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Note

Determination of DL-asparagine by gas chromatography

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A great deal of effort has been made over the last few years to analyze amino acids by gas chromatography (GC) or high-performance liquid chromatography (HPLC). Especially the resolution of DL-Asn has always been difficult. Using HPLC, separation factors (α) of 0.87 (ref. 1), 1.23 (ref. 2), 1.25 (ref. 3) and 1.5 (ref. 4) have been achieved. Using GC, the main problem has always been to avoid the degradation, due to acid hydrolysis, of the amides, Asn and Gln. Makita *et al.*[5] introduced a derivatization method in which the volatile derivatives N(O,S)-isobutoxycarbonyl(iso-Boc)-methyl esters of amino acids were prepared by two-step procedures involving iso-*n*-butoxycarbonylation with isobutyl chloroformate (iso-BCF), followed by esterification with diazomethane. The use of trimethylsilylation has been reported by Ruhlmann and Giesecke[6]. Hušek[8] reported another derivatization method, forming cyclic compounds using 1,3-dichlorotetrafluoroacetone in combination with reactive anhydrides such as heptafluorobutyric anhydride. But, due to the planar structure of oxazolidinones, no resolution of D- and L-enantiomers was obtained. In the other papers mentioned, no effort of enantiomer separation has been made; hence, it seemed necessary to find a quick and sensitive method with which even DL-Asn resolution is possible.

EXPERIMENTAL

Reagents and materials

DL- and D-asparagine monohydrate were purchased from Sigma (St. Louis, MO, U.S.A.) and DL-aspartic acid and L-asparagine were from Schwarz/Mann (Orangeburg, NY, U.S.A.). Acetylchloride was supplied by Mallinckrodt (KY, U.S.A.); 2-propanol, trifluoroacetic anhydride, acetone (Photorex) and dichloromethane were from J. T. Baker (Phillipsburg, NJ, U.S.A.).

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Sample preparation

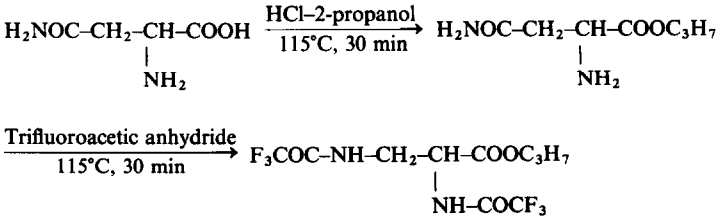
A 10-μl volume of aqueous amino acid solution (*ca.* 50 mg/ml) was evaporated either by gentle nitrogen stream or by vacuum freeze-drying. After addition of 250 μl 1.5 *N* hydrochloric acid–2-propanol, the mixture was heated at 115°C for 30 min, then cooled in an ice bath, and solvent evaporated off. A 1-ml volume of trifluoroacetic anhydride–methylene dichloride (50:50) was added and the solution heated again at 115°C for 10 min. After cooling in ice and evaporating off the excess reagents, the derivatized amino acid was dissolved in 500 μl methylene dichloride and 1-μl aliquots were injected into the gas chromatograph.

Gas chromatography

For GC analysis a Hewlett-Packard Model 5790 gas chromatograph equipped with a 60 m × 0.9 mm fused-silica Chirasil-Val column was used with helium as the carrier gas at an inlet pressure of 10 p.s.i. (0.7 bar). The nitrogen–phosphorus detector injector port was maintained at 250°C. The system was programmed as follows: injection at 40°C, after 30 sec a rapid temperature increase to 80°C, then to 180°C at 4°/min. The peaks were detected with a Hewlett-Packard nitrogen–phosphorus detector with an air flow-rate of 50 ml/min and a hydrogen flow-rate of 30 ml/min. The peaks were computed with a Hewlett-Packard 3390 A electronic integrator.

RESULTS AND DISCUSSION

Because of the mild hydrolysis conditions the asparagine is preserved and not, as is usually the case, converted into aspartic acid. It is assumed that only partial hydrolysis occurs which maintains the amide character of Asn. This leads to the di-N-trifluoroacetyl-Asn-isopropyl ester rather than to the N-trifluoroacetyl-diisopropyl ester of aspartic acid:



Their different retention times allow a clear distinction from each other as is seen in Fig. 1. By using the Chirasil-Val column and a temperature program of 1.5°C/min, baseline separation of the single enantiomers can be achieved. The Asn peaks are eluted later than the Asp peaks. The Asn:Asp ratio seems to be directly dependent on the hydrolysis conditions. Under the mildest conditions, essentially only Asn appears, whereas under the strongest conditions, only Asp is observed (Fig. 2). The best results for obtaining Asn have been achieved by treatment with 1.5 *N* hydrochloric acid and heating at 115°C for 30 min. Unfortunately, it has not been possible to obtain only Asn. Using strong hydrolysis conditions (4 *N* hydrochloric acid and heating at 115°C for 3 h) only Asp is obtained.

Since glutamine differs from asparagine only by one CH_2 group, it is expected

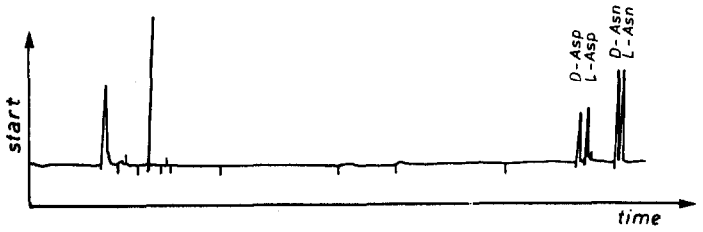


Fig. 1. Resolution of DL-asparagine and DL-aspartic acid. Aspartic acids are the by-products of asparagines; for hydrolysis, derivatization and separation conditions see text. The Asp and Asn peaks appear after ca. 30 min.

that preservation of the amide could also be possible. Similar treatment of Gln, as described for Asn, shows equivocal results (data not shown). Only once has a second peak pair been observed, but since it has not been reproducible we assume that an artifact was present. Even the mildest hydrolysis conditions seem to be too strong to preserve the amide in glutamine.

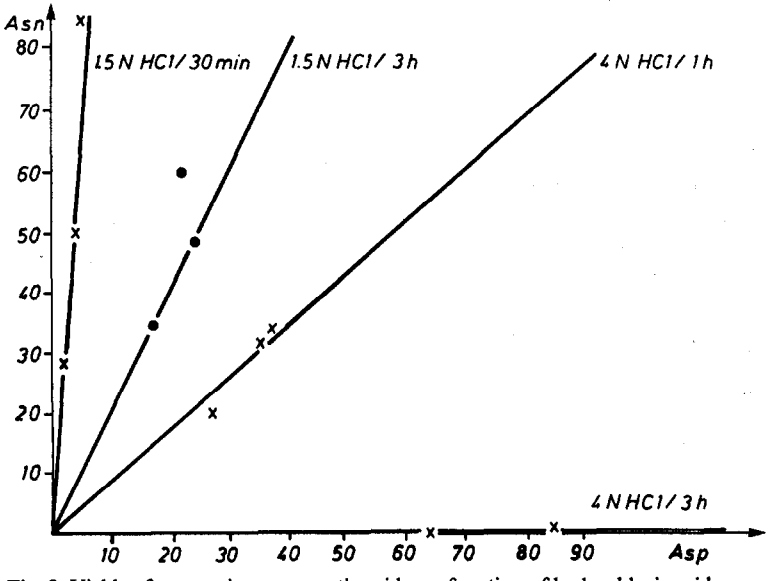


Fig. 2. Yields of asparagine *vs.* aspartic acid as a function of hydrochloric acid concentration and reaction time. The axis scales give the peak area in arbitrary units.

CONCLUSION

As the above results demonstrate, the best preservation of asparagine has been achieved by derivatizing according to our protocols with 1.5 *N* hydrochloric acid at 115°C for only 30 min. Since no reproducibility was obtained for glutamine, milder hydrolysis conditions have to be found. Because of its excellent resolving power and very high sensitivity, this method could be applied to amino acid analysis of proteins and peptides.

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