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PREPARATION OF FATTY ACID METHYL ESTERS BY DIRECT TRANSESTERIFICATION OF LIPIDS WITH ALUMINIUM CHLORIDE–METHANOL

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SUMMARY

A new and simple procedure has been developed that allows the direct transesterification of lipids, using aluminium chloride as a catalyst and methanol as the esterifying alcohol. The concentration of the salt and reaction conditions have been investigated for the different lipid classes. Comparative studies, performed with boron trifluoride–methanol, indicate that the same values are obtained when using either reagent. In addition, the method has been adapted for transesterification in the presence of silica gel and other adsorbents, thus allowing the preparation of fatty acid methyl or ethyl esters directly from samples previously fractionated by thin-layer chromatography. This new reagent is very stable and easy to handle, the fatty acids being generated in the same tube without further purification steps.

INTRODUCTION

Although direct analysis of fatty acids can be performed by gas–liquid chromatography (GLC), higher column efficiencies and better resolution are obtained when using the appropriate ester derivatives, usually their methyl esters. Several reagents and numerous procedures have been described for the preparation of fatty acid methyl esters from lipid compounds[1–47]. In most of them, hydrolysis of the ester linkage is first performed, followed by extraction and methylation of the fatty acids liberated; thus, the whole procedure is lengthy and cumbersome, with many steps involved and a high risk of losses.

In order to minimize chemical and physical processing of the sample prior to its injection in the chromatograph, a direct transesterification procedure is preferred. Methanolysis of lipids can be carried out with acidic or basic catalysts, some of which show certain drawbacks, such as high volatility, short lifetime, limitation to certain lipid classes, production of side reactions or artifacts, etc.

I have developed a new reagent and procedure, in which aluminium chloride, dissolved in the corresponding alcohol, is used as a catalyst, for the transesterification of fatty acids in any type of lipid class. This new reagent can be kept at room tem-

perature for a long time, is easy to handle, quite economical and convenient to prepare.

EXPERIMENTAL

Compounds and reagents

Aluminium chloride, anhydrous, sublimed, and aluminium chloride, hydrated, were obtained from Merck (Darmstadt, F.R.G.). Boron trifuoride–methanol (12%, w/w) was from Supelco (Bellefonte, PA, U.S.A.). 8-Anilinonaphthalene-1-sulphonate ammonium salt, methanol, ethanol, trichloroethylene, 1,2-dichloroethane, benzene and light petroleum (b.p. 60–80°C) were from Fluka (Buchs, Switzerland); tri-, di-, monoglycerides, cholesteryl esters, fatty acids and pure methyl esters were from Applied Sciences (State College, PA, U.S.A.); phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, sphingomyelin and lysophosphatidylcholine were from Sigma (St. Louis, MO, U.S.A.). Monoglyceryl laurate was a gift from Grindsted products (Copenhagen, Denmark). Silica gel, used for preparative thin-layer chromatography (TLC), was obtained as a powder from Woelm Pharma (Eschwege, F.R.G.).

Preparation of biological specimens

Biological specimens (human plasma, red cells, human and animal tissues, cereal grains, etc.) were homogenized with five volumes (w/v or v/v) of 2-propanol and, thereafter, shaken for 30 min in a mechanical shaker. After standing at room temperature for a couple of hours, the individual suspensions were centrifuged at 1000 *g* for 15 min to remove the precipitated protein, and the supernatant was transferred to small vials for further analysis. Ether-soluble products (corn oil, olive oil, marine fish oil, etc.) were dissolved directly in toluene at a concentration of 100 mg/ml.

Preparative TLC

Chromatoplates (200 mm × 200 mm) with a layer (500 μm thick) of silica gel were prepared by suspending the adsorbent in toluene and coating the plates by means of a spreader (Desaga, Heidelberg, F.R.G.). The samples were applied by means of a 25-μl Hamilton microsyringe, equipped with a repeating dispenser (Hamilton, Reno, NE, U.S.A.), each sample being applied as a band of closely spaced dots.

For the fractionation into the different lipid classes, an aliquot of the corresponding propanolic extract was applied at the origin of the chromatoplates. The individual lipid classes were separated by one-dimensional chromatography with a double developing system. The first one, which was allowed to migrate up to 80 mm from the origin, was made from 10 ml of distilled water, 52 ml absolute ethanol and 38 ml of trichloroethylene. In this system, the neutral lipids move with the front, while the various ionic species migrate at different rates along the plate. The second developing system, consisting of a single component, either benzene or 1,2-dichloroethane, was used to fractionate the various neutral lipids by allowing the solvent to migrate up to 10 mm from the top of the chromatoplate.

After evaporation of the solvent, the different lipid bands were rendered visible

by spraying the chromatoplate with a 0.1% solution of 8-anilinonaphthalene sulphonate in ethanol and examining them under ultraviolet light of long wavelength. The silica gel containing bands was scraped off from the plate and transferred to borosilicate glass tubes for the preparation of the corresponding esters.

Preparation of the reagent

Different amounts of aluminium chloride were transferred to suitable bottles containing methanol or ethanol and allowed to react with the alcohol. After complete solubilization of the aluminium chloride, the bottles were tightly closed with a screw cap and left at room temperature. Solutions containing 5, 7.5 and 10% aluminium chloride were used throughout this study. The reagent appears to be quite stable, having a shelf-life of several months.

Procedure for the transesterification of fatty acids

An aliquot of the lipid solution containing milligram amounts of the compound (usually between 2 and 10 mg) was transferred to borosilicate glass tubes. Aluminium chloride–methanol, –ethanol or aluminium chloride hexahydrate–methanol was added in different proportions, and each tube was closed with a screw cap having a PTFE liner. The tubes were then heated in a bath of boiling water for 60 min, after which time they were allowed to cool at room temperature. The corresponding esters were extracted by the addition of 5 ml of light petroleum, followed by 2 ml of a saturated solution of sodium chloride in water. The tubes were then shaken vigorously for about 30 s and allowed to stand until two distinct and clear layers were formed. The upper layer was transferred to small vials, and the solvent was evaporated under a gentle stream of nitrogen; the residue was redissolved in 0.2–1 ml of carbon disulphide, and the vials were closed with a screw cap under nitrogen.

In a few cases (milk, corn oil, olive oil, etc.), direct transesterification was performed. Thus, microlitre volumes of milk, olive oil, etc. or microgram amounts of solid foods were mixed directly with the esterifying reagent and handled in the same way as described for the lipid solutions.

Gas–liquid chromatography

The different methyl, or ethyl, esters were analyzed by GLC using fused-silica capillary columns (30 m × 0.53 mm), coated with a 1-μm layer of Supelcowax 10 (Supelco). The analyses were performed in a Perkin-Elmer 900 gas chromatograph, equipped with a flame ionization detector. The injection block temperature was kept at 250°C and the detector temperature at 260°C. Nitrogen was used as the carrier gas and the separations were performed isothermally. The signals, originating in the flame ionization detector, were evaluated and recorded by means of a Model 3380 integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

RESULTS AND DISCUSSION

Transesterification of neutral lipids

In the presence of 5, 7.5 and 10% aluminium chloride in methanol the transesterification of all neutral glycerolipids tested appears to be complete. As a typical

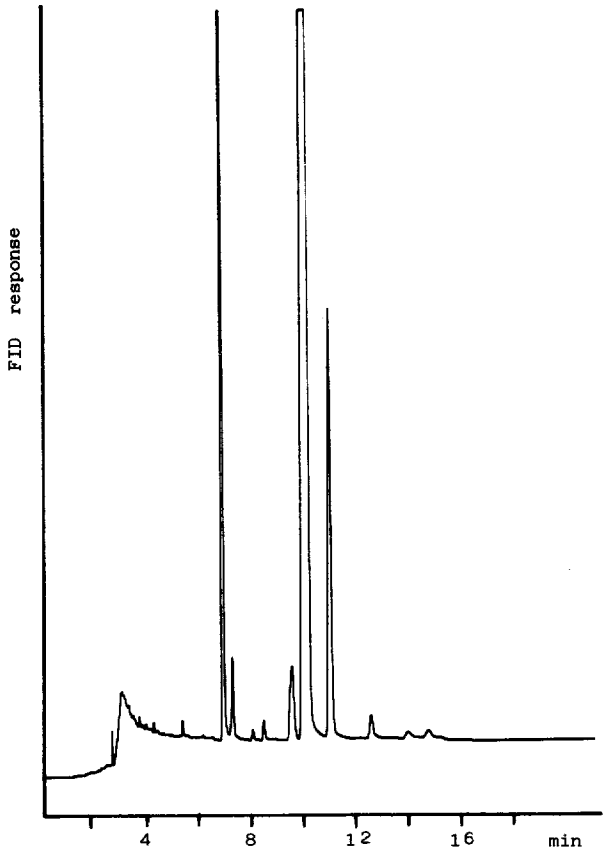

Fig. 1. Gas chromatogram of the methyl esters of fatty acids in olive oil. The methyl esters were prepared by direct transesterification with a 10% solution of aluminium chloride in methanol. For chromatographic conditions, see text.

TABLE I

FATTY ACID COMPOSITION (%) OF OLIVE OIL DETERMINED BY TRANSESTERIFICATION WITH ALUMINIUM CHLORIDE–METHANOL

Fatty acid	*Present method*	*Lit. method*[4,8]
14:0	–	6
16:0	11.1	9
16:1	1.4	–
18:0	2.2	3
18:1	73.5	77
18:2	9.1	11
18:3	0.5	–
20:4	0.2	–

TABLE II

FATTY ACID COMPOSITION (%) OF CORN OIL DETERMINED AS IN TABLE I

Fatty acid	*Present method*	*Lit. method*[48]
14:0	–	6
16:0	9.91	13
16:1	0.66	–
18:0	2.94	3
18:1	26.35	31
18:2	57.30	53
18:3	1.07	–
20:4	0.12	–

example, Fig. 1 shows the chromatographic analysis of the fatty acids present in olive oil; as can be verified from Tables I and II, the fatty acid composition of olive oil and of corn oil obtained by this method is practically identical to that reported in the literature for both types of oils.

The transesterification of triacylglycerols appears to be complete as long as the ratio of reagent to substrate is adequate to dissolve the lipid completely. I found that 4 ml of aluminium chloride solution are sufficient for the transesterification of about

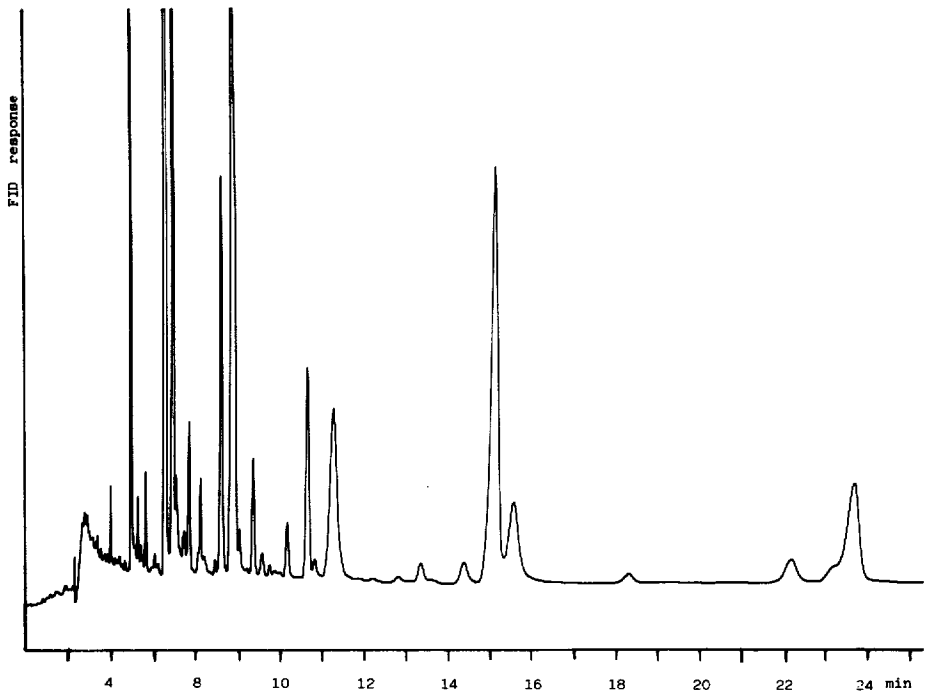

Fig. 2. Gas chromatogram of the methyl esters of fatty acids in marine fish oil. The fatty acid methyl esters were prepared by direct transesterification with a solution of 10% aluminium chloride in methanol. For chromatographic conditions, see text.

TABLE III

FATTY ACID COMPOSITION (%) OF MARINE FISH OIL DETERMINED AS IN TABLE I

Fatty acid	*Present method*	*Lit. method*[48]
14:0	8.3	6
16:0	17.4	13
16:1	9.1	13
18:0	4.3	3
18:1	14.8	20
18:2	3.3	2
18:3	3.2	–
20:1	3.5	12
20:5	14.8	9
22:1	5.7	6
22:5	2.2	2
22:6	7.4	9

10 mg of lipid at 100°C for 60 min. The reagent does not appear to affect the integrity of the highly unsaturated fatty acids, such as eicosatetraenoic, eicosapentaenoic and docosahexaenoic, as shown by the chromatogram (Fig. 2) and fatty acid composition (Table III) of marine fish oil, which is quite rich in polyunsaturated fatty acids.

It is noteworthy that, when using the proportions indicated above, it is un-

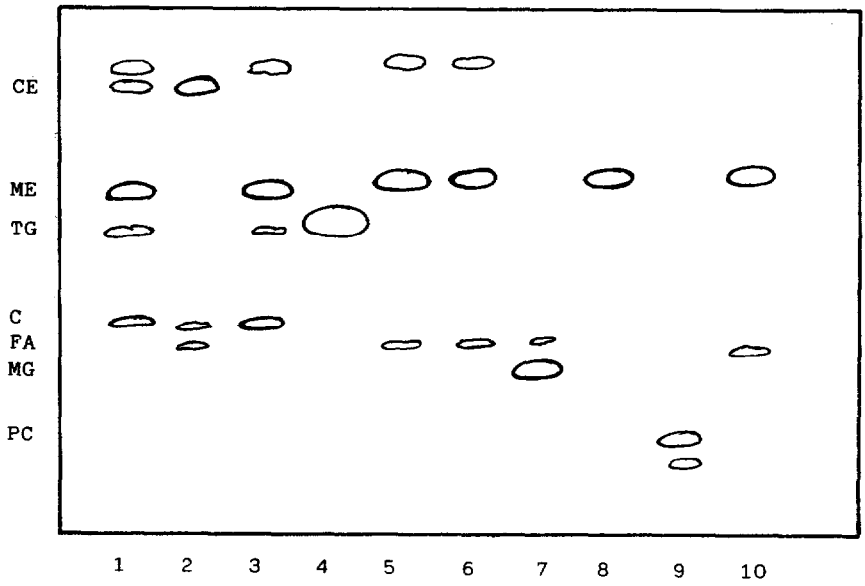

Fig. 3. TLC analysis of different lipid compounds and their corresponding derivatives after transesterification. 1 = Cholesteryl linoleate transesterified with boron trifluoride–methanol; 2 = cholestryl linoleate; 3 = cholesteryl linoleate transesterified with aluminium chloride–methanol; 4 = corn oil; 5 = corn oil transesterified with aluminium chloride; 6 = cow's milk transesterified with aluminium chloride; 7 = monoacylglycerol; 8 = monoacylglycerol after reaction with aluminium chloride; 9 = phosphatidylcholine; 10 = phoshatidylcholine transesterified with aluminium chloride.

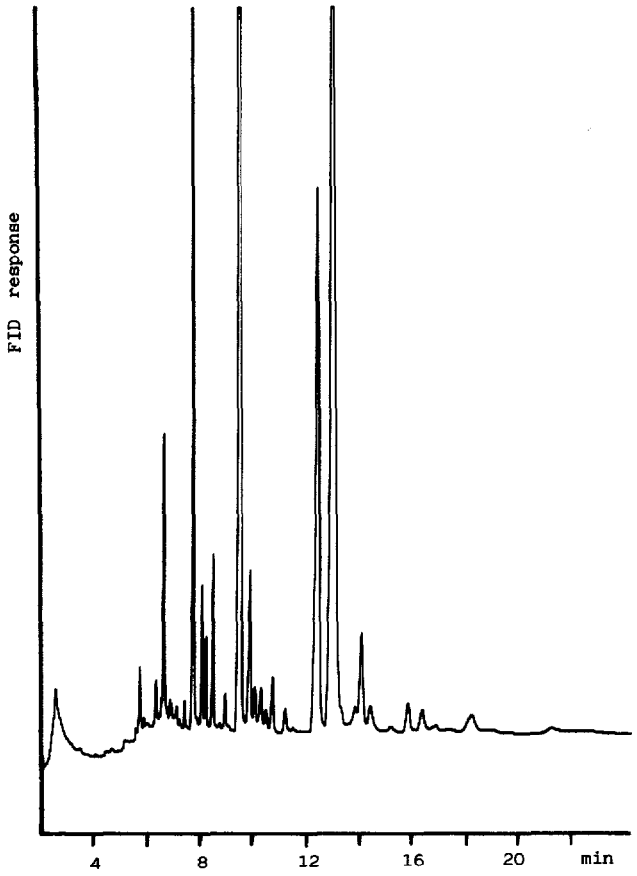

Fig. 4. Gas chromatogram of the methyl esters of the fatty acids in a sample of cow's milk reacted directly with a 7.5% solution of aluminium chloride in methanol. For chromatographic conditions, see text.

TABLE IV

FATTY ACID COMPOSITION (%) OF COW'S MILK DETERMINED AS IN TABLE I

Fatty acid	*Present method*	*Lit. method*[48]
12:0	2.96	2–6
14:0	11.38	12–6
14:1	1.53	.....
16:0	29.46	18–38
16:1	2.56	2–3
18:0	10.49	9–12
18:1	29.64	19–23
18:2	2.92	2–18

necessary to add a solvent of low polarity, *e.g.*, benzene to dissolve the corresponding glycerolipids and, thus, achieve complete methanolysis of these compounds. As Fig. shows, TLC analysis of the light petroleum extract demonstrates that the glycerolipids present in natural oils have disappeared completely, and only a main band, corresponding to the methyl esters, is seen after the reaction.

Direct transesterification of biological products, even when they contain a high amount of water, results in quantitative formation of the corresponding methyl esters. The fatty acid chromatogram obtained from a sample of cow's milk, added directly to the aluminium chloride–methanol reagent, shows that it is within the limits reported in the literature, which vary widely according to breed, climate, feed, etc. (Fig. 4 and Table IV).

Mono- and diacylglycerols are transesterified in the same way, and apparently in the same yield as the triacylglycerols. TLC analysis of the methyl esters, derived from a commercial sample of monoglyceryl laurate (Fig. 3), serves as an example. While the original product gives a strong band at the level of the monoacylglycerols, the extract obtained after the reaction with aluminium chloride–methanol shows only the band corresponding to the methyl ester.

Ionic glycerolipids (glycerophospholipids)

Similarly to the behaviour of the tri-, di- and monoacylglycerols, the different phospholipids (phosphatidylethanolamine, phosphatidylcholine, etc.) are completely

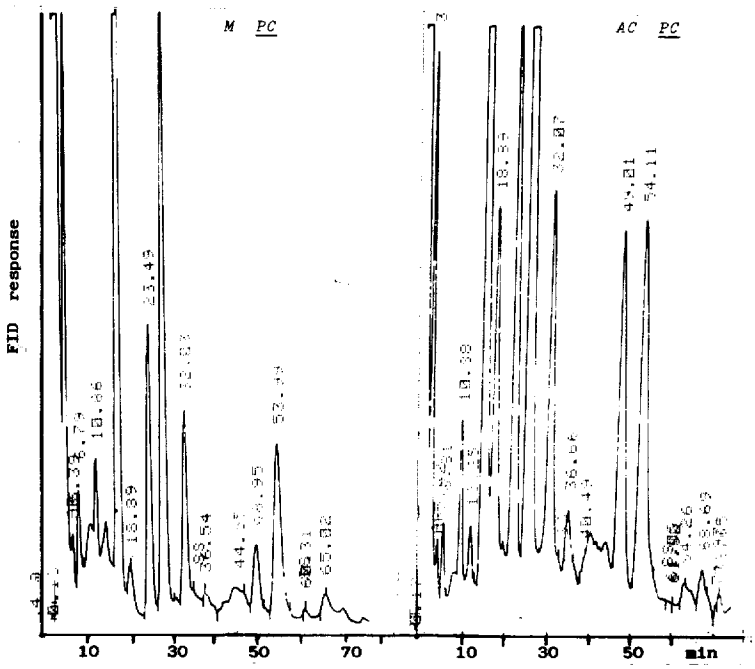

Fig. 5. Gas chromatogram of the methyl esters of the fatty acids in phosphatidylcholine obtained from a normal myometrium (M) and from an uterine adenocarcinoma (AC) in the same patient. The phospholipid, isolated by thin-layer chromatography, was subjected to direct transesterification with a 5% solution of aluminium chloride in methanol. For chromatographic conditions, see text.

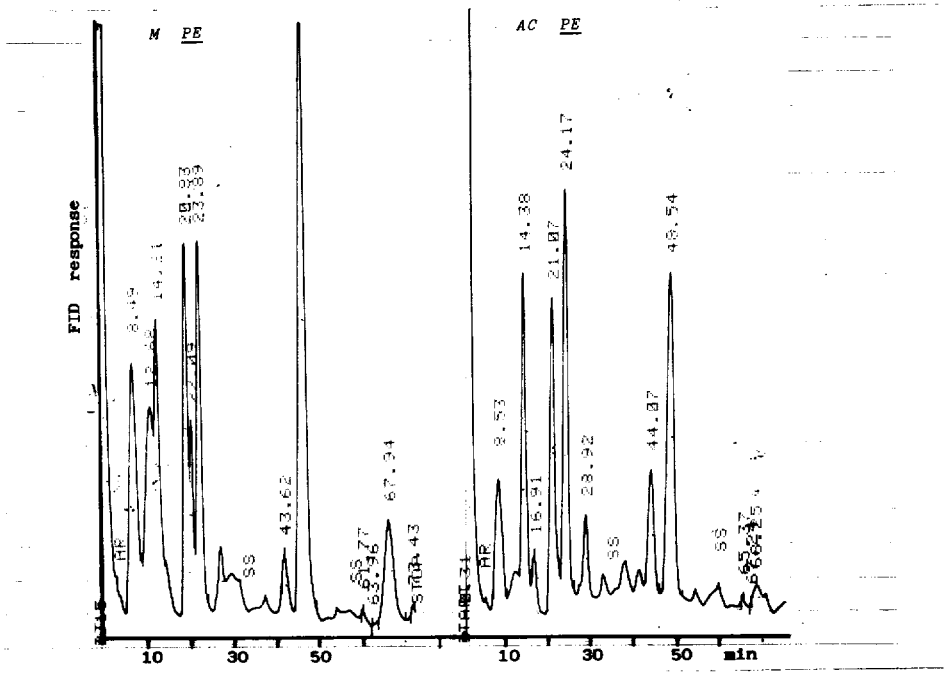

Fig. 6. Gas chromatogram of the methyl esters of the fatty acids in phosphatidylethanolamine obtained from a normal myometrium (M) and from an uterine adenocarcinoma (AC) in the same patient. The phospholipid, isolated by TLC, was subjected to direct transesterification with a 5% solution of aluminiumchloride in methanol. For chromatographic conditions, see text.

transesterified by the aluminium chloride–methanol reagent. Phosphatidylethanolamine from bovine brain, after reaction with methanol in the presence of aluminium chloride, shows a fatty acid pattern characteristic of this type of biological sample. By TLC, only the band corresponding to the methyl esters is seen after the reaction (Fig. 3).

Figs. 5 and 6 depict the fatty acid chromatogram corresponding to phosphatidylcholine and phosphatidylethanolamine, respectively, present in an adenocarcinoma of the uterus, compared to those present in a normal area of the same organ

TABLE V

FATTY ACID COMPOSITION (%) OF HUMAN PLASMA CHOLESTERYL ESTERS DETERMINED AS IN TABLE I

Fatty acid	*%*
16:0	11.77
16:1	3.63
18:0	3.35
18:1	23.84
18:2	50.84
20:4	6.54

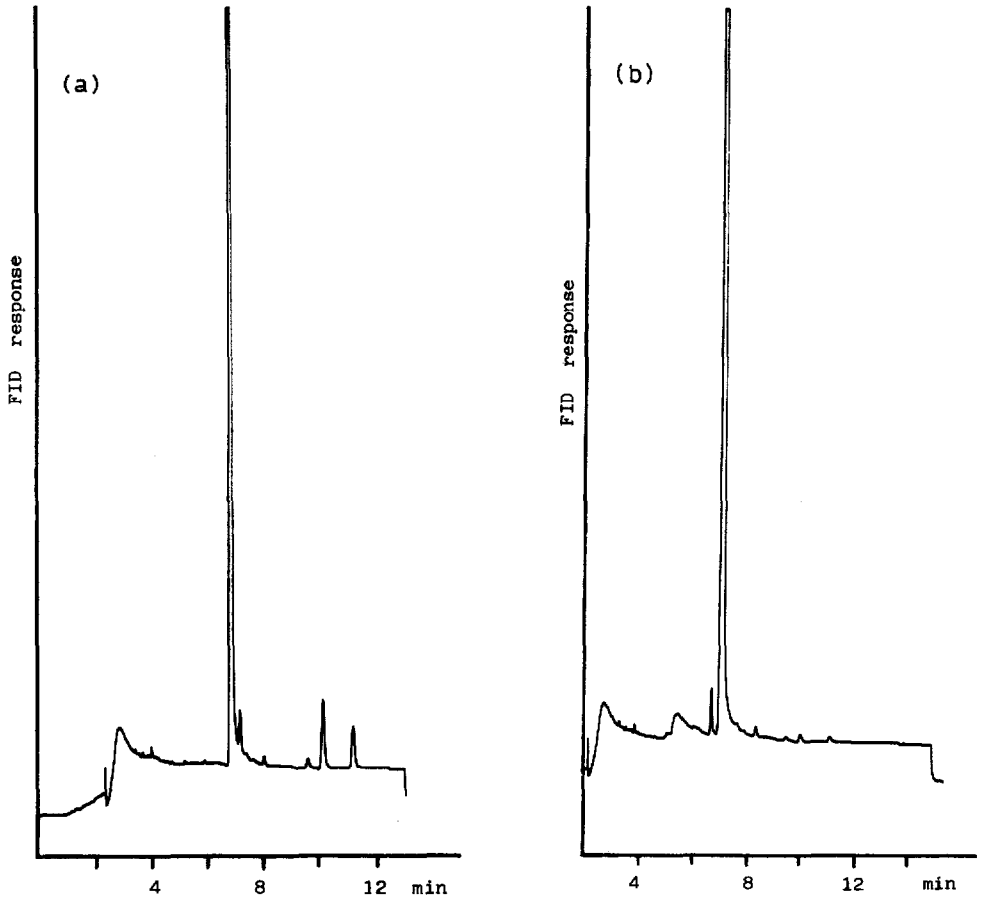

Fig. 7. Gas chromatogram of tripalmitin, directly transesterified with a 10% solution of aluminium chloride in (a) methanol and (b) ethanol. For chromatographic conditions, see text.

and subject. In this case, the analysis was performed on a packed column, containing Silar 10C as the stationary phase.

Sterol esters

Despite the fact that sterol esters are the most difficult type of compounds to be transesterified, the treatment of cholesteryl linoleate with aluminium chloride–methanol brings about the complete methanolysis of the sterol ester, while the non-esterified cholesterol remains unchanged (Fig. 3). On the other hand, GLC analysis shows the presence of only one major peak, that corresponding to methyl linoleate (Fig. 10).

The analysis of the cholesteryl esters in human plasma gave a fatty acid composition and proportions typical of those described for normal individuals (Table V).

Methylation of fatty acids

Quite surprisingly, non-esterified fatty acids (palmitic, linoleic, elaidic, etc.),

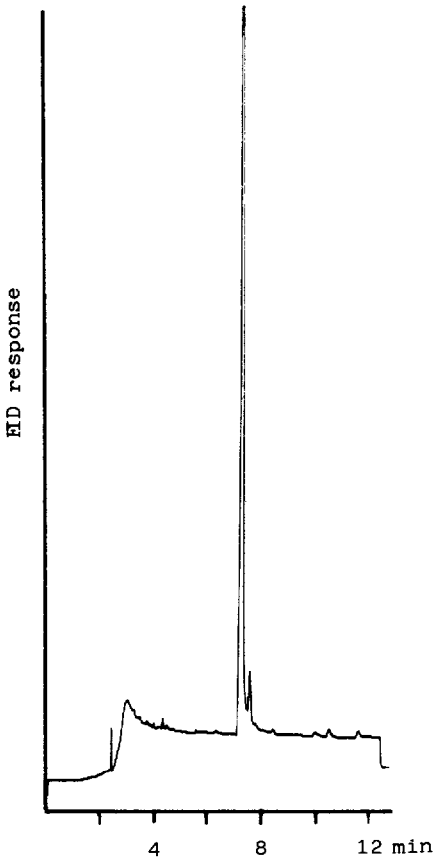

Fig. 8. Gas chromatogram of tripalmitin allowed to react directly with a 10% solution of aluminium chloride hexahydrate in methanol. For chromatographic conditions, see text.

when allowed to react with aluminium chloride–methanol, do not form the corresponding methyl esters. Variations in the temperature, duration of heating, concentration of reagent, etc., have no effect on the null yield of the reaction.

Transesterification with other alcohols

The use of ethanol, instead of methanol, with aluminium chloride as a catalyst, allows the formation of the corresponding ethyl esters. Fig. 7 shows the GLC analysis of tripalmitin, transesterified with a 10% solution of aluminium chloride in ethanol, in which a single peak, corresponding to ethyl palmitate, is seen. For comparison, the GLC analysis of tripalmitin, after reaction with aluminium chloride–methanol is also shown.

Transesterification in the presence of aluminium chloride hexahydrate

In order to determine whether the catalytic effect was due to a reaction or formation of a derivative between the anhydrous aluminium chloride and the alcohol,

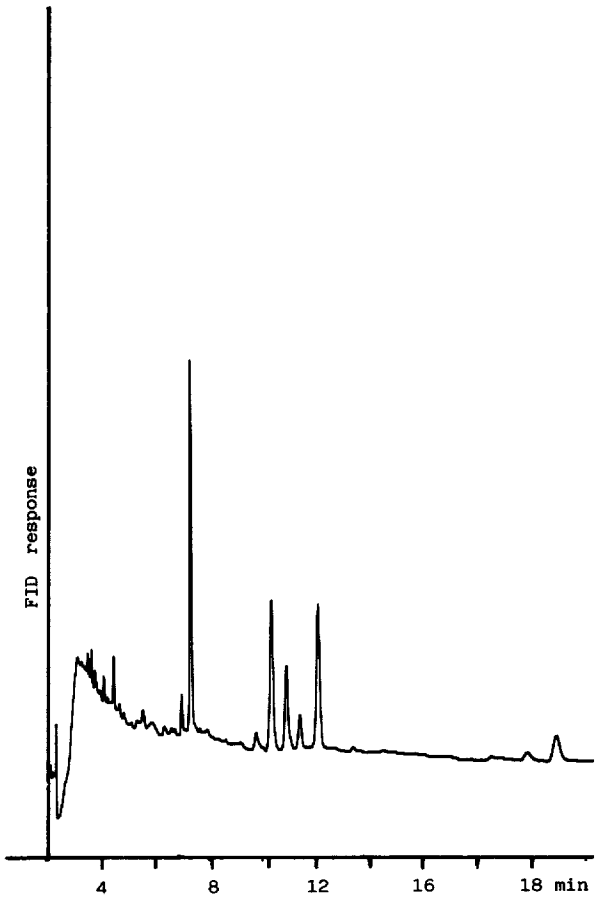

Fig. 9. Gas chromatogram of the fatty acids in the phosphatidylcholine from normal human plasma allowed to react with a 10% solution of aluminium chloride hexahydrate in methanol, in the presence of silica gel.

a 10% solution of hydrated aluminium chloride, which does not react with the alcohol, was tested with several types of lipids. As shown in Fig. 8, tripalmitin is completely transesterified in the presence of the hydrate, in the same manner as that observed with anhydrous aluminium chloride. In the same way, phosphatidylcholine obtained by preparative TLC from an extract of normal human plasma shows a normal fatty acid chromatogram in GLC analysis of the methyl esters formed in the presence of hydrated aluminium chloride (Fig. 9). On the other hand, formation of the corresponding methyl esters was not quantitative when this type of reagent was used with cholesteryl esters.

Aluminium chloride-methanol versus boron trifluoride–methanol

Transesterification with boron triflurorideñmethanol is a well established procedure; therefore, a comparison of the two reagents seemed of interest. The products

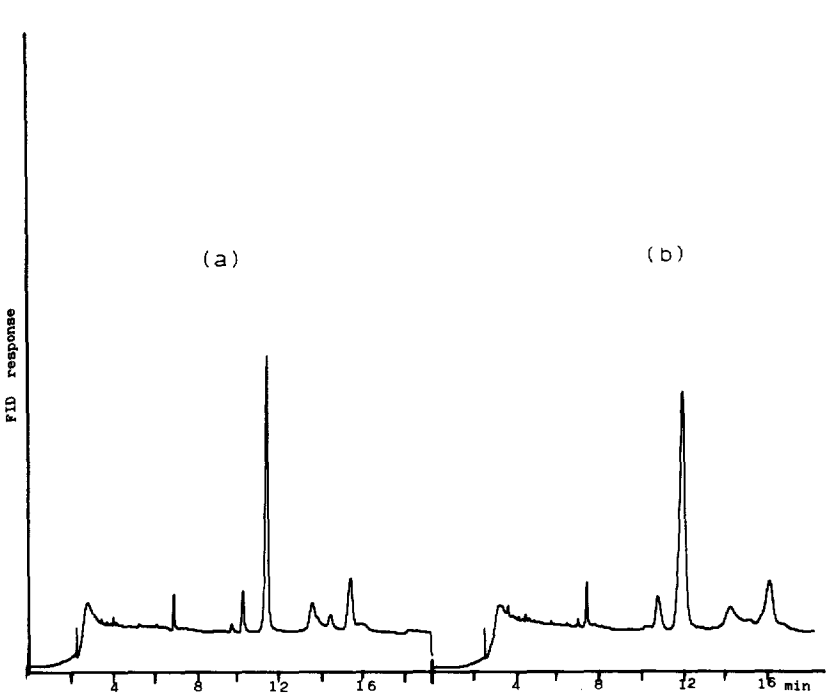

Fig. 10. Gas chromatogram of cholesteryl linoleate allowed to react (a) with a 10% solution of aluminium chloride in methanol and (b) with a 12% solution of boron trifluoride in methanol.

obtained after reaction of cholesteryl linoleate with boron trifluroride–methanol were compared with those obtained after reaction of this compound with aluminium chloride–methanol. As Fig. 10 shows, a major peak, corresponding to methyl linoleate, appears in the gas chromatogram in both cases; however, more artifacts also appear, as shown by TLC, in the sample treated with boron trifluoride than with that treated with aluminium chloride (Fig. 3).

Transesterification in the presence of silica gel

In most instances, it is convenient to prepare the corresponding methyl or ethyl esters directly on the silica gel used to fractionate the different lipids by TLC. Aluminium trichloride–methanol or –ethanol as well as boron trifluroride–methanol can be added directly to the adsorbent scraped off from the chromatoplate and containing the different lipid classes; the methyl or ethyl esters are formed in a way similar to that in a reaction in the absence of silica gel.

A method for preparing fatty acid methyl esters for GLC should ideally be "simple, rapid, and quantitative, and not give rise to unwanted structural changes or side reactions"[30]. With the reagent and procedure presented in this paper, most of the ideal conditions for the preparation of fatty acid methyl esters are fulfilled.

The reaction is performed with a minimum of glassware and instrumentation, does not require previous hydrolysis of esters and does not involve complicated or lengthy steps in the preparation of the reagent or its application. The reaction appears

to proceed quantitatively, without side reactions or formation of interfering artifacts.

Most of the catalysts used to achieve methanolysis of lipid compounds (boron trifluoride, diazomethane, perchloric acid, acetyl chloride, etc.) are quite volatile, irritating, flammable or explosive, making their use cumbersome or hazardous; in addition, some of these reagents have to be prepared freshly or stored under special conditions. In contrast, aluminium chloride, particularly its hydrated form, allows the preparation of alcoholic solutions that are very convenient to handle, can be used safely, do not generate irritating or flammable vapours and are stable for a long time at room temperature.

Initially, I thought that aluminium chloride would behave like an acid catalyst. However, the reagent made up with this salt and methanol does not form methyl esters with non-esterified fatty acids. No peaks by gas chromatography nor bands on thin-layer plates corresponding to the methyl esters have been observed, even in minimal quantities, indicating that none of the fatty acid molecules were converted into their methyl ester form. This suggests that the mechanism of the reaction is not identical to that of an acid-catalyzed process, as occurs with hydrochloric or sulphuric acid–methanol or with boron trifluoride or boron trichloride–alcoholates, which behave like strong acids.

The pH values of the different aluminium chloride–methanol solutions were found to be below 1. Nevertheless, the elimination of any volatile acidic compound, while bubbling nitrogen through the reagent, did not eliminate its capacity as a transesterification reagent, a fact that would reinforce the assumption that the mechanism of the reaction is different from that of the known catalysts.

The lack of methylation of non-esterified fatty acids with the aluminium chloride–methanol reagent, despite its drawbacks, may have some advantages. If a sample contains significant amounts of free fatty acids, additional steps are required to extract and evaluate them separately. Due to the fact that unesterified fatty acids are easily converted, even at room temperature, into their methyl esters by using acid catalysts in methanol, the determination of such components in an aliquot of the sample can be performed, followed by transesterification of the fatty acids combined with other hydroxyl groups in the different species of lipids.

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