# CHEMICAL VALORIZATION OF AGRICULTURAL BY-PRODUCTS: ISOLATION AND CHARACTERIZATION OF XYLAN-BASED ANTIOXIDANTS FROM ALMOND SHELL BIOMASS

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The isolation of non-cellulosic polysaccharides from almond shells (AS) and their solid residue (ASR) after autohydrolysis was investigated using a two-step alkaline extraction without and in combination with short ultrasonic treatment. The obtained polysaccharide preparations were characterized by yield, chemical composition, and structural features, and the antioxidant activity of the water-soluble preparations tested. The results showed that the use of ultrasound at a reduced extraction time of 10 min as compared to 60 min of the classical procedure, with a 5% NaOH solution, resulted in the greatest yield of hemicelluloses. The AOA of their water-soluble portion ranged between 48 and 80%, indicating the antioxidant potential of these materials. The xylan polymers isolated from both AS and ASR might serve as biopolymer sources in native form or after targeted modification for production of value-added substances and polysaccharide-based antioxidants applicable in food, cosmetics, and other areas.

*Keywords: Almond shells, Extraction, Ultrasound, Xylan, Pectic polysaccharides, Phenolics content, Antioxidant activity.*

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## INTRODUCTION

Commercial processing of grains and seeds yields low-value by-products such as hulls, husks, polish waste, etc. These agricultural by-products are rich in cellulose and non-cellulosic polysaccharides (hemicelluloses, pectin) as has been recently reviewed (Ebringerová et al. 2005; Popa and Volf 2006) as well as in antioxidants (Moure et al. 2001; Garrote et al., 2004), and may serve as potential raw materials for the production of high-value added substances, such as natural antioxidants, 'super gel' for wound dressing, biodegradable commodity plastics, drug carriers, xylo-oligosaccharide prebiotics, polymeric additives in functional foods, etc. In this context almond shells (AS), which may comprise from 35 to 75% of the total fruit weight and are available in large quantities, could be taken in account as a valuable agricultural by-product. Nearly one third of the AS biomass comprises xylan type- hemicelluloses (Nabarlatz et al. 2005), which are biopolymers with a broad application potential in natural form or after targeted modification, but not yet established industrial utilization (Ebringerová and Heinze 2000). AS are also rich in lignin and other phenolic compounds (Pinelo et al. 2004; Moure et al. 2006; Amarowicz et al. 2006), representing natural antioxidants, which exhibit promising biological capacities to protect the human body from free radicals,

retard the progression of many chronic diseases, and retard lipid oxidative rancidity in foods (Dizhbite et al. 2004; Vaya and Aviram 2001). Xylans containing phenolic compounds, such as free or bound phenolic acids and/or lignin, represent a very attractive group of natural antioxidants, because they combine antioxidant activity (AOA) of the phenolics with the physicochemical and functional properties of the polysaccharide moiety (Lu et al. 1998). Recently, feruloyl oligosaccharides prepared from wheat bran were shown to efficiently protect normal rat erythrocytes against free radical-induced hemolysis (Yuan et al 2005). As has been reported by Ohta et al. (1994), phenolic acids linked to carbohydrates exhibit a higher AOA than their free form.

In previous papers, the preparation and structural properties of xylo-oligosaccharides by autohydrolysis of AS (Nabarlatz et al. 2007a) and their immunostimulatory activity have been described (Nabarlatz et al. 2007b). However, the residual fiber biomass (ASR) resulting from the thermal treatment still contains ~ 15% xylan (Nabarlatz et al. 2007a). AS are harder and more rigid than wood, they have greater density and lower porosity, and their lignin content is similar to that of hardwoods (Nabarlatz et al 2007a). All these facts negatively affect the extractibility of the xylan component.

The purpose of this paper is to investigate the extractability of the hemicellulose component of both AS and ASR materials using a two-step extraction method without and with application of ultrasonic treatment, the last aimed to increase the efficiency of the extraction process. Chemical and spectral methods are used to characterize the obtained hemicellulose preparations and their antioxidant capacity.

## EXPERIMENTAL

### Materials and Methods

Starting materials were air-dried almond shells (AS) purchased from MIMSA S.A. (Lleida, Spain) and milled to pass through a 1 mm screen and the fiber residue (ASR) obtained after autohydrolytic treatment of AS (Nabarlatz et al 2007a). The free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH$^{\bullet}$) was from Sigma-Aldrich (Germany), and gallic acid was from Merck (Germany).

The analytical methods used to characterize both materials (Table 1) are described in detail in the previous paper (Nabarlatz et al 2007a). The neutral sugar composition of the isolated preparations was determined after hydrolysis with 2M TFA by gas chromatography of the alditol trifluoroacetates and the acidic sugars qualitatively by paper chromatography as described by Kardošová et al. (2004). The uronic acid content was determined according to Ahmed and Labavitch (1977) using galacturonic acid as calibration standard, and the total phenolics content (TPC) by the Folin-Ciocalteau assay (Thaipong et al. 2006) using gallic acid as calibration standard. The radical scavenging activity was tested by the DPPH$^{\bullet}$ method according to Rao and Muralikrishna (2006) using methanol/water as solvent. The scavenging effect is expressed as the concentration of the sample needed to decrease the initial DPPH$^{\bullet}$ concentration by 50% ($EC_{50}$). Its inverse value was used to express the antioxidant activity (AOA).

FT-IR spectra of the samples (2 mg/200 mg KBr) were recorded on a NICOLET Magna 750 spectrometer with DTGS detector and OMNIC 3.2 software, using 128 scans

at a resolution of 4 $cm^{-1}$. $^{13}C$ NMR spectra were recorded in $D_2O$ or DMSO-$d_6$ at 25ºC on a FT-NMR Advance DPX 300 spectrometer (at 75.46 MHz). Chemical shifts of signals were referenced to internal acetone (31.07 ppm). Ultrasonication was performed with the Ultragen system PERSON (20 kHz, at 200 W) for 10 min as described in more detail in a previous paper (Hromádková and Ebringerová 2003).

## Extraction of Hemicelluloses

A two-step extraction procedure, schematically illustrated in Fig. 1, was used with varying conditions in the first step concerning the solvent (5% NaOH or 0.5% NaOH) and extraction time (without or with short application of ultrasonication), and with constant conditions (5% NaOH at room temperature for 1 h) in the second washing step. A more detailed description of the extraction procedure is described in a previous paper (Hromádková et al. 1999). In the experiments with AS the sample-liquid ratio used was 1:40 (g/ml) and in case of ASR it was 1:10 (g/ml). After separation of the extract (A) from the first step by filtration, the solid residue was immediately subjected to the washing step and the obtained extract (B) separated by filtration. The resulting fiber residue was successively washed with distilled water acidified with dilute HCl, distilled water and ethanol, and then air-dried. Its moisture content was determined by drying at 105 °C to constant weight. The hemicelluloses from the individual extracts A and B or the combined extracts (A+B) were precipitated by four volumes of ethanol. After overnight settlement of the precipitate, the pH of the dispersion was adjusted to 7.5 with acetic acid. Then the product was separated by filtration and exhaustively dialyzed against distilled water to reach a constant conductivity value. The insoluble precipitate (wis) eventually formed during dialysis was separated from the soluble (ws) one by centrifugation (10 min at 5000 g) and both fractions were recovered by lyophilisation.

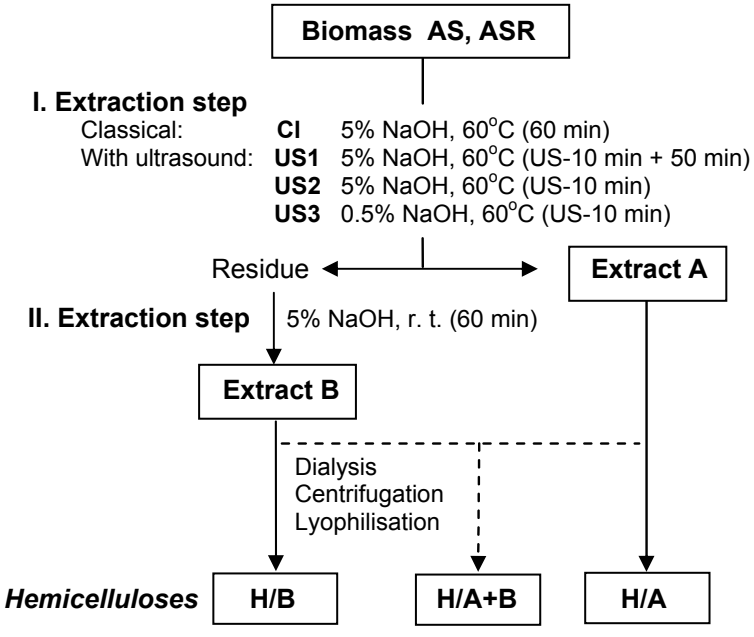


**Fig. 1.** Extraction scheme of the almond shell biomass (AS) and its fiber residue (ASR) after autohydrolysis.

## RESULTS AND DISCUSSION

The analytical data in **Table 1** indicate that xylan and the arabinan component of pectic polymers were the most susceptible to hydrolysis during the hydrothermal treatment of almond shells (Nabarlatz et al. 2007a), which resulted in accumulation of cellulose, lignin and pectin, evidenced by the uronic acid content in the fiber residue (ASR). Chromatographic analysis of the acidic sugars in the hydrolyzates of AS and ASR indicated the presence of both 4-*O*-methylglucuronic and galacturonic acid, the last predominating in that of ASR.

**Table 1.** Chemical Composition Data of Almond Shell (AS) and its Fiber Residue (ASR) after Autohydrolysis

| | Ash | N | Lignin [a] | Xylan [b] | UA [c] | Neutral sugars (Rel. mol%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | (%) | (%) | (%) | (%) | (%) | Rha | Ara | Xyl | Man | Glc | Gal |
| AS | 2.8 | 0.3 | 27.4 | 27.8 | 4.9 | 0.6 | 5.4 | 50.7 | 2.1 | 37.8 | 3.4 |
| ASR | 1.1 | 0 | 30.3 | 14.4 | 7.1 | 0 | 0.9 | 27.1 | 2.5 | 67.1 | 2.3 |

[a)] Klason lignin; [b)] Expressed as xylose content (Nabarlatz et al. 2007a); [c)] Uronic acid content.

The extractibility of the xylan component from AS and ASR was performed using a two-step alkaline extraction procedure (Fig. 1) with and without assistance of ultrasound in the first extraction step, which has been applied also for other lignocellulose materials (Hromádková et al. 1999; Hromádková and Ebringerová 2003).

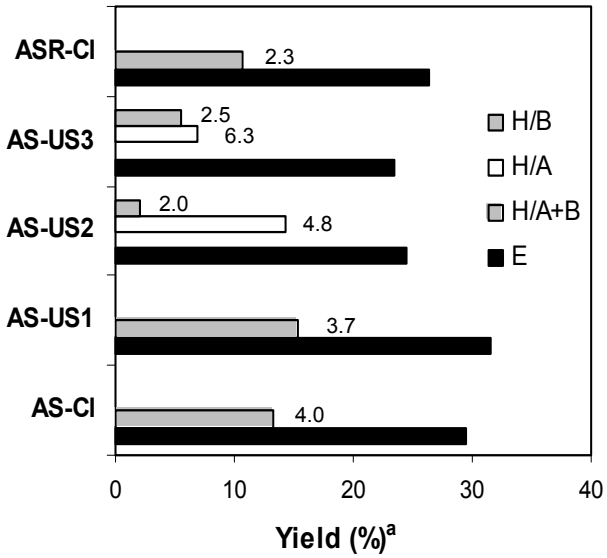


**Fig. 2.** Yield of the total extracted material (E) and of hemicellulose fractions (H) isolated from the individual extracts (A and B), and the combined extracts (A+B) according to the scheme in Fig. 1.
[a)] All yields are calculated on the basis of the starting, oven dried materials and are averages from two experiments; The number inserted denotes the yield of the water-soluble portion of the hemicellulose fraction.

As illustrated in Fig. 2, the classical extraction of AS with 5% NaOH yielded 13.2% hemicelluloses, comprising 45% of the extracted material. A short application of

ultrasound (10 min) at the beginning of the first extraction step, lasting 60 min (AS-US1), increased the yield of both the extract and the isolated hemicelluloses due to a higher amount of the wis-fraction. By performing the extraction only with 10 min ultrasonication (AS-US2), the yield of the extracted material decreased in comparison to the former experiment. However, the amount of the hemicelluloses isolated predominately in the first extraction step increased and comprised about 67% of the extract, which is higher than what is obtained in the classical and former ultrasound-assisted extraction.

The sonication effect on the extractability of plant extractives (Mason et al. 1996) and polysaccharides (Hromádková et al. 1999) is known to depend also on the type of the extractant. Therefore, the extraction under sonication was performed in 0.5% NaOH (AS-US3). The yield of the extracted material was similar as in the former experiment, but the yield of the separated hemicelluloses was lower and comprised about 54% of the extract. This can be explained by the fact that in spite of the sonomechanical effect (Mason et al. 1996), the very low NaOH concentration hinders the release of the hemicelluloses from the attacked cell matrix due to their low solubility in this extractant.

**Table 2.** Chemical Composition Data of Hemicellulosic Fractions Isolated from AS and ASR

| Sample | Fraction | TPC [a] | UA [b] | Neutral sugar composition (Rel. mol%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | (%) | (%) | Rha | Fuc | Ara | Xyl | Man | Glc | Gal |
| AS-Cl | A,B/ws | 8.9 | 9.1 | - | 4.4 | 23.2 | 56.6 | 4.9 | 6.1 | 4.8 |
| | A,B/wis | | 8.6 | - | - | 6.5 | 86.7 | - | 2.1 | 4.7 |
| AS-US1 | A,B/ws | 7.5 | 7.1 | 1.3 | 3.6 | 28.0 | 52.1 | 1.2 | 3.7 | 10.1 |
| | A,B/wis | | 12.0 | - | - | 2.5 | 89.5 | 0.6 | 2.3 | 5.1 |
| AS-US2 | A/ws | 10.1 | 11.1 | 1.1 | 1.0 | 35.5 | 41.4 | 5.4 | 3.5 | 12.1 |
| | A/wis | | 8.9 | - | - | 1.5 | 97.7 | - | 0.2 | 0.6 |
| | B/ws | 10.6 | 8.6 | 1.1 | - | 39.5 | 43.4 | 2.0 | 3.7 | 10.3 |
| AS-US3 | A/ws | 13.3 | 10.1 | 1.0 | 2.6 | 31.0 | 47.1 | 4.0 | 4.4 | 9.9 |
| | A/wis | | - | 0.5 | - | 40.1 | 42.0 | - | 7.2 | 10.2 |
| | B/ws | 8.0 | 4.1 | Tr | - | 25.1 | 68.8 | 0.5 | 1.1 | 4.5 |
| | B/wis | | 8.0 | - | - | Tr | 94.7 | - | 0.2 | 5.1 |
| ASR-Cl | A,B/ws | 6.5 | - | - | - | 27.2 | 55.3 | 4.8 | 6.0 | 6.8 |
| | A,B/wis | | - | - | - | 5.8 | 87.8 | - | 3.0 | 3.4 |

[a] Total phenolics content expressed as gallic acid equivalents; [b] Uronic acid content; The values of TPC and UA are averages from at least four experiments and that of the neutral sugar composition from two experiments; Tr, Traces.

As expected, the yield of the hemicelluloses obtained from ASR in comparison to AS was lower (Fig. 2), because most of the extractible polysaccharides were isolated during autohydrolysis of AS in form of oligosaccharides (Nabarlatz et al. 2007a). But interestingly, according to the analytical data of both preparations (Table 2), the corresponding ws- and wis-fractions showed a very similar neutral sugar composition with xylose as the main component, particularly of the wis-fractions. These results indicate that the hydrothermal treatment of AS (Nabarlatz et al. 2007a) attacked a very susceptible and accessible portion of the xylan cell wall component (Hromádková et al.

1996), whereas a part of xylan integrated into the cell wall matrix was still preserved and extractable under the studied alkaline conditions at elevated temperature.

The sugar composition of the hemicellulose preparations and their ws- and wis fractions is listed in Table 2. The results indicate that xylan is predominating and accompanied by varying amounts of pectic polysaccharides, which is evidenced by the presence of galacturonic acid, rhamnose, fucose as well as galactose and particularly arabinose, both sugars typical of the arabinan and arabinogalactan components of the pectin network (Voragen et al. 1995). In the experiments with 5% NaOH the cell wall matrix was much more attacked and split by the alkaline reagent, and both xylan and pectin polymers were released, solubilized, and accumulated in the ws-fractions. The wis-fractions represent xylans with very low pectin contamination. On the contrary, the sonochemical attack using 0.5% NaOH is milder and the wis-fraction of the isolated hemicelluloses (US3-A/wis) contained both xylan and pectic polysaccharides in about equal amounts.

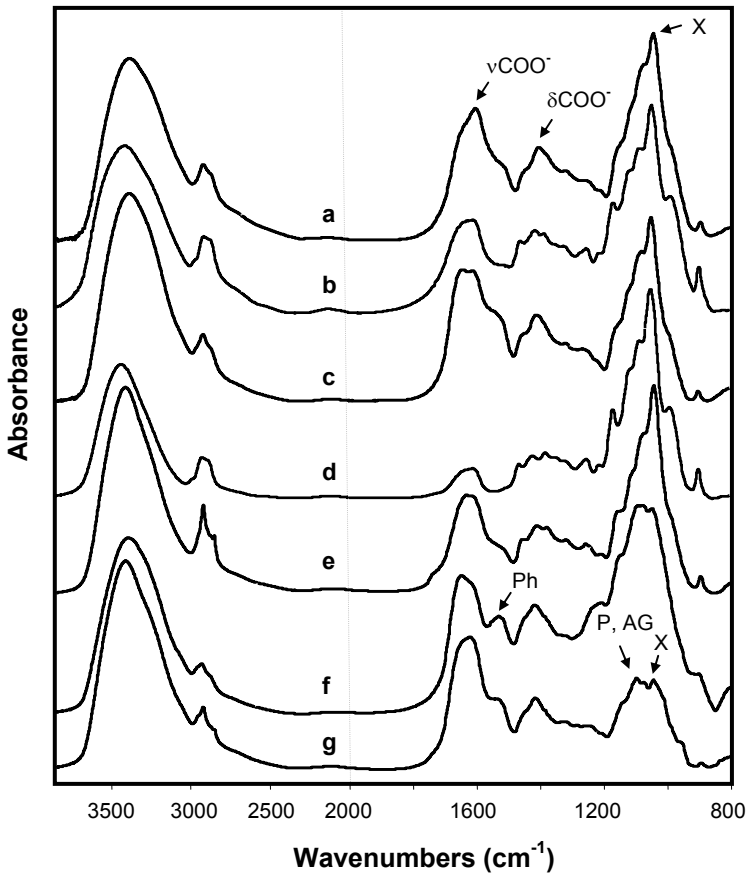


**Fig. 3.** FT-IR spectra of hemicellulose fractions (a) AS-Cl-A,B/ws, (b) AS-US2-A/wis, (c) ASR-Cl-A,B/ws, (d) AS-US3-B/wis, (e) AS-US3-B/ws, (f) AS-US3-A/ws, and (g) AS-US3-A/wis; Ph (aromatic ring of phenolics); P, pectin; AG, arabinogalactan; X, xylan.

The FT-IR spectra (Fig. 3) supported the suggestions about the presence of both xylan and pectic polysaccharides. The spectra show differences of the spectral patterns in the 1200-1000 $cm^{-1}$ region with absorption bands typical for xylan, pectin and

arabinogalactans, and ($\nu_{as}COO^-$) and ($\nu_sCOO^-$) bands of the carboxylic group of uronic acids at 1614 $cm^{-1}$ and 1414 $cm^{-1}$, respectively (Kačuráková et al. 2000).

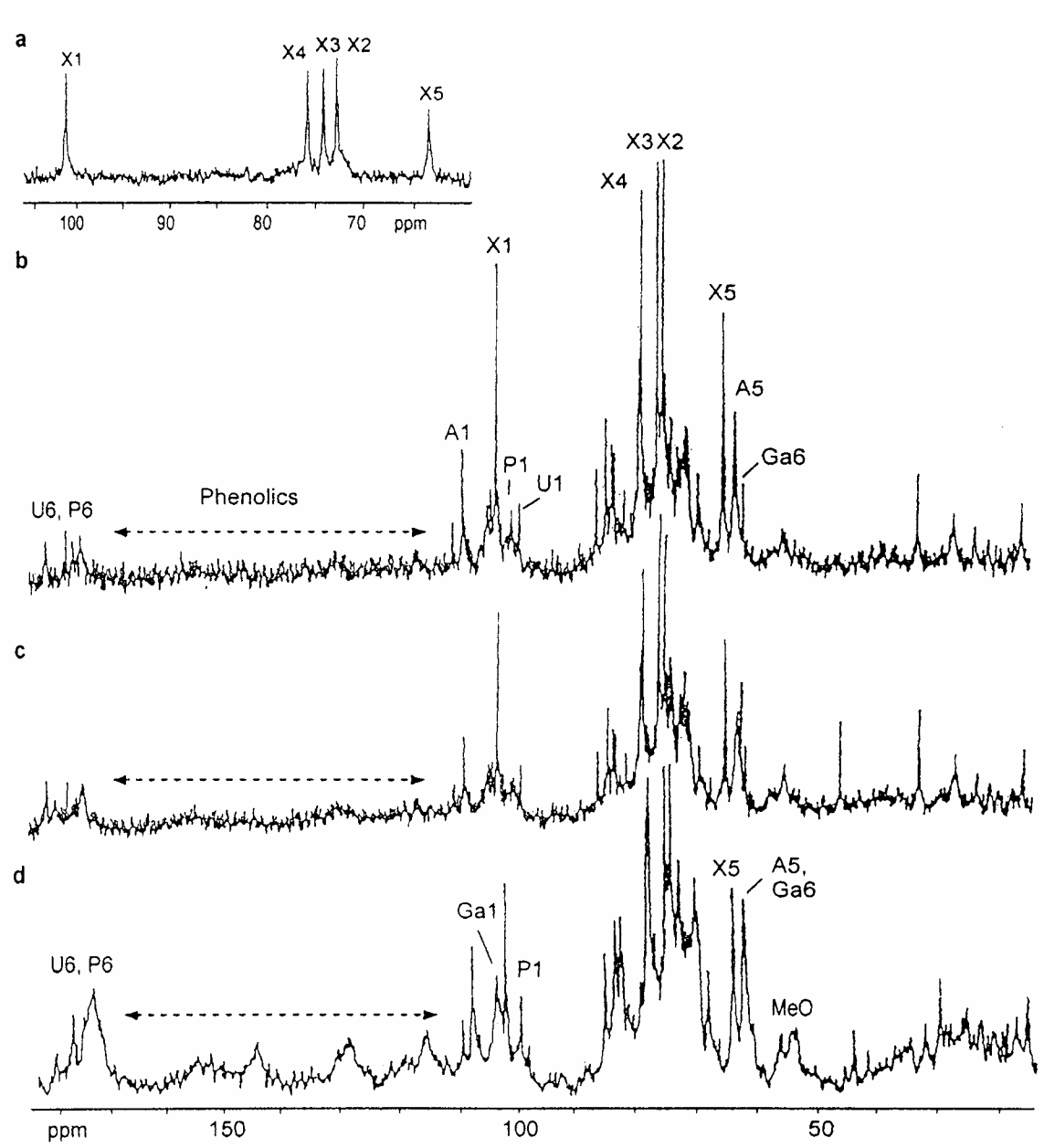


**Fig. 4.** $^{13}C$ NMR spectra of hemicellulose fractions (a) US2-A/wis in DMSO-$d_6$, and (b) Cl-A,B/ws, (c) US1-A/ws and (d) US3-A/ws in $D_2O$.

As illustrated in Fig. 4, the $^{13}C$ NMR spectra of some of the obtained hemicellulose fractions confirmed the presence and various proportions of xylan and pectic polysaccharides, indicated by the sugar analysis. In the spectrum of US2-A/wis the resonances of the 4-linked β-D-xylopyranosyl residues (X1–X5) are dominating

(Ebringerová et al. 1995). In contrast, the spectra of the ws-fractions Cl-A,B/ws, US1-A/ws and US3-A/ws showed, except for the resonances at δ 103.2, 98.1, 83.2, 76.8, 75.8, 74.0, 64.1, and 60.5 ppm, characteristic of 4-*O*-methyl-glucuronoxylan (Ebringerová et al. 1995), the signals at δ 101–99, 110–107, and ~ 105, assigned to the anomeric carbons of α-D-galacturonic acid, α-L-arabinofuranose and β-D-galactopyranose residues, respectively, which are typical of pectic acid and arabinogalactans (Keenan et al. 1985; Kardošová et al. 2004). The varying pattern of signals at δ ~ 180–170 related to the C6 of uronic acids indicated the different proportion of galacturonic and 4-O-methyl-glucuronic acid constituents, i.e. reflecting the pectin/xylan proportion. Some of carboxyl group signals might originate from contaminating phenolics, indicated by the presence of bands at ~ 1505-1515 $cm^{-1}$ of the aromatic ring vibrations in the FTIR spectra (Fig. 3) and by the total phenolics content (TPC) of the ws-fractions, ranging between 6.5–13.3 % (Table 2).

The ws-fractions were tested for antioxidant activity based on the radical scavenging effect in the DPPH• assay (Rao and Muralikrishna 2006). This effect was quantified in terms of the sample concentration needed to decrease the initial DPPH• concentration by 50% ($EC_{50}$). Its inverse value was used to express the antioxidant activity (AOA). As shown in Table 3, the AOA of the ws-fractions varied between 48-80% and it seems that the AOA activity correlates with the phenolics content ($R^2$= 0.701), except of that from fraction AS-US1/A,B. The found AOA corresponds to the lower DPPH radical scavenging activity observed by Pinelo et al. (2004) for almond shell extracts obtained by water than by organic solvents, which is in accord with the different TPC and phenolics composition of these extracts.

**Table 3.** Total Phenolics Content (TPC) and Antioxidant Activity (AOA) of Water-Soluble Hemicellulose Fractions Isolated from Almond Shells

| | AS-Cl/A,B | AS-US1/A,B | AS-US2/A | AS-US3/A | AS-US3/B |
|---|---|---|---|---|---|
| TPC (%) | 8.9 | 7.5 | 10.1 | 13.3 | 8.0 |
| $EC_{50}$ (mg/mg) [a] | 1.55 | 1.25 | 1.45 | 1.35 | 2.10 |
| AOA (%) [b] | 64 | 80 | 69 | 74 | 48 |

[a] sample/DPPH•; [b] AOA = 1/$EC_{50}$ x 100; The values are averages from at least two experiments.

However, it has to be mentioned that there are congruent reports on the relation between AOA and phenolics content (Kähkönen et al. 1999). Moreover, TPC does not reflect the composition of the present phenolics, which might be affected by the used extraction conditions. In case of sample AS-US1-A,B/ws, the alkaline extraction at 60 °C continued after the short ultrasonic irradiation by 50 min, thus enabling reactions of the formed radicals generated from the lignin as well as polysaccharides components resulting in production of other phenolics. Neverthless, the presented results indicate that the water-soluble polysaccharide-phenolic complexes isolated from AS have the potential to be used as polymeric antioxidants.

## CONCLUSIONS

1. Hemicellulose preparations composed of 4-O-methylglucuronoxylan, pectic polysaccharides, and phenolics can be isolated from both AS and ASR with dilute alkali extraction using a two-step extraction procedure. By a short application of ultrasound in the I. extraction step with 5% NaOH, not only the yield of hemicelluloses, but also the extraction time and hereby the heating time can be significantly shortened (from 60 min to 10 min), which is important from the technological point of view. The yields of the hemicelluloses ranged between 12.5-16.3% of AS and 10.7% of ASR. The recovered hemicelluloses comprised 41-67% of the extracted material, indicating losses of aqueous ethanol soluble substances (native phenolics, lignin and degradation products of lignin, and polysaccharides).
2. The water-soluble portion of the hemicellulose fractions from AS exhibited antioxidant activities in the DPPH radical scavenging test (ranging between 48-80% at doses 1.25-2.10 mg sample/mg $DPPH^{\bullet}$) indicating their potential application as polysaccharide-based antioxidants in food, pharmacy and cosmentics. This is substantiated by the fact that almond shells were not reported to contain toxic or otherwise harmful components, and the formation of novel ones are not expected under the described extraction conditions.
3. Using as extractant 5% NaOH in the I. step, the xylan component predominates in fractions of the classical as well as ultrasound-assisted extractions, and is accumulated mainly in the water-insoluble portions. The use of 0.5% NaOH in this step makes it possible to separate ws-hemicelluloses with different proportions of xylan and pectic polymers, and a very pure wis-xylan fraction in the II. step.
4. The isolated xylan-phenolics complexes from both AS and ASR are applicable wherever antioxidant activity combined with the functional properties of the polysaccharide components can be utilized. In general, the isolated hemicellulose fractions from both AS and ASR might serve as biopolymer source in its native form or after targeted modification for the production of value-added polymeric substances utilizable in various technical and non-technical areas.

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