# Identification of Caffeine as Natural or Synthetic

O. J. WEINKAUFF,[1] R. W. RADUE, and R. E. KELLER
Organic Chemicals Division, Monsanto Chemical Co., St. Louis, Mo.

H. R. CRANE
Department of Physics, University of Michigan, Ann Arbor, Mich.

Infrared absorption and radiocarbon dating methods were developed and used to identify caffeine as of natural *vs.* synthetic origin. By convention natural caffeine refers to that extracted from tea or coffee as well as that prepared by methylation of theobromine extracted from cacao beans. Synthetic caffeine includes that prepared by synthesis from urea and from 1,3-dimethylurea. Infrared identification of characteristic components in alkali-soluble residues extracted from caffeine was used to differentiate between natural and synthetic products. A radiochemical method based on measurement of $C^{14}$ in natural caffeine derived from $C^{14}O_2$ in the atmosphere was used to differentiate the natural from the synthetic compound which is composed of essentially dead carbon from petroleum or coal sources. The sample is burned, carbon dioxide is collected as calcium carbonate, and the radioactivity of the evolved gas measured. Representative caffeines from domestic and foreign sources were identified properly. The principles and techniques should serve for better identification of other organic products from natural and syntehtic sources.

MANUFACTURERS and consumers often need methods to differentiate between natural and synthetic products. Customer acceptance of caffeine depends many times upon absolute proof of its origin. No reported work has been found on the identification of caffeine as either a natural or synthetic product. Methods for differentiation of natural and synthetic organic products in general have also not been reported in the literature.

Natural caffeine as used in this study includes that extracted from coffee and tea as well as that prepared by the methylation of theobromine which has been extracted from natural products such as cocoa. Most synthetic caffeine is prepared from urea or 1,3-dimethylurea. This paper describes two approaches that have been used successfully for identification of caffeine as either natural or synthetic. A less complete study was done by Allen (*1*) almost simultaneously with our work. The first is based on separation of alkali-soluble residues from caffeine by liquid-liquid extraction and subsequent identification of characteristic residual components by infrared absorption analysis. The second approach is based on a radiocarbon dating scheme. The presence of $C^{14}$ in living matter serves as a basis for this scheme (*3, 4*). Natural caffeine from tea and coffee is composed of modern carbon containing the radioisotope $C^{14}$ in the same relative proportion to stable carbon as found in atmospheric carbon dioxide. Natural caffeine prepared by methylating theobromine obtained from cocoa can contain either all modern carbon or the equivalent of seven out of eight modern carbons (Figure 1). This results from variations in the source of methanol used to prepare either methyl chloride or dimethyl sulfate for the methylation step. On this basis, caffeine showing radioactivity equivalent to or greater than seven out of eight modern carbons or a theoretical of 87.5% as based on measurement of the $C^{14}$ isotope is considered a natural product. Likewise, any product showing an activity equivalent to less than seven modern carbons out of eight is a synthetic product. Known processes for the manufacture of caffeine from urea or 1,3-dimethylurea produce a product containing either no modern carbon or no more than four modern carbons. It is possible to synthesize caffeine which contains all modern carbon, but no such commercially available product has been found on the market. The examples (Figure 1) are included to show several possible routes for obtaining synthetic caffeine with modern carbon. The radiochemical method is based on burning the sample in a combustion tube, collection of the carbon dioxide formed, and subsequent counting for radioactivity.

In most cases, each method used alone will correctly identify caffeine as natural or synthetic. The carbon dating technique provides little information about the true source of caffeine within the natural and synthetic categories. Caffeine containing 100% modern carbon which is derived from coffee, tea, or even cocoa will be identified properly as natural product by both techniques. However, the carbon dating technique will not differentiate among the sources. On the other hand, the infrared method will, in addition to identification of these caffeines as natural, also disclose, in most instances, the exact source of the natural caffeine. With synthetic caffeine, the infrared method provides additional information about the synthetic route used. In certain cases, the infrared spectra are not characteristic of known components in residues from synthetic and natural caffeines. In this situation, the carbon dating technique is essential for the proper identification as natural or synthetic. The analytical work conducted in this study shows that the methods complement each other and that both sometimes are necessary for the accurate and conclusive identification of caffeine. It is suggested that the described techniques no doubt can be used to identify other organic products as either natural or synthetic. This assumes that impurities can be isolated from the product for infrared examination or for characterization by other techniques. The carbon dating technique should be unequivocal provided dead carbon is used in the synthetic route.

### *Experimental. Infrared Analyses*

**Sample Preparation.** A 50 (±0.1)-gram sample of caffeine was weighed into a 1-liter beaker and dissolved in 500 ml. of chloroform. The solution was cooled to 0° to 5° C. in an ice bath and then transferred to a 1-liter separa-

[1] Deceased.

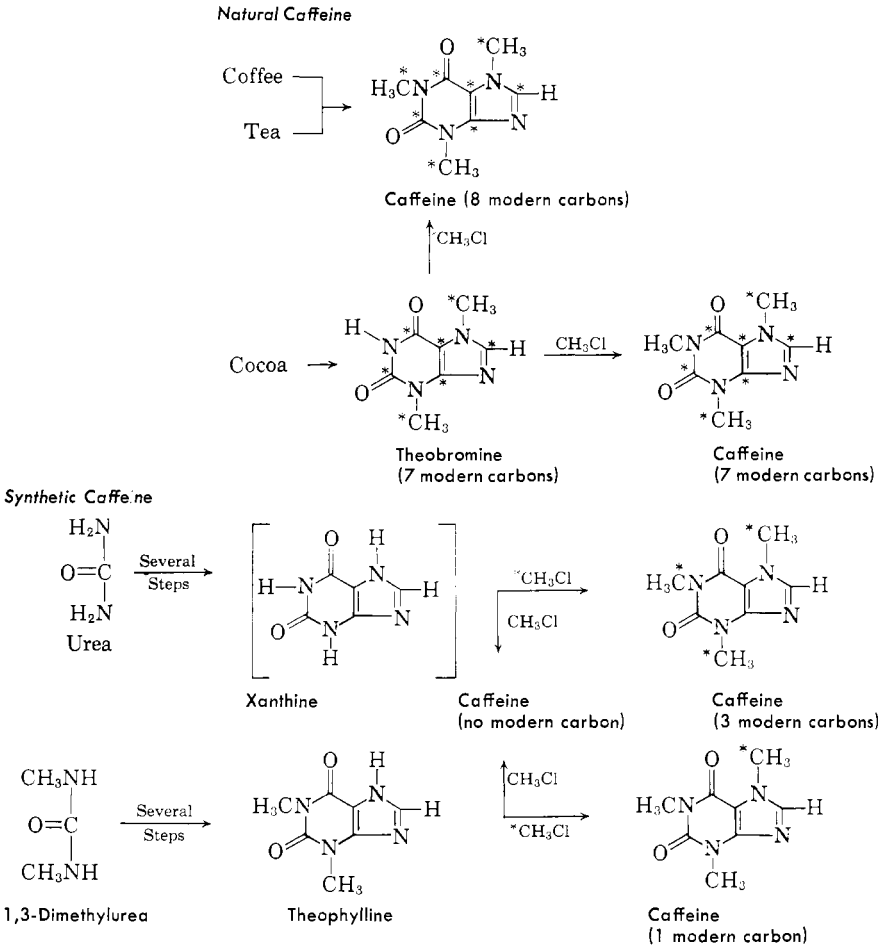


Figure 1. Modern Carbon in Caffeine

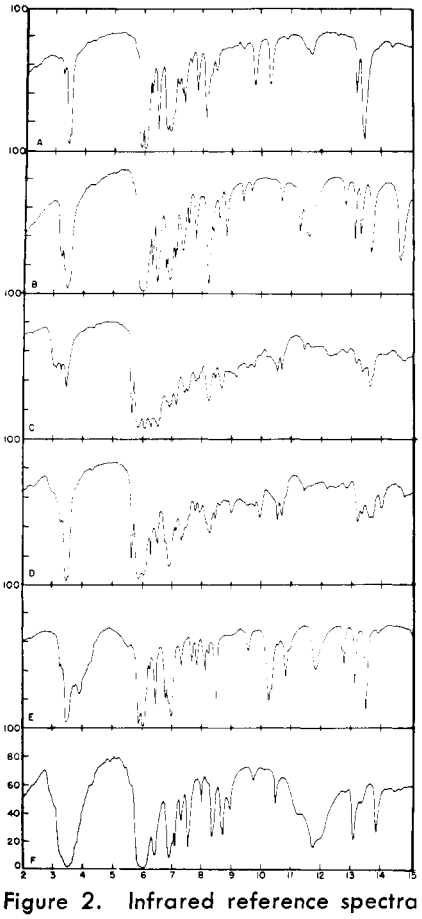

Figure 2. Infrared reference spectra

A. Caffeine
B. Theobromine
C. Residue from coffee caffeine
D. Residue from synthetic caffeine from urea
E. Theophylline
F. Xanthine

tory funnel. The chloroform solution was extracted with three 100-ml. portions of 1% sodium hydroxide solution cooled to 0° to 5° C. After each extraction, the chloroform layer was drawn off into the original 1-liter beaker for transfer back to the separatory funnel. The three alkaline extracts were combined in a 500-ml. beaker. Upon completion of the alkaline extractions, the chloroform layer was transferred to a second 1-liter separatory funnel where it was washed with 25 ml. of distilled water, then drawn off and discarded. The aqueous phase was retained in the funnel. The combined alkaline extracts were transferred to the original 1-liter funnel and extracted with six 50-ml. portions of chloroform. Each 50-ml. chloroform extract was transferred in turn to the second 1-liter funnel where it was washed with the aqueous phase in the funnel, then discarded. The aqueous phases in the two separatory funnels were combined in a 500-ml. beaker and acidified with concentrated hydrochloric acid to pH 3.0 to 3.5 using a pH meter. The acidified sample was transferred to a distillation-type, liquid-liquid extractor and it was exhaustively extracted with chloroform for 16 hours. The major portion of solvent was removed from the receiver flask by straight take-over distillation and the remaining solvent was removed by evaporation on a steam bath. The receiver containing the residue was transferred to an oven and dried at 110° C. for 1 hour. The dry residue varied in weight from 10 to 150 mg.

**Infrared Procedure.** A 1.0- to 1.2-mg. sample of the extracted residue was weighed on to a small piece of plate glass. Sufficient Nujol oil to make a 40% dispersion of the sample was added. The oil and the sample were covered with a second piece of plate glass. The glass pieces were pressed and twisted repeatedly to break down the sample. The oil containing the dispersed sample was collected by scraping with a razor blade. The mixture was redeposited on the glass pieces and the pressing and twisting were repeated until a usable mull was obtained. Part of the mulls were prepared by vibrating the glass plates with a copper-tipped Vibratool, using a heavy steel anvil for backing. The finished mull was transferred to salt plates for infrared analysis. The optimum thickness of the mulls, for good spectra, was found to be 0.01 mm. The spectra were obtained using a Perkin-Elmer Model 21 double beam spectrophotometer. The 7- to 15-micron region was used for differentiating synthetic and natural caffeine residues.

## Discussion

The residues obtained from the alkaline extracts of caffeine are often as small as 10 mg. The samples and theobromine, which is one of the components sought in the analysis, are not sufficiently soluble in the common solvents used for infrared work to permit analysis. A semimicro mulling technique is a satisfactory solution to this problem.

Many residues are too gummy or resinous to be dispersed by pressing and/or grinding techniques. The sample may be made more brittle by removing residual solvents and moisture by exposing the mulled sample on the separated glass plates to vacuum obtained with a Hyvac pump and/or by oven heating. Storing the closed plates in a cool place for several hours may also help.

As an alternative to mulls, capillary films of melts also have been run. Other samples have been analyzed by separating oils from mullable solids by extracting with solvents such as chloroform and acetone.

## Experimental. Radiochemical Analyses

**Apparatus for Combustion.** The apparatus consisted essentially of a

standard macro combustion unit normally used for carbon-hydrogen determinations, although production and isolation of carbon dioxide were of prime interest in these combustions. The 96% silica glass combustion tube, which was 39.5 inches long × 3/4 inch in I.D., had a 10-inch section packed with copper oxide, black wire, and also had rolls of copper gauze appropriately located in the tube to retain copper oxide and to isolate the combustion boats from the inlet end of the train during combustion. The exposed surfaces of the rolls of copper gauze were oxidized thoroughly to black copper oxide in preparing the tube for use. The tube was set in a combustion furnace having three movable heating elements. The unusual factors involved in the caffeine combustions were: the large size sample burned, the absorbing train and reagents used, and the procedure used for isolating the calcium carbonate produced in the combustion.

**Sample Preparation.** To provide sufficient carbon dioxide for the $C^{14}$ counting operation, it was found desirable to burn 6 to 7 grams of caffeine which is equivalent to 25 to 30 grams of calcium carbonate. The total caffeine sample was weighed into four large porcelain combustion boats. Two combustions were run to handle the total sample, with two boats being charged for each run. The initial absorber-charges were made so as to handle the entire carbon dioxide produced in the two combustions and thus the absorbers did not have to be changed between runs. The time involved in running a single combustion was 3 to 4 hours.

**Preparation of Absorbers.** The absorption train consisted of three 1000-ml. vacuum filtering flasks connected in series using glass and rubber tubing and rubber stoppers. When used the flask was connected directly to the combustion tube and kept empty to act as a safety catch pot. The following two flasks in the series were charged each with absorbing solution. In preparing the absorption train the three flasks, connected in series, but disconnected from the combustion equipment, were purged thoroughly with nitrogen, then sealed. A solution of 26 grams of anhydrous calcium chloride in 40 ml. of distilled water was prepared and cooled to room temperature. A funnel was connected to the side arm of one of the flasks to be charged with absorbing solution and the calcium chloride solution was allowed to flow into the flask followed by sufficient 28% ammonium hydroxide solution to fill the flask to a volume of 800 ml. It was necessary to vent nitrogen slowly from the system during the charging operation, but care was taken to avoid introduction of any air. The second flask to be filled received a similar charge of absorbing solution. The absorption train was connected to the combustion tube, but kept sealed until the combustion was to begin.

**Isolation of Calcium Carbonate.** After combustion of the total caffeine sample was complete and the resulting carbon dioxide had been purged through the absorption train, the two flasks containing the absorbing solution were removed from the system and gently heated on a steam bath to remove excess ammonia. In some instances, this was accomplished by digesting overnight. Removal of excess ammonia appears necessary to ensure maximum recovery of calcium carbonate. After digestion, the calcium carbonate slurry was filtered on an all-glass Büchner funnel, the filter cake washed with distilled water, and dried to constant weight in an oven at 110° C. Calcium carbonate was now ready for use in the $C^{14}$ counting procedure.

**Radiochemical Procedure.** Calcium carbonate in sufficient amount to yield approximately 2 liters of carbon dioxide was decomposed in dilute hydrochloric acid. The carbon dioxide was passed through a trap cooled by alcohol and dry ice to remove water vapor. It was then stored in a borosilicate glass flask for 1 week or longer in order to allow any slight trace of radon (half life 3.8 days) to decrease in activity. The gas was then introduced into a Geiger counter, together with 5% by volume of carbon disulfide vapor, to a total pressure of 1 atm. Each sample was counted for approximately 12 hours. Comparison runs, using modern carbon dioxide from oak wood and "dead" carbon dioxide of petroleum origin, were made. The background counting rate given by the ancient carbon dioxide was 8 per minute and the rate given by the modern carbon dioxide was 17 per minute. The resulting standard deviation in the determination of the content of modern carbon dioxide in an unknown sample was, on the basis of the number of counts, about 2%. The counting apparatus and the technique are those which are used routinely for radiocarbon dating at the University of Michigan, and are described fully (*2*).

### *Results and Discussion for Infrared Studies*

The infrared spectra of alkali-chloroform extracted residues from caffeine obtained from natural and synthetic sources can be classified according to the groups in Table I. Reference spectra used as a basis for this classification scheme are shown in Figure 2. Interpretation of the infrared spectra for the 7- to 15-micron region results in identification

**Table I. Examination of Residues Extracted from Caffeine**

| *Class* | *Source* | *Infrared Spectra Show* |
|---|---|---|
| Natural | Tea | Weak theobromine and related bands |
| | Coffee | Bands peculiar to coffee source |
| | Cocoa (theobromine) | Moderately strong to intense theobromine bands |
| Synthetic | Urea | Characteristic unidentified synthetic bands denoted as compound X and/or associated bands |
| | Dimethylurea | Theophylline bands |

**Table II. Questionable Caffeines by Infrared Analysis**

| | | *Source by Infrared* | | *Source by Carbon Dating* | |
|---|---|---|---|---|---|
| *Caffeine* | *Labeled Source* | *Residue spectra show* | *Origin* | *% modern carbon* | *Origin* |
| *A* | Natural from theobromine | No theobromine bands, urea caffeine and coffee caffeine bands found | Uncertain (not derived from theobromine) | 16 | Synthetic |
| *B* | Natural from theobromine | No theobromine bands, urea caffeine and coffee caffeine bands found | Uncertain (not derived from theobromine) | 15 | Synthetic |
| *C* | Natural from theobromine | Characteristic urea caffeine bands | Synthetic | 13 | Synthetic |
| *D* | Natural from theobromine | Characteristic urea caffeine bands | Synthetic | 48 | Synthetic |

**Table III. Specific Absorption Bands for Differentiating Tea and Cocoa Caffeines from Urea Caffeines**

| *Tea and Cocoa Sources* | *Synthetic Source from Urea* |
|---|---|
| 14.72 microns (theobromine at 14.68) | 14.51 microns |
| 14.23 weak, broad | 13.70 strong |
| 14.03 | 13.51 strong |
| 13.45 | 13.36 |
| 13.19 | 12.85 weak |
| 11.65 broad region | 11.42[a] |
| 10.83 | 10.68[a] |
| 10.45 broad | 10.54[a] (strong doublet) |
| 10.30 broad region | 9.77[a] |
| 9.97 | 9.03 |
| 8.98 | 8.45 |
| 8.13 strong | 8.26 strong |
| 7.36 (theobromine at 7.33) | 7.91 |
| | 7.76 |
| | 7.48 strong |

[a] Prominent compound X bands.
Table does not include any theobromine or theophylline bands. Bands listed for tea and cocoa are due to components that are extracted from tea and cocoa along with the theobromine. The 13.70- and 7.76-micron bands listed under synthetic are coincident with theobromine bands, but are not theobromine.

of the characteristic absorption bands peculiar to each source of natural and synthetic caffeine. Familiarity with the characteristic spectra for known components as well as for unidentified components which are peculiar to the different sources makes it possible to classify an unknown caffeine as shown (Table I). This scheme has been used to examine 36 natural and synthetic caffeines from domestic and foreign sources. With the exception of the samples reported in Table II, the identifications were conclusive.

**Natural Caffeine.** Residues of caffeine prepared from coffee produce spectra which contain unidentified absorption bands peculiar to coffee.

Natural caffeine derived from tea produces a residue that exhibits weak absorption bands corresponding to theobromine as well as related bands peculiar to tea residues. When the theobromine is no longer apparent, a series of absorption bands due to unidentified components found with theobromine can be used to identify the material as a tea or cocoa source. These bands listed in Table III do not coincide with the theobromine bands. They occur in tea and cocoa residues, but not in urea derived synthetics. The presence of most of these bands, in the absence of theobromine, will establish that the sample is derived from tea or cocoa and is not a urea synthetic. Conversely, absence of most of these bands when the theobromine is not apparent will exclude the sample from the tea and cocoa class. The method does not imply that all bands must conform because some residues are complex mixtures and the loss or gain of some minor component may add or subtract a band at any point, since most of the components involved are known to occur to some extent in all of the samples, but varying widely according to sources.

Residues from caffeine prepared from theobromine derived from cocoa contain prominent theobromine absorption bands. As the proportion of theobromine decreases, the bands of associated materials become more prominent. When the theobromine is no longer apparent a series of absorption bands due to the theobromine fellow travelers can be used to identify the material as a tea or cocoa source. These bands are listed in Table III.

**Synthetic Caffeine.** Residues from synthetic caffeine synthesized from urea show characteristic bands attributed to an unknown component denoted as compound X. This synthetic group is differentiated from the tea and cocoa group by the absence of theobromine or its related components and by the presence of compound X and/or its associated bands. Presence of most of these bands in a spectrum will establish that the residue is derived from a urea synthesis or a coffee source. Except for traces of compound X, these bands will rule out the possibility of tea or cocoa being the source. Compound X, which has not been identified, exhibits a number of absorption bands, five of which are indicated in Table III. The differentiation of synthetic caffeine (synthesized from urea) from natural caffeine derived from coffee is sometimes difficult. The following criteria are used as a basis for this identification.

Residues that are predominantly compound X are usually synthetic. Coffee sources generally contain a small amount or none.

Urea synthetics exhibit a weak band of varying intensity at 11.28 microns. This band is insignificant or absent in the spectra of mulls of coffee residues.

Three broad and poorly defined bands occur in coffee-type mulls. Absorption centering at 10.45 microns appears as a side band on the strong 10.54-micron band of compound X when it is present. A broad band centering at 8.67 microns is found in coffee residues. This overlaps the position of a similar band found in urea synthetics that centers at 8.61 microns. Coffee samples generally show strong absorption at 8.22 microns. These bands appear to be independent of each other and are probably due to oils and waxes extracted from the coffee.

The over-all appearance of the spectra of the two types differs. The spectra of the synthetics apparently are the result of a limited number of related materials, thus producing clearly defined absorption bands characteristic of the purines. The spectra obtained for coffee residues are very poorly defined, weak, and show many broad absorption regions, except for the compound X bands. This is probably due to the presence of a great number of substances. Thus, the normally strong absorption of the purines in the 13- to 14-micron region is reduced by a background of weakly absorbing materials.

Synthetic caffeine synthesized from 1,3-dimethylurea contains residues which exhibit prominent theophylline absorption bands.

### *Results and Discussion for Radiochemical Studies*

The infrared absorption method is considered a reliable and satisfactory procedure for the identification of most caffeines. However, two suspicious anomalies occurred during the infrared study which prompted the investigation of a supplementary method that could be used better to identify questionable material. A radiocarbon dating scheme based on measurement of modern carbon by radioactivity measurements was investigated and found to be a satisfactory confirmatory method. It is based on the fact that natural caffeine from growing products will contain the radioisotope $C^{14}$ in the same relative proportion to stable carbon as found in atmospheric carbon dioxide. Likewise, synthetic caffeine prepared from urea or 1,3-dimethylurea would be expected to contain essentially stable carbon, since most of the raw materials used in known syntheses come from petroleum or coal sources made up of dead carbon.

Synthetic caffeine from urea, natural caffeine from theobromine, and natural caffeine from tea were examined as reference materials for the radiocarbon method. The caffeine samples were burned in a 96% silica glass combustion tube and the carbon dioxide evolved was collected as calcium carbonate in aqueous calcium hydroxide scrubbers. Carbon dioxide was evolved from the calcium carbonate and counted for $C^{14}$ radioactivity with a Geiger counter.

Results showed that synthetic caffeine from urea contained 38% of modern

carbon, natural caffeine from theobromine 87% of modern carbon, and natural caffeine from tea 100% of modern carbon. These data confirmed that caffeine from tea contained eight modern carbon atoms and caffeine prepared by methylation of theobromine contained the equivalent of seven modern carbon atoms out of a total of eight in the caffeine structure. The synthetic caffeine prepared from urea contained the equivalent of three modern carbon atoms. The presence of this modern carbon was explained after a check with a supplier showed the methyl chloride (used in the synthesis of caffeine) to have been obtained from methanol prepared by the destructive distillation of wood. Another synthetic reference sample contained one modern carbon due to the use of formic acid made from methanol in the synthesis. Hence, the amount of modern carbon in synthetic caffeine can vary, but it never has approached the equivalent of seven or eight modern carbons, since known synthetic processes do not use raw materials totally derived from natural or modern carbon sources. On this basis, an unknown showing less than the equivalent of seven modern carbons by radioactive $C^{14}$ measurement is classified arbitrarily as synthetic material.

Questionable caffeines (Table II) which had been identified by the infrared method as from an uncertain or synthetic source instead of the natural source claimed by the supplier were examined by the radiocarbon method. Samples *A* and *B* produced residue infrared spectra containing bands peculiar to urea and coffee caffeine, but no theobromine bands. This shows that the products were not derived from theobromine. Since a positive identification could not be made, the samples were classified as unidentified. Samples *C* and *D* produced spectra possessing distinct characteristic urea caffeine bands. On this basis, these samples were classified as synthetic.

Results with the radiocarbon method showed that all the samples contained 48% or less of modern carbon and must be classified as synthetic caffeine. These findings confirmed that the samples were not as labelled. The analysis of 11 additional synthetic samples, some from an unknown source, showed an average of 13.2% of modern carbon with a spread ranging from 11 to 15%.


### Acknowledgment

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**PEANUT FLOUR CONSTITUENTS**

# Isolation and Identification of a Sterol Glucoside from Peanut Flour


**NELLE J. MORRIS and LOUISE S. LEE**

**Southern Regional Research Laboratory, New Orleans, La.**


CONSTITUENTS of the nonoil, alcohol-soluble fraction of the peanut are being studied as a part of a program to relate composition of raw peanuts to the quality of processed peanut products, with particular attention to those present in small quantities. The present report is concerned with β-sitosterol-D-glucoside.

### Experimental

In the preparation of the nonoil alcohol-soluble fraction, Spanish peanuts were blanched, freed from skins, sliced, and extracted with commercial hexane (60° to 70° C.) until the oil content of the residue was less than 0.1%. The air-dried flour was exhaustively extracted with 95% ethyl alcohol. The alcohol extract was concentrated by vacuum distillation and subsequently lyophilized to yield a dry product representing 7% of the flour, or 3.5% of the blanched peanut kernel (*B*, Figure 1).

Sufficient 50% aqueous alcohol was added to 300 grams of the dry extract to dissolve it. The resulting solution was treated with powdered neutral lead

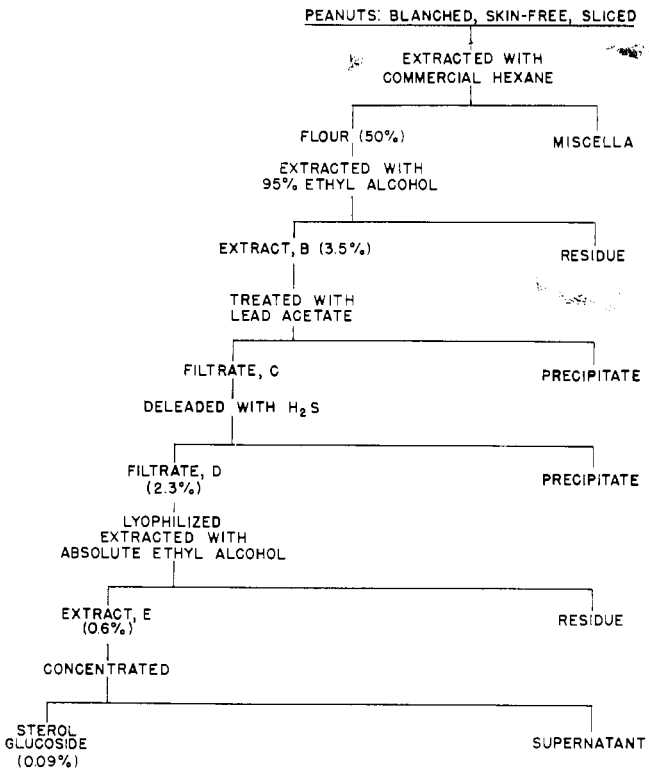


Figure 1. Schematic diagram of isolation of sterol glucoside