Journal of Chromatography, 151 (1978) 384–390

CHROM. 10,622

Note

Adaptation of a macro-scale method to the micro-scale for fatty acid methyl transesterification of biological lipid extracts

J. P. CARREAU and J. P. DUBACQ

Laboratoire de Physiologie cellulaire, Université de Paris VI, CNRS ERA 323, 12 Rue Cuvier, 75005 Paris (France)

(Received September 12th, 1977)

In recent years, the common use of gas–liquid chromatography (GLC) for the analysis of fatty acid mixtures led several workers to study different procedures for the methylation of fatty acids. In fact, most of the published methods have been applied with large amounts of lipids containing the stabler fatty acids, *i.e.*, saturated and mono-unsaturated fatty acids[1–17]. When applied to biological samples these procedures sometimes give artefacts[18,19] and it is difficult to find methods suitable for use with the usual conditions encountered in the micro-analysis of biological lipids.

In this paper we propose some procedures for the preparation of fatty acid methyl esters from unpurified complex lipids. Care was taken to utilize the actual conditions of analysis that are usually used in biological laboratories, *viz.*, a small weight of lipid extracts, the lipids being either dissolved in a solvent or scraped off as spots from thin-layer chromatographic (TLC) plates.

THEORETICAL

We started from the method developed by Loury[12] for oil analysis, which combines direct anhydrous methanolysis of glycerol exters by sodium methylate with further esterification of free or saponified fatty acids by hydrochloric acid in methanol. This method was developed by Loury for macro-amounts of oils (5 g), and we studied its application for biochemical use on the 500–20 μg scale.

Lipids are generally found as glycerides, phospho- or glycolipids, sterol esters or free fatty acids in crude extracts from either animals or plants. Alkaline methanolysis transesterifies fatty acids directly from glycerol or sterol to methanol (Fig. 1); in this reaction, the methoxyl ion is fixed on the carbo-cation of the carbonyl group of the fatty acid. The glycerol or sterol part of the lipid molecule is converted into its sodium salt. Free fatty acids of the extract are converted into anhydrous sodium salts (saponification) because of the great affinity of Na^+ for fatty acids, which is considerably greater than its affinity for methanol. Acidic esterification is then necessary in order to convert the fatty acid sodium salts into the methyl esters; an excess of hydrochloric acid in methanol is used for this purpose (Fig. 2).

This last treatment is necessary even if there are no free fatty acids in the extract. In practice, under laboratory conditions one usually cannot avoid the presence

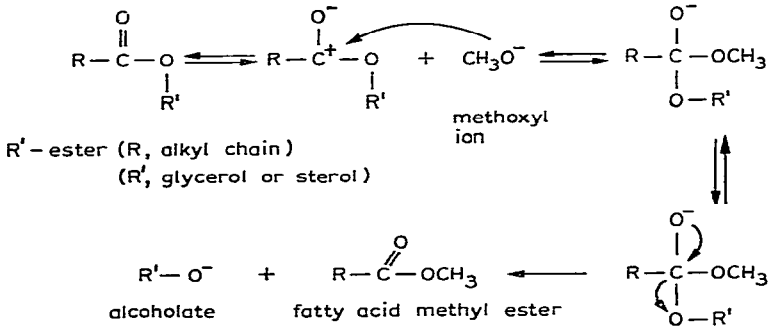

Fig. 1. Direct transesterification of a fatty acid by sodium methylate.

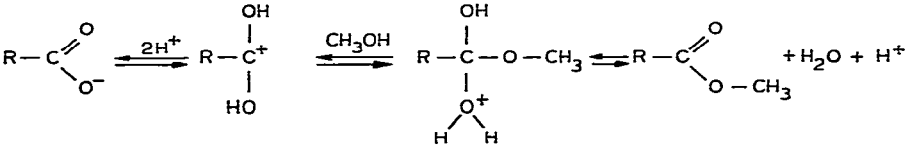

Fig. 2. Conversion of sodium salts of fatty acids by hydrochloric acid in methanol. Note the formation of water (see text).

of trace amounts of water in the reactants (either from condensation or absorption of water from the air, or from imperfectly dried samples). When water is present, a certain amount of the sodium methylate is converted into sodium hydroxide in the methanol. The hydroxide ions have a greater affinity for the carbonyl group of the glycerol esters than the methanol, and hydrolysis of the glycerol esters occurs, followed by saponification by Na^+ of the free fatty acids formed. It is therefore necessary to apply the above acidic esterification so as to ensure that no saponified fatty acids are lost in the analysis. During the last part of the methylation reaction, some water is formed (Fig. 2) and can also lead to hydrolysis of esters (Fig. 3) in the same way as external water.

We calculated that under our experimental conditions, the ratio of (moles of sodium methylate + hydrochloric acid in methanol) to moles of fatty acids is about 10^4, which means that the hydrolysis by the water produced from the reaction shown in Fig. 2 can be neglected, and that the reaction is rapid and complete (Fig. 4). Fig. 4 summarizes the whole procedure.

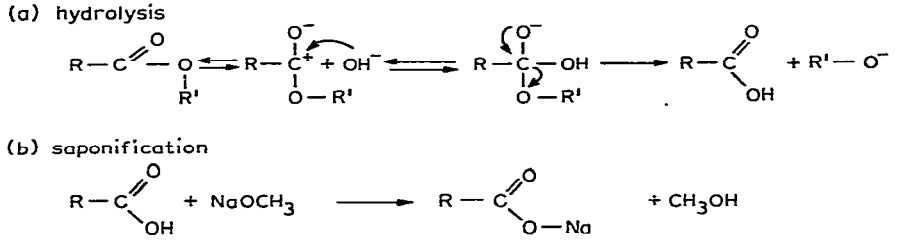

Fig. 3. Consequences of the presence of water or sodium hydroxide in the medium: (a) hydrolysis of esters; (b) saponification of free fatty acids.

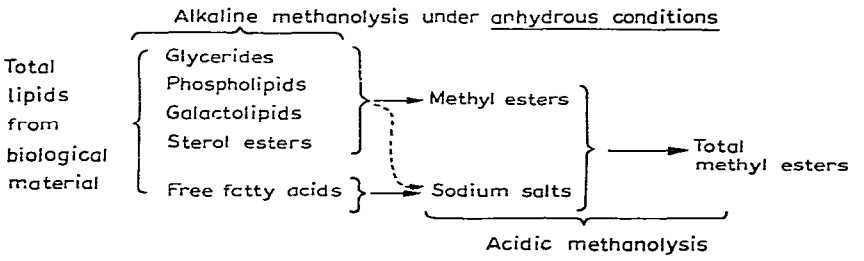

Fig. 4. General scheme of the proposed procedure. Solid arrows, under anhydrous conditions; broken arrows, with water present in the reaction medium.

EXPERIMENTAL

Materials

Sodium methylate solution (1%, w/v) is prepared by dissolution of 0.5 g of metallic sodium (well cleaned from sodium hydroxide formed on contact with wet air) in 50 ml of anhydrous methanol. The solution can be stored at 4° for several weeks. Small amounts are more convenient for storage because contact with wet air leads to absorption of water and formation of sodium hydroxide, and insoluble sodium carbonate may be formed. Low-temperature storage of the solution assists in its conservation, but may allow some condensation of water when the solution is used in open air at ambient temperature, so small well closed, bottles should be used.

Anhydrous 1 *N* hydrochloric acid in methanol is prepared by bubbling hydrogen chloride gas (J. T. Baker, Phillipsburgh, N.J., U.S.A.) into cold methanol, the correct concentration being ascertained by weight difference. The solution is stored at 4° with the same precautions as for sodium methylate.

Procedures

For kinetic studies, samples of commercial cholesteryl oleate (SRL) and sunflower seed oil were dried under nitrogen and then treated with sodium methylate followed by hydrochloric acid in methanol, being allowed to stand for various times in the reactant at 55°. After reaction, the methanol and hydrochloric acid were removed under nitrogen. The methyl esters were dissolved in a small volume of chloroform and aliquots were injected directly for GLC analysis into a Varian 1400 chromatograph fitted with a 3-m column of 4% DEGS on Chromosorb W, HMDS (80–100 mesh), with nitrogen as the carrier gas at a flow-rate of 15 ml/min. To avoid injection of NaCl into the column, the fatty acid methyl esters could be extracted with pentane.

For determination of the fatty acid content, known amounts of heptadecanoic acid were added to the extracts before methylation as an internal standard.

For studies of the stability of fatty acids in terms of the degree of unsaturation, egg yolk lipids were used under various conditions (Table I) and analysed by GLC and/or TLC on silica gel G 60 (Merck, Darmstadt, G.F.R.) layers 0.5 mm thick according to Brown and Johnston[20]. The lipids were revealed with osmium tetroxide vapour.

For studies of fatty acids from lipid spots on silica gel plates, we used spinach total lipids. Known samples of lipids were spotted on TLC plates, the spots were

TABLE I

STABILITY OF FATTY ACIDS ACCORDING TO REACTION TIMES IN THE METHYLATION PROCEDURE

Experiment No.	*Reaction time (min)*		*Fatty acid analysis (% from four fatty acids)* *			
	In sodium methylate	*In hydrochloric acid in methanol*	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
1	3	3	33.5	45.3	15.2	6.0
2	30	3	33.2	45.9	14.1	6.8
3	60	3	33.0	44.9	16.1	6.0
4	3	30	31.7	45.8	15.5	7.0
5	3	60	31.8	45.2	16.3	6.7

* Averages calculated from the five experiments: stearic acid, 32.4 ± 0.8%; oleic acid, 45.3 ± 1.0%; linoleic acid, 15.5 ± 1.1%; arachidonic acid, 6.8 ± 0.3%.

scraped off the plates with various amounts of silica gel, heptadecanoic acid was added and various treatments were carried out with the reactants (see under Results). In all instances the methyl esters were analysed by GLC, after extraction with pentane to avoid the injection of silica gel into the column.

RESULTS

Kinetic studies

Amounts of 0.24 μmole of cholesteryl oleate and 0.22 μmole of heptadecanoic acid were treated with 0.3 ml of sodium methylate for 0.5–60 min and then with 0.3 ml of hydrochloric acid in methanol for 3 min. The theoretical weight ratio of oleic acid to $C_{17:0}$ is 0.92, based on 100% purity of the commercial sample of cholesteryl oleate. This ratio is expected to remain constant when the reaction is completed. In our experiments, the maximal weight ratio was obtained within 5 min and was 0.85.

Amounts of 0.5 μmole of triglycerides from sunflower oil (which represents approximately 1.5 μmole of C_{18} fatty acids) and 0.3 μmole of heptadecanoic acid were treated as above. For the same reasons as for cholesteryl oleate, the ratio of fatty acids to heptadecanoic acid will be constant when the reaction is completed. We express this ratio in Table II as linoleic acid to heptadecanoic acid because this di-unsaturated fatty acid is the most unstable of the fatty acids present in sunflower oil. The reaction was completed within 30 sec and, as it is well known that triglycerides are the slowest glyceride species to be esterified, we can concluce that all glycerol esters will be esterified within at least 1 min by this direct transesterification method.

Stability of polyunsaturated fatty acids

Amounts of 100–150 μg of total lipids from egg yolk mixed with 30–40 μg of heptadecanoic acid were treated according to Table I, the fatty acid methyl esters being extracted and analysed by TLC (Fig. 5) and GLC. Fig. 6 shows the rapid (1 min) and complete methylation of the sample, with no degradation of fatty acids for at least 1 h. Very little variation in the total amount of fatty acids can be observed on GLC analysis (Fig. 5). The percentage of each component does not vary by more than 1%, which is less than the triangulation standard error (Table I).

TABLE II

EXPERIMENTAL CONDITIONS FOR THE METHYLATION PROCEDURE ON SILICA GEL

Experiment No.	*Weight of lipids (μg)*	*Weight of heptadecanoic (μg)*	*Weight of silica gel (mg)*	*Ratio of silica gel to lipids*	*HCl in methanol (ml)*	*Sodium methylate (ml)*
1	208	40	—	—	0.2	0.2
2	208	40	40	$0.2 \cdot 10^3$	0.2	0.2
3	208	40	400	$2 \cdot 10^3$	0.8	0.8
4	20.8	40	40	$2 \cdot 10^3$	0.4	0.4
5	208	40	800	$4 \cdot 10^3$	2.0	2.0
6	208	40	800	$4 \cdot 10^3$	4.0	4.0
7	208	40	1600	$8 \cdot 10^3$	4.0	4.0
8	20.8	40	400	$2 \cdot 10^4$	0.8	0.8

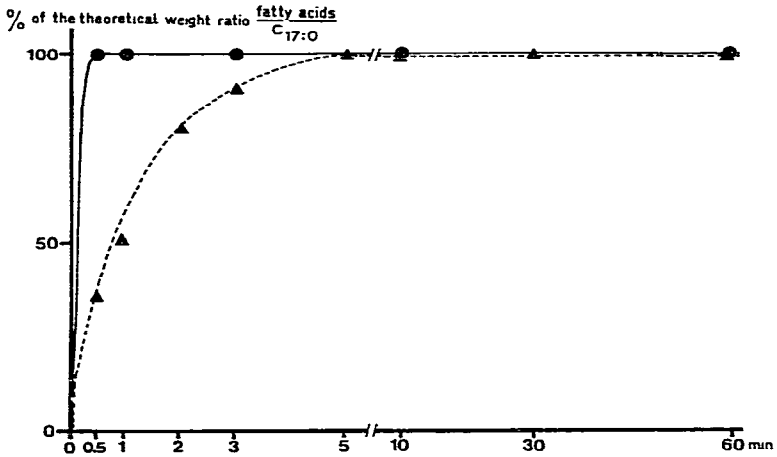

Fig. 5. Kinetics of fatty acid transesterification for different lipid extracts. Stability of products according to reaction time: ●, triglycerides from sunflower oil; ▲, cholesteryl acetate.

Direct treatment of lipids on silica gel plates

The ratio of silica gel to lipids was studied, and the stability of unsaturated fatty acids was also checked using spinach lipid extracts[21]. The samples were treated as described under Experimental and according to Table II for 5 min in each reaction mixture. After treatment, all volumes were adjusted to 8 ml with methanol and the fatty acid methyl esters were extracted and analysed by GLC.

Table III shows that few variations occur when the weight ratio of silica gel to lipids is less than $4 \cdot 10^3$. For higher ratios, the lipids are not entirely recovered and polyunsaturated fatty acids are badly extracted. For example, the recovery of linolenic acid is about halved when a ratio of $2 \cdot 10^4$ is used instead of $0.2 \cdot 10^3$. The maximal ratio for good extraction is $4 \cdot 10^3$ and corresponds to 100 μg of lipids scrapped off from a 20-cm^2 area of silica gel 0.5 mm thick. These conditions seem rarely to be obtained in biochemical analysis, in which the ratio of silica gel to fatty acids is often lower than 10^3.

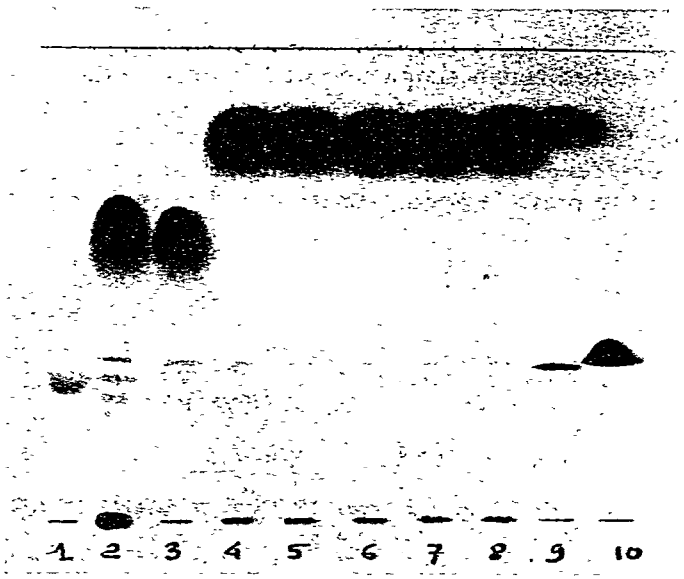

Fig. 6. Thin-layer chromatography of methyl esters[20] after treatment with sodium methylate and hydrochloric acid in methanol. 1, Free cholesterol; 2, egg yolk lipids before treatment with sodium methylate and HCl in methanol; 3, triolein; 4–8, egg yolk lipids after treatment with sodium methylate and HCl in methanol: 4, basic treatment 3 min, acidic treatment 3 min; 5, basic treatment 3 min acidic treatment 30 min; 6, basic treatment 3 min acidic treatment 60 min; 7, basic treatment 30 min acidic treatment 3 min; 8, basic treatment 60 min acidic treatment 3 min; 9, methyl oleate; 10, oleic acid.

TABLE III

RECOVERY OF FATTY ACIDS FROM THE METHYLATION PROCEDURE ON SILICA GEL

*Experiment No.**	*Total fatty acids recovered (%)*	*Recovery of individual fatty acids (% of total)*					
		$C_{16:0}$	$C_{16:3}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
1	100	13.9	7.8	0.5	4.8	11.5	58.2
2	≈ 95	14.1	8.1	0.7	5.5	12.1	57.0
3		14.4	8.3	0.8	5.2	12.1	57.3
4		15.4	7.7	1.2	7.7	10.6	55.2
5	≈ 90	13.5	7.4	0.6	5.5	11.9	58.5
6		13.7	8.1	1.0	6.5	11.2	57.0
7	≈ 55	43.0	2.6	6.6	12.1	8.8	22.1
8		46.5	1.4	5.6	14.9	8.0	17.6

* Same experiments as in Table II.

Even with heating at 60° for 60 min, no effect is noticed on fatty acids linked to sphingolipids (sphingomyelin or cerebrosides). This method therefore avoid the analysis of fatty acids from sphingolipids at the same time as fatty acids from glycerolipids.

CONCLUSION

The micro-scale method described here is simple and well adapted to the conditions used in biochemical analysis. The formation of methyl esters is very rapid and without effect on polyunsaturated fatty acids even with a reaction time of 1 h. With silica gel and under the conditions described the ratio of silica gel to lipids must not be higher than $4 \cdot 10^3$ in order to preserve good extraction and methylation of fatty acids.

REFERENCES

1 B. M. Craig and N. L. Murty, *J. Amer. Oil Chem. Soc.*, 36 (1959) 549.
2 G. G. Esposito and M. H. Swann, *Anal. Chem.*, 34 (1962) 1048.
3 E. J. Gauglitz, Jr., and L. W. Lehman, *J. Amer. Oil Chem. Soc.*, 40 (1960) 197.
4 G. V. Marinetti, *Biochemistry*, 1 (1962) 350.
5 M. E. Mason, M. E. Eager and G. R. Waller, *Anal. Chem.*, 36 (1964) 587.
6 A. K. Lough, *Biochem. J.*, 90 (1964) 4c.
7 J. B. M. Coppock, N. W. R. Daniels and P. W. R. Eggitt, *J. Amer. Oil Chem. Soc.*, 42 (1965) 652.
8 W. Stoffel, F. Chu and E. H. Ahrens, *Anal. Chem.*, 31 (1959) 307.
9 F. E. Luddy, R. A. Barford and R. W. Riemenschneider, *J. Amer. Oil Chem. Soc.*, 37 (1960) 447.
10 A Prevot and C. Barbati, *Rev. Fr. Corps Gras*, 12 (1965) 743.
11 J. I. Peterson, H. de Schmertzing and K. Abel, *J. Gas Chromatogr.*, 4 (1965) 126.
12 M. Loury, *Rev. Fr. Corps Gras*, 14 (1967) 386.
13 R. L. Glass, *Lipids*, 6 (1971) 919.
14 N. S. Radin, A. K. Hajra and Y. Akahori, *J. Lipid Res.*, 1 (1960) 250.
15 W. R. Morrison and L. M. Smith, *J. Lipid Res.*, 5 (1964) 600.
16 D. van Wijngaarden, *Anal. Chem.*, 39 (1967) 485.
17 I. Hornstein, P. Crowe and B. Ruck, *J. Chromatogr.*. 27 (1967) 485.
18 W. K. Fulk and M. S. Shorb, *J. Lipid Res.*, 11 (1970) 276.
19 E. A. Dawidowicz and T. E. Thompson, *J. Lipid Res.*, 12 (1971) 636.
20 J. L. Brown and J. M. Johnston, *J. Lipid Res.*, 3 (1960) 480.
21 E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.*, 37 (1959) 911.