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Impact of Isolation Method on the Antioxidant Activity of Rapeseed Meal Phenolics

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Rapeseed meal is the byproduct of the rapeseed deoiling process. Among oilseed plants, rapeseed contains the greatest amount of phenolic compounds. In this study, the rapeseed phenolics were isolated with aqueous methanol, aqueous ethanol, hot water, and enzymatically with ferulic acid esterase. These isolates were tested for radical scavenging and for liposome and low-density lipoprotein (LDL) model systems. The radical scavenging activities of all isolates were >60% at a concentration of 1.5 mg/mL. In the liposome model system the formation of hexanal was inhibited by all rapeseed meal isolates by >90% and the formation of conjugated diene hydroperoxides by >80% (8.4 μg/mL concentration). All rapeseed meal isolates also inhibited oxidation of LDL particles by >90% inhibition (4.2 μg/mL concentration). Isolation of rapeseed meal phenolics with either water or enzyme is a very suitable method devoid of organic solvents. Thus, rapeseed meal phenolics constitute an interesting source for food and cosmetic applications with antioxidant effect.

KEYWORDS: Rapeseed phenolics; antioxidants; enzyme-assisted extraction; oxidation model systems

INTRODUCTION

Rapeseed meal is the byproduct of the rapeseed deoiling process. It contains 40% proteins, and the amino acid content has a high nutritive value (*1*). Thus, rapeseed meal is commonly used as feed (*2*). Rapeseed meal also has a high content of fiber and several minerals such as calcium, magnesium, zinc, and copper. It contains some vitamins and other bioactive compounds such as α-tocopherol, several B vitamins, and choline, which make the meal nutritionally very valuable. The amount of residual oil in rapeseed meal depends on the processing method and varies typically between 1 and 10% (*2*−*4*). Rapeseed, especially rapeseed meal, is rich in phenolic compounds. According to Nowak et al. (*5*), among oilseed plants, rapeseed contains the greatest amount of phenolic compounds. The most significant phenolic compounds in rapeseed are sinapic acid derivatives such as sinapine [the choline ester of sinapic acid (constitutes ∼80% of the total phenolic compounds) (**Figure 1**)]. Sinapic acid in rapeseed can also exist as a glucosidic ester, glucopyranosyl sinapate (*6*). Only a small part, <16%, of sinapic acid exists as free sinapic acid (**Figure 1**) (*7*). Most of the phenolic compounds remain in the meal when the oil is pressed from the seeds (*8*). Typically, the amount of sinapic acid derivatives in rapeseed meal varies between 6390 and 18370 μg/g depending on the oilseed plant variety and the oil processing method (*7*). In crude rapeseed oil there are some phenolic compounds present, with the most abundant phenolic compound being a newly identified phenolic compound, vinylsyringol, in amounts of 245−700 μg/g (**Figure 1**) (*8, 9*). The amounts of sinapic acid and sinapine were 16 and 19 μg/g, respectively, in crude rapeseed oil. During the oil refining process, the amount of phenolic compounds decreases significantly with only up to a 25 μg/g amount of total phenolics found in commercially refined rapeseed oil (*8*).

Rapeseed phenolics are usually extracted from rapeseed with different organic solvents such as aqueous methanol (70%), methanol, or acetone (*8, 9, 11*). For compositional analysis, the extract is then hydrolyzed for releasing sinapic acid from its esters, because sinapine is not available as a commercial standard. Alkaline hydrolysis is the most often used procedure for releasing the phenolic acids, but enzymatic hydrolysis can also be employed. According to our previous study ferulic acid esterase and Ultraflo L, a β-glucanase with side activities, were as effective as sodium hydroxide in hydrolyzing rapeseed phenolic esters (*8*).

Rapeseed phenolic compounds are potent antioxidants in various environments relevant to food, cosmetic, and pharmaceutical preparations. Nowak et al. (*6*) found that rapeseed phenolic compounds, especially sinapic acid, were active in inhibiting the oxidation of emulsions. According to Wanasundara et al. (*12*) the antioxidant activity of different rapeseed phenolic fractions was lower than the activity of crude ethanolic extract due to synergism between different phenolics in a β-carotene−linoleate model system. The most active rapeseed meal phenolic fraction contained several classes of phenolic compounds including phenolic acids, flavones, and flavonols. Koski et al. (*9*) fractionated crude rapeseed oil and found the vinylsyringol-containing fraction to be the most effective

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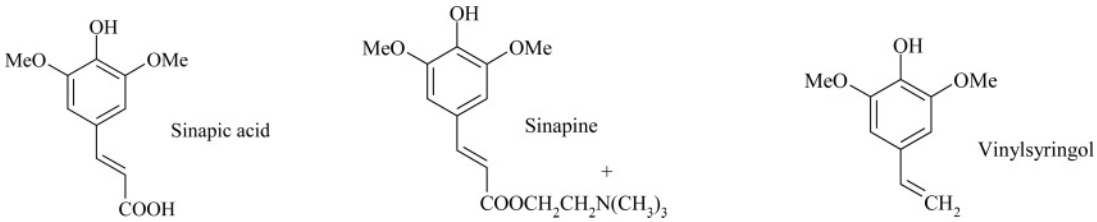

Figure 1. Structures of the main phenolics in rapeseed meal and crude rapeseed oil.

antioxidant in bulk and emulsified methyl linoleate and in lecithin−liposome systems.

The aim of this study was to compare different isolation methods for best exploiting the antioxidant properties of phenolic compounds present in rapeseed oil processing byproduct. The antioxidant activity of rapeseed meal phenolics has not previously been investigated in either liposome or low-density lipoprotein (LDL) model systems. Due to the cell membrane-resembling properties of liposomes from soybean lecithin (also used as food emulgator, E 322) and the in vitro significance of LDL oxidation as a biomarker for cardiovascular diseases (*13*, *14*), these model systems may provide useful information on the utilization of rapeseed phenolics in functional foods intended for health benefits. Although extraction with aqueous methanol is a very common procedure for isolating rapeseed phenolics, it is not suitable for food applications. Thus, isolation methods based on ethanol, water, or enzymatic extraction offer better choices for exploiting rapeseed phenolics.

MATERIALS AND METHODS

Materials. The rapeseed (*Brassica rapa*) meal (fat = 9%, not removed) used was the residue of a rapeseed deoiling process in which the oil was expelled from the seeds at elevated temperature by Mildola Ltd. Sinapic acid, pyrogallol, 1,1-diphenyl-2-picrylhydrazyl (DPPH) reagent, and catechin were from Extrasynthèse (Genay, France). All solvents were of HPLC grade and purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). A Milli-Q water purification system was used (Millipore, Bedford, MA). Ammonium dihydrogen phosphate, sodium diphosphate, disodium phosphate, cupric acetate, copper sulfate, sodium chloride, and α-tocopherol were from Merck (Darmstadt, Germany). Lecithin from soybean (containing 40% phosphatidylcholine) and LDL were from Sigma Chemical Co. (St. Louis, MO). According to HPLC analysis of phosphatidylcholine, it contained some α-, γ-, and δ-tocopherols (19, 117, and 59 μg/g, respectively). The fatty acid composition of phosphatidylcholine was similar to that of soybean oil. Ferulic acid esterase was provided by Novozymes (Bagsvaerd, Denmark).

Isolation of Phenolics. The different isolation methods tested for the extraction of rapeseed meal phenolics were extraction with solvents (methanol and ethanol), hot water, enzymatic treatment, and supercritical CO_2. All extractions were performed in triplicate.

Extraction with 70% Methanol/70% Ethanol/Water. Solvent and hot-water extraction of rapeseed phenolics was performed according to the method outlined by Cai and Arntfield (*11*). Rapeseed meal (0.2 g) and 20 mL of 70% aqueous methanol or 70% aqueous ethanol or hot water were put in a centrifuge tube that were then shaken in a water bath (75 °C) for 20 min. The clear phenolic extract was collected after centrifugation (3500 rpm, 15 min).

Enzymatic Treatment. The enzymatic treatment was carried out according to the method of Vuorela et al. (*8*). Rapeseed meal (0.2 g) and 20 mL of 0.02 M ammonium diphosphate buffer solution (pH 5.5) containing 0.1% ferulic acid esterase (calculated toward total phenolic content in rapeseed meal) were put in a shaking water bath (37 °C) for 2 h. The enzymatic reaction was stopped by boiling for 10 min. After centrifugation (3500 rpm, 20 min), the phenolic extract was collected. The ferulic acid esterase preparation was confirmed by HPLC to contain no phenolic compounds.

Extraction with Supercritical Carbon Dioxide. The supercritical carbon dioxide extraction was performed at 50 °C. The pressure used was 460 bar (CO_2 1) or 300 bar ($C0_2$ 2). The carbon dioxide flow was 0.4 mL/min. The extraction was performed by Aromtech Ltd.

Total Phenolic Content. Phenolic extract (0.2 mL) was evaporated to dryness. After that, 0.2 mL of methanol/water (1:2), 1 mL of Folin−Ciocalteu reagent (1:10), and 0.8 mL of sodium dicarbonate solution (7.5%) were added. After 30 min, the total phenolic content was measured at 765 nm by a Perkin-Elmer λ15 UV−vis spectrophotometer (Norwalk, CT) (*13*). Sinapic acid was used as standard compound.

HPLC Analysis. The HPLC analysis of rapeseed meal phenolics was performed according to the method outlined by Koski et al. (*9*). The HPLC system consisted of a 2690 separations module, a PDA 996 diode array detector, and a Millenium 2020C/S software data module. The column was Nova-Pak C18 (150 × 3.9 mm, 4 μm, Waters, Millipore, Bedford, MA) equipped with a C18 guard column. The solvent system used was as follows: solvent A, 0.02 M ammonium dihydrogen phosphate buffer (pH 2.15)/methanol (75:25); and solvent B, methanol. The gradient system was the following: 5% solvent B (0−15 min), 5−35% solvent B (15−20 min), 35−100% B (35−45 min), 100−5% B (45−50 min), 5% B (50−52 min) with an 18-min postrun period at 5% solvent B. For detection, 325 nm (for sinapic acid and sinapine) and 275 nm (for vinylsyringol) were the wavelengths recorded. The flow rate was 1 mL/min, and the temperature was 27 °C. The samples for HPLC analysis were first dried under nitrogen and then diluted with methanol/ammonium dihydrogen phosphate buffer (0.02 M, pH 2.15) (1:2). The injection volume was 100 μL. The results were expressed as sinapic acid equivalents, μg/g of SAE. Sinapine was isolated from rapeseed meal according to the method outlined by Clandinin (*16*).

Antioxidant Activity Testing. *DPPH Radical Scavenging Test.* This test was performed according to the method outlined by Kähkönen et al. (*17*). Methanolic 0.1 mM DPPH• radical solution (2.950 mL) was mixed with 0.050 mL of the phenolic extract. The concentrations of the phenolic extracts were 0.5, 1.0, and 1.5 mg/mL based on the content of total phenolics. The absorption (517 nm) was monitored every 30 s for 5 min using a Perkin-Elmer λ15 UV−vis spectrophotometer (Norwalk, CT). The results were expressed as the percentage of radicals scavenged after 4 min of reaction time. Percentage of radical scavenging activity was calculated as

$$\text{radical scavenging activity (\%)} = 100 \times (A_1 - A_2)/A_1$$

where A_1 = the initial absorbance immediately after addition of the antioxidant ($t = 0$) and A_2 = the absorbance after 4 min of reaction time.

DPPH• free radical scavenging test was performed with 0.5, 1.0, and 1.5 mg/mL phenolic concentrations and in triplicate measurements. Pyrogallol (0.5 mg/mL) was a control sample in each measurement.

LDL Model System. This test was performed according to the method outlined by Kähkönen et al. (*17*). Human LDL (Sigma) solution was diluted with 0.01 M phosphate buffer solution (pH 7.4) containing 0.15 M NaCl to 0.2 mg/mL protein concentration. The rapeseed phenolic extracts were put into headspace vials and dried under nitrogen to result in final concentrations of 0.83, 1.39, 1.94, and 4.17 μg/mL. After that, 450 μL of diluted LDL solution and 1350 μL of 0.01 M phosphate buffer (pH 7.4) were added, so that the total volume of the samples in the vials was 1.8 mL. The LDL concentration of total volume was 0.05 mg/mL. After the addition of 10 μL of 1.8 μM $CuSO_4$, the samples were sealed and stirred. Then the vials were put into a shaking water

bath (37 °C, 100 rpm). The oxidation tests were performed in triplicate. After 2 h of incubation, the formation of hexanal due to lipid oxidation was measured by static headspace gas chromatography with an automatic sampler (Perkin-Elmer HS 40XL). Vials were thermostated for 13 min at 60 °C. The gas chromatograph (Perkin-Elmer AutoSystem XL) was equipped with a capillary column (Nordibond NB-54, 25 m, 0.32 mm) and a flame ionization detector. The oven temperature was held constant at 80 °C. Identification was based on comparing the retention times of peaks with commercially available hexanal standard. The control sample contained no added antioxidants. Sinapic acid was used as a reference, and it was tested at concentrations of 5.6 and 11.2 μg/mL, which correspond to 25 and 50 μM in molarities. The inhibition of rapeseed phenolics against the formation of hexanal was calculated as

$$(A_0 - A_1)/A_0 \times 100$$

where A_0 is the area of hexanal in the control sample and A_1 is the area of hexanal in the tested sample. The inhibitions were expressed as percentages.

Liposome Model System. The liposome oxidation model system was performed according to the method outlined by Huang and Frankel (*18*). The liposomes were prepared from soybean lecithin (containing 40% phoshatidylcholine), and the concentration of phosphatidylcholine in samples was 0.8 mg/mL. Lecithin (4.2 g) was weighed into a decanter flask and suspended with deionized water (70 mL) by sonicating and stirring using a U 50 Control Ikasonic sonicator (Janke & Kunkel GmbH & Co. KG, Staufen, Germany). Tested phenolic extracts were put into Erlenmeyer flasks and dried under nitrogen. Liposome solution (4 mL) was added, and the samples were sonicated until diluted. Deionized water (26 mL) was added to the final volume (30 mL), and 30 μL of 3 μM cupric acetate was added. Finally, the samples were put into a shaking water bath (100 rpm, 37 °C) for 4 days. The oxidations were performed in triplicate. Sinapic acid was used as a reference, and it was tested at concentrations of 2.2 and 5.6 μg/mL, which correspond to 10 and 25 μM in molarities. The inhibition against liposome oxidation was calculated at day 3 by measuring the formation of oxidation products.

Measurement of Conjugated Diene Hydroperoxides. A sample of the rapeseed phenolic extract (0.1 mL) was dissolved in methanol (5 mL). The formation of conjugate dienes was measured at 234 nm by a Perkin-Elmer λ15 UV−vis spectrophotometer. The inhibition against formation of conjugated dienes was calculated as

$$(A_0 - A_1)/A_0 \times 100$$

where A_0 is the absorbance of a control sample and A_1 is the absorbance of a tested sample. The inhibitions were expressed as percentages.

Measurement of Hexanal Formation. A sample of the rapeseed phenolic extract (0.5 mL) was put into a headspace vial, and the formation of hexanal was measured by headspace gas chromatography. Liposome samples were placed in headspace vials sealed with PTFE-coated septa and aluminum caps. Liposome samples were injected with an automatic sampler (Perkin-Elmer HS 40XL). Vials were thermostated for 18 min at 80 °C. The gas chromatograph (Perkin-Elmer Autosystem XL) was equipped with a capillary column (Nordibond NB-54, 25 m, 0.32 mm) and a flame ionization detector. The oven temperature was held constant at 60 °C. Identification was based on comparison of the retention times of peaks with commercially available hexanal standard. The control sample contained no added antioxidants. The inhibition against the formation of hexanal was calculated identically as in the LDL oxidation model system.

Statistical analysis was performed using Statgraphics (STCC Inc., Rockville, MD) one-way ANOVA.

RESULTS

Isolation of Rapeseed Meal Phenolics. The total phenolic content in rapeseed meal phenolic isolates varied between 5310 and 6937 μg of sinapic acid equivalents (SAE)/g with the highest amount found in enzyme-assisted isolate and the lowest in aqueous ethanolic isolate. The main phenolics in rapeseed meal were sinapine and sinapic acid. The rapeseed meal phenolic isolates contained 17 and 3070 μg/g of sinapine and 120−1700 μg/g of sinapic acid (**Table 1**). The phenolic profile of rapeseed obtained with the use of ferulic acid esterase (FAE) was different from that with the other methods using as the main phenolic compound sinapic acid, and the content of sinapine was ~10-fold lower. Due to the enzymatic hydrolysis, nearly all sinapine had been hydrolyzed to yield free sinapic acid. However, the total amount of sinapine and sinapic acid (1870 μg/g) isolated with FAE was lower compared to the use of solvents (2260−3240 μg/g) or hot water (2020 μg/g). The use of supercritical CO_2 for the isolation of rapeseed meal phenolics was not successful. The amount of phenolics isolated with supercritical CO_2 remained low; total phenolic contents were 459 and 424 μg/g, and sinapic acid contents were 16 and 10 μg/g. The main phenolic compound in supercritical CO_2 was vinylsyringol, 250 and 250 μg/g, in the two different extraction conditions.

Table 1. Sinapine, Sinapic Acid, and Total Phenolics (Micrograms per Gram) Isolated from Rapeseed Meal Using Different Methods (Percent Inhibition, Mean ± SD)[a]

method of isolation[b]	sinapine	sinapic acid	vinyl-syringol	total phenolics
70% MeOH	3070 ± 138a	170 ± 5b		6580 ± 600ab
70% EtOH	2140 ± 50b	120 ± 3b		5310 ± 310c
H_2O	1870 ± 140c	150 ± 10b		5960 ± 710bc
FAE	170 ± 22d	1700 ± 239a		6940 ± 320a
CO_2 1	nd[c]	16	250	460
CO_2 2	nd	10	240	420

[a] Values in the same column with different letters are significantly different ($p < 0.05$). [b] Rapeseed meal phenolics isolated either with methanol (70% MeOH), ethanol (70% EtOH), hot water (H_2O), enzyme-assisted extraction with ferulic acid esterase (FAE), or two slightly different supercritical carbon dioxide techniques (CO_2 1 and CO_2 2). [c] Not determined.

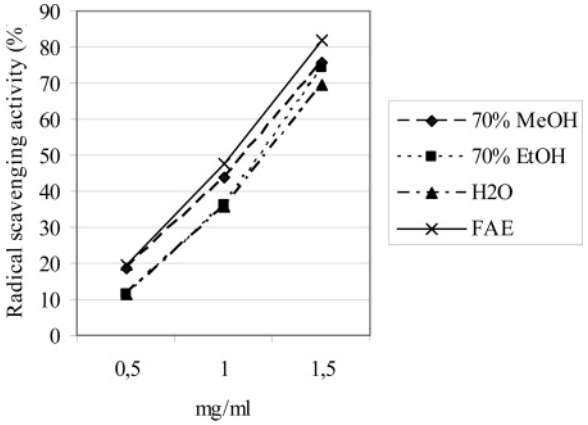

Figure 2. Radical scavenging activities of rapeseed meal phenolics isolated using different methods.

Antioxidant Activity of Rapeseed Meal Phenolics Isolated with Different Methods. *Radical Scavenging Activity.* The radical scavenging activity of rapeseed meal phenolics differed according to the isolation technique used. The rapeseed meal phenolic isolate obtained with ferulic acid esterase resulted in the best activity followed by aqueous methanol, aqueous ethanol, and water at all concentrations (0.5−1.5 mg/mL) tested (**Figure 2**). The radical scavenging was highly dependent on concentration: at a concentration of 0.5 mg/mL the activities of rapeseed meal isolates were under 20%, at 1.0 mg/mL the activities were between 27.2 and 47.6%, and at 1.5 mg/mL the activities were between 69.3 and 81.8%. There was no statistically significant

Table 2. Impact of Isolation Method of Rapeseed Phenolics on Liposome Oxidation (Percent Inhibition, Mean ± SD)[a]

	conjugated dienes			hexanal		
method[b]	1.4 μg/mL	4.2 μg/mL	8.4 μg/mL	1.4 μg/mL	4.2 μg/mL	8.4 μg/mL
70% MeOH	80.6 ± 0.94a[a]	89.9 ± 0.46a	91.9 ± 0.41a	98.0 ± 0.20a	99.3 ± 0.03a	99.3 ± 0.07a
70% EtOH	81.7 ± 0.76a	90.9 ± 0.64a	92.8 ± 0.29a	98.2 ± 0.20a	99.3 ± 0.17a	99.4 ± 0.05a
H_2O	80.7 ± 0.51a	83.0 ± 0.70a	82.7 ± 1.21b	97.4 ± 0.20a	99.0 ± 0.08a	98.4 ± 0.26b
FAE	51.7 ± 1.64b	69.3 ± 0.97b	81.9 ± 0.42b	83.7 ± 1.41b	90.3 ± 0.70b	97.6 ± 0.16c
sinapic acid	88.8[c]	89.7[c]		95.7[c]	95.5[c]	
α-tocopherol	88.5[d]			96.9[d]		
catechin	63.8[e]	83.1[e]		86.2[e]	96.4[e]	

[a] Values at the same concentration in the same column with different letters are significantly different ($p < 0.05$). [b] Method of isolation. Aqueous methanol (70% MeOH), aqueous ethanol (70% EtOH), hot water (H_2O), and enzyme-assisted extraction with ferulic acid esterase (FAE). [c] 2.2 μg/mL = 10 μM. [d] 5.6 μg/mL = 25 μM. [e] 10 and 25 μM.

difference in radical scavenging activity between aqueous methanol and enzyme-assisted isolates except at the concentration of 1.0 mg/mL, at which the enzyme-assisted isolate was better. Isolation with hot water did not significantly differ from aqueous ethanolic isolate. At concentrations of 0.5 and 1.5 mg/mL regarding radical scavenging activity, aqueous methanol did not differ from hot-water or aqueous ethanolic isolates. For comparison, the radical scavenging activity of the positive control, pyrogallol, was 92% at a concentration of 0.5 mg/mL and that of sinapic acid was 43.9% at 0.5 mg/mL, 90.8% at 1.0 mg/mL, and 95.8% at 1.5 mg/mL.

Liposome Model System. On the basis of the inhibition of formation of conjugated diene hydroperoxides and hexanal at a concentration of 8.4 μg/mL during liposome oxidation, the antioxidant effect of rapeseed meal phenolics decreased in the following order according to the method of isolation: aqueous ethanol ≈ aqueous methanol > water > enzyme-assisted extraction (**Table 2**). The antioxidant effect was significant (inhibition > 80%) also at lower concentrations, especially for phenolics extracted with solvents or with hot water. The antioxidant effect was dose-dependent only with phenolics isolated with FAE, although isolation of rapeseed meal phenolics with ferulic acid esterase generally weakened the antioxidant effect (**Table 2**). Inhibitions against the formation of conjugated dienes and hexanal of sinapic acid were 89.7 and 95.5% at a concentration of 5.6 μg/mL. α-Tocopherol inhibited 88.5% of conjugated dienes and 96.9% of hexanal formation at a concentration of 10 μM. Catechin inhibited 63.8% (10 μM) and 83.1% (25 μM) of conjugated diene formation and 86.2% (10 μM) and 96.4% (25 μM) of hexanal formation.

LDL Model System. In the LDL model system rapeseed meal phenolics inhibited the formation of hexanal at significantly high levels of 89.4−97.8% depending on the method of isolation at concentrations ranging from 1.39 to 4.17 μg/mL. At 0.83 μg/mL only enzyme-assisted extract inhibited >60% of LDL oxidation. The inhibition against the formation of hexanal of sinapic acid was 95.5% at a concentration of 5.6 μg/mL. The inhibitions of supercritical extracts were tested at concentrations of 2.2 and 5.6 μg/mL (SAE), and the inhibitions were between 32 and 97% (**Table 3**). The inhibitions of catechin were 97.7% at a concentration of 10 μM and 98.6 at a concentration of 25 μM, whereas α-tocopherol was pro-oxidative.

Table 3. Impact of Isolation Method of Rapeseed Phenolics on LDL Oxidation (Percent Inhibition, Mean ± SD)[a]

method[b]	0.83 μg/mL	1.39 μg/mL	1.94 μg/mL	4.17 μg/mL
70% MeOH	24.4 ± 5.39b[a]	96.7 ± 1.15a	90.0 ± 2.48a	94.8 ± 2.48a
70% EtOH	7.18 ± 3.52b	93.6 ± 0.33a	90.7 ± 4.04a	96.3 ± 0.33a
H_2O	2.97 ± 0.20b	95.7 ± 0.23a		93.0 ± 3.24a
FAE	61.1 ± 28.6a	96.8 ± 0.03a	89.4 ± 3.37a	97.8 ± 0.45a
CO_2 1			35.0[c]	97.0[c]
CO_2 2			32.0[c]	89.0[c]
sinapic acid			95.3[d]	95.5[d]
catechin			97.7[e]	98.6[e]
α-tocopherol			–9.0[e]	–8.8[e]

[a] Values at the same concentration in the same column with different letters are significantly different ($p < 0.05$). [b] Method of isolation. Aqueous methanol (70% MeOH), aqueous ethanol (70% EtOH), hot water (H_2O), and enzyme-assisted extraction with ferulic acid esterase. [c] 2.2 (10 μM) and 5.6 μg/mL (25 μM) sinapic acid equivalents. [d] 2.2 μg/mL (25 μM) and 5.6 μg/mL (50 μM). [e] 10 and 25 μM.

DISCUSSION

Isolation of Rapeseed Meal Phenolics. The effect of different isolation methods of rapeseed phenolics was investigated to be able to provide food grade isolates of rapeseed meal phenolics. The isolation methods selected included aqueous ethanol, hot water, enzymes, and supercritical CO_2 extraction. In our previous study we reported that ferulic acid esterase was very effective in hydrolyzing sinapic acid esters in both rapeseed meal and crude rapeseed oil (*8*). In the present study it was shown that enzymatic extraction with FAE was also a very effective procedure to extract rapeseed meal phenolics. Simultaneous release of sinapic acid may also improve the antioxidant activity as sinapic acid is a known antioxidant (*19*). Isolation of rapeseed meal phenolics using enzymatic extraction improves the analysis process, because the extraction and the hydrolysis can be performed in one step. Enzymatic isolation resulted in the highest amount of phenolics compared to the other methods of isolation, although not significantly different from that of isolation with methanol. However, there is a 2-fold difference between total phenolic content measured with Folin−Ciocalteu method and the total amount of sinapic acid and sinapic acid measured by HPLC due to methodological differences. The Folin−Ciocalteu procedure is very sensitive to all reducing hydroxyl groups such as those present not only in phenolic compounds but also in some sugars and proteins (*15*). Thus, the sum of HPLC-identified phenolic compounds such as sinapine, sinapic acid, and vinylsyringol (in the case of supercritical CO_2) provides better grounds for comparing the effect of different isolation methods. Consequently, isolation of rapeseed phenolics was significantly most efficient with 70% methanol followed by ethanol, hot water, or FAE. Moreover, there was no significant difference between isolation with 70% ethanol and isolation with hot water. Isolation of rapeseed meal phenolics with supercritical CO_2 differed from the other isolation methods, resulting in a much lower amount of phenolics, thus rendering supercritical CO_2 extraction not to be the method of choice for the isolation of rapeseed phenolics. The phenolic profile obtained with supercritical CO_2 is similar to that in post-

expelled crude rapeseed oil, in which the content of vinylsyringol formed during processing was 245−700 μg/g (*8*, *9*). Supercritical CO_2 extraction is best applied to the isolation of nonpolar compounds with the more significant rapeseed meal phenolics remaining in the solid material (*20*).

Impact of Isolation Method on the Antioxidant Activity. Rapeseed meal phenolics isolated with either solvents, ferulic acid, or hot water were quite effective in free radical scavenging and exhibited powerful antioxidant properties in inhibiting the oxidation of both LDL and liposome model systems. In addition, phenolics isolated with supercritical CO_2 also inhibited the oxidation of LDL particles.

The DPPH assay evaluates the ability of antioxidants to scavenge free radicals (*21*). Thus, the DPPH assay is a rapid screening method for the antioxidant potential of chemical substances. However, information of antioxidant activity in inhibiting lipid oxidation has to be obtained in conditions with oxidizable lipids present.

Pekkarinen et al. (*19*) investigated the radical scavenging activity of some hydroxycinnamic and benzoic acids. They found that the activity was higher in hydroxycinnamic acids with two hydroxyl groups (caffeic acid) due to electron-donating ability. In addition to one hydroxyl group, a second methoxy group in sinapic acid increased the radical scavenging activity more than hydrogen in ferulic acid. According to Kikuzaki et al. (*22*) the radical scavenging activity was higher in phenolic acids than in their ester derivatives. This finding is in accordance with the results of rapeseed meal phenolics found in this study. Rapeseed meal phenolics isolated with ferulic acid esterase resulted in a high amount of free sinapic acid that in turn contributed to better radical scavenging activity compared to the other rapeseed meal phenolics containing mostly sinapic acid esters (sinapine). According to Amarowicz et al. (*19*) the radical scavenging activity of phenolic compounds isolated from rapeseed hulls varied from 6 to 81% (1 mg/mL). However, none of the fractions contained free sinapic acid. In this study, the radical activity of sinapic acid was >90% at a concentration of 1 mg/mL.

Rapeseed meal phenolics isolated with solvents resulted in the most powerful antioxidant effect compared to the slightly weaker effect of enzyme-assisted extraction in inhibiting oxidation of liposomes. The significant difference between enzymatic extracts and other extracts was that the enzymatic extract contained mostly free sinapic acid instead of sinapic acid esters. Although sinapic acid was very effective in concentrations of 2.2 μg/mL (10 μM) and 5.6 μg/mL (25 μM), the higher sinapic acid content in the enzymatically obtained phenolic extract did not result in superior antioxidant activity toward the oxidation of liposomes. Kikuzaki et al. (*22*) compared the antioxidant activity of alkyl gallates and alkyl ferulates in a liposome model system. They found that the effective antioxidant activity requires the optimum chain length. According to them, the higher polarity of a phenoxyl group in alkyl gallates than in alkyl ferulates might require a somewhat longer alkyl chain in the alcohol part. For effective antioxidant activity it is important that the antioxidants locate near the membrane surface to act as antioxidants. In this study it was found that the antioxidant activity was stronger in those rapeseed extracts which contained sinapine, the choline ester of sinapic acid. Sinapine is a more polar compound than sinapic acid due to the positive charge in the choline part. Therefore, it can be assumed that this polar molecule can move easily to the lipid bilayer due to the lower solubility to phosphatidylcholine in liposomes. According to Castelli et al. (*23*) liposomes are a suitable model for studying the membrane structure and properties due to their structural similarity to the lipid martix of cell membranes. This allows us to speculate about the in vivo bioactivity of the compounds investigated.

The oxidation of LDL is believed to be very essential in atherogenesis, which is one of the main causes of coronary heart disease (*24*, *25*). It has been shown in many studies that dietary antioxidants can protect LDL from oxidation. In this study it was shown that all rapeseed meal isolates were very effective in inhibiting LDL oxidation. At higher concentrations there was no difference in the antioxidant activity toward LDL oxidation between the rapeseed meal phenolics isolated with different methods. At a lower concentration of 0.83 μg/mL isolation with FAE resulted in significantly better antioxidant activity.

The effect of hydroxycinnamic acids in inhibiting LDL oxidation has been investigated earlier. Meyer et al. (*24*) isolated hydroxycinnamic acids from fruits and found that the highest activity (86−97% at a concentration of 5 μM) was due to hydroxycinnamic acids with two hydroxyl groups. They found that the activity was highly related to hydroxylation and methylation. The 3-methoxy group in ferulic acid enchanced the antioxidant activity compared to the decreased antioxidant activity due to hydrogen in *p*-coumaric acid. The methoxy group with a hydroxyl group delocalizes **t**he molecule, affecting antioxidant activity. Chen and Ho (*26*) and Nardini et al. (*27*) found also that the most active antioxidants of hydroxycinnamic acids were caffeic acid with two hydroxyl groups. Sinapic acid was not among the tested compounds. Andreasen et al. (*28*) found caffeic acid with two hydroxyl groups to be more effective than sinapic acid, but sinapic acid was more effective than ferulic acid or *p*-coumaric acid. Thus, the amount of free sinapic acid in the enzymatic isolate of rapeseed phenolics may in part explain the antioxidant activity at lowest concentration.

In conclusion, the isolation of rapeseed meal phenolics with either aqueous ethanol, hot water, or ferulic acid esterase results in food grade ingredients with powerful antioxidant activity comparable to or even better than those of sinapic acid, catechin, and α-tocopherol. These phenolic ingredients may prove to be beneficial with regard to the development of health beneficial products such as foods, feeds, and cosmetic and pharmaceutical preparations.

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