FACTORS AFFECTING THE EXTRACTION OF ANTHOCYANIN FROM *Morus alba* L. AND *Oryza rufipogon* BY THE DIFFERENTIAL pH METHOD

Huynh Thi Lan Anh, Tran Thi Huyen Linh, Ngo Minh Truc,

Nguyen Thi Hai Hoa, Hoang Thi Ngoc Nhon*

Ho Chi Minh City University of Industry and Trade

*Email: *nhonhtn@huit.edu.vn*

Received: 11 June 2024; Revised: 26 July 2024; Accepted: 21 August 2024

ABSTRACT

The extraction of anthocyanin has become increasingly important due to its nutritional benefits and potential applications in the food industry. The main factors affecting the extraction anthocyanin include solvent extraction, temperature extraction, and time extraction. This study examined the effects of solvent extraction methods on the total anthocyanin content (TAC) of *Morus alba* L. (*M. alba* L.) and *Oryza rufipogon* (*O. rufipogon*). *M. alba* L. was extracted with ethanol 60%, the sample/ solvent ratio 1/20 w/v, pH = 2, temperature in 60 ℃, and time in 60 minutes (min) with TAC were 7.01 ± 0.93 mg/g. *O. rufipogon* was extracted with ethanol 60%, sample/ solvent ratio 1/15 w/v, pH = 2, temperature in 55 ℃, and time in 50 min with TAC were 6.38 ± 0.60 mg/g. The study highlights the influence of these parameters on anthocyanin extraction efficiency, providing a foundation for optimizing extraction processes and selecting anthocyanin-rich sources for further research and industrial applications.

Keywords: Anthocyanins, *Morus alba* L., *Oryza rufipogon*, solvent.

1. INTRODUCTION

The increasing demand for natural colorants in the food industry has driven extensive research into alternatives to synthetic dyes, which are often associated with adverse health effects. Anthocyanins, a class of naturally occurring pigments, have gained significative attention due to their vibrant colors and numerous health benefits. These pigments are responsible for the purple, violet, blue, and red hues found in a variety of fruits and vegetables, and they are a major subgroup of flavonoids [1]. Flavonoids such as flavones, isoflavones, flavanones, anthocyanins, and catechins have strong antioxidant capacity [2].

Morus alba L. is the fruit of a mulberry tree belonging to the genus Morus of the Moreaceae family and is widely grown in Asia [1]. In Vietnam, in terms of cultivated area, mulberry trees are found in all eight ecological regions across the country, stretching across 36 provinces and cities from North to South. Among them, the Central Highlands have the largest area of mulberry trees, reaching 10,061 hectares, accounting for 75.53% of the total mulberry tree area of the country. TAC in fresh M. alba L. is 1.18% anthocyanin, which is higher than purple cabbage (0.91%), perilla leaves (0.39%), hibiscus flowers (0.33%), and grape skins (0.56%) [2]. *M. alba* L. contains 84.71% water, 9.19% sugar, 1.8% acid (malic acid, succinic acid), and 0.16% protein. Additionally, *M. alba* L. is the source of indispensable

nutrients, including minerals such as potassium, manganese, and magnesium, as well as vitamins A, C and K. These nutrients are essential for strengthening the body's immune response and supporting the metabolic processes of carbohydrates, proteins, and lipids [3]. They contain significant amounts of bioactive compounds with potential health benefits [4].

Oryza rufipogon rice contains approximately 1.4% anthocyanins [5], and is also abundant in essential minerals, including iron, copper, magnesium, and zinc [6]. *O. rufipogon* has antioxidant properties in red blood cells, preventing the formation of Heinz bodies [7]. Besides, it also can inhibit breast cancer cells [8]. The appearance of *O. rufipogon* is black and reddish purple due to the presence of anthocyanin and long rice grains.

The color and stability properties of anthocyanins are strongly affected by solvent extraction, temperature extraction, and time extraction, which can interact with anthocyanin molecules [9]. The study aimed to determine the anthocyanin extraction conditions using the differential pH method from *M. alba* L. fruit and *O. rufipogon*, two popular materials in Vietnam containing high anthocyanin content. It also compares their anthocyanin extraction conditions. The findings will provide valuable information for future research on natural colorants.

2. MATERIALS AND METHODS

2.1. Material

Materials

M. alba L. was collected from Bao Lam commune, Bao Loc district, Lam Dong province, Vietnam, and rinsed with tap water followed by distilled water to remove surface dirt. *O. rufipogon* was collected in My Xuyen downtown, Soc Trang province, Vietnam. Both materials were cultivated according to VietGAP standards, dried at 60 ℃ until their moisture content was below 10%, then ground into powder (20-40 mesh). Both samples were stored in polyethylene (PE) plastic bags to safeguard them from light and moisture during all experimental procedures.

Chemicals

Ethanol (China, 99.5%), methanol (Merck, 99.7%), hydrochloric acid (Merck, 36.5%), potassium chloride (China, 98%), and sodium acetate trihydrate (China, 99.5%) were utilized in this study. All chemicals were of analytical grade.

2.2. Method

2.2.1. Effects of solvent on anthocyanin extraction

1 gram (g) of the sample was added with a solvent of methanol (60%), ethanol (60%), and distilled water, pH of 2 (adjusted by HCl 5%), at a sample/ solvent ratio 1/25 (w/v). After selecting the appropriate solvent, continue investigating the optimal solvent concentration of 40%, 50%, 60%, 70%, 80%). The extraction process was conducted in a laboratory thermostatic water bath (Germany, 24L, IKA) at 60 ℃ in 60 min under dark conditions. After extraction, the mixture was centrifuged at 5000 rpm for 15 minutes using a HERMLE Z206A centrifuge (Germany) to separate the solid residue and collect the supernatant enriched with anthocyanins. TAC was determined (mg/g) using the differential pH method.

2.2.2. Effects of sample: solvent and pH of solvent on anthocyanin extraction

1 g of sample was mixed with ethanol 60% at sample/ solvent ratios of 1/10, 1/15, 1/20, 1/25, and 1/30 (w/v), and pH was adjusted to 1, 2, 3, 4, and 5 using 5% HCl. The extraction was conducted at 60 ℃ in 60 min under dark conditions, followed by centrifugation to isolate the solid residue and retrieve the supernatant containing anthocyanins. TAC (mg/g) was assessed using the differential pH method.

2.2.3. Effects of temperature and time extraction on the extraction of anthocyanin

1 g of sample was mixed with 60% ethanol at a sample-to-solvent ratio of 1/25 w/v with the pH adjusted to 2 using 5% HCl. The extraction of *M. alba* L. was carried out at temperatures of 40 ℃, 50 ℃, 60 ℃, 70 ℃, and 80 ℃ for 30, 60, 90, 120, and 150 mins. The extraction of *O. rufipogon* was carried out at temperatures of 40 ℃, 45 ℃, 50 ℃, 55 ℃, and 60 ℃ for 40, 50, 60, 70, and 80 mins under dark conditions then centrifuged to remove the residue and obtain the supernatant containing anthocyanin. TAC (mg/g) was determined using the differential pH method.

2.3. Analysis of total anthocyanin content

TAC (mg/g) was quantified using the pH differential method. Anthocyanin samples were analyzed with a V-780 UV-Vis spectrometer (Jasco, Japan) at a wavelength of 520 nm. TAC was calculated in mg/g using the following equation [10]:

$$\text{TAC (mg/g)} = \frac{\text{A} \times D_f \times M_w \times \text{V} \times 10^3}{\varepsilon \times \text{L} \times \text{m}}$$

With:

A: $(A_{\lambda max} - A_{\lambda 700})_{pH = 1} - (A_{\lambda max} - A_{\lambda 700})_{pH = 4.5}$

D_f: Dilution factor; L: Length of cuvette, L = 1 cm

M_W: Molecular weight of cyanidin 3-glucoside, M_W = 449.2 g/mol

ε: Molar absorptivity ($l.mol^{-1}.cm^{-1}$), ε = 26900 $l.mol^{-1}.cm^{-1}$

V: volume of diluted samples (mL); m: the weight of the sample (g)

2.4. Data analysis

The results are presented as mean ± standard deviation, based on experiments conducted in triplicate. Statistical evaluation was performed using one-way analysis of variance (ANOVA statistical). Data processing was carried out with Microsoft Excel 2016 and Minitab Statistics 19.

3. RESULTS AND DISCUSSION

3.1. Effects of solvent extraction on anthocyanin content

Variations in the optimal extraction conditions could be attributed to the diverse polarity of individual anthocyanin compounds concerning the solvent. TAC of *M. alba* L. and *O. rufipogon* extracts were shown in Fig. 1.

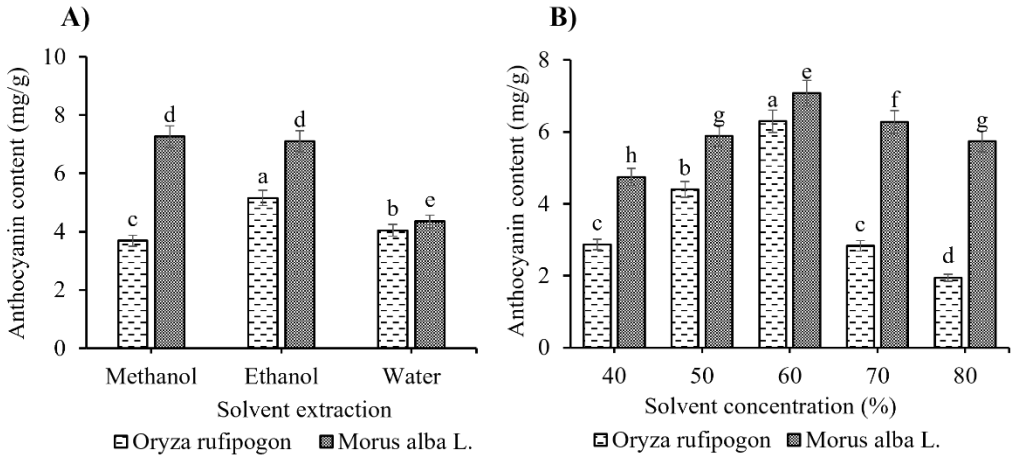

Fig. 1. Effect of solvent type (A) and ethanol concentration (B) on anthocyanin extraction from *O. rufipogon* and *M. alba* L.

Note: The different letters within same column indicate statistically significant differences at $p < 0.05$.

Based on results from Fig. 1A, TAC ranged from 3.69 ± 0.69 mg/g to 5.16 ± 0.25 mg/g for *O. rufipogon*, and from 4.34 ± 0.18 mg/g to 7.26 ± 0.03 mg/g for *M. alba* L.. Among solvents used, extraction with methanol/H2O (60/40, v/v) showed the highest TAC for *M. alba* L. extract (7.26 ± 0.03 mg/g). For *O. rufipogon* extract, the highest TAC was obtained with ethanol/ water (60/40, v/v) (5.16 ± 0.25 mg/g), followed by water (4.04 ± 0.20 mg/g) and methanol (3.69 ± 0.85 mg/g). Anthocyanins are typically extracted from plant materials using an organic solvent, with methanol being the most commonly used [11]. The anthocyanins contain aromatic rings with -OH, -C=O, -O-CH, and glucosyl groups, forming a polar molecule, that is easily extracted with organic solvents or organic solvent–water systems [12]. According to Boeing *et al*., the methanol:H2O: acetic acid (70:29.5:0.5, v/v/v) mixture yields the highest TAC among all the berries analyzed [13]. This solvent combination is effective in breaking down cell membranes, allowing for the dissolution and stabilization of anthocyanins [14]. Although methanol/water resulted in higher TAC than ethanol, however, the difference was not significant (p-value < 0.05). Therefore, ethanol was chosen for subsequent experiments due to its high anthocyanin extraction efficiency, safety, reasonable cost, and availability.

Anthocyanin possesses hydrophobic hydrocarbon radicals and exhibits solubility in organic solvents. Conversely, their polar polyphenol functional groups enable dissolution in polar solvents. Selecting an optimal ethanol concentration is crucial for maximizing anthocyanin yield while maintaining color stability during extraction [15]. TAC of *M. alba* L. and *O. rufipogon* extracts were shown in Fig. 1B.

For *O. rufipogon*, TAC increased from 2.87 ± 0.15 mg/g to 4.40 ± 0.22 mg/g as ethanol concentration increased from 40% - 50%, a maximum of 6.29 ± 0.32 mg/g of 60% ethanol. However, TAC declined significantly to 2.83 ± 0.15 mg/g at 70% ethanol and continued to decrease to 1.95 ± 0.10 mg/g at 80% ethanol. Consequently, 60% ethanol is deemed the optimal solvent concentration for extracting anthocyanins from *O. rufipogon*, ensuring optimal extraction efficiency.

Experimental data indicate that in *M. alba* L. when ethanol concentration was from 40% to 50%, the obtained TAC increased (4.75 ± 0.15 mg/g to 5.88 ± 0.25 mg/g), peaked at 60% ethanol with TAC of 7.08 ± 0.44 mg/g. At 80% ethanol, TAC obtained decreases to 5.74 ± 1.26 mg/g. Low ethanol concentrations correspond to reduced TAC due to the high water ratio, which impedes the dissolution of polyphenol compounds and leads to the extraction of protein,

polysaccharide, and other inorganic substances from the raw materials [8]. Conversely, ethanol concentration above 60% tends to decrease TAC, as changes in solvent system polarity affect anthocyanin diffusion [8]. Based on these findings, an ethanol concentration of 60% was chosen for extracting anthocyanins from *M. alba* L., to achieve the most effective extraction performance. Similarly, Gao's research on anthocyanin extraction from blueberry also identified 60% ethanol as the optimal concentration for anthocyanin content [16]. This conclusion aligns with Cacace's research on blackcurrants, which also found that the highest TAC was achieved at 60% ethanol concentration [17].

3.2. Effect of sample/ solvent ratio and pH of solvent on anthocyanin content

The sample: solvent ratio is a key parameter influencing the efficiency of active ingredient extraction. Together with particle size, this ratio determines the contact surface area between the material and solvent, as well as the material's dispersion within the solvent. The impact of the extraction ratio on TAC is illustrated in Fig. 2.

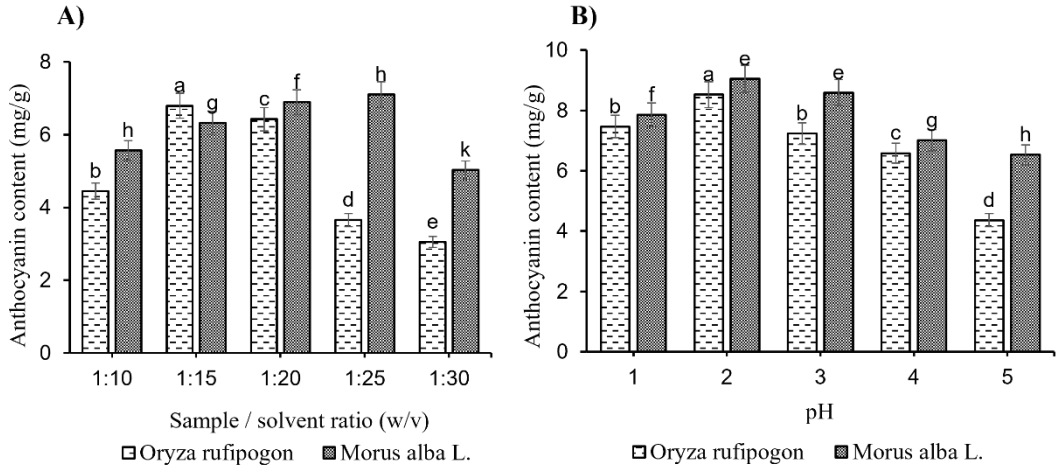

Fig. 2. Effect of sample/ solvent ratio (A) and pH (B) on anthocyanin extraction of *O. rufipogon* and *M. alba* L.
Note: Different letters within the same column indicate statistically significant differences at $p < 0.05$.

Sample/ solvent ratio is a crucial parameter that significantly TAC obtained. Figure 2A illustrates that sample/ solvent ratios of 1/10, 1/15, 1/20, 1/25, and 1/30 (w/v), increasing ratio from 1/10 to 1/15 resulted in a peak TAC of *O. rufipogon* (4.45 ± 0.32 mg/g to 6.79 ± 0.39 mg/g), a steady decrease as the ratio increased from 1/20 to 1/30 (6.89 ± 0.10 mg/g to 5.03 ± 0.15 mg/g). The TAC of *M. alba* L. reached its maximum at a ratio of 1/25 w/v. When comparing ratios of 1/20 and 1/25 w/v, there was no statistically significant difference (6.89 ± 0.10 mg/g versus 7.12 ± 1.67 mg/g). However, the 1/20 ratio is preferred as it minimizes solvent usage. During the extraction process, a sufficient volume of solvent is essential to penetrate the material and dissolve the target compounds effectively. An increase in solvent volume improves the diffusion of anthocyanins, but only up to an optimal threshold, beyond which further increases yield diminishing returns. Malik *et al.* found a sample-to-solvent ratio of 1:15 w/v effective for extracting anthocyanins from goji berries [18].

The structure, color fastness, and color of anthocyanin are influenced by pH changes [19]. The effect of pH on TAC is depicted in Fig. 2B. TAC increased rapidly with a rise in pH from 1 to 2 (7.46 ± 0.46 mg/g to 8.52 ± 0.61 mg/g for *O. rufipogon* and 7.85 ± 0.62 mg/g to 9.05 ± 0.32 mg/g for *M. alba* L.), reaching a maximum at pH = 2. However, as pH increased from 3 to 5, TAC decreased (7.23 ± 0.36 mg/g to 4.36 ± 0.22 mg/g for *O. rufipogon* and 8.59 ± 1.51

mg/g to 6.53 ± 0.74 mg/g for *M. alba* L.). This is attributed to the stability of the flavylium cation of anthocyanins under low pH conditions, though excessively low pH can lead to partial hydrolysis of acyl radicals in anthocyanins, making the polar phenolic compounds stronger and unstable. Conversely, increasing pH values lead to significant degradation of anthocyanins in the raw materials [19]. Espada-Bellido *et al*. identified that the optimal conditions for anthocyanin extraction were achieved with a solvent composition of 76% methanol in water at a pH of 3 [20]. Based on these findings, a pH of 2 was chosen as the extraction condition to achieve the highest anthocyanin content.

Temperature is a critical factor in the extraction of compounds that are sensitive to heat. Elevated temperatures can accelerate solvent diffusion and enhance mass transfer, facilitating the dissolution of the desired components. However, excessive heat may also increase the co-extraction of unwanted impurities and lead to the degradation of thermally labile compounds, including anthocyanins [20]. Fig 3 demonstrates the influence of temperature on the anthocyanin extraction process.

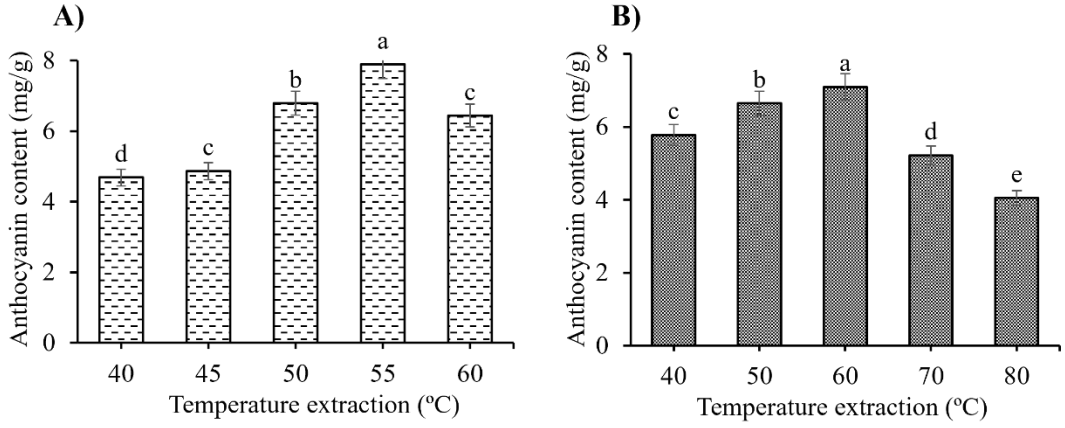

Fig. 3. Effect of temperature extraction on anthocyanin content of *O. rufipogon* (A) and *M. alba* L. (B)
Note: Different letters within the same column indicate statistically significant differences at $p < 0.05$.

For *O. rufipogon*, in Fig 3A, TAC increases rapidly within the temperature range of 40 ℃ to 60 ℃, reaching a maximum at 55 ℃ (7.89 ± 0.24 mg/g). However, further increasing the temperature to 60 ℃ results in a decrease in TAC (6.34 ± 0.19 mg/g). In Fig 3B, TAC of *M. alba* L. increases with temperature in the range of 40 ℃ to 80 ℃, peaking at 60 ℃ (7.01 ± 1.42 mg/g). However, a sharp decline in TAC is observed when the temperature is further increased from 70 ℃ to 80 ℃ (5.22 ± 1.42 mg/g to 4.05 ± 1.12 mg/g). When increased temperature enhances the diffusion and solubility of anthocyanin pigments and reduces solvent viscosity, thereby increasing the mass transfer rate and accelerating pigment extraction. Nonetheless, excessively high temperatures lead to anthocyanin oxidation, causing a loss in TAC [8]. Jing's research also determined that 50 ℃ is the optimal temperature for extracting the highest anthocyanin content from purple corn [21]. The initial increase in temperature improves the diffusion and solubility of anthocyanin pigments and decreases solvent viscosity, enhancing the mass transfer rate and accelerating pigment extraction. Yet, at excessively high temperatures, TAC diminishes due to anthocyanin oxidation.

The duration of extraction is another crucial factor influencing anthocyanin yield. Fig. 4 presents the impact of extraction time on TAC.

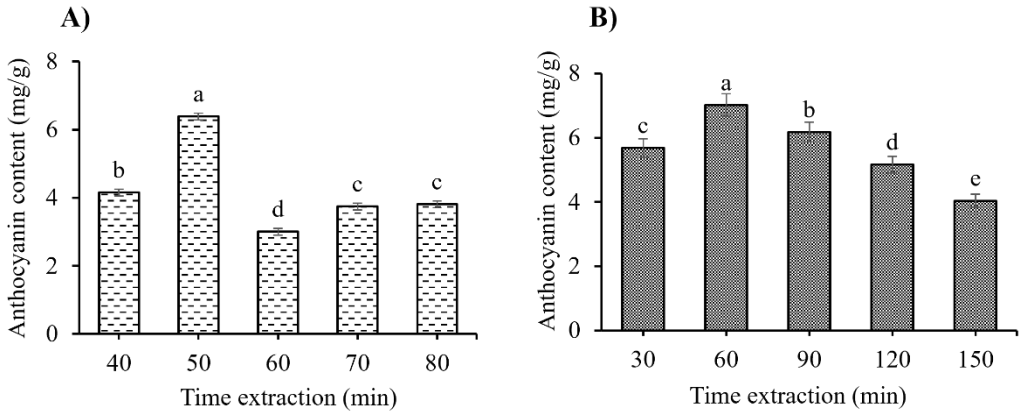

Fig. 4. Time extraction effect on TAC of *O. rufipogon* (A) and *M. alba* L. (B)
Note: Different letters within the same column indicate statistically significant differences at $p < 0.05$.

The results in Fig. 4A, for *O. rufipogon*, TAC increases with extraction times ranging from 40 to 80 min, peaking at 50 min (6.39 ± 0.19 mg/g). However, TAC diminishes to 3.01 ± 0.09 mg/g at 60 min before rising again to between 3.74 ± 0.11 mg/g and 3.81 ± 0.13 mg/g at 70 min. Fig. 4B shows that in *M. alba* L., TAC increases significantly with extraction times from 30 to 90 min, reaching a maximum of 7.01 ± 0.28 mg/g at 60 min. Beyond this point, TAC declines to 6.17 ± 0.56 mg/g at 90 min and drops substantially to 4.03 ± 1.25 mg/g at 150 min. Extended extraction times can compromise extract quality, as the solvent may extract non-target compounds and prolonged high-temperature conditions can lead to anthocyanin degradation. Additionally, extraction occurring at high temperatures for extended periods can result in anthocyanin decomposition [22]. In a study by Pedro *et al*., anthocyanins from black rice also an almost similar conclusion about the optimal extraction time of 80 min [23].

4. CONCLUSION

The findings of this study demonstrate that the extraction conditions significantly influence the TAC from *Oryza rufipogon* and *Morus alba* L.. These suitable conditions provide valuable insights into maximizing anthocyanin yield from these sources. However, the study has some limitations, including the potential variability in anthocyanin stability under different extraction conditions and the need for a more comprehensive analysis of other influencing factors, such as solvent types and extraction methods. Future research should focus on exploring these variables and examining the applicability of these findings to large-scale extraction processes. Additionally, investigating the bioavailability and functional properties of the extracted anthocyanins will be crucial for their effective application in food industry.

Acknowledgements: This study was sponsored and funded by Ho Chi Minh City University of Industry and Trade under Contract No.173/HD-DCT dated 31 August 2023.

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TÓM TẮT

CÁC YẾU TỐ ẢNH HƯỞNG ĐẾN QUÁ TRÌNH CHIẾT XUẤT ANTHOCYANIN TỪ *Morus alba* L. VÀ *Oryza rufipogon* BẰNG PHƯƠNG PHÁP pH VI SAI

Huỳnh Thị Lan Anh, Trần Thị Huyền Linh, Ngô Minh Trực,
Nguyễn Thị Hải Hòa, Hoàng Thị Ngọc Nhơn*
Trường Đại học Công Thương Thành phố Hồ Chí Minh
*Email: *nhonhtn@huit.edu.vn*

Chiết xuất anthocyanin ngày càng được quan tâm nhờ vào những lợi ích dinh dưỡng và tiềm năng ứng dụng trong ngành công nghiệp thực phẩm. Nghiên cứu này tập trung đánh giá tác động của các phương pháp chiết xuất sử dụng dung môi đến tổng hàm lượng anthocyanin (TAC) của *Morus alba* L. (*M. alba* L.) và *Oryza rufipogon* (*O. rufipogon*) thông qua phương pháp pH vi sai. *M. alba* L. được chiết xuất với ethanol 60%, tỷ lệ mẫu/dung môi 1/20 (g/mL), pH = 2, nhiệt độ 60 ℃ và thời gian 60 phút với TAC đạt 7.01 ± 0.93 mg/g. *O. rufipogon* được chiết xuất với ethanol 60%, tỷ lệ mẫu/dung môi 1/15 (g/mL), pH = 2, nhiệt độ 55 ℃ và thời gian 50 phút với TAC đạt 6.38 ± 0.60 mg/g. Quá trình chiết xuất anthocyanin chịu ảnh hưởng bởi nhiều yếu tố quan trọng như loại dung môi, nhiệt độ và thời gian chiết. Nghiên cứu làm nổi bật ảnh hưởng của các thông số này đến hiệu quả chiết xuất anthocyanin, cung cấp nền tảng cho việc tối ưu hóa quy trình chiết xuất và lựa chọn các nguồn giàu anthocyanin cho nghiên cứu và ứng dụng công nghiệp.

Từ khóa: Anthocyanin, dung môi, *Morus alba* L., *Oryza rufipogon*.