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Semimicro Determination of Total Fatty Acids and Unsaponifiable Matter[1]

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A semimicro method for determining both total fatty acids and unsaponifiable matter in fats, fatty acids, and soap is presented. The procedure involves saponification and recovery of the total fatty acids (which includes the unsaponifiables), removal of the fatty acids with anion exchange resins, and determination of the unsaponifiable matter by weight.

The total fatty acid method was evaluated on seven samples and showed a standard deviation from the mean of 0.21%. The unsaponifiable matter determination gives good reproducibility with a standard deviation from the mean of 0.06%. Since this ion exchange is a direct determination of the nonionic components, the results obtained from it should be more accurate and more nearly the true values than the macro extraction method which is empirical.

UNSAPONIFIABLE MATTER is a variable mixture of hydrocarbons, aldehydes, ketones, alcohols, steroids, pigments, and fat-soluble vitamins which may occur naturally in fats and oils or may arise from processing or degradation of the fat. The level of these materials in fats and fat products can be related to the nutritional value, or to storage conditions and handling, or to the extent and nature of the processing. Thus this analysis is an important one in characterizing a fatty sample.

Frequently in research and development the amount of sample that can be used in analytical characterizations is small because of the small quantity produced, or the number of other performance tests required, or the difficulty encountered in obtaining or isolating the material. Since the regular macro method (6) requires a 5-g. sample, a semimicro method employing only one-tenth the amount or less is necessary for the limited materials.

The reduction of macro procedures to the semimicro scale generally involves a scaling down of reagent volumes and equipment size. However it is frequently necessary to adapt or to modify solutions or conditions so that the methods are readily usable in the particular operations. The well-known functional group analyses, such as acid value and saponification value, have been established as routine semimicro procedures, using readily available equipment. Some of these semimicro methods are presented in Table I with the range of sample weights employed and the standard deviation from the mean calculated from replicates examined over a one-year period. The largest sample weight is 150 mg. except for the saponification value, which employs 200 mg. for values in the range of zero to 20. As is typical of macro methods, the sample weight decreases as the expected result increases.

The macro method for unsaponifiable matter (6) has specified sample size and volume of solvent, and any appreciable deviation from these conditions can lead to significantly different results. When the conversion of this method to the semimicro scale was attempted, considerable difficulty was encountered in achieving consistent results. It was therefore decided to try a new approach. If fats are saponified and the fatty acids and unsaponifiables are extracted, these materials could then be treated with an anion exchange resin to yield only the unsaponifiable components. Such a procedure would not be dependent upon extraction conditions nor would it require corrections for extracted fatty acids.

TABLE I

Semimicro Analyses

Analysis	Range	Mg. of sample	Standard deviation
Acid value	0–20	150	0.1
	21–100	125–25	0.5
	101–250	25–10	0.8
Hydroxyl value	0–100	150	1.2
	101–200	120–60	2.4
	201–300	60–40	3.5
Iodine value	0–20	150	0.3
	21–100	130–25	0.4
	101–150	25–15	0.7
Saponification value	0–20	200	0.5
	21–100	175–50	0.8
	101–200	40–15	1.5
Monoglyceride, %	0–30	150	0.6
	31–60	140–70	0.6
	61–100	70–30	0.8

Ion exchange separations of the fatty acids have not been reported for analytical purposes. However such separations have been used for isolating various materials. Mattson and Beck (4) separated and isolated the free fatty acids from various partial glycerides with Amberlite IRA-400. Cason *et al.* (2) purified amides by removing the free fatty acids on an ion exchange column. Chromatography of unsaponifiable extracts has also been tried; alumina columns were used to remove free fatty acids instead of performing the usual titration and correction (3, 5). But this technique has never gained favor for routine determinations.

A semimicro total fatty acid determination, which is similar to the official A.O.C.S. method for soaps (7), is a useful test for many fatty materials. If only total fatty acid is required on a sample, then as little as 50 mg. are used; when run in conjunction with the unsaponifiable-matter analysis, 200 mg. are employed.

Two types of extraction apparatus performed satisfactorily in these analyses; these are shown in Figure 1. The apparatus on the left requires multiple extractions but is faster and gives better control of the operations; however it involves more manual effort and more analyst time than the continuous unit on the right. The continuous extraction is a small-scale modification of the unit described by Buerki and Holt (1). With the left unit the soap solution is placed in the lower, narrower section, and the water is adjusted to just below the side arm. The extracts are drained through the side arm.

The advantages of this extraction unit compared

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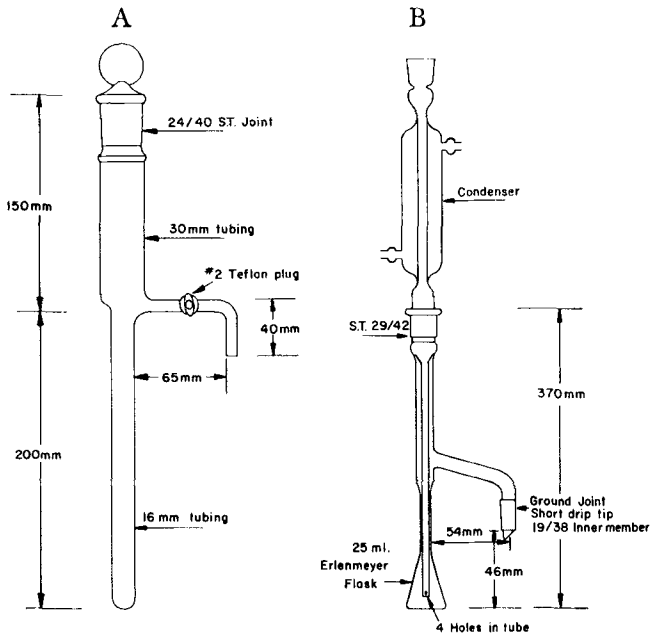

FIG. 1. Extraction apparatus: A. batch-type unit; B. continuous extractor.

to small separatory funnels are a) greater ease in handling, b) fewer transfers because only the ether solution is removed, and c) smaller interfaces at the time of separating phases but comparable interfaces during extraction.

The continuous extractor on the right is more readily suited for routine operations. Contrary to the apparent experience of other workers with macro extractors, the semimicro units have given reproducible results with less analyst time than is required for any other type of extractor. However more equipment and more handling are necessary for this technique.

The unsaponifiable-matter determination employs a strongly basic anion exchange resin. A mixed solvent was necessary for the ion-exchange treatment in order to keep the various materials in solution and to permit the fatty acids to combine with the resin. Although moist ethyl ether can be used (2), the system ethyl ether:acetone:water (5:5:1) has greater applicability.

The ion exchange columns which have been used successfully in our laboratories for this and other analyses are shown in Figure 2. The A unit with its separate reservoir and stopcock is a more versatile column. It is applicable to a greater variety of solvents and changes in conditions. The stopcocks in the A unit permit variable control of the flow rate and more uniform flow-conditions. The system is not limited to the height of solvent in the reservoir for flow rate. Thus the A column is better suited for analytical development work and special analyses.

The "B" unit is simpler in design and thereby easier to handle. With replicate analyses under the same conditions this column shows good reproducibility. The big advantage in routine operations is that the analyst does not have to worry about the column running dry while he is engaged in other laboratory operations. Thus for general routine work, this B column is better.

The detailed methods and a discussion of results are presented below.

Experimental

Reagents and Apparatus. The anion exchange resin is Dowex 2-X8, 20-50 mesh. The other reagents are reagent grade or comparable quality.

Pear-shaped acetylation flasks, 25-ml. capacity, with air condensers are employed for the saponification. Either the batch-type of extractor or the continuous extractor shown in Figure 1 may be used. A chromatographic column similar to either one shown in Figure 2 should be used for the ion exchange separations.

Mixed Solvent. The solvent employed for the ion exchange separation consists of five parts of acetone, five parts of ethyl ether, and one part of distilled water. The acetone and water are mixed first; then the ethyl ether is added.

Preparation of Ion Exchange Column. The following quantities of material are used to prepare one column. When more than one column is required, the quantities are increased proportionately.

To convert the resin to the free base form, approximately 20 ml. of Dowex 2-X8 resin are mixed with 90 ml. of 10% KOH in 80% alcohol. The alkali solution is decanted, and the resin is rinsed with distilled water, then with 80% ethanol, and finally with the mixed solvent. Extraneous materials are removed from the resin by repeating this treatment several times. Next, stearic acid (0.2 g.) in 50 ml. of mixed solvent is stirred with the resin. After decanting this solution, the resin is reprocessed with alkali as described above.

Enough of the prepared resin slurried in 80% ethanol is transferred to the chromatographic tube to make a 9 to 10-in. column. The excess alcohol is drained from the tube as required. The solution is not allowed to drain below the top surface of the resin at any time. The excess resin is stored in 80% ethanol and used to replace any resin lost in subsequent regenerations or transfers.

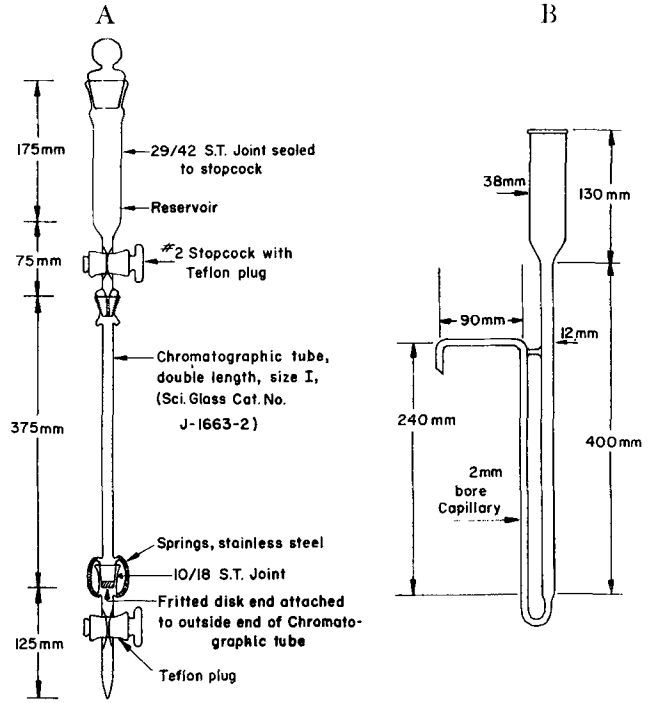

FIG. 2. Ion exchange columns: A. straight chromatographic column; B. self-leveling column.

Approximately 100 ml. of the mixed solvent are allowed to drain through the column at a slow rate of 2 ml. per minute. At the end of this treatment there should be no air bubbles in the chromatographic tube.

A solvent blank is run on each column by allowing 50 ml. of the mixed solvent to drain through the column into a tared 60-ml. weighing bottle at the rate of 8–10 ml. per minute. The solvent was evaporated by gentle heating while sweeping with nitrogen. The residue weight should be less than 0.3 mg. If not, the column is regenerated and the solvent blank is redetermined until the column is satisfactory.

The completeness of the fatty acid retention by the resin is checked by passing 0.2 g. of reagent grade stearic acid in 50 ml. of mixed solvent through the column at the rate of 8–10 ml. per minute. The eluate is evaporated to dryness and weighed. This residue weight minus the weight of the solvent blank should be less than 0.3 mg. for routine analyses. If this residue is greater than 0.3 mg., then the column is regenerated and the fatty acid retention is redetermined until the column is satisfactory. The regeneration can be performed with the resin in the column. When not in use, the resin is covered with 80% alcohol.

A well-prepared column should be satisfactory for at least 20 determinations.

Total Fatty Acid Determination. The sample (0.2 g.) and 2.0 ml. of 1N alcoholic KOH are placed in an acetylation flask with an air condenser. The mixture is heated on a steam bath for 40 min. The air condenser is then removed, and 2 ml. of distilled water and 0.5 ml. benzene are added. The mixture is heated with nitrogen sweeping to evaporate the alcohol. Occasionally it is necessary to disperse the solids around the sides of the flask by gentle tapping. The evaporation is continued until their is no odor of alcohol remaining. Evaporation to dryness, while satisfactory, is not necessary.

Ten ml. of warm distilled water are added to dissolve the soap, and the solution is transferred to the extraction apparatus. The flask is rinsed with 2.4 ml. of 1N aqueous HCl, and this solution is added to the extractor. The water level is adjusted with additional small amounts of water. After the solution is cool, it is extracted with ethyl ether and drained into a 60-ml. weighing bottle. The extraction is repeated four additional times with fresh quantities of ethyl ether. The ether is removed by mild heating, and the residue is weighed. A blank is run with each group of samples.

$$\% \text{ Total fatty acid} = \frac{(\text{residue weight} - \text{blank weight})}{\text{sample weight}} \times 100$$

Unsaponifiable Determination. The dry sample residue from the total fatty acid determination is dissolved in 30 ml. of the mixed solvent and transferred to the ion exchange column. A blank is run through the entire procedure with each group of samples. The solution is percolated through the column at 6–10 ml. per minute into a 60-ml. weighing bottle. When necessary, additional quantities of the mixed solvent are added to the column until 50–55 ml. of eluate are collected. The Teflon stopcock is rinsed after the last addition of solvent by gently pulling the plug out approximately 1/16 in. from its seat and quickly releasing so as to reseat itself.

TABLE II

T.F.A. Content of Some Fats

Sample	% Total fatty acid		Standard deviation
	Expected	Found	
Tripalmitin	95.29	95.30	0.14
Distearin	91.03	91.01	0.34
Monopalmitin	77.59	77.96	0.11
Tallow	95.53	95.05	0.25
Stearic acid	100.0	99.64	0.16
Lauric acid (SS)	100.0	99.52	0.27
Cholesterol	100.0	99.98	.....

The solvent is evaporated by mild heating under gentle nitrogen sweeping and the residue weighed.

$$\% \text{ Unsaponifiable Matter} = \frac{(\text{weight of residue} - \text{weight of blank})}{\text{sample weight}} \times 100$$

Results and Discussion

The total fatty acid (TFA) content can be determined either separately or as a part of the unsaponifiable matter estimation. If only TFA is required, then as little as 50 mg. of sample are employed; otherwise 200 mg. are used. When running low-molecular-weight fatty acids (those containing 10 carbons or less) which are volatile under the conditions employed, a modification of the described procedure is employed. The extracted acids are neutralized with 0.1N alcoholic potassium hydroxide to the thymol blue end point, evaporated to dryness, and weighed. The extract weight is corrected for the alkali used, and the TFA is calculated.

The evaluation of the total fatty acid method (which includes unsaponifiable matter) with replicate analyses of seven samples is given in Table II. The three glycerides are synthetic preparations and represent relatively pure materials of known composition. The agreement between the found values (means of replicate determinations) and the expected or calculated results is good. The last four materials are commercial samples, and the true values are not known. The lauric acid was, of necessity, run by the soda soap procedure. Cholesterol, an unsaponifiable material, was tested because it is one of the most difficult unsaponifiables to extract and therefore the most likely to give trouble. Good agreement was obtained among all found and expected values. The standard deviation from the mean calculated from at least six determinations per sample is comparable to the macro results. The over-all standard deviation is 0.21%.

The semimicro determination of unsaponifiable matter was evaluated by recovery tests with cholesterol, and a synthetic unsaponifiable mixture was added to stearic acid and then analyzed by the complete procedure including the saponification and extraction.

Cholesterol alone was added in five of the determinations, and a synthetic mixture of cholesterol, fatty alcohol, and squalene was used in the other three. The

TABLE III

Recovery of Unsaponifiables from Stearic Acid

Material added	Mg. unsap.		% Recovery
	Added	Found	
Cholesterol	1.06	1.16	109
Cholesterol	1.43	1.46	103
Cholesterol	1.55	1.47	96
Cholesterol	2.05	2.12	103
Cholesterol	2.19	2.32	106
Mixed unsap.	1.17	1.19	102
Mixed unsap.	1.92	2.00	104
Mixed unsap.	2.33	2.23	96

amount of unsaponifiables found is corrected for the 0.13% originally present in the stearic acid. The data are presented in Table III. Good recoveries were obtained in all cases, and the average deviation from theory is ± 4%. This is equivalent to a standard deviation at the 1% level of unsaponifiable of only 0.05%.

The reproducibility for replicate analyses of three different tallows, a coconut oil, and a soap is presented in Table IV, and the results are compared to the macro method values.

TABLE IV

Unsaponifiable Content of Some Fats

Sample	No. of runs	% Unsap.		
		Semimicro	A.O.C.S. Ca 6a-40	Std. dev.
Tallow A	6	0.79	0.57	0.12
Tallow B	10	0.83	0.55	0.05
Tallow C	2	0.58	0.45	0.04
Coconut oil	4	0.40	0.39	0.05
Soap	3	0.45	0.44	0.04

The semimicro method gave the same or slightly higher unsaponifiable values than the macro procedure, A.O.C.S. Ca 6a-40. Since the semimicro (ion exchange) method is a direct determination of nonionic material whereas the macro (extraction) method is empirical, the semimicro results should be more accurate and more nearly true values. The reproducibility of the semimicro method is good and is comparable to that obtained by the macro method. The standard deviation from the mean for the five samples was 0.06%.

Twenty-three additional determinations on fat and oil samples were run under routine analytical conditions. The standard deviation from the mean for the range 0–2% unsaponifiable matter was 0.06%.

When soaps are examined for unsaponifiables plus unsaponified materials, the sample is dissolved in warm water and extracted with ethyl ether without any saponification. The ion exchange procedure is the same as that in the method.

The unsaponifiable determination is applicable to all normal fats and oils (both crude and refined), to partial glycerides and esters, and to fatty acids and soaps containing no more than 22 carbon atoms. Severely oxidized fats or polymerized fatty acids cannot be analyzed because these fatty acids are not retained by the ion exchange resin. Some modification of the eluting system could possibly give better retentions.

This method has been used in various nutritional studies and should find application in clinical investigations. Where it is desirable to use this technique on a preparative scale or where larger samples are to be used, proportionate increases in reagents and equipment were found to give satisfactory results.

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Thermal Dimerization of Fatty Ester Hydroperoxides[1]

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FORMATION of conjugated fatty hydroperoxides as initial products in fat autoxidation is well established (1,6,8,22,23,28). However knowledge on the subsequent decomposition of hydroperoxides is relatively incomplete. Some of the confusion in the literature arises from the great variety of conditions of decomposition used by different workers.

Thermal decomposition of fat hydroperoxides produces a variety of scission products (3,10,13,19) and high-molecular-weight material, principally dimeric in nature (7,8,27,31,35,36). When decomposition is carried out at relatively low temperatures and in the presence of oxygen, dimeric products appear to be oxygen-linked. By molecular distillation Swern *et al.* (31) isolated polymeric fractions from methyl oleate autoxidized at 65°C. in the presence of cobalt oleate. To account for the excessive oxygen they believed that the polymers were oxygen-linked and assumed an ether linkage since the polymers were resistant to saponification. Chang and Kummerow (7) and Witting *et al.* (36) used solvent extraction to isolate polymers from ethyl linoleate and linolenate, respectively, which were autoxidized at 30°C. They were able to split the polymers into monomeric units with concentrated halogen acids, thus indicating an oxygen-linkage.

When decomposition of fat hydroperoxides is carried out at temperatures above 100°C. and in an inert atmosphere, the dimeric products appear to be linked by a carbon-to-carbon bond. Williamson (35) decomposed autoxidized methyl linoleate (33°C., U.V. light) by heating it at 100°C. in nitrogen. Dimers isolated by solvent fractionation and molecular distillation contained monomeric units linked by carbon-to-carbon bonds since they were not cleaved with hydrogen iodide. In these studies the isolation procedures were complicated by the presence of incompletely decomposed fat hydroperoxides and other products of autoxidation similar in solubility characteristics to the polymerized material. It is difficult to isolate polymerized material by solvent fractionation of autoxidized mixtures without contamination

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