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GAS CHROMATOGRAPHIC CONFIRMATION OF AMINO ACID STRUCTURE VIA DIASTEREOMER PREPARATION

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SUMMARY

The partial gas chromatographic (GC) resolution of several amino acid N-trifluoroacetyl 2-butyl ester diastereomeric derivatives on a short packed column has been used to confirm GC peak assignments in analysis of a marine sediment sample.

INTRODUCTION

Several methods have recently become available for analysis of amino acids by gas chromatography (GC). Quantitative procedures for analysis of amino acid mixtures have been well worked out by Gehrke and co-workers[1–3]. Their method has certain advantages over liquid amino acid analyzers in that a gas chromatograph is a less expensive and more versatile instrument than an amino acid analyzer. In addition, GC offers a method of conclusive peak identification in small samples if the GC eluent is subsequently introduced into a mass spectrometer. The biggest disadvantage of GC amino acid analysis is that the sample to be analyzed must be desalted before analysis[4,5], a potential source of serious contamination in working with very low levels of amino acids found in some geochemical samples[5].

GC analysis of amino acid enantiomers and diastereomers in fossils, sediments, and extraterrestrial samples have been reported[6–17]. Such analyses are of potential importance to sample dating and to determinations of paleo-temperatures[6–13] as well as to questions of whether amino acids from geochemical and extraterrestrial samples are biologically produced[14–17]. Two alternate approaches to GC determinations of amino acid enantiomer ratios have been utilized[18]. In one, an optically active stationary phase is coated on the inside of a metal capillary column[18–22]. In the other, a diastereomeric amino acid derivative is prepared by coupling the asymmetric amino acid to a reagent with an asymmetric center. For example, the amino acid N-trifluoroacetyl (N-TFA) 2-butyl esters can be prepared by substituting optically active 2-

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butanol for *n*-butanol in Gehrke's procedures[1-3]. Separation of the L-amino acid D-ester and the D-amino acid D-ester can then be accomplished on a capillary column[6,14-16,18]. Decomposition of amino acid derivatives can occur when metal GC columns are used[1,2]. Thus, most workers analyzing geochemical samples have utilized liquid chromatography for quantitative amino acid analyses and capillary GC columns for analyses of enantiomers[6,14-16]. Glass capillary GC columns would avoid the decomposition problem and allow for total analysis on one system.

The partial GC resolution of several protein amino acid enantiomers on a 6 ft. × 4 mm packed ethylene glycol adipate (EGA) column using the N-TFA 2-butyl ester diasteromeric derivatives was recently reported[23]. Since we were carrying out quantitative amino acid analyses of marine sediments on such a column, we tested whether resolution of the 2-butyl ester diastereomers would be sufficient to make quantitative analyses of amino acid enantiomers feasible. The best resolution obtained was similar to that previously reported[23] (Fig. 2). However, the procedure did offer a fast and simple confirmation of correct GC peak assignment.

An indication of the identity of a GC peak can be obtained by coinjection of the sample and a known standard to see if a single nonbroadened peak is produced. However, retention time coincidence does not ensure chemical equivalence. The problem of accidental peak coincidence can be minimized by carrying out the analysis on several GC columns with widely different liquid phases so that the chance of coincidental retention times without chemical identity is minimized. Alternately, the GC peak can be identified by subjecting the GC eluant to a second analytical method which provides structural information, for example mass spectrometry.

We were faced with the problem of confirming the identity of N-TFA *n*-butyl ester amino acid peaks when neither of these two methods was routinely available. The geochemical samples we were analyzing contain amino acids and polypeptides from many different biological sources as well as material which has been altered by the geochemical environment[6,14-16]. When this work began, the GC instrument available was a single-column instrument equipped with a glass column. We wished to avoid, if possible, the time-consuming process of changing columns and optimizing their performance.

A recent report of partial GC resolution of several amino acid N-TFA 2-butyl ester diastereomers on a column similar to ours[23] led us to devise a chemical method for confirming GC peak assignments. Specifically, several amino acids having an asymmetric center produce two partially resolved peaks for the diastereomeric forms when the GC derivative is prepared with racemic 2-butanol rather than *n*-butanol. The N-TFA *n*-butyl esters and the N-TFA 2-butyl esters have very similar retention times (Figs. 1 and 2)[23]. The methods of preparation of the *n*-butyl and 2-butyl ester derivatives are identical and can easily be run simultaneously on two aliquots of amino acid mixture. Quantitative amino acid analysis of the N-TFA *n*-butyl ester mixture is then carried out in the usual way. Confirmation of assignment of amino acid peaks is then accomplished by examining the gas chromatogram of the N-TFA 2-butyl ester mixture for partially resolved diastereomeric peaks for those amino acids which show doublets for the same compounds in the standard amino acid mixture (Fig. 2). Doublets must appear for the same peaks in both the standard mixture and the unknown mixture if the amino acid peaks are correctly assigned.

In this work, the method proved to be valuable in distinguishing between

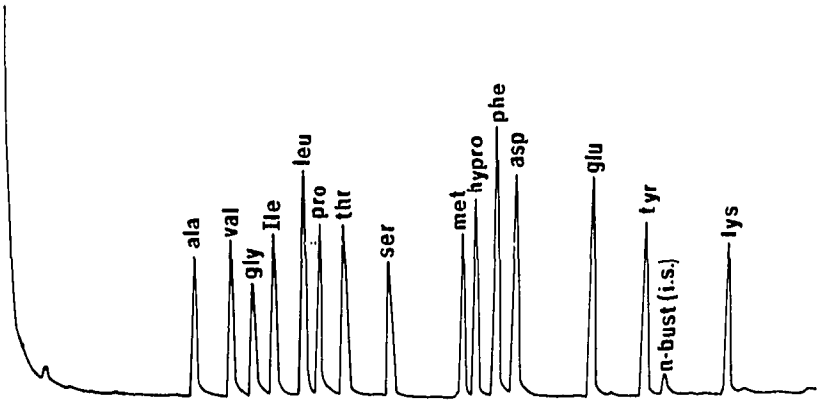


Fig. 1. Gas chromatogram of N-TFA amino acid *n*-butyl esters of a standard amino acid mixture containing fifteen amino acids. 1.5 m × 2 mm I.D. × 4 mm O.D. glass column packed with TAB-SORB (Regis). Programmed temperature: 75–210° at 4°/min. Sensitivity: 4×10^{-11} A/mV.

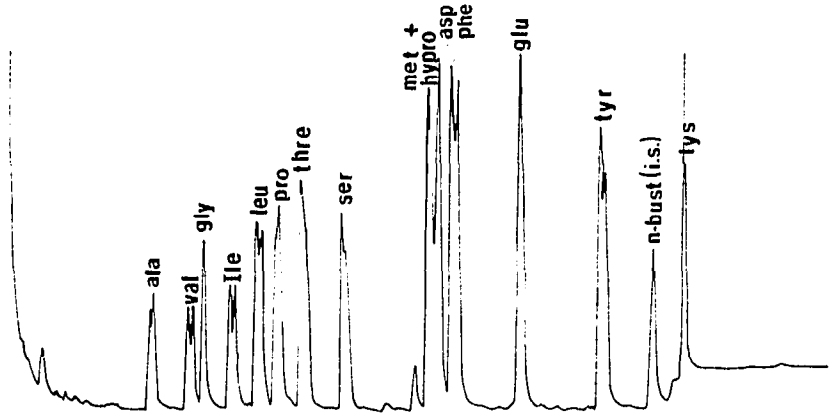


Fig. 2. Gas chromatogram of N-TFA amino acid 2-butyl esters. Same conditions as for Fig. 1, except sensitivity 8×10^{-11} A/mV (lysine: 16×10^{-11} A/mV).

γ-aminobutyric acid (GABA) and serine, which have very similar retention times on the column used. GABA, with no asymmetric center, produced one sharp peak on the gas chromatogram when derivatized with racemic 2-butanol, while serine, which has an asymmetric center, showed a doublet for the partially resolved peaks of the two diastereoisomers.

EXPERIMENTAL

Apparatus

Pyrex Reactivials (1 ml; Pierce) with PTFE-lined septum caps were used for derivatization. A Vortex Genie mixer (Scientific Industries) and a tube heater (Kontes Glass) were used in sample preparation. Small amounts of reagents were delivered with Corning micro-pipettes. It was found desirable to use silicone rubber tubing,

copper tubing, Swagelok fittings and glass tubing in all vacuum and nitrogen lines in order to avoid contamination with phthalate esters from plastic tubing.

Reagents

Doubly distilled water was used in the preparation of all reagents and solutions and in the final rinsing of all glassware. The second distillation of the water (carried out under nitrogen which had been passed through a tube containing CuO maintained at 600° or a 0.3-μm glass fiber filter) was from basic potassium permanganate through a 150 × 2 cm column packed with 8-mm Raschig rings. Trifluoroacetic anhydride reagent (25% in methylene chloride) and 3 *N* HCl in *n*-butanol were purchased from Regis (Morton Grove, Ill., U.S.A.). The reagent HCl in racemic 2-butanol was prepared by a modification of the method of Irving and Cox[24]. Concentrated hydrochloric acid (reagent grade; Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.) (25 ml) and 25 ml of racemic 2-butanol (Eastman-Kodak, Rochester, N.Y., U.S.A.) which had been redistilled were placed in separate 50-ml beakers. The open beakers were allowed to stand in a closed desiccator for about 12 h or until the 2-butanol was 3 *N* in HCl as determined by its weight gain. Initially, the desiccator had to be opened briefly every hour for about 3 h until the temperature and pressure of the system had stabilized.

The resulting reagent gave satisfactory (but not quantitative) yields in the derivatization. There was no interfering background in a GC blank of the procedure carried out on the residue from 250 ml of doubly distilled water. Methylene chloride (reagent grade; Fisher Scientific, Pittsburgh, Pa., U.S.A.) was distilled from anhydrous calcium chloride in a previously oven-dried apparatus protected with a calcium chloride drying tube and was stored in a dry bottle until used. *n*-Butyl stearate in *n*-butanol (1 mg/ml) was used as internal standard.

Standard amino acid solutions (in 0.1 *N* HCl) containing one or several amino acids were 2.5 moles/l in each amino acid. The amino acids were purchased from Sigma (St. Louis, Mo., U.S.A.) or Calbiochem (La Jolla, Calif., U.S.A.) and were determined to be pure by GC analysis.

Hydrochloric acid, ammonium hydroxide, and ammonium acetate solutions were made from doubly distilled water and Baker Analyzed Reagents (J. T. Baker, Phillipsburgh, N.J., U.S.A.).

Extraction of amino acids from marine sediments

Sediment cores were obtained with a 1 m × 21 cm diameter sphincter corer with a fiberglass barrel. All samples were sealed and stored at −20° immediately after collection. The cores were thawed, extruded, and sectioned in the laboratory rather than on the ship. The outer portions of each section, which were in contact with the barrel, were discarded. The core sections were then stored at −20° in glass jars until analysis.

A 20- to 50-g sample of wet sediment was mixed with 100 ml of 1 *N* hydrochloric acid and filtered with a medium-porosity sintered glass funnel. The sediment was washed with 25 ml of water, filtered, and mixed with 50 ml of 0.5 *M* ammonium acetate. The mixture was filtered and the sediment rinsed with 25 ml of water. The sediment was then put into a round-bottom flask equipped with a magnetic stirrer and a reflux condenser attached to a gas inlet tube which could be alternately con-

nected to vacuum (aspirator) or positive nitrogen pressure. That is, the nitrogen came into a glass Y joint. One branch of the Y tube was connected to the gas inlet tube of the extraction mixture; the other branch was connected to a bubble tube. In this manner, the reaction mixture was kept under nitrogen without having nitrogen continuously passing through it. Hydrochloric acid (6 *N*, 100 ml) was added. The stirred mixture was alternately evacuated and filled with nitrogen three times and then allowed to heat at reflux under nitrogen for 15 h. The mixture was allowed to cool to room temperature and filtered.

The three extracts (1 *N* HCl, 0.5 *N* ammonium acetate, and 6 *N* HCl) were analyzed separately after concentration (Buchi all-glass rotary evaporator, 35°, to a volume of about 25 ml) and desalting (described below). In the marine sediments examined in these laboratories, 90% or more of the amino acids were found in the 6 *N* HCl extracts. However, the three extractions were done on each sample since work with Buzzard's Bay surface sediments showed that recoveries of amino acids were greater than if the wet sediment was simply refluxed with 6 *N* HCl under nitrogen with no pre-extraction[25].

Analysis of the sediment before and after the extraction outlined above showed that this procedure removed all of the organic nitrogen from this sediment core[26].

The concentrated extract was desalted on Bio-Rad AG 50W-X8 cation-exchange resin (50–100 mesh). The resin was first cleaned by alternately washing with two portions of 6 *N* HCl, water, two portions of 7 *N* ammonium hydroxide and water. This procedure was repeated three times. A 12 × 1 cm column of the resin was then prepared. This was sequentially eluted with two 20-ml portions of 6 *N* HCl, water, (to neutrality), two 20-ml portions of 0.2 *N* ammonium hydroxide, two 20-ml portions of 2 *N* ammonium hydroxide, four 20-ml portions of 7 *N* ammonium hydroxide, and water (to neutrality). This procedure was repeated until the background from the residue of 50 ml of the 7 *N* ammonium hydroxide eluent was blank to the N-TFA *n*-butyl ester amino acid GC analysis. The column could be regenerated (after the desalting of a sample) by repeating this procedure. The regenerated columns generally showed cleaner blank analyses than freshly prepared columns.

After the column had been reacidified and brought back to neutrality with water, the concentrated marine sediment extract was applied. The column was then eluted with two 10-ml portions of water (to neutrality), two 10-ml portions of 0.2 *N* ammonium hydroxide, seven 10-ml portions of 2 *N* ammonium hydroxide (when the eluant started to become basic) and five 10-ml portions of 2 *N* ammonium hydroxide.

The 50 ml of basic 2 *N* ammonium hydroxide were collected, combined, evaporated almost to dryness, and made up to standard volume with 0.1 *N* hydrochloric acid for subsequent GC amino acid analysis. Only the amino acids from the 6 *N* HCl sediment extracts are discussed in this paper. When a standard amino acid mixture was desalted in this way, GC analysis showed that the amino acid recovery was 92%.

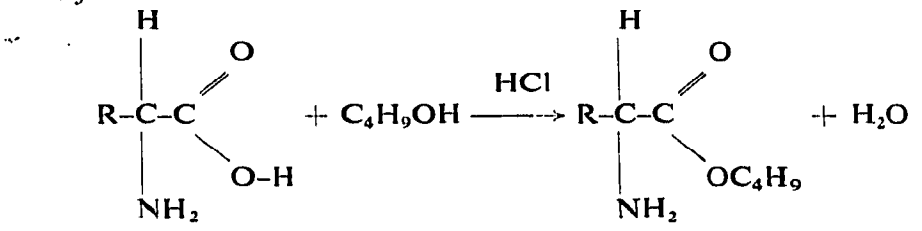
Derivatization

Nitrogen used in derivatization was dried and filtered by passing it through molecular sieves and then through a 0.45-μm Millipore gas filter.

Derivatization was carried out on the microgram level as described by Roach and Gehrke[2]. A quantity of amino acid solution (in 0.1 *N* HCl) estimated to contain

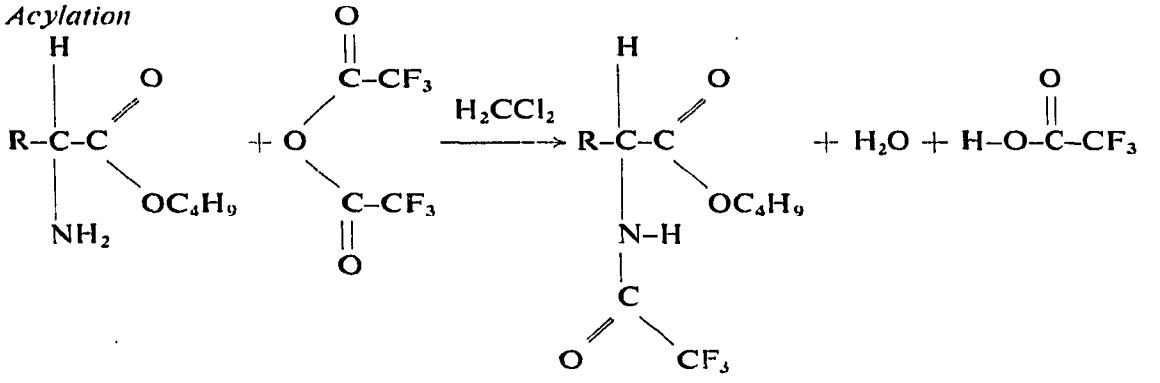
a total of about 10 μg of total amino acids was pipetted into a Reactivial and evaporated just to dryness at 100° in a nitrogen stream. Methylene chloride (300 μl) was added and the mixture evaporated under nitrogen with heat only to azeotropically remove any remaining water.

Esterification



The sample was treated with 200 μl of 3 *N* HCl in either *n*-butanol or 2-butanol. The vial was capped and the mixture heated at 100° for 30 min with occasional agitation on the Vortex mixer to assure a homogeneous solution by the end of the heating period. The butanol was evaporated at 100° in a nitrogen stream. The Reactivial was allowed to cool to room temperature and 300 μl of methylene chloride were added and evaporated by heating only to remove remaining water.

Acylation



Acylation was carried out by adding 150 μl of 25% trifluoroacetic anhydride in methylene chloride. The sample was capped and allowed to stand for 2 h at room temperature or overnight in the refrigerator prior to GC analysis.

The trifluoracetic anhydride and trifluoracetic acid in the final reaction mixture complicate GC analysis due to interfering peaks under amino acid peaks on some GC columns and by shortening drastically the useful life of the GC column. Thus, the following procedure was adopted for evaporating samples prior to GC analysis. A three-way stopcock was attached to a vacuum pump (capable of maintaining a pressure of 10–30 mm Hg), nitrogen line and a 22-gauge syringe needle as shown in Fig. 3. The needle was attached to the stopcock via a broken off syringe so that a clean needle could be used for each sample. A gentle nitrogen stream was blown through the needle as it was inserted just through the septum cap of the sealed Reactivial.

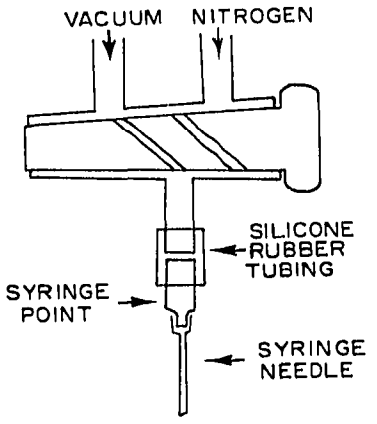


Fig. 3. Apparatus used to evaporate N-TFA amino acid butyl ester reaction mixtures.

The vial was cooled in an ice-bath and the stopcock was carefully turned to the vacuum position. If care was taken not to move the vial during this process, no bumping or foaming occurred unless some solid residue was present in the reaction mixture. The sample was allowed to evaporate just to dryness with slow lowering of the ice-bath if warming was required to complete the process. The sample was refilled with nitrogen by turning the stopcock, and the vial was removed from the syringe needle. It was kept sealed under nitrogen during all subsequent analyses and storage. For GC analysis, the sample was made up to a total volume of 30 μl with methylene chloride and 2- to 3-μl samples of the solution were used. It was found that samples so prepared and stored in the refrigerator were stable for at least a month except for the slow decomposition of the hydroxy- and sulphur-containing amino acid derivatives.

Using the procedures described below, relative peak areas of standard amino acid mixtures and amino acid yields as determined using *n*-butyl stearate internal standard were routinely reproducible to within $\pm 3\%$.

GC conditions

The most satisfactory column found for this work was a 1.5 m $\times$ 4 mm O.D. $\times$ 2 mm I.D. glass column packed with a commercially available EGA packing (TABSORB, available from Regis). EGA packing prepared in our laboratory showed neither the stability nor the resolution capability of the TABSORB. The column was conditioned for 12 h at 210° before use. In carrying out many amino acid analyses every day as described above, the useful life of this column was 8–12 weeks before sample decomposition became a problem.

On-column injection was used with the injector maintained at a temperature of 150°. The temperature of the flame ionization detector was 250°. The flow-rates were: carrier, nitrogen, 26 ml/min; hydrogen, 30 ml/min; and air, 240 ml/min. The temperature program was: 75–210° at 4°/min.

The gas chromatograph was a Varian Model 1400 single-column instrument equipped with a linear temperature programmer and a flame ionization detector. An Autolab Model 6300 digital integrator was used to measure peak areas. Mass spectra were obtained using a Finnegan System 150 gas chromatograph–mass spectrometer–computer.

RESULTS AND DISCUSSION

The partial separation of nine amino acid N-TFA 2-butyl ester diastereomers is shown in Fig. 2. This gas chromatogram was obtained with a temperature program of 4°/min. The resolution is about the same as that obtained earlier[23], but was accomplished with a faster program rate (4°/min rather than 2°/min). A slower program rate of 2°/min did not improve the resolution. As noted earlier[23], the relative position of each amino acid is the same as when using the N-TFA *n*-butyl esters except for the inversion between the phenylalanine and aspartic acid peaks. The peaks of alanine, valine, isoleucine, leucine, proline, threonine, serine, phenylalanine, and tyrosine all show doublets indicating partial resolution of the diastereomeric pairs of amino acid derivatives. Previously[23], trouble was encountered in resolving the glycine and isoleucine peaks. This separation was not a problem in this work. Doublets were not obtained for aspartic or glutamic acid although there was some peak broadening in both cases.

The procedure was then applied to the amino acid analysis of a marine surface sediment sample taken from Buzzard's Bay, Mass.[25] (Figs. 4 and 5). Even though the amino acids in the sample were less concentrated at injection than in the standard mixture, doublets can be seen for the same amino acids as in the standard mixture (Fig. 2). Quantitative amino acid analysis of this sample had previously been carried

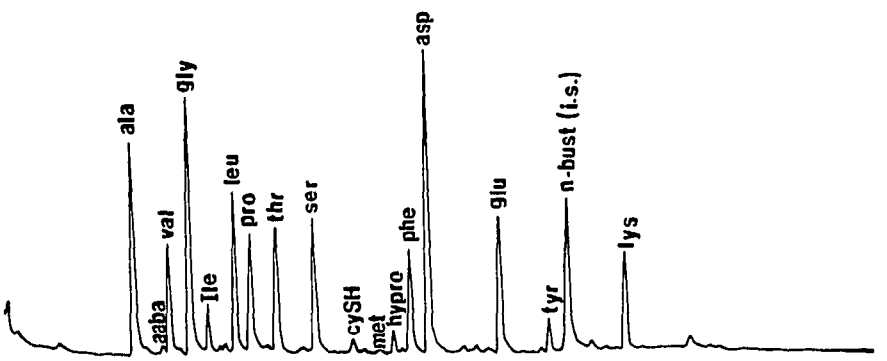


Fig. 4. Gas chromatogram of N-TFA amino acid *n*-butyl esters of Buzzard's Bay, Mass. surface sediment sample. Same conditions as for Fig. 1. aaba = *α*-Aminobutyric acid.

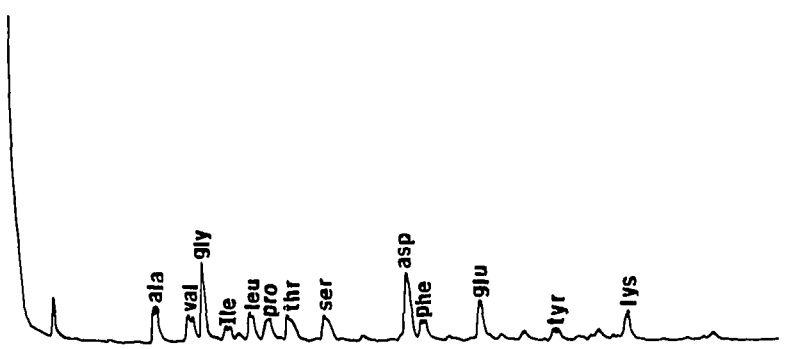


Fig. 5. Gas chromatogram of N-TFA amino acid 2-butyl esters of Buzzard's Bay, Mass. surface sediment sample. Same conditions as for Fig. 1.

out on the N-TFA *n*-butyl ester mixture (Fig. 4). The areas of peaks in the N-TFA 2-butyl ester gas chromatogram approximately equal those observed for the N-TFA *n*-butyl esters even though no precautions were taken to ensure that the 3 *N* HCl–racemic 2-butanol reagent used was anhydrous.

The technique was particularly useful in establishing the identity of amino acids in a deep ocean sediment core (K-19-4-9, Woods Hole Oceanographic Institution, taken from a water depth of 5454 m at 30° N, 60° W, approximately 300 miles southeast of Bermuda). This core is currently being analyzed for hydrocarbons and lipids as well as for amino acids, and the results will be reported elsewhere[26]. However, with regard to amino acids, preliminary analyses of two sections of this core (one at the sediment surface, 0–8 cm, and the other at a sediment depth of 16–24 cm) indicated substantial amounts of two nonprotein amino acids, *viz.* GABA and β-alanine, in addition to the normal protein α-amino acids in the surface section. The core section from a sediment depth of 16 cm shows only traces of α-amino acids, while the amounts of GABA and β-alanine are approximately equal to those present in the surface section. The interpretation of this result is unclear without additional analyses of other core sections. However, it was important to confirm the structures of these two compounds in as many ways as possible. Coinjection of standards substantiated the identity of β-alanine. However, the peaks for GABA and serine had almost identical retention times on the TABSORB column. Coinjection of standards indicated that the peak from the core was GABA rather than serine, but the retention times of the two compounds were so close that only partial resolution does not allow us to estimate the contributions of each compound. GC analysis of the N-TFA racemic 2-butyl esters of both samples showed only singlets for the peaks previously assigned to β-alanine and GABA. The gas chromatogram of the deeper section (Fig. 6) also shows weak doublets for peaks assigned to alanine and leucine. Since β-alanine and GABA do not possess asymmetric centers, they cannot produce diastereomers upon esterification with racemic 2-butanol. The identification of these two peaks as well as those of the other amino acids present was confirmed by GC–mass spectrometry.

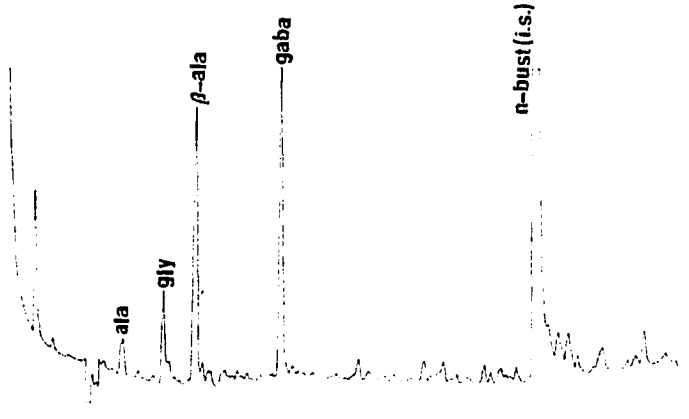


Fig. 6. Gas chromatogram of N-TFA amino acid 2-butyl esters of mid-Atlantic sediment sample Core K-19-4-9 (water depth, 5454 m; sediment depth, 16–24 cm). Same conditions as for Fig. 1.

Work is being continued to see if the method can be applied to quantitative amino acid enantiomer analysis, either by increasing diastereomer resolution on a longer column or by applying mathematical methods for integrating multiple peaks.

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