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Short Communication

Long-chain fatty acids esterified by action of alkyl chloroformates and analysed by capillary gas chromatography

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ABSTRACT

Long-chain fatty acids can be instantaneously converted into methyl, ethyl or chloroethyl esters by the action of corresponding alkyl chloroformates. The reaction is catalysed by pyridine and proceeds in acetonitrile in the presence of a small amount of the corresponding alcohol. The reproducibility is high and the yield exceeds 95%.

INTRODUCTION

Fatty acid methyl esters (FAMEs) are widely used for routine lipid analysis by gas chromatography (GC). Esterification and transesterification (methanolysis) of fatty acids is one of the most comprehensively investigated and used techniques [1]. Transesterification of neutral lipids (triglycerides, phospholipids or cholesterol esters) is much easier to achieve than their hydrolysis (saponification) to free acids [2,3], even though rapid procedures for saponification are available [4,5]. However, the saponification of the lipids to free fatty acids was not, until recently, an attractive route because a rapid procedure for their esterification was not available. This situation has now changed, with the presentation of two approaches to the immediate esterification of free fatty acids. One is based on pyrolytic methylation in hot capillary GC injectors [6], and the other on the treatment of the acids with chloroformates [7]. This paper describes the optimal reaction conditions for esterification of long-chain fatty acids to methyl, ethyl or 2-chloroethyl esters with the corresponding chloroformates.

EXPERIMENTAL

Chemicals

Methyl, ethyl and 2-chloroethyl chloroformate (MCF, ECF, CECF), methanol, ethanol, 2-chloroethanol, acetonitrile, pyridine, hexane, etc. were obtained in the best available quality from Fluka (Buchs, Switzerland). Fatty acids mixture A-NHI-D was obtained from Supelco (Gland, Switzerland). Unsaturated fatty acids, UN-10 kit (palmitooleic, petroselinic, elaidic, oleic, linoleic, linolenic, arachidonic, erucic, nerv-

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onic and docosahexanoic acids) and brassidic acid were delivered by Sigma (St. Louis, MO, USA). Fish oil was kindly donated from a lipid laboratory. Aqueous solutions of 1 *M* sodium hydrogencarbonate were prepared. Reaction vials, 1 ml in volume with rounded bases, were made from 10 mm O.D. glass tubes with 10/14 mm ground glass joints.

Procedure

An artificial mixture of the above-mentioned unsaturated fatty acids with 16–24 carbon atoms and 1–6 double bonds was dissolved, together with myristic, palmitic and stearic acids, in dichloromethane. A 5-μl aliquot (containing 10–50 nmol of each fatty acid) was treated with 100 μl of the particular reaction medium, and 5 μl of the corresponding chloroformate (MCF, ECF or CECF) were added after brief shaking (2–3 s). The medium consisted of acetonitrile–pyridine–alcohol (methanol, ethanol or 2-chloroethanol), in volume ratio 22:2:1. With MCF the same esterification yield was achieved without methanol in the medium.

After each chloroformate addition, extraction was performed by adding hexane (100 μl) and water or aqueous hydrogencarbonate solution (200 μl) to the vial. After a brief shaking, an aliquot of the upper hexane layer was injected into the gas chromatograph.

Non-esterified fatty acids in the fish oil were liberated simply by shaking its solution in dichloromethane with an equal volume of aqueous carbonate solution (0.1 *M*). The upper aqueous alkaline layer with fatty acid salts was transferred to another vial and washed twice with equal volumes of hexane. After acidification with 1 *M* HCl, the fatty acids were shaken out with hexane. An aliquot of the hexane layer was evaporated and subjected to esterification with MCF.

Chromatography

A Carlo Erba 5300 MEGA series gas chromatograph equipped for flame ionization detection (FID) and a 25 m × 0.25 mm I.D. CP-Sil 88 (0.2 μm film thickness) fused-silica capillary column (Chrompack International, Middelburg, Netherlands) was used for the analysis. Alternatively, a 25 m × 0.32 mm I.D. CP-Sil 5CB column (0.11 μm film thickness) from the same supplier was employed for studies on intra-assay reproducibility. The temperature of the injector was maintained at 270°C, and the carrier gas (helium) pressure was 80 kPa with the former and 50 kPa with the latter column. The CP-Sil 88 column was programmed from 130°C (1 min hold) at 10°C/min to 240°C for fatty acids converted into 2-chloroethyl esters. The esters were injected in split mode (1:20).

TABLE I

INTRA-ASSAY ESTERIFICATION YIELD OF FATTY ACIDS DETERMINED VIA DERIVATIZATION AND INJECTION OF TEN INDIVIDUALLY PREPARED SAMPLES UNDER THE SAME CONDITIONS

The initial weight ratio of the fatty acids in solution, *i.e.* myristic, palmitic, palmitooleic, stearic and oleic, was 12, 24, 7, 13, 44%. Column, 25-μm CP-Sil 5 CB, film-thickness, 0.11 μm, injection split ratio, 1:20.

Acid	Methyl ester		Ethyl ester		Chloroethyl ester	
	Mean yield	C.V.	Mean yield	C.V.	Mean yield	C.V.
14:0	11.91	2.34	11.86	2.92	11.95	2.86
16:0	23.64	1.94	23.7	2.19	23.45	2.14
16:1	6.91	2.64	6.88	2.39	6.98	3.07
18:0	12.68	3.08	12.81	3.32	12.67	2.91
18:1	43.7	2.13	43.52	2.09	43.41	1.87
Total	98.84	2.12	98.77	2.73	98.46	2.9

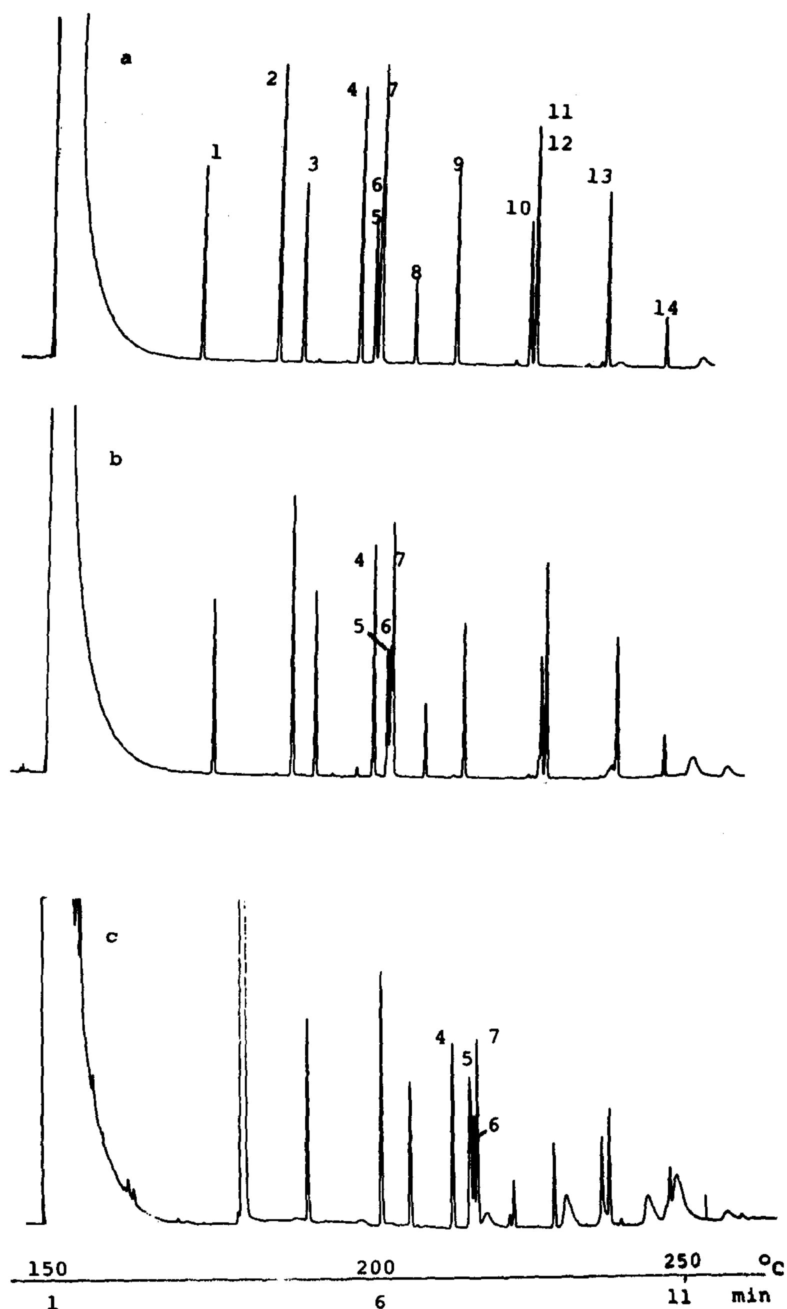

Fig. 1. GC–FID analysis of an artificial mixture of C_{16} to C_{24} fatty acids, treated with (a) MCF, (b) ECF, (c) CECF, on the CP- Sil 88 capillary column. Peaks: 1 = 14:0; 2 = 16:0; 3 = 16:1; 4 = 18:0; 5 = t18:1n9; 6 = c18:1n12; 7 = c18:1n9; 8 = 18:2; 9 = 18:3; 10 = t22:1n9; 11 = c22:1n9; 12 = 20:4; 13 = 24:1; 14 = 22:6. Injected amount, 2 nmol in total.

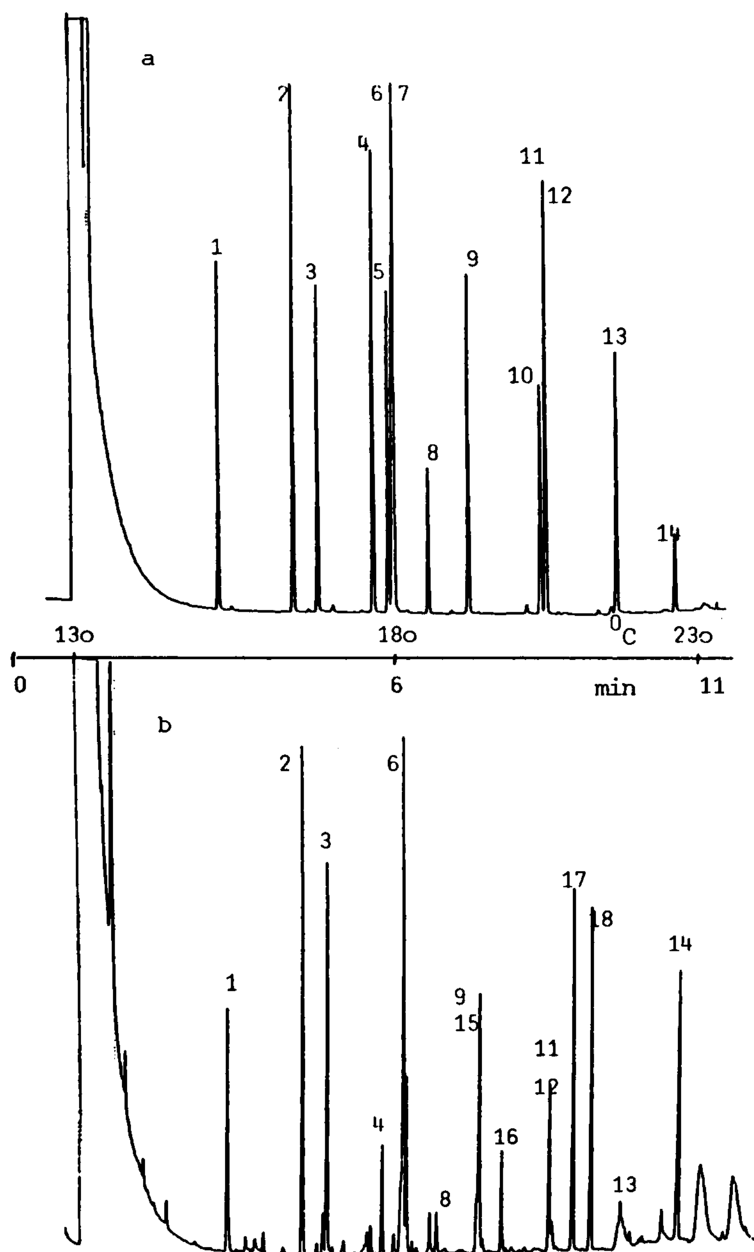

Fig. 2. GC–FID analysis of (a) the artificial mixture of fatty acids as in Fig. 1, and (b) fatty acids isolated from fish oil after treatment with MCF. Peaks: 1–14, as in Fig. 1; 15 = 20:1; 16 = 18:4; 17 = 20:5; 18 = unknown.

RESULTS AND DISCUSSION

Our earlier report [7] described a new approach to the rapid esterification of carboxylic groups by means of chloroformates. Even though the activation of carboxylic groups by chloroformates during formation of mixed anhydrides has been known for a long time [8,9], and the rate of decomposition of the anhydrides to corresponding alkyl esters has been examined comprehen-

sively [10], there has been no report on using the reaction in GC analysis. Nevertheless, without testing it on a large scale, we found that reaction conditions on the analytical scale are different from those given previously. In particular, the conversion into esters proceeds instantaneously, which is unprecedented among present procedures. There is, of course, one exception, *i.e.* the on-column methylation in hot injection port of a GC instrument [6]; however, the rate of conversion and reproducibility of this method are less satisfactory, and it cannot be used with short-chain fatty acids. The latter objection is also true for the most widely used procedure for fatty acids esterification, namely boron trifluoride-catalysed methylation [2]. Moreover, exclusion of water from the reaction medium is a pre-requisite.

These limitations do not affect our approach. Esterification proceeds in presence of water, and not only methyl esters but also ethyl and 2-chloroethyl esters are formed instantaneously [7]. The presence of water in the medium may be advantageous for the treatment of short-chain fatty acids but is useless with long-chain fatty acids. For lipophilic compounds derivatization in acetonitrile–pyridine, as given in Experimental, is the method of choice. The reproducibility and the average yield of ester formation are given in Table I. The study was done with the medium-chain fatty acids in commercial samples from Supelco. This is a representative choice, but the results with short-chain fatty acids were similar. Analysis of an artificial mixture of C_{14} to C_{24} fatty acids in form of methyl, ethyl and 2-chloromethyl esters on a highly polar phase in a 25-m fused-silica column is shown on Fig. 1. The originally 50-m column, delivered by Chrompack, was shortened to 25 m in order that the chloroethyl esters could also be eluted. However, elution of the $C_{22:6}$ fatty acid (highest retention) was not improved, and signs of column bleeding are already apparent from the record. Nor does the shorter column allow the separation of $C_{22:4}$ from $C_{22:1}$. It is interesting to note that separation of petroselinic ($C_{18:1n12}$) and oleic ($C_{18:1n9}$) acids improves with enlargement of the alkyl moiety.

Analysis of free fatty acids from fish oil in the form of methyl esters after a simple isolation step is given in Fig. 2. For comparison of retention times, a chromatogram of the methyl esters formed from the acids of the artificial mixture is included. The fatty acid composition of the biological sample is in a good agreement with previous findings [11]. Elution of the $C_{22:6}$ fatty acid, which is very abundant in the sample, proceeds well. Peak 18 could not be identified with any component of the standard sample.

CONCLUSION

A new method for the rapid and simple preparation of fatty acid esters with cheap chemicals is available. For GC analyses, it is possible to choose from three ester forms. Introducing a halogen atom into the alkyl moiety of the chloroformate can lower the detection limit in GC with electron-capture detection [12].

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