CHROM. 4136

GAS–LIQUID CHROMATOGRAPHY OF TRIFLUOROACETYLATED AMINO ACID METHYL ESTERS

DEVELOPMENT OF A MIXED STATIONARY PHASE FOR THEIR SEPARATION

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(Received April 24th, 1969)

SUMMARY

The gas–liquid chromatography of the trifluoroacetylated amino acid methyl ester derivatives is reported using the flame ionization detector. A mixed silicone stationary phase is shown to give isothermal separations, when the electron capture detector may also be used to obtain higher sensitivity. The application of temperature programming to separate all the amino acids is shown with protein hydrolyzates.

INTRODUCTION

The conversion of amino acids to volatile derivatives and methods for their subsequent separation using the technique of gas–liquid chromatography have been adequately discussed in recent reviews[1–3]. Using the trifluoroacetyl (TFA) amino acid *n*-pentyl ester derivatives DARBRE AND BLAU[4] were unable to chromatograph histidine and found arginine, tryptophan and cystine gave very long retention times when using isothermal conditions. COULTER AND HANN[5] avoided some of these difficulties by converting arginine to ornithine and histidine to aspartic acid before preparing the N-acetyl *n*-propyl ester derivatives of the amino acids for gas chromatography. They did not report on cystine.

We report here on the TFA protein amino acid methyl esters (see preliminary report by DARBRE AND ISLAM[6]). These derivatives are shown to be useful for both isothermal and temperature-programmed separations using a conventional packed column with a mixed silicone stationary phase.

MATERIALS AND METHODS

Apparatus

A MicroTek MT 220 (Techmation Ltd., 58 Edgware Way, Edgware, Middx., Great Britain) dual column gas chromatograph with flame ionization detectors was

used. Nitrogen (99.9% "white spot" from British Oxygen Co. Ltd., and "high-purity oxygen-free" from Air Products Ltd.) was used as carrier gas.

Materials

Chemicals were obtained as follows: α-amino isobutyric acid, heptadecane and tridecanoic acid (Koch-Light Laboratories Ltd., Colnbrook, Bucks., Great Britain); L(+)-arginine hydrochloride, L-phenylalanine and L-proline (Sigma Chemical Co., St. Louis, Mo., U.S.A.); L-isoleucine (*allo*-free) (Calbiochem. A.G., Lowengraben 14, 6002 Luzern, Switzerland); DL-methionine (Hopkin & Williams Ltd., Chadwell Heath, Essex, Great Britain); L-cysteine hydrochloride, L-cystine, L-histidine monohydrochloride, L-hydroxyproline, L-threonine, L-tyrosine, other DL-amino acids, octanoic, palmitic and stearic acids, insulin and trifluoroacetic anhydride (BDH Chemicals Ltd., Poole, Dorset, Great Britain).

Acid-processed pig skin gelatin was a gift from Dr. J. E. EASTOE, Dental Science Department, Royal College of Surgeons, London, W.C.2, Great Britain.

Stationary phases were obtained as follows: DC LSX 3-0295, neopentyl glycol succinate HI-EFF-3BP, XE-60 (Applied Science Labs., P.O. Box 140, State College, Pa., U.S.A.); Versilube F44 (a gift from General Electric, Silicone Products Dept., Waterford, N.Y., U.S.A.); LP-109 S.F. F-50, LP-104 polyphenyl ether OS-138 (6-ring) (F. & M. Scientific Division, Hewlett-Packard Ltd., 224 Bath Road, Slough, Bucks., Great Britain); MS-200, 100cS, MS-550, MS-710 (Hopkin & Williams Ltd.); OV-17 (Supelco Inc., P.O. Box 628, Bellefonte, Pa. 16823, U.S.A.); QC-2-0093 (Dow Corning Corp., Midland, Mich., U.S.A.); QF-1 (Midland Silicones Ltd., Barry, Glamorgan, Great Britain); SE-30, E-301 (I.C.I. Ltd., Stevenson, Ayrshire, Great Britain). Neopentyl glycol sebacate was prepared in the laboratory[7].

Our thanks are due to Dr. K. BLAU for some samples.

Support materials were obtained as follows: Anakrom, ABS (Analabs, through Fisons Scientific Apparatus Ltd., Loughborough, Leics., Great Britain); Celite 545, Celite 560, Silocel C22 firebrick (Koch-Light Laboratories); Chromosorb G, Supasorb (BDH Chemicals Ltd.); Chromosorb W, Diatoport S (F. & M. Scientific Division, Hewlett-Packard Ltd.); Diatomite CQ (W.G. Pye Ltd., P.O. Box 60, Cambridge, Great Britain); Gas-Chrom Q (Applied Science Labs.).

Coating of supports

For screening purposes support materials were coated with stationary phase using a 1-l round-bottomed flask with dimples as previously described[4]. Diatoport S 80–100 mesh was made powder-free by fluidization[8]. When the mixed stationary phase was developed methods of coating the Diatoport S were examined to find the one which gave highest efficiency. The method finally adopted was to cover the Diatoport S with butan-2-one in a flat-bottomed dish and to add the appropriate amount of a 1% stock solution of the silicones in butan-2-one. Evaporation was achieved by keeping the dish at about 60° whilst blowing warm air from a hair drier across the surface. The mixture was occasionally stirred. Residual solvent was removed *in vacuo* at about 80°. The method of blending[4] gave no better results with this mixed phase.

Packing of columns

Glass columns 3.25 m long with 2.5 mm internal diameter were used. Wider bore tubing did not give such good separations. The glass was pretreated and packing carried out as previously described[4]. The first 5 cm of the column at the injection end was not packed, but some pretreated glass yarn[4] was loosely inserted. The column was conditioned at 230° overnight with a flow of nitrogen, before connecting the outlet end to the detector. The efficiency of the column depended almost entirely on the coating of the support material and very little on the method adopted to pack the tube. Columns were unpacked and repacked several times using variations in the packing technique. There was never more than a total loss of efficiency of 5–10% and this was probably due to the production of fines. Despite the attempts to standardize procedures (see COULTER AND HANN[5]) it was not possible to eliminate variable efficiencies between columns, although the relative retention times for the derivatives remained unchanged. More than thirty columns with lengths varying from 2 m to 6 m and giving between 500 and 820 theoretical plates per foot were prepared over a period of two years. The optimum carrier gas flow rate was 15 ml/min.

Preparation of derivatives

For screening purposes the derivatives were chromatographed singly or in pairs. Protein samples were hydrolyzed with 6 *N* HCl in sealed tubes at 105° for 20 h. The acid was removed by means of the rotary evaporator. The dried residues from protein hydrolyzates were treated in the same way as single amino acids for making the TFA methyl ester derivatives[6].

RESULTS AND DISCUSSION

The TFA amino acid methyl esters are more polar than the corresponding *n*-pentyl ester derivatives and they tailed badly on many support materials. Table I

TABLE I

THEORETICAL PLATE HEIGHTS OBTAINED WITH SUPPORTS EACH COATED WITH THE SAME MIXED SILICONE PHASE

A = acid washed; B = base washed; S = silanized. HETP = height equivalent to theoretical plate. Instrument details are given in Fig. 1; various oven temperatures.

Support material	*Treatment*	*Mesh size*	*HETP (mm)*
Anakrom	ABS	80–90	1.2–0.9
Celite 545	ABS	72–80	1.5–1.0
Celite 560	ABS	72–80	1.0–0.7
Celite 560	A	72–80	3.8–3.0
Chromosorb G	AS	70–80	3.0–1.5
Chromosorb W	AS	70–80	1.0–0.8
Diatomite CQ	AS	100–120	1.0–0.8
Diatoport S	S[a]	80–100	0.6–0.4
Gas-Chrom Q	ABS	80–100	1.0–0.8
Silocel C22	AS	72–85	0.7–0.6
Supasorb	AS	80–100	1.2–0.9

[a] Other treatment not known.

gives the range of theoretical plate heights obtained with columns having support materials coated 2.5% and 5% w/w with the mixed silicone stationary phase XE 60–QF-1–MS-200, 100 cS, in the proportions 46:27:27 (w/w) respectively[6] and packed under identical conditions in the same glass column. The column efficiencies[9] were measured in each case for the alanine, leucine, and phenylalanine derivatives. Diatoport S gave the highest efficiency with minimum tailing. This was the support material used by HAGEN AND BLACK[10] for the same derivatives. However, glycine still tailed (see DARBRE AND BLAU[7]). Celite 560 ABS was the next best material and tailing was not severe. Celite 560 acid washed only gave low efficiencies, although GEHRKE and co-workers utilized successfully acid-washed Chromosorb W for the TFA amino acid *n*-butyl esters[11]. Silocel C22 caused severe tailing.

Because some of the TFA amino acid methyl esters are so volatile and losses occurred when concentrating a solution containing these derivatives[7,12] we investigated various methods of evaporating the solvent. A mixture of the amino acid methyl ester hydrochlorides in stoppered B14 test tubes was treated with 0.2 ml TFA anhydride at room temperature for 20 min and the excess reagent then evaporated. The method finally adopted to avoid losses was to use the rotary evaporator with an oil pump with the tube immersed in ice-water at 0°. Table II gives the results from an experiment in which the residual TFA amino acid methyl esters were taken up in 0.2 ml butan-2-one containing biphenyl internal standard. Changes in the peak height ratios of amino acid to biphenyl were recorded. From Table II it is seen that there were no significant losses after 12 min. Repeated experiments in which 0.2 ml of methylene chloride–TFA anhydride (4:1) was evaporated showed no losses up to 10 min. It must be stressed that the pressure measured with a mercury Pirani gauge during the evaporation was 10 torr. As a precaution preliminary experiments are therefore advisable. Most of the excess liquid was removed within 2 min and in practice we restricted the evaporation time to 4 min.

In certain cases it may be possible to dissolve the internal standard in the methylene chloride–TFA anhydride mixture[11] and by injecting an aliquot of this solution directly on to the column no evaporation is required, but we found that the TFA anhydride gave rise to long tailing solvent peaks.

Table III lists the retention times of the seven most volatile protein TFA amino acid methyl esters relative to the leucine derivative on fourteen different stationary

TABLE II

PEAK HEIGHT RATIOS OF TFA AMINO ACID METHYL ESTERS TO BIPHENYL INTERNAL STANDARD AFTER EVAPORATION OF EXCESS TFA REAGENT FOR INCREASING PERIODS OF TIME

Each figure is the average for two determinations and represents the peak height ratio of the amino acid derivative to biphenyl internal standard.

TFA amino acid methyl ester	*Time (min) on rotary evaporator at 0°*			
	1	*3*	*6*	*12*
α-Amino isobutyric acid	1.35	1.36	1.34	1.32
Alanine	0.78	0.80	0.79	0.77
Valine	0.93	0.95	0.94	0.92
Glycine	0.28	0.27	0.28	0.28
Isoleucine	0.58	0.59	0.59	0.58
Leucine	0.60	0.60	0.61	0.60

TABLE III

RELATIVE RETENTION DATA FOR TFA AMINO ACID METHYL ESTERS

The figures are retention times relative to the leucine derivatives taken as 1.0. The actual retention time in minutes for this derivative is given in brackets. The support material was Diatoport S 80–100 mesh coated with 2.5% w/w stationary phase (1.25% w/w for NPGS and NPGSeb) packed into 3.25 m × 0.25 cm I.D. glass columns. Nitrogen gas flow, 15 ml/min.

Liquid phase	*Column temperature (°C)*	*N-TFA amino acid methyl ester*					*N,O-di-TFA methyl ester*		*HETP Leu peak (mm)*
		Ala	*Val*	*Gly*	*Ile*	*Leu*	*Thr*	*Ser*	
DC-LSX	100	0.42	0.65	0.49	0.95	(23.8)	0.96	1.24	0.6
F-44	60	0.24	0.53	0.28	1.00	(36.1)	0.50	0.56	1.1
F-50	80	0.35	0.61	0.40	1.04	(16.2)	0.56	0.59	0.8
PPE	80	0.33	0.50	0.54	0.90	(25.7)	0.40	0.53	1.7
MS-200	80	0.32	0.61	0.33	1.05	(16.0)	0.58	0.63	0.6
MS-550	80	0.37	0.59	0.41	0.99	(18.0)	0.53	0.65	1.4
MS-710	80	0.38	0.58	0.46	0.98	(18.6)	0.47	0.63	1.3
OV-17	80	0.38	0.62	0.50	1.04	(17.0)	0.48	0.63	1.0
QC-2	80	0.37	0.62	0.45	0.97	(27.0)	1.07	1.33	2.5
QF-1	100	0.44	0.68	0.51	0.99	(16.0)	1.05	1.31	1.4
SE-30	80	0.33	0.62	0.38	1.05	(12.5)	0.60	0.64	0.7
XE-60	110	0.44	0.55	0.72	0.76	(11.8)	0.89	1.54	0.9
NPGS	100	0.47	0.53	0.94	0.78	(16.5)	0.79	1.75	0.6
NPGSeb	110	0.37	0.48	0.63	0.72	(20.7)	0.69	1.47	0.9

TABLE IV

RELATIVE RETENTION DATA FOR TFA AMINO ACID METHYL ESTERS

Interpretation of data and column details as for Table III.

Liquid phase	*Column temperature (°C)*	*N-TFA methyl ester*	*N,O-di-TFA methyl ester*	*N,S-di-TFA methyl ester*	*N-TFA methyl ester*	*N-TFA di-methyl ester*	*N,O-di-TFA methyl ester*	*N-TFA methyl ester*	*N-TFA di-methyl ester*	*N-TFA methyl ester*
		Leu	*Ser*	*CySH*	*Pro*	*Asp*	*Hyp*	*Met*	*Glu*	*Phe*
DC-LSX	110	0.24	0.28	0.46	0.56	0.49	1.12	0.81	(19.0)	1.03
F-44	100	0.24	0.21	0.36	0.47	0.46	0.63	0.99	(21.3)	1.74
F-50	110	0.33	0.20	0.45	0.55	0.50	0.67	1.05	(15.0)	1.80
PPE	110	0.13	0.06	0.20	0.45	0.37	0.39	0.89	(51.5)	1.55
MS-200	110	0.30	0.22	0.43	0.49	0.48	0.68	1.00	(17.3)	1.73
MS-550	100	0.21	0.14	0.31	0.50	0.43	0.62	0.96	(35.0)	1.69
MS-710	120	0.21	0.17	0.32	0.60	0.48	0.57	0.97	(17.0)	1.65
OV-17	110	0.19	0.13	0.28	0.57	0.45	0.54	0.95	(27.3)	1.65
QC-2	110	0.22	0.25	0.39	0.47	0.43	1.03	0.72	(30.2)	1.01
QF-1	120	0.25	0.32	0.46	0.53	0.47	1.16	0.83	(26.8)	1.05
SE-30	110	0.45	0.27	0.44	0.53	0.50	0.70	1.00	(11.3)	1.71
XE-60	130	0.17	0.26	0.54	0.34	0.43	0.76	0.99	(27.3)	1.12
NPGS	140	0.17	0.23	0.44	0.28	0.43	0.50	0.84	(21.2)	1.10
NPGSeb	140	0.17	0.21	0.50	0.24	0.37	0.51	1.00	(38.0)	1.38

TABLE V

RELATIVE RETENTION DATA FOR TFA AMINO ACID METHYL ESTERS

Interpretation of data and column details as for Table III.

Liquid phase	*Column temperature (°C)*	*N-TFA methyl ester*	*N,O-di-TFA methyl ester*	*N,N'-di-TFA methyl ester*			*N-tri-TFA methyl ester*	*N,N'-di-TFA methyl ester*	*N-TFA methyl ester*	*N,N'-di-TFA di-methyl ester*
		Phe	*Tyr*	*Orn*	*Lys*	*Try*	*Arg*	*His*[1]	*His*[2]	*CySScy*
DC-LSX	190	0.26	0.50	0.63	(18.0)	1.42	1.89	0.47	0.89	3.06
F-44	170	0.75	1.00	0.73	(4.0)	3.12	1.63	1.65	2.38	5.00
F-50	180	0.76	1.06	0.76	(3.3)	2.60	1.54	1.51	2.12	3.94
PPE	180	0.59	0.69	0.63	(8.0)	3.22	1.34	2.35	3.63	6.00
MS-200	170	0.80	1.02	0.75	(5.6)	2.83	1.54	1.20	2.06	4.30
MS-550	180	0.70	0.96	0.71	(4.4)	3.02	1.48	2.00	2.89	5.87
MS-710	170	0.61	0.80	0.63	(7.1)	3.12	1.38	2.46	3.95	6.12
OV-17	180	0.62	0.83	0.73	(5.8)	2.94	1.15	2.56	3.45	5.80
QC-2	180	0.30	0.60	0.70	(10.0)	1.46	1.93	1.00	1.20	3.43
QF-1	200	0.32	0.63	0.78	(6.4)	1.41	1.77	1.00	1.22	3.02
SE-30	180	0.78	1.00	0.72	(4.5)	2.89	1.55	1.96	2.80	4.23
XE-60	200	0.17	0.33	0.72	(15.0)	1.05	1.40	0.60	1.77	3.74
NPGS	200	0.25	0.40	0.76	(12.0)	1.34	2.50	0.71	2.60	5.40
NPGSeb	220	0.31	0.49	0.76	(10.3)	1.70	—[a]	—	—	4.50

[a] —, sample applied: no peak obtained.

phases. Only silicone phases with good thermal stability and which gave satisfactory peaks are reported here. Non-silicone, polar stationary phases are suspected to catalyze the breakdown of O-TFA and S-TFA groups[13] and they were not studied extensively. Polyphenyl ether has high thermal stability and gave similar retention times to the silicones. The results for the two polyester phases NPGS and NPGSeb are included because they have been used for the separation of TFA amino acid methyl ester[14–17] and TFA amino acid *n*-butyl ester[11,18] derivatives. In Table III XE-60 is the only silicone phase which behaves like NPGS and NPGSeb in separating isoleucine and leucine and also in having such a long retention time for serine.

Table IV shows the retention times of eight amino acids relative to glutamic acid. Leucine and serine are included here in order that the retention times quoted in Table III may be related to those of glutamic acid. XE-60 shows great similarity to NPGS and NPGSeb. DC-LSX, QC-2 and QF-1 may be noted for the long retention time of the hydroxyproline derivative, which carries two TFA groups. In practice it is difficult to separate methionine and glutamic acid and the best stationary phases for this purpose are DC-LSX, QC-2, QF-1 and NPGS. A comparison with data for the TFA amino acid *n*-pentyl ester derivatives[19,20] shows that the size of the ester group affects greatly the order of emergence of some amino acids from the column. Whereas with the methyl esters phenylalanine has a longer retention time than glutamic acid in the case of the *n*-pentyl esters phenylalanine always emerges from the column before

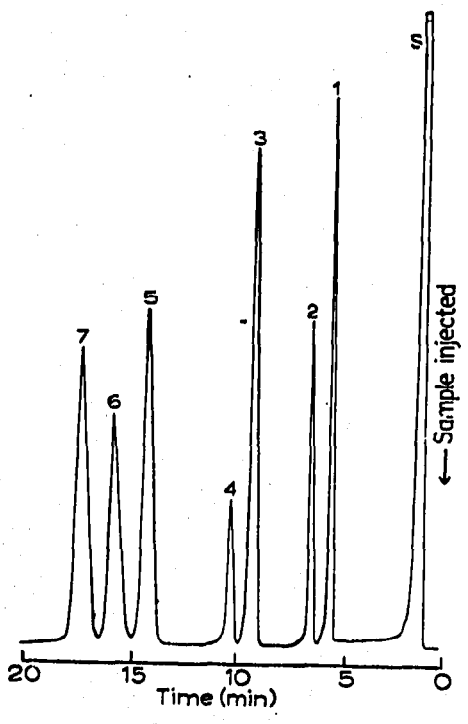

Fig. 1. Isothermal separation of a mixture of seven TFA amino acid methyl esters. The amino acids were mixed in equimolar proportions prior to derivative formation (stages 1 and 2a (ref. 6)). Instrument and conditions: MicroTek MT 220 fitted with dual flame ionization detectors; glass columns, 3.25 m × 2.5 mm I.D. packed with 2.5% (w/w) mixed stationary phase XE-60–QF-1–MS-200, 100cS, in the proportions 46:27:27 (w/w) respectively on Diatoport S 80–100 mesh; inlet heater block temperature, 230°; detector block temperature, 250°; nitrogen gas flow, 15 ml/min; sample size, 1 μl; attenuation, $10^2 \times 8$; oven temperature, 110°. 1 = α-amino isobutyric acid; 2 = alanine; 3 = valine; 4 = glycine; 5 = isoleucine; 6 = threonine; 7 = leucine. S = solvent.

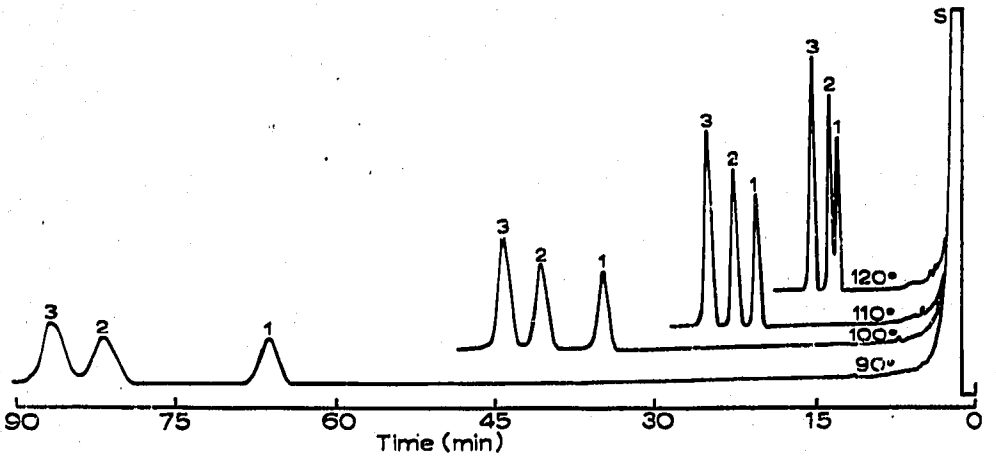

Fig. 2. Effect of temperature on the isothermal separation of N-TFA isoleucine, N,O-di-TFA threonine and N-TFA leucine methyl esters. Instrument and conditions as in Fig. 1 except for oven temperature as indicated. Peaks: 1 = isoleucine; 2 = threonine; 3 = leucine. S = solvent.

glutamic acid. Also the methyl ester derivatives of hydroxyproline and methionine appear after aspartic acid: the reverse is true with the *n*-pentyl derivatives.

Table V records the retention times for seven amino acids relative to lysine. Ornithine is included because it is reported to be a breakdown product of arginine[21]. Histidine always gives two peaks[6]. Cystine shows the expected long retention time.

The best single silicone stationary phase for separating most of the amino acids

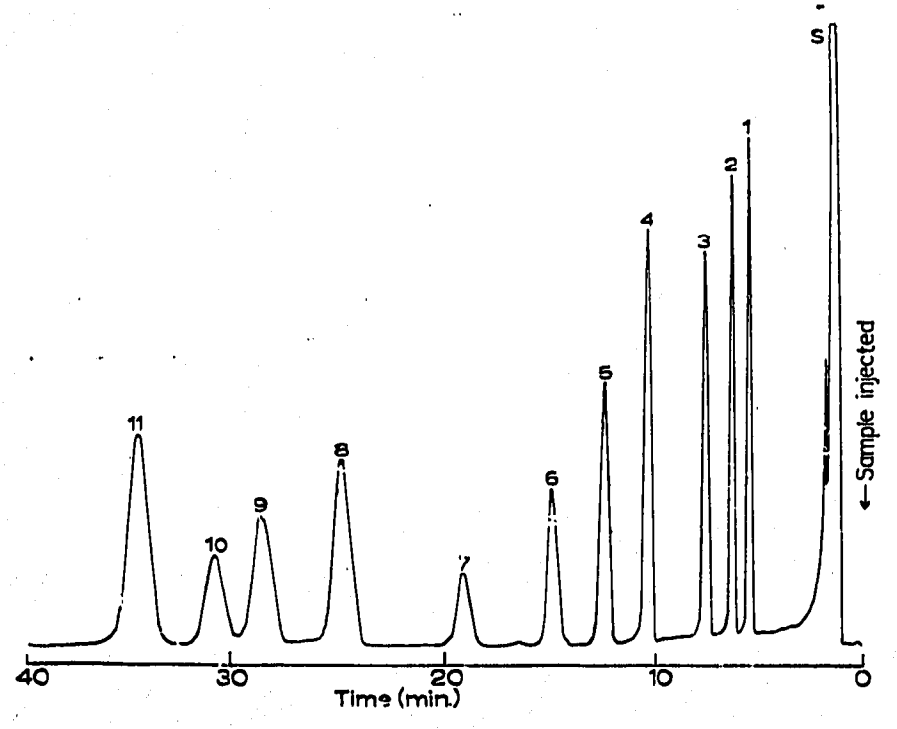

Fig. 3. Isothermal separation of a mixture consisting of equimolar proportions of eleven TFA amino acid methyl esters. Derivative formation, instrument and conditions as in Fig. 1 except oven temperature, which was 135°. 1 = leucine; 2 = norleucine; 3 = serine; 4 = proline; 5 = aspartic acid; 6 = cysteine; 7 = homoserine; 8 = hydroxyproline; 9 = methionine; 10 = glutamic acid; 11 = phenylalanine. S = solvent.

was XE-60, but incomplete resolution was obtained between glycine and isoleucine, between methionine, glutamic acid and phenylalanine and between lysine and tryptophan. By plotting the relative retention times in the manner previously described[4] it was found that a mixture of XE-60 and QF-1 in the proportions 60:40 (w/w) respectively gave the best resolution. MS-200 was then introduced in order to improve the separation between glutamic acid and phenylalanine. The best results were finally obtained with XE-60–QF-1–MS-200, 100 cSt in the proportions 46:27:27 (w/w) respectively[6]. A column length of 3.25 m was sufficient to give adequate separation. Diatoport S gave best results when coated 2.5% (w/w) with the mixed phase. Columns did not lose their resolving power easily and lasted for many months, when the maximum oven temperature was 230° for relatively short periods. Using lower concentrations of stationary phase the overall gas chromatography time was reduced but the resolution of some peaks was impaired.

Fig. 1 shows the isothermal separation of the N-TFA amino acid methyl esters of α-amino isobutyric acid, alanine, valine, glycine, isoleucine and leucine and N,O-di-TFA threonine methyl ester. When separating these protein amino acids, either α-amino isobutyric acid or octanoic acid methyl ester may be used as internal standards. The group isoleucine–threonine–leucine was difficult to resolve completely but this was aided by careful selection of the column temperature. The effect of changing column temperature on these three amino acids is shown in Fig. 2. Changing the carrier gas flow rate had little effect on their separation factors.

Fig. 3 shows the isothermal separation of N-TFA leucine, norleucine, proline,

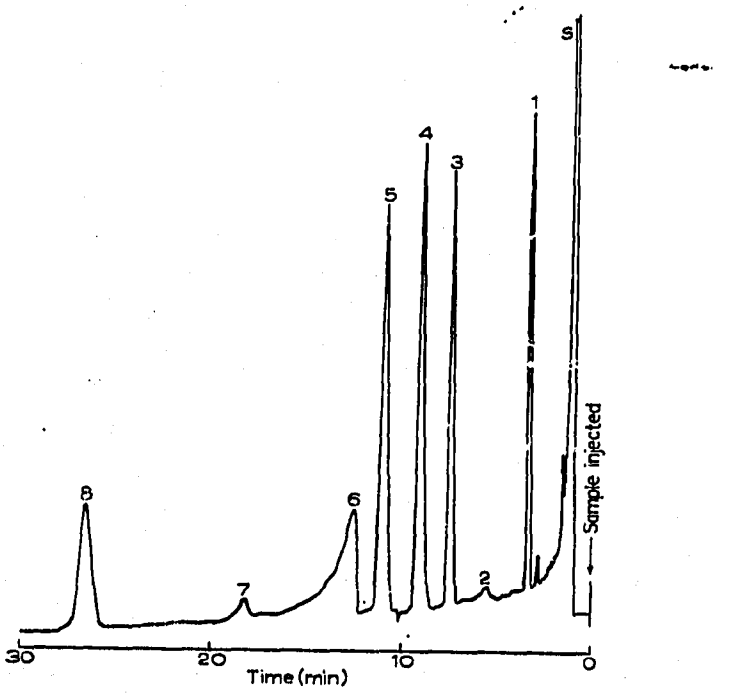

Fig. 4. Isothermal separation of a mixture of TFA amino acid methyl esters. Histidine and cystine were in 2 *M* ratio to the other amino acids. Derivative formation stages 1 and 2b (ref. 6). Instrument and conditions as in Fig. 1 except oven temperature 210° and attenuation as shown. 1 = O,N-di-TFA tyrosine; 2 = N,N′-di-TFA histidine; 3 = N,N′-di-TFA lysine; 4 = N,N′-di-TFA tryptophan; 5 = N-tri-TFA arginine; 6 = mono-TFA histidine; 7 = mono-TFA tryptophan; 8 = N,N′-di-TFA cystine. S = solvent.

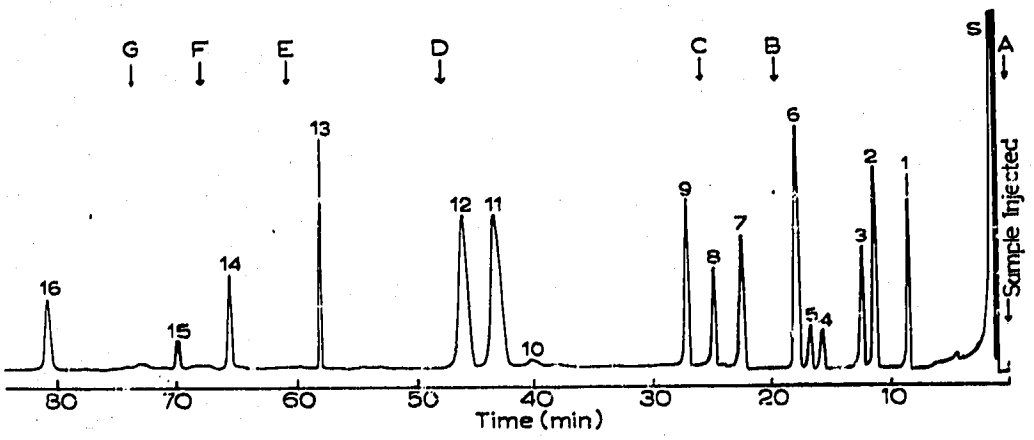

Fig. 5. Temperature-programmed separation of TFA amino acid methyl esters prepared from a hydrolyzate of insulin. Instrument and conditions as in Fig. 1. Attenuation $10^2 \times 8$ except valine and leucine $10^2 \times 16$. 1 μl injected contained derivatives equivalent to a total of approximately 50 μg amino acids. Programming details are as follows: (A) initial temperature, 100°, programmed at 1°/min; (B) programmed at 3°/min; (C) held at 140°; (D) programmed at 5°/min; (E) held at 210°; (F) programmed at 5°/min; (G) held at 230°. Peaks: 1 = alanine; 2 = valine; 3 = glycine; 4 = isoleucine; 5 = threonine, 6 = leucine; 7 = serine; 8 = proline; 9 = aspartic acid; 10 = unknown; 11 = glutamic acid; 12 = phenylalanine; 13 = tyrosine; 14 = lysine; 15 = arginine; 16 = cystine. S = solvent.

methionine and phenylalanine methyl esters, N-TFA aspartic acid and glutamic acid dimethyl esters and N,O-di-TFA serine, homoserine and hydroxyproline and N,S-di-TFA cysteine methyl esters. The most difficult group to resolve consisted of methionine, glutamic acid and phenylalanine and the overall efficiency of the column may be judged by their separation. In the absence of homoserine, phenylglycine, heptadecane or tridecanoic acid methyl ester may be used as internal standards.

The separation of the least volatile protein amino acids is shown in Fig. 4. The gas chromatography of histidine is unsatisfactory[6], but whether this is due to prob-

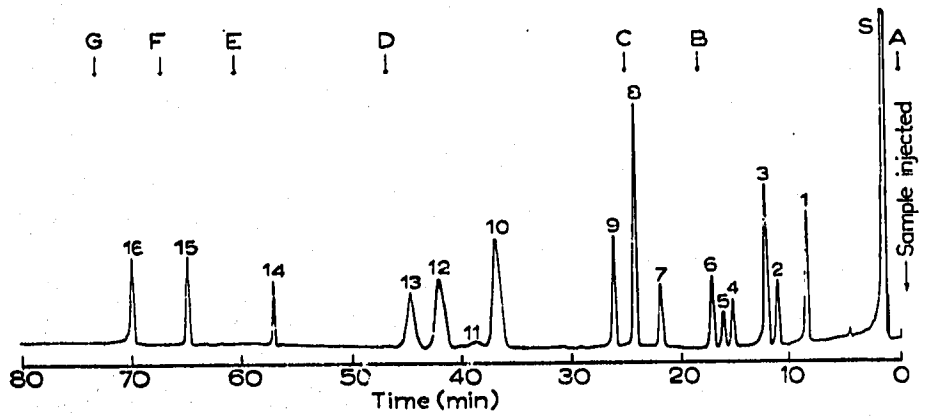

Fig. 6. Temperature-programmed separation of TFA amino acid methyl esters obtained from gelatin hydrolyzate. Instrument and conditions as in Fig. 1. Attenuation $10^2 \times 8$, except alanine, glycine, proline $10^2 \times 16$; phenylalanine, tyrosine $10^2 \times 4$. 1 μl injected contained derivatives equivalent to a total of approximately 45 μg amino acids. Programming details as in Fig. 5. Peaks: 1 = alanine; 2 = valine; 3 = glycine; 4 = isoleucine; 5 = threonine; 6 = leucine; 7 = serine; 8 = proline; 9 = aspartic acid; 10 = hydroxyproline; 11 = unknown; 12 = glutamic acid; 13 = phenylalanine; 14 = tyrosine; 15 = lysine; 16 = arginine + hydroxylysine. S = solvent.

lems of derivatization or to gas chromatography conditions has not been established. We were unable to eliminate the appearance of the first histidine peak due to the di-TFA derivative by injecting methanol or *n*-butanol onto the column[22]. A peak corresponding to mono-TFA tryptophan methyl ester sometimes appears as shown in Fig. 4. This is due either to incomplete trifluoroacetylation or to partial breakdown of the di-TFA derivative. Methyl palmitate or methyl stearate may be used as internal standards. They emerge from the column before and after tyrosine respectively.

Temperature programming was used for the separation of a synthetic mixture of TFA amino acid methyl esters[6] and is here shown for a hydrolyzate of insulin in Fig. 5 and acid-processed pig skin gelatin in Fig. 6 (compare GEHRKE *et al.*[22]). No peaks were obtained for histidine from either insulin or gelatin. Hydroxylysine which is present in this gelatin sample is not resolved from arginine in our separation. A quantitative analysis of the gelatin using a modified Moore and Stein technique was

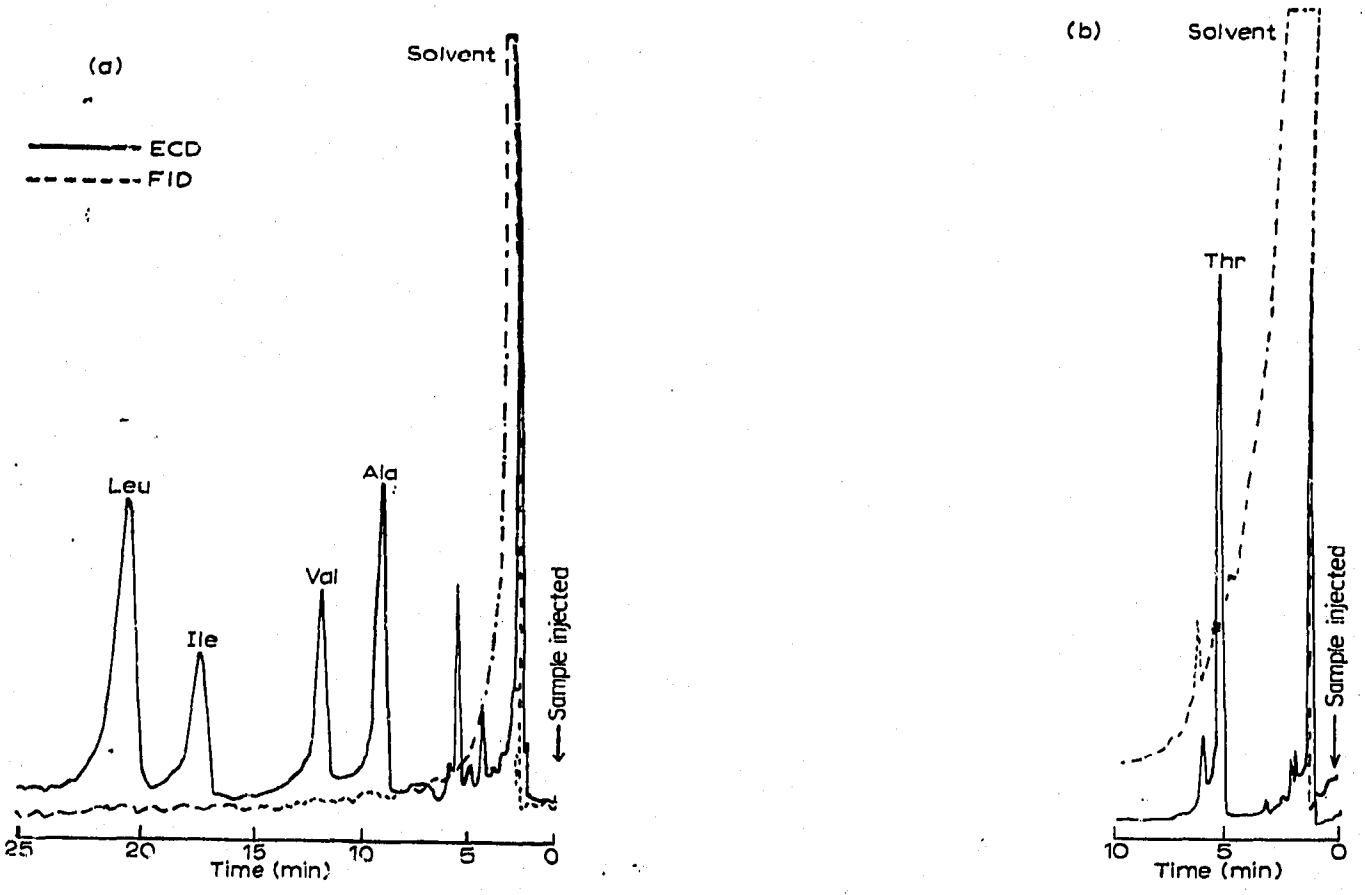

Fig. 7. (a) Separation of TFA alanine, valine, isoleucine and leucine methyl esters showing simultaneous monitoring by flame ionization and electron-capture detectors. Instrument and conditions: Pye Series 104, Model 24. ^{63}Ni ECD set at 200° with pulse interval 50 μsec. Glass column 3 m with 2.5 mm I.D. packed with Celite 560 ABS 72–80 mesh coated with mixed silicone stationary phase (2.5% w/w). Outlet splitter for N_2 carrier gas with 1:1 ratio; sample size 0.1 μl; butan-2-one solvent; oven temperature, 120°. Rikadenka 1 mV twin-pen recorder. ———, ECD amplifier setting 100 × 10^{-12} A for f.s.d.; — — —, FID 20 × 10^{-12} A for f.s.d. (b) Gas chromatogram of N,O-di-TFA threonine methyl ester with simultaneous monitoring by FID and ECD. Details as in Fig. 7a, except sample size 1 μl; oven temperature, 150°. ———, ECD amplifier setting 500 × 10^{-12} A for f.s.d.; — — —, FID 10 × 10^{-12} A for f.s.d.

published by EASTOE[23]. An unknown compound appears before methionine in Figs. 5 and 6.

The increasing use of the electron capture detector (ECD) has led to its application to fluoro compounds. Fig. 7a shows the isothermal separation of an equimolar mixture of N-TFA-alanine, valine, isoleucine and leucine methyl esters. The effluent gas stream was split in 1:1 ratio and simultaneously monitored by flame ionization detector (FID) and ECD using a twin-pen recorder. The limit of detection for the FID was approximately 20 mμg at the amplifier setting used (20×10^{-12} A for full scale deflection) and no amino acid derivative was detected. However, the peaks obtained with the ECD indicated 100–200 times greater sensitivity of detection. The peaks showing on the ECD recording and which appear before alanine were probably due to TFA artifacts. The solvent butan-2-one always gave some response due to traces of halogen.

Fig. 7b shows a similar FID and ECD recording for N,O-di-TFA threonine methyl ester. The ECD shows a sensitivity of detection about 3 500 times greater than does the FID for this compound. This is due to the presence of two TFA groups on the molecule. CLARKE *et al.*[24] reported on similar findings with TFA amine derivatives.

ACKNOWLEDGEMENTS

This work was generously supported by grants from the Medical Research Council, the Science Research Council and the University of London Central Research Fund. A.I. is supported by a grant from the Wellcome Trust. We are grateful to Professor H. R. V. ARNSTEIN for his support and encouragement.

The simultaneous recording by flame ionization and electron capture detectors was carried out in the Laboratory of the Government Chemist, Department of Scientific and Industrial Research, Stamford Street, London, S.E.1, by courtesy of Mr. E. A. WALKER, to whom we are greatly indebted for help and advice.

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