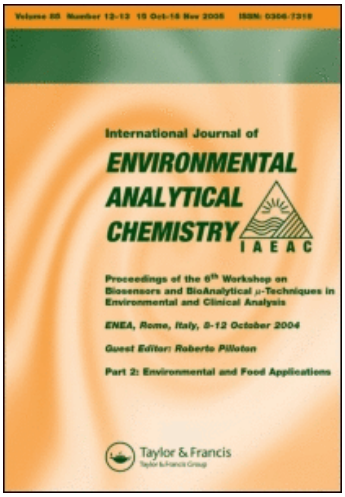

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Gas Chromatography of Amino Acids as their N-Trifluoroacetyl Methyl Esters and Comparison with the Ion-exchange Method in Maize and Oats†

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A method for the preparation of *N*-trifluoroacetyl methyl esters of amino acids has been developed, permitting the separation and determination of 20 amino acids by temperature-programmed gas chromatography on two columns. The method was applied to the analysis of the amino acids in hydrolyzed maize and in hydrolyzed oats, where a direct comparison was made with the ion-exchange method.

INTRODUCTION

The literature on the conversion of amino acids to volatile derivatives and their subsequent separation and estimation by gas chromatography has been reviewed by Weinstein,[1] by McBride and Klingman,[2] and by Blau,[3] and thus no extensive review will be undertaken here. Only a small proportion of the work reported was carried out using the *N*-trifluoroacetyl (*N*-TFA) methyl esters. Recently, Darbre and Islam[4] have reported on the preparation of these derivatives and Islam and Darbre[5] on the development of a mixed stationary phase for their separation.

We have studied the methods used in the preparation of the *N*-TFA methyl esters of the amino acids and the application of the gas chromatographic analysis to samples of maize and oats.

† Contribution Number 705.

MATERIALS AND METHODS

Apparatus

Most of the work was carried out using an F & M 700 gas chromatograph which was modified so as to shorten the distance from the ends of the columns to the flame ionization detectors.

Materials

Amino acids were purchased from the British Drug Houses and from the National Biochemical Company, and trifluoroacetic anhydride (TFAA) from Eastman-Kodak. Methanol was dried by reaction with magnesium turnings and then distilled. Methylene chloride was reagent grade from Fisher Scientific, thionyl chloride was purified by distillation from boiled linseed oil and dimethyl sulfite was used as received from Aldrich. HCl gas was used as purchased from Matheson. Norleucine was methylated and trifluoroacetylated and purified by distilling under reduced pressure (80°/0.7 mmHg).

Anal. Calc. for $C_9H_{14}O_3NF_3$ C:44.81, H:5.85. Found C:44.75, H:5.71. A 0.603 g portion was dissolved in 25 ml of methylene chloride containing ca. 0.5 ml TFAA to prevent hydrolysis.

Neopentylglycol succinate (NPGS) was obtained from Analabs and OV-17 from Applied Science Laboratories, Inc.

Columns

Glass columns were custom-built to fit in the oven of the F & M 700 so as to run directly to the $\frac{1}{8}''$ fittings which lead to the flame ionization detectors. Two columns were filled as follows: (1) A $4\frac{1}{2}' \times \frac{1}{8}''$ column with 1.1% NPGS on 80/100 Chromosorb G(AW, DMCS) and (2) A $7\frac{1}{2}' \times \frac{1}{8}''$ column with 3.3% OV-17 on 80/100 HP Chromosorb G, (AW, DMCS).

The stationary phases were prepared by the filtration method, i.e. the solid support, suspended in a solution of the liquid phase in an appropriate solvent, was filtered. The damp filter-cake was dried using an HI-EFF fluidizer (Applied Science Laboratories, Inc.) and the columns were packed under pressure, using a Column-Pac (Illinois Instrument Group, Des Plaines, Illinois) and gentle tapping. Columns were conditioned at 220° for 48 hr before use.

Preparation of methyl esters

Mixtures of amino acids (100 mcmole of each) and hydrolyzates were methylated by refluxing for 30 min with 20 ml methanol saturated with HCl gas and with the addition of 2 ml dimethyl sulfite.

Acylation

A solution of 3 ml TFAA and 1 ml methylene chloride containing the internal standard, *N*-TFA norleucine methyl ester, was added to the methyl ester hydrochlorides which had been evaporated to dryness. This was heated at 150° in a sealed tube for 6 min. In each case, a portion of the resulting solution was injected into the gas chromatograph.

Measurement of peak areas

The areas under the peaks on the chromatograms were measured by triangulation, by the use of a planimeter, or, in a few cases, by using a Dupont Curve Resolver. The areas were compared with the area of an internal standard.

Preparation of maise samples

The dry maize kernels were ground in a hammer mill to pass 20 mesh. A 1-g portion was added to 500 ml of 6N HCl and nitrogen was bubbled through the solution to remove oxygen. The solution was refluxed with stirring for 20 hr under N_2. The resulting hydrolysate was filtered and reduced to dryness on the rotary evaporator at 60°. The residue was transferred to a 100-ml flask and methylated. The methylated amino acid hydrochlorides were extracted three times with petroleum ether to remove any fatty acids and then they were acylated as described above. A 2-3 mcl portion of this was injected into the gas chromatograph.

Preparation of oats samples

Oats were ground to pass 20 mesh and a 2-g portion was extracted in a Goldfish apparatus for 4 hr with petroleum ether (b.p. 40–60°C), then it was added to 500 ml of 6N HCl and hydrolyzed as above; it was filtered, reduced to dryness, treated with 10 ml methanol and 15 ml benzene, and again concentrated to dryness. The residue was dissolved in 100 ml of water and 50 ml was put aside for analysis by ion exchange. The remaining 50 ml was again reduced to dryness, methylated, acylated, and analyzed as above.

RESULTS AND DISCUSSION

Columns

Using NPGS on silanized Chromosorb G allowed us to measure histidine but not cystine or arginine (Figure 1A). These could be determined using OV-17 on Chromosorb G, although many of the earlier peaks were not separated on this column (Figure 1B). As a result, in order to analyse all these

amino acids it was necessary to use both columns. The OV-17 column was used starting at a higher temperature and programming somewhat faster so as to bring out the desired peaks more rapidly (Figure 2).

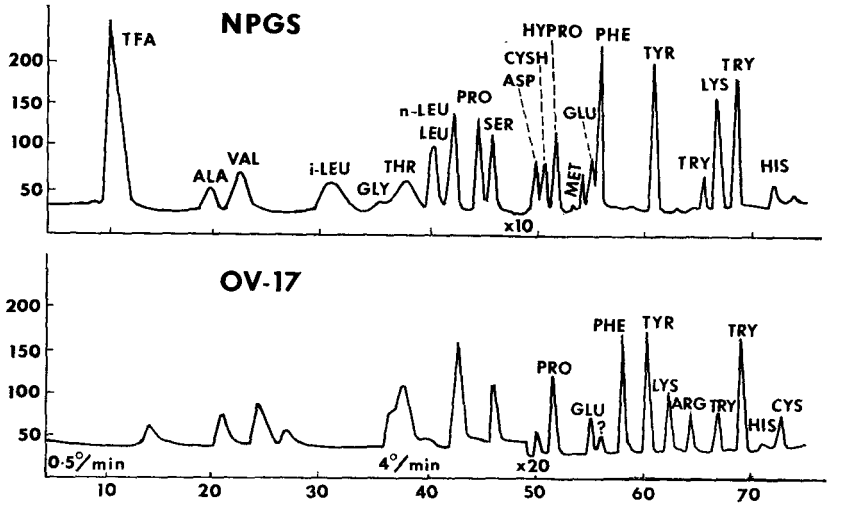

FIGURE 1 Separations of amino acids as their *N*-TFA methyl esters on columns containing (A) 1.1% NPGS on 80/100 Chromosorb G (AW, DMCS), 4.5′ × ⅛″; (B) 3.3% OV-17 on 80/100 HP Chromosorb G (AW, DMCS), 7.5′ × ⅛″.Temperature-programmed from 70° to 230°.

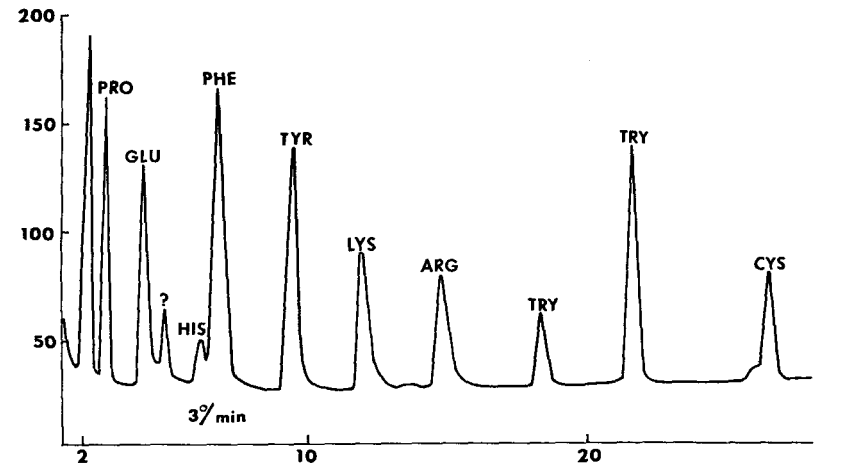

FIGURE 2 Separation of amino acids. Column as in Figure 1B. Temperature-programmed from 165° to 240°.

Derivatization

The methylation procedure used was similar to that of Cruickshank and Sheehan.[6] Trifluoroacetylation of amino acids is most conveniently carried out after methylation and using TFAA which is readily available commercially. The procedure used was essentially that of Gerhke and Stalling.[7]

Tryptophan gave two peaks on all the columns tried. The ratio of the two peaks depended partly on the method of preparation of the TFA derivatives. With an excess of tryptophan or in the presence of other amino acids a ratio of 80:20 could result, compared to ratios of 94:6 for preparations using an excess of TFAA.

Response factors

Norleucine could be readily resolved on the columns we were using and since it does not normally occur either in maize or in other cereals, it was adopted as the internal standard. Taking the response for the *N*-TFA methyl ester of norleucine as 1.00 the molar response factors of the other amino acids were determined and are listed in Table I. Note that the responses for isoleucine, leucine, and norleucine are the same, as would be expected.

TABLE I

Molar response factors for amino acids as their *N*-TFA methyl esters (Analytical conditions as indicated in Figures 1 and 2)

Amino acid	Molar response factor	Amino acid	Molar response factor
Alanine	0.43	Hydroxyproline	0.83
Valine	0.89	Methionine	0.50
Isoleucine	1.00	Glutamic acid	0.60
Glycine	0.20	Phenylalanine	1.59
Threonine	0.80	Tyrosine	1.45
Leucine	1.00	Arginine	0.77
Norleucine	1.00	Lysine	0.90
Proline	0.82	Tryptophan	1.56
Serine	0.54	Histidine	0.31
Aspartic acid	0.58	Cystine	0.59
Cysteine	0.49		

Histidine

Difficulties with the analysis of histidine have been encountered by other workers and have been reviewed by Roach *et al.*[8] We have found also that,

e.g., when histidine was analyzed on an OV-17 column, slow temperature programming gave a peak following tryptophan (Figure 1B at 72 min) which was most likely due to the mono-acylated derivative,[9,10] whereas when the initial temperature of the column was 165° and with rapid programming (Figure 2), histidine appeared at 6 min, just before phenylalanine. This is assumed to be due to the diacylated derivative. On the column containing NPGS on HP Chromosorb G, histidine appeared as a sharp peak when the column was new, but as the column aged the peak showed more and more tailing. Moreover, when using a standard containing only histidine in pure TFAA, the earlier peak, due to the diacylated derivative, was obtained. With added water or in the presence of other amino acids the latter peak, which is probably the monacylated derivative, appeared.

Cystine

Cystine gave no peak on columns containing NPGS on a sylanized support. On a short column containing NPGS on unsylanized supports peaks were obtained at first, but as the column aged the peaks disappeared.

On columns containing OV-17 on sylanized Chromosorb G, good peaks could be obtained if the initial temperature of the column was not too high and if the residence time on the column was not too long. With conditions as shown in Figure 2, reproducible cystine values could be obtained.

Arginine

There are conflicting reports in the literature on arginine. We were unable to detect arginine on NPGS columns, although when using short columns a peak could be obtained initially, which disappeared as the column aged. On column 2 (OV-17) after good conditioning, reproducible peaks were obtained for arginine.

Maize analysis

The gas chromatographic method for the determination of amino acids was applied to samples of maize. The curves are shown in Figure 3 and the results are listed in Table II, where they are given in terms of grams of individual amino acids per 100 g of protein. For purposes of comparison Table II lists values reported by Mertz *et al.*[11] and Nelson *et al.*[12] on samples of normal and opaque-2 maize, which were obtained by ion-exchange chromatography of hydrolyzed endosperm. These methods generally seemed to give higher values for most of the amino acids, although the amounts are relatively similar. Since only the endosperm, which appears to have a lower content of crude

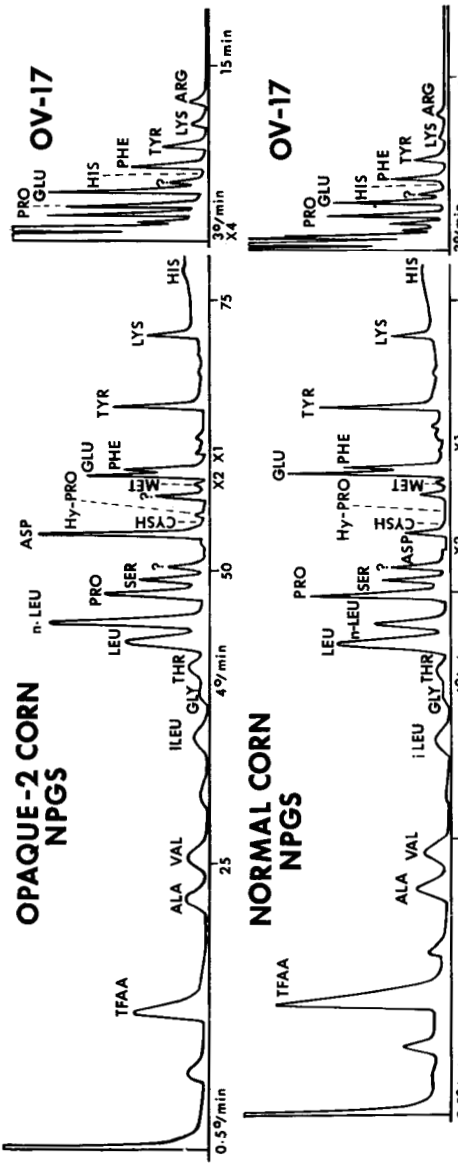

FIGURE 3 Analysis of amino acids in hydrolysates of opaque-2 and normal maize. Columns as in Figure 1; temperature-programmed from 70° to 230°, oven-cooled to 160° and programmed to 230°.

protein, was used, some differences between the ion-exchange method they employed and our gas chromatographic method might be expected. However, the wide variation of results between samples even using the same method, casts suspicion on the validity of comparing two different methods unless the same hydrolyzates are used.

Opaque-2 is a variety of maize which is nutritionally superior to normal maize since it contains larger amounts of lysine and tryptophan.[11,12] The latter is destroyed during acid hydrolysis and was not determined, but the gas chromatographic analysis confirms that lysine is present in larger amounts in the opaque-2 maize.

TABLE II

Amino acids in normal and opaque-2 maize (expressed as grams per 100 g of crude protein)

Amino acid	GLC method[a]		Mertz *et al.*[11]		Nelson *et al.*[12]	
	Normal	Opaque-2	Normal	Opaque-2	Normal	Opaque-2
Alanine	5.2	4.3	8.1	7.0	10.1	7.2
Valine	2.9	3.1	4.7	5.0	5.4	5.3
Isoleucine	2.3	2.1	3.8	3.9	4.5	3.9
Glycine	2.1	2.7	3.2	4.0	3.0	4.7
Threonine	2.0	2.6	3.5	3.9	3.5	3.9
Leucine	9.9	6.3	14.3	11.6	18.8	11.6
Proline	6.9	5.3	9.7	9.4	8.6	8.6
Serine	4.4	3.7	5.2	5.0	5.6	4.8
Aspartic acid	6.0	7.6	6.2	8.5	7.0	10.8
Cysteine	2.3	1.9	—	—	—	—
Hydroxyproline	1.8	3.5	—	—	—	—
Methionine	1.0	0.6	2.8	2.0	2.0	1.8
Glutamic acid	19.6	12.3	21.3	19.1	26.0	19.8
Phenylalanine	6.2	4.3	5.3	5.0	6.5	4.9
Tyrosine	4.9	2.7	5.3	4.7	5.3	3.9
Arginine	2.3	2.8	3.8	5.1	3.4	5.2
Lysine	1.3	2.4	2.0	3.4	1.6	3.7
Tryptophan[b]	—	—	—	—	0.3	0.7
Histidine	1.4	1.6	2.8	3.4	2.9	3.2
Cystine	1.3	0.8	1.8	2.4	1.8	(0.9)
Ammonia	—	—	3.3	3.4	—	—
Crude Protein (%)	13.7	16.0	8.69	8.69	12.7	11.1

[a] Conditions as listed in Figure 3.

[b] Tryptophan is destroyed during acid hydrolysis.

TABLE III

Amino acids in oats by ion-exchange and gas chromatographic methods
(Expressed as percent of total amino acids)

Amino acids	Ion-exchange methods								Gas chromatography method			
	Graham				Sowden							
	Stormont	OA123–1	Rodney	OA123–17	Stormont	OA123–1	Rodney	OA123–17	Stormont	OA123–1	Rodney	OA 123–17
Aspartic acid	8.3	(3.7)	8.5	8.8	8.5	8.9	8.8	8.5	5.7	5.4	7.2	7.2
Threonine	3.2	3.4	3.2	3.3	3.3	3.6	3.4	3.5	3.3	2.8	2.7	2.8
Serine	4.8	5.0	4.5	4.7	4.6	5.0	4.7	4.8	5.2	6.2	5.4	4.6
Glutamic acid	23.3	24.0	23.3	22.5	22.3	21.6	21.7	21.9	17.0	20.8	17.4	17.1
Proline	4.9	5.2	4.9	5.1	5.5	5.5	5.5	5.5	3.9	4.3	4.6	4.9
Glycine	5.0	5.6	5.4	5.2	5.1	5.2	5.2	5.0	4.5	3.6	7.5	6.3
Alanine	4.8	5.2	5.1	4.9	4.8	4.7	4.9	4.8	5.7	4.8	7.0	6.7
Valine	5.5	5.9	6.2	5.5	5.5	5.5	5.8	5.5	17.5	16.1	15.5	13.7
Cystine	2.2	1.8	1.4	2.7	2.5	2.6	2.3	2.4	1.5	2.9	1.3	3.6
Methionine	1.6	1.6	1.5	1.5	1.7	1.6	1.7	1.8	0.5	0.8	0.7	1.0
Isoleucine	4.0	4.2	4.2	4.0	4.1	3.9	3.9	4.0	5.6	2.9	4.0	4.5
Leucine	8.0	8.3	8.0	8.0	8.2	8.0	8.1	8.2	7.6	7.8	7.1	7.7
Tyrosine	3.1	3.3	3.1	3.2	4.0	4.2	4.3	4.3	4.1	1.6	3.6	4.2
Phenylalanine	5.5	5.7	5.6	5.4	5.7	5.5	5.6	5.8	5.3	5.1	5.2	4.2
Amide	2.5	2.8	2.7	2.5	—	—	—	—	—	—	—	—
Lysine	3.6	4.0	3.7	3.7	4.3	4.5	4.2	4.3	4.7	6.1	3.9	4.3
Histidine	2.3	2.2	2.3	2.0	2.6	2.4	2.5	2.4	0.0	0.0	0.0	0.0
Arginine	7.5	7.8	6.3	6.9	7.2	7.2	7.8	7.5	7.9	8.7	6.9	7.2

Oats analysis

Table III shows the ion-exchange analyses of amino acids as carried out by two analysts in different laboratories and at different times.

The analyses listed in Columns 1–4 were carried out in 1966 by Graham whereas those listed in Columns 5–8 were done in 1968 by Sowden. The agreement between the two is quite remarkable, especially since different samples as well as different hydrolysates were used.

By contrast, Columns 9–12 of Table III show the results by gas chromatography, using exactly the same hydrolysates as were used to obtain the results in Columns 5–8. Quite large differences are evident especially with valine which seems to be about three times too high by gas chromatography.

This comparison of results shows that there are obvious short-comings with the gas chromatographic technique employed, one probably being the presence of artifacts which interfere with the measurement of the amino acid peaks. This problem was investigated, and on the basis of recovery of pure amino acid mixtures by passage through an ion-exchange column improvement could be anticipated if hydrolysates were separated from non-amino acids. However, this added an extra step to the procedure, introducing possible additional errors, and a further investment of time for the analysis.

The authors believe that at present these disadvantages are sufficient to discourage use of the gas chromatographic method on samples such as cereals, which contain many interfering substances. Great improvements have been made recently with the ion-exchange method. It is now possible to run many more samples per day and the initial cost of the analyzer has decreased, making ion exchange a much more attractive method. However, it is possible that the introduction of a specific detector, such as the thermionic nitrogen detector, might make the gas chromatographic method attractive enough for a further study.

CONCLUSIONS

The studies reported here have shown that the gas chromatographic method can be made to work using mixtures of pure amino acids. However, when samples such as hydrolysates of maize or oats are used, great caution in the application of the results must be exercised. If interference from artifacts is evident, then a clean-up step will definitely be required.

Acknowledgements

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References

1. B. Weinstein, in *Methods of Biochemical Analysis*, Vol. 14, edited by D. Glick (Interscience, New York, 1966), p. 203.
2. W. J. McBride, Jr. and J. D. Klingman, in *Lectures on Gas Chromatography* 1966, edited by L. R. Mattick and H. A. Szymanski (Plenum Press, New York, 1967), p. 25.
3. K. Blau, in *Biomedical Applications of Gas Chromatography*, Vol. 2, edited by H. A. Szymanski (Plenum Press, New York, 1968), p. 1.
4. A. Darbre and A. Islam, *Biochem. J.* **106**, 923 (1968).
5. A. Islam and A. Darbre, *J. Chromatogr.* **43**, 11 (1969).
6. P. A. Cruickshank and J. C. Sheehan, *Anal. Chem.* **36**, 1191 (1964).
7. C. W. Gehrke and D. L. Stalling, *Separation Sci.* **2**, 101 (1967).
8. D. Roach, C. W. Gehrke, and R. W. Zumwalt, *J. Chromatogr.* **43**, 311 (1969).
9. S. Makisumi and H. A. Saroff, *J. Gas Chromatogr.* **3**, 21 (1965).
10. C. W. Gehrke, R. W. Zumwalt, and L. L. Wall, *J. Chromatogr.* **37**, 398 (1968).
11. E. T. Mertz, L. S. Bates and O. E. Nelson, *Science* **145**, 279 (1964).
12. O. E. Nelson, E. T. Mertz, and L. S. Bates, *Science* **150**, 1469 (1965).

Note added in proof

Since this paper was submitted, papers by Zumwalt, Roach, and Gehrke [*J. Chromatog.*, **53**, 171-193 (1970)] and Zumwalt, Kuo, and Gehrke [*ibid.*, **55**, 267-280 (1971)] have appeared in which cereal hydrolyzates were analyzed successfully for amino acids by gas chromatography. In both, interfering substances were removed by the use of ion-exchange columns.