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A micro-technique for the preparation of the methyl esters of fatty acids from complex lipids ¨

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During the last decade, a number of techniques were introduced in the field of lipid research that permit separation and examination of the lipids and their cleavage products in the micro-quantity range. They have provided the basis which makes it possible to perform this type of analysis by using minimum quantities of test substances. Except for a few instances [1,7,9], however, no one has yet worked out a technique that would make full use of the means at our disposal today and that also converts to micro units the preceding steps of preparative work. As a rule, the techniques customary in laboratory practice have been retained in principle, with certain reductions in the size of equipment and the quantity of solvents. This may do as a solution as long as the available test quantities do not fall below a critical limit of 10 - 30 mg. But if it is planned

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to start with tissue samples from which only substantially fewer lipids can be extracted, then both the loss and the change of substances caused by the wetting of large surfaces and of different glass parts (cocks, stoppers) and the impurities absorbed in the course of preparation from very large quantities of solvents and reagents, compared with the substance-samples, begin to have a disturbing effect and could lead to misinterpretations [6,8]. For these reasons, a micro-technique has been developed - originally for the chemical examination of lipids from organ-tissue biopsies (fresh weight of the tissue 50 - 100 mg)- with the intention of eliminating this type of interference, which also is suitable for the performance of serial tests.

Equipment

Centrifuging tubes. All processes are carried out in centrifuging tubes with a slight NS 14.5 concave-ground section. The dimensions vary with the centrifuge available, e.g., 16 mm outside diameter and a length of 95 mm. It has been found useful to employ centrifuging tubes with round bottoms for filtration, extraction and the performance of reactions, but to use tubes with pointed bottoms for the evaporation of solutions. For quantities of lipids in the range up to 30 mg, quantities of reagents of between 1 - 2 ml are sufficient for all conversions; this corresponds to a filling height of from 1.5 - 2 cm in the centrifuging tubes

used by us.

Stirring rods can be improvised in the lab by securing 1 mm thick wire ends (made from nails), 6 - 8 mm long, in thin-walled (!) glas tubes by melting them in, or they can be bought commercially with Teflon-coating (6 x 12 mm).

Magnet-stirrers must have sufficient rpm's for this purpose and should be as easy as possible to handle. This requirement is not met by most of such devices. We have found the "Rühromag-Combi" manufactured by K. Retsch, Haan (Rheinld.), to be useful. If such equipment is not available, a small electric motor with variable rpm's may also be used; its rotor is connected to a round magnet (used to set the temperature on contact thermometers).

Gas inlet tubes (Fig. 1a) serve as protection against oxidative changes during the extraction process and can, like the micro reflux condenser (Fig. 1b), be made up by a glass-blower.

Dropping pipettes, or so-called Pasteur pipettes, are used to transfer watery or methanolic solutions. They can easily be made from glass tubes of a suitable thickness (7-8 mm); their capacity should be 2 - 3 ml. As rubber suction devices, the commercial round suction bulbs have not been found suitable for them nor for the solvent pipettes described in the following. A safer use of the pipettes is provided by rubber hoses of

an appropriate thickness, the one end of which is closed by means of a stopper.

Solvent pipettes. Solvents having a high vapor pressure, like the ones used preferably in the chemistry dealing with lipids, are difficult to transfer with satisfactory quantitative accuracy by means of Pasteur pipettes, unless relatively high-boiling liquids are used. But in most cases, this is impractical, p. 232 undesirable, or even unsafe because of the high temperatures employed in evaporating. This situation led to the construction of a solvent pipette [2] that can also be built in the lab from a T-shaped glass tubing-connector (tube diameter 10 - 12 mm) by drawing the one end of the glass tube out to a point while the tube attached at right angles is closed by melting and bent slightly in the direction of the tip of the pipette. A closely fitting rubber hose with a stopper is fitted onto the untreated tube as suction device. The suggestion pictured in Fig. 2 for manufacture by a glass-blower, however, has the advantage of less dead space in the neck of the pipette and improved strength of the capillary tube.

Other equipment required is a small centrifuge (e.g., the model "Piccolo", made by M. Christ, Osterode/Harz), a rotary evaporator with water bath (e.g., W. Büchi, Flawil, Switzerland), and a hot-plate equipped with a thermostat, onto which an aluminium block with holes to receive the centrifuging tubes can be placed.

Chemical preparation of lipids in the micro-quantity range

A. Extraction and purification of the total lipid content from smallest tissue samples

Tissue sections with a fresh weight of from 50 - 200 mg are placed on the bottom of a round-bottomed centrifuging tube immediately after weighing. There, they are crushed by means of a glass rod, and 2 - 4 ml chloroform-methanol (2:1) or (1:1) are added. It must be pointed out that even p.a. qualities of solvents may still contain considerable amounts of impurities and

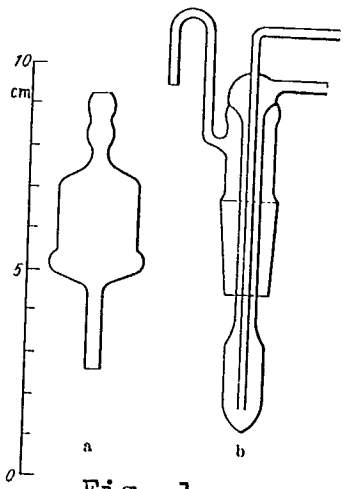

Abb. 1 Fig. 1

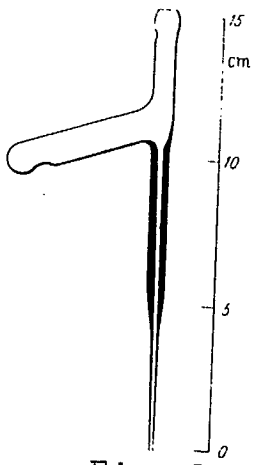

Abb. 2 Fig. 2

Fig. 1 - glass devices used in micro-technique
a gas inlet tube, b reflux condenser

Fig. 2 - solvent pipette for the transfer of solvents having a high vapor pressure

are, before use, best destilled over a short packed column, in glass containers that are absolutely clean. All glass equipment

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used in the micro-technique must be cleaned with chromosulfuric acid; cleaning with commercial special cleaning agents may have extremely disturbing effects. After fitting the micro-reflux-condenser, the contents is boiled for an hour, and the remaining, cooled-off solution is drawn off with the aid of the micro-filtration arrangement as shown in Fig. 3.

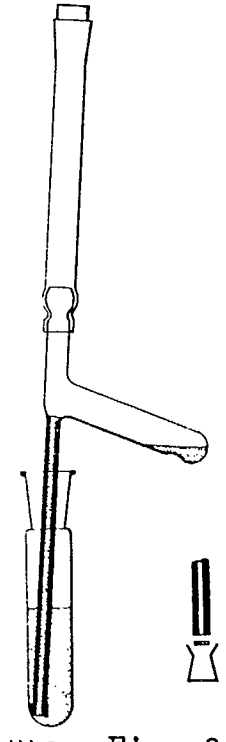

Abb. 3 Fig. 3

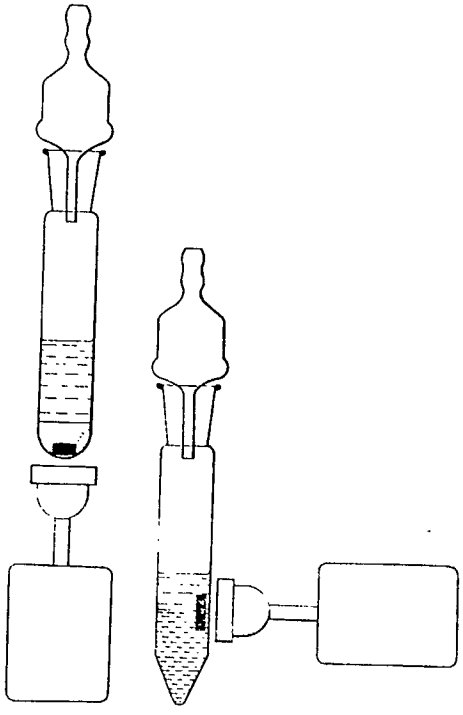

Abb. 4 a b Fig. 4

Fig. 3 - Employing a solvent pipette as a filtration device. The detailed drawing shows how the actual filter disk is attached to the capillary tube by a round clip.

Fig. 4 a, b - Arrangement for extracting in micro-scale. Whether the agitating is done from the bottom of the vessel (a) or from the side wall of the vessel (b) depends on the strength of the permanent magnet on the agitator motor, on the size of the stirring rod, and on the shape of the centrifuging glass.

This arrangement can be built by placing a disk punched out of degreased filter paper with an office punch on the capillary end of a solvent pipette that has not yet been drawn out to a point and then fastening it by clamping on a fastening device made of thin sheet metal (see detail drawing). To achieve full extraction of all lipids, this process can be repeated once more, and the lipid extract, in order to remove non-lipoid extracted substances, may be cleaned according to the method of Folch et al [5] by dispersion/distribution against (sic) water or a saline solution. For this distribution, the magnet-agitation technique described below is employed. The lipid extract is then evaporated to a dry substance in a centrifuging tube with pointed bottom. Evaporation is carried out on a rotary evaporator at a bath temperature of below 30°C p. 23/ in a water-jet pump vacuum.

B. Alcoholysis of lipids and preparation of the methyl esters of fatty acids.

Up to 20 mg of a lipid sample prepared in this or some other way are added to 2 ml 5% water-free methanolic hydrochloric acid and 2 ml methyl acetate in a round-bottomed centrifuging glass, and 2 or 3 "boiling stones", about 1 mm thick, are added (earthenware or tile chips). The mixture is boiled for one hour, employing reflux. The addition of methyl acetate not only improves the solution ratios for otherwise poorly soluble

lipids [3], but also provides an even washing of the walls of the tube by the condensing solvent. After the solution has cooled, extraction of the lipid-soluble constituents - chiefly the methyl esters in other words - takes place in the arrangement pictured in Fig. 4. The centrifuging tube is fastened to a stand by means of a test tube clamp, 1 ml water and 1.5 ml ether p.a. - petroleum ether p.a. KP 30 - 60° C (1:1)is added and an absolutely clean stirring rod is introduced (touch with tweezers only!). The contents of the tube is then stirred so rapidly with the magnet-stirrer that no more phases can be recognized. As protection against oxydation, a gas inlet tube has been placed on the centrifuging tube, through which a small stream of nitrogen is forced. The speed of flow is controlled with a simple bubble counter to prevent that some of the solvent is blown off by the gas stream. Whether agitation is carried out from the bottom (Fig. 4a) or from the side (Fig. 4b) depends on the strength of the stirring magnet and on the size of the stirring rod.

After the phase system has been mixed for 3 minutes, the phases are allowed to separate; this process can be considerably accelerated by centrifuging if the phases have a tendency to form emulsions. The petroleum-ethereal upper phase which contains the lipophilic elements is now drawn off as far as possible with the solvent pipette. Care should be taken that

none of the substance comes in contact with the ground section of the centrifuging tube. The inside wall of the tube is carefully rinsed off with 0.5 ml fresh petroleum ether, and the solvent is removed again with the solvent pipette. After adding another 1.5 ml ether - petroleum ether, the phases are again dispersed by means of the magnet stirrer in the manner described, for three minutes, and the extraction is repeated. A total of three extracting operations is sufficient to obtain all the lipoid substances. The ether - petroleum-ethereal phases are collected in a standard centrifuging tube and, by adding 1 ml saturated sodium hydrogen carbonate solution, are washed neutral again by employing the magnet-stirrer technique. The upper phase is then transferred quantitatively into a centrifuging tube with pointed bottom by means of a solvent pipette, and the solvent is removed on the rotary evaporator. In order to move the entire substance to the bottom of the tube, thus reducing its surface as a protection against oxydation, the entire inside wall of the tube is wetted with about 3 drops of petroleum ether or chloroform, and the closed tubes are centrifuged for a short time.

Removal of the acid, however, can also be achieved by using the method of Stoffel et al [9] and drying the petroleum ether extract over a spatula tip of a water-free mixture of sodium sulphate - sodium bicarbonate (4:1). The drying process

may be accelerated by keeping the drying substance agitated in the solution by means of a stirring rod (magnet-stirrer) for about 10 minutes. It is subsequently sedimented to the vessel bottom by centrifuging; the supernatant solution may then be drawn off easily without stirring up the bottom sediment.

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The operation of the solvent pipette is carried out as follows: As much of the petroleum-ethereal solution is drawn off into the pipette (located at right or obtuse angles to the surface of the liquid) so that just enough suction capacity remains to also draw the solvent situated in the suction capillary completely into the lateral collecting attachment. In learning this technique, it is advisable not to bring the tip of the pipette out of the centrifuging tube from which the solvent is to be drawn. This process can be repeated until the collecting attachment is almost filled. For emptying, the pipette, which is held tip down at an angle of about 50°, is then turned around its axis until the attachment points up. In this position, the solution collected in it empties into the actual pipette space from where it is blown out by means of the suction arrangement. It has been found advantageous to provide a slight dip in the collecting attachment as shown, in order to retain any drops of water drawn up with the extract in the attachment. The solvents run off well from the spherical drop of water.

In addition to the methyl esters of fatty acids, however, the petroleum-ethereal extract as a rule also contains unsaponifiable material like cholesterol, hydrocarbons, and similar matter. These impurities have to be removed as they would interfere in, for example, the gas-chromatographical analysis. Their separation can be achieved best by means of chromatography with small columns (length 20 - 25 cm, diameter 1 cm), which are made from suitable glass tubes and are drawn out to a point at the bottom. They are filled with 5.0 - 5.5 g silicagel, Mallinckrodt 100 mesh, which has stood over water for 3 - 5 days, as petroleum-ethereal suspension. Filling height after sedimentation is 12 - 13 cm. After placing on it an exactly fitting, degreased filter disk, the filling is compressed with a glass rod to a length of 10 - 11 cm. In this way, a convenient column is obtained possessing excellent separating characteristics. After pouring off the supernatant petroleum ether, the fatty-acid-methyl esters dissolved in 1 ml petroleum ether along with the impurities are poured into the column. One has to wait until the solution has completely penetrated into the filling. The methyl esters of fatty acids are eluted with from 30 to a maximum of 50 ml of a 2% solution of ether in petroleum ether. They are now available in pure form and can be used for analysis after the solvent has been removed.

C. Rediscovery tests on the extraction technique

As a control of the completeness of the extraction, we

placed exactly 1.0 ml of an0.2% methanolic linoleic acid solution or of an 0.2% triolein solution in acetone-methanol (1:1) with a pipette into each of 10 centrifuging tubes added 1 ml water, and extracted the fatty substances three times in the manner described. The use of an ether-petroleum ether mixture (1:1) in place of pure petroleum ether provided no advantages for these tests; however, it might be of use in some cases (extraction of polar substances). The petroleum-ethereal phases of one test series were cleaned and evaporated. The Table shows the results of the rediscovery tests.

In a further double series, the quantitative extraction of substances during the course of a saponification was to be determined. For this purpose, we placed into each of 9 centrifuging tubes 1 ml of an 0.2% triolein solution in acetone-methanol (1:1) and 1.5 ml n methanolic caustic soda solution, and boiled this for 60 min with reflux. Finally, we evaporated about 0.5 - 0.75 ml methanol, added 0.5 ml 18% hydrochloric acid, 1 ml water, and 1.5 ml petroleum ether to the cooled solution, and - after allowing the mixture to stand for 10 minutes - carried out a three-time extraction with petroleum ether of the liberated fatty acids. Here, too, preparation led to quantitative yields.

Table - Rediscovery tests on the extraction technique p. 236

Substance employed	Rediscovered
I. 3x Extraction with petroleum ether:	
10 x 2.0 mg linoleic acid	19.5 mg (97.5%)
10 x 2.0 mg linoleic acid	20.0 mg (100%)
10 x 2.0 mg linoleic acid	20.2 mg (100.5%)
10 x 2.0 mg triolein	19.0 mg (95%)
10 x 2.0 mg triolein	20.2 mg (100.5%)
10 x 2.0 mg triolein	19.9 mg (99.5%)
II. Saponification, 3x extraction of the liberated fatty acids with petroleum ether:	
9 x 2.0 mg triolein	17.5 mg (101.5%)
9 x 2.0 mg triolein	17.2 mg (100%)

Discussion of the Technique

Working in the micro-quantity range has become a very common operation today in chemical research. Particularly in the area of clinical chemistry, the fact that frequently only inadequate quantities of test substances are available for the performance of a macroanalysis has contributed to the development and to the spread of micro-techniques. And yet, these methods as a rule not only achieve an equally high accuracy, but save time, work, and material in addition. But the availability of commercia micro-quantity equipment for analyses that is easy to operate, too, has encouraged this tendency. On the other hand, there are many places where classical chemistry without separating funnels and destilling bridges can still not be imagined, and for preparative work with quantities of substance that are almost in the micro-

quantity range, this fact is not taken into consideration. The substances are being diluted with solvents until the accustomed technique can be employed. When the sample is then recovered, a concentration of the impurities contained in the added reagents takes place which may lead to considerable confusion in the subsequent tests, which are mostly only to a limited degree specific as to substances. Thus, our own observations showed that the evaporation residue of 200-ml-portions of commonly used solvents (all p.a. qualities of known suppliers) during gas-chromatography produced several, distinct bands which interfered with methyl esters of fatty acids. Another source of errors is the residue of cleaning materials on the comparatively large surfaces of standard-size glass equipment.

These and other experiences and considerations led to the development of the micro-technique of preparation described above, using as an example p. 237 a lipid-chemical preparation. This technique has meanwhile proved itself practically for a number of years. It is highly suited to the performance of serial tests since the manual operations are limited to a few simple ones which always repeat themselves and do not involve any special techniques. An example of a saponification is described elsewhere[4].

As mentioned before, the technique was originally worked

out for gas-chromatographic examinations of the composition of fatty acids in liver cylinders obtained in biopsies. Initially, various errors in interpretation had occurred that were traceable to "dirt effects", also reported by other authors, which made a reduction of solvent and reagent quantities imperative. In order to keep the loss of substance during preparation as low as possible, dispersing the substances between two phases according to the magnet-stirrer technique - as it is employed at various points during the process - seemed to offer the greater advantage as compared to a shaking in a closed vessel. Care was also taken to ensure that the liquids came into contact only with smooth, well-draining surfaces on which no "creeping" is likely to take place. At first, working with low-boiling solvents also caused some difficulty, since the Pasteur pipettes, because of the high vapor pressure of the liquid, emptied without warning during the transfer of the solvents from one vessel into another. A quantitative working was practically impossible in this way. The use of the solvent pipette has now eliminated this difficulty. In order to prevent losses of the substance, it is important to continue to try performing as many operations as possible in the same test tube.

Summary

A micro-technique is described for the preparation of lipids from very small tissue samples yielding the methyl esters

of fatty acids. Great importance was attached to avoiding loss and oxydation of material and to the prevention of interferences by concentrated impurities from solvents and reagents. The method may be applied to similar problems in organic micropreparative work and is suited for serial experiments.

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