Determination of Amino Acids in Green Beans by Derivatization with Phenylisothiocianate and High-Performance Liquid Chromatography with Ultraviolet Detection

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Abstract

A high-performance liquid chromatographic method for the determination of primary and secondary amino acids in green beans is described. Hydrochloric acid hydrolysates of 15 representative samples of bean proteins are derivatized with phenylisothiocianate, and the resulting phenylthiocarbamyl derivatives are separated on a reversed-phase column by gradient elution with sodium acetate buffer and acetonitrile–water (60:40 [v/v]) and detected by ultraviolet detection at 254 nm. Recoveries range from 93.5 to 97.5%, method precision (relative standard deviation) ranges from 1.26 to 3.98%, and detection limits range from 8.11 to 13.3 pmol/µL.

Introduction

The nutritive value of proteins, one of the most important components of the human diet, depends mainly on their amino acid composition, especially their content of the eight essential amino acids. In addition to their dietary importance, proteins also influence food organoleptic properties: proteins affect food texture, small peptides and amino acids contribute to food flavor, and all three undergo thermal and enzymic reactions during food preparation and storage that generate colored aromatic compounds (1).

The amino acid components of food proteins are mainly determined by reversed-phase high-performance liquid chromatography (HPLC) of hydrolysates. In order to effectively detect amino acids, however, it is first necessary to chemically modify them, which usually involves converting them into derivatives that absorb or fluoresce in the ultraviolet–visible (UV–vis) wavelength range. Several reagents have been developed for this purpose; a description of the most commonly used reagents follows.

Ninhydrin (2–4) is used for post-column derivatization of amino acids separated by high-resolution ion-exchange chromatography, allowing them to be detected in the visible region. This methodology was described over 25 years ago and forms the basis for many commercial amino acid analyzers. Although these instruments have been improved over the years with regard to both sensitivity and shorter analysis times, amino acid analyzers are complex and expensive and generally cannot be adapted for other uses.

Dansyl chloride (5) forms fluorescent adducts with amino acids but lacks selectivity (it reacts with both -OH and -NH_2 groups) and requires rather long reaction times and high reaction temperatures (6).

9-Fluorenylmethyl chloroformate (FMOC–Cl) (7–9) rapidily reacts with amino acids to form highly fluorescent, stable adducts. The major disadvantage of this method is the need to use excess reagent that must be extracted prior to chromatography, which often results in hydrolysis and loss of the FMOC–amino acids adducts (10).

o-Phthaldialdehyde (OPA) (11–13) is nonfluorescent itself and reacts rapidily with primary amino acids at room temperature to form highly fluorescent isoindoles. The disadvantage of this method is the lack of reactivity of OPA with secondary amino acids (14).

Phenylisothiocyanate (PITC) (15,16) reacts with both primary and secondary amino acids to yield stable phenylthiocarbamyl (PTC) derivatives that can be detected by their UV absorption at 254 nm. The main drawback of this method is its low sensivity, compared with methods based on fluorometric detection (6).

In this work, we developed a method for determination of amino acids in whole green beans (*Phaseolus vulgaris* L.) by HPLC of their PITC derivatives. PITC was chosen for derivatization because its only drawback was low sensivity, which could be countered by using a large amount of bean material.

Experimental

Samples

Green bean (*Phaseolus vulgaris* L.) samples were obtained from a commercial plantation (Pontevedra, Spain). They were planted in the open in March, and 15 samples were harvested during July and August when they were judged to have reached

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an appropriate pod length-to-bean length ratio. The samples were immediately homogenized and lyophilized, pending analysis.

Reagents

Amino acid standards (*L*-alanine, *L*-arginine, *L*-aspartic acid, *L*-glutamic acid, *L*-glycine, *L*-histidine, *L*-isoleucine, *L*-leucine, *L*-lysine, *L*-methionine, *L*-phenylalanine, *L*-hydroxyproline, *L*-proline, *L*-serine, *L*-threonine, *L*-tyrosine, and *L*-valine), the derivatizing reagent PITC, and triethylamine (TEA) were from Sigma Chemical (Madrid, Spain). HPLC-grade acetonitrile and methanol were from Scharlau (Barcelona, Spain). Reagent-grade sodium acetate trihydrate and sodium phosphate were from Merck (La Coruña, Spain), and hydrochloric acid was from Normasolv (Barcelona, Spain).

HPLC apparatus

The HPLC equipment consisted of a Spectra Physics (San Jose, CA) HPLC apparatus comprising an 8700 XR ternary pump, a 20-µL Rheodyne (Cotati, CA) injection loop, an SP8792 column heater, a 8440 XR UV–vis detector, and a 4290 integrator linked via Labnet to a computer running WINner 8086 software (operating system, MS.DOS version 3.2). For separation, a 250- × 4.6-mm column packed with 5-µm Spherisorb C_{18} (Sugelabor, Madrid, Spain) was used.

Preparation of samples and standards

Prior to derivatization, bean proteins were hydrolyzed as follows. A 0.1-g lyophilized sample was weighed into a 16- × 125-mm screw-cap Pyrex (Barcelona, Spain) tube, 15 mL of 6N hydrochloric acid was added, and the tube was throughly flushed with N_2, quickly capped, and placed in an oven at 110°C for 24 h (17). After hydrolysis, the tube contents were vacuum filtered (Whatman #541, Maidstone, England) to remove solids, the filtrate was made up to 25 mL with water, and an aliquot of this solution was further filtered through a 0.50-µm pore-size membrane (Millipore, Madrid, Spain). A standard solution containing 1.25 µmol/mL of each amino acid in 0.1N hydrochloric acid was created.

Derivatization procedure

The procedure used was a modification of the method of Elkin et al. (18). A standard solution (5, 10, 15, or 20 µL) or 50 µL of bean solution was pipetted into a 10- × 5-mm tube and dried in vacuo at 65°C. To the residue, 30 µL of methanol–water–TEA (2:2:1 [v/v]) was added and then removed in vacuo at 65°C. Next, 30 µL of the derivatizing reagent methanol–water–TEA–PITC (7:1:1:1 [v/v]) was added, and the tube was agitated and left to stand at room temperature for 20 min. Finally, the solvents were removed under a nitrogen stream, and the tube was sealed and stored at 4°C, pending analysis. Prior to injection, 150 µL of diluent consisting of 5mM sodium phosphate with 5% acetonitrile was added to each tube.

Table I. Gradient Program Employed for the Separation of PTC–Amino Acids

Time (min)	Flow rate (mL/min)	% Eluent A	% Eluent B
0	1.0	90	10
12.0	1.0	70	30
20.0	1.0	52	48
22.0	1.0	0	100
24.0	1.0	0	100
30.0	1.5	0	100
37.0	1.0	90	10

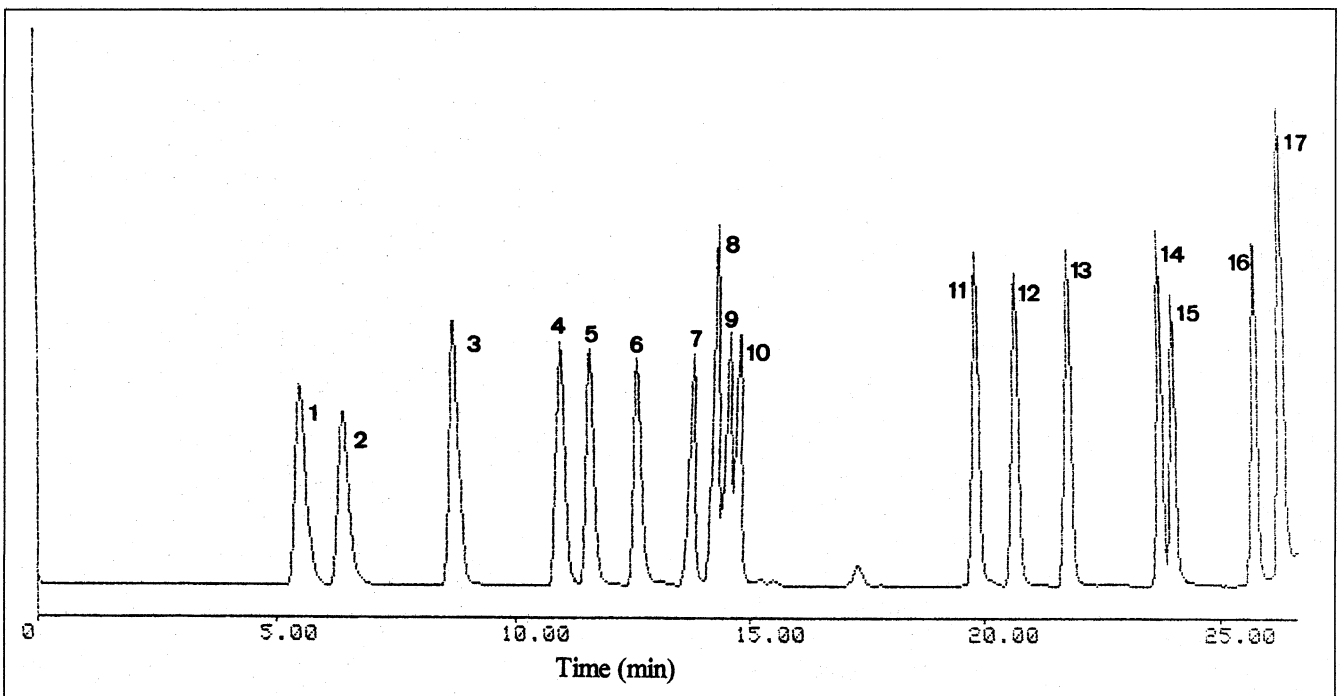

Figure 1. Chromatogram of a PTC–amino acid standard. Peaks: 1, Aspartic acid; 2, glutamic acid; 3, hydroxyproline; 4, serine; 5, glycine; 6, histidine; 7, arginine; 8, threonine; 9, alanine; 10, proline; 11, tyrosine; 12, valine; 13, methionine; 14, isoleucine; 15, leucine; 16, phenylalanine; 17, lysine.

Chromatographic procedure

Chromatography was carried out at a constant temperature of 30°C using a gradient elution as follows. Eluant A was an aqueous buffer prepared by adding 0.5 mL/L TEA to 0.14M sodium acetate and titrating it to pH 6.20 with glacial acetic acid; eluant B was acetonitrile–water (60:40 [v/v]). The gradient program is shown in Table I.

Results and Discussion

The total time required for derivatization was 1 h (Elkin's method took 4 h [18]), of which 20 min was for reaction between the amino acids and PITC. Neither longer reaction times nor a greater excess of PITC appreciably increased the intensities of the HPLC peaks due to the PTC–amino acids. The time-saving changes were motivated largely by the need to avoid possible thermal degradation of the PTC–amino acids (or even of the amino acids themselves) in the course of the derivatization procedure. Specifically, removing the excess derivatizing reagents in vacuo at a temperature between 30 and 35°C reduced the intensities of the HPLC signals due to the PTC–amino acids, particularly those of PTC–Asp and PTC–Glu, compared with the signal intensities obtained when these reagents were removed in vacuo without heating (which naturally took longer). These observations led us to: (*a*) omit repetition of the evaporation step (in Elkin's procedure [18], methanol–water–TEA [2:2:1] was twice driven off from the sample by heating under a vacuum), and (*b*) use a nitrogen stream rather than heating under a vacuum to drive off the excess derivatizing reagents. Use of the latter method allowed the isolation of a suitably clean derivatized sample within 40 min and afforded much higher recoveries of aspartic and glutamic acids (96.9 and 94.9%, respectively, compared with 82.0 and 81.0% if a vacuum is used [15]). As a further precaution, the PTC–amino acids were sealed and refrigerated pending analysis (always within 24 h of derivatization).

Figures 1 and 2 show the chromatograms of the PTC–amino acid standard mixture (each peak corresponds to 200 pmol/µL of amino acid) and a typical green bean sample, respectively. The detection wavelength used (254 nm) afforded signal intensities similar to those for the alternative wavelengths (200 and 212 nm) but was much more selective. The chromatographic run time was 37 min, which included 10 min between injections to allow elution of PITC–TEA-derived components (detected in both samples and standards). Nonetheless, these components did not interfere with detection of the PTC–amino acids when the gradient program in Table I was used. This gradient is a modification of the one used by Bidlingmeyer et al. (17). Most significantly, slight modification of the pH of the buffer (eluant A) used by Bidlingmeyer et al. (17),

Table II. Measurement and Method Precision with Recoveries and Detection Limits Calculated for Each PTC–Amino Acid

Amino acid	Precision of measurement CV* (%)	Precision of method CV* (%)	Recovery (%)	Detection limit (pmol/µL)
Asp	0.62	1.85	96.9	11.3
Glu	0.60	1.53	94.9	8.50
H-Pro	1.57	2.24	96.5	12.5
Ser	1.02	1.32	93.5	10.2
Gly	0.45	1.51	96.0	13.3
His	1.33	2.13	94.2	9.88
Arg	1.69	1.79	94.3	9.92
Thr	1.26	2.21	95.2	8.73
Ala	3.01	3.22	97.5	8.98
Pro	3.51	3.98	95.6	8.77
Tyr	1.24	1.26	95.7	8.77
Val	1.14	1.27	95.7	8.11
Met	1.03	2.75	96.1	8.18
Ile	0.86	2.09	95.9	8.61
Leu	0.36	1.58	94.5	9.37
Phe	1.75	2.42	97.1	9.44
Lys	1.01	1.90	95.9	8.65

* Coefficient of variation.

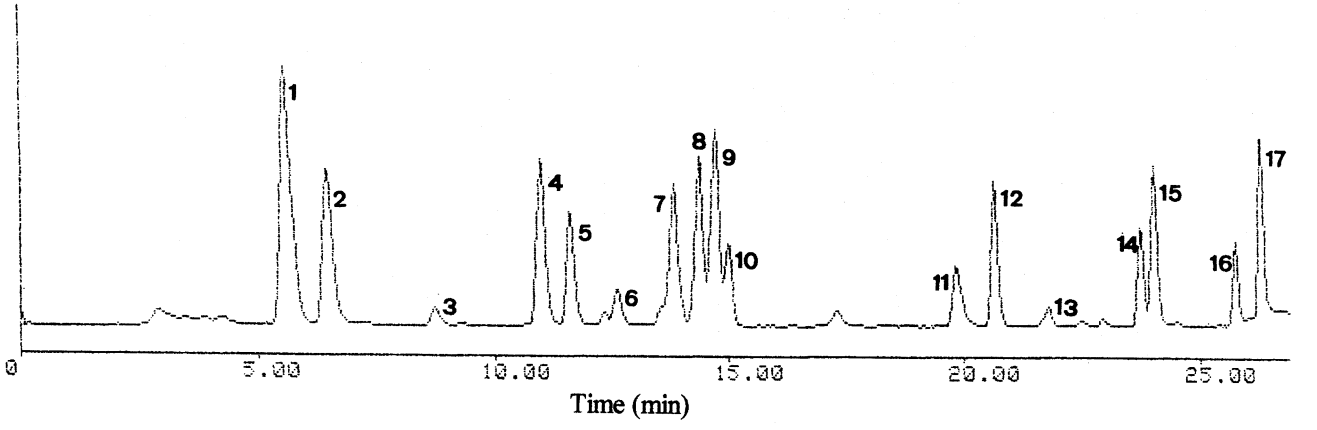

Figure 2. A typical chromatogram showing the amino acids detected by lyophilized green beans. Peaks: 1, Aspartic acid; 2, glutamic acid; 3, hydroxyproline; 4, serine; 5, glycine; 6, histidine; 7, arginine; 8, threonine; 9, alanine; 10, proline; 11, tyrosine; 12, valine; 13, methionine; 14, isoleucine; 15, leucine; 16, phenylalanine; 17, lysine.

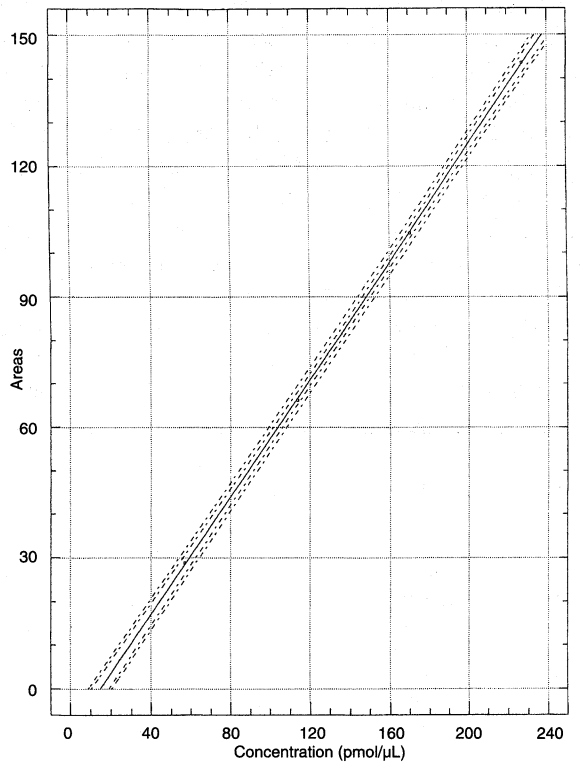

Figure 3. Calibration curve ($y = a + bx$) obtained by regression of peak area (y) on the concentration (x) of aspartic acid.

together with a slightly different gradient program and a lower column temperature, shifted the peak that eluted between PTC–Phe and PTC–Lys using Bidlingmeyer's gradient to a retention time that was safely beyond the PTC–Lys peak.

To determine the measurement precision, 10 aliquots of the same standard solution were injected. To determine the method precision, 10 aliquots of the same homogenized sample were each subjected to the complete procedure. The coefficients of variance (%) are given in Table II.

The detection limit of each amino acid was calculated in accordance with American Chemical Society guidelines (19). The results (pmol/µL) are given in Table II. Percent recoveries (Table II) were evaluated by spiking triplicate samples of bean hydrolysates at three levels with the mixed standard, and then subjecting them to the rest of the procedure.

Figure 3 shows the aspartic acid calibration curve (similar curves were obtained for all other analytes). A linear UV absorbance response was obtained in the range of analytical interest (40–200 pmol/µL). Correlation coefficients for these data exceeded 0.996. The poorest linearity was for alanine, which was probably due to integration problems.

Table III lists the mean plus or minus standard deviation (g/100g of dry weight) of amino acid contents of 15 samples in green beans. The major amino acids were aspartic acid (2.44 g/100g of dry weight) and glutamic acid (1.65 g/100g of dry weight). The amino acids tryptophan, asparagine, and glutamine were destroyed by acid hydrolysis and were not determined. Cysteine was not determined because of the rapid oxidation of this compound to form cysteic acid.

Table III. Amino Acid Content of Lyophilized Green Beans

Amino acid	Mean ± SD* (g/100g dry weight)
Asp	2.44 ± 0.52
Glu	1.65 ± 0.24
H-Pro	0.115 ± 0.017
Ser	0.927 ± 0.118
Gly	0.509 ± 0.043
His	0.340 ± 0.033
Arg	1.29 ± 0.102
Thr	0.351 ± 0.042
Ala	0.960 ± 0.077
Pro	0.728 ± 0.059
Tyr	0.491 ± 0.109
Val	0.720 ± 0.065
Met	0.122 ± 0.035
Ile	0.605 ± 0.060
Leu	0.864 ± 0.071
Phe	0.474 ± 0.048
Lys	0.951 ± 0.102

* SD = standard deviation; 15 replicates.

Conclusion

The results of the present paper suggest that reversed-phase HPLC separation and UV detection of PITC derivatives of amino acids are useful for the determination of primary and secondary amino acids in green beans. Furthermore, this method provides rapid and highly reproducible results.

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