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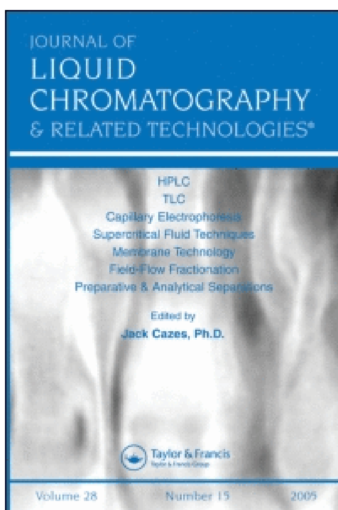

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RAPID AMINO ACID ANALYSIS IN BIOTECHNOLOGY: DETERMINATION OF ALANINE AND ASPARTIC ACID

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ABSTRACT

A fast and sensitive chromatographic method was developed to monitor the enzymatic conversion of aspartic acid into alanine in a membrane reactor. The amino acids were converted into dansyl derivatives which were separated by ion-pair chromatography on a reversed-phase column and detected by fluorimetry using only conventional HPLC equipment.

INTRODUCTION

The use of biotechnological processes to produce amino acids has recently been gaining in importance (1). Their main advantage in comparison with most chemical syntheses is that pure enantiomers are formed. L-amino acids are produced not only using cultures of microorganisms, but also with isolated enzymes as reaction catalysts (2). For example, L-amino acids are produced very successfully by using soluble enzymes in an enzyme-membrane reactor where the substrate solution is pumped continuously into a stirred tank reactor. Ultrafiltration is used to retain the enzymes in the reaction vessel and to allow the amino acids formed to be collected in the effluent (3,4). L-α-alanine is produced from L-aspartic acid by the soluble enzyme L-aspartate-β-dicarboxylase. Measuring the optical rotation of the products is one way to record the conversion (5). This method, however, is problematic in low

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concentration ranges (c < 100 mM) and is hampered by problems like bubble formation (CO_2) and protein precipitation.

Chromatographic methods can be used for measuring process kinetics if run in a repetitive way. A chromatographic method suitable for amino acid analysis of the effluent of a membrane reactor has to be fast to allow analysis of a large number of samples and sensitive to allow measurements of the beginning and end phase of the reaction with low concentrations of the end product and of the precursor. In spite of its routine use for amino acid analysis, ion-exchange chromatography is too slow to be used in the monitoring of the conversion in a membrane reactor and, furthermore, accumulation of contaminants in the column necessitates frequent regenerations. Systems with column-switching could overcome some of these problems.

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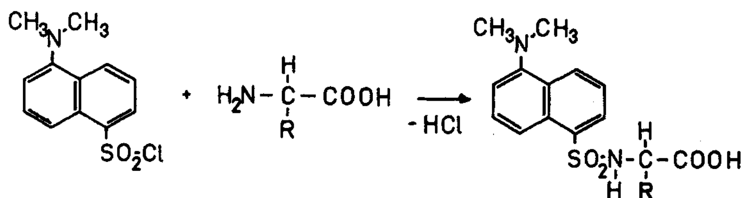
Reversed-phase columns are, by far, most frequently used in HPLC. However, compounds like polar amino acids are hardly retained on these columns. Therefore, pre-column derivatization has to be performed to convert amino acids into more unpolar derivatives which also can be more easily and sensitively detected. The main disadvantage of pre-column derivatization is the time and labor necessary to treat the samples. However, preparation time per sample reduces with increasing numbers of samples.

Conversion of amino acids into fluorescent dansyl derivatives (see equation below) has been used to improve chromatographic separations and to enhance sensitivity (6, 7).



dansyl chloride + amino acid ⟶ dansylated amino acid

There are several publications on the separation of complex mixtures of dansyl amino acids (8 - 12). The task of this investigation, however, was to find out conditions which allowed the analysis of just two amino acids in the shortest possible time. We now describe the use of the dansylation technique in combination with high-pressure liquid chromatography for the rapid and sensitive monitoring of amino acid conversion in a membrane reactor.

MATERIALS AND METHODS

Apparatus

A Waters (GmbH) M 6000A pump was connected with a U6K injector and a μ-Bondapak C18 column (30 x 0.4 cm, 10 μm particles, octadecylsilyl phase). Detection was achieved with a fluorescence detector (Du Pont 836) equipped with a 16 μl flow cell. The excitation wavelength was λ = 250 - 390 nm while emitted light passed through a cut-off filter of λ = 451 nm. Signals were recorded by a Laumann (Selb, Bavaria) recorder and processed by a Spectraphysics System I Computing Integrator.

Chemicals

Acetonitrile was p.a. grade (Merck). It was distilled prior to use. L-amino acids were purest available grade (Sigma). Other chemicals were p.a. reagents (Merck) with the exception of tetrabutylammonium bromide (Fluka).

Sample Derivatization

A modification of the method described by Tapuhi et al. (13) was used. In a glass vial with a teflon-lined screw cap, 10 μl of a sample containing up to 0.2 M amino acid were mixed with 1 ml dansyl chloride solution (3 mg/ml in acetonitrile) and 2.0 ml buffer containing the internal standard (40 mM Li_2CO_3, adjusted to pH 10 with HCl, 1.0 mM glycine). The solution was

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kept 15 min at 60 °C. Thereafter, 100 µl of proline solution (5 % in water) was added to bind the excess of dansyl chloride, and the reaction was completed at 60 °C (5 min). After cooling to room temperature, 25 µl of this mixture was injected for analysis. The derivatized samples are sufficiently stable at room temperature and in the dark.

Eluent Preparation and Chromatographic Conditions

The eluent consisted of acetonitrile (32 %, 34 %, 36 % or 39 %) and aqueous buffer. To prepare the aqueous buffer, 100 ml of a stock solution (aqueous solution of 40.8 g sodium acetate x 3 H_2O and 16.1 g of tetrabutylammonium bromide in 1000 ml) were mixed with 0.9000 to 5.400 g acetic acid and brought to 1000 ml with water. These concentrations correspond to 30 mM sodium acetate, 5 mM tetrabutylammonium bromide, and 15 - 90 mM acetic acid. The eluent selected for amino acid analysis consisted of 36 % acetonitrile (v/v) and 64 % aqueous buffer (30 mM sodium acetate, 45 mM acetic acid, and 5 mM tetrabutylammonium bromide).

HPLC was performed at room temperature (21 °C) and with a flow of 2.0 ml/min.

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Determination of Amino Acid Concentrations

A calibration curve was obtained with standard solutions of 0 - 0.2 M amino acid, derivatization as described above and by measuring the peak areas. Amino acid concentrations of samples were calculated using the program of the integrator (method 2: Internal Standard).

RESULTS AND DISCUSSION

For the reliable analysis of aspartic acid and alanine by HPLC of the dansyl derivatives, the derivatization procedure as well as the chromatographic separation had to be optimized.

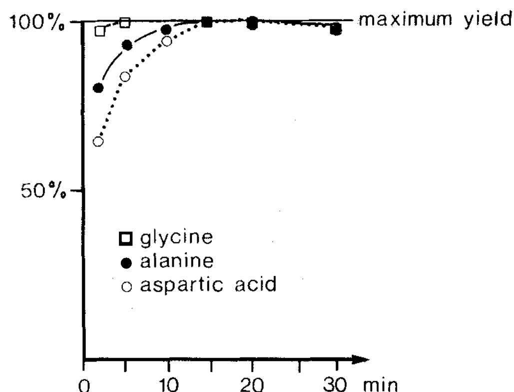

FIGURE 1. Time course of the reaction of aspartic acid, alanine and glycine with dansyl chloride.

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Previous problems with varying yields depending on the excess of dansyl chloride in relation to the amino acid concentration have been mostly overcome by the use of acetonitrile as solvent and lithium carbonate as buffer component in the derivatization procedure (13). In order to establish a stable baseline and to increase the stability of the reaction products, it was found that binding of the excess of dansyl chloride is important. The use of an internal standard increased the accuracy of the method considerably. A suitable internal standard had to show similar reactivity with all compounds analyzed. Glycine has a similar reaction rate with dansyl chloride when compared with alanine and aspartic acid (14) and was, therefore, chosen as internal standard. As can be seen from figure 1, the yield of the dansylation reaction of the amino acids reached the maximum after 5 - 15 minutes. A reaction time of 5 minutes was found sufficient for analysis. Proline, which was used to bind the excess of dansyl chloride, reacted within seconds under the reaction conditions applied.

The separation of dansyl derivatives of amino acids on reversed-phase columns has been achieved mainly using phosphate or acetate buffers with acetonitrile or methanol as organic

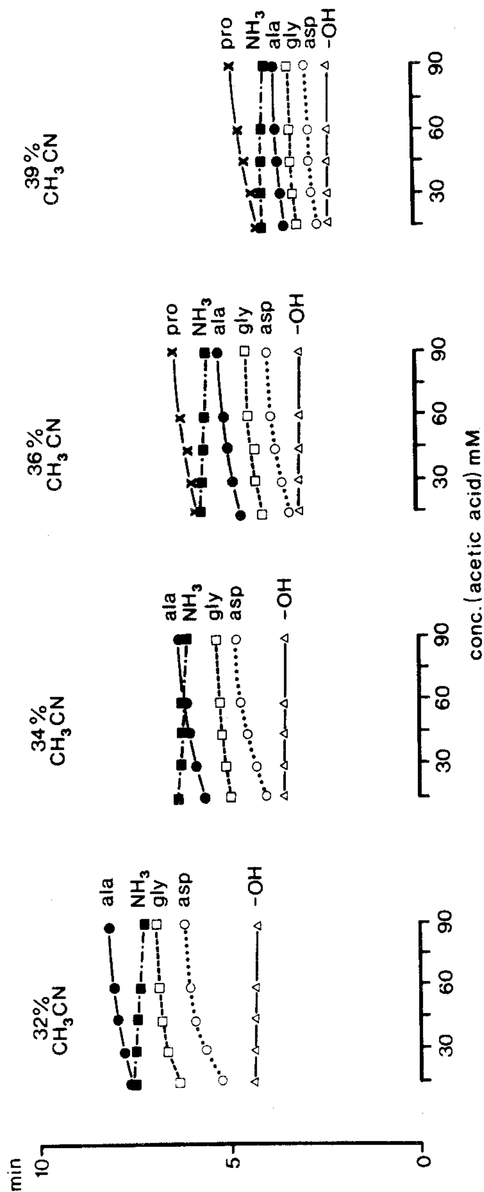

FIGURE 2. Retention of dansyl derivatives with varying eluent composition.

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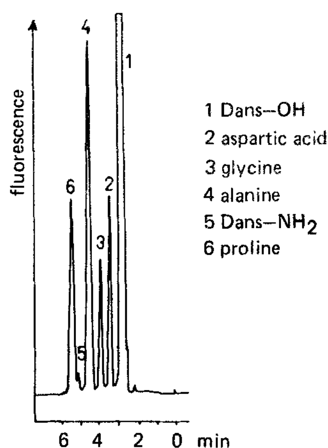

FIGURE 3. HPLC chromatogram of a dansylated sample containing alanine, aspartic acid, glycine (internal standard) and proline (dansyl chloride scavanger).

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modifiers (8 - 12). In order to resolve all 20 protein amino acids, gradient programs had to be applied. For the complete resolution of the amino acids investigated in this study in the shortest possible time more suitable separation conditions had to be found. In addition to dansyl aspartic acid, alanine and glycine (internal standard), dansyl proline (bound excess of dansyl chloride), Dans-OH (hydrolyzed dansyl chloride) and dansyl amide (side product) had to be separated. It was found that the cationic ion-pair reagent tetrabutylammonium bromide improved the peak shape of the eluted dansyl amino acids considerably. Optimum separation conditions were obtained by keeping concentrations of sodium acetate and ion-pair reagent constant and by varying acetic acid and acetonitrile concentrations.

From figure 2 it can be seen that a relatively small increase in acetonitrile concentration caused strong reductions in retention time of all compounds analyzed. Ionization of the carboxylic group of the dansyl amino acids is reduced by the addition of acetic acid, and this loss in polarity is responsible for the increased retention with increasing acid concentrations.

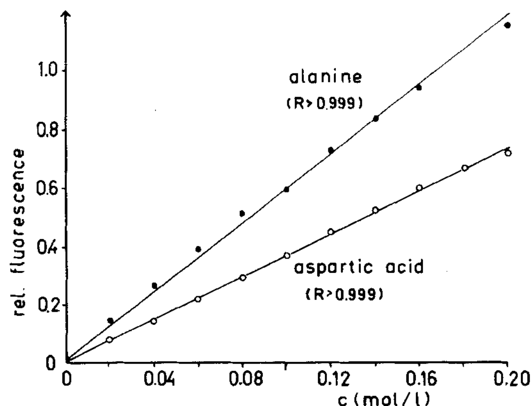

FIGURE 4. Calibration curve for the determination of aspartic acid and alanine.

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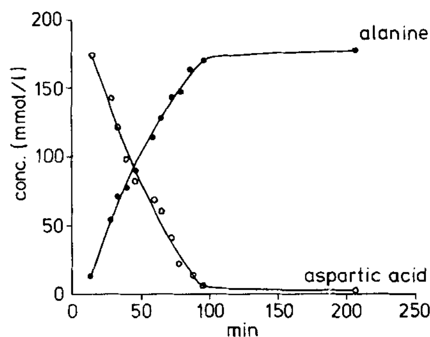

FIGURE 5. Time course of alanine production from aspartic acid in a membrane reactor.

Separation conditions were found to be optimal with 36 % acetonitrile in the solvent under the HPLC conditions used. Under these conditions all dansylated amino acids involved in the analytical system and the dansyl derivatives of ammonia and water were separated well enough to allow the analytical procedure to be established (see fig. 3). Higher acetonitrile concentrations would give even smaller retention times, but

aspartic acid would then be eluted too near to Dans-OH resulting in loss of accuracy.

With concentrations between 2.0 and 200 mM amino acid, typical for the reactor, an excellent calibration curve was obtained ($R > 0.999$; see fig. 4). The use of this calibration curve allowed the measurement of aspartic acid conversion into alanine (see fig. 5).

CONCLUSION

Using the chromatographic separation described, one run was completed within six minutes. The dansylation procedure (about 15 minutes for 10 samples) lengthened the process time per sample only slightly. Regeneration of the column after several runs yielded hardly any fluorescent material. Therefore, time-consuming regeneration procedures are not necessary adding to the convenience and speed of this method. In comparison, the analysis using a routine amino acid analyzer would require at least one hour and even with an abbreviated program not more than one injection in 30 minutes would be possible.

Although the presented method describes the determination of alanine and aspartic acid, the principles of optimization can be applied to any dansylated amino acid to be analyzed. This way, amino acids produced by fermentation or by any other process can be analyzed conveniently using standard HPLC equipment.

For semi-continuous reactor analysis automatic sample treatment would be required. Investigations are now under way to convert the method described into such a semi-continuous method for membrane reactor monitoring.

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