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SEPARATION AND QUANTITATION OF FERULIC ACID AND TYROSINE IN WHEAT SEEDS (*TRITICUM AESTIVUM*) BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Separation and quantitation of free and bound ferulic acid and tyrosine in wheat seeds were undertaken by reversed-phase high-performance liquid chromatography. The first separation on the C_{18} column was followed by one or two successive separations of the collected peaks on the same column with different compositions of the mobile phase. The separated compounds were identified from their fluorescence spectra and thin-layer chromatographic profiles and estimated from their peak heights on the chromatograms obtained from the final step of separation. This paper describes a simpler, cheaper and more rapid method with no requirement for preliminary preparation of derivatives.

INTRODUCTION

The phenylpropane series of phenolic acids is widely distributed in plants in both free and bound form. Some are the basic structural units of lignin[1] and some inhibit fungal growth around roots[2]. Ferulic acid has been found to be a constituent of the cell walls of many plants[3–6]. The amino acid tyrosine is the most ubiquitous phenylpropane naturally occurring and it is a precursor of nitrogen-free phenylpropanes through the reaction of tyrosine ammonia lyase that is characteristic of *Gramineae*[7].

Geissman and Neukom[8] postulated that an oxidative mechanism in wheat flour pentosan, involving the radical coupling of ferulic acid, causes gelation of the soluble pentosans. They detected diferulic acid in wheat flour[9]. Neukom suggested that tyrosine residues are also responsible for phenolic coupling in the same way[10].

In the present work both phenylpropanes, tyrosine with high polarity and ferulic acid with lower polarity, were separated and quantitated effectively by successive chromatography on the same column without any pretreatment.

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EXPERIMENTAL

Materials

Bran, germ, and endosperm of *Triticum aestivum*, Canadian northern white wheat, Manitoba II, were milled by Nisshin Flour Milling. These samples were defatted by extraction with petroleum ether and air-dried. The defatted bran was ground using a ball mill before the next extraction. The samples were then extracted by refluxing with 70% aqueous methanol for 1 h. The combined filtrate was concentrated under reduced pressure and lyophilized. The lyophilized material is called the extract. All manipulation of the extracts and authentic compounds was done in the dark to prevent light-induced isomerization of ferulic acid[7]. The yields were 0.11, 0.25, and 0.013 g for 1 g of bran, germ, and endosperm, respectively.

In acid hydrolysis, the extract (20–200 mg) was heated with 3 ml of 2 *N* hydrochloric acid in an evacuated sealed tube at 100 ± 1°C for 2 h. The hydrolysate was evaporated to dryness under vacuum. In alkaline hydrolysis, the extract (200 mg) was heated with 500 μl of 1 *N* sodium hydroxide in an evacuated sealed tube at 60°C for 90 min.

Reversed-phase high-performance liquid chromatography (HPLC)

The chromatographic system (Japan Spectroscopic) included a Twincle pump, a variable-loop injector, and a UVIDEC-100 III UV Spectrophotometer. An analytical column (250 × 4.6 mm I.D.) packed with Fine Sil C_{18}-10 was used, with a mobile phase of methanol–0.033 *M* potassium dihydrogen phosphate–acetic acid (pH 3.5) and a flow-rate of 1 ml/min. The column effluent was monitored by the absorbance at 280 nm. To desalt the effluent, a preparative column (250 × 10 mm I.D.) packed with Fine Sil C_{18}-20 was used, with a mobile phase of methanol–water–acetic acid (pH 3.5) and a flow-rate of 4 ml/min. The extract and the dried acid hydrolysate were each dissolved in 40% methanol. The alkaline hydrolysate was acidified to pH 3.5 with 6 *N* hydrochloric acid.

A first separation was done on the analytical column using methanol–0.033 *M* potassium dihydrogen phosphate (pH 3.5) (40:60). After evaporation of the peak fraction corresponding to tyrosine or ferulic acid, a second separation was done. The concentration of methanol in the mobile phase was 10% and 30% for tyrosine and ferulic acid, respectively. A third separation was done for the tyrosine peak fraction collected from the second one using 0.033 *M* potassium dihydrogen phosphate (pH 3.5).

Fluorescence spectrometry

Each fraction containing tyrosine or ferulic acid was collected from the final step of chromatography and desalted by HPLC. The fluorescence was measured using a recorder attached to a Shimadzu Spectrofluorometer RF 510. For the ferulic acid fraction, the fluorescence intensity was stabilized by exposure to light of 330 nm for 30–60 min prior to each run.

Thin-layer chromatography (TLC)

The peak fraction of each extract thought to contain *ca.* 0.1 μg of tyrosine or ferulic acid was obtained from the final step of chromatography and desalted. It was

concentrated to a small volume and derivatized with dansyl chloride (Pierce, Rockford, IL, U.S.A.)[11]. The derivatives were placed on one corner of a polyamide-layered sheet (5 × 5 cm, Chen-Chin Trading, Taiwan). Two-dimensional chromatography[12] was done using water–90% formic acid (200:3) as solvent I, benzene–acetic acid (9:1) as solvent II, and ethyl acetate–methanol–acetic acid (20:1:1) as solvent III. The first development was done with solvent I; the second, at 90° to the first, with solvent II; and the third, in the same direction as the second, with solvent III. Fluorescent spots were located by exposing the test sheets under UV light at 365 nm using a fluorescence inspection lamp.

Exposure of ferulic acid peak fraction to UV light

The ferulic acid peak fraction prepared for fluorescence spectrometry was concentrated and exposed to 330-nm light in a quartz tube using the same spectrofluorometer with occasional stirring for different periods of time.

Estimation of tyrosine and ferulic acid

Tyrosine and ferulic acid were assayed from the peak heights of the corresponding fractions from the final step of chromatography. The minimum detectable amount was 0.2 μg for one injection (30 μl). Tyrosine was also assayed by fluorescence at 310 nm with excitation light of 280 nm, because there was linearity between the fluorescence intensity and the concentration of tyrosine in the range 0–5 μg/ml.

RESULTS AND DISCUSSION

HPLC of non-hydrolysed extracts of wheat seed fractions

The non-hydrolysed extract of endosperm was separated with methanol–0.033 *M* potassium dihydrogen phosphate (pH 3.5) (40:60) as shown in Fig. 1. Authentic substances appeared at the positions indicated with arrows: tyrosine slightly behind the solvent front and ferulic acid at 10 min. The fractions thought to contain tyrosine and ferulic acid are shaded and dotted in the figure, respectively. They were collected separately for the next step of chromatography. The second separation of the tyrosine fraction was followed by the third one. Fig. 2 is the chromatogram obtained from the third step using 0.033 *M* potassium dihydrogen phosphate (pH 3.5). The peak at 8.5 min was well resolved, and completely overlapped the peak of authentic tyrosine. The ferulic acid fraction shown as the dotted area in Fig. 1, when submitted to the second chromatographic step using methanol–potassium dihydrogen phosphate (pH 3.5) (30:70), gave a peak at 26 min (Fig. 3). This peak was clearly resolved and completely overlapped that of authentic ferulic acid.

When the same procedures were carriet out for the tyrosine and ferulic acid fractions of non-hydrolysed extracts of germ and bran, all these fractions were separated with good resolution in the final steps of chromatography.

Chromatography of hydrolysed extracts

Both acid[13] and alkali[14] have been used to liberate phenolic acids from sugar esters. Treatment with hydrochloric acid was found to decompose ferulic acid into a substance with a shorter retention time on HPLC, whereas treatment with sodium hydroxide led to a good recovery of ferulic acid. Tyrosine was recovered completely with no degradation after acid treatment.

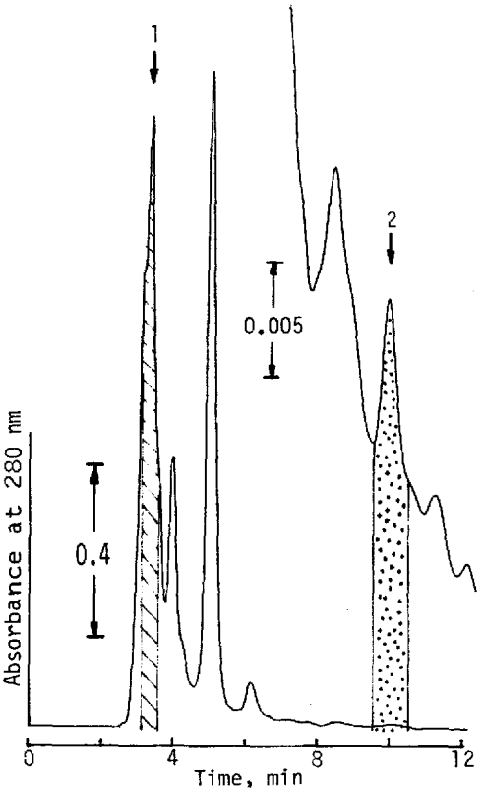

Fig. 1. HPLC profile of non-hydrolysed extract of endosperm (8 mg). Chromatographic conditions are given in the text. Authentic tyrosine (1) and ferulic acid (2) were eluted at the positions indicated with arrows.

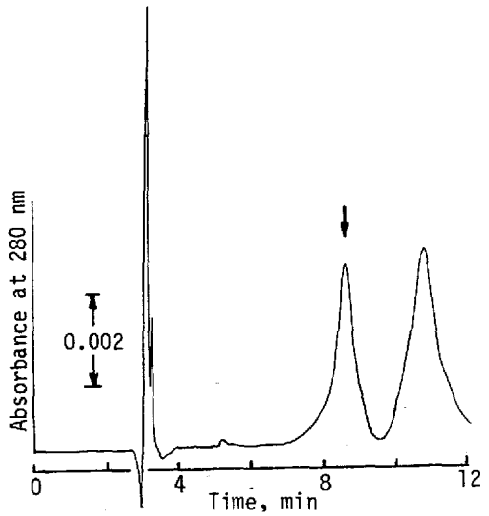

Fig. 2. Chromatogram of the third separation of the tyrosine peak collected by the successive chromatography of the first and the second step of non-hydrolysed endosperm extract (1.1 mg). Chromatographic conditions are given in the text. Authentic tyrosine was eluted at the position indicated with an arrow.

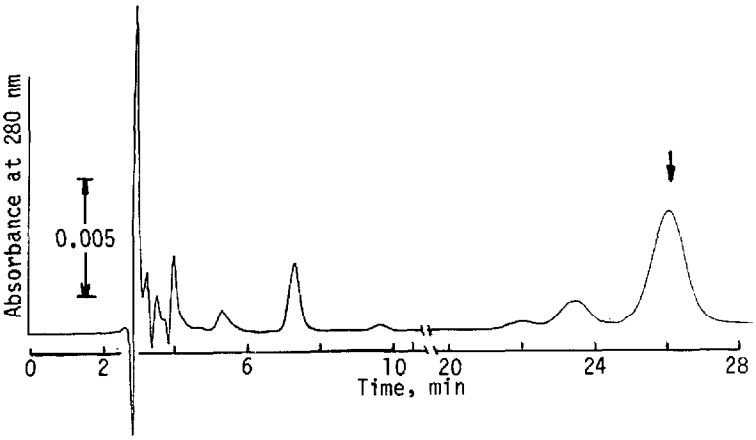

Fig. 3. Chromatogram of the second separation of the ferulic acid peak collected by the chromatography of the first step of non-hydrolysed endosperm extract (11.4 mg). Chromatographic conditions are given in the text. Authentic ferulic acid was eluted at the position indicated with an arrow.

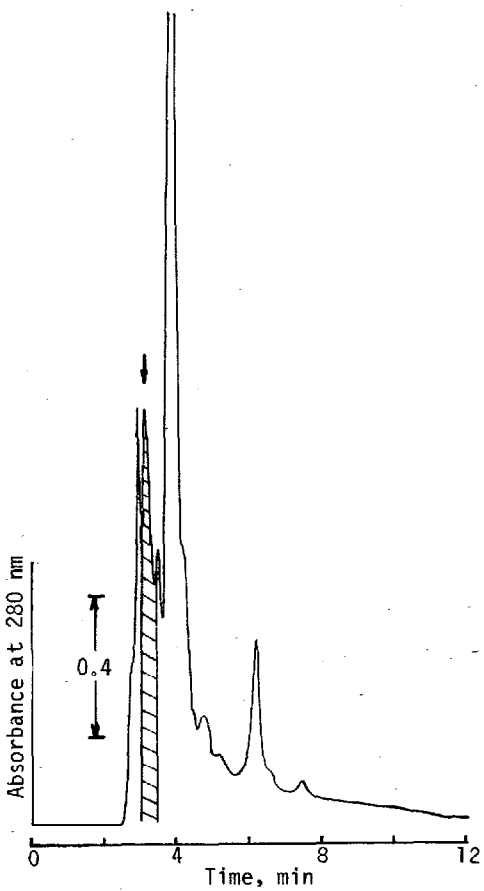

Fig. 4. HPLC profile of the acid hydrolysate of endosperm extract (4 mg). Chromatographic conditions are given in the text. Authentic tyrosine was eluted at the position indicated with an arrow.

Fig. 4 is the chromatographic profile of the acid hydolysate of the endosperm extract using the mobile phase methanol–potassium dihydrogen phosphate (pH 3.5) (40:60). The tyrosine peak fraction shown as the shaded area in the figure was successively separated by the second and third steps of chromatography. The peak at 8.5 min on the chromatogram of the third step was well resolved and completely overlapped that of authentic tyrosine.

Fig. 5 shows the chromatographic profile of the alkaline hydrolysate of the endosperm extract using the 40% methanol system. The ferulic acid fraction shown as the dotted area in the figure was collected and separated by the second step of chromatography. A well-resolved peak appeared at 26 min and overlapped the peak of authentic ferulic acid.

The tyrosine fraction from the acid-hydrolysate and the ferulic acid fraction from the alkaline hydrolysate of bran or germ were separated with good resolution in the same way.

Identification of tyrosine and ferulic acid peaks separated by HPLC

Fluorescence characteristics for the peak fractions obtained from the final step of chromatography were identical with those of the authentic compounds: 280-nm excitation and 310-nm emission maxima for the tyrosine fraction, and 330-nm excitation and 440-nm emission maxima for the ferulic acid fraction.

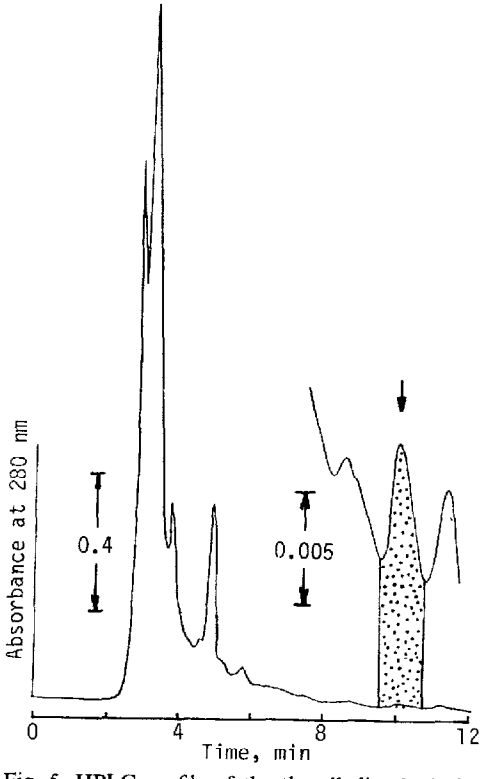

Fig. 5. HPLC profile of the the alkaline hydrolysate of endosperm extract (4 mg). Chromatographic conditions are given in the text. Authentic ferulic acid was eluted at the position indicated with an arrow.

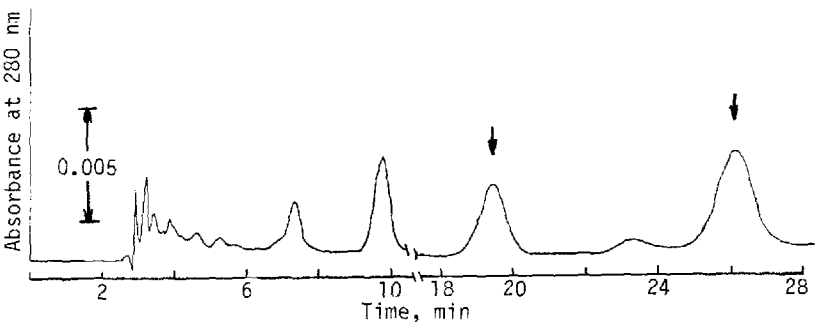

Fig. 6. HPLC profile of the ferulic acid peak of bran extract with irradiation. The peak fraction collected from the three steps of chromatography of bran extract (1.3 mg) was exposed to light of wavelength 330 nm for 30 min as described in the text. Chromatographic conditions are given in the text.

Fractions from the final separation step, when incubated with dansyl chloride and developed on the polyamide layer, gave bright yellow spots at R_F 0 and 0.74 for all tyrosine fractions coinciding with that of the dansyl derivative of authentic tyrosine. Dansyl ferulic acid was detected as orange spots at R_F 0 and 0.89 for all ferulic acid fractions and the authentic compound.

The *trans* isomer of ferulic acid is partially converted into the *cis* isomer by UV light[7,15], and this reaction was used to identify the ferulic acid peak substance. The ferulic acid peak collected from the three steps of chromatography, when submitted to HPLC with methanol–potassium dihydrogen phosphate (pH 3.5) (30:70) after 30-min irradiation with UV light, separated into two peaks. Retention times (min) were 19.5 and 26 (Fig. 6). After prolonged irradiation, the ratio of the peak height with a short retention time to that with the longer time approached the final ratio for the irradiated authentic ferulic acid (1.8).

Estimation of tyrosine and ferulic acid in endosperm, germ, and bran

The fluorescence characteristics, R_F values, and isomerization behaviour under UV irradiation all indicated that the peak fractions separated here by HPLC were tyrosine and ferulic acid. Thus, the amounts of free and methanol-soluble bound ferulic acid and tyrosine were estimated from the peak heights on the chromatograms of the final step (Table I). The amount of the bound form was estimated by sub-

TABLE I

TYROSINE AND FERULIC ACID DETERMINED FROM THE PEAK HEIGHTS SEPARATED BY SUCCESSIVE SEPARATION ON C_{18} COLUMN OF THE WHEAT SEEDS

Data are averages of four determinations ± S.D.

	Tyrosine (μg/g)		*Ferulic acid (μg/g)*	
	Free	*Bound*	*Free*	*Bound*
Bran	40.0 ± 2.6	Nil	14.2 ± 0.5	29.4 ± 1.3
Germ	37.5 ± 1.9	41.6 ± 1.83	2.80 ± 0.03	31.3 ± 1.6
Endosperm	4.06 ± 0.25	3.33 ± 0.24	0.114 ± 0.003	0.217 ± 0.008

tracting the amount of free form from that of the free plus bound form evaluated using the hydrolysed sample.

The amount of either form of ferulic acid was negligible in endosperm. The free form was present in considerable amounts in germ and bran, and the bound form was present in bran. Bran contains much cell-wall material, accounting for the higher levels of ferulic acid in this fraction. However, the levels found here were much smaller than that of the insoluble form in grain cell walls as reported in the literature: 4.8 mg/g of cell wall for wheat bran[16], 5.3 mg/g for shoots of Italian rye grass[4], and 9.1 mg/g for rice endosperm[5]. This low level of soluble ferulic acid probably arises from the formation of complexes in ripe seeds that are insoluble in the aqueous methanol used for extraction.

The amount of free tyrosine found in endosperm is comparable with that found in wheat flour by El-Dash and Johnson[17]. In germ and bran there was ten-fold more free tyrosine than in endosperm. There were similar amounts of soluble bound and free tyrosine in germ. The levels of tyrosine found in bran and germ are comparable with that of ferulic acid in bran. This may point indirectly to the function of tyrosine in the cell wall, where it may cross-link through the oxidative coupling of hydroxyl groups[10].

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