Isolation and Identification of an Antioxidative Component in Canola Meal

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Canola meal was extracted with 95% ethanol at 80 °C. The dried crude extract was dissolved in methanol and fractionated on a Sephadex LH-20 column. Seven major fractions were isolated according to UV absorption and contents of phenolics and sugars. Antioxidant activity of these fractions was evaluated in a β-carotene/linoleate model system. Fraction IV showed the best antioxidative effect by exhibiting the highest preventive activity against the bleaching of β-carotene. This fraction was further separated by thin-layer chromatography. Four major antioxidative bands were present; the band with R_f value of 0.50 was found to be most effective in preventing oxidation of β-carotene. This band was isolated and characterized by UV, MS, IR, ^{1}H NMR, and ^{13}C{^{1}H} NMR techniques. The active compound was identified as 1-*O*-β-D-glucopyranosyl sinapate.

INTRODUCTION

Antioxidants are major ingredients that protect the quality of oils by retarding oxidation. Currently BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), PG (propyl gallate), and TBHQ (*tert*-butylhydroquinone) are used as antioxidants in lipid-containing foods (Sherwin, 1990). However, there has been growing concern over their possible activity as promoters of carcinogenesis (Ito *et al.*, 1986) and a general desire to replace synthetic food additives with natural alternatives (Howell, 1986). However, tocopherols and ascorbic acid and their derivatives that are used as alternatives to BHA and BHT are much less effective antioxidants. Therefore, extraction, characterization, and utilization of natural antioxidants is desired. Such new antioxidants may also serve as potent candidates in combatting carcinogenesis and the aging process (Namiki, 1990).

Sources of natural antioxidants are primarily plant phenolics that may occur in all parts of the plant. They can be found in fruits, vegetables, nuts, seeds, leaves, flours, roots, and barks (Pratt and Hudson, 1990). The antioxidant properties of certain herbs and spices have been known for long time and have been the subject of many scientific studies (Chipault *et al.*, 1952). The extract from rosemary plant has been found to be superior in retarding the development of rancidity in lipid-containing foods (Wu *et al.*, 1982). Other antioxidants include isoflavone glycosides, phenolic acids, amino acids, and tocopherols in soybean (Hayes *et al.*, 1977), dihydroxyquercetin and taxifolin in peanut (Pratt and Miller, 1984), quercetin and rutin in cottonseed (Whittern *et al.*, 1984), sesamolinol, sesamol, and sesamolin in sesame seeds (Fukuda *et al.*, 1985), and caffeic and chlorogenic acids in chia seeds (Taga *et al.*, 1984).

Canola meal has been reported to contain 1–2% phenolic compounds (Shahidi and Naczk, 1992). These compounds include phenolic acids (Kozlowska *et al.*, 1983; Zadernowski, 1987), flavonoids (Zadernowski *et al.*, 1991), and condensed tannins (Shahidi and Naczk, 1989). However, the presence of high levels of phenolic compounds in canola meal is undesirable due to adverse effects on the nutritional and organoleptic properties of the meal (Clandinin and Robblee, 1981). Therefore, removal of phenolic compounds from canola meal and their possible use as natural antioxidants to retard lipid oxidation would present new opportunities for the canola industry.

The antioxidant activity of crude ethanolic extract of canola meal on canola oil has been reported from our laboratories (Wanasundara, 1993). The activity of such extracts was equivalent to that of TBHQ and stronger than that of BHA, BHT, and BHA/BHT/monoglyceride citrate (MGC). This paper reports results of our studies on the isolation, fractionation, and structural elucidation of compounds responsible for antioxidative activity of ethanolic extracts of canola meal.

MATERIALS AND METHODS

Preparation of Canola Extract. Canola seeds were first ground in a Moulinex coffee grinder, defatted with hexane using a Soxhlet apparatus, and then air-dried overnight. Defatted canola meal (6.0 g) was extracted with 100 mL of 95% ethanol for 20 min at 80 °C. The extraction was repeated twice, and residual meal was separated by centrifugation (10 min at 5000*g*). Ethanolic extracts were combined and evaporated to dryness under vacuum at 40 °C. This procedure was repeated to obtain a sufficient amount of canola extract. The dried extracts so obtained from several 6.0-g batches of canola meal were transferred into airtight glass vials and stored at −20 °C until used.

Sephadex Column Chromatography. The crude extract was fractionated by Sephadex LH-20 column (1.5-cm diameter and 77-cm height, particle size 25–100 μm, Pharmacia, Uppsala, Sweden) chromatography. A 0.5-g sample of dried canola extract was dissolved in 3 mL of methanol and was introduced to the top of the column. The same solvent was used for elution. Fractions (8 mL) were collected using an LKB Bromma 2112 Redirac fraction collector (Pharmacia), and their absorbance in methanol was measured at 280 nm. Absorbance values at 490 and 725 nm were also measured after color development for sugars (Dubois *et al.*, 1956) and phenols (Shahidi and Naczk, 1989), respectively. Eluates were then pooled into seven major fractions, solvent was removed, and the elvates were weighed. Content of total phenolic compounds in each fraction was determined using Folin–Denis reagent (Swain and Hillis, 1959). *trans*-Sinapic acid was used

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as a standard in this work. UV spectra were recorded using a Hewlett-Packard 8452A diode array spectrophotometer.

Evaluation of Antioxidant Activity. The antioxidant activity of the isolated fractions was evaluated using a β-carotene/linoleate model system (Miller, 1971) with modifications. A solution of β-carotene (Sigma Chemical Co., St. Louis, MO) was prepared by dissolving 2.0 mg of β-carotene in 10 mL of chloroform. One milliliter of this solution was then pipetted into a round-bottom flask. After chloroform was removed under vacuum, using a rotary evaporator at 40 °C, 20 mg of purified linoleic acid, 200 mg of Tween 40 emulsifier (Aldrich Chemical Co., Milwaukee, WI), and 50 mL of aerated distilled water were added to the flask with vigorous shaking. Aliquots (5 mL) of this prepared emulsion were transferred into a series of tubes containing 2 mg of each fraction (fractions I–VII) or 2 mg of BHA for comparative purposes. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm. Subsequent absorbance readings were recorded at 15-min intervals by keeping the samples in a water bath at 50 °C until the visual color of β-carotene in the control sample had disappeared (about 120 min).

Of the seven fractions separated, fraction IV, showed the highest antioxidative activity and was used for further investigation. Fraction IV was loaded onto thin-layer chromatographic (TLC) plates (silica gel; 60-Å mean pore diameter, 2–25-μm mean particle size, 250-μm thick ɜss; Sigma). Chromatograms were developed in a glass chamber 22 × 22 × 10 cm (Fisher Scientific Co., Toronto, ON) using chloroform/methanol/water (65:35:10 v/v/v) as the mobile phase (Amarowicz *et al.*, 1992). The antioxidant activity of separated bands was determined according to the procedure described by Whittern *et al.* (1984).

To obtain sufficient quantities of the compound with highest antioxidative activity, fraction IV was subjected to preparative TLC separation. The band with R_f value corresponding to that of the highest antioxidative compound was scraped off and extracted with spectral grade methanol. The mixture was centrifuged (3 min at 5000*g*) and the supernatant evaporated to dryness under vacuum at 40 °C. The dried residue was used for further analysis.

Acid Hydrolysis of the Most Active Antioxidative Component. Five milligrams of the most active antioxidative component, isolated as described above, was hydrolyzed at 100 °C for 1 h using 1.0 mL of a concentrated HCl/water/ethanol (6:3:9 v/v/v) mixture (Ireland and Dziedzic, 1986). After 1 h, the sample was cooled and diluted with water (1.0 mL), and the hydrophobic fraction of the molecule was extracted with diethyl ether, which was subsequently removed by evaporation.

The aqueous layer containing the hydrophilic fraction (sugar) was applied to a TLC plate (the TLC plate was enhanced by impregnating with 0.02 M sodium acetate) along with standard monosacchrides (Sigma). The TLC plate was developed using chloroform/methanol (60:40 v/v) as the mobile phase (Pifferi, 1965). After drying, bands were located and identified by spraying with aniline/diphenylamine/phosphoric acid (Pifferi, 1965).

The ether layer containing the hydrophobic fraction was applied to a TLC plate along with standard phenolic acids (Sigma). The TLC plate was developed using diethyl ether/petroleum ether/acetic acid (80:20:1 v/v/v) as the mobile phase (Zadernowski, 1987). After drying, bands were located and identified by spraying with ferric chloride/potassium ferricyanide (Barton *et al.*, 1952).

Instrumental Analysis of the Most Active Antioxidative Component. *UV Spectrophotometry.* The UV absorption spectrum of the most active antioxidative component in methanol after purification was recorded. A Hewlett-Packard 8452A diode array spectrophotometer was used.

Mass Spectrometry. All mass spectra were recorded using an electron ionization (EI) mode at 70 eV with a VG7070 HS Micromass double-focusing mass spectrometer. The source was at 200 °C, probe at 100–300 °C, and scanning at 20–25 °C.

IR Spectrometry. IR spectral data were obtained using a Mattson Polaris Fourier transform infrared spectrophotometer.

Nuclear Magnetic Resonance (NMR) Spectrometry. NMR spectra were recorded on a General Electronic 300-NB spectrometer. ^1H (at 300 MHz) and $^{13}C\{^1H\}$ (at 75.5 MHz) NMR data were collected at room temperature in deuterated methanol.

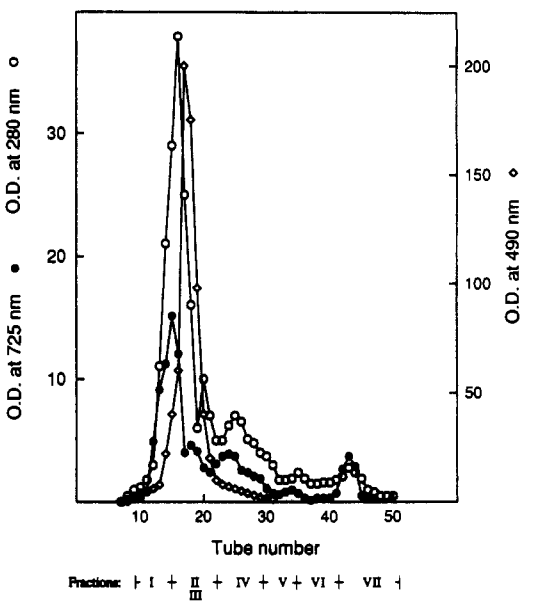

Figure 1. Eluates following Sephadex LH-20 column chromatography: UV absorbance of phenolics (280 nm), phenolics (725 nm), and sugars (490 nm) following color development.

Table 1. Percentage Recovery of Canola Extracts and Their Total Phenolic Content[a]

fraction no.	rel content, % of total	total phenolic content (mg/g) as sinapic acid equiv
I	32.3	68.0
II	10.2	22.0
III	29.9	23.0
IV	14.6	34.0
V	4.4	194.0
VI	3.5	119.0
VII	5.1	96.0

[a] Separated on Sephadex LH-20 column.

Chemical shifts were reported relative to tetramethylsilane as internal standard. In addition, attached proton test (APT) and correlation spectroscopy (COSY) experiments were performed for further elucidation of the chemical structure.

RESULTS AND DISCUSSION

The UV absorption intensity of isolated fractions at 280 nm and the content of their phenolics (absorbance at 725 nm) and sugars (absorbance at 490 nm) following Sephadex LH-20 column chromatographic separation are presented in Figure 1. Five peaks were clearly defined according to the absorbance reading at 280 nm as well as the phenolic content of samples. A large peak was observed for sugars, perhaps originating from glucosinolates (Shahidi and Gabon, 1989) and soluble sugars present in canola extracts as reported by Amarowicz *et al.* (1992). On the basis of these data, samples were separated into seven major fractions (I–VII). The Sephadex LH-20 used for fractionation of canola extracts provides an efficient medium for separation of plant phenolics (Amarowicz *et al.*, 1992). The relative proportion and the content of phenolics, as sinapic acid equivalents, in each of the seven fractions are given in Table 1. Although fractions V and VI contributed only 4.4 and 3.5%, respectively, to the total weight of extracts, they were quite rich in phenolic compounds.

The antioxidant activity of each of the seven isolated fractions and that of BHA is presented in Figure 2. Fraction IV, which contributed 14.6% to the total weight of the crude extracts, exhibited a strong antioxidative effect

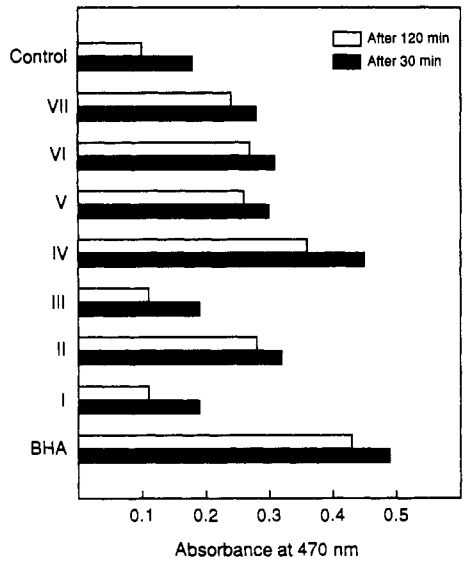

Figure 2. Antioxidant activity of individual fractions (I–VII) separated on Sephadex LH-20 column.

Table 2. UV Spectral Data of Different Fractions of Canola Extracts[a]

fraction no.	λ_{max} (nm)	λ(sh) (nm)
I	328	
II	270, 328	
III	270, 328	
IV	280, 328	276
V	328	
VI	328	
VII	280	318

[a] Separated on Sephadex LH-20 column.

by performing best preventive activity against bleaching of β-carotene. Fractions II, V, and VI showed comparatively good antioxidative effects during the first 30 min. The activity of fractions I and III was similar to that of the control. Fraction IV, which exhibited the best antioxidative activity, contained only 34.0 mg/g of phenolic compounds. However, other fractions contained comparatively higher amounts of phenolics (Table 1). This indicates that the amount of phenolic compounds is not the only factor in the consideration of antioxidative activity and that their molecular structures play an important role in their antioxidant activity (Zadernowski *et al.*, 1991). Although crude canola extract exhibited a higher antioxidative activity than BHA in canola oil (Wanasundara, 1993), all isolated fractions showed a lower antioxidant activity than BHA in the β-carotene/linoleate model system. Possible synergism of phenolics with one another or with other components present in the crude extract may be responsible for this observation.

UV spectral data of fractions I–VII (Table 2) indicated a similar absorption maximum at 328 nm, but for fraction VII did not. Fractions II–IV showed an additional absorption maximum in the range 270–285 nm. Fractions IV and VII had shoulders at 276 and 318 nm, respectively. The peak maxima of fractions I–VI at 328 nm may be due the presence of sinapic acid in the extract as reported by Kozlowska *et al.* (1983) and Naczk *et al.* (1992). The absorption maxima at 270–280 nm of fractions II–IV and VII may be due to the presence of small amounts of either *p*-hydroxybenzoic acid or flavones/flavonols in the extracts. Mabry *et al.* (1970) have reported that flavones and flavonols produced two major absorption peaks in the 240–400-nm range in methanol, one peak in the range 240–280

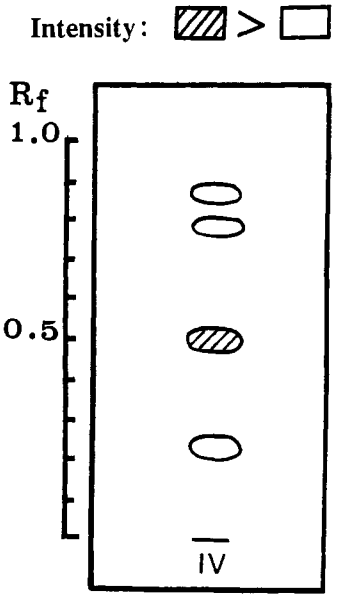

Figure 3. TLC chromatogram of fraction IV after β-carotene spray.

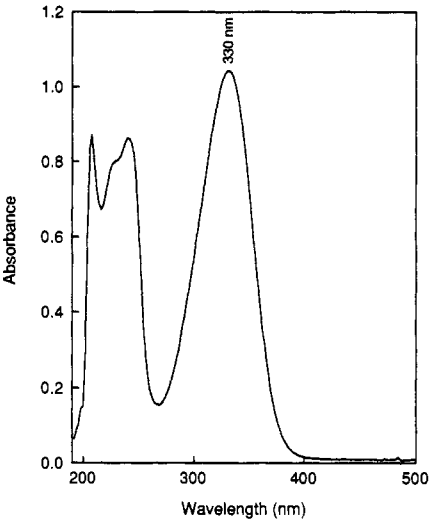

Figure 4. UV spectrum of the most active antioxidative component isolated from canola meal.

nm, and another one in the range 300–380 nm. Therefore, fraction IV, which had the highest antioxidative activity, may contain several kinds of antioxidative compounds possessing phenolic nature. Fraction IV with highest antioxidative activity was further separated to obtain the most active antioxidative component.

Figure 3 shows the antioxidative activity of individual components of fraction IV separated on a TLC plate, using a β-carotene/linoleate spray system. This fraction contained four major antioxidative compounds with R_f values of 0.20, 0.50, 0.80, and 0.90. Among the four major bands, the band with an R_f value of 0.50 was most effective in preventing oxidation of β-carotene. This band was isolated and used for structural elucidation.

The UV spectrum of the component with the most antioxidative effect showed an absorption maximum at 330 nm (Figure 4). This absorption band may be due to the presence of sinapic acid or its derivatives as reported by Kozlowska *et al.* (1983) and Naczk *et al.* (1992).

The mass spectral analyses showed two major fragment ions with *m/z* of 180 and 206. The *m/z* 180 fragment suggested the presence of a six-carbon sugar moiety in the molecule. The *m/z* 180 fragment may be produced from

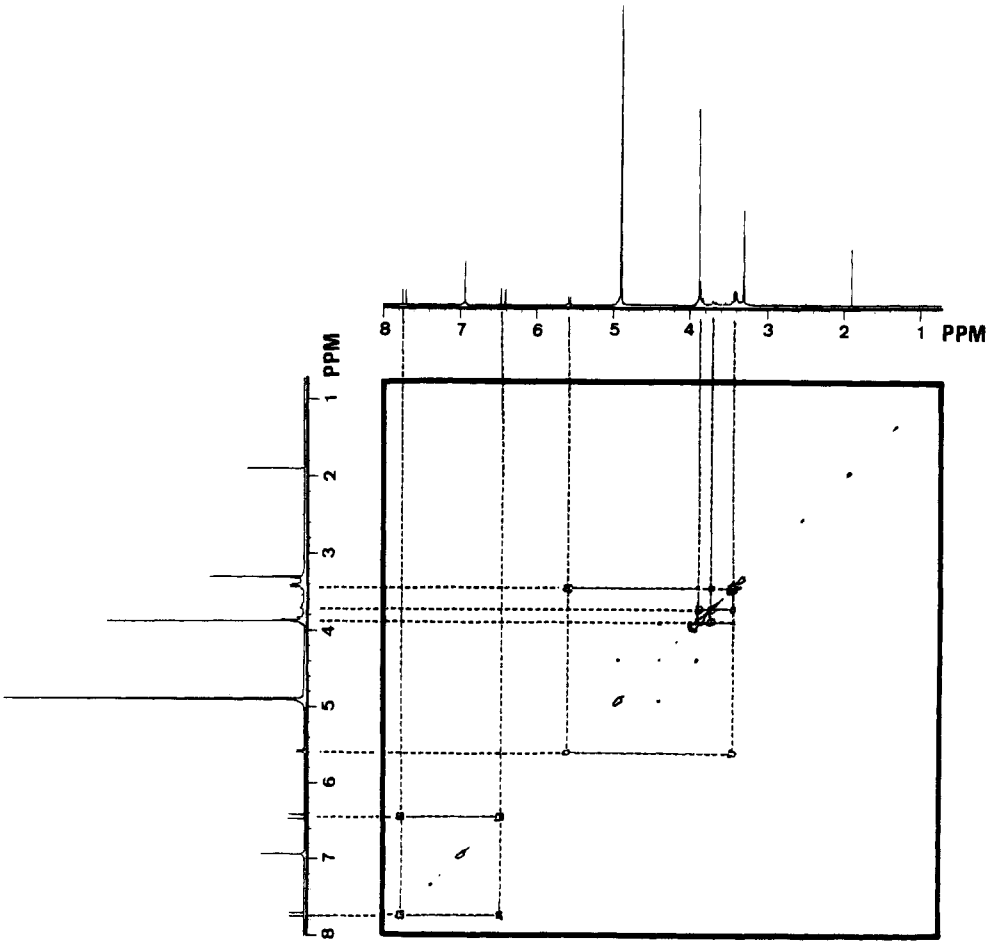

Figure 5. COSY spectrum of the most active antioxidative component isolated from canola meal.

Table 3. ^{1}H NMR Assignment of the Most Active Antioxidative Component[a]

δ	multiplicity	J (Hz)	integration	assignment
7.72	doublet	J_{ab} = 16	1H	H_b
6.93	singlet		2H	H_c
6.43	doublet	J_{ab} = 16	1H	H_a
5.57	doublet	J = 7.8	1H	H_d
3.87	singlet		6H	OCH_3
3.85	doublet–doublet	J_{ef} = 12, J_{eg} = 1.8	1H	H_e
3.68	doublet–doublet	J_{ef} = 12, J_{fg} = 4.8	1H	H_f
3.41	multiplet			other CH's from sugar and phenolic acid

[a] Assignment of coupling interactions (J) was confirmed by a COSY experiment. Subscript designations are the same as in Figure 6.

the fragmented sugar moiety with a migrated hydrogen atom. The other fragment represents sinapic acid. The total molecular mass of the suggested compound was 386 and corresponded with these and other fragment ions present in the mass spectrum of the sample, but the molecular ion itself did not appear. The IR spectrum indicated the presence of hydroxyl groups (3350 cm^{-1}), an aromatic ring (1608, 1518 cm^{-1}), and an ester bond (1769 cm^{-1}).

The ^{1}H NMR spectrum of the compound in deuterated methanol displayed eight resonances (Table 3). Some of the proton nuclei showed coupling interactions as given by a COSY experiment (Figure 5). Two doublet of doublets centered at δ 3.85 and 3.68, with a relative integration equivalent to one proton each, were diagnostic of the H_e and H_f protons of the sugar moiety, respectively. Two doublets at δ 7.72 and 6.43, with a relative integration equivalent to one proton each, were assigned to the H_b and H_a olefinic protons of the phenolic acid moiety, respectively. The assignment of the resonance due to these olefinic protons is based on the chemical shifts predicted by using the additivity rule (Pretsch *et al.*, 1989). A singlet at δ 6.93 with a relative integration equivalent to two protons was assigned to the H_c protons of the phenolic ring. The relative integration equivalent to six protons was observed for a singlet at δ 3.87, which is diagnostic of two OCH_3 groups attached to the phenolic ring. The multiplet at δ 3.41 represented all other protons of the sugar and phenolic acid moieties. An ester linkage in the molecule may be assigned on the basis of the chemical shift of H_d of the sugar moiety at δ 5.57.

The $^{13}C\{^1H\}$ NMR spectral data in deuterated methanol revealed 13 resonances from the proposed 17-carbon adduct, suggesting considerable symmetry in the molecule. A summary of the $^{13}C\{^1H\}$ NMR assignments is shown in Table 4. Assignment for the ^{13}C NMR spectrum was aided by APT data which showed the presence of three or four ipso carbons (at δ 126.4, 149.5, and 167.6), one methylene carbon (at δ 62.3), and one or two methyl carbons (at δ 56.8). The vinyl (at δ 6.43 and 7.72) and other methine protons of the phenolic acid and sugar moieties were correlated with their corresponding ^{13}C resonances. The chemical shifts of all carbon atoms of the sugar moiety were in agreement with the standard values of β-D-glucopyranose (Pretsch *et al.*, 1989). Thin-layer chromatography of the compound upon hydrolysis also indicated the presence of β-D-glucopyranose in aqueous fraction and sinapic acid in ether fraction using a set of monosaccharides and phenolic acid standards. Therefore,

Table 4. $^{13}C\{^1H\}$ NMR Assignment of the Most Active Antioxidative Component[a]

δ	assignment	δ	assignment
167.6	COO	95.8	C-1′
149.5	C-3,5	78.8	C-2′
148.4	C-7	78.0	C-3′
126.4	C-4	74.1	C-4′
115.2	C-7	71.1	C-5′
107.0	C-2,6	62.3	C-6′
		56.8	CH_3O

[a] Signal from C-1 was obscured in the noise even after overnight scanning. Carbon designation numbers are given in Figure 6.

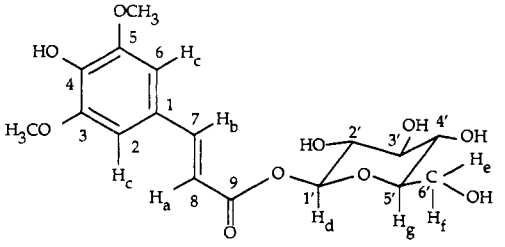

Figure 6. Structure of the most active antioxidative component of canola meal, 1-*O*-β-D-glucopyranosyl sinapate.

on the basis of UV, MS, and IR as well as ^1H, COSY, and $^{13}C\{^1H\}$ NMR spectral data and hydrolysis products, the most active antioxidative compound isolated from canola extract was identified as 1-*O*-β-D-glucopyranosyl sinapate (1-*O*-β-D-glucopyranosyl 3,5-dimethoxy-4-hydroxycinnamate) (Figure 6).

Phenolic acids of canola and rapeseed include sinapic, *p*-hydroxybenzoic, vanillic, gentisic, procatechuic, syringic, *p*-coumaric, and ferulic acids. However, sinapic acid is the major phenolic acid, representing over 73% of free phenolic acids and about 99% of phenolic acids released from esters and glycosides (Kozlowska *et al.*, 1983). The antioxidant activity of canola sinapic acid has recently been reported by Nowak *et al.* (1992). Sinapic acid is a derivative of cinnamic acid, and the presence of a –CH=CH—COOH group in the molecule ensures better antioxidant activity of the molecule in comparison with benzoic acid derivatives possessing only a –COOH group (Cuvelier *et al.*, 1992). In fact, sinapic, ferulic, and *p*-coumaric acids, all cinnamic acid derivatives, were found to be more active than benzoic acid derivatives such as procatechuic, syringic, vanillic, and *p*-hydroxybenzoic acids. This may be due to participation of the double bond (–HC=CH–) in stabilizing the aryloxy radical by resonance. Several authors (Cort, 1974; Pokorny, 1987) have shown that the antioxidant efficiency of monophenolic compounds was increased substantially by substitution of electron-donor alkyl or methoxy groups which stabilized the aryloxyl radical. In the molecular structure of sinapic acid two methoxy groups are substituted at the ortho positions relative to the hydroxyl group. Therefore, a greater antioxidant activity is expected for this molecule. Esterification of the acid group with another bulky compound may further enhance its activity.

CONCLUSION

The ethanolic extract of canola meal exhibited antioxidant activity in a β-carotene/linoleate model system. The extract contained different classes of phenolics including phenolic acids and flavonoids with varying antioxidative strengths. Separation of the crude extract afforded seven fractions; the antioxidant activity of each fraction was somewhat less than that of the original extract. Fraction IV, with the highest antioxidative activity, contained several phenolic compounds. Thin-layer chromatographic separation of this fraction allowed isolation of the most active antioxidative component of canola meal, which was identified as 1-*O*-β-D-glucopyranosyl sinapate.

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