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Sensitive determination of sugars by capillary zone electrophoresis with indirect UV detection under highly alkaline conditions

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Abstract

The separation with capillary zone electrophoresis and indirect UV detection of underivatized sugars was studied. It was found that the high pH of the background electrolyte which was required for the separation resulted in a strong increase of the low-frequency noise and baseline instability with indirect detection. It was shown that the baseline instability is related to the Joule heat production and insufficient thermostating of the capillary. Detection could be strongly improved by using narrow (25 μm) capillaries and low voltages, and by instrumental measures to promote a uniform heat dissipation along the capillary. The low-frequency noise and baseline disturbances were found also to depend on the type of monitoring and counterions in the background electrolyte. By optimization of the experimental conditions the workable pH range for the separation could be extended to 13. After optimization, limits of detection of approximately 0.05 mmol l^{-1} were obtained. Calibration plots were linear up to concentrations of 5 mmol l^{-1} . The reproducibility of migration times was within 0.4%; peak areas were reproducible within 4%. The method developed was applied to the analysis of culture medium samples.

1. Introduction

Methods for sugar analysis have found applications in many fields, such as biology, biochemistry, medicine, pharmacy and food production and control. Commonly used approaches include gas-liquid chromatography (GC) and high-performance liquid chromatography (HPLC) [1]. GC has been the method of choice when high sensitivity was required. However, this technique necessitates time-consuming derivatization procedures to convert the carbohydrates into volatile derivatives. Therefore, GC has largely been substituted by HPLC [2,3]. Underivatized sugars can be separated on different HPLC column

types, such as reversed-phase, amino-modified silica gel or cation-exchange resins, with either low-wavelength UV or refractive-index detection. A short column lifetime, insufficient separation efficiency and lack of sensitivity of detection are the major drawbacks of these HPLC methods [4]. In order to achieve better separation and detection, laborious pre- or post-column derivatization or sample preparation methods were often required in HPLC [5–7]. Sensitive analysis of underivatized carbohydrates can be obtained by ion chromatography with a sodium hydroxide mobile phase, normally at $\text{pH} \geq 13$, using pulsed amperometric detection (PAD) [8]. Investigation showed, however, that amino acids can be detected by PAD and often coelute with sugars, interfering with the sugar determi-

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nation, and should be removed from the sample with an anion–cation-exchange pre-column [9].

In recent years, capillary electrophoresis (CE), with its simple instrumentation, high separation efficiency, rapid analyses and low sample and reagent consumption, offers an alternative to HPLC in many analytical fields. The separation and detection of sugars in CE, however, are difficult because of the absence of readily ionizable functional groups and chromophore systems in mono-, di- and oligosaccharides. Moreover, the very hydrophilic property of sugars prevents the use of micellar separation systems. Different strategies have been described in the literature to solve these problems. (a) By dynamic formation of negatively charged complexes between sugars and borate at pH 8–10, a relatively low increase (2–20 fold) in UV absorbance of underivatized sugars at 195 nm has been observed [10]. However, detection at such a low wavelength is non-selective and may suffer from the interference of charged substances such as amino acids, peptides and proteins that are present with the sugars in the sample [11]. (b) By labeling sugars with a charged chromophoric or fluorophoric group through pre-column derivatization, a significant increase in sensitivity and improved separation have been achieved [12,13]. Recently, several derivatization approaches have been evaluated. It was found that a number of di- and trisaccharides as well as aldonic acids still could not be derivatized successfully [14]. (c) With an electrochemical detector, underivatized sugars can be detected sensitively by a pulsed-potential technique with a gold electrode [15] or at a constant detection potential with copper-based electrodes [16,17]. A high pH (12–13) solution is necessary for the separation as well as for the detection. (d) Sugars are weakly acidic, having a pK_a value in the range from 12 to 13. By ionizing sugars at high pH and adding a UV absorbing or fluorescent monitoring ion to the separation buffer, a charge displacement between sugars and the chromophore or fluorophore is observed that allows the indirect detection of sugars. Generally, the resolution of sugars is improved at a higher pH. With indirect UV detection, the highest utilizable pH was

reported to be pH 12.1 and the detection limit was relatively high (0.5 mmol l^{-1}) [18]. By increasing the pH, the sensitivity decreased rapidly, due to increasing competition of hydroxide ions in the displacement of the chromophore. With indirect laser-induced fluorescence detection [19], the utilizable pH range was even lower, being limited to around pH 11.5. According to the theory with respect to the detection limit in indirect fluorescence detection, a lower concentration of the fluorophore (in a certain range) gives a lower detection limit [20]. Normally, indirect fluorescence detection is performed with fluorophore concentrations below 1 mmol l^{-1} . At such low concentrations, the competition of hydroxide ions with the monitoring ions is more pronounced with rising pH.

In our recent study on noise, using indirect detection, which will be presented in another paper [21], it was found that low-frequency noise, drift and wavy baseline disturbances, as well as disturbances resembling real peaks, increased with an increasing conductivity of the background electrolyte (BGE) and led to a dramatic decrease in sensitivity and in the quantitative reliability. At pH values above 12, necessary for the separation of underivatized sugars, the conductivity increases exponentially with the pH, thereby limiting the detection possibilities for sugars.

In the work presented here, we have tried to extend the buffer pH value for indirect UV detection to 13 without compromising with the sensitivity, mainly by solving the noise problems. The method developed has been applied for the analysis of sugars in tissue culture media also containing a number of neutral and charged substances, including amino acids or proteins.

2. Experimental

2.1. Apparatus

Two CE systems have been used and compared: an ABI Capillary Electrophoresis System, Model 270A-HT (Foster City, CA, USA), and a system PRINCE (Lauer Labs, Emmen, Nether-

lands), with a Spectroflow 757 (ABI, Ramsey, NJ, USA) or a Spectra 100 (Linear, Reno, NV, USA) UV detector. The UV detector was operated at 256, 267 or 310 nm when using sorbic acid, riboflavin or 3,4-dimethoxycinnamic acid as monitoring ions, respectively.

Fused-silica capillaries of 25 μm I.D. and 350 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) were used, with a tube length of 78 cm when using the ABI system or ABI detector and 63 cm when using the Linear detector, with a 50-cm distance from the injection end to the detection window. The window for the on-column detector cell was created by burning off a small section (ca. 0.4 mm) of the polyimide coating.

2.2. Capillary zone electrophoresis

Experiments were carried out at room temperature (18.5°C). The fluctuation of the temperature is $\pm 1^\circ\text{C}$ per day and $\pm 0.1^\circ\text{C}$ per run. A new capillary was filled with BGE for at least 10 h before use. Samples were injected hydrodynamically at 167 mbar for 10 s (ABI) or at 145 mbar for 12 s (PRINCE). Electrophoresis was carried out at a voltage of +10 kV for 30 min, unless stated otherwise. After each electrophoretic run, the capillary was flushed with the BGE at 668 mbar for 9 min and at 1500 mbar for 3.5 min when using the ABI system and the modular PRINCE system, respectively. The anode buffer was replaced after 10 runs.

Electropherograms were recorded with a sample rate of 10 Hz and analyzed on a 486 DX66 PC with an appropriate ADC card and interface, using the electrophoresis data processing software CAESAR 3.0 (Lauer Labs). Analyte concentrations were quantitated by peak area.

2.3. Chemicals and solutions

Maltose monohydrate was obtained from Merck (Darmstadt, Germany). Sucrose, D-(+)-glucose, D-(-)-fructose, riboflavin, lithium hydroxide monohydrate (label content >90%, titration value 87.23%) and sorbic acid were purchased from BDH (Laboratory Chemicals

Division, Poole, UK) and 3,4-dimethoxycinnamic acid (DMC) from Fluka (Buchs, Switzerland). RPMI-1640 culture medium (pH 8.6) was obtained from Sigma (Poole, UK). In some cases 10% (v/v) fetal calf serum (FCS, pH 8.4) was added to the medium. Deionized water was used for the preparation of all solutions.

BGE solutions were prepared by dissolving appropriate amounts of hydroxide and monitoring compounds (sorbic acid, riboflavin or DMC) and adding distilled water to one litre, giving final concentrations of 31, 46, 63 and 109 mmol l^{-1} of the hydroxides and 12 mmol l^{-1} of the monitoring ions. The buffers were stored in polyethylene containers in the dark at 4°C and filtered through a 0.45- μm filter (Millipore) before use.

Stock solutions of sugars of 20 mmol l^{-1} in distilled water and RPMI media with and without 10% fetal calf serum were stored at -20°C and were thawed before use. After dilution of the samples with distilled water they were used without filtering.

3. Results and discussion

3.1. Noise reduction by instrumental optimization

Since most sugars have a pK_a value between 12 and 13, a high pH of the electrolyte is required for their separation [15–18]. The conductivity of the medium increases with the pH and the resulting high electrophoretic currents are accompanied with baseline instabilities and low signal-to-noise ratios with indirect UV detection. According to our studies on noise with indirect UV detection, low-frequency noise and baseline disturbances are closely related to an insufficient and non-uniform heat dissipation along the capillary. Since the Joule heat development in the separation capillary is proportional to the electrophoretic current, we have tried to decrease the noise problems by using small-diameter capillaries. A decrease of the capillary inner diameter from the standard 50 μm to 25 μm resulted in a decrease of the current by a

factor of four and of the low-frequency noise by a factor of ten, while the peak heights remained virtually the same when the ABI detector was used (see Fig. 1). The independence of the sensitivity on the capillary diameter was found to be a special feature of the ABI detector; with the Linear detector the signals were substantially lower when 25- μm capillaries were used. The noise could be reduced further by using 10- μm capillaries. However, with these very narrow inner diameters blocking of the capillary became a frequent problem and flushing times became unacceptably long. Therefore, 25- μm capillaries were used in subsequent work. Reduction of the electrophoretic current by reducing the applied voltage also resulted in a more stable baseline, of

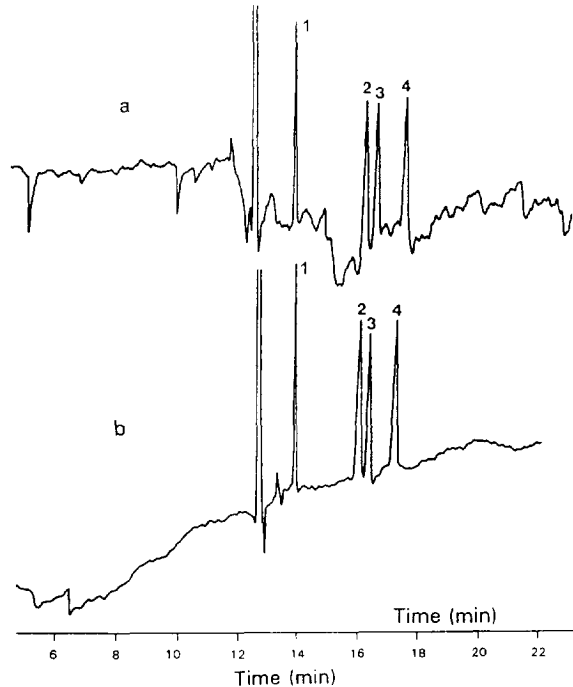


Fig. 1. Influence of the inner diameter of the capillary on baseline noise. Instrument: ABI system; capillary: 110 cm total length and 90 cm to the detection point; injection: 167 mbar for 2.5 and 10 s using 50- μm (a) and 25- μm (b) capillaries, respectively; separation: 25 kV, 35 μA (a), 11 μA (b); BGE: 12 mmol l^{-1} sorbate–63 mmol l^{-1} NaOH; sample: standard mixture of 2.5 mmol l^{-1} of sugars. Peaks: 1 = sucrose, 2 = maltose, 3 = glucose, 4 = fructose.

course at the expense of short analysis times (Fig. 2).

The importance of the thermostating of the capillary was shown by comparing the results obtained with different instruments. In Fig. 3a, the electropherogram obtained with the ABI

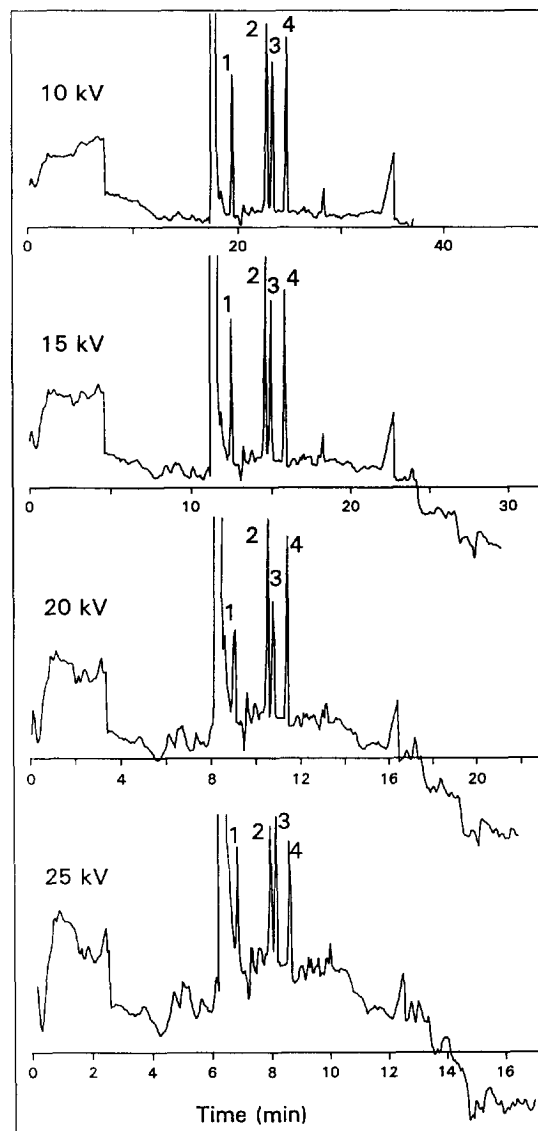


Fig. 2. Influence of the separation voltage on baseline noise. Instrument: modular PRINCE system; BGE: 12 mmol l^{-1} riboflavin–63 mmol l^{-1} LiOH; sample: standard mixture of 1.0 mmol l^{-1} of sugars as in Fig. 1.

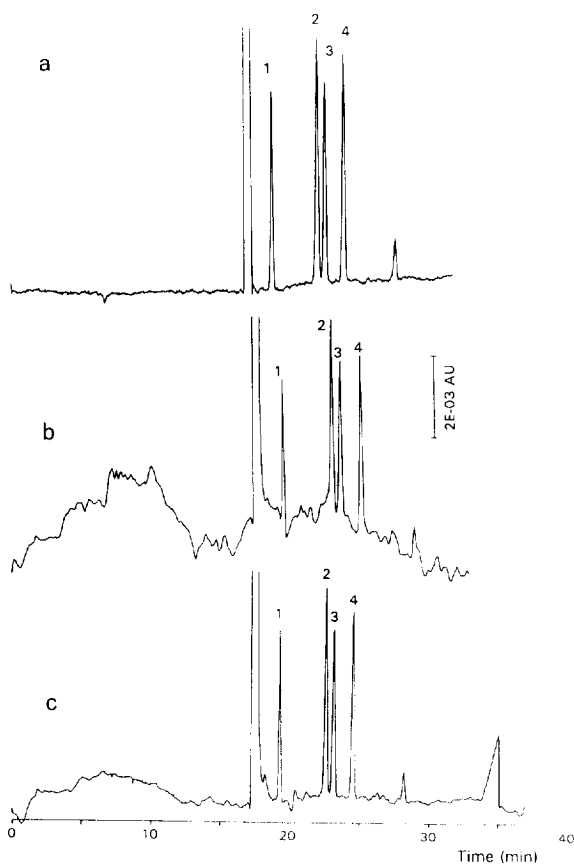


Fig. 3. Comparison of the detection sensitivities obtained with different CE systems and detectors, and the influence of thermostating of the capillary on noise and disturbances of the baseline. Instrument: (a) ABI system, (b) normal PRINCE system, (c) PRINCE system with thermostating of the capillary using sponge sleeves; BGE: 12 mmol l^{-1} riboflavin– 63 mmol l^{-1} LiOH; sample: standard mixture of 1.0 mmol l^{-1} of sugars as in Fig. 1.

system is shown. With the ABI apparatus approximately 84% of the effective length of the capillary is placed in the ventilated, thermostated area of the instrument and 10% of the length in the detector area. By making an opening of 2 cm diameter between the ventilation and the detector chambers the noise could be slightly reduced further. In Fig. 3b, the electropherogram obtained with the modular PRINCE system is shown. With this apparatus in its standard configuration only 30% of the capillary length is in the thermostated chamber. A substantial

improvement of the baseline stability could be realized by shielding the parts of the capillary exposed to ambient air with sponge sleeves (Fig. 3c).

Working at temperatures above ambient intensified the baseline instability (Fig. 4). When the temperature was increased from 22 to 40°C , the low-frequency noise and drift increased by a factor of approximately ten. Again, this can be explained by the non-uniform thermostating conditions along the capillary.

3.2. Optimization of the BGE composition

In Fig. 5, the influence of the hydroxide concentration of the BGE on the resolution of the sugars is shown. It is clear that an increase of the pH gives a better separation. A baseline separation of maltose and glucose was obtained at hydroxide concentrations of 63 mmol l^{-1} and higher.

The influence of the BGE composition on the sensitivity for the sugars tested was first studied with the help of a computer simulation program for CZE developed in our laboratory [22,23]. The results indicated that the pK_a value, the

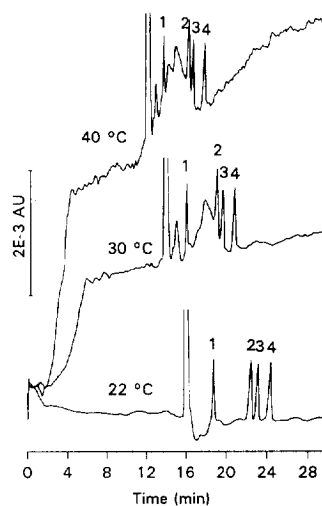


Fig. 4. Influence of the working temperature on the noise, disturbances and separation. Instrument: ABI system; BGE: 12 mmol l^{-1} sorbate, 63 mmol l^{-1} NaOH; standard mixture of 1.0 mmol l^{-1} of sugars as in Fig. 1.

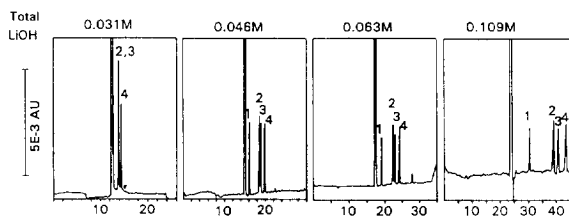


Fig. 5. Influence of the hydroxide concentration of the BGE on the resolution of the sugars. Instrument: ABI system; BGE: 12 mmol l^{-1} riboflavin + LiOH; standard mixture of 1.0 mmol l^{-1} of sugars as in Fig. 1.

ionic mobility and the concentration of the monitoring ion to be used will have little influence on the displacement ratios (sensitivities) and electromigration dispersion obtained at high pH values. Sorbic acid ($\epsilon = 27\,000$ at 256 nm), DMC ($\epsilon = 27\,000$ at 310 nm) and riboflavin ($\epsilon = 30\,000$ at 267 nm) were tested experimentally as monitoring compounds. Indeed, it was found that the sensitivities for the sugars were virtually the same with these monitoring ions. Also it was found (with riboflavin as monitoring ion) that the sensitivities did not depend on the monitoring-ion concentration in the range from 6 to 15 mmol l^{-1} with a hydroxide concentration of 63 mmol l^{-1} . However, the low-frequency noise and baseline disturbances strongly depended on the type of monitoring ion, especially when an instrument with insufficient thermostating capability was used (Fig. 6). The instability of the baseline increased with increasing mobility of the monitoring ion (sorbate > DMC > riboflavin).

According to the simulation programme an influence of the counterion mobility on the sensitivities is expected, with a higher mobility leading to higher displacement ratios. We found experimentally that the sensitivities using sodium hydroxide in the BGE were approximately two times higher than with lithium hydroxide. However, the choice of the counterion type also influences the baseline stability. Apparently, the chemical composition of the BGE controls the way in which temperature differences and changes along the capillary are translated into absorbance differences. Of the counterions tested [tetrabutyl ammonium (TBA), tetramethyl ammonium (TMA), lithium, sodium and potassium], lithium ions gave the most stable baseline,

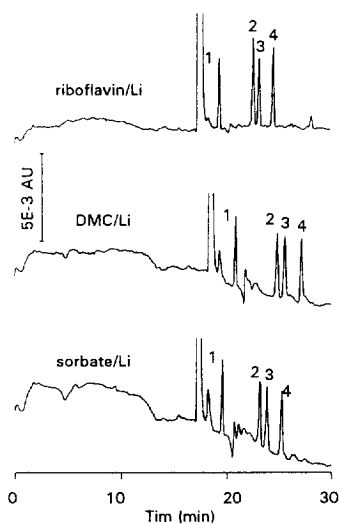


Fig. 6. Influence of the monitoring ion on noise and baseline disturbances. Instrument: PRINCE system; BGE: 12 mmol l^{-1} monitoring ion, 63 mmol l^{-1} LiOH; standard mixture of 1.0 mmol l^{-1} of sugars as in Fig. 1.

irrespective of the monitoring ion used (Fig. 7). The choice of the counterion did not influence the separation behaviour of the sugars. The electroosmotic flow-rate, however, depended strongly on the counterion type (TBA > TMA > K > Na > Li).

Generally, up to three system disturbances were observed in the electropherograms. One is the water system peak appearing just before the sucrose peak, representing the electroosmotic flow-rate. A second system disturbance appears as a triangular peak after the sugar peaks. The migration time of this peak depends on the counterion type. Since its migration time increases with the length of the capillary after the detector window, it appears as if it originates from the end of the capillary. A third system disturbance is visible as a baseline shift before the electroosmotic peak. It appears in the electropherograms after approximately four runs from the same buffer vials. Therefore, this disturbance is probably related to the change of the buffer composition in the inlet or outlet vials due to the electrochemical reaction taking place at the electrodes. None of the system disturbances interfered with the determination of the sugars tested.

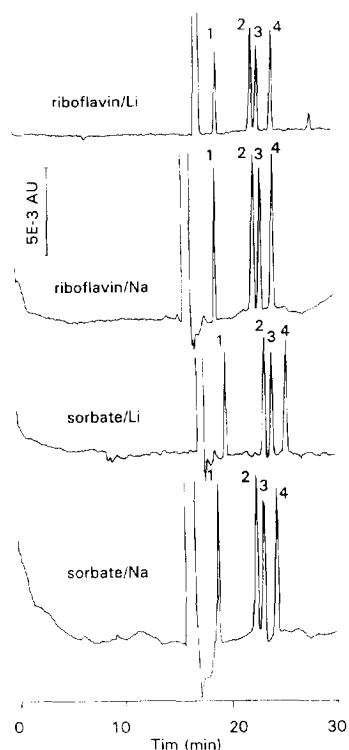


Fig. 7. Influence of the counterion on noise and baseline disturbances. Instrument: ABI system; BGE: 12 mmol l^{-1} monitoring ion, 63 mmol l^{-1} hydroxides; standard mixture of 1.0 mmol l^{-1} of sugars as in Fig. 1.

3.3. Analytical performance

From the optimization studies described above, a BGE containing 63 mmol l^{-1} LiOH and 12 mmol l^{-1} riboflavin and the PRINCE system

were selected for the evaluation of the analytical performances of the method developed in this paper, unless stated otherwise.

The sample pH did not influence peak shapes and area counts from pH 7 to pH 12.2. Thus, samples can normally be injected or diluted with water. The maximum loading capacity of the capillary without loss of resolution was determined. The limit was 3 nl, corresponding to a zone length of 5 mm or injection at 145 mbar for 12 s. The plate number was approximately 110 000 for all four sugars at concentrations of 0.5 mmol l^{-1} . Regression parameters of the calibration lines for the four sugars tested are listed in Table 1.

The upper limits of the linear range of calibration for the sugars were determined to be at least 5 mmol l^{-1} . Above it, because of the concentration overloading effect, maltose and glucose were not baseline separated.

The limits of detection (LOD) for fructose as a function of the LiOH and NaOH concentrations obtained using the ABI systems are given in Fig. 8. It is seen that sensitivity can be gained by using Na. By choosing Na, however, efficient dissipation of heat becomes more crucial.

A standard mixture of sugars was analyzed repeatedly, using the same vial for the anode electrolyte. The results for the reproducibility of the migration times are given in Table 2. As is clear from the table, the reproducibility of the migration times of the sugars is excellent, since the relative standards deviations were found

Table 1
Regression parameters and limits of detection (LOD)

Calibration lines	Sucrose	Maltose	Glucose	Fructose
Regression parameters ^a				
<i>a</i>	0.0422	0.0693	0.0529	0.0539
<i>b</i>	-0.00074	-0.0017	-0.0068	-0.00097
<i>r</i> ²	0.99994	0.99994	0.99990	0.99995
LOD (mmol l^{-1}) ^b	0.06	0.04	0.05	0.055

^a Here *a* and *b* are the constants in the calibration line $y = ax + b$, where *x* is the concentration of the analyte in mmol l^{-1} and *y* is the peak area, and *r*² is the correlation coefficient of the regression line with *n* = 7.

^b Obtained using a signal-to-noise ratio of 3.

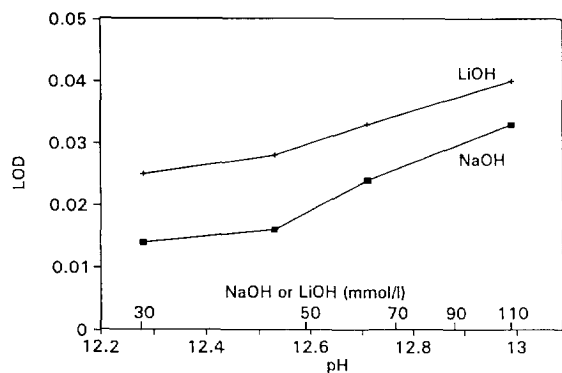


Fig. 8. Limit of detection (LOD) of fructose as a function of the hydroxide concentration. Instrument: ABI system; BGE: 12 mmol l⁻¹ riboflavin + NaOH or LiOH.

within the range from 0.1 to 0.9% for ten consecutive injections. The reproducibility of the peak areas ($n = 10$) was also acceptable, with relative standard deviations between 1 and 4% (Table 3). However, after approximately 20 injections a change in the peak areas for all sugars was observed. This can be explained as

the result of a change in the anode electrolyte composition, due to the electrochemical reaction taking place at the electrode. Therefore, it is recommended to change the anode solution after every ten injections.

Over a period of nine days a standard mixture of sugars was injected repeatedly using the same capillary. In between, various experiments had been performed with RPMI-media with and without calf serum. The reproducibility of the electroosmotic peak migration time was within 1.5%. Peak areas showed a change of -2 to -7% over nine days. Riboflavin is a photo-sensitive compound and must be kept in a dark and cool place. Separate etching procedures for the capillary appeared not to be necessary. A short flushing step after each run was sufficient.

3.4. Application to culture medium

RPMI-1640 medium contains more than 40 compounds, including almost all naturally existing amino acids, vitamins, organic acids and

Table 2
Reproducibility of the migration times with a standard mixture of sugars (1.0 mmol l⁻¹)

Peak	Migration times (min. mean \pm S.D. ^a)		
	First 10 runs	Second 10 runs	Third 10 runs
Sucrose	19.46 \pm 0.08	20.03 \pm 0.17	20.22 \pm 0.22
Maltose	22.89 \pm 0.07	23.54 \pm 0.19	23.71 \pm 0.04
Glucose	23.52 \pm 0.07	24.16 \pm 0.21	24.32 \pm 0.04
Fructose	24.89 \pm 0.05	25.26 \pm 0.20	25.72 \pm 0.04
Electroosmotic flow	17.61 \pm 0.05	18.17 \pm 0.15	18.37 \pm 0.02

^a S.D. = standard deviation.

Table 3
Reproducibilities of peak areas with a standard mixture of the sugars (1.0 mmol l⁻¹)

Sugars	Measured concentration (mmol l ⁻¹ , mean \pm S.D. ^a)		
	First 10 runs	Second 10 runs	Third 10 runs
Sucrose	0.98 \pm 0.02	0.97 \pm 0.03	0.93 \pm 0.01
Maltose	0.99 \pm 0.04	0.96 \pm 0.02	0.92 \pm 0.02
Glucose	1.02 \pm 0.02	1.01 \pm 0.02	0.97 \pm 0.04
Fructose	0.99 \pm 0.03	0.97 \pm 0.02	0.94 \pm 0.02

^a S.D. = standard deviation.

Table 4
Recovery of sugars added to culture media

Sugar	Concentration (mmol l ⁻¹)			Recovery (%)
	Present	Spiked	Found	
<i>RPMI-1640</i>				
Sucrose	0	1.0	0.98 ± 0.02	97.8 ± 2
Maltose	0	1.0	1.00 ± 0.03	103.3 ± 3
Glucose	1.16 ± 0.03	1.0	2.15 ± 0.05	99.1 ± 5
Fructose	0	1.0	0.98 ± 0.02	98.2 ± 2
<i>RPMI-1640 + 10% FCS</i>				
Sucrose	0	1.0	1.01 ± 0.01	100.1 ± 1
Maltose	0	1.0	1.01 ± 0.02	101.1 ± 2
Glucose	1.07 ± 0.02	1.0	2.10 ± 0.05	103.8 ± 5
Fructose	0.054 ± 0.004	1.0	1.09 ± 0.02	103.2 ± 2

Mean ± S.D. for three injections.

inorganic salts. The concentration of D-glucose in the medium is 2 g l⁻¹ (ca. 11 mmol l⁻¹). To some of the samples, 10% fetal calf serum was added to resemble the protein fraction of possible cultures.

The recovery of the sugars from the medium was studied by comparing peak areas obtained

with and without spiking standard mixtures to the medium. Recoveries were found to be between 98 and 104% for all sugars (Table 4).

In Fig. 9, electropherograms obtained with ten times diluted RPMI media, with and without the addition of 10% fetal calf serum are shown. From these electropherograms and recovery

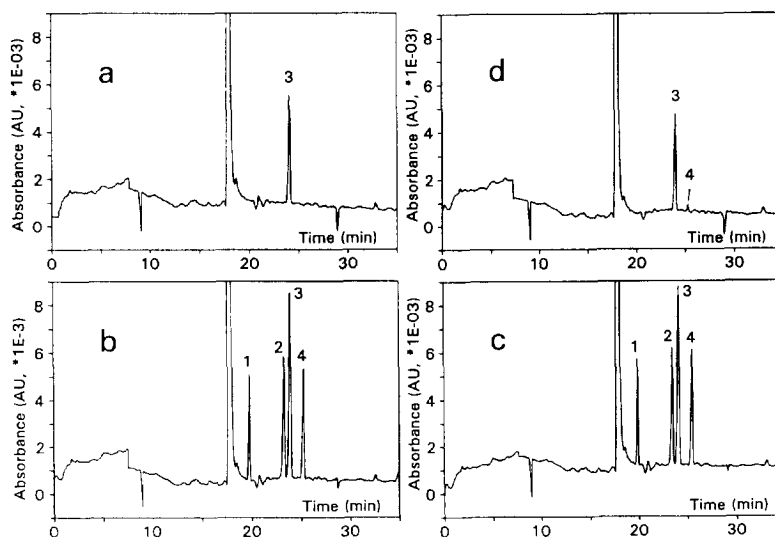


Fig. 9. Electropherograms obtained with RPMI culture media diluted with water (dilution factor 1:9): (a) RPMI-1640; (b) RPMI-1640 spiked with 1.0 mmol l⁻¹ of the sugars as in Fig. 1; (c) RPMI-1640 + 10% FCS; (d) RPMI-1640 + 10% FCS spiked with the sugars as in (b).

studies it is clear that: (1) the presence of the RPMI medium does not change the electrophoresis of the sugars; (2) the RPMI medium does not contain components (in significant concentrations) interfering with the sugar determination; (3) the addition of calf serum to the RPMI medium does not influence the electrophoretic process, and does not contain components (in significant concentrations) interfering with the sugar determination except for a small amount of fructose.

The fact that the serum proteins do not change the electroosmotic flow, as has been observed frequently in other applications of CE, may be explained by the high pH of the separation solution. At high pH absorption of proteins to the fused-silica surface of the capillary is not very likely. Moreover, the capillary wall is constantly etched by the solution during analysis.

4. Conclusions

The method developed for determination of low-molecular-mass sugars by capillary zone electrophoresis is sensitive, quantitative, reproducible and robust. Joule heat and non-uniform thermostating of the capillary are the main sources of the low-frequency noise and baseline disturbances. Moreover, the noise is influenced by the composition of the BGE. Thus, riboflavin and lithium were found to be the preferred monitoring and counterions, respectively. Both compounds had the lowest mobility of the compounds tested, which provides an explanation for the reduced noise problems observed. By reducing the low-frequency noise and the baseline disturbances, the pH of the BGE can be increased to 13 without major loss of sensitivity. The detection limits obtained with the optimized system are approximately 25 times lower at pH 12.3 when using a riboflavin–NaOH BGE and one order of magnitude lower at higher pH

(12.6–13) when using a riboflavin–LiOH BGE, compared to results cited in the literature [18].

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