Indirect UV Detection of Carbohydrates in Capillary Zone Electrophoresis

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Key Words

Capillary electrophoresis
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Summary

A new system for the rapid and sensitive analysis of underivatized carbohydrates has been established using capillary zone electrophoresis with indirect UV detection. At an applied potential of 28 kV, sugars and sugar acids could be separated by the combined effects of electroendosmosis and electrophoresis within 20 minutes in a fused silica capillary of 50 µm internal diameter and an effective length of 100 cm using 6 mM sorbic acid, pH 12.1, as both carrier electrolyte and chromophore. The alkaline pH ensured ionization of the sugars and, hence, their detection by means of charge displacement. Furthermore, the chosen concentration of sorbic acid allowed the smallest fractional change in the background signal to be measured. While the electrophoretic mobilities of the sugars were found to increase within a pH range of 11.9 to 12.3, those of the sugar acids were not affected. Due to the increasing competition of hydroxide ions in the displacement of the chromophore with rising pH, a significant loss of sensitivity is observed at pH values higher than 12.1 and this pH was found to provide sufficient resolution, optimum sensitivity, and a acceptably short analysis time. Under these conditions, a lower detection limit of 2 pmol was obtained for glucose.

Introduction

Capillary zone electrophoresis represents an alternative to the commonly used techniques for the determination of carbohydrates (thin-layer chromatography [1], gas chromatography [2] and high-performance liquid chromatography [3]). However, detection of the nanoliter sample volumes injected in capillary electrophoresis is a challenging problem especially in the case of carbohydrates which have a very low UV absorbance because of the small proportion of the carbonyl form in aqueous solution. This makes photometric detection without derivatization a difficult task. Recently, however, a 2–20 fold increase in UV absorbance of underivatized carbohydrates at 195 nm has been observed by adding borate to their aqueous solutions [4]. Under alkaline conditions, the borate ions react with vicinal hydroxyl groups with the resultant transformation of carbohydrates into negatively charged borate complexes, which are able to migrate in an electric field [5–7]. However, the observed increase in absorptivity is comparably small and detection of underivatized sugars is restricted to the nmol range. A significant increase in sensitivity was achieved upon derivatization of reducing mono- and maltooligosaccharides to N-2-pyridylglycamines [8–10]. Using on-column UV monitoring at 240 nm these derivatives can be detected at the lower pmol level. However, successful coupling of carbohydrates with 2-aminopyridine by reductive amination with sodium cyanoborohydride requires the presence of a free aldehyde group and therefore, only aldoses can be determined. A more sensitive method, which permits the analysis of both underivatized aldoses and ketoses in the fmol range, is indirect fluorescence detection with visible laser excitation [11]. However, the high cost of a laser and its non-availability in most commercial capillary electrophoretic systems precludes the use of indirect fluorescence detection in many laboratories. A more universal approach is the indirect photometric detection of analytes, which has already been applied successfully to the analysis of both inorganic [12] as well as organic anions [13]. Provided that a carrier electrolyte anion with a high molar absorptivity coefficient and an effective mobility close to the mobilities of the analytes is selected, a detection limit as low as one pmol can be achieved.

The present paper deals with the indirect photometric detection of underivatized carbohydrates in the pmol range using sorbic acid as both carrier electrolyte anion and chromophore.

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Experimental

Chemicals

Standard solutions were prepared from chromatographic or analytical reagent grade chemicals (Merck, Darmstadt, FRG; Sigma, St. Louis, MO, USA) in deionized water (NANOpure, Barnstead Co., Newton, MA, USA) at concentrations of 0.4 mg/ml. Running electrolyte solutions were prepared by dissolving appropriate amounts of sorbic acid (Sigma) in double distilled water to yield final concentrations of 2–20 mM. The pH was adjusted to values in the range of 11.9–12.3 by the addition of 0.25 M NaOH (Merck).

Apparatus

Analyses were performed either on an Applied Biosystems (ABI, San Jose, CA, USA) Model 270A or a Waters Quanta 4000 (Milford, MA, USA) capillary electrophoresis system, which were equipped with a 122 cm fused silica capillary of I.D. 50 µm and a 100 cm fused silica capillary of I.D. 75 µm, respectively. Detection was carried out by on-column measurement of UV absorption at 256 nm at 22 cm from the cathode in the case of the Applied Biosystems equipment or at 254 nm at 7.5 cm from the cathode in the case of the Waters system. Pherograms were recorded on a Shimadzu Chromatopac C-R6A integrator (Kyoto, Japan). Samples were loaded either by applying a vacuum at a pressure of 16.9 kPa (ABI) or by means of hydrostatic pressure at a 10 cm height (Waters) for a specified period. Analyses were carried out either at a constant temperature of 30 °C (ABI) or at ambient temperature (Waters).

Capillary Conditioning

Every new fused silica capillary was flushed with 1 M NaOH for one hour followed by 0.001 M NaOH for 5 min. Between runs, the capillary was washed first with 1 M NaOH for 4 min, and then with 0.001 M NaOH for 2 min. Subsequently, it was equilibrated with running buffer for 6 min. Finally, the system was prerun for 10 minutes in order to obtain a stable background signal. Overnight the capillary was stored in 0.001 M NaOH.

Results and Discussion

The selection of sorbic acid as carrier electrolyte anion and chromophore for the indirect UV photometric detection of carbohydrates separated by capillary zone electrophoresis was based on several reasons. Firstly, the molecule has a high molar absorptivity coefficient (ε = 27800 at 256 nm). Secondly, it is compatible with the solvent system used and thirdly it carries a single charge.

This ensures a good transfer ratio, which is defined as the number of chromophore molecules displaced by one analyte molecule. Fourthly sorbic acid interacts neither with the analytes nor with the capillary surface and finally, its effective mobility matches the ionic mobilities of carbohydrates, thus, avoiding peak spreading.

Carbohydrates are only weakly acidic. As an example the pK for glucose is 12.35 [14]. Therefore, the running buffer has to be made very basic before any ionization will occur. Dissociation of the sugars is a prerequisite both for their separation based on differences in migration velocity as well as for their detection by means of charge displacement. This means that the pH of the running buffer must be approaching 12 to have any substantial fraction of a carbohydrate in its ionized form. However, when the pH of the buffer solution gets this high the concentration of hydroxide ions is no longer negligible relative to the concentration of the chromophore. This results in a decrease of the transfer ratio, which can be described by the equation

$$TR_{total} = \alpha[sugar] / [C] + [OH^-]$$

where TR_{total} is the transfer ratio, α[sugar] the amount of sugar molecules ionized, [C] the amount of chromophore, and [OH^-] the amount of hydroxide ions [11].

It can be seen that at constant sugar and chromophore concentrations, α in the numerator and the [OH^-] in the denominator are competing functions of pH. As a consequence, TR_{total} goes through a maximum when it is plotted as function of pH (Figure 1). The pH at this maximum is the most sensitive pH for detection. Similar considerations preclude the use of borate due to the high concentration required (100–200 mM) for efficient complexation of sugars.

While the pH of the running electrolyte did not exert any significant impact on the electrophoretic mobilities of sugar acids, a slight increase in the mobilities of sugars could be observed (Figure 2). However, due to the increasing concentration of sodium ions in the background electrolyte with rising pH, which increases the thickness of the diffusion double layer at the inner capillary wall [15], electroendosmotic flow decreased gradually. For this

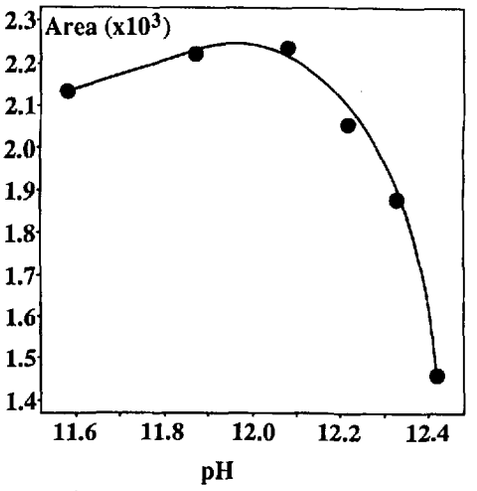

Figure 1

Effect of pH on sensitivity. Electrolyte: 6 mM sorbate; apparatus: ABI Model 270A; capillary: fused silica, L = 122 cm, l = 100 cm, ø = 50 µm; current: 6, 8, 13, 17, 20, and 24 µA at pH 11.58, 11.87, 12.08, 12.22, 12.33, and 12.42, respectively; voltage: 28 kV; temperature: 30 °C; detection: UV, 256 nm; injection: vacuum: 1.0 s; sample: mannose, 12.5 mM.

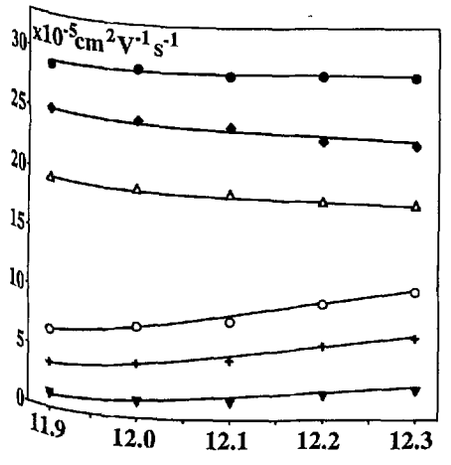

Figure 2
Effect of pH on the electrophoretic mobilities of carbohydrates: (●) mannuronic acid, (◆) gluconic acid, (Δ) N-acetylneuraminic acid, (o) mannose, (+) galactose, and (▼) raffinose. Electrolyte: 6 mM sorbate; apparatus: Waters Quanta 4000; capillary: fused silica, L = 100 cm, l = 92.5 cm; ø = 75 µm; current: 21 µA; voltage: 27.8, 23.4, 20.3, 16.4, and 13.1 kV at pH 11.9, 12.0, 12.1, 12.2, and 12.3, respectively; temperature: ambient; detection: UV, 254 nm; injection: hydrostatic, 30 s.

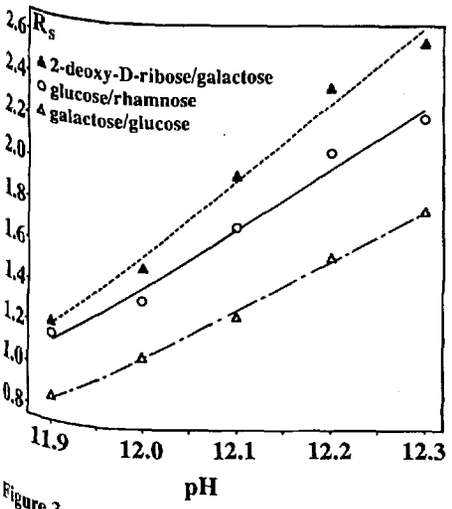

Figure 3
Effect of pH on resolution of carbohydrates: (Δ) galactose/glucose, (o) glucose/rhamnose, (▲) 2-deoxy-D-ribose/galactose. Electrophoretic conditions as in Figure 2.

reason, apparent electrophoretic mobilities decreased considerably from pH 11.9 to 12.3, which caused an increase in time of analysis for mannuronic acid from 19 to 40 minutes.

Generally, resolution of sugars improved with increasing pH, as depicted in Figure 3. However, the enhancement observed above pH 12.1 cannot be utilized since zone width became smaller due to the aforementioned decrease in sensitivity. Based on these considerations, a pH of 12.1 was chosen for all subsequent analyses, because it offered maximum sensitivity and sufficient resolution within an acceptably short time of analysis. Moreover, despite the high pH of the background electrolyte no alkaline degradation of the carbohydrates could be observed at

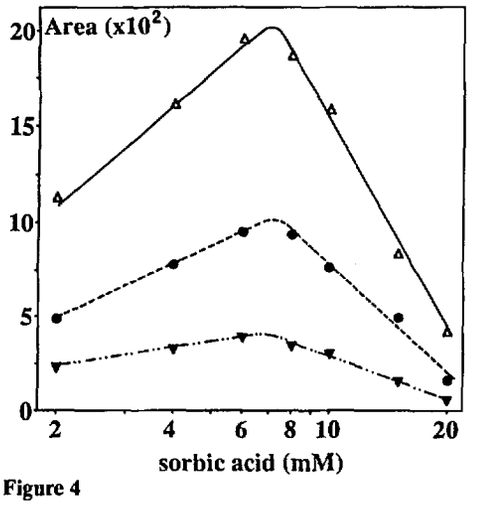

Figure 4
Effect of the concentration of sorbic acid on response: Δ = 33.3 pmol mannose, ● = 16.6 pmol mannose, ▼ = 8.3 pmol mannose. Electrolyte: sorbate, pH 12.1; apparatus: ABI Model 270A; capillary: fused silica, L = 122 cm, l = 100 cm, ø = 50 µm; voltage: 28 kV; temperature: 30 °C; detection: UV, 256 nm; injection: vacuum, 0.8 s.

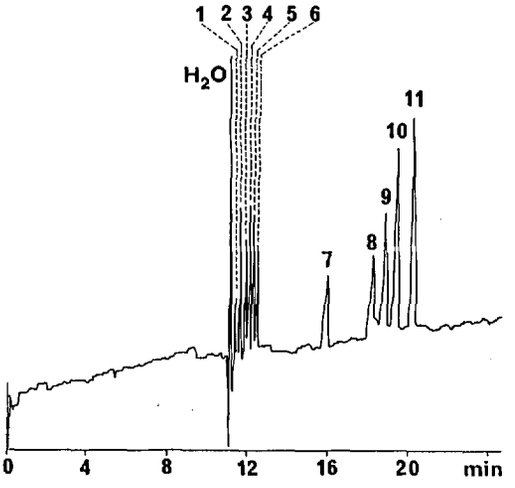

Figure 5
Capillary zone electrophoretic analysis of carbohydrates (0.95 – 2.66 mM). Electrolyte: 6 mM sorbate, pH 12.1; apparatus: ABI Model 270A; capillary: fused silica, L = 122 cm, l = 100 cm, ø = 50 µm; current: 13 µA; voltage: 28 kV; temperature: 30 °C; detection: UV, 256 nm; injection: vacuum, 2.0 s. Zone identification: **1** = raffinose, **2** = 2-deoxy-D-ribose, **3** = galactose, **4** = glucose, **5** = rhamnose, **6** = mannose, **7** = N-acetylneuraminic acid, **8** = gluconic acid, **9** = galacturonic acid, **10** = glucuronic acid, **11** = mannuronic acid.

the chosen temperature, which is in agreement with a previous study [16].

Another major consideration in optimizing indirect photometric detection in capillary zone electrophoresis is the concentration of the chromophore. As shown in Figure 4, the response of the system for a given amount of mannose has its maximum at approximately 6 mM, which permits the analysis of glucose with a lower detection limit of 2 pmol at a signal-to-noise ratio of 3. Concentration sensitivity, however, is comparatively low (~ 0.5 mM) due to the small proportion of dissociated carbohydrate

molecules. At lower concentrations of sorbic acid, sensitivity decreases due to the relatively high content of hydroxide ions in the background electrolyte. At concentrations above 6 mM, response deteriorates because less light reaches the photodiode. This reduces the ability to measure a small change on top of a large background signal [17].

Figure 5 shows the separation of a mixture of eleven sugars and sugar acids with concentrations ranging from 0.95 to 2.66 mM. At the pH value selected, the carbohydrates migrate away from the detector toward the anode. However, due to the large electroosmotic flow in the system, which is a magnitude greater than electrophoretic migration, analytes are propelled together with the bulk solution toward the cathode but at a much lower rate. Therefore, the sugars dissociated least are detected first since they are less able to migrate upstream. The sugar acids, however, are detected last due to their greater upstream migration rate as a result of the complete dissociation of the carboxyl group.

In indirect photometric detection, the range of linearity is limited both by the concentration of the chromophore and by the degree of dissociation of the analyte, which determines the number of background molecules displaced by each analyte molecule. The calibration curves showed excellent linearity and negligible Y intercepts (Figure 6). Within the range of linearity, least-square linear regression analysis provided the following equations of the regression line as well as regression coefficients for fucose, galactose, mannose and lactulose, respectively: $Y = .03X + .078$ ($R^2 = .997$), $Y = .031X - .036$ ($R^2 = .998$), $Y = .044X - .113$ ($R^2 = .999$), $Y = .036X - .026$ ($R^2 = .999$). Figure 7, however, shows that the slope of the regression lines depends on injection time as a consequence of the accidental take-up of sample when the capillary is dipped into the sample tube. Therefore, it is of great importance to use the same injection times both for calibration and for the quantitative determination of the carbohydrates contained in the samples.

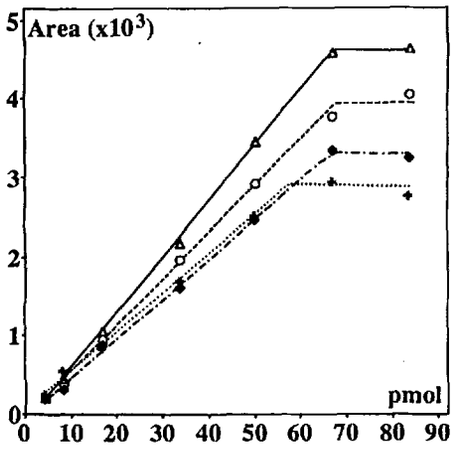

Figure 6

Calibration curves for fuctose (+), galactose (◆), mannose (o), and lactulose (Δ). Electrolyte: 6 mM sorbate, pH 12.1; apparatus: ABI Model 270A; capillary: fused silica, L = 122 cm, l = 100 cm, ø = 50 µm; current: 12 µA; voltage: 28 kV; temperature: 30 °C; detection: UV, 256 nm; injection: vacuum, 2.0 s.

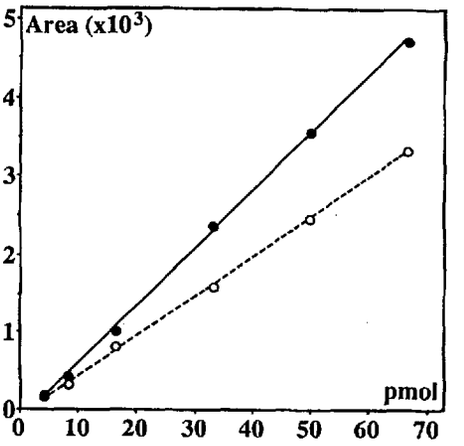

Figure 7

Influence of injection time on the slope of the calibration curve for galactose. Electrolyte: 6 mM sorbate, pH 12.1; apparatus: ABI Model 270A; capillary: fused silica, L = 122 cm, l = 100 cm, ø = 50 µm; current: 12 µA; voltage: 28 kV; temperature: 30 °C; detection: UV, 256 nm; injection: vacuum (●, 0.5 s; o, 2.0 s).

Table I. Effect of carbohydrate concentration on number of theoretical plates and resolution.

sampling time sec	sample amount pmol	carbohydrate concentration mmol/l	number of theoretical plates ($\times 10^5$)/m		resolution
0.4	12.5	12.5	galactose	1.069	2.38
	12.5	12.5	lactulose	0.767	
0.6	12.5	8.3	galactose	1.405	2.87
	12.5	8.3	lactulose	0.908	
1.0	12.5	5.0	galactose	1.732	2.96
	12.5	5.0	lactulose	1.224	
1.6	12.5	3.1	galactose	1.599	2.84
	12.5	3.1	lactulose	1.036	
2.2	12.5	2.3	galactose	1.274	2.39
	12.5	2.3	lactulose	0.791	
2.8	12.5	1.8	galactose	1.144	2.25
	12.5	1.8	lactulose	0.728	
3.2	12.5	1.5	galactose	0.926	2.15
	12.5	1.5	lactulose	0.620	

Number of theoretical plates and resolution reached their optimum at a carbohydrate concentration roughly equal to that of sorbic acid (Table I). Below and above that concentration they were found to decrease because of molecular diffusion.

Table II lists the electrophoretic mobilities of 36 carbohydrates, which have been obtained at a constant voltage of 28 kV in a fused silica capillary of 50 µm internal diameter and an effective length of 100 cm using 6 mM sorbic acid, pH 12.1, as background electrolyte. For determing the reproducibility of electrophoretic mobilities, at least five repeat runs were carried out for 10 different carbohydrates. A mean standard deviation of 0.237 was obtained. The coefficient of variation was 3.6 % and ranged from 0.6 to 11.2 % for mannuronic acid and saccharose, respectively. In order to obtain baseline resolution under the described conditions, the difference in electrophoretic mobility between two carbohydrates has to be approximately 0.9×10^{-5} $cm^2V^{-1}s^{-1}$.

A comparison with ion partition chromatography [18] reveals that sugars such as galactose, mannose and xylose, which cannot be separated by chromatography, are resolved by means of capillary zone electrophoresis. On the other hand, however, glucose and arabinose can be well resolved by means of ion partition chromatography but not by capillary zone electrophoresis. Therefore, it can be concluded that high-performance liquid chromatography and the capillary zone electrophoretic method presented here are not competing, but rather complementary techniques.

The practical applicability of capillary zone electrophoresis and indirect photometric UV detection to the determination of sugars was confirmed by the analysis of a variety of samples. Figure 8, for instance, shows the analysis of orange juice, which contains saccharose, glucose and fructose.

Table II. Mean electrophoretic mobilities of carbohydrates*.

No.	Carbohydrates	mobility x $10^{-5}cm^2V^{-1}s^{-1}$
1	raffinose	1.312
2	saccharose	1.356
3	2-deoxy-D-galactose	2.419
4	2-deoxy-D-ribose	2.798
5	D-fucose	3.096
6	lactose	4.175
7	maltotriose	4.313
8	D-galactose	4.358
9	melibiose	4.623
10	D-galactosamine	4.694
11	cellobiose	4.794
12	maltose	4.813
13	L-arabinose	5.121
14	D-glucose	5.135
15	lactulose	5.403
16	palatinose	5.594
17	D-glucosamine	5.805
18	D-xylose	6.268
19	N-acetyl-galactosamine	6.287
20	D-lyxose	6.440
21	L-sorbose	6.468
22	L-rhamnose	6.599
23	turanose	7.047
24	D-fructose	7.140
25	D-ribose	7.419
26	D-mannose	7.462
27	N-acetyl-glucosamine	8.018
28	N-acetyl-neuraminic acid	19.867
29	D-galactonic acid	24.950
30	D-gluconic acid	25.518
31	D-mannonic acid	25.677
32	D-galacturonic acid	26.789
33	D-arabonic acid	27.593
34	D-glucuronic acid	27.796
35	D-ribonic acid	28.139
36	D-mannuronic acid	29.315

* 6 mM sorbic acid, pH 12.1, 28 kV, L = 122 cm, l = 100 cm.

Conclusion

Indirect photometric UV detection using sorbic acid as background electrolyte and chromophore allows the sensitive determination of both aldoses as well as ketoses in the lower pmol range without the need for derivatization.

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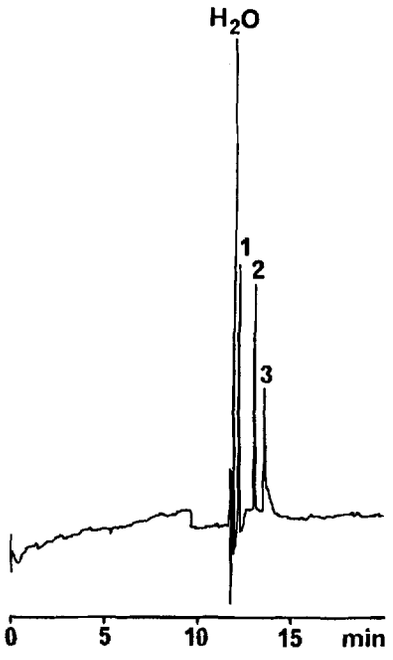

Figure 8

Capillary zone electrophoretic analysis of orange juice. Electrolyte: 6 mM sorbate, pH 12.1; apparatus: ABI Model 270A; capillary: fused silica, L = 112 cm, l = 90 cm, ø = 50 µm; current: 11 µA; voltage: 24 kV; temperature: 30 °C; detection: UV, 256 nm; injection: vacuum, 2.0 s; sample: orange juice, diluted 1:25 with bidistilled water; zone identification: **1** = saccharose, **2** = glucose, **3** = fructose.

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