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Ultraviolet Detection of Monosaccharides: Multiple Wavelength Strategy to Evaluate Results after Capillary Zone Electrophoretic Separation

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Efficient overall processing of wood biomass still lacks suitable concepts for analytical strategies. The traditional strategy is based on compound-targeted analytical tools, which, however, tend to expand the range of required methods for each unit process and restrict the amount of results gained from process monitoring. Especially, development of heterogeneous bioprocess streams would benefit from expanding the range of results gained from applied analytical methods. Therefore, this study was executed in order to demonstrate a novel strategy for the analytical methods to meet the requirements of suitable and efficient process monitoring. The advantages of a novel strategy for interpretation of chemical analysis results by exploiting capillary zone electrophoresis (CZE) with ultraviolet (UV) detection at two complementary wavelengths are presented. Electropherograms of wood-based bioprocess samples were studied as examples with a heterogeneous composition of carbohydrates. Results show that a fairly simple CZE method has the advantage of providing a thorough characterisation of the sample profiles in the preliminary identification of unknown compounds in a heterogeneous wood-based sample.

Keywords: Capillary electrophoresis; UV detection; Monosaccharides; Process monitoring; Multiple wavelengths

1. INTRODUCTION

Biorefining remains a challenge from the analytical point of view. Biorefining is the combination of a large variety of unit processes [1, 2, 3], and it necessitates a large range of process streams to be monitored and controlled. In such a process environment, gaining adequate information on the various parameters or compounds in the process with as simple input to the analytical method as

possible is essential. Process monitoring aims to evaluate only the most crucial parameters of the process. As the biorefining industry continuously seeks new products and intensified processes that demand low energy [2, 4], analytical methods suitable for the overall profiling and monitoring of heterogeneous samples are needed.

Wood material, in general, is the most utilised raw material of Scandinavian biomass in industrial scale, because it is naturally abundant in scarcely populated Nordic areas. Lignocellulosic wood raw materials are heterogeneous because of their natural variance in chemical composition [5]. This property presents both a challenge and an opportunity in the further improvement of industrial utilisation of the material. The complexity of the chemical composition of biomass raw material or plant material sets special requirements for process monitoring and development, especially in the case of enzymatic or biological processes. In Scandinavia, the most utilised species are pine, spruce and birch. As pine and spruce are both softwoods, their chemical composition is similar. By contrast, birch is a hardwood and is different from the softwood species as a raw material.

Wood contains hemicellulose composed of monosaccharides. As a raw material, pine differs from spruce by the concentration of carbohydrates [5, 6]. Monosaccharides are the desired product of many biorefinery processes, as they are platform chemicals for the production of many renewable products. The most common non-structural monosaccharides of wood raw material are glucose, mannose, galactose, arabinose and xylose (Table 1). They are found as monomers of hemicellulose polymers and as glycosides in the extractives of wood [5]. The molecular structures of common monosaccharides are similar, with some detailed differences affecting the separation by capillary electrophoresis.

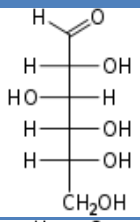
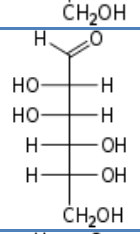
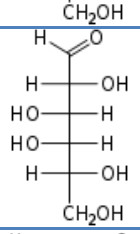
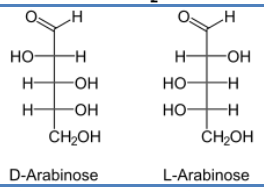
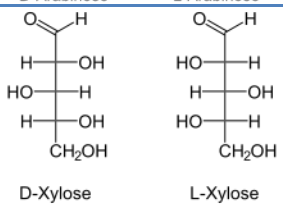
Besides monosaccharides, also other types of carbohydrates can be found through biorefining. In a recent scientific publication by Kemppainen et al. [7], industrially separated spruce bark was found to contain a high amount of non-cellulosic glucose along with tannin and white wood. The researchers concluded that, to develop a good method to remove sugars from the water extracts of spruce bark, the form of the sugars as they exist needs to be well understood. [7]

Capillary zone electrophoresis (CZE) is an analysis method with many applications for determining carbohydrates from aqueous samples. However, CZE methods typically require time-consuming derivatisation for the compounds to be detected by ultraviolet (UV) light, which leads to partial destruction of the original structures of the targeted compounds [8]. The advantage of CZE is its ability to analyse samples with heterogeneous matrices both qualitatively and quantitatively within a reasonable time scale. Concerning the CZE, UV detection is the most inexpensive option in practice [9].

A deeper understanding of the principles of UV detection helps in evaluating the disturbances from the resultant electropherogram. Recently, several research groups have tried to explain the chemistry behind the UV detection of carbohydrates, as carbohydrates commonly lack chromophore groups. Rovio et al. [10] demonstrated the ability of CE to separate monosaccharides from fruit juices in a strong alkaline background electrolyte (BGE). The high alkaline pH of the BGE enables carbohydrates to be detected by UV without derivatisation, although the actual mechanism of the formation of a UV visible structure is still under debate. Sarazin et al. [11] suggested that the detection is based on the photo-oxidation reaction of carbohydrates and polyols. Detected chromophoric

structures should be either malonaldehydes or related compounds instead of enediolates. After studying the pH of the BGE and the residence time of the compounds in the detection window, Sarazin et al. [11] found that the detection is based on the photo-oxidation reactions of carbohydrates in high pH.

Table 1. Structures, molar masses and pKa values of common monosaccharides [10] found in wood process streams.

Monosaccharide	Structure	Molar mass, g/mol	pKa
Glucose		180.16	12.35
Mannose		180.16	12.08
Galactose		180.16	12.39
Arabinose	 D-Arabinose L-Arabinose	150.13	12.34
Xylose	 D-Xylose L-Xylose	150.13	12.15

Oliver et al. [12] studied the determination of monosaccharides in plant fibres in complex mixtures through CE and high-performance liquid chromatography (HPLC). They concluded that CE could be used to determine 22% more carbohydrates than HPLC. The comparison between CE results and nuclear magnetic resonance spectroscopy results shows that the alkaline degradation suggested by Rovio et al. [10] is too slow to explain the detection of neutral carbohydrates of the method at hand [12].

Sarazin et al. [13] found that the most influential factors affecting the separation are temperature, BGE conductivity and sodium hydroxide (NaOH) concentration. Mala et al. [14] studied

the electromigration effects of carbon dioxide (CO_2) in high pH BGE. Sensitivity of the system to several types of disturbances in the detection was found to increase with increasing pH [14]. The high alkaline pH of the method emphasises the control of ambient conditions in which the equipment needs to work. When the different electropherograms from different wavelengths are compared, eliminating the disturbances from the compound peaks is possible. Nevertheless, the disturbances should primarily be minimised beforehand by regulating ambient conditions.

This work was initiated by the fact that process monitoring does not always require detailed quantifications of concentrations, contrary to the results in a recent review by Alhusban et al. [4]. Menon and Rao [15] pointed out the importance of characterisation and understanding of a typical biorefinery unit process, i.e. enzymatic hydrolysis, to process development. The aim of profiling is to characterize a selected sample within a single analytical run. Methods for the overall monitoring of process streams become especially important when the chemical composition of the raw material itself also varies.

The present study aims to provide a novel approach to the utilisation of case-specific wavelength combinations in the identification of monosaccharides in aqueous process streams. The detection of monosaccharides is discussed by comparing them with the detection of a disaccharide, i.e., sucrose. We present sample characteristics usually left out from the interpretation of analytical results of CZE electropherograms. The traditional approach of gaining analytical results by targeting individual carbohydrates from process samples occasionally leaves out essential information. Further interpretation of compound groups will add value to process monitoring by explaining the factors influencing the process or by predicting changes in the process streams.

This paper contributes to the field of process analytics by evaluating the combination of wavelengths to interpret the carbohydrate profiles of extraction samples. Selecting the suitable combination of wavelengths is the foundation of identifying monosaccharides from process samples. This investigation presents the possibilities of analysing process streams based on the requirements of the process control, instead of concentrating on the best abilities of the analytical method.

2. EXPERIMENTAL

2.1 Chemical and sample preparation

Glucose was purchased from Fluka (Buchs, Switzerland), and sucrose was purchased from BDH (Poole, England). NaOH and disodium phosphate ($Na_2HPO_4 \cdot 2\ H_2O$) were from Sigma (St. Louis, MO, USA). All chemicals used were of analytical grade. Water was obtained from an Elga purification system (Centra-R 60/120, Veolia Water). Saccharose and glucose in purified water were used as reference to confirm the detection of carbohydrates in the reference solution without possible matrix effects.

Samples were prepared in a laboratory scale by aqueous batch extraction of ground wood raw material. The applied raw material was the stem wood of pine species. Different layers of the log

cross-section were separated and then stored in the freezer fresh from felling. The applied extraction liquid was deionised water from the water purification system. No additional chemicals were used.

2.2 CZE procedure

Reference solutions and hydrolysed wood samples were analysed with an HP 3D CE system (Agilent). The inner diameter of the capillary was 50 μm, and the total length was 70 cm (61.5 cm effective length). The BGE solution consisted of 130 mM NaOH and 36 mM sodium hydrogen phosphate ($Na_2HPO_4 \cdot 2\ H_2O$). The pH of the electrolyte solution was adjusted to 12.6 with NaOH.

Prior to analyses, the capillary was conditioned daily with 0.1 M NaOH for 20 min, purified water for 20 min and the BGE for 5 min. Conditioning between analytical runs was performed with the BGE for 8 min. Samples were injected by pressure at 34.5 mbar for 8 s, and electropherograms were detected at a wavelength of 210 nm and 270 nm by a photodiode array detector. The separation voltage was 17 kV with positive polarity. Separation was conducted with air flow regulation at 25 °C. The BGE and all samples were filtered before analysis (Acrodisc PSF, GHP 0.45 μm, Pall).

2.3 Strategy of adding value to results

Real samples of pine wood were prepared by aqueous extractions to gain examples of electropherograms with several separated peaks at both selected wavelengths. To evaluate the potential of the alternative or complementary analysis strategy, the following five steps were applied:

1. Measurement of the electropherograms at the primary and secondary wavelengths
2. Grouping the detected peaks based on the carbohydrate type at the primary wavelength
3. Preliminary characterisation of the grouped peaks based on migration order
4. Comparison of the absorbance at the primary wavelength with the absorbance at the secondary wavelengths
5. Identification of the targeted peaks based on previous steps

Evaluation of the electropherograms was conducted without the commonly applied normalisation calculations of migration time or peak area [9, 16] to reflect the sample results in their original form, considering the practical aspects of bioprocesses.

3. RESULTS AND DISCUSSION

The two detection wavelengths selected for this study were 270 nm and 210 nm. The first wavelength was used previously by other research groups. Differences in the detected peaks of the extraction samples were compared with the electropherograms recorded at the two wavelengths.

3.1 Identification based on migration time

Figure 1 presents an example of an electropherogram of a wood-based sample. Two peaks from the sample electropherogram were identified after analysing the reference solutions, and additional peaks were partially identified by migration times. Sucrose (1) was identified at 12.5 min, and the first peak from the group of five (14.5 min to 15.5 min) was identified as glucose (2) with reference solutions. Confirmation of the identification was done by comparing the 210 nm electropherograms of both reference and sample solutions. After the preliminary analysis of glucose, the five peaks in the 270 nm electropherogram were selected for closer evaluation (Figure 1).

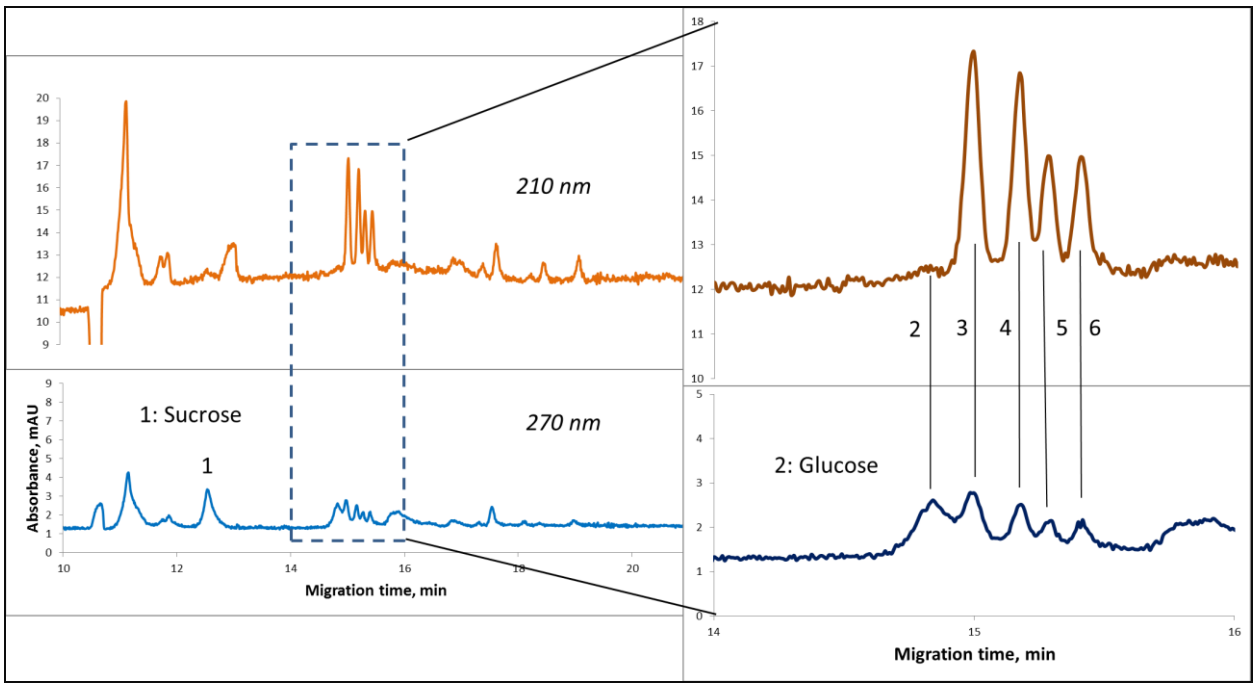


Figure 1. Carbohydrate profile of water extraction of pine wood detected at 210 nm and 270 nm.

Sucrose (1) had a barely visible peak at 210 nm (Figure 1), followed by a peak with stronger absorbance invisible at 270 nm. This peak was detected in the same size and shape in all analysed samples, including reference solutions and blanks, and was thus identified as one of the system peaks.

In Figure 1, the four following peaks (3–6) near glucose were estimated to result from other monosaccharides and roughly formed pairs of 3–4 and 5–6 based on the peak size. Reference solution of glucose showed a barely detectable absorbance at 210 nm, confirming the identification of glucose from the following four peaks. Compared with that at 270 nm, the four peaks gave a significantly stronger absorbance in the 210 nm electropherogram, and each peak was sharp. The five overlapping peaks were well separated as a group from the peaks migrating before or later in the electropherogram (270 nm). Quantifying the concentrations of monosaccharides is not included in this scientific paper, but the peaks could be better separated after further optimisation.

According to our results, glucose migrates before galactose. This result differs from those of other research groups [10, 11]. Possible reasons for this difference are under investigation, but they may originate from the nuances of e.g. the laboratory or method conditions. However, the authors consider the confirmation of the identification based on the complementary wavelengths to be as accurate, because the absorbances of the selected compounds in the sample were compared to the absorbances of the reference compounds.

3.2 Comparison of electropherogram absorbance with UV spectra

UV spectra of the following four peaks were compared to identify the compounds. The UV spectra of two peaks were selected for further investigation with a comparison to an additional carbohydrate, xylose (Figure 2). Special emphasis was given to the absorbance of the two selected wavelengths, i.e., 210 nm and 270 nm. The UV spectrum of glucose confirms that the absorbance is near maximum at 270 nm and significantly low at 210 nm. UV spectrum of sucrose shows a slightly greater difference between the two wavelengths compared with that of glucose: maximum absorbance is slightly higher and the minimum absorbance is slightly lower. UV spectrum of xylose differs from the two compounds discussed earlier. The maximum of the UV spectrum of xylose is at 210 nm, and its absorbance at 270 nm is distinctively lower than that of glucose and sucrose.

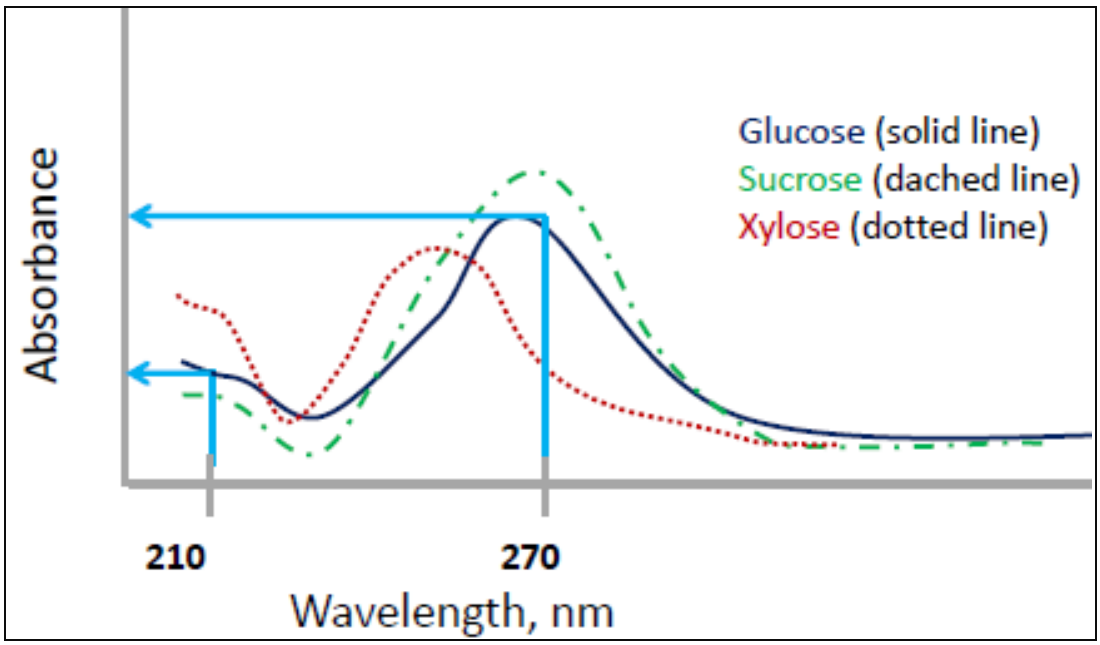


Figure 2. Principal figures of the UV spectra of sucrose [11], glucose and xylose [12].

The comparison between the two wavelengths (Figure 2) confirms the identification of a sucrose peak in the sample profile (Figure 1) as the absorbances are in accordance with those in the literature. According to the measured UV absorbance in Sarazin et al. [11], the maximum absorbance of sucrose is at 260 nm to 270 nm. At 210 nm, the absorbance is barely visible. The UV spectra of

glucose and xylose [12] confirm the similarities and differences in the absorbance of these two monosaccharides. Maximum absorbance of glucose is detected at 260 nm to 270 nm and that of xylose is at 245 nm to 255 nm. At 270 nm, the absorbance of xylose is only half of that of glucose.

The principal figures of UV absorbance (Figure 2) were interpreted to form absorbance ratios of the two detection wavelengths (270 nm/210 nm). The absorbance ratios and optimal detection wavelengths for each compound are presented in Table 2. The absorbance ratio of glucose (Table 2) is near the ratios calculated from the electropherograms of sequential repetition runs (Figure 3). In the case of xylose, the absorbance ratio indicates the peak to be larger at 210 nm than at 270 nm. Moreover, the optimum detection wavelengths of xylose at 245 nm to 255 nm confirm the selection of comparative wavelengths for each compound. The table shows that 270 nm is in the optimal range of detecting glucose and sucrose. However, xylose would be more suitable to be recorded at 250 nm because stronger absorbance usually leads to a more accurate determination of concentration.

In table 2, higher values indicate for the clarity of the identification, and thus, includes the possibility to be utilized also in the studies of separation and detection specificity in real process samples.

Table 2. Calculated ratios of absorbances (270 nm/210 nm) and optimal detection wavelengths of glucose, sucrose and xylose obtained from the UV spectra in Fig. 2.

Compound	Absorbance ratio (270 nm/210 nm)	Optimal detection wavelengths
Glucose	2.4	260 …270 nm
Sucrose	3.5	265 …275 nm
Xylose	0.75	245 …255 nm

As shown in Figure 3, the individual values of the absorbance ratios may vary between repeated analytical runs of glucose. This result is the consequence of having small peak areas at both wavelengths, leading to a deviation in the manual integration. Especially at low concentrations, the detected peak at 210 nm is nearly a detectable peak area and thus adds uncertainty to the results. Comparing the electropherograms of at least two different UV wavelengths can also be used to minimise the method-based uncertainty of the results [17], which is particularly important in regularly monitored processes of heterogeneous sample streams.

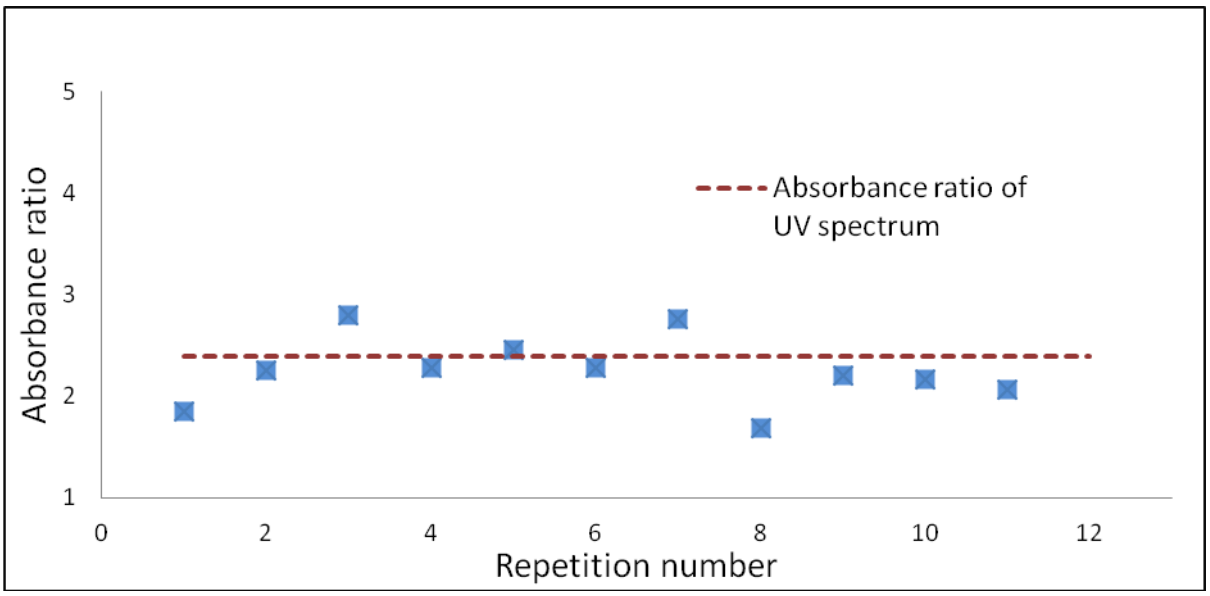


Figure 3. Calculated absorbance ratios of the glucose peak during repeated analyses of a selected concentration of reference solution (60 ppm). The dashed line represents the calculated value of absorbance ratio from the UV spectrum in Fig. 2.

3.3 Identification based on compound separation

According to the principle of separation in CZE, migration time is affected by the molar mass and the charge in the specific compound after applying high voltage in the BGE. Carbohydrates with the same molar mass separate from each other when they become charged according to their structural differences.

Similarities in the migration times indicate that peaks 3–6 (Figure 1) are monosaccharides, which are similar to glucose by their chemical structure and quality. Based on the fact that the electrophoretic separation is visible, the structures of the compounds include some distinctions from each other. The greatest difference is in the area, i.e., absorbance, of the peaks at the two wavelengths. The difference in the peak area between the first (2) and the second (3) peak indicates that their chemical structures differ by their ability to absorb UV light.

The detection and identification of glucose and saccharose were confirmed by reference solutions. The following peaks would be galactose, mannose, arabinose and xylose, as they are the most common unit monosaccharides in the wood material. Galactose and mannose are the most similar monosaccharides with glucose. Galactose and glucose have the same pKa value, which indicates similar migration time and possible overlapping of peaks. In the BGE with pH of above 12, the first compounds to migrate to the detection window are the ones with the largest pKa values and the lowest molar masses. The higher the molar mass, the slower the compound migrates. However, the larger compounds may also have more structural sites that can be charged by the electric field. In this electropherogram, the larger compounds reached the detection window earlier, and confirmation was based on the migration studies on the analyses of reference compounds. The other detected peaks in

the sample electropherogram are also from water-soluble monosaccharides that originate from hemicelluloses.

3.4 Practical issues

Changes in ambient conditions were found to affect the performance of the equipment. These changes are important in developing analytical methods for process monitoring at a site. Identifying the changes in the surroundings that can affect the CE equipment is critical if it is placed in the process environment.

In other studies, the detection signal of glucose has been noted to change as the compound band passes the detection window. [12, 13] Change in peak size among repetitions most likely indicates the alteration of glucose by the photochemical reaction. Oxygen or CO_2 can also affect peak size.

According to Oliver et al. [12], the enediolate formation suggested by Rovio et al. [10] requires a much longer reaction time. Moreover, alkaline degradation is not possible in these conditions because the reaction time is longer than the duration of the analysis [12]. Oliver et al. [12] also concluded the possible effects of oxygen in the detection.

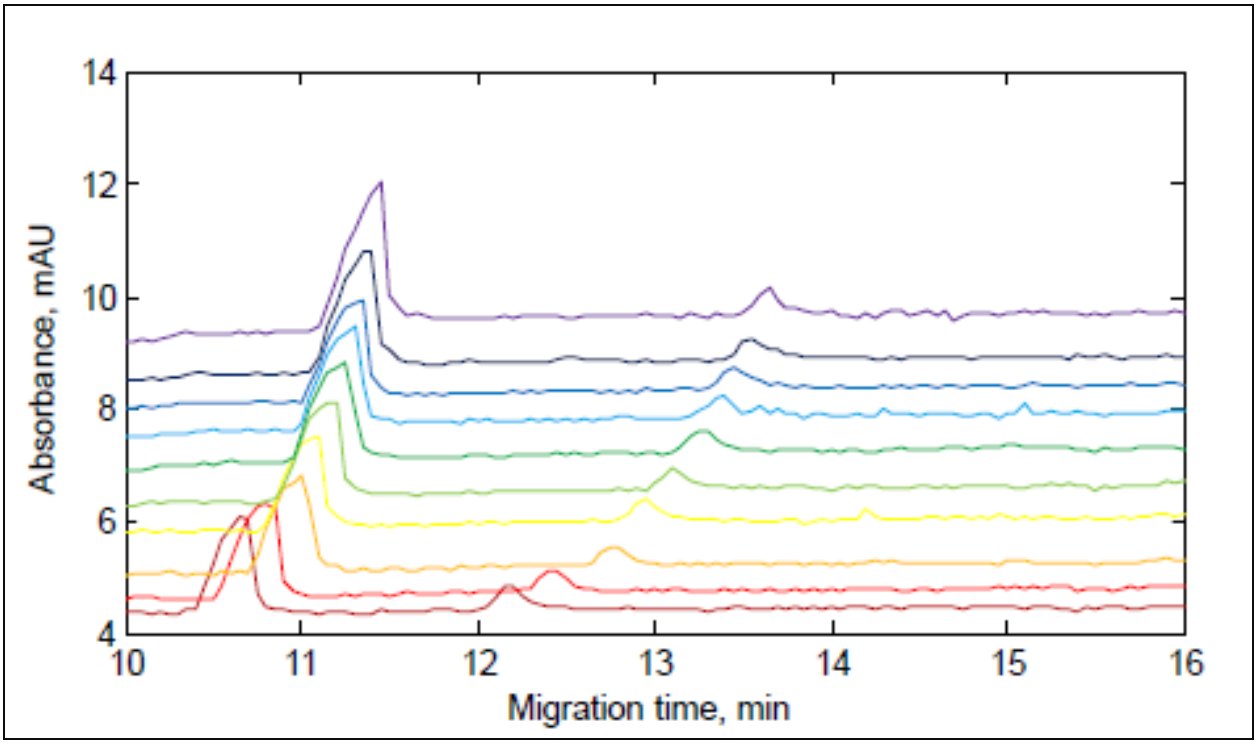


Figure 4. Examples of 10 electropherograms of sequential analytical runs. Peaks in the figure are system peak and sucrose.

Mala et al. [14] studied the effects of CO_2 on high pH CE. As CO_2 can increase the amount of interferences in the electropherogram, it may also affect the intensity of the detected peaks. The peaks of interferences are usually of equal absorbance in all detection wavelengths. CO_2 has been found to result in additional peaks and valleys in the electropherogram. These additional peaks are especially

important to consider when analysing complex matrices. CO_2 can be absorbed from air or be present in the BGE [14]. In the present study, the effects of carbonates were as expected because of the original composition of the BGE.

Interferences originating from practical issues are addressed in our recent paper [17], e.g., systematically drifting migration times (Figure 4). The peak shape and baseline issues were handled in previous studies [17]. However, these interferences in electropherograms can be minimised by several practical and mathematical strategies [16, 18, 19]. The aim of minimizing the effect of the systematic drift on the resulting electropherograms can be achieved by data normalization, or it can be acknowledged visually, in the case of sequential process monitoring.

4. CONCLUSIONS

Our results prove that by applying UV detection at multiple wavelengths, the carbohydrate identification of monosaccharides can be conducted even without standard addition. In sequential analysis run, the data normalisation step can be left out; i.e., calculations to adjust the migration times are unnecessary. The absorbance ratios calculated from repeated injections correlate well with the UV spectral absorbances from the literature, thus, confirming the hypothesis of electropherogram profiling. Additional advantage is gained by fixing the primary and secondary wavelengths according to the characteristic UV spectrum of selected compounds, and, thus, storing of the total UV spectrum can be avoided. This novel strategy shows that the absorbance ratios of complementary wavelengths can be utilized in preliminary identification of interferences as well as compounds. The difference in identifications of glucose and galactose based on our results, compared to the several previous studies, requires further investigation, in order to evaluate e.g. the possible effects of surrounding conditions. Moreover, this approach can be automated because the changes may be predicted and identified without adding reference solutions. This aspect is especially important in monitoring changes in compound profiles and concentration rates. The appearance of new compounds can be a sign of critical changes in the process; thus, these new compounds require fast identification. Based on our results, the systematic change in the peak migration time depends also on the time elapsed from sample injection. One of the applications benefiting from this type of analytical approach is the monitoring of aqueous streams of carbohydrate-related biorefinery processes.

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