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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.

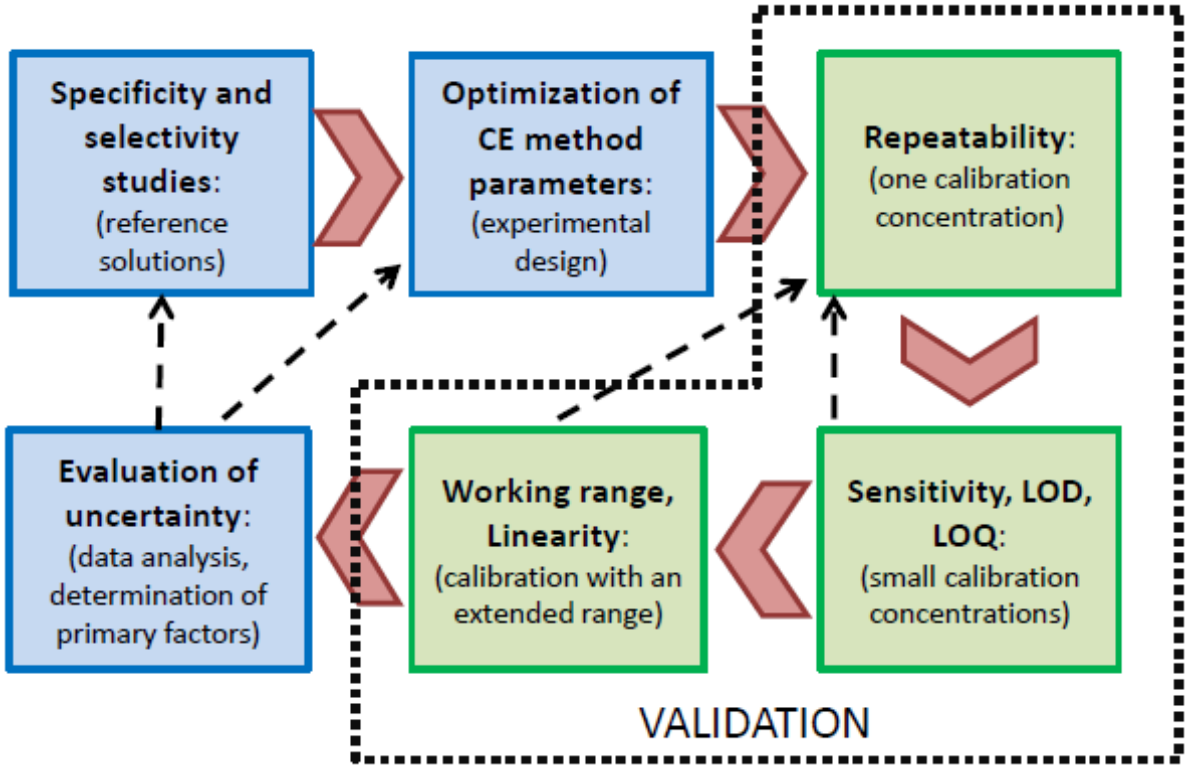

Figure 1. Schematic presentation of a practical application of an in-house validation process utilized in the evaluation of uncertainty of the capillary electrophoretic method.

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.

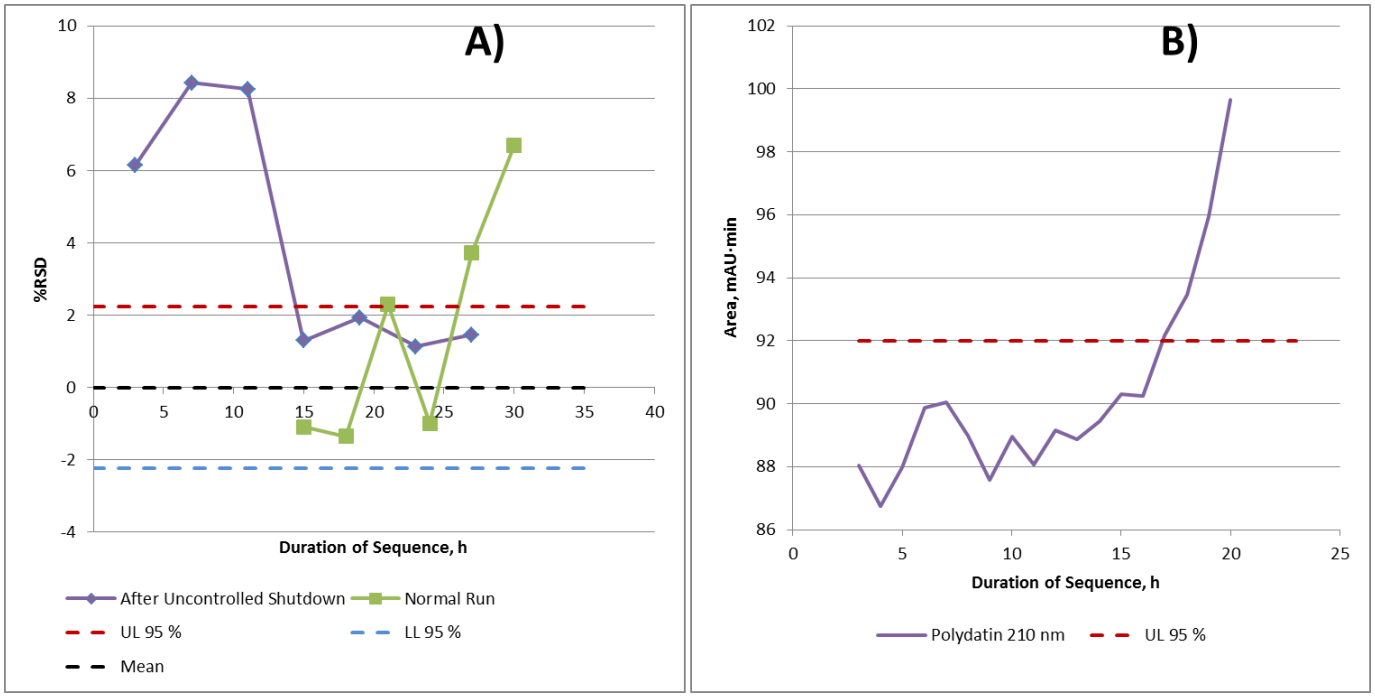

Figure 2. A) Relative standard deviation (%RSD) of the mean for polydatin samples with 95 % confidence limits, and B) area of polydatin peaks. Upper (UL) and lower limits (LL) are selected for confidence level of 95 %.

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

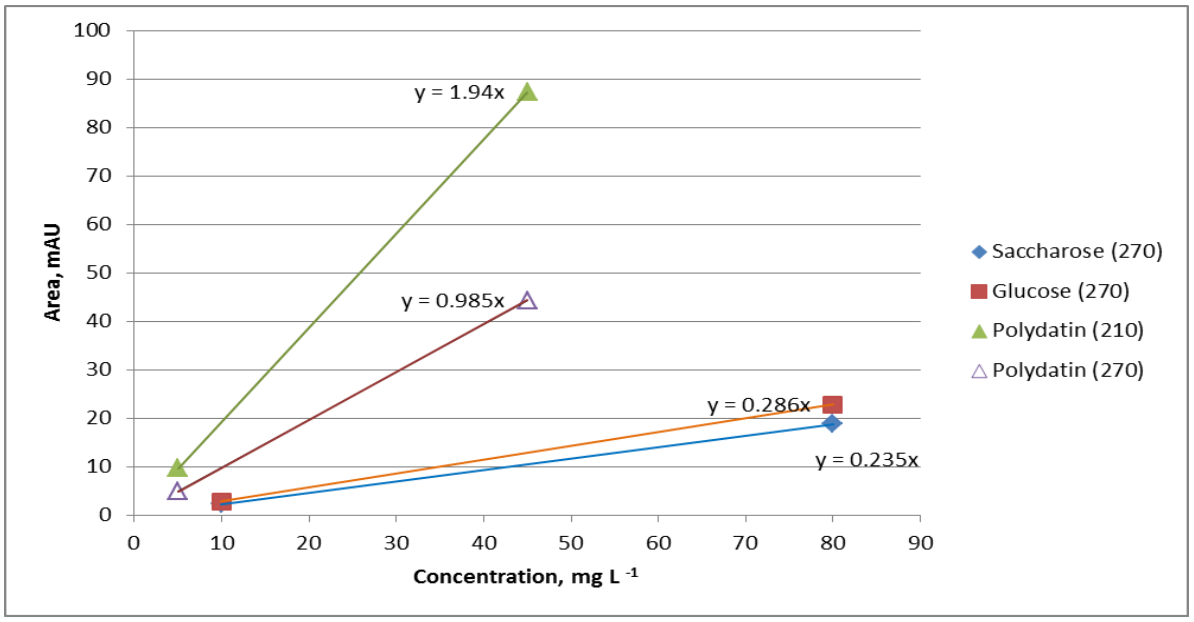

Figure 3. Correlation equations of peak area as function of concentration for glucose and saccharose at 270nm. Correlation of polydatin at 210 and 270 nm.

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.

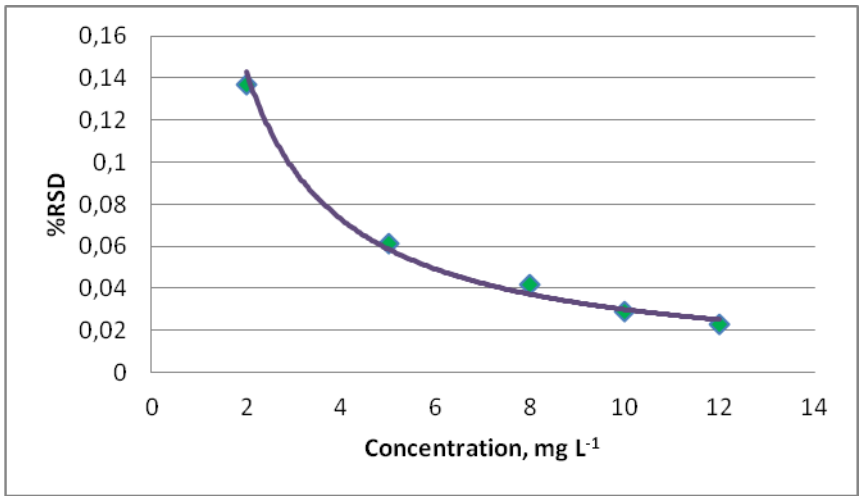

Figure 4. Definition of LOQ with RSD%. RSD% in the function of concentration of the calibration solution. %RSD was calculated from 5 parallel samples.

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.

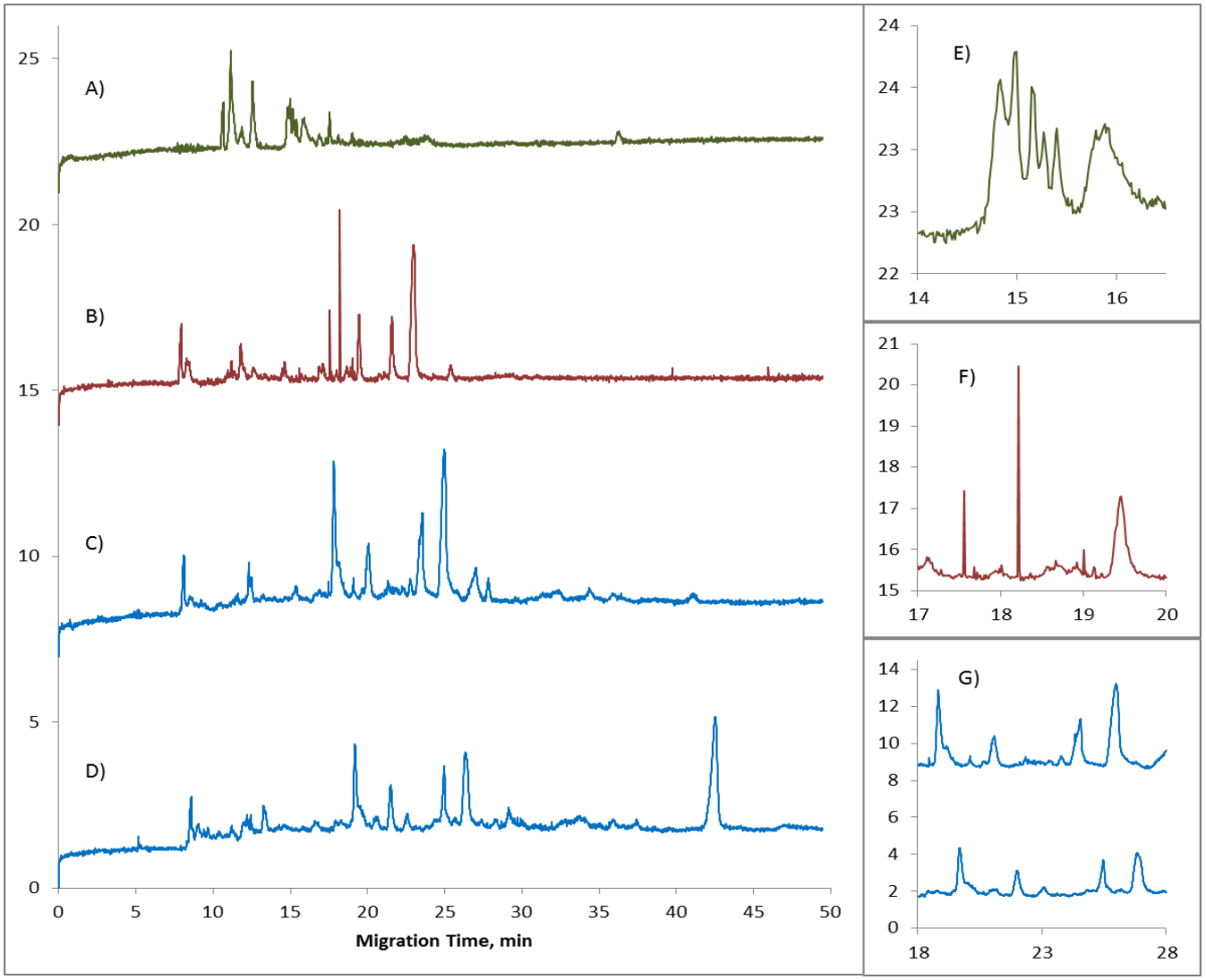

Figure 5. Example electropherograms of typical process samples. A) water extraction of pine phloem, B) water extraction of pine white wood, C) water extraction of spruce white wood, and D) microbial treatment of spruce white wood. Small figures E), F), and G) are zoomed from electropherograms A), B)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