Short Communication:

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) Quantitative Analysis of L-Arginine in Freeze-Dried Red Dragon Fruits (*Hylocereus polyhizus*)

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Abstract: Hylocereus polyrhizus *is an exotic fruit with distinct bright colors and tastes that make it popular among many regions of the world. The fruit is cultivated worldwide and firmly established as a source of nutrition, thus, making good value in terms of its potential harvesting by various methods. However, prior reviews have documented weak scientific backing sparsely around extraction efficiency and favorable conditions for optimal yield. This study aimed to quantify the crude L-arginine content for both types of dragon fruit by an RP-HPLC isocratic method, thus establishing a reliable and efficient approach for their detection. This study has revealed that short extraction times are beneficial in retaining L-arginine content since more extended extractions can result in the degradation of other components. Thus, this study opens further avenues toward exploring red dragon fruit as a natural source of L-arginine and advancing functional and nutraceutical products.*

Keywords: *L-arginine; red dragon fruit; RP-HPLC*

■ INTRODUCTION

Pitaya, commercially known as dragon fruit, is a rustic fruit from the *Cactaceae* family, specifically the *Hylocereus* genus. It is known as a dragon fruit because of the brilliant red skin and overlapping green fins that surround the fruit. Other common names for this fruit are pitahaya, dragon pearl fruit, night-blooming cereus, strawberry pear, and Cinderella plant. Fruits of distinct species may differ in shape, thorn presence, skin, and pulp color, indicating great genetic variety [1-3]. In Malaysia, consumers frequently use the peel and pulp of *Hylocereus polyrhizus* to prepare fruit juice, although the peel is typically discarded when consumed directly. Among 18 amino acids found in dragon fruit, L-arginine is well-known for its role in enhancing the flavor of dragon fruit, as it is found in the seeds and contributes to the fruit's pleasant taste. It plays a vital role in human health, supporting the cardiovascular and immune systems [4]. While other amino acids contribute to general metabolic functions, L-arginine plays a role in vasodilation, and its potential therapeutic effects in cardiovascular health make it a compelling target for quantification [5].

Despite various methods like ion-exchange chromatography, gas chromatography (GC), and capillary electrophoresis being used for quantifying L-arginine from fruits, this work required extensive derivatization, making it less effective. Another major problem in the analysis is the high susceptibility of L-arginine to degradation during extraction, and some optimization is needed to maintain its integrity [6-7]. Given the growing global consumption of dragon fruit, determining its dietary importance and possible health benefits requires analyzing accurate L-arginine concentration [3,8]. Red dragon fruit is known for its antioxidant qualities attributed to a range of phytochemicals, including flavonoids, polyphenols, and vitamin C, as well as vital minerals like calcium and magnesium [9]. According to previous report, red dragon fruit contains higher levels of antioxidants and phenolic compounds, whilst white dragon fruit contains more sugar [4].

The molecular structure of L-arginine was presented in Fig. 1. Arivalagan et al. [4] reported that there are 18 types of amino acids detected in dragon fruit, and one of them is arginine (L-arginine). Its IUPAC name of arginine is known as (2*S*)-2-amino-5-(diaminomethylideneamino) pentanoic acid with a molar mass 174.2 g/mol and a molecular formula of $C_6H_{14}N_4O_2$. The molecular structure was presented in Fig. 1. L-Arginine acts as a substrate for nitric oxide synthase (eNOS) enzyme that will produce nitric oxide (NO) and convert L-arginine to L-citrulline [10]. The L-arginine supplement helps prevent many health issues [11], and this amino acid is categorized as a conditional or semi-essential amino acid [12]. It is involved in the ornithine cycle, which is needed for the human body, and can be a nutritional supplement for medicine and an anti-obesity food [11].

Reverse-phase high-performance liquid chromatography (RP-HPLC) is a versatile technique that is still chosen because it is suitable for separating proteins and peptides. This approach is regarded for its high-resolution capabilities, ease of use, stability, and reproducibility. L-Arginine and L-citrulline can be successfully quantified using RP-HPLC with good resolution and separation peak results, according to a previous study [13]. This technique offers flexibility for various separation needs, including size-based separation when paired with mobile phases, including acetonitrile and trifluoroacetic acid, that are able to inhibit undesired interactions and improve separation efficiency [14]. The column's stationary phase must be compatible with both the physicochemical characteristics of L-arginine and the mobile phase. Good separation and quantification result from this compatibility ensures optimal interactions between analyte and stationary phase [15]. Referring to the previous work, it is proven that the Gemini C18 column was more efficient in quantifying L-arginine and L-citrulline than the Zorbax Eclipse XDB-C18 column [13].

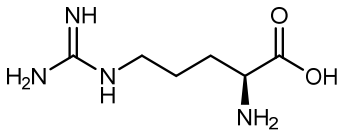

Fig 1. L-Arginine molecular structure

Thus, this study aims to determine the characteristic of L-arginine content in red dragon fruits at different extraction times and to determine the optimum extraction time with the highest yield of L-arginine content in red dragon fruits. Specifically, it seeks to determine the characteristics of L-arginine content in red dragon fruits. Additionally, it aims to identify the optimal extraction time that yields L-arginine content. This study offers important insights into improving the recovery of bioactive compounds for pharmaceutical and nutraceutical applications by improving the extraction and measurement procedure.

■ EXPERIMENTAL SECTION

Materials

L-Arginine standard was obtained from Sigma Aldrich (USA) with ≥ 98% purity. Methanol (analytical reagent grade) was provided by Fisher Scientific (UK), while 1 M hydrochloric acid (HCl) was purchased from Merck (Germany). Phosphoric acid (purity ≥ 85%) was acquired from Bionex (Malaysia). Deionized water was prepared using a Purite water purification system (UK). The experiment employed Whatman No. 1 filter paper, Eppendorf microcentrifuge tubes (1.5 and 2 mL diameters), analytical balance, glass vials with screw caps, micropipettes (20–1000 µL) with disposable tips, and volumetric flasks. All glasses and plasticware were sterilized before use.

Instrumentation

The Agilent Technologies 1100 HPLC system was used for analysis. It includes solvent reservoirs, a G1315B Diode Array Detector (DAD) (UV-vis), a G1316A Column Compartment, a G1329A Autoloader Sampler, a G1328B Manual Injector, a G13111A QuatPump, and a G1322A Degasser. The separation was carried out under isocratic conditions with RP-HPLC.

Procedure

Sample preparation

The red dragon fruits were obtained from a local market in Puncak Alam, Selangor, Malaysia. The fruit's flesh was separated gently, then cut into small cubes. The juice from both fruits was frozen for 5 d at −35 °C. The

low-temperature freezing reduces enzymatic breakdown and oxidation, protecting the quality of L-arginine and other amino acids [16]. The frozen juices were later dehydrated in a freeze dryer (ALPHA 1-4 LD Plus, Germany) for 3 d. A temperature of −20 °C was maintained for the dried juice powders. In order to conduct the testing, methanol extract samples were prepared. A known amount of dried juice powders was extracted by mixing with 30 mL of MeOH and 1 mL of 1 M HCl, and then 30 min of sonication and vortexing was proceeded. Additionally, the components were homogenized in an orbital shaker for the period of 24, 48, and 72 h, and Whatman filter paper was used for method filtration. After performing a second extraction of the residues using a different solvent, the two separate methanol extracts were mixed and then dried with a spinning vacuum evaporator at 40 °C. Then, the methanol extracts were kept at a temperature of 4.0 °C until testing.

RP-HPLC analysis isocratic standard preparation

The L-arginine standard solution was prepared in deionized water (diH_2O) with 1 mg/mL concentration and filtered using a syringe filter with pores of 0.45 µm (Bioflow, Malaysia). To make the mixed standard solution, the same volume of each standard stock solution was added together. Standard working solutions were obtained ranging from 20 to 100 µg/mL by preparing the dilution with diH_2O. Crude methanol extracts were diluted in diH_2O at 5 mg/mL and vortexed for 15 min. Before being injected into an isocratic RP-HPLC system, every extract underwent filtration using filters with a 0.45 µm particle size.

Chromatographic analysis

The evaluation was performed using an Agilent Technologies 1100 HPLC system equipped with a DAD. The column was maintained at room temperature, and the RP-HPLC column with the following dimensions and parameters being utilized: Gemini C18, 110 Å, 3 µm, 250 × 4.6 mm (USA). The analysis was conducted to quantify L-arginine in red dragon fruit methanol extracts with a mobile phase 0.1% H_3PO_4 and 0.5 mL/min flow rate. For UV-vis detection, 195 nm wavelength was used. Calculating the levels of L-arginine content requires the usage of linear curve standards and the concentration was measured in µg/mL unit of sample extracts.

Statistical analysis

The independent t-test is used to compare the mean difference between L-arginine content and the type of dragon fruits in different methanol extraction times. The ANOVA test is used to determine any significant mean difference between L-arginine content and methanol extraction time in both fruits. The significant level was set to $p \leq 0.05$.

■ RESULTS AND DISCUSSION

Standard Preparation

The initial isocratic RP-HPLC method for the L-arginine standard was performed using mobile phases conducted by previous work [13]. In this study, 0.1% H_3PO_4 was chosen as the mobile phase for analyzing the L-arginine in the red dragon fruits. The concentration of 0.05 and 0.1% (v/v) of acidic reagents was generally used to separate most peptides [17-18]. Furthermore, 0.1% of H_3PO_4, as the mobile phase, was proven to provide the best resolution for the peptide and protein separations [19]. The L-arginine standards were tested in triplicate at different concentrations ranging from 20–100 µg/mL in 0.1% H_3PO_4. The initial RP-HPLC isocratic results are shown in Fig. 2, while the subsequent are summarized in Table 1.

L-Arginine standard retention time was obtained at 4.3 min for all standard concentrations from 20 to 100 µg/mL and successfully analyzed at 4.3 min. This result was supported by the previous study that L-arginine was eluted at a retention time of 4.773 min. However, the study results also showed a slight difference in retention time caused by column temperature, which affects the retention time shift. It was set to room temperature and can be easily affected by environmental factors [20]. In the previous research, the retention time can be affected by small changes in temperature, approximately 2% for 1 °C, with an acceptable range of 0.02–0.05 min [21]. Since the results were in the acceptable range, thus L-arginine was best resolved and separated at 4.3 min retention time.

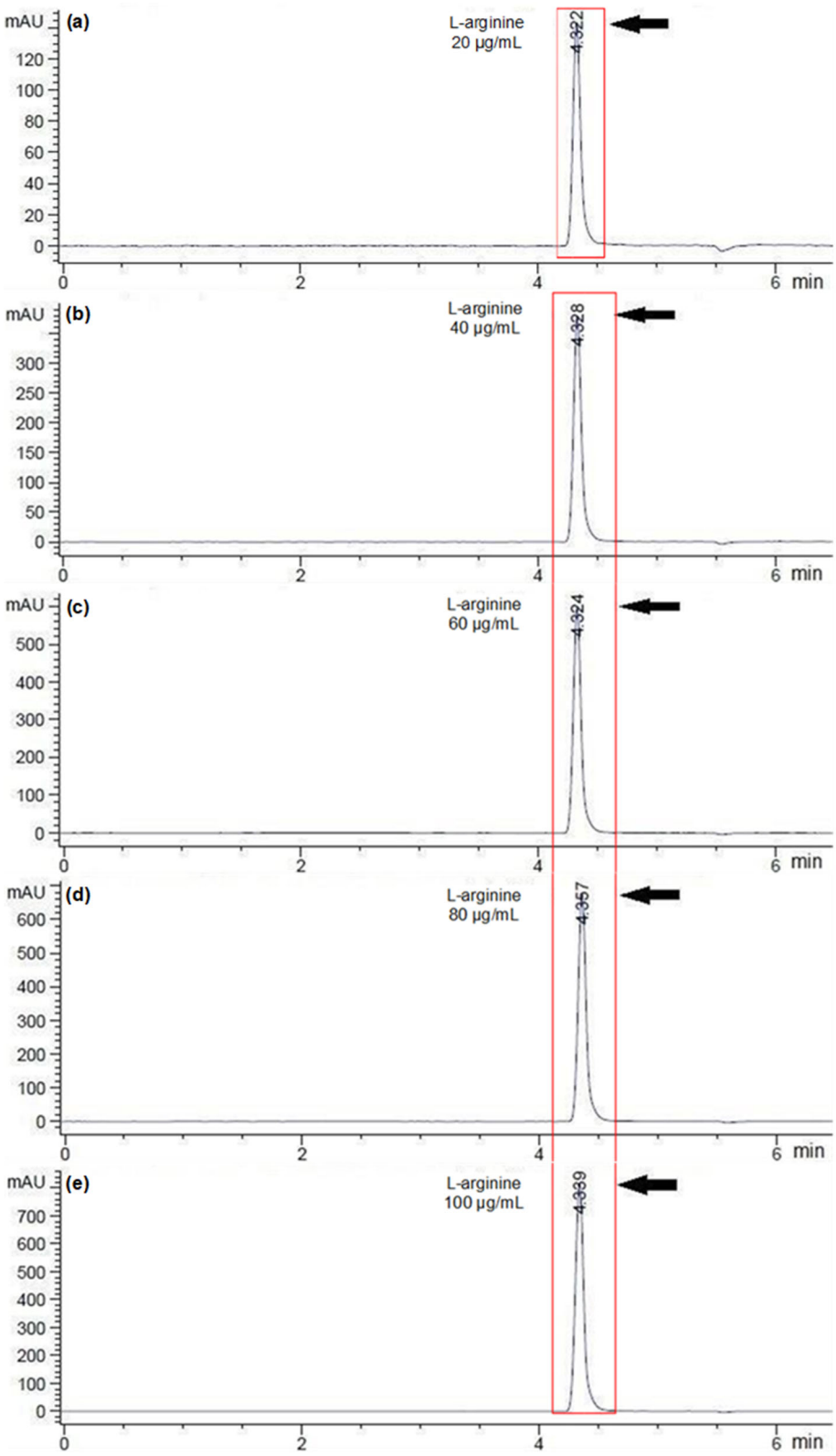

Fig 2. Comparative profile showing RP-HPLC isocratic analysis results on L-arginine standards with different concentrations ranging from: (a) 20, (b) 40, (c) 60, (d) 80, and (e) 100 µg/mL using Gemini C18 column and 0.1% H_2PO_4 mobile phase. The peak marked with an arrow represents the L-arginine

Table 1. RP-HPLC isocratic analysis results on L-arginine standards with different concentrations

Concentration (μg/mL)	Retention time (min)				mAU Average
	1st test	2nd test	3rd test	Average	
20	4.322	4.317	4.311	4.317	734.97
40	4.328	4.325	4.326	4.326	1858.97
60	4.324	4.322	4.326	4.324	2941.27
80	4.357	4.336	4.355	4.349	3377.60
100	4.339	4.336	4.334	4.336	4186.03

Clear resolution of L-arginine standard peak was obtained by using the Gemini C18 column. This is supported by the study conducted by the previous work, which indicate that the Gemini C18 column provides clear result and effective separation of polar compounds [13]. This conclusion was reached following a comparison of 3 different columns: the Gemini C18, Synergi Hydro-RP, and Zorbax SB-Aq. The Gemini C18 demonstrated the highest level of performance and resemblance. A layer of embedded silica in the Gemini C18 column aids in lowering the quantity of silanols that remain after treatment. This decrease is essential for enhancing the column's functionality by reducing undesired interactions that may compromise analyte separation [22].

Quantification of L-Arginine Content in Dragon Fruit Extract

L-arginine is an essential amino acid for our body. Consumption of dragon fruit can be one of the sources of L-arginine. L-arginine can increase protein synthesis and muscular growth [23]. It is also reported that *H. undatus* contains numerous benefits, such as vitamin C, minerals, phenolics, and antioxidant compounds [24]. Therefore, a quick, trustworthy, and effective RP-HPLC isocratic method is crucial in quantifying L-arginine content in dragon fruit extract.

The results of the chromatographic profile, which tested red dragon fruits three times for 24, 48, and 72 h extraction time, were tabulated in Table 2. The L-arginine peak first test results for red dragon fruits are presented in Fig. 2. The quantification of L-arginine was calculated based on the calibration curve of the L-arginine standard achieved with the correlation coefficient and linear regression equation of R_2 = 0.9865 and y = 42.772x + 44.544, respectively. L-Arganine standard and red dragon fruit with different extraction times are presented and tabulated in Fig. 3 and Table 3. The calibration curve was presented in Fig. 4. Based on Table 3, the results show the L-arginine content in red dragon fruits in all extraction times. L-arginine content was highest in red dragon fruit extract for 24 h (7.188 ± 0.219 μg/mL), followed by 72 h (5.976 ± 0.149 μg/mL) and lowest at 48 h (4.658 ± 0.206 μg/mL).

Fig. 5 illustrates the decreasing trend of L-arginine content in red dragon fruits with increasing extraction time. The data shows that the L-arginine content is highest

Table 2. RP-HPLC analysis results on three times testing of red dragon fruits for different extraction times

Time (h)	Sample	Retention time (min)				mAU Average
		1st test	2nd test	3rd test	Average	
24	Red	4.33	4.335	4.332	4.332	396.2
48	Red	4.337	4.319	4.321	4.326	289.67
72	Red	4.323	4.324	4.326	4.324	345.13

Table 3. Concentration of L-arginine content in red dragon fruits with three different extraction times

Extraction time (h)	Type of dragon fruit	Absorbance (mAU)	Concentration ± SD (μg/mL)
24	red	396.20	7.188 ± 0.219
48	red	289.67	4.658 ± 0.206
72	red	345.13	5.976 ± 0.149

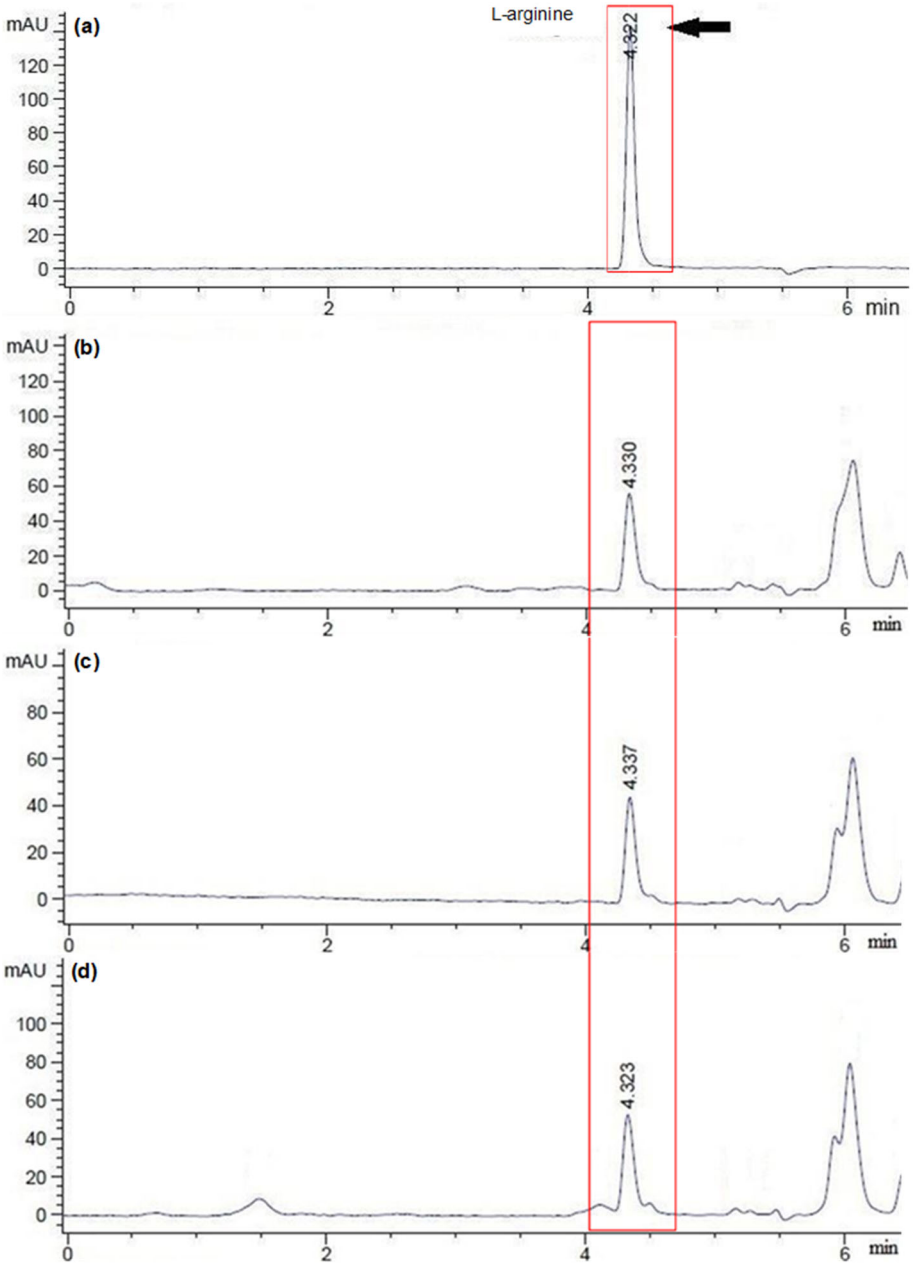

Fig 3. Comparative profile showing RP-HPLC isocratic results between L-arginine standard and red dragon fruit with different extraction times (1st test result). (a) L-arginine standard, (b) 24, (c) 48, (d) 72 h. The peak with an arrow represents L-arginine

at 24 h of extraction time with 7.188 µg/mL and lowest at 48 h with 4.658 µg/mL. In this study, 24, 48, and 72 h extraction times were used to investigate the effect of prolonged extraction on L-arginine. Longer extraction times yield the most amount of L-arginine recovered, improving the process's overall efficiency, according to the earlier study. Therefore, extractions that take less than 24 h are not performed.

The results obtained in this study were presented with a lower L-arginine content for red (7.188 ± 0.219 µg/mL) dragon fruits compared to previous research by the previous work, which showed

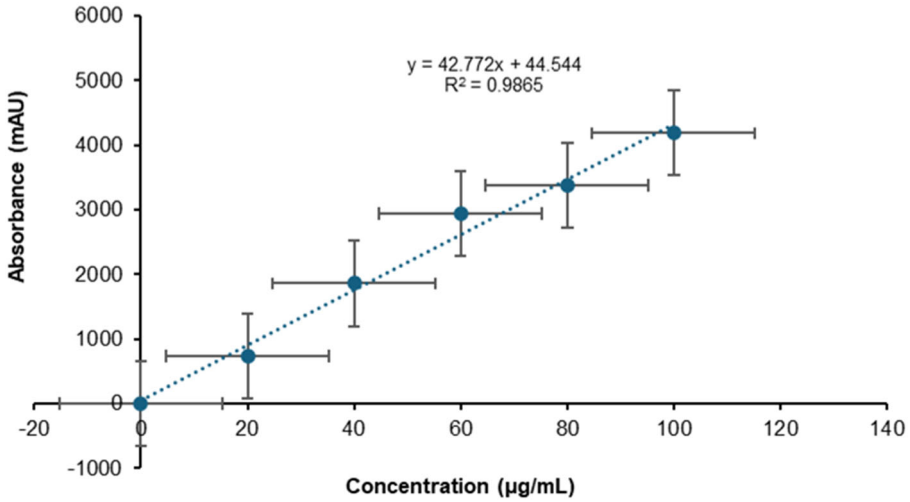

Fig 4. Calibration curve of L-arginine standard

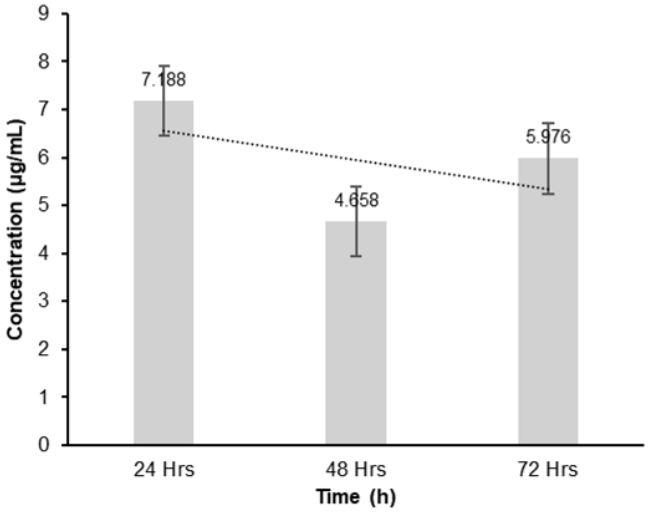

Fig 5. L-Arginine content in red dragon fruits with different extraction time

that L-arginine content in red dragon fruit was $131 \times 10^3 \pm 13.9$ µg/mL [4]. The cause may be variations in the extraction and quantification techniques employed. L-arginine content in red dragon fruit obtained in this study was lower than the daily intake. Additionally, the diet intake for protein is about 0.83 g of protein/kg of body weight, which means that dragon fruit may not be a source of essential amino acids in the diet. Furthermore, the most abundant amino acid in dragon fruit was phenylalanine, with the red variety containing $183 \times 10^3 \pm 4.1$ µg/mL [4].

Statistical Analysis

In this study, two types of statistical analysis (In this study, two types of statistical analysis (Independent t-test and ANOVA) were used to analyze the data. An independent t-test was used to compare the significant mean difference of 2 independent variables between L-arginine content and type of dragon fruits in different extraction times. ANOVA test was used to determine the significant mean difference of 3 independent variables between L-arginine content and extraction time in both fruits. A significant level was set at $p \leq 0.05$.

Based on the independent t-test results in Table 4, a mean difference of L-arginine of red dragon fruits for 24, 48, and 72 h of extraction time was proven to be significant ($p < 0.01$). Overall, the red dragon fruit shows a high L-arginine content. For 24 h extraction time, red

Table 4. Comparison of Independent t-test analysis of red dragon fruits with different extraction times

Variables	Type of dragon fruit	Mean diff[a] (95% CI)	t-stat[a] (df)	p-value
Extraction time (h)	Red (n = 3), Mean (SD)			
24	7.188 (0.219)	6.031 (4.721,7.341)	12.785 (4)	<0.001
48	4.658 (0.206)	2.616 (2.278,2.955)	21.461 (4)	<0.001
72	5.976 (0.149)	3.989 (3.073,4.903)	12.097 (4)	<0.001

[a]Independent t-test

Table 5. Comparison of ANOVA analysis of red dragon fruits with different extraction times

Variables	Extraction time (h)	Number of samples, n	Mean concentration (SD)	F-stat[a] (df)	p-value[a]
Red dragon fruit	24	3	7.188 (0.219)	128.146 (2,6)	< 0.001
	48	3	4.658 (0.206)		
	72	3	5.976 (0.149)		

[a]ANOVA test

dragon fruit shows a high L-arginine with M = 7.188, SD = 0.219, with $p < 0.001$. At 48 h of extraction, red dragon fruits presented with a high L-arginine (M = 4.658, SD = 0.206) and $p < 0.001$. Lastly for the 72 h of extraction, red dragon fruit also shows a high in L-arginine (M = 5.976, SD = 0.149) and $p < 0.001$.

Based on the ANOVA in Table 5, there is a significant mean difference between 24, 48, and 72 h of extraction time for red dragon fruit since $F(2,6) = 128.146$ and $p < 0.001$. The test proceeded with the Post Hoc (Scheffe) test. Based on the test, 24 h (M = 7.188, SD = 0.219) has a significant mean difference between 48 h (M = 4.658, SD = 0.206) and 72 h (M = 5.976, SD = 0.149) of extraction time ($p < 0.001$). Next, 48 h (M = 4.658, SD = 0.206) also has a significant mean difference with 72 h (M = 5.976, SD = 0.149) of extraction time ($p < 0.001$).

■ CONCLUSION

This study presents a novel methodological approach for quantifying amino acids, specifically L-arginine in red dragon fruits (*H. polyrhizus*), which were successfully quantified using RP-HPLC isocratic method. It delivers unique insights into L-arginine preservation and quantification by optimizing extraction time and utilizing chromatographic techniques, with the usage of a Gemini C18 column together with 0.1% H_3PO_4. Red dragon fruits are presented with high L-arginine content and extraction time with the highest L-arginine content for the red dragon fruits are best at 24 h. This study showed that dragon fruits are best extracted earlier, and a longer extraction time was not an efficient way due to L-arginine regenerative potential. Due to the presence of L-arginine and other nutrients in dragon fruits, it is suggested that dragon fruits be included in daily diet intake. However, further research on L-arginine is suggested as it can reduce and prevent any health-related disease.

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■ CONFLICT OF INTEREST

The authors declare that they have no competing interests.

■ AUTHOR CONTRIBUTIONS

Wan Mazlina Md Saad supervised the research, revised the manuscript, and provided critical feedback. Mohd Qamarul Aizat Isha, Muhamad Faizzudin Mohamad Zan, and Siti Mastura Hanim Sallehoddin conducted the experiments, collected and analyzed the data, and contributed to the manuscript's drafting. All authors gave their approval to the publication's final draft.

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