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Quantitative Determination of Amino Acids by Reversed-Phase High Performance Liquid Chromatography after Pre-column Derivatization

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A simple, accurate and reliable high performance liquid chromatographic method was developed to determine the amino acids by pre-column derivatization method using *ortho*-phthaldialdehyde. The resulting derivatives are separated on reverse phase C_{18e} column by gradient elution with sodium acetate buffer and acetonitrile-water and detected by ultraviolet detection at 340 nm. The amino acids separated in 25 min with fine resolution in the standard mixture were aspartate, asparagine, serine, glycine, histidine, arginine, threonine, tryptophan, proline, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine and lysine. Modification of the elution program allows concurrent resolution of peaks of valine, methionine, isoleucine and leucine. Thus, the proposed method shows the promise of wide applicability in estimation of amino acids in cereals.

Keywords: Amino acids, *ortho*-Phthaldialdehyde, derivatization, HPLC.

INTRODUCTION

Amino acids are the basic constituents of proteins. For that reason, qualitative and quantitative analysis of the amino acid composition of hydrolyzed samples of pure proteins or peptides is used to identify the material and to directly measure concentration. In addition, these are also intermediates in many metabolic pathways and therefore measured as elements of physiological and nutritional studies. The amino components of food proteins are mainly determined by reverse phase high performance liquid chromatography. For effective detection, the amino acids are usually converted into derivatives that absorbs in the ultraviolet-visible wavelength range. Derivatization methods of two types such as post column and pre-column are used for estimation of amino acids in HPLC system. In post column type, derivatisation was performed with ninhydrin and characterized by an ion exchange separation technique [1]. But due to low sensitivity and high instrument expenses it became unsuitable for the users. Dansyl chloride [2] forms fluorescent adducts (pre-column derivatization) with amino acids but lacks selectivity and requires long reaction times and high reaction temperatures [3]. Now-a-days, *ortho*-phthaldialdehyde (OPA) has been found as an alternative to the previously used derivatising agents [4,5]. Development of a particular pre-column derivatization step with short reaction time along with short work up procedure appears as a challenge in amino acids analysis. Furthermore in earlier methods, acid hydrolysis step was used in extraction method of amino acids which results in loss of few amino acids due to hydrolysis. Therefore the present investigation has been aimed to develop a HPLC method using *ortho*-phthaldialdehyde as pre-column derivatizing agent and determine its applicability in analysis of amino acids in cereals.

EXPERIMENTAL

ortho-Phthaldialdehyde, methanol, acetonitrile and all other reagents were procured from Merck, India. Deionized water was generated using an ultra-pure water purification system. Amino acids standard kit was procured from Sigma, India. Fresh derivatizing agent was prepared by taking 10 mg pthalialdehyde in 100 µL methanol followed by addition of 900 µL borate buffer and 50 µL β-mercaptoethanol. A standard mixture containing (10 µg/mL each) glycine (Gly), aspartate (Asp), asparagine (Asn), serine (Ser), histidine (His), arginine (Arg), threonine (Thr), alanine (Ala), proline (Pro), tyrosine (Tyr), valine (Val), methionine (Met), tryptophan (Trp), isoleucine (Ile), leucine (Leu), phenylalanine (Phe) and lysine (Lys) was prepared. 100 µL of standard mixture was added to 100 µL of derivatizing agent and was agitated and left to stand at room temperature for 15 min. Prior to injection, 800 µL of methanol was added to above solution. The amino acid derivatives were separated on Shimadzu HPLC system attached to UV detector. A 20 µL sample was injected into C_{18e} RP

column using autosampler. The concentration of the optimized mobile phase A was 0.14 M sodium acetate containing 500 µL triethylamine adjusted to pH 6.7 with glacial acetic acid and methanol (90:10). Mobile phase B was acetonitrile:water (60:40) v/v. The separation gradient used was 0-5 min (25 % B), 10 min (50 % B), 15 min (75 % B), 22 min (60 % B), 25 min (50 % B), 27 min (25 % B) and 30 min (0 % B). The amino acids were detected at 340 nm with column condition set at 38 °C. To determine the measurement precision, 5 aliquots of the same standard solution were injected. The peaks were acquired by using LC software and calculation was based on five runs at four concentrations *i.e.* 10, 5, 2.5 and 1 µg/mL. The proportional molar concentration for each amino acid was calculated based on concentration of standard amino acid. The same was used for calculating amino acids in wheat leaves.

Amino acids in dried wheat leaves (0.1 g) were extracted thrice with 2.0, 1.5 and 1 mL, respectively of 70 % aqueous methanol. This extract was vortexed (1 min), centrifuged at 8000 rpm (10 min) and filtered. The final volume of supernatant was made up to 5 mL by using 70 % methanol. The same developed procedure was adopted for amino acid analysis as described for standard mixture.

RESULTS AND DISCUSSION

Separation of amino acids: Chromatogram of *ortho*-phthaldialdehyde derivatized standard amino acid mixture is shown in Fig. 1. The retention time of different amino acids were Gly (5.63 min), Asp (9.65 min), Asn (12.6 min), His (12.9), Ser (13.3 min), Arg (13.7 min), Thr (14.0 min), Tyr (15.5 min), Val (17.3 min), Met (18.1 min), Trp (18.3 min), Ile (18.4 min), Leu (18.6 min), Phe (19.4 min), Pro (19.9 min) and Lys (21.3 min). The glycine eluted first having retention time 5.45 min and lysine was the last eluted amino acid with retention time 21.3 min. The amino acids in standard mixture were identified according to their characteristic time as revealed when injected individually. Gradient run time was optimized for better separation of different peaks; wherein all the test amino acids eluted with 30 min without interfering in next elution. The separation and resolution of peaks of all the 16 amino acids present in the standard were found satisfactory using this method. The gradient used in the study provided good separation of various peaks resulting in better identification and quantification. The peaks such as valine, methionine, isoleucine and leucine clearly separated in this method. The amino acids in the standard mixture were Gly, Asp, Asn, Ser, His, Arg, Thr, Pro, Tyr, Val, Met, Trp, Ile, Leu, Phe and Lys separated in 25 min with fine resolution. Furthermore, the high sensitivity of this HPLC method is evident from intensity of peaks of different amino acids. The total time required for derivatization was 30 min of which 15 min was for reaction between the amino acids and *ortho*-phthaldialdehyde. The intensities of HPLC peaks were not affected by longer reaction time or a greater excess of *ortho*-phthdialdehyde.

Determination of amino acids in wheat leaves: The chromatographic profile of *ortho*-phthaldialdehyde-derivatized amino acids in wheat leaves is shown in Fig. 2. Identification of each amino acid peak was made by matching its retention

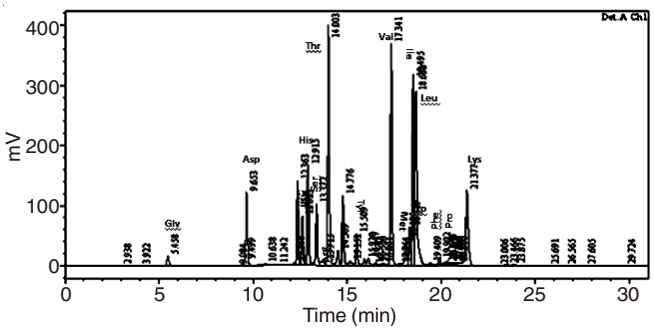

Fig. 1. A typical chromatogram showing the standard mixture of amino acids

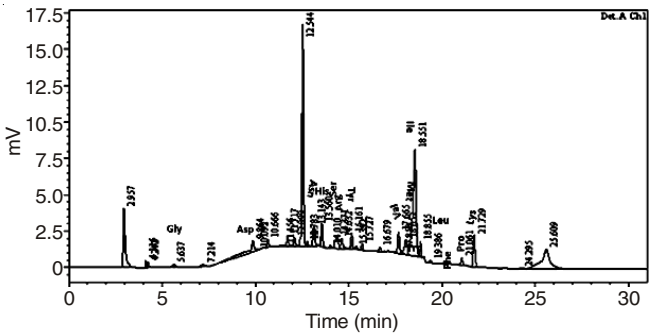

Fig. 2. A typical chromatogram showing the amino acids detected in wheat sample

time with respect to chromatogram of standard amino acid mixture. All the sixteen amino acids (Gly, Asp, Asn, Ser, His, Arg, Thr, Trp, Pro, Tyr, Val, Met, Ile, Leu, Phe and Lys) were separated with good resolution. The sequence of elution of all the test amino acids was similar to that of standard amino acid mixture. The values for the amino acids in wheat leaves such as Asp, Asn, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe and Lys were 1.33, 2.25, 0.30, 0.64, 1.90, 0.57, 2.52, 0.44, 0.42, 0.27,1.47, 3.81,0.15, 3.03, 4.35 and 0.68 nmol/g, respectively (Table-1). The standard deviation (SD) and coefficient of variance (CV %) of amino acids in wheat samples was also calculated and presented in Table-1. As the acid hydrolysis of amino acids was not done in extraction of amino

TABLE-1
RETENTION TIME, PEAK AREA AND AMINO ACIDS (nmol/g) OF WHEAT SAMPLE DERIVATIZED WITH *ortho*-PHTHALDIALDEHYDE

Amino acids	Retention time (min)	Peak area	Amino acids (nmol/g)	SD	CV (%)
Gly	5.63	1726	1.33	0.215	6.93
Asp	9.86	18740	2.25	0.310	7.25
Asn	12.7	1863	0.30	0.043	6.97
His	13.1	10794	0.64	0.080	8.06
Ser	13.5	9326	1.90	0.240	7.91
Arg	14.0	1255	0.57	0.085	6.71
Thr	14.6	5295	2.52	0.295	8.54
Tyr	15.7	2724	0.44	0.109	4.03
Val	17.6	10025	0.42	0.095	4.42
Met	17.8	1160	0.27	0.052	5.19
Trp	18.0	10299	1.47	0.198	7.42
Ile	18.5	91940	3.81	0.395	9.64
Leu	18.8	5143	0.15	0.070	3.28
Phe	19.3	1139	3.03	0.385	7.87
Pro	21.0	2999	4.35	0.496	8.77
Lys	21.7	16226	0.68	0.098	6.93

acids, the peaks of amino acids tryptophan and asparagine were also obtained. In other methods, tryptophan and asparagine destroyed by acid hydrolysis and were not determined [6,7]. The present study resulted a simple, accurate and reliable method for the amino acids analysis, which can be further useful in analysis of different amino acids in cereals.

Conclusion

The results of amino acids analysis by developed method suggest that reversed-phase HPLC separation and UV detection of *ortho*-phthaldialdehyde derivatives of amino acids are useful for determination of amino acids in wheat leaves as the accuracy of method using C_{18e} cap column is trustworthy and sensitive. Furthermore, this method provides high resolution, rapid and reproducible results.

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