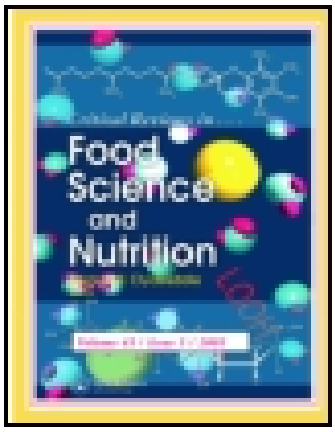


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# Technological and Analytical Methods for Arabinoxylan Quantification from Cereals

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Abstract

Arabinoxylan (AX) is the major non-starch polysaccharide contained in various types of grains. AX consists of a backbone of β-1.4-D-xylopyranosyl residues with randomly linked α-L-arabinofuranosyl units. Once isolated and included as food additive, AX affects foodstuff attributes and has positive effects on human health. AX can be classified into water-extractable and water-unextractable AX. For isolating AX out of their natural matrix, a range of methods was developed, adapted and improved. This review presents a survey of the commonly used extraction methods for AX by the influence of different techniques. It also provides a brief overview of the structural and technological impact of AX as a dough additive. A concluding section summarizes different detection methods for analyzing and quantification AX.

 

*Keywords: dietary fiber, enzymatic treatments, extraction, instrumental analysis, structure, technologic properties*

***Abbreviations***: ara, arabinose; ara/xyl, ratio arabinose to xylose; AG, arabinogalactans; AX, arabinoxylan; AX-OS, arabinoxylan-oligosaccharide; DDT, dough development time; FA, ferulic acid; G′, storage modulus; G′′, loss modulus; lG*l, complex shear modulus; HMW, high molecular weight; LMW, low molecular weight; WE-AX, water-extractable arabinoxylan; WU-AX, water-unextractable arabinoxylan; xyl, xylose; xyl*p*, xylopyranosyl; US, ultrasound.

## 1 Introduction

In recent years, pentosans which can be divided into arabinogalactan (AG) and arabinoxylan (AX) were in focus of many researches, in which especially AX was of main interest. A lot of research was done to extract, analyze and identify AX as well as to analyze its influences on human health and its functional properties in foodstuff (Glitsø, Jensen et al. 2000; Izydorczyk and Biliaderis 2007; Izydorczyk and Dexter 2008; Qiang, YongLie et al. 2009). Especially the chosen way of extraction determines different AX attributes such as molecular weight, water absorption and AX content, which were so far been underestimated.

For human diet AX is described as a helpful ingredient to prevent a lot of diseases like diabetes type 2, intestine cancer and cardiovascular disease. In the intestine, AX stimulates the growth of probiotic bacteria which additional has a positive effect on human health (Izydorczyk and Biliaderis 2007). Furthermore, AX absorbs water and increases fecal bulk which decreases the concentration of potential carcinogens in the intestine (Izydorczyk and Biliaderis 2007). Next to degradation of AX, a slight increase of acetic, butyric and propionic acid concentration in the colon was also observed, which may result in reducing colon cancer (Aura, Karppinen et al. 2005). For diabetes type 2, it is assumed that AX-OS lower the postprandial glucose level and insulin response.

There exists several publications about the positive effects of dietary fibers and how they influence the glycemic index (Torsdottir, Alpsten et al. 1991).



 

Extracted, purified and added as food additive, AX can also impart technological properties to food (e.g. bakery products) such as an increased water binding capacity, foam stability as well as modified textural properties and improved shelf life (Vinkx and Delcour 1996).

Nevertheless, the detected positive properties of AX, especially in dough, are mainly determined by the AX structures and its compounds to other grain ingredients. In addition to the purification and concentration steps, but more dominantly the methodologies of AX extraction influence the structure of the achieved AX molecules. Therefore, different laboratory extraction methods of grain cultivars are being developed and optimized as in wheat, rye, and barley as well as for AX in food matrices such as dough and bread. For AX fortification and technological use (in foods), further researches have been carried out for different extraction techniques and solvents. Furthermore, the knowledge about easy, efficient and cheap extraction methods can be helpful for later improvement of functional and technological properties of food systems with AX (e.g. bakery goods) but also to facilitate research about positive effects of AX for human health.

A lot of time and effort has been spent to develop possible ways of extraction, isolation, fractionation and detection of AX, due to; The high content of AX in different cereal grains, the proven positive effects on human health and food properties such as bakery products as well as for analyzing and characterizing the AX molecule,. The objective of this manuscript is to review present laboratory scale methods for extraction of AX. It

also summarizes the impact of the different extraction techniques in respect to the achieved AX structure and its application as dough additive.

## 2 Structural characteristics of AX

For all plant tissues and cereals the general molecular structure of AX is similar (Vinkx, Reynaert et al. 1993). The main structure is described as a linear molecule that consists of a β-1.4-D-xylopyranosyl backbone with side chains of α-L-arabinofuranosyl linked at position 2 and/or 3 of the xylose-chain. This results in four different molecular structures of AX: unsubstituted xyl*p* (xylopyranosyl), monosubstituted xyl*p* at O-2, monosubstituted xyl*p* at O-3 and disubstituted xyl*p* at O-2.3 (see **Error! Reference source not found.**) (Izydorczyk and Biliaderis 1992; Vinkx, Reynaert et al. 1993; Izydorczyk and Biliaderis 2007). The content of AX depends on its cereal source and its location in the grain. Concentration of AX increases from the inner (endosperm) to the outer layers (bran) (Lempereur, Rouau et al. 1997). Table 1 summarizes AX contents of different cereal sources out of different cereal locations.

For AX characterization, the ratio of arabinose to xylose (ara/xyl) is described and investigated for different types of grains. Generally, the ratio of ara/xyl in AX from wheat endosperm (0.50-0.60) (Hoffmann, Roza et al. 1991; Dervilly-Pinel, Rimsten et al. 2001) and rye endosperm (0.48-0.55) (Bengtsson and Åman 1990) is lower than in wheat bran (0.57-1.07) (Shiiba, Yamada et al. 1993) and rye bran (0.48-0.78) (Ebringerová, Hromádková et al. 1990; Nilsson, Saulnier et al. 1996), respectively. The ratio of ara/xyl plays an important role for the solubility of AX. Unsubstituted regions of AX show an

increased tendency of aggregation and thus become insoluble. The smooth unbranched regions without arabinose residues form aggregates due to stabilization by hydrogen bonds (Andrewartha, Phillips et al. 1979; McCleary and Prosky 2001; Courtin and Delcour 2002; Ko□hnke, O□stlund et al. 2011). Especially Andrewartha *et al.* (Andrewartha, Phillips et al. 1979) demonstrated that an ara/xyl ratio lower than ~ 0.43 causes a drastic drop in water solubility. For a more intensive investigation of the AX structure, it's necessary to be extracted. Extraction procedure depends on the type of AX since there are water-extractable arabinoxylan (WE-AX) and water-unextractable arabinoxylan (WU-AX) present in grain.

Functionality and effects concerning the structural and functional properties of AX are not only dependent on its concentration in the plant but also on the constitution of the side chains. Differences in the AX properties are based on the aforementioned kind of substituent. Izydorczyk and Biliaderis (Izydorczyk and Biliaderis 1995) found out that most of the arabinofuranosyl molecules of AX are present as monosubstituted residues. Only a small percentage of these side chains consist of more than one arabinofuranosyl substituent. These oligomeric side chains are attached to each other via 1→2, 1→3, and 1→5 linkages. Moreover, the relative amount of monosubstituted xylose at O-2 is low but especially these kinds of molecules were found in significant amounts in isolated barley and rye fractions (Ebringerová, Hromádková et al. 1990; Vinkx, Delcour et al. 1995a). In addition to α-L-arabinofuranosyl and β-D-xylopyranosyl residues, small contents of other sugar residues such as galactopyranose and 4-O-methyl-α-D-glucuronic acid were detected (Höije, Sandström et al. 2006).

Additionally, ferulic acid (FA) and related derivate such as p-coumaric acid and sinapic acid play an important role as side chain constituents. In grains, FA is connected to lignin in the cell wall matrix. AX is linked to the cell walls by an ester bond between the FA carboxyl group and the hydroxyl group at C-5 position of the α-L-arabinofuranosyl side chain (**Error! Reference source not found.**; red structure) (Nilsson, Saulnier et al. 1996; Lempereur, Rouau et al. 1997; Bataillon, Mathaly et al. 1998; Courtin and Delcour 1998; Nino-Medina, Carvajal-Millan et al. 2010). The mechanism of binding to the cell wall was described in detail by Ishii (Ishii 1997). Concerning this attachment, FA can act as cross-link between AX as well as AX and Lignin (Tan, Hoson et al. 1991). For cross linking AX side chains, two distinct mechanisms have been described: The photochemical induces dimerisation as the predominant mechanism on the other hand the oxidative coupling of dehydrodimers via peroxidases (**Error! Reference source not found.**) (Fry 1979; Ford and Hartley 1990; Hartley and Morrison 1991). FA which is not bound to dehydrodimers can also connect to other cell wall constituents such as proteins, lignin or cellulose by covalent and non-covalent interactions (Iiyama, Lam et al. 1990; Ralph, Hatfield Ronald et al. 1998; Piber and Koehler 2005). These cross-links of cell wall components influence the wall characteristics in the original plant tissue such as flexibility, adherence, extensibility and plasticity (Fry Stephen and Miller Janice 1989). Apart from that, the mentioned cross links also influence the solubility of AX. It was shown that water-unsoluble AX are 8 to 39 times higher cross-linked than water-soluble AX (Bunzel, Ralph et al. 2001). Therefore, researchers concluded that the solubility of AX does not only depend on the kind of arabinose-substituent but also on

the degree of cross-linking by diferulic acid (Michniewicz, Biliaderis et al. 1990; McCleary and Prosky 2001) and on the substitution pattern of AX (Ko□hnke, O□stlund et al. 2011). Up to now there are only few indications for a possible classification scheme of AX into WE-AX and WU-AX. In a certain range of branching, the xylan backbone is water soluble; above and below it is not soluble, but this value can be shifted by the amount of linked arabinose units and interconnections for e.g. FA. Especially the ratio of WE-AX to WU-AX has an important influence on dough and bread characteristics as described below.



## 3 Technological properties of AX

An addition of isolated AX to dough revealed a competition of AX with other flour components concerning the hydration of particles and influenced dough and bread characteristics (Michniewicz, G. et al. 1991). For measuring water absorption, many analyses were done by the farinograph. Originally, the farinograph is used for rating wheat flour characteristics such as water absorption or dough development time (DDT) by a consistency measurement. For water soluble pentosans and water insoluble pentosans, consisting of approximately 70 % AX, water holding capacities of 4.4 and 9.9 times their weight was reported for wheat flour, respectively (Kim and D´Appolonia 1977). Girhammar and Nair (Girhammar and Nair 1992) reported wheat water soluble and insoluble pentosans water holding capacities of 11 and 10 times of their weight, respectively. Jelaca and Hlynka (Jelaca and Hlynka 1971) showed that water soluble pentosans absorb 9.2 times their weight of water and water insoluble pentosans 8.0

times their weight. Worth to mention, the uncertainty in the water absorption measured by the farinograph is caused by the AX itself or by the interactions of AX with other flour components such as FA linkages which could result in a gel network and therefore in a higher water absorption as described later. Several scientists proposed different mechanisms how water is generally bound in polysaccharides. Water is associated with solutes in a number of ways such as water absorption on hydrophilic sites of the molecule by hydrogen bonding or water held by a network (Labuza and Busk 1979; Chen, Piva et al. 1984; Chaplin 2003). Thus, the determination of water absorption is basically dependent on the kind of water binding. It is assumed that these attributes are affected by the molecule structures themselves as well as by the pattern of intra-molecular and inter-molecular bonds. In addition, it is also assumed that these abilities apply equally for water-extractable and water-unextractable AX. WU-AX is not able to dissolve in water due to its pattern of structure as described before. Nevertheless, it is assumed that WU-AX are able to bind water by capillarity action and by hydrogen bonding (Chaplin 2003). Further, investigations prove that the content of potential reactants with AX such as FA also have a strong influence on the water holding capacity of AX (Pentoans) (Izydorczyk and Biliaderis 1992). Consequently, for measuring the water absorption capacity of AX, it is necessary to determine the content and the degree of connectivity of possible AX reactant. At this point, more research work is needed to analyze the process of water binding capacity.

A significant increase in DDT was shown by Microfarinograph investigations of flour, fortified with 2 % AX showed. The most effective increase was gained for water soluble

pentosans from rye. The DDT increased from 5.5 min (control) to 10 min. Determined DDTs with addition of water-extractable and water-unextractable pentosans from wheat were 8 and 7.5 min, respectively (Michniewicz, G. et al. 1991). It was further analyzed that the amount and the molecular weight of AX (HMW and LMW) as well as the AX source like wheat or rye and the layer (bran, aleurone layer or endosperm) have an important influence on the extent of these effects (Izydorczyk and Biliaderis 1992; Biliaderis, Izydorczyk et al. 1995). The fractionation of the HMW and LMW AX took place after extraction via gel permeation chromatography. In another study, it was shown that the water holding capacity can be increased by oxidative cross-linking (by $H_2O_2$/peroxidase, laccase/$O_2$ or chemical systems) of ferulic acid polymers (see **Error! Reference source not found.**) which results in water holding capacities up to 100 g of water per g of polymer (Nino-Medina, Carvajal-Millan et al. 2010). Additionally, it was further investigated, that water-extractable pentosans and also AX, treated with oxidants, formed a gel network (Izydorczyk, Biliaderis et al. 1990). The gelling potential is influenced by the molecular weight, the degree of branching, which can be separated during the extraction via ethanol or ammonium sulfate in AX with low and high ara/xyl ratios as described later, and phenolic acid content as well as the AX source (Rattan, Izydorczyk et al. 1994). Dervilly-Pinel *et al.* (Dervilly-Pinel, Rimsten et al. 2001) showed that gels from rye and barley result in stronger gels than those from wheat and triticale, this effect has also an important influence on dough rheology. Rattan *et al.* (Rattan, Izydorczyk et al. 1994) demonstrated in frequency sweep tests that AX, treated with peroxidase and $H_2O_2$ show an initial increase of storage modulus (G′) followed by a

plateau region, but generally G′ dominates over G′′. For untreated AX solutions, the loss modulus (G′′) predominates over the G′ in the frequency test range. It is also mentioned, that the rheological behavior of this AX solution changed from a viscous solution to a solid-like material. For wheat, rye and triticale AX, similar rheological behaviors have been reported (Dervilly-Pinel, Rimsten et al. 2001). In addition, Köhnke *et al.* (Ko□hnke, O□stlund et al. 2011) changed the AX pattern by the use of the specific enzyme arabinofuranosidase and demonstrated that the solubility of AX depends on its substitution pattern. With this finding, Köhnke *et al.* also demonstrated that the functional properties of AX can be changed by the use of Enzymes. For dough making, it is suggested that WE-AX have a positive effect on the dough structure and dough stability, especially when standing higher pressure in the gas cells in the beginning of the baking process (Izydorczyk and Biliaderis 2007). For WU-AX, a decrease in dough stability, loaf volume and other bread characteristics was analyzed (Courtin and Delcour 2002), it was also demonstrated that the water-unextractable part can form physical barriers and interfere directly or delay the process of gluten formation. As a conclusion, the gluten has a lower extensibility as well as a lower rate of aggregation (Wang, Hamer et al. 2003). Dornez *et al.* (Dornez, Gebruers et al. 2009) demonstrated that AX can be weakened by the use of xylanases. Beck *et al.* (Beck, Jekle et al. 2011) analyzed the effect of Transglutaminase on rye proteins cross-linking and assumed that the interaction is limited by the presence of pentosans.

In general, it can be summarized that the effect of AX on dough and bread characteristics depends on the pattern of AX structure, the ara/xyl ratio and the

molecular size of AX (Biliaderis, Izydorczyk et al. 1995). Due to the different features of WE-AX and WU-AX, a separation of these molecules is beneficial for analyzing the effect of AX on food processing and enables therefore a quality optimization.

## 4 Methods of extraction and isolation of AX

AX is identified as a useful ingredient to impart some functional properties into food beside its positive metabolic effect on human health. For the isolation of AX, especially from various types of grains, different extraction methods are known. Recently, the (most) common methods for isolating AX in labor scale involve aqueous and alkaline extraction but also enzymatic and ultrasound treatment (Elbegzaya, Hollmann et al. 2010). WE-AX are described to be bound weakly in the plant tissue cell wall and thus can easily be extracted with the help of water. The extractability of WU-AX depends on the degree of arabinose-substituents, on the substitution pattern of AX as well as on the degree of cross links between FA and other cell wall components as described above. In this case, for cleaving these bonds, more sophisticated extraction methods have to be applied. For the isolation of WU-AX from the cell wall, chemicals with a higher dissolving effect like NaOH, $Ba(OH)_2$ e.g., or enzymes like xylanase and esterase as well as ultrasound are needed. Moreover, for several methods, researchers implemented different intermediate steps to isolate AX such as: stirring, heating or enzyme treatments to increase extraction yield of AX along with the degree of purity. The knowledge about functionality and applicability of different extraction methods is

essential to gain the highest AX yield possible. Moreover, the chosen extraction methods also influence the functional properties of AX.

### *4.1 Pre-purification of AX sources*

Cereals consist of starch, non-starch polysaccharides, different proteins, lipids and other minor components. For AX recovery, these components are obstructive and thus need to be removed step by step from the whole matrix.

First, the grain samples are milled, then heat treatment is applied to inactivate sample immanent endogenous enzymes (Cleemput, Roels et al. 1993). Hartmann *et al.* (Hartmann, Piber et al. 2005) used this pre-purification step also successfully for flour and bread. For dough however, enzymes were inactivated under reflux with 90 % (v/v) ethanol for 30 min.

For the removal of proteins, Fincher and Stone (Fincher and Stone 2004) treated wheat flour with 80 % ethanol before isolating WE-AX. Such treatments result in water-soluble pentosans with about 2 % protein content. In their work, Courtin and Delcour (Courtin and Delcour 1998) analyzed a wheat pentosan concentrate, a byproduct of the industrial wheat starch-gluten separation process. Before analyzing the contained AX, proteins were removed by the addition of clay as flocculation agent. To obtain the maximal protein absorption, the wheat pentosan concentrate was suspended in water, pH was adjusted to 3.5 and afterwards the clay-protein precipitate was separated by centrifugation and discarded. Vansteenkiste *et al.* (Vansteenkiste, Babot et al. 2004) used the same purification method for solely crude wheat WE-AX. Before clay treatment, the samples were thermally purified at 100 °C for 20 min for enzyme

inactivation. In detail, Dervilly-Pinel *et al*. (Dervilly-Pinel, Rimsten et al. 2001) analyzed different flours and the effect of cooking in ethanol (80 %) prior to extraction and confirmed that ethanol-treated flours were less contaminated by proteins than untreated flours.

In a next step, after centrifugation and separation of proteins, α-amylases and glucosidases were added to degrade starch and saccharides (Nilsson, Saulnier et al. 1996). Hansen *et al.* (Hansen, Rasmussen et al. 2003) processed a sample with thermostable α-amylase at 100 °C for 1 h. Afterwards, the remaining starch fragments were completely degraded by amyloglucosidase (Hansen, Rasmussen et al. 2003). For maize bran, analogous proceedings are published (Carvajal-Millan, Rascón-Chu et al. 2007).

The mentioned pre-purification steps were instrumental in achieving more purified AX and facilitate the later extraction step. Nevertheless, these purification steps go along with exposition of AX. The subsequent process steps deal with the virtual extraction of WE-AX and WU-AX, respectively.

### *4.2 Extraction of WE-AX*

As already mentioned, most of the water soluble AX are only weakly bound in the cell wall tissue of the plant and can easily be isolated by moderate extraction methods. Here, water is the main reagent for isolating AX. The described methods slightly differ in its extraction parameters as temperature, time and amount of purification steps.

After sample extraction from different types of grains by stirring with water, most methods involve centrifugation of the aqueous suspensions, leading to aqueous

supernatant which contains the WE-AX and an insoluble residue. In some studies, the supernatant was directly taken for analyzing WE-AX (Hashimoto, Shogren et al. 1987; Lempereur, Rouau et al. 1997; Delcour, Van Win et al. 1999). In other studies, the WE-AX containing supernatant was additionally purified prior to final WE-AX quantification. The supernatant was heated (app. 90 °C) and the precipitating soluble proteins were removed by filtration, adsorbed with ammonium sulphate, by clay or by centrifugation (Cleemput, Roels et al. 1993; Rattan, Izydorczyk et al. 1994; Loosveld, Grobet et al. 1997). For rye whole meal and wheat flour, Vinkx *et al.* (Vinkx, Reynaert et al. 1993) and Rattan *et al.* (Rattan, Izydorczyk et al. 1994) hydrolyzed residual starches and other polysaccharides by addition of α-amylase and/or amyloglucosidase. After incubation, the solution was centrifuged and the supernatant was filtered with Celite or the enzymes were inactivated by heating the solution for 30-60 min at 85-95 °C. Furthermore, Rattan *et al.* (Rattan, Izydorczyk et al. 1994) dialyzed the supernatant with water instead until no sugar was detectable in the dialysate. Some other researchers skip this step and centrifuged the supernatants directly after cooling for yielding AX solutions (Loosveld, Grobet et al. 1997; Ragaee, Campbell et al. 2001).

To achieve a concentration of WE-AX, it was precipitated with ammonium sulfate solution or with ethanol (80-90 %) and dried in an oven for 24 h at 45 °C, 7 h at 40 °C, respectively (Cleemput, Roels et al. 1993; Cleemput, van Oort et al. 1995; Nilsson, Andersson et al. 2000). These two chemicals can also be used for fractional AX precipitation by stepwise addition. Nevertheless, with an increasing concentration of ethanol and ammonium sulfate solution, there was a detectable increase in the ratio of

ara/xyl in the achieved fractions of the wheat flour samples, respectively (Izydorczyk and Biliaderis 1992; Cleemput, van Oort et al. 1995). These results could not be confirmed by Courtin and Delcour (Courtin and Delcour 1998) who investigated a wheat pentosan concentrate in which the ratio of ara/xyl did not increase by an ethanol concentration.

In addition to the separation via ethanol, Cleemput *et al.* (Cleemput, Roels et al. 1993) reported, that a concentration of 65 % (v/v) yielded the best separation between the two pentosan polysaccharides AX and AG. On the other hand, Loosveld *et al.* (Loosveld, Grobet et al. 1997) reported no separation of WE-AX and water extractable Arabinogalactan-peptides by the mentioned ethanol concentration.

Another technique of AX separation involves adsorption of water-soluble hemicelluloses with tris-HCl buffer on a diethylaminoethyl (DEAE)-Sepharose (CL-6B) column. The AX concentrates from the DEAE column were loaded onto a Sephacryl (S-200) column and the achieved AX fractions were pooled and dialyzed against dest. water. For further experiments, the solution was frozen immediately and freeze-dried (Shiiba, Yamada et al. 1993). Nilsson *et al.* (Nilsson, Andersson et al. 2000) used the same technique for fractionation of a crude AX extract. For this, a DEAE-cellulose column was activated with sodium borate, the AX extract was applied on the column and eluted by dest. water. The achieved fractions were pooled, dialysed against dest. water and freeze-dried.

For the isolation of WE-AX from dough or bread, the samples were freeze-dried, milled and analyzed as described above for flour (Cleemput, Booij et al. 1997; Hartmann, Piber et al. 2005).

The extraction by water seems to be an easy way for an isolation of WE-AX. The complexity of extraction is less and no special tools or chemicals are needed which implies lower costs. Additionally, due to the effect of easy extractability of WE-AX, this fraction shows different functional properties to WU-AX such as water absorption, dough development or gel formation as described above. For dough and bread preparation the WE-AX are described as more beneficial as the WU-AX. The content of WE-AX depends on the source and is far less than the content of WU-AX. Although regarded as water soluble, the AX bound to the cell tissue are not or only in small quantities isolated by these methods.

### *4.3 Extraction of WU-AX*

For the extraction of WU-AX more complex and more sophisticated methods are necessary than for WE-AX. Beside longer stirring times, higher temperatures and more complex purification steps, the extraction solvent have an essential effect on AX yield and purity. The extraction chemicals can be differentiated into alkaline and acidic solvents.

For WU-AX determination, Gruppen *et al.* (Gruppen, Hamer et al. 1991) treated wheat flour and wheat bran samples with a series of solvents: Dimethylsulfoxide (DMSO), urea, hydroxylamine hydrochloride ($NH_2OH$-HCl) in phosphate buffer and sodium hydroxide (NaOH). After continuous stirring for 16 h at 20 °C and centrifugation, the



 

residues were re-extracted with the respective solvent, diluted with water and centrifuged again. The supernatants were combined; the pH value adjusted to 7.0 and finally dialyzed with distilled water. When using hydroxylamine hydrochloride, the samples were dissolved in sodium carbonate buffer and adjusted to pH 5.0 and 7.2.

A further extraction solvent was a sodium carbonate ($Na_2CO_3$) solution. For extraction with this solvent the samples were extracted for 16 h at 4 °C, centrifuged, neutralized, dialysed with deionized water and analyzed. The achieved residues were re-suspended in sodium carbonate and sodium borhydride, extracted for 3 h at 20 °C, centrifuged, re-extracted with solvent, water and analyzed.

The next solvent described for extraction was a saturated barium hydroxide ($Ba(OH)_2$) solution. The samples were extracted with this solvent for 16 h at 20 °C. After centrifugation, the residues were re-extracted with solvent and centrifuged again. Both supernatants were combined, neutralized and dialyzed against sodium acetate buffer and water. The achieved residues were treated with acetic acid and extracted with water. After centrifugation, the supernatants were combined, dialyzed with water and analyzed. With $Ba(OH)_2$ it was possible to extract approximately about 80 % of pure (less glucose) AX present in wheat flour and wheat bran. This was the highest concentration of AX achieved by the different extraction solvents.

Equal amounts of AX were obtained using NaOH on the contrary fewer amounts were extracted using DMSO and urea. For $NH_2OH$ - HCL solution, different yields were obtained depending on the pH such that at pH 7.2 more AX were extracted than at pH 5.0. Moreover, different FA concentrations for both pH values were observed. This

indicates that more ester linkages are broken at pH 7.2, which results in higher AX contents (Gruppen, Hamer et al. 1991). Additionally, the structure of AX and therefore their functional properties can be changed by different pH values during extraction.

Bergmans *et al.* (Bergmans, Beldman et al. 1996) applied the method of Gruppen *et al.* using $Ba(OH)_2$ as described before for selective extraction of WU-AX from wheat bran. Using this methodology, Nilsson *et al.* (Nilsson, Andersson et al. 1999) achieved an extraction of 44.3 % WU-AX in rye bran. In matters of NaOH extraction, Bataillon *et al.* (Bataillon, Mathaly et al. 1998) extracted a wheat bran sample with NaOH at different concentrations (10, 20, 40 or 80 % corresponding of NaOH/starting bran) and temperatures (20, 40, 60 and 80 °C) for 6 h after removing lignin with sodium chlorite. After centrifugation, the supernatants were cooled, the pH was adjusted to 4.8, cooled down and centrifuged. The acidic supernatants were purified by microfiltration and spray-dried. The effective concentration for the extraction of AX was 80 %, independent of extraction temperature. For lower concentrations, the temperature had a slight influence on the extraction efficiency.

For the extraction of WU-AX, chemicals with a high dissolving effect are necessary. WU-AX make up to 95 % of total AX in the different kinds of bran. In dependence on the source of plant tissue, it is possible to extract up to 80 % of total WU-AX. Among the solvents described above, $Ba(OH)_2$ seems to be the most effective solvent for extraction WU-AX out of different cell wall matrices. Conversely, the high dissolving effect of those chemicals changes the ara/xyl ratios and therefore the physiochemical properties of AX. It is reported that the ara/xyl ratio increases with ethanol, ammonium sulphate as well

as barium hydroxid concentration for WE-AX and WU-AX (Izydorczyk and Biliaderis 1992; Schooneveld-Bergmans, Beldman et al. 1999). However, these treatments are more complex and associated with higher chemicals costs.

### *4.4 Enzymatic extraction of AX*

Enzymatic treatment of plant tissue can have several aims: AX with linkage to the cell wall of plant tissue can be disconnected from that, WU-AX can be split in that way to convert them into WE-AX or WE-AX can be degraded into its molecular components, namely xylose and arabinose. For that aim, the commonly applied enzymes are (1→4)-β-endo-xylanase (EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55) or feruloyl-esterase (EC 3.1.1.2) (Benamrouche, Crônier et al. 2002).

Especially endoxylanases attack the AX backbone and change the functionality of the polymers (Dornez, Gebruers et al. 2009). Endoxylanases convert WU-AX into WE-AX and these will be further degraded into their single components: arabinose and xylose (Petit-Benvegnen, Saulnier et al. 1998; Courtin and Delcour 2002). Benamrouch *et al.* (Benamrouche, Crônier et al. 2002) investigated the influence of a (1→4)-β-endo-xylanase treatment on wheat bran. After pre-purifying a soft wheat cultivar, the resulting liquid was considered as freed from starch. Xylanase action was initiated and supported by constant stirring for 24 h at 60 °C. Afterwards, enzymes were deactivated by heating at 100 °C for 10 min and the achieved solution was centrifuged. The supernatant was assayed for sugar content. Analysis of carbohydrates accounts for 63 % of total dry matter, in which 40 % were arabinose and xylose. Furthermore, 50 % of the wheat bran AX was solubilized by enzymatic treatments. This result was confirmed by Beaugrand *et*

*al.* (Beaugrand, Reis et al. 2004), who used the same hydrolyze conditions as Benamrouch *et al*.

Another method for extracting pentosans (AX and AG) was described by Hong *et al.* (Hong, Rubenthaler et al. 1989) who used a multicomponent enzyme system, declared as Meicellase. This enzyme system includes cellulases, β-glucosidase, xylanase, β-xylosidase and α-L-arabinosidase. Extraction was done for 18 h at 30 °C in a shaking water bath. Carbohydrate measurements were done for water-soluble, enzyme-extractable and total pentosans (Hashimoto, Shogren et al. 1987; Hong, Rubenthaler et al. 1989). In this case, the pentosan content instead of the AX content was determined and by further degrading it would be possible to determine the actual AX content.

A general way for isolating AX by enzymatic treatments is given in the following clause. To make AX available for further processing, first it has to be split from lignin that constitutes the cell wall. Since ferulic acid acts as bridge between lignin and AX, feruloyl-esterase can be used to split the ester linkage between arabinose and ferulic acid. This reaction takes place in water at a moderate temperature. In this specific reaction, the content of accessible WE-AX and WU-AX in the matrix is increased. This pool of AX is further used for the determination of the whole AX content in the grain. For the transformation of WU-AX into WE-AX, highly specified enzymes such as endoxylanases, xylanases and arabinofuranosidases are used. The transformation of WU-AX into WE-AX depends on the reaction time and concentration of the chosen enzymes. To decrease the degree of inter-molecular linkages the bridge former, ferulic acid, has to be cleaved. This can be performed by the use of feruloyl-esterase.

Therefore, all accessible AX should be present as WE-AX and WU-AX without inter-molecular linkages. Now, WE-AX and WU-AX can be isolated and be used for either fortification of other food systems or can further be degraded for a quantification of AX. For further degradation, the next step comprises the removal of side chains from the xylose backbone of both WE-AX and WU-AX. For this purpose, arabinofuranosidase is commonly used. In the last step the xylose backbone is exposed to xylanases. This results in a total liquefaction of former AX into separate arabinose and xylose molecules which can further be determined quantitatively and represent the total amount of AX.

Enzymatic treatments seem to be a good alternative for isolating AX from different plant tissues. Certainly, the relevant enzymes are presently not economically available on the market for isolating AX in large-scale (Elbegzaya, Hollmann et al. 2010). Furthermore, enzymatic treatments are problematic when treating AX with high ratios of ara/xyl as well as highly branched AX since the enzymes are hindered by the side chains (Lequart, Nuzillard et al. 1999; Dervilly-Pinel, Tran et al. 2004). Additionally, the degree in hydrolyzing the AX structure depends on the specific enzyme (Courtin and Delcour 2002). Another disadvantage of the use of enzymes arises from the steric structure of the plant tissue. Other components such as lignin are known to hinder the accessibility of enzymes in the tissue. Due to that, the extraction yields of AX are less compared to methods that were mentioned in the chapters before (Lequart, Nuzillard et al. 1999; Benamrouche, Crônier et al. 2002). On the other hand, investigations mentioned by Köhnke *et al.* (Ko□hnke, O□stlund et al. 2011) demonstrate, that specific functional characteristics of AX can be adjusted by the use of specified enzymes. Enzymes can be

used for target-orientated, specific modification of biopolymers. The findings lead to the conclusion that the currently known enzymes are able to change specific AX structures by selective hydrolysis which leads to AX with defined functional properties. Especially in the case of structural and functional changing of AX (as well as biopolymers) using enzymes, yet more research is needed.

### *4.5 Extraction of AX via ultrasound*

Ultrasound (US) has been used for a gentle extraction of low-molecular materials and polymers for more than 20 years (Sun, Sun et al. 2002; Sun and Tomkinson 2002). Thus for the extraction of AX, ultrasound has successfully been applied. Elbegzaya *et al.* (Elbegzaya, Hollmann et al. 2010) analyzed the influence of US on extraction performance of non-water extractable Glucurono-AX from rye bran. The rye sample was milled, purified and the WE-AX removed. Afterwards, the achieved rye sample was treated with US in combination with alkaline hydrogen peroxide (NaOH-$H_2O_2$) and aqueous NaOH (aNaOH), respectively. For comparison, extraction of WU-AX without US was done, as well. For aNaOH determinations, 20 g purified WU-AX were suspended, 1, 2 and 3 % aNaOH was added, heated up to 50 °C and treated with US for 10 min at 120 W and 24 W $cm^{-2}$. The highest yield in AX was measured with a use of 3 % aNaOH. The results also show that the additional US treatment yields a lower ara/xyl ratio which means more cleavage of arabinose from the xylose backbone instead of breaking it. The content of extracted AX with US (131 g $kg^{-1}$ raw bran) was slightly higher than without US (128 g $kg^{-1}$ raw bran). NaOH-$H_2O_2$ treatment with and without US resulted in 175 and 170 g $kg^{-1}$ raw bran, respectively. Especially, the

reduced extraction time of 10 min with US compared to former 240 min without US was mentioned positively. It was assumed that US accelerates saponification of esters between phenolic acids and AX due to alkaline treatment. This mechanism was also mentioned by Hromádková *et al.* (Hromádková, Kováciková et al. 1999) and Sun *et al.* (Sun, Sun et al. 2004). Another explanation was given by Hollmann and Lindhauer (Hollmann and Lindhauer 2005), who describe that the extractability of AX will be influenced positively by removing lignin by $H_2O_2$-treatments. It was described, that the relative molecular weight is negatively influenced by US. Due to US exposure, the relative molecular weight was reduced from 770 kDa to 480 kDa. The reducing of molecular weight was effected by the high energy input of US to remove AX from the cell wall.

Ultrasound as a single means of extraction does not perform better in terms of extraction quantities but has enormously positive effects on reaction times.



## 5 Quantification of AX

After extraction, different analyzing methods for the detection and quantification of the AX content are commonly used. These measurements provide information about the structure and the composition and thereby deliver knowledge about the characteristics of solubility, molecule size, etc.

One method was based on the hydrolysis of AX into arabinose and xylose combined with high performance anion exchange chromatography (HPAEC). For the determination of AX content, the sample was dispersed in buffer (prepared from sodium

acetate and acetic acid or tris(hydroxymethyl)-amino-methane and hydrochlorid acid (HCl)), treated with HCl and thus hydrolyzed. After cooling, the sample was neutralized by addition of NaOH. Samples which contain starch were treated with glucose oxidase/catalase to convert glucose into gluconic acid, since a peak overlay between glucose and pentosan monosaccharides can occur. The outcome of this was a longer dwell time of gluconic acid by the HPAEC and thereby better chromatographic separation of arabinose and xylose peaks (Houben, de Ruijter et al. 1997).

Another way for the hydrolysis of AX was described by Shiiba *et al*. (Shiiba, Yamada et al. 1993) with trifluoroacetic acid. Trifluoroacetic acid was added to the AX isolates and the solution was aerated with nitrogen for one minute. After heating in an oven at 105 °C for 2 h for hydrolyzing AX, the solution was evaporated at 50 °C to remove trifluoroacetic acid from the sample. Before injection into the HPLC, the sample was dissolved in dest. water and filtered. Compared to the method of Houben *et al.* (Houben, de Ruijter et al. 1997) and Krahl *et al.* (Krahl, Müller et al. 2009), the monosaccharides were analyzed by HPLC at 80 °C. Other researchers used sulphuric acid for the hydrolysis of WE-AX with small variations in time and temperature (Izydorczyk, Biliaderis et al. 1991; Rattan, Izydorczyk et al. 1994; Beaugrand, Reis et al. 2004; Hartmann, Piber et al. 2005; Carvajal-Millan, Rascón-Chu et al. 2007).

In the study of Rantanen *et al.* (Rantanen, Virkki et al. 2007) different sample preparations and analyzing methods were arranged and compared. For HPLC sample preparation, a rye flour sample was hydrolyzed with 2 M HCl for 4 h at 100 °C and neutralized with 4 M NaOH (2 mL). The second sample was hydrolysed with 1 M

sulfuric acid for 30 min at 120 °C and hydrolysed with 10 M NaOH. As the results show, the treatment with sulfuric acid showed the highest yield in xylose (65 %). As described by the researchers, the hydrolysis with sulfuric acid gave the best recovery of the carbohydrate composition provided by the manufacturer.

Bataillon *et al.* (Bataillon, Mathaly et al. 1998) adopted a hydrolysation method for measuring AX by HPLC from Hoebler *et al*. (Hoebler, Barry et al. 1989) and Gruppen *et al.* (Gruppen, Hamer et al. 1992) with small modifications: Before hydrolysation of the monosaccharides, the samples were pretreated with 72 % (w/w) sulfuric acid for a solubilization of semi-crystalline structures, followed by hydrolysis with high concentrated sulfuric acid for 2 h at 100 °C. The monomeric sugars were analyzed by HPLC fitted with a Biorad Aminex column HPX-87H at 40 °C. Gruppen *et al.* (Gruppen, Hamer et al. 1991; Gruppen, Hamer et al. 1992) developed the instruction above for analyzing AX by gas chromatography, then the AX content was calculated by equation 1 (Houben, de Ruijter et al. 1997; Krahl, Müller et al. 2009):

$$AX = 0.88 * (\%\ \text{D-xylose} + \%\ \text{L-arabinose}) \qquad \text{(equation 1)}$$

Especially for calculating the AX content, Courtin and Delcour (Courtin and Delcour 1998) used the following equation (equation 2):

$$AX = 0.88 * [(\%\ \text{arabinose} - 0.7 * \%\ \text{galactose}) + \%\ \text{xylose}] \qquad \text{(equation 2)}$$

The factor 0.88 was applied for the conversion of free sugar residues to anhydro sugars as present in polysaccharides (Hashimoto, Shogren et al. 1987).

Another method for analyzing AX was realized by gas chromatography (GC) measurement. The GC can only be used for samples which are gaseous or, although undecomposed, vaporizable. However, the monosaccharides in their native form are not directly vaporizable, for that specific reason, the sample hydrolysats were treated with different chemicals for converting the monosaccharides into alditol acetates (highly volatile). These alditol acetates were used for GC injection as described by Englyst and Cummings (Englyst and Cummings 1984) as well as by Cleemput *et al.* (Cleemput, Roels et al. 1993). This method was also used by many other researchers with only small modifications in sample preparations (Vinkx, Reynaert et al. 1993; Rouau, El-Hayek et al. 1994; Figueroa-Espinoza, Poulsen et al. 2004).

Some researchers also used a colorimetric phloroglucinol method for analyzing AX. The samples were treated with a special extraction solution and placed in a boiling water bath for 25 min. After a rapid cooling in cold water, the absorbance at 552 nm and 510 nm was measured. Calculation of the percentage of pentosans can be achieved by equation 3:

$$P\ (\%) = (A_{550nm-510nm} * S * D * V * 0.88 * 100) * F^{-1} \qquad \text{(equation 3)}$$

Where $A_{550nm-510nm}$ is the difference of absorbance between 550 nm and 510 nm; S is the slope of the xylose calibration plots; D is the dilution factor (50); V is the volume

 

[mL] of extract; 0.88 is the polymerization factor and F is the mass [g] of the flour (dry matter). The contents of AX, determined by different analyzing methods are shown in Table 1.

The most-used method of AX characterization is the detection of its molecular weight. This determination provides information about different AX sizes from the different plant tissues as well as the extent of the extraction treatments on the molecular size. Different methods were applied by the scientists such as sedimentation test, gel filtration chromatography and size exclusion chromatography (SEC) with different detectors such as laser light scattering analyzer or refractometer. Molecular weights of two different purified fractions from wheat bran, analyzed by SEC and determined with a refractometer detector, ranged from 300.000 to 350.000 Da (Shiiba, Yamada et al. 1993). Hartmann *et al.* (Hartmann, Piber et al. 2005) define molecular weights of wheat and rye flour between 5.000 and 110.000 Da, as estimated by SEC with RI (refractive index) and UV detection. Molecular weight measurements of rye samples, analyzed by SEC and detected with a multi angel light scattering detector, resulted for AX at 124.000 (Nilsson, Andersson et al. 2000). For alkali-extractable wheat flour AX, an average molecular weight of 850.000, analyzed by laser light scattering detector, was reported (Gruppen, Hamer et al. 1992). In the same study, for the sample subfractions, the molecular weights varied between 260.000 and 650.000 Da.

The research about impact of endogenous non-starch polysaccharide hydrolyzing enzymes on molecular weight of wheat flour AX measured molecular weights between

5.500 and 853.000 for three different cultivars. The eluate was monitored by refractive index detection (Cleemput, Booij et al. 1997).

Rattan *et al.* (Rattan, Izydorczyk et al. 1994) reported a molecular weight of eight different wheat flours, measured by limiting viscosity between 134.000 and 204.000.

The results show that many suitable methods are available for AX quantification. It can be assumed that none of the methods mentioned is better suited for AX quantification than another. This statement is confirmed by comparing the achieved results of the different analyzing methods shown in Table 1. In order to evaluate the comparability of the different methods, test series with the same analyzing probe (grain, harvest year, growing area, etc.) are necessary.



## 6 Conclusion

AX represents a considerable part in cereal grain plant tissues. They protect and provide stability as well as flexibility to the cell wall tissue. The highest deposits in the grain are placed in the outer layer. They are bound by covalent and non-covalent cross-links to other plant tissue polymers such as lignin, proteins and cellulose. The ability of AX to improve food systems (e.g. bakery goods) and to affect human health positively stimulated many researchers to find effective ways of extracting AX and making it appropriate for technological application such as bread improvement. The methods mentioned are used for extraction, characterization and quantification of AX in laboratory scale. For AX extraction the chosen method, the solvents and the concentration of the solvents determines the purity, yield, ara/xyl ratio and the solubility

of the AX. Nevertheless, not all of the available AX can be isolated by the methods presented. From the process point of view it was not yet successful to isolate AX without fragmentation of isolates and consequently changes of physical properties and molecular weights. Therefore, more research is recommended to delimit extraction to AX without other impurities. In addition, especially for commercial AX extraction, more efficient (higher AX contents) and cheaper techniques are necessary to fortify and improve foodstuffs by AX such as beverages and bakery goods.

Another possibility for enhancing different kinds of especially flour based products by AX could be the addition of AX rich matrices such as finely ground bran (without extraction) to rye dough. However, up to now no information exists about the influence of the milling process on the ground bran and the final ratio of WUAX and WEAX. Currently there is less research done about the influence of wheat bran particle size on wheat dough structure and even no results are available about finely ground rye particles and their impact on the rheological behavior of rye dough. Especially, the necessity for these results was pointed out in this review. For the future development of natural and nutrition rich rye products the knowledge about the rheological influence of rye bran is essential.

A possible way to reach technological benefits could be the modification of polysaccharides structures (e.g. AX) by use of specific enzymes. For rye products, enzymatic treatment seems to be a good alternative in terms of AX modification and therefore to improve dough and bread characteristics.

For understanding and controlling the effect of enzymatic treatment more knowledge and usage of existing analyzing methods of these structures are necessary.



 

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**Table 1: AX levels of different cereals**

| Source | Total AX content [%] | WEAX content [%] | Analyzing method | Reference |
|---|---|---|---|---|
| *Wheat* | | | | |
| whole meal | 4.07 – 6.02[ab] | 0.37 – 0.56[ab] | Auto analyzer (spectrophotometer) | (Lempereur, Rouau et al. 1997) |
| whole meal | 4.0 – 9.0[b] | 0.3 – 0.9[b] | - | (Fincher and Stone 2004) |
| whole meal | n.d. | 1.0[b] | gas liquid chromatography | (Ragaee, Campbell et al. 2001) |
| whole meal | 5.5[b] | 0.8[b] | HPAEC | (Krahl, Müller et al. 2009) |
| whole meal | 2.15 – 2.77[a] | 0.50 – 0.51[a] | gas chromatography | (Dornez, Cuyvers et al. 2008) |
| flour | n.d. | 0.35 – 0.54[ab] | gas liquid chromatography | (Loosveld, Grobet et al. 1997) |
| flour | n.d. | 0.42[b] | gas chromatography | (Cleemput, van Oort et al. 1995) |
| flour | n.d. | 0.31 – 0.44[a] | gel permeation chromatography | (Courtin and Delcour 1998) |
| flour | n.d. | 0.44[ab] – 0.71[ab] | gas liquid chromatography | (Dervilly-Pinel, Rimsten et al. 2001) |
| flour | 1.77 – 2.59[ab] | 0.41 – 0.50[ab] | gas liquid chromatography | (Rouau, El-Hayek et al. 1994) |
| bran | 24.1 | 0.9 | gas liquid chromatography | (Maes and Delcour 2001) |
| bran | n.d. | 1.5[c] | HPSEC | (Schooneveld-Bergmans, Hopman et al. 1998) |
| bran | 19[b] | n.d. | HPLC | (Bataillon, Mathaly et al. 1998) |
| bran | 22.6[b] | 1.8[b] | gas-liquid chromatography | (Maes and Delcour 2002) |
| *Rye* | | | | |
| whole meal | 7.6 | n.d. | gas liquid chromatography | (Bengtsson and Åman 1990) |
| whole meal | 5.3 – 8.4[a] | 2.6 – 4.0[a] | gas liquid chromatography | (Hansen, Rasmussen et al. 2003) |
| whole meal | n.d. | 0.07 – 0.35[ab] | size exclusion chromatography | (Nilsson, Andersson et al. 2000) |
| whole meal | 7.1 – 12.2[b] | 0.6 – 2.4[b] | - | (Fincher and Stone 2004) |
| whole meal | n.d. | 1.8[b] | gas liquid chromatography | (Ragaee, Campbell et al. 2001) |
| whole meal | n.d. | 0.66 | gas chromatography | (Delcour, Rouseu et al. 1999) |

| | | | | |
|---|---|---|---|---|
| whole meal | 7.0[b] | 1.4[b] | HPAEC | (Krahl, Müller et al. 2009) |
| whole meal | 7.3[b] | n.d. | gas chromatography | (Vinkx, Stevens et al. 1995) |
| flour | n.d. | 2.10[b] | gas liquid chromatography | (Dervilly-Pinel, Rimsten et al. 2001) |
| bran | n.d. | 1.7 – 7.7 | gas liquid chromatography | (Figueroa-Espinoza, Poulsen et al. 2004) |

**Table 1 (continued): AX levels of different cereals F**

| Source | Total AX content [%] | WEAX content [%] | Analyzing Method | Reference |
|---|---|---|---|---|
| *Other cereals* | | | | |
| oat whole meal | 2.2 – 4.1[b] | 0.2[b] | - | (Fincher and Stone 2004) |
| barley whole meal | 4 – 8[b] | 0.4[b] | - | (Fincher and Stone 2004) |
| barley whole meal | 4.38 – 7.79[ab] | n.d. | gas chromatography | (Henry 1986) |
| barley flour | n.d. | 0.29 – 0.46[ab] | gas liquid chromatography | (Dervilly-Pinel, Rimsten et al. 2001) |
| triticale flour | n.d. | 0.55[b] | gas liquid chromatography | (Dervilly-Pinel, Rimsten et al. 2001) |
| malt (wheat) | 5.5[b] | 1.5[b] | HPAEC | (Krahl, Müller et al. 2009) |
| malt (rye) | 7.0[b] | 2.9[b] | HPAEC | (Krahl, Müller et al. 2009) |
| Different wheat milling fractions | 1.44 – 30.66[b] | 0.48 – 1.71[b] | gas chromatography | (Delcour, Van Win et al. 1999) |

[a] = different varieties

[b] = dry matter

[c] = glucuronoarabinoxylan

HPAEC: High Performance Anion Exchange Chromatography

HPLC: High Performance Liquid Chromatography

HPSEC: High Performance Size Exclusion Chromatography

n.d. = not determined

Figure 1: Identified AX molecules: A) unsubstituted xylp; B) monosubstituted xylp at O-2; C) monosubstituted xylp at O-3 connected with ferulic acid; D) disubstituted xylp at O-2,3

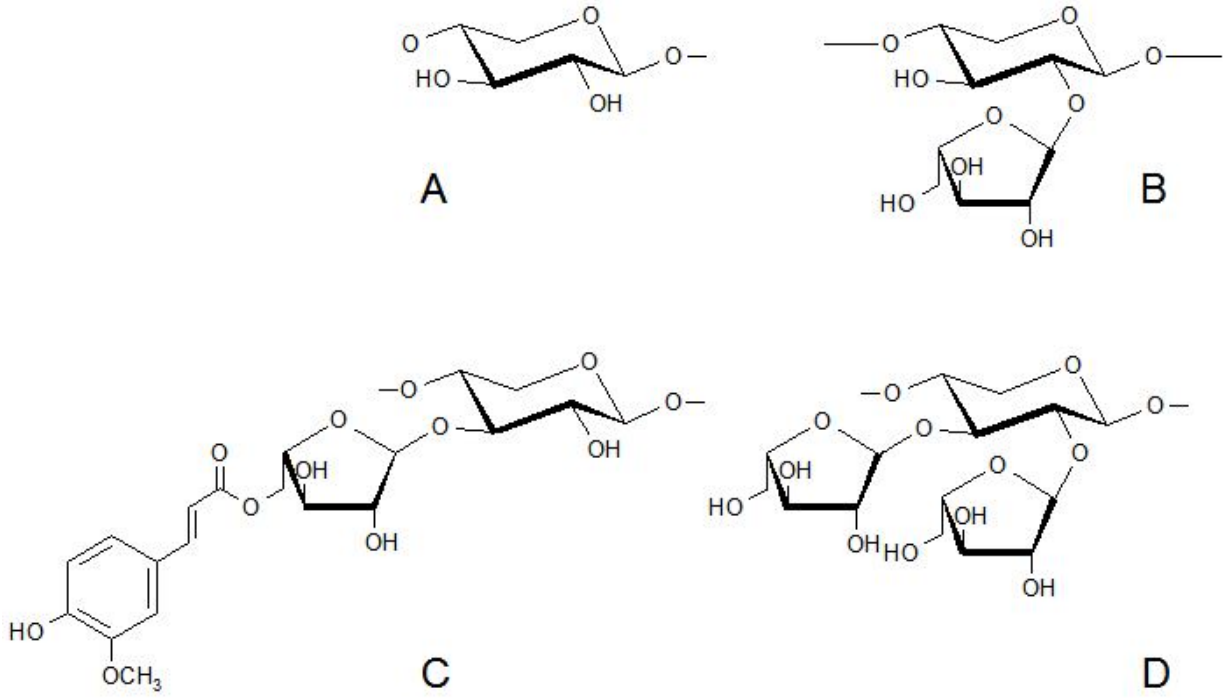


Figure 2: Identified AX structure according to Bunzel and Steinhart (Bunzel and Steinhart, 2003): Red structure: Isolated and identified ferulic acid AX fragments. Blue structure: Ferulic acid association, which can act as cross links between AX and Lignin

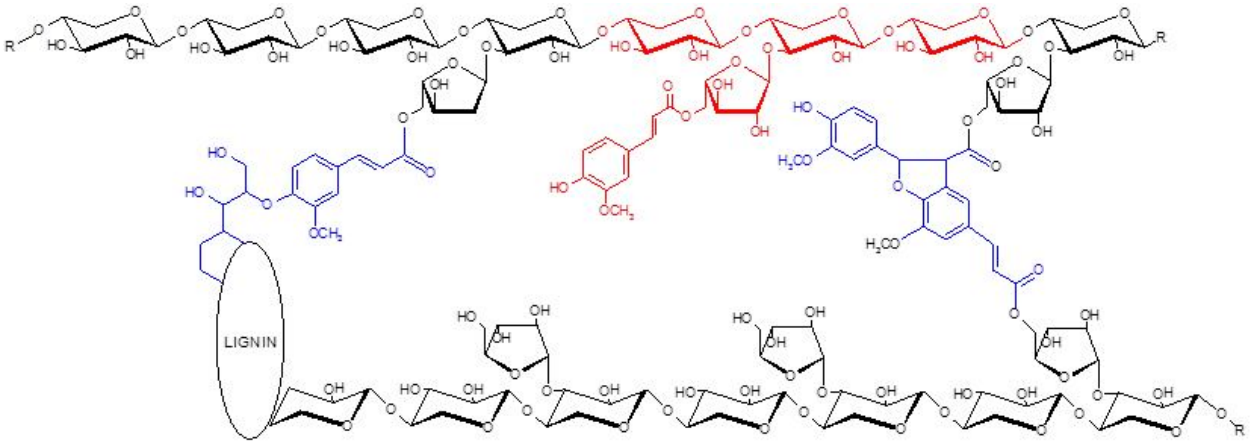

Figure 3: Oxidative coupling of ferulic acid