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AN IMPROVED TECHNIQUE FOR THE GAS–LIQUID CHROMATOGRAPHIC SEPARATION OF THE N-TRIFLUOROACETYL *n*-BUTYL DERIVATIVES OF AMINO ACIDS*

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SUMMARY

A complete separation of the twenty protein amino acids and two internal standards in 35 min has been made possible by analyzing their N-trifluoroacetyl *n*-butyl (TAB) derivatives simultaneously on an ethylene glycol adipate (EGA) column and on an OV-17 column using the same temperature programme. The detector signal from the EGA column is recorded up to the point just after the elution of TAB-aspartic acid; thereafter, the signal from the effluent of the OV-17 column is recorded. The TAB derivative of norleucine is poorly resolved from TAB-leucine and TAB-proline. Therefore, α-aminocaprylic acid has been used as the internal standard instead of norleucine. TAB-α-aminocaprylic acid appears as a separate peak between TAB-serine and TAB-cysteine.

INTRODUCTION

Because of its speed, sensitivity and high precision, gas–liquid chromatography (GLC) could be the technique of choice for routine analysis of amino acid composition when a large number of samples have to be handled as, for instance, in plant breeding or clinical studies.

The experimental conditions for the quantitative preparation, and the chromatographic requirements for the separation, of N-trifluoroacetyl *n*-butyl (TAB) derivatives of amino acids have been described in a series of publications by Gehrke and coworkers[1–13]. The TAB derivatives of the amino acids were eluted quantitatively from two separate columns containing 0.65% (w/w) ethylene glycol adipate (EGA) on Chromosorb W and 1.5% (w/w) OV-17 on Chromosorb G. Use of the OV-17 column was necessary in order to elute arginine, histidine and cystine, which were not eluted

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from the EGA column. By this procedure a complete GLC analysis of the protein amino acids takes about 55 min, as one aliquot of the sample has first to be injected into the EGA column and, after the appearance of lysine, another aliquot injected into the OV-17 column.

Our aim during the present study was to adapt the standard analytical technique of Gehrke and co-workers[9-11] to a large number of samples from single plants and even single seeds, thus enabling a quick screening of both homozygous and heterozygous populations of Brassica seeds in order to estimate the genetic and environmental control of protein composition.

This paper deals with an improvement in the standard procedure, which enables the time for a single run of all the amino acids to be reduced to about 35 min. Two internal standards (norleucine and butyl stearate) are used. The substitution of α-aminocaprylic acid for norleucine as the internal standard is suggested. During the time our work was in progress, Gehrke *et al.*[7] published a modification of their earlier procedure with two simultaneous injections on two columns (EGA and OV-17/OV-210) with the same temperature programme. The detector signals were integrated and recorded simultaneously through two electronic integrators and a dual-pen recorder. The single-column procedure published later by Gehrke and Takeda[8] does not seem to give satisfactory separations of many of the amino acids.

EXPERIMENTAL

Reagents

The standard amino acids and the stearic acid were purchased from BDH (Poole, Great Britain) and were chromatographically pure. The standard amino acid solution (2.5 μmole/ml) was obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.) and α-aminocaprylic acid was purchased from Sigma (St. Louis, Mo., U.S.A.). Trifluoroacetic anhydride was an Eastman-Kodak (Rochester, N.Y., U.S.A.) product. Butanol and methylene chloride were obtained from BDH and were refluxed and redistilled before use as described by Gehrke *et al.*[9]. The dry hydrogen chloride was purchased from Matheson (Joliet, Ill., U.S.A.) and was passed through a tube loosely packed with phosphorous pentoxide before it was dissolved in the butanol.

Apparatus

A Varian Aerograph Model 2100-20 instrument, fitted with two flame ionization detectors, a dual differential electrometer and a linear temperature programming unit, was used for most of the experiments. The gas chromatograph was connected to a dual-pen recorder (Varian Aerograph, Model 20), a digital integrator (Varian, Model 480) and a teletype printer (TT4, Model 33-TBM) with a paper-tape punching mechanism. Trials with α-aminocaprylic acid were conducted on a Pye Unicam-104 chromatograph connected to a Hitachi Perkin-Elmer (QD15) recorder and a Vidar Autolab 6300 digital integrator.

Chromatographic columns

The EGA column (1000 mm × 4 mm I.D.) was prepared by gently filling the glass U-tube with packing material, which was prepared and conditioned as described by Gehrke and co-workers[10,11]. The packing contained 0.65% (w/w) stabilized ethylene

glycol adipate (EGA) (Analabs, North Haven, Conn., U.S.A.), on 80–100-mesh Chromosorb W HP (Johns Manville, Denver, Colo., U.S.A.) heated at 140° for 12 h before impregnation. The OV-17 column (2000 mm × 4 mm I.D.) was prepared and conditioned as described by Gehrke *et al.*[9]. The packing contained 1.5% (w/w) OV-17 (Analabs) coated on 80–100 mesh Chromosorb G.

Preparation of derivatives

Aliquots of the standard amino acid solution were "spiked" with known amounts of norleucine, α-aminocaprylic acid and butyl stearate, the last of which was prepared by HCl-catalyzed esterification of stearic acid in butanol. The sample was evaporated to dryness *in vacuo* at room temperature and derivatives were prepared according to the method of Roach and Gehrke[12].

RESULTS AND DISCUSSION

The mode of operation of the dual-column system and the signal flow are shown in Fig. 1. The EGA column was connected to the detector A and the OV-17 column to detector B, both detectors being connected through the dual-channel electrometer to the digital integrator, potentiometer recorder and teletype printer. By this arrangement, signals from each of the detectors could be carried to the integrator and the recorder, as and when desired, by changing the mode selector position between A and B. This is currently done manually, but a simple timer to control this switch is to be manufactured.

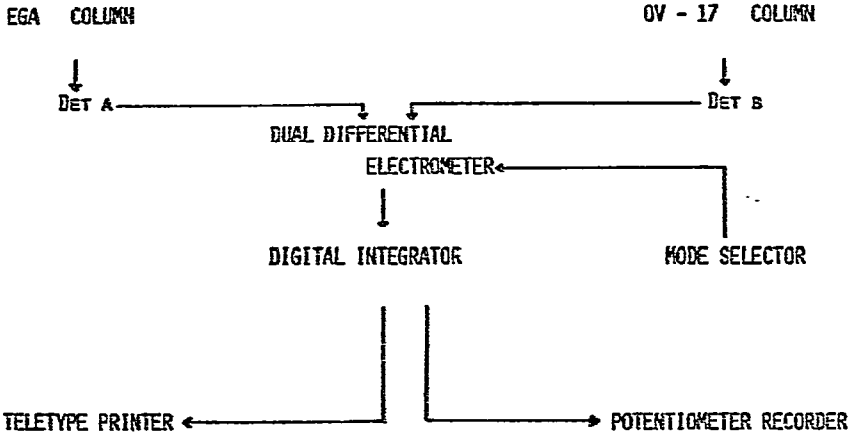

Fig. 1. Schematic diagram showing the flow of signals from the detectors to the recorder and printer.

First, a standard sample was injected into the EGA column and then, after a complete run, into the OV-17 column. The OV-17 column was deliberately made longer than that used in the standard Gehrke procedure in order to lengthen the elution time of the TAB derivatives by an increment large enough to achieve simultaneous elution of TAB-aspartic acid on both columns. By comparing the chromatographic charts (Figs. 2 and 3), one could determine the time at which the mode selector should preferably be operated. Changing the mode selector from A to B after the elution of

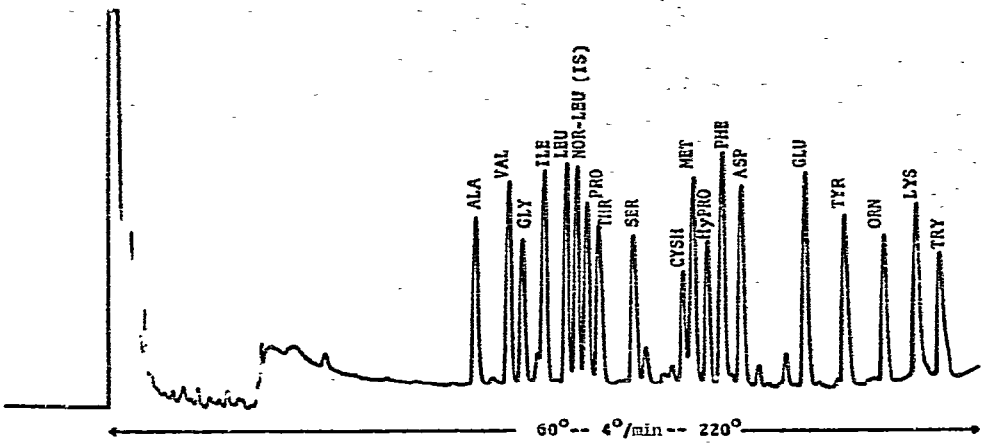

Fig. 2. Separation of 19 amino acids on an EGA column as their TAB derivatives. Conditions: 0.5 μg of each amino acid, with norleucine and butyl stearate as the internal standards; temperature programmed for 60–220° at 4°/min; carrier gas (argon) flow-rate, 50 ml/min; attenuation $10^{-10} \times 2$.

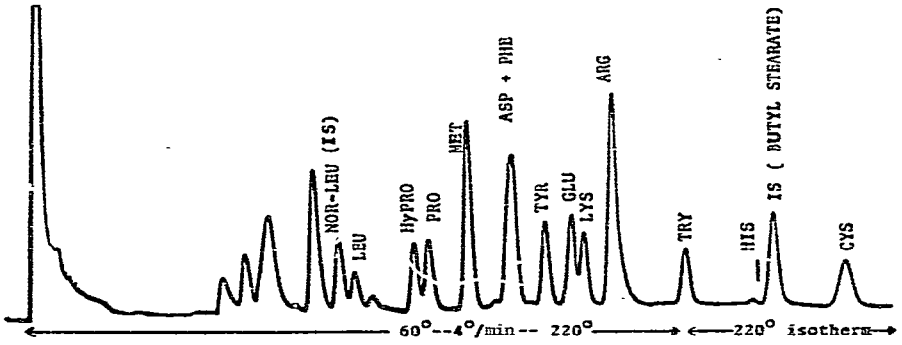

Fig. 3. Separation of tyrosine, glutamic acid, lysine, arginine, tryptophan, histidine and cystine on an OV-17 column as their TAB derivatives. Conditions as for Fig. 2.

TAB-aspartic acid allowed us to take the greatest advantage of the EGA column. By injecting first one 5-μl sample into the EGA column and then another into the OV-17 column after about 2 min and switching over after the appearance of the aspartic acid peak from the EGA column, a chromatogram with all the amino acid peaks was obtained (Fig. 4). We have used norleucine as the internal standard for the peaks recorded from the EGA column and butyl stearate for those recorded from the OV-17 column so as to allow accurate calibration and further calculation of the data. A programme for analyzing the data with a Univac 1108 computer is currently being prepared that should yield quantitative data for each of the amino acids present in the biological samples expressed, for example, as mole per mg sample or mole per 16 g N. As seen from Fig. 4, a simultaneous separation of TAB derivatives of all the 20 protein amino acids could be carried out within a period of about 35 min using the improved method described above.

In any screening of large biological populations, a reduction in the analysis

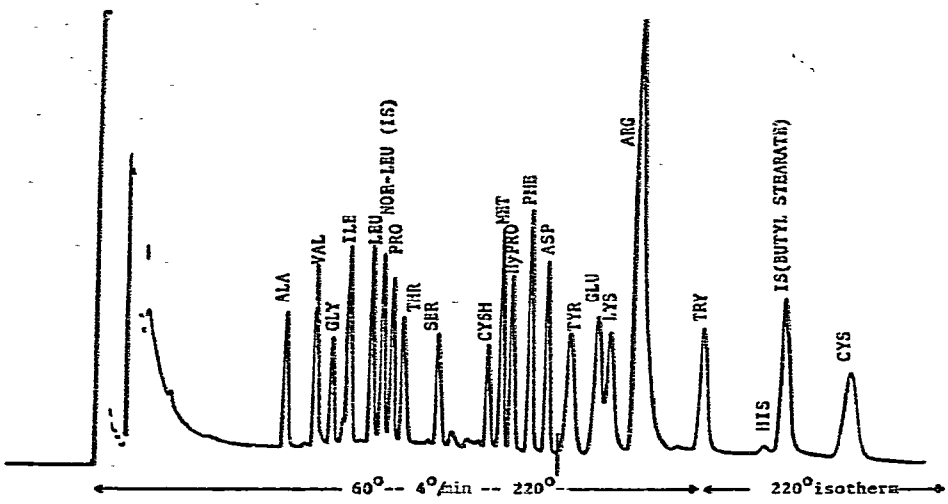

Fig. 4. Separation of all the protein amino acids from a dual-column system; 0.5 μg of each amino acid, with nor-leucine and butyl stearate as the internal standards; sample injected into the EGA column first and then into the OV-17 column simultaneously within a period 2 min before beginning the temperature programme 60–220° at 4°/min. Argon flow-rate, 50 ml/min; attenuation $10^{-10} \times 2$.

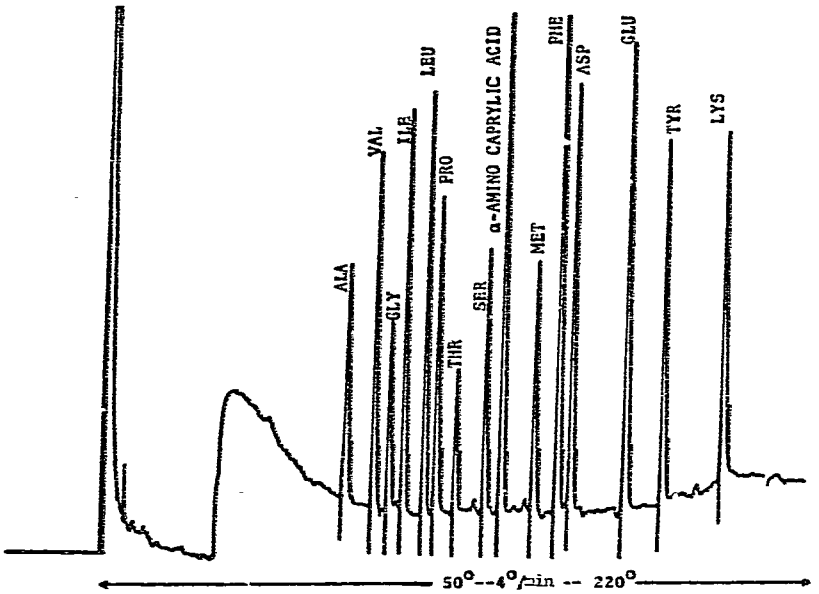

Fig. 5. Separation of the TAB derivative of α-aminocaprylic acid as the internal standard together with other protein amino acids on an EGA column.

time is most valuable. Compared with the standard technique of Gehrke *et al.*[9], our system will reduce the total GLC analysis time from about 55 to about 35 min, which indicates an approximately 50% increase in capacity of the GLC instrument. Compared with the latest dual-column technique of Gehrke *et al.*[7], our technique has the advantage that we used only one integrator and one recorder for continuous runs.

On certain occasions it was difficult to separate norleucine from the neighbouring leucine and proline. This situation sometimes arose when a column became old or when new batches of column packing were made, even though the separation of all the other amino acids was satisfactory. Moreover, it would be advantageous in seeking to obtain reliable and reproducible relative retention times for all the amino acids if the internal standard could be placed in the middle of the elution sequence. We have tried α-aminocaprylic acid and found that it could be derivatized and run in GLC under the same conditions; it eluted after serine and before cysteine from the EGA column (Fig. 5) while using the dual-column technique. α-Aminocaprylic acid eluted well before tyrosine and thus does not interfere with any of the peaks of interest in the OV-17 column (Fig. 6).

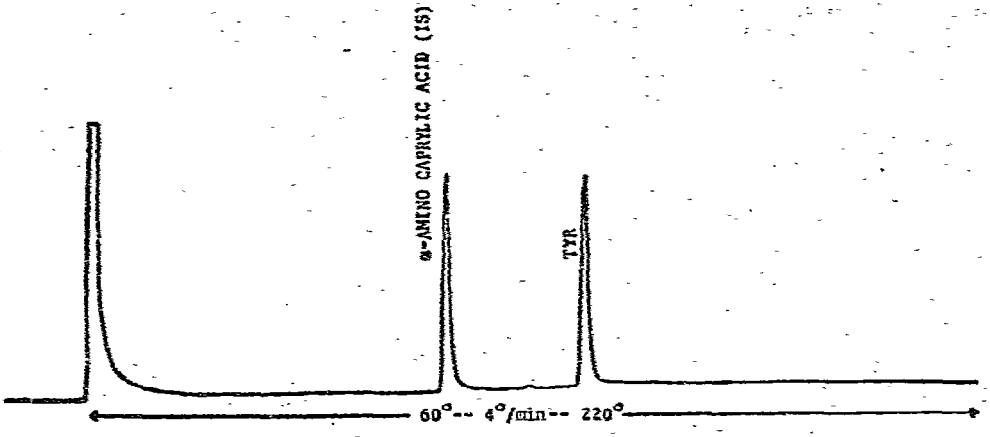

Fig. 6. Separation of TAB derivative of α-aminocaprylic acid before tyrosine, without interfering with the peaks of interest, on an OV-17 column.

ACKNOWLEDGEMENTS

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