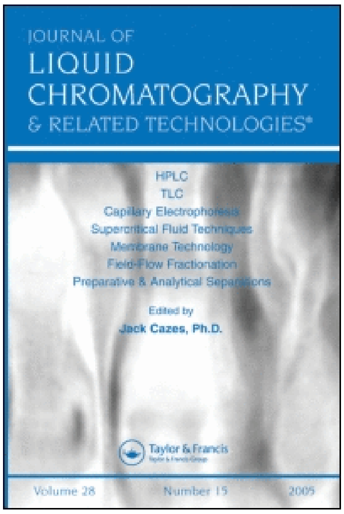

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A. Negro[a]; E. Paz[a]; B. Rabanal[a]
[a] Área Química Analítica, Facultad de C.C. Biológicas y Ambientales, Universidad de León, León, Spain

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New Electrolyte Composition for Determination of Anions by Capillary Electrophoresis with Indirect UV Detection

A. Negro,* E. Paz, and B. Rabanal

Área Química Analítica, Facultad de C.C. Biológicas y Ambientales,
Universidad de León, León, Spain

ABSTRACT

This paper proposes a new analytical method by capillary electrophoresis (CE) with indirect UV detection for determining the following anions: fluoride, chloride, bromide, nitrite, nitrate, sulphate and phosphate in water, biological fluids, or other similar media. Various substances were tried to perform the functions of background electrolyte and electro-osmotic flow (EOF) modifier. In addition, a study was undertaken of the influence over the analytical parameters of anions of variation in the main factors affecting the electrophoretic process, such as: concentration of background electrolyte and EOF modifier, pH of electrolyte, voltage, and temperature. The data obtained from this work allow the most appropriate conditions for the analysis of these anions to be fixed. These are: EOF modifier: benzethonium chloride 0.10 mM; background electrolyte: sodium thiosulphate

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*Correspondence: A.Negro, Área Química Analítica, Facultad de C.C. Biológicas y Ambientales, Universidad de León, E-24071, León, Spain; E-mail: dbbana@unileon.es.

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25.0 mM; pH = 5.00; voltage 15.0 kV; and $T = 25°C$. Under these conditions, analyses can be performed of all the anions listed, in times ranging from 3.72 min for bromide to 5.30 min for phosphate, with detection limits lying between 0.12 μg/mL for fluoride and 0.35 μg/mL for nitrite.

Key Words: New electrolyte; Anions; Capillary electrophoresis.

INTRODUCTION

Capillary electrophoresis (CE) is complementary to ion chromatography, and has been used to analyze inorganic[1–4] and small organic ions. Capillary electrophoresis is attractive due to its short analysis times, large peak capacity,[5] efficiencies of the order of 10^6 theoretical plates,[6] and minimal solvent and sample requirements. Optimum analysis times and resolution in CE require control of electro-osmotic flow (EOF), but more importantly require a mechanism that will permit controlled changes in separation selectivity.

Suppression of the reversal of EOF in the separation capillary with amines or organic solvents has been shown to enhance separation selectivity for anionic species.[7] Similarly, these same EOF modifiers can be used to shorten, dramatically, the time required for analysis of anions.[8]

In indirect UV detection, analytes without chromophores are detected by recording the decreases in absorption of the sample zone, which are caused by the displacement of the chromophoric background electrolyte ions by the sample ions. In this case, success in the detection of analytes is largely dependent upon the proper detection conditions. Usually, low molecular weight ions are spared in a co-electro-osmotic mode. For this purpose, the direction of EOF must be reversed and directed to the anode by adding an EOF modifier to the background electrolyte.

Electro-osmotic flow modifiers commonly used for dynamic modification of the fused-silica surface, include long chain alkyltrimethylammonium salts, such as cetyltrimethylammonium bromide and tetradecyltrimethylammonium bromide or hexyldiquaternaryammonium salts, such as hexamethonium hydroxide. Indirect UV detection methods require a background electrolyte. The choice of suitable background electrolytes is restricted to substances that have background UV absorbance and co-migrate with the mixture of analytes.[9,10] Many background electrolytes for the determination of anions have been described, examples being benzoate[11,12] phthalic acid, 2-sulphobenzoic acid, o-benzoylbenzoic acid[12] and chromate, among others.[13–15]

The combination of anodic EOF and indirect UV detection is most favorable for fast and complete CE analysis of low molecular weight anions.[16]

The aim of this work was to design a new analytical method for anion analysis by CE: fluoride, chloride, bromide, nitrite, nitrate, sulphate, and phosphate in water and biological fluids. To obtain a good analytical method, tests were carried out of various background electrolytes other than those mentioned in the literature on indirect UV detection at $\lambda = 254$ nm, such as: ammonium molybdate, potassium permanganate, sodium thiosulphate, and potassium oxalate. With respect to EOF modifiers, tests involved various chemicals mentioned in published work, such as tetradecyltrimethylammonium bromide,[17] hexadecyltrimethylammonium bromide,[18] dodecyldecyltrimethylammonium bromide, benzydildimethyl-tetradecylammonium chloride, benzydildimethylhexadecylammonium chloride, benzyldimethyldodecylammonium bromide, plus two further substances put forward here: benzethonium chloride: (*N*,*N*-dimethyl-*N*-[2-[2-[4-(1,1,3,3-tetramethylbuthyl)phenoxy]ethoxy]ethyl]benzenetetramethylbutylphenoxy) ethoxy]ethyl ammonium chloride) and methylbenzethonium chloride. Furthermore, in this work, a study was undertaken of the effects of variations in the principal parameters affecting separation, such as concentration of components of electrolyte, pH, voltage, and temperature, and on the basis of the data obtained, a new analytic method is proposed.

EXPERIMENTAL

Instrumentation and Equipment

All the experimental work was carried out with a P/ACE System 2100 high-performance capillary electrophoresis set-up (Beckman Coulter, Inc. Fullerton, CA). An untreated fused silica capillary tube (Beckman Coulter) was used with a 75 μm internal diameter, $L_t = 570$ mm; $L_d = 500$ mm, enclosed in a liquid-cooled cassette. Detection was performed with a UV-VIS detector, $\lambda = 254$ nm. Equipment was checked and data were processed with Beckman P/ACE Station V 1.2 software (Beckman Coulter).

Chemicals and Reagents

Tetradecyltrimethylammonium bromide, hexadecyltrimethylammonium bromide, dodecyldecyltrimethylammonium bromide, benzydildimethyltetradecylammonium chloride, benzydildimethylhexadecylammonium chloride, benzyldimethyldodecylammonium bromide, and benzethonium chloride and methylbenzethonium chloride, were obtained from Sigma (Madrid, Spain). Fluoride, chloride, bromide, nitrite, nitrate, sulphate, and phosphate were standard solutions purchased from Merck Farma y Química S.A (Barcelona, Spain). The water was purified with a Milli-Q Element purchased from Millipore (Bedford, MA).

Sample Preparation

Drinking water did not need any prior treatment. Serum was prepared from pig blood by centrifuging and the sample then processed using Ultrafree-CL (Millipore) 5000 NMWL ultrafiltration units operating a microcentrifuge at a high centrifugal speed (5000 G). The liquid obtained was diluted in water at a 1 : 4 ratio.

Capillary Preparation

In order to achieve good repeatability, it was necessary for the process to be carried out with the capillary in an identical condition on all occasions. To achieve this, an unchanging work routine had to be adopted, including a cycle of washing and renewal of the previously used capillary for every electrophoretic process. The steps in the process used were: regeneration of the capillary with NaOH 0.10 M (2 min); filling the capillary with work electrolyte (2 min); introduction of the sample into the capillary under pressure at 0.50 p.s.i. for 5 seconds; introduction of the electrolyte into the capillary under pressure at 0.50 p.s.i. for 1 second; placing the ends of the capillary into two vials with fresh buffer every ten processes in order to avoid "buffer depletion",[19,20]and initiation of the electrophoretic process. When the electrophoretic process was over, the capillary was washed with water for 2 min.

RESULTS AND DISCUSSION

Choice of Background Electrolyte and Electro-osmotic Flow Modifier

Background electrolyte trials were made of various salts that might be suited because of their high absorbance at 254 nm at low concentrations in the order of 5.00 mM. In particular, tests were carried out on sodium thiosulphate ($Ab_{254} = 0.75$) and potassium oxalate ($Ab_{254} = 0.25$). Both these salts were mixed in a concentration of 5.00 mM with either benzethonium chloride or methyl benzethonium chloride, substances being used as EOF modifiers.

Aqueous solutions containing standard samples of all the anions under study, in concentrations of 25 μg/mL, were analyzed using the former background electrolyte, and an EOF modifier dissolved in water. All the possible combinations of the substances listed were tried, and the most satisfactory results were obtained with a combination of sodium thiosulphate 5.00 mM and benzethonium chloride 0.50 mM; $pH = 6.20$; 10.0 kV, 25°C. Once the chemicals, which were to form the electrolyte, had been chosen in this way, the influence of variations of the various parameters in the process of electrophoretic separation was investigated in order to optimize all the conditions for the analytic method.

Concentration of Electro-osmotic Flow Modifier

Electrolytes were prepared by combining aqueous solutions of benzethonium chloride at different concentrations, 0.00; 0.10; 0.25, and 0.50 mM, with sodium thiosulphate 5.00 mM, $pH = 6.20$, which is what was obtained when both components are dissolved in water. Each of these electrolytes was used to analyze the sample containing all the standard solutions of the anions under study, at a concentration of 25 μg/mL. In the electropherograms obtained, no peaks appeared for any of the anions when the concentration of benzethonium chloride was 0.00 mM, as was to be expected. Peaks did appear for the anions with good resolution at concentrations from 0.10 up to 0.25 mM of benzethonium chloride. However, when the concentration of benzethonium chloride was increased to 0.50 mM, resolution was lost in the peaks corresponding to bromide, chloride, nitrate, and sulphate, and there were virtually no t_m variations for the other anions (Fig. 1). On the basis of these outcomes, a concentration of 0.10 mM of benzethonium chloride may be considered as the most suitable for carrying out such analyses, since it gives good resolution; and with this lower concentration there is no rise in the conductivity of the electrolyte. The other conditions under which this trial was performed were: $pH = 6.20$; voltage = 10.0 kV; $T = 25°C$.

Concentration of Background Electrolyte

Once the most appropriate concentration for the EOF modifier had been established, it was necessary to optimize the concentration of background electrolyte. For this purpose, electrolytes were prepared composed of aqueous solutions of 0.10 mM of benzethonium chloride and varying concentrations of sodium thiosulphate: 5.00; 10.0; 15.0; 20.0; 25.0; and 30.0 mM. It was observed that as the concentration of sodium thiosulphate increased, the resolution of the peaks corresponding to all the anions improved. In accordance with these results, the most suitable concentration of background electrolyte was considered to be 25.0 mM, rather than higher values, so as to avoid increasing electrolyte conductivity and diminishing the production of

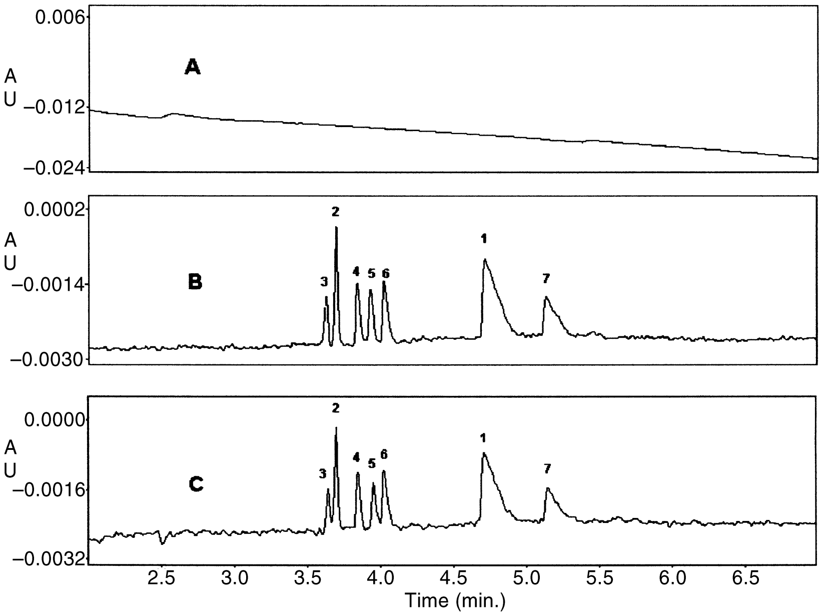

Figure 1. Electropherograms corresponding to the optimization of concentration of electro-osmotic flow modifier. (A) Benzethonium chloride 0.00 mM. (B) Benzethonium chloride 0.10 mM. (C) Benzethonium chloride 0.50 mM. In all these cases, sodium thiosulphate 5.00 mM; pH = 6.20; voltage = 10.0 kV; $T = 25$°C. (1) Fluoride, (2) Chloride, (3) Bromide, (4) Nitrite, (5) Nitrate, (6) Sulphate, and (7) Phosphate.

Joule heat.[21] The other conditions applying in carrying out this trial were: pH = 6.20; voltage = 10.0 kV; $T = 25$°C (Fig. 2).

pH of Electrolyte

Electrolyte pH is probably the parameter with the greatest influence over whether a good CE analysis is achieved, as pH is responsible for the electric charge on the analytes and, hence, for their electrophoretic mobility, if samples contain a complex mixture or several anions. The pH value also influences the charge on the walls of the capillary tube, which are composed of fused silica and have ionizable silanol groups in contact with electrolyte.[22] The pH of fused silica is about 1.50; the degree of ionization of the wall of capillary, and EOF[23] is controlled by the pH of the electrolyte.

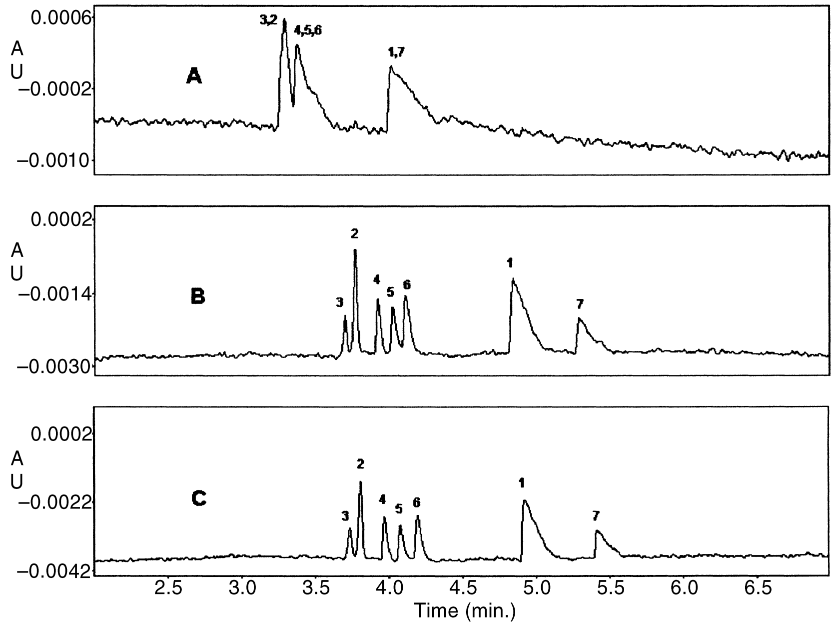

Figure 2. Electropherograms corresponding to the optimization of the background concentration of electrolyte. (A) Sodium thiosulphate 5.00 mM. (B) Sodium thiosulphate 25.0 mM. (C) Sodium thiosulphate 35.00 mM. In all these cases, benzethonium chloride 0.10 mM; pH = 6.20; voltage = 10.0 kV; $T = 25°C$. (1) Fluoride, (2) Chloride, (3) Bromide, (4) Nitrite, (5) Nitrate, (6) Sulphate, and (7) Phosphate.

To discover what was the most suitable pH for this analytic method, use was made of an electrolyte composed of benzethonium chloride 0.10 mM and sodium thiosulphate 25.0 mM; pH = 6.20. To this solution, were added quantities of sodium hydroxide 0.10 M, sufficient to achieve pH values of 7.00; 8.00, and 9.00. To bring the pH down, quantities of acetic acid 0.10 M to get pH values of 3.00; 4.00, and 5.00 were added. The other conditions were: $T = 25°C$, 10.0 kV. As the sample of anions was analyzed with electrolytes of different pH value, a progressive loss of resolution was noted for some anions as pH went down (Fig. 3). At basic pH levels, no significant difference was observed either in selectivity or in migration times for peaks. On the basis of the data obtained from this study, the most appropriate pH value was considered to be pH = 5.00.

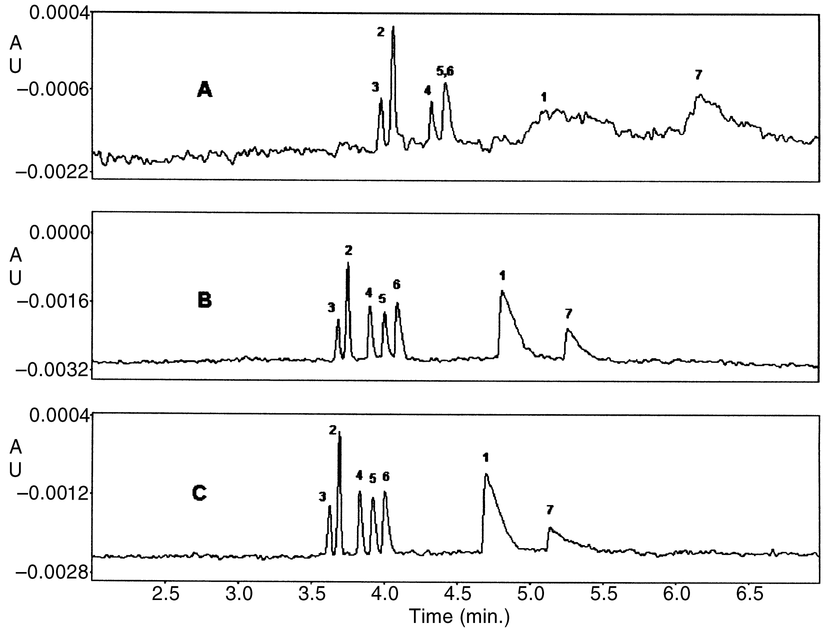

Figure 3. Electropherograms corresponding to the optimization of electrolyte pH. (A) pH = 3.00; (B) pH = 5.00; (C) pH = 9.00. In all these cases, benzethonium chloride 0.10 mM; sodium thiosulphate 25.0 mM; voltage = 10.0 kV; $T = 25°C$. (1) Fluoride, (2) Chloride, (3) Bromide, (4) Nitrite, (5) Nitrate, (6) Sulphate, and (7) Phosphate.

Voltage Optimization

Control and optimization of the voltage used, resulted in analyses being quicker and more effective with better-resolved peaks, because peak resolution is proportional to the square root of the voltage applied. This means, that to double the resolution, the voltage applied has to be quadrupled. For technical reasons, most CE equipment operates with an upper voltage limit of 30.0 kV. A further problem is Joule heating[24] with increasing voltage, which can downgrade the analysis. Hence, although voltage increases can, within certain limits, improve peak resolution, care must be taken not exceed a reasonable voltage limit. To determine the most appropriate voltage for analysis, use was made of the electrolyte previously selected as the most suitable in prior trials, composed of benzethonium chloride 0.10 mM, sodium thiosulphate 25.0 mM; pH = 5.00; $T = 25°C$. Analyses of all the anions were performed at the

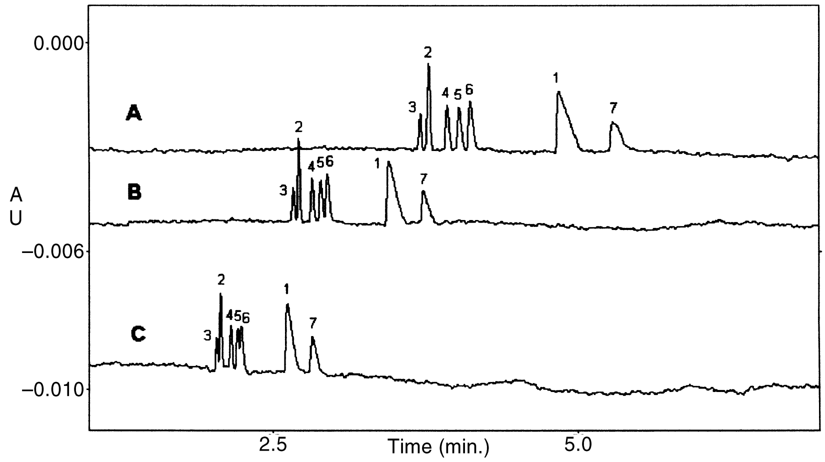

Figure 4. Electropherograms corresponding to the optimization of the voltage used. (A) 15.0 kV; (B) 20.0 kV; (C) 25.0 kV. Benzethonium chloride 0.10 mM; sodium thiosulphate 25.0 mM; $T = 25°C$; pH = 5.00.

following voltages: 10.0; 15.0; 20.0; and 25.0 kV. As the voltage increased, there was logically a rapid drop in migration times, but from 20.0 kV upwards there was also a drastic diminution in the resolution of peaks. On the basis of these data the most appropriate voltage for such analyses was considered to be 15.0 kV (Fig. 4). This is because it provides good migration times, high resolution, and no excessive Joule heat.

Optimization of Temperature

Precise control of the temperature during CE is of great importance in achieving good separation selectivity and, above all, good reproducibility of results.[25] The equipment used in this study maintained a constant temperature in the capillary, by means of circulating cooling liquid in direct contact with the capillary. To determine the optimum temperature for the electrophoretic process, the standard solutions were analyzed using an electrolyte composed of benzethonium chloride 0.10 mM, sodium thiosulphate 25.0 mM, pH = 5.00, with voltage 15.0 kV and temperatures of 20, 25, 30, and 35°C. The use of higher temperatures brings down migration times for all the anions, in the most striking way between 20 and 30°C, especially for phosphate, so that the peaks for chloride, bromide, nitrite,

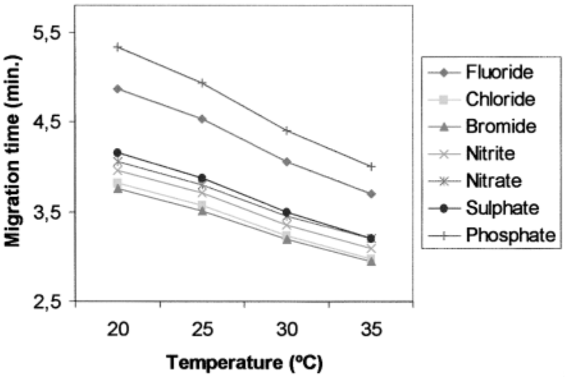

Figure 5. Data corresponding to the optimization of temperature. Migration times at: 20; 25; 30, and 35°C. Electrolyte composed of benzethonium chloride 0.10 mM; sodium thiosulphate 25.0 mM; pH = 5.00; voltage = 15.0 kV.

nitrate, and sulphate come too close together (Fig. 5). Hence, the most suitable temperature for such analyses was considered to be 25°C.

Optimal Conditions for Analyses

The data obtained from the studies detailed above permit a profound awareness of the behavior of these anions. As a result, it is possible to predict the most appropriate conditions for analyzing these anions in water, serum, and other biological fluids. The values considered most appropriate for analysis of these anions are: benzethonium chloride 0.10 mM, sodium thiosulphate 25.0 mM, pH = 5.00, 15.0 kV and 25°C.

Validation of the Method

Reproducibility

The distribution of test results under repeatability conditions, as defined in ISO 5275-1986, was estimated for retention times and for the determination of compound concentrations ($n = 10$). In this work, the repeatability RSD% for retention time was ≤ 0.08, while the repeatability RSD% for concentrations was ≤ 4.0. These values were lower than those acceptable, according to the criteria established by Huber.[26]

Linearity

The linearity of standard curves was expressed in terms of a correlation coefficient (*r*) from plots of the integrated peak area vs. concentrations of the standards: 5.00; 10.0; 25.0; 50.0, and 100.0 μg/mL. A linear equation was found ($r \geq 0.999$) with an intercept close to zero for all anions studied.

Recovery

Accuracy was calculated as the percentage of analyte by assay. Standard solutions of all anions were used; solutions in water being prepared to achieve known concentrations of the substance of 5.00; 25.0; and 100.0 μg/mL. Once the results obtained had been considered, recoveries were obtained, which in all cases, were in the range 97% to 103%.

Limits of Detection and Quantification

The limits of detection (LOD) corresponds to lowest concentration or amount of analyte that can be detected by the method. The Limits of quantification (LOQ) corresponds to the lowest concentration or amount of analyte that can be determined with suitable precision and accuracy.[27] To fix LOD, the criterion used was identification of a minimum peak with a signal-to-noise ratio of 3, each injection being repeated 6 times. The LOQ was evaluated by testing the repeatability of successive injections of standard solution at low concentration levels based on the standard deviation obtained. This was subsequently validated by replicated analysis of 6 standards prepared

Table 1. Limits of detection (LOD) and quantification (LOQ).

Anion	LOD (μg/mL)	LOQ (μg/mL)
Fluoride	0.12	0.33
Chloride	0.31	1.03
Bromide	0.33	0.97
Nitrite	0.35	0.98
Nitrate	0.30	1.00
Sulphate	0.32	1.01
Phosphate	0.31	0.98

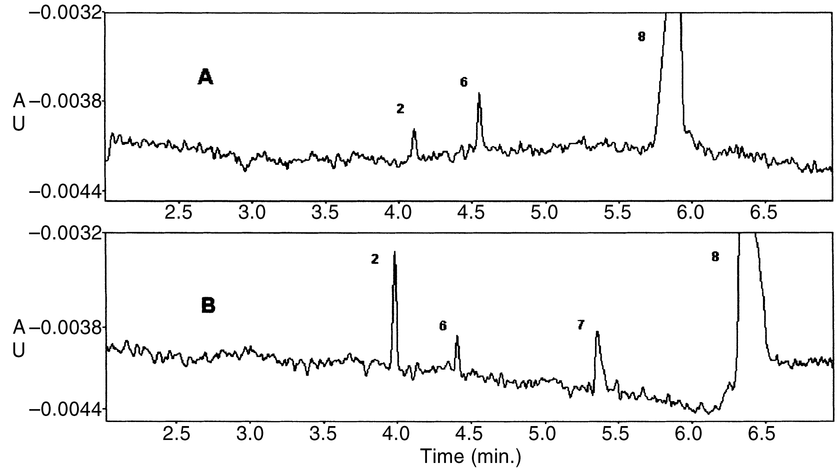

Figure 6. Electropherograms corresponding to drinking water (A) 2. Chloride: 3.70 μg/mL. 6. Sulphate: 6.50 μg/mL. 8. Carbonate: 2741.30 μg/mL. (B) Pig serum sample. 2. Chloride: 2741.30 μg/mL. 6. Sulphate: 26.60 μg/mL. 7. Phosphate: 74.50 μg/mL. 8. Carbonate: 928.00 μg/mL. Composition of electrolyte: benzethonium chloride 0.10 mM; sodium thiosulphate 25.0 mM; pH = 5.00; voltage = 15.0 kV; $T = 25°C$.

around this concentration. A relative error of ± 10% and repeatability of about 10% are acceptable for impurities at the LOQ. The results are shown in Table 1.

Application of the Method to Experimental Samples

To verify the practical utility of the analytic method being proposed here, a number of analyses of the anions present in one sample of drinking water and another of pig serum, containing some of the anions under study in known concentrations were carried out, with the expected values and related electropherograms being obtained (Fig. 6).

CONCLUSIONS

This study proposes two new substances that may effectively form part of the electrolyte used in the analysis of anions by CE. It also considers a number of the most important parameters influencing such analyses. Using the data obtained, it is possible to put forward an analytic method for these anions and, even, for other chemicals of similar characteristics in any liquid or fluid of biological interest: drinking water, waste water, soft drinks, serum, urine, and so forth. On the basis of the data obtained in this study, an overall analytic method can be proposed as a model for samples of drinking water and serum. The most suitable conditions for such an analysis by CE are, EOF modifier: benzethonium chloride 0.10 mM; background electrolyte: sodium thiosulphate 25.0 mM; $\mathrm{pH} = 5.00$; voltage 15.0 kV; $T = 25°\mathrm{C}$. Under these conditions, a rapid, precise, and reliable method is achieved, which is an alternative to other methods for analysis of anions, with limits of detection that are similar to, and in some cases better than, those consulted in published works.

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