A Rapid High-Performance Liquid Chromatographic Method for the Determination of Sinapine and Sinapic Acid in Canola Seed and Meal

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ABSTRACT: A high-performance liquid chromatographic (HPLC) method has been developed to separate sinapine and sinapic acid from other phenolics in canola seed and meal in a single run. The separation was achieved with a reverse-phase C18 column. Owing to the higher recovery of phenolics and ease of use, refluxing with 100% methanol for 20 min was selected as the extraction method for HPLC analysis and determination of total phenolics using Folin-Ciocalteu reagent. A 10-min isocratic/linear/concave gradient and a 15-min isocratic/linear gradient were selected as the best gradients for the separation of these phenolic compounds. Peak identities for sinapine and sinapic acid were verified with ion exchange separation followed by HPLC analysis. The method was calibrated using sinapine bisulfate and sinapic acid standards; correlation coefficients (R^2) for the calibration curves were 0.997 and 0.999 for sinapine bisulfate and sinapic acid, respectively. The extinction coefficient of sinapine was determined to be 1.16 times that of sinapic acid at the detector wavelength (330 nm). Applying this method to routine canola phenolic analyses can greatly reduce the cost by simplifying the procedures and reducing the time required for each determination.

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KEY WORDS: Canola, high-performance liquid chromatography (HPLC), phenolic determination, sinapic acid, sinapine.

Sinapine is a major phenolic compound in canola and rapeseed (1,2). Although sinapine may have physiological functions during germination and maturation stages of canola and rapeseed (3,4), sinapine and related phenolic compounds are responsible for the tainting of brown-shelled eggs (5–7) when the meal is used as a poultry feed and may also contribute to the dark color of the meal (8–10). The presence of sinapine and the related phenolic compounds such as sinapic acid, the hydrolyzed product of sinapine, has been a concern for oilseed breeders and processors (11,12). Rapid and sensitive methods for determining sinapine and related phenolics will facilitate canola/rapeseed breeding and processing. Thin-layer chromatography (13), which has been used extensively to separate phenolics, is difficult to quantitate. Colorimetric methods (1,14), when used to determine individual phenolics, require specific reagents for color development. Furthermore, it is difficult to avoid the interference from other compounds in these systems. Some colorimetric methods and ultraviolet (UV) spectrophotometric methods require purification procedures (15,16). Gas chromatography (2,17) requires the hydrolysis of phenolic esters and the derivatization of the resulting phenolic acids before determination. Therefore, it is an indirect method and not directly applicable to nonvolatile phenolic esters. High-performance liquid chromatography (HPLC) is a rapid and sensitive method for qualitative and quantitative analysis of plant phenolics (18).

Several HLPC methods for the determination of phenolic compounds have been developed. Clausen *et al.* (19) used a reverse-phase Nucleosil 5 C8 column and a Nucleosil 5 C18 column with a 30-min linear gradient composed of acetonitrile and a phosphate buffer to separate a group of 22 standard aromatic choline esters. Hagerman and Nicholson (20) used a Lichrosorb C8 column for a total of 45 min with two isocratic elutions of solvents composed of methanol and a sodium acetate buffer to separate hydrolyzed phenolic acids from plant extracts. Separations were excellent in both cases. These two methods, however, were tested only with pure anionic or cationic phenolics rather than plant extracts. With a Bondapak C18 column, Lattanzio (21) used a combination of isocratic and linear gradient elution to separate about 30 standard phenolic acids and flavonoids, and used two concave gradients to separate about 14 standard flavonoids in 50 min. However, only hydrolyzed phenolic acids of eggplant extracts were tested using simple phenolic acids as standards. Bjerg *et al.* (22) used a series of ion exchange columns to separate the phenolics into neutral, anionic, and cationic groups prior to HPLC analysis. Bouchereau *et al.* (3,23) used the same technique to separate phenolics into differently charged groups and then determined each group separately with a Spherisorb ODS 2 column. Although the HPLC separation for the anionic and the cationic fractions required approximately 25 and 30 min, respectively, long hours were involved in concentration and purification during sample preparation.

Most of the previous methods either involved complicated purification steps or were used only for the separation of standards and hydrolyzed phenolic acids, which would not be applicable to many instances where the intact phenolics are the interest of the analysis. New techniques with simpler sample

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preparation procedures are required for intact phenolic analysis, especially when a large number of samples are to be analyzed. Mailer *et al.* (24) used an HPLC method to qualitatively characterize the ethanol extracts from canola flours as a means of cultivar identification. However, no quantitative determinations were made. The present method took advantage of the fact that the retention time of the individual phenolic compounds in a system could be properly adjusted with a change in elution solvent composition. Since the sinapate anion and sinapine carry opposite charges and have very different hydrophobicities, they are difficult to separate with reasonable retention times in the same solvent system. It was demonstrated with this study that it was possible to separate the anionic and cationic phenolics in a single run with the same solvent system by adjusting the solvent composition. The current method separated the anionic and the cationic phenolics in either a 10- or a 15-min run with no purification steps. Therefore, it greatly increased the analytical efficiency and reduced cost. In addition, analytical results for the two phenolics with opposite charges were obtained from the same chromatogram, thus making them directly comparable.

EXPERIMENTAL PROCEDURES

Sources of materials. Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sinapine (3,5-dimethoxy-4-hydroxycinnamoyl choline) was isolated from *Sinapis alba* certified seed from Tilney mustard crop as sinapine bisulfate according to the method outlined by Clandinin (25). This sinapine bisulfate was used in place of sinapine as a standard for HPLC calibrations as no pure sinapine was available. Parkland canola seed, a variety of *Brassica campestris*, was provided by Canbra Foods (Lethbridge, Alberta, Canada). Canola meal was provided by CanAmera Foods (Fort Saskatchewan, Alberta, Canada). Canola protein isolate was prepared according to a protein micellar mass (PMM) procedure outlined by Murray *et al.* (26), using the canola meal as raw material. CM-Sephadex C-25 was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Ecteola Cellulose, capacity 0.31 meq/g, and Folin-Ciocalteu's reagent were purchased from Sigma Chemical Company (St. Louis, MO). The methanol used for canola phenolic extraction and HPLC analysis was HPLC-grade. All other chemicals were ACS-grade and were purchased from Fisher Scientific Co. (Nepean, Ontario, Canada). Deionized water was used throughout the research.

Methanol extraction of canola flour and meal. The canola flour was prepared by grinding the canola seed in a Scientific Industrial Inc. grinder (Bohemia, NY) for 1 min, extracting the ground material with hexane for 16 h with a Sohxlet apparatus, and regrinding the defatted flour for 1 min. Canola meal was ground for 1 min in the grinder. The water contents of the flour and the meal were determined to be 6.0 and 7.2%, respectively. Fifty milligrams canola flour or meal was weighed into a 20-mL capped test tube for the first three extraction conditions or a 250-mL distilling vessel connected with a distilling/condensing system for the last extraction condition. Extraction was conducted using a solvent-to-meal ratio of 100:1 using four conditions as follows: (i) 100% methanol, 50°C, 10 min; (ii) 100% methanol, 75°C, 20 min, (iii) 70% methanol, 75°C, 20 min, and (iv) 100% methanol refluxing for 20 min. Except for the reflux system, temperatures were maintained using a Haake C Water Circulator (Karlsruhe, Germany). After each extraction, the mixture was cooled to room temperature before the cap was removed. The liquid layer was then decanted and the volume measured to check for vapor loss. Under extraction conditions used in this study, there was no detectable vapor loss. The solid layer was discarded. The liquid phase was filtered through a 0.45-μm filter with a 3-mL syringe. This filtered liquid was used for both the HPLC analysis and total phenolic determination using the Folin-Ciocalteu's reagent method. The concentration of the liquid phase was assumed to be the same throughout the mixture, and the volume of the solvent originally added to the sample was used in calculations (15)

HPLC analysis. Chromatographic equipment consisted of two Waters (Milford, MA) pumps (models 501 and 510) and an automated gradient controller model 680, a Shimadzu (Kyoto, Japan) SPD-6A UV spectrophotometric detector, and a Hewlett-Packard (Avondale, PA) model HP3396II integrator connected with a peak 96 HPLC software. A reverse-phase C18 column (Supelcosyl, 3-μm particle size, 33 × 4.6 mm i.d.; Supelco, Bellefonte, PA) was used. Component A was a 0.05 M acetate buffer prepared by a 1:100 dilution of a stock 5 M acetate buffer adjusted to pH 4.7 with solid NaOH (20). Component A was filtered through a 0.45-μm filter. Component B was 100% methanol. The column was maintained at 37°C and run at a constant flow rate of 1.4 mL/min.

Two gradients were selected from a series of gradients examined, and their solvent profiles are shown in Figure 1. In gradient I, the initial solvent was 15% methanol (component B) and 85% component A. After a 3.5-min isocratic flow, a 1.5-min linear gradient was used to change the solvent composition to 45% methanol. Then a 5-min concave gradient altered the solvent composition from 45 to 100% methanol. This solvent was maintained for 2 min until another 2-min linear gradient (not shown in Fig. 1) returned the solvent to its original composition (15% methanol). Sinapic acid was eluted during the 3.5-min isocratic flow while sinapine eluted with the 5-min concave gradient. In gradient II, the initial solvent was 14% methanol (component B) and 86% component A. After a 5-min isocratic flow, a 10-min linear gradient was used to change the solvent composition from 14 to 100% methanol. This solvent was maintained for 2 min until another 2-min linear gradient (not shown in Fig. 1) returned the solvent to its original composition (14% methanol). Sinapic acid was separated during the isocratic flow while sinapine separated during the linear gradient elution. Other gradients tested included a combination of different concave and linear gradients with varying elution times but provided no improvement in separation of the phenolics.

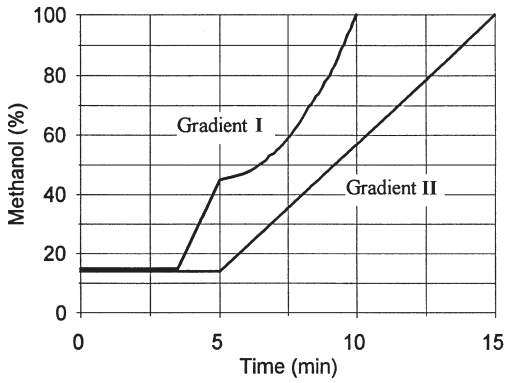

FIG. 1. Elution solvent profiles for the high-performance liquid chromatographic (HPLC) method. Gradient I: initial solvent was 15% methanol (component B) and 85% component A. After 3.5-min isocratic flow, a 1.5-min linear gradient was used to change the solvent composition to 45% methanol. Then a 5-min concave gradient altered the solvent composition from 45 to 100% methanol. This solvent was maintained for 2 min until another 2-min linear gradient returned the solvent to its orignal composition (not shown). Gradient II: initial solvent was 14% methanol (component B) and 86% component A. After 5-min isocratic flow, a 10-min linear gradient was used to change the solvent composition from 14 to 100% methanol. This solvent was maintained for 2 min until another 2-min linear gradient (not shown) returned the solvent to its original composition. Component A: 0.05 M acetate buffer, prepared as 1:100 dilution of a stock 5 M acetate buffer adjusted to pH 4.7 with solid NaOH. Component B: 100% methanol.

Ion exchange. About 2 g of CM-Sephadex C-25 resin were treated with 10 mL 1 M HCl and then washed with water until the pH of the eluant was neutral. The treated resin was then packed by gravity into a 10-mL pipette to form a cation exchange column (8 × 60 mm). About 2 g of Ecteola Cellulose was treated with 10 mL 2 M acetic acid, washed, and packed as described above to form an anion exchange column (8 × 60 mm) (22).

Fifty milligrams of the ground canola flour was refluxed with 5 mL 100% methanol for 20 min. After cooling to room temperature, the liquid layer was filtered through a 0.45-µm filter. The liquid phase was then concentrated to about 200 µL by evaporating under vacuum, applied to the CM-Sephadex C-25 cation exchange column, and washed with 10 mL water. The effluent was placed on the Ecteola Cellulose anion exchange column. Neutral phenolic derivatives were collected in the effluent from the anion exchange column. Aromatic choline esters were eluted from the cation exchange column, first with 10 mL of a mixture of 2 M acetic acid/methanol (1:1, vol/vol), and then with 10 mL 100% methanol. All the effluents were analyzed by HPLC.

Standard calibration. Stock solutions (200 µg/mL) for sinapine bisulfate and sinapic acid were prepared by accurately weighing 1 mg of each substance into 5 mL methanol. Dilution of the above stock solutions gave two sets of standard solutions of 200, 100, 50, and 25 µg/mL for sinapine bisulfate and sinapic acid, respectively. During the calibration, a 3-µL mixture of sinapine bisulfate/sinapic acid solution (2 µL sinapine bisulfate and 1 µL sinapic acid) was injected into the HPLC column using a 25-µL sample loop. Two calibration curves were obtained for sinapine bisulfate and sinapic acid, respectively, by plotting concentrations vs. peak areas. Regression equations were obtained from the calibration curves for the two compounds.

Calculation of sinapine and sinapic acid contents. Sinapine and sinapic acid contents of the flour and meal were calculated using the following equations:

$$\text{sinapine bisulfate (mg/g)} = (a + bA)\,\frac{V_c V_t}{V_S W} \quad [1]$$

$$\text{sinapine} = \text{sinapine bisulfate}\left(\frac{310.4}{407.4}\right) \quad [2]$$

$$\text{sinapic acid (mg/g)} = (a + bA)\,\frac{V_c V_t}{V_S W} \quad [3]$$

where a and b = y-intercept and slope of the standard curves for sinapine bisulfate or sinapic acid, respectively, A = peak area, V_c = injection volume for calibration (µL), V_S = injection volume for sample (µL), V_t = volume of solvent added to sample (mL), W = weight of the flour or meal (mg), 310.4 = molecular weight of sinapine, and 407.4 = molecular weight of sinapine bisulfate. The sinapine bisulfate was only used to determine the sinapine content of the canola products; sinapine bisulfate is not present in these products.

Total phenolic content. The total phenolic content was determined by Folin-Ciocalteu's reagent method according to Swain and Hillis (14) and Schanderl (27) using sinapic acid to prepare the standard curve. The total peak area from HPLC included sinapic acid, sinapine, and all other unknown peaks. Total phenolic contents estimated by this method were found to be very close to that determined by the Folin-Ciocalteu's reagent method.

UV spectra and extinction coefficients of standard sinapine bisulfate and sinapic acid. UV spectra and extinction coefficients of standard sinapine bisulfate (20 µg/mL) and sinapic acid (10 µg/mL) in methanol were determined with a Hewlett-Packard 8452 diode array spectrophotometer with MS-DOS UV-VIS software. Extinction coefficients were calculated according to the equation: $\varepsilon = A/cl$, where ε = extinction coefficient (L cm^{-1} mol^{-1}), A = absorbance, c = concentration (mol/L), and l = cell length (cm).

Statistical analysis. Where appropriate, the data were analyzed using analysis of variance (28), and Duncan's multiple range test was performed to determine significant differences between means.

RESULTS AND DISCUSSION

Selection of extraction conditions. Determination of optimal extraction conditions is a necessary prerequisite for the measurement of sinapine and sinapic acid by HPLC. Bouchereau *et al.* (3) extracted rapeseed phenolics by boiling the flour in 100% methanol. The phenolics in the extract were then separated by ion exchange chromatography and determined by

TABLE 1
Effect of Extraction Conditions on the Recovery (mg/g) of Sinapine, Sinapic Acid, and Total Phenolics from Canola Flour (dry basis)[a]

	100% methanol			70% methanol
	50°C, 10 min	75°C, 20 min	Reflux, 20 min	75°C, 20 min
Sinapine	10.85 ± 0.26^{B}	10.65 ± 0.07^{B}	12.09 ± 0.72^{A}	$11.74 \pm 0.99^{A,B}$
Sinapic acid	0.36 ± 0.05^{B}	0.34 ± 0.05^{B}	0.49 ± 0.04^{A}	$0.40 \pm 0.03^{A,B}$
Total phenolics (HPLC)	17.71 ± 2.05^{B}	16.13 ± 2.00^{B}	20.07 ± 0.74^{A}	22.58 ± 1.22^{A}
Total phenolics (Folin-Ciocalteu)	17.84 ± 1.47^{B}	17.19 ± 1.11^{B}	21.96 ± 1.94^{A}	22.90 ± 1.50^{A}

[a]Means of three replicates ± standard deviation. Row values with the same superscript letter are not significantly different ($P \leq 0.05$) based on Duncan's multiple range test following analysis of variance. HPLC, high-performance liquid chromatography.

HPLC. On the other hand, 70% aqueous methanol was used for an extraction at 75°C for 20 min during the determination of sinapine by UV spectrophotometric method in conjunction with an ion exchange separation (15). Naczk *et al.* (29) found 70% aqueous methanol extracted twice as much total phenolics from rapeseed meal as pure methanol. Since the extractions were conducted at lower solvent-to-meal ratios (10:1 and 20:1), the extraction efficiencies of these solvents at a higher solvent-to-meal ratio (100:1 in this research) were still unknown. In addition, aqueous methanol extracts traces of proteinaceous material that require removal before determination by HPLC. Owing to the uncertainty of the efficiency of these extraction methods, an evaluation is required. In particular, the condition of aqueous methanol or pure methanol was a major point that needed to be clarified.

The effect of extraction conditions on the amount of sinapine, sinapic acid, and total phenolics measured is given in Table 1. The amounts of sinapine, sinapic acid, and the total phenolics determined using the extraction condition of refluxing with 100% methanol for 20 min were not significantly different from those determined using the extraction condition of 70% methanol at 75°C for 20 min, but were significantly higher than those obtained using the other two conditions at 50 and 75°C with 100% methanol. Of the two methods giving higher phenolic extraction rates, refluxing for 20 min with 100% methanol was selected as the routine method for extraction of the phenolics, as there was not the same need for temperature control as there was for the extraction with 70% methanol.

The total phenolic contents estimated by HPLC were reasonably close to the total phenolic contents determined by the Folin-Ciocalteu's reagent method (Table 1).

Elution conditions. HPLC resolution is directly proportional to the difference between retention times of adjacent peaks over the sum of peak widths at the base of each peak (30). Retention time is a thermodynamically controlled factor, whereas peak width is a factor controlled by the kinetics of the solute. The goal of this study was to change the retention time by changing thermodynamic factors (i.e., solvent composition) to obtain retention times different enough for good resolution yet in a short chromatographic period. It was also observed that a change in solvent composition affected the peak width of sinapine.

Elution conditions were selected based on trial-and-error methods within the limits of the chromatographic system. The conditions tested included several isocratic, linear, and nonlinear gradients, and the combinations of either two or all three of these gradients. Two elution conditions, gradients I and II (Fig. 1), were selected for further evaluation.

HPLC chromatograms of different samples under these two elution conditions are shown for two different gradients

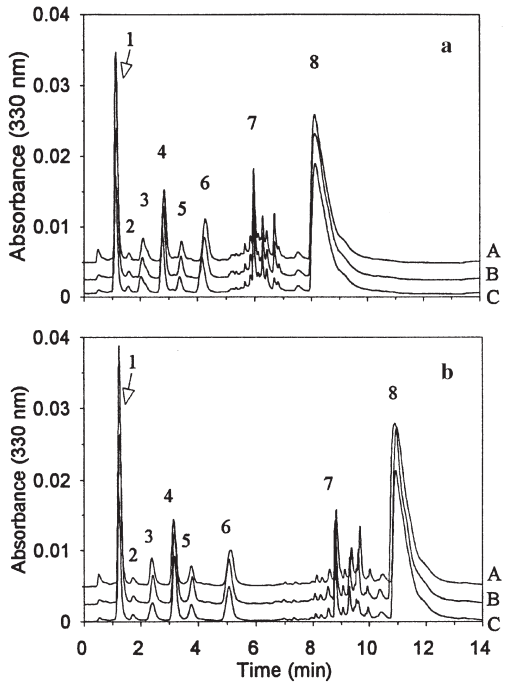

FIG. 2. HPLC chromatograms of different samples (A–C) using gradient I (upper panel) and gradient II (lower panel). Sample A, extracted with 70% aqueous methanol at 75°C for 20 min; sample B, extracted with 100% methanol refluxing for 20 min; sample C, extracted with 100% methanol at 50°C for 10 min. Peak identifications: 5, sinapic acid; 8, sinapine; 1–4, 6, 7, unidentified. For description of gradients and for abbreviation, see Figure 1.

in Figures 2a and 2b, with the three chromatograms for each gradient representing chromatograms of samples from different extraction conditions, including 70% aqueous methanol at 75°C for 20 min, 100% methanol refluxing for 20 min, and 100% methanol at 50°C for 10 min. The chromatogram of the sample extracted with 100% methanol at 75°C for 20 min resembled that of the sample extracted with 100% methanol at 50°C for 10 min and is not shown.

The number of peaks eluted by the two gradients was generally the same (Figs. 2a,b). The combination of an isocratic and a linear gradient, gradient II (Fig. 2b), gave better resolution than gradient I (Fig. 2a), which is a combination of an isocratic, a linear, and a concave gradient. However, the time required for gradient II (15 min) was longer than that for gradient I (10 min). Because resolution of sinapine and sinapic acid from other compounds was good enough in both cases and gradient I required a shorter time, gradient I was selected as a routine technique for the determination of sinapine and sinapic acid contents. Other gradients tested in this study did not provide any better resolution or any better peak shape, and they are not presented.

Although the number of peaks eluted with either gradient was also the same for all three samples extracted at different conditions (Figs. 2a,b), there were differences in peak heights and areas for different samples. The sample extracted by refluxing with 100% methanol had the largest peak area for sinapine (peak 8) and sinapic acid (peak 5), whereas samples extracted with 70% aqueous methanol had the largest areas for peak 1.

With this method, both sinapine and sinapic acid are shown in the same chromatograms, and as a result, their levels are comparable, since the ratio of their extinction coefficients at 330 nm has been determined to be 1:1.16. In addition to being fast and simple, this direct comparison is another advantage of the current method over the conventional methods.

Ion exchange. To verify the peak identities of sinapine and sinapic acid and to classify the other peaks into differently charged groups for possible peak identifications, the same extracts were separated using ion exchange columns and analyzed using gradient I by HPLC. The extract was separated into neutral, anionic, and cationic fractions before analysis by HPLC. Two peaks (corresponding to peaks 1 and 3 in Fig. 2a) were observed for the neutral fraction and were therefore considered to be neutral compounds. According to Bouchereau *et al.* (3), neutral compounds such as sinapoylglucose, 1,2-disinapoylglucose and sinapoylmalate have been found in methanol extracts of rapeseed flour. Two peaks were observed in the anionic fraction. One peak (peak 5) was identified as sinapic acid. A second peak (peak 2) could also be an anionic phenolic but was not identified, as was the case for the other small unnumbered peaks in the chromatogram. Three peaks (peaks 4, 6, and 7) were noted in first cationic fraction eluted from the cationic exchange column with 2 M acetic acid/methanol solvent. These peaks were possibly cationic compounds such as 4-hydroxybenzoylcholine (19) and sinapine-*O*-β-D-glucopyranoside (3,31,32), as these have been found in seeds of some glucosinolate-containing plants, including canola. Peak 8 from the second cationic fraction, eluted with 100% methanol, was identified as sinapine. With the ion exchange technique, it was verified that sinapic acid was in the anionic fraction while sinapine was in the cationic fraction. Other unidentifed peaks were grouped into different charged groups. Attempts were made to identify these peaks by spiking the methanol extract of canola flour with standards of ferulic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid, but the unknown peaks could not be identified as any of these compounds.

According to HPLC chromatograms from the ion exchange fractions, there was no evidence of compounds from other fractions eluting at the same time as either sinapine or sinapic acid. Coelution of this type could result in an overestimation of sinapine and sinapic acid based on peak area.

Standard chromatograms. HPLC chromatograms for the standard sinapine bisulfate and sinapic acid analyzed using gradient I are shown in Figure 3. The four curves show chromatograms resulting from using the same injection volume (2 µL sinapine bisulfate and 1 µL sinapic acid) but different concentrations. The shapes of the peaks were symmetrical with sinapic acid, but skewed to the left for sinapine bisulfate. Clausen *et al.* (19) obtained a better peak shape with a Nucleosil C5 column than a Nucleosil C18 column when separating aromatic choline esters.

The retention times from the chromatographic studies are shown in Table 2. The analysis of variance showed that the retention times for sinapine bisulfate at different concentrations were significantly different ($P \leq 0.05$). Retention time increased as concentration decreased, but the peak was far enough removed from other peaks that this did not impair identification of this peak. On the other hand, retention times for sinapic acid were not significantly different ($P \leq 0.05$).

Calibration curves. Peak areas in relation to concentrations of standard sinapine bisulfate and sinapic acid are given in Table 2. These data were used to generate calibration curves (not shown). Linear regression analysis showed that for both sinapine bisulfate and sinapic acid there was a linear

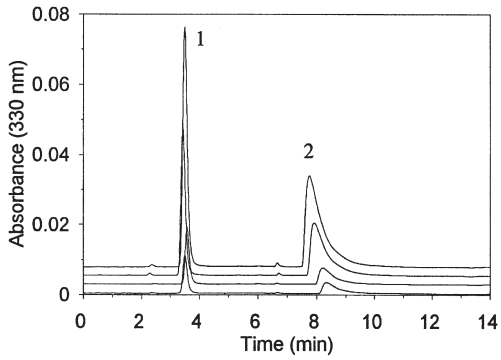

FIG. 3. HPLC chromatograms of a mixture of standard sinapine bisulfate (peak 2) and sinapic acid (peak 1). Traces, top to bottom, represent 200, 100, 50, and 25 µg/mL sinapine and sinapic acid, each in a mixture of an injection volume of 3 µL (2 µL sinapine and 1 µL sinapic acid). For abbreviation see Figure 1.

TABLE 2
Retention Times and Retention Times in Relation to Concentrations of Standard Sinapine Bisulfate and Sinapic Acid (2 μL of sinapine bisulfate and 1 μL sinapic acid solutions)[a]

Concentration (μg/ mL)	Retention time (min)		Peak area	
	Sinapine bisulfate[b]	Sinapic acid[b]	Sinapine bisulfate[b]	Sinapic acid[b]
25	8.41 ± 0.09^{D}	3.53 ± 0.12^{A}	$5.76 \times 10^5 \pm 5.1 \times 10^4$ ^{D}	$5.18 \times 10^5 \pm 4.3 \times 10^4$ ^{D}
50	8.25 ± 0.49^{C}	3.61 ± 0.17^{A}	$1.23 \times 10^6 \pm 5.6 \times 10^4$ ^{C}	$1.03 \times 10^6 \pm 8.3 \times 10^4$ ^{C}
100	8.00 ± 0.11^{B}	3.53 ± 0.20^{A}	$2.66 \times 10^6 \pm 2.4 \times 10^5$ ^{B}	$2.04 \times 10^6 \pm 1.6 \times 10^5$ ^{B}
200	7.84 ± 0.11^{A}	3.61 ± 0.08^{A}	$4.98 \times 10^6 \pm 2.2 \times 10^5$ ^{A}	$4.18 \times 10^6 \pm 3.4 \times 10^5$ ^{A}

[a]Mean of six replicates ± standard deviation.
[b]Column values with the same superscript letter are not significantly different ($P \leq 0.05$) based on Duncan's multiple range test following analysis of variance.

relationship between peak area and concentration. Therefore, the determination of concentration according to peak area is valid. Regression equations for the two compounds are as follows:

$$[\text{Spn}] = 0.030 + 3.97 \times 10^{-5}A \quad R^2 = 0.9881 \quad [4]$$

$$[\text{Spa}] = -0.945 + 4.78 \times 10^{-5}A \quad R^2 = 0.9811 \quad [5]$$

where [Spn] = sinapine bisulfate concentration (μg/mL), [Spa] = sinapic acid concentration (μg/mL), and A = peak area.

UV spectra and extinction coefficients of sinapine bisulfate and sinapic acid at detector wavelength. UV spectra of sinapine bisulfate and sinapic acid are given in Figure 4. The maximum absorbance wavelengths for sinapine bisulfate and sinapic acid were 332 and 322 nm, respectively. Since sinapine is the major phenolic component of canola/rapeseed, the detector wavelength was set close to the sinapine maximum (330 nm). In addition, this wavelength has been used in previous determinations of sinapine (15) and sinapic acid (20). For the purpose of comparing sinapine and sinapic acid on the same chromatogram, their extinction coefficients at the maximum absorbance wavelengths and at the detector wavelength were determined. These values are listed in Table 3. The extinction coefficients for sinapine bisulfate and sinapic acid at 330 nm were 2500 ± 50 and 2160 ± 60 cm^{-1} mol^{-1}, respectively. Therefore, the absorbance of sinapine was 1.16 times higher than that of sinapic acid (mole concentration basis).

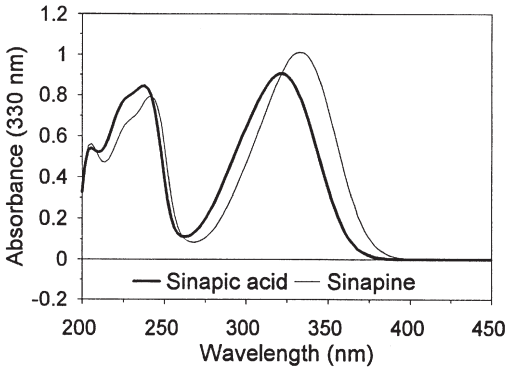

FIG. 4. Ultraviolet spectra of sinapine bisulfate (20 μg/mL; λ_{max} = 332 nm) and sinapic acid (10 μg/mL; λ_{max} = 322 nm) in methanol.

Similarly, the same peak areas for sinapine and sinapic acid on the chromatogram should indicate a mole ratio of 0.86 to 1.

Determination of the phenolic contents of the methanol extracts from canola whole seed, industrial meal, and protein isolate. The sinapine and sinapic acid contents of canola flour, industrial canola meal, and a protein isolate (PMM) were determined by HPLC using gradient I. The results are given in Table 4. The sinapine contents were 12.03, 11.38, and 0.74 mg/g for canola flour, industrial meal, and PMM, respectively. The results of the flour and meal cannot be directly compared since the sources of the materials were different. PMM was isolated from the same meal used in this research; therefore, after isolation, the PMM contained less than one-tenth of its original sinapine. The variations in retention times were consistent with those observed for the standards. Since standard sinapine bisulfate was used in place of sinapine, the results must be converted from sinapine bisulfate to sinapine. On the other hand, the sinapic acid contents were 0.39, 0.24, and 0.20 mg/g for canola flour, industrial meal, and the protein isolate, respectively. Retention times for sinapic acid were approximately 3.4 min for all three materials, similar to that seen for the standard.

A higher content of sinapine has been reported in *B. napus* rapeseed cultivars (16.5–22.6 mg/g) than in *Brassica campestris* cultivars (12.2–15.4 mg/g) (7). Between 26.7 and 28.5 mg/g of sinapine was found in defatted rapeseed and canola cotyledons (11). In a colorimetric method using titanium tetrachloride for the determination of sinapine in rapeseed, the content of sinapine was 10.4 mg/g for rapeseed and 1.1 to 1.8 mg/g for rapeseed protein concentrate (16). The levels of sinapic acid reported in the literature for canola are highly variable. The total content of free phenolic acids was only 0.06 mg/g of flour in the Indian cultivar, Yellow Sarson,

TABLE 3
Absorbance Wavelength Maxima (λ_{max}) and Extinction Coefficients of Sinapine Bisulfate and Sinapic Acid at λ_{max} and at Detector Wavelength (λ_{330}) in Methanol[a]

	λ_{max} (nm)	ε_{max} ($cm^{-1}mol^{-1}$)	ε_{max} ($cm^{-1}mol^{-1}$)
Sinapine bisulfate	332	2518 ± 53	2500 ± 53
Sinapic acid	322	2300 ± 60	2147 ± 60

[a]Mean of three replicates ± standard deviation.

TABLE 4
Retention Times and Sinapine and Sinapic Acid Contents of Canola Flour, Industrial Meal, and Canola Protein Isolate (PMM) (dry basis)[a]

	Sinapine		Sinapic acid	
	Retention time (min)	Sinapine content (mg/g)	Retention time (min)	Sinapic acid content (mg/g)
Flour	7.96 ± 0.05	12.03 ± 0.63	3.49 ± 0.05	0.39 ± 0.05
Meal	8.01 ± 0.04	11.38 ± 1.35	3.44 ± 0.04	0.24 ± 0.06
PMM	8.52 ± 0.21	0.74 ± 0.22	3.38 ± 0.11	0.20 ± 0.06

[a]Mean of five replicates ± standard deviation. PMM, protein micellar mass.

while Canadian cultivars, such as Candle and Tower, contained over 10 times this level, primarily due to the high levels of sinapic acid (2). Therefore, the sinapine and sinapic acid contents determined by the current method are in the same range as values previously reported in literature. This method, however, simplified the sample preparation and chromatographic procedures in comparison with previous methods and allowed for comparison of sinapine and sinapic acid on the same chromatogram.

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REFERENCES

1. Austin, F.L., and I.A. Wolff, Sinapine and Related Esters in Seed Meal of *Crambe abyssinica, J. Agric. Food Chem. 16*:132–135 (1968).
2. Krygier, K., F. Sosulski, and L. Hogge, Free, Esterified and Insoluble Phenolic Acids. II. Composition of Phenolic Acids in Rapeseed Flour and Hulls, *Ibid. 30*:334–336 (1982).
3. Bouchereau, A., J. Hamelin, M. Renard, and F. Larher, Structural Changes in Sinapic Acid Conjugates During Seedling Development of Rape, *Plant Physiol. Biochem. 30*:467–475 (1992).
4. Shahidi, F., and M. Naczk, *Food Phenolics: Sources, Chemistry, Effects and Applications*, Technomic Publishing, Lancaster, PA, 1995.
5. Hobson, F.A., G.R. Fenwick, R.K. Heaney, D.G. Land, and R.F. Curis, Rapeseed Meal and Egg Tainting: Association with Sinapine, *Br. Poult. Sci. 18*:539–541 (1977).
6. Goh, Y.K., D.R. Clandinin, A.R. Robblee, and K. Darlington, The Effect of Level of Sinapine in a Laying Ration on the Incidence of Fishy Odor in Eggs from Brown-Shelled Egg Layers, *Can. J. Anim. Sci. 59*:313–316 (1979).
7. Shahidi, F., and M. Naczk, An Overview of Phenolics of Canola and Rapeseed: Chemical, Sensory and Nutritional Significance, *J. Am. Oil Chem. Soc. 69*:917–924 (1992).
8. Keshavarz, E., R.K.M. Cheung, R.C.M. Liu, and S. Nakai, Adaptation of the Three Stage Extraction Process to Rapeseed Meal for Preparation of Colorless Protein Extracts, *Can. Inst. Food Sci. Technol. J. 10*:73–77 (1977).
9. Sosulski, F.W., Organoleptic and Nutritional of Phenolic Compounds on Oilseed Protein Products: A Review, *J. Am. Oil Chem. Soc. 56*:711–715 (1979).
10. Youngs, C.G., Technical Status Assessment of Food Protein from Canola, in *Research on Canola Seed, Oil and Meal, 9th Project Report*, Canola Council of Canada, Winnipeg, 1991, pp. 309–341.
11. Blair, R., and R.D. Reichert, Carbohydrate and Phenolic Constituents in a Comprehensive Range of Rapeseed and Canola Fractions: Nutritional Significance for Animals, *J. Sci. Food Agric. 35*:29–35 (1984).
12. Sosulski, F.W., and K.J. Dabrowski, Composition of Free and Hydrolyzable Phenolic Acids in the Flours and Hulls of Ten Legume Species, *J. Agric. Food Chem. 32*:131–133 (1984).
13. Ribereau-Gayon, P., *Plant Phenolics*, Oliver & Boyd, Edinburgh, United Kingdom, 1972.
14. Swain, T., and W.E. Hillis, The Phenolic Constituents of *Prunus domestica* I. The Quantitative Analysis of Phenolic Constituents, *J. Sci. Food Agric. 10*:63–68 (1959).
15. Wang, S.X., B.D. Oomah, and D.I. McGregor, Application and Evaluation of Ion-Exchange UV Spectrophotometric Method for Determination of Sinapine in *Brassica* Seeds and Meals, *J. Agric. Food Chem. 46*:575–579 (1998).
16. Ismail, F., and N.A.M. Eskin, A New Quantitative Procedure for Determination of Sinapine, *Ibid. 27*:917–918 (1979).
17. Hartley, R.D., and E.C. Jones, Effect of Ultraviolet Light on Substituted Cinnamic Acids and the Estimation of Their *Cis* and *Trans* Isomers by Gas Chromatography, *J. Chromatogr. 107*:213–218 (1975).
18. Andersen, J.M., Analysis of Plant Phenolics by High Performance Liquid Chromatography, *Ibid. 259*:131–139 (1983).
19. Clausen, S., O. Olsen, and H. Sorensen, Separation of Aromatic Choline Esters by High-Performance Liquid Chromatography, *Ibid. 260*:193–199 (1983).
20. Hagerman, A.E., and R.L. Nicholson, High-Performance Liquid Chromatographic Determination of Hydroxycinnamic Acids in the Maize Mesocotyl, *J. Agric. Food Chem. 30*:1098–1102 (1982).
21. Lattanzio, V., High-Performance Liquid Chromatography of Free and Bound Phenolic Acid in the Eggplant, *J. Chromatogr. 250*:143–148 (1982).
22. Bjerg, B., O. Olsen, K.W. Rasmussen, and H. Sorensen, New Principles of Ion-Exchange Techniques Suitable to Sample Preparation and Group Separation of Natural Products Prior to Liquid Chromatography, *J. Liquid Chromatogr. 7*:691–707 (1984).
23. Bouchereau, A., J. Hamelin, I. Lamour, M. Renard, and F. Larher, Distribution of Sinapine and Related Compounds in Seeds of *Brassica* and Allied Genera, *Phytochemistry 30*:1873–1881 (1991).
24. Mailer, R.J., J. Daun, and R. Scarth, Cultivar Identification in *Brassica napus* L. by Reversed-Phase High-Performance Liquid Chromatography of Ethanol Extracts, *J. Am. Oil Chem. Soc. 70*:863–866 (1993).
25. Clandinin, D.R., Rapeseed Oil Meal Studied. 4. Effect of Sinapine, the Bitter Substance in Rapeseed Oil Meal, on the Growth of Chickens, *Poult. Sci. J. 40*:484–487 (1961).

26. Murray, E.D., C.D. Myers, and L.D. Barker, Protein Product and Process for Preparing Same, Canadian Patent 1,028,522 (1978).
27. Schanderl, S.H., Tannins and Related Phenolics, in *Methods in Food Analysis, Physical Chemical and Instrumental Methods of Analysis*, edited by M.A. Joslyn, Academic Press, New York, 1970, pp. 701–725.
28. SAS, *SAS User's Guide*, Version 6, SAS Institute Inc., Cary, NC, 1996.
29. Naczk, M., F. Shahidi, and A. Sullivan, Recovery of Rapeseed Tannins by Various Solvent Systems, *Food Chem. 45*:51–54 (1992).
30. Macrae, R., *HPLC in Food Analysis*, Academic Press, London, 1982, p. 4.
31. Larsen, L.M., O. Olsen, A. Ploger, and H. Sorensen, Sinapine-*O*-β-D-glucopyranoside in Seeds of *Alliaria officinalis*, *Phytochemistry 22*:219–222 (1983).
32. Wanasundara, J., R. Amarowicz, and F. Shahidi, Isolation and Identification of an Antioxidative Component in Canola Meal, *J. Agric. Food Chem. 42*:1285–1290 (1994).

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