Analysis of L-citrulline and L-arginine in *Ficus deltoidea* leaf extracts by reverse phase high performance liquid chromatography

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ABSTRACT

Background: *Ficus deltoidea* (FD) is one of the native plants widely distributed in several countries in Southeast Asia. Previous studies have shown that FD leaf possess antinociceptive, wound healing and antioxidant properties. These beneficial effects have been attributed to the presence of primary and secondary metabolites such as polyphenols, amino acids and flavonoids. **Objective:** The aim was to develop a reverse phase high-performance liquid chromatography method with ultraviolet detection that involves precolumn derivatisation with *O*-phthaladehyde for simultaneous analysis of two amino acids L-citrulline and L-arginine in FD leaf extracts. **Materials and Methods:** An isocratic elution program consisting of methanol: acetonitrile: Water at 45:45:10 v/v (solvent A) and 0.1 M phosphate buffer pH 7.5 (solvent B) at A: B v/v ratio of 80:20 on Zorbax Eclipse C18 SB-Aq column (250 × 4.6 mm, 5 µm) were used. The flow rate was set at 1 ml/min and detection was carried out at 338 nm with 30 min separation time. **Results:** Good linearity for L-citrulline and L-arginine was obtained in the range 0.1-1000 µg/ml at $R^2 \geq 0.998$. The limit of detection and limit of quantification values for both L-citrulline and L-arginine were 1 and 5 µg/ml, respectively. The average of recoveries was in the range 94.94-101.95%, with relative standard deviation (%RSD) less than 3%. Intra- and inter-day precision was in the range 96.36-102.43% with RSD less than 2%. **Conclusion:** All validation parameters of the developed method indicate the method is reliable and efficient for simultaneous determination of L-citrulline and L-arginine for routine analysis of FD.

Key words: *Ficus deltoidea*, L-arginine, L-citrulline, reverse phase high-performance liquid chromatography

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INTRODUCTION

Ficus deltoidea (FD) Jack (Moraceae) is one of the native plants widely distributed in several countries in Southeast Asia. In Malaysia, FD is locally known as Mas cotek.[1,2] Traditionally this plant was used in the treatment of several diseases including gout, high blood pressure; blood circulation, pneumonia, diarrhea, skin infection and diabetes.[3] Studies have shown that FD leaf possess antinociceptive, wound healing and antioxidant properties.[1,3,4] These beneficial effects have been attributed to the presence of compounds such as polyphenols, flavonoids, amino acids, vitamins, carbohydrates, and purine alkaloids.[5]

L-arginine is classified as a semi-essential or conditionally essential amino acid [Figure 1]. It serves as a substrate for the enzyme nitric oxide synthetase (eNOS), which converts L-arginine to L-citrulline and produces nitric oxide (NO).[6] L-arginine is also very important in a number of essential processes such as cell division, ammonia removal, wound healing, immune function, and hormones release.[7,8] The metabolites of L-arginine are involved in many physiological and pathological processes. Previous studies have demonstrated that L-arginine might have an antihypertensive effect.[8,9] The creatural experiment showed that L-arginine function to protect cerebral

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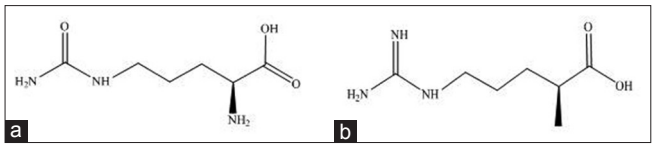

Figure 1: Structure of L-citrulline (a) and L-arginine (b)

ischemic tissues.[10] Thirteen weeks of oral administration of L-arginine in rats on a high-cholesterol diet showed that generation of vascular NO was increased, and endothelial release of superoxide anions was decreased with regression of intimal atherosclerotic lesions.[11] Intracoronary infusion of L-arginine in patients with hypercholesterolemia and in patients with microvascular angina pectoris as well as in those with atherosclerosis was normalized the defective acetylcholine-induced vasodilation of coronary microvessels.[12-14] In another study, chronic oral supplementation of L-arginine improved coronary small-vessel endothelial function in association with a significant improvement in symptoms and a decrease in plasma endothelin concentrations.[15] Citrulline, a byproduct of the NOS-catalyzed reaction, can be recycled to produce arginine in a pathway known as the citrulline–NO or arginine–citrulline.[7] Arginine can also be synthesized in cells from citrulline by the sequential action of two urea cycle enzymes, argininosuccinate synthetase and argininosuccinate lyase (ASL).[7]

L-citrulline is a nonprotein amino acid and was first identified from watermelon (*Citrullus vulgaris* Schrad).[16] L-citrulline plays a key role in mere metabolic intermediate, especially in the urea cycle.[17] Moreover, it was emerged as a product of the NO cycle and precursor for the biosynthesis of arginine.[18] L-citrulline is known as an essential amino acid for young mammals and adults with trauma, burn injury, massive small bowel resection and renal failure.[19] It is synthesized via argininosuccinate synthase and ASL in all animal cells.[16] One of the important biological functions of L-citrulline is through production of NO, which liberated during conversion of citrulline to arginine by the enzyme peptidylarginine deiminase (reference). Furthermore, this enzyme is also responsible for conversation of arginine back into citrulline.[20] Previous studies suggested that, a dietary supplement rich in L-citrulline seems to improve sexual stamina and erectile functions while the exact mechanism is not known.[21]

Limited number of chromatographic methods including HPLC, capillary electrophoretic and anion exchange chromatography for analysis of L-citrulline and L-arginine have been reported.[22] In chromatographic methods, due to the lack of a suitable chromophore in amino acid's structures it is necessary to label them using labeling reagent, such as 4-dimethylaminoazobenzene-4-sulfonyl chloride (dabsyl chloride), *O*-phthaladehyde (OPA) or phenylisothiocyanate.[23] In such a case, it involves derivatisation with precolumn or postcolumn and detection by fluorescence, ultraviolet (UV) or diode array detector.[5] Due to vast biological significance, there is a demand for rapid and effective analytical methods for the analysis of L-citrulline and L-arginine, which should be suitable across a wide range of research and practical applications. In this context, the objective of the present study is to develop simple, rapid and isocratic reverse phase high-performance liquid chromatography (RP-HPLC) method with UV detection for simultaneous identification and quantification of the two amino acids L-citrulline and L-arginine in FD leaf extracts.

MATERIALS AND METHODS

Chemicals and reagents

L-citrulline, L-arginin, OPA and β-mercaptoethanol were purchased from Sigma-Aldrich (Subang Jaya, Selangor, Malaysia). Boric acid was obtained from Agilent (Petaling Jaya, Selangor, Malaysia). Methanol and acetonitrile of HPLC grade were purchased from Merck (Petaling Jaya, Selangor, Malaysia). Deionized water for HPLC was prepared using Ultra-pure water purifier system (Elgastat, Bucks, UK).

Instrumentation

The HPLC was performed using an Agilent Technologies Series 1100 (Waldronn, Germany) System equipped with degasser (G 1379 A, Agilent), quaternary pump (G 1311 A, Agilent), auto sampler (G 1313 A, Agilent), column oven (G 1316A, Agilent) and UV detector (G 1314 A, Agilent). The detector was operated at different UV wavelengths, and the sensitivity range was set at 0.005 AUFS, output of 15 mV.

Plant samples

The FD leaves were purchased from Herbagus Sdn. Bhd. Penang-Malaysia and identified at the herbarium of School of Biological Sciences Universiti Sains Malaysia, where a voucher sample was deposited (reference number: 11204). 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

The dried ground leaves (100 g) were suspended and extracted with water under reflux conditions for 24 h. The extract was filtered and concentrated under vacuum, and it was dried using freeze dryer to obtain the water extract (FD-W). For the preparation of methanol extract,

500 g of the dried and ground leaves were extracted with methanol using soxhlet extractor for 24 h. The extract was concentrated under vacuum at 40°C using rotavapor, and it was further dried on a freeze dryer to obtain the methanolic extract (FD-M).

Preparation of standards and extracts for high-performance liquid chromatography analysis

Standards

A stock solution of L-citrulline and L-arginine was prepared in methanol at 5 mg/mL, and then filtered through a 0.45-μm syringe filter (UK). A series of working standard solutions were prepared by diluting the stock solution with methanol in the range of 0.1-1000 μg/mL.

Samples

FD-M and FD-W (100 mg) were dissolved in 5 mL of methanol and water, respectively, and sonicated for 15 min. Working sample solution at concentration of 2 mg/mL was prepared by diluting the stock solution with methanol. All samples were filtered through a 0.45-μm filter.

Analysis of L-citrulline and L-arginine in plant extracts by reverse phase high-performance liquid chromatography

Precolumn derivatization with O-phthaladehyde

The derivatization with OPA was carried out according to the method reported previously.[5] Briefly, 70 μL of FD-M and FD-W extracts (2 mg/mL) or standard solutions were mixed with 10 μL of OPA solution, and then the volume was made up to 1 mL with phosphate buffer (pH 7.5) and incubated at 25°C ± 1°C for exactly 2 min. Subsequently, the reaction mixture was immediately analyzed by HPLC. The derivatization solution was freshly prepared everyday as the following; 5 mg of OPA was dissolved in 0.05 mL of methanol, and it was added to 0.45 mL of 0.4 M boric acid/borate buffer (pH 9.5), followed by addition of 25 μL of β-mercaptoethanol.

Chromatographic conditions

The HPLC method was developed for the determination of L-citrulline and L-arginine using a Zorbax Eclipse C18 SB-Aq column (250 × 4.6 mm, 5 μm) (Agilent). The flow rate was set at 1 mL/min, injection volume was 10 μL, column temperature was set at 40°C and detection was carried out at 338 nm. The mobile phase consisted of the solvent A (methanol: Acetonitrile: Water, 45:45:10 v/v) and solvent B (phosphate buffer, pH 7.5) at a volume ratio A: B (80:20) with 30 min separation time.

Validation of high-performance liquid chromatography 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.[24] Accuracy of the HPLC method was evaluated through recovery studies by adding known amounts of standard solution (three concentrations) of each reference marker into the test samples (extract solution). The spiked extract solutions were injected three times, and the recovery was calculated with the value of detected versus added amounts. Precision of the method was determined as percentage relative standard deviation (SD) of peak area of intra-day and inter-day analysis data. Three different concentrations of the standards were analyzed per day for the intra-day analysis ($n = 6$), and on different days ($n = 6$) for inter-day analysis of precision. The resulting peak area was used to calculate SD and the relative standard deviation (%RSD). The linearity of the method was constructed using the calibration curve over eight different concentrations in the range 0.1-1000 μg/ml ($n = 3$). LOD and LOQ were measured based on the signal-to-noise ratio (S/N) method. Determination of the S/N was performed by comparing measured signals from samples at known low concentration of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. An S/N of 3 is generally considered acceptable for LOD, while LOQ is established at an S/N of 10.

RESULTS

Development and validation of high performance liquid chromatography analytical method

HPLC separation of a standard mixture was achieved using described method. The representative chromatogram of standards is shown in [Figure 2a]. For FD-M and FD-W, elution profile of reference markers was similar to that observed in their corresponding standards [Figure 2b and c]. Linearity was evaluated by determining a series of eight concentrations of the standard solution in three replicates. The linear regression equations and correlation coefficients were established from the graph by plotting the mean of peak area versus the concentration. The standard solutions of the reference markers showed good linearity over the evaluated concentration range with $R^2 > 0.998$ [Table 1]. The sensitivity of the method was evaluated by LOD and LOQ analyses. The values of LOD and LOQ for both L-citrulline and L-arginine were similar and were 1 and 5 μg/mL, respectively [Table 1]. Percentage recovery ranged from 94.94% to 101.95%, with %RSD less than 3% that indicates good accuracy of the method [Table 2]. The peak area was used to calculate the %RSD of the markers. Intra-day and inter-day precision data is shown in Table 3. The mean %RSD for L-citrulline and L-arginine is less than 2%.

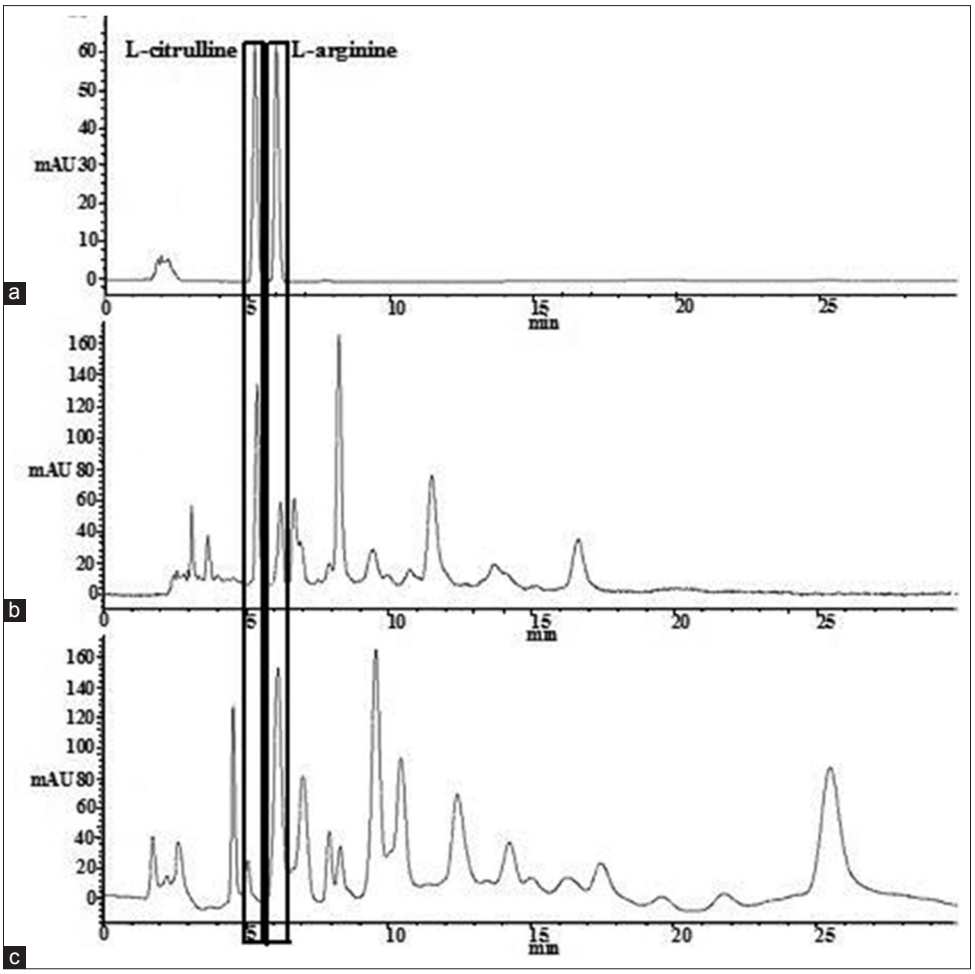

Figure 2: High performance liquid chromatography chromatogram of L-citrulline and L-arginine derivatized with *O*-phthaladehyde. (a) Standard of L-citrulline and L-arginine, (b) *Ficus deltoidea* methanol extract, (c) *Ficus deltoidea* water extract

Table 1: Calibration data of the reported HPLC method

Compounds	Regression equation	R^2	Linear range (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)
L-citrulline	Y=18.117 X+5.8669	0.9989	0.1-1000	1	5
L-arginine	Y=21.266 X+29.285	0.9988	0.1-1000	1	5

HPLC=High performance liquid chromatography, LOD=Limit of detection, LOQ=Limit of quantification

Determination and quantification of L-citrulline and L-arginine content in *Ficus deltoidea* extracts

The HPLC method was applied for analyses of L-citrulline and L-arginine in aqueous and methanolic extracts of FD leaf. As shown in Figure 2 selected markers were well separated by the developed HPLC method. Table 4 presents concentration of L-citrulline and L-arginine determined by HPLC in these extracts. Results were derived from the mean of peak area from three replicate injections. FD-M contained the highest amount of L-citrulline, while FD-W was rich in L-arginine.

CONCLUSION

Marker compounds of L-citrulline and L-arginine were determined in FD extracts in a single run HPLC using a precolumn derivatization with OPA reagent and detected at 338 nm. Some HPLC methods have been previously used for analysis of L-citrulline and L-arginine in plants. However, in those studies mostly other derivatization reagents were used such as naphthalene-2,3-dicarboxaldehyde, 4-dimethylaminoazobenzene-4'-sulfonyl chloride (dabsyl chloride) or phenylisothiocyanate and others.[12,19] In comparison, in this study, the OPA derivatives are relatively stable and prolong the life-span of the chromatography column. Most of the previous methods performed by other researchers require fluorescence detection.[25] Although, in this study UV–visible detector was used. Meanwhile, this method was used to identify and quantify L-citrulline and L-arginine in the plant extract for the first time in FD leaf extracts. This method may have the potential for the routine analysis of L-citrulline and L-arginine in commercial and biological samples.

Table 2: Recovery of L-citrulline and L-arginine

Standard	Concentration (μg/ml)	FD-M			FD-W		
		Amount (μg/ml)	Recovery (%)	RSD (%)	Amount (μg/ml)	Recovery (%)	RSD (%)
L-citrulline	20	19.78	98.92	1.93	18.99	94.99	2.45
	40	38.98	97.46	0.78	39.08	97.71	1.09
	100	100.18	100.18	0.03	100.00	100.00	0.76
L-arginin	20	18.98	94.94	2.85	19.08	95.44	0.34
	40	39.07	97.68	0.98	40.00	100.01	0.07
	100	98.04	98.04	0.67	101.95	101.95	0.26

RSD=Relative standard deviation, FD-M=*Ficus deltoidea* methanol extract, FD-W=*Ficus deltoidea* water extract

Table 3: Intra-day and inter-day data for L-citrulline and L-arginine

Standards	Concentration (μg/ml)	Intra-day (*n*=6) (%)		Inter-day (*n*=6) (%)	
		Mean	RSD	Mean	RSD
L-citrolline	10	98.456	1.975	100.965	0.346
	50	102.432	0.975	100.658	0.123
	100	97.465	0.0234	98.998	0.064
L-arginin	10	101.943	1.231	100.463	0.235
	50	97.453	1.083	98.264	1.253
	100	96.364	0.352	97.352	0.362

RSD=Relative standard deviation

Table 4: Content of markers (mg/g dry weight) of FD I extracts

Standard	FD-M (mg/g)	FD-W (mg/g)
L-citrolline	8.532	7.453
L-arginin	5.364	8.442

FD=*Ficus deltoidea*, FD-M=*Ficus deltoidea* methanol extract, FD-W=*Ficus deltoidea* water extract

In summary, effective RP-HPLC method with UV detector has been successfully developed to determine the content of two amino acids, L-citrulline and L-arginine in FD leaf extracts. The proposed method is a sensitive, reliable and efficient analytical platform for simultaneous determination of L-citrulline and L-arginine, either qualitatively or quantitatively. This method can be used for fingerprint profiling and for the standardization of herbal medicines and herbal products from FD extracts.

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