Capillary zone electrophoresis of carbohydrates derivatized with 4-aminobenzoic acid ethyl ester

Application Note

Life Sciences

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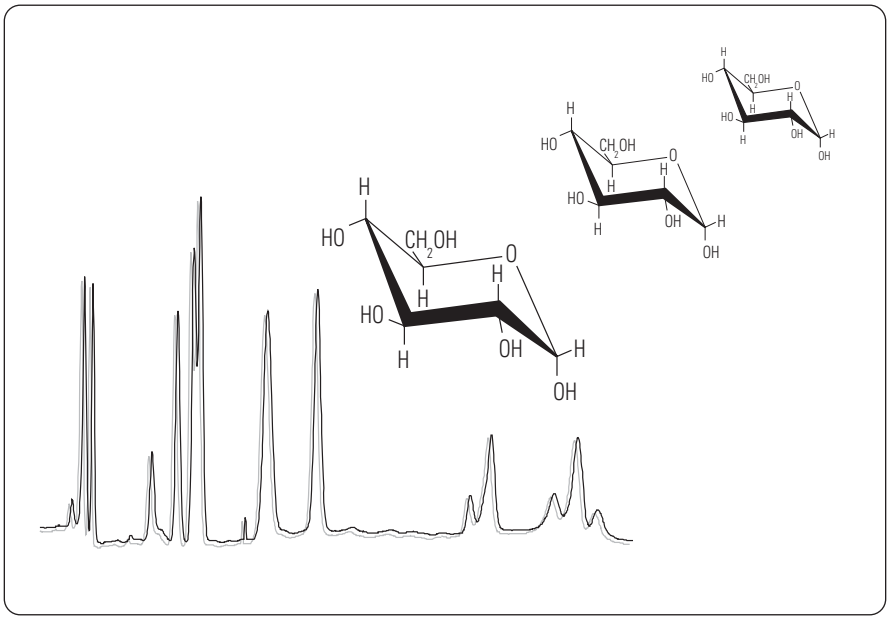

Abstract

Carbohydrates derivatized with 4-aminobenzoic acid ethyl ester were separated by capillary zone electrophoresis with an alkaline borate background electrolyte. The precision of the run-to-run migration time had a relative standard deviation of less than 0.2 %, provided an intersample wash was included. The concentration limit of detection was approximately 4 µM for glucose (signal-to-noise ratio: 5) using a 50-µm internal diameter (id) capillary with a “bubble factor” of 3.

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Introduction

The determination of the carbohydrate content of N-linked polysaccharides by capillary zone electrophoresis (CZE) is complicated by the lack of sensitive and practical detection schemes. Direct detection of sugars as their borate complexes at 195 nm is insensitive and requires a temperature of 60 ºC to effect separation.[1] This lack of a suitable chromophore has spurred the development of indirect detection,[2, 3] pulsed amperometric detection,[4, 5] and pre-capillary derivatization techniques[6-10].

Although it is a universal technique for ionic species, indirect photometric detection of sugars is complicated by their high pKa. Sugars are not sufficiently ionized to permit separation and detection until a pH of 12 is reached. At this pH, the significant hydroxide ion population interferes with indirect detection. As a result, the reported concentration limit of detection (CLOD) of 500 µM is inadequate for many applications. Selectivity may also be problematic when complex samples such as plant tissues are employed. The pulsed amperometric technique, which is sensitive down to 2 µM, is not commercially available for CZE. In addition, most of the known derivatization reagents react only with aldoses or require the use of laser fluorescence detection.

A notable exception to these problems involves the use of 4-aminobenzoic acid ethyl ester (ABEE) as a derivatization reagent. The chemical reaction, illustrated in figure 1 permits the derivatization of both aldoses and ketoses. The derivatives are separated as borate complexes by CZE.

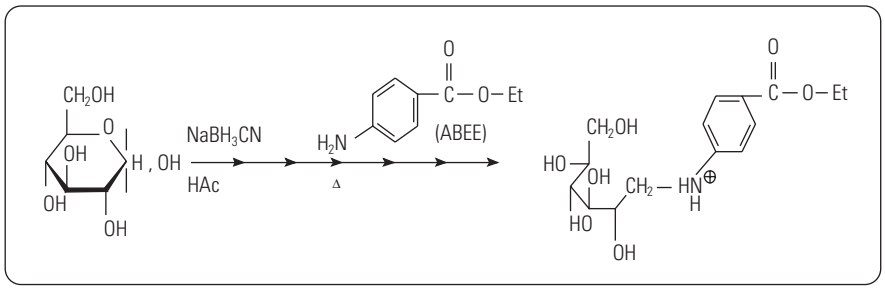

Figure 1
Reaction of sugars with ABEE.

Carbohydrates detectable		Carbohydrates not detectable
2-deoxy-D-galactose	L-sorbose	Raffinose
2-deoxy-D-ribose	L-rhamnose	Saccharose
D-fucose	D-fructose	Lactulose
Lactose	D-ribose	D-galacturonic acid
Maltotriose	D-mannose	D-gluconic acid
D-galactose	D-galacturonic acid	D-mannonic acid
Melibiose	D-glucuronic acid	D-arabonic acid
Cellobiose	D-mannuronic acid	D-ribonic acid
Maltose	Arabinose	Sialic acid
L-arabinose	N-acetyl-D-galactosamine	
D-glucose	N-acetyl-D-glucosamine	
D-xylose	D-galactosamine	
D-lyxose	N-acetyl-neuraminic acid	

Table 1
Carbohydrates tested by the ABEE method. Data from references 9 and 10, except for sialic acid.

Certain disaccharides, trisaccharides, uronic acids and amino sugars can also be measured, however the technique is not universal. A list of solutes tested by this method is given in table 1.

Experimental

Materials

Experiments were performed using an Agilent Capillary Electrophoresis system equipped with a diode-array detector. Agilent Extended Light path capillaries were used. These capillaries contain a "bubble" at the point of detection. The ratio of the extended lightpath in the "bubble" to the internal diameter of the rest of the capillary is known as the bubble factor (BF). An Agilent ChemStation was used for system control, data acquisition and postrun processing. The sugar standards, cyanoborohydride (CBH) and ABEE were purchased from Sigma-Aldrich Co. Buffer components were of the highest available purity. All buffer solutions were filtered through 0.2 µm filters before use.

Reagent solutions

Stock A: 10 % (w/v) ABEE, 10 % acetic acid in methanol. This solution is stable for several weeks at room temperature. Sugar standards: 100 mg/mL in 40 % methanol/water or water alone

Derivatization procedure

Just before use, dissolve 1 mg CBH in 100 µL of Stock A. Combine 49 µL of this solution with 1 µL sugar standard

in a sealable tube. Heat for 15 minutes (longer times may be occasionally required) at 80 ºC. For mixed standards, double the amount of Stock A. After completion of the reaction, add methanol q.s. to make the total volume 2 mL. Transfer an aliquot of this solution to an autosampler vial.*

**Note: Maintain the temperature of the autosampler at 10 ºC to minimize methanol evaporation.*

Capillary electrophoresis conditions
Background electrolyte (BGE): 175 µM borate buffer, pH 10.5.

Note: This solution must be prepared by titrating boric acid with sodium hydroxide. Use of the tetraborate salt will result in unacceptable operating current.

Voltage: 25 kV
Temperature: 30 ºC
Detection: 306 nm,
8 nm bandwidth,
no reference
Capillary wash procedure:
Between each run, flush the capillary for 5 to 7 minutes, with 1 N sodium hydroxide, water for 3 minutes, and BGE for 5 to 7 minutes.

Note: Failure to follow this wash procedure will result in migration time imprecision.

Anolyte (inlet) and catholyte (outlet) reservoirs must also be changed prior to each run using the automated buffer replenishment system.

All other experimental conditions are noted in the figure captions.

Results and discussion

An electropherogram illustrating the separation of a variety of carbohydrates from a 90-minute derivatization procedure is shown in figure 2. Several artifact peaks appear in the vicinity of the uronic acids. These are probably degradation products that can be eliminated by shortening the reaction time.

The loss of sensitivity is not substantial for aldoses since the reaction is nearly complete. For slow-to-react ketoses such as fructose, longer reaction times are best to improve sensitivity. In any event, the reaction time should be carefully controlled to optimize quantitative precision. Once the sample is cooled and diluted, the reaction is effectively quenched.

With the specified conditions, the borate-complexed derivatized sugars are anionic and electrophoretically migrate toward the positive electrode (anode). With a bare silica capillary, the electro-osmotic flow (EOF) is sufficiently high to sweep all separands toward the cathode. Under these conditions, the first eluting components are actually the least mobile.

The derivatizing reagent, ABEE is effectively neutral at the pH of the BGE. The excess reagent migrates along with the EOF and is well separated from all derivatized carbohydrate peaks. This important feature does not occur with most derivatizing reagents. Either removal of the reagent is necessary or the experimental conditions must be adjusted to allow a separation window to be found.

In addition, the UV spectra of the derivatives (determined on-line by

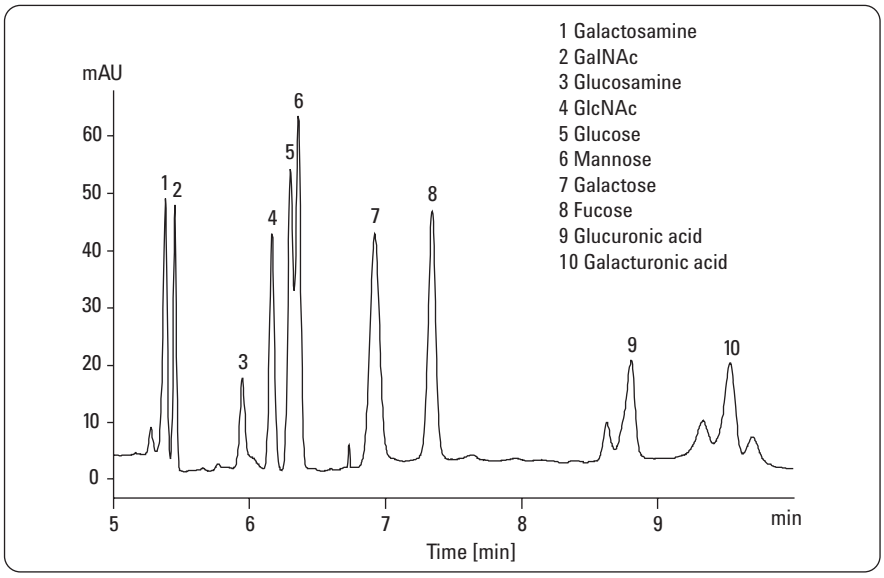

Figure 2
Separation of ABEE sugars in a 50-µg id x 56-cm capillary (BF 3).

Chromatographic conditions
Reaction time: 90 min
Injection: 150 mbar x s
Refer to Experimental section for other conditions

diode-array detection, data not shown) is shifted compared with the reagent alone. Monitoring at the optimal wavelength of the derivative helps reduce the signal from excess reagent.

The use of such a concentrated BGE for separating small molecules is quite unusual. Resolution continues to improve even at higher borate concentrations because the equilibrium is shifted toward complex formation. Joule heating is not problematic when titrated boric acid is used to prepare the BGE. Under typical conditions, the current reaches only 35 µA.

At higher borate concentrations, the EOF slows, giving lengthy separations. Adjustment of pH in the range of 9.5 to 11 can be employed to fine-tune the resolution, if required.

Impact of capillary length and diameter

Replicate runs on a 50-µm id x 72 cm BF 3 capillary are given in figure 3. Although the resolution between glucose-mannose was improved relative to figure 2, the run time increased to 24 minutes. As expected, employing a 25-µm id capillary improved the resolution, even with a capillary length of 56 cm. The use of the 25-µm capillary provided optimal resolution without a prolonged run time.

Normally, when using a 25-µm capillary, sensitivity is compromised. In this case, a BF 5 capillary increased the effective optical pathlength to 125 µm. Because of the unique band-narrowing effect of the bubble cell, combined with narrow optical slits, no extra-capillary band broadening occurred.

A series of 10 runs using the 25-µm capillary is shown in figure 4. The drifting baseline from 6 to 8 minutes is the

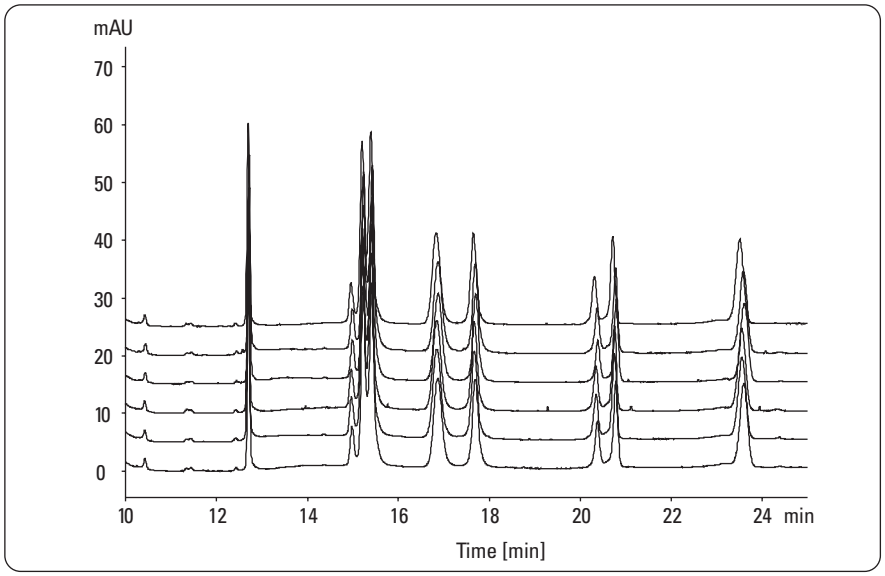

Figure 3
Replicate separations of ABEE sugars in a 50-µm id x 72-cm capillary (BF 3).

Chromatographic conditions
Reaction time: 15 min
Injection: 150 mbar x s
Refer to Experimental section for other conditions

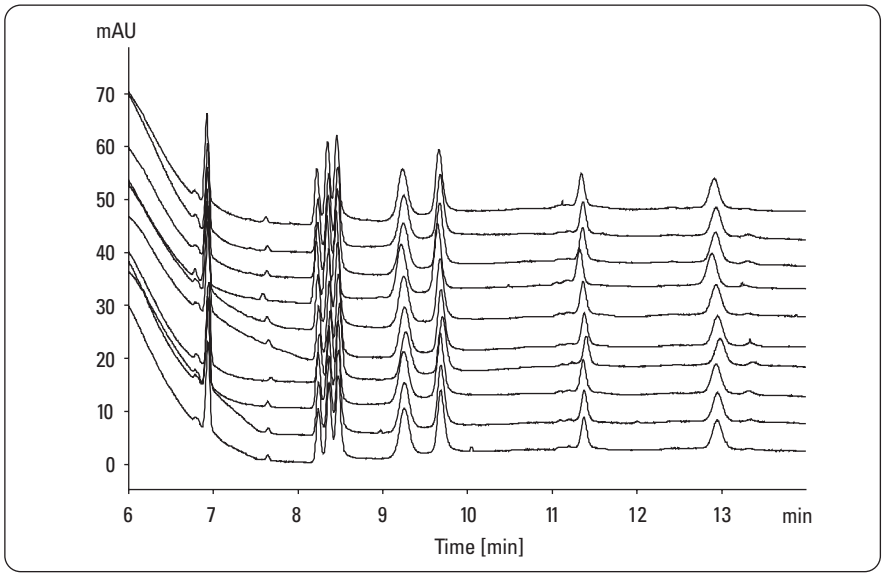

Figure 4
Replicate separations of ABEE sugars in a 50-µm id x 72-cm capillary (BF 3).

Chromatographic conditions
Reaction time: 15 min
Injection: 450 mbar x s
Refer to Experimental section for other conditions

tail of the reagent peak. This could be reduced, if necessary by lowering the injection size, increasing the capillary length or adjusting the reagent concentration.

Precision

When the capillary wash procedure was followed in conjunction with automated buffer replenishment, the relative standard deviation (RSD) of the migration time was very low. Table 2 contains data generated using both 50- and 25-µm id capillaries. These data indicate that precision is not dependent on the capillary diameter for the two internal diameters studied

Linear dynamic range

Calibration curves covering the range 200 to 2700 fmole for various sugars were measured using the 50-µm id x 56-cm capillary. One such curve is shown in figure 5 with the 95 % confidence limits bracketing the calibration curve.

Limit of detection (LOD)

The mass LOD was 40 fM glucose at a signal-to-noise ratio of 5 (data not shown). With a 10 nL injection, this produced a concentration LOD (CLOD) of 4 µM. A two- to three-fold larger injection was made without incurring substantial band broadening (data not shown). Under these conditions, the CLOD was further improved. The CLOD for ketoses was a factor of 20 higher but this was improved when longer reaction times were used.

Sugar	Average	SD	% RSD	Average	SD	% RSD
	25 µm x 56 cm			50 µm x 72 cm		
GlcNac	6.94	0.0064	0.09	12.70	0.0094	0.07
GalNac	8.24	0.011	0.13	14.97	0.013	0.08
Glucose	8.36	0.011	0.13	15.22	0.013	0.09
Mannose	8.47	0.011	0.13	15.41	0.014	0.09
Galactose	9.25	0.014	0.15	16.85	0.017	0.10
Fucose	9.68	0.016	0.17	17.68	0.019	0.11
Glucuronic acid	11.37	0.021	0.18	20.76	0.029	0.14
Galacturonic acid	12.94	0.026	0.20	23.57	0.036	0.15
	n =10			n = 6		

Table 2
Migration time precision.

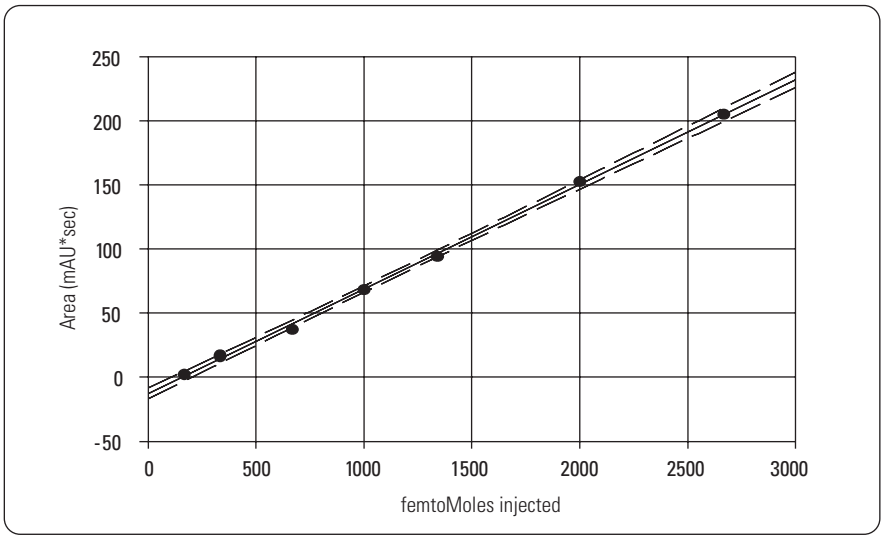

Figure 5
Calibration curve and confidence limits for mannose.

Chromatographic conditions
Reaction time: 15 min
Injection: 150 mbar x s
Refer to Experimental section for other conditions

Application

The utility of the method is shown for a separation of monosaccharides obtained from the acid hydrolysis of polysaccharides extracted from plant tissue.[10]

The extract was hydrolyzed with 6 M trifluoroacetic acid for 6 hours at 120 °C, then lyophilized to dryness and derivatized along with added 2-deoxy-D-ribose as an internal standard. The sugars identified in the electropherograms shown in figure 6 are consistent with the known carbohydrate content of the plant.

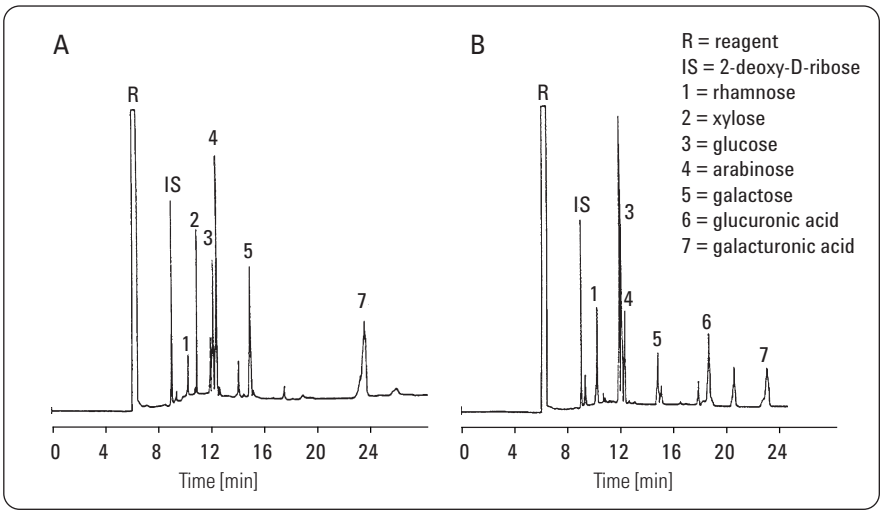

Figure 6*
Separation of the component ABEE derivatized monosaccharides in polysaccharides extracted from A) *Flos matricariae* and B) R*adix althaeae.*
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Key to abbreviations

ABEE Aminobenzoic acid ethyl ester
BF bubble factor
BGE background electrolyte
CLOD concentration limit of detection
CBE cyanoborohydride
CZE capillary zone electrophoresis
EOF electro-osmotic flow
RSD relative standard deviation

Chromatographic conditions

Capillary:	72 cm (50 cm) x 50 µg id bare silica
BGE:	200 mM borate, pH 10.5
Voltage:	25 k
Detection:	UV, 305 nm
Temperature:	30 °C

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