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GAS CHROMATOGRAPHY OF CYCLIC AMINO ACID DERIVATIVES

A USEFUL ALTERNATIVE TO ESTERIFICATION PROCEDURES

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SUMMARY

The use of 1,3-dichlorotetrafluoroacetone in combination with reactive anhydrides such as heptafluorobutyric anhydride provides a useful, quick and sensitive method of analysing the twenty protein amino acids by gas chromatography. The chemical treatment proceeds under very mild conditions without the necessity of heat so that both amides, glutamine and asparagine, are preserved and amenable to chromatographic estimation. An ordinary column (2 m) packed with OV-17 stationary phase gave excellent separations of the derivatized compounds in the temperature range of 75–230°C within 20 min. A second short column is required for complete elution of histidine, tryptophan and cystine.

INTRODUCTION

A considerable number of papers dealing with gas chromatographic (GC) or high-performance liquid chromatographic (HPLC) separation of amino acids have appeared over the last few years. While the popularity of GC analysis apparently reached its peak in the last decade, when the numerous previous investigations[1] led to several successful alternative procedures[2–4], in the 1980's the emphasis seems to be turning toward the HPLC technique, with which it is possible to analyse amino acids in their free forms[5–7]. However, with the exception of classical ion-exchange chromatography[8,9], which enables only a moderate acceleration of the separation process because of the limited compressibility of the column packing, and, perhaps, ligand-exchange chromatography[7], the most frequently used alkylated silica supports (reversed-phase, RP) are not so suitable for the separation of highly polar compounds. Thus, in HPLC, as in GC, complex mixtures of amino acids are often separated after conversion of the substances into one of several derivatives.

Precolumn derivatization reduces the chromatographic analysis time to about one-third of that required for ion-exchange amino acid analyzers and provides a greater sensitivity than postcolumn ninhydrin detection, especially if fluorescent compounds are formed. Reversed-phase HPLC is presently the most versatile method of protein sequencing, as the strongly UV-absorbing phenylthiohydantoin (PTH) deriv-

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atives of all protein amino acids can be resolved in a single chromatographic run[10–15] and without additional chemical treatment as it is necessary in the case of GC estimation[1]. However, the lengthy procedure required for the preparation of the PTH derivatives limits their usefulness for routine amino acid analysis.

Two other derivatization procedures may be employed for this purpose, *i.e.*, dansylation[16–18] (or dabsylation[19]) and treatment with *o*-phthaldialdehyde[20–22], a reaction which proceeds very rapidly (2–3 min) in aqueous solution. Strongly fluorescent derivatives are formed in each case and the reversed-phase HPLC separation can be achieved within 30 min. The short analysis time is comparable with present GC methods, but the quality of separation seems to be sacrificed for the high rate of analysis. Moreover, interferences occur due to fluorescent side-products[23], and the imino acids, proline and hydroxyproline, cannot be estimated with the second procedure.

As regards the GC estimation, the higher requirements of the derivatization process often prove to be the Achilles heel of this approach[1]. Whereas in the HPLC analysis not all the reactive groups in the amino acid molecule need to be masked, a complete derivatization is required for satisfactory GC analysis. Nearly 100 chemical treatments have been proposed for this purpose since 1956, when GC was first introduced for amino acid analysis[1]. The N(O,S)-perfluoroacyl amino acid alkyl esters have proved to be the most convenient derivatives enabling the quantitation of all twenty protein amino acids. This is essentially the result of the remarkable work of Gehrke and co-workers[2,24–26], who succeeded with the N(O,S)-trifluoroacetyl *n*-butyl esters first introduced by Zomzely *et al.*[27]. However, certain difficulties were encountered in the determination of histidine[26,28,29], so other procedures were developed with more stable derivatives[3,30,31]. One of the most popular volatile amino acid derivatives is the N(O,S)-heptafluorobutyryl isobutyl ester introduced by MacKenzie and Tenaschuk in 1974[4], which has extensively been studied[32–36] and has recently been used in biomedical applications[37,38].

Progress in capillary technology and especially in the synthesis of new optically active (chiral) phases[39,40] has resulted in an attractive procedure called enantiomer-labelling: the optical antipode to each L-amino acid serves as an internal standard after conversion of the antipodes into the N(O,S)-pentafluoropropionyl isopropyl esters[41–43]. Another novel approach involves determination of D-amino acids by means of deuterium labelling and selected ion monitoring[44]. Because of the special instrumentation required, these procedures are more expensive alternatives to the earlier ones.

Even when the GC analysis of acylated amino acid alkyl esters became routine some negative aspects of the esterification procedures persisted: (1) two incompatible reaction media with an intermediate evaporation step; (2) degradation of the amides, glutamine and asparagine, due to the HCl-catalyzer; (3) dissolution problems with amino acids in the higher alcohols; (4) employment of high reaction temperatures for both reaction steps; (5) stringent requirements of purity of the particular alcohol[45]. In order to avoid these difficulties, some years ago[46–49] the use of 1,3-dichlorotetrafluoroacetone (DCTFA) as a condensation agent was investigated. The results were promising as even the most problematic (with respect to GC estimation) amino acids, *i.e.*, histidine, tryptophan and cystine, could be analysed by this approach[50], and except of the dicarboxylic amino acids[51] even their amides could be preserved[52]. However,

problems were encountered in generalizing the reaction conditions as the various side-chain reactive groups require different chemical treatments[53]. It is now possible to so modify the conditions that all twenty amino acids can be successfully derivatized and quantitatively analysed within 1 h.

EXPERIMENTAL

Reagents and materials

DCTFA was purchased from Fluka (Buchs, Switzerland), HFBA (heptafluorobutyric anhydride) from Pierce Eurochemie (Rotterdam, The Netherlands) and methyl chloroformate from E. Merck (Darmstadt, G.F.R.). Before and after some months of use, the DCTFA was treated with phosphorus pentoxide and distilled (b.p. 45°C). The stock solutions of the reagents were kept in a refrigerator; when required, small amounts (up to 1 ml) were placed in glass vials capped with Mininert valves (Pierce) and kept at room temperature.

The organic solvents, *i.e.*, acetonitrile, pyridine, benzene, dichloromethane, methanol, heptane and petroleum ether (b.p. 60–80°C and 40–60°C), were obtained in the best available quality from Lachema (Brno, Czechoslovakia) and E. Merck. The petroleum ether was redistilled before use, and the pyridine treated with KOH and distilled after some months of use. The mixed solvents, *i.e.*, acetonitrile–pyridine (5:1), benzene–methanol (24:1) and petroleum ether (b.p. 60–80°C)–dichloromethane–methyl chloroformate (200:100:1), were prepared daily in the required amounts (1–10 ml) and kept in Reactivials with the Mininert-valve closures. For application of reagents and the organic solvents, syringes of 50–1000 μl in volume were employed.

Amino acids of grade A quality were supplied by Calbiochem (Lucerne, Switzerland) and E. Merck. The two internal standards, α-aminocaprylic acid and diaminopimelic acid, were purchased from Sigma (St. Louis, MO, U.S.A.). Equimolar mixtures of all protein amino acids, including hydroxyproline, ornithine and S-methylcysteine, were prepared by dissolving the amino acids in 0.1 *M* HCl or water (asparagine, glutamine and tryptophan) to give 10–100 nmoles of each amino acid in 10 μl of the aqueous medium. Approximately 1 *M* aqueous solutions of sodium carbonate (5 g per 45 ml) and hydrochloric acid (dilution 1:11) were prepared monthly from distilled water and chemicals of p.a. quality.

The round-bottomed reaction vials, (height *ca.* 50 mm, capacity *ca.* 2 ml) employed for derivatizations and subsequent extractions were made from 12 mm O.D. glass tubes with ground glass joints 12.5/15 mm. Solid glass or bottom-closed stoppers were used. The reaction tubes were silanized by a treatment with dichlorodimethylsilane in toluene (1:10) for 30 min, and washed subsequently with methanol and acetone.

GC

Commercial low-bleed silicone septa were employed. The bleeding was suppressed further by refluxing in acetone for 6 h and subsequent conditioning at 140°C in a chromatographic oven for 60 h (over the weekend).

The following chromatographic materials were used for the analyses: silanized diatomaceous earth supports, Chromosorb W and G of HP or AW DMCS grade (80–100 mesh); polyorganosiloxane phases, *i.e.*, the methylphenylsilicone fluid OV-17

or SP-2250 and the methylsilicone gum SE-30 or OV-1. These materials were obtained from Applied Science Europe (Oud-Beijerland, The Netherlands) and Supelco (Crans, Switzerland), and coating of a particular support with a phase was performed by the usual rotary evaporator method. The coated supports were then placed in the corresponding analytical columns, the column inlets were filled with silanized glass wool (5–6 cm) and the packings were conditioned under nitrogen (15 ml/min) at 300°C (2°/min linear increase from 60°C) overnight while maintaining the injector port temperature at 250°C.

A dual-column dual-FID Hewlett-Packard 5730 A gas chromatograph connected to a computing integrator 3380 A was employed for the linear temperature-programmed analysis (8°/min increase, injector and detector temperatures 200 and 250°C) of the cyclic amino acid derivatives. Two glass analytical columns (2 mm I.D.) of lengths 2 m (A, filled with 3% OV-17 or SP-2250 on Chromosorb W) and 0.5–0.6 m (B, filled with 1.5% SE-30 or OV-1 on Chromosorb G), operating in the temperature ranges 75–230°C (A) and 150–200°C (B) under nitrogen flow-rates of 20 ml/min (measured at 200°C with column A) and 30 ml/min, were required for a successful determination. As an alternative packing for column A, 3% OV-17–OV-22 (3:1) on Supelcoport (80–100 mesh) can be used. When used as a combustion suporter for the hydrogen flame (30 ml/min), oxygen (60 ml/min) was found to afford a doubled response of the halogen-rich molecules as compared with air (240 ml/min).

Procedure

Condensation. To the dry residue of amino acids (less than 0.3–0.4 mg in total), 70 μl of solvent (acetonitrile–pyridine, 5:1) and 30 μl DCTFA were added and the stoppered sample was held at room temperature for 15 min.

Acylation. To the condensation medium, 100 μl of benzene–methanol (24:1) and two 15-μl volumes of HFBA (approximate time interval, 1–2 sec) were added with continuous gentle mixing. The sample was left to stand for at least 30 sec.

Extraction. A 500-μl volume of extraction medium [petroleum ether (b.p. 60–80°C)–dichloromethane–methyl chloroformate, 200:100:1] followed by 400 μl of 1 *M* sodium carbonate solution were added to the sample and the mixture was shaken until the white precipitation in the organic phase had disappeared (usually after 5–10 sec). The lower aqueous solution was then removed with a Pasteur pipette joined to a microscrew on a fixed stand, and the remaining solution was shaken with 400 μl of 1 *M* hydrochloric acid until the opalescent organic phase had become clear (10–15 sec) and, finally, with 400 μl water. After removal of the aqueous layer, the organic extract was transferred into another vial and reduced in volume to a small drop (10–20 μl) at room or slightly elevated (less than 35°C) temperature using a current of nitrogen (slight moving of the surface of the liquid was decisive for setting of the gas flow). The last drop was then evaporated by manually rotating the vial along its longitudinal axis, followed by addition and evaporation of a small drop (20–30 μl) of light petroleum. This process is necessary and critical, because losses of the simplest amino acids occur if the organic extract is evaporated completely under the gas stream.

Additive acylation. To the residue of the amino acid derivatives were added 50–100 μl *n*-heptane and an aliquot was taken for GC analysis on column B. A drop of HFBA (3–5 μl) was then added to the sample and after heating at 80°C for 2–3 min an aliquot (2–3 μl) was injected on column A.

Comments on the procedure

(1) An effective extraction can be achieved by quickly striking (5–7 times per sec) the tube bottom against a pad on a table. The tubes should be closed tightly. The aqueous phases should be completely removed with the Pasteur pipette, even if a drop of the organic phase is also taken.

(2) The organic extract must be poured into another tube as a thin layer of water firmly adheres to the walls of the reaction tube. Before doing this the ground glass joint should be wiped with a piece of cotton-wool.

(3) Evaporation should be followed visually and carefully at the end. The vial was placed on a thin pile of soft papers on the surface of a sand-bath heated to 80°C. Cooling of the vial, caused by the rapid evaporation of the dichloromethane, was then compensated for by a slight temperature elevation (about 30–35°C) at the bottom of the vial.

(4) If a drop of water appears in the tube after evaporation of the organic phase it should be removed, before the heptane addition, by contacting it with a piece of blotting-paper.

(5) The silanized reaction tubes can be used continuously, provided they are washed with acetone after each derivatization, for 2–3 weeks. If during extraction some drops of water adhere to the walls, the silanization should be repeated.

RESULTS AND DISCUSSION

The separation of the protein amino acids, along with hydroxyproline, S-methylcysteine and ornithine and two internal standards, is shown in Fig. 1. As in previous cases[50,53], three protein amino acids cannot be eluted from column A because of their strong adsorption to the column packing. Another type of column material, together with a shorter column resulted in the successful analysis of these compounds[50]. Since that time, Chromosorb G has been found to be a more convenient support material because even the N^{im}-methoxycarbonyloxazolidinone of histidine could be eluted completly from column B. Thus, this column packing is recommended for analysis of histidine, tryptophan and cystine oxazolidinones.

The analysis of all the other protein amino acids was achieved on 2-m column with a milder polar silicone phase. Chromosorb W HP proved to be the best of the three high quality diatomaceous supports tested (plus Supelcoport and Gas-Chrom Q), as mere coating with the OV-17 phase afforded a satisfactory resolution of the pair proline–hydroxyproline. If Supelcoport is used instead of Chromosorb, an enhancement of the phase polarity (addition of OV-22) is required as stated in the Experimental. Broadening of peaks occurred when Gas-Chrom Q was used.

The influence of phase polarity on the retention behaviour of the derivatives is shown in Fig. 2. Even on the least polar methylsilicone phase (OV-101) the pair leucine–isoleucine is separated completely, while threonine–serine (together with leucine) and some other amino acids are co-eluted. A high-resolution capillary column could be helpful in separating these compounds. As the phenyl group content of the phase increases the separation of alanine–glycine and threonine–serine improves and all the hydroxyl-containing amino acids, together with aspartic acid, exhibit an almost linear decrease in retention times. The best separation of all the compounds is achieved on the OV-17 phase; an improved separation of proline–hydroxyproline is possible by mixing with OV-22, as recommended for Supelcoport.

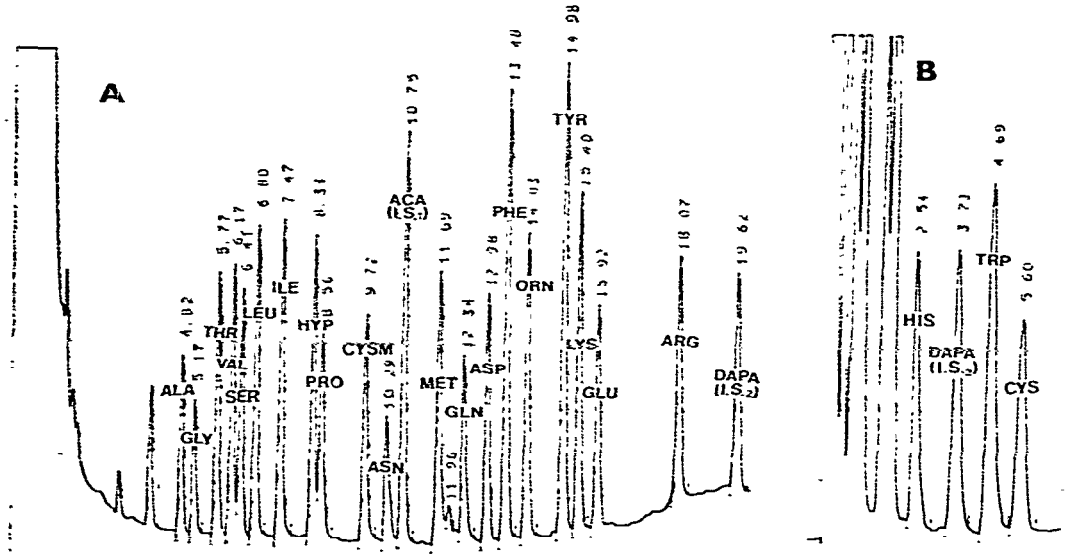


Fig. 1. GC analysis of (N,O)-heptafluorobutyryl amino acid oxazolidinones after derivatization of an equimolar mixture containing 50 nmoles of each amino acid. The brackets around N,O mean that only the reactive side-chain groups are acylated. Temperature ranges: 75–230°C for column A [2 m, 3% OV-17 on Chromosorb W HP (80–100 mesh)] and 150–200°C for column B [0.5 m, 1.5% SE-30 on Chromosorb G HP (80–100 mesh)]. Each peak represents 1 nmole of amino acid. Attenuation: 4×10^{-10} A. ACA = ε-aminocaprylic acid; DAPA = diaminopimelic acid.

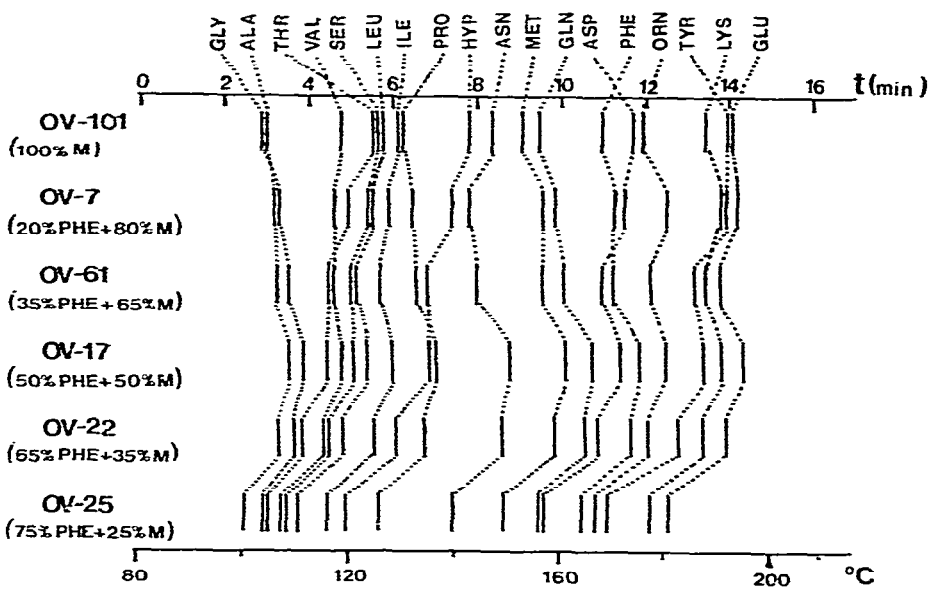
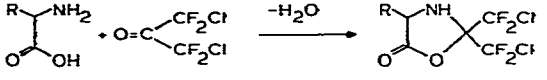


Fig. 2. Retention behaviour of (N,O)-HFB oxazolidinones of eighteen amino acids in a column (2 m × 2 mm) packed with 3% of the particular phase on Chromosorb W HP (80–100 mesh). Temperature range: 80–240°C (8 °/min). Carrier gas flow-rate: 25 ml/min. PHE = phenyl; M = methyl.

The retention behaviour of threonine and serine in the analytical column A is influenced by the carrier gas flow-rate and initial temperature: higher flow-rates result in higher retention times for these two compounds; a higher initial temperature leads to the opposite behaviour. However, the separation of the two imino acids is worse under such conditions, as hydroxyproline exhibits higher retention times and tends to merge in proline.

Instead of HFBA, TFAA (trifluoroacetic anhydride) can be used as the acylation agent. The TFAA-treated oxazolidinones have lower retention times only on the phases with lower contents of phenyl groups, while on OV-17 these derivatives are eluted later than the corresponding HFBA-treated forms[53]. Any of the phases studied enabled a complete separation of the TFAA-treated oxazolidinones, so that the employment of HFBA was preferred in general.

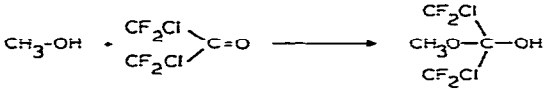
The described procedure is a result of earlier attempts[50–53] to find a chemical treatment that would deal successfully with all of the reactive side-chain groups in a mixture of the protein amino acids. The conversion of free amino acids or their hydrochloride salts into the cyclic oxazolidinones was shown to be dependent on the presence of a convenient condensation medium as the amino acids themselves were not soluble in DCTFA alone. Acetonitrile, in combination with pyridine as a catalyst, proved to be the best medium for this purpose as the presence of the base generally improved the dissolving ability of acetonitrile and the dissolved portion was simultaneously the converted one[46–48]:

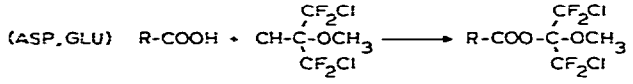


As the rate of dissolution is important, a limited amount of a material should be treated as in the Experimental. The pyridine concentration influences the derivative yields for some amino acids[51,53]. By enhancement of its amount in the solvent, the dissolution of the amino acid residue and cyclization of the two imino acids is more rapid; on the other hand, a progressive yield decline of threonine and serine is accelerated[53]. The selected reaction conditions and condensation time of 15 min represent a compromise in respect of the cyclization of proline: the formation of the cyclic form (retention time 8.56 min) was stimulated even when a small portion remained uncyclized and underwent a second reaction with HFBA to give a side-product (small peak behind methionine with a retention time of 11.96 min, see Fig. 1). Provided the amino acid sample lacks proline, a condensation time of 5 min is sufficient. Oxazolidinones of simple amino acids can be analysed by GC[53] as the polarity of the hydrogen atom in the ring is suppressed by the adjacent halogen atoms to such an extent that this hydrogen cannot be removed even by strong acylating agents[46–48].

A further chemical treatment is required for amino acids with reactive side-chain groups. When only the anhydride was employed, difficulties due to derivatization of the second carboxyl group in the molecules of aspartic and glutamic amino acids were encountered[51]. This problem was solved by addition of a definite amount of methanol to a condensation medium of lower polarity (mixed solvent of benzene in acetonitrile[51]) resulting in conversion of DCTFA into a secondary alcohol, which acts as the specific esterification agent of the additional carboxyl groups. Esterifi-

cation together with acylation of the reactive side-chain groups proceeds readily after addition of the reactive anhdride:

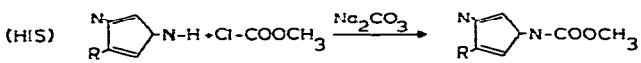




An acylation time of at least 30 sec is required for a full response of glutamine. The two-step HFBA addition improves the results for dicarboxylic amino acids; and the total volume of 30 μl proved to be sufficient for an effective treatment of asparagine. The co-addition of benzene with the alcohol before the HFBA treatment helps to lower the polarity to the extent that the previously recommended combined condensation medium is unnecessary[50–53]. It is a positive factor because acetonitrile alone is the best solvent for the condensation step. Under the chosen acylation conditions the indolyl group of tryptophan is not acylated.

Optimization of the procedure led to a lowering of the amount of methanol added to about 70% of that previously recommended[51,52] in order to get a full response for arginine. Under the specified conditions, the yields were about 10% for the dicarboxylic amino acids, 20% for glutamine and 5–7% for lysine and ornithine lower than the maximal ones[54]. The relative molar responses including the variation coefficients are given in Table I. For comparison with the absolute molar response values see ref. 54. The overall reproducibility averages 4%, the worst cases being glutamine and asparagine (8–9%) and also arginine (6–7%).

Direct evaporation of pyridine with the condensation medium leads to complete loss of the oxazolidinones of most protein amino acids. By means of a convenient extraction medium it was possible to remove the excess of the perhalogenated reagents by shaking with aqueous carbonate solution, and the pyridine salts by shaking with hydrochloric acid, while retaining the derivatives in an organic phase volatile enough to prevent evaporation losses of the simplest amino acids. The use of the higher boiling instead of the lower boiling[50–53] petroleum fraction (b.p. 60–80°C) led to more reproducible extraction yields, to which also the presence of benzene and a higher content of dichloromethane in the phase contribute. The small amount of methyl chloroformate in the extraction medium proved to be an effective means for converting the imidazolyl group in the histidine oxazolidinone to a stable analysable form:



This conversion proceeds instantaneously during shaking of the organic phase with the first alkaline wash, while the free indolyl group of tryptophan as well as the other derivatives are not attacked.

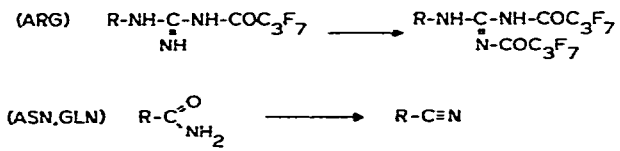
TABLE I

MOLAR RESPONSES OF AMINO ACID OXAZOLIDINONES RELATIVE TO α-AMINOCAPRYLIC ACID OR TO DIAMINOPIMELIC ACID (HIS, TRP AND CYS ONLY).

Values shown are the means for ten individually prepared samples of an amino acid calibration mixture (total amino acid content less than 0.3 mg), together with standard deviations (S.D.) and coefficients of variation (C.V.). The amino acids are ordered according to their elution from the analytical column(s).

Amino acid	*Abbrev.*	$\bar{x}$	*S.D.*	*C.V. (%)*
Alanine	ALA	0.41	0.016	3.93
Glycine	GLY	0.30	0.016	5.25
Threonine	THR	0.65	0.018	2.72
Valine	VAL	0.64	0.029	4.57
Serine	SER	0.60	0.025	4.16
Leucine	LEU	0.76	0.025	3.28
Isoleucine	ILE	0.78	0.008	1.06
Hydroxyproline	HYP	0.73	0.041	5.75
Proline	PRO	0.54	0.020	3.63
S-Methylcysteine	CYSM	0.59	0.012	2.05
Asparagine	ASN	0.36	0.031	8.61
Methionine	MET	0.70	0.030	4.24
Glutamine	GLN	0.43	0.039	8.83
Aspartic acid	ASP	0.65	0.034	5.18
Phenylalanine	PHE	1.12	0.008	0.73
Ornithine	ORN	0.76	0.031	4.08
Tyrosine	TYR	1.17	0.032	2.73
Lysine	LYS	0.84	0.029	3.44
Glutamic acid	GLU	0.59	0.020	3.38
Arginine	ARG	0.72	0.047	6.55
Histidine	HIS	0.80	0.038	4.75
Tryptophan	TRP	1.52	0.031	2.04
Cystine	CYS	0.84	0.029	3.44

Three amino acids, *i.e.*, arginine, glutamine and asparagine, cannot be analysed without additional acylation in heptane at a higher temperature, during which the guanidino group of arginine is acylated fully and the amides are converted into the nitriles (see ref. 55 for mass spectra of amino acid oxazolidinones):



As any change in the asparagine response occurred when the heating was prolonged from 3 to 10 min —unlike an earlier report[52]— short heat treatment is recommended in general. Also tryptophan undergoes a partial acylation (the derivative with the acylated indolyl group being eluted closely after arginine on column A) and the response of histidine declines due to the heat treatment. Analysis in heptane only on column B is therefore preferred. If the above three amino acids are not to be analysed, the other amino acid derivatives can be injected in column A in heptane only (Fig. 3). However,

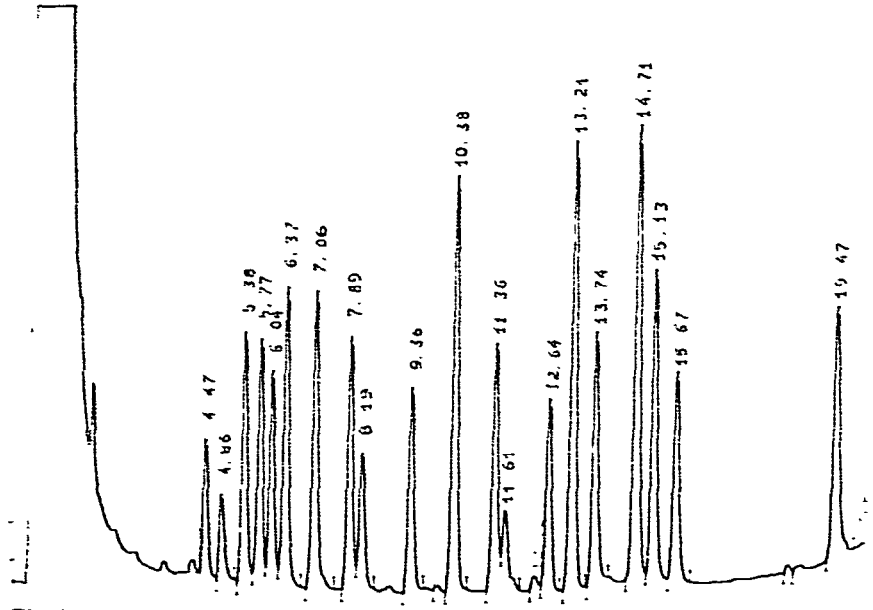


Fig. 3. Amino acid derivatives of the same equimolar mixture as in Fig. 1 analysed on column A [alternative packing: 3% OV-17–OV-22 (3:1) on Supelcoport (80–100 mesh)] in heptane only. Time of condensation shortened to 5 min (see the augmented peak for the second proline derivative, retention time 11.61 min). Nitrogen flow-fate: 25 ml min. Temperature range: 75–230°C (8° min).

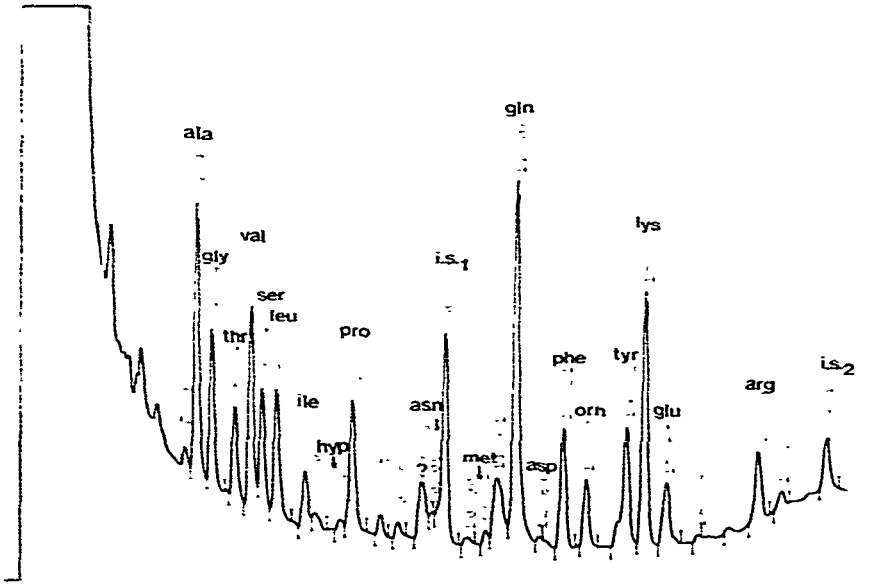


Fig. 4. Analysis of free amino acids in human control serum (100 μl taken off to the clean-up ion-exchange procedure[5a]). Internal standards added in amounts of 10 nmoles. GC conditions as in Fig. 3.

as in an earlier study[53], a partial adsorption (10–20%) of the N- and O-HFB acylated forms takes place.

The procedure was especially developed for routine amino acid analysis in minute amounts of biological materials. For this purpose, a pretreatment of the starting material, often involving a clean-up isolation step on ion-exchangers, is carried out prior to derivatization. It was found that the isolation of free amino acids from physiological fluids without precipitation of the protein[3,30] was preferred to the precipitating techniques[2,38] even when the yields for some amino acids were not as expected with the recommended cation-exchange material. By modifying the technique to give a better reproducibility[56], it was possible routinely to analyse free plasma amino acids in 50–100 μl of serum. Moreover, in comparison with the esterification procedures, this derivatization mode proved to be far less sensitive to the presence of interfering material (salts, protein, etc.) because even its one to two order higher excess did not disturb the reaction course. Some examples of biological applications are shown in Figs. 4 and 5.

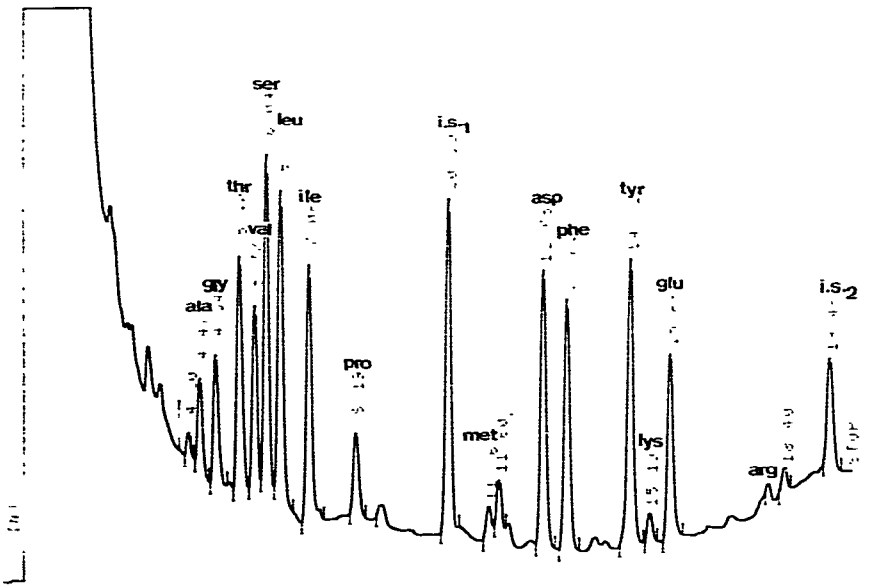


Fig. 5. Analysis of amino acids in acid hydrolysate of pepsin (initial amount hydrolysed in 6 *M* HCl at 110°C for 20 h was 20 μg). Amounts of internal standards added and GC conditions as in Figs. 3 and 4.

The application of this derivatization approach to the analysis of amino acid optical antipodes was also investigated. GC analysis of the enantiomeric amino acid oxazolidinones in a capillary column coated with the recommended chiral phase[39] confirmed a theoretical assumption that because of the planar structure of the oxazolidinone ring it should not be possible to resolve D- and L-antipodes[57].

The electron capture detector (ECD) is highly sensitive to the perhalogenated derivatives. By means of this method picogram amounts could be analysed even when problems were encountered, *e.g.*, adsorption of some perhalogenated forms on the

column packing. The GC-ECD temperature-programmed analysis of cyclic amino acid derivatives will be reported subsequently[58].

CONCLUSIONS

Compared with the esterification procedures[2–4,24–30,32–38], the formation of cyclic derivatives by treatment of amino acids with DCTFA has the following advantages: (1) both characteristic groups, the α-amino and the carboxyl, are blocked by action of a single reagent; (2) the aprotic condensation medium allows one to perform the subsequent acylation step in the same milieu; (3) both reactions proceed very smoothly at room temperature and the acylation is instantaneous; (4) glutamine and asparagine are preserved; (5) histidine, arginine, tryptophan and cystine can be estimated with a good reproducibility; (6) the total time of the chemical treatment including extraction is about half of that previously required; (7) the derivatives show an augmented ECD response and analysis in the picogram range is possible. The only drawback is the requirement for a separate column for the determination of histidine, tryptophan and cystine.

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