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Application of a new capillary electrophoretic method for the determination of carbohydrates in forensic, pharmaceutical, and beverage samples

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ABSTRACT

A new capillary electrophoresis method dedicated to the analysis of neutral underivatized carbohydrates was recently developed by our group. It involved a background electrolyte composed of 98 mM NaOH and 120 mM NaCl, and direct UV detection via the formation of an absorbing intermediate in the detection window by photooxidation. This article focuses on the validation of this method for the determination of fructose, glucose, lactose, and sucrose in forensic, pharmaceutical, and beverage samples. Intermediate precisions were about 2.3% for normalized corrected peak areas and 1.8% for normalized migration times using naphthalenesulfonate as internal standard. Limits of detection varying from 5 μM for sucrose and lactose to 7 μM for glucose and 10 μM for fructose were obtained. Potential matrix effects were statistically studied for soil, cloth, plastic, cotton, red wine, and with simulated iron, calcium, and sucrose-based matrices, containing various inorganic anions and cations, sometimes at high levels. No significant matrix effect was observed. Finally, analyses of real post-explosion residues, smoke device, cough syrup, red wine, and apple juice were successfully performed.

1. Introduction

Carbohydrates such as fructose, glucose, lactose, and sucrose are widely distributed in various food, beverage, drug, and forensic samples. Their quantitative analyses allow the evaluation of sample authenticity, the quality of the food products or, for example, provide information on the nature and the composition of carbohydrate-based explosive charges. The most widely employed techniques for carbohydrate analysis is HPLC using normal [1] or reversed phase [2] or anion-exchange modes [2,3] with direct low-UV-range or refractive index detection [4] and most often mass spectrometry or fluorescence detection after precolumn derivatization [5–7]. Important developments involving high performance anion-exchange chromatography coupled to pulsed-amperometric detection were also carried out [3,4].

In recent years, capillary electrophoresis (CE) has been established as a powerful technique for carbohydrates analysis [4,7–11] in several matrices such as fruit juices [12–17], alcohol [14,18,19], dairy products [17,20,21], plant fiber [22] or rat brain [23]. As most carbohydrates do not contain easily ionizable (pKa∼12) and chromophoric groups, many strategies have been developed in order to use the high separation efficiency and speed of CE for carbohydrate analyses such as complexation with borate anions [24,25], pre-column derivatization with a fluorophore [26–29] or the use of an anionic chromophore in alkaline conditions [15,19,30,31] or ionic liquid [32] for their indirect UV-detection. In order to avoid time-consuming and expensive derivatization steps for carbohydrate labeling or the indirect UV detection mode with high alkaline electrolytes, which is poor in sensitivity, a simple method inspired by a former work by Rovio et al. [14,22] has been developed and optimized by our group [33,34]. A separation of 9 carbohydrates involving a percolation of hexadimethrine bromide (HDMB) before each run to reverse EOF, associated with a background electrolyte (BGE) composed of 98 mM NaOH and 120 mM NaCl was optimized with a response surface strategy and a desirability analysis [34]. The direct UV detection of carbohydrates was based on the formation of an absorbing intermediate (malonaldehyde or related compounds) undergoing the low-UV radiations of a Beckman Coulter diode array detector (DAD) [33]. This article focuses on the quantitative validation of the developed method. A chemometric approach has next been involved to study the potential matrix effect with several real extracts of cloth, plastic, soil, wine, and cotton, and of simulated matrices containing high levels of sucrose, iron(II) or calcium ions. Finally, applications to forensic, pharmaceutical, and beverage real samples were carried out.

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2. Materials and methods

2.1. Standards and electrolytes

All high purity carbohydrates used as standard samples (fructose, glucose, lactose, and sucrose) were purchased from VWR (Fontenay-sous-Bois, France). HDMB used as electroosmotic flow reversal agent and naphthalenesulfonic acid (NSA) used as internal standard were purchased from Sigma-Aldrich (L'Isle-d'Abeau, France). 100 mM individual carbohydrate solutions were prepared weekly by volumetric dissolution in ultra-pure water delivered by a Direct-Q3 UV system (Millipore, Molsheim, France). A standard mixture of the carbohydrates of interest was prepared daily (0.05 mM each in ultra-pure water). BGE was composed of 98 mM NaOH and 120 mM NaCl (calculated pH, 13.0). HDMB solution at 1 g L^{-1} was prepared by dissolving the appropriate amount in ultra-pure water.

2.2. Sample preparation for forensic, pharmaceutical, and beverage samples

Real samples of red wine (vine plant Merlot from France), apple juice (Pressade, France), and cough syrup (Clarix, COOPER, France) were commercial products and were just diluted with ultra-pure water before analyses. Real forensic samples (smoke device powder and post-blast residues) and blank matrices were collected either directly or via cotton swabs. The first step of this last procedure consisted of the purification of usual hydrophilic cotton swabs by assisted solvent extraction using an ASE 200 instrument (Dionex, Voisin-Le-Bretonneux, France), with one cycle of 5 min at 100 °C and 100 bar with water and next with acetone. Cotton swabs moistened with water were next wiped over samples and were used for inorganic analysis. Post-blast residues, cotton swabs, 1 g of smoke device powder and blank samples were next extracted in boiling water placed in a sonication bath for 10 min. The obtained solutions were filtered through a 150-μm cellulose filter (Les Filtres Durieux, Marne-la-Vallée, France) then through a 0.45-μm nylon syringe filter (Teknokroma, A.I.T France, Houilles, France) and finally diluted just before the injection.

2.3. Apparatus

The CE experiments were carried out with a Beckman Coulter P/ACE MDQ system (Villepinte, France) equipped with a DAD set at 270 nm (analysis wavelength) and 350 nm (reference wavelength) and with bandwidths set at $\pm$6 nm and $\pm$40 nm, respectively. Instrument control and data acquisition were performed using Beckman 32 Karat® software.

2.4. Electrophoretic procedures

Electrophoretic separations were performed using 50 μm id × 60 cm bare fused-silica Polymicro capillaries purchased from Photonlines (Marly-Le-Roi, France). A detection window was created for the UV detection at 10 cm from the anodic end. Before first use, capillaries were conditioned by successive flushing with 1 M NaOH, 0.1 M NaOH, ultra-pure water, HDMB solution, and finally BGE, each under 2.8 bar for 3 min (12 capillary volumes), except for HDMB which were under 1.4 bar for 6 min (12 capillary volumes) in order to obtain a better coating and more reproducible separations. Between each run, HDMB layers were refreshed (1.4 bar for 6 min), and this was followed by the percolation of BGE (2.8 bar for 3 min). Injections were performed hydrodynamically under 50 mbar for 5 s (0.75% of the capillary volume). Separations were run at 26.5 °C under -14 kV. BGE was changed between each run.

2.5. Chemometric approach of matrix effect

Chemometric studies of matrix effect were carried out with five repeated injections for five different carbohydrate concentrations (between 15 and 300 μM for sucrose and lactose, and 30 and 600 μM for fructose and glucose) in ultra-pure water for the standard calibration and three repeated injections for the same five carbohydrate concentrations in given matrix extracts (cloth, plastic, soil, wine, and cotton) and in simulated matrices containing high level of sucrose, iron(II) or calcium ions. Extracts of cloth, plastic, soil, and cotton used for matrix effect study were obtained after extraction of blank matrices in boiling water under sonication. Simulated matrices were prepared by dissolution of sucrose, $FeSO_4$, and $CaSO_4$ in water. A wine sample (vine plant Merlot from France) was also selected for the matrix effect study. When matrices already contained some carbohydrates, additional spiking was performed. For simulated sucrose-based matrix, which contained a high content of sucrose, the matrix effect was studied only on fructose, glucose, and lactose. Statistical parameters of the regression lines were computed with Excel® software (Microsoft).

3. Results and discussion

3.1. Quantitative validation

The validation of the previously optimized method [34], enabling the separation of a standard mixture of 9 carbohydrates in 19 min with a 98 mM NaOH and 120 mM NaCl-based BGE, was carried out to evaluate its potential for routine determination of carbohydrates in real extracts. This validation was just performed for the 4 carbohydrates of interest for the Central Laboratory of the Prefecture de Police (fructose, glucose, lactose, and sucrose) according to the ISO/CEI 17025 norm specifications. Naphthalenesulfonate anion was used as internal standard. For the Central Laboratory of the Prefecture de Police de Paris, precision and accuracy of the method had to be better than 5% and 20%, respectively.

3.1.1. Selectivity

The electrophoretic method, optimized using a multivariate approach, led to a complete separation of nine carbohydrates, the four of prime interest plus five other carbohydrates which may be present in natural samples [34]. The most critical pair, galactose/maltose, was the only one presenting a partial resolution, but this did not impact the detection of the four carbohydrates of interest. This lends support to considering that the selectivity of the CE method was satisfactory.

3.1.2. Precision

The precision of the method was evaluated for a mixture of the four carbohydrates at a concentration of 0.05 mM each and 0.34 mM NSA, making five repeated analyses on three different days. Standard mixtures and electrolytes were prepared daily. The responses measured on each electropherogram were the normalized corrected peak areas $((A_i/t_{M,i})/(A_{NSA}/t_{M,NSA}))$ and the normalized migration time $(t_{M,i}/t_{M,NSA})$ of each carbohydrate where A_i stands for carbohydrate peak area, $t_{M,i}$, carbohydrate migration time, A_{NSA}, NSA peak area and $t_{M,NSA}$, NSA migration time. Method repeatability was characterized by the intra-day RSD and method intermediate precision was characterized by the intermediate precision RSD. One way analysis of variance (ANOVA) [35] was used to determine the intra-day (Eq. (1)) and

the day-to-day (Eq. (2)) contributions:

$$\mathrm{RSD}_{\text{intra-day}}\,(\%) = \frac{\sigma_r}{\overline{x}} \times 100 \quad (1)$$

$$\mathrm{RSD}_{\text{day-to-day}}(\%) = \frac{\sigma_d}{\overline{x}} \times 100 \quad \text{with} \quad \sigma_d^2 = \frac{q_d - q_r}{n} \quad (2)$$

where $\overline{x}$ is the mean of the response, σ_r, the intra-day standard-deviation, σ_d, the standard-deviation due to the day factor, and q_d and q_r the mean square of the day factor and the residual mean square of the ANOVA respectively. In the present study, the day factor always appeared significant ($\alpha = 0.05$). The intermediate precision RSD, corresponding to the variability of a single determination carried out on a single day (Eq. (3)) was then calculated:

$$\mathrm{RSD}_{\text{intermediate precision}}(\%) = \frac{\sqrt{\sigma_d^2 + \sigma_r^2}}{\overline{x}} \times 100 \quad (3)$$

Results for normalized areas and migration times are presented in Table 1. For absolute migration times, intermediate precision was of the order of 4.5% for all four carbohydrates but when the migration times were normalized by NSA migration time, intermediate precision did not exceed 2%. Such quite acceptable values should allow easy and fast identification of peaks. For normalized peak areas, intermediate precision was around 2.5% which is always inferior to 5%. Thus, the performances of the method complied requirements.

3.1.3. Limits of detection

For a signal-to-noise ratio of 3, LODs were determined at 5 μM for lactose and sucrose, 7 μM for glucose, and 10 μM for fructose (Table 1). This method appeared as one of the most powerful for the CE analysis of underivatized carbohydrates with direct UV detection.

3.1.4. Limits of quantitation

For a signal-to-noise ratio of 9 ($3 \times$ LODs), the LOQs were experimentally predetermined at 30 μM for fructose, 21 μM for glucose and 15 μM for lactose and sucrose. These LOQs were checked with five different solutions of carbohydrates at their LOQ concentrations injected twice. At these concentration levels, the maximum bias obtained for carbohydrates did not exceed 10%, which meets the requirement of the police laboratory for forensic applications.

3.1.5. Linearity

The linearity of the method was evaluated by plotting the normalized corrected areas for each carbohydrate against the injected concentrations. Linear regressions were performed and coefficients of determination R^2 are given in Table 1. The linearity range has for lower limit the limits of quantitation and for upper limit, the concentration for which a bias superior to 10% was observed. Thus for glucose and fructose the linearity range spanned from 21 or 30 μM, respectively, to 1000 μM, and for sucrose and lactose from 15 to 700 μM. R^2 equals to 0.998 were obtained for all carbohydrates, in these considered ranges.

3.1.6. Matrix effects

To evaluate potential matrix effects, the calibration lines obtained with standards in pure water and in a given aqueous matrix extract were compared. The comparison was carried out

Table 1
Figures of merit for the analysis of 4 carbohydrates of interest: intra-day ($n=5$), day-to-day ($n=3$) and intermediate precision, LODs ($n=15$), and detection linearity in the 30–1000 μM range for fructose, 21–1000 μM range for glucose and in the 15–700 μM range for sucrose and lactose.

Carbohydrates	Normalized corrected areas			Normalized migration times			LOD (μM)	Linearity	
	RSD (%) intra-day	RSD (%) day-to-day	RSD (%) intermediate precision	RSD (%) intra-day	RSD (%) day-to-day	RSD (%) intermediate precision		Equation	R^2
Fructose	1.6	1.9	2.1	1.1	1.8	1.7	10	0.0159*x*–0.0589	0.998
Glucose	1.8	2.2	2.3	1.0	1.8	1.8	7	0.0265*x*–0.1765	0.998
Lactose	2.0	2.2	2.3	1.1	1.7	1.8	5	0.0163*x*–0.0555	0.998
Sucrose	2.0	2.3	2.4	1.2	1.9	1.9	5	0.0135*x*–0.0435	0.998

Table 2
Initial aqueous extract composition of the studied matrices, evaluation of matrix effects based on statistical analysis of calibration lines, and maximum bias value, expressed as a percentage, that it could generate on quantitation in routine analysis of these four carbohydrates. The matrices were spiked at 30, 50, 100, 200, and 600 μM by glucose and fructose, and 15, 30, 50, 100, and 300 μM by sucrose and lactose.

Matrix	**Initial composition of the matrix aqueous extract**			**Residual variance homogeneity**	**Equality of slopes**	**Equality of intercepts**	**Matrix effect**	**Bias (%)**
	Anions (mg L^{-1})	Cations (mg L^{-1})	Carbohydrates (μM)					
Soil	NO_3^-: 33, HCO_3^-: 36, Cl^-: 15, SO_4^{2-}: 6	Ca^{2+}: 40, K^+: 8, Na^+: 18	Nd[a]	Yes	Yes	Yes	No	0
Cloth	HCO_3^-: 10, Cl^-: 8	Na^+: 12	Nd	Yes	Yes	Yes	No	0
Red wine	Cl^-: 5, HPO_4^{2-}: 406, NO_3^-: 14, SO_4^{2-}: 186	Na^+: 21, K^+: 139, Ca^{2+}: 39, Mg^{2+}: 74	Frc: 65, Glc: 50	Yes	Yes	Yes	No	0
Plastic	Nd	Nd	Nd	Yes	Yes	Yes	No	0
Simulated metal	SO_4^{2-}: 500	Fe^{2+}: 850	Nd	Yes	No	No	Yes	11
Simulated sucrose	Nd	Nd	Suc: 300	Yes	No	No	Yes	10
Simulated plaster	SO_4^{2-}: 1900	Ca^{2+}: 800	Nd	Yes	No	No	Yes	5
Cotton	HCO_3^-: 20	Nd	Nd	Yes	Yes	Yes	No	0
Cotton wiped over metal	HCO_3^-: 35, Cl^-: 8, SO_4^{2-}: 14	Ca^{2+}: 51, K^+: 7, Fe^{2+}: large amount[b]	Nd	Yes	Yes	Yes	No	0
Cotton wiped over carbonized surface	HCO_3^-: 60, Cl^-: 20, NO_3^-: 20, SO_4^{2-}: 40	Na^+: 6, K^+: 9, NH_4^+: 3, Ca^{2+}: 19, Mg^{2+}: 14	Nd	Yes	Yes	Yes	No	0

[a] Nd: not detected.
[b] Fe^{2+} was considered as interfering compound and was not quantified.

using a chemometric strategy, based on the comparison of the two least-squares regression lines [36,37]. Three successive steps were involved: (i) the residual variances of the regression must be homogeneous (bilateral F-test), (ii) in case residual variances were homogeneous, it was then possible to compare the slopes of the regression lines (bilateral t-test using the pooled standard-deviation of the slopes as denominator), and (iii) in case slopes were the same, it was finally possible to compare intercepts (bilateral t-test using the pooled standard-deviation of the intercepts as denominator). Statistically, it could be considered that two calibration lines were not significantly different only if residual variances of the regression lines, slopes, and intercepts could be simultaneously considered not significantly different. All statistical tests were carried out with a first kind risk α set at 1%. All the results and conclusions are gathered in Table 2 for the 10 tested matrices, of which the aqueous extracts varied in nature and ion and carbohydrate contents. Cation and anion concentrations were determined by CE methods previously developed in our group [38,39]. In case a significant matrix effect was observed, the consequences of neglecting this matrix effect were evaluated by the maximum bias value (expressed as a percentage) that it could generate in routine analysis for the 4 targeted carbohydrates.

Significant matrix effects were observed for matrix extracts having high ion or carbohydrate concentrations. However, even for these cases, no bias of more than 11% was observed, which can be considered as meeting the specifications of many laboratories, among which the police laboratory, for which a 20% bias is acceptable. This enables to use calibration lines obtained in ultra-pure water for quantitative determination of fructose, glucose, lactose, and sucrose in a variety of aqueous matrix extracts. It is worthy of note that with all the studied matrices, no relevant migration time shift was observed. Eventually, the developed CE method for the identification and the quantification of carbohydrates in forensic and food samples can be considered validated. Its performances were in agreement with current requirements.

3.2. Applications to real samples

3.2.1. Forensic analyses

Carbohydrates are widely used in homemade explosive or pyrotechnic compositions. Their addition to chlorate, perchlorate, or nitrate solutions creates important gaseous releases (CO_2 or CO), which induces an explosion in a confined place, or creates smoke (as in smoke device for example). Therefore their identification is needed to determine the initial composition of an explosive charge. The developed CE method was used to determine carbohydrate contents in (i) a sample collected with cotton swab wiped over a plastic surface after a homemade explosion caused by a man in his kitchen and (ii) a powder issued from a homemade smoke device. Electropherograms obtained for these two samples are presented in Fig. 1. The analysis of post-explosion extract showed the presence of fructose, glucose, and sucrose in the explosive composition (Fig. 1-B). According to the quantitative results (Table 3), sucrose was the main component with a concentration of 420 μM. The chlorate anion was also detected in the extract by anionic CE analysis developed by our laboratory [38], so the use of sucrose and chlorate salt in the explosive charge was proved. The analysis of the homemade smoke device extract confirmed the presence of lactose at 132 μM in the sample. Indeed, lactose is currently used in smoke device composition and its use was checked here.

3.2.2. Syrup analysis

The control of carbohydrate contents is very important in pharmaceutical industry. The present CE method was applied to the analysis of cough syrup (Fig. 2). Due to the high content of sucrose, an important dilution (1/5000) was carried out with ultra-pure water in order to bring the concentration into the linear range. A single peak of sucrose was detected in the syrup solution. Without dilution, no other compound was detected in the syrup composition. The amount of sucrose in the syrup was quantified at 1.31 ± 0.26 M. The sucrose concentration specified by the manufacturer was 1.40 M, which corresponds to a difference of 6%, in keeping with the differences that can be due to

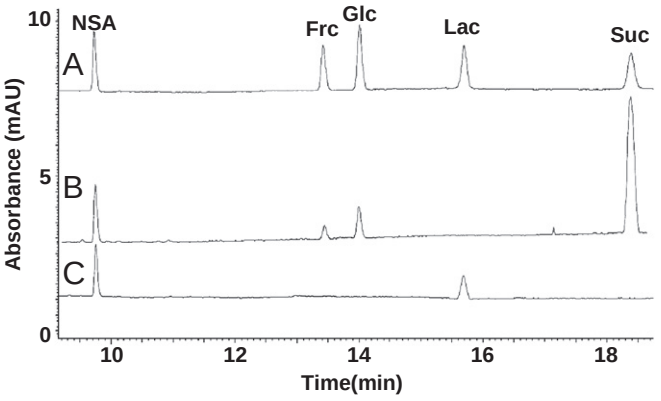

Fig. 1. CE analyses of carbohydrates in standard solution (A), in post-explosion residue extract (B), and in smoke device powder extract (1/20 diluted with ultra-pure water) (C). Bare fused-silica capillary, 50 μM id × 60 cm (UV detection at 50 cm) modified with HDMB (1 g L^{-1} in ultra-pure water). BGE: 98 mM NaOH (pH 12.99), 120 mM NaCl. Temperature, 26.5 °C. Applied voltage, −14 kV. Hydrodynamic injection, 5 s, 50 mbar. Direct UV detection at 270 nm. Analyte concentration in standard solution, 0.05 mM each in ultra-pure water. Quantitation, see Table 3.

Table 3
Contents in carbohydrates found in the studied samples, as determined by CE.

Samples	Fructose (mM)	Glucose (mM)	Lactose (mM)	Sucrose (mM)
Post-explosion extract	0.070 ± 0.007	0.12 ± 0.01	Nd[a]	0.42 ± 0.04
Smoke device extract	Nd	Nd	132	Nd
Cough syrup	Nd	Nd	Nd	1310 ± 260
Apple juice	335 ± 67	98 ± 20	Nd	53 ± 11
Red wine	2.1 ± 0.2	1.4 ± 0.1	Nd	Nd

[a] Nd: not detected.

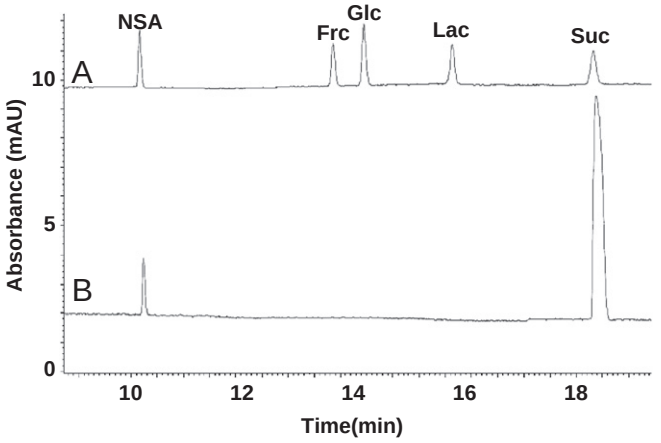

Fig. 2. CE Analyses of carbohydrates in standard solution (A) and in cough syrup (B) (1/5000 dilution with ultra-pure water). Separation conditions, see Fig. 1. Quantitation, see Table 3.

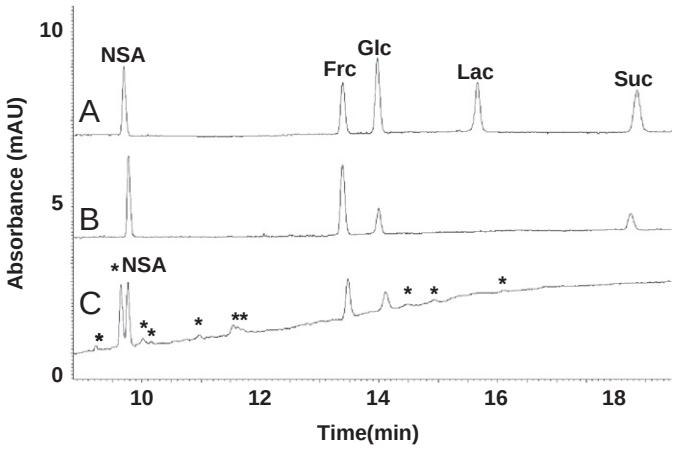

Fig. 3. CE analyses of carbohydrates in standard solution (A), an apple juice (B) (1/1000 dilution with ultra-pure water), and red wine (C) (1/20 dilution with ultra-pure water). Separation conditions, see Fig. 1. Identification: *, unknown compounds. Quantitation, see Table 3.

matrix effects. This 6% value corresponds to a bias rather high for pharmaceutical industry. Further investigations would be necessary to definitively validate this method with the criteria classically used in this field.

3.2.3. *Beverage analyses*

The content of carbohydrates is drastically controlled in beverages such as wines or fruit juices. Applications of this method to analyses of an apple juice diluted 1000 times and a red wine diluted 20 times, both with ultra-pure water are presented in Figs. 3B and 3C, respectively.

The analysis of the apple juice (Fig. 3B) showed the presence of 335 mM fructose, 98 mM glucose and 53 mM sucrose. This composition agreed with the qualitative one specified on the bottle (no concentration given). In the red wine sample, only fructose and glucose were detected at 2.1 and 1.4 mM, respectively. A number of additional components labeled with a star in Fig. 3 were detected in the red wine sample. Some of them should be aromatic carboxylic or phenolic acids. The identification of these peaks, however, was not the aim of this work. Through these examples, the versatility of the developed CE method was evidenced for beverage analyses.

4. Conclusion

The CE method previously optimized by our group for the analysis of carbohydrates was validated and the method fully complied the requirements of the police laboratory of Paris with respect to selectivity, precision, and limits of detection. It appeared that matrices containing high levels of ions or carbohydrates lead to significant matrix effects, but the maximum induced bias never exceeded 11%, which can be considered as acceptable in other fields of applications than forensics. Thus, real samples from pharmaceutical, and beverage fields were successfully analyzed.

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