# Facile Spectrophotometric Quantification Method of Sinapic Acid in Hexane-Extracted and Methanol-Ammonia-Water-Treated Mustard and Rapeseed Meals

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The contribution of sinapic acid to the free, esterified, and insoluble bound phenolics of a number of *Brassica* oilseeds, namely Triton (*B. napus*), Midas (*B. napus*), and Hu You 9 (*B. napus*) rapeseed and Domo mustard (*B. juncea*), was determined according to a simple spectrophotometric method. Sinapic acid constituted 65–86% of free and 71–97% of esterified phenolic acids of defatted meals. The contribution of sinapic acid to insoluble bound phenolic was 7–32%. Treatment of defatted meals with 10% ammonia in 95% methanol (MeOH–$NH_3$–$H_2O$) resulted in the removal of 41–75% of their total free phenolics and 45–79% of the content of sinapic acid in this fraction. This process also removed 82–93% of the total esterified phenolics and 86–93% of their corresponding sinapic acid. In all cases, MeOH–$NH_3$–$H_2O$ extraction enhanced the protein content of the resultant meals.

## INTRODUCTION

Rapeseed cultivars, including canola with low gluconinolate and erucic acid contents, have similar levels of phenolics (Krygier et al., 1982b; Kozlowska et al., 1983; Dabrowski and Sosulski, 1984; Naczk et al., 1986; Naczk and Shahidi, 1989) which are mainly located in seed cotyledons (Kozlowska et al., 1990). Phenolic acids of rapeseed exist in free, esterified, and insoluble bound forms. Phenolic esters and free phenolic acids make up approximately 80% and up to 16% of the total phenolic compounds of canola, respectively (Krygier et al., 1982b; Naczk et al., 1986).

Tentative identification of principal phenolic acids of rapeseed has been reported by Durkee and Thivierge (1975), Fenton et al., (1980), Krygier et al. (1982b), and Kozlowska et al. (1975, 1983). Sinapic acid was found to be the predominant phenolic acid in rapeseed (Clandinin, 1961); it constituted over 73% of free phenolic acids and about 99% of phenolic acids released from esters and glucosides (Krygier et al., 1982b; Dabrowski and Sosulski, 1984). Minor phenolic acids identified included *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, *p*-coumaric, ferulic, and caffeic acids. In addition, trace amounts of chlorogenic acid were found in the free phenolic acid fraction of rapeseed meals (Krygier et al., 1982b; Kozlowska et al., 1975, 1983; Lo and Hill, 1972).

Sinapine was the principal phenolic ester in rapeseed of the Midas and Echo varieties. At least seven other compounds were also identified, and their hydrolysis also yielded sinapic acid (Fenton et al., 1980). Mueller et al. (1978) reported that *Brassica napus* cultivars of rapeseed contained significantly ($P < 0.01$) higher levels of sinapine (1.65–2.26%) as compared with *Brassica campestris* cultivars (1.22–1.54%). Furthermore, Fenwick et al. (1984a) demonstrated that *B. napus* meals contained 1.17–1.83% sinapine. The content of sinapine in several cultivars of Cruciferae crops including the *Brassica* species varied from 0.4 to 1.8% (Kerber and Buchloh, 1980). However, Argentine and Polish rapeseed contained only 0.94 and 0.92% sinapine, respectively (Austin and Wolff, 1968). Blair and Reichert (1984) determined the mean content of sinapine in defatted high-glucosinolate rapeseed and canola cotyledons to be 2.67 and 2.85%, respectively.

Sinapine has a bitter taste and may contribute to unpleasant flavors in rapeseed meals. The presence of sinapine is also linked to a crabby or fishy taint note in eggs from some brown-egg-laying hens (Hobson-Frohock et al., 1973; Butler et al., 1982; Fenwick et al., 1984a) resulting from trimethylamine (TMA) production (Pearson et al., 1980). Phenolic compounds, in general, contribute to the dark color and astringency of rapeseed meals. They may also participate in complexation with enzymes and other proteins, thus lowering protein utilization. Therefore, phenolic compounds are important factors when rapeseed meal is considered as a protein source in food formulations (Kozlowska et al., 1975; Sosulski, 1979).

In this paper, a simple spectrophotometric method for determining the contribution of sinapic acid to the phenolics of hexane-extracted and methanol–ammonia–water (MeOH–$NH_3$–$H_2O$)-treated *Brassica* meals is reported. To evaluate the performance of this method, high-glucosinolate rapeseed varieties, namely Midas (*B. napus*) and Hu You 9 (*B. napus* of Chinese cultivar), and a low-glucosinolate cultivar, namely, Triton (*B. napus*), as well as mustard (*B. juncea*) were selected for this study. These varieties are known to differ in their content of sinapic acid. This methodology was then employed to determine the effect of processing on the content of sinapic acid in free, esterified, and insoluble bound phenolic acid fractions of *Brassica* seeds.

## MATERIALS AND METHODS

Hexane-extracted meal was prepared by blending 60 g of crushed seed with 400 mL of hexanes in a Waring blender at approximately 15 000 rpm for 2 min. The meal was separated from the slurry by vacuum filtration, and residual oil was further extracted from the meal with hexanes using a Soxhlet apparatus. The resultant defatted meal was dried at 50 °C in a forced-air convection oven.

Methanol–ammonia–water (MeOH–$NH_3$–$H_2O$)-treated meal was obtained as described below. Ground seed (60 g) was blended with 400 mL of 10% (w/w) ammonia in absolute or 95% (v/v)

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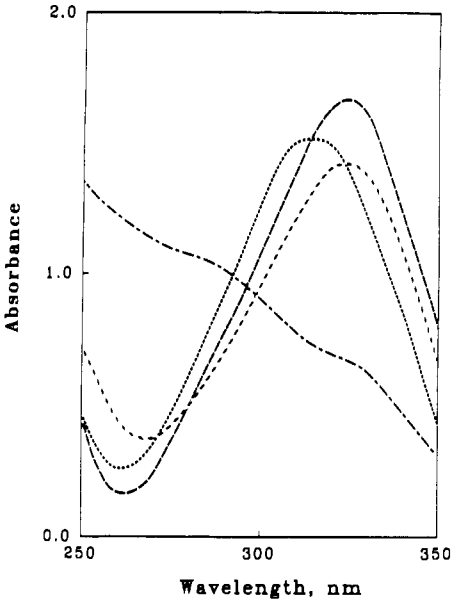


**Figure 1.** Spectra of sinapic acid: (···) in water containing 10% (v/v) methanol; (– – –) in 90% (v/v) methanol; (– – –) esterified fraction of canola phenolics in 90% (v/v) methanol; (– - –) phenolics from insoluble bound fraction in 90% (v/v) methanol.

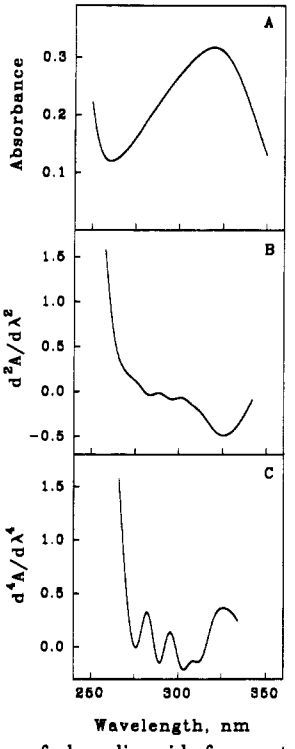


**Figure 2.** Spectra of phenolic acids from esterified fraction of Triton canola. (A) As such in the 250–350-nm region; (B) second derivative $d^2A/d\lambda^2$ ($\times 10^4$); (C) fourth derivative $d^4A/d\lambda^4$ ($\times 10^6$).

methanol in a Waring blender at approximately 15 000 rpm for 2 min. After a 15-min quiescent period, 400 mL of hexanes was added and the mixture was blended again for 2 min. The meal was separated from the slurry by vacuum filtration, rinsed three times with a total of 100 mL of absolute methanol, and dried at 50 °C in a forced-air convection oven. Residual oil was further extracted from treated meal with hexanes using a Soxhlet apparatus. The resultant defatted meal was again dried as described above.

The free phenolic acids, soluble esters, and glycosides of phenolic acids and insoluble bound phenolic acids of treated rapeseed meals were isolated according to the procedure of Krygier et al. (1982a). Free phenolics of meals (2 g) were extracted

**Table I. Precision Data for Determination of Sinapic Acid Content in the Esterified Phenolic Acid Fraction of Triton Canola Assayed on Different Days**

| assay day | sinapic acid, mg/100 g of protein | mean value | SD | relative SD, % |
|---|---|---|---|---|
| 1 | 2597<br>2559<br>2617<br>2630<br>2552 | 2591 | 34 | 1.3 |
| 2 | 2471<br>2504<br>2595<br>2494<br>2552 | 2523 | 50 | 2.0 |
| 3 | 2583<br>2599<br>2517<br>2485<br>2647 | 2566 | 65 | 2.5 |
| overall | | 2560 | 56 | 2.2 |

**Table II. Crude Protein Content (N% × 6.25) of *Brassica* Oilseed Meals[a]**

| meal | hexane extracted | MeOH–$NH_3$–$H_2O$/ hexane extracted |
|---|---|---|
| Hu You 9 | 39.1 ± 0.4 | 49.1 ± 0.9 |
| Midas | 41.2 ± 0.9 | 50.2 ± 0.8 |
| Triton | 41.2 ± 0.2 | 51.0 ± 0.3 |
| mustard | 44.2 ± 0.4 | 50.6 ± 0.6 |

[a] On a dry basis.

six times with 40 mL of a methanol–acetone–water solvent system (7:7:6) at room temperature using a Polytron homogenizer (15 s, 10 000 rpm). After centrifugation (15 min, 5000 rpm) of each treatment/extraction mixture, the supernatants were combined and evaporated at 30 °C under vacuum to approximately 40 mL. This mixture was then extracted six times with a mixture of diethyl ether–ethyl acetate (1:1 v/v). Ether–ethyl acetate extracts were combined and evaporated to dryness at 30 °C under vacuum. The extracted phenolic acids (referred to as free phenolic acids) were dissolved in methanol. The supernatant solution separated earlier still contains the esterified phenolic acids. This solution was then treated with 30 mL of 4 N NaOH under nitrogen for 4 h at room temperature. The resultant hydrolyzed solution was acidified to pH 2 using 6 N HCl, extracted six times with diethyl ether–ethyl acetate, and evaporated to dryness, as before. The extract of phenolics liberated from their esters was dissolved in methanol. The residual meal after extractions was treated with 20 mL of 4 N NaOH under nitrogen for 4 h at room temperature. The mixture was acidified with 6 N HCl to pH 2 and then centrifuged (15 min, 5000 rpm). The supernatant was extracted six times with diethyl ether–ethyl acetate, and after evaporation of solvents, as before, the acids liberated from the insoluble residues (referred to as insoluble bound phenolic acids) were dissolved in methanol. The content of phenolic acids in the methanol extracts was determined colorimetrically using the Folin–Denis reagent and by measuring the absorbance of the adduct formed at 725 nm as described by Swain and Hillis (1959). The content of free phenolic acids, phenolic acids liberated from esters and glycosides, and insoluble bound phenolic acids was expressed as *trans*-sinapic acid equivalents (milligrams per 100 g of proteins) and calculated using the equation $C = k(0.1744A_{725} - 0.0155)$, correlation coefficient $r = 0.9965$, where $k$ is a constant.

The free phenolic acids, esterified phenolic acids, and insoluble bound phenolic acids in methanol were assayed after dilution with methanol (1:10 for insoluble bound phenolic to 1:50 for phenolics liberated from esters) and analyzed for their content of sinapic acid by UV spectrophotometry at the 320 nm. The concentration of sinapic acid in the methanol extracts (reported as milligrams per 100 g of defatted dry meal) was calculated using the equation $C = 0.120kA_{320}$, correlation coefficient $r = 0.9997$, where $k$ is a constant.

**Table III. Content of Free, Esterified, and Insoluble Bound Fractions and Total Phenolic Acids, as Milligrams of Sinapic Acid Equivalents per 100 g of Protein, in *Brassica* Oilseed Meals**

| meal | free | esterified | insoluble bound | total |
|---|---|---|---|---|
| hexane extracted | | | | |
| Hu You 9 | 307 ± 15 | 3023 ± 84 | 100 ± 10 | 3429 ± 86 |
| Midas | 348 ± 10 | 3699 ± 20 | 167 ± 17 | 4214 ± 28 |
| Triton | 131 ± 2 | 2590 ± 52 | 110 ± 7 | 2831 ± 53 |
| mustard | 245 ± 11 | 3480 ± 75 | 51 ± 5 | 3775 ± 76 |
| MeOH–$NH_3$–$H_2O$/hexane extracted | | | | |
| Hu You 9 | 76 ± 2 | 209 ± 8 | 103 ± 9 | 388 ± 12 |
| Midas | 206 ± 6 | 651 ± 13 | 205 ± 8 | 1061 ± 17 |
| Triton | 61 ± 2 | 426 ± 23 | 77 ± 6 | 563 ± 24 |
| mustard | 65 ± 2 | 444 ± 8 | 90 ± 10 | 599 ± 13 |

**Table IV. Content of Sinapic Acid, in Milligrams per 100 g of Protein, on a Dry Basis, in Different Fractions of Phenolic Constituents of *Brassica* Oilseeds Determined by UV Spectrophotometry**

| meal | free | esterified | insoluble bound | total |
|---|---|---|---|---|
| hexane extracted | | | | |
| Hu You 9 | 236 ± 11 | 2913 ± 115 | 17 ± 1 | 3166 ± 116 |
| Midas | 251 ± 6 | 2624 ± 93 | 12 ± 2 | 2888 ± 94 |
| Triton | 92 ± 3 | 2504 ± 33 | 17 ± 2 | 2613 ± 34 |
| mustard | 209 ± 6 | 2525 ± 66 | 16 ± 3 | 2750 ± 67 |
| MeOH–$NH_3$–$H_2O$/hexane extracted | | | | |
| Hu You 9 | 50 ± 2 | 200 ± 2 | 16 ± 1 | 266 ± 3 |
| Midas | 143 ± 4 | 370 ± 11 | 15 ± 1 | 528 ± 12 |
| Triton | 51 ± 1 | 319 ± 3 | 13 ± 1 | 577 ± 4 |
| mustard | 49 ± 1 | 323 ± 4 | 28 ± 2 | 400 ± 5 |

All assays were conducted at room temperature (about 22 °C) using appropriate samples and blanks. The content of phenolics was then reported as milligrams of sinapic acid equivalents per 100 g of protein.

## RESULTS AND DISCUSSION

Benzoic acid derivatives show a maximum absorption at 270–280 nm, while the absorption maximum for cinnamic acid derivatives is at 305–330 nm with a shoulder at 290–300 nm (Macheix et al., 1990). According to Owades et al. (1958), simple phenolics show an absorption maximum in the region between 220 and 280 nm. Structurally related phenolics may, however, show considerable variation in their molecular absorptivities. Absorption spectra of these compounds are also affected by the nature of solvent employed and the pH of the solution.

The absorption spectra of sinapic acid in methanolic solutions, free phenolic acids, and phenolic acids liberated from esters were similar, in the range 250–350 nm with the absorption maximum at approximately 325 nm (Figure 1). However, the absorption maximum for methanolic solutions of insoluble bound phenolic acids was less pronounced. A lower concentrations of sinapic acid in this fraction and the presence of other phenolic acids were responsible for this observation (Figure 1). Examples of second ($d^2A/d\lambda^2$) and fourth ($d^4A/d\lambda^4$) derivatives of the UV spectra of esterified phenolic acids fraction isolated from Triton are shown in Figure 2. The fourth-order derivative showed that the UV spectrum was made up of four separate component bands at 282, 296, 308, and 326 nm. Mathematical analyses of these spectral derivatives were carried out using Lorentzian functions. Results indicated that about 10% of the absorption at 325 nm was due to component bands located at 296 and 308 nm. A somewhat higher contribution was found for insoluble bound phenolic acids at 325 nm (D. Pink, M. Naczk, and F. Shahidi, 1991, unpublished results). Furthermore, the precision of the data for determination of sinapic acid content with esterified phenolic acid fraction of Triton was monitored using freshly prepared extracts in three different days. Results in Table I indicate that data obtained during each day as well as those from different days were not significantly different from one another. Therefore, the above spectrophotometric methodology affords reproducible results with good accuracy.

Table II summarizes the protein content of mustard and rapeseed meals. The MeOH–$NH_3$–$H_2O$-treated meals had 14.5–25.6% more crude protein than their corresponding hexane-extracted counterparts. This increase in crude protein content is due to the removal of 8–10% of the seed solids such as carbohydrates, phospholipids, and other constituents into the polar phase (Naczk et al. 1985, 1986; Shahidi et al., 1990).

The content of total, free, esterified, and insoluble bound phenolic acids in rapeseed cultivars and Domo mustard is shown in Table III. These values are reported as milligrams of sinapic acid equivalent per 100 g of crude protein. It is the interaction of phenolics with proteins that is important when the nutritional value of the meals is considered, and this explains our deviation from the accepted norm of reporting the results on a meal basis. However, the above data could easily be recalculated, on a meal basis, using the protein content data given in Table II. *Brassica* seeds contain a considerably higher content of phenolic compounds as compared to other oleoginous meals. Hence, they are considered by a number of authors to be important factors when the usefulness of rapeseed meal as a protein source in food formulations is evaluated (Kozlowska et al., 1975; Sosulski, 1979). Formation of protein–phenolic complexes may affect the nutritional value as well as the quality of rapeseed-based protein products. Smyk and Drabent (1989) reported the complexation of protein–phenolics in a model system consisting of bovine serum albumin and sinapic acid. They found that formation of such complexes is favored in the neutral and basic pH ranges. Such complications were also reported by Kozlowska and Zadernowski (1988), who examined the amount of soluble matters which could be extracted into 80% (v/v) ethanol. These authors suggested that the undesirable taste and dark color of isolates obtained from rapeseed varieties may be due to the presence of phenolic compounds.

The MeOH–$NH_3$–$H_2O$/hexane extraction system removed 75–89% of the total phenolics from rapeseed meals (Table III). These results are in good agreement with

those reported in the literature. McGregor et al. (1983) found that gaseous ammoniation of *B. juncea* mustard meal removed up to 74% of sinapine. However, Fenwick et al. (1979, 1984b) demonstrated that treatment of *B. napus* meal with $NH_3$ or lime reduced the sinapine content by about 90%. Similarly, Kirk et al. (1966) reported a drop of up to 90% in the sinapine content of Crambe meal after treatment with gaseous ammonia; moreover, aqueous ammonia was less effective. Treatment of Candle and Tower meals with ethanol containing 0.2 M ammonia removed up to 82 and 39% of their phenolics, respectively (Goh et al., 1982). These authors, however, did not offer any explanation for the difference in the removal efficiency of phenolics from these meals.

Results presented in Tables III and IV indicate that sinapic acid constituted 65–85 and 71–97% of the free and esterified phenolic acids of hexane-extracted meals, respectively. These data are in good agreement with the gas chromatographic data reported by Dabrowski and Sosulski (1984), Krygier et al. (1982b), and Kozlowska et al. (1983) for rapeseed and mustard seed. Dabrowski and Sosulski (1984) found that sinapic acid constituted 72 and 98% of the phenolic acid fraction liberated from esters isolated from mustard (*Brassica hirta* L.) and rapeseed (*B. campestris* L.) flours. We found that the contribution of sinapic acid to phenolic acids liberated from esters was 73% for mustard and 97% for Triton meals. Thus, it is evident that the UV spectrophotometric method together with the procedure of Swain and Hillis (1959) for total phenolic acids can be used for quantification of sinapic acid in *Brassica* seeds.

The contribution of sinapic acid to the insoluble bound fraction of phenolic acids varied from 7% for Midas rapeseed to 32% for Domo mustard (Tables III and IV). While Krygier et al. (1982b) reported that contribution of sinapic acid to the insoluble bound fraction of phenolic acids of Tower hulls was 10%, Kozlowska et al. (1983) found that the contribution of sinapic acid in rapeseed and mustard flours ranged from 30 to 59%. Our data are in the range of those reported in the literature. However, the UV method may overestimate the content of sinapic acid in the insoluble bound phenolic acid fraction due to an overlap contribution from other phenolic acids to the absorption maximum at 325 nm (see above).

The MeOH–$NH_3$–$H_2O$/hexane solvent extraction system removed 45–79 and 86–93% of the original sinapic acid in the free and esterified phenolic acid fractions of treated meals, respectively (Table IV). As expected, the contribution of sinapic acid to the total insoluble bound phenolic acids was not affected to any great extent.

Data presented in Table III indicate that esterified phenolic acids are extracted more effectively than the free phenolic acids; however, we cannot offer any explanation for this observation. Removal of free phenolic acids ranged only from 41% for Midas to 75% for Hu You 9, while the level of esterified phenolics decreased by 82% for Midas and up to 93% for Hu You 9. The apparent increase in the content of insoluble bound phenolic acids in treated meals may be a direct result of dissolution of nonprotein components of seed into the MeOH–$NH_3$–$H_2O$ phase.

The extraction of canola seeds with the MeOH–$NH_3$–$H_2O$/hexane extraction system improved the color and flavor of resultant meals. These meals were bland in taste and had a light beige color. The removal of sinapic acid and other phenolic acids should also have a beneficial effect on the nutritional value of treated meals.

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**Registry No.** MeOH, 67-56-1; $NH_3$, 7664-41-7; sinapic acid, 530-59-6.