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Note

Gas–liquid chromatography of amino acids

The heptafluorobutyryl-isobutyl ester derivative of tryptophan

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In recent reports MacKenzie and Tenaschuk[1,2] have referred to the difficulty experienced by various workers in achieving successful gas chromatography (GC) of the N-heptafluorobutyryl-isobutyl ester derivative of tryptophan.

A brief discussion by these authors on the effect of changes in the esterification conditions mentions[1] the ultimate production of small quantities of products other than diacyl-isobutyl-tryptophan and states that the proportions of these other compounds do not change significantly whether esterification is carried out for 15 min or for 60 min. They subsequently refer to the importance of the acylation temperature and demonstrate[2], as might be expected, that a temperature of 75°C results in a mixture *inter alia* of mono- and diacylated tryptophans, whereas at 150°C diacylation is complete. It is then concluded that multiple peaks previously reported by other authors are likely to be due to "incomplete acylation at too low a temperature"[2].

This aspect of the GC analysis of heptafluorobutyryl-isobutyl ester derivatives has been of concern for some time, as it represents a serious drawback to an otherwise potentially good technique. The modified procedure[3] used at this Institute for routine amino acid analysis is based on that originally described by MacKenzie and Tenaschuk[4]. Despite several refinements the technique has never given a satisfactory result for tryptophan.

Careful examination and comparison of the acylation conditions in the earlier account[4] with those described in the later publication[2] reveals two important differences. First, the use of ethyl acetate as an acylating solvent has latterly been discontinued and, secondly, the amount of heptafluorobutyric anhydride (HFBA) has been greatly increased (by approximately seven to eight times) relative to the amino acid starting material. It should be noted that Siezen and Mague[5] were able to obtain fully diacylated tryptophan using an acylating mixture composed of HFBA and acetonitrile. These observations suggest that the multiple peaks often obtained in the case of tryptophan derivatization may be due to the presence of insufficient HFBA and the presence of ethyl acetate (also implied by MacKenzie and Tenaschuk[2]) rather than to acylation being carried out at too low a temperature.

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EXPERIMENTAL

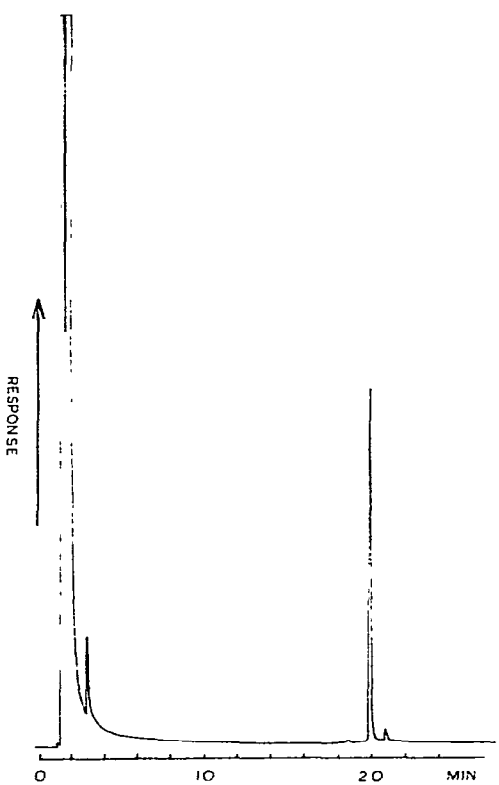
Reagents

Amino acids and heptafluorobutyric anhydride were obtained from Sigma (St. Louis, MO, U.S.A.).

Gas chromatography

Analyses were carried out using a Hewlett-Packard Model 5711 gas chromatograph fitted with "on-column" injection and a flame ionization detector. The single glass helical column (3.1 m × 2.5 mm I.D.) used was silanized and packed with 3.5% OV-101 on Supelcoport (100–120 mesh). Coating of liquid phase on the support was carried out in this laboratory. The chromatograph was linked to a Hewlett-Packard 3353B Laboratory Data System which performed data acquisition and handling.

With the exception of the changes described here, the derivatives and chromatographic conditions were the same as those reported previously[3].



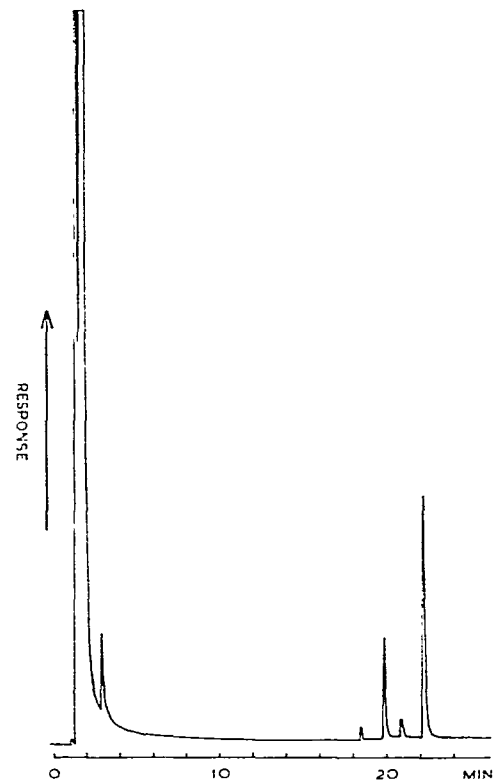


Fig. 1. Chromatogram of product from esterification of tryptophan (0.25 mg; 1.23 μmole), followed by acylation using HFBA (1.5 ml) for 10 min at 150°C.

Fig. 2. Chromatogram of product from esterification of tryptophan (0.25 mg; 1.23 μmole), followed by acylation using HFBA (200 μl) and ethyl acetate (500 μl) for 10 min at 150°C.

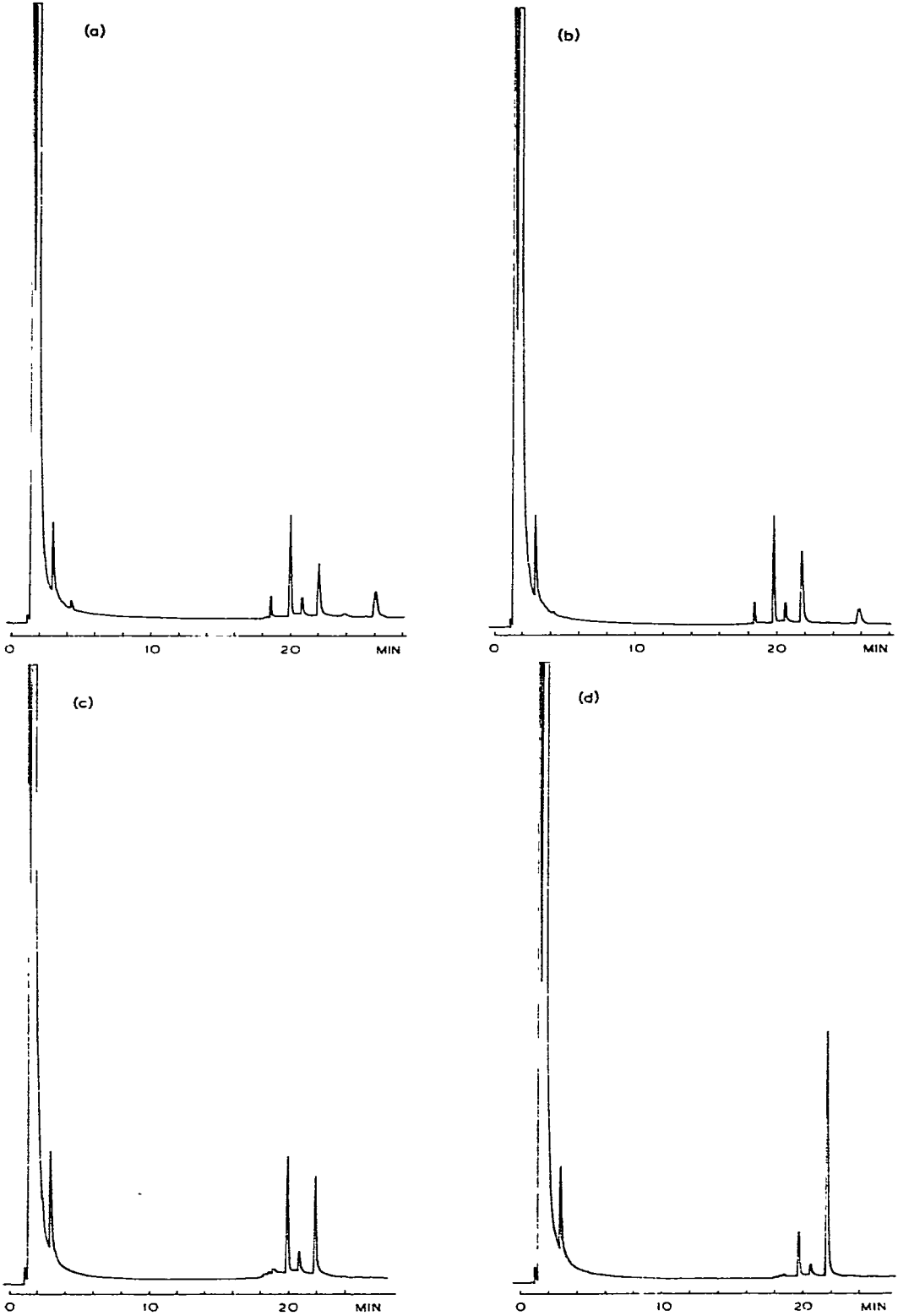


Fig. 3. Chromatograms of product from esterification of tryptophan (0.25 mg; 1.23 μmole), followed by acylation using HFBA (1.5 ml) and ethyl acetate (1.5 ml) (a), HFBA (750 μl) and ethyl acetate (750 μl) (b), HFBA (400 μl) and ethyl acetate (1 ml) (c) or HFBA (200 μl) and ethyl acetate (1.3 ml) (d).

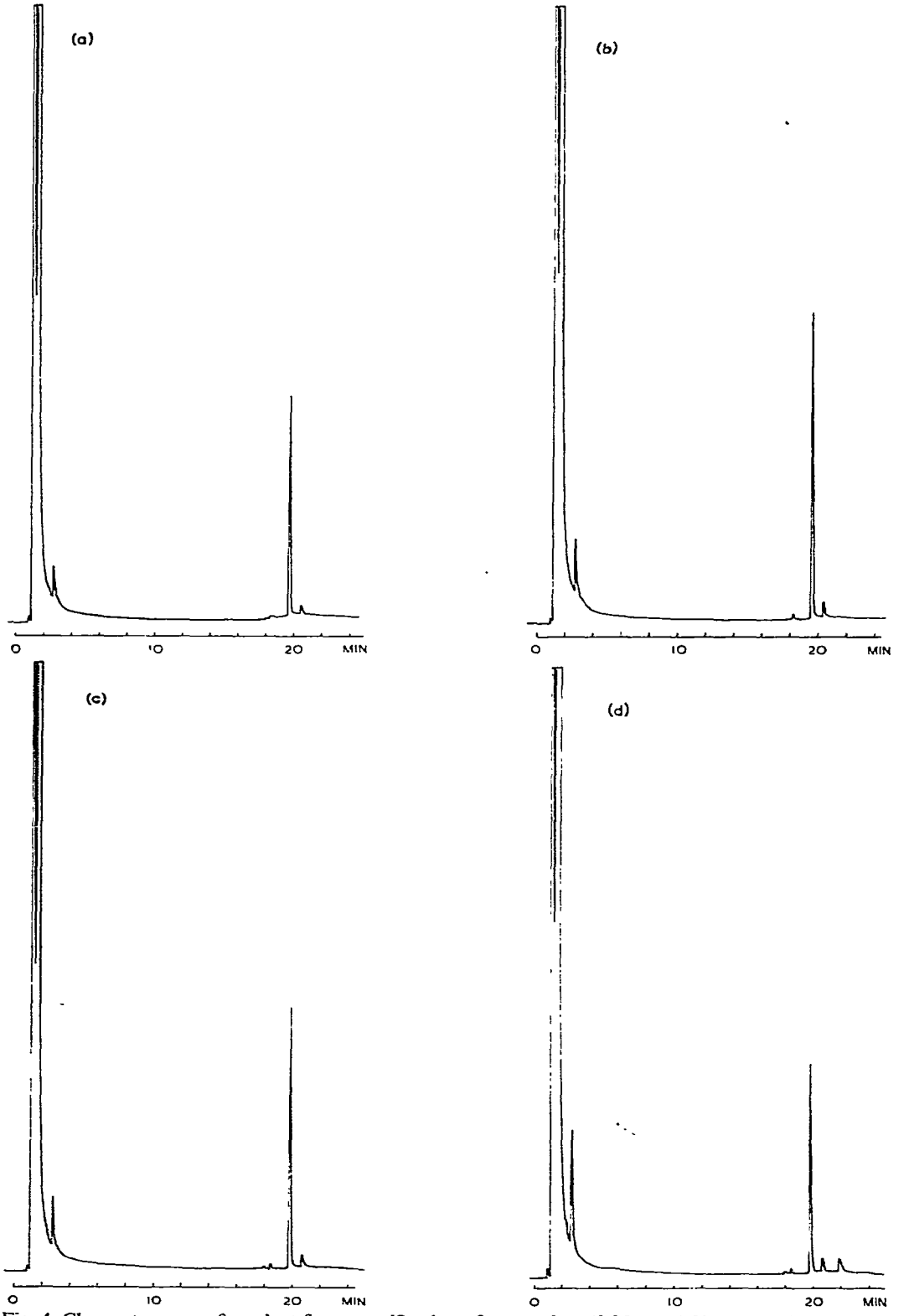


Fig. 4. Chromatograms of product from esterification of tryptophan (0.25 mg; 1.23 μmole), followed by acylation using 700 μl (a), 350 μl (b), 140 μl (c) or 70 μl HFBA (d).

RESULTS AND DISCUSSION

An initial investigation confirmed that, by using a quantity of HFBA some seven to eight times that originally used by MacKenzie and Tenaschuk[4], complete diacylation of tryptophan was obtained (Fig. 1), whereas their earlier conditions yielded the monoacylated product together with other spurious peaks (Fig. 2).

In a second experiment, the effect of varying the composition of the acylating reagent was studied with particular reference to the amount of HFBA present. Four equal aliquots of esterified tryptophan, each derived from 0.25 mg (1.23 μmole) of amino acid, were acylated at 150°C for 10 min using the reagent compositions shown in Fig. 3. It is clear from the results that reduction of the HFBA:ester ratio leads to increased quantities of the monoacyl derivative; it is also apparent, however, that the presence of ethyl acetate has a detrimental effect on the extent of acylation since the reaction conditions which lead to a totally diacylated product (Fig. 1) are similar to those in Fig. 3a, apart from the absence of ethyl acetate.

A third experiment in which acylation was carried out using only HFBA was

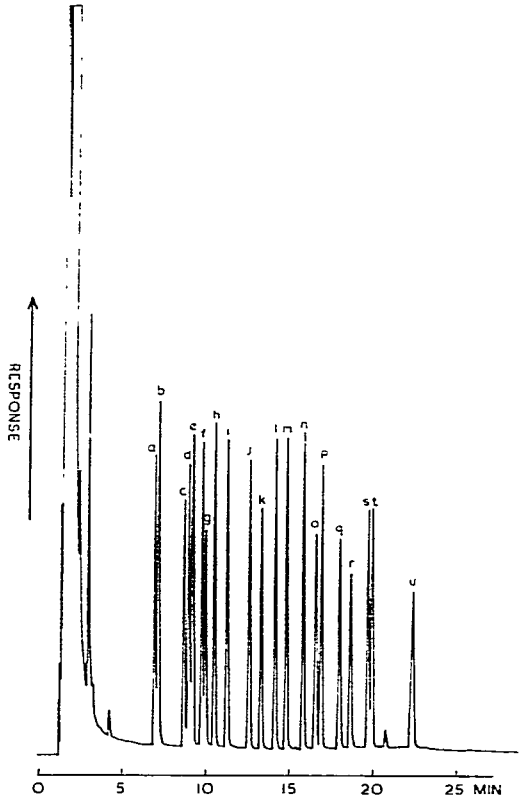


Fig. 5. Chromatogram of a standard amino acid mixture (including tryptophan) derivatized according to the modified conditions. Peaks: a = alanine; b = glycine; c = valine; d = threonine; e = serine; f = leucine; g = isoleucine; h = norleucine (internal standard); i = proline; j = hydroxyproline; k = methionine; l = aspartic acid; m = phenylalanine; n = glutamic acid; o = lysine; p = tyrosine; q = arginine; r = histidine; s = S-ethoxycarbonylcysteine; t = tryptophan; u = cystathionine.

TABLE I
REPEATED ANALYSES OF A DERIVATIZED STANDARD MIXTURE OF AMINO ACIDS (INCLUDING TRYPTOPHAN) USING THE REVISED DERIVATIZATION PROCEDURE

Amino acid	*Content in standard (%, w/w)*	*Analytical value* (%)*	*Standard deviation*	*Relative standard deviation (%)*
Alanine	4.96	4.97	0.011	0.22
Glycine	5.06	5.10	0.011	0.22
*Valine***	5.12	5.13	0.010	0.19
Threonine	4.98	5.01	0.012	0.25
Serine	4.96	5.01	0.010	0.21
Leucine	4.92	4.92	0.004	0.08
Isoleucine	5.35	5.34	0.009	0.17
Proline	5.03	5.03	0.007	0.14
Hydroxyproline	5.01	5.03	0.010	0.20
Methionine	5.01	5.01	0.007	0.15
Aspartic acid	4.96	4.94	0.008	0.16
Phenylalanine	5.03	5.04	0.020	0.40
Glutamic acid	5.15	5.12	0.037	0.73
Lysine	4.33	4.35	0.015	0.35
Tyrosine	5.09	5.08	0.012	0.25
Arginine	5.25	5.41	0.023	0.43
Histidine	4.26	4.25	0.039	0.91
S-Ethoxycarbonylcysteine	5.15	5.14	0.023	0.45
Tryptophan	5.39	5.39	0.015	0.28
Cystathionine	4.98	5.03	0.031	0.61

* Mean from six separate injections of the same sample.

** The essential amino acids are italicized.

TABLE II
REPEATED DERIVATIZATION OF A STANDARD MIXTURE OF AMINO ACIDS (INCLUDING TRYPTOPHAN) USING THE REVISED DERIVATIZATION PROCEDURE

Amino acid	*Content in standard (%, w/w)*	*Analytical value* (%)*	*Standard deviation*	*Relative standard deviation (%)*
Alanine	4.96	5.02	0.037	0.74
Glycine	5.06	5.09	0.024	0.47
Valine	5.12	5.16	0.099	1.92
Threonine	4.98	4.95	0.037	0.73
Serine	4.96	4.99	0.039	0.77
Leucine	4.92	4.93	0.008	0.17
Isoleucine	5.35	5.33	0.118	2.22
Proline	5.03	5.02	0.008	0.17
Hydroxyproline	5.01	5.00	0.016	0.31
Methionine	5.01	4.99	0.019	0.37
Aspartic acid	4.96	4.95	0.017	0.34
Phenylalanine	5.03	5.02	0.012	0.24
Glutamic acid	5.15	5.15	0.024	0.46
Lysine	4.33	4.33	0.010	0.22
Tyrosine	5.09	5.06	0.016	0.32
Arginine	5.25	5.24	0.051	0.98
Histidine	4.26	4.26	0.037	0.86
S-Ethoxycarbonylcysteine	5.15	5.15	0.025	0.48
Tryptophan	5.39	5.41	0.036	0.67
Cystathionine	4.98	5.00	0.036	0.73

* Mean from six separate standard solutions.

** The essential amino acids are italicized.

designed to determine the minimum amount of HFBA required to bring about complete diacylation of the tryptophan ester. The results (Fig. 4) show that the monoacylated product occurred only at the lowest amount of HFBA. It should be remembered, however, that these conditions apply to tryptophan alone and that the presence of other amino acid esters will also lead to the consumption of HFBA, thus necessitating the adoption of an elevated level of this reagent. Using a mixture of amino acids (1.0–3.0 μmole of each), esterification was carried out as described previously[1] and the product acylated with 250 μl HFBA for 10 min at 150°C. A typical chromatogram shows tryptophan resolved from S-ethoxycarbonylcysteine (Fig. 5). Repeated analyses of this mixture were subjected to a statistical treatment summarized in Table I. In addition, a similar treatment of the results of analyses of six separate standard solutions is shown in Table II.

It is concluded that the acylation conditions chosen lead to accurate and precise analytical results for these amino acids and for tryptophan in particular. Moreover, it is stressed that acylation of tryptophan appears to be characterized by its sensitivity to low levels of HFBA and the presence of ethyl acetate.

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