ELSEVIER

Journal of Chromatography B, 717 (1998) 57–91

JOURNAL OF CHROMATOGRAPHY B

Review

Chloroformates in gas chromatography as general purpose derivatizing agents

Petr Hušek[1]

Institute of Endocrinology, Národní Ave 8, CZ-116 94 Prague 1, Czech Republic

Abstract

Chloroformates with simplest alkyls, i.e. methyl, ethyl or isobutyl, already known as favourable reagents for treating amino groups in gas chromatography for years, were revealed randomly as exceptionally rapid esterification agents. Unlike the rather poor results achieved with chloroformate-mediated ester formation in organic chemistry, the pyridine-catalyzed esterification of carboxylic acids appeared to proceed at the analytical microscale smoothly. Along with the catalyzer, an alcohol should also be present in the medium, accompanied by acetonitrile or water, according to the character of the compounds treated. Reaction conditions were optimized for various classes of carboxylic acids and a uniquely rapid derivatization of amino acids in aqueous ethanol was shown to be possible. Most of the analytes, e.g. acidic metabolites in physiological fluids, could be treated directly in the aqueous matrix. A simultaneous analysis of, e.g., amino and fatty acids or amines and their acidic catabolytes was proven to be possible. Along with the low-molecular-mass reagents, still some others, i.e. the hexyl, menthyl or pentafluorobenzyl ones, found their application fields. Results of optimized reaction conditions and a wide range of applications of chloroformate-mediated derivatization in various disciplines have been summarized in this review.

Keywords: Reviews; Derivatization; Chloroformates; Carboxylic acids; Amino acids

Contents

[1]Fax: +420 2 24905325; e-mail: phusek@endo.lib.cas.cz

0378-4347/98/$19.00
PII: S0378-4347(98)00136-4

1. Introduction

Instrumentalized chromatographic and electromigration techniques play a predominant role in compound identification and quantification in analytical chemistry at present. Gas chromatography (GC), the oldest instrumentalized separation technique, still holds a leading position among them, as follows from the recent survey done in European laboratories [1]. The unmatched separation power of capillary GC, the availability of universal detectors and the popular combination of GC with mass spectrometry (MS) account for its widespread use.

A lot of organic compounds with reactive groups such as fatty acids and amines possess sufficient volatility and thermal stability for analysis by GC. However, their interactions with active sites present in every GC system, deteriorates the analysis as a rule, unless special selective column coatings are at hand. As a matter of fact, there are categories of compounds containing polar functional groups, many of them of considerable biomedical and environmental interest, which are practically inseparable. Some are thermally labile at the temperatures required for their separation, others are ionic and not volatile enough to change them into the gaseous state. To make these compounds amenable to GC, the technique of derivatization was developed [2–4]. Basically it is a microchemical synthesis employing chemical reagents/reactions to convert the original structure of the analyte into another (derived) molecule.

It is generally valid that the nature of the compounds to be analyzed, i.e., the variety of the reactive groups present and their chemical properties, govern the choice of the particular chemical treatment. Development of a new derivatization method requires a good knowledge of organic chemistry with a knowledge of as many reaction mechanisms as possible. This is especially true in case of derivatization of protein amino acids with so many different chemical groups present, as we have here ‘the whole Beilstein chemistry’. It does not mean, however, that the reaction conditions used by organic chemists at the macroscale are readily adaptable to analytical microscale. The discovery described here may serve as a practical confirmation of this statement.

Derivatization in GC is usually the most time-consuming and labour-intensive part of an analytical procedure. The chemical pretreatment of the sample aims at removing the active hydrogen atoms from all, or at least most, of the reactive groups. It is desirable that it should be carried out as quickly as possible with a minimum number of reagents and reaction steps. For polyfunctional carboxylic acids, there is generally two basic approaches as options. One is based on two (or more)-step procedures involving separate esterification and acylation reactions, the second involves a universal one-step, one-reagent silylation (or alkylation) treatment [2,3]. A special procedure(s) called cyclization may be used for treatment of bifunctional compounds with at least two functional groups in close proximity: by the action of the particular reagent, inactive cyclic derivatives are formed [4].

During the last decade, many of the GC derivatization methods became more or less useless. Their time-conditional invention was dictated by a lack of alternative techniques for detection of minute amounts of some analytes especially in biological fluids. Before the discovery of immunoassay techniques and the onset of sensitive HPLC detectors, the electron-capture detector (ECD) in GC was obviously the only way to reach picomolar level. At that time scientists were enormously interested in re-

agents and treatments to convert the analytes into perhalogenated products with a correspondingly high ECD response. Some of those methods persisted, many others were left. Currently, the haloalkylacyl reagents in multi-step derivatization procedures and the universal silylating reagents belong to those mostly required in treating compounds for GC analysis. Moreover, regarding speed and ease-of-sample pretreatment, the chloroformate-mediated derivatizations are supposed to open a new era in derivatization methodology.

2. Treatment of amino groups in aqueous media

Most derivatization reactions use apolar reagents that require organic solvents that are not directly compatible with aqueous material. Contrary to that, chloroformates have been widely used to convert amines into carbamates in buffered aqueous media. Such reactions proceed rapidly at room temperature and are presently of frequent use in HPLC amine and amino acid analyses. The 9-fluorenylmethyl chloroformate (FMOC) and some others with bulky aryls became increasingly popular in the methodology of sample pretreatment [5,6].

2.1. Derivatization of amines and phenols for GC analysis

Unlike labeling compounds in HPLC, where the bulky aryl-chloroformates function as UV or fluorescence detection tags, the rather low-molecular-mass reagents used in GC work primarily to volatilize the analytes. The methyl, ethyl and isobutyl chloroformates (MCF, ECF, IBCF) plus some halogen-containing reagents, e.g. the trichloroethyl and pentafluorobenzyl ones (TCECF, PFBCF), synthesized for purposes of selective ECD detection, proved their usefulness in treating phenolic and amino groups.

At the beginning of the 80s, before the onset of HPLC with electrochemical detectors, biogenic amines were often treated with MCF [7,8] or ECF [9] to prepare derivatives amenable to GC. Since alcoholic groups do not react with the reagents, unlike the phenolic hydroxyl [10], silylation was often subsequently performed. According to the reaction conditions chosen, the catechol functions, converted to carbonates, were transformed to silyl ethers or retained the carbonate moieties after the subsequent step performed.

Ahnfelt and Hartvig studied derivatization of aliphatic amines [11,12] and aminobutyric acids [13] and concluded that the TCECF was an order of magnitude more reactive than ECF or IBCF. Even the tertiary amines, treated preferably with PFBCF, underwent transformation to carbamates due to displacing the smallest one of the groups attached [14,15].

IBCF was obviously the reagent of choice leading to a compromise between a derivative of higher mass and one of greater stability. Derivatization of e.g., urinary aliphatic amines [16] or 57 amines in aqueous samples [17] was often performed by extractive alkylation with IBCF in organic solvent and the analytes in basified water. Besides also, the novel approach tailored for esterification of carboxyl, was used in treating methylimidazole in caramel colour with IBCF in acetonitrile–isobutanol–pyridine in spite of any carboxyl present [18].

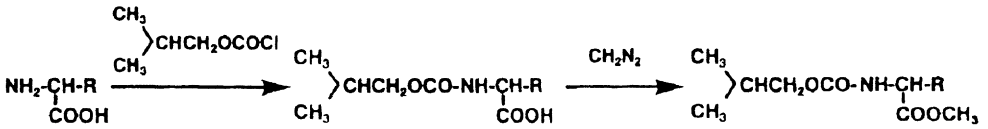
Regarding the completeness of amino group to carbamate conversion, the conclusions are not fully consistent. Some authors [7] reported maximal yields of both the phenolic and the amino groups in the pH range 7–8.5, others introduced a pH shift from 7.5 to 9 or more to provide for phenolic hydroxyl and to improve reaction of the amine functions [8,17], or treated the amines at pH 12 [16]. It was stated already earlier that the choice of pH depends on the basicity of the amine and quantitative yields since the very basic diamines required a pH$>$12 [19].

However, urinary amphetamines treated with MCF, PCF (propyl-) and BCF (butyl-) in a pH range of 9.5–14 for 1 min showed no significant effect upon the derivatization yield [20].

The recent review article on derivatization reactions for amines determined by GC affords a good survey on this important topic [21].

2.2. IBCF in two-step derivatization of amino acids

Treatment of amino acids with IBCF in aqueous carbonate was first reported two decades ago by Makita et al. [22–24]. The two-step procedure was based on alkylation of the amino group and most of



the side-chain groups with IBCF in the carbonate (10 min at 20°C) and subsequent methylation of the carboxyl groups with diazomethane in ethereal extract from the preceding step (see above).

Before done, an intermediate ethereal extraction aimed at removing the reagent excess. After acidification of the medium, the semiderivatized acidic analytes were transformed into the second ethereal extract and methylated. As a result, all the amino, imino, imidazole, phenolic hydroxyl and thiol groups were converted to isobutyloxycarbonyl (IBOC) products. The alcoholic hydroxyl groups (ser, thr, hyp), indole ring of tryptophan and amido groups of asparagine and glutamine remained untouched. Despite the latter fact, even these amino acids were successfully eluted from packed-GC columns, unlike arginine, the guanidino group of which could not be subjected to such an effective treatment to allow its determination.

Starting in the 80s, the GC technique advanced substantially and with fused-silica capillaries and chemically bonded phases, new horizons for the old procedures were opened. Kataoka and coworkers continued the studies and using the same derivatization principle, many useful applications and improvements took place. In the earlier studies, *O*-phosphoamino acids liberated from proteins were converted to *N*-IBOC methyl or trimethylsilyl (TMS) esters [25–27] and selectively and sensitively detected by GC with a flame photometric detector (FPD). The high detection limit of FPD toward P and S-atoms was utilized also in studies aimed at the determination of the important class of sulphur amino acids [28–30]. The two-step procedure involved treatment of aminogroups with isopropyl chloroformate (IPCF) in the first step and with acidified methanol in the second reaction step, after evaporation of the preceding ethereal extract. Wide-bore (0.53 mm I.D.) and relatively short capillaries succeeded in separating 14 sulphur amino acid standards and those occurring in urine [28], for determination of plasma glutathione [29] and total homocysteine [30] with related aminothiols, following reduction of the disulfidic bonds with sodium borohydride. The body fluid analytes were determined directly without a prior cleanup and noticeable interference from coexisting substances.

Nevertheless, the main emphasis was on continuous refinement and utilization of Makita's procedure. First, the reaction course of IBCF treatment was shortened to seconds by means of sonication and a properly selected capillary column allowed analysis of *N*(*O*,*S*)-IBOC methyl esters of protein amino

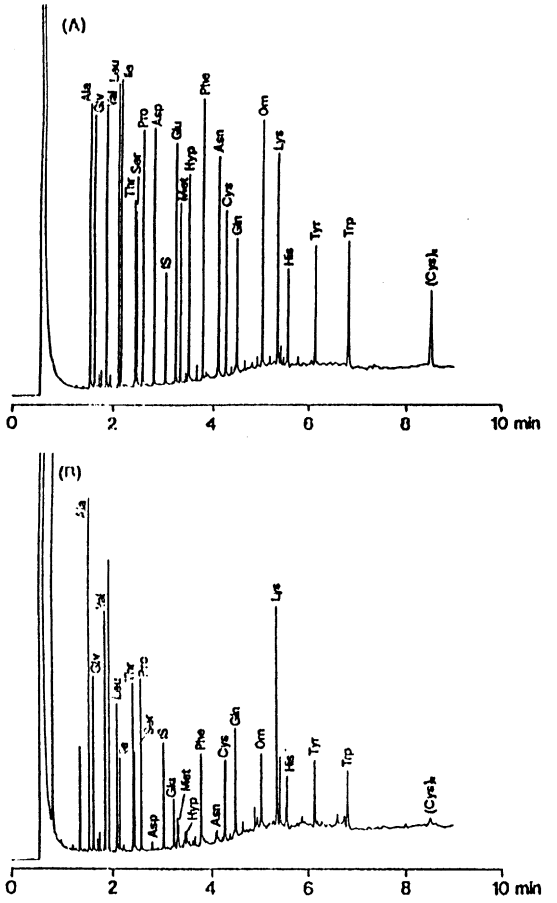


Fig. 1. GC–FID analysis of *N*(*O*,*S*)-IBOC amino acid methyl esters as chromatographic standards (A) and those from 0.1 ml-serum sample (B). Reproduced from Ref. [32].

acids within 9 min [31]. Together with a simple procedure for serum workup, involving deproteinization only, the preceding approach succeeded in rapidly determining the serum amino acids [32] as follows from Fig. 1.

Second, a suitable capillary column, along with a sophisticated pressure- and temperature-programmed operation by means of GC electronic pressure control, rendered a rapid and simultaneous analysis of protein and nonprotein amino acids possible [33]. Third, to enhance sensitivity and selectivity for determination of amino acids in biological fluids, the nitrogen–phosphorus detector (NPD) was employed alternatively to the flame ionization detector (FID) [34] and both the NPD and FID detection of urinary and serum amino acids were compared [35]. NPD detection of 54 amino acid standards and those of a urine sample is shown in Fig. 2.

As an alternative to the above procedure, silylation instead of methylation was performed as the second reaction step. Kim and others [36,37] subjected the *N*(*O*,*S*)-IBOC amino acid intermediates to a silylation with *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) in order to convert the

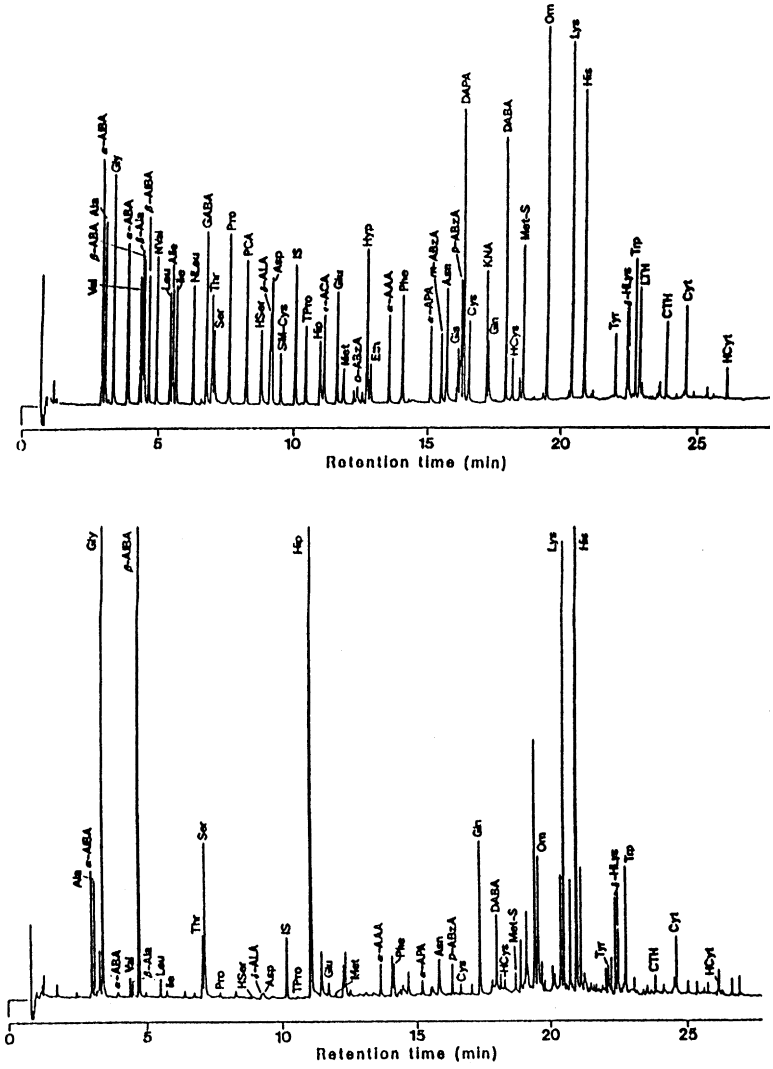


Fig. 2. GC–NPD analysis of *N*(*O*,*S*)-IBOC amino acid methyl esters of 21 protein and 33 nonprotein amino acid standards (top) and those from urine samples (10–50 μl). Reproduced from Ref. [34] with permission.

free carboxyl into the TBDMS ester. The intention was to silylate even the free alcoholic and amido group functions untouched by action of IBCF and, perhaps, to reach even arginine. The latter aim failed and serine and threonine required a longer reaction time and double-derivative formation with threonine was observed [36]. When tetrahydrofuran replaced acetonitrile in the silylating medium, the yield of both the amides declined markedly [37]. Therefore, the only advantage seemed to be a substitution of the unpleasant diazomethane by another reagent. However, these authors were the first to present a simultaneous analysis of 51 amino acids as *N*(*O*,*S*)-IBOC TBDMS esters even when the resolution was not complete on the selected capillaries [37].

3. Chloroformate-mediated transformation of carboxylic groups

The easy conversion of amino and phenolic groups to carbamates and carbonates in aqueous media was recognized in analytical chemistry early and used frequently in various applications, as discussed. In organic chemistry, however, much more attention was paid to the reaction of chloroformates with carboxylic groups leading to the formation of so-called mixed anhydrides. These reaction products appeared stable enough to sustain GC vaporization in our own testing, and reactive enough to enter reactions with groups containing active hydrogen.

Till recently, however, nothing was known about the ability of chloroformates to act as esterification agents in water-containing media. The discovery made by the author of this review at the analytical microscale randomly came without basic knowledge of the achievements made in organic chemistry and under different reaction conditions. It appeared that an immediate esterification of carboxyl might be possible under the conditions found. Let us briefly reflect on the results achieved at the organic macroscale.

3.1. Retrospection to mixed anhydrides in organic chemistry

The ability of chloroformates to form mixed (i.e. carboxylic–carbonic) anhydrides with carboxyl was described by organic chemists already at the beginning of our century [38]. They were prepared at low temperature in an inert solvent (dichlorormethane, toluene) under a catalytic influence of triethylamine (TEA) smoothly, within 30 min according to rule:

$$R_1\text{-COOH} + \text{Cl-COOR}_2 \xrightarrow[[\text{TEA},CH_2Cl_2]]{-\text{HCl}} R_1\text{-COOCOOR}_2$$

Mixed anhydrides have been found to be excellent acylating agents for the synthesis of peptides by 3 independent groups in 1951 [39–41]. The by-products of the reaction, carbon dioxide and an alcohol, were readily removed and the peptide was obtained in a high state of purity:

$$\text{X-NHCHRCOOCOOR}'' + H_2NR' \rightarrow \text{X-NHCHRCONHR}'' + CO_2 + R'OH$$

Tarbell and coworkers [42–45] studied the formation, stability and decomposition of the mixed anhydrides intensively at the end of the 50s. They found them to be more stable than previously supposed, e.g. even upon contact with aqueous bicarbonate (unlike our findings, see Section 4). *N*-Methylpiperidine (NMPIP) appeared to be a more effective catalyst than TEA in forming the mixed anhydride from a highly branched chlorocarbonate [43].

Regarding the stability and decomposition of these remarkable products, the suggestion already made much earlier [38] was confirmed experimentally [42,43]: the mixed anhydrides yield two sets of products on heating, the ester (path A) and the symmetrical acid anhydride plus the dialkyl carbonate (path B) (see below).

$$R_1COOCOOR_2 \xrightarrow{A} R_1COOR_2 + CO_2$$
$$R_1COOCOOR_2 \xrightarrow{B} (R_1CO)_2O + (R_2O)_2C{=}O + CO_2$$

In the absence of catalysts (e.g., NMPIP, sodium methoxide, dry hydrogen chloride etc.) the transformation required temperatures around 150°C but it was greatly accelerated in dimethylformamide. The proportions of A and B products were otherwise not altered by changes in solvent, temperature, or the presence of catalysts. Windholz [46] confirmed the figured pathways and reported good ester yields upon heating over 200°C. Mutual interaction of the second set of products (path B) supported the yield of the desired ester (path C):

$$(R_1CO)_2O + (R_2O)_2C = O \xrightarrow{C} R_{COOR_2} + CO_2$$

In the studies mentioned, details are given on how the structure of the alkyls involved in the process (R_1, R_2) influence the course and direction of the transformation. However, in view of preparing esters via the decomposition of mixed anhydrides, the outcome of the reaction was rather disappointing, with few exceptions. A facile conversion of the mixed anhydrides of α-keto acids into α-keto esters in the presence of TEA was reported by Domagala [47] and, half esters of malonic acid, treated with ECF and TEA in tetrahydrofurane at 4°C, underwent a spontaneous decarboxylation of the mixed anhydride to the ester within 30 min [48].

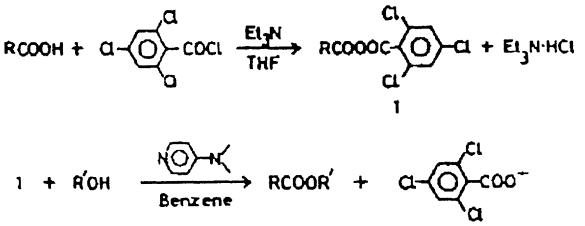
3.2. Mixed anhydrides as intermediates of ester formation in nonaqueous media

Further experimental studies of the 80s, especially those of Kim et al. [49,50], revealed that the transformation of the anhydrides to the esters can be promoted by the use of 4-dimethylamino-pyridine (DMAP) as a catalyst. Most aliphatic carboxylic acids, when treated with equimolar amounts af an alkyl chloroformate and TEA under catalytic influence of DMAP in dichloromethane at 0°C, yielded the corresponding esters in high yields within 15–60 min and, without contamination of the symmetrical acid anhydrides and the carbonates [49]:

$$RCOOH + ClCOOR' + Et_3N$$
$$\text{---[cat.DMAP, } CH_2Cl_2\text{,0°C]} \rightarrow RCOOR'$$

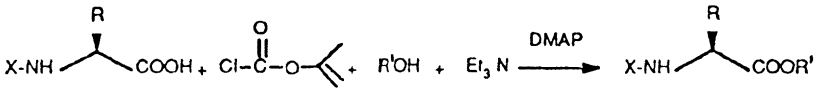
A variety of structurally different carboxylic acids and reagents were subjected to the process to determine the scope and limitations of the method. The esterification yields with aromatic acids were found to be critically dependent on the nature of the reagents. Although these acids gave a mixture of the ester, the acid anhydride and the carbonate as a rule, increased amounts of DMAP drastically lowered formation of the latter. This method reached a limit with sterically hindered acids (pivalic, mesitoic) where the formation of the acid anhydride and the carbonate was favored. With *N*-protected α-amino acids, the reaction proceeded under the given conditions smoothly, without observable racemization in most amino acids, and was complete within 30 min. However, when hydroxycarboxylic acids were treated with ECF, a mixture of the ethyl ester and the lactone was obtained in a variable ratio [50].

Next to this, others [51–54] experienced an alternative mechanism of the mixed anhydride transformation: the alcoholysis. Inanaga et al. [51] reported on a rapid and mild esterification consisting of two steps: formation of the mixed anhydride and its subsequent alcoholysis. The 2,4,6-trichlorobenzoyl chloride was found as the most satisfactory agent as regards the rate and the yield of the alcoholysis:



Aromatic hydrocarbons, such as benzene or toluene, were found to be the most suitable solvents for the alcoholysis step and DMAP proved again to be the best catalyst. In such a medium, the esters of, e.g., secondary and tertiary alcohols were prepared at room temperature in yields exceeding mostly 95% within an hour.

Ten years later, Jouin and coworkers [52–54] reported on esterification of *N*-protected amino acids via activation with isopropenyl chloroformate. In situ alcoholysis of the unstable mixed anhydride intermediate was also catalyzed by DMAP:



Competing isopropenyl ester formation was negligible when using methylene chloride as the solvent. A variety of esters from primary and secondary alcohols were obtained with good yields (60 to 96%) and the following was stated: (a) Isopropenyl chloroformate appeared to be the most versatile reagent for acid activation; (b) an appreciable amount of isopropenyl ester was noticed when *N*-protected amino acid reacted with the reagent in the absence of alcohol in tetrahydrofurane instead of in methylene chloride; (c) this method of esterification failed for amino acids with nonprotected side-chain functions such as hydroxyl or amine: thus, the presence of the free hydroxyl group in serine or threonine did not allow the preparation of the corresponding esters by this procedure (!) [52].

To conclude, the outcome was not encouraging enough to promote studies on chloroformate-mediated esterification in the field of GC derivatization procedures.

3.3. Instantaneous esterification in nonaqueous and water-containing media

TEA did not prove to function as a suitable esterification catalyst as it was outlined in the studies of organic chemists. Pyridine instead appeared to be indispensable for the instantaneous transformation of the carboxyl to the ester at the analytical microscale.

Medium-chain fatty acids with 7, 8 and 9 carbon atoms were chosen as model compounds in the introductory study [55]. Since free fatty acids usually emerge from a well-deactivated capillary, the rate of conversion to the esters was to follow in the record directly.

Chloroform proved to be a suitable reaction medium. After addition of 1 μl of MCF into chloroform with 2% pyridine, three sets of peaks appeared in the chromatogram: predominant peaks with the highest, lower peaks with the shortest and trace peaks with the middle retention times (Fig. 3a). When ECF instead of MCF was added, only the predominant and the trace peaks emerged (Fig. 3b).

Available standards enabled the confirmation of the peaks as methyl and ethyl esters, while leaving the trace peaks to free acids. The origin of the ethyl esters in the MCF-treated sample was explained by using another kind of chloroform, i.e. that stabilized with amylenes: after the addition of MCF only the methyl esters appeared (Fig. 3c). The small content of ethanol (<1%), used commonly as chloroform stabilizer, was then responsible for the formation of the ethyl esters.

Because no methanol was present in any of the chloroform used, we tried to interpret the results as follows: methyl esters were products of decarboxylation of the intermediate mixed anhydrides, i.e. the methoxycarbonyl (MOC) esters, the ethyl esters might be condensation products of carboxyl and ethanol under a catalytic influence of hydrogen chloride liberated from MCF. However, such an interpretation appeared later as rather erratic: further experiments have supported the alcoholysis pathway more. In such a case, the intermediate MOC-esters were attacked by both the ethanol-stabilizer and the traces of methanol originating from MCF due to its partial decomposition. The phenomenon of progressive reagent decomposition in the presence of pyridine excess seems to be characteristic for the reaction course.

Changing the molar ratio of reagent to pyridine in favour of the former and diminishing the solvent

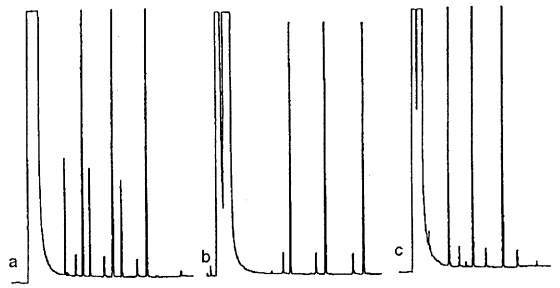


Fig. 3. GC analysis of a standard sample of fatty acids with 7, 8 and 9 carbon atoms in 100 μl chloroform with ethanol stabilizer (plus 2 μl pyridine) after treatment with 1 μl MCF (a) or 1 μl ECF (b), and after treatment with MCF in chloroform stabilized with amylenes (c). Reproduced from Ref. [55] with permission.

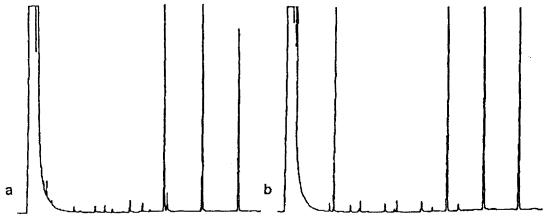


Fig. 4. Standard sample of Fig. 8 in 100 μl of hexane–chloroform (3:1) with 2% pyridine treated with 3 μl MCF (a) or ECF (b) to produce MOC or EOC esters, respectively [55].

polarity by admixture of hexane to chloroform free of alcohol, resulted in formation of the mixed anhydrides, i.e. the MOC and EOC(ethoxycarbonyl) esters (Fig. 4). They proved to be stable enough to withstand both the hot temperature of the GC injector and the shaking of the organic phase with aqueous bicarbonate in order to remove a white precipitate in it. Hexane supposingly aided in preventing decomposition of the anhydrides to the salts of carboxylic acids (not to the esters!) and carbon dioxide because in chloroform they were rapidly decomposed upon contact with the bicarbonate counterphase, in contrast to Ref. [42] as mentioned.

Further experiments have shown that acetonitrile affords still better esterification yields: the portion of unreacted fatty acids was the lowest in it (Fig. 5a). However, a partial substitution (10%) of acetonitrile by water resulted in a yield lowering to about 50%. It helped in increasing the pyridine concentration four times and the excellent yield of reacting in acetonitrile alone was restored (Fig. 5b,c). Next to this, the admixture of methanol in an amount of 4 to 5% enabled reaction even in a 1:1 ratio of acetonitrile and water without deteriorating the esterification yields.

In addition to MCF, even ECF and CECF (2-chloroethylCF) were tested as esterification agents. In the nonaqueous medium of acetonitrile and pyridine (8%), an admixture of trace amounts (4%) of ethanol or 2-chloroethanol, respectively, was necessary in order to esterify with yields of about 95%. With MCF on the other hand, the methyl esters were produced smoothly even in the absence of methanol. In water-containing media, a large differentiation among the agents regarding their reactivity occurred. The rate of conversion to the corresponding esters with ECF and CECF was much more dependent on the alcohol concentration and also the composition of the medium was shown as important: ethanol required more water and chloroethanol, more acetonitrile, as accompanying solvent. However, even under conditions close to optimum, the reaction yields were a bit lower than in the acetonitrile alone (Fig. 6). Samples containing water in the reaction medium were always subjected to hexane extraction before the injection.

Alternative catalysts like NMPIP, DMAP or TEA were found as ineffective toward ester formation in

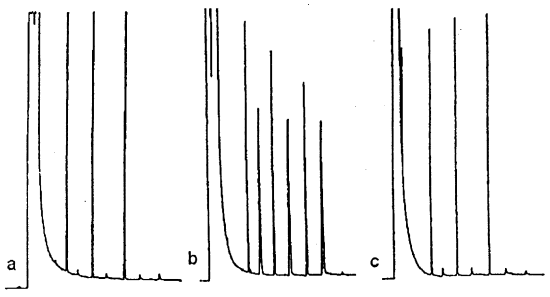


Fig. 5. Standard sample of Fig. 8 in 100 μl of acetonitrile–pyridine, 98:2 (a), acetonitrile–water–pyridine, 88:10:2 (b) after treatment with 1 μl of MCF and, in 100 μl of acetonitrile–water–pyridine, 82:10:8 (c) after treatment with 4 μl MCF [55].

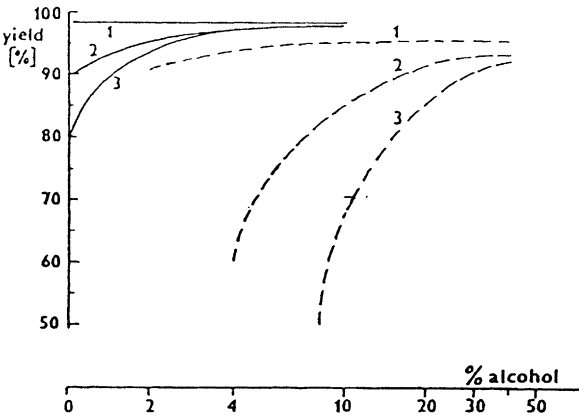


Fig. 6. Influence of alcohol concentration in the reaction medium on the esterification yield of methyl (1), ethyl (2), and 2-chloroethyl (3) esters. The corresponding alcohols added into the medium were methanol, ethanol and 2-chloroethanol, respectively. Esterification was carried out in acetonitrile alone (solid lines) and with coadmixed water (dashed lines) [55].

Table 1
Conversion of C_8, C_{10}, and C_{12} lower fatty acids with TCECF in the presence of different basic catalysts. Reproduced from Ref. [56] with permission

Basic catalyst[a]	Relative yield of		
	C_8-TCECF derivative	C_{10}-TCECF derivative	C_{12}-TCECF derivative
Diethylaniline	14	39	33
DMAP	103	96	91
N-Methylpiperidine	95	97	99
3-Picoline	93	86	69
Piperazine	0	0	0
Piperidine	0	0	0
Pyridine	100	100	100
Pyrrole	0	0	0
Tributylamine	20	20	25
Triethanolamine	7	15	14
Triethylamine	16	21	27
Tripropylamine	23	23	32

[a] 50 μl; except with DMAP (75 μl of a 1 g/ml solution in acetonitrile).

our studies. However, in a follow-up study of Dutch authors [56] some of the catalysts mentioned proved to be useful in this respect. Fatty acids with 8, 10 and 12 carbon atoms were treated with either CECF or TCECF to prepare them for the selective GC–ECD analysis. In a nonaqueous media with about 8% pyridine and 5% of the corresponding alcohol in the acetonitrile, a nearly quantitative conversion to esters was achieved in less than 20 s with the halogenated reagents and within 30–40 s with MCF, which was said to be less reactive. NMPIP, DMAP and 3-picoline were presented as equally effective as the pyridine catalyzer (Table 1). In water-containing media with up to 40% water in acetonitrile, pyridine was surprisingly found as inferior and suggested a replacement by NMPIP (Fig. 7).

As our results with MCF and ECF in treating various classes of acids did not support the findings just mentioned, a reagent-related effectiveness of certain catalysts might be assumed.

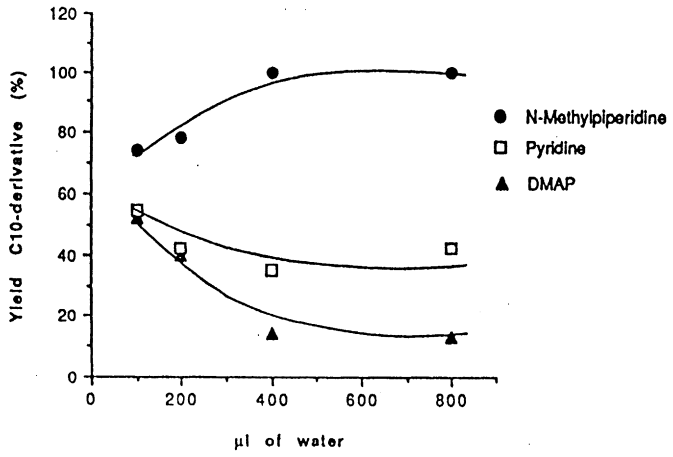


Fig. 7. Esterification of decanoic acid by TCECF in the presence of different catalysts as a function of the amount of water present in the reaction medium. Reproduced from Ref. [56] with permission.

4. Treatment of various classes of carboxylic acids with ECF and MCF

In the preceding sections, chloroformates were presented as outstanding reagents for converting the amino and phenolic groups to carbamates and carbonates rapidly and effectively — a process known already for decades. Moreover, the new discovery done at the analytical microscale revealed them as extraordinarily suitable esterification agents for transforming the carboxyl into the ester instantaneously. In the subsequent studies, various classes of carboxylic acids were subjected to treatment with the both ECF and MCF in order to optimize reaction conditions mainly for polyfunctional compounds, and to find out the extent of to which chloroformates could be employed as derivatizing agents of general utility. Included are corresponding follow-up studies in various fields of interest.

4.1. Fatty and keto acids

Fatty acids regardless of the chain length can be converted to alkyl esters virtually instantaneously [57]. At the optimum composition of the reaction medium, i.e. acetonitrile–pyridine–alcohol in a ratio of 22:2:1, the long-chain fatty acids were esterified with yields >98% and variation coefficients of up to 3% (Table 2). With MCF, the same esterification yield was achieved even without methanol in the medium.

Reaction conditions for esterification of short- and long-chain fatty acids appeared to be identical

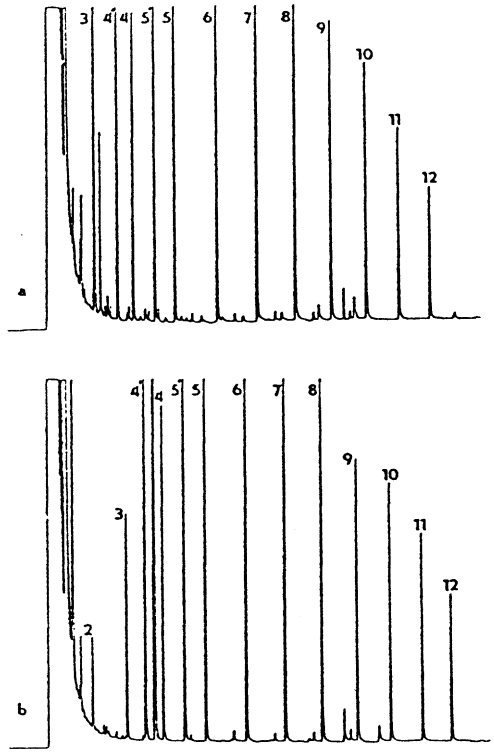


Fig. 8. Analysis of short-chain fatty acids with 2 to 12 carbon atoms as methoxycarbonyl esters (a), ethoxycarbonyl esters (b) according to Ref. [55].

[55,57]. Methyl and ethyl esters of acetic up to butyric acid were too volatile to separate from the solvent peak. However, the separation succeeded with 2-chloroethyl esters and also with MOC and EOC esters provided that reaction conditions leading

Table 2
Intraassay esterification yield of fatty acids determined via derivatization and injection of ten individually prepared samples under the same conditions. Reproduced from Ref. [57].

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	13.64	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	2.32	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

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

to the formation of mixed anhydrides were preferentiated [55]. The latter products proved to be stable enough to sustain analysis in hot injection ports of the GC instrument but they decomposed in time. For the progressive decline in response, observed with homologs >C8 (Fig. 8), either a sorption or decomposition in the column was responsible.

A latent presence of the mixed anhydrides of fatty acids >C12 in the hexane extract was confirmed indirectly, by coaddition of a small amount of ethanol to the solvent: alcoholysis took place and certain amounts of ethyl esters of the higher homologs appeared on the chromatogram. As it was not possible to elute mixed anhydrides of the long-chain acids of any of the columns tested, the range of acids, amenable to analysis in this way, remained limited to the short-chain homologs.

Next to the fatty acids also, the aliphatic keto acids could be esterified directly, i.e. without removing the keto group by oximation, as is usual with silylation procedures [58]. Several keto acids with oxygen in position 2, 3 and 4 on the aliphatic chain, among them some of physiological importance, yielded high responses corresponding to their carbon number (Fig. 9) [59].

However, the conversion yield was found to be strongly influenced by the composition of the reaction medium, in which acetonitrile should prevail, as in the case of fatty acids. Water content, if any, should not exceed 25%, since after this limit, a progressive decline of the yield occurred. Under the GC conditions chosen, the pyruvic acid was coeluted with the early peaks and the 3-ketobutyric (acetoacetic) acid always decomposed in the system if not oximated first. This was not the case with the 4-ketovaleric (levulinic) acid. The high retention of

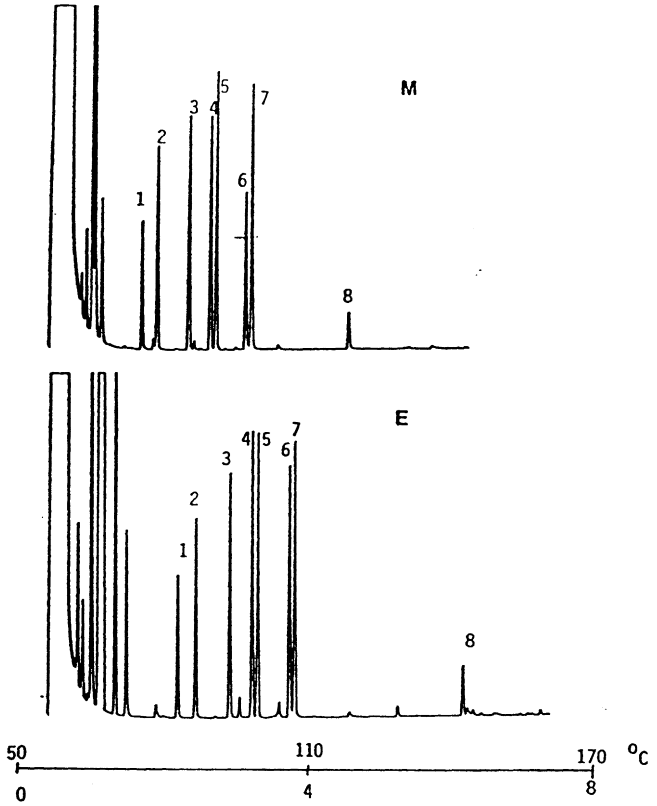


Fig. 9. Analysis of native keto acids (i.e., without any stabilization of the keto groups) after treatment with MCF (M) or ECF (E). Acids: 1=2-ketobutyric; 2=2-ketoisovaleric; 3=2-ketovaleric; 4=2-keto-3-methylvaleric; 5=2-ketoisocaproic; 6=4-ketovaleric (levulinic); 7=2-ketocaproic; 8=2-ketoacetic (glyoxylic). According to Ref. [59].

glyoxylic (2-ketoacetic) acid, being eluted on the record at the rear, was explained by means of MS: the analyte appeared to be a trimer, probably a coupling product of the activated carboxyl with the enolic hydroxyl. However, a tendency to form oligomers as reaction side-products was observed especially with another class of acids, the 2-OH-carboxylic acids, as discussed later.

4.2. Dicarboxylic acids

Unlike the fatty (monocarboxylic) acids, the di-, tri- or polycarboxylic acids usually resist a direct esterification with acidified alcohols and their convertion to TBDMS-esters using MTBSTFA is often carried out [60]. Another useful option proved to be alkylation with benzyl bromide in acetonitrile [61] employed even for organic acid analyses in body fluids [62].

In our experiments, the C2 to C12 dicarboxylic acids, including some branched and OH-substituted ones, were converted to esters with MCF and ECF smoothly and the yields were relatively independent of the composition of the medium [59]. However, when acetonitrile prevailed in the reaction medium the response of oxalic, succinic and glutaric acids including that of their substituents was negligible or low, especially with ECF (Fig. 10).

Later examinations revealed that these acids prefer to form alternative products even under conditions of ester formation with the others. To promote the formation of esters, alcohol should replace acetonitrile in the reaction medium partially or fully and the reagent should be added successively, as is presently under study.

Nevertheless, in spite of the problems encountered with the mentioned di-C4 and di-C5 acids, a report on a L-2-OH-glutaric acid urine assay appeared

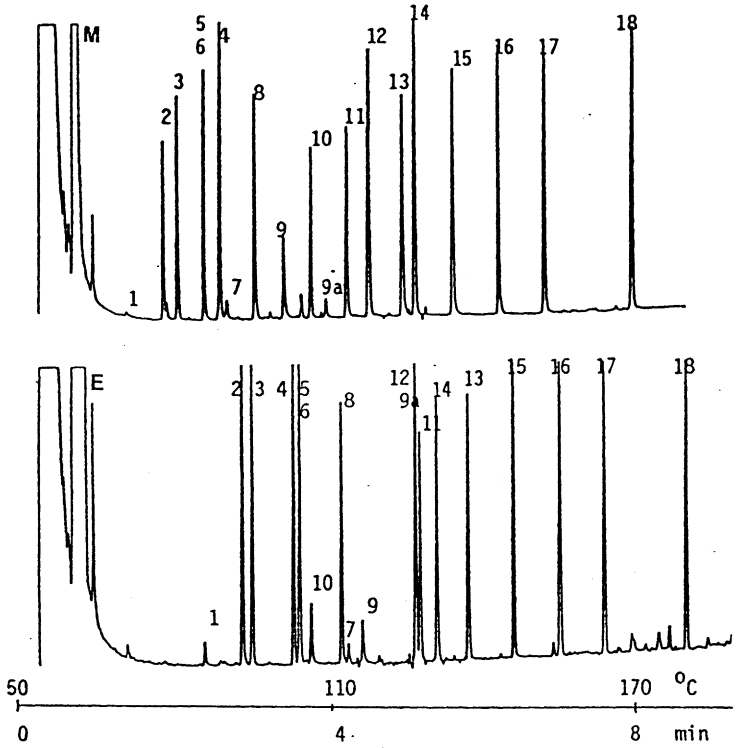


Fig. 10. Equimolar mixture of dicarboxylic acids after treatment with MCF (M) or ECF (E). The peaks belong to the following acids: 1=Oxalic; 2=malonic; 3=methylmalonic; 4=maleic; 5=fumaric; 6=ethylmalonic; 7=succinic; 8=mesaconic; 9/9a=glutaric; 10=methylsuccinic; 11=adipic; 12=3,3-dimethylglutaric; 13=pimelic; 14=3-methyladipic; 15=suberic; 16=azelaic; 17=sebacic; 18=dodecanoic. According to Ref. [59].

recently [63]. Following ethyl acetate extraction, the dry residue was treated with ECF in aqueous ethanol and the enantiomers were subjected to chiral GC separation. Some precautions toward maintaining an exact reaction time of 5 s were announced as crucial, the method was otherwise considered as quantitative.

4.3. Aromatic acids

Phenolic acids, i.e. benzoic, phenylacetic, phenylpropenoic (cinnamic), phenylglycolic (mandelic), phenylpropionic and their variously substituted forms occur partly in body fluids, partly in beverages, etc. Unlike rather disappointing results experienced with chloroformate-induced esterification of aromatic acids in organic chemistry (see 3.2.), a smooth conversion to methyl and ethyl esters was noticed with the new approach [64]. Addition of up to 30–40% of water to the acetonitrile–alcohol medium did not seem to influence the reaction yields much. The only exception was benzoic acid and some of its substituents. The reaction behaviour of this class of compounds, with a carboxylic group directly attached to the benzene ring, differed from

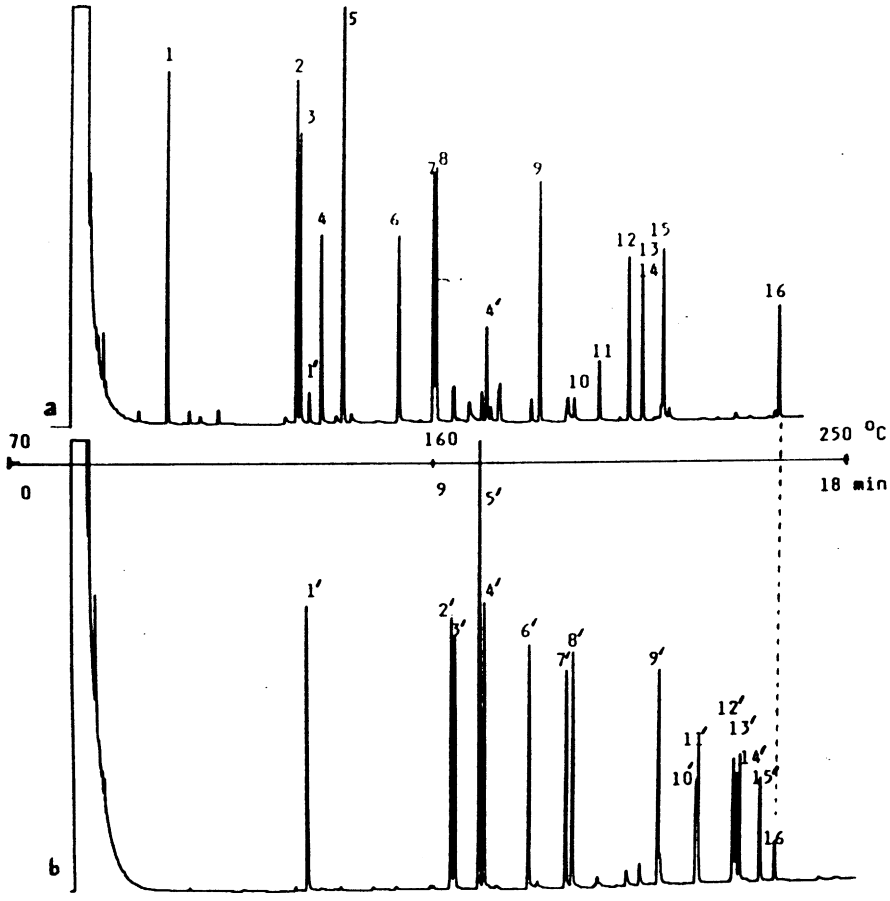


Fig. 11. Benzoic acid and its substituents in an equimolar mixture analyzed as *O*-alkylated methyl esters (a) and *O*-alkylated MOC esters (b) after treatment with MCF in the corresponding media. Peaks correspond to the following acids: 1=benzoic; 2=2-methoxy-benzoic (*o*-anisic); 3=3-methoxybenzoic (*m*-anisic); 4=4-methoxybenzoic (*p*-anisic); 5=2-acetoxybenzoic (acetylsalicylic); 6=2-hydroxybenzoic (salicylic); 7=3-hydroxybenzoic; 8=4-hydroxybenzoic; 9=4-hydroxy-3-methoxybenzoic (vanillic); 10=2,6-dihydroxy-benzoic (γ-resorcylic); 11=2,3-dihydroxybenzoic; 12=3,4-dihydroxybenzoic (protocate-chuic); 13=2,5-dihydroxybenzoic (gentisic); 14=2,4-dihydroxy-benzoic (β-resorcylic); 15=3,5-dihydroxybenzoic (α-resorcylic); 16=3,4,5-trihydroxybenzoic (gallic). Reproduced from Ref. [64] with permission.

the others. With regard to multiple-peak formation, the most problematic acids were benzoic and the 4-methoxybenzoic acid, each yielding up to four peaks on the record. Three of them were identified as the alkyl ester, the free acid and the alkoxycarbonyl ester. As follows from the record, a low response of some homologs is apparent (Figs. 11 and 12).

Even under conditions promoting formation of alkyl esters, a certain amount of alkoxycarbonyl esters (the mixed anhydrides) was always produced, especially with the homologs of lower mass and more with ECF. The yields were not influenced much by changing the composition of the medium. Acetonitrile promoted formation of the mixed anhydrides in general but lowering its content too excessively led to a diminished reaction yield.

It appeared that benzoic acid and its *o*-, *m*- and *p*-methoxy and hydroxy substituents afforded better analytical results when analyzed as mixed anhydrides. These products were easily prepared in hexane–chloroform (4:1) with 2% pyridine and 3% of either MCF or ECF added. However, after shaking

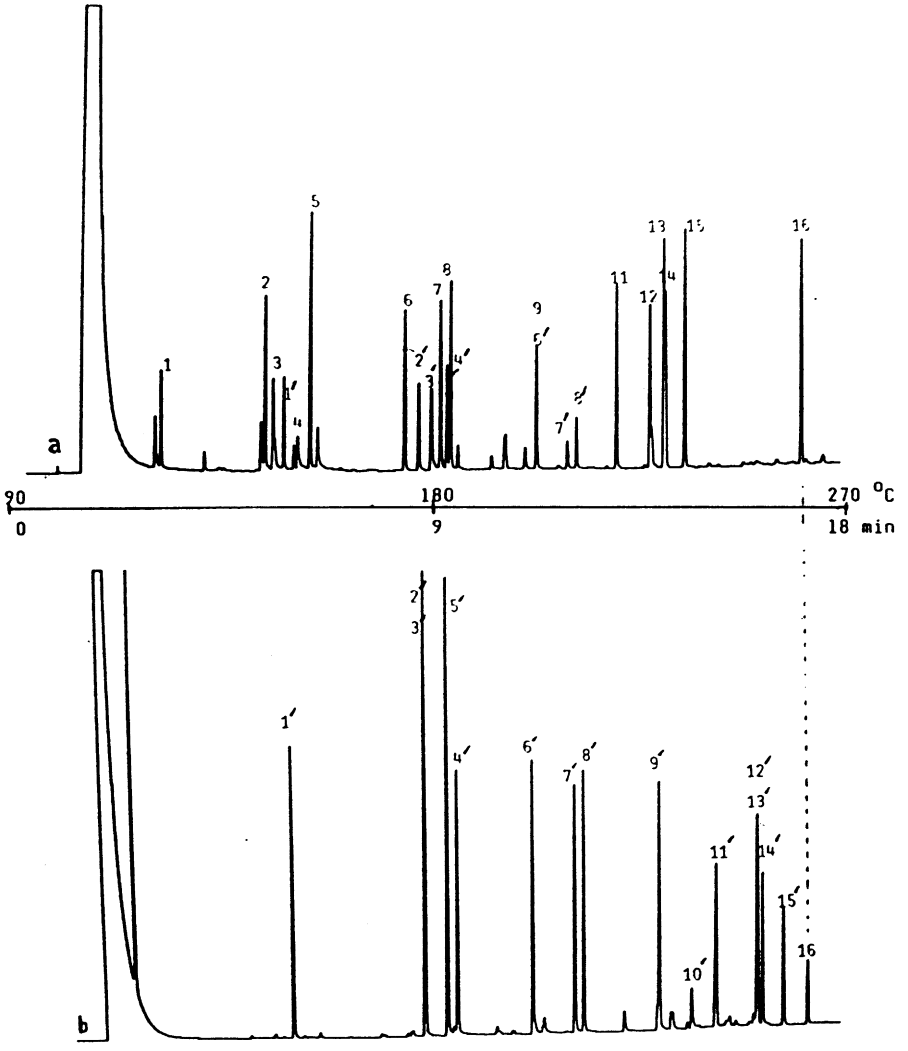


Fig. 12. Benzoic acid and its substituents in equimolar mixture (see Fig. 11) analyzed as *O*-alkylated ethyl esters (a) and *O*-alkylated EOC esters (b) after treatment with ECF in the corresponding media [64].

the organic extract with aqueous (bi)carbonate they almost fully decomposed to the salts. Such a treatment also became a way of 'clearing' the chromatogram when one wished to favour the formation of alkyl esters as the reaction products.

One methylene unit more, i.e., the one between the carboxylic group and the benzene ring, reversed the reaction course in favour of the alkyl ester formation. Thus, phenylacetic acid and its substituents, some of them being regular urine metabolites, afforded a smooth chromatographic record (Fig. 13). For the seemingly lower yields of components 3 and 6, it was not an incomplete derivatization but an internal cyclization that was responsible.

Outstanding results were achieved after treating cinnamic acid and its substituents with both MCF and ECF (Fig. 14). The records are unique in their cleanness and in calculating the methylene units, the yields can be considered as quantitative. These acids are highly abundant in beverages and their determination profits in food technology.

Acids with an alcoholic group on the aliphatic chain, e.g. the mandelic and its substituents and the phenylpropionic acids with differently positioned OH-groups, offered results similar to those achieved with hydroxycarboxylic acids, i.e., a certain portion of the 2-OH-group (<10% at reaction optimum) remained free. Moreover, the low record of atrolactic acid (peak 4) in Fig. 15 confirmed the findings of organic chemists, that with α-branched acids, the esterification yields with chloroformates were poor regardless of the reagent used.

Some of the acids mentioned here, e.g., the benzoic and *p*-methoxybenzoic one, the phenylacetic, phenylbutyric, cinnamic, acetylsalicylic and *p*-methoxyphenylacetic ones were easy to convert into

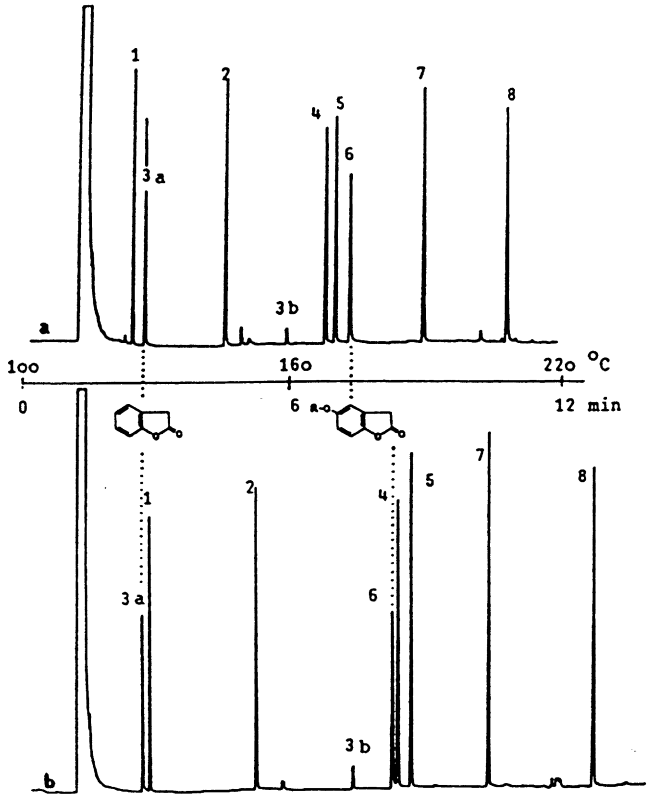


Fig. 13. Phenylacetic acid and its substituents in an equimolar mixture analyzed as *O*-alkylated methyl esters (a) and *O*-alkylated ethyl esters (b). Recorded acids are the following: 1=Phenylacetic (PA); 2=4-methoxyPA; 3=2-hydroxyPA; 4=3-hydroxyPA; 5=4-hydroxyPA; 6=2,3-dihydroxyPA (homogentisic); 7=4-hydroxy-3-methoxyPA (homovanillic); 8=3,5-dihydroxyPA. Reproduced from Ref. [64].

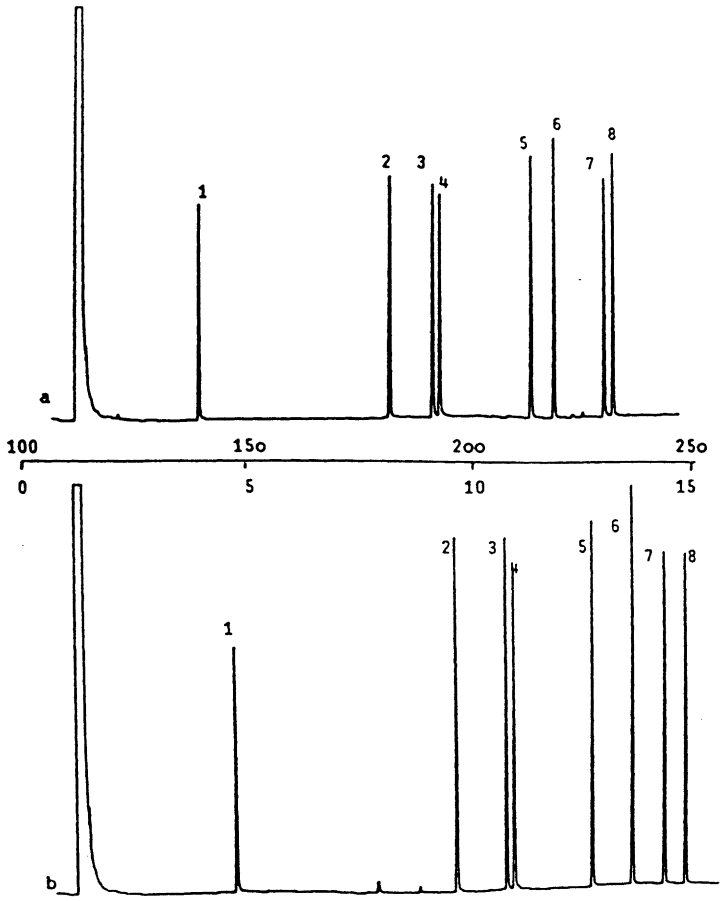


Fig. 14. Cinnamic acid and its substituents in equimolar mixture analyzed as *O*-alkylated methyl esters (a) and *O*-alkylated ethyl esters (b). The peaks belong to the following acids: 1=Cinnamic (3-phenylpropenoic); 2=2-hydroxycinnamic; 3=3-hydroxycinnamic; 4=4-hydroxycinnamic (*p*-coumaric); 5=4-hydroxy-3-methoxycinnamic (ferulic); 6=4-hydroxy-3,5-dimethoxycinnamic (sinapic); 7=3,4-dihydroxycinnamic (caffeic); 8=3,4-dihydroxydihydrocinnamic (dihydroxycaffeic). Reproduced from Ref. [64].

the mixed anhydrides under the conditions described for the benzoic acids and the products could be analysed successfully [64].

Determination of fifteen chlorophenoxy and seven other acidic herbicides of widespread use for weed control (Fig. 16) belonged to one of the first applications in the field of ecology [65]. Occurring often as residues in surface and ground waters (50–200 ng/l) they were solid-phase (RP-18) extracted and, following treatment with either MCF, ECF or BCF in acetonitrile, pyridine and the particular alcohol, the corresponding methyl, ethyl or butyl esters were determined by means of ECD or MS detection.

The authors also checked the optimum reaction conditions for the aliphatic acids with the aromatic analytes. They concluded in agreement with [55] that with a constant 2:1 alcohol-to-pyridine ratio, the optimum proportions of acetonitrile and water were reagent dependent. With other catalysts like TEA and sodium carbonate, formation of some by-products was observed and pyridine was confirmed as the base of choice.

Others treated aminosalicylates like 5-amino-salicylic (5AS) and *N*-acetyl-5-amino-salicylic acid and their radical-derived oxidation products with ECF in the corresponding medium and subjected the derivatives to GC–MS analysis [66]. Some of the analytes were considered as possible markers for

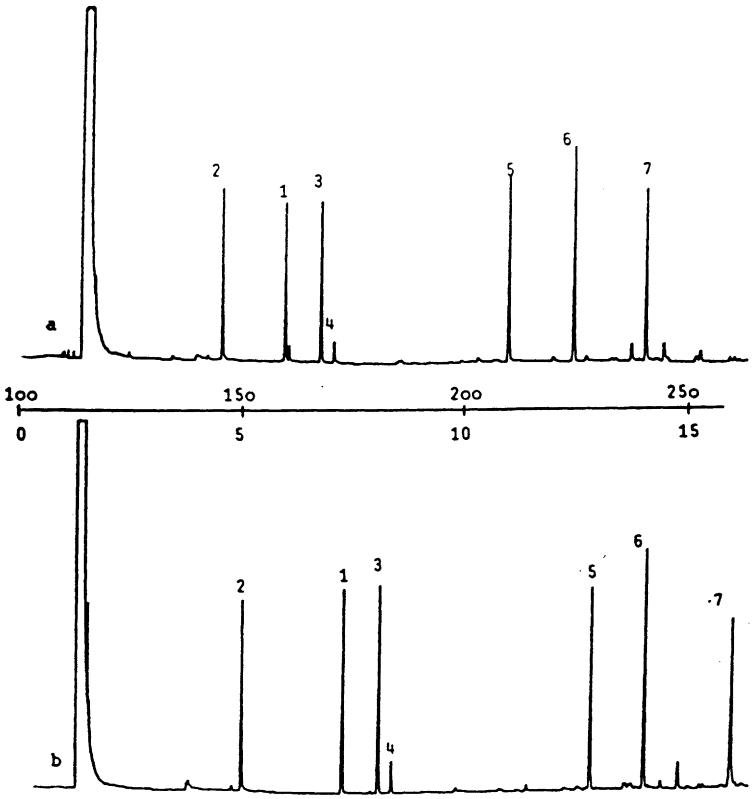


Fig. 15. Phenolic acids with hydroxyl group on the aliphatic chain in an equimolar mixture analyzed after treatment with MCF (a) and ECF (b). The acids were the following: 1=Phenylglycolic (mandelic); 2=2-phenyl-3-hydroxypropionic (tropic); 3=2-hydroxy-3-phenylpropionic (phenyllactic); 4=2-hydroxy-2-phenylpropionic (atrolactic); 5=4-hydroxymandelic; 6=4-hydroxy-3-methoxymandelic (vanillylmandelic); 7=3,4-dihydroxymandelic. Reproduced from Ref. [64].

identification of yet unknown metabolites of 5AS in biological material.

4.4. Hydroxycarboxylic acids

Phenolic hydroxyl and alcoholic groups not adjacent to the carboxyl on an aliphatic chain were no problem when treated with chloroformates: the former group is always esterified smoothly, the latter remained untouched. Unlike that, the derivatization of 2-hydroxycarboxylic acids (HAs), experienced with MCF and ECF, appeared to be the most difficult task among all classes of acids tested [67–69]. Not because of a lowered reactivity but rather due to an excessive one involving both the activated functional groups. As HAs belong to highly abundant metabolites in body fluids, especially the lactic acid in blood, reproducible derivatization yields with these analytes were of special importance.

When treated with the reagents mentioned, the HAs gave the expected *O*-alkoxycarbonyl alkyl esters (R′OCOO-CHR-COOR′) as the main reaction products. However, the main product was always accompanied by a number of side-products either with shorter (prepeak) or with longer (postpeaks) retention times. The abundance of the particular product appeared to be largely dependent on the reaction conditions chosen [67].

The by-product with shorter retention was clearly identified as alkyl ester with free hydroxyl group. The alkylation yield of the α-positioned OH-group was shown to be strongly influenced by the amount of alcohol in the reaction medium. It is apparent from Fig. 17, where HAs with 2–6 carbon atoms

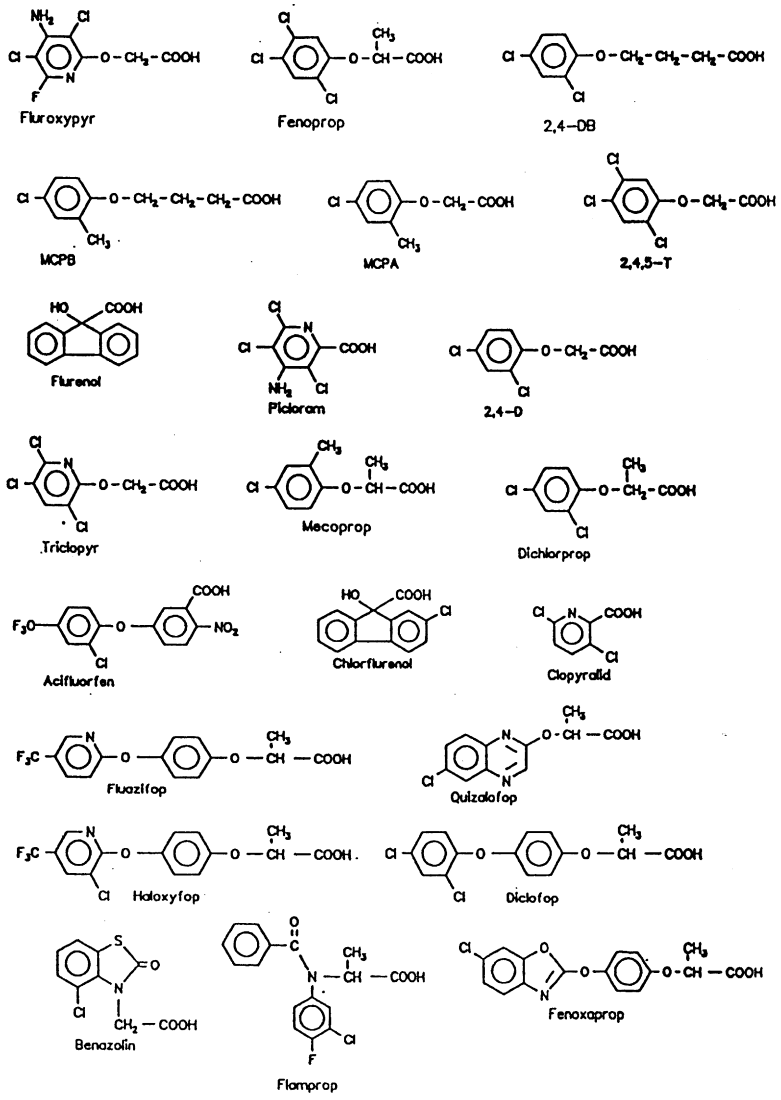


Fig. 16. Structures of pesticides investigated by means of GC–ECD or GC–MS analysis after chloroformate-mediated derivatization. Reproduced from Ref. [65].

were exposed to MCF and ECF treatment in alcohol–pyridine media.

The amount of prepeaks was highest under such conditions and higher with methanol than with ethanol. Alcohol evidently prevented an effective esterification of the hydroxyl and from this point of view, its portion in the medium should be held as low as possible. The findings made with fatty acids, that acetonitrile with 4–5% of admixed alcohol afforded the highest yield of the main product, were confirmed [68]. However, the presence of water up to 10% for treatment with MCF and up to 20% for that with ECF proved to be acceptable without noticeable changes in the yield [67]. Increasing the pyridine content to 10% or more worked towards main product formation, too. On the other hand, exclusion of alcohol, lowering the pyridine amount below that of the reagent and a partial replacement of acetonitrile by acetone resulted in formation of the mixed anhydrides (Fig. 18).

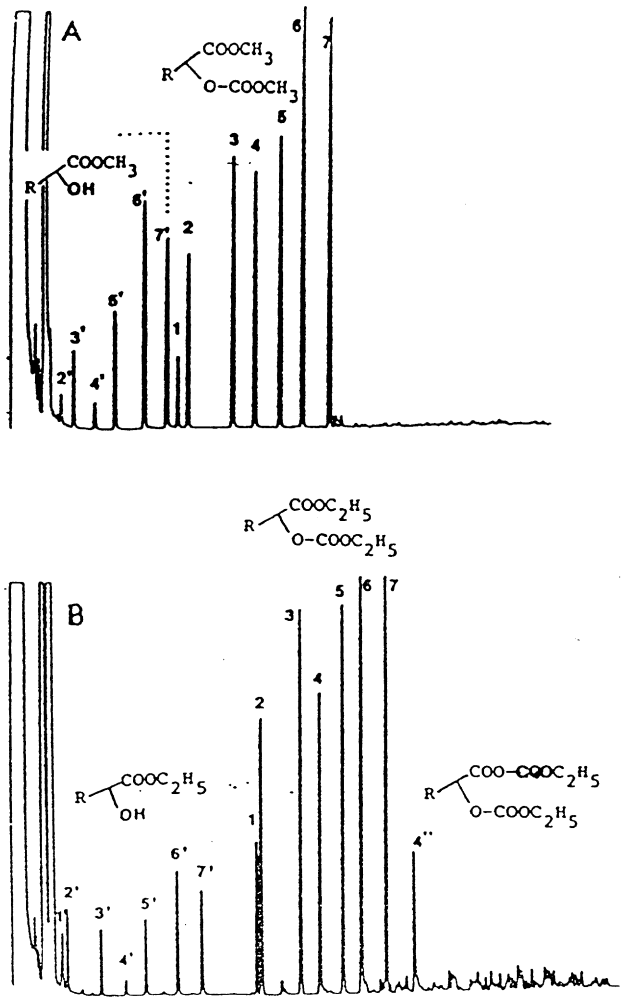


Fig. 17. GC–FID analysis of the following HAs: 1=2-OH-acetic (glycolic); 2=2-OH-propionic (lactic); 3=2-OH-butyric; 4=2-OH-isovaleric; 5=2-OH-valeric; 6=2-OH-isocaproic; 7=2-OH-caproic, after treatment with (A) MCF in methanol and (B) ECF in ethanol in the presence of 8% pyridine. Primed numbers represent side-products with a free hydroxyl group, peak 4 is the nondecarboxylated side-product, i.e. the ethoxycarbonyl ester of HIV. Reproduced from Ref. [67].

Conversion to these products was apparently not quantitative, except for the 2-OH-isovaleric acid (peak 4), which already showed a different behaviour upon treatment with ECF: here the main product was lowered at the cost of the parallelly formed mixed anhydride (Fig. 17, peak 4″). The 4-OH-butyric acid (peak 9) exerted the same retention time with MCF and ECF and a diminished response. An internal cyclization under formation of tetrahydrofuranone was confirmed and it proceeded with ECF even under conditions for ethyl ester formation [67].

Greater problems were encountered with the slow-running by-products that were responsible for the turbulent baseline at the rear of the chromatogram, notably with ECF (see Fig. 17). Initially, we took them for the mixed anhydrides [67] but further examinations with single acids revealed that more than one product emerged on the record and with retention times different to those of the anhydride. Moreover, unlike the anhydrides, the rear side-products were not decomposed upon contact with aqueous (bi)carbonate. Next to this, individual differences in HAs regarding the side-product formation

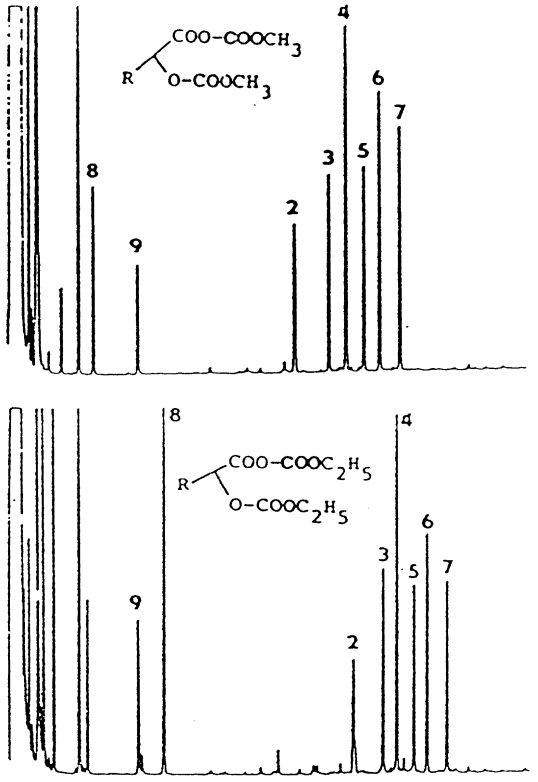


Fig. 18. HAs 1–7 (see Fig. 17) plus 3-OH-butyric (peak 8) and 4-OH-butyric (peak 9) acids analyzed as (top) *O*-MOC/MOC esters or (bottom) *O*-EOC/EOC esters after treatment with the corresponding chloroformates. The 4-HB acid was found as cyclized to tetrahydrofuranone. Reproduced from Ref. [67].

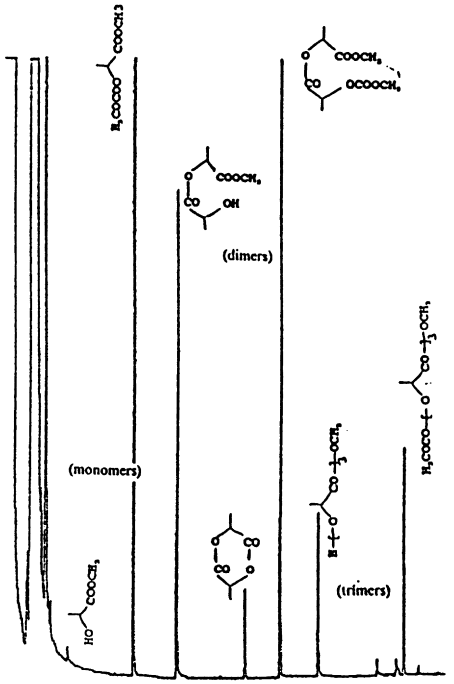


Fig. 19. Chromatographic record of the reaction products after treating lactic acid with MCF at nonoptimized conditions. Monomers, dimers and trimers with free or alkoxylated hydroxyl group plus the lactide-dimer were identified by means of MS [69].

were observed (Table 3). Least favorable results were achieved with lactic and butyric HAs [68].

By means of GC–MS [69], the products were identified as inter-ester oligomers (dimers, trimers) with a mostly derivatized and partly underivatized terminal OH-group, plus the lactide dimer (Fig. 19). With ECF, the results were similar and again, least favorable with lactic acid. In retrospect concerning the turbulent baseline in Fig. 17, the conclusion was drawn that HAs present in a mixture inter-react

Table 3
Distribution of mass of the derivatives of the particular hydroxycarboxylic acid treated with MCF in acetonitrile–water–methanol (14:5:1) and with ECF in acetonitrile–water–ethanol (2:1:1), respectively. Reproduced from Ref. [68]

Hydroxycarboxylic acid	Peak abundance (%)					
	MCF			ECF		
	Prepeak	Main peak	Postpeak	Prepeak	Main peak	Postpeak
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

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

mutually with each other leading to a variety of mixed inter-esters. The number of by-products, when working with racemic reference substances, was further increased through the formation of diastereomeric inter-esters and lactides [69].

The process of inter-ester formation might be considered as a 'self-alcoholysis' caused by mutual interaction of an activated carboxyl of one molecule with an activated 2-OH-group of another molecule. With hydroxy acids having the alcohol group not adjacent to the carboxyl, i.e. with 3-OH or 4-OH-butyric acids, the formation of inter-esters was not observed.

Complete elimination of the side-products did not appear to be possible under the chosen reagents and reaction conditions. Despite optimization, the maximum yield of lactic acid EOC ethyl ester was less than 75%, provided that the amount of pyridine was not enhanced.

However, a solution was found to improve the

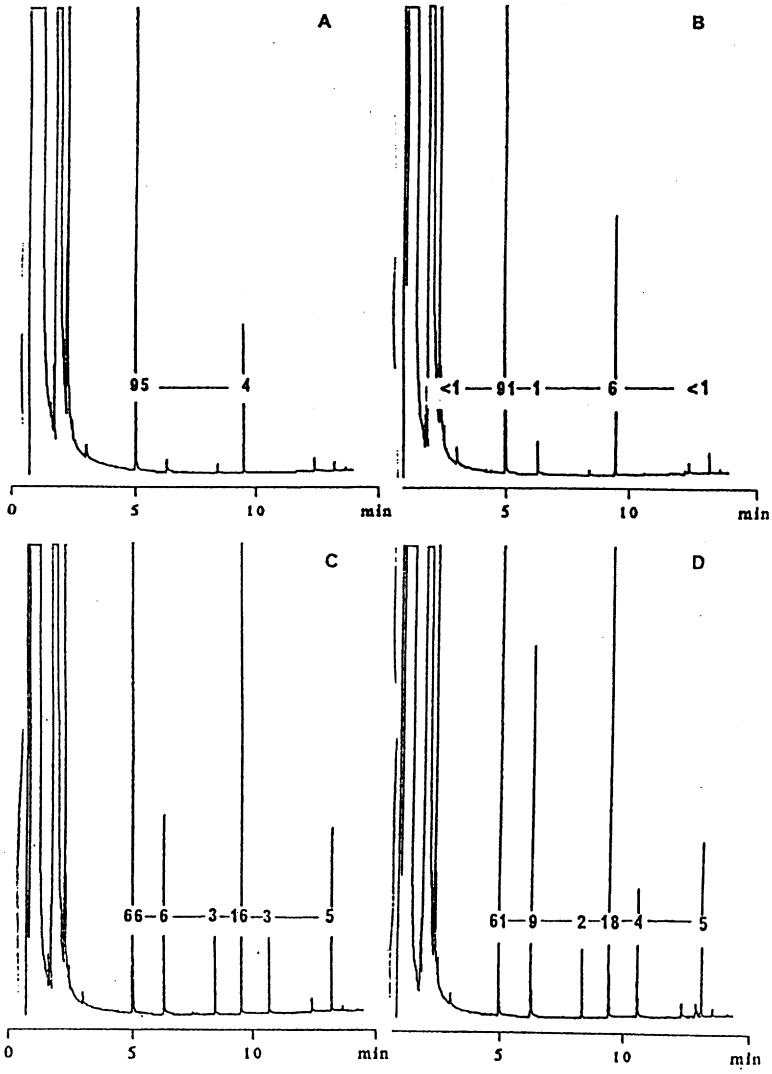


Fig. 20. GC profile of the reaction products of D-lactic acid treated with MCF according to the 'normal mode' (C,D) and the 'reversed mode' (A,B) in two different reaction media containing either 10% (A,C) or 25% (B,D) water in acetonitrile–methanol–pyridine. The percentage peak abundance are given on the abscissa. Reproduced from Ref. [68].

results substantially. It was based on altering the sequence of base and reagent addition [68]. With such a 'reversed mode' (base after reagent) addition, the yield of the main product was higher by 30% with MCF (Fig. 20) and by about 20% with ECF. Upon admixing 10 to 25% of water to the acetonitrile with 1% of methanol or 5% of ethanol for the MCF and ECF treatment, the lactic acid converted to the main product by more than 90% and a bit less with ECF. The benefit of reversing the mode of reagent-base addition is apparent by comparison with the normal (previous) mode in the figures: formation of the by-products at the rear of the chromatograms was supressed substantially (Figs. 20 and 21).

To conclude, HAs, namely the lactic one, should be treated with MCF and ECF via the reversed mode in acetonitrile with 1 to 5% of alcohol. The presence of up to 10% of admixed water is tolerable.

5. One-step treatment of amino acids in various media

A real breakthrough into the current methodology of amino acid analyses brought the finding that these polyfunctional analytes were to derivatize with chloroformates readily by one-step treatment [70,71]. The method met the basic criteria required for an ideal procedure: simplest sample handling in aqueous solutions and, derivatization in seconds with microliter amounts of reagent of negligible cost.

Modification in the reaction conditions towards

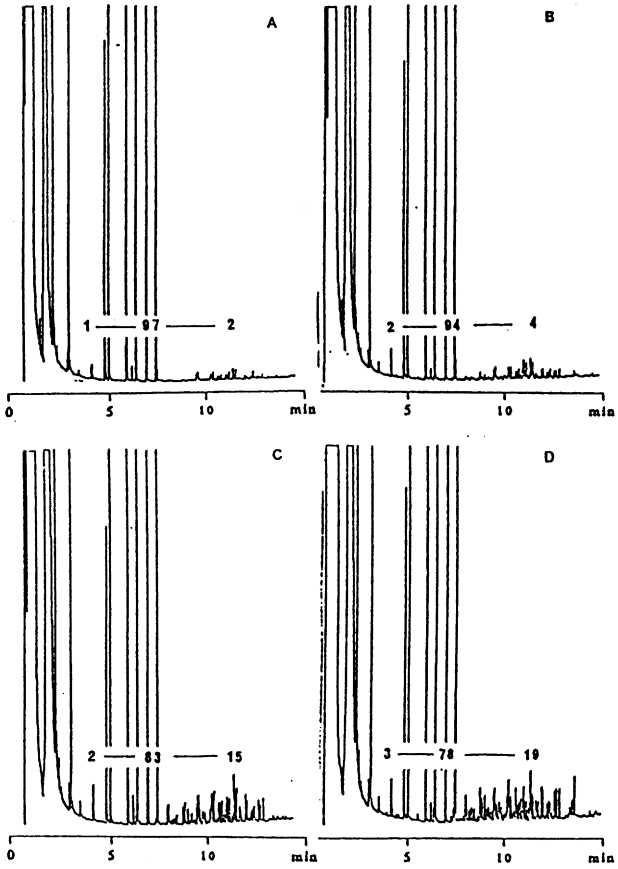


Fig. 21. GC analysis of an equimolar mixture of 6 HAs (without 2-OH caproic acid) plus 3-OH-butyric acid (the first large peak after the solvent peak) after treatment with MCF as in Fig. 20 [68].

using reagent and alcohols with different alkyls appeared to be a useful extension of the procedure. It resulted in possibly preparing a wide variety of esters tailored to the analytical needs of a specific task. The optically active menthyl chloroformate, for example, succeeded in treating and separating the amino acid optical antipodes. Finally, the employment of fluorinated alcohols or reagents enabled the sensitivity and selectivity of GC–MS detection to be enhanced significantly.

The attractiveness of the extraordinary simple approach was recognized soon and most of the applications dealt with amino acid determination in various kinds of material as follows.

5.1. Treatment with reagents and alcohols of the same alkyl

Conversion of the protein amino acids to the *N*(*O*,*S*)-EOC ethyl esters appeared to be one of the simplest. It was completed within 1 min upon addition of a few microlitres of ECF to a solution of amino acids in aqueous ethanol with pyridine [70–72]. The ECF-treated analytes afforded a good GC–FID response and a better separation on the columns tested than those treated with MCF. Details on the derivatization, stationary phase selectivity and optimization of the analysis are given in Ref. [71]. A middle-polar column of OV-1701 type coated in fused-silica capillaries of 5–10 m length enabled an excellent separation in 4–5 min at a temperature raise of 40°C/min. A rapid screening of serum amino acids after a simple ion-exchange chromatography of undeproteinized sample [70] was shown to be possible (Fig. 22).

Double-derivative formation has been observed with glutamic acid and ornithine owing to internal cyclization; however, it could be supressed by using basified medium. Arginine and citrulline did not elute from any of the columns tested. The amidogroup of asparagine was converted to the nitrile, while that of glutamine remained untouched – the reason for markedly increased retention of the latter compound. Variation coefficients of the derivative formation did not exceed 5%, except for those for glutamine and histidine (8%).

Electron-impact (EI) mass spectra together with

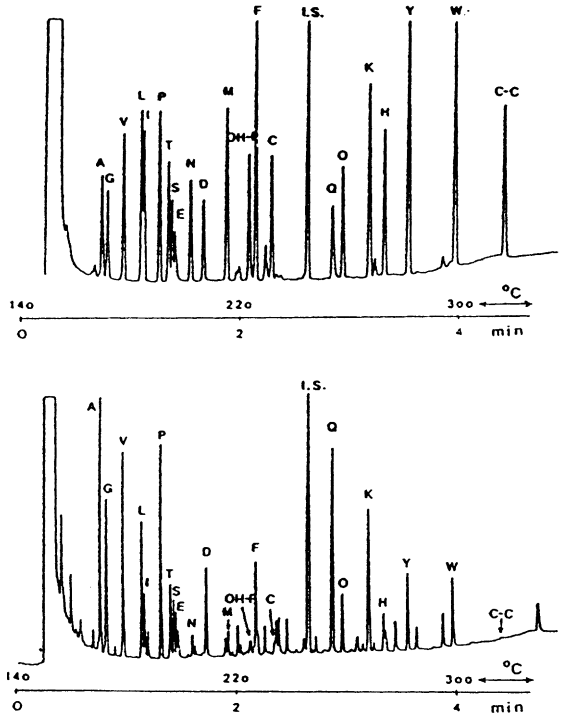
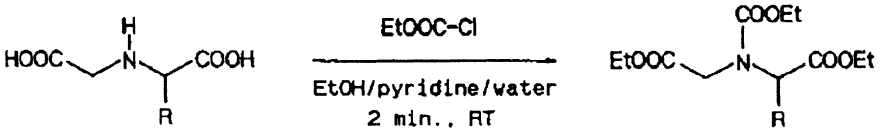


Fig. 22. GC–FID analysis of an equimolar mixture of 23 amino acids (top) and amino acids isolated from 50 μl of human serum via cation-exchange chromatography (bottom) after derivatization with ECF in aqueous ethanol–pyridine medium. Reproduced from Ref. [70].

fragmentation pathways and structures of individual amino acids were examined in detail [73]. GC–MS analysis of *N*(*O*,*S*)-EOC amino acid ethyl esters using an EI ion source was shown as convenient and reliable. Consequently, a sensitive methodology suitable for the rapid determination of specific enrichment of stable, isotopically labelled tracer amino acids in plasma and protein hydrolysates was developed [74].

The ECF–ethanol treatment succeeded also in GC–FID determination of selenium analogs of sulfur-containing amino acids such as the Se-cystine and Se-methionine, the results being in good accordance with those obtained by HPLC and fluorimetry [75]. The same kind of sample pretreatment was used effectively for derivatization of *N*-carboxymethyl (i.e. dicarboxylic) amino acids to the corresponding *N*-EOC diethyl esters:



This application has been connected with an undesirable process of product browning in food technology. Due to a nonenzymatic glycation of the primary or N-terminal and ε-lysyl amino groups in peptides and proteins with aldoses losses of the essential lysine, e.g. in milk powder, occur. Periodate oxidation of the glycated products leads to formation of *N*-carboxymethyl amino acids, the amount of which directly reflects the extent of glycosylation of the various amino sites in the products [76].

Equal treatment was chosen for converting aminomalonic acid into its *N*-EOC diethyl ester after searching for its origin in alkaline hydrolysates of *Escherichia coli* proteins [77] and for investigating cycad seeds for a possible presence of some neurotoxic amino acids [78]. GC–MS helped to identify twelve nonprotein amino acids, among them a putative neurotoxin β-*N*-methylamino-L-alanine and the known neurotoxin β-*N*-oxalylamino-L-alanine.

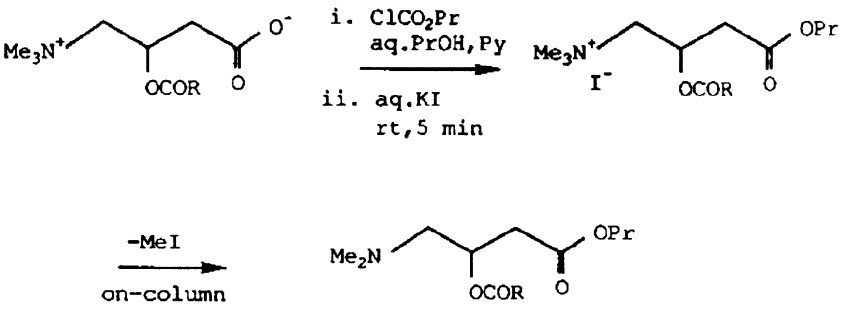
The plasma content of homocysteine, the currently followed biomarker of vascular disease, was easy to determine by GC–MS after treatment with either ECF–ethanol [79] or PCF–propanol [80]. After a deproteinization with sulfosalicylic or trichloroacetic acid, following a release of the analyte from disulfide bonds by tributylphosphine or dithiothreitol, the supernatant was treated upon admixing propanol with PCF directly [80] or by first subjecting to amino acid uptake by cation-exchange [79]. The simple sample pretreatment enabled analysis of >100 samples within one day.

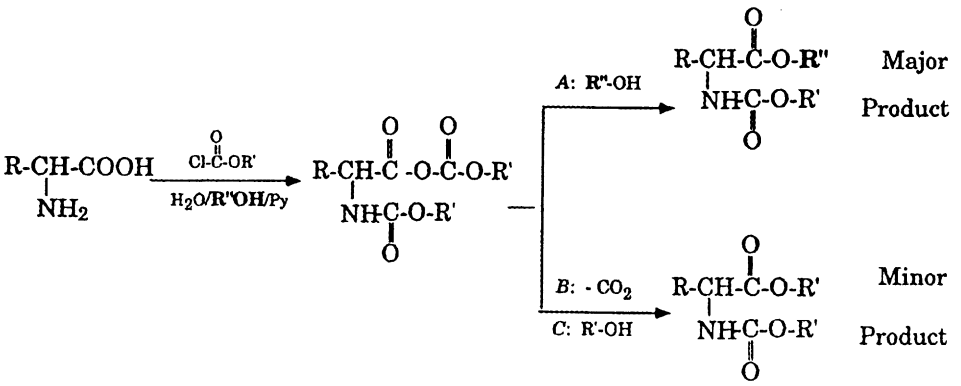
A novel approach to the analysis of (urinary) acylcarnitines [81] was based on 3 consecutive steps: (a) direct esterification of the isolated acylcarnitines using PCF–propanol; (b) ion-pair extraction with potassium iodide into chloroform; (c) subsequent on-column *N*-demethylation of the resulting acylcarnitine propyl ester iodides (see below).

The products were volatile enough to be easily analyzed by GC–MS with chemical ionization. PCF was preferred over the lower-alkyl reagents since it provided, while retaining enough volatility, more lipophilic propyl esters of especially short-chain acylcarnitines for the subsequent chloroform extraction.

5.2. Treatment with reagents and alcohols of different alkyls

A further study of the extraordinary simple procedure of amino acid pretreatment aimed at understanding the reaction mechanism in more detail was carried out. As a result, the process of ester formation via alcoholysis of the mixed anhydride was confirmed with great probability [82]. The authors examined various combinations of reagents and alcohols in mutual inter-reaction and concluded that the mixed anhydride underwent an exchange reaction





with the alcohol present (pathway A) leading to the principal product:

A small amount of minor product with the same alkyl group as that of the reagent was also found. In view of this, either the decarboxylation mechanism (route B) or the alcoholysis by the action of the alcohol, liberated from the reagent (route C), was proposed.

Additional evidence for the direct involvement of alcohol constituents in the aqueous reaction medium was given in the same study. After treating phenylalanine with IBCF in the presence of equimolar amounts of seven alcohols, the seven major peaks, corresponding to the esters of the present alcohols, were obtained. Then, various combinations of reagents and alcohols were used to generate a wide variety of *N*(*O*,*S*)-alkoxycarbonyl amino acid alkyl esters to produce, e.g., IBOC-isobutyl, -heptafluorobutyl or -trimethylsilylmethyl amino acid esters. This opened new horizons in extending the procedure to various applications.

However, even before publishing the paper just mentioned, another study on using reagents and alcohols of different alkyls preceded. The optically active menthyl chloroformate (derived from menthol, i.e. 2-isopropyl-5-methylcyclohexanol) in combina-

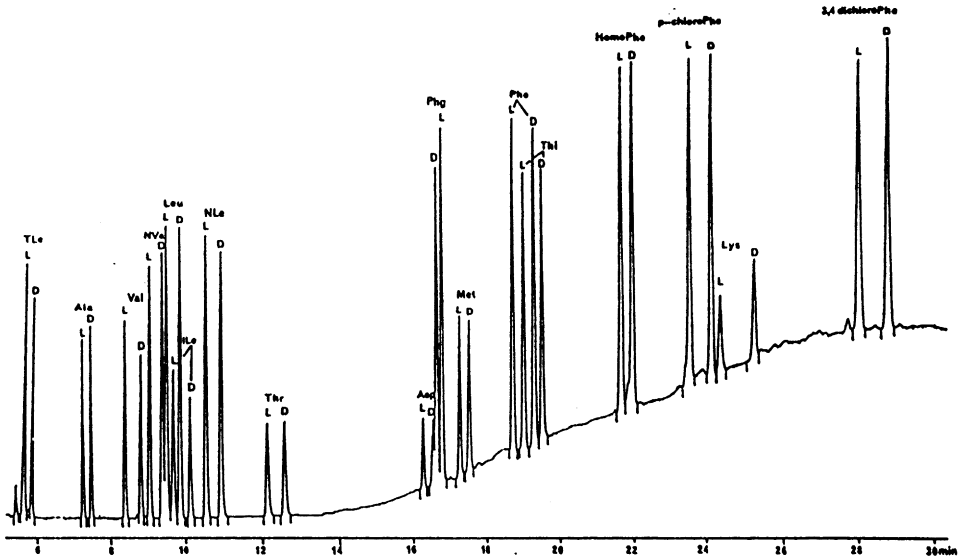


Fig. 23. Separation of DL-amino acids as diastereomers after treatment of the analytes with the optically active menthyl chloroformate in aqueous ethanol. Reproduced from Ref. [83] with permission.

tion with aqueous methanol or ethanol was employed in the chiral analysis of amino acid optical antipodes [83]. Except for arginine and histidine, good separation factors were achieved with the other protein amino acid diastereomers on normal silicone phases (Fig. 23). The relative amounts of water, alcohol and pyridine were optimized in relation to the chiral reagent in a series of experiments and the yields were said to exceed 95%. Any menthyl esters which would arise from decarboxylation of the mixed anhydride intermediate were never detected.

Others used the menthyl chloroformate in combination with various alcohols for chiral analysis of some nonprotein amino acids occurring in peptide antibiotics. The best separation factors were obtained with methyl, 2-fluoroethyl and 2-methylbutyl esters on silicone phases of DB-210 and BPX70 type [84]. The method was successfully employed in chiral analysis of amino acid residues from new natural and semisynthetically prepared cyclosporines [85].

Abe et al. [86] employed IBCF-methanol for treating amino acids in hydrolyzates of certain fossils as their approximate age can be determined from the enantiomeric ratio of the particular amino acids, especially the aspartic one. The dating studies are based on the fact that L-amino acids in living tissues undergo racemization to D-isomers once the life process has ceased. *N*-IBOC methyl esters of the enantiomeric amino acids were separated by capillary GC on an optically active stationary phase. The IBOC-methyl derivatives were said to be more stable than MOC- or EOC-methyl ones, as both the latter tended to decrease upon storage.

Some researchers dealing with derivatization of amino acids and small peptides preferred fluorinated derivatives over nonfluorinated ones for their inherent higher sensitivity in positive-ion and negative-ion chemical-ionization (PICI, NICI) modes than EI. In a series of papers, Moini and coworkers treated the protein [87] and nonprotein [88] amino acids and some small peptides [89,90] with ECF and trifluoroethanol (TFE) instead of ethanol and the *N*(*O*,*S*)-EOC amino acid TFE esters were analyzed by GC–MS using EI and NI/PI CI modes. Twenty-one out of the twenty-three nonprotein amino acids studied produced detectable ion chromatograms in both ionization modes when methane was used as the CI reagent gas. The detection limits using PICI were mostly in the femtomole range. The approach was extended to derivatization of twelve dipeptides and one tripeptide, i.e. to the hydrolytic products of Equal, a sugar substitute containing aspartame (a methyl ester of Asp-Phe) and diprotin B [89,90]. The derivatization and extraction efficiencies were checked for the first time by repeating the treatment following extraction of the derivatives into chloroform and evaporation of the first reaction medium. The former recoveries ranged from 90–99% (except 79% for aspartic acid), the latter ones were close to 100% [90].

A two-step procedure for enantiomeric amino acids with high separation factors also for proline was reported [91,92]. Based on esterification with IBCF (ECF or IPCF, alternatively) in aqueous TFE, the resulting TFE-esters were transformed into *N*-alkylamides by the action of isobutylamine or propylamine and subjected to capillary GC analysis on chiral phase (Fig. 24). The second reaction required a prolonged reaction time of 30 min.

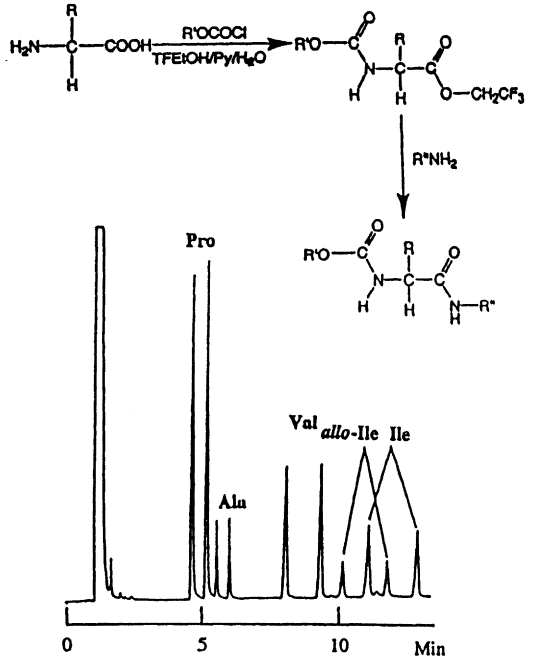


Fig. 24. Derivatization scheme of a two-step procedure for conversion of amino acids into *N*-alkyloxycarbonyl alkyl amides and GC analysis of *N*-isopropyloxycarbonyl amino acid isobutylamide enantiomers separated on a chiral phase. Reproduced from Ref. [92] with permission.

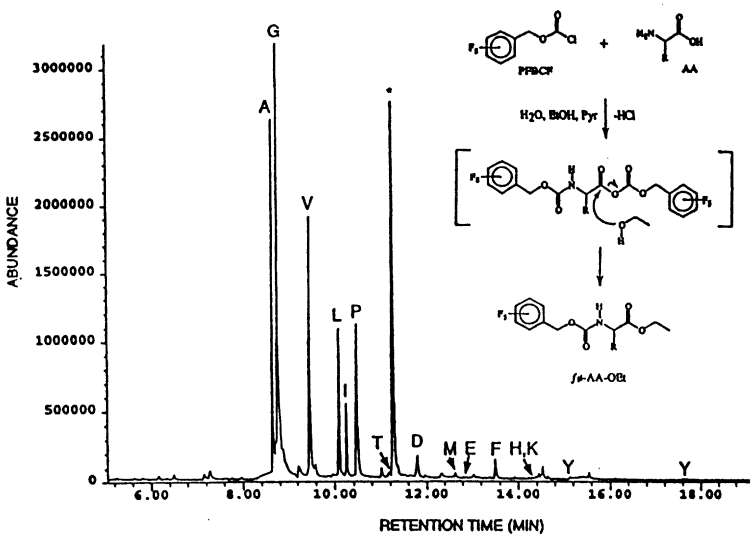


Fig. 25. Reaction scheme for treating amino acids in aqueous ethanol–pyridine solution with PFBCF and selected-ion current profiles from SIM GC–MS analysis of *N*(*O*,*S*)-PFBOC amino acid ethyl esters after treating the 10 μl-whole blood sample directly. Reproduced from Ref. [94] with permission.

Others [93,94] used a perfluorinated reagent, the only available PFBCF, for treatment of amino acids in aqueous ethanol to provide structurally relevant fragmentation in NICI–MS. To demonstrate the efficiency and sensitivity of this approach, amino acids from a finger stick were subjected to derivatization as a 10-μl whole blood sample with no prior extraction or purification. The blood was covered with 100 μl of water–ethanol–pyridine (6:3:1), treated with 10 μl of PFBCF and the analysis succeeded after extraction into 100 μl of toluene [94]. The corresponding record and the reaction scheme are shown in Fig. 25.

The following conclusions should be drawn from these studies. First, apparently low responses of the basic amino acids are recorded throughout those approaches in which the fluorinated alcohols or reagents were used. Optimization of the reaction conditions, if still possible, would be desirable. Second, a combination of ECF–methanol and MCF–ethanol was tested with amino acids in our early (unpublished) studies. Though no special profit was found, the former mixture would be more acceptable than the latter, in case even traces of methyl esters occurred. Consequently, a stronger alcohol, i.e. that of shorter alkyl, should be present as a medium component as a rule, provided that a reagent of another alkyl was employed for the treatment.

6. Treatment of highly hydrophilic compounds with hexyl chloroformate

The short-chain chloroformates were found to be ineffective in treating hydroxy groups not adjacent to the carboxyl as it was discussed. However, surprising results were achieved with *n*-hexyl chloroformate, which proved to be much more effective in treating polyhydroxycarboxylic acids and polyhydroxyamines than any other reagent of that kind [95–98].

Two advantages were connected with its use: (1) Hydrophilic compounds were converted to hexyl esters, carbonates and carbamates, i.e., to highly hydrophobic products which were quantitatively extracted in hexane in one step; (2) The reaction medium consisted of water only without any added alcohol and both pyridine and dicyclohexylcarbodiimide (DCHC) worked as effective catalysts. Although the best results were obtained in purely aqueous solutions, the presence of up to 50% of organic solvent (acetonitrile, acetone) was said not to prevent the derivatization.

In one of the studies only [96] pyridine preceded addition of the reagent and, among the experimental parameters tested, it was found crucial to introduce the reagent slowly and under sonication. In the other studies, however, the reagent was always added first and emulsified in the aqueous medium by sonication,

followed by slow stepwise addition of the pyridine. Improved reaction yields of some analytes even by factors 10 to 100 were announced for some amino-alcohols, hydroxylamines, aminophenols and other aminic substances when a saturated solution of DCHC in pyridine was employed as the catalyzer [98].

For quantification of the analytes, the GC–MS detection seemes to be a prerequisite since even with blank samples, nearly the whole area of the record is full of extraneous peaks. The hexane extracts generally contained by-products, including particularly dihexyl carbonate as well as hexyl formate, dihexyl oxalate and some polycarbonyldihexyl esters [96]. It was noted that the concentration of by-products increased significantly, when the amount of reagent used was above the concentration for full derivatization. When an unknown mixture had to be deriva-

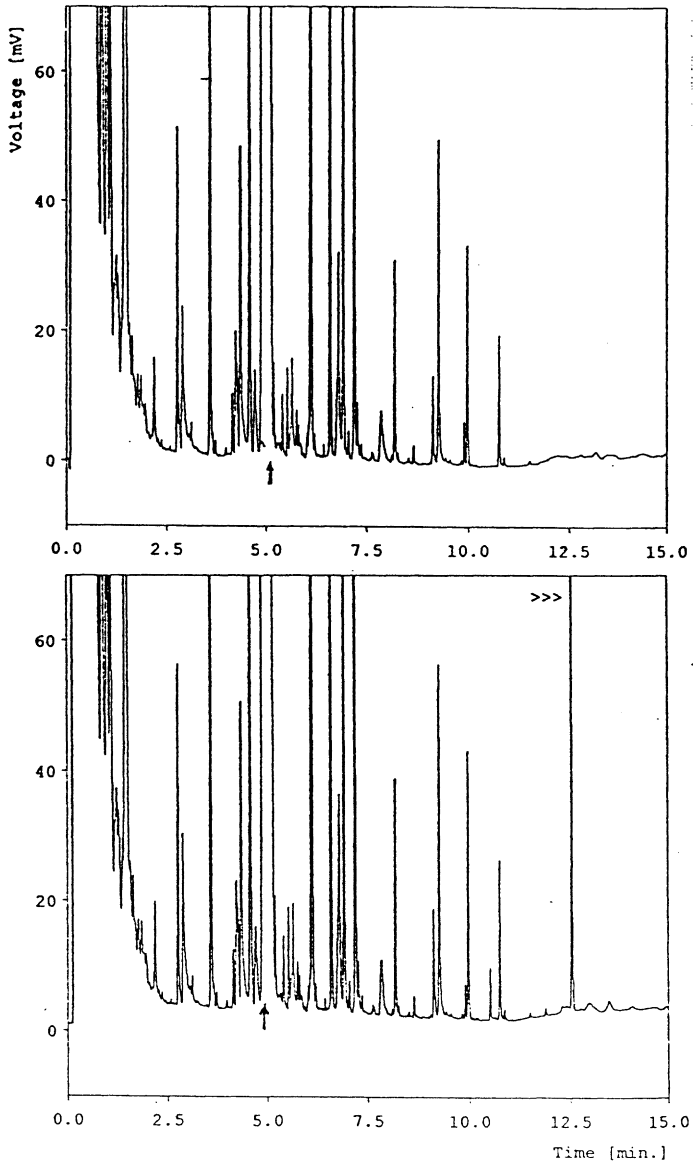


Fig. 26. GC–FID chromatographic record of side products formed after treating aqueous pyridine solutions with hexyl chloroformate [95–98]. Blank sample (top) and sample with tartaric acid (bottom) converted to di-*O*-hexyloxycarbonyl dihexyl ester.

tized, its hexane extract should be compared with that obtained from a blank, in order to rule out these reaction by-products. PICI was found as the most suitable detection mode, EI-MS was shown as inferior due to the extensive and unspecific fragmentation observed.

In order to find out the extent of the reaction by-products, tartaric acid was treated with hexyl chloroformate and analyzed by GC–FID in our own experiments. Due to an introduction of two hexyl and two hexyloxycarbonyl moieties into the molecule, the relatively heavy derivative was luckily eluted at the rear of the run (Fig. 26). The same was experienced with amino acids extracted into hexane after the performed treatment: the yields were good even with the basic ones but the first members were hidden among the jungle-peaks. This drawback must be taken into account when analytes of lower molecular masses are to be treated in this way.

7. Simultaneous treatment of amino and nonamino compounds with ECF or MCF

This was a special challenge for the chloroformate methodology going beyond the current derivatization procedures. The task was to unify the reaction conditions to such an extent that hydrophilic and hydrophobic compounds such as the amino and the fatty acids, and also amines and acids, could be determined simultaneously.

At first, we were interested in the possibility of derivatizing and analyzing biogenic amines, their deaminated acidic products and related alcohols, simultaneously. Altogether ten amines (catecholamines, metanephrines, serotonin etc.), two polyamines (spermidine, spermine), four acids (including 5-OH-indolacetic acid) and two aromatic alcohols were treated with ECF and MCF according to the novel approach in aqueous acetonitrile–alcohol medium and, alternatively, in aqueous bicarbonate [99]. Comparing the results, the yields of the amines were about the same, the polyamines were higher in the bicarbonate medium. As expected, the acids were recorded following the former procedure only. EI mass spectra and fragmentation pathways of several MCF-treated metabolites are included.

In the subsequent study, three aromatic amino acid precursors were coadded to a mixture of the four acids, six biogenic amines and two aromatic glycols and the blend was treated in a medium optimized for the novel approach [100]. Analysis succeeded upon treatment with the both ECF and MCF, however, only the ECF-treated analytes were completely separated under the chromatographic conditions used (Fig. 27).

The results were promising but of less practical use since cleanup procedures for a selective isolation of such a bunch of chemically diverse compounds from, e.g., body fluids were not at hand. However, a simultaneous treating and analyzing of amino and nonamino compounds with chloroformates proved to be possible. As the unified conditions did not require exclusion of water from the reaction media, the attractive possibility of derivatizing directly in the aqueous matrix, i.e. without preisolation of the analytes, offered itself for testing. The way for so-called metabolic profiling of biological fluids was open.

Hoffmann et al. [101–105] contributed substantially to both the methodology and the data acquisition in analysing organic acids in human fluids by capillary GC. For quantification of the acids in, e.g.,

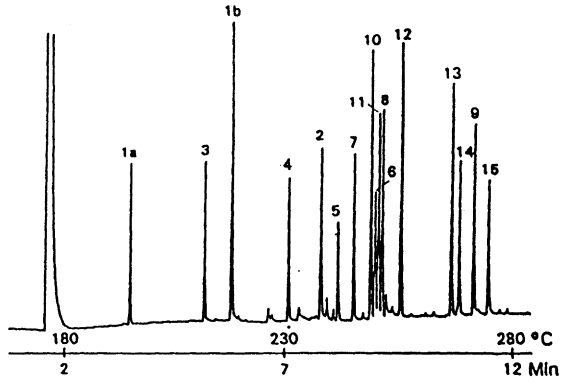


Fig. 27. GC–FID analysis of biogenic amines, their precursors and catabolytes in equimolar mixture after treatment with ECF. The peaks belong to the following analytes: 1a = 3-Methoxy-4-hydroxyphenylglycol (1b = derivatized piperazine in doubled concentration accompanying the MHPG-standard); 2 = 3,4-dihydroxyphenylglycol; 3 = homovanillic acid; 4 = 3,4-dihydroxyphenylacetic acid; 5 = vanillylmandelic acid; 6 = 5-hydroxyindole-3-acetic acid; 7 = tyrosine; 8 = tryptophan; 9 = 3,4-dihydroxyphenylalanine; 10 = metanephrine; 11 = normetanephrine; 12 = dopamine; 13 = epinephrine; 14 = norepinephrine; 15 = serotonin. Reproduced from Ref. [100] with permission.

cerebrospinal fluid and plasma [102,103], the previously elaborated methods were used [104–106]. The consecutive steps involved oximation of keto acids, lyophilization of the sample, isolation of the acids by liquid partition chromatography on silica, evaporation of the eluate and subsequent silylation of the dry residue with BSTFA (2 h at 60°C). Even when tedious and time-consuming, it was in accordance with state-of-the-art procedures.

Using ECF as derivatizing agent enabled marked simplification of the methodology of the sample preparation [107–109]. The advanced approach reduced the plasma workup to minutes because of: (a) using the same organic solvent for plasma deproteinization and subsequent reaction, (b) omitting isolation of the compounds of interest while removing neutral lipids by means of a simple extraction with hexane, (c) not requiring the oximation of keto acids.

Provided that only oxo-, hydroxy- and dicarboxylic acids were the centre of interest, the supernatant was shaken with hexane and particles of cation-exchange resin. Due to this, the free fatty and the amino acids were removed [107]. The second option left the free fatty acids in the sample while removing amino acids by pushing the supernatant through the exchanger bed [108]. When each of the described pretreatments was omitted, the whole spectrum of the plasma carboxylic acids was determined as shown in Fig. 28 [109].

The 3:1 ratio of the organic portion (acetonitrile–ethanol) to water was justified as a reasonable compromise in order not to worsen derivatization yields of most of the amino acids while leaving satisfactory yields of both the keto and hydroxy-carboxylic acids. The reverse mode of reagent-base addition (see 4. 4.) helped to suppress formation of side-products of the predominant metabolite of serum profile, the lactic acid.

Equal treatment proved to be effective also in profiling the urinary organic acids following removal of the nitrogen-containing compounds by means of exchange resin [110]. The GC–FID analysis of urine treated with ECF is given in Fig. 29. With regard to the huge amount of unidentified peaks because of the lack of necessary chromatographic standards, the GC–MS analysis would certainly be a better option.

The multidimensional enantioselective capillary GC–MS analysis of the main urinary MSUD (maple syrup urine disease) metabolites is very recent data [111]. Without any alcohol added, one mililitre of urine was simply mixed with an equal volume of pyridine and a two-fold volume of MCF was carefully added dropwise. The main metabolites of the disorder were monitored in cut-intervals in ethereal extract that was rid of pyridine by shaking it with

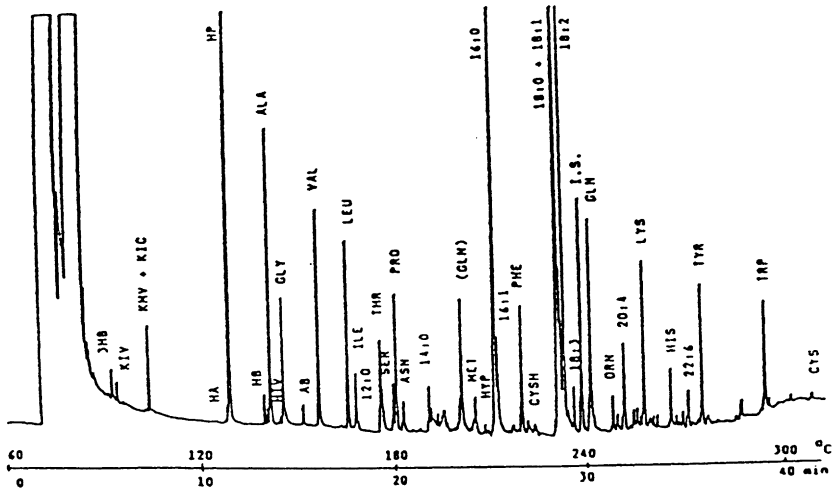


Fig. 28. Simultaneous GC–FID profiling of keto, hydroxy, fatty and amino acids in plasma after treating the supernatant, deproteinized by the action of organic solvent (acetonitrile–ethanol), with ECF. Reproduced from Ref. [109].

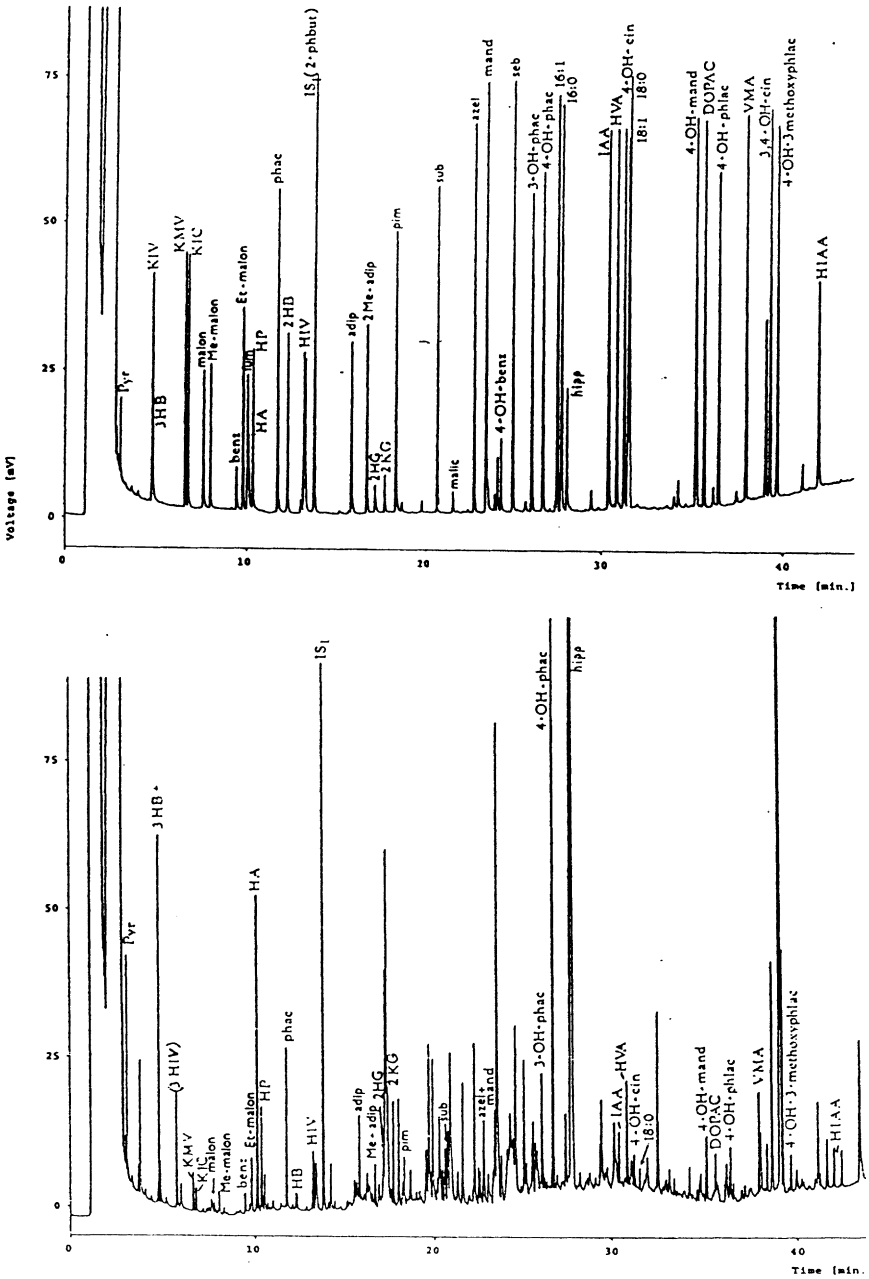


Fig. 29. Urine organic acid GC–FID profiling after removal of amines and amino acids by cation-exchange chromatography and treating urine with coadmixed organic solvents with ECF. The compound abbreviations are easy-to-follow, for details see the original paper. Reproduced from Ref. [110] with permission.

acidified and NaCl-saturated aqueous phase. As the authors succeeded in determining some of the branched-chain carboxylic, α-oxo, α-hydroxy and α-amino acids, it follows, that in spite of far-from-optimum reaction conditions, the derivatization proceeded to a certain extent even in the presence of

urea. This is additional evidence that with chloroformates, still many surprising results can be expected.

The aim of the last application presently mentioned was to identify proteinaceous and oil-binding media used in paintings from collections of art in the Region of Valencia (Spain) [112]. Samples <1 mg were treated with HCl to hydrolyze proteins and glycerides and both the dry residue after the chloroform extraction and the remaining aqueous layer were subjected to the most common ECF–ethanol treatment. Amino acids were found in the aqueous phase and C14–C18 saturated fatty acids in the organic phase. Moreover, the presence of azelaic acid was also confirmed, the derivative of it being distributed equally in the two phases. The ratio of the amino to the fatty acids found proved to be extremely useful for identifying the binding media in the paintings in view of planned conservation and restoration work.

Let us make a closing remark and express the wish that not only in the art of painting but generally, that the chloroformates could prove their usefulness in restoring derivatization methodology and conserving the GC technique for the break of the century.

8. Conclusions

In the 60s, silylating reagents of general utility were introduced into the GC derivatization methodology for treating polyfunctional compounds in one reaction step. Amino acids were not determined together with other classes of carboxylic acids, aprotic medium and heating were required as a rule.

In the 90s, alkyl chloroformates were discovered as potential general purpose reagents in analytical chemistry. Instantaneous reaction course which did not require the exclusion of water, negligible reagent costs, plus a possibly simplified sample workup making isolation of analytes from a matrix unnecessary, are the main advantages of these derivatizing agents. Moreover, a simultaneous determination of amino and nonamino polyfunctional carboxylic acids was shown to be possible. The approach was highlighted generally as ‘‘unrivalled in ease, speed and flexibility for the preparation of alkyl esters’’.

Future expected development might result in synthesizing halogenated reagents derived from perfluorinated alcohols in order to exploit the sensitivity of GC(HPLC)/NICI-MS facilities for the correspondingly prepared derivatives. The chloroformate-mediated transformation of analytes into compounds amenable to separation techniques is undoubtedly a perspective for the future.

9. Abbreviations

BCF	*n*-Butyl chloroformate
CECF	2-Chloroethyl chloroformate
DCHC	Dicyclohexylcarbodiimide
DMAP	4-Dimethylaminopyridine
ECD	Electron-capture detector
ECF	Ethyl chloroformate
EI	Electron impact
EOC	Ethoxycarbonyl
FID	Flame ionization detector
FMOC	9-Fluorenylmethyl chloroformate
FPD	Flame photometric detector
GC	Gas chromatography
HAs	2-Hydroxycarboxylic acids
HPLC	High-performance liquid chromatography
IBCF	Isobutyl chloroformate
IBOC	Isobutyloxycarbonyl
IPCF	Isopropyl chloroformate
MCF	Methyl chloroformate
MOC	Methoxycarbonyl
MS	Mass spectrometry
MSUD	Maple syrup urine disease
MTBSTFA	*N*-methyl, *N*-*tert*.-butyldimethylsilyltrifluoroacetamide
NICI	Negative-ion chemical ionization
NMPIP	*N*-methylpiperidine
NPD	Nitrogen–phosphorus detector
PCF	Propyl chloroformate
PFBCF	Pentafluorobenzyl chloroformate
PICI	Positive-ion chemical ionization
TBDMS	*tert*.Butyldimethylsilyl
TCECF	2,2,2-Trichloroethyl chloroformate
TEA	Triethylamine
TFE	Trifluoroethanol
TMS	Trimethylsilyl

Acknowledgements

The author wishes to acknowledge the cooperation of a number of coworkers especially from the Netherlands (J. Buyten from Chrompack, P. Leclercq, J. Rijks, C.A. Cramers from the Eindhoven University of Technology), Switzerland (R. Liardon and U. Richli from Nestlé), United States (C.C. Sweeley, Z.-H. Huang from Michigan State University) and Germany (H.M. Liebich, E. Gesele, H.G. Wahl, University of Tübingen).

Numerous studies in the years 1992–98 were made in the Institute of Endocrinology in Praha under financial support of Internal Grant Agency of the Ministry of Health of the Czech Republic (Grant No. 851-3, 2733-3 and 4848-3) which is highly acknowledged.

References

[1] A. Muir, LC–GC Intern. 10(4) (1997) 252.
[2] K. Blau, J.M. Halket (Eds.), Handbook of Derivatives for Chromatography, 2nd ed., J. Wiley and sons Ltd., Baffins Lane, Chichester, England, 1993.
[3] C.F. Poole, S.K. Poole, J. Chromatogr. Sci. 25 (1987) 434.
[4] C.F. Poole, A. Zlatkis, J. Chromatogr. 184 (1980) 99.
[5] H. Brückner, M. Lüpke, J. Chromatogr. A 697 (1995) 295.
[6] M. Ahnoff, S. Chen, A. Green, I. Grundevik, J. Chromatogr. 506 (1990) 593.
[7] O. Gyllenhaal, L. Johansson, J. Vessman, J. Chromatogr. 190 (1980) 347.
[8] A.P.J.M. DeJong, C.A. Cramers, J. Chromatogr. 276 (1983) 267.
[9] S. Yamamoto, K. Kakuno, S. Okahara, H. Kataoka, M. Makita, J. Chromatogr. 194 (1980) 399.
[10] M. Makita, S. Yamamoto, A. Katoh, Y. Takashita, J. Chromatogr. 147 (1978) 456.
[11] N.O. Ahnfelt, P. Hartvig, Acta Pharm. Suec. 17 (1980) 307.
[12] N.O. Ahnfelt, P. Hartvig, K.E. Karlsson, Chromatography 16 (1982) 60.
[13] N.O. Ahnfelt, P. Hartvig, Acta Pharm. Suec. 19 (1982) 367.
[14] P. Hartvig, J. Vessman, J. Chromatogr. Sci. 12 (1974) 722.
[15] P. Hartvig, J. Vessman, Anal. Lett. 7 (1974) 223.
[16] T. Lundh, B. Akesson, J. Chromatogr. 617 (1993) 191.
[17] K.R. Kim, M.J. Paik, J.H. Kim, S.W. Dong, D.H. Jeong, J. Pharm. Biomed. Anal. 15 (1997) 1309.
[18] J.O. Fernandez, M.A. Ferreira, J. Chromatogr. A 786 (1997) 299.
[19] G. Skarping, M. Dalene, T. Brorson, J.F. Sandström, C. Sangö, A. Tiljander, J. Chromatogr. 479 (1989) 125.
[20] H.G. Ugland, M. Krogh, K.E. Rasmussen, J. Chromatogr. B 701 (1997) 29.
[21] H. Kataoka, J. Chromatogr. A 733 (1996) 19.
[22] M. Makita, S. Yamamoto, M. Kono, K. Sakai, M. Shiraishi, Chem. Ind. (London) 19 (1975) 355.
[23] M. Makita, S. Yamamoto, M. Kono, J. Chromatogr. 120 (1976) 129.
[24] M. Makita, S. Yamamoto, S. Kiyama, J. Chromatogr. 237 (1982) 279.
[25] H. Kataoka, N. Sakiyama, M. Makita, J. Chromatogr. 436 (1988) 67.
[26] H. Kataoka, Y. Ueno, M. Makita, Agric. Biol. Chem. 55 (1991) 1587.
[27] H. Kataoka, K. Nakai, Y. Ueno, M. Makita, Biosci. Biotech. Biochem. 56 (1992) 1300.
[28] H. Kataoka, H. Tanaka, A. Fujimoto, I. Noguchi, M. Makita, Biomed. Chromatogr. 8 (1994) 119.
[29] H. Kataoka, K. Takagi, M. Makita, Biomed. Chromatogr. 9 (1995) 85.
[30] H. Kataoka, K. Takagi, M. Makita, J. Chromatogr. B 664 (1995) 421.
[31] S. Matsumura, H. Kataoka, M. Makita, Biomed. Chromatogr. 9 (1995) 205.
[32] S. Matsumura, H. Kataoka, M. Makita, J. Chromatogr. B 681 (1996) 375.
[33] H. Kataoka, S. Matsumura, H. Koizumi, M. Makita, J. Chromatogr. A 758 (1997) 167.
[34] H. Kataoka, Biomed. Chromatogr. 11 (1997) 154.
[35] H. Kataoka, S. Matsumura, M. Makita, J. Pharmaceut. Biomed. Anal. 15 (1997) 1271.
[36] K.R. Kim, J.H. Kim, C.H. Oh, T.J. Mabry, J. Chromatogr. 605 (1992) 241.
[37] C.H. Oh, J.H. Kim, K.R. Kim, D.M. Brownson, T.J. Mabry, J. Chromatogr. 669 (1994) 125.
[38] A. Einforn, Ber. Buneenges Phys. Chem. 42 (1909) 2772.
[39] J.R. Vaughan Jr., J. Am. Chem. Soc. 73 (1951) 3547.
[40] R.A. Boissonnas, Helv. Chim. Acta 34 (1951) 874.
[41] T. Wieland, H. Bernhard, Ann. 572 (1951) 190.
[42] D.S. Tarbell, N.A. Leister, J. Org. Chem. 23 (1958) 1149.
[43] D.S. Tarbell, E.J. Longosz, J. Org. Chem. 24 (1959) 774.
[44] E.J. Longosz, D.S. Tarbell, J. Org. Chem. 26 (1961) 2161.
[45] D.S. Tarbell, Acc. Chem. Res. 2 (1969) 296.
[46] T.B. Windholz, J. Org. Chem. 23 (1958) 2044.
[47] J.M. Domagala, Tetrahedron Lett. 21 (1980) 4997.
[48] A.L. Gutman, A. Boltanski, Tetrahedron Lett. 26 (1985) 1573.
[49] S. Kim, Y.C. Kim, J.I. Lee, Tetrahedron Lett. 24 (1983) 3365.
[50] S. Kim, J.I. Lee, Y.C. Kim, J. Org. Chem. 50 (1985) 560.
[51] J. Inanaga, K. Hirata, H. Saeki, T. Katsuki, M. Yamaguchi, Bull. Chem. Soc. Japan 52 (1979) 1989.
[52] P. Jouin, B. Castro, C. Zeggaf, A. Pantaioni, Tetrahedron Lett. 28 (1987) 1661.
[53] P. Jouin, B. Castro, C. Zeggaf, A. Pantaioni, J.P. Senet, S. Lecolier, G. Sennyey, Tetrahedron Lett. 28 (1987) 1665.
[54] C. Zeggaf, J. Poncet, P. Jouin, M.N. Dufour, B. Castro, Tetrahedron 45 (1989) 5039.
[55] P. Hušek, J.A. Rijks, P.A. Leclercq, C.A. Cramers, J. High Resol. Chromatogr. 13 (1990) 633.

[56] R.B. Vreeken, M.E. Jager, R.T. Ghijsen, U.A.T. Brinkman, J. High Resol. Chromatogr. 15 (1992) 785.
[57] P. Hušek, J. Chromatogr. 615 (1993) 334.
[58] T. Niwa, J. Chromatogr. 379 (1986) 313.
[59] P. Hušek, LC–GC Int. 5(9) (1992) 43.
[60] T.P. Mawhinney, R.S.R. Robinett, A. Atalay, M.A. Madson, J. Chromatogr. 361 (1986) 117.
[61] B. Schatowitz, G. Gercken, J. Chromatogr. 409 (1987) 43.
[62] B. Schatowitz, G. Gercken, J. Chromatogr. 425 (1988) 257.
[63] H.J. Chaves das Neves, J.P. Noronha, H. Rufino, J. High Res. Chromatogr. 19 (1996) 161.
[64] P. Hušek, Chromatographia 34 (1992) 621.
[65] S. Butz, H.-J. Stan, J. Chromatogr. 643 (1993) 227.
[66] C. Fischer, U. Klotz, J. Chromatogr. B 661 (1994) 57.
[67] P. Hušek, J. Chromatogr. 547 (1991) 307.
[68] P. Hušek, J. Chromatogr. 630 (1993) 429.
[69] H.M. Liebich, E. Gesele, H.G. Wahl, C. Wirth, J. Wöll, P. Hušek, J. Chromatogr. 626 (1992) 289.
[70] P. Hušek, FEBS Lett. 280 (1991) 354.
[71] P. Hušek, J. Chromatogr. 552 (1991) 289.
[72] P. Hušek, C.C. Sweeley, J. High Resol. Chromatogr. 14 (1991) 751.
[73] Z.H. Huang, J. Wang, D.A. Gage, J.A. Watson, C.C. Sweeley, P. Hušek, J. Chromatogr. 635 (1993) 271.
[74] J. Pietzsch, S. Nitzsche, B. Wiedermann, U. Julius, W. Leonhardt, M. Hanefeld, J. Mass Spectrom. 30 (1995) S129.
[75] J. Janák, H.A.H. Billiet, J. Frank, K.C.A.M. Luyben, P. Hušek, J. Chromatogr. A 677 (1994) 192.
[76] L. Fay, R. Badoud, U. Richli, P. Hušek, J. Chromatogr. 552 (1991) 345.
[77] S.D. Copley, E. Frank, W.E. Kirsch, T.H. Koch, Anal. Biochem. 201 (1992) 152.
[78] M. Pan, T.J. Mabry, P. Cao, M. Moini, J. Chromatogr. A 787 (1997) 287.
[79] J. Pietzsch, U. Julius, M. Hanefeld, Clin. Chem. 43 (1997) 2001.
[80] J.O. Sass, W. Endres, J. Chromatogr. A 776 (1997) 342.
[81] Z.-H. Huang, D.A. Gage, L.L. Bieber, C.C. Sweeley, Anal. Biochem. 199 (1991) 98.
[82] J. Wang, Z.H. Huang, D. Gage, J.T. Watson, J. Chromatogr. 663 (1994) 71.
[83] N. Domergue, M. Pugniere, A. Previero, Anal. Biochem. 214 (1993) 420.
[84] P. Šimek, A. Heydová, A. Jegorov, Chem. Listy 90 (1996) 969.
[85] A. Jegorov, V. Matha, P. Sedmera, V. Havlíček, J. Stuchlík, P. Seidel, P. Šimek, Phytochemistry 38 (1995) 403.
[86] I. Abe, H. Yanagi, T. Nakahara, J. High Resol. Chromatogr. 20 (1997) 451.
[87] M. Vatankhah, M. Moini, Biol. Mass Spec. 23 (1994) 277.
[88] P. Cao, M. Moini, J. Chromatogr. A 710 (1995) 303.
[89] P. Cao, M. Moini, Rapid Comm. Mass Spec. 11 (1997) 349.
[90] P. Cao, M. Moini, J. Chromatogr. A 759 (1997) 111.
[91] I. Abe, M. Kawazuma, N. Fujimoto, T. Nakahara, Chem. Lett. (1995) 329.
[92] I. Abe, T. Nakahara, J. High Resol. Chromatogr. 19 (1996) 511.
[93] J.T. Simpson, D.S. Torok, S.P. Markey, J. Am. Soc. Mass Spectrom. 6 (1995) 525.
[94] J.T. Simpson, D.S. Torok, J.E. Girard, S.P. Markey, Anal. Biochem. 233 (1996) 58.
[95] C. Minero, M. Vincenti, E. Pelizzetti, Annali Chim. (Rome) 83 (1993) 511.
[96] C. Minero, M. Vincenti, S. Lago, E. Pelizzetti, Fres. J. Anal. Chem. 350 (1994) 403.
[97] M. Vincenti, C. Minero, S. Lago, C. Rovida, J. High Resol. Chromatogr. 18 (1995) 359.
[98] S. Angelino, V. Maurino, C. Minero, E. Pelizzetti, M. Vincenti, J. Chromatogr. A 793 (1998) 307.
[99] P. Hušek, Z.H. Huang, C.C. Sweeley, Anal. Chim. Acta 259 (1992) 185.
[100] P. Hušek, J. Microcol. Sep. 5 (1993) 101.
[101] W. Meier-Augenstein, G.F. Hoffmann, B. Holmes, J.L. Jones, W.L. Nyhan, L. Sweetman, J. Chromatogr. 615 (1993) 127.
[102] G.F. Hoffmann, C.K. Seppel, B. Holmes, L. Mitchell, H.J. Christen, F. Hanefeld, D. Rating, W.L. Nyhan, J. Chromatogr. 617 (1993) 1.
[103] G.F. Hoffmann, W. Meier-Augenstein, S. Stockler, R. Surtees, D. Rating, W.L. Nyhan, J. Inherit. Metab. Dis. 16 (1993) 648.
[104] G.F. Hoffmann, S. Aramaki, E. Blum-Hoffmann, W.L. Nyhan, L. Sweetman, Clin. Chem. 35 (1989) 587.
[105] G.F. Hoffmann, L. Sweetman, Clin. Chim. Acta 199 (1991) 237.
[106] L. Sweetman, F.A. Hommes (Ed.), Techniques in Diagnostic Human Biochemical Laboratories: A Laboratory Manual, Wiley-Liss, N. York, 1991, p. 143.
[107] P. Hušek, P. Matucha, J. Chromatogr. B 993 (1997) 499.
[108] P. Hušek, H.M. Liebich, J. Chromatogr. B 656 (1994) 37.
[109] P. Hušek, J. Chromatogr. B 669 (1995) 352.
[110] P. Hušek, Clin. Chem. 43 (1997) 1999.
[111] F. Podebrad, M. Heil, S. Leib, B. Geier, T. Beck, A. Mosandl, A.C. Sewell, H. Böhles, J. High Resol. Chromatogr. 20 (1997) 355.
[112] R.M. Castro, M.T.D. Carbó, V.P. Martínez, J.V.G. Adelantado, F.B. Reig, J. Chromatogr. A 778 (1997) 373.