C-Glycosylflavones from Hard Red Spring Wheat Bran[1]

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ABSTRACT

Two flavonoids were isolated from bran of hard red spring wheat (Len variety). They were identified as apigenin-6-C-arabinoside-8-C-hexoside, which is the major compound, and apigenin-6-C-hexoside-8-C-pentoside, which is the minor compound. The flavonoids were extracted from bran with dilute NaOH solution (pH 11). They were purified by chromatography on XAD-2 resin and Sephadex G-15 columns, followed by paper chromatography with three different solvents, and finally by thin-layer chromatography. Six ultraviolet spectra of the bran flavonoids in methanol and methanol plus five different shift additives indicated apigenin-type flavones. The ^{13}C-NMR spectrum supported the same conclusion. Mass spectra showed two sugar molecules, a pentose and a hexose, bound to a flavone ring by carbon-carbon bonds. For the major flavonoid the pentose was at C-6 and hexose at C-8, and for the minor flavonoid the hexose was attached to C-6 and the pentose to C-8.

Flavonoids are widely distributed plant pigments. During the past 30 years flavonoids in many edible plant products have been isolated and characterized because of their influence on color of plant leaves and flowers and on the flavor and nutritional quality of foods.

Bran flavonoids may be important for the miller because bran specks are introduced into flour during the milling process. Increasing amounts of bran will decrease the grade of the flour. Recently, the importance of dietary fiber in the human diet has been emphasized as a beneficial influence on health (Callaghan 1985). Many foods with wheat bran as a source of fiber have come on the market.

It is generally believed that the flavonoids in wheat grain were first recognized by Simpson in 1928 (Markley and Bailey 1935), but no papers were published on this work. In 1932, a very small amount of trihydroxydimethoxy flavone, tricin, was isolated from wheat leaves (Anderson 1932). This flavonoid is usually cited as the one in wheat grain. King (1962) isolated two flavonoids from commercial wheat germ and identified them as apigenin glycosides, but the exact chemical structure and the bonding between the apigenin and sugar could not be determined. Information on the chemical structure of bran flavonoids has not been found in the literature. The purpose of the present research was to isolate and determine the structure of flavonoid(s) in wheat bran.

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MATERIALS AND METHODS

Preparation of Bran Sample

Len variety hard red spring (HRS) wheat was grown at Hettinger, ND, in 1986. The wheat sample was cleaned on a Carter dockage tester and milled on a Buhler automatic laboratory mill, type MLU-202. The bran was cleaned on a Buhler laboratory bran finisher, type MLU-302, to remove the endosperm clinging to the bran. The cleaned bran was ground through a 0.8-mm screen on a hammer mill (Falling Number laboratory mill 3100).

Extraction and Purification Procedures Developed for Bran Flavonoids

Bran (100 g lots) was extracted twice with 4 L of water adjusted to pH 11 with NaOH. Each time the mixture was shaken on an Eberbach shaker at room temperature for 12 hr. The two extracts from each batch were decanted from settled insolubles and combined. The combined extracts were adjusted to pH 5 with acetic acid (HAC), and the resultant white precipitate was removed by centrifuging (5,000 × g at 20° C) for 20 min. A glass column (8.5 × 45 cm) was packed with 500 g of Amberlite XAD-2 (Eastman Kodak, Rochester, NY), and the column was washed with 4 L of H_2O. The acidified extract from each batch of bran was added to

the column, and the adsorbed flavonoid fraction was washed with 8 L of distilled water to remove nonphenolic water soluble impurities. The flavonoids were eluted with 2 L of methanol. The methanol eluate was concentrated to about 20 ml under vacuum on a rotary evaporator using a 35° C water bath, and then 10 ml of the concentrated solution was applied to the top of a column (3 × 6 cm) packed with Sephadex G-15 (Pharmacia Inc., Piscataway, NJ). The column was eluted with water at a flow rate of 2 ml/min, and the 120–400-ml fraction containing the flavonoids was evaporated to dryness on a rotary evaporator using a 40° C water bath. The elution time of the flavonoid was determined in a preliminary run where 20-ml fractions were collected and evaporated to dryness. The flavonoids, if present in these fractions, were separated as a purple flavonoid band, as seen under ultraviolet (UV) light (366 nm), by paper chromatography using 15% HAC as solvent. The residue from the 120–400-ml fraction that contained the flavonoids was dissolved in a minimum volume of methanol.

For further purification, the flavonoids were separated by paper chromatography, first with ethyl acetate/formic acid/H_2O (66:14:20, v/v), then with 15% HAC, and finally with *tert*-butanol/HAC/H_2O (3:1:1, v/v) as solvents. The flavonoid fraction was then separated by thin-layer chromatography on silica gel with $CHCl_3$/hexane (1:1, v/v) solvent. Flavonoids on chromatograms were viewed under UV light (366 nm). The fluorescent dark purple band that changed to fluorescent yellow green in the presence of ammonia was eluted from the paper or thin-layer chromatograms with 80% methanol.

Identification of Flavonoids

UV analysis. The reagents and procedures are those described by Mabry et al (1970). The UV spectra were determined by scanning a flavonoid solution (1 cm light path) from 225 to 525 nm at a speed of 1,200 nm/min on a Beckman BU-7 spectrophotometer. The purified flavonoids were dissolved in about 10 ml of methanol, and the concentration was then adjusted to a major peak absorption of 0.6 to 0.8 using spectroscopic methanol as the reference.

Acid hydrolysis of the flavonoids. The procedure of Markham (1982) for hydrolysis of *O*-glycosides was used. About 2 mg of isolated flavonoids in 10 ml of 2*N* HCl was refluxed on a steam bath for 2 hr. The hydrolysate was concentrated to about 1 ml on a rotary evaporator with a water bath (35° C), and the concentrated hydrolysate and unhydrolyzed flavonoids were analyzed by paper chromatography using 15% HAC as solvent. The R_f values of both hydrolyzed and unhydrolyzed flavonoids were determined after observation under UV light.

Mass spectral analysis of flavonoids. The purified flavonoids were permethylated as described by Brimacombe et al (1986). A $CHCl_3$ solution of permethylated flavonoids was concentrated to about 200 μl under N_2, and the derivative applied as a band on a high-performance thin-layer chromatography silica plate (Whatman). The chromatogram was developed with $CHCl_3$: acetone (4:1, v/v) to the top of the plate. After drying, the plate was developed again using the same solvents. Two violet bands, a major one at R_f 0.38 and a minor one at R_f 0.61, were scraped off and eluted with 10 ml of acetone. The acetone solution was evaporated under N_2 to dryness, and the residue was dissolved in about 10 μl of $CHCl_3$ for mass spectral analysis. The mass spectrum was obtained on a Varian MAT CH7 (70 eV) mass spectrometer.

^{13}C-Nuclear Magnetic Resonance Spectroscopy

Purified flavonoid (5 mg) was dissolved in D_2O, deuterium oxide (heavy water), and a ^{13}C-nuclear magnetic resonance (NMR) spectrum was obtained at room temperature with a JEOL FX-90Q Fourier transform NMR spectrometer. Acetonitrile (1.3 δ) was used as a reference.

RESULTS AND DISCUSSION

Isolation of Flavonoids

The extraction, isolation, and purification procedures are outlined in Figure 1. Bran flavonoids were extracted with alkaline NaOH solution (pH 11) rather than the traditional solvent, alcoholic water. Bran flavonoids could not be extracted by 20 or 100% methanol. The alkaline condition may have been necessary to disrupt bonding between the flavonoids and other components of the bran. Also, flavonoids are weakly acidic polyphenols, which would be more soluble in alkaline solution.

In the first purification step of acid precipitation, dissolved protein and other materials were removed. The dried precipitate contained 45% protein by Kjeldahl analysis. More than 90% of the protein in the crude extract was removed by this procedure. Other water-soluble materials were removed by passage through a column of nonionic Amberlite XAD-2. Flavonoids in the supernatant were adsorbed on the column and other water-soluble impurities were washed off with water. The adsorbed flavonoids were eluted with methanol. A short column of Sephadex G-15 was used to remove high molecular weight impurities, followed by a series of paper and thin-layer chromatography steps to remove lower molecular weight impurities. The bran flavonoids are rather strongly absorbed by Sephadex G-15, as has also been reported for flavonoids in biscuits prepared with wheat and cottonseed flours (Blouin et al 1981). The purified compounds contained two flavonoids, but no solvent systems were found to separate them by paper chromatography and thin-layer chromatography. Later experiments indicated that they could be separated after methylation.

Identification of Flavonoids

UV spectra. Six UV spectra of flavonoids in methanol and methanol containing five different shift additives are shown in Figure 2; the magnitudes of the shifts are given in Table I. According to the UV spectra interpretations summarized by Mabry et al (1970), the two methanol peaks and the peak shifts with five shift reagents indicated apigenin is the aglycone of the bran flavonoids. The methanol spectrum showed maximum absorption at 273 and 336 nm, similar to that reported for flavones. The bathochromic shift induced by NaOMe (band I + 60 nm) and NaOAC (band II + 9 nm) suggested the presence of free 4′- and 7-hydroxyl groups, respectively. Moreover, the bathochromic shifts with AlCl (band I + 40 nm) and $AlCl_3$/HCl (band I + 46 nm) showed the presence of a free 5-OH on the A-ring.

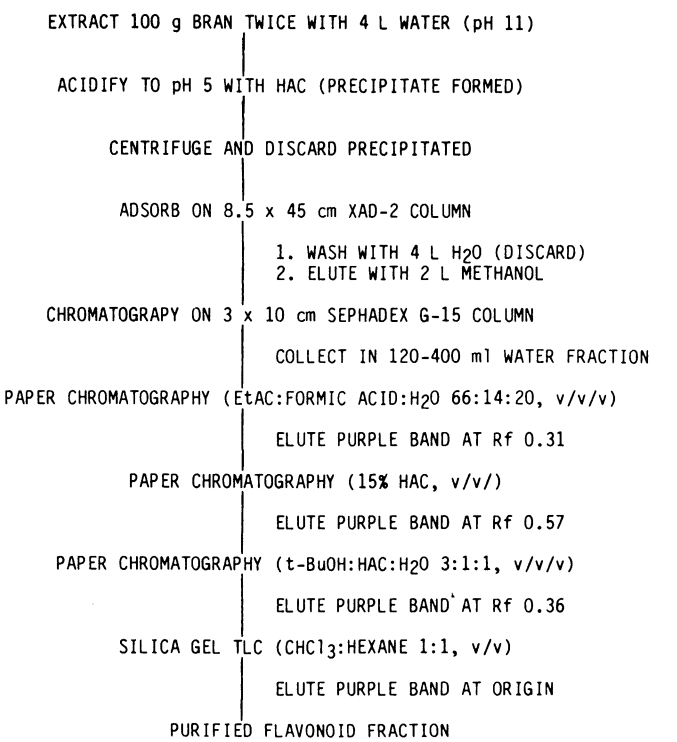

Fig. 1. Scheme for purification and isolation of flavonoids from bran of Len variety wheat.

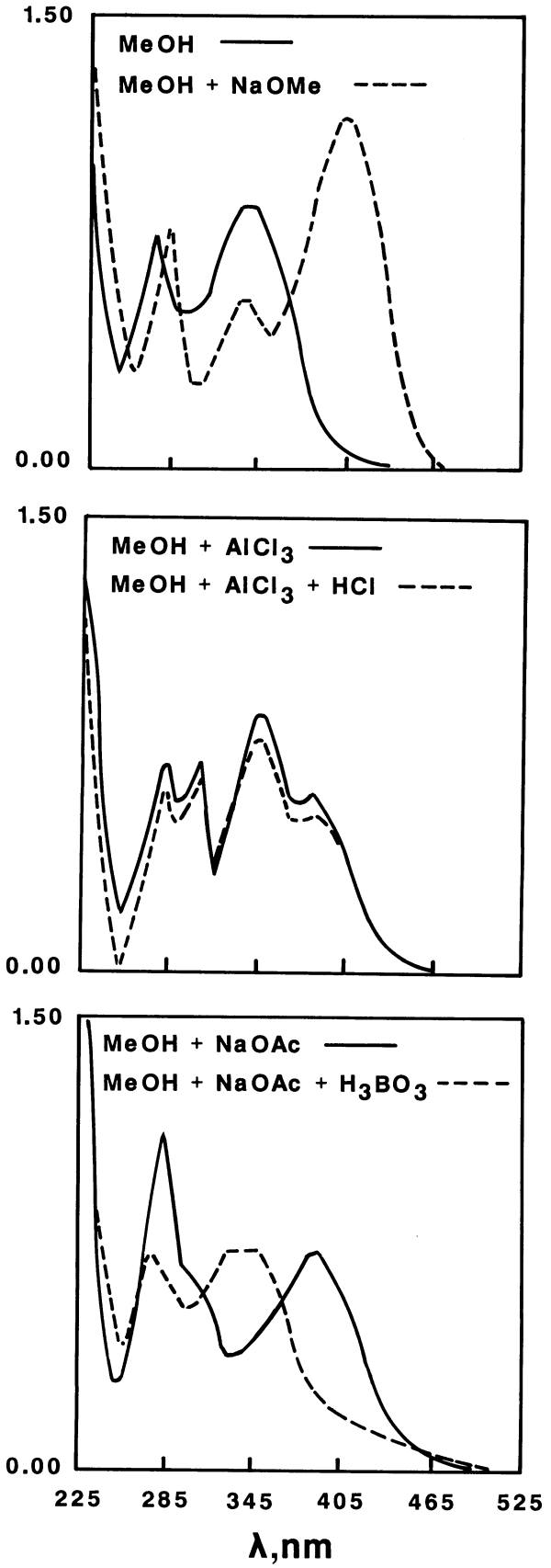

Fig. 2. Six ultraviolet (UV) spectra of flavonoids isolated from Len bran. UV spectra peaks were as follows: MeOH = 273,335; NaOMe = 282,334,396; $AlCl_3$ = 280,304,344,376; AlCl3/HCl = 280,304,343,382; NaOAc = 282,300,390; NaOAc/H3BO3 = 276.339.

^{13}C-NMR spectrum. The chemical shift data of the bran flavonoid sample are given in Table II. The ^{13}C chemical shifts of the purified fraction of flavonoids were similar to those reported for schaftoside, 6-C-glycosyl-8-C-arabinosylapigenin (Markham and Chari 1982). This suggested that bran flavonoids are glycosylapigenins. The chemical shifts of the sugar carbons could not be assigned with certainty because the sample contained two flavonoids that we could not separate without derivatization. The presence of more than two different sugars makes it difficult to identify the sugar residues by ^{13}C-NMR due to overlapping peaks. However, all the shifts observed are consistent with an apigenin glycoside structure.

Acid hydrolysis. The R_f values of acid hydrolyzed and unhydrolyzed bran flavonoids were both 0.57, which means that no actual hydrolysis had occurred. This indicated that the flavonoids are C-glycosides because the C-glycosylflavonoids are resistant to acid hydrolysis (Mabry et al 1970).

Mass spectra. The mass spectra of the two permethylated flavonoids from bran are given in Figure 3 (major flavonoid) and Figure 4 (minor flavonoid). Both have unfragmented ions (M^+) at m/z of 704 and fragments with molecular weight losses of M-15, M-31 (base peak), M-47, M-61, M-119, M-131, M-145, M-163, M-175, M-189, M-205, and M-221, which are characteristic peaks of 6,8-di-C-glycosylflavones (Bouillant et al 1975). Because the aglycone moiety is known to be apigenin from UV and ^{13}C-NMR data, the unfragmented ion of 704 indicated that the sugars linked to the nuclei are one pentose and one hexose (Markham 1982). The mass spectrum of the major bran flavonoid (Fig. 3) shows that the fragment at M-131 (pentose loss) is more intense than the M-175 fragment (hexose loss). Therefore, according to Chopin et al (1982), the hexose is at the C-8 position and the pentose at the C-6 position because the C-6 sugar is easier to fragment than the C-8

TABLE I
Ultraviolet Spectral Interpretation of Bran Flavonoids

Shift Reagent	Wavelength Shift Observed		Interpretation
	Band I[a]	Band II[a]	
NaOMe	+60 nm	...	4′-OH
NaOAc	...	+9 nm	7-OH
NaOAc/H_3BO_3	...	...	...
$AlCl_3$/HCl	+46 nm	...	5-OH
$AlCl_3$	+40 nm	...	...

[a] In methanol the wavelengths for band I and band II of bran flavonoids are 336 and 273 nm, respectively.

TABLE II
^{13}C-Nuclear Magnetic Resonance (NMR) Chemical Shifts of a Flavonoid Fraction Isolated from Bran of Hard Red Spring Wheat[a]

Agylcone Carbons	Chemical Shift (ppm)	Sugar Carbons	Chemical Shift (ppm)
Carbon			
2	163.6	6-C-sugar	
3	102.2	carbon 1	74.5
4	182.2	carbon 2	71.1
5	159.8	carbon 3	...
6	107.8	carbon 4	69.7
7	160.0	carbon 5	80.0
8	104.0	carbon 6	61.7
9	156.0	8-C-sugar	
10	103.5	carbon 1	75.9
1′	121.6	carbon 2	...
2′	128.6	carbon 3	...
3′	115.8	carbon 4	...
4′	160.0	carbon 5	71.1
5′	115.8	carbon 6	...
6′	128.2	...	...

[a] The ^{13}C-NMR assignments were made with reference to the published ^{13}C-NMR spectrum of schaftoside (Markham and Chari 1982).

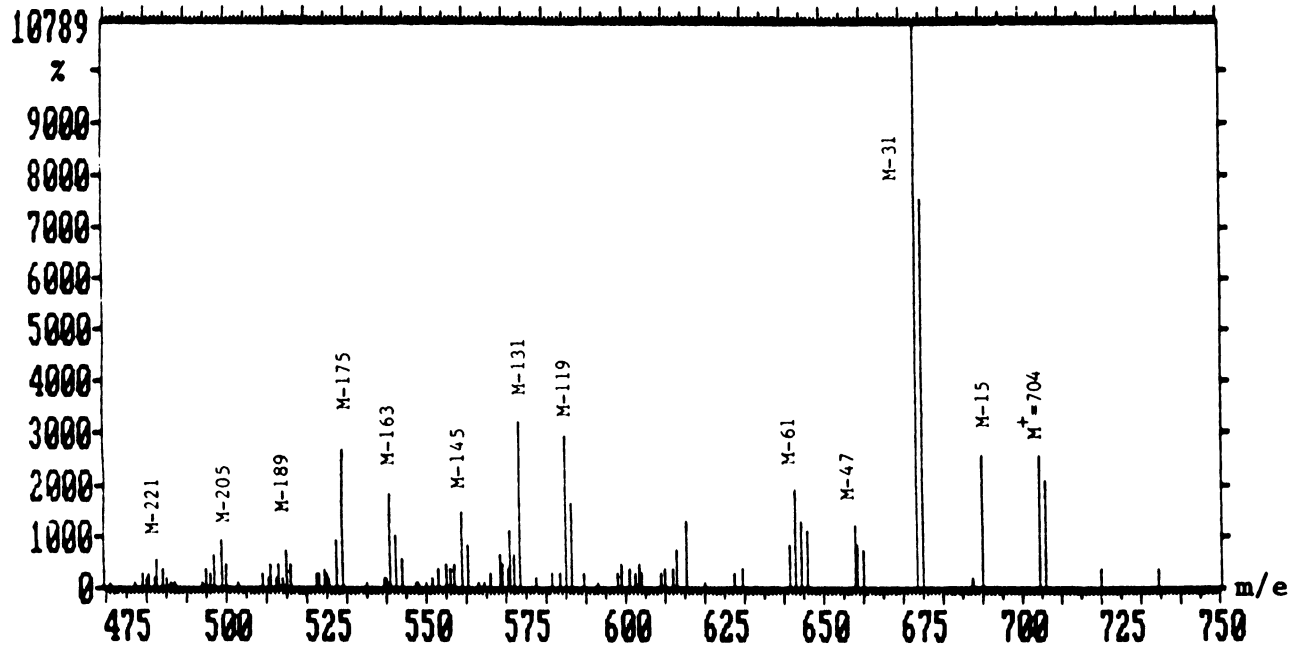

Fig. 3. Mass spectrum of major Len bran flavonoid: Permethyl apigenin-6-C-arabinoside-8-C-hexoside.

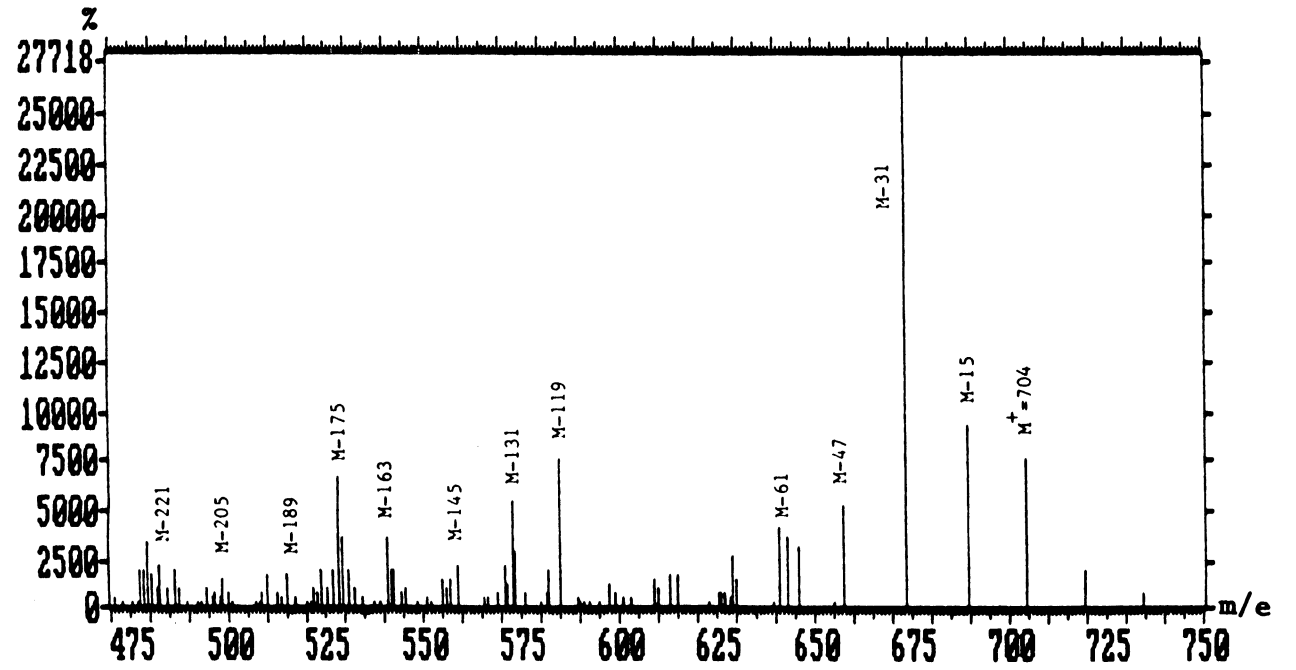

Fig. 4. Mass spectrum of minor Len bran flavonoid: Permethyl apigenin-6-C-hexoside-8-C-pentoside.

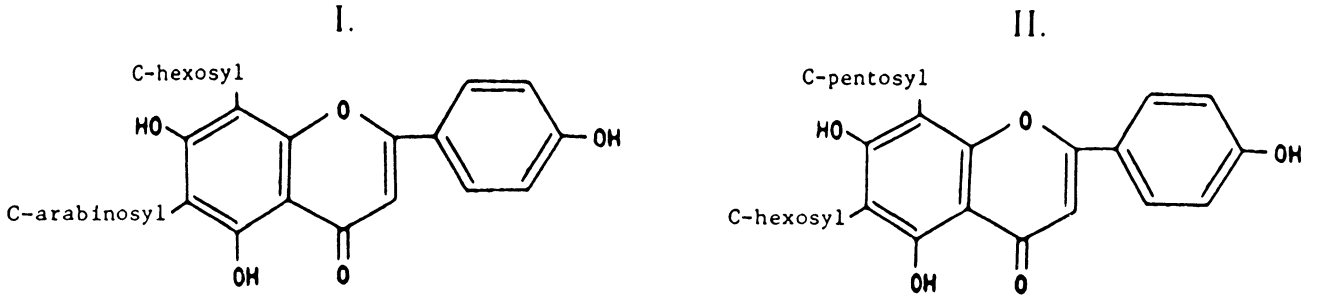

Fig. 5. The chemical structure of Len bran flavonoids. **I,** Major flavonoid of Len bran: apigenin-6-C-arabinoside-8-hexoside. **II,** Minor flavonoid of Len bran: apigenin-6-C-hexoside-8-C-pentoside.

sugar. The mass spectrum of the minor glycosylflavonoid shows that the M-175 fragment is higher than the M-131 fragment, and indicates that the hexose is at C-6 and the pentose is at C-8. Also, if the peak intensity of M-131 > M-119 > M-145, as is the case with the major bran flavonoid, one may conclude that the pentose is arabinose (Bouillant et al 1975).

The mass spectrum strongly indicated the two bran flavonoids are apigenin-6-C-arabinoside-8-C-hexoside (major flavonoid) and apigenin-6-C-hexoside-8-C-pentoside (minor flavonoid) with only one sugar of the major flavonoid being identified (I and II, respectively, in Figure 5).

CONCLUSION

Two C-glycosylflavones were found in bran of HRS wheat. They were identified as 6-C-pentosyl-8-C-hexosyl- and 6-C-hexosyl-8-C-pentosylapigenin by mass spectrometry, ^{13}C-NMR, and UV spectroscopy.

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