Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

Ultramicrodetection of Amino Acids by GLC

Gerhard Ertingshausen[a]; Charles W. Gehrke[a]; Walter A. Aue[a]
[a] EXPERIMENT STATION CHEMICAL LABORATORIES, UNIVERSITY OF MISSOURI, COLUMBIA, MISSOURI

To cite this Article Ertingshausen, Gerhard , Gehrke, Charles W. and Aue, Walter A.(1967) 'Ultramicrodetection of Amino Acids by GLC', Separation Science and Technology, 2: 5, 681 — 689
To link to this Article: DOI: 10.1080/01496396708049731
URL: http://dx.doi.org/10.1080/01496396708049731

SEPARATION SCIENCE, 2(5), pp. 681–689, Nov., 1967

Ultramicrodetection of Amino Acids by GLC*,†

GERHARD ERTINGSHAUSEN,‡ CHARLES W. GEHRKE, and WALTER A. AUE

EXPERIMENT STATION CHEMICAL LABORATORIES
UNIVERSITY OF MISSOURI
COLUMBIA, MISSOURI

Summary

A method has been developed for the separation and qualitative detection of 11 amino acids at the picogram level, which uses a derivatization new to the area of gas-chromatographic analysis. Phosphorus-containing derivatives were selected to allow selective and hypersensitive determination with a modified version of the alkali-flame detector. This method comprises the formation of the *N*-diethyl phosphate amino acid methyl esters and subsequent gas-chromatographic separation and analysis. The minimum detectable limit for alanine (response–noise, 2:1) was found to be ca. 5×10^{-12} g injected. The method should be applicable to the investigation of very small amounts of naturally occurring amino acids and peptides, as was shown with gramicidin-S. A gas-chromatographic analysis of 250 pg of gramicidin-S was found to yield clearly discernible peaks for valine, leucine, proline, and phenylalanine.

The increasing demand for the determination of amino acids in biological samples has led to the development of various chromatographic methods. Generally, these methods require microgram quantities of substance; however, much greater sensitivities would be needed to cope with a wide variety of biological problems in which the available amount of substance is the limiting factor, e.g.,

* Missouri Agricultural Experiment Station Journal Series No. 5139. Approved by the Director.

† Supported in part by Grant No. GM 08525-06 from the National Institutes of Health and Grant No. GB 4564 from the National Science Foundation.

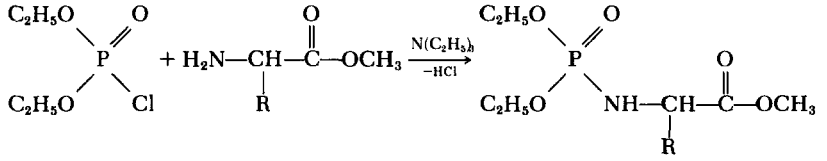
‡ Postdoctoral Fellow.

studies involving hormones, enzymes, and other biologically important molecules at the cellular level.

In recent years, gas chromatography has become increasingly important because of its speed and relative sensitivity, as illustrated by contributions from this laboratory (*1–4*). To still significantly lower the limit of detection (MDL) for amino acids, we have undertaken studies that employ the hypersensitive, selective response of the alkali-flame detector (*5,6*) as modified by Aue and Gehrke et al. (*7,9*), toward phosphorus-containing compounds. This response has been shown to be four to five orders of magnitude higher than that afforded by a normal flame ionization detector. Consequently, the objective of this study was to find phosphorus-containing derivatives of amino acids that would prove sufficiently stable and sufficiently volatile for gas-chromatographic analysis.

Pilot experiments, in which the methylesters of some simple amino acids like glycine, alanine, and leucine were reacted with diethylchlorophosphite in analogy to the method of Anderson (*8*), showed that the readily formed reaction products were easily oxidized in air to the corresponding phosphates and thus proved unsuitable for our purpose.

To eliminate this complication, diethylchlorophosphate was used as the phosphorylating agent in ether or acetonitrile solution in the presence of triethylamine as HCl acceptor.



The use of pyridine, as described for other phosphorylations, resulted in unsatisfactory yields of the desired derivatives.

We have limited this investigation to monophosphorylated derivatives since our experiments have shown that further studies would be required for the gas chromatography of di- or multiphosphorylated compounds (as those derived from serine, cysteine, tryptophan, arginine, etc.), for which the gas-chromatographic conditions described in this paper proved inadequate.

The work on monophosphorylated derivatives, then, comprised the following steps:

1. Synthesis of pure derivatives for yield and identity studies.

2. Comparison of these synthesized reference derivatives with the collected gas-chromatographic effluents.
3. Response of the alkali-flame detector to submicroamounts of derivatives and determination of MDL.
4. Derivatization of amino acid mixtures and gas-chromatographic separation.
5. Pilot experiment using a naturally occurring peptide, gramicidin-S.

EXPERIMENTAL

Reagents

The methylesters of alanine, leucine, glycine, methionine, and glutamic acid, as well as proline, valine, phenylalanine, aspartic acid, and gramicidin-S, were obtained from Mann Research Laboratories; β-alanine was purchased from Nutritional Biochemicals Corporation. Methanol "Fisher certified reagent" was dried by refluxing over magnesium turnings prior to distillation. Ninety-nine per cent pure hydrogen chloride gas was purchased from The Matheson Co., Inc. Diethylchlorophosphate from Aldrich Chemical Co., Inc., was used without further purification. Diethyl ether was A.R. grade, dried over sodium.

Apparatus

A Barber–Colman Model 5000 gas chromatograph with temperature-programming facilities was used in this study. The regular

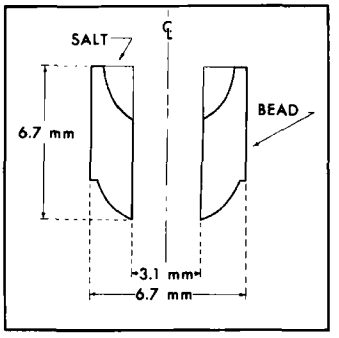


FIG. 1. Modified alkali source, Rb_2SO_4 bead. Saxonburg Ceramics P-879.

Barber-Colman flame ionization detector was modified by placing a ceramic ball-and-socket-type bead on the jet tip (Fig. 1). The concave side of the bead was filled with highly purified rubidium sulfate (9). The regular single-loop electrode was replaced by a triple-loop 4-mm-i.d. platinum spiral occupying the area between 3 and 8 mm above the rubidium sulfate surface. The detector proved sensitive to changes in hydrogen flow and displayed its optimum performance in continuous operation. Consequently, it was not turned off overnight. Prepurified nitrogen obtained from Air Products was used as carrier gas after further purification by passing through a Linde molecular sieve, 5-A.

IR spectra were obtained either directly from the pure substance placed between KBr plates or by using a micropellet method with thallium bromide as the matrix. The latter method was used with gas-chromatographic effluents employing an Aerograph Model 1520 equipped with hydrogen flame detector, 1:40 splitter, microswitch actuated collection facilities, and 1 m × 4 mm i.d. coiled glass column. The NMR spectra were recorded in $CDCl_3$ solution with a Varian A-60 Spectrometer.

Preparation of Derivatives

The amino acid methylester hydrochlorides were either purchased or prepared according to the method of Curtius and Goebel (*10*). The following synthesis of the *N*-diethylphosphate derivative of the glycine methylester is representative for the general derivatization procedure.

Glycine methylester hydrochloride, 125.1 mg (1 mmole), was suspended in 5 ml of anhydrous ether, 202.4 mg (2 mmoles) of triethylamine was added, and the mixture magnetically stirred for 1 hr at room temperature. Then, 172.6 mg (1 mmole) of diethylchlorophosphate in 1 ml of anhydrous ether was added, and after 30 min of stirring, the mixture was filtered through a glass frit, dried over anhydrous sodium sulfate, and the solvent removed in vacuum. The residue was distilled using a Kontes Microdistillation Apparatus at 10^{-4} mm Hg and a bath temperature of 65°C. Yield 45%.

The colorless liquid gave the IR spectrum shown in Fig. 2.

Characteristic bands (cm^{-1}): 3230 (NH); 2960 (CH_3); 2930 (CH_2);

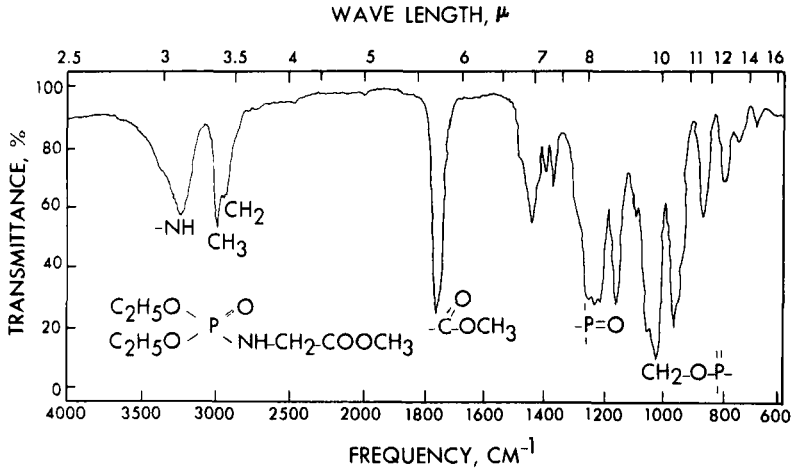


FIG. 2. Infrared spectrum of *N*-diethylphosphate derivative of glycine methylester, neat film, Beckman IR-10.

1755 (—C(=O)—O—CH_3); 1230 (—P(=O)—); 1159 and 1025 (CH_2—O—P(=O)—).

The NMR spectrum is shown in Fig. 3.

Analysis: Calculated for $C_7H_{16}O_5NP$: C, 37.34%; H, 7.16%; N, 6.22%; P, 13.75%
Found for $C_7H_{16}O_5NP$: C, 37.69%; H, 7.33%; N, 6.28%; P, 14.03%

Derivatization of a Gramicidin-S Hydrolyzate

Gramicidin-S (1.5 mg) was hydrolyzed for 48 hr with 6 *N* HCl at 105°C in a closed tube. The water was evaporated at 60°C under reduced pressure and the residue dried by repeatedly adding and evaporating methanol and benzene. Five milliliters of anhydrous methanol containing 1.20 ± 0.10 meq/ml of anhydrous HCl was added and the solution stirred for 30 min at room temperature. The methanol was completely evaporated and 10 mg of triethylamine in 1 ml of anhydrous ethyl ether was added. The mixture was ultrasonically stirred for 10 min. After addition of 6 mg of diethyl-

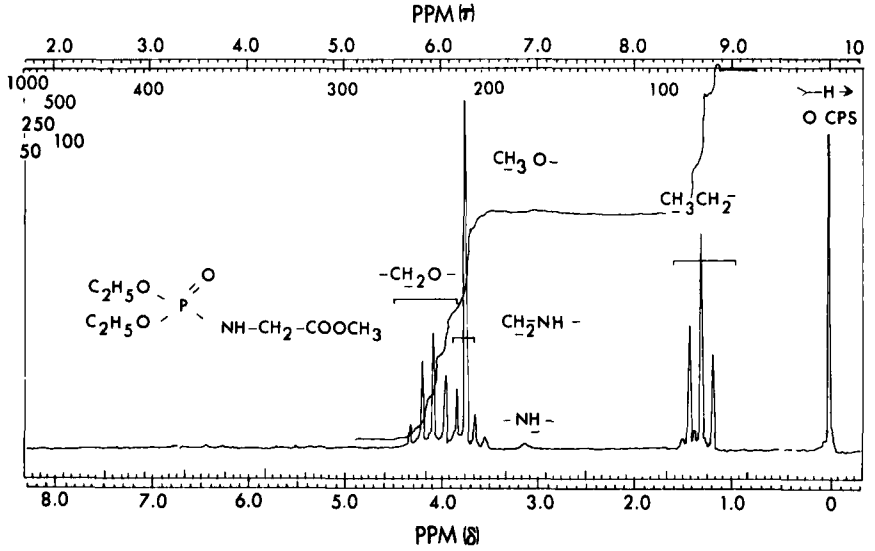


FIG. 3. NMR spectrum and integral of *N*-diethylphosphate derivative of glycine methylester at 60 mcps relative to tetramethylsilane in $CDCl_3$.

chlorophosphate, the reaction mixture was allowed to stand at room temperature for 30 min and then analyzed.

Results and Discussion

The synthesis of the pure reference derivatives did not present any problem. The IR and NMR spectra, chromatographic behavior, and elemental analysis of the diethyl phosphate derivative of glycine methylester were in agreement with the assumed structure. The IR spectrum of the derivative collected from the gas-chromatographic effluent was identical to that of the injected compound.

Several liquid phases were evaluated according to their ability to separate the amino acid derivatives. Carbowax 20M and OV-17 showed satisfactory performance at the conditions indicated. Derivatives were prepared from α- and β-alanine, valine, leucine, isoleucine, glycine, proline, aspartic acid, glutamic acid, methionine, and phenylalanine. Each of these compounds yielded a single, symmetrical peak. Compared to the commonly used acylated and esterified derivatives, the GLC behavior of the phosphorylated compounds is predominantly determined by the phosphate group. Thus the retention times are quite similar and, while

separation poses certain probelms, the analysis can be conducted isothermally. This is especially advantageous since the alkali-flame detector is difficult to operate at ultrahigh sensitivity with temperature programming above 200°C. At a somewhat lower sensitivity level, temperature programming proved useful, as shown by the separation of 11 derivatized amino acids in Figs. 4 and 5.

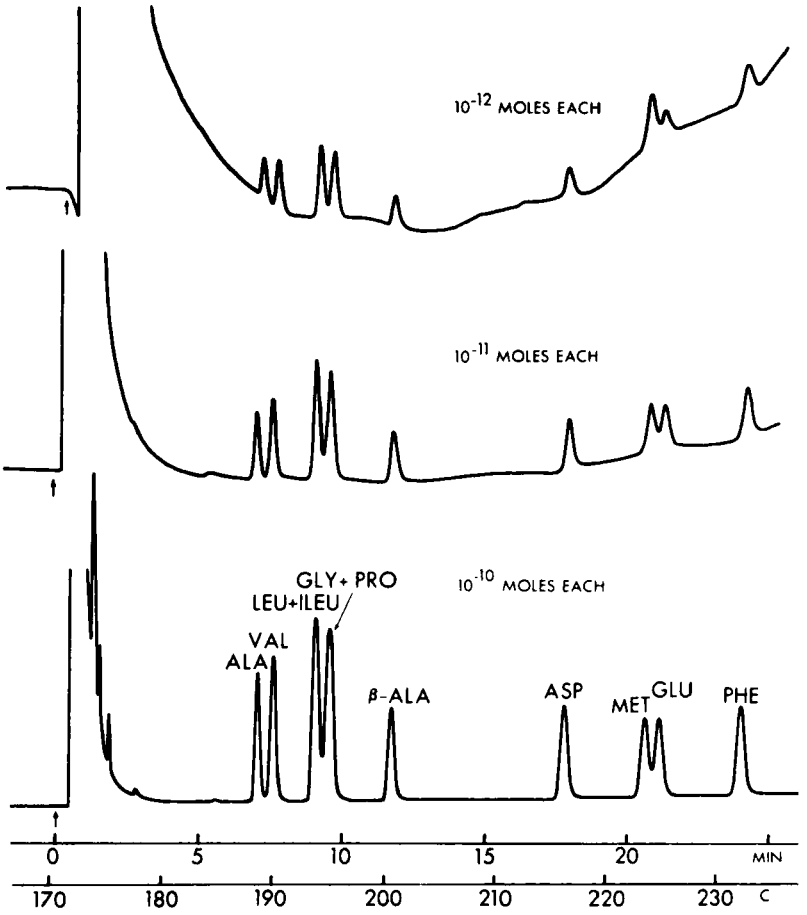


FIG. 4. GLC of *N*-diethylphosphate amino acid methylester derivatives. The injected mixture contained 10^{-12} (top), 10^{-11} (middle), and 10^{-10} (bottom) moles of each amino acid and was determined by a Rb_2SO_4 alkali-flame detector. Column: 5% Carbowax 20M on Chromosorb W, DMCS treated, 60/80 mesh; Pyrex U-tube 2m × 2.5 mm i.d. N_2, 16 ml/min; H_2, 40 ml/min; air, ca. 200 ml/min.

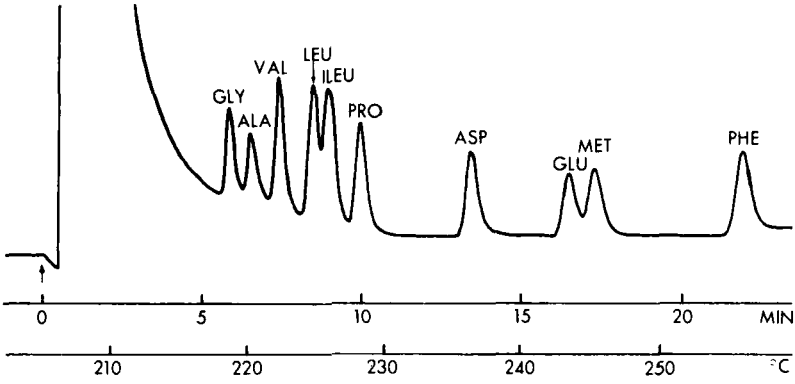


FIG. 5. GLC of *N*-diethylphosphate amino acid methylester derivatives. The injected mixture contained 5×10^{-11} moles of each amino acid and was determined by a Rb_2SO_4 alkali-flame detector. Column: 6% OV-17 on Chromosorb G, HP, 80/100 mesh; Pyrex U-tube 2 m × 3.5 mm i.d. N_2, 19 ml/min; H_2, 40 ml/min; air, ca. 200 ml/min.

Since the response of the alkali-flame detector is determined almost exclusively by the phosphate group, and, furthermore, since the amino acids are present in equimolar amounts, the peaks obtained are of similar area. This indicates that the derivatization procedure is quite efficient and encourages further studies in quantitation.

The significant IR absorptions of the amino acid derivatives were similar in all the cases investigated and in accordance with the assumed structure—as is the NMR spectrum of the glycine derivative. The two overlapping quadruplets characteristic for the methylene protons of the ethyl groups occur in phosphorus compounds of similar structure (*11*).

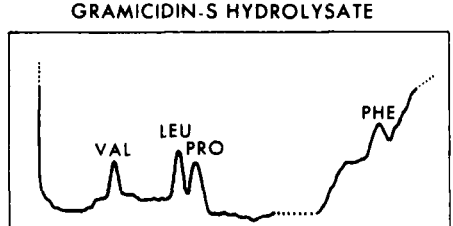


FIG. 6. Conditions, retention times, and temperatures as given in Fig. 4. Injected amount represents 2.5×10^{-10} g of unhydrolyzed peptide.

The minimum detectable limit obtainable under the described conditions was determined using the alanine derivative. At a peak-to-noise ratio of 2:1 it corresponds to somewhat less than 5×10^{-12} g of alanine injected.

To show the applicability of the developed method to a naturally occurring peptide, gramidicin-S was hydrolyzed and esterified in the usual way and the resulting mixture of amino acid methylesters subjected to phosphorylation. Gramicidin-S is a cyclopeptide with five amino acids in the following order: L-val, L-orn, L-leu, D-phe, L-pro. The above unit occurs twice in a closed peptide chain. Clearly discernable peaks were obtained for the monofunctional amino acid derivatives from an injected amount representative of 250 pg of original peptide. The peaks representing valine, leucine, proline, and phenylalanine have similar areas, as shown in Fig. 6, since the amino acids are present in equimolar amounts. A chromatographic peak for ornithine was not obtained.

Acknowledgment

We express our appreciation to Mr. Larry L. Wall for collecting GLC effluents for IR analysis.

REFERENCES

1. W. M. Lamkin and C. W. Gehrke, *Anal. Chem.*, **37**, 383 (1965).
2. C. W. Gehrke, W. M. Lamkin, D. L. Stalling, and F. Shahroki, *Biochem. Biophys. Res. Commun.*, **13**, 328 (1965).
3. C. W. Gehrke and D. L. Stalling, *Separation Sci.*, **2**(1), 101 (1967).
4. D. L. Stalling, C. W. Gehrke, and G. Gille, *Anal. Biochem.*, **1**, 118 (1967).
5. A. Karmen and L. Giuffrida, *Nature*, **201**, 1204 (1964).
6. L. Giuffrida, N. F. Ives, and D. C. Bostwick, *J. Assoc. Offic. Anal. Chemists*, **49**, 8 (1966).
7. W. A. Aue and C. W. Gehrke, paper presented at the *152nd National ACS Meeting, New York, Sept. 1966.*
8. C. W. Anderson, T. Blodinger, R. W. Young, and A. D. Welche, *J. Am. Chem. Soc.*, **74**, 5304 (1952).
9. W. A. Aue, C. W. Gehrke, R. C. Tindle, D. L. Stalling, and C. D. Ruyle, *J. Gas Chromatog.*, **5**, 381 (1967).
10. J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Vol. 2, Wiley, New York, 1961, p. 925.
11. J. Ross, personal communication, 1967.

Received by editor September 26, 1967
Submitted for publication October 2, 1967