Peanut Science (1983) 10, 40-43

A Preliminary Classification of Selected White Testa Peanuts (*Arachis hypogaea* L.) by Flavonoid Analysis

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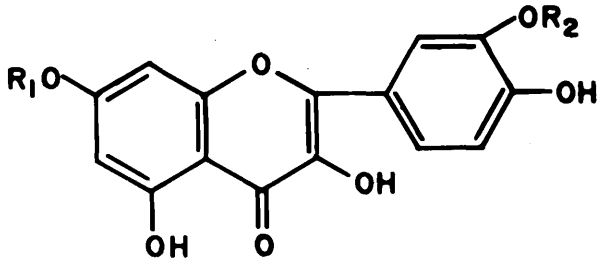
ABSTRACT

The testa and defatted flours of 32 white testa and the krinkleleaf tan spanish peanut (*Arachis hypogaea* L.) genotypes were analyzed for flavonoids in order to classify these peanuts for breeding purposes. Flavonoids were extracted from the testa and flours with aqueous methanol. These extracts were analyzed for flavonoids by high pressure liquid chromatography. UV spectrometry was used to identify the compounds. These flavonoids were principally sugar derivatives of the aglycones quercetin, rhamnetin, and isorhamnetin. Two other aglycones were detected and tentatively identified as isoflavones. From these data, it was possible to classify the 32 white testa and the krinkleleaf genotypes into several groups based upon the presence, absence, or ratio of various flavonoid compounds.

Key Words: Groundnut, chemotaxonomy, HPLC, genotypes, testa, flour.

Flavonoids include all natural plant constituents with a structure based on the aromatic heterocyclic 3-phenylbenzopyrone, flavone. Three examples of flavonols, one of several classes of flavonoids are shown in Figure 1. Variation within each class in the number and position of hydroxyl substituents and ether derivatives (sugars, methyl, etc.) make a large number of flavonoids possible and some six hundred have been identified in plants. Such ubiquity and complexity in structures should make ideal chemical markers (1).

In the early reports (4-6, 10-17) on the pigments of peanut (*Arachis hypogaea* L., cultivar not specified), the methods of isolation and identification of flavonoids were not very sophisticated resulting in some contradictory data. The indication that a dihydroflavonol or flavonol



$R_1 = R_2 = H$ QUERCETIN (Q)

$R_2 = H; R_1 = CH_3$ RHAMNETIN (R)

$R_1 = H$ $R_2 = CH_3$ ISORHAMNETIN (I)

FLAVONOLS

Fig. 1. Molecular structure of representative flavonols.

exists in peanuts was substantiated by Skikora's work on extracts of raw spanish peanut (8). Skikora also suggested the presence of a flavonone-dihydroquercetin. Turner et al. (18) isolated and identified 5, 7-dimethoxyisoflavone as a component of peanut cotyledons. However, a number of cultivars were used and the authors did not specify which cultivar had more or less of this type of flavonoid. An important property of this flavonoid was that it inhibited growth of *Aspergillus flavus* (Link) Fr., and *Trichoderma viride* Pers. ex Fr. Gibbs (2). The most significant work in the field of chemotaxonomy and peanut was published by Seeligmann (7). He found the leaves of four species of *Arachis* contained a C-glycoside flavone and tentatively identified it as vitexin. While paper and thin layer chromatography have been the usual modes of chromatographic analyses of flavonoids, a recent paper by

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Stewart et al. (9) described the application of high pressure liquid chromatography (HPLC) to flavonoid analysis. Both qualitative and quantitative differences were shown in an intraspecific analysis of poinsettia cultivars. In this paper, the application of HPLC to flavonoid analyses of the methanolic (80%) extracts of the testa and flours of 32 white testa and one krinkleleaf peanut genotypes are reported. From these analyses, the genotypes were classified into seveal groups based upon the presence, absence, or ratio, or various flavonoid compounds.

Materials and Methods

A Waters Associates Liquid Chromatograph equipped with a Model 440 Absorbance Detector, 254 nm wavelength; two 6000A pumps and a Model 660 solvent programmer was used. The output from the detector was monitored on a Hewlett-Packard 3380A Integrator. The column was 30 cm by 3.9 mm. I. D. packed with μ Bondapak C_{18}, 10μ, preceded by a micro-guard silica column.

Solvents were filtered using a glass Millipore system with a 0.45μ filter and degassed at room temperature under vacuum with magnetic stirring. The elution solvent was water:acetic acid (495 ml:5 mL) from pump A and methanol from pump B. The flow rate was 2 mL/min. A ten min linear gradient, pump A providing 90 to 65% and pump B, 10 to 35% of the solvent mixture was used. For the extracts after hydrolysis, an isocratic mixture of water-acetic acid-methanol (51:1:48) was used.

UV spectra were obtained using the conditions outlined by Mabry et al. [3] with a Beckman Model 25 spectrophotometer. The aglycones were identified by comparing the UV spectra with standard spectra. Identification was confirmed by HPLC using retention times and spiking techniques. Aglycone ratios were determined by counts as measured by the Hewlett-Packard 3380A Integrator.

All the genotypes were grown under similar conditions at Tifton, Ga. (1980) and stored in a refrigerator (3 C). After prepartion the samples were put into septum vials and stored in a freezer (-5 C).

White testa peanut have been differentiated by geneticists from the tan and/or flesh, pink, and red peanut, but color standards have never been published. The testa color of Table 1 only serve to show visual differences among the genotypes.

Preparation of Testa Extracts. The peanut seed (75 g) were placed between wet sheets of absorbent paper toweling for five minutes and then deskinned by hand. The testa (dry wt. 2 g) were stirred overnight (18 hr) at room temperature in 180 mL of 20% aqueous methanol. The methanol was evaporated at room temperature by a rotary evaporator under vacuum. The solution was lyophilized, the freeze-dried material taken up in 2 mL of HPLC methanol, and filtered using a Millipore system with a 0.45μ filter.

Preparation of Flour Extracts. Deskinned peanut seeds (25g) were homogenized in 250 mL of hexane in a Waring blender at high speed for 2 min. The homogenate was filtered, then rehomogenized in 125 mL of hexane. Flavonoids were then extracted from the homogenate (flour) by the procedure outlined above. The freeze-dried material was suspended in 25 mL of a citrate-Na_2HPO_4 buffer (pH = 3.3). Polyvinylpyrollidone (PVP) (0.6 g) was added and the mixture stirred for 1 hr at room temperature. The mixture was poured into a small column (11 cm X 1 cm ID), then washed with water (pH = 6.0). Flavonoids were eluted with basic (pH = 8-10) methanol until the eluting solvent was no longer yellow (50-100 mL). The pH of the solution was adjusted to 6-7 with 2N HCL and evaporated to dryness with a rotary evaporator at room temperature. The dried material was dissolved in 2 mL of HPLC methanol and filtered. The PVP procedure is necessary to remove material with is incompatible with the initial HPLC solvent system.

Hydrolysis of Extracts. The flavonoid extract (0.5 mL) is a sealed tube was hydrolyzed with 2N HCl (5 mL) on a steam bath for 45 min. The HCl was removed on a rotary evaporator and the dried material taken up in 2 mL HPLC methanol, then filtered.

Identification of Chromatographic Peaks. Aglycone Determination. The flavonoid peaks displayed by both testa and flour extracts were individually isolated from the HPLC and hydrolyzed by the procedure previously described to an aglycone structure. By comparison with standard UV spectra and the HPLC retention times and spiking techniques, the aglycone was identified and the correlation of derivative peak with aglycone was made.

Results and Discussion

Testa. After examination of their chromatograms, the 32 white testa and one tan testa genotypes were classified into five groups with one group being subdivided (Table 1). Groups I and IA did not contain any flavonoids. In groups II and III the amount of flavonoids is so small it could be considered negligible. Group IV seems to represent transition peanut - a number of flavonoids present but none in large concentration. Group V contains a large number and concentration of flavonoids. The chromatogram of Spanwhite (Figure 2) is typical of group V peanut. The large peak at 22.5 min represents a quercetin derivative and the peaks at 33.5 and 36.0 min are isorhamnetin derivatives.

Flavonoid content appears to be correlated with testa color. Groups I and IA chromatograms show nothing in the flavonoid region. They differ, however, in that Group I displays a large number of peaks in the 0-5 min portion of the chromatogram while group IA displays a large amount of peaks in the 1-15 min portion. This difference between groups I and IA appears to be correlated with testa color-

Table 1. Flavonoid Analysis of White Testa from Peanut.

Group	PI No.	TRIVIAL NAME	FLAVONOID ANALYSIS	TESTA COLOR
I	408745	Sen. 61-58	No Flavonoids	Cream
	-	Alba		Bright White
IA	-	Krinkleleaf	No Flavonoids	Tan
	329225	Albo		Flesh
II	338497	AH6742	One derivative	Cream
	338498	AH6644	of quercetin,	Cream
	288160	SR57	low	Bright White
	408729	Sen. 57-149	concentration	Cream
	408732	Sen. 58-522		Cream
	408734	Sen. 58-670		Cream
	393516	Tifrust-8		Reddish
	313151	Taiwan 0323		Bright White
	315611	Pearl White		Bright White
III	408737	Sen. 59-230	2 Flavonoids-	Pinkish
	408741	Sen. 59-236	probably quercetin	Pinkish
	298115	Israel 136	derivatives	Dirty White
IV	376050	U/12/47/1	3-5 flavonoids in	Cream
	376051	U/2/24/4	low concentration	Greenish
V	-	Spanwhite	Numerous	Yellow
	299468	Oakes Col. 471	flavonoids	Yellow
	408728	Sen. 55-117	Quercetin & Iso-	Yellow
	408730	Sen. 57-161	rhamnetin	Yellow
	408733	Sen. 58-644	derivatives	Yellow
	408735	Sen. 59-216		Dirty White
	408738	Sen. 59-231		Dirty White
	408747	Sen. PI851		Brownish
	408748	Sen. PI886		Yellow
	408749	Sen. CTH 71/667		Brownish
	393517	Tifrust-9		Greenish
	408722	Uganda B-13		Yellow
	306228	Sen. 57-204		Yellow
	314817	Tifrust-14		Greenish
	338502	AH259		Yellow

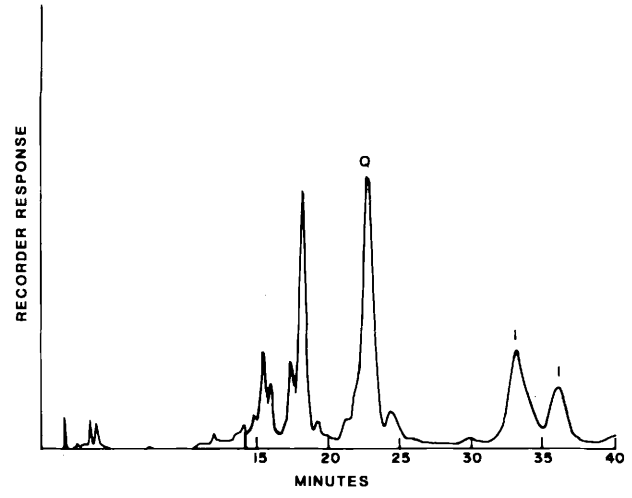

Fig. 2. Chromatogram of Spanwhite skin extract. (Flow rate 2 ml/min, 10 min linear gradient [90-65% of 1% HOAC and 10-35% of methanol] on a 30 cm X 3.9 mm μ Bondapak C_{18} column). Q = quercetin derivative; I = isorhamnetin derivative.

cream or bright versus flesh.

Yellow, brownish, or dirty testa color indicate a large concentration of flavonoids; cream or bright, none or very little. There are some anomalies such as Israel 136, Tifrust 14, Tifrust-8, and Tifrust-9.

Although testa color and flavonoid content appears to be associated, sharp lines of demarcation could not be drawn based on the data from these 33 genotypes. Flavonoid content of peanut testa, therefore, may not be a good chemotaxonomic marker.

Flour. Classification of the 32 white and one tan testa peanut by flavonoid analysis of the flours was more feasible than by flavonoid analysis of the testa, as large differences were noted between the flours.

Table II was arranged in the same manner as Table 1, i.e., the higher group number indicates an increase in the number and/or amount of flavonoids. The major flavonoid aglycones and their ratio to one another were the means of classification into groups.

The first two groups might be considered "rhamnetin peanut" since the principal aglycone is rhamnetin. They differ, however, because group II also contains an unidentified aglycone, UNK. 1 (retention time of derivative, 21 min). The spectrum of UNK. 1 resembled that of an isoflavone but it was not identical to any reported in the literature. The retention time range of the rhamnetin derivative(s) is 17-20 min. These peaks do not completely represent rhamnetin derivative(s) because aglycone determinations afforded much smaller amounts of rhamnetin in relationship to derivative peak size. Group IV represents the transition between the "rhamnetin peanuts" of groups I-III and the "isorhamnetin peanut" of V-VIII. In groups V-VIII, the unknown aglycone (UNK. 2) (retention time of derivative(s), 41 min) was combined with rhamnetin for ratio purposes since they could not be separated chromatographically under the conditions used. The unknown aglycone had a retention time of 24.7 min. and rhamnetin is represented by the shoulder on this peak (retention time, 26.5 min).

The flour chromatograms of Sen. 57-204 and Sen. 61-58 showed that the ratio of the two peaks (retention time 33.5 and 36 min) associated with isorhamnetin derivatives are different. This difference was not taken into account for classification purposes as only the ratio of aglycones were used.

Table 2. Flavonoid Analysis of White Testa Peanut Flours.

GROUP	PI NO.	TRIVIAL NAME	MAJOR FLAVONOID AGLYCONES	RATIO	MINOR FLAVONOID AGLYCONES
I	329225	Albo	R		UNK.1
	408734	Sen. 58-670	R		UNK.1
	376050	U/12/47/1	R		UNK.1,UNK.2
	408732	Sen. 58-522	R		UNK.1,UNK.2
	T1920	Alba	R		UNK.1,Q
	-	Krinkleleaf	R		Q,UNK.1
	315611	Pearl White	R		UNK.1,Q
	288160	Sr 57	R		UNK.1,Q,I
II	408729	Sen. 57-149	R:UNK.1	1.2:1	---
	338497	AH6742	R:UNK.1	1.2:1	---
	313151	Taiwan 0323	R:UNK.1	1.2:1	I
	408738	Sen. 59-231	R:UNK.1	1.3:1	I
	393516	Tifrust-8	R:UNK.1	2:1	I
	298115	Israel 136	R:UNK.1	1:1	I,UNK.2
III	338498	AH6644	R:UNK.1,Q	2.4:1:0.3	---
	408737	Sen. 59-230	R:UNK.1,Q	1:3.5:1	I,UNK.2
IV	408733	Sen. 58-644	I:R	1:1	Q
	408728	Sen. 55-117	I:R	1:1	Q
	408741	Sen. 59-236	I:R:UNK.1	2.9:1.5:1	Q,UNK.2
V	338502	AH259	I:Q	1:1	UNK.2,R.
	306228	Sen. 57-204	I:Q	1.4:1	UNK 1,UNK.2,R
	408745	Sen. 61-58	I:Q	1.6:1	UNK.1,UNK.2,R.
	408722	Uganda B-13	I:Q	1.7:1	UNK.2,R
	408749	Sen. CTH 71/667	I:Q	1.2:1	UNK.2,R,UNK.1
VI	408747	Sen. PI851	I:Q:[UNK.2,R]	1.1:1:1	--
	408730	Sen. 57-161	I:Q:[UNK.2,R]	1.6:1:1	--
	376051	U/2/24/4	I:Q:[UNK.2,R]	1.6:1:1.4	--
	C32W	Spanwhite	I:Q:[UNK.2,R]	1.6:1:1.5	--
VII	408748	Sen. PI886	I:Q:[UNK.2,R]	3:1:2	--
	T2373	Tifrust-14	I:Q:[UNK.2,R]	4.2:1:3.1	--
	393517	Tifrust-9	I:Q:[UNK.2,R]	6.5:1:3.1	--
VIII	408735	Sen. 59-216	I:Q	27.6:1	UNK.2,R
	299468	Oakes Col. 471	I:Q:[UNK.2,R]	1:1.4:0.5	--

R = Rhamnetin; I = Isorhamnetin;
Q = Quercetin; UNK.1 = unknown 1,
UNK.2 = unknown 2, aglycones

This study demonstrates the possibility of using the flavonoid content of peanut flours rather than of their testa as taxonomic markers. Future studies are planned in which the flavonoid analyses will be quantitative rather than qualitative.

Acknowledgment

We gratefully acknowledge the technical support of Dorselyn C. Chapital and George I. Pittman, for drawing the figures.

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slh: 07/12/82, shl: 08/03/82, laq: 09/10/82, jaf: 09/30/82, slh: 03/17/83, jaf: 03/18/83

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Accepted April 16, 1983