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Carbohydrate Research 339 (2004) 2037–2043
Carbohydrate RESEARCH

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# An optimized CZE method for analysis of mono- and oligomeric aldose mixtures

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Received 17 March 2004; accepted 10 June 2004
Available online 26 June 2004

**Abstract**—An optimized capillary electrophoresis (CZE) method to analyze complex mixtures of aldoses was developed. The approach allows simultaneous quantitative analysis of all four isomeric aldopentoses, eight aldohexoses, as well as xylo- and cello-oligosaccharides up to the tetraoses. UV tagging with 4-aminobenzoic acid ethyl ester (ABEE) in combination with reductive amination was used as pre-column derivatization. With optimum baseline separation and short run times, the method is very robust, and especially suited to follow reaction and isomerization kinetics of monosaccharides.


*Keywords*: Capillary electrophoresis; Aldose; 4-Aminobenzoic acid ethyl ester; Cellodextrins; Xylodextrins; Reductive amination

The separation of complex mono- and oligosaccharide mixtures is a challenging analytical task for numerous reasons. At first, the analytes show differences only in the configuration of the hydroxyl groups and possess therefore very similar separation properties. Furthermore, they generally lack UV-absorbing or fluorescent functions as well as readily ionizable charged groups. At a first glance, capillary electrophoresis (CE) may not be a very obvious choice for carbohydrates, but successful strategies have turned it into a widely used tool for mono- and oligosaccharide analysis. The introduction of charge into the analyte molecules, as a pre-requisite to CE separation, can easily be achieved by chelation of the carbohydrate with a suitable ion, such as borate, which is most commonly used for this purpose. Direct detection of the complexes at a low wavelength (i.e., <200 nm) or indirect detection by the addition of an absorbing species in the running buffer is then possible.

For most related analytical procedures, however, derivatization of the carbohydrates is required, especially since the introduction of a chromophoric moiety can substantially improve the sensitivity. Furthermore, a suitable tagging of the saccharide usually results in enhanced resolution and less matrix disturbances. Pre-column derivatization using reductive amination is a well-known technique for labeling reducing saccharides that works in highly polar solvent systems over a wide pH range.[1–4] A large number of reagents has been tested, among them 4-aminobenzoic acid ethyl ester (ABEE), which has been successfully applied to quantitative CZE–UV analysis of some commonly occurring aldopentoses and hexoses.[5–7] This particular reagent was first introduced for carbohydrate analysis by Wang et al.[8] and shows good properties regarding extinction coefficient, separation, stability, reaction, and availability.

A reaction scheme for the ABEE labeling of reducing sugars is shown in Figure 1. The reaction produces an acid-labile imine derivative intermediate (Schiff's base) in the first step, which in a second step is efficiently reduced to a stable secondary amine by sodium cyanoborohydride.

The aim in this work was to present an optimized, powerful, and robust analytical technique capable of simultaneously separating all aldopentose and

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doi:10.1016/j.carres.2004.06.003

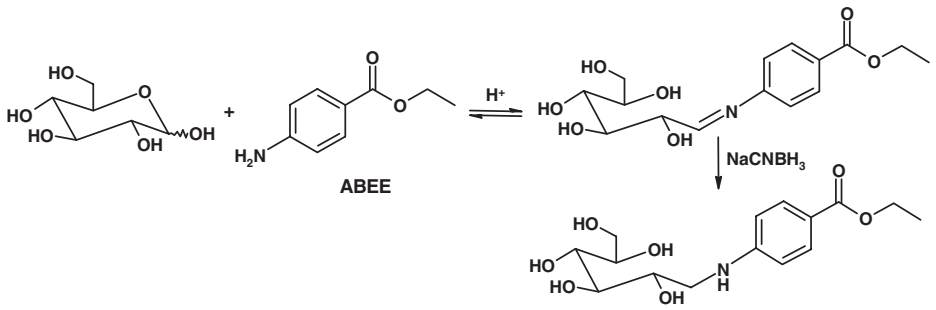


**Figure 1.** Derivatization of an aldose (D-glucose as example) with 4-aminobenzoic acid ethyl ester (ABEE).

aldohexose stereoisomers. A requirement was that the method should be well suited to follow and record the kinetics of isomerization reactions of monosaccharides, such as those occurring under alkaline conditions or in a melt of *N*-methylmorpholine-*N*-oxide (NMMO).[9]

## 1. Experimental

### 1.1. Chemicals

4-Aminobenzoic acid ethyl ester (ABEE) 98%, sodium cyanoborohydride 95%, boric acid 99.5+%, D-arabinose 99%, D-xylose 99+%, and D-glucose 99+% were obtained from Aldrich (Milwaukee, WI, USA). D-Lyxose 99+%, D-galactose 99+%, D-allose 99+%, D-altrose 99+%, D-talose 99+%, cellobiose 99+%, cellotriose 98+%, and cellotetraose ~95% were obtained from Fluka (Buchs, Switzerland). Xylobiose (estimated purity ~90%) was kindly provided as a gift. Xylotriose ~95% and xylotetraose 90+% were purchased from Megazyme (Sydney, Australia). D-Mannose 98+% was from E. Merck (Darmstadt, Germany). D-Ribose 99+%, D-gulose 95%, and L-idose 95% were supplied by Sigma (St. Louis, MO, USA). The water used was purified (>17 MΩ/cm) utilizing a HQ5 filter apparatus (REWA, Gladbeck, Germany).

### 1.2. Pre-column derivatization with 4-aminobenzoic acid ethyl ester (ABEE)

The procedure for reductive amination of saccharides suggested by Oefner and coworkers[5] was employed with some modifications. Temperature and time was chosen according to the initial work by Wang et al.[8] The precipitation step to remove the main part of excess non-reacted ABEE prior to analysis was performed as proposed by Dahlman et al.[6]

To a stock solution of ABEE (100 mg/mL) and acetic acid (100 mg/mL) in methanol, cyanoborohydride (10 mg/mL) was added immediately before mixing 1:1 (v/v) with an aqueous sample containing the reducing sugars in a maximum concentration of about 60–70 mM. Thus, the reagent excess was always at least tenfold. After 1 h at 80 °C the main part of the non-reacted ABEE was precipitated by adding 1–1.5 parts of an alkaline borate buffer (450 mM, pH 8.6) to the hot reaction mixture, which thereafter was vigorously vortex mixed for a few seconds. The precipitate was removed after cooling to room temperature and prior to analysis by a syringe membrane filter (13 mm 0.22 μm PVDF, Rotilabo®, Carl Roth GmbH, Karlsruhe, Germany).

### 1.3. Capillary electrophoresis conditions

A Hewlett–Packard $^{3D}$CE instrument equipped with a UV-DAD detector was employed (Agilent Technologies, Palo Alto, CA, USA). The operation of the CE system, data acquisition, and area integrations were performed using the software 3D-CE ChemStation for Windows NT 4.0 (© Agilent Technologies, Rev. A.08.03). The wavelength used for detection and quantification of the derivatized carbohydrates was 305 nm with a bandwidth of 30 nm. The detector response time was set to 0.1 s and the data collection rate was 20.8 Hz. A roll of fused-silica capillary having an internal diameter (ID) of 30 μm was supplied by Skandinaviska Genetec (Göteborg, Sweden). A 48.5 cm piece was cut using a CE(C) Column Cutter (Hewlett–Packard), and a 2–4 mm length detection window was created by removing the polyimide coating by burning, its center being located 8.5 cm from the outlet end. The capillary was fitted into the cassette and the temperature control was set to 20 °C. The capillary was initially flushed for 10 min with 0.1 M sodium hydroxide followed by running buffer (10 min). The running buffer was an aqueous solution of boric acid (450 mM) and sodium hydroxide (300 mM), pH 9.7. The washing procedure between the runs consisted of three flushing steps: 1.5 min with 0.1 M sodium hydroxide, 1 min with water and finally 3 min with running buffer. Positive pressure was used for sample injection into the capillary at the anodic end; commonly 30 mbar was applied for 5 s. In the positive polarity mode a constant current of 100 μA was applied immediately after the injection and was kept constant until the run was stopped. The resulting voltage was close to 28 kV.

For capillary comparisons, a pre-cut capillary with an internal diameter of 25 μm equipped with a so-called bubble cell detection window and a roll of 50 μm capillary were obtained from Agilent Technologies.

## 2. Results and discussion

### 2.1. Separation of aldopentose and hexose derivatives

For quantification of monosaccharides by selective, sensitive, and robust CE methods, pre-column derivatization is among the most popular approaches. In the present case, reductive amination with 4-aminobenzoic acid ethyl ester (ABEE) as the UV-active tag was chosen, which works in highly polar solvent systems over a wide pH range. The UV label affords stable secondary amines after reductive attachment to the carbohydrate.

An excess of UV label and reductant were used to guarantee optimal conversion into the labeled saccharide derivative. It has been stated that reductive amination also works for ketoses,[5] such as sorbose and fructose, but the derivatization reaction resulted in poor yields for ketoses (<2% of the conversion achieved for aldoses). Therefore, at least in our hands, the method was found to be unsuitable for quantitative CZE analysis of ketoses.

In procedures for separation of ABEE-derivatized carbohydrates, 200 mM borate buffer at pH 10.5 and standard 50 μm ID capillaries are generally recommended.[10] In complex monosaccharide mixtures, however, signal overlaps must be expected under such conditions. For example, the ABEE derivatives of the common carbohydrates glucose, mannose, and arabinose are difficult to resolve, as already addressed in the first work using the ABEE method for CZE separations.[5] By the use of high borate concentrations in the running electrolyte, a separation of glucose, mannose, and arabinose derivatives has been accomplished.[11,12,6]

Figure 2 shows a typical CZE separation of derivatized pentoses under the conditions described in the experimental part. The forwardly directed electroosmotic flow, EOF, drives the negatively charged pentose-ABEE borate complexes past the detection window, and the separation depends upon the oppositely directed electrophoretic mobility (counter-electroosmotic separation mode), which in turn is related to how well the particular carbohydrate derivative forms borate complexes.[13] In this particular separation lyxose showed the lowest electrophoretic mobility, and consequently migrated first. The derivatives of xylose, ribose, and arabinose followed, all well separated.

In Figure 3, a corresponding separation of all aldohexoses as their ABEE derivatives is shown. The migration order of the derivatives was gulose, talose, glucose, mannose, idose, allose, altrose, and galactose. The identity of all signals has been verified through spiking experiments. Nonreacted ABEE remaining after the precipitation had a migration time of about 3.3 min; its signal could be used as an EOF marker.

A simultaneous separation of the 12 aldopentose and hexose isomers is presented in Figure 4a. To our knowledge, this is the first report showing a single-run CE separation method for all aldopentoses and hexoses. The electrophoretic mobilities, efficiencies, and resolutions, including relative standard deviations (RSDs) for this particular separation, are given in Table 1. The efficiencies ($N$>10,000) and resolutions ($R_s$>1) obtained proved that the method was well suited for quantitative analysis even of very complex sugar mixtures. The

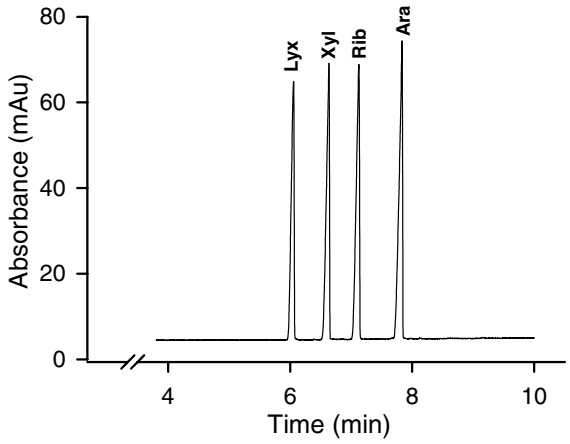


**Figure 2.** Separation of aldopentoses (1.5 mg/mL each) as their ABEE derivatives. Capillary: 48.5 cm × 30 μm fused silica; buffer: 450 mM borate, pH 9.7; applied current: 100 μA; cartridge temperature: 20 °C; detection: UV absorbance at 305 nm. Abbreviations are explained in Table 1.

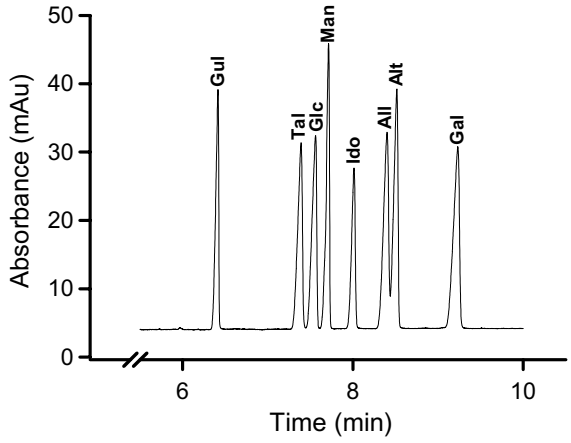


**Figure 3.** Separation of aldohexoses (0.75 mg/mL each) as their ABEE derivatives. CE conditions were the same as in Figure 2. Abbreviations are explained in Table 1.

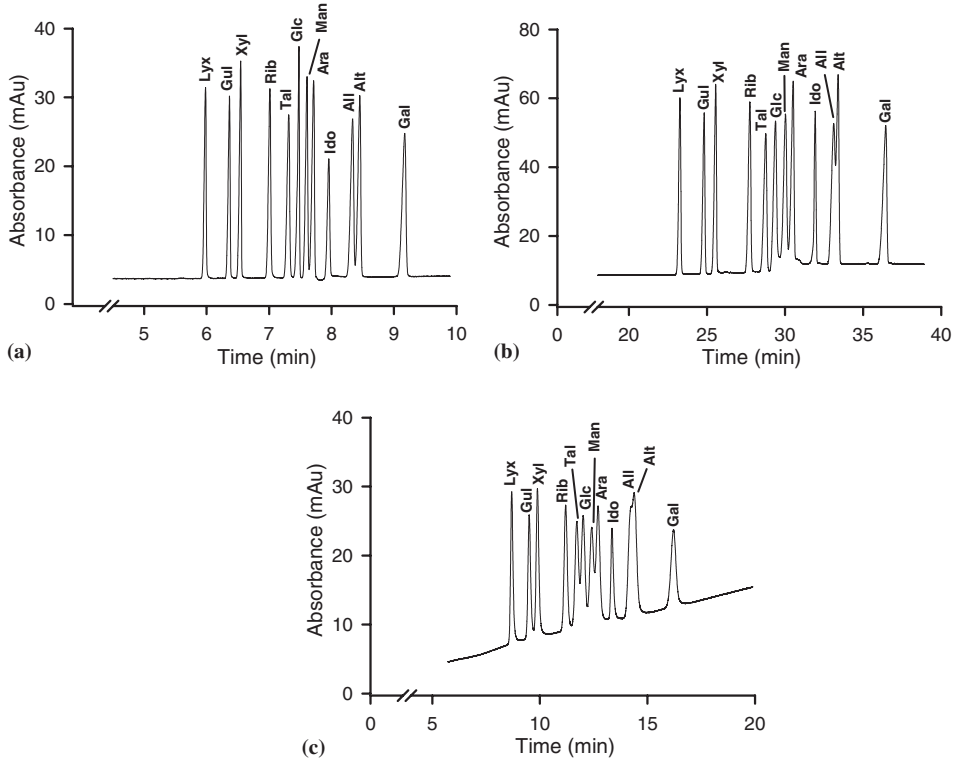


**Figure 4.** Comparison of separation of aldopentoses and hexoses (0.50 mg/mL each) as their ABEE derivatives (~1 mg/mL per derivative) in three different fused-silica capillaries under otherwise similar conditions (buffer: 450 mM borate, pH 9.7; applied current: 100 μA): (a) ID = 30 μm, $L = 48.5$ cm; (b) ID = 50 μm, $L = 64.5$ cm; (c) ID = 25 μm, extended light path capillary (bubble factor 5), $L = 48.5$. Abbreviations are explained in Table 1.

average mobility of the ABEE according to the EOF was $3.54 \times 10^{-4}$ cm$^2$/V s.

A narrow bore diameter was found to be essential for a successful separation: Figure 4b illustrates the difficulties regarding resolution and separation time using a standard-sized capillary with ID 50 μm. In the following, a capillary with an internal diameter of 25 μm equipped with a so-called bubble cell detection window resulting in an extended light path length was tested. The use of this capillary was supposed to improve the signal-to-noise ratio, and at the same time to replace the somewhat unusual 30 μm ID capillary without resolution

**Table 1.** Electrophoretic mobility ($\mu_e$) and resolution between adjacent peaks ($R_s$) of the ABEE derivatives of aldopentoses and hexoses (0.50 mg/mL of each saccharide) in a fused-silica capillary (ID = 30 μm, $L = 48.5$ cm) using a borate buffer (450 mM, pH 9.7)

| No | Carbohydrate | Abbrev. | Electrophoretic mobility,[a] $\mu_e$ (cm$^2$/V s) | | Efficiency,[b] $N$ | | Resolution,[c] $R_s$ | |
|---|---|---|---|---|---|---|---|---|
| | | | $\times 10^{-4}$ | RSD | $\times 10^5$ | RSD | | RSD |
| P1 | Lyxose | Lyx | −1.54 | 0.15 | 1.5 | 14 | 6.4 | 6.4 |
| H1 | Gulose | Gul | −1.67 | 0.18 | 1.8 | 7.7 | 3.2 | 3.5 |
| P2 | Xylose | Xyl | −1.72 | 0.19 | 1.8 | 8.9 | 8.4 | 4.1 |
| P3 | Ribose | Rib | −1.85 | 0.22 | 1.4 | 7.1 | 3.9 | 3.3 |
| H2 | Talose | Tal | −1.92 | 0.21 | 0.9 | 4.3 | 1.8 | 1.9 |
| H3 | Glucose | Glu | −1.96 | 0.20 | 1.3 | 6.6 | 2.1 | 3.5 |
| H4 | Mannose | Man | −1.99 | 0.19 | 1.0 | 7.3 | 1.0 | 3.1 |
| P4 | Arabinose | Ara | −2.01 | 0.22 | 2.1 | 5.3 | 3.8 | 6.1 |
| H5 | Idose | Ido | −2.06 | 0.14 | 1.6 | 5.7 | 4.9 | 3.3 |
| H6 | Allose | All | −2.13 | 0.16 | 1.1 | 7.0 | 1.2 | 2.9 |
| H7 | Altrose | Alt | −2.15 | 0.16 | 1.4 | 6.2 | 8.3 | 3.4 |
| H8 | Galactose | Gal | −2.27 | 0.12 | 1.5 | 14 | 6.4 | 6.4 |

Averages and relative standard deviations from 10 consecutive runs (injection pressure: 30 mbar for 5 s, applied current: 100 μA) are presented.
[a] $\mu_e = lLV^{-1}(t_m^{-1} - t_{EOF}^{-1})$, where $l$ = length to detector, $L$ = total length, $V$ = applied voltage, $t_m$ = migration time, $t_{EOF}$ = EOF time.
[b] $N = 5.54(tw_{1/2})^2$, where $w_{1/2}$ = peak width at half height.
[c] $R_s = 0.5N^{1/2}(\mu_2 - \mu_1)(\mu_2 + \mu_1)^{-1}$ for two adjacent peaks 1 and 2.[14]

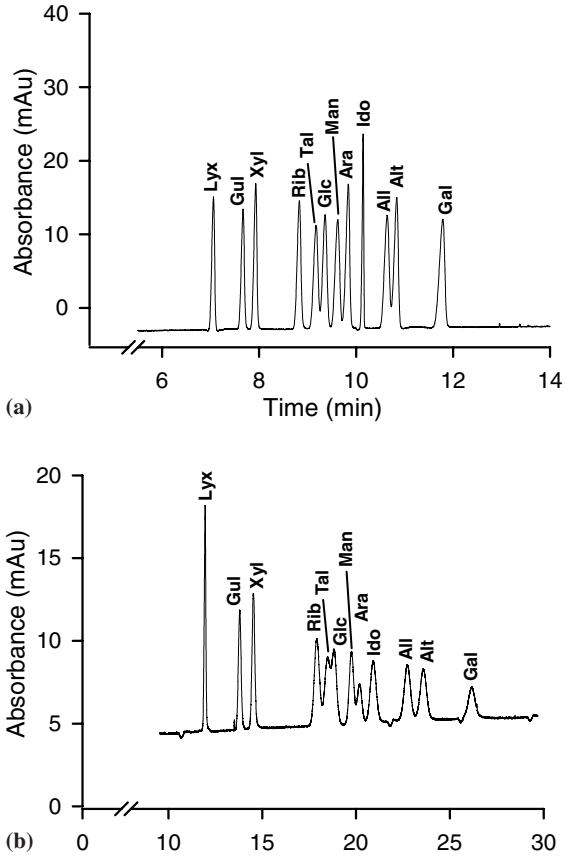


**Figure 5.** Separation of aldopentoses and hexoses as their ABEE derivatives in 450 mM borate buffer adjusted with 1 M sodium hydroxide to (a) pH 10.0 and (b) pH 11.6. Conditions were otherwise the same as in Figure 4a.

penalty. Unfortunately, also the bubble cell capillary did not give satisfactory results. Furthermore, it was troublesome to obtain a stable baseline (Fig. 4c). To summarize, the 30 μm capillary proved to be best suited for separations of the ABEE-aldose derivatives.

The influence of the pH of the running borate buffer was also investigated, and pH 9.7 was found to give sufficient peak resolutions. From the electropherograms in Figure 5 it can be seen that already an increase to pH 10 caused a slightly decreased peak resolution (cf. Fig. 4a). At pH 11.6, adjusted with 1 M sodium hydroxide, an even worse separation resulted, taking more than 25 min.

## 2.2. Separation of xylo- and cellooligosaccharides

Under conditions close to those presented here 6-aminoquinoline derivatives of xylooligomers up to xylohexaose were separated under 6 min by Rydlund and Dahlman.[11] A simultaneous separation of derivatized xylo- and cellooligosaccharides up to tetraoses with 4-aminobenzonitrile (ABN) as the labeling reagent was

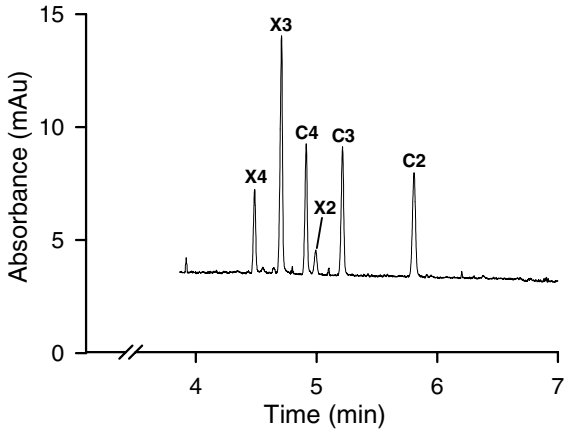


**Figure 6.** Separation of xylo- and cellooligosacharides as their ABEE derivatives. Capillary: 48.5 cm × 30 μm fused silica; buffer: 450 mM borate, pH 9.6; applied current: 100 μA. X2 = xylobiose, X3 = xylotriose, X4 = xylotetraose, C2 = cellobiose, C3 = cellotriose, and C4 = cellotetraose.

also carried out by Sartori et al.[15] The ABN derivatives were separated in coelectroosmotic mode, so that higher oligomers migrated at later times. Complete separation was not achieved—cellotetraose and xylobiose derivatives comigrated—and a run took 16 min.

Here, a complete separation of both xylo- and cellooligosaccharides up to tetraoses in about 6 min is presented. Using the same conditions (derivatization procedure and analytical parameters) as for the analysis of the aldoses discussed above, the cello- and xylooligomers were separated fast and efficiently (Fig. 6). The larger the oligomers, the shorter the migration time since the separation is carried out in counter-electroosmotic flow mode with positive polarity.

## 2.3. Simultaneous separation of aldoses and oligosaccharides

A separation of all 12 aldopentoses and hexoses together with xylo- and cellooligosaccharides up to tetraoses as their respective ABEE derivatives was achieved, taking less than 10 min for a single run, as shown in Figure 7. Only one overlap between the derivative of gulose and cellobiose was observed, otherwise the separation resolution of adjacent peaks was greater than 1. The overlap between cellobiose and gulose can be expected to cause less trouble than the overlap with xylose, which was a problem in the ABEE approach presented by Nguyen et al.[12] In total, 17 saccharide derivatives were separated. This number could easily be increased, especially since reducing monosaccharides having a carboxylic group (i.e., uronic acids) will form derivatives migrating later than the neutral saccharide derivatives due to the additional negative charge of the dissociated carboxylic group.

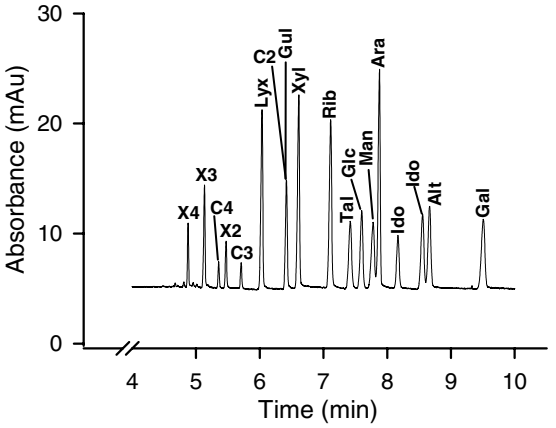


**Figure 7.** Separation of aldopentoses, aldohexoses, and oligosaccharides as their ABEE derivatives. Capillary: 48.5 cm × 30 μm fused silica; buffer: 450 mM borate, pH 9.7; applied current: 100 μA. Abbreviations are explained in Table 1 for the monosaccharides and in the caption of Figure 6 for the oligosaccharides.

**Table 2.** Calibration constants $y = k \times x$, with $x$ being the concentration of the saccharide in millimolar, and $y$ being the ratio between the corrected areas for the saccharide and the internal standard

| Saccharide | Calibration constants | |
|---|---|---|
| Int. std | Galactose, 9.96 mg/mL | Ribose, 9.94 mg/mL |
| Lyxose | 0.4619 | |
| Xylose | 0.4562 | |
| Ribose | 0.4605 | |
| Arabinose | 0.4605 | |
| Glucose | | 0.3455[a] |
| Mannose | | 0.3721 |
| Allose | | 0.3658 |
| Altrose | | 0.3722 |
| Galactose | | 0.3711 |

The concentrations were 0.1, 1, and 5 mM, with an additional data point (50 mM) for xylose and glucose. Three measurements on each concentration level were carried out. The regression coefficients, $r^2$, were >0.999 for the 0.1–5 mM range, and >0.993 for the 0.1–50 mM range.

[a] 0.3828 up to 5 mM.

### 2.4. Repeatability and quantitation

Run-to-run and day-to-day repeatability were established using a mixture of ABEE-derivatized hexoses and pentoses (1–12 in Table 1, electropherograms in Fig. 4). The run-to-run repeatability of migration time and peak areas was obtained from ten consecutive replicate runs. The evaluation of day-to-day repeatability was based on five replicate runs collected on six different days, the samples having been stored at room temperature, re-diluted, and re-filtered before analysis. The average relative standard deviations (RSDs) for run-to-run were 0.6% and 4.0% for migration time and peak areas, respectively, 3.9% for normalized relative peak areas. The migration times varied slightly more between different days, with the average RSD being 2.1%. The day-to-day average RSD value for normalized peak areas was 2.5%. Without peak normalization this figure would increase to 15%, caused by evaporation during the storage and subsequent re-dilution during sample preparation. The sample stability was excellent, which is proven by the fact that one month after the repeatability runs the test mixture—which was stored in a capped vial at room temperature—still showed normalized relative peak areas that did not differ more than 0.1% units from the average figures obtained in the day-to-day repeatability runs.

Although the run-to-run area repeatability was good, there are many advantages in using an internal standard for quantitation. With little effort variations of different kinds (injection volume, yield of derivatization reaction etc.) are corrected. A further advantage is that samples containing an internal standard are easier to handle thanks to being largely independent of dilution and evaporation effects. Our study of isomerization kinetics of monosaccharides in NMMO[9] required beside an efficient separation method also a robust quantification procedure for all isomeric aldopentoses and aldohexoses. For this purpose linear calibration curves in the concentration range of 0.1–5 mM were established—for xylose and glucose even up to 50 mM—corresponding to a linear range of about 3.5 orders of magnitude. Two different internal standards were used: ribose for the hexoses and galactose for the pentoses. A large linear range was observed, in agreement with previous reports.[6]

In Table 2, the constants $k$ of the linear calibration curves $y = k * x$ are given (zero was included as a point), where $x$ is the monosaccharide concentration, and $y$ the ratio of the monosaccharide and the internal standard area, corrected by division through migration time. After correction for the molecular weight difference between the internal standards used, the calibration factors obtained showed that the UV absorbance values of the different derivatized sugars were nearly equal. This is reasonable since the absorbance of the ABEE chromophore is supposed to be largely independent of the attached nonabsorbing carbohydrate. Absorbance was measured in the wavelength region of $305 \pm 30$ nm. Therefore, an average from the $k$-values of the other aldohexose derivatives was used for the rare aldohexoses (gulose, talose, and idose).

## 3. Conclusions

An efficient simultaneous CZE separation of ABEE derivatives of all aldopentoses, aldohexoses, and xylo- and cellooligosaccharides up to tetraoses in less than 10 min was demonstrated. The optimized method was found to be well suited to examine reaction mixtures and to record isomerization kinetics of aldoses. The average

run-to-run and day-to-day repeatability of normalized peak areas were 1.9% and 2.5%, respectively. Linear calibration curves were established in the range of 0.1–5 mM (up to 50 mM for glucose and xylose). The UV responses were found to be very similar for the different ABEE-saccharide derivatives.

## Acknowledgements

The authors are grateful to Prof. Dr. Dietmar Haltrich, Department of Food Sciences and Food Technology at the University of Natural Resources and Applied Life Sciences for the donation of rare monosaccharides. They also thank the Austrian Christian-Doppler-Research Society, the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (Project P-14687, 'Chemistry of amine oxides'), and the Lenzing AG, Austria, for financial support.

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