Fast Esterification of Fatty Acids with Alkyl Chloroformates

Optimization and Application in Gas Chromatography

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Summary
Alkyl chloroformates with methyl, ethyl, and 2-chloroethyl substituents can instantaneously esterify fatty acids under proper reaction conditions. Apart from the formation of the corresponding alkyl esters, even the alkoxy carbonyl esters can be prepared. These derivatives are useful for the analysis of short-chain fatty acids. As alkoxy carbonyl ester, even acetic acid can already be separated from the solvent peak. The reaction conditions were examined, and the influence of solvent polarity and reagent concentration on the conversion was studied. Quantitative conversion of acids to their esters was achieved in non-aqueous solutions, but even in the presence of water the yields were acceptable.

1 Introduction
Fatty acids and/or their esters can nowadays be determined by gas chromatography (GC) or high-performance liquid chromatography (HPLC). It is, e.g., possible to apply a universal derivatization method for use in GC as well as in HPLC in combination with the most frequently used detection methods [1], or not to derivatize at all and apply either of the separation techniques [2].

Using GC, the most popular derivatives are still the methyl esters [3, 4]. With short-chain fatty acids, however, it is impossible to separate the most volatile homologs from the solvent peak. Analysis of acids in the free form is, therefore, a viable alternative [5]. With HPLC, on the contrary, the analysis of non-esterified fatty acids is of a low importance because the analytes do not possess detection properties required for convenient trace level monitoring. Fluorescent labeling is often the method of choice to create derivatives for fluorescence detection [1, 6, 7]. In this way, of course, the advantage of HPLC as a technique not requiring a preceding chemical step is lost. Moreover, the necessary reagents are rather exotic and expensive.

Concerning the methyl ester formation for GC analysis, the most commonly used and one of the oldest procedures is the boron trifluoride catalyzed methylation [8, 9]. This is now the basis of a number of internationally accepted procedures, e.g., by the American Oil Chemist’s Society in 1969. Less used is the methanol-HCl catalyzed esterification [10]. The use of acetyl chloride as a catalyst for both methanolysis and methylation [11, 12] deserves more attention. At the beginning of the 1960’s there were a lot of new approaches dealing with methyl ester formation. For instance, 2,2-dimethoxypropane was found to be a good substitute for methanol in the HCl catalyzed esterification. The advantage of this reagent is that only free fatty acids are methylated, whereas fatty acids bound in neutral lipids remain untouched [13]. Almost instantaneous conversion of fatty acids into methyl esters in the hot injector of a GC instrument, so-called “on-column” methylation, can be obtained by the catalytic influence of tetraalkylammonium hydroxide in methanol [14, 15]. However, this method is not very reliable from a quantitative point of view, and inconvenient for short-chain fatty acids. Furthermore, selective methylation of free fatty acids can be performed by means of methyl iodide in a polar aprotic solvent [16, 17].

Some of the above-mentioned procedures [12, 14] are also effective in transesterification, i.e. methyl ester formation from fatty acids bound in neutral lipids. The base-catalyzed transesterification using sodium methoxide in methanol [18] or, more recently, a methanolic NaBH4/NaOH solution [19] is — as a single-step procedure — more attractive than two-step techniques using saponification followed by methylation of the liberated fatty acids. One reason for this is certainly the simple fact that none of the esterification approaches is rapid enough to justify the preceding, mostly lengthier, saponification step.

A completely new approach to esterification of carboxylic groups for analytical purposes resulted from an investigation of old reagents [20]. Chloroformates have been known in organic chemistry as a possible source of ‘mixed anhydride’ formation since the beginning of this century [21]. These reagents were investigated comprehensively in the 1960’s [22–25]. Chloroformates have been used in analytical chemistry for the treatment of amino and hydroxy groups only [26–28]. To our knowledge, the application of chloroformates to the derivatization of carboxylic groups has not been reported yet. In this paper it will be shown that alkyl or alkoxy carbonyl esters of carboxylic acids can be formed instantaneously and almost quantitatively. The optimization of reaction conditions for the treatment of fatty acids with methyl, ethyl, and 2-chloroethyl chloroformate, in order to create the corresponding alkyl or alkoxy carbonyl esters, is subject of this study.
2 Experimental

2.1 Chemicals
Methyl-, ethyl-, and 2-chloroethyl chloroformate (MCF, ECF, CECF), methanol, ethanol and 2-chloroethanol, acetonitrile, pyridine, hexane, chloroform (with or without ethanol as a stabilizer), all chemicals in best available quality, were obtained from Fluka AG (Buchs, Switzerland). Short-chain fatty acids with 2 to 12 carbon atoms, i.e., acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic, enanthic, caprylic, pelargonic, caprinic, undecanoic and lauric acid were obtained from Chrompack (Middelburg, The Netherlands) and Sigma (St. Louis, MO, USA) and dissolved in acetone to give a 0.1% solution of each.

A separate solution of the same concentration was prepared with enanthic, caprylic, and pelargonic acid. An aqueous solution of sodium bicarbonate was prepared in a concentration of 1 mol/l.

The reaction vials, 2 ml in volume and with rounded bases, were made from 10 mm o.d. glass tubes with 10/14 mm ground glass joints.

2.2 Procedure
The reaction conditions for esterification of the three model fatty acids are given in the legends to the Figures 1–5.

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Figure 1
Chromatograms of fatty acids with 7, 8, and 9 carbon atoms (5 μg each in 5 μl acetone = STANDARD SAMPLE) in 100 μl chloroform (stabilized with 1% ethanol) with 2% pyridine were injected (a) in this solvent, (b) after addition of 1 μl MCF, (c) after addition of 1 μl ECF (25 m CP-Sil 5 CB capillary column, temperature program 70–170 °C, 10°/min).

Figure 2
Standard sample in 100 μl of hexane-chloroform (3:1) with 2% pyridine treated with (a) 3 μl MCF, (b) 3 μl ECF (separation conditions as in Figure 1).

Figure 3
Standard sample in (a) chloroform with ethanol stabilizer, (b) chloroform without ethanol, (c) acetonitrile. To each solvent were added 2% pyridine and 1% MCF and analysis was performed as in Figure 1.
Formation of alkyl esters (I)

Procedure A: esterification in non-aqueous solvents

Fatty acids (2–10 μl of the acetone solution) are esterified simply by adding 5 μl of the appropriate chloroformate to 100 μl of the reaction medium with shaking (2–3 s). The medium consists of acetonitrile-pyridine alcohol (i.e., methanol, ethanol, or 2-chloroethanol) in a volume ratio of 22:2:1. Option: conversion to methyl esters proceeds smoothly even in the absence of methanol.

Procedure B: esterification in the presence of water

To 50 μl of a water-pyridine (5:1) solution is added 50 μl of an acetonitrile-methanol (5:1) solution for MCF treatment or 50 μl of an acetonitrile-ethanol (2:3) solution for ECF treatment. For treatment with CECF 25 μl of a water-pyridine (2:1) solution is mixed with 75 μl of acetonitrile-chloroethanol (2:3).

After each chloroformate addition, extraction is performed by adding hexane (100 μl) and water (200 μl) to the vial. After brief shaking an aliquot of the upper hexane layer is injected.

Formation of alkoxy carbonyl esters (II)

Methoxycarbonyl esters: 100 μl of hexane-chloroform (2:1) with 0.8 μl pyridine are treated with 1 μl MCF. Ethoxycarbonyl esters: 25 μl of acetonitrile-water-pyridine (8:3:1) plus 100 μl of hexane-chloroform (2:1) are treated with 3 μl ECF. For extraction, 200 μl of the aqueous bicarbonate solution is added instead of water, and the white precipitate is removed by shaking.

2.3 Chromatography

A Carlo Erba HRGC 5300 MEGA series gas chromatograph equipped with flame ionization detector (Carlo Erba Instruments, Milano, Italy) and a 25 m x 0.32 mm fused silica capillary column CP-SIL 5 CB (0.11 μm; Chrompack, Middelburg, The Netherlands) was used for the analysis. Injection was carried out via split mode (1:20) at 240 °C inlet temperature, and a helium pressure of 50 kPa.

3 Results and Discussion

As mentioned already in the Introduction, fatty acids can be submitted to GC analysis in the free form, provided that a well

Figure 4
Standard sample dissolved in 100 μl of acetonitrile-water (9:1) plus 2–4–8 μl pyridine and 1–2–4 μl MCF (a–b–c). Analyzed as in Figure 1.

Figure 5
Standard sample dissolved in 100 μl of acetonitrile-water mixed in the ratios (a) 5:1, (b) 3:2, (c) 1:1 plus 4–8–5 μl pyridine and 4–4–5 μl methanol (a–b–c) and treated always with 2 μl MCF. Analysis as in Fig. 1.
deactivated capillary with an appropriate stationary phase is used. A fused silica capillary CP-SIL 5 CB column from Chrompack was one of the best columns tested among those from various suppliers. As the free acids emerged from the column smoothly, the rate of conversion of the acids to their esters could be followed on the record (Figures 1–5).

Medium-chain fatty acids, with 7, 8, and 9 carbon atoms, were used as model compounds in the studies of carboxylic group esterification. The analytes were dissolved in chloroform, in which 0.5 to 1% of ethanol as stabilizer was added by the supplier. Pyridine in an amount of 2 vol% (0.25 mol/l) was added to each sample. The non-esterified fatty acids could be satisfactorily eluted during temperature programming from 70 °C to 170 °C (10 °C/min) in less than 7 min (Figure 1a).

The adventure of discovering new derivatization horizons started by the mere addition of 1 µl MCF to the mentioned solution. Three sets of peaks appeared in the chromatogram (Figure 1b): peaks with identical retention times as those of the free acids (lowest amount), peaks with shorter and peaks with longer retention times, the latter being predominant. Replacing MCF by RCF resulted in the disappearance of the peaks with shortest retention (Figure 1c).

Subsequently, the influence of the addition of larger amounts of reagents was studied. Changing the molar ratio of chloroformate to pyridine in the reagent (3 µl added), and diminishing the solvent polarity by addition of hexane to chloroform free of ethanol, resulted in formation of derivatives with highest retention (Figure 2a, b).

Reviewing the preceding chromatograms the following conclusions, confirmed by mass spectrometric analysis, can be drawn:

I. Methoxy carbonyl esters

(so called ‘mixed anhydrides’-derivatives in Figure 2a) are produced by coupling of the reagent to the carboxylic group:

\[
\text{R-C-} + \text{Cl-C-OC}_{2}H_{5} + \text{HCl} \rightarrow \text{R-C-OC}_{2}H_{5}
\]

II. Methyl esters are formed by decarboxylation of the former ones:

\[
\text{R-C-OC}_{2}H_{5} \rightarrow \text{R-C-} + \text{CO}_{2}
\]

III. Ethyl esters are formed by activation of the present trace amount of ethanol by the liberated hydrogen chloride from the chloroformate:

\[
\text{R-C-} + \text{HO-C}_{2}H_{5} + \text{HCl} \rightarrow \text{R-C-OC}_{2}H_{5}
\]

The following questions had to be answered experimentally:

a) Is the presence of alcohol necessary for the esterification?

b) Is chloroform the best medium for quantitative ester formation?

c) Under which conditions is the formation of alkoxycarbonyl esters promoted?

d) Is the esterification and/or decarboxylation catalyzed by the presence of pyridine?

These questions were answered by series of experiments, as demonstrated briefly in Figure 3. It was again confirmed that the trace amount of ethanol in chloroform is responsible for the ethyl ester formation (Figure 3a, largest peaks with highest retention), regardless of the kind of chloroformate used. With MCF, a smaller portion of methyl esters is produced as well. However, the presence of the particular alcohol, with an alkyl group corresponding to that of the chloroformate, is not decisive for the esterification as it proceeds in the absence of alcohol, too (Figures 3b, c). Acetonitrile proved to be the best reaction medium among the organic solvents tested. The portion of unreacted fatty acids was the lowest in acetonitrile (less than 3%, Figure 3c).

As the polarity of acetonitrile is one of the highest among organic solvents, which is beneficial for the reaction course, it seemed logical to test a medium with an even higher polarity, i.e., to esterify in the presence of water. Success in this respect would be of great advantage as the short-chain fatty acids and numerous other carboxylic acids are water-soluble. The results of this study are presented in Figures 4 and 5.

The presence of pyridine is required in the reaction medium. Below a certain concentration the reaction ceases. This threshold concentration is dependent on the composition of the medium. The presence of water has an adverse effect on the esterification yield, as is apparent on comparison of Figures 3c and 4a. With the same pyridine concentration and equal amount of MCF added, a partial substitution of acetonitrile by water (10%) results in lowering the esterification yield to about 50% only. However, this change dramatically when the pyridine concentration is doubled or, even better, increased four times (Figures 4b, c). In the latter case the ester formation is nearly quantitative and approaches that of Figure 3c.

There is still another possibility of improving the esterification yield in water-containing media. That is the addition of alcohol with an alkyl group corresponding to that of the chloroformate. This approach is presented in Figure 5, where methanol was admixed (in an amount of 4–5%) to a medium with various ratios of acetonitrile, water, and pyridine before the MCF treatment. The results are rather surprising as the best yield was obtained with aqueous ratios of acetonitrile and water (45:45 vol%) and pyridine-methanol (5:5 vol%), Figure 5c. Preference of acetonitrile to water (Figure 5a) or pyridine to methanol (Figure 5b) is of no benefit.

To conclude, methylated fatty acids with MCF requires pyridine as a catalyst, and acetonitrile (non-aqueous conditions) or acetonitrile-water-methanol as reaction medium. Under the specified conditions the esterification proceeds instantaneously and quantitatively.

Besides methyl esters, ethyl- and 2-chloroethylesters can be prepared using the particular chloroformate with the corresponding alcohol. The retention times are correspondingly higher, as apparent from Figure 6, where series of C6 to C12 fatty acids were submitted to the particular esterification procedure and analyzed as shown. It is noteworthy that the reaction conditions for effective ECF and CECF treatments differ from those for MCF treatment as given in the Experimental section and demonstrated by Figure 7. In acetonitrile-pyridine alone, the same rate of conversion as with MCF is not achieved. Addition of the corresponding alcohol to the reaction medium was necessary in order to esterify successfully. With acetonitrile it sufficed to add trace amounts (4%) of alcohol only and the esterification yield exceeded 95%. 

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Figure 6
Analysis of short-chain fatty acids with 2 to 12 carbon atoms as (a) methyl, (b) ethyl and (c) 2-chloroethyl esters, prepared according to procedure IA (column temperature program 50 °C, 1 min hold to 200 °C, 16/min). Peak numbers refer to the chain lengths of the acids (4', 5' are isobutyric and isovaleric acids; r = reagent-pyridine product).

For the average esterification yields and the reproducibility of the procedure the reader is referred to a report on long-chain fatty acid esterification [29]. The rate of conversion was much more dependent on the alcohol concentration in water containing media, mainly with ethanol and chloroethanol. Also the composition of the medium was important: ethanol required more water and chloroethanol more acetonitrile as accompanying solvent.

Figure 7
Influence of alcohol concentration in the reaction medium on the esterification yield of methyl (1), ethyl (2), and 2-chloroethyl (3) esters. The corresponding alcohols added into the medium are methanol, ethanol and 2-chloroethanol, respectively. Solid lines: esterification in acetonitrile only (procedure A), dashed lines: esterification in the presence of water (procedure B).

Figure 8
Analysis of short-chain fatty acids with 2 to 12 carbon atoms as (a) methoxycarbonyl esters, (b) ethoxycarbonyl esters, prepared according to procedure II. Analytical conditions as in Figure 5.
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However, even under optimal conditions the reaction yield did not reach that achieved with acetonitrile alone. The amount of alcohol should be held as low as possible respecting the findings with hydroxy acids [30]. The residual portion of free fatty acids (5–10%) can be shaken out with aqueous bicarbonate solution in order not to interfere in the chromatogram.

Methyl and ethyl esters of acetic to butyric acids are too volatile to be separated from the solvent peak. Separation is possible with chloroethyl esters only. However, there is still another possibility, even with MCF and ECF, to determine also these most volatile fatty acids. This is the formation of mixed anhydrides, i.e. alkoxy carbonyl esters (Figure 8).

For this reaction course it is necessary to add chloroformate in excess to pyridine, and to exclude alcohol from the medium, which is composed of organic solvents of low polarity. The mixed anhydrides are thermally stable enough to be analyzed successfully but they decompose in time. However, decomposition does not result in the corresponding alkyl esters by decarboxylation. The latter are formed only and instantaneously under the specified conditions.

The decline in yield with higher homologs, apparent already from Figure 8, is caused partially by solvent effects along with split injection and partially, perhaps, by column sorption. The former reason can be eliminated by splitless or on-column injection. The latter fact is unavoidable and means that the range of acids, which can be analyzed in this way, is limited to more volatile compounds.

The utilization of this approach for GC analysis of various organic acids with or without additional reactive groups in the molecule is under study at present.

References


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