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A new method to determine biological sample volume by short end multiple injection capillary electrophoresis: Application in determination of nitrate and thiocyanate in human saliva

Luciano Vitali, Valfredo T. Fávere, Gustavo A. Micke*

Department of Chemistry, Federal University of Santa Catarina, Campus Universitário Trindade, 88040-900 Florianopolis, SC, Brazil

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ABSTRACT

The aim of this study was to develop a simple and rapid method of capillary electrophoresis using a short end multiple injection in free solution to determine simultaneously the biological sample volume and analytes concentration. The method consists of a sequence of injection steps with an internal standard as the reference for correction of the volume of sample collected. The procedure was applies in the determination of NO_3^- and SCN in saliva samples. The background electrolyte was composed of 12 mM tris(hydroxymethyl)aminomethane and 8.5 mM sulfuric acid, at pH 2.5. The internal standard used was BrO_3^-. A fused silica capillary (48.5 cm total length, 8.5 cm effective length and 75 μm i.d.) coated with chitosan was used in a short-end injection configuration. Modification of the electroosmotic flow (EOF) using dynamic coating resulted in a controlled and stable EOF, contributing to the rapid separation of anions (0.36 min) in co-electroosmotic mode. The validation of the method for correcting the volume of saliva collected with a swab showed a difference of less than 3.5% compared with the predicted value and a correlation of 0.999. The limits of detection for NO_3^- and SCN^- were 0.13 and 0.23 mg L^{-1}, respectively. The inter-day precision of the method determined for both analytes was less than 5% and the recovery ranged between 97 and 102%.

1. Introduction

Capillary electrophoresis (CE) is a very important analytical technique which has received increasing attention in recent years in relation to the separation of analytes due to its high versatility [1]. The diversity of possible separation modes in CE enables its application to the analysis of different substances in various types of samples. However, for each type of sample different preparation procedures are required to enable the analyses [2]. In the methods of analysis commonly applied to biological fluids samples, the steps involved in the experimental procedure include: sample collection, protein precipitation using an organic solvent when appropriate; centrifugation and/or filtration to remove solid particles; and dilution of the sample by pipetting a discrete volume [3]. Each of these steps increases the complexity of the process and the time spent on sample preparation. CE technique allows the use of different strategies to overcome some of these drawbacks using a short end multiple injection mode.

In biological matrices, analytes of high and low molecular weight can be separated and determined by CE, whether neutral or charged, for example, inorganic anions and cations, proteins, drugs, and pharmaceuticals [4]. However, analysis of biological material by CE requires some care, since many samples may contain high concentrations of salts and proteins. Both of these can cause problems in CE analysis and therefore the composition of a biological sample is decisive in determining which analytical approach is most appropriate. In most CE modes the separation occurs in solution in a fused silica capillary, and interaction between the components of the sample and capillary walls is undesirable. The proteins found in many biological samples can bind strongly to the surface of the silica capillary under neutral or moderately acidic or basic pH conditions. This adsorption manifests in the separation through changes in the electroosmotic flow (EOF), peak broadening and formation of a peak tail, which in the case of proteins can inhibit their quantification and can cover a large part of the electropherogram, as well as alter the base line [5]. There are a variety of options available to prevent protein adsorption, such as sample preparation by precipitation with organic solvent, or non-covalent coating of the capillaries using physically adsorbed surfactants and polymers. The non-covalent coating of the capillaries, besides preventing protein adsorption, has the advantages of generally involving a simple procedure with good stability of the EOF and allowing modification and control of the EOF thus enabling the simulation of separation in specific software programs, which eliminates the use of reagents in

* Corresponding author. Tel.: +55 48 3721 6852x215; fax: +55 48 3721 6852.
E-mail address: gustavomicke@qmc.ufsc.br (G.A. Micke).

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this step. Additionally, the analysis rate can be increased due to the possibility of separation in co-electroosmotic mode. Another feature that can be observed in these systems is the need for a short capillary conditioning time with running electrolyte between runs, which reduces considerably the total analysis time [6,7].

Saliva is a biological fluid produced daily by humans in amounts ranging from 500 to 1500 mL, usually consisting of 98% water and 2% of other substances such as inorganic (Na^+, K^+, Ca^{2+}, Cl^-, HCO_3^- PO_3^{2-}) and organic (uric acid, bilirubin, creatinine, glucose, cholesterol, linoleic acid, lactate) constituents, proteins and enzymes (amylase, albumin, lysozyme, transferrin) and hormones (cortisol, testosterone, progesterone, estradiol) [8,9]. Saliva can be used as a biological matrix for the analysis of different substances, to study drug release [10–12], and to identify of drugs of abuse. Component analysis of saliva can also be used in the diagnosis of local and systemic disease [5]. In the latter case, saliva constitutes an alternative source of medical information which has been relatively unexplored and which may increase the accuracy of diagnoses, sparing the patient some discomfort associated with invasive procedures such as blood tests. Moreover, many of the constituents of blood are also present in saliva, making this biological matrix an indicator of the state of the blood as well as the rest of the body. Many substances used as biomarkers or indicators of biological conditions can be easily found in saliva [9]. One example of the use of saliva as a bioindicator is the determination of NO_3^-, which is related to the levels of oxidative and nitrosative stress, and of SCN^-, which is a marker of smoke exposure in smokers and nonsmokers, as a result of detoxification of hydrocyanic acid present in cigarette smoke by an enzyme called rodanase [3,13]. Although this biological fluid does not contain a very high amount of protein, for the determination of inorganic anions in saliva by CE some measures of adsorption prevention are usually adopted, such as the precipitation of proteins with acetonitrile [13], the use of sodium hydroxide and sodium dodecyl conditioning between runs [14], the use of hydroxypropyl cellulose as an additive in the electrolyte [15], and treatment of the capillary with successive layers of ionic polymer [3].

In this study, a simple and rapid method was developed to determine simultaneously a liquid biological sample volume and analyte concentrations using a short end multiple injection procedure. The methodology was applied in saliva samples. In the collect saliva samples, was determining the saliva volume collected and NO_3^- and SCN^- concentrations in this matrix using a capillary coated with chitosan. After characterizing the EOF in the coated capillary, the separation conditions and the analytes were selected using the Peakmaster software.

2. Experimental

2.1. Chemicals and solutions

Chitosan (CTS) with a deacetylation degree of 90% and molecular weight of 122.740 was acquired from Purifarma (Sao Paulo, Brazil) and glutaraldehyde (GLU – 25% water) was purchased from Vetec (Duque de Caxias, Brazil). A stock solution of 1.0% (w/v) CTS in 1.0% (v/v) acetic acid was prepared by stirring at room temperature and centrifuged at 6000 rpm for 10 min. Phosphoric acid, sodium dihydrogen phosphate, tris(hydroxymethyl)aminomethane (Tris), 2-hydroxy-isobutyric (HIBA), acetic acid, morpholine ethanesulfonic acid (MES), sodium hydrogen carbonate and sodium carbonate, all with analytical grade purity, were used in the preparation of buffer solutions to measure the EOF. Buffer solutions, and their respective pH values, used to measure the EOF were: phosphate (pH 2.4 and 2.8); Tris/HIBA (pH 3.3–4.2; and pH 7.6–8.9); Tris/acetic acid (pH 4.7 and 5.3); Tris/MES (pH 5.9–7.4); and carbonate (pH 9.5), all with ionic strength of around 20 mM and buffering capacity above 6.0 mM. Stock solutions of sulfuric acid (20%, v/v) and Tris (60.4 mM), reagents purchased from Labsynth (Diadema, Brazil), were used to prepare the running electrolyte for analysis of NO_3^- and SCN^-. The concentrations of electrolyte components optimized were 12 mM Tris and 8.5 mM sulfuric acid, at pH 2.5. Standard stock solutions (10 mM) of potassium nitrate (KNO_3), potassium thiocyanate (KSCN) and potassium bromate ($KBrO_3$), all purchased from Labsynth (Diadema, Brazil), were prepared by dissolving in deionized water. The calibration solutions of the anions were prepared by appropriate dilution of the stock solution. Water deionized in a Milli-Q system (Millipore, Bedford, MA, USA), with a resistivity of 18.2 M Ω cm, was used to prepare all solutions.

2.2. Saliva sampling procedure

The procedure used in the preparation of saliva samples was as follows: saliva samples from volunteer smokers and nonsmokers were collected using swabs with Rayon tips sterilized by gamma radiation with a total length of 15 cm, manufactured by Alamar Techno Scientific Ltd. (Diadema, Brazil). Immediately after collection, the piece of the swab containing the absorbed sample was carefully cut (3 cm) and immersed in 1.0 mL of 25.6 mg L^{-1} BrO_3^- solution (internal standard), contained in a 2.0 mL flask with a cap, stirred vigorously for 1.0 min and then centrifuged at 6000 rpm for 10 min. An aliquot of the centrifuged solution was removed and reserved for electrophoretic separation.

2.3. Apparatus and analytical methods

All experiments were performed in a capillary electrophoresis instrument (Agilent Technologies model HP^{3D} CE, Palo Alto, CA, USA) equipped with a diode array detector. The measurements were performed at 25 °C in a fused silica capillary with external coating of polyacrylate (Microtube, Araraquara, Brazil) with dimensions of 48.5 cm (8.5 cm to detector) × 75 μm i.d. × 365 μm o.d. The capillary was treated by dynamic coating with CTS and GLU following the procedure used by Huang et al. [16]. The EOF measurements for the characterization of changes were taken according to the procedure described by Williams and Vigh [17] using acetone as a neutral marker. Prior to the determination of NO_3^- and SCN^- in the standard solutions and saliva samples, the capillary was washed between runs for 30 s at a pressure of 1 bar (wash equivalent to two volumes of capillary) with a running electrolyte of 12 mM Tris/8.5 mM sulfuric acid. The standards and samples were introduced at the short end of the capillary and injected by hydrodynamic pressure (40 mbar = 3997.0 Pa) according to the following steps: −40 mbar/4 s (internal standard); −40 mbar/3 s (electrolyte); −40 mbar/4 s (sample or standard); −40 mbar/3 s (electrolyte). The separation voltage applied was 25 kV with negative polarity in the injection. The acquisition and data processing were performed using HP Chemstation software.

3. Results and discussion

3.1. Procedure for determination of sample volume using multiple injection

Fig. 1 shows an alternative method for collecting saliva, correcting the sample volume collected and determining NO_3^- and SCN^- by CE. Fig. 1(I) details the method for the collection of saliva samples. A stock acceptor solution (S_1) containing a given concentration of standard internal is prepared and a known volume is transferred to a flask with a cap. A swab containing an unknown amount of saliva sample collected from the volunteer is inserted into this flask giving solution S_2. Taking into consideration that the peak area of

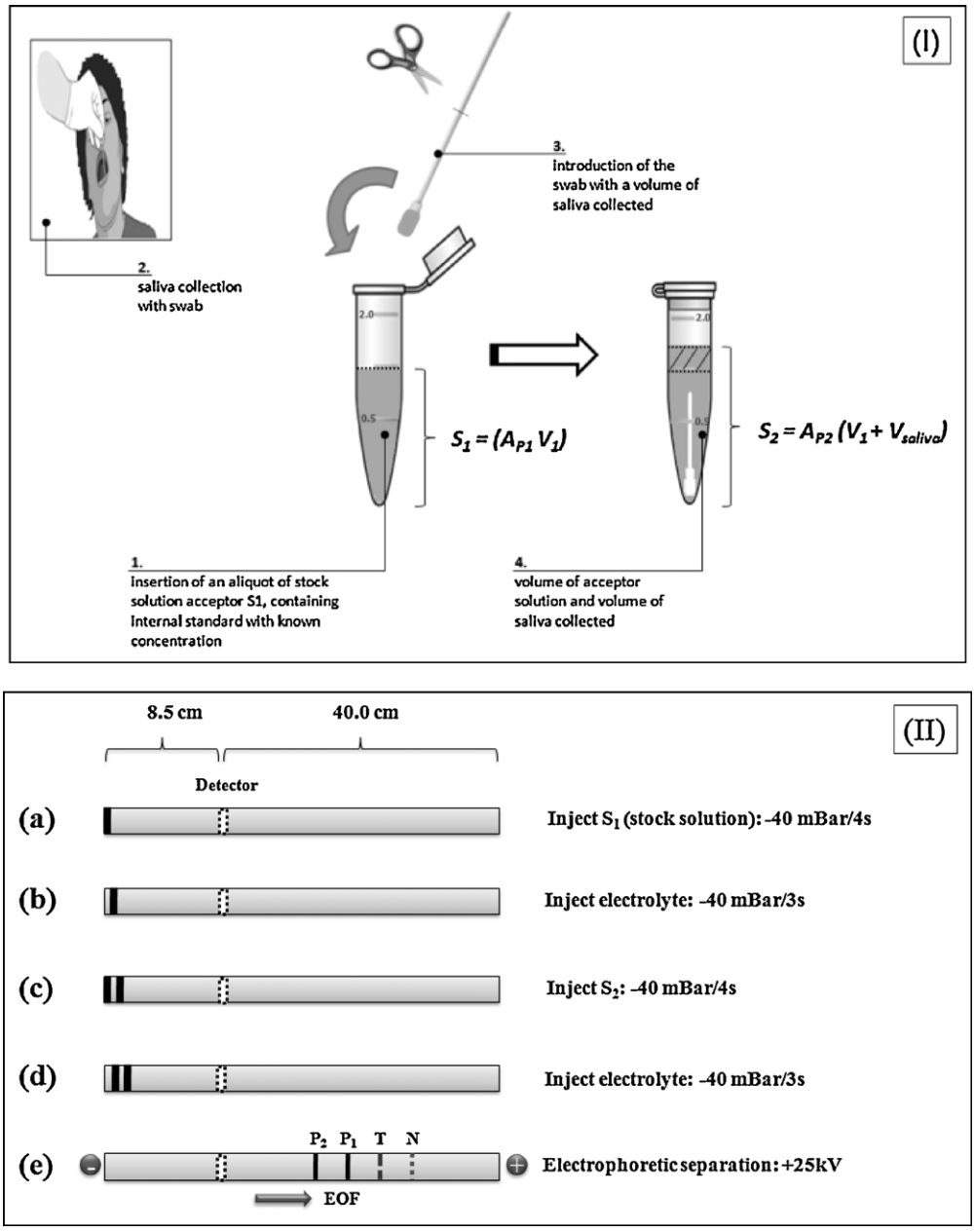

Fig. 1. Scheme representing the steps involved in the method for correction of volume of saliva sample collected using internal standard procedure and determination of NO_3^- and SCN^- by capillary electrophoresis. (I) Procedure for saliva collection and (II) steps to determine the volume of saliva collected by CE.

the internal standard A_{P1} is proportional to the concentration of the internal standard, and the variation of the area of internal standard is directly related to the change in the volume of the acceptor solution, it follows that:

$$S_{1(n\,IS)} = S_{2(n\,IS)}$$

where $S_{1(n\,IS)}$ is the number of moles of internal standard in solution S_1 and $S_{2(n\,IS)}$ is the number of moles of internal standard in solution S_2. As $S_{1(n\,IS)} = A_{P1}\,V_1$, $S_{2(n\,IS)} = A_{P2}\,V_2$, and $V_2 = (V_1 + V_{saliva})$, the following equation can be used to calculate the volume of saliva collected:

$$A_{P1}V_1 = A_{P2}(V_1 + V_{saliva})$$

Rearranging the above equation gives:

$$V_{saliva} = V_1 \left(\frac{A_{P1} - A_{P2}}{A_{P2}}\right)$$

where V_{saliva} is the volume of saliva collected, V_1 is the volume of the aliquot removed from the stock acceptor solution; A_{P1} is the area of internal standard in the stock acceptor solution and A_{P2} is the area of internal standard in the acceptor solution containing the sample of saliva. To determine the areas of the internal standard A_{P1} and A_{P2}, a CE method was developed that allows the acquisition of these data in a single run. Fig. 1(II) shows the sequence of steps for this procedure. Initially, a plug of solution S_1 is injected into the capillary (a). The electrolyte spacer (b) is then injected followed by injection of solution S_2 (internal standard solution containing an unknown amount of saliva) (c). Electrolyte spacer is again injected (d) and, finally, voltage is applied for the electrophoretic separation (e). The injection of electrolyte in the last step aims at obtaining a good repeatability of the results. The electropherogram obtained will consist of four peaks: 1 – NO_3^- (N); 2 – SCN^- (T); 3 – internal standard shown in S_2 (P1); and 4 – internal standard of acceptor solution containing saliva collected (P2). By varying the injection

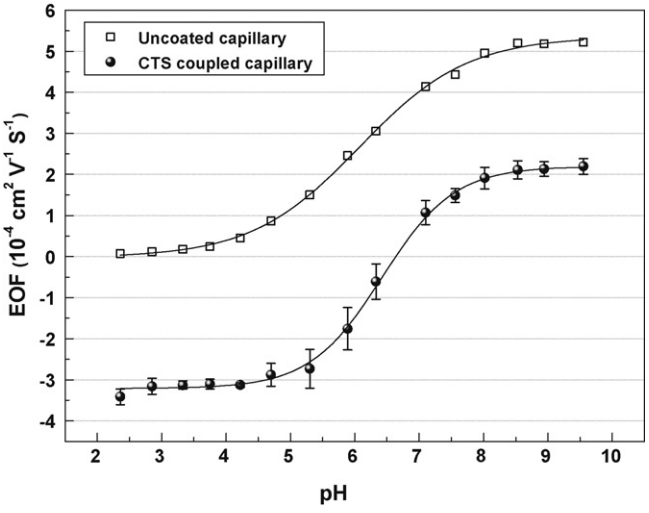

Fig. 2. Variation of the EOF as a function of pH. Capillary coated with CTS: measurements for $n = 4$ performed in two capillaries treated exactly as described by Huang [16]. Uncoated capillary: measurements for $n = 1$ in one capillary conditioned with 1 M NaOH and deionized water. Conditions for EOF measurement: electrolyte with ionic strength of 20 mM, flushing with electrolyte for 3 min before the measurements, acetone as a flow marker, migration time 120 s, and voltage ±15 kV.

time of the spacer it is possible to determine the migration time of P1, so that this peak migrates to the desired position in the electropherogram resulting in the proper resolution of this peak in relation to the other peaks on the electropherogram. The P1 migration time is calculated according to the following equation:

$$t_{P1} = \frac{L_{det} - L_{inj}}{\mu_{P1} E}$$

where,

$$L_{inj} = \frac{\Delta P t_{int} r^2}{8 L_{tot} \eta} \quad \text{and} \quad \mu_{P1} = (\mu_{osm} + \mu_{eff})$$

Thus,

$$t_{P1} = \frac{L_{det} - ((\Delta P t_{int} r^2)/(8 L_{tot} \eta))}{(\mu_{osm} + \mu_{eff}) E}$$

where t_{P1} and t_{int} are the migration time of P1 and total time of pressure application during the injection of all plugs, respectively, in seconds; L_{det}, L_{int} and L_{tot} are the length of capillary to the detector, the length of the injected plug and the total length of capillary, respectively, in meters; μ_{p1}, μ_{osm}, and μ_{eff} are the apparent mobility, mobility of the electroosmotic flow and effective mobility of the internal standard, respectively, in $m^2 V^{-1} s^{-1}$; E is the electric field applied, in $V m^{-1}$; ΔP is the injection pressure, in $N m^{-2}$ (1 mbar = 99.92 N m); and η is the viscosity of the solution at 25 °C, in $N s m^{-2}$.

3.2. Electroosmotic flow characterization

The preparation of the coating of the fused silica capillary with CTS and GLU used in this study was identical to that described by Huang. However, the variation observed in the EOF profile differed. Fig. 2 shows the variation of EOF as a function of pH performed in capillaries treated with 0.2% CTS and 12.5% Glu and in an uncoated capillary. The profile observed in both cases was sigmoidal. In the case of the capillary treated with CTS, the EOF remained constant and inverted at pH below 4.7, with an inflection at pH 6.3, which starts at pH 5.3 (inverted EOF) and ends at pH 7.5 (normal EOF), remaining constant and normal above pH 8.0. In contrast, the profile obtained by Huang showed an almost linear increase in the EOF with increasing pH in the pH range of 1.8–5.0 with an inverted EOF at pH values below 4.0 and a constant and normal EOF above pH 6.0. The behavior of the EOF as a function of pH in this study is consistent with that expected considering the p*K*a values for the CTS (6.3–6.7) [18] and silica (4.0–6.0) [19]. While at pH below 5.0 the CTS (coated capillary) is protonated characterizing an inverted EOF, silica (uncoated capillary) has a low ionization, leading to a normal EOF. Comparison of the EOF values at pH above 7.0 determined for the coated and uncoated capillaries shows that the lower intensity of the EOF observed for the coated capillary is not due to the CTS, which has a neutral pH, but rather to the amount of remaining ionized silanol groups of the silica that did not interact with the CTS. Thus, at pH above the p*K*a value of the CTS, the polymer acts only by reducing the number of silanol groups present on the capillary wall, which explains the difference in the EOF compared to the uncoated capillary, which has a larger number of silanol giving a normal EOF with a higher value. Based on this information it is possible to calculate the percentage of silanol groups replaced after coating with CTS which was 59.8 ± 3.6% (average percentage of substitution ± standard deviation), calculated considering EOF values of pH 8.0, 8.5 and 9.0, for an uncoated capillary and two other capillaries coated with CTS.

3.3. Selection of experimental conditions

3.3.1. Influence of several counter-ions on electroosmotic flow employing coated capillary

In the capillary coated with CTS at pH values below 6.5 the co-ion component of the running buffer which influences the separation of the analytes at the same time acts as a counter-ion of the wall also influencing the EOF value measured. The verification of this influence on the EOF is important because it allows the simulation of the separation of NO_3^- and SCN^- under conditions closer to real conditions using the Peakmaster software. The anions with mobility close to that of the analytes, which prevents the peak asymmetry phenomenon, and therefore can be selected as co-ion components of the running electrolyte are chloride, sulfate and perchlorate. Thus, it is necessary to measure the EOF with each of these anions acting as counter-ions of the modified capillary wall. The results showed that varying the co-ion significantly modified the EOF, since with chloride (-3.2×10^{-4} $cm^2 V^{-1} s^{-1}$) and perchlorate (-2.9×10^{-4} $cm^2 V^{-1} s^{-1}$) the intensity of the observed EOF was around three times higher than with sulfate (-1.1×10^{-4} $cm^2 V^{-1} s^{-1}$). One possible explanation for this behavior is that at pH 2.5 sulfate has a greater charge density than chloride and perchlorate, due to the almost complete dissociation of the second proton of sulfuric acid. Sulfate has a greater effect of ionic interaction with the CTS of the wall than chloride and perchlorate, which causes a significant decrease in the intensity of the EOF. According to Zheng and coauthors, who studied the type of interaction between sulfate and CTS membranes, this effect reflects ionic crosslinking of the CTS by sulfate ions. However, in the same study it was found that crosslinking occurs only with the sulfate ions located between two NH_3^+ groups of neighboring chains of the polymer [20].

3.3.2. Peakmaster simulations

The characteristics of a suitable running electrolyte include a good buffering capacity and appropriate selection of the co-ion and counter-ion to minimize the peak asymmetry. In addition, other parameters influencing the separation are easily obtained using Peakmaster [21–23]. In the simulation of the separation the use of a UV detector should be considered, given that the analytes NO_3^- and SCN^- absorb in this region of the spectrum, and thus the components of the running electrolyte should not absorb, particularly at the same wavelength as the analytes.

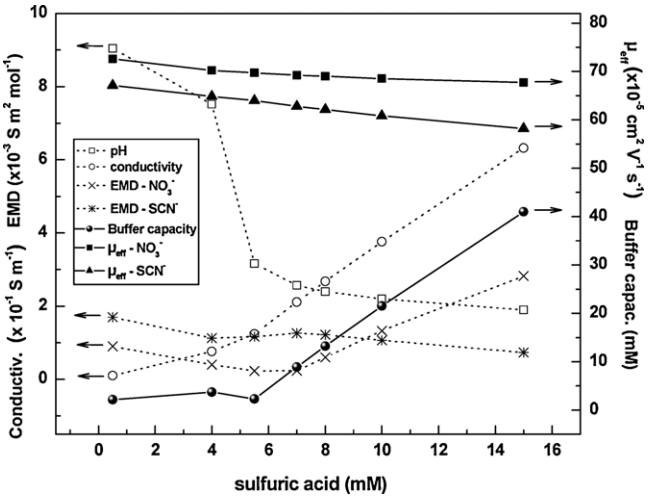

Fig. 3. Simulation of the conditions for the separation of analytes using sulfate as co-ion. Data obtained using Peakmaster with a fixed concentration of 10 mM Tris and considering the average EOF value with sulfate as co-ion. Simulation conditions: total capillary length 48.5 cm, effective capillary length 8.5 cm, voltage 25 kV, pH 2.50, ionic strength 23 mM (correction with sodium). Electromigration by dispersion (EMD) expressed as a module.

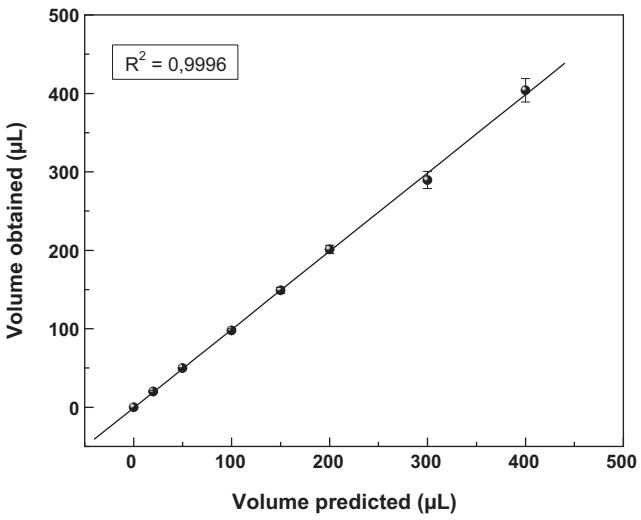

Fig. 4. Validation of the method proposed for correction of the saliva sample volume using an acceptor solution containing the internal standard and a capillary coated with CTS. Results expressed as mean and standard deviation of solutions prepared in triplicate and determined in duplicate. A volume of 5.0 mL of saliva was collected and centrifuged at 6000 rpm for 10 min; different aliquots of the sample were removed to be added to acceptor solution and placed in different containers. Other conditions: see Section 2.

The simulation for the selection of the co-ion component of the electrolyte was performed using Tris as the counter-ion and testing three different co-ions (chloride, perchlorate and sulfate), considering the EOF characteristic of each co-ion in a capillary treated with CTS. The EOF values used were: -3.2×10^{-4} cm^2 V^{-1} s^{-1} for the chloride; -2.9×10^{-4} cm^2 V^{-1} s^{-1} for perchlorate and -1.1×10^{-4} cm^2 V^{-1} s^{-1} for sulfate. It was observed that of the three co-ions tested in the simulation sulfate gave the best conditions for separation of the analytes. The average value for the electromigration dispersion (EMD) observed for sulfate was similar to that for perchlorate, but better than that for chloride, and the buffering capacity was greatest for sulfate due to the pH of the electrolyte in the simulation (2.5) being close to the p*K*a value of sulfuric acid. Furthermore, the resolution of NO_3^- and SCN^- using sulfate as the co-ion was the highest, as might be expected since the separation occurs in the co-electroosmotic mode and thus the resolution is favored by the lower EOF value. Therefore, sulfate was chosen as the running electrolyte.

Fig. 3 shows the simulation used to select the best conditions for the separation of NO_3^- and SCN^- using Tris as the counter-ion and different concentrations of sulfate. It can be observed that the proportion of Tris/sulfate that resulted in the best conditions for the separation was 1.4:1.0 (v/v). For this composition the solution pH was around 2.5 with a sufficient buffer capacity (~9.0 mM), acceptable conductivity value (~0.2 S m^{-1}) and, most importantly, low EMD values (NO_3^- ~ 0.2 and SCN^- ~ 1.3 S m^2 mol^{-1}) for both analytes. Thus, the concentrations of the components of the Tris and sulfuric acid electrolyte prepared for use in the separations were 12 mM and 8.5 mM, respectively.

The choice of a suitable internal standard for the separation was also based on a simulation carried out using the Peakmaster software. The anion selected as the internal standard should have the following characteristics: high effective mobility, absorbance in the UV region, no reaction with the analytes and not present in the sample. An anion that meets all these requirements is BrO_3^-, which was thus chosen as the internal standard.

3.4. Validation of method for correction of saliva volume collected

Fig. 4 shows the validation of the correction of the saliva volume collected by the proposed method using a known volume of pipetted saliva, a reference volume, which was added to a known volume of acceptor solution containing the internal standard. The values for the volume of pipetted saliva corresponded to the values determined by the volume correction method with a correlation coefficient of 0.999 and the difference between the expected and obtained values was less than 3.5% with a relative standard deviation obtained for the volumes of less than 3.8%, showing that the method can be used to correct the volume of saliva collected with a swab.

In Fig. 5 a typical electropherogram of a sample of saliva collected using a swab can be observed, in which the migration time of the BrO_3^- reference peak P1 was calculated for $\mu_{osm} = -1.1 \times 10^{-4}$ cm^2 V^{-1} s^{-1} and $\mu_{eff} = -5.33 \times 10^{-4}$ cm^2 V^{-1} s^{-1} (the data taken from the Peakmaster software, considering the

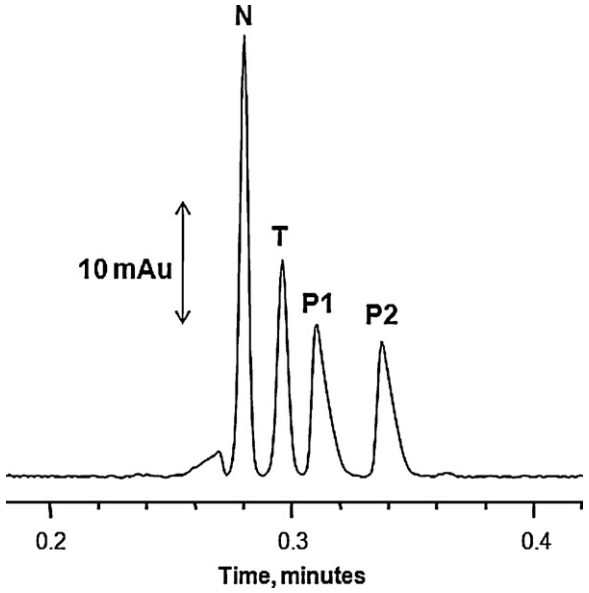

Fig. 5. Electropherogram of a sample of saliva collected with a swab employing the optimized method in a capillary coated with CTS. Data acquired at 200 nm.

Table 1
Figures of merit of the optimized method for the determination of NO_3^- and SCN^- in saliva by CE using a capillary coated with CTS. For other conditions see Section 2.

Figure	Analyte	
	NO_3^-	SCN^-
Linearity-calibration range (mg L^{-1})[a]	6.2–62	5.8–58
Linearity-slope (L mg^{-1}) [a]	1.55	1.04
Slope standard deviation[a]	0.01	0.01
Linearity-intercept[a]	0.38	0.33
Intercept standard deviation[a]	0.01	0.06
Linearity-coefficient determination (R^2)[a]	0.999	0.999
Detection limit, LOD (mg L^{-1})[b]	0.81	1.0
Quantification limit, LOQ (mg L^{-1})[b]	2.5	3.2
Instrumental precision, RSD (%) – peak area[c]	1.2	1.7
Instrumental precision, RSD (%) – migration time[c]	0.8	1.3
Intra-day precision, RSD (%) – peak area[c]	2.6 (3.7)	1.8 (2.1)
Intra-day precision, RSD (%) – migration time[c]	2.4 (1.4)	2.4 (1.4)
Inter-day precision, RSD (%) – peak area[c]	3.1	2.6
Inter-day precision, RSD (%) – migration time[c]	4.0	4.6
Number of plates (N m^{-1})[d]	15,450	15,004
Peak symmetry[e]	1.07	0.921
Peak asymmetry factor[e]	0.95	1.16
Peak tailing factor[e]	0.98	1.04
Resolution (NO_3^-: SCN^-)[f]	1.2	

[a] Values for $n = 6$, each point on the curve measured in triplicate. Calibration curve without internal standard correction.
[b] Limits of detection and quantification calculated according to the equations: LOD = $(3.3 \times s)/S$; LOQ = $(10 \times s)/S$, where s is the linear coefficient and S is the slope of the analytical curve equation, respectively.
[c] Relative standard deviation calculated with the equation: RSD = $(P/\bar{u}) \times 100$, where P is the absolute standard deviation and $\bar{u}$ is the arithmetic mean of the analyte area or arithmetic mean of the migration time. RSD values for instrumental precision measured in the same solution, for the intra-day precision with 10 preparations at the same concentration and the inter-day precision with 10 preparations on one day and 10 the next day. Instrumental precision $n = 14$, intra-day precision $n = 10$ and inter-day precision $n = 20$. Intra-day RSD values in brackets refer to a saliva sample diluted 10 times for $n = 10$.
[d] Number of plates calculated according to the equation $N = 16(t_i/w_{hi})^2$, where t_i is the migration time of analyte given in minutes and w_{hi} is the bandwidth at the baseline, in the same units t_i. Concentration of NO_3^- and SCN^- around 25 mg L^{-1} and $n = 3$.
[e] Symmetry, asymmetry and tailing factor of the peaks for $n = 3$.
[f] The resolution is calculated as: $Rs = 2(t_n - t_{n-1})/(w_n + w_{n-1})$, where t is the peak migration time and w is the width of the base. Concentration of NO_3^- and SCN^- around 25 mg L^{-1} and $n = 3$.

ionic strength of the electrolyte), $n = 8.9 \times 10^{-4}$ N s m^{-2} and other conditions which are given in Section 2. Thus, the t_{P1} calculated for $t_{int} = 14$ s, due to the sequence of plugs injected, was 18.8 s which corresponds to 0.313 min, a value that is close to the time of P1 migration observed in the electropherogram (0.310 min; data acquired from HP Chemstation software). With knowledge of the migration time calculated from the BrO_3^- reference and the base width of the peaks of the anions it is possible to predict the resolution of BrO_3^- P1 in relation to the SCN^- and BrO_3^- P2, since the width of the peak bases for BrO_3^- P1 and P2 will be very similar. The resolutions provided for the combinations T:P1 and P1:P2 were 0.92 (0.67–1.2; considering the EOF deviation value $-1.1 \pm 0.1 \times 10^{-4}$ cm^2 V^{-1} s^{-1} in the calculation) and 1.1 (0.87–1.3; considering the EOF deviation value $-1.1 \pm 0.1 \times 10^{-4}$ cm^2 V^{-1} s^{-1} in the calculation), while the resolutions obtained were 0.83 and 1.3, respectively.

Finally, measurements were taken in two solutions with the same concentration of NO_3^- and SCN^-, the first without dipping the swab in the sample collector and the second by immersing the swab in the solution for 1 h, in order to verify whether there is interference caused by the material which comprises the swab. The results showed a relative standard deviation of 3.4% and 0.65% for NO_3^- and SCN^-, respectively (area of analyte/area of internal standard), showing that the swab material is suitable for the sample collection method proposed and does not cause interference.

3.5. Analytical validation

3.5.1. Figures of merit

The applicability of the proposed method for the determination of NO_3^- and SCN^- in saliva samples was performed according to the figures of merit of the method presented in Table 1. The calibration range for both analytes was 6–60 mg L^{-1} with a coefficient of determination higher than 0.99. The limits of detection and quantification of the method were suitable for determination of NO_3^- and SCN^- in saliva samples. The RSD values for the peak area of the analytes and the migration time were less than 5%. Likewise, the RSD value for the intra-assay precision using a saliva sample was as low as those obtained for the standard solutions.

3.5.2. Accuracy and samples analysis

The selectivity of the proposed method for determination of NO_3^- and SCN^- in saliva samples was measured by the standard addition method and the results are presented in Table 2. The recoveries obtained for five different concentrations of NO_3^- and SCN^- were within the range of 97–102%, indicating good selectivity of the method. The application of the optimized method for the collection and determination NO_3^- and SCN^- was carried out using saliva samples from six volunteers and measured concentrations are shown in Table 2. The sample from each volunteer was collected and injected in triplicate.

Table 2
Verification of the selectivity of the method for determination of NO_3^- and SCN^- in saliva samples using standard addition and results for the determination of these analytes in saliva samples collected from volunteers using the optimized method for the collection and determination. For other conditions see Section 2. Data acquired at 200 nm. Correlation coefficients > 0.99.

Analyte	Concentration (mg L^{-1})[a]		Recovery (%)[b]	Concentration determined sample[c] (mg L^{-1})					
	Added	Found [b]		1	2	3	4	5	6
NO_3^-	0.620	0.622	100.4	14.8 (±0.3)	394.5 (±8.8)	42.8 (±6.5)	18.3 (±3.0)	39.9 (±1.5)	175.5 (±9.5)
	6.20	6.03	97.3						
	12.40	12.37	99.7						
	24.80	25.06	101.1						
	37.21	37.08	99.6						
SCN^-	0.581	0.593	102.1	30.2 (±3.1)	55.4 (±1.2)	54.6 (±1.3)	9.99 (±2.6)	98.6 (±4.4)	26.3 (±3.6)
	5.81	5.69	98.0						
	11.62	11.53	99.2						
	23.23	23.51	101.2						
	34.85	34.71	99.6						

[a] Recovery using saliva sample diluted 10 times. Concentration of analytes in saliva: 25.3 mg L^{-1} NO_3^- and 12.7 mg L^{-1} SCN.
[b] Average results for two replicates ($n = 2$) determined in duplicate for each concentration.
[c] Results expressed as mean with confidence limit of 95% for $n = 3$.

Table 3
Comparative analysis of some characteristics of the method presented in this paper with other methods.

Modifier	Preconditioning time (min)	Separation time (min)[a]	LOD NO_3^- (mg L^{-1})	LOD SCN^- (mg L^{-1})	References
Without modifier	3	6.2	–	0.041	[13]
Polybrene	10.5	2.8	1.1	5.4	[3]
NaOH and sodium dodecyl sulfate	11	7	0.21	–	[14]
Hydroxypropyl cellulose	4	9	4.6×10^{-3}	3.8×10^{-3}	[15]
Chitosan	0.5	0.36	0.81	1.0	This work

[a] Final time of separation considering the migration time of the last interest anion (NO_3^- or SCN^-) or standard internal used in the method.

3.6. Comparison with other methods

The comparative analysis (summarized in Table 3) of some characteristics of the method presented in this paper with other methods [3,11,13,14] employed to determine anions in saliva, especially NO_3^- and SCN^-, showed that the proposed method using CTS as the EOF modifier required less conditioning time between runs and a shorter separation time. Also, the LOD values for NO_3^- and SCN^- were as good as or better than those obtained with other methods. Moreover, the average total analysis time (TAT) [7,24] of the method was measured for $n = 5$ resulting in 2.81 min per analysis which represents a sampling rate of 21 runs per hour. Of the TAT, 29% (0.81 min) relates to the flush procedure of the capillary, 58% (1.64 min) to the injection time and 13% (0.36 min) to the electrophoretic separation. Thus, most of the time required for the analysis is related to instrumental speed. Another important point related to the analysis rate is the stability of the analytes in the sample. According to a study by Tanaka and co-authors, the anionic concentration of some components in saliva samples, including NO_3^- and SCN^-, are stable at room temperature for 4 h and with refrigeration at 3 °C this stability could be maintained for 48 h [3]. Thus, by employing a rapid method for the collection and determination several samples can be analyzed without the need for refrigeration.

4. Conclusions

Capillary electrophoresis (CE) has been shown to be an even more powerful separation technique through the development of a new strategy to reduce the number of steps in the experimental procedure, allowing analysis with minimal sample pretreatment. The coated capillary prevents the adsorption of proteins present in saliva samples, requiring only a few seconds of rinsing with electrolyte between runs, which also contributes to the increased throughput of the method. Another advantage is the possibility of its use with a mass detector, since the modifier is not present in the electrolyte but is fixed to the wall of the capillary. The proposed method has advantages that could be extended to determine other substances in saliva, since this biological fluid contains several markers used in diagnostics and as indicators of drug abuse. Additionally, the strategy proposed for sample volume correction could also be implemented and applied to other samples that are difficult to pipette due to their viscosity or even other biological fluids that are difficult to collect, such as tears and sweat.

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