vivo activity at least when administered in the diet, (2) in vivo inhibition correlates well with synergism of methyl paraoxon, (3) lack of antiacetylcholinesterase activity by most compounds make them useful tools for selective inhibition of certain B-esterases, and (4) the degree of synergism is too low to be of economic value in boll weevil control.

Registry No. 1, 1642-44-0; 2, 85480-01-9; 3, 78-48-8; 4, 78788-15-5; 5, 3819-72-5; 6, 68598-42-5; 7, 68598-41-4; 8, 2797-64-0; 9, 68598-40-3; 10, 68598-39-0; 11, 68598-38-9; 12, 68598-37-8; 13, 85480-02-0; 14, 85480-03-1; 15, 85480-04-2; 16, 68598-36-7; 17, 85480-05-3; 18, 68598-35-6; 19, 85480-06-4; 20, 85480-07-5; 21, 85480-08-6; 22, 26115-85-5; 23, 26115-86-6; 24, 24067-01-4; 25, 85480-09-7; 26, 24067-02-5; 27, 85480-10-0; 28, 30299-04-8; 29, 85480-11-1; 30, 4081-23-6; methyl paraoxon, 950-35-6; B-esterase, 9016-18-6.

Supplementary Material Available: A listing of data from IR and ^{1}H NMR spectra of esters 1–30 and MS of esters 3, 8, 18, 21, and 24 (7 pages). Ordering information is given on any current masthead page.

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Quantitation of Free and Hydrolyzable Phenolic Acids in Seeds by Capillary Gas–Liquid Chromatography

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Free phenolic acids were initially extracted with tetrahydrofuran, followed by extraction of soluble phenolic esters with methanol–acetone–water. Alkaline hydrolysis was employed to release aglycons from soluble phenolic esters (2 N NaOH) and residue (4 N NaOH). The Me_3Si derivatives of the released phenolic acids were quantitated by capillary gas–liquid chromatography within an elution time of 20 min. Greater precision was achieved by using methyl heptadecanoate instead of *n*-tetracosane as the internal standard. Although operations were conducted in the dark under nitrogen, recoveries of *p*-hydroxybenzoic, vanillic, and syringic acids exceeded 90%, but those of *p*-coumaric, ferulic, and sinapic acids were 87, 82, and 78%, respectively, and that of caffeic acid was only 17%.

Phenolic compounds occur widely as microconstituents in plant foods and there is increasing interest in their effects on food quality. Among the various forms of phenolic compounds, the free phenolic acids, esters, and glycosides that contain an acrylic acid group conjugated with the aromatic ring are of particular concern. These monocyclic phenolics are readily oxidized, leading to the formation of quinones which further react to form polymers or bind to proteins and carbohydrates (Sabir et al., 1974; Van Sumere et al., 1975). The *o*-dihydroxyphenols such as caffeic acid are particularly reactive and, thus, are found in only low concentrations in seeds (Sosulski, 1979). However, esters of these phenolic acids, such as chlorogenic acid (3-caffeoylquinic acid), occur widely in plant tissues and represent 3–4% of the defatted flour from sunflower. Sabir et al. (1974) demonstrated that, at neutral pH, one-third of the chlorogenic acid in slurries of sunflower flour was covalently bonded to low molecular weight proteins and polypeptides. Under alkaline conditions, oxidation and bonding of chlorogenic acid with sunflower proteins may be extensive, developing dark green colors that oxidize further to a dark brown on prolonged storage.

Simple phenolic acids have been characterized as having sour, bitter, astringent, and phenol-like flavors (Arai et al., 1966; Maga and Lorenz, 1973). However, it is the choline ester of sinapic acid, sinapine, which occurs in concentrations of 0.5–1.5% in defatted crambe (Austin and Wolff,

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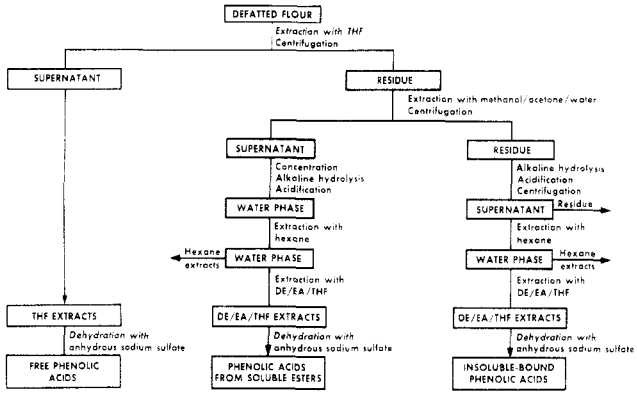

Figure 1. Procedure for the extraction and separation of free, esterified, and residue phenolic compounds and their hydrolysis to phenolic acids for analysis by capillary GLC (DE/EA/THF = diethyl ether–ethyl acetate–tetrahydrofuran, 1:1:1).

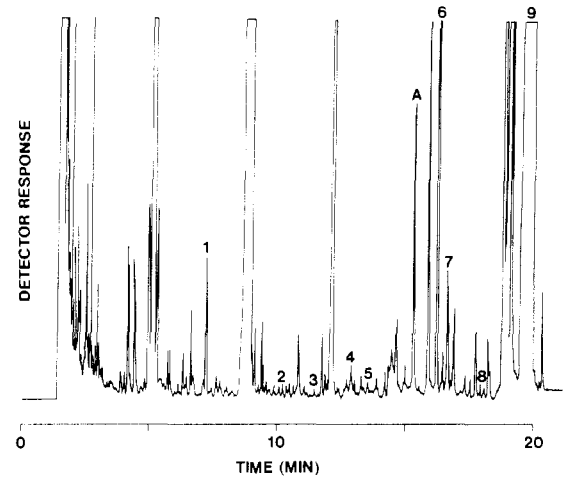

Figure 2. GLC chromatogram of Me_3Si derivatives of phenolic acids released on hydrolysis of the soluble ester fraction in rapeseed flour using a WCOT capillary column of fused silica (0.2 mm i.d. × 25 m) coated with OV-1. 1 = *p*-hydroxybenzoic, 2 = vanillic, 3 = protocatechuic, 4 = syringic, 5 = *trans*-*p*-coumaric, 6 = *cis*-sinapic, 7 = *trans*-ferulic, 8 = *trans*-caffeic, and 9 = *trans*-sinapic acids. A = methyl heptadecanoate as the internal standard.

1968) and rapeseed (Kozlowska et al., 1975). Glycosides of sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) are also present in *Brassica* meals (Fenton et al., 1980). These esters of sinapic acid are responsible for the strong bitterness and astringency of rapeseed flours and protein concentrates. The relative order of bitterness of equimolar concentrations of the ester and its components were sinapine > sinapic acid > choline (Ismail et al., 1981).

Simple phenolic compounds usually do not bind as extensively with proteins as the polymeric tannins, and there are few reports of antinutritional effects on protein utilization. There are literature reports of phenolic acids binding with enzymes (Loomis and Battaile, 1966). Pierpoint (1969) has elucidated the mechanism of chlorogenic and caffeic acid binding to the ϵ-amino group of lysine or thiol group of cysteine. In leaf protein concentrates, the reductions in available lysine during extraction were attributed to reactions with reducing sugars or quinones (Allison et al., 1973).

Phenolic acid esters can be quantitated directly by HPLC (Ong and Nagel, 1978). Alternatively, Krygier et al. (1982a) developed a procedure for fractionating the total phenolic constituents of flours into free, soluble ester, and hydrolyzable-residue forms and, after hydrolysis, determining the relative proportions of the released phenolic acids by GLC. Rapeseed flours contained 6–98 mg/100 g free phenolic acids, 768–1196 mg/100 g phenolic acids from hydrolyzed esters, and no phenolic acids from the residues (Krygier et al., 1982b). The total phenolic acid contents of wheat, rice, and oat flours ranged from 71 to 87 ppm while corn flour contained 309 ppm and potato flour 410 ppm (Sosulski et al., 1982). The principal phenolic compounds were esterified sinapic acid in rapeseed, bound ferulic acid in the cereal flours, and free chlorogenic acid in potato flour.

The objectives of the present investigation were to improve the procedures for quantitating the free phenolic acids in seed materials, avoiding hydrolysis of esterified acids during the extraction process. The effectiveness of alternate solvent and purification systems was evaluated by thin-layer chromatography (TLC), gel filtration (GF), capillary gas–liquid chromatography (GLC), and mass spectroscopy (MS). Recoveries of the common phenolic acids under the proposed extraction and hydrolysis procedures were determined. Reference phenolic acids and, because of their wide distribution of phenolic acids and esters, rapeseed flours were utilized in the various determinations. The modified procedure was then applied to the analysis of defatted flours from 10 oilseed species (Dabrowski and Sosulski, 1984) and the flours and hulls of 10 legume species (Sosulski and Dabrowski, 1984).

EXPERIMENTAL SECTION

Sample Preparation. Reference phenolic acids from ICN Pharmaceuticals, Inc., and Sigma Chemical Co. (salicylic, *trans*-cinnamic, *p*-hydroxybenzoic, gentisic, protocatechuic, vanillic, syringic, *trans*-*o*-coumaric, *trans*-*m*-coumaric, *trans*-*p*-coumaric, *trans*-isoferulic, *trans*-ferulic, *trans*-caffeic, and *trans*-sinapic acids) were dissolved in tetrahydrofuran in known concentrations. Prior to silylation, aliquots were evaporated under nitrogen in vials to which the internal standard, *n*-tetracosane or methyl heptadecanoate, was added. Phenolic acids were silylated by slight warming with *N*,*O*-bis(trimethylsilyl)-acetamide Formula D (Pierce Chemical Co.).

Rapeseed flours used in the study were from the low glucosinolate cultivars of *Brassica napus* (Tower) and *Brassica campestris* (Candle). The seeds were flaked and dehulled by hand before oil extraction with hexane. Sinapine was isolated as the bisulfate salt from a portion of the Candle meal by the method of Clandinin (1961).

Extraction Conditions. To avoid light-mediated isomerization and oxidation of phenolic compounds the extraction, hydrolysis, and concentration steps were conducted in the dark under nitrogen atmosphere.

Free phenolic acids were extracted from the rapeseed flours with tetrahydrofuran, tetrahydrofuran–acetone (1:1), and acetone–methanol (1:1) as well as the methanol–acetone–water (7:7:6) solvent for free phenolic acids and esters. In each case, 1 g of flour was extracted 6 times with 20 mL of solvent by using a Polytron mixer (Brinkmann Instruments, Inc.). The combined extracts were centrifuged, and the supernatant was evaporated to dryness under vacuum at 30 °C and stored for GLC analysis. In parallel experiments, the supernatants were concentrated to 10 mL under nitrogen at room temperature for fractionation by TLC and GF.

Procedure for Flours. Initially, the scheme of Krygier et al. (1982a) for extraction and separation of free, esterified, and residue phenolic acids was followed. The final procedure adopted involved the separate extraction of free

Table I. Precision of Gas Chromatographic Determination of Me_3Si Derivatives of Reference Phenolic Acids Using Two Internal Standards (Means of Eight Determinations)

			n-tetracosane				methyl heptadecanoate			
phenolic acid	RRT[a]	quantity applied, μg	quantity recovered, μg	recovery, %	SD, μg	SD, % of mean	quantity recovered, μg	recovery, %	SD, μg	SD, % of mean
salicylic	0.33	113.9	113.0	99.2	11.4	10.0	110.3	96.8	9.2	8.3
trans-cinnamic[b]	0.35	112.0	114.8	102.5	12.4	10.8	111.9	99.9	5.1	4.5
p-hydroxybenzoic	0.47	134.0	136.3	101.7	12.5	9.2	133.0	99.2	6.1	4.6
vanillic	0.66	188.0	190.6	101.3	13.2	6.9	190.9	101.5	5.1	2.7
gentisic	0.70	186.0	184.9	99.4	11.3	6.1	181.6	97.6	4.6	2.5
trans-*o*-coumaric	0.71	129.1	132.0	102.2	8.5	6.4	127.9	99.1	2.5	1.9
protocatechuic	0.75	129.1	129.2	100.0	6.9	5.3	129.0	99.9	2.5	1.9
trans-*m*-coumaric	0.80	170.9	174.2	101.9	9.2	5.3	172.3	100.8	3.6	2.1
syringic	0.84	193.7	197.5	101.9	9.8	5.0	197.0	101.7	2.0	1.0
trans-*p*-coumaric	0.89	193.7	198.2	102.3	8.9	4.5	196.4	101.4	4.4	2.2
trans-isoferulic	1.07	208.9	213.5	102.2	7.6	3.6	208.7	99.9	2.3	1.1
trans-ferulic	1.09	199.4	201.5	101.0	6.7	3.3	203.4	102.0	3.2	1.6
trans-caffeic	1.16	237.3	238.7	100.6	4.4	1.8	236.4	99.6	3.9	1.6
trans-sinapic	1.28	364.6	366.5	100.5	9.7	2.6	363.1	99.0	9.3	2.6

[a] Relative retention time. [b] Not a phenolic acid.

phenolic acids with tetrahydrofuran (Figure 1). This solvent was also included with diethyl ether–ethyl acetate (1:1:1) to transfer the hydrolyzed phenolic acids from the aqueous phase in the analysis of phenolic acid esters and residue phenolics.

One gram of defatted flour was extracted 6 times for 5 min each with 20 mL of tetrahydrofuran in the Polytron mixer, and the combined supernatants were tested for free phenolic acids as above. The residue was then extracted 6 times with 20 mL of methanol–acetone–water (7:7:6) at room temperature. After centrifugation, the combined supernatants were evaporated under vacuum at 40 °C to obtain a water suspension containing the soluble phenolic esters and glycosides. The aqueous suspension was combined with an equal volume of 4 N NaOH for hydrolysis at 2 N NaOH for 4 h under nitrogen at room temperature. The hydrolysate was adjusted to pH 2 with 6 N HCl and extracted in a separatory funnel 5 times with hexane at a hexane to water phase ratio of 1:1 to remove free fatty acids and other lipid contaminants. The released phenolic acids were then extracted 6 times with diethyl ether–ethyl acetate–tetrahydrofuran (1:1:1) at a solvent to water phase ratio of 1:1. The ether–ethyl acetate–tetrahydrofuran extracts were dried with anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum at 30 °C. Methyl heptadecanoate (250 μg), dissolved in tetrahydrofuran, was added to the dry residue and the solvent was evaporated under nitrogen at room temperature.

The moist residue from methanol–acetone–water extraction was hydrolyzed directly with 10 mL of 4 N NaOH under the same conditions as above. Further purification steps for the released acids were the same as for the above hydrolysates.

Gas–Liquid Chromatography. The Me_3Si derivatives of the phenolic acids were separated on a Hewlett-Packard Model 5880A gas chromatograph equipped with flame ionization detector and connected to a Spectra-Physics SP 4100 computing integrator. A WCOT capillary column of fused silica (0.2 mm i.d. × 25 m), coated with OV-1, was used. Initial temperature of 112 °C was held for 3 min, after which the temperature was programmed to 260 °C at 4.5 °C/min. The injector temperature was 250 °C and detector temperature 300 °C. Phenolic acids were identified on the basis of relative retention times of Me_3Si derivatives in the sample and Me_3Si derivatives of reference acids. The results were confirmed by GLC–MS techniques as described by Krygier et al. (1982a).

Thin-Layer Chromatography. The TLC plates were coated with silica gel IB2-F containing fluorescent indicator UV-254 (J.T. Baker Chemical Co.). A solvent system of butanol–acetic acid–water (40:7:32) was applied for separation of rapeseed extracts and reference compounds. Phenolic compounds were visualized on the TLC plates as blue fluorescent spots under long-wave (366-nm) UV light.

Gel Filtration. Gel chromatography of tetrahydrofuran extracts from rapeseed and reference sinapic acid was conducted according to Lattanzio and Marchesini (1981) using Sephadex LH-20 resin (Pharmacia Fine Chemicals AB, Sweden). The column (bed volume 19 × 2.5 cm) was equilibrated by using an aqueous ethanolic (50%) solution acidified to pH 2 with concentrated H_3PO_4. A 5-mL aliquot of rapeseed extract or 1 mL (2.0 mg) of reference sinapic acid was applied to the column. The elution solution was the same as above with a flow rate of 39 mL/h. The fractions (3 mL) were collected and the absorbance read at 332 nm.

RESULTS AND DISCUSSION

Capillary GLC. The WCOT capillary column of fused silica coated with OV-1 used in this study gave an excellent separation of Me_3Si derivatives of phenolic acids (Figure 2). Total chromatographic analysis of phenolic acids, including sinapic acid, could be performed in 20 min.

Reference Standard. Poor repeatability of reference phenolic acid determinations was observed when *n*-tetracosane was used as an internal standard. Among three other internal standards tested, methyl heptadecanoate proved to be most suitable for phenolic acid analysis. Table I shows that higher precision was obtained with methyl heptadecanoate than with *n*-tetracosane. Also, the relative standard deviations of GLC determinations of reference phenolic acids were decreased by approximately half when methyl heptadecanoate was used instead of *n*-tetracosane. This could be due to the better solubility of the former in the silylation reagent.

Extraction Conditions. The methanol–acetone–water used to extract the soluble phenolic acids and esters by Krygier et al. (1982a) was effective in the solubilization of 16 standards. Only *trans*-sinapic acid required slight warming and shaking for the solubilization of 10 mg of acid in 0.20 mL of solvent. However, the subsequent separation of the free acids from the esters gave variable results. The

Table II. Apparent Free Sinapic Acid Content in Candle Flour Determined Using Four Solvent Systems for Initial Extraction

alternate solvent system	mg/100 g of flour	% of the total in flour[a]
acetone–methanol–water (7:7:6)	75.3	6.5
water	80.5	6.9
tetrahydrofuran–water	74.2	6.4
tetrahydrofuran	trace	0.0

[a] Based on the total sinapic acid in flour (Table III).

difficulty was overcome by using tetrahydrofuran as the solvent for the initial extraction of the free acids (Figure 1). Most of the phenolic acids (*p*-hydroxybenzoic, salicylic, *trans*-cinnamic, vanillic, gentisic, *o*-coumaric, protocatechuic, *m*-coumaric, *p*-coumaric) were immediately soluble in tetrahydrofuran; the remainder (syringic, *trans*-isoferulic, *trans*-ferulic, *trans*-caffeic, *trans*-sinapic) dissolved readily with shaking. On the other hand, chlorogenic acid and sinapine were completely insoluble in this solvent. As a further test of the solvent, tetrahydrofuran used in six 1-min extractions with the Polytron was effective in extracting 95.3% of sinapic acid added to rapeseed flour, but no sinapine or added chlorogenic acid was recovered. The solubility of free phenolic acids was not improved by combining tetrahydrofuran with acetone.

The rapeseed flour samples were extracted with fresh tetrahydrofuran 6 times for 5 min each in the Polytron mixer or in a single 24-h extraction using a magnetic stirrer and beaker. Essentially no free phenolic acids were detected in the rapeseed flours, except for occasional traces of sinapic acid. These results were confirmed by TLC and GF of the extracts and GLC–MS of the peaks in the GLC chromatograms. The low concentrations of free phenolic acids found in the investigations of Krygier et al. (1982b) and others (Fenton et al., 1980; Kozlowska et al., 1975) may have been artifacts of the extraction and purification procedure.

Stepwise analysis of the fractionation scheme revealed that the initial steps, including extraction, concentration, and acidification of the aqueous extract to pH 2 (Figure 1), had caused partial hydrolysis of esters from which sinapic acid was liberated. This phenomena occurred in several solvent systems in which water was a component (Table II). In these conditions 74–80 mg of sinapic acid/100 g of flour (constituting about 7% of total sinapic acid present in flour in form of esters) was released. Similarly, chlorogenic acid was solubilized in methanol–acetone–water, and after evaporation of the organic solvents and adjustment to pH 2, quinic and caffeic acids were found in the GLC chromatograms (Figure 3).

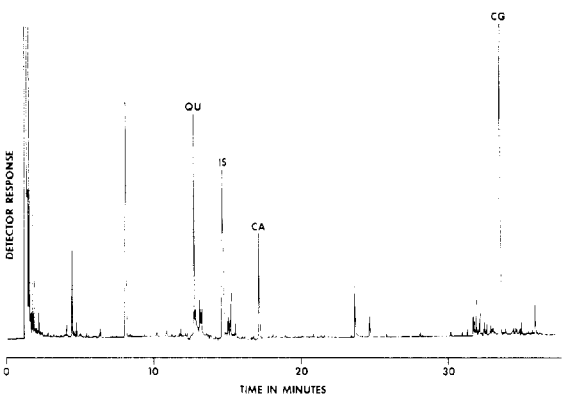

Figure 3. GLC chromatogram of Me_3Si derivatives of compounds extracted in the free phenolic acid fraction after subjecting chlorogenic acid to acidic (pH 2) conditions of the Krygier et al. (1982a) procedure. QU = quinic acid, IS = internal standard, CA = caffeic acid, and CG = chlorogenic acid.

Isomerization and oxidation of chlorogenic acids (Sondheimer, 1964) would account for some of the small peaks that eluted near the chlorogenic acid peak in Figure 3.

As a result of these observations, the Krygier et al. (1982a) procedure has been modified to include an initial extraction of the defatted flour or other plant material with tetrahydrofuran to detect and quantitate the free phenolic acids only (Figure 1). The residue would then be extracted for soluble esters and glycosides with methanol–acetone–water, with the organic solvents being removed by evaporation in order to conduct the hydrolysis under aqueous conditions. It was also found that the transfer of the hydrolyzed phenolic acids from the aqueous phase was improved by the addition of tetrahydrofuran to the diethyl ether–ethyl acetate extracting solution. The recoveries of some minor phenolic acids were increased severalfold, permitting quantitation of most peaks (Figure 2; Table III). Also, the isomerization of *trans*-phenolic acids to the cis form was reduced substantially.

Recovery of Phenolic Acids. As mentioned previously, no free phenolic acids were found in the Candle or Tower flours nor were any acids recovered from the hydrolysates of the residues. However, nine phenolic acids were released on hydrolysis of the soluble ester fraction in both rapeseed flours (Figure 2; Table III). Sinapic acid containing compounds predominated in the ester fraction; the small quantity of *cis*-sinapic likely arose via isomerization of the natural trans form. *trans*-Ferulic and *p*-hydroxybenzoic acids appeared in much greater concentrations than obtained by Krygier et al. (1982b).

Previously, Krygier et al. (1982a) reported that the alkaline hydrolysis conditions (2 N NaOH) employed in the procedure would destroy about two-thirds of the caffeic

Table III. Phenolic Acids Released on Hydrolysis of the Soluble Ester Fraction from Candle Flour and Recoveries of Admixtures of Authentic Phenolic Acids with Candle Flour after Subjection to Alkaline Hydrolysis Conditions

phenolic acid	present in flour, μg/g of flour	admixed, μg/g of flour	total recovered, μg/g of flour	recovery of added phenolic acids,[a] %
p-hydroxybenzoic	56 ± 5[a]	184	223	91.0
vanillic	7 ± 2	161	156	91.4
protocatechuic	trace			
syringic	11 ± 2	164	160	90.4
trans-*p*-coumaric	5 ± 2	235	209	86.7
trans-ferulic	150 ± 26	280	380	82.3
cis-sinapic	330 ± 46		304	
trans-sinapic	11 107 ± 1033	11 000	19 716	78.3
trans-caffeic	5 ± 2	281	52	16.7

[a] Based on four determinations.

acid and one-third of the sinapic acids in rapeseed flour. Appropriate quantities of the authentic phenolic acids present in rapeseed were added to the flour (Table III). The tetrahydrofuran extraction step was bypassed in order to subject the phenolic compounds to the alkaline hydrolysis conditions. Recoveries of *p*-hydroxybenzoic, vanillic, and syringic acids exceeded 90%. Increasing losses were evident for *p*-coumaric, *trans*-ferulic, and *trans*-sinapic acids, and correction factors would be justified in reporting data on their composition. Nearly all of the added caffeic acid was lost under the conditions of the hydrolysis, and no estimate of the composition of caffeic acid esters could be made by the present procedure, including that of chlorogenic acid. HPLC methods for quantitation of these phenolic acid esters would be recommended (Ong and Nagel, 1978).

The low recovery of caffeic acid (3,4-dihydroxycinnamic acid) can be attributed to the instability of the *o*-dihydroxycinnamyl moiety in the presence of an alkaline reagent (Harborne, 1964; Sutherland, 1958). The rapid oxidation rate of caffeic acid reflected its low oxidation potential of +0.35 V in 1 M acetate at pH 4.7 (Felice et al., 1976). The oxidation potentials of other phenolic acids were as follows: for *p*-hydroxybenzoic, +0.99; for vanillic, +0.72; for syringic, +0.55; for *p*-coumaric, +0.73; for ferulic, +0.57. The oxidation of syringic acid (Table III) was less than predicted from the oxidation potential because two of the three hydroxyl groups were methylated as in the case of sinapic acid.

Salomonsson et al. (1978) have recommended the use of enzymes rather than alkali for hydrolysis of alkali-labile phenolic esters. The addition of reducing agents such as sodium tetraborohydrate prior to the alkaline treatment resulted in high recoveries of compounds containing the 3,4-dihydroxycinnamyl moiety (Schroeder, 1967). Herrmann and co-workers (Schmidtlein and Herrmann, 1975; Schulz and Herrmann, 1980a,b) have used this reducing agent to suppress the formation of *o*-quinones in TLC and GLC analysis of caffeic and sinapic acids; their recoveries were in the range of 87–88%. The present authors were unable to improve the recoveries of caffeic or sinapic acids through the use of sodium tetraborohydrate but this approach is still being investigated.

ACKNOWLEDGMENT

Technical assistance was provided by H. Braitenbach and L. Hogge.

Registry No. Salicylic acid, 69-72-7; *trans*-cinnamic acid, 140-10-3; *p*-hydroxybenzoic acid, 99-96-7; vanillic acid, 121-34-6; gentisic acid, 490-79-9; *trans*-*o*-coumaric acid, 614-60-8; protocatechuic acid, 99-50-3; *trans*-*m*-coumaric acid, 14755-02-3; syringic acid, 530-57-4; *trans*-*p*-coumaric acid, 501-98-4; *trans*-isoferulic acid, 25522-33-2; *trans*-ferulic acid, 537-98-4; *trans*-caffeic acid, 501-16-6; *trans*-sinapic acid, 7362-37-0; *cis*-sinapic acid, 7361-90-2.

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