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Research Article

Analysis of drugs of forensic interest with capillary zone electrophoresis/time-of-flight mass spectrometry based on the use of non-volatile buffers

The present work is aimed at investigating the influence of the background electrolyte composition and concentration on the separation efficiency and resolution and mass spectrometric detection of illicit drugs in a capillary zone electrophoresis-electrospray ionization-time of flight mass spectrometry (CZE-ESI-TOF MS) system. The effect of phosphate, borate and Tris buffers on the separation and mass spectrometry response of a mixture of 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, methadone, cocaine, morphine, codeine and 6-monoacetylmorphine was studied, in comparison with a reference ammonium formate separation buffer. Inorganic non-volatile borate and Tris buffers proved hardly suitable for capillary electrophoresis-mass spectrometry (CE-MS) analysis, but quite unexpectedly ammonium phosphate buffers showed good separation and ionization performances for all the analytes tested. Applications of this method to real samples of hair from drug addicts are also provided.

Keywords:
Capillary electrophoresis / Drugs of abuse / Non-volatile buffer / Phosphate / Time-of-flight mass spectrometry
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1 Introduction

In forensic toxicology, capillary electrophoresis (CE), after more than a decade of neglect, has recently started attracting the interest of a few researchers. Indeed, this miniaturized analytical technique, because of orthogonal separation mechanisms as compared to gas and liquid chromatography and high versatility of application, looks suited to face a variety of toxicological problems spanning from inorganic ions to large biopolymers [1–3]. As it occurred in gas and liquid chromatography, also in CE a giant leap forward in the practical application to the analysis of complex matrices, as it is usual in analytical toxicology, was the availability of hyphenated systems combining CE with mass spectrometry. To this aim, the crucial point that attracted the attention of the CE researchers was the development of sound and reliable interfaces to couple this separation technique with MS, the recognized "gold standard" for compound identification in forensic analysis. This coupling, when successful, provides a powerful integration of technologies combining the high efficiency and high versatility typical of CE with the high sensitivity and specificity of MS [4, 5].

By far, the favorite ionization source in CE-MS is the electrospray, which is characterized by high ionization efficiency and soft nature of the ionization process. The interfacing of the CE separation compartment with the electrospray ionization-mass spectrometry (ESI-MS) section has been achieved by different means, namely sheath-flow, sheathless and liquid-junction interfaces, the former being, until recently, the only commercially available and for this reason predominately used. As widely discussed in the literature, the crucial parameters to be optimized for an effective CE-MS coupling are typically represented by: (i) the composition and the flow rate of the sheath liquid, (ii) the nebulizing gas flow rate, (iii) the applied voltage and (iv) the injection conditions [3, 6, 7]. Indeed, one of the major points of weakness of the ESI source comes from the possibility that the ionization process is affected by nature and concentration of the compounds entering the ion source. This typically poses high restrictions on the composition and concentration of the buffers used in the separation process, as it is well known in liquid chromatography-mass spectrometry (LC-MS), where the traditional mobile phases containing phosphate, borate, carbonates, bicarbonates, triethylamine, Tris, etc., are banned in favor of few organic buffers. Indeed, non-volatile buffer constituents are reported to be highly problematic for causing suppression of the analytes' ionization and contamination of the ion source and optics of the mass spectrometer.

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Abbreviations: **COC**, cocaine; **COD**, codeine; **FASS**, field-amplified sample stacking; **6-MAM**, 6-monoacetylmorphine; **MDA**, 3,4-methylenedioxyamphetamine; **MDMA**, 3,4 methylenedioxymethamphetamine; **MORP**, morphine; **MTD**, methadone

This, in short, negatively affects the ESI-MS sensitivity, accuracy and reproducibility, especially for the analytes with low m/z ratio [8]. Moreover, as widely reported in the literature, co-eluting compounds present in the matrix may also affect analyte ionization in the ESI source, causing signal suppression or enhancement.

In comparison with liquid chromatography, CE deals with much smaller amounts of sample (only few nanoliters are injected) and much lower separation buffer flow rates (fractions of μL/min) and consequently looks, in principle, very suitable for limiting the above-mentioned causes of ESI instability, which are highly dependent by the whole amount of ions entering the ion source. Also, when CE is hyphenated to MS through a sheath liquid interface, the dilution (1:20–1:30) exerted by the sheath flow and the liquid sheath effect (background electrolyte (BGE) inorganic anions migrate toward the injection end) [9] may further reduce the ion suppression phenomena. Notwithstanding the above-mentioned considerations, in the real practice, the choice of buffer electrolytes in CE-MS, mimicking an obligate approach of high performance liquid chromatography-mass spectrometry (HPLC-MS), is traditionally limited to those with high volatility, such as ammonium acetate and formate. Unfortunately, the CE separation efficiency and resolution are not always maintained when using volatile buffers, showing peak defocusing during the electrophoretic separation process.

On the basis of these considerations, recently a few laboratories have published contributions on the possibility of using non-volatile buffers in CE-MS in different fields [10–14]. Particularly, in 2003 the group of de Jong reported the feasibility of introducing non-volatile buffers and surfactants into ESI by testing the direct infusion of mebeverine [15]. Also, Van Wijk et al. [16] reported the successful application of CZE-MS with non-volatile buffers for pharmaceutical impurity profiling, using 100 mM Tris adjusted to pH 2.5 with phosphoric acid. Chien et al. [17] used 60 mM ammonium phosphate buffer at pH 3.5 in the CE-MS separation of antihistamines. The authors concluded that phosphate can be used in this application, but with same limitations, including the substitution of the sodium ion by ammonium ion, the use of an acidic separation condition and the use of a sheath liquid containing a low concentration of phosphoric acid. Under these conditions, the buffer generated ion suppression was significantly decreased.

More recently, the group of Her proposed the application of a new liquid- junction-low flow interface designed by their group, to limit the ion suppression caused by the non-volatile buffers (borate, phosphate) in the CE-MS analysis of antihistamines, perfluorocarboxylic acids and gangliosides [18, 19]. The results of this approach showed that phosphate-related ion suppression was successfully controlled by using the proposed interface without degrading the CE separation efficiency.

In the forensic field, only few papers have been presented on the use of non-volatile buffers for the CE analysis of drugs of abuse. Recently, the group of Boatto [20, 21] proposed the separation of amphetamines congeners in urine or plasma extracts by CE–ESI-MS using a non-volatile buffer electrolytes (10 mM sodium phosphate monobasic adjusted to pH 4.5 or 2.5 with phosphoric acid) without sacrificing analytical sensitivity [limit of detections (LODs) ranging from 3.98 to 4.64 ng/mL in urine, from 11 to 23 ng/mL in plasma].

In order to investigate more systematically the possibility of using non-volatile buffer electrolytes traditionally used in CE-UV also in CE-MS, in the present study, different electrolyte systems have been compared for the optimization of the CE–ESI-MS analysis of a mixture of selected forensic drugs, in terms of separation efficiency and detection sensitivity. Moreover, to demonstrate the practical feasibility of this approach, applications of non-volatile buffers to the separation of drugs of abuse in real samples of hair are presented.

2 Materials and methods

2.1 Chemicals

Standards of 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), methadone, morphine, codeine, 6-monoacetylmorphine (6-MAM) and cocaine were purchased from Salars (Como, Italy). Stock solutions of each standard were prepared in methanol at a concentration of 2 mg/mL and stored at −20°C. Isopropanol, ammonium formate, boric acid, Tris (hydroxymethyl)aminomethane, formic acid, hydrochloric acid, phosphoric acid and ammonia used for the preparation of the buffer electrolytes, the ESI sheath liquid and for the procedures of sample extraction were of HPLC or "analytical" grade (Carlo Erba, Milan, Italy). Ultrapure water was supplied by a Milli-Q RG purification unit (Millipore, Bedford, MA, USA). Ready-to-use tubes for liquid-liquid extraction (Toxi-Tubes A) of basic compounds from biological samples were supplied by Varian (Lake Forest, CA, USA). All the electrolyte solutions for the CE separation were stored in glass bottles at + 4°C and filtered under vacuum through a 45 μm cellulose filter (Sartorius, Hannover, Germany). The sheath liquid and the separation electrolyte were degassed by sonication (10 min) before use.

2.2 Background electrolytes preparation

Formate buffers were prepared by adding to a 25 mM solution of formic acid the suitable amount of a 1 M ammonia solution in order to reach the working pH (9.5 or 5). Borate and Tris buffers were prepared by adding ammonia to a 25 mM solution of boric acid or Tris (for obtaining the pH of 9.5) or hydrochloric acid (for pH 5). In the experiments evaluating the influence of the pH on separation performance, 25 mM solutions of phosphate buffer at the starting pH of 1.8 were titrated with ammonia (1 M) to match the pH of 2.5, 5.0, 6.5 and 9.5. For each buffer, the sheath liquid was composed of isopropanol/water mixture (50:50 v/v) containing 0.5% v/v of the same acid which was contained in the separation buffer electrolyte, except for Tris for which formic acid was used.

2.3 Sample collection and preparation

Hair samples were cut from the scalp of drug abusers with an average length of 2.5 cm in the area named "vertex posterior" of the head and processed has fully described in a previous paper [22]. In short, hair samples (100 mg) were washed with an aqueous solution of 0.3% Tween-20, in order to remove the potential contaminants present on the surface. Then hair were then cut into small fragments and incubated overnight in 1 mL 0.1 M HCl at 45°C. Finally, the incubation mixtures were neutralized with equimolar NaOH and extracted into organic phase with Toxi-Tubes A. The organic layers were evaporated under a stream of air and finally the dried residues were reconstituted in 250 μL of water with thorough vortex mixing.

2.4 Instrumentation

2.4.1 CZE

On the basis of previous experiences [22] for the separation of analytes, plain CZE was chosen as the most compatible with ESI-MS. For the sake of completeness, molecular weight and pKa of each analyte are summarized in Table 1. CZE experiments were performed with an HP ^{3D}CE system (Agilent, Palo Alto, CA, USA) equipped with a diode-array detector. Separations were performed at 20°C in uncoated fused-silica capillary (Composite Metal Service Worcester, UK) with ID of 75 μm, OD of 360 μm and a total length of 100 cm. The UV detection window was placed at 22 cm from the inlet end of the capillary. Experiments were carried out in "normal polarity" mode (anode at the capillary inlet) by applying a constant voltage of 15 kV during analyses (~20 μA).

For an evaluation of the performance of the technique in terms of efficiency and resolution, analytes were dissolved in the running buffer to a final concentration of 2.5 μg/mL and introduced into the capillary by hydrodynamic injection (50 mbar for 20 s). When, instead of hydrodynamic injection, field-amplified sample stacking (FASS) injection was performed, analytes were dissolved in ultrapure water at 50 ng/mL. FASS injections were carried as follows: the injection end of the capillary was dipped into water for 1 s (external rinse step), then a plug of water was hydrodynamically injected for 1 s at 35 mbar and finally the sample was injected electrokinetically for 30 s at 7 kV.

Table 1. Chemical-physical properties of the analytes object of the present study

Analyte	Molecular weight	pKa
Cocaine	303.4	8.6
Codeine	299.4	8.2
MDA	179.2	10.1
MDMA	193.3	10.4
Methadone	309.4	8.3
Morphine	285.3	8.0
6-MAM	327.4	8.0

2.4.2 MS

High-resolution MS measurements were performed with a micrOTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer fitted with an ESI ion source. The interfacing of the CE instrument with the mass spectrometer was affected by a coaxial sheath liquid interface (Agilent Technologies). The sheath liquid was delivered at a flow rate of 4 μL/min by a syringe pump (Cole-Parmer, Vernon Hill, IL, USA) and consisted of an isopropanol/water mixture (50:50 v/v) containing 0.5% v/v of the same acid which was contained in the separation buffer electrolyte (i.e. formic, phosphoric or boric). This strategy was adopted in order to avoid the development of a moving ionic boundary inside the capillary as it could be expected if the BGE contains different co-ions than the sheath liquid [9]. The ESI capillary voltage was set at 4000 V. A nebulizing gas pressure of 0.6 bar was applied to assist the spraying. Drying gas flow rate and drying gas temperature were set at 5 L/min and 200°C, respectively. Electrospray voltage and nebulizing gas were turned off during injection in order to avoid a pressure-induced flow during injection. The mass spectrometer was operated in the positive ion full scan mode from 50 to 800 m/z. External mass calibration was obtained by infusing for 1 min (at the beginning of each run) a solution composed of 10 mM sodium hydroxide in isopropanol and 0.2% formic acid in water (1:1, v/v), using seven calibration ions corresponding to the formulas Na(NaCOOH)x, with x ranging from 2 to 9. The external calibration provided accurate mass values (better than 5 ppm) with a nominal resolution of the instrument of 10 000 (FWHM). Identification of the peak of interest was performed by using both accurate mass measurement and isotopic pattern. The similarity matching between the theoretical and measured isotopic patterns was expressed by the so-called sigma value (the lower the value, the better the matching), generated by a Bruker Daltonics proprietary algorithm. Data acquisition and data handling were carried out with the MicrOTOF Control and Data Analysis software (Version 3.4, Bruker Daltonics).

3 Results and discussion

In the present work, in order to make possible a comparison of the analyte ionization yield in non-volatile BGEs versus a typical volatile buffer (ammonium formate), already applied in the same context [22], all the working parameters of both CE and MS [particularly the CE position relative to MS, the nebulizing gas flow rate (0.6 bar) and the position of the capillary tip into the interface (0.2 mm of protrusion)] remained unchanged when the BGEs were switched.

3.1 Effect of the background electrolyte on the *S/N* ratio and separation efficiencies of drug analytes

As reported by several authors [17, 18] the co-ion of the BGE (particularly sodium and potassium) could strongly affect the ionization of small molecules, particularly when using the "normal polarity" mode, since interfering cations are drawn by the electric field into the ion source. Consequently, ammonium was chosen as the suitable co-ion in different electrolyte buffers based on boric acid, phosphoric acid or Tris, which were compared to ammonium formate, which was previously used for the CE separation of the selected drugs of abuse [22]. Suitable buffers were prepared from solutions of non-volatile acids by titration with ammonia to match the working pH as described in Section 2.2.

As discussed in the same previous report [22], a value of 9.5 was used as the most suitable pH of the CE separation buffer, because being close to the pKa of the analytes, subtle differences in their ionization may contribute to achieving different individual electrophoretic mobilities. Moving from this starting point, the non-volatile electrolytes (formic acid, phosphoric acid, boric acid and Tris) were initially tested while keeping constant both buffer concentration (25 mM) and pH (at 9.5 with ammonia), in order to mimic the conditions already found suitable for efficient drug separation [22]. A mixture composed of MDMA, cocaine, morphine, methadone and codeine, dissolved at the individual concentration of 2.5 μg/mL in the separation buffer, was used as the test solution. The analysis was performed on a 75 μm ID capillary (100 cm of total length), by injecting hydrodynamically the test mixture for 20 s under 50 mbar of positive pressure at the capillary inlet.

Figure 1 shows the effect of the buffer composition on S/N ratio of the molecular ions of the tested compounds. As expected, ammonium formate gave in general the best results in terms of MS response, but with some important exceptions: for cocaine, morphine and codeine ammonium phosphate, borate and Tris showed S/N ratios comparable to formate. The results are shown in Fig. 1 [average values of three different injections ± standard deviations].

The separation efficiencies were comparable for ammonium formate and phosphate, being in the range 6000–10 000 for all the analytes, while for Tris and borate buffer the efficiencies were slightly worse, with *N* ranging from 3500 to 6500 for borate and from 4500 to 10 000 for Tris.

It is well established that in bare silica capillaries, at low pH, the electroosmotic flow (EOF), oriented toward the capillary outlet, is highly reduced. Therefore, it is expected that, under acidic conditions, the migration of buffer anions, such as phosphate or borate, is directed toward the inlet (anode) dragging them in direction opposite to ESI source, thus preventing them from affecting the ESI process [9]. Such considerations were verified in the following experiments in which separation buffers based on phosphoric acid/ammonia, boric acid/hydrochloric acid and Tris/hydrochloric acid (each at a concentration of 25 mM and at pH 5.0) were tested for with the usual test mixture in comparison to 25 mM ammonium formate at pH 5.0.

As shown in Fig. 2, quite surprisingly, phosphoric acid 25 mM adjusted to pH 5 with ammonia gave the best results, even better than those obtained with ammonium formate for all the analytes. In particular the gain in S/N ratio for MDMA, cocaine and methadone was more than four folds. On the contrary, for morphine and codeine the results observed when using formate and phosphate buffers were comparable. When considering the separation efficiencies the results were slightly better for ammonium phosphate (*N* values ranging from 9300 to 11 500), rather than ammonium (range 7200–12 000 for all the analytes). The efficiencies calculated with borate buffer were lower, being in the range of 1800 to 3500; while Tris buffer still allows for acceptable separation efficiencies (between 4 700 and 10 000) although lower than those obtained with ammonium formate and phosphate.

Since lowering the pH is known to improve the ionization efficiency of the selected basic compounds, probably due to an increased net charge, phosphate buffer was tested over a broad range of pH in order to meet the best conditions for the separation of the analytes of interest. In this experiment, the S/N ratios of the analytes were calculated when using 25 mM phosphate buffer at the following pH: 1.8, 2.5, 5.0, 6.5 and 9.5 (with ammonia) and compared to those obtained with ammonium formate 25 mM, adjusted at pH 9.5 with ammonia.

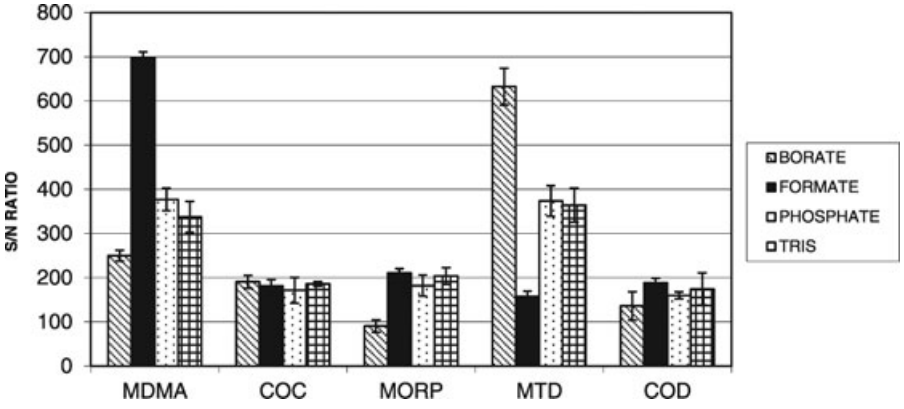

Figure 1. *S/N* ratios of the selected compounds in the different electrolyte buffers, all at 25 mM at pH 9.5. The results are shown as average values of three different injections ± standard deviation.

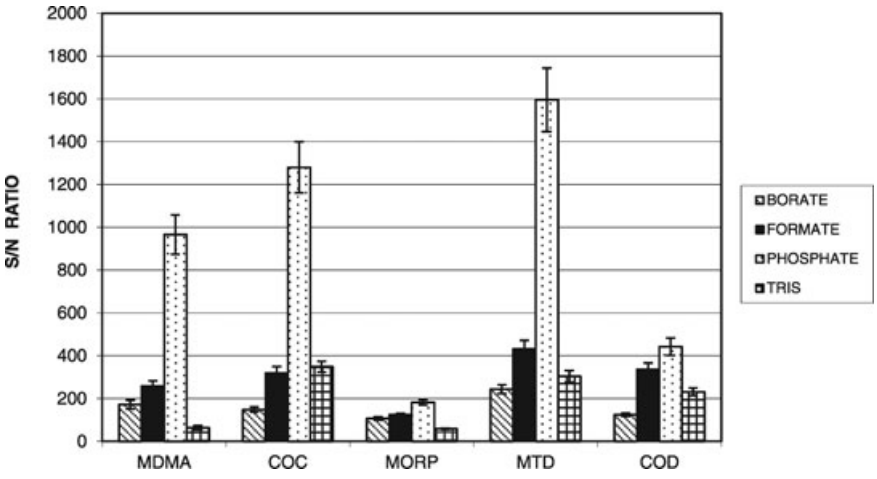

Figure 2. S/N ratios of the selected compounds in the different electrolyte buffers, all at 25 mM at pH 5.0. The results are shown as average values of three different injections ± standard deviation.

As depicted in Fig. 3, although with differences among the analytes, in general the best results in terms of S/N ratio were obtained with a BGE composed of phosphoric acid at pH 5.0–6.5. The separation efficiencies were in the range of 10000–15000 with pH $\leq$ 5, while they were comparable for ammonium formate and phosphate both at pH 9.5, being in the range 6000–10000 for all the analytes. The best results in terms of efficiencies (N = 47000–54000) were obtained when using ammonium phosphate at pH 6.5.

3.2 Effect of the phosphate concentration on separation efficiency and resolution of drugs

One of the major drawbacks of volatile buffers typically used in CE-MS is represented by a somewhat poor separation efficiency and resolution, if compared to the results with the inorganic BGE's, such as phosphate or borate. Further experiment were then carried out to test the performance of the system in terms of efficiency and resolution, while using phosphate buffer instead of formate in the BGE. In these tests, S/N ratio and resolution of the target compounds were evaluated using BGEs composed of phosphate solutions at pH 6.5 at concentrations of 25, 50, 100 and 150 mM). Overall, the separation efficiencies for all the compounds ranged between 100 000 and 150 000 theoretical plates at any phosphate concentrations tested, with slightly better results with a phosphate concentration of 50 mM (data not shown).

The resolution of adjacent peaks at different concentrations of ammonium phosphate (i.e. 25, 50, 100 and 150 mM) at pH 6.5 was in general good, being $\geq$1.5 for most of compounds (except for morphine, codeine and 6-MAM) using a BGE concentration ranging 50–100 mM. Only the couple MDA/MDMA showed a poor resolution at any BGE concentration.

From the considerations mentioned above phosphate was chosen as a suitable alternative to organic BGE constituents for the separation of drugs of abuse. The analytical sensitivity obtained with the 50 mM ammonium phosphate buffer at pH 6.5, expressed by the LODs (calculated with S/N $\geq$3), corresponded to 0.01 ng/mg for cocaine, MDA and MDMA; 0.002 ng/mg for morphine and codeine; 0.006 ng/mg for 6-MAM; 0.008 ng/mg for methadone. These

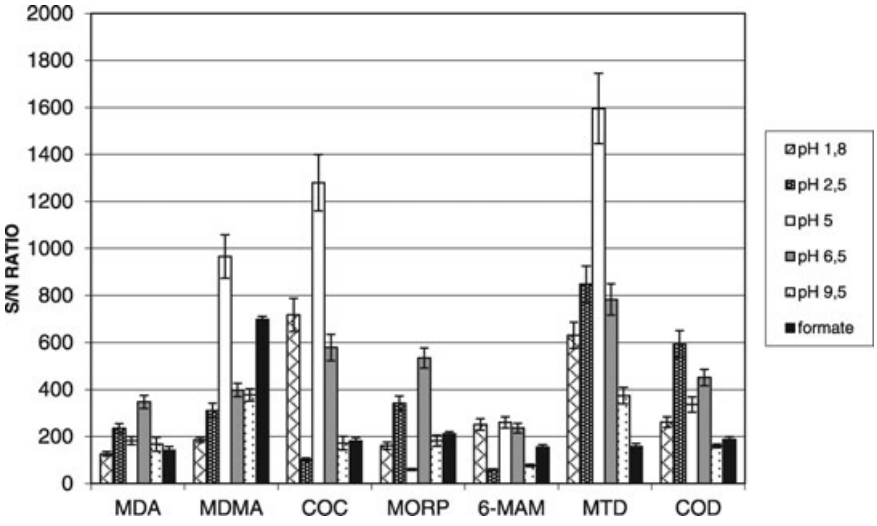

Figure 3. Influence of the phosphate buffer pH on the S/N ratio of the selected compounds at a concentration of 25 mM, in comparison with 25 mM ammonium formate at pH 9.5 (reference method). The results are shown as average values of three different injections ± standard deviation.

sensitivity limits resulted slightly better than those obtained by using the 25 mM ammonium formate at pH 9.5 [22], but for opiates, for which the phosphate buffer allows for a ten-fold increase of the analytical sensitivity. Although conditions to achieve the best resolution and the highest signal intensity and S/N ratio have been found mutually conflicting, a good compromise was obtained with 50 mM ammonium phosphate buffer at pH 6.5. Moreover, the orthogonal ESI interface used in this study presented an excellent tolerance to the non-volatile buffers. Indeed, the contamination of the source was minimal, allowing for the use of the phosphate electrolyte for several days without noticing any relevant reduction of the performance of the ESI source, in terms of signal intensity. As a precaution, ion source cleansing every week was applied in order to assure reproducible detector response.

3.3 Application to real samples

On the basis of the results discussed above, 50 mM phosphoric acid/ammonia buffer, pH 6.5, was tested as a suitable BGE for the CZE-ESI-TOF MS analysis of drugs of abuse in hair samples of real drug users. Figure 4 shows the electropherogram of a hair sample from a cocaine abuser. In the figure, the total ion electropherogram is shown (above) together with the extract ion electropherograms corresponding to cocaine (m/z 304.1543 err. 2.1 ppm, sigma 0.0104, concentration 3.06 ng/mg), benzoylecgonine (m/z 290.1287, err. −4.8 ppm, sigma 0.0045, concentration 0.47 ng/mg), cocaethylene (m/z 318.1700, err. 3.5 ppm, sigma 0.0102), ecgonidine methylester (m/z 182.1176, err. 4.0 ppm, sigma 0.0256) and ecgonine methylester (m/z 200.1281, err. −3.5 ppm, sigma 0.0126).

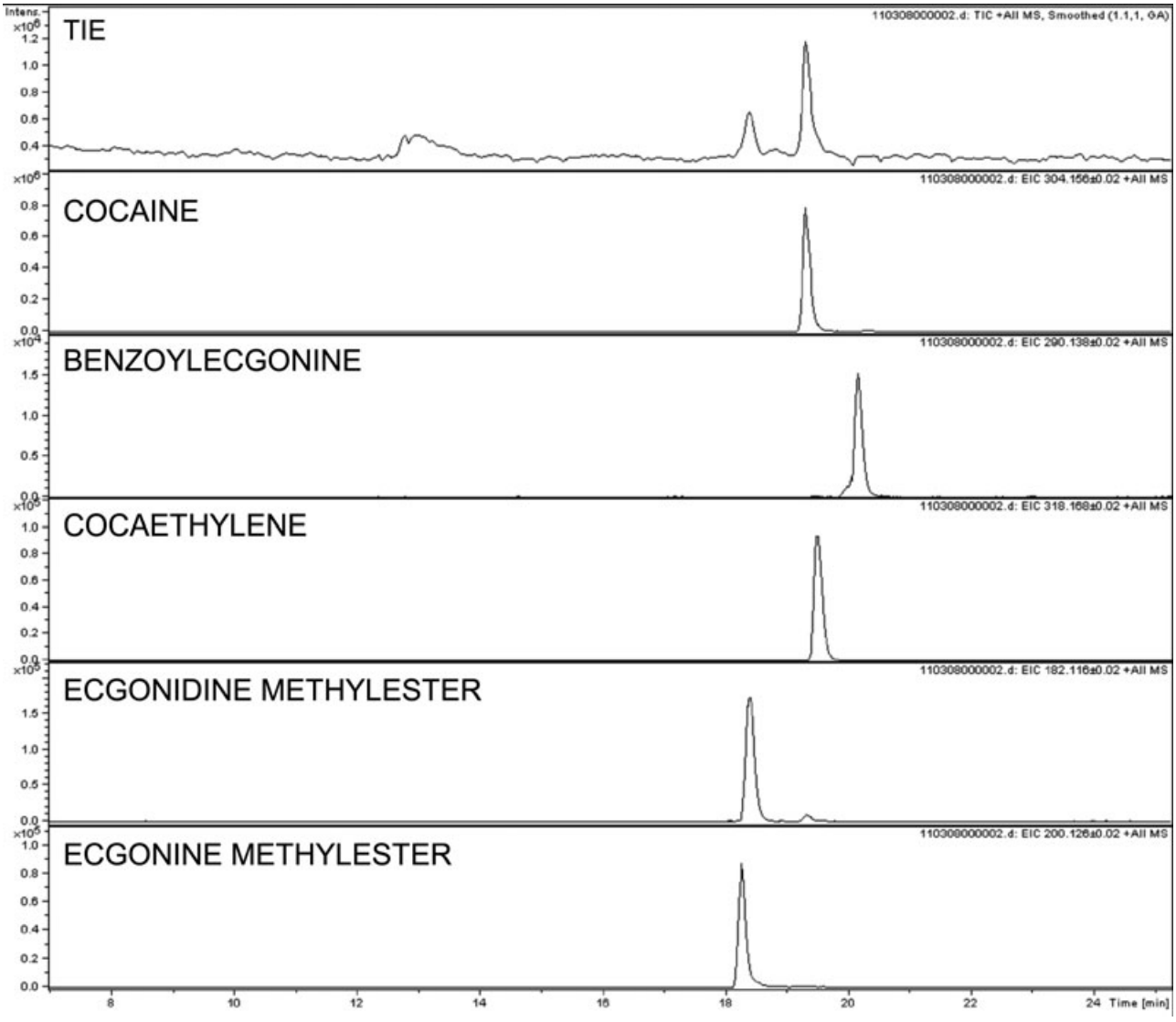

Figure 4. Total ion (TIE) and extracted ion electropherograms of a hair sample of a cocaine addicted. Analytical conditions: BGE 50 mM ammonium phosphate, pH 6.5; separation +15 kV, electrokinetic injection 7 kV for 30 s.

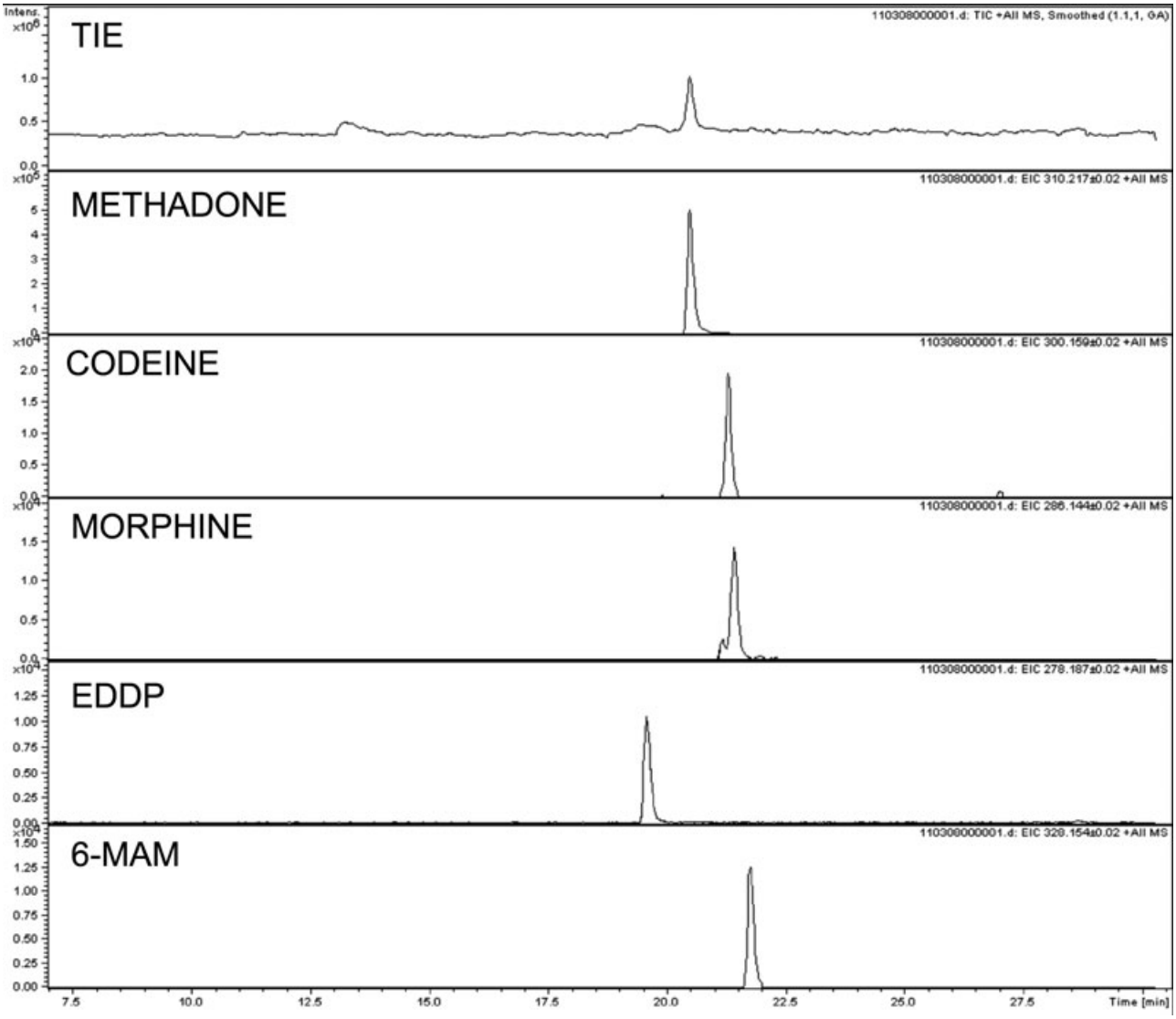

Figure 5. Total ion (TIE) and extracted ion electropherograms of a hair sample of a heroin addicted treated with methadone. Analytical conditions: BGE 50 mM ammonium phosphate, pH 6.5; separation +15 kV, electrokinetic injection 7 kV for 30 s.

The presence of all these compounds can be clearly referred to the addiction history.

The example depicted in Fig. 5 shows the electropherogram of a hair sample from a heroin addict undergoing a detoxification with methadone. The figure shows the total ion electropherogram (above) and the extract ion electropherograms corresponding to the abused drugs and to those prescribed in the detoxification treatment, namely, morphine (m/z 286.1437, err. −2.6 ppm, sigma 0.0256, concentration 1.93 ng/mg), 6-MAM (m/z 328.1549, err. 4.9 ppm, sigma 0.0112, concentration 0.69 ng/mg) and codeine (m/z 300.1549, err. 2.9 ppm, sigma 0.0082, concentration 0.54 ng/mg) were clearly identified. The electropherogram recorded also the presence of methadone (m/z 310.2165, err. 3.5 ppm, sigma 0.0221) and its metabolite EDDP (m/z 278.1902, err. 1.2 ppm, sigma 0.0321).

4 Concluding remarks

The results herein described show the feasibility, under controlled conditions, of coupling CE and MS without the mandatory need of using volatile buffers. Advantages in using inorganic buffers can be found in better buffering capacity, easier transferability of methods, better efficiency, etc. This increases the versatility of CE-MS, in comparison to HPLC-MS. The higher tolerance of the ESI source toward non-volatile ions when coupled to CE is justified by the extremely low flow rate entering the source and by the dilution exerted by the sheath flow. This advantage, in theory, should also limit the ion suppression phenomena caused by interfering compounds present in the injected mixture, which typically plague the reliability of HPLC-MS of complex biological matrices. Against this background, CE-MS looks potentially very

suitable for forensic analysis where the non-standardization of the samples and their contamination is an almost constant handicap.

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