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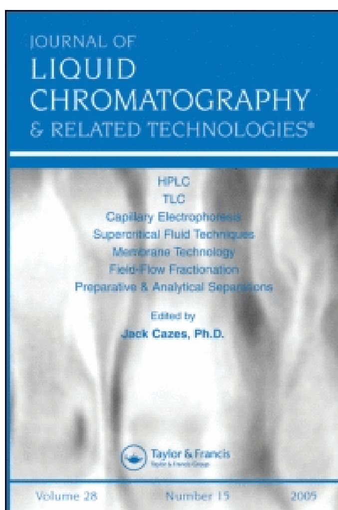

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Determination of Glutamine and Asparagine by Isocratic Elution Reverse Phase Liquid Chromatography with Fluorescent Detection

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DETERMINATION OF GLUTAMINE AND ASPARAGINE BY ISOCRATIC ELUTION REVERSE PHASE LIQUID CHROMATOGRAPHY WITH FLUORESCENT DETECTION

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ABSTRACT

A method was developed specifically for the determination of glutamine and asparagine in the presence or absence of other amino acids. The amino acids were derivatized by o-phthalaldehyde/ 2-mercaptoethanol and separated by isocratic elution with a mobile phase consisting of acetonitrile and sodium acetate buffer. An application of the method for the analysis of glutamine and asparagine in the enzymatic hydrolysate of cottonseed protein is described.

INTRODUCTION

In routine procedure for the determination of amino acid compositions of proteins and peptides, glutamine and asparagine are deamidated to glutamic acid and aspartic acid, respectively, during acid hydrolysis. It has therefore been customary in the analysis of protein hydrolysates to sum glutamine and glutamic acid as Glx and to sum asparagine and aspartic acid as Asx. The recovery of

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glutamine and asparagine in protein hydrolysis is difficult. Equally difficult is the analysis of glutamine and asparagine because there is a lack of standard methods for the identification and quantitation of these amino acids.

Glutamine and asparagine can normally be obtained by enzymatic hydrolysis. After they are separated from the protein, the free glutamine and asparagine can then be analyzed by the use of lithium buffers on ion-exchange resins (1). This ion-exchange method, though useful, is more complicated than desirable unless there is interest in the other amino acids as well, or alternately, if there are no other amino acids present.

Recently high performance liquid chromatography (HPLC) has been widely employed for the analysis of amino acids (2-7). This technique, especially with reversed-phase columns, offers greater efficiency, ease of use, and higher flow rates than the conventional ion-exchange techniques. However, for the complete analysis of protein hydrolysates, the effectiveness of HPLC in terms of reliability and reproducibility is still limited. HPLC is more suitable for the analysis of a few specific amino acids. Most reports deal with the HPLC analysis of all the amino acids, and the information is generally incomplete and insufficient in regard to the analysis of glutamine and asparagine.

We are interested in the analysis of glutamine and asparagine because our research concerns oilseed proteins which are rich in these amino acids. The ability to separate and quantitate these amino acids is very desirable for the characterization of the

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oilseed proteins.

In this study we (a) report a fast and reliable method for the analysis of glutamine and asparagine by HPLC, (b) evaluate the use of enzymes in the hydrolysis of protein, and (c) estimate the ratios of glutamine : Glx and of asparagine : Asx in the enzymatic hydrolysate of cottonseed protein.

MATERIALS AND METHODS

Apparatus

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Analyses were performed on a Beckman Model 324 gradient liquid chromatograph equipped with a Beckman Model 421 microprocessor-controller and a Krato Model FS970 liquid chromatographic fluorometer. The following fluorometer settings were used for detection: 5 μl flow cell, excitation monochrometer at 330 nm, the emission measured with a 418 nm cut-off filter, time constant of 0.5 sec, and a sensitivity dial setting at 4.7 units.

All sample injections were performed with a Beckman Model 210 sample injector, fitted with a 5 μl loop. A Beckman Ultrasphere ODS column (150 x 4.6 mm; particle size, 5 μ) fitted with a guard column (70 x 4.6 mm) packed with CO:PELL ODS sorbent (particle size, 30-38 μ) (Whatman) was used for chromatographic separations. Chromatographic peaks were recorded on a Beckman Model BD-41 recorder, and integrated by A/D converters in a Hewlett Packard 3345B laboratory automation system. Isocratic elution was used, unless otherwise indicated, in the chromatographic separation, and

the flow rate was 1.0 ml/min. The elution of the amino acids of our interest was complete in 20 min. In the case of protein hydrolysate, the elution was programmed at the end of 20 min to change to 100% acetonitrile in 5 min to wash off the remaining amino acids, followed by changing back to the original solvent in 5 min and then equilibrating for at least 10 min before conducting another injection.

Reagents and Solutions

Amino acids, 2-mercaptoethanol (ME), and o-phthalaldehyde (OPA) were purchased from Sigma Chemical Co. Storage protein of glandless cottonseed flour (Southern Regional Research Center) was prepared according to the method of Zarins and Cherry (8). Viokase was obtained from Viobin Incorporated. All other chemicals were reagent grade.

The o-phthalaldehyde/2-mercaptoethanol derivatizing solution was prepared as follows: o-phthalaldehyde (250 mg) was dissolved in MeOH (6.3 ml). 2-Mercaptoethanol (250 µl) and 0.4 M potassium borate (pH 10.5, 56 ml) were then added. The mixture was flushed with nitrogen and stored in the refrigerator. 2-Mercaptoethanol (100 µl) was added each day to help maintain the reagent strength. The reagent solution was stable for approximately one week.

Acetonitrile was distilled-in-glass grade (Burdick and Jackson) and used without further treatment. High purity water was obtained with a system from Millipore. The acetate buffers were prepared by mixing different ratios of stock solutions of 0.2 M acetic acid and 0.2 M sodium acetate to obtain various pH values and then diluting

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with water to desired concentrations. The eluting solvents were prepared by adding acetonitrile to acetate buffers, degassed for 30 min in an ultrasonic bath, and filtered through a 0.5 μm Millipore filter.

Derivatization Procedures

OPA/Me derivatives were prepared as follows: to a solution of 80 nmol amino acid or 400 ug protein hydrolysate in 0.5 ml water was added 0.2 ml OPA/ME solution. After 1 min at room temperature, 0.5 ml 0.1 M potassium phosphate (pH 4.0) was added, followed by 3.0 ml MeOH. The solution was mixed, filtered, and 5 μl injected onto the column.

Viokase-hydrolysis of Protein

The protein sample (30 mg) was suspended in 1.0 ml water, and 1.0 ml of 0.1 N NaOH was added to effect complete dissolution. To this solution, 5.0 ml of 0.04 M tris-(hydroxymethyl)aminomethane (pH 8.2 Tris buffer) in 0.11 M NaCl were added, plus 2.0 ml of dialyzed Viokase suspension (40 mg/ml Tris buffer). A drop of toluene was added and the mixture was incubated at 38°C for 24 hrs. A control was conducted following exactly the same procedure except for omission of the protein sample. At the termination of incubation, enzyme solids were removed by centrifugation, and the pH of the supernatant liquid was adjusted to pH 10.0 before lyophilization. The lyophilized hydrolysate was dissolved in 20.0 ml water and assayed by the HPLC method detailed above.

RESULTS AND DISCUSSION

The use of acetonitrile and sodium acetate buffer as the mobile

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phase has been most effective for our purposes. Sometimes, persistent coelutions could be resolved by the use of gradient elutions or by the addition of a third component such as tetrahydrofuran to the acetonitrile-acetate system. However, since simpler and more direct methods were always more reproducible, we kept the elution isocratic when possible and maintained a binary system of acetonitrile and acetate buffer as the mobile phase.

Amino acids reacted instantly with OPA in the presence of ME, but the products were unstable. There are several ways to overcome the lack of stability of the OPA/ME derivatives. The use of ethanethiol to replace mercaptoethanol (7) or the addition of sodium dodecyl sulfate (9) in the derivatization have been claimed to improve product stability and to enhance fluorescent sensitivity. We found neither method satisfactory; ethanethiol was no better than mercaptoethanol and the sodium dodecyl sulfate treatment caused precipitation problems. Instead, the addition of relatively large amounts of MeOH to the OPA/ME products immediately after the products were prepared appeared to effect improved stability and reproducibility. The derivatives of glutamine and asparagine were stable for at least one hour after preparation, whereas those of the others were stable for 10 min.

With the use of acetonitrile-acetate as the mobile phase, the OPA/ME amino acid derivatives were eluted from the reversed-phase column roughly in three groups. As shown in Fig. 1, the first group to come out generally include derivatives of asparagine (Asn), aspartic acid (Asp), serine (Ser), glutamine (Gln), histidine (His),

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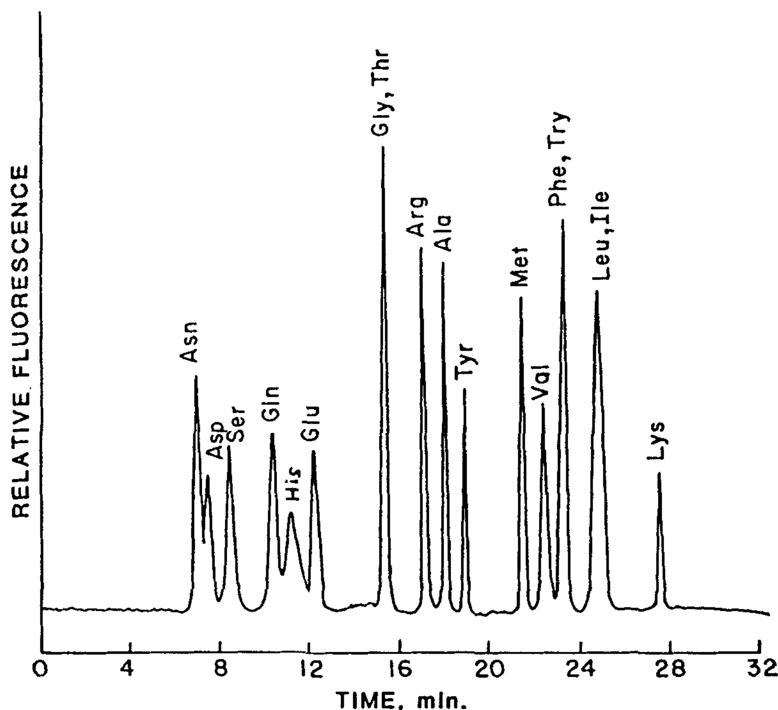

Figure 1. Elution profile of amino acid standards derivatized by the reaction with OPA/ME. Each peak represents 0.1 nmol. Elution conditions: Solvent A, acetonitrile : 0.04 M sodium acetate (pH 4.75), 12 : 88; Solvent B, acetonitrile; gradient program, isocratic at 100% A of 10 min duration, linear step to 50% B in 20 min, isocratic at 50% B of 10 min duration.

and glutamic acid (Glu). In the last group were usually methionine (Met), valine (Val), phenylalanine (Phe), tryptophan (Try), leucine (Leu), isoleucine (Ile), and lysine (Lys). Scattered in the middle were glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala) and tyrosine (Tyr).

We were interested in the analysis of four amino acids (glutamine, asparagine, glutamic acid, and aspartic acid) which,

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according to the above mentioned groupings, could be done by dealing with only the first group of derivatives. To achieve the best separation for the six derivatives of the first group, experiments were conducted to investigate effects of acetonitrile and pH in the mobile phase on the retention time. When the derivatives were eluted with solvents of constant pH but various acetonitrile concentrations, the retention time decreased and peaks began to coelute as the percentage of acetonitrile increased. The concentration of acetonitrile at 12% appeared to give the most effective separations; at higher concentrations, column resolution was poor, whereas, at lower concentrations, zone spreading became a problem and column efficiency decreased.

Another series of isocratic elutions was conducted at a constant acetonitrile concentration (12%) in buffers of various pH values (4.35-5.15). All six OPA/ME derivatives in the first group and the derivative of glycine in the second group were investigated. The resulting retention times of these seven derivatives as a function of pH are shown in Fig. 2. Glycine being normally the first to elute in the second group of derivatives, the curve representing glycine served to mark the elution front of the rest of the amino acids. The retention times of asparagine, serine, glutamine, and glycine displayed similar pattern of changes from pH 4.35 to pH 5.40; the curves declined slightly as pH increased from 4.35 to a minimum at about pH 4.60 and then climbed to a maximum at about 5.15 before decreasing again. The OPA/ME derivatives of glutamic acid and aspartic acid were the most sensitive to pH

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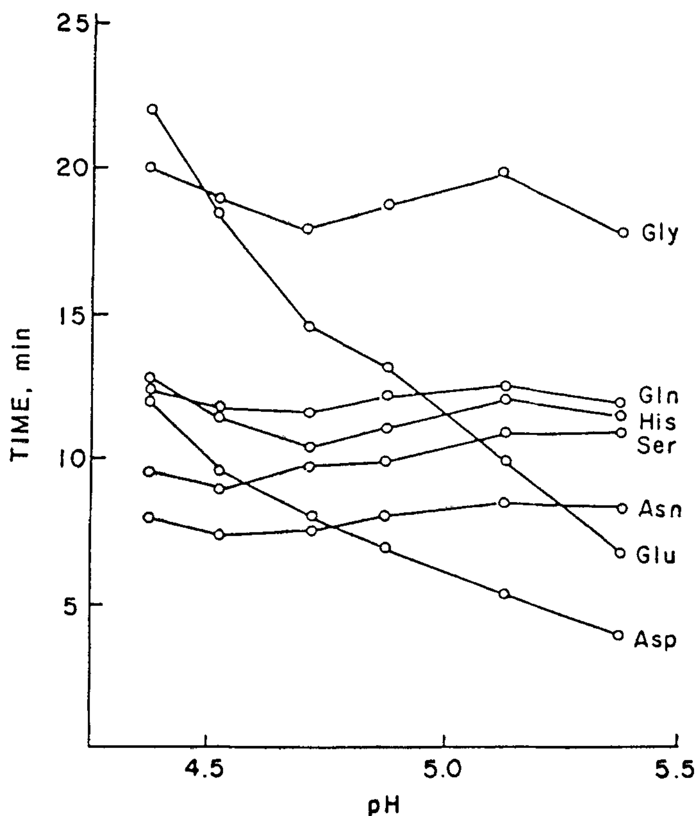

Figure 2. Influence of pH on the retention time of OPA/ME amino acid derivatives. Retention times were obtained by isocratic elutions with solvents consisting of 12% acetonitrile and 88% sodium acetate buffers (0.04 M, pH 4.40-5.40).

changes; the curves of these two amino acids dropped drastically in the pH range of 4.45 to 5.40 crossing over curves of other derivatives. The best separation of these seven derivatives appeared to occur at pH 4.85. A profile of elution under these conditions (12% acetonitrile and pH 4.85) is shown in Fig. 3.

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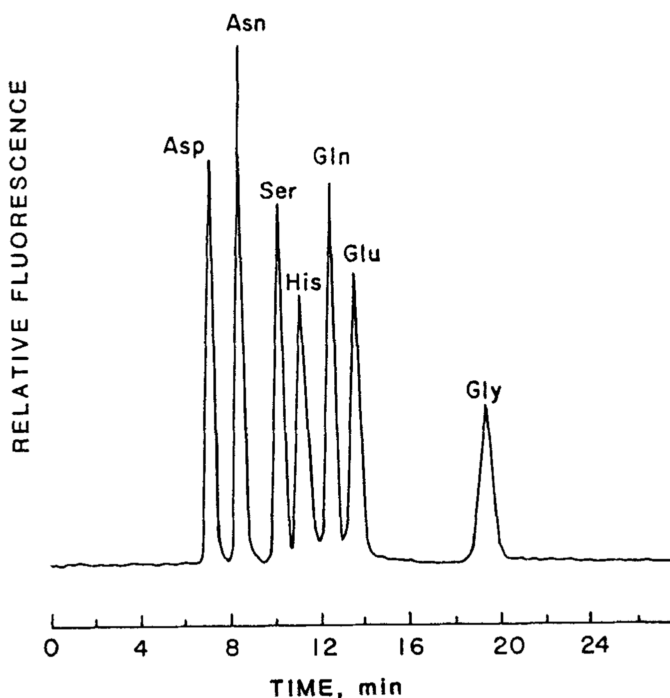

Figure 3. Elution profile of seven amino acid standards derivatized by the reaction with OPA/ME. The mobile phase of the isocratic elution consisted of 12% acetonitrile and 88% sodium acetate buffer (0.04 M, pH 4.85).

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Baseline separations for all seven amino acids were obtained. In the concentration range of 50-250 pmol, the OPA/ME derivatives of glutamine, asparagine, glutamic acid, and aspartic acid showed a linearity of response, and the quantitation of these four amino acids was remarkably reliable.

Attempts were made to determine the composition of glutamine and asparagine in the storage protein of cottonseed. The protein was hydrolyzed by various enzyme systems. A multi-step treatment,

based on the method of Hill and Schmidt (10), in which the protein was first hydrolyzed to small peptides by proteases (pepsin or papain) and then to amino acids by aminopeptidase and prolidase was unsatisfactory; the results were inconsistent. The use of Viokase (11), a one step reaction, was found by far the simplest and most consistent in the hydrolysis of cottonseed protein. Fig. 4a shows a front portion of the elution profile of the Viokase-hydrolyzed cottonseed protein as analyzed by the above described HPLC method. The elution of the amino acids of our interest was complete in 20 min and the remaining amino acids were washed off by acetonitrile. Fig. 4b shows another profile of the same protein which had been completely hydrolyzed by acid (6N HCl at 110° for 24 hrs). The peaks of glutamic acid and aspartic acid in Fig. 4b represent the total Glx and Asx, respectively, in the cottonseed protein. Calculations show that, at equivalent amounts of protein under hydrolysis, the sum of glutamine and glutamic acid in the enzyme hydrolysate is smaller than the Glx in the acid hydrolysate. Exactly the same ratio in difference is found between the sum of asparagine and aspartic acid in the enzyme hydrolysate and the Asx in the acid hydrolysate, indicating that the Viokase-catalyzed hydrolysis is incomplete. The value of 62.0% hydrolysis was obtained as an average of three analyses with a standard deviation of 1.88%; this hydrolysis valute was confirmed by the calculation of the ratio of nitrogen recovery between Viokase and acid hydrolysates from conventional ion-exchange analysis. According to Tower et. al (11), most proteins were hydrolyzed by Viokase to 50-80% completion.

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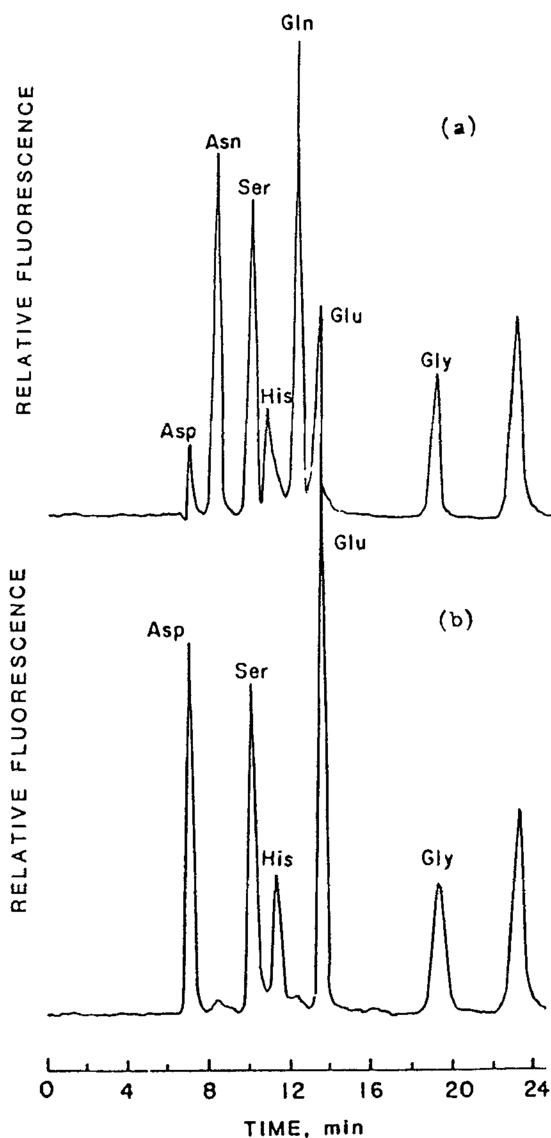

Figure 4. Elution profiles of cottonseed protein which had been (a) Viokase-hydrolyzed and (b) completely hydrolyzed by HCl. The injection of 5 ul OPA/ME derivatized hydrolysate, representing 0.56 ug protein in the case of Viokase-hydrolysis or 0.45 ug protein in the case of acid-hydrolysis, was eluted isocratically for 20 min with the mobile phase consisting of 12% acetonitrile and 88% sodium acetate bufer (0.04 M, pH 4.85), followed by a change to 100% acetonitrile in 5 min to wash off the remaining amino acids.

They argued strongly that the incomplete hydrolysis was not because of the existence of an unhydrolyzable "core" in the protein; they demonstrated that the extent of hydrolysis could be increased to over 90% by dialysing the hydrolysate and reincubating the sac contents several times. Consequently, by using the mean percentage of hydrolysis value, the contents of the various residues found experimentally by enzymatic hydrolysis were corrected to 100% hydrolysis.

According to the calculation method of Tower et al., the glutamine and asparagine contents in cottonseed protein were estimated to be 13.3% and 8.0%, respectively. However, we are not quite satisfied with the generalization of the "coreless" theory in the calculation. Investigations are being conducted in our laboratory for complete enzymatic hydrolysis of oilseed proteins. For the time being we could only report that 62.0% of cottonseed protein was consistently hydrolyzed by the Viokase enzyme system to amino acids in which 55.8% Glx was glutamine and 75.1% Asx was asparagine.

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CONCLUTION

We have demonstrated that HPLC technique is very effective in the analysis of glutamine and asparagine. The method can be used in the analysis of protein hydrolysate. When the storage protein of cottonseed was hydrolyzed by the enzyme system Viokase, HPLC analyses showed that, by comparing with the complete hydrolysis with acid, the enzymatic hydrolysis was not complete. The contents of

glutamine and asparagine in cottonseed protein can be estimated by correcting the experimental results to 100% hydrolysis, provided that the protein composition is homogeneous.

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