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Determination of Protein Amino Acids as the N-TFA N-Butyl Esters by Gas Liquid Chromatography

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Gas Liquid Chromatography에 의한 단백질 아미노산의 분석

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Abstract

For effective separation of the N-TFA n-butyl ester amino acids on the stainless steel column by GLC, dual column of the mixed stationary phases, 3.36% OV-17 + 3.0% SE-30 (column 1) and 1% NPGS + 0.5% OV-17 + 0.5% SE-30(column 2) on chromosorb W HP 100-120 mesh, were used. On the column 1, the nineteen amino acids except histidine were obtained. However, alanine and valine peaks were not separated by this column. On the column 2, the sixteen amino acid peaks showed good separation, but tryptophan, arginine, histidine, and tyrosine peaks were not obtained. Calibration graphs for all amino acids obtained by the plotting the ratios of their peaks hights to that of internal standard versus the micro mole of the amino acids in the range 1.25×10^{-3} μmol-1.0×10^{-2} μmole showed linearity and passed through the origin.

Kew words: N-TFA n-butyl ester, mixed stationary phases, protein amino acids

Introduction

In the amino acids analysis, ion exchange chromatography (IEC) has been the most widely used because of the construction of fully automated devices. Automated IEC amino acid analyzer are applicable to the analysis of carbohydrates, amines and other compounds with slight modification. However, the defects of IEC method is their low sensitivity and relatively long analaysis time in comparision with GLC method. These drowbacks are still completely unresolved in spite of the application of fluorescamine, pyridoxal or radioactive reagents instead of ninhydrin for detection, and spherical ion exchangers for column packing.[1]

The analysis of amino acids by gas liquid chromatography have been the subject of extensive researches because the qualitative and quantitative determination of amino acids by GLC method can be achieved rapidly, accurately, and cheaply in comparision with other techniques. Moreover, GLC methods have tremendous adventages in resolution, versatility, and reliability, and sensitivity in small amount of samples such as biological liquid for measurement of amino acids.[1-4] However, the advance of a practical GLC method has been limited by the difficulty of preparing a volatile derivatives and of separating all natural amino acids with a single column. In the analysis of amino acids by GLC a complete derivatization is essential. Due to the differences of chemical structure and reactivity of each amino acid, the quantitative derivatization of all the fuctional groups by one step reaction has many problems.[4] Moreover, a complete resolution of the twenty protein amino acids on a single has been extremely difficult to achieve even with any derivatization methods.

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It was the purpose of this study to develope a liquid phase which can resolve the twenty amino acids as their N-trifluoroacetyl (N-TFA) n-butyl ester derivatives, the most widely using method. Twenty liquid phases known to resolve amin acids were examined with one or combination of two or three, but the stationary phase resolving twenty protein amin acids on single stainless steel column were not found in this study. Two stationary phases showed good resolution by the combination of dual column are reported.

Materials and Methods

Reagents

The amino acids used in this research were obtained from Sigma (ST. Louis, USA). N-butanol and methylene chloride were first refluxed over anhydrous calcium chloride and redistilled from an all glass system and stored in a dessicator to protect from atmospheric moisture. Trifluoroacetic anhydride (TFAA) was obtained from Aldrich (Milwaukee, USA). Pure anhydrous HCl gas was generated by slow addition of concentrated sulfuric acid into concentrated hydrochloric acid. The generated HCl gas was passed through two drying towers containing concentrated sulfuric acid and then slowly bubbled into n-butanol until 3N in HCl. The generating rate of HCl gas was controlled by addition rate of concentrated sulfuric acid. The normality of n-butanol-HCl was checked by titration with standard NaOH. Liquid phases were obtained from Althech (Deerfield, USA). The supporting material, chromosorb W HP, was obtained from Sigma (ST. Louis, USA).

Preparation of chromatographic column packings

Column 1; 2 m × 3 mm I.D. Stainless steel column

Packing; 3.36% OV-17 + 3% SE-30 on chromosorb W HP 100-120 mesh

Column 2; 2 m × 3 mm I.D. Stainless steel column

Packing; 1% Neopentyl glycol succinate (NPGS) + 0.5% OV-17 + 0.5% SE-30 on chromosorb W HP 100-120 mesh

Derivatization, esterification and acylation

Derivatization, esterification and acylation were carried out by a slightly modified procedure from that of Gehrke *et al.*[5] Individual amino acid standard solution was prepared to 0.25m mole/100 m*l*. Each 2 m*l* of individual amino acid standard solution was placed in a 125 m*l* round bottom flask. Five milliliters of the internal standard solution (0.1 mg/m*l* of ornithine in 0.1N HCl) were added. The mixture of standard solution was evaporated to dryness with a rotary evaporator under vacuum in a 60 °C water bath. Fifteen milliliters of n-butanol-HCl were added and then a magnetic stirring bar was placed in the flask. A glass drying tube containing $CaSO_4$ anhydrous was attached to the mouth of the flask. The flask was placed in an ultrasonic bath for 1 min and then into a 100 ± 2 °C oil bath on a magnetic hot plate for 15 ± 1 min to esterification by stirring. After esterification, the flask was cooled to room temperature and the contents were dried with rotary evaporator under vacuum in the 60 ± 5 °C water bath. Five milliliters of methylene chloride were added to azeotrope any remaining moisture and dried again as above. The flask was cooled to room temperature. Two milliliters of methylene chloride and 1 m*l* of trifluoroacetic anhydride were added and the flask was tightly stopped with glass stopper winded with polytetrafluoroethylene (PTFE) thread seal tape, and placed on the magnetic stirrer. The mixture was stirred for 1 min and then transferred into the pyrex glass tube of 10 m*l* with the screw stopper. The tube was closed securely with a PTFE tape-lined screw stopper. The tube was immersed into the 150 ± 2 °C oil bath for 5 min to acylation of n-butyl ester. Following acylation, the derivatized amino acids were cooled to room temperature and injected to the column immediately for analysis.

Results and Discussion

Chromatograms of the columns

The chromatogram shown in Fig. 1 was obtained with the column of the 3.36% OV-17 + 3.0%

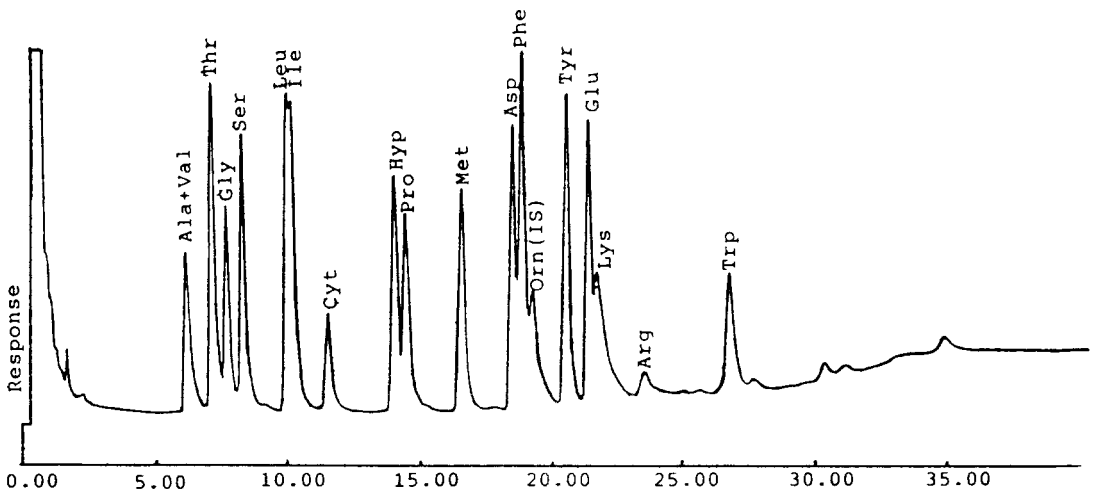

Fig. 1. Analysis of the standard amino acids in the form of N-TFA-n-butyl esters.
Column 1: 2 m × 3 mm I.D. stainless steel, with the mixed phases of 3.36% OV-17 and 3.0% SE-30 on 100-120 mesh chromosorb W HP. Temperature programme: 85°C-245°C, 5°C/min. Carrier gas N_2, 60 ml/min. FID injection amount: 5.0 $\times 10^{-3}$ μmole (I.S: ornithine, 1.2×10^{-3} μmole).

SE-30. This column provided the separation of eighteen amino acids except alanine, valine, and histidine. The alanine and valine were not separated by this column, but histidine peak was not obtained. Gehrke *et al.*[6] elucidated the interaction of the histidine, arginine, and cystine derivatives with the supporting materials and liquid phases. Gehrke *et al.*[7] also concluded that the histidine derivative was destroyed by the polar liquid phases, such as ethylene glycol adipate (EGA) and that the derivatives of arginine, histidine, and cystine were all subject to complex, temperature dependent interactions with polar liquid phases and supporting materials. They found that these derivatives could be separated on the glass columns of siloxane liquid phases prepared by deactivated supporting materials or by capillary column. As the N-TFA n-butyl esters were found to decompose in the heated metallic injection ports and on the heated metallic column surface,[10] direct injection on glass column was reported to be obligatory.[11] But arginine was separated on the column 1 of the siloxane mixed stationary phase of OV-17 and SE-30 in spite of stainless steel column. The histidine derivatives may be decomposed on the heated metallic column surface as the histidine did not appear by the column 1 despite of the non-polar liquid phases. The cystine may be destroyed to cysteine by HCl during derivative preparation process because the cystine peaks appeared at the same position of cysteine. Glutamine and asparagine also were converted to glutamic acid and aspartic acid.

The chromatogram of the column 2 is given in Fig. 2. This column showed the good resolution for the sixteen amino acids except tryptophan, histidine, tyrosine, and cystine. In this column cystine also appeared at the cysteine position. From the start of the GLC analysis of N-TFA n-butyl ester, the polyester stationary phases were mainly used rather than the other phases.[1] In the first study,[8] all protein amino acids except only unresolved pair of aspartic acid and phenylalanine were almost completely separated by the column of 1% NPGS on Gas-chrom A. With the other study,[9] the column of 0.5% NPGS on chromosorb G AW further improved the separation charactristics than above column. But these columns were all glass columns. In the others studies showed good resolution of protein amino acids the glass columns were almost used. Moreover, Gehrke *et al.*[10] reported that the reproducibly and quantitatively elution of the N-TFA n-butyl esters of the arginine, histidine, and cystine on the polyester

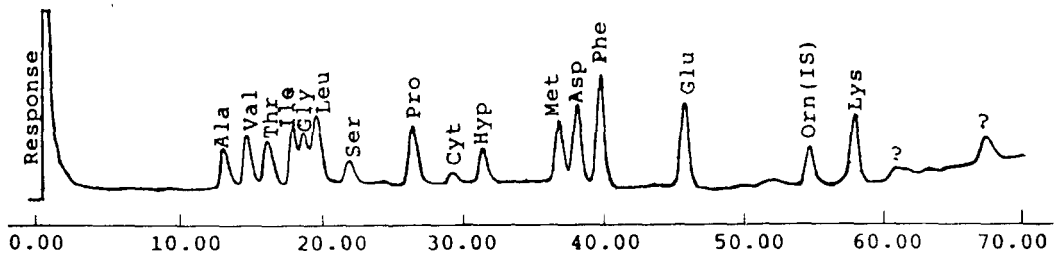

Fig. 2. Analysis of the standard amino in the form of N-TFA-butyl esters.
Column 2;2 m×3 mm I.D. stainless steel, with the mixed phases of 1% NPGS+0.5% OV-17+0.5% SE-30 on 100-120 mesh chromosorb W HP. Temperature Programme: 85°C-230°C, 2°C/min. Carrier gas; N_2, 75 ml/min. FID. Injection amount: 5.0×10^{-3} μ mole (I.S.: ornithine, 1.2×10^{-3} μ mole)

columns was very difficult problem even by glass column. In this study, column 2 was attempted to separate with the addition of NPGS, polyester liquid phase, alanine and valine unresolved on column 1. The alanine and valine were separated, but tryptophan, tyrosine, and arginine peaks obtained on column 1 were not found. NPGS, the polyester liquid phase, may be responsible for the destruction of these derivatives.

Galibration graphs for all amino acids

Calibration graphs for all amino acids obtained by plotting the ratios of their peak heights to that of the internal standard versus the mole of amino acids in the range 1.25×10^{-3} μ mole-1.0×10^{-2} μ mole showed linearity and passed through the origin (Fig. 3 and 4).

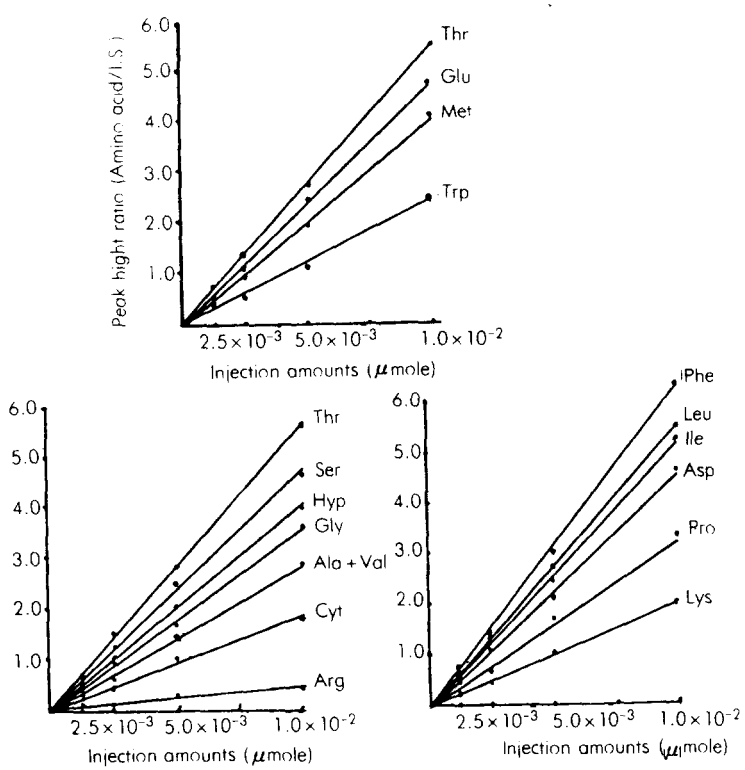

Fig. 3. Calibration curves for amino acids in the range 1.25×10^{-3}-1.0×10^{-2} μ mole on column I. Internal standard, L-ornithine; 1.2×10^{-3} μ mole.A spot is the average of three determinations.

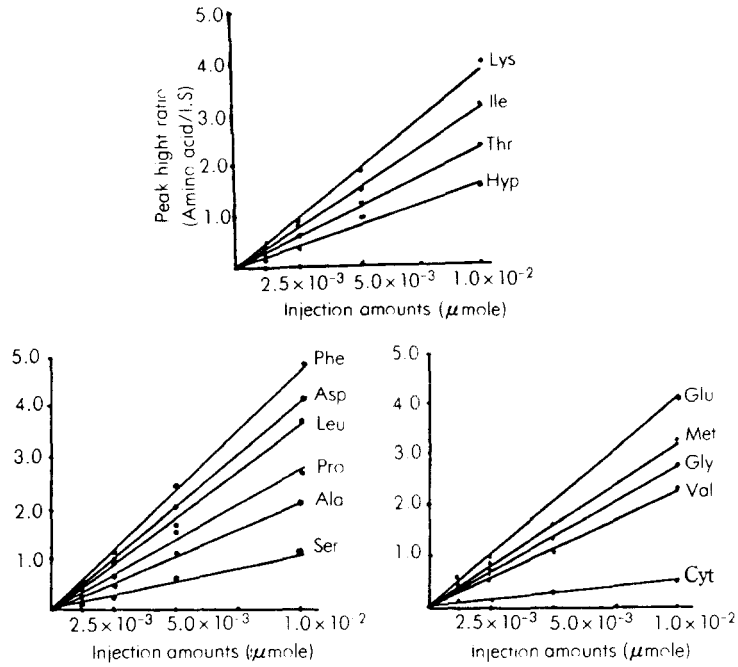

Fig. 4. Calibration curves for amino acids in the range 1.25×10^{-3} μmole. A spot is the average of three determinations.

요 약

GLC를 이용 N-TFA n-butyl ester 유도체화에 의한 아미노산 분석시 stainless steel packed column으로 효과적인 분리를 할 수 있는 고정상을 찾기 위하여 다음과 같은 2개의 column을 개발하여 표준 아미노산의 N-TFA n-butyl ester 유도체를 분석하였다.

Column 1 ; 3.36% OV-17+3.0% SE-30 on chromosorb W HP 100-120 mesh. Stainless steel column (2 m×3 mm I. D.)

Column 2 ; 1% NPGS+0.5% OV-17+0.5% SE-30 on chromosorb W HP 100-120 mesh. Stainless steel column (2 m×3 mm I. D.)

Column 1에서 histidine을 제외한 19개의 아미노산 peak가 나타났으나 alanine과 valine peak는 분리되지 않았다. Colume 2에서는 15개의 아미노산이 좋은 분리를 이루었고 column 1에서 분리되지 않은 alanine과 valine이 잘 분리되었으나 column 1에서 분리된 tryptophan, arginine 및 tyrosine이 나타나지 않았다. 분리된 모든 아미노산은 column 1, 2를 막론하고 아미노산 주입량 1.25×10^{-3} μmole−1.0×10^{-2} μmole의 범위에서 각 아미노산 peak의 높이와 내부표시제 peak 높이와의 비에 대한 주입량으로 나타낸 검정선이 선형을 나타내었고 모두 원점을 지났다. Glutamine, asparagine 및 cystine은 column 1, 2에서 모두 glutamic acid, aspartic acid 및 cysteine peak와 일치하는 것으로 보아 N-TFA n-butyl ester 유도체를 만드는 과정에서 염산에 의해 각각 glutamic acid, aspartic acid 및 cysteine으로 전환되는 것으로 사료된다.

References

1. Husek, P. and Macek, K.: Gas chromatography of amino acids. *J. Chromatogr.*, 113, 139 (1975).
2. Makita, M., Yamamoto, S. and Kono, M.: Gas-liquid chromatographic analysis of protein amin acids as N-iso-butyl oxycarbonyl amino acid methyl esters. *J. Chromatogr.*, 120, 129 (1976).
3. Gehrke, C.W., Wall, L.L., Absheer, J.S., Kaiser, F.E. and Zumwalt, R.W.: Sample preparation for chromatography of amin acids: Acid hydrolysis of

proteins. In *amino acid analysis by gas chromatography,* Zumwalt, R.W., Kuo, K.C.T. and Gehrke, C.W. (ed), CRC press, Inc. Boca Raton, Florida, Vol. 1, p. 2 (1987).

4. Zumwalt, R.W., Roach, D. and Gehrke, C.W.: Gas-liquid chromatography of amin acids in biological substances. *J. Chromatogr.,* **53**, 171 (1970).
5. Gehrke, C.W., Kaiser, F.E., Kuo, K.C.T. and Zumwalt, R.W.: Gas-liquid chromatography of amino acids as the N-TFA n-butyl esters. In *amino acid analysis by gas chromatography,* Zumwalt, R.W., Kuo, K.C.T. and gehrke, C.W. (ed), CRC press, Inc. Boca raton, florida, Vol. 1, p. 31 (1987).
6. Gehrke, C.W., Younker, D.R., Gerhardt, K.O. and Kuo, K.C.: Gas-liquid chromatography of histidine, arginine, and cystine. Interactions with the liquid and solid support phase. *J. Chromatogr. Sci.,* **17**, 301 (1979).
7. Gehrke, C.W., Kuo, K.C. and Zumwalt, R.W.: Research on the N(O,S)-TFA n-butyl esters and trimethylsilyl derivatives: Derivatization, chromatography, mass spectrometry, interactions, and application. In *amino acid analysis by gas chromatography,* Zumwalt, R.W., Kuo, K.C.T. and Gehrke, C.W. (ed), CRC press, Inc. Boca raton, Florida, Vol. 1. p. 31 (1987).
8. Zomzely, C., Marco, G. and Emergy, E.: Gas chromatography of the N-trifluoroacetyl-n-butyl derivatives of amin acids. *Anal. Chem.,* **34**, 141 (1962).
9. Gehrke, C.W. and Stalling, D.L.: Quantitative analysis of the twenty natural Protein amin acids by gas-liquid chromatography. *Sep. Sci.,* **2**, 101 (1967).
10. Lamkin, M.W. and Gehrke, C.W.: Quantitative gas chromatography of amino acids. *Anal. Chem.,* **37**, 383 (1965).
11. Stalling, D. L. and Gehrke, C.W.: Quantitative analysis of amino acids by gas chromatography: Acylation of arginine. *Biochem. Biophys. Res. Commun.,* **22**, 329 (1966).

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