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Gas–liquid chromatography of twenty protein amino acids on a single column*

The polar nature of the naturally occurring amino acids prohibits their direct analysis by gas–liquid chromatography (GLC) at temperatures at which they are stable. Modification of the polar groups in the molecule, however, produces a stable derivative which is amenable to analysis under normal conditions of GLC. Extensive studies[1–10] have been made to develop methods for preparing suitable derivatives and to develop GLC column materials for separating the derivatized amino acids. However, none of the investigators have reported the successful separation of the twenty protein amino acids on a single column. We have now achieved this separation with the N-heptafluorobutyryl *n*-propyl derivatives of the twenty amino acids by using a siloxane column (OV-1) as the liquid phase.

Materials and methods

Gas chromatographic analyses were made by using a Perkin-Elmer Model 900** instrument equipped with flame ionization detectors. Samples were analyzed on a 12 ft. × 1/4 in. O.D. coiled glass column packed with 3% OV-1 coated on 80–100 mesh, acid washed, DMCS-treated, high performance Chromosorb W (Applied Science Laboratories, State College, Pa.). The injection port temperature was 250° and the detector temperature 290°. The column was maintained at 100° for 5 min after injection of the sample and then temperature-programmed to 250° at 4°/min. The electrometer range was 10 with an attenuation of 16. This range resulted in a full-scale deflection of 3×10^{-10} A with a 1 mV recorder. Helium was used as the carrier gas at a flow rate of approximately 40 cc/min.

Amino acids were obtained from Sigma Chemical Co. (St. Louis, Mo.). Solutions containing 2.5 μmoles/ml of each individual amino acid and a standard mixture consisting of 2.5 μmoles/ml of each of twenty amino acids were prepared in 0.1 *N* HCl. The N-heptafluorobutyryl (N-HFB) *n*-propyl ester derivatives were prepared for GLC. The propylation step was carried out according to the procedures described by COULTER AND HANN[10]. The apparatus, however, was modified to accommodate a high temperature acylation tube (Regis Chemical Co., Chicago, Ill.) in which all the derivative steps were performed. After propylation, 0.2 ml of heptafluorobutyric anhydride (Pierce Chemical Co., Rockford, Ill.) and 0.1 ml of ethyl acetate were added, and the acylation tube was sealed. The tube was placed in an oil bath at 150° for 10 min and then cooled to room temperature. The contents of the tube were evaporated just to dryness under a gentle stream of dry nitrogen. The dried N-HFB *n*-propyl ester derivatives were dissolved in 0.1 ml of ethyl acetate for injection into the GLC. Three μl of sample, injected simultaneously with 2 μl of acetic anhydride, were analyzed by GLC.

Results and discussion

The most extensive studies of the use of GLC for analysis of amino acids have been made by GEHRKE *et al.*[1–5]. These studies have led to the development of pro-

* *Editor's note*: See also: C. W. GEHRKE, K. KUO AND R. W. ZUMWALT, *J. Chromatogr.*, 57 (1971) 209, which had not yet appeared when this paper was submitted.

** Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

cedures for the quantitative analysis of amino acids as their N-trifluoroacetyl (N-TFA) *n*-butyl ester derivative[2]. With the exception of histidine, conversion of other protein amino acids to their N-TFA *n*-butyl ester derivative appears to be complete. Separation of the derivatized amino acids, however, requires a two-column GLC analysis with two different liquid phase materials. Of the many liquid phase materials which have been investigated[1–10], a two-column system using ethylene glycol adipate (EGA) and OV-17 appeared to give the most satisfactory results[1].

In our laboratory, we have used EGA, OV-17, Tabsorb (Regis Chemical Co.) and other liquid phases for GLC analysis of amino acids from micro-organisms and from other biological materials. Like others, we found that neither of these liquid phases, when used alone, was effective for complete separation of the twenty protein amino acids as their N-TFA *n*-propyl derivative. Recently, however we have used an OV-1 liquid phase material and have observed that separation of the N-TFA *n*-propyl derivatives on this column was much better than on the OV-17 column. Eighteen of the twenty protein amino acids were well separated; various attempts to improve this separation by adjusting instrument parameters (temperature, carrier gas flow) were unsuccessful. With these results, however, we felt that the twenty protein amino acids could be separated on the OV-1 column by preparing a derivative with a boiling point slightly greater than that of the N-TFA *n*-propyl derivative. This was accomplished at the acylation step by substituting heptafluorobutyric anhydride for trifluoroacetic anhydride.

The N-HFB *n*-propyl derivatives were prepared as described above and injected into the OV-1 column along with 2 μl of acetic anhydride. The results are presented in Fig. 1. The chromatogram shows excellent separation of the twenty protein amino acids. The first peak, alanine, is well separated from the solvent peak and the last peak, cystine, is eluted from the column after only 43 min. The excellent resolution of peaks and the low bleed from the column also indicate that precise quantitative measurements can be made. Only a small, broad peak at a retention time of 37 min was observed when histidine alone was injected into the column. This

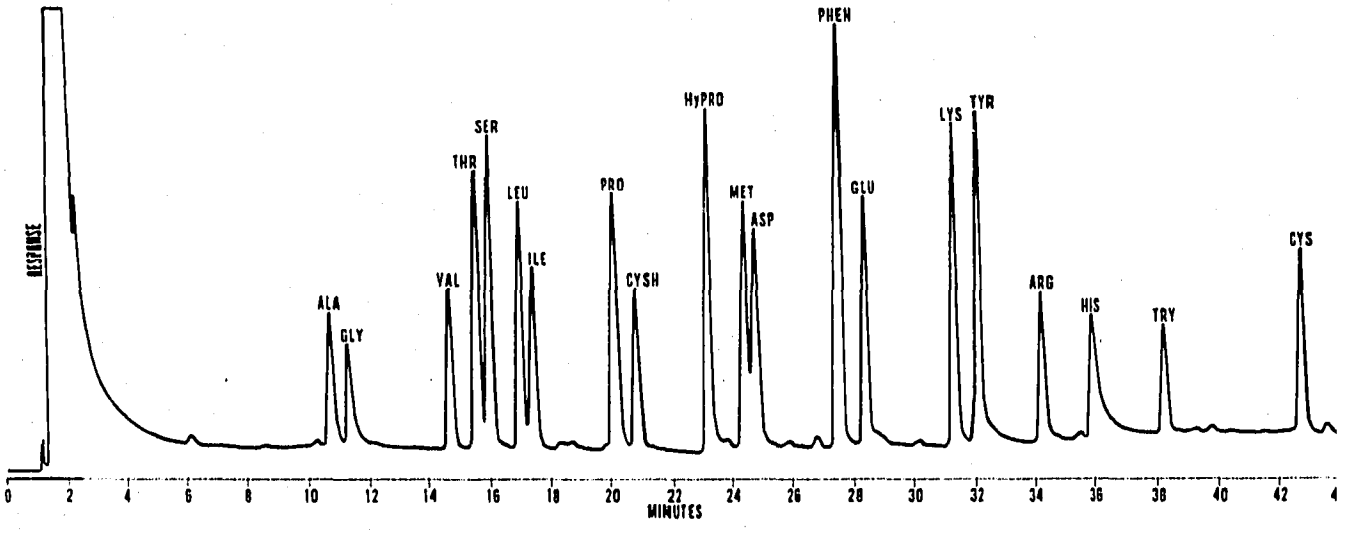

Fig. 1. Gas chromatogram of the N-HFB *n*-propyl derivatives of the twenty protein amino acids on a 3% OV-1 column. Each peak represents 0.015 μmole of amino acid.

peak probably represents the mono-HFB *n*-propyl derivative of histidine, which, as discussed by ROACH *et al.*[5], is converted "on column" to a diacyl derivative by injection with an anhydride. When acetic anhydride was injected simultaneously with sample, the broad peak was removed and converted completely to a single, sharp peak at a retention time of 36 min (Fig. 1); the retention times of the other amino acids were unchanged. The use of acetic anhydride for "on column" derivatization is also desirable from the standpoint that it elutes from the OV-1 column along with the solvent peak, and thus well ahead of the first amino acid (Fig. 1).

OV-1 is a common GLC stationary phase material in laboratories involved in GLC studies. It is a non-polar silicone material noted for its high thermal stability and low bleed rate. For these reasons, it is an excellent GLC column material for amino acid and other analyses, which require temperature programming to relatively high temperatures. We have performed over one hundred amino acid analyses on this column and have observed no significant changes in the retention time or peak resolution.

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