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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FERULIC ACID IN WHEAT MILLING FRACTIONS AS A MEASURE OF BRAN CONTAMINATION*

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SUMMARY

A high-performance liquid chromatographic method was developed for selective determination of ferulic acid in 7 min in the extracts from wheat flour and ground whole wheat at typical levels of 50 and 500 μg/g, respectively. Recovery of 99.9% was obtained when ferulic acid was extracted into dilute sulfuric acid, followed by enzymatic treatment of the extract with an α-amylase preparation. The chromatographic system included a 100-mm column packed with Hypersil 5 μm reversed-phase ODS operating isocratically with 12% methanol–citrate buffer (pH 5.4) mixture. The selectivity and sensitivity of both ultraviolet diode array and fluorescence detectors was investigated. The optimum wavelengths selected were 320 nm and 312 nm/418 nm respectively. Relative standard deviations of the anlytical procedure were 2.43% and 5.10% for whole wheat and flour samples, respectively.

INTRODUCTION

Flour milling attempts to physically separate the starchy endosperm of wheat from the germ and bran. Assessment of the efficiency of separation, requires a sensitive analytical procedure chemically specific for the bran. Previous work in fluorescence microscopy of wheat indicated that methods based on determination of ferulic (4-hydroxy-3-methoxycinnamic) acid should serve that purpose[1,2]. The work described here includes development of a high-performance liquid chromatographic (HPLC) method with selective fluorescence detection of ferulic acid in the presence of other materials found in the extracts of wheat flour and milling fractions.

Fluorescence microscopy combined with microchemical staining techniques applied to wheat kernels[1,3,4] showed that ferulic acid occurred in high concentrations in the aleurone cell walls, to a lesser extent in the seed coat and embryo, and only as traces in the starchy endosperm.

Spectrofluorimetry[5] of tediously dissected botanical parts including both per-

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icarp and aleurone fractions of the bran, in addition to the endosperm, corroborated the microscopic observations. Botanical part determination based on multiterm empirical relationships of spectrofluorescence data from slurries of finely ground plant material was suggested. The work of plant researchers indicates that the nature of morphological deposits in wheat makes chemically specific determination preferable over indirect bran impurity tracing methods based upon minerals (ash on ignition) or color (from pigments predominant for bran) observed visually or with a reflectance color meter. The non-selectivity and relative insensitivity of these methods makes them unsuitable[6].

Ferulic acid determination by gas chromatography requires a derivatization step[7,8] and results reported for flour indicate that recovery may be imcomplete[8]. Modern liquid chromatographic (LC) techniques for quantitating phenolic compounds of vegetable origin that were developed through 1980 have been reviewed[9] and retention data have been compiled[10,11]. Systematic studies of the effect of organic solvent content and pH on reversed-phase separation have been reported[12–14]. There are several reported reversed-phase separations of *cis* and *trans* isomers of cinnamic acid derivatives[15–18]. Reversed-phase methods applied to mixtures of flavonoids and phenolic acids required gradient elution[10,11]. For chromatographing mixtures of members of benzoic or cinnamic acid classes or both, binary[19], ternary[20], and concave[21] gradients or complex solvent mixtures with multistep gradients[22] have been employed. Isocratic systems[23,24] with 10 μm ODS columns lacked the desired peak characteristics. Ultraviolet (UV) detection was used almost exclusively, but the wavelength choice was inconsistent. In contrast, one isocratic but relatively complex solvent system designed for use on extracts of commercial beverages[25] used electrochemical detection.

All of the preceding methods were designed to separate several phenolic acids from each other. Thus, they were not necessarily developed or optimized for rapid ferulic acid recovery and separation in the matrix of concern. The purpose of this work has been the development of a simple, rapid, accurate HPLC method for ferulic acid, which is a major phenolic acid in cereal grains, to provide a true indicator of bran contamination (*i.e.*, endosperm purity) in milling fractions.

EXPERIMENTAL

Standards and chemicals

The benzoic and *trans*-cinnamic acids obtained from Sigma (St. Louis, MO, U.S.A.) were checked for purity by HPLC and were used without further purification. A 100-μg/ml ferulic acid stock solution was prepared daily by dissolving 10.0 mg of ferulic acid in 5 ml of methanol, and diluting to 100 ml in a volumetric flask with distilled water. Working standards were prepared by further dilution with HPLC-grade water and protected from light until used. Water for use in the mobile phase was redistilled from alkaline permanganate solution in an all-glass apparatus. Methanol was dehydrated by treatment with magnesium activated by iodine. The mobile phase was a mixture of 12% methanol and a sodium citrate buffer. A buffer solution was prepared by adjusting 0.01 *M* citric acid to pH 5.40 with concentrated sodium hydroxide solution. Prior to use, the mobile phase was filtered under vacuum through a 0.45-μm microporous filter (HATF 04700, Millipore, Bedford, MA, U.S.A.).

Chromatography

A Hewlett-Packard 1084B liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) was used with either a Hewlett-Packard 1040A photo diode array detector or a Kratos FS 970 fluorescence detector with variable excitation wavelength (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.). The analytical column was made from 100 mm × 4.6 mm I.D. 316 stainless-steel tubing (Handy and Harmon Tube Company, Norriston, PA, U.S.A.) equipped with Uptight 1/4 in. zero dead volume column end fittings (C-201, Upchurch Scientific, Oak Harbor, WA, U.S.A.). The column was slurry packed in our laboratory with ODS-Hypersil (5 μm, Shandon Southern Instruments, Sewickley, PA, U.S.A.). A 20 mm × 20 mm I.D. pre-column (Upchurch Scientific) was packed with 10 μm ODS packing material. The HP 1040A detector was set at 320 nm with an optical bandwidth of 4 nm. Absorption at 550 nm (100 nm bandwidth) was used as a reference wavelength. The monochromator of the FS 970 fluorescence detector was set to an excitation wavelength of 312 nm, with a 418 nm cutoff filter installed on the emission side of the flow cell.

Fluorescence microscopy

The fluorescence microscope used in this study was a Zeiss universal microscope equipped with a III RS epi-illuminating condensor and 100-W mercury arc illuminator. An excitation filter with a maximum transmittance at 365 nm and an emission filter with a cutoff below 418 nm were used to examine all samples.

Extraction of ferulic acid from wheat and milling fractions

The samples of 1985 hard red winter (HRW) wheat grown in Kansas were ground through a Cyclotec sample mill (UD Corporation, Boulder, CO, U.S.A.) equipped with a 1-mm screen. The flour samples were comprised of 23 fractions obtained from the KSU pilot mill (Department of Grain Science and Industry, Kansas State University) and were used without additional grinding.

A sample of ground wheat or flour (2.000 g for samples with a ferulic acid content of less than 100 μg/g or 0.500 g with a ferulic acid content of greater than 100 μg/g) was weighed into a 100 ml centrifuge tube, to which 35 ml of 0.1 *M* sulfuric acid was added. After the sample was slurried with the dilute acid, the tube was placed into a boiling water bath for 30 min. The sample extract was cooled by placing the tube under running tap water. A total of 5.0 ml of a 2% (w/v) suspension of Clarase fungal amylase preparation (Miles Labs., Elkhart, IN, U.S.A.) in 2.5 *M* aqueous sodium acetate solution was added to the tube, and the extract was incubated in 55°C water bath for 60 min with periodic agitation. The sample was then centrifuged for 15 min at a minimum of 2000 *g* and the supernatant was decanted into a 50-ml volumetric flask and diluted to volume with distilled water. Prior to injection into the chromatograph, an aliquot of the extract was pressure-filtered through a 0.45-μm microporous filter (HATF 01300, Millipore, Bedford, MA, U.S.A.) by use of a Swinny adapter fitted to a 5-ml hypodermic syringe. Exposure to light was minimized by storing extracts in low actinic glassware, or in standard laboratory glassware covered with aluminum foil. The extracts were protected from UV light and stored in the refrigerator prior to analysis.

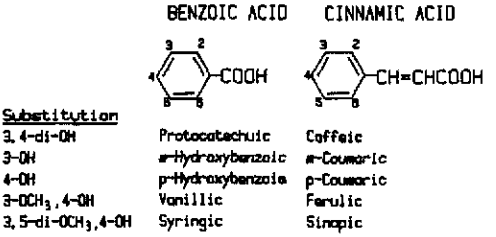

Fig. 1. Chemical structures of hydroxy and hydroxy-methoxy derivatives of benzoic and cinnamic acids.

RESULTS AND DISCUSSION

Separation of ferulic acid from other common phenolic acids occurring in cereal grains

The structures of some common hydroxy and hydroxy-methoxy derivatives of benzoic and cinnamic acids found in cereal grains are shown in Fig. 1. To optimize time and resolution in the separation of phenolic acid mixtures, different mobile phases and stationary phases were investigated. As a stationary phase, the performance of C_{18} reversed-phase (5 μm) was preferred in comparison with 5 μm C_8 and C_6 or C_{18} of 10 μm particle size.

Generally, mobile phases for the reversed-phase separation of phenolic acids have been mixtures of distilled water, an organic solvent, and weak organic acid.

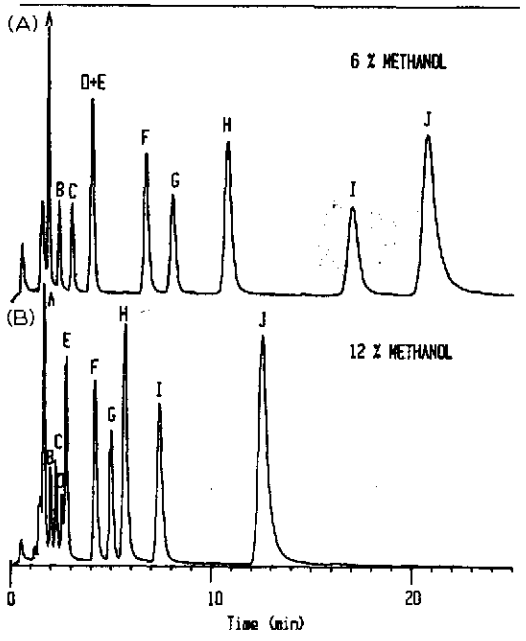

Fig. 2. Separation of standard phenolic acids on a 5 μm ODS-Hypersil (100 mm × 4.6 mm I.D.). Mobile phase (A) 6% methanol, (B) 12% methanol–0.01 *M* citrate buffer (pH 5.40). Flow-rate 1.0 ml/min. UV detection at 280 nm. Peaks: A = protocatechuic acid, B = *p*-hydroxybenzoic acid, C = vanillic acid, D = syringic acid, E = caffeic acid, F = *p*-coumaric acid, G = *m*-coumaric acid, H = ferulic acid, I = sinapic acid, and J = cinnamic acid.

Organic acids assist in pH control, which is necessary to prevent serious deterioration of resolution. The pH of the mobile phase was controlled in the range of 5.0 to 5.5 by a citrate buffer to optimize the analysis time and separation between *p*-coumaric, ferulic, and sinapic acids. Varying the concentration of citric acid from 0.01 *M* to 0.02 *M* did not significantly affect the resolution or the retention times. Buffer strength was high enough to enhance the separation but low enough to avoid precipitation of the salt.

Fig. 2A illustrates the separation of ten standard phenolic acids by a 10-cm reversed-phase (ODS) 5 μm column with UV detection at 280 nm. The mobile phase is 6% methanol and 0.01 *M* citric acid (adjusted to pH 5.4 with sodium hydroxide). Retention data for these compounds are listed in Table I. The flow-rate of 1.0 ml/min resulted in an elution time of 22 min for this mixture of 10 phenolic acids. Elution was in order of decreasing polarity, as expected in reversed-phase LC. A description of the effect of phenolic acid structure on retention[14] stated that the presence of the ethylenic side chain of cinnamic acid decreases polarity and the addition of a methoxy group or the loss of a hydroxy group decreases polarity within each class (benzoic *vs.* cinnamic). A plot of the logarithm of the retention times from Table I of members of a benzoic acid class and cinnamic acid class *vs.* the characteristic of the functional

TABLE I

RETENTION TIMES, t_R, CAPACITY FACTORS, k', RELATIVE RETENTIONS, α, OF PHENOLIC ACIDS ON ODS-HYPERSIL (5 μm)

Flow-rate = 1.0 ml/min; t_0 = 0.49 min; BA = benzoic acid; CA = cinnamic acid. Values reported are means of triplicate runs.

Compounds	*6% Methanol*			*12% Methanol*		
	t_R *(min)*	k'	α	t_R *(min)*	k'	α
Protocatechuic acid 3,4-di-OH BA	1.81	2.69	1.32	1.60	2.26	1.31
p-Hydroxybenzoic acid 4-OH BA	2.32	3.55	1.47	1.94	2.96	1.19
Vanillic acid 4-OH, 3-OCH_3 BA	3.05	5.22	1.39	2.21	3.51	1.17
Syringic acid 4-OH, 3,5-di-OCH_3 BA	4.14	7.45	1.03	2.51	4.12	1.10
Caffeic acid 3,4-di-OH CA	4.24	7.65	1.72	2.72	4.55	1.65
p-Coumaric acid 4-OH CA	6.94	13.16	1.21	4.16	7.49	1.21
m-Coumaric acid 3-OH CA	8.33	16.00	1.37	4.95	9.10	1.15
Ferulic acid 4-OH, 3-OCH_3 CA	11.23	21.92	1.62	5.63	10.49	1.33
Sinapic acid 4-OH, 3,5-di-OCH_3 CA	17.87	35.47	1.20	7.35	14.00	1.75
Cinnamic acid CA	21.34	42.55		12.48	24.47	

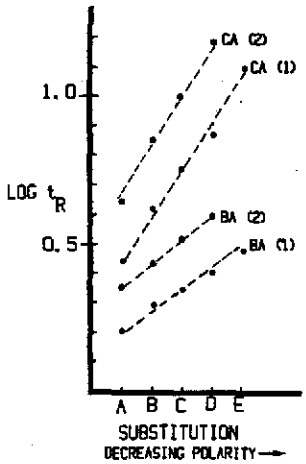

Fig. 3. Effect of decreasingly polar substituted phenolic acid on their retention behaviors. Plots of the logarithm of the retention times (t_R) of members of benzoic acid (BA) class and cinnamic acid (CA) class *versus* the characteristic of the substitution groups: A = 3,4-di-OH; B = 4-OH, 3-OCH_3; D = 4-OH, 3,5-di-OCH_3; and E = none. The separation conditions (1) 12% methanol–0.01 *M* citrate buffer (pH 5.40); 10 cm ODS-Hypersil column. (2) 4% acetonitrile–0.01 *M* citrate buffer (pH 5.88); 20 cm ODS-Hypersil column. Flow-rate = 1.0 ml/min.

groups by decreasing polarity is illustrated in Fig. 3. Parallel linear plots were obtained for each class of compounds in two solvent systems.

Optimization of the mobile phase began with a mixture of citrate buffer and methanol or acetonitrile. Acetonitrile resulted in shorter retention times. For example, at a pH of 5.80, the capacity factor k' of ferulic acid was 9.06 and 4.55 for 4% methanol and acetonitrile, respectively. However, methanol seemed preferable to acetonitrile because of its non-toxic nature and because a higher content of organic solvent could be used in the mobile phase to prevent deterioration of the reversed-phase column. In order to optimize the analysis time, methanol concentration was increased to 12% (Fig. 2B). The effect of solvent strength on the retention behavior was consistent with earlier reports[13,14]. The cinnamic acids decreased much faster in retention time than the benzoic acids, indicating that the separation factor between any cinnamic and any benzoic acid decreases with increasingly methanolic solvents. The extra non-polar ethylenic linkage in cinnamic acids apparently results in a greater solubilizing action of methanol for this compound class than for the benzoic acids. For a mixture of hydroxylated phenolic acids of a given class, relative retention did not change as the percentage of methanol increased. Vanillic, syringic, ferulic, and sinapic acids, with their methoxy groups, are affected more by a methanolic solvent than simple hydroxylated cinnamic acids. Lower α values of these acids and the preceding peaks were observed when methanol content of the mobile phase was increased (Table I).

Optimization of the detection system

The UV absorption spectrum of each peak in the chromatogram of standard phenolic acids was collected during the chromatographic run by the use of an HP

1040A-photodiode array detector. The larger bathochromic effect due to the ethylenic linkage in cinnamic acids allows their selective detection at higher wavelengths. Three different chromatographic signals (254, 280 and 320 nm) from the same run are shown in Fig. 4. At 320 nm, cinnamic acid derivatives can be detected without any intereference from benzoic acid derivatives, which have higher responses at 254 nm. However, detection at 280 nm is the best among the three for the determination of both classes of phenolic compounds.

Because of the presence of the ethylenic side chain in cinnamic acid derivatives, fluorescence can be used for selective detection of cinnamic acid derivatives. The optimum excitation wavelength and cut off filter were chosen from fluorescence excitation and emission spectra of ferulic acid dissolved in the mobile phase. The fluorescence spectra were obtained on a Perkin-Elmer MPF-44A fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.).

Chromatography of wheat extracts

Identification of the chromatographic peaks was performed by comparing the retention times and absorption spectra with authentic compounds. Ferulic acid was positively identified (the normalized spectra superimpose exactly) in extracts of wheat and wheat hull (Fig. 5A); *p*-coumaric acid was identified only in the extract of wheat hull (Fig. 5B).

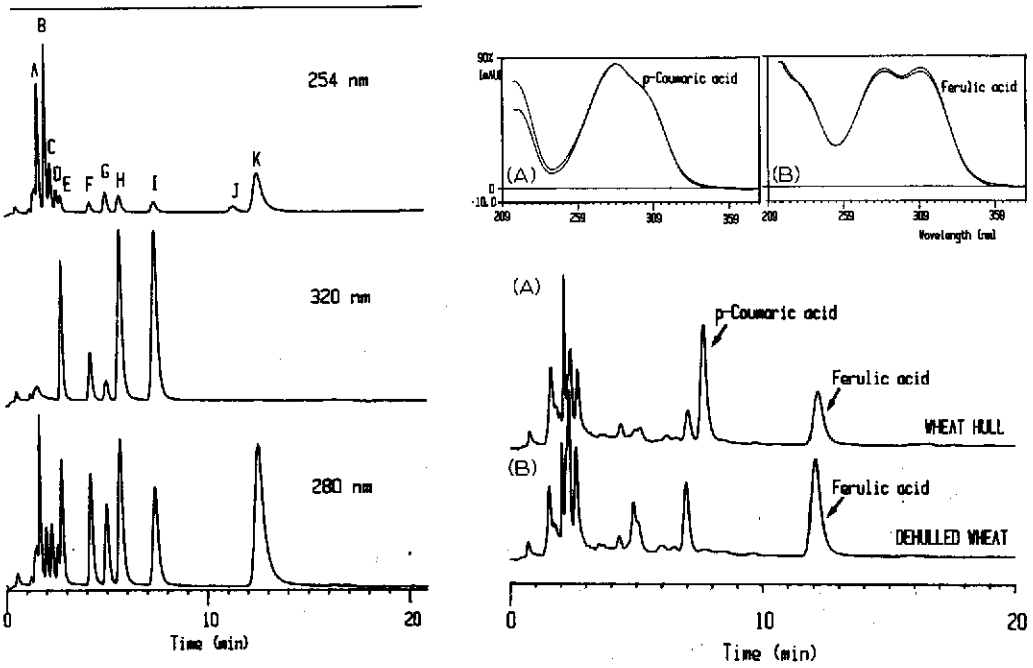

Fig. 4. Chromatograms of phenolic acids with UV detection at 254, 280 and 320 nm. Separation conditions as in Fig. 2B. Peaks: A–I identified as in Fig. 2; J = hydrocinnamic acid and K = cinnamic acid.

Fig. 5. Chromatograms of extracts from (A) wheat hull and (B) dehulled wheat. Separation conditions as in Fig. 2A.

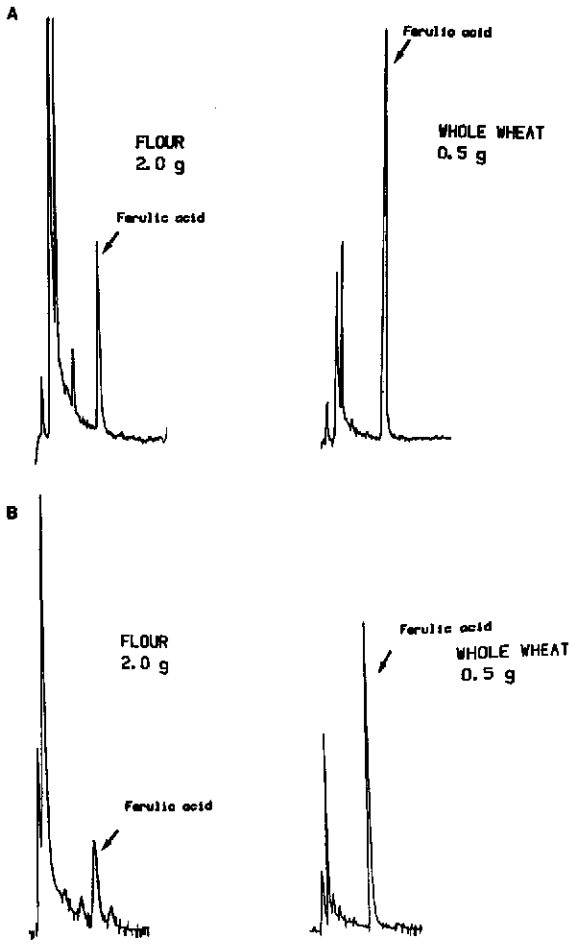

Fig. 6. Chromatograms of extracts from wheat and flour samples. Column: 5 μm ODS-Hypersil (100 mm × 4.6 mm I.D.). Mobile phase: 12% methanol–0.01 *M* citrate buffer (pH 5.40). Flow-rate: 1.0 ml/min. (A) UV detection at 320 nm. (B) Fluorescence detection with ex = 312 nm and em >418 nm.

Three chromatographic signals from the same run of an extract of a flour stream with high bran content (6th midds) were examined and the absorption was measured at 254, 280 and 320 nm, which are the most common wavelengths for a fixed-wavelength detector. Detection at 280 nm provided the maximum sensitivity for ferulic acid. However, when working with samples of low ferulic acid content, detection at 310–320 nm is preferred because of greater selectivity and ease of quantitation. Fig. 6 shows chromatograms of wheat and flour exracts using the chromatographic conditions optimized for speed, allowing determination of ferulic acid in 7 min. A highly sensitive UV detector operating at 320 nm or a fluorescence detector with excitation at 312 nm and emission cutoff >418 nm can be used to provide good sensitivity and selectivity in the resulting matrix.

TABLE II

RECOVERY OF FERULIC ACID AFTER DILUTE ACID HYDROLYSIS AND ENZYME INCUBATION

Hydrolysis time (min)	*Recovery (%)*
10	59.67
15	79.63
30	99.89
60	81.22
90	76.34
120	71.26

Extraction of ferulic acid from wheat and milling fractions

For the detemination of total phenolic acids, the phenolic acids esters are usually hydrolyzed into their consituent phenolic acids. The most significant losses of the phenolic acids occur during hydrolysis and during liquid–liquid extraction of the hydrolyzate. Destruction of phenolic acids in the hydrolysis step would seriously reduce recovery and contribute new products to complicate the sample mixture. Earlier workers reported that alkaline hydrolysis gave better recoveries of phenolic acids than did acid hydrolysis[26,27]. The sample preparation technique that we employed was a modification of the extraction used by Wehling and Wetzel[28] for water-soluble vitamins from cereal products. Equal amounts of standard ferulic acid

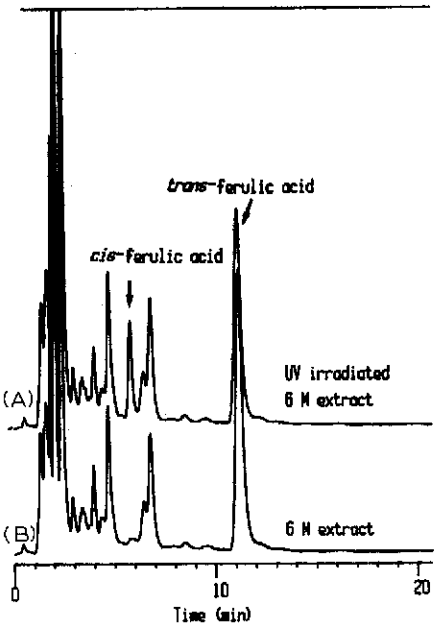

Fig. 7. Chromatograms of (A) 6th midds extract (KSU Pilot Mill) and (B) the extract after 30 min UV irradiation. Chromatograms were obtained using UV detection at 280 nm and chromatographic conditions identical to Fig. 6.

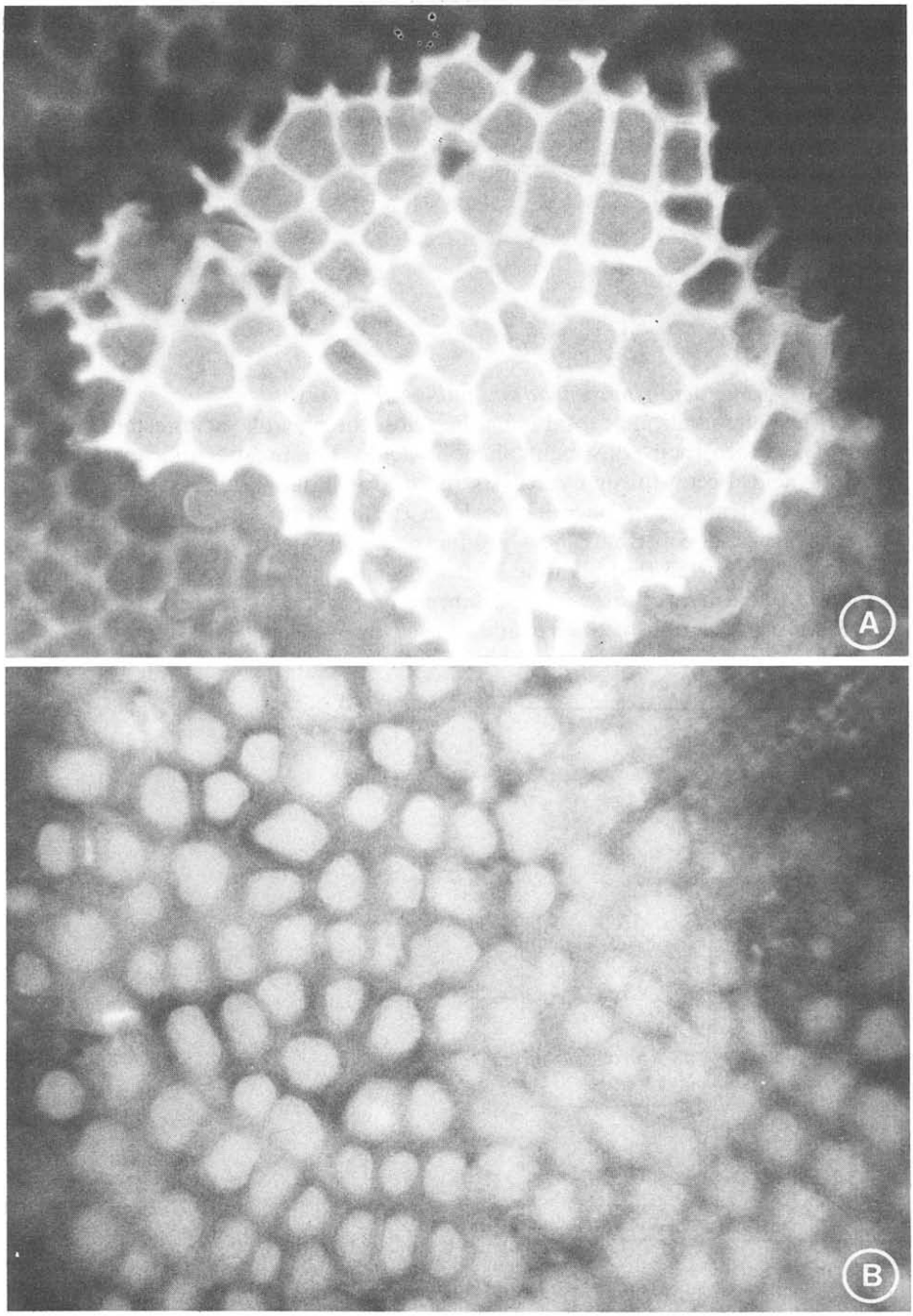

Fig. 8. Photomicrographs of the aleurone cell walls (surface view) of a ground wheat sample (A) before and (B) after the extraction. Both figures at the same magnification.

were subjected to dilute acid (0.1 *M* sulfuric acid) in a boiling water bath for different periods of hydrolysis time and were then carried through the extraction procedure described. From recoveries calculated by comparison peak areas to an unhydrolyzed standard, we found no destruction of ferulic acid from time 0 to 30 min (Table II). The optimum hydrolyis time was found to be 30 min with a recovery of 99.9%. After the hydrolysis step, α-amylase enzyme was used to clarify the extract and centrifugation was used in place of filtration. Liquid–liquid extraction of the hydrolyzate was avoided and with sensitive detection, pre-concentration was not necessary.

During sample preparation, the formation of artifacts can contribute new products to the sample mixture. Because of the presence of a vinyl group in the side chain of cinnamic acids, these acids can be partially converted to the *cis* form by ultraviolet light[29]. To answer the question regarding possible formation of the *cis* form of ferulic acid during sample work-up in daylight, which would cause a lower recovery of ferulic acid, identification of the *cis*-ferulic acid peak was necessary. Fig. 7A illustrates the chromatogram of the 6th midds extract with no *cis*-ferulic acid. The extract was then irradiated in a Vycor tube at a distance of 5 cm from the light source. The irradiation experiment was carried out using a mercury lamp with phosphor coat (GE No. 4TF/BL; 4 W), which provided a maximum excitation at 360 nm. Fig. 7B shows a decrease of the *trans*-ferulic acid peak and appearance of the *cis*-ferulic acid peak after the extract was irradiated for 30 min. The advantage of using acid hydrolysis is that in an acidic medium, undissociated ferulic acid has a higher stability to *cis–trans* rearrangement than in the ionized form. The conversion of *trans*-ferulic acid into the *cis*-form was noted in samples stored for 48 h, even when refrigerated and in the dark. For this reason, it is recommended that samples be analyzed within 24 h after extraction.

The completeness of extraction can be shown by fluorescence microphotographs of the tissues before and after extraction. Fig. 8A shows a typical blue autofluorescence of ferulic acid of aleurone cell walls. After the extraction, the residue was washed with distilled water and when examined under the microscope, all the blue fluorescent color of ferulic acid had disappeared (Fig. 8B).

TABLE III

REPRODUCIBILITY OF ANALYTICAL PROCEDURE

Sample No.	*Ferulic acid (μg/g)*	
	Whole wheat	*Flour*
1	491.4	40.7
2	498.7	41.3
3	492.8	44.3
4	513.9	43.4
5	521.3	40.6
6	489.9	39.4
7	495.7	38.3
Mean	500.5	41.1
S.D.	12.2	2.1
R.S.D.	2.4	5.1

Reproducibility of analytical procedure

The reproducibility of the chromatography and integration was evaluated by making a series of seven injections of a given sample extract, and measuring the relative peak areas. Relative standard deviations (R.S.D.) of 0.74% and 1.51% were obtained from these replicate injections of whole wheat and flour extracts, respectively. Quantitation reproducibility was tested with both the previously described fluoresence and diode array UV detection systems and was found to be equivalent[30]. No difference in linearity was noted for the two detection modes in the concentration range of concern.

To determine the reproducibility of the overall analytical procedure, seven replicate samples were analyzed. The average value from duplicate injections of each sample was used to calculate the precision (Table III). R.S.D. values of 2.43% and 5.10% were obtained from whole wheat and flour, respectively.

TABLE IV

DISTRIBUTION OF FERULIC ACID AND MINERAL CONTENT EXPRESSED AS ASH IN MILLING FRACTIONS (KSU PILOT MILL)

Abbreviations: Prebreak (PBK); 1st break–5th break (1BK–5BK); 1,2,3, break reduction (BKRD); bran and shorts duster (BSD); suction (SUC); 1st tailings (1T); 2nd quality (2Q); coarse sizings top and bottom (CST and CSB); fine sizings top and bottom (FST and FSB); 1st middlings–6th middling reduction (1M–6M).

Fraction	*Wheat A*		*Wheat B*	
	FA (μg/g)	*Ash (%)*	*FA (μg/g)*	*Ash (%)*
PBK	24.4	0.38	33.3	0.51
1BK	16.9	0.35	21.1	0.45
2BK	17.3	0.41	21.2	0.42
3BK	24.4	0.41	23.9	0.43
BKRD	26.3	0.35	24.0	0.40
4BK	38.1	0.51	37.0	0.52
4BK	81.6	0.89	72.1	0.82
BSD	265.7	1.94	287.2	1.95
SUC	44.2	0.48	55.0	0.54
1T	69.6	0.67	52.1	0.62
2Q	45.1	0.43	41.0	0.48
CST	20.5	0.30	20.6	0.35
CSB	28.3	0.29	24.3	0.32
FST	19.4	0.29	15.7	0.32
FSB	21.5	0.27	17.7	0.29
1MT	15.0	0.25	13.9	0.28
1MB	16.6	0.26	14.2	0.29
2MT	15.9	0.26	12.8	0.30
2MB	24.3	0.26	21.3	0.30
3M	32.1	0.30	34.0	0.35
4M	76.2	0.54	72.7	0.55
5M	181.9	1.01	180.0	1.13
6M	433.8	2.11	733.5	3.32

Distribution of ferulic acid in wheat milling fractions

The HPLC method we developed can be used to quantitatively determine ferulic acid in all milling fractions. Table IV shows a distribution of ferulic acid in different milling fractions from the Kansas State University pilot flour mill. A wide range of ferulic acid values between streams with high bran contamination and those with low contamination is observed. The low correlation between ferulic acid and mineral content of streams with relatively low bran content could be explained by the fact that the ferulic acid determination has a higher sensitivity to bran than the gravimetric ash procedure. Specificity of ash to bran is limited, since mineral content of the endosperm itself is approximately 20% of the total. A small change in bran contamination could not be detected by the gravimetric ash method. The results show that ferulic acid can be used as an excellent indicator of endosperm purity in milling fractions. Purity determination based on this method makes it possible to assess processing efficiency in milling production and experimentation with an accuracy not previously available. This measure also should prove useful in the related areas of wheat breeding and baking.

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