# A Specific, Robust, and Automated Method for Routine At-Line Monitoring of the Concentration of Cellulases in Genetically Modified Sugarcane Plants

**Ruchi Gupta · Sara J. Baldock · Peter R. Fielden · Bruce D. Grieve**

**Abstract** Bagasse is one of the waste crop materials highlighted as commercially viable for cellulosic bio-ethanol production via enzymatic conversion to release fermentable sugars. Genetically modified sugarcane expressing cellobiohydrolases (CBH), endoglucanase (EG), and β-glucosidases (BG) provide a more cost-effective route to cellulose breakdown compared to culturing these enzymes in microbial tanks. Hence, process monitoring of the concentration profile of these key cellulases in incoming batches of sugarcane is required for fiscal measures and bio-ethanol process control. The existing methods due to their non-specificity, requirement of trained analysts, low sample throughput, and low amenability to automation are unsuitable for this purpose. Therefore, this paper explores a membrane-based sample preparation method coupled to capillary zone electrophoresis (CZE) to quantify these enzymes. The maximum enzyme extraction efficiency was obtained by using a polyether-sulfone membrane with molecular cut-off of 10 kDa. The use of 15 mM, pH 7.75, phosphate buffer resulted in CZE separation and quantification of CBH, EG, and BG within 10 min. Migration time reproducibility was between 0.56% and 0.7% and hence, suitable for use with automatic peak detection software. Therefore, the developed CZE method is suitable for at-line analysis of BG, CBH, and EG in every batch of harvested sugarcane.

**Keywords** Bio-fuel · Sugarcane · Enzyme expression · Process monitoring · Capillary zone electrophoresis

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## Introduction

Global energy consumption continues to increase while fossil-fuel reserves are diminishing. To avert a looming energy crisis, the development of renewable energy sources is becoming a priority. In particular, bio-ethanol is considered to have a potential to decrease our dependence on petroleum-derived transportation fuels [1]. While the use of crops for bio-ethanol production has been controversial, the production of bio-ethanol from crop wastes (such as bagasse) does not create pressure on existing food and land resources. In comparison to other crop wastes, the use of sugarcane bagasse is particularly attractive with the most positive energy balance in the near term [2]. Furthermore, dry bagasse consists of 40–50% cellulose, thereby presenting a lucrative commercial opportunity for bio-ethanol production [3]. It is estimated that just 50% of the current Australian sugarcane bagasse crop could produce approximately 1.2 billion liters of bio-ethanol per annum [4] which is equivalent to about 2% of the country's gasoline consumption in 2006 [5]. However, the hydrolysis of cellulose in pre-treated bagasse to release fermentable sugars requires a synergistic action of cellobiohydrolase (CBH), endo-β-glucanse (EG), and β-glucosidase (BG). The production of these enzymes in industrial reactors is expensive (approximately USD 0.10/l of ethanol) and is the key barrier to economic production of ethanol from bagasse. Genetically modified (GM) plants expressing multiple cellulases have the potential to provide a more cost-effective source of cellulosic enzymes [6].

Currently, mills screen each incoming batch of sugarcane and make payments to growers based on sucrose content of their sugarcane. But an additional parameter, i.e., quantification of BG, CBH, and EG has to be incorporated to the payment scheme of GM sugarcane growers. This is because the exact concentration of enzymes is likely to be dependent upon environmental factors, cane variety, cultivation, and harvesting conditions despite the incorporation of "gene switches" [7]. In addition, the hydrolysis efficiency of a particular enzyme is dependent on the concentration of other enzymes. Therefore, it is imperative to obtain a concentration profile of individual enzymes to assess a mixture's cellulosic conversion efficiency. The information obtained could also be used to provide a feedback to farmers to assist with their agronomic practices, i.e., their cultivation and harvesting conditions to maximize the expression of enzymes in planted GM cane. Furthermore, the data on the concentration of expressed enzymes could be used to determine the amount of processing or means of processing required before they could be added to the hydrolysis tank. While online analysis has an ability to enable near real-time monitoring, it presents more technical challenges such as blockages due to solid particles (such as sand, fiber particles, and so on) and surface adsorption of organic matter (e.g., sucrose, glucose, fructose, salts, proteins, starch, dextran, waxes, and phosphatides) [8]. Therefore, at-line analysis is a simple, low cost option for measuring the concentration of BG, CBH, and EG in every incoming batch of sugarcane.

Today the total protein concentration is measured by using the bicinchoninic acid (BCA) protein assay. But sensitivity of reagents used in conventional BCA assays towards reducing sugars results in inaccurate estimation of enzymes in the juice [9]. In addition, BCA assay is not suitable to obtain a profile of individual enzymes in an enzyme mixture. Various enzymatic assays have been reported in the literature as a solution to this problem. The viscometric method though specific for EG [10] is time-consuming, labor intensive, and limits the number of samples that can be handled [11]. Therefore, to decrease the analysis time, synthetic substrates such as p-nitrophenyl-β-D-cellobioside and p-nitrophenyl-β-D-glucopyranoside have been used successfully for the measurement of EG and BG activities,

respectively [12]. However, there is no standard assay to measure the activity of CBH in cellulase complexes [13].

A straightforward idea to quantify multiple cellulase enzymes in a mixture is to separate them before quantification. Gel electrophoresis-based separation methods such as SDS-PAGE and isoelectric focusing (IEF) are difficult to automate and are subject to operator interpretation due to the visual nature of the output data. In addition, most staining dyes are unable to stain glycoproteins and have a poor linear dynamic range making quantitative analysis difficult [14]. Although IEF in capillaries (cIEF) offers higher automation capabilities, the added complexity of the mobilization process has been an obstacle in achieving reproducible cIEF separations [15]. Multi-step analysis and a long analysis time of the combined fast protein liquid chromatography and ion-exchange chromatography [16–18] for the separation of cellulases make chromatography-based techniques unsuitable for the accurate and high-throughput quantification of enzymes in numerous samples. Unlike the parabolic profile of the hydrodynamic flow in chromatography, the characteristic plug-like flow profile of a capillary zone electrophoresis (CZE) separation leads to lower peak dispersion resulting in higher separation efficiency. Therefore, CZE has a potential to separate and quantify CBH, EG, and BG in a single step. One of the major problems of using CZE for biological samples is unpredictable shifts in migration time due to the sample matrix. But migration time is crucial for compound identification especially when multiple species are present. Hence, routine analysis of biological samples by using CZE demands stringent sample preparation [19].

For the first time, this work reports a membrane-based sample preparation method for the CZE separation of cellulosic enzymes in sugarcane juice. The effect of molecular weight cut-off (MWCO) point of the membrane on the enzyme extraction efficiency has been investigated. The effect of pH and concentration of buffer on CBH and EG (from *Trichoderma longibrachiatum*) and BG (from *Aspergillus niger*) is studied and optimized in this study. The above-mentioned enzyme mixture meets the minimum conditions required for efficient hydrolysis of cellulose and expressing at those levels would not produce detrimental effects on plants during their growth and development at ambient temperatures as their temperature optima are between 40°C and 70°C [20]. In addition, the knowledge on the regulation mechanism of these enzymes in *Trichoderma* and *Aspergillus* could be used to control their expression in GM plants [21, 22]. The quantitative details on method validation such as reproducibility of migration times of these enzymes are also presented. Finally, the calibration plots and limit of detection of cellulosic enzymes are included. It should be noted that while cellulase enzymes have been separated by Khaled et al. [23] at pH 9.2, only a single research group has quantified cellulases by CZE [24–26]. However, the work conducted by Jørgensen et al. [24–26] used the enzymes from *Penicillium brasilianum* Batista IBT 20888 instead of *Trichoderma* and *A. niger*. In addition, no reports were made on the effect of a wider range of pH and concentration of the buffer on the separation of cellulases. The analysis time of the method reported in this paper is at least 40% lower than the separation time reported in the previous studies.

## Experimental

### Materials

All samples and electrolytes were prepared using 18.2 MΩ water (Direct-Q™ 5 Ultrapure Water Systems, Millipore, Hampshire, UK).

In order to make standard enzyme solutions for peak identification and calculation of extraction efficiency of sample preparation and to obtain calibration curves, CBH and EG from *T. longibrachiatum* and BG from *A. niger* in 3.2 M ammonium sulfate and 0.02% sodium azide were bought from Megazyme (Bray, Ireland). It should be noted that BG enzyme suspension was supplied with approximately 1 mg/ml of bovine serum albumin (BSA) to alleviate stability problems due to the dilution of BG. The molecular weights of BG, CBH, and EG used in this work are 121, 65, and 57 kDa [27], respectively. Since the calculated molecular weights based on amino acid sequence [28] disregard the contribution of saccharides attached to these enzymes [29], the reported molecular weights are higher than those calculated based on their amino acids sequence alone. Hence, desalting of enzymes was performed by using 10 kDa MWCO Vivaspin™ 6 polyethersulfone columns (Sartorius, Surrey, UK) according to the manufacturer's instructions. The concentration of desalted CBH, EG, and BG were measured by using (a) RC-DC™ protein assay (Bio-Rad, Hertsfordshire, UK) using BSA as a standard and (b) absorbance at 280 nm using NanoDrop™ 2000 (Thermo Scientific, Hampshire, UK) following the manufacturer's instructions.

Samples of fresh, raw sugarcane juice were shipped from the Queensland University of Technology, Australia in the frozen state. The sample was defrosted in a water bath at 40°C, divided into aliquots of ~1.5 ml and stored at −4°C. Each aliquot was defrosted and centrifuged at 14,000×g for 3 min (to remove solid particles such as sand) after which the supernatant was collected to perform CZE experiments.

The phosphate buffer used in this work was made by using the following chemicals: sodium phosphate monobasic (99.6%, Sigma-Aldrich, Gillingham, UK), and disodium hydrogen orthophosphate dodecahydrate (99.85%, Fisher Scientific, Loughborough, UK).

Sample Preparation

Purification of enzymes in sugarcane juice was performed by using columns with 30 and 10 kDa MWCO polyethersulfone (Vivaspin™ 500, Sartorius) membranes. Basically, 0.1 ml of juice containing CBH, EG, and BG was added to the column. The column was then centrifuged at 14,000×g for 20 min reducing the volume of the concentrate to ~50 μl. The column was topped up with 50 μl of water and centrifuged (14,000×g for 20 min) to reduce the volume to ~20 μl. Finally, 80 μl of water was added to the filter. The sample was collected by using a pipette.

A stock solution of volume 0.2 ml was prepared by adding 2, 2, and 7 μl, respectively, of standard CBH, EG, and (BG+BSA) enzyme solutions to the sugarcane juice. The stock solution was divided into two aliquots of 0.1 ml each. Both of these aliquots underwent filtration using different MWCO PES filter membranes. The corrected peaks areas of BG, CBH, and EG were used to estimate the concentration of these enzymes after sample preparation and to calculate the extraction efficiency of the method.

Capillary Electrophoresis

A P/ACE™ MDQ (Beckman Coulter, High Wycombe, UK) was used for the CZE separations presented in this paper. A neutral capillary of 50 μm I.D. and total length of 63 cm with an online detection window at 11 cm from the end was housed in a cartridge which allowed liquid cooling to maintain a constant capillary temperature of 25°C. A neutral capillary was chosen because its walls are coated with polyacrylamide which decreases electroosmotic flow (EOF) and reduces wall interaction with proteins and basic

samples. The capillary was prepared by sequentially flushing with $H_2O$, 0.1N HCl, $H_2O$, and buffer by applying a pressure of 20 psi for 2, 1, 5, and 20 min, respectively. The capillary was washed with buffer for 5 min between runs. The sample plug was introduced by applying a pressure of 0.5 psi for 5 s following which the ends of the capillary were dipped in water to avoid sample carry-over. Short-end injection was used unless otherwise specified. The separation was performed at 15 kV, and components were detected at 214 nm. Thirty-two Karat™ analysis software (Beckman Coulter) was used to calculate retention times, peak widths, corrected peak areas, and resolution.

## Results and Discussion

The CZE experiments presented in this paper were performed by using sodium phosphate buffer due to its low absorbance at the detection wavelength, i.e., 214 nm. In addition, a broad and degraded peak of BG was observed at pH 8.5, which suggested that the enzyme is unstable at high buffer pH values (Fig. 1). Therefore, buffers with a pH up to 8.0 were studied.

Experiments were also performed to obtain an Ohm's law plot for various concentrations of phosphate buffer at pH 7.5. A linear relationship between current and voltage was observed up to 25 mM phosphate buffer concentration [$I$=1.29–0.47 V (I, current and V, voltage), $R^2$=0.99]. This suggests that capillary temperature was not adequately maintained at higher concentrations of the buffer. Therefore, considering the heat dissipating capacity of the P/ACE™ MDQ CZE-system, the investigation into the effect of buffer pH was conducted using 25 mM phosphate buffer at 15 kV.

Effect of Buffer pH on the Separation of Enzymes

The effect of pH on the separation of CBH, EG, and BG in sugarcane juice is presented in Fig. 2. The identities of the peaks were assigned by spiking. BG was found to exhibit the
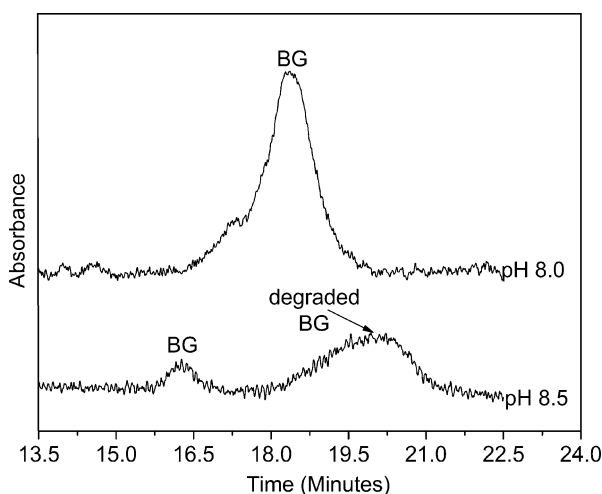


Fig. 1 Comparison of electropherograms of BG at pH 8.0 and 8.5 (50 mM phosphate buffer, long-end injection)
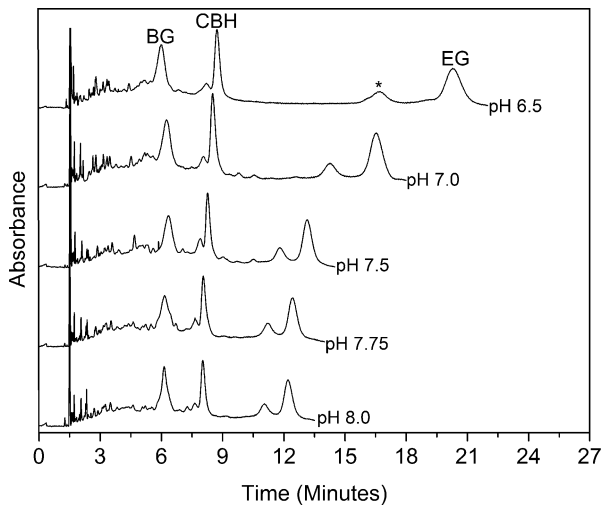
**Fig. 2** Effect of pH on CZE separation of enzymes in sugarcane juice (25 mM phosphate buffer; *single asterisk* indicates unknown contaminant in EG)

highest mobility despite its greatest molecular weight which suggests that it is heavily charged. In addition, though the mobility of EG was increased at higher pH, no appreciable change in the mobility of BG and CBH was observed between pH 6.5 and 8.0. But BG and CBH possess a number of amino acid residues [28] with α-NH$_2$ terminal group (pKa, 6.4 or 6.68) and a few histidine (pKa, 7.6 or 8) residues [30]. Therefore, BG and CBH possess ionizable groups over the pH range used and it is likely that the two enzymes are fully ionized.

It is also clear from Fig. 2 that as the pH increases from 6.5 to 8.0, the analysis time decreases from 22 to 13 min. In addition, as the elution time of EG decreases, the time available for the diffusional broadening reduces. This results in increasing sharpness of the peaks. The effect was most notable on the peak width of EG which was reduced from 1.94 to 1.09 min. But as the time scale contracts, shoulders begin to develop before and after the BG peak at pH 7.75 and pH 8.0 suggesting that BG is not fully separated from interfering components in the juice. In other words, the resolution of separation is hampered at these pH values. Considering the above-mentioned trade-offs, the use of phosphate buffer at pH 7.5 was determined to be the most suitable.

Following this, multiple runs were performed at pH 7.5 to check if the migration times of BG, CBH, and EG are reproducible. The results are shown in Fig. 3. The model presented in [31] predicted that variation in EOF is the major cause of these shifts. As the number of runs increased, there was a buildup of a layer of unidentified residues from the sugarcane juice on the inside walls of the capillary, resulting in increased EOF, and hence, an increase in migration times and peak widths. The relative standard deviations (RSD, $n=3$) of the migration times of BG, CBH, and EG were 1.8%, 2.4%, and 6.6%, respectively. Though it is possible to use this method for the routine analysis of BG, CBH, and EG, the enzymes were purified from the juice to reduce the RSD of their migration times to enable automatic peak identification to be used.

The RSD ($n=3$) of migration times of BG, CBH, and EG were found to be 0.84%, 0.64%, and 0.79%, respectively, after sample preparation (electropherograms not shown) which is in comparison to which has been reported in the literature [32]. Therefore,
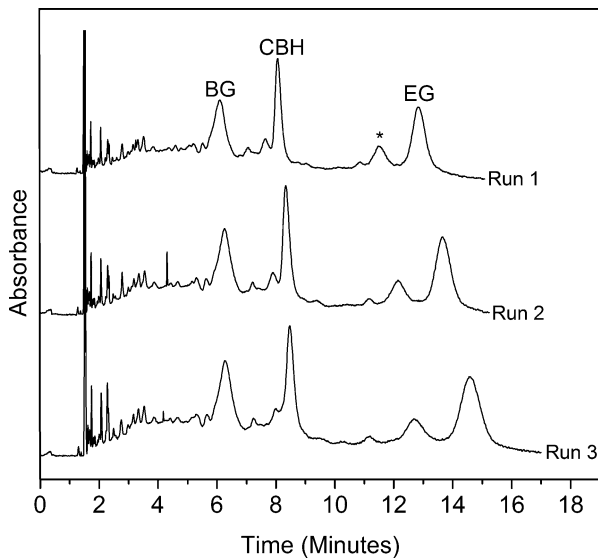
**Fig. 3** Shift in migration time of BG, CBH, and EG due to sample matrix effects (phosphate buffer, pH 7.5, 25 mM; *single asterisk* indicates unknown contaminant in EG; *Run 1* to *Run 3* are sequential with 5 min intermediate wash)

coupling of the developed sample preparation method with the CZE method ensures peak identification using software. This in turn reduces the requirement of having trained analysts for interpretation of the results, thereby making it more suitable for at-line analysis in a process environment. Furthermore, purification of proteins from sugarcane juice allowed an increase of the buffer pH to 7.75, thereby reducing the analysis time further, without degrading the resolution of separation (Fig. 4).
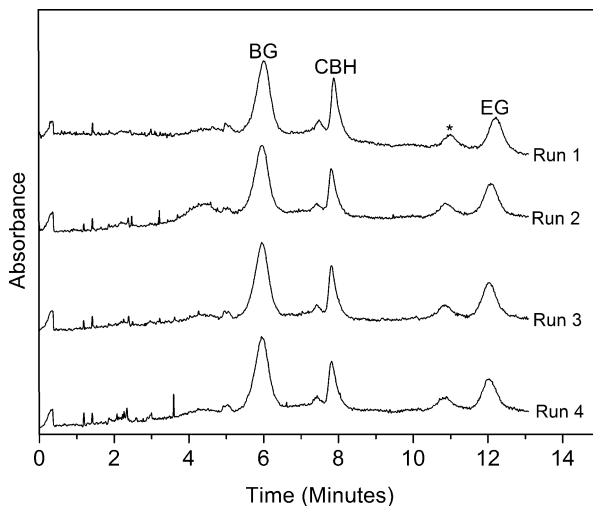


**Fig. 4** Electropherograms of purified enzymes (phosphate buffer: pH 7.75, 25 mM; sample preparation: Vivaspin™ 500, 30 kDa MWCO, PES membrane; *single asterisk* indicates unknown contaminant in EG; *Run 1* to *Run 4* are sequential with 5 min intermediate wash)

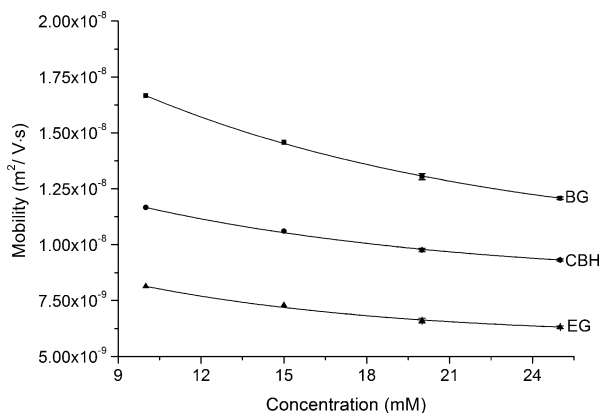Effect of Concentration of Buffer on the Separation of Enzymes

Figure 5 illustrates an exponential dependence of enzyme mobilities on the concentration of phosphate buffer. This effect is observed due to an exponential increase in the effective mobility with respect to square root of ionic strength [33]. The greater rate of increase in the mobility of BG with respect to buffer concentration further verifies that BG possesses a greater number of charged groups than CBH and EG [33].

Therefore, an important factor in favor of the use of a lower concentration of buffer is a decrease in separation time (Fig. 6). But in a 10 mM phosphate buffer, BG and BSA are not baseline separated. Therefore, sodium phosphate buffer of 15 mM and pH 7.75 was found to be optimum for the separation of CBH, EG, and BG. Under these optimized conditions, the analysis was completed within 10 min. Hence, the CZE method reported in this paper is suitable for at-line analysis of BG, CBH, and EG in every batch of harvested sugarcane.

Quantification of Enzymes

It is necessary to know the initial concentration of standard enzyme solutions to obtain calibration curves and the limit of detection (LOD). The results on the concentrations of desalted cellulosic enzymes are summarized in Table 1.

In order to decide if the results obtained by using the two methods are significantly different, statistical $t$ and degree of freedom values for CBH, EG, and MIX were calculated. It is clear from Table 1 that the null hypothesis is retained for CBH and EG, i.e., it has not been demonstrated that the concentration of these enzymes using RC-DC™ protein assay and NanoDrop™ 2000 are different. However, the experimental $t$ value for MIX is greater than the critical value of 2.31. Therefore, the estimated concentrations of MIX using the two methods are significantly different. A probable cause for the observed discrepancy in the concentration of MIX could be the widely different absorption characteristics of BG and BSA making it difficult to estimate the net absorption coefficient of their mixture. The concentration of BG was estimated to be 0.7 mg/ml in the MIX of concentration 1.7 mg/ml based on the concentration ratio of BG and BSA in ammonium sulfate suspensions. To



**Fig. 5** Effect of phosphate buffer concentration on enzyme mobility ($n=3$, *error bars* indicate ± standard deviation and RSD is below 1.65%)
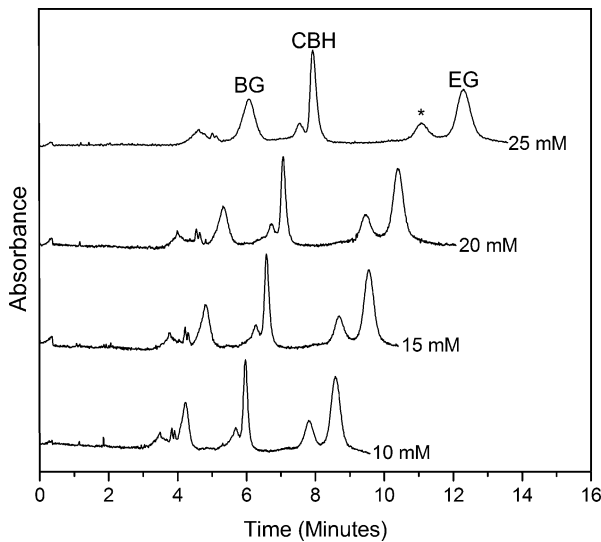
**Fig. 6** Effect of buffer concentration on separation of CBH, EG, and BG (pH 7.75; *single asterisk* indicates unknown contaminant in EG)

summarize, the starting concentration of reference solutions of CBH, EG, and BG were 8.1, 10.41, and 0.7 mg/ml, respectively.

The linearity of the method was tested in the range of 20 to 250, 20 to 100, and 20 to 150 mg/L for CBH, EG, and BG, respectively. The enzyme standards at different concentrations were ran as triplicates, and the results are summarized in Table 2.

The RSDs of the migration times of cellulosic enzymes were between 0.56% and 0.7%. The calibration curves for the enzymes were plotted using corrected peak area to account for variations in migration time [34] and resulted in regression coefficients ($R^2$) above 0.99. The RSD of the peak areas between the three replicates was on average 5.3±3.3% for samples with enzyme concentration above 20 mg/l. This is comparable to the results obtained by Jørgensen et al. using their CZE method for quantification of cellulases from *P. brasilianum* Batista IBT 20888 [24]. The LODs for enzymes were estimated by using the blank signal ($y_B$) plus three times the standard deviation associated with this value [35]. But the negative values of $y_B$ do not have a physical meaning. Since $y_B=0$ corresponds to the minimum concentration of the enzyme required to obtain a measurable absorbance signal, $y_B$ was assumed to be zero to calculate the LODs of the enzymes. Using this definition, the LODs for CBH, EG, and BG are 6.4, 40.35, and 4.25 mg/l, respectively. The broader peak of EG compared to the other two enzymes could be a probable cause of the higher LOD of EG, though the exact cause of the problem is not known for certain. It should also be noted

**Table 1** Concentrations of desalted enzymes using two methods and parameters for statistical tests (based on definitions in [34])

| Enzyme | RC-DC™ ($n=9$) | NanoDrop™ 2000 ($n=9$) | $|t_{exp}|$ | Degrees of freedom | $|t_{crit}|$ ($P=0.05$) |
|---|---|---|---|---|---|
| CBH | 8.1±0.65 | 8.82±1.64 | 1.224 | 10 | 2.23 |
| EG | 10.48±0.95 | 10.41±0.34 | 0.208 | 10 | 2.23 |
| MIX=BG+BSA | 1.7±0.45 | 1.16±0.034 | 3.589 | 8 | 2.31 |

**Table 2** LOD, linearity, and RSD of migration times of CBH, EG, and BG

| Enzyme | Regression line | $R^2$ | LOD (mg/l) | Average migration time (min) | % RSD of migration time |
|--------|-----------------|-------|------------|------------------------------|-------------------------|
| CBH | 0.015×−0.048 | 0.999 | 6.4 | 6.68 | 0.56 ($n=27$) |
| EG | 0.017×−0.212 | 0.998 | 40.35 | 9.65 | 0.7 ($n=15$) |
| BG | 0.046×−0.154 | 0.998 | 4.25 | 3.69 | 0.7 ($n=15$) |

that the developed method is actually capable of detecting lower concentrations of CBH, EG, and BG than their stated LODs because the LOD has been overestimated due to the assumption that $y_B=0$.

Transgenic rice has been reported to accumulate EG to levels of ~5% of total soluble proteins [36]. In addition, sugarcane juice contains ~1,000 mg/L of soluble proteins [37]. The commercially available enzyme products used for efficient hydrolysis of biomass consists of approximately 75–80% of CBH, 20% of EG, and 2–5% of BG [3]. Therefore, the concentration of CBH, EG, and BG expressed in sugarcane is expected to be 190–200, 50, and 5–13 mg/l, respectively. To summarize, the developed method is suitable to monitor the expected concentration of enzymes in GM sugarcane plants.

Sample Preparation—Ultrafiltration and Extraction Efficiency

In the past 10 years, ultrafiltration (UF) has emerged as a promising alternative to conventional sugarcane juice clarification [38]. As a result, most mill operators are highly likely to have experience in using UF. This makes UF an attractive sample preparation technique for at-line monitoring of the concentration of cellulases in sugarcane juice. Besides sugars (such as glucose, fructose, and sucrose) and salts, cane juice contains soluble solids in the form of coloring pigments (such as anthocyanin and chlorophyll), polysaccharides, proteins, and so on [37]. These high molecular mass species are not removed due to UF. But the RSD in migration times of enzymes was reduced after sample preparation. Hence, it is likely that sugars and salts were affecting the EOF by changing the surface characteristics of the CZE capillary and ionic strength, respectively. But injecting a plug of sugarcane juice into the CZE capillary did not have any effect on the observed current. This suggests that the presence of sugars in the juice is the likely cause of variations in migration time of CBH, EG, and BG. In comparison to coloring pigments, polysaccharides, and proteins, the variations in the amount of sugars and salts in sugarcane juice are much higher due to differences in cane variety and age as well as due to external factors (e.g., soil type, fertilizers, climate, and water) [39]. Therefore, the use of UF for

**Table 3** Percentage extraction efficiency of the enzymes using different MWCO PES membranes ($n=3$)

| Enzyme | PES | | | |
|--------|-----|---|---|---|
| | 10 kDa | | 30 kDa | |
| | Concentration (mg/ml) | Extraction efficiency (%) | Concentration (mg/ml) | Extraction efficiency (%) |
| CBH | 61.4±4.49 | 75.8±5.5 | 46.69±11.38 | 57.76±14.05 |
| EG | 56.33±3.56 | 56.33±3.56 | 39.42±7.07 | 39.42±7.07 |
| BG | 19.46±0.64 | 79.43±2.61 | 19.13±4.15 | 78.08±16.9 |

cellulosic enzymes preparation eliminates variable matrix effects on their CZE-based separation and quantification.

Using the information on the volume of enzymes added to sugarcane juice and the initial concentration of standard cellulase solutions, the concentration of CBH, EG, and BG in cane juice was estimated to be 81, 100, and 24.5 mg/ml, respectively, before sample preparation. A comparison of their concentration and percentage extraction efficiency by using PES membranes of different MWCO is presented in Table 3.

From Table 3, it is clear that a PES membrane with a MWCO of 10 kDa resulted in maximum extraction efficiency of enzymes from the juice. It should be noted that regenerated cellulose-based membranes are unsuitable for the purpose because the desired enzymes degrade cellulose. Therefore, the use of a 10 kDa PES membrane is recommended for the purification of CBH, EG, and BG in sugarcane juice.

## Conclusions

The effect of pH and concentration of the buffer on the CZE-based separation of CBH, EG, and BG was studied. The optimized method offers adequate dynamic range and limits of detection to measure the concentration of cellulases in GM sugarcane. The use of 10 kDa MWCO, PES membrane improved the robustness of the method by increasing the reproducibility of the migration times making it suitable for use in an automated analysis environment. While this study used centrifugal/gravitational force as the driving force behind the removal of undesirable particles through the membrane, the method is equally applicable with the use of other forces such as electrical, hydraulic, electromagentic, and so on. Thus the sample preparation method reported in this paper could be further developed for online sampling, thereby alleviating the requirements of manual removal of the sample.

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