GAS CHROMATOGRAPHY OF VOLATILE AMINO ACID DERIVATIVES

I. ALANINE, GLYCINE, VALINE, LEUCINE, ISOLEUCINE, SERINE AND THREONINE

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INTRODUCTION

Gas chromatographic methods of analysis are increasingly being used for biological problems because of their speed and convenience, and their application to the analysis of the amino acids is receiving much attention. In particular it is of great potential value for peptide analysis.

The conversion of the amino acids to suitable volatile derivations has been achieved in a number of ways: conversion of the amino acids to the corresponding aldehyde by the action of ninhydrin[1–5], decarboxylation to the corresponding amines[6], conversion of the amino acid to the α-chloro derivative[7] and to the α-hydroxy derivative[8,9]. Amino acid methyl esters have also been used[10,11]. A number of N-trimethylsilyl amino acid trimethylsilyl esters were prepared and separated by RÜHLMANN AND GIESECKE[12]. N,N-Dimethyl amino acid methyl esters have been investigated[13]. YOUNGS used the N-acetyl amino acid *n*-butyl esters[14] while the N-acetyl *n*-amyl esters (and the N-acetyl esters of some of the lower alcohols) were studied extensively by JOHNSON, SCOTT AND MEISTER[15] and by SHLYAPNIKOV, KARPEISKY AND LITVIN[16]. GRAFF, WEIN AND WINITZ[17] prepared the N-acetyl *n*-propyl esters. LOSSE, LOSSE AND STÖCK[18] prepared and separated some N-formyl amino acid methyl esters. The N-trifluoroacetyl (TFA) amino acid methyl esters were prepared by a number of workers[19–25], whilst N-TFA *n*-butyl esters were used by ZOMZELY, MARCO AND EMERY[26]. Like ourselves[13,27], TEUWISSEN, LENAIN, DORLET AND LEONIS[28] obtained best results with N-TFA amino acid *n*-amyl esters. PISANO, VANDENHEUVEL AND HORNING[29] chromatographed a number of phenylthiohydantoin derivatives of amino acids, as well as their N-2,4-dinitrophenyl methyl esters (see also refs. 30, 31).

Since the ultimate objective is to develop a method which is capable not only of identifying but also of estimating the amino acids in a mixture, quantitative aspects have had prior consideration in our choice of derivatives. The final choice was based on considerations of (a) applicability to all the common α-amino acids, (b) volatility, (c) yield, (d) ease of separation and (e) practical convenience in making and handling the derivatives.

The present work reports the results of an investigation of the most volatile of the derivatives used by us (the TFA amino acid *n*-amyl esters) namely those of alanine, glycine, valine, leucine, isoleucine, serine and threonine.

Temperature programming was not used, because the nature of the detector, which we consider to be the most satisfactory for quantitative work, restricted us to isothermal separations. This has involved a search for a stationary phase which enables these seven derivatives to be separated unequivocally and without oves-lapping of peaks. All phases we have tried are mentioned, since some of the novel oner might prove useful in other applications, being suitable in all senses except the specific retention properties needed for this work, while others can usefully be excluded from further consideration.

MATERIALS AND METHODS

Abbreviations

DDS = dichlorodimethylsilane

H.E.T.P. = height equivalent to a theoretical plate calculated from the formula:

$$h = 330 \left(\frac{\text{width at half height}}{\text{relative retention time}}\right)^2 \text{ for the columns used.}$$

PVP = polyvinylpyrrolidone

TFA = trifluoroacetyl.

Apparatus

A D6 gas chromatograph (Griffin & George Ltd., Alperton, Middlesex, Great Britain) with a ga-density balance detector was used. Columns consisted of two stainless steel tubes of 5 mm I.D., connected at their lower end by a stainless steel capillary U-tube. Packed length was 182 cm. Nitrogen (99.9 % "White spot", British Oxygen Co., Wembley, Middlesex, Great Britain) was used as carrier gas. Integrations were carried out with an Instron Integrator (Instron Ltd., High Wycombe, Bucks. Great Britain).

Preparation of the packed columns

(*a*) *General.* It was found necessary to pretreat supports to prevent tailing of the peaks. Glass beads were tried, but the peaks obtained on such packings showed poor efficiency, even when fine Celite (Johns-Manville "Super-floss", L. Light & Co. Ltd., Colnbrook, Bucks.) was added[32]. Fluoropak 80 used as a support had the same disadvantage. Celites (Johns-Manville grades 545 and 560; L. Light & Co. Ltd.) and crushed Silocel C22 firebrick (L. Light & Co. Ltd.) were tried as supports, all deactivated with dimethyldichlorosilane by the method of SJÖVALL, MELONI AND TURNER[33]. Of these, C22 firebrick was best, both in terms of peak efficiencies and also because much higher ratios of stationary phase could be used without the packing losing its free flowing quality. Deactivation with hexamethyldisilazane[34], diphenyldichloro-silane, polyvinylpyrrolidone (PVP) and "Saran" were also tried, the last two using the method of VANDENHEUVEL, GARDINER AND HORNING[35]. PVP gave slightly higher peak efficiencies compared with the method of SJÖVALL *et al.*[33], but appeared to catalyse the decomposition of the serine derivative and probably the threonine derivative (see Table IV and Discussion). "Saran" was not very effective for deac-

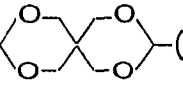
tivating the C22 firebrick. Diphenyldichlorosilane was as good as dimethyldichlorosilane, but less convenient in practice, since a toluene solution of phenol had to be used for reacting with residual chloro groups, and the excess phenol had to be washed out with toluene.

(*b*) *Preparation of the support.* Silocel C22 firebrick was crushed in a mortar, and graded dry using British Standard test sieves and a mechanical shaker (Endecotts (Filters) Ltd., Lombard Road, London, S.W. 19). The graded fractions were soaked in conc. HCl overnight and washed with acid until free of iron. After washing with distilled water until the washings were free of chloride, the support was dried in an oven at 100°. The individual acid-washed fractions were then re-graded before deactivation by the method of SJÖVALL *et al.*[33]. While peak efficiencies were improved by either acid washing or DDS deactivation, the highest efficiencies were obtained when both were carried out.

(*c*) *Stationary phases.* QF-1 (fluorosilicone fluid FS 1265, 10,000 cs) was obtained from Midland Silicones Ltd., Barry, Glamorgan. Other MS silicones (Midland Silicones Ltd.) were obtained from Hopkin & Williams Ltd., Chadwell Heath, Essex. F-50 (methyl chlorophenyl silicone) and XE-60 (cyanoethyl silicone gum) were obtained from F & M Scientific Europa N.V., Leidsestraat 67, Amsterdam, M & B "Embaphase" (dimethyl polysiloxane) from May and Baker, Ltd., Dagenham, Essex, and SE 30 (dimethyl silicone elastomer E 301) from I.C.I. Ltd., Stevenston, Ayrshire. SG (stopcock grease) and HVG (high vacuum grease) (Edwards High Vacuum Ltd.) are generally available; HVG was purified by the method of NELSON AND MILUN[36]. BDA (butane-1,4-diol–adipic acid polyester) and BDS (butane-1,4-diol–succinic acid polyester) were prepared by the method of FARQUHAR, INSULL, ROSEN, STOFFEL AND AHRENS[37]. Hi-Eff-8B (cyclohexane dimethanol–succinic acid polyester) was obtained from Applied Science Laboratories Inc., P.O. Box 140, State College, Pa., U.S.A. DEGA (diethylene glycol–adipic acid polyester), NPGS (neopentyl glycol–succinic acid polyester), NPGG (neopentyl glycol–glutaric acid polyester) and NPG Seb (neopentyl glycol–sebacic acid polyester) were prepared by the method of FARQUHAR *et al.*[37]. PVP (polyvinylpyrrolidone) was obtained from British Drug Houses Ltd.; "Saran" (Saran resin F 220) from R. W. Greeff & Co. Ltd., 31 Gresham St., London E.C. 2. PEG-A (polyethylene glycol adipate) was obtained from W. G. Pye & Co. P.O. Box 60, Cambridge, and PPS (polypropylene sebacate) and EGS (ethylene glycol distearate) from Griffin & George Ltd. GMHS (glyceryl monohydroxystearate), GML (glyceryl monolaurate), GMO (glyceryl monooleate), GMR (glyceryl monoricinoleate), GMS (glyceryl monostearate), WOL (polyglycerol–polyricinoleic acid polyester), Admul 19 (polyglycerol ester of mixed fatty acids) and Admul S 57 (polyglycerol ester of hardened tallow fatty acids) were obtained from Advita Ltd., New Zealand House, Wellington Avenue, Walton-on-Thames. WOL and Admul S57 were purified by suspending in aqueous acetone and boiling with 1 g of Bio-deminrolit (Permutit Ltd.) per 2 g of polyglycerol ester. After filtration and washing the resin with aqueous acetone the solvent was removed from the combined filtrates on a rotary evaporator. The residues were dried in a vacuum desiccator. TWEEN 60 and 61 (polyoxyethylene Sorbitan monostearate), TWEEN 65 (polyoxyethylene Sorbitan tristearate), TWEEN 80 and 81 (polyoxyethylene Sorbitan monooleate), TWEEN 85 (polyoxyethylene Sorbitan trioleate), SPAN 20 (Sorbitan monolaurate), SPAN 40 (Sorbitan monopalmitate), SPAN 60 (Sorbitan monooleate)

and SPAN 85 (Sorbitan trioleate) were obtained from L. Light & Co. Ltd.

Acetyl SPAN 80 was prepared from SPAN 80 by leaving it for 72 h dissolved in 10 volumes of acetic anhydride. Excess of the anhydride was removed under vacuum and the residue was used as Acetyl SPAN 80.

U 1 (triphenyl germanium oxide), U 2 (tri-*n*-hexylgermanium oxide), U 3 $[\text{-Ge}(C_6H_5)\text{-}C_6H_4\text{-Ge(Me)}_2\text{-}C_6H_4]_n$, U 4 (dibutyltin sulphide), U 5 (tetra-*β*-cyanoethyl-tin), U 6 (dibutyltin dilaurate), U 7 (tetra-*n*-octyl-tin), U 8 $[Sn(C_6H_5)_2\text{-CH=CH-}(CH_2)_6\text{-CH=CH}]_n$, U 9 $[Sn(C_4H_9)_2\text{-CH=CH-}(CH_2)_5\text{-CH=CH}]_n$, U 10 $[Sn(C_3H_7)_2\text{-}CH_2\text{-CH}(C_6H_5)]_n$, U 11 $[Sn(C_6H_5)_2\text{-}(CH_2)_3\text{—O-CO-}CH_2\text{-O-}CH_2\text{COO-}(CH_2)_3]_n$ and U 12 $[Sn(C_6H_5)_2\text{-}(CH_2)_2$  $(CH_2)_2\text{—}]_n$ were donated by Dr. J. W. MARSMAN of Utrecht University. Tetraphenyltin was obtained from Eastman Organic Chemicals (through Kodak Ltd., Kirkby, Liverpool) and TPMT (triphenyl-methyl-tin) was donated by Dr. G. A. LUIJTEN of Utrecht University. Methyl cyclohexyl titanate and triethanolamine titanate were donated by Mr. P. BROOKS of Griffin & George Ltd., triethanolamine titanate stearate was purchased from Griffin & George Ltd., and tetrastearyl titanate from K & K Chemicals Inc. (through Kodak Ltd.). DSPO (Duo-Seal pump oil, Pyror S.A., Geneva) was supplied through the courtesy of Camlab (Glass) Ltd. Cambridge. Ethanolamine adipate was prepared according to the method of FARQUHAR *et al.*[37]. EGCNSS-S(ethylene glycol succinate–cyanoethyl silicone copolymer) and EGSS-X (ethylene glycol succinate–methyl silicone copolymer) were obtained from Applied Science Laboratories Inc. KEL-F No. 90 grease (chloro-fluorocarbon, Minnesota Mining & Mfg. Co.) was obtained from British Drug Houses Ltd., Poole, Dorset. PEG 1540 and PEG 20M (polyethylene glycols) were obtained from Union Carbide Ltd. Grafton Street, London, W.1. PEG-T (TFA Carbowax PEG 20M) was made by treating 1 g PEG 20M dissolved in 20 ml trifluoroacetic acid with 5 ml of trifluoroacetic anhydride for 18 h at room temperature. Removal of excess reagents *in vacuo* gave 2.1 g of a viscous syrup, which was kept in a vacuum desiccator. TTP (tri-*o*-tolyl phosphate) was obtained from British Drug Houses Ltd. PEG-L (polyethylene glycol lauryl ether) was obtained from Honeywill & Stein Ltd., Mayfair Place, London W.1. Versamid 900 (polyamide from dimerised linoleic acid and ethylene diamine) was obtained from F & M Scientific Europa N.V., and TAS 10 (tetra-*o*-tolyl silicate) from Monsanto Chemicals Ltd., Victoria St., London S.W.1., was prepared from TAS 10A, a solution in decalin, by removing the solvent by vacuum distillation. TAS 190 (phenyl resorcinyl silicate), TAS 1,000 (tetraphenyl silicate) and HT-180S (phenyl-triphenoxy-silane) were obtained from Hygrotherm Engineering Ltd., Pebblecombe Research Station, Pebblecombe Hill, Dorking Road, Tadworth, Surrey.

(*d*) *Coating the support.* The correct weight of stationary phase was dissolved in 200 ml of a suitable solvent in a 500 ml round-bottomed flask with four dimples round the side. The correct weight of deactivated support was added, and the solvent removed under vacuum on a rotary evaporator (at low speed to avoid abrasion of the support particles).

(*e*) *Packing the columns.* Columns were cleaned by passing successive plugs of fine grade steel wool through them until the surface was burnished, and finishing off with successive plugs of cotton wool, first soaked in benzene and subsequently dry. Packings were poured continuously through a funnel into each arm of a column which

was kept vibrating by means of an electric motor. Final settling was achieved by manual tapping with a piece of wood. A plug of glass yarn (900-5/5 HT sewing thread, Fibreglass Ltd., 63, Piccadilly, London) was put at each end of the column, which was checked for leaks before insertion in the machine, and then conditioned for at least 12 h at the temperature of operation with a slow flow of nitrogen passing through it.

Preparation of the volatile derivatives

(*a*) *TFA amino acid methyl esters.* 2 mg of the amino acid was placed in a B 14 test tube with 2 ml methanol. The tube was kept in an oil bath at 70° and a continuous stream of dry HCl gas was passed through the alcohol through a Pasteur pipette for 20 min. The alcohol was evaporated using a rotary evaporator and a hot water bath. To the residue, 0.1 ml TFA anhydride was added, and the tube stoppered and left at room temperature for one hour. Excess of the anhydride was removed on the rotary evaporator at room temperature. The N-TFA amino acid methyl ester was taken up in 100 or 200 μl methyl ethyl ketone or nitromethane.

The preferred method was to treat the amino acid with 0.1 ml of TFA anhydride in a B 14 test tube, and to leave the tube stoppered at room temperature for one hour. The excess anhydride was removed as before, and to the residue was added 1–2 ml of an ethereal solution of diazomethane (CARE: carcinogenic agent), prepared by the method of DE BOER AND BACKER[38]. After about 10 min, excess ethereal reagent was removed on a warm water bath before dissolving up the residue as above.

(*b*) *Ethyl, propyl and butyl esters.* The ethyl ester was made like the methyl ester, only at 80° instead of 70°, while 108° was used for all higher aliphatic alcohols.

(*c*) *Benzyl esters.* These were made using phenyldiazomethane prepared either by the method of YATES AND SHAPIRO[39] or of OVERBERGER AND ANSELME[40]. The benzyl esters were then treated with TFA anhydride as above.

(*d*) *Amyl esters.* The method of JOHNSON *et al.*[15] was tried, using HCl gas instead of HBr, and was found to give variable yields. Poor yields were obtained when a strongly acidic resin was used as catalyst[23]. The method used by ZOMZELY *et al.*[26] was tried with amyl alcohol and di-*n*-amyloxy-propane[41–43] but was not very successful. Finally the method outlined in (b) above was used, passing HCl gas at 108° for 25 min. The alcohol was removed on a rotary evaporator under vacuum (oil-pump), and the amino acid ester hydrochloride trifluoroacetylated as described above. The 99 % 1-pentanol used was obtained from Union Carbide Chemicals Co., Texas City, Texas.

RESULTS

General

The retention times of four amino acids as their N-acetyl and N-TFA ester derivatives are given in Table I on both a polar and a non-polar stationary phase. It can be seen that the retention time increases as the ester group becomes larger. Also, the non-polar QF-1 column shows shorter retention times than the polar PEG-A, whilst the N-acetyl derivatives are always much slower than the corresponding N-TFA derivatives[44,45]. It will be noted that glycine is disproportionately retarded on PEG-A relative to the other three amino acids. This anomaly has been repeatedly observed

TABLE I

RETENTION DATA FOR N-TFA AND N-ACETYL AMINO ACID ESTERS

The figures are the retention times relative to the leucine derivative taken as 1.00. The actual retention time in minutes for this derivatives is given in brackets.
Column temp. 150°. Flow rate 38 ml/min. 5% PEG-A and 5% QF-1 on HCl-washed, DDS-treated 150–200 mesh Silocel C22.

Amino acid ester	*5% PEG-A*	*5% QF-1*	*Amino acid ester*	*5% PEG-A*	*5% QF-1*
N-TFA Ala methyl	0.61	0.63	N-Acetyl Ala methyl	0.52	0.49
N-TFA Val methyl	0.63	0.70	N-Acetyl Val methyl	0.67	0.84
N-TFA Gly methyl	1.21	0.62	N-Acetyl Gly methyl	0.86	0.51
N-TFA Leu methyl	(10.6)	(7.9)	N-Acetyl Leu methyl	(66.7)	(29.3)
N-TFA Ala ethyl	0.67	0.53	N-Acetyl Ala ethyl	0.53	0.47
N-TFA Val ethyl	0.69	0.63	N-Acetyl Val ethyl	0.66	0.73
N-TFA Gly ethyl	1.28	0.63	N-Acetyl Gly ethyl	0.90	0.55
N-TFA Leu ethyl	(10.6)	(9.4)	N-Acetyl Leu ethyl	(70.0)	(31.7)
N-TFA Ala amyl	0.62	0.51			
N-TFA Val amyl	0.60	0.73			
N-TFA Gly amyl	1.39	0.59			
N-TFA Leu amyl	(31.4)	(26.6)			

when comparing relative retention times for glycine on polar and non-polar phases (see also DORLET[45]). It may be that the active hydrogen atoms on the α-carbon of glycine interact with the groupings that confer polar character on the stationary phase. Since the TFA group is more electronegative than the acetyl group one might expect this effect to be accentuated with the N-TFA derivatives of glycine, and indeed Table I shows that for these compounds the increased activity of the hydrogens on the α-carbon is reflected in the increased relative retention times on PEG-A.

The peaks obtained on chromatography of the N-TFA amino acid methyl esters were always satisfactory, and since these derivatives are the most volatile of those shown in Table I they afforded the most likely chance for working at moderate temperatures, which was of potential value particularly for the derivatives of the less volatile amino acids. However, a study of quantitative yields in the preparation of the derivatives of the more volatile amino acids revealed such wide variation that it was evident that extensive losses were occurring, which could be traced to the process of evaporating the excess ethereal diazomethane after esterification of the N-TFA amino acid. This occurred with the derivatives of alanine, valine, glycine, leucine and isoleucine. In the case of the alanine derivative, sublimation was observed as it was being evaporated to dryness in a rotary evaporator under vacuum. The high vapour pressure of N-TFA amino acid esters was used by WEYGAND and his school[20,46] to achieve partial resolution into groups by vacuum sublimation.

Table II shows the results of an experiment in which the volatility of the N-TFA esters of alanine are compared. Known amounts of the derivatives were kept in a gentle stream of gas at room temperature for varying times, and the percentage of the starting material that remained was determined by gas chromatography. It is clear that losses occur under these conditions, and indeed, by using a more vigorous stream of gas, N-TFA alanine methyl ester may be evaporated completely, and extensive losses occur even with the corresponding derivatives of glycine and leucine.

TABLE II

LOSSES OF AMINO ACID DERIVATIVES OWING TO VOLATILITY

Pretreatment: Argon 250 ml/min, diameter of jet 1.1 mm, distance from ester 3 cm in a tube 1.0 cm I.D.
Column temp. 108°. Flow rate 38 ml/min. 1 % PEG-A on 100–120 mesh HCl-washed, DDS-treated Celite 560.

N-TFA alanine ester	*Percentage recovery after exposure to stream of gas*		*Retention time (min)*
	5 min	*20 min*	
Methyl	66	21	3.1
Ethyl	61	6	3.2
n-Propyl	100	61	4.9
n-Butyl	96	73	7.0
n-Amyl	99	93	11.5

Only the *n*-amyl esters give a reasonable assurance that losses will not occur during handling. It was found to be safe to use a rotary evaporator for the removal of solvents.

The cyclohexyl esters of the N-TFA amino acids always gave multiple peaks for each amino acid. The benzyl esters, prepared by either of the two methods quoted[39,40] contained, in addition to the expected derivative, bibenzyl and another unidentified volatile compound. The production of more than one peak from each amino acid is confusing, and therefore these derivatives were not used.

By restricting ourselves to the TFA amino acid amyl esters, and by removing excess solvents on the rotary evaporator no losses by evaporation were detected during the preparation of the volatile derivatives.

NOTES ON TABLES III TO VII

Table III

The relative retention times for the phases in this table are broadly similar in pattern, although a number of sub-groups may be recognised: those like SE-30 (MS 200, SG, MS Antifoam A) and those like MS 550 and F-50. The pattern of MS 710 is more like that of the silicates TAS 10 and HT-180S in Table VII.

Using the relative retention time for the glycine derivatives as an approximate guide to the polarity of a phase, XE-60 may be seen to be the most polar, and SE-30 the least polar of the silicones. By this criterion many phases mentioned in later tables are more polar than XE-60. The pattern on XE-60 resembles those for the polyesters in Table IV. Serine and threonine have very similar retention times on MS 115, MS 200, M & B and F-50. Leucine and isoleucine run together on many of these silicones. The relative retention times of the aromatic compounds on QF-1 are the lowest we have found.

Efficiencies obtained with SG, HVG and MS Antifoam A were low.

Table IV

The patterns of relative retention times on these phases varied widely, and no

TABLE III

RELATIVE RETENTION DATA FOR SILICONE TYPE STATIONARY PHASES

The figures are retention times relative to the leucine derivative taken as 1.00. The actual retention time in minutes for this derivative is given in brackets.

Liquid phase	*% w/w liquid–solid*	*Column temp. (°C)*	*Gas flow (ml/min)*	*N-TFA amino acid n-amyl ester*					*O,N-DiTFA n-amyl ester*		*Biphenyl*	*Bibenzyl*	*H.E.T.P. (mm) Leu peak*
				Ala	*Val*	*Gly*	*Ileu*	*Leu*	*Thr*	*Ser*			
MS 115	5	130	38	0.41	0.69	0.47	1.00	(47.8)	0.59	0.65	0.66	1.33	0.9
MS 200	5	140	38	0.44	0.73	0.49	1.01	(16.6)	0.57	0.61	0.66	1.23	1.4
MS 550	2	141	38	0.45	0.67	0.57	1.04	(16.9)	0.57	0.68	0.86	1.67	1.2
MS 710	5	159	45	0.48	0.70	0.62	1.00	(10.5)	0.46	0.60	1.21	n.r.	1.7
M & B	5	130	40	0.41	0.68	0.49	1.03	(17.8)	0.63	0.69	0.69	1.32	1.0
F-50	5	150	40	0.48	0.51	0.78	1.15	(13.2)	0.58	0.61	0.85	n.r.	0.9
QF-1	5	150	38	0.51	0.73	0.59	0.60	(26.6)	0.88	1.09	0.30	0.45	0.7
SE-30	5	132	38	0.39	0.68	0.46	1.01	(37.0)	0.54	0.64	0.64	1.23	1.1
XE-60	5	150	38	0.53	0.61	0.92	0.82	(26.5)	0.81	1.47	0.37	0.67	1.1
S.G.	2	132	38	0.48	0.67	0.55	1.17	(10.8)	0.62	0.74	0.66	1.20	2.4
H.V.G.	2	130	38	0.52	0.72	0.57	1.00	(11.5)	0.63	0.71	0.71	1.19	1.7
MS Anti-foam A.	2	132	38	0.44	0.69	0.53	1.11	(9.0)	0.63	0.72	0.59	1.11	2.0

n.r. = compound not run.

TABLE IV

RELATIVE RETENTION DATA FOR POLYESTER-TYPE PHASES

The figures are the retention times relative to the leucine derivative taken as 1.00. The actual retention time in minutes for this derivative is given in brackets.

Liquid phase	*% w/w liquid–solid*	*Column temp. (°C)*	*Gas flow (ml/min)*	*N-TFA amino acid n-amyl ester*					*O,N-DiTFA n-amyl ester*		*Biphenyl*	*Bibenzyl*	*H.E.T.P. (mm) Leu peak*
				Ala	*Val*	*Gly*	*Ileu*	*Leu*	*Thr*	*Ser*			
BDA	5	150	38	0.59	0.59	1.22	0.76*	(30.7)	0.62	1.32	0.89	1.49	0.8
BDS	5	160	40	0.69	0.63	1.42	0.77*	(14.1)	0.65	—	1.11	n.r.	1.0
Hi-Eff-8B	5	150	38	0.58	0.58	1.17	0.77*	(24.5)	—	—	1.11	1.77	0.8
DEGA	2	145	38	0.60	0.60	1.19	0.79	(15.2)	0.79	1.45	n.r.	1.28	1.0
NPGS	10	152	50	0.57	0.57	0.85	0.65*	(66.0)	0.60	1.14	0.53	n.r.	0.7
NPGS + PVP	9 / 1	150	38	0.59	0.59	1.17	0.77*	(63.5)	0.80	—	0.61	1.04	0.6
NPGG	5	150	38	0.57	0.57	1.11	0.78	(31.4)	0.68	—	0.67	1.13	1.4
NPGSeb	5	150	38	0.50	0.57	0.90	0.78*	(33.5)	0.58	—	0.69	1.28	0.8
PEG-A	5	150	38	0.62	0.61	1.39	0.74*	(31.4)	0.70	1.43	0.96	1.52	0.8
PPS	5	150	38	0.47	0.56	0.89	0.78*	(48.5)	—	—	0.68	1.21	0.7

* Shows partial resolution of alloiso- and isoleucine.
n.r. = compound not run.
— = compound applied but gave no peak.

definite regularities are immediately apparent. These phases, in general, exhibit high efficiencies (except NPGG) and symmetrical peaks, and some of them are able to effect a partial resolution of the isoleucine peak into alloisoleucine and isoleucine, which usually emerge in that order. No phase was found which resolved these two completely.

If one applies the criterion of polarity mentioned before (relative retention time for the glycine derivative) these polyesters are generally more polar than the silicones. BDS is the most polar, and NPGS the least polar.

Table V

The behaviour of the derivatives of serine and threonine on columns of this type is significant. Most of these surface active agents appear to be active also as catalysts in the decomposition of the derivatives of serine and threonine. The extent of the decomposition is a function of the time the derivatives are in contact with the phase, so that serine, with longer retention times, is always more affected than threonine.

Detailed studies with EGS have shown that when conditions are used where retention times are short (low proportion of stationary phase, higher temperatures, rapid flow rates) both derivatives emerge from the column, but when retention times are progressively increased first the serine and then the threonine peaks fail to come off. This is discussed more fully later.

Many of these phases are able to effect partial resolution of the isoleucine peak. TWEENS 20, 40, 60, 80 and acetyl SPAN 80 are highly polar (see glycine).

Studies with the SPAN and TWEEN series might offer an opportunity for analysing the interaction of compounds with stationary phases which vary in a controlled manner to see whether any underlying regularities or generalisations can be detected. Acetylating SPAN 80 shows the effect of a relatively simple chemical modification on the polarity of a stationary phase.

Table VI

With the exception of U 4, U 6 and TPMT, efficiencies of the peaks obtained on these organometallic phases were low. As one might expect from the variety of structure that is to be found within this group, there are also wide variations in polarity. The most polar was U 12, where no peak for glycine was observed after 60 min, and U 5 also showed high polarity. Threonine and serine derivatives were also affected in many cases.

A number of other organometallic compounds were investigated in addition to those quoted. A tin compound designated U 9 $[Sn(C_4H_9)_2\text{-}CH{=}CH\text{-}(CH_2)_5\text{-}CH{=}CH\text{-}]_n$ had the property of catalysing the complete destruction of all the amino acid derivatives, although biphenyl and bibenzyl emerged normally. The same behaviour was noted with the four titanium compounds methyl cyclohexyl titanate, tetrastearyl titanate, triethanolamine titanate and triethanolamine titanate stearate.

Table VII

Although it is clearly unprofitable to expect any regularities where there are no chemical relationships, certain affinities may be recognised. Thus there is a resemblance between DSPO and TAS 10 and Kel-F has a resemblance to silicone MS 550 in Table III.

TABLE V

RELATIVE RETENTION DATA FOR SURFACTANTS

The figures are the retention times relative to the leucine derivative taken as 1.00. The actual retention time in minutes for this derivative is given in brackets.

Liquid phase	*% w/w liquid–solid*	*Column temp. (°C)*	*Gas flow (ml/min)*	*N-TFA amino acid n-amyl ester*					*O,N-DiTFA n-amyl ester*		*Biphenyl*	*Bibenzyl*	*H.E.T.P. (mm) Leu peak*
				Ala	*Val*	*Gly*	*Ileu*	*Leu*	*Thr*	*Ser*			
EGS	5	150	38	0.40	0.62	0.59	0.88	(38.8)	—	—	0.91	1.76	0.8
GMHS	2	152	38	0.49	0.63	0.70	0.93	(7.0)	0.56	0.83	0.84	1.54	2.0
GML	2	152	38	0.49	0.68	0.65	0.93*	(12.0)	0.53	0.76	0.83	1.49	0.8
GMO	2	152	38	0.45	0.66	0.70	0.88	(12.0)	0.55	0.83	0.85	1.57	0.8
GMR	5	155	38	0.47	0.61	0.84	0.89*	(31.0)	0.51	—	n.r.	1.62	0.5
GMS	5	150	38	0.40	0.64	0.64	0.88*	(56.5)	0.49	—	0.85	1.62	0.4
WOL	2	130	38	0.41	0.56	0.71	0.80*	(55.0)	0.49	0.84	0.75	1.50	0.5
Admul 19	2	130	38	0.42	0.57	0.76	0.90	(37.7)	0.48	—	0.71	1.37	0.8
Admul S57	2	150	38	0.46	0.61	0.73	0.91	(14.0)	0.56	—	0.85	1.61	1.2
TWEEN 20	5	150	38	0.61	0.56	1.56	0.74*	(28.5)	—	—	1.12	1.83	0.5
TWEEN 21	5	149	38	0.58	0.58	1.19	0.77*	(49.2)	—	—	0.73	1.27	0.9
TWEEN 40	5	150	38	0.60	0.57	1.53	0.75*	(30.7)	—	—	1.04	1.72	0.9
TWEEN 60	5	150	38	0.61	0.56	1.45	0.77*	(31.0)	—	—	1.00	1.69	0.8
TWEEN 61	5	150	38	0.49	0.58	0.99	0.79*	(39.0)	0.46	—	0.87	1.52	0.6
TWEEN 65	5	150	38	0.52	0.58	1.10	0.79*	(38.6)	0.45	—	0.69	1.24	0.7
TWEEN 80	2	150	38	0.64	0.63	1.50	0.78	(9.9)	—	—	1.07	1.68	0.8
TWEEN 81	5	150	38	0.49	0.56	1.02	0.78	(20.9)	0.53	—	0.86	1.50	1.2
TWEEN 85	5	150	50	0.53	0.60	1.16	0.80*	(34.7)	0.68	—	0.97	1.70	0.6
SPAN 20	5	150	38	0.47	0.60	0.85	0.85	(30.7)	—	—	0.73	1.35	0.7
SPAN 40	5	150	38	0.47	0.61	0.77	0.88*	(21.0)	—	—	0.84	1.57	0.6
SPAN 60	2	150	55	0.50	0.64	0.80	0.91	(8.2)	0.50	—	0.88	n.r.	1.0
SPAN 65	2	150	53	0.51	0.58	0.72	0.92	(6.5)	0.46	0.66	0.91	n.r.	1.4
SPAN 80	5	140	38	0.44	0.58	0.73	0.85*	(49.7)	—	—	0.78	1.50	0.6
SPAN 85	5	150	38	0.49	0.60	0.88	0.84	(20.3)	0.48	—	0.87	1.53	0.8
Ac SPAN 80	5	150	60	0.58	0.57	1.40	0.74*	(18.2)	0.49	—	1.05	n.r.	1.0

* Shows partial resolution of alloiso- and isoleucine.

n.r. = compound not run.

— = compound applied but gave no peak.

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TABLE VI

RETENTION DATA FOR METALLO-ORGANIC STATIONARY PHASES

The figures are the retention times relative to the leucine derivative taken as 1.00. The actual retention time in minutes for this derivative is given in brackets.

Liquid phase	*% w/w phase–support*	*Column temp. (°C)*	*Gas flow (ml/min)*	*N-TFA amino acid n-amyl ester*					*O,N-DiTFA n-amyl ester*		*Biphenyl*	*Bibenzyl*	*H.E.T.P. (mm) Leu peak*
				Ala	*Val*	*Gly*	*Ileu*	*Leu*	*Thr*	*Ser*			
U1	2	150	38	0.62	0.74	0.72	1.02	(7.4)	0.61	—	1.39	2.34	2.1
U2	2	152	38	0.49	0.74	0.57	1.07	(6.1)	0.54	—	1.11	2.13	1.4
U4	2	131	38	0.46	0.63	0.76	1.09	(19.7)	0.53	0.76	1.19	2.62	0.7
U5	5	150	40	0.73	0.66	1.54	0.79	(14.3)	—	—	0.85	1.20	1.2
U6	2	145	38	0.42	0.60	0.67	0.91	(13.0)	—	—	n.r.	1.71	0.9
U7	2	131	38	0.57	0.71	0.46	1.00	(24.9)	0.45	0.50	1.09	2.27	2.2
U8	2	123	38	0.40	0.62	0.63	1.00	(19.5)	—	—	1.70	3.49	1.9
U10	2	152	38	0.56	0.75	0.60	0.98	(4.8)	—	—	1.04	1.81	
U11	5	151	38	0.61	0.61	1.31	0.79	(12.4)	—	—	0.86	1.15	1.5
U12	5	150	38	0.53	0.65	—	0.89	(13.4)	—	—	1.46	2.34	2.1
TPMT	10	153	40	0.43	0.62	0.67	1.00	(31.5)	—	—	1.48	n.r.	0.5

n.r. = compound not run.

— = compound applied but gave no peak.

TABLE VII

RETENTION DATA FOR MISCELLANEOUS TYPES OF STATIONARY PHASES

The figures are the retention times relative to that for the leucine derivative taken as 1.00. The actual retention time in minutes for this derivative is given in brackets.

Stationary phase	*% w/w phase–support*	*Column temp. (°C)*	*Gas flow (ml/min)*	*N-TFA amino acid n-amyl ester*					*O,N-DiTFA n-amyl ester*		*Biphenyl*	*Bibenzyl*	*H.E.T.P. (mm) Leu peak*
				Ala	*Val*	*Gly*	*Ileu*	*Leu*	*Thr*	*Ser*			
DSPO	5	151	30	0.39	0.45	0.68	1.05	(32.0)	0.42	0.47	1.38	2.75	0.6
EAA	5	153	35	0.80	0.55	2.09	0.69	(6.5)	0.61	—	0.91	n.r.	2.6
EGCNSS-S	5	141	38	0.67	0.59	1.54	0.76	(20.8)	0.74	—	0.76	1.18	1.4
EGSS-X	5	158	60	0.84	0.68	1.66	0.82	(4.4)	0.66	—	1.25	n.r.	2.3
KEL-F 90	5	150	40	0.47	0.58	0.75	1.03	(7.7)	0.69	0.78	0.73	n.r.	2.0
PEG 1540	1	130	60	0.63	0.44	1.81	0.67	(7.9)	—	—	0.85	1.32	1.8
PEG 20M	10	201	40	0.83	0.70	1.76	0.83	(6.3)	—	—	1.92	2.63	1.2
PEG-T	5	152	55	0.73	0.56	1.97	0.73	(4.2)	—	—	1.16	1.77	1.0
PEG-L	5	150	38	0.64	0.56	1.64	0.74*	(25.0)	—	—	1.12	1.84	1.0
TTP	5	152	60	0.47	0.51	0.61	0.70*	(86.5)	1.01	—	0.99	n.r.	0.7
Versamid 900	5	170	60	0.58	0.58	1.08	0.80	(10.0)	1.10	—	n.r.	1.50	1.7
TAS 10	5	153	35	0.58	0.72	0.80	0.98	(7.1)	0.44	0.62	1.95	n.r.	2.9
TAS 190	5	153	26	0.51	0.65	0.77	1.00	(11.0)	0.45	0.55	n.r.	n.r.	1.8
TAS 1,000	5	144	50	0.48	0.66	0.71	0.95	(8.9)	0.41	0.54	1.49	n.r.	0.8
HT-180S	5	141	37	0.45	0.66	0.68	0.96	(24.7)	0.40	0.54	1.66	3.36	1.9

* Shows partial resolution of alloiso- and isoleucine.

n.r. = compound not run.

— = compound run but gave no peak.

The two Carbowax phases are similar, and show a resemblance to acetylated SPAN 80, and to some of the TWEENS (Table V). Other members of this group are PEG-T, PEG-L, EAA and the silicone-polyesters EGCNSS-S and EGSSX.

Just as the polarity of SPAN 80 was affected by acetylation so that of PEG 20M is affected by trifluoroacetylation, but to a lesser degree. The O-TFA group is not so stable as the N-TFA group, and it was found that the PEG-T deteriorated in use.

The figures quoted for TAS 10 are of interest since with this phase threonine emerges first, very much earlier than alanine. The chemically related aromatic silicates (TAS 190, TAS 1,000 and HT-180S), and TWEEN 65, SPAN 65 and Ac SPAN 80 also show this effect but to a lesser extent. The resolution of the other amino acid derivatives is quite good with TAS 10, but all the aromatic silicates appear to have insufficient stability and are too volatile for routine use as stationary phases above 120°.

DISCUSSION

As has already been mentioned earlier, serine and threonine derivatives are subject to decomposition catalysed by some of the stationary phases, due probably to the labile nature of the O-TFA group, and evidence has been obtained indicating that the product of such a reaction is the mono-substituted N-TFA derivative. This is still volatile and its retention time is about double that of the O,N-di-TFA derivative on XE-60. However, owing perhaps to the possibility of N → O acyl shifts (WEYGAND AND RINNO[47]), these N-mono-TFA derivatives are also heat-labile, so that after destruction no volatile products are recovered. Polar polyesters of the surfactant type appear to be the most active catalysts. It is in fact possible to obtain peaks of the O,N-di-TFA derivatives of serine and threonine from even the most catalytically active phases by using higher temperatures and fast flow rates, *i.e.* conditions where the time that the derivative is in contact with the phase is minimised. This phenomenon has practical significance in quantitative studies.

One factor which must be noted when studying the retention data of Tables III-VII is that in many cases retention times were obtained with individual amino acid derivatives, or pairs of derivatives. However, when applied in the full seven-component mixture retention times sometimes change from the figures quoted in the tables, so that some peaks coalesce, and the full resolution is not attained.

In most cases attempts have been made to optimise resolutions by variations not only of the operating temperatures and flow rates, but also by varying the ratio of the amount of stationary phase to support. It has been found, in agreement with other workers, that at higher values of this ratio higher peak efficiencies are obtained. There seems to be, for each phase, a critical value for the ratio of phase to support below which the H.E.T.P. rises steeply (peaks become broad): thus for XE-60 this is about 5 %, while for WOL it is 2 % (both on C22 firebrick).

No single column packing has been found which allows the seven most volatile amino acids to be separated completely under the isothermal conditions used. However, some useful separations can be recommended. Fig. 1 shows the resolution of alanine, threonine, valine, glycine, isoleucine and leucine, on 2 % WOL at 130°. The isoleucine peak is not quite resolved into alloisoleucine and isoleucine; the serine peak, however, comes directly on top of these two. Fig. 2 shows the resolution of

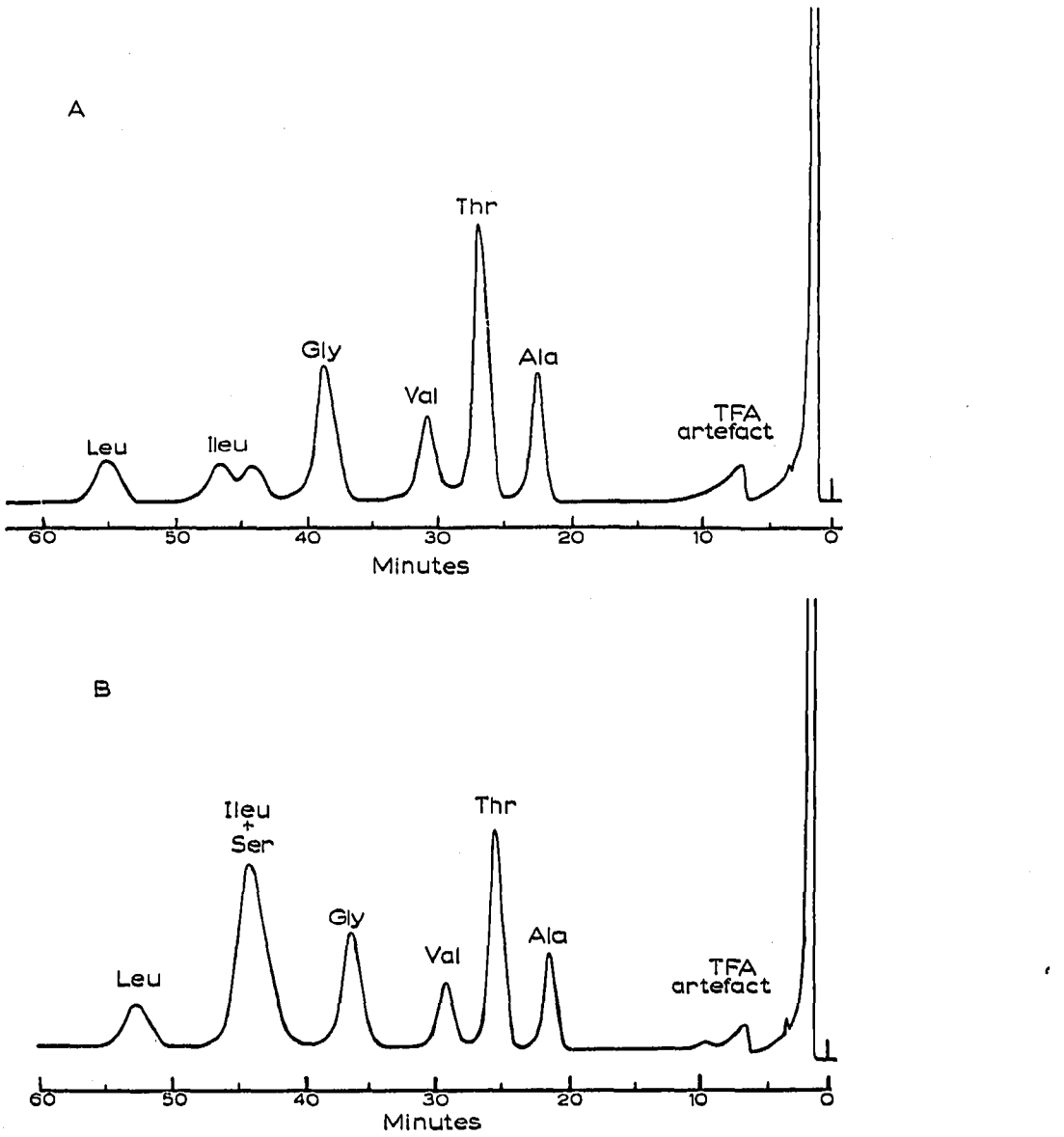

Fig. 1. Separation of N-TFA amino acid *n*-amyl esters (O,N-di-TFA derivatives of serine and threonine) on WOL. Conditions: 2% WOL on 150–200 mesh, HCl-washed, DDS-deactivated Silocel C22 firebrick; 130°; 1.45 kg/sq.cm; 40 ml N_2/min. A: without serine; B: with serine.

alanine, valine, glycine, isoleucine and leucine on 5 % SPAN 80 at 150°. Threonine and serine are completely decomposed under these conditions unless present in high concentration. The isoleucine also appears in the partially resolved form. Fig. 4 shows good resolution of alanine, valine, isoleucine, glycine, leucine and serine on 10 % XE-60 at 140°. Threonine here coincides with isoleucine. Isoleucine is not split on this phase, although the alloisoleucine forms a shoulder on the peak.

A study of the retention data might suggest that it would be possible to achieve better resolutions by using columns in which more than one phase is present, either by putting lengths of different packings in the columns, by blending mixtures of packings, or by preparing packings in which the two (or more) different phases are applied to the support dissolved in the same solvent. All these methods of using

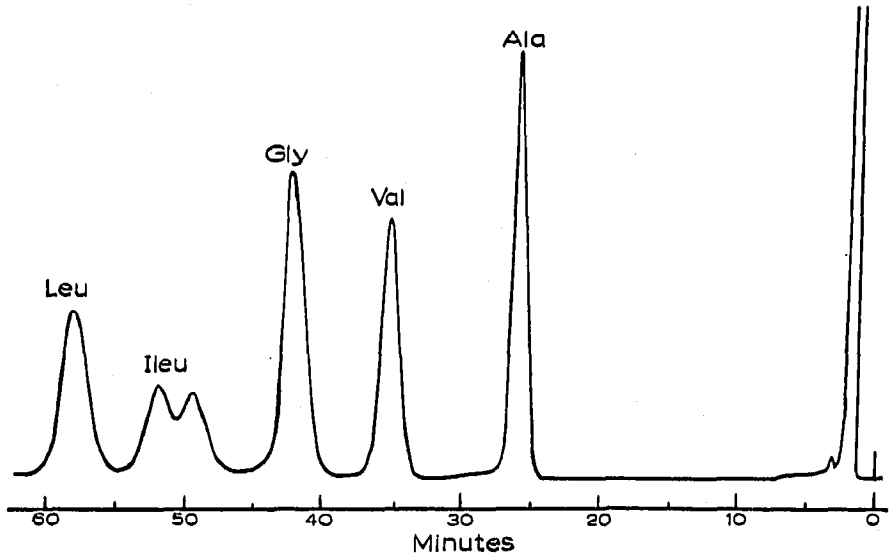

Fig. 2. Separation of N-TFA amino acid *n*-amyl esters on SPAN 80. Conditions: 5% SPAN 80 on 120–150 mesh, HCl-washed, DDS-deactivated Silocel C22 firebrick; 152°; 1.05 kg/sq.cm; 30 ml N_2/min.

mixed phases were in fact tried out, and it was found that the results were equivalent to what one would expect if the retention and other properties (*e.g.* efficiencies) behaved in an additive manner. However, it was not readily possible to find a combination capable of achieving a perfect resolution of the seven-component mixture, and this approach is being explored further.

For a purely qualitative separation it is not necessary to insist on resolution of such high quality, but if quantitative results are ultimately to be achieved, it is necessary for satisfactory integration that each peak should return to a stable baseline

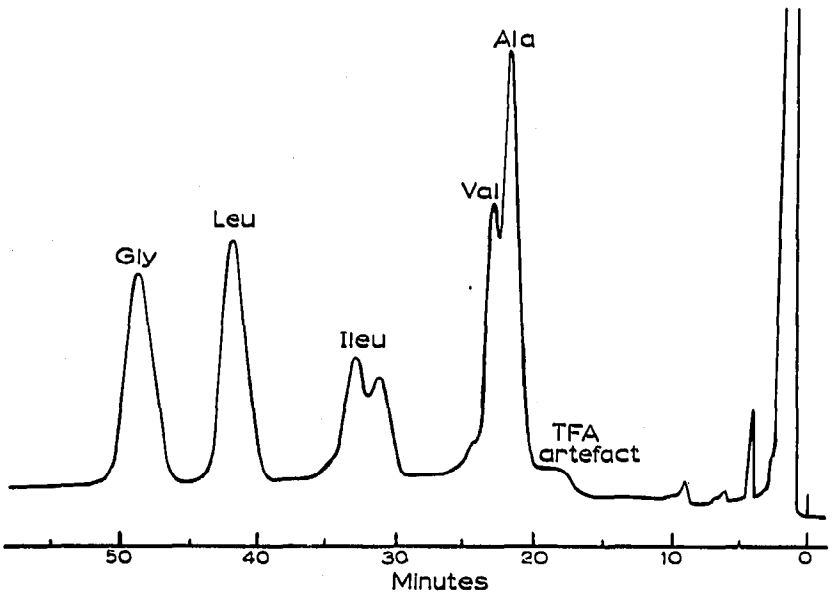

Fig. 3. Separation of N-TFA amino acid *n*-amyl esters on TWEEN 85. Conditions: 5% TWEEN 85 on 60–85 mesh, HCl-washed, DDS-deactivated Silocel C22 firebrick; 140°; 0.62 kg/sq.cm; 38 ml N_2/min.

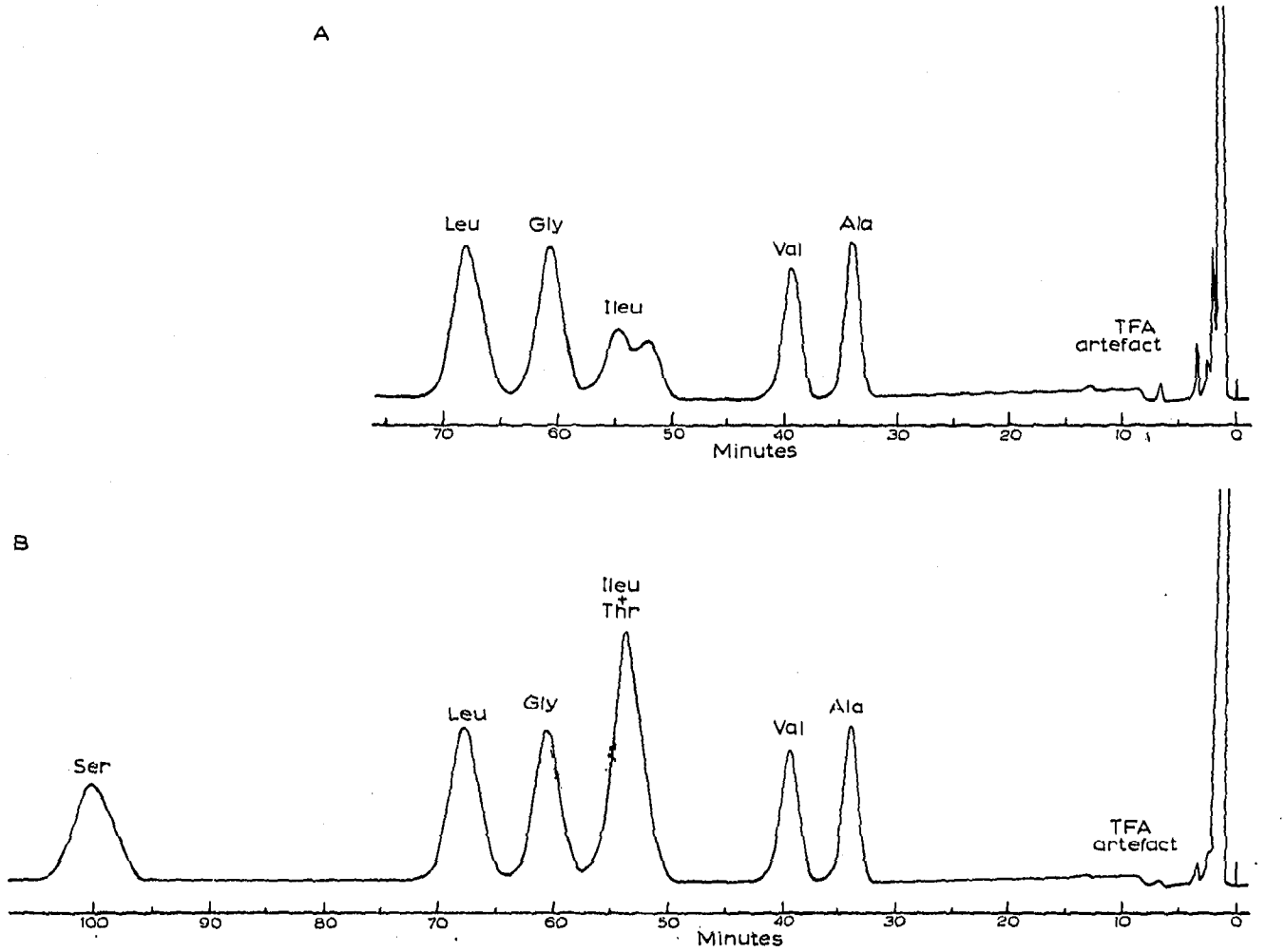

Fig. 4. Separation of N-TFA amino acid *n*-amyl esters (O,N-di-TFA derivatives of serine and threonine) on XE-60. Conditions: 10% XE-60 on 60–85 mesh, HCl-washed, DDS-deactivated Silocel C22 firebrick; 140°; 0.78 kg/sq.cm; 50 ml N_2/h. A: without serine and threonine; B: with serine and threonine.

before the emergence of the following peak. From the figures shown it is evident that for the derivatives used a quantitative estimation of all these seven amino acids cannot be obtained with a single run. However, it is possible to do so from runs on two different columns, by a process of subtraction. The problem therefore resolves itself into choosing suitable stationary phases for either threonine or isoleucine. For isoleucine, in addition to the SPAN 80 column in Fig. 2 one can also use TWEEN 85 (Fig. 3), but since alanine and valine are not resolved one looses some of the advantage of obtaining duplicate results.

The separations in Figs. 1–4 show rather slower runs than could be achieved, but under these conditions one obtains peaks with large areas, and therefore high counts on the integrator, which is of great potential advantage for accurate quantitative work.

The peak labelled "TFA artefact" is due to excess trifluoroacetic acid, and is only found when the last traces have not been removed after trifluoroacetylation, so that its appearance is sporadic. It has been included for illustrative purposes.

In practice we prefer to base quantitative work on XE-60 (Fig. 4), because of its high thermal stability, and to derive the isoleucine figures from SPAN 80, for in this way duplicate results are also obtained for alanine, valine, glycine, and leucine, while the use of these two columns aids unequivocal identification of the amino acids.

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SUMMARY

Methods are described for the preparation of volatile derivatives of amino acids, which permits analysis by gas chromatography. Alanine, glycine, isoleucine, leucine, serine, threonine and valine were converted to the trifluoroacetyl amino acid *n*-amyl esters, and the separation of these seven compounds was investigated on a wide variety of stationary phases. No single phase capable of achieving complete and unequivocal resolution was found, but the separation necessary for quantitative work was obtained by the use of two columns packed with different phases.

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