cient predominates in okra seed oil, thus giving a lower calculated value for linoleic acid. At present it is impossible to say which is the correct assumption. However, the evidence seems to favor the belief that a position isomerism, instead of a cis-trans type, exists in the linoleic acid of okraseed oil.

So far as is known the natural occurrence of an isomer of linoleic acid which will not isomerize to the conjugated position when heated with alkali in the manner described above has not been reported before. Edwards and Miller (3) have mentioned a similar discrepancy between the values obtained on okraseed oil by the spectrophotometric method and those obtained by other accepted methods of analysis, but no data are given. Similar discrepancies have been obtained by other investigators during spectrophotometric studies on sesameseed oil. Such an isomer, 9,15-linoleic acid, has been postulated as being present in reverted hydrogenated soybean and linseed oils and is believed to arise from the partial hydrogenation of the linolinate fractions of these oils. The natural occurrence of this, or a similar isomer with isolated double bonds, is entirely a reasonable possibility and is strongly indicated by our observations on okraseed oil.

We are continuing our investigations on the fatty acid composition of okraseed oil for the purpose of proving or disproving the presence of such a linoleic acid isomer in this oil and to positively identify the other unsaturated acids accounting for the amount of iodine absorbed.

If such an isomer occurs naturally in oils, it may place limits on the applicability of the spectrophotometric method of fat analysis or may necessitate modifications in the presently accepted technique.

Summary and Conclusions

Okraseed have been analyzed for their principal constituents. The characteristics of the oil have been determined, and it has been analyzed to determine its fatty acid composition. Characteristics of the oil as determined in this laboratory agree well with values previously reported by other investigators. However, our study of the oil components shows a considerably different fatty acid composition from that recorded in the literature. Myristic acid is reported present for the first time, and the possible presence of palmitoleic acid is indicated. The amount of linoleic acid determined spectrophotometrically does not agree with the percentage arrived at by other accepted methods of analysis. Evidence exists which suggests the presence of an isolated double bond isomer of linoleic acid in okraseed oil. Further work is in progress.

Okraseed can be produced in sufficient yields to make it a profitable oilseed crop (3). The oil is suitable for edible purposes, particularly when hydrogenated and used in shortening or margarine manufacture (3). Extracted okraseed meal should be a desirable protein rich food, being somewhat richer in protein than cottonseed and is not known to be toxic.

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Micro-Methods in Lipide Chemistry

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MOST of the methods of lipide chemistry have generally been applied to large samples of material and very little attention has been centered on micro-analysis. Techniques for the analysis of small samples of lipides have distinct and definite purposes in biology. In the analysis of small biological samples it is impossible to use classical lipide methods since these determinations require much more material than can often be obtained from a biological sample. Recently there has been considerable literature stressing the micro-analysis of lipides and their component parts. Due to the fact that this is the case and that the literature concerning these micro-analyses is widely scattered, it was felt that this is an opportune time to review the present status of the literature and to point out the way in which some of these analyses can be applied to biology.

This discussion will include the micro-analysis of main categories of lipide substances, the analysis of their component parts, and finally the analysis and determination of fatty acids.

Reviews which take up this work in part have been presented by Piskur (1) and Thannhauser (2).

Extraction

Stetten (3) has devised a liquid-liquid extractor which can be used for samples of about 10 mgms. in size and utilizes the principles employed in the Soxhlet extractor. Stetten employed this micro-extractor for various fatty acids and obtained complete extraction, in most cases, in three hours. The apparatus was applied, in this laboratory, to re-extract an alcohol-ether extract of lipide with petroleum ether (boiling point 30°-60°C.). Complete extraction was obtained in approximately two hours.

Bloor (4) has suggested a procedure for the extraction of approximately 50 mgms. of sample as follows

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the sample is poured in a fine stream (this extraction obviously can be utilized only for fluids or tissue suspensions) into a flask containing Bloor's mixture (3 parts 95% alcohol to 1 part of peroxide-free ethyl ether). The sample is boiled in Bloor's solution for about 10 minutes during which time a watch glass is placed on the top of the flask to keep the air out and to retain an atmosphere of alcohol and/or ether in the flask. After this extraction the alcohol-ether is evaporated down to almost dryness and the sample is re-extracted with petroleum ether. The petroleum ether can then be placed in a receptacle such as a tared 10-ml. volumetric flask and the weight of the lipide can be obtained after evaporation of the solvent under reduced pressure.

A micro-method for the extraction of fatty acids from one mg. of tissue has been presented by Schmidt-Nielsen (5). The tissue is treated with alcoholic KOH at 80°-100°C. for about two minutes in an ampulla, after which time 20 μl of toluene are added to extract the unsaponifiable fraction, and then the ampulla is sealed. The ampulla is centrifuged and the toluene is decanted. The soaps formed are acidified with 2.5 μl of a 5 N HCl and the fatty acids so obtained are extracted with 20 μl of toluene. Although in this procedure 1 mg. of tissue is utilized, a disadvantage exists in that special equipment is required for the extraction, i.e., micro-burettes, ampullae, and a micro-centrifuge.

Fats

The triglyceryl radical is the most distinctive constituent found universally in all natural fat molecules. Fat can be separated from the phospholipide by precipitating the phospholipides with acetone from alcohol, and the glycerol in the alcohol soluble portion can be determined. Through the determination of the glycerol portion of a fat an approximation of the amount of fat present can be obtained.

Glycerol can be determined by saponifying the fat, acidifying, and extracting the fatty acids from the aqueous phase with an appropriate organic solvent. The glycerol remains in the aqueous phase. It is possible with Bloor's method (6) to obtain an approximation of the amount of glycerol present by the use of chromate oxidation.

A spectrophotometric method was reported by Whyte (7) which can be used in the determination of 1,100-1,800 mgm. of glycerol per liter at a wave length of 630 mμ. The basis of this method is in the fact that glycerol forms a blue color when it is in the form of sodium cupriglycerol. In this method Whyte works with approximately 120-170 mg. of glycerol per 100 ml. Since there is now a micro-adapter for the Beckman quartz spectrophotometer (8) the volume can be decreased to 1 ml. in order that 1.20 to 1.70 mgms. of glycerol can be determined. There is also the possibility that this type of complex has a higher absorption in the ultra-violet spectrum which may aid in the use of smaller samples than the amounts cited.

Blix (9) and Voris (10) have reported methods for the estimation of neutral fat by determining glycerol oxidatively. These methods were successfully used on 5-20 mg. of fat.

Phospholipides

This major group of lipides is almost equally as important as triglycerides. The phospholipides lecithin, cephalin, and sphingomyelin are essentially the same as far as their chemical makeup is concerned. Probably the only chemical differences exist in the types of organic bases present and in the constituent fatty acids. On the basis of solubility the phospholipides can be partially separated from each other, but since no gravimetric micro-methods are being considered in this discussion, the identification of the various phospholipides is based primarily on the identification of their organic bases. It is also possible to determine phospholipides by utilizing the Fiske and SubbaRow method for phosphorus (11) which requires 12 micrograms of phosphorus, or the Ma-Zuzaga (12) micro-Kjeldahl which utilizes 50 micrograms of nitrogen for the determination.

The phospholipides which are most commonly found in nature are the lecithins and the cephalins. Lecithin contains the organic base choline, and it is by the determination of this substance that the amount of lecithin can be estimated. Glick (13) presented a method in which the choline is precipitated by Reinecke salt from a solution which is made just acidic to a 1% alcoholic solution of thymolphthalein, with glacial acetic acid. The precipitate is then collected on a sintered-glass funnel, washed with n-propanol, and finally dissolved in acetone and read at 526 mμ. By reading at this wave length it is possible to estimate as low as 1 mg. of choline. Winzler (14) showed that the Reinecke-choline complex also has a maximum absorption at 327 mμ and since the absorption coefficient was very high, as little as 100 micro-grams could be estimated with ease. It is possible by the use of small volumes to estimate as little as 20 micrograms of the choline reineckate. Since lecithin is the only phospholipide besides sphingomyelin which contains choline, it is possible to obtain a fairly good estimate of the amount of lecithin present by the use of the methods outlined by Schmidt *et al.* (15). Schmidt indicated that the phosphorus of monoaminophosphatides is or can be made acid soluble whereas that of sphingomyelin cannot be made acid soluble. If the lipide or phospholipide extract is treated with the N KOH first, and then with an excess of HCl and trichloracetic acid, the phosphorus from the monoaminophosphatides will be in the acid filtrate. Data on mixtures of phospholipide are presented to prove this point and the results agree within 3%. Thus the acid soluble phosphorus can be determined and subtracted from the total phosphorus to obtain an estimate of sphingomyelin. It is possible then to subtract from the total choline determination the amount of choline due to sphingomyelin, and the remaining choline is then equal to the amount of choline contributed by lecithin. From the choline an estimation of lecithin can be obtained. Thus it is possible to subtract from the acid soluble phosphorus that phosphorus contributed by lecithin. The remaining phosphorus is that contributed by cephalin. In a recent article by Burmaster (16) a micro-method is presented for the determination of serine and ethanolamine. This method utilizes the fact that the nitrogen in serine and ethanolamine is of an alpha amino type whereas that of choline is ammonium in nature. A micro-diffusion apparatus was constructed according to the directions of Bandemer and Schaible (17). The amino nitrogen is released through the action of periodic acid and is collected in .005 N HCl. With this method approximately 0.05-0.300 mgms. of amino nitrogen resulting from serine or ethanolamine can

be determined. The serine nitrogen may be determined by making use of the ninhydrin reaction and the ethanolamine nitrogen obtained by difference. Thus various cephalins can be separated through the determination of the amino nitrogen. Burmaster cites an experiment in which he adds lecithin and recovers little if any of the choline nitrogen.

Glycolipides

A class of compound lipides which have been little studied as far as methodology is concerned are the glycolipides even though their appearance is observed in many pathologic conditions.

Probably the composite method as presented by Macy (18) is the simplest. In this, 2.8 mg. or less of cerebrosides are treated and emulsified with alkyl sulfate and hydrochloric acid. The emulsion is neutralized with NaOH to chlorophenol red. The samples are then treated with zinc sulfate, filtered, oxidized with ferricyanide, and titrated with ceric sulfate. Either alkali fast erio green or phenanthroline ferrous complex is used as an indicator. Galactose is thus determined and Macy reports that the factor for galactose is constant over the range of 0.1-0.6 mg. The factor from 33 determinations is .147 mg. per ml. of ceric sulfate. Cerebroside is calculated from the equation of $4.55 \times 0.147\ (A-B)-(C-D)$ in milligrams. In the previous equation A, B, C, and D are respectively the volumes of .003 N ceric sulfate solution required to titrate the hydrolyzed lipide sample, hydrolyzed blank, unhydrolyzed lipide sample, and the unhydrolyzed blank. The factor 4.55 is based on the assumption that there are equal amounts of phrenasin and kerasin present.

Sterols

Sterols, primarily cholesterol and cholesterol esters, are found in various tissues and have been studied for many years.

Hunter (19) reports studies using the Kelsey (20) and the Schoenheimer and Sperry (21) methods. Both of these methods combine the principles of the Liebermann-Burchard color reaction and the Windaus digitonin precipitation.

Hunter found that the Kelsey method resulted in erratic results and therefore devised a modification of the Schoenheimer and Sperry method. The digitonide procedure consisted essentially of the Schoenheimer and Sperry method except that it was adapted to the Evelyn Photoelectrometer. Lipide was treated with 2 ml. of acetone-alcohol and 0.4 per cent digitonin. The precipitate was spun down, washed, and dried. Glacial acetic acid was added and the color developed. The colorimeter tubes were then stoppered, placed at 16°C., and kept in the dark. After 50 minutes the intensity of the color which developed was read in the Evelyn using the 620 mμ filter. This method requires 1.0-0.1 mg. of cholesterol.

Saifer (22) has modified the Liebermann-Burchard method so that it could be used as a photometric determination of total cholesterol. The main advantage to this method is that all the water is removed from the sample of plasma when it is heated with acetic anhydridedioxane (3:2 by volume) in a boiling water bath for 30 minutes. Dioxane-acetic anhydride simultaneously extracts cholesterol and cholesterol esters, precipitates protein, and converts water into acetic acid. Saifer emphasizes that if all the water present is not removed, low values will be obtained.

Fatty Acids

Probably the most interesting constituents of lipides are the fatty acids for they endow the lipides with a wide variety of characteristics.

Many micro-methods for the determination of total fatty acids have been devised in the past 25 years. Prominent among these is Bloor's method of chromate oxidation (23) which recently has been supplanted by a colorimetric method (24) and by other colorimetric and titrametric methods which will be discussed briefly.

Bloor's colorimetric method (24) consists of reacting 0.1-0.9 mg. of cholesterol of fatty acid with a concentrated sulfuric acid solution of silver dichromate. In a specially built colorimeter the disappearance of the dichromate color can be measured when the sample is read against the full color of dichromate as a blank. After the addition of potassium iodide these samples can also be titrated with thiosulfate with starch as an indicator.

Another colorimetric determination of fatty acids in the range of 0.05-1.0 mg. is that proposed by Hill (25). In this method an ester is warmed with hydroxylamine in an alkaline medium to form hydroxamic acid which with ferric iron forms a bright red or lavender color. Fatty acids do not form hydroxamic acid directly but must first be esterified to a methyl ester with methanol. The red color developed from the reaction of ferric iron with the hydroxamic acid can be read at a wave length of 520 mμ.

Other methods for the determination of fatty acids have been reported by Stewart and White (26) and Stoddard and Drury (27). Both of these methods utilize the principle of titration of free fatty acids with alkali and can be used to determine the fatty acids in 5-60 mgs. of fat.

A unique method in the field of fatty acid determinations is the one worked out by Schmidt-Nielsen (28). With this determination it is possible to detect 0.01 mgms. of fatty acid. The fat to be analyzed is saponified in a sealed ampulla with alcoholic KOH. After saponification the ampulla is opened, acidification is accomplished with HCl, toluene is added, and the ampulla is resealed. The ampulla is then centrifuged, and an aliquot of toluene is pipetted off for titration. Evaporation of the toluene is accomplished at about 30 mm. Hg. During the titration the sample is protected from CO_2 with a micro-desiccator containing soda lime. Tetramethylammonium hydroxide in alcohol is used as the base instead of NaOH since NaOH deposits minute crystals of carbonates on the walls of the micro-burettes. The carbonates of the $(CH_3)_4NH_4OH$ are soluble in alcohol so these carbonates are removed from the walls of the microburettes during the titration.

Once having isolated the fatty acids and having determined the amount of fatty acids, a few other lipide constants such as the iodine value and the saponification value can be determined on a micro-level.

Yasuda (29) modified the Rosenmund-Kuhnhenn iodine value for the determination of 3-5 mgms. of fatty acid. Theoretical values were obtained for cholesterol and slightly lower values for the more highly unsaturated fatty acids. Kretchmer (30) by the use of micro-techniques was able to measure the iodine value of .01-0.10 mgms. of fatty acid with a modification of the Rosenmund-Kuhnhenn method. The

iodine values obtained compared favorably with those determined by the Wijs method. Schmidt-Nielsen (31) adapted the Kaufman method to 0.01 mgms. of fatty acid. There is little evaporation of the lipide solvent during the titration since cellosolve, which has a low vapor pressure, is used. Schmidt-Nielsen utilized various micro-techniques and micro-apparatus for the measurement of the iodine addition.

Marcali (32) has reported a micro-saponification number method which is essentially an adaptation of a semimicro saponification number method reported by Chargaff (33). Actually Marcali's method does not use quantities as small as most of the other methods that have been discussed. For this determination quantities of the order of magnitude of 1 to 100 milligrams were used. In the lower part of this range Marcali uses a double indicator and a micrometer buret. The double indicator technique consists of first titrating to the disappearance of the pink color of phenolphthalein and then continuing the titration until the green end point of bromophenol blue.

Beadle (34) recently reported the advantages in the use of spectroscopic methods in the determination of fatty acids that may be treated to form conjugated double bond systems. Cohodas (35) has reported that it is possible to cook 0.5 to 1.0 milligrams of fatty acid in alkali and examine spectrophotometrically the acids so conjugated. Briefly, the method consists of cooking the fatty acids in 6% KOH at 180°C. in nickle crucibles for fifteen minutes. At the end of this time the samples are removed, made up to a volume of 10 ml. with water, and examined in the Beckman Quartz spectrophotometer at 235 mμ, 275 mμ, 315 mμ, and 350 mμ. In this method no evaluation could be obtained for the pentaene and higher unsaturated systems since no acids higher than arachidonic acid were available for the determination of the necessary analytical constants. From ultra-violet examination of the conjugated materials the concentration of unsaturated fatty acids containing either two, three, or four double bonds can be calculated as follows (34, 36):

$$1.)\quad \frac{E^x3050}{E^s3050} \times 100 = \%\ \text{4-bond fatty acids}$$

$$2.)\quad \frac{E^x2725 - \left[\frac{E^x3050}{E^s3050} \cdot E^s2725\ (4)\right]}{E^s2725\ (3)} \times 100 = \%\ \text{3-bond fatty acids.}$$

$$3.)\quad \frac{E^x\,2350 - \left[\frac{E^x2725}{E^s2725} \cdot E^s2350\ (3) + \frac{E^x3050}{E^s3050} \cdot E^s2350\ (4)\right]}{E^s2350\ (2)} \times 100 = \%\ \text{2-bond fatty acids.}$$

E^x = absorption coefficient at a noted wave length for the unknown.
E^s = absorption coefficient at the noted wave length for the standard.
(2), (3), and (4) signifies the number of double bonds.

After calculating the 2-, 3-, and 4-double bond acids, it is a simple matter to combine these data with the iodine value and thus obtain an estimate of 1-double bond acids. Total unsaturated fatty acids can be subtracted from 100 to obtain an approximation of the saturated fatty acids.

Typical Analysis

A few of the methods discussed above have been applied in our laboratory to analyze small amounts of lipides obtained from certain liver extracts. In order to conserve as much sample as possible and to perform the methods satisfactorily, it was necessary to plan a flow sheet of analysis (Fig. 1). In this manner, less than 10 mg. of sample are necessary in the complete analysis for all of the components that have been discussed. Most of the lipide sample in this analysis was used for the determination of nitrogen and phosphorus. If finer micro methods are used for the determination of nitrogen and phosphorus, much less of the lipide sample is necessary for this portion of the analysis.

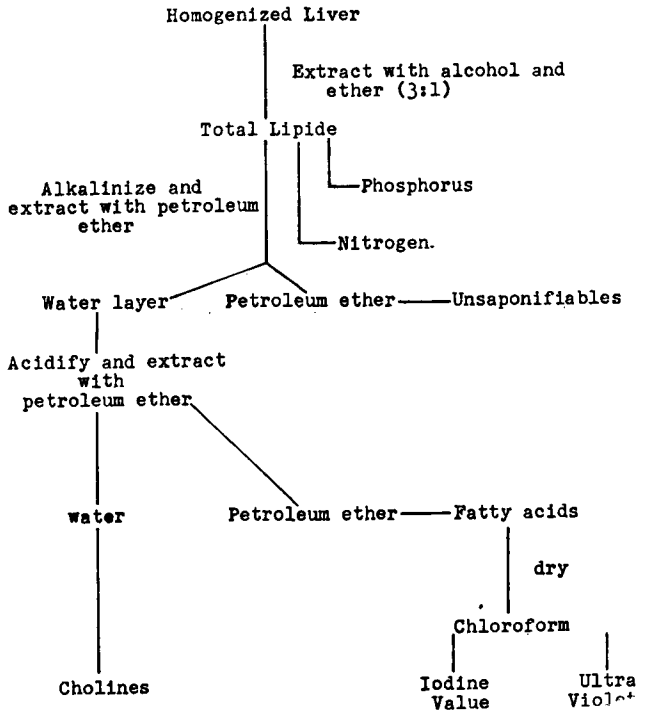

FIG. 1. A flow sheet for the lipide analysis of homogenized liver.

Summary

When dealing with biological tissues, it has become more and more necessary to deal with small samples. The field of lipide analysis on a micro level has been reviewed and an example has been given of how a small sample of lipide can be analyzed for its component parts.

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Application of the Line-Width Method to the Spectrochemical Analysis of Oils, Fats, and Related Substances

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IN a recent paper (3) the present authors described a method for the preparation of the ash of oils and fats together with a method of spectrochemical analysis of the ash for traces of certain metallic elements having sensitivity of about 2 to 4 parts of individual metals per million parts of the fat or oil. However, a sensitivity of about 1 part of a metallic trace element in 10 million parts of a sample is essential in studies on the stability of oils and fats. The present communication describes a shortened and improved procedure for the preparation of ash and the application of the newly proposed line-width method (2) for the evaluation of the spectrograms. Data are presented from critical studies of the sensitivity, precision, and accuracy of the procedure for the quantitative determination of copper, iron, manganese, nickel, and tin in various types of vegetable oils and fats with a sensitivity of about 1 part in 10 million.

Preparation of Ash

Wet washing methods, involving the use of mineral acids and chemical glassware, were investigated and proved unsatisfactory. The recently adopted tentative method of the American Oil Chemists' Society for the determination of ash in oils and fats (1), in which the oil is heated and then ignited on the surface, was studied in some detail. As shown in Table I, use of this method for ashing and subsequent determination of the trace metals by spectrochemical analysis showed consistently low results in comparison with a more adequate ashing procedure.

The ashing procedure previously proposed (3) was reinvestigated for the purpose of simplification and increasing the sensitivity. Through the use of the line-width method (2) of evaluation of spectrograms the incorporation of internal standards into the ash is not required. By the elimination of this step the over-all time of ashing can be reduced from about 72 to about 48 hours with the need of less personal attention.

The most obvious means of increasing the sensitivity is to increase the size of the sample and at the same time reduce the amount of the magnesium nitrate used to perform the triple function of ashing-aid, carrier, and spectroscopic buffer. The ratio of 2.5 grams of oil to 1.25 grams of magnesium nitrate has been changed to 16.67 grams of oil and 0.50 gram of magnesium nitrate, providing approximately a twenty-fold increase in this ratio, and permitting the quantitative determination to a sensitivity of about 1 part of metal in 10 million parts of sample.

The abbreviated ashing procedure follows. A sample of exactly 16.67 grams of the oil or fat is accurately weighed into a 100-ml. Vycor dish (90 mm. across the top) and 0.50 gram of magnesium nitrate in alcoholic solution [2 ml. of a solution of 250 grams $Mg(NO_3)_2 \cdot 6H_2O$ per liter of 95% C_2H_5OH] is added. The dish is covered with an inverted short-stemmed Pyrex funnel, whose maximum diameter is less than the maximum diameter of the dish. The sample is heated on a hot plate and the temperature gradually and cautiously raised until the maximum temperature of the hot plate, approximately 300°C., is attained, and the heating is continued overnight. The charred sample is then ashed in a muffle furnace, with an initial temperature of 225° and increasing in increments of 25° at 30-minute intervals until a temperature of 450° is reached. The samples are held at this temperature in the muffle furnace overnight and then cooled and quantitatively transferred to a small mortar with the aid of a camel's hair brush. The ash is finely ground and very thoroughly mixed and is then ready for the spectrochemical analysis.

Spectrochemical Analysis by the Line-Width Method

The spectrograms were prepared exactly as described in the earlier paper (3) except that a step-sector was no longer required. All the precautions previously discussed in detail—such as complete absence even of traces of the elements to be determined from all reagents, from glassware and from the carbon rods after their preparation and handling, and very careful mixing of the prepared ash—must be maintained if accurate results are to be obtained. Fig. 1 shows the spectrograms of a number of vegetable oils photographed on a single plate for evaluation by the line-width method. As the step-sector,

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