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Pilot-scale isolation of glucuronoarabinoxylans from wheat bran

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Abstract

The major dietary-fiber component of wheat bran, glucuronoarabinoxylans, have been isolated from purified wheat bran in a pilot-scale procedure. Wheat bran, milled from the wheat variety Bussard, was purified with boiling 70% ethanol following removal of water-extractable arabinoxylans by extraction with water. Glucuronoarabinoxylans were subsequently extracted with 2% hydrogen peroxide of pH 11 at 40 °C. Residual proteins and mixed linked (1-3)(1-4)-β-D-glucans were removed enzymatically. The final product was precipitated with ethanol and had a purity of 70–80%. X/A-ratio was 1.25, ferulic acid content was below 0.1%, HPSEC analysis with LALS detection showed a single peak with an apparent MW of 100–110 kDa. ^{13}C-NMR analysis showed the presence of mono- and di-substituted xylose residues of the xylan core. The procedure allowed the production of 350 g of glucuronoarabinoxylans within 1 week, using low priced chemicals of low toxicity. It can be easily upscaled to industrial scale.
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Keywords: Arabinoxylans; Wheat bran; Non-starch polysaccharides

1. Introduction

The epidemiological EPIC study has demonstrated that an increased consumption of fruit and vegetables and a high intake of dietary-fiber reduce the risk of colon cancer (Bingham & Riboli, 2004). Whole grains of cereals are a rich source of fermentable carbohydrates including dietary-fiber, resistant starch and non-digestible oligosaccharides. Dietary-fiber is concentrated in the outer layers of the grain, thus wheat bran can contain 45–50% (dm) of fiber. The nutritional value of a regular consumption of wheat bran in terms of to having any protective effect against colon cancer is still a matter of debate. Despite several laboratory animal model studies having shown results which were interpreted as having protective effects (Harris & Ferguson, 1999), several large epidemiological studies (Sengupta, Tjandra, & Gibson, 2001) have not given any conclusive evidence for a risk reduction for colon cancer. This is difficult to understand due to sound epidemiological data provided by the EPIC study, which clearly showed a statistical significant risk reduction for colon cancer by the regular consumption of a high-fiber diet. The health-promoting roles attributed to dietary-fiber in the human colon include: reduction of caloric intake, fecal bulking, dilution of potentially harmful substances and a decreased intestinal transit time of colonic contents, thereby reducing the bioavailability of food-borne carcinogens, binding of secondary bile acids and formation of short chain fatty acids (SCFA, mainly acetate, propionate and butyrate) through microbial fermentation. Intestinal anaerobic fermentation of wheat bran produces butyrate that inhibits proliferation, induces differentiation and enhances apoptosis in colon cancer cells and acts as a growth factor to the healthy colon epithelium.

Wheat bran sustains butyrate levels throughout the complete lumen of the colon in contrast to most fiber sources. It is hypothesized that the protective effects of wheat bran against colon cancer may be associated with increased colonic butyrate.

The arabinoxylans of wheat bran (25–28% dm) are a major source of fermentable and butyrate producing

Abbreviations: HPSEC, high-performance size-exclusion chromatography; dm, dry matter; WEAX, water-extractable arabinoxylans; AEAX, alkali-extractable arabinoxylans; GAX, glucuronoarabinoxylans; HPAEC, high-performance anion exchange chromatography; X/A, xylose to arabinose-ratio.

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polysaccharides. Bacterial fermentation of arabinoxylans from wheat and sorghum has been studied in vitro (Van Laere, Hartemink, Bosveld, Schols, & Voragen, 2000). It was concluded from these studies that various intestinal species of bacteria could ferment this substrate to some extent. These studies did not allow any predictions about the fermentation in vivo. For such in vivo studies or any other studies aimed at the evaluation of the nutritional properties of arabinoxylans from wheat bran preparative amounts of these compounds must be provided.

The objective of this study was to develop an economically viable procedure for the isolation of the glucuronoarabinoxylans from wheat bran in a pilot-scale. This procedure differed from other procedures in the literature in that arabinoxylans of high purity are produced and low price and low toxicity chemicals are used. The procedure allows an easy upscaling for production of these polysaccharides at industrial scale. It may open up opportunities for the application of this wheat-processing by-product as a health improving food supplement, or for other purposes.

2. Experimental

2.1. Isolation and purification of glucuronoarabinoxylans

Grains of the wheat cultivar Bussard (harvested in 2001) were milled on a pilot plant mill (MIAG/Buehler AG, Switzerland). The bran fraction (mean particle diameter 0.5 mm) was collected and analyzed according to ICC standard methods (ICC, 1991) or procedures described below. All extraction experiments were carried out in water-jacketed vessels made of high-grade steel, having a total volume of 50 l. The jacket was fed with tap water for a fast and efficient cooling of the vessel contents. For removal of low molecular weight contaminations and inactivating arabinoxylan-degrading enzymes wheat bran was treated with boiling aqueous ethanol. Five kilogram batches of wheat bran were suspended in 30 l of 70% ethanol and heated to 80 °C for 4 h with stirring. After cooling the bran was collected on a 0.40 mm sieve, washed two times with 3 l of 70% ethanol and then dried in a dryer with a recycled air system for 24 h. For the removal of water-extractable arabinoxylans (WEAX) from the purified wheat bran 3.5 kg of purified bran was suspended in 40 l of water and heated for 2 h at 40 °C with constant stirring. Subsequently, the bran particles were recovered by sieving, washed with 5 l of cold water and dried for 24 h at 40 °C in a dryer with recycled air system. For the isolation of glucuronoarabinoxylans 2 kg of purified and WEAX-free wheat bran was suspended in 40 l of 2% hydrogen peroxide adjusted to pH 11 with 25% sodium hydroxide. 6 ml (0.015%, v/v) of an anti-foaming agent (Witafrol 7456 C) were added to suppress foaming. The suspension was heated to 40 °C under constant stirring and kept at this temperature for 4 h. After cooling to room temperature the pH was adjusted to 8.5 with concentrated sulfuric acid and 50 ml Alcalase 2.4L (Novozymes, Denmark) were added. After stirring for 12 h at room temperature, the extract was heated (10 min at 100 °C), cooled to room temperature and the pH adjusted to 7. The bran particles were sieved with a 0.40 mm sieve and washed with water. The collected filtrate was concentrated by ultrafiltration using a Labstak Module 38 on fluoro polymer flat sheet membranes with a MWCO of 10 kDa (Danish Separation Systems AS, Denmark) to 1/5 of its inital volume. Ninety-six percent ethanol was added to the retentate with stirring up to a final concentration of 65%. Stirring was discontinued and precipitated material allowed to settle overnight at 4 °C. The supernatant was decanted, the residue collected by centrifugation (10 min at 2000*g*), washed with 96% ethanol then with acetone and air-dried for 12 h. β-Glucans were removed enzymatically by a combination of lichenase at pH 6.5 and β-glucosidase at pH 4.0, respectively. After thermal inactivation of the enzymes, and centrifugation, the supernatant was dialyzed exhaustively against distilled water and freeze-dried.

2.2. Materials

Hydrogen peroxide, sodium hydroxide, sulfuric acid, acetone were from Merck (Darmstadt, Germany), industrial 96% ethanol, denatured with 1% petroleum ether, was from Alkohol Handelskontor (Lippstadt, Germany), the anti-foaming agent Witafrol 7456 C was from Sasol GmbH (Witten, Germany). The following enzymes were used: Lichenase (E.C. 3.2.1.73) from *Bacillus subtilis*, and β-glucosidase (E.C. 3.2.1.21) from *Aspergillus niger* were kindly provided by Gamma-Chemie, München, Germany.

The protease Alcalase 2.4L was kindly provided by Novozymes A/S, Bagsvaerd, Denmark.

2.3. Analytical methods

Moisture, ash, fat, protein content of wheat bran, purified wheat bran and of water- and alkali-extractable fractions were determined by ICC standard methods No. 109/1, 104/1, 136, and 105/2 (ICC, 1991). The (1-3)(1-4)-β-D-glucan content was determined by a mixed-linkage β-glucan assay procedure (MEGAZYME, 1998). The relative amounts of monosaccharides in wheat bran and polysaccharide fractions thereof were quantified by high-performance anion exchange chromatography (HPAEC) on a DIONEX BioLC system (Dionex Corporation, Sunnyvale, USA). The system consisted of a gradient pump GS50 and an electrochemical detector ED50. The HPLC system was equipped with a DIONEX CarboPac PA1 column (2×250 mm) in combination with a CarboPac PA1 guard column (4×50 mm) and a PAD cell. A reference Ag/AgCl electrode was used with a working gold electrode with the following pulse potentials and durations: E_1 0.1 V for 40 s, E_2 2.0 V for 2 s E_3 0.6 V for 1 s and E_4 0.1 V for 6 s.

Samples of 15–20 mg were hydrolyzed at 105 °C with 1 M H_2SO_4 for 2 h and neutralized with 0.25 M barium hydroxide. The hydrolyzed samples (25 μl) were eluted with 20 mM sodium hydroxide at 1.0 ml/min and 20 °C within 25 min. Standard monosaccharides (Ara, Gal, Glc, Man, Xyl) were of highest purity (>95%) available and obtained from Sigma (Taufkirchen, Germany). The arabinoxylan content of samples was calculated as 0.88 (%arabinose + %xylose). All analyses were conducted in duplicate, and values were required to be within a 5% reproducibility range.

Total uronic acids were quantified after subsequent reactions with sulfuric acid/tetraborate and *m*-hydroxybiphenyl (Blumenkrantz & Asboe-Hansen, 1973). Phytic acid was quantified by the colorimetric method of Latta and Eskin (1980). For the determination of ferulic acid and *p*-coumaric acid, samples (70–80 mg) were saponified with 5 ml of 2 M sodium hydroxide at 20 °C in the dark for 18 h in a helium atmosphere. 0.1 mg of cinnamic acid was added as an internal standard, samples were acidified with 4 M hydrochloric acid to pH 2 and four times extracted with 2 ml portions of ethylacetate. The organic phases were separated from the aqueous phase by centrifugation (3000*g* for 10 min), combined and dried with anhydrous sodium sulfate and evaporated to dryness in a rotary evaporator at 30 °C. The residue was dissolved in 10 ml of methanol/water (90:10, v/v) and filtered (0.45 μm-filter) prior to HPLC/DAD analysis of hydroxycinnamic acids. Chromatographic analysis was performed with a combination of a Merck 6200A Intelligent pump and a L-6000 Intelligent pump and a L-4500 diode array detector (Merck, Darmstadt, Germany). UV detection was carried out at 300 nm. The extracted samples (25 μl) were analyzed using a Maxsil™ RP-C18 column, 250×4.6 mm, 5 μm endcapped, equipped with a SecurityGuard™ C18 column (Phenomenex, Aschaffenburg, Germany) using an aqueous acetonitrile gradient. Linear gradient elution was performed by acetonitrile/acetic acid (99:1, v/v) and water/acetic acid (99:1, v/v) with a pump rate of 0.9 ml/min at 35 °C from 10:90 to 20:80, respectively, in 20 min, from 20:80 to 60:40 in 10 min, from 60:40 to 100:0 in 5 min and maintained for 15 min. Standards of *trans*-ferulic acid and *p*-coumaric acid were used to quantify the amounts present in the samples.

During sample work-up isomerization of the *trans*-isomer to the *cis*-isomer may occur. Amounts of ferulic acid encompass the sum of both isomers.

2.4. High-performance size-exclusion chromatography

Molecular weight distribution of the isolated arabinoxylans were determined by HPSEC using two ViscoGel GMPWXL Mixed Bed (7.8×300 mm) columns in series (Viscothek, Weingarten, Germany). 100 μl samples (1.8 mg/ml) were injected and eluted at 35 °C with 0.2 M lithium nitrate at a flow rate of 0.6 ml/min. The average molecular weight and intrinsic viscosity were determined using a triple detection system TDA 302, composed of a dual refractometer/viscometer detector in combination with a right angle laser light scattering detector (Viscothek, Weingarten, Germany). Molecular weights and viscosities were calculated using the calibration modules of the Trisec software (Viscothek). The system was calibrated with pullulan standards and $dn/dc = 0.147$.

2.5. ^{13}C-NMR analysis

Proton-decoupled ^{13}C-NMR spectra of glucuronoarabinoxylans were recorded with a Varian INOVA 500 spectrometer at 333 K at a frequency of 125 MHz. Samples were measured in DMSO-d_6 at a concentration of 2% (w/v). The solvent served as an internal reference at 39.5 ppm. Spectra were recorded by Inverse-Gated-Decoupling-Technique, spectral width was set to 25 kHz, the pulse delay was 3 s and the data acquisition time 1.3 s. Twenty thousand scans were accumulated within 24 h.

2.6. Removal of β-glucans

To remove β-glucans from the isolated glucuronoarabinoxylans, e.g. 10 g portions of arabinoxylans were dissolved with constant stirring in 1000 ml of 20 mM PBS, pH 6.5. Two millilitre of lichenase (50 U/ml, Gamma-Chemie, München, Germany) were added and the solution kept for 1 h at 40 °C. Subsequently the pH was adjusted to 4.0 with 4 M hydrochloric acid, 10 ml β-glucosidase (20 U, Megazyme, Wicklow, Ireland) were added and the mixture kept at 40 °C for 20 min. Following heat inactivation of the enzymes (10 min, 100 °C) the solution was centrifugated (15 min at 3000*g*) and arabinoxylans precipitated at 65% final concentration of ethanol for 12 h at 4 °C. The precipitate was collected, washed with 96% ethanol and air-dried at 40 °C in an forced-air oven. The β-glucan content of the precipitate was quantified by ICC standard method No. 166 (ICC, 1991).

3. Results and discussion

In order to isolate glucuronoarabinoxylans in a pilot-scale procedure from wheat bran in good yield and high purity the wheat bran milled from wheat grain had to contain merely the outer layers of the grain. Purification of the bran with hot aqueous ethanol was another prerequisite. The chemical composition of the bran fraction is shown in Table 1. The starch content of 8.8%, the protein content of 17.7% and the low amount (<1%) of water-extractable arabinoxylans indicated the presence of a high share of grain outer layers in the bran. By extraction with hot 80% ethanol before isolation of glucuronoarabinoxylans the bran was freed from coloured contaminants, fat and low molecular weight carbohydrates. Arabinoxylan-degrading enzymes were inactivated (data not shown). No arabinoxylans were

Table 1
Chemical constituents of bran of the wheat cultivar Bussard

Ash (%)[a]	7.6
Protein (%)	17.7
Starch (%)	8.8
Water (%)	11.5
∑Cellulose + lignin (%)	27.7
Arabinoxylans (%)[b]	25.0
Water-extractable arabinoxylans (%)	2.8
β-Glucans (%)	2.8
TDF (%)[c]	53.5
Fat (%)	3.6
Arabinose (%)	12.0
Xylose (%)	14.1
Mannose (%)	n.d.
Glucose (%)	21.4
Galactose (%)	1.2
Phytic acid (%)	4.9
Uronic acid (%)	3.1
p-Coumaric acid (mg/100 g)	n.d.
t-Ferulic acid (mg/100 g)	0.4
Lignans (mg/100 g)[d]	3.4

All values were determined in duplicate. n.d., Not detectable.
[a] [%] = Weight percentage (dm) of bran.
[b] Arabinoxylans = 0.88 (%Xyl + %Ara).
[c] TDF, Total dietary-fibre.
[d] $\text{Lignans} = \sum \text{Secoisolariciresinol} + \text{Matairesinol} + \text{Lariciresinol} + \text{Pinoresinol} + \text{Syringaresinol} + \text{Isolariciresinol}.$

extracted by this purification step. Besides glucuronoarabinoxylans (AEAX), which can only be extracted under strong alkaline conditions, wheat bran also contains arabinoxylans which are extractable with water at neutral pH (WEAX) (Fincher & Stone, 1974). Therefore, the ethanol treated wheat bran was stirred with water at 40 °C in order to remove WEAX. Wheat bran of the variety Bussard contained only up to 1% of dry mass of the WEAX. The ferulic acid and *p*-coumaric acid content of this arabinoxylan type was below 0.1% which was still below the 0.2% reported by Schooneveld-Bergmans, Dignum, Grabber, Beldman, and Voragen (1999). The xylose/arabinose-ratio was 2.5 and thus higher than the value of 1.6–1.9 reported for arabinoxylans of wheat flour by Suckow, Abdel-Gawad, and Meuser (1983), and Izydorcyk, Biliaderis, and Bushuk (1990). The botanical origin of water-extractable arabinoxylans remains unclear.

It cannot be excluded that during the purification of the bran L-arabinose substituents were split off from the xylan core. Neither uronic acids or ferulic acid nor *p*-coumaric acid could be detected. The maximum yield of glucuronoarabinoxylans was recovered by extracting a 5% suspension (w/v) of purified and WEAX-free wheat bran with 2% hydrogen peroxide at pH 11. While suspending the bran in the alkaline hydrogen peroxide solution the suspension warmed up to 40 °C. This temperature was maintained during the whole extraction process. Higher temperatures did not increase the yields of the extracted products (data not shown). Due to the decomposition of the hydrogen peroxide strong foaming occured, which could efficiently be suppressed by addition of an anti-foaming agent (Witafrol 7456 C), which is allowed as an additive according to European food regulations. Solubilized glucuronoarabinoxylans were contaminated by coextracted proteins which were enzymatically degraded at room temperature at slightly alkaline pH with Alcalase 2.4L. Following ultrafiltration and precipitation, the arabinoxylans had a protein content of 3% and a β-glucan content of 7.4 up to 9.0%. The latter polysaccharides were removed by a combined action of lichenase and β-glucosidase in buffered solution. Table 2 lists the analytical data of the purified arabinoxylans.

This pilot-scale procedure allowed the isolation of roughly 50% of the glucuronoarabinoxylans from wheat bran. So it was possible to isolate from 3 kg of wheat bran, containing 25–28% (dm) of AEAX, up to 400 g of GAX having a purity of 70–75%. Removing β-glucans from this product gave around 350 g of 80% purity. So half the amount of the arabinoxylans of the outer layers of the wheat grain remain unextractable under the chosen alkaline conditions.

This was also reported by other authors (Maes & Delcour, 2001; Schooneveld-Bergmans, Hopman, Beldman, & Voragen, 1998). Fig. 1 shows the gel permeation pattern of the HPSEC analysis of the GAX with its characteristic broad asymmetric and homogenous molecular weight distribution profile. The molecular weight of the GAX was calculated to be 100–110 kDa. The xylose/arabinose-ratio was 1.25 and was significantly lower than the corresponding value for the water-extractable arabinoxylans. This indicates a greater degree of branching of the xylan core. The uronic acid content amounts to 2.5%. Ferulic acid and *p*-coumaric acid could not be detected. This result was supported by viscosity measurements of an aqueous solution of arabinoxylans before and after addition of hydrogen peroxide and

Table 2
Yields and composition of glucuronoarabinoxylans extracted from purified, WEAX-free wheat bran with 2% hydrogen peroxide, pH 11

Yield (%)[a]	12.8
Protein (%)	3.1
Starch (%)	n.d.
Pectin (%)	n.d.
β-Glucans (%)	n.d.
Arabinoxylans (%)[b]	81
Arabinose (%)	47.2
Xylose (%)	45.0
Galactose (%)	1.4
Glucose (%)	n.d.
X/A[c]	1.25
Uronic acid (%)	2.5
p-Coumaric acid (mg/100 g)	n.d.
t-Ferulic acid (mg/100 g)	n.d.

Values represent mean values from four different extractions. All values from each extraction were determined in duplicate. n.d., Not detectable.
[a] [%] = Weight percentage (dm) of glucuronoarabinoxylans.
[b] Arabinoxylans = 0.88 (%Xyl + %Ara).
[c] X/A, Xylose–arabinose-ratio.

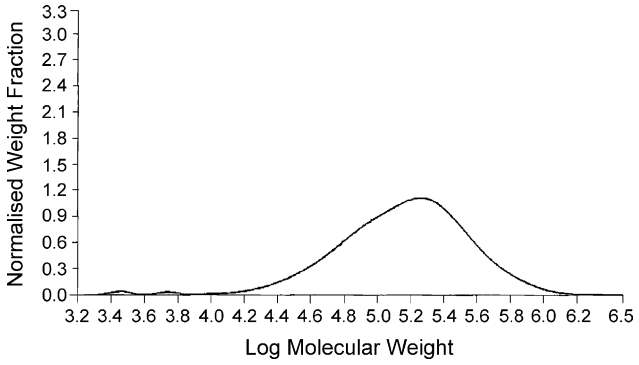

Fig. 1. High-performance size-exclusion chromatography of glucuronoarabinoxylans, isolated from purified wheat bran.

a peroxidase. No increase of viscosity could be observed (data not shown). It can be expected that these substituents of the arabinose moieties of the xylan core were removed under the alkaline extraction conditions during isolation from the cell walls of the wheat bran particles. Arabinoxylans are known to contain differently substituted xylose residues by L-arabinose moieties. In order to get a deeper insight into the branched structure of the GAX, a ^{13}C-NMR-spectrum of the β-glucan free GAX was recorded as shown in Fig. 2. The major carbon resonance signals were assigned with reference to ^{13}C-NMR-spectra of arabinoxylans from wheat (Annison, Choct, & Cheetham, 1992). Table 3 contains a list of the resonances measured. The polysaccharide is confirmed to be a linear polymer of β-D-xylopyranose monomers. The xylan core is substituted with α-L-arabinofuranose residues at O2 or O3 with arabinofuranose. Disubstituted β-D-xylopyranose residues having α-L-arabinofuranose substituents at O2 and O3 are present as well.

In conclusion a pilot-scale procedure for preparative isolation of glucuronoarabinoxylans from wheat bran has been developed. Wheat bran is a by-product of wheat-flour processing and accounts for 15–20% of the weight of the grain. It is a rich source of dietary-fibre (45–50% of dry matter) and contains 23–28% of (glucurono)arabinoxylans which are the major dietary-fiber component of the bran. The main part of the produced wheat bran is marketed as animal feed. Glucuronoarabinoxylans from wheat bran, due to their structural features, could find industrial applications as viscosity enhancers, foam stabilisers and fat replacers.

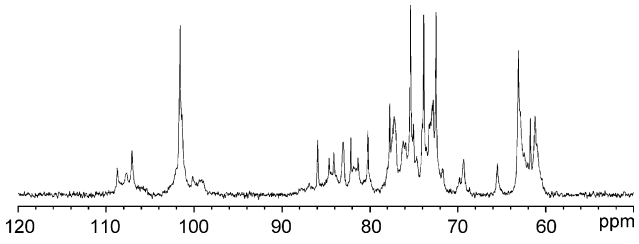

Fig. 2. ^{13}C-NMR spectrum of glucuronoarabinoxylans, isolated from purified wheat bran. The chemical shifts were assigned relative to dimethylsulfoxide.

Table 3
Resonances in the ^{13}C-NMR spectrum of glucuronoarabinoxylans, isolated from purified wheat bran

Chemical shift (ppm)	Assignment	Chemical shift (ppm)	Assignment
108.7	C1-Ara-O2-Xyl (O3-Ara)	77.7	C3-Ara
107.7	C1-Ara-O3-Xyl (O2-Ara)	75.4	C4-Xyl
107.1	C1-Ara-O3-Xyl	73.9	C3-Xyl
101.6	C1-Xyl (O2,3-Ara)	72.8	C2-Xyl
101.6	C1-Xyl (O3-Ara)	72.5	C2-Xyl (O3-Ara)
101.6	C1-Xyl (terminal)	69.8	C4-Xyl
100.2	C1-Ara-O3-Xyl	69.3	C4-Xyl (terminal)
85.9	C4-Ara	65.5	C5-Xyl (terminal)
–	C3-Xyl (O2,3-Ara)	63.1	C5-Xyl
81.4	C3-Xyl (O3-Ara)	61.8	C5-Ara
80.2	C3-Xyl (O2-Ara)	61.2	C5-Ara

Furthermore, these non-starch polysaccharides could be added to processed foods to increase the content of dietary-fibre, i.e. to increase health benefit. Arabinoxylans are plant cell wall components and are not hydrolyzed by mammalian digestive enzymes. Their digestion in the digestive tract, especially in the colon, is not very well understood. A reduction in the postprandial glucose response by consumption of arabinoxylan enriched white bread in normoglycemic subjects was reported by Lu, Walker, Muir, Mascara, and O'Dea (2000), and recently by Grasten et al. (2003). Whether (glucurono)arabinoxylans have any prebiotic effect is still speculative. The main reason for the relatively scarce knowledge of their nutritional impact on the human body is that these polysaccharides are still not available in quantities to perform any animal or human studies. In fact, no human intervention studies have yet been performed with glucuronoarabinoxylans. The pilot-scale procedure described here, allowed the production of around 350 g of glucuronoarabinoxylanes having a purity of at least 70% (dm) from 3 kg of wheat bran within 1 week.

This process should be easy to scale up, and thus make it possible to produce arabinoylans in sufficient quantities for research and application studies. A major aim of this work was to develop an economic procedure that was also environmentally friendly. Thus, the procedure employed low priced chemicals with inherent low toxicity. This excluded the application of chemicals like barium hydroxide for the extraction as used by other authors like Bergmans, Beldman, Gruppen, and Voragen (1996).

The organic solvent used, could be recycled, as least partially, by distillation. This is a basic prerequisite for a pilot-scale procedure to be scaled up to in industrial-scale level.

This newly developed extraction procedure for the isolation of glucuronoarabinoxylans from a rather cheap wheat milling by-product could open up new ways for marketing wheat bran and value-added products isolated from it.

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