Simple Procedure for the Routine Assay of *Dioscorea* Tubers

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A project for the evaluation and domestication of wild, high-yielding species of *Dioscorea* required an accurate assay method suitable for the routine analysis of several thousand samples. A method which meets these requirements consists of three basic steps: maceration of the fresh tuber, acid hydrolysis, and extraction. The results obtained by this simple method are within +5% of the estimated theoretical values. A single operator can assay from 12 to 24 samples per day.

DIOSGENIN, a steroid, now a commercial product of considerable importance, is the starting material for the preparation of several very potent and widely used pharmaceutical products. At present, diosgenin is obtained from the tuberous roots of wild, tropical plants of the genus *Dioscorea*.

During the past few years several independent groups have started agronomic programs which may lead to the domestication of a high yielding species of *Dioscorea*. The research at this station has been under way for 6 years, and some of the initial observations have been published (*3*).

At the beginning of the program, the problem was encountered of periodically assaying several hundred samples representing 63 types. A simple, rapid method was needed, which could be carried out with accuracy by relatively unskilled technicians. These requirements could not be met by any of the published methods. This paper presents a laboratory procedure which was devised and found to be very satisfactory for routine assay. Samples whose dry weights were less than 2.0 grams have been satisfactorily assayed.

The principles on which this method is based, and the importance of their sequence (maceration, hydrolysis, and extraction), have been known in the trade for many years, but have not been previously incorporated and published as a simple laboratory procedure, suitable for routine assays. Rothrock and coworkers (*6*) have recently described a commercially practical procedure based on the same principles as the procedure presented here, and have given a thorough review of the literature on the isolation of diosgenin. Gould *et al.* (*1*) and Hershberg *et al.* (*2*) were recently granted patents on commercial processes based on the same principles as this laboratory procedure.

Procedure

Fresh tubers are sliced, and macerated with an equal weight of water in a high-speed blender for 5 minutes. A 100-gram sample, for a moisture determination, is poured into a 250-ml. porcelain dish, and heated at 90° to 100° C. for 12 hours. The loss in weight is recorded and the sample is discarded. A second 100-gram sample is poured into a 500-ml. Erlenmeyer flask that can be fitted with a ground-glass joint. Then 115 ml. of 3.5*N* hydrochloric acid are added. The mixture will be 1.9 to 2.1*N*, depending on the moisture content of the tuber being analyzed. It is refluxed for 3 hours, 250 ml. of water are added; then it is cooled to room temperature and filtered. A 9-cm. Büchner funnel is very satisfactory for this filtration. The residue is washed on the filter paper 8 to 10 times with 50-ml. portions of water, and both are dried overnight at 65° to 70° C. Drying for shorter periods at higher temperatures is also satisfactory. The dry residue and filter paper are placed in a 25 × 80 mm. Soxhlet thimble and extracted for 8 hours with petroleum ether (boiling point 30° to 60° C.). A 125-ml. receiver with 100 ml. of solvent is satisfactory for this extraction. After the extraction is completed, the volume of the solvent is reduced to approximately 10 ml. The flask is then stoppered and allowed to stand at room temperature for 2 hours. At this point the diosgenin will be in the form of white crystals, often clinging together in a large single mass. The mixture is transferred to a fritted glass, filtering crucible. The solvent is removed by suction. The crystals are then washed twice with 3-ml. portions of fresh solvent, allowed to stand at room temperature for 30 minutes, dried at 105° C. for 30 minutes, and weighed. The percentage diosgenin on a dry weight basis is calculated from this weight, and the dry weight of the sample taken for moisture determination.

If there are more tubers than can be analyzed in a single day, they should be macerated and sampled, and the samples dried and stored for future analysis. If an accurate assay is desired, on tubers containing less than 1% diosgenin, the extract must be treated according to another procedure indicated in this paper.

Discussion

The assay method just presented consists of three basic steps: maceration of the sample, hydrolysis of the sample in acid solution, and extraction of the diosgenin from the remaining residue.

Preliminary trials indicated that the treatment given tubers prior to acid hydrolysis would significantly affect the amount of diosgenin that could be recovered. In order to determine the treatment necessary for the highest possible yields, identical tuber samples were given one of six different preparatory treatments prior to hydrolysis. Table I presents data which show the effects of the various treatments involved. These treatments include only those simple ones which can be carried out in the ordinary laboratory. The data demonstrate the importance of several factors concerning sample preparation. Maceration of the fresh tuber is necessary if the highest yields are to be obtained. Drying of unmacerated tubers significantly decreased the amount of diosgenin that could be isolated. The decrease of diosgenin caused by the drying of macerated tubers was so small that 10 replicate analyses of both dried and undried samples were required to estimate its magnitude. Grinding of the dried, macerated material did not increase yields, nor did maceration time in excess of 5 minutes. These observations led to the adoption of a 5-minute maceration period prior to hydrolysis.

The acid hydrolysis of the macerated tubers serves two purposes. It liberates diosgenin from the saponin dioscin, and converts all but a small fraction of the starchy tuber into water-soluble components, leaving a residue that contains up to 35% diosgenin. The diosgenin then can be isolated readily in a highly

Table I. Effect of Sample Preparation on Yields of Diosgenin[a]

Preparation Prior to Hydrolysis	% Diosgenin of Dry Weight		
	a	b	c
Sliced, 1 mm.	2.76	2.79	2.86
Sliced, dried, ground to 80 mesh	3.56	3.60	3.62
Macerated 5 minutes in high-speed blender	4.31	4.50	4.56
Macerated 5 minutes, dried	4.20	4.26	4.30
Macerated 5 minutes, dried, ground to 80 mesh	4.16	4.20	4.26
Macerated 10 minutes	4.40	4.50	4.54

[a] All samples were hydrolyzed for 2 hours in 2*N* hydrochloric acid; extracted for 16 hours with petroleum ether. Yields based on recrystallized diosgenin melting at 198° to 203° C.

Table II. Effects of Acid Concentrations and Reflux Times on Yields of Diosgenin[a]

Reflux Time, Hours	Normality of Hydrochloric Acid			
	1	2	3	4
1.0	2.45	4.30	4.05	3.91
2.0	3.65	4.12	3.45	3.80
2.5	3.68	4.33	3.92	3.60
3.0	4.23	4.46	3.86	3.23
3.5	4.19	4.32	3.98	3.45
4.0	4.49	4.19	4.02	3.56

[a] % of dry weight, and mean of three determinations.

Table III. Effect of Hydrolysis Time on Rate of Diosgenin Extraction with Petroleum Ether[a]

Extraction Time, Hours	Hydrolysis Time, Hours		
	1	2	3
	Extraction Rate, %		
0.5	35 ± 5	43 ± 5	47 ± 3
1.0	52 ± 7	61 ± 6	66 ± 4
1.5	63 ± 7	73 ± 4	78 ± 4
2.0	72 ± 7	80 ± 4	85 ± 3
4.0	88 ± 5	92 ± 2	96 ± 1
6.0	95 ± 3	96 ± 1	98.8 ± 0.2
8.0	98 ± 1	99 ± 1	99.9 ± 0.1
16.0	100.00	100.00	100.00
Yields	4.77	4.76	4.81

[a] Values are means of 4 replicates and are percentages of totals extracted after 16 hours. All samples were hydrolyzed in 2*N* hydrochloric acid at 102° to 104° C.

purified state by extraction with a nonpolar solvent.

The hydrolysis of dioscin in acid solutions has been investigated by Rothman, Wall, and Walens (*5*) and by Rothrock, Hammes, and McAleer (*6*). The first group operated at temperatures of 75° to 78° C., and used an acid concentration of 4*N*. This group found that a 4-hour hydrolysis period was required for the optimum recovery of diosgenin. Rothrock and his coworkers used 2*N* acid at temperatures slightly above 100° C. (probably 102° to 104° C.), and found that a 2-hour hydrolysis period gave optimum yields. Because neither group used the conditions and the apparatus which the present authors had found most satisfactory for routine assay, a series of analyses was carried out where both the acid concentration and the reflux time varied over wide ranges. The data obtained are shown in Table II, and indicate that a fairly wide range of conditions leads to the same satisfactory result. It appears that often hydrolysis is complete under conditions less drastic then previously reported necessary. The acid concentration and reflux time recommended for assay work—i.e., 2*N* acid and a 3-hour reflux period—were selected because they appear to be near the mid-point of the permissible range. These conditions should ensure the complete hydrolysis of the dioscin in unusual samples, and decrease the restrictions placed on sample preparation. At the same time, neither the acid concentration nor the reflux time appears to be highly critical. Slight variations in the normality of the acid or of the reflux time do not cause an appreciable error, and do not lower the quality of the product isolated. Present observations are in close agreement with those made by Rothrock (*6*). The longer reflux periods and the higher acid concentrations recommended by Rothman (*5*) are simply explained by the fact that he operated at much lower temperatures than those recommended above.

It was observed that for any given species, the weights of residues obtained from samples weighing 100 grams varied little if any, although the acid concentration and the reflux time varied over wide ranges. Eighteen identical, fresh samples, each weighing 100 grams were hydrolyzed in 2*N* acid. Six replicates were removed after 1 hour, six others were removed after 2 hours, and the remaining six were taken out after 3 hours. The mean weight of the first group was 4.23 ± 0.08 grams, that of the second was 4.17 ± 0.05 grams, and that of the third group 4.15 ± 0.05 grams. These data indicate that most of the hydrolytic reactions were completed during the first hour of refluxing.

One of the important reasons for selecting a 3-hour hydrolysis period is related to the fact that the hydrolysis time significantly affects the time required for the extraction of the diosgenin. The stability of diosgenin under the conditions recommended for hydrolysis was demonstrated in a very simple manner. Samples of purified diosgenin weighing 1 gram each were suspended in 200 ml. of 2*N* acid, refluxed for 3 hours, filtered, and recrystallized. In six trials the losses never exceeded 3% of the original weights.

The extraction of diosgenin from the hydrolysis products was carried out easily and conveniently with a Soxhlet extractor and petroleum ether. It was observed that the time required for the complete extraction of diosgenin was related to the reflux time during hydrolysis. Samples that were hydrolyzed in 2*N* acid for 3 hours were completely freed of diosgenin in 6 hours. Samples hydrolyzed for 1 and for 2 hours were freed of diosgenin only after 12 to 16 hours of extraction. Because the yield and quality of the products were the same in all cases, the 3-hour hydrolysis period was adopted in order to shorten the extraction time. Eight hours of extraction were recommended to ensure against any losses and to permit the extraction to be completed in one day. Overnight periods of 12 to 16 hours led to the same results, and are permissible if the inherent hazards are duly considered. Table III contains data which show the effect of the duration of the hydrolysis on the time required for extraction.

In order to determine the simplest, acceptable method for handling the final product, several procedures were investigated. The complete evaporation of the solvent, followed by the weighing of the diosgenin without further treatment, led to large positive errors. With tubers containing 4 to 8% diosgenin these errors were between 5 and 10%. This procedure was considered unsatisfactory, because of these large errors. The most accurate and precise method required: (1) reduction of the volume of the extract between 15 to 20 ml.; (2) cooling; (3) filtration; (4) washing of the diosgenin; (5) recrystallization and weighing of the diosgenin; and (6) repetition of steps 1 to 5 on the mother liquor and on the combined washings. This procedure yielded a very pure product, but proved to be tedious and time-consuming. The method finally adopted was a simple one which gave highly satisfactory results. The extract was evaporated to a small volume and filtered, and the diosgenin washed free of oils and weighed without recrystallization. The results obtained by this procedure were never significantly different from those obtained by the more laborious method, where the mother liquors and washings were treated quantitatively. Obviously, the accuracy obtained with the simplified procedure is due partially to the compensating action of two small errors which are always small and com-

pensating in nature. The simplified procedure was adopted as part of the assay method.

The method described above has been in use in these laboratories for 3 years. The analysis of over 1000 samples has permitted the segregation of high and low yielding varieties from a collection of 63 types. One operator can assay from 12 to 24 samples per day. A single determination usually gives a result which is within ±5% of the mean value of triplicate determinations run by the most accurate and precise method available. The results obtained by different laboratories have been in very close agreement. Three analysts assayed a tuber that contained 6.2% diosgenin and reported the following results: (*a*) 5.8, 6.0, 6.1%; (*b*) 5.9, 6.1, 6.1%; (*c*) 5.9, 5.9, 6.2%.

A recent investigation by Peal (*4*) demonstrated that diosgenin loses a molecule of water when refluxed in 4*N* hydrochloric acid for 2 hours, and that maximum yields of diosgenin are obtained with 2*N* hydrochloric acid and a reflux period of 2 hours. These observations are in complete agreement with the data presented herein.

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CITRAL DETERMINATION

Specific Quantitative Colorimetric Method of Analysis for Citral in Lemon Oil

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The aldehyde, citral, is the component in lemon oil responsible for the typical lemon aroma. Methods of analysis used in the past for determining the citral content of lemon oils, however, were nonspecific and measured only total carbonyl content. A specific method for citral in the presence of other aldehydes and ketones has been developed. It is based on the discovery that citral with a reagent mixture of vanillin and piperidine in absolute alcohol forms an alcohol-soluble green comp'ex (absorption maximum 605 mμ). Other carbonyls produce yellow, orange, or red colors. Only dihydrocitral and pseudoionone interfere. The method provides a highly sensitive and selective objective tool for evaluation and standardization of lemon oil quality and should be useful in following the effects of process variables and agronomic conditions on the composition of lemon oils.

The typical fragrant aroma of lemon peel oil has been ascribed to the terpene aldehyde citral (*2*). However, other aldehydes (octanal, nonanal, decanal, lauryl aldehyde, citronellal, and an unknown aldehyde, $C_{10}H_{18}O$) and a ketone (methyl heptenone) have also been reported in lemon oil (*2*). The relative concentrations of carbonyl compounds in lemon oils are not known, but it has been assumed among oil processors and essential oil dealers that citral represents about 80% of the total carbonyl content.

In evaluating the quality of samples of lemon oil and folded or concentrated lemon oils, the practice has been to determine total carbonyl with hydroxylamine (*7*) or phenylhydrazine (*4*) reagents and to report the results as per cent citral. Methods of analysis specific for citral in the presence of other aldehydes and ketones have been sought, particularly by processors who are interested in determining the effects of processing changes on the quality of lemon products.

A method which eliminates interference from ketones, proposed by Chace (*1*), employed the fuchsin aldehyde reagent and was reported to be best adapted for lemon extracts. It was of limited value, because the other aldehydes interfered. More recently a method employing benzidine was reported to be specific for citral (*8*). However, positive tests with this reagent also were obtained in this laboratory with crotonaldehyde and 2-hexenal. This evidence led to the assumption that the benzidine method could serve only as a general test for the α,β-unsaturated aliphatic aldehydes.

A general test for the detection of aldehydes and ketones was reported by Levine and Taterka (*6*). They found that the saturated aliphatic aldehydes and ketones gave yellow or red solutions when heated with vanillin and potassium hydroxide in aqueous or alcoholic solution. Testing the reaction with a series of known aldehydes, it was found that saturated aldehydes including citronellal gave yellow solutions and that α,β-unsaturated aldehydes, including citral, gave red solutions. Unfortunately, the method was not adaptable to quantitative colorimetric analysis of higher molecular weight aldehydes, because in alcohol-water mixtures suitable for dissolving the higher molecular weight aldehydes the addition of alkali caused an undesirable cloudiness.

The difficulty was overcome by substituting piperidine for the potassium hydroxide and using absolute alcohol (methanol or ethyl alcohol) as solvent. The vanillin-piperidine system in alcoholic solution produced yellow solutions with saturated aldehydes and red solutions with the α,β-unsaturated aldehydes as obtained previously with the vanillin-potassium hydroxide reagent. Citral, dihydrocitral, and pseudoionone, on the other hand, developed an intense emerald green color. These latter compounds are unique in having a branching methyl group on the terminal carbon atom of a double bond system conjugated with the carbonyl group. Citronellal, having no double bond at the position