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Highlights:

Headspace NTD sampling to determine VOCs in whole blood is studied.

Matrix effect is very significant in complex biological matrices such as blood.

LODs at ng·L^{-1} are easily achieved by concentration with active HS-NTD sampling.

HS-NTD is a robust, sensitive and simple methodology for VOC analysis.

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1 # A Headspace Needle-Trap Method for the
2 # Analysis of Volatile Organic Compounds in
3 # Whole Blood

4

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18 **Abstract**

19 Needle trap devices (NTDs) are a relatively new and promising tool for headspace (HS)
20 analysis. In this study, a dynamic HS sampling procedure is evaluated for the
21 determination of volatile organic compounds (VOCs) in whole blood samples. A full
22 factorial design was used to evaluate the influence of the number of cycles and
23 incubation time and it is demonstrated that the controlling factor in the process is the
24 number of cycles. A mathematical model can be used to determine the most appropriate
25 number of cycles required to adsorb a prefixed amount of VOCs present in the HS
26 phase whenever quantitative adsorption is reached in each cycle. Matrix effect is of
27 great importance when complex biological samples, such as blood, are analyzed. The
28 evaluation of the salting out effect showed a significant improvement in the
29 volatilization of VOCs to the HS in this type of matrices. Moreover, a 1:4 (blood:water)
30 dilution is required to obtain quantitative recoveries of the target analytes when external
31 calibration is used. The method developed gives detection limits in the 0.020-0.080
32 $\mu g \cdot L^{-1}$ range (0.1-0.4 $\mu g \cdot L^{-1}$ range for undiluted blood samples) with appropriate
33 repeatability values (RSD<15% at high level and <23% at LOQ level). Figure of merits
34 of the method can be improved by using a smaller phase ratio (i.e., an increase in the
35 blood volume and a decrease in the HS volume), which lead to lower detection limits,
36 better repeatability values and greater sensibility. Twenty-eight blood samples have
37 been evaluated with the proposed method and the results agree with those indicated in
38 other studies. Benzene was the only target compound that gave significant differences
39 between blood levels detected in volunteer non-smokers and smokers.

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40 **Keywords:** Needle trap, Headspace analysis, Blood, Volatile organic compounds,

41 Matrix effect

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43 **1. Introduction**

44 Volatile organic compounds (VOCs) are a group of contaminants of great interest as
45 they are encountered in the workplace, in daily routines, widely used consumer
46 products, and are ubiquitous in both outdoor and indoor air. Inhalation of VOCs is the
47 most common route of exposure [1,2], but they can also be absorbed through the skin
48 [3-5] and, in some cases, oral uptake may be of considerable importance [6]. In order to
49 evaluate the individual exposure of a person to VOCs and the resulting health risk, it is
50 necessary to determine the internal exposure by analyzing biological fluids. Different
51 occupational studies have revealed that levels of VOCs and their metabolites in blood
52 tend to correlate highly with the corresponding air levels [7].

53 In normal healthy subjects many VOCs are detected in blood at very small
54 concentrations (tens to hundreds of $ng \cdot L^{-1}$) [8-13], except acetone (few $mg \cdot L^{-1}$) [8].
55 Measuring such low levels in human biological fluids accurately and reproducibly
56 presents a complex analytical problem that requires special techniques and great care
57 [14,15]. In forensic analytical toxicology, the separation of organic compounds from
58 biological matrices is one of the most important and complex aspects of the entire
59 analytical procedure. Another difficulty observed is that some volatile substances are
60 present at concentrations below the detection limits of the available instrumentation
61 [16].

62 Headspace (HS) analysis is the most common technique for the determination of VOCs
63 in biological fluids [17]. Conventional HS-GC is a routine technique for VOCs present
64 at higher concentrations in blood (mainly methanol and ethanol) [18-22], with LODs in
65 the 0.2-20 $mg \cdot L^{-1}$ range. The use of HS-GC with large volume collection from the

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66 headspace [23], sometimes with cryogenic oven trapping [24,25] and cryo-focussing
67 [26], has been used to improve sensitivity (LODs at ng·L^{-1} level). However, this
68 technique does not permit the evaluation of the presence of VOCs that can be used as
69 endogenous or exogenous markers.

70 The determination of VOCs by purge-and-trap (PT) makes it possible to significantly
71 reduce the LODs to the ng·L^{-1} level [8,9,12,27,28]. Unfortunately, PT-GC is not suitable
72 for blood samples as it results in foaming and the clogging up of the gas flow routes.
73 The use of an antifoam agent, added at a level that is high enough to prevent foaming, is
74 essential in the PT analysis of blood samples [15], but can lead to sample contamination
75 unless the antifoaming agent is heated under vacuum [8,29].

76 The most common concentration technique for the determination of minority VOCs in
77 blood is solid-phase microextraction (SPME). HS-SPME allows LODs to be obtained at
78 ng·L^{-1} without the foaming problem [10,11,16,21,30-32].

79 HS sampling is heavily dependent on the sample matrix and so results can vary
80 significantly [19,20,33]. Blood is very complex and varies from one person to another,
81 and it has a strong matrix effect due to its rich protein content [19]. It is therefore
82 necessary to compensate the matrix effect in HS blood analysis to obtain reliable
83 quantitative results. A well-established and accepted method in forensic medicine for
84 this purpose is to use an internal standard (IS) based matrix-matched calibration method
85 [19-22], although it has been found that the use of an IS in itself does not generally
86 eliminate the matrix effect and systematic errors may still occur [19,34].

87 The dilution of blood with water is the simplest method to reduce matrix effects as it (i)
88 minimizes the effect of matrix proteins, which can bind analytes, and (ii) reduces the
89 matrix viscosity, which increases the diffusion coefficients, allowing greater extraction

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90 efficiency. The main drawback of dilution is that it leads to an increase in detection
91 limits. The most common dilution ratio used in blood analysis is 1:1 with water or an
92 aqueous solution of an IS [16-18,22-24,27]. Some authors have indicated that 1:1
93 dilution avoids matrix interference by endogenous compounds in blood [16,18].
94 Unfortunately, there are no common criteria for the evaluation of the effect of dilution
95 on the matrix effect and some authors suggest greater dilution (e.g., 1:5 [21] and 1:2
96 [31]). On the other hand, in many other cases blood samples are analyzed directly
97 without dilution [8-11,26,28].

98 In HS analysis, the addition of salts has a greater influence on the distribution of highly
99 hydrophilic components towards the gas phase than temperature [35]. Moreover,
100 compensation of the salting-out effect is essential in biological fluids where the ionic
101 strength, which influences solubility, may vary considerably from one sample to another
102 [36]. In this situation, the added salt compensates for any ionic-strength effect. Houte et
103 al. [27] found a dramatic improvement in the recovery of VOCs when a salting-out
104 substance was used. Gottzein et al. [31] reported no influence of adding salts by SPME
105 analysis, but the amount of salt added was very small and was insufficient to
106 compensate for random salt concentrations in different biological samples. As in the
107 case of sample dilution, there are no agreed criteria for the salting-out effect. Some
108 studies have taken into account the salting-out effect [16,21,22,27] whereas others have
109 not [8-11,18-20,23,31].

110 Another parameter to considerer in HS sampling is the temperature applied during the
111 equilibration of VOCs in the gas phase. Although the distribution coefficient of
112 hydrophilic compounds is favored at increased temperatures in HS, protein denaturation
113 takes place at T>43ºC. Therefore, HS temperatures below this value are recommended

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114 for VOC analysis in blood samples [16]. Higher temperatures can result in undesired
115 changes to the blood samples caused by thermal stress within the heated vial.

116 It is necessary to develop simple and sensitive methods for managing difficult
117 biological matrices such as blood. In this study, we have evaluated the behavior of
118 needle trap devices (NTDs) for this purpose. This is a relatively new, simple and robust
119 methodology that has shown promising results in the analysis of VOCs from aqueous
120 solutions [37]. Different effects (e.g., dilution and salting-out) have been evaluated in
121 order to find the best experimental conditions to obtain quantitative recoveries of all the
122 target compounds.

123

124 **2. Experimental**

125 2.1. Materials

126 Carboxen 1000 (60/80 mesh, specific surface area of 1200 $m^2 \cdot g^{-1}$ and a density of 0.47
127 $g \cdot mL^{-1}$) and Tenax TA (60/80 mesh, specific surface area of 35 $m^2 \cdot g^{-1}$ and a density of
128 0.25 $g \cdot mL^{-1}$) were used as sorbent materials (Supelco, Bellefonte, PA, USA). Reagents
129 (purity >97%, Table 1) were supplied by Sigma-Aldrich (Steinheim, Germany).

130 22-gauge (22G, O.D. 0.71 mm, I.D. 0.41 mm, 51 mm length) stainless steel (metal hub)
131 needles with point style 5 were from Hamilton (Bonaduz, Switzerland). Gold wire of
132 100 μm diameter (Supelco) was used to prepare the spiral plugs and hold sorbent
133 particles inside the needles. 20 mL crimp-cap HS vials, Teflon/silicone septum and caps
134 were purchased from Fisher Scientific Spain (Madrid).

135 Stock solutions were freshly prepared daily by spiking milli-Q water (Millipore Iberica,
136 Barcelona, Spain) with 50 μL of a methanolic solution containing the compounds at

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137 320-590 mg·L^{-1}. These solutions were transferred to HS vials, filling them to avoid any
138 remaining headspace. The vials were then closed and stored at 4ºC. Working solutions
139 were prepared by the appropriate dilution of the stock solution in milli-Q water. In order
140 to prevent VOC losses during the preparation of the solutions and samples, glass
141 syringes (Hamilton) were used for sample transfer avoiding the formation of any gas
142 space in the syringe [38].

143

144 2.2. Sampling by needle-trap device

145 Each NTD was prepared by taking a 51-mm long, 22G, stainless steel needle and filling
146 it with the sorbent materials The protocol used to immobilize sorbent particles inside the
147 needle has been previously described [37,39]. Each NTD was conditioned in the GC
148 injector at 300ºC for 2-3 hours with a permanent helium flow to remove impurities.
149 Finally, the tip end was sealed with the help of a Teflon septum and the upper part of
150 the needle was closed with a push-button syringe valve (SGE Europe Ltd, Milton
151 Keynes, UK) to prevent contamination during storage.

152 Sample extraction was performed with a bi-directional syringe pump (New Era Pump
153 System Inc., Farmingdale, NY, USA). The pump was programed to complete 1 mL
154 sampling cycles at 2 mL·min^{-1}. The air drawn from the vial was injected back through
155 the NTD at 2 mL·min^{-1} to maintain the pressure conditions inside the vial.

156 Desorption and transfer of VOCs into the GC column was performed taking advantage
157 of the desorptive flow produced by the internal air expansion inside the needle at the hot
158 desorption temperatures of the GC injector [39]. The NTD was inserted into the
159 injection port in the splitless mode for one minute. After opening the split valve, the
160 needle was kept in the hot injector for one minute. Blank runs were carried out every

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161 five samples, analyzing the NTD just after a desorption step and before sampling again,
162 and no carry over was observed.
163
164 2.3 GC-MS analysis
165 Component separation was achieved by the use of a 30 m long TR-Meta.VOC column
166 with 0.25 mm I.D. and 1.5 μm film thickness (Teknokroma, Barcelona, Spain). A Focus
167 GC (Thermo Scientific, Waltham, MA, USA) with a mass spectrometer detector (DSQ
168 II, Thermo Scientific) was used.
169 The injector (desorption) temperature was maintained at 300ºC to ensure complete and
170 fast desorption of target VOCs [39-41]. The oven temperature program was 40ºC for 4
171 min, then ramped at 5ºC·min^{-1} to 150ºC, followed by a ramp at 10ºC·min^{-1} to 225ºC and
172 held for 2 min. Helium carrier gas was used with a constant inlet flow of 0.8 mL·min^{-1}
173 after purification for water vapor, hydrocarbons and oxygen. MS analyses were carried
174 out in full-scan mode, with a scan range of 40-250 uma, electron impact ionization was
175 applied at 70 eV, and the transfer line was maintained at 230ºC. Chromatographic data
176 was acquired by means of Xcalibur software (v. 1.4, Thermo Electron).
177
178 2.4. Blood samples
179 Whole blood samples were collected by venipuncture into vacutainers containing EDTA
180 as the anticoagulant (BD Vacutainer, Trenton, NJ, USA). Whole blood samples were
181 refrigerated at 4ºC within 10 minutes [8,15,32]. Freezing blood samples is not
182 recommended as it lyses the red cells and may change the equilibrium within the matrix
183 [15,32]. All measurements were performed within 14 days of collection [8,32].

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184 For the VOC measurements, 0.5 mL blood was mixed with 2.0 mL water and 0.4 g
185 NaCl (i.e., 0.16 g·mL^{-1} salt content) in the HS vials. Vials were first incubated for 5
186 minutes in a dry bath at 30ºC (Model FB15101, Fisher Scientific, Loughborough, UK).
187 Afterwards, 20 cycles (a total volume of 20 mL) were programed to collect the VOCs
188 on the sorbents. All samples were determined three times.

189

190 2.6. Experimental design and statistical analysis

191 A full factorial design was performed to evaluate the influence of the parameters on the
192 extraction of VOCs from blood samples with the NTDs. This allowed us to determine
193 the influence of the experimental variables studied and also to ascertain the interactions
194 between them. For each analyte, we considered two variable factors that can affect the
195 extraction yield: volume of gas sample passed through the NTD quantified as the
196 number of cycles (n) and the equilibration time before starting the sorption process (t).
197 We then selected a 2^2 full factorial design. Table 2 shows the experimental range for
198 each factor. The central point (10 cycles, 25 min) was also measured and considered as
199 an experiment. All experiments were duplicated randomly, except for the central point
200 that was triplicated. Minitab v14 software was used for data manipulation and
201 calculations [42]. SPSS for Windows (v. 15.0) was used for other statistical analyses.

202

203 **3. Results and discussion**

204 3.1. Sampling methodology

205 The evaluation of different HS sampling methodologies with NTDs [37] showed that
206 this methodology can reach limits of detection (LODs) at the ng·L^{-1} level. A dynamic
207 sampling procedure was applied in the present study. Eom et al. [43] compared three

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208 syringe pump assisted dynamic HS procedures for collecting a fixed volume of
209 headspace sample. They found that using various sorption cycles and returning the
210 extracted gas volume to the vial after each cycle resulted in a significant improvement
211 in the sensitivity compared to a conventional purge-and-trap method. They suggest that
212 the increase in the amount extracted was due to the minimum dilution effect resulting
213 from recycling the air. Therefore, a similar recycling system was used in the present
214 study. Instead of returning the filtered air through a separate channel, our system
215 recycles it back through the sorbent bed, eliminating the need for a distribution valve so
216 simplifying the instrumentation required. The binding of the analytes to the bed is
217 sufficiently strong as to avoid any analyte loss when recycling a small volume of gas
218 through it [44].

219 The percentage of analyte trapped in each cycle can be determined by a simple
220 preliminary calculation. If a 15 mL HS volume (V_{HS}) is used and each cycle collects 1
221 mL (V_c) of this volume through the trap, the amount of the analyte retained in each
222 cycle will be 6.7% of the VOC content present in the HS (assuming that all the VOCs
223 present in the volume passed through the trap are adsorbed):

$$\frac{100 \times V_c}{V_{HS}} = \frac{100 \times 1\ mL}{15\ mL} = 6.7\% \qquad \text{(eq. 1)}$$

224 Therefore, it can be determined that the percentage of analyte remaining in the HS after
225 each cycle ($C_{i,HS}$) is:

$$C_{i,HS} = C_{i-1,HS}\left(1 - \frac{V_c}{V_{HS}}\right) \qquad \text{(eq. 2)}$$

226 and the percentage of analyte retained by the sorbent trap ($C_{i,T}$) after each cycle is:

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$$C_{i,T} = C_{i-1,T} + \frac{V_e}{V_{HS}} C_{i-1,HS} \qquad \text{(eq. 3)}$$

227 In these conditions, 34 cycles will be required to transfer 90% of the analytes in the HS
228 to the trap (Figure 1). A preliminary study was performed to determine the percentage
229 of compound extracted at different numbers of cycles for the target compounds
230 evaluated. As can be seen in Figure 1, the experimental retention percentages found for
231 all VOCs fitted with the proposed model, which confirms that no-breakthrough took
232 place and that quantitative adsorption was obtained in each cycle.

233 The sampling temperature also has a significant effect on the sorption mechanism of the
234 volatile compounds by NTDs [37,43]. Although an increase in the temperature increases
235 the partition of volatile compounds through the gas phase, breakthrough was observed
236 for benzene at sampling temperatures above 40ºC [43]. Increase in the headspace
237 temperature results in a competitive desorption of the most volatile compounds from the
238 sorbent, which is more important when a large volume of gas phase is transported
239 through the material [37]. Moreover, temperatures <47ºC are required when
240 determining VOCs from blood samples [16]. A temperature of 30±0.1 ºC was selected
241 for the measurements. The use of low temperatures reduces the formation of water
242 vapor in the gas phase, which would otherwise compete with the VOCs for the sorption
243 sites of the carbon molecular sieves (Carboxen 1000) [45].

244

245 3.2. Study of the sampling conditions

246 An experimental domain was defined to ascertain the influence of incubation time and
247 the number of cycles on the extraction of the target compounds from aqueous solutions
248 (Table 2). A full two-level factorial design was implemented to check for the presence
249 of interactions and evidence of curvature effects that could not be detected using a

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250 classical procedure based on the evaluation of each variable individually. Absolute peak
251 areas were analyzed and the results obtained are summarized in Table 3 with *p*-values.
252 The sign next to each variable name indicates the optimal level to maximize the
253 response. The results obtained show that no statistically relevant interactions occurred
254 between the variables evaluated in any compound. The corresponding *p*-values for
255 single interactions are always much smaller than those for the double interaction, even
256 in the cases of 2,5-dimethylfuran, *o*-xylene and 1,2-dichlorobenzene, where the double
257 interactions are significant by themselves but are not when compared with the single
258 ones. In all the cases, the factor that clearly controls the process is the number of cycles
259 (see Supplementary Materials for the complete results). For a fixed number of cycles,
260 factor time does not have a significant effect. Clearly relevant curvature effects were not
261 detected, except the small interaction found in Furan.

262 Although 40 cycles are theoretically required to retain >90% of VOCs in the conditions
263 used (17.5 mL HS and 1 mL cycle), the number of cycles was fixed at 20 (expected
264 total extraction ~70%) and the incubation time chosen was 5 minutes to reduce the total
265 analysis time. In these conditions, a total sampling time of 25 minutes (5 min
266 incubation, and 30 seconds for sampling and 30 seconds for recycling in each cycle) is
267 required for each analysis. This allows sufficient time to condition the NTD and
268 perform a new sorption while a prior analysis is run.

269

270 3.3. Salting-out effect

271 As indicated in the introduction section, compensation of the salting-out effect is
272 necessary in biological fluids as the ionic strength of blood may vary considerably from
273 one sample to another [36]. Furthermore, the effect of the ionic-strength in fortified
274 blood samples was evaluated to determine whether adding a salt improves the extraction

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275 efficiency of the NTDs. Sodium chloride was added to the samples in order to obtain
276 0.16 g·mL^{-1} of salt content. The extraction efficiency of all compounds improved
277 significantly with the addition of salt ($p<0.05$, t-test, one-side), except for carbon
278 tetrachloride ($p=0.167$) (Figure 2). In the case of the two most polar compounds, ethyl
279 acetate was not detected and acetone was detected close to its detection limit without the
280 addition of salt, but they were both clearly detected once salt was added. Therefore, the
281 salting-out effect is required for the HS-NTD methodology to improve the sensitivity of
282 the method and to compensate the different ionic strengths of blood samples.

283

284 3.4. Matrix effect

285 The complexity of blood requires the evaluation of matrix effects. The use of external
286 calibration is the simplest and fastest quantification method. However, this can be
287 strongly affected by matrix effects. This problem can often be overcome with complex
288 biological samples by diluting the sample.

289 A pool of different blood samples was used as a matrix solution for recovery studies.
290 Recoveries were calculated by analyzing both the pooled blood sample, with the
291 dilution ratio required in each case, and the same sample fortified at a fixed mass for
292 each compound. The recovery percentage was determined as the ratio between the
293 calculated mass difference obtained and the spiked mass in the fortified sample.

294 Figure 3 shows the recoveries obtained with different diluting rates. There are strong
295 interactions between the target compounds and the matrix components as none of the
296 compounds evaluated yielded quantitative recoveries without dilution of the blood
297 sample or with a 1:1 (blood:water) dilution. The results indicate that the greater the
298 volatility of the compound, the lower the interaction with the matrix. Most volatile

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299 compounds evaluated (furan, carbon tetrachloride and benzene) yielded quantitative
300 recoveries with a 1:2 dilution. Intermediate volatiles (2,5-dimethylfuran, 1,2-
301 dichloropropane and toluene) gave reasonable recoveries (>75%) with a 1:3 dilution.
302 Other less volatile compounds (ethylbenzene, xylenes and styrene) required a 1:4
303 dilution to obtain good recoveries. The least volatile compound evaluated (1,2-
304 dichlorobenzene) did not yield adequate recoveries in any of the dilutions. The tendency
305 observed suggests that this compound suffers a strong matrix effect that cannot be
306 solved simply by dilution. Therefore, 1:4 dilution is required to analyze target VOCs,
307 except 1,2-dichlorobenzene. The use of blood dilution has the disadvantage of
308 increasing the detection limits of VOCs in blood.

309

310 3.4. Figures of merit of the HS-NTD methodology

311 Calibration standard mixtures (n=6, each measured twice) in the 0.2 to 50 $\mu g \cdot L^{-1}$ range
312 for each compound were analyzed (corresponding to concentrations in the undiluted
313 blood samples in the 1 to 250 $\mu g \cdot L^{-1}$ range). Acetone, hexane, ethyl acetate, chloroform,
314 and carbon tetrachloride were excluded from the calibrations due to blank
315 contamination as they are solvents that are commonly used in adjacent laboratories.

316 Table 4 shows the figure of merits obtained in these experiments. LODs were calculated
317 by analyzing a standard at 0.05-0.1 $\mu g \cdot L^{-1}$ (n=5) with the SD obtained being taken as the
318 SD of the blank [46,47]. The $3SD_{blank}$ criterion was then applied to calculate LODs.
319 Positive detection was confirmed by preparing standards and fortified blood samples at
320 the calculated values and then measuring them with the HS-NTD method. When the
321 conventional signal-to-noise ratio (S/N=3) was used, the LODs obtained were one order
322 of magnitude lower than those of the first option (ranging from 0.003 to 0.018 $\mu g \cdot L^{-1}$).

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323 However, these values only correspond to the instrumental detection limit and do not
324 take into account blank contaminants, which are a ubiquitous problem with some
325 compounds at $ng \cdot L^{-1}$ level (see Figure S1 in Supplementary Materials). We decided to
326 use the first method as it takes into account blank contamination and repeatability at low
327 concentrations. Blank analyses always gave values below LODs when this procedure
328 was applied.

329 Limit of quantification (LOQ) values in Table 4 correspond to the first calibration
330 standard used that gave a signal $>10SD_{blank}$ [46,47]. Linearity was confirmed in the
331 range of LOQ to 50 $\mu g \cdot L^{-1}$ by evaluating residual distribution. Good fits were achieved
332 for all compounds ($R^2>0.983$, except for styrene).

333 Recoveries were evaluated from blood samples fortified at the levels indicated in Table
334 5. The results obtained were adequate for all compounds, except for
335 1,2-dichlorobenzene, which yielded a 30% recovery. Precision (repeatability) was
336 determined at high and LOQ levels. All compounds gave repeatability values within the
337 precision limits suggested by the ICH (precision not to exceed 15% except for the LOQ
338 level, where it should not exceed 20%) [46,47].

339 The trueness of the achieved results was determined by evaluating five blood samples in
340 triplicate with the proposed NTD methodology and a conventional SPME method to
341 analyze VOCs in blood. The same compounds were identified in each sample for both
342 methods. A paired t-test was performed for those compounds giving concentration
343 values above LOQs and no significant differences were obtained in any of the
344 comparisons ($p>0.1$).

345 The large phase ratio ($\beta=7$) used in this study is a disadvantage for the detection of most
346 volatile compounds as their concentrations in the gas phase are reduced due to the large

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347 volume in this phase. Some experiments were performed using a higher volume of
348 sample (V_s=10 mL, β=1.0). In these conditions, the percentage of extraction is expected
349 to increase from ~69% to ~88% (calculated from eq. 1, 2 and 3). The results obtained
350 confirmed a significant increase in the extraction efficiency of the NTDs (slope in the
351 calibration curves increased by a factor of between 1.4 and 2.1). An improvement in the
352 precision and the detection limits were also observed (Table S1 in Supplementary
353 Materials).

354 Two IS were evaluated (d-furan and d-benzene) to see whether the precision and
355 calibration response could be improved. Determination coefficients in the calibration
356 curves measured for the two phase ratios indicated previously (β=7 and β=1) showed a
357 significant improvement for the most volatile compound (furan) when d-furan was used
358 as the IS (R^2 increased from 0.985 to 0.994 with a β=7 and from 0.968 to 0.995 with a
359 β=1). No other compound showed any improvement with any of the IS evaluated. This
360 seems to indicate that the use of an IS with NTDs is important for the most volatile
361 compounds. It should be noted that only the target VOC that cannot be quantitatively
362 retained by the hydrophobic Tenax TA sorbent (furan), and which we would expect to
363 be retained by the stronger sorbent (Carboxen 1000, a carbon molecular sieve), showed
364 an improvement in the results with the use of an IS. This effect could be attributed to
365 the fact that the distance that this compound has to travel inside the NTD after thermal
366 desorption is relatively large, and so there may be a less reproducible desorption process
367 and slow transport to the GC column. The use of an IS with the same
368 sorption/desorption characteristics seems to be useful in avoiding these reproducibility
369 problems.

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371 3.5. Analysis of blood samples

372 Twenty-eight samples from different individuals (12 non-smokers, 7 ex-smokers and 9
373 smokers) were evaluated with the developed HS-NTD methodology. The method with
374 the lower phase ratio (β=7) was chosen for the analysis of samples as there were some
375 difficulties in obtaining large volumes of blood from some of the volunteers. Figure 4
376 shows the chromatogram obtained for a sample from a smoker.

377 Three of the target compounds (furan, 2,5-dimethylfuran and 1,2-dichloropropane) were
378 not detected in any of the samples. In the case of benzene, two samples gave outlying
379 results and were excluded from the analysis. This compound was detected in 17 samples
380 (65.4%). Toluene was detected in all samples (100%). Ethylbenzene was detected in 15
381 samples (53.6%). *p*-xylene was detected in 19 samples (68%). *o*-xylene was detected in
382 7 samples (25.0%). Styrene was detected in 21 samples (75.0%). Ethylbenzene,
383 *o*-xylene and styrene were always detected below LOQs. Table 6 shows the summary of
384 the results obtained together with those obtained in other studies where blood levels in
385 non-exposed individuals were evaluated [7-9,48-51]. As can be seen, the results agree
386 with those found in previous studies.

387 Despite the limited number of samples, a preliminary statistical evaluation of the data
388 was performed to evaluate the possible existence of differences between smokers,
389 former smokers and non-smokers. For statistical analysis, a value of ($LOD/\sqrt{2}$) was
390 used in the case of non-detected compounds. Benzene was the only compound that gave
391 significant differences between the three sub-groups evaluated (p=0.005, ANOVA test,
392 Figure 5). All other target compounds gave non-significant differences (p>0.05). In the
393 case of benzene, the use of a post-hoc test (Tukey B) showed that the smokers group
394 gave significantly higher levels than non-smokers (p=0.005) and ex-smokers (p=0.030),
395 and that these two non-smoking groups did not differ between them (p=0.888).

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397 **4. Conclusions**

398 The NTD methodology has been evaluated for its use in the analysis of VOCs from
399 blood samples of unexposed individuals. The complexity of blood samples results in a
400 significant matrix effect that can be eliminated by the dilution of the blood samples,
401 although this leads to an increase in the detection limits. The LODs obtained in the
402 conditions evaluated are relatively large for an adequate quantification of VOCs in
403 unexposed individuals. This problem can be overcome by decreasing the phase ratio
404 during the HS process (i.e., increasing the volume of the sample) or by increasing the
405 number of cycles for the sorption process. The results obtained show that the HS-NTD
406 technique is a good alternative to conventional SPME methods for the analysis of VOCs
407 in complex biological matrices. It can be easily automated and it is very robust. More
408 than 200 consecutive analyses have been performed with the same needle trap without
409 any significant variation in the precision and sensitivity.

410

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502 **Figure Captions**

503 **Figure 1**. Increase in the sorption of VOCs with the number of cycles (1 mL HS
504 sampling each cycle) using the proposed dynamic HS-NTD system (calculated from eq.
505 3). Experimental values obtained for furan (♦), benzene (■) and toluene (×). Model
506 evaluated for a phase ratio β=3 (5 mL sample and 15 mL HS). Three replicates for each
507 value.

508 **Figure 2.** Salting-out effect on the adsorption of VOCs with the dynamic HS-NTD
509 system. Black blocks without the addition of salt, white blocks with an 0.16 $g \cdot mL^{-1}$
510 NaCl content. Three replicates each.

511 **Figure 3.** Recoveries obtained without blood dilution and different blood:water dilution
512 ratios to assess matrix effects. Pooled blood sample fortified in the range 15-20 $\mu g \cdot L^{-1}$
513 range for each VOC, β=7, 0.16 $g \cdot mL^{-1}$ NaCl, 20 cycles. Three replicates each.

514 **Figure 4.** Extracted chromatogram (m/z= 57,63,68,71,78,83,91,96,104,106,117,146)
515 obtained in the analysis of a blood sample from a smoker. Numbers by peaks
516 correspond to the compound numbers in Table 1.

517 **Figure 5.** Box plots of data obtained for benzene. The bottom and top of the box are
518 25th and 75th percentiles, the line inside the box is the median (50th percentile), and the
519 whiskers indicate the lowest and highest data within the 1.5 interquartile range.

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521 **Table 1.** List of volatile compounds evaluated

#	Compound name	Retention time (min)	Characteristic masses [a]
1	Furan	4.1	**68**
2	Acetone	4.2	43,58
3	Hexane	7.4	57,69,85
4	Ethyl acetate	8.0	43,71,86
5	Chloroform	8.2	83,85
6	Carbon tetrachloride	9.6	117,119
7	Benzene	10.0	77,**78**
8	2,5-dimethylfuran	11.5	81,95,**96**
9	1,2-dichloropropane	11.8	**63**,112
10	Toluene	14.4	**91**,92
11	Ethylbenzene	18.4	**91**,106
12	*m*-, *p*-xylene	18.6	91,105,**106**
13	*o*-xylene	19.8	91,105,**106**
14	Styrene	19.9	78,**104**
15	1,2-dichlorobenzene	25.9	111, **146**, 148

522 [a] Quantification masses in bold

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524 **Table 2.** Factor levels considered in optimizing the experimental design.

Variable	Low level (-)	Medium level (0)	High level (+)
Number of cycles (n)	1	10	20
Equilibration time (t, min)	5	25	45

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526 **Table 3**. Statistical results for the experimental design. Significance *p*-values are given for main effects, double interactions and for curvature
527 evidence. Relevant single and double variable terms effects are also shown (the signs indicate the optimal variable level). When the double
528 interaction is relevant, the *p*-values attached to each single interaction are also given.

529

Compound	Single variable effects		Double variable effects		*p*-value for curvature evidence
	p-value	Most significant terms (*p*-value)	*p*-value	Significant terms	
Furan	<0.001	+n	0.102		0.024
Carbon tetrachloride	0.008	+n	0.895		0.354
Benzene	0.001	+n	0.895		0.400
2,5-dimethylfuran	<0.001	+n(<0.001) +t(<0.001)	0.001	+nt	0.509
1,2-dichloropropane	<0.001	+n	0.305		0.508
Toluene	<0.001	+n	0.452		0.654
Ethylbenzene	<0.001	+n	0.346		0.887
m-, *p*-xylene	<0.001	+n	0.241		0.943
o-xylene	<0.001	+n(<0.001) +t(0.019)	0.044	+nt	0.292
Styrene	<0.001	+n	0.252		0.716
1,2-dichlorobenzene	<0.001	+n(<0.001) +t(0.007)	0.021	+nt	0.832

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537 **Table 4.** Linearity parameters, determination coefficients (R^2) and limits of detection
538 (LOD) and quantification (LOQ) for the target VOC with the HS-NTD methodology
539 (method with β=7).

Compound	**slope (SD) (·10⁴)**	**R^2**	**LOD [a] (μg·L⁻¹)**	**LOQ [a] (μg·L⁻¹)**
Furan	194 (8)	0.985	0.04 (0.2)	0.20 (1.0)
Benzene	1067 (33)	0.995	0.08 (0.4)	0.24 (1.2)
2,5-dimethylfuran	448 (27)	0.983	0.02 (0.1)	0.28 (1.4)
1,2-dichloropropane	223 (12)	0.987	0.04 (0.2)	0.36 (1.8)
Toluene	1519 (79)	0.987	0.04 (0.2)	0.28 (1.4)
Ethylbenzene	1528 (113)	0.989	0.04 (0.2)	0.28 (1.4)
m-, *p*-xylene	525 (36)	0.987	0.06 (0.3)	0.26 (1.3)
o-xylene	637 (28)	0.991	0.04 (0.2)	0.26 (1.3)
Styrene	589 (47)	0.970	0.02 (0.1)	0.28 (1.4)
1,2-dichlorobenzene	541 (42)	0.990	0.05 (0.25)	0.28 (1.4)

540 [a] Limit values in undiluted blood samples are given between brackets
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542 **Table 5.** Recoveries and repeatability obtained with the HS-NTD methodology proposed.

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Compound	Fortified level ($\mu g \cdot L^{-1}$)	Recovery (SD) (%) (n=3)	Repeatability (RSD, %) (n=5)			Reproducibility[c] (RSD, %) (n=5)
			high level[a]	LOQ level	blood samples[b]	high level[a]
Furan	15	99 (9)	7	15	ND	13
Benzene	14	117 (9)	10	14	4-22 (15)	15
2,5-dimethylfuran	14	109 (12)	9	18	ND	14
1,2-dichloropropane	18	115 (14)	10	21	ND	17
Toluene	14	109 (15)	12	12	4-20 (11)	18
Ethylbenzene	14	97 (13)	11	23	<LOQ	17
m-, *p*-xylene	14	93 (12)	9	20	6-23 (14)	15
o-xylene	14	90 (11)	10	22	<LOQ	16
Styrene	14	75 (8)	12	11	<LOQ	18
1,2-dichlorobenzene	21	29 (2)	8	20	ND	16

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554 [a] repeatability obtained with a spiked sample at a concentration equal to the value indicated in the "fortified level" column

555 [b] range of repeatabilities obtained in the measure of blood samples. Mean RSD obtained from all quantified blood samples is indicated between
556 brackets

557 [c] Measured in five consecutive days

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558 **Table 6.** Main statistical parameters of blood concentrations of target VOCs (μg·L^{-1}).

Mean	Median	Minimum	Maximum	n	LOD	Reference
			benzene			
<LOQ	<LOQ	ND	2.610	26	0.400	This study
0.176	0.102	0.032	0.728	287	0.024	7
0.072	--	--	--	12	0.032	8
0.262	0.194	ND	2.241	431	0.015	9
0.094	0.062	0.033	0.487	61	0.016	48
0.226	0.106	0.046	1.187	25	0.016	49
--	0.062	--	1.880	796	0.030	50
--	<LOQ	--	0.480/0.320	837/1345	0.024	51
			2,5-dimethylfuran			
ND	ND	ND	ND	28	100	This study
0.029	0.002	--	0.373	61	--	48
--	ND	--	180	1221	12	51
			toluene			
1.543	1.150	<LOQ	3.100	28	0.200	This study
0.442	0.234	ND	4.880	292	0.025	7
1.200	--	--	--	13	0.088	8
1.100	0.559	0.120	6.040	25	0.043	49
--	0.281	--	6.767	575	0.092	50
--	0.160/0.096	--	1.430/0.880	954/1336	0.025	51
			ethylbenzene			
<LOQ	<LOQ	ND	0.690	28	0.200	This study
<LOQ	<LOQ	ND	0.949	251	0.024	7
0.120	--	--	--	13	0.012	8
0.231	0.145	ND	0.596	25	0.022	49
--	<LOQ	--	3.731	606	0.020	50
--	<LOQ	--	0.180/0.120	879/1299	0.024	51
			m-, p-xylene			
<LOQ	<LOQ	ND	1.750	28	0.300	This study
0.261	0.174	ND	5.300	285	0.034	7
0.540	--	--	--	13	0.010	8
0.719	0.457	<LOQ	1.713	25	0.052	49
--	0.117	--	33.057	1018	0.033	50
--	0.150/0.130	--	0.890/0.400	962/1346	0.034	51
			o-xylene			
<LOQ	ND	ND	<LOQ	28	0.200	This study
<LOQ	<LOQ	ND	2.260	298	0.024	7
0.350	--	--	--	13	0.024	8
--	0.101	--	3.487	628	0.040	50
--	ND	--	0.180/<LOQ	981/1365	0.049	51
			styrene			
<LOQ	<LOQ	ND	0.600	28	0.100	This study
0.050	--	--	--	13	0.010	8

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--	<LOQ	--	4.006	624	0.019	50
--	ND	--	0.260/0.130	950/1245	0.030	51

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560 ND: <LOD

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Figure 1

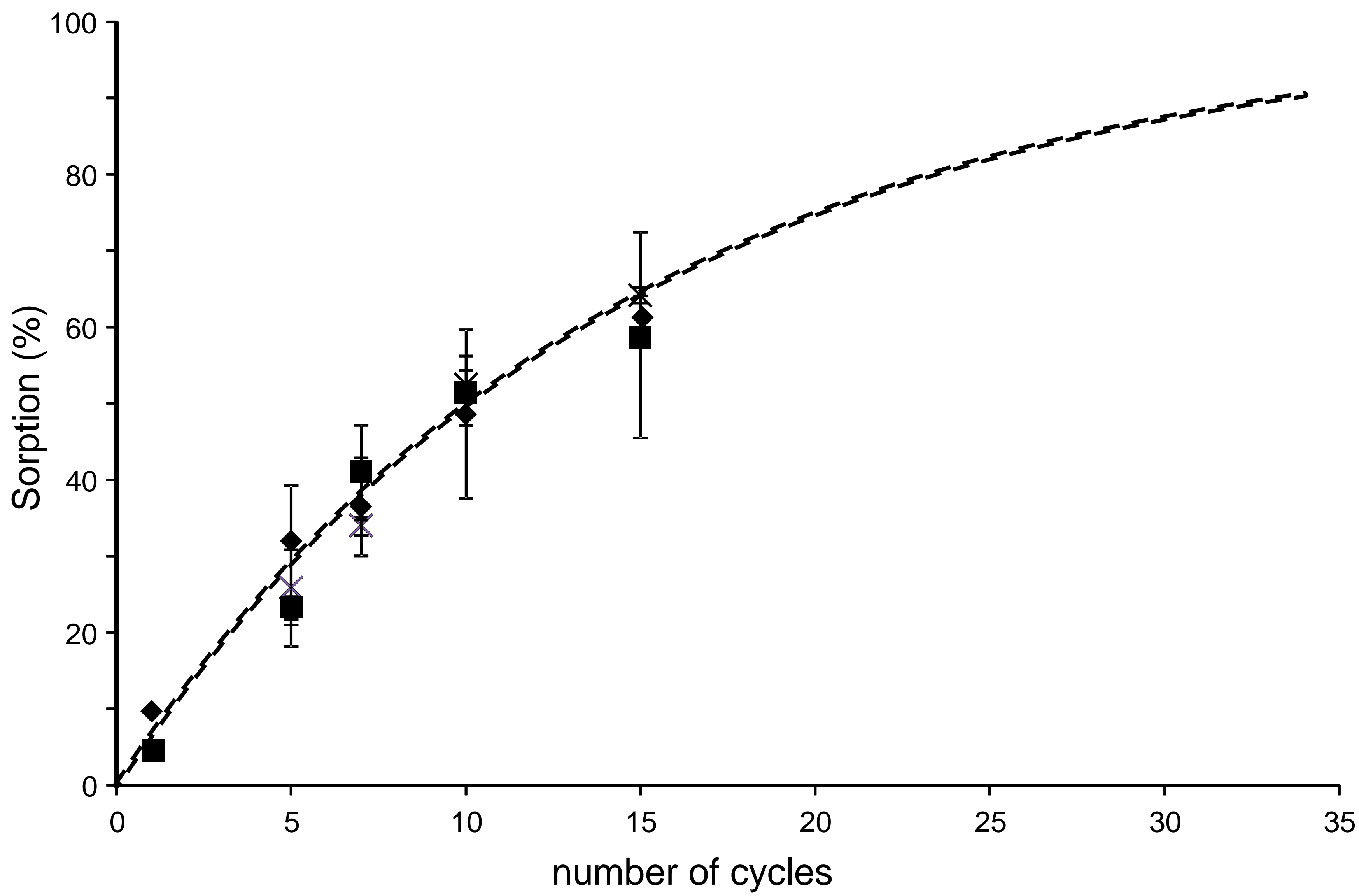

Figure 2

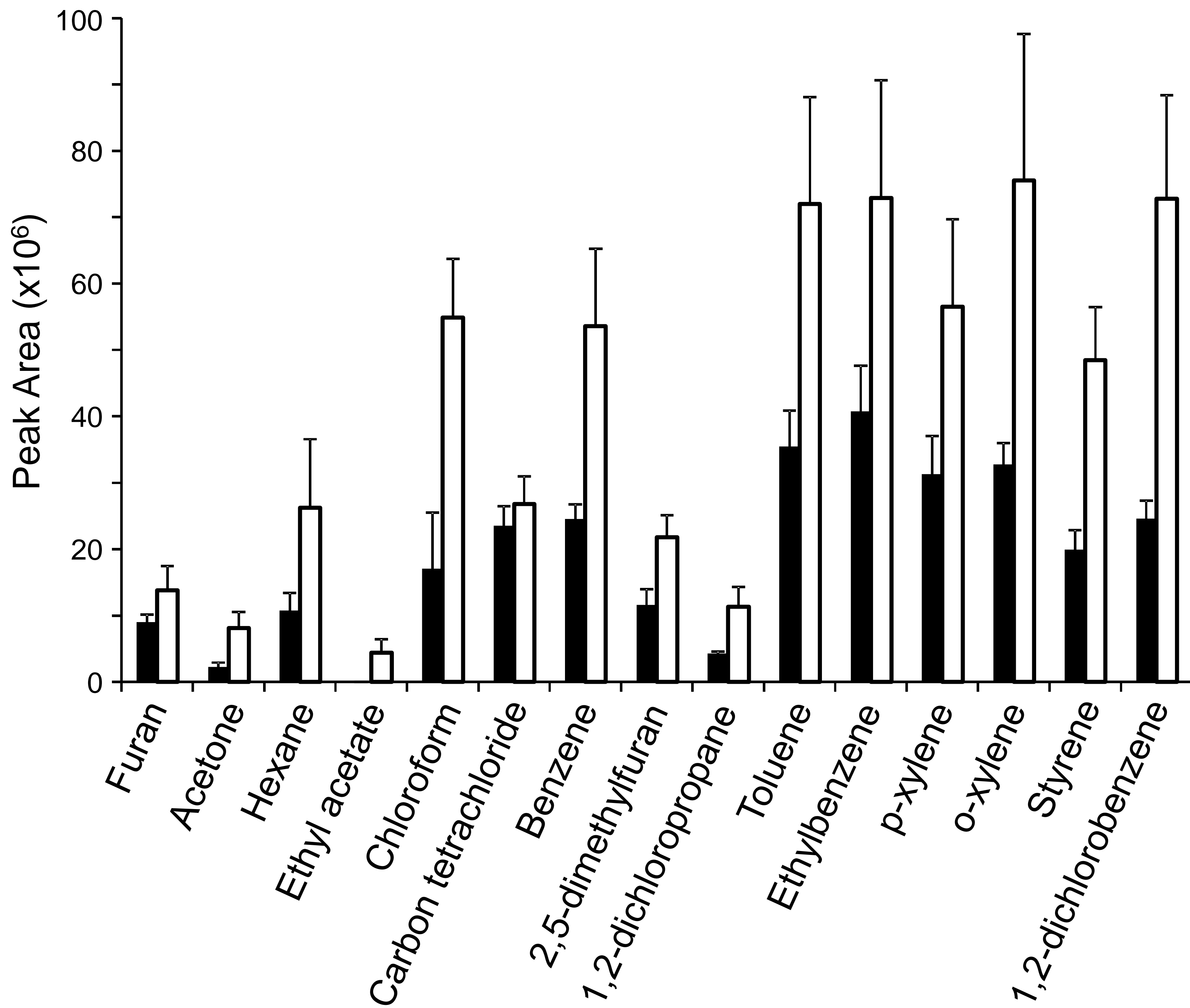

Figure 3

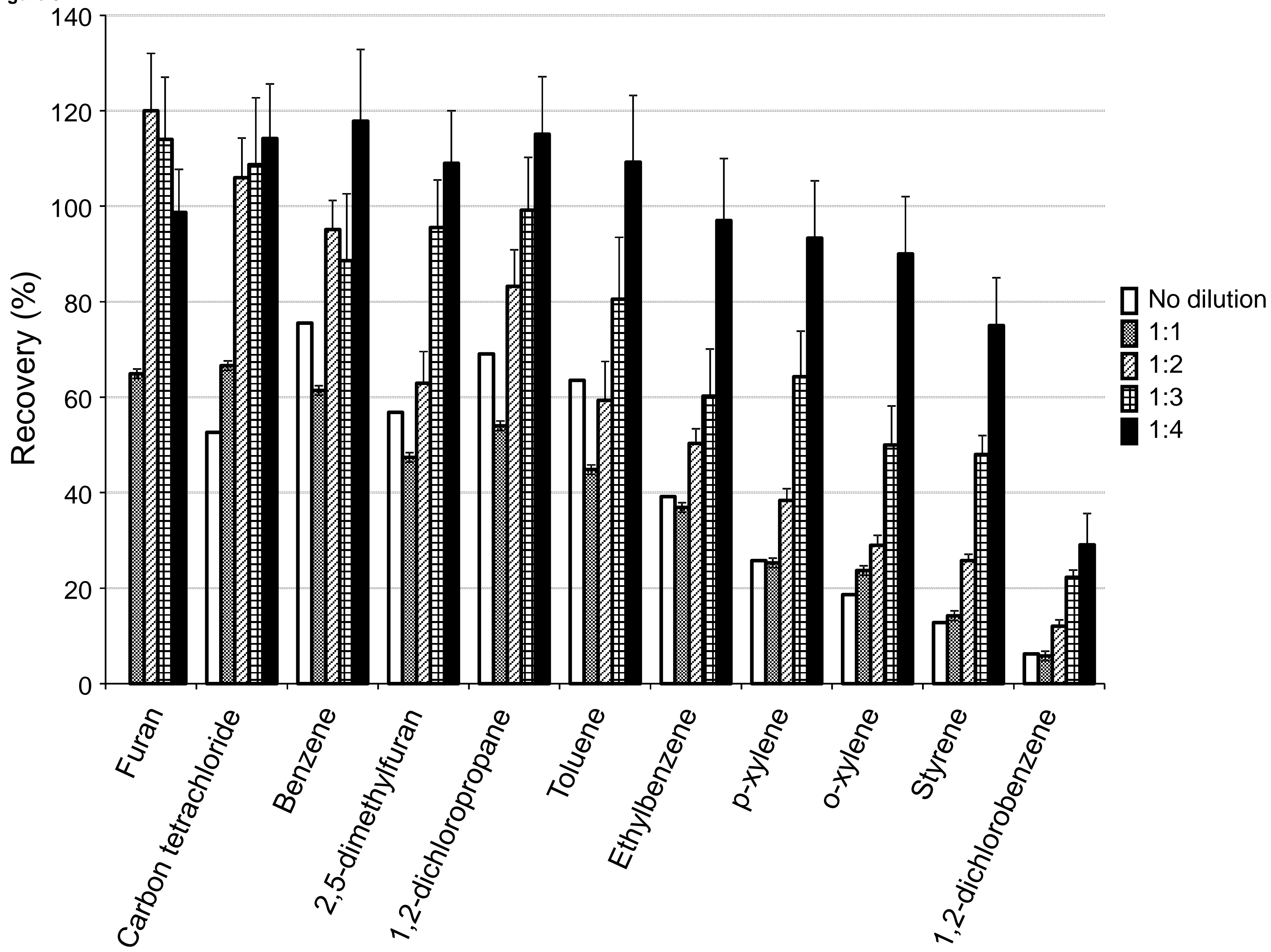

Figure 4

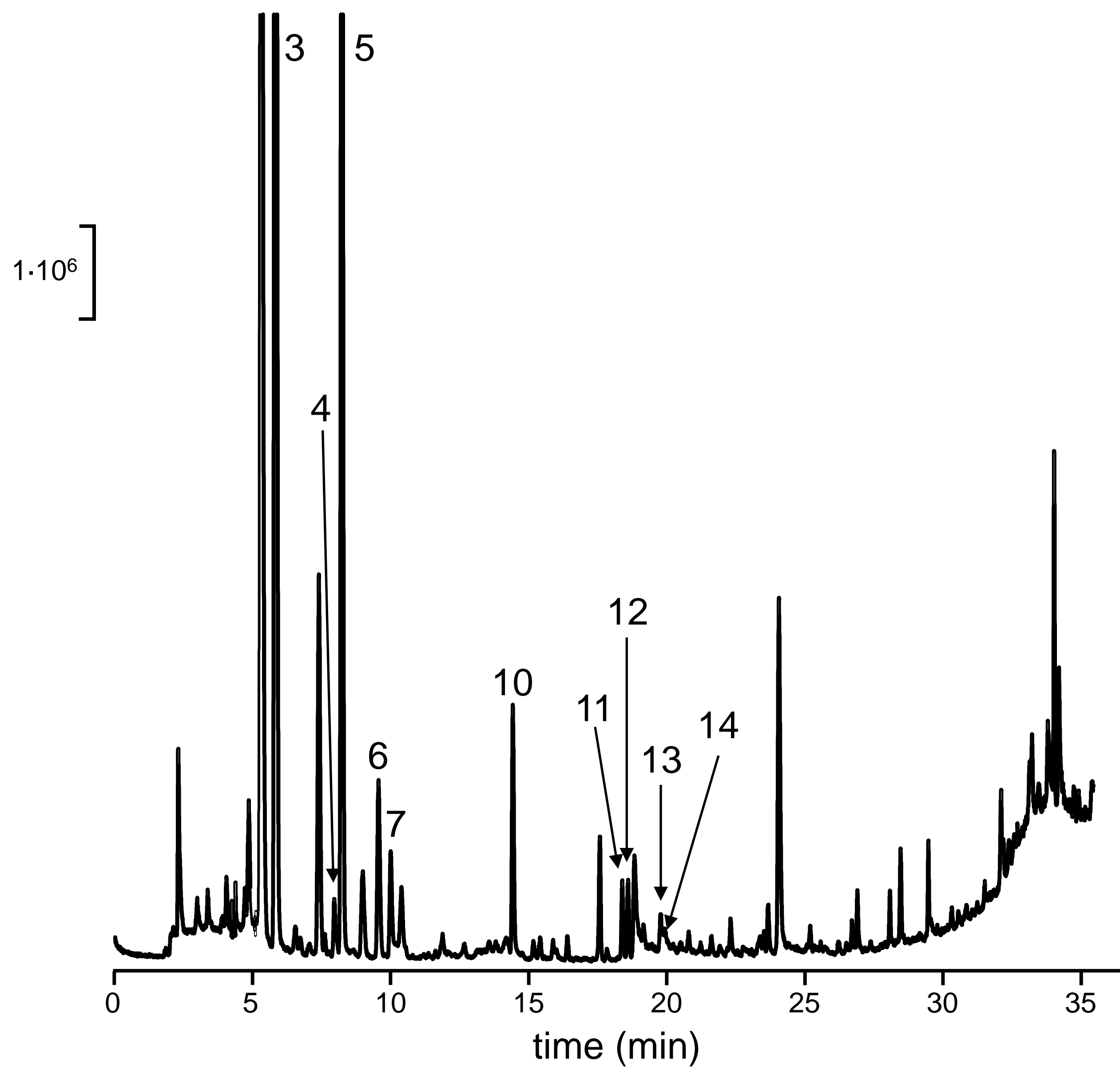

Figure 5