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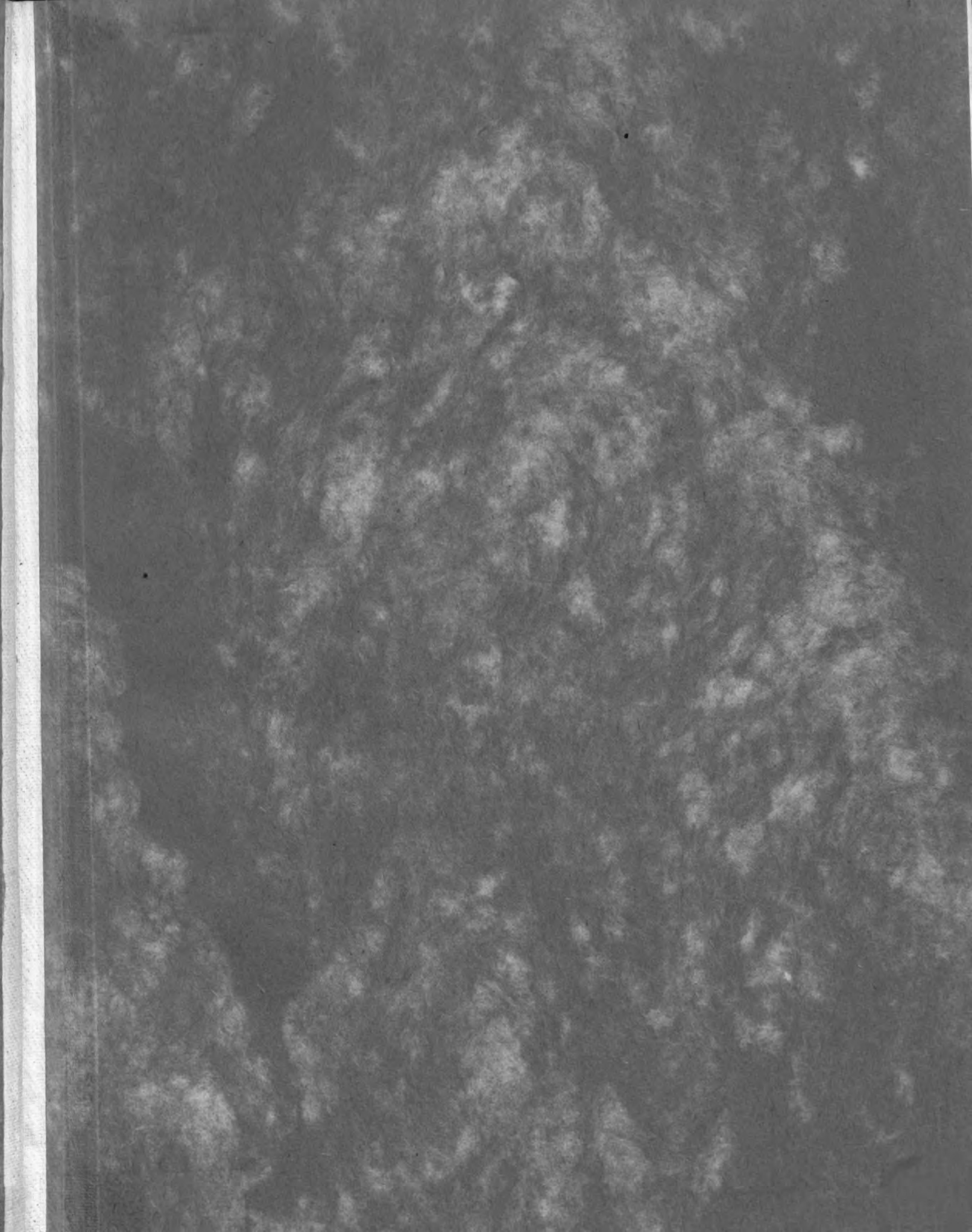

A STUDY OF THE METHODS FOR DETERMINATION OF THE LIPIDE CONTENT OF PLANT TISSUES

A Thesis

Submitted to the Faculty of Michigan State College of Agriculture and Applied Science in Partial Fulfillment of the Requirements for the Degree of Master of Science

by
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Clarence Hieserman

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INTRODUCTION

The methods used for the determination of lipides and fat content of biological materials are numerous and quite different in many respects. None of the methods employed up to the present time gives any accurate measurement of the true fat content or the content of the entire fat-like substances.

The purpose of the research connected with this problem was to compare the values obtained by the various methods, and then modify the procedures in such a way as to obtain a more desirable procedure for the determination of the lipide content of plant materials.

The nomenclature and classification of the ether soluble, fat-like substances are at the present time quite confusing. The term "lipide" as used in this paper shall include those substances which are water insoluble, ether or alcohol soluble, and which are either ester-like combinations with fatty acids or capable of forming such combinations (1). The term "fat" shall include only those fatty substances which are glycerol esters of fatty acids.

HISTORICAL

Early investigators considered an ether extract of most tissues to consist mainly of neutral fats, therefore, there was thought to be no need for further separation of the ether extract. Later investigations showed that the ether soluble substances present in plant and animal tissues were a mixture of several substances. For example, the ether extract of vegetable products contains not only glycerides of fatty acids, but also lecithins, chlorophylls, xanthophylls, carotenes, waxes, alkaloids, and sterols. The ether extraction method (Official Method of the Association of Agricultural Chemists) is still used today, although it is now known that this method neither extracts all of the lipides (Schulze and Steiger (2), Maxwell (3), Rather (4), Hertwig (5), and Rask and Phelps (6)), nor is the extracted material by any means pure lipide. Chibnall and Channon (7) stated that it had been their experience that the presence of a small amount of water in ether would allow a small but definite amount of inorganic matter, amino-compounds, carbohydrates, and possibly tannins to go into solution.

Waldemar Koch (8) made an attempt to remedy the incompleteness of the extraction of the lipides from animal tissues. It was found by this investigator that a more complete extraction could be obtained if the ether extraction was preceded and followed by alcoholic extractions. Later, F. C. Koch (9) introduced modifications which adapted the

method to plant tissue analysis. This method was one of the first attempts made to extract the lipide material from fresh plant materials without going through the process of drying. The method devised by Koch also made it possible to store the plant material (without drying) for a period of time before the actual analysis had to be made. Boyd (10) found that no appreciable change occurred in the total free fatty acid content during the storage period of the lipides, but that there did occur a significant decrease in phospho-lipides between one and three months storage. Between three and six months storage, there also occurred a further significant decrease in cholesterol esters, total cholesterol and total lipides.

Shulze and Stieger (2) justified the use of a combination of solvents for lipide extraction by claiming that the phospho-lipides can only be completely removed from seed material if the ether extraction is followed by an alcohol extraction.

The use of solvents other than ether and alcohol has been recommended by various investigators. Schlesinger (11) and Rosenfeld (12) used chloroform as a solvent following a preliminary extraction with alcohol. Kumagawa and Suto (13) have pointed out that the more efficient removal of lipides was due to the alcoholic extraction rather than the chloroform.

Carbon disulphide has been recommended because of its high solvent powers. Bryant's (14) results indicated that carbon tetrachloride was a very rapid solvent and that in two

hours time the extraction was complete. Wiley (15) claimed the last traces of carbon tetrachloride are removed with difficulty and that in the presence of traces of moisture the solvent breaks down into hydrochloric acid, which may act upon the fat. Nevertheless, this solvent does have two distinct advantages in that it is non-inflammable and rather inexpensive in pure form.

Benzene, trichloroethylene, and acetone are also known to have high solvent powers for fats and other organic compounds (9).

A number of investigators, Kumagawa and Suto (13), Horwit, Cowgill and Mendel (16), and Rather (17) have made attempts to obtain the fat content on the basis of the amount of fatty acids present after saponification and subsequent acidification. Various methods have also been submitted for the determination of the unsaponifiable fraction (Kerr and Sorber (18), Kumagawa and Suto (13), and the Fat Analysis Committee of the Division of Industrial Chemists and Engineers of the American Chemical Society (19)). The desire to separate the ether soluble substances arose because of the need for this information in regard to nutritional studies. During recent years the unsaponifiable fraction has become biologically important because it contains Vitamin E, and the precursors of Vitamin A and Vitamin D. The investigations of Langworthy and Holmes (20) and Holmes (21) in 1915 definitely showed that practically all true fats are digested and assimilated by man.

Only recently, however, has the digestibility of the other ether soluble substances been investigated. Sinclair (22) in 1934 showed conclusively that the lecithins were readily hydrolysed by the digestive lipases and were thereby rendered available to the animal body.

Some of the pigments present in plant materials are apparently not utilized by the animal body. According to Fischer and Heidschel (23) the chlorophylls are only slightly changed in the digestive tract of human beings. The carotenoid pigments can be absorbed according to Hess and Meyers (24) but apparently they are not available for energy metabolism because Salomon (25) reported that adsorption bands of carotene and xanthophyll were found in the fecal extract even after nine days of pigment-free diet.

As the phytosterols cannot be resorbed in significant amounts through the intestional wall of the usual laboratory animals, they have probably no significance for the sterol metabolism of man and higher animals (26). The phytosterol esters in the plant tissue are probably not determined by the present saponification methods of lipide analysis, since Drummond, Channon, and Coward (27) found that alcoholic potassium hydroxide did not hydrolyse esters of cholesterol and some of the higher alcohols. According to these investigators, sodium ethylate was necessary for the hydrolysis of these substances.

Horwit, Cowgill and Mendel (16) through the application

of their saponification method, showed that more than fifty-five per cent of the material obtained from spinach by ether extraction and sometimes called "fat" was indigestible in the animal body. Rather (17) in his studies of the digestibility of the various ether soluble fractions of feedstuffs, showed that the fatty acids not extracted by ether, but extracted by alcoholic soda, had a digestibility in sheep ranging from 0 in Sudan grass to 36.7 per cent in moth-bean hay. After making molecular weight determinations on the fatty acids, Rather came to the conclusion that the indigestibility of the fatty acids extracted by the alcoholic soda, but not by ether was due to the form of combination of the acids in the feed or to the presence of difficultly soluble incrustations rather than to inherent unavailability.

Horwit, Cowgill and Mendel (16) made investigations with the enzyme lipase in an attempt to determine the availability of the fat-soluble fraction. All experiments were failures.

The great differences found in the procedures used for the determination of the fatty substances has been due in part to the different viewpoints from which the work has been attacked. Those investigators interested in the fatty substances from a strictly nutritional viewpoint desired a determination which would give a value from which energy values could be calculated. This, of course, stimulated the development of methods which determine the amount of fatty

acids present after hydrolysis of the plant material. On the other hand, the investigators interested in plant metabolism were often interested in a determination which would include all of the lipide substances, since in this case it is not known whether the synthesis of sterols, for example, is distinctly a different process from the synthesis of fatty acids.

EXPERIMENTAL

The experimental work was divided into three parts: first, the determination of total lipides in several plant materials by standard procedures; second, the study of various modifications in the standard procedures, and the significance of the different steps in the analysis; and, third, the separation of the unsaponifiable and the fatty acids from the crude lipide extracts by standard and modified procedures.

The materials used for the experimental work were fresh and dried alfalfa leaves, and soybean seed, variety Manchu.

For fresh material, alfalfa stems were collected from the field, and immediately carried to the laboratory. The leaves were stripped off a portion of the stems and placed in a refrigerator. During the following 24 hours the fresh leaves were weighed out on a pulp balance in 25 gram samples. The samples were immediately transferred to individual 200 cc. beakers and enough 95 per cent ethyl alcohol was added to bring the alcoholic content to approximately 80 per cent. (Since the leaves contained approximately 70 per cent moisture, 93 cc. of 95 per cent alcohol was added.) The leaves were boiled for 15 minutes on a steam bath to arrest enzymatic activity. The boiled samples, which were covered with watch glasses, were set aside and saved for future determinations. Moisture determinations were also run on the fresh alfalfa leaves by weighing out 10 gram samples in aluminum dishes and

drying in a vacuum oven at 80° C. for 6 hours. The samples were cooled in a desiccator and weighed. The weighing was continued for two-hour intervals until the weight remained constant.

For dried material, the remainder of the alfalfa stems collected from the field were air dried in a room at 60° C. As the leaves were needed, the stems were withdrawn from the drying room and stripped of their leaves. The leaves were ground in a mortar.

Soybean meal was prepared from soybean seed by finely grinding in a Hobart electric mill. The meal was allowed to remain in contact with the atmosphere for two days and then was placed in an air tight bottle to be stored until needed during the course of the next few months.

Standard Methods of Lipide Analysis

I. Official Method of the Association of Agricultural Chemists (A. O. A. C. Method) (23)

The air dried, ground alfalfa leaves, previously described, were transferred to a vacuum oven at 60° C., dried for a period of 6 hours, and cooled in a desiccator. The same drying procedure was followed for the soybean meal. Five gram samples of the vacuum dried plant materials were weighed rapidly and transferred to dry 33 x 94 mm. Schleicher and Schull fat-free paper thimbles. The thimbles were immediatedly placed into moisture-free Pyrex Soxhlet extractors. The tops of the condensors of the extracting apparatus were equipped with calcium

chloride tubes to keep out all moisture. The flasks containing several glass beads to prevent bumping of the solvent during extraction were thoroughly dried and rinsed with the solvent to be used in the extraction process. A portion of the solvent, 100-150 cc., was poured into the flasks, which were then connected with the extractors holding the samples.

After the extraction was complete, the ether extracts containing the extracted lipides were transferred quantitatively to tared beakers. The ether was evaporated off on a steam bath, and the lipide material was dried at 100° C. for 30 minutes, cooled in a desiccator, and weighed. The drying was continued for 15-minute intervals until the weight remained constant.

Moisture determinations were made on the plant materials used by weighing out 5 gram samples in weighing bottles and drying at 100° C. for 5 hours. The samples were cooled in a desiccator and reweighed. The drying was continued for 2-hour intervals until constant weights were obtained.

II. Modified Koch Procedure (9)

The plant materials used in this experiment were the previously described fresh and dried alfalfa leaves, and soybean meal.

The fresh alfalfa leaves which were previously heated in alcohol were transferred to 33 x 94 mm. Schleicher and Schull fat-free paper thimbles. This process was accomplished by fitting the extraction thimbles into glass filter funnels held in turn in clean suction flasks. The leaves in the extraction

thimbles were rinsed with a jet of hot 95 per cent alcohol and covered with a piece of quantitative filter paper. The separate alcoholic filtrates were saved and later combined with other portions of the extract. Five gram samples of the previously prepared dried alfalfa leaves and soybean meal were weighed, transferred to paper extraction thimbles and covered with pieces of quantitative filtered paper.

The thimbles containing the plant materials were placed in individual continuous Pyrex Soxhlet extractors and extracted for 10 hours with 95 per cent alcohol. The alcoholic extraction was followed with an ether extraction for a period of 8 hours. Following the ether extraction the sample was freed of ether and powdered as finely as possible in a small coffee mill. The powdered material was then carefully collected and replaced in the apparatus and extracted further for a period of 12 to 14 hours with 95 per cent alcohol. The several alcohol and ether extracts including the orginal alcoholic preserving filtrate were then combined. In the case of the ether extract most of the solvent was removed by evaporation and the residue transferred to the alcoholic portions with aid of hot alcohol. Two ml. of a hydroquione solution in ether containing 0.2 mg. hydroquinone was added, and the combined extracts were then evaporated on a steam bath, without overheating, until all the liquid was driven off. The residue was finally dried in a vacuum oven for 3-4 hours at a temperature of 50° C.

The drying of the lipide residue was performed to facilitate a subsequent extraction with ether of the dry

residue. This extraction was carried out by adding successive quantities of anhydrous ether to the residue, heating just to boiling on an electric hot-plate with stirring, and quickly filtering the separate portions of ether through an ashless filter-paper into a weighed 150 cc. beaker. The filtered extract was evaporated to dryness on the steam bath and the residue dried at 100° C. as in the "Official Method".

Moisture determinations were made on the original plant materials as in the "Official Method".

III. Kumagawa-Suto Method (9)

The published procedure was modified slightly in regard to the initial extraction. The original procedure called for either direct saponification of material or extraction with alcohol. The procedure used in this work consisted of extractions with alcohol, ether, and again with alcohol as previously described in Koch's Method (9).

The plant materials used in this experiment were the previously described alfalfa leaves and soybean meal. Five gram samples of the soybean meal and dried alfalfa leaves were weighed and placed in 33 x 94 Schleicher and Schull fat-free paper thimbles. In the case of the fresh material, the previously prepared alfalfa leaves were transferred to the extraction thimbles in the usual manner (9).

The samples were extracted for 10 hours with 95 per cent alcohol. The alcohol extraction was followed with an ether extraction for a period of 8 hours. Following the ether extraction the sample was freed of ether and powdered

as finely as possible in a small coffee mill. The powdered material was then carefully collected and replaced in the apparatus and extracted further for a period of 12 to 14 hours with 95 per cent alcohol. The several alcohol and ether extracts, including the original alcoholic preserving fiftrate in case of fresh material, were combined. The combined extracts were evaporated to a volume of about 100 cc. and 7 to 8 cc. of a strong sodium hydroxide solution (sp. gr. 1.5) was added to the alcoholic extract. The alcohol was allowed to evaporate on a steam bath, the saponification continuing meanwhile. When the alcohol had been removed, the residue was taken up with a little warm water and quantitatively transferred to a separatory funnel. To the mixture in the separatory funnel, 30 cc. of a 20 per cent hydrochloric acid solution (sp. gr. 1.1) was slowly added, with cooling. When thoroughly cool, the acid liquid was vigourously shaken with successive portions of ether. For the first shaking, 70 to 100 cc. of ether was used, while the second and third required only 5 to 10 cc. The aqueous layer was drawn off each time and the ether layers collected in a beaker. During the foregoing shaking operations, a precipitate formed in the funnel at the zone of partition of the two layers. This precipitate was dissolved in 5 cc. of N. sodium hydroxide solution. The alkaline solution was shaken with 30 to 50 cc. of ether. Then the first aqueous acid liquid was added and the whole vigorously shaken. The separated ether layer was added to the ether portion and the united extracts evaporated. The

dry residue was then redissolved in absolute ether. The ethereal solution was filtered and evaporated to dryness. The residue was dried at 50° C. for 4 hours in a vacuum oven at 50° C. To the residue while still warm 30 to 40 cc. of petroleum ether was added and gently rotated. The beaker was covered with a watch glass and set aside for 30 minutes to permit the settling of resinous matter. The fatty acid solution was filtered through asbestos and well washed with petroleum ether. The filtrate and washings were collected in a weighed 100 cc. beaker and evaporated. The residue was dried to constant weight at 50° C. in a vacuum oven. This weight represented crude fatty acids.

The results obtained by these three methods on soybean meal and alfalfa leaves are shown in Tables I and II respectively.

Table I

Total Lipides In Soybean Meal

By Standard Procedure
(Calculated on Dry Weight Basis)

A. O. A. C. (% Moisture = 1.22) (% Lipides)	Koch (Ave. % Moisture = 6.92) (% Lipides)	Kumagawa - Suto Method (Ave. % Moisture = 6.92)	
		% Crude Fatty Acid	% Fat (Calc.) (Fatty acid x 1.045)
19.31 19.38 19.24 19.31 19.32 19.28	22.01 22.05 22.25 22.15 22.09 22.11	19.76 19.91 19.78 19.73 19.71	20.65 20.81 20.67 20.62 20.60
19.31	22.11		20.67

Table II

Total Lipides In Alfalfa Leaves

By Standard Procedure
(Calculated on Dry Weight Basis)

Fresh Leaves (% Moisture - 67.02 %)			Dried Leaves (Air Dried at 60°) (% Moisture - 4.27%)			
Koch (% Lipid)	Kumagawa-Suto		A.O.A.C.* (% Lipid)	Koch (% Lipid)	Kumagawa-Suto	
	% crude fatty acids	% fat(calc.) (fatty acids x 1.046)			% crude fatty acids	% fat(calc. fatty acid x 1.046)
9.35	4.65	4.86	2.62	7.42	3.32	3.47
9.07	4.43	4.63	2.75	7.86	3.34	3.49
8.92	4.25	4.45	2.72	7.55	3.32	3.47
	4.17	4.36	2.69		3.35	3.50
			2.67		3.28	3.43
			2.64			
Ave. 9.11		4.58	2.68	7.61		3.47

*A.O.A.C. Samples were dried at 60° in vacuum oven (% Moisture = 2.35%)

Modifications of Standard Methods of Lipide Analysis

The previously described plant materials were used for the experimental work in the following modifications. Five gram samples of the dried alfalfa leaves and soybean meal were weighed and transferred to Pyrex Soxhlet extractors in the usual manner. The previously prepared fresh alfalfa leaves were likewise transferred to Soxhlet extractors.

I. Substitution of acetone for alcohol in initial extraction.

The modified Koch procedure (9) was followed with the exception of the fact that acetone was substituted for alcohol in the first Soxhlet extraction.

The results obtained on alfalfa leaves and soybean meal by this modified procedure are shown in Tables III and IV respectively.

TABLE III

Comparison of Acetone and Alcohol In

Initial Extraction (Alfalfa Leaves)
(Calculated on Dry Weight Basis)

Fresh Leaves (% Moisture = 67.02%)		Dried Leaves (Air Dried at 60°) (% Moisture = 4.27%)	
Acetone-Ether-Alochol Final Anhydrous Ether Purification (% Lipides)	Comparison with Koch (Ave.=9.11%)	Acetone-Ether-Alcohol Final Anhydrous Ether Purification (% Lipides)	Comparison with Koch (Ave.=7.61%)
8.35	- .76	5.65	- 1.96
7.87	- 1.24	5.75	- 1.86
8.27	- .84	5.84	- 1.77
Ave. 8.16	- .95	5.75	- 1.86

TABLE IV

Comparison of Acetone and Alcohol In

Initial Extraction Soybean Meal - 6.92% Moisture
(Lipide Calculations on Dry Weight Basis)

Acetone-Ether-Alcohol	Comparison with Koch (Ave.=22.11%)	Acetone-Ether	Comparison with Koch (Ave.=22.11%)
22.53	+ .42	19.62	- 2.49
22.01	- .10	19.63	- 2.48
22.63	+ .53	19.29	- 2.82
22.19	+ .08		
22.07	- .02		
22.18	+ .07		
22.15	+ .04		
Ave. 22.25		19.51	

II. Procedure for the determination of the amount of fat extracted by various individual solvents.

The plant materials were extracted in Soxhlet extractors using the same combinations of solvents as formerly, namely, alcohol-ether-alcohol and acetone-ether-alcohol. In this experiment the individual extracts were not combined, but instead the fat content was determined on them individually using the Kumagawa-Suto saponification method (9).

The results obtained on alfalfa leaves are shown in Table V.

TABLE V

Amount of Fat Extracted by Various Solvents

Determined as Fatty Acids
(Dried Alfalfa Leaves)

(Fat Calculated on Dry Weight Basis)
(Fatty Acids x 1.046 = Fat)

Kumagawa-Suto Method											
Alcohol-Ether-Alcohol		Alcohol-Ether		Final Alcohol		Acetone-Ether-Alcohol		Acetone-Ether		Final Alcohol	
% crude fatty acids	% fat (calc.)	% crude fatty acids	% fat (calc.)	% crude fatty acids	% fat (calc)	% crude fatty acids	% fat (calc)	% crude fatty acids	% fat (calc)	% crude fatty acids	% fat (calc)
3.32	3.51	3.19	3.34	.13	.14	3.09	3.23	2.81	2.94	.28	.29
3.34	3.50	3.16	3.31	.18	.19	3.10	3.24	2.77	2.90	.33	.34
3.32	3.47					3.06	3.20				
3.35	3.50					3.06	3.20				
3.28	3.43					3.02	3.16				
Ave. =	3.48		3.33		.17		3.21		2.92		.32

III. Procedure for the comparison of anhydrous ethyl ether and U. S. P. ethyl ether for lipide extraction.

The standard A. O. A. C. Method (28) was used in the one case while wet ether (U. S. P.) was used in the other case. In the latter case the samples were not vacuum dried before extraction.

The results are shown on soybean meal in Table VI.

TABLE VI

Comparison of Anhydrous Ethyl Ether and U. S. P. Ether for Lipide Extraction

Soybean Meal
(Lipides Calculated on Dry Weight Basis)

Anhydrous Ethyl Ether (% Moisture = 1.22%)	U. S. P. Ether (% Moisture = 7.18%)
19.31	19.36
19.38	19.73
19.24	19.46
19.31	19.86
19.32	19.89
19.28	19.79
Ave. 19.31	19.68

IV. Procedures for comparison of methods used for final extraction.

A. Comparison of purification with anhydrous ethyl ether with that of saponification.

All samples received the same initial extraction, namely, acetone, ether, followed by final alcohol. The final alcoholic extraction was shorter than usual (approximately 4 hours). One-

half of the samples were dried and purified by redissolving in anhydrous ethyl ether as was done in the Modified Koch Method (9). The other half of the samples were saponified and the fatty acids collected as in the standard Kumagawa-Suto Method (9).

The results obtained on soybean meal are shown in Table VII.

TABLE VII

Comparison of Methods Used for Final Extraction of Lipides

Soybean Meal - 6.92% Moisture
(% Lipide and Fat Calculated on Dry Weight Basis)

Initial Extraction: Acetone-Ether-Alcohol

Estimation by Purification with Anhydrous Ethyl Ether	Estimation by Saponification (Kumagawa-Suto Method)	
	% crude fatty acids	% fat (calc.) (fatty acids x 1.045)
21.34 21.38 21.02	19.24 19.41 19.16	20.11 20.28 20.02
21.25		20.14

B. Comparison of anhydrous petroleum ether and anhydrous ethyl ether for final extraction.

All samples received the Modified Koch (9) initial extraction, namely, alcohol-ether-alcohol. The solvents containing the extracted lipides were combined, evaporated, and made up to a volume of 250 cc. Four 50 cc. aliquot samples were pipetted into individual beakers. The ether was evaporated off, and the lipide residue was dried in a vacuum oven at 50°C.

Two of the aliquot samples were subjected to several 15 to 20 cc. successive hot anhydrous ethyl ether extractions. All of the extractions from each sample were filtered through a fat-free filter paper into a tared beaker. The ether was evaporated off on a steam bath. The lipide material was dried at 100° C. for 30 minutes, cooled in a desiccator and weighed. The drying was continued for 15-minute intervals until the weight remained constant.

The other two samples received the same general treatment except that anhydrous petroleum ether was used in making the final extraction in place of ethyl ether.

The results obtained on soybean meal are shown in Table VIII.

C. Comparison of anydrous ethyl ether purification with Rohrig tube purification.

All of the samples received the Modified Koch (9) initial extraction, namely, alcohol, ether, followed by alcohol. The solvents containing the extracted lipides were combined and evaporated to a volume less than 100 cc., and then made up with ether volumetrically to 100 cc.

Two 20 cc. aliquots were pipetted into individual beakers, the ether evaporated off on a steam bath, and the lipide residues dried in a vacuum oven at 50° C. These samples were extracted with several 15 to 30 cc. successive portions of hot anhydrous ethyl ether. All of the extractions from each sample were filtered through a fat-free filter paper into a tared beaker.

The ether was evaporated off on a steam bath. The lipide material was dried at 100° C. for 30 minutes, cooled in a desiccator and weighed. The drying was continued for 15-minute intervals until the weight remained constant.

Two other aliquot samples were taken from the original sample and transferred to Rohrig tubes. The standard Rohrig tube procedure (29) as given below was applied to these samples.

The results of this method on soybean meal are shown in Table VIII.

D. Rohrig tube procedure (29).

The Modified Koch initial extraction, namely, alcohol, ether, alcohol, was used to extract all the lipide material from the plant tissue. The combined extracts were evaporated on steam bath to a small volume and transferred quantitatively to a Rohrig tube. Two cc. of concentrated ammonium hydroxide solution was added to the tube and mixed by inversion. The tubes were allowed to stand until the upper layer became perfectly clear (15 min.). Most of the upper layer was drained off by opening the stop-cock and tipping the tube. The ether-fat solution was run through a small filter paper into a tared beaker. The extraction was repeated twice using only 15 cc. of each ether. The ether was evaporated off of the combined extracts. The lipide material was dried at 100° C. for 30 minutes, cooled in a desiccator, and weighed. The drying was continued for 15-minute intervals until the weight remained constant.

The results obtained by this method on alfalfa leaves are shown in Table IX.

TABLE VIII

Comparison of Methods Used for Final Extraction

Soybean Meal - 7.14% Moisture

Initial Extraction: Alcohol-Ether-Alcohol
(Values Obtained by Use of Aliquot Samples)

% Lipides Calculated on Dry Weight Basis

Sample No.	Anhydrous Ethyl Ether	Anhydrous Petroleum Ether	Rohrig Tube
1	21.27 21.97	22.58 22.29	
2	21.12 20.95	21.19 21.00	
3	22.12 23.06	24.00 23.61	
4	21.99 22.01		22.95 23.10

TABLE IX

Amount of Lipides Extracted

By Use of Rohrig Tube

Alfalfa Leaves
(Calculated on Dry Weight Basis)

FRESH LEAVES (% Moisture = 67.02%)		DRIED LEAVES Room Dried at 60° C. (% Moisture = 4.27%)	
Rohrig Tube (% Lipides)	Comparison with Koch (Ave.=9.11%)	Rohrig Tube (% Lipides)	Comparison with Koch (Ave.=7.61%)
8.26	- .85	7.29	- .32
8.23	- .88	7.35	- .26
8.35	- .76	7.20	- .41
8.19	- .92	7.25	- .36
8.25	- .86		
Ave. 8.26	- .85	7.27	- .34

Procedures for the Methods of Determining Unsaponifiable and Fatty Acids

I. Kumagawa-Suto Method (9).

The Kumagawa-Suto Method as given in connection with total lipide determination can be continued to give a separation of the true fatty acids from the unsaponifiable. The procedure for the determination as far as the "crude fatty acid" stage has already been described. The crude fatty acids obtained, the results of which are shown in Tables I and II, included the unsaponifiable plus the true fatty acids. The following procedure gives the operations involved to separate out the unsaponifiable.

The soybean meal used for the experimental work was the same used in previous experiments, however, the alfalfa leaves were not the same. A collection of leafy alfalfa stems was made from alfalfa growing in a greenhouse. The fresh leaves were prepared in the same manner as previously described in connection with the total lipide determinations.

Fifteen gram samples of the fresh alfalfa leaves and 2.5 gram samples of soybean meal were weighed and carried through the Kumagawa-Suto procedure (9) to the "crude fatty acid" stage.

The crude fatty acids were dissolved in petroleum ether and introduced into a separatory funnel. To dissolve completely the residue and to transfer the solution quantitatively from the beaker to funnel required 50 to 70 cc. of petroleum ether. Thirty to 40 cc. of N/5 nearly absolute alcoholic potash were

added to the solution in the funnel which was then well shaken once. A clear solution resulted. A quantity of water was added to this equal to the volume of alcoholic potash used, whereby a separation occurred with the 50 per cent alcoholic liquid as the bottom layer and the petroleum ether solution as the top layer. The unsaponifiable substances remained in the top layer, while the soaps went to the dilute alcoholic layer. After separation of the solution, the dilute alcoholic layer was again extracted with 30 to 50 cc. of new petroleum ether. After evaporation of the petroleum ether from the combined extractions the residue was freed from the small traces of fatty acids. This separation was accomplished by dissolving the residue in a little absolute alcohol, adding 0.5 to 1.0 cc. N/10 nearly absolute alcoholic soda, evaporating on the water bath, drying the residue 15 to 30 minutes at 100° C., and then extracting with hot petroleum ether. The petroleum ether extract was filtered through asbestos into a weighed flask, evaporated and dried to constant weight at 100° C. This weight represented total unsaponifiable matter. It was subtracted from the weight of the total crude fatty acids previously obtained to find the quantity of true fatty acids. The weight of the true fatty acids obtained from alfalfa multiplied by the factor 1.046 gave the weight of the neutral glycerides. The factor 1.045 was used to convert the true fatty acids of soybean meal to the neutral glycerides.

Two modifications of the standard Kumagawa-Suto Method were tried in order to shorten the procedure. It was desired

to know if the final steps taken to remove the last traces of fatty acids from the petroleum ether solution were necessary.

In the first modification the final steps to remove the last traces of fatty acids from the unsaponifiable were not made. The first petroleum ether extraction was evaporated to dryness and weighed. In the second modification the steps to remove the last traces of fatty acids were changed. The first petroleum ether extract was washed four times with small portions of water. The ether extract was transferred to a tared beaker, the ether evaporated off, and the residue dried at 100° C. until constant weights were obtained.

The results obtained by the standard Kumagawa-Suto Method, and modifications of this method on alfalfa leaves and soybean meal are shown in Tables X and XI respectively.

The efficiency of the method in extracting the fatty acids was determined by adding 0.5 grams of trimyristin to a 2.5 gram sample of standard soybean meal. The sample was carried through the entire Kumagawa-Suto procedure to determine the amount of fatty acids and unsaponifiable matter. The amount of the trimyristin recovered as myristic acid was obtained by subtracting from the total weight of fatty acids recovered, the average weight of the fatty acids obtained from a 2.5 gram sample of soybean meal to which nothing was added.

The trimyristin added was prepared from nutmegs. The fat was recrystallized twice from 95 per cent ethyl alcohol,

and had a melting point of 54°-55° C.

The results in regard to the recovery of the trimyristin by the standard Kumagawa-Suto Method are shown in Table XII.

II. Horwit-Cowgill-Mendel Method (16).

The materials used for the experimental work were soybean meal and fresh alfalfa leaves, described previously under the Kumagawa-Suto Method (9).

The Horwit-Cowgill-Mendel Method was modified in regard to the initial extraction. The method described accomplished the initial extraction by boiling the plant material in an alcohol, ether (4:1) mixture for 10 minutes and then extracting the residue for 15 minutues with three successive portions of the alcohol, ether mixture.

In this experiment Koch initial extraction (9) was used. The alcohol, ether, alcohol extracts were combined and evaporated on a steam bath to a volume of about 100 cc. Twenty cc. of approximately 10 N potassium hydroxide solution was added and the mixture was saponified on a steam bath until the volume was reduced to about 30 cc. At this point 30 cc. of water were added and the saponification continued until the total time elapsed on the steam bath was one hour.

The total saponified mixture was acidified with hydrochloric acid. The acidified mixture was transferred to a separatory funnel and extracted with petroleum ether; twice with 50 cc. and twice with 30 cc. This extract was filtered and evaporated to a volume of 50 cc.

The petroleum ether extract was extracted with 50 cc. of N/10 potassium hydroxide solution in 50 per cent alcohol. This in turn was twice extracted with 25 cc. of alcoholic potassium hydroxide solution. Most of the yellow pigments were left behind in the petroleum ether fraction.

The combined potassium hydroxide fractions were acidified with hydrochloric acid using a trace of brom cresol purple as indicator. (The use of phenolphthalein as called for in the original procedure was found unsatisfactory because the fatty acids are not liberated at the end-point of phenolphthalein.) Extractions with 50 cc., 25 cc., and 20 cc. portions of warm petroleum ether were made. The petroleum ether extract of the fatty acids were evaporated to dryness in a tared beaker and dried at 100° C. for 30 minutes, cooled in a desiccator, and weighed. The drying was continued for 15-minute intervals until the weight remained constant.

A modification of the above method was made in order to try to obtain a more complete removal of the fatty acids and unsaponifiable matter from the acid saponified material during the petroleum ether extraction process. In the initial petroleum ether extraction following the acidification of the saponified material, tne extraction was made from a warm (60° C.) solution instead of from cool solution as was indicated in the described procedure.

The final petroleum ether extraction was also modified by extracting from warm alcoholic solution (60° C.) instead of

using warm petroleum ether.

The results are shown on alfalfa leaves and soybean meal in Tables X and XI respectively.

III. Modification for the determination of unsaponifiable and fatty acids.

Due to the difficulty or inconvenience in the determination of the fatty acids and unsaponifiable by the previously described methods, a modification was thought worthwhile.

The plant material used was the same soybean meal as that used in previous work. Two and one-half gram samples were weighed out and transferred to paper extraction thimbles and subsequently to Pyrex Soxhlet extraction flasks. The samples were extracted with alcohol, ether, and alcohol according to the modified Koch (9) initial extraction. The combined extracts were evaporated to a volume of about 100 cc. To this solution 20 cc. of approximately 10 N potassium hydroxide was added. The mixture was saponified on a steam bath in an open beaker until the volume was reduced to about 40 cc. At this time, 30 cc. of water was added and the saponification was continued until the total saponification period had been approximately one hour.

The unsaponifiable matter was now extracted before acidification. Two alternatives were possible for this operation. The Kerr-Sorber Method (30) or the F. A. C. Method (19). Subsequent to the determination of the unsaponifiable, the fatty acids were determined as described in a following section.

Separation of the unsaponifiable by Kerr-Sorber Method (30).

The saponified material was cooled, transferred to a separatory funnel, 50 cc. of ethyl ether added, and the mixture was mixed thoroughly. The saponification flask was washed with 100 cc. of an approximately 2N potassium hydroxide solution and poured into the separatory funnel in a slow steady stream. The funnel was rotated very gently to secure better contact of the solution but shaking was avoided because at this stage shaking would have produced very stubborn emulsions. The liquids were allowed to separate completely and the soap solution was slowly drawn off. The volume of the ether was kept at about 150 cc. by replacing that dissolved by the wash solutions. The ether solution was further treated with two successive 100 cc. portions of dilute potassium hydroxide solution in the manner described previously. Thirty cc. of water were added to the ether and the liquid layers were rapidly rotated. When the layers were completely separated, the water was drawn off and the treatment repeated until the washings were free from alkali, as shown by testing with phenolphthalein. The ether solution was transferred quantitatively through a pledget of cotton in a stem of a funnel to a weighed beaker. The ether was evaporated off on a steam bath and the dry residue was dried at 100° C. until no further loss in weight occurred.

Separation of the unsaponifiable by F. A. C. (Fat Analysis Committee of the Division of Industrial Chemists and Engineers of the American Chemical Society) Method (19).

The saponified material was transferred to a separatory funnel and washed to 40 cc. volume with 95 per cent alcohol. The transfer was completed, first with warm, then with cold water, until the total volume was 80 cc. The flask was rinsed with 50 cc. of petroleum ether and the rinsings were added to the contents of the funnel previously cooled to room temperature. The mixture was shaken as vigorously as possible for a minute and allowed to settle until both layers were clear. The petroleum ether was drawn off as closely as possible and placed in a separatory funnel of 500 cc. capacity. The extraction was repeated six more times, using 50 cc. of petroleum ether for each extraction. The combined extracts were washed in the separatory funnel three times with 25 cc. portions of 10 per cent alcohol by volume, shaking vigorously each time. The petroleum ether extract was transferred to a weighed beaker and the ether evaporated off on a steam bath. The flask with residue was heated at 100° C. in an oven, cooled in a desiccator and weighed. The final residue was tested for solubility in 50 cc. of petroleum at room temperature. The extract was filtered and thereby freed from any insoluble residue. The ether was evaporated off and the unsaponifiable matter was weighed as before until constant weights were obtained.

Modification for the determination of fatty acids.

The combined basic alcoholic extracts from one of the above extractions, depending on the method used for the unsaponifiable determination, were collected in a separatory

funnel and acidified with hydrochloric acid. The solution was cooled, and the free fatty acids were extracted by the use of four portions of ethyl ether; two 60 cc. and two 30 cc. portions. Each time the top ether layer was collected in a 250 cc. beaker while the alcoholic layer was returned to the separatory funnel to be re-extracted. The ether was evaporated off the combined extracts on a steam bath. The fatty acid residue was dried in a vacuum oven at 50° C. for four hours. The fatty acids were redissolved in four 15-30 cc. portions of boiling anhydrous petroleum ether, and filtered through a fat-free filter paper into a tared beaker. The ether was evaporated off on a steam bath. The beaker containing the fatty acids was dried at 100° C. for 30 minutes, cooled in a desiccator, and weighed. The drying was continued for 15-minute intervals until the weight remained constant.

The results obtained by the modified methods are shown in Table XI.

TABLE X

Determination of Unsaponifiable and Fatty Acids

Fresh Alfalfa Leaves*
(% Moisture - 68.94%)
(Fat Calculated on Dry Weight Basis)

Horwit-Cowgill-Mendel Method		Kumagawa-Suto Method	
% Fatty Acids	% Unsaponifiable	% Fatty Acids	% Unsaponifiable
1.13	.45	3.82	1.78
1.37	.39	3.87	1.76
Ave. 1.25	.42	3.85	1.77

*The alfalfa leaves used in this experiment were of different origin from the alfalfa material used in the other experiments.

TABLE XI

Determination of Unsaponifiable and Fatty Acids

Soybean Meal - 6.75% Moisture

(Calculated on Dry Weight Basis)

Kumagawa-Suto Method				Horwit-Cowgill-Mendel			Modified Methods		
Unsaponifiable			%	%	% Fatty Acids		% Unsaponifiable		%
Standard Method	Modification No.1	Modification No.2	Fatty Acids (Calc.)	Unsaponifiable	Standard Procedure	Modification	Kerr-Sorber	F.A.C.	Fatty Acids
.36			19.62	.26	14.27		2.06		17.52
.40			19.71	.22	15.44		2.15		17.41
.35			19.70	.26		16.92		.56	19.40
	2.62		17.11	.26		18.29		.54	19.41
	2.67		17.04					.57	19.46
		1.81	17.51						
		1.75	17.86						
.37	2.65	1.78		.25	14.65	17.61	2.11	.56	

TABLE XII

Amount of Myristic Acid Recovered from .5 gram Addition of Trimyristin to Soybean Meal Sample

Kumagawa-Suto Method

Weight of Fatty Acid from Sample Containing .5 gram Trimyristin	Weight of Trimyristin Recovered (Soybeans contained .4121 grams fatty acid)	% Recovered (Theoretical Amount Myristic Acid = .4735 grams)
.8870	.4744	100.2
.8748	.4622	97.6
Ave. .8809	.4683	98.9

DISCUSSION OF RESULTS

From observation of tables I and II, the data clearly show that the term lipide is merely a relative term since in the case of dry alfalfa leaves the lipide value varies from 2.68 per cent to 7.61 per cent. The range in lipide values for the soybean meal by different standard methods is from 19.31 per cent to 22.11 per cent. The lowest value in each case is given by the Official Method of the Association of Agricultural Chemists, while the highest value is given by the Modified Koch Method. The Kumagawa-Suto Method, which involves the determination of fat by saponification, gives an average value of 3.47 per cent for alfalfa leaves and 20.67 per cent for soybean meal. The weight of the fatty acids recovered in each case is greater than the weight of the ether extract obtained by the "Official Method"; a fact definitely proving that all of the fatty material in the plant tissue is not extracted by one ether extraction.

The high value obtained by the Modified Koch Method apparently is due in part to the presence of non-fatty substances, such as various pigments. The pigments probably constitute a much larger per cent of the ether extract in the case of the alfalfa leaves than in soybean meal, therefore, the greater difference between the values obtained by the Kumagawa-Suto Method and Modified Koch Method in the case of alfalfa leaves is justified.

The difficulty encountered in obtaining constant values by the Modified Koch Method in the analysis of alfalfa leaves

is due to the fact that pigments are redissolved by the final anhydrous ethyl ether purification. The degree to which the chlorophyll pigments are broken down during the concentration and drying processes will affect the amount of lipide residue finally obtained. Also, much difficulty is met with in the filtration process. If the crude lipide extracts are properly dried, more colloidal material will be suspended in the anhydrous ether extract. This colloidal matter clogs the filter paper and makes filtration almost an impossibility. Asbestos filters are found to be no better than filter paper.

The data in table II also shows the effect of drying of plant materials on the amount of lipide extracted. The fat content of the dried alfalfa leaves determined by the Kumagawa-Suto Method is only 75.8 per cent of the value obtained on fresh leaves. The Modified Koch Method on the dried leaves gives 83.3 per cent of that obtained from the fresh leaves. The results show that there is a decided drop in the amount of fatty acids and total lipides due to the drying process. The decrease may be due to decomposition of the fatty substances or to their conversion to anhydrides or polymers which would render them less soluble and consequently put them in a form more difficult to extract from the tissues. The latter explanation seems more logical.

The comparison of the amount of lipides extracted by acetone and alcohol in the initial Soxhlet extraction gives with the former (acetone-ether-alcohol) an average of only 89.5 per cent of that extracted by the Koch solvents

(alcohol-ether-alcohol). The reduction in the amount of lipides extracted by the substitution of acetone for alcohol in the first extraction is much more pronounced in the case of dry alfalfa leaves; the former gives only 74.2 per cent as much as the latter. The larger amount extracted in the case of fresh leaves can be explained by the fact that they received a short initial alcoholic extraction during the boiling of the leaves to stop enzymatic activity. The phospholipides and closely allied substances are insoluble in acetone, hence, they are not liberated by an initial extraction by acetone; since much of the fatty material in the tissues is probably bound as phospholipides, the subsequent extraction by ether is likely to be very inefficient in the removal of the fatty compounds.

The substitution of acetone for alcohol in the first extraction is found to increase very slightly the amount of lipide extracted from soybean meal. The results shown in table IV, however, show that the omission of alcohol entirely from the extraction will allow a large amount of lipides to remain in the tissues unextracted. The alcoholic extraction following the extractions made by acetone and ether is found to remove on an average an additional 2.7 per cent lipides from soybean meal.

From the data on hand it appears that the substitution of acetone for alcohol in the initial extraction process on plant materials in general is not advantageous. The slight increase in material extracted in the case of soybean meal

is probably due to the extraction of some non-lipide substance, because the samples which received an acetone extraction are found to always darken upon heating to dryness.

The amount of fat extracted from dry alfalfa leaves by the separate individual solvents shows that the amount of fat removed by the final alcohol extraction, following a previous acetone and ether extraction, is almost twice that obtained when the final alcohol extraction is preceded by alcohol and ether extractions. The data gives support to the belief that a final alcoholic extraction following the ether extraction is necessary to remove all of the lipide material.

According to the data in table V, we are forced to conclude that a large proportion of the lipides hydrolysable to fatty acids are removed by either of the combinations of solvents, namely, alcohol-ether-alcohol, or acetone-ether-alcohol; the latter extracts on the average 92.2 per cent as much of the lipides as the former. The decidedly lower values of total lipides obtained by the substitution of acetone for alcohol in the extraction process (table III) is, therefore, probably due to the smaller amount of non-lipide material extracted rather than to a decided failure of removal of the material hydrolysable to fatty acids.

The necessity for maintaining a very anhydrous condition in the extraction of lipides by the Official Method is shown in table VI. The results shown in this table agree with the work of Chibnall and Channon (7). In the case of

soybean meal an additional 0.37 per cent of material is extracted by use of U. S. P. (wet) ether over that of anhydrous ether.

The data in table VII shows the results of a comparison made of several modifications for the final extraction. The first comparison made between purification by redissolving the dried extracted lipide residue with anhydrous ether, on the one hand, and saponifying and determining fat on the basis of fatty acids, on the other hand, shows that the former method gives an average of 1.1 per cent more lipide. The total lipide values are approximately 1 per cent lower than normal due to a shorter final alcoholic extraction period.

Table VIII shows the results obtained by the use of aliquot samples (attempted in order to insure uniformity of samples) in determining the relative solvent powers of petroleum ether and ethyl ether. The petroleum ether is found to redissolve a slightly larger percentage of dried lipide residue than ethyl ether. This finding is contrary to the general belief that ethyl ether has a wider solubility range than petroleum ether. A crystalline substance isolated from the soybean meal is found to be more soluble in petroleum ether than in ethyl ether. Otherwise the solvent action of these two ethers on the dried lipide extract of soybean meal is found to be so near the same that no appreciable advantage can be gained by the substitution of petroleum ether for ethyl ether in the purification process.

The data obtained by the use of the Rohrig tube, shown in table VIII for soybean meal and in table IX for alfalfa leaves, indicates a slightly larger per cent of lipides are extracted over the Modified Koch Method in the case of soybean meal and a slightly lower percent of lipides in the case of alfalfa leaves. The lower value in the case of alfalfa leaves is probably due to the removal of a smaller amount of the pigments by the ether extraction due to their greater solubility in the alcoholic layer. This method appears to be more desirable than the Modified Koch Method. The following advantages over the Modified Koch are observed: first, probably a smaller amount of non-lipide material is extracted from the alfalfa leaves; second, the drying of the lipide extract is not necessary, thereby eliminating errors due to the process of drying; and third, the method does not require as much time.

In the separation of the unsaponifiable and the fatty acids as shown in tables X and XI, the Horwit-Cowgill-Mendel Method is found to give very inconsistent values and also lower than the values obtained by the other methods. The value for the per cent fatty acids in alfalfa is less than one-half the value obtained by the Kumagawa-Suto Method. The fatty acid content of soybean meal as determined by the Horwit-Cowgill-Mendel Method is increased greatly by extracting the petroleum ether soluble substances from a warm solution. It is also observed that the last extraction is still yellow in color after the specified number of petroleum

ether extractions are made from the cool, acidified, saponified material. This indicates that the extraction of unsaponifiable matter is incomplete. The analytical results coincide with this observation because the unsaponifiable fraction is found to be lower than that obtained by the "Official Methods" (Kerr-Sorber and F. A. C.).

The F. A. C. Method for the determination of the unsaponifiable is found to be more easily carried out than the Kerr-Sorber Method. In the Kerr-Sorber Method, emulsions are formed which are found almost impossible to break completely. It is also very difficult to remove the last traces of fatty acids from the unsaponifiable fraction by washing with water. No difficulties are encountered in the execution of the F. A. C. procedure in the determination of unsaponifiable matter. The last traces of fatty acids are readily removed by the dilute alcohol washing called for in the procedure.

In the determination of unsaponifiable matter, the Kumagawa-Suto Method gives results lower than the F. A. C. Method; this is thought to be due to the destruction of the carotenoid pigments by the final purification, a treatment which necessitates the heating of the unsaponifiable fraction with alkali at 100° C. for 30 minutes. In the event that the last purification process is not used, the unsaponifiable value is quite high due probably to the insufficient removal of all the fatty acids. This observation is shown in table XI, in which modification number one

receives no washings, while in the modification number two, the petroleum ether is washed with four successive portions of water. The washing of the ether extract probably removes all of the fatty acids present as sodium salts, however, the free fatty acids formed by hydrolysis during the washing process are not removed.

The true fatty acid content in the case of the Kumagawa-Suto Method is not determined experimentally but by the subtraction of the unsaponifiable from the crude fatty acids, therefore, the method used for the unsaponifiable determination affects greatly the true fatty acid value. It can be observed from the data in table XI that the sum of the unsaponifiable and the true fatty acids, in the case of the Kumagawa-Suto Method, is approximately the same regardless of the method used for the unsaponifiable determination. In spite of this indirect estimation of fatty acids, the method appears to be reliable as is shown in table XII. In this case an average of 98.9 per cent of the calculated amount of myristic acid is recovered from 0.5 gram additions of trimyristin to the soybean samples.

In the case of the saponification methods, the fat content of the plant tissues is calculated by multiplying the determined weight of fatty acids by the factor 1.046. The factor is obtained by assuming that on saponification an equal quantity of each of the three fatty acids, oleic, stearic and palmitic acids, is obtained from the corresponding triglycerides. The factor used may vary from this normal

value if the actual composition of the fatty material is known. The factor 1.045 is used to convert the true fatty acids obtained from the soybean meal to the neutral glycerides. This factor was calculated on the basis of the composition of the soybean oil given by Baughman, Brauns, and Jamieson (31).

From a study of the results obtained in connection with the work on this problem, the following facts are observed: first, the Modified Koch initial extraction insures the most complete removal of lipides from plant tissues; second, the Kumagawa-Suto procedure for the separation of the fatty acids gives best results; and third, the F. A. C. Method gives what is considered the most ideal unsaponifiable value. With these facts in mind the following method is proposed for the determination of the lipide content in plant tissues: The Modified Koch (9) initial extraction is used. The alcoholic and ether extracts are evaporated to a volume of about 100 cc. To this solution 20 cc. of approximately 10 N potassium hydroxide solution are added. The mixture is saponified on a steam bath in an open beaker until the total volume has been reduced to about 40 cc. At this time, 30 cc. of water are added and the saponification continued until the total saponification period has been approximately one hour.

Transfer the saponified material to an extraction cylinder or separatory funnel and wash to 40 cc. volume

with 95 per cent alcohol. Complete the transfer, first with warm, then with cold water, until the total volume is 80 cc. Rinse the flask with 50 cc. of petroleum ether and add the rinsings to the contents of the funnel previously cooled to room temperature. Shake as vigorously as possible for one minute and allow to settle until both layers are clear. Draw off the petroleum ether layer as closely as possible and place in a separatory funnel of 500 cc. capacity. Repeat the extraction at least six more times, using 50 cc. of petroleum ether for each extraction. Wash the combined extracts in the separator funnel three times with 25 cc. portions of 10 per cent alcohol by volume, shaking vigorously each time. Transfer the petroleum ether extract to a weighed beaker and evaporate off the ether on a steam bath. Heat the beaker with residue for 30-minute intervals at a temperature of 100° C. until a constant weight is obtained. Test the final residue for solubility in 50 cc. of petroleum ether at room temperature. Filter and wash free from any insoluble residue. Dry in the same manner as before and weigh. This weight represents total unsaponifiable matter.

The combined basic alcoholic extracts from the above extractions are collected in a separatory funnel and acidified with hydrochloric acid. The solution is cooled, and the free fatty acids are extracted by the use of four portions of ethyl ether; two 60 cc. and two 30 cc. portions. Each time the top ether layer is collected in a 250 cc. beaker while the alcoholic layer is returned to the separatory

funnel to be reextracted. The ether is evaporated off the combined extracts on a steam bath. The fatty acid residue is dried in a vacuum oven at 50° C. for four hours. The fatty acids are redissolved in four 15-30 cc. portions of boiling anhydrous petroleum ether, and filtered through a fat-free filter paper into a tared beaker. The ether is evaporated off on a steam bath. The beaker containing the fatty acids is dried at 100° C. for 30 minutes, cooled in a desiccator, and weighed. The drying is continued for 15-minute intervals until the weight remains constant. This weight represents true fatty acids. The weight of true fatty acids multiplied by the factor 1.046 gives the weight of neutral glycerides.

The method proposed is thought to have the following advantages: first, it gives the most complete removal of lipides from the plant tissues; second, both the unsaponifiable and the fatty acids are experimentally determined, which does not permit errors in the unsaponifiable determination to reflect upon the fatty acid value as is the case in the Kumagawa-Suto method; third, the method is the shortest, simplest method which gives responsible results; and fourth, the most accurate value for the unsaponifiable is given by this method.

It is planned to give this proposed method further investigation in regard to its suitability for the determination of lipides in other plant materials.

CONCLUSIONS

1. The Modified Koch procedure for the initial extraction was found to be the most efficient in the removal of the lipide material.
2. The values obtained by the Modified Koch Method include a large per cent of non-lipide compounds, especially when chlorophyll pigments are present.
3. The substitution of acetone for alcohol in the initial extraction was found to be just as effective a solvent for the lipides in soybean meal but not as good for alfalfa leaves.
4. The final alcohol extraction following a previous alcohol-ether extraction was found necessary to completely remove the lipides from dried alfalfa leaves.
5. The final alcohol extraction following a previous acetone-ether extraction removed an additional fourteen per cent.
6. The use of the Rohrig tube was found more rapid, and to include less non-lipide matter in heavily pigmented materials than the Modified Koch Method.
7. The "Official Method" for lipide determination was always found to give the lowest lipide values of any of the methods used.
8. The percentage of fatty acids obtained by the saponification method exceeded the total ether extract by the "Official Method" (A. O. A. C.).

9. A small but definite amount of additional non-lipide material was present in the ether solution if moisture was introduced.
10. An appreciable loss in solubility of the lipides was observed when the plant material was dried before extraction.
11. Ethyl ether and petroleum ether redissolve approximately the same amount of the dried extracted lipide residue from soybean meal.
12. Although the Kumagawa-Suto Method involved a very long procedure, the method gives good results.
13. The F. A. C. Method was found to be the simpliest and most accurate procedure for the determination of unsaponifiable matter.
14. Modifications attempted to shorten the Kumagawa-Suto Method were found unsatisfactory.
15. The use of the F. A. C. Method for unsaponifiable determination, following an extraction such as outlined in the Modified Koch Method and a determination of fatty acids as outlined in the Kumagawa-Suto Method, gave the best results of any of the methods tried.

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