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Polymeric fractions containing phenol glucosides in flaxseed

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Abstract

Extract rich in phenolic compounds was obtained from flaxseed with 1,4-dioxane:ethanol (1:1, v/v). This extract (whole polymer) was fractionated by solid-phase extraction into three "polymeric" fractions of comparable polarity. HPLC analyses of the base hydrolysates of the three polymeric fractions showed that they contain the same UV-absorbing components, though at different levels and all contained substantial amounts of a lignan, secoisolariciresinoldiglucoside (SDG). Fractionation of the base hydrolysates by column chromatography, followed by high performance liquid chromatography (HPLC) yielded two pure hydroxycinnamic acid derivatives; 4-O-β-D-glucopyranosyl-*p*-coumaric acid and 4-O-β-D-glucopyranosylferulic acid, whose structures were identified by nuclear magnetic resonance spectroscopy (NMR). NMR analysis showed all three polymeric fractions to have phenolic and aliphatic components and, in line with HPLC, suggested some structural variations between these fractions. The results of this study suggest that the glucosylated phenolic compounds of flaxseed exist in polymeric structure(s) containing ester linkages.

Keywords: Flaxseed; Phenol glucoside polymers; Secoisolariciresinol diglucoside; *p*-Coumaric acid glucoside; Ferulic acid glucoside

1. Introduction

Flaxseed (*Linum usitatissimum*, Linn., Linaceae) is an interesting raw material for food applications within the emerging concept of functional foods (Oomah & Mazza, 1998) because it is a valuable source of omega-3 fatty acids, fibre and lignans with beneficial health effects (Cunnane & Thompson, 1995). Defatted flaxseed flour was shown to contain 1–3% of a lignan, secoisolariciresinoldiglucoside (SDG, Fig. 1, Bakke & Klostermann, 1956; Johnsson, Kamal-Eldin, Lundgren, & Åman, 2000; Westcott & Muir, 1996a). Matairesinol is another lignan which is present in flaxseed in relatively low levels compared to SDG but yet in high levels compared to other food raw materials (Mazur & Adlercreutz, 1998). Secoisolariciresinol and matairesinol are believed to exert phytoestrogenic effects by acting as precursors of two metabolites: enterodiol and enterolactone (Borriello, Setchell, Axelson, & Lawson, 1985). Other lignans found in flaxseed include a SDG isomer (Bambagiotti-Alberti, Coran, Ghiara, Ginnellini, & Raffaelli, 1994a, 1994b), pinoresinol diglucoside (Qiu et al., 1999) and isolariciresinol (Meagher, Beecher, Flangan, & Li, 1998).

Besides lignans, flaxseed was reported to contain some phenylpropanoids, e.g. *p*-coumaric, *o*-coumaric, ferulic, *p*-hydroxybenzoic, gentisic, vanillic, and sinapic acids in free and/or bound forms (Babrowski & Sosulski, 1984; Kozlowska, Zadernowski, & Sosulski, 1983). Both *p*-coumaric acid glucoside and ferulic acid glucoside (Fig. 1) were reported by Westcott and Muir (1996b). *p*-Coumaric acid glucoside has been isolated (Klosterman, Smith, & Clagett, 1955) and has been named linocinnamarin. On the other hand, there is no sound evidence in the literature supporting the presence of ferulic acid glucoside in flaxseed but there is one report on an isomeric compound which was named linusitamarin (Luyengi, Pezzuto, Waller, Beecher, & Fong, 1993). In this paper we provide evidence, from nuclear magnetic resonance (NMR), for the presence of a ferulic acid glucoside in flaxseed.

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Secoisolariciresinol and other phenolic compounds in flaxseed are present in bound forms with both glucosidic and ester bonds (Westcott & Muir, 1996b) and extracts obtained from flaxseed with mixtures of ethanol and 1,4-dioxane or ethanol and water were reported to be polymeric in nature (Bakke & Klostermann, 1956; Westcott & Muir, 1996b). Phytoestrogenic, antioxidant, anticarcinogenic and cardioprotective effects were attributed to SDG and other phenolic compounds (Clifford, 2000; Cunnane & Thompson, 1995; Oomah & Mazza, 1998; Westcott & Muir, 2000). Since revelation of the complex structures of bioactive compounds is of importance for their physical properties, nutritional value and applicability as functional ingredients in foods, this paper was aimed at initiating investigations on the polymeric structure of the phenolic antioxidants in flaxseed.

2. Materials and methods

2.1. Extraction and fractionation of the flaxseed polymer

Substantially oil-free flaxseed cake was milled to pass a 0.5-mm sieve (Retsch type ZM 1, Haan, Germany) and the extra oil was removed by extraction with *n*-hexane (1:4 w/v, 2 × 24 h). The flaxseed polymer was extracted from defatted flaxseed flour (DFF, obtained from Alternativ Förädling AB, Glanshammar, Sweden) by the method of Klosterman et al. (1955). Briefly, 50g of DFF were extracted twice with 200 ml 1,4-dioxane/ethanol (1:1 v/v) on a hot plate (60 °C) with continuous stirring for 7 h. After standing overnight at room temperature, the suspension was filtered through a Buchner funnel and the solid phase was re-extracted using the same method. The combined extract was centrifuged for 20 min at 2000 rpm (850 × ***g***), to obtain a solution of a polymeric flaxseed extract. The yield of this extract was determined gravimetrically, in triplicate, by extraction of 10 g DFF with ethanol/1,4-dioxane (1:1 v/v, 2 × 40 ml) using the same method as mentioned above.

Part of the polymeric extract, hereafter called whole polymer, was fractionated using a 1 g pre-packed reversed-phase (C18) solid phase extraction column (Mega Bond Elut®, Varian, Harbor City, LA, USA). The column was conditioned with 5 ml of methanol followed by 5 ml of water. An aqueous solution of the polymeric extract (0.1 g/ml, 5 ml) was loaded onto the column and eluted with 5 ml of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% methanol in water to obtain 11 fractions.

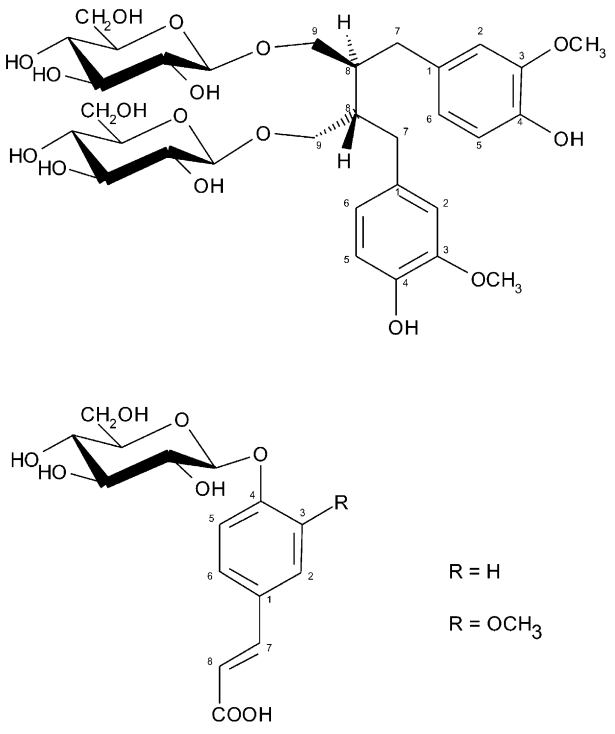

Fig. 1. The chemical structures of Secoisolariciresinol diglucoside (SDG, upper panel), and 4-O-β-D-glucopyranosyl-*p*-coumaric acid; R = H, and 4-O-β-D-glucopyranosyl ferulic acid; R = OCH_3 (lower panel).

2.2. High performance liquid chromatography (HPLC) of the polymeric fractions

The whole polymer and the 11 fractions obtained above were analysed by HPLC (Hp series 1100 system, Hewlett Packard, Avondale, PA, USA) at 25 °C on an Econosil C18 column (250 mm × 4.6 mm, 5 μm particles, Alltech, Deerfield, IL, USA). The mobile phase was a gradient of methanol and 1% acetic acid in water mixed under the following conditions: 0–10 min (38:62, v/v), 40–45 min (80:20, v/v) and the flow rate was 1 ml/min. Peaks were detected by ultraviolet-diode array detector (UV-DAD, between 215 and 400 nm) and chromatograms were recorded at 280 nm using the HP Chemstation software (Hewlett Packard GmbH, Waldbronn, Germany). The fractions eluted by solid phase extraction with 50, 60 and 70% methanol were found to contain UV-absorbing extracts, hereafter called polymeric fractions.

2.3. Analysis of the monomeric components of the polymeric fractions

The whole polymer and the three polymeric fractions with UV-absorbing materials were analysed for constituent sugars by the method described by Theander, Åman, Westerlund, Andersson, and Pettersson (1995). To investigate the phenolic components in the flaxseed polymer(s), the whole polymer, as well as its three fractions, were subjected to base hydrolysis of ester bonds

(0.3 M NaOH, room temperature, 2 days) and analysed by HPLC as described before (Johnsson, Kamal-Eldin, Lundgren, & Åman, 2000). SDG was identified by comparison with an authentic standard (Johnsson et al., 2000). The identities of two other major peaks in the hydrolysed fractions were investigated by liquid chromatography–mass spectrometry (LC-MS) and by nuclear magnetic resonance spectroscopy (NMR), as explained below. LC-MS was performed using the same column and mobile phase as before (Johnsson et al., 2000) and a positive mode electrospray ionisation technique. The analysis was performed on a Waters Alliance 2690 system with a Waters Zspray™ mass detector (mass range 60–900 Da, Waters, Milford, MA) and a MassLynx 3.1 software for data processing (Micromass Ltd., Manchester, UK).

A hydrolysed extract, from 50 g DFF, was run through solid phase extraction columns (Isolute™, C18 RP, 60cc/10grm, Hengoed, Mid-Glamoran, UK), previously activated with methanol (50 ml), followed by water (50 ml). The samples were eluted with water (100 ml), 20% aqueous methanol (100 ml) and then pure methanol (100 ml). The fractions were analysed by HPLC (Johnsson et al., 2000) to identify the fraction(s) most suitable for further purification. The major parts of the two peaks assigned to *p*-coumaric acid glucoside and ferulic acid glucoside were found in the water extracts. Salt was removed from this extract by drying the sample in vacuo and re-dissolving it in methanol. Methanol was then evaporated in vacuo and the sample was dissolved in the solvent mixture used as a mobile phase for the chromatographic column separation, i.e. chloroform: methanol: water: glacial acetic acid (60:20:2:1, v/v/v/v). Further purification was performed on a 20×2 cm glass column packed with silica gel 60 (32–65 mm particle size, Merck, Dermstadt, Germany). Fractions were collected with a fraction collector, checked with TLC (Si-60, F_{254}, Merck) and those showing the same spots were combined, concentrated and analysed by HPLC (Johnsson et al., 2000). Fractions containing most of the two compounds of interest were subjected to final purification using an Econosil RP C18 HPLC-column (250×4.6 mm, 5 μm, Alltech, Deerfield, IL) and an isocratic mobile phase (acetonitrile:0.1% acetic acid (12:88, v/v); 1ml/min). The two isolated compounds were analysed by NMR.

2.4. NMR

Before use for the NMR analysis, samples from the whole polymer and its three fractions were dried using a rotary evaporator, re-dissolved in D_2O (deuterated water) and re-dried three times to exchange water, followed by overnight incubation in a vacuum oven. All spectra (^{1}H NMR, COSY, HSQC-DEPT and HMBC) were recorded on a Bruker DRX 400 (at frequencies of 400 (^{1}H) and 100 (^{13}C) MHz) or a Bruker DRX 600 (at frequencies of 600 (^{1}H) and 151 (^{13}C) MHz) instrument (Karlsruhe, Germany). Measurements were performed in CD_3OD at 30 °C for the isolated hydroxycinamic acid glucosides and in a D_2O:CD_3OD mixture (1:1, v/v) at 60 °C for the flaxseed polymers. ^{1}H NMR chemical shifts were referenced to TMS while ^{13}C chemical shifts were referenced to the methyl group signals from residual methanol ($\delta = 49.0$ ppm). A sequence with presaturation of the water peak was used for ^{1}H-NMR. HMBC was run with a mixing time of 70 ms.

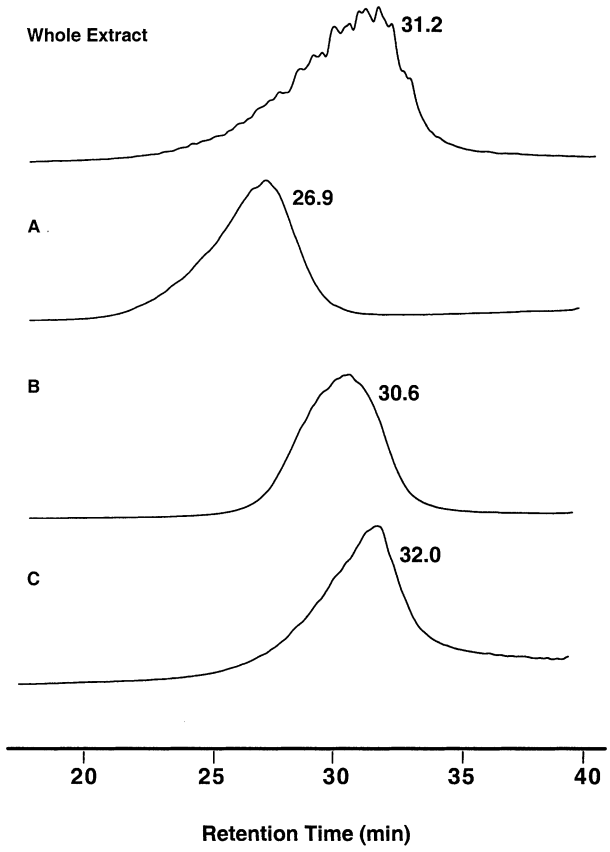

Fig. 2. HPLC analysis of the whole polymeric extract, and the three polymeric fractions separated by reversed-phase solid-phase extraction: (A) 50% MeOH:H_2O, (B) MeOH:H_2O, and (C) 70% MeOH:H_2O (retention times are shown on the top of the peaks, for conditions, see Section 2).

3. Results and discussion

The yield of the extract from the DFF sample analysed in this study was 2.9%; in accordance with the values (2–4%) reported by Klosterman and Clagett (1954). Sugar analysis showed that the only sugar residue present in the polymer was glucose. When the whole polymer was fractionated by solid phase extraction, three fractions were obtained by elution with 50, 60 and 70% methanol in water. Fig. 2 shows HPLC chromatograms of the whole polymer and these three polymeric

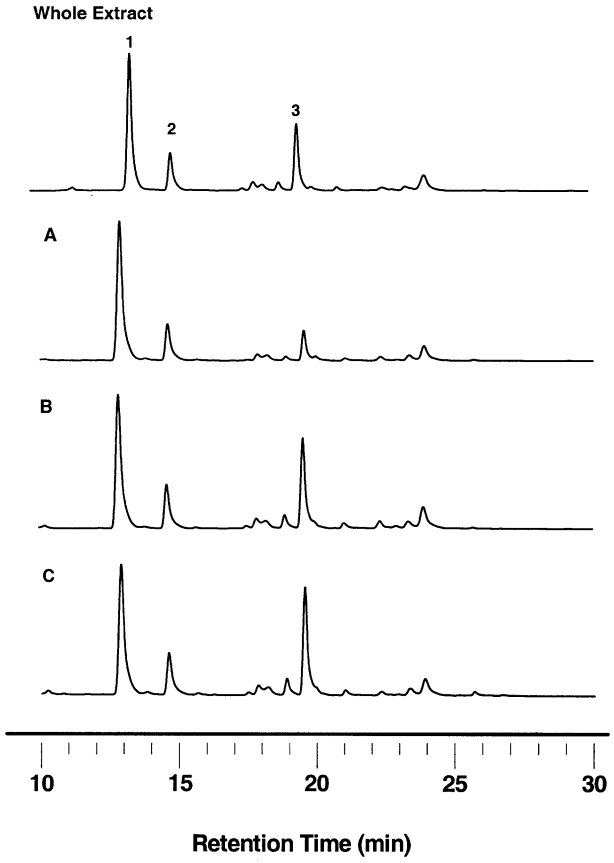

Fig. 3. HPLC analysis of the base-hydrolysates from the whole polymeric extract, and the three polymeric fractions separated by solid-phase extraction: (A) 50% MeOH:H_2O, (B) 60% MeOH:H_2O, and (C) 70% meOH:H_2O (for conditions, see Section 2). Peaks: 1, *p*-coumaric acid glucoside; 2, ferulic acid glucoside and 3, SDG; secoisolariciresinoldiglucoside (for structures, see Fig. 1).

fractions. On basis of the peak area, the 60% fraction was the largest fraction (≈ 64%), followed by the 50% fraction (≈ 23%) and the 70% fraction (≈ 13%). Broad peaks were obtained for the whole polymer (peak maximum of 31.2 min) as well as for its three polymeric fractions (peak maxima at 26.9, 30.6 and 32.0 min, respectively). The broadness of the peaks cannot be attributed to the chromatographic method used because these peaks are transformed into sharp peaks upon hydrolysis (see later). The broad nature of the peaks suggests a variation (e.g. differences in molecular weight, substituent and/or substitution pattern) in the structure of this polymer(s).

Fig. 3 presents the HPLC chromatogram of the hydrolysates of the whole polymer and its three fractions obtained by SPE (viz. 50, 60 and 70% fractions). The peak eluting at 19.5 min was identified as SDG by comparison with authentic standards (Johnsson et al., 2000) and by LC-MS where it showed an ion at m/z 709, corresponding to the molecular weight of SDG (m/z 686) plus a sodium ion (m/z 23) picked from the mobile phase. LC-MS of the first two major peaks in the chromatograms of the hydrolysed polymers (eluting at 12.8 and 14.6 min) showed ions corresponding to 4-O-β-D-glucopyranosyl-*p*-coumaric acid (m/z 349 = 326 + 23) and 4-O-β-D-glucopyranosyl-ferulic acid (m/z 379 = 356 + 23). Due to lack of standards, these compounds were separated by column chromatography, purified by HPLC and their structures identified by two dimensional NMR (COSY, HSQC-DEPT, and HMBC, Table 1). Luyengi et al. (1993) previously isolated, from flaxseed, two compounds with NMR chemical shifts comparable with those obtained in this study. The compound here assigned as 4-O-β-D-glucopyranosyl-ferulic acid has a typical ^{1}H NMR pattern for 1,3,4-substitution at 600 Mhz (Table 1) in contrast to Luyengi et al. (1993) who interpreted the signals from C-4, H-4, and C-5 & H-5 differently, assigned an isomeric structure with 1,3,5-substitution and named it linnusitamarin. Although Luyengi et al. (1993) assigned the right structure to 4-O-β-D-glucopyranosyl-*p*-coumaric acid, their assignments for H-2 and H-3, H-5 and H-6 were also different. Our interpretation of the proton and carbon signals is well supported by the two dimensional techniques and is in agreement with the interpretation of the NMR spectra of 4-O-β-D-glucopyranosyl-*p*-coumaric acid (Cui, Tezuka, Kikuchi, Nakano, Tamaoki, & Park, 1990; Terahara, Saito, Honda, Toki, & Osajima, 1990). The presence of 4-O-β-D-glucopyranosyl-*p*-coumaric acid and 4-O-β-D-glucopyranosyl-ferulic acid in flaxseed was previously mentioned in the patent by Westcott and Muir (1996b) without spectroscopic evidence. Recently, Westcott, Hall, and Muir (2000) obtained NMR evidence supporting the structure of 4-O-β-D-glucopyranosyl-ferulic acid methyl ester against that of linnusitamarin. With different extraction and NMR methodology, results presented here are in agreement with those of Westcott et al. (2000).

HPLC analysis of the base-hydrolysates (Fig. 3) showed that the whole polymer and its three fractions were similar with regard to the nature of their components absorbing in the UV range used for detection (210–400 nm). There were, however, differences in the relative ratios of the different components between the three polymeric fractions. In accordance with the fact that the 60% fraction was the major part of the polymer, the HPLC chromatogram of its hydrolysate was most similar to that of the hydrolysate of the whole polymer. The 70% fraction was rather similar to the 60% fraction but had a slightly higher proportion of SDG compared to other components. The 50% fraction, on the other hand, was clearly different and it contained a much smaller proportion of SDG and a much higher proportion of the *p*-coumaric acid and ferulic acid glucosides.

Fig. 4 presents the ^{1}H NMR spectra of the whole polymer and its three fractions. All polymeric fractions showed phenolic and aliphatic components but in

Table 1
NMR assignments for *p*-coumaric acid glucoside and ferulic acid glucoside compared with those of Luyengi et al. (1993)[a]

Position	*p*-Coumaric acid glucoside				Ferulic acid glucoside			
	This work		Luyengi et al. (1993)		This work		Luyengi et al. (1993)	
	δ_H (ppm)[b]	δ_c (ppm)	δ_H (ppm)	δ_c (ppm)	δ_H (ppm)[c]	δ_c (ppm)	δ_H (ppm)	δ_c (ppm)
1	–	130.8	–	127.7	–	131.1	–	130.4
2	7.49 (d)	129.9	7.07	129.9	7.24 (d)	112.2	7.24	112.6
3	7.09 (d)	117.7	7.69	116.1	–	151.1	–	150.1
4	–	160.0	–	159.1	–	149.7	7.16	117.4
5	7.09 (d)	117.7	7.69	116.1	7.17 (d)	117.6	–	151.0
6	7.49 (d)	129.9	7.07	129.2	7.14 (dd)	123.2	7.16	123.5
7	7.46 (d)	142.4	7.66	144.1	7.56 (d)	145.0	7.63	146.1
8	6.40 (d)	122.1	6.54	115.5	6.40 (d)	119.5	6.43	116.5
3-OMe	–	–	–	–	3.90 (s)	56.7	3.88	56.8
COO	–	nd[d]	–	nd	–	171.6	–	nd
1′	4.95 (d)	101.7	4.96	99.9	4.96 (d)	102.4	4.98	102.2
2′	3.38–3.49	74.6	3.1–3.9	73.1	3.50 (t)	74.8	3.2–3.7	74.8
3′	3.38–3.49	77.8	3.1–3.9	76.5	3.40–3.53	77.9	3.2–3.7	77.8
4′	3.38–3.49	71.1	3.1–3.9	69.6	3.40–3.53	71.2	3.2–3.7	71.2
5′	3.38–3.49	77.8	3.1–3.9	77.0	3.40–3.53	78.2	3.2–3.7	78
6′*a*	3.70 (dd)	62.2	3.1–3.9 (a + b)	60.6	3.70 (dd)	62.4	3.7–3.9 (a + b)	62.5
6′*b*	3.89 (dd)				3.87 (dd)			

[a] For experimental conditions, see Section 2.
[b] The coupling constants (Hz) for ^{1}H NMR of *p*-coumaric acid glucoside are: $J_{2,3}=J_{5,6}=8.8$, $J_{7,8}=16.1$, $J_{1',2'}=7.3$, $J_{5',6'a}=5.5$, $J_{5',6'b}=2.2$, $J_{6'a,6'b}=12.1$.
[c] The coupling constants (Hz) for ^{1}H NMR of ferulic acid glucoside are: $J_{2,6}=1.7$, $J_{5,6}=8.4$, $J_{7,8}=15.9$, $J_{1',2'}=7.5$, $J_{5',6'a}=5.5$, $J_{5',6'b}=2.0$, $J_{6'a,6'b}=12.1$.
[d] nd, Not detected.

agreement with the HPLC results, they presented some structural variations. The 60% fraction was most similar to the whole polymer. The ^{1}H NMR signals were broad, which might be due to signal overlap and/or high relaxation rate. The aromatic part of the ^{1}H NMR spectra showed peaks from SDG elements (δ 6.4–6.7 ppm) as well as from other phenolic compounds, such as *p*-coumaric acid and ferulic acid moieties (δ 6.1–7.7 ppm). The ratio of SDG elements to these phenylpropanoids was larger in the whole polymer and in the 60 and 70% polymeric fractions compared to the 50% fraction, in agreement with results from the HPLC analysis of the hydrolysed fractions. The ^{1}H NMR showed that the SDG elements gave sharp signals while the other compounds gave broad signals in the aromatic regions of the spectra. Thus, both HPLC and ^{1}H NMR analyses of the whole polymer and its three fractions showed a variation in the structure of the polymer.

This study shows that flaxseed polymer contains moieties of SDG, *p*-coumaric acid glucoside, ferulic acid glucoside and other phenolic compounds, present most probably in acylated forms, since they are easily released by alkaline treatment. It is not yet known whether this flaxseed polymer is based on structural variations in one structure or whether it is based on several structures of comparable polarities. Studies to further reveal the structure(s) of the flaxseed polymer(s) are ongoing in our laboratory.

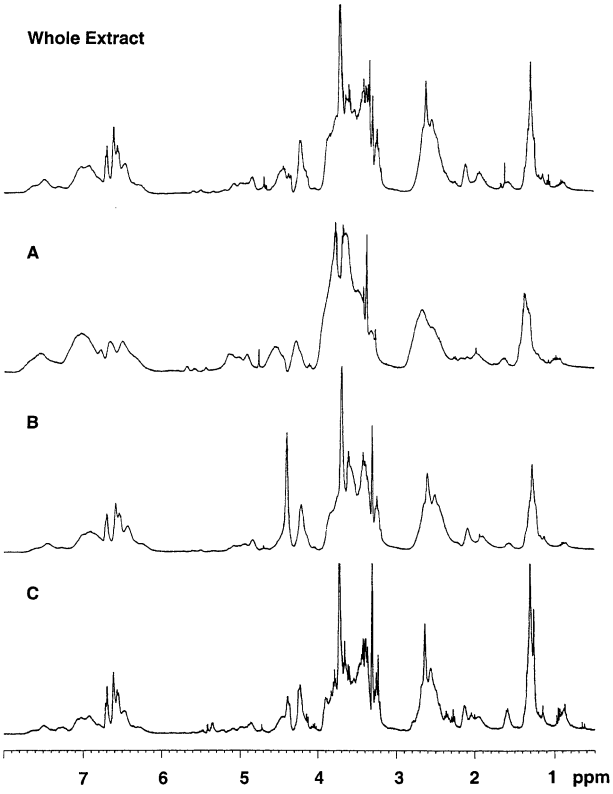

Fig. 4. ^{1}H NMR spectra of the whole polymeric extract, and the three polymeric fractions separated by solid-phase extraction: (A) 50% MeOH:H_2O, (B) 60% MeOH:H_2O, and (C) 70% MeOH:H_2O (for conditions, see Section 2).

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