Free, Esterified, and Insoluble-Bound Phenolic Acids. 1. Extraction and Purification Procedure

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A rapid procedure for the accurate quantitation of free, esterified, and insoluble-bound phenolic acids in oilseeds is described. Phenolic acids and esters were extracted with methanol–acetone–water; the esterified and bound phenolic aglycons were released by alkaline hydrolysis. The extracts and hydrolysates were purified by washing with hexane and extraction in diethyl ether–ethyl acetate before silylation and gas–liquid chromatography.

Phenolic acids and their derivatives are widely distributed in plants, many being essential metabolites. A few specific compounds occur in relatively high concentrations in certain oilseeds and legume seeds and are implicated in the development of adverse tastes and colors in food products or in decreasing the protein nutritive value (Sosulski, 1979). The contents of chlorogenic acid in sunflower, gossypol in cottonseed, and sinapine in *Brassica* species have been quantitated by several investigators. However, the composition of minor phenolic constituents in cereals, oilseeds, and legumes has received little attention. Where quantitation has been done, the concentrations are frequently reported in only relative terms (Arai et al., 1966; Durkee and Thivierge, 1975; Fenton et al., 1980).

The soluble phenolic constituents in oilseed flours such as soybean (Arai et al., 1966), soybean, cottonseed and peanut (Maga and Lorenz, 1974), sunflower (Milic et al., 1968; Mikolajczak et al., 1970; Sabir et al., 1974), and rapeseed (Kozlowska et al., 1975) have been investigated. Some bound phenolic constituents in seeds have also been identified (Austin and Wolff, 1968; Durkee, 1977; Durkee and Thivierge, 1975; Kawakishi and Maramatsu, 1966).

Earlier separations of phenolic compounds in oilseed flours were by paper or thin-layer (TLC) chromatography, but recent procedures involve the use of gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GLC–MS). Generally the phenolic compounds were extracted with aqueous solutions of alcohol or acetone. After removal of organic solvents, the aqueous solution of phenolic acids and soluble esters was fractionated by lengthy extraction with diethyl ether or ethyl acetate. Phenolic acid esters remaining in the aqueous phase were hydrolyzed by alkali or acid to liberate the acids for quantitation as for free phenolic acids. In most cases these procedures were lengthy, and exposure to daylight and air or application of boiling conditions would result in chemical deterioration of the more reactive phenolic acids. Recently, Fenton et al. (1980) reported that previous investigators had failed to consider cis and trans isomerization of phenolic compounds, interference by lipids, and formation of artifacts during extraction and separation procedures.

The objective of this investigation was to develop a more rapid procedure for the accurate estimation of the free, esterified, and insoluble-bound phenolic acids. Although analyses were conducted on a rapeseed sample, the procedures are being employed for the determination of phenolic compounds in other oilseeds, cereals, and legumes.

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EXPERIMENTAL SECTION

Sample Preparation. The analyses were conducted on seed samples of low glucosinolate cultivars of *Brassica napus* (Tower) and *Brassica campestris* (Candle). The seed coat color of Tower was essentially black while the hulls of Candle were yellow-brown.

However, the flaked seeds were dehulled completely by hand before grinding and defatting the meats with hexane. After evaporation of solvent at room temperature, the meal was remilled and defatted again with hexane.

Procedure. The final procedure adopted for the isolation of phenolic acids is outlined in Figure 1. One gram of defatted flour was extracted 6 times in a polytron with 20 mL of 70% methanol–70% acetone (1:1) at room temperature. After centrifugation, the combined supernatants were analyzed for free phenolic acids and soluble phenolic acid esters, and the residue was reserved for determination of insoluble-bound phenolic acids. The combined supernatants were evaporated under vacuum at 45 °C to a 20-mL volume. The aqueous suspension was adjusted to pH 2 (6 N HCl) and centrifuged to separate a cloudy precipitate. The clear supernatant was extracted 5 times with hexane at a hexane to water phase ratio of 1:1 to remove free fatty acids and other lipid contaminants. The free phenolic acids were then extracted 6 times with diethyl ether–ethyl acetate (1:1) at a solvent to water phase ratio of 1:1. The ether–ethyl acetate extracts were dehydrated with anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum at 30 °C. The dry residues were transferred into vials containing a known amount of internal standard (*n*-tetracosane) by using ether–ethyl acetate as the solvent and dried before silylation under nitrogen at room temperature.

The moist sodium sulfate was washed free of phenolic acid esters, and the washings were combined with the above water phase and the cloudy precipitate. The esters were hydrolyzed with 20 mL of 4 N NaOH for 4 h under nitrogen and at room temperature. The hydrolysate was acidified to pH 2 before removing the free fatty acids with hexane. The liberated phenolic acids were then extracted with ether–ethyl acetate as described above.

The residues from the methanol–acetone extractions were hydrolyzed directly with 10 mL of 4 N NaOH under the same conditions as the esters. After acidification and centrifugation, the clear supernatants were extracted with hexane and then with ether–ethyl acetate as described above.

Gas–Liquid Chromatography. Phenolic acids were silylated by slight warming with Tri-Sil/BSA Formula D (Pierce Chemical Co.). The Me_3Si derivatives of the

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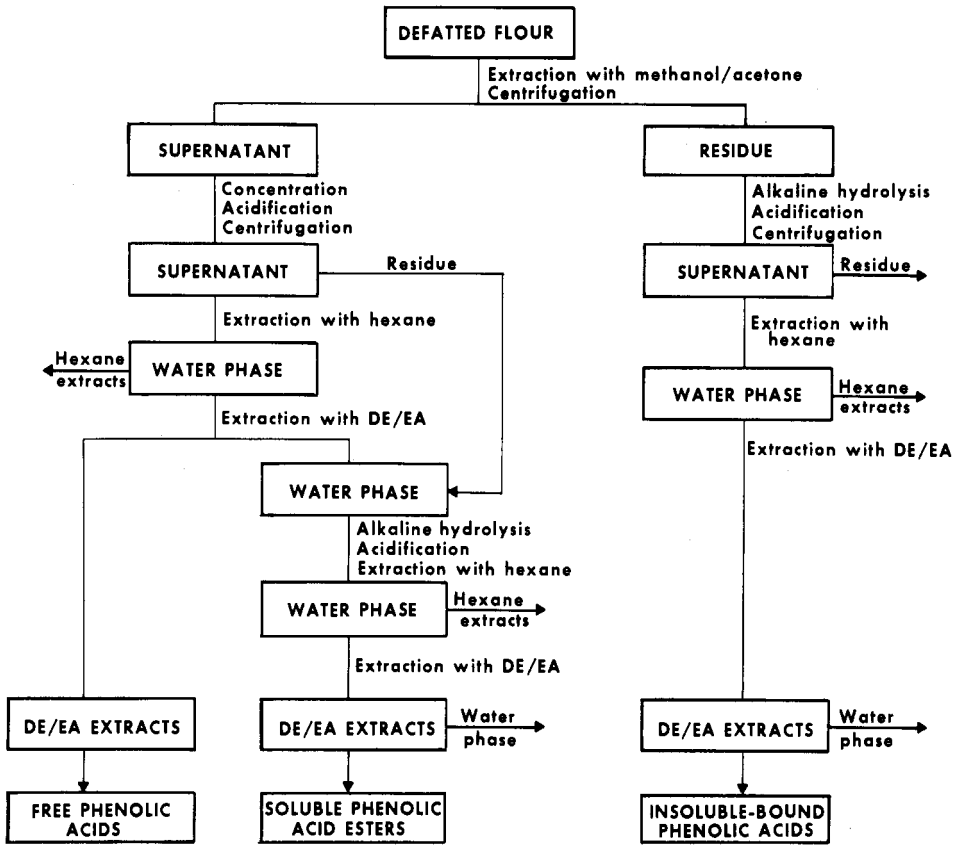

Figure 1. Procedure for the extraction and separation of the free, esterified, and insoluble-bound phenolic compounds and their hydrolysis to phenolic acids (DE/EA = diethyl ether–ethyl acetate, 1:1).

phenolic acids were separated on a Hewlett-Packard Model 5710A/30A gas chromatograph equipped with a flame ionization detector and peak area integrator. The 2.0 × 2 mm o.d. glass column was packed with 3% OV-1 on 80–100-mesh Chromosorb W (HP). The flow rate of the carrier gas was 40 cm^3/min of nitrogen. The injection and detector temperatures were 300 °C. The oven temperature was programmed at the start of the run from 120 to 300 °C at 4 °C/min. Phenolic acids were identified by comparing the retention times of the Me_3Si derivatives with the values of retention times of the Me_3Si derivatives of standard acids. The results were confirmed by TLC and GLC–MS techniques.

Thin-Layer Chromatography. The TLC plates were coated with silica gel IB2-F containing fluorescent indicator UV-254 (J. T. Baker Chemical Co.). The solvent system employed for separation of phenolic acids, fatty acids, and other contaminants was benzene–methanol–acetic acid (20:4:1). All compounds were visualized on the TLC plates as dark spots under short-wave (254-nm) UV light and the phenolic acids as blue fluorescent spots under long-wave (360-nm) UV light. All acids appeared as yellow spots after spraying the plates with a 0.02% ethanol solution of bromocresol green.

Gas Chromatography–Mass Spectrometry. The GC–MS system was a Finnigan Model 4000 instrument interfaced with an Incos Model 2300 data acquisition system. A WCOT capillary column of fused silica (0.2 mm i.d. × 24 m) coated with OV-101 was employed. The temperature was programmed 40 to 150 °C at 40 °C/min and then from 150 to 300 °C at 4 °C/min after which the temperature was held at 300 °C for up to 10 min (50-min total time). The injector temperature was 300 °C; the separator oven was at 280 °C. Mass spectra were taken at an electron energy of 70 eV and a scan time of 2.5 s from mass 40 to mass 800.

Table I. Residual Esterified Sinapic Acid Extracted by Acetone or Methanol after Five Consecutive Extractions of Tower Flour with the Alternate Solvent

	trans-sinapic acid		*cis*-sinapic acid		total sinapic acids	
alternate solvent	mg/100 g of flour	% of total in flour[a]	mg/100 g of flour	% of total in flour[a]	mg/100 g of flour	% of total in flour[a]
acetone	3.0	0.3	6.8	9.7	9.8	1.0
methanol	16.3	1.8	3.9	5.3	20.2	2.1

[a] Based on the total sinapic acids in flour (Table IV).

RESULTS AND DISCUSSION

Extraction Conditions. Previous investigators have extracted phenolic compounds with aqueous ethanol (Arai et al., 1966; Durkee, 1977; Durkee and Thivierge, 1975; Felice et al., 1976; Salomonsson et al., 1978), methanol (Kozlowska et al., 1975; Maga and Lorenz, 1974; Sabir et al., 1974), and acetone (Durkee and Thivierge, 1975; Hobson-Frohock et al., 1975; Steela and Bolan, 1972). Extraction conditions varied from room temperature to reflux or boiling conditions. The three solvents were evaluated under each of these extraction conditions on Tower flour. No single solvent was found to give a complete extraction of soluble phenolic compounds. After five consecutive extractions with 80% methanol, a single extraction of the residue with 70% acetone yielded 9.8 mg of esterified sinapic acid/100 g of original Tower flour (Table I). The yield was even greater (20.2 mg of sinapic acids) when a methanol extraction was conducted on the residues from five extractions with 70% acetone. Maximum yields of soluble phenolic acids and esters, with minimal losses due to thermal degradation and polymerization, were obtained by six extractions (20:1 v/w) with

Table II. Losses of Authentic Hydroxycinnamic Acids When Subjected to Acidic (1 N HCl; Boiling; 30 min) and Alkali (2 N NaOH; Room Temperature; 4 h; under Nitrogen) Conditions

	losses in %	
phenolic acid	acid	alkali
o-coumaric	15.1	7.5
p-coumaric	72.9	2.7
trans-isoferulic	50.3	2.7
trans-ferulic	78.0	4.8
caffeic	87.3	66.7
trans-sinapic	91.7	36.5

an equal mixture of 70% methanol and 70% acetone (methanol–acetone–water, 7:7:6) at room temperature by using the polytron.

Purification Procedure. After evaporation of methanol or acetone, the phenolic acids are usually extracted from the acidified solution by liquid–liquid extraction with diethyl ether for 24 h (Ribereau–Gayon, 1972). Refluxing under the strongly acidic environment (pH 2) for 24 h was found to cause losses of esters (chlorogenic acid) and increased free phenolic acid values. In addition, the aqueous phase contained traces of ferulic acid at the end of the extraction period. A mixture of diethyl ether and ethyl acetate (1:1) proved to be an excellent solvent for the range of phenolic compounds present in rapeseed. The extraction time was shortened substantially by extraction of the acidified solution (pH 2) with ether:ethyl acetate (about 1:1) using a separatory funnel. Six extractions of 5 min each gave essentially complete transfer of the soluble phenolic compounds into the organic solvents without evidence of hydrolysis of esters. This was confirmed by liquid–liquid extraction of the residual water phase and GLC–MS of the diethyl ether extract to show no traces of phenolic constituents in the water phase.

Dehydration of Ether–Ethyl Acetate Extracts. Further losses of phenolic acids were avoided by thorough dehydration of the extracts before evaporation to dryness, silylation, and chromatography. Anhydrous sodium sulfate was used to dehydrate the ether–ethyl acetate phase. After filtration, the moist sodium sulfate was analyzed for adsorbed phenolic compounds. Free phenolic acids were not found, but hydrolysis of the filtered moist salt with 4 N NaOH yielded sinapic acid. Apparently there was some contamination of esters in the traces of water in the ether–ethyl acetate extracts. Tentatively, the problem was corrected by washing the moist sodium sulfate with water and adding the washings back to the aqueous (pH 2) phase (Figure 1) before analysis for the phenolic acid esters.

Hydrolysis of Esters. Phenolic acid esters are usually hydrolyzed into their constituent phenolic acids for analysis as the Me_3Si ether derivatives. Hydrolysis conditions may be with NaOH at room temperature or HCl under reflux or boiling temperatures. When exposed to 1 N HCl in a boiling water bath for 30 min, the losses of a range of hydroxycinnamic acids varied from 15 to 92% (Table II). The same authentic acids, except for caffeic and sinapic acids, were relatively stable to treatment with 2 N NaOH for 4 h under nitrogen at room temperature, and the losses did not exceed 10%. The losses of caffeic acid were expected because of the reactive nature of the *o*-dihydroxyphenols (Sosulski, 1979). The reasons for disappearance of sinapic acid during the alkali treatment are still being investigated as the loss of one-third of the acid was high.

Quantitation by TLC. TLC has been employed successfully to separate and identify the phenolic acids in rapeseed meals (Durkee and Thivierge, 1975; Fenton et al., 1980); however, only relative concentration values were obtained. Several solvent systems were investigated in the present study, but, typically, the large sinapic acid spot overlapped those of the minor phenolic constituents (Figure 2; Table III). In addition, GLC–MS analysis demonstrated that syringic acid occurred in several peaks and *trans*-sinapic acid was found in bands 2 and 3.

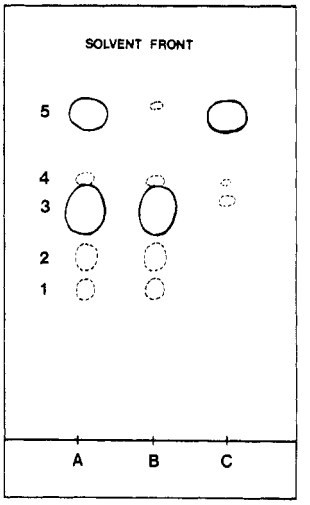

Figure 2. Thin-layer chromatogram of diethyl ether–ethyl acetate extracts of the hydrolysate of phenolic acid esters in the water phase. Samples were (A) without hexane extraction, (B) with hexane extraction, and (C) hexane extract. The spots are identified in Table III.

Table III. R_f Values and Identity of Phenolic Acids Liberated from Soluble Esters and Separated by TLC (Figure 2)

band no.	R_f	identity (GLC-MS)
1	0.37	syringic (trace)
2	0.44	syringic *trans*-sinapic
3	0.58	*trans*-sinapic *cis*-sinapic syringic *p*-hydroxybenzoic *p*-coumaric *cis*-ferulic
4	0.62	syringic *cis*-ferulic *trans*-ferulic vanillic
5	0.76	fatty acids

Quantitation by GLC. Sinapine, the sinapic acid choline ester, is the principal phenolic compound in rapeseed (Tzagoloff, 1963). The hydrolysates of the soluble esters yielded a large *trans*-sinapic acid peak which was typically asymmetric or a double peak (Figure 3). Similar results were obtained by Kozlowska et al. (1975) for a Span rapeseed flour. During GLC–MS analysis of the phenolic acids, using a capillary column (0.2 mm × 24 m), oleic and other C_{18} fatty acids were found to be present in the hydrolysates, their retention times being close to that of *trans*-sinapic acid (Figure 4). Similarly, the C_{16} fatty acid, palmitic, was identified as occurring in the same region as *cis*-sinapic, ferulic, and isoferulic acids in the chromatogram.

The hydrolysates of the water phase containing the phenolic esters were fractionated by TLC to demonstrate the presence of a large fraction of free fatty acids (Figure 2). Polar lipids represent a high proportion of the residual lipids in rapeseed flours (Sosulski et al., 1981). Apparently these were extracted by the methanol–acetone solvent and remained in the water phase to be hydrolyzed by the alkali

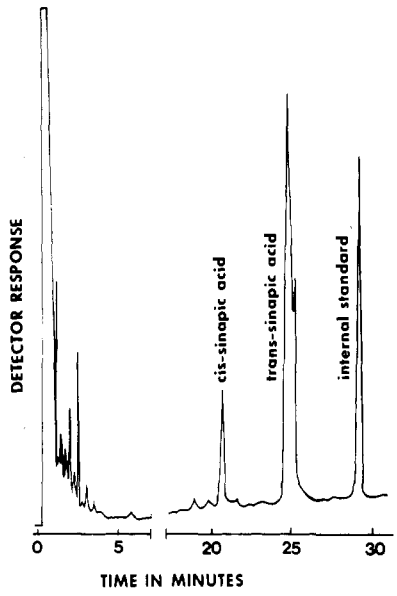

Figure 3. GLC chromatogram of the Me_3Si derivatives of phenolic acids liberated from soluble phenolic acid esters in Tower flour (2.0 m × 2 mm glass column packed with 3% OV-1 on 80–100-mesh Chromosorb W (HP).

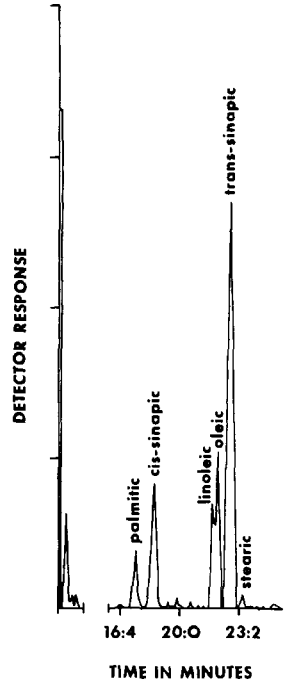

Figure 4. GLC chromatogram of the Me_3Si derivatives of phenolic and fatty acids liberated from the water-soluble ester fraction in Tower flour. Separation was on a capillary column (0.2 mm × 24 m) coated with OV-101.

treatment. Hexane extraction of the acidified water phase removed the free fatty acids effectively (Figure 2). The hexane extract showed two minor spots with retention times which were similar to those of phenolic acids. GLC–MS analyses showed that neither compound(s) was (were) phenolic in nature. Further, the solubility of phenolic acids in hexane was checked by preparing a solution of 13 phenolic acids (salicylic, *p*-hydroxybenzoic, vanillic, gentisic, *o*-coumaric, protocatechuic, syringic, *p*-coumaric, *trans*-isoferulic, *trans*-ferulic, caffeic, *trans*-sinapic, and *trans*-chlorogenic acids) and adjusting to pH 2. The acidified solution was extracted with hexane in the same way as the previous sample (5 times in a separatory funnel at a hexane to water phase ratio of 1:1). Only traces of

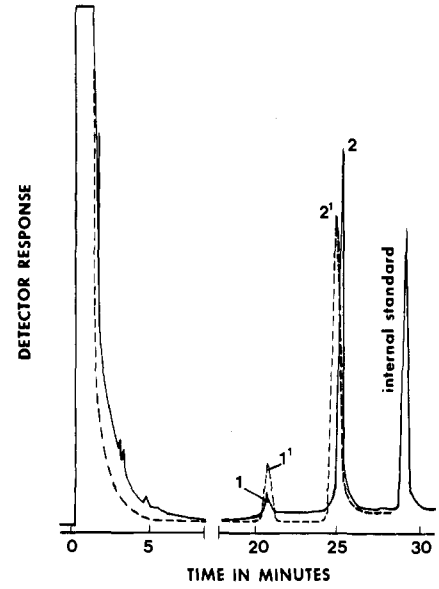

Figure 5. GLC chromatograms of the Me_3Si derivatives of the phenolic and fatty acids in the hexane (dashed line) and ether–ethyl acetate (solid line) extracts of the soluble ester fraction in Candle flour (1 = *cis*-sinapic acid; 2 = *trans*-sinapic acid; 1′ = palmitic acid; 2′ = oleic acid).

Table IV. Contents of Sinapic Acids Liberated from Soluble Esters with (A) and without (B) Hexane Extraction of Free Fatty Acids

phenolic compound	Candle		Tower	
	A	B	A	B
cis-sinapic, mg/100 g of flour	72.1	176.6	73.6	87.6
trans-sinapic, mg/100 g of flour	1116.2	1586.4	894.9	1479.6
sinapine, % of flour	1.74	2.57	1.41	2.29

vanillic acid were found in the hexane extract by GLC–MS analysis.

A comparison of the GLC chromatograms of the hexane and ether–ethyl acetate extracts of the hydrolyzed phenolic acid esters in Candle flour show how palmitic and oleic acids would interfere with the quantitation of *cis*- and *trans*-sinapic acids (Figure 5). The contents of *cis*- and *trans*-sinapic acids which were liberated from soluble esters in Candle and Tower rapeseed were 1188 and 968 mg/100 g of defatted flour (Table IV). When calculated as the choline ester, using the factor of 1.46, the total sinapine content was 1.74 and 1.41%, respectively, of the flour for the two varieties. Without hexane extraction, the values would have been 2.57 and 2.29%, respectively. If it is assumed that the losses of *cis*- and *trans*-sinapic acids were the same (Table II), the corrected sinapine levels in the flours would be 2.74 and 2.22% in Candle and Tower rapeseed. Based on several determinations, the standard deviations for the total sinapic acid contents of the two flours were 7–9% of the sample mean.

In the present samples, free fatty acids did not appear to interfere with the quantitation of the free phenolic acids (Figure 1). However, free fatty acids can be present in seeds and flours, especially after long storage. Also, alcohol solvents, such as methanol–acetone used in the present study, can disrupt lipid–protein complexes to release fatty acids. While the amount of liberated fatty acids may not be high, the quantities of free phenolic acids in a sample

are also minute. Therefore, the hexane extraction step has been introduced for the purification of each phenolic fraction in the analytical scheme proposed in Figure 1. In general, the extraction and isolation procedures proposed require only one-fifth the time involved in previous methods (Kozlowska et al., 1975; Sabir et al., 1974). The procedure should greatly reduce the opportunity for formation of artifacts during the extraction and separation procedures (Fenton et al., 1980).

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Received for review March 5, 1981. Accepted December 2, 1981. This investigation was supported by the Canola Council of Canada under the Canola Utilization Assistance Program.

Free, Esterified, and Insoluble-Bound Phenolic Acids. 2. Composition of Phenolic Acids in Rapeseed Flour and Hulls

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The composition of free, esterified, and insoluble-bound phenolic acids in rapeseed cultivars was determined by gas–liquid chromatography and mass spectrometry. Rapeseed hulls contained no free phenolic acids and relatively low levels of soluble ester and bound phenolics. Sinapic acid was the principal phenolic acid released by hydrolysis of the soluble esters in the hulls while protocatechuic acid was the major phenolic acid in the residues. The flours contained 6–98 mg/100 g of free phenolic acids, 768–1196 mg/100 g of phenolic acids from hydrolyzed esters, and no phenolic acids in the residues. Sinapic acid represented a high proportion of the free phenolic acids and 99% of acids released from esters in the flours. Minor phenolic acids included *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, *p*-coumaric, and ferulic acids in the various fractions and cultivars.

During the past decade, the quality of rapeseed has been improved markedly by plant breeding to reduce the levels of erucic acid in the oil and thioglucosides in the meal. Unfortunately, the "canola" type of rapeseed meal has limited feed applications due to its dark appearance, high proportion of hulls, and strong flavor. Yellow-seeded cultivars including the mustards are significantly lower in crude fiber content than black-seeded rapeseed (Sosulski, 1979). Recently a yellow/brown-seeded cultivar was developed in which hull and crude fiber levels are intermediate.

In addition to the reduction in meal fiber, plant breeders are seeking to improve the palatability of the canola-type meals by selecting for low levels of sinapine. Sinapine is the choline ester of sinapic acid which occurs in rapeseed meals at levels of 1.0–2.5% under Canadian conditions (Mueller et al., 1978). In addition to its adverse effects on meal flavor and palatability, sinapine is responsible for fishy odors in brown-shelled eggs when incorporated into poultry rations (Hobson-Frohock et al., 1975). Numerous other phenolic compounds have been identified in rapeseed meals and flours (Durkee and Thivierge, 1975; Kozlowska et al., 1975; Lo and Hill, 1972), but there is little quantitative information available on which to base a plant breeding and selection program.

The objectives of this investigation were to obtain qualitative and quantitative information on the phenolic acids in rapeseed by gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GLC–MS) methods. Since many of the important phenolic acids and esters are highly reactive, the analyses were designed to separate the free, soluble ester, and insoluble-bound phenolic acids present in the flours and hulls based on the fractionation procedure of Krygier et al. (1982). The study was conducted on the defatted flours of yellow-, yellow/brown-, and black-seeded cultivars of rapeseed, as well as the hulls of the black cultivar.

EXPERIMENTAL SECTION

Materials. Analyses were conducted on freshly harvested seeds of the cultivars Yellow Sarson, strain R-500 (*Brassica campestris* L.), Candle (*B. campestris* L.), and Tower (*Brassicanapus* L.). The seeds were flaked, the hulls separated by hand, and meats ground and defatted

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0021-8561/82/1430-0334$01.25/0