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Short Communication

High-performance liquid chromatographic separation of phenylthiocarbamyl derivatives of amino acids from protein hydrolysates using a Partisphere C_{18} column

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ABSTRACT

The conditions for the rapid analysis of amino acids in protein hydrolysates are given for the Whatman Partisphere C_{18} cartridge column. Amino acids from protein hydrolysates are derivatized to phenylthiocarbamyl (PTC) derivatives and separated within 22 min. The Partisphere column is generally less expensive than specialty columns designed for the PTC derivative. The "void sealing" capability of this column, in addition to its guard column attachment, increases the life span of the column.

INTRODUCTION

Analyzing amino acids as phenylthiocarbamyl (PTC) derivatives has gained increased popularity over the past several years due to the fact that the derivatization is simple and stable (one-step derivatization at room temperature); does not require special detection apparatus (uses a standard UV detector at 254 nm); and is very adaptable to most HPLC systems [1]. Specialty columns are available on market for PTC-amino acid analysis [1]. But, generally these specialty columns are among the most expensive columns available. It would be more convenient and less expensive to use more common, multipurpose columns for these analyses. In this respect, the conditions for several of these columns have been reported [2–5]. However, unique separation conditions must be generated for each new column and these conditions take considerable time to develop. In this report, separation conditions are presented for the analyses of PTC derivatives of amino acids from protein hydrolyses using a 125×4.6 mm Partisphere C_{18} cartridge columns available from Whatman (Clifton, NJ, USA).

EXPERIMENTAL

Amino acid hydrolysis

A 10-mg sample of oven-dried (59°C) mixed fish feed (65% crude protein) was placed in a 10-ml hydrolysis tube (Pierce, Rockford, IL, USA). Norleucine and α-aminobutyric acid internal standard (10 μmol each in 100 μl distilled water) were added to the tube; 900 μl of 6 *M* HCl was then added; the tube was flushed three times with nitrogen followed by evacuation; and the sample was hydrolyzed for 24 h at 105°C

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under vacuum. A 50-μl aliquot of hydrolyzate was removed and dried under nitrogen; 50 μl of 50% triethylamine in methanol was added to neutralize the acid; and the sample was again dried under vacuum. Norleucine, α-aminobutyric acid and amino standards (AA-S-18) were purchased from Sigma (St. Louis, MO, USA).

Derivatization

Samples were derivatized according to Bidlingmeyer *et al.* [1]. Briefly, 20 μl of methanol–phenylisothiocyanate–triethylamine–water (7:1:1:1) were added and left for 20 min at room temperature. Phenylisothiocyanate was purchased from Pierce. Methanol and triethylamine was purchased from Fisher Scientific (Santa Clara, CA, USA). Solvents were removed under vacuum and the sample was reconstituted in 2 ml of solvent A (below) and filtered through a nylon-66 0.45-μm filter (Fisher Scientific) before injection.

Chromatography

Chromatography was performed using a Shimadzu HPLC system (Kyoto, Japan) consisting of two LC-6A pumps, column oven (CTO-6A), pump controller (SCL-6A), auto injector (SIL-6A) and variable-wavelength UV detector (SPD-6AV). Data were collected and processed with an Axxiom (Model 727) chromatography data system (Calbasas, CA, USA). UV detection was at 254 nm and 20 μl were injected. The HPLC solvents were similar to that described by Ebert [3]. Solvent A was composed of 50 m*M* sodium acetate and triethylamine (4 ml/l) adjusted to pH 6.4 with phosphoric acid. Solvent B was composed of 50% solvent A, 40% acetonitrile and 10% methanol. All solvents were purchased from Fisher Scientific. All solvents were filtered through an Acrodisc 0.45-μm filter (Fisher Scientific) before use. The column was a 5 μm, 12.5 cm × 4.6 mm Whatman Partisphere C_{18} column equipped with a Whatman reversed-phase guard column and maintained at a temperature of 25°C. The separation conditions were as follows: flow-rate, 1.5 ml/min; initial solvents, 14% solvent B; linear rise to 20% solvent B in 7 min; 20% solvent B for 3 min; linear rise to 41% solvent B in 4 min; 41% solvent B for 7 min; 100% solvent B for 4.5 min. Re-equilibration to 14% solvent B required 4.5 min for a total analysis time of 30 min.

RESULTS AND DISCUSSION

The amino acid hydrolysis procedure was a fairly standard procedure and is not dealt with in this communication. Several excellent reviews are available on this subject [6–9]. PTC derivatives of amino acids are formed from a reaction of phenylisothiocyanate (Edman reagent) with the amino group of amino acids at room temperature. This step is essentially the first step in the Edman degradation that is used in amino acid sequencing of polypeptides. Koop *et al.* [10] are credited with the first use of PTC derivative in amino acid analysis. However, Heinrikson and Meredith [2] and Bidlingmeyer *et al.* [1] are the first to describe the complete analysis of amino acids in protein hydrolysates using phenylisothiocyanate. Heinrikson and Meredith [2] used both an IBM octadecylsilane (ODS) and Altex Ultrasphere octylsilane columns for separating 18 amino acids common to protein hydrolysis within 35 min. Bidlingmeyer *et al.* [1] used a specialty column (Picotag) developed by Waters and achieved a separation time of less than 12 min. This is still the fastest separation time to our knowledge. There is considerable published literature on the Picotag hydrolysate column [1,11–18]. However, specialty columns tend to be expensive. Several other PTC-amino acid analysis systems have been developed using multipurpose columns. These columns have included Spherisorb ODS-2 [3,4,19,20]; Hypersil ODS [5,20]; Nova-Pak C_{18} [21]; Altex Narrow Bore C_{18} [22,23]; IBM C_{18} [2,20], Supersphere C_{18} [24]; Zorbax C_{18} [25] and Altex Ultrasphere C_{18} [26]. The separation times for the majority of these columns have ranged from 15 to 35 min. Separation of PTC-amino acids from a standard mixture of amino acids and from hydrolysates of a feed sample using the present Partisphere C_{18} column is illustrated in Figs. 1 and 2, respectively. The separation time is approximately 23 min and is among the faster elution times. Analyses can also be run at near room temperatures

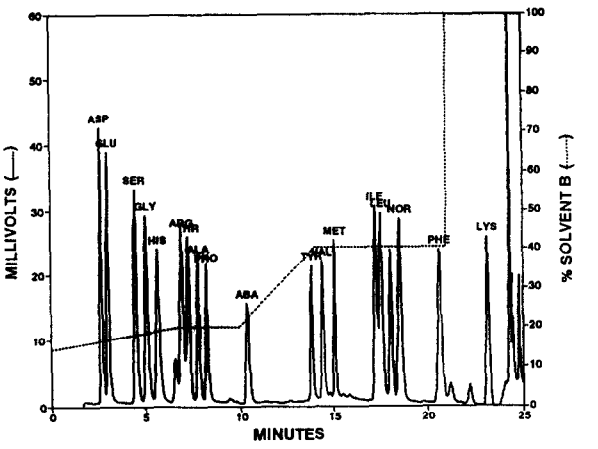

Fig. 1. Chromatogram of PTC-amino acids from standard amino acids mixture at an equimolar concentrations (100 pmol each). ABA = α-Aminobutyric acid (internal standard); NOR = norleucine (internal standard). Chromatographic conditions: column, Whatman Partisphere C_{18} (5 μm) (125 × 4.6 mm I.D.); Whatman reversed-phase guard column; column temperature, 25°C; eluent A, 50 m*M* sodium acetate, triethylamine (4 ml/l), pH 6.4; eluent B, 50% eluent A, 40% acetonitrile, 10% methanol; flow-rate, 1.5 ml/min; gradient, 0 min 14% B, 7 min 20% B, 10 min 20% B, 14 min 41% B, 21 min 41% B, 21 min 100% B; UV detection, 254 nm; injection volume, 20 μl.

eliminating the need for a column heater. Mixtures of standard amino acids were injected onto the column in the range of 10 to 1000 pmol to measure the sensitivity of the method. Linear responses in peak areas were found for all amino acids within the range of 50 to 1000 pmol. Linear responses in peak area below 50 pmol were found for alanine, methionine and norleucine but the other amino acids showed a curvilinear response within this range. The linear response of norleucine and the curvilinear responses of threonine and valine are illustrated in Fig. 3. Threonine and valine were selected in particular because they showed the greatest curvilinear response in the range of 10 to 50 pmol. This sensitivity is similar to that found with specialty columns [1]. The relative molar responses of the amino acids relative to α-aminobutyric acid and norleucine internal standards are given in Table I. These responses were consistent through at least first 300 analyses and a concentration range of 50 to 1000 pmol. The Partisphere C_{18} column is also among the less expensive columns presently on the market. Additionally, Whatman columns have void sealing capabilities as a result of movable inlet frits. These movable frits effectively remove column inlet dead volumes which causes loss of theoretical plates created by prolonged column use. As a result, theoretical plates are partially restored and the column life span increased. In our laboratory we have been able to perform over 300 runs per column with movable inlet frit adjustments at approximately 60–70 h of constant use. The amino acid analysis procedure described here will add more flexibility in analyses that can be performed in lab-

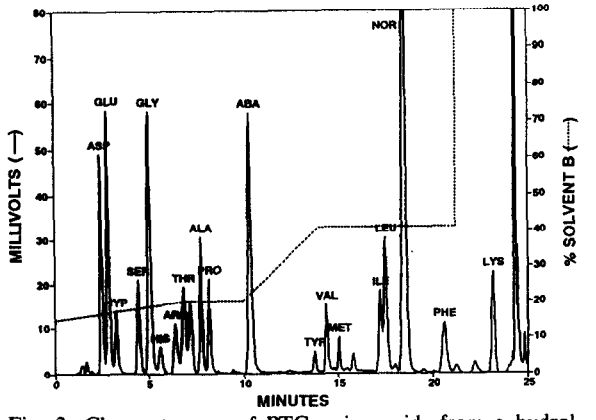

Fig. 2. Chromatogram of PTC-amino acids from a hydrolysate of a mixed fish feed (65% protein). Conditions as in Fig. 1.

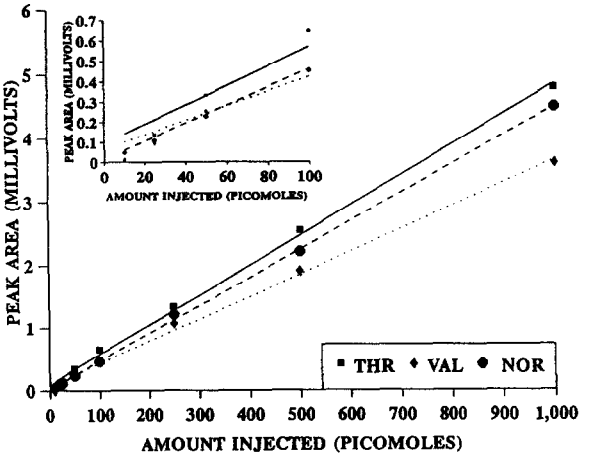

Fig. 3. Linearity of peak area responses of amino acids in the concentration range of 10 to 1000 pmol.

TABLE I

RELATIVE MOLAR RESPONSES (RMR) OF AN EQUIMOLAR MIXTURE OF AMINO ACIDS RELATIVE TO α-AMINOBUTYRIC ACID (ABA) AND NORLEUCINE (NOR) INTERNAL STANDARDS

RMR is calculated by dividing the area of the amino by the area of the internal standard at equimolar concentrations.

Amino acid	RMR_{ABA}	RMR_{NOR}
Aspartic acid	1.01	0.948
Glutamic acid	1.13	1.07
Serine	1.16	1.10
Glycine	1.31	1.23
Histidine	0.894	0.842
Arginine	1.11	1.05
Threonine	1.52	1.43
Alanine	1.23	1.16
Proline	1.33	1.26
α-Aminobutyric acid	1.00	0.941
Tyrosine	1.04	0.980
Valine	1.07	1.01
Methionine	1.16	1.10
Isoleucine	0.898	0.845
Leucine	1.35	1.27
Norleucine	1.06	1.00
Phenylalanine	1.07	1.00
Lysine	2.06	1.86

oratories which currently use the Partisphere C_{18} column.

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