Journal of Chromatography, 446 (1988) 231–236
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1340

GAS CHROMATOGRAPHIC ANALYSIS OF HISTIDINE: EFFECT ON OTHER AMINO ACIDS PRESENT

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SUMMARY

Earlier proposals aimed at increasing the detector responses of histidine in gas chromatographic analysis have been compared and contrasted with regard to their effect on the other amino acids present. Twenty-one amino acids have been measured in the form of their N,O,(S)-trifluoroacetyl (TFA) isobutyl esters. Detector responses of the amino acid N,O,(S)-TFA isobutyl esters of dichloromethane solutions alone and mixed with acetic anhydride were recorded and compared with those of other TFA esters. Reproducibility data for chosen representative amino acid N,O,(S)-TFA isobutyl esters are presented.

INTRODUCTION

The optimum parameters for the gas chromatographic (GC) analysis of histidine have remained an open question in amino acid assays[1–7]. According to MacKenzie[6], "several methods have been used to overcome this problem, but the final solution is still being sought". Earlier, Gehrke and co-workers[1,2] described three methods for the analysis of histidine as its trifluoroacetyl(TFA) *n*-butyl ester: (i) as the monoacyl derivative after removal of the excess of acylating reagent, trifluoroacetic anhydride (TFAA); (ii) as diacyl derivatives after converting the monoacyl derivative into the diacyl form with an on-column injection of TFAA and (iii) as trimethylsilyl derivatives.

MacKenzie and Tenaschuk[3–6] and Moodie *et al.*[7] analyzed amino acids as their heptafluorobutyric (HFB)isobutyl esters. For the estimation of histidine as its mixed acylated ester (MAE = N^{I}-HFB, N^{II}-acetyl isobutyl ester), they used two different pretreatments. After removing the excess of acylating agent, Moodie *et al.*[7] preferred the preparation of a stock solution with acetic anhydride (AA), whereas MacKenzie and Tenaschuk[3–6] injected the residue dissolved in ethyl acetate. Formation of the MAE occurred on the column by simultaneous injection of AA in the volume ratio, stock solution/AA = 1/0.2.

So far, neither comparative studies concerning the most advantageous derivatization and elution procedure for histidine determination, nor the effect of the proposed procedures for histidine on the analysis of the other amino acids present

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have been reported. In this paper we compare the previously suggested[1–7] procedures for increasing the histidine responses in relation to the responses of other amino acids present.

MATERIALS AND METHODS

Reagents

All reagents and standard amino acids were of analytical purity obtained from Reanal (Budapest, Hungary).

Apparatus

A Chromatron G.C.H.F. 18.3 gas chromatograph (VEB Chromatron, Berlin, G.D.R.), equipped with a flame-ionization detector and a 2 m × 4 mm I.D. stainless-steel column, was used. Nitrogen was the carrier gas at a flow-rate of 60 cm^3/min. The column packing consisted of 3% SE-30 on Chromosorb W (100–120 mesh) (Supelco, Bellefonte, PA, U.S.A.). The column temperature was increased from 90 to 250°C at 4 or 6°C/min. The temperatures of the injector and detector were 250 and 300°C, respectively.

Derivatization

A 1–2 cm^3 volume of the 1 *M* hydrochloric acid stock solution of amino acids (each 1.5–5 mg/cm^3) was pipetted into a 25-cm^3 vessel, which could be fitted with either a vacuum distillation device or a reflux condenser, via a ground-glass joint and evaporated to dryness under vacuum. To the residue was added 1 cm^3 of isobutanol containing 1 *M* thionyl chloride. A reflux condenser was then fitted to the flask and the apparatus placed in a water-bath. Esterification took place at 100°C for 60 min. After cooling to room temperature, the solution was evaporated to a syrupy consistency under vacuum, in a water-bath at 60°C. The residue was transferred quantitatively with 5 × 0.1 cm^3 dichloromethane to a 5-cm^3 Pierce Reacti-Vial. A 1.0-cm^3 volume of TFAA was added and the acylation was carried out for 5 min at 150°C. Thereafter, the solution of the acylated derivatives was transferred quantitatively to a glass-stoppered, calibrated test-tube (also connected to the vacuum evaporator). The solution of the N,O,(S)-TFA esters was evaporated to dryness in an ice-bath.

The derivatization was performed in six replicates. To the residues of the samples, dichloromethane and AA were added, and a stock solutions of 1.5 cm^3 was prepared from each. Aliquots (5–10 μl) were injected into the gas chromatograph (see also the footnote to Table I).

The amino acid N,O,(S)-TFA *n*-butyl and *n*-propyl esters were prepared as described above, with the exception that, instead of isobutanol, *n*-butanol or *n*-propanol was used.

RESULTS AND DISCUSSION

After exhaustive studies concerning the most advantageous form for GC analysis, the N,O(S)-TFA isobutyl esters were chosen (Fig. 1A–C).

To our knowledge, except for our recently published paper[8], no other reports

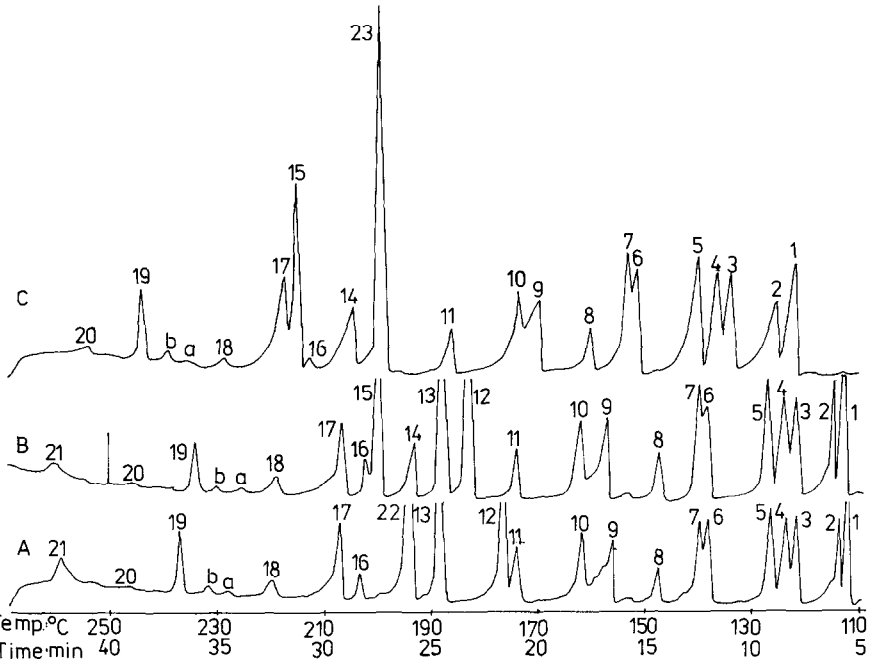

Fig. 1. Chromatograms of the N,O,(S)-TFA *n*-propyl (A), isobutyl (B) and *n*-butyl (C) esters of amino acids. Peaks: 1 = alanine; 2 = glycine; 3 = threonine; 4 = serine; 5 = valine; 6 = leucine; 7 = isoleucine; 8 = cysteine; 9 = proline; 10 = hydroxyproline; 11 methionine; 12 = aspartic acid; 13 = phenylalanine; 14 ornithine; 15 = glutamic acid; 16 = tyrosine; 17 = lysine; 18 = arginine; 19 = tryptophan; 20 = histidine; 21 = cystine; 22 = ornithine + glutamic acid; 23 = aspartic acid + phenylalanine; a and b = tryptophan.

have been made on the quantitation and GC analysis of amino acids as their N,O,(S)-TFA isobutyl esters: while the cost of TFAA is about one sixth of that of HFBA, the results with the two acylated products under our conditions were the same.

The advantage of the N,O,(S)-TFA isobutyl esters over the corresponding *n*-butyl and *n*-propyl esters is seen in Fig. 1: the *n*-propyl esters of ornithine + glutamic acid (Fig. 1, peak 22) and the *n*-butyl esters of aspartic acid + phenylalanine (Fig. 1, peak 23) are not resolved.

It is remarkable that removal of the excess of TFAA and dissolution of the residue in dichloromethane resulted in a considerable decrease in detector responses (Fig. 2A,B). As shown, the decreases in sensitivity (Fig. 2B) are on the average about 25–35% of those obtained with an excess of TFAA (Fig. 2A) with the exceptions of the most unstable amino acids, *e.g.*, arginine (93% less), tyrosine (55% less) and cysteine (36% less).

The reproducibility of the determinations, performed with two levels of selected* amino acids, is presented in Table I.

* *i.e.*, with members the detector responses of which are not altered by subjecting them to treatments (a–f) used for increasing the histidine responses (more detailed data in Table I).

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF CHOSEN REPRESENTATIVES OF AMINO ACID N,O,(S)-TFA ISOBUTYL ESTERS

a(a_1)–f = Detector responses of the stock solution of N,O,(S)-TFA isobutyl esters, obtained according to the derivatization procedure, after removal of the excess of TFAA and dissolution of the residue in dichloromethane (*a–d*) or after removal of the dichloromethane and dissolution of the residue in acetic anhydride (AA; e,f.). a–f, Freshly derivatized; a_1–d_1, 1 week later. a, Dichloromethane solution; b,c, 10 μl of dichloromethane solution with simultaneous injection of 1 (b), 2 (c) and 4 μl AA(d), respectively; e,f, solution of N,O,(S)-TFA isobutyl ester of amino acids, dissolved in AA(e) and heated at 130°C for 20 min (f). Peak areas obtained (in arbitrary units), equivalent to 1 μg of substance.

Amino acid	*Injected amount (μg)*	*Pretreatment*										*Mean*	*S.D.*	*R.S.D. (%)*
		a	a_1	*b*	b_1	*c*	c_1	*d*	d_1	*e*	*f*			
Alanine	14.5	367	371	371	372	372	374	373	380	373	373	373	3.2	0.9
	29.0	373	374	377	378	371	370	374	373	367	376			
Glycine	14.5	449	445	441	443	445	450	441	436	443	447	447	10.3	2.3
	29.0	449	442	436	440	466	458	439	432	463	470			
Serine	14.7	292	295	283	280	280	285	294	292	291	291	286	7.4	2.6
	29.4	280	277	285	281	294	283	298	288	268	289			
Proline	16.4	483	483	478	482	471	469	480	467	483	484	475	6.1	1.3
	32.8	474	477	476	463	475	474	475	474	469	468			
Hydroxyproline	13.5	322	322	329	307	325	332	322	301	322	319	314	12.3	3.9
	27.0	306	310	304	297	320	319	292	292	320	327			
Aspartic acid	24.0	407	405	403	402	402	393	399	388	408	418	393	13.3	3.4
	48.0	389	390	399	379	391	381	372	363	381	398			
Phenylalanine	21.6	548	541	534	543	538	527	546	550	546	555	541	7.9	1.5
	43.2	545	539	541	541	543	548	540	528	533	537			
Glutamic acid	21.3	336	337	323	331	339	335	331	325	335	337	330	5.6	1.7
	42.6	331	336	329	334	329	324	329	324	320	325			
Lysine	12.0	280	280	266	271	276	277	280	273	295	301	274	10.1	3.7
	24.0	270	270	269	267	263	264	(238)	267	265	268			

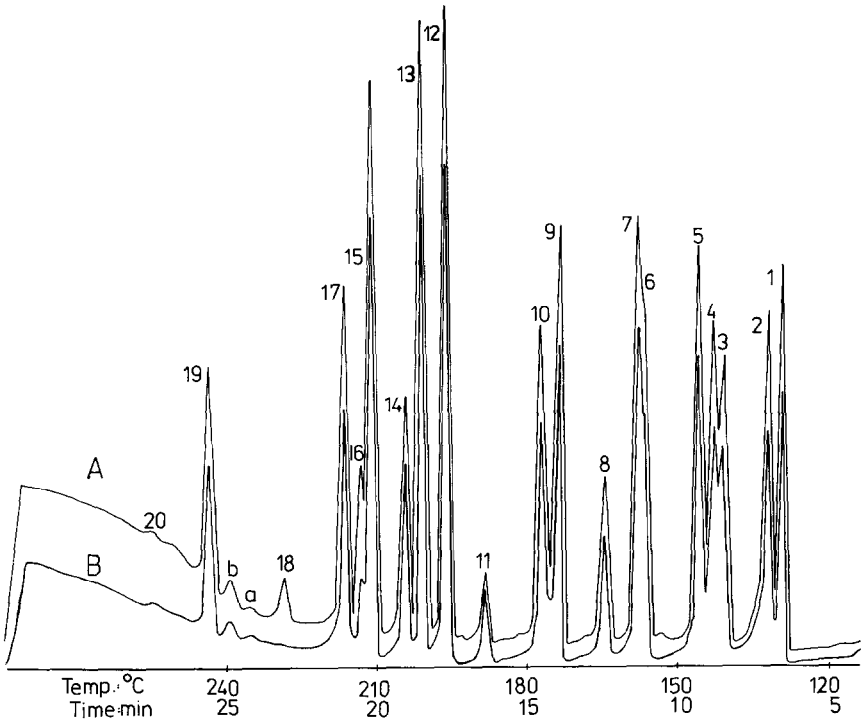

Fig. 2. Chromatograms of the N,O,(S)-TFA isobutyl esters of amino acids obtained in the presence of excess of TFAA (A) and after removal of the excess of TFAA and redissolution of the residue in dichloromethane (B). Peaks as in Fig. 1B. Detector responses calculated from chromatogram B expressed as % of those obtained from chromatogram A were as follows: peak 1, 75; 2, 76; 3, 75; 4, 71; 5, 77; 6 + 7, 79; 8, 65; 9, 74; 10, 76; 11, 100; 12, 77; 13, 78; 14, 85; 15, 79; 16, 45; 17, 70; 18, 7; 19, 79; 20, 70%.

The detector response of histidine as a result of various pretreatments (Fig. 3A–E) was not considerably improved: (i) the trifluoroacylated histidine esters were eluted as two peaks (Fig. 3A, peaks 20/1, 20/2) probably due to the monoacylated (peak 20/2) and to the diacylated (peak 20/1) forms, respectively; (ii) the detector responses of MAE (Fig. 3B–E) occurred at the same retention time as those of the diacylated form (Fig. 3A, peak 20/1) independent of the handling of the stock solutions. On the average, they were the same, but entailed the following disadvantages: (i) the detector responses of arginine and tyrosine as a result of pretreatments (b–c) were further decreased compared to the detector responses obtained upon elution of the dichloromethane solution of the N,O,(S)-TFA isobutyl esters (Fig. 1B), although under the conditions of pretreatments d and e the amount of tyrosine became greater (Fig. 3E,D); (ii) the use of AA was accompanied by a considerable increase in the baseline (see the trend in Fig. 3A–E).

Based on all the above-mentioned details, we prefer the analysis of N,O,(S)-TFA isobutyl esters with solutions containing an excess of TFAA.

We are continuing our investigations in order to find better GC conditions for the separation of the N,O,(S)-TFA isobutyl esters and to extend the lifetime of the coated support material.

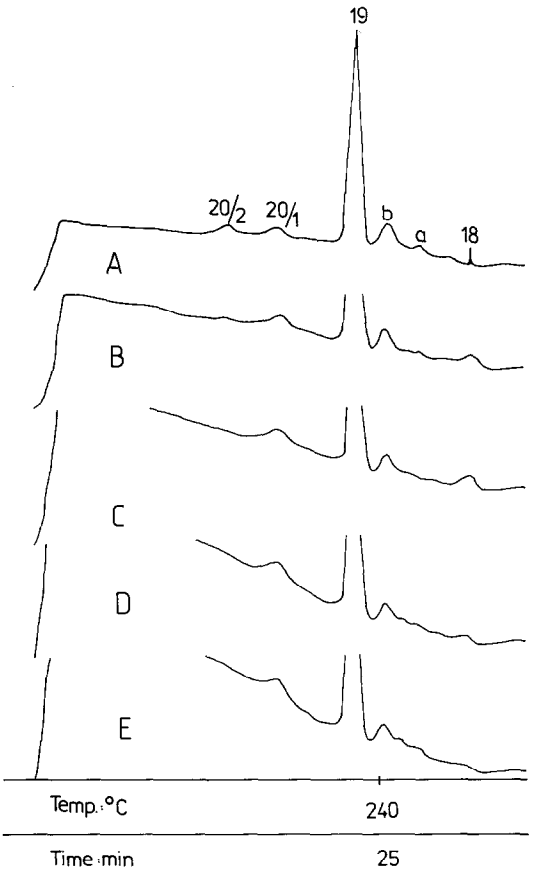

Fig. 3. Chromatograms of the N,O,(S)-TFA isobutyl esters of arginine (18), tryptophan (19a,b) and histidine (20/1 = diacylated, 20/2 = monoacylated ester) after different pretreatments of the stock solution of the N,O,(S)-TFA isobutyl esters. Chromatograms A–E correspond to the pretreatments *a–e*, as detailed in Table I.

ACKNOWLEDGEMENT

This work was supported by the State Office of Managing Department for Protein Researches, Budapest, Hungary.

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