Identification of Kaempferol-3-Rhamnoglucoside and Quercetin-3-Glucoglucoside in Cottonseed[1]

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The flavonol glycosides, quercetin-3-glucoglucoside and kaempferol-3-rhamnoglucoside, have been separated from crushed, delinted cottonseed (kernel and hull) by extensive use of paper chromatography, ultraviolet spectrophotometry, and qualitative and quantitative analysis of their hydrolysis products. Details of the separation and identification are described.

NO PREVIOUS REPORT has been made of the presence of kaempferol glycosides or of quercetin-3-glucoglucoside in cottonseed although these have been reported in other natural products (1,2). Perkin (3) identified flavonoid compounds in the flowers of cotton, but Boatner (4) has stated that there is little evidence that these pigments occur in the seed. The investigations of Pratt and Wender (5) however have revealed the presence of at least six flavonoid pigments in cottonseed. Two of these flavonoids have been identified previously (5) as isoquercitrin (quercetin-3-glucoside) and rutin (quercetin-3-rhamnoglucoside). The present paper reports our identification of two additional flavonoid compounds as kaempferol-3-rhamnoglucoside and quercetin-3-glucoglucoside. Kaempferol is 3,4′,5,7-tetrahydroxyflavone, and quercetin is 3,3′,4′,5,7-pentahydroxyflavone. Identification has been achieved through paper chromatography and ultraviolet spectrophotometry of the glycosides and their hydrolysis products.

Experimental

Separation of the Glycosides from Cottonseed. The concentrated 85% isopropyl alcohol-water extracts of 5 kg. of mechanically-delinted cottonseed, which had been crushed (kernel and hull) with a Waring Blendor, were washed four times with water. The aqueous layers were combined and again concentrated *in vacuo*. This concentrated extract was streaked on Whatman No. 3 MM chromatography paper (18 x 22½ in.) and the papers were developed for 12–16 hrs. in n-butyl alcohol-acetic acid-water (6:1:2 v/v). Three main zones were found on the resulting chromatograms. These were labelled 1, 2, and 3; Zone 1 had the smallest R_f value.

Zone 3 was cut out and sewn onto new sheets of chromatography paper; the papers were developed in 15% acetic acid-water for 5–6 hrs. Two zones, 3A and 3B, were now present. Zone 3A contained isoquercitrin, and Zone 3B, with higher R_f value, contained two new glycosides. For further purification and identification, Zone 3B was cut out, sewn onto new sheets of chromatography paper, and developed a second time in 15% acetic acid-water. Two zones now appeared and were labelled 3B-1 and 3B-2. The zone with the smaller R_f value, 3B-1, again contained isoquercitrin. Zone 3B-2, containing the desired compounds, was cut out and sewn onto new chromatograms; the papers were developed once more in 15% acetic acid-water. This time only one zone appeared (3B-2A). Zone 3B-2A was cut out and sewn onto new papers; each was developed 14–18 hrs. in the n-butyl alcohol-acetic acid-water system. Two zones now appeared, 3B-2A-1 and 3B-2A-2. Each of these zones was cut out, sewn onto new papers, and developed again in the n-butyl alcohol-acetic acid-water until each zone contained only one compound.

Hydrolysis Products of the Kaempferol Glycoside. Ten mg. of compound 3B-2A-2 were dissolved in 30 ml. of 50% ethyl alcohol-water solution, containing 2% v/v sulfuric acid and refluxed for 2.5 hrs. The ethyl alcohol was distilled off, and the hydrolysis mixture was extracted twice with ethyl acetate. The ethyl acetate solution was concentrated and co-chromatographed with authentic kaempferol and found to be identical with it in the n-butyl alcohol-acetic acid-water, 15% acetic acid-water, 60% acetic acid-water, and nitromethane-benzene-water (3:2:5 v/v) solvent systems. An ultraviolet absorption curve of the aglycone, resulting from the hydrolysis of 3B-2A-2, was identical with one of reference kaempferol.

The aqueous layer of the extracted hydrolysate was passed through a column that contained a mixed bed ion exchange resin (Amberlite MB-1), and the neutral sugar solution was concentrated to about 2 ml. *in vacuo*. In the n-butyl alcohol-acetic acid-water and n-butyl alcohol-pyridine-benzene-water (5:3:1:3 v/v) systems, this concentrate was found to contain the sugars glucose and rhamnose.

Identification Studies on the Kaempferol Glycoside. The glycoside 3B-2A-2, now shown to contain kaempferol, glucose, and rhamnose, was compared with authentic samples of kaempferol-3-rhamnoglucoside on one- and two-dimensional paper chromatograms, using all the solvent systems previously listed in this paper, alone and in combinations of two. In every case 3B-2A-2 corresponded to kaempferol-3-rhamnoglucoside (Table I). Two-dimensional mixed chromato-

TABLE I

R_f Values of Isolated Compounds and Known Standards

Compound	Solvent Systems[a] (1)	(2)	(3)	(4)
Isoquercitrin	0.68	0.48	0.69	0.89
Quercitrin	0.80	0.58	0.75	0.95
Rutin	0.51	0.60	0.71	0.88
3B-2A-1	0.59	0.67	0.76	0.91
Kaempferol-3-rhamnoglucoside	0.60	0.79	0.80	0.93
3B-2A-2	0.60	0.80	0.80	0.92
Quercetin	0.77	0.07	0.39	.....
Aglycone from 3B-2A-1	0.75	0.07	0.38	.....
Kaempferol	0.84	0.14	0.49	.....
Aglycone from 3B-2A-2	0.84	0.15	0.48	.....

[a] Solvent systems: (1) n-butyl alcohol-acetic acid-water (6:1:2 v/v); (2) 15% acetic acid-water; (3) 60% acetic acid-water; and (4) 60% isopropyl alcohol-water.

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grams of 3B-2A-2, superimposed on the authentic kaempferol-3-rhamnoglucoside, using the n-butyl alcohol-acetic acid-water system in one direction and the 15% acetic acid-water system in the second direction, gave only one spot.

Determination of the ultraviolet absorption spectrum of 3B-2A-2 in ethyl alcohol, using the Beckman DK-1 recording spectrophotometer, produced the same curve as that obtained for authentic kaempferol-3-rhamnoglucoside.

Identification of Quercetin-3-glucoglucoside. Ten mg. of the chromatographically-pure 3B-2A-1 was hydrolyzed by the procedure used for the kaempferol glycoside. By paper chromatography the products were found to be quercetin and glucose.

R_f values of 3B-2A-1 before hydrolysis (Table 1) indicated that this glycoside of quercetin likely contained 2, rather than 1 or 3, units of monosaccharide. For proof of the ratio of glucose to quercetin in this glycoside another 10 mg. of 3B-2A-1 were hydrolyzed as described previously except that the organic layer was evaporated to dryness and then dissolved to make 5 ml. of solution in 95% ethyl alcohol. The neutral aqueous layer was concentrated to exactly 2 ml. An aliquot (0.6 ml.) of the aqueous solution was streaked on Whatman No. 1 chromatography paper and analyzed quantitatively by a modified procedure of Timell (6).

Of the 5-ml. solution of aglycone in 95% ethyl alcohol, an aliquot (0.4 ml.) was chromatographed, using the n-butyl alcohol-acetic acid-water system. The quercetin zone was cut out and eluted with 95% ethyl alcohol and made to exactly 10 ml. in volume. A blank chromatography paper strip was treated by the same procedure except that no quercetin was present. The absorbance of the quercetin was measured against its blank at 374 mμ, using 1-cm. silica cells and the Beckman spectrophotometer, Model DU. The quantity of quercetin present was determined from a standard quercetin curve. To obtain this standard curve five samples of different, but known, concentrations of quercetin were processed through the same procedure already described for the quercetin in the glycoside hydrolysate. A straight-line curve was obtained by plotting the absorbance against micrograms of quercetin originally streaked at the beginning of its paper chromatography. By this method, glycoside 3B-2A-1 was found to have a ratio, within experimental error, of one quercetin to two glucose units.

Spectral Studies. In order to locate the position or positions of attachment of the two glucose units a spectral shift study was made by the procedures of Jurd (7) and of Jurd and Horowitz (8). Since the aglycone was quercetin, the sugar linkages could occur at positions 3,3′,4′,5, or 7 of quercetin. First, the ultraviolet spectrum of compound 3B-2A-1 in absolute ethyl alcohol was determined by using the Beckman DK-1 recording spectrophotometer. Maxima were at 260 mμ and 370 mμ. For analysis of position 7 excess anhydrous fused sodium acetate was added to the sample cell and to the blank, and, after 5–10 min. the spectrum was determined again. The first maximum had shifted from 260 mμ to 272 mμ. This indicated that the Number 7 hydroxyl group was not substituted by a glucose unit.

To 2 ml. of the 3B-2A-1 stock solution (approximately 0.0001 M) in absolute ethyl alcohol were added 2 ml. of a saturated solution of boric acid in absolute ethyl alcohol. The solution was diluted to 10 ml. with absolute ethyl alcohol, and an excess of sodium acetate was added. After the solution was shaken and allowed to settle for 10–20 min., the spectrum of the solution was recorded on the same graph as the untreated sample. The 260 mμ peak had shifted 23 mμ toward shorter wavelengths. The 370 mμ maximum had shifted to 385 mμ. These shifts indicated that the 3′,4′-o-dihydroxy groups of the quercetin are not substituted by sugar in the glycoside studied.

The reaction of the glycoside with aluminum chloride indicated that the sugar was not on the Number 5 position. This leaves the Number 3 position as the point of attachment of glucose. Actually the substitution of the Number 3 hydroxyl group by the glucose had already been evidenced by the experimental fact that this quercetin glycoside fluoresces brown, rather than yellow, under long wavelength (3660 Å) ultraviolet light.

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Report of the Uniform Methods Committee, May, 1961

THE MEETING of the Uniform Methods Committee of the American Oil Chemists' Society was held in St. Louis on May 2, 1961. E.F. Sipos, R.A. Marmor, R.J. Houle, K.E. Holt, and J.H. Benedict (representing E.M. Sallee, editor *ex officio*), and D.L. Henry were present. Vistors were Edward Handschumaker, and T.D. Parks. The following matters were discussed and decisions were made as indicated:

Report of the Soap and Synthetic Detergent Analysis Committee, J.C. Harris, chairman—The Soap and Synthetic Detergent Analysis Committee recommended advancement of Tentative Method Da 17–52 and Dd 7b–55 to official status, and to correct Db 8–48 by making a change in section C. 1, line 2 Da 12–42 to Da 12–48. Their report was accepted by the Uniform Methods Committee, and the recommendations were approved.

Report of the Seed and Meal Analysis Committee, M.H. Fowler, chairman—The Seed and Meal Analysis Committee recommended the adoption of a revised Crude Fiber Method to replace Ba 6–49. Their report was accepted by the Uniform Methods Committee, and the recommendation was approved.

Report from the chairman of the Oxirane subcommittee,