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GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF N(O,S)-TRIFLUOROACETYL *n*-PROPYL ESTERS OF PROTEIN AND NON-PROTEIN AMINO ACIDS

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SUMMARY

Chromatographic conditions for the determination of protein and non-protein amino acids as their N(O,S)-trifluoroacetyl *n*-propyl esters are given which allow the separation of about 30 amino acids within 19 min using nitrogen or within 17 min using helium as the carrier gas. Retention times and responses are given for 42 amino acids or related compounds. Only few of them elute together when using a 2 m × 2 mm I.D. glass column filled with 0.65% ethylene glycol adipate on Chromosorb W AW (80–100 mesh). The acylation of the *n*-propyl esters was investigated with respect to optimal reaction conditions and was found to be best performed at 150°C for 5 min.

INTRODUCTION

The utility of gas–liquid chromatography (GLC) for amino acid analysis has already been demonstrated by many workers and was reviewed by Husek and Macek[1] in 1975. The type of derivative most often used today is prepared by esterification of the carboxylic groups and acylation of the other functional groups. Frequently *n*-butanol–hydrochloric acid has been used for esterification, and trifluoroacetic anhydride (TFAA) for subsequent acylation to yield the N(O,S)-TFA *n*-butyl esters[2]. Alternative methods make use of heptafluorobutyric anhydride (HFBA) for acylation, after esterification either with *n*-propanol–[3] or isobutanol–hydrochloric acid[4].

In many instances, chromatographic analysis is performed on non-polar stationary phases.

Bengtsson and Odham[5] described the successful separation of 19 protein amino acids as their N(O,S)-HFB isobutyl esters using either OV-101, SE-30 or a mixture of OV-101 and OV-17. The time required for these separations was 1 h, but could be reduced to 30 min by the use of a capillary column[6]. Moodie[7] reported the separation of the same derivatives on a column packed with OV-101 within 24 min. SE-30 was also used by MacKenzie and Tenaschuk[4] and Guarino *et al.*[8], the latter

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authors using the N(O,S)-TFA *n*-butyl esters. A mixture of OV-101 and OV-17 was used by March[9] to separate the N(O,S)-HFB *n*-propyl esters of 20 protein amino acids within 55 min.

Ethylene glycol adipate (EGA), a rather polar stationary phase, has been widely used for separation of the N(O,S)-TFA *n*-butyl esters[10,12]. The separation of 17 protein amino acids within 14 min was reported by Roch and Gehrke[10], but difficulties with the elution of arginine, cystine and histidine led to the development of two-column systems as described by Gehrke *et al.*[11] and Kaiser *et al.*[12]. Here, the basic amino acids and cystine are eluted from various silicone phases[11–13]. Simultaneous operation of two columns allows the separation of all protein amino acids within 25 min[11].

A mixture of polar stationary phases containing Carbowax 20M, Silar 5CP and Lexan was used for the single-column separation of all protein amino acids as their N(O,S)-acetyl *n*-propyl esters[14] within 15 min.

In contrast to methods involving hydrochloric acid-catalysed esterification the additional determination of the amides glutamine and asparagine is possible when using the oxazolidinone derivatives of amino acids[15], or the N(O,S)-isobutyloxycarbonyl methyl esters[16].

Most of the methods described to date are restricted to the analysis of protein amino acids. Only a few publications have dealt with the determination of non-protein amino acids in the presence of protein amino acids as found in biological fluids.

Lewis *et al.*[17] described the determination of whole blood and plasma amino acids as their N(O,S)-acetyl *n*-propyl esters, but the analysis of arginine, histidine, cysteine and tryptophan was not possible. The time required for the separation of 16 amino acids was 18 min.

Guarino *et al.*[8] used N(O,S)-TFA *n*-butyl esters for clinical investigations. Analysis on 3% SE-30 allowed the separation of 25 amino acids within 30 min, including those which are not eluted from an EGA column. Using the latter column, the separation of 17 protein and 20 non-protein amino acids was reported[18], but the analysis time was 80 min. The chromatographic behaviour of protein and non-protein amino acids was also reported, using either EGA[19] or OV-17 as the stationary phase[20].

Fifty amino acids were analysed as the N(O,S)-HFB isobutyl esters on a column packed with 3% OV-101. Separation of 32 amino acids could be achieved within 35 min by use of this system[21]. Desgrees *et al.*[22] described a quantitative method based on the above system for analysing physiological fluids, but used a capillary column instead of a packed column. The separation of 32 amino acids took 1 h.

N(O,S)-HFB *n*-propyl esters have been used only for protein amino acid analysis[3,9].

In our work on defects in amino acid metabolism, we searched for a method suitable for quantitative amino acid screening. As cost and speed become important points within a screening programme, GLC seems to be best suited as a fast, inexpensive and quantitative method.

In order to fulfil these requirements, TFAA was chosen for acylation, as it is much less expensive than HFBA, and propanol was chosen for esterification, as the

propyl esters would be expected to have lower retention times than butyl esters.

The N(O,S)-TFA *n*-propyl esters have not been described before exept for the analysis of four amino acids for comparative studies[23]. Therefore, we investigated the chromatographic behaviour of these derivatives and some parameters that are important for their formation.

Chromatographic data and separation properties of 42 amino acids are reported, and also the influence of reaction time and temperature during the acylation step. Considering the straightforward and time-saving derivatization and the rapid separation of a large number of amino acids, this method should be a good supplement to those previously described.

EXPERIMENTAL

Ethyl acetate, acetyl chloride, 1-propanol, acetic acid and ammonia were of analytical-reagent grade, obtained from Merck (Darmstadt, G.F.R.). Methylene chloride, "gold label" grade, was purchased from Aldrich (Beerse, Belgium), and trifluoroacetic anhydride, reagent grade, from Pierce (Rotterdam, The Netherlands). Dowex 50W-X8 (100–200 mesh) was obtained from Bio-Rad Labs., (Vienna, Austria). Pipecolic acid, cystathionine, citrulline, homoarginine, 3-methylhistidine (A-grade) and α-aminocaprylic acid (B-grade) were obtained from Calbiochem (San Diego, CA, U.S.A.), phosphoethanolamine from Fluka (Buchs, Switzerland) and cystine from Sigma (Munich, G.F.R.). The other amino acids were purchased from Serva (Heidelberg, G.F.R.) and were chromatographically pure.

Stock solutions, 2.5 m*M* in 0.1 *M* hydrochloric acid, were prepared for single amino acids and various mixtures, and stored at 4°C.

Glass columns, 2 m × 2 mm I.D., and the stock packings of 0.65% EGA on Chromosorb W AW (80–100 mesh) were obtained from Supelco (Crans, Switzerland) and gases from ASW (Graz, Austria).

For GLC, a Hewlett-Packard gas chromatograph with two injection ports, one flame-ionization detector (FID) one nitrogen–phosphorus (NP) FID, an HP 7672A automatic sampler and a calculator equipped with BASIC capability was used. Injections were made with Hamilton syringes.

Derivatization was carried out in 100 × 16 mm glass tubes with PTFE-lined screw-caps (Sovirel). Heating was performed using a block heater (Pierce) and an aluminum block in which holes of depth 3 cm and diameter 17 mm were drilled. For evaporation, a Büchi rotavapor with a special attachment allowing simultaneous evaporation of ten tubes was used. They were connected to the rotavapor by PTFE-lined seals and screw-caps with a hole.

Sample preparation

Urine samples were purified according to the method described by Adams[14], except that the tin(II) chloride for cystine reduction was omitted.

Derivatization

Details of the derivatization procedure are given elsewhere[24]. Samples containing up to 10 μmole of amino acids were evaporated. Esterification with 300 μl of 3.5 *M* acetyl chloride in 1-propanol was achieved by heating to 110°C for 25 min. After

cooling and evaporating excess of reagent at 40°C, acylation was performed using 200 μl of TFAA and 400 μl of a solvent (methylene chloride was normally used, but acetonitrile and chloroform have also been tested). The reaction mixture was heated at 150°C for 5–6 min, or various other times as described later. After cooling and evaporation to dryness at 30°C, the residue was dissolved in an appropriate amount of ethyl acetate, depending on the amino acid concentration.

Chromatographic analysis

The gas supply for the detectors was 30 ml/min of hydrogen and 450 ml/min of air for the FID and 3 ml/min of hydrogen and 60 ml/min of air for the NP-FID. Other chromatographic conditions are given in the legends of the figures. Complex mixtures need two- or three-step linear temperature programmes as described in Figs. 1 and 5, whereas the separation of a smaller number of amino acids can be obtained with a single-step temperature programme (Fig. 2).

In spite of the multiple-step temperature programmes normally used, retention times remain constant for up to many weeks and several hundred injections. The injections made by the autosampler are highly reproducible, and therefore a single analysis of the sample is usually sufficient. This also allows the calculation of molar responses as described below.

Quantitation

Internal standardization with norleucine was used for this step. Relative molar responses (RMR) were calculated as peak area (amino acid)/peak area (norleucine) when using equimolar standard solutions. Molar responses, expressed as peak area (amino acid)/concentration (amino acid), were used in derivatization studies. However, they are not included in this paper, because these values will not be comparable for different working conditions.

RESULTS AND DISCUSSION

Chromatography

As EGA was found to give the best separation of the TFA *n*-butyl esters[10] and the *n*-propyl esters were expected to show similar properties, this stationary phase was used to separate the TFA *n*-propyl esters. Indeed, separation of these derivatives proved to be much better than the separation achieved on a mixture of 2% OV-17 and 1% OV-210, which has been used for elution of arginine, histidine and cystine as the TFA *n*-butyl esters[12,25].

The chromatographic properties of 42 amino acid derivatives are given in Table I. Fig. 1 shows the separation of a complex mixture completed within 20 min, and also the chromatographic conditions used (also for the data in Table I). The influence of the carrier gas and differences between the two columns, in spite of identical dimensions and column fillings, account for the different relative retention times (RRT), as seen in Table I. The retention times are, however, very constant on a single column.

Separation of the amino acid pairs Ile/β-AIBA, Pip/Norleu, Asp/HPro, Trp-2/1-Me-His and MetSulphon/HLys is poor or nil. If pipecolic acid is expected in the sample, another stationary phase or internal standard (*e.g.*, α-ACA) should be used.

TABLE I

RETENTION TIMES (RT), RELATIVE RETENTION TIMES (RRT), RELATIVE MOLAR RESPONSES (RMR) AND COEFFICIENTS OF VARIATION (C.V.) OF RMR OF THE N(O,S)TFA *n*-PROPYL ESTERS OF AMINO ACIDS

RMR values calculated relative to norleucine. RMR values are the means of at least six independent determinations.

Amino acid	*FID*				*NP-FID*			
	RT	*RRT*	*RMR*	*C.V. (%)*	*RT*	*RRT*	*RMR*	*C.V. (%)*
α-AIBA	1.19	0.33	0.71	6.3	0.84	0.22	1.17	8.1
Ala	1.43	0.40	0.60	4.8	1.00	0.35	1.32	5.9
Sarc	1.63	0.46	0.56	3.0	1.15	0.41	1.70	3.5
α-ABA	1.80	0.50	0.71	3.0	1.27	0.45	1.12	3.7
Val	1.95	0.55	0.91	2.7	1.39	0.49	1.04	5.2
Gly	2.27	0.64	0.49	2.1	1.61	0.57	1.25	3.7
PEA*	2.65	0.74	0.06	–	1.91	0.67	0.25	–
Norval*	2.66	0.75	1.08	–	2.01	0.71	1.35	–
Ile	2.77	0.78	0.94	3.0	2.03	0.72	0.90	5.5
β-AIBA*	2.84	0.80	0.85	–	2.13	0.75	0.54	–
β-Ala	3.07	0.86	0.64	0.6	2.30	0.81	0.71	2.8
Leu	3.31	0.93	1.05	0.5	2.57	0.91	1.08	1.1
Pip	3.47	0.97	0.69*	–	2.74	0.97	0.54	5.7
Norleu	3.57	1.00	1.00	–	2.83	1.00	1.00	–
Pro	3.73	1.04	0.90	1.1	3.01	1.06	1.48	2.0
Thr	4.14	1.16	0.48	1.1	3.47	1.23	0.52	1.2
SER**	4.80	1.34	0.15	3.5	4.20	1.48	0.15	5.1
γ-ABA	4.91	1.38	0.80	1.8	4.27	1.51	1.29	2.1
α-ACA*	5.29	1.48	1.50	–	4.67	1.65	1.04	–
CysH***	5.81	1.63	–	–	5.27	1.86	–	–
Met	5.98	1.68	0.83	1.1	5.39	1.90	0.90	2.7
Asp	6.12	1.71	1.07	1.4	5.55	1.96	1.13	3.8
HPro*	6.18	1.73	1.02	–	5.61	1.98	1.20	–
Phe	6.51	1.82	1.45	1.5	5.93	2.1	0.98	3.1
Cit	6.94	1.94	0.07	10.5	6.35	2.24	0.12	3.4
Glu	7.23	2.03	1.15	1.6	6.68	2.36	1.02	3.3
α-AAA	7.85	2.20	1.25	1.9	7.31	2.58	0.98	3.7
Tyr**	8.42	2.36	0.36	14.1	7.85	2.77	–	–
3-Me-His	8.85	2.48	0.75	1.5	8.33	2.94	2.37	3.7
Orn	9.03	2.53	0.92	3.1	8.42	2.98	1.66	3.7
Arg§	9.05	2.54	–	–	8.51	3.01	0.16	–
His-2§	–	–	–	–	8.54	3.02	–	–
Lys	9.57	2.68	1.08	8.7	8.97	3.17	1.75	4.7
HomoArg§	9.56	2.68	–	–	8.98	3.17	0.24	–
DOPA**	–	–	–	–	9.18	3.24	0.05	–
Trp-2	10.10	2.83	0.65	16.5	9.39	3.32	0.48	9.5
1-Me-His	10.28	2.88	1.16	4.2	9.43	3.33	1.99*	–
MetSulphon	11.02	3.09	0.96	4.8	10.05	3.55	0.97	3.0
HLys 1*	11.00	3.08	0.25		10.13	3.58	0.48	–
HLys 2*	11.19	3.13	0.20	–	10.27	3.63	0.41	–
His-1	11.51	3.22	0.46	5.3	10.42	3.68	1.31	11.7
Trp-1	13.48	3.78	0.52	5.2	11.90	4.20	0.48	6.0
CTT	13.87	3.89	0.87	1.5	12.51	4.42	1.00	0.9
HomoCys	18.65	5.22	1.29	5.8	16.35	5.78	1.30	7.6

* Values from single determinations only.

** Response is strongly enhanced by addition of TFAA to the final solvent, but the RMR values given were obtained without TFAA addition, except for DOPA.

*** Cysteine normally does not appear in the chromatogram (only when inorganic salts were present during derivatization; see text).

§ The fully acylated derivatives are destroyed during evaporation of the acylating reagent; see text.

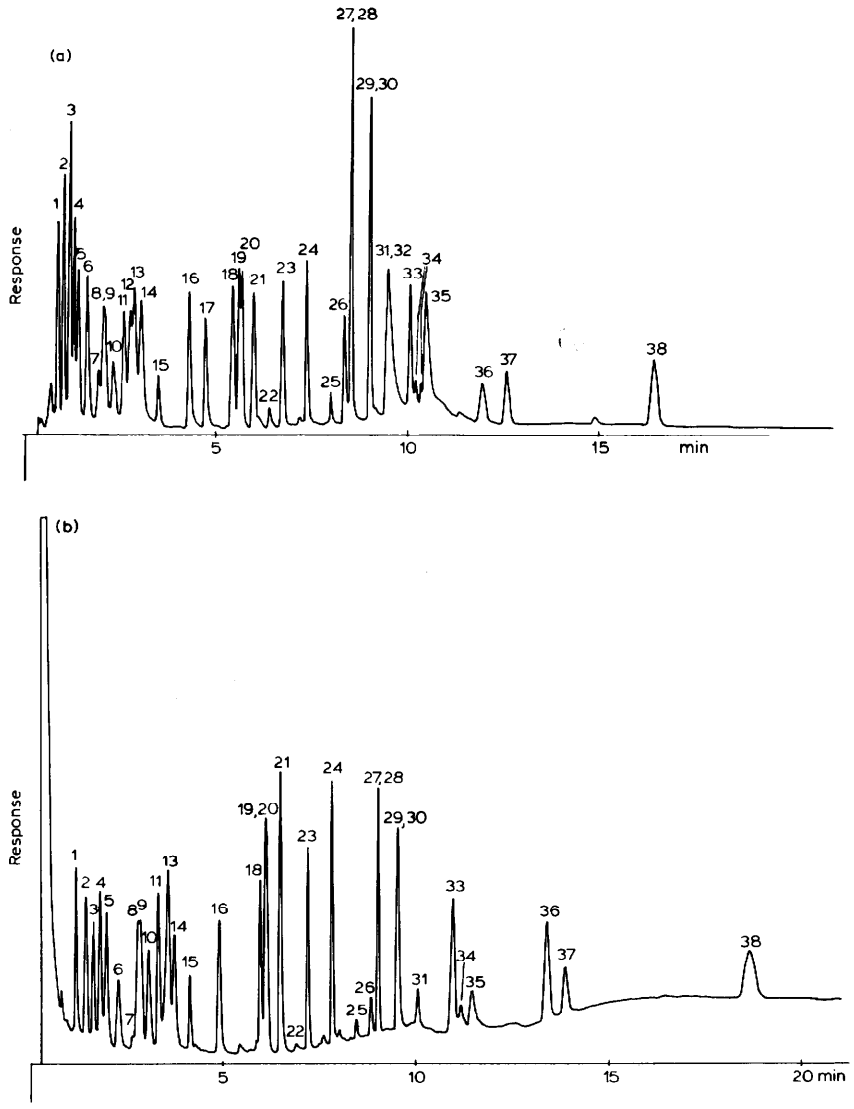

Fig. 1. Separation of standard solutions using (a) helium as carrier gas and NP-FID or (b) nitrogen and FID. Injection port temperature, 250°C; detector temperature, 300°C. Columns were both glass, 2 m × 2 mm I.D., filled with 0.65% EGA on Chromosorb W AW (80–100 mesh). Oven temperature profile: 115°C for 1 min, then increased at 3°C/min to 118°C followed by 15°C/min to 210°C. This temperature was held for 3 min, then increased at 10°C/min to 240°C, and this temperature was finally held constant for another 10 min. Chart speed, 1 cm/min; attenuation 2^5; volume injected, 3 μl. The standard solutions used contained about 0.25 nmole/μl of each amino acid except 3-Me-His (0.074 nmole/μl) and HLys (0.14 nmole/μl). The solution used in (a) contained additionally Pip, 1-Me-His and α-ACA. The carrier gas flow-rate was set at 20 ml/min for both columns. Peaks: 1 = α-AIBA; 2 = Ala; 3 = Sarc; 4 = α-ABA; 5 = Val; 6 = Gly; 7 = PEA; 8 = Ile; 9 = β-AIB; 10 = β-Ala; 11 = Leu; 12 = Pip; 13 = Norleu; 14 = Pro; 15 = Thr; 16 = γ-ABA; 17 = α-ACA; 18 = Met; 19 = Asp; 20 = HPro; 21 = Phe; 22 = Cit; 23 = Glu; 24 = α-AAA; 25 = Tyr; 26 = 3-Me-His; 27 = Orn; 28 = Arg; 29 = Lys; 30 = Homoarg; 31 = Trp-2; 32 = 1-Me-His; 33 = MetSulphon; 34 = HLys (*dl,dl-allo*); 35 = His-1; 36 = Trp-1; 37 = CTT; 38 = HomoCys.

The cysteine derivative is probably only formed when inorganic salts containing anions other than Cl^- are present during derivatization. Additionally, a peak with a very short retention time (RRT 0.22) appeared when cystine or cysteine were derivatized. This may be due to the formation of degradation products, as has been observed for cysteic acid when derivatized to the HFB isobutyl ester[26]. A peak with the same retention time as found for cysteine in a standard solution (derivatized in the presence of phosphate or carbonate salts) was often seen in normally treated urine samples. Reproducible analysis of cystine or cysteine needs further investigation as no adequate explanation can be offered for these observations.

The response of serine, tyrosine and DOPA is very low, owing to the hydrolysis of O-TFA bonds[27], but can be increased by addition of TFAA to the final solvent (as has been previously proposed[2]). Using 10% TFAA in ethyl acetate, the RMR for serine is 0.55 (FID) and 0.97 (NP-FID), and the RMR of tyrosine is 1.53 (FID) and 0.97 (NP-FID). DOPA was detected only when 10% TFAA and the NP-FID were used.

The poor response of citrulline is caused by its partial breakdown to ornithine[22], but is also influenced strongly by the addition of TFAA to the solvent; 10% TFAA led to the elution of a sharp peak, with an RMR of 0.51 at the position marked in the chromatograms and a second peak at the position of ornithine, which represented about 75% of the total area.

Tryptophan is probably partially destroyed, as mentioned previously[21,22], but incomplete formation of the diacyl derivative, which was studied by Moodie[7] for the HFB isobutyl ester, also accounts for the higher coefficients of variation. This will be discussed together with the derivatization studies.

The derivatives of arginine, homoarginine and histidine obviously lose one TFA group during evaporation of the acylating reagent. Nevertheless, a peak for histidine is eluted from the EGA column, the identity of which was tested by loading experiments. Probably this peak represents the monoacyl derivative (the diacyl derivative may be expected to have a shorter retention time, and a small peak near the position of ornithine was indeed seen at high histidine concentrations). That the TFA *n*-butyl ester of arginine does not elute on this column has already been reported[10]. A non-polar stationary phase can be used for the elution of the arginine TFA *n*-propyl ester (as shown in Fig. 3). Homoarginine was not tested on this column. Co-injection of TFAA was not successful in eluting arginine on EGA, as there were problems with the baseline and, therefore, quantitation when using the FID.

The separation speed is higher than with previously published methods. Compared with the results of Siezen and Mague[21], the analysis time is halved, whereas amino acid analysers based on ion-exchange chromatography take about eight times longer (120 min) for a similar separation. The analysis time can be further reduced when fewer amino acids are determined. Chromatographic conditions for the separation of 14 amino acids within 9 min are listed in Fig. 2.

Owing to the short separation time and the simplicity and cheapness of derivatization, this method is an improvement for the analysis of samples containing many non-protein amino acids, whereas for the analysis of protein hydrolysates other methods are preferable, owing to the failure of arginine and cystine to elute and the poor resolution of aspartic acid and hydroxyproline.

Detection

In most instances, the amino acid derivatives are detected with a flame-ionization detector. The HFB derivatives have also been detected with electron-capture detectors[5,6,28]. The use of a nitrogen-selective detector has been described by Adams *et al.*[29].

The NP-FID offers the advantage of giving signals only for compounds that contain nitrogen or phosphorus. When analysing biological samples by GLC, one must consider the possibility of compounds other than amino acids eluting in the same temperature range. This has been demonstrated for various sugars[30] using the HFB isobutyl esters. Of course, the best method for identifying an unknown compound is mass spectrometry combined with GLC, but this is often not available. A comparison of the FID and NP-FID signals of an unknown peak gives some information about the nature of the compound.

Another important feature is the approximately 100-fold enhancement of sensitivity using the NP-FID[29], allowing easy detection of picomole amounts. The sensitivity can be adjusted according to the nature of the analytical problem by varying the alkali element temperature, so that exact values of sensitivity enhancement cannot be given. Contamination and destruction of the alkali pearl result in lower sensitivity with time. However, readjustment is not necessary for several weeks when using internal standardization.

Finally, this type of detector also eliminates the solvent peak. This allows higher column oven starting temperatures, therefore reducing the analysis time.

The application of the NP-FID to the analysis of standard solutions is shown in Figs. 1a and 2, allowing a comparison of the FID and NP-FID signals.

Derivatization

Esterification. Esterification was performed according to Adams[14] with some modifications. A 3.5 *M* acetyl chloride solution in 1-propanol was used instead of 8 *M* hydrochloric acid, because this reagent is more convenient to prepare, and the high hydrochloric acid concentration did not offer any advantages. In addition, instability of 8 *M* acid has been noted[9]. The 8 *M* hydrochloric acid solution, which was used for comparison, was prepared as described previously[14].

The effect of hydrochloric acid concentration in 1-propanol was investigated by March[9], who found that esterification could be performed under various conditions with similar results. Lower hydrochloric acid concentrations must be compensated for by longer reaction times.

Adams[14] proposed the use of a water scavenger during esterification, but we observed no significant effect on responses. This agrees with previously reported results[9,31].

Acylation. No investigations on the acylation step of the *n*-propyl esters of amino acids appear to have been published. Therefore, we studied the effect of reaction time at a constant reaction temperature of 150°C and the effect of reaction temperature while keeping the reaction time constant at 6 min.

The influence of reaction temperature was investigated on a mixture of 17 protein amino acids and norleucine. Volumes of 200 µl were evaporated, esterified as described and acylated using acetonitrile as solvent. The derivatives were dissolved in 1 ml of ethyl acetate. For separation, the conditions employed were as described in

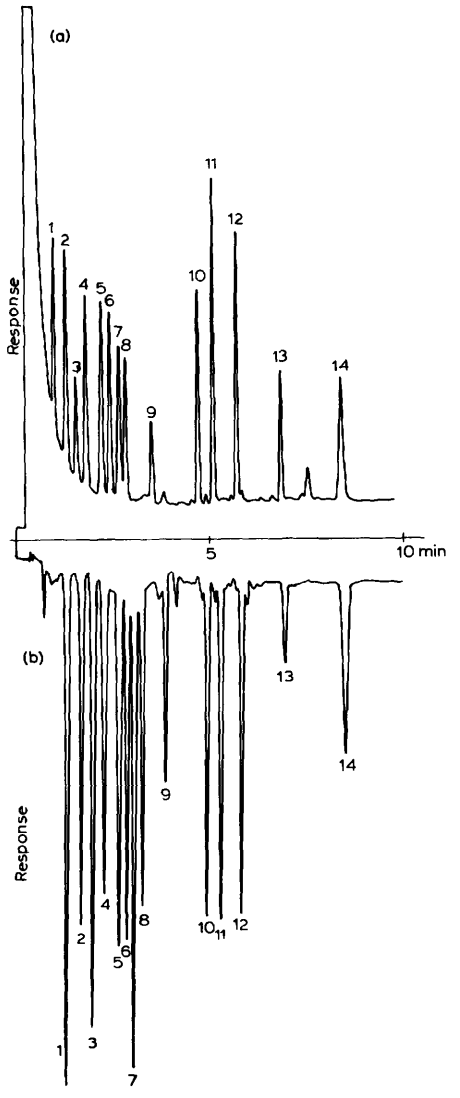

Fig. 2. Separation of thirteen protein amino acids within 9 min using a single-step temperature programme. Injection port temperature, 250°C; detector temperature, 300°C. Columns as described in Fig. 1. Carrier gas (helium) flow-rate, 30 ml/min. Oven temperature profile: 125°C for 1 min, then increased at 20°C/min to 210°C, and finally held constant for 5 min. Chart speed, 1 cm/min. The standard solution contained about 0.5 nmole/μl of each amino acid. The response of serine and tyrosine was enhanced by addition of 1% of TFAA to the ethyl acetate. (a) FID: attenuation 2^6; each peak represents 1.5 nmole. (b) NP-FID: attenuation 2^8; each peak represents 0.5 nmole. Peaks: 1 = Ala; 2 = Val; 3 = Gly; 4 = Ile; 5 = Leu; 6 = Norleu; 7 = Pro; 8 = Thr; 9 = Ser; 10 = Asp; 11 = Phe; 12 = Glu; 13 = Tyr; 14 = Lys.

Fig. 1, resulting in the appearance of only 16 peaks. Arginine and cystine cannot be analysed on this column, and histidine was not included in the standard solution.

The results are shown in Table II. Most of the amino acids are fully acylated at 30°C and probably also at room temperature after 6 min. Serine, tyrosine, lysine and tryptophan show increasing responses when using higher temperatures, whereas the

TABLE II

EFFECT OF REACTION TEMPERATURE ON THE ACYLATION OF THE *n*-PROPYL ESTERS OF AMINO ACIDS

RMR values calculated relative to norleucine are the means of four determinations, the coefficient of variation ranging between 2 and 6% in most instances. Trp represents the diacyl-*n*-propyl ester of tryptophan.

Amino acid	*Relative molar response*				
	30°C	*50°C*	*80°C*	*110°C*	*150°C*
Ala	0.68	0.71	0.66	0.66	0.63
Val	0.91	0.92	0.87	0.87	0.90
Gly	0.49	0.51	0.48	0.48	0.47
Ile	0.89	0.90	0.89	0.90	0.93
Leu	1.07	1.07	1.06	1.06	1.07
Pro	0.92	0.92	0.93	0.93	0.92
Thr	0.46	0.45	0.45	0.45	0.49
Ser	0.12	0.12	0.12	0.12	0.15
Met	0.83	0.82	0.81	0.81	0.79
Asp	1.07	1.09	1.08	1.09	1.07
Glu	1.13	1.14	1.12	1.13	1.14
Tyr	0.26	0.30	0.29	0.29	0.36
Lys	0.88	0.88	0.89	0.98	1.09
Trp	0.60	0.61	0.59	0.62	0.67

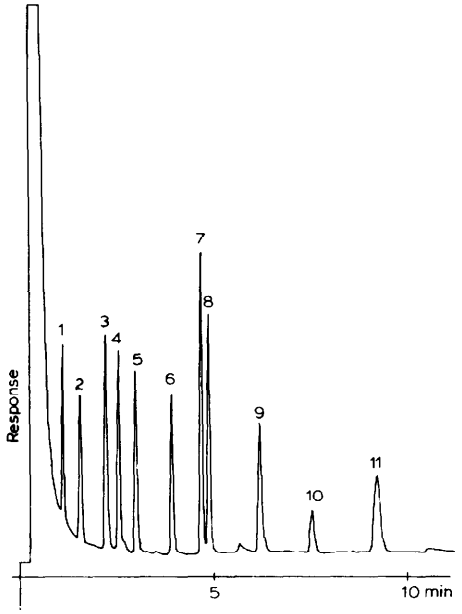

Fig. 3. Separation of ten amino acids on 2% OV-17 and 1% OV-210 for acylation studies. Injection port temperature, 220°C; detector temperature, 300°C. Column: glass, 2 m × 2 mm I.D., filled with 2% OV-17 and 1% OV-210 on Supelcoport (100–120 mesh). Carrier gas (helium) flow-rate, 30 ml/min. Oven temperature profile: 125°C for 0.5 min, then increased at 5°C/min to 135°C followed by 20°C/min to 220°C and finally held constant for 10 min. Chart speed, 1 cm/min; attenuation, 2^6; volume injected, 3 μl; amino acid concentration 0.5 *M* each. Peaks: 1 = Ala; 2 = Ser; 3 = Leu; 4 = Norleu; 5 = Pro; 6 = Met; 7 = Phe; 8 = Glu; 9 = Arg; 10 = Trp-2; 11 = Trp-1.

response of alanine and methionine decreases with higher temperatures. Although most amino acids are acylated at room temperature, 150°C seems to be necessary for the acylation of some. The same temperature is frequently used for trifluoroacetylation of *n*-butyl esters and has also been proposed for the acylation of isobutyl esters with HFBA[32].

The reaction time was varied from 1 to 90 min, keeping the reaction temperature at 150°C. Long exposure of the reaction mixture to this temperature resulted in a significant decrease in the molar responses of the amino acids tested. Therefore, times between 1 and 15 min were used in the final experiments. A mixture of ten representative amino acids, 2.5 m*M* each, (listed in Fig. 3) was derivatized as described and analysed on a column filled with 2% OV-17 and 1% OV-210 on Supelcoport under the conditions shown in the legend of Fig. 3. This column allows the elution of arginine. Histidine was investigated separately.

The results are shown in Fig. 4. The RMRs are nearly constant for many amino acids, whereas the MR and RMR decreases significantly for methionine and, to a lesser extent, for histidine. No peak for the methionine derivative was observed after 15 min of acylation.

Similar results have been obtained using HFBA for the acylation of the *n*-propyl derivatives[9] or the isobutyl derivatives[30]. Oxidative degradation of these amino acids when acylated for 12 min[9] or 10 min[30] could be avoided only by using an antioxydant (2,6-di-*tert.*-butyl-*p*-cresol, BHT) during acylation.

On the other hand, arginine and, to a lesser extent, serine are not fully acylated after short reaction times. At least 5 min must be used for the acylation of these amino acids.

Tryptophan gives two peaks, which probably belong to the mono- and diacyl derivatives. The relative area of the peak with the shorter retention time increases with longer acylation times, and therefore probably represents the diacyl derivative. The formation of two derivatives with the relative amount depending on acylation time (and probably also of TFAA concentration) explains, at least partially, the higher standard deviation found for the RMR of this amino acid.

Difficulties with the derivatization of tryptophan using the HFB isobutyl ester have been reported previously[30]. This was explained by insufficient levels of HFBA and "the presence of ethyl acetate" during acylation[7]. Other workers have reported reproducible analyses of the HFB isobutyl ester[32] and the acetyl *n*-propyl ester[14] of this amino acid.

From the investigations on the acylation step, it became clear that the proposed reaction conditions (150°C for 5 min) are a compromise rather than the optimal reaction parameters. Optimal conditions cannot be produced for all amino acids simultaneously, but those given above allow derivatization with fairly good results, providing constant reaction parameters.

The conditions proposed are very similar to those often used for trifluoroacetylation of the *n*-butyl esters; 150°C was also used for acylation of the *n*-propyl ester[9] for 12 min or the isobutyl esters[32] with HFBA for 10 min. The alcoholic group esterified to the carboxylic groups of the amino acids probably does not influence the acylation reaction significantly.

The overall procedure applied to the preparation of the TFA *n*-propyl derivatives of amino acids includes esterification with 3.5 *M* acetyl chloride in *n*-propanol for

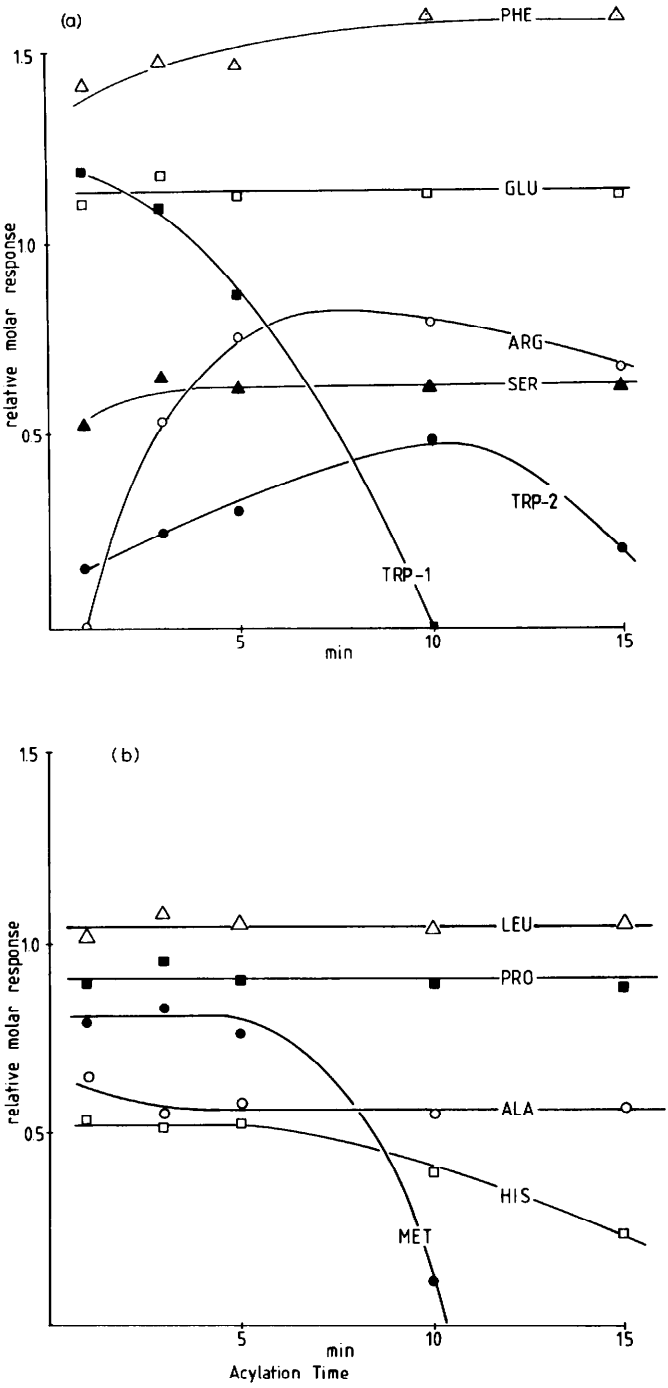

Fig. 4. Relative molar responses of the N(O,S)-TFA *n*-propyl esters of amino acids as a function of acylating time. Conditions as in Fig. 3. The values represent the means of two determinations.

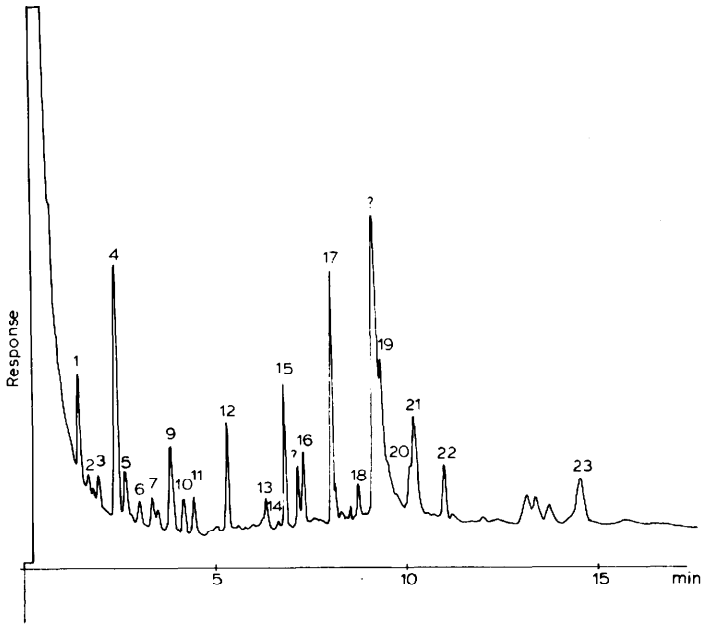

Fig. 5. Analysis of a normal urine sample. Derivatives prepared from 1 ml of urine, finally dissolved in 1 ml of ethyl acetate. Internal standard represents 0.5 nmole. Column as described in Fig. 1. Carrier gas (helium) flow-rate, 30 ml/min; injector temperature, 250°C; detector temperature, 300°C. Oven temperature profile: 115°C for 0.5 min, then increased at 1.5°C/min to 118°C followed by 15°C/min to 210°C and finally held constant for 10 min. Chart speed, 1 cm/min; attenuation, 2^6. Peaks: 1 = Ala; 2 = Sarc; 3 = Val; 4 = Gly; 5 = PEA; 6 = β-AIBA; 7 = β-Ala; 8 = Leu; 9 = Norleu; 10 = Pro; 11 = Thr; 12 = Ser; 13 = CysH?; 14 = Met; 15 = Asp, HPro; 16 = Phe; 17 = Glu; 18 = α-AAA; 19 = Tyr; 20 = 3-MetHis; 21 = Orn; 22 = Lys; 23 = His.

25 min at 110°C and acylation with 200 μl of TFAA and 400 μl of solvent at 150°C for 5 min. When analysed on a 0.65% EGA column these derivatives offer a good compromise among simplicity and cheapness of preparation, speed of separation and the information one can obtain about biological samples in a single run.

Examples of the application of the method to the analysis of human urine samples are given in Figs. 5 and 6. The abnormal amount of methylhistidines seen in Fig. 6 was verified by two-dimensional thin-layer chromatography, of course without exact quantitative comparison as quantitation of two-dimensional thin-layer chromatograms is not easily possible.

CONCLUSIONS

Analysis of the N(O,S)-TFA *n*-propyl esters of amino acids by GLC on a polar stationary phase proved to be a method well suited to the quantitative analysis of samples containing many non-protein and protein amino acids. Up to 33 amino acids can be separated in a single run within 17–19 min, thus providing a separation speed that could not be achieved by previously described methods. In addition, preparation of derivatives is at least as simple as for other frequently used methods involving esterification and acylation, but is the least expensive with respect to the chemicals

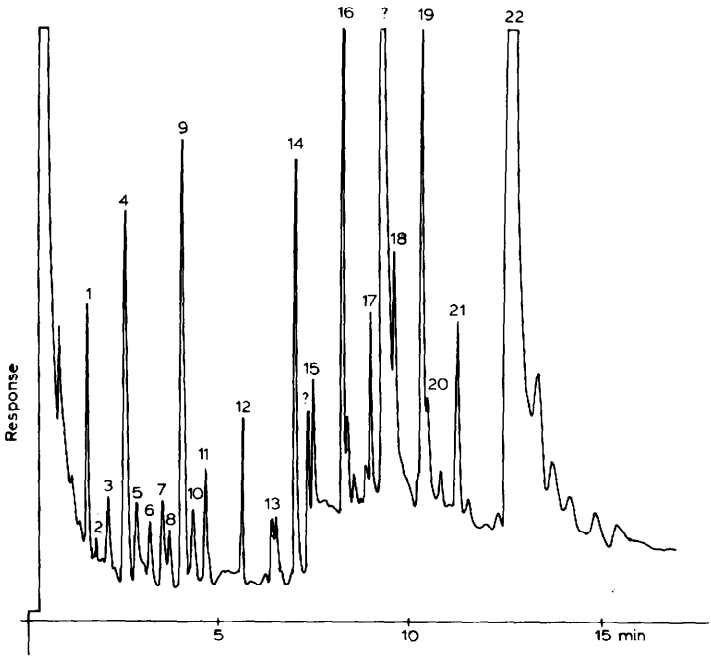

Fig. 6. Analysis of an abnormal urine sample. Conditions as in Fig. 5, except that the derivatives were finally dissolved in 0.5 ml of ethyl acetate, and the internal standard peak represents 2 nmole. Attenuation, 2^5. Peaks: 1–13 as in Fig. 5; 14 = Asp, HPro; 15 = Phe; 16 = Glu; 17 = α-AAA; 18 = Tyr; 19 = 3-MetHis; 20 = Orn; 21 = Lys; 22 = 1-MetHis.

employed. The use of a packed column results in a simplification over methods using capillary columns, whereas multi-step temperature programming is necessary for a reduction of separation time. A nitrogen-selective detector increases the sensitivity, permitting the easy detection of picomole amounts without the need for solvent-free injection techniques[33]. The disadvantage of the failure of some amino acid derivatives to elute can be overcome, at least partially, by the use of other stationary phases.

Considering the features summarized above, the application of this method to the analysis of samples containing many amino acids, particularly non-protein amino acids, or in screening programmes involving quantitative analysis of large numbers of samples should be advantageous.

ABBREVIATIONS

α-AIBA = α-aminoisobutyric acid; Sarc = sarcosine; α-ABA = α-aminobutyric acid; PEA = phosphoethanolamine; β-AIBA = β-aminoisobutyric acid; Pip = pipecolic acid; γ-ABA = γ-aminobutyric acid; α-ACA = α-aminocaprylic acid; HPro = hydroxyproline; α-AAA = α-aminoadipic acid; 3-Me-His = 3-methylhistidine; 1-Me-His = 1-methylhistidine; His-2 = histidine diacyl derivative; His-1 = histidine monoacyl derivative; Trp-2 = tryptophan diacyl derivative; Trp-1 = tryptophan monoacyl derivative; HLys-1 and -2 = *dl*- and *dl-allo*-hydroxylysine; DOPA = dihydroxyphenylalanine; MetSulphon = methionine sulphone; CTT = cysta-

thionine; TFAA = trifluoroacetic anhydride; HFBA = heptafluorobutyric anhydride; EGA = ethylene glycol adipate.

FID = flame-ionization detector; NP-FID = nitrogen–phosphorus-selective flame-ionization detector.

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