. Such
180 behavior has been already described in the literature for some other chemicals ¹³. In terms of
181 analytes' properties, strongly acidic compounds, such as perfluorobutanoic acid, presented
182 acceptable retention at all the studied conditions, with a few compounds (e.g. monomethyl
183 phthalate) showing the above mentioned dual HILIC-like and RP mechanisms depending on the
184 percentage of organic modifier (data not shown). Neutral analytes, e.g. sucralose, showed low
185 retention times at almost all studied conditions (see **Figure 2**), as the only available mechanism,
186 RP, is not strong enough for these polar neutral compounds. Cationic and basic compounds were
187 well-retained and the behavior of amphoteric chemicals was highly dependent on the conditions
188 and the nature of each analyte. Thus, bipyridilium compounds (permanently positively charged),
189 such as diquat and paraquat, phosphonic and amino acid group-containing compounds, e.g.
190 glyphosate, and mono-substituted phosphates were too strongly retained at the studied
191 conditions.

192 In summary, evaluating the isocratic behavior of model analytes we observed that 1) most of
193 compounds presented high retention at the lowest pH, i.e. 3.5; and 2) high buffer concentration
194 could improve the elution of the most retained analytes (e.g. glyphosate) ³⁰ but led to strong ion
195 suppression in LC-MS. On the basis of the isocratic results, a gradient elution program was tested
196 in order to obtain compromise elution conditions covering a wide range of model analytes. In this
197 case, the pH value was fixed at 5.5 and a simultaneous gradient of buffer (from 10 mM to 40 mM
198 ammonium acetate) and organic modifier (from 2% to 80%) was programmed in 10 minutes, using
199 a final isocratic step at maximum acetonitrile percentage of 15 min. The obtained retention factors
200 are represented in **Figure S3**. More than 80% of model analytes elute in less than 15 min, with
201 adequate peak shapes, showing a retention factor lower than 8. As previously mentioned,
202 bipyridilium compounds, phosphonic and amino acid group-containing compounds and mono-

203 substituted phosphates were too strongly retained and would need dedicated approaches. Thus,
204 in further experiments, these 8 analytes were not further considered and since then, the model
205 compounds mixture consisted of 37 compounds. Determination of those 8 strongly retained
206 chemicals could be improved in the future by increasing the buffer concentration (not highly
207 compatible with LC-MS), decreasing the column length below 5 cm or by selecting other MMLC
208 column chemistries.

209 Comparison of MMLC and RPLC

210 The retention of the model compounds under optimal conditions in the MMLC column was
211 compared to that obtained with a Waters Symmetry Shield RP18 (3.5 μm ; 2.1 mm \times 100 mm)
212 column. For RP column a common water/acetonitrile (with 5 mM of ammonium acetate in both
213 phases) gradient was selected. This gradient was programmed from 2% to 100% acetonitrile in
214 10 min, with the same final isocratic time than the MM column. **Figure 3** presents the obtained
215 differences in the retention factor for both columns. From the model analytes, 62% of them
216 showed better retention when the MMLC method was used, 16% presented similar and low
217 retention in both approaches and the remaining 22 % model compounds were more efficiently
218 retained in the RP column. Moreover 2 chemicals presented retention factors <1 in MMLC (5%),
219 5 in RPLC (14%) and 7 in both MMLC and RPLC (19%). According to the acid/base character,
220 the neutral species were the poorly retained in both approaches, whereas strongly acidic species
221 performed better with MMLC. Also, the MMLC approach produced better results for basic and
222 cationic compounds which were retained through the strong cationic exchange (SCX)
223 mechanism. Retention of some neutral chemicals could be improved on MMLC by exploiting the
224 HILIC-like behavior of the column, but this would produce shorter retentions of most of the
225 remaining compounds, thus, since the aim was the development of a wide scope screening
226 method, this possibility was not exploited.

227 As a final step in method development and in order to decrease the chromatographic run time,
228 and also to further decrease the concentration of ammonium acetate arriving to the LC interface,
229 a shorter MM column was tested, which was not commercially available when the study began.
230 Thus, a 50 mm Acclaim Trinity P1 column was used instead the original 100 mm one (all other
231 characteristics of the columns remained the same). The pH value and organic modifier gradient
232 were maintained but the 10 min dual buffer/acetonitrile gradient was changed from 5 mM to 20

233 mM (instead of 10-40 mM used with the 100 mm column) with a final isocratic step and re-
234 equilibration time of 15 and 10 min, respectively. The obtained separation was quite similar, with
235 phenylbenzimidazole sulfonic acid as the last eluted compound, with a retention time of 12 min.
236 Thus, the 50 mm column and the diluted buffer gradient were employed in screening experiments,
237 since separation was faster and lower buffer is preferred in ESI-MS.

238 **Sample preparation and method performance**

239 In order to extract polar analytes from water, SPE using different ionic exchange cartridges was
240 tested. This technique was applied by several authors and the eluted fractions from RP, CX and
241 AX sorbents were combined before the analysis or injected separately in the LC-QTOF system
242 for screening studies.^{14, 31-35} However, development of such SPE methods for mixtures of different
243 strong/weak acid/base chemicals is challenging and time-consuming. Thus, we tested the
244 applicability of a freeze-drying procedure as a non-discriminant sample preparation approach.
245 This technique is certainly more time-consuming. Nevertheless, since several samples can be
246 processed simultaneously the real invested time per sample decreases and it avoids
247 discrimination between polar analytes with different acid/base properties. Thus, a high
248 concentration factor was achieved and compounds with different chemical properties could be
249 screened for simultaneously.

250 The figures of merit for the developed method are given in **Table 1**. iLODs ranged between 0.5
251 and 300 ng/mL, whereas the mean mLOD obtained for surface water was 51.5 ng/L, for the 34
252 model analytes considered. These values are at the same order of magnitude than those reported
253 with different protocols for some of these model analytes³⁴⁻³⁵ and were obtained by a 9-years old
254 QTOF system. Therefore, they could be easily improved by the newest high-end HRMS
255 instruments. Both instrumental and method mean RSD were lower than 15%. The recovery of the
256 whole process was evaluated by spiking river water samples as detailed in experimental section.
257 Thus, the recovery values presented do not exclusively represent possible losses during sample
258 preparation, but also matrix effects (mainly signal suppression) occurring during ionization in LC-
259 MS. Up to 50% of model compounds presented a recovery higher than 60% (see **Figure S2**).
260 These recoveries were considered acceptable taking into account that the goal of this screening
261 method was to detect and identify the compounds, not their quantification and that it includes

262 matrix effects.⁵ Actually, these values are even higher than those reported in the literature using
263 SPE protocols for some targets as metformin and acephate³⁶ and quite similar to those obtained
264 in many quantitative methods for environmental water samples, due to the inherent matrix
265 effects.³⁷⁻³⁹

266 **Screening of real samples**

267 The developed method was used to process 15 real water samples as detailed in experimental
268 section. **Figure 1** shows the total number of unique compounds in each step of the suspect
269 screening approach using the two selected databases. Blank experiments were performed and
270 only those analytes which presented an area ratio sample/blank higher than 10 were considered.
271 Finally, 22 organic contaminants were identified to occur at least in one of the samples, as
272 presented in **Table 2** (**Table S2** contains further details on identifiers, physico-chemical properties
273 and usage of the detected compounds). From them, 21 were unequivocally identified
274 (confirmation level 1) and the remaining compound, the carbamazepine metabolite, was a
275 probable positive (confirmation level 2), since its presence could not be confirmed with a standard,
276 according to the confirmation levels proposed in the literature⁴⁰.

277 Most of the detected chemicals can be classified as PMOCs as the log D at pH7.4 of the identified
278 species ranged between -6 and 4 (**Table S2**), with an average value of logD of -0.9, but some
279 less polar chemicals were also detected. Confirmation of tentatively identified chemicals was
280 performed as explained in the experimental section. Initially, those suspects without standard
281 available in the laboratory were tentatively identified by MS/MS spectra comparison with the
282 commercial databases *Forensics and Pesticides Libraries* from Agilent, or open web resources,
283 namely METLIN, MassBank or m/zCloud. If spectra were not available in such libraries, tentative
284 identification was made on the basis of HRMS/MS spectra interpretation. In subsequent steps,
285 standards were purchased and from the list of initially tentatively identified analytes, 21 were
286 confirmed by the injection of the corresponding standard. Only 10,11-trans-dihydroxy-10,11-
287 dihydrocarbamazepine, a carbamazepine metabolite, could not be identified with authentic
288 standard, since it was not commercially available. However the MS/MS spectrum was compared
289 to that reported in the MassBank (ref. AU203901) and MzCloud (ref. 1005) databases and four
290 common product ions were found. **Table 2** contains the retention time and product ions obtained

291 in MS/MS experiments and used for identification by comparison with databases and in most
292 cases later with standards, along with the estimation of concentration ranges, according to the
293 procedure described in the experimental section. The estimated concentration levels of each
294 individual sample are detailed in **Table S3**.

295 The majority of the identified pollutants in the processed samples were pharmaceuticals (**Table**
296 **S2**). This result is in accordance with several reports, as e.g. Wode et al.,²⁶ on the high occurrence
297 of pharmaceuticals through the water cycle, which can even reach ground and drinking water.
298 Within this group, the most abundant species is metformin (estimated 0.1-12 µgL⁻¹), a common
299 antidiabetic drug that was actually used as a model chemical in the method development. This
300 pharmaceutical was also described as the most abundant pharmaceutical in previous literature
301 works sampling river water³⁴ and wastewater³⁵, with concentration ranges in accordance with
302 those found in this paper. The presence of other pharmaceuticals, such as lidocaine, amisulpride,
303 venlafaxine, etc, has also been previously reported.^{26, 34-35}

304 Within the remaining non-pharmaceuticals identified pollutants, saccharin and acesulfame are
305 common sweeteners whose presence in water has been reported in other screening studies.^{10,}
306 ³⁴⁻³⁵ Screening estimated concentration ranges (Table 2) are also in agreement with previous
307 findings in the literature.⁴¹⁻⁴²

308 Perfluoroalkyl substances are also common polar water pollutants. Although the presence of
309 PFOS, PFBS and PFOA was detected in procedural blank samples, their signals in the real water
310 samples were over ten times higher. Concerns regarding the presence of these substances in the
311 water cycle have been growing and PFOS has been recently included in the list of Priority
312 Substances of the European Water Framework Directive (WFD).⁴³ The mLOD for this substance
313 using our proposed method (0.5 ng/L, **Table 1**) is at the same order than that from the ISO method
314 25101 used for quantification issues, thus, low enough for a screening method. However, it would
315 be insufficient for monitoring of maximum allowable concentrations according to the Directive
316 2013/39/EU.

317 As an example, **Figure 4** shows the identification data for a pollutant that, to the best of our
318 knowledge, was not previously reported in water: 1,3-di-o-tolylguanidine. This pollutant was
319 detected in 4 of analysed samples, two groundwaters (DE-GW-1 and CH-GW-2), one surface

320 water and the finished drinking water produced from that (FR-SW-2 and FR-DW-2), at estimated
321 concentrations between 0.3 and 15 $\mu\text{g/L}$ (see **Table 2** and **Table S3**). Firstly, the compound was
322 identified by the software using the database built within the PROMOTE consortium with a MS
323 score of 96%, (**Figure 4a**), from the extracted ion chromatogram automatically generated by the
324 software (**Figure 4b**) and its MS spectrum (**Figure 4c**). Then, in order to obtain structural
325 information, MS/MS experiments were conducted at 15 and 30 V of collision energy. The MS/MS
326 spectra at 30 V (**Figure 4d**) was firstly interpreted, since it was not available in the HRMS libraries,
327 with 5 product ions matching the compound structure, and finally confirmed by injection of the
328 pure standard (**Figure 4e**). This chemical is a substance commonly used in polymerization and
329 vulcanization processes in industry, thus likely to occur in water. Since, this compound has also
330 been characterized as having antidepressant properties ⁴⁴, it may well have some
331 ecotoxicological relevance and deserves future studies.

332 Some of the remaining chemicals detected are sulfonates. Among them, trifluoromethane sulfonic
333 acid had a high detection frequency (41%) in the analysed samples (see **Figure S4** for
334 identification details). This compound is commonly used in industry as a catalyst and appeared
335 in 41% of the analysed samples with estimated concentrations in the 0.04 to 6.3 $\mu\text{g L}^{-1}$ range.
336 However, very scarce information on its occurrence in environment exist to date, excepting the
337 very recent report from Zahn et al. ⁴⁵ who reported the presence of this pollutant in the same
338 sampling campaign (with some additional samples) by using a HILIC-based approach. Due to its
339 high polarity ($\log D < -3$) it could have skipped RPLC based screening approaches. As an example
340 **Figure S5** presents the chromatograms obtained by MMLC and RPLC for 3 of the detected
341 chemicals, of which trifluoromethane sulfonic acid shows very poor retention and peak shape by
342 RPLC, while a well-retained symmetrical peak is obtained by MMLC. Similar results are obtained
343 for basic polar chemicals (e.g. melamine in **Figure S5**).

344 Finally, two organophosphorus triesters were detected. The flame retardant tris(2-butoxyethyl)
345 phosphate (TBEP) is the less polar pollutant detected in in this study ($\log D$ at pH 7.4 = 3.4). The
346 presence of this substance in water has been widely reported in the literature.^{24, 26}. Although this
347 compound could be indeed detected by both RPLC and MMLC (**Figure S5**), the advantage of
348 MMLC is that it allows the simultaneous detection of polar and non-polar chemicals.

349

350 MMLC-HRMS, based on trimodal RP/CX/AX columns, was proven to be an alternative to
351 traditional RPLC methods for the analysis of polar organic pollutants with very different chemical
352 properties. Actually, this approach permitted the identification of 22 polar chemicals, among which
353 trifluoromethanesulfonic acid had only previously been reported once, whereas, di-o-
354 tolylguanidine is reported here for the first time. Although this compound is primarily used in
355 industrial applications, it has pharmaceutical activity, so it will be further studied. Both compounds
356 require further investigation, as to obtain more information on their fate in the water cycle and
357 (eco)toxicological implications.

358

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368 that was used for screening purposes.

369

370 **Conflicts of interest**

371 The authors declare no competing financial interest

372

373 **Supporting information**

374 A pdf file contains:

375 Table S1. Description of model compounds, properties and LC-QQQ parameters used for
376 chromatographic method development.

377 Figure S1. Scheme of the screening process workflow

378 Figure S2. Histogram of recoveries and accumulated frequency (%) obtained for the 37 model
379 analytes from a spiked river water sample

380 Figure S3. Retention factors obtained for the model compounds using the 100 mm-length MMLC
381 column
382 Table S2. Description of detected compounds, properties and uses
383 Table S3. Concentration ranges of detected compounds per sample (ng/L)
384 Figure S4. Trifluoromethanesulfonic acid identification.
385 Figure S5. Chromatograms of 3 of the detected chemicals obtained by MMLC and RPLC.
386 Furthermore, Tables S1 and S2 are also given as excel spreadsheets.

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Table 1. Instrumental and method relative standard deviation (RSD, %) and limits of detection (LODs).

Model Compound		iLOD	mLOD	iRSD	mRSD
Name	CAS	ng/mL	ng/L	%	%
Acesulfame	33665-90-6	5	7	1.0	3.9
Benzenesulphonamide	98-10-2	175	187	11	12
BP-4	4065-45-6	50	84	10	11
Cyclamate	100-88-9	50	86	1.9	2.8
Diatrizoic Acid	117-96-4	100	103	18	20
Dibutylphosphate	107-66-4	15	24	9.4	10
Diphenylphosphate	838-85-7	20	28	10	18
Ethyl sulphate	540-82-9	5	3	4.1	8.1
Monoethylphtalate	2306-33-4	50	71	4.2	5.9
Monomethylphtalate	4376-18-5	50	74	11	11
PFBS	45187-15-3	1	2	2.5	2.5
PFBA	375-22-4	1	2	2.2	4.1
PFOS	1763-23-1	1	0.5	2.1	18
PFOA	335-67-1	2	3	4.2	6.9
PBSA	27503-81-7	50	94	6.9	21
Sacharin	81-07-2	15	21	12	15
Trichloacetic Acid	76-03-9	15	22	16	20
5-Fluoracil	51-21-8	35	42	16	23
Acephate	30560-19-1	3	3	3.7	6.3
Acrilamide	79-06-1	85	10	6.4	20
Cyanuric Acid	108-80-5	75	165	9.1	24
Cytarabine	147-94-4	160	234	1.0	11
Ethylenthiourea	96-45-7	20	8	2.6	21
Iohexol	66108-95-0	100	102	4.6	6.6
Methylisothiazoline	2682-20-4	5	5	6.5	19
N,N-dimethylsulphamide	3984-14-3	300	159	19	17
p-Toluenesulphonamide	70-55-3	250	170	1.0	19
Sucralose	56038-13-2	10	4	3.3	16
Thiourea	2062-56-6	120	48	8.0	20
Chlormequat	999-81-5	1	1	4.7	7.4
Tetrapropylammonium	13010-31-6	0.5	1	1.3	2.3
Melamine	108-78-1	2	0.3	12	25
Metformin	657-24-9	1	2	1.4	4.2
NDMA	9075-31-4	180	88	6.5	12
Quadrol	102-60-3	0.5	0.5	1.9	26
Aspartame	22839-47-0	5	0.1	8.5	14
Gabapentin	60142-96-3	50	50	5.0	8.0
Mean		54.3	51.5	6.7	13.3

iRSD and mRSD evaluated at 500 ng/mL (n=3) and 500 ng/L (n=3), respectively.

Table 2. Chemicals identified in the real water samples using the MMLC-HRMS suspect screening method.

Compound	DATABASE	Ionization mode	Rt (min)	Product ions (m/z)	Detection rate (%)	Estimated Conc. Range (ng/L)
Tris(2-butoxyethyl)phosphate	[1]	ESI (+)	13.56	299.1607, 199.0710, 98.9839	65%	10-2013
Sacharin	[2]	ESI (-)	15.13	105.9594, 93.0342, 79.9555	29%	135-26727
Trifluoromethanesulfonic acid	[2]	ESI (-)	14.66	98.9543, 79.9576, 68.9963	41%	44-6325
m-Xylenesulfonic acid	[2]	ESI (-)	15.88	121.0664, 79.9578	29%	6-109727
p-Toluenesulfonic acid	[2]	ESI (-)	15.65	107.0501, 79.9574	12%	37342-537948
1-Naphthalenesulfonic acid	[2]	ESI (-)	16.39	143.0494, 79.9578	76%	21-14571
2-amino-4,5-dichlorobencenesulfonic acid	[2]	ESI (-)	16.99	177.9626, 159.9688, 79.9568	12%	438-6065
1,3-di-o-tolylguanidine	[2]	ESI (+)	12.79	223.1221, 133.0756, 108.0804	24%	326-15325
Aminophenazone	[1]	ESI (+)	10.47	113.1069, 98.0837	12%	48-312
Amisulpride	[1]	ESI (+)	12.65	242.0476, 196.0053, 112.1121	29%	6-403
Azithromycin	[1]	ESI (+)	15.06	434.3177, 375.2627, 158.1133, 116.1037	12%	62-2766
10,11-trans-Dihydroxi-10,11-dihydrocarbamazepine	[1]	ESI (+)	10.68	180.0803, 210.0900, 236.0700	6%	standard not available
Lidocaine	[1]	ESI (+)	12.28	142.1583, 100.1116, 86.0962	12%	35-1065
Metformin	[1]	ESI (+)	12.18	85.0509, 71.0604, 60.0560	29%	139-12039
Tributylphosphate	[1]	ESI (+)	12.72	155.0473, 98.9841	6%	266-1662
Venlafaxine	[1]	ESI (+)	12.68	260.2011, 97.0648	12%	54-403
Trichloroacetic acid	[1]	ESI (-)	15.33	116.9070, 68.9941	53%	87-11610
Acesulfame	[2]	ESI (-)	14.39	112.9856, 82.0302, 77.9657, 68.9951	76%	6-8506
Bentazon	[1]	ESI (-)	15.37	132.0302, 59.0134	24%	6-169
Amobarbital	[1]	ESI (-)	11.87	182.1135, 68.9956	12%	189-1662
Perfluorobutanesulfonic acid	[1]	ESI (-)	15.11	168.9899, 68.9967	71%	8-870
Perfluorooctanesulfonic acid	[1]	ESI (-)	16.32	368.9776, 168.9889, 68.9968	65%	12-7247

[1] Database created from Wode et al. ²² ; [2] Database build within the PROMOTE consortium ^{27,28}

Caption to figures:

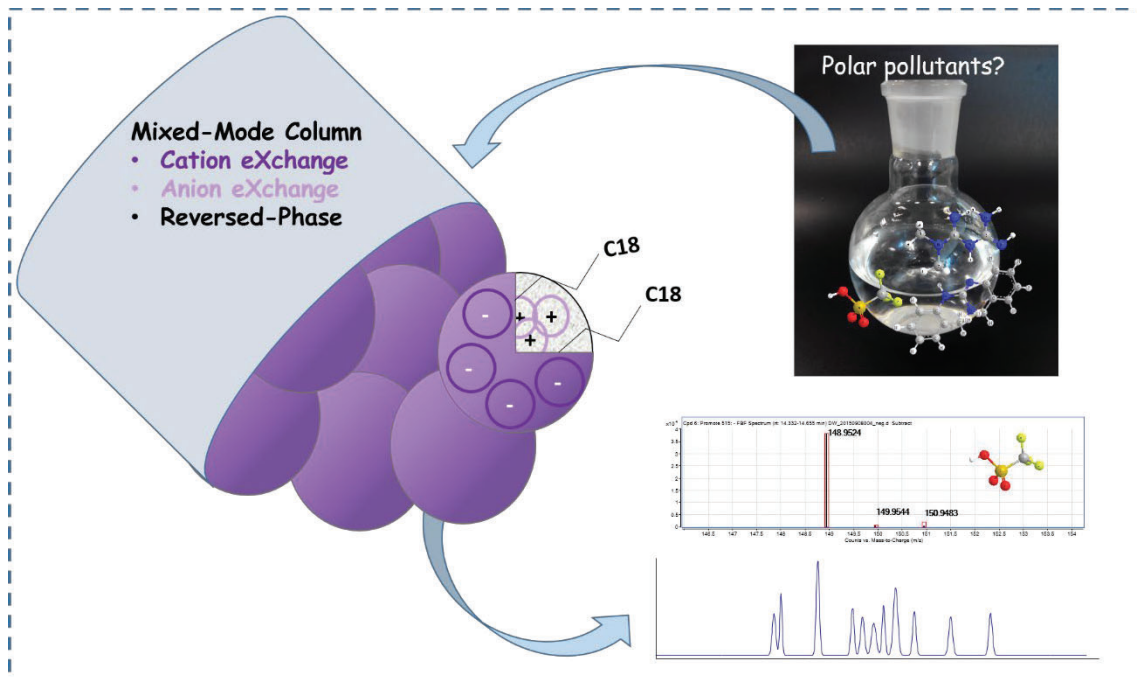
Figure 1: Workflow scheme of the suspect screening method and number of chemicals tentatively identified after each subsequent step

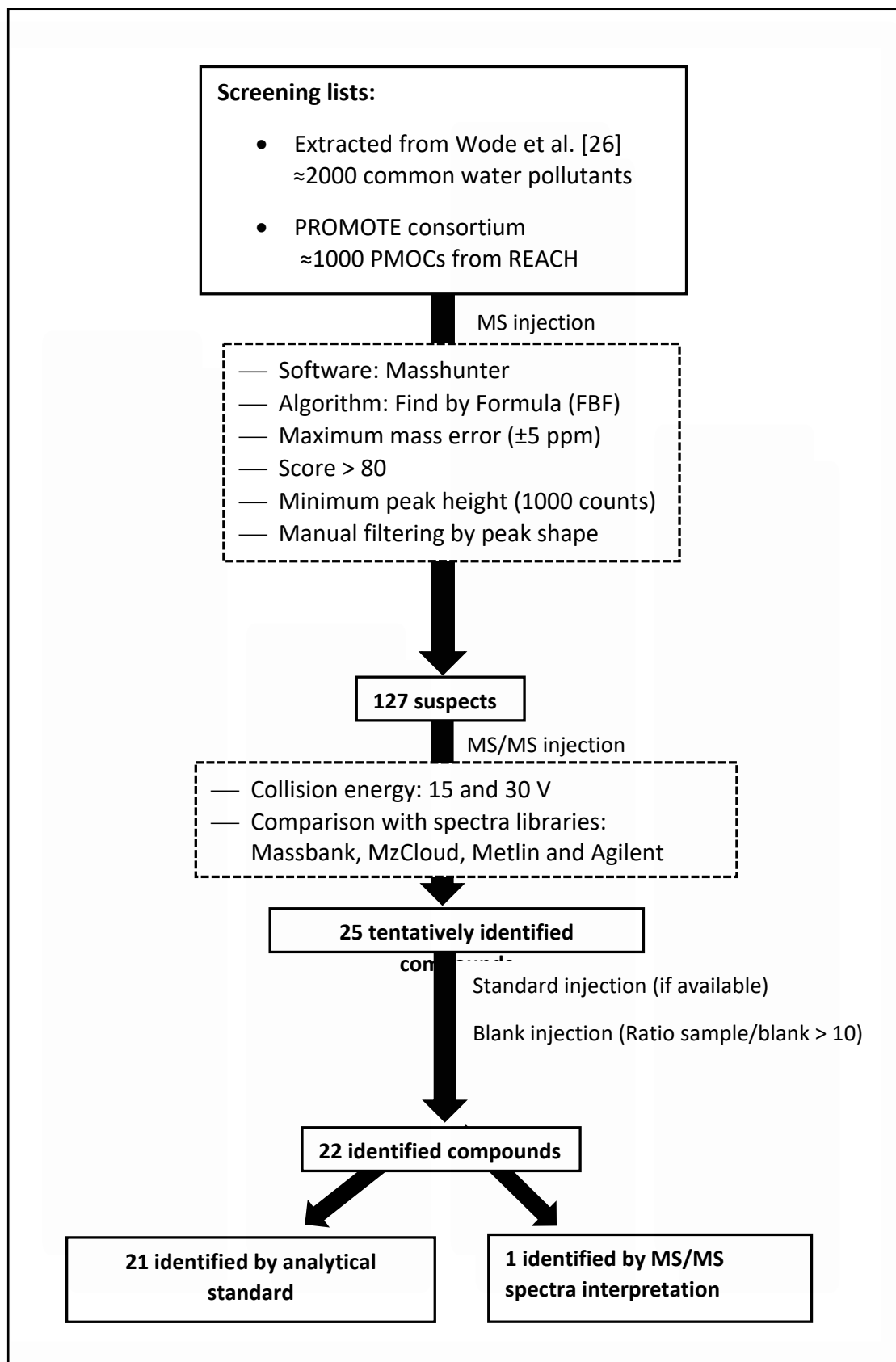
Figure 2: Examples of retention behavior of model compounds in MMLC according to their chemical class

Figure 3: Differences in retention factors of MMLC Vs. RPLC. For those compounds with retention factors < 1 this is indicated with "a" when occurred in RPLC, "b" when occurred in MMLC and "c" when occurred in both RPLC and MMLC.

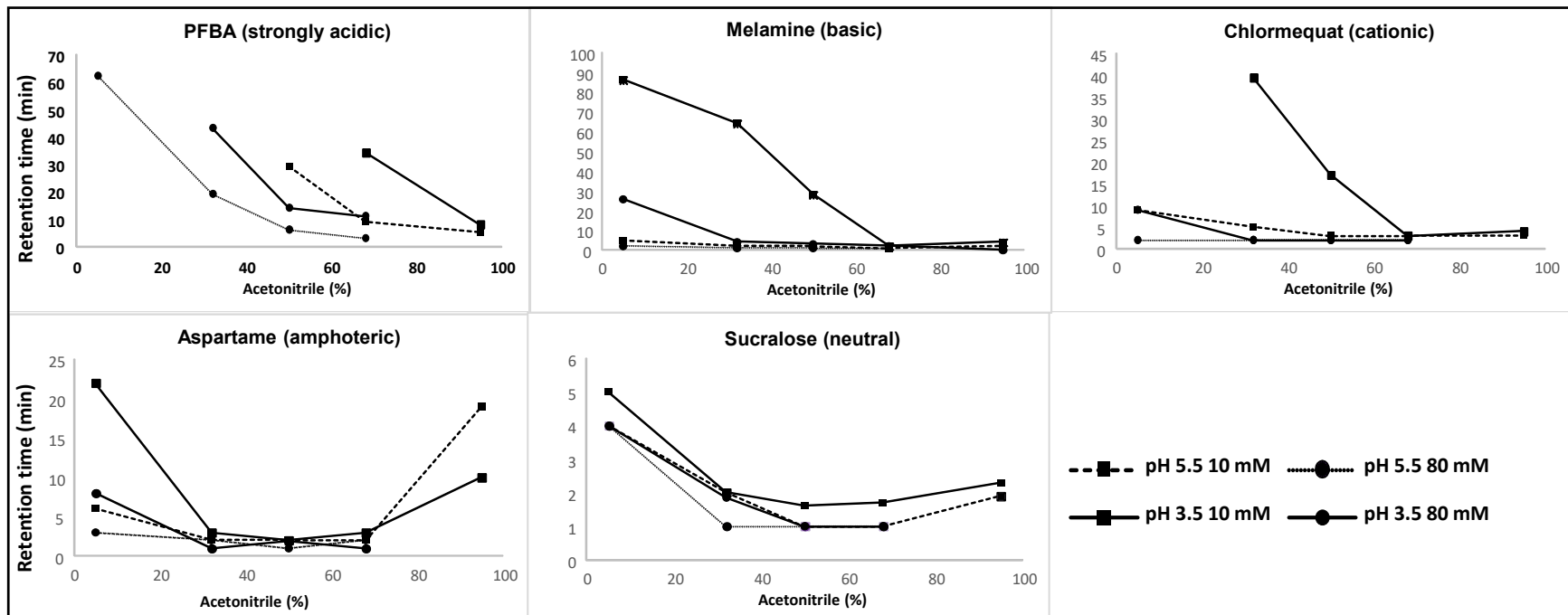
Figure 4: Identification of 1,3-di-o-tolylguanidine in a real drinking water sample.

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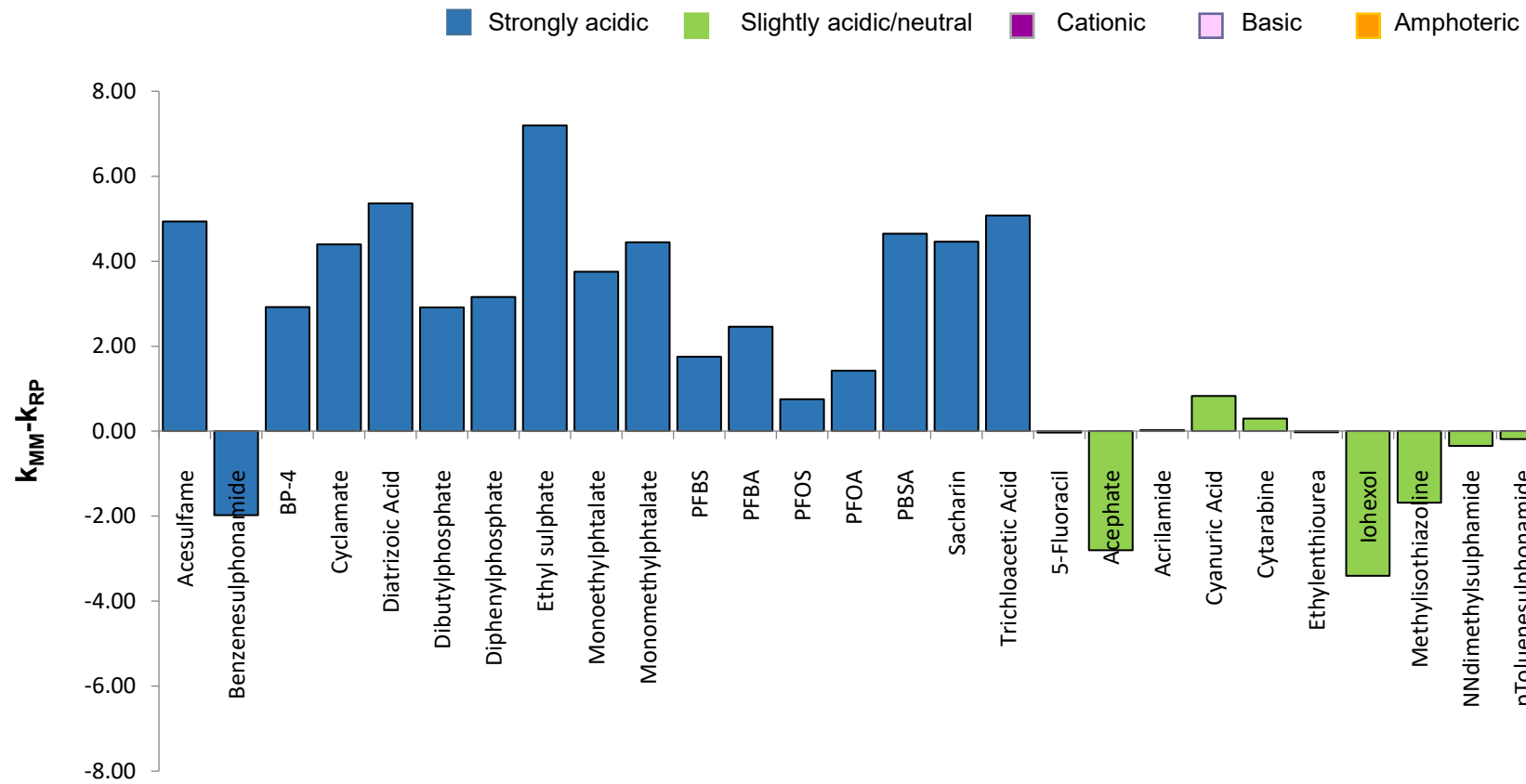




Montes et al. Fig. 1



Montes et al. Fig. 2



Montes et al. Fig. 3