Bitter Principles of the Peanut. Isolation, General Properties, and Distribution in the Seed

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A concentrate of the bitter principles of peanut hearts has been prepared, which possesses the general properties of the saponins. Glass paper chromatography indicates at least four components, each stained reddish purple with concentrated sulfuric acid and altered by acid hydrolysis. Aglycones obtained by acid hydrolysis can be separated into at least six components by chromatography on glass paper treated with monopotassium phosphate. Each gives a reddish purple color with sulfuric acid. The sugar moiety from the hydrolytic cleavage products gives four spots when chromatographed. These spots correspond in R_f value and color to glucose, xylose, rhamnose, and glucuronolactone.

NORMALLY, PEANUT hearts, the embryo without the cotyledons, have a bitter taste. Under certain field conditions, not completely understood, the cotyledons also taste bitter. An investigation of flavors that adversely affect peanut quality included the nature of the peanut bitter principles, with initial emphasis on those normally present in peanut hearts. The present paper describes the isolation of some of these substances and some of their general chemical and physical properties.

Experimental and Results

Assay. The primary test for the bitter principles in peanut hearts was by taste. A small aliquot of an aqueous or aqueous alcohol solution of the sample is placed in the palm of the hand, the excess alcohol evaporated, and the residue tasted. A brief interval of time is often required to permit the sample to reach the back area of the tongue, which is sensitive to the bitter substances. Owing to the variable sensitivity toward the bitter principles between individuals and in a single individual at different times of the day, a negative test for a given fraction was not considered final until it was tested by several persons known to be able to taste the bitter principle and until concentrated solutions had been tried. This assay is only qualitative.

Isolation of Bitter Principles. A concentrate of the bitter principles was prepared essentially as shown in Figure 1. The details of the procedure are as follows: Eight kilograms of hearts from lightly roasted peanuts were extracted twice with 16-liter portions of 80% ethyl alcohol. In each case the peanut hearts were extracted at room temperature for about 48 hours and then filtered first through a canvas bag and then through a bed of Celite, to remove suspended matter. All the bitter material was removed by this process, although the peanut hearts were not comminuted prior to extraction.

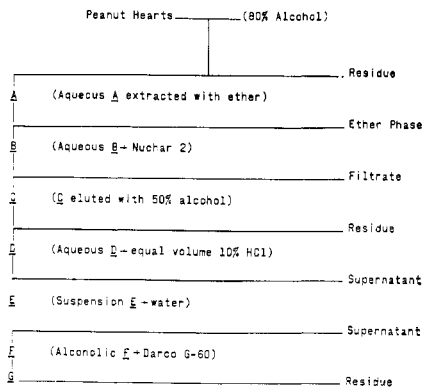

Figure 1. Schematic diagram of the preparation of the bitter concentrate

After the filtrates were combined and concentrated to a thin sirupy consistency to remove the alcohol, the sirup was diluted with about 2 liters of distilled water. The resulting aqueous solution was extracted with successive amounts of ethyl ether until practically all the colored materials which passed into the ether phase had been removed. None of the bitter material could be detected in the ether extract by the taste test. The aqueous phase was boiled to remove the ethyl ether, and the bitter materials were adsorbed on Nuchar 2 charcoal. The charcoal adsorption step was repeated until the filtrate was colorless. In each step Celite was added to facilitate the filtration. Even after concentration to a sirup, the filtrate was not bitter to the taste.

The combined charcoal-Celite cakes were broken up and extracted several times with 50% ethyl alcohol to elute the bitter materials. The combined eluates were concentrated to 200 ml. to remove the alcohol, cooled to room temperature, and mixed with an equal volume of 10% hydrochloric acid. The fluffy, brownish white precipitate was collected by centrifugation. It was broken up and washed with small volumes of 10% hydrochloric acid to remove impurities and finally, the precipitate was resuspended several times in small volumes of distilled water to remove the acid. The washed precipitate was dissolved in absolute ethyl alcohol and decolorized with Darco G-60 charcoal. Upon evaporation of the alcohol, 4.5 grams of white, amorphous powder was obtained, representing a yield of about 0.056%. This fraction will be referred to as the bitter concentrate.

General Properties of Bitter Concentrate. A dilute aqueous solution of the bitter concentrate froths on shaking and is intensely bitter. Alcoholic solutions of the bitter material are nearly colorless. The dry material gives a red color with concentrated sulfuric acid (*11*). The bitter concentrate is only slightly soluble in water, but dissolves readily in dilute alkali or potassium bicarbonate solutions. It is soluble in aqueous alcohol and absolute ethyl alcohol, but is insoluble in chloroform, ethyl ether, benzene, iso-octane, and other similar solvents. The concentrate was tested for flavonoids by the ammonia test (*6*); it gave negative results. Elemental analysis indicated the presence of carbon and hydrogen, but failed to reveal nitrogen, sulfur, phosphorus, or ash.

Crystallization of Bitter Concentrate. The bitter fraction was crystallized according to the following procedure. Water was added dropwise to a methanol solution of the bitter material until the solution became slightly turbid. Then, methanol was added dropwise until the turbidity just disappeared. Crystals in the form of thin plates appeared on standing. The melting point of the crystals which had previously been dried over sulfuric acid for

24 hours was not sharp. The crystals began to soften at 226° C. and were completely melted at 232° C.

Analysis of Bitter Concentrate by Glass Paper Chromatography

The equipment and general technique for glass paper chromatography were essentially those described by Dieckert and Reiser (*5*) and modified by Dieckert and Morris (*3*). All solvents were ACS grade or its equivalent and no attempt was made to purify them.

Chromatography of the bitter materials was carried out on glass paper treated with 0.1*M* monopotassium phosphate (*7*). Organic impurities were removed from the paper as described by Dieckert, Ory, Carney, and Morris (*4*). The glass paper treated with monopotassium phosphate was made as follows. 3 × $7^1/_2$ inch strips of glass paper, which were suspended from $^3/_8$-inch capacity binder clips, were dipped in 0.1*M* monopotassium phosphate. The excess solution was removed by wiping the suspended strip with a glass rod and the wet sheets were dried by hanging them on a glass rod in an oven set at 70° to 100° C. The dry sheets of paper were stored in a covered tray to keep dust out.

Usually about 0.3% solutions of the bitter materials were applied to the origin of the chromatogram in 2- to 5-μl. amounts. For demonstrating some of the minor components in the bitter concentrate solutions as high as 1 to 2% were used. At high concentrations of the bitter materials the two major spots overlapped. After the chromatogram was developed with a suitable solvent, the solvent was allowed to evaporate and the chromatogram was sprayed with concentrated sulfuric acid. The chromatographed substances became visible on heating the chromatogram over a hot plate. With mild heating, reddish purple colors were developed which faded as the substances were charred. After strong heating the chromatographed substances appeared as black spots on a white background. The spots were best viewed by transmitted light.

A number of different solvent systems were successfully used to develop the chromatograms. Because all of them seemed to give about the same degree of separation, only the solvent system most often used is reported. This system was acetone–*n*-propyl alcohol–water: : 50 : 40 : 10 (*7*). As shown in Figure 2, four distinct spots were observed with this system. The spot with the next to highest R_f value appeared to represent a minor component of the bitter concentrate and in certain preparations was difficult to detect. All four spots stained reddish purple with sulfuric acid when heated. Under favorable circumstances the long spot having the lowest R_f value was seen to be composed of several overlapping spots. Each of these stained reddish purple with sulfuric acid, upon heating the chromatogram gently.

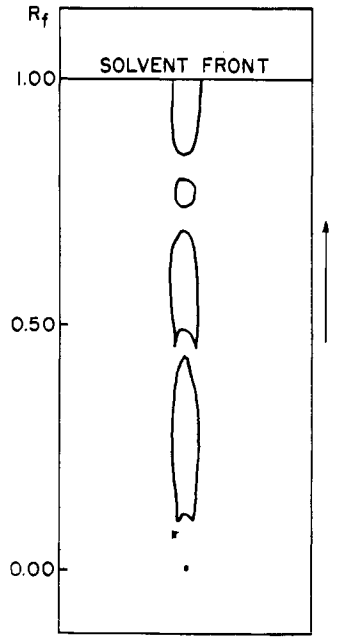

Figure 2. Chromatography of the bitter concentrate on glass paper treated with monosodium phosphate

Acetone–*n*-propyl alcohol–water: :50:40:10

Preparation of Aglycones from Bitter Concentrate. About 2 grams of the bitter concentrate was dissolved in 250 ml. of ethyl alcohol and 40 ml. of concentrated hydrochloric acid. After refluxing for 30 hours, the reaction mixture was diluted with 2 liters of distilled water and the aglycones were extracted from the reaction mixture with ethyl ether. The ether extract was scrubbed with distilled water until a negative 1-naphthol test indicated the absence of free sugars in the water washings. Upon evaporation of the ether, 0.964 gram of aglycones was obtained, representing a 50% yield.

Analysis of Aglycones by Glass Paper Chromatography. The aglycones which were obtained as outlined in the previous section were chromatographed on glass paper treated with 0.1*M* monopotassium phosphate. A 0.1 to 0.2% solution of the mixed aglycones was applied to the origin of the chromatogram. Two-dimensional chromatography on a $7^1/_2$ × $7^1/_2$ inch sheet of the paper was used with benzene as the first system and benzene–ethyl ether–ethyl alcohol: : 100:3:2 as the second.

At least six chromatographically distinct aglycones are present in the aglycone mixture, as can be seen from the chromatogram illustrated in Figure 3. One series of the aglycones could be separated on the phosphate-treated glass paper with benzene. A second series remained at the origin in the benzene system, but could be separated in the benzene–ethyl ether–ethyl alcohol: :100: 30:2 solvent system. In each case, the spots made visible by the charring action of sulfuric acid matched one for one the spots giving reddish purple colors with sulfuric acid under mild heat.

Preparation of Sugar Moiety of Bitter Concentrate. The bitter concentrate was subjected to a milder acid hydrolysis for the preparation of the sugar moiety than for the aglycone moiety in order to reduce the chance for degrading these acid-labile substances. A 1-gram sample of the bitter concentrate was dissolved in 250 ml. of ethyl alcohol and 25 ml. of about 8*N* aqueous hydrochloric acid. The solution was refluxed for 6 hours, diluted with 5 volumes of distilled water, and extracted with ethyl ether until the aqueous phase was clear. The aqueous phase which contained the sugar fraction was heated on a steam bath until the excess ether was removed, and then was passed through a column packed with enough Dowex 3 ion exchange resin to remove the hydrochloric acid. Freeze drying was used to remove the relatively large amounts of water from the sugars. Approximately 0.5 gram of the sugar fraction was obtained.

Analysis of Sugar Fraction by Paper Chromatography. A 2% aqueous solution of the sugar fraction obtained as described in the previous paragraph was prepared and analyzed by descending paper chromatography on Whatman No. 1 filter paper according to the technique of Partridge (*11*). The solvent system used was *n*-butyl alcohol–acetic acid–water: : 4:1 : 5. The sugars were located by dipping the air-dried chromatogram in a specially designed tray (*8*) containing the solution, *p*-anisidine phosphate reagent (*9*)–acetone: : 1:3. The air-dried chromatogram was heated in an oven at 95° C. for 3 minutes to develop the spots.

The results of the analysis by paper chromatography of the sugars and sugar derivatives obtained from the hydrolytic cleavage products of the bitter concentrate appear in Table I. Four major spots were observed on the chromatogram. When authentic samples of glucose, xylose, rhamnose, and glucuronolactone were examined concurrently with the unknown sample, spots with corresponding R_f values were found for the unknown. In addition, the unknown substances which corresponded in R_f value to glucuronolactone and its acid showed the characteristic pink color of these compounds under the action of *p*-anisidine phosphate. There was a similar agreement between the colors developed with the other known sugars and the substances which had the corresponding R_f values in the unknown mixture.

Examination of Raw Cotyledons and Hearts for Components of Bitter Concentrate. The components of the bitter concentrate can be detected easily in a

Table I. Identification of Components of Sugar Moiety of Bitter Concentrate by Paper Chromatography[a]

Compound	R_f Value: Authentic samples	R_f Value: Sugar moiety
Glucuronic acid	0.17	0.17
Glucose	0.20	0.20
Xylose	0.28	0.28
Glucuronolactone	0.37	0.36
Rhamnose	0.40	0.38

[a] Whatman No. 1 filter paper and *n*-butyl alcohol–acetic acid–water: :40:10:50.

1-gram sample of peanut hearts by first preparing a concentrate of the bitter materials, then chromatographing the concentrate on glass paper treated with monopotassium phosphate as described earlier. The process for preparing the concentrate was as follows. A 1-gram sample of hand-picked raw peanut hearts was ground in a Henry slicer, extracted with ethyl ether in a Butt extractor (*1*) for 8 hours, after which the sample was pulverized and extracted again by the same process. The meal was extracted with 80 ml. of 80% alcohol overnight, made up to 100 ml. with 80% ethyl alcohol, and filtered. The alcohol and water were removed from a 50-ml. aliquot in a thin film evaporator; the residue was taken up in distilled water. An aqueous solution of neutral lead acetate was added until no further precipitation occurred. The precipitate was collected by centrifugation, resuspended in distilled water, and again collected by centrifugation. This process was repeated twice more and the residue was extracted with 50% ethyl alcohol several times. The combined extracts were concentrated to dryness, taken up in 0.5 ml. of 50% aqueous ethyl alcohol, and examined chromatographically on glass paper treated with 0.1*M* monopotassium phosphate using acetone–*n*-propyl alcohol–water: :50:40:10 as the developing solvent.

The method for preparing the bitter concentrate from peanut cotyledons was the same as above except for the following changes. A 20-gram sample of raw peanut cotyledons was used. To ensure that all of the peanut heart had been removed from each cotyledon, the region of the cotyledon joining on the heart was carved out with a razor blade or scalpel. The defatted cotyledons were extracted with 180 ml. of 80% ethyl alcohol and then diluted to 200 ml. with more 80% ethyl alcohol. The resulting mixture was filtered as before and a 100-ml. aliquot was used to prepare the lead salts as described. The final residue was dissolved in 0.5 ml. of aqueous ethyl alcohol as before and examined by glass paper chromatography as was done for the original bitter concentrate.

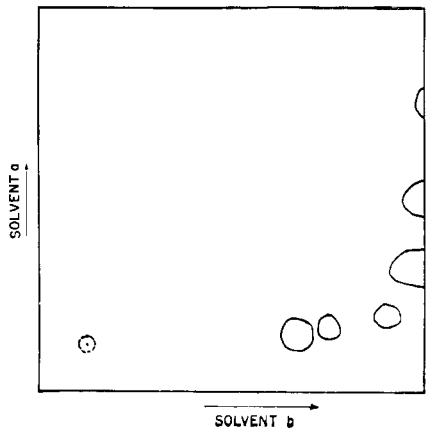

Figure 3. Two-dimensional chromatography of the aglycone moiety of the bitter concentrate on glass paper treated with monopotassium phosphate

Solvent *a*. Benzene. Solvent *b*. Benzene–ethyl ether–ethyl alcohol : : 100:30:2

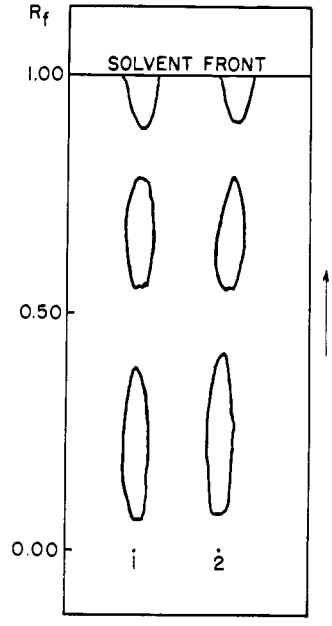

Figure 4. Detection of components of the bitter concentrate in peanut cotyledons by glass paper chromatography

1. Bitter concentrate from hearts
2. Concentrate from cotyledons

It may be seen by comparing Figures 2 and 4 that chromatographically similar substances are present in cooked and in raw peanuts as well as in the raw cotyledons. The corresponding spots on each chromatogram showed the characteristic reddish purple color for the bitter concentrate.

Discussion

A number of data reported in this paper suggest that the components of the bitter concentrate are saponins. One of the classical properties of the saponins is the frothing action of their dilute aqueous solutions (*2*). Other general properties of various classes of saponins are: precipitation from aqueous solution with neutral lead acetate (*2*); solubility in water and aqueous alcohol, but insolubility in ether, benzene, and related solvents (*2*); reddish coloration when a dry sample is mixed with concentrated sulfuric acid (*10*); presence of carbon and hydrogen, but not nitrogen, sulfur, or phosphorus; and a bitter taste (*2*). All of these properties were found for the bitter concentrate. Saponins are considered glycosides having one or more sugar derivatives and an aglycone. Acid hydrolysis of the bitter concentrate yields a mixture of ether-soluble aglycones and a mixture of sugars and sugar derivatives.

Neither of the fractionation procedures described for saponin isolation yields single substances, as is made clear by the chromatographic examination. The various components in the mixture, however, are principally saponins, because each of the chromatographic entities stains reddish purple with sulfuric acid and is destroyed by acid hydrolysis. This multiple nature is also in agreement with the finding that at least six aglycones are obtained on acid hydrolysis of the bitter concentrate.

It would seem that one or more of the substances which stain reddish purple on the glass paper chromatogram, under the action of sulfuric acid, are bitter agents in peanuts. Bitter concentrates prepared by two distinct processes give paper chromatograms which are similar with respect to both the distribution of the spots and the reddish purple color developed in each of the spots upon treating the chromatogram with strong sulfuric acid. Upon acid hydrolysis, the resulting cleavage products no longer taste bitter; and concomitantly, the characteristic spots which are associated with the chromatography of the bitter concentrates on glass paper disappear. The cotyledons from good grade peanuts fail to taste bitter and have much reduced levels of the substances found in the bitter concentrate from hearts as indicated by the fact that 20 times by weight as much cotyledons as hearts were required to get comparable chromatograms in the two cases. Finally many substances, which are known to be bitter and to occur in nature, are absent from the bitter concentrate of peanut hearts. Thus, the alkaloids and cyanophoric glycosides must be absent, since no nitrogen is found in the bitter concentrate. The flavonoid glycosides are probably absent, as the bitter concentrate gives a negative ammonia test.

Under some poorly understood conditions peanuts develop bitter flavors in the cotyledons and at least by taste, these bitter substances appear to be identical with those in the peanut hearts. No distinctly bitter cotyledons were available at the time these investigations were made, so it was decided to look for the components of the bitter concen-

trate in cotyledons from nonbitter peanuts on the supposition that these substances occur there normally, but at low levels. Substances that stain reddish purple with sulfuric acid plus heat and which chromatograph like the components of the bitter concentrate from hearts were found in the cotyledons. It took 20 times as much cotyledons as hearts to give roughly an equivalent amount of material as measured by glass paper chromatography. Small fragments of the peanut hearts which had adhered to the cotyledons could account for the substances observed. This possibility was ruled out by carving out and discarding the region of the cotyledon joining the heart and cotyledon together.

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TUNA MEAT PIGMENT STUDIES

Spectral Reflectance Studies of the Heme Pigments in Tuna Fish Flesh. Some Characteristics of the Pigments and Discoloration of Tuna Meat

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During investigations on the "greening" in certain yellowfin tuna on precooking, information regarding the characteristics and reactions of the heme pigments in tuna meat was revealed, largely through application of the technique of spectral reflectance. Spectral evidence indicated a predominance of hemoglobin rather than myoglobin in the flesh pigments, an increase in methemoglobin and denaturation on storage, and a greater solubility of methemoglobin in aqueous media in contrast to oxyhemoglobin. Greening is an actual color condition due to an anomalous heme protein oxidation. Related are high concentrations of methemoglobin, some denaturation, and a slightly high fat peroxide content in the raw meat. Oxygen starvation due to the exhaustion of the fish during landing does not appear to produce factors that lead to greening, but changes that occur even in the frozen state seem to be responsible.

THE DISCOLORATION of meatstuffs is a problem familiar to the packers and processors of such products. Present knowledge of the cause and probable mechanisms producing a variety of such conditions has been summarized by Watts (*24*). One similar off-color variation recognized in fish meat is the undesirable "green" color that develops in the flesh of certain tuna on precooking, prior to canning, and results in considerable economic loss to the fisherman and to the industry (*19*).

The problem has been the subject of intensive research by the Japanese (*16*). Matsuzaka and Takahashi (*15*) have reported a correlation between the greening of tuna and the concentration of a flesh pigment identified spectrophotometrically by them as myoglobin. More recently Brown, Tappel, and Olcott (*5*) have reported on work which indicates that the pigment responsible for greening is a hemichrome.

The advantages of spectral reflectance applied to biochemical problems have been pointed out (*4*, *18*). Application of this method to the present investigation was obvious, because it made possible the study of the normal or anomalous protein pigments in raw and cooked flesh, and of the changes induced by the variation of experimental conditions.

Materials and Methods

Samples used in this research were carefully trimmed sections of the loins of yellowfish tuna (70 to 200 pounds), *Neothunnus macropterus* (Temminck and Schlegel), caught on long line by research vessels of the Pacific Oceanic Fishery Investigations of the U. S. Fish and Wildlife Service in the central equatorial Pacific. The fish were frozen on board, as soon as possible after capture. Yellowfin up to 15 pounds were caught by trolling in local waters and sampled while fresh.

To obtain precooked samples for the evaluation of color, frozen fish were thawed in tanks at Hawaiian Tuna Packers, Ltd., and cut into loins. One loin from each fish was retained as a raw sample. The remaining loins were put through the regular commercial precooking process of that company. Color grade of the precooked samples was judged by the company's food technologist. Specific samples characteristic of their types (normal, green, pink, etc.) as subjectively judged, were used in much of the work.