Journal of Chromatography, 441 (1988) 443–447
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 486

Note

High-performance liquid chromatography of cottonseed flavonoids

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(Received March 11th, 1988)

Cottonseed flour is a potential source of edible protein. However, when it is used in certain food products it causes an undesirable yellow color. It was established that this coloration was caused by flavonol glycosides present in the flour in a concentration of approximately 0.5% (ref. 1). Seven of these flavonoids were isolated by column and thin-layer chromatography and identified as isoquercetin, rutin, quercetin 3-O-neohesperidoside, kaempferol 3-O-neohesperidoside, quercetin 3-O-glucoglucoside, kaempferol 3-O-glucoglucoside and quercetin 3-O-robinoside. The presence of other minor flavonoids was indicated by two-dimensional cellulose thin-layer and Sephadex LH-20 column chromatography. El-Negoumy *et al.*[2] recently identified from the seeds of *Gossypium barbadense*, the following flavonoids: quercetin 3-O-glucoside, 3-O-galactoside, 3-O-rutinoside, 3-O-glucogalactoside, and 3-O-glucorutinoside and kaempferol 3-O-glucoside and 3-O-glucorutinoside. No quantitation of cottonseed flavonoids has been reported. This communication reports on the quantitation of the individual flavonoids of glandless cottonseed (*Gossipium hirsutum*) flour by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Apparatus

A Beckman HPLC system consisting of two Model 112 pumps, Model 421 controller, Model 340 organizer and Model 165 variable-wavelength detector was used for the identification and quantitation of flavonoids. The detector was connected to an analog-to-digital converter, which was on-line with a Hewlett-Packard laboratory automation system, HP3357.

HPLC conditions

Separations were performed on a Beckman Ultrasphere ODS 3-μm column (7.5 cm × 4.6 mm I.D.). A gradient of 5% acetic acid (A) and 100% methanol was used: 1 min with 100% A, then a linear gradient to 80% A in 25 min, then a linear gradient to 73% A in 14 min and held constant for 10 min. A flow-rate of 0.5 ml/min and a 20-μl injection loop were used. The eluate was monitored at 254 nm and spectra of eluted components were obtained by scanning from 200 to 400 nm.

Materials

Glandless cottonseed kernels were flaked, extracted four times with hexane, air-desolventized and milled to a flour by the Food Protein Research and Development Center, Texas a & M University, College Station, TX, U.S.A. The flour was extracted 24 h at room temperature with light petroleum at solids to solvent ratio of 1:10. The mixture was filtered on a fritted glass funnel, the solids were washed with an equal volume of light petroleum (b.p. 35–60°C) and the solid residue was air dried. The flour residue was then extracted 4 h at room temperature in 70% aqueous ethanol with vigorous mechanical stirring at a solids to solvent ratio of 1:10. The mixture was filtered on a fritted glass funnel, the solids were washed with an equal volume of 70% aqueous ethanol, and the extract and washings were combined. The ethanol was removed on a rotary evaporator and the aqueous solution was freeze dried.

Standards

The standards were cottonseed flavonoids isolated in a previous study[1]. Although these compounds appeared to be pure by two-dimensional thin-layer chromatography (TLC), all but two were only 80–90% pure when examined by the current HLPC method. These compounds were used to identify the peaks in the HPLC chromatogram of the aqueous ethanol extract of the cottonseed flour. Two of the flavonoids, quercetin 3-O-neohesperidoside and kaempferol 3-O-glucoglucoside, were better than 95% pure by HPLC and were used as standards for quantitation of the flavonoid mixture.

RESULTS AND DISCUSSION

The HPLC chromatogram of the aqueous ethanol extract of cottonseed flour is shown in Fig. 1. The first major flavonoid eluted at about 33 min. Ten major flavonoids were present (Peaks 1–10). By addition of flavonoid standards to the aqueous ethanol extract, peak identifications of the flavonoids in the extract were made and are presented in Table I. Spectra of peaks 1 and 2 were almost identical to those of the other quercetin flavonoids. Peaks 1 and 2 were, therefore, tentatively described as quercetin 3-O-diglycosides. Peaks 5 and 10 gave spectra significantly different from the spectra of the other flavonol glycosides Peak 5 gave maxima at 253, 263 (sh), 297, 328 and 360 (sh) nm and peak 10 at 263, 299, 320 and 365 (sh) nm. The shapes of the maximum peaks in the short-wavelength region and the retention times suggested that peaks 5 and 10 were quercetin and kaempferol glycosides, respectively.

With the previously isolated quercetin 3-O-neohesperidoside as a standard for the quercetin derivatives and kaempferol 3-O-glucoglucoside as the standard for the kaempferol derivatives, quantitation of the major flavonoids of the aqueous ethanol extract of cottonseed flour was performed. Peak areas of the standards and unknowns at 254 nm, measured by the laboratory automation computer system, were the basis for the calculations. The results are presented in Table II. The quercetin glycosides are the predominant species, about 87% of the major flavonoids. The quercetin neohesperidoside and the glucoglucoside are the major flavonoids, 28% and 20% respectively. The total concentration in the flour was 0.51%.

To get more information about the structure of peaks 5 and 10, which had

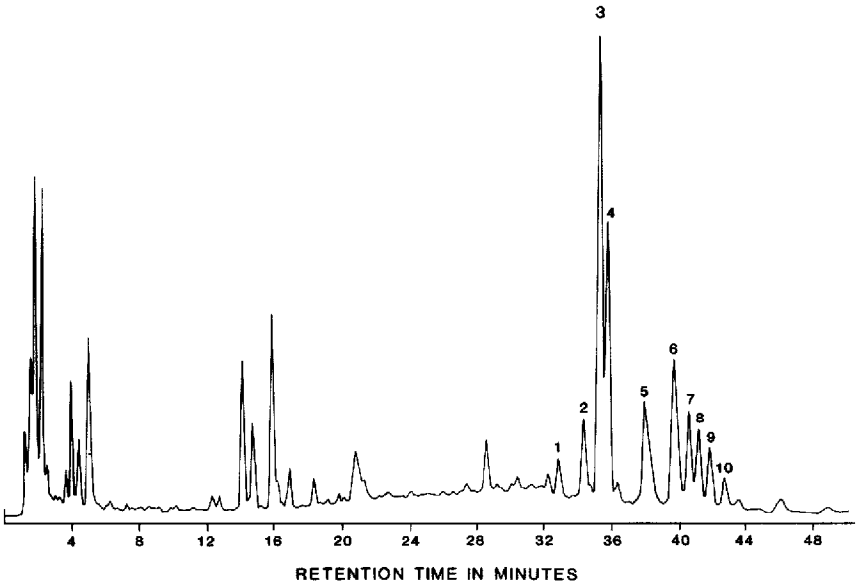

Fig. 1. Chromatogram of aqueous ethanol extract of cottonseed flour on an Ultrasphere ODS column developed with a acetic acid–methanol gradient.

rather unusual UV spectra, a small amount of peak 5 was isolated as follows. On a Sephadex LH-20 column with 50% methanol as the eluent, a fraction containing a mixture of peaks 2, 3, 5, 8 and 10 was obtained. Peaks 8 and 10 were removed from the mixture on cellulose thick-layer plates developed with *n*-butanol–acetic acid–water (4:1:5, uper phase). Peak 5 was separated from 2 and 3 on silica gel plates developed with ethyl acetate–methyl ethyl ketone–formic acid–water (5:3:1:1). The

TABLE I

IDENTIFICATION OF FLAVONOID PEAKS IN HPLC OF THE AQUEOUS ETHANOL EXTRACT OF COTTONSEED FLOUR

Peak No.	*Retention time (min)*	*Identification*
1	32.0	Quercetin diglycoside (tentative)
2	34.4	Quercetin diglycoside (tentative)
3	35.3	Quercetin 3-O-neohesperidoside (2-O-α-L-rhamnosyl-β-D-glycoside)
4	35.8	Quercetin 3-O-glucoglucoside
5	38.0	Quercetin acylated 3-O-glucoglucoside (tentative)
6	39.7	Quercetin 3-O-robinoside (6-O-α-L-rhamnosyl-β-D-galactoside)
7	40.6	Quercetin 3-O-rutinoside (6-O-α-Lrhamnosyl-β-D-glucoside)
8	41.2	Kaempferol 3-O-neohesperiodoside
9	41.9	Kaempferol 3-O-glucoglucoside
10	42.8	Kaempferol acylated 3-O-diglycoside (tentative)

TABLE II

QUANTITATION OF THE MAJOR FLAVONOIDS OF COTTONSEED FLOUR

Peak No.	*% in flour*	*% of total flavonoids*
1	0.015	3.0
2	0.027	5.2
3	0.142	27.6
4	0.103	20.1
5	0.055	10.8
6	0.065	12.6
7	0.037	7.2
8	0.031	6.0
9	0.025	4.9
10	0.014	2.6

material isolated from the silica gel plates was again passed through a Sephadex LH-20 column to remove contaminants extracted from the plates. Less than a milligram of peak 5 was obtained.

Diagnostic UV spectral studies[3] were performed on the isolated peak 5 component [band I (300–380 nm) shifts: sodium methoxide–methanol 47 nm, $AlCl_3$/HCl–methanol 40 nm, $AlCl_3$/HCl–$AlCl_3$ 31 nm, sodium acetate/H_3BO_4–methanol 12 nm and band II (240–280 nm) shift: sodium acetate–methanol 15 nm]. The data indicated that peak 5 was a 3-O-substituted quercetin. These studies also indicated that the maxima at 297 and 320 nm of peak 5 were not the flavonoid absorption peaks and suggested that this flavonoid is acylated. The spectrum of peak 5 resembled a superimposition of an aromatic acid absorption on the normal flavonoid spectral bands[4].

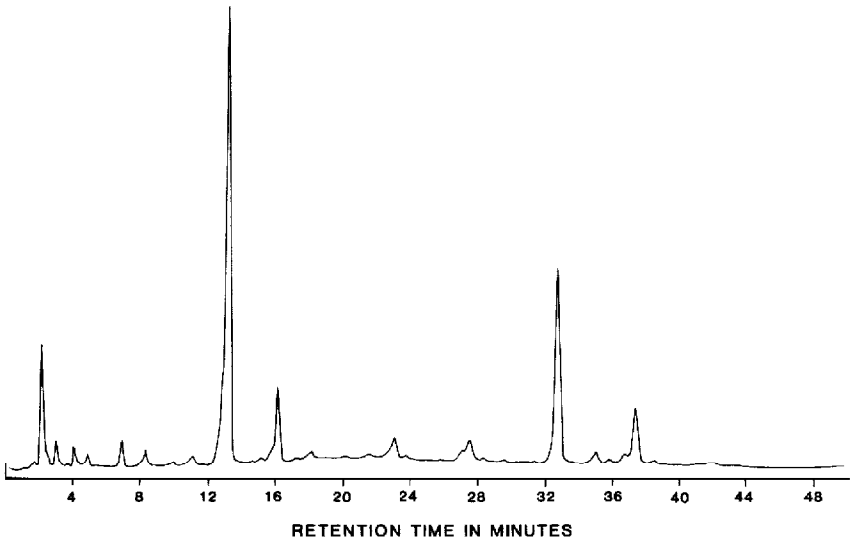

Fig. 2. Chromatogram of ethyl acetate extract of base hydrolysis of peak 5 (Fig. 1) on an Ultrasphere ODS column developed with a 5% acetic acid–methanol gradient.

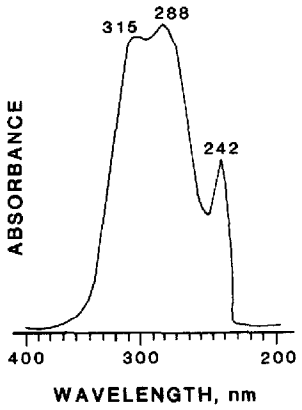

Fig. 3. Spectrum of peak at 13.24 min in Fig. 2.

Acid hydrolysis of the isolated peak 5 flavonoid and TLC of the hydrolysate indicated quercetin as the aglycone and glucose as the sugar moiety. A UV fluorescent component with R_F 0.48 (cellulose plate, ethyl acetate–pyridine–water, 12:5:4) was also evident in the acid hydrolysate, suggesting the presence of a phenolic acid. Base hydrolysis (3 h, 2 *M* sodium hydroxide), acidification to pH 2–3 with hydrochloric acid and extraction with ethyl acetate gave a mixture with the HPLC curve presented in Fig. 2. The peak at 32.77 min was a flavonoid and co-chromatography with the original aqueous ethanol extract indicated this flavonoid to be the same as peak 4 (quercetin 3-O-glycoglucoside) of Fig. 1. The peak at 13.24 min had the spectrum given in Fig. 3, suggestive of a phenolic acid. None of the phenolic acid standards on hand (gallic, α-resorcylic, protocatechuic, γ-resorcylic, gentisic, *p*-hydroxybenzoic, *m*-hydroxybenzoic, vanillic, caffeic, isovanillic, phloretic, syringic, *p*-coumaric, veratic, salicylic, *m*-coumaric, ferulic, *o*-coumaric, isoferulic and sinapic acids) gave a retention time and spectrum matching this base-hydrolyzed component. The data, however, suggest that peak 5 is an acylated flavonoid and that peak 10 with its similar spectrum is also an acylated flavonoid.

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