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ORIGINAL ARTICLE

Analysis of Free Amino Acids in Different Extracts of *Orthosiphon stamineus* Leaves by High-Performance Liquid Chromatography Combined with Solid-Phase Extraction

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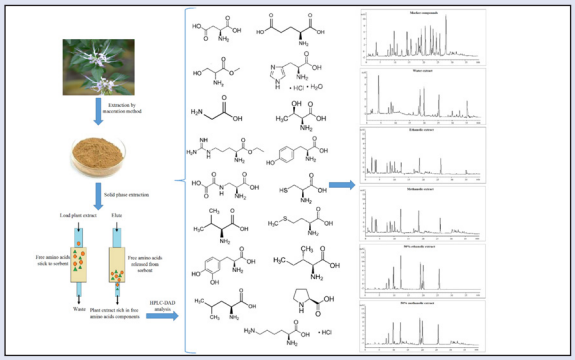
ABSTRACT

Background: *Orthosiphon stamineus* (OS) Benth is a medicinal plant and native in Southeast Asia. Previous studies have shown that OS leaves possess antioxidant, cytotoxic, diuretic, antihypertensive, and uricosuric effects. These beneficial effects have been attributed to the presence of primary and secondary metabolites such as polyphenols, amino acids, and flavonoids. **Objective:** To develop and validate an high-performance liquid chromatography (HPLC)-diode array detector (DAD) method combined with solid-phase extraction that involves precolumn derivatization with *O*-phthaladehyde for simultaneous analysis of free amino acids in OS leaves extracts. **Materials and Methods:** OS leaves were extracted with water (OS-W), ethanol (OS-E), methanol (OS-M), 50% ethanol (OS-EW), and 50% methanol (OS-MW). The extracts were treated by C18 cartridge before derivatization, resulting in great improvement of separation by Zorbox Eclipse XDB-C_{18} column. **Results:** The HPLC–DAD method was successfully developed and validated for analyzing the contents of free amino acids in OS extracts. The results showed that l-aspartic acid with 0.93 ± 0.01 nmol/mg was the major free amino acid in OS-W extract. However, in OS-E, OS-M, OS-EW, and OS-MW, l-glutamic acid with 3.53 ± 0.16, 2.17 ± 0.10, 4.01 ± 0.12, and 2.49 ± 0.12 nmol/mg, respectively, was the major free amino acid. Subsequently, l-serine, which was detected in OS-W, OS-E, and OS-M, was the minor free amino acid with 0.33 ± 0.02, 0.12 ± 0.01, and 0.06 ± 0.01 nmol/mg, respectively. However, l-threonine with 0.26 ± 0.02 and 0.19 ± 0.08 nmol/mL in OS-EW and OS-MW, respectively, had the lowest concentration compared with other amino acid components. **Conclusion:** All validation parameters of the developed method indicate that the method is reliable and efficient to simultaneously determine the free amino acids content for routine analysis of OS extracts.

Key words: Derivatization, free amino acids, HPLC-DAD, *Orthosiphon stamineus*, solid-phase extraction

SUMMARY

- The HPLC-DAD method combined with solid phase extraction was successfully developed and validated for simultaneous determination and quantification of 17 free amino acids in *Orthosiphon stamineus* (OS) Benth extracts
- OS extracts were found to be rich in free amino acid content
- L-aspartic acid was the major free amino acid in OS water extract while, in OS ethanol, methanol, 50% ethanol and 50% methanol extracts, L-glutamic acid was the major free amino acid
- L-serine was the minor free amino acid in OS water, ethanol and methanol extracts while, in OS 50% ethanol and 50% methanol extracts, L-threonine had the lowest concentration compared to other amino acid components.



Abbreviations used: HPLC-DAD: High-Performance Liquid Chromatography with Diode-Array Detection, OS: *Orthosiphon stamineus*, OS-W: *Orthosiphon stamineus* water extract, OS-E: *Orthosiphon stamineus* ethanol extract, OS-M: *Orthosiphon stamineus* methanol extract, OS-EW: *Orthosiphon stamineus* 50% ethanol extract, OS-MW: *Orthosiphon stamineus* 50% methanol extract, OPA: *O-phthaladehyde*, SPE: Solid Phase Extraction, UV: Ultraviolet, LOD: Limit of Detection, LOQ: Limit of Quantification, RSD: Relative Standard Deviation.

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INTRODUCTION

Orthosiphon stamineus (OS) Benth. (Lamiaceae) is a medicinal plant grown in Southeast Asia and currently cultivated in Indonesia and Malaysia. The local name of OS in Malaysia is *Misai Kucing*. The leaves of OS have been used traditionally in treating angiogenesis-related diseases, urinary lithiasis, edema, inflammation, eruptive fever, influenza, hepatitis, jaundice, rheumatism, diabetes, and hypertension.[1] OS leaves are used commonly in Southeast Asian and European countries as herbal tea, known as "Java tea." Various chemical constituents including flavonoids, terpenoids, saponins, hexoses, organic acids, chromene, myo-inositol, and polyphenols have been identified in OS leaves.[2-5] Numerous studies reported that OS leaves possess potential therapeutic properties such as antioxidant, cytotoxic, diuretic, antihypertensive, and uricosuric effects in rats.[6-9] These beneficial effects have been attributed

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to the presence of compounds such as polyphenols, flavonoids, amino acids, and carbohydrates.[6-9]

OS leaves have been reported to contain high content of proteins.[10] Proteins constitute the major nitrogen reserve in the plant extract, representing approximately 60% of the total nitrogen.[11] The free amino acid is a composition of the total protein fraction obtained from plant extracts.[12] Numbers of free amino acids have been identified in tea leaves such as theanine, γ-glutamylethylamide, or 5-*N*-ethyl glutamine.[13] Previous studies have shown beneficial pharmacologic and physiologic activities of these compounds such as promoting relaxation, inhibiting caffeine's negative effects, reducing blood pressure, enhancing antitumor activity, neuroprotection, and anti-obesity activity.[14-16] Consequently, because of vast biologic significance, there is a demand for rapid and effective analytical methods for analyzing amino acids.

A numbers of methods have been developed to determine the presence of various amino acids in plant extracts. The total content of amino acids can be determined by ninhydrine or 2,4-dinitrofluorobenzene colorimetric method.[17] Limited number of chromatographic methods have been reported for analyzing amino acids, namely high-performance liquid chromatography (HPLC), capillary electrophoretic, and anion exchange chromatography.[18-22] In chromatographic methods, it is necessary to label amino acids using labeling reagent, such as 4-dimethylaminoazobenzene-4-sulfonyl chloride (dabsyl chloride), *O*-phthaladehyde (OPA), and phenylisothiocyanate, due to lack of suitable chromophore in their structures. In such a case, it involves derivatization with precolumn or postcolumn and detection by fluorescence, ultraviolet, or diode array detector (DAD).[18] However, according to a previous report, clear chromatograms of free amino acids in Chinese green tea could not be obtained even after precolumn derivatization with OPA. This was due to the existence of other soluble compositions in plant extract that were extracted out together with free amino acids.[18] These soluble compositions may affect the derivatization or separation of the amino acids by HPLC. Therefore, it is necessary to treat plant extract before derivation by liquid-liquid or solid-phase extraction (SPE). Since SPE is rapid, economical, and sensitive, it has been widely used. In addition, different cartridge with great variety of sorbent can be used.[23,24] However, there is little information about application of SPE for the sample preparation in the analysis of free amino acids in plant extracts. In this report, therefore, we introduce SPE combined with high-performance liquid chromatography (HPLC)-DAD for the determination of 17 free amino acids including l-aspartic acid, l-glutamic acid, l-serine, l-histidine hydrochloride monohydrate, glycine, l-threonine, l-arginine, l-alanine, l-tyrosine, l-cystine, l-valine, l-methionine, l-phenylalanine, l-isoleucine, l-leucine, l-lysine hydrochloride, and l-proline in different extracts of OS leaves.

MATERIALS AND METHODS

Chemicals and reagents

Solutions of 17 amino acids in five concentrations (0.01-1 nmol) and OPA reagent (*O*-phthalaldehyde and 3-mercaptopropionic acid) in 0.4 M borate buffer at concentration of 10 mg/mL were obtained from Agilent, USA. Methanol and acetonitrile of HPLC grade were purchased from Merck (Petaling Jaya, Selangor, Malaysia). Deionized water for HPLC was prepared using ultrapure water purifier system (Elgastat, Bucks, UK).

Instrumentation

The HPLC was performed by an Agilent Technologies series 1260 infinity (Waldronn, Germany) system equipped with quaternary pump (G 1311 C), autosampler (G 1329 B), column oven (G 1316A), and DAD (G 1315 D).

Plant samples

OS leaves were purchased from specialized supplier of herbal products in Malaysia (Herbagus Trading, Kepala Batas, Pulau Pinang) and identified at the herbarium of School of Biological Sciences Universiti Sains Malaysia, where a voucher sample was deposited (reference number: 11009). The leaves were pulverized into a fine powder using milling machine (Retsch GmbH, Germany) and saved in air-tight plastic bags until used.

Preparation of plant extracts

In preparing of extracts, 100 g plant powder was extracted with 500 mL water (OS-W), ethanol (OS-E), methanol (OS-M), 50% ethanol (OS-EW), and 50% methanol (OS-MW) using maceration method for 48 h at 50°C. After cooling, extracts were filtered using Whatman filter paper No.1 (Whatman, England), concentrated at 50°C under vacuum using a rotary evaporator (RE121 Buchi, Switzerland), and dried using a freeze-drying system (Labconco, USA).

Treatment of extracts by SPE

OS-W, OS-E, OS-M, OS-EW, and OS-MW extracts (100 mg) were dissolved in 5 mL distilled deionized water. Then the extracts were treated by SPE using Bond Elute-C18 cartridge (Agilent, USA) according to the method described by Wang *et al*.[18] Briefly, the Bond Elute-C18 cartridge was conditioned by rinsing with 30 mL of methanol and 10 mL of distilled deionized water. The extracts (5 mL) were loaded onto the conditioned cartridge and the retained compounds were eluted with 5 mL of 10% ethanol. The eluted compounds were then collected and evaporated to dryness in the oven at 60°C. The residue was dissolved in 1 mL distilled deionized water and filtered through a 0.45 μM (Whatman).

Precolumn derivatization

The precolumn derivatization with OPA reagent was carried out according to the previously described method.[18] Briefly, 70 μL of extracts or standard solutions were mixed with 10 μL of OPA solution at 25°C for exactly 2 min. Subsequently, the reaction mixture was immediately analyzed by HPLC.

Chromatographic condition

The HPLC separation was carried out according to the previously described method[25] using a Zorbax Eclipse XDB-C_{18} column (150 × 4.6 mm internal diameter, 5 μm particle size) (Agilent, USA). The flow rate was 0.8 mL/min, injection volume was 10 μL, and the temperature of column oven was set at 40°C. The analysis was examined by DAD at signal A (338 nm, 10 nm bandwidth and reference wavelength 390 nm, 20 nm bandwidth), signal B (262 nm, 16 nm bandwidth and reference wavelength 324 nm, 8 nm bandwidth), and signal C (338 nm, 10 nm bandwidth and reference wavelength 390 nm, 20 nm bandwidth). The responses of the reference markers were compared at different signals. The strongest response (signal A) was taken as appropriate signal for detection and quantification. The mobile phase consisted of solvent A (methanol:acetonitrile:water 45:45:10 v/v) and solvent B (phosphate buffer, pH 7.5). Elution was performed with a linear gradient as shown in [Table 1].

Validation of HPLC method

The developed HPLC method was validated for accuracy, precision, linearity range, limit of detection (LOD), and limit of quantification (LOQ) according to the ICH guideline.[26] Linearity was performed by injecting a series of standard solutions (0.01–1 nmol). Calibration curves were established on five data points covering the concentration range for each amino acid standard using HPLC condition as described above. The calibration curves were obtained by plotting the peak area (y-axis) of each amino acid standard versus the concentration (x-axis). Intraday and

interday analyses were used to evaluate precision of the method. Different concentrations of the amino acid standards were analyzed using HPLC. The standards were injected five times per day (intraday) and once per day for five consecutive days (interday). The resulting peak area was used to calculate the percentage of relative standard deviation (%RSD). Accuracy of proposed HPLC method was evaluated by recovery studies. Known amounts of amino acid standard solutions (five concentrations) were added to all extract solutions. The spiked extract solutions were injected three times to HPLC and the recovery was calculated with the value of detected versus added amounts. LOD and LOQ were estimated at a signal-to-noise ratio (S/N) of 3 and 10, respectively, through the slope and standard deviation method according to the following formula:

$LOD = (3.3 \times \delta)/S$

$LOQ = (10 \times \delta)/S$

where

δ = standard deviation of the y-intercept of the linear regression equations

S = slope of the linear regression equations

Identification of free amino acids in the extracts

The chromatographic peaks of free amino acids in the extracts were confirmed by comparing their retention time and ultraviolet spectrum with those of amino acids standards and spiking small amount of the amino acid standards to the extracts solutions.

Quantification of free amino acids in OS extracts by HPLC method

The validated HPLC method was applied to determine the selected amino acids compounds of OS extracts. All prepared extract solutions were injected (three replicates) in the HPLC system using described chromatographic condition. The peak area corresponding to the marker compounds in the extracts was recorded. The linear regression equations were calculated using the regression parameters obtained from the calibration curve. These equations were applied to calculate the concentration of free amino acids compounds found in the extract according to the following formula and expressed in nanomole standards per milligram:

$AA\ (nmol/mg) = (VC)/W$

where

AA = amount of standard amino acids compounds in the extract

V = final volume of the extract solution in milliliter

W = weight of the extract

C = concentration of standard compounds obtained from calibration curve

Table 1: Scheme of elution gradient for HPLC analysis.

Time(min)	Solvent A (%)	Solvent B (%)
0	2	98
0.84	2	98
33.4	57	43
33.5	100	0
39.3	100	0
39.4	2	98
40	2	98

RESULTS

Development and validation of HPLC method

HPLC separation of the amino acids standard mixture was achieved using the stated method. The representative chromatogram of standards is shown in [Figure 1A]. For OS extracts, elution profile of reference markers was similar to the observed one in their corresponding standards [Figure 1]. The calibration curves were constructed using solutions of 17 amino acids in five concentrations. The calibration curves were constructed by plotting peak area versus concentrations of each standard compound. A linear regression equation and correlation coefficients were appointed from the graph by plotting the mean of three replicates peak areas versus the concentration. The results show a good linearity of all reference compounds versus concentration ranges with $R^2 > 0.98$ [Table 2]. LOD and LOQ were determined to evaluate sensitivity of the method. The LOD is defined as the lowest detectable concentration of analyst that analytical system can reliably differentiate from the background level. However, LOQ is defined as the lowest quantifiable level of analyst that can be measured with a standard level of confidence. In [Table 2], results of LOD ($S/N = 3$) and LOQ ($S/N = 10$) of each amino acid compound are listed.

The intraday and interday precision were determined using multisamples preparation. In intraday analysis, five concentrations of reference

Table 2: Linear correlation between peak area and concentration of amino acids reference compounds, limit of detection (LOD) and limit of quantification (LOQ) of the reported HPLC method.

Components	LOD (nmol/mL)	LOQ (nmol/mL)	Linearity range (nmol/mL)	Equation	R^2 Value
L-aspartic acid	0.01 ± 0.00	0.03 ± 0.00	0.01-1	Y = 154.4X + 2.375	0.999
L-Glutamic acid	0.06 ± 0.00	0.20 ± 0.00	0.01-1	Y = 65.30X + 3.934	0.992
L-serine	0.01 ± 0.00	0.03 ± 0.00	0.01-1	Y = 110.9X - 0.241	0.998
L-Histidine hydrochloride monohydrate	0.03 ± 0.00	0.10 ± 0.00	0.01-1	Y = 127.8X + 5.598	0.998
Glycine	0.01 ± 0.00	0.04 ± 0.00	0.01-1	Y = 95.25X + 4.308	0.992
L-Threonine	0.01 ± 0.00	0.04 ± 0.00	0.01-1	Y = 77.24X + 3.222	0.990
L-arginine	0.01 ± 0.00	0.04 ± 0.00	0.01-1	Y = 80.84X + 2.885	0.990
L-Alanine	0.01 ± 0.00	0.03 ± 0.00	0.01-1	Y = 81.03X + 2.372	0.996
L-Tyrosine	0.01 ± 0.00	0.05 ± 0.00	0.01-1	Y = 124.9X + 2.897	0.995
L-Cystine	0.009 ± 0.00	0.02 ± 0.00	0.01-1	Y = 189.4X + 2.673	0.994
L-Valine	0.008 ± 0.00	0.02 ± 0.00	0.01-1	Y = 108.4X - 1.502	0.996
L-Methionine	0.006 ± 0.00	0.02 ± 0.00	0.01-1	Y = 198.8X + 0.203	0.999
L-Phenylalanine	0.01 ± 0.00	0.04 ± 0.00	0.01-1	Y = 149.5X + 9.568	0.990
L-Isoleucine	0.01 ± 0.00	0.03 ± 0.00	0.01-1	Y = 124.8X - 1.633	0.997
L-Leucine	0.01 ± 0.00	0.05 ± 0.00	0.01-1	Y = 44.24X + 2.285	0.984
L-Lysine Hydrochloride	0.01 ± 0.0	0.03 ± 0.00	0.01-1	Y= 69.28X + 1.579	0.992
L-Proline	0.007 ± 0.00	0.02 ± 0.00	0.01-1	Y = 124.5X + 0.359	0.998

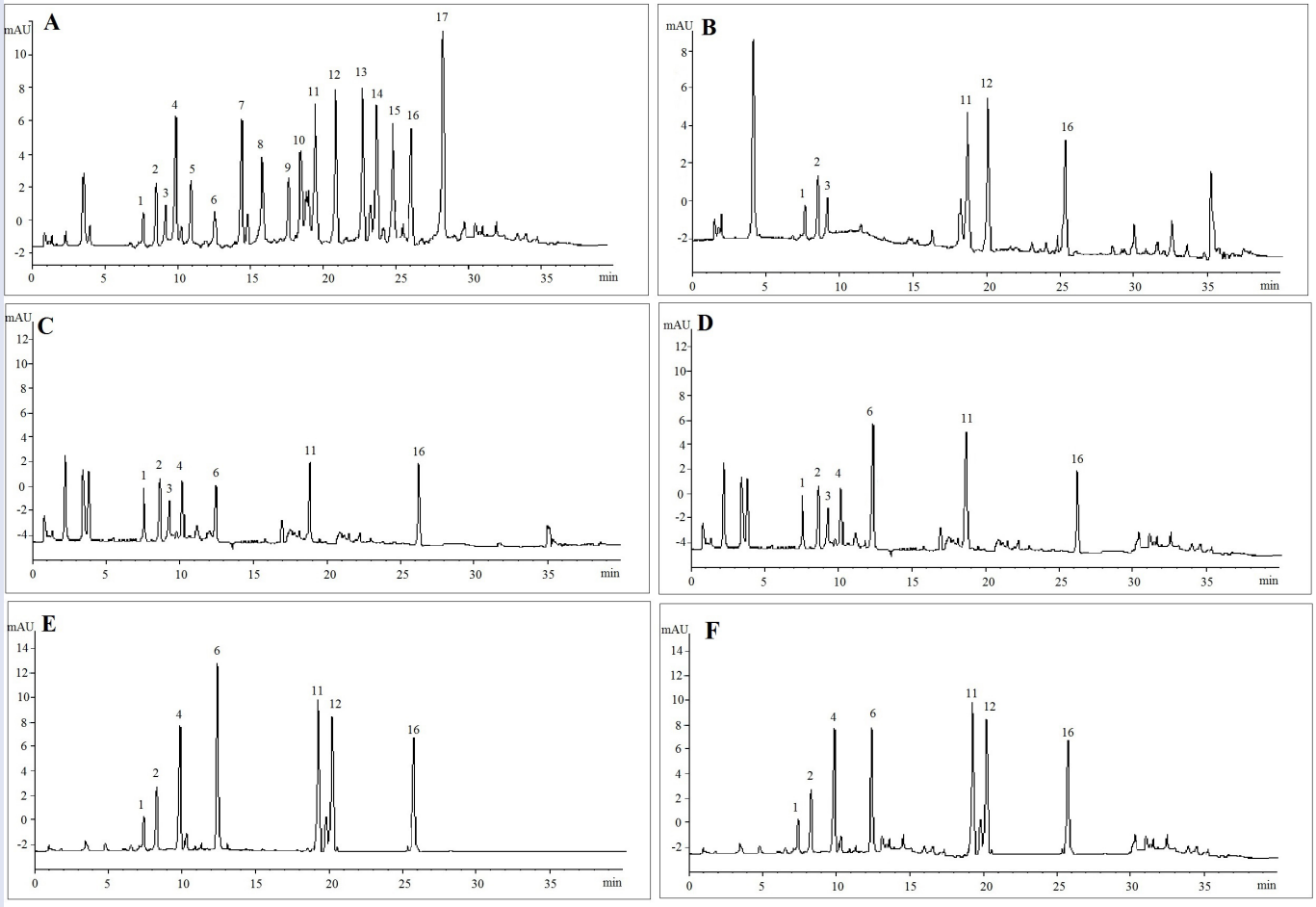

Figure 1: (A) Chromatogram of amino acid standards: (1) l-aspartic acid, (2) l-glutamic acid, (3) l-serine, (4) l-histidine hydrochloride monohydrate, (5) glycine, (6) l-threonine, (7) l-arginine, (8) l-alanine, (9) l-tyrosine, (10) l-cystine, (11) l-valine, (12) l-methionine, (13) l-phenylalanine, (14) l-isoleucine, (15) l-leucine, (16) l-lysine hydrochloride, and (17) l-proline. (B) *O. stamineus* water extract (OS-W). (C) *O. stamineus* ethanolic extract (OS-E). (D) *O. stamineus* methanolic extract (OS-M). (E) *O. stamineus* 50% ethanolic extract (OS-EW). (F) *O. stamineus* 50% methanolic extract (OS-MW)

compounds were analyzed for five replicate in a single day, while interday precision value was obtained by one replicate analysis for five consecutive days. The peak areas were used to calculate %RSD of each reference compound to predict method precision and repeatability. The results show that the %RSD of intraday and interday of peak area for all reference markers are less than 6%. This indicates that the method is precise and repeatable [Table 3].

The accuracy of HPLC method was evaluated by recovery study on those amino acids compounds, which were presented in the extract. The recovery rates were calculated at four different concentrations of amino acid compounds in the extracts and in three replicates. The mean of percentage recoveries for each reference compound in different extracts of OS are presented in [Table 4]. These values are in the range of 94.93-104.43% with %RSD less than 6 for all reference compounds in all extracts, indicating that the method is accurate.

Quantification of free amino acids in OS extracts by HPLC method

The validated HPLC method was applied for analyzing free amino acids in different extracts of OS. The chromatograms of amino acid standard compounds and different extracts revealed good separation profiles of selected markers [Figure 1]. Identification of amino acids standard compounds in the extracts was appraised through comparing their retention time and spiking technique. The mean of peak areas from three replicates injections were used to calculate the free amino acids content in the extracts [Table 5]. The results demonstrate that l-aspartic acid with 0.93 ± 0.01 nmol/mg is the major free amino acid in OS-W extract. However, in OS-E, OS-M, OS-EW, and OS-MW, l-glutamic acid with 3.53 ± 0.16, 2.17 ± 0.10, 4.01 ± 0.12, and 2.49 ± 0.12 nmol/mg, respectively, is the major free amino acid. Subsequently, l-serine, detected in OS-W, OS-E, and OS-M, is the minor free amino acid with 0.33 ± 0.02, 0.12 ± 0.01, and 0.06 ± 0.01 nmol/mg, respectively. However, l-threonine, with 0.26 ± 0.02 and 0.19 ± 0.08 nmol/mL in OS-EW and OS-MW, respectively, possesses the lowest concentration compared with the other amino acid components. Furthermore, glycine, l-arginine, l-alanine, l-tyrosine, l-cystine, l-phenylalanine, l-isoleucine, l-leucine, and l-proline are not detected in all OS extracts [Table 5].

CONCLUSION

The HPLC–DAD method combined with SPE was successfully developed and validated for determination and quantification of 17 free amino acids, namely, l-aspartic acid, l-glutamic acid, l-serine, l-histidine hydrochloride monohydrate, glycine, l-threonine, l-arginine, l-alanine, l-tyrosine, l-cystine, l-valine, l-methionine, l-phenylalanine, l-isoleucine, l-leucine, l-lysine hydrochloride, and l-proline in OS extracts. Due to lack of

Table 3: Precision of the developed HPLC-DAD method for determination of free amino acids compounds in *Orthosiphon stamineus* leaf extracts.

Components	Concentration	Intra-day		Inter-day	
	nmol/mL	Accuracy (% of true value)	RSD (%)	Accuracy (% of true value)	RSD (%)
L-aspartic acid	0.025	104.27	2.47	103.75	2.57
	0.1	99.12	3.33	96.53	3.78
	0.25	102.60	3.61	104.15	1.46
	1	102.29	2.37	104.10	4.06
L-Glutamic acid	0.025	98.62	3.82	96.17	5.45
	0.1	108.47	5.55	108.47	5.55
	0.25	108.82	5.56	108.82	5.56
	1	97.73	2.63	97.73	2.63
L-serine	0.025	104.41	5.27	98.86	4.89
	0.1	108.75	3.13	105.14	2.42
	0.25	97.31	3.43	99.33	5.12
	1	102.85	3.39	104.81	2.02
L-Histidine hydrochloride monohydrate	0.025	102.53	1.65	98.77	1.86
	0.1	95.47	2.51	97.04	5.55
	0.25	104.41	4.33	106.26	4.09
	1	98.02	1.04	101.15	4.86
Glycine	0.025	96.25	5.76	92.05	5.10
	0.1	107.42	6.10	105.32	1.44
	0.25	104.96	4.76	105.74	3.67
	1	98.19	2.48	95.82	2.99
L-Threonine	0.025	106.57	2.07	105.54	3.45
	0.1	101.57	5.79	102.48	1.87
	0.25	106.76	3.35	100.88	5.41
	1	96.45	2.32	96.91	3.27
L-arginine	0.025	99.70	5.94	99.70	5.94
	0.1	106.81	1.88	103.12	1.68
	0.25	106.95	3.30	106.16	3.94
	1	97.09	2.10	97.84	3.05
L-Alanine	0.025	103.07	5.39	96.16	4.74
	0.1	95.12	5.12	92.90	3.84
	0.25	103.80	3.82	97.87	3.76
	1	98.22	1.29	96.79	2.78
L-Tyrosine	0.025	94.89	5.98	100.65	0.90
	0.1	105.54	4.76	104.90	2.89
	0.25	102.17	5.44	103.58	4.25
	1	99.00	5.29	100.21	4.58
L-Cystine	0.025	97.71	2.16	93.91	4.02
	0.1	103.20	3.52	103.09	4.70
	0.25	96.14	2.60	95.09	3.68
	1	98.82	0.96	100.39	2.89
L-Valine	0.025	100.88	4.38	98.59	5.73
	0.1	103.70	4.08	104.81	3.40
	0.25	103.03	3.90	100.22	1.86
	1	100.44	1.48	102.01	2.05
L-Methionine	0.025	101.75	3.45	103.36	5.23
	0.1	104.51	3.81	104.31	4.81
	0.25	105.58	1.73	96.17	3.53
	1	99.92	1.24	100.53	0.51
L-Phenylalanine	0.025	100.38	3.33	104.13	2.85
	0.1	100.94	5.81	102.02	4.45
	0.25	104.11	3.01	105.50	5.65
	1	99.21	1.20	98.20	2.84
L-Isoleucine	0.025	103.36	4.49	99.77	5.65
	0.1	107.95	2.43	105.55	1.99
	0.25	96.06	3.56	95.04	5.64
	1	102.07	2.89	103.52	1.77
L-Leucine	0.025	100.45	3.49	99.00	5.69
	0.1	103.86	5.37	104.31	4.91
	0.25	97.06	5.19	98.32	4.25
	1	97.50	2.79	96.86	5.18

Contd...

Table 3: Contd...

L-Lysine Hydrochloride	0.025	91.28	4.79	100.51	4.94
	0.1	105.38	2.68	105.38	3.76
	0.25	96.65	1.98	97.92	1.92
	1	103.06	4.35	104.47	3.54
L-Proline	0.025	99.63	3.29	102.84	2.51
	0.1	104.58	3.89	104.58	3.27
	0.25	98.95	4.79	101.01	4.10
	1	99.59	0.69	100.59	0.71

Table 4: Recovery of spiked reference compounds in different extracts of *Orthosiphon stamineus.*

		OS-W		OS-E		OS-M		OS-EW		OS-MW	
Component	**Concentration**	**Recovery**		**Recovery**		**Recovery**		**Recovery**		**Recovery**	
	nmol/mL	**Mean (%)**	**RSD (%)**	**Mean (%)**	**RSD (%)**	**Mean (%)**	**RSD (%)**	**Mean (%)**	**RSD (%)**	**Mean (%)**	**RSD (%)**
L-aspartic acid	0.025	97.58	0.38	103.62	3.06	99.20	3.28	103.62	1.93	99.30	1.62
	0.1	98.22	2.08	99.30	3.09	101.46	4.32	99.30	2.26	101.46	1.90
	0.25	101.9	3.02	101.89	4.90	101.89	2.42	99.30	1.08	99.30	5.09
	1	100.92	2.07	101.25	0.59	103.84	1.51	98.01	1.22	102.11	1.07
L-Glutamic acid	0.025	102.09	4.68	102.09	3.01	98.02	0.87	97.99	0.72	102.10	3.11
	0.1	103.11	2.45	99.54	4.76	102.09	0.38	98.51	1.56	100.05	2.06
	0.25	101.48	3.27	100.05	2.43	98.01	4.31	98.00	0.20	100.05	3.21
	1	98.36	0.53	97.49	2.38	101.58	3.15	97.49	0.30	102.60	2.85
L-serine	0.025	102.18	1.90	96.19	3.41	98.57	5.48	-	-	-	-
	0.1	101.59	3.20	99.19	3.96	102.19	5.48	-	-	-	-
	0.25	97.26	2.74	96.18	2.82	98.58	5.05	-	-	-	-
	1	101.20	1.39	99.48	1.85	100.39	0.84	-	-	-	-
L-Histidine	0.025	-	-	101.29	2.10	104.34	2.96	100.14	1.30	103.29	2.73
hydrochloride	0.1	-	-	99.11	2.37	99.11	1.25	101.71	2.33	101.72	4.62
monohydrate	0.25	-	-	101.20	2.28	102.24	3.07	101.19	4.22	103.28	1.34
	1	-	-	102.50	2.47	100.93	0.83	103.02	0.80	102.24	1.78
L-Threonine	0.025	-	-	98.39	2.83	94.93	2.31	96.68	3.28	101.83	3.64
	0.1	-	-	103.57	2.96	104.43	4.69	103.57	5.43	104.00	5.91
	0.25	-	-	101.84	4.90	103.05	4.11	100.12	5.85	96.49	2.58
	1	-	-	100.12	1.12	102.27	1.19	101.41	3.56	101.32	1.65
L-Valine	0.025	103.30	0.56	100.87	4.08	102.10	1.91	98.38	1.98	98.40	2.89
	0.1	101.47	2.49	102.09	2.94	98.40	4.41	101.47	2.59	98.40	2.51
	0.25	98.39	2.44	98.40	2.68	102.09	2.38	97.16	4.11	100.86	2.23
	1	98.40	2.29	99.63	2.20	99.93	1.05	99.32	1.92	98.093	1.69
L-Methionine	0.025	100.61	1.16	-	-	-	-	100.60	3.79	100.58	3.16
	0.1	100.60	4.32	-	-	-	-	102.28	2.95	102.27	2.79
	0.25	103.95	3.05	-	-	-	-	103.95	1.31	103.95	3.20
	1	99.59	0.94	-	-	-	-	102.11	1.86	100.1	2.14
L-Lysine	0.025	96.20	1.29	96.21	4.42	96.21	5.47	96.24	4.39	101.99	0.84
Hydrochloride	0.1	96.22	3.07	101.03	3.18	98.14	2.75	101.04	3.81	101.03	4.60
	0.25	100.07	5.96	98.15	4.82	102	5.61	103.92	4.52	102	2.82
	1	97.67	2.75	100.55	2.21	102.96	2.29	103.92	3.34	103.92	0.92

suitable chromophore in the amino acid structure, it is necessary to label amino acids using labeling reagent. Some HPLC methods have previously been used for analyzing free amino acids in plants. However, due to extraction of other soluble compositions, especially phenolic compounds together with free amino acids, the clear chromatogram cannot be obtained.[19,21,22] Furthermore, in those studies, other derivatization reagents were mostly used, including naphthalene-2,3-dicarboxaldehyde, 4-dimethylaminoazobenzene-4'-sulfonyl chloride (dabsyl chloride), or phenylisothiocyanate that require fluorescence detection.[27] In comparison, in this study, SPE was successfully applied to eliminate interfering substances from OS extracts. The precolumn derivatization by OPA reagent was used for labeling the free amino acids presence in OS extracts. OPA derivatives are relatively stable and prolong the lifespan of chromatography column. In addition, in this study, DAD was used. This method was applied to identify and quantify amino acids in plant extract for the first time. Therefore, this study is pioneer in determining free amino acids in OS leaves extracts. This method has the potential for the routine analysis of amino acids in commercial and biological samples.

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Conflicts of interest

There are no conflicts of interest

Table 5: The content of free amino acids in *Orthosiphon stamineus* extracts.

Components	OS-W nmol/mg	OS-E nmol/mg	OS-M nmol/mg	OS-EW nmol/mg	OS-MW nmol/mg
L-aspartic acid	0.93 ± 0.01	1.17 ± 0.03	1.11 ± 0.06	1.32 ± 0.07	1.07 ± 0.03
L-Glutamic acid	0.68 ± 0.04	3.53 ± 0.16	2.17 ± 0.10	4.01 ± 0.12	2.49 ± 0.12
L-serine	0.33 ± 0.02	0.12 ± 0.01	0.06 ± 0.01	ND	ND
L-Histidine hydrochloride monohydrate	ND	1.83 ± 0.05	1.47 ± 0.03	2.09 ± 0.04	1.60 ± 0.08
Glycine	ND	ND	ND	ND	ND
L-Threonine	ND	0.14 ± 0.03	0.06 ± 0.01	0.26 ± 0.02	0.19 ± 0.01
L-arginine	ND	ND	ND	ND	ND
L-Alanine	ND	ND	ND	ND	ND
L-Tyrosine	ND	ND	ND	ND	ND
L-Cystine	ND	ND	ND	ND	ND
L-Valine	0.53 ± 0.04	0.34 ± 0.02	0.33 ± 0.02	0.44 ± 0.03	0.46 ± 0.02
L-Methionine	0.40 ± 0.01	ND	ND	0.32 ± 0.03	0.37 ± 0.03
L-Phenylalanine	ND	ND	ND	ND	ND
L-Isoleucine	ND	ND	ND	ND	ND
L-Leucine	ND	ND	ND	ND	ND
L-Lysine Hydrochloride	0.62 ± 0.06	0.35 ± 0.04	0.28 ± 0.03	0.47 ± 0.06	0.52 ± 0.04
L-Proline	ND	ND	ND	ND	ND

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