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GAS–LIQUID CHROMATOGRAPHY OF AMINO ACIDS

A SOLUTION TO THE HISTIDINE PROBLEM

IAIN MacARTHUR MOODIE

Fishing Industry Research Institute, University of Cape Town, Rondebosch, Cape Province (Republic of South Africa)

SUMMARY

Histidine may be derivatized to yield N^{α}, N^{im}-bis(trifluoroacetyl) *n*-butyl histidinate, a compound whose stability and hence suitability for use in gas chromatographic analysis is considered doubtful.

The reactivity of this material is discussed and indicates in the light of experience that an alternative derivative should be sought.

Model reactions led to the conclusion that by using a well-known protein modification reagent, a more suitable and stable derivative, N^{α}-trifluoroacetyl-N^{im}-carbethoxy *n*-butyl histidinate, is obtained. The preparation and identity of this material is established and its use as a suitable derivative for gas chromatography is demonstrated both qualitatively and quantitatively.

INTRODUCTION

Since the classical work on amino acid analysis carried out by Moore and Stein[1] on ion-exchange resins some two decades ago, many improvements have been forthcoming both in technique and instrumentation until at the present time it is possible to perform an analysis on a protein hydrolysate in approximately 3 h.

However, at the same time the scope of gas chromatography as a quantitative analytical tool has rapidly broadened and during the mid 1960s there appeared the start of a series of reports on the separation of amino acids as their derivatives by gas chromatography. This was followed by an increasing number of publications on the use of different derivatives and, indeed, research work in this field has been devoted almost exclusively to seeking the optimum combination of derivates and liquid phase for quantitative determination of amino acids. This work has been ably reviewed by Blau[2].

The potential advantages offered by gas chromatographic methods over those based on ion exchange are greater speed and sensitivity of analysis together with a lower capital cost for equipment, which offers flexibility of application. These considerations have contributed extensively to the great upsurge of research in this field.

At this time, there are two techniques which have been developed to any worth-

while extent. They are those reported by (a) Darbre and Islam[3,4] involving the separation of eighteen of the protein amino acids as their N-trifluoroacetyl methyl ester (TAM) derivatives on a single set of columns, and (b) Roach and Gehrke[5] who claim separation and quantitation for all twenty protein amino acids as their N-trifluoroacetyl *n*-butyl ester (TAB) derivatives on a dual set of columns.

Initial studies in our laboratories prompted us to adopt the latter technique as the methyl esters are rather too volatile and losses may easily be incurred during manipulation of these derivatives particularly when the operation is placed on a routine basis.

During the implementation of amino acid analysis by gas chromatography using the TAB derivatives and methods described by Gehrke[5], a particular problem has arisen, namely the successful chromatographic analysis of histidine.

A survey of the literature on the gas chromatography of amino acid derivatives reveals that only Gehrke's group appears to have overcome the problem for in all other work histidine is either omitted or derivatization has not been successful. However, even Gehrke and co-workers experienced difficulty as in an earlier paper[6] they describe its determination following on-column conversion either to the N^{α}-trifluoroacetyl *n*-butyl histidinate (monoacyl derivative) by a subsequent injection of *n*-butanol or to the N^{α},N^{im}-bis(trifluoroacetyl) *n*-butyl histidinate (diacyl derivative)

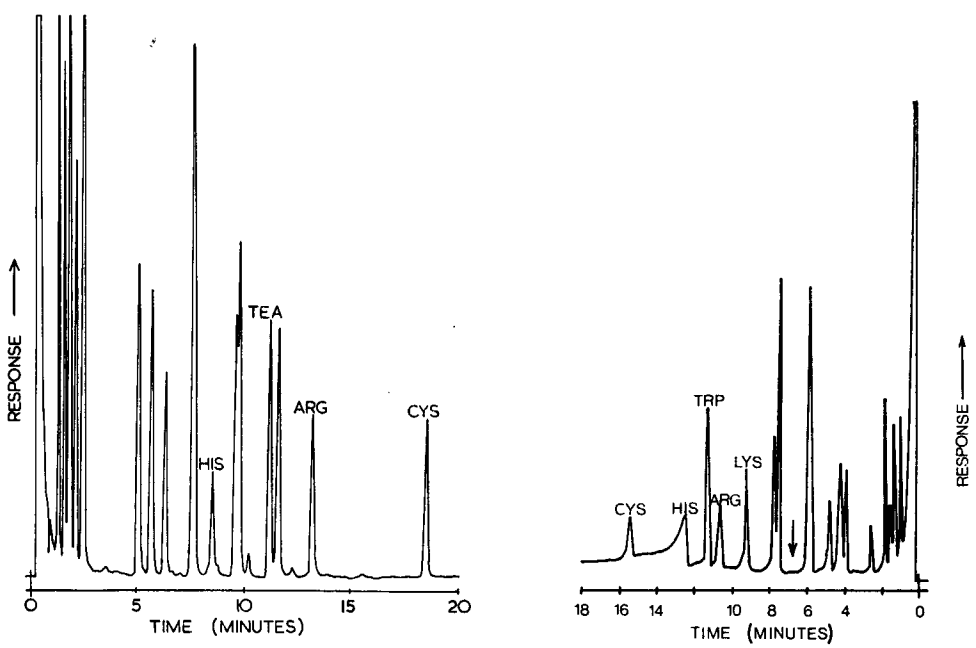


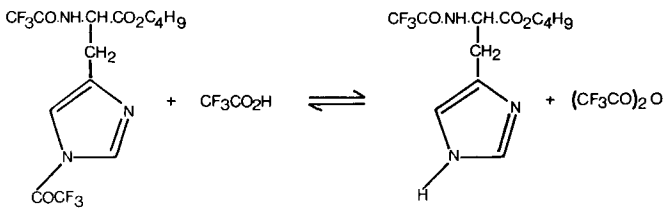
Fig. 1. Mixture of standard amino acids derivatized and analysed using 2% (w/w) OV-17 and 1% (w/w) OV-210 on Gas-Chrom Q (100–120 mesh) in 1.85-m columns. Carrier flow, *ca.* 50 ml/min; initial temperature, 125°; programme rate, 6°/min; final temperature, 235°; internal standard, tranexamic acid (TEA).

Fig. 2. Mixture of standard amino acid TAB derivatives analysed using 2% (w/w) OV-17 and 1% (w/w) OV-210 on Chromosorb W (AW-DMCS; 100–120 mesh) in 1.5-m columns. Carrier flow, *ca.* 70 ml/min. Initial temperature, 120°; programme rate, 6°/min; final temperature, 225°.

by a similar injection of trifluoroacetic anhydride (TFAA), neither procedure proving satisfactory. In 1971, Gehrke *et al.*[7] described the use of a mixed silicone phase 2% (w/w) OV-17, 1% (w/w) OV-210 on silanized support (Supelcoport) for the separation and quantitative determination of arginine, cystine and histidine as its diacyl derivative. A typical chromatogtam is shown in Fig. 1.

In our hands, all attempts, using the specified conditions, to obtain a peak at the retention shown in Fig. 1 have failed, no peak for histidine being observed in this position. Interestingly, the commercially available standard only exhibits a peak whose retention time corresponds to that of the monoacyl derivative[5] (Fig. 2).

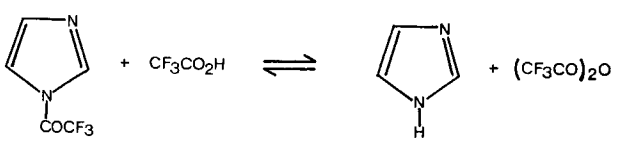
Careful consideration of certain aspects of the chemistry of the two histidine TAB derivatives and their relationship to each other leads to a prediction that successful quantitative determinations of either must be doubtful.



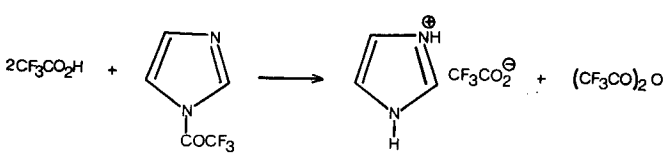
I II

The stability of the diacyl derivative (I) must be considered to be very poor in the light of the effectiveness of N-perfluoroacyl imidazoles as potent acylating agents[8], the trifluoroacetyl group being particularly labile. The equilibrium is thus inclined to favour a preponderance of the monoacyl derivative (II).

However, in the presence of a large excess of TFAA[6] this tendency diminishes as is corroborated by Staab *et al.*[9] who describe a similar equilibrium in the case of imidazole:



In addition, however, Staab *et al.*[9] also indicate that a further reaction takes place in which the N-trifluoroacetyl imidazole deacetylates in the presence of trifluoroacetic acid to form the trifluoroacetate salt of imidazole:



Thus it appears that the histidine diacyl derivative (I) readily undergoes deacetylation and salt formation with its co-product, trifluoroacetic acid, and has, at best, a transient existence when there is an absence of (or the derivative becomes separated from) a large excess of TFAA.

Similar behaviour has been reported independently by Makisumi and Saroff[10] when they prepared N^{α}-trifluoroacetyl methyl histidinate trifluoroacetate.

On the other hand, the monoacyl derivative (II), in the absence of trifluoroacetic acid, should be stable but is inclined to undergo derivative–packing interaction as evidenced by our observations (Fig. 2) of severe tailing.

Clearly, then, successful quantitative determination of histidine still remains an elusive problem and this paper sets out a means of overcoming the problem.

The aim was to obtain a stable derivative that would exhibit a satisfactory non-tailing peak on the gas chromatogram (*i.e.* no column–derivative interaction) and whose ready preparation at the same time would dove-tail with the established methodology.

EXPERIMENTAL

Apparatus

A Hewlett-Packard 7611 gas chromatograph equipped with a four-column oven, four hydrogen flame ionization detectors, two dual differential electrometers and a double-channel recorder was used. This unit was coupled to a Hewlett-Packard 3370B electronic integrator system and later to a Hewlett-Packard 3352B laboratory data system. Mass spectrometry was carried out using a Varian Model CH7 mass spectrometer.

High-purity grade nitrogen was used as carrier gas and, like the hydrogen, was passed through filters containing charcoal pellets, indicating silica gel and molecular sieve pellets, Type 5A.

Esterification, acylation and carbethoxylation reactions were conducted in Pyrex screw-top culture tubes (120 × 15 mm) whose caps were fitted with a thin PTFE gasket as seal. Solvents were removed either at 100° (oil-bath) with a current of filtered dry nitrogen taking the sample just to dryness or at 50–60° (water-bath) *in vacuo* using a rotary film evaporator.

Reagents

Amino acids were obtained from BDH, Poole, Great Britain; E. Merck, Darmstadt, G.F.R.; and Sigma, St. Louis, Mo., U.S.A. The internal standard, *trans*-4-(aminomethyl)cyclohexanecarboxylic (tranexamic) acid was obtained from A. B. Kabi, Stockholm, Sweden, as Cyclokapron.

n-Butanol was obtained from E. Merck and methylene chloride from May & Baker, Dagenham, Great Britain. Each was distilled in an all-glass system, the first and last 10% being rejected.

Anhydrous hydrogen chloride was prepared by addition of pelletized ammonium chloride (Analar grade) to concentrated sulphuric acid (reagent grade). The hydrogen chloride produced was further dried by bubbling through concentrated sulphuric acid and thence through a sinter surrounded by glass wool forming a spray trap.

The butylation reagent was formed by passing the appropriate weight of dry hydrogen chloride into chilled (ice–water bath) *n*-butanol.

TFAA was obtained from E. Merck and the acylating reagent prepared by addition of the TFAA to distilled methylene chloride in 1:3 (v/v) ratio.

Ethoxyformic anhydride (EFA) was obtained from Bayer, Leverkusen, G.F.R., in the form of a commercial preparation, Baycovin.

Reference amino acid standard TAB derivatives were obtained from Regis Chemical Co., Chicago, Ill., U.S.A.

Alumina (Brockman grading II-III) was obtained from E. Merck.

Chromatographic columns

Stabilized ethylene glycol adipate (EGA) was obtained from Analabs, North Haven, Conn., U.S.A. and the silicone liquid phases OV-17 and OV-210 from J.J.'s (Chromatography) Ltd, Norfolk, Great Britain.

The polar (EGA) and non-polar (OV-17/OV-210) column packings were prepared and conditioned exactly as described by Gehrke[7,11] except that the commercially available 80–100 mesh and 100–120 mesh acid-washed Chromosorb W, were further acid washed, graded and dried prior to (i) coating the 80–100 mesh with 0.65% (w/w) stabilized EGA and (ii) silanizing and coating the 100–120 mesh with 2% (w/w) OV-17, 1% (w/w) OV-210. The packings were finally dried as a fluidized bed with a current of warm dry filtered nitrogen. Silanized glass U-shaped columns (4 mm I.D.) were used in all analyses.

Preparation of amino acid TAB derivatives

The "direct esterification–acylation" procedure described by Roach and Gehrke[5] was used.

Preparation of N^{α}-trifluoroacetyl-N^{im}-carbethoxy n-butyl histidinate

Having carried out the "direct esterification–acylation" procedure[5] on histidine (0.005 m*M*), the solution was taken almost to dryness using a current of filtered

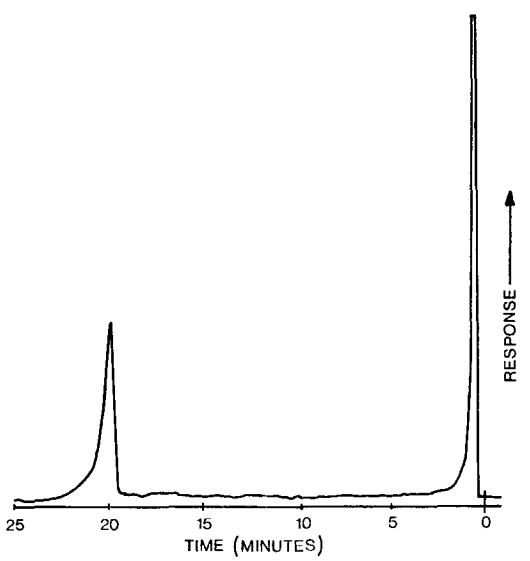


Fig. 3. Chromatogram of N^{α}-trifluoroacetyl *n*-butyl histidinate. Analysis using 2% (w/w) OV-17 and 1% (w/w) OV-210 on Chromosorb W (AW-DMCS; 100–120 mesh) in 1.5-m columns. Carrier flow, *ca.* 60 ml/min. Initial temperature, 85°; programme rate, 6°/min; final temperature, 225°.

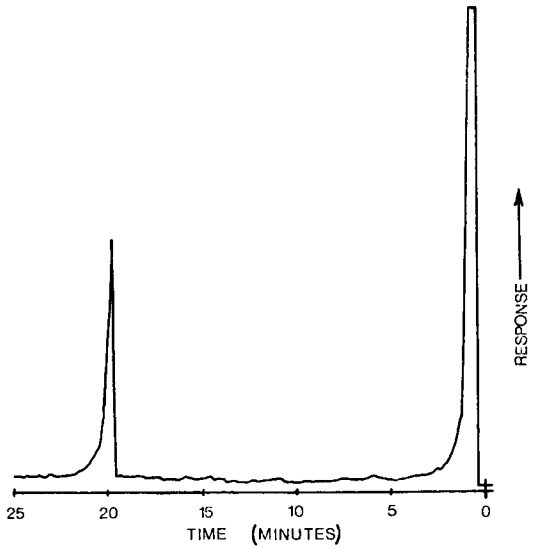


Fig. 4. Chromatogram of commercial histidine TAB standard. Analysis conditions as detailed in Fig. 3.

dry nitrogen, thereby forming N^{α}-trifluoroacetyl *n*-butyl histidinate as described by Gehrke *et al.*[6]. A chromatogram of this material (Fig. 3) is identical with that of the commercially available material (Fig. 4). Methylene chloride (1 ml) was added, followed by EFA (1 μl; 0.007 m*M*) and after flushing with nitrogen the tube was sealed. After 45 min a sample of the product exhibited the chromatogram shown in Fig. 5.

In a second similar experiment the sealed reactants were heated at 150° (oil-

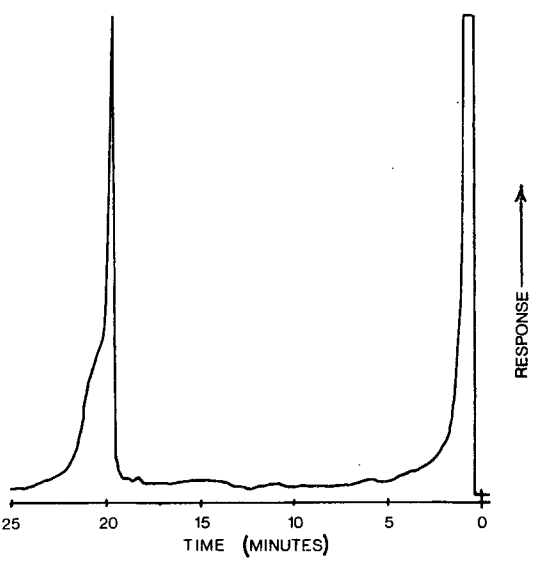


Fig. 5. Chromatogram of low-temperature reaction product from treatment of N^{α}-trifluoroacetyl *n*-butyl histidinate with EFA. Analysis conditions as detailed in Fig. 3.

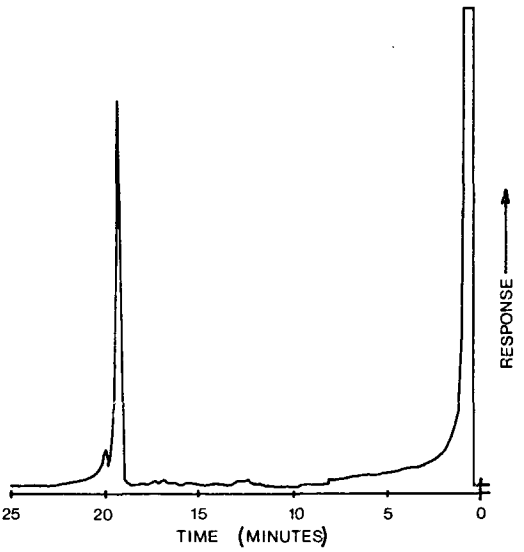


Fig. 6. Chromatogram of high-temperature reaction product from treatment (10 min) of N^{α}-trifluoroacetyl *n*-butyl histidinate with EFA. Analysis conditions as detailed in Fig. 3.

bath) for 10 min yielding a product whose chromatogram is shown in Fig. 6. Heating of this material for a further 10 min gave a chromatographically pure material (Fig. 7).

A third experiment was conducted on a 2.5-m*M* scale and the product, a pale yellow oil, was subjected to chromatographic clean-up. The reaction product in methylene chloride–benzene (1:1) was placed on a column (8 × 1 cm) of alumina. The column was eluted with methylene chloride in 20-ml aliquots, the eluate fractions

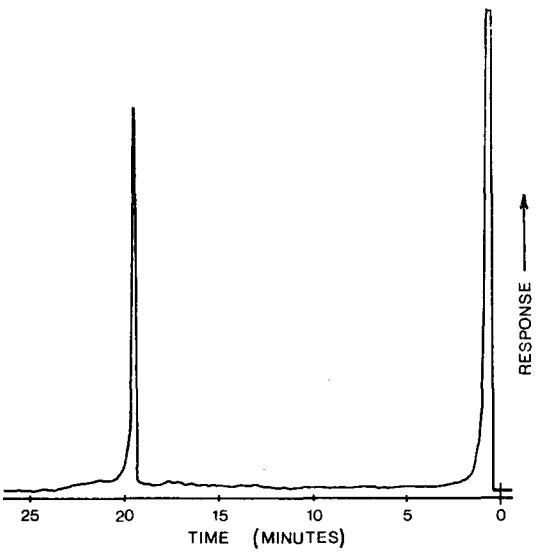


Fig. 7. Chromatogram of high-temperature reaction product from treatment (20 min) of N^{α}-trifluoroacetyl *n*-butyl histidinate with EFA. Analysis conditions as detailed in Fig. 3.

being concentrated and monitored by gas chromatography. When no further quantities of the derivatives were eluted, those fractions (all but first three) containing pure material (correct retention time) were combined and the solvent removed to yield a pale yellow oil which on micro-analysis gave: C, 47.4%; H, 5.6%; N, 11.6%. Calculated for $C_{15}H_{20}N_3O_5F_3$: C, 47.5%; H, 5.3%; N, 11.1%.

Mass spectral analysis showed principally a mass ion at *m/e* 379 with losses at *m/e* 278 ($-CO_2C_4H_9$) and *m/e* 206 ($-CO_2C_4H_9 + -CO_2C_2H_5$). The spectrum also shows an ion at *m/e* 153 (N-carbethoxy imidazolyl fragment).

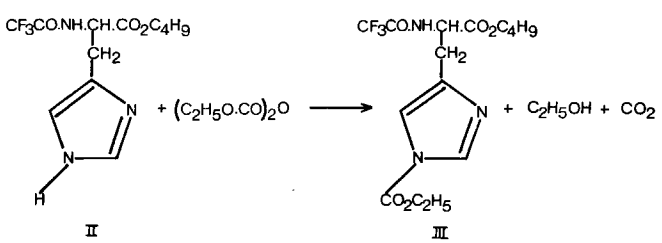
RESULTS AND DISCUSSION

Model reactions with reagents capable of rapid N-substitution of imidazole had been studied[12] and acetic anhydride or acetyl chloride in the presence of lithium carbonate, as acid scavenger, reacted with imidazole to yield N-acetyl imidazole, the former reaction proceeding almost quantitatively. However, though more stable than its N-trifluoroacetyl analogue, the acetyl group was also still too labile. In addition, treatment of N-trifluoroacetyl *n*-butyl histidinate with acetic anhydride yielded a product that was shown by gas chromatography[12] to be identical with the starting material.

N-carbethoxy imidazole is reported[13] to be some two orders of magnitude more stable than N-acetyl imidazole and this system seemed to be worth studying since it clearly offered potential when considered in the context of the histidine monoacyl derivative. However, reaction of imidazole with ethyl chloroformate gave N-carbethoxy imidazole hydrochloride and similar treatment of the monoacyl histidine derivative (II) yielded a product whose gas chromatogram exhibited severe tailing rendering it useless for quantitative chromatography.

To obtain the N-carbethoxy imidazole without, at the same time, liberating acid which might lead to salt formation, EFA was used. This compound is a well-known reagent for protein modification[14–16], reacting with α-amino and imidazole groups in histidine and in one instance[17] it is used as the basis for a specific method of estimating the histidine content of a protein. However, its use in non-aqueous media has so far not been described.

The reaction of EFA with the monoacyl derivative (II) of histidine was expected to lead to the desired product, N^{α}-trifluoroacetyl-N^{im}-carbethoxy *n*-butyl histidinate (III) with no accompanying reactive by-products:



II III

Room-temperature conditions were observed to lead to a marked reaction but those involving elevated temperature produced a single chromatographically pure

material identified by micro-analysis and mass spectrometry as N^{α}-trifluoroacetyl-N^{im}-carbethoxy *n*-butyl histidinate.

The latter procedure was extended to a mixture of some commercial standard TAB derivatives including the monoacyl histidine (II), and prior to treatment (Fig. 2) this component shows a typical badly tailing peak. However, following treatment with EFA at 150° for 20 min the sharp peak ascribed to the new derivative (III) has appeared at a shorter retention time (Fig. 8). Confirmation of its origin was obtained by "spiking" the mixture with the monoacyl derivative (II), re-treatment with further EFA leading to an enhancement of the new peak (Fig. 9).

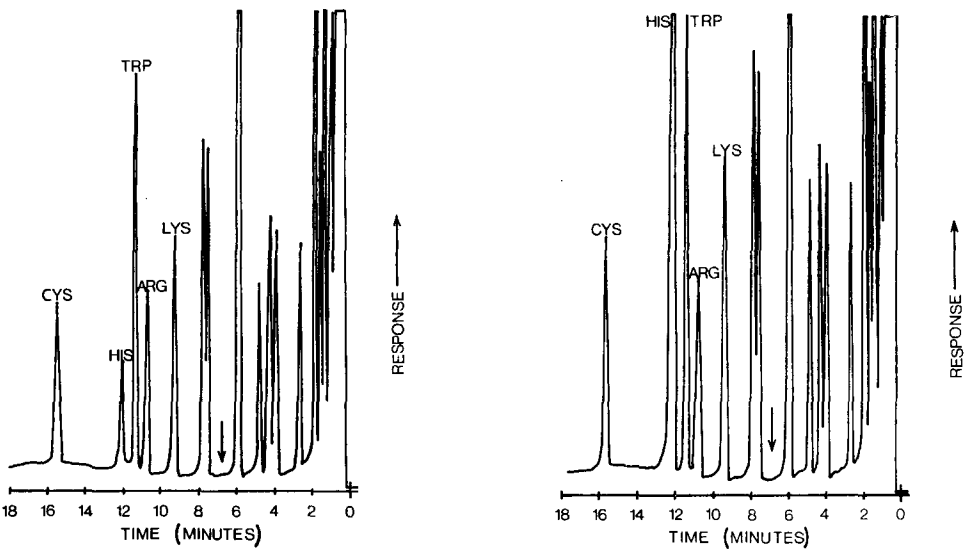


Fig. 8. Mixture of standard amino acid TAB derivatives treated with EFA for 20 min at 150° and chromatographed using conditions detailed in Fig. 2.

Fig. 9. Mixture of standard amino acid TAB derivatives "spiked" with N^{α}-trifluoroacetyl *n*-butyl histidinate, treated with EFA for 20 min at 150° and chromatographed using conditions detailed in Fig. 2.

Having thus established a suitable derivatization step and qualitative technique, the quantitative aspects were studied, relative molar response (*RMR*) values for all twenty protein amino acids being shown in Table I. Analyses 1–5 are derived from separate amino acid mixtures and show a good measure of agreement. Meaningful figures for histidine were unobtainable in this group but in analyses 6–9 for which the EFA treatment was used, good results were obtained. However, the EFA appears to affect the response for some of the other amino acid derivatives, particularly those of methionine, arginine and tyrosine, and the usual analysis[5] should therefore be followed by the EFA procedure and analysis to avoid this interference.

The pronounced effect in the case of the tyrosine derivative may be attributed to the reactivity, previously discussed by Darbre and Blau[18], of the O-TFA group, which may undergo scission in the presence of EFA.

TABLE I

RELATIVE MOLAR RESPONSE (*RMR*) DATA FOR TWENTY PROTEIN AMINO ACIDS

Amino acid	*Analysis No.**								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Alanine	0.47	0.49	0.48	0.50	—	0.52			
Valine	0.64	0.69	0.67	0.69	0.69	0.69			
Glycine	0.37	0.39	0.39	0.40	0.40	0.41			
Isoleucine	0.73	0.78	0.75	0.76	0.78	0.77			
Leucine	0.74	0.78	0.75	—	0.80	0.79			
Proline	0.63	0.68	0.64	—	—	0.68			
Threonine	0.56	0.59	0.68	0.70	0.61	0.57			
Serine	0.38	0.42	0.41	0.43	0.47	0.38			
Cysteine	0.34	0.19	0.19	0.21	0.21	0.21			
Methionine	0.68	0.68	0.66	0.68	—	0.51			
Hydroxyproline	0.68	0.69	0.68	0.71	0.72	0.76			
Phenylalanine	1.05	1.10	1.06	1.11	1.10	1.14			
Aspartic acid	0.83	0.88	0.85	0.89	0.88	0.89			
Glutamic acid	1.07	0.98	1.02	0.99	1.07	0.97			
Tranexamic acid	1.00	1.00	1.00	1.00	1.00	1.00			
Ornithine	0.70	0.74	0.72	0.76	0.74	0.73			
Lysine	0.79	0.80	0.77	0.81	0.80	0.83			
Tryptophan	0.78	0.78	0.76	0.79	0.79	0.76			
Tyrosine	1.51	1.35	1.27	1.26	1.25	0.48	0.40	0.37	0.40
Arginine	1.07	0.91	0.80	0.92	0.92	0.59	0.60	—	0.63
Histidine						0.59	0.59	0.55	0.60
Cystine	—	0.56	0.59	0.60	—	—	—	0.66	0.64

* Analyses Nos. 1–5: no EFA treatment; analyses Nos. 6–9: EFA treated.

CONCLUSION

Although adding to the time required to complete the derivatization and analysis of the protein amino acids, this new EFA treatment enables a reliable assessment of histidine to be made.

ACKNOWLEDGEMENTS

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