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Analysis of free amino acids in natural waters by liquid chromatography-tandem mass spectrometry

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Abstract

This paper reports a new analytical method for the analysis of 18 amino acids in natural waters using solid-phase extraction (SPE) followed by liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) operated in multiple reaction monitoring mode. Two different preconcentration methods, solid-phase extraction and concentration under reduced pressure, were tested in development of this method. Although concentration under reduced pressure provided better recoveries and method limits of detection for amino acids in ultrapure water, SPE was a more suitable extraction method for real samples due to the lower matrix effects for this method. Even though the strong cation exchange resin used in SPE method introduced exogenous matrix interferences into the sample extracts (inorganic

26 salt originating from the acid-base reaction during the elution step), the SPE method still
27 incorporates a broad sample clean-up and minimised endogenous matrix effects by reducing
28 interferences originating from real water samples. The method limits of quantification
29 (MLQ) for the SPE LC-MS/MS method in ultrapure water ranged from 0.1 to 100 µg L^{-1} as
30 N for the different amino acids. The MLQs of the early eluting amino acids were limited by
31 the presence of matrix interfering species, such as inorganic salts in natural water samples.
32 The SPE LC-MS/MS method was successfully applied to the analysis of amino acids in 3
33 different drinking water source waters: the average total free amino acid content in these
34 waters was found to be 19 µg L^{-1} as N, while among the 18 amino acids analysed, the most
35 abundant amino acids were found to be tyrosine, leucine and isoleucine.

36

37 **1. Introduction**

38 Total organic carbon (TOC) or dissolved organic carbon (DOC) is commonly used to
39 measure the amount of organic material in drinking water source waters and to indicate
40 the concentration of disinfection by-product (DBP) precursors. Dissolved organic
41 nitrogen (DON) is a subset of DOC which includes any nitrogen-containing compounds
42 present in water [1]. The concentration of DON in surface waters, such as seawater,
43 lakes, rivers, is reported to typically range from 0.1 to 10 mg L^{-1} as N, which is
44 approximately 0.5 - 10 % of the DOC content [2]. The major sources of organic nitrogen
45 in surface waters are algal breakdown products, agricultural runoff, urban runoff [2,3]
46 and wastewater input. Examples of nitrogen-containing functional groups within DON
47 include amines, amides, nitriles and amino acids [2].

48

49 Amino acids are reported to be major constituents of DON, contributing up to 75 % of
50 DON in surface waters [4]. The concentrations of total amino acids in seawaters,

51 groundwaters and lakes were reported to range from 20 to 6000 μg L^{-1} [5], while the
52 concentrations of free amino acids ranged from 1 to 80 μg L^{-1} as N [5-7]. Glycine, alanine
53 and serine have often been reported to be detected in the highest concentrations [6,7]. The
54 structure and physical properties [8,9] of 20 of the 22 proteinogenic amino acids are
55 presented in Table 1; selenocysteine and pyrrolysine were not included in this study as they
56 were not commercially available. Amino acids have been reported to be precursors for
57 several classes of N-DBPs, including halonitriles [10] and cyanogen halides [11], as well as
58 some odorous DBPs, such as *N*-chlorophenylacetaldimine [12]. The concentration of amino
59 acids in natural water samples can be related to algal blooms and can also affect the level of
60 other natural organic matter in the water [13]. Amino acids are also an important source of
61 carbon for marine bacteria [14]. However, little information is reportedly available on amino
62 acids in various natural waters partially due to the relatively low concentration of amino acids
63 in the environment and difficulties with analytical methods [6]. In addition, when
64 chlorinated, amino acids demonstrate a breakpoint curve phenomenon similar to ammonia,
65 resulting in a higher chlorine demand for the distributed waters [15]. If inorganic chloramine
66 is used as the disinfectant, the presence of amino acids introduces a risk of overestimation of
67 disinfection capabilities [16]. Therefore, knowledge of the occurrence of amino acids in
68 source waters is important in understanding the formation and occurrence of N-DBPs,
69 understanding the impact of algal blooms on water treatment and also to ensure that sufficient
70 disinfectant is added during water treatment.
71
72 Given their polarity, reversed-phase liquid chromatography is commonly used for the
73 separation of amino acids, followed by spectroscopic (UV-visible or fluorescence)
74 detection with pre- or post-column derivatisation [17,18], as amino acids are generally
75 neither chromophores nor fluorophores [19]. However, derivatisation may result in

76 inconsistent results caused by varying stability of the derivatives and incomplete
77 derivatisation [19]. In addition, spectroscopic detection may lack the selectivity and
78 sensitivity required for trace analysis, which is essential for the analysis of amino acids in
79 natural waters. Mass spectrometry (MS) is more selective than spectroscopic techniques,
80 as spectroscopic techniques can only differentiate between compounds by their retention
81 time, while MS is also able to differentiate between compounds by their unique isotopic
82 mass and fragmentation pattern. The separation and detection of 20 free amino acids in
83 ultrapure water by liquid chromatography-electrospray ionisation-mass spectrometry
84 (LC-ESI-MS) was first reported by Chaimbault *et al.* [20]. The same research group later
85 also introduced a method using tandem mass spectrometry for the analysis of free
86 amino acids in ultrapure water [21]. Since then, amino acids have been analysed
87 by LC-MS in a range of applications involving a variety of matrices, including foods
88 [22,23] and biological samples [24,25]. However, there are no reports to date on the
89 application of LC-MS for the analysis of amino acids in natural waters, particularly
90 waters containing natural organic matter.
91
92 As the concentrations of amino acids in natural waters have been reported to be in the
93 microgram per litre range [5-7], a preconcentration method is needed for analysis of amino
94 acids in natural waters to concentrate the analytes and, if possible, to also remove matrix
95 interferences before analysis. Concentration under reduced pressure is one of the most
96 common techniques used for concentration of non-volatile analytes in an aqueous matrix,
97 however it is a time consuming process due to the low volatility of water. Solid-phase
98 extraction (SPE) is a preconcentration technique known to provide sufficient sample
99 concentration for sub-nanogram per litre analysis in environmental samples [26]. In addition,
100 SPE is also able to remove some matrix species [26] and isolate the analytes from the sample.

101 However, SPE is costly, can be time consuming and often suffers from low recoveries due to
102 loss of analytes during the loading or the washing step [27]. In addition, the likely
103 interferences in the water matrix are often similar to the analytes in terms of polarity and
104 retention [28] and so may not be separated during the SPE stage. Concentration under reduced
105 pressure has been reported to be used for concentration of amino acids in natural waters [6,7].
106 However, there have been no reports to date of the use of SPE for the extraction and isolation
107 of amino acids from natural waters, even though SPE has previously been reported for the
108 extraction of amino acids from various matrices, including plant roots [29], tea leaves [30]
109 and human plasma [24]. Concentration under reduced pressure and SPE were chosen to be
110 trialled for the preconcentration and isolation of amino acids from natural waters in the
111 current study.
112
113 In this study, a novel method for the analysis of amino acids in natural waters was
114 developed using liquid chromatography-electrospray ionisation-tandem mass
115 spectrometry (LC-ESI-MS/MS) with solid-phase extraction (SPE) pre-treatment. The
116 method was successfully developed and validated for the analysis of 18 out of 20
117 proteinogenic amino acids in natural waters. The suitability of SPE for analyte extraction
118 of amino acids and matrix removal for natural waters was also compared to the more
119 traditional approach of concentrating samples under reduced pressure.
120
121 **2. Experimental**

122 *2.1 Sampling and sample pre-treatment*

123 Grab samples were collected from a river in South Perth, Western Australia (River water),
124 from a tap located in the Curtin Water Quality Research Centre laboratory (Tap water)
125 and a groundwater sample from a local groundwater bore (Groundwater). Surface water

126 samples (Surface water A-C) were collected from the raw water inlets of various
127 drinking water treatment plants in Western Australia (basic characteristic
128 presented in Table S1). All water samples were collected in amber glass bottles,
129 previously annealed at 550 °C overnight and rinsed with the sample several times prior to
130 sample collection. Samples were kept cold with ice packs during transport. On arrival at
131 the laboratory, all natural water samples were filtered through 0.45 μm polyethersulfone
132 membrane filters (Pall Life Science, Michigan, USA) and stored at 4 °C until extraction
133 to prevent analyte degradation.
134
135 *2.2 Analytical standards and chemicals*
136 The amino acids, alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glycine,
137 glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline,
138 serine, threonine, tryptophan, tyrosine and valine, were purchased from Sigma Aldrich (New
139 South Wales, Australia). The purity of all analytical standard compounds was ≥97 %. The
140 surrogate standards, [^{2}H3] alanine (alanine-d_3), [^{2}H3] leucine (leucine-d_3), [^{2}H3] glutamic
141 were purchased from acid (glutamic-d_3 acid), were purchased from CDN Isotopes (Quebec,
142 Canada, distributed by SciVac, Hornsby, Australia); [^{2}H2] glycine (glycine-d_2) and [^{2}H5]
143 phenyl [^{2}H3] alanine (phenyl-d_5-alanine-d_3) were purchased from Sigma Aldrich (New
144 South Wales, Australia). Methanol (MeOH) and acetonitrile (ACN) (ChromHR grade) were
145 purchased from Mallinckrodt Baker (New Jersey, USA). Formic acid (purity 99%),
146 concentrated hydrochloric acid (32 %, HCl) and ammonium solution (28 % ammonium) were
147 purchased from Ajax FineChem (New South Wales, Australia). Ultrapure water (H_2O) was
148 purified using an ion exchange system (IBIS Technology, Perth, Australia), followed by an
149 Elga Purelab Ultra system with a 0.2 μm filter (Elga, High Wycombe, UK). Single standard
150 stock solutions (1000 ng μL^{-1}) of the 20 amino acids and mixed working solutions of all 20

151 amino acids (100 ng μL^{-1} and 1 ng μL^{-1}) were prepared using 30:70 (*v:v*) MeOH:H_2O
152 solvent. Individual surrogate standard stock solutions (1000 ng μL^{-1}) and a mixed surrogate
153 standard working solution (100 ng μL^{-1}) were prepared in 30:70 (*v:v*) MeOH:H_2O. All
154 solutions were kept at -13 °C to avoid degradation.
155
156 *2.3 Solid-phase extraction preconcentration and isolation procedure*
157 Two types of reversed-phase stationary phases (Oasis HLB from Waters, Mildford, USA;
158 Strata-E from Phenomenex, New South Wales, Australia) and one type of strong cation
159 exchange stationary phase (Strata-X-C from Phenomenex) solid-phase extraction (SPE)
160 cartridges were trialled for analytes preconcentration and isolation. All cartridges had 500
161 mg of resin and a 6 mL bed volume. Strata-X-C cartridges were selected for the
162 preconcentration and isolation of amino acids in natural waters as they provided the highest
163 recoveries and precision among the 3 types of SPE cartridge trialled. The pH of the water
164 samples was adjusted to 1.3 using concentrated HCl solution before loading the samples
165 onto the SPE cartridges. An automated Aspec XLi extractor (Gilson, Middleton, USA) was
166 used for the conditioning, washing and elution of the cartridges, as described in Table 2.
167 After cartridge conditioning, samples were loaded onto the SPE cartridges using two 8-
168 channel off-line peristaltic pumps (Gilson, Middleton, USA) at a flow rate of 2 mL min^{-1}.
169 The cartridges were dried under vacuum of 20 mmHg for 5 min to remove excess
170 moisture. Analytes were then eluted into 12 mL glass test tubes in the Aspec XLi
171 collection rack. Analytes were eluted with a delay of 1 min between each aliquot of solvent
172 dispensed to ensure that the stationary phase was efficiently soaked with the eluent. The
173 eluent (11.5 mL) from each sample was evaporated to dryness under a gentle stream of
174 nitrogen using a dry block heater fitted with nitrogen blowdown (Ratek 30D, Boronia,
175 Australia), set at 40 °C. The dried samples were re-dissolved in 500 μL of 30:70 (*v:v*)

176 MeOH:H_2O. Sample extracts were then transferred via pipette into 2 mL screw cap amber
177 glass vials (Agilent, USA) and stored in a freezer at -13 °C until analysis.
178
179 *2.4 Concentration of amino acids under reduced pressure*
180 The concentration under reduced pressure procedure was adapted from the method by Chinn
181 and Barrett [6] with some modifications. Each sample (500 mL) was placed into a round-
182 bottom flask and concentrated to approximately 10 mL using rotary evaporation (Heidolph
183 Instrument, Schwabach, Germany) at 60 °C under 9 mbar of vacuum. The reduced pressure
184 allowed faster concentration time (~ 2 h per sample) and minimised the heating of the sample
185 which could have resulted in some thermal degradation of amino acids [31]. The concentrate
186 (~10 mL) was then evaporated to dryness using a dry block heater fitted with nitrogen
187 blowdown, set at 40 °C and under a gentle stream of nitrogen. The samples were then
188 redissolved using 500 µL of 30:70 (*v:v*) MeOH:H_2O and transferred via pipette to a 2 mL
189 screw cap amber glass vial and stored in a freezer at -13 °C until analysis.
190
191 *2.5 Separation and detection of amino acids by LC-MS/MS*
192 Unless otherwise stated, all liquid chromatography and tandem mass spectrometry conditions
193 adopted in this work were the same as we have previously reported in Swann *et al*. [25].
194 Briefly, chromatographic separation was achieved using an Agilent 110 HPLC system (Palo
195 Alto, CA, USA) and the amino acids were separated using a Gemini C18 column from
196 Phenomenex® at a flow rate of 150 µL min^{-1}. The amino acids were detected using a Triple
197 Quadrupole Mass Spectrometer (Micromass Quattro, Manchester, UK) fitted with an
198 electrospray ionisation (ESI) interface operated in positive ion mode.
199

200 Retention time was the main parameter used to identify analytes, and multiple reaction
201 monitoring (MRM) ratio was used as a second confirmation for analytes where two stable
202 transitions were monitored. In order to increase the sensitivity of the analytical assay, the
203 MRM transitions were grouped into three separate windows based on their retention times.
204 Moreover, given that 14 analytes and three surrogate standards eluted in the first 10 min of the
205 chromatographic run, in order to optimise the sensitivity, as well as to increase the number of
206 points collected across each chromatographic peak by the MS, only one transition (single
207 reaction monitoring, SRM) was monitored for each analyte in the first window.

208

209 Analytes were quantified using the ratio of the analyte peak area to the surrogate standard
210 (Table S3) peak area and using an external calibration curve obtained by diluting working
211 standards with MeOH:H_2O (*v:v*) 30:70. Deuterated amino acids were used as surrogate
212 standards and the corresponding surrogate standard with its analytes are listed in Table S2.
213 Data processing was carried out using MassLynx NT4.0 software, while data
214 quantification was performed using QuanLynx 4.0.

215

216 **3. Results and discussion**

217 We have previously developed a method for the analysis of amino acids and amines in
218 mammalian decomposition fluids by LC-MS/MS [25]. In this method, direct injection of
219 samples was used. However, since the concentrations of amino acids in natural waters were
220 expected to be much lower than those in mammalian decomposition fluids, direct injection was
221 not appropriate for natural water samples. Therefore, a preconcentration method was required.
222 The matrix characteristics for natural waters, as compared to mammalian decomposition fluids,
223 must also be studied. In addition, only 15 amino acids of interest were analysed in our earlier

224 method, thus there was a need to include a more complete suite of amino acids in the current
225 method.
226
227 *3.1 Optimisation of tandem mass spectrometry conditions for additional analytes*
228 Infusion experiments were conducted to determine the MRM transitions of the amino acids
229 not analysed previously [25], i.e., alanine, aspartic acid, cysteine, glycine, glutamine and the
230 deuterated surrogates, alanine-d_3, leucine-d_3, glutamic-d_3 acid, glycine-d_2 and phenyl-d_5-
231 alanine-d_3, so that they could be incorporated into the present analytical method. The parent
232 ion to product ion transition data for the remaining amino acids were as obtained previously
233 [25]. A significant improvement on the previous method was the introduction of deuterated
234 standards as surrogate standards to account for matrix effects and recovery, and also for
235 quantification. One deuterated standard was assigned to multiple amino acid species as not all
236 homologue deuterated standards for the amino acids were commercially available. The parent
237 ion to product ion transitions used for SRM or MRM were selected based on their intensities
238 in the MS/MS spectra (Table S2).
239
240 *3.2 Instrumental linearity, detection limits and peak identification criteria*
241 Instrument performance data is reported in Table S3. Instrumental linearity and instrumental
242 detection limits were determined from analysis of 13 calibration standards ranging from
243 0.002 ng μL^{-1} to 20 ng μL^{-1}. Calibration curves showed good linearity (R^2>0.990) up to
244 maximum concentrations that ranged between 5 to 20 ng μL^{-1} for all analytes. Instrumental
245 detection limits, estimated at signal-to-noise (S/N) ratios of 3, ranged from 1 to 190 pg on
246 column (0.2 - 38 pg μL^{-1}), which is consistent with our previous study [25]. The variabilities of
247 retention time and MRM ratio were calculated from repeat injections (n=10) of a solution
248 containing 1 ng μL^{-1} of each amino acid. In general, the standard deviation (SD) of the

249 retention time (t_R) was less than 20 s, indicating repeatable chromatography. However,

250 leucine, isoleucine, tyrosine and phenylalanine showed higher SDs (ranging from 35s to 65

251 s). The reason for this variability of t_R is not known, but it was also observed in our

252 previous work [25]. The relative standard deviation (RSD%) of the MRM ratios (peak area

253 ratio between the two MRM transitions) was generally less than 5 %, indicating repeatable

254 fragmentation of parent ions in the collision cell.

255

256 *3.3 Optimisation of the solid-phase extraction procedure*

257 *3.3.1 Selection of the type of solid-phase extraction cartridge*

258 Two types of reversed-phase, polymeric (Oasis HLB, Waters®) and octadecyl silica

259 (Strata C18-E, Phenomenex®), and one type of strong cation exchange phase (Strata-X-C,

260 Phenomenex®), solid-phase extraction (SPE) cartridges were trialled to determine the

261 most suitable stationary phase for the extraction and concentration of amino acids from

262 aqueous samples. As a preliminary comparison, one laboratory ultrapure water blank

263 (1000 ng of deuterated surrogate standards in 500 mL of ultrapure water), two low

264 concentration standards (250 ng amino acid standards + 1000 ng of deuterated standards

265 in 500 mL of ultrapure water), and two high concentration standards (1000 ng amino

266 acid standards + 1000 ng of deuterated standards in 500 mL of ultrapure water) were

267 separately loaded onto the three types of SPE cartridges without pH modification, as

268 advised from the SPE manufacturer, and the analytes were extracted using the

269 procedures outlined in the supporting information (Table S4). Without pH adjustment,

270 the pH of the sample was approximately 6.5, and thus most of the amino acids have no

271 significant net charge. Neither the deuterated surrogate standards nor most of the amino

272 acid standards (16 out of 20) were recovered from the reversed-phase cartridges at either

273 the low or high concentrations tested (data not shown). Leucine-d_3 and phenyl-d_5-

274 alanine-d_3 and 19 amino acids were recovered (average recovery = 23 %) when using
275 the Strata-X-C cartridge. These preliminary results suggested that strong cation
276 exchange cartridges were more suitable than reversed phase cartridges for the extraction
277 of amino acids, in agreement with the work previously reported by Spanik *et al.* [32].
278 The poor recoveries of analytes from the reversed-phase cartridges can be attributed to the
279 fact that amino acids are polar at neutral pH, characteristics that reduce the retention of
280 analytes under reversed-phase conditions, with the likely outcome that the analytes
281 remained in the aqueous phase and were not retained on the SPE cartridges. The polar
282 nature of the amino acids makes them more amenable to retention on the strong cation
283 exchange cartridges. In addition, strong cation exchange packing material is polymeric
284 and therefore designed to provide additional retention through reversed-phase
285 mechanisms (e.g. π-π bonding, hydrogen bonding and hydrophobic interactions). The
286 mixed-mode retention properties of the ion exchange packing material explained the
287 improved retention of the amino acids when compared to the reversed-phase cartridges,
288 where only π-π bonding, hydrogen bonding and hydrophobic interactions between the
289 resin and the amino acids were possible. However, depending on the structure and
290 isoelectric point (pI) of the amino acids, some of them (17 out of 20) are overall
291 negatively charged at neutral pH, which results in the quite poor recoveries observed on
292 the strong cation exchange resin.

293

294 It was noted that some of the amino acids tested were not recovered at all by the sample
295 preconcentration methods (recovery data presented in Section 3.5). Arginine was not
296 recovered by any of the cartridges trialled for the SPE method, while cysteine was not
297 recovered by either the SPE or the concentration under reduced pressure method. As both
298 arginine and cysteine are polar amino acids, they were not expected to be retained on the

299 reversed-phase SPE cartridge and therefore they were not expected to be recovered by this
300 method. It was expected that arginine would be well-retained by the cation exchange phase
301 because its pI value of 10.76 indicates that it will be positively charged at neutral pH, but,
302 since the pH of the eluting solution was around pH 10, it is likely that the arginine was
303 retained on the cartridge in a cationic form and was not eluted, resulting in minimal recovery.
304 The fact that cysteine was not recovered by the concentration under reduced pressure method
305 suggests that cysteine was not stable in the concentration step or that it has a limit of
306 detection higher than the working concentration range. Higher concentrations of cysteine
307 were not tested as the concentration of cysteine in natural waters is unlikely to be more than 2
308 mg L^{-1}.
309
310 The strong cation exchanger SPE cartridge provided the best recoveries among all the 3 types
311 of SPE cartridges trialled and a lower pH will improve the recoveries of amino acids when
312 using strong cation exchanger SPE by ensuring that all amino acids are in their cationic
313 (positively charged) forms.
314
315
316 *3.3.2 Optimisation of pH for solid-phase extraction on the strong cation exchange resin*
317 Given the importance of charge for the retention of analytes on the strong cation
318 exchange resin, a series of experiments were undertaken to investigate the effect of pH on
319 the on the recovery of amino acids. In these experiments, the pH of the samples of
320 amino acids in water was adjusted to pH 2 using concentrated HCl prior to application
321 strong cation exchange SPE cartridge. The results showed that reducing the sample pH
322 to 2 significantly improved the recovery of the amino acids (Table 3). For example, the
323 recovery of proline increased from less than 1 % at neutral pH to 80 % at pH 2. This

324 finding is in agreement with Spanik *et al.* [32], and also expected since the lowest pI
325 value of the amino acids studied was 2.77 (Table 1), and most of the amino acids have
326 pI less than 6 (Table 1), such that at pH 2, all amino acids should be predominantly in
327 their positively charged form. Lowering the pH has the effect of protonating the
328 amino group while the carboxylic acid group undissociated, resulting in an overall
329 positive charge on the amino acids. This promotes the interaction between the
330 negatively charged resin and the positively charged amino acids, improving retention
331 and recoveries.
332
333 The use of buffer has been reported to increase the recoveries of amino acids where 30
334 mM phosphate buffer (pH 2.7-3.3) was found to be the most effective for the SPE
335 extraction of amino acids [23]. Therefore, the effect of a 30 mM phosphate buffer on the
336 recoveries of the amino acids was investigated at both 2.5 and pH 6.5. The pH of the
337 acidic buffer should be made near to the unbuffered acidic solution of pH 2 to minimise
338 differences in recovery due to pH differences, however, an excessive volume of
339 concentrated HCl solution would be required to lower the pH of the phosphate buffer to
340 below pH 2.5. Amino acid recoveries were generally lower in buffered samples than in
341 non-buffered samples (Table 3). The lower recoveries may be explained by competition
342 between buffer cations and the amino acids on the SPE cartridge or by the slightly
343 higher pH of the buffered samples under acidic conditions. The concentration of
344 potassium ions was calculated to be 1000 times higher than that of the amino acids in
345 the solutions tested. The effect of this competition could be reduced by using a lower
346 ionic strength buffer solution or an acidified non-buffered solution. An acidified non-
347 buffered solution was chosen for the final method to reduce the chance of cation
348 competition from the buffer solution.

349

350 While reducing pH had the effect of improving the recoveries, a loading pH of 2.5
351 (buffered solution) was not sufficiently low to protonate some of the amino acids. Some
352 of the amino acids are overall negatively charged at pH 2.5, since they all have acidic pKa
353 values below 2.5 (Table 1). Therefore the carboxylic acid groups in these amino acids
354 would still be dissociated at pH 2.5, and the retention mechanism for these amino acids
355 will be based on reversed-phase interaction rather than cation exchange. In order to
356 further improve the recoveries of the amino acids, the loading pH was further reduced to
357 1.3, below the lowest acidic pKa of 1.70 for all of these amino acids (Table 1), using
358 concentrated HCl solution. This pH guaranteed all amino acids to be positively charged,
359 and thus better interaction between the amino acids and the cation exchange resin could
360 be achieved. The reduction in pH from 2.5 to 1.3 significantly improved the recoveries
361 and precision of analysis of the amino acids (Tables 3 and 4). The final procedure for the
362 SPE extraction of amino acids to optimise their recoveries is shown in Table 2.

363

364 *3.3.3 Optimisation of solid-phase extraction cartridge washing conditions*

365 Despite modification to the pH of the sample in the loading step to optimise the SPE
366 cation exchange process, recoveries of many of the amino acids were still very low
367 (Table 3). In order to determine whether the analytes were not retained on the cartridges
368 during loading, or whether they were being eluted in the washing step (3 mL of 0.1 mol L^{-}
369 ^{1}HCl in H_2O followed by 3 mL of 0.1 mol L^{-1} HCl in MeOH), the eluent from the
370 washing step was collected, concentrated to dryness, redissolved in MeOH:H_2O (*v:v*)
371 (30:70) mixture, and analysed by LC-MS/MS. Serine, alanine, asparagine, glutamine,
372 aspartic acid and glutamic acid were all detected in the extract from the washing step
373 eluent (data not shown). According to the manufacturer [33], the eluent from the

374 washing step should contain acidic and polar compounds previously retained on the
375 resin, and this corresponds to the nature of the amino acids detected. Detection of these
376 acidic and polar amino acids in the washing step eluent suggested that retention of
377 amino acids was by both reversed-phase and cationic interaction. Thus, the use of
378 50 % methanol in the washing step eluted the compounds. In order to determine if
379 the removal of the washing step would prevent the loss of analytes, an experiment
380 to analyse the amino acids was conducted with the washing step removed. However,
381 without the washing step, high ion suppression was observed.
382 In order to reduce the matrix effects but avoid loss of analytes, a series of experiments were
383 conducted. A gentler washing step (10 mL of ultrapure water followed by 5 mL of 2.5 %
384 MeOH in ultrapure water) was tested and was found to reduce ion suppression. Four different
385 organic washing solvent systems, 2.5 and 4.5 % of isopropyl alcohol or MeOH in ultrapure
386 water, were also trialled to further optimise the washing step. Cartridges were loaded with
387 ultrapure water containing 2 μg L^{-1} of amino acids with the pH of the solution adjusted to 1.3
388 using concentrated HCl solution. The cartridges were then washed with 10 mL of ultrapure
389 water and one of the different organic washing solvent systems. The eluent from each organic
390 solvent wash was evaporated to dryness and redissolved using 500 μL of 30:70 (*v:v*)
391 MeOH:H_2O. Each solution was then analysed for amino acids. No amino acids were detected
392 in the four organic solvent systems investigated (Table S5), indicating that the amino acids
393 were lost during the washing by the first 10 mL of ultrapure water, resulting in low recoveries
394 for the amino acids. In the procedure for strong cation exchange, a basic solvent is used to
395 elute the retained analytes and it is possible that, ultrapure water, with a pH of 7.3, may also
396 have been able to elute the amino acids that were not strongly retained by the strong cation
397 exchange resin. It was also noted that the less polar organic solvent (isopropyl alcohol)
398 resulted in lower loss of analytes than the more polar organic solvent (MeOH) and a higher

399 percentage of organic solvent in the washing solvent system also reduced the loss of analytes
400 (Table S5). In order to reduce the loss of analytes due to the aqueous and/or the organic
401 washing solution, an acidic wash of 5 mL of 0.1 mol L^{-1} HCl in 4.5 % MeOH was tested. The
402 results showed a general increase in recovery compared to washing the cartridges at neutral
403 pH, especially in the first window where the losses of analytes were up to 100 % for some of
404 the amino acids when using a neutral washing step (Table S5). This indicated that the pH of
405 the washing step needed to be similar to that of the loading step to prevent the loss of
406 analytes.
407
408 Although it was found that the washing step resulted in significant loss of some analytes,
409 this step was required to reduce the matrix effect. A washing step using 5 mL 0.1 mol L^{-1}
410 HCl in 4.5 % MeOH was chosen as it resulted in the lowest loss of analytes.
411
412 *3.4 Matrix effects and choice of surrogate standards*
413 In LC-MS/MS, the signals for analytes can either be suppressed or enhanced by the matrix due
414 to the competition between analytes and the matrix for the primary ions produced in the LC-
415 MS/MS interface [34,35]. Ion suppression can result in the loss of sensitivity, accuracy and
416 precision; while ion enhancement can result in the loss of accuracy and precision [34,35].
417 Many methods have been suggested to account for the matrix effect [26,27,34], the use
418 of deuterated standards being one of them. Deuterated standards usually co-elute with the
419 homologue analytes and are subjected to almost identical matrix effects [26], therefore
420 deuterated standards represent the most effective way to account for matrix effects.
421 However, not all homologue deuterated standards for the amino acids were commercially
422 available and, to also minimise costs, a total of five deuterated standards were chosen for this

423 analytical method to correct potential matrix effects, with one to three deuterated standards
424 assigned to each monitoring window (Table S2).
425
426 *3.4.1 Matrix effects caused by real water samples*
427 In order to investigate the impact of matrix effects on both the SPE and concentration
428 under reduced pressure preconcentration methods, peak areas of standards added into
429 MeOH:H_2O water 30:70 (*v:v*) were compared to peak areas of standards added into a
430 surface water, a tap water and a groundwater at 2 µg L^{-1} after filtration. The
431 introduction of the real water sample matrices resulted in shifts in retention times of 30-
432 60 s and caused ion suppression for most analytes for both SPE and concentration
433 under reduced pressure preconcentration procedures. When concentration under
434 reduced pressure was used as the preconcentration method, the ion suppression for
435 most analytes was close to 100 % for each of the three water samples (Figure 1).
436 However, when the final SPE procedure was used as the preconcentration method, ion
437 suppression was lower than with the concentration under reduced pressure method
438 (Figure 1). The suppression of signals for analytes in the river water, especially those
439 analytes that eluted in the first 10 min, was expected since the river water was brackish,
440 containing up to 30 parts per thousand of inorganic salts.
441
442 The high signal suppression experienced by samples pre-concentrated using the
443 concentration under reduced pressure method results from the fact that this method only
444 removes volatile compounds, with non-volatile compounds like inorganic salts being
445 concentrated to the same extent as the analytes during the process. In addition, many of
446 the non-volatile compounds are likely to be polar/ionic in nature and therefore elute in
447 the first 10 min of the chromatogram, resulting in high interferences and high signal

448 suppression in this part of the chromatogram. SPE is a preconcentration and separation
449 technique which provides a higher level of sample clean-up. Therefore, the SPE method
450 was found to be more suitable as a preconcentration method for LC-MS/MS to reduce
451 matrix effects.
452
453 *3.4.2 Matrix effects caused by the SPE procedure*
454 While SPE gave better clean-up of the sample matrix, it appeared to also introduce
455 additional matrix that was not present in the samples from the concentration under
456 reduced pressure method. Histidine, glycine and serine could only be detected, but not
457 quantified, in SPE extracts, as these analytes co-eluted with an interference peak that
458 could not be resolved from the analyte peaks. This co-elution was only observed in
459 samples that were pre-concentrated using SPE, and not those pre-concentrated using the
460 concentration under reduced pressure method, suggesting that the interferences were
461 contributed by the SPE method, presumably from compounds leaching from the cation
462 exchange resin.
463
464 The impact of such an interference peak can be reduced by improving the separation of
465 the analytes from the interference peak or by increasing the selectivity of the detection
466 method. As previously mentioned, the disadvantage of SPE is that the matrix that is not
467 removed during clean-up is likely to have similar chromatographic properties to the
468 analytes. Therefore, it is unlikely that changing the mobile phase and/or the elution
469 gradient of the LC separation would significantly change the retention of the analytes
470 and their separation from the interference peak. For example, using a mobile phase
471 gradient with 90 % water at the beginning of the HPLC analysis is designed in part to
472 flush out inorganic salts. However, some amino acids are very polar and therefore have a

473 similar retention time to the inorganic salts. A number of additional modifications were
474 tested to improve the separation of the amino acids with the interference, including the
475 use of a cation exchange column. However, no significant improvement in the separation
476 of the amino acids and the interference was observed compared to our previously
477 published LC method [25].
478
479 A comparison of ion suppression caused by SPE sample and solvent blank (cartridges
480 subjected to the whole SPE procedure using ultrapure water as the sample) and SPE
481 solvent blank (cartridges subjected to only conditioning and elution, without the sample
482 loading step) showed that the SPE solvent blank could contribute up to 86 % of ion
483 suppression and the SPE sample and solvent blank could contribute up to 96 % ion
484 suppression (Figure 2). The ion suppression might be caused by the ammonium chloride
485 produced during the elution step where the basic solvent neutralised the acid from the
486 sample and/or damage of the resins of the cartridges from the low pH during the
487 conditioning and loading step. Even though the low pH had an adverse effect on the
488 analysis, it was not possible to extract the amino acids at a higher pH, as the low pH was
489 required to maintain the recovery and precision of the method (Section 3.3.2).
490
491 As ammonium chloride (5 mg mL^{-1}) is likely to be produced during the SPE extraction of
492 amino acids from water samples, an experiment was conducted to investigate the impact
493 of ammonium chloride on the analysis of amino acids. Ammonium chloride (6 mg) was
494 dissolved in 500 µL of MeOH:H_2O (*v:v*) 30:70 solvent, containing 2 ng μL^{-1} of amino
495 acid standards and surrogate standards.
496

497 The peak areas of the analytes in the sample with added ammonium chloride were, on
498 average, 30 % lower in the first window and were, on average, 10 % lower in the second
499 and third windows as compared to a standard solution without ammonium chloride.
500 When comparing the chromatograms (Figure 3), interference peaks similar to those found
501 from samples that have gone through SPE were observed in the sample with added
502 ammonium chloride. This suggested that ammonium chloride may have been formed
503 during the eluting step in the SPE procedure and indicated that the matrix effect would be
504 reduced if the ammonium chloride was removed or the formation was prevented.

505

506 Although an acidic washing step resulted in the formation of ammonium chloride,
507 which was an interfering species for analytes eluting at the same time as the ammonium
508 chloride (histidine, glycine and serine), an acidic wash in the SPE procedure was
509 required to ensure higher recoveries and precision for analysis of the other amino acids.
510 Therefore, the acidic washing step was utilised in the final SPE method.

511

512 The signal suppression/enhancement for the standards was similar to that of the
513 surrogate standards (Table S6), indicating that the surrogate standards chosen were
514 suitable for this application.

515

516 *3.5 Method validation*

517 The recovery and precision of the two methods using SPE and concentration under
518 reduced pressure preconcentration were determined using standard solutions of amino
519 acids prepared in ultrapure water (Tables 4 and 5). The recovery was expressed as the
520 percentage recovery relative to the surrogate standards, and the precision (repeatability)
521 was expressed as the relative standard deviation (RSD) of the measured concentrations of

522 amino acids after analysis. Results for recoveries and precision are presented as averages over
523 three concentrations (2, 5 and 10 μg L^{-1}), each analysed in triplicate (*n*=9).
524

525 The relative recoveries of amino acids using the SPE preconcentration method had a median
526 value of 105 % and the precision varied from 5% to 25 % (Table4). The recoveries of amino
527 acids using the concentration under reduced pressure preconcentration method had a median
528 value of 95 % and a precision comparable to the SPE method, varying from 5% to 35 %
529 (Table5). Little information on the analytical recoveries of free amino acids from natural
530 waters has been previously published, so comparison of the recoveries achieved in this
531 method to previously published methods is not possible.
532

533 As the samples were loaded on the SPE cartridge at low pH, the amide functional groups of
534 glutamine and asparagine would have been partially hydrolysed into the corresponding
535 carboxylic acid groups, forming glutamic acid and aspartic acid, respectively, resulting in
536 high relative recoveries of glutamic acid and aspartic acid in the SPE method (Table 4).
537

538 Cysteine was not detected in either preconcentration method and arginine was not detected in
539 the SPE method due to poor recoveries (Section 3.3.1). Histidine, glycine and serine were not
540 quantified due to interference from ammonium chloride formed during the SPE procedure
541 (Section 3.4.2)
542

543 The method validation was performed in ultrapure water; for analysis of amino acids in real
544 water samples, QA/QC was assured by standard addition of amino acids to selected real
545 water samples to ensure good recoveries and precision for each batch of samples processed.
546

547 Preconcentration of amino acids using the two methods, concentration under reduced
548 pressure and SPE, gave comparable recoveries and precision, indicating that both methods
549 could potentially be used for the preconcentration of amino acids in real water samples.
550
551 *3.6 Method limits of quantification*
552 For the two methods using SPE and concentration under reduced pressure, the method
553 limits of quantification (MLQ) were calculated for triplicate (n=3) analysis of amino acids
554 (2, 5 and 10 μg L^{-1}) in ultrapure water (Tables 4 and 5). Method limits of quantification were
555 determined as the concentrations equivalent to signal to noise (S/N) = 10 by manual S/N
556 calculation on unsmoothed chromatograms. The MLQ of the amino acids in the method
557 using SPE as the preconcentration method were 0.1-100 μg L^{-1} as N (median: 20 μg L^{-1} as
558 N) (Table 4), with the exception of arginine and cysteine which were not detected
559 (discussed in Section 3.4.1) and histidine, glycine and serine which were not quantified
560 (discussed in Section 3.4.2). The MLQ of most amino acids using the concentration under
561 reduced pressure preconcentration method in ultrapure water was 0.1-40 μg L^{-1} as N
562 (median: 1 μg L^{-1} as N) (Table 5), with the exception of cysteine which was not detected.
563 The MLQ of amino acids using the concentration under reduced pressure preconcentration
564 method was lower than the MLQ of amino acids using the SPE method due to the better
565 absolute recovery of amino acids when using concentration under reduced pressure. Both
566 analytical methods, therefore have the potential to be used for the analysis of amino acids in
567 natural waters, since free amino acids have previously been found to be present in natural
568 waters in the range of 1 - 80 μg L^{-1} as N [5-7]. For real water samples, QA/QC, including the
569 recoveries of amino acids, was assured by standard addition of amino acids to selected real
570 water samples for each batch of samples processed.
571

572 Solid phase extraction was chosen for the preconcentration of amino acids in the rest of this
573 study due to fact that SPE included a sample clean-up which reduced the ion suppression
574 caused by real water samples and thus was more suitable for use as the preconcentration
575 method for the detection of amino acids using mass spectrometry.

576

577 *3.7 Application of the SPE LC-MS/MS method to drinking water source waters*

578 The free amino acid concentrations of three different surface waters (Surface waters A-C)
579 were measured (Table 6) using the developed analytical method of SPE preconcentration
580 followed by LC-MS/MS, with six amino acids present above their MLQs and the total free
581 amino acid concentrations being 15, 16 and 26 μg L^{-1} as N for Surface Waters A, B and C,
582 respectively. The developed method therefore shows promise for the detection and
583 determination of amino acids in natural waters.

584

585 These concentrations are higher than total free amino acid concentrations measured in
586 previous studies on surface waters in the USA analysed by spectroscopic detection with
587 derivatisation, i.e., 7 μg L^{-1} as N [6] and 0.69 μg L^{-1} as N [7]. The three amino acids present
588 in highest concentrations in Surface Waters A-C were tyrosine, leucine and isoleucine,
589 however the three amino acids present in highest concentrations in the previous studies [6,7]
590 were alanine or histidine, serine and glycine.

591

592 Natural variation and/or analytical variation may account for the differences in measured
593 amino acid concentrations in natural waters. According to Chinn and Barrett [6], the
594 concentrations of amino acids in water bodies change over time, and a single analysis may
595 not capture all variations. The composition and concentration of natural organic matter, and
596 thus naturally occurring amino acids, were reported to be very specific for each natural water

597 source [36] and strongly depended on biological activity (algae bloom) and season. In terms
598 of analytical variation, the use of derivatisation followed by UV detection in previous studies
599 [6,7] may have resulted in lower concentrations of amino acids measured due to incomplete
600 derivatisation, compared to the current mass spectrometric detection method without
601 derivatisation. Another possible reason for the higher concentrations of amino acids detected
602 in the current study may be due to the beneficial use of surrogate standards which allowed for
603 correction from matrix effects and recoveries. In addition, 18 amino acids were analysed in
604 the current study, as compared to only 16 amino acids analysed in previous studies [6,7],
605 possibly resulting in differences in concentration and composition of total amino acids.
606 Histidine, serine and glycine were the most abundant amino acids in natural waters in
607 previous studies [6,7]; however, these amino acids were not quantified in this study, resulting
608 in differences in composition of total free amino acids in this study compared to previous
609 studies.
610

611 ## 4. Conclusions

612 A novel analytical method for the analysis of amino acids in natural waters, using SPE as the
613 extraction and preconcentration method followed by separation and detection using LC-
614 MS/MS, was developed and optimised. In the method, 18 out of the 20 amino acids tested
615 could be successfully analysed, however, histidine, glycine and serine could only be semi-
616 quantified due to exogenous matrix effects from the SPE cartridge. An alternative
617 preconcentration method using concentration under reduced pressure was tested and it
618 allowed for the analysis of 19 amino acids in ultrapure water. However, it is not suitable as a
619 preconcentration method for natural waters as it does not incorporate a sample clean-up step,
620 which could result in up to 100 % signal suppression for almost all amino acids. Although
621 preconcentration using concentration under reduced pressure provided better recoveries,

precision, and MLQs in ultrapure water, SPE was found to be a more suitable extraction and preconcentration method, as it incorporates a sample clean-up step, thus minimising matrix effects from real water samples. The developed analytical method using the SPE preconcentration step was successfully applied to the analysis of free amino acids in three surface water samples used as drinking water source waters. The average total free amino acid concentration in the natural water samples in this study was found to be 19 µg L^{-1} as N and the most abundant amino acids were found to be tyrosine, leucine and isoleucine. Since the concentrations of amino acids vary in different source waters, it is necessary to characterise the amino acids in each water source to be able to optimise treatment methods to minimise the formation of DBPs from amino acids and prevent overestimation of disinfection capacity.

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Table 1. Selected amino acids, their structure, molecular weight, isoelectric point (pI) and acid-base constants.

Name	Classification	Structure [9]	Molecular weight (Da)	pI [8]	pK_a [8]	pK_b [8]	pK_c [8]
Alanine	Non-polar		89.1	6.00	2.33	9.71	
Glycine	Non-polar		75.1	5.97	2.34	9.58	
Isoleucine	Non-polar		131.2	6.02	2.26	9.60	
Leucine	Non-polar		131.2	5.98	2.32	9.58	

Methionine	Non-polar		149.2	5.74	2.16	9.08	
Phenylalanine	Non-polar (aromatic)		165.2	5.48	2.18	9.09	
Proline	Non-polar		115.1	6.30	1.95	10.47	
Tryptophan	Non-polar (aromatic)		204.2	5.89	2.38	9.34	
Valine	Non-polar		117.2	5.96	2.27	9.52	
Asparagine	Polar		132.1	5.41	2.16	8.73	
Glutamine	Polar		146.2	5.65	2.18	9.00	
Serine	Polar		105.1	5.68	2.13	9.05	
Thereonine	Polar		119.1	5.60	2.20	8.96	
Arginine	Basic		174.2	10.76	2.03	9.00	12.1
Histidine	Basic		155.2	7.59	1.70	9.09	6.04
Lysine	Basic		146.2	9.74	2.15	9.16	10.7
Aspartic acid	Acidic		133.2	2.77	1.95	9.66	3.71
Cysteine	Acidic		121.2	5.07	1.91	10.28	8.14
Glutamic acid	Acidic		147.1	3.22	2.16	9.58	4.15
Tyrosine	Acidic (Aromatic)		181.2	5.66	2.24	9.04	10.1

642

643 **Table 2**. Optimised SPE conditions adopted for the recovery and clean-up of amino acids in water

Step	Solvent and dispensed volumes
Conditioning	4.5 mL of MeOH 9 mL of 0.1 mol L^{-1} HCl solution (pH 1.3) in ultrapure water
Loading	500 mL of sample (pH 1.3) at 2 mL min^{-1}
Washing	5 mL of 4.5% MeOH in 0.1 mol L^{-1} HCl solution

Elution	11.5 mL of 5% ammonium hydroxide in MeOH

644

645 646 **Table 3.** Accuracy (recovery %) and precision (RSD %) of recovery experiments of amino acids (2, 5 and 10 μg L^{-1}) conducted at acidic and neutral pH from buffered (30 mM phosphate) and unbuffered ultrapure water samples

Name	pH 2.5 (buffered)	pH 2 (unbuffered)	pH 6.5 (buffered)	pH 6.5 (unbuffered)
Lysine	10±15	80±20	<1	<1
Histidine	3±20	70±15	<1	<1
Arginine	<1	<1	<1	<1
Glycine-d_2	N.D.	25±50	N.D.	<1
Glycine	15±20	20±25	N.D.	<1
Serine	<1	10±25	N.D.	<1
Alanine-d_3	N.D.	30±10	N.D.	<1
Alanine	1±25	35±5	N.D.	<1
Asparagine	<1	10±1	N.D.	<1
Glutamine	1±30	5±10	N.D.	<1
Thereonine	1±25	15±10	1±30	<1
Glutamic – d_3 -acid	2±0	1±5	N.D.	<1
Aspartic acid	1±20	1±30	<1	<1
Cysteine	N.D.	N.D.	N.D.	N.D.
Glutamic acid	2±15	5±10	<1	<1
Proline	20±20	80±20	<1	<1
Valine	10±80	95±10	<1	<1
Methionine	10±40	10±5	N.D.	30±10
Isoleucine	30±60	80±10	<1	60±5
Leucine	50±45	40±1	<1	25±5
Leucine-d_3	45±20	50±5	N.D.	105±5
Tyrosine	60±10	165±10	<1	100±20
Phenyl-d_5-alanine-d_3	60±15	60±10	1±15	60±2
Phenylalanine	55±10	85±10	2±10	105±0
Tryptophan	60±10	80±10	15±5	55±5

647 N.D. - not detected.

648

649 650 651 **Table 4.** Accuracy, precision and method limit of quantification (MLQ) achieved in analysis of amino acids (2, 5 and 10 μg L^{-1}) in ultrapure water using the strong cation exchange SPE preconcentration method. Recoveries are presented as average over the 3 concentrations in triplicate (*n*=9) of all analyses.

Name	Recovery (%)	Precision (RSD %)	MLQ (μg L^{-1} as N)
Lysine	90	10	80
Arginine	N.D.	N.D.	N.D.
Histidine	N.Q.	N.Q.	N.Q.
Glycine	N.Q.	N.Q.	N.Q.
Serine	N.Q.	N.Q.	N.Q.
Asparagine	80	20	65
Alanine	175	5	100

Glutamine	80	25	90
Thereonine	135	20	70
Glutamic Acid	245	5	30
Aspartic Acid	280	5	20
Cysteine	N.D.	N.D.	N.D.
Proline	90	10	5
Valine	130	20	5
Methionine	80	15	55
Isoleucine	100	10	0.5
Leucine	110	5	0.5
Tyrosine	120	10	0.5
Phenylalanine	105	5	0.1
Tryptophan	85	10	0.5
Median	**105**	**10**	**20**

N.Q. - not quantified; N.D. - not detected

Table 5. Accuracy, precision and method limit of quantification (MLQ) achieved in analysis of amino acids (2, 5 and 10 µg L^{-1}) in ultrapure water using the concentration under reduced pressure preconcentration method. Recoveries are presented as average over the 3 concentrations in triplicate (n=9) of all analyses.

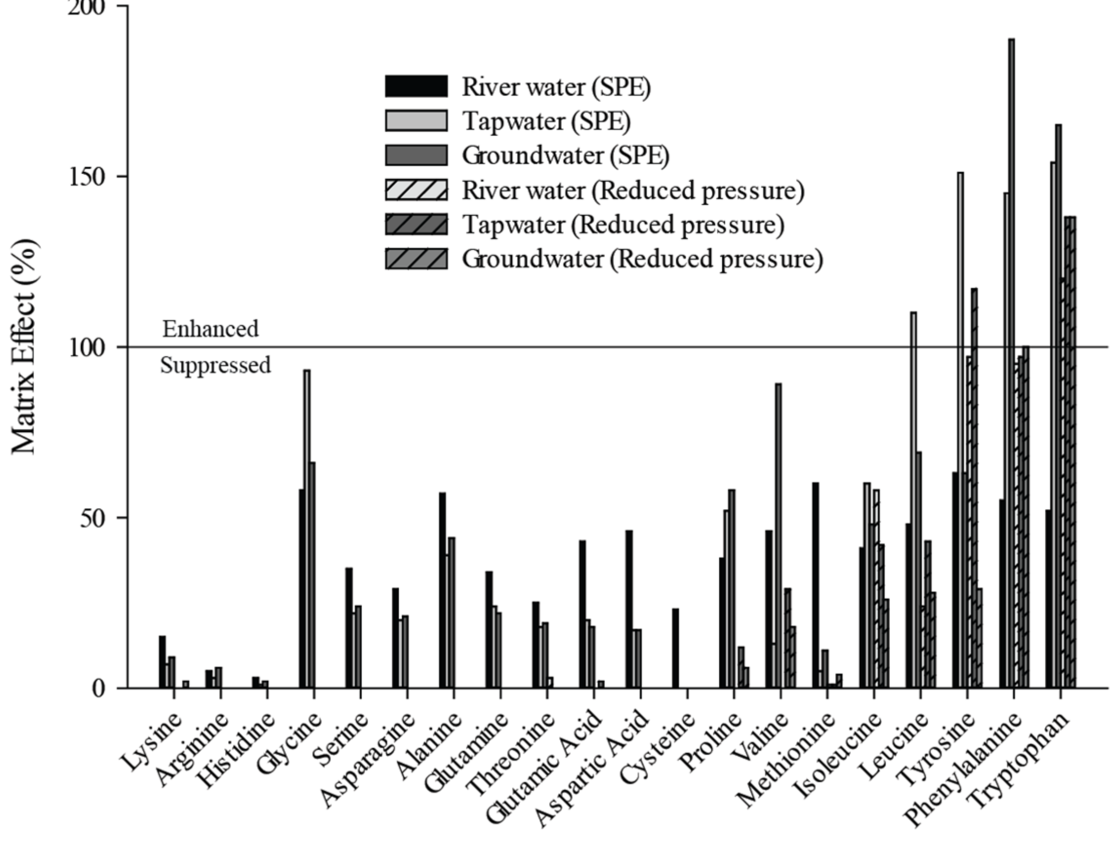
Name	Recovery (%)	Precision (RSD %)	MQL (µg L^{-1} as N)
Lysine	20	35	30
Arginine	25	25	10
Histidine	20	35	40
Glycine	120	10	10
Serine	120	20	5
Asparagine	110	15	1
Alanine	90	10	5
Glutamine	95	20	1
Thereonine	115	20	1
Glutamic Acid	125	15	0.5
Aspartic Acid	85	10	5
Cysteine	N.D.	N.D.	N.D.
Proline	85	10	0.5
Valine	100	10	0.5
Methionine	90	40	1
Isoleucine	105	10	1
Leucine	100	10	1
Tyrosine	90	5	0.5
Phenylalanine	105	10	0.5
Tryptophan	70	10	0.1
Median	**95**	**10**	**1**

N.D. - not detected.

659 Table 6. Concentration (μg L^{-1} as N) of measured free amino acids for surface waters A, B and C. Arginine and cysteine were below their
660 method limit of detection, while the other amino acids not listed were detected, but were present in concentrations below their respective
661 method quantification limit

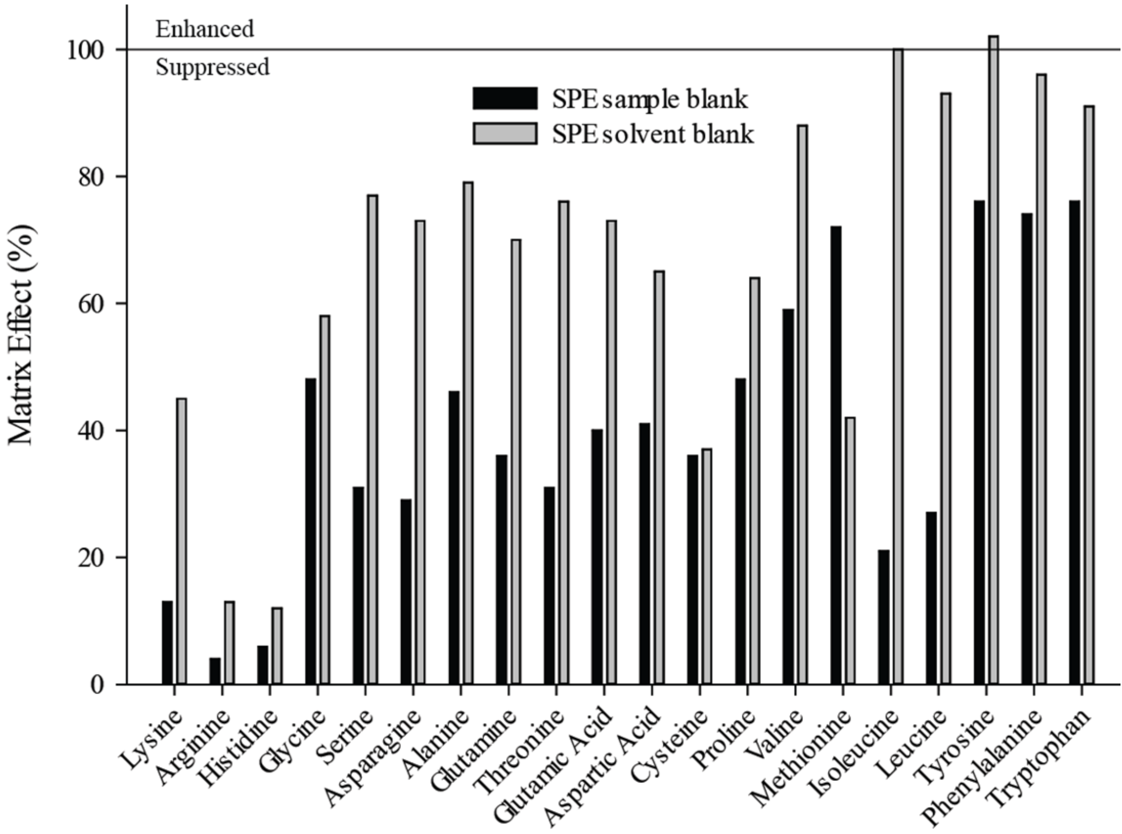
Name	Surface water A	Surface water B	Surface water C
Proline	<2	2	2
Isoleucine	3	3	5
Leucine	4	4	8
Tyrosine	4	4	7
Phenylalanine	3	3	4
Tryptophan	1	2	1
Total free amino acids	**15**	**16**	**26**

662



663

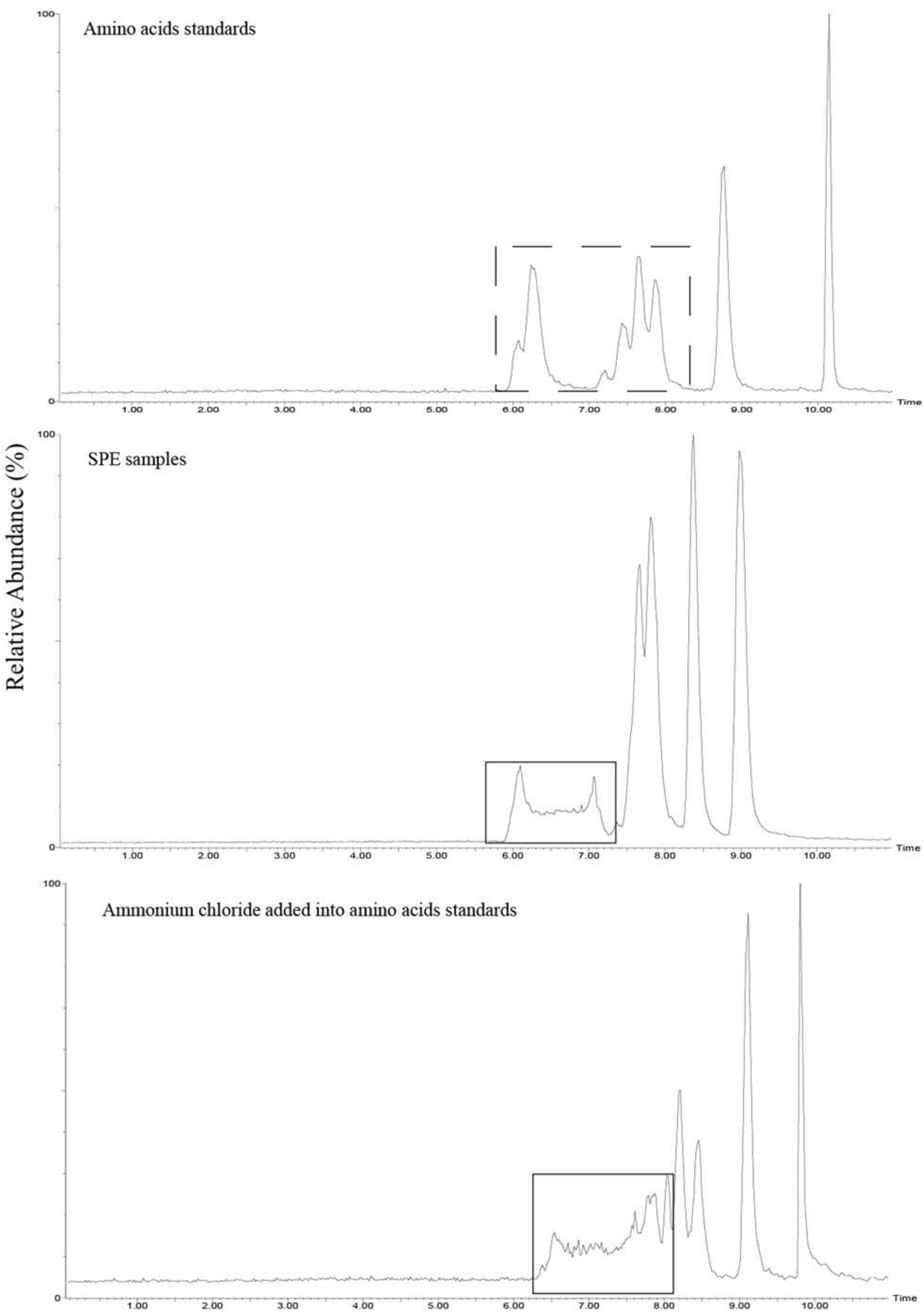
664 **Figure 1.** Matrix effects (%) from SPE and concentration under reduced pressure pre-
665 concentrated samples (n=2) of river water, tap water and groundwater containing 2 ng μL^{-1} of
666 amino acids. The matrix effects less than 100 % represent ion suppression, while a percentage
667 more than 100 % represents ion enhancement. The suppression/enhancement effect of each
668 matrix was determined by comparison to a standard solution of amino acids (2 ng μL^{-1})
669 dissolved in 70:30 MeOH: H_2O.
670



671

672 **Figure 2 .** Matrix effects (%) by SPE sample and solvent blank with 2 ng μL^{-1} of amino
673 acids. The matrix effects less than 100 % represent ion suppression, while a percentage more
674 than 100 % represents ion enhancement. The suppression/enhancement effect of each matrix
675 was determined by comparison to peak areas from a standard solution of amino acids (2 ng
676 μL^{-1}) in 70:30 MeOH: H_2O.
677

678



679 **Figure 3.** Impact of ammonium chloride on the chromatograms in the 10 min first window.
680 The chromatogram from analysis of amino acids in ultrapure water with added ammonium

681 chloride showed similar interference to the SPE sample and solvent blank, indicating that
682 ammonium chloride was likely to be produced in the SPE elution step. Regions affected by
683 matrix effects are highlighted by a solid line, as compared to the standard in a dashed line.

684

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