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QUANTITATIVE GAS–LIQUID CHROMATOGRAPHY OF NON-PROTEIN AMINO ACIDS

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SUMMARY

The quantitative gas–liquid chromatographic analysis of non-protein amino acids, in the presence of protein amino acids, is described. The amino acids were determined as their N-trifluoroacetyl *n*-butyl esters on an ethylene glycol adipate column. The relative molar responses of 38 amino acids are reported.

INTRODUCTION

During the course of a study on the chemical constituents of marine algae, which is currently being carried out in our laboratory, a sensitive and accurate method for the identification and quantitation of protein and non-protein amino acids was needed.

The gas chromatographic method developed by Gehrke and co-workers[1–5], which requires the derivatization of the amino acids as their N-trifluoroacetyl *n*-butyl esters, proved to be a valid alternative to ion-exchange chromatography. Although derivatization of the sample is required, it is generally more rapid than automated amino acid analysis.

In this paper, we describe the gas chromatographic behaviour of 30 non-protein amino acids in the presence of protein amino acids. Some of these amino acids have not been investigated before, some have been examined under different experimental conditions[6,7] and some have been studied only qualitatively[8].

EXPERIMENTAL

Apparatus

A Varian Model 2740 dual-column gas chromatograph with hydrogen flame detectors, a differential electrometer and a linear temperature programmer was used.

Reagents

Most of the amino acids were obtained from Calbiochem (Los Angeles, Calif., U.S.A.) or ICN (Cleveland, Ohio, U.S.A.). Pyrrolidine-2,5-dicarboxylic acid, N-

methylmethionine and baikiain were available from previous work[9] and were chromatographically pure.

n-Butanol and methylene chloride of reagent grade (Carlo Erba, Milan, Italy) were further purified by re-distillation over potassium carbonate and calcium chloride, respectively. Trifluoroacetic anhydride (TFAA) was obtained from Fluka (Buchs, Switzerland) and Chromosorb W (AW) and ethylene glycol adipate (EGA) from Merck (Darmstadt, G.F.R.).

Derivative formation

Volatile derivatives of amino acids were obtained according to the method described by Gehrke *et al.*[2]. The conversion was usually carried out using *ca.* 20 mg of total amino acids.

n-Butanol, 3 *N* in hydrochloric acid, was added to the amino acids (1 ml of acidified *n*-butanol per 30 μmol of amino acids) and the mixture, protected from atmospheric moisture, was refluxed for 1 h in a silicone oil bath. Excess of *n*-butanol and hydrogen chloride were removed under reduced pressure at 60° and a 1:3 mixture of TFAA and anhydrous methylene chloride was added to the residue. The reaction mixture was kept at room temperature for 2 h and then injected into the gas chromatograph.

Gas–liquid chromatographic conditions

A Pyrex glass column, 1.5 m long × 3 mm I.D., was used. The column was packed with 80–100 mesh Chromosorb W (AW) as support material and 0.325% (w/w) of EGA as the stationary phase. The Chromosorb had been previously heat-treated for 12 h at 140°.

The experimental conditions used to effect the separation of amino acids are reported in Table I.

Determination of relative molar responses

The molar responses of the amino acids as their N-trifluoroacetyl *n*-butyl

TABLE I

INSTRUMENT SETTINGS

Condition	*Value*
Hydrogen flow-rate	30 ml/min
Air flow-rate	300 ml/min
Nitrogen flow-rate	40 ml/min
Initial temperature	80°
Programming rate	2°/min (25 min) 4°/min (20 min)
Final temperature	210°
Upper limit interval	6 min
Detector temperature	230°
Injector temperature	220°
Chart speed	1 cm/min
Attenuation	$32 \cdot 10^{-10}$
Sample injected	8 μl

esters were determined from the gas chromatogram of a mixture of a single amino acid and a reference compound (leucine).

Relative molar response (*RMR*) were calculated from the equation

$$RMR_{aa/leu} = \frac{A_{aa}\, M_{leu}}{A_{leu}\, M_{aa}}$$

where A is the area measured on the chromatogram and M the corresponding number of moles. As we used equimolar amounts of amino acids and the reference compound, $RMR_{aa/leu} = A_{aa}/A_{leu}$.

TABLE II

RETENTION TIMES (t_R) AND RELATIVE MOLAR RESPONSES ($RMR_{aa/leu}$) OF N-TFA *n*-BUTYL ESTERS OF 38 AMINO ACIDS

Amino acid	*Abbreviation*	t_R *(min-sec)*	$RMR_{aa/leu}$
α-Aminoisobutyric acid	α-AIBA	9-05	0.79
Alanine	ALA	10-35	0.59
Sarcosine	SARC	11-55	0.48
α-Aminobutyric acid	α-ABA	12-15	0.73
Valine	VAL	13-05	0.78
Glycine	GLY	14-05	0.53
Norvaline	NORVAL	15-30	0.80
Isoleucine	ILEU	15-50	0.98
β-Aminoisobutyric acid	β-AIBA	16-30	0.65
β-Alanine	β-ALA	17-05	0.56
Leucine	LEU	17-45	1.00
1-Amino-1-cyclopropanecarboxylic acid	ACCA	18-00	0.75
Norleucine	NORLEU	18-35	1.05
Baikiain	BAI	18-35	0.93
Proline	PRO	20-20	0.94
Threonine	THR	20-45	0.91
Serine	SER	23-40	0.64
γ-Aminobutyric acid	GABA	24-30	0.86
L-Azetidine-2-carboxylic acid	A2C	24-30	0.63
N-Methylmethionine	MET-N-CH_3	27-35	0.85
Cysteine	CYSH	27-45	0.85
Methionine	MET	28-15	0.69
Hydroxyproline	HYPRO	29-05	0.48
Phenylalanine	PHE	29-30	1.19
Homoserine	HOMOSER	30-00	0.48
5-Hydroxypipecolic acid	HPA	30-00	0.80
ε-Aminocaproic acid	ACA	30-30	0.58
Aspartic acid	ASP	30-50	0.94
Allohydroxyproline	AHYPRO	31-10	0.47
Glutamic acid	GLU	34-10	1.11
Pyrrolidine-2,5-dicarboxylic acid	PDC	34-25	1.80
2,4-Diaminobutyric acid	DABA	35-00	0.53
α-Aminoadipic acid	AAA	36-15	1.09
Tyrosine	TYR	36-30	0.91
α-Aminopimelic acid	APA	37-55	1.54
Kainic acid	KAI	39-30	1.35
α,α′-Diaminopimelic acid	DAPA	43-55	0.92
Methionine sulphone	MET-SO_2	47-35	0.52

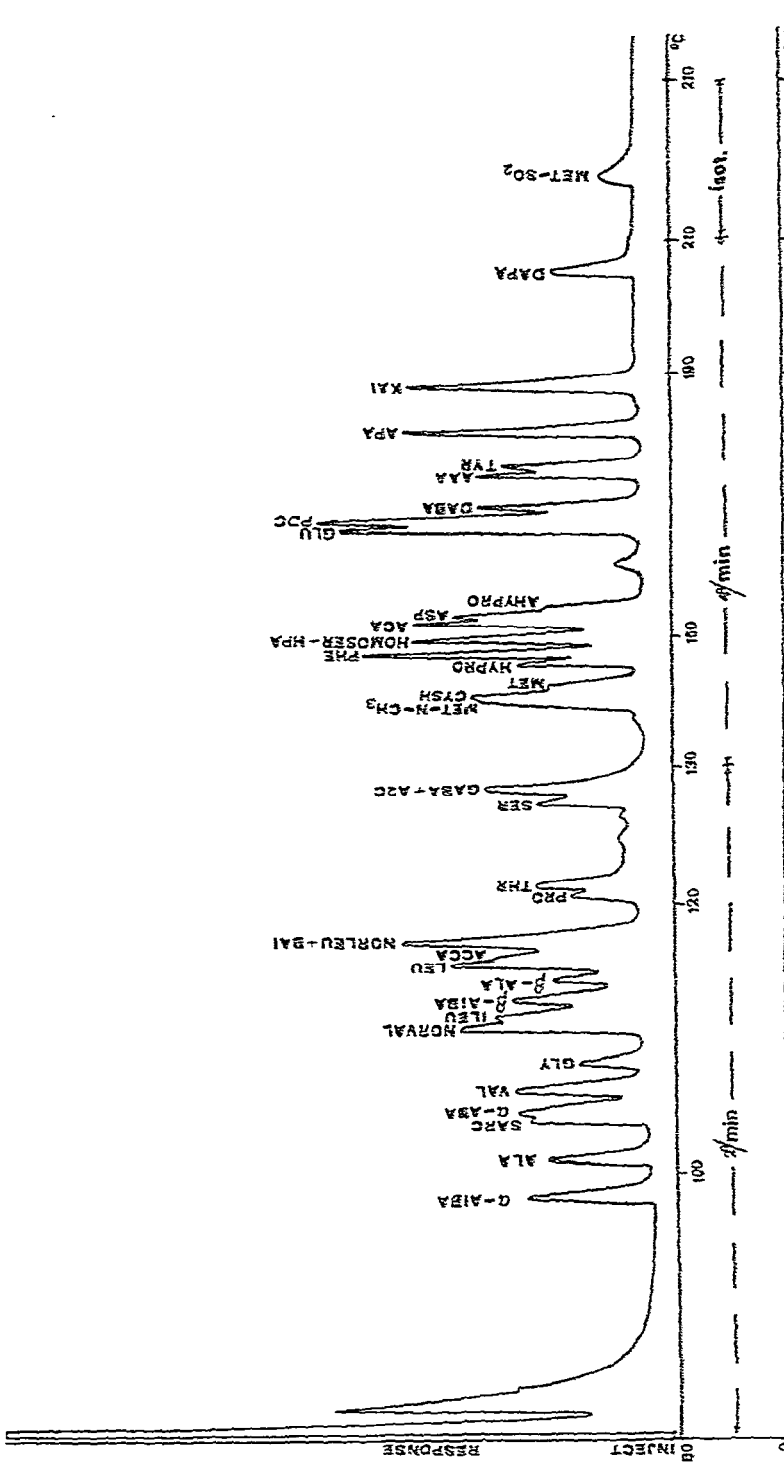

Fig. 1. GLC of an equimolar mixture of 24 non-protein and 14 protein amino acids. Column: 0.325% (w/w) EGA on 80–100 mesh Chromosorb W (AW), 1.5 m × 3 mm I.D., glass. Conditions: initial temperature, 60°; programming rate, 2°/min (25 min) then 4°/min (20 min); final temperature, 210°, held for 6 min. Attenuation: $32 \cdot 10^{-10}$.

RESULTS AND DISCUSSION

Thirty non-protein and 14 protein amino acids were examined. Under the experimental conditions adopted, several compounds, *viz.*, L-djenkolic acid, allocystathionine, S-2-aminoethyl-L-cysteine, δ-hydroxylysine, glycocyamine and L-α-aminoguanidinopropionic acid were not eluted from the EGA column.

In preliminary experiments, the effect of different temperature programmes was evaluated. As expected, improved overall resolution was achieved by using slower programming rates. However, at rates of 2°/min or less, amino acids above methionine showed a marked tendency to give peaks of poor shape. The best results were obtained by operating the column at 2°/min for an initial period of 25 min (to 130°) and subsequently at 4°/min to a final temperature of 210° and this programme was therefore adopted throughout this work.

Amino acid derivatives were each first chromatographed individually and then added successively to the mixture of the derivatives of the protein amino acids, thus allowing peak identification.

The chromatogram of the total mixture (Fig. 1) shows 25 adequately separated peaks, corresponding to amino acids that are not co-eluted, *viz.*, α-aminoisobutyric acid, alanine, sarcosine, α-amino *n*-butyric acid, valine, glycine, norvaline, isoleucine, β-aminoisobutyric acid, β-alanine, proline, threonine, serine, hydroxyproline, phenylalanine, ε-aminocaproic acid, glutamic acid, pyrrolidine-2,5-dicarboxylic acid, 2,4-diaminobutyric acid, α-aminoadipic acid, tyrosine, α-aminopimelic acid, kainic acid, α,α′-diaminopimelic acid and methionine sulphone. However, owing to the complexity of the mixture, complete separation was not achieved and some pairs of amino acids, *viz.*, leucine and 1-amino-1-cyclopropanecarboxylic acid, baikiain and norleucine, γ-aminobutyric acid and L-azetidine-2-carboxylic acid, homoserine and 5-hydroxypipecolic acid, and aspartic acid and allohydroxyproline, each gave a single peak or at best were very poorly resolved. The same is true for the three amino acids N-methylmethionine, cysteine and methionine. Methionine sulphoxide, which was not separated from methionine, probably on account of its reduction during derivatization, was not added to the total mixture.

Table II lists name, abbreviation, retention time (minutes from injection) and molar responses relative to leucine ($RMR_{aa/leu}$) of each amino acid under the conditions adopted.

CONCLUSION

Temperature-programmed gas chromatography of a number of non-protein amino acids, in the presence of protein amino acids, was studied on EGA, two different programming rates being used in sequence: 2°/min from 80° to 130° and 4°/min from 130° to the final temperature of 210°. The derivatization process was that described by Gehrke *et al.*[2]. The 24 non-protein amino acids that were eluted from the column (α-aminoisobutyric acid, sarcosine, α-aminobutyric acid, norvaline, isoleucine, β-aminoisobutyric acid, β-alanine, 1-amino-1-cyclopropanecarboxylic acid, norleucine, baikiain, γ-aminobutyric acid, L-azetidine-2-carboxylic acid, N-methylmethionine, homoserine, 5-hydroxypipecolic acid, ε-aminocaproic acid, *allo*-hydroxyproline, pyrrolidine-2,5-dicarboxylic acid, 2,4-diaminobutyric acid, α-aminoadipic acid,

α-aminopimelic acid, kainic acid, α,α′-diaminopimelic acid and methionine sulphone) were all distinguishable from protein amino acids and were not co-eluted, apart from norleucine, which was co-eluted with baikiain, homoserine with hydroxypipecolic acid, and γ-aminobutyric acid with L-azetidine-2-carboxylic acid.

This method has been used in our laboratory for the routine quantitative analysis of amino acids extracted from marine algae. In this study, α-aminoisobutyric acid, which is absent from all of the seaweeds examined so far, was used as the internal standard.

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