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Short Communication

Improved procedure for the derivatization and gas chromatographic determination of hydroxycarboxylic acids treated with chloroformates

P. Hušek

Institute of Endocrinology, Narodni trida 8, 116 94 Prague I (Czechoslovakia)

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ABSTRACT

Hydroxymonocarboxylic acids with a hydroxyl group adjacent to the carboxylic group can easily be converted with alkyl chloroformates into derivatives amenable to gas chromatography. However, the main reaction product, the 0-alkoxycarbonylalkyl ester, is always accompanied by a certain amount of reaction side-products, by altering the sequence of addition of the organic base and the reagent. Under optimum conditions, $\geqslant 94\%$ of the mass of the analyte can be converted into the desired main product.

INTRODUCTION

Considering chloroformates as possible reagents for organic acid profiling of biological fluids, we were especially interested in the determination of hydroxycarboxylic acids (HA), as these compounds are of prime analytical importance mainly in diabetes [1–3]. A rapid and reliable method for serum lactic acid is still required [4,5].

According to a previous study [6], HA can easily be converted into 0-alkoxycarbonylalkyl esters [R′OCOO–(R)CH–COOR′] by reaction with alkyl chloroformates. However, the main reaction product was always accompanied with a certain amount of at least two side-products, one with a lower and the other with a higher retention time than the main product. The conclusion was drawn that the side-product with the higher retention time was the non-decarboxylated product, the alkoxycarbonyl ester [mixed anhydride, R′OCOO–(R)CH–COO-COOR′], which was prepared preferentially under altered reaction conditions.

However, the preceding statement had to be corrected as a subsequent GC-MS study [7] revealed that more than one peak emerged on the chromatogram after the main reaction product. Moreover, none of them had a retention time identical with that of the mixed anhydride prepared alternatively. In addition, in the previous paper [6] a mistake in the procedure was made in that the addition of chloroform should be accompanied by addition of sodium hydrogencarbonate solution to perform the extraction. In that connection, it was observed that the mixed anhydride decomposed progressively by action of the hydrogencarbonate whereas the side-products did not.

Correspondence to: P. Hušek, Institute of Endocrinology, Narodni trida 8, 116 94 Prague 1, Czechoslovakia.

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In this work, paper the reaction conditions were re-examined in order to minimize the side-product formation and a new approach to achieving of this goal is presented.

EXPERIMENTAL

Apparatus

A Carlo Erba MEGA Series 5000 gas chromatograph with a flame ionization detector and a Hewlett-Packard Model 3396A integrator were employed. The injector and detector temperatures were 240 and 270°C, respectively. The analysis was carried out on a 25 m × 0.25 mm I.D. FS-OV-1701-DF-0.25 fused-silica capillary column (Macherey–Nagel, Düren, Germany) in the temperature range 60–200°C or *80* (3-min hold)-240°C programmed at 10 or 15°C/min. Helium was used as the carrier gas with a head pressure of 100 kPa and sample size 2 μl with a splitting ratio of 1:20.

Chemicals

Methyl and ethyl chloroformate (MCF, ECF), pyridine, acetonitrile, methanol, 96% ethanol and chloroform (stabilized with amylenes) were obtained from Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany). The HA, i.e., 2-hydroxyacetic (glycolic), D-2-hydroxypropionic lithium salt (D-lactic), 2-hydroxybutyric sodium salt (HB), 2-hydroxyisovaleric (HIV), 2-hydroxyvaleric (HV), 2-hydroxyisocaproic (HIC), 3-hydroxybutyric (3HB) and 4-hydroxybutyric (4HB) acid, were

TABLE I

INFLUENCE OF REACTION CONDITIONS ON THE SIDE-PRODUCT FORMATION OF LACTIC ACID DERIVATIZED WITH MCF (I) OR ECF (II)

The values are means of five derivatization carried out at the particular composition of the medium; the relative standard deviation was below 5% on average.

(I)	Reaction medium for MCF treatment (%, v/v)			Peak abundance (%)						
	A	W	M	0	1	2	3	4	5	6
a	99	1	0	(<1)	80	*3.4*	11.1	2.2	<1	1.7
b	80	20	0	(2)	62	*4.8*	16.2	8.0	2.5	4.5
c	95	1	4	(4)	81	*4.4*	7.2	<1	<1	2.4
d	94	5	1	(2)	77	*5.1*	11.2	2.1	<1	1.7
e	90	5	5	(5)	81	*4.5*	7.0	1.0	<1	1.5
f	70	25	5	(6)	68	*7.3*	11.5	1.4	2	3.7
g	70	25[a]	5	(8)	74	*4.3*	9.9	<1	<1	1.8
h	46	46	8	(10)	65	*8.0*	8.5	1.5	2.5	4.5

(II)	Reaction medium for MCF treatment (%, v/v)			Peak abundance (%)				
	A	W	E	0	1	2	3	∑ (minor peaks)
a	99	1	0[b]	1	93	1.5	3	2
b	80	20	0[b]	1	68	4	15	12
c		1	4	1	91	2	3.5	2
d	95	10	10	2	84	5	7	2
e	0	2	98	17	70	6	5	2
f	33	33	33	6	64	13	14	3
g	50	25	25	5	72	9	11	3
h	17	50	33	8	74	6	7	5

[a] Acidified water (0.5 *M* HCl).
[b] Incomplete derivatization.

obtained from Aldrich. An equimolar mixture (without 4HB) of HA was prepared in water (100 μmol of each HA in 1 ml); individual HA were dissolved in water at the same concentration.

Procedures

General approach. A 2-μl volume of the aqueous solution of HA was covered with 200 μl of the corresponding medium (see Table I) composed of acetonitrile (A), methanol (M) or ethanol (E) and water (W, replaced with 0.5 *M* HCl in some instances), and pyridine in an amount of 10% (v/v) (7.5515% in some instances) was added. Subsequently, MCF or ECF in half the amount of pyridine was added, followed by 100 μl of chloroform and 200 μl of 1 A4 HCl. By briefly shaking the tube for about 10 s, the pyridine was transferred into the upper aqueous phase, which was removed by means of a push-button pipette. The chloroform phase was subsequently shaken with 200 μl of 1 *M* aqueous hydrogencarbonate solution and an aliquot was injected into the column. In some instances or for special purposes the wash of the organic phase with aqueous HCl or hydrogencarbonate solution was omitted; the results were not influenced by this change.

Special approach. The reaction medium for the highest yields of the main product consisted of acetonitrile containing 10% of water and 1% of methanol or 5% of ethanol for treatment with MCF or ECF, respectively (A and C in the figures). A parallel medium consisted of acetonitrile with containing 25% of water and 2.5% of methanol or 25% of ethanol, respectively (B and D in the figures). Then, 200 μl of the corresponding medium were mixed with either (i) 20 μl of pyridine and 10 μl of MCF or ECF were added (= normal mode: reagent after base, C and D); or (ii) 10 μl of MCF or ECF and 15 μl of pyridine were added (= reversed mode: base after reagent, A and B).

Following addition of 100 μl of chloroform the extraction was carried out as in the preceding section.

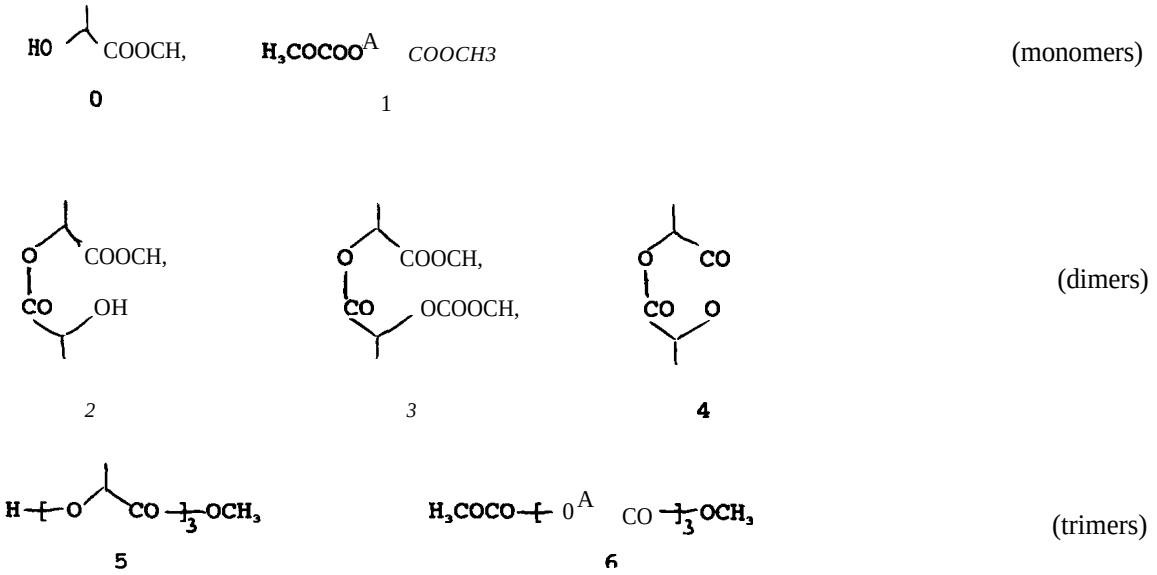
RESULTS AND DISCUSSION

General approach

The derivatization yields of HA treated with chloroformates were studied previously [6] using an equimolar mixture of the analytes. Under optimum reaction conditions the side-product formation was minimized but could not be fully eliminated.

With the individual HA at higher concentrations the occurrence of side-products was augmented. The most problematic HA proved to be lactic acid, affording largest amount of accompanying side-products, as is apparent from Fig. 1C and D.

The structures of the peaks were identified in the preceding study as follows:



Apart from the main reaction product, the O-methoxycarbonyl methyl ester (1), the activated molecules conjugate to form iactides or interester oligomers with alkoxycarbonyl-treated and with untreated hydroxyl groups.

Regarding the distinct side-products, the influence of the composition of the reaction medium on the reaction yield was re-examined. The results are summarized in Fig. 2 and Tables I and II, where the pre-peak, main peak and post-peaks are represented by 0, 1 and 2-6, respectively.

First, the previous findings were confirmed,

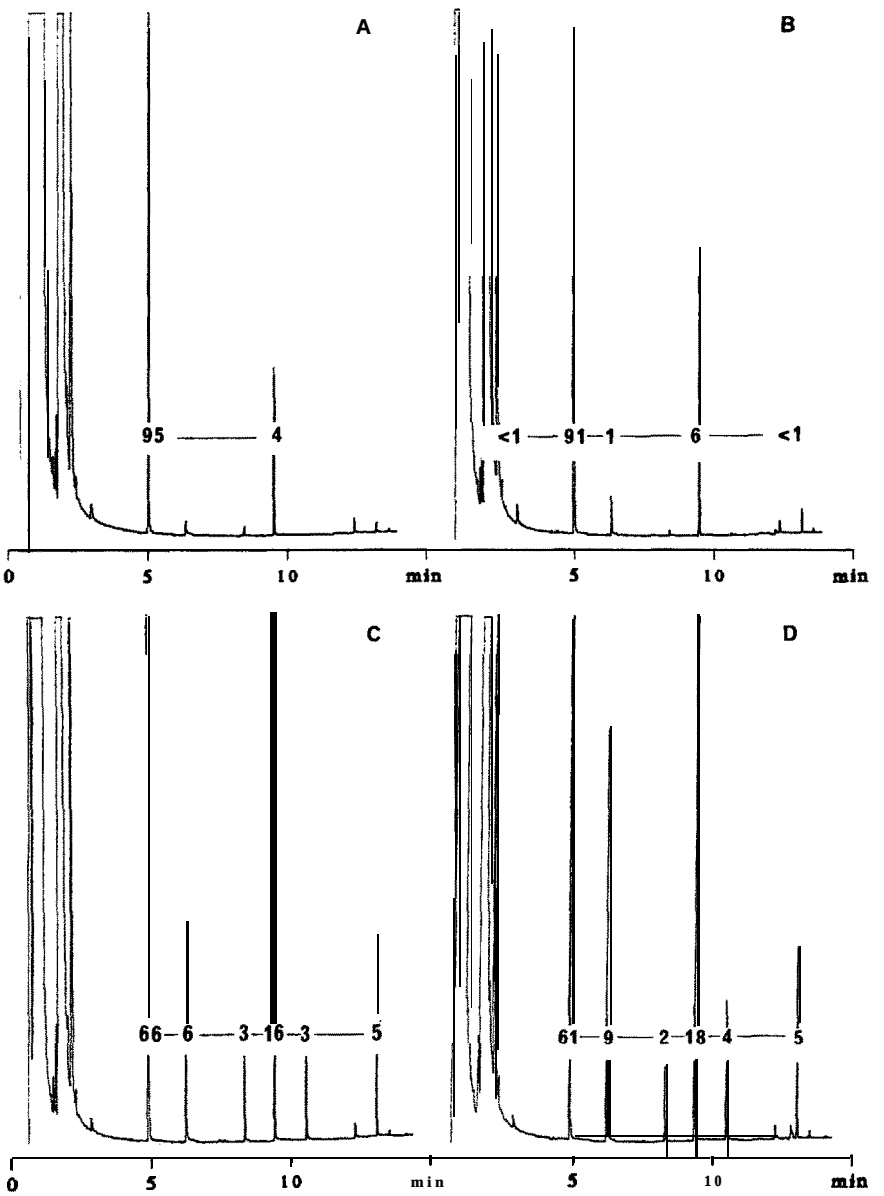


Fig. 1. GC profile of the reaction products of D-lactic acid trated with MCF according to the "normal mode" (C,D) and the "reversed mode" (A,B) in two different reaction media (A,C and B,D; see Experimental). The percentage peak abundance are given on the on the abscissa.

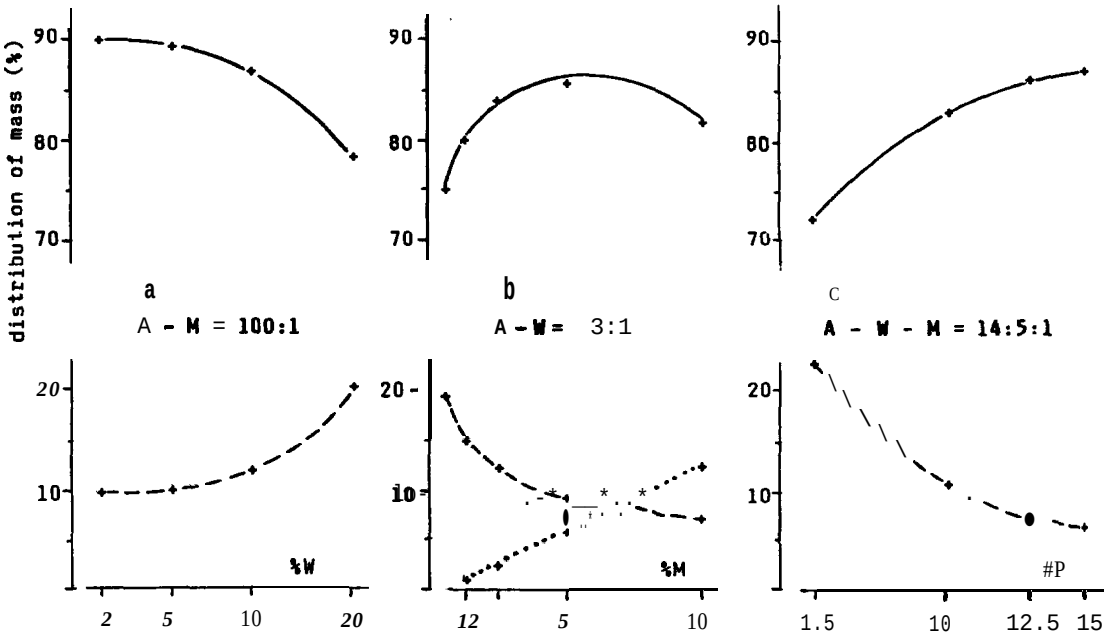


Fig. 2. Influence of water, methanol and pyridine concentration changes in the corresponding reaction media (a,b,c) on the distribution of mass of the MCF-derivatized HA (seven in total) between the main product (full line) and the side-products (dashed line = post-peaks, dotted line = pre-peak).

namely that acetonitrile should predominate in the reaction medium for the highest yields of the main product. When partially replaced with acetone, a small amount of mixed anhydride was always present on the chromatogram, confirming the results of the previous study [6]. Addition of water to acetonitrile causes a rapid decrease in the yield with a concomitant increase in the rear side-products. Moreover, the reaction with ECF does not proceed to completness in acetonitrile-water or acetonitrile

TABLE II

DISTRIBUTION OF MASS OF THE DERIVATIVES OF THE PARTICULAR HYDROXYCARBOXYLIC ACID TREATED WITH MCF IN A-W-M (14:5: 1) AND WITH ECF IN A-W-E (2: 1:1), RESPECTIVELY

The values are means of five identically derivatized samples; the relative standard deviations were less than 5% on average.

Hydroxycarboxylic Acid	Peak abundance (%)					
	MCF			ECF		
	Pre-peak	Main peak	Post-peaks	Pre-peak	Main peak	Post-peaks
Glycolic	6	87	6	5	82	12
Lactic	8	74	17	5	72	22
Butyric	8	75	16	12	77	10
Isovaleric	1	95	4	2	95	2
Valeric	6	89	5	6	87	6
Isocaproic	9	79	12	9	83	7
All (on average)	6	84	10	7	83	10

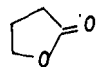
alone. Addition of ethanol is necessary, in general. Optimum yields with both MCF and ECF were achieved when the amount of alcohol added to acetonitrile was about 4–5%, and this is just the same reaction medium as for the treatment of fatty acids [8]. When water is added to replace acetonitrile partially in the medium, the yield of the main product declines, provided that alcohol is not co-added (Fig. 2a). From Fig. 2b it follows that even 25% of water is are tolerable when the amount of methanol is increased from 1 to about 5%. However, an increase in methanol concentration causes a rise of peak 0 (the values are in parentheses for MCF-treated lactic acid in Table I, as this peak more than less co-elutes with the front peaks and its amount is difficult to estimate), so that for an optimum yield of the main product a compromise between pre-peak and post-peak sizes, in relation to the content of alcohol, has to be found.

With ECF the best yields are achieved with 4–5% of ethanol in acetonitrile, and an increase in water content should be accompanied by the addition of an approximately equal amount of alcohol. It is interesting that even with 50% of water in the medium the third best results with lactic acid can be obtained (IIh, Table I). In contrast, the lowest yield of the main product was noted with equal amounts of the three solvents in the medium.

The preceding experiments were done with 10% of pyridine in the medium. However, the yields were improved by increasing the pyridine concentration to 15%, as shown in Fig. 2c and Table I (Ig). The same applies for treatment with ECF.

In Table II, individual differences in HA with regard to side-product formation are presented. As is apparent, lactic and HB acids are the most problematic, affording the highest abundance of the side-products. HIV and HV acids, on the other hand, give only small amounts of side-products. Hence the derivatization yield proved to be compound dependent to a certain extent.

The main reaction products of 3HB and 4HB acids have the hydroxyl group underivatized, as found previously [6]. However, about 5–8% are also alkylated on the hydroxyl group. This is true for MCF treatment; with ECF only 3HB acid behaves as described. When 4HB acid was subjected to treatment with ECF, the product formed was found to be the internal ester:



Special approach

The distribution of mass between the main and side reaction products can be positively influenced in favour of the main product by reversing the order of addition of the organic base and the reagent. With this "reversed mode", ***i.e.,*** when reagent is added to the reaction medium first followed by addition of pyridine, the formation of side-products is substantially reduced. This concerns especially lactic acid treated with MCF (Fig. 1), where the yield of the main product is 30% higher. With the ECF-treated lactic acid the difference is about 20% (Fig. 3), which means that the effect of reversing the order of addition is not so marked here. With the MCF-treated 2HB the yield of the main reaction product is relatively high even with the "normal mode"; the improvement after the reversal is about 10%. Moreover, the occurrence of double peaks points to the formation of diastereomeric interesters and lactides due to the racemic reference substance used.

Considering to the baseline at the rear of the chromatogram in Fig. 4, where an equimolar mixture of 7HA was analysed after treatment with MCF, the improvement with the reversed mode is also clearly apparent. Another phenomenon is the number of rear peaks, which are eluted almost continuously one after the other. With a high probability it can be concluded that interesters are formed not only by conjunction of two molecules of the same HA but even by conjunction of various members in the mixture. Hence the difficulty of minimizing the formation of side-products increases with increasing number of analytes in a mixture of HA, and this is of prime importance when a quantitative analysis is taken in account.

Under the optimum reaction conditions (main-product yields of 94–98%), the reproducibility was high enough to make the procedure suitable for quantitative analysis. The relative standard deviations were below 3% and a linear relationship was observed between the peak height and the amount of the lactic acid in the range l-1000 nmol. Moreover, the same accuracy and linearity were maintained even under the less favourable conditions

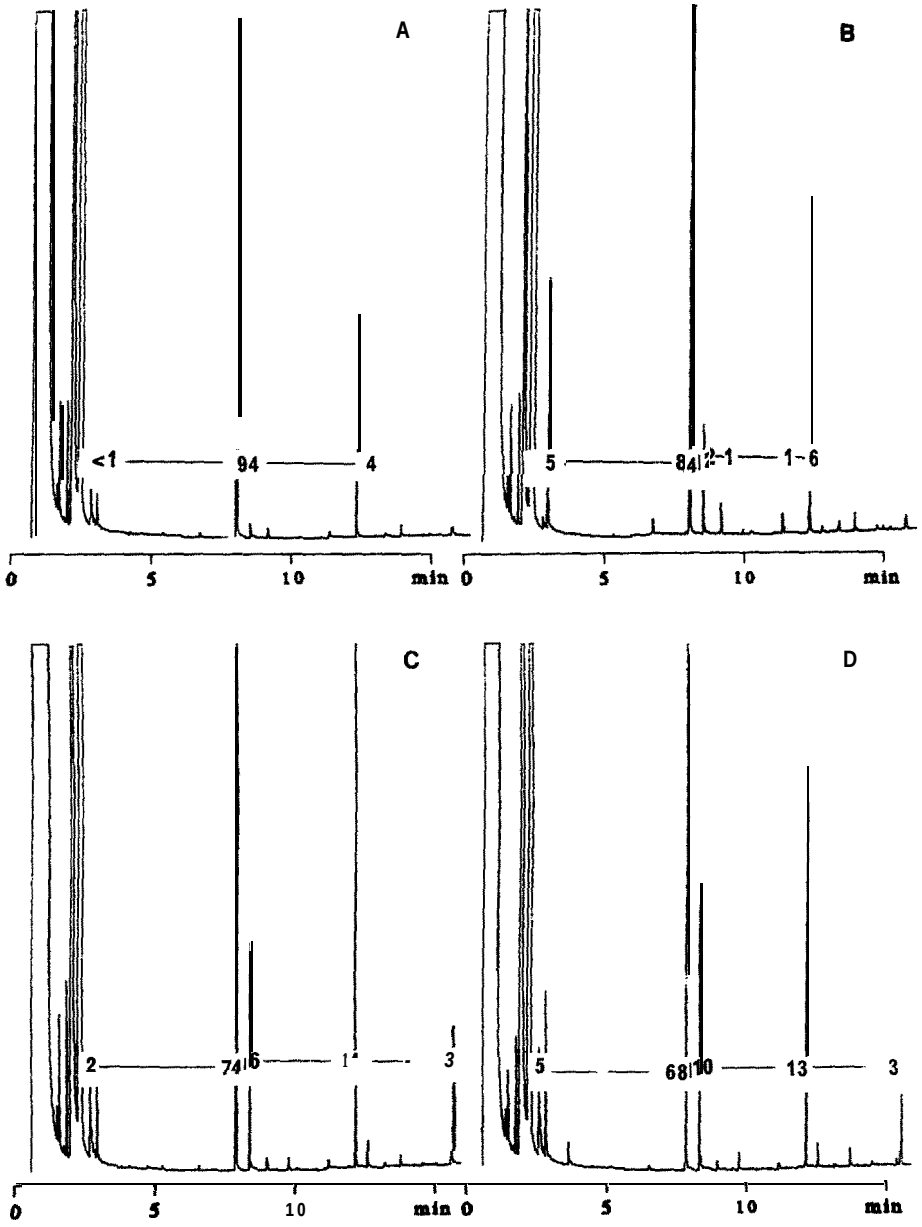


Fig. 3. GC profile of the reaction products of D-lactic acid trated with ECF in the same manner as with MCF in Fig. 1.

with a higher water content in the medium, which are, however, convenient for practical work up of biological fluids.

CONCLUSIONS

In previous papers concerning the derivatization of carboxylic acids with chloroformates, it was

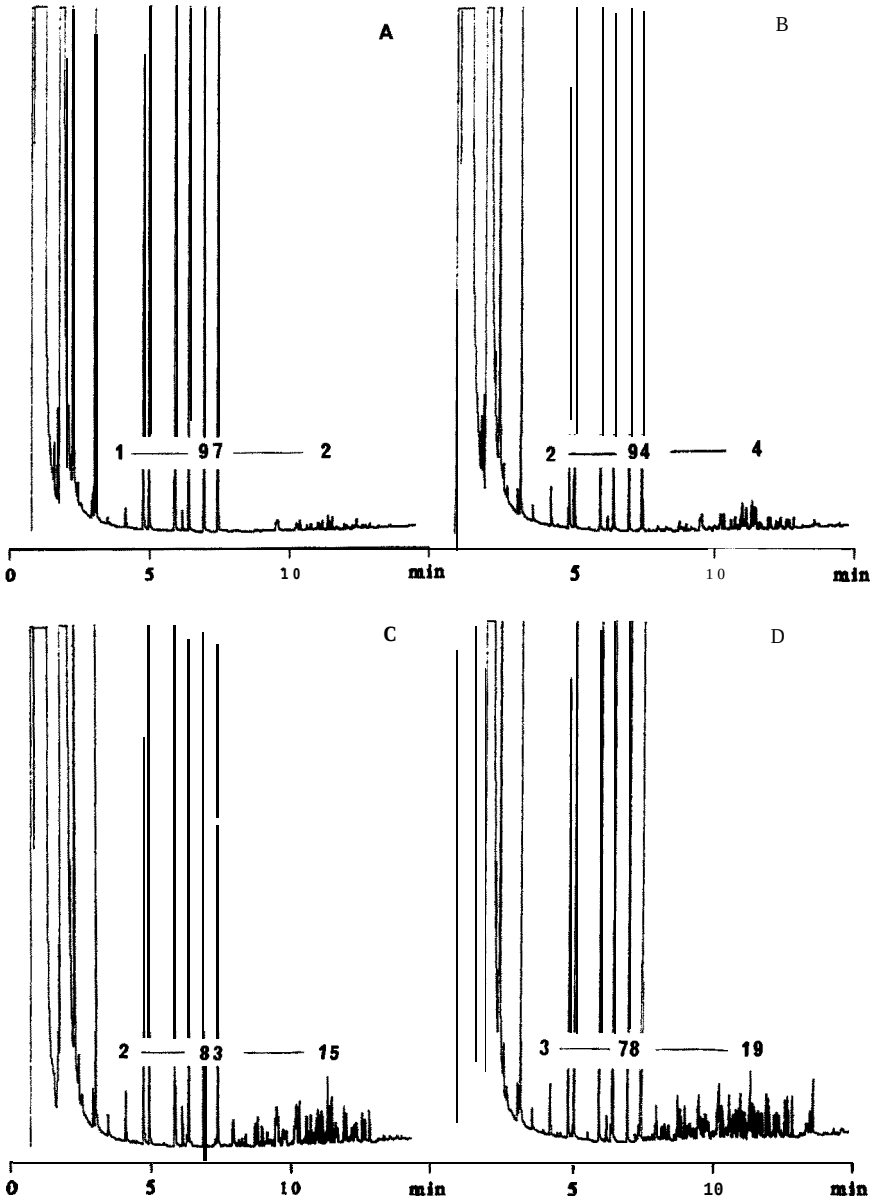


Fig. 4. GC analysis of an equimolar mixture of six 2-hydroxycarboxylic acids plus 3-hydroxybutyric acid (the first large peak after the solvent peak) after treatment with MCF as in Fig. 1.

demonstrated that with hydroxymonocarboxylic acids the optima of reaction conditions are especially important. Evidence is presented here that addition of the reagent to the reaction medium prior to the organic base, being the catalyst of the reaction, improves the yield of the main reaction product substantially and makes this procedure suitable even for quantitative analysis. The formation of oligomers by interesterification is greatly supressed by this way, especially when methyl chloroformate is used.

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