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Research Article

Analysis and differentiation of paper samples by capillary electrophoresis and multivariate analysis

This work reports an investigation for the analysis of different paper samples using CE with laser-induced detection. Papers from four different manufactures (white-copy paper) and four different paper sources (white and recycled-copy papers, adhesive yellow paper notes and restaurant serviettes) were pulverized by scratching with a surgical scalpel prior to their derivatization with a fluorescent labeling agent, 8-aminopyrene-1,3,6-trisulfonic acid. Methodological conditions were evaluated, specifically the derivatization conditions with the aim to achieve the best S/N signals and the separation conditions in order to obtain optimum values of sensitivity and reproducibility. The best conditions, in terms of fastest, and easiest sample preparation procedure, minimal sample consumption, as well as the use of the simplest and fastest CE-procedure for obtaining the best analytical parameters, were applied to the analysis of the different paper samples. The registered electropherograms were pretreated (normalized and aligned) and subjected to multivariate analysis (principal component analysis). A successful discrimination among paper samples without entanglements was achieved. To the best of our knowledge, this work presents the first approach to achieve a successful differentiation among visually similar white-copy paper samples produced by different manufactures and paper from different paper sources through their direct analysis by CE-LIF and subsequent comparative study of the complete cellulose electropherogram by chemometric tools.

Keywords:
Capillary electrophoresis / Cellulosic samples / Fluorescent labeling / Forensic Analysis / Principal component analysis DOI XXX

1 Introduction

To date, most of the documents daily used around the world are produced from paper. This fact involves these paper documents are a common part of many crime scenes, thus a deep study of the common paper sources becomes crucial. Forensic studies on documents are focused on searching similarities and differences of color, size, shape, composition, fibers, etc. among papers. However, associating one paper sample with another is still an extremely hard issue. The paper manufacturing processes are improved to fabricate homogeneous batches along time. Moreover, the analysis and examination of questioned documents require nondestructive or minimal destructive techniques [1, 2]. As a consequence, an exhaustive and minimal destructive analysis of paper is pursued in the forensic field to differentiate among these practically identical samples.

Paper sources are mainly made of cellulose, a natural polymer composed of linear chains of D-glucopyranose units linked by β (1→4) bonds. Consequently, the knowledge of cellulose properties and its analytical determination are an important part of document examination, combined with the posterior generation of high-quality information for the discrimination among paper sources, which is very useful in the forensic field. However, most analytical methodologies proposed for the analysis of paper focus on studying its elemental composition, avoiding the cellulose investigation. In this respect, infrared [3–6] and Raman [7] spectroscopy as well as ICP MS [8–12], X-ray diffraction [13, 14], GC [15], and image analysis [16] have been proposed to perform these investigations. Only X-ray diffraction has been used to determine differences between paper sources based on the differences of crystallinity that cellulose presents in each kind of sample [13]. In contrast, traditional analysis of paper has involved a deep examination of cellulose through the use of microscopy for the examination of cellulose fibers, in terms of thickness,

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Abbreviations: APTS, 8-aminopyrene-1,3,6-trisulfonic acid; **CBH**, cyanoborohydride; **PCA**, principal component analysis

Colour Online: See the article online to view Figs. 1 and 4–6 in colour.

strength, and morphology [17, 18]. However, this approach is highly dependent on the expertise of the examiner and is also insufficient to detect statistical differences among paper sources [18]. Surprisingly, the forensic study of cellulose was scarcely performed by separation techniques and has never been approached by CE.

CE is an appropriate analytical technique for the study of cellulose. Additionally, CE is a technique of choice in terms of versatility, sample miniaturization, reduced solvent consumption, and well adaptation for analyzing minimal amounts of sample, which is a critical aspect in forensic investigations [19]. This analytical technique presents a high performance in terms of selectivity, efficiency, and resolving power which may be enhanced by using a sensitive detector such as the LIF detector. CE has been used to investigate cellulose as carbohydrate, the components of cellulose fibers, and also the degradation products generated after aging or pulp processes of the cellulose polymer [20–26]. Nevertheless, CE has never been employed for the direct detection of cellulose contained in ordinary papers and its posterior discrimination of the entire CE electropherograms by multivariate analysis.

To study these neutral compounds with high levels of selectivity and sensitivity using CE, a previous derivatization stage with a labeling reagent was necessary. Among the most commonly used labeling agents for oligosaccharides, 8-aminopyrene-1,3,6-trisulfonic acid (APTS) was selected in this work due to the fact that its derivatives have an excitation wavelength (absorption at 488 nm) which is appropriate for the CE-LIF instrument available in our laboratory (Ar-ion laser at 488 nm of excitation) [27,28]. APTS reacts with the reducing D-glucopyranose polymer end by reductive amination, providing three negative charges from the three sulfonate groups of the APTS molecule. Moreover, APTS reagent provides fluorescent properties with an intense absorption at 488 nm. Both characteristics have allowed the selective and sensitive separation of a wide range of monosaccharides and oligosaccharides from different samples such as glycoproteins, drug compositions, food, and pathogens [29] as well as standard carbohydrate mixtures containing glucose, mannose and galactose [27–29], and different kind of starches [30] using CE-LIF methodologies.

Based on the information provided above, in this study we proposed the separation of cellulose from paper using CE-LIF, assisted with a previous labeling process with APTS. The proposed methodology was evaluated in terms of sample preparation and separation conditions. The electropherograms registered for all kind of cellulosic samples were subjected to a posterior multivariate analysis with the aim to find statistical differences that allowed the paper differentiation among samples. To the best of our knowledge, this work presents the first approach for the CE-separation and posterior discrimination of similar white-copy office papers produced by different manufactures and different ordinary (everyday-use) paper samples through the comparative study of their complete electropherogram using multivariate analysis.

2 Materials and methods

2.1 Instrumentation

A Beckman P/ACE MDQ CE system from Beckman Coulter (Fullerton, CA, USA) equipped with LIF detection (4 mW argon-ion laser with excitation at 488 and 520 nm emission filter) was employed. LIF detector sampling rate at 4 Hz was established for all analyses. Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, USA) of 50 μm id and 375 μm od and total length of 50.2 cm (40.0 cm to the detector) were used in all experiments. New capillaries were conditioned prior to their first use by hydrodynamic injection at 20 psi of commercial methanol for 5 min; Milli-Q water for 2 min; 1 M NaOH for 30 min; Milli-Q water for 2 min; 0.1 M HCl for 10 min; Milli-Q water for 2 min, and finally the BGE for 15 min.

Samples were introduced into the capillary by hydrodynamic injection under 0.5 psi during 5 s. Between runs, rinsing cycles by sequential pumping at 20 psi through the capillary of 0.1 M HCl during 2 min, Milli-Q water for 2 min, and BGE during 4 min were performed. The optimum CE separation conditions selected for all analyses were a sample temperature of 15°C (stored inside the CE system), a separation temperature of 25°C inside the capillary, and −20 kV of voltage. Buffer solution of 1.0 M formic acid adjusted to pH 2.0 with 1.0 M NaOH was used as BGE. All solutions were prepared with Milli-Q water and all buffers were filtered through 0.45 μm nylon membrane filters before their use. The BGE was renewed after four runs. Instrument control and data acquisition was performed using 32 Karat™ software (Beckman Coulter).

Samples were weighted on a Discovery DV214CD analytical balance (Ohaus, USA) with a precision value of ±0.01 mg.

2.2 Chemicals and reagents

All reagents were of analytical or reagent grade. Hydrochloric acid and methanol were supplied from Scharlab (Barcelona, Spain). Urea and boric, formic and acetic acids were purchased from Sigma (St. Louis, MO, USA). APTS was obtained from Fluka (St. Louis, MO, USA). Cellulose powder (high purity) and sodium cyanoborohydride (CBH) 1.0 M in tetrahydrofuran were from Aldrich (Milwaukee, WI, USA). Sodium hydroxide pellets were from Scharlau Chemie (Barcelona, Spain). Liquid nitrogen was provided by Statebourne Cryogenics (England, UK). Ultrapure water purified through a Milli-Q system from Millipore (Bedford, MA, USA) was used to prepare all solutions.

2.3 Sample preparation

To achieve an optimal APTS derivatization procedure, solid samples with small particle size were necessary. Since

standard cellulose is commercialized as fine powder, any sample treatment prior to its derivatization process was needless. However, for paper samples and also prior to their derivatization, the edges of the samples were scratched with a surgical scalpel (blade number 20) until approximately 3 mg of fine paper powder were collected for each kind of paper sample. Similar samples of A4 80 gsm white-copy paper from four different manufacturers (three Auchan, three Eroski, three Folder, and three UPM samples) were pulverized as previously to perform their analysis. Five A4 80 gsm white-copy paper samples (Smart, Multioffice, Logic, Copimax and Mondy Economy), five A4 80 gsm recycled-copy paper samples (Natur Papel, Q-Connet, University of Alcalá, DHL and Xerox), six adhesive yellow-note paper samples (Disnak, Jiacheng, Impega, Tartan, Grafoplas and Post-it), and six paper serviettes from several Spanish restaurants were pulverized for the analysis of different paper sources.

For APTS-labeling of samples, 0.30 mg of standard cellulose and 0.30 mg of each commercial paper sample were derivatized with 2 μL of 1×10^{-4} mg/mL APTS solution (15% v/v) in glacial acetic acid. Then, 2 μL of 1.0 M CBH in tetrahydrofuran were added in each sample vial. Vials were closed and protected from light to avoid a possible decomposition of the labeling agent. These solutions were heated in a thermostatic bath at 65°C during 6 h. After the derivatization time and when vials were at room temperature, 46 μL of Milli-Q water were added. Finally, the samples were slightly stirred with the aid of a micropipette.

2.4 Data treatment

The measurement of S/N and LODs were determined at the highest-intensity peak of each electropherogram and when the concentrations gave peak heights three times the noise of the sample. The noise value was measured as the maximum deviations of the baseline obtained in the first 3 min of the electrophoretic profiles. Electropherograms were prepared using Origin Pro v8.6 software (Originlab, Northampton, MA, USA). In order to perform a proper comparison of the electropherograms, a pretreatment of the electrophoretic profiles was initially made with SpecAlign v2.4.1 (University of Oxford, UK). In this pretreatment, baseline correction and multialignment by correlation and peak matching combined method were performed. Data normalization and multivariate data analysis were achieved using the Unscrambler X v10.2 (Camo, Norway) software.

3 Results and discussion

3.1 Method optimization

In order to determine the best conditions for approaching the CE analysis of the commercial paper sources, the sample preparation procedure was investigated. To optimize this step a sample of white office paper was employed. First, a procedure based on pulverization of frozen samples with liquid nitrogen and subsequent pulverization on a ceramic mortar was evaluated. It turned out that this procedure was inadequate for the pulverization of paper samples. Hence, different devices such as meat mincer, blender, cheese grinder, paper shredder, and surgical scalpel were tested. The most successful pulverization process was achieved by using a surgical scalpel. Its use allowed less paper consumption and the finest powder collection compared to the other devices.

Due to the structural similarities among cellulosic and nitrocellulosic compounds, a derivatization and CE separation protocol specifically developed for the study of nitrocellulose [31] was considered as starting point for the determination of cellulosic samples by CE-LIF. Briefly, in this protocol 0.30 mg of sample were derivatized with a fluorescent and charged label-agent by adding 2 μL of APTS and 2 μL of CBH 1.0 M in tetrahydrofuran. The mixture was kept at 65°C for 4 h in a thermostatic bath. Finally, 46 μL of ultrapure water was incorporated in the mixture for diluting and stopping the derivatization reaction. However, since significant differences exist among non-nitrated and nitrated carbohydrates, the derivatization and CE separation parameters were investigated in order to improve the efficiency and sensibility of this methodology focusing on cellulosic compounds. Changes in volume of derivatization agent (4 μL instead of the 2 μL APTS previously used) and derivatization temperature (values of 55 and 75°C were also tested) provided unremarkable changes for APTS-labeled cellulose CE signals. However, an increase of the derivatization time from 4 to 6 h allowed to observe improvements in S/N of about 1.2 orders of magnitude (results not shown). To investigate the influence of CE separation parameters in the determination of cellulose, BGE composition, voltage, and temperature of separation were also evaluated. Borate and formate buffers at pH 2.0 were tested, being 1.0 M formate the buffer which produced the best results regarding the shape and sensitivity of signal peaks and baseline stabilization. Additionally, with the aim of improving the separation resolution of the cellulose signals based on an increment in the buffer density, urea at concentrations from 1.0 to 5.0 M was incorporated in the BGE. Contrary to expectations, no improvement in the electrophoretic separation was observed and losses of signal resolution were registered, as well as an APTS band broadening, which caused an increase in the migration time of the APTS-labeled cellulose peaks of about 2 min. In consequence, urea was eliminated from BGE. Finally, different run voltage (−15, −20, −30 kV) and run temperature (15, 25, 35°C) values were also checked. Maximum S/N were registered at −20 kV and 25°C. Improvements in S/N values of about 6 and 20 orders of magnitude were registered for temperature and voltage, respectively.

Considering these derivatization and electrophoretic conditions, standard cellulose was analyzed. Figure 1 shows its electropherogram. The broad band registered from 4.5 to 6 min was identified as the signal corresponding to the excess of the APTS present in the sample solution while the cellulose signal peaks started at about the 6th min and

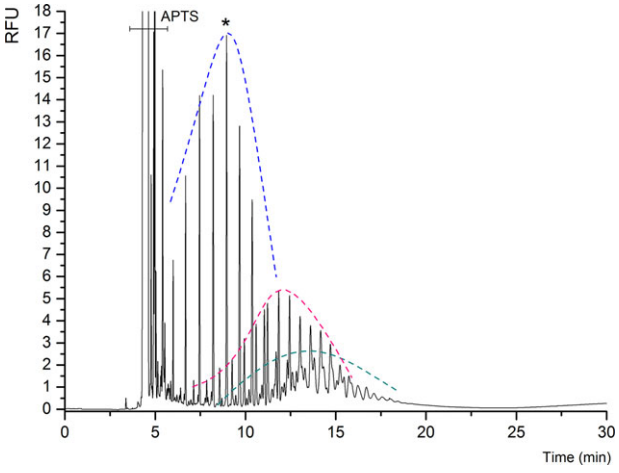

Figure 1. Electropherogram of the APTS-labeled cellulose showing the presence of different Gaussian-shaped distributions (marked for clarifying). Sample: 0.30 mg ATPS-labeled standard cellulose. Derivatization conditions: 2 μL APTS and 2 μL CBH; temperature, 65°C; time, 6 h. CE conditions: uncoated fused-silica, 50 μm id × 50.2 cm (detection window at 40.0 cm); BGE: 1.0 M formate buffer adjusted at pH 2.0 with sodium hydroxide; hydrodynamic injection, 0.5 psi during 5 s; LIF detection at 488 nm (λ_{ex}) and 520 (λ_{em}). (*) peak used for S/N calculations.

continued up to the 20th min. As can be seen, the cellulose electrophoretic profile presented a good peak resolution where at least three different Gaussian-shaped peak distributions were visible. Because it was not the purpose of this work, the cellulose peaks from the Gaussian-shaped distributions were not identified. Besides, these distributions have been referenced before in the literature [32], and they were attributed to the differences of length and DP present in the oligosaccharides due to their polymeric structure.

In order to test the optimized analytical method in practice, the qualitative analysis of 12 similar samples of white-copy paper of four different manufacturers and 22 paper samples from different paper sources (five white-copy, five recycled-copy, six adhesive yellow-notes, and six serviettes paper samples) was performed. Table 1 collects the main characteristics of the analytical procedure that was applied for the analysis of these cellulosic samples. For this purpose, 0.30 mg of each pulverized paper sample were weighted and subjected to the previously described APTS derivatization process. As expected, the electrophoretic profiles (Fig. 2) revealed that all the analyzed samples followed electrophoretic patterns similar to that previously observed for the standard cellulose. The high baseline resolution and the well-defined distributions obtained demonstrated that the sample treatment and methodology was appropriate for studying real paper samples through cellulose APTS-derivatization. LOD values of 6.0 μg/mL for white-copy paper, 1.5 μg/mL for recycled-copy paper, 3.0 μg/mL for adhesive yellow-note, and 17.0 μg/mL for serviette paper samples were calculated.

Despite being the CE-LIF analysis of real paper samples a new analytical method itself, an attempt to discriminate among similar and different types of papers was also made in order to potentially identify the source of paper from a crime evidence and detect statistically significant variations between the different paper samples analyzed in this work.

To assure the correct comparison of the data, the intra- and inter-day precision was studied evaluating the repeatability of a sample in a day and in the different working days of a week (from Monday to Friday). An APTS-labeled paper sample was injected in quintuplicate on the same day and in triplicate for a week (Monday to Friday). As expected, lower values of the RSD (RSD∼5% for the peak area of the first twelve peaks) were obtained for the results collected in a day than those registered during a week (RSD ∼9% for the first twelve peaks). Since the differentiation between white-copy papers were studied in more detail, as an example, Fig. 3 depicts the intra- and inter-day mean and SD obtained for a white-copy paper sample.

3.2 Differentiation of paper samples

A principal component analysis (PCA) model was created with the electropherograms registered at better reproducibility conditions, that is, measured on the same day, to examine the differences among paper samples. For each of the PCA model described for differentiation among similar and different paper samples the weighting was first calculated from 1/ (StdDev) equation to assure representative comparison among classes. Additionally, mean centering and cross validation were applied, and nonlinear iterative partial least squares algorithm was used in order to handle the missing values and evaluate the first few factors of the dataset.

First, since in forensic cases it is frequently necessary to determine the degree of differentiation between two similar pieces of papers, a PCA model considering visually similar pieces of white-copy paper from different manufactures was constructed to evaluate the capability of the CE-LIF method to discriminate them. White-copy paper samples were selected as they are the most common type of papers used worldwide. The PCA 3D scores plot showed (see Fig. 4) a

Table 1. Characteristic of the analytical procedure developed for the analysis of paper samples

Sample preparation	Derivatization with APTS	CE-LIF procedure
Pulverization assisted by a surgical scalpel	Sample : 0.30 mg Reagents: 2 μL APTS + 2 μL CBH Time: 6 h Temperature: 65°C	BGE: 1.0 M formate at pH 2 Injection: 0.5 psi during 5 s Separation: −20 kV, 25°C LIF detection: λ_{ex} = 488 nm, λ_{em} = 520 nm

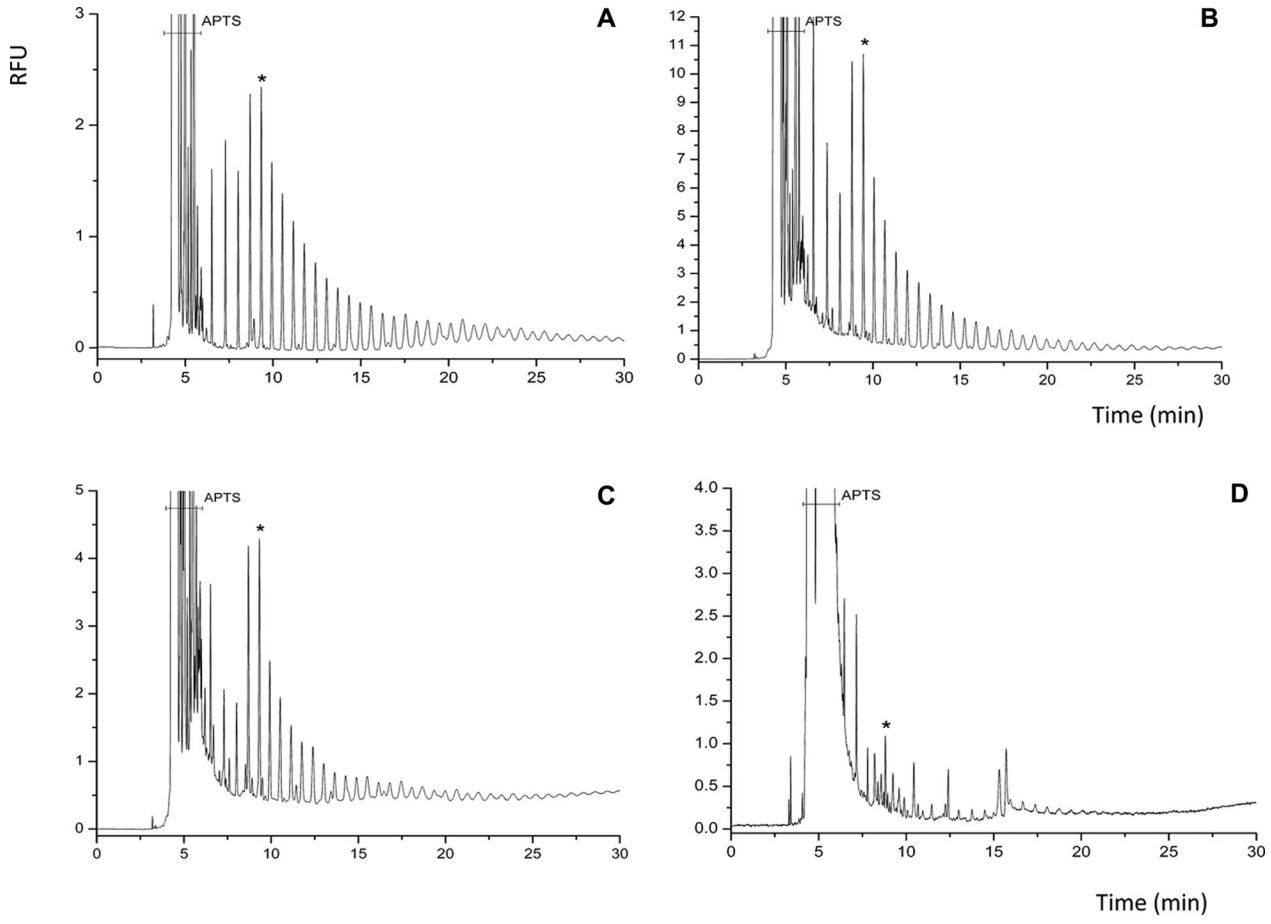

Figure 2. CE-LIF electropherograms of (A) white-copy paper; (B) recycled-copy paper; (C) adhesive yellow-note; and (D) serviette. Derivatization and CE conditions indicated in Fig. 1. (*) peaks used for S/N calculations.

clear separation of the classes with no entanglements. In this PCA analysis, the score plots for PC-1, PC-2, and PC-3 were used to study the class separation since they explain the 93% of data variation, i.e. 50, 31, and 12%, respectively. Each type of white-copy paper was located in a different class apart from the others. In fact, it was possible to distinguish among the four types white-copy paper samples analyzed.

Differences among white-copy, recycled-copy, adhesive yellow-notes, and serviettes paper sources were then studied. As can be observed in Fig. 5, four different groups of samples were clearly visible in the PC scores plot corresponding to the first, second, and third component (PC-1, PC-2, and PC-3), which explained 78% of the PCA model (41, 27, and 10% for PC-1, PC-2, and PC-3, respectively). White-copy, recycled-copy, adhesive yellow-notes, and serviettes paper samples defined four compact and separate groups without entanglements. Statistical differences among cellulose electropherograms were detected and surprisingly, the characteristics of cellulose electrophoretic profiles were common for each type of paper source. These differences were probably influenced by the several treatment stages that each type of paper had during its manufacturing process. The higher dispersion registered for the serviette class was attributed to the variety of samples. Each serviette was collected from a different restaurant and all of them presented differences in aspect, texture, and density. In consequence, the cellulose composition should be slightly different, producing the observed dispersion in the serviette class.

Because the PCA model described for the different paper sources presented lower data explanation (78%) than the PCA model described for the similar white-copy paper samples (93%) the loading and score plots were also studied in order to explain the differences observed among classes. The comparison between scores and loadings plots has the advantage that it allows to establish relationships between samples and variables in a general mode and at a glance. Figure 6 displays the weight and significance of the CE signal in the paper samples classification. On the one hand, Fig. 6A shows that the adhesive yellow-notes samples and also one serviette sample had a negative weight. Accordingly, from Fig. 6B it is possible to see that the signal peaks located in the negative part of the plot are those which appeared at the times corresponding to the derivatization agent, APTS, from 4.5 to 6 min. Subsequently, these paper samples were the most intensely influenced by the APTS CE-LIF signal peaks. On the other hand, the

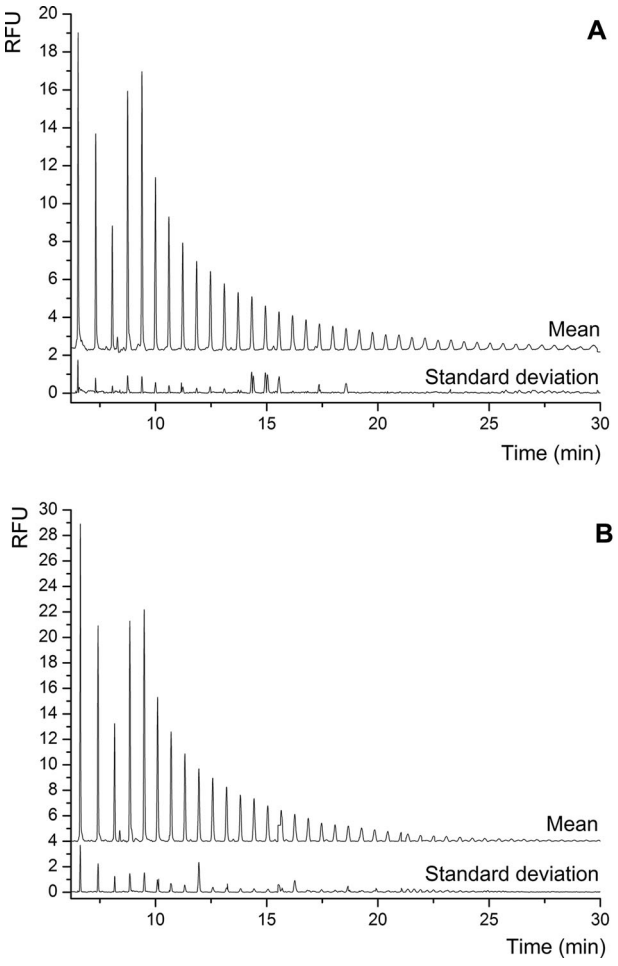

Figure 3. Mean and SD for APTS-labeled white-copy paper electropherograms obtained for (A) successive injections ($n = 5$) of the sample in a day and (B) periodic injections ($n = 15$, five days and triplicate analysis) of the sample during a week.

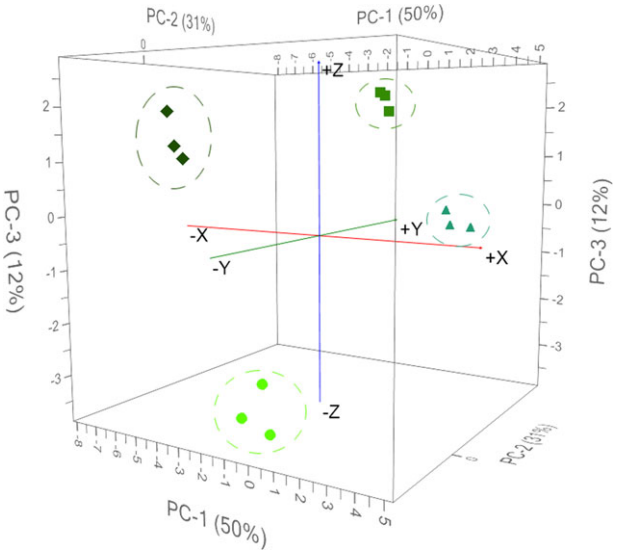

Figure 4. Three-dimensional score plot of first, second, and third principal components (PC-1, PC-2, and PC-3) showing the classification of white-copy paper samples produced by four different manufactures. Groups are marked for clarity.

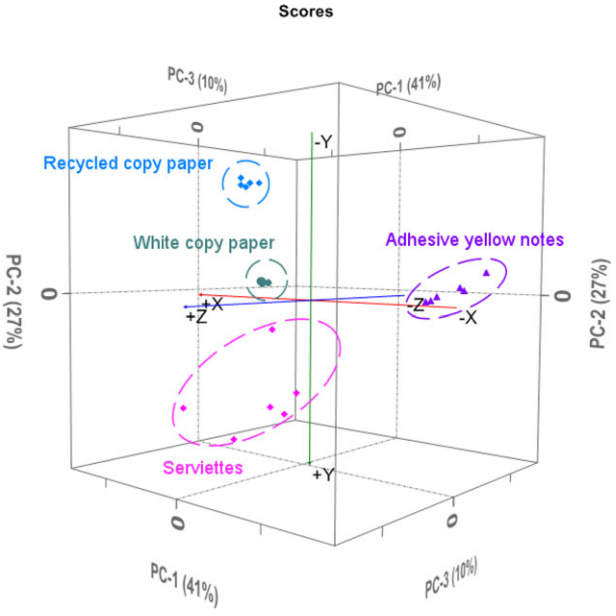

Figure 5. Three-dimensional score plot of first, second, and third principal components (PC-1, PC-2, and PC-3) showing the classification of white-copy, recycled-copy, adhesive yellow-notes, and serviettes paper samples. Groups are marked for clarity.

remaining paper samples showed a positive weight in Fig. 6A and thus, they were mainly influenced by the cellulose CE-LIF signal peaks (from the 6th min to the end), which were located in the positive part of the plot. This means that the most derivatized agent-consuming samples (white and recycled office papers and some paper serviettes) presented more influence on cellulose signals and consequently had more cellulose molecules available for reaction with the derivatizing agent. On the contrary, the less agent-consuming samples presented a negative weight in the score plot and more influence on the APTS signals and consequently less quantities of cellulose molecules available for derivatization. These differences allowed the successful differentiation between the different types of paper samples studied.

4 Concluding remarks

An analytical methodology for the determination of similar and different papers using CE-LIF after their derivatization has been developed in this work. The proposed method allowed the analysis of paper samples through the use of a reductive amination process with APTS. In this study, a simple, fast, economical, and minimal destructive sample treatment based on a unique pulverization step using a surgical scalpel for collecting paper samples was developed.

Finally, multivariate analysis was successfully applied for the discrimination among paper samples. The PCA score

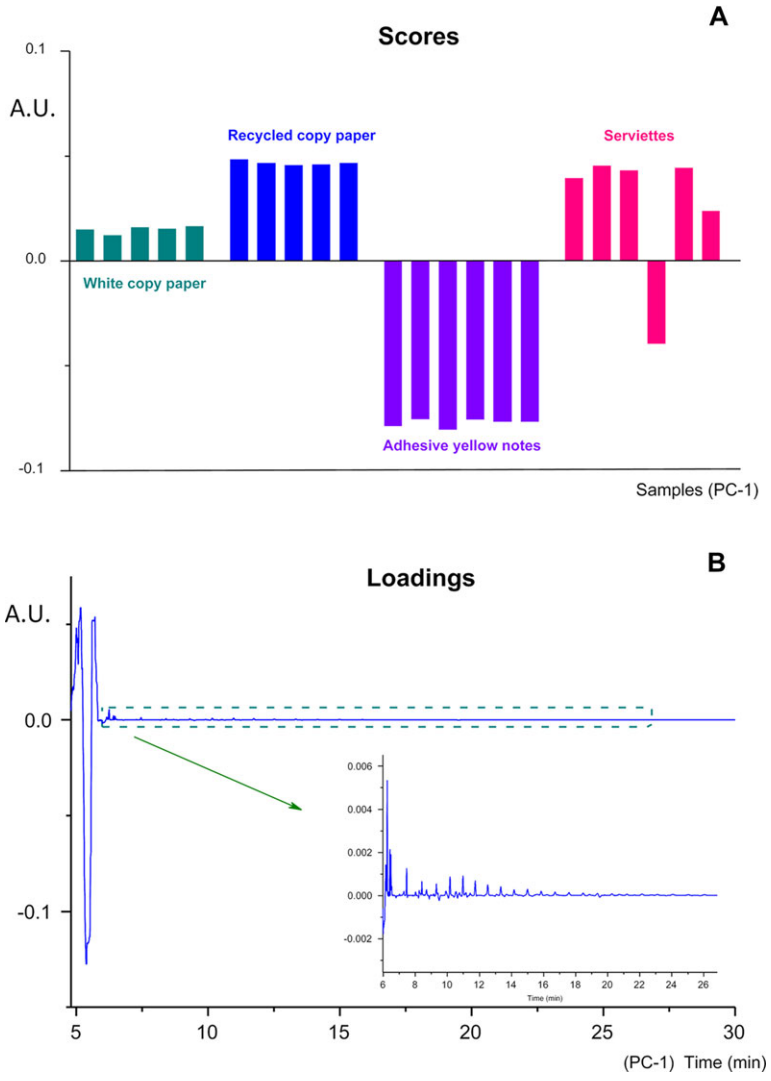

Figure 6. Scores (A) and loadings (B) plots for the white-copy, recycled-copy, adhesive yellow-notes, and serviettes paper samples. The upward position of the papers in the scores plot is influenced by the variables lying upwards in the loadings plot and conversely. A.U. stands for arbitrary units.

plots clearly showed the differentiation among similar white-copy paper samples and also among different paper sources such as white-copy paper, recycle-copy paper, yellow paper notes, and serviettes. The analysis of cellulosic samples by CE-LIF assisted with an APTS derivatization has shown its potential to discriminate between similar paper samples such as white-copy office papers produced by different manufactures. In case of different paper sources, since the PCA model presented lower data explanation than the PCA model for the similar white-copy paper samples, the loadings and score plots gave the explanation about this differentiation based on the characteristics of cellulose CE profiles. These differences have been statistically evaluated on the basis of good results (RSD values for peak areas below 10%) obtained for intra and inter-day repeatability.

In fact, the proposed methodology could be considered as a complementary tool for forensic investigations of paper samples, which would allow a successful discrimination among papers with the advantage of avoiding the needed expertise of the examiner and its inherent subjectivity, indispensable characteristic for the currently used microscopic analyses. However, to fully implement this method for routine analysis further investigations considering more paper samples produced by different manufactures, the study of real forensic cases of paper matching, and the evaluation of the proposed CE-LIF method effectiveness in comparison to the currently established methods, should be performed.

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