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QUANTITATIVE FORMATION OF N(O,S)-HEPTAFLUOROBUTYRYL ISOBUTYL AMINO ACIDS FOR GAS CHROMATOGRAPHIC ANALYSIS

II. ACYLATION*

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SUMMARY

The effect of temperature (75–150°) and time (5–30 min) on the reaction of heptafluorobutyric anhydride with the isobutyl esters of protein amino acids has been studied. Acylation of most of the amino acids was complete after heating at 75° for 10 min. However, the procedure of Pearce (heating at 110° for 10 min) was shown to be inadequate for quantitative acylation of arginine. Therefore, heating at 150° for 10 min is recommended.

Acylation at 150° for 10 min was shown to produce quantitative (>97%) formation of diacyltryptophan.

Acylation using ethoxyformic anhydride was originally developed to overcome the problem of acylating the imidazole nitrogen of histidine. This technique was compared with and concluded to offer no advantage over acylation with carboxylic acid anhydrides.

INTRODUCTION

Amino acids, being multifunctional, require derivatization of reactive groups before they can be analysed by gas–liquid chromatography (GLC). By far the most commonly used derivatives are N(O,S) acylated amino acid alkyl esters. Considerable study of this type of derivative led to the successful quantitation of the protein amino acids and some non-protein amino acids by Gehrke and co-workers[1–3]. Later investigators in this field are particularly indebted to this group for a sustained and thorough study of the conditions required for quantitative derivatization, specifically of the N-trifluoroacetyl (TFA) *n*-butyl esters. However, the chromatographic separation of these derivatives requires two columns[3]. In certain circumstances, additional information can result from two columns but for most routine analyses the need for two columns increases the overall analysis time. Subsequent investigations led to the

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development of separations of protein amino acid derivatives using a single column[4–7]. All of these procedures present practical alternatives to amino acid analysis by cation-exchange chromatography (CIE). Amino acid analysis by GLC possesses inherent advantages over CIE in speed of analysis, resolution of components, cost of equipment and the capability of analysing extremely small amounts of material. Furthermore, and perhaps most important, the equipment need not be dedicated to only one type of analysis. The advantages of analytical speed are particularly relevant in the analysis of physiological samples which contain many non-protein amino acids and which require several hours for analysis by CIE.

Despite the development of excellent single-column techniques which have much in common, unanimity has not been achieved in certain problem areas. For example, the imidazole nitrogen of histidine cannot be readily acylated using the perfluorocarboxylic acid anhydrides.

Several solutions to this problem have been suggested. Roach *et al.*[8] used on-column acylation by co-injecting the sample with trifluoroacetic anhydride (TFAA) and variations of this procedure were subsequently used by others[4,6,9]. An alternative procedure, developed by Moodie[10], involves the reaction (150°, 10 min) of N^{α}-TFA *n*-butyl histidinate with ethoxyformic anhydride (EFA) to form N^{α}-TFA, N^{π}-carbethoxy *n*-butyl histidinate which was claimed to be a stable derivative suitable for gas chromatography. Because this procedure caused considerable degradation of the derivatives of methionine, arginine and tyrosine, two separate analyses were required, one after the normal acylation of the amino acid esters and another, specifically for histidine, after the reaction with EFA. Although the peak obtained using EFA was sharper than that generally obtained using on-column injection with acetic anhydride, no data comparing the reproducibility of the two techniques were presented.

The EFA technique was later applied by Pearce to the N-heptafluorobutyryl (HFB) isobutyl ester of histidine[11]. The reaction conditions (110°, 5 min) were milder than those of Moodie and were claimed to cause less severe degradation of methionine, arginine and tyrosine N(O,S)-HFB isobutyl esters, but the claim was not documented. Furthermore, the chromatographic peak tailed considerably and, despite the use of a capillary column, was no sharper than was achieved by the same author using on-column acetylation. Again, no data were presented comparing the reproducibility and precision of the two procedures.

Tryptophan does not survive acid-catalysed hydrolysis of proteins. Consequently, it must be determined either by a spectrophotometric method or by using appropriate hydrolytic conditions. Its subsequent analysis by GLC as an N-acyl ester involves exposure to a strong acid. The derivatization of tryptophan has been found by some workers to result in multiple peaks and it has been claimed[11] that tryptophan derivatives cannot be chromatographed reproducibly. Nevertheless, both Gehrke and Takeda[12] and Adams[7] have demonstrated the precise, reproducible quantitation of tryptophan derivatives. Zanetta and Vincendon[5] also obtained an excellent peak for the N-HFB isoamyl ester of tryptophan. In an earlier publication from this laboratory[13], the formation of the N-HFB isobutyl ester of tryptophan was shown to produce only minor amounts of other compounds, none of which could be identified by gas chromatography–mass spectrometry (GC–MS) as monoacyltryptophan. In the context of a mass spectrometric study, no attempt was made to explore the quantitative aspects of the derivatization. In the hands of another worker[11], our procedure has been

declared to produce "at least two major peaks and one minor peak". None of these compounds was identified nor were relative proportions given or illustrated. In Part I[14] we again showed that, using our procedure, only minor amounts of compounds other than di-HFB isobutyl tryptophan are formed. We have further demonstrated that the proportions of these other compounds are not dependent on the time of exposure to 3 *M* hydrochloric acid. In this work we have investigated the effect of variation in the acylation conditions on the formation of di-HFB isobutyl tryptophan.

The acylation of arginine has been amply demonstrated to require a temperature much higher than that for the acylation of the other amino acids. This has been shown for the N-TFA *n*-butyl ester[15] and the N-HFB isoamyl ester[5,16]. Although the effect of temperature was not specifically investigated, elevated temperatures were used without deleterious effect for the formation of arginine N-HFB *n*-propyl ester[4,17–19], arginine N-TFA methyl ester[20,21] and arginine N-HFB isobutyl ester[9]. However, the use of a temperature of 150° for 10 min has been claimed to result in degradation and 110° has been recommended for the formation of N-HFB isobutyl arginine[11]. Because this recommendation conflicts directly with our earlier results we have studied the acylation of isobutyl arginine under a variety of conditions.

In Part I[14], we studied in detail the esterification reaction in the preparation of N-HFB amino acid isobutyl esters and discussed some of the features essential for reproducible derivatization and chromatography. We present here the results of a study of the acylation reaction and of the related problems described above.

EXPERIMENTAL

Reagents

The amino acid standard containing 2.5 μmoles/ml of each amino acid was obtained from Chromatographic Specialties (Brockville, Canada). Heptafluorobutyric anhydride (>99%) was obtained from Fluka (Buchs, Switzerland). *tert.*-Butylhydroxyanisole (BHA), 2,6-di-*tert.*-butylhydroxytoluene (BHT) and ethoxyformic anhydride (EFA) was obtained from Sigma (St. Louis, Mo., U.S.A.). Ethyl acetate and isobutanol were purified as described previously[6]. Acetic anhydride, propionic anhydride and butyric anhydride were purified by fractional distillation. All other reagents were used as received.

Derivatization

Esterification and acylation were generally performed as previously described[6,14,22]. However, to study the acylation, 125 μl (0.3125 μmole) of the amino acid standard were evaporated to dryness and esterified by heating at 120° for 30 min in 250 μl of 3 *M* hydrochloric acid–isobutanol in a 1-ml Reactivial (Pierce, Rockford, Ill., U.S.A.). After esterification and cooling of the vial to temperature, 50 μl aliquots were dispensed into each of four 1-ml Reactivials. After evaporating excess of reagents at 50° using a stream of dry nitrogen, each sample was acylated in 50 μl of HFBA. All vials in each batch of four were acylated at the same temperature but for times ranging from 5 to 30 min (Table I). Thus, for any one acylation temperature all samples were identically esterified. Other variations of acylation conditions are described where appropriate in the text.

Following acylation, excess of reagent was evaporated at room temperature

using a stream of dry nitrogen. The sample was then dissolved in ethyl acetate containing *n*-hexadecane (half the concentration of the amino acids) and co-injected with acetic anhydride (0.5 μl of acetic anhydride per microlitre of sample) as previously described[14].

In addition to the standard co-injection procedure, the proportion of acetic anhydride used for co-injection was varied. Acetic anhydride was also added to the sample in various proportions. Corresponding experiments were also conducted using propionic anhydride and butyric anhydride.

Esterified and acylated amino acid standards were also made to react with EFA (1 μl in 25 μl of ethyl acetate) for 5 min at 110° and for 10 and 20 min at 150°. Derivatized standards were also co-injected with EFA.

Chromatography

Chromatography was performed as described in Part I[14]. All quantitation was performed using a Hewlett-Packard Model 3354 Laboratory Data System and all samples were analysed at least in triplicate.

RESULTS AND DISCUSSION

In Part I, we discussed some of the critical factors in the formation of the N(O,S)-HFB isobutyl esters of protein amino acids with particular emphasis on the esterification reaction[14]. We also discussed some of the factors that affect the chromatographic reproducibility. It is therefore relevant to consider some factors that affect reproducible acylation.

Apart from the formation of N-acetyl derivatives[7], the acylation reaction is performed at an elevated temperature. Therefore, we cannot overemphasize the need for pure reagents. We have routinely used HFBA of purity >99% while some other workers have used HFBA of all too frequently unspecified quality. Felker[23] has reported reduced threonine and tyrosine responses as a result of an unsatisfactory batch of HFBA. We have had similar experiences when using reagent-grade HFBA but not using a grade of purity >99%. In addition, we have observed a considerably reduced arginine response when using reagent-grade HFBA; this observation is discussed in more detail later.

It is important that the ethyl acetate be anhydrous. This can readily be achieved by a standard procedure[6]. Felker[23] observed the accumulation of peroxides in ethyl acetate and demonstrated that peroxides had a negative effect on the acylation of methionine and arginine. We have specifically tested redistilled reagent-grade ethyl acetate after storage at 0° for several months and have been unable to detect the presence of peroxides. Nevertheless, a routine test for peroxides would be a wise precaution.

Earlier we reported that we acylated amino acid isobutyl esters using 20 μl of HFBA and 50 μl of ethyl acetate. More recently we have adopted the use of 50 μl of HFBA only[19]. Although the results are identical, this procedure simplifies the acylation system and, provided that the HFBA is of good quality, avoids potential problems resulting from heating the esters in the presence of any contaminants which may be present in the ethyl acetate.

The results of acylating amino acid isobutyl esters at 75, 100, 125 and 150°

TABLE I

RELATIVE MOLAR RESPONSES OF N(O,S)-HFB ISOBUTYL AMINO ACIDS AS A FUNCTION OF ACYLATION TEMPERATURE AND TIME

The molar responses are expressed relative to norleucine.

Amino acid	*Temperature (°C)*															
	75				*100*				*125*				*150*			
	Time (min)															
	5	*10*	*20*	*30*	*5*	*10*	*20*	*30*	*5*	*10*	*20*	*30*	*5*	*10*	*20*	*30*
Ala	0.57	0.63	0.64	0.64	0.64	0.65	0.66	0.64	0.65	0.64	0.64	0.65	0.66	0.66	0.65	0.66
Gly	0.58	0.59	0.60	0.59	0.60	0.60	0.59	0.60	0.59	0.61	0.59	0.58	0.59	0.60	0.59	0.60
Val	0.85	0.88	0.90	0.89	0.89	0.92	0.91	0.91	0.89	0.91	0.90	0.91	0.92	0.93	0.92	0.92
Thr	0.98	0.98	0.98	0.98	0.99	0.99	0.98	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Ser	0.88	0.88	0.88	0.88	0.89	0.89	0.88	0.89	0.89	0.89	0.89	0.89	0.88	0.89	0.89	0.89
Leu	1.00	1.02	1.01	1.01	1.01	1.02	1.02	1.02	1.01	1.01	1.01	1.01	1.01	1.02	1.01	1.01
Ile	1.04	1.07	1.08	1.06	1.08	1.08	1.08	1.08	1.08	1.07	1.07	1.08	1.07	1.08	1.08	1.08
Pro	0.93	0.93	0.93	0.93	0.91	0.92	0.93	0.92	0.92	0.92	0.93	0.93	0.92	0.94	0.94	0.94
Pip	0.87	0.97	0.96	0.96	0.97	0.97	0.96	0.97	0.97	0.98	0.97	0.97	0.97	0.98	0.97	0.97
Met	0.91	0.90	0.90	0.90	0.90	0.90	0.90	0.89	0.90	0.91	0.90	0.88	0.90	0.90	0.89	0.88
Asp	1.19	1.19	1.18	1.19	1.20	1.20	1.19	1.19	1.18	1.20	1.19	1.18	1.18	1.19	1.19	1.19
Phe	1.36	1.37	1.37	1.37	1.37	1.36	1.36	1.37	1.37	1.38	1.38	1.38	1.37	1.38	1.37	1.38
Glu	1.26	1.25	1.26	1.27	1.25	1.25	1.26	1.25	1.25	1.25	1.26	1.26	1.25	1.25	1.24	1.25
Lys	1.12	1.12	1.13	1.14	1.13	1.13	1.13	1.12	1.12	1.13	1.12	1.09	1.12	1.13	1.12	1.12
Tyr	1.37	1.37	1.38	1.38	1.37	1.38	1.38	1.40	1.38	1.39	1.40	1.40	1.40	1.40	1.39	1.38
Arg	0.05	0.09	0.14	0.21	0.27	0.48	0.88	1.10	0.91	1.13	1.17	1.17	1.16	1.17	1.17	1.17
His	0.99	0.99	0.97	0.96	0.96	0.96	0.95	0.95	0.95	0.96	0.96	0.94	0.95	0.96	0.96	0.93
Cys* | Cys	1.04	1.03	1.04	1.05	1.05	1.04	1.05	1.05	1.04	1.05	1.06	1.05	1.06	1.06	1.05	1.04
Nle**	0.58	0.58	0.58	0.58	0.57	0.58	0.58	0.57	0.57	0.57	0.57	0.58	0.58	0.58	0.58	0.58

* The cystine concentration in the amino acid standard was expressed as 2.5 μmoles half-cystine per millilitre. The response has been adjusted to moles of cystine.

** The molar response of norleucine is expressed relative to cetane.

for 5–30 min are shown in Table I. Acylation of most of the esters was essentially complete after heating at 75° for only 10 min. However, heating for 5 min at 75° was inadequate for complete acylation of valine, isoleucine and pipecolic acid. Heating for an unnecessarily long time (30 min) at 150° resulted in a small decrease in the relative molar response (RMR) of methionine. The other amino acids were not affected. In general, the results are consistent with those reported from other laboratories. For instance, Felker and Bandurski[16], referring to the acylation of amino acid isoamyl esters, reported that "within limits, time and temperature of acylation are not critical". We agree with that conclusion with one important exception. The acylation of arginine is incomplete at 75 and 100° even after heating for 30 min, and at 125° heating for 20 min is required for complete acylation (Table I and Fig. 1).

The preponderance of evidence in the literature demonstrates clearly that, except for N-acetylation, an elevated temperature is required for the N-acylation of arginine alkyl esters[4–6,9,14–24]. More specifically, we reported earlier that heating at 120° for 10 min is insufficient for the quantitative formation of N-HFB isobutyl arginine[22]. Nevertheless, Pearce[11] reported difficulty in acylating arginine using our

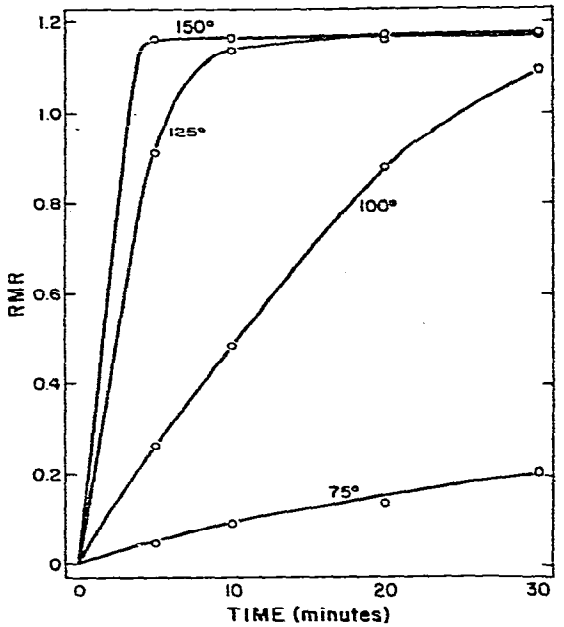

Fig. 1. Effect of acylation temperature and time on the RMR (Nle = 1) of N-HFB isobutyl arginine.

procedure and presented results which suggested that arginine was degraded by heating at 150°. A temperature of 110° was claimed to be optimal, but the RMR values obtained (0.86–0.91, after correction of the data presented) were significantly lower than we have obtained by heating at 150°. The results presented in Table I and Fig. 4 clearly support our earlier report. It is also clear from interpolation of the data that acylation at 110° for 5 min would produce, at the most, 60% of the maximal response for arginine. It is equally clear that the degradation reported by Pearce was not a function of temperature *per se*.

It is not possible to explain unambiguously the anomalous acylation of arginine described by Pearce. There are several differences between the procedure described by us and that used by Pearce and therefore several possible sources for the problem encountered by Pearce. The most likely cause is contamination of one of the reagents, particularly in view of the decreased response with increased temperature. Pearce prepared 3 *M* hydrochloric acid–isobutanol by the addition of acetyl chloride to isobutanol. In the absence of specific information on the quality of the acetyl chloride, we must assume that reagent-grade material was used. It is therefore possible that high-boiling contaminants were carried over from the esterification. A more likely cause of incomplete acylation of arginine lies in the quality of the HFBA. Felker[23] has noted degradation of threonine and tyrosine as a result of using unsatisfactory HFBA. We have also noted a poor response for arginine with individual batches of reagent-grade HFBA. A typical chromatogram of this type is illustrated in Fig. 2. The presence of protons was confirmed by nuclear magnetic resonance spectroscopy but could not be attributed to HFB acid. None of the compounds other than HFBA could be unequivocally identified but the peaks at spectra numbers 28.

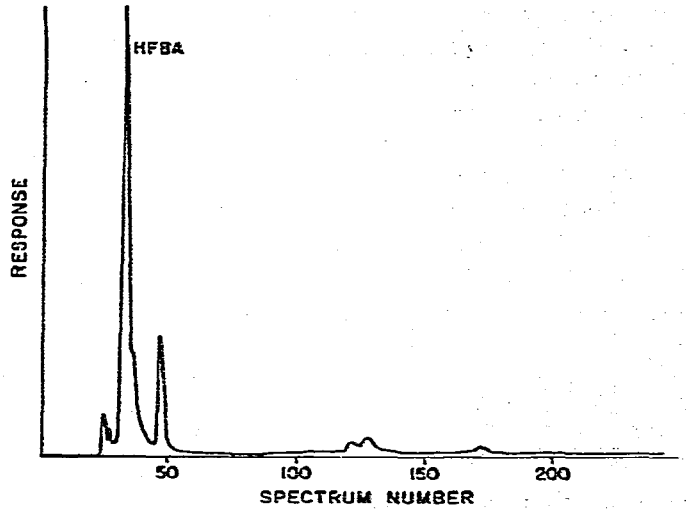

Fig. 2. Total ion chromatogram of a reagent-grade sample of heptafluorobutyric anhydride analysed using a Finnigan Model 3300 gas chromatograph–mass spectrometer. The column (3.2 m × 2 mm I.D.) was packed with 100–120-mesh Chromosorb W HP coated with 3% SE-30. The oven temperature was programmed from 70° at 4°/min.

47 and 128 had molecular ions of 416, 242 and 410, respectively. Furthermore, the compounds at spectrum numbers 28 and 172 contained chlorine.

In Part I[14] we described the effect of varying esterification conditions on the formation of isobutyl tryptophan. The small amounts of components other than di-HFB isobutyl tryptophan did not vary and none could be identified by GC–MS as monoacyl isobutyl tryptophan. There was, however, a significant variation in the RMR of di-HFB isobutyl tryptophan with variation in esterification conditions. The effects of varying the acylation temperature are shown in Fig. 3. The peak with a retention time (t_R) of 30.7 min represents di-HFB isobutyl tryptophan and that at 34 min is mono-HFB isobutyl tryptophan. The identities of both compounds were confirmed by GC–MS. Methane was used as the carrier gas and operation of the mass spectrometer in the chemical ionization mode permitted unequivocal identification of the molecular ions. The peak at t_R 32.4 min had a molecular ion (652) identical with di-HFB isobutyl tryptophan and a similar fragmentation pattern but could not be further identified.

The RMR of di-HFB isobutyl tryptophan (Nle = 1) increased with increasing acylation temperature to a maximum of 1.15 while the proportion of mono-HFB isobutyl tryptophan decreased to less than 3% of the diacyl derivative at 150° (Fig. 4). The proportion of the unknown compound, which may represent an impurity in the original sample, remained constant relative to the internal standard.

Acylation in the presence of the antioxidants BHA or BHT had no significant effect on the RMR of di-HFB isobutyl tryptophan, unlike the observations of Felker[23]. Furthermore, there was no significant effect on the RMR of the corresponding histidine[11] or methionine[17] derivatives. The RMR of 1.15 for di-HFB isobutyl tryptophan corresponds to a relative weight response of 0.74. Felker obtained values of 0.71–0.79 using an antioxidant but values of 0.60 were more typical.

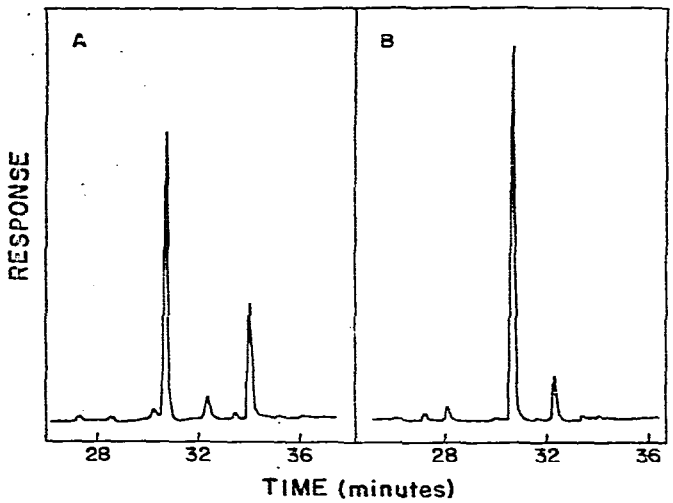

Fig. 3. Chromatograms illustrating the effect of acylation temperature on the formation of N-HFB isobutyl tryptophan. (A) Results of acylation at 75°; (B) results of acylation at 150°. The peak with a retention time of 30.7 min represents di-HFB isobutyl tryptophan and that with a retention time of 34 min represents mono-HFB isobutyl tryptophan.

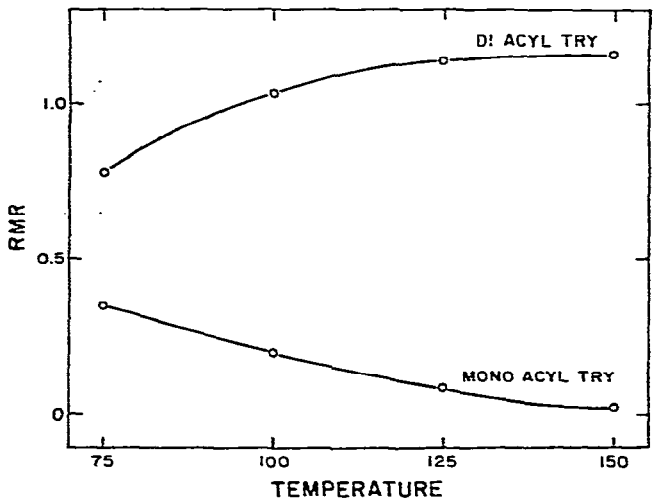

Fig. 4. Effect of acylation temperature on the formation of mono- and di-HFB isobutyl tryptophan. The RMR is expressed relative to norleucine.

The above results and the establishment of other reproducible methods for quantitating tryptophan as the N-acetyl *n*-propyl esters[7] and N-TFA *n*-butyl esters[12] directly contradict the contention that "it has not been possible to chromatograph tryptophan reproducibly"[11]. Furthermore, it appears that the conclusion "at least two major peaks and one minor peak are produced as a result of the derivatization procedure" may be a consequence of incomplete acylation at too low a temperature.

The procedures normally used to form N-perfluoroacyl alkyl esters of amino acids are not entirely satisfactory for the quantitation of histidine because of the

difficulty in acylating the imidazole nitrogen, and because of the lability of the N^{π}-acyl bonds. Roach *et al.*[8] achieved a relative standard deviation of 1.3% by co-injection of N^{α}-TFA *n*-butyl histidine with trifluoroacetic anhydride. Moodie[10] found this procedure to be unsatisfactory and developed an alternative procedure by reacting the sample with ethoxyformic anhydride to form N^{α}-TFA,N^{π}-carbethoxy *n*-butyl histidine[10]. However, no data were presented to demonstrate that this approach was more reproducible than on-column acylation. Furthermore, this method introduces a third reaction which increases the total time necessary for derivatization. In addition, the reaction with EFA degrades the derivatives of methionine, arginine and tyrosine by about 25, 69 and 34%, respectively. Consequently, two separate analyses are required for a complete analysis of the protein amino acids. Reaction with EFA has also been favoured by Pearce for the quantitation of N-HFB isobutyl histidine[11]. However, the resultant peak tailed considerably, despite the use of a capillary column, and the reproducibilities of the two techniques were not compared.

In our studies of the N-HFB isobutyl amino acid esters we adopted the on-column acetylation procedure. Because the on-column acetylation technique does not appear to be widely accepted, we examined the procedure in greater detail than before. We have previously used[6] an anhydride to sample ratio of 1:2. The effect of the proportion of the anhydride is illustrated in Fig. 5, which demonstrates that 0.2 μl of acetic anhydride per microlitre of sample is sufficient to attain the maximal response for N^{α}-HFB,N^{π}-acetyl isobutyl histidine. Similar results were obtained by adding comparable proportions of acetic anhydride directly to the sample, thus simplifying the injection technique and rendering on-column acylation compatible with automatic sample injection. Equivalent experiments conducted using butyric anhydride gave similar results although the RMR was greater because of the additional carbon atoms. Propionic anhydride (not shown) gave responses intermediate between the acetic and butyric derivatives. The relative retentions of N^{π}-acetyl, *n*-propyl and *n*-butyl N^{α}-HFB isobutyl histidine are illustrated in Fig. 6A, B and C. There was no significant difference in the shapes of the peaks representing the three different acyl derivatives and in all instances the degree of tailing was acceptable. The precision for

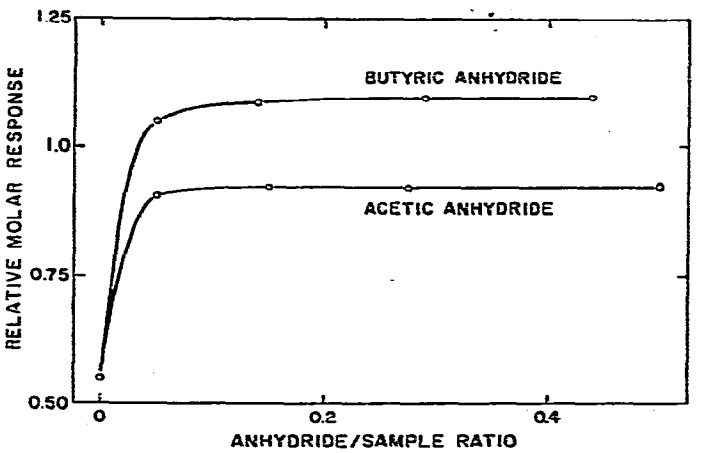

Fig. 5. Effect of anhydride to sample ratio on the RMR (Nle = 1) of N^{α}-HFB,N^{π}-acetyl isobutyl histidine and N^{α}-HFB,N^{π}-*n*-butyryl isobutyl histidine.

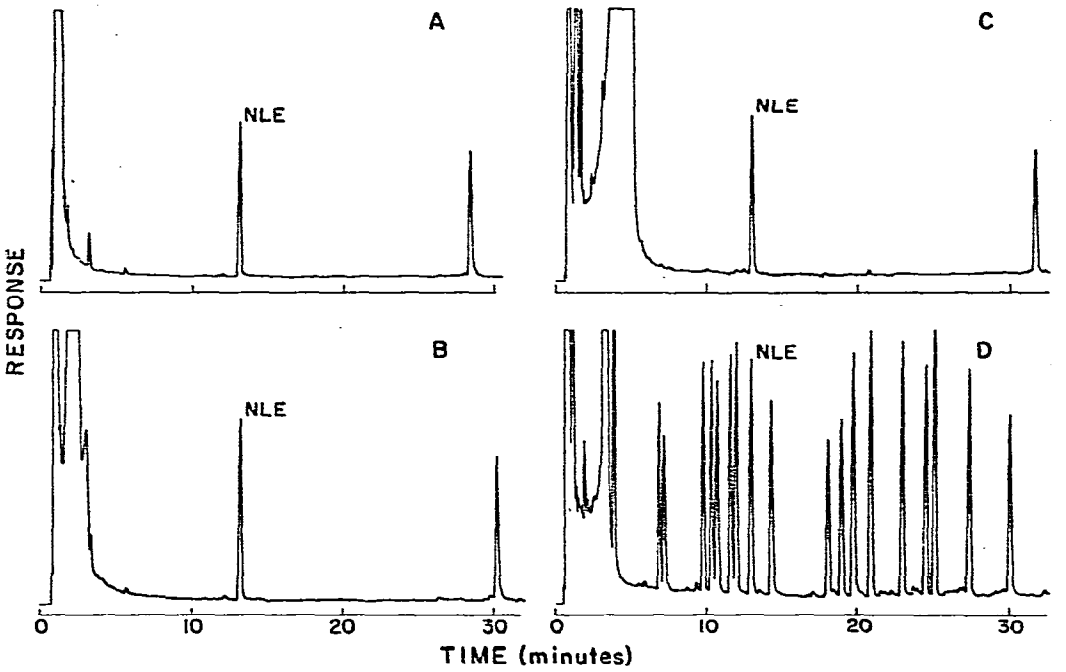

Fig. 6. Illustration of chromatographic properties of various N^{π}-substituted derivatives of N^{α}-HFB isobutyl histidine. (A) N^{π}-Acetyl,N^{α}-HFB isobutyl histidine with a retention time of 28.4 min; (B) N^{π}-propionyl,N-HFB isobutyl histidine with a retention time of 30.2 min; (C) N^{π}-butyryl,N^{α}-HFB isobutyl histidine with a retention time of 31.6 min; (D) N^{π}-carbethoxy,N^{α}-HFB isobutyl histidine with a retention time of 30 min.

each derivative was within $\pm$ 1.5% and none of the anhydrides, if sufficiently pure, had any effect on any of the other amino acids. However, acetic or propionic anhydride is preferred because tailing of butyric anhydride may interfere with the quantitation of alanine and glycine.

The reaction of N^{α}-HFB isobutyl histidine with EFA (1 μl of EFA in 25 μl of ethyl acetate; 150°, 5 min) resulted in a histidine derivative which chromatographed as illustrated in Fig. 6D. The RMR (Nle = 1) was 0.95, which is substantially greater than the values reported by Moodie[10] and Pearce[11]. In addition, the peak tailed much less than was shown by Pearce. There was no significant difference in the degree of tailing of the various histidine derivatives shown in Fig. 6. Reaction with EFA caused a 15% loss of methionine but, unlike earlier reports, there was no significant loss of tyrosine and arginine[10,11].

Given the chromatographic similarity of the various histidine derivatives, we see no benefit in reacting N^{α}-HFB isobutyl histidine with EFA. Results of comparable precision may be achieved by using any one of the techniques but we favour the anhydrides because EFA is difficult to handle. If desired, a third reaction with an anhydride may be performed *in vitro* rather than on-column.

Methods for preparing acyl esters of histidine generally result in the formation of only the monoacyl derivatives. Because these tail badly, studies on the quantitative chromatography of histidine have focused primarily on the formation of stable

mixed acyl derivatives. The role of the chromatographic system and in particular of the support material has often been ignored. In preliminary studies comparing the effect of different supports on the chromatography of N^{α}-HFB isobutyl histidine we have obtained a moderately good peak using 3% SE–30 coated on Chromosorb W HP (prepared in our own laboratory) when other supports resulted in no peak being observed. The peak following on-column acetylation was correspondingly sharper. It appears at this stage that there are subtle but significant differences between the various high-quality supports currently available. This will be discussed in greater length in a subsequent paper.

Unsilanized glass-wool has been clearly shown to produce artefacts[25]. However, the degradative effects of glass-wool are less clear. Consequently, we invariably use silylated glass-wool. Other factors which may effect the chromatography of labile compounds have been adequately considered elsewhere. When sharper histidine derivative peaks can be obtained on a packed column (Fig. 6) than on a capillary column[11], the tailing cannot be considered to be solely a function of the derivative or derivatization procedure. Chromatographic factors, albeit subtle and as yet relatively unexplored, must also be considered.

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