Journal of Chromatography, 131 (1977) 233–238

CHROM. 9414

QUANTITATIVE ANALYSIS OF PROTEIN AND NON-PROTEIN AMINO ACIDS BY GAS–LIQUID CHROMATOGRAPHY

VINCENZO AMICO, GIOVANNA ORIENTE and CORRADO TRINGALI
Istituto di Chimica Organica dell'Università di Catania, Catania (Italy)
(Received May 11th, 1976)

SUMMARY

The quantitative analysis of protein and non-protein amino acids by gas–liquid chromatography is described. The amino acids are determined as their *n*-butyl N-trifluoroacetyl esters on an OV-17 (methylphenyl silicone polymer) column. The relative molar responses of 16 protein and 30 non-protein amino acids are reported.

INTRODUCTION

In a previous paper[1] we reported a method for the gas–liquid chromatographic (GLC) analysis of protein and non-protein amino acids, which was employed in our laboratory for the determination of the free amino-acid content of marine algae[2]. We applied the well-known technique developed by Gehrke *et al.*[3], which requires the derivatization of the amino acids as their *n*-butyl N-trifluoroacetyl (BTFA) esters. In the preceding analyses, a column packed with 0.325% of ethylene glycol adipate (EGA) on Chromosorb W AW (80–100 mesh) was used; this liquid phase allows the separation of 17 protein amino acids and of many non-protein amino acids. However, some amino acids are poorly resolved on the EGA column; moreover, the limited thermal stability of the polyester phase[4] hinders the analysis of the BTFA derivatives having high boiling points.

For these reasons, we describe in this paper the gas chromatographic behaviour of 30 non-protein and 16 neutral protein amino acids on a column packed with OV-17, a silicone polymer. Silicones have been mainly used as liquid phases for the analysis of BTFA protein amino acids by Gehrke and his co-workers[3,5–12] and by other workers[13–15]. Only a few of the non-protein amino acids examined in this work have been previously analysed on OV-17 columns[14], and quantitative data have never been reported.

EXPERIMENTAL

Apparatus

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

Reagents

Amino acids were obtained from Calbiochem (Los Angeles, Calif., U.S.A.), ICN Pharmaceutical Inc. (Cleveland, Ohio, U.S.A.), Sigma (St. Louis, Mo., U.S.A.) and E. Merck (Darmstadt, G.F.R.), with the exceptions of pyrrolidine-2,5-dicarboxylic acid and baikiain which were available from previous work[16] and were chromatographically pure. *n*-Butanol and methylene chloride of reagent grade purity (Carlo Erba, Milan, Italy) were further purified by redistillation over potassium carbonate and calcium chloride, respectively. Trifluoroacetic anhydride (TFAA) was obtained from Fluka (Buchs, Switzerland). The column packing materials were purchased from Mela (Genoa, Italy).

Volatile derivatives of amino acids were obtained as described previously[1], according to the method of Gehrke *et al.*[3]. The conversion was carried out using 1.25 μmole of each amino acid.

Gas–liquid chromatography

A Pyrex glass column (1.5 m × 3 mm I.D.) was packed with 1.5% of OV-17 as stationary phase on Chromosorb G HP (80–100 mesh). The experimental conditions used to effect the separation of the amino acids are given in Table I. It is to be noted that the upper limit interval (235° held for 3 min) was programmed only for the analysis of non-protein amino acids.

TABLE I

INSTRUMENT SETTINGS

Parameter	*Value*
Hydrogen flow-rate	30 ml/min
Air flow-rate	300 ml/min
Nitrogen flow-rate	60 ml/min
Initial temperature	80°
Programming rate	4°/min
Final temperature	235°
Upper limit interval	3 min
Detector temperature	250°
Injector temperature	220°
Chart speed	0.5 cm/min
Attenuation	$16 \cdot 10^{-10}$
Sample injected	5 μl

The relative molar responses (*RMR*) of the amino acids as their BTFA esters were determined as previously reported[1], using valine (VAL) as reference compound. Equimolar amounts of amino acids and the reference compound were used, so that $RMR_{aa/VAL} = A_{aa}/A_{VAL}$, where A is the area measured on the chromatogram.

RESULTS AND DISCUSSION

The quantitative gas chromatographic analysis of 30 non-protein and 16 protein amino acids was carried out. The following compounds were not eluted from the

OV-17 column: δ-hydroxylysine, α-amino-β-guanidinopropionic acid, 2,3-diaminosuccinic acid, α,β-diaminopropionic acid and allocistathionine.

Chromatographic data are not reported for homoserine, which gave two peaks, and for methionine sulphoxide which gave a peak at the position of methionine, probably on account of its reduction during derivatization. Each amino acid was first individually chromatographed as a mixture with VAL; then, all of the amino acids were added successively to VAL, thus allowing peak identification.

Fig. 1 and Fig. 2 illustrate the gas chromatographic behaviour of protein and non-protein amino acids, respectively; in all of the cases of complete co-elution, only one of the amino acids having the same retention time was included in each mixture. In order to permit ease of comparison between the two chromatograms, the analyses were effected under the same conditions, operating at 4°/min from 80 to 235°; 3 min at the upper limit of 235° were required for the mixture of non-protein amino acids. Nevertheless, the separation of protein amino acids could be effected at 10°/min from 80 to 235°, with 3 min at the upper limit, thus reducing the time required for the analysis to 18 min.

Fig. 1 shows 14 adequately separated peaks, corresponding to the following

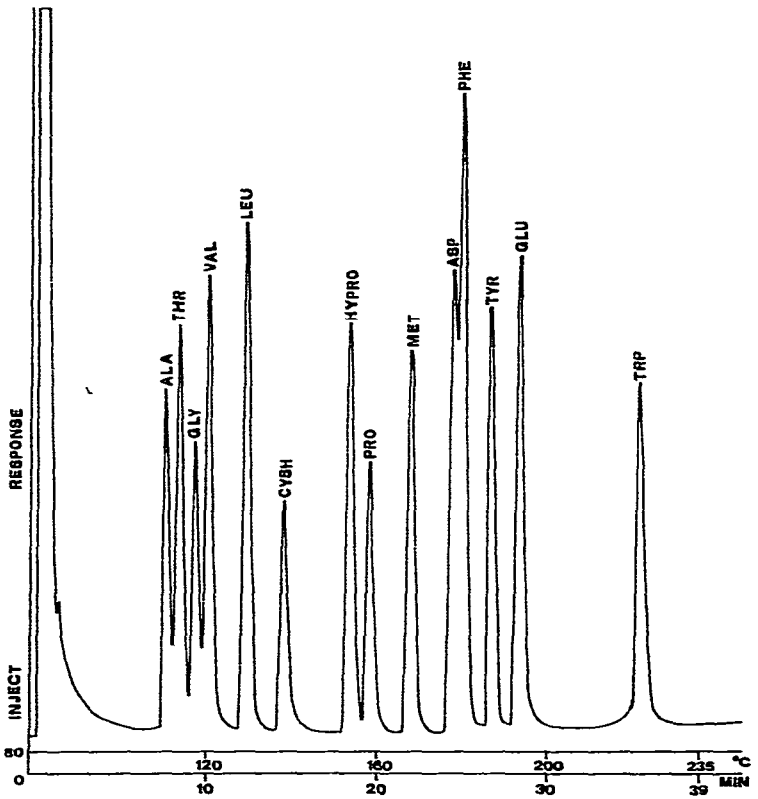

Fig. 1. GLC of an equimolar mixture of 14 protein amino acids. Column (1.5 m × 3 mm I.D.), glass, packed with 1.5% OV-17 on Chromosorb G HP (80–100 mesh). Conditions: initial temperature, 80°; programming rate, 4°/min; final temperature, 235°. Attenuation: $16 \cdot 10^{-10}$.

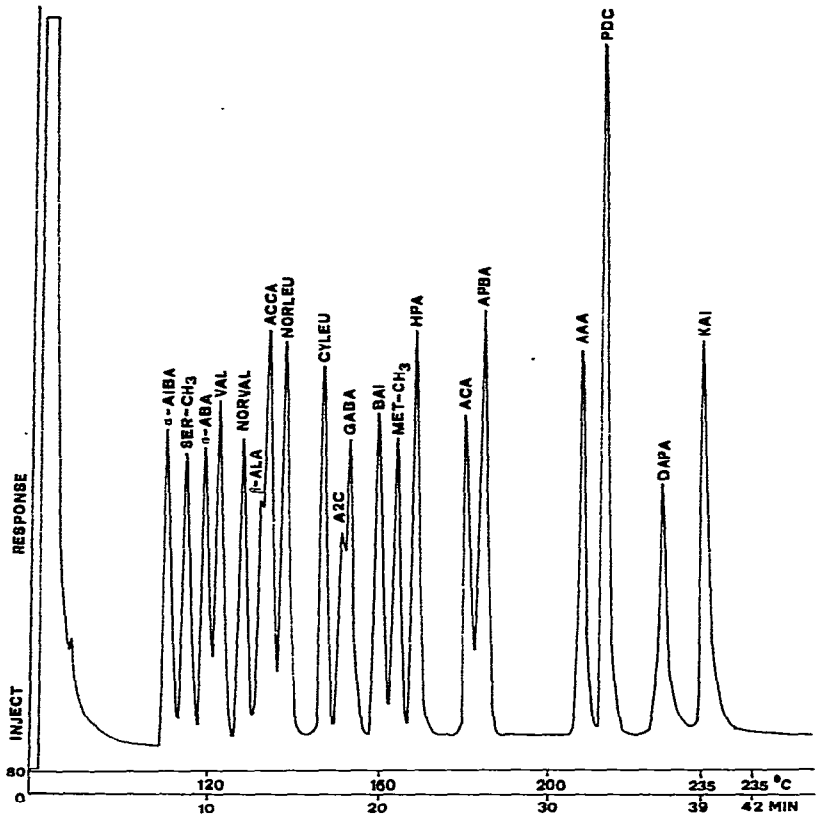

Fig. 2. GLC of an equimolar mixture of 20 non-protein amino acids. Column and conditions as in Fig. 1.

amino acids: alanine (ALA), threonine (THR), glycine (GLY), VAL, leucine (LEU), cysteine (CYSH), hydroxyproline (HYPRO), proline (PRO), methionine (MET), asparagine (ASP), phenylalanine (PHE), tyrosine (TYR), glutamine (GLU) and tryptophan (TRP). In preliminary experiments, a complete co-elution was observed for the two pairs: GLY and serine (SER), LEU and isoleucine (ILE).

The mixture of non-protein amino acids gave the chromatogram of Fig. 2 which shows the peaks corresponding to the following 20 amino acids: α-aminoisobutyric acid (α-AIBA), α-methylserine (SER-CH_3), α-aminobutyric acid (α-ABA), VAL (internal standard), norvaline (NORVAL), β-alanine (β-ALA), 1-amino-1-cyclopropanecarboxylic acid (ACCA), norleucine (NORLEU), cycloleucine (CYLEU), azetidine-2-carboxylic acid (A2C), γ-aminobutyric acid (GABA), baikiain (BAI), α-methylmethionine (MET-CH_3), 5-hydroxypipecolic acid (HPA), ε-aminocaproic acid, (ACA), 2-amino-3-phenylbutanoic acid (APBA), α-aminoadipic acid (AAA), pyrrolidine-2,5-dicarboxylic acid (PDC), α,α′-diaminopimelic acid (DAPA) and kainic acid (KAI). The following other amino acids were not included in the mixture because in preliminary experiments they were not separated from the compound in parentheses: sarcosine, SAR (NORVAL); β-aminobutyric acid, β-ABA, β-aminoisobutyric

acid, β-AIBA, and isoserine, ISER (β-ALA); glycocyamine, GLYCO, and pipecolic acid, PIP (BAI); allohydroxyproline, AHYPRO (HPA); ornithine, ORN (APBA); S-2-aminoethylcysteine, SAEC, and α-aminopimelic acid, APA (PDC), methionine sulphone, MET-SO_2 (DAPA).

Table II lists the retention times (minutes from injection) and molar responses relative to VAL ($RMR_{aa/VAL}$) of the 16 protein amino acids quantitatively analysed, and the corresponding values of the non-protein amino acids. As is evident from a comparison of the protein and non-protein amino acids there are several cases of complete co-elution among the amino acids examined on OV-17; however, different retention times have been obtained for pairs of amino acids which are scarcely separated on the EGA column[1]: *viz.*, SAR and α-ABA, NORVAL and ILE, NORLEU and BAI, PRO and THR, GABA and A2C, CYSH and MET.

TABLE II

RETENTION TIMES (t_R) AND RELATIVE MOLAR RESPONSES ($RMR_{aa/VAL}$) OF BTFA ESTERS OF 16 PROTEIN AND 30 NON-PROTEIN AMINO ACIDS*

Amino acid	t_R *(min-sec)*	$RMR_{aa/VAL}$	*Amino acid*	t_R *(min-sec)*	$RMR_{aa/VAL}$
Protein amino acids					
ALA	7-30	0.62	HYPRO	18-30	0.96
THR	8-30	0.89	PRO	19-55	0.70
GLY	9-35	0.57	MET	22-00	0.80
SER	9-35	0.75	ASP	25-00	0.98
VAL	10-15	1.00	PHE	26-00	0.97
LEU	12-50	1.15	TYR	26-40	0.86
ILE	12-50	1.06	GLU	28-20	1.10
CYSH	14-30	0.55	TRP	36-10	0.80
Non-protein amino acids					
α-AIBA	7-30	0.86	PIP	19-55	0.83
SER-CH_3	8-30	0.80	GLYCO	19-55	0.30
α-ABA	9-35	0.85	MET-CH_3	21-00	0.82
NORVAL	12-00	0.95	HPA	22-10	1.10
SAR	12-00	0.70	AHYPRO	22-10	1.05
β-ALA	12-50	0.57	ACA	25-00	0.93
β-AIBA	12-50	0.88	APBA	26-00	1.40
β-ABA	12-50	0.85	ORN	26-00	0.51
ISER	12-50	0.70	AAA	31-45	0.92
ACCA	13-30	0.72	PDC	33-00	1.97
NORLEU	14-25	1.18	SAEC	33-00	0.49
CYLEU	16-40	1.14	APA	33-00	0.55
A2C	17-50	0.50	DAPA	36-10	0.68
GABA	18-15	0.65	MET-SO_2	36-10	0.66
BAI	19-55	0.97	KAI	39-30	1.11

* See text for abbreviations.

CONCLUSIONS

The gas-chromatographic analysis of 30 non-protein and 16 protein amino acids on the OV-17 column is complementary to a previous study of the GLC of non-

protein amino acids in the presence of protein amino acids on an EGA column. The present study has yielded chromatographic data on 10 other non-protein amino acids, including glycocyamine and S-2-aminoethylcysteine which are not eluted from the EGA column. The retention times on OV-17 are generally different from those obtained on EGA; thus identification of non-protein amino acids, even in the presence of the common protein amino acids, is facilitated by using both the EGA and silicone polymer columns. The results obtained confirm that the "dual column system" is particularly suitable for the analyses of free amino-acid mixtures in biological samples.

REFERENCES

1 V. Amico, G. Oriente and C. Tringali, *J. Chromatogr.*, 116 (1976) 439.
2 V. Amico, G. Oriente, M. Piattelli, C. Tringali, E. Fattorusso, S. Magno, L. Mayol, C. Santacroce and D. Sica, *Biochemical Systematics and Ecology*, 4 (1976) 143.
3 C. W. Gehrke, R. W. Zumwalt and L. L. Wall, *J. Chromatogr.*, 37 (1968) 398.
4 P. Hušek and K. Macek, *J. Chromatogr.*, 113 (1975) 139.
5 D. Roach, C. W. Gehrke and R. W. Zumwalt, *J. Chromatogr.*, 43 (1969) 311.
6 D. Roach and C. W. Gehrke, *J. Chromatogr.*, 44 (1969) 269.
7 R. W. Zumwalt, D. Roach and C. W. Gehrke, *J. Chromatogr.*, 53 (1970) 171.
8 R. W. Zumwalt, K. Kuo and C. W. Gehrke, *J. Chromatogr.*, 55 (1971) 267.
9 R. W. Zumwalt, K. Kuo and C. W. Gehrke, *J. Chromatogr.*, 57 (1971) 193.
10 C. W. Gehrke, K. Kuo and R. W. Zumwalt, *J. Chromatogr.*, 57 (1971) 209.
11 C. W. Gehrke, R. W. Zumwalt, D. L. Stalling, D. Roach, W. A. Aue, C. Ponnamperuma and K. A. Kvenvolden, *J. Chromatogr.*, 59 (1971) 305.
12 F. E. Kaiser, C. W. Gehrke, R. W. Zumwalt and K. Kuo, *J. Chromatogr.*, 94 (1974) 113.
13 E. D. Pellizzari, J. H. Brown, P. Talbot, R. W. Farmer and L. F. Fabre, Jr., *J. Chromatogr.*, 55 (1971) 281.
14 M. Sakamoto, K.-I. Kajiyama and H. Tonami, *J. Chromatogr.*, 94 (1974) 189.
15 I. MacArthur Moodie, *J. Chromatogr.*, 99 (1974) 495.
16 G. Impellizzeri, S. Mangiafico, G. Oriente, M. Piattelli, S. Sciuto, E. Fattorusso, S. Magno, C. Santacroce and D. Sica, *Phytochemistry*, 14 (1975) 1549.