Hydration of wheat flour water-unextractable cell wall material enables structural analysis of its arabinoxylan by high-resolution solid-state ^{13}C MAS NMR spectroscopy

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ABSTRACT

To enable its structural characterization by nuclear magnetic resonance (NMR) spectroscopy, the native structure of cereal water-unextractable arabinoxylan (WU-AX) is typically disrupted by alkali or enzymatic treatments. Here, WU-AX in wheat flour unextractable cell wall material (UCWM) containing 40.9% ± 1.5 arabinoxylan with an arabinose-to-xylose ratio of 0.62 ± 0.04 was characterized by high-resolution solid-state NMR without disrupting its native structure. Hydration of UCWM (1.7 mg H_2O/mg UCWM) in combination with specific optimizations in the NMR methodology enabled analysis by solid-state ^{13}C NMR with magic angle spinning and ^{1}H high-power decoupling (^{13}C HPDEC MAS NMR) provided sufficiently high resolution to allow carbon atom assignments. Spectral resonances of *C-1* from arabinose and xylose residues of WU-AX were here assigned in solid-state. The proportions of un-, mono-, and di-substituted xyloses were 59.2%, 19.5%, and 21.2%, respectively. ^{13}C HPDEC MAS NMR showed the presence of solid-state fractions with different mobility in the UCWM. This study presents the first solid-state NMR spectrum of wheat WU-AX with sufficient resolution to enable assignment without prior WU-AX solubilization.

KEYWORDS

Solid-state NMR, water-unextractable arabinoxylan, wheat flour, ^{13}C NMR, controlled hydration

INTRODUCTION

Arabinoxylan (AX), the predominant dietary fiber in wheat kernels, makes up 60-70% of the cell walls of its starchy endosperm.[1,2] It has a backbone of β-1,4-linked D-xylopyranosyl (Xyl) residues that are either un-, mono-, or disubstituted (uXyl, mXyl, dXyl, respectively) with α-L-arabinofuranosyl (Ara) residues.[3,4] In wheat, mono-substitution of Xyl with Ara is mainly restricted to the *O-3* position of Xyl, whereas di-substitution occurs at the *O-2* and *O-3* positions of Xyl residues.[5,6] In turn, Ara can carry a ferulic acid (FA) residue on its *O-5* position via an ester linkage.[7,8] FA plays a role in crosslinking AX molecules to each other or to other cell wall components via its participation in dehydrodiferulic acid (diFA) bridges.[9]

The AX population in wheat consists of a group of structurally heterogeneous molecules. Typically, 20-30% of the AX in wheat flour is water-extractable (WE-AX), the complement being water-unextractable AX (WU-AX).[10,11] Although WE-AX and WU-AX have similar structures, WU-AX molecules on average have a higher molecular weight,[5,12] a higher Ara-to-Xyl (A/X) ratio,[5,13,14] and higher FA and diFA contents.[5,15,16] The higher A/X ratio of WU-AX goes hand in hand with a higher level of mXyl and a lower level of uXyl, while dXyl levels of WE-AX and WU-AX are similar.[13,14,17]

The more pronounced diFA crosslinking in WU-AX than in WE-AX is considered to be the major cause of its un-extractability.[5,16] Additional causes are that uXyl residues more likely occur as clusters in WU-AX and hence are prone to form intermolecular hydrogen bonds, while they are rather isolated in WE-AX.[13,18] In addition, the higher A/X ratio in WU-AX has been speculated to be at the basis of enhanced physical entanglement.[19] Interactions and reactions between AX and

other endosperm cell wall polysaccharides may result in retention of WU-AX in the matrix.[2,5] AX extractability differs from AX solubility, as AX-AX interaction or AX interaction with the matrix can cause inherently soluble AX molecules to not be extractable with water.

Evidently, the unextractable nature of WU-AX makes its structural characterization challenging. A way to address this challenge is to solubilize WU-AX under alkaline conditions[12,17,20,21] or to perform partial enzymatic hydrolysis.[14,22] Although such procedures have allowed structural characterization through high performance size exclusion chromatography and liquid-state nuclear magnetic resonance (NMR) spectroscopy, they disrupt the native WU-AX structure by breaking covalent and non-covalent bonds.[23]

Ha *et al.*[24] have examined the polymers in destarched and defatted cell walls of wheat bran by solid-state ^{1}H-^{13}C cross-polarization magic angle spinning (CP MAS) NMR. Peak assignment was possible for *C-1* carbons of AX [Ara (δ 109 ppm) and Xyl (δ 102 ppm)] and cellulose (δ 105 ppm). The authors reported an increase in spectral resolution upon sample hydration. Mense *et al.*[25] performed solid-state ^{1}H-^{13}C CP MAS NMR to study wheat bran containing comparable AX and cellulose contents [each about 24% w/w of the dry matter (DM)]. Here, sample hydration resulted in an increased resonance intensity, and enzymatic removal of starch and proteins from wheat bran further improved spectral resolution and reduced overlap between *C-1* signals of glucose in cellulose and Xyl in AX. The reduced signal overlap was attributed to an increase in effective concentration of AX and cellulose (about 43% and 28% w/w of DM, respectively) after removal of starch, protein, and water extractables.[25]

Although solid-state ^{1}H-^{13}C CP MAS NMR experiments have been performed on isolated AX or wheat cell wall material fractions, a full and detailed structural characterization of AX therein has

in all cases been impaired by signal overlap and low spectral resolution. To the best of our knowledge, ^{13}C MAS NMR spectra with a resolution allowing structural analysis have not yet been obtained for wheat flour unextractable cell wall material. We here reasoned that applying ^{13}C HPDEC MAS NMR on partially hydrated samples could yield high resolution spectra and thus detailed structural characteristics which further on can benefit studies of the role of WU-AX in flour processing and its impact on health. In what follows, we report on the isolation of wheat flour unextractable cell wall material (UCWM) and, following limited hydration thereof[25], analysis of its WU-AX by high-resolution ^{13}C HPDEC MAS NMR in a highly optimized environment.

MATERIALS AND METHODS

Materials

Kernels of the hard wheat (*Triticum aestivum* L.) cultivar Evina were kindly supplied by Limagrain (Avelgem, Belgium). Unless specified otherwise, all chemicals, solvents, and reagents were at least of analytical grade and purchased from Sigma-Aldrich (Overijse, Belgium). Used enzymes (with units as defined by Sigma-Aldrich) were α-amylase (Termamyl® 120) from *Bacillus licheniformis* (type XII-A, saline solution, ≥500 units/mg protein, reference A3403) and papain from *Carica papaya* (powder, ≥3 units/mg, reference 76220). The enzyme preparations were free from xylanase activity as determined by the Xylazyme AX method (Megazyme, Bray, Ireland). Deuterium oxide (D_2O) had an isotopic purity of 99.9 atom% D (Sigma-Aldrich reference 151882). The cellulose used was microcrystalline Avicel PH-101 (Sigma-Aldrich reference 11365).

Methods

Wheat milling and flour characteristics

Wheat was conditioned in-house to reach a moisture content of 16.5% w/w and subsequently milled using a Bühler (Uzwil, Switzerland) MLU-202 Laboratory Mill (milling yield 72%) as in Delcour *et al.*[26] Moisture (14.9%) and ash (0.44% of DM) contents of the flour were determined as described in AACC Approved Methods 44-15.02 and 08-01.01[27], respectively. The flour protein content (11.9% of DM) was determined following an adaptation of AOAC method 990.03[28] to an automated elemental analyzer 1108 (Carlo Erba Instruments, Hindley Green, UK) micro-Dumas protein analysis system.

Isolation of unextractable cell wall material from wheat flour

UCWM was isolated in triplicate from wheat flour essentially as described by Frederix *et al.*[29] and outlined in Figure 1. Doughs prepared from 100.0 g of flour and 58.0 mL of water by mixing (4 min) in a mechanical pin mixer (National Manufacturing, Lincoln, NE, USA) were washed with 500 mL deionized water to separate the gluten fraction from the remaining soluble and dispersed flour constituents. The dispersions were centrifuged (5,000 *g*, 10 min, 15 °C), yielding from top to bottom supernatant, a squeegee starch fraction (SQSF, gelatinous layer, also often referred to as squeegee starch or starch tailings), and prime starch. SQSF was manually removed from the prime starch using a spatula, suspended in deionized water (1:10 w/v), heated to 75 °C, and then incubated with α-amylase (500 units/g SQSF, 90 °C, 60 min, continuous mechanical shaking). The suspensions were then allowed to cool to 15 °C and centrifuged (10,000 *g*, 15 min, 15 °C), yielding

supernatants and residues. The latter were re-suspended in deionized water (1:5 w/v), treated with papain (0.3 units/g SQSF, 25 °C, 5 h, continuous mechanical shaking), boiled (15 min) to inactivate enzymes and cooled to room temperature. Centrifugation as above yielded supernatants and residues. The latter were washed with deionized water (1:2 w/v) and again centrifuged as above to recover the residues that were freeze dried and weighed. The pooled freeze dried residues are further referred to as UCWM.

Determination of arabinoxylan contents and arabinose-to-xylose ratios

Contents and A/X ratios of total AX and WE-AX in flour, gluten, prime starch, SQSF, and UCWM were determined in triplicate by gas chromatographic analysis of the monosaccharides released upon acid hydrolysis as described by Gebruers *et al.*[10] and Courtin *et al.*[30]. Accurately weighed flour aliquots (5.0-10.0 mg) were submitted to hydrolysis in 5.0 mL trifluoroacetic acid (TFA, 2.0 M) for 60 min at 100 °C. Aliquots (5.0-10.0 mg) of freeze-dried gluten, SQSF, prime starch, and UCWM materials were also accurately weighed and then were subjected to hydrolysis as above. Reduction of the obtained monosaccharides into alditols was with sodium borohydride (30 min, 40 °C) and subsequent acetylation with acetic acid anhydride. The alditol acetates were then submitted to gas chromatography as described in De Man *et al.*[31]

For the WE-AX content of flour, an accurately weighed quantity (about 0.5 g) was first heated in 10 mL 80% v/v ethanol at 90 °C in open Falcon tubes until all ethanol was evaporated to inactivate potentially present endogenous xylanases. Subsequent extraction of WE-AX was with 10.0 mL deionized water (30 min, continuous shaking, room temperature), followed by centrifugation

(1,500 *g*, 15 min) and filtration. To 2.5 mL filtered supernatant, 2.5 mL 4.0 M TFA was added prior to hydrolysis (60 min, 100 °C). Subsequent reduction, acetylation, and separation of the obtained alditol acetates were as above.

From the total and water-extractable Ara, Xyl, and galactose (Gal) composition, the contents and A/X ratios of total AX, WE-AX, and WU-AX were calculated as described elsewhere,[10,30,31] thus using the following set of equations:

$$[total\ AX] = 0.88 \times ([Ara] - 0.7 \times [Gal]_{extractable} + [Xyl]) \quad (1)$$

$$[WE\text{–}AX] = 0.88 \times ([Ara]_{extractable} - 0.7 \times [Gal]_{extractable} + [Xyl]_{extractable}) \quad (2)$$

$$[WU\text{–}AX] = [total\ AX] - [WE\text{–}AX] \quad (3)$$

$$A/X_{total\ AX} = ([Ara] - 0.7 \times [Gal]_{extractable})/[Xyl] \quad (4)$$

$$A/X_{WE\text{–}AX} = ([Ara]_{extractable} - 0.7 \times [Gal]_{extractable})/[Xyl]_{extractable} \quad (5)$$

$$A/X_{WU\text{–}AX} = ([total\ AX] \times {}^{A}/_{X_{total\ AX}} - [WE\text{–}AX] \times {}^{A}/_{X_{WE\text{–}AX}})/[WU\text{–}AX] \quad (6)$$

In doing so, a correction was made for Ara originating from water-extractable arabinogalactan peptide (AGP) by assuming that all extractable Gal was from the latter and that it had an Ara to Gal ratio of 0.70.[32,33] As the gluten, prime starch, and SQSF fractions still contained water, and thus extractable AGP, a correction was made for the latter as well based on the moisture content and remaining Gal content in these fractions (Figure 1). No correction was made for extractable Gal in UCWM, assuming that no remaining water-extractable AGP was present after the various extraction steps. Since hydrolysis with trifluoroacetic acid under the conditions used here hardly releases monosaccharides from cellulose,[34] the total non-cellulosic carbohydrate content was

calculated here as the sum of the released pentoses and hexoses multiplied by 0.88 and 0.90, respectively.[21] Protein content (%, N x 6.25) of the isolated UCWM was determined as above.

Solid-state NMR spectroscopic analysis of isolated unextractable cell wall material

$^{13}C\{^{1}H\}$ MAS NMR experiments of the pooled UCWM were carried out on a wide bore 500 Avance III spectrometer (Bruker Biospin, Ettlingen, Germany; 11.4 T, ^{1}H Larmor frequency of 500.87 MHz) and ^{13}C Larmor frequency of 125.95 MHz, using a Bruker 4 mm triple-channel (HXY) VT CP MAS probe head. The spectrometer was located in a temperature-controlled room (22 °C, maximum fluctuation 0.1 °C over a 24 h timespan) with vibration-lean floor. Prior to the analysis, WU-AX samples were hydrated by progressive addition of small aliquots of ultrapure water (MilliQ, Millipore) and gentle mixing to achieve a partially hydrated sample. In total, 115 mg of H_2O was added to 67 mg of UCWM. Microcrystalline Avicel PH-101 cellulose was hydrated in the same way. Immediately following this hydration step, samples were packed into 4 mm zirconia rotors and capped vespel snap-on drive caps and spun up to 15 kHz for the double-resonance experiments. The temperature was controlled at 295 K using a Bruker cooling unit II. ^{13}C High-power decoupling (HPDEC) MAS experiments were performed at 15 kHz spinning speed and 295 K sample temperature using $\pi/2$ ^{13}C RF pulses at 75 kHz, a recycle delay of 2 s and SW_f-SPINAL[35] ^{1}H decoupling at 15 kHz. 61440 transients were collected for the HPDEC experiment. ^{1}H-^{13}C CP MAS experiments were performed using a pi/2 ^{1}H excitation pulse at 83 kHz in combination with ramped (100 – 70) and square contact pulses of 1000 µs at 71 kHz and 54 kHz on ^{13}C and ^{1}H respectively, using SW_f-SPINAL ^{1}H decoupling at 40 kHz during detection. 20480 transients were recorded using a recycle delay of 2 s. The decoupled $^{13}C\{^{1}H\}$ INEPT experiment

was done, accumulating 20480 number of transients, with pulses of RF strength 70 kHz and recycle delay of 2 s. ^{13}C chemical shift referencing was performed using adamantane resonance at 38.5 ppm as a secondary standard with respect to tetramethylsilane (TMS).

Statistical analyses

All statistical analyses were performed using JMP Pro 14 (SAS Institute, Cary, NC) software. To detect significant differences, one-way variance (ANOVA) was performed followed by a Tukey multiple comparison test ($P = 0.05$).

RESULTS & DISCUSSION

Arabinoxylan characteristics in wheat flour

The total AX content of Evina flour was $2.0 \pm 0.1\%$ of DM and its A/X ratio was 0.67 ± 0.01. WE-AX and WU-AX contents were 0.7 ± 0.1 and $1.3 \pm 0.1\%$ of DM, respectively, resulting in an AX extractability of $34.3 \pm 1.2\%$. The A/X ratio of WE-AX (0.50 ± 0.02) was significantly ($P < 0.05$) lower than that of WU-AX (0.76 ± 0.04). In literature, total AX, WE-AX, and WU-AX contents in wheat flour have been reported to range from 1.4% to 2.9%, 0.3% to 1.4%, and 1.2% to 2.3% of DM, respectively, with corresponding A/X ratios ranging from 0.50 to 0.70, 0.40 to 0.55, and 0.51 to 0.67.[10,14,36–39] The obtained results are thus largely within the range found in literature.

Isolation of unextractable cell wall material

As a result of the execution of the procedure outlined in Figure 1, 72.7% ± 8.8, 20.3% ± 3.2, and 7.2% ± 0.5 of DM of the flour WU-AX were present in SQSF, prime starch, and gluten fractions, respectively. The A/X ratio of WU-AX in SQSF (0.66 ± 0.02) was significantly ($P < 0.05$) lower than that of WU-AX in the prime starch (1.25 ± 0.12) and gluten (1.17 ± 0.02) fractions. That highly substituted WU-AX was retained in prime starch and gluten fractions explains the slightly lower A/X ratio of WU-AX in SQSF as compared to that of the original WU-AX in flour. Evidently, the supernatant fraction contained the WE-AX.

SQSF was further submitted to enzymatic breakdown of starch and protein to obtain a fraction enriched in WU-AX (*i.e*. UCWM, Figure 1). About 33% of the flour AX initially considered water-unextractable was rendered extractable during this treatment (data not shown). Since neither the flour nor the used enzymes contained xylanase, their increased extractability was the result of changes in the matrix accompanying squeegee starch and/or protein breakdown. In addition, the increased temperature during the α-amylase treatment may have contributed to solubilization of some AX. Earlier, Cyran *et al.*[40] reported that the extractability of AX from rye flour increases with temperature.

The total non-cellulosic carbohydrate polymer content in UCWM amounted to 49.8% ± 1.5 of DM, its protein content to 12.6% ± 1.0 of DM. These results are similar to those obtained by Maes and Delcour[21] for wheat bran UCWM. Cell walls isolated from wheat flour have been reported to also contain non-endosperm material,[41] and thus cellulosic material from bran is likely to be present in the obtained UCWM. Recently, Gartaula et al.[42] found significant levels of cellulose to

be present in the wheat starchy endosperm. Although cellulose could not be quantified by acid hydrolysis with TFA, it can be assumed that the obtained UCWM also contained some cellulose. Of all analyzed non-cellulosic carbohydrate polymers in UCWM, the major fraction (82.1% ± 4.0) consisted of AX with an A/X ratio of 0.62 ± 0.01. The latter was comparable to that of AX in SQSF, which suggests that the AX population rendered extractable during the enzyme treatments was representative for that in SQSF, and average wise was not particularly highly or lowly substituted. About 72.7% ± 8.8 and 24.3% ± 1.8 of the flour WU-AX (1.3%) ended up in SQSF and UCWM, respectively. The other non-cellulosic carbohydrate polymers in UCWM mainly consisted of glucose (11.8% ± 0.7) and thus probably were residual starch material and β-D-glucan. The low proportions of mannose (3.8% ± 0.2) and Gal (2.2% ± 0.1) probably originated from mannan[43], galactomannan, and/or glucomannan.[16,21]

Solid-state NMR of unextractable cell wall material

^{13}C MAS NMR spectra of dry biopolymers such as AX[25,44] or cellulose[25,45] typically have broad features resulting from a combination of chemical shift and dipolar broadening. Controlled hydration of the polymers assisted to increase resolution by increasing the molecular mobility of the polymer chains. The ^{13}C MAS NMR spectra of both a dry and a hydrated UCWM sample in Figure 2 readily revealed sharpening of the spectral resonances as a result of hydration. Comparison of a ^{13}C HPDEC MAS NMR spectrum of the hydrated sample with its ^{1}H-^{13}C CP MAS NMR spectrum (Figure 3a,b) highlights the hydration induced changes in the spectral resonances even more. Indeed, as long as the repetition delay exceeds 5*t1 of the slowest relaxing

resonance, ^{13}C HPDEC MAS NMR quantitatively measures all ^{13}C signals, irrespective of the local chemical environment and properties of the nuclei,[46] while ^{1}H-^{13}C CP MAS relies on the presence of dipolar interactions to transfer spin polarization from ^{1}H to ^{13}C nuclei. This results in spectral editing, with ^{1}H-^{13}C CP MAS selectively highlighting the more rigid, highly dipolar coupling affected components of the sample, while ^{13}C HPDEC MAS NMR measures all nuclei quantitatively.[47] Conversely, refocused insensitive nuclei enhanced by polarization transfer (INEPT) and distortionless enhancement by polarization transfer (DEPT) pulse sequences can also be exploited for spectral editing and signal enhancement. These pulse sequences rely on J-couplings to transfer spin polarization from ^{1}H nuclei to the ^{13}C nuclei they are attached to, and hence selectively highlight the most mobile, solid components of the sample (Figure 3d).

In the present case, controlled hydration of UCWM in combination with ^{13}C HPDEC MAS NMR with spinal-64 ^{1}H decoupling provided quantitative spectra with a resolution allowing detailed spectral assignment using earlier assignments of WE-AX carbons (Table 1).[48] For AX, resonances at δ109.5 ppm and δ108.8 ppm correspond to *C-1* of two neighboring Ara residues linked to *O-2* and *O-3* of a dXyl residue, respectively. The resonance at δ108.4 ppm is that of *C-1* of an Ara residue linked to *O-3* of a Xyl residue (mXyl). The resonance at δ102.5 ppm is assigned to both *C-1* of mXyl and *C-1* of a uXyl residue without neighboring mXyl or dXyl. The resonances at δ102.0 ppm and δ100.7 ppm are assigned to *C-1* of a uXyl residue with neighboring mXyl or dXyl and *C-1* of a dXyl residue, respectively. The assignment of *C-2* to *C-5* carbons are further presented in Table 1. Integration of the Ara *C-1* carbons assigned to mXyl (δ 108.4 ppm) and dXyl (δ109.5 and δ108.8 ppm) resulted in a dXyl/mXyl ratio of 1.1. Combining this with the A/X-ratio determined by gas chromatographic analysis, the relative proportion of uXyl, mXyl, and dXyl in

WU-AX were determined to amount to 59.2%, 19.5%, and 21.2%, respectively. These observations are in line with earlier findings that wheat flour WU-AX on average contain about 61% uXyl, 21% mXyl, and 19% dXyl.[14] To the best of our knowledge, this is the first time that such detailed peak assignment could be performed on WU-AX in solid-state. A comparative study could establish insights on the effects of WU-AX solubilization on its structure, and might provide knowledge on validation or possible shortcomings of WU-AX solubilization prior to liquid-state NMR spectroscopic analysis.

In the here obtained ^{13}C MAS NMR spectrum of dry UCWM (Figure 2), the broad peak centered around δ100 – 110 ppm may be a convolution of resonances from both WU-AX and cellulose. In the ^{13}C HPDEC MAS NMR spectrum of hydrated UCWM, on the other hand, no peaks of cellulose (e.g. *C-1* at δ 105 ppm)[49] were visible (Figure 3a). Since the effect of cellulose hydration on the spectrum may have been different from that of hydration of WU-AX, Avicel PH-101 cellulose was subjected to solid-state NMR before or after hydration [see supporting information (SI)]. No major differences were observed between ^{1}H-^{13}C CP MAS NMR spectra and the ^{13}C HPDEC MAS NMR spectra of Avicel PH-101 cellulose in dry or partially hydrated form (Figure S2, Figure S3). While hydration clearly affected the WU-AX resonances in UCWM, the ^{13}C HPDEC MAS NMR spectra of hydrated Avicel PH-101 cellulose recorded under similar experimental conditions as the UCWM sample did not show any significantly sharpened resonances (Figure S3). Hence it is clear that cellulose hydration does not occur in a way similar to that of WU-AX present in UCWM. Experimental conditions were only optimized for the sharp resonances of hydrated WU-AX. The more limited signal-to-noise-ratio obtained for the broader contributions could still lead to an

underestimation of the cellulose fraction in UCWM. If such information needs to be extracted, acquisition of spectra with an even higher signal-to-noise-ratio could be considered.

It is important to stress that the high resolution spectra of hydrated UCWM provided in Figure 3 correspond with the solid polymer, as hydration of WU-AX does not solubilize the polymer. The enhanced resolution in these spectra results from a combination of sample preparation (controlled hydration), NMR methodology (magic angle spinning and efficient ^{1}H decoupling) and optimized NMR spectrometers and their environment (see SI). As revealed by the ^{1}H-^{13}C CP MAS spectrum for adamantane (Figure S1, SI), the spectrometer used in this experiment was optimized to achieve sub 1 Hz full width at half maximum (FWHM) for ^{13}C resonances on suitable solid-state samples, allowing to fully resolve the ^{13}C satellites in a single scan.

Detailed inspection of the ^{13}C HPDEC MAS NMR spectra shown in Figure 3 reveals both narrow and broad resonances, indicating the presence of both more and less mobile solid fractions (Figure 3a). The narrow resonances in HPDEC spectra of hydrated UCWM exhibit FWHM in the range of 8 – 10 Hz. These components can selectively be highlighted, though at the expense of both internal and external quantitativity by spectral editing using INEPT or DEPT based pulse sequences (Figure 3d). The broad components visible at foot of the narrow resonances in ^{13}C HPDEC MAS NMR spectrum (Figure 3a), exhibit FWHM close to 1000 Hz and can in turn be highlighted by spectral editing (Figure 3b) using a ^{1}H-^{13}C CP MAS pulse sequence, again at the expense of both internal and external quantitativity.

The narrow components in the hydrated UCWM sample result from enhanced mobility of the polysaccharide chains upon hydration. This is related to the motional narrowing observed for the ^{13}C resonances in solid-state adamantane, a plastic crystal. While only the ^{13}C HPDEC MAS NMR

spectrum yields a quantitative description of the system, accounting for mobile and immobile fractions, the ^{1}H-^{13}C CP MAS spin-edited spectrum yields information about the immobile fraction of the polysaccharide network (most likely high molecular weight crystalline or amorphous parts). INEPT based spin editing in contrast reveals information on the low molecular weight fraction. Subtraction of the ^{1}H-^{13}C CP MAS spectrum from the ^{13}C HPDEC MAS NMR spectrum after scaling to fit the broad foot of the HPDEC NMR spectrum resulted in a spectrum containing mostly narrow components, representative of the mobile low molecular weight fractions and resembling the INEPT spectrum (Figure 3d). More detailed spectral deconvolution and principal component analysis can be used to obtain quantitative information of each of these fractions.

In conclusion, wheat flour UCWM mainly containing WU-AX was isolated with retention of its unextractable nature. ^{13}C HPDEC MAS NMR spectroscopy on solid samples after controlled hydration yielded high-resolution ^{13}C NMR spectra, allowing in depth structural characterization of WU-AX present in UCWM. Thus, high spectral resolution could be achieved in solid-state NMR experiments on wheat flour WU-AX, allowing a detailed structural elucidation. Additionally, ^{13}C HPDEC MAS NMR indicated the presence of both more and less mobile solid-state fractions in the sample. Cellulose resonances were underestimated in UCWM by means of ^{13}C HPDEC MAS NMR, probably due to spin-editing effects induced by different hydration behavior of cellulose and WU-AX present in UCWM. To the best of our knowledge, this was the first time that high-resolution solid-state NMR spectra were obtained on flour WU-AX with sufficient resolution for its structural analysis.

ABBREVIATIONS

AGP, arabinogalactan peptide; Ara, α-L-arabinofuranosyl; AX, arabinoxylan; A/X, arabinose-to-xylose ratio; CP, cross polarization; DEPT, distortionless enhancement by polarization transfer; diFA, dehydrodiferulic acid; dXyl, disubstituted xylose; SI, supporting information; FA, ferulic acid; FWHM, full width at half maximum; Gal, galactose; HPDEC, high-power decoupling; INEPT, insensitive nuclei enhanced by polarization transfer; MAS, magic angle spinning; mXyl, monosubstituted xylose; NMR, nuclear magnetic resonance spectroscopy; SQSF, squeegee starch fraction; TFA, trifluoroacetic acid; TMS, tetramethylsilane; UCWM, unextractable cell wall material; uXyl, unsubstituted xylose; WE-AX, water-extractable arabinoxylan; WU-AX, water-unextractable arabinoxylan; Xyl, ß-1,4-linked D-xylopyranosyl.

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SUPPORTING INFORMATION DESCRIPTION

Optimization of NMR equipment to obtain high resolution; influence of hydration on cellulose resonances.

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FIGURE CAPTIONS

Figure 1. Isolation of unextractable cell wall material (UCWM) containing water-unextractable arabinoxylan (WU-AX) from wheat flour by performing gluten-starch separation and subsequent squeegee starch fraction (SQSF) processing. The procedure was slightly adapted from that based on Frederix *et al.*[29]

Figure 2. ^{1}H decoupled ^{13}C MAS NMR spectra of unextractable cell wall material (UCWM) from Evina flour, under dry (black) and hydrated (grey) conditions. The data was collected in a 500 MHz NMR spectrometer at an MAS frequency of 15 kHz. Recycle delay of 2 s (hydrated) and 30 s (dry), accumulating 2048 transients.

Figure 3. (a) ^{13}C HPDEC MAS (blue) together with the assignment of chemical shifts of *C-1* carbons of un-, mono-, and di-substituted xylopyranose (⬣, red dots) by arabinofuranose (⬠, green dots) residues, (b) ^{1}H-^{13}C CP MAS (red), (c) difference spectra of HPDEC and CP MAS (green), and (d) ^{13}C INEPT (pink) MAS NMR spectrum of hydrated unextractable cell wall material (UCWM) from Evina flour.

TABLES

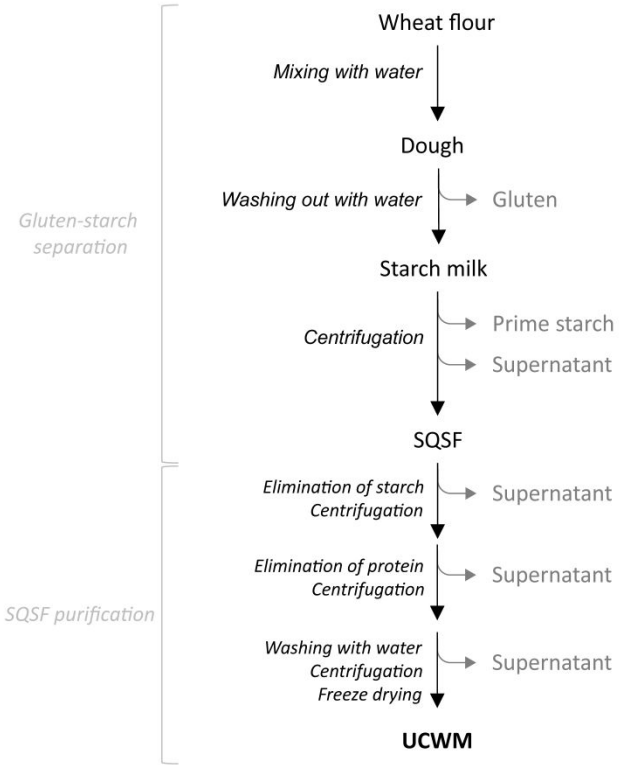
Table 1 Assignment of chemical shifts (ppm) of xylopyranose (Xyl) and arabinofuranose (Ara) residues from water-unextractable arabinoxylan (WU-AX) in isolated unextractable cell wall material (UCWM) by ^{13}C HPDEC MAS NMR, based on Hoffman *et al.*[48]*

Residue	**Chemical shift (ppm)**				
	C-1	*C-2*	*C-3*	*C-4*	*C-5*
Unsubstituted					
Xyl (isolated)	102.5	73.6	74.7	77.2	63.8
Xyl (neighboring branched Xyl)	102.0	73.7	74.7	77.3	63.8
Mono-substituted					
Xyl	102.5	74.4	78.2	78.2	63.6
Ara *O-3*	108.4	81.7	78.2	85.7	62.3
Di-substituted					
Xyl	100.7	74.7	78.2	78.2	63.3
Ara *O-2*	109.5	82.3	77.7	85.1	62.2
Ara *O-3*	108.8	82.0	78.2	85.3	62.2

*****Resonances at δ 70.2 and δ 66.1 ppm were not assigned by Hoffman et al. [48], but have respectively been associated with *C-4* and *C-5* of terminal non-reducing Xyl residues in WE-AX by Izydorczyk and Biliaderis [50] and Sun et al. [51]. Furthermore, Vaneeckhaute et al. [52] detected these resonances in AX oligosaccharides, more specifically for the non-reducing terminal xylose of a xylotriose compound. This raises the question whether low molecular weight AX fragments could be associated with UCWM components.

532 **FIGURES**

533 **Figure 1**



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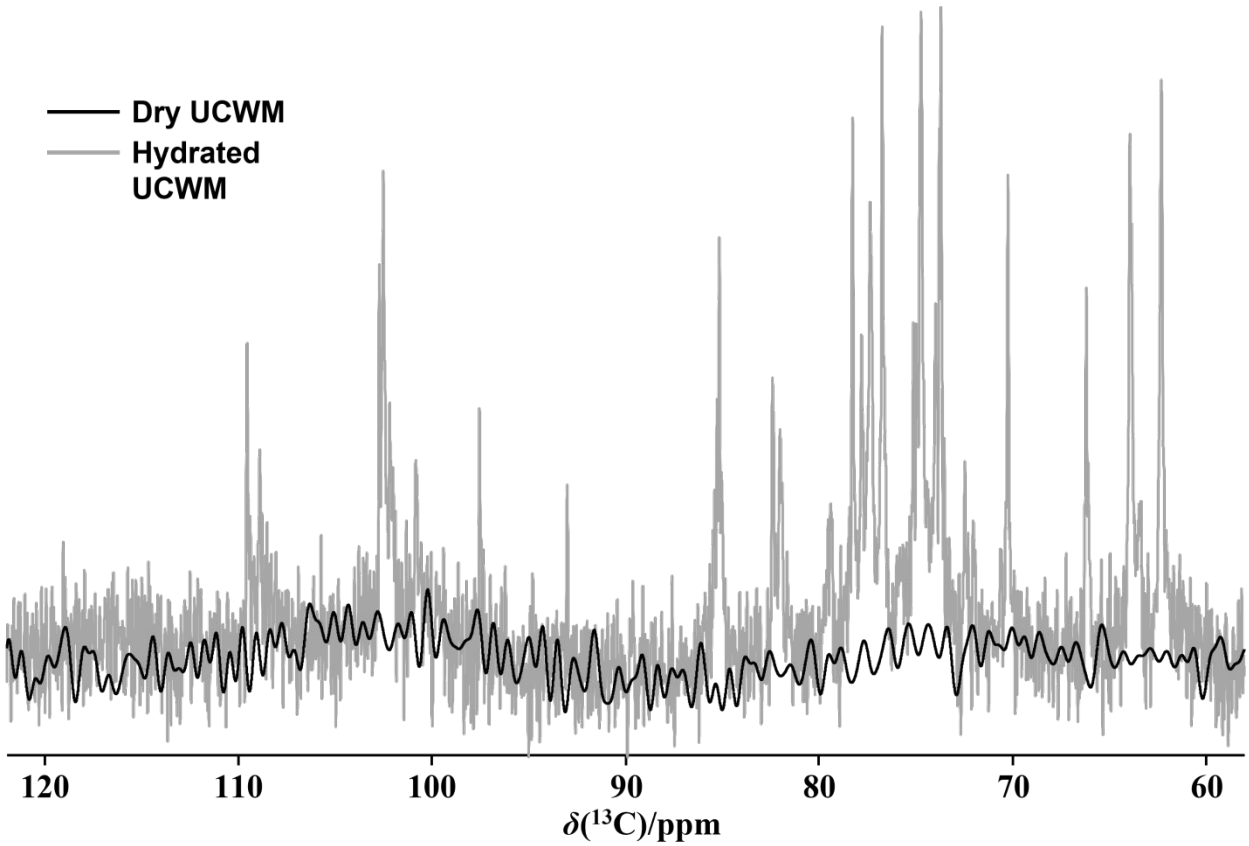
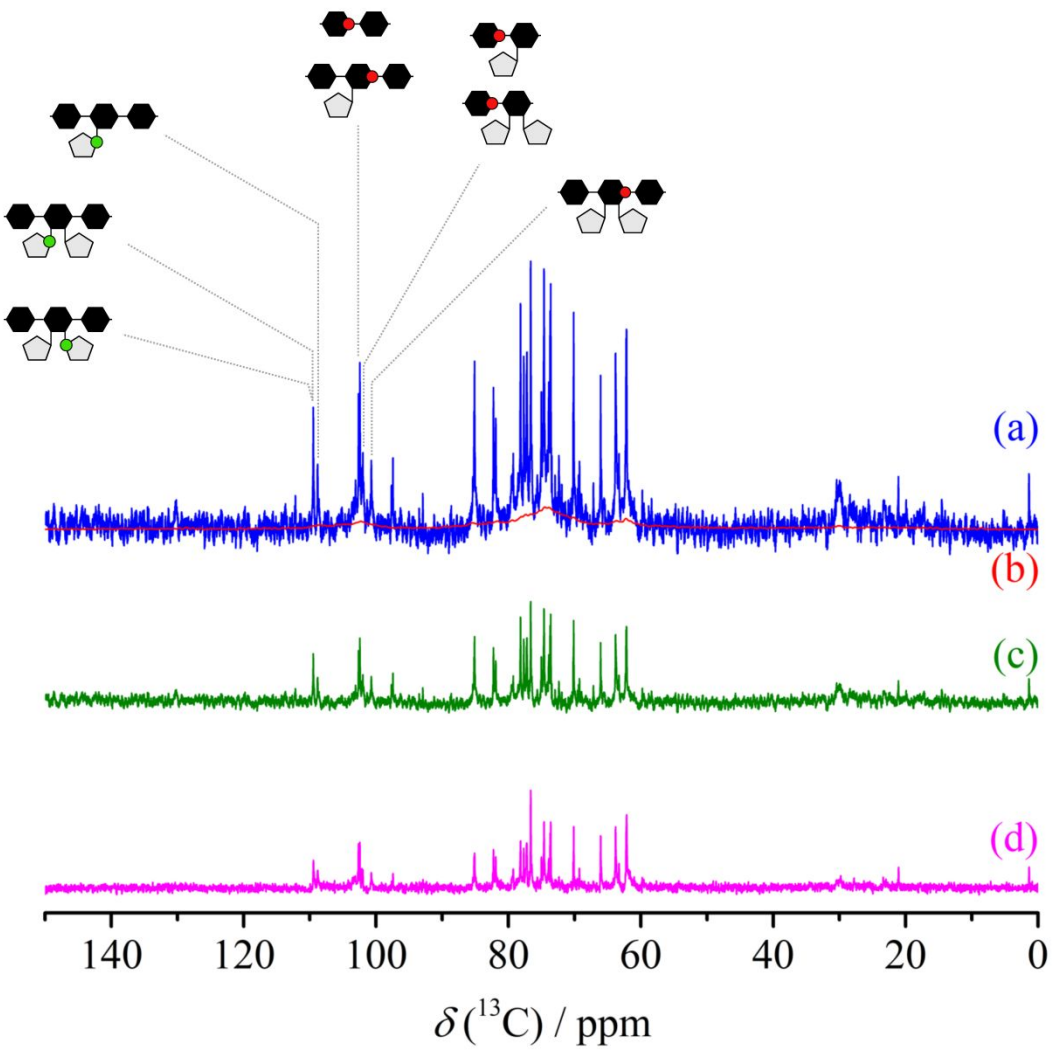
535 **Figure 2**



536

537 **Figure 3**



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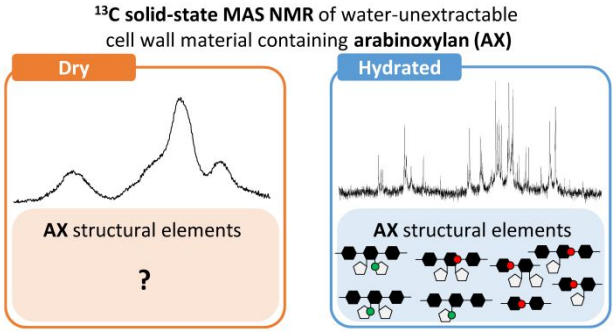
539 **GRAPHIC FOR TABLE OF CONTENTS (TOC graphic)**



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