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Analysis of amino acids by gas chromatography–flame ionization detection and gas chromatography–mass spectrometry: Simultaneous derivatization of functional groups by an aqueous-phase chloroformate-mediated reaction

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Abstract

The one-step ethyl chloroformate derivatization of amino acids in an aqueous medium is extended with the use of a variety of alkyl chloroformate reagents. This provides a new and convenient procedure for preparing esters with different alkoxy groups. A new mechanism for esterification during chloroformate derivatization is proposed based on the formation of an intermediate mixed carboxylic–carbonic acid anhydride followed by the exchange with an alcohol. Among the different reagents investigated, isobutyl chloroformate derivatized amino acids were found to provide more sensitivity for analyses by GC–flame ionization detection and GC–MS relative to derivatives prepared by other alkyl chloroformates.

1. Introduction

Analysis of protein hydrolysates for usual amino acids by gas chromatography is now routine. The foundation for the most commonly used method was laid by Gehrke and co-workers [1] who developed a procedure for quantitative derivatization to provide N(O,S)-trifluoroacetyl (TFA) amino acid *n*-butyl esters (TAB amino acids). Other perfluoroacyl alkyl esters [2,3] have also been useful in the vapor phase analysis of amino acids. Procedures for preparation of TAB and related derivatives require two reactions.

An alternative approach for the analysis of amino acids by GC was introduced by Hušek [4–6] and fatty acids [7]. Briefly, the Hušek procedure uses an aqueous medium in which ethyl chloroformate (EtCF) reacts instantaneously with the amino, carboxyl, and the side-chain functional groups (excluding the guanidino- and aliphatic hydroxyl groups). Results of a comprehensive study on the mass spectrometric fragmentation of this family of derivatives have been reported by this laboratory [8].

Here we report an extended and improved chloroformate derivatization method by applying

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alternate alkylchloroformate reagents, as well as different combinations of reagents to derivatize amino acids prior to analysis by GC and GC–MS. Specifically, the incorporation of different alcohols in the reaction mixture provides a convenient means to prepare a variety of different esters. Some of these newly described derivatives provide higher sensitivities than the ethyl chloroformate amino acid derivatives. A reaction mechanism for carboxyl group derivatization is proposed which differs from that suggested by Hušek [4–7].

2. Experimental

The amino acids were purchased from Sigma (St. Louis, MO, USA), alkyl chloroformates and alcohols were purchased from Aldrich (Milwaukee, WI, USA). A solution of 20 amino acids in 0.1 *M* HCl at a concentration of 0.5 μg/μl each was prepared.

The N(O,S)ethoxycarbonyl ethyl ester of amino acids (ECEE) were prepared by adding 10 μl of the amino acid mixture to a H_2O–ethanol (EtOH)–pyridine (Py) (60 μl–30 μl–10 μl) solution. 5–10 μl of ethylchloroformate (EtCF) were then added and the reaction mixture was vortexed for 5–10 s and extracted with 100–200 μl $CHCl_3$. A 1-μl aliquot of the $CHCl_3$ layer was injected for analysis. Other chloroformate derivatives were prepared by adding the amino acids to a solution of H_2O–alcohol–Py (80 μl–30 μl–10 μl) and 10 μl of the chloroformate reagent following the same procedure.

Analyses by GC–MS were carried out on a JEOL AX-505H double focusing mass spectrometer coupled to a Hewlett-Packard 5890J gas chromatograph. GC separation was achieved on a DB-1701 (15 m × 0.25 mm I.D.) fused-silica capillary column with a 0.25 μm film coating from J&W Scientific (Rancho Cordova, CA, USA). Direct (splitless) injection was used. Helium gas flow was approximately 1 ml/min. MS conditions were as follows: interface temperature 275°C, ion source temperature *ca.* 150–200°C, electron energy 70 eV, scan rate of the mass spectrometer 1 s/scan over the range of *m/z* 50–750. GC–FID (flame ionization detection) was carried out on the same gas chromatographic column with injector and detector temperatures 260°C and 280°C, respectivelt; N_2 was the carrier gas.

3. Results and discussion

Initial experiments using different combinations of alkyl chloroformate reagents and various alcohols in the reaction medium indicated that the type of ester formed during the derivatization process with chloroformate reagents is directly dependent upon the type of alcohol present in the reaction medium. When using an alcohol with an alkyl group different from that in the alkyl chloroformate, the alkoxy group found in the ester derivative corresponds to the alcohol in the reaction medium, and not to the alkyl group of the chloroformate reagent. For example, when phenylalanine reacts with isobutyl chloroformate (iBuCF) in an aqueous medium also containing trimethylsilylmethanol [$(CH_3)_3SiCH_2OH \equiv TMSCH_2OH$], the derivative produced is that in which the carboxylic group is esterified with the trimethylsilylmethyl group, not the isobutyl group. The mass spectrum of this derivative and its structure are shown in Fig. 1. Similarly, when Phe is derivatized by reaction with isobutyl chloroformate in the presence of *n*-heptafluorobutanol in an aqueous reaction medium, the ester derivative formed includes a heptafluorobutoxyl group. The mass spectrum of this derivative and its structure are shown in Fig. 2.

The results presented in Figs. 1 and 2 are in conflict with those expected for carboxyl group derivatization based on the mechanism proposed by Hušek *et al.* [4–7]. According to the Hušek mechanism, the mixed anhydride formed by reaction between the alkyl chloroformate and carboxyl group should decarboxylate ($-CO_2$) to yield the ester containing the alkyl group derived from the alkyl chloroformate. Rather, the results reported here are consistent with a mixed anhydride–alcohol exchange mechanism for derivatization of the carboxyl group as illustrated in

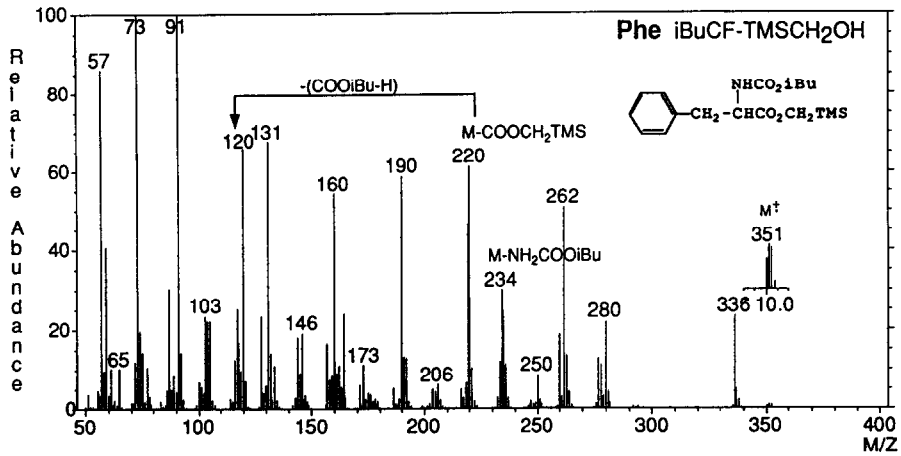

Fig. 1. EI mass spectrum of Phe derivatized by reaction with iBuCF–TMSCH$_2$OH.

Fig. 3. The carboxylic–carbonic acid mixed anhydride proposed as an intermediate in Fig. 3 has precedence in classical organic synthesis utilizing chloroformate reagents. The mixed anhydride reacts with the alcohol in the reaction medium to undergo an exchange reaction as illustrated via pathway A leading to the principal product. A small amount of derivative is also found in which the alkyl group in the ester moiety is the same as that in the alkyl chloroformate reagent. It is likely that this minor product derives from one or both of the possible routes indicated as B and C in Fig. 3. Via route B, it is possible that there is some decarboxylation of the mixed anhydride that takes place to produce the minor product. It is also possible, however, that the mixed anhydride reacts with a small amount of alcohol (having the same alkyl moiety as the chloroformate reagent) produced *in situ* from hydrolysis of the chloroformate reagent in the reaction medium.

Additional evidence for the direct involvement of alcohol constituents in the aqueous reaction medium containing the chloroformate reagent is given in Fig. 4. These data were obtained during analysis of Phe after treatment with isobutyl chloroformate in an aqueous solution containing equimolar amounts of seven alcohols: pentaflu-

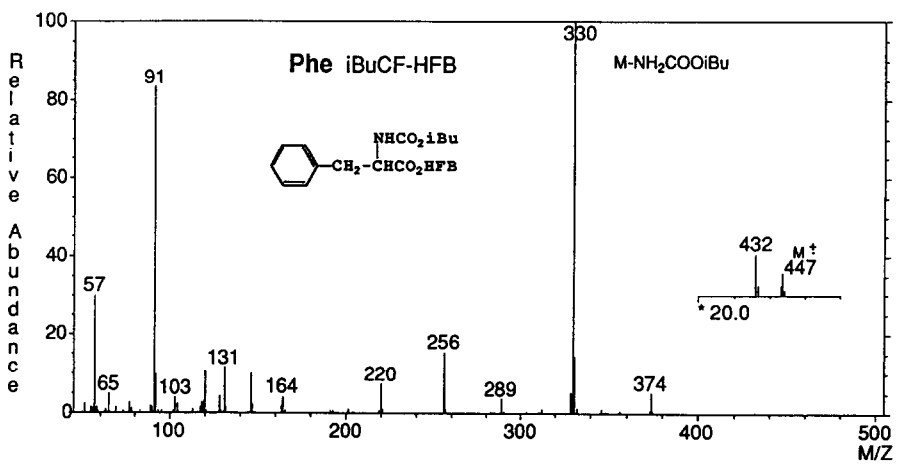

Fig. 2. EI mass spectrum of Phe derivatized by reaction with iBuCF–HFB.

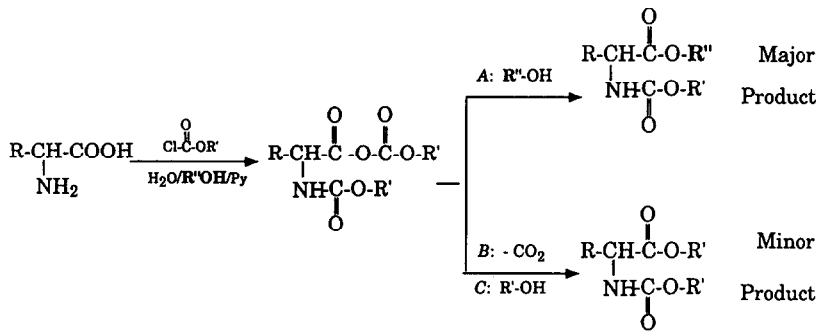

Fig. 3. Mixed anhydride–alcohol exchange mechanism for carboxyl group derivatization of amino acids reacting with ClCOOR' in a medium of H_2O–R"OH–pyridine.

oropropanol (PFP), heptafluorobutanol (HFB), trifluoroethanol (TFE), methanol, ethanol, propanol, and trimethylsilylmethanol. Fig. 4 is a reconstructed total ion current chromatogram resulting from analysis of the reaction mixture by GC–MS. Seven major peaks are obtained, corresponding to the esters formed by reactions with the alcohols in the reaction medium. The different intensities of the peaks result from the differential reactivity of these alcohols with the mixed anhydride and/or responses of the corresponding derivatives under EI–MS conditions.

Various combinations of chloroformate reagents and alcohols were used to generate a wide variety of N(O,S)-alkoxycarbonyl amino acid alkyl esters for analyses by GC or GC–MS with the objectives of optimizing the chromatographic separation of the amino acid derivatives, and evaluating the influence of the alkyl group of the chloroformate (alkyl carbamate in the derivative) and also the structure of the alcohol (alkoxyl group in the ester of the derivative) in the reaction medium on the response of the derivatives detected by FID or by EI-MS. These different combinations of the chloroformate reagents and alcohol systems are tabulated in Table 1 in groups I–III for ease of discussion. Within group I, the derivatives formed by the

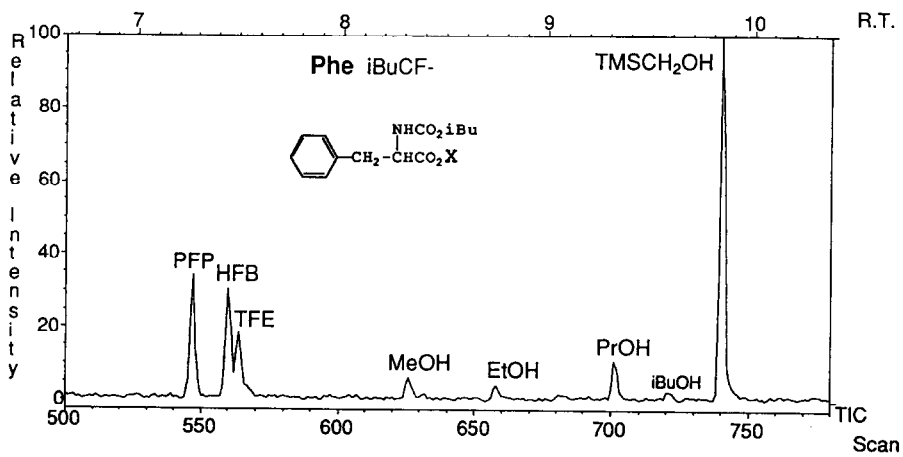

Fig. 4. Reconstructed TIC from analysis by GC–MS of the reaction mixture of Phe and iBuCF in an aqueous solution of an equimolar mixture of seven alcohols. The peaks correspond (as proved from corresponding mass spectra, not shown) to esters formed by exchange reaction with pentafluoropropanol (PFP), heptafluorobutanol (HFB), trifluoroethanol (TFE), methanol, ethanol, propanol, and trimethylsilylmethanol ($TMSCH_2OH$). The peak labeled iBuOH represents a trace of the ester product formed with the alkoxyl group corresponding to the alkyl group of the chloroformate reagent (see text)

Table 1
Composition of chloroformate reagent–alcohol systems studied

Reagent	Derivative
Group I	
EtCF–EtOH [a]	N(O,S)-ethoxycarbonyl ethyl ester
PrCF–PrOH [b]	N(O,S)-propoxycarbonyl propyl ester
iBuCF–iBuOH [c]	N(O,S)-isobutoxycarbonyl isobutyl ester
Group II	
EtCF–TFE	N(O,S)-ethoxycarbonyl trifluoroethyl ester
–PFP	N(O,S)-ethoxycarbonyl pentafluoropropyl ester
–HFB	N(O,S)-ethoxycarbonyl heptafluorobutyl ester
PrCF –TFE	N(O,S)-propoxycarbonyl trifluoroethyl ester
–PFP	N(O,S)-propoxycarbonyl pentafluoropropyl ester
–HFB	N(O,S)-propoxycarbonyl heptafluorobutyl ester
iBuCF–TFE	N(O,S)-isobutoxycarbonyl trifluoroethyl ester
–PFP	N(O,S)-isobutoxycarbonyl pentafluoropropyl ester
–HFB [d]	N(O,S)-isobutoxycarbonyl heptafluorobutyl ester
Group III	
iBuCF–TMSCH_2OH [e]	N(O,S)-isobutoxycarbonyl trimethylsilymethyl ester
–TMS$(CH_2)_2$OH	N(O,S)-isobutoxycarbonyl trimethylsilyethyl ester
–TMS$(CH_2)_3$OH	N(O,S)-isobutoxycarbonyl trimethylsilypropyl ester

The order of response by GC–FID and by GC–MS: $e \sim d \sim c > b > a$.

reagents generates a response in MS analysis and by FID that increases with the size of the alkyl groups for the groups studied: (isobutyl > propyl > ethyl) in the chloroformate reagent as well as in the derivatizing alcohol. Thus, the responses of the iBuCF–iBuOH amino acid derivatives are higher than those of EtCF–EtOH derivatives. In group II, for any particular chloroformate reagent (EtCF, PrCF, or iBuCF), the detectability increases with the size of the perfluoroalcohol (HFB > PFP > TFE). Also in group II, for any particular alcohol (TFE, PFP, or HFB), the detectability increases with the size of the chloroformate (iBuCF > PrCF > EtCF). In group III, when using iBuCF and various TMS alcohols similar results as those in group II were found, but an increase in the size of the alcohol (TMS$(CH_2)_3$OH > TMS$(CH_2)_2$OH > TMSCH_2OH) causes greater production of the derivatives esterified with the alkyl group of the chloroformate reagent. The reason for increased competition from this side reaction is unclear, although it may result from slower alcohol–mixed anhydride exchange reactions for bulkier alcohols. In general, the formation of minor products from hydrolysis/exchange (pathway B + C, Fig. 3) were typically much less than 10% as indicated by the total ion current chromatogram of the derivatives; however, the yields of side products were not systematically investigated.

The highest detectability and the best chromatographic separations are produced by iBuCF–iBuOH, iBuCF–HFB, and iBuCF–TMSCH_2OH derivatization reagents. Fig. 5 shows that the GC–FID responses of the 20 amino acids derivatized with iBuCF–iBuOH, iBuCF–HFB, and iBuCF–TMSCH_2OH are higher than those prepared with EtCF–EtOH. Tyr and Hyp also can be derivatized and separated from the other 20 amino acids (even though these two were not included in the mixture represented in Fig. 5). The guanidino group on the side chain of Arg is not derivatized by the reaction mixture described here as verified by detection with FAB; Arg in this form cannot be eluted from the GC column. A quantitative comparison of the detector response to

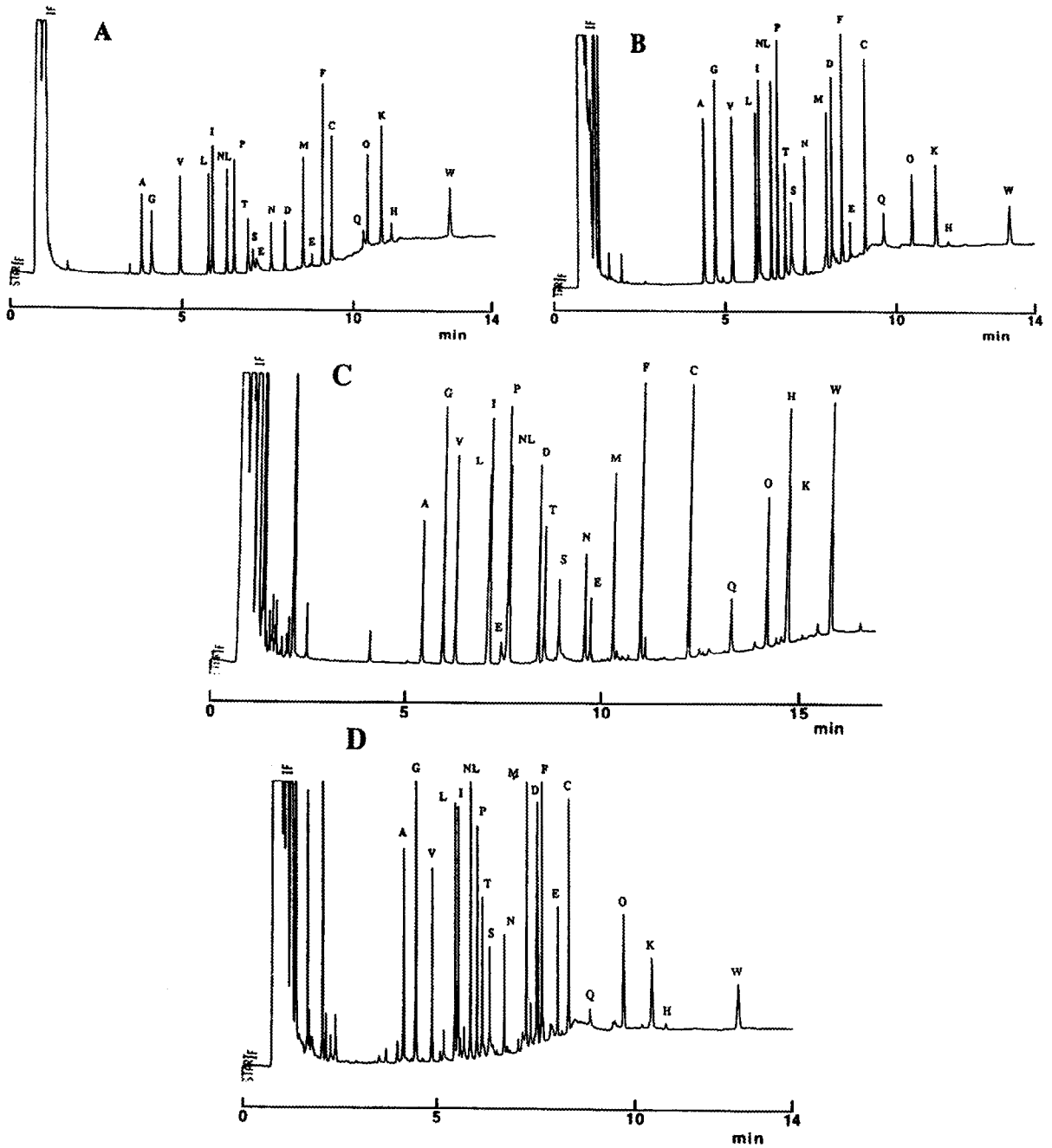

Fig. 5. Comparison of GC–FID chromatograms of derivatives of 20 amino acids prepared from four different chloroformate derivatization mixtures: (A) EtCF–EtOH; (B) iBuCF–iBuOH; (C) iBuCF–HFB; (D) iBuCF–TMSCH$_2$OH. The chromatograms result from injection of an aliquot of the reaction mixture containing 50 ng of each amino acid on to a 15m × 0.25 mm I.D. column containing a 0.25-μm film of DB-1701. Temperature programs: (A) from 110°C to 180°C at 10°C/min, then 30°C/min to 280°C; (B) from 140°C to 200°C at 10°C/min, then 30°C/min to 280°C; (C) from 100°C to 200°C at 10°C/min, then 15°C/min to 280°C; (D) from 150°C to 200°C at 10°C/min, then 40°C/min to 280°C. Norleucine (NL) is also included in the mixture. Tyrosine (Tyr) and hydroxyproline (Hyp)can also be derivatized, although the hydroxyl group of the latter is not modified. Arg is derivatized at the N- and C-terminus, but not on the side chain, and, as such, cannot be eluted from the GC column.)

Table 2
Ratio of peak area on GC–FID of indicated derivatives relative to those prepared with EtCF–EtOH

	PrCF–PrOH /EtCF–EtOH	iBuCF–iBuOH /EtCF–EtOH	iBuCF–HFB /EtCF–EtOH
Ala	1.5	2.0	1.4
Gly	1.8	2.6	2.4
Val	1.2	1.6	1.5
Leu	1.3	1.7	1.4
Ile	1.1	1.6	1.4
n-Leu	1.5	1.7	1.9
Pro	1.2	1.7	1.2
Thr	1.3	1.7	1.5
Ser	1.8	1.3	2.0
Asn	1.5	1.9	1.6
Asp	0.5	3.1	2.9
Met	1.7	1.5	1.4
Glu	1.3	2.2	4.3
Phe	1.2	1.5	1.4
Cys	1.3	1.4	2.0
Gln	4.1	3.2	4.5
Orn	1.1	1.2	1.4
Lys	1.0	1.1	1.2
His	0.7	0.4	5.7
Trp	0.9	1.2	2.7

Responses for EtCF–EtOH derivatives are the averages of results from triplicate analyses; responses for the indicated derivatives are the averages of results from duplicate analyses.

various derivatives is given in Table 2, which lists the ratio of GC–FID peak area produced by the derivatives made from PrCF–PrOH, iBuCF–iBuOH or iBuCF–HFB to those made from EtCF–EtOH. For derivatives prepared with PrCF and iBuCF, pyro-Glu is not resolved from isoleucine. Table 3 compares the reconstructed TIC responses of different groups of derivatives relative to those prepared from EtCF–EtOH. In nearly all cases, derivatives prepared from EtCF–EtOH gave a lower response. The mass spectra of the amino acid derivatives reported herein are produced through fragmentation pathways similar to those described earlier for mass spectra of EtCF–EtOH derivatives of amino acids [8]. The amino acid derivatives described in this paper are expected to have recoveries similar to those of amino acids derivatized with EtCF–EtOH [4–6]. Although quantitative experiments were not conducted, the inclusion of a suitable internal standard, such as norleucine (see Fig. 5) or stable isotope labelled amino acids, would make the approach described here suitable for quantitative analyses. Derivatization by iBuCF–iBuOH has been applied to the determination of isotopic amino acid incorporation into photosynthetic proteins of Synechocystis PCC 6803 [9].

4. Conclusions

The one-step chloroformate derivatization of amino acids in an aqueous medium has been extended with the use of a variety of alkyl chloroformate and alcohol reagents. It was discovered that the ester moiety of the amino acid derivatives is directly dependent upon the type of alcohol used in the aqueous reaction medium. Based on these findings, a new mechanism for ester formation is proposed to involve an alcohol exchange reaction with an intermediate mixed anhydride of the carboxyl group. These results have provided new insight into the one-step

Table 3
Ratio of both peak area and peak height of reconstructed TIC corresponding to the indicated derivatives relative to those made with EtCF–EtOH from analyses by GC–MS (EI)

	PrCF–PrOH /EtCF–EtOH		iBuCF–iBuOH /EtCF–EtOH		iBuCF–HFB /EtCF–EtOH	
	Area	Height	Area	Height	Area	Height
Ala	0.99	0.95	1.79	2.09	2.05	1.93
Gly	1.13	1.13	1.98	2.24	3.33	3.02
Val	0.90	0.81	1.09	1.21	1.70	1.57
Leu	0.97	1.01	1.49	1.58	2.24	2.96
Ile	0.78	0.81	0.87	0.91	1.37	1.75
Leu	0.98	0.92	1.42	1.45	2.01	1.83
Pro	1.06	1.16	1.60	1.92	1.88	1.88
Thr	1.14	1.39	1.58	1.70	1.87	2.02
Ser	0.85	0.67	1.10	1.08	1.05	1.43
Asn	1.23	1.18	1.69	1.72	2.49	2.42
Asp	0.37	0.37	1.88	2.66	3.51	2.81
Met	1.81	1.55	1.52	1.53	1.84	1.52
Glu	1.20	1.30	0.94	0.83	2.33	2.16
Phe	1.22	1.79	1.41	1.90	1.83	1.82
Cys	1.48	1.85	0.62	0.83	2.02	2.25
Gln	2.03	1.96	1.70	1.68	1.40	1.22
Orn	1.22	1.87	1.01	1.58	1.02	1.27
Lys	1.07	1.42	0.95	1.14	0.71	0.67
His	1.68	1.65	1.17	0.83	2.93	3.17
Trp	1.10	0.84	1.20	0.86	1.52	1.05

derivatization reaction and have provided the basis for preparing a variety of derivatives that can be assessed for optimizing the analysis of amino acids by GLC with FID or by GC–MS. Discovering the influence of the alcohol on the chloroformate reaction in an aqueous medium opens the possibility for preparing a wide variety of ester derivatives that can be tailored to the analytical needs of a specific problem. A variation of the chemical procedure reported here to prepare perfluorinated derivatives with chloroformate–alcohol reagents is being undertaken to facilitate analyses by electron capture negative ionization mass spectrometry.

5. Acknowledgements

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