

A STUDY OF THE ACIDIC CONSTITUENTS  
OF THE HULLS OF ENGLISH WALNUTS (JUGLANS REGIA L.)

by

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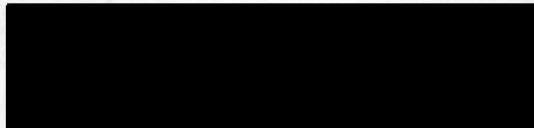
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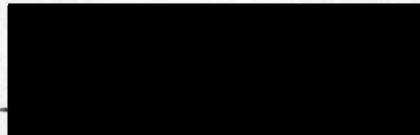
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INTRODUCTION

Considerable work has been done on the shells of the English walnut (*Juglans regia*) in an effort to find a way of utilizing this waste material from the walnut industry. Ramarao (15) found that on destructive distillation of one ton of the shells he obtained 7400 cu. ft. of gas, 740 lbs. of charcoal, as well as lesser amounts of tar, acetic acid, and methanol. Others (8, 9, 14, 16) have determined the furfural, pentosans, cellulose, lignin, and ash content. The by-products of destructive distillation procedures appear most promising.

Although this work has been done on the shells, there is little or no information in the literature in regard to the hulls of walnuts. A.R.C. Haas, who has made extensive horticultural studies on walnuts, mentions that there are very little data in regard to the composition of walnut kernels, shells, and husks. He reported the pH to be 4.7-6.8 and made determinations of the amounts of dry matter, reducing sugars, pectin, sodium, potassium, and magnesium in the hulls in a study of the resistance of various walnuts to the walnut husk fly.

It is a common experience of one who has handled these hulls to observe the characteristic brown stain which is left after contact. Yet it is surprising to find that there is in the literature practically no information regarding

the nature of this stain. Mell (9) suggests that it may be a useful dye. However the more reliable synthetic dyes have probably crowded such materials out of commercial consideration.

Paessler (10) reports that there are 22.2% of apparently usable tannins in the hulls of walnuts and suggests that they be used in the manufacture of leather. Other than this no mention has been made of the tannins in the hulls.

Gergelezhiu (4) has reported that green walnuts and the green shells are very rich in vitamin C, containing from 500-2459 milligrams of ascorbic acid per 100 grams fresh weight, which, he states, is 40-50 times that found in oranges and lemons. Hennig and Ohshe (7) found 950-1000 milligrams per 100 grams of green shell; while Natadze reported 11,000-13,000 vitamin C units per kilogram, which is approximately 650 milligrams per 100 grams. In a recent investigation E. Hansen (6), at the Oregon Agricultural Experiment Station, has shown that there are 487-605 milligrams of ascorbic acid per 100 grams fresh weight of the hulls, which, he points out, might make them valuable for nutritional purposes.

In view of this information a study of the acid constituents of the hulls of English walnuts (*Juglans regia*) was undertaken in this laboratory.

## EXPERIMENTAL

I. Vitamin Assays

The hulls which were used in this investigation were obtained from a grove in the Willamette Valley, near Dayton, Oregon, in the fall of the year, just as they were beginning to crack and fall. About 60 lbs. of the green hulls were collected, frozen, and stored in a dark refrigerated room at 0°F. They kept very well under these conditions, retaining their green color and showing no signs of mold or decay.

A one kilogram sample of the hulls was dried in the oven at 110°C. to a constant weight. They were found to be 88% moisture, which is in good agreement with the data given by Hansen (6).

This material was subjected to several vitamin assays in an attempt to determine its nutritional value.

The ascorbic acid assays were carried out according to the method of Evelyn et al (3). Samples consisting of 25 grams of the frozen hulls were blended with 350 ml. of water in a Waring blender. A 10 ml. aliquot of this mixture was made up to 250 ml. with one per cent meta phosphoric acid, and filtered samples of this were analyzed with an Evelyn photoelectric colorimeter. Assays were also carried out on samples extracted with meta phosphoric acid, as well as on samples of each of the above mentioned extracts which had been steamed in the autoclave for one hour. Another water extract was assayed by titrating with the 2,6-di-

chlorophenol-indophenol dye.

Micro-biological assays as developed by Williams and co-workers (18) at the University of Texas were used to determine the amounts of various B vitamins present in extracts of the hulls. Those for which assays were performed are: thiamin, riboflavin, inositol, pantothenic acid, and nicotinic acid. Samples of the hulls were hydrolyzed, using enzyme digestion for twenty-four hours, autoclaving in 0.1N HCl for thirty minutes, autoclaving in 1.0 N HCl for thirty minutes, and refluxing in 6.0N HCl for six hours so that different conjugated forms of several of the vitamins might be hydrolyzed. The results of these assays are tabulated below.

#### Vitamin C

<u>Treatment of extract</u>	Mg/100g fresh weight	
Water extract	538-548	
HPO <sub>3</sub> extract	485	
Water extract-steamed	452-463	
HPO <sub>3</sub> extract-steamed	423	
Water extract-titrated	550-600	
B vitamins	♂/g fresh weight	♂/g dry weight
Thiamin-enzyme hydrolysis	3.	25.
Riboflavin-enzyme hydrolysis	1.1	9.1
Riboflavin-0.1N HCl hydrolysis	1.0	8.3
Inositol-enzyme hydrolysis	3700.	31,000.
Inositol-6N HCl hydrolysis	2500.	21,000.
Pantothenic acid-enzyme hydrolysis	4.3	36.
Nicotinic acid-enzyme hydrolysis	3.7	31.
Nicotinic acid-1.ON HCl hydrolysis	1.5	12.5

### II. Malic Acid

About two kilograms of fresh walnut hulls were extracted with alcohol, using a continuous extractor similar to that described by Morton (11). The extraction was continued until the extracting liquid came over colorless. The resulting alcohol-water mixture was then centrifuged, and a black, insoluble, amorphous solid was obtained. This material proved to be insoluble in nearly all solvents and gave no tests for functional groups. The centrifugant was evaporated to near dryness, with a brown, sticky, water-soluble residue remaining. This residue had the odor of caramel, a very sour taste, and contained a large amount of reducing sugars. An aqueous solution of this material could be readily decolorized with activated charcoal, and when treated with a solution of neutral lead acetate a flocculent white precipitate resulted. Further investigation was centered around the lead precipitate.

A sample of the lead precipitate was suspended in water and the suspension treated with hydrogen sulfide. The resulting black lead sulfide was removed by filtration and the colorless filtrate evaporated to dryness. A light brown, sticky, very sour material remained. This substance was taken up with a small amount of water, and the water was allowed to evaporate slowly. No difference was observed in the residue. The residue was dried in a vacuum oven at 50° C. for about two hours with no apparent change. This

semi-solid acidic material was very soluble in water and insoluble in absolute alcohol and in ether.

Attempts were made to prepare an anilide and an amide of this material, but no product was obtained in either case. It was believed that decomposition took place in drying this material because the solution resulting after the removal of the lead sulfide was perfectly colorless, but the solid which was obtained was light brown and gave a light brown solution on being redissolved. With this in mind another sample of the lead salt was prepared, treated with hydrogen sulfide, and the resulting clear solution evaporated to a volume of about ten milliliters on a water bath. This solution was then neutralized with sodium hydroxide, made slightly acid with dilute hydrochloric acid, and then an equal volume of alcohol followed by a small amount of p-nitrobenzyl bromide was added. This mixture was refluxed for an hour and a half on a water bath. On cooling, two fractions of crystals were obtained, one being the unreacted p-nitrobenzyl bromide, and the other, apparently, the derivative. This material was recrystallized from alcohol, white feathery crystals being formed. M.P. 123-124°C.

This procedure was repeated, using 14.4 grams of the extraction product to obtain more of the derivative. Five grams of p-nitrobenzyl bromide were used and the mixture refluxed six hours. A yield of 2.2 grams was obtained which melted at 123-124°C. after recrystallization.

Using the same method and amounts, a derivative of p-bromophenacyl bromide was prepared in a yield of 2.2 grams. M.P. 179° C.

These two values check with those given in the literature for l-malic acid.

The isolation of the acid was undertaken by evaporating the solution resulting from the hydrogen sulfide treatment of the lead salt, in vacuo at room temperature to a syrupy consistency. After the bottom of the container had been scratched and the contents dried further in a vacuum desiccator, small white crystals appeared in the syrup; these were dried on a porous plate. M.P. 100° C. These crystals were very hygroscopic, and as they were extremely soluble in water, recrystallization was very difficult. They were, however, only slightly soluble in ether and in absolute alcohol. In order to recrystallize them, they were dissolved in a small amount of water to which an equal volume of methyl alcohol was added; then dry ether was added, with shaking, until a slight turbidity was produced. The same product was obtained by treating an aqueous extract of the hulls with neutral lead acetate and then following a slightly modified procedure; namely, the solution resulting from the hydrogen sulfide treatment was treated with alcohol and an insoluble fraction removed.

p-Nitrobenzyl ester

Calcd. for  $C_{18}H_{16}O_9N_2$ : C, 53.48; H, 3.99  
Found: C, 53.40; H, 3.98

p-Bromophenacyl ester

Calcd. for  $C_{20}H_{16}O_7N_2$ : C, 45.48; H, 3.05

Found: C, 45.45; H, 3.00

### III. Ascorbic Acid

In view of the recent articles in the literature coupled with preliminary experiments which indicated the presence of a large amount of ascorbic acid, the isolation of this material was undertaken. The method described by Svirbely and Szent-Gyorgyi (17) was used. About 9.7 kg. of the hulls, which would correspond to approximately 55g. of the desired ascorbic acid, were ground in a meat grinder, treated with water and a barium acetate solution, and then pressed to express the juices from the pulp. The above mentioned procedure was then followed closely. Considerable difficulty was encountered during the concentration of the aqueous solution in vacuo at room temperature. This step took several days to accomplish and was carried out in an atmosphere of carbon dioxide as much as possible. The liquid remaining in the final step was placed in a large crystallizing dish and stored in a desiccator to await the crystallization of the product. When the required time had elapsed, no change was noticed and no crystals were present.

Various modifications of the isolation procedure were tried, using variations of the methods of Svirbely and Szent-Gyorgyi, Baumann and Metzger (1), and Waugh, Bessey, and King (20). It was observed that the lead salt of the

ascorbic acid was stable and that, on being hydrolyzed, the ascorbic acid still maintained its ability to reduce the dye. As a result of this stability of the lead salt, 6 kg. of the frozen hulls were extracted and taken through the precipitation of the lead salt of ascorbic acid. This brown precipitate was used for subsequent experiments.

A sample of pure ascorbic acid was purchased so that the properties and reactions of the material could be studied. The 2,4-dinitrophenylhydrazone of the ascorbic acid was prepared. Several attempts were made to prepare the acetone derivative (19), but all were unsuccessful. A 100 mg. sample of the ascorbic acid was dissolved in water, to which ammonium hydroxide and then neutral lead acetate were added. The white precipitate thus obtained was washed with water, then with acetone, suspended in methyl alcohol, and treated with concentrated hydrochloric acid. The lead chloride precipitate was removed by centrifuging, and the resulting clear solution evaporated to dryness in vacuo. A thick syrup resulted which crystallized on being stirred. The crystals were suspended in acetone and the acetone removed by evaporation in vacuo. A yield of 70 mg., or 70%, was obtained, showing that ascorbic acid can be recovered by this procedure. In another experiment a sample of ascorbic acid was dissolved in methanol on a watch glass. The methanol was then evaporated by placing the watch glass over a water bath; this process

was repeated three times, and the final product was a white crystalline material melting at 180-182°*C.*, which is a little low for ascorbic acid. The sample had been subjected to heat for over half an hour, with only a slight lowering of the melting point. An attempt to acetylate some of the ascorbic acid, using pyridine and acetic anhydride, resulted in a small amount of brown oil.

The precipitate obtained with the lead acetate was shown to have an activity of 1.5-2.5 mg./g. It was found that it could be concentrated by dissolving the precipitate in 6*N* acetic acid and then reprecipitating it by the careful addition of ammonium hydroxide. Thus the concentration of the ascorbic acid was increased to 7.5 mg./g.

Several attempts were made to isolate the ascorbic acid from the lead salt which had been prepared. The solution resulting after precipitating the lead as the sulfate or chloride was always very dark brown. To overcome this, the aqueous solution was extracted several times with n-butyl alcohol, which left the aqueous solution light brown or yellow in color. On evaporation, a dark brown, gummy material was the only product. Several trials ended in the same manner.

Another series of experiments was carried out by suspending the lead salt in methyl alcohol and treating with dry hydrogen chloride gas followed by a vacuum evaporation of the alcohol. On several trials a small amount of white

crystals which had a melting point of 220-222°C. were obtained, so it was concluded that they were not ascorbic acid. On continued evaporation, a brown viscous residue was again obtained. This residue showed an activity of about 3% as measured by titrating with the dye. The preparation of the 2,4-dinitrophenylhydrazone was undertaken using the residue, but no product could be detected. Attempts were made to prepare this derivative from the lead salt of our material, but they were also to no avail.

It was considered likely that the hydrogen chloride was causing decomposition of the desired material and was leaving these gummy residues which were most likely furfural or furfural derivatives. To overcome this possibility, hydrogen sulfide was used to remove the lead. When this method was used, the solution did not darken nearly as much as previously. Also, since we had shown that the pure ascorbic acid dissolved in methyl alcohol could be heated over a water bath with little effect, it was decided to speed up the process by evaporating the methyl alcohol over a water bath rather than in vacuo at room temperature. When we used these two procedures together, that is, the hydrogen sulfide and the water bath, the time was shortened from several days required by the vacuum evaporation to less than one, and a product was obtained which was much lighter in color and had a potency of 22%. However, no crystalline material was isolated from this.

#### IV. Inositol

In the discussion on ascorbic acid it was mentioned that a white solid settled out which melted at 220-222°<sup>C</sup>. The desiccator in which it was hoped the ascorbic acid would appear in the original isolation experiment was found to contain a considerable amount of a brown amorphous solid after standing for about four months. After the brown amorphous solid stood on a porous plate for about an hour, white crystals remained. These crystals also melted at 220-222°<sup>C</sup>. Initial experiments indicated that this material might be inositol. It was very soluble in water, but insoluble in methyl alcohol and ether. It was recrystallized by dissolving in a small amount of water, then adding a little over an equal volume of methyl alcohol. Dry ether was added, with stirring, until a slight turbidity was produced. After it stood for several hours, crystals could be obtained. If too much ether was added, the material precipitated out, taking all of the colored impurities out of solution with it.

A mixed melting point was taken with a sample of ash-free inositol, and no depression was observed. A microbiological assay was run by making a solution of 70 micrograms in 10 ml. of water and using aliquots of that. The yeast *Saccharomyces cerevisiae* "Gebruder Mayer" were used. The assay showed the material to have the same effect on yeast growth as the standard made up with ash-free inositol.

## DISCUSSION

I. Vitamin Assays

The ascorbic acid content of walnut hulls was found to be very high, as had been reported by various investigators, although it is not found to be as high as reported by Gergelezhiu. It was shown that steaming the pulp for one hour reduces the amount of ascorbic acid by about 12-18%.

The amount of thiamin as shown by the micro-biological assay was slightly higher than in many common fruits and vegetables. The amount of riboflavin was just average. It is of interest to note that the acid-hydrolyzed hulls have a slightly lower riboflavin content than those which were hydrolyzed with the enzymes. The amount of inositol shown to be present is extremely high, being about 2-2.5 times that of oranges and grapefruit, which are considered very rich in this factor. It is surprising that the acid-hydrolyzed hulls have a lower inositol content than the enzyme-hydrolyzed because it has been shown (2) that inositol can be refluxed for several hours with 6N HCl without decreasing its activity. The pantothenic acid content is slightly above average, while the nicotinic acid content is rather low compared with most fruits and vegetables. Again it is surprising to note that the acid-hydrolyzed material gave a lower value than that hydrolyzed with the enzymes. This is a very rare phenomenon in nicotinic acid assays.

When considered on the dry weight basis, the vitamin

content is unusually rich and would have a high vitamin value for nutritional purposes.

The fact that all of the assays on acid-hydrolyzed hulls were lower than the corresponding enzyme-hydrolyzed material might suggest some acid labile highly active growth factor.

## II. Malic Acid

Due to the deliquescence of malic acid and difficulty in preparing dry samples, carbon and hydrogen values only were determined on its derivatives. These results coupled with M.P. data, however, should furnish ample proof to establish its existence in walnut hulls.

## III. Ascorbic Acid

As has been indicated, all attempts to isolate ascorbic acid were unsuccessful, although a mixture containing 22% ascorbic acid was finally prepared.

Since the assay of ascorbic acid depends on the addition of 2,6-dichlorophenol-indophenol indicator, the method is not entirely specific. Using the method of Svirbely and Szent-Gyorgyi on a pure ascorbic acid solution, a 70% recovery of the pure acid was attained. Thus, these results may be attributed in part either to unremoved impurities in this particular substance or to possible existence of some other easily oxidized compound as yet unknown. That oxalic was not found in residue from ascorbic

acid isolation lends support to such a view.

#### IV. Inositol

A small amount of inositol was obtained during the attempted isolation of ascorbic acid. It was shown to be inositol by its melting point, mixed melting point, and a micro-biological assay.

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