Variation in Ferulic Acid Concentration among Diverse Barley Cultivars Measured by HPLC and Microspectrophotometry

J. M. Zupfer,[†] K. E. Churchill,[†] D. C. Rasmusson,[‡] and R. G. Fulcher*[,†]

Department of Food Science and Nutrition and Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, Minnesota 55108

Ferulic acid (FA) is a low molecular weight phenolic acid that is a common component of the outer layers of cereal grains. It has been implicated in resistance to both insect and fungal pathogens. The FA concentrations of 18 cultivars of 2-row and 6-row barleys, grown in 2 locations, were quantified by HPLC analysis following acid hydrolysis. The FA concentrations ranged from 365 to 605 μg/g dry weight. The concentration of FA varied significantly among the different cultivars of barley. A genetic basis for FA levels is inferred by the similar ranking of the cultivars in both locations. FA levels were also measured by the absorbance at 340 nm of the acid-hydrolyzed extract, and in situ detection of fluorescence intensity of the ground grain using a UMSP80 microspectrofluorimeter. Both of these methods are more rapid than HPLC and may allow the practical screening of FA concentrations in numerous barley lines.

Keywords: *Ferulic acid; phenolics; fluorescence; UV absorption; barley*

INTRODUCTION

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is the major low molecular weight phenolic in barley (Nordkvist, 1984) and in many other common cereal grains. It has been examined in wheat (Fulcher et al., 1972; Pussayanawin, 1986), rice (Shibuya, 1984), sorghum (Hahn et al., 1983), oats (Durkee and Thivierge, 1977), corn (Serratos et al., 1987), and pearl millet (Emiola and De La Rosa, 1981). FA is concentrated in the cell walls of the outer coverings of seeds where it is mainly esterified to the arabinose backbone of arabinoxylans (Faush et al., 1963; Geissmann and Neukom, 1973; Hartley, 1973; Meuller-Harvey et al., 1986; Shibuya, 1984). These esterified phenolics form cross-links between cell wall polymers. The formation of cross-links is mediated by extracellular peroxidases which form diferulic acid (Biggs and Fry, 1987). The additional structural integrity added by these cross-links may be responsible for the correlations found between kernel hardness and bound ferulate (Classen et al., 1990). Also, it was reported that hardness in wheat kernels had a close association with natural fluorescence attributed to phenolic acids in the grain (Irving et al., 1989).

The cross-linking of phenolic compounds also is thought to provide a physical barrier to invasive disease development and consumption by insects. Considerable evidence exists linking the presence of phenolic acids in the caryopsis to disease resistance; cultivars with elevated levels of phenolics show increased resistance to mold damage in sorghum (Hahn et al., 1984) and to insect consumption of corn (Arnason et al., 1992; Classen et al., 1990; Serratos et al., 1987). In barley, FA levels have been found to increase in response to aphid infestation, thereby reducing the severity of new infestations (Cabrera et al., 1995).

FA may also serve roles in the caryopsis other than cell wall cross-linking. In its free form, FA is astringent and may, consequently, act as an antifeedant to insects and animals (Arnason et al., 1992; Magna and Lorenz, 1973). FA also has antioxidant properties (Daniels and Martin, 1967; Marinova and Yanishlieva, 1992) that may protect the lipid-rich aleurone cell interior it encases; this may be a desirable attribute in the initial stages of beer production (Maillard and Berset, 1995).

FA also may be used to predict end-use quality. For example, cultivars of wheat with elevated FA concentration generally had reduced amounts of extractable endosperm (Pussayanawin, 1986). It is to be expected that higher aleurone cell wall volume would result in less extractable endosperm. The presence of elevated levels of FA in the outer coverings makes its presence a useful indicator of the nonendosperm tissues in various milling fractions (Pussayanawin et al., 1988).

Various methods are available to measure FA concentration. HPLC is commonly employed for separating and quantifying phenolic compounds (Pussayanawin, 1986; Sen et al., 1991). However, this method is too time-consuming to allow practical screening of a large number of samples. In contrast, the absorbance (measured at 347 nm) of a wheat bran extract correlated well with FA concentrations in HPLC profiles (Smith and Hartley, 1983). The fluorescence of FA has also been used extensively to map the location of FA within cereal grains (Akin, 1995; Fincher, 1976; Fulcher et al., 1972). Fluorescence also provides an opportunity for the in situ quantification of FA in whole grain flours, which has been demonstrated in wheat (Symons and Dexter, 1996) and maize (Sen et al., 1991).

Phenolic acid concentrations vary considerably within a given cereal crop and correlate well with agronomic

* Address correspondence to this author at the Department of Food Science and Nutrition, University of Minnesota, 1334 Eckles Ave., St. Paul, MN 55108. Telephone: (612) 626-1220. Fax: (612) 625-5272. Email: fulch001@maroon.tc.umn.edu.

[†] Department of Food Science and Nutrition.

[‡] Department of Agronomy and Plant Genetics.

S0021-8561(97)00810-8 CCC: $15.00
Published on Web 03/20/1998

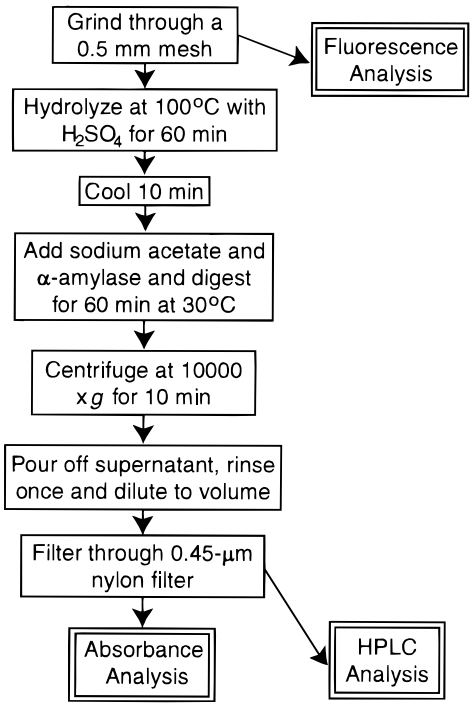

Figure 1. Sample preparation for three methods of ferulic acid measurement.

factors in corn, sorghum, and wheat (Arnason et al., 1992; Hahn et al., 1983; Irving et al., 1989). Barley is another cereal crop in which relationships between phenolic acid concentration and certain grain quality attributes may exist. The purposes of this study were to (1) determine the range of FA concentrations in diverse cultivars of barley and (2) evaluate two different methods for the measurement of FA in an effort to develop protocols which will enable the rapid screening of a wide range of barley samples in breeding or processing programs.

MATERIALS AND METHODS

Barley Samples. To assess variation in the concentration of phenolic acid present in diverse barley grains, 18 different malting and feed barleys of U.S., Canadian, and European origin were evaluated. They were grown as a part of the Minnesota Experiment Station barley project that was designed to assess genetic traits of each barley in the distinctly different environments of St. Paul and Crookston, MN. Typically, the two locations receive different amounts of annual rainfall and have different soil types. The cultivars were grown in a randomized block design with two replicates. Twelve of the barley varieties were six-row cultivars: Morex, Robust, Excel, M62, Steptoe, Vantage, M84-824, M85-68, M85-84, M86-589, M60, and M86-85, all originating from the Minnesota barley breeding program. Six were two-row cultivars: Harrington, Prisma, Cheri, Betzes, Glacier, and Wabet. Prisma and Cheri are European malting cultivars, and Harrington is a Canadian malting cultivar while Betzes and Wabet were developed in Montana. Wabet is a Betzes progeny with a naked caryopsis and waxy starch. Glacier is a unique cultivar among these samples, having a high amylose content. Mature kernels having 8.4−10.4% moisture were ground in a Retsch mill (model ZM-1, Glen Mills Inc., Clifton, NJ) through a 0.5 mm screen at 10 000 rpm in preparation for analysis. The analytical methods are summarized in Figure 1.

Extraction by Acid Hydrolysis of Ferulic Acid. Samples were prepared using the procedure of Pussayanawin et al. (1988) with some modifications (Figure 1). Samples of 0.8−0.9 g ground grain were combined with 15 mL of 0.2 N H_2SO_4 in 50 mL centrifuge tubes and heated in a boiling water bath for 1 h. Hydrolysis was terminated by cooling in an ice−water bath for 10 min before the addition of 2.14 mL of a 2.5 M aqueous sodium acetate solution that contained 2% (w/v) α-amylase from *Aspergillus oryzea*, type X-A (Sigma Chemical Co., St. Louis, MO). The samples were incubated at 30 °C for 1 h and then centrifuged at 10000*g* for 10 min. Samples from St. Paul received an additional treatment prior to centrifugation, of boiling for 5 min followed by the addition of 5 mL of methanol to inactivate the α-amylase. This additional treatment was performed to improve the consistency of extraction of other minor phenolic acids, but it had little appreciable effect on FA concentrations. The supernatant was collected and the surface of the pellet rinsed once with buffer, and the resulting fractions were pooled before the appropriate dilution was made. Prior to injection into the HPLC, the samples were filtered through a 0.45-μm nylon filter and then diluted 3 to 1 with HPLC mobile phase. All extractions and injections were performed in duplicate with control barley samples run with each analysis set.

HPLC Determination of Ferulic Acid. An isocratic procedure developed by Pussayanawin (1986) was used with a mobile phase of 0.01 M sodium citrate containing 13% methanol, pH 5.4. Separations were obtained using a Waters piston pump (1 mL/min) and a 15 cm × 4.6 mm, C18 column with 5 μm Hypersil packing (Vydac). A 20 μL injection loop was used on the sample injector. Peaks were detected with a Waters Model 440 UV−Vis absorbance detector operating at 280 nm. Although this wavelength is not the optimum for FA, it allowed the simultaneous detection of several phenolic acids. A Hewlett-Packard integrator (Model QA-1) was used to record retention times and peak areas.

Absorbance Detection of Ferulic Acid. FA extract absorbance was measured at 340 nm in a double-beam instrument. These measurements were made on the same extracts as were evaluated by HPLC.

Fluorescence Detection of Ferulic Acid. Relative fluorescence intensities (RFI) were measured on the Zeiss computerized, Universal Microspectrophotometer Model 80 (UM-SP80). "Lambda Scan" software was used to define the fluorescence emission spectra of barley aleurone (Fulcher and Collingwood, 1987). The filter cube used for analysis of aleurone contains an exciter filter (λ_{max} 365 nm), a dichroic reflector (λ_{max} > 395 nm), and a barrier filter (λ_{max} > 420 nm).

The measuring parameters included a Neofluar 10 × objective with a 0.32 mm measuring spot and a 0.63 mm luminous field diaphragm. Ground flour was placed in a 4 × 6 × 1 cm sample holder with a glass top, which fits onto the microscope stage. Areas of 5000 μm^2 were scanned in increments of 100 μm across the sample holder, giving a total of 2601 fluorescence intensity readings. The scans were performed on 12 different flour samples of each grain variety used. These data were used to obtain a mean RFI. A uranyl glass standard was used to adjust the photomultiplier to ensure photometric consistency. The uranyl glass standard does not fade and provides reference so that the drift of the photomultiplier can be monitored and readjusted as necessary. With the use of this standard, multiple samples from the same experiment can be compared with little significant drift in the photomultiplier adjustment.

Analysis of variance and Duncan multiple comparison tests were run on a Statistical Analysis System (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

The primary objective of this study was to evaluate the FA concentrations in the mature kernels of barley cultivars with diverse genetic backgrounds. The levels of phenolic acids could then be assessed as to potential associations with important quality traits. The average FA concentration of the different cultivars across both locations ranged from 343 to 580 μg of ferulic acid/g of ground grain (Table 1). The Duncan Multiple Range

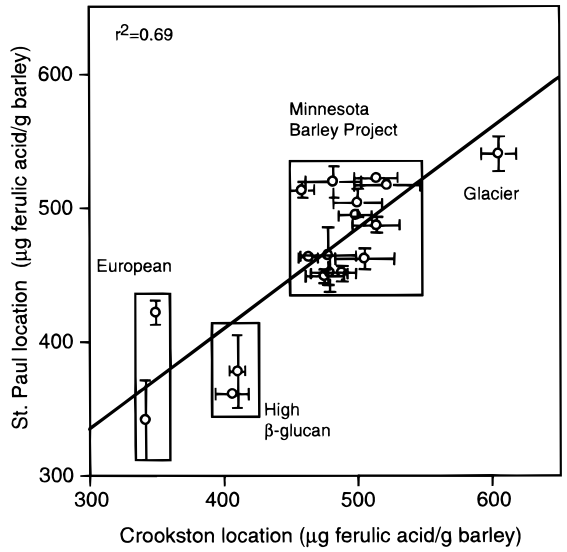

Figure 2. Ferulic acid concentration of barley cultivars measured using HPLC, grown at two locations.

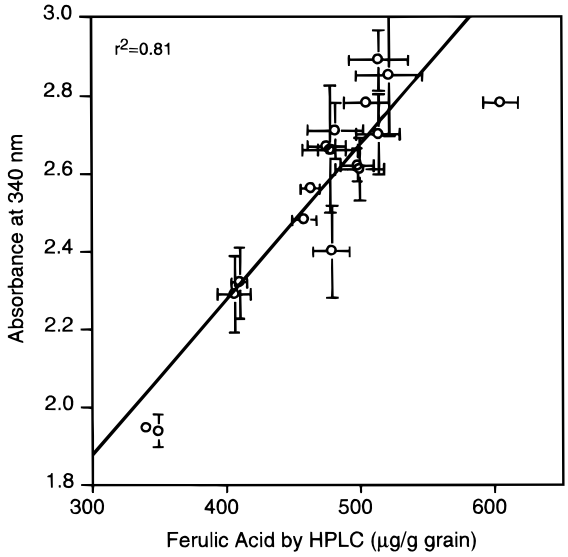

Figure 3. Ferulic acid concentration of 18 barley cultivars determined by absorbance at 340 nm and HPLC.

Table 1. Ferulic Acid Concentration Determined by HPLC

variety	1000 kernel wt (g as is)	ferulic acid (μg/g dwb)	ferulic acid Duncan grouping, α = 0.05
Glacier	42.5	579.7	A
Excel	37.0	545.0	B
M84-824	33.2	541.6	B C
Vantage	32.0	527.3	B C D
M85-68	35.9	521.8	B C D
M85-84	39.0	521.6	B C D E
Robust	40.5	514.6	C D E F
M62	36.1	509.4	C D E F
M86-589	34.6	507.8	C D E F
Morex	34.3	497.0	D E F
M86-85	40.7	493.0	D E F
M60	42.4	486.2	E F
Steptoe	42.4	483.1	E F
Harrington	37.0	477.0	F
Prisma	42.5	397.7	G
Wabet	37.9	397.6	G
Betzes	34.4	385.6	G
Cheri	36.9	343.2	H
average	37.7	477.5	

Test placed the samples in eight groups (A−H). While several groups overlapped, there were significant differences among cultivars. In the HPLC procedure, other phenolics were identified, but their concentrations did not affect the overall correlations. We are exploring more detailed analysis of these compounds at this time.

Almost all of the cultivars that clustered between 486 and 545 μg of ferulic acid/g of ground barley (a range of 59 μg/g) are products of the Minnesota barley project. It is not surprising that a group of 12 closely related barleys would exhibit a tight grouping of phenolic acid concentration. Four cultivars were much lower in FA concentration. Two of these are two-rowed European malting cultivars, Prisma and Cheri, and the other two, Betzes and Wabet, were also two-rowed varieties developed in Montana. Glacier, the high amylose cultivar, had the highest FA concentration. These results, which exhibit a similar ranking between the two locations, indicate a strong genetic basis for FA concentrations. Figure 2 illustrates the consistency in the ranking of the 18 cultivars at the 2 locations and shows the grouping of related lines.

The genetic or cultivar differences in FA concentration could result from differences in the caryopsis structure. For example, this might include an increased frequency of FA substitution of the arabinoxylan backbone, or a proportional increase in aleurone cell wall volume, in turn causing an increase in both arabinoxylan and FA concentration in the caryopsis. Smaller grains have a higher surface-to-volume ratio, and as a result, they might have a higher percentage of aleurone and germ and thus have a higher FA concentration. Measurements of 1000-kernel weights were made to determine which cultivars had larger kernels, since heavier grains are better filled. A comparison between kernel weight (Table 1) and FA concentration revealed no relationship, however. Kernel weight is affected to a great extent by environmental conditions, and the lack of correlation between kernel weight and FA concentration also points more toward genetic control of phenolic acid levels in the grain.

Absorbance. In order for FA to be used as a parameter for selection in a plant breeding program, there must be a reasonably rapid and inexpensive method for analysis of many samples. The absorbance of the hydrolyzed extract is proposed here as a relatively inexpensive and readily accessible technique. The spectra of the unseparated HPLC extracts were evaluated spectrophotometrically, and after comparing these spectra and the spectra of many of the pure phenolic acids, it appeared that the intensity of 340 had the highest correlation with FA in the HPLC profiles. Minor hydroxycinnamic acids, such as sinapic acid and *p*-coumaric acid, would only contribute a very small amount to the total absorbance measured. The absorbance was then compared to the HPLC FA concentration of the 18 cultivars grown at the Crookston location.

The absorbance method showed sizable differences among cultivars (Figure 3). There was good agreement between absorbance and HPLC FA concentration. The correlation value was $r^2 = 0.81$. The small discrepancies are probably caused by absorbing species other than FA. It is possible to run 40−50 samples through the acid hydrolysis and measure absorbances in about 5 h, compared to 8 samples per day using HPLC analysis. Cultivars of special interest could then be evaluated by

Table 2. Comparison of Ferulic Acid Concentrations for Six Cultivars (Two Replicates of Each) Determined by RFI and HPLC Methods Using Samples Grown at Crookston, MN

variety	RFI	Duncan groups, α = 0.05			ferulic acid (μg/g dwb)	Duncan groups, α = 0.05		
Glacier	28.94	A			605	A		
Wabet	23.60		B		410		C	
Robust	19.87			C	498		B	
Excel	19.36			C	D	510		B	
Cheri	17.70			C	D	341			D
Prisma	16.71				D	349			D
average	21.03					452			

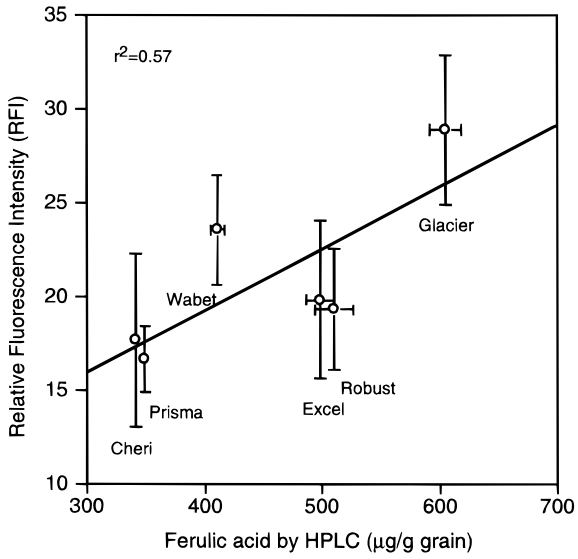

Figure 4. Ferulic acid concentration of six barley cultivars determined by HPLC and RFI.

the more rigorous HPLC method of phenolic acid determination.

Solid Sample Fluorescence Reflectance. A number of attempts have been made to determine FA concentration using whole grain samples (Sen et al., 1991; Symons and Dexter, 1996). This procedure is based on in situ fluorescence detection of FA in unextracted ground grain. The method is rapid because it eliminates the extraction procedure, which could also improve the accuracy of the assay. This technique has been used successfully in determining FA concentration in different millstreams of milled wheat (Pussayanawin et al., 1988). Fulcher and Collingwood (1987) observed significant differences among barley cultivars in RFI at 365 nm, but FA concentrations were not measured for comparison. Six samples representing the highest and lowest FA concentrations, as determined by HPLC, were chosen to determine if RFI could distinguish these samples. The six cultivars differed significantly for both measures of FA, RFI and HPLC (Table 2; Figure 4). The range among cultivars was almost 2-fold for both RFI and HPLC values. It was encouraging that the two methods resulted in large differences and agreed in identifying the high and low FA cultivars. On the other hand, the error bars (Figure 4) show high standard deviations for the RFI measurements. This indicates that cultivar means based on analyzing 2600 data points are estimated with relatively large errors.

Dipix Technologies (Maztech Microvision Ltd., Ottawa, ON) has developed instrumentation designed to collect many more data points over a much larger surface area to distinguish small concentration differences in biological samples. Their instrument also eliminates the problem of drift in the mercury light source and allows for standard calibration of fluorescence readings (Harrigan, 1996). Improved solid sample fluorescence instrumentation may reduce error and make RFI measures of FA concentration more competitive with other procedures.

CONCLUSIONS

Ferulic acid concentrations in the mature grains of a diverse set of barley cultivars differed by almost 2-fold. These differences, measured using HPLC, were consistent across two environments, St. Paul and Crookston, MN. The 5 2-rowed cultivars ranked below the 11 6-rowed cultivars in FA content. The sizable cultivar difference and the consistent ranking of cultivars across the two environments indicate substantial opportunity to select cultivars for either high or low FA content.

Detection of FA concentration using extract absorbance has several features, which make it attractive, and in these experiments it gave encouraging results. It detected sizable differences among the 18 barley cultivars, and these differences were highly correlated with differences observed using the HPLC procedure. While the RFI procedure detected cultivar differences in FA concentration, measurement errors were large. The RFI procedure deserves additional attention in light of the prospect for improved instrumentation.

LITERATURE CITED

Akin, D. E. Microspectrophotometric characterization of aromatic constituents in cell walls of hard and soft wheats. *J. Sci. Food Agric.* **1995**, *68*, 207−214.

Arnason, J. T.; Gale, J.; Conilh de Beyssac, B.; Sen, A.; Miller, S. S.; Philogen, B. J. R.; Lambert, J. D. H.; Fulcher, R. G.; Serratos, A.; Mihm, J. Role of phenolics in resistance of maize grain to the stored grain insects *Prostephanus truncatus* and *Sitophilus zeamais*. *J. Stored Prod.* **1992**, *28*, 119−126.

Biggs, K. J.; Fry, S. C. Phenolic cross-linking in the cell wall. In *Physiology of cell expansion during plant growth: proceedings of the second annual Penn State Symposium in Plant Physiology*; Cosgrove, D. J., Knievel, D. P., Eds.; American Society of Plant Physiologists: Rockville, MD, 1987; pp 46−57.

Cabrera, H. M.; Muñoz, O.; Zúñiga, G. E.; Corcuera, L. J.; Argandoña, V. H. Changes in ferulic acid and lipid content in aphid-infested barley. *Phytochemistry* **1995**, *39* (5), 1023−1026.

Classen, D.; Arnason, J. T.; Serratos, J. A.; Lambert, J. D. H.; Nozzolillo, C.; Philogene, B. J. R. Correlation of phenolic acid content of maize to resistance to *Sitophilius zeamais*, the maize weevil, in CIMMYT's collections. *J. Chem. Ecol.* **1990**, *16*, 301−315.

Daniels, D. G. H.; Martin, H. F. Antioxidants in oats: Monoesters of caffeic and ferulic acids. *J. Sci. Food Agric.* **1967**, *18*, 589−595.

Durkee, A. B.; Thivierge, P. A. Ferulic acid and other phenolics in oat seeds. *J. Food Sci.* **1977**, *42*, 551.

Emolia, L. O.; De La Rosa, L. C. Characterization of pearl millet nonstarchy polysaccharides. *J. Food Sci.* **1981**, *46*, 781.

Faush, H.; Kundig, W.; Neukom, H. Ferulic acid as a component of a glycoprotein from wheat flour. *Nature* **1963**, July 20, p 287.

Fincher, G. B. Ferulic acid in barley cell walls: A fluorescence study. *J. Inst. Brew.* **1976**, *82*, 347−349.

Fulcher, R. G.; O'Brien, T. P.; Lee, J. W. Studies on the aleurone layer. I Conventional and fluorescence microscopy of the cell wall with emphasis on phenol−carbohydrate complexes in wheat. *Aust. J. Biol. Sci.* **1972**, *25*, 23−34.

Fulcher, R. G.; Collingwood, K. Quantitative microscopy in barley research. In *Proceedings Australian Barley Technical Symposium;* NSW Department of Agriculture: Wagga Wagga, New South Wales, 1987; pp 222−228.

Geissmann, T.; Neukom, H. A note on ferulic acid as a component of the water-insoluble pentosans of wheat flour. *Cereal Chem.* **1973**, *50*, 414−416.

Hahn, D. H.; Faubion, J. M.; Rooney, L. W. Sorghum phenolic acids, their HPLC separation and their relation to fungal resistance. *Cereal Chem.* **1983**, *60* (4), 255−259.

Harrigan, K. Flour power. *Cereal Foods World* **1996**, *24*, 14−19.

Hartley, R. D. Carbohydrate esters of ferulic acid as components of cell walls of *Lolium multiform*. *Phytochemistry* **1973**, *14*, 661−665.

Irving, D. W.; Fulcher, R. G.; Bean, M. M.; Saunders, R. M. Differentiation of wheat based on fluorescence, hardness and protein. *Cereal Chem.* **1989**, *66* (6), 471−477.

Magna, J. A.; Lorenz, K. Taste threshold values for phenolic acids which can influence flavor properties of certain flours, grains and oilseeds. *Cereal Sci. Today* **1973**, *18*, 326.

Maillard, M.-N.; Berset, C. Evolution of antioxidant activity during kilning: Role of insoluble bound phenolic acids of barley and malt. *J. Agric. Food Chem.* **1995**, *43*, 1789−1793.

Marinova, E. M.; Yanishlieva, N. V. Inhibited oxidation of lipids II: Comparison of the antioxidative properties of some hydroxy derivatives of benzoic and cinnamic acids. *Fett. Wiss. Technol.* **1992**, *94* (11), 428−432.

Mueller-Harvey, I.; Harley, R. D.; Harris, P. J.; Curzon, E. H. Linkage of *p*-coumaroyl and feruloyl groups to cell-wall polysaccharides of barley straw. *Carbohydr. Res.* **1986**, *148*, 71−85.

Nordkvisk, E.; Salomonssen, A.; Åman, P. Distribution of insoluble bound phenolic acids in barley grain. *J. Sci. Food Agric.* **1984**, *35*, 657−661.

Pussayanawin, V. High Performance Liquid Chromatographic Studies of Ferulic Acid in Flour Milling Fractions. Ph.D. Dissertation, Kansas State University, 1986.

Pussayanawin, V.; Wetzel, D. L.; Fulcher, R. G. Fluorescence detection and measurement of ferulic acid in wheat milling fractions by microscopy and HPLC. *J. Agric. Food Chem.* **1988**, *36* (3), 515−520.

Sen, A.; Miller, S. S.; Arnason, J. T.; Fulcher, R. G. Quantitative determination by high performance liquid chromatography and microspectrofluorimetry of phenolic acids in maize grain. *Phytochem. Anal.* **1991**, *2*, 225−229.

Serratos, A.; Arnason, J. T.; Nozzolillo, C.; Lambert, J. D. H.; Philogene, B. J. R.; Fulcher, R. G.; Davidson, K.; Peacock, L.; Atkinson, J.; Morand, P. The factors contributing to resistance of exotic maize populations to maize weevil *Sitophilus zeamais*. *J. Chem. Ecol.* **1987**, *13*, 751−762.

Shibuya, N. Phenolic acids and their carbohydrate esters in rice endosperm cell walls. *Phytochemistry* **1984**, *23*, 2233−2237.

Smith, M.; Hartley, R. D. Occurrence and nature of ferulic acid substitution of cell wall polysaccharides in graminaceous plants. *Carbohydr. Res.* **1983**, *118*, 65−80.

Symons, S. J.; Dexter, J. E. Aleurone and pericarp fluorescence as estimators of mill stream refinement for various Canadian wheat classes. *J. Cereal Sci.* **1996**, *23*, 73−83.

Received for review September 17, 1997. Revised manuscript received February 4, 1998. Accepted February 10, 1998.

JF9708103