Title: A Microdestructive Capillary Electrophoresis Method for the Analysis of Blue-pen-ink Strokes on Office Paper

Author: Matías Calcerrada Miguel González-Herráez Carmen Garcia-Ruiz

PII: S0021-9673(15)00598-1
DOI: http://dx.doi.org/doi:10.1016/j.chroma.2015.04.036
Reference: CHROMA 356456

To appear in: *Journal of Chromatography A*

Received date: 26-2-2015
Revised date: 16-4-2015
Accepted date: 20-4-2015

Please cite this article as: M. Calcerrada, M. González-Herráez, C. Garcia-Ruiz, A Microdestructive Capillary Electrophoresis Method for the Analysis of Blue-pen-ink Strokes on Office Paper, *Journal of Chromatography A* (2015), http://dx.doi.org/10.1016/j.chroma.2015.04.036

- Simple CZE-DAD method for the concurrent separation of acid and basic dyes
- Appropriate analytical performance assessed for qualitative analyses
- Successful application to blue pen strokes of diverse ink nature
- Visual differentiation between brands, models and batches from the same model
- Useful microdestructive separation method for the analysis of questioned documents

A Microdestructive Capillary Electrophoresis Method for the Analysis of Blue-pen-ink Strokes on Office Paper

Matías Calcerrada, [1] Miguel González-Herráez, [2] and Carmen Garcia-Ruiz, [1*]

[1] *Department of Analytical Chemistry, Physical-Chemistry and Chemical Engineering, Multipurpose Building of Chemistry, University of Alcalá, Madrid 28871, Spain*

[2] *Department of Electronics, Polytechnic School, University of Alcalá, Madrid 28871, Spain*

* Corresponding author: Carmen Garcia-Ruiz, Department of Analytical Chemistry, Physical Chemistry and Chemical Engineering, Multipurpose Building of Chemistry, University of Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid) Spain.

e-mail: carmen.gruiz@uah.es

Phone: + 34918856431

Abstract

This manuscript describes the development of a capillary electrophoresis (CE) method for the detection of acid and basic dyes and its application to real samples, blue-pen-ink strokes on office paper. First, a capillary zone electrophoresis (CZE) method was developed for the separation of basic and acid dyes, by studying the separation medium (buffer nature, pH and relative amount of additive) and instrumental parameters (temperature, voltage and capillary dimensions). The method performance was evaluated in terms of selectivity, resolution (above 5 and 2 for acid dyes and basic dyes, respectively, except for two basic dye standards), LOD (lower than 0.4 mg/L) and precision as intraday and interday RSD values of peak migration times (lower than 0.6 %). The developed method was then applied to 34 blue pens from different technologies (rollerball, ballpoint, markers) and with different ink composition (gel, water-based, oil-based). A microdestructive sample treatment using a scalpel to scratch 0.3 mg of ink stroke was performed. The entire electropherogram profile allowed the visual discrimination between different types of ink and brands, being not necessary a statistical treatment. A 100% of discrimination was achieved between pen technologies, brands, and models, although non-reproducible zones in the electropherograms were found for blue gel pen samples. The two different batches of blue oil-based pens were also differentiated. Thus, this method provides a simple, microdestructive, and rapid analysis of different blue pen technologies which may complement the current analysis of questioned documents performed by forensic laboratories.

Keywords

Blue ink, blue pen, capillary electrophoresis, dyes, ink differentiation, profiling

1. Introduction

Questioned documents and their analysis are of relevant importance for forensic laboratories, as different crimes such as forgery, frauds, suicide letters, or even terrorist attacks involve them as evidence. Proof of the wide range of analyses required in these samples is demonstrated in a recent review [1]. The determination of ink composition from blue pens is of special importance, probably due to the number of caseworks leaving them as evidence. Despite the existence of novel analytical instrumentation, compared to the past years, the analysis of questioned documents is often a difficult task. Basically, this adversity is not originated in the analysis itself, but in the results interpretation considering the sample background. One of the most important compounds present in a questioned document are often the compounds contained in the inks. As long as ink formulations are under patent, the identification of compounds can be tedious. In addition, modern manufacturing processes are able to produce large batches of pens with identical ink composition, which makes the differentiation or individualization challenging. These facts, together with other conditions affecting the sample, such as the environment (temperature, light), interferences from the support (office paper for example) or simply the pressure applied during the writing process, make complex the interpretation of the results. Besides, it should be noted that inks degrade over time, and this uncontrollable process must be considered [2]. Finally, these samples play an important role in forensic caseworks related to forgery, and therefore destruction must be minimal in order to preserve the evidence for the court or future analysis.

Given the challenges in ink analysis, different methodologies have been developed [1]. Each technique can provide different results and has its limitations, advantages and disadvantages. Mass spectrometry (MS) techniques have the possibility of unequivocally identifying the species contained in inks, and sometimes are non-destructive methods, but these are expensive and require more resources than other techniques [3]. Recently, spectroscopic techniques have shown their potential to characterize inks, although they might not have sufficient discrimination power, and their sensitivity is lower. For these techniques, the use of the entire spectra without the identification of compounds, also called spectral fingerprint, is a common practice for discrimination purposes [4]. Separation techniques have a destructive character, but they can separate compounds in complex mixtures, and even traditional modes, such as thin layer chromatography, still constitute the official method for ink analysis [5].

Focusing on separation techniques, capillary electrophoresis (CE) is attractive for the analysis of questioned documents: it possesses high versatility, is less expensive than other separation techniques, needs minimal amount of sample, and also provides higher sensitivity than other techniques like vibrational spectroscopy, which can be of interest for differentiation processes; yet CE has been less explored for the discrimination of pen inks than other techniques as HPLC or MS [1]. Various CE modes have been proposed for the analysis of dyes, pens and ink strokes on paper. Reader can consult Table SM1 in the supplementary material file for details on the number of samples, quantity needed, preparation, and separation media employed for these studies. Regarding non-aqueous CE (NACE), few studies have been carried out to analyze dyes and pens. A. Fakhari et al. optimized a NACE method which allowed the separation of different basic dyes (standards). Then, 8 different ballpoint and fiber tip pens (7 blue and 1 black) were analyzed. With the developed method, RSD values lower than 2% in migration times were obtained. These standards were also identified in real samples when using MS as a complementary technique. [6]. H. Zou et al. also employed a NACE method with a similar background electrolyte (BGE). 120 black ballpoint pens were clustered in 6 groups, and good RSDs in migration times (0.63%) and peak areas (3.38%) were obtained [7]. Another possibility is the use of micellar electrokinetic capillary chromatography (MECK). J. A. Zlotnick et al. showed preliminary data on the analysis of black rollerball pens using a borate buffer with SDS. Despite being a limited set, the method proved to be another alternative for the analysis of these samples [8]. Later, J. Mania et al. developed a procedure for the analysis of blue and black ballpoint pens and fountain pens. The method was firstly applied to different blue and black standard dyes. The optimized extractant (water:pyridine 1:1 (v/v)) was useful for the extraction of ballpoint pens (22 samples) and fountain pens (9 samples) but invalid for gel pens (10 samples). Different electropherogram profiles were obtained depending on the type of pen [9]. More recently, J. D. Brewer et al. developed two methods to analyze black and blue inks (10 samples) from ballpoints [10, 11]. However, each method consisted in two different CE analysis of the same sample with different conditions in order to analyze separately anions and cations. Besides, the BGE employed in both cases was quite complex. The cationic method provided more discrimination. However, the UV-Vis and MS spectra were needed to unequivocally differentiate between the samples. On the other hand, with the anionic method, no significant differences were found for the set of blue ballpoint pen samples. Note that apart from dyes, other compounds such as guanidine or copper phtalocyanine were detected and identified [10]. Finally, MECK has also been used for the analysis of inks from inkjet printers. During the last years, P. Kościelniak et al. have developed comprehensive MECK methodologies for the analysis of color and black inkjet printing inks [12-

15], as well as CE-methods for the examination of black inkjet printing inks [16]. As can be seen in Table SM1, the BGE, containing SDS, has also been used to successfully extract the inks from the paper. On the basis of the UV-Vis spectrum of each peak, dyes were clustered in cyan, magenta and yellow, and other peaks (from additives) were considered for discrimination. Colored samples were clustered in 25 different groups, whilst black printing inks were grouped in 3 different groups, demonstrating also a reproducible method.

Regarding capillary zone electrophoresis (CZE), K. Tsutsumi et al. applied a method based on a borate buffer to analyze water-soluble black pens (including rollerball and marking pens). This method allowed the differentiation among most of the samples despite obtaining poor reproducibility in the peak areas, identifying also methyl violet and direct black 154 dyes for the set under study [17]. Later, C. Vogt et al. analyzed blue and black fountain pens by CZE and UV-Vis and fluorescence detection. Separation was performed in a basic medium to assure ionization of the species. 12 different pens (6 blue and 6 black) were analyzed. A poor reproducibility was obtained for migration times, especially for the last peaks. In addition, interferences from the paper were evidenced when using UV-Vis detection. Thus, stacking procedures were recommended for sample preconcentration [18]. Subsequently, authors applied the method to a set containing 5 more samples, obtaining similar results [19]. On the other hand, C-M Shin et al. compared two CE methods (one MECK and other CZE) to analyze one blue ink taken directly from a printing cartridge. Authors found the CZE method useful for ageing purposes, while the MECK allowed more discrimination power. However, this methodology was only tested in one blue ink taken from the pen cartridge [20]. Finally, A. M. López-Montes et al. developed a CZE method to analyze synthetic dyes, by employing a BGE composed of 50 mM ammonium acetate and 15% (v/v) acetonitrile. Despite not being applied to pens for forensic purposes, the method showed a wide range of dyes successfully detected [21].

The methods above described have proved the successful performance of CE for the analysis of dyes and pens, showing the versatility of the technique in terms of modes and separation media employed. However, some of the reported methods have focused solely in the determination/study of the dyes; others have analyzed cationic or anionic species alone; or have focused only in one pen technology and/or type of ink. Also, during the last years, attention has been fundamentally paid to complex modes of CE [6-16], being the most basic mode (CZE) less used. Therefore, this study aims to develop and evaluate a simple CZE-DAD method for the separation of blue inks, and its application to the microdestructive analysis of ink strokes written on office paper. To achieve this, three specific objectives were pursued:

(i) The development of a new CZE-DAD method for the separation of acid and basic dyes by studying the separation medium and instrumental parameters;

(ii) The evaluation of the analytical performance of the developed method in terms of selectivity, LODs and precision; and

(iii) The application of the method to ink strokes written on office paper from blue pens of diverse nature (gel, water-based and oil-based inks).

2. Material and Methods

2.1. Instrumentation and software

The optimization of the method, as well as the application to real samples, was performed in a CE commercial equipment PA 800 plus from Beckman Coulter Inc. (Brea, CA, US). Detection of the species was carried out through a DAD detector equipped with a deuterium lamp ranging from 190 to 600 nm, also from Beckman Coulter inc. (Brea, CA, US).

For the separation of the species, two different capillaries were employed. A conventional fused-silica polyimide-coated capillary of 50 µm internal diameter (id) and another of 25 µm id (both capillaries with 360 µm outer diameter) from Polymicro Technologies (Lisle, IL, US) were employed. For the measurement of the id of the capillaries, an optical microscope from AM Scope (Irvine, CA, US) equipped with a digital camera was used.

For the preparation of real samples, an analytical balance from Ohaus (Parsippany, NJ, US) an ultrasound bath from VWR (Radnor, PA, US) a thermo agitator from Optic Ivymen system Selecta (Barcelona, Spain) and a centrivap concentrator from Labconco (Kansas, MO, US) were employed. The apparent pH of the BGEs was adjusted by using standards at pH 4, 7 and 9 in a 781 pH/ion meter from Metrohm (Herisau, Switzerland). Finally, for the UV-Vis spectra acquisition, a UV-Vis spectrometer Cintra 202 from GBC scientific equipment (Hampshire, IL, US) was used.

Data obtained and analytical parameters were acquired with the Software Karat 32 from Beckman Coulter Inc. (Brea, CA, US). Excel from Microsoft (Redmond, WA, US) was employed to depict the electropherograms in the manuscript.

2.2. Reagents and CE method

For the developed methodology the following reagents were employed: ultrapure-grade water, henceforth as water (Millipore, Billerica, Massachusetts, US), Methanol (MeOH)

analytical grade (Labkem, Barcelona, Spain), Ethanol (EtOH) analytical grade (Labkem, Barcelona, Spain), Hydrochloric acid (HCl) ACS reagent, 37% (Sigma Aldrich, St. Louis, MO, US), and sodium hydroxide (NaOH) pellets, >99% purity (Scharlau, Barcelona, Spain). In addition, for the BGEs preparation, Acetic acid (AcOH) ReagentPlus®, ≥99%, (Sigma Aldrich, St. Louis, MO, US), Ammonia solution (NH_4OH) 32% (v/v), (Sigma Aldrich, St. Louis, MO, US), Acetonitrile (AcN), gradient analytical grade, (Scharlau, Barcelona, Spain) and tris(hydroxymethyl)aminomethane (tris) ACS reagent, ≥99.8%, (Sigma Aldrich, St. Louis, MO, US) were employed.

The 25 µm capillary was washed for the first time by flushing it with EtOH, water, HCl, water, NaOH, water, and the BGE, during 10, 1, 20, 1, 40, 1 and 20 minutes, respectively, applying a pressure of 30 psi and capillary temperature of 40 °C. Besides, the capillary was daily washed with EtOH, water, HCl, water, NaOH, water and BGE for 10, 1, 6, 1, 10, 1 and 15 minutes, respectively, applying a pressure of 30 psi and capillary temperature of 15 °C. Between runs, the capillary was flushed with EtOH, water, NaOH, water and BGE, during 1, 1, 2, 1 and 4 minutes, respectively, applying a pressure of 30 psi and capillary temperature of 15 °C. After each run, the capillary was flushed with water for 1 min at 30 psi and 15 °C.

The final conditions for standards and real samples were a 25 µm id (27.8 µm measured) conventional capillary of 42 cm total length (32 cm length to the detector) for the CE separation. Samples were introduced hydrodynamically at 1 psi during 7 s (1.48 nL of sample, 0.76% of the capillary total length). Both sample tray and capillary were thermostatized at 15 °C. Applied voltage for the CE separation was established at +15 kV (anode in the capillary inlet and cathode near the detection window).

2.3. Standard dyes, real samples and preparation procedure

For the method optimization, 11 different dyes were selected: Basic Violet 3 (BV3), Basic Violet 4 (BV4), Basic Blue 26 (BB26), Basic Blue 7 (BB7), Basic Blue 9 (BB9), Basic Blue 11 (BB11), Acid Blue 9 (AB9), Acid Violet 17 (AV17), Acid Red 2 (AR2), Solvent Blue 38 (SB38) and Phenol Red (PR) all from Sigma Aldrich (St. Louis, MO, US). Table SM2 from the supplementary material file provides more information about these dyes, such as other common names, as well as their color index (C.I.) numbers, molecular masses, chemical structures and UV-Vis spectra. The UV-Vis spectrum of each blue dye was acquired from the UV-Vis spectrophotometer for comparative purposes with the spectra acquired from the DAD

detector. After identifying each standard dyes, mixtures at different concentrations (from 1 to 5 mg/L) were made and used as working range of the method.

Real samples of blue pens were purchased in different shops in Spain and UK, and also samples from different factories, which were facilitated by the Criminalistic Service of Guardia Civil (Spain). Table 1 shows relevant information of each one of the samples under study. Note that some information could not be obtained for some samples, as no information was found on the pens. On the one hand, the analysis of inks from the pen cartridges was performed. In this case, circled-strokes were written on polystyrene weighing pans during 30 s, and 100 µL of the extractant mixture (MeOH:water, 1:1 (v/v)) was applied and mixed until solution of the ink. Note that these samples were only prepared for comparative purposes to identify the interferences from the paper. Besides, most of the ink remained in the polystyrene, thus concentrations were not calculated. Fig. SM1a in the supplementary material file may be consulted to observe the comparison of the pans with ink before and after extraction for a randomly selected sample. 20µL of this solution was added to vials containing 100 µL of extractant. This second solution was directly analyzed in the CE equipment. On the other hand, strokes written on office paper were analyzed (see Fig. S1b from the supplementary material file). Two Ink strokes of 3 cm length from the same pen were written on pieces of multi-purpose paper from DA Alizay (Alizay, France). One of the strokes (top) from each pen remained intact for comparative purposes. The other stroke (bottom) was scratched to collect approximately 0.3 mg of paper containing the ink. Note that most of the powder was composed of paper, thus amounts< 0.3 mg of ink were expected. This method, compared to previous approaches [7, 9-19], avoided cutting the sample, thus the stroke remained with less intensity on the document, but preserving ink and the shape of the stroke. The powder was collected in microcentrifuge tubes from Sorenson (West Salt Lake City, UH, US) and 300 µL of extractant mixture (MeOH: water, 1:1 (v/v)) was added. The sample was then sonicated during 8 minutes for the gel pens and 5 minutes for the liquid and ballpoint pens. Afterwards, samples were stirred during 5 minutes establishing a temperature of 25 °C. A centrivap was finally employed to centrifuge the samples for 5 min. 120 µL of the supernatant was placed in vials (LabBox, China) and introduced in the CE system for their analysis. Despite not achieving a total extraction of the ink from the paper, this approach allowed the detection of several peaks for all the samples analyzed assuring a minimal degradation of the samples.

3. Results and Discussion

In a recent review, it was evidenced that the most employed dyes in ink formulations, independently of the color, have been acid and basic dyes [1]. Bearing in mind the limited knowledge of the ink formulations (often under patent), 11 different standards (6 basic dyes, 4 acid dyes, and 1 solvent dye) were selected for the method optimization. Table SM2 in the supplementary material file collects detailed information of these dyes. From the standards selected, 9 of them were blue or violet, whereas two of them were red, as some blue-ink formulations can add non-blue dyes to obtain the final color. Note that these standards were only used to optimize the electrophoretic and instrumental parameters, and also to evaluate the analytical performance of the method. The optimized methodology was then applied to the real samples targeted in this study.

3.1. Method development

The above-mentioned dye standards were used to optimize a CZE-DAD method able to perform their concurrent separation. For this, a CZE method based on previous literature [21] was tested. A standard mixture of the dyes was prepared at concentrations of 15 mg/L. The BGE was composed of 50 mM AcOH adjusted to an apparent pH 9.00 with NH_4OH, in aqueous solution with 15 % AcN (v/v). This pH allowed a basic medium and the ionization of the species under study, except the standard dye SB 38, which attached to the capillary walls during the voltage application, and was detected after flushing the capillary under high pressure conditions. Thus, it was discarded for subsequent analysis. However, the resulting peaks from the remaining dyes were not well resolved, overall for basic dyes whose migration times were very similar due to their similar chemical structures (as can be seen in Fig. SM2a from the supplementary material file).

To overcome this issue, it was decided to evaluate the separation media, specifically decreasing the pH of the medium (which would allow an increase in resolution between similar species despite obtaining longer run times) and the AcOH and AcN relative amounts. Thus 6 different BGEs were prepared, by changing the concentrations of AcOH (50, 100, 100, 50, 40, 40 mM), AcN (15, 15, 15, 15, 15, 25 % (v/v)), and apparent pH (8, 8, 7.6, 7.6, 7.8, 7.8), respectively. Finally, the separation medium composed of 40 mM AcOH at pH 7.8, and 25 % (v/v) AcN was selected as it provided the most acceptable separation performance, as depicted in Fig. SM2b from the supplementary material file. Nevertheless, through the separation medium optimization, low reproducibility was evidenced, even for subsequent replicate

analyses with previous capillary washing. Note that, however, the working pH of the method (7.8, based on previous literature [21]) was not inside the buffering range of the BGE selected. Consequently, it was decided to change the BGE nature by using a salt with buffering capacity in the desired pH (7.8). Tris was finally selected due to its low and regular current during the voltage application and its buffering pH capacity at pH 7.8 (pKa 8.06). This change allowed repeatable analysis for the subsequent method optimization.

After selecting the separation medium, acid dyes were resolved enough (with resolution between peaks higher than 5), whereas the basic dyes were partially resolved in one peak and one cluster of 4 non-resolved peaks (see Fig. SM2b from the supplementary material file). Instrumental parameters were evaluated in order to achieve the most efficient separation among the basic dyes. The capillary temperature (15, 20 and 25 °C), sample temperature (15, 20 and 25 °C) and separation voltage (30, 25, 20 and 15 kV) were firstly tested. On the one hand, capillary and sample temperature did not affect significantly to the separation, so they were established at 15 °C to avoid sample degradation and solvent evaporation. On the other, final voltage was 15 kV to enhance separations, to enlarge the separation process (overall the cationic zone), and also to maintain a low current between the extremes of the capillary (20 µA), assuring a high stability in the separation medium. Subsequently, the dimensions of the capillary were modified. First, the total length of the capillary was increased, from 30 to 42 cm length. Because of this increase, the separation among these species was considerably improved (see Fig. SM2c from the supplementary material file). Nonetheless, migration times were very similar for BB11, BB7, BV4 and BB26 cationic dyes. Since a good separation of these species was not possible under these conditions, one of them was rejected for subsequent analysis. BV4 was eliminated as it was the less employed in ink formulations, as previous literature reported [1]. Secondly, the id of the capillary was decreased, from 50 to 25 µm id. Despite BB7 and BB4 presented similar migration times, this change allowed the separation of these two dyes from BB11. Thus, this capillary was selected for further optimization.

Finally, instrumental parameters related to the DAD detector were also studied. The scan range selected was the maximum allowed (from 190 to 600 nm). After observing the entire wavelength scan, three different wavelengths were selected: 210, 217 and 598 nm. At 210 and 217 nm, a significant improvement in signal to noise ratio was visually observed. At 598 nm, low signals were obtained. However, most of the blue dyes absorbed around this region, and therefore this wavelength was used as a “confirmation” of blue dyes. This was possible due to the UV-Vis spectra of blue dyes (see Table SM2 from the supplementary material file) which were different from magenta standard dyes and other non-dye compounds

306 possibly found in ink formulations under these CE conditions. Note that, despite relating the
307 spectra to identify dye standard samples [10,11], using the spectra for identification of dyes in
308 real samples was not considered, as lower absorbances were expected and UV profiles of
309 different dyes were very similar (see Table SM2 from the supplementary material file).
310 Between 210 and 217 nm, there were not considerable differences, thus 210 nm was selected,
311 since slightly larger signals were obtained. Note that at this wavelength, non-dye signals and
312 other interferences were expected when analyzing real samples. However, these peaks would
313 be used for discrimination between samples. In addition, other DAD parameters were studied.
314 Bandwidth was tested at 6, 10 and 30 nm. It was evidenced an increase in sensitivity when
315 selecting 6 nm, thus this bandwidth was used for the subsequent analysis.

316 After the CZE method optimization, standard dye mixtures were prepared in the
317 extracting agent selected (MeOH: water, 1:1 (v/v)) at concentrations ranging from 1 to 5 mg/L.
318 Fig. 1 shows a representative example of one of the electropherograms from the standard dye
319 mixture at 3 mg/L and using a separation medium based on tris 100 mM, containing 25% (v/v)
320 AcN (apparent pH 7.8). The instrumental parameters selected were a capillary of 25 µm id and
321 42 cm total length, a capillary temperature of 15 °C, a separation voltage of +15 kV, DAD
322 detection at 217 nm and 6 bandwidth.

323

324 3.2. Evaluation of the method analytical performance

325 Under these selected conditions, the analytical performance of the CZE-DAD method
326 was assessed in terms of selectivity and LOD, parameters to evaluate a qualitative method for
327 the characterization of blue pens through their electrophoretic profiles. Besides, intraday and
328 interday precision was also evaluated. Table 2 summarizes the parameters calculated for a 3
329 mg/L standard dye mixture.

330 Selectivity of the method varied depending on the region under study (cationic or
331 anionic). For the anionic dyes region (from min. 7 to the ending) selectivity of the method was
332 excellent (see Fig. 1). Selectivity was assessed through the resolution between peaks from this
333 region, always above 5 (see Table 2). For the cationic dyes region (from min. 0 to min. 6),
334 selectivity was more limited, as resolution values were lower (see Fig. 1), and even two
335 standards (BB11 and BB7) presented similar migration times, being the resolution between 0.7
336 and 1, due to their similarity in chemical structure (see Table SM2 from the supplementary
337 material file). In fact, given the resolution values between these peaks, they were not
338 considered for the remaining analytical performance evaluation.

Next, LOD was obtained considering a signal-to-noise ratio of 3 for the 3 mg/L standard dye solution. As can be seen in Table 2, LOD values ranged from 0.1 to 0.4 mg/L, demonstrating the high sensitivity of the method. Note that these low values correspond with the high absorption of the chromophore groups in the structures of the dye standards.

Finally, the 3 mg/L standard was employed to calculate the intraday (n=10 during the same day) and interday (n=15 for a period of 15 days, one analysis per day, before the analysis of real samples) precision of the method, as RSD of the migration times of all the standard dyes, as well as the analyte signal (intraday RSD of the peak areas and intensities). RSD values for migration times were in all cases lower than 0.6 %, both for the intraday (n=10) and interday (n=15) precision. These values were complemented by calculating the confidence intervals (95% confidence level) of the intra and inter-day migration times for all the dyes, using the test T-student, as can be seen in Table 2. On the other hand, acceptable precision was obtained regarding areas and intensity peaks, with RSDs lower than 14% for and 11%, respectively.

3.3. Application to real samples

The developed CZE-DAD method was applied to a total of 34 blue pens from different technologies (see Table 1). In order to identify possible interferences, every sample was analyzed directly from the cartridge. For confirmation purposes, analyses of the substrate with no ink (only office paper) were also carried out, showing no significant interferences for most of the analyses (As can be seen in Fig. SM3a from the supplementary material file).

Then, in order to assure a minimally destructive methodology, we proposed a new sample preparation which avoided cutting the sample (usual practice evidenced in previous literature) [7, 9-19]. Considering a previous work, where office paper was pulverized in order to obtain higher derivatization yields of cellulose with a derivatization agent [22], the idea of scratching the ink strokes emerged. Only 0.3 mg of sample was needed to observe signals above the LOD (note that most of this mass came from the paper, thus ink quantity was in fact less than 0.3 mg). By using this rapid method, a microdestructive sample preparation was achieved, as can be seen in Fig. SM1b from the supplementary material file, which shows the lines before and after scratching, for the blue gel samples. In addition, scratching the ink stroke allowed a higher extraction efficiency of the ink. A mixture of MeOH:water 1:1 (v/v) was selected for extraction. MeOH was selected, as it had proven to be an efficient extractant for blue inks on paper [6, 7, 10, 11, 18, 19]. However, and taking into account the purpose of our

method (being applicable for a wide variety of samples with different ink nature) water was included in the extractant, to assure solution of water-based samples. Only 5 min of sonication for blue liquid and ballpoint pen samples was needed, whereas 8 min were needed for blue gel pens. After sonication, samples were stirred for 5 min to maximize the extraction efficiency. This procedure was efficient enough to extract most of the ink sample, without long sonication times and high temperatures which could degrade the samples.

After selecting the extraction procedure, analyses were made in triplicate for each one of the samples. The entire CE-profile obtained for each sample was used for characterization and discrimination purposes. Results obtained have been organized as a function of the pen type, and a final discussion on the discrimination power of the method is subsequently summarized.

3.3.1. Blue gel pens

Five different blue gel pens were analyzed (see Table 1, from G1 to G5). Note that for one of the brands (Zande) there were two different models. Fig. 2. Shows the results of two representative samples (G3 and G2, for the latter the triplicate analyses are shown in the same graphic), whereas Fig. SM3 from the supplementary material file can be consulted for detailed results on the remaining samples. As can be seen, the analysis of the substrate alone (Fig. SM4a) did not provide any signal, thus no interferences from the paper were observed. Due to the variety of results obtained for the gel pens, different areas in the electropherogram were considered. Firstly, basic dyes or other positively charged species (which should appear before the EOF) were not significant for these samples. However, the anionic species provided more information about the samples. Fig. 2a and Fig. SM3b depict the electropherograms of two samples from the same brand (Zande) and different model (G3 and G4). Their profiles were rather similar, with a cluster of peaks around min 8 (zones a_1 and b_1 in the figures). Fig. SM3c and Fig. 2b show the profiles of samples G1 and G2 respectively. These electrophoretic profiles, contrary to samples G3 and G4, have a unique peak at min 8 (zones c_1 and b_1) and other less reproducible zone from min. 12 onwards (zones c_2 and b_2). In the inset of Fig. 2b the three replicates of sample G2 are showed to demonstrate the low reproducibility due to the presence of uncontrollable spikes. However, as can be seen in the inset of Fig. SM3c, their UV-Vis spectrum corresponded to a spectrum similar to those from blue species. Thus, spikes may appear due to the presence of non-soluble blue components, acting as pigments from blue-gel pen ink formulations. Sample G2 also showed a peak around min. 10 (Fig. 2b). Finally, sample G5 (Fig. SM3d) showed two differentiable zones: a cluster of peaks around min. 8 similar to samples G3 and G4 (zone f_1) and other cluster starting from min. 16 (zone f_2). Therefore, a

406 complete differentiation between brands was obtained for these samples. Nevertheless,
407 studies of samples from the same model and different batch were not carried out due to the
408 poor repeatability obtained in some zones of the electropherograms.

409 *3.3.2. Blue liquid pens*

410 Nine pens with different technologies containing blue-liquid inks were analyzed (see
411 Table 1, from L1 to L9). Note that for Pilot samples, there were two different models and for
412 model V7 Hi-Techpoint, 5 specimens were analyzed (2 pens and three cartridges, all of them *a*
413 *priori* from the same batch). Just as the case of gel pens, water-based pens were analyzed with
414 and without paper in order to compare the electropherograms, and also blank samples from
415 the substrate (office paper) were analyzed. No interferences were detected from the
416 substrate. Fig. 3 shows the resulting electropherograms for some of the blue liquid ink strokes
417 on paper under analysis. Fig. SM4 from the supplementary material material shows the
418 resulting electropherograms for the remaining samples. Migration times of some signals,
419 corresponding to blue dyes, were indicated in the electropherograms for comparative
420 purposes, while the other signals corresponded to unidentified charged species from the blue
421 liquid ink formulations, which despite not being identified, facilitated differentiating the
422 samples. As can be seen, blue liquid pens provided, for all the cases, signals after the EOF, thus
423 they comprised acid or anionic dyes in their formulations. Therefore, as blue gel pens, the
424 cationic zone (before the EOF) was not considered for characterization of the inks. Each
425 sample was analyzed in triplicate, and RSD of migration times for all the peaks were lower than
426 0.6, except for sample L5 and L7, whose signals presented RSDs lower than 3% and 0.9%,
427 respectively. Thus, contrary to gel pens, the method did provide repeatable results for blue
428 liquid inks. Sample L1 (Fig. SM4a) was the ink with more blue dyes in its formulation and easily
429 differentiated from the remaining samples in this set. Samples L2 and L3 (Fig. 3a and Fig. 3b)
430 presented two blue dyes (peaks at 9.6 and 9.4, and 15.4 and 14.8 min, respectively). They
431 showed similar CE profiles except for the signal at min. 9.5 in Fig. 3b corresponding to a non-
432 dye charged specie found only in sample L3, which allowed the differentiation between them.
433 Finally, samples from L4 to L9 (Pilot samples) corresponded to Fig. 3c-d and Fig. SM4b.
434 Differentiation between the two models was possible, since L4 (model Vball grip) only
435 presented a blue dye (peak at min 8.5 in Fig. 3c) whilst L5-9 (model V7 Hi-Techpoint) possessed
436 two blue dyes (peaks at min 8.7 and 8.5, and 16.1 and 14.9 in Fig. 3d and Fig. SM4b). As can be
437 seen, differentiation between samples from the same model and batch was not possible under
438 visual comparison. Electropherograms from samples L5 and L6 (Fig. 3d and Fig. SM4b)
439 provided similar patterns and specific signals were not found in any of the electropherograms.

Besides, the analysis of the cartridges from this model (which corresponded to samples L7, L8 and L9) also resulted in similar CE profiles (electropherograms not shown).

Regarding the intra-batch differentiation, peak intensities were corrected by normalization of the signals in order to eliminate the variation originated from the ink collection during the sample preparation process and perform statistical discrimination of the samples. However, once normalized, similar profiles were obtained with slight variation in the migration times (note that precision of migration times was up to 3% for one sample). As these variations only occur with some signals, the correct alignment of the electropherograms was not possible. Therefore, statistical treatments were not applied to the entire electropherogram, as it would cause a false differentiation of the samples inside a batch.

3.3.3. Blue ballpoint pens

Finally, the set of twenty blue ballpoint pens (see Table 1) was analyzed and results showing representative electropherograms of some samples are in Fig. 4. Detailed information on the remaining samples can be consulted in Fig. SM5 from the supplementary material material. For the 20 samples, RSDs in migration times (triplicate) ranged from 0.01 to 0.05, providing the most precise results among the samples selected for this study. Migration times of specific signals for each sample are indicated in the electropherograms. Contrary to the rest of samples, ballpoint pens provided signals in both sides of the electropherogram, thus these samples possessed cationic and anionic species, ionized in the separation medium selected. All samples, except BP15-17 in Fig. 4c (one batch of Bic Cristal) showed an intense peak at min 4.2, which was attributed to the dye BV3, typical in oil-based samples. Sample BP15-17 possessed another unidentified blue dye with a different migration time (4.0 min, indicated with an asterisk in Fig. 4c). Other blue dyes as well as other compounds detected in the samples allowed further differentiations. Samples BP1 (Papermate, Fig. SM5a), BP2 and BP3 (Pentel Kachiri, Fig. SM5b and Fig. SM5c), and BP4-6 (Study Office School, Fig. SM5d) were visually different from the remaining samples in the set. Despite coming from the same model (Pentel Kachiri), BP2 and BP3 showed different CE profiles since their color was different (blue and violet inks). Besides, sample BP4-6 (Fig. SM5d) showed a cascade of decreasing peaks near the BV3 signal (see the inset of the figure), which may correspond to the degradation products of this dye, previously reported in the literature [1]. Considering that the samples were acquired in December 2014, this degradation could come from the manufacturing process, or the samples were sold in a considerable period of time after manufacturing. Samples BP7-9 (Erichkrauss, Fig. SM5e) and BP10-12 (Sainsbury's, Fig. SM5f) provided similar patterns. However, when observing the UV-Vis spectra of the signals, peaks at 4.2 and 4.5 min in Fig.

SM5f corresponded both to blue dyes, whereas in Fig. SM5e only the peak at min. 4.6 was a dye, being the peak at min. 4.6 another component from the ink formulation. Finally, Bic samples provided the electropherograms shown from Fig. 4a-d. For Bic Samples, three different models were selected (Cristal Fine, 4 Colors, and Cristal), and for one of them (Cristal), two different batches were analyzed (years 2010 and 2013). Differentiation between batches (Samples BP15-17 in Fig. 4c and BP18-20 in Fig. 4d) was clear, as one of the batches only showed a signal and it did not correspond to the dye BV3 but to another unidentified blue dye (peak at min. 4.0 marked with an asterisk). However, differentiation between one of the batches and the other two models was not possible under visual comparison. As can be seen, the three electropherograms showed two peaks at the same migration times, for the three cases (see Fig. 4a, Fig. 4b, and Fig. 4d). It was decided to calculate ratios of the peak intensities between these two peaks for the three samples. These ratios were different for the three models (0.34±0.01, 0.62±0.03, 0.52±0.03, see Fig. 4). As the precision of the method and the repeatability of these samples was high enough, ratios for each model did not vary and the differentiation among the models was also possible in this way.

3.3.4. Discrimination power

Regarding the ink technology, a clear differentiation in the electropherograms was evidenced, as each ink nature provided different results (100% discrimination). Oil-based samples were the only showing signals from blue dyes in the cationic zone, apart from the anionic species. Water-based and gel-based samples, on the contrary, only provided significant signals in the anionic zone. Among these two ink types, the differences were clear, as blue-gel pens showed non-reproducible signals in some zones of the electropherograms, possibly from non-soluble components, such as blue pigments, usually contained in this type of ink. Besides, differences in the RSDs of the samples, demonstrated that ink nature play an important role for discrimination purposes.

Focusing on the brand, all samples from different brands and the same technology were visually differentiated (100 % discrimination). For water-based inks, differences were mainly originated by the use of different blue dyes. For the oil-based samples, the dye BV3 was common for most of the samples, and the appearance of other signals from other components in the chemical composition of the inks allowed the differentiation.

When attempting the discrimination between models of the same brand, various Pilot samples (water-based inks) and Bic samples (oil based inks) were analyzed (100 % discrimination). Specific signals from other components (no blue dyes) for Pilot samples were

507 enough to visually discriminate among the models. Despite being possible the differentiation
508 between models for the Bic pens, the use of peak ratios was needed.

509 Finally, two batches from the same brand and model (Bic Cristal) were subjected to
510 analysis. Surprisingly, a clear differentiation was evidenced among this set of samples, even
511 clearer than the differentiation between models from Bic Samples which required the use of
512 peak ratios.

513 Samples from the same brand, model, and batch, have been analyzed for both water-
514 based and oil-based inks. Despite encountering slight differences in peak intensities and
515 migration times, the similarity of the electropherograms was evidenced. As commented
516 before, the slight variation in migration times for some peaks did not allow the correct peak
517 alignment, and statistical treatment was not performed to avoid false differentiations. Thus,
518 no differences between specimens from the same brand, model and batch were found in this
519 way.

520

521 4. Concluding remarks

522 Throughout this manuscript a CZE method has been optimized with different dye
523 standards and has been applied for the microdestructive analysis of blue ink strokes from
524 different nature and different pen technologies, extracted from office paper. The following
525 conclusions are extracted from the results obtained.

526 On the one hand, the optimized method shows relevant advantages over the previous
527 reported literature, since it:

528 (i) Can detect concurrently acid and basic dyes

529 (ii) Uses the simplest CE mode, which facilitates routine analysis

530 (iii) Has been qualitatively evaluated, obtaining excellent selectivity for acid dyes
531 and acceptable selectivity for basic dyes, with LODs <0.5 mg/L and assessing
532 precision of the results.

533 On the other hand, regarding the applicability of the method, some advantages are
534 relevant:

535 (i) The proposed microdestructive sample preparation avoids cutting the sample,
536 which is crucial in forensic casework.

(ii) The method is suitable for a wide variety of blue pen technologies and inks of diverse nature.

(iii) A visual discrimination has been successfully applied to differentiate among inks from different nature, brands, models, and batches from the same model, considering the set under study. Discrimination power is similar to other spectrometric and spectroscopy techniques reported in the literature such as LA-ICP-MS and Raman spectroscopy.

Regarding the future perspectives on this issue, differentiation between samples from the same brand, model and batch seems a challenge. Note that, however, these samples are suspected from possessing the same ink, and therefore there would not be differences among them. This, together with the background of the samples (uncontrollable), and the large quantity of specimens with identical ink formulations, make the individualization a difficult and questionable practice. Instead, an effort must be made not in the individualization, but in the correct comparison of strokes for a proper result interpretation.

To conclude, the potential of this method makes it attractive for the forensic analysis of questioned documents, as a wide range of ink-nature samples can be analyzed in a rapid, simple and microdestructive way and avoiding statistical treatment. Therefore, this methodology may be used as a complementary technique for the analysis of blue pen inks in forensic laboratories.

Acknowledgements

M. C. thanks the Ministry of Education (Spain) for his predoctoral grant and also the help provided by Dr. Javier J. Centelles for the UV-Vis spectra acquisition and Ignacio Jimeno for the editing process of figures. Authors also acknowledge the Criminalistic Service of Guardia Civil (Spain) for providing the dyes and Bic samples.

562

563 References

564 [1] M. Calcerrada, C. García-Ruiz, Analysis of questioned documents: a review, Anal. Chim.
565 Acta 853 (2015) 143-166.

566 [2] C. Weyermann, B. Spengler, The potential of artificial aging for modelling of natural
567 aging processes of ballpoint ink, For. Sci. Int. 180 (2008) 23–31.

568 [3] F. Alamilla, M. Calcerrada, C. García-Ruiz, M. Torre, Forensic discrimination of blue
569 ballpoint pens on documents by laser ablation inductively coupled plasma mass spectrometry
570 and multivariate analysis, For. Sci. Int 228 (2013) 1-7.

571 [4] A. Braz, M. López-López, C. García-Ruiz, Studying the variability in the Raman signature
572 of writing pen ink, For. Sci. Int 245 (2014) 38-44.

573 [5] C. Neumann, P. Margot, New perspectives in the use of ink evidence in forensic
574 science: Part I. Development of a quality assurance process for forensic ink analysis by HPTLC,
575 For. Sci. Int. 185 (2009) 29–37.

576 [6] A. Reza-Fakhari, M. C. Breadmore, M. Macka, P. R. Haddad, Non-aqueous capillary
577 electrophoresis with red light emitting diode absorbance detection for the analysis of basic
578 dyes, Anal. Chim. Acta 580 (2006) 188-193.

579 [7] H. Zou, Z. Wang, N. Ye, R. Xu, R. Zhu, B. Wang, X. Gu, NACE Discrimination of Black
580 Ballpoint Pen Inks, Chromatographia 67 (2008) 483-486.

581 [8] J. A. Zlotnick, F. P. Smith, Separation of some black rollerball pen inks by capillary
582 electrophoresis: preliminary data, For. Sci. Int. 92 (1998) 269-280.

583 [9] J. Mania, J. Bis, P. Kościelniak, An evaluation of the application of capillary
584 electrophoresis to forensic examination of inks, Prob. Forensic Sci. 51 (2002) 71–86.

585 [10] J. D. Brewer, K. H. Hagan, J. M. Egan, Forensic analysis of blue ballpoint pen inks using
586 capillary electrophoresis, For. Sci. Comm. 7 (2005). Webpage: http://www.fbi.gov/about-
587 us/lab/forensic-science-communications/fsc/july2005/research/2005_07_research02.htm
588 (visited in April 2015)

589 [11] J. M. Egan, K. H. Hagan, J. D. Brewer, Forensic analysis of black ballpoint pen inks using
590 capillary electrophoresis, For. Sci. Comm. 7 (2005). Webpage: http://www.fbi.gov/about-
591 us/lab/forensic-science-

592 communications/fsc/july2005/index.htm/research/2005_07_research01.htm (visited in April
593 2015)

594 [12] M. Szafarska, A. Solarz, R. Wietecha-Posłuzny, M. Woźniakiewicz, P. Kościelniak,
595 Extraction of colour inkjet printing inks from printouts for forensic purpose, Acta Chim. Slov. 57
596 (2010) 963-971.

597 [13] M. Szafarska, R. Wietecha-Posłuzny, M. Woźniakiewicz, P. Kościelniak, Application of
598 capillary electrophoresis to examination of color inkjet printing inks for forensic purposes, For.
599 Sci. Int. 212 (2011) 78-85.

600 [13] M. Szafarska, R. Wietecha-Posłuzny, M. Woźniakiewicz, P. Kościelniak, Examination of
601 colour inkjet printing inks by capillary electrophoresis, Talanta 84 (2011) 1234-1243.

602 [15] M. Król, A. Kula, R. Wietecha-Posłuzny, M. Woźniakiewicz, P. Kościelniak, Examination
603 of black inkjet printing inks by capillary electrophoresis, Talanta 96 (2012) 236-242.

604 [16] A. Kula, M. Krol, R. Wietecha-Posłuzny, M. Woźniakiewicz, P. Kościelniak, Application of
605 CE-MS to examination of black inkjet printing inks for forensic purposes, Talanta 128 (2014) 92-
606 101.

607 [17] K. Tsutsumi, K. Ohga, Capillary zone electrophoresis of water-soluble black pen inks,
608 Anal. Sci. 12 (1996) 997-1000.

609 [18] C. Vogt, J. Vogt, A. Becker, E. Rohde, Separation, comparison and identification of
610 fountain pen inks by capillary electrophoresis with UV-visible and fluorescence detection and
611 by proton-induced X-ray emission, J. Chromatogr. A 781 (1997) 391-405.

612 [19] E. Rohde, C. Vogt, W. R. Heineman, The analysis of fountain pen inks by capillary
613 electrophoresis with ultraviolet/visible absorbance and laser-induced fluorescence detection,
614 Electrophoresis 19 (1998) 31-41.

615 [20] C-M. Shih, J-T. Liu, B-H. Chen, C-H. Lin, Separation of crystal violet dyes and its
616 application to pen ink analysis using CZE and MEKC methods, J. Sep. Sci. 31 (2008) 893-897.

617 [21] A. M. López-Montes, A-L. Dupont, B. Desmazières, B. Lavédrine, Identification of
618 synthetic dyes in early colour photographs using capillary electrophoresis and electrospray
619 ionisation-mass spectrometry, Talanta 114 (2013) 217-226.

620 [22] M. A. Fernández de la Ossa, F. Ortega-Ojeda, C. García-Ruiz, Discrimination of non-
621 explosive and explosive samples through nitrocellulose fingerprints obtained by capillary
622 electrophoresis, .J. Chromatogr. A, 1302 (2013) 197-204.

623

624 Figure Captions

625 Figure 1. Electropherograms of the mixture of standards at 3 mg/L concentration: BB9, Basic
626 blue 9; BV3, Basic Violet 3; BB11, Basic Blue 11; BB7, Basic Blue 7; BB26, Basic Blue 26; AB9,
627 Acid Blue 9; AR2, Acid Red 2; PR, Phenol Red; AV17, Acid Violet 17. Electrophoretic conditions:
628 BGE, Tris 100 mM, 25 % (v/v) AcN, apparent pH 7.8 adjusted with HCl; capillary, 25 µm id, 42
629 cm total length; temperature capillary 15 °C; injection, hydrodynamic at 1 psi during 7s;
630 separation voltage, +15 kV, DAD detector: 217 nm, 6 bandwidth. (In color on the web only).

631 Figure 2. Electropherograms of two of the six gel pens (gel-based inks) analyzed in this study.
632 For each sample three replicates were made and one of them is showed as representative
633 example. In b) three replicate analyses for sample G2 to show the irreproducible zone (b_2).
634 Resulting electropherograms of the remaining samples can be consulted in Fig. SM3 from the
635 supplementary material file. Electrophoretic conditions as in Fig. 1. (In color on the web only).

636 Figure 3. Electropherograms of the liquid pens (water-based inks). For each sample three
637 replicates were made and one of them is showed as representative example. Resulting
638 electropherograms of the remaining samples can be consulted in Fig. SM4 from the
639 supplementary material file Electrophoretic conditions as in Fig. 1. (In color on the web only).

640 Figure 4. Electropherograms of the ballpoint pens (oil-based inks). For each sample three
641 replicates were made and one is showed as representative example. In c) and d) specimens
642 from the same brand, model, and batch are depicted by one representative electropherogram
643 (BP15-17 and BP18-20). For a), b) and d), ratios between the two indicated peaks in the
644 electropherogram are showed. Resulting electropherograms of the remaining samples can be
645 consulted in Fig. SM5 from the supplementary material file Electrophoretic conditions as in Fig.
646 1. (In color on the web only).

647

648 Table 1. Available information of real samples analyzed under the proposed methodology.

Name	Brand	Model[1]	Technology	Type of ink	Brand Country[3]	Date (mm/yy)[3]
G1	Study Office School		rollerball	gel	-	-
G2	Grappa				-	--/2012
G3	Zande				China	-
G4	Phondex				China	-
G5	Uniball	Jetstream			Japan	12/2005
L1	Inoxcrom		rollerball	water-based (liquid)	Spain	-
L2	Aihao		rollerball		China	--/2001
L3	Auchan		marker		France	-
L4	Pilot	Vball grip	rollerball		Japan	03/2013
L5		V7 Hi-Techpoint	rollerball			-
L6						-
L7[2]			-			04/2013
L8[2]						
L9[2]						
BP1	Papermate	Inkjoy	ballpoint	oil-based	US	02/2013
BP2	Pentel	Kachiri			Japan	01/2014
BP3						
BP4	Study Office School				-	-
BP5					-	-
BP6					-	-
BP7	Erichkrauss				Germany	02/2013
BP8						
BP9						
BP10	Sainsbury's				Great Britain	-
BP11						-
BP12						-
BP13	Bic	Cristal Fine			France	04/2011
BP14		4 Colours				03/2012
BP15		Cristal				03/2010
BP16						
BP17						
BP18						02/2013
BP19						
BP20						

649 [1] Samples from brands different from Uniball, Pilot, Papermate, Pentel and Bic had not specific model.
650 [2] Samples L7, L8 and L9 were stains made with blue liquid ink from cartridges and no ink technology is specified.
651 [3] Samples with hyphen in brand country or date columns indicate that these data was neither available nor found.

652
653 Table 2. Analytical parameters obtained for the developed CZE-DAD method applied to a 3
654 mg/L standard dye mixture.

	Selectivity (as Rs)[1]	LOD (mg/L)[2]	Precision					
			Migration time (min)		Dye signal			
			Intraday (1 day) (n=10)	Interday (30 days) (n=15)	Area		Intensity (AU)	
			x±CI[3]	x±CI[3]	x	RSD	x	RSD
Basic Blue 9	-	0.4	3.913±0.007	3.92±0.01	749	10	449	8
Basic Violet 3	>5	0.1	4.21±0.01	4.24±0.02	3970	12	2153	8
Basic Blue 11	>2	0.1	4.33±0.02	4.36±0.02	2816	10	2816	10
Acid Blue 9	>15	0.4	8.95±0.04	8.98±0.03	3352	11	575	4
Acid Red 2	>20	0.1	12.33±0.06	12.37±0.05	9117	12	1664	10
Phenol Red	>15	0.2	15.22±0.08	15.25±0.07	7642	12	1160	11
Acid Violet 17	>5	0.1	16.43±0.05	16.29±0.09	15120	14	1857	11

655 [1] Rs value indicated in the table corresponded to the minimum obtained for the 3 mg/L standard dye mixture. BB7
656 and BB26 are not showed in the Table as they were not used to calculate these parameters. Rs values between
657 these two dyes ranged from 0.7-1.
658 [2] LOD is calculated by considering a 3S/N for the corresponding 3 mg/L standard dye mixture.
659 [3] Confidence interval (95% confidence level) applying the test T-Student.

Figure-1

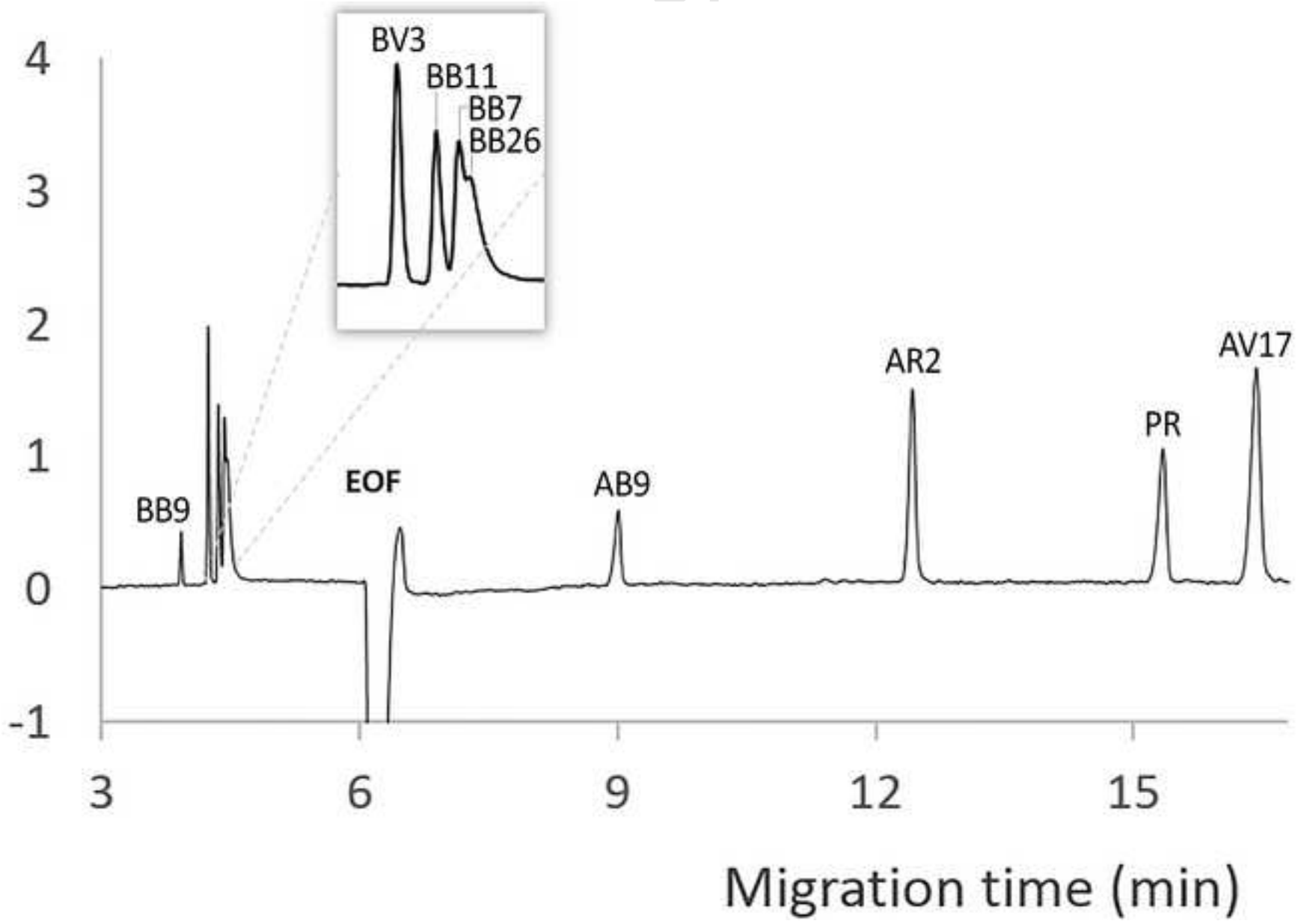

BV3
BB11
BB7
BB26
BB9
EOF
AB9
AR2
PR
AV17
4
3
2
1
0
-1
3
6
9
12
15
Migration time (min)

Figure-2

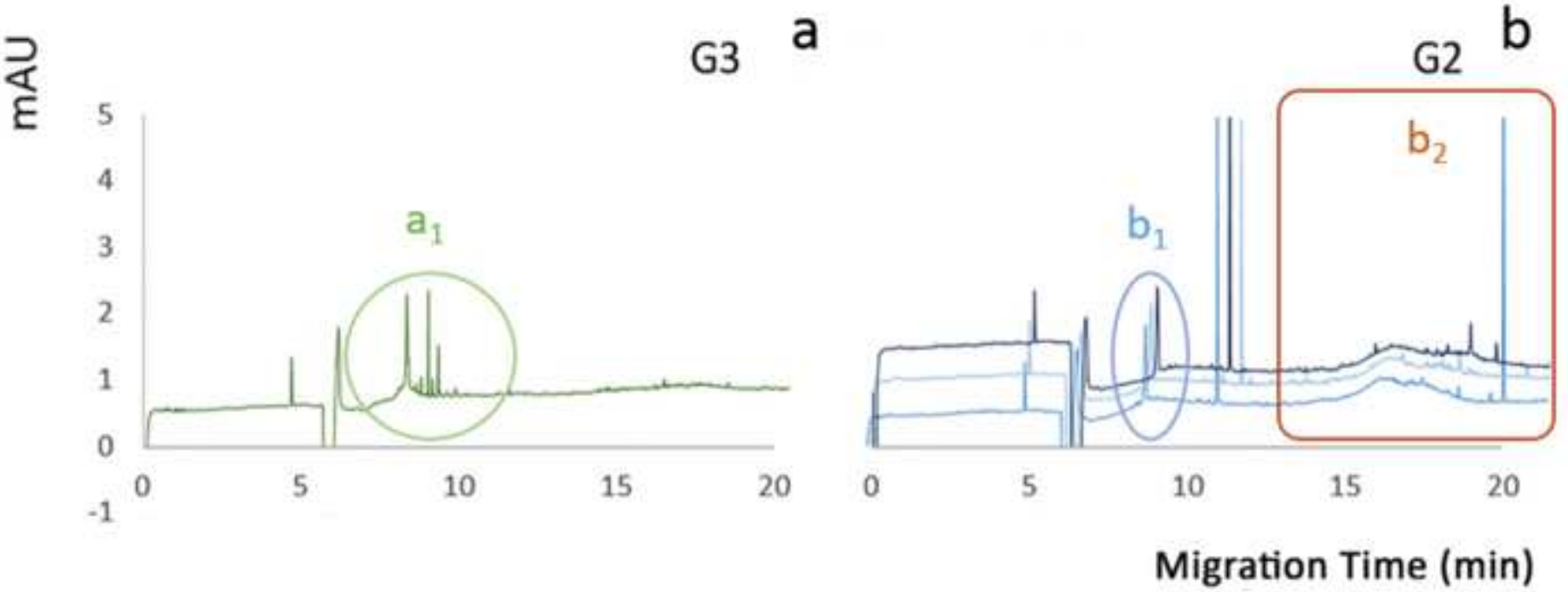

mAU
a
G3
a_1
5
4
3
2
1
0
-1
0
5
10
15
20
b
G2
b_2
b_1
0
5
10
15
20
Migration Time (min)

Figure-3

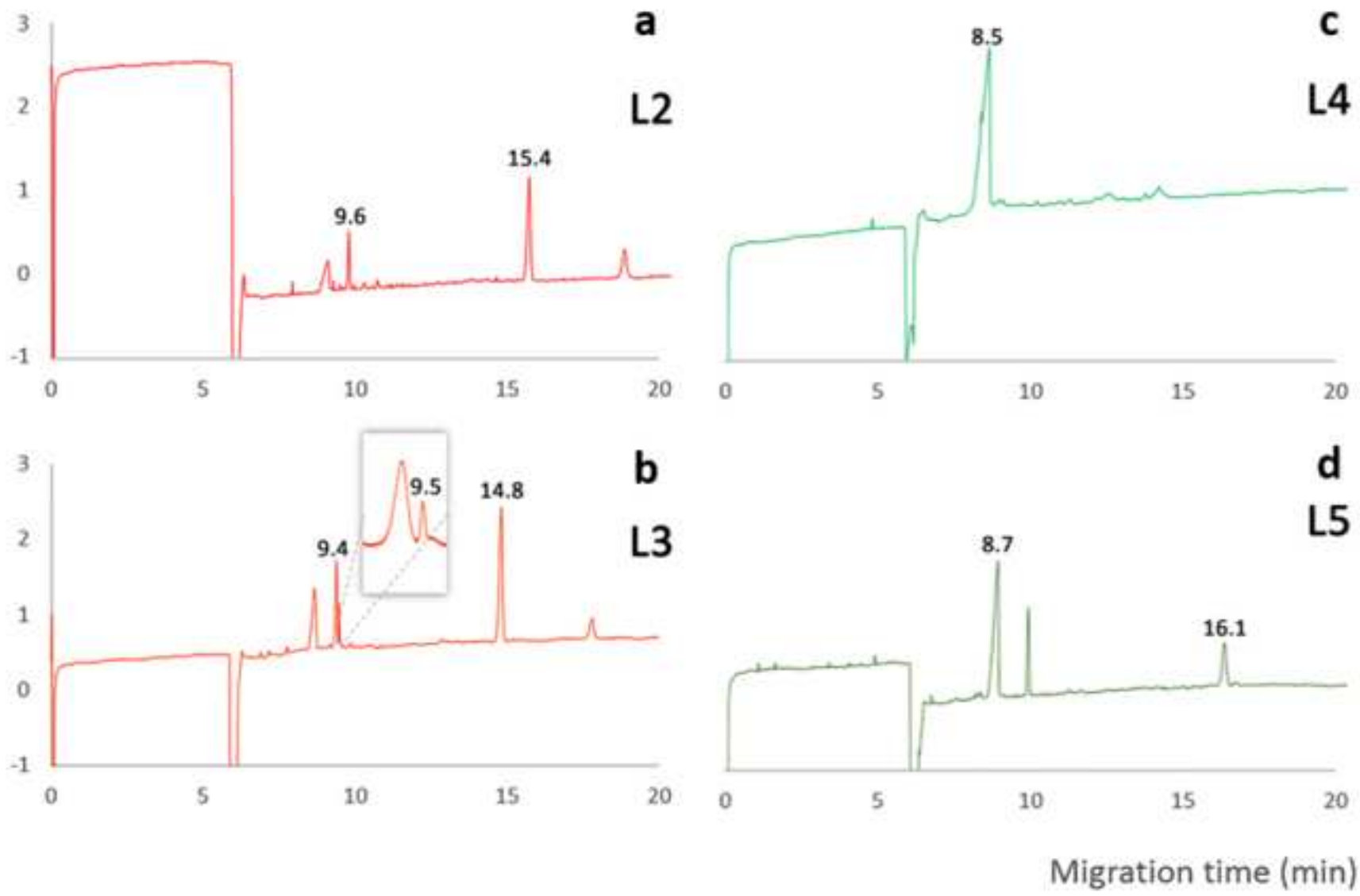
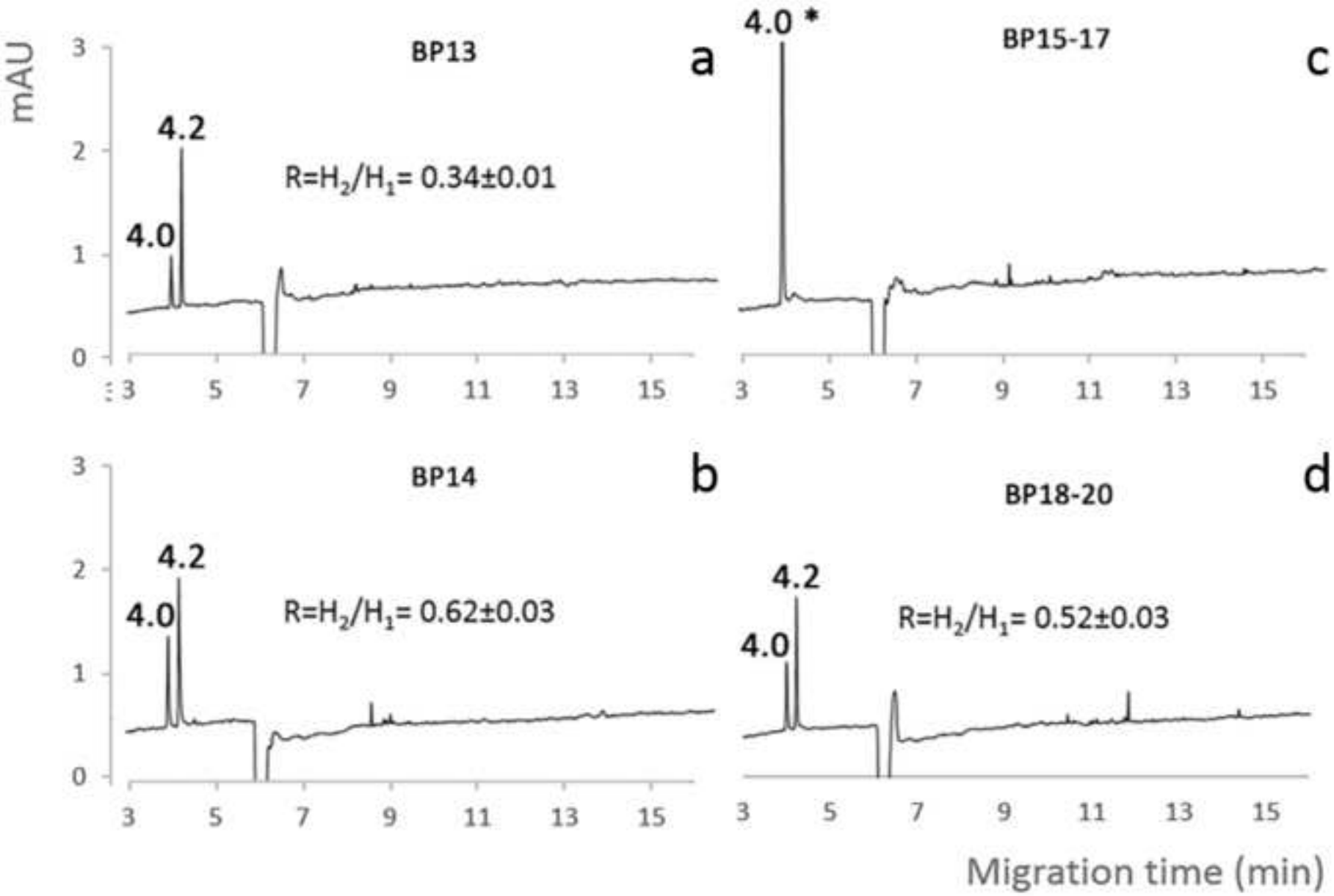

a
L2
15.4
9.6
c
L4
8.5
b
L3
9.5
14.8
9.4
d
L5
8.7
16.1
3
2
1
0
-1
0
5
10
15
20
Migration time (min)

Figure-4


mAU
BP13
a
4.2
4.0
R=H2/H1= 0.34±0.01
BP15-17
c
4.0 *
BP14
b
4.2
4.0
R=H2/H1= 0.62±0.03
BP18-20
d
4.2
4.0
R=H2/H1= 0.52±0.03
Migration time (min)