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**ANTHOCYANIN RECOVERY FROM BLACK RICE BRAN USING
ALTERNATIVE TECHNOLOGIES AND IMPROVING STABILITY THROUGH
ENZYMATIC ACYLATION**

Florianópolis

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O presente trabalho em nível de Doutorado foi avaliado e aprovado, em 22 de novembro de 2024, pela banca examinadora composta pelos seguintes membros:

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RESUMO

Durante todo o ciclo de vida dos alimentos, algumas partes dos produtos podem ser desperdiçadas, gerando resíduos ou subprodutos. Cerca de 20% da casca, 10% do farelo e 14% do arroz quebrado são produzidos durante o processamento do arroz negro (ou arroz preto). Moléculas de valor agregado podem ser obtidas de subprodutos alimentares do arroz negro, principalmente as antocianinas. Relatos desses compostos com efeitos antiobesidade, antidiabéticos, antimicrobianos, anticancerígenos, neuroprotetores e em doenças cardiovasculares foram apresentados. A extração de antocianinas pode ser uma alternativa viável para o reaproveitamento do material gerado durante o beneficiamento do arroz negro, principalmente utilizando técnicas alternativas, como extração por líquido pressurizado (PLE), extração assistida por ultrassom (UAE) e extração assistida por micro-ondas (MAE). No entanto, esses compostos são susceptíveis a condições notáveis de processamento de luz, temperatura e oxigênio. A acilação (esterificação de um ácido orgânico ao glicosídeo da molécula de antocianina) pode ser uma abordagem para melhorar a estabilização desses compostos. A acilação enzimática tem alta regioseletividade e reações moderadas, sendo considerado o método preferido para a produção de derivados flavonoides acilados. Dessa forma, este trabalho propõe investigar a recuperação de antocianinas de farelo de arroz negro usando tecnologias emergentes (PLE, UAE e MAE) e posterior acilação enzimática de antocianinas recuperadas para melhorar a estabilidade desses compostos. Foram realizadas análises de termoestabilidade, citotoxicidade e medidas da sustentabilidade através das métricas verdes: Certificado Verde e EscalaEco. Além disso, para os compostos purificados e acilados, foi realizada a análise de transporte epitelial utilizando cocultura Caco2:HT29-MTX. Todos os métodos alternativos de extração quando otimizados apresentaram recuperação de antocianinas superior a 80%, não tendo diferença significativa entre si ($p < 0.05$). Entretanto, o extrato obtido por UAE foi o que apresentou maior quantidade de cianidina-3-glicosídeo (C3G), identificado por HPLC. Nenhum dos extratos apresentou citotoxicidade (500 $\mu\text{g/mL}$) para as células L929 e todos apresentaram efeito citoprotetor quando submetido ao estresse oxidativo com H_2O_2 . Todas as técnicas foram consideradas ecologicamente verdes pelo Certificado Verde (A) e métodos excelentes pela EcoScale (pontuações maiores que 75), destacando-se o extrato obtido por UAE. Para a acilação enzimática, foi considerado o método por UAE para dar continuidade ao trabalho devido ao seu maior teor de C3G e melhores resultados obtidos pelo Certificado Verde e EcoScale. Foi possível obter cerca de 53% da formação do produto acilado com ácido octanóico (C3G-C8), ao utilizar acetonitrila:DMSO (10:1), temperatura de 60 °C e 15 g/L de enzima lipase CalB. Além disso, foi observado que o composto acilado apresentou maior termoestabilidade que o purificado (C3G), sendo verificado pelos maiores valores de tempo de meia-vida para os pHs 3, 5 e 7. Ambos compostos não apresentaram citotoxicidade e apresentaram cerca de 1,2% de transporte epitelial em cocultura com Caco2:HT29-MTX, não diferenciando entre si ($p < 0.05$). Portanto, o extrato do farelo do arroz negro tem grande potencial para aplicação como ingrediente em alimentos funcionais, suplementos ou formulações farmacológicas. A acilação enzimática de antocianinas demonstrou-se uma técnica promissora para aumento da sua termoestabilidade e lipofilicidade, permitindo a aplicação em produtos de matrizes lipídicas.

Palavras-chave: *Oryza sativa* L.; extrato rico em antocianina, métodos de extração; corantes naturais, estabilidade.

RESUMO EXPANDIDO

Introdução

Os resíduos ou subprodutos provenientes das indústrias alimentícias podem apresentar potencial em várias aplicações de acordo com o material gerado. No processamento do arroz, parte dos produtos gerados que são descartados são o farelo (10%) e arroz quebrado (14%). No caso do arroz negro, esses subprodutos possuem em sua composição antocianinas das quais são conhecidas por apresentarem alto valor biológico. Uma maneira para recuperar esses compostos é através da extração, sendo técnicas alternativas como extração por líquido pressurizado (PLE), extração assistida por ultrassom (UAE) e extração assistida por micro-ondas (MAE) abordagens a serem avaliadas. No entanto, esses compostos são susceptíveis à degradação dependente das condições de processamento, como luz, temperatura e oxigênio. Alguns métodos foram utilizados para manter a estabilidade das antocianinas: spray-dryer, liofilização, encapsulamento, copigmentação e acilação. A acilação ocorre pela esterificação de um ácido orgânico ao glicosídeo da molécula de antocianina. Tanto a acilação química quanto a enzimática foram investigadas, sendo a química não recomendada devido à baixa regioseletividade, obtendo-se diversos produtos que dificultam a purificação. Em contraste, a acilação enzimática apresentou alta regioseletividade e reações moderadas, sendo o método preferido para a produção de derivados flavonoides acilados. Além disso, as antocianinas aciladas apresentaram maior estabilidade quando comparadas às não aciladas. Poucos estudos avaliaram tanto diferentes métodos da extração das antocianinas do farelo do arroz negro quanto a acilação enzimática visando melhora na estabilidade, com isso, a estratégia de otimização da extração e acilação enzimática dos compostos obtidos do subproduto do arroz negro são interessantes a fim de reduzir seu desperdício além de agregar valor para o mesmo.

Objetivos

O objetivo geral deste trabalho é a recuperação de antocianinas do farelo de arroz negro através da otimização utilizando técnicas emergentes, como PLE, UAE e MAE. Além disso, realizar acilação enzimática das antocianinas recuperadas na extração a fim de aumentar sua termoestabilidade para possíveis aplicações.

Metodologia

As extrações foram otimizadas por meio da metodologia de superfície de resposta, utilizando modelo Box-Behnken. O extrato rico em antocianinas foi caracterizado por meio da concentração de antocianinas monoméricas totais pelo método do pH diferencial, porcentagem de antocianina recuperada em relação ao total presente na matriz, compostos fenólicos totais, cor polimérica, atividade antioxidante, citotoxicidade, atividade antitumoral e antocianinas individuais por HPLC-DAD e HPLC-MS. Além disso também foi avaliada a sustentabilidade da técnica através do Certificado Verde e Escala Eco, ferramentas semiquantitativas para mensurar quão “sustentável” as técnicas de extrações podem ser. O extrato rico em antocianinas foi purificado utilizando sílica C18 e submetido a otimização da acilação enzimática com ácido octanóico (C8) como grupo acil doador. Foi verificada a formação do produto através das técnicas de HPLC-DAD, HPLC-MS e RMN. Foi realizada análise de termoestabilidade, lipofilicidade, citotoxicidade e estudo de transporte epitelial (utilizando cocultura Caco2:HT29-MTX) para ambos compostos (acilado e não acilado). Além disso, também foi avaliado o processo de acilação enzimática quanto a sustentabilidade através das métricas verdes: Certificado Verde e EscalaEco.

Resultados e Discussões

As condições ótimas para extração de antocianinas por PLE foram: 55 °C, vazão de 5 mL/min e solução etanol:ácido cítrico 0,1 mol/L (50:50, v/v). PLE recuperou cerca de 80% do total de antocianinas (2,33 mg/C3GE g), enquanto HSE recuperou 46% (1,17 mg/C3GE g). Cianidina-3-O-glicosídeo (C3G) foi identificada (m/z 449) e quantificada, 1,63 mg/g para PLE e 1,07 mg/g para HSE. Para UAE, 83,73% das antocianinas totais (2,44 mg C3G/g) foram recuperadas, usando como parâmetros temperatura de 50°C, frequência de 380 W e solvente 60:40 (v/v) ácido cítrico 0,1 M:etanol. Para o MAE, foi possível recuperar 81,44% das antocianinas totais (2,37 mg C3G/g), usando parâmetros temperatura de 55°C, ácido cítrico 0,1 M:etanol (50:50, v/v) e massa de 0,8 g (1:24, g/mL). As técnicas PLE, UAE e MAE foram consideradas ecologicamente verdes pelo Certificado Verde (A) e métodos excelentes pela EcoScale (pontuações maiores que 75). Os extratos ricos em antocianina não apresentaram citotoxicidade para células L292 (500 µg/mL) e apresentaram citoproteção quando submetidos ao estresse oxidativo com H₂O₂. Com relação à acilação enzimática, o extrato obtido por UAE foi purificado com C18, obtendo a cianidina-3-glicosídeo (C3G) purificada. Foi possível obter cerca de 53% de formação de antocianina acilada utilizando ácido octanoico (C8) como grupo doador acila, como solvente Acetonitrila:DMSO (10:1), temperatura de 60 °C e concentração de enzima de 15 g/L. Com relação à estabilidade, foi possível observar um aumento na meia-vida para antocianina acilada (C3G-C8) em diferentes pHs (3, 5 e 7) em comparação com C3G. Também foi verificado aumento da lipofilicidade da antocianina acilada (log P = 1,37) em comparação com seu precursor (log P = -1,59). Os compostos também foram avaliados quando à citotoxicidade em cocultura Caco2:HT29-MTX (9:1) até a concentração de 200 µM durante 4 horas, não apresentando citotoxicidade em nenhuma concentração para ambas as amostras. Para o estudo de transporte epitelial, foi verificado que para a cocultura Caco2:HT29-MTX, cerca de 1,2% de antocianinas foram transportadas ao final do período de 4 h, sendo que as amostras não se diferenciaram entre si. O processo de acilação enzimática foi considerado nota B para o Certificado Verde e considerado aceitável para a EscalaEco (valor entre 50 e 75).

Considerações Finais

Observou-se que todas as técnicas emergentes (PLE, UAE e MAE) utilizadas apresentaram recuperação próxima a 80% e não diferiram entre si (p<0,05). Entretanto, em relação às antocianinas quantificadas individualmente, a amostra extraída por UAE apresentou maior quantidade de C3G (2,41 mg/g), resultado importante para a etapa de acilação enzimática. Além disso, os extratos também apresentaram resultados semelhantes para TPC e PC. Todos os extratos não apresentaram citotoxicidade (500 µg/mL). Verificou-se também que todos os processos utilizados foram ecologicamente corretos por meio das medidas do Certificado Verde e EscalaEco, nas quais UAE apresentou melhor resultados para ambas as escalas, 95 e 84,9, respectivamente. Já para a acilação enzimática, foi possível obter cerca de 53% da formação do composto acilado (C3G-C8) ao otimizar os parâmetros do processo. Além disso, o composto acilado apresentou melhor termoestabilidade (verificado pelo tempo de meia-vida) e aumento da lipofilicidade (medido pelo coeficiente de partição) quando comparado com o seu precursor. Os compostos não apresentaram citotoxicidade e foi verificado transporte de cerca de 1,2% dos compostos em cocultura Caco2:HT29-MTX, avaliados através do transporte epitelial. Também foi observado que uma parte das antocianinas aciladas ficaram adsorvidas nas células/mucos, das quais parte delas (cerca de 70%) foi convertida para C3G. Mais estudos são necessários para entender melhor sobre esse comportamento.

Palavras-Chave: *Oryza sativa* L., extrato rico em antocianina, métodos de extração, lipofilização, termoestabilidade.

ABSTRACT

Some parts of the products may be wasted throughout the food life cycle, generating residues or by-products. Approximately 20% of the husk, 10% of the bran, and 14% of the broken rice are produced during the processing of black rice. Value-added molecules can be obtained from black rice food by-products, mainly anthocyanins. Reports of these compounds with anti-obesity, anti-diabetic, antimicrobial, anti-cancer, neuroprotective, and cardiovascular disease effects have been presented. Anthocyanin extraction may be a viable alternative for the reuse of the material generated during the processing of black rice, mainly using alternative techniques, such as pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE). However, these compounds are susceptible to notable light, temperature, and oxygen processing conditions. Acylation (esterification of an organic acid to the glycoside of the anthocyanin molecule) may be an approach to improve the stabilization of these compounds. Enzymatic acylation has high regioselectivity and moderate reactions and is the preferred method for producing acylated flavonoid derivatives. Therefore, this project proposes to investigate the recovery of anthocyanins from black rice bran using emerging technologies (PLE, UAE, and MAE) and subsequent enzymatic acylation of recovered anthocyanins to improve the stability of these compounds. Thermostability, cytotoxicity, and sustainability measures were analyzed through Green Certificate and EscalaEco green metrics. In addition, epithelial transport analysis was performed using Caco2:HT29-MTX coculture for the purified and acylated compounds. All emerging extraction methods, when optimized, showed anthocyanin recovery greater than 80%, with no significant difference between them ($p < 0.05$). However, the extract obtained by UAE presented the highest amount of cyanidin-3-glucoside (C3G) identified by HPLC. None of the extracts presented cytotoxicity (500 $\mu\text{g/mL}$) for L929 cells, and all presented cytoprotective effects when subjected to oxidative stress with H_2O_2 . All techniques were considered ecologically green by the Green Certificate (A) and excellent methods by EcoScale (scores higher than 75), highlighting the extract obtained by UAE. The UAE method was considered to continue the work with enzymatic acylation due to its higher C3G content and better results obtained by the Green Certificate and EcoScale. It was possible to obtain approximately 53% of the formation of the product acylated with octanoic acid (C3G-C8) when using acetonitrile:DMSO (10:1), temperature of 60 °C and 15 g/L of lipase enzyme CalB. Furthermore, it was observed that the acylated compound presented greater thermostability than the purified one (C3G), as verified by the higher half-life values for pHs 3, 5, and 7. Both compounds did not present cytotoxicity and presented approximately 1.2% epithelial transport in coculture with Caco2:HT29-MTX, not differentiating between them ($p < 0.05$). Therefore, black rice bran extract has great potential for application in functional foods, supplements, or pharmacological formulations. The enzymatic acylation of anthocyanins proved a promising technique for increasing their thermostability and lipophilicity, allowing the application in lipid matrix products.

Keywords: *Oryza sativa* L.; anthocyanin-rich extract, extraction methods; natural colorants, stability.

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LIST OF ABBREVIATIONS

HSE – Heating-stirring extraction	PC – Polymeric color
PLE – Pressurized-liquid extraction	TPC – Total phenolic content
UAE – Ultrasound-liquid extraction	t_{1/2} – Half-life time
MAE – Microwave-liquid extraction	k_d – Degradation rate
HPLC – High-Performance Liquid Chromatography	E_a – Activation energy
RMN – Ressonância magnética nuclear	ΔH – Activation enthalpy
MNR – Nuclear magnetic resonance	ΔG – Free energy of activation
DMSO – Dimethyl sulfoxide	ΔS – Activation entropy
C3G – Cyanidin-3-glucoside	DMEM – Dulbecco's Modified Eagle Medium
C3G-C8 – Cyanidin-3-glucoside octanoic ester	RPMI – Roswell Park Memorial Institute 1640 medium
BRB – Black rice bran	Acet – Acetonitrile
DES – Deep eutectic solvent	C6 – Hexanoic acid
CAGR – Compound annual growth rate	C8 – Octanoic acid
C3R – Cyanidin-3-glucoside	C10 – Decanoic acid
M3G – Malvidin-3-glucoside	C12 – Dodecanoic acid
SLE – Solid-liquid extraction	2M2B – 2-methylbutan-2-ol
LLE – Liquid-liquid extraction	MW – Molecular weight
CE – Conventional extraction	
NADES – Natural deep eutetic solvente	
SWE – Supercritical water extraction	
GRAS – Generally recognized as safe	
DPPH – 2,2-diphenyl-1-picrylhydrazyl	
ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)	
FRAP – Ferric Reducing Antioxidant Power	
PBS – Phosphate buffered saline	
AR – Anthocyanin recovery	
TMA – Total monomeric anthocyanin	

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CONCEPTUAL DIAGRAM

Why?
• There is a great demand for natural products, which are considered healthy and economically viable; • To the detriment of this food demand, the development of sustainable methodologies has been used in order to give a better destination to by-products and residues from the industry; • Most by-products and residues from the food industry have interesting nutritional properties, with recovery potential and possible application in several areas; • Black rice bran is a by-product with high biological value and which has not yet been much explored, having in its composition several interesting compounds, mainly anthocyanins; • To perform the recovery of these bioactive compounds, few studies have used emerging technologies, such as pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE); • Anthocyanins are known for not showing good stability (temperature, light, oxygen, etc.) and for this reason some stabilization methods have been used, such as enzymatic acylation; • The optimization of enzymatic acylation of anthocyanins from black rice bran (BRB) and transepithelial transport experiments has not yet been studied.
What has already been done?
• The extraction of anthocyanins from BRB using MAE, in which the parameters were time, power energy, and concentration of citric acid in water; • For the extraction of anthocyanins from BRB by UAE, the methodology was used using a water bath (parameters temperature, time, solvent, and solid/liquid ratio) and using a tip with natural deep eutectic solvents (DES) as solvents; • In the case of PLE extraction, no study was found in the literature to extract anthocyanins from BRB; • The conventional methodology was carried out regarding the enzymatic acylation of anthocyanins; however, the parameters of the process were not optimized; • Acylated compounds were evaluated for their thermostability and lipophilicity.
Hypotheses
• Alternative methodologies for recovering anthocyanins from BRB may be more efficient compared to the conventional one, as it uses less time and less solvent; • Extracts obtained by optimizing the recovery of anthocyanins from BRB may have high biological value without showing cytotoxicity; • These emerging recovery methodologies (PLE, UAE, and MAE) can be considered eco-friendly; • The enzymatic acylation of anthocyanins can be a viable alternative to be explored to improve the stability of these compounds; • The optimization of the enzymatic acylation process can improve the product formation yield; • Acylated anthocyanin alters and/or improves transepithelial transport.
Methodologies
• Optimization of anthocyanin extraction process from BRB using Box-Behnken experimental design by PLE, UAE, and MAE; • Comparison of techniques through anthocyanin yield, polymeric color, total phenolic compounds, antioxidant activity, and Green certificate and Ecoscale tool; • Optimization of enzymatic acylation of anthocyanins extracted from BRB; • Verification of enzymatic acylation through HPLC-DAD, HPLC-MS and NMR spectroscopy; • Evaluation of acylated anthocyanins and their precursors concerning thermostability, lipophilicity, cytotoxicity, and transepithelial transport experiments.
Responses
• Define the method with the highest yield in the extraction of anthocyanins from BRB; • Check the cytotoxicity of extracts for possible use in food and pharmaceutical formulations; • Establish enzymatic acylation of BRB anthocyanin; • Verify the possible increase in thermostability through the enzymatic acylation of anthocyanins; • Check the cytotoxicity and transepithelial transport of the acylated product.

THESIS GRAPHICAL ABSTRACT

CHAPTER 1: INTRODUCTION

POTENTIAL OF BLACK RICE BY-PRODUCTS



HEALTHY LIFESTYLE



OBJECTIVES



CHAPTER 2: LITERATURE REVIEW

GAPS:

ANTHOCYANIN EXTRACTION

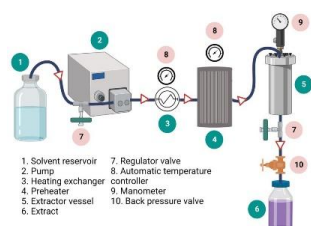
➤ EMERGENT TECHNOLOGIES

ENZYMATIC ACYLATION

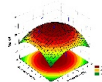
➤ PROCESS OPTIMIZATION



CHAPTER 3: PLE



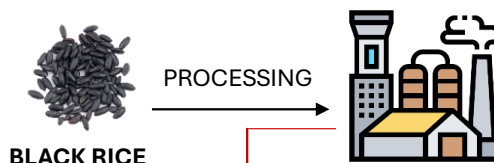
OPTIMIZATION ANTHOCYANIN RECOVERY



Box-Behnken design

- Temperature
- Solvent (etanol:water)
- Flow rate

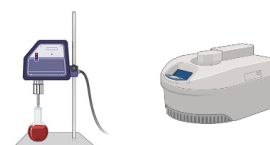
SAMPLE PREPARATION



BLACK RICE BRAN

- Milling
- Standardization (20- 32 mesh)

CHAPTER 4: UAE & MAE



OPTIMIZATION ANTHOCYANIN RECOVERY

Box-Behnken design



UAE

- Temperature
- Solvent
- Frequency power

MAE

- Temperature
- Solvent
- Sample mass

CHARACTERIZATION OF ANTHOCYANIN-RICH EXTRACTS

- % recovered (AR)
- Total monomeric anthocyanin (TMA)
- Total phenolic content (TPC)
- Polymeric color (PC)
- Antioxidant activity (AA)
- Individual anthocyanin

STABILITY ASSAY

LIGHT

- Incandescent



TEMPERATURE

- 65, 80, and 95 °C



CHARACTERIZATION OF ANTHOCYANIN-RICH EXTRACTS

- % recovered (AR)
- Total monomeric anthocyanin (TMA)
- Total phenolic content (TPC)
- Polymeric color (PC)
- Antioxidant activity (AA)
- Individual anthocyanin

STABILITY ASSAY

TEMPERATURE

- 65, 80, and 95 °C



LIGHT

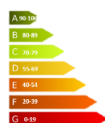
- Incandescent

CELL CULTURE

- Cytotoxicity (L929 cells)
- H₂O₂-Induced Oxidative Stress in L929
- Antitumoral activity

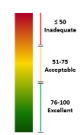


GREEN METRIC TOOLS



GREEN CERTIFICATE

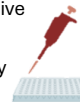
ECOSCALE



GREEN METRIC TOOLS

CELL CULTURE

- Cytotoxicity (L929 cells)
- H₂O₂-Induced Oxidative Stress in L929
- Antitumoral activity

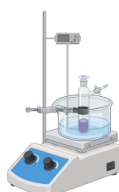


CHAPTER 5: ANTHOCYANIN ACYLATION (extract from UAE, chapter 4)

OPTIMIZATION ANTHOCYANIN ACYLATION

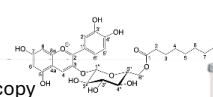
PARAMETERS

- Solvent
- Temperature
- Acyl donor
- Enzyme concentration



IDENTIFICATION ACYLATED COMPOUND

- HPLC-DAD
- HPLC-MS
- NMR spectroscopy



LIPOPHILICITY

- Partition coefficient determination

THERMOESTABILITY ASSAY

TEMPERATURE

- 65, 80, and 95 °C

pH

- 3, 5, and 7

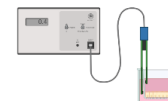


GREEN METRIC TOOLS

- Green Certificate & EcoScale

CELL CULTURE

- Cytotoxicity
- Transepithelial Absorption Experiments



PLE: Pressurized-liquid extraction, UAE: Ultrasound-assisted extraction, MAE: Microwave-assisted extraction.

1 INTRODUCTION

Anthocyanins are naturally found in fruits, vegetables, flowers, cereals, roots, grains, and tubers (ARRUDA et al., 2021; TENA; MARTÍN; ASUERO, 2020). They are considered water-soluble natural dyes belonging to the group of flavonoids, which have bioactive properties due to their antioxidant properties (LUZARDO-OCAMPO et al., 2021; TARONE; CAZARIN; MAROSTICA JUNIOR, 2020). The intake of foods rich in anthocyanins is related to health benefits, having anti-obesity, anti-diabetic, antimicrobial, anticancer, neuroprotective, and cardiovascular disease effects (TENA; MARTÍN; ASUERO, 2020).

According to Meticulous Research (2020), the natural colorants market will reach the \$3.2 billion mark in 2027, with a compound annual growth rate of 8.4% from 2019 to 2027. Among these colors, anthocyanins are gaining more attention from the population, mainly due to the concern about using artificial ingredients (CASTAÑEDA-OVANDO et al., 2009).

Black rice is known to be rich in anthocyanins, and the product's coloring is due to this compound's presence (MOIRANGTHEM et al., 2020). In addition, this type of rice is known for the presence of nutritional compounds interesting to human health, such as tocopherols, tocotrienols, oryzanol, vitamin B complex, and other phenolic compounds (THAKUR et al., 2022).

In Brazil, black rice began to be studied in 1994, and in the 1996-1997 harvest, a strain showed agronomic stability and great productive potential (ITO; LACERDA, 2019). With the increased demand for the product, more by-products and waste are generated during its processing (FERNANDES et al., 2021). The extraction of phenolic compounds, especially anthocyanins, may be a viable alternative for reusing this material generated during the processing of black rice. However, using solvents to extract bioactive compounds in a conventional way can be long, costly, and still have low efficiency (HALEE et al., 2020). Therefore, alternative technologies may be an interesting alternative to recover anthocyanins.

Microwave-assisted extraction was the most widely used among the emerging technologies for extracting anthocyanins from black rice (ABDEL-AAL et al., 2014; HALEE et al., 2020; JHA; DAS; DEKA, 2017; MOIRANGTHEM et al., 2021) followed by ultrasound-assisted extraction (JHA; DAS; DEKA, 2017; THAKUR et al., 2022). However, these technologies have been little explored in literature, although they have great potential.

Although anthocyanin-rich extracts present great potential for application, they offer low stability mainly due to their sensitivity to temperature, pH, light, oxygen, and other substances (enzymes, pigments, and metal ions) (CASTAÑEDA-OVANDO et al., 2009;

YANG et al., 2020a). Due to the high water solubility, there is a limitation of use in lipophilic matrices (CRUZ et al., 2015; YANG et al., 2018). Therefore, for anthocyanins to be widely used, it is necessary to compensate for these deficiencies to maintain their coloration and health-giving properties (YANG et al., 2020a).

Some methods were used to maintain the coloration of the anthocyanins: spray-dryer, freeze-drying, encapsulation, copigmentation, and acylation (CAI et al., 2022; HUANG et al., 2021; TAN et al., 2021; TARONE; CAZARIN; MAROSTICA JUNIOR, 2020). In addition to these methods, the acylation of anthocyanins has become a promising process to increase the stability of these compounds (LUO et al., 2017; ZHAO et al., 2017a), since studies have reported that acylated anthocyanins show greater stability than non-acylated ones (FEI et al., 2021; FENGER et al., 2019; GAMAGE; LIM; CHOO, 2021; GRAS et al., 2018; PRIETTO et al., 2017; XU et al., 2015). Acylation occurs by the esterification of an organic acid to the glycoside of the anthocyanin molecule, which can be classified as aromatic or aliphatic, depending on the presence of an acid group (aromatic or aliphatic) as the acyl group (CHATHAM; PAULSMEYER; JUVIK, 2019; GAMAGE; LIM; CHOO, 2021).

Both chemical and enzymatic acylation were investigated, with chemical acylation not recommended due to low regioselectivity, yielding several products that make purification difficult (CRUZ et al., 2018; GRAJEDA-IGLESIAS et al., 2017). In contrast, enzymatic acylation has high regioselectivity and moderate reactions, being considered the preferred method for the production of acylated flavonoid derivatives (LUO et al., 2013). Recent studies have shown that enzymatic acylation is easily accomplished and significantly increases the stability of anthocyanins (TU et al., 2019; YAN et al., 2016; ZHANG et al., 2021).

Thus, this study proposes to investigate the recovery of anthocyanins from black rice bran using emerging technologies, these never being used (such as PLE) or little explored (such as UAE and MAE), and evaluate enzymatic acylation of the recovered anthocyanins to improve their stability, a technique still little explored.

1.1 OBJECTIVES

The general objective of this work is to recover anthocyanins from black rice bran (BRB) and increase their stability through enzymatic acylation.

To achieve this objective, the following steps, which can be seen as specific objectives, will be carried out:

- Optimize the recovery process of anthocyanins using pressurized liquid extraction (PLE), ultrasonic-assisted extraction (UAE), and microwave-assisted extraction (MAE);
- Evaluate the polymeric color, antioxidant activity, and total phenolic compounds present in the extract obtained at optimized process conditions;
- Investigate the thermostability of the extracts at different temperatures (65, 80, and 95 °C) and by fluorescent light;
- Evaluate the cytotoxicity, oxidative stress (by H₂O₂) and antitumoral activity of the extracts;
- Optimize enzymatic acylation using fatty acids as acyl groups;
- Evaluate enzymatic acylation by HPLC-DAD, HPLC-MS, and NMR techniques;
- Evaluate the thermostability and partition coefficient of acylated and non-acylated compounds;
- Investigate the cytotoxicity and transepithelial transport of acylated and non-acylated compounds.

2 LITERATURE REVIEW

This chapter presents a review of the literature with emphasis on the extraction of anthocyanins from black rice and its by-products and enzymatic acylation as an approach to stabilize anthocyanins, based on an overview of the development of this area in recent years. Part of the information contained in this chapter was recently published as scientific articles entitled "*Black rice and its by-products: anthocyanin-rich extracts and their biological potential*" (doi: 10.1080/10408398.2023.2211169) and "*A review on enzymatic acylation as a promising opportunity to stabilizing anthocyanins*" (doi: 10.1080/10408398.2022.2041541), both in the journal Critical Reviews in Food Science and Nutrition (Qualis A1; Impact Factor 7.3).

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REVIEW ARTICLE



Black rice and its by-products: anthocyanin-rich extracts and their biological potential

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CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
<https://doi.org/10.1080/10408398.2022.2041541>



REVIEW



A review on enzymatic acylation as a promising opportunity to stabilizing anthocyanins

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2.1 INTRODUCTION

During the entire life cycle of food, some parts of the products can be wasted, generating residues or by-products. The highest food waste is produced by household activities (42%) and by industrial processing (39%) (KUMAR et al., 2017). Agro-industrial by-products are usually used for animal feed or as fertilizers. However, this material generated by industrial food processing is a rich source of bioactive compounds (ARRUDA et al., 2021; KUMAR et al., 2017).

Several value-added molecules can be obtained from food by-products, such as anthocyanins. These compounds have attracted the attention of the general population, researchers, and industry due to their technological application characteristics and biological properties (ARRUDA et al., 2021). Considering the circular economy concept, many recent studies have focused on using these plant residues as sources of natural pigments characteristic of anthocyanins (BENUCCI et al., 2022). Anthocyanins are compounds naturally found in many foods, such as fruits, vegetables, flowers, cereals, grains, roots, and tubers (ARRUDA et al., 2021; TENA; MARTÍN; ASUERO, 2020). In the food industry, these compounds are considered important because they can be used as water-soluble natural colorants. In addition, they belong to the flavonoid group, which has bioactive properties due to their antioxidant properties (LUZARDO-OCAMPO et al., 2021; TARONE; CAZARIN; MAROSTICA JUNIOR, 2020). The intake of foods rich in anthocyanins is related to health benefits, having anti-obesity, antidiabetic, antimicrobial, anticancer, neuroprotective, and anti-cardiovascular disease effects (TENA; MARTÍN; ASUERO, 2020).

Due to the concern for synthetic ingredients, anthocyanins have been gaining more attention regarding their use as natural dyes (CASTAÑEDA-OVANDO et al., 2009). According to Meticulous Research (2020), the natural dyes market will reach the mark of 3.2 billion dollars in 2027, with a compound annual growth rate (CAGR) of 8.4% from 2019 to 2027.

The color of black rice is due to the presence of anthocyanins (MOIRANGTHEM et al., 2020; PRATIWI; PURWESTRI, 2017). In addition, this type of rice is known for the presence of nutritional compounds interesting to human health, such as tocopherols, tocotrienols, oryzanol, vitamin B complex, and other phenolic compounds (Dias et al., 2017; Poonia & Pandey, 2022; Thakur et al., 2022). Black rice has been increasingly consumed as a functional food due to its beneficial health effects (POONIA; PANDEY, 2022). Except for white and red, all varieties of the colored pericarp are generally, considered black rice

(POONIA; PANDEY, 2022). Furthermore, according to Ito & Lacerda (2019), black rice is also known as imperial rice and forbidden rice (for historical reasons), purple rice, black japonicak rice, Chinese black rice, Thai black rice, and Indonesian black rice.

Black rice is mainly produced in Asian countries (SANGMA; PARAMESHWARI, 2023). With the higher demand for the product, consequently, higher production of by-products and waste are generated during its processing (FERNANDES et al., 2021). The extraction of phenolic compounds (mainly anthocyanins) from the residue of black rice processing can be a viable alternative for reuse. However, conventional solvent extraction of antioxidant compounds can be long, expensive, and still have low efficiency (HALEE et al., 2020).

Based on these aspects, the relevance of the subject, and the lack found in literature, a systematic study that summarizes the extraction of black rice and its by-products were carried out. In addition, based on the bioactive potential of these extracts (mainly anthocyanins), a study focusing on enzymatic acylation was also performed.

2.2 BLACK RICE (BR) COMPOSITION

The largest producing region of black rice is Asia, especially China, India, Indonesia, and Sri Lanka (PRATIWI; PURWESTRI, 2017; SANGMA; PARAMESHWARI, 2023). According to Zelenskaya et al. (2018), black rice is produced in Africa, America, Australia, and Southern Europe in addition to Asia. Although 90% of black rice production is in Asia, some studies showed the expansion of this product. Studies using black rice grown in different countries were reported in the literature: USA (HIEMORI; KOH; MITCHELL, 2009; LAOKULDILOK et al., 2011), France (PEREIRA-CARO et al., 2013); Italy (MELINI et al., 2019), Russia (TUMANIAN et al., 2020; ZELENSKAYA et al., 2018), Brazil (DITTGEN et al., 2019; FERNANDES et al., 2021), and Australia (CALLCOTT et al., 2018a).

More than 200 black rice varieties are found worldwide (PRATIWI; PURWESTRI, 2017). Although white (unpigmented) rice is the most consumed worldwide, interest in pigmented rice, such as red, purple, and black, has increased in recent years (PITIJA et al., 2013; YAMUANGMORN; PROM-U-THAI, 2021). The rice color comes from the pigmentation of the rice caryopsis in the pericarp and/or sclerenchyma, associated with antioxidant activity, as phenolic compounds are usually the main ones responsible for the different colors (PITIJA et al., 2013; TUMANIAN et al., 2020). In addition to these phenolic compounds, pigmented rice is a source of fat-soluble bioactive compounds such as c-oryzanols, vitamin E isomers, and carotenoids (VENI, 2019).

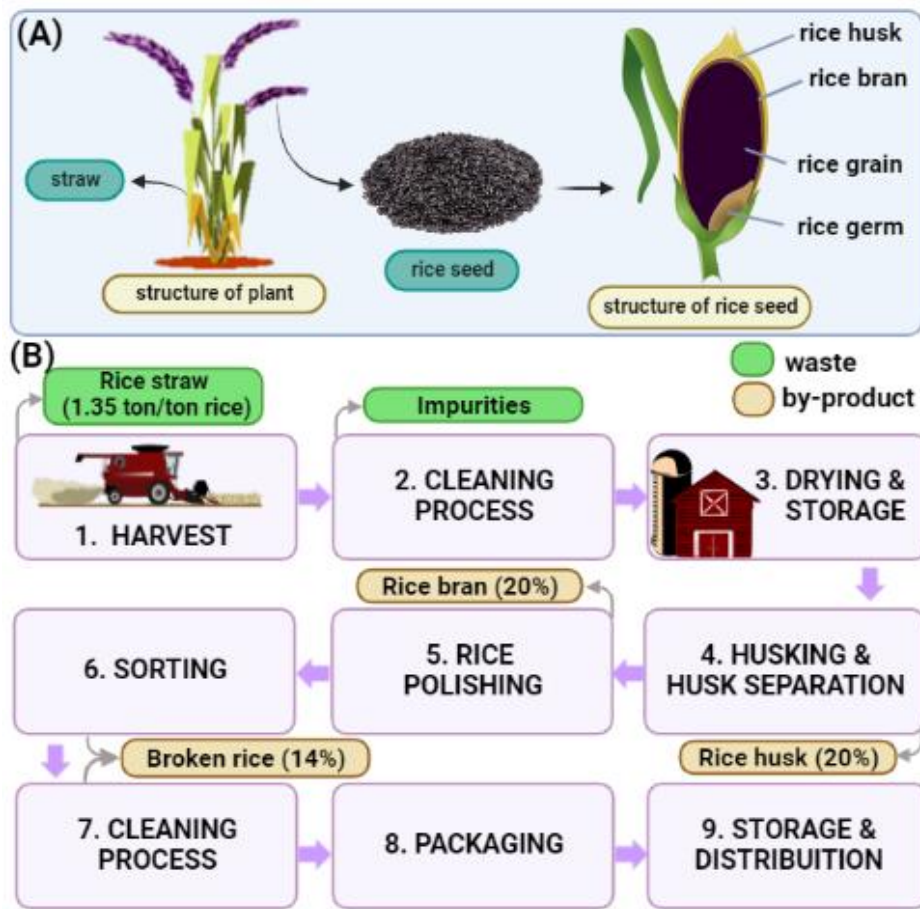
Among the compounds in black rice, anthocyanins are usually the main ones, about 90% being cyanidin-3-glucoside (C3G) and small traces of peonidin-3-glucoside (P3G), cyanidin-3-rutinoside (C3R), and malvidin-3-glucoside (M3G) (ITO; LACERDA, 2019). Black rice anthocyanins were reported with anti-inflammatory activity (FABRONI et al., 2016), antidiabetes by inhibiting the α -amylase activity and α -glucosidase (reduced risk of type 2 diabetes) (JI et al., 2021; SUI; ZHANG; ZHOU, 2016), protection against liver fibrosis (JIANG et al., 2015), and antimetastatic properties (CHEN et al., 2015; FAN et al., 2015). In addition, black rice presents other phenolic compounds (ferulic, coumaric and caffeic acids) related to low postprandial blood glucose response (ITO; LACERDA, 2019). The compound c-oryzanol (unsaponifiable lipid) showed anti-adipogenic activity (decreased lipid accumulation) (MINATEL et al., 2016). Studies focusing on the bioactive compounds in black rice were reviewed by Dias et al. (2017), Ito & Lacerda (2019), and Poonia & Pandey (2022); in our overview will be focus on anthocyanins as the main bioactive from black rice.

The chemical and proximate composition of black rice depends on several factors, mainly the different varieties and the environment conditions (climate, soil, management). In general, the proximate composition of black rice for moisture ranged from 9-13%, carbohydrates from 70-78%, lipids from 2-3%, and protein varied from 8-10% (AMRINOLA et al., 2021; FATCHIYAH et al., 2020; ITO; LACERDA, 2019; MELINI et al., 2019; SOMPONG et al., 2011; VENI, 2019).

2.3 BLACK RICE PROCESSING

Figure 2.1A shows the structure containing the rice plant parts and the grain. Figure 1B shows the processing of rice, suggested by Dias et al. (2011) and Moraes et al. (2014), with adaptations. Rice processing can be summarized in steps: harvesting, pre-cleaning, drying, shelling, milling/polishing, and finally, selection and classification. The main solid wastes generated in this process include straw, husk, bran, and broken rice. In rice harvest, about 1.35 tons of straw is left in the field for each ton of grain harvested (MORAES et al., 2014). During black rice processing, about 20% of rice husk, 10% in rice bran, and 14% in broken rice are produced (MORAES et al., 2014; NAGRALE; HAJARE; MODAK, 2012; VAN HOED et al., 2006). Depending on the process and its efficiency, bran and broken rice can be generated in different ratios (MORAES et al., 2014).

Figure 2.1 (A) Black rice plant and grain structure. (B) Black rice processing steps, adapted from Dias et al. (2011) and Moraes et al. (2014). Created with BioRender.com.



Aforementioned, black rice is a source of bioactive compounds. Its color refers to anthocyanins present in the grain, more than 90% being C3G (DIAS et al., 2017; HOU et al., 2013; LEE, 2010). In addition, by-products from rice processing (mainly bran and husk) also had a high concentration of anthocyanins (DAS; GOUD; DAS, 2018; FERNANDES et al., 2021; HALEE et al., 2020; HUANG; LAI, 2016; JHA; DAS; DEKA, 2017). However, much of this material is usually for animal nutrition (DAS; GOUD; DAS, 2018; HALEE et al., 2020; PEANPARKDEE; IWAMOTO, 2019).

The extraction of bioactive compounds can be a way to add value to black rice by-product (HALEE et al., 2020). The extraction of bioactive compounds from natural sources (e.g., anthocyanin-rich extracts) depends on the method, and process conditions applied (TEIXEIRA et al., 2021). The following topic summarizes the extractions of anthocyanins from black rice and their residues and by-products.

2.4 ANTHOCYANIN EXTRACTION FROM BR

Anthocyanins are sensitive compounds, being a challenge to extract, analyze and storage due to their low stability to temperature, light, pH, and solvent (THAKUR et al., 2022). Therefore, the choice of the extraction method (preferably eco-friendly methods), process conditions, and purification steps (free of harmful solvents) are essential to recovery them. Hence, toxicity analyses are necessary to highlight any risks for food or pharmaceutical purposes. Regulatory agencies and society also require effective sustainability standards for the process to be environmentally friendly (ARRUDA et al., 2021; FERNANDES et al., 2021).

In May (2022), a search was carried out using the keywords "anthocyanin" AND "black rice" on the SCOPUS database. The search was limited to research articles and reviews in the English language. A total of 347 documents were found. In Figure 3.2A, the increase in research on black rice with anthocyanins is observed, being more expressive in recent years, and in 2022, 13 studies have already been published.

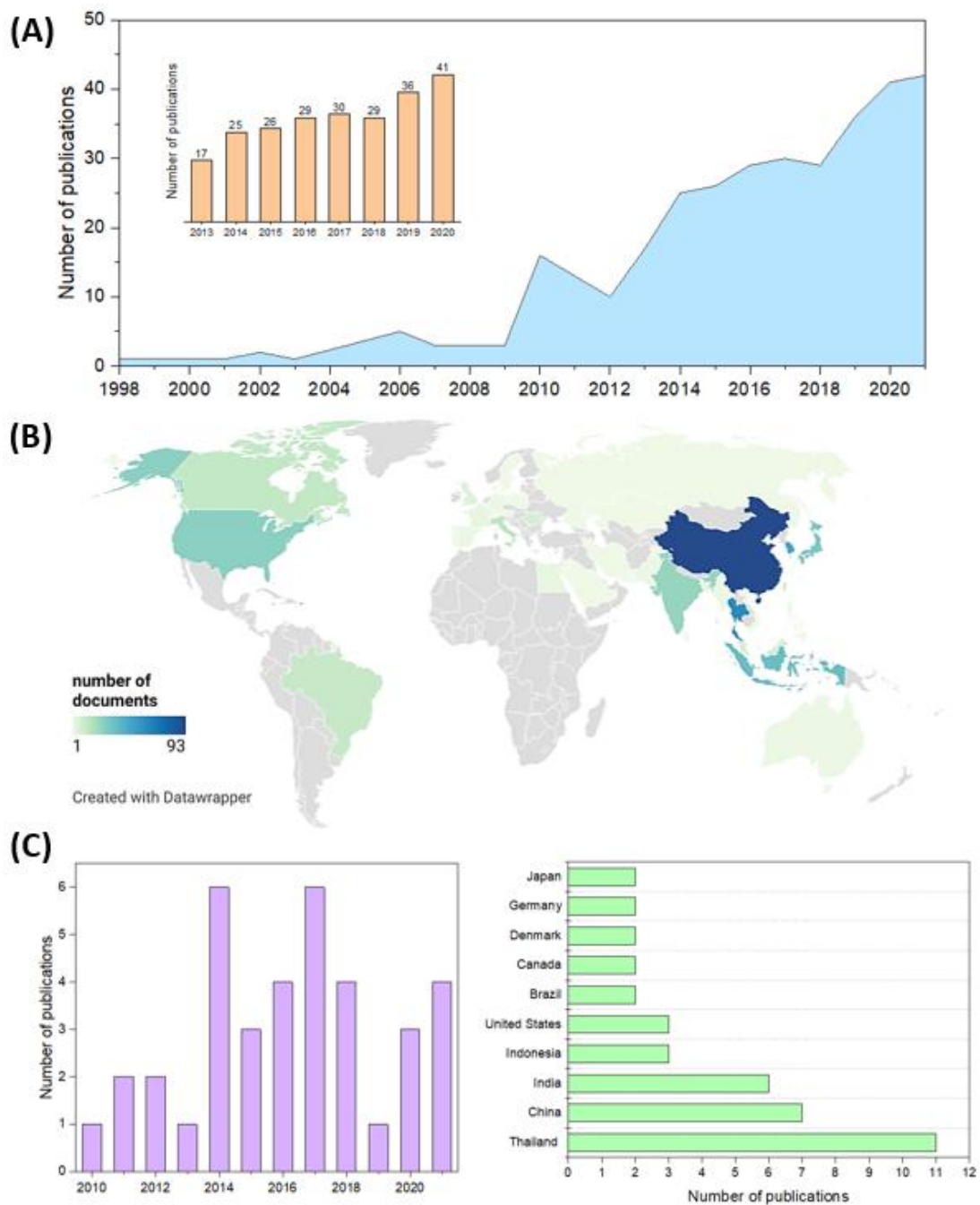
Figure 2.2B shows the main countries responsible for these publications. China is responsible for publishing 93 documents, followed by Thailand (61), South Korea (47), Indonesia (36), and Japan (31). The countries in America that presented the most documents were the United States (27 documents), Canada (13 documents), and Brazil (12 documents). The countries that stood out in Europe were Italy (14 documents) and the United Kingdom (6 documents). It can be observed that the studies of black rice are carried out mainly by the Asian regions, in which the production of this product is higher when compared to the other continents. America also presented many documents, with three countries among the ten producing the most studies. The use of grain is still a focus in most of these studies. However, there is a greater need for studies to focus on grain processing by-products to encourage biorefinery.

A new search was carried out in the SCOPUS database about recovering anthocyanins from black rice to evaluate the studies that extracted these compounds from the product or its by-products and/or residues. The keywords "anthocyanin" AND "black rice" AND "extraction" were used, limited to articles and reviews in English. It was observed that there is a slow increase in studies on the extraction of anthocyanins from black rice, with Asian countries being the largest responsible for these, followed by the countries of America (Figure 2.2C)

Forty-one studies with the keywords mentioned above were found. All that presented extraction of anthocyanins from black rice and its by-products as their objective were presented in Tables 2.1 and 2.2, respectively. Conventional extraction methods commonly used for the

recovery of bioactive compounds are maceration, solid-liquid extraction (SLE) or Soxhlet extraction, and liquid-liquid extraction (LLE) (ALARA; ABDURAHMAN; UKAEGBU, 2021; BENVENUTTI; ZIELINSKI; FERREIRA, 2021).

Figure 2.2 (A) Number of documents and (B) main countries found using the keywords "anthocyanin" AND "black rice" in the SCOPUS database. Created with datawrapper.de (C) Number of documents and main countries found using the keywords "anthocyanin" AND "black rice" AND "extraction" in the SCOPUS database.



The conventional extraction method (maceration) was the most used method to recover anthocyanins from black rice and its by-products and residues, as shown in Tables 2.1 and 2.2. Although this method has been the most used compared to emerging technologies, it is known that they have low efficiency, spend more time, and consumes more solvents (HALEE et al., 2020). Among the emerging methods, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) were the most used, which are better explained in the following topics. Figure 2.3 shows all the alternative methods used to extract anthocyanins from black rice. Among the alternative methods used, as seen in Figure 2.3, the immobilized enzyme (D) and acid hydrolysis (E) used only the rice grain to extract anthocyanins. The microwave-assisted extraction (B) used rice and all the by-products from the rice processing. The ultrasound-assisted extraction (A) and PEG solution (C) methods also used rice processing by-products as raw material to perform the extraction of bioactive compounds.

Table 2.1 Extraction methods, process parameters, and the main results obtained in the extracting anthocyanins from black rice.

Extraction	Solvent	Process parameters	Main anthocyanins	Reference
Maceration	3% aqueous trifluoroacetic acid (TFA)	Shaking at 5 °C for 24 hours	Cyanidin 3-glucoside (94.0%) Peonidin 3-glucoside	Ichikawa et al. (2001)
Maceration	Methanol containing 1% HCl	Overnight room temperature	Total anthocyanin 31.3 mg/g Cyanidin 3-glucoside (97.9%) Peonidin 3-glucoside	Zawistowski et al. (2009)
Maceration	A mixture of 3 mL methanol and 1 M hydrochloric acid (85:15, v/v)	Under rotation at 40 rpm for 30 min and subsequently centrifuged (repeated twice)	Cyanidin-3-glucoside: 0.2-1.4 mg/g Peonidin-3-glucoside: 0.1-0.19 mg/g	Frank et al. (2012)
Accelerated solvent extraction (ASE) Microwave-assisted extraction (MAE)	Acetone:water (40:60)	ASE: 50 °C, 2500 psi, 10 min using 5 cycles, and 100% flush MAE: 50 °C, 1200 W, and 20 min	ASE: 2.4 mg/g Cyanidin 3-glucoside (87.9%) Peonidin 3-glucoside (4.9%) Cyanidin 3-rutinoside (1.1%) MAE: 2.0 mg/g Cyanidin 3-glucoside (88.1%) Peonidin 3-glucoside (6.0%) Cyanidin 3-rutinoside (1.2%)	Abdel-Aal et al. (2014)
Maceration	Solid/liquid ratio of 1:10 (w/v): acidified water, 70% methanol, acidified 70% methanol, 70% ethanol, acidified 70% ethanol, 70% acetone, and acidified 70% acetone	2 h at 25 °C in a shaking water bath	Acidified 70% (v/v) methanol (pH 1.0) resulted in the highest anthocyanin extract: 461.72 mg/L	Kang et al. (2014)
Maceration (M1) Acid hydrolysis (M2)	M1: extracted twice with 95 g/100 g ethanol, at a ratio of 1:3 (w/v) M2: 1.5 N HCl in 95 g/100 g ethanol at a ratio of 1:3 (w/v)	1 h at 25 °C in a mechanical shaker	The best result for Kum variety (total anthocyanins) M1 ≈ 221 mg/g and M2 ≈ 449 mg/g	Maisuthisakul & Changchub (2014)
Maceration	0.1 N HCl in methanol	-	Cyanidin 3-glucoside (5.69 mg/g) Peonidin 3-glucoside (11.46 mg/g) Cyanidin chloride (12.60 mg/g)	Pengkumsri et al. (2015)
Maceration	Ethanol and citric acid 1.0 mol/L in the ratio of 80:20	Temperatures of 10, 30 and 50 °C, and extraction times of 20, 50, and 80 min with continuous stirring	The optimal condition (1.16 mg/ g of anthocyanins): extraction at 34.7 °C for 80 min using a solid:solvent ratio of 1:30: extract	Pedro et al. (2016)

Maceration	pH values varying from 2.0 to 6.2 prepared with 0.2 M disodium hydrogen phosphate and 0.1 M citric acid	40-60 °C for 60-100 min	The optimal condition: liquid-solid ratio 12:1, temperature 50 °C, time 80 min and pH 3.2 with extraction ratio $0.99 \pm 0.01\%$	He et al. (2017)
Maceration	Methanol-HCl 0.1% (v/v) at a ratio 1:10 (w/v)	100 rpm for an hour	1.05 mg eq/g	Raharjo et al. (2019)
Maceration	Methanol	Samples were vortexed for 30 s followed by incubation at 60 °C for 20 min (samples vortexed twice during the incubation)	1.72 mg eq/g	Moirangthem et al. (2021)
Enzyme-immobilized nanobiocatalysts extraction	-	20-70 °C for 30-180 min at pH 4.5-7.0	The optimal condition (anthocyanin content 2.66 mg/g): enzyme-immobilized, 30 °C with pH 5.0-5.5	Yi et al. (2021)

2.4.1 Conventional extraction (CE) methods

Conventional methods are those techniques that have been used for a long time, generally based on solvent or heat extraction or their combination (SAGAR et al., 2018). Among the conventional techniques for extracting bioactive compounds, the main ones are (a) Soxhlet extraction, (b) hydro-distillation, (c) and maceration (SAGAR et al., 2018; SILVA et al., 2017a; ZIELINSKI et al., 2021).

The Soxhlet extraction takes place in the “Soxhlet apparatus”, in which cyclic and continuous repetitions of the extraction are carried out with the solvent mixed with the solid powder for a determined time (TENA; ASUERO, 2022). This type of extraction is generally applied for extracting oils and other phenolic compounds, although it is rarely used for extracting anthocyanins (KHANH, 2015). Hydro-distillation involves three physicochemical processes: hydro-diffusion, hydrolysis, and decomposition by heat. These processes are not advisable for extracting thermolabile compounds since the high temperatures lead to degradation (SAGAR et al., 2018).

The maceration technique is widely used in extraction because it is simple, mixing the sample with solvent at room temperature for a specific time (usually days) (KHODDAMI; WILKES; ROBERTS, 2013; LEZOUL et al., 2020; TENA; ASUERO, 2022). Some studies used higher temperatures; however, below boiling (in this case, the extraction is called decoction) (LEZOUL et al., 2020; NAVIGLIO et al., 2019). In addition, occasional or frequent agitation can be applied (ALARA; ABDURAHMAN; UKAEGBU, 2021; SAGAR et al., 2018). It can be seen in Tables 2.1 and 2.2 that most studies use maceration as a strategy to extract anthocyanins from black rice.

From these Tables, one can also observe that over the years, higher temperatures were chosen and, consequently, shorter times in the extraction by the maceration method. This time reduction is necessary since emerging technologies have better extraction results in much faster times. Although maceration still presents more studies, it can be observed that more studies are using alternative methods for anthocyanin recovery (mainly MAE and UAE), optimizing this process to obtain more compounds at less cost.

Table 2.2 Extraction methods, parameters, and results obtained in extracting anthocyanins from black rice by-products and waste.

Extraction method	Solvents	Parameters	Anthocyanin	Reference
Maceration (Rice Bran)	Methanol acidified with 1.0 N HCl (85:15 v/v)	Shaking at 4 °C for 24 h	Total anthocyanin 1.13-2.56 mg/g Cyanidin 3-glucoside (58-95%) Peonidin 3-glucoside	Laokuldilok et al. (2011)
Rice Bran pre-treated with ohmic heating-assisted (OHM) and extracted by Maceration	Water:95% ethanol (1:1), and acidified with 0.1 M HCl (pH 2.5)	At 100 rpm for 90 min	Total anthocyanins: 10.8 mg/g Cyanidin 3-glucoside 7.7 mg/g (70.9%)	Loypimai et al. (2015)
Maceration (Rice Bran)	2 mL of 80% ethanol (cold)	10 minutes at room temperature with continuous stirring	Black rice Thailand: Cyanidin 3-glucoside (10.6 and 6.6 mg/g) Peonidin 3-glucoside (0.81 and 0.40 mg/g) Cyanidin 3-rutinoside (0.52 and 0.36 mg/g)	Huang & Lai (2016)
Ultrasound-assisted extraction (UAE) (Rice Bran)	20-60% ethanol (v/v)	30-60 °C for 10-60 min	The optimal condition for extraction (31.9 mg/L of anthocyanin): 23.78% (v/v) ethanol concentration, 35.97 °C temperature, 22.84 min, and pH 2.52	Das et al. (2017)
Microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) (Rice Husk)	40-70% ethanol (v/v)	UAE: working frequency 35 kHz, and power frequency 160/640 W MAE: constant frequency 2450 MHz	The optimal condition (anthocyanin content 0.03 mg/g): 10.02 min sonication time, 49.46 °C, 1:40.79 (w/v) solute-solvent ratio, 67.34% ethanol concentration, for 31.11 s microwave time	Jha et al. (2017)
Maceration (Rice Bran)	25-100% ethanol or acetone (v/v)	30-60 °C for 10-60 min	The optimal condition (18.9 mg/L of anthocyanin): 43.74% (v/v) ethanol concentration, 37.5 °C temperature, 22.5 min, and 8.8 mL/g solvent to solid ratio	Das et al. (2018)
Maceration (Rice Bran)	Ethanol or methanol containing citric acid (0.05-0.1 M)	100 rpm for two hours at room temperature	The methanolic extract with 0.1 M. of citric acid provided the highest anthocyanin content: 6.18 mg/g	Halee et al. (2018)
MAE (Rice Bran)	Water containing citric acid (0.033-0.117 M)	Power energy from 298-800 watts for 13-147 seconds	The optimal condition of extraction (anthocyanins 18.82 mg/g): 648 watts of power energy, 0.076 M of citric acid, and 83 s	Halee et al. (2020)

Poly(ethylene glycol) (PEG) solution Broken Rice (BBR), Rice Bran (BRB), and Rice Husk (BRH)	PEG solution with 1.0 mol/L HCl (pH 1.0)	25-45 °C for 5-25h at 200 rpm	The optimal condition was time 10 h, PEG concentration 36.59% (m/m), and anthocyanin source mass 1.4 g (BBR: 1.3 mg/g, BRB: 8.8 mg/g, BRH: 1.48 mg/g)	Fernandes et al. (2021)
Maceration (MA) Microwave-assisted subcritical water extraction (MA-SWE)	MA: Methanol	MA: Samples were vortexed for 30 s followed by incubation at 60 °C for 20 min (vortexed twice) MA-SWE: room temperature for 2 min and then heated (temperatures 25–200 °C) for 5 min	MA: Bran (7.83 mg eq/g), Leaf (0.12 mg eq/g), Straw (0.63 mg ed/g) MA-SWE: Straw (maximum efficiency (85.8%) at 90 °C for 5 min): 0.54 mg eq/g	Moirangthem et al. (2021)
UAE using natural deep eutectic solvents (DES) (Rice Bran)	NADES: lactic acid:glucose (5:1) lactic acid:glycerol (1:1) lactic acid:fructose (5:1) fructose:glucose:sucrose (1:1:1) citric acid:fructose (1:1)	30 °C for 5-30 min	Lactic acid:fructose (anthocyanin ~30 mg/L): with time 11.25 min, water 12.63%, solvent-to-solid ratio 18.75 ml/g, amplitude 21.31%	Thakur et al. (2022)

2.4.2 Ultrasound-assisted extraction (UAE)

In food processing, ultrasonic waves can be divided into categories based on the difference in sound intensity and frequency, being an important index to determine the yield of reactions (BIMAKR et al., 2017; CHEMAT; ZILL-E-HUMA; KHAN, 2011; DING et al., 2021). High-frequency ultrasound (from 2 to 20 MHz) is used in food quality analysis, medical imaging, and non-destructive inspection. High-intensity ultrasound operating at lower frequencies (from 20 to 100 kHz) is used to improve the process of extracting bioactive compounds (BIMAKR et al., 2017; CHEMAT; ZILL-E-HUMA; KHAN, 2011).

Ultrasound-assisted extraction (UAE) is a research topic that affects several fields of modern plant-based chemistry. Many reported applications have shown that ultrasound-assisted extraction (Figure 2.3A) is a green and economically viable alternative to conventional food and natural product techniques (CHEMAT et al., 2017; CHEMAT; ZILL-E-HUMA; KHAN, 2011). It has several benefits, such as faster mass transfer, thus decreasing extraction time, low temperature, selective extraction, low solvent consumption, low installation cost, and ease during operation (CHEMAT et al., 2017; CHEMAT; ZILL-E-HUMA; KHAN, 2011; GALLO; FERRARA; NAVIGLIO, 2018). As a disadvantage, it presents inhomogeneous heating in the extraction (GEOW et al., 2021; LIANFU; ZELONG, 2008).

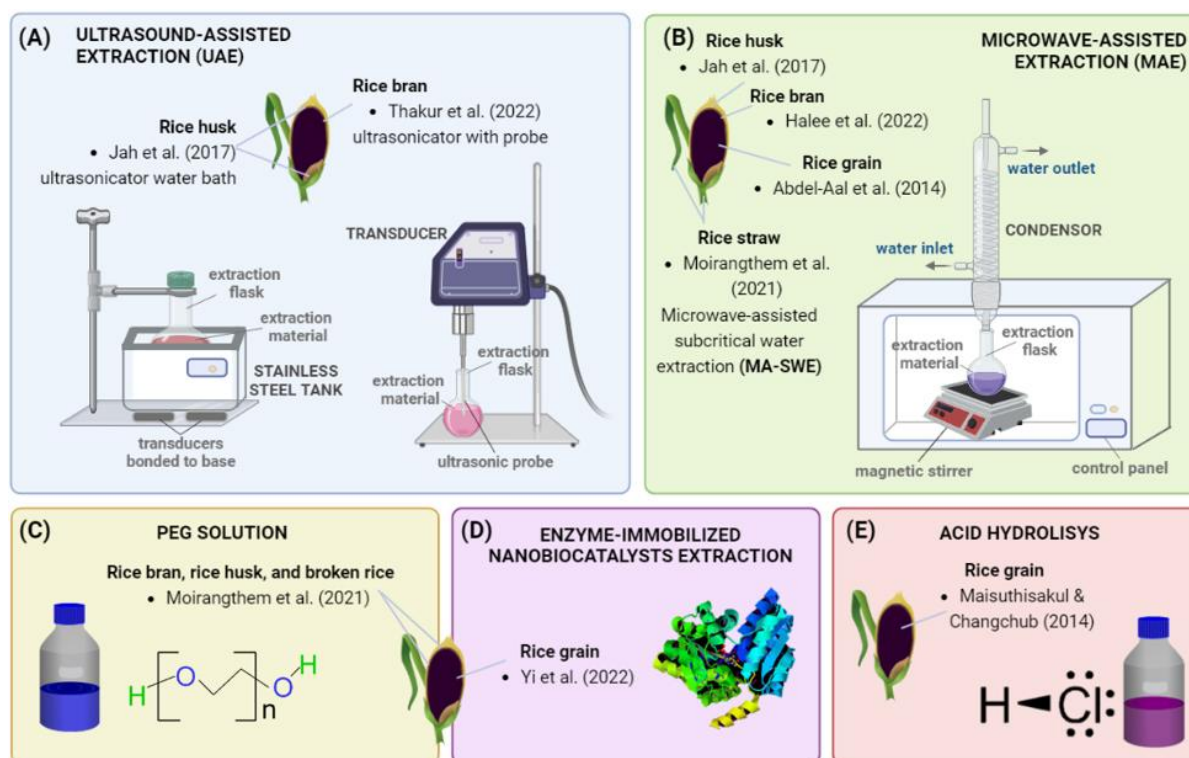
The frequency of ultrasound in the extraction is efficient due to the breakdown of the cell wall; the implosion of cavitation bubbles generates microturbulence, high-speed collisions between particles, and internal diffusion (PERERA; ALZAHRANI, 2021; SHIRSATH et al., 2017). Furthermore, the power of ultrasound agitates the extraction solvent, thus increasing the contact between the solvent and the target compounds, significantly improving the extraction efficiency (CHEN et al., 2007; TENA & ASUERO, 2022).

In the study by Das et al. (2017), the authors optimized anthocyanin extraction from black rice using UAE (ultrasonic water bath, Bandelin Sonores). While for UAE, the recovery of anthocyanins was 30.40 mg/L and 35.56 mg/L for black and purple rice, respectively, for the conventional method, it was 18.75 mg/L and 21.82 mg/L for black and purple rice, respectively.

Thakur et al. (2022) used, combined with ultrasound extraction (ultrasonicator with the probe, Model UW2070; Bandelin electronic, Germany), natural deep eutectic solvents (NADES), considered green solvents according to the authors. Among the combinations of NADES used (glucose (5:1), lactic acid:glycerol (1:1), lactic acid:fructose (5:1), fructose:glucose:sucrose (1:1:1), and citric acid:fructose (1:1)), lactic acid:fructose led to the most promising result, as shown in Table 2.2. The authors also performed a kinetics study using the optimized parameters (Table 2.2) in a conventional extraction method with NADES

and the UAE method with NADES. It was observed that in both ways, about 15 minutes was enough to extract the anthocyanins; however, while the conventional method recovered about 70 mg/L, the UAE method extracted about 110 mg/L.

Figure 2.3 Alternative methods used to extract anthocyanins from black rice, (A) adapted from Alara et al. (2021), and (B) adapted from Chaturvedi (2018). Created with BioRender.com.



Ultrasound can be used both with a probe (THAKUR et al., 2022) and in a water bath (Figure 2.2A) (DAS; GOUD; DAS, 2017). It can be observed that when using a probe, even when using water in the extraction, superior results were obtained by Thakur et al. (2022) than when using a water bath in the study by Das et al. (2017). UAE using the probe have better results because it has greater mass transfer, produces more energy, and accelerates chemical reactions (WEN et al., 2018). In the case of using the UAE in a water bath, the ultrasound waves need to pass through the liquid and the wall of the container and only then reach the sample, consequently decreasing its intensity (KUMAR; SRIVASTAV; SHARANAGAT, 2021). Generally, the use of the probe is preferable for the extraction of bioactive compounds since, although an ultrasonic bath is cheaper and easier to use, it has low reproducibility, which restricts its use (KUMAR; SRIVASTAV; SHARANAGAT, 2021; WEN et al., 2018).

Although UAE has several advantages listed above, such as green and economically viable, easy to handle, moderate use of solvents, and reproducibility, filtration is also necessary

at the end of the process, which can cause degradation of the extracted compounds (CHEMAT et al., 2017; MEDINA-TORRES et al., 2017).

2.4.3 Microwave-assisted extraction (MAE)

MAE is an emerging technique developed to extract antioxidant compounds, pigments, aromas, essential oils, and other organic compounds from solid samples (ALVI; ASIF; IQBAL KHAN, 2022). The method requires less time, less solvent consumption, and a greater focus on thermolabile constituents (BAGADE; PATIL, 2021). Microwaves are non-ionizing electromagnetic waves within the range of 300 MHz to 300 GHz located between the radio-frequency field at the lowest frequency and infrared at the highest frequency of the electromagnetic spectrum (ROUTRAY; ORSAT, 2012). The effect of microwaves on the material occurs as follows: (a) heating of the internal water of the material leading to cell drying, (b) stretching of the cell structure due to increased internal pressure and temperature, and (c) disruption of the cell wall releasing the bioactive compounds (TENA; ASUERO, 2022).

MAE is classified as multi-mode and focused-mode (mono-mode) systems. As its name suggests, the focused system allows focused microwave radiation in a restricted zone in the cavity, while the multi-mode allows for random dispersion (CHAN et al., 2011). Figure 2.3B shows the MAE system (mono-mode). For the extraction to work by both methods (focused and multi-mode), it needs (a) a microwave generator (magnetron): responsible for generating energy, (b) a waveguide: responsible for the propagation of microwaves, and (c) the applicator: in which the sample is deposited, and (d) circulator: responsible for allowing the wave to move only forward (MANDAL; MOHAN; HEMALATHA, 2007; ROUTRAY; ORSAT, 2012). Generally, the focused mode is associated with the system employed under atmospheric pressure, while the multimode is related to high pressure (CHAN et al., 2011).

The use of MAE as an extraction method can be divided into categories (MAE for thermolabile substances, Microwave heating extraction, Green extraction without solvent, and Microwave extraction with no solvent pressure); according to the different proposals for using the method (e.g., Microwave-assisted extraction (NPMAE), vacuum microwave-assisted extraction (VMAE), focused microwave-assisted Soxhlet extraction (FMASE), ultrasonic microwave-assisted extraction (UMAE), and others) (TENA; ASUERO, 2022).

Four studies addressed this methodology in the case of anthocyanin extraction by the MAE method (ABDEL-AAL et al., 2014; HALEE et al., 2020; JHA; DAS; DEKA, 2017; MOIRANGTHEM et al., 2021). In the study by Abdel-Aal et al. (2014), the authors used the CEM model MARS-X equipment (CEM Corporation, Matthews, N.C., U.S.A.) to extract the

compounds, varying the temperature (50 and 70 °C), time (10 and 15 min) and power (300 and 1200 W). The authors obtained 2.0 mg/g of anthocyanins with the parameters presented in Table 1. This result showed similar efficacy to accelerated solvent extraction (ASE). The extraction method takes minutes by using high pressures and temperatures above the solvent's boiling point (AHMAD; AHMAD; SHEHZAD, 2020).

In the study performed by Jha et al. (2017), the authors used the UAE (Sonorex RK510H, Bandelin, Germany) followed by MAE (MC-8087ABR, LG Electronics, India) to obtain anthocyanins from black rice husk. The authors recovered 0.03 mg/g of anthocyanins from the husk of black rice (Table 2.2). This value is lower when compared to the rice bran obtained by Halee et al. (2020) in a microwave reactor, equal to 0.18 mg/g. The rice bran, being part of the grain, is richer in anthocyanins when compared to the husk, a result also reported by Fernandes et al. (2021). Moirangthem et al. (2021) used microwave-assisted subcritical water extraction (MA-SWE) (Monowave 300 microwave synthesis reactor, Anton Paar, Germany) to extract anthocyanin from black rice processing straw. Although the result obtained (53.9 mg eq/100 g) was inferior to the conventional method (Table 2.2), the time used was three times shorter. According to the studies, it is possible to observe that all by-products from the BR processing have considerable amounts of anthocyanins, especially bran.

MAE is considered helpful in extracting short-chain polyphenols (phenolic acids and flavonoids); however, in the case of polymeric polyphenols such as anthocyanins and tannins, it can lead to degradation due to heat sensitivity and also destroy polyphenols with several hydroxyl-type substituents (ALARA; ABDURAHMAN; UKAEGBU, 2021). Thus, for using MAE, the best types would be nitrogen-protected microwave-assisted extraction (NPMAE) and vacuum microwave-assisted extraction (VMAE), categorized as MAE for thermolabile substances, according to Tena & Asuero (2022).

2.4.4 Acid hydrolysis extraction, Enzyme-immobilized nanobiocatalysts extraction, and PEG solution extraction

Acid hydrolysis is a process commonly used to treat lignocellulosic materials, aiming at obtaining sugars for ethanol production (LOOW et al., 2016; RINALDI; SCHÜTH, 2009; SUN; CHENG, 2002; TAHERZADEH; KARIMI, 2007). In addition, this technique has been used for the production of levulinic acid (Kang et al., 2018) and lactic acid (LAOPAIBOON et al., 2010) and in the production of nanoparticles (HARUN et al., 2022). However, concentrated acids can be toxic, corrosive, and dangerous, and there is a high cost in acid recovery (AKHTAR et al., 2016; SUN; CHENG, 2002).

Maisuthisakul & Changchub (2014) used acid hydrolysis as an alternative method to extract anthocyanins from black rice. Although the authors obtained positive results (about 449 mg/g of anthocyanins), this method used a large amount of acid (1.5N HCl, in 95 g/100 g ethanol at a ratio of 1:3, w/v), which may have the problems mentioned above.

Another alternative method used for extraction was using immobilized enzymes (YI et al., 2021). Enzyme-assisted biomolecule extraction has been gaining attention due to its efficient, sustainable, and eco-friendly extraction, and this technology is a potential alternative to conventional solvent extraction methods (NADAR; RAO; RATHOD, 2018). The authors immobilized enzymes using magnetic nanoparticles to facilitate their application and enzyme recovery, having already verified the potential for juice clarification and starch hydrolysis (YI et al., 2021). The authors obtained 2.26 mg/g of anthocyanins from BR extraction, having the parameters presented in Table 1 as the optimal point.

It was also used as an alternative method of extracting anthocyanins from BR aqueous solutions of poly(ethylene glycol) (PEG) (FERNANDES et al., 2021). This solution is known for its low flammability, low cost, nontoxic, and involatile (MANSOUR; ZHOU; DANIELSON, 2015; ZHOU et al., 2011). The authors obtained high amounts of anthocyanins from all rice processing residues and by-products evaluated (Table 3.2), especially rice bran (8.83 mg/g) (FERNANDES et al., 2021).

The use of new emerging techniques may be viable alternatives to obtain these compounds since these are sustainable and help in the circular economy (POONIA; PANDEY, 2022). The leading green technologies that can be used are electric pulse-field assisted extraction, supercritical water extraction (SWE), and pressurized liquid extraction (PLE). It is also essential to highlight the use of biologically-based solvents (produced from biomass raw materials), as the use of emerging technologies (considered sustainable) must be combined with the availability of renewable solvents (CLARK et al., 2015; CLAUX et al., 2021).

However, anthocyanins are unstable compounds easily degraded by temperature and light, making their use in nutraceutical products difficult. Therefore, methods to increase the stability of these compounds can be used, highlighting encapsulation (TARONE; CAZARIN; MAROSTICA JUNIOR, 2020), copigmentation (CAI et al., 2022), acylation (LEONARSKI et al., 2022), and using protective agents (HUANG et al., 2021). Among these anthocyanin stabilization methods, acylation has recently been widely studied, especially enzymatic acylation, which has advantages over chemical acylation.

2.5 ACYLATION AS A PROMISING OPPORTUNITY TO STABILIZING ANTHOCYANINS

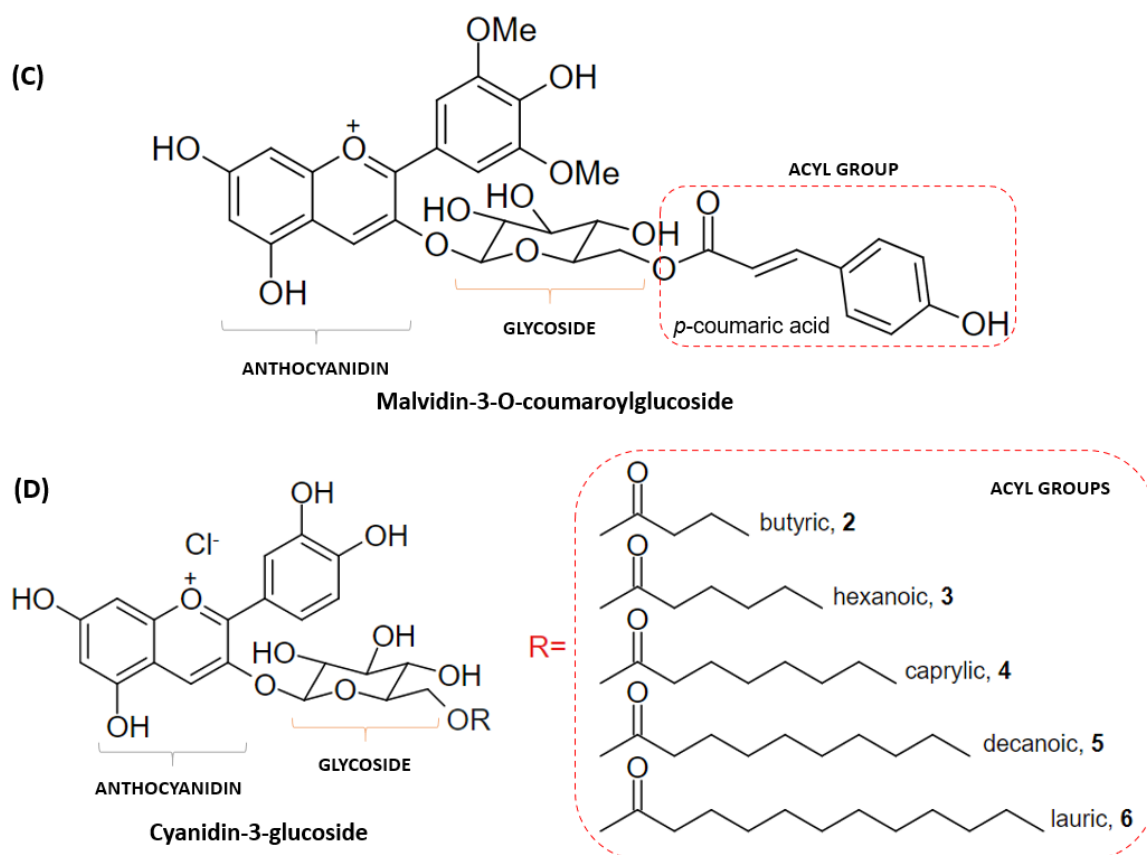
As already mentioned, there are some methods to maintain the anthocyanins coloration: spray-dryer, freeze-drying, encapsulation, copigmentation, and acylation (CAI et al., 2022; HUANG et al., 2021; TAN et al., 2021; TARONE; CAZARIN; MAROSTICA JUNIOR, 2020). In recent years, anthocyanins acylation has become a fundamental process to increase the stability of these compounds (Yang et al., 2018; Zhao et al., 2017). Several studies have reported that acylated anthocyanins show higher stability than non-acylated anthocyanins (FEI et al., 2021; FENGER et al., 2019; GAMAGE; LIM; CHOO, 2021; GRAS et al., 2018; PRIETTO et al., 2017; XU et al., 2015). Acylation occurs by esterifying an organic acid to the glycoside of the anthocyanin molecule, which can be classified as aromatic or aliphatic, depending on the presence of an aromatic (Figure 2.4A) or aliphatic acid group (Figure 2.4B) as the acyl group (CHATHAM; PAULSMEYER; JUVIK, 2019; GAMAGE; LIM; CHOO, 2021). Esterification is a way to improve this hydrophobic nature, and this acylation may be from natural sources, however, these modifications are not as common as glycosylation and generally do not cover long hydrophobic molecules (PLAZA et al., 2014). In addition, compared to non-acylated anthocyanin, in some cases, this acylation induced an increase in biological and compound properties, mainly due to the increased stability (ARAÚJO et al., 2017; GAMAGE; LIM; CHOO, 2021; MARQUEZ-RODRIGUEZ et al., 2021). A water-soluble compound is acylated by an esterification reaction with a fatty acid or fatty alcohol, known as lipophilization (MARQUEZ-RODRIGUEZ et al., 2021). This reaction can be accelerated using lipases, characterized as enzymatic lipophilization.

Both enzymatic and chemical acylation have already been investigated. The chemical acylation was not recommended due to low regioselectivity, obtaining several products which make purification difficult (CRUZ et al., 2018; GRAJEDA-IGLESIAS et al., 2017). In contrast, enzymatic acylation has high regioselectivity, and moderate reactions are considered the preferred method for producing acylated flavonoid derivatives (LUO et al., 2013). Recent studies have shown that enzymatic acylation is easily performed, in addition to significantly increasing the stability of anthocyanins (TU et al., 2019; YAN et al., 2016; ZHANG et al., 2021).

Furthermore, the acylation improves the biological properties of anthocyanins, as previously reported, favoring their application as natural colorants in food and pharmaceutical products, an alternative to artificial colors. Based on these aspects, the relevance of the subject,

and the lack found in the literature on a systematic study on state of the art and gaps for enzymatic acylation of anthocyanins, this review aims to report the use of this strategy to obtain natural colorants with better stability (temperature and light), and also to evaluate the antioxidant activity and lipophilicity of these acylated compounds.

Figure 2.4 Anthocyanin acylated with (A) aromatic and (B) aliphatic acids.



2.6 ANTHOCYANIN ENZYMATIC ACYLATION

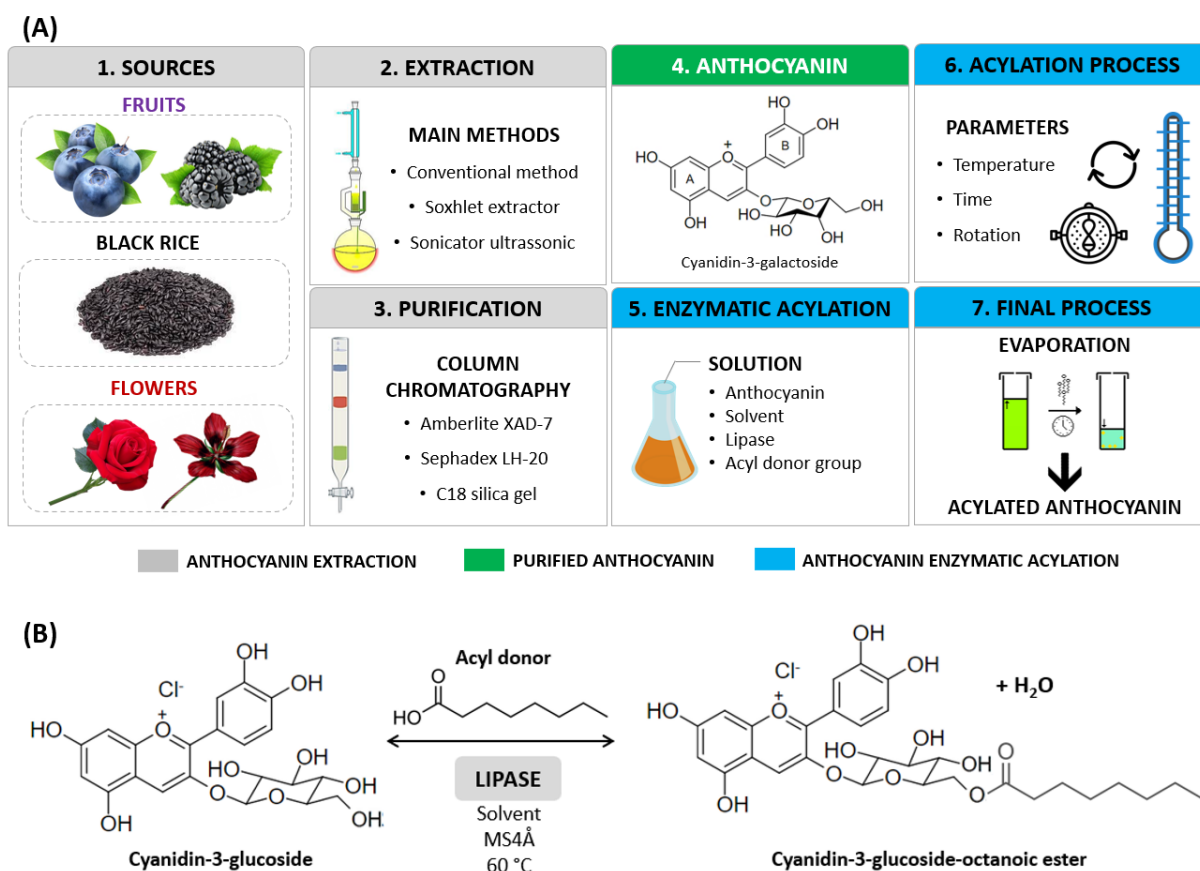
In recent years the enzymatic acylation of anthocyanins has emerged as a method to increase the stability of these compounds. Table 2.3 shows studies that performed enzymatic acylation of anthocyanins and the materials and parameters used in the process.

2.6.1 Source of Anthocyanins

It can be seen (Table 2.3) that several sources of anthocyanins are used in the studies, mostly coming from berries, fruit, and or skin/peel (73.3%). In addition, flowers (20%), black rice (13.3%), among others, were also used. Figure 3.5 summarizes the steps to carry out the enzymatic acylation of anthocyanins. A source of this pigment is needed (1), so extraction (2) and purification (3) are performed, obtaining purified anthocyanin (4). For the acylation process, the mixture (5) of previously purified anthocyanin with solvent, enzyme, and acyl

donor group is performed, after which it is submitted to the acylation process (Figure 2.5B) in which it is necessary to control some parameters (6): temperature, time and rotation. After the acylation process, evaporation (7) is carried out to obtain the acylated compounds, and, if necessary, another purification step can be carried out. Among all the studies, only three (LIU et al., 2020; LUO et al., 2017; ZHANG et al., 2021) did not extract anthocyanins because they used commercial extracts.

Figure 2.5 (A) A brief about the main steps to carry out the enzymatic acylation of anthocyanins, and (B) Schematic of anthocyanin enzymatic acylation reaction.



The extraction methods used were: (a) conventional using methanol (GUIMARÃES et al., 2018, 2020; SARI; SETIAWAN; SISWOYO, 2015), (b) Soxhlet using methanol (Castro et al., 2014), (c) sonication using methanol (FERNANDEZ-AULIS et al., 2020), (d) conventional using ethanol (YAN et al., 2016), (e) sonication using ethanol (Marquez-Rodriguez et al., 2021; Yang et al., 2018, 2019), (f) conventional with acidified water (CRUZ et al., 2018), conventional with acidified water and added with disodium hydrogen phosphate (Yang et al., 2020), and conventional using acetone (MARATHE et al., 2021). After extraction, some works carried out purification of these extracts, using Amberlite XAD-7 column (MARATHE et al., 2021; SARI; SETIAWAN; SISWOYO, 2015; YAN et al., 2016), Amberlite XAD-7HP column

(Cruz et al., 2018), column chromatography with reverse-phase C18 silica gel (GUIMARÃES et al., 2018, 2020), column packed with 25 g of Sephadex LH-20 (Yang et al., 2018, 2019), Sephadex LH-20 column chromatography using 0.1% of TFA (FERNANDEZ-AULIS et al., 2020), and column chromatography loaded with RP-C18 gel (MARQUEZ-RODRIGUEZ et al., 2021). The extracts containing the compounds must be acylated in high purity, so this step is crucial.

Among the anthocyanins studied, cyanidin was the most used for enzymatic acylation, except in Marquez-Rodriguez et al. (2021), where delphinidin was used, and peonidin was also used in one study. Cyanidin and delphinidin represent 50% and 12%, respectively, of the distribution of the six most common anthocyanidins (CASTAÑEDA-OVANDO et al., 2009).

Table 2.3 Source of anthocyanins, anthocyanin type, enzymes, acyl donor, solvents, parameters, and the products of enzymatic acylation.

Source ^{(reference)*}	Anthocyanin	Enzyme	Acyl donor	Solvents	Parameters	Acylated anthocyanin
Jaboticaba peel ^a	Delphinidin-3-glucoside Cyanidin-3-glucoside	Lipase CalB enzyme	Hexadecanoic acid	2-methyl-2-butanol (2M2B)	200 mbar at 50 °C/48 h and agitation at 600 pm	Delphinidin-3-glucoside monoester Cyanidin-3-glucoside monoester
Jambolan ^b	Native jambolana anthocyanins (not identified)	Lipase CalB enzyme	Cinnamic acid	Acetone and 10% dimethyl sulfoxide (DMSO)	40 °C/48 h at agitation	Acylated jambolana anthocyanin
Black rice ^c	Cyanidin-3-glucoside	6.67 g/L of lipase CalB enzyme	Methyl benzoate Methyl salicylate Methyl cinnamate	Pyridine	900 mbar at 40 °C/48 h and agitation at 30 pm	Cyanidin 3-(6"-benzoate)-glucoside Cyanidin 3-(6"-salicylate)-glucoside Cyanidin 3-(6"-cinnamate)-glucoside
Blueberry ^d	Cyanidin-3-galactoside	10 mg/10 g of lipase CalB enzyme	Polyoxyethylene stearate	-	50 °C/36 h and agitation at 200 pm	Cyanidin-3-galactoside oleate Cyanidin- 3-galactoside palmitate
Blackcurrant skin ^e	Delphinidin-3-glucoside Delphinidin-3-rutinoside Cyanidin-3-glucoside Cyanidin-3-rutinoside	20 g/L of lipase CalB enzyme	Octanoic acid	Acetonitrile:DMSO 10:1	60 °C/9 h at agitation	Delphinidin-3-glucoside-6"-O-octanoate; Cyanidin-3-glucoside-6"-O-octanoate. Cyanidin and delphinidin rutinosides were not acylated
Blackberry ^f	Cyanidin-3-glucoside	20 g/L of lipase CalB enzyme	Butyric acid Hexanoic acid Caprylic acid Decanoic acid Lauric acid	2M2B	60 °C/6-12 h at agitation	Cyanidin-3-glucoside–fatty acid conjugates: butyric, hexanoic, caprylic, decanoic, and lauric
Alpine Bearberry ^g	Cyanidin-3-galactoside	10 g/L of lipase CalB enzyme	Lauric acid Tetradecanoic acid Hexadecanoic acid Octadecanoic acid	Acetone Acetonitrile <i>Tert</i> -butanol 2M2B	40, 50 and 60 °C/72 h and agitation at 250 rpm	Cyanidin-3-(6"-dodecanoyl)galactoside
Blackcurrant ^h	Delphinidin-3-glucoside, Delphinidin-3-rutinoside, Cyanidin-3-glucoside, Cyanidin-3-rutinoside	10 g/L of lipase CalB enzyme	Lauric acid	<i>Tert</i> -butanol	60 °C/72 h and agitation at 250 pm	Delphinidin-3-glucoside derivative Delphinidin-3-rutinoside derivative Cyanidin-3-glucoside derivative Cyanidin-3-rutinoside derivative
Purple corn-husk ⁱ		5, 10, and 20 g/L of lipase CalB enzyme,	Vinyl cinnamate			Cyanidin-3-(6"-cinnamoyl)glucoside
Tiliapo	Cyanidin-3-glucoside		Dihydrocinnamic acid	Pyridine		Cyanidin-3-(4"-cinnamoyl)rutinoside Cyanidin- 3,5-(6"-cinnamoyl)diglucoside
Trueno	Cyanidin-3-rutinoside	C-LECTA, and	Cinnamic acid	<i>Tert</i> -butanol	60 °C/48 h and	Cyanidin -3-(6"-dihydrocinnamoyl)glucoside
Plum husk	Cyanidin-3-diglucoside	lipase from <i>C.</i> <i>cylindraceae</i>	Dihydroferuloyl	2M2B	agitation at 300 pm	Cyanidin-3-(6"-dihydroferuloyl)glucoside Cyanidin-3-(6"-dihydrosinapoyl)glucoside
Botterbrush flower						

			acid Dihydrosinapic acid			
				2M2B Ionic liquids (ILs): 1-butyl-3-methylimidazolium trifluoromethanesulfonate; 1-butyl-3-methylimidazolium hexafluorophosphate; 1,3-dimethylimidazolium methylsulfate; 1-ethyl-3-methylpyridinium trifluoromethanesulfonate	Conventional method: 60°C/30 h Microwave-Assisted: 2M2B power 150 W (cycles of 10s of irradiation) followed by 40 s at 50-60 °C/3 h ILs: 20 W at to 58 °C /3 h	
Blackberries waste ^j	Cyanidin-3-glucoside	20 g/L of lipase CalB enzyme and 34 g/L of CalB-rich extract	Octanoic acid			Cyanidin-3-glucoside-octanoic
Blueberry ^k	Native blueberry anthocyanins (not identified)	10 g/L of lipase CalB enzyme	p-coumaric acid, caffeic acid	Tetrahydrofuran (THF)	60 °C for some time to graft acyl donors	p-coumaric acid and caffeic acid were grafted on the 6-OH of glucoside and galactoside and the 5-OH of arabinose
Black rice ^l	Cyanidin-3,5-diglucoside Cyanidin-3-glucoside Cyanidin-3-rutinoside Peonidin-3-glucoside Peonidin-3-rutinoside	Lipase CalB enzyme	Lauric acid	2M2B	56 °C/28 h and agitation at 100 pm	Cyanidin-3,5-(6''-dodecanoyl)-diglucoside Cyanidin-3-(6''-dodecanoyl)-glucoside Cyanidin-3-(4''-dodecanoyl)-rutinoside Peonidin-3-(6''-dodecanoyl)-glucoside Peonidin-3-(4''-dodecanoyl)-rutinoside
Floral waste ^m (Rose petals)	Cyanidin-3,5-diglucoside	20 g/L of lipase CalB 1000 enzyme	Caprylic acid Lauric acid Hexadecanoic acid	Viz. acetone Tert-butanol 2M2B Acetonitrile	40, 50, 60, and 70 °C/24 h and agitation at 150 pm	Caprylic acid esters of anthocyanins Lauric acid esters of anthocyanins Palmitic acid esters of anthocyanins
<i>Hibiscus sabdariffa</i> flower ⁿ	Delphinidin 3-sambubioside	20 g/L of lipase CalB enzyme	Octanoic acid	2M2B	60 °C for 48 h at agitation	Delphinidin-3-sambubioside-octanoic
Pure anthocyanin ^o	Cyanidin-3-glucoside	20 g/L of lipase CalB enzyme	Methyl butyrate Methyl n-octanoate Methyl laurate Methyl myristate Methyl palmitate Methyl stearate	2M2B	60 °C/24 h and agitation at 300 pm	Cyanidin-3-(6''-n-octanoyl)-glucoside Cyanidin-3-(6''-lauroyl)-glucoside Cyanidin-3-(6''-myristoyl)-glucoside

***a**: Castro et al. (2014); **b**: Sari et al. (2015); **c**: Yan et al. (2016); **d**: Luo et al. (2017); **e**: Cruz et al. (2018); **f**: Guimarães et al. (2018); **g**: Yang et al. (2018); **h**: Yang et al. (2019); **i**: Fernandes-Aulis et al. (2020); **j**: Guimarães et al. (2020); **k**: Liu et al. (2020); **l**: Yang et al. (2020); **m**: Marathe et al. (2021); **n**: Marquez-Rodríguez et al. (2021); **o**: Zhang et al. (2021).

2.6.2 Enzyme

Due to the enzyme's regioselectivity, it is mainly intended to lead to the formation of the acylated product (in contrast to the chemical process that leads to the formation of several products) and catalyzing a reaction. For acylation, the immobilized enzyme CalB lipase (lipase produced by *Candida Antarctica*) has been commonly used, except for the study by Yang et al. (2020) that did not inform the enzyme used in their research. In addition, some studies have specified the enzyme as Novozym 435. Guimarães et al. (2020) used, in addition to the immobilized enzyme, lipase CalB in powder form (CalB-rich extract), adding 34 g/L (Table 3.3), being equivalent to 20 g/L of the enzyme (the protein extract containing 60% of lipase). Fernandez-Aulis et al. (2020), in addition to immobilized lipase CalB, used two more enzymes: C-LECTA and lipase from *Candida cylindrical*.

In the study by Guimarães et al. (2020), the use of the powdered CalB enzyme in enzymatic acylation showed a lower concentration of acylated anthocyanins than CalB immobilized, in both methods (conventional and microwave-assisted) In the case of the traditional method and using 2M2B as the solvent, the concentration of acylated compounds was approximately 0.03 g/L using CalB enzyme immobilized in about 14h, while for CalB powder it was around 0.02 g/L in about 25h. When using the microwave-assisted method, the concentration of acylated anthocyanins with immobilized CalB was about 0.045 g/L, while with CalB powder, it was about 0.025 g/L, less than two hours.

The enzymatic acylation process was also carried out using ionic liquid (Table 2.3) for conventional and microwave-assisted methods. Ionic liquids have received significant attention in recent decades due to their negligible vapor pressure, non-flammability, thermal stability, and wide conductivity window (ZHOU et al., 2018). However, although they do not contaminate the air (low vapor pressure), these compounds can contaminate water and soil due to their composition (BUBALO et al., 2014). Of all the ionic liquids tested by Guimarães et al. (2020) (Table 2.3), only one could carry out enzymatic acylation 1-butyl-3-methylimidazolium trifluoromethanesulfonate. For the conventional method, CalB reached maximum formation of acylated anthocyanins in 18h, around 0.035 g/L, while CalB in powder reached 24 h, around 0.015 g/L. The concentration did not differ between powdered and immobilized enzymes (about 0.016 g/L) for the microwave-assisted method. However, the immobilized enzyme reached this production in about 1.7h, while the powdered enzyme in about 2.3 h. The immobilization of lipases has advantages over non-immobilized enzymes, such as improved thermal and chemical stability, easy recycling and reuse, lower operating costs, and more prolonged enzyme survival (Ismail & Baek, 2020; Liu et al., 2020).

The study performed by Fernandez-Aulis et al. (2020) used three concentrations (5, 10, and 15 g/L) of three immobilized enzymes to carry out the process of enzymatic acylation anthocyanins, using tert-butanol as a solvent. The best results for each enzyme were using 20 g/L, with CalB showing 74.1% of conversion yield, followed by C-LECTA lipase with a conversion yield of 59.9%, and finally with the lipase from *Candida cylindrical* with a yield conversion rate of only 21.3%. The immobilized Lipase CalB again showed better results; however, little has been explored regarding using other lipases to perform enzymatic acylation. Therefore, this field still needs to be further explored.

The main factor that possibly affects the enzymatic acylation of anthocyanins concerning lipase (in addition to the type of lipase, with or without immobilization) is its concentration. The use of the immobilized CalB enzyme showed good anthocyanin acylation results, mainly at concentrations ranging from 10 g/L to 20 g/L. Furthermore, it is essential to evaluate the type of acyl donor group used in the process since this is responsible for forming the acylated compound. Also, according to the authors, the enzyme showed to have regioselectivity, in the anthocyanin molecules that have glycosides, all bonds occurred at the 6''-OH position of glucose (CRUZ et al., 2018; FERNANDEZ-AULIS et al., 2020; LIU et al., 2020; MARQUEZ-RODRIGUEZ et al., 2021; YAN et al., 2016; YANG et al., 2018, 2019; ZHANG et al., 2021), when the molecule has rutinoside, the acylation occurred at 4''' -OH of the rhamnose (FERNANDEZ-AULIS et al., 2020; YANG et al., 2019), and when the molecule has galactoside at the 6''-OH position of galactose (LIUan et al., 2020; YANG et al., 2018). The mainly monosaccharide anthocyanins have easy access to the enzyme's active site, possibly due to their rotation configuration (MARQUEZ-RODRIGUEZ et al., 2021).

Immobilized enzymes have been widely applied in various industries, such as food, pharmaceutical, wastewater treatment, textile, etc., due to the main advantages already mentioned above: easy recovery of the enzyme, rapid termination of enzyme assay, and repeated enzyme assay (reduce cost) (BASSO & SERBAN, 2019; LIU et al., 2018). Among the advantages of reusing the enzyme to improve the cost of the process has not yet been explored, being one of the perspectives to be analyzed in future studies. In addition, other types of lipases have been little explored and may also be studied in future research.

2.6.3 Acyl donors

Among the most studies, the acyl donors used for the enzymatic acylation of anthocyanins are the aliphatic acids, highlighting lauric and octanoic (or caprylic) acids (Table 2.3). Several works used lauric acid as an acyl donor group; however, other fatty acids were

also studied. Guimarães et al. (2018) reported intermediate to lower conversion values when using fatty acids as the acyl donor group. Conversion results were 47%, 40%, 40%, 30%, and 25% for butyric, hexanoic, caprylic, decanoic, and lauric acids, respectively, with the processing time ranging from 6 h to 12 h, varying by size chain (the longer the chain, the longer the process). In this case, it can be observed that the greater the number of fatty acid chains, the higher the conversion yield, and therefore, long-chain fatty acids are possibly better as donor acyl groups for the enzymatic acylation of anthocyanins when compared to acids short-chain fatty acids.

However, in the study by Yang et al. (2018), initially, four acyl donor groups were tested (Table 2.3), and the group with the best result was lauric acid (conversion of 41%), significantly differentiating from the others. It is interesting to note that lauric had the lowest number of chains of the fatty acids used. Therefore, it can be suggested that there is a limit on the number of chains in the molecule that results in better enzymatic acylation results of anthocyanins. In the previous work (GUIMARÃES et al., 2018), it was observed that lauric acid was the best acyl donor group compared to fatty acids with fewer chains than lauric acid. In work by Yang et al. (2018), it was found that lauric acid presented better conversion yields when compared to fatty acids with more chains. This factor may have been crucial for choosing lauric acid as the acyl donor group for further work.

Still regarding the work by Yang et al. (2018), the best result (with a significant difference $p < 0.05$) was obtained by the 1:10 ratio (anthocyanin: lauric acid), of 73% in 72h, however, in 24h this conversion was already 68% and therefore due to cost efficiency this time was used. In the subsequent work by Yang et al. (2019), the authors further optimized the use of lauric acid as an acyl donor group, obtaining conversion yield values between 63-88%, varying 20% the ethanol fraction in MQ-water. In work by Marathe et al. (2021), the best condition was a ratio of 1:100 (anthocyanin:lauric acid), equal to 63% in 24 h.

Some studies that used other acyl donor groups also had good results for conversion yield. In the study by Yan et al. (2016), the conversion values after 48h were 91% for methyl benzoate as a donor, 84% for methyl salicylate as a donor, and 68% for methyl benzoate as acyl donor, with the equilibrium reaching between about 30h-40h. In work by Fernandez-Aulis et al. (2020), the donor acyl groups shown in Table 1 were evaluated. Of these, the one with the best conversion yield was vinyl cinnamate (74.1%), followed by dihydrocinnamic acid (67.3%), and dihydroferuloyl acid (45.5%), dihydrocinapic acid (21.8%), and finally cinnamic acid (18.1%).

Low conversion yield values were also observed. In the study by Liu et al. (2020), the authors calculated the degree of acylation through experimental analysis (all other studies performed the conversion yield analysis by HPLC-MS) for p-coumaric and caffeic acids as acyl donor group (Table 2.3), obtaining degrees of acylation of 5.38%, and 5.68%, respectively. These values are much lower than other studies; this may have occurred because the methodology used differs from other studies, and the anthocyanin has not been identified. Marquez-Rodriguez et al. (2021) performed enzymatic acylation with octanoic acid as acyl donor (Table 2.3). In the acylation process, the formation of two products was verified: delphinidin-3-glucoside-octanoic, and delphinidin-3-O-sambubioside-octanoic, with conversion yields of 28% and 15%, respectively. According to the authors, this lower conversion compared to other studies may be due to the spatial configuration and steric hindrance of the molecule delphinidin-3-sambubioside, which may have difficulty accessing the enzyme's active site. In the study by Zhang et al. (2021), the authors used six acyl donor groups (Table 2.3). The results obtained for the conversion were low, from 3.4% to 7.3%, being higher for the methyl stearate group. The authors report that these low conversion values were possibly due to the non-optimization of the process.

It can be observed that lauric acid in the proportion of 1:100 (anthocyanin:lauric acid) showed promising results for conversion yield in the enzymatic acylation process of anthocyanins, which is considered one of the main donor acyl groups to be used in the acylation processes. On the other hand, it was observed that there is a need for optimization whenever an acyl donor group is used for the acylation process since low results were verified when the optimization was not performed.

2.6.4 Solvents

It is necessary to verify the solubility of the substrates used to carry out the chemical reactions, which must be compatible with the substrates and the esterification reactions. In addition to being soluble, these solvents must not degrade the compound formed (FERNANDEZ-AULIS et al., 2020). Among the studies that evaluated different types of solvents (Table 2.3), Yang et al. (2018) reported better results using *tert*-butanol and 2M2B (at temperatures of 50 °C and 60 °C), with 2M2B showing the best results. Fernandez-Aulis et al. (2020) observed better results using the *tert*-butanol solvent. In the case of the work by Guimarães et al. (2020), the authors used ionic liquids to perform enzymatic acylation (Table 3.3), which are considered more sustainable compounds when compared to organic solvents. Of these solvents, only 1-butyl-3-methylimidazolium trifluoromethanesulfonate formed

acylated compounds, verified both by the conventional method and by the microwave-assisted process. Marathe et al. (2021) reported the formation of acylated compounds only when acetonitrile was used as a solvent, is also tested *viz.* acetone, *tert*-butanol, and *tert*-amyl alcohol. Possibly, the fact that none of these solvents were able to carry out the enzymatic acylation of anthocyanins is due to the lack of work optimization since these solvents have already presented positive results using cyanidin-3-glucoside, the same anthocyanin used in work.

Solvents tend to show similar results regardless of the type of anthocyanin because they have similar molecules. This can be seen because it was possible to carry out enzymatic acylation using 2M2B solvent for the different kinds of anthocyanins used: cyanidin, delphinidin, and peonidin. According to Marathe et al. (2021), due to the better solubility of non-polar substrates, greater thermal stability of the enzyme, and easy product recovery, the use of organic solvents is advantageous. However, according to Guidance Q3C Impurities: Residual Solvents of the Food And Drugs Administration (2017) and (2020) (FDA), of the seven solvents used, four (2-methyl-2-propanol, acetonitrile, pyridine, and tetrahydrofuran) belong to class 2 (limited use, being allowed in specific cases and conditions), two solvents (acetone and DMSO) belong to class 3 (permitted use as they do not include any risk to human health), while this guidance does not yet classify 2-methyl-2-butanol. Alder et al. (2016) presented the GKS Solvent Sustainability Guide. This Guide shows divided categories that measure the sustainability of solvents, measured in few known issues, some know issues, and major known issues. Although not yet classified by the FDA, 2-methyl-2-butanol (or *tert*-amyl alcohol) has few known issues for the solvents used in the studies. In contrast, 2-methyl-2-propanol, acetone, acetonitrile, and DMSO have some known issues, and finally, pyridine and tetrahydrofuran have major known issues. The solvent most used for enzymatic acylation of anthocyanins was the one with the best sustainability, according to this Guide.

The use of solvents that do not affect the environment has been a challenge for industries; however, this is a field to be explored to make processes more and more sustainable. In the case of enzymatic acylation of anthocyanins, the use of sustainable solvents becomes paramount to develop a method with the least possible harm to the environment.

2.6.5 Process parameters

The parameters used for enzymatic acylation varied for each study. Only two studies used reduced pressure (Table 2.3), and the most current studies did not control pressure. Castro et al. (2014) and Yan et al. (2016) used reduced pressures following the result obtained by Passicos et al. (2004), in which he reported an increase in the conversion yield of enzymatic

acylation (using CalB) from 32% (atmospheric pressure) to 85% (pressure reduced to 200 mbar). Furthermore, one of the interesting properties of enzymes is precisely their ability to act as catalysts in aqueous media under mild conditions, that is, at low temperatures and pressures (ORTIZ et al., 2019). Only Yan et al. (2016) verified the conversion yield of acylated compounds. The authors showed the best conversion yield (91% using methyl benzoate as donor) of all studies. The pressure reduction had a positive effect on the enzymatic acylation of anthocyanins.

Temperature is one of the most critical parameters for enzymatic acylation of anthocyanins because, as mentioned above, these compounds are more unstable as temperature increases (Patras et al., 2010). In addition to anthocyanins, the temperature rise can influence the enzyme, and it has been reported that increasing it decreases the reactive activity of the lipase CalB (Novozym 435) (Souza et al., 2018; Talbert et al., 2014). Most of the experiments were reported as being carried out with agitation, varying the temperature from 40 °C to 60 °C, with the majority being used close to 60 °C. Among the works that evaluated different temperatures, Yang et al. (2018) verified better results with temperatures of 50 °C and 60 °C; however, they chose to use the temperature of 60 °C due to the acylation time being 24 h. The authors observed that the increase in temperature (from 40 °C to 50 °C) significantly affected the solubility of anthocyanin, with solubility being an important parameter to carry out enzymatic acylation.

Marathe et al. (2021) found better temperatures equal to 40 °C and 50 °C (in 24 h), opting for the lower temperature due to the cost of the process. At 60 °C and 70 °C, there was a decrease in molar conversion (%); according to the authors, this may have occurred due to decreased enzyme activity when using higher temperatures. The temperature parameter must be evaluated before carrying out the enzymatic acylation process of anthocyanins to obtain the best conversion yield of acylated compounds. According to the authors who optimized this temperature, 50 °C got better conversion values for both works.

The process of enzymatic acylation of anthocyanins takes considerable time, according to Table 2.3. Yan et al. (2016) evaluated 48h of the process but obtained better results between 30h to 40h. Yang et al. (2018) evaluated 72h and found that 24h was enough to reach the maximum yield practically. Marathe et al. (2021) also assessed the time (36h), finding maximum conversion values in 24h. As with the other parameters, the time must be evaluated to obtain the best conversion values in a shorter period. After reaching the maximum conversion yield, the process must be interrupted mainly due to the cost. For the traditional method of enzymatic acylation of anthocyanins, generally, the best period to reach the maximum

conversion yield is around 24 hours. However, some authors used lower time values: 6h to 12 h (GUIMARÃES et al., 2018) and 9 h (Cruz et al., 2018).

In all studies, it was possible to carry out the enzymatic acylation of anthocyanins, the conventional method efficient for forming these compounds. Only an alternative method (microwave-assisted) was also used (GUIMARÃES et al., 2020), which may be a promising aspect for the acylation of anthocyanins, that is, using unconventional techniques to form these new compounds. A perspective to be considered is the use of alternative processes to the conventional one that can optimize some parameters, as in the case of the use of microwave-assisted, which significantly reduced the processing time.

2.7 FINAL CONSIDERATIONS

Black rice and its by-products have a high content of phytochemical compounds in their composition, mainly anthocyanins. Some emerging technologies were applied to recover anthocyanin compounds; however, few of them performed kinetics and subsequent optimization of the process. In addition, some alternatives have not yet been explored for extracting anthocyanins from black rice as electric pulse-field assisted extraction and pressurized liquid extraction (PLE). However, anthocyanins are unstable compounds easily degraded by temperature and light, and for that, some approaches for stabilizing these compounds have been studied, such as enzymatic acylation.

Enzymatic acylation of anthocyanins affects the stability of anthocyanins. Enzymatic acylation of anthocyanins affects the stability of anthocyanins. Adding an acyl group to the anthocyanin molecule usually occurs at the 6''-OH position for glucoside and galactoside, while for rutinoside in the 4'-OH. Bonding at these positions on the glucose molecule allows for the folding of aromatic acyl rings or aliphatic acids and consequently protects anthocyanin from degradation. These compounds have affinity in a lipophilic medium and can be applied in lipid matrix products. There are still some gaps (emerging techniques, enzyme types and green solvents) in the enzymatic acylation processes that need to be elucidated.

Guimarães et al. (2020), in addition to the conventional process, used the microwave-assisted process for enzymatic acylation. The use of emerging techniques is also an important alternative to be evaluated regarding the application of this process, since these techniques can bring better results, present results with less energy expenditure and, consequently, be less harmful to the environment. A possibility to be evaluated in future studies is the use of alternative enzymes (cheaper) to carry out the acylation since practically all the works used only the CalB enzyme. Furthermore, the reuse of this immobilized enzyme has not yet been

explored and should be evaluated in future works. Another possibility concerns the use of organic solvents for enzymatic acylation, except for the study by Guimarães et al. (2020), in which they used ionic liquids as solvents to carry out the process. The use of green solvents has been highly explored in recent years; therefore, the replacement of organic solvents may be another alternative to be studied in the future for enzymatic acylation.

Considering this, the first part of our study is related to optimizing anthocyanin extraction from black rice bran using PLE, UAE, and MAE technologies to obtain higher yields with less time and less solvent consumption compared to the conventional process. The second part of our study is to carry out the enzymatic acylation of anthocyanins from BRB, extracted by the emergent method selected in the first part, and verify the thermostability.

3 PRESSURIZED LIQUID EXTRACTION AS AN ECO-FRIENDLY APPROACH TO RECOVER ANTHOCYANIN FROM BLACK RICE BRAN

The information in this chapter was compiled in a scientific article entitled "*Pressurized liquid extraction as an eco-friendly approach to recover anthocyanin from black rice bran*" (doi: 10.1016/j.ifset.2023.103372), which was published in the journal "Innovative Food Science and Emerging Technologies" (Qualis A1; Impact Factor 6.3). The aim of this study was to optimize the recovery of anthocyanin-rich extract from black rice bran by the pressurized liquid extraction (PLE) method. The temperature and fluorescent light stability of anthocyanin-rich extracts obtained by PLE was also evaluated, as well as cytotoxicity and antitumoral activity. Therefore, this chapter contains both the methodology and the respective results.

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Pressurized liquid extraction as an eco-friendly approach to recover anthocyanin from black rice bran

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ABSTRACT

This study aimed to recover the anthocyanin-rich extract from black rice bran by pressurized liquid extraction (PLE) and compare with the conventional process (heating-stirring extraction - HSE). PLE performs fast and efficiently and results in high yields in less time and less solvent, being considered eco-friendly. The extracts were characterized for thermal and light stabilities, cytotoxicity, antitumoral activity, and cytoprotective effect. Optimum PLE conditions were: 55 °C, a flow rate of 5 mL/min, and solution ethanol: citric acid 0.1 mol/L (50:50, v/v). PLE recovered about 80% of total anthocyanins (2.33 mg/C3GE g), while HSE recovered 46% (1.17 mg/C3GE g). Cyanidin-3-O-glucoside (C3G) was identified (m/z 449) and quantified, 1.63 mg/g for PLE and 1.07 mg/g for HSE. Both anthocyanin-rich extracts in low temperature showed high thermostability, did not present cytotoxicity (normal fibroblast L929 cells), and showed cytoprotection of L929 cells when subjected to oxidative stress with H₂O₂. Overall, the anthocyanin-rich extracts obtained by PLE showed potential to be applied as natural colorants and possibly to improve wound healing.

Industrial relevance: PLE method is an eco-friendly technology that performs fast and efficient extractions under high temperature and pressure conditions. This study provides information about the extraction of anthocyanin-rich extracts from black rice bran by green PLE process under optimized conditions. The process allowed for obtaining antioxidant-rich extracts with no cytotoxicity and a high potential for application in the food, cosmetic, and biomedical industries.

Keywords: Anthocyanin-rich extracts, Box-Behnken design, *Oryza sativa* L., Stability, Cytotoxicity.

3.1 INTRODUCTION

Black rice is becoming popular recently, being consumed as a functional food due to its health potential (ITO; LACERDA, 2019; POONIA; PANDEY, 2022). However, from rice processing, about 20% of rice husk is produced, 10% of rice bran, and 14% of broken rice (MORAES et al., 2014; NAGRALE; HAJARE; MODAK, 2012; VAN HOED et al., 2006). Although these by-products have a high content of bioactive compounds, they are generally intended for animal nutrition (DAS; GOUD; DAS, 2018; HALEE et al., 2020; PEANPARKDEE; IWAMOTO, 2019) and one way to add value to the black rice by-product is extracting its bioactive compounds (HALEE et al., 2020).

Among the mentioned by-products, black rice bran has a high amount of bioactive compounds, mainly anthocyanins, usually containing around 64-90% of cyanidin-3-O-glucoside (HUANG; LAI, 2016). Anthocyanins are water-soluble natural colorants belonging to flavonoids, which are considered bioactive due to their antioxidant properties. They are related to health benefits, having anti-obesity, anti-diabetic, antimicrobial, anti-cancer, neuroprotective, and cardiovascular disease potential (LUZARDO-OCAMPO et al., 2021; TENA; MARTÍN; ASUERO, 2020).

The extraction of phenolic compounds, mainly anthocyanins, can be a viable alternative for reusing this material generated during the processing of black rice. However, using solvents to extract antioxidant compounds in a conventional process can be slow, expensive, and still present low efficiency (HALEE et al., 2020). Therefore, innovative technologies can be an alternative to achieve better yields in the recovery of bioactive compounds. Among them for extracting anthocyanins from black rice, microwave-assisted extraction was the most used (ABDEL-AAL et al., 2014; HALEE et al., 2020; JHA; DAS; DEKA, 2017; MOIRANGTHEM et al., 2021) followed by ultrasound-assisted extraction (JHA; DAS; DEKA, 2017; THAKUR et al., 2022).

One of the alternative methods with fast and efficient extraction is pressurized liquid extraction (PLE), which uses conditions of high temperature and pressure, ensuring that the solvent remains in a liquid state (above the boiling point but below its critical point) (MUSTAFA; TURNER, 2011; ZIELINSKI et al., 2021). High pressure improves solvent diffusion (high solvation power). Mass transfer increases due to the decrease in surface tension and viscosity under the applied conditions (PICOT-ALLAIN et al., 2021). Also, it results in high yields in less time and less solvent due to a facilitated rupture of solid matrix cells (ZIELINSKI et al., 2021).

Furthermore, the extraction efficiency depends on the analyte to be extracted, the location of the analyte within the matrix, and the nature of the sample matrix (MUSTAFA; TURNER, 2011). The main solvents used are ethanol and water and their mixtures, which result in high solubility of the pigments and meet environmental requirements, being considered GRAS (Generally Recognized as Safe) solvents (VIGANÓ et al., 2022; ZIELINSKI et al., 2021).

Using the PLE technique with water and/or ethanol as solvents is considered an eco-friendly approach widely used for obtaining anthocyanins from fruits, vegetables, and their by-products (ZIELINSKI et al., 2021). Therefore, the technique used is essential to enhance the use of black rice bran, which, although it has a high nutritional value, is usually used as animal feed. To the best of our knowledge, this is the first study to optimize the recovery of anthocyanin-rich extract from black rice bran by PLE. The stability to temperature and fluorescent light of anthocyanin-rich extracts obtained by PLE was also evaluated, as well as the cytotoxicity and antitumoral activity of anthocyanin-rich extracts.

3.2 MATERIAL AND METHODS

3.2.1 Materials

Ruzene supplied black rice bran (BRB) (Pindamonhangaba, Brazil). The black rice was harvested in the February-May (2022) crop, and the bran comes mainly from the polishing stage of rice processing. Analytical standard of anthocyanin (cyanidin-3-O-glucoside), the Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid), TPTZ (2,4,6-tri(2-pyridyl)-s-triazine), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), Folin-Ciocalteu, Sodium carbonate were purchased from Sigma-Aldrich (Steinheim, Germany). Dulbecco's Modified Eagle's medium (DMEM), TrypLE Express enzyme, and phosphate buffered saline (PBS) were purchased from Gibco. CellTiter 96® AQueous One Solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] was purchase from Promega Biotecnologia do Brasil.

3.2.2 Sample preparation

Black rice bran was ground in a Wiley mill (DeLeo, Brazil) and sieved (particle size between 20 to 32 mesh). The proximate composition of black rice bran (BRB) was moisture

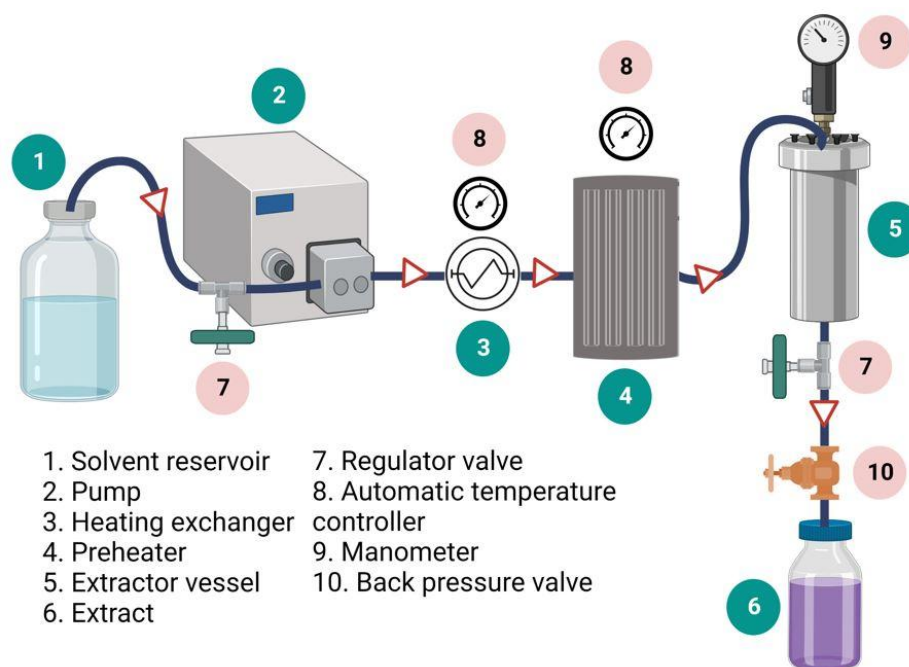
$12.68 \pm 0.08\%$, $1.49 \pm 0.02\%$ fixed mineral residue (ash), $2.69 \pm 0.25\%$ total fat (lipids), protein $7.87 \pm 0.11\%$, and $75.27 \pm 0.45\%$ carbohydrates.

3.3 PRESSURIZED LIQUID EXTRACTION (PLE)

3.3.1 Obtention of the anthocyanin-rich extract

In dynamic conditions, a self-assembled instrumented unit (Figure 3.1) was used to extract anthocyanin from black rice bran by PLE. The customized apparatus was developed by Rodrigues et al. (2019) in the Laboratory of Thermodynamics and Supercritical Technology (LATESC) at UFSC. Ground dry black rice bran (5 g) was placed in the extraction vessel of 90 mL (internal diameter of 25 mm and height of 180 mm) and about 40 g of glass spheres inside the extraction column forming a fixed bed. The extraction begins only after filling the extractor and consequently increasing the pressure (10 MPa).

Figure 3.1 Customized pressurized liquid extraction (PLE) system. Created with BioRender.com.



The assays were performed continuously, with a pressure set at 10 MPa and a time of 8 min (defined through the extraction kinetics based on the concentration of anthocyanins TMA (Section 3.5.1) performed with the parameters set at the central point) (Figure S1 – Appendix B). The process conditions were optimized following a Box-Behnken design, with 15

experiments performed randomly. The factors evaluated (independent variables) were temperature (x_1 : 30, 45 and 60 °C), flow rate (x_2 : 4, 5 and 6 mL/min) and solvent concentration (x_3 : 20:80, 50:50 and 80:20, citric acid 0.1 M:ethanol, v:v).

3.3.2 Optimization of anthocyanin recovery

Anthocyanin Recovery (AR) was the dependent variable used in the optimization methodology by response surface methodology (RSM). For this, a second-order polynomial model was used to fit the experimental data, according to Equation (3.1):

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{j \leq i \leq 1}^k \beta_{ij} x_i x_j \quad (3.1)$$

Where, y is the predicted response, β_0 is the intercept, β_i , β_{ii} , and β_{ij} are the regression coefficients for linear, quadratic, and interaction terms, respectively, and x_i and x_j are the independent variables. The evaluation of the model was initially carried out by evaluating the significant factors. Non-significant effects (ANOVA, $p > 0.05$) were removed, and parameters were refitted. To assess the adequacy and quality of the fit, the parameters p-lack of fit and coefficient of determination (R^2 and R^2 adjusted) were investigated.

3.4 HEATING-STIRRING EXTRACTION (HSE)

The HSE method was also used to obtain anthocyanin extract from BRB for comparative purposes. The optimal condition for extraction was based on study published by Das et al. (2018): 43.74% (v/v) ethanol concentration, 37.5 °C, 22.5 min extraction time, and 8.80 (mL/g) solvent to solid ratio.

3.5 CHARACTERIZATION OF ANTHOCYANIN-RICH EXTRACTS

3.5.1 Total monomeric anthocyanin (TMA), Anthocyanin recovery (AR), and Polymeric Color (PC)

The total monomeric anthocyanins (TMA) concentration was determined according to the differential pH methodology (GIUSTI; WROLSTAD, 2001). For the analysis, 20 μ L of extract in microplates were added to 280 μ L of a buffer solution with pH 1.0 (potassium chloride 0.025 mol/L) and pH 4.5 (sodium acetate 0.4 mol/L). Then the absorbances were read at 520 and 700 nm (Multileader Infinite M200 TECAN, ZH, Switzerland). The concentrations of TMA were calculated using Equations 3.2 and 3.3:

$$A = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5} \quad (3.2)$$

$$TMA = \frac{A \cdot MW \cdot DF \cdot 1000}{\varepsilon} \quad (3.3)$$

where A is the calculated absorbance, MW is the molecular weight of anthocyanin standard (cyanidin-3-glucoside: 449.2 g/mol), DF is the dilution factor (15), and ε is the molar absorptivity of cyanidin (26900).

The recovery of anthocyanins (AR) was performed according to the methodology of Gurak et al. (2014) with modifications. For total anthocyanin content (TAC), three sequential extractions were performed using 50 mL of 80% ethanol solution acidified with 0.1 mol/L of HCl for 0.5 g of the sample and stirring at room temperature for 30 min. Afterward, another extraction was performed using 50 mL of 70% acetone solution, also under stirring at room temperature for 30 min. To calculate AR (%), the TMA result (Equation 3.3) obtained for each assay was used and finally calculated by Equation 3.4:

$$\%AR = \left(\frac{TMA}{TAC} \right) \cdot 100 \quad (3.4)$$

The polymeric color (PC) was measured by diluting the extracts in potassium chloride (pH 1.0) and subsequently bleaching them with potassium metabisulfite (0.9 M). For the calculations, the metabisulfite bleached sample (PC) and the control sample (DC) were used, calculated by Equation 3.5, in which A₄₂₀, A₅₂₀, and A₇₀₀ are the absorbances at 420, 520, and 700 nm, respectively. The % PC was determined by Equation 3.6:

$$DC \text{ or } PC = [(A_{420} - A_{700}) + (A_{520} - A_{700})] \cdot DF \quad (3.5)$$

$$\%PC = \left(\frac{PC}{DC} \right) \cdot 100 \quad (3.6)$$

Where DF is the dilution factor.

3.5.2 Total phenolic content (TPC)

The total phenolic content (TPC) was evaluated based on the colorimetric methods described by Singleton et al. (1999), adapted for microplates. Gallic acid (GAE) was used as standard, and TPC was expressed as mg GAE per g of dry sample (mg GAE/g).

3.5.3 Antioxidant activity

DPPH method performed antioxidant activity analysis according to Brand-Williams et al. (1995), ABTS according to Re et al. (1999), and FRAP according to Benzie & Strain (1996), all methods adapted for microplates. Trolox was standard, and the antioxidant activity was expressed as μ mol of Trolox equivalent per g of dry sample (μ mol TE/g).

3.6 INDIVIDUAL ANTHOCYANIN BY HPLC-MS

The freeze-dried extracts were resuspended in formic acid 0.1% and filtered through a 0.22 μm nylon syringe filter. The samples (10 μL) were injected in high-performance liquid chromatography (model LCMS-2020, Shimadzu, Kyoto, Japan) system consisting of an LC-20AD binary pump, a photodiode array detector (PDA), a SIL-20AC HT autosampler, a central controller, and a single-quadrupole MS detector with electrospray ionization (ESI) interface (Shimadzu). The system was equipped with a Kromasil® C18 column (100Å, 300 mm x 4.6 mm i.d.). The HPLC condition was performed as follows: the solvent system was formed from 0.1% formic acid as fluid phase A and acetonitrile as fluid B. Gradient conditions: 0–25 min, 15–45% B linear; 25–30 min, 45–60% B linear; 30–35 min, 60–10% B linear. The eluates were detected at 25 °C. The flow rate was 0.7 mL/min with an injected column of 10 μL . In both cases, a drying gas of 15 L/min and a nebulizing gas of 1.5 L/min were applied to ionization under nitrogen conditions.

3.7 COLOR MEASUREMENT

The color parameters were determined according to Cárdenas-Pérez et al. (2017), with minor adaptations. Configured with zoom 45X, a digital camera (Nikon, Model - D5500, Tokyo, Japan) was used in automatic mode. The photos were taken inside a camera with a white background, illuminated by a fluorescent lamp source. The images were treated with the Color Space Converter plugin by the ImageJ 1.53t software (National Institutes of Health, USA) to convert colors from RGB to CIELab scale (ALVES et al., 2021).

3.8 STABILITY OF THE PLE ANTHOCYANIN-RICH EXTRACT

For both analyses (thermostability and photostability), the lyophilized crude anthocyanin-rich extract was diluted in distilled water (10 mg/mL), and the pH was adjusted to 3.5 (with NaOH or HCl).

3.8.1 Thermostability

The extracts were distributed in sealed tubes and finally subjected to temperatures (of 65, 80, and 95 °C) in a thermostatic bath. At 0, 30, 60, 120, 180, 240, and 300 min, samples were removed and immediately cooled on ice. These points were quantified in terms of TMA content (Section 3.5.1) to obtain their isothermal degradation. A first-order kinetic model was

fitted according to Equation 3.7 (BENVENUTTI; ZIELINSKI; FERREIRA, 2022; TEIXEIRA et al., 2021):

$$\frac{C_t}{C_0} = e^{-k_d \cdot t} \quad (3.7)$$

where C_t is the TMA concentration with time t , C_0 is the TMA initial concentration, k_d is the degradation rate (min^{-1}), and t is the time (min).

The time of half-live ($t_{1/2}$) of anthocyanin degradation was determined by Equation 3.8, and the activation energy (E_a) was determined through the Arrhenius model by Equation 3.9.

$$t_{1/2} = \frac{\ln(2)}{k_d} \quad (3.8)$$

$$\ln(k_d) = \ln(A) - \frac{E_a}{R \cdot T} \quad (3.9)$$

where A is the frequency factor (min^{-1}), R is the ideal gas constant ($8.314 \text{ J/mol} \cdot \text{K}$), and T is the temperature (K).

The activation enthalpy (ΔH), the free energy of activation (ΔG), and the activation entropy (ΔS) were calculated by Equations 3.10, 3.11 and 3.12, respectively:

$$\Delta H = E_a - R \cdot T \quad (3.10)$$

$$\Delta G = -R \cdot T \cdot \ln \left(\frac{k_d \cdot h}{k_B \cdot T} \right) \quad (3.11)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (3.12)$$

where E_a is the activation energy for the degradation reaction (J/mol), R is the ideal gas constant ($8.314 \text{ J/mol} \cdot \text{K}$), T is the temperature (K), k_d is the kinetic rate constant (s^{-1}), k_B is the Boltzmann constant ($1.3806 \cdot 10^{-23} \text{ J/K}$), and h is Planck's constant ($6.6262 \cdot 10^{-34} \text{ J/s}$).

3.8.2 Photostability

The effect of light was determined at room temperature ($25 \pm 2 \text{ }^\circ\text{C}$) in test tubes containing the extracts prepared according to Section 3.8. The tubes were exposed to direct light from a white fluorescent lamp (20 W) in a closed wooden chamber (435 x 435 mm) for three days. At 0, 90, 180, 270, 360, 450, 540, 1440, 2880, and 4320 min (3 days), samples were removed and quantified in terms of TMA content (Section 3.5.1) to obtain their isothermal degradation. The constant rate for anthocyanin degradation (k) and its half-life ($t_{1/2}$) was calculated as described by Equations 3.7 and 3.8.

3.9 CELL CULTURE

3.9.1 Culture condition

Cell lines were grown in DMEM (Dulbecco's Modified Eagle Medium), and were supplemented with 10% (v/v) Fetal bovine serum (FBS) and 1% antibiotic (penicillin and streptomycin). Cells were kept under a moist atmosphere at 37 °C with 5% CO₂. The cell culture medium was refreshed every two days until cells reached 75–90% confluence. Cells were detached using Tryplex, counted, and plated.

3.9.2 Cytotoxicity assay

The cytotoxicity of anthocyanin-rich extract (MA and PLE) was evaluated by measuring the metabolic activity of normal fibroblast cell (L929) (BCRJ, Brazil) following the ISO 10993-5:2009 (2009) by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] colorimetric assay (Promega, Brasil). Cells were placed in 96-well plates at a density of 5×10^3 cells/well and cultured for 24 h. After 24 hours for complete adhesion the extract was added (500 µg/mL) and incubated for 24 and 48 h. The control wells contained culture medium only. Optical density (OD) was measured by spectrophotometry (490 nm, Molecular Devices). The cell viability (%) was determined concerning the control setup as 100% (AGUILAR et al., 2021).

3.9.3 Hydrogen Peroxide-Induced Oxidative Stress in L929 Fibroblast Cells

The evaluation of cell viability after oxidative stress (H₂O₂) was performed according to Pitz et al. (2016). L929 fibroblast cells were seeded at a density of 5×10^3 cells/well into a 96-well plate containing DMEM culture medium supplemented with 10% FBS and incubated overnight at 37 °C in a humidified 5% CO₂ atmosphere. After incubation, DMEM with 10% FBS containing 250 µg/mL (dry weight) of anthocyanin-rich extracts (MA and PLE) was used to treat cells exposed concomitantly to 1.0 mM of H₂O₂ for 24 h (dose that decreases 80% of cell viability from cell L929 in 24 h). Cell viability was evaluated using MTT assay as described in Section 3.9.2.

3.9.4 Antitumoral activity

The cell viability of anthocyanin-rich extracts (MA and PLE) was measured using MTS colorimetric assay. The cells (human breast (MDA-MD-231), embryonic Kidney (HEK-293), lung adenocarcinoma (A549), melanoma (B16F10), and malignant skin melanoma (SK-MEL-28)) were seeded in 96-well plates at a density of 1×10^4 cells/well and kept for 24 h at 37 °C

in a CO₂ incubator for attachment. After 24 h, the cells were treated with 500 µg/mL of MA or PLE. Doxorubicin (10 µg/mL) was used as a positive control, DMSO was negative control, and the blank control used only the culture medium. The treated cells were incubated for 24 h and 48 h at 37 °C in a CO₂ incubator. After the incubation period, the culture medium was removed, and the cells were washed twice with PBS. A mixture of 100 µL of medium and 20 µL of MTS reagent was added to each well and maintained for 2 h in an incubator with 5% CO₂ at 37 °C. The solution was transferred into 96-well plates for measurement of the optical density (OD) at a wavelength of 490 nm using a spectrophotometer (Molecular Decives) (Sousa et al., 2020).

3.10 STATISTICAL ANALYSIS

All results were presented as mean $\pm$ standard deviation. The dataset was evaluated by one-way analysis of variance (ANOVA), and the significant differences were determined using Tukey's Test at a probability level of less than 5% ($p < 0.05$). ANOVA also determined the statistical significance of the models used. The quality and adequacy of the mathematical models were assessed by the determination coefficient (R^2), adjusted R^2 , and the lack of fit (p). All statistical procedures were performed using Statistica v. 13.5 software (TIBCO Software Inc., Palo Alto, CA, USA).

3.11 RESULTS AND DISCUSSION

Black rice bran comes from the milling process in which the bran layer and germ are separated (ITO; LACERDA, 2019; MORAES et al., 2014). Bioactive compounds (mainly anthocyanins) are found in different layers of the bran, accumulated mainly in the pericarp (outermost layer of the grain) (CALLCOTT et al., 2018b; ITO; LACERDA, 2019).

3.11.1 Optimization of PLE extraction conditions

The results ranged from $29.74 \pm 2.27\%$ to $77.94 \pm 3.61\%$ for AR (%) (Table 3.1), equal to 0.86 mg C3GE/g up to 2.27 mg C3GE/g of total monomeric anthocyanins (TMA). It can be observed that PLE presented a wide range in the extraction of anthocyanins from BRB, presenting a high recovery value ($\sim 80\%$). Moreover, it is much faster than conventional extractions evaluated by Pedro et al. (2016) and He et al. (2017), which used an extraction time of 80 min.

According to the data obtained through experimental design (Table 3.1), mathematical models were built to describe the behavior obtained from the extraction of anthocyanins from BRB using PLE. In addition, the response surfaces shown in Figure 3.2 were also generated.

The proposed model for AR presented R^2 equal to 0.90, did not present a lack of fit ($p = 0.059$), and was able to explain 86% of the total variance of the experimental data (adjusted $R^2 = 0.864$). For PC, the proposed model showed R^2 equal to 0.84, presented no lack of fit ($p = 0.208$), and explained 78% of the total variance (R^2 adjusted = 0.779).

The effects that were significant for AR were the positive linear effect of temperature (x_1), the positive linear effect of flow rate (x_1), the negative quadratic effect of flow rate (x_2^2), and the positive quadratic effect of solvents concentration (citric acid:ethanol) (x_3^2) (Equation 3.13). In association with the response surfaces (Figure 3.2A-C), it is possible to observe that the highest values of AR were reached when the temperature and flow parameters were high, however, the quadratic negative effect indicates that the significant increase or decrease in flow can lead to a decrease in the extraction yield. In the studies by Teixeira et al. (2021) and Kuasnei et al. (2022), significant temperature effects were verified when extracting anthocyanins from black bean hulls by PLE. Teixeira et al. (2021) found a positive and negative quadratic effect when using temperatures between 30 °C and 70 °C, while Kuasnei et al. (2022) found a negative effect of temperature (temperatures between 90 and 130 °C). Generally, higher temperatures tend to lead to the degradation of anthocyanins (FAROOQ et al., 2020; SILVA et al., 2017b). In the study by Benvenuti et al. (2022), extracting anthocyanins from jaboticaba by-product with DES by PLE showed a positive linear effect and a negative quadratic effect for flow rate. The flow used in the study was between 2.3 to 5.7 mL/min, and for the optimization, the ideal flow was equal to 5.3 mL/min.

The effects that were significant for PC were the negative linear effect of solvents (citric acid:ethanol) (x_3), the positive quadratic effect of solvents (x_3^2), the negative synergic effect between temperature (x_1) and solvents (x_3), and the negative synergic effect between quadratic temperature (x_1^2) and solvents (x_3) (Equation 3.14). The high polymeric color of the extract may be associated with polymerization reactions (WANG et al., 2022). Therefore, lower PC indices indicate less degradation of anthocyanins by polymerization. With the help of the response surfaces (Figures 3.2D and 3.2F), we can observe that the highest PC values were reached when the concentration of ethanol content in the solvent was high (80%). It was also observed (Figure 3.2E) that temperature and flow rate have no significant effects on PC, however, the interaction between temperature and solvent had a negative effect.

$$y = 52.41 + 22.57 \cdot x_1 + 12.53 \cdot x_2 - 8.36 \cdot x_2^2 + 16.62 \cdot x_3^2 \quad (3.13)$$

$$y = 45.11 - 6.29 \cdot x_3 + 4.29 \cdot x_3^2 - 6.94 \cdot x_1 x_3 - 4.55 \cdot x_1^2 x_3 \quad (3.14)$$

Table 3.1 Box-Behnken experimental design and PLE results of recovery (AR) and polymeric color (PC).

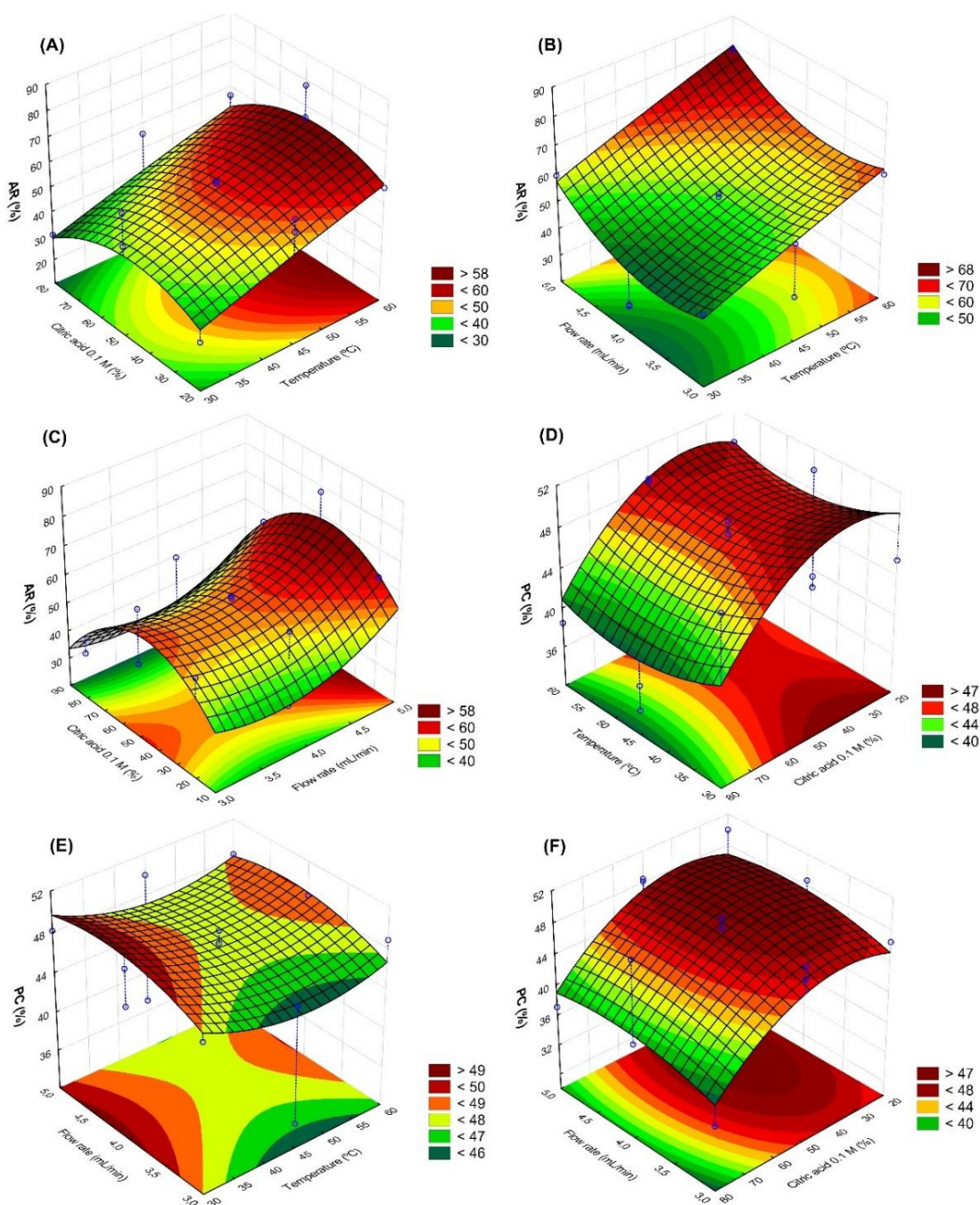
Assay	Independent factors			Response	
	Temperature (°C)	Flow Rate (mL/min)	Citric acid: Ethanol (v/v)	AR (%)	PC (%)
1	0 (45)	-1 (3)	1 (80:20)	36.31 ^f ± 1.93	34.53 ^d ± 1.62
2	1 (60)	1 (5)	0 (50:50)	77.94 ^a ± 3.61	48.47 ^a ± 1.27
3	0 (45)	-1 (3)	-1 (20:80)	55.26 ^{cd} ± 0.52	46.37 ^{ab} ± 2.59
4	-1 (30)	0 (4)	-1 (20:80)	29.74 ^f ± 2.27	45.45 ^{abc} ± 2.62
5	1 (60)	0 (4)	1 (80:20)	56.30 ^{cd} ± 2.15	38.45 ^{bcd} ± 3.02
6	0 (45)	0 (4)	0 (50:50)	54.46 ^{cd} ± 2.91	48.92 ^a ± 0.75
7	0 (45)	1 (5)	-1 (20:80)	60.35 ^{bc} ± 4.30	49.94 ^a ± 1.91
8	-1 (30)	0 (4)	1 (80:20)	30.28 ^f ± 2.91	49.14 ^a ± 2.38
9	0 (45)	0 (4)	0 (50:50)	52.77 ^{de} ± 2.13	46.48 ^{ab} ± 1.87
10	1 (60)	-1 (3)	0 (50:50)	65.18 ^b ± 3.55	48.77 ^a ± 3.14
11	0 (45)	0 (4)	0 (50:50)	55.33 ^{cd} ± 3.51	47.68 ^a ± 2.53
12	1 (60)	0 (4)	-1 (20:80)	56.04 ^{cd} ± 3.80	48.65 ^a ± 1.04
13	0 (45)	1 (5)	1 (80:20)	55.55 ^{cd} ± 1.06	37.05 ^{cd} ± 2.63
14	-1 (30)	1 (5)	0 (50:50)	59.11 ^{bcd} ± 4.01	48.14 ^a ± 3.81
15	-1 (30)	-1 (3)	0 (50:50)	46.06 ^e ± 2.79	47.11 ^{ab} ± 2.94

Note: Results are presented as mean ± standard deviation. AR: anthocyanin recovery; PC: polymeric color. Different letters in the same column show significant differences between the assays ($p < 0.05$) by the Tukey test.

Although the temperature has a linear positive behavior for the recovery of anthocyanins, several studies have shown that temperatures above 60 °C using PLE can lead to the degradation of these compounds (PEREIRA et al., 2019; TEIXEIRA et al., 2021). In the case, for the extraction of anthocyanins from black rice, the optimized temperatures ranged from 30 to 50 °C (DAS; GOUD; DAS, 2017; HE et al., 2017; JHA; DAS; DEKA, 2017; PEDRO; GRANATO; ROSSO, 2016; YI et al., 2021). High temperature leads to the degradation of anthocyanins by their polymerization, hydrolysis, or ring fission (RODRIGUEZ-AMAYA, 2019). It was observed that higher ethanol concentration (Figure 3.2D-F) could lead to higher polymerization of anthocyanins. It was also observed (Figure 3.2B-C) that solvent concentrations close to 50% of citric acid solution (0.1 M) and ethanol presented higher AR values. Le et al. (2019) related that citric acid might act against the polymerization of anthocyanin-rich extracts. Usually, a pH between 1.0 to 3.0 increases anthocyanins' stability and improves the extraction of these compounds due to cell disruption by acid hydrolysis (ZIELINSKI et al., 2021). Lao & Giusti (2018) observed that the proportion of ethanol from 40 to 60% showed higher values in the TMA extraction, with 50% also showing a higher content of total phenolic compounds. Therefore, regarding the solvent, the proportion containing 50% citric acid (0.1 M) and 50% ethanol was considered ideal for the extraction of

anthocyanins (pH $\approx$ 2.8), aiming at a higher extraction yield and less degradation of these compounds. Although pH was higher than 1.0 for solutions containing 80% citric acid 0.1 M, the maximum extraction was not obtained. For solutions containing 20% acid, the pH was higher than 3.0 and impaired anthocyanin extraction yield.

Figure 3.2 Three-dimensional (3D) response surfaces show the effect of independent variables (temperature, flow rate, and solvents) on the dependent variables AR (A, B, and C) and PC (D, E, and F) for anthocyanin-rich extraction by PLE.



Therefore, combining the results obtained by the optimization models and considering the practicality of the method, the optimal extraction conditions were: temperature of 55 °C, a flow rate of 5 mL/min, and 50:50 (v/v) of 0.1 M citric acid:ethanol. The optimization point was externally validated, providing value within the predicted intervals (solving equation 3.13) for a 95% confidence level (observed: $79.22\% \pm 2.68$, predicted: 75.86%). With that, the proposed model can predict the AR response.

3.11.2 Comparison between extraction methods

In terms of comparison, the heating-stirring extraction (HSE) method was also performed, considered a conventional method. The process parameters used are shown in Section 3.4 and were optimized by Das et al. (2018). The result obtained for AR was $45.59 \pm 2.02\%$, corresponded to 1.17 mg/g of anthocyanins, being practically half of that recovered by PLE (2.30 mg/g). Laokuldilok et al. (2011) extracted between 1.13 to 2.56 mg C3GE/g of anthocyanins from BRB using stirring at 4 °C for 24 h (acidified methanol as solvent). Regarding emerging technologies, Abdel-Aal et al. (2014) extracted 2.0 mg C3GE/g of anthocyanins from black rice by microwave-assisted extraction (MAE).

The cyanidin-3-O-glucoside (C3G) is the main anthocyanin from black rice bran (HUANG; LAI, 2016; LOYPIMAI et al., 2015), containing at least 70% of all. In our study, the extracts showed 1.07 mg/g and 1.63 mg/g of C3G for the HSE and PLE extracts, respectively. In the study by Laokuldilok et al. (2011), three different species of black rice bran obtained C3G values between 0.66 mg/g and 2.31 mg/g (58% to 95% of total anthocyanin). Related to TMA results, about 70% of anthocyanins are C3G for PLE, while for HSE extract, about 92% are C3G.

In addition to these values, Table 3.2 also presents data on PC, total phenolic content (TPC), antioxidant activity (DPPH, ABTS, and FRAP), and the color of these optimized extracts. In addition to the TMA value having shown a significant difference ($p < 0.05$), all the other parameters also presented superior values that differed significantly (except for color). Regarding PC (Table 3.2), PLE showed a higher value than HSE, suggesting lower maintenance of the anthocyanin integrity (BENVENUTTI; ZIELINSKI; FERREIRA, 2022). Agcam et al. (2021) reported that the main factors affecting the polymeric color of anthocyanin-rich extracts are pressure, temperature, and time. However, Vergara-Salinas et al. (2013) reported that the time used (5-30min) did not affect the degradation of extracts evaluated by PC. Therefore, although HSE has a longer extraction time (22 min), it uses room temperature and does not use pressure variation, which explains the lower PC value.

Table 3.2 Characterization of the extracts obtained by HSE and optimized PLE.

Analysis	HSE	PLE
AR (%)	45.59 ± 2.08 ^b	79.22 ± 2.68 ^a
TMA (mg C3GE/g DW)	1.16 ± 0.05 ^b	2.30 ± 0.08 ^a
C3G (mg/g DW)	1.07 ± 0.01 ^b	1.63 ± 0.02 ^a
PC (%)	35.37 ± 2.77 ^b	44.52 ± 0.51 ^a
TPC (mg GA/g DW)	4.24 ± 0.19 ^b	10.74 ± 0.12 ^a
DPPH (μmol TE/g DW)	46.03 ± 1.25 ^b	65.24 ± 1.44 ^a
ABTS (μmol TE/g DW)	65.90 ± 6.90 ^b	103.3 ± 7.29 ^a
FRAP (μmol TE/g DW)	96.43 ± 3.63 ^b	260.79 ± 5.08 ^a
L*	24.25 ± 0.80 ^a	14.01 ± 0.29 ^b
a*	38.75 ± 0.50 ^a	29.44 ± 0.42 ^b
b*	26.05 ± 0.70 ^a	10.68 ± 0.42 ^b
C*	46.70 ± 0.79 ^a	31.32 ± 0.51 ^b
°h	33.92 ± 0.43 ^a	19.95 ± 0.56 ^b

Note: Results showed a mean ± standard deviation. TMA: total monomeric anthocyanins; AR: anthocyanin recovery; C3GE: cyanidin-3-glucoside equivalents; TPC: total phenolic compounds; GAE: gallic acid equivalents; C3GE: cyanidin-3-glucoside; TE: Trolox equivalents; HSE: heating-stirring extraction and PLE: pressurized liquid. Different letters on the same line indicate significant differences ($p < 0.05$) between the experiments using by t test.

Regarding the total phenolics content (TPC), it was observed that the extraction by PLE showed a value about 64% higher than that extracted by HSE. Pedro et al. (2016) reported values close to TPC for black rice, equal to 5.2 mg GA/g, also obtained by the HSE method. Das et al. (2018), when using a conventional extraction process in a water bath, related 8.52 mg GAE/g of TPC from BRB, however in another study by Das et al. (2017), using UAE as an emerging technique, the authors showed higher TPC values (19.57 mg GAE/g).

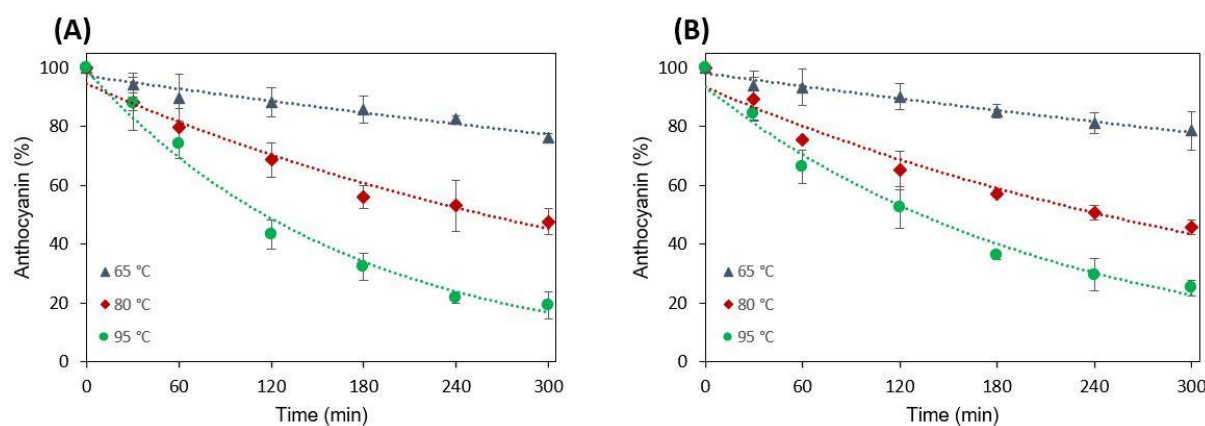
By the DPPH, ABTS, and FRAP methods, the antioxidant activity of the extracts was measured. Higher values (Table 3.2) were presented by the extract obtained by PLE ($p < 0.05$). Antioxidant activity by DPPH was also evaluated by Pang et al. (2018), in which they reported values of seven different species of black rice between 13.5 μmol TE/g up to 35.5 μmol TE/g (extraction by mechanical shaker for 30 min at room temperature). Halee et al. (2020) optimized the extraction by MAE of antioxidant compounds from black rice, obtaining a value for DPPH equal to 109.4 μmol TE/g. For the antioxidant activity measured by ABTS, the values obtained for PLE in our study were higher than those reported by Finocchiaro et al. (2010), which reported a maximum value equal to 94.81 μmol/g for a species of black rice. As for FRAP, the value obtained by HSE was similar to the study by Halee et al. (2018), equal to 65.14 ± 1.36 μmol TE/g and 70.48 ± 0.91 μmol TE/g by conventional extraction, using acidified water or acidified ethanol (both with 0.1 M citric acid) as the solvent, respectively. For the extract obtained by PLE, there was a significant increase ($p < 0.05$) in the content of antioxidant activity measured by FRAP.

Regarding the color parameters (Table 3.2), PLE showed a lower luminosity value (L^*) than HSE, indicating that the sample is darker. Positive a^* values indicate a tendency towards red colors attributed to the high content of anthocyanins. In addition, positive b^* values indicate a tendency towards yellow. However, a significant influence on the colors of the extracts was not noticed. Chromaticity (C^*), which measures the color saturation or intensity, and hue angle value ($^{\circ}h$) related to color had a high value for both samples. The lowest $^{\circ}h$ and high C^* and a^* (red color intensity) from PLE extract is possibly due to the low pH and high concentration of TMA, which maintains the conformation of anthocyanin color, predominantly flavylum cation (BRITO et al., 2017; MOJICA; BERHOW; GONZALEZ DE MEJIA, 2017).

3.11.3 Anthocyanin stability

Figure 3.3 (A and B) shows the results obtained from the temperature degradation of the extracts obtained by HSE and PLE, respectively. The thermal degradation curves obtained the kinetic (Table 3.3) and thermodynamic (Table 3.4) parameters.

Figure 3.3 Thermostability of anthocyanin-rich extracts obtained by HSE (A) and PLE (B) at 65, 80, and 95 °C. The dashed lines correspond to the fit to a first-order degradation model.



The degradation increased as the temperature increased, and both extracts showed similar degradation kinetics, with slight variation in half-life time. Guimarães et al. (2018) reported half-life times for C3G of 9.23, 3.30, and 2.0 h at 65, 80, and 95 °C, while Yan et al. (2016) reported half-life times of 24.93, 8.02, and 4.48 h at temperatures of 65, 80, and 95 °C. Our extracts showed values between both cited studies, and the differences in half-life times possibly occurred due to the different concentrations of added extract and the final pH of extracts. According to Benvenuti et al. (2022), the hydrolysis of the glycosidic bond leads to the thermal degradation of anthocyanins, forming cyanidin (aglycone), which is a more unstable compound and consequently discolors more quickly than the glycosidic form.

According to Yang et al. (2019) and Teixeira et al. (2021), activation energy (E_a) is a parameter used to describe the energy required to reach the transition state of a reaction. The values of $\ln k_d$ (Table 3.3) were used against $1/T$ and fitted by the Arrhenius equation to obtain E_a values. It was observed (Table 3.4) that the extract obtained by HSE presented an E_a value 11.5% higher than the extract obtained by PLE.

A result similar to that of the PLE extract was presented by Yan et al. (2016) for C3G from black rice. E_a is the energy required to perform the degradation (LEONARSKI et al., 2022); therefore, the sample obtained by HSE showed better stability compared to PLE. According to Hou et al. (2013), the activation energy tends to decrease with increasing pH due to its loss of stability as the pH approaches neutral. The authors reported E_a values between 67.38 kJ/mol and 68.16 kJ/mol for C3G from black rice extract, which is close to those reported in our study in which pH 3.5 was used.

Table 3.3 Kinetics parameters of thermal and light stability of anthocyanin-rich extracts obtained by HSE and PLE.

	T (°C)	k_d (min ⁻¹)	$t_{1/2}$ (h)	R^2	Light	k_d (min ⁻¹)	$t_{1/2}$ (h)	R^2
HSE	65	0.00076	15.20	0.934	Fluorescent	0.00446	6.48	0.807
	80	0.00250	4.62	0.974				
	95	0.00590	1.96	0.989				
PLE	65	0.00077	15.00	0.976	Fluorescent	0.00656	4.40	0.967
	80	0.00260	4.44	0.963				
	95	0.00470	2.46	0.985				

Note: HSE: heating-stirring extraction; PLE: pressurized liquid extraction; k_d : degradation rate; R^2 : determination coefficient; $t_{1/2}$: time of half-live.

Table 3.4 provides the results for the activation enthalpy (ΔH), the Gibbs free energy of inactivation (ΔG), and the activation entropy (ΔS) for the degradation of anthocyanin-rich extracts obtained by HSE and PLE extractions.

The activation enthalpy (ΔH) value is a measurement of the endothermic state between the reactant and the activated complex (CONDURACHE et al., 2021); that is, it is a measure of the energy barrier that must be overcome by the reactant molecules, representing to the strength of the bonds that are broken and rebuilt during the transition state (BENVENUTTI; ZIELINSKI; FERREIRA, 2022). Positive values for both extracts (HSE and PLE) indicate that the degradation reaction was endothermic (PERON; FRAGA; ANTELO, 2017), and in addition, the lower the ΔH values, the lower the potential energy barrier, indicating that these

are more susceptible to being transformed into other states, and in our case, more susceptible to degradation (CHEOK; RAGUNATHAN, 2022). Benvenuti et al. (2022) presented values between 59-63 kJ/mol for anthocyanin-rich extracts from jaboticaba (*Myrciaria cauliflora*) extracted with water or water acidified by the PLE method, while Cheok & Ragunathan (2022) related ΔH values close to 51 kJ/mol for anthocyanin extract from hibiscus (*Hibiscus sabdariffa* L.). Regarding ΔG , positive values indicate nonspontaneous reaction (PERON; FRAGA; ANTELO, 2017). The close values obtained by both extractions can be explained by the fact that anthocyanins are from the same source. Negative ΔS values represent that this thermal degradation process is irreversible and that the transition molecules are more organized than those in the initial reaction (CONDURACHE et al., 2021; SHARMA; DASH, 2022).

Table 3.4 Thermodynamic parameters of the anthocyanin degradation in the extracts obtained by HSE and PLE.

	E_a (kJ/mol)	R^2	Temperature (°C)	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (kJ/mol K)
HSE	70.82	0.995	65	68.01	206.47	-0.459
			80	67.89	213.02	-0.459
			95	67.76	220.26	-0.460
PLE	62.68	0.971	65	59.86	205.55	-0.477
			80	59.74	213.30	-0.479
			95	59.62	219.85	-0.478

Note: HSE: heating-stirring extraction; PLE: pressurized liquid extraction; E_a : activation energy; ΔH : activation enthalpy; ΔG : free energy of activation; ΔS : activation entropy.

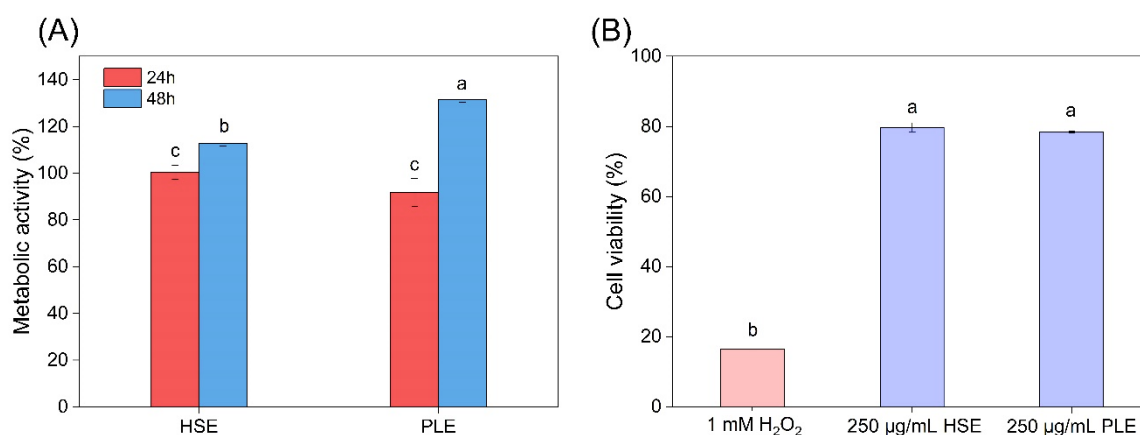
Table 3.3 also shows the results obtained for the degradation of the extracts obtained by HSE and PLE submitted to fluorescent light (Section 3.8.2). It was observed by the half-life times that the extract obtained by HSE presented higher stability when compared to PLE. Yan et al. (2016) presented a half-life time for C3G obtained from black rice of 4.72 days, which is close to that obtained in our study for the extract obtained by PLE.

3.11.4 Cell culture

Cytotoxicity assays are a quick and straightforward way to perform initial acute toxicity assessments (LOPEZ-CORONA et al., 2022; SONI et al., 2021). Direct contact tests with L929 cells evaluated the cytotoxicity of the anthocyanin-rich extracts, and the results are shown in Figure 3.4A. L929 cells (derived from the normal tissue of the mouse) are commonly used as reference cells to test the cytotoxicity of polymers or natural plants (Wang et al., 2017). It can

be observed that both extracts (500 µg/mL) did not lead to a decrease in the metabolic activity of the L929 cell, indicating that they do not present cytotoxicity in 48 h. Wang et al. (2017) reported that blueberry anthocyanins (12.5–800 µg/mL) did not show cytotoxicity for L929 cells in 72 h, being extracted by the maceration method (24 h at room temperature in the dark.). In the study by Aprodu et al. (2019), the authors evaluated the cytotoxicity of the microencapsulated black rice extract, showing no effect on L929 cells (10 and 500 µg/mL) in 48 h. On the other hand, concentrations of 750–1000 µg/mL induced a decrease in cell viability (80%). In the study by Sangkitikomol et al. (2010), UAE was used as the extraction method; the authors reported that the anthocyanin-rich extract showed cytotoxicity at high doses (≥ 800 mg/L). According to ISO 10993-5:2009 (2009), the minimum cell viability for L929 cells is 70%; therefore, the extracts did not show cytotoxicity at the evaluated concentrations.

Figure 3.4 (A) Metabolic activity of L929 cells treated with the anthocyanin-rich extract (HSE and PLE) for 24 and 48h. (B) Viability of L929 cells treated with the anthocyanin-rich extract (HSE and PLE) with concomitantly H₂O₂ exposure for 24 h.



Note: Different letters indicate significant differences by Tukey test ($p < 0.05$).

Antitumoral activity of extracts was performed on human breast cells (MDA-MD-231), embryonic kidney cells (HEK-293), lung adenocarcinoma cells (A549), melanoma cells (B16F10), and malignant skin melanoma cells (SK-MEL- 28) presented in the Appendix (Figure S3 - Appendix D). No antitumor activity was found at the extract concentrations used (500 µg/mL), except for the HSE extract in MDA-MD-231 cells, which showed a slight decrease (13.1%) at 48h. Although it did not show activity in our study, Luo et al. (2014) reported that at a concentration of 100-200 µg/mL anthocyanin-rich extracts from black rice have anticancer potential. The authors obtained these results through analyzes of migration, adhesion, motility, and invasion inhibited by the extracts in MDA-MB-453 cells in a

concentration-dependent manner. Liu et al. (2013) reported that both compounds (peonidin-3-O-glucoside (P3G) and cyanidin-3-O-glucoside) isolated from black rice extracts also have antitumor activity by inducing HER2-positive breast cancer cells apoptosis.

The cytoprotective effect of black rice extracts on H₂O₂-induced oxidative stress in L292 cell are shown in Figure 3.4B. It can be observed that both extracts (500 µg/mL) showed cytoprotection (values close to 80%) and showed no significant difference ($p < 0.05$). In the study by Sangkitikomol et al. (2010), the authors reported that concentrations >600 mg/L of anthocyanin-rich extract from black rice significantly reduced oxidative stress in HepG2 cells. In the study by Boonyanuphong & Tobgay (2022), the oxidative stress in HT-29 cells was significantly reduced when using a concentration of 1.25 mg/mL of black rice extract.

The protective effect of HSE and PLE extracts is due to their antioxidant activity (corroborating with the results shown in Table 3.2), mainly coming from anthocyanins. H₂O₂ molecules are free radicals that can result from oxidative reactions, playing an important role in tissue damage (XU et al., 2013). Due to this, it is speculated that anthocyanins can improve wound healing (PITZ et al., 2016; XU et al., 2013). Therefore, future studies aiming at applying these extracts in this field can be evaluated.

3.12 CONCLUSION

It was possible to obtain high concentrations of anthocyanin-rich extracts from black rice bran by PLE using the Box-Behnken design. About 80% of total monomeric anthocyanins (2.30 mg C3GE/g) were recovered using the optimal point (55 °C, 5 mL/min flow rate, and ratio (50:50, v/v) of citric acid 0.1 M: ethanol). The recovery of anthocyanins was higher by PLE when compared to extraction by heating-stirring extraction (HSE), in which it obtained about 44%. Both extracts showed high stability at lower temperatures. There was no evidence of cytotoxicity in the PLE, and HSE extracts evaluated by L929 cells. The extracts showed cytoprotection of L929 cells when subjected to oxidative stress with H₂O₂. The cytoprotective effect is directly linked to the antioxidant effect present in the extracts.

The recovery of anthocyanins from black rice bran by PLE proved efficient, being a method considered eco-friendly and adding value to a by-product that would be discarded. The obtained extract showed a high biological value and can be applied in several areas, such as food, cosmetic, and biomedical industries. Due to its cytoprotective factor, future studies can be carried out to identify effects on wound healing.

4 ULTRASOUND AND MICROWAVE ASSISTED EXTRACTIONS AS GREEN AND EFFICIENT APPROACHES TO RECOVER ANTHOCYANIN FROM BLACK RICE BRAN

The information brought in this chapter was compiled in a scientific paper entitled "*Ultrasound and Microwave assisted extraction as green and efficient approaches to recover anthocyanin from black rice bran*", which was published in the journal "Biomass Conversion and Biorefinery" (Qualis A4; Impact Factor 3.5). This study aimed to optimize the recovery of anthocyanin-rich extract from black rice bran by ultrasound-assisted (UAE) and microwave-assisted extraction (MAE) methods. The temperature and fluorescent light stability of anthocyanin-rich extracts obtained by UAE and MAE were also evaluated, as well as cytotoxicity activity. Therefore, this chapter contains both the methodology and the respective results.

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ORIGINAL ARTICLE



Ultrasound and microwave-assisted extractions as green and efficient approaches to recover anthocyanin from black rice bran

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Abstract

Extraction techniques are one way to separate bioactive compounds from agro-industrial material. Emerging technologies for the extraction of bioactive compounds, such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), are preferable to conventional extraction techniques because they are more efficient, spend less time, and are environmentally friendly. This study compared ultrasound (UAE) and microwave (MAE) assisted extraction techniques to recover anthocyanins from black rice bran. By optimizing using Box Behnken design, for UAE, 83.73% of the total anthocyanins (2.44 mg C3G/g) were recovered, using as parameters temperature 50°C, a frequency 380 W, and a solvent 60:40 (v/v) citric acid 0.1 M:ethanol. For MAE, it was possible to recover 81.44% of the total anthocyanins (2.37 mg C3G/g), using parameters temperature 55°C, citric acid 0.1M:ethanol (50:50, v/v), and a mass of 0.8 g (1:24, g/ml). Both techniques were considered ecologically green by the Green Certificate (A) and excellent methods by EcoScale (scores higher than 75). Anthocyanin-rich extracts did not show cytotoxicity for L292 cells (1000 µg/mL) and showed cytoprotection (> 92% using 500 µg/mL) when subjected to oxidative stress with H₂O₂. Both extracts showed technological potential as a natural dye and as an ingredient of nutraceutical products.

ABSTRACT

Extraction techniques are one way to separate bioactive compounds from agro-industrial material. Emerging technologies for the extraction of bioactive compounds, such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), are preferable to conventional extraction techniques because they are more efficient, spend less time, and are environmentally friendly. This study compared ultrasound (UAE) and microwave (MAE) assisted extraction techniques to recover anthocyanins from black rice bran. By optimizing using Box Behnken design, for UAE, 83.73% of the total anthocyanins (2.44 mg C3G/g) were recovered, using as parameters temperature 50 °C, a frequency 380 W, and a solvent 60:40 (v/v) citric acid 0.1 M:ethanol. For MAE, it was possible to recover 81.44% of the total anthocyanins (2.37 mg C3G/g), using parameters temperature 55 °C, citric acid 0.1M:ethanol (50:50, v/v), and a mass of 0.8 g (1:24, g/ml). Both techniques were considered ecologically green by the Green Certificate (A) and excellent methods by EcoScale (scores higher than 75). Anthocyanin-rich extracts did not show cytotoxicity for L292 cells (1000 µg/mL) and showed cytoprotection (> 92% using 500 µg/mL) when subjected to oxidative stress with H₂O₂. Both extracts showed technological potential as a natural dye and as an ingredient of nutraceutical products.

Keywords: Anthocyanin-rich extracts, Box-Behnken design, *Oryza sativa* L., Stability, Cytotoxicity.

4.1 INTRODUCTION

Some parts of plant-based products can be wasted during the food process, generating residues or by-products. These by-products have greatly increased in recent years. However, they generally present high concentrations of bioactive compounds and have been investigated as a cheap and available source of interest compounds for food and pharmaceutical applications. (DAS; GOUD; DAS, 2017; LEONARSKI et al., 2023a). In the milling step during black rice processing, about 10% is lost, producing black rice bran, which is generally used as animal feed (HALEE et al., 2020). Among the main compounds in black rice, anthocyanins are considered one of the main ones due to their great biological potential. Anthocyanins are water-soluble natural colorants, and the intake of that compounds are related to health benefits: anti-obesity, antidiabetic, antimicrobial, anticancer, anti-cardiovascular, and neuroprotective effects (LUZARDO-OCAMPO et al., 2021; TENA; MARTÍN; ASUERO, 2020).

Anthocyanins are water-soluble natural colorants, and the intake of those compounds is related to health benefits: anti-obesity, antidiabetic, antimicrobial, anticancer, anti-cardiovascular, and neuroprotective effects (LUZARDO-OCAMPO et al., 2021; TENA; MARTÍN; ASUERO, 2020). In the literature, anthocyanins are generally quantified as total monomeric anthocyanins by spectrophotometric methods, and the main compounds are identified through mass chromatography (LEE; RENNAKER; WROLSTAD, 2008), with cyanidin-3-glucoside (C3G) being one of the majority anthocyanins in several products rich in anthocyanins, including black rice (LEONARSKI et al., 2023b).

Thus, extraction techniques are one way to separate these bioactive compounds (LEONARSKI et al., 2023b). Conventional extraction techniques generally consume a lot of solvents, in addition to being time-consuming and not very efficient (HALEE et al., 2020) and therefore, new extraction methods have been developed to be more efficient with less time, in addition, to being environmentally friendly (DAS; GOUD; DAS, 2017; FERNANDES et al., 2021).

Among the emerging technologies for the extraction of bioactive compounds, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) stand out. In the case of UAE, the extraction mechanism occurs through cavitation, in which bubble formation occurs, generating microturbulence and leading to cell wall breakdown (PERERA; ALZAHIRANI, 2021; SHIRSATH et al., 2017). In general, the generation of sound waves leads to the production of bubbles, and the growth of these bubbles is related to the expansion and compression cycles that affect the cell structure, leading to the separation of the material

molecule (MOREIRA et al., 2019). This process consists of a strong vibration between the molecules of the sample, which causes the rupture of bubbles in the cell wall, damaging its structure and facilitating mass transfer (MOREIRA et al., 2019; TENA; ASUERO, 2022). The main advantages of this technique are the greater penetration into the cellular material, which achieves better yields in shorter times, causing low solvent consumption. In addition, it allows the extraction of thermolabile compounds, uses less energy, and is considered an ecological technology (CHEMAT et al., 2017; MOREIRA et al., 2019).

In the study by Thakur et al. (2022), the authors extracted anthocyanins from black rice bran using UAE, with different concentrations of natural deep eutectic solvents (NADES), obtaining the best efficiency (anthocyanin ≈ 30 mg/L) using lactic acid:fructose as the solvent, water 12.63%, solvent-to-solid ratio 18.75 mL/g, amplitude 21.31%, and 11.25 min. In addition to the parameters already studied, the temperature and solvent concentrations (0.1 M citric acid: ethanol, v/v) will be evaluated in our study.

In the case of MAE, the generated electromagnetic radiation is absorbed by the polar molecules present in the solvent and food, producing a dipolar rotation in the molecules and the migration of ions. Cell rupture occurs due to the high pressure generated by the vaporization of water from the microwaves that act on the cells (COELHO et al., 2020; TENA; ASUERO, 2022). The main advantages of this technique are similar to UAE, shorter extraction times, higher yields, lower costs with solvent, and being environmentally friendly (BAGADE; PATIL, 2021).

Among the studies that carried out extraction of black rice and its by-products by MAE, in the study by Moirangthem et al. (2021), microwave-assisted subcritical water extraction was used to extract anthocyanins from the straw, varying the temperature (90 °C and 100 °C), with a maximum efficiency of about 0.52 mg C3G/g. In the study by Abdel-Aal et al. (2014), for the extraction of anthocyanins from black rice, the authors varied temperature, power frequency, and time and used acetone-water (40/60, v/v) as the solvent, obtaining a maximum efficiency of 2.0 mg/g. In the study by Jha et al. (2017), the authors extracted polyphenols from black rice husk UAE followed by MAE, obtaining anthocyanins equal to 0.03 mg/g. In this study, using MAE to extract anthocyanins from black rice bran will be optimized by varying the temperature, solvent concentration (0.1 M citric acid:ethanol), and sample mass (g) (mass/volume ratio, g/mL).

Therefore, this study aims to optimize the extraction of anthocyanins from black rice bran by UAE and MAE techniques as green extraction approaches. Green Certified and EcoScale analyses were carried out to verify the UAE and MAE techniques as eco-friendly

approaches. Furthermore, these extracts' temperature and fluorescent light stability were also evaluated, as well as the cytotoxicity and antitumoral activity of anthocyanin-rich extracts.

4.2 MATERIAL AND METHODS

4.2.1 Materials

The black rice bran was harvested in the February-May (2022) crop, and the bran comes mainly from the polishing/milling stage of rice processing, supplied by Ruzene (Pindamonhangaba, Brazil). Analytical standard of anthocyanin (cyanidin-3-O-glucoside), the Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid), TPTZ (2,4,6-tri(2-pyridyl)-s-triazine), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), Folin-Ciocalteu, Sodium carbonate were purchased from Sigma-Aldrich (Steinheim, Germany). Dulbecco's Modified Eagle's medium (DMEM), TrypLE Express enzyme, and phosphate-buffered saline (PBS) were purchased from Gibco. CellTiter 96® AQueous One Solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] was purchase from Promega Biotecnologia do Brasil.

4.2.2 Sample preparation

The proximate composition of black rice bran (BRB) and sample preparation are described by (Leonarski et al., 2023b). The sample used has a particle size between 20 to 32 mesh.

4.3 ANTHOCYANIN EXTRACTION

4.3.1 Ultrasound-assisted extraction (UAE)

An ultrasonicator with a probe (Eco-Sonics, Ultronique Q3.0/37A, Brazil) was used to extract anthocyanin from black rice bran. Initially, was used 0.5 g of BRB and 15 mL of solvents (1:30) for kinetics (using the center point parameters, temperature 45°C, frequency power 300 W, and solvent (50:50, v/v) citric acid 0.1 M:ethanol) to evaluate the best time to extract anthocyanins from BRB. The extraction assays following a Box-Behnken design, with 15 experiments performed randomly. The factors evaluated (independent variables) were temperature (x_1 : 30, 45, and 60 °C), frequency power (x_2 : 150, 300, and 450 W), and solvent concentration (x_3 : 20:80, 50:50 and 80:20, citric acid 0.1 M:ethanol, v:v).

4.3.2 Microwave-assisted extraction (MAE)

For MAE, the extractions were conducted in a microwave reactor (Monowave™ 200, Anton Paar). The extraction time was defined through a kinetics study (temperature 45 °C, solvent (50:50, v/v) citric acid 0.1 M:ethanol, and mass sample 0.67 g (1:30). The extraction assays following a Box-Behnken design, with 15 experiments performed randomly. The factors evaluated (independent variables) were temperature (x_1 : 40, 55, and 70 °C), solvent concentration (x_2 : 20:80, 50:50 and 80:20, citric acid 0.1 M:ethanol, v:v), and sample mass (x_3 : 0.33, 0.67, and 1.0 g).

4.3.3 Optimization of anthocyanin recovery

The dependent variable used in the optimization methodology by response surface methodology (RSM) was Anthocyanin Recovery (AR). A second-order polynomial model was used to fit the experimental data, according to Equation (5.1):

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{j \leq i \leq 1}^k \beta_{ij} x_i x_j \quad (5.1)$$

Where y is the predicted response, β_0 is the intercept, β_i , β_{ii} , and β_{ij} are the regression coefficients for linear, quadratic, and interaction terms, respectively, and x_i and x_j are the independent variables. Through the significant factors, the model was initially evaluated. Non-significant effects (ANOVA, $p > 0.05$) were removed, and parameters were refitted. The parameters p -lack of fit and coefficient of determination (R^2 and R^2 adjusted) were investigated to assess the adequacy and quality of the fit. The optimized values' relative error (RE) was calculated according to Benvenuti et al. (BENVENUTTI; ZIELINSKI; FERREIRA, 2022).

4.4 CHARACTERIZATION OF ANTHOCYANIN-RICH EXTRACTS

4.4.1 Total monomeric anthocyanin (TMA), Anthocyanin recovery (AR), and Polymeric Color (PC)

For the analysis of TMA, 20 μ L of extract in microplates were added to 280 μ L of a buffer solution with pH 1.0 (potassium chloride 0.025 mol/L) and pH 4.5 (sodium acetate 0.4 mol/L), according to the differential pH methodology (GIUSTI; WROLSTAD, 2001). Then the absorbances were read at 520 and 700 nm (Multileader Infinite M200 TECAN, ZH, Switzerland). The concentrations of TMA were calculated using Equations (5.2) and (5.3):

$$A = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5} \quad (5.2)$$

$$TMA = \frac{A \cdot MW \cdot DF \cdot 1000}{\epsilon} \quad (5.3)$$

where A is the calculated absorbance, MW is the molecular weight of anthocyanin standard (cyanidin-3-glucoside: 449.2 g/mol), DF is the dilution factor (15), and ϵ is the molar absorptivity of cyanidin (26900).

The anthocyanins recovery (AR) was performed according to Leonarski et al. (2023b). Three sequential extractions were performed using 50 mL of 80% ethanol solution acidified with 0.1 mol/L of HCl for 0.5 g of the sample and stirring at room temperature for 30 min. Afterward, another extraction was performed using 50 mL of 70% acetone solution, also under stirring at room temperature for 30 min, for total anthocyanin content (TAC). To calculate AR (%), the TMA result (Equation 5.3) obtained for each assay was used and finally calculated by Equation (5.4):

$$\%AR = \left(\frac{TMA}{TAC} \right) \cdot 100 \quad (5.4)$$

The polymeric color (PC) was measured by diluting the extracts in potassium chloride (pH 1.0) and subsequently bleaching them with potassium metabisulfite (0.9 M). The metabisulfite bleached sample (PC) and the control sample (DC) were used for the calculations by Equation 5.5, in which A_{420} , A_{520} , and A_{700} are the absorbances at 420, 520, and 700 nm, respectively. The % PC was determined by Equation 5.6:

$$DC \text{ or } PC = [(A_{420} - A_{700}) + (A_{520} - A_{700})] \cdot DF \quad (5.5)$$

$$\%PC = \left(\frac{PC}{DC} \right) \cdot 100 \quad (5.6)$$

Where DF is the dilution factor.

4.4.2 Total phenolic content (TPC)

Adapted for microplates, the total phenolic content (TPC) was evaluated based on the colorimetric methods described by Singleton et al. (1999). TPC was expressed as mg GAE (gallic acid equivalent) per g of dry sample (mg GAE/g).

4.4.3 Antioxidant activity

The antioxidant potential was evaluated by DPPH and ABTS radical scavenging methods according to Brand-Williams et al. (1995) and Re et al. (1999), respectively, with adaptations to the microplate reader.. The antioxidant activity was expressed as μ mol of Trolox equivalent per g of dry sample (μ mol TE/g).

4.4.4 Individual anthocyanin by HPLC-MS

The individual anthocyanins were determined by high-performance liquid chromatography (HPLC) was described by Leonarski et al. [5]. The HPLC system (model LCMS-2020 Prominence, Shimadzu, Kyoto, Japan) consisted in a degassing unit (DGU-20A3),

binary pump (LC-20AD), autosampler (SIL-20AC HT), column oven (CTO-20A), photodiode array detector (PDA, SPD-M20A) and a single-quadrupole MS detector with electrospray ionization (ESI) interface (MS 2020, Shimadzu). The system was controlled by a CBM-20A unit. Chromatographic analysis and treatment of generated data was performed with the LabSolutions® (Shimadzu, Japan) v. 5.109 software.

To achieve separation, a Kromasil® C18 column (100Å, 300 mm x 4.6 mm i.d.) was used as stationary phase, while the mobile phase consisted of aqueous 0.1 % formic acid (eluent A) and acetonitrile (eluent B). Gradient conditions: 0–25 min, 15–45% B linear; 25–30 min, 45–80% B linear; 30–35 min, 80–15% B linear. The flow rate was 0.7 mL/min and the oven was set to 25°C. Samples consisted in 10.0 µL (LC-PDA-only) or 0.1 µL (LC-PDA-MS) of the freeze-dried extracts resuspended in formic acid 0.1% and filtered through a 0.22 µm nylon syringe filter.

PDA detection parameters were: analysis range 190 – 600 nm, with reference (correction) wavelength at 590 ± 10 nm, the cyanin-3-glucoside was detected at 530 nm, eluting with a retention time of 7.9 minutes. MS ESI alternated between positive and negative modes. The interface, desolvation line and heat block temperatures were 350, 250 and 200°C, respectively. Capillary voltage were the same as the autotune (+4.5 kV and -3.5 kV in positive and negative modes, respectively). Nitrogen was used as both nebulizing (1.5 L/min) and drying gas (15 L/min). The detection program cycle consisted of four events, each with a duration of 0.55 seconds: 1. Scan (positive mode, m/z 350 to 590), 2. ion selective mode (SIM, positive mode, m/z = 450 and 491 corresponding to $[M+H]^+$ and $[M+H+MeCN]^+$, accordingly), 3. Scan (negative mode, same range as 1), 4. SIM (negative mode, m/z = 448, $[M-H]^-$ and 490, $[M-H+MeCN]^-$). Full widths at half maximum (FWHM) were set at 0.6 per autotune procedure.

4.4.5 Green metric tools

The sustainability of the extraction processes proposed was evaluated by two green metric tools, Green Certified and EcoScale analyses, as described by Sajid & Plotka-Wasyłka (2022) and Van Aken et al. (2006), respectively. These tools consider the process environmentally safe with a maximum score (100) and consider Penalty Points (PP) to evaluate this process according to tabulated values for different parameters. For Green Certificate, the parameters are the amount and environmental hazard of the solvents, energy, and generated waste. The EcoScale combines the above parameters with process yield, safety, economic and environmental aspects of the extraction.

4.4.6 Cell culture

The methodology for culture condition, cytotoxicity assay (L929 fibroblast cells), Hydrogen peroxide-Induced oxidative stress in L929 fibroblast cells, and antitumoral activity were described by Leonarski et al. (2023b). In this study, concentrations of extracts between 10 and 1000 µg/mL were used (10, 50, 100, 250, 500, 750, and 1000 µg/mL).

4.5 STABILITY OF THE ANTHOCYANIN-RICH EXTRACTS

4.5.1 Thermostability

The extracts were distributed in sealed tubes and finally subjected to temperatures (of 65, 80, and 95 °C) in a thermostatic bath, removed, and immediately cooled at 0, 30, 60, 120, 180, 240, and 300 min. These points were quantified regarding TMA content to obtain their isothermal degradation. A first-order kinetic model was fitted according to Equation (5.7) (BENVENUTTI; ZIELINSKI; FERREIRA, 2022; TEIXEIRA et al., 2021):

$$\frac{C_t}{C_0} = e^{-k_d \cdot t} \quad (5.7)$$

where C_t is the TMA concentration with time t , C_0 is the TMA initial concentration, k_d is the degradation rate (min^{-1}), and t is the time (min).

The time of half-live ($t_{1/2}$) was determined by Equation 5.8, and the activation energy (E_a) was determined through the Arrhenius model by Equation 5.9.

$$t_{1/2} = \frac{\ln(2)}{k_d} \quad (5.8)$$

$$\ln(k_d) = \ln(A) - \frac{E_a}{R \cdot T} \quad (5.9)$$

where A is the frequency factor (min^{-1}), R is the ideal gas constant ($8.314 \text{ J/mol} \cdot \text{K}$), and T is the temperature (K).

For the activation enthalpy (ΔH), the free energy of activation (ΔG), and the activation entropy (ΔS), Equations (5.10), (5.11) and (5.12), respectively, were used for calculations:

$$\Delta H = E_a - R \cdot T \quad (5.10)$$

$$\Delta G = -R \cdot T \cdot \ln \left(\frac{k_d \cdot h}{k_B \cdot T} \right) \quad (5.11)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (5.12)$$

where E_a is the activation energy for the degradation reaction (J/mol), R is the ideal gas constant ($8.314 \text{ J/mol} \cdot \text{K}$), T is the temperature (K), k_d is the kinetic rate constant (s^{-1}), k_B is the Boltzmann constant ($1.3806 \cdot 10^{-23} \text{ J/K}$), and h is Planck's constant ($6.6262 \cdot 10^{-34} \text{ J/s}$).

4.5.2 Photostability

The effect of light was determined at room temperature (25 ± 2 °C). The tubes were exposed to direct light from a white fluorescent lamp (20 W) in a closed wooden chamber (435 x 435 mm) at 0, 90, 180, 270, 360, 450, 540, 1440, 2880, and 4320 min (3 days). The samples were removed and quantified regarding TMA content to obtain their isothermal degradation. The constant rate for anthocyanin degradation (k) and its half-life ($t_{1/2}$) was calculated as described by Equations (5.7) and (5.8).

4.6 STATISTICAL ANALYSIS

The results were presented as mean $\pm$ standard deviation. The dataset was evaluated by one-way analysis of variance (ANOVA), and the significant differences were determined using Tukey's Test at a probability level of less than 5% ($p < 0.05$). The quality and adequacy of the mathematical models were assessed by the determination coefficient (R^2), adjusted R^2 , and the lack of fit (p). ANOVA also determined the statistical significance of the models used. All statistical procedures were performed using Statistica v. 13.5 software (TIBCO Software Inc., Palo Alto, CA, USA).

4.7 RESULTS AND DISCUSSION

4.7.1 Optimization of UAE and MAE extraction conditions

The results of the experimental design obtained by the Box-Behnken design for anthocyanin recovery (AR) showed a significant difference ($p < 0.05$) among all treatment conditions for both techniques, UAE (Table 4.1) and MAE (Table 4.2). The results for AR ranged from 17.56% to 77.13% for UAE and from 29.14% to 86.44% for MAE.

Through the data (Tables 4.1 and 4.2), mathematical models were built to describe the behavior obtained in the extraction of anthocyanins from black rice bran. For the extraction by UAE, the proposed model for AR presented R^2 equal to 0.93, did not present a lack of adjustment ($p = 0.053$), and was able to explain about 90% of the total variance of the experimental data (adjusted $R^2 = 0.89$). As for extraction by MAE, the model also did not show a lack of fit ($p = 0.102$), with R^2 equal to 0.99, representing 98% of the data (adjusted $R^2 = 0.98$). Furthermore, response surfaces were generated, shown in Figure 4.1 (A-C) for UAE and Figure 4.1 (D-F) for MAE.

Table 4.1 Box-Behnken experimental design and UAE results of anthocyanin recovery (AR).

Assay	Independent factors			Response
	Temperature (°C)	Frequency Power (W)	Citric acid : Ethanol (v/v)	AR (%)
1	0 (45)	-1 (150)	1 (80:20)	25.59 ^d ± 1.72
2	1 (60)	1 (450)	0 (50:50)	76.77 ^{ab} ± 4.14
3	0 (45)	-1 (150)	-1 (20:80)	18.11 ^d ± 1.96
4	-1 (30)	0 (300)	-1 (20:80)	38.58 ^c ± 4.17
5	1 (60)	0 (300)	1 (80:20)	59.45 ^b ± 4.77
6	0 (45)	0 (300)	0 (50:50)	68.11 ^{ab} ± 2.93
7	0 (45)	1 (450)	-1 (20:80)	77.56 ^{ab} ± 4.51
8	-1 (30)	0 (300)	1 (80:20)	57.87 ^b ± 4.89
9	0 (45)	0 (300)	0 (50:50)	69.69 ^{ab} ± 2.78
10	1 (60)	-1 (150)	0 (50:50)	26.77 ^{cd} ± 1.47
11	0 (45)	0 (300)	0 (50:50)	66.14 ^b ± 2.76
12	1 (60)	0 (300)	-1 (20:80)	73.23 ^{ab} ± 3.98
13	0 (45)	1 (450)	1 (80:20)	79.13 ^a ± 1.90
14	-1 (30)	1 (450)	0 (50:50)	62.20 ^b ± 4.29
15	-1 (30)	-1 (150)	0 (50:50)	27.56 ^{cd} ± 2.93

Note: Results are presented as mean ± standard deviation. AR: anthocyanin recovery. Different letters in the same column show significant differences between the assays ($p < 0.05$) by the Tukey test.

The effects that were significant for AR (Eq. 4.13) were the positive quadratic effect of temperature (x_1^2), the positive linear effect of frequency power (x_2), the positive quadratic effect of frequency power (x_2^2), and the synergic negative effect between temperature (x_1) and solvent concentration (x_3) ($x_1 \cdot x_3$). Through the significant effects and the response surfaces, it was possible to observe that the higher temperature (Fig. 4.1B) and higher frequency power (Fig. 4.1C) provided the better anthocyanin yield. Regarding both significant effects, frequency power had a superior effect (Fig. 4.1A), as described by Eq. 4.13. However, increasing (to more than 70 °C) or decreasing the temperature and solvent (0.1 M citric acid) could decrease AR yield; that is, the temperatures and solvent concentration close to the central point are the ones that presented the best results. The increase in power frequency, and temperature in our study (which causes an increase in the shock wave by repeating cycles of compression and rarefaction) led to an increase in the effectiveness of anthocyanin extraction due to the rupture of the cell wall caused by both mechanical and thermal reactions, as observed by Boateng et al. (2023).

In the study by Zhang et al. (2020), the increase in frequency power from 200 to 450 W did not result in a significant increase ($p < 0.05$) in the concentration of anthocyanins recovered by UAE from purple barley bran. However, in the study by Rocha & Noreña (2020), in which

anthocyanins were extracted from grape pomace by UAE, it was reported that at the frequency between 250 W and 450 W, there is an increase in yield, with a maximum being reached between 350 and 450 W. This result is close to related in this study, in which the highest frequencies showed the best AR yields.

$$y = 53.43 + 12.50 \cdot x_1 + 49.41 \cdot x_2 + 12.65 \cdot x_2^2 - 16.53 \cdot x_1 x_3 \quad (4.13)$$

Table 4.2 Box-Behnken experimental design and MAE results of anthocyanin recovery (AR).

Assay	Independent factors			Response
	Temperature (°C)	Citric acid: Ethanol (v/v)	Sample mass (g)	AR (%)
1	0 (55)	0 (50:50)	0 (0.67)	79.25 ^a ± 0.91
2	-1 (40)	0 (50:50)	-1 (0.34)	33.77 ^c ± 1.61
3	1 (70)	1 (80:20)	0 (0.67)	57.30 ^b ± 3.16
4	0 (55)	0 (50:50)	0 (0.67)	78.86 ^a ± 3.42
5	0 (55)	0 (50:50)	0 (0.67)	77.58 ^a ± 3.15
6	-1 (40)	1 (80:20)	0 (0.67)	55.03 ^b ± 0.97
7	-1 (40)	-1 (20:80)	0 (0.67)	51.19 ^b ± 2.86
8	1 (70)	0 (50:50)	-1 (0.34)	33.28 ^c ± 1.47
9	-1 (40)	0 (50:50)	1 (1.0)	83.49 ^a ± 2.79
10	1 (70)	0 (50:50)	1 (1.0)	86.44 ^a ± 1.63
11	0 (55)	-1 (20:80)	-1 (0.34)	29.14 ^c ± 4.38
12	1 (70)	-1 (20:80)	0 (0.67)	56.12 ^b ± 1.69
13	0 (55)	1 (80:20)	-1 (0.34)	36.80 ^c ± 1.00
14	0 (55)	-1 (20:80)	1 (1.0)	83.94 ^a ± 2.46
15	0 (55)	1 (80:20)	1 (1.0)	82.60 ^a ± 3.58

Note: Results are presented as mean ± standard deviation. AR: anthocyanin recovery. Different letters in the same column show significant differences between the assays ($p < 0.05$) by the Tukey test.

For the extraction of anthocyanins, very high temperatures are generally not used. In the case of studies that extracted these compounds by UAE, the best temperatures for extraction were equal to 35 °C for haskap berries (CELLI; GHANEM; BROOKS, 2015), 47.1 °C for epicarp jabuticaba (ALBUQUERQUE et al., 2020), and 55.1 °C for eggplant peel (DRANCA; OROIAN, 2016). For black rice, temperatures between 30 °C to 55 °C are generally used (Das et al., 2017; Jha et al., 2017; Leonarski et al., 2023b). Concerning the solvent, it was reported in the extraction of anthocyanins from *Hibiscus sabdariffa* addition of about 40% ethanol (PINELA et al., 2019), while for jambolan and haskap berries, the addition reached 80% (CELLI; GHANEM; BROOKS, 2015; SABINO et al., 2021). In our previous study, when extracting anthocyanins from black rice by pressurized liquid (PLE), it was observed that the proportions close to 50% ethanol and 50% citric acid 0.1 M were the ones that presented better extraction and less degradation (measured by the polymeric color). Therefore, the optimization

was carried out using a temperature of 50 °C, a frequency of 380 W, and a solvent in the ratio of 60:40 (citric acid 0.1 M:Ethanol).

In the case of extraction by MAE, the significant effects were the positive quadratic effect of temperature (x_1^2), the positive quadratic effect of solvent concentration (x_2), the positive linear and (x_3) quadratic effect (x_3^2) of sample mass (Eq. 4.14). The response surfaces (Fig. 4.1D) and the equation 4.14 indicate that both the temperature and the solvent have less influence than sample mass (Fig. 4.1E-F). According to Routray & Orsat (2012), the efficiency of microwave extraction generally increases with increasing temperature until achieving an optimal temperature (along with a solvent/solid ratio). After this temperature, efficiency tends to decrease. This fact can be observed in Figure 4.1D, in which, close to 50 °C, greater extraction efficiency is obtained, and after this temperature, it was observed a decrease in yield. The cell wall rupture occurs due to the high pressure generated through temperature, with heat being produced from the inside to the outside of the material in the opposite direction to what occurs in UAE extraction (TENA; ASUERO, 2022). The conditions that resulted in the better results were similar to those reported in the extraction by UAE: temperature close to 50 °C and solvent in the proportion of 50:50 of citric acid 0.1 M and ethanol.

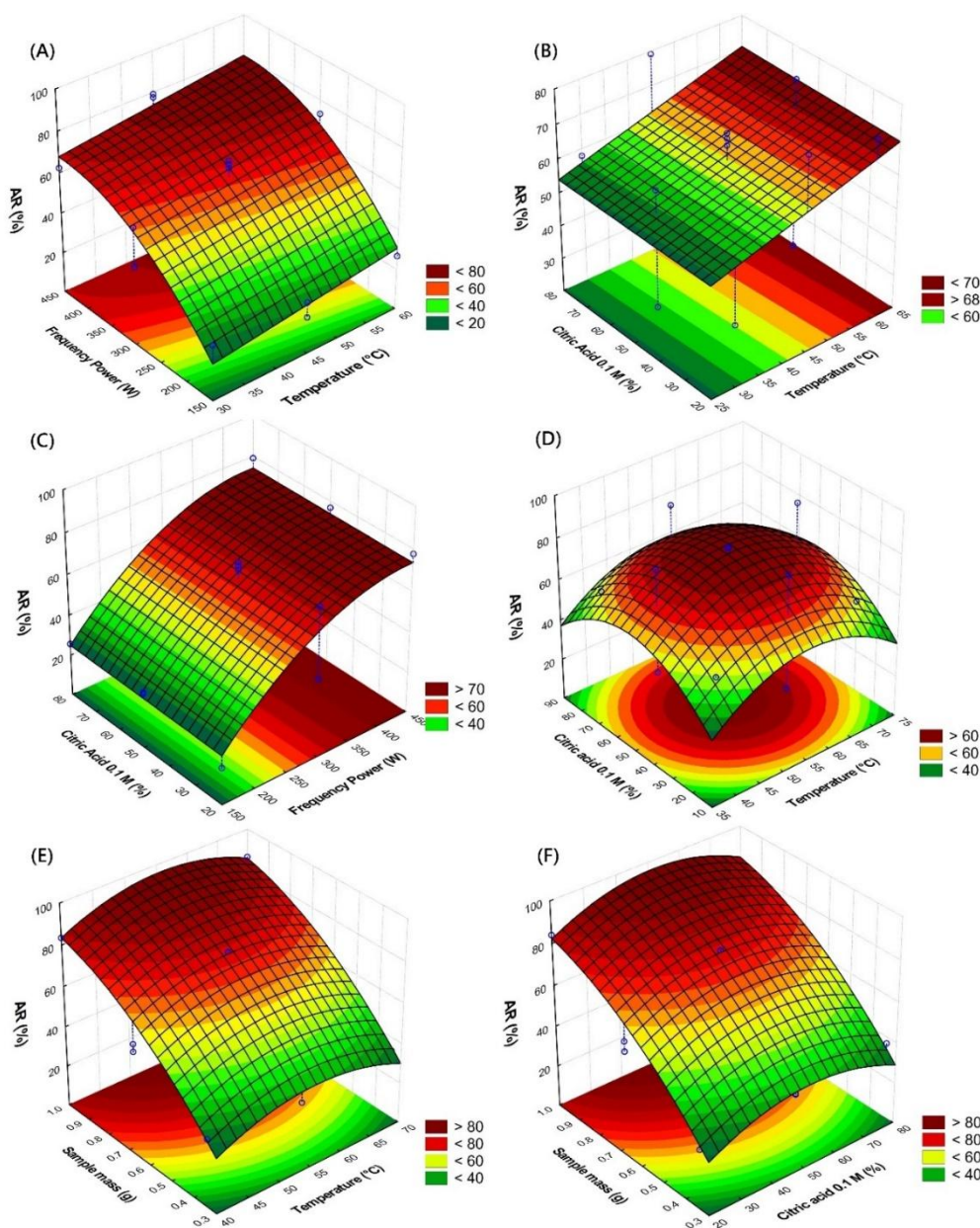
$$y = 57.42 + 11.26 \cdot x_1^2 + 12.38 \cdot x_2^2 + 50.87 \cdot x_3 + 8.05 \cdot x_3^2 \quad (4.14)$$

Regarding the temperature for anthocyanin extraction, as well as for the UAE and evidenced in this study, temperatures close to 50 °C have been reported for optimizations in the extraction of anthocyanins from saffron tepal's (48 °C) (JAFARI; MAHDAVEE KHAZAEI; ASSADPOUR, 2019), blueberry powder (47 °C) (ZHENG et al., 2013), and *Clitoria ternatea* flowers (60 °C) (IZIRWAN et al., 2020). For solvents, generally, a proportion between 40% and 60% of ethanol (LAO; GIUSTI, 2018) and acidity between 1.0 and 3.0 (ZIELINSKI et al., 2021) present better yields in the extraction of anthocyanins. In this study, the 50:50 ratio of 0.1 M acetic acid and ethanol showed the best results (pH about 2.6-2.8).

Regarding the mass parameters of the sample, in some studies, such as solid/solvent ratio (g/mL), the increase in mass resulted in higher yields in the extraction of anthocyanins (Fig 4.1 E-F). The added solid/solvent ratio depends on the type of sample, being equal to 1:15 (g/mL) for *Clitoria ternatea* flowers (IZIRWAN et al., 2020), 1:34 (g/mL) for blueberry powder (ZHENG et al., 2013), 1:34 (g/mL) for blueberry powder (Zheng et al. al., 2012), and about 1:23 (g/ml) for *Hibiscus rosa-sinensis* (ASHITHA; PRINCE; SANJAY, 2020). This study obtained the best results with the solid/solvent ratio between 1:20 (1 g) to 1:30 (0.67 g). Based

on these results, for the optimization of the extraction, a temperature of 55 °C, citric acid 0.1M:ethanol (50:50, v/v), and a mass of 0.8 g (1:24, g/mL) were chosen.

Figure 4.1 Three-dimensional (3D) response surfaces show the effect of UAE independent variables (temperature, frequency power, and solvents) on the dependent variable AR (A, B, and C); and MAE independent variables (temperature, solvents, and sample mass) on the dependent variable AR (D, E, and F).



The optimization point was externally validated for UAE and MAE, providing value within the predicted ranges (solving Eq. 4.13 and Eq. 4.14, respectively) for a 95% confidence level. For UAE, the AR result obtained was $83.73\% \pm 3.14$ (predicted: 85.67%), while for MAE,

the AR value obtained was $81.44\% \pm 1.69$ (predicted: 79.06%). Thus, both proposed models can predict the AR response.

4.7.2 Comparison between extraction methods

Regarding the comparison between the methods, as observed previously by the optimization, the anthocyanins recovery (AR) obtained by both extraction methods (UAE and MAE) was similar, equal to 83.73% and 81.44% (Table 4.3). In terms of total anthocyanins, UAE presented a value of 2.44 mg C3GE/g, and MAE showed 2.37 mg C3GE/g, not differing from each other ($p < 0.05$). Concerning studies that performed extraction by UAE of anthocyanins from black rice bran, Das et al. (2017) presented a result of 31.9 mg/L, using 23.78% (v/v) ethanol concentration, 35.97 °C temperature, pH 2.52, and 22.84 min. According to the authors, an ultrasonic bath was used, while an ultrasonic probe was used in this study, reaching a maximum value of 81.19 mg/L of anthocyanins. In addition, the authors used time as an optimization parameter, which resulted in a longer time than in this study. To extract anthocyanins by MAE from black rice, Abdel-Aal et al. (2014) used 50 °C, 1200 W, and 20 min reported values of 2.0 mg C3GE/g, close to those reported in this study.

Table 4.3 Characterization of the extracts obtained by optimized UAE and MAE.

Analysis	UAE	MAE
AR (%)	83.73 ± 3.14^a	81.44 ± 1.69^a
TMA (mg C3GE/g DW)	2.44 ± 0.09^a	2.37 ± 0.04^a
C3G (mg/g DW)	2.41 ± 0.01^a	2.11 ± 0.01^b
PC (%)	44.35 ± 1.44^b	48.47 ± 2.37^a
TPC (mg GA/g DW)	11.80 ± 0.14^b	11.45 ± 0.35^a
DPPH ($\mu\text{mol TE/g DW}$)	61.55 ± 1.38^a	63.05 ± 1.45^a
ABTS ($\mu\text{mol TE/g DW}$)	72.86 ± 3.13^a	60.80 ± 3.77^b

Note: Results showed a mean $\pm$ standard deviation. TMA: total monomeric anthocyanins; AR: anthocyanin recovery; C3GE: cyanidin-3-glucoside equivalents; TPC: total phenolic compounds; GAE: gallic acid equivalents; C3G: cyanidin-3-glucoside; TE: Trolox equivalents; UAE: ultrasound-assisted extraction and MAE: microwave-assisted extraction. Different letters on the same line indicate significant differences ($p < 0.05$) between the experiments using by t test.

The main anthocyanin of black rice is cyanidin-3-glucoside (C3G), corresponding to about 90% of total anthocyanins (ITO & LACERDA, 2019; LEONARSKI et al., 2023b). In this study, it was identified that for UAE, 97.3% of the total anthocyanins correspond to C3G (2.37 mg/g), while for MAE, 89.1% of the total anthocyanins correspond to C3G (2.11 mg/g). In the study by Laokuldilok et al. (2011), was identified after extracting anthocyanins from black rice with methanol acidified with 1 N HCl (85:15, v/v) at 4 °C for 24 h, C3G values

between 58% and 95%. In the study by Abdel-Aal et al. (2014), by extracting anthocyanins by MAE, the authors reported 88.1% of C3G, a value close to that reported in this study.

Concerning polymeric color (PC), it can be observed that both samples presented close results, with these values comparable to those reported by Chañi-Paucar et al. (2021) on the extraction of polyphenols from grape waste (46.6%). The higher the PC values, the greater the degradation of anthocyanins by polymerization (WANG et al., 2022). Generally, this degradation of anthocyanins occurs by increasing temperature and leads to the production of chalcones that are subsequently degraded into brown products (such as melanoidins) (AGCAM et al., 2021; JIANG et al., 2019).

For total phenolic compounds (TPC), both samples showed values close to 11.5 mg GA/g, not differing from each other ($p < 0.05$). These phenolic compounds come from anthocyanins and also from phenolic acids, mainly protocatechuic acid, vanillic acid, 4-hydroxybenzoic acid, and syringic acid (DAS; GOUD; DAS, 2017; SETYANINGSIH et al., 2016). In the study by Leonarski et al. (2023b), when extracting anthocyanins from black rice bran by PLE, TPC equal to 10.7 mg GAE/g was reported, while in the study by Das et al. (2018), when extracting phenolic compounds from black rice bran by conventional technique (water bath), a value equal to 8.5 mg GAE/g was reported, both values close to those reported in this study.

Regarding the antioxidant activity, there was no difference between the results obtained by the DPPH radical ($p < 0.05$) for the samples extracted by both techniques. Both samples showed values close to 62 $\mu\text{mol/g}$, close to that reported by Leonarski et al. (2023b) for black rice bran extracted by PLE (65.2 $\mu\text{mol/g}$). For ABTS, the sample extracted by UAE showed a higher value than MAE, differing from each other ($p < 0.05$). Finocchiaro et al. (2010) reported values of antioxidant activity by ABTS for two species of black rice 42.01 $\mu\text{mol/g}$ and 94.81 $\mu\text{mol/g}$, values close to those reported in this study.

UAE and MAE extractions are considered eco-friendly approaches. Considering this, the green metric tools Green Certificate and EcoScale (Section 4.6) were applied to assess how eco-friendly these methods are. For using the green certificate, penalties related to the solvent, energy, and residue generated in the extraction are assessed (Figure 4.2A). The methods showed close values (95 for UAE and 93 for MAE), and both methods were considered highly effective green (Green certificate), in which the scale varies from A to G (Figure 4.2A), with A being considered the greenest process (ARMENTA; GARRIGUES; DE LA GUARDIA, 2015; GALLART-MATEU et al., 2015). This difference in green certificate values was due to the higher addition of ethanol in MAE extraction since both techniques have a low energy cost.

The study by Espino et al. (2018), when extracting phenolic compounds from *Larrea cuneifolia* by UAE and using NADES as solvent (mixture of lactic acid with dextrose), presented a green certificate of 95.99. In the study by Abd-Elsalam et al. (2021) using the MAE extraction of quercetin from red onion, the authors reported a value of 85.

Regarding the EcoScale, six parameters presented in Figure 4.2B are considered. It can be observed that both samples showed similar values (84.9 for UAE and 80.7 for MAE), being higher for the UAE. However, values above 75-80 are considered excellent, while values below 50 are considered inadequate (ARMENTA; GARRIGUES; DE LA GUARDIA, 2015; DENEYER; ENNAERT; SELS, 2018). Benvenuti et al. (2022), when performing anthocyanin extraction from jaboticaba by-product by PLE, reported higher EcoScale values (69.4-72.5) when using NADES as solvent (mixture of choline chloride and propylene glycol and mixtures of choline chloride and malic acid).

Figure 4.2 (A) The Green Certificate and the (B) EcoScale of the UAE and MAE techniques.

(A)

UAE		MAE	
Reagents	PPs	Reagents	PPs
Etanol (< 10 mL)	4	Etanol (> 10 mL)	6
Acidified water	0	Acidified water	0
Instruments	PPs	Instruments	PPs
Energy consumption (≤ 1.5 kWh)	0	Energy consumption (≤ 1.5 kWh)	0
Waste (< 1 g)	1	Waste (< 1 g)	1
Total PPs	5	Total PPs	7
Green certificate	95 (A)	Green certificate	93 (A)

Green effectiveness

A 90-100

B 80-89

C 70-79

D 55-69

E 40-54

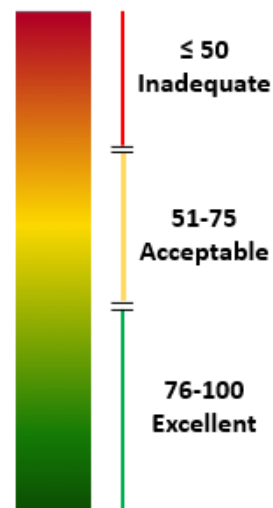
F 20-39

G 0-19

Not green effectiveness

(B)

UAE		MAE	
Parameters	PPs	Parameters	PPs
Yield (100 – %yield)/2	8.1	Yield (100 – %yield)/2	9.3
Costs (< \$10)	0	Costs (< \$10)	0
Security	5	Security	5
Technical Setup	0	Technical Setup	3
Temperature/time (Heating, < 1 h)	2	Temperature/time (Heating, < 1 h)	2
Workup and purification	0	Workup and purification	0
Total PPs	15.1	Total PPs	19.3
EcoScale	84.9	EcoScale	80.7

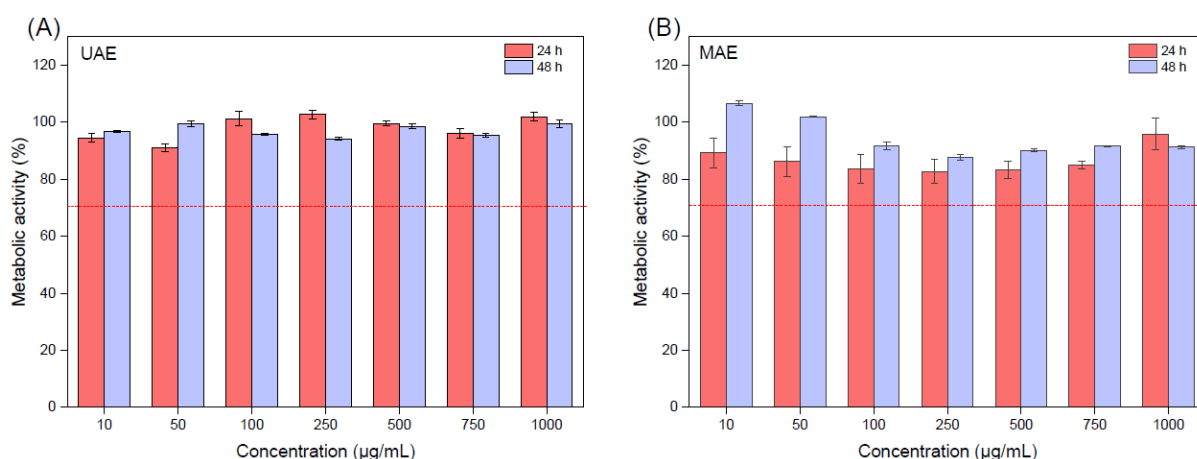


4.7.3 Cell culture

Figure 4.3 shows the cytotoxicity results for L929 cells (normal tissue of the mouse) at 24h and 48h for both extracts (UAE and MAE). According to ISO 10993-5:2009 (ISO 10993-5:2009, 2009), the minimum cell viability must be 70% for the extracts not to present cytotoxicity. None of the extracts showed cell viability lower than 80%, showing no cytotoxicity. Furthermore, it can be observed that in 72 h, there was a significant increase ($p < 0.05$) in cell viability for both extracts. In the study by Aprodu et al. (2019), microencapsulated black rice extract did not show cytotoxicity (10-1000 $\mu\text{g/mL}$), similar to those reported in this study.

According to results presented in the Appendix (Figure S4 – Appendix E), no antitumoral activity (500 $\mu\text{g/mL}$) was observed for the cells: lung adenocarcinoma cells (A549), rat glioma cells (C6) and mouse glioma cells (GL261). Antitumoral activity was also not verified for A549 cells using anthocyanin-rich extract (500 $\mu\text{g/mL}$) from pressurized liquid extraction (PLE) and heat-stirring extraction (HSE) (LEONARSKI et al., 2023a). The results on H_2O_2 -induced oxidative stress in L929 cells showed that when using 500 $\mu\text{g/mL}$ of sample, both extracts showed a cytoprotective effect close to 93%, equal to 94.5% for UAE and 92.9% for MAE. In contrast, the sample without extract showed a cytoprotection of only 4.6%. In the study by Leonarski et al. (LEONARSKI et al., 2023a), the authors reported about 82% for 250 $\mu\text{g/mL}$ extracts rich in anthocyanins.

Figure 4.3 (A) Metabolic activity of L929 cells treated with the UAE anthocyanin-rich extract and (B) MAE anthocyanin-rich for 24 and 48 h.



4.7.4 Stability of UAE and MAE anthocyanin-rich extracts

The stability of anthocyanins is one of the factors most affected by thermal processing (LEONARSKI et al., 2022). Tables 4.4 and 4.5 show the kinetic and thermodynamic

parameters, respectively, obtained through the thermal degradation curves. It can be observed that the extract obtained by MAE (pH $\approx$ 2.8) presented slightly higher $t_{1/2}$ than the extract obtained by UAE (pH $\approx$ 2.6). In the study by Guimarães et al. (2018), the author reported a $t_{1/2}$ value for C3G (pH 3.0) extracted from frozen blackberries of 9.23, 3.30, and 2.0 h at 65, 80, and 95 °C, respectively, values close to those reported in this study. According to Oancea (2021), the increase in temperature shifts the balance of anthocyanins to the tautomeric open-form, that is, the colorless chalcone prevails in monoglycosidic anthocyanins and does not have a substituent in the C3 position.

Table 4.4 Kinetics parameters of thermal and light stability of anthocyanin-rich extracts obtained by UAE and MAE.

	T (°C)	k_d (min ⁻¹)	$t_{1/2}$ (h)	R ²	Light	k_d (min ⁻¹)	$t_{1/2}$ (days)	R ²
UAE	65	0.0014	8.13	0.986	Fluorescent	0.013	2.22	0.876
	80	0.0027	4.23	0.974				
	95	0.0066	1.75	0.991				
MAE	65	0.0012	9.39	0.987	Fluorescent	0.0015	1.93	0.982
	80	0.0025	4.62	0.978				
	95	0.0044	2.62	0.943				

Note: UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; k_d : degradation rate; R²: determination coefficient; $t_{1/2}$: time of half-live.

Regarding the thermodynamic parameters (Table 4.5), it can be seen that the activation energy (E_a) is higher for the extract obtained by UAE. Teixeira et al. (2021) presented an E_a value of 40.56 kJ/mol for an anthocyanin-rich bean peel extract obtained by PLE. In the study by Guimarães et al. (2018), the authors presented E_a for C3G of 52.5 kJ/mol. The higher E_a values, the lower the susceptibility to anthocyanin degradation.

Table 4.5 Thermodynamic parameters of the anthocyanin degradation in the extracts obtained by UAE and MAE.

	E_a (kJ/mol)	R ²	Temperature (°C)	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (kJ/mol K)
UAE	52.85	0.986	65	50.04	206.22	-0.462
			80	49.92	213.61	-0.464
			95	49.79	220.07	-0.463
MAE	44.02	0.998	65	41.21	206.62	-0.489
			80	41.08	213.83	-0.489
			95	40.96	221.31	-0.490

Note: UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; E_a : activation energy; ΔH : activation enthalpy; ΔG : free energy of activation; ΔS : activation entropy.

A positive activation enthalpy (ΔH) indicates an endothermic reaction for both extracts (UAE and MAE). For an anthocyanin-rich extract from hibiscus (*Hibiscus sabdariffa* L.), Cheok & Ragunathan (2022) presented ΔH values close to 51 kJ/mol, results close to the extract obtained by UAE. The ΔH is a measure of the energy barrier that must be overcome by the reacting molecules, representing the strength of the bonds that are broken and rebuilt during the transition state; therefore, higher values indicate that these are more likely to transform into other forms (degradation) (BENVENUTTI; ZIELINSKI; FERREIRA, 2022; CONDURACHE et al., 2021).

Positive ΔG values indicate that the degradation is non-spontaneous, and negative ΔS means that the reaction is irreversible and the molecules are more organized than in the initial reaction (BENVENUTTI; ZIELINSKI; FERREIRA, 2022; CHEOK; RAGUNATHAN, 2022; CONDURACHE et al., 2021). In studies by Leonarski et al. (2023), authors presented ΔG values close to 215 kJ/mol for anthocyanin-rich extracts from black rice bran extracted from PLE, result close to those reported in this study.

Regarding the stability of fluorescent light, both samples showed a $t_{1/2}$ of approximately 2 days. These values were close to those reported in the study by Junior et al. (2023) for anthocyanin-rich extract from grumixama berries, which showed a $t_{1/2}$ of 1.9 days (45.61 h).

4.8 CONCLUSION

Using UAE and MAE techniques with the Box-Behnken design tool efficiently optimized the recovery of anthocyanins from black rice bran. When optimized, both extraction methods showed similar anthocyanin recovery, equal to 83.73% for UAE and 81.44% for MAE. UAE showed a higher amount of cyanidin-3-glucoside (2.41 mg/g) when compared to MAE (2.11 mg/g). Both methods were considered eco-friendly, presenting a green certificate above 90 (A) and EcoScale considered excellent (above 75). There was no evidence of cytotoxicity in the UAE, and MAE extracts were evaluated by L929 cells at concentrations of 10 $\mu\text{g/mL}$ to 1000 $\mu\text{g/mL}$ of extract. A slight advantage is observed for the UAE method due to the ease of the technique compared to MAE, in which high pressure is required. The extracts obtained by both methods have interesting characteristics for use in several areas, such as natural coloring, cosmetics, and biomedical industries.

Regarding the extractions (Chapters 3 and 4), it was observed (Table 4.6) that all emerging techniques (PLE, UAE, and MAE) used showed recovery close to 80% and did not differ from each other ($p < 0.05$). However, regarding anthocyanins quantified individually, the sample extracted by UAE showed a higher amount of C3G (2.41 mg/g), an important result for

the next study stage. In addition, the extracts also showed similar results for TPC and PC. All extracts did not show cytotoxicity (up to 500 µg/mL). It was also verified that all the processes used were eco-friendly through the Green Certificate and EcoScale measures, in which UAE presented better results for both scales, 95 and 84.9, respectively. In contrast, MAE and PLE (Figure S5 – Appendix F) presented values for the Green certificate of 93 and 91 and EcoScale of 80.7 and 80.1, respectively. Therefore, for the next steps of our study, the extract obtained through the UAE was used.

Table 4.6 Characterization of the extracts obtained by optimized HSE, PLE, UAE, and MAE.

Analysis	HSE*	PLE	UAE	MAE
AR (%)	45.59 ± 2.02 ^b	79.22 ± 2.68 ^a	83.73 ± 3.14 ^a	81.44 ± 1.69 ^a
TMA (mg/g)	1.17 ± 0.06 ^b	2.30 ± 0.08 ^a	2.44 ± 0.09 ^a	2.37 ± 0.04 ^a
C3G (mg/g)	1.07 ± 0.01 ^d	1.63 ± 0.02 ^c	2.41 ± 0.01 ^a	2.11 ± 0.01 ^b
PC (%)	35.37 ± 2.77 ^c	44.52 ± 0.51 ^{ab}	44.35 ± 1.28 ^b	48.47 ± 1.94 ^a
TPC (mg/g)	4.21 ± 0.19 ^b	12.30 ± 0.14 ^a	11.80 ± 0.14 ^a	11.45 ± 0.35 ^a
DPPH (µmol TE/g)	46.03 ± 1.25 ^c	65.24 ± 1.44 ^a	61.55 ± 1.38 ^b	63.05 ± 1.45 ^{ab}
ABTS (µmol TE/g)	65.90 ± 6.90 ^{bc}	103.3 ± 7.29 ^a	72.86 ± 3.13 ^b	60.80 ± 3.77 ^c

5 ENZYMATIC ACYLATION OF CYANIDIN-3-GLUCOSIDE FROM BLACK RICE BRAN: THERMOSTABILITY, LIPOPHILICITY, CYTOTOXICITY AND ABSORPTION STUDIES

The information brought in this chapter was compiled in a scientific paper entitled "*Enzymatic acylation of cyanidin-3-glucoside from black rice bran: thermostability, lipophilicity, cytotoxicity, and absorption studies*", which was submitted to the journal "Food Chemistry" (Qualis A1; Impact Factor: 8.5) and it is currently in under review. The work was developed at the University of Porto, oriented by Professor Luís Cruz and Hélder Oliveira. This study aimed to isolate anthocyanin (cyanidin-3-glucoside) from the black rice extract and perform enzymatic acylation of this compound to increase its stability. The NRM technique was performed to verify the acylation, and the stability (temperature), partition coefficient, cytotoxicity, and transport studies were also evaluated. Therefore, this chapter contains both the methodology and the respective results.

ABSTRACT

The enzymatic acylation has been a promising alternative to reduce anthocyanin's thermal and chemical instability and increase their lipid affinity. This study aimed to perform enzymatic acylation of cyanidin-3-glucoside (C3G) from black rice bran extract with octanoic acid (C8), and to evaluate its lipophilic conjugate in terms of thermostability at different pHs, octanol-water partition coefficient, cytotoxicity and absorption through cell culture transepithelial transport studies. C3G, the main anthocyanin present in black rice bran, was extracted by ultrasound and purified using C18 silica. Regarding the experimental parameters optimization (solvent, enzyme concentration, temperature), it was possible to obtain around 53% formation of acylated anthocyanin using octanoic acid (C8) as the acyl donor group, as solvent Acetonitrile:DMSO (10:1), with temperature of 60 °C and enzyme concentration of 15 g/L. For the stability, it was possible to observe an increase in the half-life time for acylated anthocyanin (C3G-C8) at different pHs (3, 5, and 7) compared to C3G. The octanol–water partition coefficients confirmed the acyl compound's increased lipophilicity ($\log P = 1.37$) compared to its precursor ($\log P = -1.59$). No cytotoxicity was observed for both compounds at concentrations up to 200 μM for NCI cells and Caco2:HT29-MTX coculture. Both anthocyanins presented similar transepithelial transport efficiency using the coculture (about 1.2%). The enzymatic acylation method for the green metric tools was considered acceptable for EcoScale (61.3) and with a score of 89 (B) for the Green Certificate. Acylated anthocyanin showed potential for application in lipid matrix products, properties not observed in its precursor and can be added to the food and cosmetic industries.

Keywords: lipophilization, thermostability, natural dyes, transport studies.

5.1 INTRODUCTION

Consumer awareness of food safety has advanced in recent years, such as interest in using anthocyanins (natural pigments) instead of synthetic colorants (GUIMARÃES et al., 2018). Anthocyanins are natural water-soluble compounds responsible for the red, blue, and purple colors of various foods such as fruits, vegetables, cereals, flowers, grains, roots, and tubers (ARRUDA et al., 2021; GUIMARÃES et al., 2018; TENA; MARTÍN; ASUERO, 2020).

Black rice has been consumed as a functional food due to its high content of bioactive compounds (mainly anthocyanins) (ITO; LACERDA, 2019). During rice processing, a large part of the product is discarded (about 20% of rice husk is produced, 10% of rice bran, and 14% of broken rice), and this residue generated (generally intended for animal nutrition) has a high potential for the extraction of anthocyanins (MORAES et al., 2014; NAGRALE; HAJARE; MODAK, 2012; VAN HOED et al., 2006). Black rice bran is one of these residues that has been identified as having a high potential for extracting natural pigments, having in its composition around 64-90% cyanidin-3-*O*-glucoside of total anthocyanins (HUANG; LAI, 2016; LEONARSKI et al., 2024a).

However, although it has great potential, some disadvantages have limited the use of anthocyanins in industrial applications, such as temperature, sensitivity to light, chemical balance due to pH variation, and low solubility in lipophilic media (CRUZ et al., 2017; FERNANDEZ-AULIS et al., 2020). Therefore, to compensate anthocyanins stability issues, some strategies are necessary to be followed to maintain or improve their color and beneficial human health properties (Yang et al., 2020). Some methods already used included: spray-dryer, freeze-drying, encapsulation, copigmentation and acylation (HOANG et al., 2024; LEONARSKI et al., 2022; OLIVEIRA et al., 2023; TAN et al., 2023). Regarding acylation, studies have reported that acylated anthocyanins have greater thermal stability than non-acylated ones (FEI et al., 2021; GAMAGE; LIM; CHOO, 2021; TENG et al., 2022), therefore, the acylation of anthocyanins has become a promising tool to increase the chemical, chromatic and thermal stability of these compounds (LEONARSKI et al., 2022; ZHAO et al., 2017b).

Acylation has been described to occur mainly by the esterification or transesterification of an organic acid or ester, respectively, to the glycoside of the anthocyanin molecule, which can be classified according the presence of the acyl group, as aromatic or aliphatic (CHATHAM; PAULSMAYER; JUVIK, 2019; GAMAGE; LIM; CHOO, 2021). When a water-soluble compound is acylated by an esterification reaction with a fatty acid or fatty alcohol, known as lipophilization (MARQUEZ-RODRIGUEZ et al., 2021), this reaction can be

accelerated by lipases, characterized as enzymatic lipophilization. The enzymatic approach has high selectivity compared with chemical catalysts since the reaction occurs preferentially at primary alcohols of the glycoside residue of anthocyanin (CRUZ et al., 2018; GRAJEDA-IGLESIAS et al., 2017; LUO et al., 2013).

Three studies performed the enzymatic acylation of C3G using octanoic acid as the donor acyl group (CRUZ et al., 2018; GUIMARÃES et al., 2018; LIN et al., 2022); however, none of these works focused on optimizing this process. Some studies have also evaluated the transepithelial absorption of acylated anthocyanins extracted from black carrot (KAMILOGLU et al., 2017), red wine (HAN et al., 2020), black carrot and strawberry (CARRILLO et al., 2020), and grape skin (LIU et al., 2022). However, in these studies, anthocyanins are found naturally in their respective raw materials, and none of them are acylated with fatty acid. In addition, no work has been performed regarding the transepithelial transport study for enzymatic acylated anthocyanin.

Therefore, this work aims to optimize the enzymatic acylation of anthocyanins recovered from black rice bran with octanoic acid (C8) as the acyl donor group to increase their thermal stability and improve liposolubility and color properties in lipid-based media. Furthermore, evaluate the cytotoxicity and epithelial transport studies of the acylated anthocyanin and their precursor.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Fatty acids (hexanoic acid (C6), octanoic acid (C8), decanoic acid (C10), dodecanoic acid (C12)), acetonitrile (Acet), 2-methyl-2-butanol (2M2B), *tert*-butanol (*Tert*-B), dimethylsulfoxide (DMSO), molecular sieves 4 Å (MS 4Å), *Candida antarctica* lipase B (*CalB*) immobilized in an acrylic resin (≥ 5000 U/g, recombinant, expressed in *Aspergillus niger*), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (Madrid, Spain). Silica gel RP-18 (40–63 μm) LiChroprep was provided from Merck (Darmstadt, Germany). Cyanidin-3-*O*-glucoside (C3G) was obtained by extraction and purification from black rice bran.

5.2.2 Extraction and purification of C3G from black rice bran

A study was carried out to scale up the extraction of anthocyanins from black rice bran since a volume of 15 mL (Chapter 4) was used for optimization (Leonarski et al., 2024b). The study was published in the journal *Foods* (Qualis A2; FI: 4.7) (Figure S6 - Appendix G).

Ultrasound-assisted extraction (UAE) of anthocyanins from black rice bran was performed according to the procedure described in Leonarski et al. (2024b) namely, the temperature was set to 50 °C and solvent ethanol:acetic acid 0.1 mol/L (40:60) (optimized conditions from Chapter 4). A probe ultrasound (Bandelin Sonopuls HD 2070 Ultrasonic, Berlin) was used, in which the ultrasound frequency was 20 kHz, with an amplitude of 80% and a total volume of 300 mL. A kinetic-based study of the concentration of total monomeric anthocyanins (according LEONARSKI et al., 2024a) was performed, and the time was defined as 10 min. After extraction, the liquid was separated from the black rice bran through filtration and subjected to rotary evaporation to eliminate ethanol.

After ethanol removing, the extract was subjected to liquid-liquid extraction twice with chloroform (1:1) and twice with ethyl acetate (1:1). Then, the extract was rotary evaporated again to eliminate any remaining solvents. The aqueous fraction was concentrated and purified in a Buchner funnel loaded with RP-C18 silica gel. A water/methanol 90:10 (v/v) fraction acidified with HCl 2% was used to elute the anthocyanins. Subsequently, the methanol was rotary evaporated, and the extract lyophilized.

5.2.3 Enzymatic acylation of C3G

For the enzymatic acylation process, 4 mg of purified extract (90.2% of C3G), 100 g/L of activated MS 4Å, 250 eq. of fatty acid (octanoic acid), and 20 g/L of the *CalB* enzyme were mixed. Initially, acylation tests with different solvents (2 mL volume): 2M2B (2-methyl-2-butanol), 2M2B:DMSO (10:1), *tert*-butanol, *tert*-butanol:DMSO (10:1), acetonitrile, and acetonitrile:DMSO (10:1) were carried out for a period of 12 h, these parameters were obtained through previous work (CRUZ et al., 2018; GUIMARÃES et al., 2018; MARQUEZ-RODRIGUEZ et al., 2021) and laboratory tests. After 12h, the samples were centrifuged and evaluated by HPLC-DAD (Section 5.2.4). The control of the product formation yield (%) was calculated using the area of acylated C3G (A_1) divided by the area of the initial C3G (A_0), according to equation (5.1):

$$Product\ formation\ (\%) = \frac{A_1}{A_0} \times 100 \quad (5.1)$$

After defining the best solvent, new enzymatic acylation reactions of C3G were carried out to evaluate different temperatures (40, 50, 60, and 70 °C) and enzyme concentrations (10,

15, 20, 25, and 30 g/L), both for a period of 12 h. Once the best parameters were defined, kinetics were performed using different fatty acids (C6, C8, C10, and C12) for a period up to 20 h.

5.2.4 Purification of acylated extracts

After enzymatic acylation, filtration was carried out to remove molecular sieves and enzyme, and then the extract was rotary evaporated. Afterward, acidified methanol (HCl 2%) was added to the extract, and then three extractions were carried out with heptane and saturated NaCl solution to remove impurities and unreacted fatty acids.

The extract was evaporated and diluted in a 20% methanol solution (acidified with HCl 2%), then purified by chromatography on a column loaded with reversed-phase C18 silica gel (150 mm × 16 mm i.d.). The non-acylated C3G were obtained with an acidified methanol solution between 20 and 40%, while the acylated products were obtained with about 60% acidified methanol. Then, the methanol was removed, and the acylated C3G were lyophilized and stored at −18 °C (CRUZ et al., 2017).

5.2.5 Identification and quantification of acylated compounds by HPLC-DAD

Acylated compounds were evaluated according to Cruz et al. (2017) on HPLC (Hitachi LaChrom Elite HPLC System, Tokyo, Japan) equipped with a diode array detector (L-2455) and a reversed-phase column (250 mm × 4.6 mm i.d., 2.7 µm, Poroshell 120 EC-C18, Agilent) thermostated at 25° C. Detection was performed at 520 nm. The solvents were (A) water/formic acid (99:1, v/v) and (B) acetonitrile/formic acid (99:1, v/v), with the following gradient: 0-20% B over 5 min, 20-100% over 10 min and isocratic 100% B for 15 min at a flow rate of 0.4 mL/min. The sample injection volume was 20 µL.

5.2.6 Identification of acylated compounds by HPLC-MS

Samples were analyzed according to Cruz et al. (2017) on a Finnigan Surveyor series liquid chromatograph equipped with an LCQDECA XP MAX mass detector (Finnigan Corporation, San Jose, CA). An atmospheric pressure ionization source (API) and an electrospray ionization interface (ESI) were used using the same reverse phase column, solvents, injection volume, gradients, and flow rate referred to in HPLC-DAD analyses. Online dual detection was performed by photodiode spectrophotometry and mass spectrometry. The vaporizer and capillary voltages were 5 kV and 4 V, respectively. The capillary temperature

was set at 325 °C. Nitrogen was used as sheath and auxiliary gas at flow rates of 90 and 25 arb units, respectively. Spectra were recorded in positive and negative ion modes from m/z 250 to 1500.

5.2.7 Nuclear magnetic resonance (NMR) analysis

The ^1H NMR (600.13 MHz) of C3G-C8 conjugate was analyzed on a Bruker-Avance 600 spectrometer at 303 K. The sample was dissolved in DMSO- d_6 /TFA (9:1) with TMS as an internal standard (chemical shifts (δ) in parts per million, coupling constants (J) in hertz). The ^1H chemical shifts were assigned using 2D NMR of correlation spectrometry (COSY) experiment. The delay for the long-range C/H coupling constant was optimized to 7 Hz.

5.2.8 Thermostability assays

The thermostability of C3G and its acylated conjugate (C3G-C8) at 1 mM and different pHs (3, 5, and 7) was carried out on a 0.1 M SDS solution. The diluted compounds were added to tubes and subjected to temperatures of 65, 80, and 95 °C for a period between 1 and 10 h, with an absorbance reading between 400 at 600 nm on a Varian-Cary 100 Bio spectrophotometer (Palo Alto, CA). A first-order kinetic model was fitted according to Equation (5.2) (TEIXEIRA et al., 2021; YANG et al., 2020):

$$\frac{A_t}{A_0} = e^{-k_d \cdot t} \quad (5.2)$$

where A_t is the absorbance with time t , A_0 is the initial absorbance, k_d is the degradation rate (h^{-1}), and t is the time (h).

The time of half-live ($t_{1/2}$) was determined by Equation 5.3, and the activation energy (E_a) was determined through the Arrhenius model by Equation 5.4.

$$t_{1/2} = \frac{\ln(2)}{k} \quad (5.3)$$

$$\ln(k_d) = \ln(A) - \frac{E_a}{R \cdot T} \quad (5.4)$$

where A is the frequency factor (h^{-1}), R is the ideal gas constant (8.314 J/mol·K), and T is the temperature (K).

For the activation enthalpy (ΔH), the free energy of activation (ΔG), and the activation entropy (ΔS), Equations (5.5), (5.6) and (5.7), respectively, were used for calculations:

$$\Delta H = E_a - R \cdot T \quad (5.5)$$

$$\Delta G = -R \cdot T \cdot \ln \left(\frac{k_d \cdot h}{k_B \cdot T} \right) \quad (5.6)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (5.7)$$

where E_a is the activation energy for the degradation reaction (J/mol), R is the ideal gas constant (8.314 J/mol·K), T is the temperature (K), k_d is the kinetic rate constant (s^{-1}), k_B is the Boltzmann constant ($1.3806 \cdot 10^{-23}$ J/K), and h is Planck's constant ($6.6262 \cdot 10^{-34}$ J/s).

5.2.9 Partition coefficient determination

The methodology was performed according to Marquez-Rodriguez et al. (2021). The lipophilicity of the acylated conjugate and its respective precursor was measured by calculating the octanol-water coefficient ($\log P_{o/w}$) by the shake flask method. The n-octanol was pre-saturated with acidified water (0.1 M HCl) for 24 h. Then, the samples were dissolved in 1 mL of n-octanol, and the same volume of water (HCl 0.1 M) was added until reaching a final concentration of 0.17 mM. Subsequently, the flask was shaken for 1 h until reaching equilibrium (24 h). Then, each phase will be analyzed by UV-Vis spectroscopy, and the absorbance of the compounds at the maximum wavelength will be analyzed. The $\log P_{o/w}$ was calculated using Equation 5.8:

$$\log P_{o/w} = \log_{10} \frac{A_{octanol}}{A_{water}} \quad (5.8)$$

5.2.10 Cell culture

Gastrointestinal human epithelial cell lines NCI-N87 (gastric carcinoma), Caco-2, and HT29-MTX (intestinal carcinoma) were grown in monolayers and maintained at 37 °C in an atmosphere of 5% CO₂ and 90% relative humidity. The NCI-N87 cells were maintained in RPMI medium (RPMI 1640 (LifeTechnologies) supplemented with 10% (v/v) fetal bovine serum (FBS, LifeTechnologies), non-essential amino acids (MEM, LifeTechnologies) and a mixture of 100 U/mL Penicillin and 100 µg/mL Streptomycin (LifeTechnologies). Caco-2 and HT29-MTX cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Sigma® St. Louis, MI, USA, F4135), 1% sodium pyruvate (Sigma® S8636), 1% transferrin (Sigma® T8158), 1% antibiotic/antimycotic solution (100 units/mL of penicillin, 100 mg/mL of streptomycin, and 0.25 mg/mL of amphotericin B) (Sigma® A5955), and 1% L-glutamine (Biowest®, Nuaille, France, X0551-100). Every 2 to 3 days, the cell culture medium was changed, and the cells were kept in exponential growth in T75 flasks.

5.2.10.1 *Cytotoxicity*

An MTT assay was performed for the NCI-N87 cells, and coculture Caco2:HT29-MTX cells (9:1) to evaluate the cytotoxicity of the C3G and its precursor (C3G-C8). In a 96-well plate, the cells were seeded at a concentration of 6.2×10^4 cells/mL, prepared in RPMI (for NCI-N87 cells), and a concentration of 5.0×10^4 cells/mL prepared in DMEM (for Caco-2:HT29-MTX (9:1) cells). Such concentrations were selected to ensure the confluency of the wells after 7 days. These plates were then incubated at 37 °C in an atmosphere of 5% CO₂ and 90% relative humidity. 200 µM C3G and C3G-C8 solutions were prepared in RPMI and DMEM media containing 0.1% DMSO. Through serial dilutions, different concentrations were added to the plates (200 µM, 100 µM, 50 µM, 25 µM, and 12.5 µM) and kept in incubation for 240 min. Then, the medium was removed, and a solution of MTT at a concentration of 0.5 mg/mL (diluted in RPMI or DMEM medium) was added to the plates, and finally, these were incubated for approximately 60 min until the development of the MTT-derived formazan insoluble crystals. Then DMSO was added to solubilize the crystals, and the absorbance was registered in a microplate reader (FlexStation® 3 Multi-Mode Microplate Reader) at 570 nm.

5.2.10.2 *Transepithelial Absorption Experiments*

The transepithelial absorption was developed according to Han et al. (2020), with adaptations. Coculture Caco2:HT29-MTX (9:1) cells were plated on 12-well Transwell® polycarbonate permeable inserts (Corning Costar, Corning, NY, USA) at 5×10^4 cells/mL. The cells were cultured for 25 days, and the medium was changed every 2-3 days at 37 °C in an atmosphere of 5% CO₂. Using a MILLICELL-ERS epithelial voltammeter (Millipore Co., Bedford, MA, USA) with “chopstick” electrodes, the transepithelial electrical resistance (TEER) was measured. The transepithelial absorption experiments were performed after 25 days, as the TEER values stabilized around 250–280 Ω.cm². The medium was removed, and cells were washed with Hanks (pH 7.4). For the apical side of the wells, a total of 0.5 ml compound solution (200 µM of C3G or C3G-C8) in Hanks (HBSS) pH 7.4 was added, while for the basolateral side of the wells was added 1.5 ml Hanks pH 7.4 containing 2% FBS. At 30, 60, 120, 180, and 240 min of incubation, samples (150 µL) were taken from the basolateral side and replaced by fresh Hanks containing 2% FBS. At 240 min, the samples were collected from the apical side, and the adsorbed fraction was collected with acidified CH₃OH.

5.2.11 Green Metric Tools

Analysis of Green Certified, according to Sajid & Plotka-Wasyłka (2022), and EcoScale, according to Van Aken et al. (2006), were used to evaluate the sustainability of the enzymatic acylation process. Penalty points (PP) with tabulated values were considered to evaluate this process, with a maximum score of 100 (considered environmentally safe).

5.2.12 Statistical analysis

The results were evaluated by analysis of variance (ANOVA) and the significant differences were determined using Tukey's Test at a probability level of less than 5% ($p < 0.05$).

5.3 RESULTS AND DISCUSSION

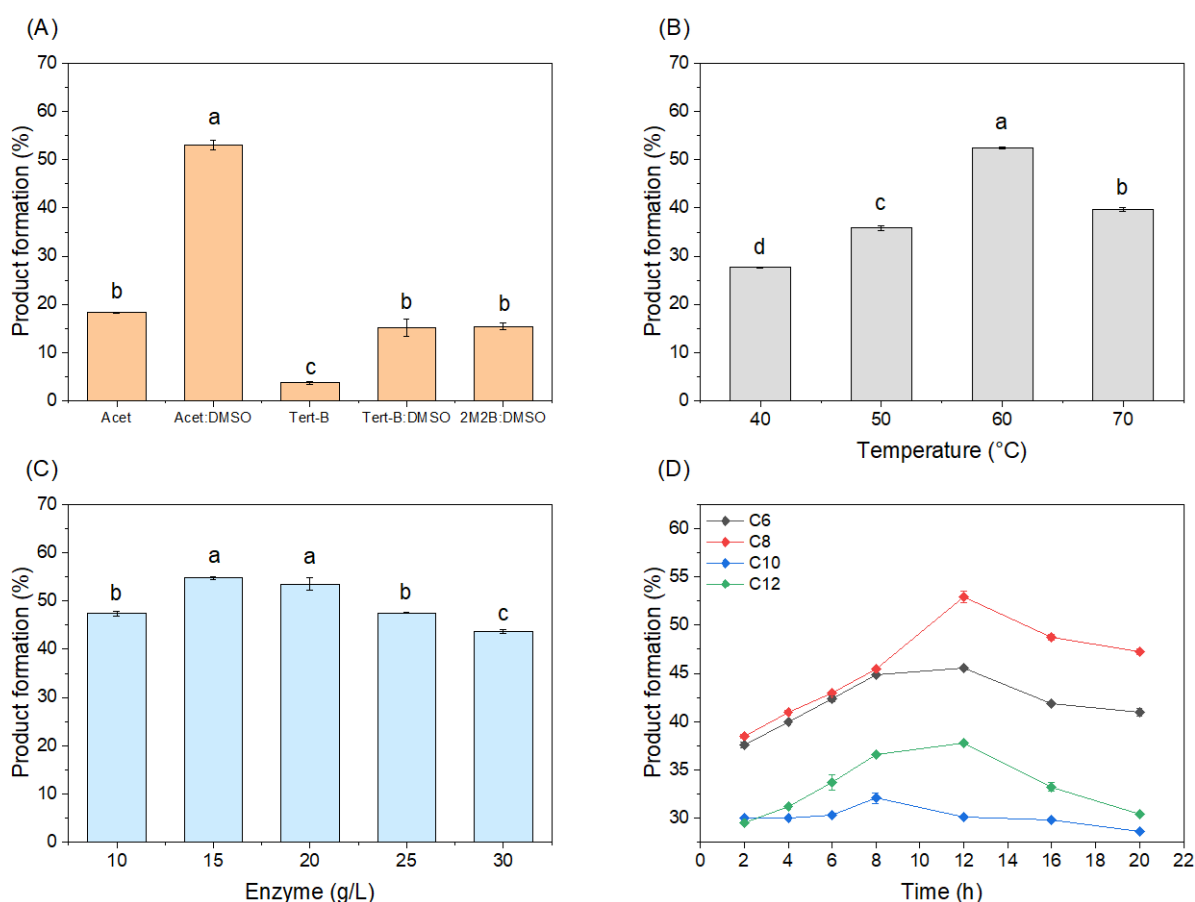
5.3.1 Enzymatic acylation of C3G-rich extract

A study was carried out using different experimental conditions to optimize the enzymatic acylation of C3G (Figure S7B - Appendix H) from black rice bran extract, previously purified according to Section 5.2.1, and using octanoic acid (C8) as the acyl donor group (Figure S7A - Appendix H). Firstly, acylation was carried out using solvents Acet, 2M2B, and Tert-B. Using 2M2B, the formation of the acylated compound was not verified through HPLC, while for the Tert-B solvent, only 3.7% of the product formation was verified. Product formation was higher using Acet as the solvent, equal to 18.3% (Figure 5.1A). In the study by Marathe et al. (2021), the authors performed acylation using acetone, t-butanol, t-amyl alcohol, and acetonitrile as solvents. The anthocyanin enzymatic acylation was verified only with acetonitrile as solvent.

Due to the high hydrophilicity of anthocyanins, there is difficulty in solubilizing these compounds in apolar solvents. The use of small proportions of a polar aprotic solvent, such as DMSO, helps catalyze enzymatic acylation, as reported by Cruz et al. (2018). Then, mixtures of Acet:DMSO (10:1, v/v), 2M2B:DMSO (10:1, v/v), and Tert-B:DMSO (10:1, v/v) were tested in order to increase the formation of the acylated product. Therefore, an improvement on the yield of enzymatic acylation was observed compared to the pure solvents. The formation of the product for 2M2B:DMSO (10:1, v/v) and Tert-B:DMSO (10:1, v/v) mixtures presented similar values, around 15%, while the mixture containing Acet:DMSO (10:1, v/v) allowed to obtain the maximum product formation around 53% (Figure 5.1A).

After solvent screening, enzymatic acylation was carried out using different temperatures. As observed in the Figure 5.1B, the temperature increased from 40 to 60 °C, and the yield of product formation also increased. However, when the temperature was extended to 70 °C, a decrease in yield was observed. In the study performed by Yang et al. (2018) the solubility of cyanidin is correlated with the conversion of the product, with the best results being obtained at 60 °C for the solvent tert-butanol, while for Acet, the best temperature was 40 °C. In our study, it was observed that the use of Acet with DMSO improved the solubility of the reaction and thus obtained the best conversion yield compared to the solvents 2M2B and tert-butanol. As the temperature increases, there is a decrease in product conversion, also verified by Marathe et al. (2021). This decrease might be correlated a reduction of enzymatic activity and/or anthocyanin degradation as well.

Figure 5.1 Percentage of acylated product formation using different solvents at 60 °C (A), different temperatures using Acet:DMSO (9:1) (B), different enzyme concentrations using Acet:DMSO (9:1) at 60 °C (C), and different fatty acids using Acet:DMSO (9:1) at 60 °C, and 15 g/L of enzyme (D).



Regarding the enzyme concentration, it was observed that increasing concentration (from 10 to 20 g/L) increases product yield, with higher values achieved using 15 and 20 g/L,

around 53% of acylated anthocyanin (Figure 5.1C). Concentrations of 25 and 30 g/L led to a decrease in product yield, around 6 and 10%, respectively. In the study by Marathe et al. (2021), when carrying out enzymatic acylation of anthocyanins from floral waste, the authors found that as the enzyme concentration increases to 20 g/L, the conversion of anthocyanin to the acylated product increases; however, at higher concentrations, the conversion yield decreases. In the Fernandez-Aulis et al. (2020) study, concentrations between 10 and 20 g/L of enzyme did not demonstrate differences in conversion yield, while a concentration lower than 5 g/L showed a decrease in yield. The decrease in conversion yield with increasing concentration may result from an increase in the viscosity of the medium, which can hinder mass transfer (MARATHE et al., 2021).

Enzymatic acylation kinetics was performed (Figure 5.1D) with different fatty acids as acyl donor groups (C6, C8, C10, and C12) using the solvent and parameters selected previously (solvent Acet:DMSO (10:1), 15 g/L of enzyme concentration, and 60 °C of temperature). The acylated compounds were analyzed in LC-DAD–ESI-MS (Figure S8 – Appendix I) in positive ion mode (Table 1). The chromatographic peak products' molecular ions were observed to correspond to the respective cy3glc-fatty acid conjugates (C6-C12), and MS² verified that the fragmentation occurred only at the glucose residue.

Table 5.1 Mass data of C3G and C3G-fatty acid derivatives (C6-C12) in positive ion mode.

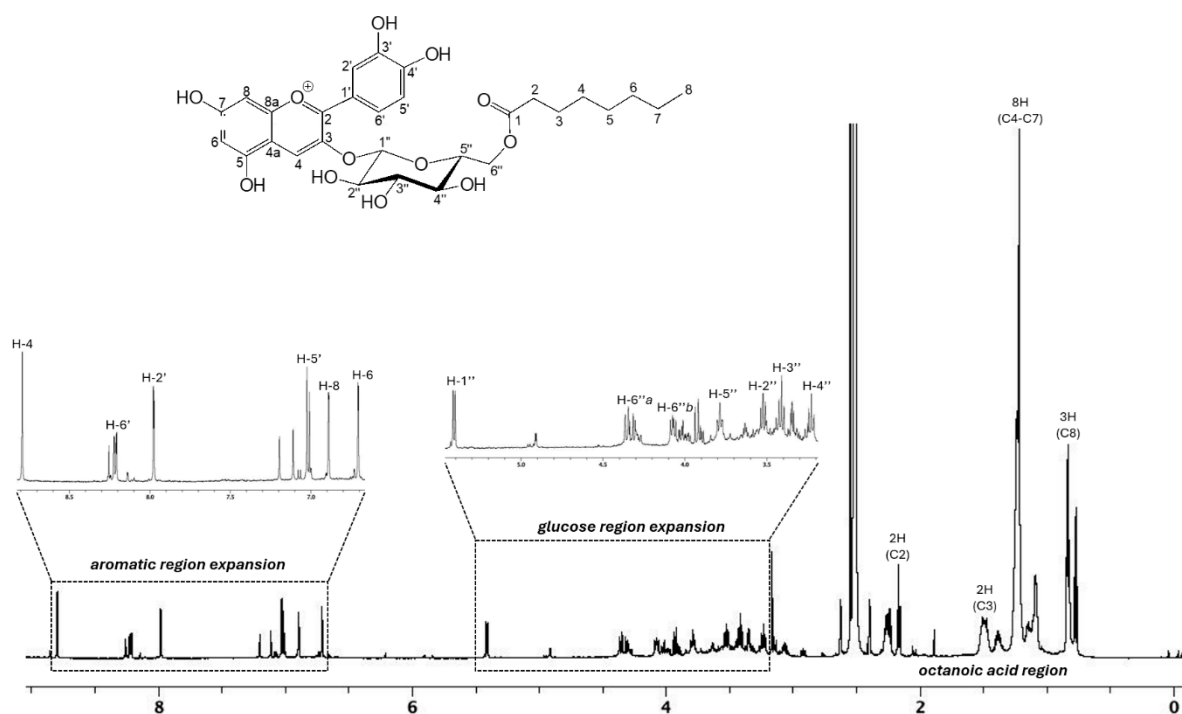
Compound	MW (g/mol, Cl ⁻)	Rt (min)	λ_{max} (nm)	m/z	
				[M] ⁺	MS ²
C3G	484	14.03	516	449	287
C3G-C6	583	16.13	524	548	287
C3G-C8	610	16.76	526	575	287
C3G-C10	638	17.77	527	604	287
C3G-C12	666	18.88	528	632	287

It was possible to observe that the maximum yield was achieved after 12 h of reaction using the C8 acid, obtaining around 53% conversion of C3G into acylated product. Compared with studies that performed enzymatic acylation of C3G with octanoic acid, the optimization allowed an increase in product conversion by 13% compared to the study by Guimarães et al. (2018) and 6% compared to the study by Lin et al. (2022). The other acids showed higher yields between 8 and 12 h, being equal to around 46% for C6 (12 h), 32% for C10 (in 8 h), and 38% for C12 (12 h). Similar results were obtained in the study by Cruz et al. (2017), in which enzymatic acylation of malvidin-3-glucoside was carried out with different fatty acids for 6 to

12 h, obtaining a higher yield for the C8 acid (40%), followed by the C4 (40%), C12 (35%), and C10 (30%). In the study by Guimarães et al. (2018), in the enzymatic acylation of C3G with different fatty acids for 12 h, the highest yield was obtained for the C4 acid (47%), followed by the C6 and C8 acids (40%), C10 (30%) and C12 (25%). The results show a trend for lowering conversion rates as the fatty acid chain increases. Fatty acids with shorter chains are generally better for carrying out the enzymatic acylation of anthocyanins. Therefore, the acylated compound was purified with octanoic acid, according to section 2.6, and was subsequently characterized by HPLC-DAD, HPLC-MS (Figures S7C – Appendix H, and S8B – Appendix I) and NMR spectroscopy (Figure 5.2 and Table S2 – Appendix J).

The chemical structure of the C3G-C8 was confirmed using 1D and 2D NMR techniques (Figure 5.2). Downfield chemical shifts (~ 0.2 – 0.5 ppm) (Table S1) of the 6''a-H and 6''b-H protons of glucose occurred in C3G-C8 compared to its precursor C3G (LIN et al., 2022). Therefore, the NMR data confirmed that the fatty acid took place at 6''-OH of glucose residue (Figure 5.2), as already reported in other related works (CRUZ et al., 2018; LIN et al., 2022; MARQUEZ-RODRIGUEZ et al., 2021).

Figure 5.2 ^1H NMR spectrum (600.13 MHz) of C3G-C8 conjugate recorded in $\text{DMSO-}d_6/\text{TFA}$ (9:1).

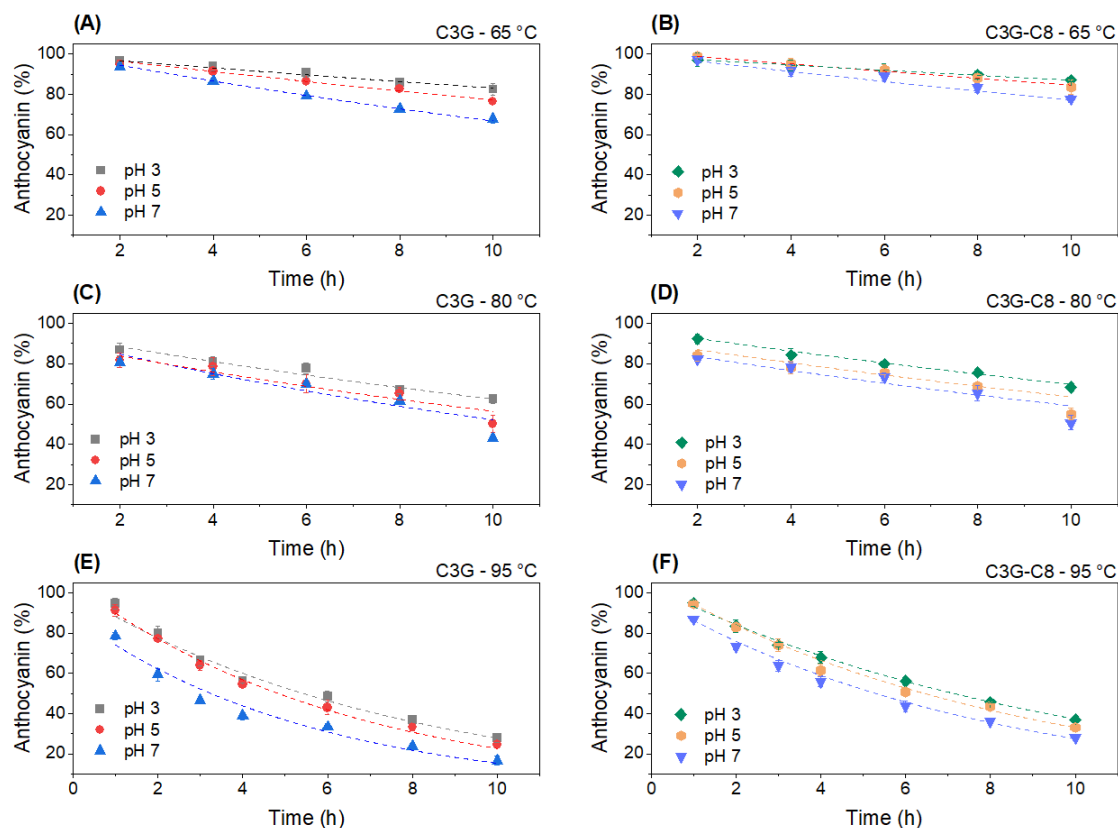


5.3.2 Thermostability

An accelerated degradation process was applied to assess the thermostability of C3G and acylated derivative, and thermodynamic parameters, such as the degradation rate constant (k) and half-life time ($t_{1/2}$), were evaluated by kinetic models (LEONARSKI et al., 2022). Figure 5.3 shows that increasing temperature causes an increase in the degradation of anthocyanins. Both C3G and C3G-C8 presented better stability in acidic conditions (pH 3) and decreased as the pH increased. In addition, it was possible to verify that the degradation decreased for the acylated compound.

According to Guimarães et al. (2018), for C3G and its acylated precursors (with C4, C6, C8, C10, and C12 fatty acids) at pH 3, the flavylium cation is the main species present at the equilibrium. At pH 5, the flavylium cation of C3G is reduced to a molar fraction of 70%, while for the acylated conjugate, the flavylium cation species is still presented at 90%. This possibly explains the greater degradation of the non-acylated compound with increasing temperature. At pH 7, no flavylium cation is found; therefore, thermal degradation is higher than other pHs (3 and 5). Generally, at this pH, the thermal degradation mechanism occurs in several stages due to the presence of multiple electron-rich species such as quinoidal bases and chalcones.

Figure 5.3 Thermostability of C3G in pH 3 (A), pH 5 (C), and pH 7 (E), and of C3G-C8 in pH 3 (B), pH 5 (D), and pH 7 (F) at 65, 80, and 95 °C. The dashed lines correspond to the fit to the degradation model.



The results presented in Table 5.2 showed that the fatty acid acylation of C3G increased the half-life times of the acylated derivative enhancing their thermostability in lipophilic media. Some authors, when carrying out acylation of cyanidin-3-glucoside from different sources such as blackcurrant (YANG et al., 2019), blackberries (GUIMARÃES et al., 2018), and husk of tilapo and corn (FERNANDEZ-AULIS et al., 2020) also showed an improvement in the thermostability of the acylated compounds through an increase in the half-life times when compared to the non-acylated compounds. It can be also observed that anthocyanins' stability is superior in more acidic media, as the electron polarization due to the increase in pH by deprotonation increases, taking the absorption maxima towards higher wavelengths (HOUGHTON; APPELHAGEN; MARTIN, 2021).

This improvement on stability is dependent on the acyl donor group. According to the study by Guimarães et al. (2018), it was possible to observe that the longer the fatty acid chain, the longer the half-life time of the compound, suggesting greater resistance in the hydrolysis of the glycosidic bond which would lead to the degradation of anthocyanins. However, the enzymatic acylation of anthocyanins has lower formation yields as the fatty acid chain

increases. Therefore, medium-chain fatty acids may present a better balance when assigned to enzymatic synthesis since they will present both good yields and improved thermostability.

Table 5.2 Kinetic and thermodynamic parameters of thermal stability of C3G and C3G-C8 from black rice bran.

Compound	T (°C)	pH	<i>k</i> (h ⁻¹)	<i>t</i> _{1/2} (h)	<i>E</i> _a (kJ/mol)	Δ <i>H</i> (kJ/mol)	Δ <i>G</i> (kJ/mol)	Δ <i>S</i> (kJ/mol.K)
C3G	65	3	0.0203	34.18	63.63 (0.9827)	60.82	210.25	-0.442
	80		0.0430	16.12		60.69	217.50	-0.444
	95		0.1290	5.37		60.57	223.50	-0.443
C3G-C8	65	3	0.014	50.48	69.11 (0.9978)	66.30	211.35	-0.429
	80		0.0360	19.25		66.17	218.02	-0.430
	95		0.1020	6.80		66.05	224.22	-0.430
C3G	65	5	0.0267	25.98	57.58 (0.9957)	54.78	209.48	-0.458
	80		0.0580	11.95		54.65	216.62	-0.459
	95		0.1420	4.88		54.53	223.21	-0.458
C3G-C8	65	5	0.0206	33.65	58.7 (0.9998)	55.89	210.21	-0.456
	80		0.0490	14.15		55.77	217.12	-0.457
	95		0.1130	6.13		55.64	223.91	-0.457
C3G	65	7	0.0416	16.67	46.13 (0.9863)	43.32	208.23	-0.488
	80		0.0730	9.50		43.20	215.95	-0.489
	95		0.1590	4.36		43.07	222.86	-0.488
C3G-C8	65	7	0.0275	25.20	52.17 (0.9979)	49.35	209.40	-0.473
	80		0.0570	12.16		49.23	216.67	-0.474
	95		0.1250	5.55		49.11	223.60	-0.474

T: temperature, *k*: degradation rate, *t*_{1/2}: time of half-live, *E*_a: activation energy, Δ*H*: activation, enthalpy, Δ*G*: free energy of activation, Δ*S*: activation entropy.

The activation energy (*E*_a) was obtained by the ln *k* (Table 5.2) values used against 1/*T* and fitted by the Arrhenius equation. *E*_a is a parameter that describes the energy required to reach the transition state of a reaction (TEIXEIRA et al., 2021). The higher the activation energy, the more difficult it becomes to degrade the compound. In our study, the acylated product showed slightly higher *E*_a than the native C3G, with a similar behavior reported by Yang et al. (2019).

Positive values of activation enthalpy (Δ*H*) indicate that the reaction and degradation was endothermic since this parameter measures the endothermic state between the reactant and the activated complex (CONDURACHE et al., 2021). It represents the strength of the bonds that are broken down and reconstituted during the transition process to be overcome by reacting

molecules (BENVENUTTI; ZIELINSKI; FERREIRA, 2022). The ΔH value of C3G for pH 3 was similar to the C3G of black rice obtained by Hou et al. (2013).

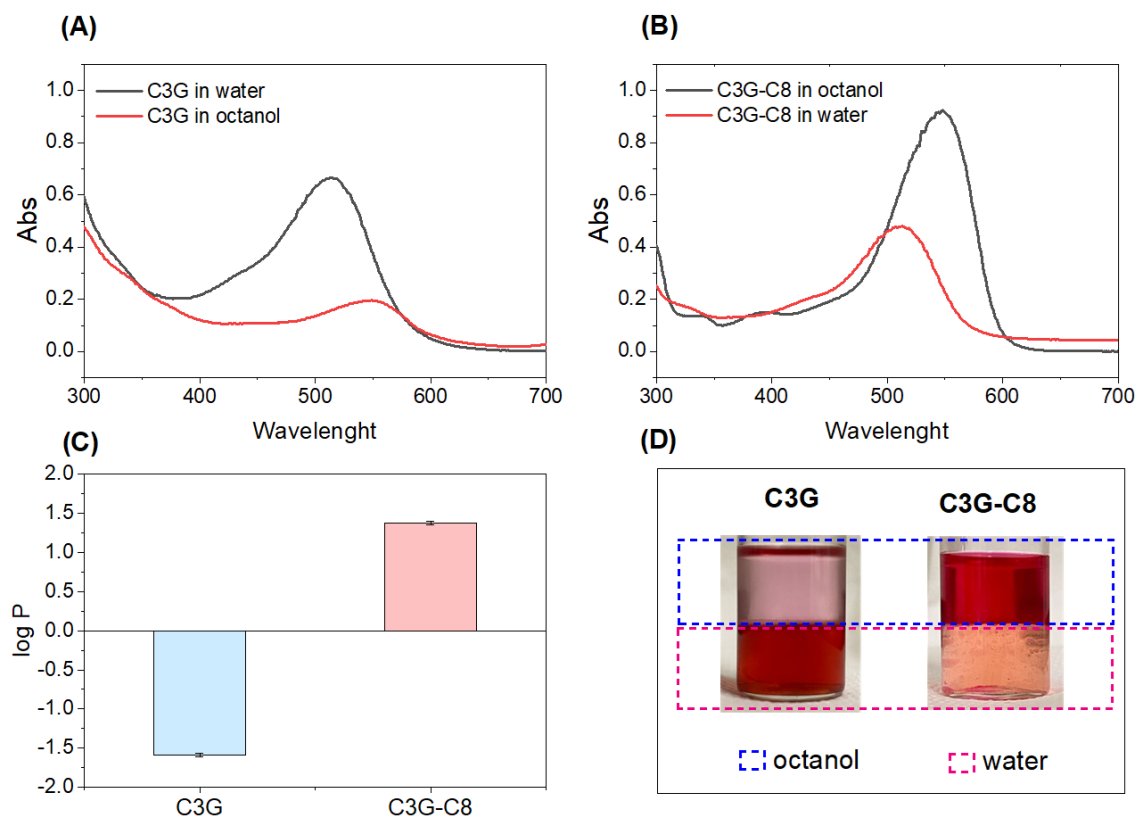
Regarding ΔG and ΔS , positive ΔG values indicate a nonspontaneous reaction and ΔS negative values show that the reaction is irreversible and the molecules are more organized than in the initial reaction (CHEOK; RAGUNATHAN, 2022; CONDURACHE et al., 2021; PERON; FRAGA; ANTELO, 2017). Both anthocyanins C3G and C3G-C8 presented similar values to both parameters.

5.3.3 Partition coefficient determination

For the partition coefficient (Figure 5.4), confirmation of the greater lipophilicity of the acylated compound (C3G-C8) concerning its precursor (C3G) can be verified by the results presented in Figures 5.4A and 5.4B, in which C3G presented greater absorbance in the phase containing water. In contrast, C3G-C8 presented greater absorbance in the phase containing octanol. In Figure 5.4D, this division between the phases can be better observed.

Regarding $\log P$ (Figure 5.4C), higher values generally represent greater lipophilicity. For C3G, the value was -1.59, while for C3G-C8, the value was equal to 1.37. In the study by Zhang et al. (2021), the authors presented a value for C3G equal to -1.42. After acylation (with methyl butyrate, methyl n-octanoate, methyl laurate, methyl myristate, methyl palmitate, and methyl stearate), the values changed to 0.7–1.2. In the study by Yang et al. (2019), the authors reported a C3G value of around -1.2, while the compound acylated with lauric acid presented a value of around 1.3. Altering the hydrophilic-lipophilic balance allows the use of such derivatives in more lipid-based matrices (YANG et al., 2018).

Figure 5.4 Spectral of (A) Cyanidin-3-glucoside (C3G) and (B) Cyanidin-3-glucoside-octanoic ester (C3G-C8) in water (HCl 0.1 M) and octanol. (C) Log P values and (D) biphasic mixture in an octanol (upper phase)-water 0.1 M HCl (lower phase) for C3G and C3G-C8.



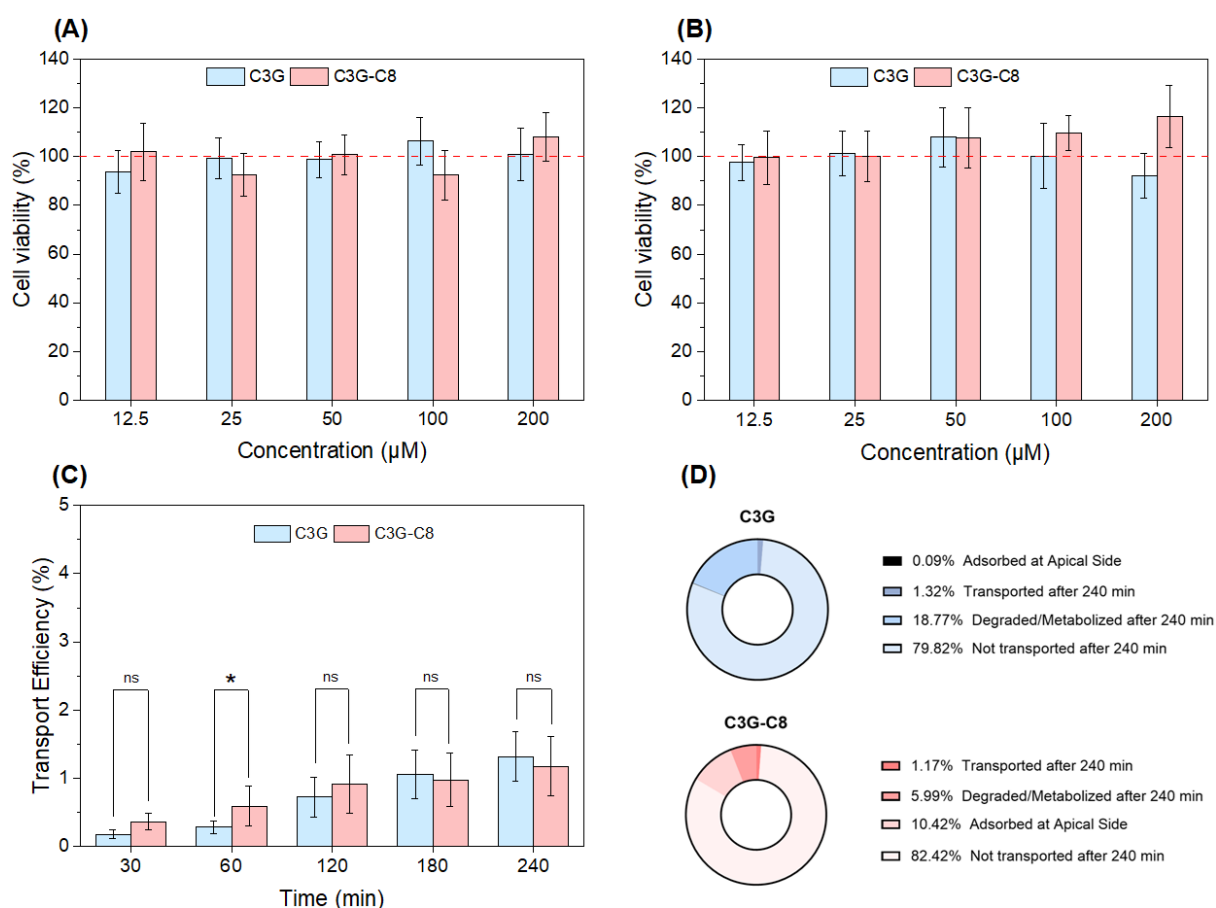
5.3.4 Cell culture

5.3.4.1 Cytotoxicity by MTT assay

Initially, a cytotoxicity assessment was performed for NCI-N87 cells and the Caco2:HT29-MTX coculture cells. This step is crucial for the absorption experiments, since the anthocyanin concentration used must preserve the integrity of the cell monolayers and consequently not interfere with the results of the experiments. Although they are natural compounds, high concentrations can be toxic to cells. Cyanidin was not considered toxic up to a concentration of 200 μM for Caco2, NHF, and Hela cells (LAZZE et al., 2004). In the study by Liu et al. (2022), the authors did not observe cytotoxicity at a concentration of 200 μM of wine anthocyanins; however, at 400 μM , the compounds were reported to be harmful to Caco2 cells. Jing et al. (2020) verified that up to 200 μM bilberry anthocyanins were not cytotoxic to MC38 tumor cells and normal cells (L929), but at 500 μM the extract was cytotoxic. Through viability of gastric (MKN-28) and intestinal (Caco-2) cell assays, Han et al. (2020) verified that the use of 200 μM anthocyanins is recommended for carrying out transepithelial transport experiments.

It can be observed in Figure 5.5A-B that for both cells, neither concentration showed a decrease in cell viability, and they were not cytotoxic, therefore, for the next step, a concentration of 200 μM of the compounds was used. In the study by Teixeira et al. (2024), the authors did not observe cytotoxicity for 0.8 mg/mL edible flowers' purified anthocyanin extracts on a Caco-2 cell line. In the study by Zhang et al. (2024), the authors did not verify cytotoxicity for up to 100 μM of anthocyanin enzymatically acylated with oleic acid for carcinoma cell (HepG2 cell), and normal cell line (QSG-7701 cell).

Figure 5.5 Cytotoxicity assay for (A) NCI-N87 cells and (B) Caco2:MTX-HT29 cells over 4 hours using 200 μM C3G and C3G-C8; (C-D) Transport Efficiency (%) by Transepithelial absorption experiments for Caco2:MTX-HT29 cells using 200 μM C3G and C3G-C8.



5.3.4.2 Transepithelial Absorption Experiments

For the transport study, for a more accurate simulation of the intestine, cocultured Caco-2 and HT29-MTX (9:1) cells were used since they provide a monolayer of enterocytes and goblet cells, which are the two main phenotypes of the human intestinal epithelium (JAILANI; WILLIAMSON, 2014). The presence of mucus (produced by goblet cells) under normal conditions tends to limit the absorption of compounds. Therefore, the absorption of compounds

is decreased for the cocultured cell model (Caco-2 and HT29-MTX) when compared to monolayer Caco-2 cells (ZOU et al., 2024).

The transport efficiency of anthocyanins was evaluated using the Caco2:HT29-MTX coculture model. Transepithelial transport was time-dependent, increasing throughout 4 h (Figure 5.5C). Only at 60 min there was a significant difference between the acylated anthocyanin and its precursor. At the end of the time, just over 1.1% of transport was observed for both compounds. Kuntz et al. (2015), when evaluating the transport of anthocyanins from red grapes, verified a transport of 0.35% for malvidin-3-glucoside using a model with Caco2 cells, and this value decreased to only approximately 0.05% when they used the Caco2:HT29-B6 coculture model. In the study by Kamiloglu et al. (2017), the authors reported the transport efficiency of acylated and undigested anthocyanins from black carrots to a model with Caco2 cells between 0.8% and 2.1%, values close to those reported in our study. Han et al. (2020), when carrying out an epithelial transport study on wine anthocyanins, the authors reported transport efficiencies of approximately 9% for malvidin-3-O-glucoside and malvidin-3-O-(6-O-coumaroyl)-glucoside-5-O-glucoside (acylated compound) for gastric MKN-28, and approximately 3% for intestinal Caco-2 cells (not differentiating between them, $p < 0.05$). These values are higher than those reported in our study because, as previously mentioned, models using cocultures tend to be more complex to simulate the intestine better.

It was also verified that approximately 80% of the anthocyanins were not transported after 4 h (Figure 5.5D). Kamiloglu et al. (2017) reported 56-98% values for apical recoveries of acylated anthocyanins from black carrots. Among these compounds that were not transported, only $0.09\% \pm 0.05$ was adsorbed on the apical side to C3G, while $18.8\% \pm 3.2$ was degraded. As for the acylated compound, only $5.9\% \pm 3.1$ of the compounds were degraded, while $10.42\% \pm 0.9$ were adsorbed on the apical side, and in addition, approximately $7.4\% \pm 0.7$ (71% of the total) was converted to C3G. Hahm et al. (2021) verified that acylated anthocyanins were relatively more stable than non-acylated ones during intestinal absorption (evaluated in Sprague-Dawley rats). Although C3G-C8 was converted to C3G, the transport of these compounds during these 4 hours was not verified, but this possibility should be considered when the compounds are exposed for more time.

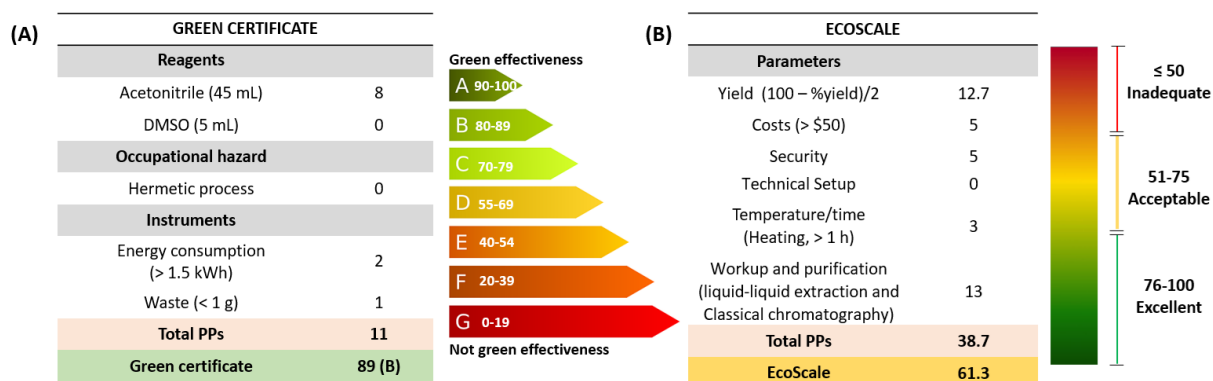
In the study by Jailani & Williamson (2014), the authors found that the addition of oils with a high proportion of saturated, monounsaturated, and polyunsaturated fatty acids can decrease the transport in Caco-2:HT29-MTX models of flavonols (in the case of galangin, the most hydrophobic), or increased the transport/conjugation (in the case of quercetin and kaempferol). Also, according to the authors, the lipid matrix has the potential to modify the

transport, with the effect being dependent on the structure and hydrophobicity. In our study, acylation with fatty acid did not affect the transport. Still, it provided adsorption on the apical side, which was converted to its precursor (C3G) and can potentially be transported to the basolateral side.

5.3.5 Green metric tools

Analytical processes must be linked to environmental issues, and therefore, it is essential to investigate the actions that chemicals can have on the environment, experimentally and commercially (IMAM; ABDELRAHMAN, 2023). Therefore, researchers have developed detailed tools to ensure process greenness and sustainability using green chemistry principles (CETINKAYA; KAYA; OZKAN, 2023). The enzymatic acylation of anthocyanins is a complex process, and to better understand this method, metric tools (Green Certificate and EcoScale, Section 5.2.11) were applied to assess its eco-friendliness. For the Green Certificate, the solvents, occupational hazards, instruments, and waste of the process were evaluated (Figure 5.6A). The results obtained for the enzymatic acylation of anthocyanins was 89 (B) on a scale ranging from A to G, with A being considered the greenest (ARMENTA et al., 2015). Different parameters are evaluated for EcoScale, with security, cost, and purification presenting the most penalties (Figure 5.6B). The scale of this method is from 0 to 100, with the result obtained being 61.3, which is considered acceptable. Considering that the enzymatic acylation of anthocyanins will grow in the upcoming years, it is important that, in addition to increasing the product yield, to focus also on enhancing the sustainability of whole process.

Figure 5.6 (A) The Green Certificate and the (B) EcoScale of the enzymatic acylation technique.



5.4 CONCLUSION

It was possible to obtain around 53% of the formation of acylated anthocyanin (C3G-C8) by optimizing the parameters, using Acet:DMSO (10:1, v/v) as the solvent, 60 °C and 15 g/L of CalB lipase. Furthermore, an increase in half-life time was observed (between 18% and 51%) in the stability of these compounds at different pHs (3, 5, and 7); therefore, the acylation increased the anthocyanin's thermostability. An increase in lipophilicity was verified for C3G-C8 ($\log P = 1.37$) when compared to C3G ($\log P = -1.59$). Furthermore, no cytotoxicity (200 μ M) was verified for both compounds, and the transepithelial transport study was similar for both anthocyanins (about 1.2% transport efficiency). However, the degradation observed by the apical side was lower for the acylated anthocyanin. The enzymatic acylation method for the green metric tools presented a score of 89 (B) for the Green Certificate and was considered acceptable for EcoScale (61.3). Therefore, the acylated compound (C3G-C8) showed greater thermostability than anthocyanin (C3G) and has potential for application in lipid matrix products.

6 FINAL CONSIDERATIONS & FUTURE PERSPECTIVES

This study evaluated different techniques for the recovery of anthocyanins from black rice bran, considered a by-product of rice processing. It was possible to obtain approximately 80% of anthocyanins through optimization by alternative techniques (PLE, UAE, and MAE), a result higher than the conventional method (about 44%). Furthermore, the extracts obtained by alternative methods presented similar contents of total phenolic compounds (about 21 mg GA/g) and antioxidant capacity (DPPH, ABTS, and FRAP). All extracts presented first-order degradation models and similar thermodynamic parameters. Furthermore, they did not present cytotoxicity for L929 cells (500 $\mu\text{g/mL}$), did not present antitumor activity (A549 lung adenocarcinoma cells, C6 rat glioma cells, and GL261 mouse glioma cells), and presented cytoprotection of approximately 90% for H_2O_2 -induced oxidative stress in L292. In general, the extracts from all technologies showed great application potential. For the continuation of our studies, the extract obtained by UAE was utilized since it presented a higher quantity of C3G, an essential compound for the enzymatic acylation step. Through enzymatic acylation, it was possible to optimize the process using octanoic fatty acid (C8) as the acyl donor group, obtaining up to 53% product conversion, verified by HPLC-DAD and HPLC-MS. The NMR data confirmed that the fatty acid took place at 6''-OH of glucose moieties. This acylated compound (C3G-C8) showed better stability, as verified thermally and at different pHs (3, 5, and 7), and during the epithelial transport process in intestinal cocultures, in which C3G showed superior degradation. No cytotoxicity was observed for both anthocyanins at concentrations up to 200 μM for NCI-N87 cells and Caco2:HT29-MTX coculture. Both presented similar transepithelial transport efficiency using the coculture (about 1.2%). Increased lipophilicity was also verified for the acylated compound, thus increasing the range of application of this compound.

In addition to epithelial transport with intestinal cocultures, a study should also be conducted on gastric cultures to better elucidate these compounds' absorption process (study on going). The transformation of the C3G molecule to C3G-C8 allowed greater affinity to lipid matrices, which may facilitate the application of these compounds in emulsions in both the cosmetic and food areas. Furthermore, the use of alternative techniques for performing enzymatic acylation is an approach to be addressed, as in extraction, to obtain a higher yield in the formation of products using less time and greener solvents. The use of alternative enzymes is also an approach to be evaluated. Therefore, two paths still need to be explored: alternative

methods for the enzymatic acylation process and the application of these compounds in lipid matrices.

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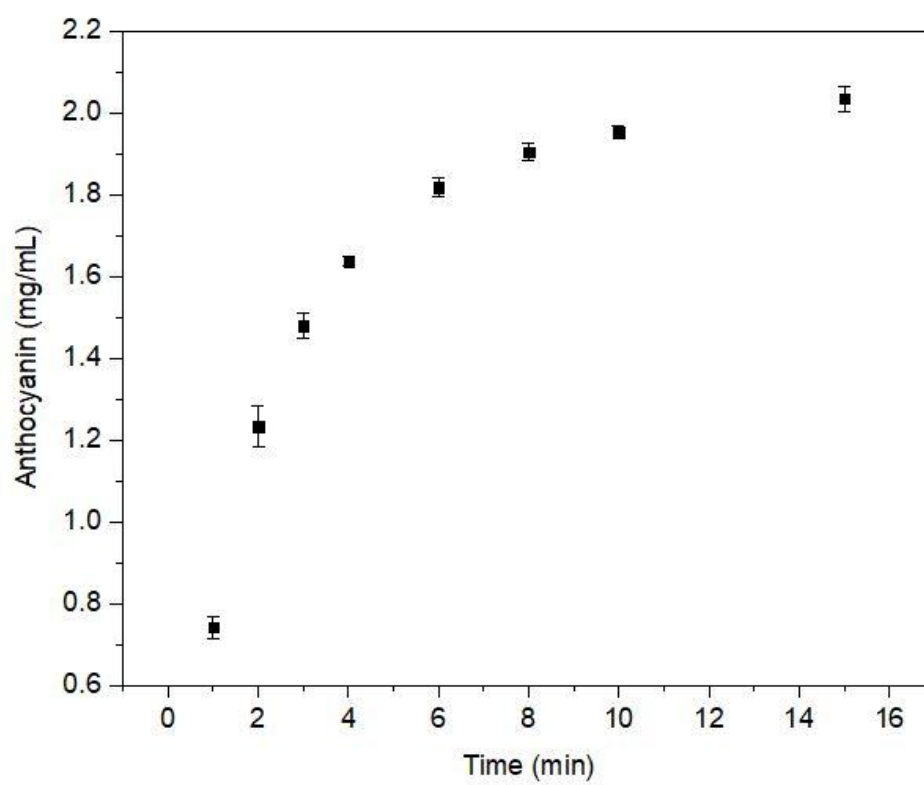
APPENDIX A

Table S1 – Regression coefficients and statistical parameters of multi-regression adjustment of each response variable in the Box-Behnken Design.

Response variables	Factors	Regression coefficient	Standard error	t-value	p-value	- 95 confidence	+ 95 confidence
AR (%)	Constant	52.41211	1.305830	40.13703	0.000000	49.5025	55.32168
	x ₁	22.56722	3.329225	6.77852	0.000049	15.1492	29.98520
	x ₂	12.53232	3.329225	3.76434	0.003696	5.1143	19.95030
	x ₂ ²	-8.36582	2.442983	-3.42443	0.006499	-13.8091	-2.92251
	x ₃ ²	10.61704	2.442983	4.34593	0.001453	5.1737	16.06035
R ²	0.90						
adjusted R ²	0.86						
p model	< 0.01.						
p lack of fit	0.059						
PC (%)	Constant	45.11138	0.610761	73.86092	0.000000	43.7505	46.47224
	x ₃	-6.29280	1.703331	-3.69441	0.004147	-10.0881	-2.49754
	x ₃ ²	4.24297	1.182734	3.58742	0.004950	1.6077	6.87826
	x ₁ .x ₃	-6.94228	2.285259	-3.03785	0.012508	-12.0342	-1.85040
	x ₁ ² .x ₃	-4.55521	1.615922	-2.81895	0.018193	-8.1557	-0.95471
R ²	0.84						
adjusted R ²	0.78						
p model	< 0.01						
p lack of fit	0.209						

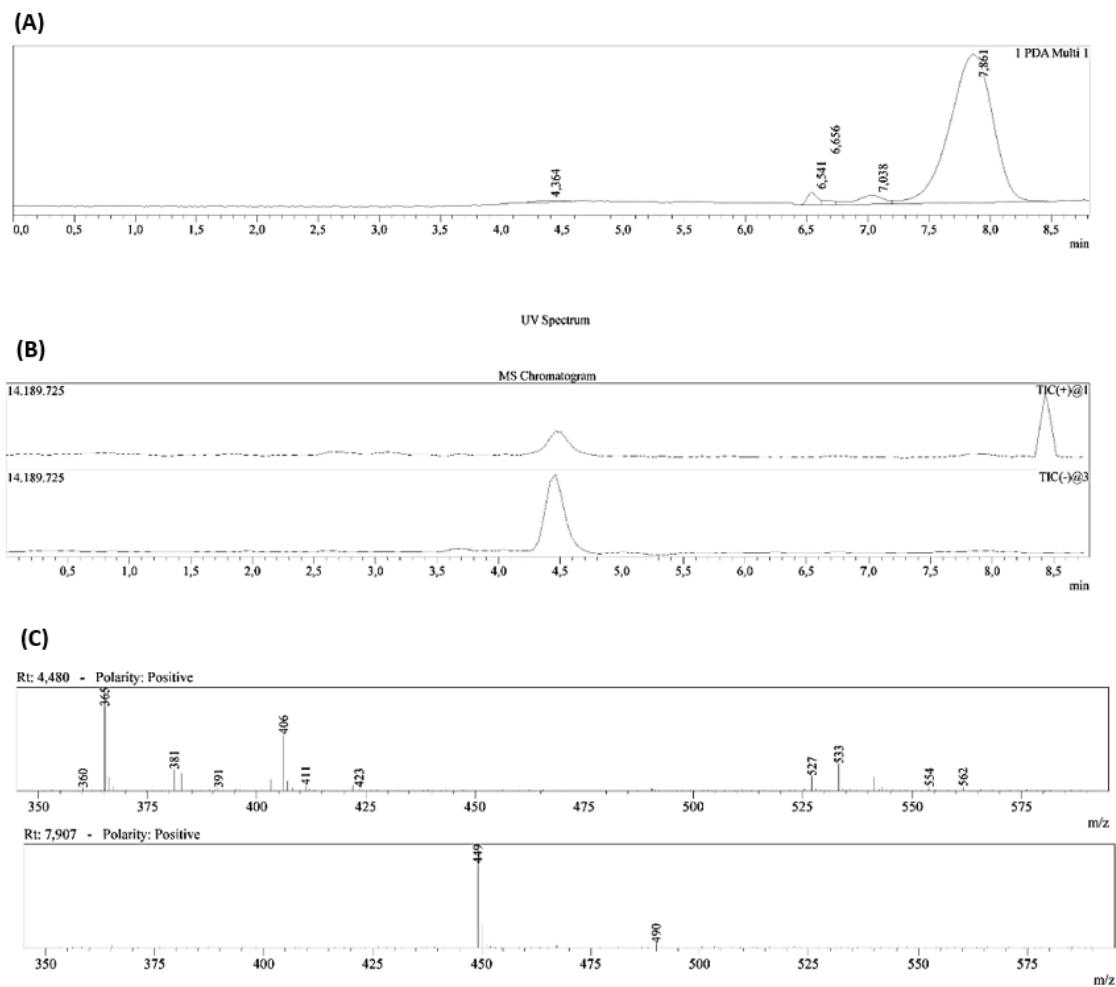
Note - x₁: temperature, x₂: flow rate, x₃: solvents (Citric acid: Ethanol), AR – anthocyanin recovery, PC – polymeric color.

APPENDIX B

Figure S1. Extraction kinetics of total monomeric anthocyanin (ATM) content.

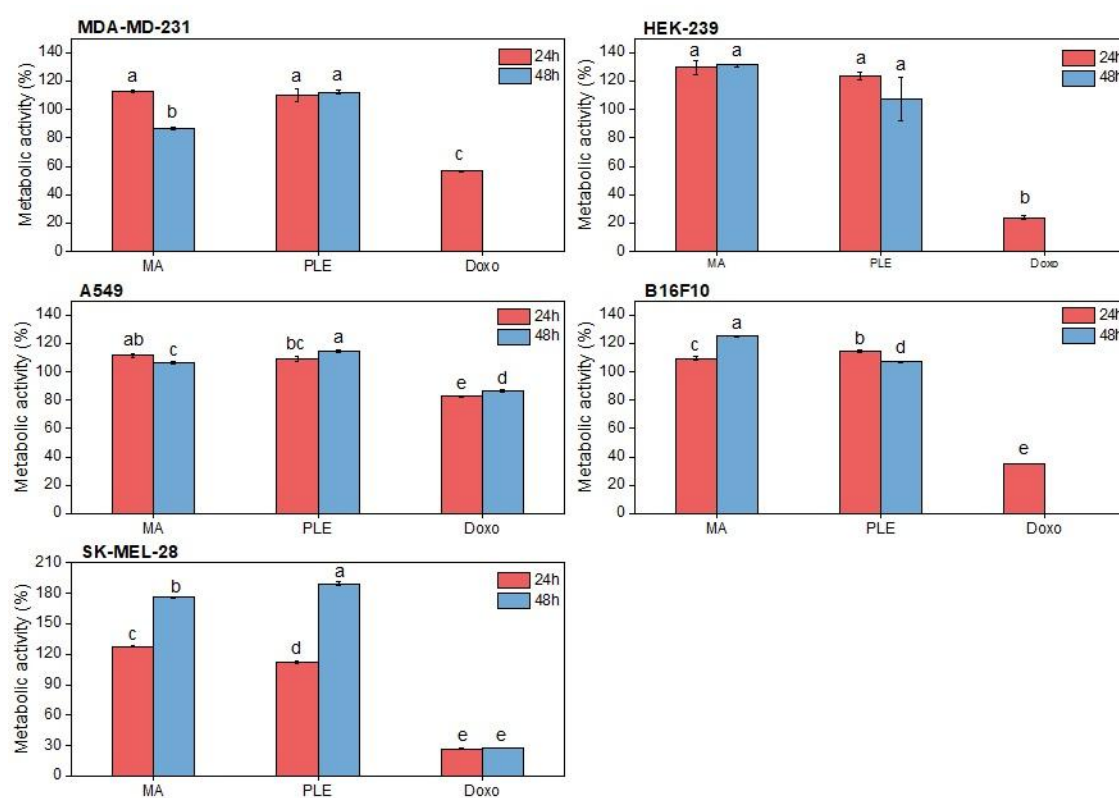
APPENDIX C

Figure S2. Representative LC chromatogram of anthocyanin extract: (A) UV, (B) Mass spectra and (C) retention time. The 449 m/z peak corresponds to C3G.



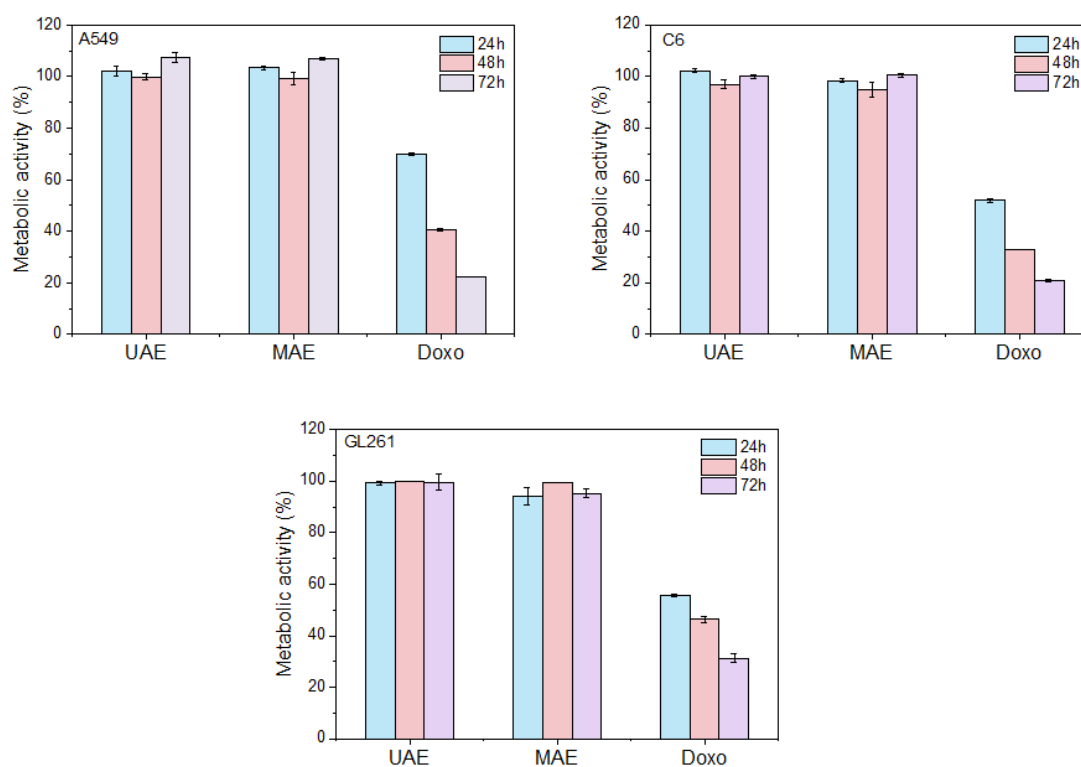
APPENDIX D

Figure S3. Antitumoral activity (%) of PLE and MA extracts in human breast (MDA-MD-231) cells, embryonic Kidney (HEK-293) cells, lung adenocarcinoma (A549) cells, melanoma (B16F10) cells, and malignant skin melanoma (SK-MEL-28) cells.



APPENDIX E

Figure S4. Antitumoral activity (%) of UAE and MAE extracts in lung adenocarcinoma (A549) cells, rat glioma cells (C6) and mouse glioma cells (GL261).



APPENDIX F

Figure S5. (A) The Green Certificate and the (B) EcoScale of the HSE and PLE techniques.

(A)

	HSE	PLE	Green effectiveness
Reagents			A 90-100
Ethanol	4	6	B 80-89
Acidified water	0	0	C 70-79
Instruments			D 55-69
Energy consumption (≤ 1.5 kWh)	1	0	E 40-54
Waste (< 1 g)	1	3	F 20-39
Total PPs	6	7	G 0-19
Green certificate	94 (A)	91 (A)	Not green effectiveness

(B)

	HSE	PLE	
Parameters			
Yield $(100 - \%yield)/2$	27.2	9.9	≤ 50 Inadequate
Costs ($< \$10$)	0	0	
Security	5	5	51-75 Acceptable
Technical Setup	0	3	
Temperature/time (Heating, < 1 h)	2	2	
Workup and purification	0	0	76-100 Excellent
Total PPs	34.2	19.9	
EcoScale	65.8	80.1	

APPENDIX G

Figure S6. Published article about scale-up for anthocyanin recovery from black rice bran.



Article

The Potential of Crude and Partially Purified Black Rice Bran Extracts Obtained by Ultrasound-Assisted Extraction: Anti-Glycemic, Cytotoxicity, Cytoprotective, and Antitumoral Effects

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Abstract: Recovering anthocyanins from black rice bran is a way of valuing this byproduct, by obtaining an extract with biological potential. The objective of this study was to recover anthocyanins using ultrasound-assisted extraction. Some of the extract was partially purified, and both (crude and partially purified) extracts were evaluated for their anthocyanin content, antioxidant activity, antidiabetic and antitumoral activities, cytotoxicity, and oxidative stress. An increase in the laboratory scale was also achieved, making possible to increase the extraction volume up to 20 times without significantly changing the content of anthocyanins (1.85 mg C3G/g DW). It was found that the purified sample presented a 4.2 times higher value of total anthocyanins compared to the crude sample. The best IC₅₀ values for the purified sample were verified by DPPH and ABTS (0.76 and 0.33 mg/mL). The best results for antidiabetic activity were obtained for the partially purified sample: 0.82 µM C3G for α-glucosidase and 12.5 µM C3G for α-amylase. The extracts demonstrated protection (~70%) when subjected to the oxidative stress of L929 cells. An antitumoral effect of 25–30% for both extracts was found in A459 cells. The crude and partially purified extracts of black rice have antidiabetic and anticancer effects and more studies are needed to explore their potential.

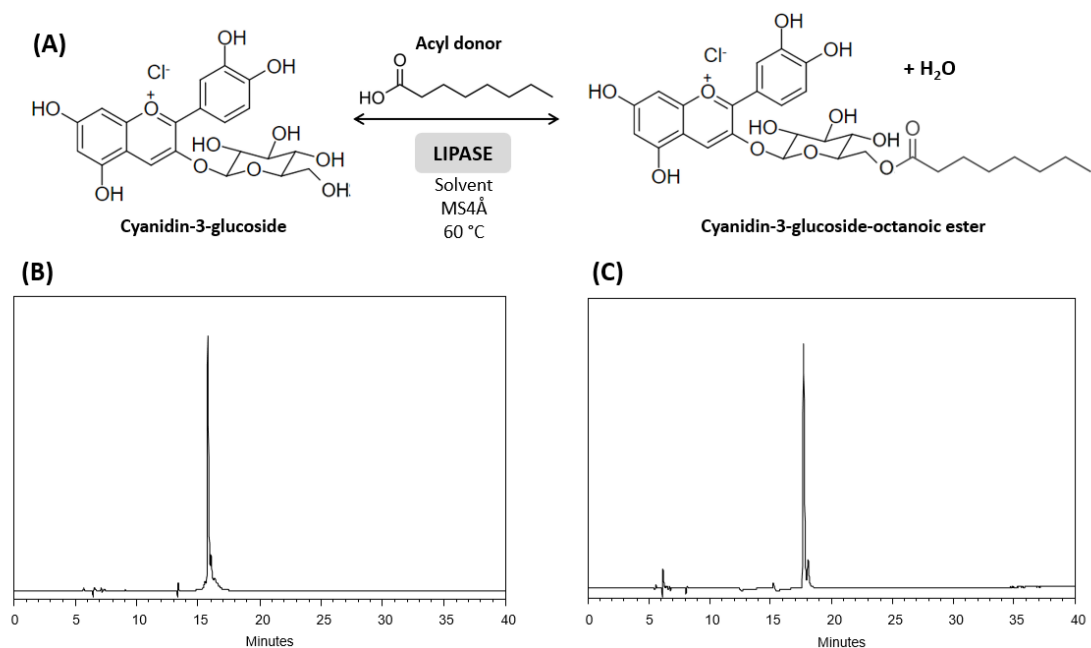
Keywords: *Oryza sativa* L.; purification; oxidative stress; antidiabetic potential



Citation: Leonarski, E.; Kuasnei, M.; Santos, E.H.; Moraes, P.A.D.; Cesca, K.; Oliveira, D.d.; Zielinski, A.A.E. The Potential of Crude and Partially Purified Black Rice Bran Extracts Obtained by Ultrasound-Assisted Extraction: Anti-Glycemic, Cytotoxicity, Cytoprotective, and Antitumoral Effects. *Foods* **2024**, *13*, 597. <https://doi.org/10.3390/foods13040597>

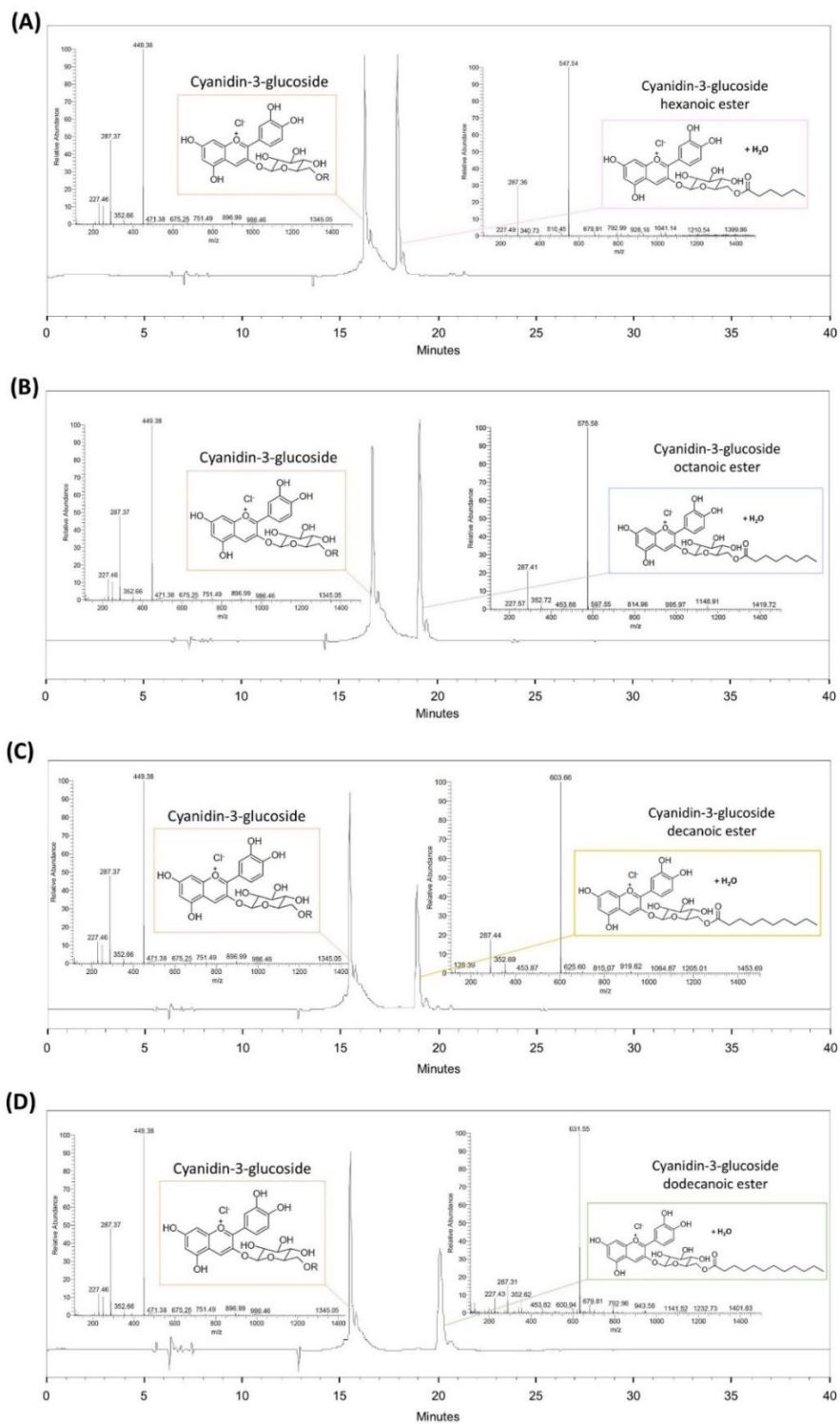
APPENDIX H

Figure S7. (A) Enzymatic acylation of Cyanidin-3-glucoside (C3G) with octanoic acid; (B) C3G and (C) C3G-C8 after purification quantified in HPLC-DAD.



APPENDIX I

Figure S8. The liquid phase spectra and mass spectra of C3G and the acylated compounds with C6 (A), C8 (B), C10 (C), and C12 (D) as acyl donors.



APPENDIX J

Table S2. ^1H NMR data of Cy3glc-C8 conjugate in DMSO- d_6 /TFA (9:1).^a

Position	δ (^1H); J (Hz)
<i>Benzopyrylium ring</i>	
2	-
3	-
4	8.78; s
4a	-
5	-
6	6.70; d, 1.8
7	-
8	6.88; d, 1.8
8a	-
1'	-
2'	7.97; d, 2.3
6'	8.20; dd, 2.3/8.7
3'	-
4'	-
5'	7.00; d, 8.7
<i>Glucose</i>	
1''	5.40; d, 7.7
2''	3.52; m
3''	3.43; m
4''	3.23; m
5''	3.77; m
6'' <i>a</i>	4.35; m
6'' <i>b</i>	4.05; m
<i>Octanoic acid</i>	
1 C=O	-
2 CH ₂	2.15; t, 7.4
3 CH ₂	1.48, *
4-7 (CH ₂) ₄	1.21, *
8 CH ₃	0.82, t, 6.8

^aKey: s, singlet; d, doublet; dd, double of doublets; t, triplet; m, multiplet.