Simultaneous determination of cereal monosaccharides, xylo- and arabinoxylo-oligosaccharides and uronic acids using HPAEC-PAD

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Highlights

- The simultaneous determination of mono and oligosaccharides using HPAEC
- The validation of the presented HPAEC method
- Screening the released oligosaccharides released from wheat bran with endoxylanase
- The activity of commercial xylanase on oligosaccharides standards X5 and X6
- The studied xylanase showed a preference

Abstract

Xylo- and arabinoxylo-oligosaccharides (XOS and AXOS) are of interest for their prebiotic activity. The production of these oligomers might be accompanied with monosaccharides. The measurement of both oligosaccharides and monosaccharides usually requires two methods. The current work presents an HPAEC-PAD method based on gradient elution of aqueous solvents sodium hydroxide and sodium acetate, in contrast to conventional isocratic elution, for the simultaneous separation of 16 standards of monosaccharides, xylo-oligosaccharides, arabinoxylo-oligosaccharides and uronic acids using CarboPac PA 200 column. The presented method showed a stable baseline and high-resolution separation of the standards. The method showed acceptable accuracy and precision. Limits of Detection and Quantitation (LOD and LOQ) were estimated for all the standards. The method was applied to measure the activity of a commercial endoxylanase on wheat bran; a steady release of

xylose monosaccharide was observed. Enzyme action on oligosaccharide standards showed a preference for the larger oligosaccharides.

Keywords

Arabinoxylo-oligosaccharides (AXOS), xylo-oligosaccharides (XOS), HPAEC-PAD, xylanase activity, oligosaccharide separation, wheat bran

1-Introduction

Arabinoxylans (AX) are an abundant non-starch polysaccharide that occurs in the outer-layers and endosperm cell walls of cereals grains, associated with cellulose and lignin to provide the structural framework of the grain (Biliaderis *et al.*, 1995; Vinkx and Delcour, 1996). AX consist of a linear chain of β-D-xylopyranosyl with α-L-arabinofuranoside residues attached to the xylose units at O-3, O-2 and/or both O-2, 3 positions (Izydorczyk and Dexter, 2008; Saulnier *et al*., 2007). The breakdown of AX polymers to arabinoxylan-oligosaccharides (AXOS) and the linear xylo-oligosaccharides (XOS) has become of interest due to the impact of these oligomers on human and animal health via prebiotic activity. AXOS and XOS are fermented by *Bifidobacterium* strains to produce metabolically active compounds such as short chain fatty acids, thus invigorating the activity of the gut bacteria and improving gut health, reducing infections and suppressing colon cancer (Van Craeyveld *et al.*, 2008; Broekaert *et al.*, 2011; Immerzeel *et al.*, 2014). Increasing consumption of these prebiotics could enhance human and animal nutrition. Xylanases are already used in bread formulations to improve dough rheological properties by hydrolysing large AX molecules, leading to some production of AXOS; judicious use of enzymes to enhance this AXOS production while retaining good dough handling properties could increase AXOS consumption in human diets (Damen *et al.*, 2012). Similarly, xylanases are traditionally added to animal feed in order to reduce the viscosity of the feed in the animal digestion system, but recent research has highlighted the additional prebiotic benefits arising from XOS and AXOS, and attention is now focussed on understanding and exploiting these prebiotics in animal feed formulations (González-Ortiz et al., 2019).

Because of its interest as a prebiotic material, there have been a number of studies investigating production of XOS from a range of substrates. Most research to date has focussed on XOS rather than AXOS, the latter being harder to analyse due to the lack, until

recently, of readily available standards. Table 1 illustrates the range of materials, pretreatments and enzyme combinations that have been investigated and the resulting yields of XOS. Bagasse, corncobs and the various components of wheat have been of interest as they naturally arise within biorefineries and offer a ready feedstock for further co-product production, while birchwood, tobacco stalks and cotton stalks have also been investigated. Pretreatments have included steam explosion, alkaline and acidic extraction, and ultrasound. Other enzymes have been deployed alongside endoxylanases: feruloyl esterases to aid release of AX by breaking ferulic acid crosslinks; arabinofuranosidases to shave off the arabinose units; and cellulases, glucosidases and xylosidases to help break down the structure to release AX. Yields have varied according to substrate, pretreatment and enzymes, with the highest yields from purified birchwood xylan following endoxylanase treatment (Falck *et al*., 2013; Nieto-Domínguez *et al.*, 2017) and from Kenaf stems (Wan Azelee *et al.*, 2016).

Table 1. Yields of XOS from various biomass substrate and enzyme combinations reported in the literature.

Biomass	Pre-treatment	Enzymes	Yields of XOS from original material*	Reference
Sugarcane bagasse	Aqueous ammonia	Bacterial arabinofuranosidase + β-xylosidase	*	(Reddy and Krishnan, 2016)
Sugarcane bagasse	Alkaline extraction	Bacterial cellulase + endoxylanase	6%	(Xue *et al.*, 2016)
Wheat bran	Destarching	Fungal endoxylanase + Feruloyl esterase	**	(Wu *et al.*, 2017)
Wheat chaff	Ultrasound	Endoxylanase	8% (+2-3% xylose)	(Antov and Đorđević, 2017)
Wheat straw	Alkaline extraction	Bacterial endoxylanase	11%	(Faryar *et al.*, 2015)
Wheat straw	Steam explosion	Commercial endoxylanase + glucosidase	8.9%	(Álvarez *et al.*, 2017)
Birchwood	-	Fungal endoxylanase GH11	28.8%	(Nieto-Domínguez *et al.*, 2017)
Birchwood, Rye	-	Bacterial endoxylanase	20% birchwood 3.3% rye	(Falck *et al.*, 2013)
Kenaf stems (*Hibiscus cannabinus*)	$Ca(OH)_2$ followed by peracetic acid	endoxylanase and arabinofuranosidase	35.2%	(Wan Azelee *et al.*, 2016)
Corncob	Alkaline extraction	Bacterial xylanase	**	(Gowdhaman and Ponnusami, 2015)
Corncob	Alkaline extraction	Fungal β-xylosidase	10.7%	(Chapla *et al.*, 2012)
Corncob	Acidic hydrolysis	Cellulase	14% (+ 32.8% glucose, 14.8% xylose)	(Zhang *et al.*, 2017)
Corncob	Hydrothermal + 16% alkali	Commercial endoxylanase	9%	(Samanta *et al.*, 2015)
Corncob	Alkali/acid pressure cooking	Fungal endoxylanase	**	(Aachary and Prapulla, 2009)
Tobacco stalk	Alkaline extraction	Commercial endoxylanase	11%	(Akpinar *et al.*, 2010)
Cotton stalk	Alkaline extraction	Commercial fungal endoxylanase	10.6%	(Akpinar *et al.*, 2007)

* Yields are measured and reported in a variety of ways and with varying precision, and are indicative rather than directly comparable.

** Insufficient data

Studies of enzymatic XOS production have only occasionally reported the accompanying release of monosaccharides. Uçkun Kiran *et al.* (2013) reported that two commercial endoxylanases released different amounts of undesired xylose monosaccharide in the extracts. Antov and Dorđević (Antov and Đorđević, 2017) advised that the endoxylanase treatment time is crucial to minimise the production of monosaccharides.

Generally, the activity of endoxylanases on oligosaccharides is not well understood. For example, only a limited number of contradictory reports in the literature discuss whether endoxylanases produce monosaccharides or not (Antov and Đorđević, 2017; Cürten *et al.*, 2018; Uçkun Kiran *et al.*, 2013). Also, the action or preference of endoxylanases for larger or smaller oligosaccharides chains has not previously been reported, as the interest in the hydrolysing enzymes has been primarily their efficacy in breaking down the polysaccharides either all the way to the constituent monosaccharides (as a fermentation feedstock, for example) or just to reduce viscosity. Now, with the increasing interest in oligosaccharides arising from their prebiotic functionality, it has become essential to investigate in detail the mode of action of xylanases on the production and degradation of small xylo- and arabino-oligosaccharides. However, such investigations faced the difficult technical challenges of measuring these oligosaccharides.

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been the method of choice for carbohydrate analysis (Cui and Brummer, 2005). The utilisation of PAD enables a sensitive detection, which is indispensable in the case of complex samples. Recently, the commercial availability of XOS standards up to DP6 and a smaller number of AXOS standards has facilitated the separation of these analytes in a simple and direct analysis using an HPAEC-PAD system. The accurate determination of xylanase activity by directly measuring the produced oligosaccharides has thus become a routine analysis. Falck *et al.* (2015) determined the XOS and AXOS produced by different xylanases

using an HPAEC system with a CarboPac PA200 column and a fixed 100 mM sodium hydroxide aqueous solution with a linear increase of sodium acetate from 0 to 120 mM within a 30-minute run. Cürten *et al.* (2018) reported a faster method for the analysis of XOS using a CarboPac PA100 column with a more concentrated mobile phase composition. Other reports have showed the effective usage of HPAEC-PAD for the quantification of XOS and/or AXOS variously using CarboPac columns PA1, PA100 and PA200 to measure XOS in the range DP2-6 (Arruda *et al.*, 2017; Bian *et al.,* 2013, 2014; Courtin *et al.,* 2009; Gullón *et al.,* 2008; Lafond *et al.,* 2011; Li *et al.,* 2013; Zhang *et al.,* 2017). All of these used a fixed (isocratic) concentration of sodium hydroxide, with gradient elution of sodium acetate, in varying proportions and molarities.

The fast and reliable analysis together with the availability of standards have made this technique the favoured choice for oligosaccharides analysis. However, the isocratic elution of sodium hydroxide solution, as recommended by the manufacturers (Dionex Corporation, 2004), severely reduces the resolution of the monosaccharides and to some extent the oligosaccharides; these recommendations are probably inherited from the first generations of ion exchange techniques when RI detectors were the only option. With isocratic elution, the analysis of monosaccharides requires different conditions from those for oligosaccharides; hence, to get the full picture of enzyme hydrolysis activity, samples need to be analysed twice under different conditions and with two different columns and mobile phases, to quantify the monosaccharides and the oligosaccharides separately (Mathew *et al.,* 2017). Furthermore, the content of the uronic acids (glucuronic and galacturonic acid, which are prevalent in wheat-derived AX) has not been considered in conjunction with oligosaccharide analysis in the literature, as it has also required different conditions. Due to these various technical limitations, the precise activity of xylanases in terms of their activity on oligosaccharides has not been well described in the literature.

Therefore, the current work introduces the use of gradient elution of both sodium hydroxide and sodium acetate to give a high resolution method for the simultaneous determination of cereal monosaccharides (arabinose, galactose, glucose and xylose), along with xylo-oligosaccharides in the range DP2-6, and arabinoxylo-oligosaccharides (2^3-α-L-arabinofuranosyl-xylotriose (A2XXX), 3^3-α-L-arabinofuranosyl-xylotetraose (XA3XXX), 2^3-α-L-arabinofuranosyl-xylotetraose (XA2XXX) and 2^3,3^3-di-α-L-arabinofuranosyl-xylotriose (A2A3XXX)), as well as the uronic acids (glucuronic and galacturonic acid). The method allows a comprehensive description, quantitative and qualitative, of the modes of action of xylan-degrading enzymes as well as the substrate specificity of the enzymes.

This paper describes the materials and instrument used, then evaluates the performance of the method in terms of validation and system suitability parameters. The method is then applied to follow the sugars released from wheat bran following treatment with a commercial endoxylanase. The method is further illustrated in a selected result from a study of the endoxylanase activity on small oligosaccharide standards, in which the evolving oligosaccharide profile was followed as a function of the relative preference of the enzyme for different sizes of oligosaccharide substrate.

2- Materials and Methods

2.1- Materials

Standards of monosaccharides fucose (as internal standard), arabinose, galactose, glucose and xylose were obtained from Sigma UK. Xylan oligosaccharides (arabinose, galactose, glucose and xylose), xylo-oligosaccharides (xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6)) and arabinoxylo-oligosaccharides (2^3-α-L-arabinofuranosyl-xylotriose (A2XXX), 3^3-α-L-arabinofuranosyl-xylotetraose (XA3XXX), 2^3-α-L-arabinofuranosyl-xylotetraose (XA2XXX) and 2^3,3^3-di-α-L-arabinofuranosyl-xylotriose (A2A3XXX)) were purchased from Megazyme (Bray, Ireland). Glucuronic acid,

galacturonic acid, sodium acetate, sodium hydroxide (52%), citric acid and sodium phosphate mono-basic were obtained from Fisher scientific UK. Commercial xylanase Econase XT 25L (160.000 BXU/g) was supplied by AB Vista (Marlborough, UK). Wheat bran was kindly provided by Biorenewables Development Centre, York, UK.

2.2- Chromatography System

The HPAEC-PAD system used was a Dionex ICS–3000 Ion Chromatography System with gold electrode (Dionex Corporation, CA, USA) consisted of an ICS-3000 dual pump, an AS50 autosampler and an ICS-3000 DC (Detector/Chromatography) compartment with an electrochemical detector with a gold working electrode, an Ag/AgCl reference electrode. The separation was performed on a CarboPac PA200 (3 mm × 250 mm) with a CarboPac PA200 guard (3 mm × 50 mm) column and a CarboPac PA20 (3 mm × 150 mm) with a CarboPac PA20 guard (3 mm × 30 mm) column. Chromeleon® (6.8) software was utilised for data processing.

2.3- Chromatographic conditions

Gradient elution of two mobile phases was performed: Solution A comprising 10 mM sodium hydroxide; and Solution B comprising a mixture of 200 mM sodium hydroxide and 125 mM sodium acetate. The elution was performed starting with 100% Solution A at time 0 with a linear increase of Solution B to reach 100% after thirty minutes, then held at 100% B for five minutes (Alyassin, 2019). Column equilibration was performed by maintaining starting conditions (100% A) for at least 15 min before sample injection. The mobile phase was sparged with helium and kept under helium during the analysis. The elution flow rate was maintained at 0.3 mL min^{-1} and the injection volume was 20 μL. Pulsed wave form "carbohydrates (standard quad)", wave form description "carbohydrates (std. quad. Potential)" was used for the detection. Fucose was used as an internal standard.

2.4- Preparation of standard solutions

A primary stock solution of each standard was prepared in Milli-Q (18.2 MΩ cm) water to obtain a concentration of 2 mg mL^{-1}. Then a secondary stock solution of a mixture of all the standards was prepared at concentrations of 100 mg L^{-1} each. Serial dilutions with water were performed to prepare the calibration curve for each standard. The concentration range for working standard solutions was 1-20 mg L^{-1}. The standards solution was found to be stable when kept refrigerated at 4°C.

2.5- Xylanase action on wheat bran

Wheat bran was washed with water at 70°C with continuous stirring for 60 minutes, then strained over a 250-micron sieve three times to ensure the removal of any residual starch. The washed bran was oven-dried overnight at 75°C.

A sample of 20.0 g of the washed dried bran was weighed into a flask with 200 mL of citrate/ phosphate buffer at pH 4.5 and placed in a shaking water bath at 50°C. One mL of the enzyme was added equivalent to 50 mL per kg of dry sample or 500× the commercial dosage, to accelerate changes, after which 10 mL samples of the solution were taken after 0, 30, 60 and 120 minutes and submerged in an ice bath to halt the enzyme activity. The samples were diluted and filtered over 0.45-micron syringe filters and analysed in the HPAEC system. Samples were injected manually directly after removal from the ice bath, thus samples remained cold such that enzyme activity did not resume prior to analysis.

2.6- Enzymatic degradation of XOS standards

Solutions containing a mixture of xylohexaose and xylopentaose standards at 100 mg L^{-1} each were incubated with the endoxylanase in pH 4.5 phosphate/citrate buffer in a water bath at 50°C. The enzyme was added at a dosage of 50 mL per kg of dry sample. Samples were

taken at 10, 20, 40, 60 and 120 minutes, to evaluate the XOS production and degradation over time. The samples were diluted using phosphate-citrate buffer (at pH 7) and boiled for 20 minutes to deactivate the enzyme permanently to allow queuing in the autosampler without further enzyme activity, then kept at room temperature until analysis.

3- Results and discussion

3.1- Chromatography and specificity

Figure 1(a) shows a chromatogram of the standards mixture of the studied analytes, each at a concentration of 20 mg mL^{-1}, obtained using the CarboPac PA200 column. The chromatographic run time of 35 minutes was sufficient for the elution of all standards. The baseline was smooth and stable in all the analysed samples, and the gradient elution gave a high resolution separation and did not affect the baseline. In fact, the linear gradient elution of the alkaline aqueous solvent not only enhanced the separation of oligosaccharides, it also increased the capability of the columns used for the separation. The CarboPac PA20 is commercialised as the column of choice for mono- and disaccharides, but with the applied conditions the PA20 was also able to separate the oligosaccharides efficiently although over a longer time, as illustrated in Figure 1(b). The PA200 was used for the remainder of the current work as it allowed faster run times.

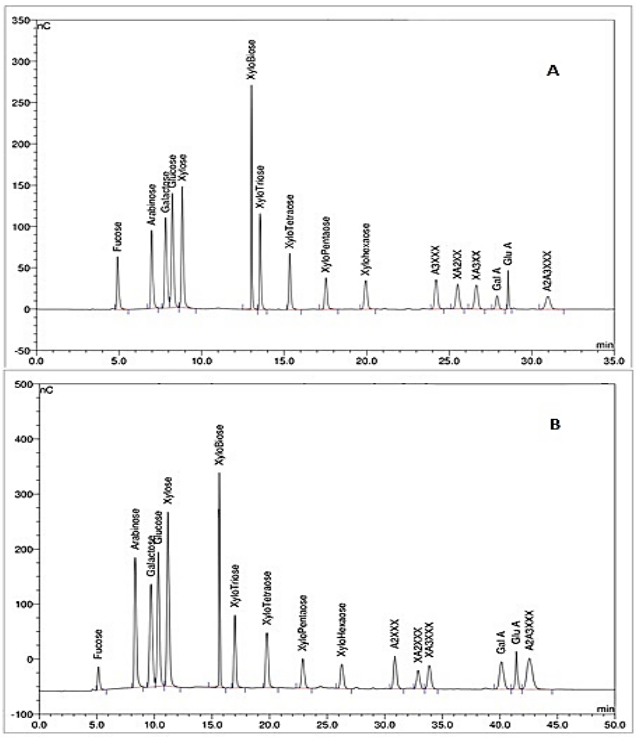

Figure 1 Chromatogram of mono and oligo saccharides mixture using (a) CarboPac PA200, and (b) CarboPac PA20.

3.2- Linearity

To assess linearity, calibration curves were plotted using the ratio of the analyte peak area to the internal standard peak area against concentrations of different dilutions (n = 9) covering the range 1-100 mg mL^{-1} for monosaccharides and 1-20 mg mL^{-1} for XOS, AXOS and uronic acids. The correlation coefficient values were acceptable for all the standards and ranged between 0.985 and 0.999. The correlation coefficients (R^2) are reported in Table 2, along with Limits of Detection (LOD) and Quantitation (LOQ).

Table 2. The HPAEC method linearity, LOD, LOQ, resolution, and separation efficiency parameters for the studied monosaccharides, oligosaccharides and uronic acids.

	R^2	LOD mg L^{-1}	LOQ mg L^{-1}	Resolution factor R_s	Asymmetry A_s	Capacity factor k'	Theoretical plates
Arabinose	0.999	0.03	0.08	3.63	1.35	2.676	17620
Galactose	0.996	0.08	0.1	1.71	1.1	3.114	15701
Glucose	0.996	0.08	0.1	2.64	1.08	3.334	18981
Xylose	0.997	0.04	0.1	10.23	1.26	3.649	26455
Xylobiose	0.984	0.001	0.008	26.6	1.1	5.851	1081600
Xylotriose	0.983	0.05	0.1	9.46	1.23	6.132	112710
Xylotetraose	0.981	0.2	0.4	9.15	1.33	7.071	80127
Xylopentaose	0.985	0.3	0.5	8.25	1.09	8.228	69836
Xylohexaose	0.986	0.03	0.1	13.27	1.04	9.5	61511
A3XXX	0.994	0.04	0.08	3.91	1.1	11.737	91312
XA2XXX	0.993	0.01	0.03	3.13	0.95	12.43	82712
XA3XXX	0.990	0.01	0.03	3.69	0.95	13.026	82668
A2A3XXX	0.995	0.08	0.2	23.52	1.05	15.298	57549
Gal A	0.984	0.06	0.2	3.06	1.07	13.684	130671
Glu A	0.997	0.06	0.1	7.47	1.37	14.035	793131

3.3- Limits of detection (LOD) and quantitation (LOQ)

LOD and LOQ measure analytical method sensitivity to low concentrations of analyte. LOD is the lowest concentration detected and LOQ is the minimum quantifiable concentration measured by the method (Uhrovčík, 2014). Signal-to-noise (S/N) ratios of 3:1 and 10:1 were used to define LOD and LOQ, respectively, and were calculated by the Chromeleon® software. The peaks were clear to identify after the dilutions and had acceptable precision. LOD and LOQ values for all the analytes are listed in Table 2. The table shows variation in the detection and quantification limits of the standards, which is a reflection of the variation in the peak heights and areas for the same concentrations, as evident in Figure 1. In general the monosaccharides showed lower detectable concentrations than the oligosaccharides, except for xylobiose which is more readily detected than xylose and gave the lowest values of LOD and LOQ (Alyassin, 2019). However, it was noted that the values of LOD and LOQ are

subject to the fitness of the of the PAD; in other words, the gradual recession of the gold electrode (electrode fouling) can cause loss of detector signal over time, which requires regular cleaning of the gold electrode.

3.4- Accuracy and precision

The accuracy of the method was studied by analysing triplicates of control samples covering three concentration levels (5, 10, 15 ppm); the results are listed in Table 3 as percentage recovery. The calculated recoveries for all the studied standards ranged acceptably between 97-103%. The precision of a method reflects the agreement among individual test results. Intra-day precision (repeatability) was estimated by analysing seven replicates of a control sample in the same run. Inter-day precision (intermediate precision) was assessed from nine triplicates over three consecutive days (three runs per day) using the same instrument with different batches of the mobile phase. Both repeatability and intermediate precision results are listed in Table 3 as the percentage coefficient of variation (CV %). Both the precision and the intermediate precision are in acceptable ranges for all the analytes.

262 *Table 3. Method accuracy (reported as percentage recovery) and precision (reported as CV%).*

		Arabinose	Galactose	Glucose	Xylose	Xylobiose	Xylotriose	Xylotetraose	Xylopentaose	Xylohexaose	A2XXX	XA2XXX	XA3XXX	Gal A	Glu A	A2A3XXX
Recovery %	15 ppm	101.02±0.64	100.46±1.46	100.28±0.82	100.42±1.2	100.81±0.78	99.99±1.23	100.34±0.31	100.4±0.86	102.67±3.2	102.13±3.33	102.07±2.42	100.01±1.12	100.4±1.02	100.26±0.41	100.12±0.6
	10 ppm	101.21±2.52	100.91±1.22	102.38±3.42	99.89±2.73	101.23±1.93	103.29±4.63	99.68±1.08	101.14±2.4	100.59±0.64	99.41±1.1	101.55±1.41	99.97±0.9	99.81±2.1	101.2±1.15	100.26±2.25
	5 ppm	100.19±3.03	100.5±3.07	101.95±2.87	100.09±0.87	97.7±2.83	103.91±4.61	100.2±1.88	99.6±1.76	100.08±0.33	99.57±0.51	101.33±3.17	100.79±2.97	99.55±0.92	100.74±2.18	100.07±1.83
Precision CV%		0.006	0.015	0.008	0.012	0.008	0.012	0.003	0.009	0.031	0.033	0.024	0.011	0.01	0.004	0.006
Intermediate precision CV%	Day 1	0.03	0.017	0.047	0.005	0.014	0.026	0.011	0.002	0.05	0.049	0.018	0.018	0.019	0.017	0.017
	Day 2	0.015	0.014	0.012	0.025	0.01	0.024	0.017	0.015	0.041	0.022	0.004	0.02	0.016	0.003	0.014
	Day 3	0.006	0.015	0.008	0.012	0.008	0.012	0.003	0.009	0.031	0.033	0.024	0.011	0.01	0.004	0.006

3.5- System suitability tests

The fitness of the chromatography system was estimated by a range of system suitability test parameters: Capacity factor (retention factor) *k'*, Resolution factor (R_s), number of theoretical plates (Column efficiency) and Asymmetry (A_s), as calculated by the Chromeleon® software. The results of these parameters for each analyte are listed in Table 2. The values of the resolution factor reflect a high-resolution separation of the analytes except for galactose where the R_s was only 1.71. In general, all the calculated parameters gave acceptable values; the asymmetry factor was between 0.95 and 1.37, the capacity factor was more than 2 and the number of theoretical plates was greater than 15000 for all the analysed standards.

3.6- Results of xylanase treatment of wheat bran

The oligosaccharides released from wheat bran by endoxylanase treatment were measured and estimated as a percentage of the original wheat bran on a weight basis. Figure 2 shows a chromatogram of the released oligosaccharides from wheat bran after 30 minutes of enzyme treatment.

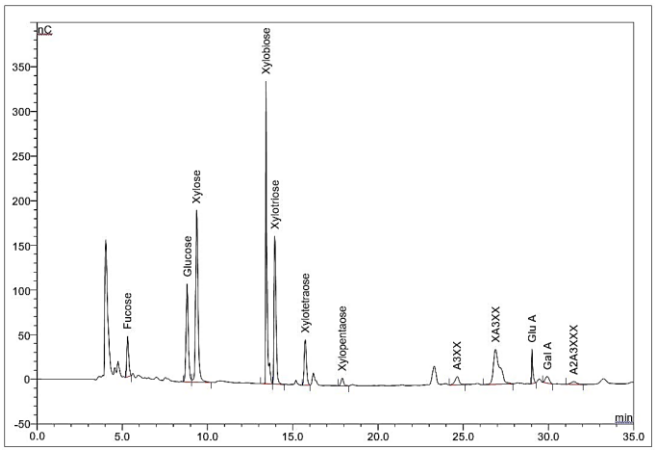

Figure 2 chromatogram of the released mono- and oligosaccharides from wheat bran by xylanase activity using HPAEC-PAD system with Carbopac PA200 column

Figure 3 (a) illustrates the evolution of X1-6 over time. Clearly xylose increased over time, xylobiose (X2) also increased monotonically, but by contrast, X3 and X4, after an initial

burst of production, decreased over time as they were further degraded by the xylanase. Small amounts of X5 and X6 also appeared briefly but were degraded even more quickly. The results indicate a pattern of the creation and degradation of the xylan oligosaccharides as initially fragments are released by enzyme action on larger AX polysaccharides, then the enzymes turn their action onto the released oligos. Thus the majority of the oligos were produced in the first 30-60 minutes, including the brief appearance of X6 and X5 during this time. Thereafter, a balance of producing new small oligos (X2) and degrading the larger oligos (X3, X4, X5 and X6) becomes evident. The steady increase of X2 during the treatment suggests that this endoxylanase cannot act on the disaccharide. On the other hand, the increasing amounts of xylose, which is not expected for endoxylanases as they attack the middle of xylan chains, could be explained by three hypotheses: endoxylanases could have a side activity as exoxylanases; or there could be some contaminating exoxylanase in the enzyme preparation.

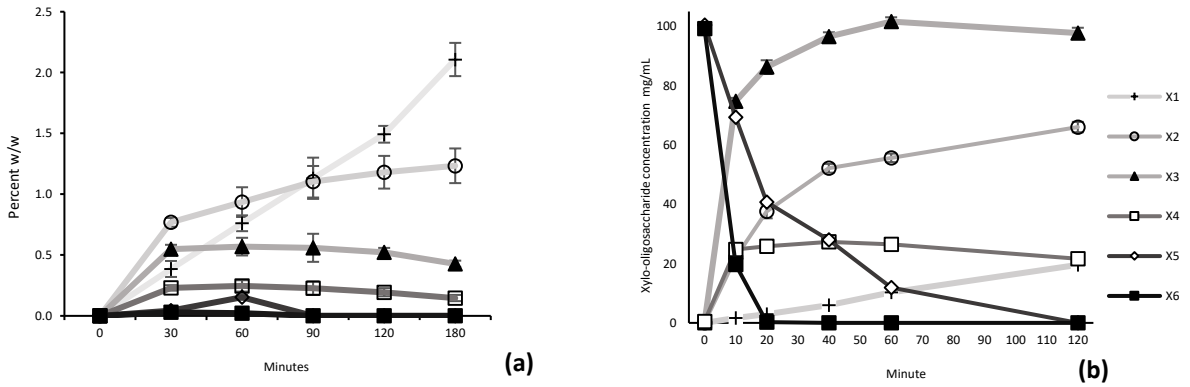

Figure 3.(a) Evolution of XOS from wheat bran over time following xylanase treatment (b) Evolution of xylo-oligosaccharides following Econase treatment of xylohexaose and xylopentaose standards.

3.7- Results of enzymatic degradation of XOS standards

The method was also applied to follow the degradation kinetics of xylo-oligosaccharide standards by the xylanase. Figure 3(b) shows the evolution of X1-X4 following treatment of xylohexaose and xylopentaose (X6 and X5) with endoxylanase. Clearly, the degradation of X6 is faster than of X5; the enzyme evidently prefers the larger oligosaccharide. The predominant product is the X3 followed by X2, which agrees with the expectation that endoxylanases act within the polysaccharide chain; cleavage of an X6 in the middle would produce two X3s, while cleavage of X5 would produce an X3 and an X2. Significant amounts of X4 were also produced initially from X6 cleavage, with corresponding X2, and with the X4 then also slowly degrading further to X2, the enzyme apparently preferring the X4 as a substrate to the X3, hence the latter only beginning to degrade, and apparently more slowly, towards the end of the two-hour period. Full understanding of the dynamics underpinning this evolving profile would require detailed population balance modelling based on a more extensive data set. Such understanding would enable the XOS with the greatest prebiotic benefits to be optimised for human or animal nutrition.

The appearance of an increasing amount of xylose monomer (X1) suggests that the enzyme can attack the terminal units of the larger oligos; the degradation of X3 also supports this, although this is unexpected for this endoxylanase and, as above, may indicate a side activity or, more likely, some contaminating enzyme. A practical application indicated by the evolving profile is that using endoxylanases to produce particular oligos such as X2 and X3, which are believed to have the greatest prebiotic value (Gullón *et al.*, 2008; Van Craeyveld *et al.*, 2008) might be enhanced if performed under a separation system such that the produced oligos are immediately removed from the enzyme, in order to avoid further degradation and control the profile of degradation products.

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4- Conclusion

This work presents a novel HPAEC-PAD method for rapid and accurate quantification of cereal monosaccharides, xylo- and arabinoxylo-oligosaccharides and uronic acids. Gradient elution of the mobile phase allowed high-resolution separation of all the analytes, using both PA200 and PA20 CarboPac columns. The method is applicable for characterising xylan-degrading enzymes and for measuring the production of mono- and oligosaccharides in foods and animal feeds. Application of the method to test endoxylanase activity on wheat bran and XOS standards revealed dynamic evolution of the XOS profile as the enzyme produces and then degrades the oligosaccharides to monosaccharide. The ability to measure the range of mono- and oligosaccharides arising from xylanase activity on cereal feedstocks will enhance the development and exploitation of enzyme products for food and feed products to enhance human and animal health.

Conflict of interest

The authors state not having any conflict of interest in the publication of this article.

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