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# INFLUENCE OF TRIFLUOROACETIC ANHYDRIDE CONCENTRATION DURING DERIVATIZATION AND CHROMATOGRAPHY OF N(O,S)-TRIFLUOROACETYL AMINO ACID *n*-PROPYL ESTERS

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## SUMMARY

When trifluoroacetic anhydride (TFAA) concentrations of less than 66.6% are employed to acylate the *n*-propyl ester of tryptophan, both the mono- and diacyl derivatives are formed. The use of higher TFAA concentrations results in the formation of the diacylated form only. To determine tryptophan as the trifluoroacetyl (TFA) *n*-propyl ester derivative in a single peak, a higher TFAA concentration is required than for the acylation of the other amino acid esters.

The partial breakdown of the O–TFA bond in tyrosine can be prevented by adding a small amount of TFAA to the solvent used for injection. The responses of some other amino acids, however, are diminished by the presence of 0.5% of TFAA in the ethyl acetate during injection. Thus, co-injection or addition of TFAA prior to injection is disadvantageous in most instances.

## INTRODUCTION

The gas–liquid chromatography (GLC) of amino acids is a convenient method for the analysis of large numbers of samples, but involves derivatization to obtain volatile compounds prior to injection. Among the many methods described[1–4], those involving esterification of the carboxylic groups and subsequent acylation of the other functional groups are most often employed[5–10].

An inexpensive and rapid method developed in our laboratory makes use of the N(O,S)-trifluoroacetyl (TFA) *n*-propyl esters of the amino acids[11].

The propylation occurs without problems under various conditions[12]. The result of the acylation step, however, depends on the reaction parameters and the reaction must be carefully controlled to obtain good reproducibility. The effect of acylation time and temperature in the preparation of the TFA derivatives of the *n*-propyl esters has been described previously and reaction at 150°C for 5 min was found to be the best compromise[13].

No investigations of the effect of the trifluoroacetic anhydride (TFAA) concentration on the derivatization of the *n*-propyl amino acid esters have been pub-

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lished. Moodie[14] described the influence of the anhydride concentration and the solvent on the acylation of the isobutyl amino acid esters with heptafluorobutyric anhydride (HFBA), particularly in the preparation of the tryptophan derivative. This amino acid forms two derivatives during acylation when the anhydride concentration is too low. From his work, one can conclude that the relative amounts of the two derivatives depend on the anhydride concentration. The lower the HFBA level, the lower is the amount of the diacylated form obtained. HFBA concentrations up to 50% proved to be insufficient for full acylation of this amino acid. Unfortunately, no attempts were made to determine the lowest anhydride concentration that results in the diacyl derivative only.

Similar to these findings, I reported[7] the apperance of both mono- and diacyl tryptophan *n*-propyl esters when using 33.3% TFAA in methylene chloride, resulting in higher standard deviations for this amino acid. To obtain better reproducibility, the influence of the TFAA concentration during acylation was investigated and almost complete acylation was obtained with TFAA concentration greater than 66.6%. The responses of the other amino acids are not significantly affected except for a slight increase in the responses of serine and tyrosine.

Attempts were made to reduce the instability of the TFA *n*-butyl esters of hydroxylated amino acids by addition of the anhydride prior to injection[15]. Thus, the effect of adding 0.5% (v/v) of TFAA to the ethyl acetate was tested using a mixture of TFA amino acid *n*-propyl esters. Only the response of tyrosine can be enhanced by the presence of a low concentration of anhydride during injection; the other amino acid responses remain unchanged or decrease.

## EXPERIMENTAL

Materials were obtained from Merck (Darmstadt, F.R.G.) and were of analytical grade. Methylene chloride (Gold Label quality) was purchased from Aldrich (Beerse, Belgium), trifluoroacetic anhydride (reagent grade) from Pierce (Rotterdam, The Netherlands), amino acids from Serva (Heidelberg, F.R.G.) and α-aminocaprylic acid (B-grade) from Calbiochem (San Diego, CA, U.S.A.).

Chromatographic materials were obtained from Supelco (Crans, Switzerland) and chromatography was performed using a Hewlett-Packard HP 5880 A gas chromatograph equipped with a flame-ionization detector and an HP 7672 A autosampler.

Various standard solutions containing either one amino acid and norleucine, each 1.25 m*M* in 0.1 *M* HCl, or complex mixtures of amino acids in 0.1 *M* HCl, were esterified to the *n*-propyl esters. Details of the derivatization procedure have been given previously[11]. Various TFAA concentrations were employed during acylation.

Samples were analysed on a 6 ft. × 2 mm I.D. glass column filled with 0.65% ethylene glycol adipate (EGA) on Chromosorb W AW (80–100 mesh). Nitrogen at a flow-rate of 20 ml/min was used as the carrier gas. The flame-ionization detector was supplied with 30 ml/min of hydrogen and 450 ml/min of air. The injector temperature was 250°C and the detector temperature was held at 300°C. Other chromatographic parameters are given in the legends of the figures.

Results were obtained either by calculation of the responses relative to norleucine or by quantitation with α-aminocaprylic acid as an internal standard. The latter

amino acid was chosen because it does not interfere with any other amino acid. Norleucine, in contrast, coelutes with pipecolic acid.

## RESULTS AND DISCUSSION

To investigate the influence of the TFAA concentration during the acylation step, 200 μl of various standard solutions containing either tyrosine, serine, methionine sulphone or tryptophan together with an equimolar amount of norleucine, or a complex amino acid mixture were derivatized. Acylation was performed with either 33.3%, 50%, 66.6% or 83.3% TFAA in methylene chloride, the total amount of acylating reagent remaining constant at 600 μl. Samples were evaporated after derivatization and dissolved in 1 ml of ethyl acetate and 3 μl of this solution were injected on to the column.

Fig. 1 shows the slight increase in the serine and tyrosine response with increasing TFAA concentration. The methionine sulphone response remains unchanged. Low anhydride concentrations result in the formation of both the mono- and diacyl derivatives of tryptophan. If the TFAA concentration is increased, the amount of the diacylated form increases until a maximum response of 1.35 relative to norleucine is achieved. Almost no monoacyl derivative is seen at TFAA levels higher than 66.6%. The relative molar response calculated from the sum of the two derivatives remains nearly unchanged on varying the anhydride concentration (1.36 with 33.3%, 1.35 with 50%, 1.25 with 66.6% and 1.36 with 83.3% of TFAA).

The results for the acylation of the tryptophan *n*-propyl ester corresspond well with those obtained by Moodie[14] for the formation of the heptafluorobutyryl isobutyl ester of this amino acid. He also observed incomplete acylation with 50% HFBA in ethyl acetate, but only the diacylated form was obtained when using the pure anhydride for acylation. No doubt it was not the presence of ethyl acetate (or any other solvent), but only the low anhydride concentration that led to incomplete acylation in his work.

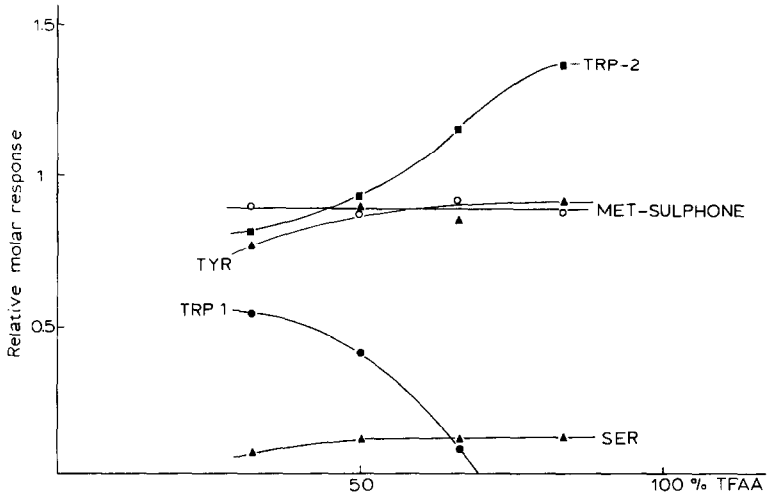


Fig. 1. Relative molar responses *versus* anhydride concentration during acylation. The values are the means of two samples run in parallel.

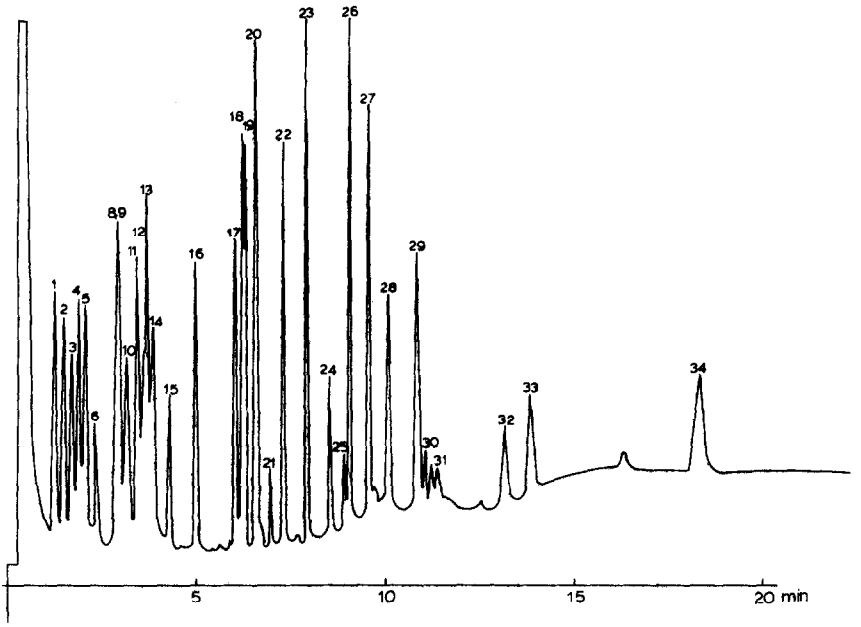


Fig. 2. Complex amino acid mixture acylated with 33.3% TFAA in methylene chloride. The oven temperature was 115°C for 1 min, then increased at 3°C/min to 118°C, followed by an increase of 15°C/min to 220°C. This temperature was held for 3 min, then increased at 10°C/min up to 240°C. This temperature was held for a further 15 min. The chart speed was 1 cm/min and the attenuation was set at 32. Each peak represents *ca.* 0.75 nmole, exept 3-methylhistidine (0.22 nmole) and hydroxylysine (0.42 nmole). Peaks: 1 = α-aminoisobutyric acid (α-AIBA); 2 = Ala; 3 = sarosine (Sarc); 4 = α-aminobutyric acid (α-ABA); 5 = Val; 6 = Gly; 7 = phosphoethanolamine; 8 = Ile; 9 = β-AIBA; 10 = β-Ala; 11 = Leu; 12 = pipecolic acid; 13 = norleucine; 14 = Pro; 15 = Thr; 16 = γ-ABA; 17 = Met; 18 = Asx; 19 = Hyp; 20 = Phe; 21 = Cit; 22 = Glx; 23 = α-aminoadipic acid; 24 = Tyr; 25 = 3-methylhistidine; 26 = Orn; 27 = Lys; 28 = diacyl-Trp; 29 = methionine sulphone (MetS); 30 = Hyl; 31 = His; 32 = monoacyl-Trp; 33 = cystathionine; 34 = homocystine.

The shift to only the diacylated form of the tryptophan derivatives can also be seen from the chromatograms in Figs. 2 and 3. Fig. 2 was obtained after acylation with 33.3% TFAA in methylene chloride and Fig. 3 from the same solution, but acylated with 83.3% TFAA.

As with the single standard solution, the increase in the tyrosine response can be seen in the chromatograms of the complex amino acid mixture. Further, the response of phosphoethanolamine is also increased when higher TFAA levels are used during the acylation. In contrast, the response of citrullin decreases with increasing TFAA concentration. As reported previously, this amino acid is partially destroyed during derivatization to yield ornithine[16]. In the present investigation, a decrease in citrulline response was observed, with a corresponding increase in the degradation product ornithine as the TFAA concentration was increased. The other amino acid responses in this mixture remained almost unaffected by changes in the TFAA level during acylation.

As previously mentioned[7], the responses of serine, tyrosine and citrulline can be enhanced by addition of TFAA to the final solvent prior to injection. Addition

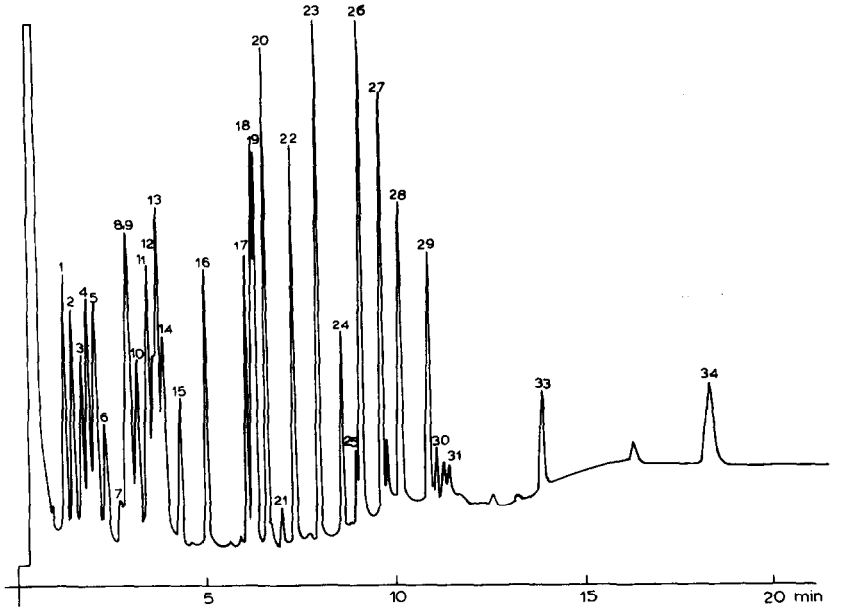


Fig. 3. Results for the same mixture as in Fig. 2, but acylated with 83.3% TFAA. Chromatographic parameters and peaks as in Fig. 2.

TABLE I

PRECISION AND REPRODUCIBILITY OF SAMPLES DERIVATIZED IN A SINGLE BATCH

| *Amino acid* | *Content in the standard (mg per 100 ml)* | *Analytical result (mg per 100 ml)** | *Standard deviation (mg per 100 ml)* | *Coefficient of variation (%)* |
|---|---|---|---|---|
| α-AIBA | 25.0 | 24.9 | 0.2 | 0.9 |
| Ala | 22.4 | 22.3 | 0.2 | 0.7 |
| Sarc | 31.2 | 30.9 | 0.6 | 1.8 |
| α-ABA | 26.5 | 26.9 | 1.3 | 4.7 |
| Val | 29.1 | 29.5 | 1.0 | 3.4 |
| Gly | 23.8 | 23.7 | 0.1 | 0.5 |
| Ile | 33.1 | 32.7 | 0.8 | 2.5 |
| β-AIBA | 26.5 | 26.7 | 0.1 | 0.5 |
| β-Ala | 22.0 | 22.1 | 0.1 | 0.5 |
| Leu | 33.5 | 33.6 | 0.2 | 0.5 |
| Pro | 30.0 | 30.1 | 0.1 | 0.3 |
| Thr | 29.3 | 29.5 | 0.2 | 0.6 |
| Ser | 28.1 | 27.3 | 0.5 | 1.7 |
| γ-ABA | 27.2 | 27.2 | 0.1 | 0.4 |
| Met | 37.2 | 37.1 | 0.1 | 0.3 |
| Asp | 32.9 | 33.1 | 0.3 | 0.8 |
| Phe | 41.3 | 41.2 | 0.2 | 0.4 |
| Glu | 35.5 | 35.6 | 0.1 | 0.3 |
| Tyr | 44.7 | 47.6 | 3.9 | 8.1 |
| Orn | 43.9 | 43.6 | 0.6 | 1.4 |
| Lys | 45.4 | 44.8 | 0.5 | 1.1 |
| Trp | 50.4 | 50.3 | 0.6 | 1.1 |
| Hyl | 21.1 | 19.8 | 1.5 | 7.6 |
| MetS | 44.6 | 43.5 | 5.3 | 12.1 |

* Mean values for six samples.

of 10% of TFAA to the ethyl acetate led to high responses for these amino acids, but also to a poor baseline, which prevents reproducible integration by the calculator except when using a nitrogen-selective detector. Therefore, the question of whether a low anhydride concentration in the ethyl acetate can prevent the breakdown of the O–TFA bonds without affecting the baseline was investigated. A 100-$\mu$l volume of the amino acid mixture usually employed for calibrating the method was derivatized using 66.6% TFAA in methylene chloride during acylation. Finally, the dried derivatives were dissolved in 1 ml of ethyl acetate. Six samples were run in parallel.

The results for the first set of runs are given in Table I and represent the mean values for the six samples, which were prepared within one batch. The table also includes the standard devations, the relative standard deviations and the amino acid concentrations originally present in the solution.

TFAA was then added to these samples with a Hamilton syringe to yield a final concentration of 0.5% (v/v) of TFAA in the solvent, and the samples were analysed again immediately. Quantitation was performed with the relative responses obtained from the first set of runs. Therefore, the differences in the analytical values given in Table II are due only to the TFAA addition and its effect on the chromatogram.

TABLE II

EFFECT OF 0.5% (v/v)TFAA PRESENT DURING INJECTION

The samples from Table I were supplemented with TFAA and analysed again.

| *Amino acid* | *Analytical result (mg per 100 ml)** | *Standard deviation (mg per 100 ml)* | *Coefficient of variation (%)* |
|---|---|---|---|
| α-AIBA | 24.4 | 0.2 | 0.6 |
| Ala | 22.9 | 1.9 | 8.1 |
| Sarc | 30.5 | 1.8 | 6.0 |
| α-ABA | 25.9 | 0.8 | 2.9 |
| Val | 28.6 | 0.8 | 2.9 |
| Gly | 24.7 | 0.8 | 3.2 |
| Ile | 32.6 | 1.5 | 4.7 |
| β-AIBA | 25.5 | 0.4 | 1.6 |
| β-Ala | 22.4 | 0.5 | 2.2 |
| Leu | 36.6 | 1.1 | 3.0 |
| Pro | 30.1 | 0.3 | 0.9 |
| Thr | 25.7 | 0.6 | 2.3 |
| Ser | 22.1 | 1.0 | 4.5 |
| γ-ABA | 27.2 | 0.1 | 0.3 |
| Met | 37.6 | 0.1 | 0.3 |
| Asp | 31.8 | 0.2 | 0.7 |
| Phe | 40.8 | 0.1 | 0.3 |
| Glu | 34.6 | 0.2 | 0.5 |
| Tyr | 86.9 | 4.9 | 5.7 |
| Orn | 46.5 | 1.3 | 2.7 |
| Lys | 44.3 | 1.8 | 4.1 |
| Trp | 83.3 | 7.5 | 9.1 |
| Hyl | 15.3 | 1.4 | 8.9 |
| MetS | 32.9 | 3.6 | 11.0 |

* Mean values for six samples.

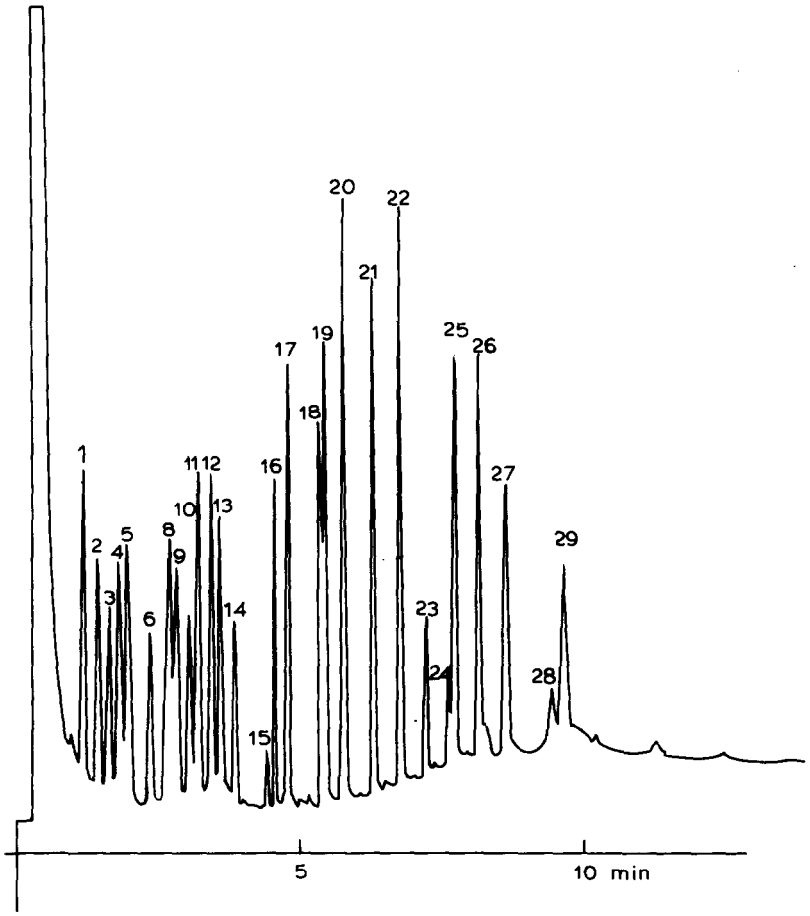


Fig. 4. Separation of the amino acid mixture used for calibration, with injection without TFAA. The oven temperature was held at 115°C for 1 min, then increased to 120°C at 5°C/min, followed by an increase of 20°C/min up to 220°C. This temperature was finally held for a further 15 min. The chart speed was set at 1 cm/min and the attenuation at 32. Peaks: 1 = α-AIBA; 2 = Ala; 3 = Sarc; 4 = α-ABA; 5 = Val; 6 = Gly; 7 = phosphoethanolamine; 8 = Ile; 9 = β-AIBA; 10 = β-Ala; 11 = Leu; 12 = norleucine; 13 = Pro; 14 = Thr; 15 = Ser; 16 = γ-ABA; 17 = α-aminocaprylic acid; 18 = Met; 19 = Asx; 20 = Phe; 21 = Glx; 22 = α-aminoadipic acid; 23 = Tyr; 24 = 3-methylhistidine; 25 = Orn; 26 = Lys; 27 = Trp; 28 = Hyl; 29 = MetS.

The results in Tables I and II indicate that the values do not change significantly for most of the amino acids tested. However, the baseline becomes poorer, particularly at the position of tryptophan. The tailing of this amino acid caused by the presence of TFAA during injection obviously precludes exact integration, and the results for this amino acid become too high. This is confirmed by comparing the tryptophan peaks in Figs. 4 and 5, which represent the chromatograms of one sample before (Fig. 4) and after (Fig. 5) TFAA addition. Further, the standard deviation is increased as a consequence of the poor integration.

In contrast, there is a real increase in both the tyrosine peak heights and the calculated amounts of this amino acid. This is the only amino acid whose response

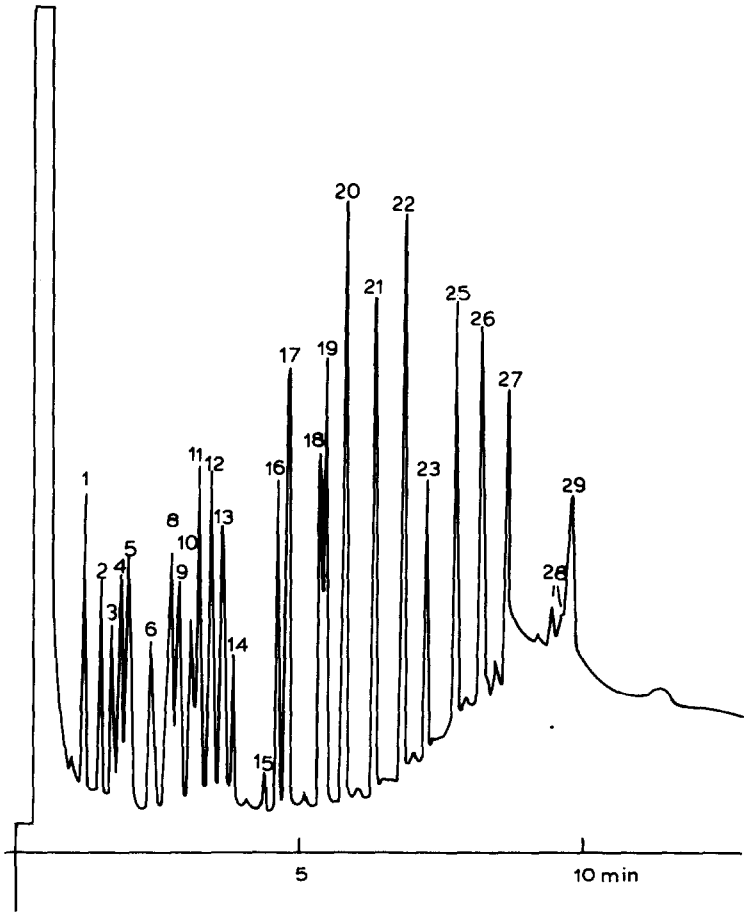


Fig. 5. Results for the same sample as in Fig. 4 after addition of 0.5% (v/v) TFAA. Chromatographic conditions and peaks as in Fig. 4.

can be increased by on-column acylation with only a small amount of TFAA present in the injection solvent.

The responses of serine, threonine, hydroxylysine and methionine sulphone decrease when the injection solvent contains 0.5% of TFAA. Further, the peak of 3-methylhistidine disappears completely.

The precision and reproducibility of amino acid analysis using the TFA *n*-propyl esters are reasonably good, as shown in Table III, which contains the means of four independent determinations of the calibration mixture, derivatized in different batches. Almost all amino acids, except the hydroxylated compounds, approach the theoretical values closely. The calculated amounts of serine, threonine and tyrosine differ, however, from batch to batch. For the precise determination of these amino acids it is therefore necessary to include one calibration sample in each batch, which consists of ten samples in our case. This calibration sample is then used to recalibrate the method before analysing the other samples in the batch. Within one batch, one

TABLE III

PRECISION AND REPRODUCIBILITY FOR SAMPLES DERIVATIZED IN DIFFERENT BATCHES

Original amino acid content as in Table I.

| *Amino acid* | *Analytical result (mg per 100 ml)** | *Standard deviation (mg per 100 ml)* | *Coefficient of variation (%)* |
|---|---|---|---|
| α-AIBA | 27.5 | 3.0 | 10.9 |
| Ala | 22.5 | 1.3 | 5.8 |
| Sarc | 31.3 | 1.8 | 5.8 |
| α-ABA | 26.5 | 1.1 | 4.2 |
| Val | 29.3 | 1.1 | 3.8 |
| Gly | 23.5 | 1.0 | 3.8 |
| Ile | 33.9 | 3.1 | 9.1 |
| β-AIBA | 26.6 | 0.5 | 1.9 |
| β-Ala | 21.1 | 0.3 | 1.4 |
| Leu | 33.6 | 0.9 | 2.7 |
| Pro | 30.0 | 0.4 | 1.3 |
| Thr | 32.5 | 2.2 | 6.8 |
| Ser | 32.7 | 6.6 | 20.1 |
| γ-ABA | 27.1 | 0.2 | 0.7 |
| Met | 37.0 | 0.6 | 1.6 |
| Asp | 33.1 | 0.2 | 0.6 |
| Phe | 41.3 | 0.2 | 0.5 |
| Glu | 35.5 | 0.2 | 0.6 |
| Tyr | 64.6 | 29.8 | 46.0 |
| Orn | 43.1 | 3.6 | 8.3 |
| Lys | 45.2 | 1.7 | 3.8 |
| Trp | 51.1 | 0.7 | 1.4 |
| Hyl | 21.4 | 2.8 | 13.1 |
| MetS | 41.4 | 5.3 | 12.8 |

* Mean values for four samples.

has to consider the standard deviations listed in Table I. A short Basic program can be used to perform the recalibration when the autosampler is employed.

## CONCLUSIONS

In contrast to some previous reports on difficulties with the reproducible analysis of acylated tryptophan esters by GLC[17,18], this work confirms the poossibility of achieving the precise determination of this amino acid as described by some workers[6,12,14,19], provided that appropriate acylation conditions are used.

Acylation of the *n*-propyl ester of tryptophan with at least 66.6% of TFAA in methylene chloride is necessary in order to obtain a single peak. Other amino acid responses are not affected by an increase in TFAA concentration from 33% to 83% during acylation, exept citrulline, the response of which is decreased, and serine and tyrosine, the responses of which slightly increase. Thus, only if tryptophan is to be determined should a high anhydride concentration be employed.

The addition of small amounts of TFAA to the solvent used for injection,

although resulting in an increase in tyrosine response, offers no real advantage because the responses of some other amino acids decrease.

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