

Review

Profiling of carbohydrate polymers in biotechnology using microdialysis sampling, high performance anion exchange chromatography with integrated pulsed electrochemical detection/mass spectrometry

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The analysis of carbohydrate polymers is very demanding and challenging because of the similar physical and chemical properties they possess. Enzymatic hydrolysis is employed to cleave the polymers. The use of enzymes in analytical chemistry requires an analytical system that has on-line capability, is fast, reproducible, robust, and offers sensitive detection. More importantly the system employed should offer *in-situ* sampling and sample clean-up so as to reduce the sample handling steps and the analysis time. One such system consists of microdialysis sampling, high performance anion exchange chromatography with integrated pulsed electrochemical detection/mass spectrometry. The on-line system offers sampling, sample clean-up, separation and detection of saccharides as a way of profiling carbohydrate polymers. The wealth of information attained include carbohydrate polymer sequencing, glycosidic linkages, anomericity and result in the unequivocal characterisation of the polymers. This review focuses on the applications of a total on-line system for the profiling of carbohydrate hydrolysates. The above-mentioned analytical system has been employed to carbohydrate polymers and hydrolysates from starch, lignocellulosic material and legumes.

Key words: Profiling, carbohydrates, sampling, separation, detection, microdialysis, enzymes.

INTRODUCTION

The depletion of natural resources has necessitated research trends towards the direction of renewable resources to reduce consumption and environmental pollution. Carbohydrates are polymers that can be used in the production of renewable resources. In order to use these polymers, one must be able to characterise and profile them. This requires fast and efficient hydrolysis, sampling and sample clean-up, separation and detection steps.

Because of the large sizes of these polymers, it is essential to breakdown the polymer and this can be achieved either chemically or enzymatically. Enzymatic

hydrolysis of the polymers is preferred because it is environmentally friendly and selective. Most hydrolysis steps are not performed on-line and this tends to be time-consuming. A volume is extracted from the bioreactor, heated to destroy the enzymes so as to stop the reaction and then filtered to prevent blockage of the separation columns (Buchert et al., 1993; Ostergaard et al., 2001). The use of microdialysis as a sampling technique allows on-line hydrolysis and real-time monitoring of the hydrolysis process. Microdialysis sampling is carried out without depleting the enzymes and the membrane introduces selectivity of a specific molecular weight cut-off and inhibits the diffusion of the enzymes and ensures prolonged use of the column with no fear of contamination.

Separation of carbohydrates is carried out mainly using chromatographic techniques; especially size exclusion

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chromatography (SEC) (Richardson et al., 1999; Nilsson et al., 1996; Nilsson et al., 1999; Nilsson et al., 2001) and high performance anion exchange chromatography (HPAEC) (Clogston et al., 1993; Panagiotopoulos et al., 2001; Sullivan and Douek, 1994; Marko-Varga et al., 1994; Torto et al., 1996; Torto et al., 1995; Schiller et al., 2002). Recently, capillary electrophoresis (CE) is emerging as an alternative means of separation of carbohydrate hydrolysates and polymers (Linhardt and Pervin, 1996; Hu et al., 2001; Osthoff et al., 2000; Bazzanella and Bachman, 1998). Several detection systems are utilised in carbohydrate analysis and these include light scattering detection systems, which are used mainly with size exclusion chromatography, integrated pulsed electrochemical detection (IPED) used frequently with HPAEC. CE is employed with ultraviolet spectrophotometers or laser induced fluorescence detection (Evangelista et al., 1995). The modes of detection used with CE require that the carbohydrates should be derivatised before analysis (Rassi and Smith, 1995). Several reagents exist for the derivatisation to improve the detectability of the substrates (Kakehi et al., 1999). This review will highlight aspects in sampling and sample clean-up, separation and detection of carbohydrates. The future trends in carbohydrate analysis will also be discussed.

METHODS IN CARBOHYDRATE ANALYSIS

Enzymatic Hydrolysis

For the hydrolysis of carbohydrate polymers, glycosyl hydrolases are mainly employed (Ademark, 2000). These enzymes are proteins usually obtained from fungi, bacteria, plants and animals (Ademark, 2000). The different sources produce enzymes that differ in terms of size, isoelectric points, temperature tolerance, pH range and hydrolytic action. A cocktail of enzymes employed for hydrolysis may result in synergism that quickens the hydrolysis (Rumbold et al., 2002) though a single enzyme can be used together with state-of-the-art instrumentation for carbohydrate analysis (Okatch et al., 2003a; Okatch et al., 2003b), leading to characterisation. The action of an enzyme (Palmqvist et al., 1998) can be inhibited by the architecture of the polymeric material with respect to chain length and substitution patterns on the polymeric backbone.

Microdialysis

Microdialysis (MD) sampling is a technique that was introduced by Delgado et al. (1972) and developed by Ungerstedt et al. (1974) for application in neurochemistry (Elmqvist and Sawchuk, 1997) and pharmacokinetic (Gunaratna and Kissinger, 1997) studies but its use has

evolved to other areas that include biotechnological (Tsai et al., 2000) and environmental (Torto et al., 2000b; Mogopodi and Torto, 2003; Torto et al., 2002) applications.

Microdialysis is a semi-permeable membrane-based sampling technique that achieves on-line dilution (Torto et al., 1999), on-line clean-up (Torto et al., 2000a), *in-situ* sampling in both in-vivo and in-vitro applications. In some cases, the use of microdialysis results in the delivery of a protein-free analyte (Stenken et al., 2001; Dempsey et al., 1997). The diversity of MD is recognised through its capability to be coupled on-line to analytical instrumentation like capillary electrophoresis (Hogan and Stobaugh, 1994), liquid chromatography (Torto et al., 1995) and also to biosensors (Rhenreev-Boom et al., 2001; Palmisano et al., 2001). Microdialysis sampling is governed by diffusion. Analytes are transported from areas of high concentration (bioreactor) to areas of low concentration through a semi-permeable membrane (Khrarov and Stenken, 1999). The membrane offers selectivity to the microdialysis sampling technique (Torto et al., 1996) due to the molecular weight cut-off (MWCO). Only the free analytes with smaller molecular weight than the MWCO of the membrane will diffuse across the membrane and into the outlet. Figure 1 shows a diagram of a tunable concentric microdialysis probe and its components.

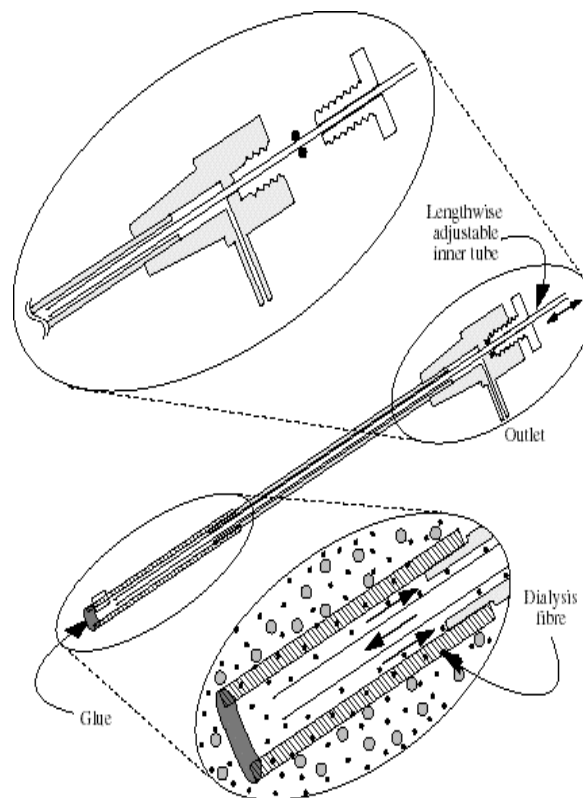


Figure 1. A tunable concentric microdialysis probe.

Advantages of using enzymes with MD

Microdialysis can be used as a sampling technique and for sample clean-up in enzymatic reactions. This is due to several benefits attributed to this method. This combination eliminates two steps that are otherwise present in other enzymatic reactions thereby reducing the analysis time. The heating or freezing step, which is usually carried out to denature the enzyme and hence stop the reaction is one of the steps eliminated. The filtration step is also eliminated since this is incorporated in microdialysis by way of the membrane. In addition, sample loss is not a problem when microdialysis is employed since no sample is drawn from the bioreactor. Microdialysis offers *in-situ* sampling and can sample from complex matrices without depleting the enzymes and without perturbing the reaction (Torto et al., 1998a).

A study carried out by Torto et al., showed that polysulphone membranes that are employed in MD can withstand temperatures as high as 90°C and can be used for periods of over 24 h (Torto et al., 1997b).

The use of microdialysis allows on-line hydrolysis since the microdialysis probe can be coupled to analytical instrumentation such as liquid chromatograph, capillary electrophoresis and mass spectrometer. This means that no further sample clean-up steps are required which makes the use of MD attractive and allows real-time monitoring of the hydrolysates. Besides the use of MD in the analysis of carbohydrates, MD can be extended to the characterisation of enzymes; with respect to the purity (Richardson et al., 1999), substrate specificity (Torto et al., 1995; Torto et al., 1997b), rate of enzymatic hydrolysis, and in the study of enzyme cooperation (Rumbold et al., 2002).

High performance anion exchange chromatography (HPAEC) with integrated pulsed electrochemical detection (IPED)

HPAEC allows the separation of carbohydrates in their enolate form at high alkaline pH. Direct detection is carried out and therefore does not require any derivatisation steps. This method is highly sensitive and allows the detection of the saccharides in the picomole level. HPAEC-IPED can be applied to a wide range of carbohydrates including monosaccharides, oligosaccharides and acidic carbohydrates. HPAEC-IPED has been used for the isocratic separation of monosaccharides and disaccharides for five common carbohydrates in fruit juices (Zook and LaCourse, 1998) in less than 15 minutes. In combination with enzymes to release N-glycans from glycoproteins, the profiling of the glycans is then carried out (Spellman, 1990; Basa and Spellman, 1990). Likewise O-glycans have been analysed with this system, an example being the analysis of the O-glycans on a fragment on a murine monoclonal

antibody (Hagmann et al., 1998). Separation and detection of acidic saccharides such as sialic acids (Dionex, 1997), sulphated carbohydrates and phosphorylated carbohydrates have also been investigated. HPAEC-IPED can be applied to studies of chain-length distribution analysis of oligo/polysaccharides such as starch (Torto, 1999), examination of hydrolytic conditions (Kiang et al., 1997) and examination of enzymatic reactions (Rumbold et al., 2002; Nilsson et al., 2001). HPAEC has also been applied in the study of glycoproteins (Clogston et al., 1993), in marine studies (panagiotopoulos et al., 2001), pulp and paper (Sullivan and Douek, 1994), fermentation (Marko-Varga et al., 1994) and biotechnology (Torto et al., 1996; Torto et al., 1995) and in pharmaceutical samples (Schiller et al., 2002).

Size Exclusion Chromatography (SEC) with light scattering detection

These systems are commonly used for the determination of the molecular weight distribution of polymers and for fractionation. A wide range of stationary phases is available. The stationary phase material, usually silica-based or polymeric sorbents or cross-linked gels, is constructed into a three dimensional network and is porous thereby allowing the analytes to penetrate through. The degree of penetration depends on the size of the analyte with respect to that of the pores of the stationary phase material (Churms, 1996; Richardson, 2001). Usually two or three columns are used in series or a mixed bed column is employed (Nilsson, 1999). In the study of carbohydrates, SEC is used for the determination of the molecular weight distribution of amylose, debranched amylopectin, and enzymatic hydrolysates of starch and cellulose (Nilsson et al., 2001; Rassi, 2002; Nilsson et al., 1996), and for the fractionation of intact starch and cellulose. SEC can be coupled to HPAEC for improved selectivity (Snortti, 1997).

Refractive index detection is mostly employed after SEC but the limitations associated with this have prompted the use of alternative detectors. The light scattering detectors, both laser light scattering and multi-angle light scattering detectors have been employed in carbohydrate analysis after SEC.

Mass spectrometry

Mass Spectrometry (MS) is an analytical technique designed to give molecular weight information of analytes in their ionic gaseous phase. The evolution of the ionisation methods from electron impact (Watson, 1997), a harsh ionisation method, to matrix assisted laser desorption ionisation (MALDI) and atmospheric pressure

ionisation (API) have enabled the use of MS for the analysis of non-volatile and thermally labile compounds such as carbohydrates.

Matrix assisted laser desorption ionisation, MALDI

This technique requires the carbohydrate analyte to be mixed in a matrix that possesses high absorptivity for laser radiation such as 2,5-dihydroxy benzoic acid (Chapman, 1993). Laser radiation is directed at the matrix/analyte mixture in pulses. The matrix molecules absorb the radiation energy and transfer the energy indirectly to the sample resulting in ionisation without degradation. Protonated ions are formed exclusively, though small amounts of dimeric and doubly charged molecules are observed as well. A study has been carried out to evaluate matrices best suited for oligosaccharides analysis especially with respect to sialic acid residue loss (Papac, 1996). MALDI is used mainly with time-of-flight mass analysers because of its ability to cope with pulses.

MALDI is used for the analysis of large carbohydrates in the femtomole range. The use of MALDI has been demonstrated in the analysis of lipopolysaccharides (Therisod, 2001) after TLC separation. However, MALDI is also used for the direct analysis of oligosaccharide binding lectins (Tseng, 2001) without any prior separation. The use of liquid matrices is applicable to MALDI and has been used in the sequencing, branching and linkage of fully intact sialylated oligosaccharides (von Seggen et al., 2003). The efficiency of a new sample clean-up method of N-glycans released from glycopeptides was demonstrated from the MALDI-TOF-MS spectra (Nakano et al., 2003). Before the clean-up, the peaks associated to the glycans were suppressed by the high intensity peaks of the peptides, however, after the clean-up method was employed, the glycan peaks were dominant in the spectra.

Atmospheric pressure ionisation, API

The advent of API, atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI), allows mass spectrometric detection of fluid flowing analytes and interfacing of chromatographic techniques to mass spectrometry because the ionisation of the analytes is carried out at atmospheric pressure. The analytes can either be introduced into the ionisation source by direct infusion or coupling LC to MS. APCI can carry out ionisation in the gas phase through charge-transfer, electron capture and ion-molecule reactions. APCI is used for the analysis of glycosides in crude plant extracts and a few examples are, the structural characterisation of flavone glycosides in soybean pods (Boue et al., 2003), the analysis of the glycosidic

flavonoid isomers (Waridel et al., 2001) and the analysis of acylated xanthone glycosides (Rezanka and Dembitsky, 2003). However, most carbohydrate analysis has been carried out using ESI and further discussions will focus on this mode of ionization.

ESI is a soft ionisation technique that is applicable to polar high molecular mass compounds like proteins and carbohydrates (Watson, 1997; Chapman, 1993; Skoog et al., 1996; Ashcroft, 1997). This type of ionisation generates multiply charged ions and is applicable to polar compounds of different molecular weight including those whose molecular weight is greater than the m/z range. The protonated ions are poorly detected and derivatisation with sodium or transition metals is usually carried out (Kohler and Leary, 1995; Smith and Leary, 1996; Smith et al., 1997). Since ESI is a very soft ionisation technique, there is very little or no fragmentation of the ions formed thereby creating clean spectra and leading to easy identification of the pseudomolecular ions. However, fragmentation can yield useful information for structural elucidation and this is attained through collision induced dissociation (CID). CID can be employed for the qualitative identification of saccharides on the basis of their fragmentation pattern. Fragmentation allows the structural elucidation of oligosaccharides with respect to degree of polymerisation, sequencing, branching, linkage positions (Harvey, 2000) and determination of the anomericity of the reducing sugars. Fragmentation depends on the energy transferred to the ions, nature of the adducts (protonated, sodiated) and the lifetime of the ion before detection and can occur by cleavage at the glycosidic bond or cross-ring cleavage.

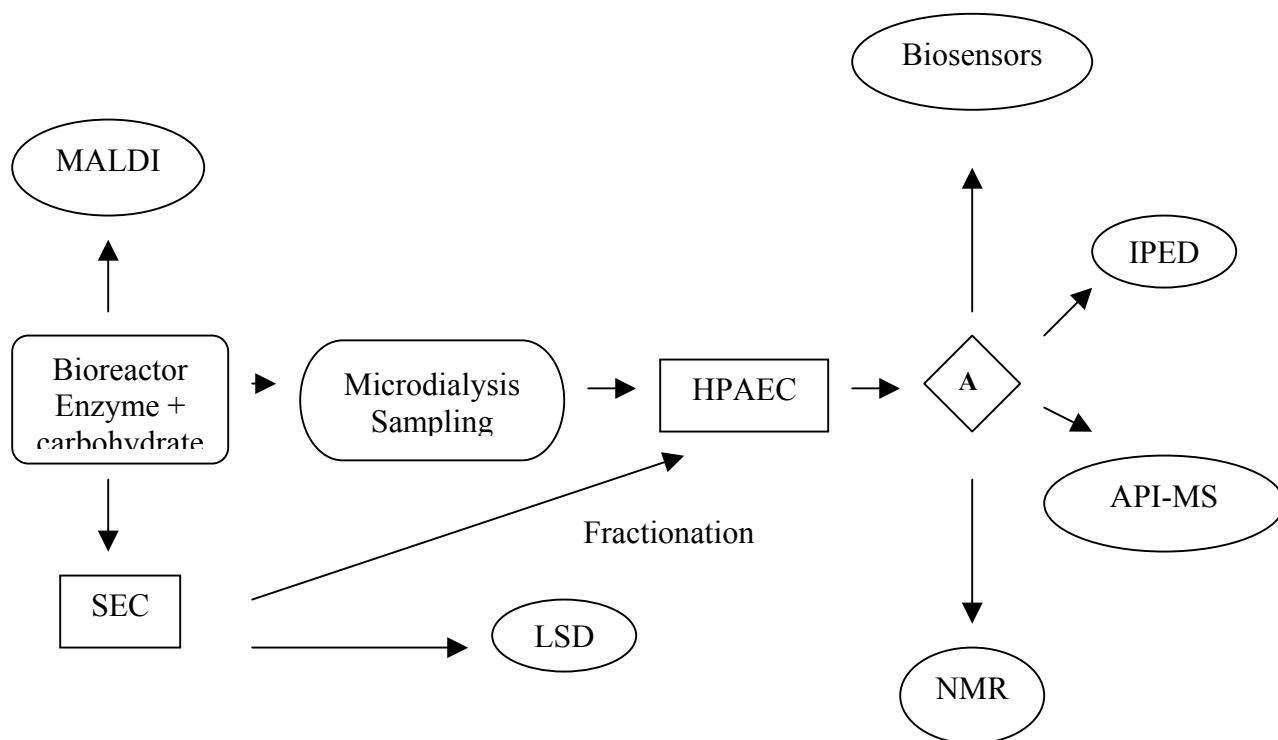
Total analysis system to profile carbohydrates

An appropriate set-up for an on-line system showing total hydrolysis, characterisation and profiling of carbohydrates is shown in Scheme 1. The scheme shows pathways for analysis of carbohydrates from the point of hydrolysis where *in-situ* sampling is achieved using microdialysis to the point of detection.

The different detection systems can be coupled to HPAEC through an interface, which is different for each detector. For the use of IPED, no interface is required; however coupling HPAEC to MS requires the use of a desalting device.

APPLICATIONS

The use of enzymes in the hydrolysis of carbohydrates with microdialysis sampling is very widespread and has been applied to starch (Richardson et al., 1999; Nilsson et al., 2001), ivory nut mannan (Torto et al., 1995; Torto et al., 1996), lignocellulosic material (Rumbold et al.,



Scheme 1. Illustration of pathway showing points of sampling, separation and detection. A represents an interface that can be changed based on the detection system in use.

2002; Breccia et al., 1998) and legumes (Okatch et al., 2003a, b). Microdialysis sampling and sample clean-up of hydrolysates after enzymatic hydrolysis has been carried out for starch from different sources including maize and potato (Nilsson, 2001). A cocktail of enzymes were used; pullulanase, isoamylase and β -amylase. The products were analysed using HPAEC-IPED. As a result of the use of microdialysis sampling, it was possible to investigate the short chain length fractions of debranched starch in the presence of amylose without prior fractionation. The molecular weight cut-off of the membrane selects against the diffusion of the large polysaccharide. The system was also used for the determination of the A:B chain ratio and the β -limit value. Torto et al. (1997a) used termamyl to hydrolyse two different types of substrates; cereal starch and native wheat starch. Using a system comprising of microdialysis sampling, HPAEC-IPED, hydrolysates of up to DP 18 were observed for the cereal starch. Incorporating a column switching device introduced selectivity to the detection of the column effluent resulting in uncomplicated spectra.

Torto et al. (1995; 1996) monitored the enzymatic hydrolysates of ivory nut mannan using microdialysis sampling with HPAEC-IPED. From this work, it is evident that through the use of microdialysis, an enzymatic reaction can be followed even though the reaction pH is different from the detection pH. The ivory nut mannan was hydrolysed using endo- β -mannanase from both

Trichoderma reesei and *Aspergillus niger* (Torto et al., 1995). The hydrolysis was monitored for 11 h and the chromatograms obtained for each strain of enzyme were compared. The mannanase from *T. reesei* showed a chromatogram different from that of the mannanase for *A. niger*. However, for both enzymes, DP 4 was the most abundant throughout the hydrolysis period and DP 5 was the least. The quantitative analysis of ivory nut mannan was carried out in different microdialysis sampling modes (Torto et al., 1996). The continuous flow microdialysis sampling mode was found not to be suitable for enzyme reactions and the stopped flow microdialysis sampling mode with stopped stirring or stopped flow microdialysis sampling mode with continuous stirring were found to be better suited in monitoring hydrolysis reactions.

The robustness of the on-line system employing microdialysis, HPAEC-ESI-MS was demonstrated in the characterisation of the carbohydrate polymer in legumes (Okatch et al., 2003a; Okatch et al., 2003b). Both beans (Okatch et al., 2003a) and bambara groundnuts (Okatch et al., 2003b) comprise a heteropolymer of galactomannans. A single enzyme, endo- β -mannanase, was used to hydrolyse the galactomannan polymer resulting in the production of hydrolysates of different degrees of polymerisation (DP). For the bean samples (Okatch et al., 2003a), hydrolysates of DP 2-6 were observed for the mung bean (*Phaseolus mungo*) and the chromatogram is shown in Figure 2. Chromatograms B-F correspond to DP 2-6 respectively. From chromatogram

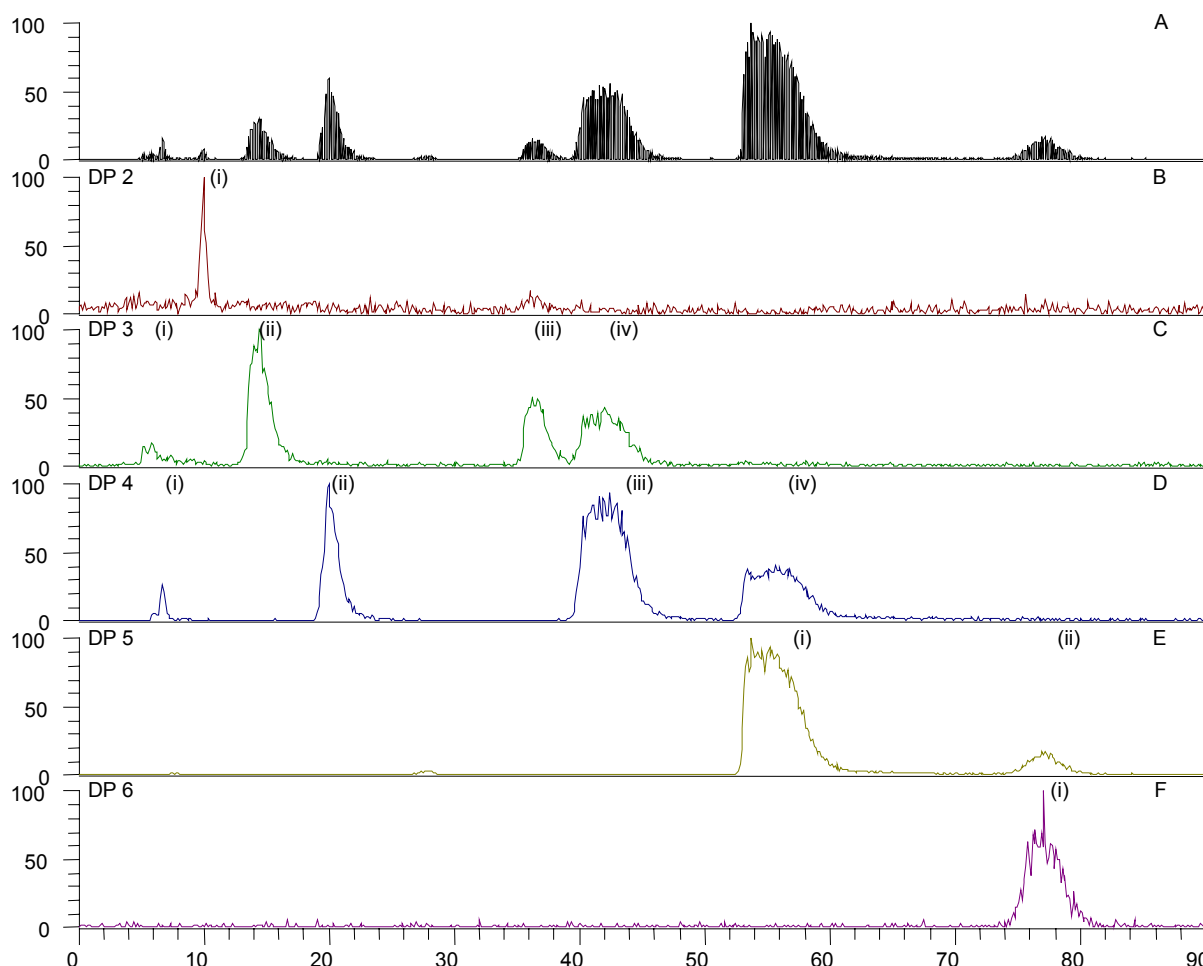


Figure 2. Mass chromatogram of mung bean hydrolysates analysed after a 12-h hydrolysis period. The chromatograms represent: (A) the total hydrolysates detected after 12-h hydrolysis (B) DP 2, (C) DP 3, (D) DP 4, (E) DP 5, and (F) DP 6, respectively. For DP 3-5, the several peaks observed indicate either a different glycosidic linkage or a different degree of branching for the detected saccharides.

C, it is evident that there is more than one type of trisaccharide present. There are four types of DP 3 and this is observed due to the ability of the mass spectrometer to carry out analysis in the single ion monitoring mode, which has better sensitivity. The presence of more than one hydrolysate for DP 3 suggests that the hydrolysates have different glycosidic linkages.

The galactomannan in bambara groundnuts were characterised by the same method and the associated chromatograms showed less hydrolysates per DP. The chromatograms obtained in the single ion monitoring mode showed that the order of elution is not the expected order and the larger hydrolysates in some cases are eluted before the smaller ones (Okatch et al., 2003b). In addition, the use of MS illustrated that there is co-elution of the hydrolysates, which is otherwise not observed with IPED. IPED relies on retention time for identification, which is not sufficient especially in situations where spiking is used as this may lead to peak broadening

(Okatch et al., 2003a; Okatch et al., 2003b; Torto et al., 1998).

A complete system of MD-HPAEC-MS was used by Torto et al. (1998b) and demonstrated the ability to hydrolyse and detect wheat starch hydrolysates up to DP 17 in their sodiated form. The difficulty in coupling LC to MS was solved by the use of a cation exchange membrane desalting device to remove the sodium, which would otherwise block the inlet capillary to the ionisation source. The same system was used for analysis of lignocellulosic substrates (Torto et al., 1998c). The use of microdialysis was compared to normal filtration and the chromatogram obtained for the wood hydrolysates with direct injection after filtration showed broad peaks. However the hydrolysates injected after microdialysis sampling showed sharper peaks due to the inherent dilution associated with the technique. This chromatogram was cleaner than the previous chromatogram and suggested that less contaminants reached the detector and/or biosensor and thereby the

use of microdialysis eventually reduces the effects of fouling of the detector/biosensor.

Although little work has been done in the characterisation of enzymes by the use of microdialysis, it is clear that this technique can be adopted in biotechnology studies. Rumbold et al. (2002) used the analytical set-up constituting of microdialysis, HPAEC-IPED/MS to monitor the hydrolysates from dissolving pulp and sugar cane bagasse. The polymer was cleaved with a cocktail of enzymes; xylanases, phenolic hydrolases, endo-glucanases and cellobiohydrolases which were added sequentially every 8 h. For the dissolving pulp, the observed chromatogram in Figure 3 shows that upon addition of the esterase, the release of DP 1 was observed. The addition of endo-glucanase and cellobiohydrolase resulted in the production of DP 2 and 3 and seem to have no impact on the production of DP 1.

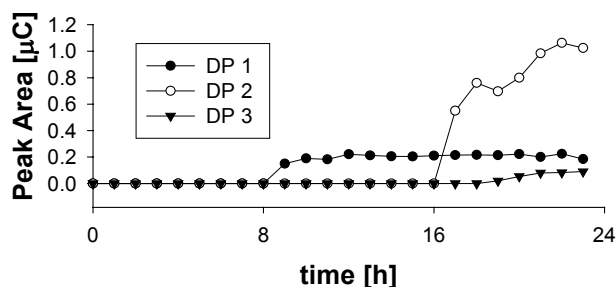


Figure 3. Hydrolysis profile during the enzymatic hydrolysis of dissolving pulp. Enzymes were added sequentially starting with xylanase after 0 h, phenolic acid esterase after 8 h and endoglucanase and cellobiohydrolase after 16 h, respectively.

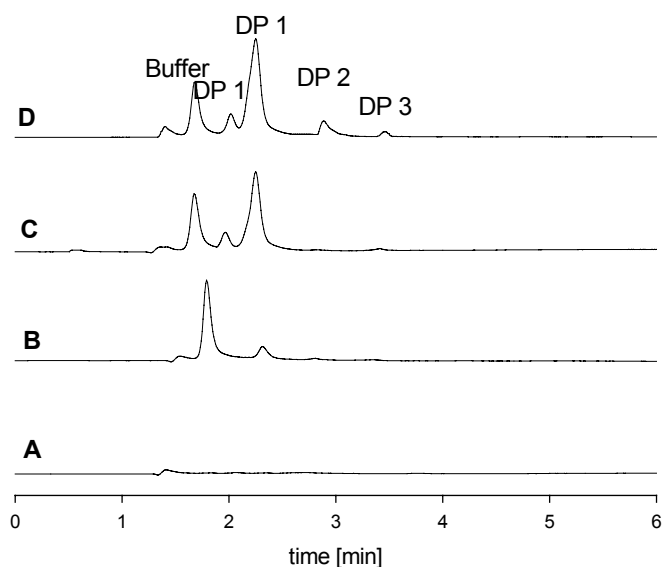


Figure 4. Chromatographic profile of sugar cane bagasse before the addition of enzyme, 0 h (A), after the addition of xylanase, 8h (B), after the addition of esterase, 16 h (C) and finally after the addition of endoglucanase and cellobiohydrolase, 23 h (D).

For the sugar cane bagasse, the profile was different as shown in Figure 4. The addition of xylanase results in the production a DP 1 sugar as shown in chromatogram B. Upon addition of the esterase, the DP 1 peak significantly increases and another peak is observed identified as a pentose. The addition of the last two enzymes lead to production of DP 2 and DP 3 hydrolysates and a further increase in the DP 1 peak. It is evident that the substrate architecture affects the hydrolytic action of enzymes.

Currently, our research group is studying the hydrolytic properties of a cocktail of lignocellulosic degrading enzymes from different sources; *Aspergillus niger*, *T. reesei* and *A. pullulanase*. In a different study by Richardson et al. (1999), a microdialysis-based assay was used to determine the purity of β -amylases and pullulanases from different sources. The purity of the enzymes was determined by microdialysis sampling with HPAEC-IPED. β -amylase cannot hydrolyse maltose to glucose units, but when monitored, two of the four enzymes showed significant amount of glucose and suggest impurities of α -glucosidase and/or α -amylase in the enzyme preparation.

CONCLUSION

The developments in improving analytical systems has led to the birth of hyphenated systems that can be applied in the profiling of carbohydrates in biosamples. The on-line system of microdialysis sampling, high performance anion exchange chromatography/size exclusion chromatography with integrated pulsed amperometric detection/light scattering detection/mass spectrometry is utilised in this area of research. This combination of techniques is well suited for this cause because it can handle small amounts of the enzyme.

The above-mentioned system can be used for rapid and reproducible analysis. The detection modes used do not require derivatisation and are highly sensitive.

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