SOME FACTORS AFFECTING THE DETERMINATION OF FATTY ACIDS AND UNSAPONIFIABLE MATTER IN PLANT MATERIALS

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INTRODUCTION

The research connected with this problem was originally undertaken to evaluate a procedure for lipide analysis proposed by C. E. Hierserman (1). As the work progressed the method was recognized as superior in some respects to the existing procedures for lipide determination. The work was then adopted as a thesis problem and an attempt was made to eliminate certain undesirable points that were encountered, and to determine what effects some variable factors had on the determination of the unsaponifiable and fatty acid fractions.

As the terminology of ether soluble, fat-like substances is quite confusing, one of the better classifications must be chosen and followed for sake of clearness and uniformity. The term "lipide" as used in this paper shall include all substances soluble in such solvents as ethyl ether, chloroform, petroleum ether; and insoluble in water and which are either ester-like combinations with fatty acids or capable of forming such combinations (2). The term "fat" shall include only those fatty substances which are glyceryl esters of fatty acids.

HISTORICAL

When plant tissues are extracted with ethyl ether or any of the other similar solvents, the extract contains a wide variety of substances some of which yield fatty acids on hydrolysis. The most of these fatty acid-yielding substances are the glyceryl esters, sterol esters, phosphatides, and cerebrosides. Also appearing in the fat solvent extract are compounds of such varied chemical composition as the wax alcohols, sterols, essential oils, hydrocarbons, resins, lignins, chlorophylls, and carotenoids.

The choice of the solvent used is one of the deciding factors of the crude lipide extract. Unfortunately the solvents such as petroleum ether that extract a minimum of non-lipide material give a poor yield of true lipides while the solvents that do give a high yield of lipides generally extract large amounts of non-lipide material. As no "ideal solvent" for lipide has been found the trend in recent years has been to employ the solvent or combination of solvents that give a maximum yield of crude lipides and then to determine the amount of the desired constituents in the crude extract.

Hence a logical division for an approach in the field of lipide analysis is indicated; namely the extraction of the crude lipide material and secondly the purification and fractionation of the lipide material.

Ethyl ether is probably the most widely used laboratory fat solvent for extraction of lipides. The common procedure

is to run a sixteen hour continuous extraction and report the total yield as "crude lipides" or "crude fat". However, in this crude extract obtained from many plant tissues by the use of ether, the non-lipide substances may amount to more than the total weight of true lipides (3). The purity of the ether employed definitely affects the quality of the crude extract. Chibnall and Channon (4) state that the presence of alcohol and water will allow the extraction of small amounts of inorganic matter, amino compounds, carbohydrates and possibly tannins. Hieserman (1) also reported a small but definite increase in the yield of crude lipides when moisture-containing ether was used. This source of error is eliminated in the official A.O.A.C. (5) method for crude lipide determination by the use of C. P. anhydrous ethyl ether on previously dried samples.

Even the use of C. P. anhydrous ethyl ether has three decided disadvantages. First, that all lipide material is not extracted has been shown by numerous workers (6) (7) (8) (9) (10), second, many non-lipide materials such as pigments, hydrocarbons, essential oils, alkaloids, and resins appear in the ether extract (3) and third, it is assumed but without justification that drying has no effect on the lipides. However, in total crude lipide determination of most plant tissue these factors tend to off-set one another. Hence the values obtained by the relatively simple A.O.A.C. method frequently approximate the values for true fat of the more involved and tedious methods.

Although petroleum ether extracts the smallest amounts of non-lipide material of any of the fat solvents (3), the yields of true lipides are quite low. Its use for total lipide extraction is not recommended.

Carbon tetrachloride, being non-inflammable and because it extracts most lipides with ease, possesses many characteristics of a most desirable solvent. Bryant (11) claims that a two hour extraction with carbon tetrachloride gives similar yields of lipides to those obtained by a sixteen hour ethyl ether extraction. However, Wiley (12) found that the last traces of this solvent are removed with the greatest difficulty and in the presence of even minute traces of moisture it is partly decomposed into hydrochloric acid which has a detrimental effect upon extracted lipides. The observation of Schindelmeiser (13) that many alkaloids are readily soluble in even cold carbon tetrachloride further discredits its use as a solvent for crude lipide determinations.

Kumagawa and Suto (14) state that the efficient removal of lipides in the method advanced by Rosenfeld (15) is not due to the more energetic action of chloroform but to the pre-treatment with alcohol.

Although carbon disulfide is a liquid of high solvent power for fatty materials it has not come into general use for quantitative lipide estimation. This is probably because of its poisonous properties, its unpleasent odor and its liability to decomposition when used for the extraction of certain lipides (3).

Acetone has a wide range of solvent power for many classes of organic compounds including lipide material but its solvent action is too general to permit its use for quantitative crude lipide determinations (3).

Kaye (et. al.) (16) reported that isopropyl ether was a desirable solvent for fatty materials in feces. Isopropyl ether, being an immiscible liquid, permits the simultaneous determination of moisture and lipides. Monaghan (17) attempted to modify the method for lipide extraction in plant material but his results indicate this solvent to be very ineffective for plant tissues. Satisfactory values for the moisture content of plant materials were obtained, however.

Benzene and trichlorethylene have also been suggested for crude lipide determination but are seldom used. The last traces of the former are very difficult to remove from the lipide residue whereas the latter has too great a range of solvent power to be of value.

Where the extracted lipide material is subsequently to be fractionated and purified, more general solvents, combinations of solvents, or other methods that facilitate a more complete removal of the lipides can be employed as most impurities will be removed by later fractionation treatment.

W. Koch (18) reported that a more complete lipide extraction from animal tissues could be obtained if the ethyl ether extraction was preceded and followed by alcoholic extractions. Later F. C. Koch (19) introduced modifications

which adapted the method for analysis of plant tissue. This modified procedure makes it possible to extract the lipide material from fresh plant tissues without first subjecting it to a harsh drying treatment. It also permits the storing of fresh plant material (without drying) for a period of time before the actual analysis has to be made. Further evidence as to the value of a combination of solvents for lipide extraction was reported by Shulze and Stieger (10). Their results indicate that the phospholipides can only be completely removed from seed material if the ether extraction is followed by one of alcohol.

Acetone has been recommended (3) in place of alcohol in the modified method of Koch. The results of Hieserman (1) however, indicate that this substitution cannot always be favorably made. In the extraction of lipides from such a typical plant tissue as alfalfa it was found that acetone was definitely inferior to alcohol.

A number of investigators, Leathes (20), Lieberman and Szekely (21), Rosethal and Trowbridge (22), and Rather (8), have reported procedures which are based on the extraction of the fatty acids. The entire sample is saponified with some suitable reagent such as alcoholic potash, then acidified, and the resulting fatty acids are extracted, dried and weighed.

Early investigators considered an ether extract of most tissues to consist mainly of neutral fats and other simple glycerides. Later it was definitely established that a con-

siderable portion of the fat soluble extract was non-lipide in nature and indigestible in the animal body (23). This led to the separation of crude ether extract into the saponifiable and unsaponifiable fractions with the interest centering on the saponifiable or fatty acid fraction because of its energy value in animal metabolism. During recent years the unsaponifiable fraction has become biologically important because it contains vitamins E and K, and the precursors to vitamins A and D. These factors have led to an increased demand for better and more rapid means of fractionation of the crude lipide extract.

The majority of the methods proposed to date call for saponification of the sample or crude extract as a means of separation of the fatty acids. Liebermann and Szekely (21) in 1898 reported the use of saponification as a means of lipide analysis. Their proposed method called for the saponification of the entire sample and following acidification the liberated fatty acids were extracted with ethyl ether and dried to constant weight. Kumagawa and Suto (14) modified and improved the above method in 1908. The product obtained in the method of Liebermann and Szekely was redissolved in boiling alcohol and saponified. The unsaponifiable material was extracted from the soap mixture with petroleum ether. After acidification of the alcoholic soap solution the fatty acids were extracted with ethyl ether, evaporated to dryness, and dried to constant weight. An approximation of the neutral

glycerides originally present was obtained by multiplying the weight of fatty acids by an empirical conversion factor.

An improved method for the determination of the unsaponifiable fraction was developed by the Fat Analysis Committee of the Division of Industrial Chemists and Engineers of the American Chemical Society. This procedure, commonly known as the F. A. C. method (24), was adopted as official for unsaponifiable by the Association of Official Agriculture Chemists in 1926. In brief the method calls for extraction of the unsaponifiable with petroleum ether following the saponification of a five gram fat sample. After evaporation of the solvent the residue is tested for solubility in petroleum ether. Any insoluble material is filtered off, the solution evaporated to dryness and dried to constant weight. Hieserman (1) in 1939 adapted the F. A. C. method for determination of the unsaponifiable in plant tissue.

In 1936 Horwit, Cowgill and Mendel (23) introduced minor modifications in the method of Kumagawa and Suto whereby they were able to run a positive determination of both the unsaponifiable and fatty acids in plant tissues. These workers demonstrated that in some cases over fifty per cent of a crude lipide extract of plant tissue was unavailable for animal nutrition.

F. Werr (25) in 1938 advanced a simplified method for determining the lipide content of poppy, rape, hemp, linseed and other oil seeds. One half gram of the seeds was ground

in a mortar for five minutes by rubbing with twice this amount of sand and some anhydrous sodium sulfate. The ground mass was transferred to a centrifuge tube and 5 ml. of benzine (B.P. 80-90°) were added, then shaken for 3-5 minutes and centrifuged at 3,000 r.p.m. for 5-10 minutes. One ml. of the clear solution was transferred to a weighed filter paper, the solvent evaporated off and the sample weighed. Werr claimed the method was sufficiently accurate and especially useful in plant breeding experiments. Z. Huber (26) in 1940 reported the results of lipide determination on various seeds by the Werr method and described it as preferable to all others. The method was checked by the more time consuming extraction procedures of Soxhlet and others on eight different kinds of seeds, poppy, sunflower, sinapis, hemp, linseed, soybean, etc.

After studying the various methods available for lipide analysis, Hieserman (1) outlined a procedure in which the initial Koch extraction (alcohol-ether-alcohol), the F. A. C. method for removal of the unsaponifiable, and finally the Kumagawa and Suto technic for determination of the fatty acids are employed. As proposed, the method is somewhat involved but gives positive, reproducible values for the unsaponifiable and fatty acids in plant tissues.

Results published by Holwech (27) in 1940 following an extensive series of investigations show that increasing the concentration of soap in aqueous phase increases the solubility of the unsaponifiable in the aqueous soap phase over that of the petroleum ether phase, thereby requiring a greater

number of extractions, or a larger volume of petroleum ether or both in order to completely extract all the unsaponifiable. He states that the soap concentration in the aqueous phase should be kept below 8 gm per 100 ml. But since a concentration of but five per cent of this value is rarely reached, in fact it is usually but one per cent in lipide determination on plant tissues, this factor can practically be disregarded.

Numerous methods have been proposed for the estimation of the individual fatty acids present in a given sample of fat. But since this thesis is limited to the separation of a crude lipide extract into the unsaponifiable and fatty acid fractions these methods will not be discussed except to mention two procedures that might be adapted to a crude lipide extract. The first is the crystallization of the fatty acids from an acetone solution at temperatures from -40° to -50° C. Earle and Milner (28) reported in 1940 that very rapid and effective separation of the saturated from the unsaturated fatty acids could be accomplished in this way. The second method involves the use of the adsorption column. Kaufmann has published a series of articles (29) (30) (31) in which the use of silica gel, alumina and animal charcoal are reported for the adsorption of fatty acids from solution. Kaufmann stated that in most cases complete separation of the individual fatty acids was obtained by the selective action of the above adsorbents.

EXPERIMENTAL

As this problem was initially undertaken to evaluate the procedure for lipide analysis in plant tissue as proposed by Hieserman (1), the first part of the work, after the preparation of samples and moisture determinations, was concerned with fractionation of the lipide extract into unsaponifiable and fatty acids according to the Hieserman method. This procedure was checked in turn by the A.O.A.C. method for crude lipides. The second part was primarily an effort to eliminate as far as possible the undesirable features encountered above. And third, an attempt was made to shorten the extraction process by running a simultaneous alcohol-ether extraction.

Part I. The plant materials upon which the determinations were run were as follows: spinach, cabbage, carrots, leaf lettuce, peas, green beans, carrot tops, corn, oats, alfalfa meal, and the stock ration used for the rat colony in the local vitamin assay laboratory. After thoroughly washing the fresh samples, the carrots and green beans were sliced and the leafy materials, (cabbage, lettuce and spinach) were separated to aid in drying. Drying was accomplished by placing in a 45°C. room in which circulation of the air was maintained by an electric fan. After 36 hours the samples were removed and ground to 40 mesh in a Hobart electric mill. The samples were then allowed to stand for 24 hours to reach an equilibrium with

the air in the laboratory whereupon they were bottled and tightly stoppered.

The moisture content was determined as follows; triplicate 2 g. samples were placed in covered aluminum dishes and dried in a vacuum oven at 90-95° C. for 6 hours and then weighed. The drying was then continued for 2-hour periods until a constant weight was obtained. The loss in weight was recorded as the moisture content. The results are shown in Tables I, II and X. These moisture free samples were then carefully transferred to 33 x 94 mm. fat free paper thimbles and used for the crude lipide determination as outlined below.

The paper thimbles containing the dried samples were placed in individual dry Soxhlet extractors, equiped with calcium chloride tubes, and extracted for 16 hours with C. P. anydrous ethyl ether. After distilling off the solvent the samples were dried at 100° C. for 30 minutes and weighed. The drying was continued as above for 15-minute intervals with intermittant weighings until a constant weight was obtained. The values for crude lipides are recorded in Tables I, II and X.

The fatty acids and unsaponifiable content of the prepared samples were determined by the Hieserman method (1). Triplicate 5 gram samples of the dried and finely ground plant tissues were transferred to 33 x 94 mm. paper thimbles and covered with discs of fat-free filter paper. The paper thimbles were placed in individual Soxhlet extractors and extracted for 10 hours with 95 per cent ethyl alcohol.

The alcohol extraction was followed by an 8-hour ethyl ether extraction and finally another 10-14-hour alcohol extraction. Two ml. of hydroquinone solution in ether containing 0.2 mg. hydroquinone were added to the combined extracts which were then transferred to a 250 ml. beaker and evaporated on a steam bath, without overheating, to a volume of about 100 ml. Approximately 20 ml. of 10 N. sodium hydroxide were added. The mixture was saponified on a steam bath in an open beaker until the total volume had been reduced to about 40 ml. At this time 30 ml. of water were added and the saponification continued until the total saponification period had been approximately one hour.

The saponified material was transferred to a separatory funnel and washed to a 40 ml. volume with 95% alcohol. The transfer was completed first with warm, then with cold water, until the total volume was 80 ml. After rinsing the flask with 50 ml. of petroleum ether the rinsings were added to the contents of the funnel previously cooled to room temperature. It was then shaken as vigorously as possible for one minute and allowed to settle until both layers were clear. The petroleum ether layer was drawn off as closely as possible and placed in a separatory funnel of 500 ml. capacity. The extraction was repeated six more times, using 50 ml. of petroleum ether for each extraction. The combined extracts in the separatory funnel were washed three times with 25 ml. portions of 10% alcohol by volume and shaken vigorously each time. The petroleum ether extract was transferred to a weighed beaker and evaporated to dryness on a steam bath.

The beaker with residue was heated for 30-minute intervals at a temperature of 100° C. until a constant weight was obtained. The final residue was tested for solubility in 50 ml. of petroleum ether at room temperature. The solution was filtered and washed free from any insoluble residue and dried in the same manner as before and weighed. This weight represented the total unsaponifiable matter.

The combined basic alcoholic extracts from the above extractions were collected in a separatory funnel and acidified to a pH of about 6 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 ml. and two 30 ml. portions. Each time the top ether layer was collected in a 250 ml. beaker while the alcoholic layer was returned to the separatory funnel to be re-extracted. The ether was evaporated off from the combined extracts on a steam bath. The fatty acid residue was dried in a vacuum oven at 50° C. for 4 hours. The fatty acids were then redissolved in four 15-30 ml. 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. This weight represented the true fatty acids multiplied by the factor 1.046 gave the weight of the neutral tri-glycerides. The results are shown in Tables I and II.

TABLE I

Moisture, Crude Lipide, Unsaponifiable and Fatty Acid Determination on Carrot Top, Cabbage, Spinach and Carrot

(Recorded as percentage composition on dry weight basis)

Material	Moisture	Crude Lipides (A.O.A.C. method)	Unsaponifiable (Hieserman's method)	Fats (calc.) (% fatty acids x 1.046)
Carrot tops Sample 1	5.63	2.66	0.75	1.88
2	5.62	2.48	0.76	2.10
3	5.37	2.54	0.77	2.02
Average	5.54	2.56	0.76	2.00
Cabbage Sample 1	13.20	1.62	0.75	1.49
2	13.14	1.60	0.69	1.63
3	13.07	1.59	0.77	1.49
Average	13.14	1.60	0.74	1.54
Spinach Sample 1	5.43	3.74	1.43	2.82
2	5.06	3.64	1.45	2.99
3	5.04	3.71	1.38	2.83
Average	5.18	3.70	1.42	2.88
Carrot Sample 1	6.93	1.70	0.37	1.04
2	6.58	1.62	0.41	1.09
3	6.83	1.71	0.41	.92
Average	6.78	1.68	0.40	1.02

TABLE II

Moisture, Crude Lipide, Unsaponifiable and Fatty Acid Determinations on Corn, Oats, Alfalfa, and Stock Ration

(Recorded as percentage composition on dry weight basis)

Material	Moisture	Crude lipide (A.O.A.C. method)	Unsaponifiable (Hieserman's method)	Fat (calc.) (% fatty acids x 1.046 (Hieserman's method)
Corn Sample 1	9.99	4.57	0.43	4.66
2	9.73	4.53	0.46	4.91
3	9.61	4.55	0.41	4.61
Average	9.78	4.55	0.43	4.73
Oats Sample 1	9.59	9.51	0.50	9.10
2	9.99	9.45	0.46	9.05
3	9.98	9.39	0.53	9.40
Average	9.85	9.45	0.50	9.18
Alfalfa Sample 1	5.21	3.84	1.06	2.35
2	5.28	3.96	1.00	2.11
3	5.43	3.89	1.04	2.24
Average	5.30	3.89	1.03	2.24
Stock ration Sample 1	7.08	13.45	0.76	12.38
2	7.20	13.32	0.80	12.23
3	7.12	13.38	0.83	11.96
Average	7.13	13.38	0.80	12.19

Part II. In order to determine the efficiency of the above saponification and fractionation procedures in the recovery of known amounts of pure fats, .075 g. of trimyristin and .025 g. of myristic acid were added to individual 5 g. samples of alfalfa. The extraction and fractionation were carried out as above. The results are shown in Table III.

TABLE III

The Recovery of Myristic Acid and Trimyristin by the Hieserman Method

Alfalfa Moisture -- 5.30%

(Recorded as weight in grams)

Unsaponifiable		Fatty acids	
5 g. alfalfa	5 g. alfalfa .025 g. myristic acid .075 g. trimyristin	5 g. alfalfa	5 g. alfalfa .025 g. myristic acid .075 g. trimyristin
0.0500	0.0459	0.1067	0.2146
0.0473	0.0492	0.0957	0.2152
0.0492	0.0472	0.1016	0.2136
Ave. 0.0488	0.0474	0.1013	0.2145

In order to determine the effect of the chlorophylls upon the fractionation of the crude extract and to determine, if possible, what role they play in the formation of the difficult emulsions encountered in analysis of chlorophyll containing tissues, .050 g. of crystalline chlorophylls were added to each of three 5 g. samples of corn. The extraction and

fractionation were carried out as outlined above. The results are given in Table IV.

TABLE IV

The Effect of Chlorophyll upon the Fractionation of the Crude Lipide Extract by the Hieserman Method

Corn Moisture -- 9.78%

(Recorded as weight in grams)

Unsaponifiable		Fatty acids	
5 g. corn	5 g. corn .050 g. chlorophyll	5 g. corn	5 g. corn .050 g. chlorophyll
0.0196	0.0433	0.2012	0.2005
0.0211	0.0414	0.2121	0.2094
0.0184	0.0401	0.1991	0.1949
Ave. 0.0197	0.0416	0.2041	0.2009

In an attempt to eliminate the emulsions encountered in the above determinations several modifications of the Hieserman method were tried. The first consisted of adding 5 ml. of concentrated hydrochloric acid to the combined crude extracts of alfalfa after they had been evaporated to a volume of 100 ml. After heating on the steam bath for 10 minutes, saponification and fractionations were carried out as in the original procedure except that an additional amount of base, sufficient to neutralize the hydrochloric acid, was added.

The results are shown in Table V.

TABLE V

The Effect of Pre-treatment with Acid on the Fractionation of the Crude Lipide Extract

Alfalfa Moisture -- 5.30%

(Recorded as weight grams from 5 g. sample)

Unsaponifiable		Fatty acids	
Original Hieserman method	Modification	Original Hieserman method	Modification
0.0500	0.0449	0.1067	0.1092
0.0473	0.0474	0.0957	0.1117
0.0492	0.0445	0.1016	0.1065
Ave. 0.0488	0.0456	0.1013	0.1091

The second modification consisted of adding various amounts of ethyl alcohol to the crude extracts after saponification. To each of three saponified samples from 5 grams alfalfa were respectively added 0, 5, and 10 ml. of 95% ethyl alcohol. Due to persistant emulsions encountered in sample one, 15 ml. of alcohol were added thereby giving three samples containing 5, 10, and 15 ml. of alcohol respectively. The fractionation was carried out as in the original Hieserman procedure. The results are given in Table VI.

TABLE VI

The Effect of the Amount of Alcohol upon the Fractionation of the Crude Lipide Extract

Alfalfa Moisture -- 5.30%

(Recorded as weight in grams from 5 g. sample)

Sample	Unsaponifiable	Fatty acids	Remarks
5 ml. alcohol	0.0522 g.	0.1276	Slight emulsions
10 " "	0.0549 "	0.1302	Practically no emulsions
15 " "	0.0508 "	0.1278	Slight emulsions

The third modification consisted of evaporating the alcohol-ether extracts of triplicate 5 gram samples of alfalfa to dryness before saponifications. The lipide material in this dry residue was extracted by heating and subsequent decantation with five 50 ml. portions of petroleum ether. To the combined, decanted extracts were added 100 ml. of ethyl alcohol. After evaporation of the samples to 100., the saponification and fractionation were carried out as above. The results are given in Table VII.

TABLE VII

The Effect of Drying upon the Fractionation of the Crude Lipide Extract

Alfalfa Moisture -- 5.30%

(Recorded as weight grams from 5 g. sample)

Unsaponifiable		Fatty acids	
Hieserman method	Modification	Hieserman method	Modification
0.0500	0.0454	0.1067	0.0964
0.0473	0.0462	0.0947	0.0942
0.0492	------	0.1016	0.0993
Ave. 0.0488	0.0458	0.1013	0.0966

The fourth modification was concerned with maintaining as low a volume of solution as possible during the extraction of the unsaponifiable and fatty acids. This was accomplished by evaporating the combined extracts of 5 g. of alfalfa on a steam bath to a volume of 20 ml. At this point 3 ml. of 10 N. sodium hydroxide were added. The heating was continued until the volume had been reduced to 10 ml. whereupon 10 ml. of water were added and the heating was continued until the total saponification period had been one hour. The saponified material was transferred with the aid of first hot then cold water to a 250 ml. separatory funnel to which were added 10 ml. of 95% ethyl alcohol. The volume at this point was between 40 and 45 ml. The rest of the fractionation followed the procedure as outlined by Hieserman. The results are listed in Table VIII.

TABLE VIII

The Effect of the Fractionation Volume upon the Extraction of Unsaponifiable and Fatty Acids

Alfalfa Moisture 5.30%

(Recorded as weight in grams from 5 g. sample)

Unsaponifiable		Fatty acids	
Hieserman method	Modification	Hieserman method	Modification
0.0500	0.0621	0.1067	0.1178
0.0473	0.0589	0.0947	0.1228
0.0492	0.0590	0.1016	0.1191
Ave. 0.0488	0.0600	0.1013	0.1199

The fifth modification was made to determine further whether or not the above modification facilitated the extraction of the unsaponifiable matter. The extraction of the 5 g. alfalfa sample and the subsequent saponification of the crude extracts were carried out as described under the fourth modification. However, in the extraction of the unsaponifiable material only five extractions with 50 ml. of petroleum ether were made instead of the seven as prescribed in the Hieserman method. Also a fifth extraction of the fatty acids using 30 ml. of ethyl ether was made to determine the completeness of the removal of this fraction in the original procedure. This fifth extraction of the fatty acid from each of three samples was combined and the total yield was

determined. The results are shown in Table IX.

TABLE IX

The Efficiency of Five Extractions with Petroleum ether in the Removal of the Unsaponifiable

(Recorded as weight in grams from 5 g. samples of Alfalfa)

Unsaponifiable		*Fatty acids	
Hieserman method	Modification	Hieserman method	Modification
0.0560	0.0593	0.1067	0.1212
0.0473	0.0587	0.0947	0.1187
0.0492	0.0612	0.1016	0.1224
Ave. 0.0488	0.0597	0.1013	0.1200

*A combined fifth extraction of the fatty acids in the samples yielded 0.0014 gms. or 0.0005 gms./sample.

For the further check on the second fourth, the fifth modifications, determination of the unsaponifiable and fatty acid content of triplicate 5 g. samples of peas, lettuce, and green beans were made. These modifications summarized as follows: After adding 2 ml. of a solution of hydroquinone in ether containing 0.2 mg. of hydroquinone, the combined extracts were evaporated to 20 ml. on the steam bath. Then 3 ml. of 10 N. sodium hydroxide were added, the heating was continued until the volume was reduced to 10 ml. whereupon 10 ml. of water were added and the saponification continued until the total saponification period had been one hour. The

saponified material was then transferred with first hot and then cold water to a 250 ml. separatory funnel. After increasing the volume to 35 ml. with water, 10 ml. of 95% ethyl alcohol were added. The unsaponifiable material was extracted by shaking five times with 50 ml. portions of petroleum ether. The combined extracts were washed with three 20 ml. portions of 10% ethyl alcohol. After evaporation of the petroleum ether extract to dryness the amount was determined as in the original Hieserman method. The combined basic alcoholic extracts from the above extractions were acidified with hydrochloric acid. Extraction of the fatty acids was accomplished by means of two 60 ml. and two 30 ml. portions of ethyl ether. The evaporation of the extract and the weight of the fatty acids were determined as in the original procedure. The results are in Table X.

TABLE X

Moisture, Crude Lipide, Unsaponifiable, and Fatty Acid Determinations on Leaf Lettuce, String Beans and Peas by Modified Method

(Recorded as percentage composition on dry weight basis)

Material	Moisture	Crude Lipide (A.O.A.C. method)	Unsaponifiable	Fats (calc.) (% fatty acids 1.046)
			Modified method	
Lettuce Sample 1	17.90	4.84	1.25	3.83
2	17.82	5.02	1.19	3.88
3	17.88	4.85	1.13	3.83
Average	17.87	4.91	1.19	3.85
Peas Sample 1	12.24	3.41	0.52	3.67
2	12.30	3.39	0.51	3.84
3	12.43	3.41	0.54	3.96
Average	12.32	3.40	0.52	3.82
Beans Sample 1	16.24	2.46	0.59	2.31
2	16.06	2.52	0.57	2.42
3	16.23	2.59	0.63	2.42
Average	16.18	2.52	0.59	2.38

Part III. The Third phase of the experimental work was an attempt to shorten and simplify the initial extractions by means of a simultaneous alcohol and ether extraction. For this purpose a solution consisting of 40% ethyl ether and 60%

ethyl alcohol was used. By trial it was found that a solution of alcohol and ether in the above ratio gave, on the partial distillation desired, a distillate composed of approximately equal portions of each solvent.

Triplicate 5 g. samples of alfalfa were placed in individual Soxhlet extractors. To the distillation flask were added 90 ml. of alcohol and 60 ml. of ethyl ether. The samples were extracted continuously for 16 hours. The crude extracts were treated by the modified procedure as outlined above. The results are listed in Table XI.

Triplicate samples of both alfalfa and carrot tops were handled in the same manner as above except that the extraction was continued for 24 hours. The results are given in Table XII.

TABLE XI

Simultaneous Alcohol and Ethyl Ether Extraction

Sixteen Hour Extraction

(Recorded as weight in grams from 5 g. samples of alfalfa)

	Unsaponifiable		Fatty acids	
	Hieserman method	Modification	Hieserman method	Modification
	0.0500	0.0502	0.1067	0.1079
	0.0473	0.0528	0.0947	0.1096
	0.0492	0.0493	0.1016	0.1088
Ave.	0.0488	0.0508	0.1013	0.1088

TABLE XII

Simultaneous Alcohol and Ethyl Ether Extraction

Twenty Four Hour Extraction

Alfalfa Moisture - 5.30%

(Recorded as weight in grams from 5 g. samples of alfalfa and Carrot tops)

Unsaponifiable		Fatty acids	
Hieserman method	Modification	Hieserman method	Modification
0.0500	0.0614	0.1067	0.1132
0.0473	0.0524	0.0947	0.1164
0.0492	0.0535	0.1016	0.1232
Ave. 0.0488	0.0558	0.1013	0.1179
	Carrot Tops Moisture -- 5.54%		
0.0354	0.0357	0.0952	0.0581
0.0358	0.0328	0.0951	0.0612
0.0363	0.0321	0.0913	0.0583
Ave. 0.0358	0.0335	0.0905	0.0596

DISCUSSION

The materials that were used in this work were chosen because of their importance in animal and human nutrition and because they were available throughout the year. These materials also represented a variation in content of lipide, carbohydrate, pigment, and nitrogenous substances. The carrots provided a material high in carbohydrates and low in lipides. The lettuce, peas, beans, alfalfa, spinach and carrot tops provided a variety of chlorophyll-containing substances that contained relatively large amounts of non-lipide substances in the "ether extract". The oats and corn were used because of their high fat and low unsaponifiable content. The stock ration, was included because of its varied content. It was composed of 30% whole milk powder, 30% cornmeal, 30% oatmeal, 5% bone meal, 4% alfalfa, and 1% inorganic salts.

The air drying of the above samples was aided by maintaining constant circulation of the air in the drying room by means of an electric fan. Grinding of the materials was first attempted in a Wiley mill but was found to be unsuitable because many of the samples gummed up the mill. The Hobart electric mill was found to be very rapid and satisfactory for the grinding of all the materials used.

For moisture determination the A.O.A.C. method was adopted. The high moisture values for peas, leaf lettuce, and string beans (Table X) were probably due to the high

humidity of the air at the time the samples were prepared. Because of the brownish chared appearance of the various samples after drying it is believed that a temperature of 90-95° C. under vacuum causes considerable oxidation and decomposition.

In the use of the Soxhlet extractors in the subsequent lipide extractions, it was observed that each extraction was merely diluted by the following one due to the slowness with which the solvent passed through the samples. Complete siphoning of the solvent from the extraction chamber was accomplished by placing 3 cm. lengths of large diameter glass tubing beneath the paper thimbles.

In the crude lipide determinations a few minor points should be noted. Determinations were made on the same samples that were used for moisture determinations. As the samples were dried at 90-95° C. before extraction some alteration through oxidation might have resulted. The crude lipide extracts were dried and weighed in the extraction flasks. It was found that reproducible weighings within 0.5 mg. could be obtained on these 250 ml. flasks. The ethyl ether therefore could be recovered after the extraction was complete by simply replacing the paper thimble with a glass tube of the same design and continuing the distillation. It was also found that by allowing the flasks to cool in a desiccator for 3 to 4 hours after drying at 100° C. that constant weights could usually be obtained after the second heating.

The A.O.A.C. method for crude lipides gives highly reproducible results (Table I, II and X). However, in nine of the eleven materials analysed the crude lipide values were lower than the combined fats and unsaponifiable values of the Hieserman procedure. By simple observation it was apparent that the crude lipide extracts contained most of the chlorophylls or their breakdown products present in the original sample whereas in the latter method, the final products were practically free of chlorophylls. Hence any nutritional studies concerned with the utilization of fat in plant tissues, based on feeding of crude extracts as lipides, would be subject to appreciable errors.

The Hieserman method was found to give relatively high yields of unsaponifiable and fatty acids (Table I, II). The unsaponifiable fractions were yellow to orange in color, indicating an absence of chlorophyll, and were waxy in nature. The fatty acids had the appearance of colorless oil when heated, and when cooled, assumed a more or less fatty acid-crystalline nature, thus indicating a fairly high degree of purity.

However, the method was found to be quite tedious and time consuming, especially when tissues containing chlorophylls were analysed. In the fractionation of chlorophyll-containing samples, persistant emulsions formed which separated only when allowed to stand for long periods of time. Since a total of fourteen extractions were required for the complete fractionation of the crude extracts, the undesira-

bility of allowing ten to forty minutes for each emulsion to break is readily apparent.

Hieserman's procedure for handling the combined crude extracts was indefinite and it was found to be physically impossible to follow his detailed directions at this point.

"----evaporation of the alcoholic and ether extracts to a volume of 100 ml. To this solution 20 ml. of approximately 10 N. potassium hydroxide solution is added. The mixture is saponified on a steam bath in an open beaker until the total volume has been reduced to about 40 ml. At this time, 30 ml. of water are added and the saponification continued until the total saponification period has been approximately one hour. The saponified material is transferred to an extraction cylinder and washed to a 40 ml. volume with 95 per cent alcohol."

The evaporations called for required over two and one half hours and still left a final volume of over 40 ml. Because of this inconsistancy in volume, in the determinations recorded in Tables I and II no alcohol was added as it was thought that adding an indefinite amount of alcohol would introduce a variable factor with adverse effects on the results. The 20 ml. of base used for saponification was recognized as being an unnecessarily large excess. This portion of Hieserman's procedure was taken directly from the F.A.C. method in which a 5 g. sample of fat was to be saponified.

The data in Table III indicate that recovery of added fat is very complete by the Hieserman method. Possibly the fat that was added had a favorable effect upon the extraction of the fatty acids thereby giving the higher than theoretical yield.

The indication that the chlorophylls were largely responsible for the persistant emulsions encountered was confirmed by the results in Table IV. In the initial analysis of corn very little trouble was encountered with emulsions but when pure chlorophylls were added, the characteristic emulsions of all the green materials analysed were present. The increase in unsaponifiable was probably due to the phytol alcohol that was split off the chlorophylls. The lower yield of fatty acids probably was a result of emulsions encountered in the fractionation of the crude extract.

In an attempt to alter the chlorophyll molecule and thereby lessen its emulsifying effect, concentrated hydrochloric acid was added to the crude extracts before saponification. The results (Table V) indicated a small loss in the unsaponifiable with a slightly larger gain in fatty acids. However, no lessening of emulsion-formation was noticed.

The results listed in Table VI indicated that the presence and the amount of alcohol have a very favorable effect upon the extraction of both the unsaponifiable and fatty acids. This favorable action of alcohol was partially due to the elimination of troublesome emulsions. In most cases separation of the layers was complete in 5-10 seconds. In spite of the limited nature of this experiment it was assumed

that approximately 10 ml. of alcohol provided the optimmal concentration for this particular determination. In connection with the high yield of fatty acids it should be mentioned that the final product appeared to be quite impure. This could have been caused by insufficient heating of the crude fatty acid extracts allowing thereby a resolution of chlorophylls and other impurities.

The fourth modification wherein the combined extracts were evaporated to dryness (Table VII) proved impractical because a thick gummy mass was formed which made re-extraction of the lipides difficult. The extracting solvent had to be decanted as the amorphous nature of the material made filteration impossible. The low yields were undoubtedly due to the occlusion of lipide material by the gummy precipitate. Furthermore much of the chlorophyll was redissolved.

In some of the previous determinations the volume of the alcoholic potash solution was increased past the 80 ml. mark in effecting a complete transfer of the solution to the separatory funnel. Fractionations made on these solutions indicated that emulsion formation increased as the volume increased. In the fifth modification (Table VIII) 3 ml. of 10 N. base were used for saponification and the fractionation volume was reduced to approximately 45 ml. The results show a very high yield of unsaponifiable with a fairly high yield of fatty acids. Although the yield of fatty acids was lower than those shown in Table VI, they appeared to be somewhat freer from chlorophyll. Furthermore the unsaponifiable

(Table VIII) was much higher indicating that the lower volume permitted a more complete removal of this fraction. It was noted that the sixth and seventh extraction of the unsaponifiable was colorless while the fifth one was nearly so. The values in Table IX indicate that five extractions are sufficient to remove the unsaponifiable. The results also indicate that four extractions with ethyl ether removed practically all the fatty acids.

The results recorded in Table X with materials of high chlorophyll content show that in each case the combined values of the purified fatty acids and unsaponifiable were higher than that for the crude extract even though the latter contained most of the chlorophyll present in the original sample. Furthermore, since only slight emulsions were encountered, the determinations were completed in a much shorter period than would have been possible by the original Hieserman procedure.

In an attempt to shorten the extraction time required by the original procedure, and, to eliminate the necessary of alternating the solvent, simultaneous extraction with alcohol and ethyl ether was tried. The composition of the solvent (40% ether, 60% alcohol) was such that would yield a distillate composed of approximately equal parts of each solvent. Whether of not this ratio is the most efficient for the removal of lipides in plant tissues was not determined. The results listed in Table XI show that a sixteen hour extraction, when fractionated by the modified procedure,

gave values that compared favorably with those of the Hieserman extraction and fractionation procedure. However, upon comparison with Table IX it is evident that a sixteen hour extraction period is insufficient to remove all the lipide known to be present in this sample of alfalfa. Table XII shows that, although a twenty-four hour extraction gives still higher yields than the sixteen-hour extraction, the values are still slightly low for alfalfa (Table IX). The values for carrot top were low, especially for the fatty acids, as to indicate that the simultaneous use of alcohol and ether could not be universally used for lipide extraction in plant tissue. Hence a longer extraction period was not tried. Perhaps a different ratio of the two solvents might be more efficient. Since many phospholipides are soluble in hot but not cold alcohol, the lower temperature of the mixed solvent in the extraction chamber over that when alcohol is used alone might also explain the ineffective solvent action.

From a study of the results obtained in connection with the work on this problem it is believed that, with certain modifications in the procedure, the Hieserman method permits a highly accurate determination of the fatty acids and unsaponifiable on any type of plant tissue regardless of its nature or lipide content. Furthermore, the method is suitable for the analysis of fresh plant tissues as well as dried or otherwise altered tissue.

The method in detail, with proposed modifications, is as follows: (1) Approximately 25 g. samples of fresh plant tissues are transferred to beakers and enough 95% ethyl al-

cohol is added to bring the alcoholic content to about 80%. The tissue is then boiled for 15 minutes on a steam bath to arrest enzymatic activity. The fresh tissue is then transferred to 33 x 94 mm. paper thimbles by fitting the extraction thimbles into glass filter funnels held in turn in clean suction flasks. The tissue is washed with a jet of hot 95% alcohol and covered with a piece of filter paper. When dried tissue is to be used, the material is finely ground, then 5 g. are placed in paper thimbles and covered with pieces of filter paper. The thimbles are then placed in the extraction chamber of continuous Soxhlet extractors each of which have been previously equipped with 3 cm. lengths of large-bore glass tubing. Following 10 hours of continuous extraction with 95% alcohol, an 8-hour ethyl ether extraction is made, followed by a second 10-12-hour ethyl alcohol extraction. When fresh tissue is to analysed, the material is freed from ether and finely ground before the final alcohol extraction. After adding 2 ml. of a hydroquinone solution in ether, containing 0.2 mg. of hydroquinone, the combined extracts are evaporated on a steam bath in an open beaker to a volume of about 20 ml. This and subsequent evaporations are made in a swiftly moving current of air produced by means of a small electric fan. (This was found to prevent overheating, facilitate evaporation, and to prevent creeping on and over the sides of the beaker). To this solution 3 ml. of about 10 N. sodium hydroxide are added and the mixture saponified on a steam bath for 30 minutes. At this time 10 ml. of water are added and

the saponification continued for an additional 30 minutes. The volume should now be approximately 15-20 ml.

(2) The saponified material is transferred to a 250 ml. separatory funnel with the aid of small quantities of hot water until the total volume is 35-40 ml. The beaker is rinsed with 50 ml. of petroleum ether which is added to the contents of the separatory funnel previously cooled to room temperature. Ten ml. of 95% alcohol are added and the separatory funnel is shaken as vigorously as possible for one minute. When complete separation of the two layers has taken place, the bottom layer is run into a second separatory funnel containing 50 ml. of petroleum ether and the top ether layer is transferred to a third separatory funnel (500 ml. capacity). The above extraction is repeated at least 4 more times using 50 ml. of petroleum ether for each extraction. The combined extracts in the separatory funnel are washed by shaking vigorously three times with 25 ml. portions of 10% alcohol. However, if the sample being analysed contains chlorophylls, persistant emulsions can be eliminated by gently rotating the initial washing thus removing the emulsifying agents. The washings are returned to the basic alcohol solution. The petroleum ether extract is transferred to a weighed beaker and evaporated to dryness on a steam bath. The beaker is heated for 30-minute intervals until a constant weight is obtained. The residue is tested for solubility in 50 ml. of petroleum ether at room temperature then filtered and washed free from any insoluble residue. The purified product is dried in the same manner as before and weighed. This

weight represents the total unsaponifiable matter and should be free from chlorophyll.

(3) The basic alcohol extracts are acidified to a pH of about 6 with hydrochloric acid. Brom cresol purple was found to be a satisfactory indicator for this reaction. The solution is then cooled and the free fatty acids extracted by shaking with four portions of ethyl ether, two 60 ml. and two 30 ml. portions. Each time the bottom layer is collected in a second separatory funnel containing the next portion of ethyl ether and re-extracted. The top layers are collected in a beaker and evaporated to dryness on a steam bath. The fatty acid residue is dried in a vacuum oven at 50° C. until the chlorophyll has been rendered insoluble in petroleum ether. This usually requires 4-6 hours. The fatty acids are redissolved in four 15-30 ml. portion of boiling anhydrous petroleum ether, and filtered from chlorophyll and other products thru 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 the true fatty acids. The weight of true fatty acids multiplied by the factor 1.046, assuming that the average molecular weight of the fatty acids is 274, gives the weight of neutral triglycerides.

CONCLUSIONS

1. The Hieserman procedure was found to give quite accurate and reproducible results. However, very persistant and troublesome emulsions were encountered in the fractionation of lipides obtained from many tissues.
2. Since persistant emulsions were encountered with chlorophyll-containing plant tissues and because the addition of crystalline chlorophylls to other samples produced stable emulsions, it was concluded that chlorophylls were the main causative agent.
3. Heating the crude extract with acid, evaporation to dryness with subsequent heating and re-extraction, and other attempts to eliminate or lessen the emulsifying power of chlorophylls were founed ineffective or useless.
4. It was found that persistant emulsions could be eliminated by maintaining a low fractionation volume consisting of approximately 20% ethyl alcohol by volume.
5. An attempt to shorten the extraction time by employing a mixed solvent of ethyl alcohol and ethyl ether was unsuccessful.
6. The combined values for the purified fatty acids (calculated as triglycerides) and unsaponifiable obtained by the proposed method were higher than those obtained by A.O.A.C. method for crude lipides. In view of the fact that the A.O.A.C. crude extract, according to Horwitt (et.al.) is known to consist of as high as 55%

non-lipide material, it must be concluded that the method is unreliable for lipide determinations.

7. The proposed method, although quite long is thought to be superior to existing methods for lipide determinations. It can be used on either fresh or dried plant tissues and gives reproducible yields of purified fatty acids and unsaponifiable.

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Some factors affecting the determination of fatty acids and unsaponifiable matter in plant materials.

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