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COMPARATIVE DETERMINATION OF PROTEIN AMINO ACIDS IN PLANT MATERIALS BY AUTOMATED CATION EXCHANGE AND GAS–LIQUID CHROMATOGRAPHY OF THE AMINO ACID N-HEPTAFLUOROBUTYRYL, *n*-PROPYL ESTERS

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SUMMARY

Gas–liquid chromatographic (GLC) analysis of the amino acid N-heptafluorobutyryl, *n*-propyl ester derivatives seems to offer the best potential for a single packed column technique of analyzing amino acids by GLC. A 25-min analysis on a 3.5 m × 2 mm I.D. glass column, packed with 3% SE-30 on 80–100 mesh Gas-Chrom Q, gave good separation of all protein amino acids, and quantitative results for all except histidine. Procedures for amino acid analysis by GLC and automated cation exchange (CIE) were compared for accuracy, repeatability, and application to protein amino acid analysis in sorghum. In the latter instance, automated CIE and GLC were used to determine the dependence of protein quality in sorghum on nitrogen and sulphur fertilization. Identical conclusions were drawn from experimental data derived from both methods of analysis.

INTRODUCTION

Analysis of amino acids by gas–liquid chromatography (GLC) is now standard laboratory practice. The best documented technique[1–5], developed by Gehrke and co-workers at the University of Missouri, is based on dual column analysis of the amino acid N-trifluoroacetyl, *n*-butyl ester derivatives. About 10 or 12 other methods[6–18], making use of a range of amino acid derivatives, have also been described. Even so, GLC has yet to fulfil two important requirements.

The first is for a single-column technique utilizing a highly stable liquid phase. Such a development would permit approximately 75% reduction in capital expenditure on a gas chromatograph capable of protein amino acid analysis, bringing the cost into line with that of a good pH meter. The second requirement is for ultrasensitive analysis of amino acids specifically localized in plant or animal tissues, or in single enzyme bands separated by gel electrophoresis. In this context, selective

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detection by electron capture of a halogen-rich amino acid derivative seems, in principle, to offer the best solution.

Both of the above requirements may be potentially resolved by analysis of the amino acid N-heptafluorobutyryl (N-HFB), *n*-propyl ester derivatives. One previous publication[19] described the GLC analysis of the N-HFB, *n*-propyl ester derivatives on a packed column, but no data were given on applications. The technique of using the heptafluorobutyryl group to promote volatility of amino acids for gas chromatographic analysis was first reported by Pollock[20]. Pollock analyzed the amino acid N-HFB, *n*-butyl esters on a 150 ft. × 0.02 in. Carbowax 20M column. Eleven amino acids were analyzed, but no details were given on quantitative aspects of the analysis, nor on the method of preparing the column. Moss *et al.*[19] made a 43-min separation of all 20 protein amino acids as their N-HFB, *n*-propyl esters on a 12 ft. × 0.25 in. O.D. glass column packed with 3% OV-1 on 80–100 mesh acid-washed DMCS-treated high-performance Chromosorb W. This technique was claimed to yield precise quantitative measurements, although no supporting data were given. Jönsson *et al.*[21] have made the most extensive study of the N-HFB, amino acid *n*-propyl esters to date. They provided statistically validated data on the choice of derivatization conditions, stability of the derivatives, response factors and analysis of amino acids in protein hydrolyzates. Their use of a 6 m × 0.02 mm I.D. glass capillary column, however, may be considered to be a disadvantage, as such columns are expensive and difficult to prepare reproducibly.

An attractive alternative to capillary column analysis is provided by packed column analysis using a highly stable liquid phase. In the study reported here, single packed column analysis of the N-HFB, *n*-propyl amino acid ester derivatives was performed, using SE-30 as the stationary liquid phase. Both SE-30 and OV-1, employed earlier by Moss *et al.*[19], are non-polar dimethyl silicone gums with virtually identical separation characteristics. They differ in average molecular weight, and in the lower temperature limits at which they can be used (70 *vs.* 100), although both phases are stable up to 350 .

Part of our work in this laboratory involves the measurement of protein quality in food plants as it relates to climate and agronomic practice. Hence the GLC method was tested in application to the analysis of protein amino acids in 6 *N* hydrochloric acid hydrolyzates of sorghum, previously grown under treatment with different amounts of nitrogen and sulphur fertilizers. Results were compared with those obtained by automated cation exchange analysis (CIE).

EXPERIMENTAL

Apparatus

A two-column Bendix 2522-2 gas chromatograph, equipped with a dual differential hydrogen flame detector, and a dual electrometer, was used. Peak areas were determined with a Vidar 6300 digital electronic integrator. A Leeds and Northrup XL 680 flat-bed potentiometric recorder, 1 mV full-scale, completed the GLC analytical system.

Amino acids were analyzed by automated cation exchange on a Technicon TSM-1 amino acid AutoAnalyzer.

Hydrochloric acid was removed from sample hydrolyzates using a Büchi

rotary evaporator, Rotavapor Model EL/S, equipped with a specially constructed six-arm spider adapter that could be butt-jointed to hydrolysis tubes or flasks using Sovirel (Paris, France) screw-connectors with PTFE–rubber sealing rings. This allowed simultaneous evaporation of up to six samples at a time.

Amino acid calibration standards, made up in 0.1 *N* hydrochloric acid, and hydrolyzates that had been evaporated once and taken up in 0.1 *N* hydrochloric acid, were frozen and dried on a Virtis automatic freeze-drier.

Hydrolysis of plant samples was conducted in 200 × 22 mm glass tubes (Sovirel, No. 611-58) or in 100-ml flat bottomed flasks (Sovirel, No. 231-40). Propyl esterification of amino acids was carried out in 100 × 16 mm glass tubes (Sovirel, No. 611-52), and acylation in 1.5-ml conical glass Mini-Aktors (Applied Science Labs., State College, Pa., U.S.A.). All tubes and flasks were fitted with PTFE-lined or PTFE screw-caps.

Derivatization solvents and reagents were removed under nitrogen with tubes placed in a Techne (Cambridge, Great Britain) temperature-controlled heating block, converted for use as a sand-bath.

A Dawe 50 W ultrasonic bath, obtained from Griffin and George, Wembley, Great Britain, was used to de-gas hydrochloric acid solutions before hydrolysis, and to effect dissolution of amino acid hydrochlorides in *n*-propanol–hydrochloric acid.

Reagents and materials

Amino acids used in calibration and testing were purchased chromatographically pure as an amino acid reference kit from BDH, Poole, Great Britain.

Reagent-grade *n*-propanol and acetic anhydride were obtained from Koch-Light, Colnbrook, Great Britain. New bottles of *n*-propanol were opened on delivery, and *ca.* 50 g of anhydrous granulated calcium sulphate were added per litre. The 3.5 *M* hydrochloric acid–*n*-propanol reagent was made by bubbling dry hydrochloric acid into dry *n*-propanol, free from calcium sulphate.

Heptafluorobutyric anhydride, silylation-grade acetonitrile and Dri-Film, used for silanizing the interior of glass columns, were obtained from Pierce, Rockford, Ill., U.S.A.

Ready-prepared 3% SE-30 on 80–100 mesh Gas-Chrom Q was acquired from Applied Science Labs.

Preparation of columns

Glass columns, 3.5 m × 2 mm I.D., were cleaned with methanol, acetone and *n*-hexane, and then silanized with Dri-Film according to the manufacturer's instructions. After silanization, each column was filled with methanol and allowed to stand for 30 min, then rinsed with methanol to ensure removal of hydrochloric acid produced during treatment. The column was rinsed further in acetone, then dried in a stream of dry nitrogen. Column packing was carried out immediately after cleaning and silanization, using the coated support as received. The column was packed with the aid of pressure from a nitrogen cylinder, and vibrated intermittently during packing with a Burgess Vibro-Tool. Due care was taken to avoid packing the inlet arm of the column in the injector zone.

Glass columns were connected to the gas chromatograph using Mini-grip front ferrules obtained from Packard, Downers Grove, Ill., U.S.A. Each front ferrule

was backed by a reversed brass back ferrule, and sealed with a brass nut. PTFE closures or silicone O-rings failed to give a lasting seal at relatively high temperatures of operation.

Column conditioning was carried out for 2 h at 250° with a nitrogen flow-rate of 20 ml/min, followed by a 2 h no-flow period at 300°, and then 24 h at 300° with the nitrogen flow-rate re-adjusted to 20 ml/min. Periodic reconditioning at 300° was required during normal operation to reduce base signal from the analytical column at high temperatures, and also to eliminate shadow components appearing in blank or control chromatograms. Columns prepared as above lasted for several hundred analyses. Signs of deterioration included decreasing resolution of leucine and isoleucine, and a decline in the relative response of the basic amino acids, particularly arginine. Both of these problems could usually be remedied by repacking the inlet arm of the column.

Normally, GLC analysis based on flame ionization detection requires that two columns should be packed and conditioned simultaneously to give a matched pair, each of which can be used either as a reference column or as an analytical column. Electronic matching of the signals from the reference and analytical columns is employed to eliminate positive baseline drift due to substrate bleed from the analytical column. However, as SE-30 is extremely stable, with an upper-temperature limit of 350°, provision of a reference column is not strictly necessary, unless one wishes to analyze unusually small amounts of amino acids at high instrument sensitivity.

Standards

Three types of amino acid standard were needed. The first, S1, was used to calibrate the gas chromatograph and to determine the reproducibility of analysis for each amino acid during sequential analysis on different days. Standard S1 is normally referred to as an external standard. Use of the second standard, S2, which in this case was a simulated biological sample (wheat grain), permitted recovery and accuracy values to be calculated for the method as it applies to a given sample type. The third standard, S3, was an internal standard. By means of the internal standard, results on the amino acid analysis of an unknown sample could be calculated, based on the response of a known amount of the internal standard added to each sample at some stage before analysis.

Standard S1 was made up to contain 50 mg of each of the amino acids shown in Table I, dissolved in 100 ml of 0.1 *N* hydrochloric acid. As at least one external standard was analyzed on each day of routine operation, it was found convenient to distribute separate 1-ml aliquots to about one hundred 100 × 16 mm Sovirel tubes, which were then dried and held in storage over phosphorus pentoxide in a vacuum desiccator until required.

Standard S2 contained the amounts in milligrams of each of the amino acids shown in Table II, dissolved in 100 ml of 0.1 *N* hydrochloric acid, giving a total amino acid content of 1000 mg. The percentage composition of S2 thus corresponds to the milligram content divided by 10, and approximates that of wheat grain protein. Individual S2 samples were prepared as for S1.

S3, the internal standard, was prepared by dissolving 250 mg of norleucine in 500 ml of 0.1 *N* hydrochloric acid. The internal standard solution was decanted into

the reservoir bottle of a repeating dispenser, and refrigerated when not in use. A 1-ml aliquot of S3 was added to each sorghum sample directly after hydrolysis, and batch-wise to tubes containing S2, which, for the purpose of obtaining the data in Table II, was treated as a synthetic unknown.

Sample preparation for protein amino acid analysis by gas–liquid chromatography and automated cation exchange

Hydrolysis. A 100-mg amount of sorghum meal (*ca.* 10% protein) plus 10 ml of 6 *N* hydrochloric acid were placed in a 200 × 22 mm screw-capped glass tube. The solution was de-gassed under vacuum with accompanying sonication, and the tube flushed with nitrogen before closure. Hydrolysis was conducted for 21 h at 110°.

Addition of internal standard. After hydrolysis, 1 ml of an internal standard solution, containing 0.5 mg/ml of norleucine, was added and the contents of the tube were mixed by shaking or sonication.

Filtration. Each hydrolyzate was filtered through a grade 4 (pore diameter 10–20 μm) sintered-glass funnel and collected in a glass tube with a side-arm connected to vacuum. A manifold was constructed to allow simultaneous filtration of up to six samples. Filtrates were transferred to 100-ml flat-bottomed flasks, which were in turn connected to the arms of an evaporator spider.

Evaporation and drying. Filtered hydrolyzates were evaporated to dryness at 50° under vacuum. An aerial heating fan was suspended to one side of the water bath to speed evaporation, and to prevent condensation in the chamber of the spider adapter. The evaporation step was repeated after addition of a few millilitres of distilled water, and the dry residues were taken up in 5 or 10 ml of 0.1 *N* hydrochloric acid.

Anion-exchange clean-up for CIE. An appropriate amount of sample from the evaporation and drying stage, containing about 5 mg of amino acids, was pipetted on to 3–5 ml of Dowex 2-X8 (100–200 mesh) placed in a 20 × 1.5 cm glass column. The sample was washed through the resin with several small portions of 0.1 *N* hydrochloric acid, and exactly 10 ml of eluate were collected in a stoppered, graduated cylinder. This procedure served to remove humins. An aliquot containing 3–5 μg of total amino acids was added to each sample cup of the Technicon AutoAnalyzer for analysis. The instrument was calibrated with 50 μl of a solution containing 0.025 micromoles of each amino acid.

Cation-exchange clean-up for GLC. The method of Zumwalt *et al.*[3] was applied, using 7 *N* ammonia solution to elute amino acids from approximately half the sorghum hydrolyzate, pipetted on to *ca.* 3 g (moist weight) of Dowex 50-X8 (100–200 mesh) resin held in a 20 × 1.5 cm column. Following elution with five 5-ml volumes of 7 *N* ammonia solution plus five 5-ml volumes of distilled water, the collected eluate was dried by rotary evaporation, redissolved in 0.1 *N* hydrochloric acid and finally freeze-dried in 100 × 16 mm glass tubes.

Derivatization and analysis by GLC. A 2-ml volume of *n*-propanol–3.5 *M* hydrochloric acid reagent was added. The tube was shaken or sonicated until the solution cleared, and then placed in an oven at 110° for 30 min. After cooling, 1 ml of the solution was transferred into a 1.5-ml conical reaction tube and evaporated just to dryness under a stream of dry nitrogen, while maintained at 90–100° in a sand-bath. To the residue in the tube were added 0.2 ml of heptafluorobutyric

anhydride plus 0.1 ml of acetonitrile. Acylation was conducted at 150° for 5 min, with the tube immersed to the inside liquid level in an oil-bath at the desired temperature.

For analysis, about 3 μl of acetic anhydride was drawn into a 10-μl syringe, followed by a similar volume of the acylation mixture, and the combined solutions were injected into the gas chromatograph.

Summary of chromatographic conditions. The column was 3.5 m × 2 mm I.D. borosilicate glass, packed with 3% (w/w) SE-30 on 80–100 mesh Gas-Chrom Q. The carrier gas was nitrogen, dried and de-oxygenated in-line, and gas flow-rates were: nitrogen, 20 ml/min; hydrogen, 40 ml/min; and air, 1600 ml/min, split two ways. Pressures were as follows: inlet, 5 kg/cm^2; outlet, atmospheric; hydrogen cylinder, 2.8 kg/cm^2; air cylinder, 5 kg/cm^2. The injection port temperature was 225° and the detector temperature 280°.

The column conditions were as follows: initial isothermal (hold) period of 3 min at 75°, then temperature programmed from 75° to 250° at 5 or 6 /min. The chart speed was 1 cm/min. A dual differential flame ionization detector was used, operated in the single mode, except for high-sensitivity analysis. The minimum detection limit was *ca.* $1 \cdot 10^{-9}$ g injected and the minimum quantifiable limit was *ca.* $1 \cdot 10^{-8}$ g.

Calculations

From the integrator print-out, in arbitrary units, peak areas were determined for each amino acid on a chromatogram of the external standard, S1. Each peak area thus obtained was divided by the corresponding value for norleucine to obtain equal weight response (EWR) factors for the amino acids, based on norleucine.

The ratio of the experimental peak area for an amino acid in an unknown to the norleucine peak area in the same sample was divided by the appropriate $EWR_{a.a./nor}$ value, obtained from at least two S1 analyses, run either on the same day or within the same series of analyses.

The quotient was then multiplied by the amount of the internal standard (norleucine) originally added to the unknown. Results referred to the absolute content of amino acid in the sample, but could readily be converted to give amino acid composition expressed as per cent (w/w), relative mole per cent, or milligrams per gram of sample.

RESULTS AND DISCUSSION

Separation

All the amino acids separated completely (Fig. 1) during a 25-min analysis, apart from leucine and isoleucine, which were only about 80% resolved. With extended column use, the resolution of these two amino acids gradually declined to about 50%. At this point in the history of the column, decreasing separation of the amino acid pairs serine–leucine, methionine–aspartic acid and lysine–tyrosine also became evident. Changing the separation characteristics at no time led to impaired measurement.

When helium was tried as a carrier gas, complete separation of the amino acids occurred in less than 15 min. However, owing to the difficulty of establishing a

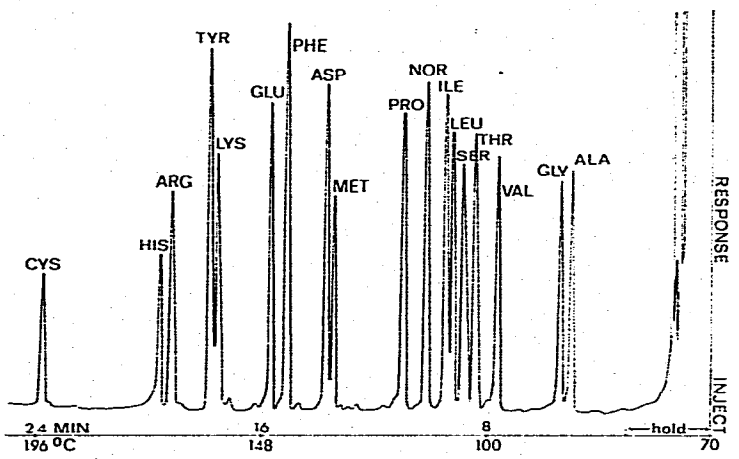

Fig. 1. GLC analysis of an equiponderate amino acid standard: amino acid N-HFB, *n*-propyl esters.

leak-tight system with helium, nitrogen was used as carrier gas for routine purposes.

The di-HFB derivative of histidine gave a satisfactory peak when injected alone, but separated poorly from glutamic acid in mixtures. As plant samples usually contain proportionately large amounts of glutamic acid, the method of Moss *et al.*[19], whereby di-HFB histidine is converted on-column into the corresponding mono-HFB derivative, was adopted for histidine analysis. The mono-HFB derivative produced a peak appearing penultimately in chromatograms between arginine and cystine.

Data were not compiled on the analysis of hydroxyproline, cysteine and tryptophan, because these amino acids are not normally detectable in acid hydrolyzates of plant materials. Nevertheless, inclusion of these amino acids in an S1 standard gave three well resolved peaks, as reported by Moss *et al.*[19] for analysis on an OV-1 column.

Relative molar response and equal weight response factors

It may be argued that, for those workers whose interests lie outside molecular biochemistry, analysis quoted on a weight basis imparts a clearer intuitive understanding than does an expression of molar composition. Accordingly, the term equal weight response (EWR) has been introduced, which refers to the ratio of the peak area for a given unit amount of amino acid divided by the peak area for the same amount of a standard, in this case norleucine, injected simultaneously.

The $EWR_{a.a./nor}$ values used later in the study to prepare data on per cent (w/w) content and milligrams of amino acids per gram of sorghum meal are given in Table I. For the purpose of comparing the response factors with other values appearing in the literature, specifically those reported for the amino acid N-HFB, *n*-propyl esters by Jönsson *et al.*[21], relative molar response (RMR) factors were also calculated, and are presented in Table I.

On investigating the most effective means of converting amino acids in to their N-HFB, *n*-propyl ester derivatives, Jönsson *et al.*[21] recommended a method incorporating two-step propylation in 8 *M* hydrochloric acid–*n*-propanol. Table I includes their RMR data obtained by this procedure. Although Jönsson *et al.*[21] tried esterifica-

TABLE I

RELATIVE MOLAR RESPONSE AND EQUAL WEIGHT RESPONSE OF THE N-HFB AMINO ACID *n*-PROPYL ESTERS

Amino acid	*EWR**		*RMR**		*RMR***
	Glu	*Nor*	*Glu*	*Nor*	*Nor*
Alanine	1.03	0.99	0.62	0.67	0.56
Glycine	0.97	0.93	0.50	0.53	0.52
Valine	1.08	1.03	0.86	0.92	0.76
Threonine	1.04	1.00	0.84	0.91	0.95
Serine	1.03	0.99	0.74	0.79	0.90
Leucine	1.13	1.09	1.01	1.09	1.02
Isoleucine	1.00	0.96	0.89	0.96	0.75
Norleucine	1.04	1.00	0.93	1.00	1.00
Proline	0.96	0.92	0.75	0.81	0.79
Methionine	0.70	0.68	0.71	0.77	0.73
Aspartic acid	1.00	0.96	0.90	0.97	1.04
Phenylalanine	1.14	1.10	1.27	1.39	1.47
Glutamic acid	1.00	0.96	1.00	1.08	1.20
Lysine	1.05	1.01	1.04	1.12	0.97
Tyrosine	1.08	1.03	1.33	1.42	1.58
Arginine	0.89	0.86	1.05	1.14	1.19
Histidine	0.41	0.40	0.43	0.47***	0.72§
Cystine	0.50	0.48	0.82	0.88	0.92

* Average of 12 determinations.
** Literature values[21].
*** Monoacyl histidine derivative.
§ Diacyl histidine derivative.

tion in *n*-propanol–3.5 *M* hydrochloric acid, they concluded that it gave low yields of the basic amino acid derivatives. Paradoxically, in the present study, in which esterification was conducted in *n*-propanol–3.5 *M* hydrochloric acid, arginine gave the same value as that cited by Jönsson *et al.*[21], and the RMR for lysine was significantly higher. Glycine and cystine also gave the same RMR values in both cases. Data were tested at the 95% significance level, using the *t*-distribution to establish confidence limits (figures not shown) from the values of standard deviation in Table IV. Most amino acid RMR factors reported in the two investigations differ significantly, although not by a sufficient margin to imply gross errors in either derivatization or chromatography.

Experimental RMR values for serine and tyrosine were more than 10% lower than those obtained by Jönsson *et al.*[21], although Tables II, III and IV reveal that the accuracy and precision of analysis for serine and tyrosine were good. Moreover, comparable analyses of serine and tyrosine in sorghum were obtained by GLC and CIE (Tables V and VI). Other amino acid RMR values were approximately the same or higher than those obtained by Jönsson *et al.*[21], implying in general that esterification of amino acids occurs at least as effectively in 3.5 *M* hydrochloric acid–*n*-propanol as in 8 *M* hydrochloric acid–*n*-propanol.

An important question is resolved by the comparison of packed column analysis with the capillary column analysis of the amino acid N-HFB, *n*-propyl esters of Jönsson *et al.*[21], namely the potential for derivative–column interaction. Such

TABLE II

PRECISION AND ACCURACY IN THE GLC ANALYSIS OF AMINO ACIDS AS THEIR N-HFB, *n*-PROPYL ESTER DERIVATIVES

Amino acid	*Added (mg)*	*Found* (mg)*	*Confidence limits*	*R.S.D.***	*Error (%)*
Alanine	32.0	30.3	±0.88	1.17	−2.6
Glycine	84.0	87.6	±2.31	1.06	+1.5
Valine	37.0	35.7	±1.74	1.96	—
Threonine	26.0	25.0	±0.88	1.41	−0.5
Serine	78.0	80.8	±5.69	2.83	—
Leucine	67.0	63.9	±2.88	1.81	−0.3
Isoleucine	28.0	25.3	±2.31	3.67	−1.4
Proline	100.0	107.5	±5.62	2.10	−1.9
Methionine	15.0	12.6	±0.95	3.02	−9.7
Aspartic acid	50.0	48.8	±1.04	0.86	−0.3
Phenylalanine	45.0	43.5	±0.43	0.40	−2.4
Glutamic acid	287.0	299.4	±6.87	0.92	+1.9
Lysine	30.0	26.9	±0.18	0.26	−9.7
Tyrosine	19.0	19.7	±1.28	2.61	—
Arginine	55.0	54.2	±2.07	1.54	—
Histidine	28.0	19.1	±7.70	16.22	−4.3
Cystine	19.0	18.0	±4.35	9.72	—

* Average of 3 determinations, based on calibration with a mixture containing equal weights of the amino acids.

** Relative standard deviation $= \frac{\text{standard deviation}}{\text{mean}} \cdot 100.$

TABLE III

COMPARISON OF AUTOMATED CIE AND GLC: PERCENTAGE RECOVERIES OF AMINO ACIDS FROM A SIMULATED WHEAT SAMPLE*

Amino acid	*CIE***		*GLC***
	Peak height	*Peak area*	
Alanine	102.2	105.5	95.2
Glycine	94.5	91.8	101.2
Valine	99.6	101.9	97.2
Threonine	98.3	104.6	96.9
Serine	90.9	89.8	102.3
Leucine	95.8	94.6	95.9
Isoleucine	100.9	95.0	92.3
Proline	96.9	94.9	106.6
Methionine	102.3	101.7	84.7
Aspartic acid	99.0	95.1	97.8
Phenylalanine	93.7	92.3	96.6
Glutamic acid	96.0	91.4	103.8
Lysine	103.0	109.3	89.5
Tyrosine	98.4	97.4	102.4
Arginine	104.8	113.1	99.1
Histidine	111.2	112.7	73.2
Cystine	105.8	112.6	99.2

* Sample made up to contain proportionately identical amounts of amino acids to those listed in Table II.

** Two determinations.

TABLE IV

COMPARISON OF AUTOMATED CIE AND GLC: SEQUENTIAL CALIBRATION ON DIFFERENT DAYS*

Amino acid	*Relative standard derivation*	
	CIE	*GLC*
Alanine	2.80	7.62
Glycine	3.56	3.91
Valine	4.80	2.23
Threonine	2.51	1.64
Serine	1.92	1.65
Leucine	2.86	2.41
Isoleucine	3.21	3.46
Proline	11.70	2.00
Methionine	-- **	5.53
Aspartic acid	3.47	0.90
Phenylalanine	3.96	1.41
Glutamic acid	2.47	1.92
Lysine	3.07	1.35
Tyrosine	4.98	2.33
Arginine	2.52	4.90
Histidine	3.33	12.59
Cystine	-- **	8.54

* Ten determinations.

** Available data did not include figures for methionine and cystine.

interactions, occurring principally between polar liquid phases and derivatives of the basic amino acids plus cystine, have complicated the search for a single packed column method of analyzing amino acids by GLC, because the best separations usually occur on polar columns. But, as it is extremely unlikely that derivative–column interaction occurred in the capillary column used by Jönsson *et al.*[21], and as lysine, arginine and cystine yielded similar RMR values on both types of column, it appears that derivative–column interaction did not take place in the SE-30 packed column used in this study.

The RMR for histidine obtained on repeated calibration with an S1 standard was poorly reproducible (Table IV). Because the peak for di-N-HFB histidine failed to separate from that for glutamic acid, histidine was analyzed as the putative[21] 3- or 5-N-acetyl-*n*-N-HFB derivative. On-column conversion into the desired compound was effected by simultaneous injection of acetic anhydride, without prior removal of heptafluorobutyryl anhydride (HFBA) and acetonitrile from the sample. Although Moss *et al.*[19] recommended the latter step, no evidence of the di-N-HFB histidine could be discovered in chromatograms when evaporation was omitted.

The molar response of the presumed mono-N-HFB, N-acetyl histidine derivative relative to norleucine was determined as 0.47, compared with 0.72 for the corresponding di-N-HFB derivative analyzed by Jönsson *et al.*[21]. As carbonyl carbon atoms and trifluoromethyl groups do not contribute to the output of the flame ionization detector[22], the replacement of a heptafluorobutyryl group by an acetyl group should slightly augment the histidine response. On the other hand, injection of the di-N-HFB derivative with acetic anhydride may have led to the removal of

TABLE V

AUTOMATED CIE DETERMINATION OF PROTEIN AMINO ACIDS IN SORGHUM* GROWN UNDER TREATMENT WITH DIFFERENT AMOUNTS OF NITROGEN AND SULPHUR FERTILIZER

Amino acid	*Nitrogen (mg/pot)*											
	50				*150*				*450*			
	Sulphur (mg/pot)											
	10	*30*	*100*	*300*	*10*	*30*	*100*	*300*	*10*	*30*	*100*	*300*
Alanine	6.4	6.6	5.7	7.1	9.8	8.7	8.7	10.5	10.9	13.9	13.9	16.9
Glycine	2.6	2.6	2.4	2.9	3.2	3.0	3.0	2.6	2.9	3.6	3.9	4.8
Valine	2.6	2.6	2.3	3.0	3.5	3.3	3.7	3.7	4.2	5.9	6.3	7.0
Threonine	2.4	2.4	2.1	2.6	2.9	2.9	3.1	3.1	3.3	4.3	4.5	5.0
Serine	3.6	3.6	3.4	4.0	5.3	4.6	4.8	5.3	5.3	6.7	6.9	8.4
Leucine	8.1	8.1	7.6	9.4	12.3	11.5	12.1	14.4	15.2	19.9	—	24.4
Isoleucine	1.8	1.8	1.6	2.1	2.6	2.4	3.1	2.9	3.7	4.7	4.5	5.5
Proline	5.8	6.9	5.5	6.9	8.3	8.1	9.0	8.5	8.3	12.0	11.0	17.5
Methionine**	1.6	1.6	1.6	1.3	1.9	1.6	1.8	1.8	1.3	2.1	2.2	2.5
Aspartic acid	4.8	4.8	4.5	5.1	6.7	5.9	6.4	—	14.1	9.3	9.3	13.6
Phenylalanine	3.3	3.3	3.3	3.6	4.6	4.0	5.0	5.6	5.3	7.3	7.6	8.6
Glutamic acid	14.7	13.5	12.7	15.6	20.6	19.4	19.7	—	25.0	31.7	—	38.5
Lysine	1.5	1.7	1.5	2.1	2.1	1.5	2.1	2.3	2.3	2.3	2.6	3.8
Tyrosine	2.5	2.9	2.5	2.9	3.3	3.6	3.6	4.0	4.0	5.8	5.8	6.9
Arginine	2.4	2.8	2.4	3.1	3.1	2.8	3.5	3.5	—	3.8	4.5	6.6
Histidine	1.6	1.6	1.6	2.2	2.2	1.9	2.2	2.2	2.5	3.7	2.8	5.3
Cystine**	1.7	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.2	2.2	2.2	2.7
Total	67.4	68.6	62.5	75.7	94.2	87.0	93.6	98.3***	113.0	139.2	139.6***	178.0

* Milligrams of amino acid per gram of sorghum meal.
** Determined as methionine sulphone and cysteic acid in performic acid-oxidized samples.
*** Includes estimated values for missing data.

one heptafluorobutyryl group without replacement, in a manner analogous to the production of mono-N-trifluoroacetyl histidine for GLC analysis, which was described by Roach *et al.*[23]. In this case, no change in RMR would be expected. The argument may be approached from a more theoretical standpoint by assuming a weight per ionizable carbon for histidine of 25.9 and 16.4 for norleucine, based on expected detector output for their N-HFB, *n*-propyl derivatives. This gives a molar response for histidine relative to norleucine of 0.74, approximately equal to the experimental value obtained by Jönsson *et al.*[21]. Low experimental RMR values obtained in this study, considered in conjunction with poor reproducibility, make the method at present unsatisfactory for histidine analysis.

Precision and accuracy of GLC analysis

Analysis of standard S2, a simulated wheat grain sample, was used to provide data on accuracy and precision of analysis (Table II), based on calibration with an S1 standard containing equal weights of all the amino acids, including norleucine. Standard deviations, relative standard deviations and confidence limits at the 95% significance level were calculated for each amino acid. An error could be inferred when the range of the value found did not include the true value. The percentage

TABLE VI

GLC ANALYSIS OF PROTEIN AMINO ACIDS IN SORGHUM* GROWN UNDER TREATMENT WITH DIFFERENT AMOUNTS OF NITROGEN AND SULPHUR FERTILIZERS

Amino acid	*Nitrogen (mg/pot)*											
	50				*150*				*450*			
	Sulphur (mg/pot)											
	10	*30*	*100*	*300*	*10*	*30*	*100*	*300*	*10*	*30*	*100*	*300*
Alanine	7.5	6.7	6.1	7.7	9.9	9.1	9.6	9.0	12.2	16.3	16.6	19.8
Glycine	2.2	2.7	1.7	2.2	2.4	1.9	2.6	2.5	2.4	3.8	3.9	3.9
Valine	2.2	2.2	2.0	2.7	3.2	3.3	3.5	3.4	3.3	5.6	5.5	7.1
Threonine	2.3	2.2	2.0	2.5	2.6	2.7	3.0	2.9	3.5	4.4	4.4	5.3
Serine	3.6	3.6	3.3	4.1	4.6	4.5	4.9	4.8	5.9	7.5	7.6	8.8
Leucine	9.0	8.5	7.6	9.5	13.3	12.0	12.1	11.7	16.0	20.8	21.2	27.1
Isoleucine	1.5	1.6	1.6	2.1	2.3	2.1	2.5	2.4	3.1	4.3	4.2	4.7
Proline	6.5	6.8	5.4	6.8	8.8	7.8	8.4	8.1	9.2	13.5	13.6	16.2
Methionine**	0.9	1.0	0.7	1.0	1.0	1.0	1.2	1.5	1.1	1.6	1.8	2.2
Aspartic acid	5.0	5.2	4.4	5.8	6.3	5.9	6.7	6.2	16.7	10.4	10.8	12.6
Phenylalanine	3.4	3.4	2.8	3.8	5.2	4.2	4.6	4.4	5.8	7.6	8.0	9.6
Glutamic acid	17.0	15.5	13.7	17.8	22.2	20.6	21.3	21.1	27.6	34.7	36.8	43.3
Lysine	1.0	1.2	1.1	1.4	1.3	1.4	1.5	1.4	1.7	2.2	2.0	2.4
Tyrosine	2.9	2.8	2.6	3.1	3.8	3.6	3.6	3.5	4.3	5.3	6.0	7.4
Arginine	1.6	2.2	1.7	2.1	2.7	2.3	2.4	2.4	3.1	3.6	3.3	4.8
Histidine	—	1.1	1.1	—	1.6	—	—	—	1.0	—	—	1.8
Cystine**	1.5	1.6	1.9	1.4	1.6	1.5	1.3	1.8	1.4	1.6	2.1	2.5
Total	68.1	68.3	59.7	74.0	92.8	83.9	89.2	87.1	118.3	143.2	147.8	179.5

* Milligrams of amino acid per gram of sorghum meal, duplicate hydrolyses.
** Determined *per se* in 6 *N* hydrochloric acid hydrolyzates.

error is taken as the difference at the extreme of the range between the value found and the true value.

It is clear from Table II that good reproducibility does not predicate freedom from error in a result based on several analyses, as exemplified by lysine. Nor does good accuracy necessarily infer high precision (cystine). Data for histidine analysis were dismissed because of both poor accuracy and poor reproducibility. Except for methionine and lysine, the accuracy of the remaining results was regarded as acceptable, although most of the amino acids present in lower concentrations than in the calibration standard gave slightly low recoveries, whereas those in higher concentrations gave slightly higher values than expected. This phenomenon may, although improbably, have resulted from poor detector linearity. An alternative, more likely, explanation is that complete conversion of amino acids into their corresponding derivatives did not occur when relatively small amounts were present in the original sample. The hypothesis is supported by interpretation of data given by Jönsson *et al.*[21], who found low recoveries for methionine (−16.8%) and lysine (−27.2%), as well as for some other amino acids, on calibration with an eight-fold concentrated standard. It is possible that reaction inhibitors, such as moisture in the original sample or heptafluorobutyric acid in heptafluorobutyric anhydride, prevented complete derivatization at low amino acid concentrations. The resultant errors could, in

practice, be almost completely avoided by calibration with a standard of about the same composition as the sample type, a procedure that would be particularly useful in application to screening analysis conducted on large numbers of samples of a given type.

Discrepancies in recoveries for amino acids in low concentrations and erratic histidine results emphasize the desirability of continuing the investigations on the chemistry of derivatizing the amino acids to their N-HFB, *n*-propyl esters.

Comparison of automated CIE and GLC

Evaluation of a new procedure for amino acid analysis is not complete without some form of comparison with an established method. Accordingly, the Technicon AutoAnalyzer and GLC procedures were compared for accuracy (Table III), repeatability (Table IV) and application to sorghum analysis (Tables V and VI and Fig. 2). Sorghum was chosen as a test material because analyses were expected to show certain specific variations in amino acid content as the result of varied nitrogen and sulphur fertilization.

Although peak area measurement is usually carried out manually in CIE analysis, electronic integration has become the standard ancillary in GLC. In this respect, comparison of analytical results from CIE and GLC, as carried out in this study, was not strictly valid. Multi-channel electronic integration of a given chromatographic signal has shown[21] that the integrator accounts for only a small fraction of the total variability in results. On the other hand, estimates of relative standard deviation based on manual methods of peak area measurement are two to five times larger than those derived from electronic integration[21], assuming the same chromatographic contribution in each case. This leads to much greater variability in manual than in electronic techniques. It seems clear that, to some extent, the results in Tables III and IV will reflect this discrepancy.

Comparison of peak height and peak area methods of quantifying results from the Technicon AutoAnalyzer (Table III) suggested that the peak height method was

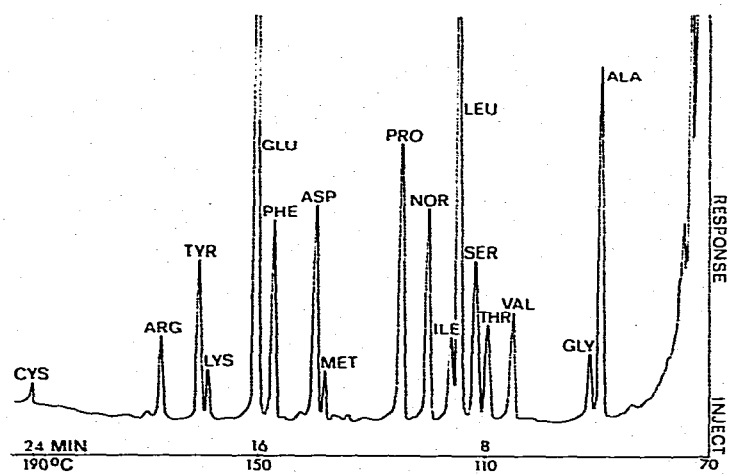

Fig. 2. GLC analysis of a sorghum meal hydrolyzate, from the 150 mg of nitrogen–30 mg of sulphur per pot treatment, cation-exchange cleaned: amino acid N-HFB, *n*-propyl esters.

superior. With one analysis completed each 76 min, peaks were sharp, and greater error derived from measurement of peak width than peak height. Analyses of amino acids in the simulated wheat grain standard (Table III) from peak height determinations in CIE, and integrator readings in GLC, produced comparable results. Five amino acids gave errors of greater than 5% in both instances. The suggestion made earlier with reference to GLC, *i.e.*, that calibration with a standard approximating the composition of the unknown would yield improved results, probably applies with equal validity to automated CIE analysis.

Determination of the variability implicit in sequential calibration with equimolar (CIE) or equiponderate (GLC) standards (Table IV) has limited application. The seemingly slight superiority of GLC over CIE probably relates to the difference in methods of peak area measurement. Calibration for proline in CIE evidently deserves special care. In GLC, alanine, methionine, arginine and cystine show rather high variability but, during the analysis of unknowns, conjunctive calibration reduces the variability to an acceptable level (Table II) for all amino acids except cystine and, of course, histidine. In general, it may be concluded that frequent calibration is desirable in both CIE and GLC, but is certainly more important for some amino acids than others.

The most desirable information stemming from a comparison of automated CIE and GLC concerns the identity of conclusions drawn from experimental data. In a typical test situation, the results of protein amino acid analysis conducted on sorghum meal by both automated CIE and GLC were used to determine the dependence of protein quality in sorghum on sulphur and nitrogen fertilization. In particular, conclusions were sought on changes in the relative content of lysine, methionine and cystine. Practical details of the sorghum experiment and its agronomic implications have been given elsewhere[25] together with a more rigorous treatment of results.

Most analyses were performed by CIE and GLC on identical samples taken from duplicate hydrolyzates. For methionine and cystine analysis by CIE, the traditional method for analyzing these amino acids as methionine sulphone and cysteic acid in performic acid-oxidized hydrolyzates was adopted. However, methionine and cystine were analyzed directly by GLC in hydrolyzates of samples that had undergone preliminary vacuum degassing with accompanying sonication[26].

Tables V and VI compare results for the protein amino acid analysis in sorghum obtained by CIE and GLC. Each value refers to milligrams of amino acid per gram of sorghum meal. Analysis of glutamic acid by CIE gave values lower than those from GLC, whereas results for lysine and arginine were considerably higher. Histidine only occasionally gave a measurable peak in GLC analysis. Amino acid totals from corresponding individual treatments were approximately the same, although somewhat lower in Table VI than in Table V for the 50 and 150 mg/pot nitrogen treatments.

Although Tables V and VI show differences in the absolute amounts of most amino acids determined by the two methods of analysis, both sets of data revealed a similar internal consistency. Conversion of data in the tables to per cent (w/w) content eliminated some irregularities, leaving lateral differences which denoted the effects of changing nitrogen and sulphur fertilization on protein quality. This is made more explicit in Tables VII and VIII for the amino acids lysine, methionine and cystine.

TABLE VII

METHIONINE, LYSINE, AND CYSTINE PERCENTAGES IN SORGHUM PROTEIN, AVERAGED FOR ALL SULPHUR-TREATMENTS AT GIVEN FIXED LEVELS OF NITROGEN, AND FOR ALL NITROGEN-TREATMENTS AT FIXED LEVELS OF SULPHUR

Amino acid	*Amino acid in sorghum protein (%)*													
	*Determined by CIE**							Determined by GLC**						
	N-level			*S-level*				*N-level*			*S-level*			
	50	*150*	*450*	*10*	*30*	*100*	*300*	*50*	*150*	*450*	*10*	*30*	*100*	*300*
Methionine	2.25	1.86	1.40	1.83	1.87	2.03	1.63	1.35	1.35	1.10	1.10	1.27	1.33	1.33
Lysine	2.47	2.15	1.92	2.17	1.97	2.17	2.43	1.78	1.63	1.45	1.50	1.67	1.73	1.64
Cystine	2.60	1.93	1.45	1.83	2.10	2.13	1.90	2.42	1.80	1.28	1.73	1.77	2.03	1.80

* Methionine and cystine determined in performic acid hydrolyzates.
** Methionine and cystine determined in 6 *N* hydrochloric acid hydrolyzates.

Data from CIE analysis or from GLC led to essentially the same conclusions. Amino acid percentages (Table VII) for all sulphur treatments at given fixed levels of nitrogen (columns 2–4 and 9–11) indicated that the relative contents of methionine, cystine and lysine decreased with increasing nitrogen fertilizer application. Amino acid percentages averaged for all nitrogen treatments at given fixed levels of sulphur (columns 5–8 and 12–15) indicated that, in general, the percentage of methionine, lysine and cystine in sorghum protein increased with increasing sulphur application up to 100 mg per pot. The data in Table VIII specify these conclusions more clearly for methionine and cystine. At any given nitrogen level, 100 mg of sulphur per pot produced the most desirable ratio of methionine + cystine in total protein.

TABLE VIII

PERCENTAGE CONTENT OF METHIONINE + CYSTINE IN SORGHUM PROTEIN, DETERMINED BY CIE AND GLC

*Nitrogen (mg)**	*Sulphur (mg)**	*Methionine + cystine*	
		*CIE***	*GLC****
50	10	4.9	3.5
	30	4.9	3.9
	100	5.5	4.4
	300	4.1	3.3
150	10	3.9	2.9
	30	3.9	3.0
	100	3.8	3.2
	300	3.6	3.5
450	10	2.2	2.1
	30	3.1	2.2
	100	3.2	2.6
	300	2.9	2.6

* Nitrogen and sulfur in milligrams per pot of growing sorghum.
** Determined in performic acid hydrolyzates.
*** Determined in 6 *N* hydrochloric acid hydrolyzates.

Measurement of methionine and cystine in 6 *N* hydrochloric acid hydrolyzates yielded results consistent with those obtained from performic acid-oxidized hydrolyzates. Although methionine values in 6 *N* hydrochloric acid hydrolyzates were apparently low, this may have been the result of incomplete derivatization, or the reaction of methionine with a specific component of sorghum meal, for example tannin. Details of an effective technique for vacuum degassing and sonication of biological samples, permitting complete recovery of methionine and cystine, are given in a recent paper by Gehrke's group[27].

As inferred previously, calibration with an amino acid standard similar in composition to sorghum protein would have led to improved accuracy in the GLC analysis of other amino acids. But the evidence suggests that, in the sorghum experiment, impaired accuracy in no way prejudiced the conclusions drawn. The practising chromatographer sometimes errs in assuming that high accuracy is an invariable and necessary premise of sound investigation.

CONCLUSIONS

GLC analysis of amino acids as their N-HFB, *n*-propyl esters constitutes a single packed column technique which is simple in execution, rapid and precise. Improved accuracy can be attained by calibration with a standard similar in composition to the unknown. Further work is evidently required to specify exactly the derivatization conditions capable of giving quantitative results over a wider linear range of amino acid concentration, particularly for histidine. Because of poor results for histidine, this method cannot at present be recommended as a satisfactory alternative to that based on the GLC analysis of amino acid N-trifluoroacetyl, *n*-butyl esters, except when dual column instrumentation is not available.

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