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

I. ESTERIFICATION*

S. L. MacKENZIE and D. TENASCHUK

Prairie Regional Laboratory, National Research Council of Canada, Saskatoon, Saskatchewan S7N 0W9 (Canada)

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SUMMARY

The effect of temperature, reaction time and HCl concentration on the formation of the isobutyl esters of protein amino acids has been studied. Quantitative esterification is achieved by heating at 120° for 30 min in 3 *M* HCl–isobutanol. Experimental procedures have been examined in detail and potential sources of error in derivatisation and chromatography have been identified.

INTRODUCTION

The quantitative analysis of amino acids by gas–liquid chromatography (GLC) requires the conversion of the amino acids to derivatives which are volatile and stable under the conditions used for chromatography. The ideal procedure would require only one chemical reaction and the derivatives should be separable using a single chromatographic column. The first requirement has been fulfilled by silylation[1] but problems such as multiple derivative formation have prevented the development of silylation into a routine technique. However, a variety of satisfactory two-step derivatisation procedures have been developed, the most popular of these usually involving the formation of amino acid alkyl esters followed by the formation of the N(O,S)-acyl derivatives[2–7]. Such procedures are usually performed in a single vessel without transfers and are generally more convenient than procedures requiring multiple extractions[8]. In several instances, the procedures have been coupled with a single column separation which is more than adequate for most routine analyses[3–5,7,9].

Despite the relative ease of derivative formation and the widespread availability of gas chromatographic equipment, the potential of GLC as a method for amino acid analysis has not yet been realised. While this is no doubt due in part to a lack of familiarity with micro-derivatisation techniques, the problem is compounded by the

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large number of variations in derivatisation conditions and procedures suggested by different authors. For example, some authors prepare acidic alcohol by the addition of dry HCl gas[2,5] while others generate HCl *in situ* using acetyl chloride[10,11]. Water scavengers, such as dimethoxypropane, have been used to remove the water formed during derivatisation. Ultrasonic agitation[2,12] have been used to facilitate dissolution of the amino acids. Traces of water[2,13] and other solvents[14] have been removed azeotropically. The concentration of acid catalyst has varied depending on the derivative being synthesised but there have been very few studies of the effect of varying the acid concentration[2,14–16]. Variations in chromatographic technique have also been reported. For instance, most authors use all-glass systems while others report no problems as a result of using stainless steel columns[7].

While many of the variations described are no doubt valid in the hands of their proponents, the validity of and necessity for some of the procedures has not been rigorously examined. The problems of duplicating precise analytical methods have also been compounded by the lack of procedural details which are very important to the neophyte both in gas chromatography and microscale derivatisation.

Earlier we reported a method for GLC analysis of protein amino acids based on the separation of their N(O,S)-heptafluorobutyryl (HFB) isobutyl esters[5]. The derivatisation procedure was based largely on conditions developed for the derivatisation of the N-TFA *n*-butyl esters[2] while the chromatographic conditions were based on the work of Moss *et al.*[3] and Zanetta and Vincendon[4]. Subsequently, we studied some of the variables involved in isobutyl ester formation purely from the point of view of minimising the time required[6]. However, the conditions required to form quantitatively the isobutyl esters of protein amino acids have not been studied thoroughly. Furthermore, the various factors involved have not been critically evaluated so that important variables may be identified. Such an evaluation of derivative formation and chromatographic conditions is particularly necessary because difficulty in duplicating our results has been reported[11]. We therefore investigated, *ab initio*, the process of amino acid isobutyl ester formation and present the results herein. Likewise, the acylation reaction has been studied and the results will be described in a later report[26]. The derivatisation and chromatographic procedures are described in detail to facilitate the application of the technique by other analysts.

MATERIALS AND METHODS

Reagents

The amino acid standard mixture containing 2.5 μmoles/ml of each amino acid was obtained from Chromatographic Specialties, Brockville, Canada. Heptafluorobutyric anhydride (HFBA) (>99%) was obtained from Fluka, Buchs, Switzerland. Ethyl acetate and isobutyl alcohol were purified as previously described[5]. Methylene chloride and acetic anhydride were purified by fractional distillation.

Isobutanol–HCl was prepared by bubbling anhydrous HCl through two successive traps containing concentrated sulphuric acid and then into isobutanol at 0°. The apparatus used was constructed of glass and the use of Tygon tubing was kept to a minimum[12]. The appropriate amount of HCl was determined by weighing and verified by titration. The main stock of this reagent was stored at 0° under nitrogen.

Amounts suitable for weekly requirements were transferred to a separate vessel, the contents of which were discarded in the event of contamination.

Isobutanol–3 *M* HCl was also prepared by the addition of acetyl chloride to isobutanol at 0° (272 μl acetyl chloride/ml isobutanol). The HCl concentration was

All reagents were stored at 0° but were allowed to equilibrate to room temperature before use.

Derivatisation

All samples were heated using a TECAM, Model DB-3H Driblock heater. Bath wax was added so that each hole in the block constituted a miniature oil bath[13]. Vials were immersed in the oil only to the level of the fluid inside the vial. Thus a vertical temperature gradient was established in the vial and consequently the reactants refluxed.

The amino acid standard mixture (25 μl) was dispensed into a 1-ml Reactivial (Pierce, Rockford, Ill., U.S.A.) and excess solvent was evaporated at 50° using a stream of dry nitrogen (100–200 ml/min). Norleucine and pipecolic acid were added in amounts equivalent to the other amino acids and the solvent was again evaporated using dry nitrogen. Isobutanol–HCl (100 μl) was added and the solution was heated. The times and temperatures of heating are indicated later. After about 5 min, the vial was removed from the heater and, while hot, the contents were agitated for 15–30 sec using a Vortex-Genie (Scientific Industries, New York, N.Y., U.S.A.). The vial was then heated for the remainder of the required period. The vial was cooled to room temperature before being opened and excess reagent was evaporated at 50° using a stream of dry nitrogen. HFBA (50–75 μl) was added and the vial was heated as described above at 150° for 10 min. The vial was then cooled to room temperature and evaporated just to dryness using a stream of dry nitrogen. Hexadecane dissolved in ethyl acetate was added in an amount equivalent to half the molar concentration of each amino acid. This amount of hexadecane subsequently gave a peak comparable in size to that of the amino acids. Ethyl acetate was then added to give a total volume of 25 μl and the whole was agitated using a Vortex-Genie. Appropriate aliquots of this solution were applied to the chromatographic column.

Chromatography

Chromatography was performed using a Hewlett-Packard (Avondale, Pa., U.S.A.), Model 5711 gas chromatograph equipped with dual flame-ionisation detectors and using a Pyrex glass column (3.1 m × 6.35 mm O.D. × 2 mm I.D.) the configuration of which permitted direct on-column injection. The column packing consisted of 3% SE-30 on 100–120 mesh Chromosorb W HP. The support was coated using the tray method and was sieved before and after coating to remove fines and agglomerated particles. Before use the column was baked at 275° until negligible baseline rise was evident during temperature programming. Overnight baking was usually sufficient. During normal operation the oven temperature was maintained at 125° overnight while the column was purged with 5–10 ml/min of carrier gas. After 2 min at 100°, the oven temperature was programmed from 100° to 250° at 4°/min. Other chromatographic conditions were: injector temperature, 250°; detector temperature, 300°; carrier gas (helium), 25 ml/min; hydrogen, 30 ml/min; and air,

300 ml/min. The carrier gas was dried by passage through a molecular sieve (Hydro-Purge, Coast Engineering Laboratory, Gardena, Calif., U.S.A.) and then through a hydrocarbon filter (Chemical Research Services, Addison, Ill., U.S.A.). All quantitation was performed using a Hewlett-Packard, Model 3354 Laboratory Data System and all samples were analyzed at least in triplicate.

Co-injection of sample and acetic anhydride necessary for the on-column acylation of histidine, was achieved by first loading the syringe with 0.5 μl acetic anhydride. The plunger was withdrawn to create a 1 μl air gap which was followed by 2 μl of sample. The sample was then withdrawn into the barrel of the syringe. On injection, the sample was thus followed rapidly by the acetic anhydride and broadening of the solvent peak was minimised.

RESULTS AND DISCUSSION

Chromatographic techniques

Before discussing the results obtained it is pertinent to first consider some aspects of the chromatographic techniques used because they are important if accurate, reproducible results are to be obtained.

Glass columns have been used most frequently for the separation of N-acyl amino acid esters[2–5]. Short (3 ft.) stainless-steel columns have been used for the separation of the N-acetyl *n*-propyl amino acids without specific comment on whether or not the stainless steel had a deleterious effect on the chromatographic behavior of the derivatives[7]. We have observed considerable tailing and considerably reduced responses for the basic amino acids when using stainless steel columns. Although we have obtained satisfactory results using nickel columns, for the present we recommend only glass or glass-lined columns for the analysis of the N-HFB isobutyl amino acids. We have not detected any significant advantage in silylating the glass provided the columns have been thoroughly cleaned using a variety of polar and non-polar organic solvents. Since destruction of some amino acid derivatives occurs on hot-metal injectors[17] we further recommend that, where appropriate, glass liners be used in the injection system. A preferable alternative is to inject the sample directly on the column.

With one exception[7], good single-column separations of N-acyl amino acid esters[3–5] have been obtained on non-polar stationary phases, for example SE-30. It is normal chromatographic practice with such stationary phases operated over a typical temperature program of 100° to 250° to use a compensating column to counteract column bleed. However, this procedure has not precluded accurate analysis of amino acid derivatives. In our experience, a virtually flat baseline can be obtained and maintained provided the columns are properly conditioned and the carrier gas flow is carefully controlled. Under such conditions, a reference column is not strictly necessary[18].

The resolution obtained in gas chromatographic analysis is a function of the support particle size. However, the improvement in resolution possible with 100–120 mesh compared with the more commonly used 80–100 mesh support is obtained at the price of increased inlet pressure and difficulty in maintaining control of carrier gas flow, particularly with long columns. Thus, we routinely sieve column packings to remove fines and have been able to operate glass columns up to 14 ft. long without excessive back pressure while maintaining flow control. Felker[19] has noted that

TABLE I

EFFECT OF TEMPERATURE PROGRAM RATE ON RESOLUTION OF N(O)-HFB ISOBUTYL AMINO ACIDS

Resolution is defined as the ratio of the distance between the peak maxima to the average peak width.

Amino acids	*Temperature program rate (°/min)*		
	4	*8*	*16*
Val–Thr	1.8	1.5	1.0
Thr–Ser	1.3	1.2	1.0
Leu–Ile	1.1	1.1	1.0
Lys–Tyr	1.7	1.7	1.6

commercial packings of nominal particle size 125–149 μm may contain as much as 25% of material of size less than 125 μm.

The resolution of N-HFB isobutyl amino acids previously reported by us[5], has not always been reproduced[11] due, at least in part, to the use of 80–100 mesh rather than 100–120 mesh support. Another factor possibly contributing to difficulty in reproducing our procedure and resolution has been the use of a temperature program rate of 20°/min[11]. In our earlier work, we described the separation obtained at a program rate of 4°/min and observed that program rates up to 10°/min could be used with little effect on precision. However, at that time we did not examine the resolution in detail. The effect of program rate on the resolution of the most poorly resolved N-HFB isobutyl amino acids is shown in Table I in which resolution has been computed as defined by Harris and Habgood[20] and Horvath[21]. A resolution, R, of 1.5 corresponds to 99.7% separation of two components and "for practical purposes $R = 1$ (98% separation) is usually adequate"[21]. The increase in temperature program rate had most effect on the resolution of valine–threonine and threonine–serine. Although the separation appears considerably inferior at elevated program rates (Figs. 1–3), the actual resolution is scarcely affected except for valine–threonine because of increased peak sharpness. However, it is clear from the data in Table I that optimum resolution is not obtained at 20°/min as was claimed by Pearce[11].

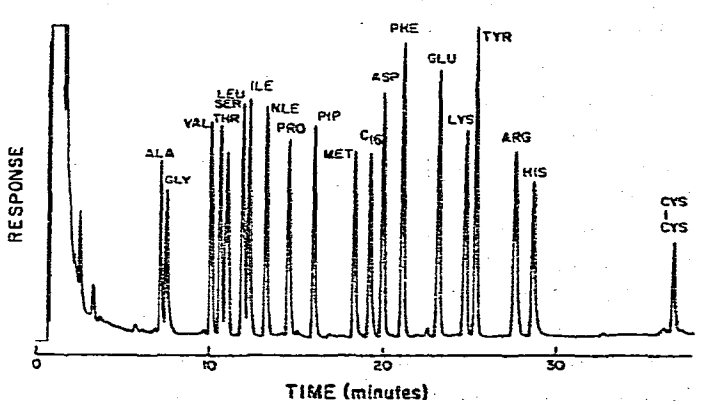

Fig. 1. Gas chromatographic separation of N(O,S)-HFB isobutyl esters of amino acids. Temperature program rate 4°/min. Other GLC conditions are given in text.

Furthermore the overall separation and particularly the separation of lysine and tyrosine illustrated in Fig. 1 is superior to that described by Pearce. Clearly the separation demonstrated by Pearce does not "represent the best resolution obtainable"[11] using packed columns.

The chromatographer is always confronted by the conflicting demands of speed and resolution. In the context of the separation of N-acyl amino acid esters it has been suggested that the packed column technique offers "no scope for varying speed or resolution"[11].The times for the analyses illustrated in Figs. 1–3 were 37, 22 and 14.5 min at temperature program rates of 4, 8 and 16°/min, respectively. Allowing in each case an additional 6 min for equilibration of the oven temperature before the next analysis, the use of a temperature program rate of 16°/min permits the analysis time to be shortened by 50% compared with using a program rate of 4°/min while maintaining adequate resolution. The consequent effect on the precision of peak area measurement is discussed later.

The resolving power of capillary columns is undoubtedly advantageous for the analysis of samples which cannot be adequately resolved using packed columns and several excellent separations of N-acyl amino acid alkyl esters have been obtained using capillary columns[11,14,22,23]. The further advantages of speed of analysis and baseline resolution have also been attributed to capillary columns. However, when applied to N-acyl alkyl esters of the protein amino acids the analyses times have been 20–25 min[22], 30 min[11], 32.5–37.5 min[23] and 35 min[14] most of which times are readily achieved on packed columns with moderate program rates and excellent resolution. The advantage of baseline resolution is presumably increased accuracy and/or precision. However, documentary data have either not been provided[23] or have not been demonstrated to be significantly better than can be achieved on a packed column[11,14,22]. In some rather unusual circumstances[11], a capillary column may be necessary for the analysis of relatively small amounts of certain amino acids. Furthermore, the advantages of capillary columns for ultramicro analysis[22] are unquestioned. In general, however, we do not consider the additional expense and delicacy of technique required with capillary columns to outweigh the simplicity, cheapness and ruggedness of the packed column for the routine analysis of protein amino acid derivatives.

Some authors[11,19] have found the packed column system for the analysis of N-HFB isobutyl amino acids[5] to be unstable. Felker[19] observed "increasingly poor resolution of lysine and tyrosine as well as severe tailing of glycine (...) after as few as eight injections" but did not specify the type of sample that created this problem. Pearce[11] also observed that "resolution between lysine and tyrosine was the most susceptible to deterioration of the column and became very poor" but no further information on sample or relevant time-scale was provided. Conversely, Kirkman[18] in a rigorous study of the quantitative factors involved in the analysis of amino acid N-HFB *n*-propyl esters noted that "changing the separation characteristics at no time led to impaired measurement". In our experience, poor resolution of lysine and tyrosine occurs only after gross contamination of the column and is not a function of premature column aging. We have readily maintained columns in operation for six months and several hundred analyses. However, one injection of a "dirty" sample is sufficient to contaminate this or any other type of column. When contamination, resulting in the creation of active sites at the front of the column, has occurred, the

standard chromatographic practise of removal of the glass wool and the first few centimeters of packing has only rarely failed to rejuvenate the column. We have always injected the sample directly on column and the sample has preferrably been deposited on the glass wool. Consequently, involatile material does not reach the column and frequently a column can be rejuvenated simply by changing only the glass-wool.

Derivatisation techniques

Some authors[10,11,19] have found it more convenient to prepare the alcohol by the addition of acetyl chloride to the alcohol rather than by the addition of acetyl gas[5]. Daily preparation of this reagent has been recommended[10]. In principle, the method of preparation of HCl should not affect the esterification provided no undesirable contaminants are introduced. We are aware of only one study[11] of the effect of preparing amino acid isobutyl esters using acidified alcohol produced by each of the above methods. However, the comparison was not conducted under identical HCl concentrations and alcohol–amino acid ratios since the HCl produced *in situ* by the addition of acetyl chloride was only about 2.3 *M* (200 μl acetyl chloride/ml alcohol). It was concluded that the "RMR's were either insignificantly different or a little greater for all except cystine whose mean RMR is considerably greater". However, the data were inverted so that the relative molar response (RMR) for alanine appeared as 1.5 when the alanine peak was, in fact, smaller than the internal standard, norleucine. When the data are correctly expressed, the RMR values of threonine, serine, methionine and cystine using acetyl chloride-produced HCl decreased by 9, 12, 12 and 24% respectively of the values obtained using dry HCl gas. There was a corresponding increase of 8% and 11% for the RMR values of respectively lysine and tyrosine. In our experiments comparing 3 *M* HCl–isobutanol prepared by our standard procedure[5] and by the addition of redistilled acetyl chloride to isobutanol at 0° (270 μl acetyl chloride/ml isobutanol) we have observed a decrease of 8–10% in the RMR values (norleucine = 1) of serine, lysine and cystine when using the latter procedure. No unambiguous explanation can be offered for these observations. However, it should be noted that the alcohol–amino acid ratios were not strictly comparable. Furthermore, it is important to note that the introduction of another reagent, *viz.* acetyl chloride into the system increases the potential for the introduction of impurities with deleterious effects on the esterfication.

For esterification, we have routinely used 200 μl of 3 *M* HCl–isobutanol and a molar ratio for alcohol to total amino acids of 1800:1, while other authors have regarded 100 μl to be sufficient[13]. We therefore esterified 25 μl amounts of the amino acid standard using 100 and 200 μl of 3 *M* HCl–isobutanol and acylated each normally. No significant difference in the RMR values of the amino acid derivatives was observed and in all subsequent analysis 100 μl of acidified alcohol was used.

We also examined the effect of evaporating excess isobutanol at 50° and 75°. Compared with room temperature evaporation, no significant losses were observed following evaporation at 50° but at 75° there was significant loss of the more volatile amino acid derivatives. The loss decreased from 8% for alanine to 2.5% for pipecolic acid. Loss of the other less volatile amino acids was not significant. The time required to evaporate 200 μl was reduced from 18 min at room temperature to 10 min at 50°. In

conjunction with a decrease in the volume of esterifying reagent to 100 μl we therefore reduced the time of evaporation after esterification to 5 min.

Esterification was also studied using water scavengers such as dibutoxy propane and dimethoxypropane but the resultant relative molar responses were not significantly different from those obtained without the use of water scavengers. The addition of methylene chloride to the acidic isobutanol had no significant effect on the relative molar responses in contrast to the findings of Cancalon and Klingman[12] for the formation of amino acid N-trifluoroacetyl *n*-butyl esters.

Heating of the esterification reactants is most often done in a 1 ml Reactivial or its equivalent placed in a heating block[7,13]. However, an oven has also been used[5,11]. Under the latter conditions there would not be a longitudinal temperature gradient in the vial and refluxing of the solvents would be less efficient. However, we have compared the results of heating the samples by each method and there has been no significant difference in the resultant RMR values. Nevertheless, we now prefer to use a heating block because we believe heat transfer to be more efficient.

Derivatisation and chromatographic variability

The reproducibility of retention times was assessed using 10 analyses taken at random from analyses performed over a two month period. Apart from alanine (0.34%) and glycine (0.31%) the coefficient of variation (C.V.) was 0.25% or less for all the amino acid derivatives. The C.V. of relative retention times (Nle = 1) was 0.15% or less for all the amino acid derivatives. These observations apply to analyses using a specific column. Because of differences in packing greater variability would be expected in data derived from different columns.

Typical C.V. values encompassing several derivatisation and chromatographic conditions are shown in Table II. The data for triplicate chromatographic analysis of one sample (column A) were selected at random from the experiments used to compile Tables III–V. The variability in peak area reproducibility was frequently better than that shown; deterioration in peak area reproducibility usually signified a need to clean the front of the column. However, we were consistently able to maintain peak reproducibility of $\pm$ 0.5% or better with the exception of histidine and cystine which could usually be measured to better than $\pm$ 1%. The larger error in histidine measurement may be related to the need for on-column injection with acetic anhydride.

The data shown in Table II, column B were derived from three standards derivatised using identical conditions and each analysed in triplicate. In most cases, the variability is not substantially different from the values in column A.

The reproducibility of peak area measurements obtained while analysing a hydrolysate of pea meal is shown in Table II, column C. The variability reflects the relative amounts of the amino acids in the sample. Glutamic and aspartic acids were present in large amounts and thus precise measurement of these amounts was relatively easy. However, the sample contained little methionine and the difficulty in measuring a very small peak is reflected in a greater variability. Nevertheless, the general range of variability is entirely consistent with accurate measurement of the amino acid composition of samples in which the amino acids are not present in equimolar amounts.

Although increasing the temperature program rates results in decreased

TABLE II

COEFFICIENTS OF VARIATION OF RELATIVE MOLAR RESPONSES OBTAINED DURING GAS CHROMATOGRAPHY OF N(O,S)-HFB ISOBUTYL AMINO ACIDS

A = triplicate analyses of one derivative; B = triplicate analyses of each of three separately derivatised standards; C = triplicate analyses of a pea meal hydrolysate; D = triplicate analyses of one derivative, temperature programmed at 8°/min, analysis time 23.5 min; E = triplicate analyses of one derivative, temperature programmed at 16°/min, analysis time 15.5 min; F = variability in RMR over 6 months.

Amino Acid	*C.V.*					
	A	*B*	*C*	*D*	*E*	*F*
Ala	0.33	0.43	0.76	0.30	0.25	1.02
Gly	0.43	0.96	0.40	0.48	0.29	1.29
Val	0.27	0.40	0.37	0.07	0.57	1.25
Thr	0.31	0.46	0.42	0.38	0.55	1.16
Ser	0.26	0.55	0.59	0.56	0.64	0.88
Leu	0.41	0.66	0.36	0.22	0.55	0.59
Ile	0.38	0.45	0.60	0.25	0.48	0.78
Nle	0.24	0.35	0.30	0.18	0.22	0.63
Pro	0.42	0.49	0.30	0.52	0.11	0.56
Pip	0.38	0.41	—	0.15	0.32	0.72
Met	0.45	0.50	1.10	0.55	0.65	1.77
Asp	0.47	0.55	0.34	0.70	0.72	1.14
Phe	0.50	0.50	0.61	0.38	0.49	1.37
Glu	0.67	0.67	0.22	0.73	0.36	1.18
Lys	0.48	0.80	0.37	0.50	0.37	1.00
Tyr	0.44	0.63	0.25	0.45	0.43	1.43
Arg	0.49	0.55	0.32	0.65	0.78	1.60
His	0.60	0.67	1.50	0.64	1.30	2.15
Cys | Cys	0.86	1.35	1.71	0.90	1.60	2.66

resolution particularly of leucine and isoleucine, the variability at 8°/min (Table II, column D) and at 16°/min (column E) is, for most amino acids, not significantly greater than at 4°/min and thus entirely acceptable results can be obtained in analyses requiring only 20 min.

The variability in RMR values over a period of six months is indicated in Table II, column F.

Difficulty in reproducing results using our procedure has been attributed to the incomplete resolution of all the amino acids on a packed column system[11]. Lack of reproducibility is not a consequence of the packed column system *per se*. It is clear from the data in Table II that precise, reproducible amino acid analysis can be performed using a packed column. In this context it is relevant to note that peak area reproducibility using ion-exchange amino acid analyses is in the order of 2–3% and that the resolution in ion-exchange chromatographs is inferior to that possible using GLC.

The RMR values of the N(O,S)-HFB isobutyl esters of the protein amino acids are shown in Table III as a function of time and HCl concentration at 100°. Pipecolic acid has been included to permit comparison with our earlier work which

TABLE III

RELATIVE MOLAR RESPONSES OF AMINO ACID N-HFB ISOBUTYL ESTERS AS A FUNCTION OF ESTERIFICATION TIME AND HCl CONCENTRATION AT 100°*

Amino acid	*HCl concentration*											
	1 M				*3 M*				*5 M*			
	Time (min)											
	15	*30*	*45*	*60*	*15*	*30*	*45*	*60*	*15*	*30*	*45*	*60*
Ala	0.64	0.64	0.65	0.64	0.62	0.64	0.65	0.65	0.63	0.63	0.65	0.65
Gly	0.60	0.58	0.59	0.59	0.53	0.59	0.59	0.59	0.58	0.60	0.60	0.59
Val	0.50	0.71	0.80	0.88	0.77	0.90	0.93	0.92	0.87	0.91	0.92	0.90
Thr	0.80	0.93	0.96	0.97	0.93	0.97	0.97	0.97	0.95	0.97	0.97	0.97
Ser	0.86	0.87	0.87	0.88	0.76	0.88	0.88	0.88	0.85	0.88	0.89	0.88
Leu	1.00	1.01	1.01	1.01	1.00	1.00	1.01	1.01	1.01	1.01	1.01	1.01
Ile	0.43	0.67	0.79	0.93	0.77	1.01	1.02	1.07	0.94	1.06	1.07	1.06
Pro	0.94	0.90	0.90	0.91	0.90	0.90	0.91	0.90	0.91	0.90	0.90	0.90
Pip	0.99	0.97	0.97	0.97	0.96	0.97	0.97	0.97	0.97	0.97	0.97	0.97
Met	0.86	0.86	0.86	0.86	0.94	0.89	0.89	0.90	0.90	0.89	0.88	0.89
Asp	1.16	1.18	1.18	1.19	1.12	1.19	1.19	1.19	1.20	1.21	1.19	1.20
Phe	1.38	1.36	1.35	1.36	1.41	1.36	1.36	1.36	1.50	1.38	1.36	1.37
Glu	1.24	1.22	1.22	1.22	1.22	1.23	1.23	1.23	1.26	1.25	1.23	1.24
Lys	1.15	1.11	1.10	1.11	0.87	1.12	1.11	1.12	1.11	1.15	1.12	1.13
Tyr	1.41	1.39	1.37	1.38	1.47	1.40	1.39	1.42	1.45	1.44	1.41	1.41
Arg	1.18	1.17	1.15	1.15	0.94	1.17	1.16	1.16	1.17	1.19	1.18	1.18
His	0.94	0.94	0.94	0.94	0.67	0.94	0.93	0.94	0.92	0.95	0.96	0.94
Cys** | Cys	1.01	1.03	1.05	1.04	0.75	1.04	1.03	1.04	1.05	1.04	1.03	0.94
Nle***	0.53	0.57	0.57	0.57	0.48	0.56	0.57	0.57	0.54	0.56	0.57	0.56

* The molar responses are expressed relative to norleucine.

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

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

was based on pipecolic acid as an internal standard. To facilitate comparison with the results obtained in other laboratories[13,19] the responses are expressed relative to norleucine. Since the optimum conditions for the formation of isobutyl norleucine have not previously been defined, the RMR of norleucine is expressed relative to cetane which was used as an external standard. Although we would normally desire to minimise the esterification time, the reaction was studied for up to 60 min to detect any degradation occurring, particularly at the more elevated temperatures discussed later. The most pronounced effect observed was the incomplete esterification of valine and isoleucine in 1 *M* HCl–isobutanol even after 1 h of heating at 100°. A similar but less pronounced effect was observed for methionine. In general there was no significant effect due to the variation in HCl concentration with the exception of valine and isoleucine which, for equivalent times, clearly reacted more quantitatively as the concentration of HCl increased. The response of cystine decreased significantly after 1 h in 5 *M* HCl. The apparent decrease in response with time in 1 *M* HCl of proline, pipecolic acid, lysine, tyrosine and arginine is due to the increase in response of the internal standard, norleucine.

TABLE IV

RELATIVE MOLAR RESPONSES OF AMINO ACID N-HFB ISOBUTYL ESTERS AS A FUNCTION OF ESTERIFICATION TIME AND HCl CONCENTRATION AT 120°*

Amino acid	*HCl concentration*															
	1 M				*2 M*				*3 M*				*5 M*			
	Time (min)															
	15	*30*	*45*	*60*	*15*	*30*	*45*	*60*	*15*	*30*	*45*	*60*	*15*	*30*	*45*	*60*
Ala	0.64	0.65	0.66	0.66	0.63	0.63	0.65	0.66	0.65	0.66	0.66	0.65	0.66	0.66	0.65	0.65
Gly	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.61	0.60	0.61	0.60	0.61	0.61	0.61	0.60	0.61
Val	0.77	0.90	0.90	0.91	0.86	0.91	0.93	0.93	0.90	0.93	0.93	0.92	0.93	0.93	0.92	0.92
Thr	0.96	0.97	0.99	0.99	0.97	0.97	0.97	0.98	0.98	0.99	0.98	0.97	0.98	0.97	0.96	0.96
Ser	0.86	0.87	0.89	0.88	0.88	0.88	0.88	0.88	0.89	0.89	0.89	0.88	0.90	0.88	0.89	0.89
Leu	1.01	1.00	1.01	1.00	1.01	1.01	1.01	1.01	1.01	1.02	1.02	1.01	1.01	1.02	1.02	1.01
Ile	0.77	1.00	1.03	1.05	0.92	1.05	1.06	1.07	1.04	1.08	1.07	1.08	1.07	1.08	1.07	1.07
Pro	0.89	0.90	0.90	0.90	0.94	0.94	0.93	0.94	0.93	0.94	0.94	0.94	0.90	0.90	0.90	0.89
Pip	0.96	0.96	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97
Met	0.89	0.89	0.89	0.89	0.90	0.89	0.88	0.86	0.90	0.90	0.89	0.88	0.91	0.89	0.89	0.89
Asp	1.15	1.15	1.18	1.19	1.20	1.20	1.20	1.19	1.18	1.19	1.19	1.19	1.19	1.19	1.18	1.18
Phe	1.36	1.36	1.38	1.37	1.37	1.37	1.37	1.36	1.37	1.38	1.37	1.38	1.38	1.38	1.38	1.39
Glu	1.21	1.21	1.24	1.24	1.23	1.24	1.24	1.24	1.26	1.26	1.26	1.25	1.22	1.25	1.25	1.25
Lys	1.12	1.12	1.13	1.13	1.12	1.14	1.14	1.12	1.13	1.13	1.13	1.11	1.12	1.12	1.12	1.12
Tyr	1.39	1.39	1.42	1.42	1.40	1.41	1.40	1.41	1.43	1.42	1.42	1.41	1.40	1.39	1.38	1.39
Arg	1.13	1.15	1.16	1.16	1.18	1.20	1.17	1.20	1.19	1.19	1.17	1.17	1.19	1.19	1.14	1.12
His	1.00	0.98	0.96	0.97	0.92	0.98	0.97	0.94	0.97	0.97	0.97	0.97	0.96	0.95	0.95	0.96
Cys**																
|	1.06	1.08	1.07	1.06	1.08	1.07	1.05	1.03	1.08	1.08	1.04	1.03	1.04	1.04	0.99	0.95
Cys																
Nle***	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.56

* The molar responses are expressed relative to norleucine.

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

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

The RMR values of the N(O,S)-HFB isobutyl amino acid esters as a function of time and HCl concentration at 120° are shown in Table IV. Valine and isoleucine reacted more rapidly as the concentration of HCl increased and, as observed at 100°, did not derivatise completely in 1 *M* HCl. Derivatisation of both was complete after 30 min in 3 *M* HCl. Derivatisation of cystine was completed rapidly at 120° but the responses decreased with extended time particularly in 5 *M* HCl. The response for arginine was generally lower in 5 *M* HCl and decreased as the esterification time was increased.

Esterification at 140° generally produced slightly lower responses than at 120° when measured against an external standard. When expressed relative to norleucine (Table V) this decrease is not entirely evident because the response of norleucine was also reduced. Valine and isoleucine were not completely esterified after heating for 15 min at 140° in 1 *M* HCl–isobutanol. The RMR values of some amino acids decreased with esterification time particularly in 5 *M* HCl. This was most noticeable for the derivatives of threonine (6%), methionine (16%), histidine (14%) and cystine

TABLE V

RELATIVE MOLAR RESPONSES OF AMINO ACID N-HFB ISOBUTYL ESTERS AS A FUNCTION OF ESTERIFICATION TIME AND TEMPERATURE AT 140°*

Amino acid	*HCl concentration*											
	1 M				*3 M*				*5 M*			
	Time (min)											
	15	*30*	*45*	*60*	*15*	*30*	*45*	*60*	*15*	*30*	*45*	*60*
Ala	0.65	0.65	0.64	0.65	0.62	0.64	0.66	0.66	0.63	0.65	0.67	0.66
Gly	0.61	0.61	0.60	0.61	0.60	0.61	0.61	0.62	0.60	0.60	0.61	0.61
Val	0.90	0.93	0.93	0.93	0.91	0.92	0.90	0.91	0.91	0.92	0.93	0.92
Thr	0.98	0.99	0.98	0.99	0.99	0.99	0.98	0.98	0.98	0.97	0.96	0.96
Ser	0.90	0.91	0.91	0.91	0.90	0.92	0.91	0.91	0.90	0.88	0.89	0.89
Leu	1.02	1.03	1.03	1.03	1.02	1.03	1.03	1.04	1.02	1.02	1.03	1.04
Ile	0.98	1.08	1.07	1.09	1.07	1.08	1.07	1.09	1.08	1.06	1.07	1.06
Pro	0.90	0.91	0.91	0.91	0.90	0.92	0.93	0.93	0.90	0.92	0.93	0.94
Pip	0.96	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97
Met	0.93	0.92	0.92	0.90	0.95	0.93	0.90	0.90	0.95	0.86	0.83	0.83
Asp	1.17	1.18	1.19	1.18	1.19	1.20	1.19	1.15	1.17	1.18	1.20	1.20
Phe	1.37	1.38	1.39	1.39	1.39	1.40	1.40	1.39	1.40	1.38	1.39	1.39
Glu	1.21	1.23	1.24	1.23	1.27	1.28	1.28	1.25	1.24	1.26	1.26	1.26
Lys	1.12	1.13	1.14	1.14	1.15	1.15	1.14	1.10	1.15	1.13	1.14	1.13
Tyr	1.40	1.42	1.43	1.44	1.45	1.45	1.48	1.46	1.45	1.43	1.48	1.48
Arg	1.13	1.15	1.15	1.13	1.15	1.14	1.13	1.13	1.14	1.15	1.16	1.17
His	1.04	1.06	1.06	1.02	1.08	1.03	1.00	1.00	1.04	1.02	0.98	0.92
Cys** | Cys	1.05	1.06	1.04	0.92	1.02	0.95	0.82	0.77	1.02	0.78	0.75	0.70
Nle***	0.56	0.55	0.55	0.53	0.55	0.54	0.54	0.54	0.55	0.55	0.54	0.53

* The molar responses are expressed relative to norleucine.

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

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

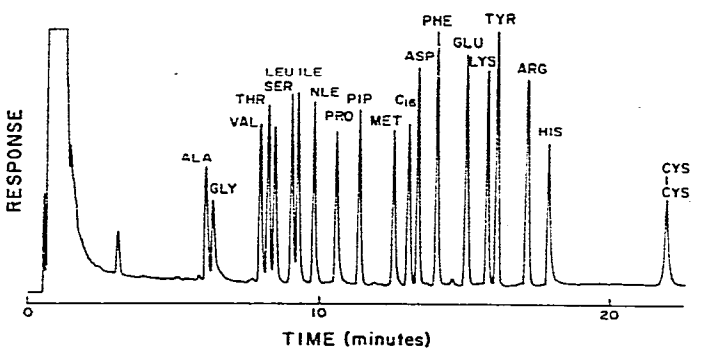

Fig. 2. Gas chromatographic separation of N(O,S)-HFB isobutyl esters of amino acids. Temperature program rate 8°/min. Other GLC conditions are given in text.

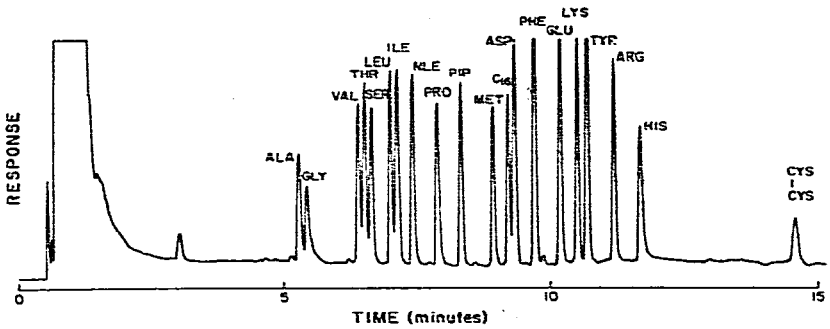

Fig. 3. Gas chromatographic separation of N(O,S)-HFB isobutyl esters of amino acids. Temperature program rate 16°/min. Other GLC conditions are given in text.

(32%) when expressed in terms of the external standard. The values given in Table IV (norleucine = 1) result in apparent decreases which are slightly lower because of the decrease in the response of norleucine. Proline, glutamic acid, aspartic acid and arginine appeared to be uneffected by extended exposure to 5 *M* HCl at 140°.

From the data presented in Tables III–V, the optimum conditions for quantitative formation of the isobutyl esters of protein amino acids is heating at 120° for 30 min in 3 *M* HCl–isobutanol. Heating for only 20 min[6] would produce small losses of some amino acids but the effect of these losses would be minimised by conjunctive calibration.

Because it is acid labile, most methods for formation of amino acid esters using acid catalysis are assumed to result in considerable destruction of tryptophan. However, Gehrke and Takeda[24] studied the formation of N-TFA *n*-butyl amino acids derived from tryptophan-containing peptides, and demonstrated that quantitative analysis of tryptophan by GLC is possible and that "no serious destruction of tryptophan had occurred". Adams[7] also demonstrated quantitative recovery of tryptophan as the N-acetyl *n*-propyl esters.

The formation of N-HFB isobutyl tryptophan has previously been shown to produce small quantities of products other than diacyl isobutyl tryptophan[25]. None of these products was identified as monacyl tryptophan[19]. The effect of varying the esterification time in 3 *M* HCl at 120° on the RMR of tryptophan is shown in Table VI. The values compare favourably with those reported elsewhere[7,15,19]. Surprisingly, the proportions of the other compounds did not change significantly during esterification over 15 min to 1 h. Therefore, the compounds either are contaminants in the original sample or are artefacts of the acylation. This facet of the derivatisation of tryptophan will be more fully explored in a later report[26].

TABLE VI

RELATIVE MOLAR RESPONSE OF N-HFB ISOBUTYL TRYPTOPHAN

Amino acid	*Time (min)*			
	15	*30*	*45*	*60*
Trp	1.03	1.16	1.20	1.14
Nle	1.00	1.00	1.00	1.00

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