Findings in the hair of drug abusers using pressurized liquid extraction and solid-phase extraction coupled in-line with capillary electrophoresis

Tatiana Baciu, Francesc Borrull, Carme Aguilar*, Marta Calull

Department of Analytical Chemistry and Organic Chemistry, Universitat Rovira i Virgili, Sescelades Campus, Marcel·lí Domingo, 1, 43007 Tarragona, Spain

Corresponding author

* Dr. Carme Aguilar
Department of Analytical Chemistry and Organic Chemistry,
Universitat Rovira i Virgili,
Sescelades Campus, Marcel·lí Domingo, 1, 43007 Tarragona, Spain
Tel.: (+34) 977 55 86 29
Fax: (+34) 977 55 84 46
E-mail: carme.aguilar@urv.cat

Abstract

A suitable method has been developed and validated for simultaneously determining cocaine and its major metabolite, benzoylecgonine, 6-acetylmorphine, codeine, morphine and the methadone enantiomers in human hair samples by the in-line coupling between SPE and CyD-assisted CE with a previous sample pretreatment procedure based on pressurized liquid extraction. Optimal separation was achieved on a fused silica-capillary of 50 µm i.d. and 80 cm total length using 11 mM α-CyD in an aqueous solution of 80 mM sodium phosphate at pH 2.5 as the separation medium and an applied voltage of 30 kV. The SPE-CE device consisted of a short length of capillary packed with Oasis HLB sorbent, which was inserted near to the inlet end of the CE capillary. Several parameters affecting the in-line preconcentration were evaluated. The LOQs reached for hair samples were in the range of 0.3–2.5 ng/mg with satisfactory analytical precision in both intraday and day-to-day experiments (RSDs <13%). Relative recoveries greater than 80% were obtained. The method has successfully been applied to the determination of these drugs of abuse in segmented hair from drug abusers who were undergoing methadone maintenance treatment. The results were consistent with the patients' statements, indicating that the method established herein can be used for verifying a history of drug abuse.

Keywords: chiral CE, cyclodextrin, segmental hair analysis, drugs of abuse, pressurized liquid extraction, in-line SPE

1 INTRODUCTION

Opiates and cocaine (COC) are among the most widely abused drugs due to their ability to give the user a feeling of euphoria. As such, they represent a major social and health problem worldwide [1]. In this context, one of the most important measures to help those who become trapped in the web of addiction is the continuous establishment of detoxification treatment centres, as well as the development of new analytical methods able to monitor prescribed and non-prescribed drug use efficiently and, conversely, to verify drug abstinence during the drug treatment programmes for the evaluation of compliance and success of the treatment.

Compared to more traditional biological matrices, such as blood and urine, hair offers particular advantages, which have already been discussed elsewhere [2,3]. Generally, scalp hair grows at an average rate of 1 cm per month and this means that each centimeter of hair records about 1 month of the individual's use or exposure to a drug [4]. Therefore, 1 cm length segmental hair analysis can provide a map of the drug consumption over time, being very useful in detoxification treatment centres. With this in mind, the purpose of this paper was to establish an analytical method for determining several illicit drugs, particularly 6-acetylmorphine (6-AM), codeine (COD), methadone (MTD), morphine (MOR), and COC and its major metabolite, benzoylecgonine (BZE), in the hair of subjects undergoing MTD maintenance treatment.

Among the analytical techniques currently used, such as GC and LC, CE has also successfully been applied to a broad range of drugs, both therapeutic and illicit, and their metabolites in a wide range of biological matrices, including human hair [3,5]. However, as is well known, one of the most commonly cited limitations of CE is its inherent poor sensitivity. To overcome this issue, different strategies have been developed including in-line coupling between SPE and CE, which has proven to be highly useful for improving the sensitivity of CE in the analysis of different kinds of compounds, including drugs of abuse [5-7].

Hair consists of a complex structure in which the incorporation of xenobiotics is affected by its melanin content and by the lipophilic/basic nature of the drug. Within this context, various strategies have been proposed for isolating different drug groups from the hair matrix [3,8].

To extract basic drugs, such as 6-AM, BZE, COC, COD, MOR and MTD, from hair, procedures based on dissolving by incubating/digesting the hair matrix with various extraction solvents have been widely used, such as methanol (MeOH), diluted hydrochloric acid (HCl), MeOH-acid mixtures and aqueous phosphate buffer solutions. Enzymes have also been proposed as reagents for the isolation of basic drugs from hair [3,8]. However, recently, attention has focused on assisted extraction techniques, such as pressurized liquid extraction (PLE), to address some of the shortcomings of the extraction methods mentioned above. Within this framework, the extraction of several drugs of abuse belonging to different chemical classes from the hair matrix has successfully been performed by PLE, with extraction recoveries between 85% and 100% [9,10].

MTD possess a stereogenic centre allowing the existence of two enantiomers (*R* and *S*) of which, as is well demonstrated today, the opioid activity is attributed to the *R*-enantiomer. In the MTD maintenance therapy of heroin addicts, both racemic MTD and *R*-MTD are applied [11]. Therefore, separate determination of each enantiomer in hair would be useful in the monitoring of patients, particularly those patients taking therapeutically the *R*-MTD and detecting both isomers in their hair samples will indicate an additional abuse of the drug.

Nowadays, CE using CyDs as chiral selectors represents an attractive technique for resolution of enantiomers and numerous applications have been found in the analysis of different chiral drugs of abuse, including MTD, in biological samples (mainly urine) [12]. However, to the extent of our knowledge, only one paper describes the enantioselective detection and quantification of MTD in hair by CE [13].

In view of these facts, the present work aimed to develop a cheap, simple and environmentally friendly method suitable for the simultaneous determination of 6-AM, BZE, COC, COD, MOR and the enantiomers of MTD in human hair through the in-line coupling between SPE and CyD-assisted CE with a prior sample pretreatment procedure based on PLE. The validated method was used to determine the concentrations of these drugs in hair segments from four patients undergoing a racemic MTD treatment programme with the purpose of monitoring the possible illicit use of drugs over the course of four months.

2 EXPERIMENTAL

2.1 Reagents and standards

All reagents used were of analytical-reagent grade. Ultrapure reagent water obtained from a water purification system (Millipore, Bedford, MA, USA) was used throughout this work. 6-AM, BZE, COC, COD, MOR, (*R*,*S)*-MTD and *R*-MTD were purchased from LGC Standards S.L.U. (Barcelona, Spain) as hydrochloride salts. Dichloromethane and MeOH were HPLC grade and were acquired from J.T. Baker (Deventer, the Netherlands). Ammonium hydroxide 28%, formic acid 98%, phosphoric acid 85%, HCl 37%, sodium phosphate, sodium hydroxide (NaOH), α-CyD and β-CyD were obtained from Sigma-Aldrich (MO, USA). Oasis HLB with an average particle size of 60 µm was purchased from Waters (Milford, MA, USA).

Stock standard solutions of 1,000 µg/mL for each compound were prepared in MeOH and stored at -18 ºC. From these standard solutions, working standard solutions of the mixture of all of the compounds at a concentration of 100 µg/mL were prepared weekly by diluting in MeOH and stored at 4 ºC. The solutions with a lower concentration were prepared daily by diluting appropriate volumes of the working standard stock solution in ultrapure water.

2.2 Instrumentation

160 The electrophoretic system was an HP^{3D} CE instrument (Agilent Technologies,
161 Waldbronn, Germany) equipped with DAD. The analytes were detected at 200 nm. Bare
162 fused-silica capillary with an i.d. of 50 and 150 µm were purchased from Polymicro
163 Technologies (Phoenix, AZ, USA). The capillary chamber was set at 25 ºC for all of the
164 experiments. For pH measurements, a Lab pH-meter Basic 20+ (Crison, Barcelona,
165 Spain) was used. PLE was carried out with an ASE 200 Accelerated Solvent Extraction
166 system from Dionex (Sunnyvale, CA, USA).
167
168 ## 2.3 Hair samples
169
170 Hair samples were provided by the CAS Tarragona Drug Addiction Monitoring and
171 Support Centre (GIPPS Health) in Tarragona, Spain. The samples were collected from
172 four subjects (A, B, C and D) with a history of drug abuse and, at the time of sampling,
173 they were undergoing a MTD maintenance treatment in the aforementioned centre.
174 Adult scalp hair grows at an average rate of 1 cm per month and this means that each
175 centimetre of hair keeps a record of about one month of drug usage, so the specimens
176 were collected during four consecutive months, starting the sampling in March 2015.
177 The hair collection procedure was performed as described in a recently published
178 review [3]. Briefly, for each subject, a strand of hair was taken and, once this was fixed
179 with a piece of string, the hair was cut as close as possible to the scalp from the
180 posterior vertex of the head. The samples were then rolled into aluminium foil with the
181 root end marked and subsequently stored in paper envelopes at room temperature in a
182 dry place until analysis.
183
184 ## 2.4 Sample preparation
185
186 To prepare a hair sample for analysis, a washing procedure was applied to eliminate any
187 possible external contamination, followed by the extraction of the analytes of interest
188 from the hair matrix by PLE. For the sectional analysis, the strands of hair obtained were
189 divided into several segments about 1.5 cm long from the site of cutting to the end of
190 the strand. These were individually decontaminated twice by shaking them in 2 mL
191 dichloromethane for 5 min each time by a vortex. After being washed, the hair

192 segments were dried under a gentle flow of nitrogen at room temperature, weighed
193 (100 mg each segment) and subsequently cut with scissors into segments of 1-2 mm.
194
195 To prepare blank and calibration samples, pooled hair was used, prepared by mixing
196 hair collected from several non-addicted female and male volunteers.
197
198 ### 2.4.1 PLE procedure
199
200 100 mg of pooled blank hair, cut into 1-2 mm segments, was thoroughly homogenized
201 with 1 g inert diatomaceous earth with a mortar and pestle. The mixture was then
202 placed in an 11 mL stainless steel cell sealed at both ends with cellulose filters. The
203 extraction was carried out with a single extraction cycle using water adjusted to pH 2.0
204 with HCl 37% (0.1% v/v) as the extraction solvent at 80 ºC and 1,500 psi for 5 min. The
205 preheating time was 1 min, flush volume was 0% of the cell volume and purge time was
206 1 min. The acidic PLE extract (12-13 mL) was transferred to a 25 mL volumetric flask, 50
207 µL of 28% ammonium hydroxide was then added and, finally, it was brought up to
208 volume with Milli-Q water (final pH 9.1). Lastly, 2 mL of this solution was filtered
209 through a 0.45 µm nylon filter, which was directly collected in a microvial for the in-line
210 SPE-CE analysis described below (Section 2.7).
211
212 ## 2.5 CE separation without preconcentration
213
214 The CE separation was performed on a fused-silica capillary measuring 80 cm in total
215 length (71.5 cm effective length) with an i.d. of 50 µm and an o.d. of 360 µm. The
216 separation voltage was 30 kV (positive polarity). The background electrolyte (BGE)
217 consisted of an aqueous solution of 80 mM sodium phosphate and 30 mM of
218 phosphoric acid at pH 2.5 containing 11 mM α-CyD. Prior to the first use, the capillary
219 was conditioned with 1 M NaOH for 40 min and Milli-Q water for 10 min. At the
220 beginning of each day, the capillary was washed with 0.1 M NaOH for 5 min and Milli-Q
221 water for 5 min. The capillary was then conditioned by flushing with BGE for 10 min.
222 Between experiments, the capillary was conditioned with 0.1 M NaOH for 4 min, Milli-Q

water for 4 min and running buffer for 4 min. Standard samples were injected using the hydrodynamic mode applying a pressure of 50 mbar for 10 s.

2.6 Construction of the analyte concentrator

The first step in the construction of the in-line SPE-CE device consisted of cutting 2 mm of bare fused-silica capillary of 150 µm i.d. and 360 µm o.d. A proper cut on both sides of the capillary is essential to obtain an optimum performance of the concentrator. This small piece of capillary named analyte concentrator (AC), was then introduced 1 mm into a 5 mm piece of a polytetrafluoroethylene (PTFE) tubing (Grupo Taper S.A., Madrid, Spain) with an i.d. of 250 µm. PTFE materials can expand hence easy to fit the o.d. of the bare fused-silica capillary. Next, a 7.5 cm segment of bare fused-silica capillary (50 µm i.d. × 360 µm o.d.) was introduced at the other end of the PTFE tubing until connecting with the AC and the free end of this capillary of 7.5 cm was connected to a vacuum pump using a syringe. Afterwards, the AC was introduced into a vial containing the Oasis HLB sorbent with an average particle size of 60 µm and this was loaded into the AC. To guarantee that the particles size of the sorbent is greater that the i.d. of the separation capillary this had previously been sieved through a 50 µm steel sieve. Later, the capillary of 7.5 cm and the AC were moved until the concentrator was placed in the half-way of the PTFE tubing. Finally, the CE separation capillary of 71.5 cm (50 µm i.d. × 360 µm o.d.) was introduced into the other part of the PTFE tubing until to join the other side of the AC. The entire process of fabricating the concentrator was monitored under a microscope.

2.7 In-line SPE-CE procedure

Before injection, the capillary with the analyte concentrator was conditioned at 930 mbar with MeOH for 5 min and Milli-Q water (adjusted to pH 9.1 with 28% ammonium hydroxide) for 5 min. The different stages of the in-line SPE procedure proposed in this paper for CE analysis of the studied drugs are described below. The first step involves the injection of standard solutions or hair extract (adjusted to pH 9.1 with 28% ammonium hydroxide) using a pressure of 930 mbar for 30 min. A sample clean-up is

255 then performed with BGE solution by applying 930 mbar for 2 min. This step allows the
256 removal of untrapped molecules and ensures adequate starting conditions for the
257 separation. Afterwards, the retained analytes are eluted by injecting a plug of MeOH
258 with 2% (v/v) of formic acid at 50 mbar for 25 s. The elution plug is then displaced from
259 the analyte concentrator with the running buffer at 50 mbar for 250 s. Finally, a voltage
260 of 30 kV is applied for the CE separation of the analytes.
261
262 ## 3 RESULTS AND DISCUSSION
263
264 ### 3.1 Optimization of the CZE separation
265
266 According to the *SciFinder* database, COC and (*R*,*S*)-MTD have a p*Ka* of 8.97 and 9.05,
267 respectively, while, for 6-AM, the p*Ka* values are 8.03 and 9.41, for BZE, the p*Ka* values
268 are 3.35 and 10.83, for COD the p*Ka* values are 8.23 and 13.40, and finally, in the case
269 of MOR, the p*Ka* values are 8.25 and 9.48 [14]. Therefore, CZE separation is possible at
270 pH values below 3.35 since, at this pH, the analytes are positively ionized so they can
271 migrate towards the cathode.
272
273 However, previous authors have reported that opiate alkaloids and their derivatives (6-
274 AM, COD, heroin, MOR, narcotine, noscapine, papaverine, etc.) have very similar size-
275 to-charge ratios under acidic conditions and are difficult to separate by CZE [15-18]. To
276 solve this issue, the addition of a CyD such as α-CyD and β-CyD to the BGE has proven to
277 be highly useful for improving the electrophoretic separation of certain alkaloids [15-
278 18].
279
280 Moreover, one of the goals of our study was to develop a CE-based method that allows
281 the simultaneous determination of 6-AM, BZE, COC, COD, MOR and the enantiomers of
282 MTD. As is well known, enantioselective CE separations are generally achieved by
283 dissolving a CyD in the CE buffer.
284
285 Taking these considerations into account, the effect of adding α-CyD and β-CyD to the
286 BGE was investigated in order to determine the most appropriate for our purpose. To

do so, based on the research works cited above, α-CyD and β-CyD at an initial concentration of 6 mM were individually prepared in 80 mM sodium phosphate at pH 2.5 and were then evaluated. The study was carried out by loading standard solutions containing the analytes at a concentration of 30 μg/mL at 50 mbar for 10 s and the applied voltage was 30 kV. Figure 1A shows the electropherograms obtained.

From this figure, it can be observed that the best results in terms of resolution were obtained when α-CyD was added to the running buffer, although complete separation of *S*-MTD and *R*-MTD (peaks 5 and 6, respectively) was not accomplished. In view of these preliminary results, α-CyD was chosen to be added to the BGE for further studies.

Then, the concentration of α-CyD was examined between 6 and 12 mM in order to check if an improvement in the separation of the MTD enantiomers could be achieved. The results indicated that the chiral separation of the racemic MTD increased when the α-CyD concentration was raised to 11 mM, whereas, over this value, a decrease in the resolution achieved thus far was observed. This is explained because the separation can be affected by the decrease in the inclusion constant, which depends on the concentration of CyD. Therefore, the concentration of α-CyD selected was 11 mM.

The study of the separation voltage indicates that the application of a voltage below 30 kV led to higher migration times with no improvement in the resolution, so the separation voltage was kept at 30 kV for further experiments.

Figure 1B shows a standard electropherogram obtained under the optimal CE conditions.

3.2 Preconcentration by in-line SPE-CE

Oasis HLB was selected as the SPE sorbent to enrich the studied analytes due the good results obtained for COC and its major metabolite, BZE, in our previous work focused on the analysis of hair [6]. As is well known, Oasis HLB is a polymeric sorbent with a polar group, with both hydrophilic and lipophilic retention characteristics, and it is therefore

319 able to extract acidic, neutral and basic compounds over a wide pH range. Based on our
320 previous research, in which it was demonstrated that basic analytes are better retained
321 in this sorbent at high pH [6], as well as considering the p*Ka* values of the investigated
322 drugs, the pH of the sample was alkalinized to 9.1 with 28% ammonium hydroxide to
323 ensure the best conditions for retaining the analytes, since, at this pH, these are
324 uncharged (COC and (*R*,*S*)-MTD) or zwitterionic (6-AM, BZE, COD and MOR) and their
325 retention in the Oasis HLB is favoured.
326
327 MeOH containing 2% (v/v) of formic acid was chosen as the elution solvent, since, under
328 these conditions, all of the studied drugs become positively charged and therefore
329 poorly retained and more easily released from the Oasis HLB. This choice was also made
330 based on our previous paper, in which the potential of acidified MeOH to desorb drugs
331 of abuse retained in the Oasis HLB was successfully demonstrated [6].
332
333 Then, in order to achieve high sensitivity in terms of peak area, the elution volume was
334 studied by introducing MeOH containing 2% (v/v) of formic acid for different periods of
335 time from 5 to 30 s at 50 mbar. The results indicated that the peak area for the different
336 compounds increased with the increment of the elution plug time up to 25 s, whereas,
337 beyond this point, the analytical responses in terms of the peak area remained relatively
338 constant, probably because a plug of 25 s is enough to desorb all of the target
339 compounds completely. Thus, 25 s was selected as the elution time for further studies,
340 which corresponds to about 24 nL (calculated using the Poiseuille equation) of organic
341 solvent needed for eluting all of the analytes. This makes the proposed method an
342 attractive and environmentally sustainable analytical tool.
343
344 Injecting large volumes of sample is the simplest way to increase sensitivity and
345 therefore obtain lower LODs. The sample loading time was evaluated between 10 and
346 40 min, injecting a 0.5 ng/mL mixture solution of the analytes at 930 mbar. As can be
347 observed in Fig. 2, in general, the peak areas increased with the sample loading time up
348 to 40 min and this means that the breakthrough volume of the SPE sorbent was not
349 exceeded. However, in the case of MOR, for injection times higher than 30 min, the
350 analytical response in terms of the peak area remained relatively constant. As such,

injection times higher than 40 min were not tested. In the end, to avoid prolonging the overall analysis time excessively, it was decided that a sample loading time of 30 min was reasonable and, therefore, this time was selected to validate the analytical method.

3.3 Hair treatment by PLE

Recently, Sergi *et al.* [9] have shown the potential of PLE for the extraction of several illicit drugs from hair, including COC and derivatives, amphetamines and opiates, using water-MeOH (80:20, v/v) as the extraction solvent followed by analysis by LC-MS/MS. With this in mind, one of our purposes was to develop a PLE procedure for the extraction of 6-AM, BZE, COC, COD, MOR and MTD enantiomers from hair suitable for combination with the method proposed in this paper, which involves a preconcentration step based on SPE coupled in-line with CE.

With the aim of simplifying the sample treatment procedure, our intention was to inject the hair extracts obtained after the PLE process directly into the in-line SPE-CE system for their analysis. To do so, the extraction solvent for the PLE process has to be compatible with the previously established in-line SPE procedure. However, the mixture reported by Sergi *et al.* [9] as the most suitable extracting medium in PLE for the extraction of drugs of abuse from hair is not an appropriate solvent to be used for our purpose because the presence of MeOH while the PLE extracts are loaded in the in-line SPE may lead to the undesired elution of the analytes from the SPE sorbent.

In view of this, to achieve efficient extraction of the target compounds from the hair matrix using PLE combined with in-line SPE-CE, the solvent composition was studied. Other parameters such as the temperature and static time were also researched. Pressure, flush volume and purge time could also have been investigated, but it is well known that these variables do not have a significant effect on the extraction efficiency and, therefore, they were adopted for our purposes from the work cited above. Thus, the pressure, flush volume and purge time were 1,500 psi, 0% and 1 min, respectively.

3.3.1 Solvent

Based on the fact that water penetrates the hair matrix, producing swelling and easy release of the drugs to the hair surface [19], pure water was investigated as the extracting medium. In addition, water adjusted to pH 2.0 was also examined in order to identify the most appropriate extraction solvent. This choice was based on a recently published study, in which a water-MeOH mixture adjusted to pH 2.0 was used as the PLE solvent, providing high extraction recoveries for several drugs of abuse, including the drugs determined in our work, from soil and suspended particulate matter samples [20]. HCl was selected as the acidulant because it is commonly used in acidic digestions of the hair matrix to extract illicit drugs, such as 6-AM, BZE, COC, COD, MOR and MTD [3,8].

To carry out the experiments, 100 mg of pooled blank hair, cut into 1-2 mm segments, were spiked with the drugs at a concentration of 1.25 ng/mg for 6-AM, BZE, COC, COD and (*R*,*S*)-MTD and 2.5 ng/mg for MOR. The extraction was carried out with a single extraction cycle at 40 ºC for 5 min. Extracts with water (12-13 mL) were brought up to a final volume of 25 mL with Milli-Q water and subsequently adjusted to pH 9.1 with 28% ammonium hydroxide. Moreover, extracts containing water acidified with HCl 37% (0.1% v/v) were transferred to a 25 mL volumetric flask, 50 µL of 28% ammonium hydroxide was added and then brought up to volume with Milli-Q water (final pH 9.1).

Figures 3A and B show the electropherograms of the in-line SPE-CE analysis of the hair extracts obtained using pure water and water adjusted to pH 2.0 with HCl 37% as the PLE extraction solvent, respectively. As can be seen, generally, in the case of 6-AM (peak 4) and BZE (peak 7), there was very little change in both solvents tested. In contrast, COC (peak 1) showed higher peak intensity when was extracted using acidified water, while COD (peak 3) and MOR (peak 2) provided higher peak intensities when extracted using pure water. On the other hand, *S*-MTD and *R*-MTD (peaks 5 and 6, respectively) were only recovered when acidified water was used for extraction.

In view of these preliminary results and, taking into account that, when pure water was used as the extractant, no peaks were observed for *S*-MTD and *R*-MTD, water adjusted

to pH 2.0 with HCl was selected as the extraction solvent to be used in the PLE system to achieve the simultaneous determination of 6-AM, BZE, COC, COD, MOR and the enantiomers of MTD.

3.3.2 Temperature and static time

Four extraction temperatures between 40 ºC and 100 ºC were tested to find out the most appropriate for our purposes using the optimal solvent. The other PLE conditions were the same as described above. As shown in Figure 4, overall, the analytical responses in terms of the peak area for all of the tested drugs increased with the extraction temperature up to 80 ºC. Above this value, in the case of BZE and MOR, the peak areas remained relatively constant, whereas, in the case of the others drugs, a slight decrease was observed. Within this framework, temperatures higher than 100 ºC were not tested and 80 ºC was selected as the optimal extraction temperature. Similarly, Baker *et al.* [20] investigated a temperature range of 40–120 ºC in the extraction of several illicit drugs by PLE, including 6-AM, BZE, COC, COD, MOR and MTD, from soil and suspended particulate matter samples using water-MeOH at pH 2.0 as the PLE solvent. The optimal temperature of 80 ºC was selected due to the decreasing recovery of various compounds above this temperature.

Four static times between 5 and 20 min were evaluated using the optimal solvent and temperature, while the other extraction conditions were the same as mentioned above, since this parameter may affect the extraction yield. The results showed that, in our case, the static time does not have a significant impact on the extraction of the compounds from hair because, in all cases, the analytical responses regarding peak area remained relatively constant. As such, 5 min was chosen as the static time.

In view of the values chosen as optimal for extraction temperature and static time, it can be concluded that the time required to complete the pretreatment procedure developed in this paper is about 15 min (2 min for mixing with the diatomaceous earth and 13 min for PLE), which makes this methodology a reasonably attractive candidate

for routine toxicological analysis in comparison with the conventional hair sample preparations mentioned in the introduction.

3.4 Figures of merit for hair samples

Figure 5 shows the electropherograms obtained under the optimal PLE/in-line SPE-CE conditions of pooled blank hair (A) and pooled blank hair spiked with the studied compounds at a concentration of 1.25 ng/mg for 6-AM, BZE, COC, COD and (*R*,*S*)-MTD and 2.5 ng/mg for MOR (B). From the figure, it can be seen that no matrix peaks were found co-migrating with the analytes and clean electropherograms with little chemical background noise were obtained. This shows the specificity of the developed PLE/in-line SPE-CE method.

The proposed PLE/in-line SPE-CE method was validated in pooled blank hair samples in terms of linearity, repeatability, reproducibility, LOD, LOQ and relative recoveries, and the obtained values are presented in Table 1.

The study of linearity was carried out using pooled blank hair spiked with known amounts of each drug ranging from 0.3 to 25 ng/mg of hair, by performing three replicates of each concentration level. The calibration graphs (Table 1) with n=6 data points were constructed by plotting peak areas as a function of the concentration and were linear over the ranges shown in Table 1, with a correlation coefficient for all the analytes of >0.99.

The LODs were determined as the concentrations corresponding to three times the noise signal (n=5) and the LOQs as the lowest calibration level of each compound. The corresponding values, expressed as ng/mg of hair, are also listed in Table 1. In the case of abusers, most drugs of abuse are found in hair in the ng/mg range [3]. For example, in one comprehensive study, 34,626 samples of hair from different sources have been analysed, such as medical and legal, workplace, clinical, research, police (forensics), schools and insurance [21]. Amounts of 6-AM, BZE, COC, COD, MOR and MTD were detected in the hair specimens in the range of 0.2–63.8, 0.1–36.1, 0.2–159.9, 0.2–12.7,

0.2–41.1 and 0.8–98.2 ng/mg, respectively. In view of this and the data provided in Table 1, it can be concluded that the method developed in this work shows suitable sensitivity for determining illicit drug use in therapeutic drug monitoring, drug rehabilitation programmes, workplace and even in forensic cases.

The precision was examined at three different concentrations. Repeatability was evaluated by injecting three replicates of each concentration level in duplicate on the same day and under identical experimental conditions. Intermediate precision was assessed over five consecutive days by injecting three replicates of each concentration level each day. The results presented in Table 1 are expressed as the %RSD of the peak areas. The recovery study was carried out by comparing the peak areas corresponding to pooled blank hair spiked with three different concentrations, with the peak areas obtained for standard solutions containing the same concentration levels, all analysed by using the developed PLE/in-line SPE-CE method. The mean extraction yields values were calculated from three independent analyses. Relative recoveries greater than 80% were obtained (Table 1). The RSD values were less than 15% (data not shown). As can be seen, the method proposed in the present work provides satisfactory results in terms of recoveries and precision.

Compared to other hair analysis methods for drug testing published in the literature [3], the method proposed in this paper is faster, simpler and more cost-effective, as well as highly environmentally friendly, although a relatively large amount of hair (100 mg) is required, which may be considered as a drawback of this approach when sample availability is limited.

3.5 Hair samples from drug abusers

Sixteen hair samples from four subjects (A, B, C and D) undergoing an MTD maintenance treatment programme were collected over a four-month period, from March to June 2015 (at the beginning of each month), and examined for the drugs of abuse under study. All participants started their rehabilitation when they stopped using heroin.

509 Therefore, all subjects were administered with racemic MTD throughout this study.
510 Detailed data concerning these individuals are presented in Table 2.
511
512 Based on that scalp hair grows on average 1 cm per month, 6-AM, BZE, COC, COD, MOR,
513 *S*-MTD and *R*-MTD concentrations were investigated in hair segments 1.5 cm long from
514 the site of cutting (proximal segment) to the end of the strand. Each hair segment was
515 extracted and analysed as described above. The identification of the drugs was
516 performed taking into consideration their migration times and absorption spectra. The
517 results obtained from the real cases are shown in Fig. 6 and 7, and discussed in detail
518 below.
519
520 ### 3.5.1 Findings in the hair of Volunteer A
521
522 In March 2015, a strand of hair of about 1.5 cm long proximal to the scalp, which
523 corresponds to recent drug use, was taken and then analysed. Figure 6 shows the
524 electropherogram obtained. The results, displayed in Fig. 7, indicated that the hair
525 sample tested positive for *S*-MTD and *R*-MTD with concentrations of 4.9 and 7.2 ng/mg,
526 respectively. This finding is consistent because racemic MTD was the prescribed drug.
527
528 Moreover, the hair sample also tested positive for MOR, with a concentration of 2.6
529 ng/mg. Heroin, once ingested, is primarily metabolized in the body to 6-AM, which is
530 further hydrolysed to MOR. However, MOR may also be originated from the ingestion
531 of therapeutic drugs such as the COD and MOR itself [22]. In this context, the Society of
532 Hair Testing recommends differentiating heroin use from COD or MOR use by testing
533 for the presence of 6-AM [23]. MOR was the only opiate drug detected, indicating MOR
534 use on this occasion, although, according to interview data, the individual did not report
535 having consumed this illicit substance.
536
537 In addition to opiates, COC and BZE with concentrations of 5.6 and 7.2 ng/mg,
538 respectively, were also found. To prove ingestion of COC, the Society of Hair Testing
539 recommends the detection of COC and at least one of its metabolites, mainly BZE, as
540 well as a metabolite-to-parent drug ratio greater than 0.05 [23]. The BZE/COC ratio was

of 1.3, suggesting polydrug use by this subject. This finding is in accordance with the data provided by the individual at the time of sampling.

In April, May and June 2015, the hair samples were always proximal to the scalp, which, as mentioned above, corresponds to recent drug consumption. As can be seen in Fig. 7, all of the hair samples analysed were positive for BZE (2.2−11.6 ng/mg), COC (1.5−10.2 ng/mg), MOR (3.8−12.3 ng/mg), *S*-MTD (2.0−6.3 ng/mg) and *R*-MTD (3.1−8.5 ng/mg). The BZE/COC ratios ranged from 1.0 to 1.6.

The detection of COC, its metabolite BZE, and MOR proves that, in spite of abstinence requirements, the patient had been using COC and MOR throughout the whole period of this study.

3.5.2 Findings in the hair of Volunteer B

In March 2015, a strand of hair of about 1.5 cm long proximal to the scalp was taken and then analysed. The results obtained are shown in Fig. 7 and indicate that the hair sample tested positive for 6-AM with a concentration of 0.8 ng/mg, which is a specific marker of heroin use. However, according to interview data, this patient had stopped consuming heroin about two months before our first sampling. Shen *et al*. [22] recently studied the disappearance of 6-AM, MOR and COD from hair after discontinuation of opiate abuse and reported that these substances could be detected in the hair of former drug users for 3−4 months after abstinence began, probably because of the delayed incorporation from tissue deposits and/or sweat.

COC and BZE with concentrations of 0.4 and 0.7 ng/mg, respectively, and, *S*-MTD and *R*-MTD with concentrations of 2.3 and 4.2 ng/mg, respectively, were also found. The BZE/COC ratio was 1.7. Subject B reported using COC during the MTD treatment and, therefore, our findings are reasonable and in accordance with information given by the volunteer.

When this subject was tested again in April 2015, as shown in Fig. 7, the 6-AM concentration (0.6 ng/mg) in the segment proximal to the scalp, which corresponds to recent drug use, had decreased with respect to the previous month and, in May 2015, the hair sample taken proximal to the scalp gave a negative response for this drug. The hair sample collected in June 2015 proximal to the scalp was reported as negative for 6-AM too. This can only be explained by the fact that incorporation from the blood supply had stopped, suggesting that this patient had abstained from using heroin for at least the previous four months. However, the results obtained revealed that, although enrolled in a detoxification programme, this patient has not stopped using COC. This drug and its metabolite with concentrations of 0.5–5.0 and 3.7–10.1 ng/mg, respectively, were quantified in the hair samples taken in April, May and June 2015. The BZE/COC ratios ranged from 0.7 to 11.0. *S*-MTD and *R*-MTD were found with concentrations of 3.7–4.5 and 6.8–11.1 ng/mg, respectively.

3.5.3 Findings in the hair of Volunteer C

In March 2015, a strand of hair of about 9 cm long was taken and divided into six sections of 1.5 cm in length, and then each segment of hair was individually analysed. All of the investigated hair segments tested positive for 6-AM with concentrations of 1.1–2.5 ng/mg. According to interview data, this patient joined to the rehabilitation treatment programme and had stopped consuming heroin about two weeks prior to our first sampling, which means that the segments of hair examined represent the period when the subject was consuming heroin and, therefore, our findings are consistent.

When this patient was tested again in April, May and June 2015, as illustrated in Fig. 7, *S*-MTD and *R*-MTD were found in the segments proximal to the scalp with concentrations of 1.9–5.1 and 3.2–9.1 ng/mg, respectively. These results are reasonable because the hair sample collected in April 2015, represent the beginning of the treatment with racemic MTD and the hair samples collected in May and June 2015, represent the period in the rehabilitation centre.

Moreover, even though this individual had stopped consuming heroin about two weeks before our first sampling, 6-AM was still found in the proximal segments analysed in April, May and June 2015 with concentrations of 1.5, 0.9 and 0.6 ng/mg, respectively. This evidence once again supports the results provided by Shen *et al*. [22], mentioned in Section 3.5.2. In addition, as can be observed, 6-AM concentration has gradually decreased over the three months and this can only be explained by the fact that incorporation from the blood supply had stopped because the volunteer had abstained from using heroin.

3.5.4 Findings in the hair of Volunteer D

In March 2015, a strand of hair of about 8 cm long was taken and divided into five sections of 1.5 cm in length, and then each segment of hair was individually analysed. All of the investigated segments only tested positive for *S*-MTD and *R*-MTD, with concentrations of 4.7–18.5 and 7.6–20.7 ng/mg, respectively. The hair samples taken in April, May and June 2015 proximal to the scalp, which corresponds to recent drug use, also only tested positive for *S*-MTD and *R*-MTD, with concentrations of 7.9–11.3 and 13.8–20.2 ng/mg, respectively. The results obtained were in accordance with the attempted detoxification and this volunteer's compliance with the therapy. In the case of the segments of hair positive for *S*-MTD and *R*-MTD with concentrations out of the calibration range, twofold dilution of the hair extracts obtained after the PLE process was prepared and analysed with the aim to properly quantify.

From the results obtained, it can be concluded that the concentrations found in all cases studied in this work are within the range of those published in the literature. For example, Lendoiro *et al.* [24] determined opiates, COC and their metabolites, among others, in 13 hair samples from forensic cases and reported concentrations between 0.02 and 20.0 ng/mg. Cordero *et al.* [25] analysed hair samples from four polydrug users enrolled on a drug rehabilitation programme. The range of 6-AM, BZE, COC, COD and MOR concentrations found in their hair was from 1.3 to 13.0 ng/mg. Frost *et al.* [13] determined a concentration of 11 ng/mg for *R*-MTD in the specimen of one patient that was undergoing a *R*-MTD treatment. Another published work showed *R*-MTD and *S*-

MTD concentrations in the hair samples of 20 patients undergoing an MTD treatment programme between 0.37 and 1.69 ng/mg and between 0.09 and 1.06 ng/mg, respectively [26].

4 Concluding remarks

A simple and environmentally friendly CyD-assisted CZE method has been developed for simultaneously determining 6-AM, BZE, COC, COD, MOR and the enantiomers of MTD in human hair. The method was fully validated and successfully applied for the quantification of these drugs of abuse in segmented hair samples from drug abusers that were undergoing MTD maintenance treatment. The results obtained showed that this novel strategy, in conjunction with segmental hair analysis, provides an effective tool for determining drug abuse histories, being very useful in the monitoring of patients undergoing rehabilitation and addiction treatment programmes.

Acknowledgements

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765 Figures captions

766

767 **Fig. 1A.** Effect of the type of CyD added to the BGE on the separation of a standard
768 solution containing the target compounds at a concentration of 30 µg/mL. **Figure 1B**.
769 Electropherogram obtained under optimal CE separation conditions of a standard
770 solution containing the target compounds at a concentration of 30 µg/mL. Experimental
771 conditions are reported in the text. Peak assignments: **1**) COC; **2**) MOR; **3**) COD; **4**) 6-
772 AM; **5**) *S*-MTD; **6**) *R*-MTD and **7**) BZE.

773

774 **Fig. 2.** Effect of sample loading time on the peak area of the analytes by in-line SPE-CE.
775 Experimental conditions are reported in the text. The concentration of the analytes in
776 standard samples was 0.5 ng/mL (*n*=3).

777

778 **Fig. 3.** Electropherograms obtained using water (A) and water adjusted to pH 2.0 with
779 HCl 37% (B) as the PLE extraction solvent. Other experimental conditions are reported
780 in the text. Pooled blank hair was spiked with the examined drugs at a concentration of
781 1.25 ng/mg for 6-AM, BZE, COC, COD and (*R*,*S*)-MTD and 2.5 ng/mg for MOR. Peak
782 assignments: **1**) COC; **2**) MOR; **3**) COD; **4**) 6-AM; **5**) *S*-MTD; **6**) *R*-MTD and **7**) BZE.

783

784 **Fig. 4** Effect of the temperature used in the PLE system on the peak area of the analytes
785 by PLE/in-line SPE-CE. Experimental conditions are reported in the text. Pooled blank
786 hair was spiked with the examined drugs at a concentration of 1.25 ng/mg for 6-AM,
787 BZE, COC, COD and (*R*,*S*)-MTD and 2.5 ng/mg for MOR (*n*=3).

788

789 **Fig. 5.** Electropherograms obtained under optimal PLE/in-line SPE-CE conditions of
790 pooled blank hair (A) and pooled blank hair spiked with the studied compounds at a
791 concentration of 1.25 ng/mg for 6-AM, BZE, COC, COD and (*R*,*S*)-MTD and 2.5 ng/mg for
792 MOR (B). Experimental conditions are reported in the text. Peak assignments **1**) COC; **2**)
793 MOR; **3**) COD; **4**) 6-AM; **5**) *S*-MTD; **6**) *R*-MTD and **7)** BZE.

794

795 **Fig. 6.** Electropherogram obtained under optimal PLE/in-line SPE-CE conditions for the
796 hair sample of Volunteer **A** collected in March 2015. Peak assignments **1**) COC; **2**) MOR;
797 **5**) *S*-MTD; **6)** *R*-MTD and **7)** BZE.
798
799 **Fig. 7.** Concentrations (ng/mg) of the drugs found in the hair specimens from Volunteers
800 **A**, **B** and **C** collected in the different months.
801
802
803
804

Table 1. Regression equations, repeatability and reproducibility values, LODs and relative recoveries obtained for hair samples by PLE/in-line SPE-CE.

	COC	MOR	COD	6-AM	*S*-MTD	*R*-MTD	BZE
Linearity (ng/mg)	0.3–12.5	2.5–25.0	0.4–12.5	0.5–12.5	0.5–12.5	0.5–12.5	0.3–12.5
Calibration curve [a]	y=15.531x+5.871	y=1.180x+2.110	y=14.252x+4.486	y=9.920x+5.472	y=7.483x+2.750	y=7.463x+3.307	y=20.524x+7.127
r^2	0.9939	0.9985	0.9969	0.9908	0.9945	0.9981	0.9981
LOD (ng/mg)	0.10	1.00	0.12	0.13	0.13	0.13	0.10
Intra-day RSD of peak area (%, *n*=6)							
0.5 ng/mg	9.68	–	9.70	8.62	7.29	7.84	9.08
2.5 ng/mg	7.39	2.72	2.51	5.54	6.54	5.31	3.61
10.0 ng/mg	8.33	3.07	2.48	5.99	4.60	4.93	3.03
Inter-day RSD of peak area (%, *n*=15)							
0.5 ng/mg	11.31	–	8.80	12.78	10.42	10.46	8.43
2.5 ng/mg	8.40	7.16	9.12	8.94	9.83	8.81	8.01
10.0 ng/mg	8.70	8.62	7.08	7.45	8.52	9.43	7.16
Relative recoveries (%, *n*=3)							
0.5 ng/mg	86.45	–	87.85	84.43	82.74	87.79	91.31
2.5 ng/mg	89.09	82.93	88.07	88.12	90.51	94.56	89.02
10.0 ng/mg	81.15	84.23	95.32	94.05	93.23	92.10	93.27

[a] y: peak area value (mAU x seconds); x: concentration (ng/mg)

Table 2. Characteristics of the four individuals under study.

Subject	Age	Sex	Hair treatment	Substance declared used
A	30–40	M	No	Heroin in use for years; stopped intake about 7-8 months prior to March 2015; COC in use at the time of sampling
B	40–50	M	No	Heroin in use for years; stopped intake about two months prior to March 2015; COC in use at the time of sampling
C	30–40	F	No	Heroin in use for four years; then, from Christmas 2014, sporadic use; stopped intake about two weeks prior to March 2015
D	40–50	M	No	Heroin in use for years; stopped intake about 7-8 months prior to March 2015

Figure 1

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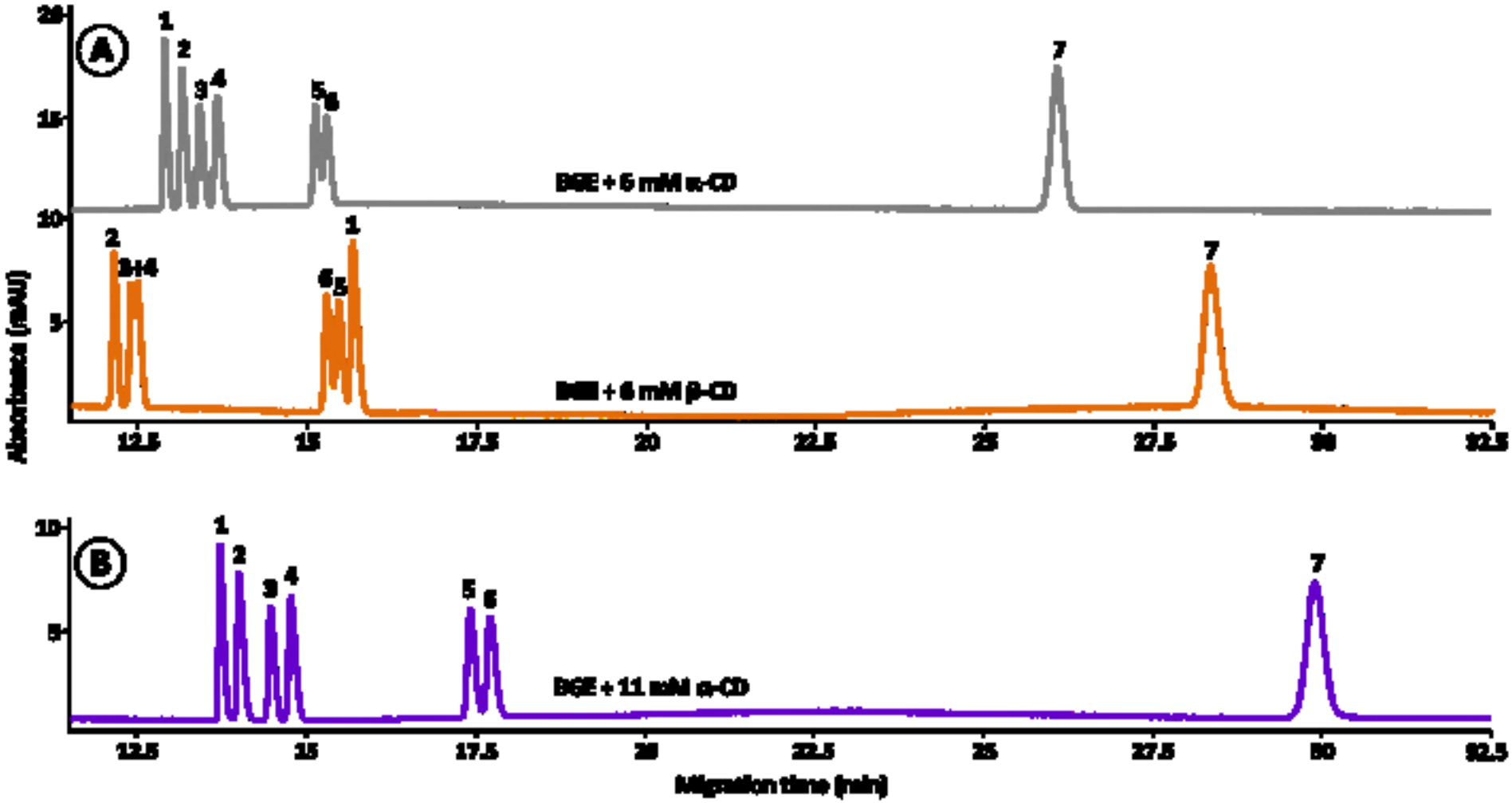

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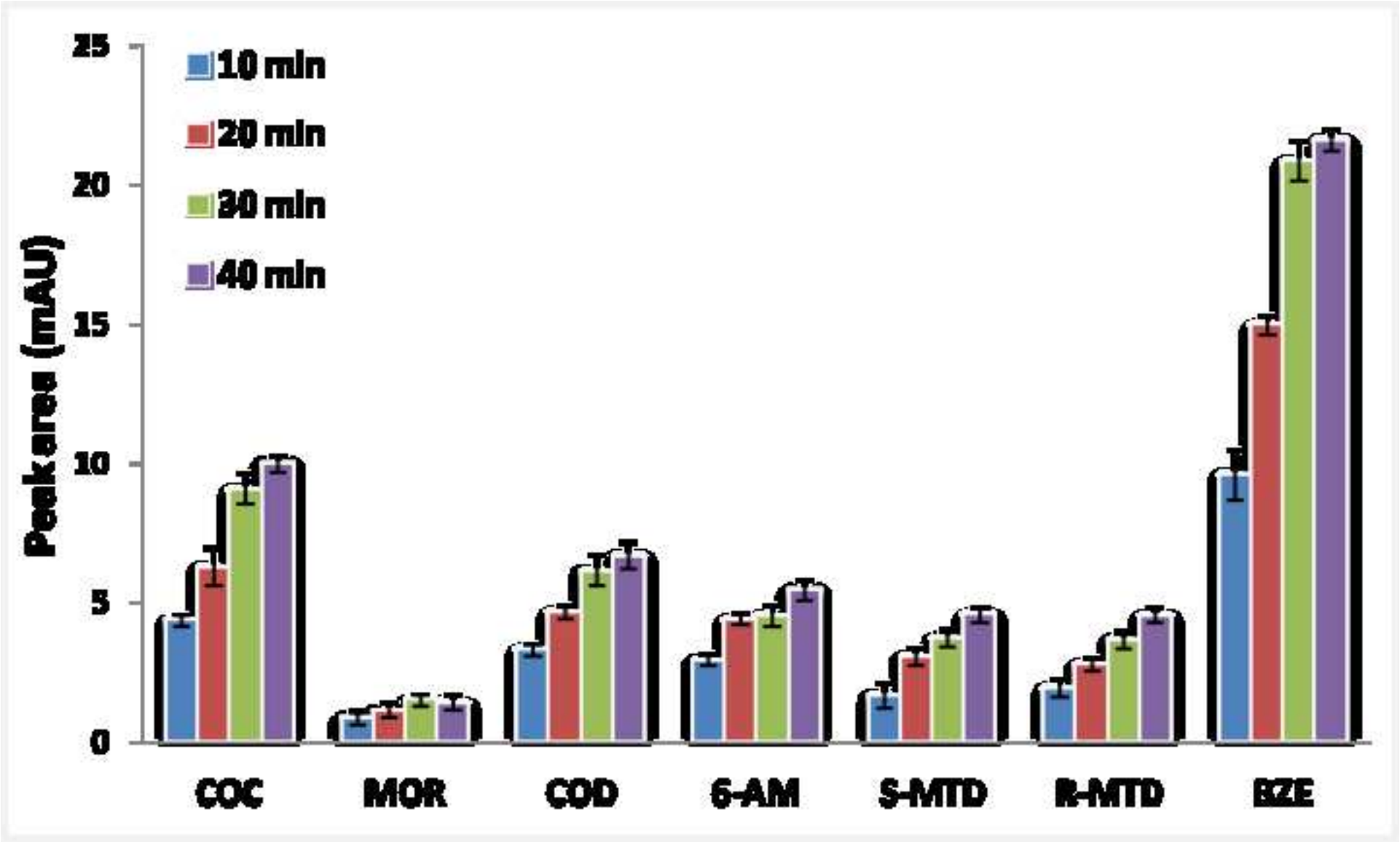

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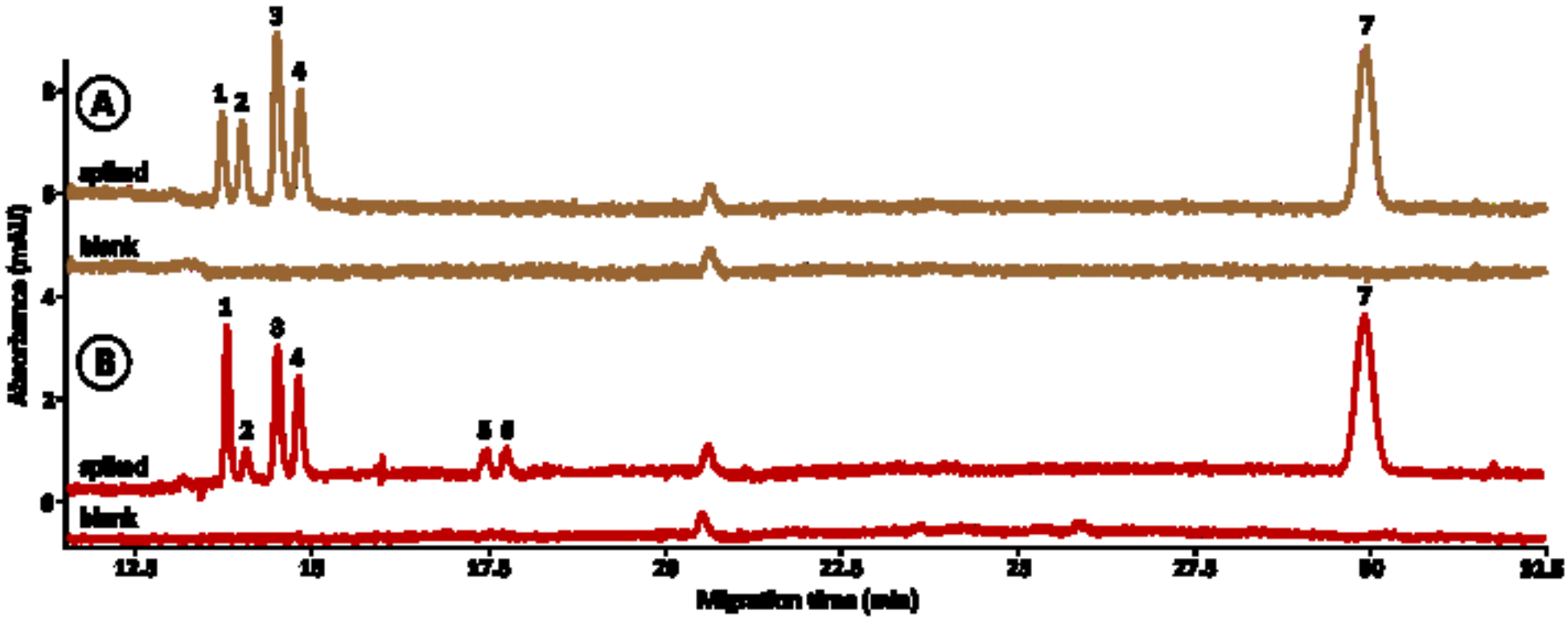

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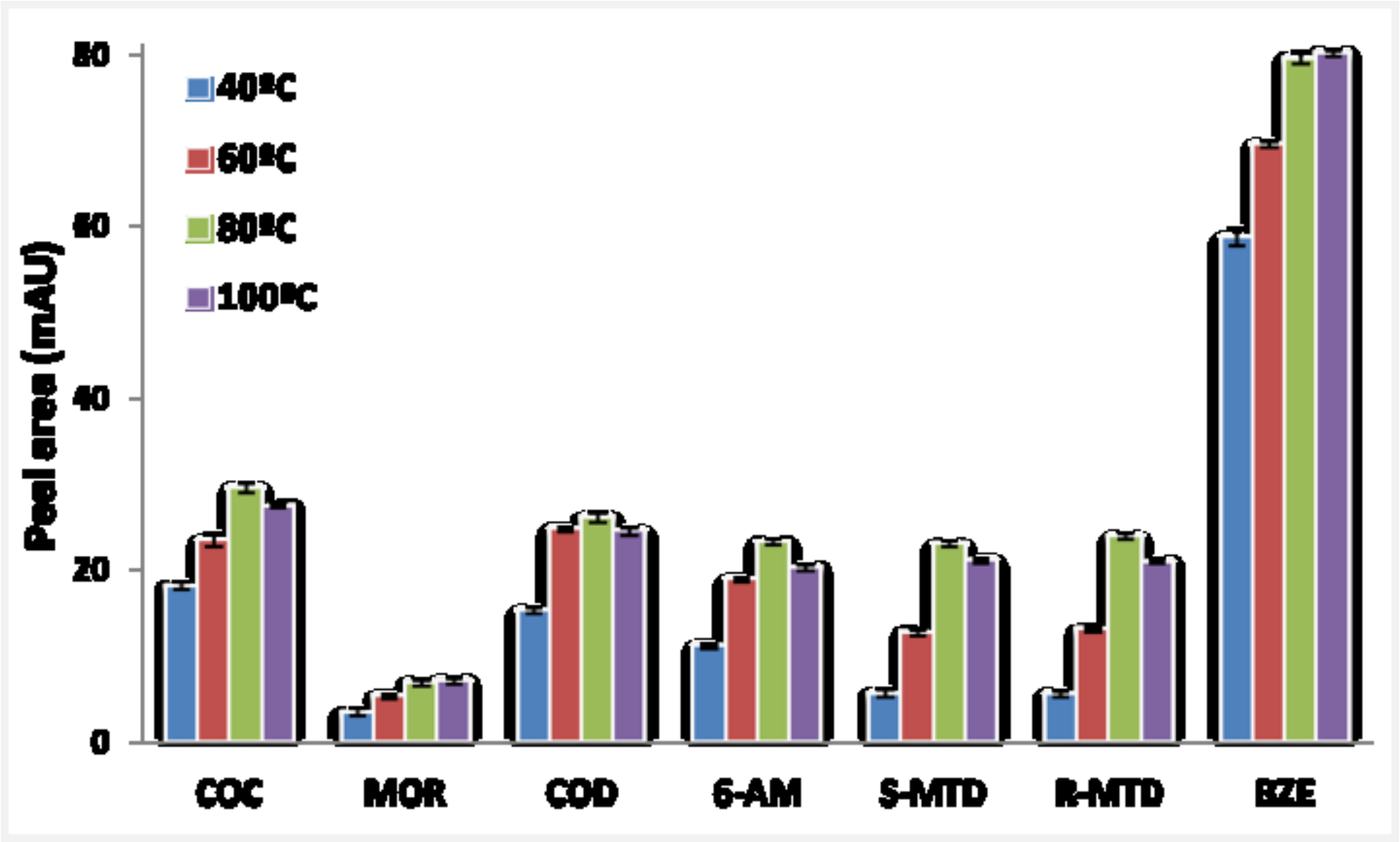

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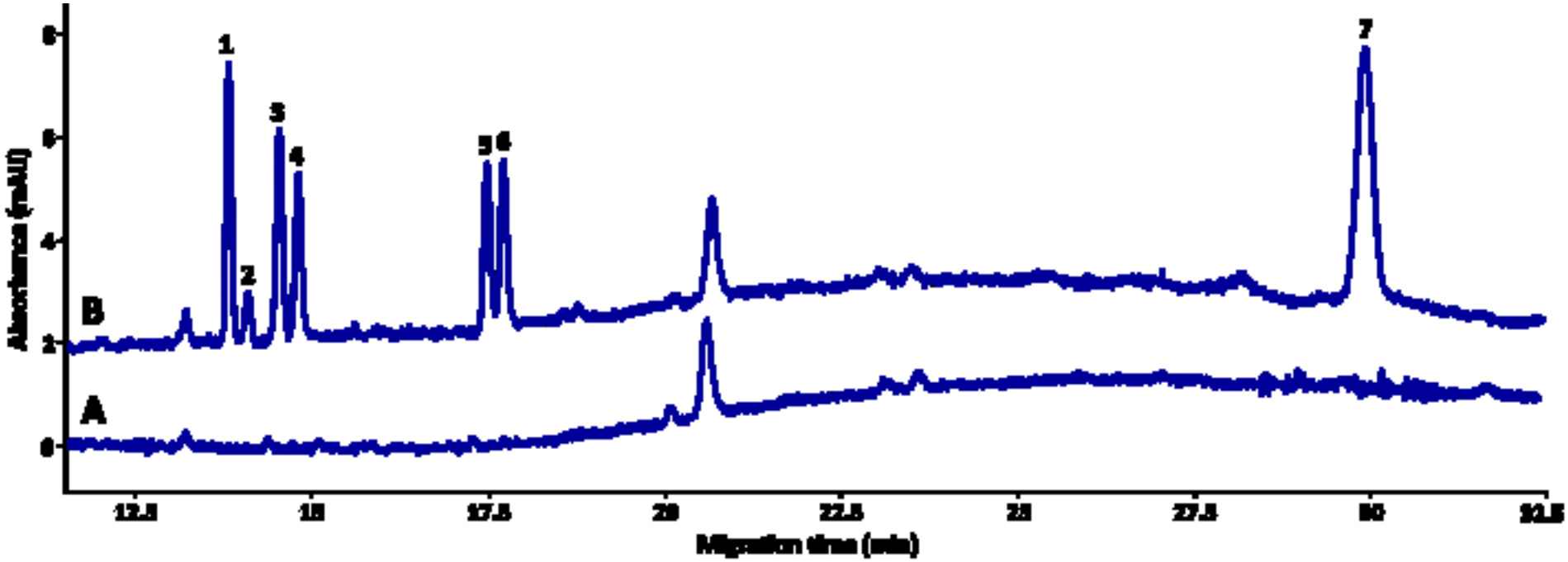

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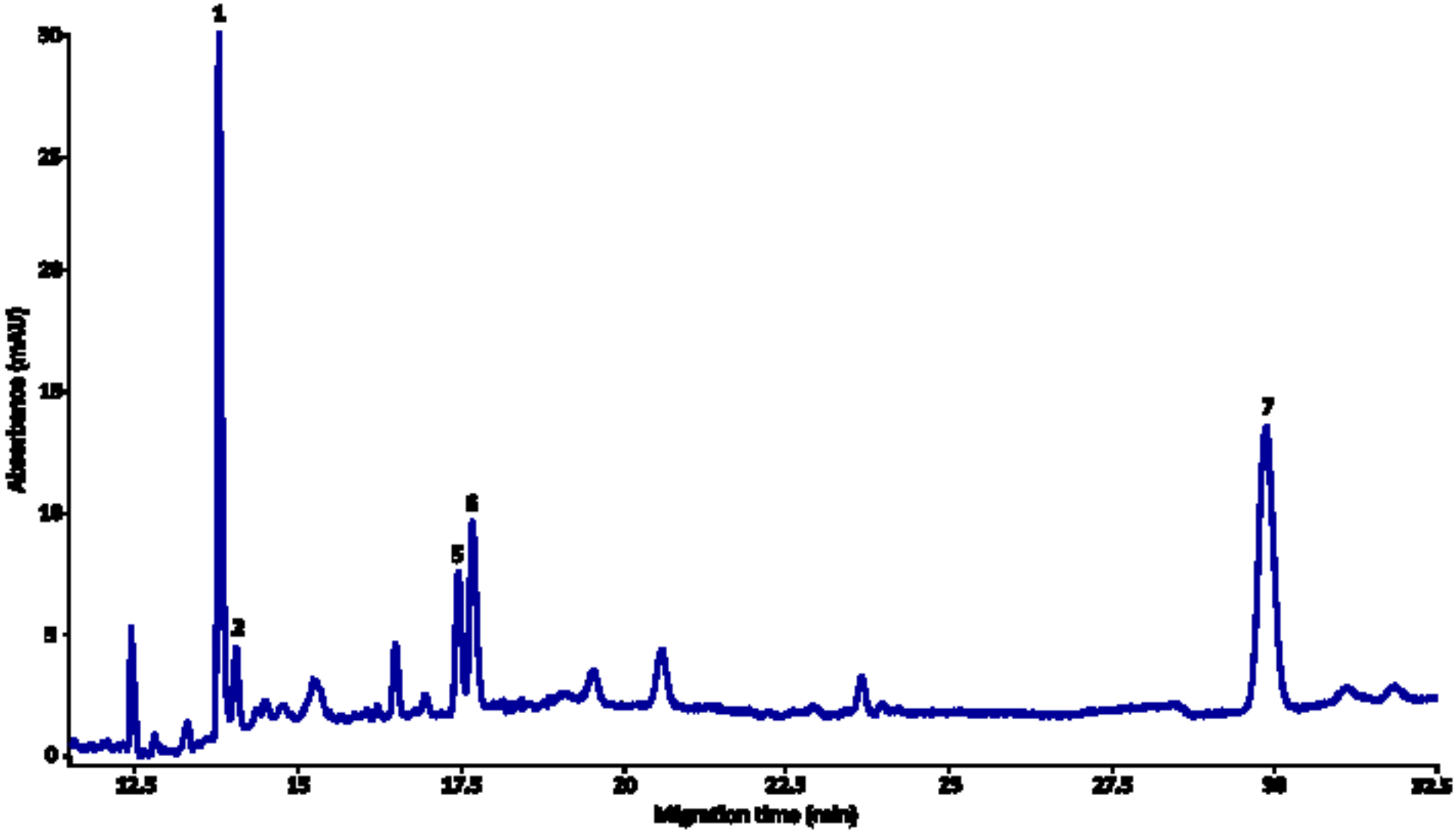

Figure 7

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