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Research Article

Chromatographic Method for Determination of the Amino Acid Content in *Dioscorea bulbifera* L. Tubers by RP-HPLC

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ABSTRACT

Background: The present study was carried out for determination of amino acid content in tubers of *Dioscorea bulbifera* using reverse-phase high-performance liquid chromatography.

Methods: The method involved the vapor phase hydrolysis of the sample, automated derivatisation of the amino acids with the aid of AccQ-Fluor reagent kit, separated on a high performance liquid chromatography equipped with photo diode array (HPLC-PDA) at 254 nm having column temperature of 37 ºC.

Results: The proportional molar concentration for each amino acid was calculated based on the concentration of standard amino acids and expressed as µg amino acid/mg sample. Methionine, aspartic acid and leucine were major components while as tyrosine was found minor from the plant on dry weight basis.

Conclusion: The method is reliable, simple and economical for determining the amino acid content of *Dioscorea bulbifera* tubers.

Introduction

Dioscorea bulbifera L. (Dioscoreaceae) locally known as yam or air potato, widely distributed in the Africa, Asia and northern Australia. This plant is distributed throughout India except in the dry north-western regions ascending up to 1800 m in the Himalayas and parts of Bihar, Orissa, Uttar Pradesh, Sikkim, Assam, West Bengal, Orissa, Bihar, Jharkhand, Madhya Pradesh, Maharashtra and Tamil Nadu and is known for its high nutritional and functional values. It is used for the treatment of wide variety of ailments in Ayurveda, Unani and Siddha systems of medicines to cure gastric cancer, rectum carcinoma, goitre and sore throat. The bulb extracts possess many biological activities including antihyperlipidemic, antioxidant, antitumor, analgesic, anti-inflammatory and antihyperglycemic.[1,2] In addition to medicinal uses, various parts of the plant (tubers, bulbils) are consumed in various types of foods through diverse processing methods such as conventional drying, roasting, fermentation, frying, boiling and steaming.[3]

Amino acids represent a class of biologically active primary metabolites with universal distribution. They are considered important and active constituents for the growth and development of human beings. Plant proteins in the human diet constitute major portion of primary metabolites in diverse range, which vary in composition and digestibility. They are perfectly capable of satisfying human needs for all ages when consumed in appropriate mixtures.[4-7] Amino acid analysis plays an important role in the field of biochemistry, pharmacy and biomedicine.[9,10] Numerous methods are available in the literature for the determination of amino acids[11-15] but such methods are either tedious or lack sensitivity, specificity and are cost intensive. However, liquid chromatography, in its high performance mode (HPLC) appears the instrumental approach of choice for the quantative estimation and method development of amino acids from *Dioscorea bulbifera*. In continuation of our previous study[16-21] on application of planar chromatography for fingerprinting and chromatographic method development to assure quality of medicinal plants, a validated analytical method has been developed and various validation parameters of the analysis like linearity, specificity, recovery, precision, robustness, limit of detection (LOD) and limit of quantification (LOQ) have been measured.

To the best of our knowledge there are no reports so far in the literature of this plant with regard to quantification and chemobiological standardisation by HPLC. Therefore, in this communication we report first time method validation, identification and simultaneous quantification of 14 amino acids from the *Dioscorea bulbifera* by HPLC.

Materials and Methods

Plant material

The plant *D. bulbifera* was collected from Budhi

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Chanderi, Ashok Nagar, India which was identified by the taxonomists of our institute (Institute of Ethnobiology Jiwaji University, Gwalior) and voucher specimen (IOE-451) was deposited in the herbarium for future reference.

Sample preparation

The plant has been shade dried and crushed into a powder by using electric grinder, 2 g of the sample was accurately weighed and transferred into a beaker. The beaker containing plant sample was then placed in a water bath at 40ºC. Digested sample was then transferred into a 50 ml flask and the volume was made up to the mark by adding distilled water. The beaker was kept in an ultrasonicator bath at 40ºC for 30 min. The extract was centrifuged followed by the addition of 30 ml of distilled water to the residue, which was sonicated for 30 min. The filtrate was transferred to a 100 ml flask and volume was made to make up the volume by adding distilled water. Finally, this solution was passed through a 0.22 µm Millipore membrane and the filtrate was used for further experiments.

Hydrolysis of proteins/peptides into amino acids

The vacuum-dried samples were hydrolyzed with 200 µL of constant boiling 6N HCl and 40 µL of phenol through vapor-phase hydrolysis. The samples were dried in an oven at 112-116 ºC for 20-24 h. After completion of hydrolysis, excess HCl was wiped off and the tubes were vacuum dried for 90 min. The plant sample was reconstituted with 100 µL of 20 mM boiling HCl.

Derivatization of amino acids

The reconstituted 20 µL samples were derivatized with AccQ-Fluor reagent kit (WAT052880- Waters Corporation, USA). AccQ-Fluor borate buffer (60 µL) was added in the sample tube with micro pipette and vortexed. Thereafter, 20 µL of AccQ-Fluor reagent was added and immediately vortexed for 30 Sec. and the contents were transferred to maximum recovery vials. The vials were heated for 10 min in a water bath at 55 ºC before separation of amino acids using HPLC.

Chromatographic conditions and procedure

The AccQ-Fluor amino acid derivatives were separated on an HPLC System (Young Lin ACME 9000) attached to a PDA. A 10 µL sample was injected into reverse phase AccQ Tag Silica-bonded Amino Acid Column C18 using an auto sampler. The Waters AccQ Tag Eluent A was diluted to 10% in Milli-Q water and used as eluent A, and 60% acetonitrile as eluent B in a separation gradient with a flow rate of 1.0 ml/min. The separation gradient used was 0-2 min (100% A), 2.0 min (98.0% A), 15.0 min (93.0% A), 19.0 min (90.0% A), 32.0 min (67.0% A), 38.0 min (0.0% A), and 56.0 min (100.0% A). The amino acids were detected using PDA at 254 nm with the column condition set at 37 ºC. The amino acid peaks obtained were analyzed, and were contents were calculated based on amino acid calibration standard run at five concentrations 20, 40, 60, 80 and 100 ng/ml having 2.5 µ mol/ml of L-forms of Asp, Glu, Ser, Gly, Thr, Arg, Ala, Tyr, Val, Met, Phe, Ile, Leu, Lys and 1.25 µ mol/ml of cystine in 0.1N HCl. Amino acid assignments were visually checked to verify the peak assignment. Injections (10 µL) of 10, 20, 30, 40 and 50 ng/ml of amino acid standard produces a calibration curve. The proportional molar concentration for each amino acid was calculated based on the concentration of standard amino acids and expressed as µg amino acid/mg sample.

Results

Qualitative and quantitative analysis

The tested amino acids were identified by comparing their retention times with that of standards used.

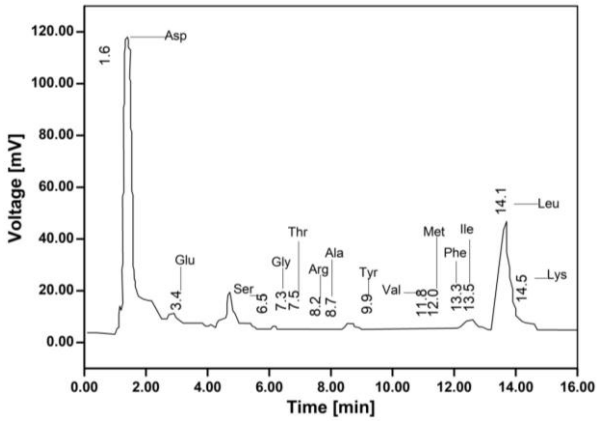

Figure 1. HPLC chromatograph of extract of *Dioscorea bulbifera* tuber.

Table 1. Regression curve equation and correlation coefficient.

S. No.	Amino acid	Calibration range (ng/ml)	Linear regression equation	Correlation coefficient (R^2)
1.	Aspartic acid (Asp)	10-100	y = 15.37x + 0.53	0.995
2.	Glutamic acid (Glu)	10-100	y = 15.25x - 40.06	0.994
3.	Serine (Ser)	10-100	y = 16.46x + 33.78	0.998
4.	Glycine (Gly)	10-100	y = 16.90x + 58.29	0.997
5.	Threonine (Thr)	10-100	y = 15.44x - 55.43	0.992
6.	Arginine (Arg)	10-100	y = 16.52x - 124.8	0.995
7.	Alanine (Ala)	10-100	y = 17.00x - 18.19	0.995
8.	Tyrosine (Tyr)	10-100	y = 15.17x - 111.5	0.996
9.	Valine (Val)	10-100	y = 16.93x - 77.91	0.998
10.	Methionine (Met)	10-100	y = 15.85x - 8.53	0.999
11.	Phenylalanine (Phe)	10-100	y = 16.63x + 2.19	0.999
12.	Isoleucine (Ile)	10-100	y = 16.91x - 87.99	0.999
13.	Leucine (Leu)	10-100	y = 17.59x - 37.31	0.999
14.	Lysine (Lys)	10-100	y = 16.95x - 58.01	0.997

In curve equation, Y= the ratio of the peak area of substance measured to the peak area of internal standard. X = the ratio of the concentration of substance measured to the concentration of internal standard.

The obtained amino acid peaks were analyzed and contents were calculated based on amino acid calibration standards run at different concentrations. Quantitative data were obtained by plotting the peak concentrations of the standard solution against the peak concentration of the sample solution. Chromatogram showing the amino acid peaks of the sample solution is shown in Figure 1.

Table 2. Intra- day and inter-day precision of HPLC method of fourteen amino acids.

Amount	Intra-day precision (n=6)		Inter-day precision (n=6)	
	Mean area	%RSD	Mean area	%RSD
ASP				
100	271.42	1.89	301.92	1.20
200	609.01	1.20	639.81	2.79
300	991.31	1.67	1001.29	2.97
GLU				
100	992.42	1.90	998.61	1.25
200	1911.88	1.63	1972.21	1.31
300	2941.57	1.06	2941.27	1.09
SER				
100	589.31	1.85	562.32	1.90
200	1101.21	1.80	988.37	1.85
300	1621.67	1.90	1502.31	1.70
GLY				
100	590.96	1.35	422.61	0.92
200	1021.32	0.54	831.21	1.31
300	1499.31	1.36	1381.00	1.01
THR				
100	412.72	0.10	622.62	1.50
200	791.66	0.31	1210.09	1.45
300	1212.01	1.93	1921.92	1.92
ARG				
100	900.12	0.59	602.11	0.68
200	1923.42	1.44	1172.73	1.02
300	2935.62	0.67	1799.97	1.32
ALA				
100	765.61	0.54	212.14	1.31
200	1464.22	0.69	392.99	0.77
300	2292.12	1.82	599.71	0.89
TYR				
100	825.61	1.32	672.12	0.89
200	1698.22	1.11	1190.32	0.72
300	2700.32	1.02	1786.41	0.52
VAL				
100	370.42	0.89	401.92	1.01
200	710.01	1.01	739.81	0.79
300	1092.31	0.67	1101.29	0.97
MET				
100	1086.42	1.21	998.61	1.25
200	2020.89	1.03	1972.21	1.31
300	3040.50	1.10	2941.27	1.09
PHE				
100	590.31	0.81	562.62	0.92
200	1110.21	0.87	988.37	0.89
300	1625.67	0.91	1502.31	0.73
ILE				
100	589.96	0.35	422.61	0.92
200	1024.32	0.54	831.21	1.31
300	1500.31	0.36	1381.00	1.01
LEU				
100	369.42	0.89	401.92	1.01
200	710.01	1.01	739.81	1.70
300	1095.32	0.67	1101.29	1.25
LYS				
100	593.94	0.35	422.61	1.92
200	1025.34	0.54	831.21	0.31
300	1500.31	0.36	1381.00	1.11

Methionine, Aspartic acid and Leucine were found to be the major components (31.87±0.19 µg/mg, 20.51±0.17 µg/mg and 20.28±0.72 µg/mg, respectively) and Tyrosine was found in least concentrations (1.42±0.42 µg/mg) (Table 3).

Validation of the analysis method

Linearity

Linearity data were calculated by investigating the area *vs.* concentration of amino acid plots. The obtained regression equations and correlation coefficients are presented in Table 1. The linearity between peak areas and concentrations was good, and the regression coefficients (R^2) were around 0.990 for all the curves, which revealed an excellent linearity of the calibrations.

Precision

Precision was presented in terms of coefficient of variation. The data show good agreement between the individual test results. The replicability was determined by chromatographic analysis of three replicate samples of the extracts (Table 2). Analysis of amino acids in the sample provided a good coefficient of variation. Relative standard deviation (RSD) was lower than 2.0% in all the cases, which gives the confirmation of the reliability of the derivatization method.

Application of the method for the analysis of amino acids

The amino acid contents (ng/mg) of the analyzed samples are reported in Table 3. Values shown represent the mean of three observations.

Table 3. Amino acid content (µg/mg) in the analyzed sample (dry weight basis).

Amino acids	Average retention time (Rt), min.	Amino acid content (µg/mg)
Aspartic acid (Asp)	1.6	20.51±0.17
Glutamic acid (Glu)	3.4	14.64±0.83
Serine (Ser)	6.4	9.17±0.62
Glycine (Gly)	7.3	18.49±0.47
Threonine (Thr)	7.5	13.52±0.49
Arginine (Arg)	8.2	10.83±0.35
Alanine (Ala)	8.7	16.34±0.27
Tyrosine (Tyr)	9.9	1.42±0.42
Valine (Val)	11.8	4.64±0.31
Methionine (Met)	12.0	31.87±0.19
Phenylalanine (Phe)	13.3	4.94±0.45
Isoleucine (Ile)	13.5	4.15±0.68
Leucine (Leu)	14.1	20.28±0.72
Lysine (Lys)	14.5	5.67±0.62

Data represented as the mean value of three replicates.
Wavelength: 254 nm at 37 °C.
The quantity of amino acid was calculated by the equation obtained in standard curve.

Discussion

Many of the extraction and amino acid analysis techniques currently used do not offer a complete analysis of all the amino acids. The purpose of this study was to incorporate extraction and analysis procedures into a reliable method to measure the complete amino acid profile of yam tubers using HPLC.

Table 4. Robustness testing of HPLC method for 14 amino acids.

Parameters*	Asp	Glu	Ser	Gly	Thr	Arg	Ala	Tyr	Val	Met	Phe	Ile	Leu	Lys
Mobile phase composition	1.56	1.28	1.26	1.99	1.44	1.87	1.55	0.99	0.23	0.55	0.88	0.95	0.57	0.87
Amount of mobile phase	2.00	1.66	1.85	1.45	1.66	1.87	1.96	1.54	1.63	1.55	1.54	2.00	1.65	1.90
Temperature	1.20	1.55	1.88	1.55	1.77	1.44	1.90	1.66	1.00	1.47	1.88	1.66	2.00	2.66
Relative humidity	1.54	1.86	1.45	1.49	1.63	1.44	1.90	1.32	1.44	1.88	1.45	1.44	1.54	1.58

***Results are expressed in %RSD

The goal of the study was to develop sensitive and rapid chromatographic method for determination of 14 amino acids present in edible tubers of *D. bulbifera*. HPLC of amino acid derivatives allowed their quantification in tubers of *Dioscorea bulbifera*. The method proposed was validated as per the ICH guidelines. The identical Rf values and absorbance/reflectance spectra for 14 amino acids between standard and sample track proved the specificity of the method. Further, the peak purity analysis confirmed the non-interference in the analysis of amino acids using this method. The high value of correlation coefficient (>0.99) and the SD for intercept value (<2%) of regression line established the linearity of calibration graphs for the amino acids. The repeatability studies ensured precision of the sample application device. On the basis of % RSD values for intra-day and inter-day precision studies, the method was found to be precise. The robustness of this method was tested using experimental design approach (Table 4). Using Photo Diode Array detector, the method showed linearity over a wide range of concentrations. Thus, this method represents a better approach to amino acid analysis. The advantages of this method are its ability to separate all amino acids present in a plant sample in one run, in a very short time, and the wide range of analytic measurement obtained using a PDA detector.

Conclusion

Herein an optimized analytical method for the separation and quantification of 14 amino acids in *D. bulbifera* tubers has been developed. The method has sufficient reproducibility and accuracy to allow the determination of amino acid content in *D. bulbifera*. This analytical method is based on the pre-column derivatisation of plant samples using AccQ-Fluor reagent kit. The acid hydrolysis with 200 µL of constant boiling 6N HCl and 40 µL of phenol through vapour-phase hydrolysis at 112-116ºC for 20-24 h resulted in the chromatograms that were suitable the quantification of amino acids in the plant sample. The developed method is highly specific, precise and shows linearity across the analytical range.

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Conflict of interests

The authors claim that there is no conflict of interest.

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