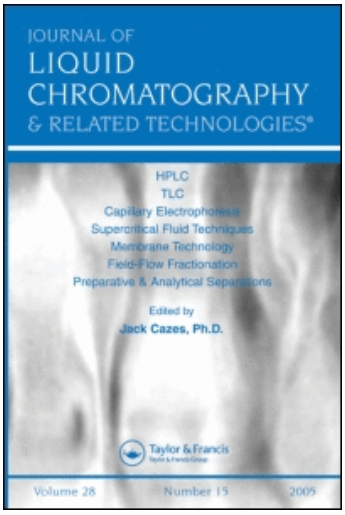

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE AMINO ACIDS IN SHRIMP

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ABSTRACT

A reverse phase high performance liquid chromatographic method for the analysis of total free amino acids in shrimp, utilizing pre column fluorescence derivatization is described. The primary amino acids were treated with o-phthalaldehyde (OPA). The reaction products were separated on a Microsorb Short-ones 3-μm reversed-phase column with gradient elution development. 15-OPA amino acids were separated in 21 min. Secondary amino acids were reacted with 4-chloro-7-nitrobenzofurazan (NBD). The separation was carried out on a Lichrosorb RP-C18, 5μm column. Wild and cultured Mexican shrimp species (Penaeus vannamei) were analyzed. Free amino acid contents of glycine, alanine and proline were higher than those of other amino acids. Total Free amino acid content was significantly higher in cultured than wild shrimp. The OPA-retention time could be measured within ±0.1 relative standard deviation and the relative peak areas, based on the internal standard calculation methodology, were within ±3% or less. The coefficient of variation of 18 kinds of amino acid samples were about 10%, the detection limit was 100 fmol.

INTRODUCTION

Dissolved free amino acids (DFAAs) are important nutrients in marine food webs. DFAAs concentrations are most often studied by high pressure liquid chromatography of fluorescent derivatives with O-phthalaldehyde (OPA) (1).

Amino acid analysis is an important technique which finds many applications in analysis of sea foods, and related fields. HPLC has been used for the determination of free amino acids in uterine fluid and blood plasma samples of four species of viviparous sharks (2); HPLC-OPA free amino acid determination in seawater (3); determination of free amino acid composition in muscle and hemolymph of prawn (4) and quantitative distribution of free amino acids in the white and red muscle of tuna (5). However, only one report has appeared describing the use of HPLC techniques for the quantitative of free amino acid analysis in shrimp (6), and others have described the quantitative determination of free amino acids in seafood but using an amino acid analyzer (7).

The purpose of this article was to report the separation, base line resolution, and quantitation of the OPA and NBD amino acid derivatives in shrimp. This methodology can be utilized in determining free amino acids as important contributors to quality flavor in seafood; osmoregulators in crustaceans and environmental factors during growth.

MATERIALS AND METHODS

Sample Preparation

Extracts for analyses were prepared by blending the shrimp tissue in a waring blendor homogenizer at a ratio of 1.0 g shrimp to 2.0 ml of trichloroacetic acid (TCA). The extracts were centrifuged and part of the supernatant analyzed (7).

Primary Amino Acids

OPA derivatizing solution

To 10 mg OPA dissolved in 250 µl of methanol, 37.5 µl 30% Brij 35, 25 µl of 2-mercaptoethanol and 3 ml of 0.5 M potassium borate buffer pH=10.4 were added. This solution was diluted to 10 ml with borate buffer, and mixed well. It was then stored under refrigeration in the dark and allowed to stand for 24 h before use. The preparation was made one day prior to use.

Sample derivatizing preparation

A sample of TCA extract was diluted with 5 ml of sodium citrate buffer pH=2.2, filtered (whatman 934-AH) and diluted with alfa-aminobutiric acid (2.5µM/ml) as an internal standard (IS) as follows:

100µl TCA extract + 40µl IS --> 1 ml

Sample derivatization.- Immediately prior to loading the injection loop, a combination of 0.5 ml of OPA solution and 0.5 ml of sample or amino acid standard solution containing IS was prepared in a small test tube and mixed. It was injected within 2 min.

Secondary Amino Acids

Preparation of the NBD derivatives

Equal volumes of TCA extract sample, 0.4 M borate buffer, and the NBD solution (2 mg/ml in methanol) were combined and the mixture was heated for 5 min at 60°C in a closed screw capped vial. The reaction was stopped by cooling the mixture to 0°C. 10µl were injected onto the column. The quantification was made using an external standard.

Chromatographic Equipment

The instrument used consisted of a Varian Model 5000 microprocessor controlled high performance liquid chromatograph coupled to a Fluorichrom fluorescence detector equipped with a deuterium lamp. The detector was connected

to a Model Vista 401 data collection system (Varian Associates, Inc. USA). The OPA and NBD derivatives were detected with the monochromator set at 330 nm and 418 nm cut-off filter. The sensitivity was set at 1 μA full scale. The sample was introduced with a Rheodyne Model 7120 valve (Berkeley, CA, USA) equipped with a 10 μl loop.

Primary amino acid separations were carried out on a 10cm x 4.6 mm I.D. Microsorb Short-ones diameter spherical HPLC column packed with 3μm Reversed-phase C-18 octadecyl dimethylsilane particles (Rainin Instrument Co. Inc., Emeryville, CA, USA) connected to a pre-column (3 cm x 4.6 mm I.D.) packed with the same material.

A Lichrosorb RP-C18, 5μm, (30 cm x 4.6 mm ID) column was used for determination and separation of secondary amino acids (Alltech/Applied Science, USA).

Statistical Analysis

The data were analyzed by descriptive statistics and one way ANOVA method to test for significant differences among shrimp samples, followed by testing of specific mean differences using Tukey's multiple comparison procedure (8).

RESULTS AND DISCUSSION

The excitation and emission wavelengths for OPA-amino acids are 340 nm, 455 nm and 220 nm, 370 nm for NBD-iminoacids, respectively.

Solvent composition, buffer concentration, and pH are major factors affecting reverse phase chromatography.

Fully automated HPLC equipment allowed unattended determination of amino acid profile within 21 min. Precise control of operating conditions and temperature (23°C) are essential to achieve maximum resolution. The

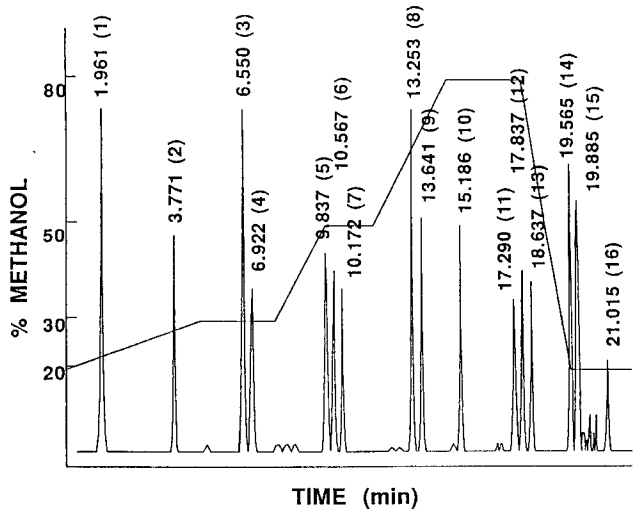

FIGURE 1. Separation of OPA-amino acid standard. Operating conditions: Column Microsorb Short-ones. Flow rate 1.2 ml/min. Solvent A: 0.1 M sodium acetate buffer + 1% tetrahydrofuran, pH=6.2. Solvent B: Methanol. Sample loop 10µl. Exitation at 330 nm, emission filter 418 nm. Peaks: 1=aspartic acid, 2=glutamic acid, 3=serine, 4=histidine, 5=glycine, 6=threonine, 7=arginine, 8=alanine, 9=tyrosine, 10=alfa-aminobutiric acid (IS), 11=methionine, 12=valine, 13=phenylalanine, 14=isoleucine, 15=leucine, 16=lysine.

reproducibility of the derivatization method and HPLC technique was checked using two samples. The results indicated that the coefficient of variability was less than 10%. The method did not yield higher or lower results and relative standard deviation systematically.

Figure 1 shows a representative chromatogram of the standard OPA free amino acids and a profile of the multistep mobile phase gradient. Linear relationships between peak areas and concentrations for each amino acid are shown. The gradient was composed of a 7-step solvent program which increased the separation efficiency of amino acids.

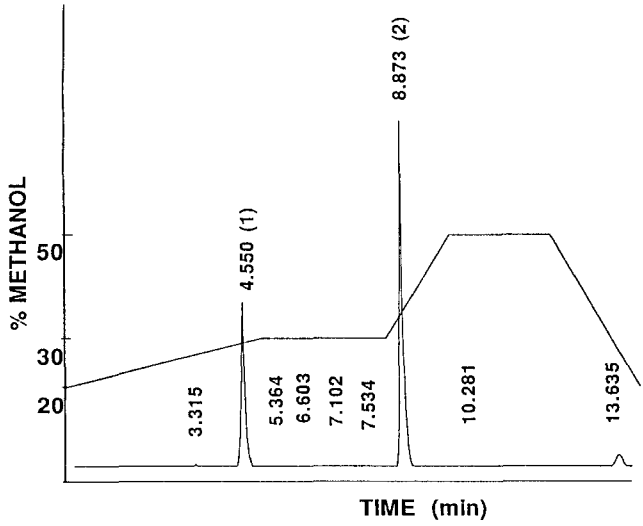

FIGURE 2. Separation of NBD-amino acid standard. Operating conditions: Column Lichrosorb RP-C18. Flow rate 1.2 ml/min. Solvent A: 0.1 M sodium acetate buffer + 1% tetrahydrofuran, pH=6.2. Solvent B: Methanol. Sample loop 10µl. Exitation at 220 nm, emission filter 370 nm. Peaks: 1=hydroxyproline, 2=proline.

Figure 2 shows a typical chromatogram of standard NBD iminoacids and its elution gradient. The elution gradient was the first part of OPA-aminoacids. The solvent programming was performed automatically with a microprocessor unit. The last OPA-amino acid eluting under these conditions was lysine, which has a retention time of 21 min. The NBD amino acids were eluted in 9 min.

Although shrimp samples contain components which might interfere with amino acid analysis, no extra peaks were observed on the chromatogram. Presumably, the specificity of the fluorometric procedure and selectivity of the solvent system eliminated this type of interference.

Precision was examined by reacting and analyzing five replicate standard amino acid mixtures and comparing peak areas for the respective mixtures. Coefficients of variation ranged from 10% for lysine to 3% for the rest of the

amino acids, at injection levels of 0.5 µmol in 10 µl. The OPA-retention time could be measured within ±0.1 relative standard deviation and the relative peak areas, based on the internal standard calculation methodology were within ±3 or less. The coefficient of variation of 18 kinds of amino acid samples was about 10%, the detection limit was 100 fmol (9, 10). This result is in agreement to that reported by Wang, et al., (3), but their final time of analysis was 40 min.

The correlation coefficients for concentrations versus response was >0.998 for all derivatives, except for lysine (r=0.991).

A typical separation of OPA- and NBD-amino acids can be seen in figure 3 and 4. The Table I shows the individual content of free amino acids in wild and cultured shrimp.

The results agree with Simpson, et al., (11) and McCoid et al.(7). However, this method of analysis was effective in separating serine and threonine; also, threonine was present in trace concentrations (Fig 3, Tr=10.501). On the other hand, fluorometry is known to be about one hundred times more sensitive than colorimetry (12), and the classical amino acid analyzers were based in ion-exchange operated in post column derivatization mode and utilized ninhydrin.

The separation of components in the raw extract of shrimp was: glycine (22.18-37.88%); arginine (22.28-32.67%) and proline (12.57-29.15%), which were present in the highest concentration. This result agrees with Takada et al., (1988) for free amino acid contents in eighteen species of imported frozen shrimps. These data represent approximately 27.55%, 26.36% and 20.81% of the total amino acid content, respectively, and comprised 72.58-76.87% of free amino acid pool. Aspartic acid content was the lowest and only had a mean of 3.07 mg%. It was followed by phenylalanine and hydroxyproline.

Cultured shrimp had higher concentration of the total free amino acid concentration than wild shrimp.

Wing et al., (1990) constructed an automated continued monitoring system for DFAAc, and stated that "DFAAc concentrations are most often studied by HPLC of fluorescent derivatives with OPA. This procedure is laborious, requires

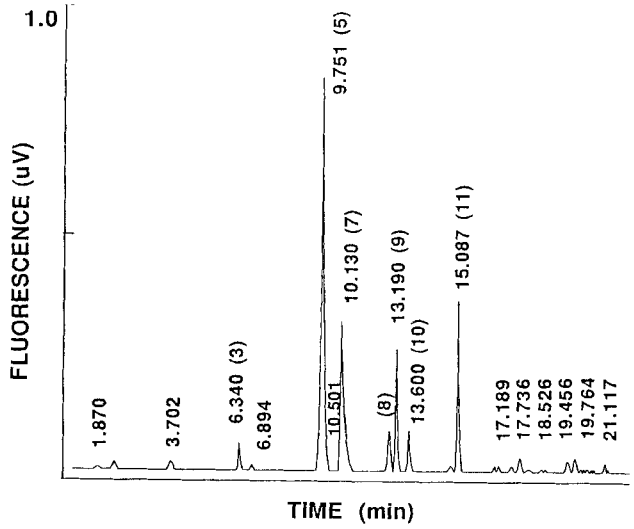

FIGURE 3. Amino acid analysis of wild shrimp. Operating conditions same as in Fig. 1. OPA-aminoacids;3=serine, 5=glycine, 7=arginine, 8=taurine, 9=alanine, 10=tyrosine, 11=alfa-aminobutiric acid (IS).

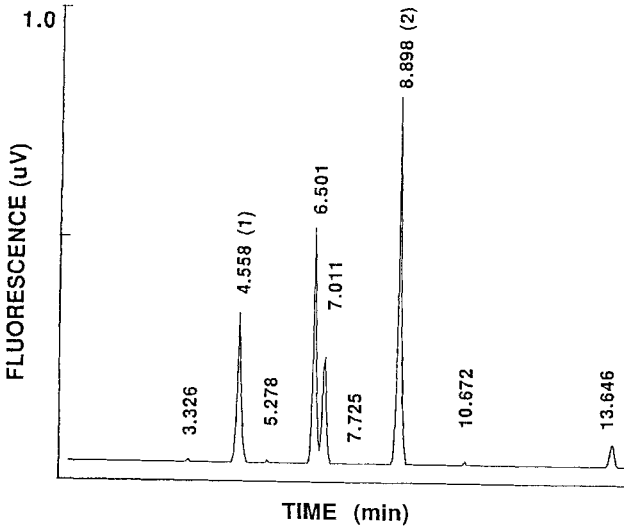

FIGURE 4. Amino acid analysis of wild shrimp. Operating conditions same as in Fig. 2. NBD-aminoacids. Peaks: 1= hydroxyproline, 2= proline.

TABLE 1

Free Amino Acids in White Srimp (P. Vannamei) Wild and Cultured 1[1]

Free Amino Acids	Wild[2]	Cultured 1[2]	Cultured 2[2]
		OPA-Amino Acids	
Aspartic	2.43 ± 0.29 a	2.72 ± 0.13 a	4.05 ± 0.24 b
Glutamic	19.99 ± 1.38 a	19.54 ± 1.64 a	20.51 ± 1.28 a
Serine	20.86 ± 1.69 a	10.41 ± 0.39 b	19.54 ± 1.75 a
Histidine	68.27 ± 20.27 a	138.23 ± 22.42 b	101.10 ± 20.37 b
Glycine	367.55 ± 10.66 a	373.15 ± 22.11 a	575.14 ± 36.79 b
Arginine	439.13 ± 21.87 a	429.61 ± 34.14 a	389.84 ± 37.01 a
Taurine	71.51 ± 4.27 a	22.54 ± 1.94 b	88.57 ± 7.99 c
Alanine	73.23 ± 3.87 a	94.48 ± 8.07 b	82.42 ± 8.81 ab
Thirosine	20.24 ± 3.62 a	24.84 ± 2.04 a	27.74 ± 3.35 a
Methionine	12.13 ± 0.78 a	5.56 ± 0.43 b	11.94 ± 1.03 a
Valine	18.91 ± 1.44 a	18.68 ± 0.52 a	22.93 ± 1.15 b
Phenylalanine	8.23 ± 0.86 a	4.76 ± 0.61 b	10.18 ± 0.41 a
Isoleucine	12.45 ± 0.78 a	8.92 ± 0.71 b	14.80 ± 0.96 a
Leusine	18.53 ± 1.48 a	13.52 ± 1.38 b	20.51 ± 1.87 a
Lysine	21.74 ± 1.24 a	17.49 ± 2.04 a	20.40 ± 2.84 a
		NBD-Amino Acids	
Proline	168.94 ± 12.61 a	490.29 ± 54.24 b	334.66 ± 31.67 c
Hydroxyproline	11.92 ± 1.27 a	7.23 ± 0.61 b	5.16 ± 0.56 c
Total Free Amino Acids	1344.14 a	1681.97 b	1749.40 c

[1] mg/100g.
[2] a,b,c = significative differences (P<0.05).

extensive sample handling, and yields results hours or days after sampling". However, with our method, seawater can be analyzed directly and results can be obtained in 21 min for OPA-DFAAc or 9 min for NBD-DFAAc.

The described method has been used in our laboratory to assess types and amounts of amino acids in regional foods, fish meal, and complex food samples.

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