

Isolation and Characterization of the Non-Starch Polysaccharides of Amaranth Seeds

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The isolation of water-soluble polysaccharides (WSF) solubilized during separation of starch and protein from whole grain amaranth flour by wet sieving in a very mild alkaline medium as well as of polysaccharides (ASF), extracted from the insoluble cell wall material (ISCW-1) by hot dilute alkali, is described. The yields of the separated starch, protein, WSF, and ISCW-1, considered as the "fibre" component of amaranth, were 32.9, 29.3, 1.6, and 8.0 %, respectively. Sugar analysis of the isolated polysaccharide fractions in combination with FTIR spectroscopy indicates the presence of highly branched arabinoxylan and arabinogalactan, in addition to the prevailing nonseparated starch and protein. Glucuronoarabinoxylan was evidenced in ASF, but not in the WSF fractions.

Amaranth represents an important pseudocereal with a great application potential in food industry.

Non-starch polysaccharides (NSP) of cereal grain are believed to have important influence on the baking performance of flours and the quality of baked as well as other flour-based products. A number of detailed studies on the structure and properties of NSP, arabinoxylans in particular, have been reported for grain of wheat, rye, oat, barley, rice, corn, and sorghum [1–5]. Nowadays, pseudocereals such as amaranth, quinoa or buckwheat have entered the modern food industry [6]. However, data concerning the chemical and structural properties of NSP of pseudocereals are rather rare. Some studies on the cell wall material isolated from sorghum endosperm [7, 8] and husks [9] as well as from the proso millet [10] have been reported, but no data could be found concerning seeds of amaranth which belongs to pseudocereals.

Amaranth seeds were used for diet in the ancient cultures of South America about 5000 years B.C. [6, 11]. Nowadays, attention has been paid to the seeds due to their higher dietary quality in comparison to that of the common cereals. They contain 15–19 % of proteins with a higher proportion of essential amino acids such as lysine, methionine, and tryptophane, 65–75 % of starch, 5–7 % of lipids, and 3–4 % of minerals [6]. They have 2–3 times higher content of saccharose in comparison to wheat grain. Such properties have great importance for the gluten-free diet [12, 13]. Though the dietary fibres comprise 4–5 % of the seeds, no information is available on their non-starch polysaccharide components.

With respect to the great application potential of amaranth in food industry and the lack of information concerning its non-starch components, a project has been started dealing with possible effects of the hemicellulose components on the baking properties of the flour. The main objectives of the present study were: 1. to separate the starch from the insoluble fibrous material of the amaranth seeds; 2. to isolate the polysaccharides released during starch separation; 3. to isolate the alkali-extractable polysaccharides from the insoluble fibrous material; 4. to determine the carbohydrate and non-carbohydrate components of the isolated non-starch polysaccharide fractions.

EXPERIMENTAL

The seeds of the amaranth hybride (*Amaranthus hybridus* K-343, SPU Nitra, Slovakia, 1997 harvested) were ground, using a laboratory mill (LMIM QC109, Hungary), to yield the flour (particle size of $\approx 300 \mu\text{m}$).

Moisture content was determined by drying the sample at 105 °C to constant mass. All yields and composition calculations were made on moisture-free basis. Concentrations of aqueous solutions were performed under reduced pressure at a bath temperature not exceeding 40 °C. All centrifugations were performed for 10 min at 3000 min^{-1} . Lipids were determined as a petroleum ether extract [12]. Protein was calculated from the nitrogen content assayed on the elemental analyzer Model 240 (Perkin—Elmer): $(w(\text{N})/\%) \times 6.25$. To determine the sugar composi-

tion, the flour and fibre residues were hydrolyzed using the two-step method: 72 % H₂SO₄ for 1 h at 0°C in the first and 4 % H₂SO₄ for 4 h under reflux in the second step. The hydrolysis of the isolated polysaccharide fractions was performed with 2 M trifluoroacetic acid (TFA) for 2 h under reflux. The hydrolyzate was separated into neutral and acidic sugars by ion-exchange technique on Dowex 1 × 8 (acetate form). Descending paper chromatography (PC) of the hydrolyzates was performed on Whatman No. 1 paper in the solvent systems S1 ethyl acetate—pyridine—water ($\varphi_r = 8:2:1$) for neutral sugars and S2 ethyl acetate—acetic acid—formic acid—water ($\varphi_r = 8:3:1:4$) for acidic sugars. The reducing sugars were detected with anilinium hydrogen phthalate. The neutral sugars were determined by GLC of the prepared alditol trifluoroacetates, conducted on a Hewlett—Packard 5890 Series II chromatograph equipped with a PAS-1701 column (25 mm × 0.32 mm i.d.) at a temperature program of 110—125°C (2°C min⁻¹) to 165°C (20°C min⁻¹) and a flow rate of hydrogen of 20 cm³ min⁻¹. FTIR spectra were measured in KBr pellets using a Nicolet-Magna 750 spectrophotometer operating at 4 cm⁻¹ resolution with the DTGS detector (software OMNIC 3.2). All the above-mentioned analytical methods were reported in a previous paper [14].

Isolation of Water-Soluble Polysaccharides

To amaranth flour (125 g) 0.25 % NaOH (750 cm³) was added and the slurry was mixed for 6 min in a laboratory mixer. After staying overnight at ambient temperature, the mixture was filtered over a silky sieve and washed with distilled water (4 × 750 cm³). The residue on the sieve was removed and recovered by drying at ambient temperature as the dilute-alkali insoluble cell wall material (code: ISCW-1). The filtrate was centrifuged and the obtained supernatant was separated from two distinct layers: an upper layer rich in proteins and a lower layer rich in starch. The supernatant was treated with 0.125 M-H₂SO₄ (260 cm³) to achieve the coagulation maximum of proteins which were further separated by centrifugation and dried at 60°C (code: Protein 1). The resulting supernatant was neutralized with 0.5 M-NaOH, concentrated *in vacuo* at 40°C, and dialyzed in a cellophane bag. The non-dialyzable part was lyophilized to yield the polysaccharide fraction (code: WSF-1). After repeated (3—5 times) decantation of the lower and upper layers with water followed by centrifugation, the obtained supernatants were neutralized with 0.5 M-NaOH to pH = 7, concentrated *in vacuo*, combined, dialyzed, and lyophilized to yield the polysaccharide fraction (code: WSF-2). The sediment from the upper layer was dried on air yielding protein (code: Protein 2) and that from the lower layer contained crude starch which was further washed with five volumes of 70 vol. % ethanol (500 cm³) and recovered by centrifugation and dry-

ing on air (code: Starch). The resulting supernatant gave after evaporation to dryness the polysaccharide fraction (code: WSF-3).

Isolation of Alkali-Soluble Polysaccharides from Fibre Residue ISCW-1

To a part of ISCW-1 (10 g) 1.0 % NaOH (100 cm³) was added and the dispersion was heated at 60°C for 1 h under vigorous stirring. The insoluble residue was separated by centrifugation and treated again with 1.0 % NaOH (100 cm³) at ambient temperature for 1 h. After centrifugation, the residue was neutralized with 6 M-CH₃COOH, washed with ten volumes of distilled water, and dried at 105°C to yield the fibre residue (code: ISCW-2). The supernatants from the alkaline extraction and washing steps were combined and treated with three volumes of ethanol. The precipitated hemicelluloses were decanted 2—3 times with 80 % ethanol, then acidified with 6 M-CH₃COOH to pH = 6, washed with 80 % ethanol and filtered. Then the precipitate was dispersed in distilled water, neutralized with 5 % NaOH, and dialyzed. The non-dialyzable portion was lyophilized yielding the polysaccharide fraction (code: ASF).

RESULTS AND DISCUSSION

Analysis of the amaranth flour (Table 1) showed that the content of non-carbohydrate components, *i.e.* protein, lipids and ash, agrees with the data reported previously for amaranth species [6, 12, 13]. The content of proteins and lipids in amaranth seeds is higher than in common cereals such as various rye varieties

Table 1. Characterization of the Amaranth Hybride K-343 Flour and the Insoluble Fibre Residues after Separation of Starch and Protein (ISCW-1) and Isolation of the Alkali-Soluble Hemicelluloses (ISCW-2)

Analysis	Sample		
	K-343	ISCW-1	ISCW-2
<i>w</i> (Moisture)/% ^a	7.8	7.9	7.8
<i>w</i> (Yield)/% ^b	100	8.0	1.2
<i>w</i> (Protein)/% ^c	17.4	11.1	0
<i>w</i> (Lipid)/% ^c	7.4	nd	nd
Neutral sugar composition	<i>x_i</i> /mole %		
Rha	0	0.7	2.9
Ara	2.5	4.4	19.1
Xyl	2.1	1.8	23.3
Man	tr	2.4	2.3
Glc	92.8	89.3	44.6
Gal	2.7	1.5	7.8
<i>x_r</i> (Ara—Xyl)	1.19	2.44	0.82

^a) On air-dry flour; ^b) on absolutely dry flour; ^c) on absolutely dry sample; nd - not determined; tr - trace amount.

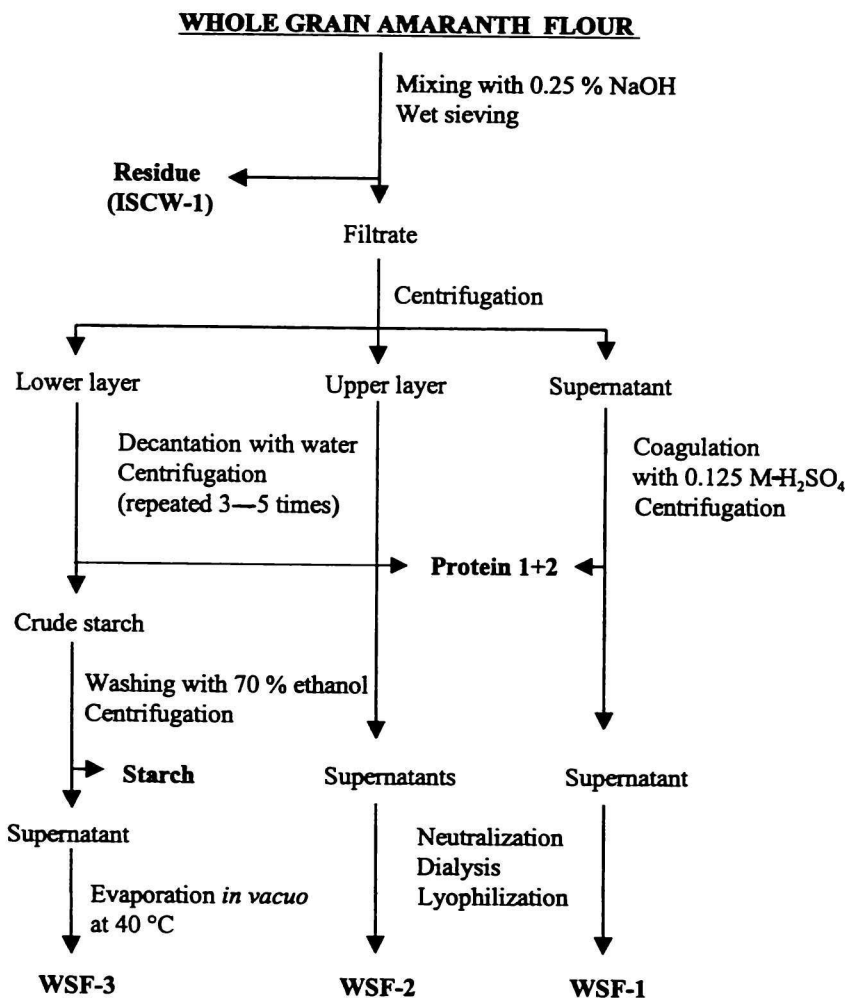


Fig. 1. Isolation scheme of water-soluble polysaccharide fractions WSF-1—3 from whole grain amaranth flour during starch and protein separation.

which were reported to contain 8.3—11.0 % protein and 1.5—1.8 % lipids [15], or in sorghum with 9.9 % protein and 3.9 % lipids [8]. Of neutral sugars, determined after acid hydrolysis of the sample, glucose is the predominating component, which is in accord with the high starch content of amaranth flours ranging between 62—69 % [16]. However, a part of glucose originates from cell wall polysaccharides, *i.e.* cellulose and hexoglycan-type hemicelluloses. Another part together with some mannose was formed during the hydrolysis and reduction steps from the reported [11] saccharose component of amaranth flour. Only about 7 % of the neutral sugars accounts for xylose, arabinose, and galactose, presumably present as hemicelluloses of the arabinoxylan and arabinogalactan types, known to occur in grain of grasses and cereals [17].

Fig. 1 shows the scheme of the extraction procedure, used to separate starch and protein from the insoluble cell wall material of flours [18] which was modified for the isolation of the simultaneously dissolved polysaccharides. Due to the mild alkaline conditions used, the release of easy accessible, water-soluble

non-starch polysaccharides from the cell walls may be enhanced in comparison to cold or warm water treatments usually applied to isolate the water-soluble polysaccharides from cereal flours [3, 4, 19]. Water-soluble fractions (WSF-1—3) have been isolated from the three formed effluents. Their analytical characteristics are summarized in Table 2.

As expected, the highest amount of solubilized polysaccharides was isolated from the filtrate after wet sieving of the flour (WSF-1), accounting for 86 % of the total amount of obtained WSF fractions. Minor amounts were recovered from the water and dilute ethanol washings of starch-rich and protein-rich fractions. The total isolated WSF represents 1.6 % of the amaranth flour. However, the real amount might be higher because of significant losses occurred during the processing of the effluents. Whereas, the WSF-1 and WSF-2 fractions contain more than 50 % protein of undetermined origin, WSF-3 contains minor amount. In accord, the FTIR spectra of WSF-1 and WSF-2 (Fig. 2) exhibit strong absorption bands at 3300 cm^{-1} ($\nu(\text{NH})$), 1657 cm^{-1} ($\nu(\text{C}=\text{O})$, amid I),

Table 2. Yield and Composition of the Water-Soluble (WSF) and Alkali-Soluble (ASF) Polysaccharides Fractions from the Flour of Amaranth Hybride K-343

Analysis	Fraction			
	WSF-1	WSF-2	WSF-3	ASF
$w(\text{Yield})/\%^a$	1.38	0.14	0.08	4.2
$w(\text{Protein})/\%^b$	52.0	61.5	1.5	0
Neutral sugar composition	$x_i/\text{mole } \%$			
Rha	0.7	5.2	0	0
Ara	14.5	19.9	12.9	2.1
Xyl	9.3	15.4	21.3	2.6
Man	2.7	5.0	11.9	2.5
Glc	67.7	49.2	49.0	90.7
Gal	5.1	5.3	4.8	2.1
$x_r(\text{Ara—Xyl})$	1.6	1.3	0.6	0.9

a) On absolutely dry flour; b) on absolutely dry sample.

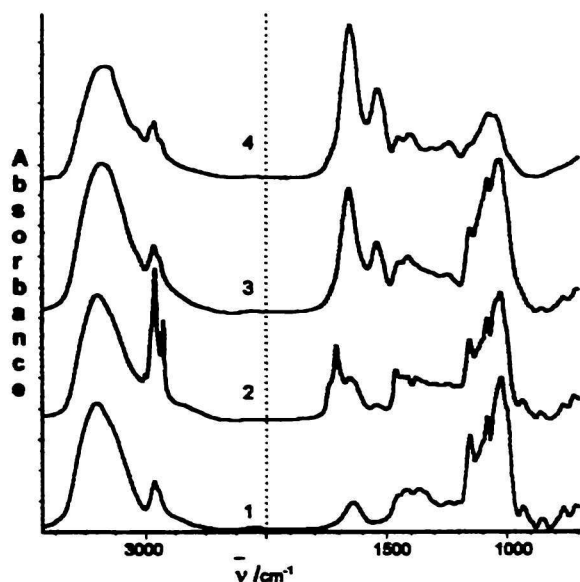


Fig. 2. FTIR spectra of the polysaccharide fractions from whole grain amaranth flour 1. ASF, 2. WSF-3, 3. WSF-1, and 4. WSF-2.

and 1545 cm^{-1} ($\delta(\text{NH})$, amid II) typical of proteins [20]. WSF-3 contains also strong absorption bands at 2926 cm^{-1} , 2858 cm^{-1} , 1768 cm^{-1} , and 1714 cm^{-1} assigned to $\nu_{\text{as}}(\text{CH}_2)$, $\nu_{\text{s}}(\text{CH}_2)$, and $\nu(\text{C}=\text{O})$ vibrations of lipids and triglycerides [20].

The sugar analysis of the WSF fractions revealed a high glucose content (49–68 %). Recently, the FTIR spectra of structurally well defined polysaccharides were reported [21] to have typical spectral patterns with certain absorption bands in the $1000\text{--}1200\text{ cm}^{-1}$ region which is dominated by ring vibrations overlapped with stretching vibrations of (C—OH) side groups and the (C—O—C) glycosidic bond vibrations. These bands can be used for an approximate iden-

tification of various polysaccharides in plant materials when combined with chemical analysis data. Except of WSF-2, the polysaccharide fractions are rich in starch. It was deduced from the well resolved absorption bands of the glycosidic bond vibrations $\nu(\text{C—O—C})$ at 1155 cm^{-1} , and at 1082 cm^{-1} , 1026 cm^{-1} , and 931 cm^{-1} assigned to ring and side group vibrations (Fig. 2). The band at 850 cm^{-1} originates from the (C1—H) vibration of the α -glucopyranose anomer [21].

Arabinose, xylose, and galactose account for hemicelluloses of the arabinoxylan and arabinogalactan types. The latter are more accumulated in the first two fractions, as deduced from the relatively high $n(\text{Ara})/n(\text{Xyl})$ ratios ($x_r(\text{Ara—Xyl}) = 1.6$ and 1.3). Although water-soluble cereal arabinoxylans are highly substituted, the $x_n(\text{Ara—Xyl})$ usually varies from 0.5 only up to 1.1 [3, 4]. Arabinogalactans occur in significantly lower amounts in water-soluble polysaccharides of cereal grains than arabinoxylans, and their $x_n(\text{Ara—Gal})$ ranges from 0.6 to 0.8 [4, 22, 23]. Rhamnose is usually connected with pectin, however, the corresponding galacturonic acid could not be detected in the hydrolyzate. Mannose may originate rather from glycoproteins than from released cell wall glucomannan which is closely associated with cellulose and extractable at stronger alkaline extraction conditions. The presence of arabinoxylan in all WSF fractions is indicated by the absorption band at 1045 cm^{-1} and the weak shoulder at 987 cm^{-1} which suggests a backbone highly substituted at position C-3 by arabinofuranosyl units [24].

The yields of the separated starch, protein, water-soluble polysaccharides (sum of WSF-1–3), and the insoluble cell wall material (ISCW-1) were 32.9 %, 29.3 %, 1.6 %, and 8.0 %, respectively, as illustrated in Fig. 3. The separation was accompanied with considerable losses of material (about 28 %) originating mainly from the starch component. The amount of isolated starch was considerably low when compared to reported values ranging between 62 % and 69 % for amaranth starches separated by wet and dry milling processes [16]. In contrast, the yield of separated protein was much higher than the protein content of the amaranth flour (17.4 %) calculated from the nitrogen content by the use of the conversion factor 6.25 (Table 1). However, the isolated protein fraction contains considerable amounts of unseparated starch, which was confirmed by the low protein content (9 %, calculated from the nitrogen content) and strong indicative absorption bands of starch in the $1000\text{--}1200\text{ cm}^{-1}$ region (Fig. 4).

The yield of ISCW-1 which may be equated with the “dietary fibre” of amaranth is higher than the already reported values of 4–5 % [12], but comparable to the non-starch polysaccharides, estimated by a conventional method based on sugar analysis [25], for various cereal grain flours [2] and sorghum whole grain

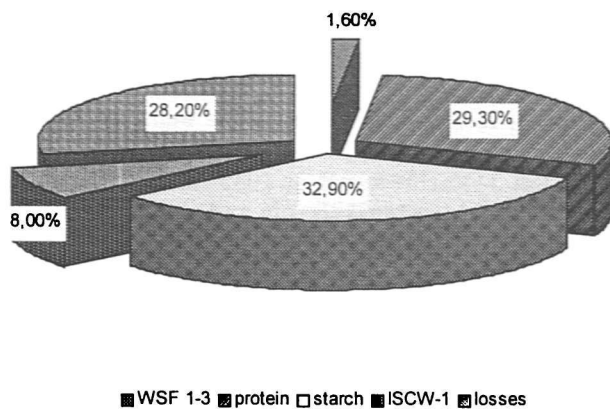


Fig. 3. Yields of starch, protein, hemicelluloses, and insoluble cell wall material (ISCW-1) isolated from amaranth K-343 flour, expressed in % ($w = (m(\text{dry solid})/m(\text{dry flour})) \cdot 100$).

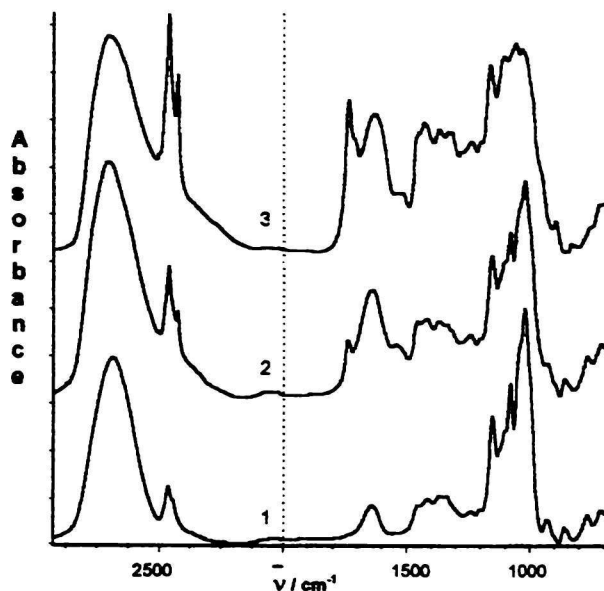


Fig. 4. FTIR spectra of starch (1), protein (2), and the alkali-insoluble residue ISCW-2 (3).

flours [8] in amounts of 3.4–7.3 %. ISCW-1 still contained 11 % proteins, which represents about 1 % of its content in the flour. This material could be structural proteins or glycoproteins in origin, similarly as in other cereal flours [26]. Considering the relatively high amount of arabinose and $x_r(\text{Ara—Xyl})$ value of 2.4 in ISCW-1, a considerable part of this carbohydrate may be an integral part of glycoproteins as found in wheat flour [22, 27, 28]. Also the galactose content is too low to affect this ratio by arabinose bound in arabinogalactans having $x_r(\text{Ara—Gal})$ values about 0.6 [23]. It is to be mentioned that galactose might be also an integral part of highly branched arabinoxylans, present in the cell walls of the outer layers of cereal grain (bran material) [3, 29], as the extraction of amaranth

had been performed on flour prepared from the whole grain. These polysaccharides need stronger extraction conditions (higher alkali concentration and temperatures) to be released from the complex polymer network of cell walls. The significantly high proportion of glucose can be attributed mainly to the cellulose component enriched in the fibre residue.

To gain further information about the hemicellulose component of the NSP of amaranth, the residue ISCW-1 was subjected to a hot alkali treatment according to the scheme in Fig. 5. The isolated polysaccharide fraction ASF comprises 53.3 % of the fibre residue, which represents 4.2 % of the amaranth flour. As can be seen in Table 2, ASF is free of protein and contains all neutral sugars detected in the WSF fractions, except of rhamnose. The $x_r(\text{Ara—Xyl})$ value of 0.9 is typical for highly branched arabinoxylans which are present in a very low amount. Glucose is the prevailing neutral sugar component, originating mainly from starch. As documented by the FTIR spectrum of ASF (Fig. 2), the absorption bands of starch predominate. This suggests that the alkaline extractant released together with hemicelluloses also starch closely associated with the cell wall polymers. Similar starch-rich hemicellulose fractions were isolated also from the delignified cell wall material of corn hulls [14].

Qualitative paper chromatography analysis of acidic sugars of the WSF and ASF fractions revealed trace amounts of D-glucuronic acid and its 4-O-methyl ether in the former and a slightly higher amount in the latter. As this sugar is a constituent of glucuronarabinoxylans which occur in outer layers of the cereal grains [30], it indicates such xylan-type to be present in the cell walls of amaranth seeds as well. Further studies on the isolated hemicellulose fractions are in progress.

The alkali-insoluble residue ISCW-2 (Fig. 5), representing the crude cellulosic component, was obtained in the yield of 1.2 % (related to the amaranth flour). In comparison to the yields of the starting ISCW-1 (8.0 %) and isolated fraction ASF (4.2 %) it means that about 30 % of the extracted material was not recovered and it was lost as solubles in the aqueous ethanolic effluents which were not analyzed. The FTIR spectrum of ISCW-2 (Fig. 4) confirmed the cellulose to be the main carbohydrate component. This was indicated by the strong absorption bands at 1162 cm^{-1} , 1061 cm^{-1} , and 1035 cm^{-1} attributed to vibrations of the glycosidic bond, (C—OH), (C—C), and ring [21]. The FTIR spectrum of ISCW-2 contains, similarly as that of the aqueous ethanol-soluble WSF-3 fraction, very strong absorption bands assigned to vibrations of lipids and triglycerides [20]. Evidently, lipids present in the amaranth flour were in part released during the extraction procedures and recoverable by ethanol, whereas the rest remained in the fibre extraction residue. ISCW-2 contains also lignin as indicated by the absorption band at 1521 cm^{-1} .

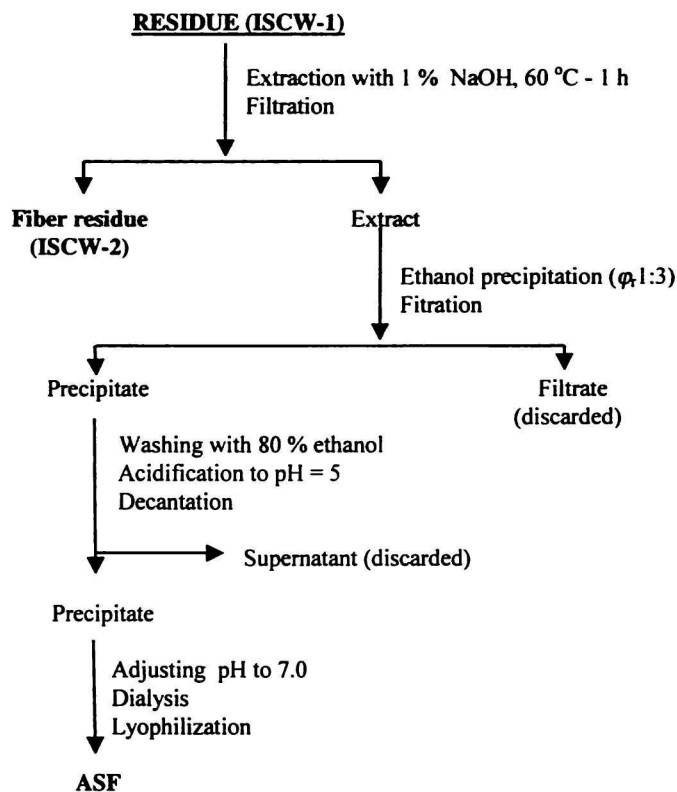


Fig. 5. Extraction scheme of alkali-soluble polysaccharide fraction (ASF) from the dilute alkali-insoluble cell wall material (ISCW-1) of whole grain amaranth flour.

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NEWS

13th International Symposium on Homogeneous Catalysis

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