Journal of Chromatography, 97 (1974) 19–24

CHROM. 7583

GAS–LIQUID CHROMATOGRAPHY OF N-HEPTAFLUOROBUTYRYL ISOBUTYL ESTERS OF AMINO ACIDS*

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(Received April 10th, 1974)

SUMMARY

The N-heptafluorobutyryl isobutyl esters of the protein amino acids have been separated by gas–liquid chromatography using a single column of 3% SE-30 on Gas-Chrom Q. The separation is superior to that of previous single-column methods and is particularly suitable for the analysis of plant seed proteins. The method is also applicable to glycoproteins.

INTRODUCTION

The analysis of amino acids by gas–liquid chromatography (GLC) can be performed only after the conversion of the amino acids to suitably volatile and stable derivatives. The most extensively investigated derivatives have been the esters and acyl derivatives of the polar groups. Due largely to the work of Gehrke and his associates[1–4], a procedure was developed for the separation and quantitation of the protein amino acids as the N-trifluoroacetyl butyl esters using two columns and two different liquid phases[5]. Subsequently, the same amino acid derivatives were separated in about 45 min using a single column[6]. An excellent single-column separation of the protein amino acids has also been achieved by Moss *et al.*[7], using the N-heptafluorobutyryl (HFB) *n*-propyl esters. More recently, Zanetta and Vincendon demonstrated the separation of the N-HFB isoamyl esters using a single-column procedure and showed that the method could be applied to acid-hydrolysed proteins or glycoproteins[8].

In our studies of seed proteins we have used both of the above methods but have found that each method presents certain disadvantages. Seed proteins commonly contain substantial amounts of aspartic and glutamic acids but relatively little methionine[9]. However, the amount of methionine in a seed protein is frequently of considerable interest. In the method of Moss *et al.*[7] the accurate quantitation of a small methionine peak close to a large aspartic acid peak is difficult. Similarly, using the method of Zanetta and Vincendon[8], the quantitation of lysine and tyrosine is made difficult by the proximity of a large glutamic acid peak. We have found that these problems are avoided by using the N-HFB isobutyl esters, the single-column GLC separation of which we now report.

* NRCC No. 14189.

EXPERIMENTAL

Reagents

Standard amino acid mixtures were obtained from Hamilton (Whittier, Calif., U.S.A.) and heptafluorobutyric anhydride was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.).

Methanol, isobutyl alcohol and ethyl acetate were refluxed for 3–4 h over magnesium turnings, decanted, refluxed for 3–4 h over calcium hydride and redistilled from an all-glass apparatus. Dry HCl gas was added to methanol and isobutyl alcohol, in the appropriate amounts, after passing the gas through a sulfuric acid tower. The normality was verified by titration. The reagents were stored at −20°.

Preparation of derivatives

All derivatization steps were performed in a 1-ml Reactivial (Pierce, Rockford, Ill., U.S.A.). All evaporations were performed using a stream of dry nitrogen at room temperature. All residues were dispersed into solution by placing in an ultrasonic bath for 30 sec.

Inter-esterification

Samples containing 0.05–0.25 μM of each amino acid were dried either by freeze drying or under a stream of dry nitrogen. The last traces of water were removed azeotropically using methylene chloride. The residue was dissolved in 200 μl of methanol–1.25 N HCl and placed in an ultrasonic bath for 30 sec. After 30 min at room temperature the reagent was evaporated, 200 μl methylene chloride was added and again evaporated. The residue was dissolved in 200 μl of isobutyl alcohol–1.25 N HCl and the vial heated at 110° for 150 min in an oven. After cooling the reagent was evaporated. The residue was dissolved in 50 μl of ethyl acetate, 20 μl of heptafluorobutyric anhydride was added and the vial was heated for 10 min at 150° in an oil bath. The vial was then thoroughly cooled, the reagent evaporated just to dryness and the residue was dissolved in an appropriate volume of ethyl acetate. An appropriate volume of the sample was injected along with acetic anhydride in the sample to anhydride ratio of 2:1.

Direct esterification

Direct esterification, using the procedure of Roach *et al.*[10], was also performed. Samples containing 0.05–0.25 μM of each amino acid were dried. The residue was dissolved in 200 μl of isobutyl alcohol–3 N HCl. After heating at 110° for 1 h and evaporating the excess reagent, the sample was acylated as described above.

Chromatography

All analyses were performed using a Hewlett-Packard, Model 7611 gas chromatograph equipped with dual-flame ionisation detectors. The column packing (3% SE-30 on Gas-Chrom Q, 100–200 mesh) was obtained from Applied Science Labs., State College, Pa., U.S.A.). Pyrex columns (11–12 ft. × 2.5 mm I.D., thin-walled) were filled with the stationary phase by gentle tapping under suction and conditioned overnight with a carrier gas (nitrogen) flow-rate of 30 ml/min. The chromatographic conditions were as follows: temperature program, 90°–240° at 4°/min; injector tem-

perature, 250°; detector temperature, 280°; air flow-rate, 300 ml/min; hydrogen flow-rate, 30 ml/min; nitrogen flow-rate, 30 ml/min. Quantitation was performed using an Infotronics Model CRS 208 automatic digital integrator.

RESULTS AND DISCUSSION

The separation of the N-HFB isobutyl esters of the protein amino acids shown in Fig. 1 and the retention temperatures shown in Table I represent the results obtained by using a temperature program of 4°/min. However, the separation was only marginally decreased by programming at 6°/min and a complete analysis could thus be obtained in less than 30 min. The order of elution of the amino acids was identical to that observed for the N-HFB propyl esters by Moss *et al.*[7]. However, methionine was completely separated from aspartic acid and phenylalanine was completely separated from glutamic acid. The order of elution of the N-HFB isobutyl esters is somewhat different from that of the N-HFB isoamyl esters[8]. The main difference is in the separation of lysine, tyrosine and glutamic acid. The removal of glutamic acid from close proximity to lysine and tyrosine is important for the accurate quantitation of

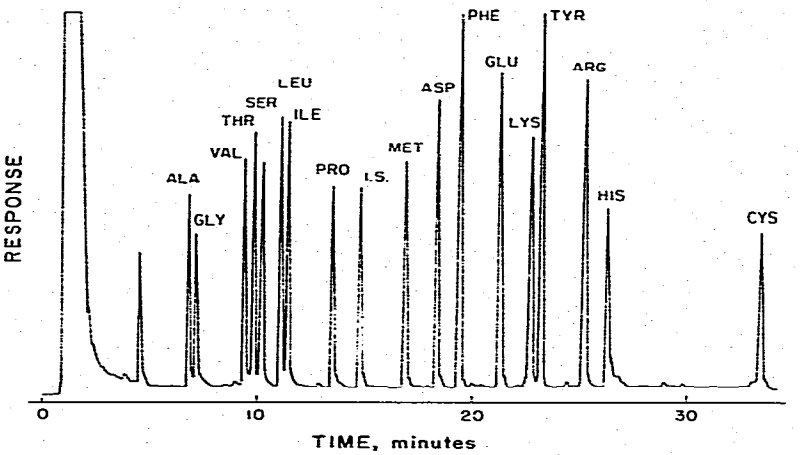

Fig. 1. Gas chromatogram of the N-HFB isobutyl esters of the protein amino acids. I.S. = internal standard, pipecolic acid used in an amount of 0.8 mole per mole of each of the other amino acids. The other peaks each represent 0.0075 μM of amino acid. GLC conditions are given in the text.

TABLE I

RETENTION TEMPERATURES OF THE N-HEPTAFLUOROBUTYRYL ISOBUTYL ESTERS OF AMINO ACIDS

Amino acid	R_T (°C)	*Amino acid*	R_T (°C)	*Amino acid*	R_T (°C)
Alanine	119.0	Histidine	204.5	Pipecolic acid	153.5
Arginine	200.0	Hydroxyproline	158.5	Proline	148.0
Aspartic acid	170.0	Isoleucine	138.5	Serine	133.5
Cysteine	149.0	Leucine	137.0	Threonine	132.0
Cystine	236.5	Lysine	189.5	Tyrosine	191.5
Glutamic acid	183.0	Methionine	163.5	Valine	130.5
Glycine	120.5	Phenylalanine	175.0		

these amino acids. This is particularly important when analysing proteins such as plant proteins which contain substantial amounts of glutamic acid. The flow-rate was not critical for the separation of any of the amino acids. These points are illustrated by the chromatographic separation of the amino acids from a typical seed source shown in Fig. 2.

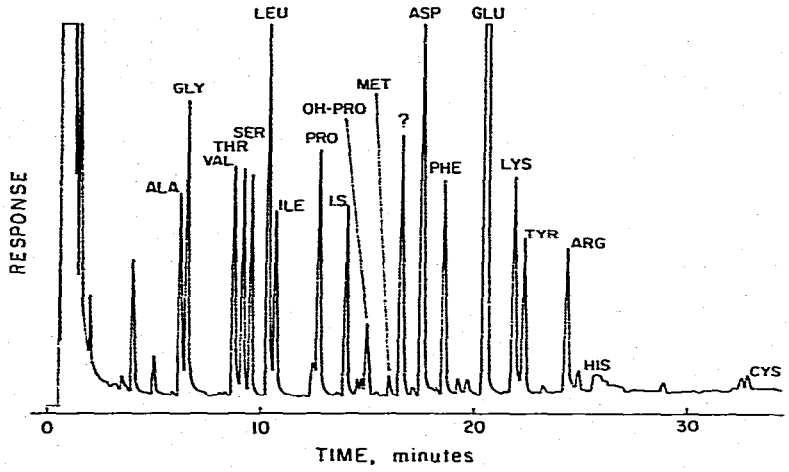

Fig. 2. Gas chromatogram of the N-HFB isobutyl esters of the amino acids in an acid hydrolysate of defatted mustard seed (*Brassica hirta* Moench). I.S. = internal standard. Temperature program, 95 –240 after a 1-min hold. Other GLC conditions are given in the text. No acetic anhydride was injected.

The relative molar responses of the N-HFB isobutyl esters of the protein amino acids relative to that of the corresponding derivative of the internal standard, pipecolic acid, are shown in Table II. The responses obtained by the direct esterification procedure compared favourably with those obtained by the more tedious procedure of inter-esterification. Since, for most of the amino acids maximum or near maximum relative responses were obtained after 1 h of direct esterification, this time was used for routine amino acid analysis. This procedure appeared to be the best compromise between the most rapid derivatisation and optimum response. The variability of the relative molar responses was less than ± 2%.

Zanetta and Vincendon[8] experienced loss of the more volatile components during evaporation when concentrating the *n*-butyl trifluoroacetyl and the *n*-propyl heptafluorobutyryl derivatives. We have not experienced this problem but we have routinely performed all evaporations in the production of these derivatives at room temperature. However, when using the isoamyl-HFB derivatives we have experienced losses of alanine and glycine when evaporations were performed at 50 and 80 as directed[8]. We therefore evaporated at room temperature after methylation and at 40 after inter-esterification. For the N-HFB isobutyl ester method, no loss of the more volatile derivatives was observed when all evaporations were performed at room temperature. Unlike the N-HFB isoamyl esters of the amino acids, the N-HFB isobutyl esters were not more soluble in acetonitrile than in ethyl acetate. The latter

TABLE II

RELATIVE MOLAR RESPONSE OF AMINO ACID DERIVATIVES AS A FUNCTION OF ESTERIFICATION TIME AND DERIVATISATION PROCEDURE*

Amino acid	*Direct esterification*				*Inter-esterification*	
	15 min	*30 min*	*60 min*	*120 min*	*60 min*	*150 min*
Ala	0.63	0.63	0.59	0.59	0.50	0.67
Gly	0.55	0.54	0.49	0.49	0.43	0.50
Val	0.83	0.85	0.85	0.85	0.75	0.85
Thr	0.89	0.88	0.87	0.87	0.84	0.87
Ser	0.73	0.73	0.74	0.74	0.70	0.75
Leu	0.99	0.98	0.98	0.98	0.93	0.99
Ile	0.90	1.00	1.00	1.01	0.96	1.04
Pro	0.84	0.84	0.83	0.83	0.82	0.79
Met	0.93	0.94	0.94	0.94	0.90	0.92
Asp	1.15	1.18	1.18	1.18	1.17	1.17
Phe	1.43	1.45	1.45	1.45	1.43	1.38
Glu	1.30	1.32	1.34	1.37	1.30	1.30
Lys	1.22	1.23	1.24	1.24	1.20	1.22
Tyr	1.48	1.50	1.50	1.50	1.49	1.50
Arg	1.28	1.31	1.41	1.42	1.46	1.40
His	0.82	0.84	0.87	0.89	0.88	0.75
Cys	0.60	0.60	0.60	0.61	0.84	0.57

* Molar response relative to the internal standard pipecolic acid. Each value represents an average of at least six determinations.

solvent was therefore used as the acylation solvent to minimise the preparation of anhydrous solvents.

The chromatography of the N-HFB isobutyl ester of histidine was similar to that of the N-HFB propyl ester[7]. A sharp peak was observed only when the sample was co-injected with acetic anhydride and thus, presumably, on-column acylation took place[11]. The difficulties in derivatising histidine were confined to the acylation step as the isobutyl ester was readily prepared in a large-scale derivatisation which was monitored by methods such as infrared spectroscopy. We have not been able to chromatograph histidine as the isoamyl ester, unlike the observations of Zanetta and Vincendon[8], without co-injecting with acetic anhydride. The derivatisation of histidine was observed to be particularly sensitive to the presence of water in any of the solvents or reagents.

The acyl derivatives of glucosamine and galactosamine had retention temperatures of respectively 146.5° and 149° and the method would thus appear to be applicable, without modification, to the analysis of the protein moieties of glycoproteins.

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