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RAPID DERIVATIZATION AND GAS CHROMATOGRAPHIC ESTIMATION OF DICARBOXYLIC AMINO ACIDS

PETR HUŠEK and VLADIMÍR FELT

Research Institute of Endocrinology, Národní třída 8, 116 94 Prague 1 (Czechoslovakia)

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SUMMARY

A simple procedure was devised for derivatization of the second carboxyl group in the molecule of dicarboxylic amino acids converted to 2,2-bis(chlorodifluoromethyl)-1,3-oxazolidinones in a preceding reaction stage. After condensation of the amino acids with 1,3-dichlorotetrafluoroacetone under catalytic influence of pyridine, the ketone was treated with a small amount of methanol, and the immediately formed halogenated alcohol esterified the second carboxyl group after a reactive anhydride such as heptafluorobutyric anhydride or trifluoroacetic anhydride was added. Factors influencing the reaction yields of a homologous series of dicarboxylic amino acids, with four to seven carbon atoms, were studied and the possible side-product pathways discussed. A simple extraction procedure was devised to obtain the derivatives free of reactants in organic solvent. Molar responses relative to phenyl-alanine (internal standard) are given and the gas chromatographic analysis on a methyl silicone phase is demonstrated.

INTRODUCTION

Condensation of amino acids with 1,3-dichlorotetrafluoroacetone (DCTFA) was reported[1,2] to proceed readily under the catalytic influence of pyridine, and the cyclic derivatives, formed by ring closure involving the α-amino and carboxyl group, exert good chromatographic properties[3]. Moreover, this derivatization method enables the remaining polar groups to be treated with reactive anhydrides, such as heptafluorobutyric anhydride (HFBA) or trifluoracetic anhydride (TFAA), directly in the condensation medium. When applied to the derivatization of the protein amino acids there arises the problem of how to treat the second carboxyl group in molecules of ASP and GLU. Employment of additional derivatization reagents would be impractical. Brooks *et al.*[4] reported recently the simultaneous esterification of carboxyl and hydroxyl groups with alcohol and HFBA for the analysis of hydroxy-acids by gas chromatography. A reagent mixture consisting of HFBA, pyridine and ethanol was used to esterify carboxyl groups with the alcohol and to derivatize hydroxyl and amine groups with excess of the anhydride. HFBA catalyzed esterification of carboxyl

groups and pyridine catalyzed derivatization of carboxyl, hydroxyl and amine groups. Good results were obtained in a few minutes without heating the sample.

In the present study we examined the possibility of using this approach in derivatization of the second carboxyl group in the molecules of just-condensed di-carboxylic amino acids. In order to obtain more definitive results, the two higher homologues with 6 and 7 carbon atoms, the α-aminoadipic (AAA) and α-amino-pimelic (APA) acids, were treated together with ASP and GLU. The second reaction step was carried out directly in the condensation medium so that the reaction conditions were already partially determined. The presence of DCTFA in the medium was found to be substantial since any addition of alcohol results in immediate formation of alcohol–DCTFA adduct:

$$(CF_2Cl)_2C=O + R-OH \rightarrow (CF_2Cl)_2C{\Large\begin{matrix} \diagup O-R \\ \diagdown OH \end{matrix}}$$
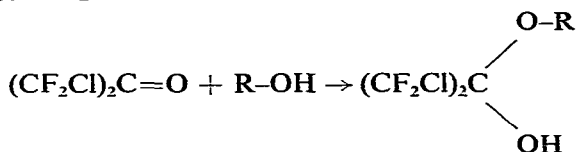
The formed alkoxy alcohol with a secondary hydroxyl group then acts as the active esterification agent of the second carboxyl group after a reactive anhydride such as HFBA or TFAA is added. The influences of medium polarity, pyridine, DCTFA, alcohol and anhydride amount on the reaction yield were studied, and the exact reaction conditions were determined. The derivatized compounds were taken, after a simple extraction procedure, to gas chromatographic analysis on OV-101 methyl silicone and molar responses relative to PHE were estimated. The method was found to be perfectly suitable for rapid analysis of the dicarboxylic amino acids.

EXPERIMENTAL

Reagents and chemicals

1,3-Dichlorotetrafluoroacetone was obtained from Fluka (Buchs, Switzerland), the heptafluorobutyric and trifluoroacetic anhydrides were purchased from Pierce Eurochemie (Rotterdam, The Netherlands). All the reactive and moisture-sensitive reagents were placed in appropriate 1–5-ml glass vials, capped with Mininert valves (Pierce) and kept at room temperature. When signs of deterioration occurred, the reagents were treated with phosphorus pentoxide and distilled. Methanol, absolute ethanol, isopropanol and all organic reagents (light petroleum 40–60°, dichloro-methane, acetonitrile, pyridine, benzene, hexane, tetrachloromethane) were of p.a. quality and obtained from Lachema (Brno, Czechoslovakia) or E. Merck (Darm-stadt, G.F.R.).

DL-Aspartic acid, L-glutamic acid and L-phenylalanine, all of grade A quality, were purchased from Calbiochem (Lucerne, Switzerland). DL-α-Aminoadipic acid and 2-aminopimelic acid were obtained from Serva (Heidelberg, G.F.R.) and Fluka, respectively. Equimolar amounts of all mentioned amino acids were dissolved in water, 2 M ammonia or 1 M HCl, the concentrations being between 10–100 nmoles of each amino acid in 10 μl of water medium.

Aqueous solutions (1 M) of sodium carbonate (10.6 g/100 ml), sodium bi-carbonate (8.4 g/100 ml) and hydrochloric acid were prepared from chemicals of p.a.

quality. Anhydrous sodium sulfate, used for the drying of the organic layer after extraction of derivatives, was of p.a. grade.

Glassware, apparatus and chromatographic conditions

Reaction vials employed for derivatization and subsequent extraction of the derivatives were made from glass tubes 10 mm O.D. with ground glass joints 10/14 or 15 mm. The bases were rounded and the vials were approximately 40 mm high to provide a capacity of 2 ml. Solid glass stoppers or bottom-closed hollow stoppers with 10/14 joints were selected.

A Hewlett-Packard 5730 A gas chromatograph connected to a computing integrator 3380 A was employed for linear temperature-programmed analysis in the range 150–230° (rate 8°/min) using flame ionization detection. The temperatures of the detector and the injection port were set to 250 and 200°. A glass column 1.5 m × 3 mm I.D. with 3% OV-101 on Supelcoport (80–100 mesh) was used, and the carrier gas flow-rate (nitrogen) was set to 40 ml/min. The corresponding rates for hydrogen and air were 40 and 240 ml/min. The attenuation was set at $4 \cdot 10^{-10}$ A when 0.5 nmoles (2.5 to 3.0 μl) of each amino acid in the mixture were injected. The total time of the C_4–C_8 dicarboxylic amino acid analysis was 10 min.

Derivatization studies

An equimolar mixture of the dicarboxylic amino acids and phenylalanine was treated with DCTFA in the presence of acetonitrile (alone or in mixture with benzene)
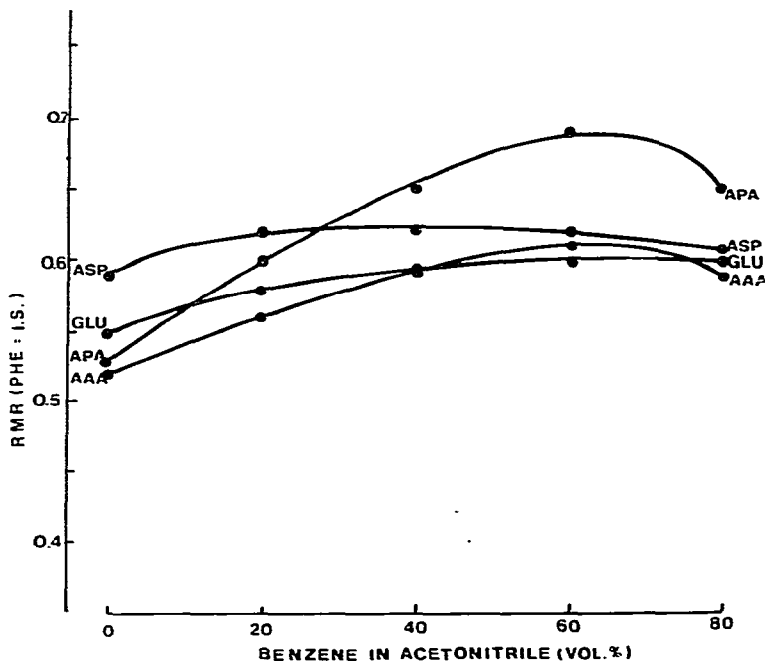

Fig. 1. Effect of medium polarity on *RMR* values. Five solvents consisting of 0, 20, 40, 60 and 80% (v/v) of benzene in acetonitrile were prepared and 100 μl of the appropriate solvent were taken to condensation with 20 μl DCTFA and 8 μl of pyridine. In the following step 4 μl of methanol and 12 μl HFBA were added to each vial.
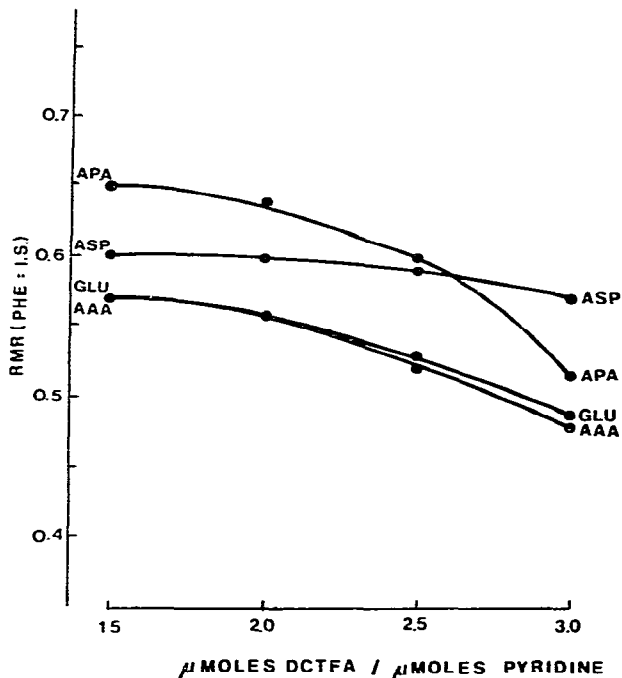
Fig. 2. Effect of DCTFA–pyridine ratio on *RMR* values. The amount of DCTFA in the reaction medium (60 μl benzene, 40 μl acetonitrile and 6 μl pyridine) was altered from 15 to 20, 25 and 30 μl. After condensation, 3 μl of methanol and 9 μl HFBA were added to each vial.
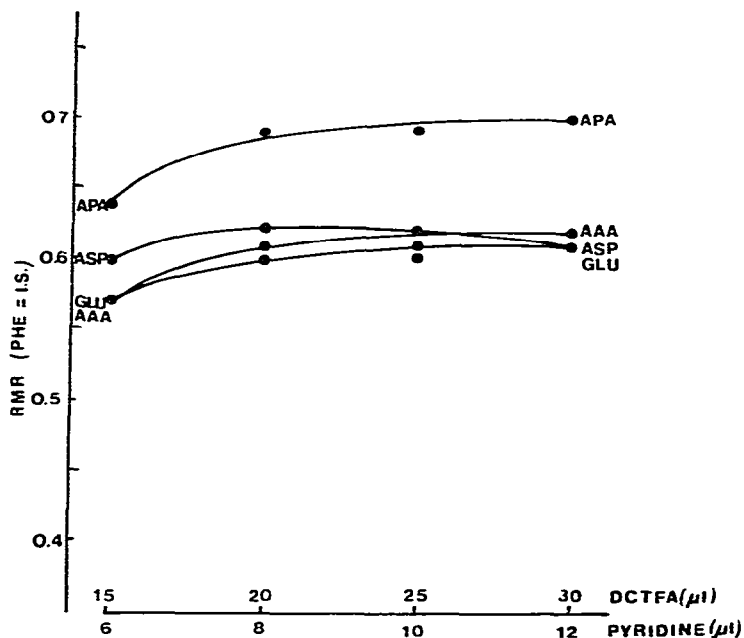

Fig. 3. Effect of proportional increase of pyridine and DCTFA in the reaction medium on *RMR* values. Solvent amount was held constant (60 μl benzene plus 40 μl acetonitrile) whereas amount of pyridine and DCTFA in four samples was proportionally increased: 6, 8, 10 and 12 μl of pyridine were mixed with 15, 20, 25 and 30 μl DCTFA. After the condensation step 3, 4, 5 and 6 μl of methanol followed by 9, 12, 15 and 18 μl HFBA were added.

as the solvent and pyridine as the catalyzer. The condensation was carried out at 40°
and was completed after 5 min. As the derivatization yields were found to be influenced
by medium polarity as well as the amounts of DCTFA and pyridine in the medium, the
effects of these factors were studied by changing the concentration of reactants in the
medium and diminishing its polarity by partial substitution of acetonitrile with
benzene. (For details see the legends to Figs. 1–3).

  The second carboxyl group in the amino acid side chain was esterified by
simple addition of methanol followed by HFBA or TFAA into the medium. The
sample was left at room temperature for at least 10 sec before it was submitted to the
extraction procedure (see the following section). The reaction yields were found to be
influenced strongly by the methanol–pyridine and HFBA–methanol ratios, and to a
lesser extent, by an increase in the amount of benzene in the medium before esterifi-
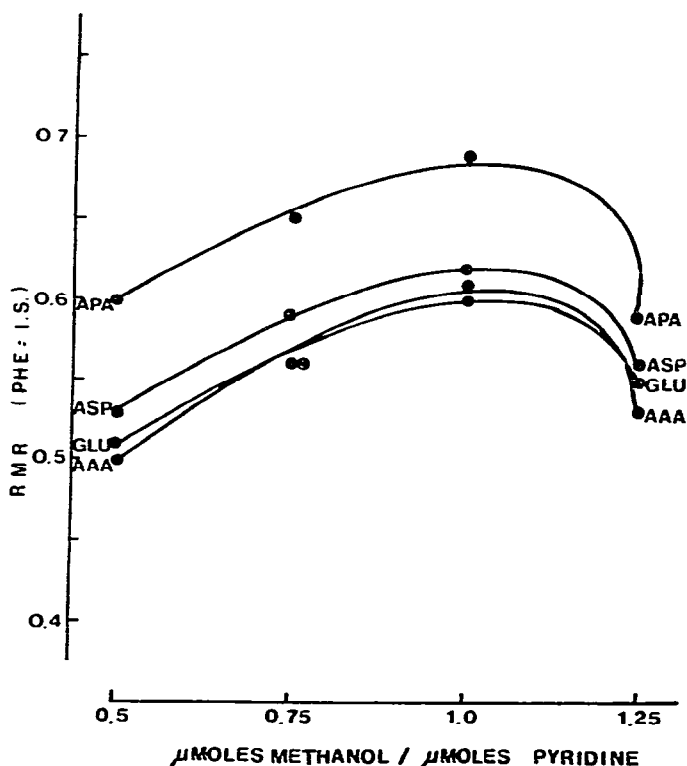cation (see legends to Figs. 4–6).

Fig. 4. Effect of methanol–pyridine ratio on *RMR* values. Condensation was carried out in a medium
consisting of 60 μl benzene, 40 μl acetonitrile and 8 μl pyridine in presence of 25 μl DCTFA. In the
subsequent step 2, 3, 4 and 5 μl of methanol followed by 6, 9, 12 and 15 μl HFBA were added.

Recommended procedure

  From 10 to 100 nmoles of each amino acid in equimolar mixture with the
others in aqueous solution (free, ammonia or hydrochloride form) were taken to
dryness at 70° under a stream of air or nitrogen, traces of water being removed azeo-
tropically with dichloromethane. The residue was treated with 100 μl of mixed
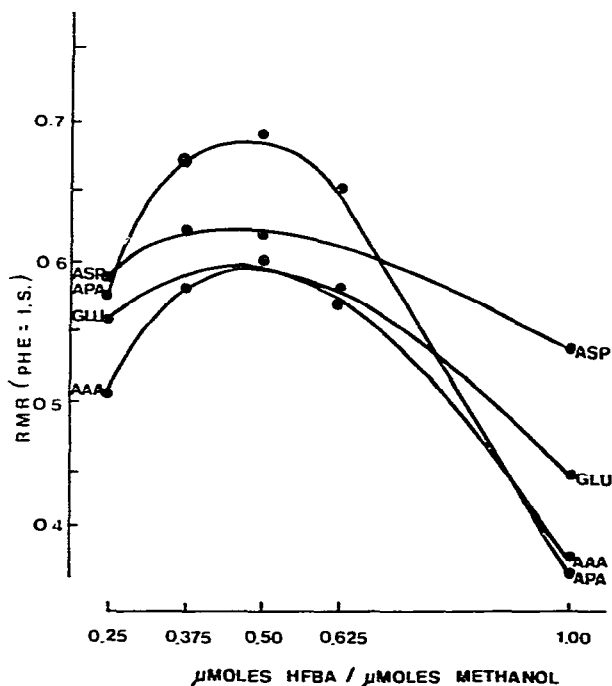
Fig. 5. Effect of HFBA–methanol ratio on *RMR* values. To the condensation stage 20 μl DCTFA and 60 % (v/v) of benzene in acetonitrile together with 8 μl of pyridine were added. After the first reaction step 4 μl of methanol were added to each vial followed by 6, 9, 12, 15 and 24 μl of HFBA.

solvent consisting of benzene, acetonitrile and pyridine in volume ratio 60:32:8 and 20 μl DCTFA. After the sample was held (stoppered) at 40° for 5 min 4 μl of methanol alone or in mixture with benzene (20 μl of 1:4 or 40 μl of 1:9, v/v) were added. The ketone which condensed on the walls was rinsed by shaking, and 12 μl HFBA (or 7 μl TFAA) were introduced into the reaction tube. The tube was left open and after at least 10 sec, 500 μl of extraction medium (20 %, v/v, of dichloromethane in light petroleum) were added. The organic phase was shaken (10–15 sec) against 400 μl each of 1 *M* aqueous solution of sodium carbonate, 1 *M* hydrochloric acid (twice) and 1 *M* sodium bicarbonate. The water phase was always removed with a Pasteur pipet joined to a microscrew, and the organic layer was finally desiccated with anhydrous sodium sulfate. Before injection into the chromatographic column the sample was reduced in volume, or better, while leaving the sulfate, the organic phase was transferred into another vial. The original tube was then washed with 0.2 ml of extraction medium and the combined extracts were evaporated just to dryness at room temperature using a current of air or nitrogen (excessive blowing of gas into the dry residue may cause losses, especially of phenylalanine). The residue was dissolved in hexane or tetrachloromethane (100 μl) and an aliquot was taken for gas chromatographic analysis.

Fig. 6. Effect on *RMR* values of further benzene addition to the medium, before the esterification with HFBA. Condensation was performed with the same medium as in Fig. 5. Before addition of 12 µl HFBA to each reaction vial the condensation medium was treated with a mixture of methanol and benzene in ratio 1:1 (8 µl), 1:4 (20 µl), 1:9 (40 µl) and 1:19 (80 µl).

RESULTS AND DISCUSSION

Preliminary experiments performed on the polyfunctional amino acids revealed that employment of reactive perfluorated anhydrides is the best way to treat polar grouping in the side chain of amino acid oxazolidinones[1]. The derivatization can be performed directly in the condensation medium and the interfering pyridine salts can be removed by simple extraction, while leaving the derivatives in a volatile organic solvent, without deterioration. However, when oxazolidinones of ASP and GLU are treated with HFBA or TFAA the results are unsatisfactory. This is apparent in Fig. 7 where ASP produces a very small peak (retention time 2.09), GLU gave two peaks (t_R 3.27 and 3.61, the latter being co-eluted with PHE) and the higher retention times correspond to main and side-products of the two higher homologues. It seems probable that dicarboxylic amino acids with 5–7 carbon atoms in the molecule form bicyclic derivatives, in which the second carboxyl group is attached to the oxazolidinone ring due to the strong dehydratative action of HFBA or TFAA:
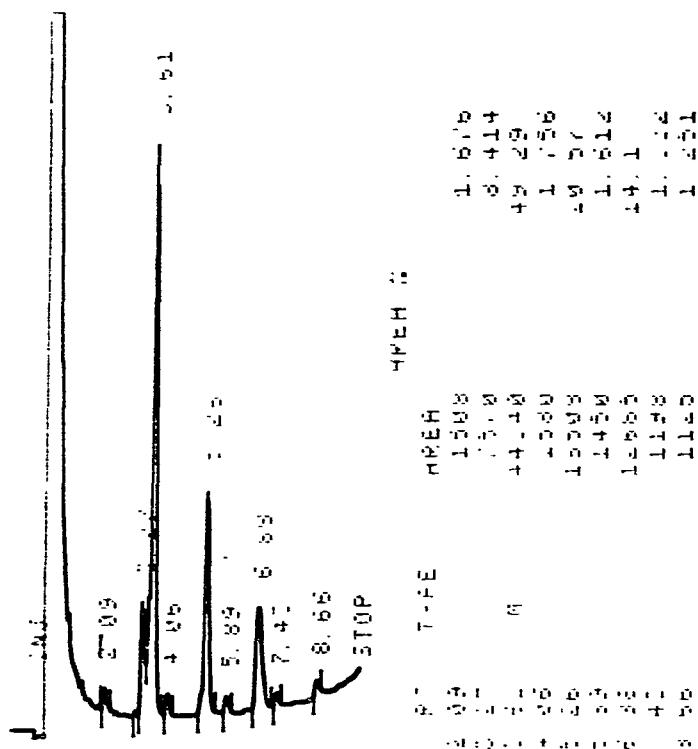
Fig. 7. Mixture of C_4 to C_7 dicarboxylic amino acids and phenylalanine (t_R 3.61 min) condensed according to *Recommended procedure* and treated with either HFBA or TFAA (10 μl) without prior alcohol addition. The derivatives were extracted and analyzed as given in the text.

This also explains why the main products have the same retention times regardless of the anhydride used. In comparison, ASP produces no peak, as the formation of a 4-membered ring is energetically improbable. It is interesting to note that the condensation medium turns red after HFBA or TFAA addition when larger amounts of ASP are present. As an alternative to the above process it is possible for acylation of the adduct of DCTFA with the second carboxyl group to occur along another pathway:
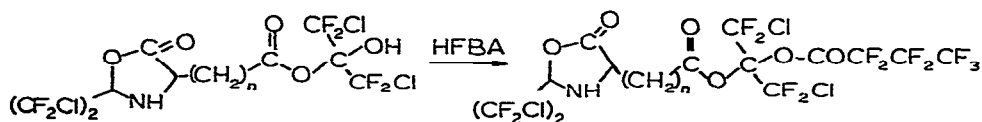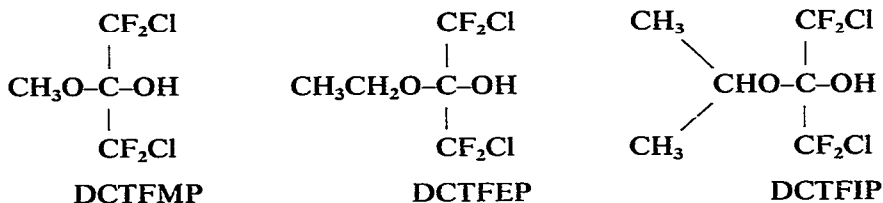
At a later stage, it is intended to explain this side-product formation, using mass spectrometry.

Addition of a lower aliphatic alcohol to the condensation medium just before the anhydride addition alters the results substantially. Methanol, ethanol and isopropanol were tested as potential esterification agents. They act indirectly, as already mentioned, and after addition of the appropriate alcohol into the medium, DCTFA is immediately converted to 1,3-dichloro-1,1,3,3-tetrafluoro-2-alkoxy-propan-2-ol so

that methoxy (M)-, ethoxy (E)- or isopropyloxy (I) groups will be attached to the second carbon atom:

$$\underset{\text{DCTFMP}}{\overset{\displaystyle CF_2Cl}{\underset{\displaystyle CF_2Cl}{|}}\text{CH}_3\text{O–C–OH}|}$$

$$\underset{\text{DCTFEP}}{\overset{\displaystyle CF_2Cl}{\underset{\displaystyle CF_2Cl}{|}}\text{CH}_3\text{CH}_2\text{O–C–OH}|}$$

$$\underset{\text{DCTFIP}}{\text{CH}_3\text{CHO–C–OH}}$$

The corresponding halogenated alkoxy-alcohol does esterify the second carboxyl group in the presence of pyridine and added anhydride. Figs. 8 and 9 and Table I show that HFBA gives slightly higher and more consistent results than TFAA (Fig. 8B, note presence of small interfering peaks) and that except for ASP the esterification of the higher homologues was better with DCTFMP than with the ethanol- and isopropanol-DCTFA adducts. However, the molar responses from ASP to APA do not apparently agree with their carbon numbers. The higher value found for ASP could be explained by the fact that formation of the bicyclic side-product in this case is highly improbable.
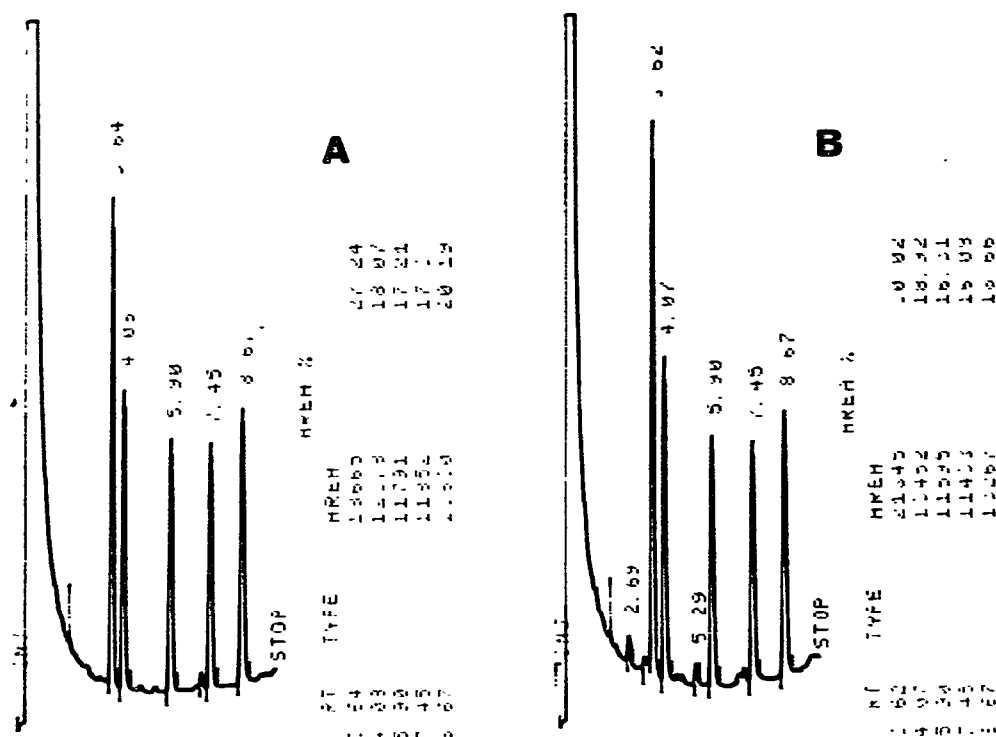

Fig. 8. Analysis of the mentioned amino acid mixture prepared according to Recommended procedure. (A) HFBA treatment, (B) TFAA treatment. The first peak is PHE followed by 4 peaks of C_4 to C_7 homologues.
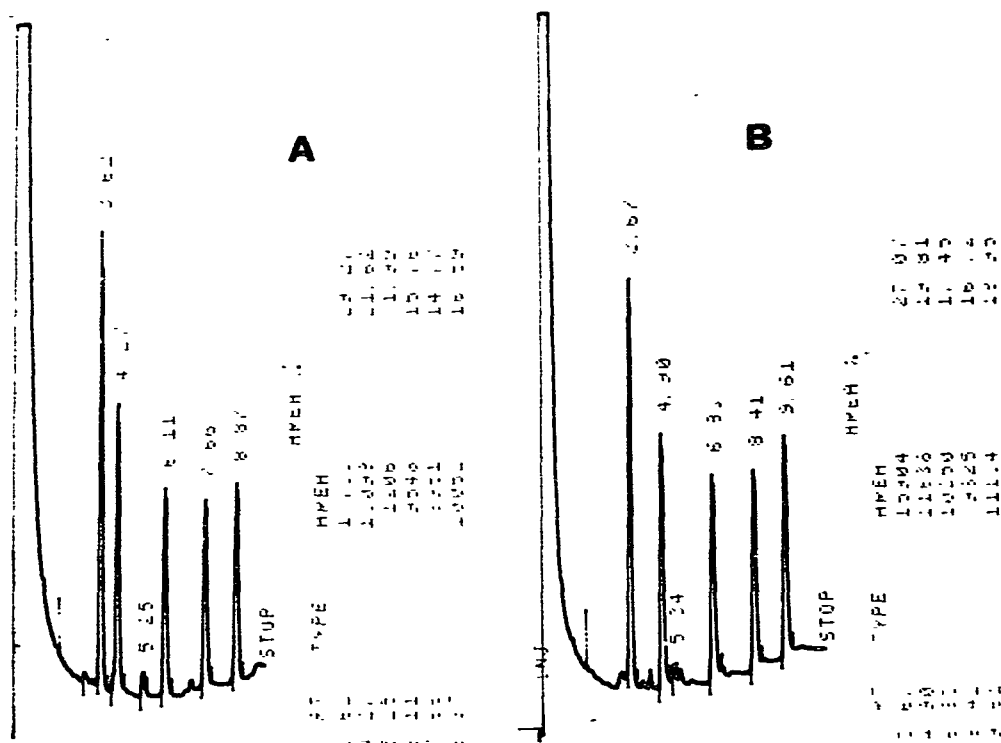
Fig. 9. Amino acids prepared and analyzed according to *Recommended procedure* with one exception: instead of 4 µl methanol, 5.8 µl of absolute ethanol (A) or 7.5 µl of isopropanol (B), *i.e.* 100 µmoles of each alcohol were added before the HFBA addition (12 µl). The corresponding derivatives exert proportionally longer retention times.

The optimal reaction conditions described in the section *Recommended procedure* result from experimental studies aimed at finding the highest reaction yields. The factors influencing esterification yields were studied separately and the results of the experiments are given graphically in Figs. 1–6. Methanol and HFBA were taken for all the studies. The medium polarity was found to influence reaction yields, the effect being more pronounced with AAA and APA (Fig. 1). The best values were obtained

TABLE I

RELATIVE MOLAR RESPONSES (PHENYLALANINE = INTERNAL STANDARD) OF C_4–C_7 DICARBOXYLIC AMINO ACIDS

Esterification with methanol-, ethanol- or isopropanol-DCTFA adduct under catalytic influence of HFBA or TFAA. (In case of methanol and HFBA treatment values are the average from 15 experiments so that standard deviations are given.)

Treatment		RMR value			
Alcohol	Anhydride	ASP	GLU	AAA	APA
Methanol	HFBA	0.63 ± 0.02	0.60 ± 0.02	0.61 ± 0.02	0.69 ± 0.04
	TFAA	0.60	0.56	0.54	0.64
Ethanol	HFBA	0.74	0.54	0.51	0.57
Isopropanol	HFBA	0.73	0.64	0.62	0.70

with a benzene–acetonitrile volume ratio 3:2. Whether the condensation proceeds in acetonitrile only or in its mixture with benzene is unimportant. The only decisive factor is the medium composition at the moment of anhydride addition. Excessively low ($<$ 1) or high ($>$ 3) values of benzene–acetonitrile volume ratio (see Fig. 6) result in diminution of the relative molar response (RMR) values even for the less susceptible amino acids, ASP and GLU.

The molar amounts of DCTFA and pyridine should be close together (Fig. 2). However, as the molar amount of methanol added subsequently corresponds with that of pyridine, the molar amount of DCTFA chosen must be a little higher (preferably 1.5 to 2.0). The reason for this is the fact that, during condensation, a certain amount of DCTFA evaporates. If a molar amount of alcohol equal to the original amount of DCTFA is added, excess of alcohol occurs and partially esterifies the second carboxyl group (Fig. 10). Thus, the molar ratio of DCTFA to added alcohol should also be near the value 1.5.
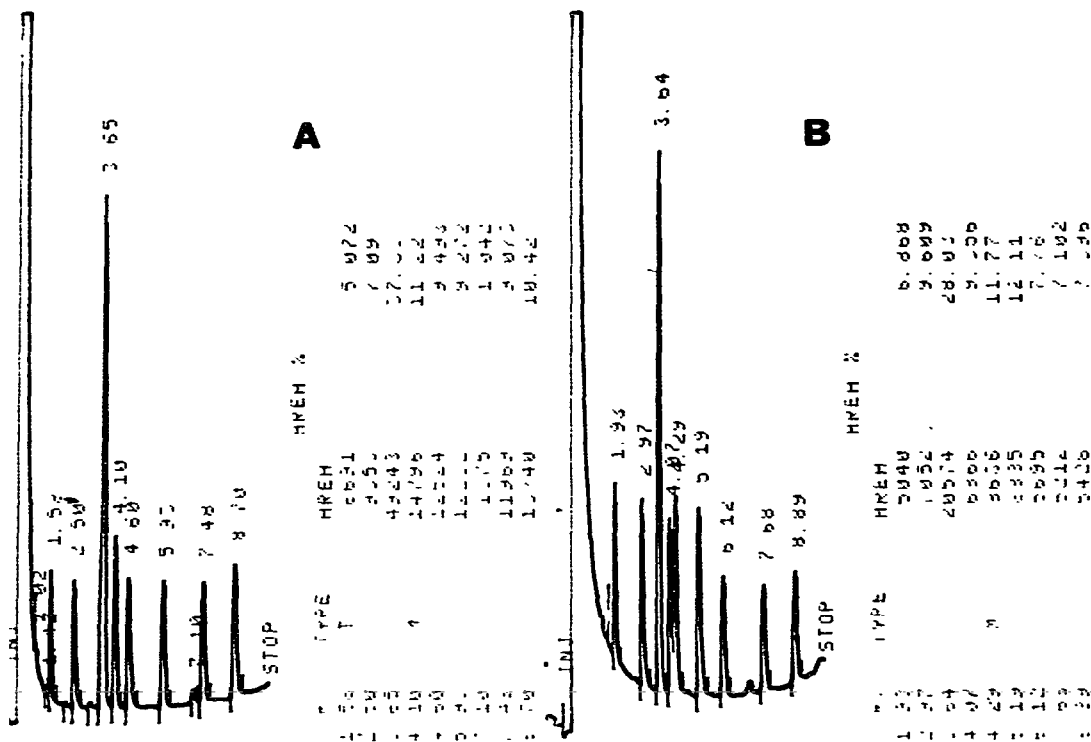

Fig. 10. Amino acid mixture was condensed as usual, however, the medium was subsequently treated with excess of alcohol (150 μmoles of alcohol were added into medium with 150 μmoles DCTFA and 150 μmoles of pyridine) followed by 50 μmoles HFBA. As well as the expected peaks, products of direct esterification of the second carboxyl group with methanol (A) or ethanol (B) are present (lower retention times).

Fig. 3 indicates that excessive amounts of pyridine and DCTFA in the solvent are unnecessary. Except for 75 μmoles of pyridine (6 μl) in the medium, which resulted in yields approximately 10% lower, the 100 μmoles and higher pyridine amounts

produced the same values. From consideration of Figs. 1–3 it can be concluded that 8 μl (100 μmoles) of pyridine together with 20 μl (150 μmoles) DCTFA in a solvent with an approximate benzene to acetonitrile ratio of 2 is the best reaction medium with regard to the subsequent esterification step.

The reaction yields were found, however, to be dependent mostly on the ratios of methanol to pyridine (Fig. 4) and anhydride to methanol (Fig. 5). Excess or lack of pyridine in relation to alcohol results in decline of the values. Also, the molar amount of anhydride to methanol (and to pyridine) should be near or equal to 0.5. Any heating of the sample or additional HFBA application, provided that the interval between first and second HFBA addition is at least 10 sec, no longer affects the values. Finally, it can be concluded that the best results are obtained when alcohol-pyridine-anhydride are in molar ratios 1:1:0.5. In case of methanol and HFBA it means a volume ratio 1:2:3, and with TFAA, 4:8:7.

The use of other organic solvents, e.g. dichloromethane or chloroform, to replace benzene in the mixture with acetonitrile, leads to diminution of the reaction yields to about 90% or 80%, respectively. The presence of acetonitrile in the medium is essential for good dissolution of the amino acids in the medium with DCTFA. The reaction yields were independent of whether the amino acids existed in free form or as a salt (NH_4 or HCl). Conversely, the derivatized compounds proved to be soluble even in organic solvents of low polarity such as hexane or tetrachloromethane.

The described extraction procedure was elaborated with respect to derivatization and gas chromatographic estimation of the protein amino acids converted to oxazolidinones. Thus, it will be discussed in more detail later.

The presented method of dicarboxylic amino acid analysis by gas chromatography seems to be superior to the procedures previously published[5] especially with regard to simplicity and rapidity. Finally, this derivatization technique enables the dicarboxylic amino acids and their amides, asparagine and glutamine, to be distinguished[6], a fact which may prove important from a number of aspects.

ACKNOWLEDGEMENTS

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