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Use of eosin as a fluorophore in capillary electrophoresis with laser detection☆

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Abstract

Eosin has been used to generate the background signal for indirect fluorimetric detection of inorganic and organic ions, simultaneously separated by capillary zone electrophoresis (CZE). This reagent provides constant fluorescence over the pH range of 5–10 and is compatible with the excitation by an argon ion laser at 488 nm with emission at 520 nm. The use of esosine as fluorophore, H_3BO_3, and $Na_2B_4O_7$ as electrolyte and diethylentriamine as modifier of the electroosmotic flow in CZE were optimised. The analytical potential of the studied buffer was tested on a group of 12 anions, used as model compounds. Both, hydrodynamic and electrokinetic injection mode were optimised. The detection limits determined by the last injection mode, were in the range 0.008–0.037 mg l^{-1}. By using this method, the quantitation of the common anions in tap and mineral water has been carried out successfully.

Keywords: Fluorimetric detection; Detection, electrophoresis; Water analysis; Eosin; Inorganic anions; Organic acids

1. Introduction

Capillary electrophoresis (CE) is a rapidly expanding separation technique which has been successfully used in a wide range of analytical applications involving inorganic and organic anions, cations and neutral or potentially ionisable molecules [1,2]. All commercial instruments have UV absorbance detection as standard, and several now have diode array detection (DAD). A few selected instruments also offer the possibility of fluorescence or laser-induced fluorescence (LIF) detection. The majority of CE methods employ UV absorbance detection. The potential and applications of LIF detection are less investigated compared with DAD. In principle, LIF approaches allow extremely sensitive detection of fluorescently labelled compounds with up to six orders of magnitude higher sensitivity than UV absorbance. Direct as well as indirect fluorescence detection can be utilised.

In the direct fluorescence approach derivatisation is frequently required since few molecules have native fluorescence. Both pre- and post-capillary derivatisation are possible to enhance CE detection [3]. There are several fluorescent derivatisation reagents but not all are compatible with the laser used in this work, only fluorescein isothiocyanate, thiazole orange and 5-bromomethyl fluorescein have excita-

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tion and emission wavelengths at 488/520 nm. Indirect detection is an alternative mode of detection, which could be used in CE. Several reasons for developing indirect detection schemes have been mentioned. First, indirect detection is universal, and can be used for compounds, which lack fluorophores or chromophores. Second, it is possible to broaden the applicability of highly sensitive but selective detectors by implementing indirect detection. Third, indirect detection is non-destructive since no chemical manipulation has been introduced. On the other hand, the disadvantages of indirect detection methods are that the linear dynamic range tends to be rather limited, the limits of detection with laser sources can often be poorer than indirect absorption detection with a highly stable light sources. Another problem with indirect fluorescence is the low ionic strength of the electrolyte solution. This can result in peak asymmetry at the upper end of the linear dynamic range. Because of the intensity of the laser light source, a low additive concentration is required. Due to these issues, the use of indirect fluorescence with a laser source is not routinely used in laboratories [4].

A CE instrument equipped with laser-induced fluorescence detection has spurred the characterisation of reagents for indirect LIF and this topic has been reviewed [5]. The majority of fluorescent compounds presented an excitation wavelength in the UV region. Reagents like salicylate and quinine can be excited using a UV laser and they have been reported for the indirect detection of amino acids [6], inorganic anions [7] and cations [8]. Fluorescein sodium salt is one of the few fluorescent compounds, which has an excitation wavelength in the visible region. The commercial CE instrument used in this work has excitation and the emission wavelengths at 488 and 520 nm, respectively. After reviewing the literature, it could be confirmed that this kind of laser has been used essentially in the biological field [9–13] and the majority of the papers include derivatisation reactions [14–18]. However, inorganic cations such as EDTA complexes [19], inorganic anions [20], organic acids [20,21], anionic surfactants [21] cyanide plus related compounds [22] and phenols [23] have been determined using indirect LIF with fluorescein. Only one paper proposed another reagent for this kind of detection, namely flavin mononucleotide (FMN) or riboflavin-5′-monophosphate, to separate various classes of anions [24].

The aim of this work deals with the use of eosin as a new fluorophore, to generate the background signal in indirect detection of inorganic and organic anions as alternative for fluorescein. Eosin provides a good match with a commercial laser-based CE instrument with excitation at 488 nm and emission at 520 nm and is stable in fluorescence response from pH 5 to 10. Diverse studies have been carried out to characterise the new buffer. A separation of 12 inorganic anions and organic acids was possible in less than 14 min.

2. Experimental

2.1. Reagents

All solutions, electrolytes and standards, were prepared using 18 MΩ water generated by a Millipore Milli-Q water purification system. The electrolytes used were prepared from solutions made up fresh each day from $5 \cdot 10^{-3}$ *M* stock solution of eosin, 0.1 *M* of H_3BO_3, 0.5 *M* of $Na_2B_4O_7$ and 0.05 *M* of diethylenetriamine (DETA). The final composition of the buffer solution was 70 m*M* H_3BO_3, 5 m*M* $Na_2B_4O_7$, 2 m*M* DETA and 10^{-6} *M* eosin. 1000 mg l^{-1} stock standard solutions of different anions in water were prepared from: sodium sulfate, sodium nitrate, sodium fluoride, sodium chloride, sodium bromide, sodium nitrite, malic acid, sodium citrate, sodium hydrogencarbonate, tartaric acid, sodium succinate and sodium oxalate supplied by Merck in all cases. Working standard solutions of mixtures were obtained by appropriate dilution in water of the stock standard solutions and they were prepared fresh daily.

2.2. Apparatus

A Beckman capillary electrophoresis instrument P/ACE 5500 equipped with an argon-laser-based fluorescence detector ($\lambda_{excitation}$=488 nm, $\lambda_{emission}$= 520 nm) was used and the capillaries were also from Beckman. Control and data processing were carried out with the Gold software. Samples were loaded by

Table 1
Different buffers used in CE with indirect fluorimetric detection (488–520 nm)

Analytes	Buffer	pH	Refs.
Inorganic anions	0.1 *M* H_3BO_3 20 m*M* $Na_2B_4O_7$ $8 \cdot 10^{-5}$ *M* fluorescein sodium salt 10^{-5} *M* trimethyltetradecylammonium bromide	8.6	[28] [20]
Inorganic cations	2.5 m*M* $Na_2B_4O_7$ 10^{-5} *M* fluorescein sodium salt	9.2	[29]
Inorganic cations	$5 \cdot 10^{-3}$ *M* EDTA NaOH 10^{-5} *M* fluorescein sodium salt	7.5	[19]
Organic anions	5 m*M* $Na_2B_4O_7$ 10^{-5} *M* fluorescein sodium salt (with and without 20% acetonitrile)	9.2	[28] [21]
Phenols	15 m*M* $Na_2B_4O_7$ 10^{-3} *M* fluorescein sodium salt	9.9	[23]
Free fatty acids	Methanol–acetonitrile (1:1) 0.1 m*M* fluorescein sodium salt KOH		[30]
Arsenic compounds	1.5 m*M* fluorescein sodium salt	9.8	[31]
Fatty acids (C5–C10)	5 m*M* $Na_2B_4O_7$ 10^{-5} *M* fluorescein sodium salt 10% 2-propanol or 20% acetonitrile	9.2	[28]
Fatty acids (C7–C18)	5 m*M* $Na_2B_4O_7$ 10^{-5} *M* fluorescein sodium salt 40% ethanol	9.2	[28]
CN^- and relative compounds	10^{-4} *M* fluorescein sodium salt	10	[22]
Polysaccharides (high molecular mass)	1 m*M* fluorescein sodium salt	11.5	[32]
Inorganic anions	0.1 *M* H_3BO_3 $3 \cdot 10^{-5}$ *M* riboflavin	7.8	[24]

pressure and electrokinetic injection into a fused-silica capillary. Before use the following solutions were flushed through the capillary, in the order given: 10 min of 1 *M* NaOH, 5 min of water and 5 min of buffer. Between each injection the capillary was rinsed with 2 min of water, 3 min of buffer and 2 min of 0.1 *M* NaOH.

The untreated fused-silica capillaries were 57 cm× 75 mm I.D. Injections were done at the high voltage cathode and anions were eluted to the anode electrode. The temperature of the capillary was 20°C. The separation voltage was 20 kV, resulting in an electrophoretic current of 16 μA. The standards were injected by hydrodynamic mode for 15 s and by the electrokinetic mode applying a voltage of 10 kV during 7 s.

3. Results and discussion

To optimise the new buffer proposed in this work, using a set of inorganic and organic anions as testing

sample carried out the following steps. Firstly, a fluorescent reagent was sought that gave a large fluorescence background at all times. Secondly, an electrolyte was studied which provides electrical conductivity so that a constant electric field gradient can be maintained along the length of the capillary. And finally, an electroosmotic flow (EOF) modifier to reverse the direction of the EOF was studied. In Table 1, some of the different buffers used with indirect laser detection found in the literature are summarised. As it can be seen, almost all of them use fluorescein sodium salt as fluorescent reagent.

3.1. Selection of the fluorescent reagent

As mentioned above, because of the characteristic of the laser-induced fluorimetric detector utilised, at both the excitation and emission wavelengths, there is only a limited choice of fluorescence agents that could be used to provide the background signal. To the best of our knowledge, only two fluorophores have been previously used. Dèsbene and co-workers used fluorescein sodium salt [19,21] and riboflavin-5′-monophosphate was used by Shamsi et al. [24].

Eosin provides a good agreement with commercial laser-based CE instruments with excitation at 488 nm and emission at 520 nm. This reagent also presents a stable fluorescence from pH 5.0 to 10. Moreover, the eosin is readily soluble in water.

To verify the potential use of eosin, a comparison between different buffers containing fluorescein, riboflavin, calcein and the proposed buffer (with eosin) was carried out. The obtained electropherograms are shown in Fig. 1. Only the buffers with eosin and fluorescein separate the 12 anions of the test mixture. Using the buffers with riboflavin and calcein did not separate citrate peak. Note that the used concentration of fluorescein sodium salt was

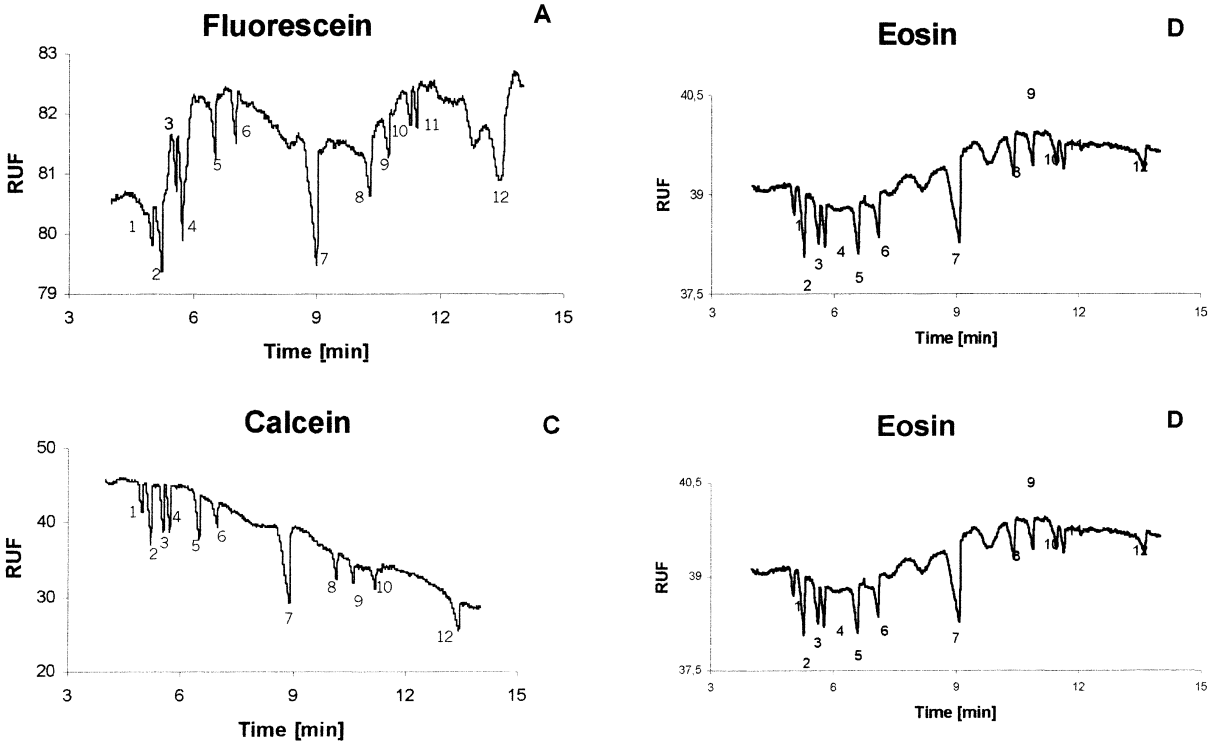

Fig. 1. Comparison of different buffers, electropherograms of a standard mixture of anions (2 mg l^{-1}). Peaks: 1=bromide, 2=chloride, 3=nitrite, 4=nitrate, 5=sulfate, 6=oxalate, 7=fluoride, 8=succinate, 9=malate, 10=tartrate, 11=citrate, 12=hydrogencarbonate. Buffers: 70 m*M* H_3BO_3, 5 m*M* $Na_2B_4O_7$, 2 m*M* DETA and (A) 10^{-7} *M* fluorescein; (B) $3{\cdot}10^{-5}$ *M* riboflavin; (C) $5{\cdot}10^{-4}$ *M* calcein; (D) 10^{-6} *M* eosin. The pH in all buffers was 8.56. RUF: relative fluorescence units.

10^{-7} *M*, two times lower than the concentration proposed in the literature due to the characteristics of the laser used in this work, which showed a saturated signal when 10^{-5} *M* of fluorescein was used.

By using calcein as fluorophore to generate the background signal, the obtained results are in agreement with the other reagents tested. This fluorophore could be used as a component of a buffer for the indirect determination of a group of anions. One of the disadvantages presented when calcein was used, as a component of the buffer, was the quantification of the different anions checked. The selectivity between peaks was good enough (see Fig. 1) but the signals were not reproducible. Further studies of the buffer with calcein as fluorophore could resolve this problem.

3.2. Effect of H_3BO_3 concentration

The concentration of H_3BO_3 was varied over the range 10–150 m*M* (pH 9.12–8.12) with a fixed concentration of $Na_2B_4O_7$ (20 m*M*), DETA (2 m*M*) and eosin ($1{\cdot}10^{-6}$ *M*). As can be seen in Fig. 2, with 100 m*M* H_3BO_3, the migration time was lower, but the signal is higher when 70 m*M* was used, so this concentration was selected in order to improve the sensitivity. Concentrations lower than 30 m*M* gave noisy backgrounds and the integration of the peaks were not possible.

3.3. Effect of $Na_2B_4O_7$ concentration

The concentration of $Na_2B_4O_7$ was studied between 5 and 30 m*M* (pH 8.3–8.8), with the selected concentration of H_3BO_3 (70 m*M*) and the same concentration of DETA (2 m*M*) and eosin ($1{\cdot}10^{-6}$ *M*) (see Fig. 3). In this case, the migration time did not present differences and the concentration was selected according to the major signal obtained, a concentration of 5 m*M* was chosen. By using concentrations of 25 and 30 m*M*, an increase in the baseline noise was observed and the separation presented a poor resolution (R_s), values of $R_s<1$ were not considered appropriate for the separation of two adjacent compounds.

3.4. Effect of eosin concentration

The optimisation of this concentration was carried out between $1{\cdot}10^{-6}$ and $5{\cdot}10^{-5}$ *M*, working with the optimum values for the concentration of H_3BO_3 (70 m*M*) and $Na_2B_4O_7$ (5 m*M*). A concentration of $5{\cdot}10^{-5}$ *M* produces the saturation of the signal. With concentrations of 10^{-5} and 10^{-6} *M* the obtained signals were similar (when it was used a standard mixture of 10 mg l^{-1}). Lower concentrations of this test mixture were prepared and better resolution between peaks was achieved by using a concentration of 10^{-6} *M* of eosin.

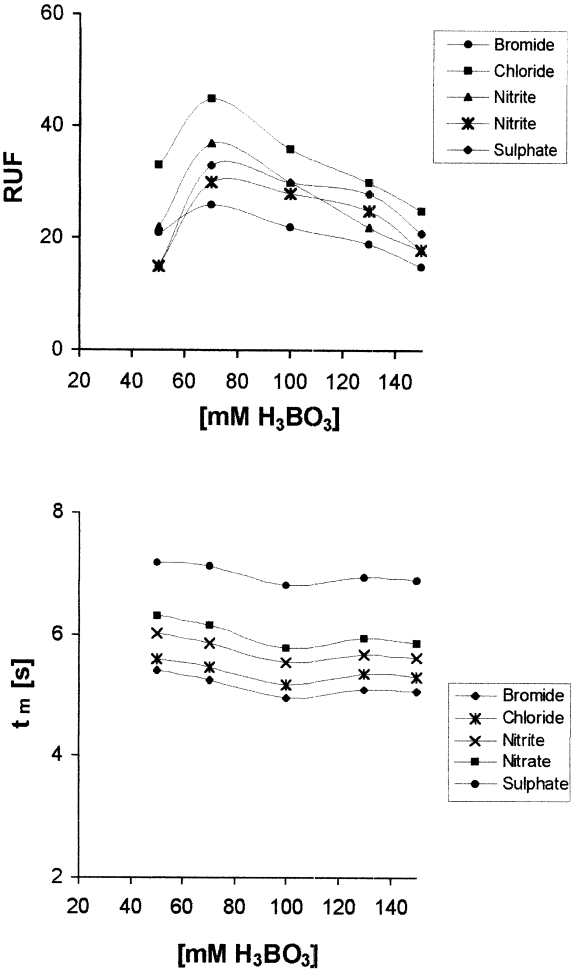

Fig. 2. Optimisation of H_3BO_3 concentration present in the buffer. RUF: relative fluorescence units.

3.5. Effect of EOF modifier

As is well known, to separate anions by CE, the

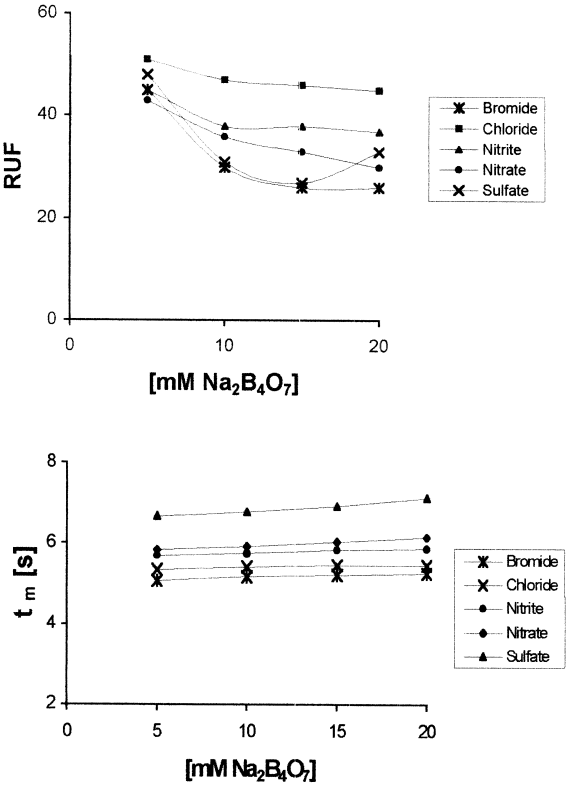

Fig. 3. Optimisation of $Na_2B_4O_7$ concentration present in the buffer. RUF: relative fluorescence units.

EOF must be at least decreased, or even reversed, in order to shorten the analysis time. The most frequently used modifiers or reverses of EOF are quaternary ammonium surfactants such as tetradecyltrimethylammonium bromide (TTAB) [25]. However, this modifier can also be DETA [26]. First of all, different modifiers of EOF were tested, such as hexadecyltrimethylammonium bromide (CTAB), hexadecyltrimethylammonium chloride (CTAC), tetrabutylammonium bromide (TBAB) and DETA in the same concentrations (2 m*M*). The best results were obtained by using DETA as EOF modifier.

The studied concentration range of DETA was between 0.5 and 3 m*M*. The optimum value was 2 m*M* because at this concentration an adequate integration of the peaks was achieved.

3.6. Instrumental variables

Increasing the applied voltage, the analysis time can be decreased. Short analysis times were achieved

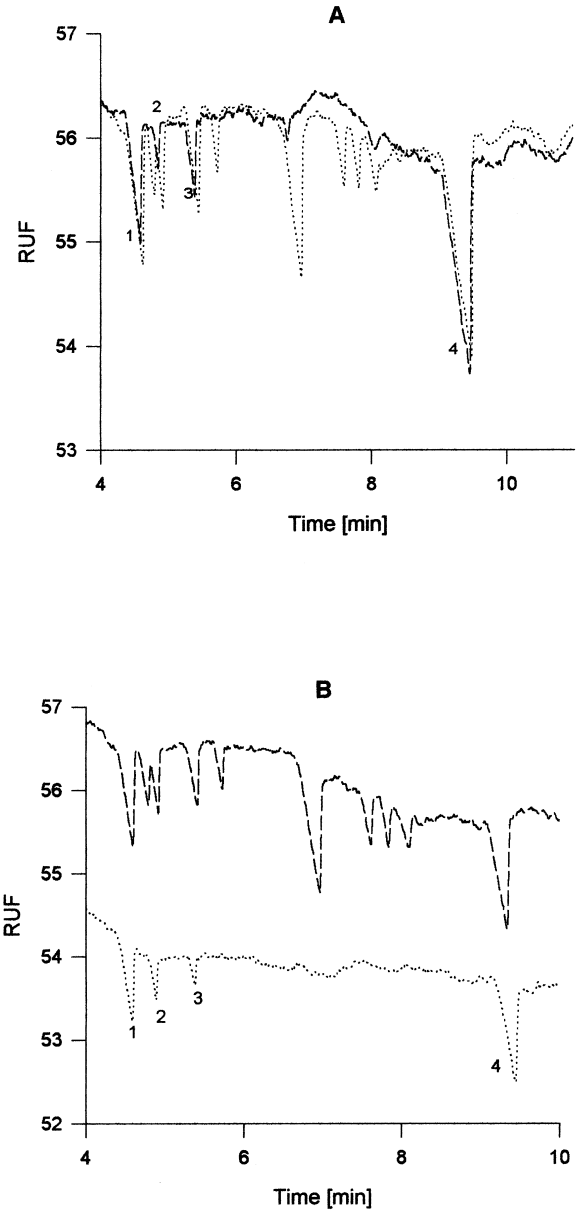

Fig. 4. Electropherogram of real samples: (A) (----) Tap water, (·····) tap water spiked with 2 mg l^{-1} of test mixture. (B) (·····) Mineral water, (----) mineral water spiked with 2 mg l^{-1} of test mixture.

by running the separation at 20 kV, with this voltage a good resolution between peaks were achieved.

The influence of temperature on the resolution of the anions was also tested. On the basis of the results obtained, it has been concluded that good resolution was achieved at 20°C.

Table 2
Figures of merit for the hydrodynamic and electrokinetic injection modes

Injection mode	Anions	Equations	Linear range (mg l^{-1})	r	R^2	$S_{y/x}$	LOD (mg l^{-1})	LOQ (mg l^{-1})	RSD (%) (t_m)	RSD (%) (peak area)
Hydrodynamic	Bromide	$a=1.42\pm0.37$ $b=15.89\pm0.12$	0.3–6	0.9996	99.92	0.99	0.07	0.23	0.37	4.5
	Chloride	$a=-2.76\pm1.57$ $b=59.36\pm0.52$	0.3–6	0.9995	99.90	4.26	0.08	0.26	0.22	4.0
	Nitrite	$a=-1.91\pm0.92$ $b=34.32\pm0.30$	0.3–6	0.9995	99.90	2.49	0.08	0.27	0.34	3.9
	Nitrate	$a=-0.86\pm0.82$ $b=22.90\pm0.27$	0.3–6	0.9998	99.93	1.43	0.1	0.36	0.55	3.3
	Sulfate	$a=11.9\pm1.01$ $b=21.92\pm0.33$	0.3–6	0.9985	99.70	2.75	0.14	0.46	0.36	4.7
	Oxalate	$a=4.62\pm1.62$ $b=21.19\pm0.53$	0.3–6	0.9959	99.19	4.39	0.22	0.76	0.51	6.2
	Fluoride	$a=-10.77\pm6.08$ $b=130.75\pm4.32$	0.3–6	0.9985	99.70	16.45	0.14	0.47	0.85	7.0
	Succinate	$a=8.60\pm1.83$ $b=22.16\pm0.60$	0.3–6	0.9953	99.06	4.94	0.25	0.82	0.71	6.7
	Malate	$a=3.51\pm1.07$ $b=14.03\pm0.35$	0.3–6	0.9990	99.81	1.67	0.22	0.76	0.68	8.3
	Tartrate	$a=21.04\pm0.74$ $b=11.61\pm0.24$	0.3–6	0.9972	99.43	2.01	0.19	0.64	0.76	7.0
	Citrate	$a=6.72\pm0.53$ $b=5.77\pm0.17$	0.3–6	0.9942	99.18	1.43	0.27	0.92	0.52	6.7
	Hydrogencarbonate	$a=41.1\pm3.00$ $b=17.86\pm1.72$	0.6–6	0.9880	97.70	7.65	0.50	1.5	0.77	6.9
Electrokinetic	Bromide	$a=20.81\pm0.63$ $b=84.75\pm3.42$	0.025–0.5	0.9935	98.71	1.95	0.022	0.074	0.56	6.8
	Chloride	$a=32.43\pm2.07$ $b=356.6\pm12.3$	0.025–0.5	0.9950	99.05	7.04	0.017	0.056	0.43	7.2
	Nitrite	$a=9.41\pm1.24$ $b=164.17\pm4.67$	0.025–0.5	0.9967	99.36	2.66	0.023	0.076	0.61	5.4
	Nitrate	$a=9.07\pm1.07$ $b=135.5\pm4.03$	0.025–0.5	0.9960	99.29	2.3	0.024	0.079	0.87	7.0
	Sulfate	$a=7.63\pm1.45$ $b=247.72\pm5.46$	0.025–0.5	0.9980	99.61	3.12	0.018	0.059	0.62	4.3
	Oxalate	$a=7.65\pm0.53$ $b=192.4\pm1.99$	0.025–0.5	0.9990	99.91	1.14	0.008	0.026	0.73	3.9
	Fluoride	$a=7.08\pm2.02$ $b=406.88\pm7.62$	0.025–0.5	0.9986	99.72	4.35	0.015	0.050	1.2	5.3
	Succinate	$a=13.4\pm3.12$ $b=244.28\pm10.5$	0.05–0.5	0.9944	98.90	5.29	0.038	0.128	0.94	5.5
	Malate	$a=8.93\pm1.21$ $b=97.14\pm4.57$	0.05–0.5	0.9910	98.25	2.61	0.037	0.120	0.99	7.1
	Tartrate	$a=5.75\pm0.68$ $b=54.83\pm0.79$	0.05–0.5	0.9989	99.79	0.49	0.037	0.124	0.98	8.3
	Citrate	$a=20.06\pm0.41$ $b=67.32\pm1.54$	0.025–0.5	0.9979	99.58	0.88	0.018	0.061	0.85	3.6
	Hydrogencarbonate	$a=6.29\pm2.53$ $b=247.88\pm9.54$	0.05–0.5	0.9940	98.83	5.45	0.031	0.100	1.02	5.9

a: Intercept; b: slope; $S_{y/x}$: standard deviation of residuals, r: regression coefficient; R^2: curve fitting level (in %) obtained by analysis of variance (ANOVA) for the validation of the model; LOD: limit of detection (calculated as $3S_a/b$); LOQ: limit of quantification (calculated as $10S_a/b$); S_a: standard deviation of intercept; RSD: relative standard deviation (in %); t_m: migration time.

Table 3
Analysis of real samples by the proposed method

Anion	Type of sample	Concentration added (mg l^{-1})	Concentration found (mg l^{-1})	Recovery (%)	Concentration[a] (mg l^{-1})
Chloride	Tap water	1.2	1.27	106	43.79
		3.0	2.75	92	
		6.0	6.09	102	
	Mineral water	1.2	1.26	105	16.1
		3.0	3.03	101	
		6.0	5.97	99.6	
Nitrate	Tap water	1.2	1.26	105	10.36
		3.0	3.46	115	
		6.0	5.86	98	
	Mineral water	1.2	1.12	94	3.8
		3.0	2.73	91	
		6.0	6.2	103.4	
Sulfate	Tap water	1.2	1.26	104	17.15
		3.0	3.22	107	
		6.0	5.89	98.2	
	Mineral water	1.2	1.06	89	2.5
		3.0	3.3	109	
		6.0	5.88	98	
Hydrogencarbonate	Tap water	1.2	1.08	90	11.0
		3.0	3.46	115	
		6.0	5.8	96.7	
	Mineral water	1.2	1.22	102	10.3
		3.0	2.86	95	
		6.0	6.04	100.7	

[a] Concentration obtained by using standard addition method.

The two general modes of injection were tested. The duration of hydrodynamic injection was studied to achieve a higher sensitivity for the anions. It was observed that long injection times resulted in poor peak shapes because of disturbance of the sample. Finally, an injection time of 15 s was chosen. The determination range obtained for the test mixture by using this mode of injection was 0.3 to 6 mg l^{-1}. Electrokinetic injection was used when higher sensitivity is required. The best combination, according to resolution parameters, was 10 kV for 7 s. When electrokinetic injection was used, the response was linearly dependent on concentration in the range 0.025–0.5 mg l^{-1}.

3.7. Application to the simultaneous determination of inorganic and organic anions

Calibration graphs were obtained by injecting standard solutions of the inorganic and organic anions. External calibration was used because no improvement was observed when an internal standard was used. Calibration curves using both, hydrodynamic injection mode and electrokinetic injection mode and other characteristic parameters for the determination of these anions are shown in Table 2. The linear determination coefficient varying from 97.70 to 99.93% for calibration curves using hydrodynamic injection mode and from 98.71 to 99.91% for calibration curves with electrokinetic injection mode. By using the hydrodynamic injection, the relative standard deviations (RSDs) were between 0.22% for chloride and 0.85% for fluoride, for the migration times and from 3.3 to 8.3% for the peaks areas, calculated from 11 independent experiments (Table 2). With the electrokinetic injection mode the lower RSD for migration time was 0.43% for chloride and the higher value was 1.2% for fluoride.

For the peaks areas this values corresponded to 3.9% for oxalate and 8.3% for tartrate (Table 2).

Two real samples (2 ml of mineral and tap water) were analysed to demonstrate the applicability of this method. Fig. 4 shows the electropherograms of these samples and in Table 3 are summarised the obtained results when standard addition method was applied. As can be seen, these results are in agreement with the values reported by Spanish Legislation [27].

4. Conclusions

The results presented here show that the electrophoretic system based on the utilisation of eosin in a basic medium is suitable for the analysis of analytes lacking fluorophoric groups, which at present can be detected routinely only by using indirect detection in CE. It is well known that a baseline drift using negative polarity is a major reason why detection limits for small ions are not improved using indirect LIF. In spite of this, the limits of detection achieved in this work are in a good agreement with those obtained by DAD. Eosin has the appropriate characteristics to be used as a fluorophore and it is a good alternative to fluorescein. The new CE method developed is rapid, sensitive and quantitative and can be readily applied to real samples for quantitative analysis.

The use of this fluorimetric buffer can be expanded to other analytical applications using CE with indirect LIF detection.

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