Journal of Chromatography, 268 (1983) 403–415
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,058

GAS CHROMATOGRAPHY OF VARIOUS N(O,S) ACYL ALKYL ESTERS OF AMINO ACIDS

GERNOT GAMERITH
Institut für Medizinische Biologie und Humangenetik, Universität Graz, Harrachgasse 21/8, A-8010 Graz (Austria)
(Received April 11th, 1983)

SUMMARY

The chromatographic behaviour of various N(O,S) acyl alkyl esters of amino acids was compared. Shorter retention times were found for the propanol esters than for the corresponding butanol esters, and esters containing a secondary alcohol were eluted more quickly than those containing a primary alcohol. The trifluoroacetyl derivatives were eluted before the heptafluorobutyryl (HFB) derivatives on three different stationary phases; however, on Carbowax 20M the HFB-amino acid esters were eluted first. Differences in retention times caused by variation of the ester or the acyl group are greater with the derivatives of the small amino acids. More "compressed" chromatograms are obtained using butanol instead of propanol or the 1- instead of the 2-alcohol for esterification and heptafluorobutyric instead of trifluoroacetic anhydride for acylation. The influence of the trifluoroacetylation time on the relative molar responses does not vary significantly between the various esters tested. Methionine esters and the monoacyl derivative of the tryptophan esters are less stable to acylation when esterified with a primary alcohol.

INTRODUCTION

In general, gas chromatography (GC) offers the advantages of high speed, good resolution and low costs compared to other quantitative chromatographic techniques. This is particularly important in screening programs involving large numbers of samples. Although amino acid analysis by GC necessitates the preparation of volatile derivatives, this technique has proved superior to classical ion-exchange chromatography for quantitative screening[1]. However, the cost of chemicals, handling of the reagents, simplicity of the procedure and the chromatographic behaviour of the resulting derivatives have to be considered carefully.

The most frequently used methods of derivatization involve esterification of the carboxylic groups with an acidified alcohol, and, after evaporation of the esterification reagent, acylation of the other functional groups with an anhydride. Many other methods have also been described[2–5]. The trifluoroacetyl (TFA) *n*-butyl esters[6,7] and the heptafluorobutyryl (HFB) isobutyl esters[8,9] have previously gained the widest

0021-9673/83/$03.00

attention, but many other combinations of acyl and alkyl groups have been used for the derivatization of amino acids[10–15].

Differences in the absolute and relative retention times of various acyl alkyl esters of a single amino acid have been reported[16–18]. Investigations on the N(O,S) acetyl derivatives of amino acid esters established similar retention times for the methyl, ethyl and propyl esters, whereas the butyl esters had markedly longer retention times[16]. Comparing various acetylated amino acid esters the following elution order was found: isobutyl, *n*-butyl, isopentyl and *n*-pentyl ester[17]. The separation of fourteen protein amino acids proved to be better using the *n*-butyl esters than with their isobutyl analogues, whereas the isopropyl esters were better separated than the *n*-propyl esters[18].

The effect of various acyl groups on the retention times and the separation is not as clear: the N(O,S) TFA derivatives exhibited shorter retention times than the corresponding N(O,S) acetyl derivatives, and the differences depended on the stationary phase employed[16]. Moss *et al.*[19] noted the lower volatility of the HFB *n*-propyl esters compared to the TFA analogues. Pollock[20], however, found about 35% shorter retention times on Carbowax 20M for the HFB derivatives than for the TFA-amino acid esters. No other comparisons of TFA and HFB derivatives of amino acid esters seem to have been published.

In the course of my investigations on methods for quantitative amino acid screening a simplified procedure for derivatization of amino acids to give the acyl alkyl esters was developed[21]. Further experiments have now been conducted to clarify the chromatographic behaviour of various acyl alkyl esters. The TFA and HFB derivatives of the *n*-propyl, isopropyl, *n*-butyl and isobutyl esters of some representative protein amino acids were compared.

EXPERIMENTAL

Methylene chloride, gold label quality, was obtained from Aldrich (Beerse, Belgium). Trifluoroacetic anhydride (TFAA) and heptafluorobutyric anhydride (HFBA), both reagent grade, were purchased from Pierce (Rotterdam, The Netherlands). Chromatographically pure amino acids were purchased from Serva (Heidelberg, F.R.G.). The other chemicals were analytical grade and obtained from E. Merck (Darmstadt, F.R.G.). Stock solutions of amino acids, each 2.5 m*M*, were prepared in 0.1 *M* HCl and stored at 4°C.

Chromatographic equipment including columns and column packings was purchased from Supelco. Heating was performed in an aluminium block heater (Pierce) and samples were evaporated using a Büchi Rotavapor equipped with an attachment allowing simultaneous evaporation of ten tubes. Chromatography was conducted with a Hewlett-Packard gas chromatograph HP 5880A equipped with an automatic sampler HP 7672A.

Derivatization

The derivatization procedure has been described previously[21]. Usually, 200 µl of a mixture of amino acids, each 2.5 m*M*, were evaporated. Esterification was performed using 300 µl of 3.5 *M* acetyl chloride in an alcohol (either 1- or 2-propanol or butanol) by heating to 110°C for 25 min. After evaporation of the esterification

reagent, the esters were acylated at 150°C either with TFAA for 5 min or with HFBA for 10 min. The acylation reagent contained 200 μl of the anhydride and 400 μl of a solvent (acetonitrile or methylene chloride). After evaporation, the residue was dissolved in 1 ml ethyl acetate to give a final concentration of 0.5 m*M* of each amino acid, 3 μl were sufficient for analysis.

Chromatography

If not otherwise stated, 6 ft. × 2 mm I.D. glass columns were used. The carrier gas was helium, with a flow-rate of 30 ml/min. The flame ionization detector was supplied with 30 ml of hydrogen and 450 ml of air, and the nitrogen–phosphorus selective detector was operated with 3 ml of hydrogen and 60 ml of air. The chart speed was 1 cm/min and the attenuation 2^7. Usually, 3 μl were injected, corresponding to an absolute amount of 3.75 nmoles of each amino acid. The other chromatographic conditions and the column packings used are listed in the figure legends and tables.

Two-step linear oven temperature gradient programs were used including a low temperature gradient at the beginning to get good separation of the more volatile amino acid derivatives, and a high gradient after a few minutes to reduce the retention times for the larger and more polar amino acid derivatives. In this way the separation time for a mixture of approximately ten amino acids was kept within 15 min for the derivatives tested. The retention times did not vary on a given column to any significant extent and, therefore, no standard deviations of retention times are given.

For quantitative studies, internal standardization with norleucine was used. Owing to the equimolarity of the standard solutions, the relative molar response can be expressed as area (amino acid)/area (norleucine).

TABLE I

INFLUENCE OF THE ESTER GROUP: ANALYSIS OF TFA DERIVATIVES ON 2% OV-17–1% OV-210

A 6 ft. × 2 mm I.D. glass column was used; the support was Supelcoport (100–120 mesh). Injector temperature: 220°C. Flame ionization detector set to 300°C. Oven temperature: raised from 125 to 135°C at 10°/min, thereafter at 20°/min to 220°C, where it was held constant for 10 min. t_R = Retention time; RRT_1 calculated with reference to norleucine, RRT_2 with reference to the TFA-*n*-butyl derivative of the amino acid.

Amino acid	*Isopropyl ester*			*n-Propyl ester*			*Isobutyl ester*			*n-Butyl ester*		
	t_R *(min)*	RRT_1	RRT_2	t_R *(min)*	RRT_1	RRT_2	t_R *(min)*	RRT_1	RRT_2	t_R *(min)*	RRT_1	RRT_2
Ala	0.90	0.42	0.56	1.11	0.44	0.69	1.38	0.47	0.86	1.60	0.50	1
Ser	1.29	0.61	0.61	1.56	0.61	0.74	1.87	0.61	0.88	2.12	0.66	1
Leu	1.82	0.85	0.63	2.22	0.81	0.77	2.60	0.89	0.91	2.87	0.90	1
Norleu	2.13	1	0.67	2.54	1	0.79	2.93	1	0.92	3.20	1	1
Pro	2.52	1.18	0.69	2.98	1.17	0.81	3.40	1.16	0.93	3.66	1.14	1
Met	3.48	1.63	0.77	3.92	1.54	0.86	4.28	1.46	0.94	4.54	1.42	1
Phe	4.08	1.92	0.78	4.67	1.84	0.89	5.00	1.71	0.95	5.25	1.64	1
Glu	4.29	2.01	0.72	4.87	1.92	0.82	5.50	1.88	0.92	5.97	1.87	1
Arg	5.82	2.73	0.87	6.18	2.43	0.92	6.48	2.21	0.96	6.72	2.10	1
Trp-2	7.09	3.33	0.85	7.55	2.97	0.91	7.90	2.70	0.95	8.31	2.60	1
Trp-1	8.45	3.97	0.81	9.20	3.62	0.88	9.81	3.35	0.94	10.44	3.26	1

TABLE II

INFLUENCE OF THE ESTER GROUP: ANALYSIS OF HFB DERIVATIVES ON 2% OV-1–1% OV-210

Same column as in Table I. Injector temperature: 280°C. Detector temperature: 300°C. Oven temperature: 120°C for 0.5 min, then raised to 135°C at 5°/min, then to 220°C at 15°/min, final hold time 13 min. RRT_1 calculated as in Table I, RRT_2 with reference to the HFB-*n*-butyl derivative of the amino acid.

Amino acid	*Isopropyl ester*			*n-Propyl ester*			*Isobutyl ester*			*n-Butyl ester*		
	t_R *(min)*	RRT_1	RRT_2	t_R *(min)*	RRT_1	RRT_2	t_R *(min)*	RRT_1	RRT_2	t_R *(min)*	RRT_1	RRT_2
Ala	1.19	0.37	0.52	1.52	0.39	0.66	1.94	0.42	0.89	2.30	0.45	1
Ser	2.35	0.72	0.59	2.91	0.74	0.79	3.44	0.75	0.87	3.97	0.78	1
Leu	2.64	0.83	0.59	3.32	0.84	0.74	3.98	0.86	0.88	4.51	0.88	1
Norleu	3.20	1	0.63	3.94	1	0.77	4.61	1	0.90	5.11	1	1
Pro	3.79	1.18	0.67	4.64	1.18	0.82	5.24	1.14	0.92	5.68	1.11	1
Met	5.56	1.74	0.79	6.25	1.59	0.88	6.70	1.45	0.95	7.08	1.39	1
Phe	6.74	2.11	0.94	7.25	1.84	0.91	7.63	1.66	0.96	7.98	1.56	1
Glu	6.50	2.03	0.73	7.51	1.91	0.85	8.27	1.79	0.93	8.86	1.73	1
Arg	–	–	–	9.31	2.36	0.96	9.50	2.06	0.98	9.74	1.91	1
Trp-2	10.05	3.14	0.91	10.44	2.65	0.94	10.70	2.32	0.97	11.06	2.16	1
Trp-1	11.16	3.49	0.88	11.77	2.99	0.92	12.22	2.65	0.96	12.73	2.49	1

RESULTS

Influence of ester group on retention times

The *n*-propyl, isopropyl, *n*-butyl and isobutyl esters of some representative amino acids (Table I) were prepared as described and acylated either with TFAA or with HFBA. The derivatives were separated on 2% OV-17–1% OV-210 coated onto

TABLE III

INFLUENCE OF THE ESTER GROUP: ANALYSIS OF HFB DERIVATIVES ON 0.31% CARBOWAX 20M–0.28% SILAR 5 CP–0.06% LEXAN

Support: Chromosorb W AW (120–140 mesh). Injector temperature: 280°C. Detector temperature: 300°C. Oven temperature program: 120°C for 0.5 min, raised at 5°/min to 135°C and then at 15°/min to 220°C, held constant for 10 min. RRT_1 and RRT_2 calculated as in Table II.

Amino acid	*Isopropyl ester*			*n-Propyl ester*			*Isobutyl ester*			*n-Butyl ester*		
	t_R *(min)*	RRT_1	RRT_2	t_R *(min)*	RRT_1	RRT_2	t_R *(min)*	RRT_1	RRT_2	t_R *(min)*	RRT_1	RRT_2
Ser	0.91	0.28	0.55	0.64	0.16	0.39	1.44	0.30	0.87	1.66	0.33	1
Ala	1.44	0.45	0.50	1.94	0.48	0.67	2.49	0.52	0.86	2.88	0.48	1
Leu	3.05	0.94	0.63	3.62	0.90	0.75	4.43	0.93	0.92	4.84	0.96	1
Norleu	3.23	1	0.64	4.02	1	0.79	4.76	1	0.94	5.07	1	1
Pro	4.18	1.29	0.72	4.89	1.22	0.84	5.45	1.15	0.94	5.82	1.15	1
Met	6.21	1.92	0.84	6.70	1.67	0.91	7.06	1.48	0.96	7.36	1.45	1
Phe	6.82	2.11	0.87	7.32	1.82	0.94	7.50	1.58	0.96	7.81	1.54	1
Glu	7.20	2.23	0.81	7.95	1.98	0.90	8.56	1.80	0.97	8.87	1.75	1
Trp-2	13.86	4.29	0.84	15.00	3.73	0.91	15.90	3.34	0.96	16.59	3.27	1

TABLE IV

INFLUENCE OF THE ACYL GROUP: ANALYSIS ON 2% OV-17–1% OV-210

Conditions as in Table II.

Amino acid n-propyl esters	*TFA*		*HFB*	
	t_R *(min)*	*RRT*	t_R *(min)*	*RRT*
Ala	1.22	0.36	1.52	0.39
Ser	1.86	0.55	2.91	0.74
Leu	2.84	0.84	3.32	0.84
Norleu	3.38	1	3.94	1
Pro	4.19	1.24	4.64	1.18
Met	5.86	1.73	6.25	1.59
Phe	6.96	2.06	7.25	1.84
Glu	7.30	2.16	7.51	1.91

Supelcoport (chromatographic conditions in Tables I and II). The HFB derivatives were additionally analysed on a mixed phase containing 0.31% Carbowax 20M, 0.28% Silar 5CP and 0.06% Lexan on Chromosorb W AW (120–140 mesh) (Table III).

The tables list the absolute and two different relative retention times: RRT_1 is calculated with reference to norleucine as the usual method of internal standardization, whereas RRT_2 is obtained as the ratio of the retention times of an N(O,S) acyl amino acid ester and the *n*-butyl ester of the same N(O,S) acyl amino acid derivative.

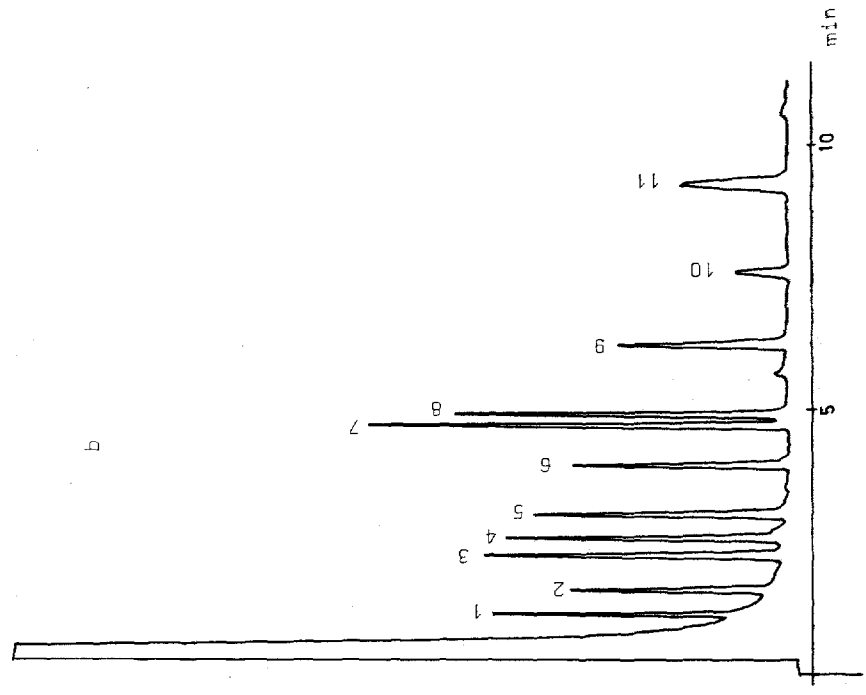
As expected, the various esters of an acylated amino acid have different retention times, with the isopropyl esters eluting first, then the *n*-propyl esters, the isobutyl esters and the *n*-butyl esters. This order remains constant on all stationary phases tested as well as for the two different acyl groups. The effect of different ester groups

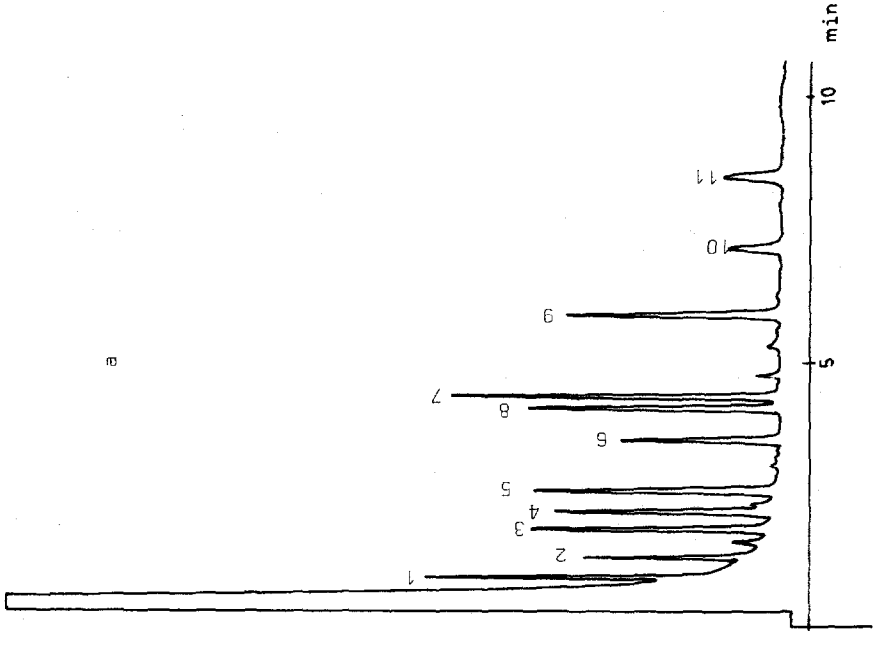
TABLE V

INFLUENCE OF THE ACYL GROUP: ANALYSIS ON 0.65% EGA

EGA was coated on Chromosorb W AW (80–100 mesh). Temperatures: injector, 250°C; detector, 300°C. Oven temperature: held at 115°C for 1 min, then raised to 118°C at 1.5°/min and to 210°C at 15°/min, then held for 15 min.

Amino acid isopropyl esters	*TFA*		*HFB*	
	t_R *(min)*	*RRT*	t_R *(min)*	*RRT*
Ala	1.58	0.35	2.05	0.40
Ser	2.52	0.56	4.08	0.79
Leu	3.75	0.84	4.46	0.86
Norleu	4.49	1	5.19	1
Pro	5.25	1.17	5.77	1.12
Met	6.95	1.55	7.37	1.42
Phe	8.10	1.80	8.41	1.62
Glu	7.89	1.76	8.12	1.57


min
10
5
11
10
9
8
7
6
5
4
3
2
1
b


min
10
5
11
10
9
8
7
6
5
4
3
2
1
a

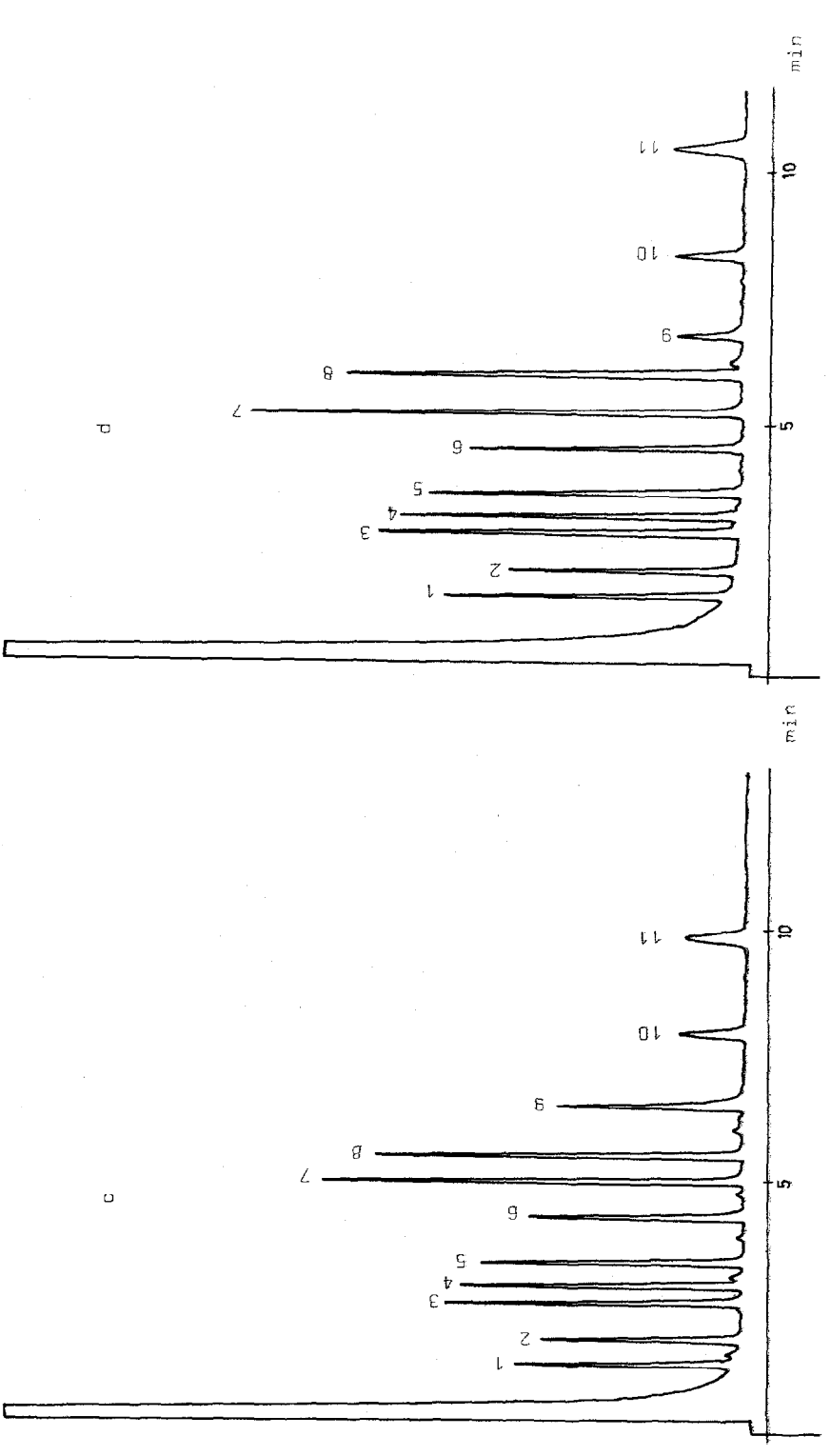


Fig. 1. Separation of TFA amino acid esters on 2% OV-17–1% OV-210. All chromatograms were obtained after 5 min of trifluoroacetylation. Conditions for separation as in Table I. a, TFA-isopropyl esters; b, TFA, TFA-*n*-propyl esters; c, TFA-iso butyl esters; d, TFA-*n*-butyl esters. Peaks: 1 = Ala; 2 = Ser; 3 = Leu; 4 = Norleu; 5 = Pro; 6 = Met; 7 = Phe; 8 = Glu; 9 = Arg; 10 = diacyl derivative of Trp (Trp-2); 11 = monoacyl derivative of Trp (Trp-1).

TABLE VI

INFLUENCE OF THE ACYL GROUP: ANALYSIS ON 3% SP 2100

Support: Supelcoport (100–120 mesh). Injector temperature: 250°C. Detector temperature: 300°C. Oven temperature: 120°C, increased to 130°C at 5°/min and to 220°C at 15°/min, held constant for 15 min.

Amino acid n-butyl ester	*TFA*		*HFB*	
	t_R (min)	*RRT*	*t_R (min)*	*RRT*
Ala	1.12	0.35	1.58	0.40
Ser	1.66	0.52	2.95	0.74
Leu	2.62	0.82	3.39	0.85
Norleu	3.19	1	4.00	1
Pro	3.99	1.25	4.59	1.15
Met	5.40	1.69	5.94	1.49
Phe	6.39	2.00	6.83	1.71
Glu	7.42	2.33	7.69	1.92

is more pronounced with small amino acids, as is seen from the value of RRT_2 which is about 0.5 for alanine, but up to 0.9 for larger amino acids. Using a small ester group and/or the 2-alcoholic group instead of the l-alcoholic analogue, the chromatograms become more spaced out, which may be useful when analysing complex amino acid mixtures.

Influence of acyl group on retention times

Retention times of the TFA and the HFB derivatives of various amino acid esters were compared. The samples were prepared as described. The *n*-propyl esters were analysed on 2% OV-17–1% OV-120 on Supelcoport (Table IV), the isopropyl esters on 0.65% EGA on Chromosorb W AW (Table V) and the *n*-butyl esters on either 3% SP 2100 (Table VI) or 5% Carbowax 20M (Table VII). The chromatographic conditions are given in the tables.

TABLE VII

INFLUENCE OF THE ACYL GROUP: ANALYSIS ON 5% CARBOWAX 20M

The stationary phase was coated on Supelcoport (100–120 mesh). Temperatures: injector, 250°C; detector, 300°C. Oven temperature: held at 130°C for 0.5 min, then raised to 135°C at 5°/min and to 220°C at 15°/min, held for 13 min.

Amino acid n-butyl ester	*TFA*		*HFB*	
	t_R (min)	*RRT*	*t_R (min)*	*RRT*
Ala	3.82	0.79	2.55	0.72
Leu	4.44	0.92	3.16	0.89
Norleu	4.81	1	3.56	1
Pro	6.41	1.33	5.24	1.47
Met	7.93	1.65	6.72	1.89
Phe	8.53	1.77	7.30	2.05
Ser	8.84	1.84	7.63	2.14
Glu	9.42	1.96	7.97	2.24
Arg	11.75	2.44	9.71	2.73

On three of these column fillings the TFA derivatives exhibit shorter retention times than their HFB analogues, whereas on Carbowax 20M the HFB derivatives are eluted before the TFA derivatives. The retention times relative to norleucine are similar for the TFA and HFB derivatives. However, the timespan between the first and the last eluting amino acids of a given derivative type is somewhat less for acylation with HFBA than with TFAA. So the chromatograms of the HFB derivatives seem to be more "compressed" compared to those obtained after acylation of the same esters with TFAA.

Influence of acylation time on relative molar responses of some TFA amino acid esters

A 200-μl volume of a mixture containing ten amino acids (Fig. 1) was esterified with one of four alcohols and then acylated with TFAA at 150°C for 1,3,5,10 or 15 min. Two samples were run in parallel. Histidine was investigated separately. The derivatives were analysed on 2% OV-17–1% OV-210 using the chromatographic conditions listed in Fig. 1, where the chromatograms were all obtained after 5 min of acylation.

As mentioned above, the TFA isopropyl esters exhibit the shortest retention times and the *n*-butyl esters are eluted last.

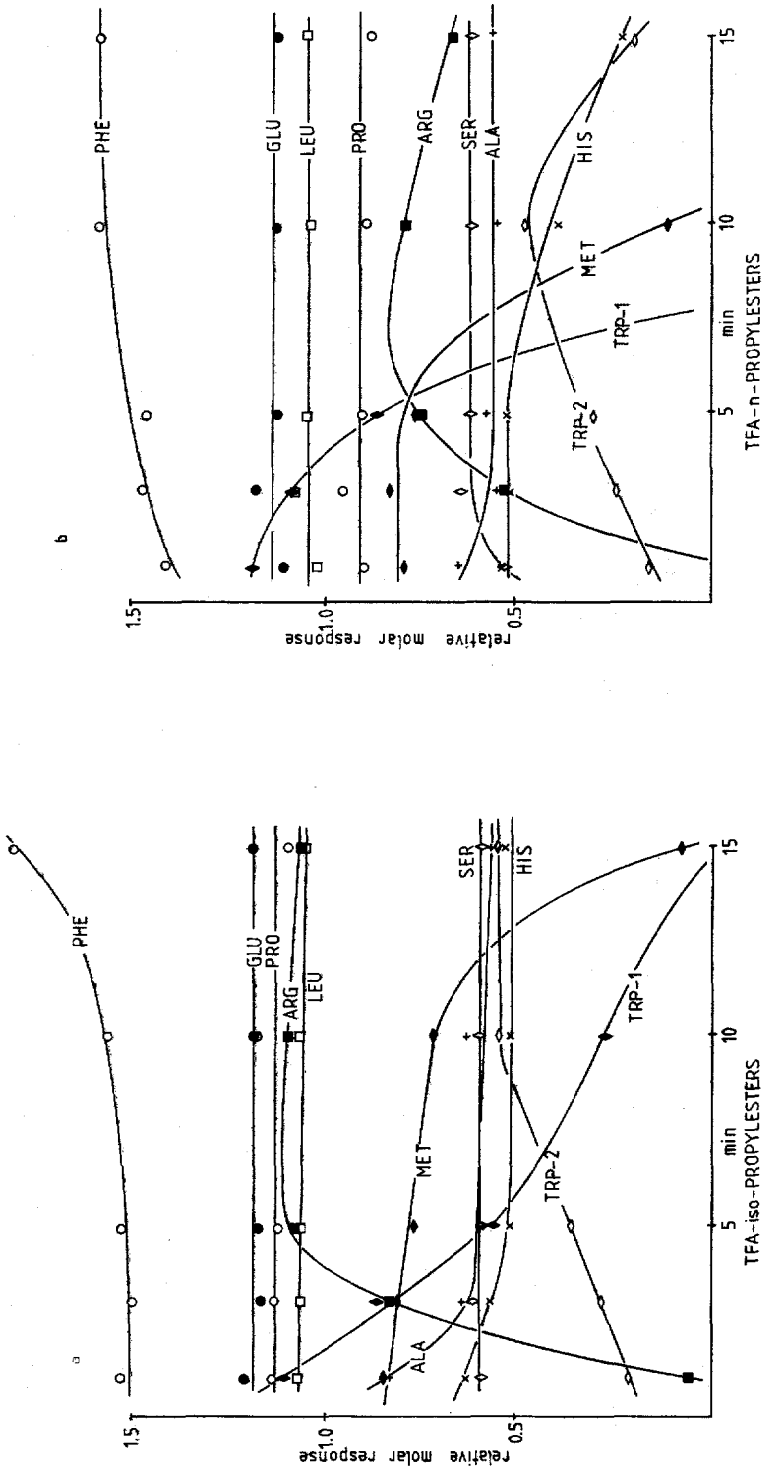
The influence of the acylation time on the relative molar responses (RMRs) is approximately the same for all esters tested (Fig. 2). The values of RMR differ slightly between the propyl and butyl esters owing to the different molecular weights of the ester groups.

The RMRs of some amino acids do not change with different acylation times, whereas phenylalanine, serine and the diacyl derivative of tryptophan show increasing RMRs with acylation time. The responses of methionine, histidine and the monoacyl derivative of tryptophan decrease with increasing acylation time. Arginine exhibits a maximum response after about 5 min of acylation. The methionine derivatives containing the primary alcoholic group obviously are more sensitive to the acylation conditions than those containing the secondary alcohol. The monoacyl derivatives of tryptophan respond similarly to acylation, but to a lesser extent. The ratio of the two tryptophan derivatives formed during the acylation varies greatly with the acylation time.

DISCUSSION

The influence of the ester group on the retention times is in good agreement both with theoretical expectations and previously reported results[16–18], A small ester group resulting in short retention times may be used to reduce separation time, or, alternatively, to permit lower oven temperatures during separation, resulting in lower (if any) column bleeding and extended column lifetime. However, the relative retention times and even the elution order may change when altering the ester group.

Care must be exercised in the choice of preparation technique for the derivatives, since the high volatility of derivatives containing a small ester group may otherwise result in losses of the more volatile amino acid derivatives during the evaporation steps. Differences in the previously used procedures probably, at least partially, account for the lack of agreement concerning their usefulness for the preparation of various acyl alkyl esters. Some workers prefer the HFB-isopentyl derivatives, because


a
b
PHE
GLU
PRO
ARG
LEU
MET
ALA
SER
HIS
TRP-2
TRP-1
relative molar response
1.5
1.0
0.5
5
10
15
min
TFA-iso-PROPYLESTERS
TFA-n-PROPYLESTERS

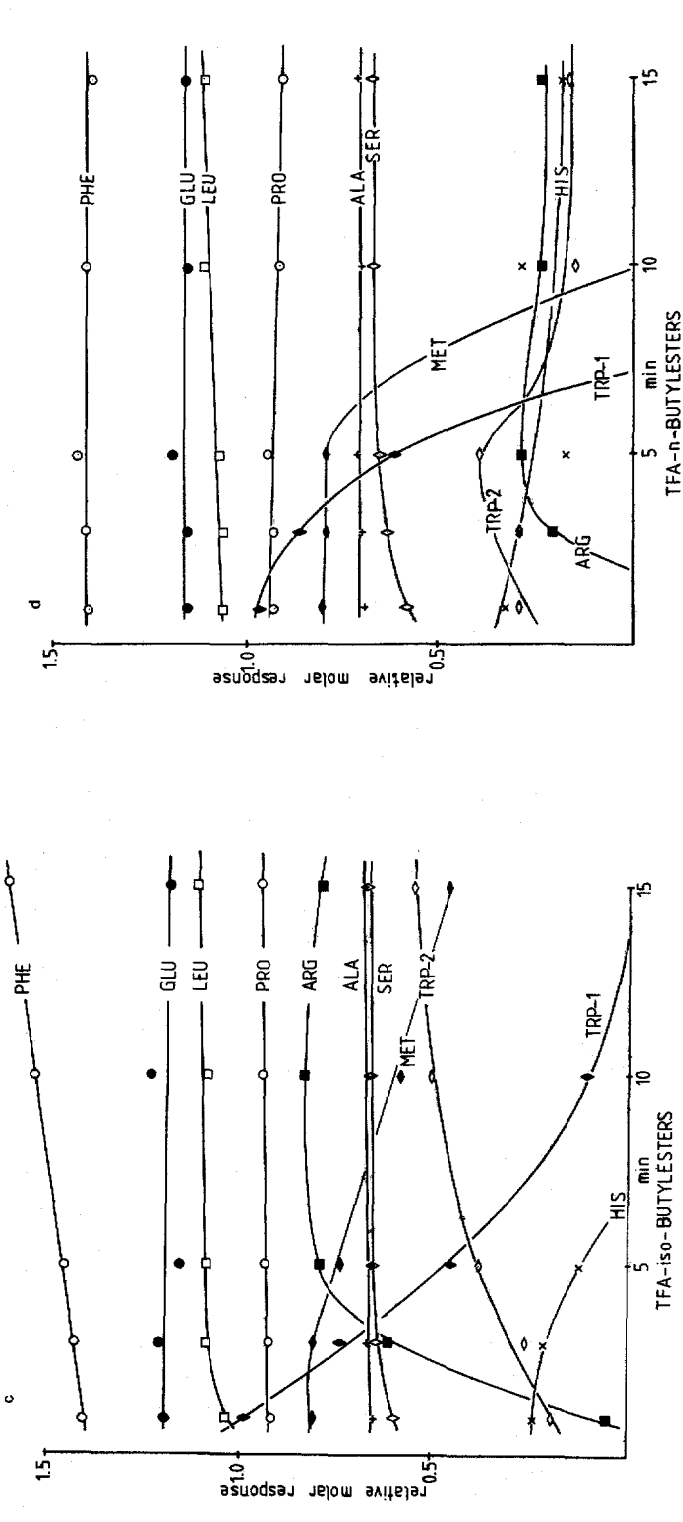


Fig. 2. Relative molar response *versus* acylation time. Responses of the TFA amino acid esters have been calculated with reference to norleucine and represent the mean of two determinations. Chromatography was performed as described in Table I. a, TFA-isopropyl esters; b, TFA-*n*-propyl esters; c, TFA-isobutyl esters; d, TFA-*n*-butyl esters.

in their opinion more highly volatile derivatives cannot be concentrated without substantial losses prior to analysis[22,23]. However, various other acyl alkyl esters have been shown to allow reproducible analysis of amino acids[10–15,24–26].

Shorter retention times for the HFB than for the TFA derivatives have been observed on Carbowax 20M[20]. This was also found during the present investigations, but on three other stationary phases the TFA derivatives exhibited shorter retention times. Obviously, no general statement about the influence of the acyl group on the retention times can be given. Besides, the stationary phase does not only influence the retention times of the TFA and the HFB derivatives, but also changes the elution order of the amino acid derivatives of a given type. So, for instance, the order of elution of a hydroxylated amino acid relative to the non-hydroxylated analogue will depend on the polarity of the stationary phase. The higher is the polarity, the longer will be the retention time of the hydroxylated amino acid derivative. This was demonstrated here in the case of the TFA-*n*-propyl esters, but similar results can be found from the work of Gehrke, if one compares the separation of the TFA-*n*-butyl esters on either EGA[6] or Apiezon[27].

The support material may also influence the separation of acyl alkyl amino acid esters. Separation quality varied on differently treated supports of the same type (Chromosorb)[28]. Comparing the chromatograms obtained on the same stationary phase (EGA), but coated on either Chromosorb W AW or Supelcoport, different elution patterns for the same sample were found in the course of my investigations. As reported previously[10], the separation of the TFA-*n*-propyl esters of aspartic acid, hydroxyproline and methionine is poor or absent on 0.65% EGA on Chromosorb W AW. However, using 0.65% EGA on Supelcoport, these amino acids are well separated, whereas a different elution order at the beginning of the chromatograms results in the failure of alanine to separate from valine and glycine from norleucine. Another important finding was that the elution pattern did not vary significantly when raising the loading from 0.65% to 2% EGA on Supelcoport. This is contrary to earlier findings using Chromosorb as support, where the elution order varied greatly with the percentage loading[29].

From the results of the experiments on the acylation kinetics, I conclude that the acylation reaction with TFAA is not influenced by the alcohol which has been used for the esterification of the carboxylic groups of the amino acids. The only exceptions are the methionine and tryptophan derivatives which are more stable to the acylation conditions when esterified to a secondary alcohol than to the corresponding l-analogue. This might explain the necessity for the addition of an antioxidant when preparing the HFB-*n*-propyl esters of methionine as described by March[14]. In the preparation of the HFB-isobutyl esters this does not seem to be necessary[30], but a slight decrease in RMR was observed in that study, and Pearce[31] also proposed the use of an antioxidant in the preparation of the latter derivatives. Owing to the short acylation time necessary when using TFAA, no serious problems arise from the derivatization of methionine to the TFA ester.

Problems with the quantitation of tryptophan have sometimes been reported[31]. These may be due either to the lability of this amino acid in hydrochloric acid, leading to oxidation of the indole ring[32], or to incomplete acylation owing to low levels of anhydride and the use of an inadequate solvent[33]. Another reason for poor reproducibility emerged from the acylation studies presented in this paper: the incomplete

diacylation of this particular amino acid resulted in two derivatives. As shown for the TFA-*n*-propyl ester, only one of these may be detected on some stationary phases, *e.g.*, EGA[10]. Because the ratio of the diacyl to the monoacyl derivative is very sensitive to the acylation time, large standard deviations will be found if this parameter is not held constant. However, tryptophan can be analysed reproducibly[10,24,30,33], provided that a suitable procedure is used.

The differences in the chromatographic behaviour of various acyl alkyl esters of amino acids should be considered in separation problems where the samples contain many protein and non-protein amino acids. It is not difficult to produce a number of different derivatives simultaneously, since the conditions for each of the preparation steps are almost always identical. Since the retention times can be varied by altering the acyl and alkyl groups, more than one type of derivative may be employed to solve difficult separation problems.

ACKNOWLEDGEMENTS

The author thanks Professor W. Rosenkranz for the opportunity of doing this work in his institute, Professor F. Paltauf for correction of this manuscript and Ms. M. Schuster for technical assistance during the laboratory work.

REFERENCES

1 M. Redford-Ellis, A. C. C. Gibbs, S. A. Haider and A. Holzel, *Lancet*, ii (1981) 10.
2 P. Husek and K. Macek, *J. Chromatogr.*, 113 (1975) 139.
3 C. W. Gehrke and K. Leimer, *J. Chromatogr.*, 57 (1971) 219.
4 P. Husek, V. Felt and M. Matucha, *J. Chromatogr.*, 252 (1982) 217.
5 M. Makita, S. Yamamoto and S. Kiyama, *J. Chromatogr.*, 237 (1982) 279.
6 D. Roach and C. W. Gehrke, *J. Chromatogr.*, 43 (1969) 303.
7 J. Gabryś and J. Konecki, *J. Chromatogr.*, 222 (1981) 345.
8 S. L. MacKenzie and D. Tenaschuk, *J. Chromatogr.*, 97 (1974) 19.
9 J. Chauhan and A. Darbre, *J. Chromatogr.*, 236 (1982) 151.
10 G. Gamerith, *J. Chromatogr.*, 256 (1983) 267.
11 W. A. König, W. Parr, H. A. Lichtenstein, E. Bayer and J. Orô, *J. Chromatogr. Sci.*, 8 (1970) 183.
12 A. J. Cliffe, N. J. Berridge and D. R. Westgarth, *J. Chromatogr.*, 78 (1973) 333.
13 A. M. Lewis and C. Waterhouse, *Clin. Chem.*, 26 (1980) 271.
14 F. J. March, *Anal. Biochem.*, 69 (1975) 420.
15 A. G. Goldhirsh, A. M. Hogg and F. M. Wolfe, *J. Agr. Food Chem.*, 30 (1982) 320.
16 A. Darbre and K. Blau, *J. Chromatogr.*, 17 (1965) 31.
17 D. E. Johnson, S. J. Scott and A. Meister, *Anal. Chem.*, 33 (1961) 669.
18 S. V. Shlyapnikov and M. Ya. Karpeiskii, *Biokhimiya*, 29 (1964) 1076.
19 C. W. Moss, M. A. Lambert and F. J. Diaz, *J. Chromatogr.*, 60 (1971) 134.
20 G. E. Pollock, *Anal. Chem.*, 39 (1967) 1194.
21 G. Gamerith, *J. Chromatogr.*, 256 (1983) 326.
22 J. P. Zanetta and G. Vincendon, *J. Chromatogr.*, 76 (1973) 91.
23 P. Felker and R. Bandursky, *Anal. Biochem.*, 67 (1975) 245.
24 R. F. Adams, *J. Chromatogr.*, 95 (1974) 189.
25 F. E. Kaiser, C. W. Gehrke, R. W. Zumwalt and K. C. Kuo, *J. Chromatogr.*, 94 (1974) 113.
26 J. Desgres, D. Boisson and P. Padieu, *J. Chromatogr.*, 162 (1979) 133.
27 C. W. Gehrke and H. Takeda, *J. Chromatogr.*, 76 (1973) 63.
28 B. Thom and J. W. Parsons, *J. Chromatogr.*, 90 (1974) 370.
29 M. Stefanovic and B. L. Walker, *Anal. Chem.*, 39 (1967) 710.
30 S. L. MacKenzie and D. Tenaschuk, *J. Chromatogr.*, 173 (1979) 53.
31 R. J. Pearce, *J. Chromatogr.*, 136 (1977) 113.
32 T. Ohta, S. Suzuki, M. Todo and T. Kurechi, *Chem. Pharm. Bull.*, 29 (1981) 1767.
33 I. Mac Arthur Moodie, *J. Chromatogr.*, 208 (1981) 60.