LIPIDES AND THEIR ESTIMATION IN VEGETABLE TISSUES

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When plant tissues are extracted with ether or any of the other fat solvents, the extract contains among other things a large number of substances which yield fatty acids on hydrolysis. The commonest of these and perhaps the most important from a biochemical standpoint are the fats, fatty oils, waxes, sterol esters, phosphatides and cerebrosides. There are also associated with these classes of compounds in various extracts many other substances, some of which are the wax alcohols, sterols, essential oils, hydrocarbons, resins and coloring matters. Some of these accompanying substances are similar to the fats and other fatty acid compounds in possessing a greasy feel but they bear little or no resemblance from a strictly chemical standpoint. Notwithstanding this divergence in chemical makeup, many writers include them in a single large group. It is evident that such a grouping is based almost entirely on the common physical property of being soluble in one or more of the ordinary fat solvents.

Early investigators considered an ether extract of most tissues to consist mainly of neutral fats and other simple glycerides. Later, however, it was definitely established that certain other substances resembling the fats in their solubilities also were removed by ether. To these "fat-like" compounds, the term "lipoids" was applied and is still recognized in this sense by many notwithstanding the fact that the term was first suggested to denote only unsaponifiable substances and has since been extended to include in addition to the "fat-like" substance also the fats and other simple glycerides. Two other terms, "lipins" and "lipides," have also been proposed in connection with the substances under consideration. It will be seen from the following brief discussion of these terms that the nomenclature of the fats and fat-like compounds is still rather confusing.

The term "lipoids" was introduced nearly 70 years ago by KLETZINSKI (77) to denote unsaponifiable materials extracted from animal tissues by alcohol and ether. Later, OVERTON (64) used the same term to designate those tissue substances which were similar to the fats in being soluble in organic solvents such as ether, chloroform, and alcohol. Since OVERTON’s time the term has been employed in several different ways. It has been used in a restricted sense to indicate only the phosphatides and cerebrosides. Reference to fats and lipoids have therefore been made on the assumption that these classes of substances properly belong to separate categories.

Many have considered the word "lipoids" a convenient designation for a

1 A review written at the request of the Committee on Methods of Chemical Analysis for the American Society of Plant Physiologists.
chemically heterogeneous group of substances which are characterized primarily by their physical property of common solubility in one or more of the ordinary fat solvents. Leathes (41) looks upon the term as "a cloak for ignorance and an indefinable limbo into which any one can thrust anything of which he knows little or nothing, including often what is not a compound of any fatty acid at all." Recently, Levine (47) in proposing a new classification of fatty compounds soluble in ether or alcohol-ether and closely associated substances ignores the broader significance which had been attached to the term "lipoids" and limits its application to the sterols and essential oils.

The designation "lipins" was suggested by Leathes (42) in 1910 for those derivatives of fatty acids which contain nitrogen but no phosphorus or carbohydrate group—an indefinite class of substances previously called "amidolipotides" by Thudichum (87, 88). Later, Rosenbloom and Gies (73) employed the term in place of "lipoids" as a group name for the fats and fat-like compounds yielding fatty acids on hydrolysis and also included under lipins a large number of diversified, non-fatty compounds and artificial products such as soaps, alcohols, sterols, cholates, chromolipins, triacetin and lead oleate. McLean (56) has used the word "lipins" in a limited sense to embrace only the phosphatides and cerebrosides which he considers substances of fat-like nature yielding on hydrolysis fatty acids or derivatives of fatty acids and containing in their molecule either nitrogen or nitrogen and phosphorus.

"Lipides" is the third generic name which has been proposed in the general nomenclature of the substances under consideration. It was first suggested and adopted in 1925 by the Committee on the Reform of the Nomenclature of Biological Chemistry at the International Congress of Chemistry meeting at Cambridge (19). It was recently used by Bloor (6) in a modified classification in preference to "lipoids" as a group-heading for ether or alcohol soluble compounds yielding fatty acids on hydrolysis and certain derived substances such as fatty acids and sterols. Since this term has been used only in the broad significance to include both the fats (including oils and waxes) and the lipoids, as used in the restricted sense to denote the "fat-like" substances, it appears to be the best one suggested so far.

Various classifications of fats and "fat-like" compounds

A strictly chemical grouping of these substances has proved a difficult matter, especially in view of the diversity of the chemical relationships of the compounds. For purposes of comparison, the more important attempts to classify the fats and "fat-like" compounds are listed here.
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THUDICHUM’S classification, 1884 (87, 88)

Phosphatides or phosphorized principles:
A. **Mononitrogenized monophosphatides** (N:P::1:1)
   lecithins, kephirins, paramyelins, myelins.
B. **Dinitrogenized monophosphatides** (N:P::2:1)
   amidomyelins, amidokephelins, sphingomyelins.
C. **Dinitrogenized diposphatides** (N:P::2:2)
   assurin.
D. **Nitrogenized phosphatide-sulphatides**
   cerebro sulphatide.
E. **Nonnitrogenized monophosphatides**

Nitrogenized nonphosphorized principles:
A. Cerebrosides
   phrenosin, kerasin.
B. Cerebrinacids
   cerebrinic acid, spheracerebrin, etc.
C. Cerebro sulphatides
D. Amidolipotides
   bregenin, krinosin.

ROSENHEIM’S classification, 1909 (77)

Lipoids:
1. **Cholesterin group**
   cholesterol, phytosterins, lipochromes, etc.
2. **Cerebro-galactosides**
   phrenosin, kerasin.
3. **Phosphatides**
   **Monamino-monophosphatides** (N:P::1:1)
   lecithins, kephirins, vesalthin.
   **Diamino-monophosphatides** (N:P::2:1)
   sphingomyelin.
   **Triamino-monophosphatides** (N:P::3:1)
   neottin.
   **Triamino-diphosphatides** (N:P::3:2)
   **Monoamino-diphosphatides** (N:P::1:2)
   eorin.

LEATHES’ classification, 1910 (42)

Substances entering into the composition of fats:
A. **The fatty acids**
B. **Glycerol and the glycerides**
   fats and oils.
C. **Other alcohols and their fatty acid esters**
   cetyl alcohol, myricyl alcohol, cholesterol, phytosterol, waxes,
   esters of cholesterol, etc.
D. **Phospholipins**
   compounds of fatty acids containing N and P., e.g., lecithin.
   **Galactolipines**
   compounds of fatty acids containing N and galactose, e.g.,
   cerebrome.
Lipines
compounds of fatty acids containing N but no P nor carbohydrate group, e.g., sphingosine.

Bang's classification, 1911 (2)

Lipoids:
1. Fats
2. Cholesterols
3. Phosphatides
   A. Unsaturated phosphatides
      Monaminomonophosphatides
      lecithin, kephalin, paramyelin, vesalthin.
      Monaminodiphosphatides
      euorin, etc.
      Triaminodiphosphatides
      sahiddin, etc.
   B. Saturated phosphatides
      Diaminomonophosphatides
      sphingomyelin, aminomyelin, apomyelin, etc.
      Triaminomonophosphatides
      neottin, carnaubon.

4. Cerebrosides
   phrenosin, kerasin, cerebron, etc.

Cramer's classification, 1911 (12)
Lipoids (with the exception of the cholesterols):
1. Phosphatides
   N-containing fatty acid esters of glycerophosphoric acid. Some may have the glycerol substituted by an unknown alcohol. Lecithin, kephalin, sphingomyelin, etc.
2. Galacto-phosphatides
   N-containing fatty acid esters of phosphoric acid, with galactose and alcohol groups. Carnaubon.
3. Cerebrosides
   N-containing fatty acid esters with galactose but no P. Cerebron, cerebrin, homocerebrin, etc.
4. Phospho-cerebrosides
   cerebrosides with P-containing groups. Protagon.

Rosenbloom and Gies's classification, 1911 (73)
Lipins, organic substances insoluble in concentrated saline solutions, soluble in hot alcohol or in warm ether or in both.

1. Natural aliphatic lipins
   A. Simple lipins
      a. Fatty acids
      b. Salts and esters of 1-A-a
         soaps, waxes, fats and fatty oils.
      c. Alcohol (mono- and di-hydroxy)
         cetyl alcohol, myricyl alcohol.
B. Conjugate lipins
   a. Proteolipins
      lecitho-protein like substances (may be mixtures).
   b. Glycolipins
      THUDICHUM’s cerebrosides.
   c. Phospholipins
      THUDICHUM’s phosphatides.
   d. Glyco-phospholipins
      phospholipins containing carbohydrate group.

2. Natural carboxyclic lipins
   A. Sterols
      natural terpeno-alcoholic derivatives, nonsaponifiable, and form esters, cholesterol, sitosterol, etc.
   B. Esterols
      natural terpeno-aliphatic waxes, e.g., cholesteryl palmitate
   C. Cholates
      a. Cholic acids
      b. Bile acids

3. Natural lipins of undetermined constitution
   A. Chromolipins
   B. Miscellaneous lipins

4. Artificial lipins
   tri-acetin, lead oleate, etc.

MATHEW’S CLASSIFICATION, 1914 (54)

Lipins—constituents of protoplasm with greasy feel; soluble in alcohol-ether.
1. Fats
   Fatty acids
2. Fatty oils
   drying oils, semi-drying oils, non-drying oils.
3. Essential oils
   volatile, generally odoriferous substances of oily and of varied chemical nature, being aldehydes, acids, terpenes, alcohols, etc.
4. Waxes
   esters of sterols and fatty acids.
5. Sterols
   alcohols, generally of the terpene group, solid at ordinary temperature; oxidation products are terpenic acids.
6. Phospholipins (phosphatides of THUDICHUM)
   Mono-amino-monophospholipins, lecithin, cephalin.
   Di-amino-monophospholipins
   Mono-amino-dipospholipins
7. Glycolipins (cerebrosides of THUDICHUM), cerebron, phrenosin, etc.
8. Sulpholipins—not well characterized.
9. Aminolipins—not well characterized.

MACLEAN’S CLASSIFICATION, 1918 (56)

Substances in an ether or alcohol extract of a tissue:
A. Neutral fat and fatty acids
B. Substances of varying chemical nature having no relation to fat such as cholesterol and certain pigments.

Lipins: "fat-like" bodies, sometimes referred to as "lipoids"

C. Phosphatides
1. Monoaminomonophosphatides (N: P:: 1:1), lecithin, kephalin.

D. Cerebrosides
phrenosin, kerasin.

LEVINE'S CLASSIFICATION, 1925 (47)

True lipins, compounds soluble in ether or alcohol-ether yielding on hydrolysis fatty acids and alcohol.

1. Simple lipins, compounds which yield on hydrolysis, fatty acid and glycerol or fatty acid and a monohydric alcohol of high molecular weight.
   a. Fats
   b. Fatty oils
   c. Waxes

2. Conjugated lipins, compounds which yield on hydrolysis, not only fatty acid and alcohol but some other complex such as sulphuric acid, phosphoric acid, monosaccharide, amino-acid, or some organic base like choline.
   a. Phospholipins, lecithin, cephalin, sphingomyelin.
   b. Glycolipins, cerebrin, phrenosin, kerasin.
   c. Glycophospholipins, jecorin.
   d. Sulpholipins
   e. Sulpho-phospholipins
   f. Aminolipins, bregenin.
   g. Proteolipins
   h. Chromolipins

3. Derived lipins, compounds other than phosphoric acid, sulphuric acid, amino acid or monosaccharide, obtained as a result of the decomposition of lipins.
   a. Fatty acids
   b. Alcohols, glycerol, myricyl alcohol, etc.
   c. Organic bases, choline, neurine, etc.

Lipoids, compounds not esters of fatty acid and alcohol, but which are closely associated with lipins and resemble them in their solubility in ether or alcohol-ether.

1. Sterols, cholesterol, phytosterol, etc.
2. Essential oils

BLOOR'S CLASSIFICATION, 1925 (6)

Lipides, substances having the following characteristics: (a) insolubility in water and solubility in fat solvents, such as ether, chloroform, benzol, (b) relationship to the fatty acids as esters, either actual or potential, (c) utilization by living organisms.

Simple lipides. Esters of the fatty acids with various alcohols.
Fats
Waxes
Compound lipides. Esters of fatty acids containing groups in addition to an alcohol and fatty acid.

- Phospholipides, containing phosphoric acid and nitrogen; lecithin, cephalin, sphingomyelin.
- Glycolipides, containing carbohydrate and nitrogen but no phosphoric acid; cerebrosides.
- Aminolipides, sulpholipides, etc., not at present sufficiently well characterized for classification.

Derived lipides. Substances derived from the above groups by hydrolysis.

Fatty acids
- Sterols, mostly high molecular weight alcohols, found in nature combined with fatty acids and which are soluble in the fat solvents; cholesterol, myricyl alcohol, etc.

It is not within the scope of this paper to discuss the relative merits of the various classifications, but it must be noted in passing that a great deal of confusion and difference of opinion exists in the literature as to what properly constitutes a single group of closely related fatty compounds and as to what terms should be used in their designation.

In the present paper, the use of the term “lipides” is preferred because of the fact that the several meanings which have been attached to the other two terms tend to create a state of disorder in an already difficult subject and therefore constitute an objection which does not apply in the case of “lipides.” On the basis of chemical relationships, the Bloor scheme appears more nearly to meet the requirements of a good classification than the others. The writer does not agree with Bloor, however, in always considering the sterols as being derived from ester-like compounds.

According to Bloor’s definition, lipides are water insoluble, ether or alcohol soluble substances which are either ester-like combinations with fatty acids or are capable of forming such combinations. These substances are separated into three main groups. The fats and waxes are considered as simple lipides, being esters of the fatty acids with various alcohols. The phospholipides, glycolipides and certain insufficiently characterized substances constitute the compound lipides or esters of fatty acids containing groups in addition to an alcohol and fatty acid. The free fatty acids and sterols represent the derived lipides, which are defined as substances obtained from the other groups by hydrolysis. For a thorough discussion of the various types of fatty compounds which are included in the foregoing classification and for their physical and chemical properties, one may refer to Bloor’s original paper (6).

A great deal of confusion would be avoided if plant physiologists and biochemists in general would adopt the Bloor scheme and designate as lipides those tissue constituents which fall under this classification. When obtained from tissues in the form of an impure extract by means of a fat solvent they could be reported as “crude lipides.”
The estimation of lipides

Nearly all methods for determining lipides are based on the fact that these substances, or the fatty acids derived therefrom by saponification, are largely separated from the other tissue constituents by means of certain solvents which exert at least a partial preferential solubility for the fatty compounds. Petroleum ether, especially, and ether, to a less degree, are better suited for this purpose and therefore are used more extensively than other solvents for lipide estimation. While all lipides are more or less soluble in a number of so-called fat solvents, not all of these extract the lipides with equal facility (37) or extract the same non-lipide substances. With dry oat kernels (5), for example, if the total 15-hour ether extract is considered equivalent to 100, the quantities of material extracted by other fat solvents are as follows: petroleum ether 97.07, carbon tetrachloride 104.24, chloroform 109.78, acetone 112.71, benzene 113.15 and absolute alcohol 127.93. In this particular case petroleum ether, of all the solvents, removes the smallest quantity of extract, while absolute alcohol removes the largest. It should be recognized that in most instances the extract obtained from plants by the use of petroleum ether contains the least quantity of non-lipide substances. Ether, carbon tetrachloride, chloroform, and the other solvents are all known to extract increasing quantities of impurities such as resins, alkaloids, coloring matters and other organic substances. Regardless of the fact that petroleum ether is probably the more suitable solvent to use for the extraction of lipides in many cases, many plant physiologists use ether instead when extracting dried plant tissues. In fact, this has become the most generally used fat solvent in the laboratory. However, in the crude lipide 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. This fact introduces a considerable error in case the extract is reported as “crude fat” or “lipoids” as is very often done by investigators working in agronomic and horticultural fields.

Next to the careful selection of solvent it is very important that attention be paid to the purity of that solvent. It is well known, for example, that impure ether, containing water and alcohol, will yield a greater quantity of extract than the pure anhydrous solvent yields. Such extracts are contaminated with more non-lipide substances than extracts obtained by the use of a pure solvent.

The foregoing statements are sufficient to emphasize the importance of the solvent in the extraction and estimation of lipides from vegetable tissues. On this account, it seemed desirable to include in this paper general data dealing with the common fat solvents2, including a table of physical

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2 Compare also the following: (53, 8, 97, 52).
constants. It must be remembered, however, that due to accompanying substances which exert an influence on their behavior, the solubilities of the pure lipides in pure solvents do not necessarily hold when lipides are extracted from tissues.

**Lipide solvents**

**Petroleum ether** \((C_nH_{2n+2})\).—Petroleum ether (b. p. 40–60°) readily dissolves most fats, oils, waxes, phospholipides, fatty acids and sterols; some of these even in the cold. Hydroxy acids and glycerides of these acids, for example, castor oil, are insoluble or nearly so in this solvent. Glycerides of the solid fatty acids are more difficultly soluble than those of the liquid acids. Pure tristearin, therefore, is only slightly soluble, but it becomes more so in the presence of soluble glycerides. Of the fatty acids, those of higher melting point are the least soluble. Hydrocarbons, essential oils, certain coloring matters to a slight extent, such as chlorophyll “a” and carotin, and a few other organic substances are soluble in petroleum ether. Despite the fact that this solvent dissolves certain non-lipide substances, it may be said to remove from plant materials the least quantity of these impurities in comparison with other solvents. This is particularly true with respect to coloring matters and compounds of a resinous nature.

**Ethyl ether** \((C_2H_5-O-C_2H_5)\).—Practically all lipides are fairly readily soluble in ether with the exceptions of sphingomyelin and the cerebrosides. Fats are more easily soluble the higher the content of glycerides of unsaturated fatty acids and those of low molecular weight. Pure tristearin is difficultly soluble but is more so in the presence of other glycerides. Lecithin is generally considered soluble but its complete removal from plant tissues with ether, especially from leguminous seeds is difficult. The extraction of this and similar substances which are but slowly removed even on prolonged extraction is greatly facilitated by alcoholic pre-treatment (7). It is generally known that ether dissolves more non-lipide materials from plant tissues than petroleum ether. Among the soluble substances may be mentioned hydrocarbons, essential oils, coloring matter (including chlorophylls, carotinoids, free flavones and flavonols), many alkaloids, organic acids, resins and related compounds. CHIBNALL and CHANNON (11) state it has been their experience that the presence of a small amount of water in ether will allow a small but definite amount of inorganic matter and amino-compounds to go into solution. The same statement holds true with respect to carbohydrates and possibly tannins.

**Carbon tetrachloride** \((CCl_4)\).—Carbon tetrachloride dissolves most lipides with the same ease as ether, but it probably removes more impurities \((70)\). The observation of SCHINDELMEISER (80) that many alkaloids are soluble in cold carbon tetrachloride indicates that these substances would
## TABLE I

**Table of Constants for Pure Fat Solvents**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Boiling Point</th>
<th>Specific Gravity</th>
<th>Vapor Pressure (mm. Hg.)</th>
<th>Heat of Vaporization (cal./gm.)</th>
<th>K cal./mol</th>
<th>Solvent in Water</th>
<th>Water in Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>36.3°</td>
<td>0.6454%</td>
<td>420.2</td>
<td>610.9</td>
<td>85.0</td>
<td>At 22° = 0.227 per cent.</td>
<td>At 22° = 0.50 per cent.</td>
</tr>
<tr>
<td>n-pentane</td>
<td>68.6°</td>
<td>0.6794%</td>
<td>120.0</td>
<td>185.4</td>
<td>79.2</td>
<td>(Petroleum ether of sp. gr. = 0.6646)</td>
<td></td>
</tr>
<tr>
<td>Ether</td>
<td>34.0°</td>
<td>0.7191%</td>
<td>538.0</td>
<td>914.0</td>
<td>84.5</td>
<td>At 20° = 1.22 per cent.</td>
<td>At 20° = 6.48 per cent.</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>76.7°</td>
<td>1.6319%</td>
<td>91.3</td>
<td>141.1</td>
<td>46.4</td>
<td>At 20° = 0.08 per cent.</td>
<td>At 30° = 5.04 per cent.</td>
</tr>
<tr>
<td>Chloroform</td>
<td>61.0°</td>
<td>1.4989%</td>
<td>158.4</td>
<td>240.0</td>
<td>61.4</td>
<td>At 20° = 0.085 per cent.</td>
<td></td>
</tr>
<tr>
<td>Trichlorethylene</td>
<td>88.0°</td>
<td>1.471%</td>
<td>36.0</td>
<td>92.0</td>
<td>58.0</td>
<td>At 20° = 0.617 per cent.</td>
<td>At 20° = 0.10 per cent.</td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>46.3°</td>
<td>1.2922%</td>
<td>298.0</td>
<td>717 (40°)</td>
<td>86.7</td>
<td>At 22°, 100 cc. of water dissolves 0.08 gm. or 0.5 cc.</td>
<td></td>
</tr>
<tr>
<td>Benzene</td>
<td>79.8°</td>
<td>0.8709%</td>
<td>75.0</td>
<td>118.0</td>
<td>94.9</td>
<td>At 25° = 0.218 per cent.</td>
<td>At 25° = 8.76 per cent.</td>
</tr>
<tr>
<td>Acetone</td>
<td>56.6°</td>
<td>0.8123%</td>
<td>184.8</td>
<td>282.7</td>
<td>125.0</td>
<td>At 25° = 0.113 per cent.</td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>78.3°</td>
<td>0.7093%</td>
<td>44.0</td>
<td>78.06</td>
<td>216.5</td>
<td>At 25° = 0.023 per cent.</td>
<td></td>
</tr>
</tbody>
</table>

*a Compiled from the following: (40, 97, 8).*
be readily removed from plant tissues on continuous extraction with the hot solvent. For a more detailed account of the solvent properties, refer to BASKERVILLE and Riederer (3). BRYANT (10) claims carbon tetrachloride extracts the fats and similar substances in a much shorter time than is required for some of the other solvents. His results seem to indicate that this solvent removes in 2 hours the same quantity of fat (lipides) obtained in 16 hours by the use of ether and in 4 hours by carbon disulphide. WILEY (94) says "carbon tetrachloride would be a most desirable solvent, owing to the fact that it is not inflammable and that it dissolves nearly all the fats and oils with ease, if it were not that the last traces of it are removed with the greatest difficulty and also that in the presence of even minute traces of moisture it is partly decomposed into hydrochloric acid, which of course may act upon the fat and make it useless for further determinations."

CHLOROFORM (CHCl₃).—Chloroform is a good solvent for most lipides. However, it dissolves the same and probably more non-lipide substances than ether. The fact that it dissolves more substances than ether does is readily shown by extracting plant materials exhaustively with ether and then following with chloroform, in which case the chloroform will be found to remove a further appreciable quantity of soluble substances. SCHLESINGER (81) and ROSENFELD (76) use chloroform as solvent in lipide determination methods. In the ROSENFELD method, the material is first heated on the water-bath with alcohol, then extracted in a continuous extractor with chloroform. Finally the dry residue, obtained after the evaporation of the solvent, is extracted with absolute ether. KUMAGAWA and SUTO (38) have pointed out that the energetic solvent involved in the more efficient removal of lipides in this method is mainly the alcohol and not the chloroform.

CARBON DISULPHIDE (CS₂).—This solvent is a liquid of high solvent power for a wide range of substances. It readily dissolves oils, fats, waxes, resins, and many other organic substances. It has not come into general use for quantitative lipide estimation, probably because of its poisonous properties, its unpleasant odor and its liability to decomposition when used for the extraction of certain lipides.

BENZENE (C₆H₆).—Benzene is a good solvent for fats, oils, waxes, sterols and phospholipides. Cerebrosides are soluble in the hot solvent. Fatty acids in general are easily soluble and to a higher degree than in petroleum ether. Here also the solid fatty acids are less soluble than the liquid unsaturated ones. For example, oleic acid is soluble in all proportions, but stearic acid only to the extent of 0.22 parts in 1 of benzene at 23° C. Soaps are taken up in not inconsiderable quantities if free fatty acids or neutral oils are present. Benzene dissolves hydrocarbons, certain coloring matters, resins and many other organic compounds. Last traces of benzene are very difficult to remove from a lipide residue.
Trichlorethylene \((\text{C}_2\text{HCl}_3)\).—This solvent dissolves most lipides very readily, but it also dissolves many other organic compounds, among which may be mentioned \((21)\) benzoic, salicylic, and cinnamic acids, acetaldehyde, benzoaldehyde, vanillin, camphor, alizarin, asparagine, glycine, caffeine and theobromine. Many of these non-lipide substances are doubtless left behind when the lipides in a sample are being determined by simple shaking with cold trichlorethylene and evaporation of an aliquot part of the fat solution.

Acetone \((\text{CH}_3\text{COCH}_3)\).—Acetone has a wide range of solvent power for many classes of organic compounds. The fats, oils, and waxes are only slightly soluble in the cold, but readily soluble in the hot solvent. The cerebrosides and fatty acids are soluble, but lecithins and cephalins are practically insoluble. Sphingomyelin is insoluble or nearly so in cold but somewhat soluble in hot acetone. Hydrocarbons, sterols, many resins, coloring matters, certain glucosides, tannins and other compounds are all more or less soluble in this solvent. Its solvent action is too general to permit its use for quantitative work. It has been recommended in place of alcohol to extract the lipides where the residue obtained is subsequently to be extracted with ether or petroleum ether to separate impurities.

Alcohol \((\text{C}_2\text{H}_5\text{OH})\).—Alcohol is a most excellent and energetic solvent for many classes of organic compounds. In this respect it may be said to possess a wider range of solvent power for plant constituents than any other solvent with perhaps the single exception of water. It is known to extract many compounds belonging to the following classes: lipides, carbohydrates, alkaloids, glucosides, tannins, saponins, resins, organic acids, bases, proteins (a few are soluble), coloring matters, etc. With respect to fats, oils, and waxes it may be stated that they are only slightly soluble in cold, but are more readily dissolved in the hot solvent. The solubility of fats and oils varies with the nature of the combined fatty acids. Glycerylides of lower, unsaturated fatty acids being more soluble than those of the higher, saturated ones. Lewkowitsch \((48)\) points out that the solubility of most fats and oils in absolute alcohol at \(15^\circ\) does not exceed 2 per cent. while in 95 per cent. alcohol the solubility is still less. Castor oil is a notable exception, being readily soluble in all proportions at ordinary temperature. Palmitin, stearin, and olein are almost insoluble in 91 per cent. alcohol. At \(35^\circ\) myristin is little soluble, while laurin is easily soluble. At higher temperatures the solubilities increase. The presence of free fatty acids also influences solubility, the presence of 50 per cent. making solution complete. Free fatty acids are soluble in alcohol, although from palmitic upwards all are sparingly soluble. For example, oleic acid and most unsaturated acids are readily soluble, but only 9.3 parts of palmitic and 2.5 parts of stearic acid are soluble in 100 parts of alcohol. Cerotic acid is almost insoluble at \(20^\circ\),
but soluble in boiling alcohol. The majority of oils and fats are soluble in absolute alcohol. In the use of alcohol at its boiling point for the removal of lipides, there is some likelihood of loss due to decomposition of the more sensitive lipides. The use of alcohol under reduced pressures as a means of extracting lipides from vegetable tissues without decomposition is well worthy of consideration. The possibility of the solvent reacting with the dissolved fatty acids at the ordinary boiling point of alcohol must not be overlooked in the use of this solvent to extract plant materials. Holland (30) claims boiling with alcohol causes a drop in the neutralization number of fatty acids, particularly stearic and myristic acid. It was observed by Emerson and Dumas (17) that 0.3 per cent. of palmitic acid was esterified in the short time necessary for the solution of this acid in alcohol on the water-bath and the evaporation of the alcohol. Of the phospholipides, cephalin is characterized by its insolubility in alcohol. Sphingomyelin is only slightly soluble in the cold, but soluble in hot alcohol. Lecithins are soluble. The glycolipides (cerebrosides) and sterols are readily soluble in hot alcohol.

**Crude lipide determination methods**

The principles involved in the extraction of lipides are practically the same whether one is dealing with animal or plant tissues. We may therefore conveniently separate the more important methods for the estimation of lipides in both classes of material into two general groups.

**First group.**—In the first group may be placed those methods which are based on the removal of lipides from the tissues in a condition as little altered as possible. The methods in this group may be subdivided into (a) those based on the direct extraction of the material with a fat solvent or a combination of solvents, and (b) those in which the material is subjected to a mild pretreatment such as pepsin digestion, or treatment with dilute acid or alkali, followed by extraction with a fat solvent.

The more important methods under “a” are those of shaking with ether, petroleum ether, or trichlorethylene according to Loges (51), Schütte (83), Monhäupt (57, 58, 59), Grimme (22), Neumann (61, 62, cf. also Phillips, 68) and Grossfeld (23, 24, 25, 26); the ether extraction methods of Soxhlet (85, cf. also Bömer, 9) (with modifications adopted as the “Official method” by the Association of Official Agricultural Chemists (96)), Lehmann (44) and Völitz (91); the petroleum ether extraction methods of Glikin (20), Lepper and Waterman (46); the carbon tetrachloride extraction method of Bryant (10); the alcohol-ether, alcohol-petroleum-ether, or alcohol-chloroform methods of Bogdanow (7), Frank (18), Voit (90), Noel-Paton (63), Hertwig (29), Rosenfeld (74, 75, 76); and the modified
Koch method (applicable to fresh tissues (34, 35, 36)) (cf. also Appleman (1), Culpepper, Foster and Caldwell (13), Jones (32), Walster (92), and Schertz (79)).

Under "b" are placed the acid-pretreatment methods of Pflüger (67), Palmquist (66), Polenske (69), Weibull (93); the ammoniacal-alcohol method of Rask and Phelps (71); and the pepsin-digestion method of Dormeyer (15, 16) (cf. also Schlesinger (81), Beger (4), Müller (60), and Dieselhorst (14)).

Second group.—In the second group may be placed those methods which are based on the extraction of the fatty acids produced on saponification of the lipides originally present in the tissues. This group includes the methods of Lieberman and Székely (49, 50) (cf. also Leathes (43), Rosenthal and Trowbridge (78), Kumawaga and Suto (37), cf. also Kumawaga (39), Inaba (31), Schimidzu (84), Lemland (45), and Terroine, Lepage, Véchot and Wolff (86), and of Rather (72)).

Not all of the methods included in the two foregoing groups have been employed for plant tissues. It is therefore not known whether they may be used without further modifications to meet the special needs of plant materials. Of those methods which are applicable to vegetable tissues only the three following will be discussed in detail.

1. OFFICIAL METHOD OF THE ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS (96)

Abstract of method.—Two or more grams of properly dried and ground material are extracted for 16 hours with anhydrous ether. The extract, after evaporation of the ether, is dried for 30 minutes at the temperature of boiling water, cooled in a desiccator and weighed. The drying is continued for 30-minute periods until the weight of the residue remains constant.

Details of operation.—For the extraction of lipides from solid plant materials, according to the "Official method," the Soxhlet apparatus or one of its many modifications is generally used, although any of the numerous types of continuous extractors which are now available may serve equally well. The quantity of dried material to be extracted will vary from 2 to 10 grams or even more, depending upon the percentage of fatty substances contained therein. The sample, carefully selected for uniformity, should be dried, preferably at 60° in a partial vacuum, and finely ground. The accurately weighed substance is placed either in a Schleicher and

4 For a discussion of automatic devices for the extraction of powdered material see Wiley (95) and Palkin and Walkins (65).

5 See recommendations of Committee on Methods of Chemical Analysis for American Society of Plant Physiologists (89).
Schüll fat-free paper thimble, in a Schott and Company "glass filter" (carried by the Empire Laboratory Supply Company) or in a porcelain cup (27) and covered with a fat-free paper or cotton. The thimble and sample are placed in the extractor6 and elevated, if necessary, by means of a glass tube of sufficient height so that the surface of the material is about 1 cm. under the top of the siphon tube. This adjustment insures a more complete extraction of the sample.

The flask, containing several glass beads to prevent bumping of the solvent during extraction, is thoroughly dried by heating, cooled over sulphuric acid in a desiccator, and weighed with the aid of a counterpoise of nearly the same size and shape. A portion of the solvent, about 50 to 150 cc., is poured into the flask, which is then connected with the extractor holding the sample. A further quantity of solvent is carefully poured over the material in the extractor until the height of the liquid nearly corresponds to the level of the top of the siphon tube. A tube containing anhydrous calcium chloride to prevent absorption of water by the solvent is attached to the top of the condenser.

The solvent is boiled during the period of extraction, either on an electric hot-plate or on a steam bath. The vapors of the solvent liquify in the condenser, the condensate flows into the extractor, penetrates the material and dissolves the lipides. When the liquid reaches the top of the siphon tube, it should automatically return to the flask. With some materials it is desirable to discontinue the extraction after the greater portion of lipides has been extracted and to reduce the sample to a finer state for a second and final period of extraction. The material, after drying to remove traces of the solvent, is ground in a mortar with 1/2 to 2/3 of its weight of pure quartz sand and the finely ground mixture quantitatively replaced in the thimble. Final traces in the mortar are obtained by removing with fat-free cotton, which must be added to the main sample in the thimble. The extraction is then continued as before. In some plant tissues the removal of lipides is nearly complete in 4 to 6 hours, in others 16 hours or longer are required. No definite time limit can be prescribed in all cases. This must be experimentally determined. In general, it may be stated that the size of particles and degree of porosity of sample are important factors influencing the rapidity and completeness of the extraction with any particular solvent.

Removal of the solvent from the extraction flask upon completion of the extraction is easily performed by using the apparatus itself for the distillation. After removal of the sample and glass tube, the boiling is continued until most of the solvent is distilled into the extractor. If necessary the solvent is siphoned off into a second container and the process continued

6 Directions indicated apply to the Soxhlet apparatus.
until nearly all of the liquid in the flask has been evaporated. The final portions of solvent must be evaporated on a steam bath, care being exercised to prevent any spattering of the contents of the flask. Last traces of the solvent may be eliminated with a stream of pure dry carbon dioxide.

The residue in the flask is dried for 30 minutes at the temperature of boiling water, cooled over sulphuric acid in a desiccator and weighed. The drying is continued for 30-minute intervals, the flask being cooled and weighed each time, until the weight remains constant. If the material is very hygroscopic it may be necessary to dry at room temperature over calcium chloride in a vacuum desiccator until constant weight has been attained. If exactly 10 grams of dried substance are used as the original sample, the weight of the residue multiplied by 10 gives directly the percentage of fatty residue. This is sometimes reported as "fat" or "crude fat." It would be more nearly correct, however, to express the results as "crude lipides" or "ether extract."

General remarks.—When considering the ether extraction of dried samples according to the "Official method," it should be borne in mind that a portion of the lipides present in the fresh tissues inevitably suffers partial decomposition during desiccation. If the drying is carried out at the temperature of boiling water, and with exposure of the material to air, changes occur due to the partial oxidation of compounds containing unsaturated fatty acids. Such changes are minimized but not entirely eliminated if the drying is accomplished at reduced pressures or in an atmosphere of neutral gas.

Another error in the ether extraction method is due to the varying quantities of impurities which are removed from the tissues along with the true lipides and reported as such. The determination of lipides in cacao products furnishes an illustration. Here the use of ether results in the extraction of significant quantities of the alkaloid, theobromine. Other impurities such as hydrocarbons, coloring matters, and resin compounds are sources of error. Many of these may be determined and corrected for, where the quantity of residue is sufficient to justify an examination for unsaponifiable matter. In plant physiological work one is often limited with respect to quantity of available material, consequently the residue obtained from an ether extraction is usually too small to attempt further examination. In such cases it is desirable to have the residue represent as pure an extract of lipides as possible.

It is generally recognized that a small loss of lipides occurs through incomplete extraction. A portion of the lipides either becomes firmly entangled with carbohydrate and protein matter during the drying of the original sample, or the solvent is unable to penetrate the material properly
within a reasonable length of time. That the removal of ether soluble constituents is nearly always incomplete was shown by Pflüger (67) and by Dormeyer (16) in the case of dried animal materials. The difficulties experienced in removing lipides from plant tissues are not as serious as in the case of animal materials, since it is well known that lipides are held more tenaciously in animal tissues. Schulze and Steiger (82) and Maxwell (55) reported that phospholipides (phosphatides) can be completely removed from seed material only if the ether extraction is followed by successive extractions with alcohol at 60°. That an ether extraction of plant materials may be incomplete has also been pointed out by Rather (72), Hertwig (28) and by Rask and Phelps (71).

2. MODIFIED KOCH METHOD

Abstract of Method, First Procedure.—A sample of fresh material, weighing from 50 to 150 grams, is introduced into a sufficient quantity of boiling 95 per cent. alcohol to insure a final concentration of at least 80 per cent., after allowance for the original water content of the tissue. The mixture is boiled 15 minutes. The cold alcoholic liquid is filtered through extraction cups, and the residue exhaustively and successively extracted with alcohol, ether, and again with alcohol. The first alcoholic preserving filtrate and the combined alcohol and ether extracts are added together and evaporated to dryness. The residue is then thoroughly extracted with anhydrous ether. The extract, after the evaporation of ether, is dried to constant weight. The dried residue represents "crude lipides."

Details of Operation.—The fresh plant material to be analyzed is collected, rapidly cut into small pieces or otherwise reduced to finely divided state and thoroughly mixed to insure uniformity. Immediately after the material has been mixed, 50 to 150 gram duplicate samples are quickly and accurately weighed out and introduced into sufficient redistilled 95 per cent. boiling alcohol to bring the final concentration to at least 80 per cent., after allowance for the water originally present in the tissue. Boiling is continued for 15 minutes to arrest enzyme activity. Preserved in this manner, the material may be stored several months before the analytical work is begun, although it might be advisable to reheat the samples at least once or twice during the storage period.

The original Koch method was first employed in the analysis of animal tissues by Waldemar Koch (34, 35, 36). Later F. C. Koch, University of Chicago, introduced changes which were embodied in his unpublished student outline for the analysis of tissues. Students of plant physiology have made further modifications (1, 13, 32, 92, 79). The modified Koch method provides for the separation of the constituents of tissues into a number of fractions which are separately examined. In the present paper discussion is confined to the so-called "lipoid" fraction.
For the extraction of the alcohol-preserved material, the insoluble residue is quantitatively transferred to Schleicher and Schüll fat-free paper extraction thimbles or to porcelain cups which are perforated on the bottom and fitted with filter-paper. If paper thimbles are used they may be placed upright in funnels in such a way that the liquid drips through the cups into flasks. The porcelain cups are suspended by clamps over the flasks or beakers to accomplish the same purpose. In transferring the contents of the containers into the cups, the greater part of the material is poured into a beaker and the supernatant liquid from this used to transfer adhering matter from the container to the cup. The whole sample is then carefully removed to the cups, the last trace of substance being washed out with a jet of hot 95 per cent. redistilled alcohol. The alcoholic filtrate is set aside to be added later to the other portions of the extract.

The well-drained material in the extraction cup is covered with fat-free cotton or filter-paper, placed in a continuous extractor and extracted 4 to 12 hours with 95 per cent. alcohol. The alcoholic extraction is followed with an ether extraction for a period of 1–12 hours. Following the ether extraction the sample is freed of ether and powdered as finely as possible in an agate or glass mortar or small coffee mill. The powdered material is then carefully collected and replaced in the apparatus and extracted further for a period of 12 to 24 hours with redistilled 95 per cent. alcohol. The several alcohol and ether extracts including the original alcoholic preserving filtrate are now combined. In the case of the ether extract most of the solvent is removed by evaporation and the residue transferred to the alcoholic portions with aid of hot alcohol followed by a little hot water. Any solid matter adhering to the flask after this treatment may be removed with the aid of a little chloroform and finally hot water. The combined extracts are then evaporated to small bulk as rapidly as possible, without over-heating, on the steam bath, quantitatively transferred to an Erlenmeyer flask, and the evaporation continued until all of the liquid is driven off. Evaporation should be hastened by the use of a suction tube extending into the mouth of the flask and by adding absolute alcohol from time to time. The residue is finally dried over calcium chloride in a vacuum desiccator. At frequent intervals the desiccator should be filled with dry air and again exhausted.

The transfer to the Erlenmeyer flask is performed to facilitate subsequent extraction with ether of the dry residue. This extraction may be 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 flask. The filtered extract is evaporated to dryness
on the steam bath and the residue dried to constant weight at the temperature of boiling water as in the "Official method." From the weight of the residue so obtained the percentage of "crude lipides" in the original sample may be calculated. A moisture determination must be made on a duplicate sample of the original material if a dry weight calculation is desired.

Abstract of Method, Second Procedure.—The sample is preserved in 80 per cent. alcohol as described under the first procedure. The insoluble residue, after separation from the alcoholic preserving liquid, is successively extracted with alcohol, ether, water and again with alcohol. The combined extracts are evaporated and the residue made into an emulsion with water. The aqueous emulsion is treated with chloroform and dilute hydrochloric acid. The mixture is well shaken and allowed to remain in a cool place until the aqueous portion becomes clear. This is drawn off through a filter. The lipide portion remains dissolved in the chloroform layer or as insoluble matter adhering to the walls of the flask and to the filter-paper. These three separations are brought together with aid of hot alcohol and the entire volume of liquid evaporated to dryness in a weighed flask, or made up to definite volume with hot alcohol after the evaporation of the chloroform, and aliquot portions taken for dry weight determination.

Details of Operation.—Following the ether extraction as described under the first procedure, the insoluble residue is finely ground in an agate or a glass mortar or coffee mill. The powdered material is transferred to a flask and heated on the steam bath with about 100 cc. of water. Sufficient 95 per cent. alcohol is then added to bring the final concentration of alcohol to about 85 per cent. and the heating continued for another period. The mixture is then filtered through paper extraction thimbles, the residue being quantitatively transferred from the flask to the thimbles and extracted continuously with fresh 95 per cent. alcohol for 12 to 24 hours. The united alcohol, ether and water extracts are then evaporated on the steam bath at 75° to a syrupy consistency. The residue is taken up with water and the aqueous emulsion is transferred to a glass-stoppered mixing cylinder or a volumetric flask having a capacity of one liter for each 100 grams of the sample. To wash adhering substances from the evaporation dish, small portions of chloroform are used which are added to the emulsion. The total volume of these washings should not amount to more than 20 or 25 cc. The flask or cylinder containing the emulsion is then filled to the mark with aqueous hydrochloric acid of such strength that the final volume

8 Certain modifications are necessary from this point on if it is the intention to make determinations other than lipides on the same material, as outlined in the original method. For these consult Culpepper, Foster and Caldwell (13).
of liquid will contain 2 or 3 cc. of concentrated acid for each 250 cc. The mixture is vigorously shaken at frequent intervals for 2 hours, then placed in an ice-box for 24 hours or until the aqueous layer has separated and become quite clear. This is drawn off by gentle suction through a long tube of small bore. By exercising great care, the aqueous solution can be separated almost completely from the chloroform layer. Should any solid matter be drawn off, it should be collected by filtering through a dry ashless filter-paper and washed with a little water. The lipide matter will be found in the chloroform layer and adhering to the walls of the precipitation flask and to the filter-paper. The tip of the latter is perforated and any solid matter adhering to the paper washed into the precipitation flask with a jet of hot 95 per cent. alcohol. The contents of the flask are then quantitatively transferred to a weighed flask and evaporated to dryness on the steam bath. The residue is dried to constant weight at the temperature of boiling water, or else at room temperature in a vacuum desiccator over calcium chloride. This represents the total quantity of "crude lipides." (The method of determining the lipide fraction by separating the aqueous layer from the chloroform fraction as described is not applicable to all materials. In such cases it is necessary to follow the procedure indicated in Koch's student outline for the analysis of tissues to which reference has previously been made). In many cases it is desirable to determine in addition, the lipide phosphorus, lipide nitrogen and other constituents. For this purpose, the residue is dissolved in hot 95 per cent. alcohol and transferred to the volumetric flask. Ordinary redistilled alcohol is then added until the flask is about half full and then absolute alcohol to about two thirds full. The mixture is heated with gentle agitation on the steam bath until solution is uniform throughout, when the flask is filled to the mark with hot 95 per cent. alcohol. Aliquot portions are collected immediately for the various determinations, care being taken that the pipette for removing the liquid is first heated to the proper temperature in an oven or over a water-bath. Another modification which may be considered as a means of separating the lipide material from the other active ingredients is that of shaking the water emulsion of the alcohol-ether soluble material with pure ether at least four times or until a fresh ether extract no longer contains lipide material. The combined ether extracts are then shaken with distilled water to remove traces of water-soluble constituents. The ether extract is finally evaporated to dryness and the residue brought to constant weight as previously indicated.

General remarks.—The fact that the modified Koch method makes use of fresh instead of dried material is the chief point in its favor. If the sample is heated immediately with alcohol as described there is very little
The likelihood of autolytic changes. Partial oxidation of certain unsaturated compounds may go on to a slight degree even with hot alcohol treatment, but the error from this source is probably much less than in any method where it is necessary to dehydrate the sample by exposure to heat and air.

Another distinct advantage of the method is the provisions which are made for the systematic extraction and separation into definite fractions for the determination of other substances, in addition to the lipides, from the same material. These determinations have been discussed elsewhere.

The use of both alcohol and ether as the first stage in the removal of lipides is perhaps preferable to the use of ether alone, since it is generally recognized that extraction with ether, no matter how prolonged, fails to completely separate all of these substances, especially the phospholipides, from the other tissue constituents such as the carbohydrates and proteins. The incomplete extraction is particularly evident with material high in starch. Leathes (43) states in referring to the estimation of fats that "the use of the alcohol for the first stage of the extraction has the advantage of removing water, and leaving the tissue in a condition to give up its fat more completely to ether; but, besides this, alcohol seems effectually to liberate fat from cells in a way not clearly understood, possibly counteracting the tendency for some fatty substances to adhere to surfaces in the presence of water, much as alcohol will remove a dye that has adhered to charcoal suspended in water."

One objection to the use of alcohol is the fact that it simultaneously removes along with the lipides most of the soluble carbohydrates which, unfortunately, interfere to a certain extent with the subsequent direct extraction of the syrupy residue with ether. This difficulty is not encountered in the second procedure in which the fatty compounds are emulsified and then separated from other constituents with chloroform and dilute hydrochloric acid. The use of dilute hydrochloric acid at this stage does not appear to have any hydrolytic effect on the lipides (33), but the whole second procedure is rather lengthy and bothersome.

3. KUMAGAWA-SUTO method

Abstract of method.—The fresh or dried plant material, or an alcoholic extract thereof, is saponified several hours with caustic soda solution. The mixture is then neutralized with acid, cooled, and thoroughly shaken out with ether to remove the fatty acids formed. The filtered ether extract is evaporated and the residue again taken up in absolute ether. The dry residue from this extract is finally dissolved in petroleum ether, the solvent evaporated, and the residue dried to consistent weight at 50° in a vacuum, cooled and weighed. This represents total crude fatty acids. A determination of unsaponifiable matter is made on this residue and the weight so
obtained subtracted from the total crude fatty acids, which gives the weight of true fatty acids. This figure multiplied by the factor 1.046 gives the approximate weight of the corresponding neutral glycerides.

Details of operation.—Either of two procedures may be followed in the method, (a) direct saponification of the material, and (b) alcoholic extraction with subsequent saponification of the alcohol extract. For direct saponification the material may be either fresh or in powder form. A sample of fresh material, weighing from 5 to 20 grams, is treated with 7 to 8 cc. of strong sodium hydroxide solution (sp. gr.=1.5). If 5 grams are used, an additional 14 cc. of water are added. With 10 grams, 10 cc. of water are added, while above 20 grams it is unnecessary to make any further additions of water, the quantity of moisture present in the original tissues being sufficient. With dried material, 2 to 5 grams are treated with 25 cc. 5 N. sodium hydroxide solution (20 grams per 100 cc. of water).

The material and alkali in a 150 to 200 cc. beaker are placed on a boiling water-bath and covered with an open bell jar. At the end of 2 hours the warm mixture is quantitatively transferred to a 250 cc. separatory funnel. The last traces are removed from the beaker with 2 or 3 washings with small quantities of warm water. To the mixture in the separatory funnel, 30 cc. of a 20 per cent. hydrochloric acid solution (sp. gr.=1.1) are slowly added, with cooling. When thoroughly cool, the acid liquid is vigorously shaken with successive portions of ether. For the first shaking, 70 to 100 cc. of ether are used. The second and third require only 5 to 10 cc. for each shaking. The aqueous layer is drawn off each time and the ether layer collected in a beaker. During the foregoing shaking operations, a precipitate forms in the funnel at the point of partition of the two layers. This precipitate is dissolved in 5 cc. of N. sodium hydroxide solution. The alkaline solution is shaken with 30 to 50 cc. of ether. Then the first aqueous acid liquid is added and the whole vigorously shaken. The separated ether layer is added to the other ether portion and the united extracts evaporated. The dry residue is then redissolved in absolute ether. The ethereal solution is filtered through a specially constructed asbestos filter (39) and evaporated to dryness. The residue is dried at 50° for 1 to 2 hours. Drying longer than this period should be carried out in a vacuum. The residue contains, besides fatty acids, also hydrocarbons, sterols, coloring matters and other substances. Some of these impurities are removed at this stage. To the residue while still warm 30 to 40 cc. of petroleum ether are added and gently rotated. The beaker is then covered with a watch glass and set aside ½ to 1 hour to permit the settling of resinous matter. The fatty acid solution is filtered through asbestos which is well washed with petroleum ether. The filtrate and washings are collected in a weighed
100 cc. beaker and evaporated. The residue is dried to constant weight at 50°, preferably in vacuo. This weight represents crude fatty acids.

It is particularly important that care be taken to sufficiently dry the residue from the ether extract before extraction with petroleum ether, in order to obtain the fatty acids in pure colorless form. The ether used for the last extraction, as well as the petroleum ether, should be pure and anhydrous. The time for drying the residue at 50° must not exceed 1 to 2 hours unless the drying is carried out under reduced pressure.

With many plant materials, especially those rich in starch and other polysaccharides, it is necessary first to make an alcoholic extract (b) before proceeding with the saponification of the lipides. The sample is extracted with absolute alcohol in the KUMAGAWA and SUTO hot extractor (39) until all but traces of lipides have been removed. After the addition of 7 to 8 cc. of a strong sodium hydroxide solution (sp. gr. = 1.5) to the alcoholic extract, the mixture is boiled under a reflux condenser for an hour. The alcohol is then allowed to evaporate, the saponification continuing meanwhile. When the alcohol has been removed, the residue is taken up with a little warm water and quantitatively transferred to a separatory funnel. The fatty acids are separated as described under the direct saponification procedure (a).

The residue of the original material which has been extracted with alcohol may still retain a small quantity of unextracted lipides, but in most cases this is too small to be significant. It may be recovered, however, by treating the residue essentially according to the direct saponification procedure (a), the only modification being that after neutralization of the saponification mixture the starch is hydrolyzed with strong acid to liberate any retained lipides.

To separate unsaponifiable matter from the final residue obtained as described in either method, the residue is redissolved 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 requires 50 to 70 cc. of petroleum ether. Thirty to 40 volumes of N/5 nearly absolute alcoholic potash are added to the solution in the funnel which is then well shaken once. A clear solution results. A quantity of water is added to this equal to the volume of alcoholic potash used, whereby a separation occurs with the 50 per cent. alcoholic liquid as the bottom layer and the petroleum ether solution as the top layer. The unsaponifiable substances remain in the top layer, while the soaps go to the dilute alcoholic layer. After separation of the solution, the dilute alcoholic layer is again extracted with 30 to 50 cc. of new petroleum ether. After evaporation of the petroleum ether from the combined shakings the residue is freed from the small traces of fatty acids which it contains. This separation is accom-
plished 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° and then extracting with hot petroleum ether. The petroleum ether extract is filtered through asbestos into a weighed flask, evaporated and dried to constant weight at 100°. This weight represents total unsaponifiable matter. It must be subtracted from the weight of total crude fatty acids previously obtained to find the quantity of true fatty acids. The weight of true fatty acids multiplied by the factor 1.046 gives the weight of the neutral glycerides.

The above-described method of KUMAGAWA and SUTO has been modified by LEMLAND (45). Very briefly, the LEMLAND procedure consists in extracting the material 8 hours in the KUMAGAWA and SUTO extractor with alcohol, evaporating and drying to constant weight in vacuo, saponifying the residue, extracting and weighing the unsaponifiable matter, acidifying and extracting the residual soap solution and drying to constant weight and weighing the fatty acids. According to TERROINE, LEPAGE, VÉCHOT and WOLFF (86) this method is directly applicable to vegetable products provided they have been finely ground before extraction.

**General Remarks.**—To convert numbers representing the weight of fatty acids into numbers representing the weight of neutral fat, KUMAGAWA and SUTO employ the factor 1.046. This 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 corresponding triglycerides. The following, which is taken from KUMAGAWA and SUTO (37) indicates how the factor was obtained:

| Molecular weight of triolein | 884.8 |
| Molecular weight of tristearin | 890.9 |
| Molecular weight of tripalmitin | 806.8 |

\[
\text{average} = \frac{884.8 + 890.9 + 806.8}{3} = 860.8
\]

\[
\text{(oleopalmitostearate)}
\]

| Molecular weight of oleic acid | 282.3 |
| Molecular weight of stearic acid | 284.3 |
| Molecular weight of palmitic acid | 256.3 |

\[
\text{total} = \frac{860.8 + 860.8}{2} = \frac{882.9}{2} = 1.046 = \text{factor}
\]

Strictly speaking, not all of the insoluble fatty acids, as determined by saponification, are derived from simple triglycerides, nor are the quantities of these fatty acids always present in equal proportions. Furthermore,
fatty acids other than the three mentioned may occur in the extract. In many cases an appreciable percentage of the total fatty acids may be obtained by hydrolysis of waxes and phospholipides which contain different percentages of fatty acids than do the simple glycerides or fats. These are sources of errors which probably cannot be overcome since it is impossible to know definitely the relative proportions of different classes of lipides giving rise to fatty acids on saponification.

In this method, the volatile fatty acids are lost and only the high-molecular fatty acids are determined. KUMAGAWA and SUTO point out that in the saponification method of LIEBERMANN and SZÉKELY much too great a quantity of low fatty acids are determined, and furthermore about 9 per cent. of the high-molecular acids escape determination.

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