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CAPILLARY ELECTROPHORESIS: APPLICABILITY AND METHOD VALIDATION FOR BIOREFINERY ANALYTICS

Thesis for the degree of Doctor of Science (Technology) to be presented with due permission for public examination and criticism in the Auditorium 1381 at Lappeenranta University of Technology, Lappeenranta, Finland on the 18th September, 2015, at noon.

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Abstract

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Wood-based bioprocesses present one of the fields of interest with the most potential in the circular economy. Expanding the use of wood raw material in sustainable industrial processes is acknowledged on both a global and a regional scale.

This thesis concerns the application of a capillary zone electrophoresis (CZE) method with the aim of monitoring wood-based bioprocesses. The range of detectable carbohydrate compounds is expanded to furfural and polydatin in aquatic matrices. The experimental portion has been conducted on a laboratory scale with samples imitating process samples.

This thesis presents a novel strategy for the uncertainty evaluation via in-house validation. The focus of the work is on the uncertainty factors of the CZE method. The CZE equipment is sensitive to ambient conditions. Therefore, a proper validation is essential for robust application.

This thesis introduces a tool for process monitoring of modern bioprocesses. As a result, it is concluded that the applied CZE method provides additional results to the analysed samples and that the profiling approach is suitable for detecting changes in process samples.

The CZE method shows significant potential in process monitoring because of the capability of simultaneously detecting carbohydrate-related compound clusters. The clusters can be used as summary terms, indicating process variation and drift.

Keywords: Capillary zone electrophoresis, Analytical profiling, UV detection, Biorefining, Softwood, Carbohydrates, Polydatin

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August 25th 2015
Lappeenranta

Laura Kajanen

To
The Winds
That lift my wings
And
Light my fire

Contents

List of publications

This thesis is based on the following papers. The rights have been granted by publishers to include the papers in dissertation.

I. Kaijanen, L., Reinikainen, S.-P., Pietarinen, S., Sirèn, H., and Jernström, E. (2015). Detection of carbohydrate-related compounds in aqueous samples using a capillary electrophoretic profiling method. *Separation Science and Technology*, 50, pp. 911-919. DOI:10.1080/01496395.2014.966202.

II. Kaijanen, L., Pietarinen, S., Sirèn, H., Jernström, E., and Reinikainen, S.-P. (2014). In-house validation of a novel capillary electrophoretic method: Uncertainty factors. *International Journal of Electrochemical Science*, 9, pp. 5438-5453.

III. Kaijanen, L., Metsämuuronen, S., Reinikainen, S.-P., and Jernström, E. (2015). Profiling of water-soluble carbohydrates in pine and spruce extracts by capillary zone electrophoresis with direct UV-detection. *Wood Science and Technology*, 49, pp. 795-809. DOI 10.1007/s00226-015-0729-5.

IV. Kaijanen, L., Paakkunainen, M., Pietarinen, S., Jernström, E., and Reinikainen, S.-P. (2015). Ultraviolet detection of monosaccharides: Multiple wavelength strategy to evaluate results after capillary zone electrophoretic separation. *International Journal of Electrochemical Science*, 10, pp. 2950-2961.

Author's contribution

Paper I: Laura Kaijanen is the principle writer and investigator. Kaijanen conducted and supervised the experiments and run the analyses.

Paper II: Laura Kaijanen is the principle writer. Kaijanen supervised the experiments and the analyses.

Paper III: Laura Kaijanen is the principle writer and investigator. Kaijanen conducted and supervised the experiments and run the analyses.

Paper IV: Laura Kaijanen is the principle writer and investigator. Kaijanen conducted the experiments and run the analyses.

Nomenclature

Latin alphabet

R^2	correlation coefficient
u_c	total combined uncertainty
u_i	standard uncertainty factor for i^{th} error source, i=1,2,…,n

Abbreviations

BGE	background electrolyte
CE	capillary electrophoresis
CZE	capillary zone electrophoresis
DAD	diode array detection
FIA	flow injection analyses
GC	gas chromatography
HPAEC	high performance anion-exchange chromatography
HPLC	high performance liquid chromatography
IR	infrared
LL	lower limit
LOD	limit of detection
LOQ	limit of quantitation
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometer
NMR	nuclear magnetic resonance
RSD	relative standard deviation
S/N	signal-to-noise ratio
TOF	time-of-flight
UL	upper limit
UV	ultraviolet

1 Introduction

1.1 Background

Fulfilling the global need for energy and chemicals has resulted in a quest for alternatives to fossil resources. Biomass-based economy has established a significant role on the global scale, providing a constantly increasing amount of alternatives to oil-based products and energy (Amidon & Liu 2009). In Finland, the traditional forest sector forms the basis for the bio-industry by providing knowledge of the raw materials and unit processes. The bio-industry is currently one of the most promising fields in developing new businesses and products for export, therefore creating jobs and improving well-being, especially through a circular economy (Sitra 2014).

The bio-industry is based on various types of processes, which utilize multiple types of bio-materials. As a concept, biorefining includes all processes of fractionating and converting biomass into energy and chemicals. Globally, however, the utilization of wood still depends on fuel and construction applications. New types of biorefineries have originated from agricultural industry (Holmbom 2011), including processes developed in the production of food and feed. Biorefining is currently studied from many aspects; both unit processes and over-all concepts are studied to add understanding of the processes, to develop new products and to improve the efficiency and productivity of processes. Also, energy and environmental aspects are under evaluation, along with a zero-waste circular economy (Sitra 2014). Figure 1.1 illustrates the need for novel analytics in the development of biorefinery processes in relation to other fields of industry.

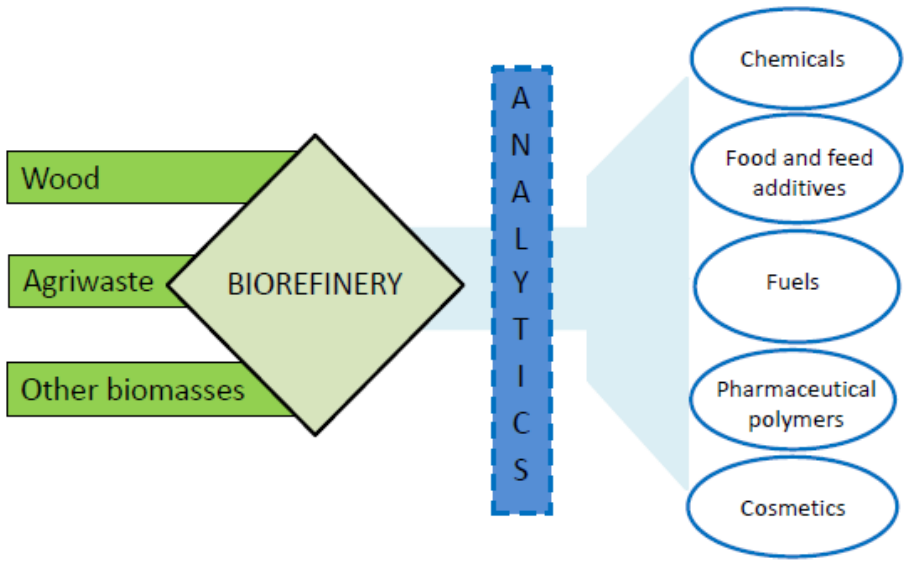

Figure 1.1 Schematic presentation of the need for novel analytics in integrating traditional industry and biorefinery processes.

Biorefining is characteristically a multi-dimensional combination of variations in raw materials and processes parameters. Even the simplest and most highly controlled unit processes can provide many different streams of products, depending partly on the raw materials. Wood is the most abundant source of biomaterial in Scandinavia, and the utilisation is conducted in various fields of industry. Chemical composition of wood is more heterogeneous than the other biomasses. Because of the complexity of the composition throughout the morphological structure, wood is considered highly challenging as a raw material. Wood also presents a broad range of possibilities for utilisation. (Amidon & Liu 2009; Liu 2010, Holmbom 2011; Menon & Rao 2012)

In large-scale industrial processes, the raw material and solvent selections aim at lowering the energy consumption and harmful impacts for the environment, but improving the yield of easily separable primary products. Process development and monitoring rely on the suitability of the selected analytical methods to determine specific features in the process stream.

1.2 Aims and scope

The purpose of this dissertation is to create a novel analytical strategy based on a widely applied capillary zone electrophoretic (CZE) method with ultra violet (UV) detection. A further target is to evaluate the suitability of the method in analysing heterogeneous wood-based bioprocess samples. The theory includes (i) the summary of CZE as a promising technique for process sample analyses, (ii) in-house validation as a ground-setting tool for process analytics of heterogeneous samples, and (iii) some sample stream characteristics concentrating on the wood-based bioprocess as the main application field.

Traditional analytical strategies consist of narrow, targeted methods to determine specific compound concentrations. Such techniques as high-performance liquid chromatography (HPLC) and gas chromatography (GC), often combined with mass spectrometer (MS), concentrate mostly on a narrow range of selected compounds, simultaneously ignoring other compounds and a large amount of data concerning sample matrices and process variables. Currently, the evaluation of process streams in biorefining is based on the interpretation of specific concentrations of compounds gained by utilising several separate analytical methods.

Capillary electrophoresis (CE) is a well-established and versatile technique with a wide range of possibilities. The simplest form of CE is the CZE, or free-solution capillary electrophoresis. The technique presents a future potential as a process-monitoring instrument. The profiling approach of the aqueous process sample takes into account the total phenomena in the process, as an alternative to applying several individual analytical methods concentrating on specific compounds. Typically, profiling methods provide more information for the evaluation of the carbohydrate matrix of the process after one single run.

Validation was targeted for the determination of the most important factors effecting the uncertainty of the results. An in-house validation procedure for the CZE method was studied that aimed at the practical implementation of validation for industrial purposes.

In general, a thorough validation is a required procedure in evaluating the quality of the gained results. Specifics of the validation requirements are based primarily on the ability of the method to detect the desired analyte in the sample. From the analytical point of view, low values of detection and quantitation limits are desired, as well as low values of uncertainty. From the industrial point of view, the requirements of the method and its applicability are determined by the practical aspects of process control and monitoring.

Process development in biorefining requires knowledge of a wide range of parameters affecting the chemical reactions. Thus, the need for analytical tools for the overall evaluation of aqueous samples consisting of different types of carbohydrates is evident. Wood-based bioprocesses were selected for this study because they are recognised as one of the most important fields considering material efficiency.

With respect to experimentation, raw materials of pine and spruce were applied in water extractions. Although biorefinery concepts commonly include use of other types of solvents, the water streams were selected as the main focus of this research. Water-based processes in low temperatures are currently considered to meet the challenges in several economic and political demands. For example, lower process temperatures require less energy. Water is an environmentally friendly solvent which can be purified, reused and discharged via relatively simple processes. Yeast and fungus were applied to extractions gain variation in the carbohydrate matrices of the samples and process changes in time.

The specific research objectives are presented in Table 1.1.

Table 1.1 Main research objectives of the scientific publications for this thesis work.

Paper I	Novel CZE analytics: Is it possible to expand the detection to furfural and polydatin among other compounds of interest?
Paper II	Recommendations for in-house validation: What are the uncertainty factors related to the validation procedure of the method?
Paper III	Extended process monitoring strategy: Can the CZE electropherogram be used to identify changes in composition of heterogeneous wood-based matrices?
Paper IV	Towards deeper understanding of UV detection: Can the UV spectra be applied to provide additional information on the characterisation of the sample profile?

The application of the studied method for process monitoring at a specific site is to be studied in the future. For example, stability and practical applicability should be studied further concentrating especially on the equipment itself and the surrounding conditions.

2 Wood-based biorefining

Biorefining uses ligno-cellulosic biomass in the production of biofuels, platform chemicals and by-products. Biorefining of wood includes a vast range of alternative conversion processes. Current trends in bioconversion of lignocellulose include products categorised as biofuels and platform chemicals. As a concept, biorefinery consists of hybrid technologies from process engineering, agriculture and polymer chemistry. This focuses also on research in the field of biotechnology. (Turley et al. 2006; Holmbom 2011; Menon & Rao 2012)

2.1 Wood raw material

The Scandinavian forest industry uses mainly two softwoods: Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*), because of their availability as raw materials and their chemical compositions. Softwoods contain 40% cellulose and 25-30% lignin, along with 25-30% hemicelluloses (Holmbom 2011), which consist of up to 16% glucomannan and 10% glucuronoxylan of the dry mass of wood (Liu 2010).

Chemical composition of softwood differs from hardwood mainly because of the detailed composition of hemicellulose, (i.e. percentages of xylan and glucomannan in dry mass of wood), as shown in Figure 2.1 (Stenius 2000). Softwood bark consists of 15% hemicellulose and 15% extractives (Valentín et al. 2010).

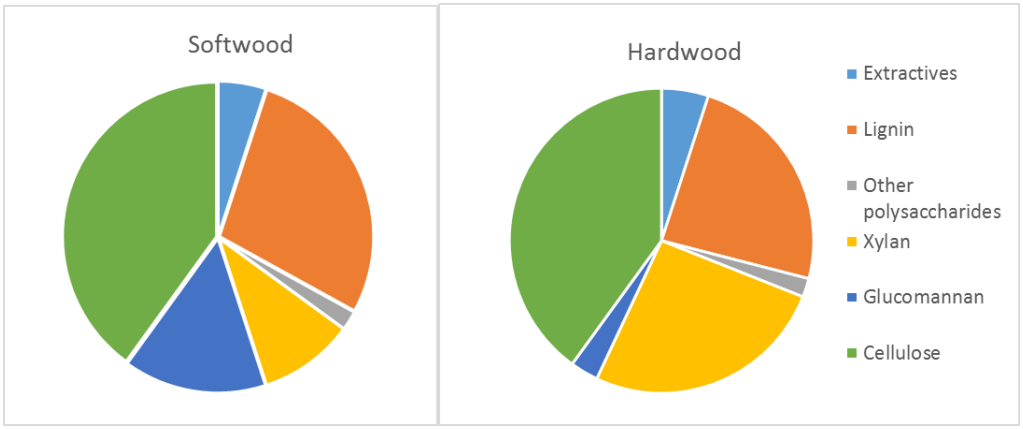

Figure 2.1 Average chemical composition of softwood and hardwood (adapted from Stenius 2000).

Practical availability of hemicelluloses is highly dependent on the solvent penetration through the macroscopic structures. Softwood hemicellulose is situated in the middle of the cell wall of the fibre-like cells, tracheids (Gullichsen 2000). However, softwood hemicelluloses are soluble in water and alkali, and they can be degraded into monomeric units of glucose, galactose, mannose, xylose, arabinose, and glucuronic acid (Peng et al. 2012).

In addition to mono-, di- and oligosaccharides, the process streams of wood-based bioprocesses may consist of other types of compounds originating from the wood material. Along with the three main components, wood material also contains 5% of extractives, which are mainly situated in the bark. Extractives include several types of aromatic compounds, which are easily separable by organic solvents or water (Holmbom 2011).

Polydatin, astringin, and isorhapontin are examples of aromatic glycoside compounds found in wood material (Latva-Mäenpää et al. 2013; Stolter et al 2009; Solhaug 1990), in addition to wines and herbal extracts (Stevanovic et al. 2009). These compounds have been demonstrated to have antimicrobial properties as aglycones (i.e. in the absence of the glucose moiety). Polydatin (also named piceid) is a secondary metabolite and biologically active glycoside of resveratrol, which has recently been detected in solutions derived from spruce (Co et al. 2010), and its formation has been shown to be enhanced by fungi. According to the research performed by Hammerbacher et al. (2011), resveratrol is also involved in the formation of astringin and isorhapontin (Hammerbacher et al. 2011). Predicting the possible appearance or increased presence of additional compounds is an important asset for process monitoring, as well as for process development.

2.2 Biorefining process characteristics

Biorefining processes are dynamic and complex by nature (Alhusban et al 2013), and the unit processes are similar to e.g. food production (Garcia-Canas et al. 2014). Processing of biomass includes many chemical and biochemical reactions; new compounds are formed as desired or unwanted. (Menon & Rao 2012; Garcia-Canas et al. 2014). For example, furfural can be formed from pentoses degradation and in the biorefining of wood raw material (Peng et al. 2012). Wood-based bioprocess samples typically consist of various carbohydrate-type compounds, and, thus, a complex sample matrix can be expected in most of the unit processes (Willför et al. 2005; Willför et al. 2009; Rasmussen et al. 2014).

Hemicelluloses are the most easily separable main component of wood-based biomasses (Amidon & Liu 2009; Liu 2010). Saccharides can be separated from hemicelluloses with membrane or enzymatic processing after auto-hydrolysis (Rivas et al. 2013). Hemicelluloses are utilised as raw material in many bioprocesses. Their molar mass and degree of polymerisation are affected by the temperature, pH, and processing time, and depend on isolation and purification methods inside the unit processes (Menon & Rao 2012; Song et al 2011). Also other compound types in the process react with hemicelluloses. For example, a decrease in the carbohydrate contents of spruce wood chips was observed in bacterial cultivations (Kallioinen et al. 2003), indicating a correlation between the reactions of carbohydrates with lipophilic wood extractives.

In addition to carbohydrates, furfural (Rasmussen et al. 2014) and polydatin (Co et al. 2010; Hammerbacher et al. 2011) are examples of important compounds in wood-based bioprocesses. Furfural presents a product compound of pentoses, and polydatin presents

a possible source material for production of resveratrol. Detection of several compounds or compound types in a single analytical run provides novel possibilities for monitoring products, waste and source materials simultaneously.

Water is generally considered to be a promising solvent in various wood extractions because it is inexpensive, non-toxic, and easy to handle. For example, a relatively simple procedure with hot water has been demonstrated to yield polymeric non-cellulosic carbohydrates in just 20 minutes (Krogell et al. 2013).

Characterisation, along with understanding and overcoming challenges related to enzymatic and microbial treatments, is essential in developing economically feasible processes (Menon & Rao 2012; Garcia-Canas et al. 2014). For complex production processes, such as fungal incubation (Valentín et al. 2010) and bacterial treatments (Kallioinen et al. 2003), monitoring a narrow range of compounds is not an optimal strategy. Especially when the critical reactions depend on several additional parameters (Alhusban et al 2013), there is a need for analytical monitoring methods of critical parameters in the unit processes (Garcia-Canas et al. 2014; Rasmussen et al. 2014). In addition to quality and purity assessment in bioprocesses, analytical methods should also be extended to characterization and monitoring (Alhusban et al 2013).

3 Process monitoring and analyses

Process monitoring and analyses aim at maintaining the functionality of the process but also at adjusting and improving the process in harsh conditions. (Aumala 1998; Huskins 1981) Monitoring of industrial processes requires measuring of both physical and chemical quantities.

Understanding the operational characteristics of the process and the limitations of the analytical equipment are required. In theory, every laboratory analysis can be transformed and applied in process monitoring, but in practice, the applications require expertise of both the laboratory analysis and the industrial conditions (Huskins 1981). Characteristics are differently emphasized for e.g. methods based on bulk properties, continuous online process control and automated chemical or elemental analysers (Huskins 1981; Willard et al. 1988). Continuous analysis and large sample throughput are typical challenges in the process monitoring in general (Willard et al. 1988). However, off-line analyses are performed in the laboratory after appropriate sampling and transfer, which both add time to the total procedure from process to applicable results (Merbel et al. 1996).

3.1 General

Developing the bioprocess yields has been followed by the interest in controlling the physicochemical and biochemical conditions during various processes (Merbel et al 1996). Also, as the interest in developing new wood-based products has increased (Holmbom 2011), the need for specific analytical methods has gained more attention. Adequate measurement techniques, which are able to provide a selected overall view of the sample and, therefore, characterise the heterogeneous and dynamic bioprocess streams (Lubbert&Jorgensen 2001; Holmbom 2011; Pinero et al. 2011; Konstantinidis et al. 2013) are needed. A general overview of the process stream could also serve as a preliminary determination procedure before more specific analyses.

Process analysers are intended to be used repeatedly at the specific locations of the stream, whereas laboratory analysers are intended to be utilized for the analyses of several types of samples from different locations of the process. One of the critical steps in transformation of an analytical strategy to industrial process conditions is sampling from the process pipelines, for both on-line and off-line laboratory methods (Huskins 1981; Merbel et al 1996; Aumala 1998; Holmbom et al. 2000; Alhusban et al. 2013). Also pretreatment procedures determine the practical suitability of the analytical methods (Pinero et al. 2011; Holmbom et al. 2000).

In process monitoring, straight forward types of analytical methods, by accurately and reliably functioning instruments, are preferred. Requirements of process analysers of a batch process are different from those of a continuous process (Huskins 1981), but minimal risks of contamination is generally required. Time acquired for the total analytical procedure should be minimized in relation to the process duration (Merbel et

al. 1996), but also long, maintenance-free operating times are considered advantages. Selecting appropriate analysis methods and equipment for process analysis is typically based on equipment availability and personnel experience on analysis. (Huskins 1981)

3.2 Sensory techniques

Typical physical quantities and parameters are e.g. flow, concentration of solids, temperature, pressure, pH, pO_2, liquid height or rotation speed (Aumala 1998; Beutel&Henkel 2011), which are executed in-line by sensors (Harms et al. 2002).

Sensor techniques have maintained their position as typical applications in the modern bioprocess monitoring. Sensor techniques are preferred, because the measurements can be performed without sampling procedures, and, thus, the risks of altering the process stream can be minimized (Beutel&Henkel 2011). Bioprocesses are typically harsh conditions for sensors (Harms et al. 2002), which are prone to fouling by the solutes in the process stream (Merbel et al 1996). The traditional way to analyze chemical composition of liquid process samples is to apply off-line methods, into process streams (Holmbom et al. 2000), but chemical compounds can be identified with specified probes or sensory equipment also (Aumala 1998).

3.3 Chemical analyses

Currently, typical methods for chemical analysis of bioprocess samples include liquid chromatography (LC), gas chromatography (GC), flow injection analyses (FIA), UV-Vis spectrometry, infrared- (IR), fluorescence- and Raman spectrometry, nuclear magnetic resonance (NMR) spectroscopy, high performance anion-exchange chromatography (HPAEC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Merbel et al. 1996; Holmbom et al. 2000; Schugerl 2001; Marchart & Kopp 2003; Willför et al. 2005; Willför et al. 2009; Kara et al. 2011; Latva-Mäenpää et al. 2013). Chemical composition analyses are mainly qualitative and less quantitative in on-line applications of process monitoring (Aumala 1998).

Most utilized methods are LC and GC, including both offline and online applications. For example, GC has been utilized for off-line carbohydrate analyses (Willför et al. 2009), and Raman spectrometry has been utilized in continuous monitoring of ethanol and glucose in biorefining batch processes (Ewanik et al. 2014). Commercial FIA methods have been applied for measuring concentrations of e.g. glucose, ammonium, sucrose and lactate (Harms et al. 2002). Chromatography can be utilized in monitoring several compounds in a single run which makes it useful in process control of complex process matrices. However, one of the disadvantages of the HPLC and GC methods in bioprocess analyses is the requirement for sample pre-treatments, such as derivatization of the selected compounds. Most on-line systems have been applied in the laboratory but not in industrial conditions (Merbel et al. 1996).

Continuous process analysers have utilized refractometry, GC, potentiometry and IR spectroscopy for a long time (Willard et al. 1988; Aumala 1998). On-line applications of monitoring bioprocesses with CE have been studied in recent years (Tahkoniemi et al. 2006; Turkia 2013). However, industrial applications are rarely available.

4 CZE with UV detection

CE is a simple technique of separation analyses in the liquid phase inside a narrow tube. CZE is the simplest sub-class of capillary electrophoresis (Whatley 2001).

4.1 Basics

Electric field, applied into a background electrolyte (BGE), causes the compounds to get charged and, thus, to migrate in separated bands in the BGE to the electrode probe at the end of the capillary. Bands of compounds go past the on-capillary window, which acts as the location of detection (Whatley 2001). The principle of CE separation is in the different migration of solutes in an electric field. CZE separation depends on the analytes charge-to-mass ratios, and it is a common mode of CE (Suntornsuk 2010). The technique is straightforward by nature, but it is also highly selective, fast to operate, and cheap to use (Altria & Elder 2004). CE is versatile, efficient, fast, and fully automated as an analytical method (Garcia-Canas et al. 2014).

Characteristically, the CE equipment is simple by configuration (Figure 4.1). In addition to the capillary, the main components are the high-voltage power supply, the outlet and inlet reservoirs and the detector. Commercial equipment is fully automated and controlled by computer software (HPCE Primer, Agilent Technologies, 2010).

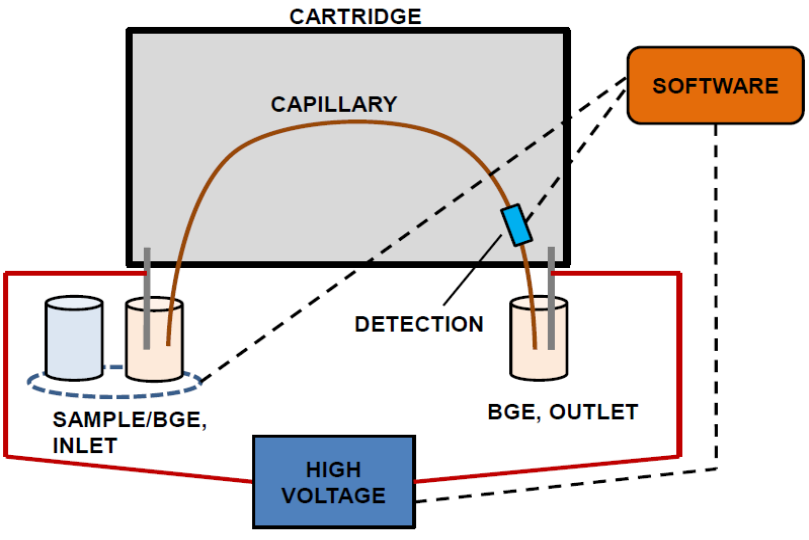

Figure 4.1 Configuration of CE equipment.

Although the equipment itself is relatively simple, the understanding of a separation mechanism and the evaluation of detection results require special expertise on liquid chemistry resulting from a combination of many parameters. CE is a versatile technique and, thus, the possibilities of method development enable the analyses of almost any type of compounds (Altria 1999). In addition to typical pharmaceutical applications of CE, methods for bioaffinity, environmental analysis, and materials have been developed (Geiger at al.). Recent review has covered advances of CE methods in food analysis and foodomics applications (Garcia-Canas et al. 2014). CE in pharmacological control and assurance applications (active pharmaceutical ingredients, APIs) is of equal importance to HPLC (Suntornsuk 2010).

4.2 Carbohydrate analyses

The investigation of natural distributions and biological roles of carbohydrates has been acknowledged to require robust analytical methods (Wang et al. 2012). Recent studies on the determination of carbohydrates with CZE have concentrated on the UV detection mechanism of underivatized compounds (Sarazin et al. 2011; Oliver et al. 2014) and on the high-throughput abilities of the equipment (Khandurina et al. 2004 a; Khandurina et al. 2004 b) for bioprocess applications. Carbohydrate detection is challenging, but the problem has been overcome by derivatisation of the compounds or applying indirect UV detection through method development (Garcia-Canas et al. 2014). Carbohydrate analysis by CE in wood and pulps were studied by Dahlman et al. (2000) The method included sample pre-treatment (derivatization) with 4-aminobenzoic acid ethyl ester, and the applied samples were extractive-free and delignified (Dahlman et al. 2000). Compositional carbohydrates in polysaccharides and foods were detected by CE-UV after compound derivatisation with 1-phenyl-3-methyl-5-pyrazolone (Wang et al. 2012).

The hydroxyl groups of carbohydrates ionise at high pH (above pH 10). Determination of carbohydrates can be performed by applying sample derivatisation or indirect UV-detection or by combining with MS detection (Altria & Elder 2004). Rovio et al. studied the direct UV detection of carbohydrates in a highly alkaline BGE solution (Rovio et al. 2007). Method development and detection evaluation of carbohydrates in a highly alkaline BGE were also studied by Sarazin et al. (2011), focusing on the detection mechanism and on the validation of the method for the analysis of forensic, pharmaceutical and beverage samples (Sarazin et al. 2012).

The CE method has also been developed further for the determination of glucuronic and galacturonic acids in fruit extraction samples (Xia et al. 2010). CE determination of xylo-oligosaccharides in hydrolysed pulp has been demonstrated to provide more selectivity and higher resolution in the separation and quantification of xylo-oligosaccharides than HPLC (Metsämuuronen et al. 2013). Compared to HPLC, CE is able to quantify 22% more carbohydrates from complex mixtures of hemicellulose degradation (Oliver at al. 2013).

4.3 CZE in bioprocess monitoring

Typically biorefining can be considered as high throughput processes, therefore requiring a short time and small sample amount for the applied analytical method (Konstantinidis et al. 2013). Sample matrix plays a crucial role in the method development of CE (e.g. strong acid or organic solvent in the matrix affect the separation). Scientific literature on CE method development still lack fully validated evaluations of matrix effects and behaviour of analytes in real sample matrices. Also the possible dilution solvents must be compatible with the BGE parameters. (Pinero et al. 2011)

CE methods for monitoring bioprocesses, mainly focusing on food industry-related fermentation processes, have recently been reviewed by Alhusban et al. (2013). Capillary electrophoresis is typically applied in laboratory analysis of food processing samples, which include same compounds (e.g. carbohydrates, small organic and inorganic compounds and polyphenols) as modern wood-based bioprocesses (Garcia-Canas et al. 2014). Chemical characterization has also been discussed with relation to analytes and metabolites.

Although, a multiple capillary instrument has been developed to analyse several samples simultaneously (Altria & Elder 2004), the feature is required for only selected types of process analyses. Simultaneous analysis of samples in parallel and the sequential monitoring of process samples are necessary, but the latter is more important in wood-related biorefining because the large-scale processes typically take hours.

For example, CE was utilized in laboratory conditions for qualitative and quantitative on-line analysis of organic acids in complex mixtures, and, as a result, provided reliable information of two fermentation processes (Ehala et al. 2001). Khandurina et al studied the automated carbohydrate profiling by CE. However, the method included fluorophore labelling and laser-induced fluorescence detection. As the implementation of some bioconversion processes require high-throughput analysis of analytes (Khandurina et al. 2004a), process monitoring in biorefining seldom proceeds faster than on hourly bases. In the study of Khandurina et al. the aims of application included processes of enzymatically treated biomasses requiring massive screening, justifying the use of the multi-capillary equipment. On-line monitoring of metals and organic acids in a batch fermentation process has been successfully executed with CE-UV including an integrated filter probe, and a simpler injection procedure without derivatization pretreatment (Tahkoniemi et al. 2006).

Recently, two dissertations (Rovio 2012; Turkia 2014) were conducted on monitoring wood-based bioprocesses by CE methods. Compounds under investigation were carbohydrates (Rovio 2012), carboxylic acids (Rovio 2012; Turkia 2014), phenolic acids and amino acids (Turkia 2014). Rovio showed the applicability of CZE with strong alkaline buffers and direct detection in the determination of currently typical product compounds, i.e. mono-, di- and oligosaccharides and polyols (Rovio 2012). Turkia

applied a CE method in online monitoring of cultivation processes by modifying a commercial instrument with constructing a flow-through sample cell. The study showed the applicability of the method in determination of complex bioprocess samples both offline and online (Turkia 2014). Latest study by Hyvärinen et al. (2014) showed a broader applications of the CE method by determination of carbohydrates and carbohydrate derivatives in ionic liquid media obtained from lignocellulosic biomass. In recent studies, the process monitoring applications of CE have been mainly applied to batch processes.

5 Validation

Validation of an analytical method involves the evaluation of the suitability of a method for its intended use. The requirements of validation for industrial purposes differ from the ones for analytical purposes. Industrial validation is targeted to be robust, simple and automated. In industrial applications, the maximum time spent on analytical procedures depends on the requirements of the process. In an iterative method development procedure, validation follows the preliminary studies on specificity, selectivity and optimisation of method parameters (Figure 5.1), as applied in this case.

Validation is the key to determine the quality of an analytical measurement. Validation of an analytical method is intended to point out the restricting conditions of performing the analyses or interpretation of the gained results. Validation of an analytical method is required in suitability evaluations and also in further method development (Fabre & Altria 2001).

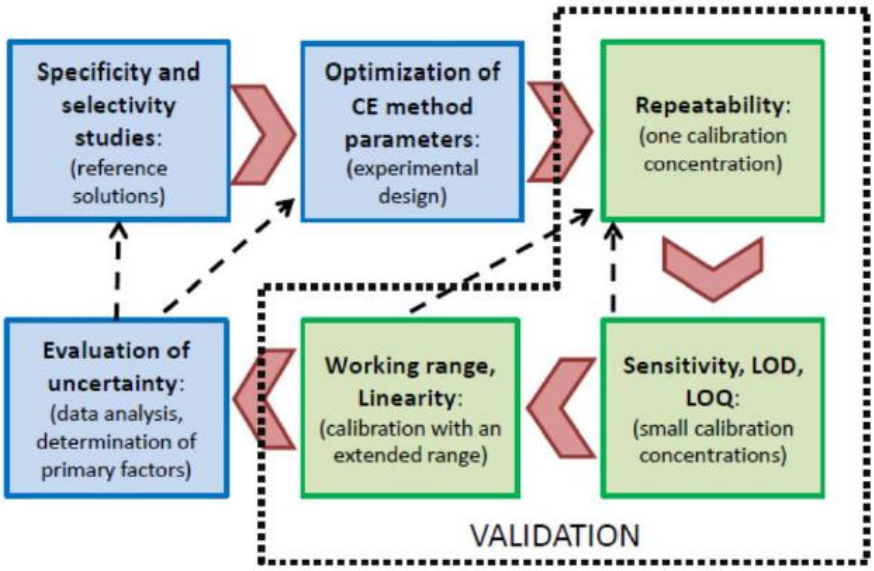

Figure 5.1 Diagram of method development procedure (adapted from Paper II).

5.1 Suitability

Suitability of an analytical method for a selected purpose is evaluated and defined in each case. In addition to practical experience of a certain method, calculated parameters should also be included in the evaluation (ISO/IEC 17025; Ellison et al. 2000).

In-house validation is the basis of suitability tests, with the purpose of demonstrating the basic features of the method through such factors as selectivity, accuracy, and

repeatability, along with limits of detection and quantification. For each case study, sampling and applying the analytical method and interpretations of the gained results should be considered (EURACHEM 1998**;** Feinberg et al. 2004). In brief, the in-house validation includes measurement of repeatability; sensitivity; limit values of detection (LOD); limit values of quantitation (LOQ) and working range and linearity as the main factors.

The purpose of method validation and measurement uncertainty is to improve the quality of chemical data. The quality of an analytical method must meet the requirements of metrological and socio-economic aspects. In practice, this includes the evaluation of uncertainty of the measurements and the proposal of a cost-effective analysis service (Feinberg et al. 2004).

5.2 Uncertainty evaluation

Uncertainty evaluation forms a crucial connection between the results from the chemical analysis of a sample and the validation of the analytical method. The use of uncertainty evaluations is a critical step in the assessment of a method suitability, because the purpose of the procedure is to identify and remove significant effects. Knowledge of the analytical method and sampling of the selected process are the bases of uncertainty evaluations (Ramsey & Ellison 2007). After specifying the primary focus of the analytical method, identification of the sources and correlations of uncertainty factors in a specific case can be executed by applying a cause-and-effect (fishbone) diagram (Figure 5.2).

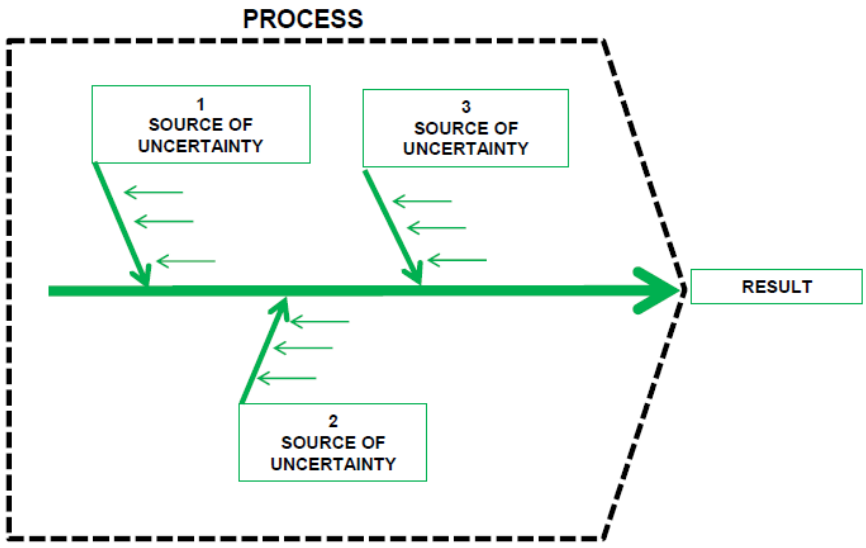

Figure 5.2 Cause-and-effect diagram of combining uncertainty factors of a production process (adapted from Ellison et al. 2000).

As a procedure, each identified source of uncertainty is treated separately to gain the contribution of the source to the total uncertainty. The sizes of the uncertainty sources are typically evaluated or quantified based on past experience and experimental data (Ellison et al. 2000).

Calculation of the total combined uncertainty consists of case-dependent uncertainty components from identified sources (Equation 1) (Ellison et al. 2000).

$$u_c = \sqrt{u_1^2 + u_2^2 + \cdots + u_n^2} \quad (1)$$

in which u_c total combined uncertainty
u_i standard uncertainty factor for i^{th} error source, i=1,2,…,n

5.3 Practical considerations

Method validation in CE uses similar methodology as in HPLC. Capillary variation, electrolyte stability, instrument transfer and capillary rinsing are special characteristics, which differ from HPLC. These need to be considered when improving the precision of the CE method (Mayer 2001). In CE, the integrated area of the detected peak is utilised in the calculations of LOD and LOQ (Oliver et al. 2013). Sufficient S/N ratio of peak area was considered to be 10 in the present study. However, recent studies related to clinical analyses suggest at peak area determination of at least 50 to achieve the S/N ratio. (Meyer et al. 2012).

Studies on large scale carbohydrate analyses by CE have shown capillary-to-capillary and run-to-run variations in migration times and signal intensities. One way to overcome the challenge is to develop data normalisation tools. (Khandurina et al. 2014b) Migration times are typically adjusted by utilising internal standard additions and calculations (Sarazin et al. 2011; Sarazin et al. 2012a), but in process monitoring this pre-treatment step for resulting data is unnecessary. Biorefining processes typically proceed in time scales of hours.

Critical factors, such as repeatability and intermediate precision, are emphasised in the cases of routine analyses of process samples (EURACHEM 1998; Suntornsuk 2010; Sarazin et al. 2012a). Emphasising the concentrations on the lower and upper ends of the calibration expands the practical working range of calibration (EURACHEM 1998). The higher the concentration the larger the peak area, but at some point the linear correlation of the peak area to the compound concentration starts lacking. With CE, the range of good analytical correlation is relatively narrow, which needs to be taken into consideration in the process-driven optimisation of the method.

Basic knowledge of the electrophoretic system is needed before planning an effective multivariate strategy for method optimisation studies. Experimental design is utilized widely in the method optimisation studies of CE, but the statistics are rarely reported in a way that would enable the best possible comparison between methods (Orlandini et al.

2014). Review of analytical characterisation of wine by CE concluded that the chemometric pattern recognition techniques have been widely applied, but very few scientific publications have been presented of multivariate analyses related to CE. The study revealed the possibility of using chemometrics (e.g. for studying the biological activity of new compounds) (Gomez et al. 2012).

Sensitivity is strongly related to the principle of detection. The best detection system is mainly determined by the selected analyte, but in practice the detection is selected based on the availability of the equipment (Simonet et al. 2003). The correlation coefficients from calibration need to be considered in the uncertainty evaluations because they could be applied for determinations of specific concentrations of the selected compounds.

Long-term analyses with CE present some challenges for validation experiments. Parameters, such as sample carryover, buffer handling and capillary breakage (Mayer & Muller 2001) need to be considered especially in the validation studies of process-motoring applications.

6 Experimental

Experimental procedures are described in Papers I-IV in more detail.

6.1 Method development

6.1.1 Apparatus and method parameters

Analyses of the aqueous samples in the Papers I-IV were conducted with an HP 3D CE system (Agilent), including a Chemstation software for data recording. The CE equipment was fully automated and included a high-voltage power supply, a carousel for auto-sampling, an injection system, an on-capillary diode array detector (DAD) with the UV range from 190 to 600 nm, and a capillary cartridge.

The BGE for the separation was composed of 130 mM of sodium hydroxide (NaOH) and 36 mM of sodium hydrogen phosphate ($Na_2HPO_4 \bullet 2\ H_2O$) in deionised water (pH 12.6). The BGE was degassed by ultrasound and filtered before CE analyses.

The separation of compounds was conducted in a fused silica capillary, which was placed in a hollow, temperature-controlled capillary cartridge. The effective length of the capillary was 61.5 cm (a total length of 70 cm) with the inner diameter of 50 µm. Because of the configuration of the cartridge, 50.5 cm (82 %) of the effective length of the capillary was under temperature control by air.

Detection was conducted with a DAD through a handmade window on the capillary. The DAD enabled the detection of the same analytical run by five specific UV wavelengths with a given band width.

The capillary was conditioned with a daily sequence of 0.1 M NaOH, purified water, and the BGE for 20 minutes, 20 minutes, and 5 minutes, respectively. The samples were pressure-injected at 34.5 mbar for 8 seconds and detected at 270 and 210 nm. The separation voltage was 17 kV with positive polarity, and the separation temperature was 25 °C. The carousel temperature depended on the ambient temperature (20-23°C) in the laboratory. The total time for detection of each separation of compounds was 50 minutes. Capillary conditioning between the sample injections was performed with the BGE for 8 minutes.

The samples were prepared by filtering 1.5 mL of the sample into injection vials (2 mL) and sealed with plastic caps. All sample vials of the sequence were loaded on the sample carousel simultaneously. As a maintenance procedure, stainless steel electrodes were cleaned with methanol before each sequence to minimise the interference of accumulated contamination.

6.1.2 Reference solutions

Furfural, polydatin, glucuronic acid, NaOH, and disodium phosphate ($Na_2HPO_4 \bullet 2\ H_2O$) were purchased from Sigma (St. Louis, MO, USA); glucose was purchased from Fluka (Buchs, Switzerland); and sucrose and inositol were purchased from BDH (Poole, England). The reference compounds (Table 6.1) were diluted in purified water, which was obtained from an Elga purification system (Centra-R 60/120, Veolia Water).

Reference solutions for calibrations were diluted from stock solutions to the concentrations of 500 or 1000 mg L^{-1} with purified water. The reference chemicals and working solutions were stored in a refrigerator or a freezer during the laboratory experiments.

Table 6.1 Molecular masses of the compounds in reference solutions.

Compound type	Model Compound (purity)	M, g mol^{-1}	pK_a (reference)
1: furan derivative	Furfural (99%)	96.1	NA
2: sugar alcohol	Inositol (99%)	180.2	12.9 (Sarazin et al. 2011)
3: disaccharide	Sucrose (99%)	342.3	12.51 (Lagane et al. 2000)
4: monosaccharide	Glucose (99%)	180.2	12.35 (Rovio et al. 2008)
5: polyphenol glucoside	Polydatin (95%)	390.4	9.40 (Zhang et al. 2007)
6: uronic acid	Glucuronic acid (98%)	194.1	3.20 (Zemann et al. 1997)

6.2 Validation and optimisation

Suitability of the CZE method was evaluated by an in-house validation procedure, and the requirements of the equipment for biorefinery-related applications were demonstrated in the laboratory. In-house validation of a method to determine polydatin is described here to illustrate some of the important validation parameters of the applied CZE method.

6.2.1 Total uncertainty evaluation

Total uncertainty was combined with individual uncertainty factors from different sources. Two main types of uncertainty factors were identified: components which can be evaluated statistically and components which can be defined based on experience. In this work, the uncertainty was evaluated according to the Eurachem Guide of Quantifying Uncertainty in Analytical Measurement (Ellison et al. 2000). The main focus was the uncertainty originating from the CZE method procedure and equipment in relation to analyses of real samples. Uncertainty arising from personnel, environment and sampling was also evaluated to some extent.

The variance components of uncertainty factors are additive according to the equation of combined uncertainty (Equation 1) and depends mostly on the factor which has the highest absolute value. Estimates for the values of uncertainty factors are often needed for practical applications.

Variance components are case-dependent on the applied procedures and methods. Some of the individual components can be minimized or even discarded by method optimisation, validation, and normal maintenance procedures.

6.2.2 In-house validation

The suitability of the methods for analyses of bioprocess samples was evaluated by performing an in-house validation process. The unique characteristics of CZE validation originate from the principle of CE separation and are affected by the details in the apparatus configuration. Thus, specificity and working environment were evaluated as critical uncertainty factors before the in-house validation.

The in-house validation procedure was evaluated in three main steps of analysis sequences:

i) measurements for repeatability
ii) sensitivity, LOD, and LOQ
iii) working range and linearity

The calibration solutions of polydatin (30 repetitions) and glucose (20 repetitions) were executed with concentrations of 60 mg L^{-1} and 50 mg L^{-1}, respectively. The smaller concentrations of polydatin (1, 2, 5, and 8 mg L^{-1}) and glucose (2, 5, 10, and 20 mg L^{-1}) in deionised water were determined, in order, to calculate sensitivity, LOD and LOQ. The determinations of linearity and working ranges were evaluated for each compound, along with analyses of blank samples.

The time-dependent variation of dynamic processes is a critical issue, and in this work it was verified with reference solutions. The two main sources for a dynamic uncertainty factor after validation are the ones arising from the equipment and from process dynamics. These uncertainty compounds have a tendency to increase systematically and, thus, cannot be minimised with increased repetitions. This may also increase selectivity and specificity. The automation of the commercial equipment offers possibilities for continuous measurements, even for days, but the reliable time window for measurements (e.g., originating from the BGE deterioration) might be only a few hours from calibration.

6.3 Process samples preparation

Process samples were prepared in laboratory scale from chipping of the raw material to the separation after extractions. The liquid process samples were prepared via water

extractions and aqueous fungal treatments. Details of the sample preparations are presented in Papers I-IV.

Samples for the analysis were prepared in laboratory scale as a part of preliminary process development studies. Raw material and process parameters were selected in this work to produce samples with heterogeneous carbohydrate compositions. Also the connection to the current products of biorefining processes was considered important. Furfural, inositol, sucrose, glucose, polydatin, and glucuronic acid were selected as reference compounds.

Selected wood raw materials were typical Scandinavian trees used by the forest industry; spruce (*Picea abies*) and pine (*Pinus sylvestris*). The selected solid raw material, referred to as 'white wood' included mainly heartwood and sapwood, excluding bark of the wood. Commercially available (Hankintatukku Oy, Helsinki, Finland) dried oyster mushroom (*Pleuretus ostreatus*) was applied (1 g in 100 mL of deionized water) for fungal treatments.

Samples were prepared under laboratory conditions. Preparations consisted 5 g of wood material in 100 mL of water in Erlenmeyer flasks on a shaker or 15 g of wood material in 500 mL of deionised water in a batch reactor with blade agitation and a temperature of 25°C. Extraction time of 24 h included constant agitation and an ambient temperature.

Liquid samples were filtered with a syringe filter (Acrodisc PSF, GHP 0.45 µm, Pall) before analyses. Compound peaks in the electropherograms of three repetitions were integrated manually using the CE equipment software.

The profiling strategy was applied for batch process samples of water extraction of pine chips and added fungus. The profiling strategy of example samples was started by grouping the detected peaks in the electropherogram into three groups after identifications of the selected reference compounds (i.e., furfural, inositol, glucose, fructose, glucuronic acid, and polydatin).

7 Results and discussion

In this work, the suitability of the analysis strategy, based on the extension of the CZE method, was studied. The focus of the target requirements was based on the method development and in-house validation experiments simulating industrial processes, aiming namely at heterogeneous bioprocess monitoring.

The main focus of the in-house validation was selected to be the evaluation of the uncertainty factors, because it was considered to be one of the crucial aspects of the requirements for process monitoring in general. To detect differences in the compositions of the samples, the method was applied for analysing the carbohydrate profiles of example samples gained from treating different wood species. The method was also expanded to the detection of novel compounds, such as polydatin. The results of the work are summarised in this chapter, and the details are found in Papers I-IV.

The most important results discussed in this research are related to:

- the advances in the UV detection of carbohydrates
- the industrially targeted uncertainty evaluation via the in-house validation
- the detection of carbohydrate profile characteristics in the process samples
- the novel application of important wood-based compounds

7.1 Method development

In this work, the aim of method development was to expand the range of detectable compounds of the selected CZE method. Also, the utilisation of UV detection in sample characterization and compound identification aiming at process monitoring was desired. Visual evaluation of the recorded electropherograms was justified, because of the practical nature of applying commercial equipment to process monitoring. Calibrations of the reference solutions were executed with a mixture of carbohydrate compounds representing different types of compounds in process streams. Detection of each compound was evaluated at two comparative wavelengths at 210 nm and 270 nm.

Two novel types of carbohydrate-related compounds were detected from reference solutions with the method at hand in less than 20 minutes (Paper I); namely, furfural and polydatin (piceid). The compounds were detected for the first time simultaneously with inositol, sucrose, and glucose, with the CZE-UV method originally developed for analysing mono- and disaccharides. In addition, glucuronic acid was detected in the reference mixture along with other saccharides.

The migration order of the selected compounds is presented in Table 7.1. In the reference mixture, furfural was identified as the first peak in the electropherogram and mostly overlapping the systematic water peak visible also in a blank water sample. Thus, the detection of furfural is possible in the same analytical run, but the quantification includes

restrictions in the lower range of concentrations. The detected furfural peak was very sharp and significantly larger than the systematic water peak.

Table 7.1 Migration order, sensitivity coefficients (with standard uncertainties), and correlation coefficients (R^2) of the selected reference compounds.

Peak identification	Compound	Migration time* (min)	Sensitivity coefficient**	R^2
1	furfural	6.9	2.3426 (0.024)	0.9996
2	inositol	7.5	1.3684 (0.062)	0.9939
3	sucrose	8.6	0.2379 (0.0042)	0.9984
4	glucose	10.5	0.2311 (0.0009)	0.9999
5	polydatin	18.4	0.4540 (0.0080)	0.9985
6	glucuronic acid	20.3	0.1992 (0.0248)	0.9418

*: from an example electropherogram (10% variation was allowed)
**: from the first injection

Examples of migration times are presented as they are shown in the recorded electropherogram. The shift in the migration time (Figure 7.1) was noted to be longer for compounds migrating later in the electropherogram. Polydatin was detected several minutes after glucose. A long migration time was expected because of the structure and size of the compound. However, the migration time is in a suitable range for process monitoring. The applied method also provides shorter migration times for glucose and glucuronic acid (11 and 20 minutes, respectively) compared to the method developed by Wang et al. (2012) for glucose and glucuronic acid (26 and 49 minutes, respectively).

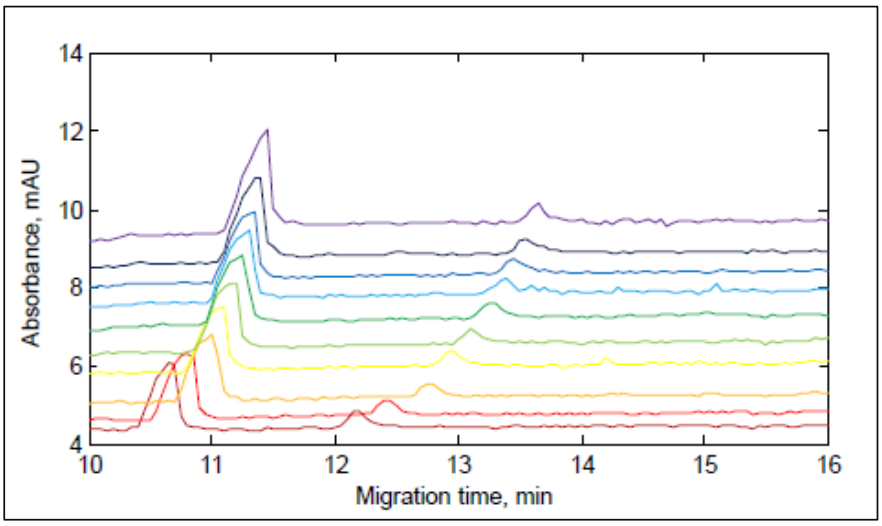

Figure 7.1 Examples of 10 electropherograms of sequential analytical runs. Peaks in the figure are system peak and sucrose.

In this work, the correlation coefficient R^2 of glucuronic acid (0.9418) is the lowest compared to other selected reference compounds, and also lower than the value (0.9866) determined by the CZE method developed by Xia et al. (2010). However, the quantification of glucuronic acid within this method extension can be further optimized.

The highest calculated calibration correlation (0.9999) was calculated for glucose. Correlation values (R^2) for furfural and polydatin were determined to be 0.9996 and 0.9985, respectively. The values were similar to the R^2 values of glucose and sucrose, indicating an equally good correlation between detected absorbance and fixed reference concentrations. The R^2 value of polydatin was found to be almost equal to the value of a determination by HPLC (0.9998) (Zhang et al. 2007), but the LOQ was calculated to be significantly lower (4.8 mg L^{-1}) than that determined by the HPLC method (20 mg L^{-1}).

Sensitivities, LOD, and LOQ are presented in Table 7.2 for sucrose, glucose and polydatin. LOQs were determined by calculation based on the minimum peak area, which was visually evaluated to significantly differ from the electropherogram baseline. This calculation, however, excluded the overlapping of furfural with the systematic water peak. Thus, the lower limit of the working range (10 mg/L) is significantly higher than the calculated LOQ concentration of furfural.

Table 7.2 Correlation coefficients (R^2) and calculated LOD and LOQ for sucrose, glucose and polydatin.

Peak identification	Compound	R^2	LOQ (mg L^{-1})	LOD (mg L^{-1})
3	sucrose	0.99	9.2	9.4
4	glucose	0.99	9.5	7.7
5	polydatin	0.99	4.8	2.2

The results confirmed the novel detection of furfural and polydatin with the applied CZE method. This suggests also the possibility to detect and identify a range of other furan derivatives and glycosides. Thus, the results show the ability of the applied method to show a wider range of detectable compounds compared to a method developed for the determination of 13 saccharides (Wang et al. 2012).

Practical application of an extended UV detection was evaluated by recording electropherograms of two wavelengths (210 and 270 nm) and comparing them to the values calculated from spectra found in literature (Figure 7.2) (Paper IV). The absorbance ratio of glucose after repeated analysis (Figure 7.3) was found comparable to the calculated absorbance ratio (2.4) based on the spectrum in the literature (Oliver et al. 2013).

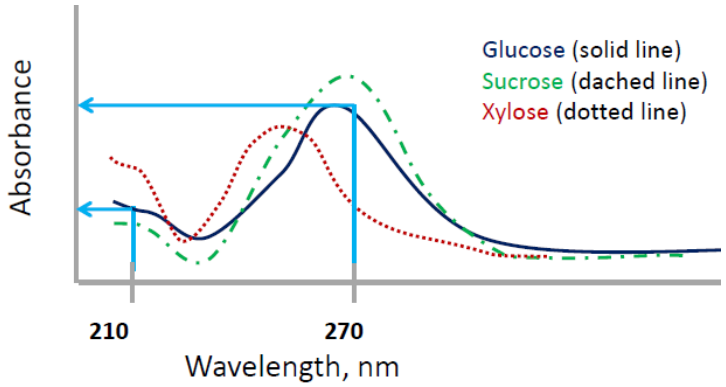

Figure 7.2 UV absorbance of glucose, sucrose and xylose (adapted from Sarazin et al. 2011; García-Pérez et al. 2008).

Optimal detection wavelengths of glucose (Sarazin et al. 2011) and xylose (García-Pérez et al 2008), based on maximum absorbance ranges discovered in the literature, were found to be consistent with the applied wavelengths. The differences in the UV absorbance of xylose and glucose at 270 nm are confirmed from the UV spectra. This confirms the practical relevance of utilising multiple wavelengths in the analyses of monosaccharides.

Table 7.3 Calculated ratios of absorbance (270 nm/210 nm) and optimal detection wavelengths of glucose, sucrose and xylose obtained from the UV spectra in Figure 7.2.

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 a result, the comparison of wavelengths showed the novel possibilities of interpretation of DAD signals without the large space needed for saving the data of a total UV spectra of certain sample. Ratios of absorbance were utilised for comparison of UV spectra of compounds to the peak areas in the electropherograms.

The comparison between the UV absorbance of different types of compounds showed significant differences, which may be utilized in process monitoring applications. Glucose was not visible at 210 nm in the calibration concentrations selected for these experiments. This invisibility may also be used in the identification of similar or closely migrating compounds in a process sample. For polydatin, the absorbance ratio of UV absorbance (Zhang et al. 2007) at 210 nm and 270 nm were in accordance with the peak

absorbance detected in the electropherograms. In addition to the wavelengths selected for this study, maximum absorbance at 305 nm was identified as a potentially comparative detection wavelength for further method development.

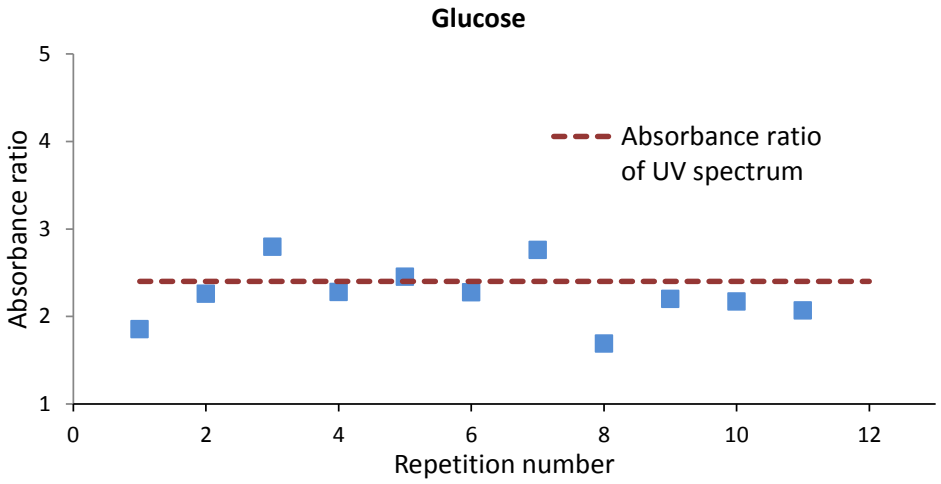

Figure 7.3 The calculated absorbance ratios of the glucose (60 ppm) peak during sequentially repeated analyses. The dashed line represents the calculated value of the absorbance ratio from the UV spectrum in Table 7.3.

Application of the comparative and multiple wavelengths for detection was found to expand the utilisation of the analytical method. This strategy can be further utilized in other CZE methods as well. Multiple wavelengths may also be useful in the future in analyses which are targeted at profiling or fingerprinting complex samples.

7.2 Validation

In-house validation of the CE method was executed to gain general information of the suitability of the method for industrial applications. In practice, the variation of the electropherographic profiles at 270 nm of polydatin and glucose was executed.

For the total uncertainty evaluation, the primary validation parameters were selected to include

(i) selectivity and specificity
(ii) repeatability and precision
(iii) LOD and LOQ
(iv) linearity and working range

The total uncertainty of the results was determined and the values of factors affecting uncertainty were estimated, as presented in Paper II.

Although the typical CE procedures include calculations for compound mobility, availability of migration times (as recorded by the software) may provide critical information of the analysed samples. The higher accuracy of the traditional approach is acknowledged, but the aim of this work to evaluate the practical aspects of CZE justifies the suitability of the novel approach.

With the in-house validation procedure, the change in the migration time was found to change significantly, depending on the functioning of the equipment (Figure 7.4). The malfunction of the equipment immediately resulted in a significant increase in the %RSD value of polydatin; however, in addition, the high level of %RSD maintained above normal through several runs before stabilising.

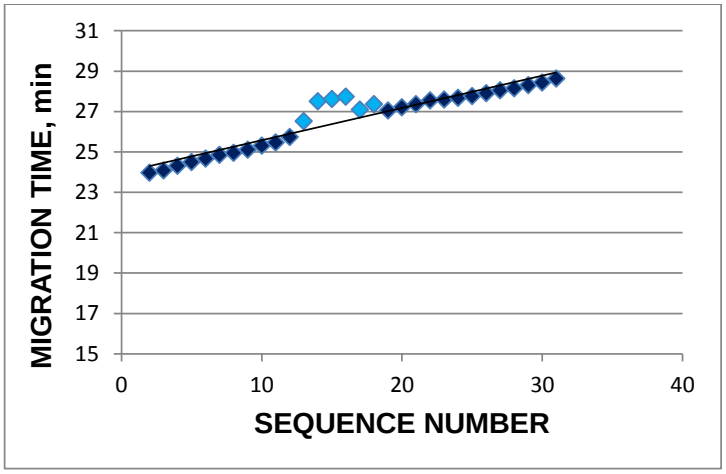

Figure 7.4 Recorded migration times of polydatin during an analytical sequence.

The in-house validation procedure, followed by the total uncertainty evaluation, was found suitable in the development and optimisation of the CZE method. The most significant factor affecting the total uncertainty of the results was the duration of the analysis sequence.

Sensitivity of the method to detect the selected compounds was good at 270 nm, in general, and the coefficients were between 0.20 and 2.3, with uncertainties between 0.0009 (glucose) and 0.062 (inositol).

Migration times for the example samples were set between 45 and 60 minutes in the experiments focusing on the total duration of optimal running sequences. Migration times for the six selected reference compounds were between 6 and 25 minutes, as the total detection time was set for a minimum of 45 minutes. With industrial on-line applications

the suitability of the method depends on the total working time from the sampling, through the chemical analysis, to the result evaluations.

Uncertainty of selectivity and specificity were found to mainly depend on the sequence duration, in addition to compound and sample characteristics, with the upper limit of 3-5%. Repeatability and intermediate precision were mostly affected by ambient conditions, and the uncertainty was evaluated to be 2.5% maximum. Uncertainty from calibration (2%) was combined from the uncertainty values of LOD, LOQ, linearity and working range. Uncertainty arising from the stability and storage conditions of the compound solutions provided an estimated uncertainty of 1 %. A total combined uncertainty of 6% was concluded from the uncertainty components in Table 7.4, according to Equation 1.

Table 7.4 Selected uncertainty factors and their upper limits (%).

Validation factors	**Components of uncertainty (estimated values)**	**Upper limit (UL) for uncertainty u_i**
Selectivity and specificity	- Compound characteristics - Method parameters - Sequence duration (<10 %) - BGE solution (pH and composition) - Sample matrix	3-5 %
Repeatability and intermediate precision	- Ambient conditions (<5 %) - Stability and storage conditions of the compounds, chemicals, and solutions (<1%)	2.5 % 1 %
LOD and LOQ	- Compound characteristics - Method of detection - Separation efficiency (<15%) - Peak identification and integration (<10%) - Baseline noise (<5%)	2 % (total uncertainty factors from calibration)
Linearity and working range	- Calibration correlation - Concentration and type of matrix and separated compounds - Sample loading	

The most significant factor affecting the total uncertainty was the sequence duration. Changes in the capillary wall, the BGE, or the ambient conditions may combine together and cause an increase in the total uncertainty, partially through the sequence duration. By optimising the sequence (i.e., the amount of samples loaded at the same time to be automatically analysed), the total combined uncertainty was decreased to 3%.

7.3 Process sample applications

After acknowledging the uncertainty factors and restrictions of the combination of the analytical method and the particular process stream (Figure 7.5), the uncertainty evaluation sets the limits for interpretations of the results. In addition, the focus of validation can be selected to include the levels of practical measurements and the changes in the process.

This method was found suitable in applying for the determination of wood-based extraction samples. The additional level of information was gained after identifying the characteristic features of the analytical results. The uncertainty evaluation results showed the possibilities of gaining additional and supporting data through analytical profiling strategy.

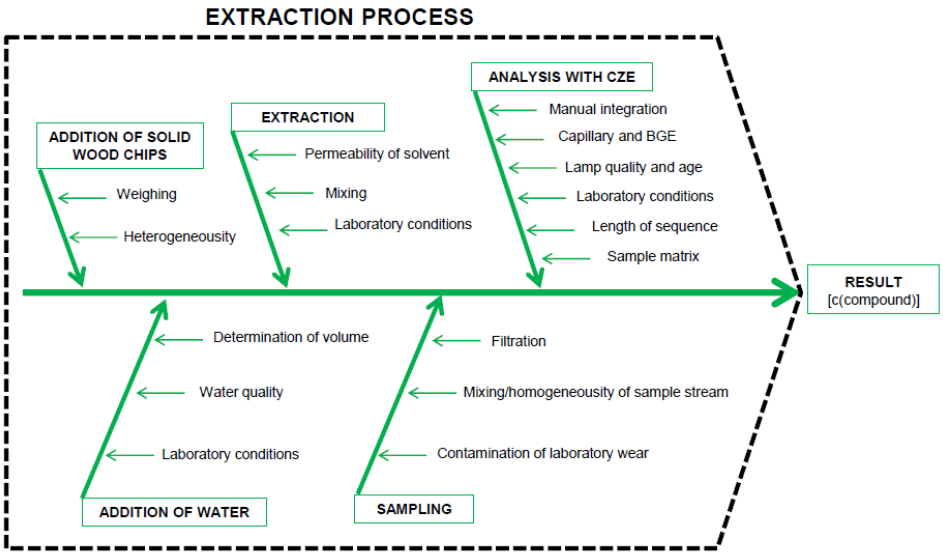

Figure 7.5 Cause-and-effect diagram of the uncertainty factors affecting the capillary electrophoretic determination of water extraction samples.

As the first level of analysis, a profiling strategy was applied to determine levels of possibly interesting compounds in liquid samples prepared in the laboratory. Compounds were divided into three groups by the detection time, and the detected peak areas were used as indications of concentration changes in the process. In this particular process, the concentrations of groups A and B showed significant decreases, according to the detected peak areas. Group C was noted to stay at the same level. However, closer evaluation of the electropherogram revealed changes in the composition of the peaks. After identifying the most important group of compounds, the method can be further optimised to concentrate on a specific group.

Carbohydrate compounds were grouped by their type of chemical structure and their migration order, to characterise the profile of examples of wood-based samples. Migration times for each peak were compared to reference compounds as approximations because the migration times in these experiments shifted forward between different analytical runs. At best, grouping the carbohydrate compounds in the electropherograms into detectable carbohydrate types assists in the identification of compounds at one glance of an electropherogram. Monitoring the process would be easier because the level of the analytical results answers to the information required to control and adjust the process.

Table 7.5 presents an example case of a wood-based sample after fungal treatment. Based on the areas, the levels of grouped compounds have been determined from a batch extraction process.

Table 7.5 Peak areas of the compound groups in electropherograms of three pine samples extracted with water and added fungus. Compounds in the groups are: A) furan derivatives and sugar alcohols, B) mono- and disaccharides, and C) uronic acids and glycosides.

Pine+fungus	Area, mAU*min			
Extraction time, h	A	B	C	tot
24	310	500	310	1120
48	30	90	280	400
168	30	20	270	320

This cannot, however, be utilized in evaluating the concentrations between different groups or peaks, because the correlation with absorbance (i.e., sensitivity) is different for each compound.

Detection at several characteristic wavelengths was noted to confirm the identification of the compound peaks without standard addition of reference solutions. Taking into active consideration the comparative wavelengths, the electropherogram can be interpreted further than traditionally. The peak groups can be further analysed and the peaks in the group can be identified by utilising the comparative UV wavelengths of the detection. The wider range of useful and utilised information of the process stream adds value to the analytical strategy and to the method.

The challenge in peak identification, especially with industrial process samples, grows as the heterogeneity of the sample grows. Separation of compounds, which differ only slightly by their structure, is challenging because of the risk of peak overlapping in the electropherogram. In addition to the reference compound used in the study, many other peaks were detected in the electropherograms. Four samples were selected for closer investigation and to be presented as examples to explain the strategy of profiling. Differences in both concentrations and compositions of carbohydrates were detected from the four example samples.

Identification of the compound peaks requires the consideration of peak shape, electric field and electroosmotic flow, in addition to determination of migration time. From the profile of the samples, other characteristic can also be identified and they can be used as target points (e.g., in group identifications). The BGE, in many cases, includes characteristic peaks or other shapes which are formed from the BGE itself and appear in all electropherograms of the method. An example of this type of characteristic is the systematic water peak detected at 270 nm.

Optimal run time depends on the process sample as well as the method, and typically the reactions within biorefining processes occur in time scale of hours.

The profiling strategy applied to the samples resulted in a preliminary level of results, which indicated changes in the example process. Thus, the results formed the basis for the further development of the CZE method, targeted, for example, fingerprinting, which is routinely utilised by HPLC applications (Funari et al. 2014).

8 Conclusion

Bio-based economy presents a platform of opportunities for the development of sustainable raw material utilisation globally. Process development relies on the suitability of the analytical methods utilised for determination of chemical compositions. In particular, the wood-based bio-processes present challenges for analytical methods because the process streams are very heterogeneous due to the complex nature of the raw material.

This thesis focuses on the applicability and validation of the capillary electrophoretic method, in addition to recent dissertations by Rovio (2012) and Turkia (2013). Previous studies have included optimizations of the CE methods for the identification of typical product compounds, but they concentrated on monitoring laboratory scale processes and selected compounds. The aim is to extend the analytical method for detecting additional compounds in bioprocess samples, but also to evaluate the applicability of the CE method for industrial scale applications.

Results show the applicability of the capillary electrophoretic analysis method in the identification of two novel compound types; stilbenoid glucosides (polydatin as a model) and heterocyclic aldehydes (furfural as a model). The CZE-UV method was found to be suitable for the determination of a wide range of carbohydrates and carbohydrate-related compounds. These compounds represent co-existing compounds which can be either desired products or side products in wood-based process streams, in addition to mono- and disaccharides.

Absorbance at complementary wavelengths can be utilised in the identification of compounds migrating very close to each other or even partially overlapping. The UV-detected carbohydrates can be divided into groups in the electropherogram using their migration order/time. Separation can be further optimised after preliminary characterisation of the process stream. Preliminary identification of compound type can be done even if the conditions inside the capillary change, causing alterations (e.g., in the baseline of the electropherograms). Carbohydrate profiles were characterised from aqueous samples with heterogeneous matrices with the help of multiple wavelengths in UV detection.

An in-house validation procedure was developed for the novel CZE method aiming at monitoring phenomena in the biorefinery. In addition to repeatability; selectivity and reproducibility are crucial parameters affecting uncertainty. The evaluation of total uncertainty is emphasised when analysing process samples, especially when analysis equipment is located in process surroundings.

Analyses of real laboratory-scale samples show that the CZE method is adjustable to heterogeneous wood-based samples, providing a large range of results to be utilised in process monitoring and development. The profiling approach of interpreting UV-detected electropherograms can be applied in evaluations of process samples without quantifying individual compounds, and, thus, can save the laboratory working time.

The profiling method described here enables the simultaneous detection of several compounds and, thus, it minimizes the need for conducting separate analyses. Profiling can be used in the evaluation of environmental and industrial process samples from water extractions. However, sensitivity of the equipment to changes in the ambient conditions requires further development.

Current trend in CE equipment development is towards miniaturization and utilizing new nanomaterials, which, however, seems to be practical for monitoring small-scale parallel batch processes only. Further research could, for example, include a practical application of online equipment to a continuous process stream. Also the hyphenated utilisation of full UV spectra together with migration time would offer an enlarged identification tool, in addition to inter-laboratory validation of the methods.

As a water based comprehensive analysis method CE has potential for process monitoring of modern biorefineries and may prove to be, if developed further, of great help in developing sustainable processes.

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Publication I

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Detection of novel carbohydrate-related compounds in aqueous samples using a capillary electrophoretic profiling method

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Detection of Novel Carbohydrate-Related Compounds in Aqueous Samples Using a Capillary Electrophoretic Profiling Method

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Detection of Novel Carbohydrate-Related Compounds in Aqueous Samples Using a Capillary Electrophoretic Profiling Method

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The aim of this research was to extend an existing capillary electrophoresis (CE) method, originally developed for the determination of mono- and disaccharides, to the determination of alternative carbohydrate compounds, namely furfural and polydatin. Empirical validation confirms that this novel method can be applied for the determination of analyte concentrations from complex matrices, and the evaluation of their carbohydrate composition. It is concluded that the approach has validity as an analytical procedure and has the ability to determine industrially important analytes from a heterogeneous biological sample matrix, and thus, the method adds value to the development of large scale separation processes. However, some additional optimization is required before online applications.

Keywords analytical profiling; capillary electrophoresis; carbohydrates; polydatin

INTRODUCTION

Economic necessity has led to increased effort in the development of innovative, high-value, wood-based products such as speciality chemicals, food additives, and pharmaceuticals (1). Global interest in biorefining has resulted in the need for an effective and comprehensive analytical method for high-throughput screening of biomass samples. The chemical composition of Scandinavian wood material, although well studied, has mainly been determined from the paper and pulp industry point of view (2, 3), and the analytical methods applied have therefore been targeted at a relatively narrow range of compounds (4, 5, 6). Furthermore, the effect of different chemical and enzymatic treatments on the complex biological matrix of wood material is not fully understood (7, 8). The main focus here is on an analytical tool for the simultaneous detection of the profile of the main carbohydrates, i.e., the composition profile of furfural, inositol, sucrose, glucose, polydatin, and glucuronic acid.

All wood-based material contains a wide range of biologically active and water-soluble organic compounds; including mono- and disaccharides, flavonoids, phenols, sterols, and tannins (1, 2, 4). One of the monosaccharides is glucose, which is utilized in many biological processes (9). Due to the increasing global demand for energy, the role of glucose in biorefining is focused on the production of ethanol, through hydrolysis and fermentation. Glucose can also be used as a raw material for the production of many other chemicals and polymers. In addition, glucose, along with other monosaccharides, can also be used in the production of carboxylic acids, amino acids, antibiotics, and enzymes (1).

The primary constituents, and most utilized parts of wood material, are cellulose and hemicelluloses (1, 2). Cellulose and hemicelluloses can constitute up to 35-40% of wood dry mass, respectively. Glucose is the monomeric unit in cellulose, and it is one of the constitutional repeating monomeric units of hemicelluloses in soft wood materials. Furfural can be formed during processing of wood material and is therefore important.

Polydatin is a glycoside conjugate of a stilbene, resveratrol. Resveratrol is a plant defensive substance found in several fruits and vegetables, and its glucoside, polydatin (piceid), can be found naturally in spruce (10). Polydatin and resveratrol, also present, for example, in wine, have gained attention because of their positive effects on human health (11). Furfural is discussed in this study only as an additional compound in the reference solution for method development, because the extraction samples of soft wood material include furfural only in minor content.

Traditional methods for the analysis of these compounds are gas chromatography (GC), high performance liquid chromatography (HPLC), and high-performance anion-exchange chromatography (HPAEC) (4, 12, 13). HPLC and GC are often combined with mass spectrometry, but, in most cases, they are time-consuming and sometimes need very complex derivatization of the compounds (4, 12). The major drawback of

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these methods is that they are only able to detect one or two types of compounds, e.g., monosaccharides or uronic acids, in a single analysis run. A further limitation is that the pretreatments required for these methods often degrade complex compounds into simpler structures so that the actual composition of polymeric structures cannot be determined. In view of these constraints, a multicomponent method with nondestructive pretreatment steps would be highly advantageous. Efficient isolation technologies and determination methods would permit improvements to production processes and allow additional valuable products in side streams to be determined and characterized.

In previous studies, the authors applied capillary electrophoresis to the determination of antioxidative and pharmacologically active compounds in plants (14). Aldehydes, ketones, organic acids, and uronic acids were identified, using CE from hydrolyzed wood samples (15, 16) and wines (17). The method presented by Rovio et al. (16, 17) was further developed by Sarazin et al. (18, 19, 20) for the determination of carbohydrates through modifications to the background electrolyte (19). Wood samples, after chemical and enzymatic treatment, have been analyzed with CE, but the applied methods require derivatization of samples with 6-aminoquinoline or 4-aminobezoic acid ethyl ester (15, 21).

For biorefinery applications, an analytical procedure, without pretreatment, is needed in order to evaluate the actual composition of heterogeneous fluids during chemical and enzymatic treatment. Fast and comprehensive, sample screening analysis methods are important, as the bioprocessing industry often consists of continuous processes or many separate, but integrated, unit processes in one factory. For industrial processes, it is furthermore highly desirable that results are obtained in one single run. Single run analyses save time and make it possible to adjust production processes with less delay. Within the context of monitoring industrial processes, rapid application of profiling methods is more necessary than determination of very accurate quantities for specific compounds. Measurements of industrial process streams can be executed on-line and the limitations of profile characteristics can be set, based on the peak areas. The monitoring of process streams from and within biorefining processes would benefit from comprehensive profiling of raw material and process compounds. Two schematic examples of process applications are presented in Fig. 1. Example A) presents a laboratory or pilot scale experiment of a batch process and B) presents a full-scale process stream with online sampling.

The aim of this research was to develop further an existing CE method (16) originally developed for the determination of mono- and disaccharides, to the identification of different types of carbohydrates in complex, aqueous sample matrices. To our knowledge, this paper presents the detection of furfural and polydatin for the first time with this separation method.

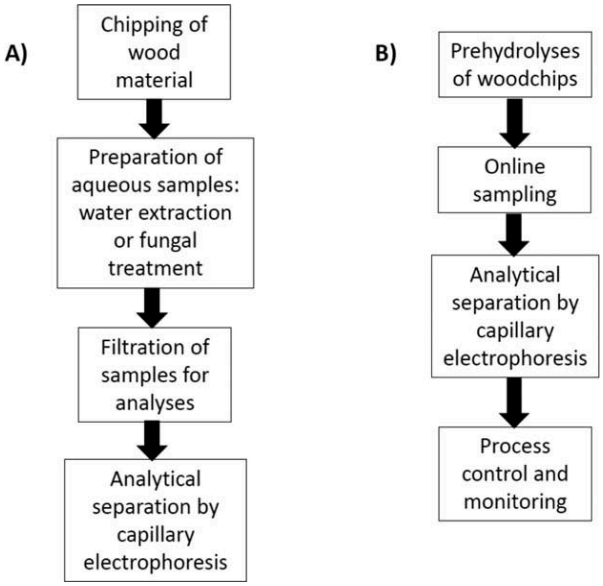

FIG. 1. Schematics of two examples of applications in separation processes. (A) Laboratory scale experiment and (B) large scale process with online sampling.

Selecting the suitable analytes and analytical strategy is a crucial part in process development and monitoring, and, thus, here the extension of the method has been driven by control requirements of modern biorefineries in practice. The paper contributes to the field of developing separation process applications by adding a new overall strategy to the evaluation of the composition of aqueous process samples by capillary electrophoresis. This work is an essential part of evaluation of separation processes in practice as it provides fundamental knowledge, which is needed to enable the CE applications for online process monitoring.

EXPERIMENTAL

Materials

Glucose was purchased from Fluka (Buchs, Switzerland), and sucrose and inositol were purchased from BDH (Poole, England). Furfural, polydatin, and glucuronic acid were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide (NaOH) and disodium phosphate ($Na_2HPO_4 \cdot 2\ H_2O$) were also from Sigma. All chemicals used were of analytical grade. Water was obtained from an Elga purification system (Centra-R 60/120, Veolia Water). The reference solution included furfural, inositol, sucrose, glucose, polydatin, and glucuronic acid in deionized water.

Process samples were prepared from chipped pine and spruce wood by water extractions and aqueous fungal treatments. The wood material included heartwood, sapwood, inner bark and bark from a piece of round wood. Commercially available (Hankintatukku Oy, Helsinki, Finland) dried oyster mushroom (*Pleuretus ostreatus*) was applied for fungal treatments.

Capillary Electrophoresis Procedure

The reference solutions and hydrolyzed wood samples were analyzed with a 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 background electrolyte solution (BGE) consisted of 130 mM sodium hydroxide (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 the addition of NaOH. The ionic strength of the BGE was 0.217 M.

The capillary was conditioned daily, prior to analyses, with 0.1 M NaOH, purified water and the BGE (for 20 min, 20 min, and 5 min, respectively). Conditioning between analyses was performed with the BGE for 8 min. Samples were pressure injected at 34.5 mbar for 8 seconds and detected at a wavelength of 270 nm by a photodiode array detector. Separation voltage was 17 kV, with positive polarity. Separation was conducted at 25°C and the temperature was regulated with air flow. All samples were filtered before analysis (Acrodisc PSF, GHP 0.45 μm, Pall). Peaks were manually integrated from the electropherograms of samples.

Method Validation

Validation of an analytical CE method is essential in order to evaluate the uncertainties in the obtained results. In-house validation is especially important with sophisticated analytical techniques such as capillary electrophoresis. Method validation studies should always rely on the determination of overall method performance parameters. Studies should be representative of the normal operation of the CE method, and if an analytical procedure is of interest, it should cover the complete method (a representative range of sample matrices and a representative range of analyte concentrations).

The novel method, in this case, has been adopted in the laboratory after partial validation. An in-house validation procedure was applied to evaluate the suitability of the method for the analyses, and also to demonstrate the requirements of the equipment in the laboratory for this particular application. Thus there are several quantificates, or standard uncertainty components, still to be estimated, such as limit of determination linearity, repeatability, etc.

In-house validation of a method to determine polydatin is described here briefly, as an example, to illustrate some of the important validation parameters of the capillary electrophoretic method applied in this research.

Sample and Reference Solutions

Liquid samples were received from laboratory scale extraction processes. Experiments for samples A, B, and D were made in Erlenmyer flasks with 5 g of wood material in 100 mL of deionized water. Flasks were placed on a shaker for an extraction time of 24 h. Constant agitation and ambient temperature were applied to all example samples. Sample C was extracted in a batch reactor (15 g of wood material in 500 mL of deionized water) with blade agitation and temperature of 25°C. Samples A, B (*Pinus sylvestris*) and C and D (*Picea abies*) were from water extraction of wood material. The amount of applied microbial material in samples B and D was 1 g in 100 mL of deionized water. These samples of softwood materials, having undergone hydrolysis procedures, were considered to be representatives of the main and side streams of forest products industry processes (3), either currently in use or to be used in the future.

The target was to make a reference mixture that included compounds containing a carbohydrate moiety (Fig. 2). An additional reason for this selection of reference material was the connection to biorefining processes. Solutions of reference chemicals included furfural, inositol, sucrose, glucose, polydatin, and glucuronic acid. Glucose was used as a comparison to a previous application of the CE method (16). Molar masses and pK_a values of reference compounds are presented in Table 1.

Stock solutions were made to 1000 or 500 mg L^{-1} of each reference compound. Reference solutions were mixed from the stock solutions and diluted to the appropriate concentration

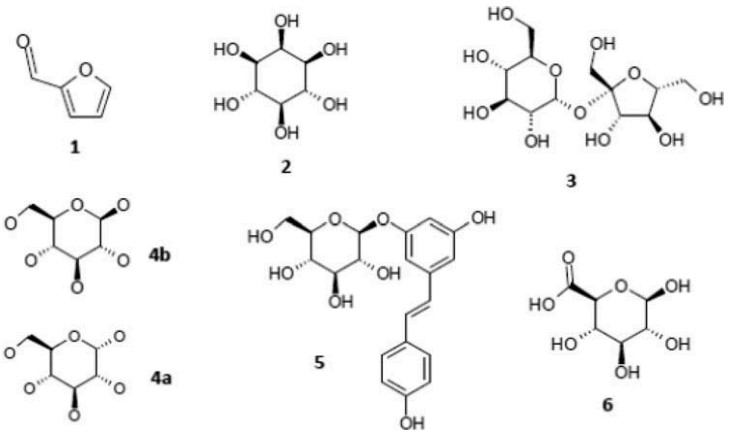

FIG. 2. Structural formulas of the reference compounds. (1) furfural, (2) inositol, (3) sucrose, (4a, 4b) glucose, (5) polydatin, (6) glucuronic acid.

TABLE 1
Molar masses of the compounds in reference solutions

Migration order	Compound	Molar mass, g mol^{-1}	Dissociation constant, pK_a (reference)
1	Furfural	96.1	NA
2	Inositol	180.2	12.9 (18)
3	Sucrose	342.3	12.51 (29)
4	Glucose	180.2	12.35 (6)
5	Polydatin	390.4	9.40 (30)
6	Glucuronic acid	194.1	3.20 (23)

*NA = not available.

with deionized water. Glucose, polydatin, and glucuronic acid were quantified, after identification in the wood-based extraction samples.

After analyses of the reference solutions, the CE method was applied to the identification of the compounds in the liquid samples produced from wood-based materials. As the focus of this work was not on the comparison of different process samples, but rather on the applicability of the CE method to complex matrices, the sample procedures are described only briefly. All reference solutions and samples were analyzed three times.

RESULTS AND DISCUSSION

Reference Solutions

Calibrations were determined in mixtures of compounds in deionized water. The working ranges of compounds in the calibration samples are shown in Table 2. Calibrations were done at five concentrations between 10 and 150 mg L^{-1} for each analyte. The electropherograms of the reference solution and reference compounds are presented in Fig. 3. The migration order of compounds in the mixtures was confirmed with comparison of single component solutions and dissociation constants (pK_a in Table 1).

Due to various factors affecting the sample matrices, migration times of the compounds may change between runs, in comparison to the reference solutions, as well as to other process samples. This is a very well noted characteristic of capillary electrophoresis, and usually it is managed with an internal standard procedure, when optimization of the buffer is not feasible. Optimization of the buffer should be done for each process matrix, and therefore it is not applied in the present process examples. In order to minimize interference and other artifacts caused by the internal standard addition to process samples, the utilization of internal standards were avoided in this work. The analytical procedure with internal standard addition was also considered to be highly time consuming in the routine analyses of process samples.

The migration order of the compounds (Table 1, Fig. 3) correlates with their pK_a values. For furfural, the exact pK_a value was not found from the literature, but it can be assumed to be the largest one obtained, based on its chemical structure (22).

TABLE 2
Working ranges, sensitivities (standard uncertainties in parenthesis), and calculated limits of quantitation (LOQ) of reference compounds

Peak identification	Compound	Working range (mg L^{-1})	Migration time* (min)	Sensitivity coefficient**	P value	R^2	LOQ (mg L^{-1})
1	Furfural	10-150	6.9	2.3426 (0.024)	6.54×10^{-8}	0.9996	0.94
2	Inositol	10-100	7.5	1.3684 (0.062)	0.000203	0.9939	1.6
3	Sucrose	10-150	8.6	0.2379 (0.0042)	3.29×10^{-8}	0.9984	9.2
4	Glucose	10-150	10.5	0.2311 (0.0009)	1.51×10^{-9}	0.9999	9.5
5	Polydatin	10-100	18.4	0.4540 (0.0080)	3.2×10^{-8}	0.9985	4.8
6	Glucuronic acid	10-100	20.3	0.1992 (0.0248)	0.001296	0.9418	11.0

*: from an example electropherogram (10% variation was allowed) **: from the first injection.

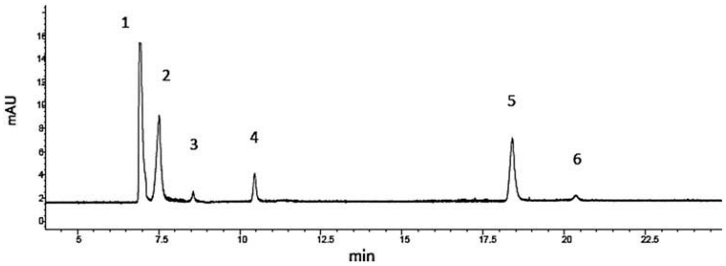

FIG. 3. Electropherogram of a reference solution of 50 mg L^{-1} for identification. Identification of peaks: (1) furfural, (2) inositol, (3) sucrose, (4) glucose, (5) polydatin, and (6) glucuronic acid.

In addition to the migration times of compounds (Table 2), peak identification (Fig. 3) from the profiles can be confirmed from features such as peak shape (Fig. 3), electric field, and electroosmotic flow.

All results are extremely significant based on the obtained P values (Table 2). The sensitivity of an analytical method is depended upon the capability of the method to discriminate small differences in concentration, or mass, of the test analyte. Sensitivity coefficients are presented in Table 2. Sensitivity is defined as the slope of the calibration curve that is obtained by plotting the peak area (y) against the analyte concentration (x). The significance of the slope and intercept was tested at the significance level of 95%, and all intercept constants were removed as they were not statistically significant. The sensitivity coefficients were obtained using equations of the form: $y = kx$, and are presented in Table 2.

Sensitivity is often confused with the limit of detection (LOD) or limit of quantitation (LOQ). LOD can be defined as the point at which a measured value is calculated to be larger than the uncertainty associated with it. It can be also defined visually from an electropherogram. In the present polydatin case, the LOQ value was defined as 5 times the area of LOD. LOQ values for other compounds were calculated based on the results of polydatin, assuming constant peak area with their calibration curves. The calculated LOQ values were justified by comparing them to the size and the shape of the peaks in the electropherograms.

According to the calculated LOQ values, the smallest polydatin calibrant could be 5 mg L^{-1}. For other compounds, the LOQ values varied from 1 to 11 mg L^{-1}. Figure 2 shows that the peak responses of the compounds are highly different from one another. However, the LOQ values seem to be in agreement with the variation.

The working range of a CE procedure is defined as the interval from the upper to the lower concentration of analytes in the sample. In Table 2, working ranges are defined as practical operating ranges. Linearity, the stability of the compound under consideration, ionic strength, etc., have to be taken into account as limiting factors when defining an upper limit for a working range.

For example, an increase in the variance and uncertainty of repeated injections was detected at the upper part of working range of glucose and polydatin, although the linear range seemed to continue into higher concentrations.

From an analytical point of view, all calibration curves have squared correlation coefficients (R^2) greater than 0.99, with the exception of glucuronic acid, for which the value is 0.9418. The response area gained from glucuronic acid is relatively small and the area can be significantly affected by background noise. Although the working range was proven to be linear in the ranges presented in Table 1, it was determined that the variance in measurements tends to increase near the upper working limit.

With an in-house validation procedure, repeatability, under the normal operation conditions, without interruptions in the sequence, was estimated to be 0.8-1.5% for polydatin. During laboratory experiments it was noted that the uncertainty of the measurements increases dramatically in the case of equipment malfunction. In practice, this uncertainty factor is usually minimized with thoroughly executed maintenance procedures. Factors that can affect reproducibility include room temperature and humidity, temperature control of the equipment, variation in experimental details, operators having different experience, and thoroughness, etc. These factors have to be taken into account, and optimized or kept constant if possible, not only within a particular laboratory, but also between different laboratories.

Application

The CE method used in the analyses gives a good overview of saccharide-bearing compounds in a single run, while requiring less than 60 minutes of detection time. Two examples of such profiles are presented in Figs. 4 and 5, which present the electropherograms of liquid samples from hydrolyzed wood materials.

Samples isolated from wood-based matrices are highly variable. Depending upon, for example, the wood species and the biological and chemical treatments, the samples may contain many different types of carbohydrates. In this study, the aim was to demonstrate the suitability of a method for the analysis of extraction samples after validation. The samples are examples of aqueous extraction solutions that have differences in concentrations and compositions of different types of carbohydrates.

Furfural, sucrose, glucose, and polydatin can be calibrated using reference solutions in the range of 10-100 mg L^{-1}. These compounds can also be quantified after identification from water extraction samples. Since the concentrations of the compounds vary depending on the raw material, for example differences in wood species or growing location (2), pretreatment and process parameters, the result values gained here are

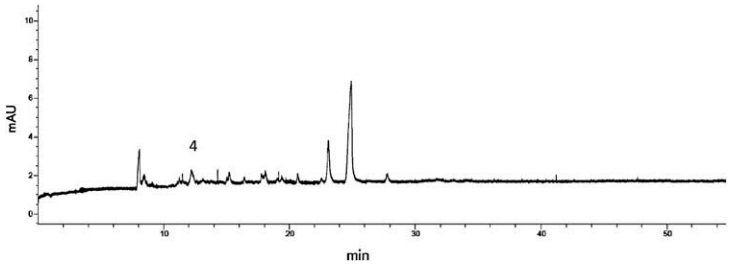

FIG. 4. Profile of a sample from water extraction of pine material (A). Peak numbering as in Fig. 3.

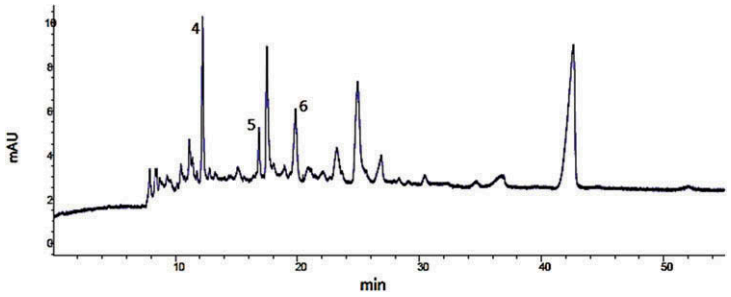

FIG. 5. Profile electropherogram of a sample after an microbial treatment of spruce material (D). Peak numbering as in Fig. 3.

not comparable to values of wood-based samples from other studies. Example electropherograms (Figs. 4 and 5) present samples which have one or more of the same compounds as the calibration solutions. Detection of furfural and inositol in the softwood extraction samples was considered to be highly unlikely. However, the presence of the two compounds in the reference solution was justified as the preliminary target for the method utilization was for applicability to all wood-based materials, including hardwood.

Sample A (Fig. 4) was collected from a water extraction process of chipped pine log with bark. Glucose (25 mg L^{-1}) was identified and quantified from the sample. Sucrose, polydatin, or glucuronic acid were not quantified in the pine extraction sample. Absence of glucuronic acid is mainly due to it being a component of polymer structure in water-soluble arabinogalactans found in pine heartwood (23). In water extraction without microbial treatment, glucuronic acid is also not liberated and it therefore is not analyzed. The same applies for glucose, as it is present as a component in water soluble hemicelluloses but found as a monomer only in trace amounts. On the other hand, polydatin is not present in Scots pine. Pinosylvin, a stilbene resembling resveratrol, is found in pine heartwood, but as an aglycone.

Sucrose, glucose, and glucuronic acid were quantified at concentrations of 1100, 860, and 90 mg L^{-1}, respectively, from sample B. This difference to sample A is due to the different pretreatment processes for the wood material from same species. In addition to glucose (35 mg L^{-1}) also 110 mg L^{-1} of glucuronic acid was detected from a water extraction method applied for spruce material (sample C). Glucose, polydatin, and glucuronic acid were quantified from sample D (Fig. 5) in concentrations of 150, 100, and 400 mg L^{-1}, respectively. The concentrations of identified compounds are listed in Table 3.

Additional peaks in the sample electropherograms (Figs. 4 and 5) were not identified in this research. However, according to the method capabilities, they include water-soluble wood-based carbohydrates, such as polyols, mono- and disaccharides and glycosides. The concentration of the additional compounds cannot be evaluated based on the comparison of the peak sizes without calibrations, and thus, their proportion in the sample was not evaluated in this work.

As predicted, based on the differences in both the raw material and the applied pretreatment processes, the profiles of the liquid samples are different from one another. Samples were analyzed without any adjustments for targeting different compounds. The concentrations were determined from an electropherogram obtained from a single run. Concentrations of analytes in the liquid samples depend on the pretreatment process used for the raw material. The small peak sizes in the electropherogram indicate low concentrations of the compounds and, thus, the sample profiling focused on the more common carbohydrates of biorefining processes. Sucrose was not quantified in the electropherograms of the water extraction samples because the detected peak area was under the quantitation limit. In addition to furfural, inositol was also not detected from the samples.

Alkasrawi et al. (24) determined the composition of wood-based samples using HPLC and found that the samples included glucose and furfural at concentrations of 17,000 and 1,200 mg L^{-1}, respectively. Since the concentrations of these carbohydrates fit into the calibration range after proper dilution, it is evident that the method used in this study could also be applied to such samples.

The profiles of the samples analyzed with this method were as expected. The raw material and the pretreatment process had an influence on the profile of the electropherogram. Aqueous samples and their matrices could be evaluated, based on the characteristics of the electropherogram profile, indicating the total carbohydrate composition. In pine, stilbenes are present as aglycones and thus they are not detectable with the method applied in this research (25).

The CE method used was found to be suitable for the simultaneous determination of several saccharide-bearing compounds in aqueous samples extracted from wood raw materials. Glucose, polydatin, and glucuronic acid could be identified and quantified from wood-based process samples. A similar method has been used by Metsämuuronen et al. (22) for the determination of oligosaccharides in hydrolyzed pulp. In addition to the

TABLE 3
Concentrations (mg L^{-1}) of the analytes identified from four examples of wood-based aqueous extraction samples

	Process sample concentration, mg L^{-1}			
Identified compound	A Pine water extraction	B Pine after microbial treatment	C Spruce water extraction	D Spruce after microbial treatment
3 Sucrose	–	1100	–	–
4 Glucose	25	860	35	150
5 Polydatin	–	–	–	100
6 Glucuronic acid	–	90	110	400

-: not quantified.

studied compounds, other structurally similar compounds could be identified from wood-based samples with this method. Based on the results presented in this study, the method is applicable for analyses of wood samples that typically contain mono- and disaccharides as well as their complexes. The results suggest that, in addition to the reference compounds used in this study, secondary metabolites of trees could also be analyzed with this method. Identifying these compounds would add value to both the CE method and the process, since the metabolites may be as much as one third of the dry weight of the material (26). These compounds include water-soluble flavonoids, steroids, and stilbenoids, which typically are present as glycosides in plants.

Wood-based samples have a heterogeneous carbohydrate matrix, and the pretreatment process is a critical issue affecting compound distribution and analytics. Concentration ranges depend on the process conditions, parameters and chemicals used in the pretreatment, which can be targeted for a specific matrix or particular compounds. Oyster mushroom (*Pleuretus ostreatus*) applied for this research has been studied as a potential fungus on industrial wood degradation (27). In the study of Chi et al. (27) the fungal lignin-degrading systems were stimulated successfully and, thus, an improved method of co-culturing fungi for biopulping was suggested.

CE is a suitable method for the monitoring or optimization of pretreatment methods and can be applied to monitor liquid phases; streams of raw materials, processes, and final products. Low sample volumes and consumption of electrolyte are typical benefits of electrophoresis, but in the case of process streams, the volume of samples might not be a critical issue. Analysis with a single capillary is currently relatively slow, but optimization of the method could include, for example, a multiple capillary equipment (28), capable of analyzing several process samples simultaneously. Additionally, the parameters can be optimized with changes in the composition of the background electrolyte. Physical changes in the process, e.g., changes in temperature and pH, need to be considered because they can influence capillary electrophoretic separation.

CONCLUSIONS

Previously, analysis of samples of the type considered in this study has concentrated mainly on mono-, di-, and oligosaccharides. However, the results presented here clearly show that the analytical compound range can be extended to other, commercially interesting, compounds. The examples studied demonstrate the applicability of the analytical separation method for determination of furfural and polydatin in aqueous samples of complex matrices. The method has potential for the determination of complex compounds in their native form in the process stream, in contrast to existing methods. The method adds value to the development of large scale separation processes as it provides the possibility of detecting both desired products and undesired compounds in the same analytical run. Some issues do, however, still need to be addressed—at the implementation stage, method optimization needs to be done for each CE apparatus; and, at the running stage, the migration times need to be calibrated daily and the total run time needs to be optimized frequently. Especially, the latter two have and will be studied and discussed in other scientific publications by the authors (31).

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In-house Validation of a Novel Capillary Electrophoretic Method: Uncertainty Factors

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A critical discussion of the in-house validation procedure presents the benefits of an application of a capillary electrophoresis (CE) method in aqueous process samples consisting of various types of carbohydrates. This study emphasized the complexity of CE via validation procedure, in the case of heterogeneous processes. An in-house validation procedure of a capillary electrophoretic method aiming at analysis of aqueous process samples with heterogeneous matrices was evaluated. The validation parameters were discussed through an example case of a CE method, developed for the determination of saccharose, glucose and polydatin, applied in calibration solutions and process samples. The validation data was used in evaluation of uncertainty components. The results from the in-house validation procedure showed that the most critical parameter in the determination of uncertainty was repeatability. Selectivity and reproducibility are also critical, particularly in the case of analyzing heterogeneous samples with changing composition. Especially in process analytical applications the evaluation of uncertainty factors was concluded to be essential, as in addition to process conditions the sample composition itself caused variation.

Keywords: Capillary electrophoresis; Carbohydrates; In-house validation; Uncertainty factors

1. INTRODUCTION

Evaluating uncertainty in terms of metrology sets a perspective to the limitations of the interpretation of an analytical result. Defining uncertainties in any analytical measurement increases confidence in the validity of a measurement result, but it is also essential in comparative research. Uncertainty can be evaluated and calculated only from a well-documented research. In addition to proper documentation, uncertainty evaluation requires basic knowledge on the analytical method and sampling [1, 2]. Uncertainty factors should be evaluated thoroughly for the case at hand, taking into

account the total procedure starting from sampling, applying an analytical method, and, to finally, interpretation of measured data [3]. The evaluation should also include estimations of the values of the factors that can be calculated [4].

Validation is the key in the evaluation of the suitability of a method for the intended use, and it is also required when the conditions change inside or outside the analytical equipment [5]. CE method is known to be sensitive for humidity, temperature, pressure, constant electric supply, etc. All these external factors should be considered and optimized at first step in designing proper environment for the measurements in process conditions. When these critical factors have been optimized, a validation procedure for the CE methods can begin.

In contrast to common interpretation in process analytics, validation is not a decision between good and bad methods but rather a guide for interpretation of results. Validation is particularly important in fine analyses, for example in pharmaceutical, clinical, or food-related applications, but also analytical methods utilized in monitoring industrial bulk processes require at least partial validation, that reveals the basic restrictions of the method, and its' sensitivity for, e.g., matrix chances in a dynamic bulk process.

In-house or "*in-situ*" validation is an important step in gaining solid ground for further development of the method, especially in cases where inter-laboratory measurements are not possible or feasible to be executed. In-house validation procedures enable the determination of uncertainty level of the result. Experiments performed need to be well documented and demonstrated for appropriate evaluation of the suitability of the method. There are several guides for choosing validation parameters for the task at hand. Typical parameters for an in-house validation are selectivity/specificity, accuracy/trueness, limit of detection (LOD), limit of quantitation (LOQ), linearity, and repeatability [3,6]. Also robustness [6], intermediate precision, and sensitivity [1] are utilized.

Capillary electrophoresis (CE) is a well-established and unique type of analytical technique based on the application of electric current to a sample injected into an electrolytic solution. The separation and identification of chemical compounds are based on the differences in the migration speed affected by the sizes and charges of the compounds in the sample. Commonly recognized advantages of CE as an analytical tool are a rapid separation procedure and low liquid volumes needed for operation [7], as well as versatility and efficiency [8]. However, the capability of CE to determine also changes in the sample matrix makes it, not only a sensitive complex tool for heterogeneous matrices, but a very interesting analytical tool in monitoring process dynamics. Typical applications of CE at present are related to the determination of nucleic acids, proteins and peptides, carbohydrates, metabolites, pharmaceuticals, cells, and organelles. Also applications related to bioaffinity, environmental analysis, and materials have been developed [9]. Typically the current applications still focus on determination of concentrations of individual chemical compounds or groups of them. The authors predict that in future the capacity of CE monitoring or identification of certain sum parameters, or process matrix dynamics will be increased. Although CE procedures are used in ever-increasing fields of applications, validation procedures of this demanding method are seldom presented in other than pharmaceutical applications.

As the CE is unique considering the amount of regulatory parameters in method development, the challenges of the technique are emphasized in the application of real samples. Variables, such as

the electric field, pH and composition of the electrolyte solution, capillary surface, temperature, etc., affect the separation and detection of compounds [7]. To overcome the challenges of multiple variables in method development, an experimental design is suggested by Orlandini et al. [10] to ensure robustness. However, the use of an experimental design for validation is seldom described in research papers concerning capillary electrophoretic methods [10]. Even in the present example method only basic 2^k factorial design was applied in optimization phase to confirm basic control parameters.

The aim of this study is to evaluate and estimate the factors affecting total uncertainty brought up by an in-house validation due an example case aiming at determination of carbohydrates. Special emphasis is made in evaluation of uncertainty factors which have the tendency to increase when analyzing real samples with heterogeneous process matrices. The example case is presented using three structurally different carbohydrate compounds, which are typically found in wood extractives and have commercial potential. Parameters which affect the separation and performance of the equipment are discussed, and the sample preparation procedures are presented briefly. This paper also presents a novel procedure for the evaluation of the primary uncertainty factors to be recognized in method development of the capillary electrophoretic determination of glucose, saccharose, and polydatin in a simple matrix, i.e. deionized water. This approach assists in the evaluation of the most influential variables or sum variables involved in analyzing real process samples, and explain the most critical factors influencing the uncertainty of the capillary electrophoretic method. In the present case, optimization of the applied method was done before the validation studies, and inter-laboratory tests were not included. The methods validated for more robust processes need to be studied further, and thus the aspects of industrial processes are emphasized in this work.

2. EXPERIMENTAL

2.1 Apparatus

This laboratory work was executed with HP 3D CE equipment (Agilent) with Agilent ChemStation software. The equipment includes a high-voltage power supply, a carousel for autosampling, an injection system, an on-capillary diode array detector, and a capillary cartridge. The introduction and injection of samples are automatized procedures in this equipment.

The separation of compounds was conducted in a fused silica capillary, which was placed in a hollow capillary cartridge with temperature control by air flow. The inner diameter of the capillary was 50 µm and the total length was 70 cm (61.5 cm effective length). Due to the configuration of the cartridge, most (50.5 cm, or 82 %) of the effective length of the capillary was temperature-controlled.

Detection was conducted with a diode array detector (DAD), which enables the detection of the same analytical run by five specific UV wavelengths with a given band widths. Also a full spectrum (from 190 to 600 nm) of the separated compounds in background electrolyte can be detected. The detection window was hand-made by burning the polyimide coating on the capillary surface with an electrical devise, and the coating residue was cleaned from the surface with methanol.

2.2 Electrophoretic procedures

The background electrolyte (BGE) for the capillary electrophoretic separation was composed of 130 mM sodium hydroxide (NaOH) and 36 mM sodium hydrogen phosphate ($Na_2HPO_4 \cdot 2\ H_2O$) in deionized water. The pH of the BGE solution was adjusted to 12.6 with NaOH.

The inner wall of the capillary was conditioned prior to analyses with a daily sequence of NaOH (0.1 M), purified water, and the BGE for 20 min, 20 min, and 5 min, respectively. Conditioning between the sample injections was performed with the BGE for 8 min. The samples were pressure-injected at 34.5 mbar for 8 seconds and detected at the ultra violet range (270 and 210 nm) by a diode array detector (DAD). The separation voltage was 17 kV with positive polarity, and the separation temperature was 25 °C, maintained with air flow inside the capillary cartridge. The carousel temperature depended on the laboratory ambient temperature, which was estimated to vary between 20 to 23 °C. Total time for detection of each separation of compounds was 50 min.

The samples were prepared by filtering (syringe filter, GHP 0.45 µm, Acrodisc, Pall) a volume of 1.5 mL into injection vials (2 mL) and sealed with plastic caps. The vials were placed on a sample carousel before the start of the sequence. All sample vials needed for the sequence were loaded simultaneously on the sample carousel. Stainless steel electrodes were cleaned with methanol before each sequence of analyses to minimize the interference of accumulated contamination.

2.3 Samples and chemicals

Calibrant glucose and saccharose were selected as example compounds for being typical representatives of the carbohydrate compounds commonly found in biomass-based process solutions. Glucose was also utilized in the previous studies of method development by Rovio et al. [11]. Polydatin (a glycoside of a stillbene) is an example of a biologically active compound, including health-promoting effects of its aglycone, resveratrol. Polydatin is commonly found in wines [12], but it has been recently detected in wood-based solutions as well [13].

Glucose and saccharose were purchased from Fluka (Buchs, Switzerland) and BDH (Poole, England), respectively. Polydatin, sodium hydroxide (NaOH) and disodium phosphate ($Na_2HPO_4 \cdot 2\ H_2O$) were from Sigma (St. Louis, MO, USA). Purified water was obtained from an Elga purification system (Centra-R 60/120, Veolia Water). All the chemicals were of the analytical grade. The reference chemicals and working solutions were stored according to the recommendation of the suppliers. The calibration samples were diluted to the appropriate concentration from stock solutions of glucose, saccharose, and polydatin (1000, 1000, and 500 mg L^{-1}, respectively) with deionized water.

The process samples were prepared in laboratory scale. The selected wood raw materials were spruce and pine, which are typical Scandinavian tree species utilized by the forest industry. The process samples were prepared from chipped wood materials by water extractions and aqueous fungal treatments.

2.4 Method validation

The suitability of the method for the intended use is done by performing a validation process. The validation of CE methods has unique characteristics caused by the principle of CE separation and details in the apparatus configuration [7]. The capillary electrophoretic method, developed originally for the determination of mono- and disaccharides [11], was applied and optimized before validation.

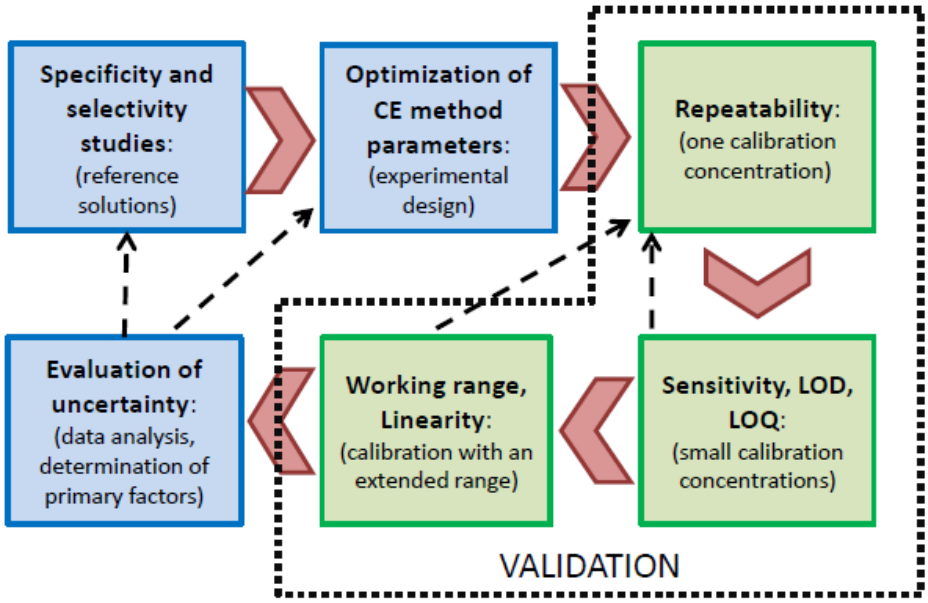

Figure 1. Schematic presentation of a practical application of an in-house validation process utilized in the evaluation of uncertainty of the capillary electrophoretic method.

For a dynamic process the time dependent variation is a critical issue, and it should be verified with a reference solution. Even after careful optimization there are two main sources for a dynamic uncertainty compound to be aware of: one arising from the equipment, and another one from process dynamics. These uncertainty compounds tend to be systematically increasing and cannot be minimized with increased repetitions, and therefore a reference solution is recommended, if target is estimation of concentration. Dynamic changes may have also effect on selectivity and specificity due to changes in migration time, physical conditions and in chemical composition. The available automation offers a possibility for continuous measurements even for days, while the reliable time window for measurements might be just few hours from calibration.

The core of the in-house validation can be evaluated after three main steps of analysis sequences (Fig. 1): measurements for repeatability; sensitivity, LOD, and LOQ and; working range and linearity. The first sequence included repetitions of the analysis of a calibration solution with 60 mg L^{-1} of polydatin (30 repetitions) and 50 mg L^{-1} of glucose (20 repetitions). In the second sequence,

the small concentrations of polydatin (1, 2, 5, and 8 mg L^{-1}) and glucose (2, 5, 10, and 20 mg L^{-1}) in deionized water were determined. The third sequence aimed at the determination of linearity and working ranges for each compound. Blank samples were determined within each sequence. The validation process and sequences are described in Fig. 1. It is worth noting that the optimization of the method had been done before the validation studies, and the time frame of a sequence was optimized based on preliminary tests.

2.5 Total uncertainty

Total uncertainty comprises individual uncertainty factors from different sources [1]. Uncertainty factors are of two types: some of the components can be evaluated statistically, while others can be defined based on experience or other information. In this work, the uncertainty was evaluated according to the Eurachem Guide of Quantifying Uncertainty in Analytical Measurement [1], including the preliminary division of sources of uncertainty according to Fig 1. The main focus was in the uncertainty originating from the CE method and equipment (repeatability, sensitivity, LOD, LOQ, working range, linearity), also in relation to real samples. Uncertainty arising from personnel, environment and sampling [3] are assumed minimal in this study.

The variance components of uncertainty factors are additive according to the equation of combined uncertainty (Eq. 1) [1]. Estimates for the values of uncertainty factors are often needed or even required for practical applications of total uncertainty calculation. According to Eq.1, the total uncertainty depends mostly on the factor which has the highest absolute value:

$$u_c = \sqrt{u_1^2 + u_2^2 + \cdots + u_n^2} \quad (1)$$

in which u_c total combined uncertainty

u_i standard uncertainty factor for i^{th} error source, i=1,2,…,n

Total combined uncertainty includes many types of variance components, which are case-dependent on the applied procedures and methods. Some of the individual components can be minimized or even discarded by method optimization, validation, and normal maintenance procedures. Some of the components discussed in this study were identified beforehand in the experimental design and method development steps.

3. VALIDATION RESULTS AND DISCUSSION

3.1 Quantitative validation

Quantitative validation is evaluated in this study based on recommendations found in the literature [1, 4, 6, 10]. For this work, factors utilized in other studies of validation of capillary electrophoretic methods were selected for closer examination. As the method optimization and validation steps are overlapping procedures in practice, also selectivity, specificity, accuracy, and sensitivity of real samples are discussed briefly in this study. Repeatability, intermediate precision, LOD, LOQ, and linearity were quantified with calibration solutions. Linearity and working range are

discussed also in the application of heterogeneous process samples. The electropherogram profiles of calibration solutions and process samples are discussed here to illustrate the practical application of the theoretical uncertainty factors. Comparison between different laboratories, i.e. reproducibility, is not quantified in this in-house validation study. Systematic errors or uncertainties will be acknowledged, but not discussed here.

3.1.1 Specificity and selectivity

In the present case, it was experimentally found out that not only the area and shape, but also the migration time of the peak changed systematically. The migration time during the measurement session showed clear linear increase as a function of the duration of measurements. The migration time for the detection of a certain chemical compound is one of the most important parameters in peak identification, because it is compound specific. It is desirable to keep it constant for each compound due to operational reasons in interpretation of measurements. However, in practice the migration time depends on several factors, and can change over a measurement session due to changes in the capillary, physical conditions or in changes in the chemical composition of the sample. Therefore the linear dependence of migration time for time can be seen a typical behavior in dynamic processes, as well as, in laboratory conditions.

In CE, selectivity is mainly controlled by the composition and pH of the background electrolyte solution. In addition other optimized separation conditions, such as capillary length, injection, voltage, and temperature, affect the selectivity [7].

Separation studies were carried out to the three compounds; glucose, saccharose, and polydatin. While glucose and saccharose are located next to each another, and can be easily misidentified with other similar compounds, the polydatin was well-separated from the other compounds, and it showed a good absorbance with the UV detector. In real samples, the specificity of polydatin is most probably affected by similar glycoside compounds, e.g. carbohydrate conjugates of stilbenes or flavonoids. However, their presence in wood-based process samples is relatively rare.

Typically, specificity and selectivity need to be determined for each compound and for each sample matrix and optimized in the method development step, i.e. before validation. In practice, and especially in the case of heterogeneous and varying process streams, this requires an iterative approach in combining optimization and validation steps. The iteration between validation and optimization can be regarded as a controlling procedure, which is desired to continue afterwards in the routine analyses.

3.1.2 Repeatability and intermediate precision

Method repeatability was evaluated from the detected electropherograms of calibration solutions gained from a repeated procedure in limited time intervals. In the example case experiments the repeatability of the migration time was determined by analyzing the reference sample (60 mg L^{-1}) 20 times in a sequence. The migration time was found to change more (7 %) in the case of polydatin, which is slower to migrate due to its higher molar mass. The migration times of the two other

compounds changed (in total) between sequences 1.9 and 0.6 min for polydatin and glucose, respectfully.

In this example case the repeatability estimated based on 3 repeated measurements over a 6 hours period of normal operating conditions (NOC) with the 95 % limits was 2.2 % for polydatin at 210 nm (Fig. 2). However, several factors have an effect on it. Fig. 3.A illustrates an example that after uncontrolled breakdown the uncertainty level stays high for over 10 hours, and even after that the standard deviation stays near the upper limit (Fig. 2.A). The lack of negative deviation indicates systematic change and increasing trend in measurements. The relative standard deviation of the peak area tends to increase outside the variance limits. An example illustrating that uncertainty is increasing after 24 hours continuous run is shown in Fig. 2.A. The Fig. 2.A shows relative standard deviation for the peak area. Fig. 2.B illustrates similar behavior with measured peak area. The peak area seems to have slowly increasing trend, until after 18 hours the area suddenly increases over the upper control limit. Other research groups [14, 15] have reported repeatabilities by RSD values from 1.0 to 4.1 % in CE determinations of saccharides, which are in consensus with values of the present example namely when RSD is reported as mean value of 3-5 repeated measurements in NOC.

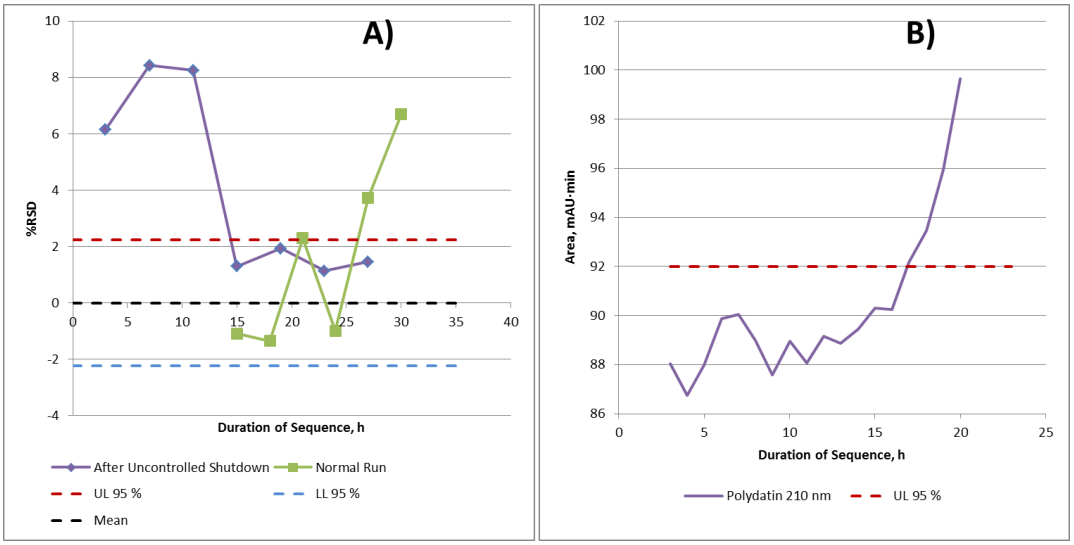

Figure 2. A) Relative standard deviation (%RSD) of the mean for polydatin samples with 95 % confidence limits, and B) area of polydatin peaks. Upper (UL) and lower limits (LL) are selected for confidence level of 95 %.

In addition to the detected response area of a compound peak, the evaluation of repeatability of the applied CE method can be evaluated by including the repeatability of the migration time. This approach is justified by the practical application of monitoring heterogeneous process samples and the importance of the peak identification in real samples. Although the migration time is typically adjusted

by utilizing internal standards and calculations [16, 17] the approach is not practical in the case of monitoring process samples, but add steps in the samples pretreatment and the interpretation of measurements. They are also targeted for individual compounds, but leave out useful information from the matrix profile.

In this study, intermediate precision (the long-term variability of the measurement process) was evaluated by comparing calibration data of sequences from separate days with paired T-test for independent samples. The intermediate precision of the calibration curves was calculated to be between 7 and 15 % depending on time interval between calibrations. In the study of Sarazin et al [16], the intermediate precision of saccharide determinations was calculated to be 4.5 % before normalized corrections of peak areas.

3.1.3 Limit of detection and limit of quantitation

Limit of detection (LOD) was defined based on the standard deviation of blank samples. This integrated magnitude of analytical background response from five repeated measurements was treated as "noise", which was multiplied with a chosen S/N ratio, i.e., constant, *k*, to gain a "signal" area to define LOD [1, 11, 16]. In the present approach LOD was confirmed with visual inspection of electropherograms, and with test samples in the specific concentration range near LOD.

In the case of CE, the integrated area is utilized for determination of LOD and LOQ (limit of quantitation) instead of peak height, because the peak height decreases as the width increases within repetitions [18]. Baseline noise was found to be both similar and constant for each wavelength, which also supported the utilization of a peak area. However, defining the values for LOD and LOQ empirically for a large number of chemical compounds by analyzing several samples is considered time-consuming. In routine work, it would be preferable to utilize a mathematical approach based on peak areas and calibration curves or sensitivity coefficients.

The primary optimization for LOD area was made with polydatin. However, after evaluation the same LOD area was utilized also for the determination of the other compounds in the electropherogram. This method of determination was expanded to other detection wavelengths, and the concentrations of LOD were calculated from the calibrations. Table 2 shows LOD values gained with this procedure and evaluated with visual detection of a small set of test samples. The constant, *k*, for LOD was experimentally set to three.

The LOD of polydatin was calculated to be 2.2 and 1.1 mg L^{-1} at 270 and 210 nm, respectively, with the presented method. The LODs for glucose and saccharose were calculated to be about four times higher than for polydatin, which is reasonable due to lower sensitivities. Sensitivity coefficient increases with the increasing migration times of compounds. The increasing magnitude of the sensitivities indicates that the compound of higher molecular weight benefits from the long migration time, in these method conditions. The determined sensitivity coefficients along with calculated LOD values for saccharose, glucose, and polydatin are presented in Table 1. The correlation coefficients (R^2) of the equations are above 0.99 for each determined compound (Table 1), which can be considered a good correlation in CE analyses. Also the low p-values in Table 1 showed the extremely high

significance of the calibration equations. Comparison of sensitivity coefficients in Table 1 and in Fig. 3 present that the method was more sensitive in the case of polydatin than it was in the case of glucose or saccharose.

Table 1. Sensitivities (standard uncertainties in parenthesis), p values, correlation coefficients (R^2), and calculated limits of detection (LOD) of reference compounds.

Compound (detection wavelength, nm)	Migration time* (min)	Sensitivity coefficient**	p value	R^2	LOD (mg L^{-1})
Saccharose (270)	12.3	0.235 (0.01)	6.1×10^{-16}	0.9916	9.4
Glucose (270)	14.4	0.286 (0.01)	8.1×10^{-17}	0.9937	7.7
Polydatin (270)	25.8	0.985 (0.02)	1.5×10^{-16}	0.9931	2.2
Polydatin (210)	25.9	1.94 (0.04)	1.2×10^{-17}	0.9952	1.1

*: from an example electropherogram (10% variation was allowed)

**: three repetitions

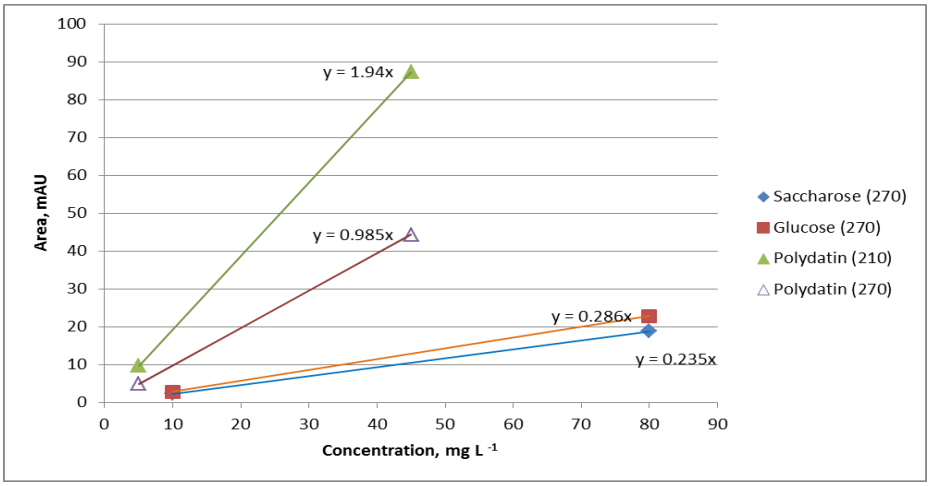

Figure 3. Correlation equations of peak area as function of concentration for glucose and saccharose at 270nm. Correlation of polydatin at 210 and 270 nm.

The LOD and LOQ values depended highly on the detection wavelength, which were in consensus to the fact that different chemical carbohydrates give different UV absorption at different wavelengths. [11, 17]

LOQ is typically defined as multiplying standard deviation of blank by 10. The main definition for LOQ is that it should be statistically distinguished from blank with some confidence limit. In the present case it was found out that peaks having area 5·blanks area, could be significantly distinguished from background at risk level of 5 %. In an alternative approach the empirically determined relative

standard deviation (%RSD) can be determined based on repeated samples and plotted versus calibrant concentration. LOQ is then defined according to the precision desired. Fig. 4 presents an example of determination of LOQ for polydatin, where samples having small concentration have been analyzed and the empirical %RSD plotted as a function of concentration. A curve has been fitted into the data and it can be utilized in the estimation of LOQ. If the LOQ uncertainty value is set to be e.g. 2.5%, the minimum concentration for LOQ would be 11…12 mg L^{-1}. On the other hand, if the uncertainty is set to be 5%, the minimum concentration would be 6 mg L^{-1}. Comparing results with the LOD (2.2) presented in Table 1, it can be suggested that LOD values are to be multiplied with a constant between 3,…,6 to gain LOQs (constant to multiply the blank area would be 9…18). The relationship seems reasonable.

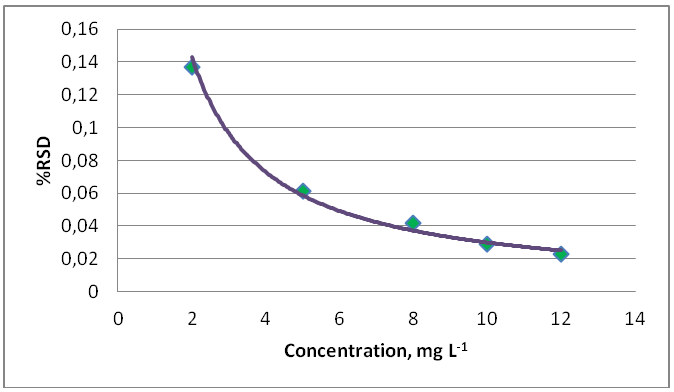

Figure 4. Definition of LOQ with RSD%. RSD% in the function of concentration of the calibration solution. %RSD was calculated from 5 parallel samples.

3.1.4. Linearity and working range

In the present case, the working range was determined from an expanded range of calibration concentrations, emphasizing the concentrations on the lower and upper ends of the calibration [6]. The recommended working range of the calibration of polydatin was determined from single compound solutions with three repetitions. The working range was finally determined from the calibration curves to be from 5 to 45 mg L^{-1}. The working range of polydatin is narrower than the working range of saccharose and glucose. This indicates that the compounds of lower sensitivity coefficients possess the advantage of wider working range in practice. The first, second, and third injections of calibration solutions were compared, and it was noticed that the uncertainties increased with repeated injections. Thus, the maximum amount of repetition of injections can be recommended to be three. This gave the relative deviation 0.75-1.5% in the normal working conditions. The concentration was detected also outside the working range, but the uncertainty of the measurement increased outside the limits. This suggests that there occurs a critical maximum concentration where the standard deviation of instrument response increases. Linear area for the example compound would have been wider (5…80 mg L^{-1}). In

process applications, the working range is more useful from the uncertainty point of view, as the total analysis time limits the amount of sample repetitions.

3.2 Application to process samples

The in-house validated method was applied to samples representing aqueous process samples of wood-based materials in order to evaluate the uncertainty in analyzing real samples. The samples were selected on the basis of differences in the raw materials and pretreatments used. In natural wood extracts the observed sugar composition is highly dependent on the process conditions during the extraction processes. Temperature, pH and processing time cause alteration in the polymeric structure of sugars and hemicelluloses [20]. In bioprocessing the selection of bacterial strain has an effect on the utilization of carbohydrates or wood extractives as energy sources for bacteria [21].

In general, the presence of various carbohydrate-type compounds in wood-based process samples can be expected [22]. In this study, the example electropherograms of four process samples (Fig. 5) illustrate some of the differences in the water-soluble carbohydrate profiles of wood-based process samples. The signals are from water extraction samples of pine phloem flour (A), pine white wood (B), and spruce white wood (C). Signal D is from a fungal treatment process with spruce white wood as raw material.

In addition, visual interpretation of the sample electropherograms is important in the in-house validation phase of method development. Besides the possible presence of identified compounds, the profile electropherograms of carbohydrates of four example samples (Fig. 5) include additional information of the sample matrices. The amount of detected peaks shows the range of different carbohydrate compounds in the sample, and the peak areas are an indication of the concentrations of the detected compounds. The electropherograms of the profiles of the process samples typically include more detected peaks than the calibration solutions, indicating the presence of a larger range of compounds in the sample. The compound peaks were preliminarily identified by comparing the total profile to the migration times. Overlapping peaks were present in the carbohydrate profiles of wood-based samples, originating partly from the raw material. Unit processes, e.g. filtration or enzymatic treatments, affect also the carbohydrate profiles [17, 20].

In addition to the determination of the traditional validation factors, the total evaluation of the sample electropherograms was applied for the process samples. Differences in raw materials, e.g. specific part of wood (Fig. 5.A and B) or wood species (Fig. 5.B and C) were detected. Overlapping peaks add uncertainty to the identification and quantitation of the desired compounds, especially in higher concentrations. Four overlapping peaks migrate near glucose in the pine phloem sample (Fig. 5.E) around 15 min, which is an indication of the presence of structurally similar compounds. This group of structurally similar compounds, i.e. monosaccharides, can be combined to a summary variable. The number of detected peaks at the migration time zone of monosaccharides (from 14 to 17 min) is higher in the phloem sample than in the white wood sample (Fig. 5.B). Differences in the water-soluble carbohydrate composition of pine (Fig. 5.B) and spruce (Fig. 5.C) white wood are clearly present in the profiles around the migration time of 18 min. This was concluded based on the

fact that the peaks are of different sizes and also the migration times are different. Signals C and D (Fig. 5) originate from the samples of the same raw material but have been prepared through different types of processes.

The specificity and selectivity of separating the desired compounds in the process samples were lower compared to the calibration solutions. The determination of migration time combined with total profile interpretation assisted in peak identification, particularly in the case of process samples. Consecutive characteristics in the profiles of the process samples (Fig. 5.G) illustrate typical examples of the fluctuation in migration time between day-to-day analyses. Indications and consequences of disturbances on the detection signal during the screening of the carbohydrate profiles of samples were acknowledged. Profile B in Fig. 5 includes an example of two peaks, indicating unexpected disturbance in the signal around 18 min (Fig. 5.F). The disturbance peak is sharper and narrower than the typical example of a detected compound peak at 19.5 min.

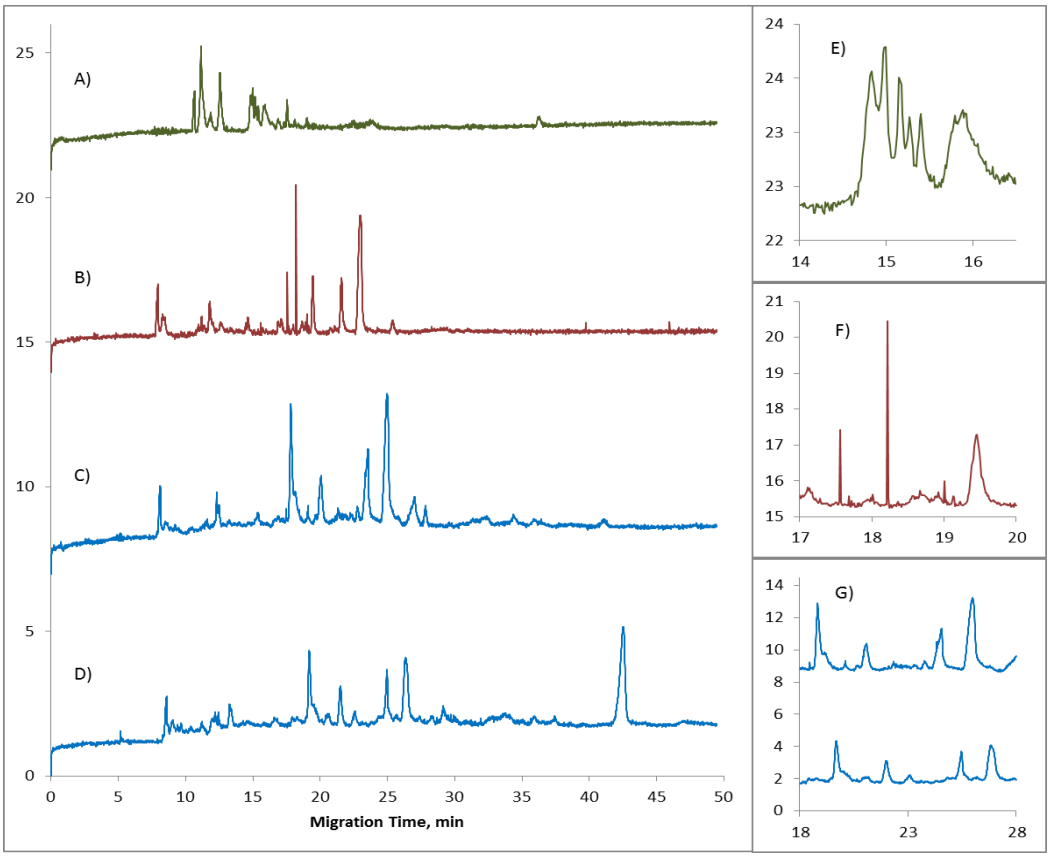

Figure 5. Example electropherograms of typical process samples. A) water extraction of pine phloem, B) water extraction of pine white wood, C) water extraction of spruce white wood, and D) microbial treatment of spruce white wood. Small figures E), F), and G) are zoomed from electropherograms A), B) and C&D).

The probability of detecting several carbohydrate compounds in sample matrix is high in this type of process samples [21, 22]. On the other hand, overlapping peaks are a very probable source of uncertainty. However, the method can be used for preliminary screening before more targeted method development for e.g. specific monosaccharides or wood grade classifications, but the evaluation of uncertainty needs to be included in all phases of development.

3.2.1 Uncertainty components in process samples

From the process point of view, the most critical factors affecting uncertainty, after appropriate method optimization, were repeatability, intermediate precision, and working range. Repeatability and intermediate precision are critical factors [6, 8, 16] especially for methods aiming at routine analysis of process samples. The uncertainty components affecting validation factors of the specific CE method are listed in Table 3. The estimations of these two uncertainty terms can be utilized in the evaluation of the process, even though the values are higher for process samples than for calibration solutions. The optimization step included the evaluation of accuracy, specificity, selectivity, and robustness. The background electrolyte solution composition and pH, along with other method parameters, have been optimized by applying the experimental design.

Sensitivity, LOD, and LOQ are useful in detecting changes in the sample streams, but the effects of ambient conditions on the performance of the equipment are crucial. For example, disturbances in the power supply or breaking of the capillary may lead to a shut-down of the equipment. From additional studies it was concluded that a shut-down affects not only the present sequence but also the first sequence after the start-up, which emphases the adequate maintenance procedures and analyzing appropriate control samples regularly.

Table 2. Recommended upper limits (UL) for the most important uncertainty components from the in-house validated CE method applied to real samples.

Validation factors	Components of uncertainty (estimated value)	Upper limit (UL) for uncertainty u_i
Selectivity and specificity	- Compound characteristics - Method parameters - Sequence duration (<10 %) - Background electrolyte solution (pH and composition) - Sample matrix	3-5 %
Repeatability and intermediate precision	- Ambient conditions (<5 %) - Stability and storage conditions of the compounds, chemicals, and solutions (<1%)	2.5 % 1 %
LOD and LOQ	- Compound characteristics - Method of detection - Separation efficiency (<15%) - Peak identification and	2 % (total uncertainty factors from calibration)

	integration (<10%) - Baseline noise (<5%)
Linearity and working range	- Calibration correlation - Concentration and type of matrix and separated compounds - Sample loading

The uncertainty components of LOD, LOQ, linearity and working range are partially overlapping (Table 2) and thus, the upper limit for total uncertainty is combined as uncertainty of calibration. The recommended upper limits of uncertainty factors in Table 2 lead to 6 % of total combined uncertainty (u_c) in the CE determination of carbohydrates in heterogeneous process samples. Extended uncertainty [1] would be 12 %, in this case. This seems a reasonable estimate for a process sample. Most significant factor (UL 5 %) affecting total uncertainty was found to be the sequence duration in the validation of selectivity and specificity. For example, if the equipment is not taken care of properly or the method is not adjusted to ambient changes, the uncertainty can easily be doubled. On the other hand, if the sequence duration is verified in the optimized method and ambient conditions, the total combined uncertainty can be decreased to even 3 %.

4. CONCLUSIONS

In this study, the uncertainty factors identified via the in-house validation procedure were successfully evaluated. It can be concluded that in the case of process samples the most significant uncertainty factor, in addition to heterogeneity, was the duration of the analysis sequence. The authors recommend the sequence duration to be optimized with reference solutions for each process and sample type. However, monitoring should also cover control procedures for identifying and monitoring process conditions requiring re-optimization of the sequence duration. Unlike equipment performance and practical operating skills, the critical uncertainty factors cannot be totally neglected at any circumstances, and, when occurring they have a destructive effect on the reliability of the measurement results. The application of real process samples indicated that the method has promising characteristics in the monitoring of heterogeneous process streams. Acknowledging the uncertainty factors through the in-house validation procedure sets ground for a wider range of CE methods to be applied in process analytics. Further studies will concern e.g. additional optimization or the robustness of the detection method, and more advanced evaluation of wood-based fractions.

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Publication III

Kaijanen, L., Metsämuuronen, S., Reinikainen, S.-P., Pietarinen, S., and Jernström, E.,
Profiling of water-soluble carbohydrates in pine and spruce extracts by capillary zone electrophoresis with direct UV detection

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ORIGINAL

Profiling of water-soluble carbohydrates in pine and spruce extracts by capillary zone electrophoresis with direct UV detection

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Abstract Monitoring wood-related biorefinery process streams is important as the composition of raw material and its variations have a great impact on the production outcome as well as on the process control. In this paper, the applicability of a simple type of capillary zone electrophoresis method to profiling heterogeneous wood-based samples was studied by grouping the peaks in the overall electropherograms. A comparison between 210 and 270 nm as an example confirmed the identification of a monosaccharide peak in the carbohydrate electropherogram. The analytical method applied in this study was found suitable, firstly, in profiling the samples based on correlation of multiple detection wavelengths and, secondly, in characterizing the total carbohydrate composition by grouping detected peaks by the compound type. Compounds were detected in their extracted form without time-consuming pretreatment procedures. Differences in the raw material and process conditions were successfully identified by comparing characteristic peaks in the sample electropherograms of an example case.

Introduction

Monitoring biorefinery process streams is important as the composition of the raw material and its variations have a great impact on process parameters concerning, e.g., equipment and on the production yield and quality. The general requirement for the monitoring method is to show changes in predefined parameters fast and reliably. On the other hand, the absolute level of a parameter can be defined with

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more accurate laboratory measurements. Secondly, the monitoring method should be able to alert when there are unwanted compounds in the process. There is still a lot of research needed in determining the characteristics of heterogeneous process streams in biorefining.

All wood materials are considered challenging raw materials due to their differences in structural parts and heterogeneous composition (Liu 2010). As the actual composition of the raw material varies from batch to batch, and the process conditions also fluctuate, monitoring of the process requires the application of analytics capable of adjusting to the changes in the process stream. Variation may occur with different raw material, for example, as a contamination of softwood raw material with hardwood raw material, presence of bark due to insufficient debarking (Kemppainen et al. 2014), or degradation of woody material due to inoculation with fungus (Sirén et al. 2014). In addition, small changes in process conditions, such as pH, temperature or residence time, can cause alterations in the final product (Wei et al. 2011). In order to monitor these changes, a robust and fast analysis method is needed.

Wood-based water extracts include a wide variety of carbohydrate compounds, which are separated, utilized or fragmented as the overall process proceeds. Monitoring these reactions enables the evaluation of the raw material composition as well as the process chemistry. Controlling the process outcome requires in-depth knowledge of the chemistry within the unit processes. In order to understand the chemistry and the degradation or synthesis of the carbohydrate compounds in practice requires selection of suitable analytical tools. Industrial scale synthesis of bio-based chemicals is of commercial interest for biorefineries. The reaction mechanisms of glucose and xylose during hydrothermal pretreatment have recently been reviewed by Rasmussen et al. (2014) with the conclusion that a wide range of possible routes of degradation and synthesis exists and that the product profile is mainly dependent on the reaction conditions (Rasmussen et al. 2014).

In some cases, the total concentration of carbohydrates is sufficiently determined by UV spectrophotometry. The applicability of the method is, however, restricted to samples that have no UV absorbance in their native state and require pretreatment (Albalasmeh et al. 2013). However, as the bioprocesses considered here are typically combinations of unit processes, the aqueous process streams also need to be monitored as the overall process proceeds. Today, typical analytical methods applied to characterize aqueous wood-based process samples are liquid chromatography (HPLC), size-exclusion chromatography (HPSEC) (Rivas et al. 2013), anion-exchange chromatography (HPAEC) and gas chromatography (GC) (Willför et al. 2009), which, however, are highly selective concerning compounds and, thus, limit their applicability to profiling analysis.

Capillary electrophoresis (CE), as a versatile technique, enables determination of compounds in their natural state in the sample (Oliver et al. 2013; Hyvärinen et al. 2014). CE is a well-established analytical method with various applications in the fields of pharmaceutical, environmental and food analysis (Altria 1999; Whatley 2001; Piñero et al. 2011; Deeb et al. 2014). The technique is based on applying high electric voltage over a background electrolyte solution to separate any charged compounds (Altria 1999). The separation of the compounds depends on the friction

(size)-to-charge ratio of the compounds. Most common detection methods used in CE rely on the UV absorbance of the compounds. The equipment is fully automated from sample injection to data processing (Whatley 2001). The advantages of CE in general are low reagent consumption, speed and high separation efficiency (Sarazin et al. 2012; Altria 1999).

A recent review by Deeb et al. (2014) showed that the number of advances and applications to pharmaceutical analysis by CE is continuously increasing. Method development strategies, validation and instrument qualification can be used for overcoming challenges related to low sensitivity or precision (Deeb et al. 2014), but these are not the main concerns in the process analyses considered here. Requirements for monitoring high-volume bulk processes differ from those for monitoring highly regulated, sophisticated processes, such as pharmaceutical chemicals. A holistic view of the sample matrix is emphasized when analyzing process samples, because it yields additional information on how the process proceeds, whether the monitoring is subjected to a continuous or a batch process (Deeb et al. 2014; Krogell et al. 2013).

A number of studies focusing on real samples with a CE method have recently been carried out (Oliver et al. 2013; Rovio et al. 2008). However, the majority of the scientific papers on CE concentrate on the possibilities of the different methods through the separation of different analytes. The matrix effects appear especially in cases where sample pretreatments are inadequate for the utilized method conditions, which can be altered, for example by dilution solutions, background electrolyte or capillary wall (Piñero et al. 2011). These aspects should be taken into consideration when applying a CE method to real process samples. Evaluation of the sample matrix as thoroughly as possible helps in identifying the causes for changes in the process stream.

In addition to the carbohydrates in the hemicelluloses, wood materials also contain various carbohydrates bound to phenolic compounds, which together form glycosides (Table 1). The phenolic compounds related to carbohydrates are considered important as their structure partly determines their activity in the process. Phenolic compounds are mostly analyzed by gas chromatography (GC) with flame ionization detection (FID) or gas chromatography–mass spectrometry (GC-MS). Typical methods for identifying and quantifying phenolic compounds require partially destructive pretreatments and determine only aglycone structures, which, thus, may leave out, e.g., information related to possible compound activity in the process.

The aim of this research was to clarify the wide potential of the CZE method in providing the required results of both individual compounds and overall carbohydrate profiles, simultaneously. This paper contributes to the field of monitoring aqueous processes of wooden raw material by presenting an extended approach to the profiling of carbohydrate matrices by CZE. The new approach includes a distinction from previous studies in the form of higher number of detected compound types. Novelty is also presented regarding commercially available equipment by showing the advantages of interpreting the recorded migration times and the correlation of multiple wavelengths in identifying compounds.

Table 1 Phenolic compounds determined from pine and spruce raw materials

Raw material	Part	Compounds (References)
Scots pine	Stemwood (debarked)	Stilbenes, pinocembrin (Willför et al. 2003b), total phenolics (Willför et al. 2003a; Venäläinen et al. 2004; Harju et al. 2003; Hovelstad et al. 2006; Kähkönen et al. 1999), total pinosylvins (Venäläinen et al. 2004; Hovelstad et al. 2006; Lindberg et al. 2004; Pietarinen et al. 2006), pinosylvin, PMME, PDME3 (Karppanen et al. 2008; Valentín et al. 2010), lignans (Willför et al. 2003a; Pietarinen et al. 2006), oligolignans, 3,4-dihydroxybenzoic acid, catechin, vanillic acid (Valentín et al. 2010)
	Bark	Total phenolics (Kähkönen et al. 1999; Stolter et al. 2009; Kanerva et al. 2008), apigenin-7-glucoside, catechin, catechin derivative, gallocatechin, dicoumarylastragalin, quercitrin, dicoumarylisoquercitrin derivatives (Stolter et al. 2009; Roitto et al. 2008), mono-coumaroyl-isoquercitrin derivatives, quercetin-3-galactoside, kempferol-3-glucoside, taxifolinmonoglycoside (Stolter et al. 2009), benzoic acid glycoside (Stolter et al. 2009; Kanerva et al. 2008; Roitto et al. 2008), condensed tannins, myricetin-3-galactoside, hyperin, neolignans (Roitto et al. 2008)
	Needles and twigs	Taxifolin, taxifolin-3′-glucoside, quercetin, quercetin-3-glucoside, quercetin-3′-glucoside (Oleszek et al. 2002), total phenolics (Willför et al. 2003a; Malá et al. 2011 Zeneli et al. 2006), astringin (Hammerbacher et al. 2011; Zeneli et al. 2006), isorhapontin, piceid (polydatin), catechin (Zeneli et al. 2006), lignans (Willför et al. 2003a)
Norway spruce	Stemwood (debarked)	Lignans, stilbenes, flavonoids (Välimaa et al. 2007), astringin (Hammerbacher et al. 2011; Evensen et al. 2000), isorhapontin (Hammerbacher et al. 2011), phenolic acid glycosides of phenolics (Malá et al. 2011), hydroxymatairesinol (Välimaa et al. 2007), catechin, taxifolin, resveratrol (Evensen et al. 2000)
	Bark	Total phenolics (Kähkönen et al. 1999; Kanerva et al. 2008, Zeneli et al. 2006; Ignat et al. 2013), astringin (Hammerbacher et al. 2011; Zeneli et al. 2006; Solhaug 1990; Latva-Mäenpää et al. 2013; Viiri et al. 2001; Lieutier et al. 2003), isorhapontin, isorhapontigenin (Solhaug 1990; Latva-Mäenpää et al. 2013; Viiri et al. 2001; Lieutier et al. 2003), catechin, epicatechin (Zeneli et al. 2006; Ignat et al. 2013; Evensen et al. 2000), taxifolin, taxifolin glycoside (Evensen et al. 2000; Lieutier et al. 2003), vanillyl alcohol, vanillic acid (Lieutier et al. 2003), piceid, resveratrol (Latva-Mäenpää et al. 2013; Viiri et al. 2001; Lieutier et al. 2003), total stilbene glycosides, total stilbene aglycones (Viiri et al. 2001), flavonoids, flavonols, gallic acid, tannins (Ignat et al. 2013)
	Needles and twigs	Total phenolics (Kähkönen et al. 1999; Stolter et al. 2009; Kanerva et al. 2008), condensed tannins (Stolter et al. 2009; Kanerva et al. 2008), catechin, protocatechuic acid, dicoumarylastragalin, taxifolinmonoglycoside, dicoumarylisoquercitrin derivative, chlorogenic acid derivatives, vanillic acid derivative, picein, picein aglycone (Stolter et al. 2009), astringin (Solhaug 1990)

Materials and methods

Chemicals and reference solutions

The reason for the selection of reference materials was their potential importance in wood-based processes. Each selected reference compound represents a larger range of similar types of possible compounds. For example, furfural is an example of a degradation product of xylan that forms as a result of pH variation in the process. Inositol, saccharose and glucose are examples of possible products (Holmbom 2011). Di- and monosaccharides are present in hydrolyzed hemicelluloses extracts, and uronic acids indicate side cleavage of hemicelluloses polymer. Polydatin is regarded as a marker of bark extractives. Glucose was used as comparison for another application of the CE method (Rovio et al. 2007). Molecular masses and pK_a values of the selected reference compounds are presented in Table 2.

Glucose (99 %) was purchased from Fluka (Buchs, Switzerland), and saccharose (99 %) and inositol (99 %) were purchased from BDH (Poole, England). Furfural (99 %), polydatin (95 %) and glucuronic acid (98 %) were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide (NaOH, 97 %) and disodium phosphate ($Na_2HPO_4{\cdot}2H_2O$, 99 %) were also purchased from Sigma. Water was obtained from an Elga purification system (Centra-R 60/120, Veolia Water). Reference solutions were prepared from the stock solutions and diluted into concentrations of 5–150 mg L^{-1} with deionized water.

Capillary electrophoresis procedure

The reference solutions and hydrolyzed wood samples were analyzed with a HP 3D CE system (Agilent). The total length of the capillary was 70 cm (61.5 cm effective length) with an inner diameter of 50 μm. The pH of the background electrolyte solution (BGE) (36 mM sodium hydrogen phosphate, $Na_2HPO_4{\cdot}2H_2O$ and 130 mM sodium hydroxide, NaOH) was adjusted by addition of solid NaOH to pH over 12, measured by Orion model 410A pH meter (Thermo Scientific, Waltham, MA, USA) with a pH electrode model 622–1782 (VWR, Radnor, PA, USA).

Table 2 Molecular masses of the compounds in reference solutions

Compound type	Model compound	M (g mol^{-1})	pK_a [T] (References)
1. Furan derivative	Furfural	96.1	NA
2. Sugar alcohol	Inositol	180.2	12.9 [ND] (Sarazin et al. 2011)
3. Disaccharide	Saccharose	342.3	12.51 [25 °C] (Lagane et al. 2000)
4. Monosaccharide	Glucose	180.2	12.35 [25 °C] (Rovio et al. 2008)
5. Polyphenol glucoside	Polydatin	390.4	9.40 [ND] (Zhang et al. 2009)
6. Uronic acid	Glucuronic acid	194.1	3.20 [25 °C] (Zemann et al. 1997)

NA not available

To condition the capillary, it was flushed daily with a sequence of 0.1 mol L^{-1} NaOH, purified water and the BGE (20, 20 and 5 min, respectively) prior to analyses. Between analyses, the capillary was rinsed with the BGE for 8 min. Samples were pressure-injected at 34.5 mbar for 8 s and detected at wavelengths of 210 and 270 nm by a photodiode array detector. The separation voltage was 17 kV (positive polarity). The separation was conducted at 25 °C, and the temperature was regulated with air flow. All samples were filtered before analysis (Acrodisc PSF, GHP 0.45 μm, Pall). All reference solutions and samples were analyzed three times. Peaks were manually integrated from the sample electropherograms utilizing the tools in the software (ChemStation, Agilent Technologies, Palo Alto, CA, USA).

Samples

After preliminary method development and validation studies (Kaijanen et al. 2014, 2015), the CE method was applied to the liquid samples produced from wood-based materials. These samples of hydrolyzed softwood were considered to be case examples and representatives of the primary and secondary streams of processes in forest products industry (Holmbom 2011) either currently utilized or under research to be applied in the future.

Liquid samples in the example case were obtained from laboratory-scale extraction experiments. Experiments to gain the samples were run in two types of reactors with variations in the operating parameters, i.e., solid/liquid ratio, temperature, reaction time and agitation. Samples were made either in Erlenmeyer flasks with 5 g of wood material in 100 mL of deionized water or in a batch reactor with 15 g of wood material in 500 mL of deionized water. Flasks were placed on a shaker for the duration (4, 24, 48 and 168 h) of the extraction, and agitation was induced with a blade in the batch reactor. Ambient or near-ambient temperatures were applied due to the fungal treatment. Raw material species were either spruce (*Picea abies*) or pine (*Pinus sylvestris*) and included white wood consisting of the trunk without bark and branches. The added fungal material (1 g in 100 mL of deionized water) was a commercial product of dried oyster mushroom (*Pleuretus ostreatus*) (Hankintatukku Oy, Helsinki, Finland).

Results and discussion

CE profiles of carbohydrates

The carbohydrate electropherograms were profiled by dividing the characteristic peaks into peak groups A, B and C. The identification of, e.g., furfural, inositol, glucose, fructose, glucuronic acid and polydatin was conducted by reference solutions in preliminary studies. Migration times and the order of different carbohydrate-related compounds were determined from the profiles. The different types of carbohydrates were grouped by the migrating order: (A) furan derivatives (1) and sugar alcohols (2), (B) mono- (4) and disaccharides (3), and (C) uronic acids

(6) and glycosides (5). Glycosides were observed to migrate partially overlapping the uronic acids.

Profile electropherograms of samples, taken after 24, 48 and 168 h of water extraction of pine with added fungus, are presented in Fig. 1. To clarify Fig. 1, only peak groups have assignments, because each of the identified groups consists of several separated peaks. The three electropherograms of the batch process illustrate the change in the carbohydrate composition as the process proceeds. Group A included one major peak in addition to two smaller ones in the 24-h sample. Group B included also a major peak which was distinctively smaller in the 48-h sample and absent in the 168-h sample. Group C, however, showed an increase in the size of certain peaks and decrease in the size of other peaks during the process.

Migration time and peak area

As the actual migration time varies from run to run and sequence to sequence (Sarazin et al. 2011; Oliver et al. 2013), applying the electropherogram profiling method was studied. Emphasizing the industrial aspects of practical utilization of CZE, in this case, the readily available migration times in the electropherograms were used for the characterization of type of compounds, although the calculation of electrophoretic mobility is scientifically accurate procedure for the identification of individual compounds (Weinberger 2000). Peaks in the electropherogram were used

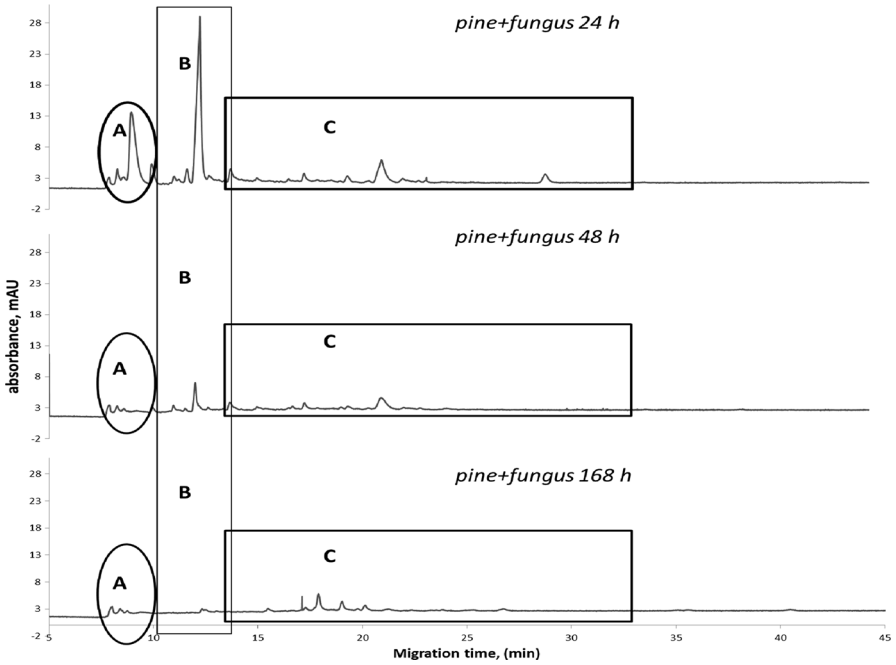

Fig. 1 Electropherograms of chipped pine extracted with a mixture of water and fungus (oyster mushroom) for 24, 48 and 168 h. Compound groups in the profile: *A* furan derivatives and sugar alcohols, *B* mono- and disaccharides and *C* uronic acids and glycosides

Table 3 Manually integrated peak areas of the compound groups in electropherograms of three pine samples extracted with water and added fungus

Pine + fungus	Area (mAU min[a])			
Extraction time (h)	A	B	C	Tot
24	310 ± 19[a]	500 ± 30[a]	310 ± 9[a]	1120 ± 67[a]
48	30 ± 2[a]	90 ± 5[a]	280 ± 7[a]	400 ± 24[a]
168	30 ± 2[a]	20 ± 1[a]	270 ± 6[a]	320 ± 19[a]

Compounds in the groups are as follows: (A) furan derivatives and sugar alcohols, (B) mono- and disaccharides, and (C) uronic acids and glycosides

[a] Uncertainty (6 %) based on analytical validation (Kaijanen et al. 2014)

in forming groups of which the peak area is to be monitored, e.g., as the process proceeds (Fig. 1). This was done because process samples typically contain a range of different organic compounds.

In addition to electropherogram profiles of the example samples, the total area of carbohydrates was found to decrease during the time period (from 1120 to 320 mAU min) as the area of the uronic acids and glycosides stayed at the same level (Table 3). As the peak area is related to the compound concentrations in the sample, the concentration of furan derivatives and sugar alcohols (Group A) decreased during the extraction from 24 to 168 h. After 168 h, the peak area of mono- and disaccharides (Group B) was considerably decreased compared to the 24-h extraction result. Peak areas of compounds in Group C (uronic acids and glycosides), on the other hand, did not show a significant change during the same time period. However, the change in the compound distribution of Group C was visible in the electropherogram profiles of the samples (Fig. 1). For further identification of compounds and their concentrations, a reference method is essential (Oliver et al. 2013; Sarazin et al. 2011).

Additional wavelengths

The specificity and selectivity of desired compounds depend on the detection: Wavelengths of the strongest possible absorbance in the selected background electrolyte solution are preferred. The detection wavelength of maximum absorbance for each compound can be measured with a UV spectrophotometer. This, however, does not necessarily take into account the effect of the BGE or other separation conditions inside the capillary. Evaluation of the most suitable wavelengths for simultaneous detection of several types of compounds is typically a compromise between optimized wavelengths.

In this study, the obtained electropherograms were compared to the principle figures (Fig. 2) of UV spectra of glucose, glucuronic acid and polydatin found in the literature (Rovio et al. 2007; Zhang et al. 2007; Xia et al. 2011). Polydatin typically shows a characteristic UV spectrum with the highest absorbance at about 310 nm. At 270 nm, which is the optimal detection wavelength of glucose (Fig. 2), the

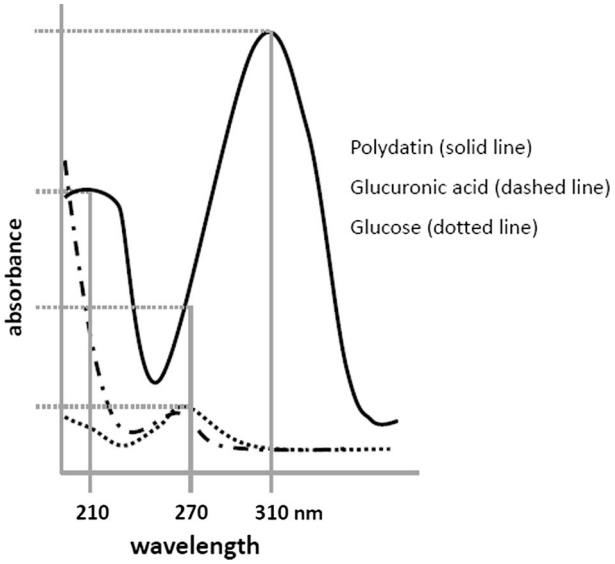

Fig. 2 Characteristic UV spectra of polydatin, glucose and glucuronic acid (Zhang et al. 2007; Sarazin et al. 2011; Xia et al. 2011)

absorbance of polydatin is approximately half of the absorbance at 210 nm. Differences in absorbance of these two compounds are clear; the maximum absorbance of polydatin is about seven times higher than the maximum absorbance of glucose. UV absorbance of glucuronic acid at 270 nm is similar to that of glucose (approximately 10 mAU for both). The difference, however, can be seen at 210 nm where glucose shows very low absorbance, but glucuronic acid absorbance is at least three times higher than the absorbance at 270 nm. In this case, the differences in the UV spectra of selected compounds are clear. However, similar compound types migrating close together have also similar UV spectra. For example, the UV spectra for the photo-oxidized products of mannose, galactose and glucose are the same, which restrict their identification, e.g., in wood-based samples.

In CE, identification of the analyte is done by comparing the electropherogram of a sample with the electropherogram of a reference solution. Typically, the identification is confirmed by spiking the sample with a known amount of the reference compound, but the procedure is considered time-consuming, taking into account the total sample preparation and analysis time.

Comparison of the carbohydrate profiles (at 210 and 270 nm) of the reference compounds in deionized water is illustrated in Fig. 3. Glucose and saccharose migrate at 12.6 and 15.0 min, respectively, but the compounds were detected only at 270 nm. A clearly separated peak of saccharose was detected at 12.5 min in the 270-nm profile. As expected, there was no peak with the same migration time in the 210 nm profile. Compound in the 210 nm profile, which migrates near the saccharose peak, is of different shape and originates mainly from the background electrolyte. System peaks were detected in preliminary studies with blank samples.

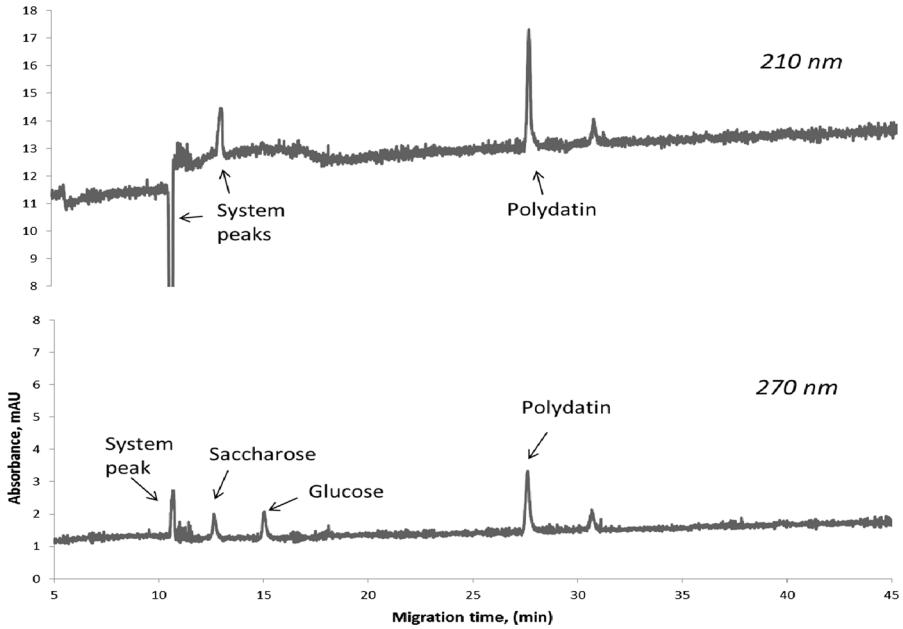

Fig. 3 Calibration solution containing glucose, saccharose and polydatin, detected simultaneously

Polydatin is a glycoside consisting of a glucose unit attached to a resveratrol aglycone, which makes it possible to be separated and detected with a method developed for the determination of monosaccharides. Polydatin (two peaks: 27.9 and 30.7 min) was detected at both wavelengths (210 and 270 nm), but the peak areas were bigger at 210 nm.

Figure 4 shows an example of using an additional detection wavelength for the identification of a poorly separated peak. Identification and integration of the glucose peak are challenging because of the following four peaks in the profile electropherogram (270 nm, Fig. 4). The identification was confirmed with the help of the additional detection wavelength. As expected, glucose does not show a peak at 210 nm.

In comparison with the previous sample profile (Fig. 3), the electropherogram in Fig. 5 includes less peaks, which indicates the presence of fewer carbohydrates. Based on the calibration solution profile, there is no glucose or saccharose of detectable concentrations in the pine white wood sample. The shape and migration times of the peaks indicate sharp separation of carbohydrates. In the pine white wood sample, there are also two peaks (at 18 min) indicating disturbances in the detection signal. As the extraction procedure is the same for both raw materials, the differences in the profiles indicate the differences in the applied raw material.

The spruce and pine samples have partially similar profiles (Fig. 5), but the spruce sample profile includes more detected peaks in total. The three peaks detected in the pine sample are also detected in the spruce sample. In addition, the spruce sample profile includes a group of detected compounds at 18 min.

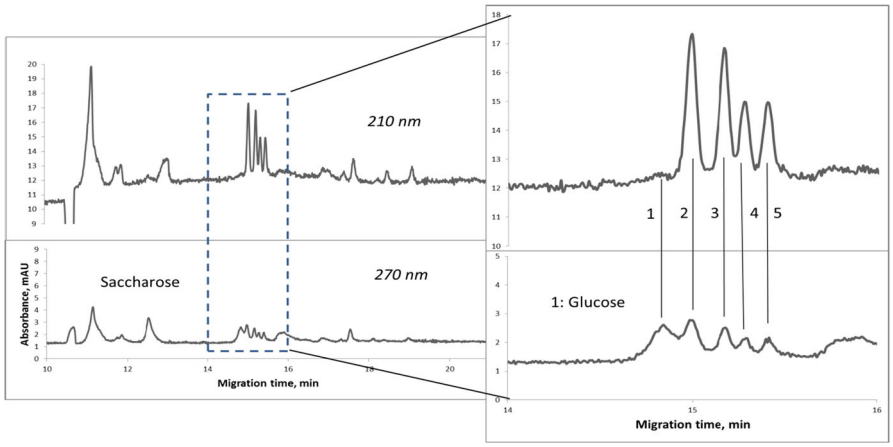

Fig. 4 Carbohydrate profile of water extraction of pine phloem. *Peaks 2–5* were identified as monosaccharides

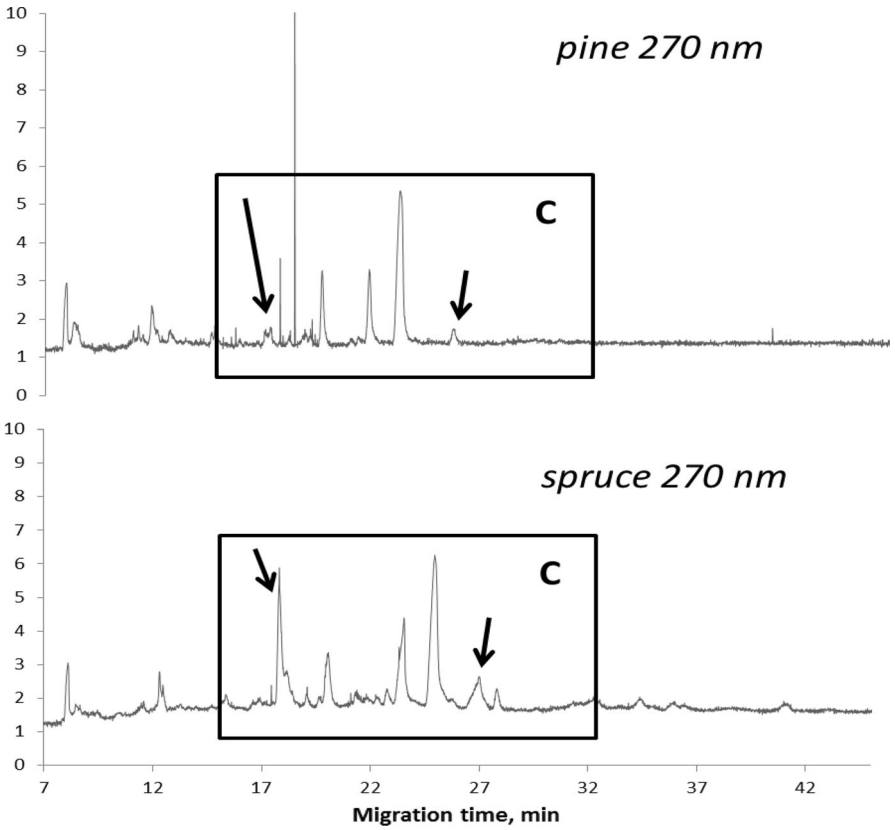

Fig. 5 Example case of identifying differences in water extractions (4 h) of two types of raw materials, pine and spruce

Three similarly detected peaks in the electropherograms are detected at both wavelengths. Ratio of each detected peak area between the two wavelengths is different. The ratio of peak areas (210/270 nm) are for the first peak 1.7 and 2.9 (pine and spruce, respectively), for the second peak 3.0 and 5.6 (pine and spruce, respectively), and for the third peak 5.2 and 5.2 (pine and spruce, respectively). Migration time of the third peak is 23 min and 25 min for pine and spruce, respectively.

Pine and spruce wood are typical raw materials for the forest industry in Scandinavia and North America. In their dry state, both species consist of roughly 41 % cellulose, which can be depolymerized to glucose units. Also, hemicelluloses are target compounds in many wood-related studies (Krogell et al. 2013; Rivas et al. 2013; Holmbom 2011), because they are an easily separated major component of wood biomass (Liu 2010) during cooking or refining processes.

Differences in the carbohydrate profiles of two softwood species are more challenging to detect than the differences between a softwood and a hardwood, because softwood species (pine and spruce) differ significantly from hardwood species (e.g., birch) by their hemicelluloses composition. Hardwood species include 28 % of glucuronoxylan and 2 % of glucomannan, whereas, in softwoods, hemicelluloses consist of 9 % glucuronoxylan and 16 % glucomannan (Liu 2010; Peng et al. 2012). Xylans and mannans are polymers which can be depolymerized into monomeric carbohydrate units and further converted into more valuable products (Peng et al. 2012).

Conclusion

Profiling carbohydrates in heterogeneous wood-based samples is essential for the process control and is achieved with the present method. Confirmation of the peak composition of the case sample was gained via simultaneous utilization of multiple wavelengths. The optimal selection for a combination of detection wavelengths for a certain method takes into account the UV absorbance of each selected compound. The differences in the raw material or process conditions were successfully detected in the sample electropherograms by monitoring the profile of the sample solution and identifying the characteristic peaks. The method applied in this study was found suitable for detecting differences, based on migration times and peak sizes, in the electropherograms of aqueous samples of pine and spruce raw materials. The method also shows potential in monitoring wood-based solutions by profiling carbohydrate composition of the matrix and analyzing the aqueous solution streams of wood-based processes.

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Publication IV

Kaijanen, L., Paakkunainen, M., Pietarinen, S., Jernström, E. and Reinikainen, S.-P.

Ultraviolet detection of monosaccharides: multiple wavelength strategy to evaluate results after capillary zone electrophoretic separation

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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.

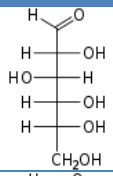
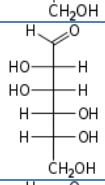
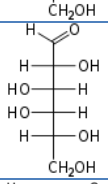
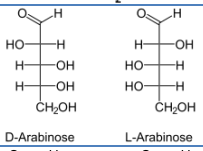
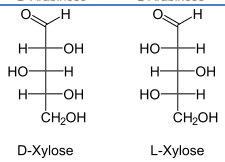
Besides monosaccharides, also other types of carbohydrates can be found through biorefining. In a recent scientific publication by Kemppainen et al. [7], industrially separated spruce bark was found to contain a high amount of non-cellulosic glucose along with tannin and white wood. The researchers concluded 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).

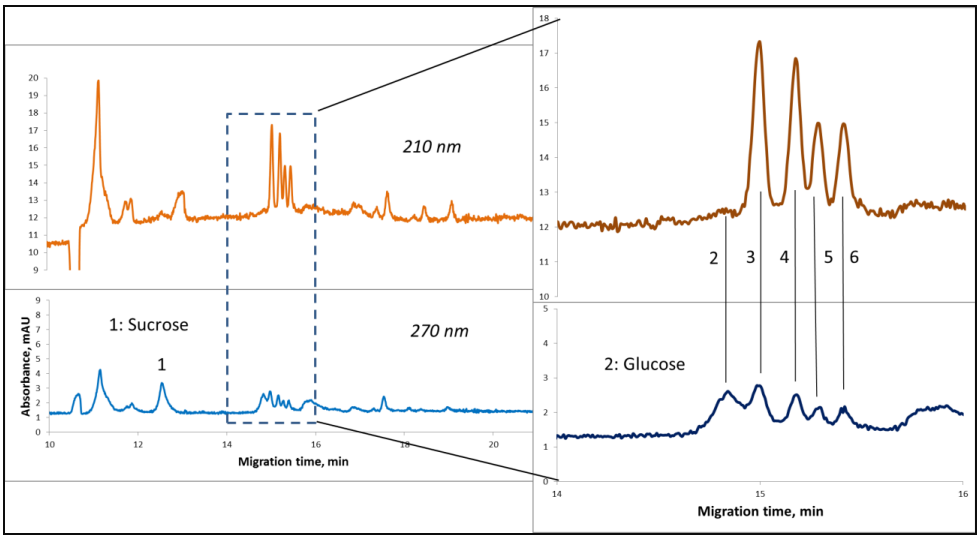


Figure 1. Carbohydrate profile of water extraction of pine wood detected at 210 nm and 270 nm.

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.

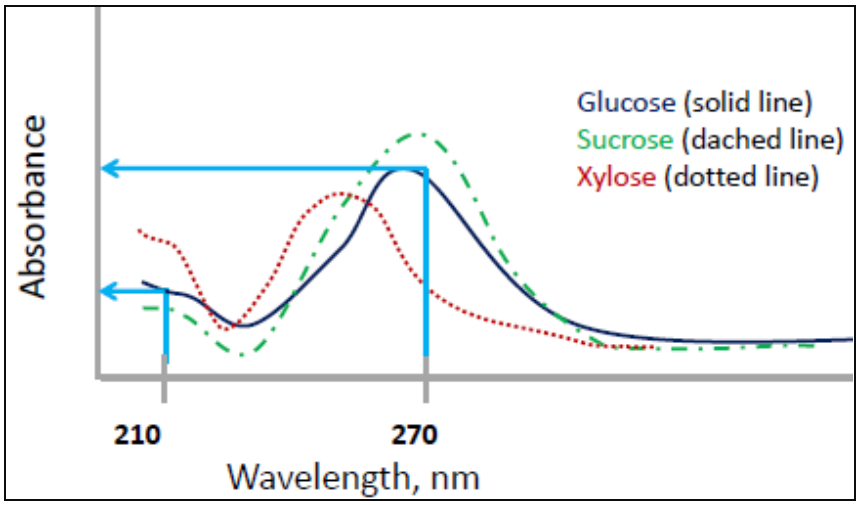


Figure 2. Principal figures of the UV spectra of sucrose [11], glucose and xylose [12].

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.

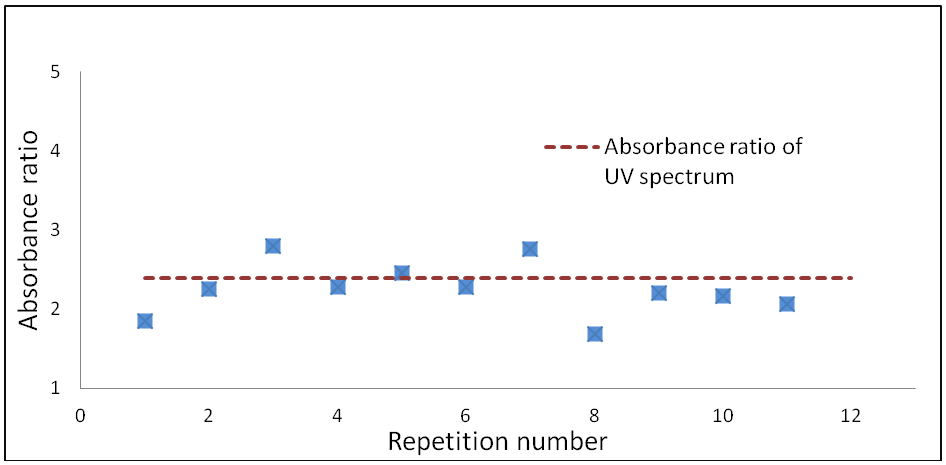


Figure 3. Calculated absorbance ratios of the glucose peak during repeated analyses of a selected concentration of reference solution (60 ppm). The dashed line represents the calculated value of absorbance ratio from the UV spectrum in Fig. 2.

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.

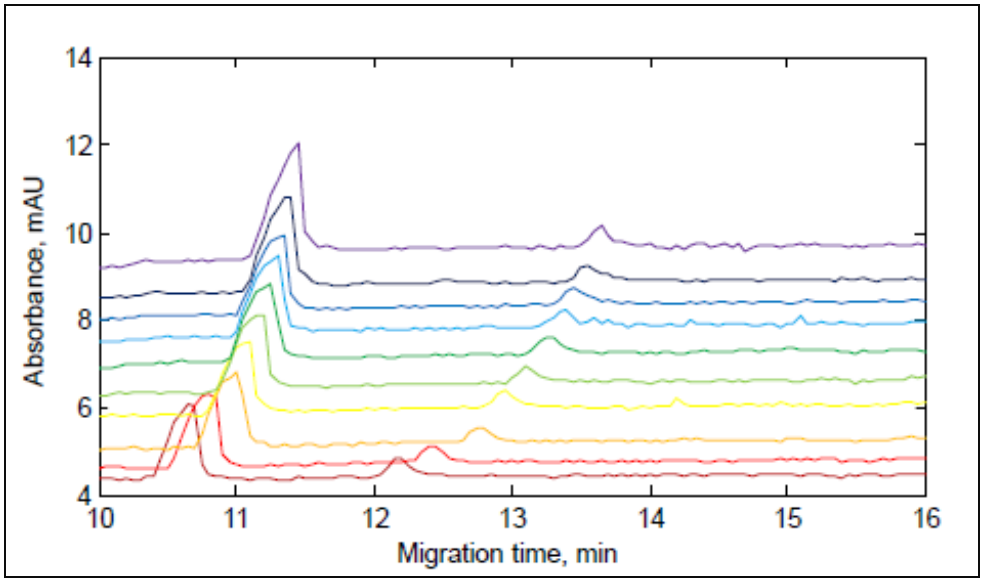


Figure 4. Examples of 10 electropherograms of sequential analytical runs. Peaks in the figure are system peak and sucrose.

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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