Journal of Chromatography, 136 (1977) 113–126

CHROM. 9863

AMINO ACID ANALYSIS BY GAS–LIQUID CHROMATOGRAPHY OF N-HEPTAFLUOROBUTYRYL ISOBUTYL ESTERS

COMPLETE RESOLUTION USING A SUPPORT-COATED OPEN-TUBULAR CAPILLARY COLUMN

R. J. PEARCE

CSIRO Dairy Research Laboratory, P.O. Box 20, Highett, Victoria 3190 (Australia)

(First received October 5th, 1976; revised manuscript received December 3rd, 1976)

SUMMARY

Amino acids have been separated by gas–liquid chromatography as their N-heptafluorobutyryl isobutyl esters. Complete resolution of derivatives of all the common amino acids has been achieved using a high-performance support-coated open-tubular capillary column. The analysis time was 30 min. Modifications to the derivatization procedure of MacKenzie and Tenaschuk have been introduced. Acylation by heating at 150° was shown to be destructive; 110° has been selected for routine preparation. To obtain a volatile histidine derivative it has been found necessary to add an antioxidant and to heat samples with ethoxyformic anhydride prior to injection. Amino acid analysis of β-lactoglobulin after 6 *N* HCl digestion yielded results in good agreement with those obtained by the conventional ion-exchange method. The method has also been successfully applied to estimation of the different caseins in whole casein and in purified fractions by amino acid analysis of residues liberated by carboxypeptidase digestion.

INTRODUCTION

The development of gas–liquid chromatographic (GLC) analysis of amino acids has been pursued for some time to produce a more sensitive, more rapid and less expensive method than conventional ion-exchange chromatography. Conversion of amino acids to stable volatile derivatives has been investigated extensively[1–11].

Zanetta and Vincendon[7] found that all the common amino acids could be separated on a single column as their N-heptafluorobutyryl isoamyl esters. However, in attempting to apply their method I became aware of deficiencies in it (also noted by MacKenzie and Tenaschuk[8]). Certain peaks were not completely resolved; it was difficult to transesterify certain amino acid esters; evaporation of solvents at elevated temperatures resulted in losses of the more volatile derivatives. MacKenzie and Tenaschuk[8] overcame some of these problems by chromatographing amino acids as

their N-heptafluorobutyryl isobutyl esters. These could be prepared by direct esterification and solvent evaporation at room temperature.

I have found, however, in using their method that accurate estimation of certain amino acids was difficult owing to incomplete peak resolution, notably between alanine and glycine, threonine and serine, leucine and isoleucine, and lysine and tyrosine. This was particularly apparent when one of the pair was in considerable excess over the other as occurred when the relative proportions of the various caseins in casein preparations were determined by the Ribadeau-Dumas method[12].

In this paper modifications to the derivatization procedure of MacKenzie and Tenaschuk[8] are reported together with the steps necessary to obtain a volatile histidine derivative. The alternative esterification procedure proposed by Felker and Bandurski[9] has also been examined and comparative results are shown. The most significant modification was the use of a support-coated open-tubular (SCOT) capillary column in an endeavour to obtain complete resolution of all the common amino acids.

EXPERIMENTAL

Reagents

Standard amino acids were purchased from Mann Research Labs. (New York, N.Y., U.S.A.). Heptafluorobutyric anhydride (HFBA), ethoxyformic anhydride (EFA), and acetyl chloride were obtained from Merck (Darmstadt, G.F.R.). 2,6-Di-*tert.*-butylhydroxytoluene (BHT) was kindly donated by Dr. B. Milligan, CSIRO Division of Protein Chemistry, Melbourne, and *tert.*-butylhydroxyanisole (BHA) was obtained from Watts Winter (Melbourne, Australia). Other reagents were prepared according to MacKenzie and Tenaschuk[8].

Isobutanol–3 *N* HCl was stored at room temperature in a polypropylene bottle fitted with a fine dropper, the aperture of which was normally covered with a polypropylene cap.

Preparation of derivatives

All derivatization steps were performed in 1-ml Reactivials (Pierce, Rockford, Ill., U.S.A.). Solvents and excess reagents were evaporated under a stream of dry nitrogen at room temperature.

Samples containing 25–250 nmoles of each amino acid were freeze dried and residual moisture removed azeotropically by shaking with dry methylene chloride (100 μl) and evaporating. Isobutanol–3 *N* HCl (5 drops = approx. 100 μl) was added and after flushing the headspace with nitrogen, the vial was capped and heated at 110° in an oven for 30 min. After cooling to room temperature excess reagent was evaporated and the residue dried azeotropically as above. A white suspension was formed on addition of dry ethyl acetate (25 μl) containing BHT at a level approximately equimolar with respect to each amino acid. This suspension cleared when HFBA (10 μl) was added. The headspace was again flushed with nitrogen, the vial capped and heated in an oven at 110° for 10 min and then cooled. If histidine determination was not important the sample was evaporated almost to dryness and then redissolved in ethyl acetate (30 μl). If histidine determination was required, the sample was evaporated as above, EFA added (5 μl) together with ethyl acetate (30 μl) and, after reflushing the headspace with nitrogen, heated at 110° for 5 min. Finally this was evaporated almost to dryness and redissolved in ethyl acetate (30 μl).

Alternative esterification reagent

Instead of preparing isobutanol–3 *N* HCl as described by MacKenzie and Tenaschuk[8] it was made as required by slowly pipetting redistilled acetyl chloride (200 μl) into dry isobutanol (1 ml) at 0° as described by Felker and Bandurski[9]. 100 μl of this esterifying reagent were added to each vial.

Chromatography

Analyses were performed using a Packard-Becker Model 419 gas chromatograph fitted with flame ionisation detectors. Columns and chromatographic conditions were either:

System A. The 3.5 m × 2 mm I.D. Pyrex columns, packed with 3% SE-30 on Gas-Chrom Q 80–100 mesh, were obtained from Supelco (Bellafonte, Pa., U.S.A.). The temperature programme was 100° 3 min, 20°/min, 250° 5 min; the injector temperature was 260° and the detector temperature 270°; the carrier gas (nitrogen) flow-rate was 20 ml/min, the air flow-rate 300 ml/min, and the hydrogen flow-rate 30 ml/min. The sample injection volume was 1–5 μl.

System B. The high-performance (60 m) glass SCOT capillary column (I.D. 0.5 mm, O.D. 1 mm), using SE-30 as stationary phase, silanised and deactivated, was supplied by Scientific Glass Engineering (Melbourne, Australia). The column was installed with a glass-lined stainless-steel, splitless injection system and similar tubing to both injector and detector. A T-piece at the effluent end of the column was fitted to allow the gas flow through the detector to be varied to maximise flame ionisation detection (FID) sensitivity. The temperature programme was 100° 0 min, 6°/min, 250° 5 min. The injector temperature was 260°, and the detector temperature 270°; the carrier gas (helium) flow-rate was 4 ml/min, the make-up gas (helium) flow-rate 16 ml/min, the air flow-rate 300 ml/min, and the hydrogen flow-rate 30 ml/min. The sample injection volume was 0.1–0.5 μl.

Retention times, peak areas, relative molar response factors (*RMR*) and relative concentrations were obtained either manually or using an Autolab I computing integrator (Spectra Physics).

Sample preparation

A standard amino acid mixture (5 μmoles/ml of each amino acid) was used for calibration. Norleucine (nor) was used as internal standard.

Protein hydrolysates were prepared by digesting in 6 *N* HCl at 110° in sealed evacuated tubes.

Carboxypeptidase digests of casein were prepared according to Ribadeau-Dumas[12] except that digestion was performed in borate buffer pH 8.5 instead of Tris buffer, since the latter yielded a volatile derivative which eluted between glycine and valine.

RESULTS

Effect of acylation temperature

Varying the esterification conditions yielded only small differences in *RMR*s. An investigation into the acylation conditions, not previously reported, produced unexpected results. Batches of identically esterified samples of amino acid calibration

mixture were acylated by heating with HFBA for 5 min at a temperature from 22–150° in a heating block. Amino acid derivatives were separated on the packed column System A. A typical chromatogram is shown in Fig. 1. *RMR*s, determined at each temperature, are shown in Table I.

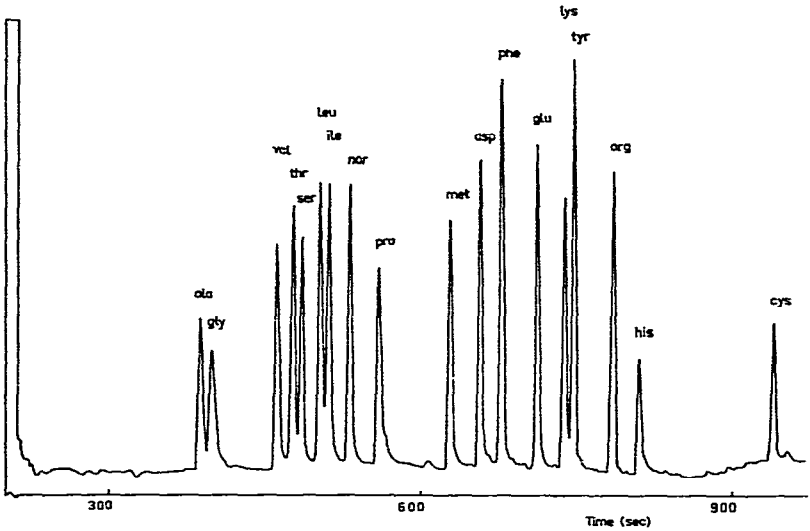

Fig. 1. Chromatogram obtained using the packed column (System A) of the amino acid calibration mixture. Chromatographic conditions as described in Experimental. Sample size, approx. 3 nmoles of amino acid. Internal standard, norleucine, denoted nor.

Consistent *RMR* values were obtained for each amino acid at each temperature except for methionine and arginine acylated at 150°. Histidine was not observed since the EFA addition technique had not then been established. It is clear that many of the amino acids yielded maximum *RMR*s after room temperature acylation, some showed decreased values after heating at elevated temperatures, some were unaffected by heat treatment but only a few required heating during acylation, and only proline gave an increased *RMR* up to 150°. As a consequence of these results and a desire to simplify the procedure and apparatus, all subsequent acylations have been performed at 110°.

Chromatography on the packed column

The chromatogram shown in Fig. 1 indicates that estimation of each amino acid could be achieved with a fair degree of accuracy using a packed column if approximately equimolar amounts of each amino acid were present as in the ideal situation represented by the calibration mixture. In addition, this chromatogram was obtained early in the life of the packed column and probably represents the best resolution obtainable with that column. The programme conditions appear at first sight to be good, that is, optimum resolution when programmed at 20°/min, yielding a total analysis time of 1000 sec. However, the column offered no scope for varying speed or resolution.

TABLE I

EFFECT OF ACYLATION TEMPERATURE ON AMINO ACID *RMRs**

Data obtained by chromatography on the packed column (System A).

Amino acid	*RMR*			
	22°	*80°*	*120°*	*150°*
Ala	0.56	0.55	0.61	0.59
Gly	0.48	0.40	0.45	0.46
Val	0.86	0.88	0.87	0.85
Thr	1.20	1.16	1.02	0.95
Ser	1.01	1.01	0.89	0.84
Leu	1.16	1.17	1.09	1.03
Ile	1.09	1.07	1.04	1.01
Nor	1.00	1.00	1.00	1.00
Pro	0.57	0.58	0.65	0.73
Met	0.90	0.83	0.79	0.36–0.75
Asp	1.19	1.20	1.11	1.04
Phe	1.48	1.47	1.32	1.27
Glu	1.19	1.21	1.09	1.11
Lys	0.94	1.04	0.98	0.94
Tyr	1.68	1.67	1.38	1.26
Arg	0.64	0.96	0.72	0.44–0.66
His	—	—	—	—
Cys	0.19	0.18	0.21	0.19

* *RMRs* calculated with respect to Nor by peak height measurement. Each value represents the mean of at least six independently derivatized samples with standard deviations better than ± 0.06, with the exception of those indicated by a range.

It may be seen that several peaks are incompletely separated. Resolution between lysine and tyrosine was the most susceptible to deterioration of the column and became very poor. The resolution between leucine and isoleucine and serine and threonine fell to less than 50%.

Column bleed was found to be so high above about 200° that differential operation was required. However, as the columns deteriorated, differential operation became progressively less satisfactory.

Chromatography on the SCOT column

A typical chromatogram of the amino acid calibration mixture obtained using the SCOT column, System B, in single column operation is shown in Fig. 2. Certain features apparent in this chromatogram demonstrate the several advantages of this system over the packed column, namely, greatly improved resolution, reduced peak tailing with the exception of histidine, and less column bleed.

It may be seen that peaks which were incompletely resolved on the packed column (Fig. 1) are now resolved completely to the baseline on the SCOT column (Fig. 2); leucine and isoleucine and lysine and tyrosine, previously the most troublesome pairs, now present no problem. The calibration mixture has been supplemented with some less commonly encountered amino acids: hydroxyproline, ornithine, S-carboxymethylcysteine (SCM-cys), and kynurenine (kyn). These are also well resolved. Tryptophan was not included in this mixture; so far, it has not been

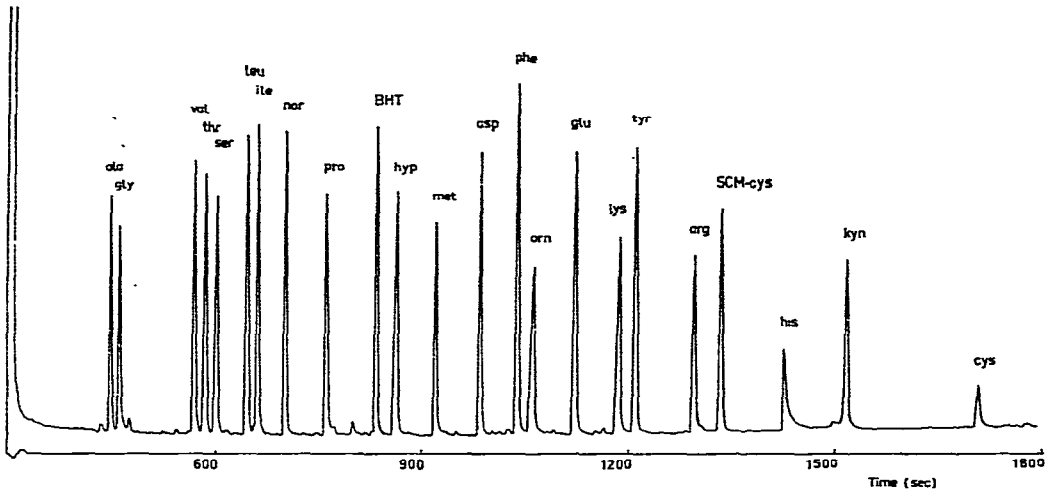

Fig. 2. Chromatogram obtained using the SCOT column (System B) of the amino acid calibration mixture. Chromatographic conditions as described in Experimental. Sample size, approx. 3 nmoles of amino acid.

possible to chromatograph tryptophan reproducibly. At least two major peaks and one minor peak are produced as a result of the derivatization procedure.

In Fig. 1 it may be seen that several peaks appeared to tail badly, namely, glycine, proline, and methionine. The greatly increased resolution obtained with the SCOT column (Fig. 2) permits recognition of minor unidentified components eluting closely after these amino acid derivative peaks, which may account for the apparent tailing on the packed column. Histidine is the peak most subject to tailing and in this case it does appear to be due to polar interactions.

An important feature of the SCOT column is the very low stationary phase bleed from the column even at temperatures greater than 200°. This is advantageous for at least three reasons: (i) a flat baseline facilitates peak area estimation whether performed manually or via an integrator; the integrator does not need sophisticated systems for handling variably sloping baselines; (ii) very small samples require low attenuation; thus a low baseline gradient, which is correspondingly attenuated, enables one to work satisfactorily with smaller sample size; (iii) low bleed indicates small loss of stationary phase from the column and correspondingly persistent high column efficiency and extended column life.

The SCOT column, like the packed column, is susceptible to progressive deterioration characterised by peak broadening and tailing promoted by passing excessive amounts of HFBA through the column; EFA does not seem to have the same deteriorative effect. However, the SCOT column readily responds to rejuvenation with silanizing reagents whereas, following the same treatment, the packed column recovers to a lesser extent. After three months of continuous use the SCOT column is still performing satisfactorily.

The transition from packed column to SCOT column chromatography necessitated certain modifications to the derivatization procedure. It was not possible to obtain a histidine peak using on-column acylation with EFA, which was successful

on the packed column, nor was it possible after preheating the sample in the oven at 110° for 5 min before injection. A similar result was obtained when the antioxidant BHT was added prior to the acylation with HFBA as suggested by March[10]. However, combination of the antioxidant addition and heating with EFA before injection enabled a histidine peak to be obtained. BHA was also tested and proved to be equally effective as an antioxidant and chromatographed between norleucine and proline as shown in Fig. 4. Small but consistent losses of methionine, arginine, and tyrosine resulted after heating with EFA at 110° for 5 min in agreement with Moodie[13], however, these conditions are much milder than those employed by Moodie[13] and do not cause such severe degradation.

In preliminary trials with the SCOT column nitrogen was used as carrier gas. Although improved resolution over the packed column was obtained, it was not as good as tests performed by the manufacturers suggested possible. Helium was substituted as carrier gas; the column then yielded the expected resolution. Carrier gas and make-up gas flow-rates have not been carefully optimised for resolution and FID response. However, even the working conditions presently employed permit choice of speed and accuracy previously not possible with the packed column. For this paper all chromatograms have been obtained at a relatively fast programme rate, 6°/min, thereby sacrificing a little resolution and hence accuracy. Reducing the heating rate to 3°/min improved resolution further but with a correspondingly slower analysis time. Resolution demonstrated here exceeds any previously recorded.

In Table II are shown retention times and *RMR*s for the amino acid derivatives. Values are shown for seven individually prepared samples of the calibration mixture as used for Fig. 2. It may be seen that retention times are consistent in all cases with standard deviations (σ) better than 1% of mean retention time, thus facilitating the recognition of peaks by a computing integrator. *RMR*s were also satisfactory, the amino acids having the least volatile derivatives, arginine to cystine, yielding the largest standard deviations.

Routinely 2–5 nmoles of each amino acid have been loaded on the column. Samples of 100 pmoles have been chromatographed successfully.

Alternative esterification procedure

The results for the amino acid esterification procedures employing isobutanol and acetyl chloride are shown in Table II. Chromatograms appeared to be almost identical to those obtained using the isobutanol–3 *N* HCl esterification procedure. *RMR*s were either insignificantly different or a little greater for all except cystine, whose mean *RMR* is considerably greater and with a much larger standard deviation.

Chromatography of sugars

Glucose, galactose, glucosamine, and galactosamine were subjected to the amino acid derivatizing procedure, to investigate whether sugars, which commonly occur in the carbohydrate moieties of glycoproteins, yield volatile derivatives. Each was run separately with norleucine to determine the relative retention times of derivative peaks. In Fig. 3 is shown a chromatogram obtained from an equimolar mixture of the sugars and equimolar with respect to norleucine. It may be seen that more than four peaks in addition to norleucine and BHT are obtained. The nature of these sugar derivatives has not been investigated; it is possible that each sugar may yield separate

TABLE II

AMINO ACID DERIVATIVE RETENTION TIMES AND *RMRs* AFTER ESTERIFICATION WITH ISOBUTANOL–HCl AND ISOBUTANOL–ACETYL CHLORIDE

Values shown are mean and standard deviations for seven individually prepared samples of amino acid calibration mixture.

Amino acid	*Retention time (sec)*		*RMR*			
			Isobutanol–HCl		*Isobutanol–acetyl chloride*	
	$\bar{X}$	σ	$\bar{X}$	σ	$\bar{X}$*	σ
Ala	463	4.5	1.50	0.08	1.56	0.06
Gly	476	4.6	1.57	0.08	1.66	0.09
Val	583	3.8	1.13	0.04	1.18	0.02
Thr	598	3.8	1.02	0.03	1.12	0.03
Ser	614	3.8	1.13	0.03	1.28	0.05
Leu	658	3.5	0.95	0.02	0.97	0.02
Ile	674	3.4	1.00	0.02	0.99	0.02
Nor	713	3.2	—	—	—	—
Pro	773	3.0	1.10	0.02	1.08	0.04
BHT	844	2.8	—	—	—	—
Hyp	872	3.0	1.01	0.03	1.06	0.07
Met	929	2.8	1.29	0.07	1.46	0.06
Asp	994	3.0	0.90	0.03	0.88	0.06
Phe	1049	3.1	0.75	0.03	0.72	0.05
Orn	1071	3.3	1.04	0.08	1.03	0.15
Glu	1131	3.0	0.84	0.05	0.80	0.08
Lys	1194	3.3	0.96	0.09	0.89	0.08
Tyr	1219	2.8	0.82	0.07	0.74	0.06
Arg	1305	3.2	1.16	0.22	1.10	0.10
SCM-Cys	1344	3.1	1.08	0.10	0.93	0.09
His	1433	3.6	1.79	0.34	1.86	0.10
Kyn	1525	3.3	1.31	0.18	1.29	0.10
Cys	1718	4.3	3.55	0.29	4.64	1.1

* Values from six individually prepared samples of calibration mixture.

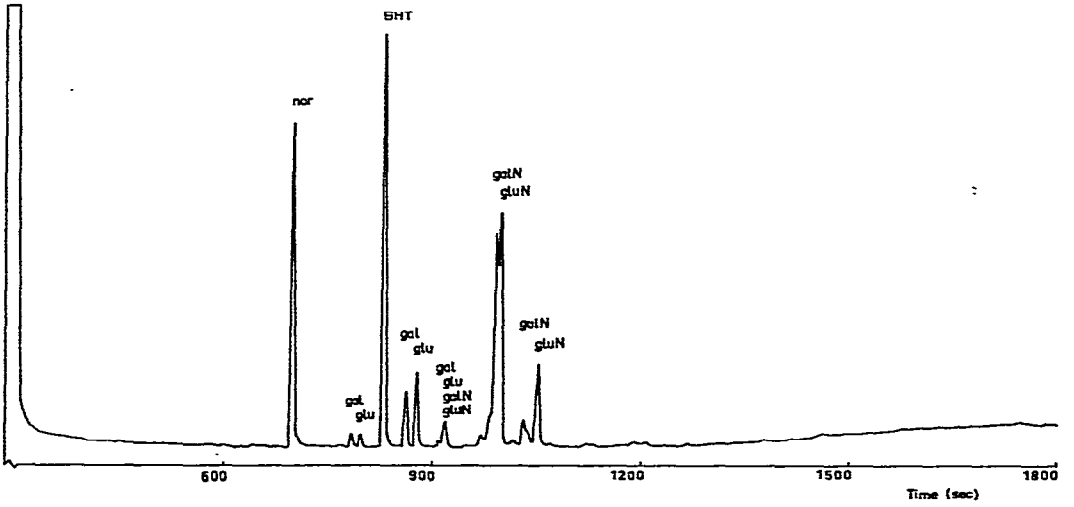

Fig. 3. Chromatogram of a mixture of glucose (glu), galactose (gal), glucosamine (gluN), and galactosamine (galN), subjected to the amino acid derivatization procedure and chromatographed on the SCOT column. All components except BHT were equimolar with respect to norleucine.

peaks from the α- and β-enantiomers. Because of the multiplicity of peaks it has not been possible to ascribe significant *RMR*s for these derivatives, but Fig. 3 shows that only the major glucosamine and galactosamine peaks yield responses comparable to that of norleucine.

In Fig. 4 the amino acids, sugar, and antioxidant peaks have been represented diagrammatically to indicate the extent to which sugar derivatives and antioxidant may interfere in the chromatography of amino acids. Lengths of lines are not quantitative representations of *RMR*s, except in the case of the sugars, where major, intermediate and minor peaks have been differentiated. The only significant interference may be between the major galactose peak and hydroxyproline. The combined galactosamine and glucosamine peak is resolved from aspartic acid. Other sugars have not been examined.

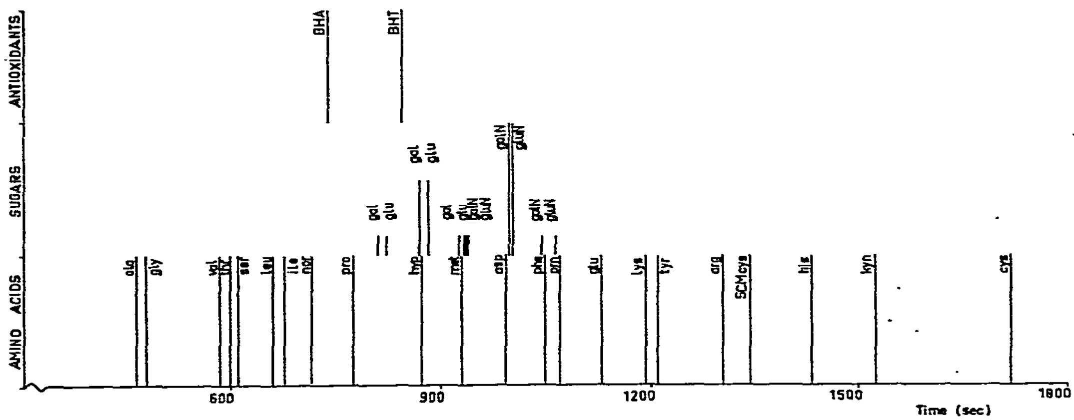

Fig. 4. Diagrammatic representation of the relative retention times of amino acids, sugars and antioxidants. *RMR*s are not represented, but sugars are indicated by long, medium or short lines, according to their possible significance in an amino acid analysis.

Amino acid analysis of β-lactoglobulin

Purified samples of β-lactoglobulin (A and B variants) were hydrolysed with 6 *N* hydrochloric acid for 2–40 h. Hydrolysates were derivatized and analysed by GLC. Fig. 5 shows a chromatogram obtained from an 8-h digest and the results of the analysis are presented in Table III, which also includes amino acid analysis data obtained by conventional ion-exchange chromatography for β-lactoglobulin. The alternative values obtained by ion-exchange chromatography represent residues in either A or B variants, thus values for mixed variants would be expected to be between these limits.

In general, agreement is extremely good. Values for a few amino acids, namely valine, leucine, isoleucine, and methionine, are a little low, relative to the ion-exchange data which were obtained for 96-h digestion[14]. The values for the residues are still increasing after 40 h digestion and hence after this time hydrolysis is probably incomplete. The chromatogram for the 8-h digest (Fig. 5) shows a number of minor peaks in the higher temperature region of the chromatogram. These are probably

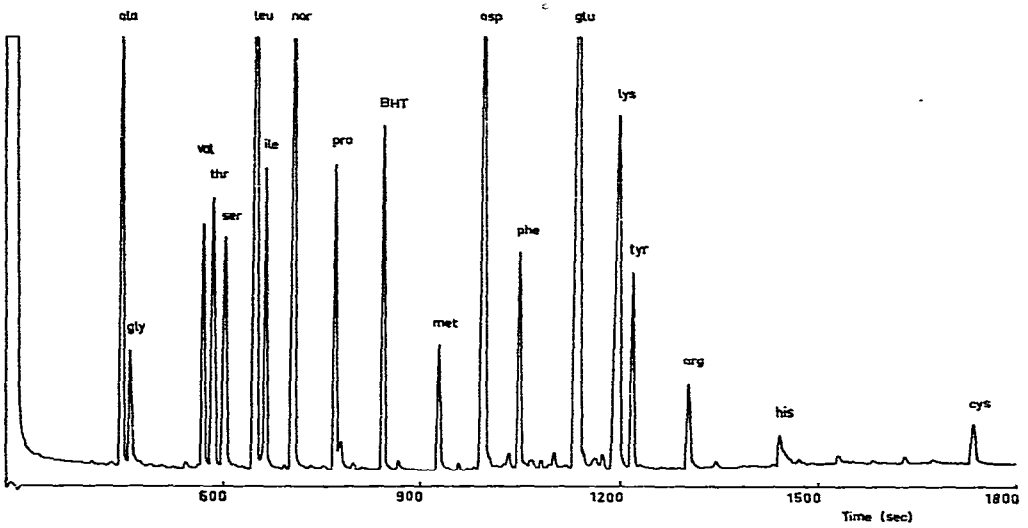

Fig. 5. Chromatogram, obtained using the SCOT column (System B), of a β-lactoglobulin acid hydrolysate (8-h digestion). Chromatographic conditions as described in Experimental.

TABLE III

AMINO ACID ANALYSIS OF β-LACTOGLOBULIN*

Amino acid	*Digestion time (h)*				*Amino acid composition by GLC***	*Amino acid*** *composition by ion exchange*
	2	*8*	*20*	*40*		
Ala	11.9	12.9	13.8	14.1	14.1	14 or 15
Gly	3.7	3.8	4.0	3.9	4.0	3 or 4
Val	2.9	5.6	8.6	8.9	8.9	9 or 10
Thr	4.3	7.3	7.6	7.5	7.6	8
Ser	5.6	6.9	7.0	6.7	7.0	7
Leu	12.6	17.1	19.5	19.7	19.7	22
Ile	3.3	6.0	8.5	8.8	8.8	10
Pro	5.6	7.2	7.5	7.4	7.5	8
Met	1.9	3.2	3.3	3.4	3.4	4
Asp	12.0	14.3	14.9	14.7	14.9	15 or 16
Phe	2.3	3.5	3.8	3.7	3.8	4
Glu	18.0	21.1	24.5	23.8	24.5	24 or 25
Lys	9.8	12.7	15.0	14.1	15.0	15
Tyr	2.5	3.6	4.1	3.9	4.1	4
Arg	1.2	2.6	2.9	2.9	2.9	3
His	1.8	2.3	1.1	1.0	2.3	2
Cys	2.4	4.6	4.7	4.0	4.7	5
Trp	not determined					2
NH_2	not determined					15

* Mixed A and B variants.
** Maximum values obtained.
*** Values from Piez *et al.*[14].

due to derivatives of small partially hydrolysed peptides, since these are more pronounced in a 2-h digest but have virtually disappeared from a 40-h digest.

Relative casein concentrations by amino acid analysis of carboxypeptidase digests

Whole casein and an α_{S1}-casein preparation were digested with carboxypeptidase and the liberated amino acid residues analysed by GLC. Chromatograms obtained from each preparation are shown in Figs. 6 and 7.

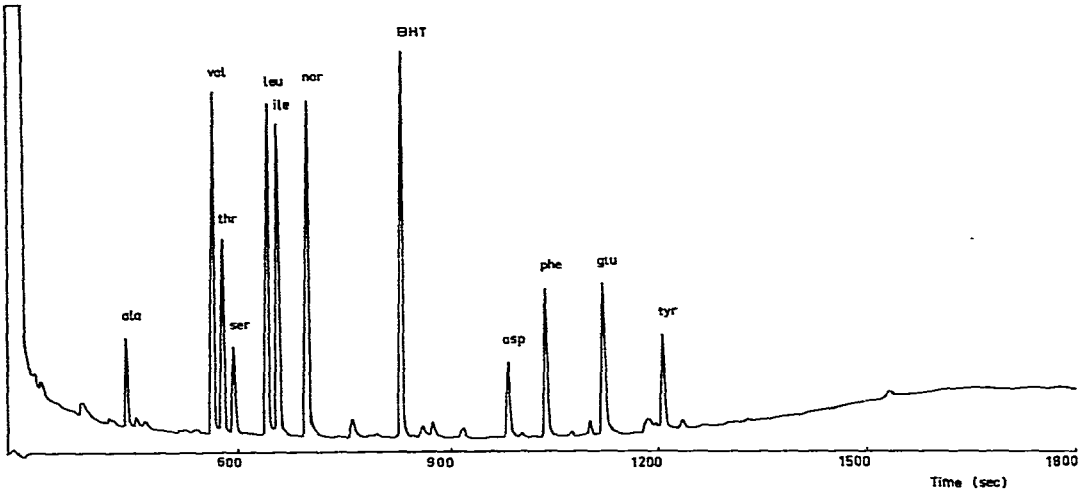

Fig. 6. Chromatogram, obtained using the SCOT column (System B), of amino acids liberated from isolated whole casein after carboxypeptidase digestion. Chromatographic conditions as described in Experimental.

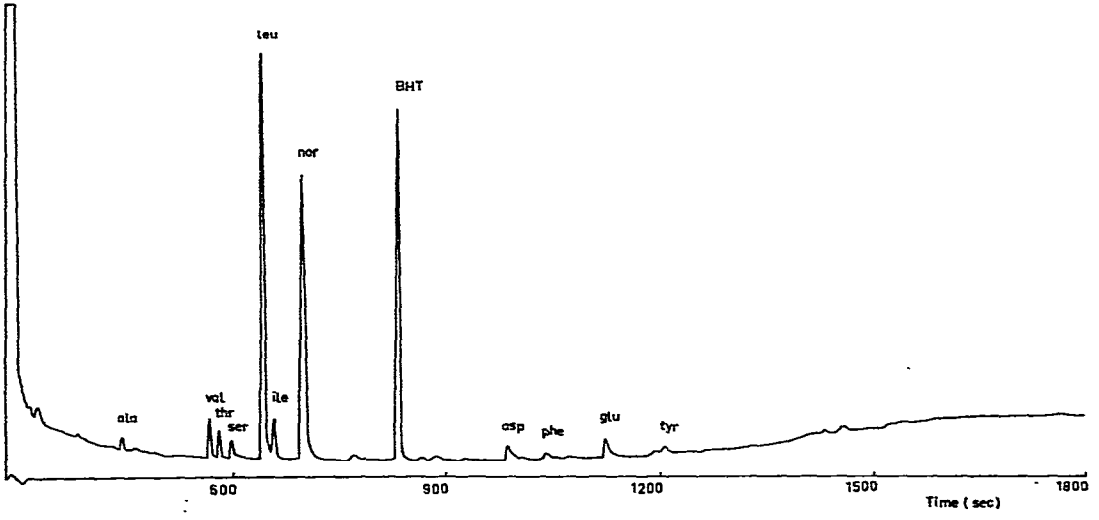

Fig. 7. Chromatogram, obtained using the SCOT column, of amino acids liberated from purified α_{S1}-casein after carboxypeptidase digestion. Chromatographic conditions as described in Experimental.

Because of short distinguishing C-terminal sequences after the last proline, consideration of leucine, isoleucine, alanine, phenylalanine, and tyrosine enables relative concentrations of the caseins to be determined[12]. The complete resolution of alanine and glycine, leucine and isoleucine, and lysine and tyrosine using the SCOT column enables accurate estimation of these residues, which was previously not possible with the packed column. Analyses of prepared casein fractions such as the α_{S1}-casein, where only a very small isoleucine peak occurs close to a large leucine peak, were previously impossible by GLC techniques.

DISCUSSION

Preparation of volatile amino acid derivatives has been achieved most frequently by their esterification and acylation[1–4,7–10], although other methods have been used[11]. For direct esterification with isobutanol–3 *N* HCl, variations in the temperature and time of heating from 110–130° and 10 min–2 h, respectively, produced only small variations in *RMR*[8,15]. An alternative esterification procedure using an alcohol–acetyl chloride mixture was described by Felker and Bandurski[9], but without comparative results. Data reported here have shown that this method yields, generally, comparable *RMR*s for all the amino acids except cystine. The simplicity of the procedure relative to the difficulties of preparation and storage of isobutanol–3 *N* HCl commends the isobutanol–acetyl chloride mixture as an alternative esterifying reagent. However, if a more reliable cystine determination is required, the isobutanol–3 *N* HCl method is probably preferable.

The acylation procedure has previously received much less attention. A variety of anhydrides have been employed but apart from early reports describing acylation at room temperature[1,2], all subsequent papers have described acylation at 150°[4,7–11]. Felker and Bandurski[9] reported that "within limits, time and temperature of acylation (with HFBA) are not critical". My early attempts to achieve reproducible, quantitative results by the method of MacKenzie and Tenaschuk[8] were not successful. The study of the effect of acylation temperature reported above showed (i) that this parameter is important for most amino acids, (ii) that 150° was certainly not the optimum acylation temperature, and (iii) that 80–120° was preferable.

Degradation, probably oxidative, of amino acid derivatives clearly occurs with excessive heating. Felker and Bandurski[9] have reported deterioration of the acylating properties of HFBA after storage in air for a month; I have not found this to be the case. However, it is now clear that HFBA has severe oxidative effects on the histidine derivative although these did not manifest themselves until the SCOT column was used. The reason for this is obscure. Nevertheless, the problem of oxidation was overcome by including an antioxidant in the acylation mixture as employed by March[10]. No benefit had been observed by introducing the antioxidant before the esterification step.

The advantages of using the N-heptafluorobutyryl isobutyl esters of the amino acids have been described by MacKenzie and Tenaschuk[8]. However, a packed column does not offer a high enough effective theoretical plate number to enable complete separation of closely similar molecules such as the derivatives of leucine and isoleucine. The SCOT capillary glass column offers effective theoretical plate numbers 10 to 100 times greater than an average-sized packed column and approaching that

obtainable from small-bore wall-coated open-tubular (WCOT) columns. It has added advantages over the WCOT column in that a higher sample loading, up to 0.5 μl, is tolerable, permitting use of splitless injection and a higher, more easily controlled carrier gas flow-rate.

For long life of the column the amount of HFBA loaded with each sample should be minimised. Complete removal of HFBA after the acylation step should be performed with caution. Previous methods[7,8] recommended evaporation until just dry, but it has been reported[9] that the excessive evaporation may result in loss of arginine, and this has been confirmed. The apparent contradiction in these two requirements does not present a problem, however, if the subsequent ethoxyformylation of histidine with EFA is performed. The HFBA does not need to be completely removed before adding EFA and is preferentially evaporated at the final stage. A small residual volume of EFA seems to protect the arginine derivative without damage to the column. The necessity to heat derivatives with EFA prior to injection, in contrast to the ability to ethoxyformylate "on-column" for the packed column[13], may be attributable to the higher linear gas flow-rate through the SCOT column and the low contact area of the reagent over the histidine derivative. At 20 ml/min the linear gas flow-rate in the packed column is about 10 cm/sec, whereas the linear rate in the SCOT column at 4 ml/min is about 34 cm/sec. Moreover, injection on the packed column spreads the sample directly on the stationary phase, whereas the SCOT column has, before the support-coated glass capillary, an uncoated glass-lined tube on which derivative may condense. The use of a short, coated precolumn is being investigated.

Possible interference by the carbohydrate moieties of glycoproteins has been briefly examined. The two neutral sugars and two amino sugars investigated all produced peaks after the amino acid derivatization procedure. The amino sugars yield *RMR*s comparable to amino acids but are fortunately well separated from them; neutral sugars gave smaller peaks. These results contrast with those of Zanetta and Vincendon[7], who reported hexosamines giving weak peaks and carbohydrate being almost completely degraded. The difference may be a consequence of the antioxidant added during acylation. Although all the possibilities have not been studied, it appears, at present, that glycoproteins should present no real difficulty for amino acid analysis.

The data obtained for the amino acid composition of β-lactoglobulin compare well with published data obtained by conventional ion-exchange chromatography[14]. Analysis of carboxypeptidase digests of casein have also been performed satisfactorily and rapidly. The high sensitivity of GLC amino acid analysis is well suited to this task, where only small amounts, approximately 2–10%, of the protein are liberated as free amino acid.

ACKNOWLEDGEMENTS

I gratefully acknowledge discussions with Ms. G. Urbach and Mr. W. Stark at this Laboratory, also Dr. C. Roxburgh and Mr. D. McMahon at the CSIRO, Division of Protein Chemistry.

REFERENCES

1 C. W. Gehrke, W. M. Lamkin, D. L. Stalling and F. Shahrokhi, *Biochem. Biophys. Res. Commun.*, 19 (1965) 328.
2 J. R. Coulter and C. S. Hann, *J. Chromatogr.*, 36 (1968) 42.
3 D. Roach and C. W. Gehrke, *J. Chromatogr.*, 43 (1969) 303.
4 D. A. Shearer and R. N. Warner, *Int. J. Environ. Anal. Chem.*, 11 (1971) 11.
5 J. J. Pisano and T. J. Bronzert, *Anal. Biochem.*, 45 (1972) 43.
6 K. M. Williams and B. Halpern, *Anal. Lett.*, 6 (1973) 839.
7 J. P. Zanetta and G. Vincendon, *J. Chromatogr.*, 76 (1973) 91.
8 S. L. MacKenzie and D. Tenaschuk, *J. Chromatogr.*, 97 (1974) 19.
9 P. Felker and R. S. Bandurski, *Anal. Biochem.*, 67 (1975) 245.
10 J. F. March, *Anal. Biochem.*, 69 (1975) 420.
11 P. Husek and K. Macek, *J. Chromatogr.*, 113 (1975) 139.
12 B. Ribadeau-Dumas, *Biochim. Biophys. Acta*, 168 (1968) 274.
13 I. M. Moodie, *J. Chromatogr.*, 99 (1974) 495.
14 K. A. Piez, E. W. Davie, J. E. Folk and J. A. Gladner, *J. Biol. Chem.*, 236 (1961) 2912.
15 S. L. MacKenzie and D. Tenaschuk, *J. Chromatogr.*, 111 (1975) 413.