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Determination of small anions by capillary electrophoresis using indirect UV detection with sulphonated nitrosonaphthol dyes

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Abstract

A capillary zone electrophoretic method was developed for routine separation of non-UV-absorbing inorganic anions, phosphonic compounds and organic acids. The background electrolyte solutions were prepared from three new dye reagents normally used for the complexation of metals, namely, the sodium salts of 1-nitroso-2-naphthol-3,6-disulphonic acid (Nitroso-R salt), 1-nitroso-2-naphthol-6-sulphonic acid (126NNS) and 2-nitroso-1-naphthol-6-sulphonic acid (216NNS). The optimized separations were carried out in 0.5 mM dye solution at pH 8.00, with UV detection of the analytes at 254 nm. All analytes in the sample mixtures were separated from each other within 20 min. The currents under the optimized separation conditions were only 1.5 μA , 0.9 μA and 0.9 μA in Nitroso-R, 126NNS and 216NNS, respectively. The detection limits of the compounds injected from vials were 1 $\mu\text{g}/\text{ml}$ at 254 nm. The method performed well for the analysis of inorganic anions and for screening of by-products in the production of clodronate. It was also promising for the screening of organic acids in fluids formed in the production of sugar. The repeatability of the analyses was evaluated in experiments involving nine replicate injections, replenishment of the background electrolyte solution after each run and temperature control at 25°C. The standard deviations of the absolute migration times of the compounds were from 0.1 to 2%, but also up to 9% when slowly moving analytes with broad sample zones were analysed.

Keywords: Background electrolytes; Anions; Nitrosonaphthols; Phosphonates; Organic acids; Sulfonic acids

1. Introduction

Small anions constitute a wide spectrum of compounds, common in matrices such as foods, drugs, biomaterials and the environment. Of the many chromatographic methods available for screening of small anions, capillary electrophoresis (CE) with its high resolving power, is a method of choice. In CE the signal detected depends not only on the optical

(electrochemical) properties of the analytes, but also on their charge and electrophoretic mobilities [1].

Although small anions typically lack a suitable chromophore of their own, CE methods have been devised allowing them to be detected indirectly. Furthermore, although the standard fused-silica capillaries used in CE are not ideal for separation of small anions because of an electroosmotic flow caused by negatively charged sites, a number of indirect UV-detection methods have been developed for their screening and determination with unmodified capillaries.

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Both laboratory-made and commercially available electrolyte solutions have been used in the indirect detection of small anions in CE [2]. Either the co-ion or the counterion of the solution, or both, can be employed to provide the background signal. The usual background electrolytes (BGE) have been benzoic acid, imidazole, β -alanine or mixtures of potassium chromate–cetyltrimethyl ammonium bromide (CTAB), potassium chromate–CIA-Pak OFM Anion BT or tricine–sodium salt of polyvinylsulphonic acid (PVA)–CTAB [3–5]. Ribonucleotide electrolytes, adenosine, cytosine, guanosine and uridine monophosphates have also been tested [6], and UV–Vis-absorbing substituted naphthalenes have proven to be excellent background electrolytes for the indirect detection of all kinds of ions [3,6–9]. An important feature of all these last compounds is that they are suitable for analyses throughout the UV region. The solutions used in screening have not, however, always been good enough for sensitive analyses. The problems associated with buffers containing potassium chromate have been attributed to reaction of the anionic analytes with chromate, which then reduces the intensity of the signal. It has been suggested that only working electrolytes where the background signal is due to the co-ion should be used.

There are many reasons for preferring indirect detection methods. For example, no special conditions are required to detect the analytes, and sensitive transfer is possible: sample preparation is usually not needed. In addition, derivatisation steps which change the physicochemical properties of the analytes will not be needed. Therefore, also the loss of the sample in preparation will be minimized. Although it may be difficult to achieve the same low detection limit for indirect detection as for direct detection, since noise tends to be greater in the presence of a strong background signal, quantitation may be easier with indirect detection [10].

The CE separation of inorganic anions and of organic acids has proven to be demanding because of the minimal differences in their electrophoretic mobilities, charges and molar masses [1]. Phosphate and fluoride, for example, have been fully resolved at pH 8.0, but since they are totally dissociated at higher pH values phosphate migrates earlier than fluoride [1]. Some organic acids also tend to comigrate.

Clodronate, a synthetic analogue of pyrophosphate recently introduced for the treatment of hypercalcaemia implicated in osteolytic metastases and malignancies [11], has not previously been analysed by CE. Screening for it and the substances used in producing it, is important in the drug control [12]. Also, the existence of organic acids must be confirmed in the production of sugar from plants.

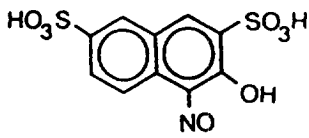
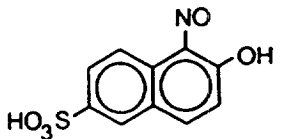
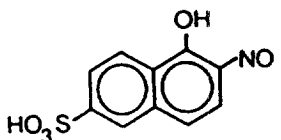
In this study we have validated a capillary zone electrophoretic method for the separation of three mixtures: one containing inorganic anions, one phosphonic compounds (phosphonates) and one organic acids. In the case of phosphonic compounds the aim was to separate clodronate, disodium(dichloromethylene)diphosphonate tetrahydrate, from inorganic phosphate and phosphite and from by-products generated in its synthesis, and in the case of the organic acids the long-term goal was simultaneous screening of food products.

2. Experimental

2.1. Instrumentation

The electropherograms were generated by using the following instruments: Beckman 2050 P/ACE System 2000 with a UV–Vis detector (available wavelength 260 nm) and a liquid cooling system for the capillary (Beckman Instruments, Fullerton, CA, USA), Waters Quanta 4000 (Millipore, Waters Chromatography Division, Milford, MA, USA) and HP 3D CE with a UV detector (Hewlett-Packard, Avondale, PA, USA) with a diode-array detector and an air cooling system. The temperatures during the separation were 25°C, ambient, and 25°C in Beckman, Waters and HP instruments, respectively. The dimensions of the non-coated fused-silica capillaries (Composite Metal Services, Worcestershire, UK) were 50 μm I.D. \times 360 μm O.D. and the analytical lengths were from 60 to 80 cm. The injection times were 3 s (pressure, 35 mbar) with Hewlett-Packard and Beckman instruments and 20 s (siphoning, at 10 cm height) with Waters instrument. The voltages during the separations were varying from 20 to 25 kV. A Jenway 3030 pH meter and an electrode (Jenway, Felsted, UK) containing 3 M KCl in

Table 1
 Sulphonated nitrosonaphthols used as the BGE solutions in indirect-UV detection in CZE

Electrolyte solution	Symbol	Molecular mass	p <i>K_a</i> value	Structure of the acid
Sodium salt of 1-nitroso-2-naphthol-3,6-disulphonic acid	Nitroso-R salt	377.3	7.52	
Sodium salt of 1-nitroso-2-naphthol-6-sulphonic acid (1H ₂ O)	126NNS	292.2	7.22	
Sodium salt of 2-nitroso-1-naphthol-6-sulphonic acid (3H ₂ O)	216NNS	328.3	7.02	

p*K_a* values taken from Ref. [13].

saturated AgCl solution were used to adjust the pH of the electrolyte solutions.

2.2. Materials

Clodronate was obtained from Leiras, Research and Development (Turku, Finland). Nitroso-R salt (1-nitroso-2-naphthol-3,6-disulphonic acid, *M_r* 377.3) was obtained from Sigma (St. Louis, MO, USA). Both 126NNS (sodium salt of 1-nitroso-2-naphthol-6-sulphonic acid, 1H₂O, *M_r* 292.22) and 216NNS (sodium salt of 2-nitroso-1-naphthol-6-sulphonic acid, 3H₂O, *M_r* 328.25) were synthesised in our laboratory. Disodium hydrogenphosphate, disodium hydrogenphosphite and the sodium salts of chloride, sulphate, sulphite and fluoride were obtained from Merck (Darmstadt, Germany). Hippuric acid, isovaleric acid, butyric acid, propionic acid, acetic acid, malonic acid, pyruvic acid, oxalic acid, glycolic acid, pentanedionic acid and lactic acid were purchased from Merck and from Fluka (Buchs,

Switzerland). Distilled water was purified with a Water-I system from Gelman Sciences (Ann Arbor, MI, USA) and further purified by filtering through 0.45- μ m membrane filters (Millipore, Molsheim, France). The pH meter was calibrated with CONVOL standard buffer solutions purchased from BDH (Poole, UK).

2.3. Electrolyte solutions

The standard stock electrolyte solutions (200 mM) of the sulphonates were prepared by dissolving known weights of reagents in purified and filtered water (18 M Ω). Before water was added to the final volumes, the pH was adjusted to 8.00 with 0.1 M NaOH. The working electrolyte solutions for indirect UV detection of the ions contained 0.5 mM of sulphonated nitrosonaphthols made from the stock solutions. By buffering the BGE solutions with 30 mM phosphate at pH 8.00 reduced the sensitivity and therefore the analytes could not be detected.

2.4. Procedures

The electropherograms were run at 254 nm, which is one of the wavelength maxima for Nitroso-R-salt, 126NNS and 216NNS. The most intensive absorption band could not be used because of the absorption of the analytes at similar low UV-wavelength. Since their pK_a values were around 7.00, the background electrolytes stayed dissociated at pH 8.00, to maintain the buffer capacity (Table 1).

2.5. Sample mixture solutions

The 1000 $\mu\text{g}/\text{ml}$ stock solutions of the analytes (anionic compounds) were prepared individually for each compounds in distilled, ionized and filtered water. The sample mixtures (inorganic anions; phosphate, phosphite and clodronate mixture; organic acids) were prepared from the 1000 $\mu\text{g}/\text{ml}$ solutions, each containing at least five analytes at concentrations varying between 1 and 100 $\mu\text{g}/\text{ml}$.

2.6. Instrumental operation

The capillary was flushed for 10 min with 0.1 M KOH and then for 10 min with deionized distilled water. Finally, it was equilibrated with the operating buffer for 30 min. Between runs the capillary was flushed for 10 min with the buffer.

2.7. Electrophoretic separations

The final sample solutions were analysed nine times each, with fresh electrolyte solution introduced after each injection. The migration times were optimized by using methanol and formic acid as the test compounds to obtain reproducible detection window. The detection limits were calculated by using standard mixtures having five times higher detection signal than concentrations needed for a signal-to-noise ratio of two.

3. Results and discussion

Study was made of the usefulness of capillary electrophoresis with indirect UV detection for the separation and identification of inorganic anions, phosphoric compounds and organic acids. In terms of electrophoretic resolution, separation and detection, 0.5 mM Nitroso-R salt solution was found to be an excellent choice for the electrolyte and the separation of the analytes, since in the sample the concentrations of the anions were 1 $\mu\text{g}/\text{ml}$ and the small amount of it (analytes in volume of approx. 10 nl) could be detected. The UV absorption spectrum of the Nitroso-R salt showed that the absorptions of the analytes at 230, 254 and 280 nm were all high enough for the indirect detection of non-absorbing compounds. Due to the similar spectra of 126NNS and 216NNS also the detection of the test com-

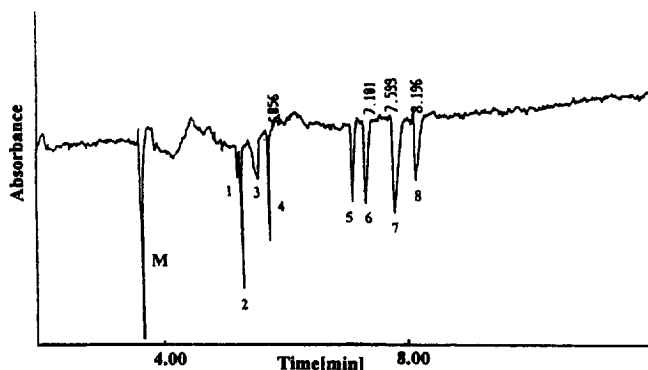


Fig. 1. Electropherogram obtained from a mixture containing (2) 105 nmol PO_4^{3-} , (3) 526 nmol F^- , (4) 127 nmol PO_3^{2-} , (5) 161 nmol NO_3^- , (6) 217 nmol NO_2^- , (7) 282 nmol Cl^- and (8) 104 nmol SO_4^{2-} in 1 ml (10 $\mu\text{g}/\text{ml}$). M is methanol. Peak (1) was not identified. BGE solution: 0.5 mM Nitroso-R salt, pH 8.00. Instrument: Waters Quanta 4000. T ambient, voltage 25 kV, current 1.5 μA , injection 20 s and λ 254 nm.

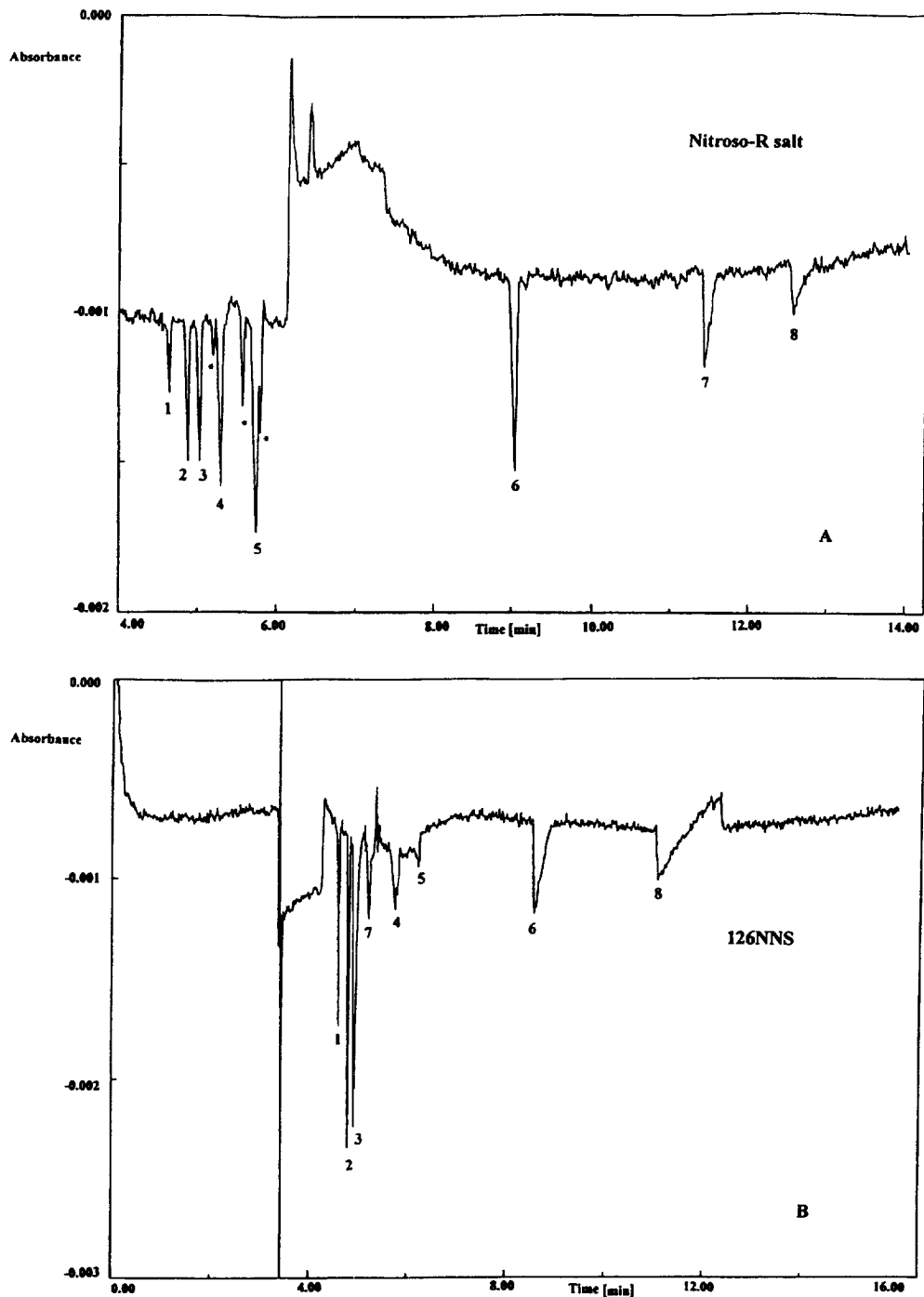


Fig. 2. Electropherograms of a 10 µg/ml organic anion standard mixture obtained with the 0.5 mM Nitroso-R salt (A) 0.5 mM 126NNS (B) and 216NNS (C) pH 8.00. Peaks: 1=hippuric acid (217 nmol), 2=isovaleric acid (98 nmol), 3=butyric acid (114 nmol), 4=propionic acid (135 nmol) 5=acetic acid (167 nmol), 6=malonic acid (75 nmol), 7=pyruvic acid (114 nmol) and 8=oxalic acid (111 nmol) in 1 ml. Hydrodynamic injection for 3 s (35 mbar), 20 kV applied for separation, currents 1.5 µA (A) and 0.9 µA (B and C), λ 260 nm, L_{tot} 67 cm, L_{det} 60 cm, T 25°C. Instrument: Beckman P/ACE 2050.

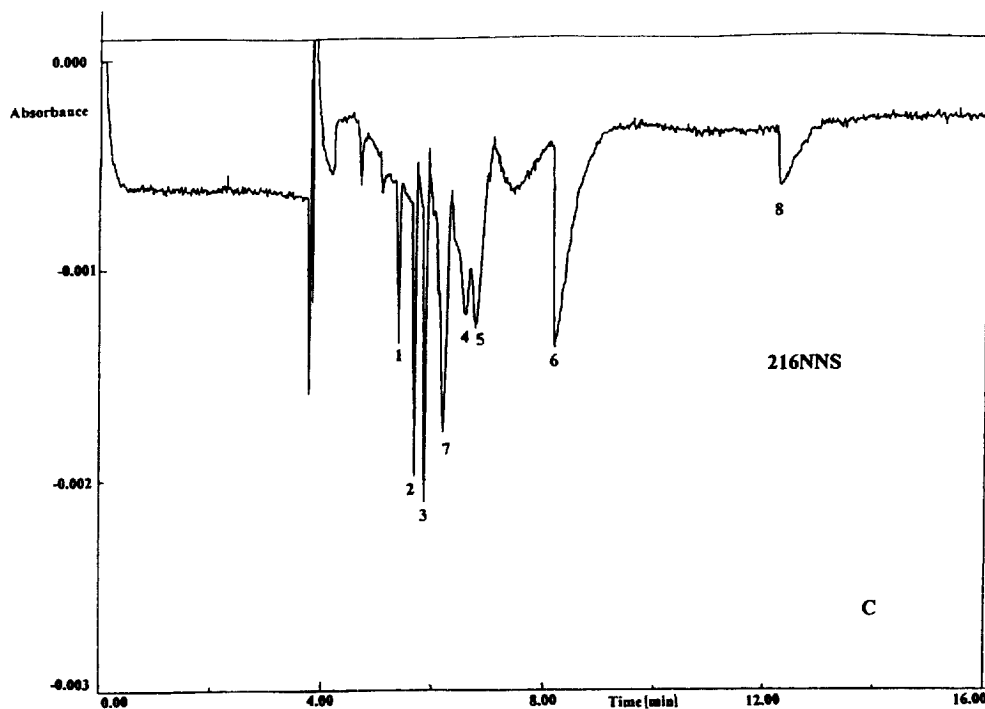


Fig. 2. (continued)

pounds was similarly obtained. The electrophoretic mobilities of the BGE solutions were tested in 30 mM disodium phosphate–sodium dihydrogen phosphate buffer (20 kV, 25.6 μ A, $L=67$ cm, injection 2 s at 50 mbar, $\lambda=260$ nm) were 10.43, 10.39 and 11.38 min for 126NNS, 216NNS and Nitroso-R salt, respectively. The validation was carried out for Nitroso-R salt by optimizing the concentration and pH of the electrolyte, the BGE co-ion, the effective length of the capillary, the detection wavelength, injection technique and applied voltage. After numerous laboratory experiments, a validated screening system was obtained for inorganic anions, phosphonic acids and organic acids.

The same experimental and optimized conditions could be used when 126NNS and 216NNS supplied the BGE solution, since these reagents dissociate in a similar way in the neutral pH range. The optimum detection wavelength of 254 nm was chosen for all the three electrolytes by extracting the dissociated and undissociated structures' spectra in the pH 8.00. The sulphonated nitrosonaphthol BGE solutions in this study were promising since they gave lower

minimum detectable concentration (c_{lim}) of the UV background providing ion than chromate, being $2 \cdot 10^{-8}$ M, $3 \cdot 10^{-7}$ M and $9 \cdot 10^{-7}$ M for aqueous Nitroso-R salt, 126NNS and 216NNS, respectively, when calculated with equation $c_{lim} = AN/\epsilon L$ (AN is the absorbance noise, ϵ is the molar absorptivity of the BGE and L is the path length). As to chromate, it gave c_{lim} of $2 \cdot 10^{-6}$ M with the best currently available UV detectors achieving noise levels [14]. With low BGE concentration of the sulphonated nitrosonaphthols the currents were low (0.9–1.5 μ A) and the serious Joule heating problems at higher ionic strengths could be avoided.

Fig. 1 shows the electropherogram of the baseline separation of seven inorganic anions in Nitroso-R salt achieved within 9 min. Only fluoride did not give a symmetrical peak. The organic acids could not all be analysed simultaneously since, for example, hippuric and pentanic acids comigrated under the separation conditions. Migration behaviour depended on the molar mass/ pK_a ratio, with the monocarboxylic acids migrating in order of decreasing ratio except formic acid and the dicarboxylic acids in

order of increasing ratio: the migration order of the monoanionic compounds tested was isovalerianic acid, butyric acid, propionic acid and acetic acid with M_r/pK_a values of 21.41, 18.32, 15.20 and 12.60 with exception of formic acid (12.27, $I=0$). The corresponding values for dianionic acids were malonic acid and oxalic acids with M_r/pK_a ratios of 18.26 and 21.49 and for the ketoacids, hippuric acid and pyruvic acid the values were 14.66 and 14.19, respectively.

The indirect mode, although practical for the screening of non-absorbing analytes, does not allow identification of compounds since spectra cannot be obtained. Therefore, in our analyses we applied a spiking technique to allow peak identification. Other compounds in the sample perhaps interfere more in the indirect method than in normal CE methods.

Sodium and potassium did not interfere in the separation of the small ions since, as cations, they

migrated first. However, heavy metals may cause problems through complexing with the BGE ion. With sulphonated nitrosonaphthol buffers at pH 8.00, stable baselines and repeatable separations were obtained for all the analytes tested Figs. 2 and 3.

Fig. 4 displays the separation of clodronate, phosphate and phosphite where phosphate and phosphite were present in low concentration in samples containing 1000 $\mu\text{g/ml}$ clodronate. In high concentration, PO_4^{3-} and PO_3^{3-} dissociated, giving rise to three to four peaks. Our studies showed the resolution in CZE to be sufficient for separation of the dissociated states of the phosphonates. Moreover, responses for the phosphonates at wavelength 254 nm were strong with all three dye solutions. The only difficulty in CZE was that, at about 2000 $\mu\text{g/ml}$ clodronate level the peaks in the electropherograms were split. Clodronate could be quantified in all lower concentrations, however.

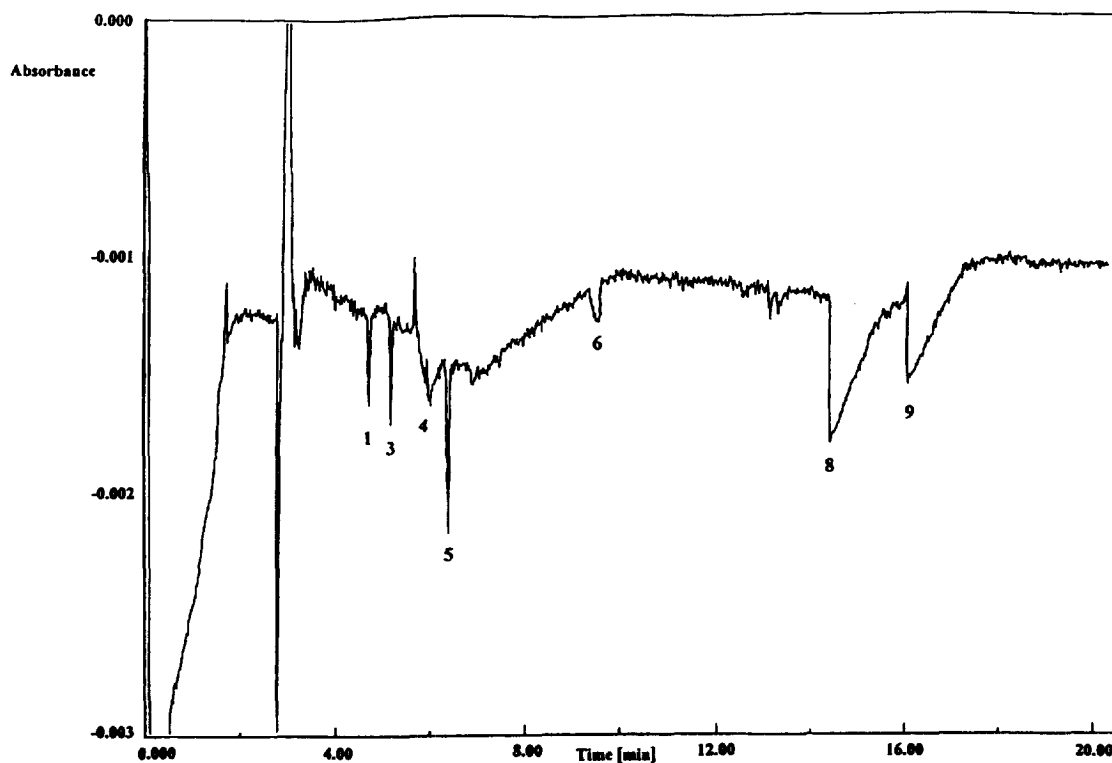


Fig. 3. Electropherogram of fluid from sugar production. Peaks: 1=hippuric acid, 3=butyric acid, 4=propionic acid, 5=acetic acid, 6=malonic acid, 8=oxalic acid, 9=not identified. BGE: 0.5 mM 126NNS solution, pH 8.00, injection 3 s (35 mbar), 20 kV, 0.9 μA , λ 260 nm, L_{tot} 67 cm, L_{det} 60 cm, T 25°C. Instrument: Hewlett-Packard 3D CE.

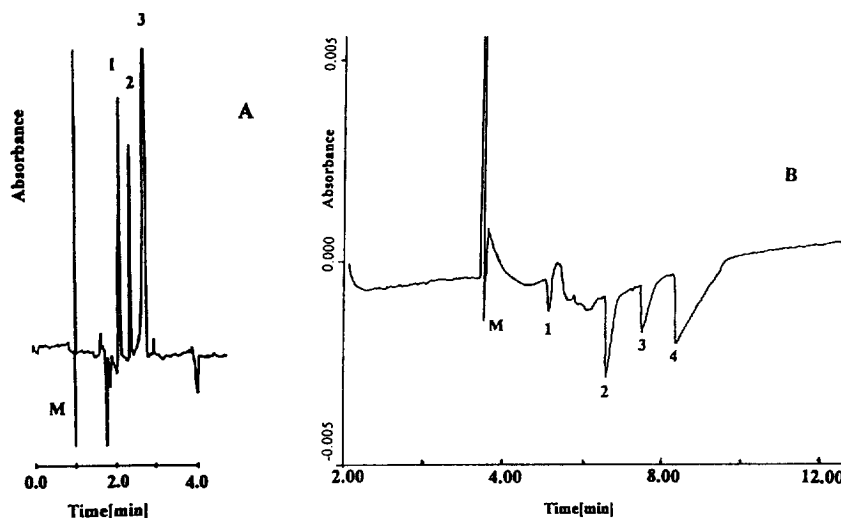


Fig. 4. Electropherograms obtained at pH 8.00 from mixtures containing phosphate, phosphite and clodronate. (A) 0.5 mM Nitroso-R salt solution. Peaks (1) 30 $\mu\text{g/ml}$ (315 nmol) PO_4^{3-} , (2) 30 $\mu\text{g/ml}$ (381 nmol) PO_3^{3-} and (3) 1000 $\mu\text{g/ml}$ (3.47 mmol) clodronate. Experimental conditions: Waters Quanta 4000 instrument, λ 254 nm, 30 kV, 10 s injection, L_{tot} 48 cm, L_{det} 40 cm, 1.5 μA , T ambient. (B) 0.5 mM 126NNS solution. Peaks (1) not identified, (2) 44 $\mu\text{g/ml}$ PO_4^{3-} , (3) 44 $\mu\text{g/ml}$ PO_3^{3-} and (4) 600 mg/ml clodronate. Peak M is methanol. Experimental conditions: Hewlett-Packard CE instrument, λ 260 nm, 20 kV, 4 s injection (35 mbar), L_{tot} 67 cm, L_{det} 60 cm, 0.9 μA , T 25°C.

The repeatability of the analyses was good. The intra-day tests were performed with nine replicate injections of the sample mixtures and replenishment of the background electrolyte solution after each run. The low conductivity of the BGE solutions made repeatable separations possible. Therefore, for example the repeatabilities in absolute migration times of the test compounds were good with the all the sulphonated nitrosonaphthol buffers. The number of sulphonic groups did not affect the efficiency of BGE. However, the slowest moving anions appeared as tailing peaks in the electropherograms. The standard deviations of the absolute migration times of the compounds were from 0.1 to 2%, but also up to 9% when slowly moving analytes with broad sample zones were analysed. Low standard deviations and variation confirmed these electrolyte solutions to be suitable for purity screening of the clodronate and other phosphonates. Clodronate could be separated from phosphate and phosphite when these were present at 0.1% impurity level. At higher concentrations, PO_4^{3-} , PO_3^{3-} and other phosphonates gave

more than one peak in the electropherogram due to their individual dissociation states.

4. Conclusion

Sulphonated nitrosonaphthols, Nitroso-R salt, 126NNS and 216NNS, suited well for the BGE compound in indirect-UV absorption studies with capillary zone electrophoresis. The conditions were optimized with Nitroso-R salt, after which they were similar in the other electrolyte solutions. Seven inorganic anions, eight organic acids and clodronate from its by-products could be separated within 15 min. The detection limits were 1 $\mu\text{g/ml}$ at 254 nm wavelength. The repeatability of the analysis was good.

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