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Gas–liquid chromatography of thirty-five amino acids and two amino sugars

The value of gas chromatography for the qualitative and quantitative analyses of mixtures of protein amino acids is well known from the work of GEHRKE and others[1–4]. Either the N-trifluoroacetyl *n*-butyl ester or the trimethylsilyl derivatives[5] can be used to achieve volatility. In connection with current research on the accumulation of peat in the Everglades of Florida, we wished to determine not only the common protein amino acids, but also amino acid degradation products and the unusual amino acids found in muco-substances of bacterial cell walls. Certain amino acid fractions from peat in fact show well over thirty peaks. The purpose of this note is to report that such complex mixtures can be analyzed by GLC and to give specific details of the chromatographic behavior of those non-protein acids whose separation on the same column along with the protein amino acids has not previously been reported.

Experimental

Amino acids used in this work were purchased from Mann Research Laboratories and were found to be chromatographically pure. Reagents used for the derivatization technique were purchased from Regis Chemical Company. A Hewlett-Packard research chromatograph, model No. 5750, equipped with a dual hydrogen flame ionization detector, was used in this study.

The derivatization technique for protein amino acids, introduced by LAMKIN AND GEHRKE[1] in 1965, was used to prepare the protein and non-protein amino acids for gas chromatography as their N-trifluoroacetyl *n*-butyl esters. The procedure for derivatization is a three-step process: (1) a ½-h room temperature esterification with 1.25 *N* HCl in methanol, (2) a 2½-h interesterification at 100° with 1.25 *N* HCl in *n*-butanol, (3) a high temperature sealed tube acylation (150°) for 5 min. Each step must be done under strictly anhydrous conditions. ROACH AND GEHRKE have recently introduced a much faster derivatization procedure which involves a direct esterification with 3 *N* HCl in *n*-butanol[6].

TABLE I

CONDITIONS USED TO EFFECT SEPARATION OF AMINO ACIDS

Column liquid phase	0.325 w/w% EGA	1.0 w/w% OV-17
Column solid support	AW, heat-treated Chromosorb G	HP Chromosorb G
Hydrogen flow, ml/min	40	40
Nitrogen flow, ml/min	60	60
Air flow, ml/min	450	450
Initial temperature, °C	75	115
Post-injection interval, min	5	4
Programmed temperature operation, °C/min	4	6
Final temperature, °C	210	235
Upper limit interval, min	10	7
Detector temperature, °C	250	250
Injection port temperature, °C	200	200
Chart speed, in./min	0.5	0.5

In order to effect separation of the amino acids, two glass columns, 4 mm I.D. were needed; a 1.5-m column packed with 0.325 w/w% ethylene glycol adipate (EGA) on acid-washed (AW), specially heat-treated Chromosorb G (ref. 7), and a 1-m column packed with 1.0 w/w% OV-17 on high-performance (HP) Chromosorb G. Both packings can be obtained from Regis Chemical Company. Because we prefer single-column operation and because we often run the instrument at 10 × 2 (range × attenuation), we found it necessary to condition the EGA column at 210° for 48 h and the OV-17 column at 235° for 24 h.

Various parameters required for operation of the two columns are reported in Table I. Once conditioning is complete, both columns can be put into the same oven. As soon as the EGA column run is completed, the oven is cooled to 115° and the sample is injected onto the OV-17 column. Care must be taken not to subject the EGA column to temperatures above 235° for any extended period of time. Glass injection liners must be used at all times since some of the amino acids degrade on contact with hot metals[4].

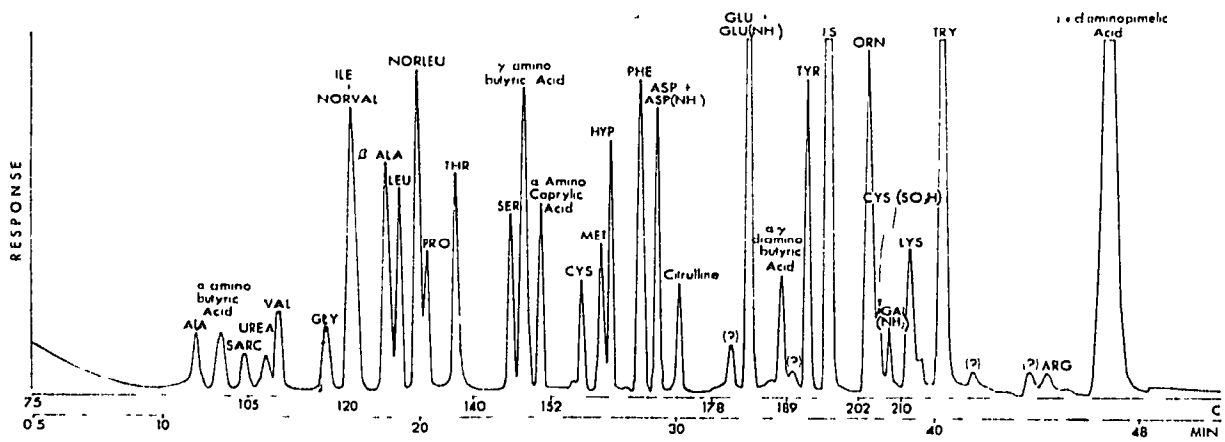

Fig. 1. Separation of amino acid N-TFA *n*-butyl esters. Liquid phase, 0.325 w/w % EGA, support, 80–100 mesh AW heat-treated Chromosorb G, column dimensions, 1.5 m × 4 mm I.D., glass; initial temperature, 75°, 4°/min, 10 × 8.

Results and discussion

The elution times and temperatures for the various amino acids investigated on the EGA and OV-17 columns are reported in Figs. 1 and 2. When thirty-three amino acids were injected on to the EGA column, thirty peaks resulted. The loss of three peaks resulted from the facts that: (1) isoleucine did not separate from norvaline and (2) asparagine and glutamine did not usually separate from their parent acids. A 6 *N* HCl hydrolysis is needed to liberate the acids from peat fractions, and this destroys most of the asparagine and glutamine; hence the problem of incomplete separation does not affect our work. It did seem that when the concentrations of glutamic acid and glutamine were similar, they could be separated. The same was true for asparagine and aspartic acid.

In some cases the resolution of urea and sarcosine was poor, but if a post injection interval of 5 to 10 min was used, they could be separated. The upper limit interval of 10 min was necessary when using the EGA column to effect separation of α,ε-diaminopimelic acid, an amino acid found solely in microorganisms.

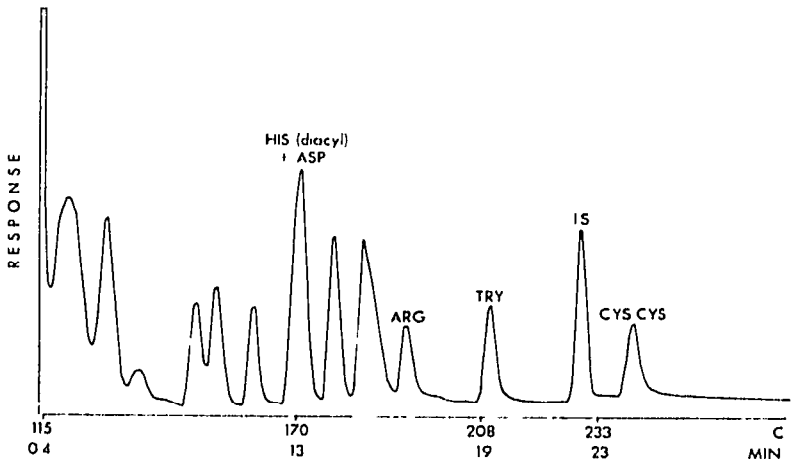

Fig. 2. Separation of amino acid N-TFA *n*-butyl esters. Liquid phase, 1.0 w/w % OV-17, support, 80–100 mesh HP Chromosorb G, column dimensions, 1.0 m × 4 mm I.D., glass, initial temperature, 115°, 6°/min, 10 × 16.

The only problem encountered using the OV-17 column concerned the analysis of histidine. Using the derivatization procedure of GEHRKE *et al.*[7], one obtains histidine as the diacyl derivative, which elutes with aspartic acid. If 7 μl of *n*-butanol are injected on to the column immediately after the sample injection, then the monoacyl derivative is formed on the column and is well separated from any other amino acid[8]. However, we prefer using the diacyl derivative since the quantitation of histidine as its monoacyl derivative is dependent on too many parameters that are hard to control[8]. Knowing the relative molar responses of aspartic acid on OV-17 and EGA columns and its amount from the chromatogram on the EGA column, one can calculate the amount of histidine present from the composite peak on OV-17 (ref. 9). If purely qualitative analysis is being done, then we should prefer forming the monoacyl derivative of histidine. Although tryptophan and arginine can be separated from the other amino acids on the EGA column, which could therefore be used for qualitative purposes, the quantitation of these two amino acids on this column has been poor since apparently there is some interaction with the column packing[8,10]. Therefore for quantitative determination, the OV-17 column must be used.

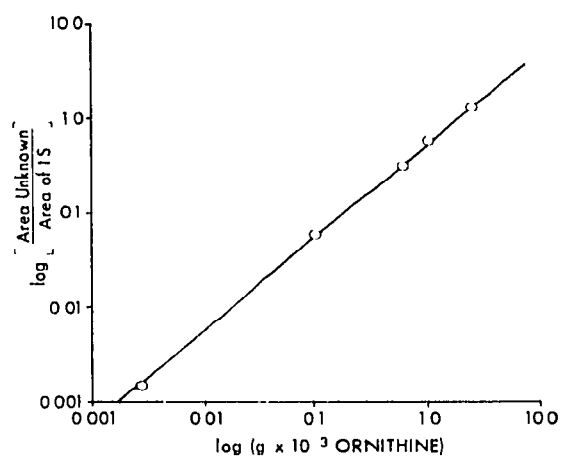

Fig. 3. Standard curve for derivatized concentrations of ornithine.

The excellent quantitative accuracy obtainable with the protein amino acids has already been demonstrated by GEHRKE *et al.*[4,7]. A full statistical study of the accuracy obtainable with the non-protein acids has not yet been carried out by us. However the calibration curve for ornithine, shown in Fig. 3, illustrates the linearity obtained with all the acids. Each point represents three independent experiments. As can be seen the points do fit a straight line. The maximum % standard deviation of any point on the curve is 4.2%.

It is interesting and potentially useful that galactosamine and glucosamine subjected to the same derivatization procedure as the amino acids and injected on to the EGA column gave distinct peaks, that of galactosamine appearing immediately after the elution of cysteic acid (Fig. 1), while that of glucosamine eluted with ornithine. Presumably the derivatives were 3,4,6-tri-O-trifluoroacetyl-N-trifluoroacetyl-1-O-methyl-2-amino-2-deoxy-hexoses.

If one should wish to determine the amino sugars in a mixture containing amino acids, it would be desirable to separate the amino sugars from the amino acids on an ion-exchange column before derivatization and GLC analysis. Although a prior ion-exchange separation is necessary if one is to determine glucosamine in the presence of amino acids, it is of practical convenience to use the same derivatization reagents and procedure for both classes of amino compounds. This possibility has not been previously reported.

The analysis of amino acids from hydrolyzates of peat fractions presents a number of problems to the investigator. The identification of the number of peaks by itself represents a formidable challenge. There are still three or four relatively small peaks in our chromatograms that have not yet been identified, so that further work may well define the chromatographic behavior of a number of additional acids. Using a gas chromatograph–mass spectrometer would of course facilitate the identification of these peaks.

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