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Analytical Methods

# Fast and green universal method to analyze and quantify anthocyanins in natural products by UPLC-PDA

Leonardo M. de Souza Mesquita<sup>a,\*</sup>, Letícia S. Contieri<sup>a</sup>, Vitor L. Sanches<sup>a</sup>, Renan Kamikawachi<sup>b</sup>, Filipe H.B. Sosa<sup>c</sup>, Wagner Vilegas<sup>b</sup>, Maurício A. Rostagno<sup>a,\*</sup>

<sup>a</sup> Multidisciplinary Laboratory of Food and Health (LabMAS), School of Applied Sciences (FCA), University of Campinas, Rua Pedro Zaccaria 1300, Limeira, São Paulo 13484-350. Brazil

<sup>b</sup> UNESP – Universidade Estadual Paulista, Faculdade de Ciências Farmacêuticas de Araraquara, Araraquara, São Paulo, Brazil

<sup>c</sup> Department of Chemistry, CICECO – Aveiro Institute of Materials, University of Aveiro Campus Universitário de Santiago, Aveiro 3810-193, Portugal

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#### ABSTRACT

This work developed a universal UPLC-PDA method based on safe reagents to analyze anthocyanins from different foods. Nine foods were studied by the developed chromatographic method, which was constructed using a solid core C18 column and a binary mobile phase composed of (A) water (0.25 mol<sub>citric acid</sub>.L<sub>sol</sub><sup>-1</sup>, and (B) ethanol. A total running time of 6 min was obtained, the faster comprehensive method for anthocyanins analysis. Mass spectrometry analysis was employed to identify a comprehensive set of 53 anthocyanins comprising glycosylated and acylated cyanidin, pelargonidin, malvidin, peonidin, petunidin, and delphinidin derivatives. Cyanidin-3-O-glucoside ( $m/z^+$  449) and cyanidin-3-O-rutinoside ( $m/z^+$  595) were used as standards to validate the accuracy of the developed method. The analytical parameters were evaluated, including intra-day and inter-day precision, robustness, repeatability, retention factor (k), resolution, and peak symmetry factor. The current method demonstrated excellent chromatographic resolution, making it a powerful tool for analyzing anthocyanins pigments.

#### 1. Introduction

The development of sustainable techniques to improve the accuracy of analytical methods is closely connected to several of the Sustainable Development Goals, emphasizing the significance of promoting sustainable practices while upholding industry standards for reproducibility and precision outcomes (Tintrop, Salemi, Jochmann, Engewald, & Schmidt, 2023). Recently, several articles have sought greener methodologies using sustainable and environmentally conscious methods, decreasing or eliminating dangerous solvents and reagents while minimizing waste production and envisioning minor energy consumption (Constable, 2021). Green Analytical Chemistry (GAC) originated as a subfield of Green Chemistry, aiming to reduce the environmental impact of analytical chemistry approaches, including sample preparation, separation resolution, and detection precision (Tobiszewski, Mechlińska, & Namieśnik, 2010). One consequence of GAC's focus on sustainability is the development of metrics that can be used to assess the environmental impact of analytical chemistry practices, which guide the optimization of more eco-friendlier approaches

#### (Pena-Pereira, Wojnowski, & Tobiszewski, 2020).

Numerous analytical techniques have been used for identifying and quantifying chemicals obtained from natural sources, with chromatography and spectrophotometry being the most widely used. Ultraperformance liquid chromatography (UPLC - or even their variations HPLC, UHPLC) coupled with UV-Vis and mass spectrometry (MS) detections are the preferred methods owing to their high sensitivity, specificity, and capacity to isolate complex mixtures (Rostagno et al., 2011). Nonetheless, these techniques have a significant environmental impact, mainly due to volatile organic solvents as mobile phases and long-time analysis. Thus, selecting the appropriate mobile phase is critical for achieving precise and consistent results in chromatographic analysis with a high reproducibility between samples (Płotka et al., 2013). Nevertheless, the use of high-volatile organic solvents (VOS), such as methanol (MeOH) and acetonitrile (ACN), as well as buffering agents (mainly acetic, phosphoric, boric, and formic acid) are associated with substantial environmental and health impacts.

As a result, alternative eco-friendly solvents, including ethanol (EtOH), (deep) eutectic solvents [(D)ES], and ionic liquids (ILs), have

\* Corresponding authors. *E-mail addresses:* mesquitalms@gmail.com (L.M. de Souza Mesquita), mauricio.rostagno@fca.unicamp.br (M.A. Rostagno).

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gained popularity as promising substitutes for VOS (Cannavacciuolo et al., 2022; Dogan, Eylem, & Akduman, 2020; Soares et al., 2016). These solvents are generally non-toxic, biodegradable, and possess a lower environmental footprint. However, there are relatively few studies on developing analytical methods employing these benign alternatives compared to traditional ones, making optimizing new green analytical methods difficult. Furthermore, creating a chromatographic analysis method that can be universally applied is a daunting task due to the extensive range of structural diversity in natural compounds. Therefore, it is essential to develop universal methods step-by-step, beginning with studying a particular class of compounds.

In this context, anthocyanin pigments from several foods were selected as the target compounds to develop a universal analysis method. Anthocyanins are a group of water-soluble pigments widely distributed in nature and responsible for the reddish-purple colors of many fruits, vegetables, and flowers (Giampieri, Cianciosi, Alvarez-Suarez, Quiles, Forbes-Hernández, Navarro-Hortal, Machì, & del Casanova, 2023; de Souza Mesquita, Sosa, et al., 2023b). Typically, anthocyanins have been extracted using aqueous-based solvents or volatile organic solvents (Tan et al., 2022). Subsequently, the extracted anthocyanins are concentrated using evaporation or freeze-drying methods to eliminate the solvent. Finally, chromatographic analysis of anthocyanins provides the requested data for the food, pharmaceutical, and cosmetic industries (Constantin & Istrati, 2022). Therefore, their accurate characterization and quantification are essential for developing new products.

The resulting extract can be further analyzed or utilized in various applications such as food coloring, pharmaceuticals, or nutraceuticals. However, there is no universally applicable chromatographic method for analyzing anthocyanins due to optimal conditions dependent on the analytes and matrix variations. Although some general guidelines can be followed, such as using a reverse-phase chromatography column (C18) and a binary mobile phase comprising water and VOS, with buffers added to adjust/keep the pH and improve chromatographic resolution (Constantin & Istrati, 2022). These clues provide a starting point for anthocyanin analysis. Still, it is essential to note that specific sample matrices and analytes may require modifications to achieve optimal separation and detection (Garcia-Oliveira, Pereira, Fraga-Corral, Lourenco-Lopes, Chamorro, Silva, Garcia-Perez, Barroso, Barros, Ferreira, Simal-Gandara, & Prieto, 2021). This means that the quality of the analysis is significantly impacted by various factors such as the solvent used to prepare the extract, purity & presence of interferents, and anthocyanins diversity. Therefore, developing a universal method is highly desirable but still challenging.

This work aimed to develop a fast and robust green chromatographic method to characterize and quantify anthocyanins found in most natural products. Combining the performance of UPLC-PDA with the advantages of solid core particles for the analysis of extracts from different anthocyanins extracts is an excellent tool to quantify and characterize the anthocyanins profile. Thus, in this work, nine representative anthocyanin extracts from different sources were used as target models: black grape, blueberry, blackberry, strawberry, pomegranate, Brazilian berry (jaboticaba), eggplant, red onion, and red cabbage. In order to reduce dependence on solvents such as VOS and toxic buffers, the optimization development method replaced them with ethanol (HPLC grade) and citric acid (buffer). This replacement was made in conjunction with envisioning a maximum chromatographic run time of 10 min. The green chromatographic method was developed using aqueous grape extracts as a starting point to address the high complexity and variety of anthocyanins. Then, the optimized method was applied to the other eight anthocyanins-rich extracts. Finally, green analytical metrics were used to assess whether the developed method is a promising eco-friendlier alternative.

#### 2. Material and methods

#### 2.1. Chemicals and reagents

Citric acid (buffer) was purchase from Dinâmica (99%), and phosphoric and acetic acid (HPLC-grade) were acquired from Sigma-Aldrich Brazil Ltda (São Paulo, Brazil). HPLC grade methanol (meOH) and ethanol (etOH) were purchased from J.t. Baker. Ultra-pure water was supplied by a Milli-Q Advantage water purifier system (Purelab Elga, UK). The reference standard of cyanidin-3-O-glucoside (analytical standard, >99%) was purchased from Sigma-Aldrich Brazil Ltda (São Paulo, Brazil).

#### 2.2. Samples

Nine representative extracts of anthocyanins from different sources were used as target models: black grape, blueberry, blackberry, strawberry, pomegranate, Brazilian berry (*jaboticaba*), eggplant, red onion, and red cabbage. All the samples were acquired in a regional supermarket (Limeira, São Paulo, Brazil).

#### 2.3. Extraction of anthocyanins

The anthocyanins extracts were prepared without any prior pretreatment of the samples. Thus, 0.15 g of each sample was soaked in 3 mL of water containing  $0.25 mol_{citric\ acid.L_{solvent}}^{-1}$  for 24 h (simple maceration). Afterward, the samples were filtered in a nylon syringe filter (0.22  $\mu m \times 25$  mm – Nova Analítica – São Paulo), diluted with the same solvent used in the extraction, and injected into the UPLC system (autosampler). A green chromatographic method was developed using grape extracts as a starting point.

#### 2.4. UPLC-PDA method development

The analysis was conducted in a UPLC-PDA system (Waters Corp, Acquity H-Class, Milford, Massachusetts, EUA). Compounds were separated in a solid-core column (Kinetex, 100×4.6 mm i.d.; 2.6 µm; Phenomenex, Torrance, CA, USA). The anthocyanins were monitored in UV-Vis absorbance at 280 and 520 nm. Different compositions of organic mobile phases were tested, composed of ACN, meOH, and etOH. Different buffers were tested in the aqueous mobile phase, mainly citric, acetic, and phosphoric acids. Different column temperatures and flow rates were also evaluated. The System Suitability software (Waters) comprises confirmatory test procedures and parameters to ensure that the chromatographic system functions align with the international guidelines, especially meeting the United States Pharmacopeia (USP). The parameters evaluated were (retention time, peak capacity -k-prime, selectivity, symmetry factors, resolution of the markers, and the number of plates). Besides, the analytical method's repeatability (intra-day) and intermediate precision (inter-day) were assessed. All the chromatographic analyses were monitored using the software for instrument control and data acquisition Empower 3 (Waters Corp, Milford, Massachusetts, EUA).

#### 2.5. Linearity, limit of detection (LOD), and limit of quantification (LOQ)

Regression equations and correlation coefficient  $(R^2)$  were calculated using Microsoft Excel software (office 365) using the mean of the peak area of three different injections of the external standards (cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside). Limit of detection (LOD) and limit of quantification (LOQ) were calculated following the International Union of Pure and Applied Chemistry (IUPAC) method. LOD and LOQ were calculated for the cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside curves and determined by analyzing sample blanks and estimating the following equation Eq. (1) and Eq. (2), respectively.

$$LOD = \frac{3 \times Sb}{m} \tag{1}$$

$$LOQ = \frac{10 \times Sb}{m}$$
(2)

Where Sb corresponds to the standard deviation of the noises measured for each sample, and m is the slope of the calibration curve.

#### 2.6. Uplc-Pda-MS/MS

The optimized method was hyphenated with mass spectrometry detection (UPLC-PDA-MS/MS) to identify the peaks in the samples. Initially, to provide the fragmentation profile of the anthocyanins extracted from black grape, blueberry, blackberry, strawberry, pomegranate, Brazilian berry (jaboticaba), eggplant, red onion, and red cabbage, flow injection analysis (FIA) was performed using a Thermo Fisher Scientific ion trap mass spectrometry (San Jose, Ca, USA) equipped with an electrospray ionization source. Afterward, MS and MS/MS analysis was performed in positive ionization mode (100-1500 Da), with the following operational conditions: flow rate 0.5 mL.min<sup>-1</sup>, the capillary voltage between -25 to -35 V, spray voltage 5 kV, tube lens offset 75 V, capillary temperature 250-300 °C, sheath gas (N2) flow rate 8 (arbitrary units). Data were acquired and processed using Xcalibur software (version 2.2 SPI.48). After optimization of the UPLC-PDA green method, a UPLC-MS/MS analysis was conducted to identify the extracted anthocyanin peaks from each sample precisely. However, once the green buffer (citric acid) used in the current green chromatographic method was not allowed for MS analysis, it was replaced with acetic acid (0.1% v/v).

#### 2.7. Green metrics analysis

One of the parameters evaluated to optimize the chromatographic method is the Green chromatographic fingerprinting response (GCFR, Eq. (3). This index is useful for achieving excellent chromatographic resolution within a short timeframe. A higher GCFR score indicates better global chromatographic optimization *per* time (*t*). GCFR is calculated based on the number of peaks (*n*), the ratio between the number of peaks in the half-part of the chromatogram with fewer peaks (FP), and MP, which represents the number of peaks in the other half-part of the chromatogram with more peaks.

$$GCFR = n^2 \times \left(\frac{FP}{MP}\right) \times \left(\frac{n}{t}\right)$$
(3)

After optimizing the green chromatographic method, we assessed its performance using the analytical green metric approach (AGREE index), a more robust green index. This modern tool employs suitable software to evaluate all 12 green analytical principles (Pena-Pereira et al., 2020). The AGREE circle is segmented into 12 parts, each representing a green principle, and the corresponding AGREE value is depicted in the circle's centre. The AGREE scores range between 0 and 1, with a score closer to 1 indicating better adherence to green principles. The AGREE index was used to evaluate the current universally developed chromatographic method and compare its performance with other recent methods developed for the same purpose.

#### 2.8. Statistical analysis

All the statistical analysis was performed using excel software (office 365). All the results were conducted at least in triplicate and expressed as mean  $\pm$  standard error. The repeatability (intra-day) and intermediate precision (inter-day) of the analytical method were assessed by the relative standard deviation ( $%_{RSD}$ ) of the cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside concentration of three repeated injections for three levels of concentrations 150, 50, and 10 ppm in two different days.

To investigate the chemical similarities between the anthocyanins extracted from the samples and the presence of the primary aglycones (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin), a non-metric multidimensional scaling (nmMDS) analysis was conducted (Primer 6.0 software). A 2D plot was acquired to categorize the samples based on their anthocyanin composition.

#### 3. Results and discussion

#### 3.1. Optimization of the method variables

The strategy for developing the method involved identifying the optimal parameters for UPLC-PDA and the respective identification of the extracted anthocyanins by mass spectrometry analysis. Thus, to achieve rapid and high-quality separation of several anthocyanins, initially, we opted to study an aqueous extract from black grapes, considered one of the most complex sources of anthocyanins among all those studied in this work. According to the report of de Souza Mesquita, Sosa, et al. (2023b), anthocyanins extracted from black grapes have four main aglycones – delphinidin, malvidin, petunidin, and peonidin – composed in their glycosylated and acylated forms. Thus, to attain optimal resolution in a shorter time frame, the efficiency of chromato-graphic separation was improved by evaluating different solvent compositions, column temperatures, and flow rates.

The green chromatographic fingerprint response (GCFR-index) was used as a guide to assess the efficiency during the method development process. Still, to achieve a high chromatographic resolution, a C18 stationary phase composed of a solid-core particle (2.6  $\mu$ m) with a relatively short column (150 mm) and narrow i.d. (4.6 mm) was selected. During the evaluation of the solvent composition of the mobile phase and to create a more environmentally friendly analytical method, ethanol (etOH) was chosen as the organic phase to replace MeOH and ACN. However, a significant challenge is imposed due to the resultant increase in system backpressure, primarily due to ethanol's relatively high viscosity (compared to other commonly used organic solvents). Consequently, the chromatographic resolution may be adversely affected, resulting in peak broadening and coelution (Yabré, Ferey, Somé, & Gaudin, 2018). Thus, it is essential to carefully optimize the use of etOH as a mobile phase to mitigate those drawbacks.

Buffers are generally added to the mobile phase to improve chromatographic resolution and peak symmetry. Acetic and phosphoric acid is often the preferred options for this task. However, these acids are hazardous and require additional safety measures and proper disposal (Morales-Gonzalez, Zhang, Li, & Hessel, 2019). The use of acetic acid, in particular, can be problematic as it is corrosive (pictograms GHS02, GHS05) and can damage UPLC equipment over time, resulting in increased maintenance and replacement costs. Additionally, the pungent odor associated with acetic acid may require additional safety precautions and make it uncomfortable for workers. Thus, alternative solvents have replaced these negative features, mainly neoteric and nonvolatile solvents, such as ILs and (D)ESs (Soares et al., 2016; Sutton et al., 2018; Treder, Bączek, Wychodnik, Rogowska, Wolska, & Plenis, 2020).

Nevertheless, these solvents are not the cheapest alternatives despite good options. Therefore, considering cost-effectiveness and safety, citric acid represents a promising alternative for use as a mobile-phase buffer. In this study, a concentration of 0.25 moL<sub>citric acid</sub>.L<sub>solvent</sub> was added to the aqueous mobile phase. This decision was based on the recommendations of the column manufacturer for pH maintenance of the mobile phase. In addition, citric acid is a commonly used food additive that aids in maintaining the anthocyanins' color, further highlighting its exceptional biocompatibility with these compounds (Gençdağ, Özdemir, Demirci, Görgüç, & Yılmaz, 2022). This characteristic makes it a suitable choice as an appropriate green buffer in the mobile phase for anthocyanins analysis.

In order to develop the proposed green chromatographic method, a



**Fig. 1.** Representative UPLC chromatograms of samples recorded at 500 nm. (A) black grape, (B) blueberry, (C) blackberry, (D) strawberry, (E) pomegranate, (F) Brazilian berry, (G) eggplant, (H) red onion, and (I) red cabbage. UPLC conditions: Solid-core column, Phenomenex C18 ( $50 \times 4.6$  mm,  $2.7 \mu$ m); mobile phase composed of (A) water ( $0.25 \text{ mol}_{citric acid} L_{solvent}^{-1}$ ) and (B) ethanol in the following gradient program: 0 min (90% A), 1.5 min (85% A), 2.0 min (85% A), 2.2 min (84% A), 2.3 min (83% A), 2.5 min (80% A), 2.75 min (78% A), 3 min (78% A), 3.5 min (75 min A), 4.0 min (75% A), 4.25 min (74% A), 4.5 min (73% A), 4.75 min (70% A), 5.0 min (65% A), 6.0 min (90% A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

trial-and-error strategy was used, switching the chromatographic variables step by step. As mentioned, an aqueous extract of black grape anthocyanins was used to establish an initial starting point. First, to achieve a quick process within the 10 min, a flow rate of 1 mL<sub>mobile phase</sub>-min<sup>-1</sup> was selected. This choice is because higher flow rates can cause an increase in the back pressure system. In contrast, lower flow rates are associated with loss of peak symmetry and longer running times (Contieri et al., 2023). Also, to achieve this goal, the column temperature was set to 55 °C (the highest possible), reducing the mobile phase's viscosity, and facilitating the release of analytes from the column, consequently favoring a fast analysis (Kim, Kim, Lee, & Jang, 2019).

A linear gradient series ranging from 95% to 50% water (0.25mol<sub>citric</sub> <sub>acid</sub>.L<sup>-1</sup>) was conducted in 10 min. However, a no-peak resolution is obtained when a linear gradient is performed, and consequently, a GCFR index = 0 (data not shown). This data suggested that a more complex gradient method is required to separate the anthocyanins accurately. Considering it, an adaption of our previously published conventional method for analyzing anthocyanins from black grapes was selected as a starting point (de Souza Mesquita, Sosa, et al., 2023b). The conventional approach is also composed of a binary mobile phase; (A) acidified water (phosphoric acid 5% v/v), and (B) acidified acetonitrile (phosphoric acid 4% v/v). The gradient used was set as follows: 0 min (97% A), 1 min (87% A), 2 min (85% A), 2.5 min (85% A), 3 min (82% A), 3.50 min (80% A), 4 min (75% A), 4.5 min (73% A), 5 min (72% A), 6 min (70% A), 7 min (70% A), and 9 min (97% A). Then, applying this same method, but now replacing ACN with etOH, and buffering the water with citric acid, nine peaks were observed instead of the 12 commonly found, besides a GCFR index = 64.8, suggesting that modifications are still needed to improve the chromatographic resolution.

Once the already studied gradient was not wholly satisfactory, especially between 2 and 4 min (low chromatographic resolution was

observed due to the coelution of multiple peaks), a new, more complex gradient was optimized. The best separation gradient profile was achieved following the conditions: 0 min (90% A), 1.5 min (85% A), 2.0 min (85% A), 2.2 min (84% A), 2.3 min (83% A), 2.5 min (80% A), 2.75 min (78% A), 3 min (78% A), 3.5 min (75 min A), 4.0 min (75% A), 4.25 min (74% A), 4.5 min (73% A), 4.75 min (70% A), 5.0 min (65% A), 6.0 min (90% A), as detailed in Fig. S1 (supplementary material). These conditions enhanced the resolution of multiple anthocyanins released during the chromatographic run, especially between 2 and 3 min. The gradient employed also included re-equilibration of the initial conditions (between 5.0 and 6.0 min), with a total running time of 6.0 min. Thus, it was possible to analyze 12 primary anthocyanins commonly found in black grapes using this gradient while achieving a high green score, as evidenced by a GCFR index of 205.72. Fig. 1A, it is possible to see the separation of the extracted anthocyanins from black grapes.

Before determining whether the optimized chromatographic method is suitable for the comprehensive analysis of anthocyanins, some parameters were evaluated, including linearity, LOD, LOQ, accuracy, and robustness. Linearity is crucial in calculating the concentration of extracted compounds, where an excellent  $R^2$  value of 1 is expected. To assess linearity, both cyanidin-3-O-glucoside (Cv3G) and cyanidin-3-Orutinoside (Cy3R), common found in several food matrices, were used as an external standard, and several concentrations of Cy3G (205, 102.5, 51.3, 25.6, 12.8, 6.4, 3.2, and 1.6 ppm) and Cy3R (195, 97.5, 48.8, 24.4, 12.2, 6.1, 3.0, and 1.5 ppm) were injected. An R<sup>2</sup> value of 0.9996 and 0.9983 weres obtained, respectively, indicating a highly linear relationship between the concentration of both standards and the detector response (Figs. S2 and S3 - supplementary material). The experiment was repeated the following day, and an excellent R2 values of 0.9992 -Cy3G and 0.9988 - Cy3R were achieved, confirming the method's reliability in quantifying anthocyanins (Figs. S2 and S3 - supplementary

#### Table 1

Retention time (RT), molecular ion ( $[M]^+ - m/z$ ), fragmentation pattern (MS/MS), and putative identification of the anthocyanins detected in the aqueous extract obtained from black grape, blueberry, blackberry, strawberry, pomegranate, Brazilian berry (*jaboticaba*), eggplant, red onion, and red cabbage.

Peak	Retention	Molecular ion	MS/MS	Aglycone	Putative identification	Food matrix	Reference
ID	time (RT -min)	$[M]^+ (m/z)$					
1	1.87	773	611, 465,	Delphinidin	Delphinidin-3-O-rutinoside-	eggplant	(Zhang et al., 2020)
2	1.91	627	303 465, 303	Delphinidin	Delphinidin-3,5-O-	Pomegranate	(Gardeli, Varela, Krokida, &
3	1.99	773	611, 449.	Cyanidin	Cyanidin-3-O-(sinapoyl)-	Red cabbage	(Murador, Mercadante, & de Rosso,
4	2.07	611	287 449, 287	Cyanidin	Cyanidin-3,5-O-diglucoside	Pomegranate	(Kostka, Ostberg-Potthoff, Briviba,
							Matsugo, Winterhalter, & Esatbeyoglu, 2020)
5	2.08	627	465, 303	Delphinidin	Delphinidin-3,5- <i>O</i> - diglucoside	Grape	(de Souza Mesquita et al., 2023b)
6	2.19	465	303	Delphinidin	Delphinidin-3-O-galactoside	Blueberry	(Li, Meng, & Li, 2016)
7	2.25	465	303	Delphinidin	Delphidin-3-O-glucoside	Pomegranate	(Kostka et al., 2020)
8	2.26	595	449, 287	Cyanidin	Cyanidin-3-O-rutinoside	Brazilian berry	(de Souza Mesquita, Contieri et al., 2023a)
9	2.27	465	303	Delphinidin	Delphinidin-3-O-glucoside	Blueberry	(Li et al., 2016)
10	2.29	979	817, 449, 287	Cyanidin	Cyanidin-3-O-(sinapoyl)- diglucoside-5-O-glucoside	Red cabbage	(Murador et al., 2016)
11	2.3	465	303	Delphinidin	Delphinidin-3-O-glucoside	Grape	(de Souza Mesquita et al., 2023b)
12	2.33	697	535, 449, 287	Cyanidin	Cyanidin-3-O-(malonoyl)- glucoside-5-O-glucoside	Red onion	(Wu & Prior, 2005)
13	2.34	611	465, 303	Delphinidin	Delphinidin-3-O-rutinoside	eggplant	(Zhang et al., 2020)
14	2.4	449	287	Cyanidin	Cyanidin-3-O-galactoside	Blueberry	(Li et al., 2016)
15	2.5	641	479, 317	Petunidin	Petunidin-3,5-O-diglucoside	Grape	(de Souza Mesquita et al., 2023b)
16	2.51	449	287	Cyanidin	Cyanidin-3-O-glucoside	Blackberry, Pomegranate, Brazilian berry, Strawberry,	(Kostka et al., 2020)
						Red onion,	
17	2.54	435	303	Delphinidin	Delphinidin-3-O-arabinoside	Blueberry	(Li et al., 2016)
18	2.58	625	463, 301	Peonidin	Peonidin-3,5-O-diglucoside	Grape	(de Souza Mesquita et al., 2023b)
19	2.62	611	449, 287	Cyanidin	Cyanidin-3-O-diglucoside	Blackberry, Blueberry	(Li et al., 2016)
20	2.03	011	287	Cyanidin	Cyanidin-3-O-laminaribioside	Red onion Streambarry Domographic	(Wil & Prior, 2005) (Vestive et al. 2020: Lense de Silve
21	2.83	433	2/1	Pelargonidin	Pelargoniain-3-O-giucosiae	Strawberry, Pomegranate	de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2002)
22	2.91	535	287	Cyanidin	Cyanidin-3-O-(3"-malonoyl- glucoside)	Red onion	(Wu & Prior, 2005)
23	2.93	1081	919, 449, 287	Cyanidin	Cyanidin-3-O-(caffeoyl)(p- coumaroyl)-diglucosides-5-O- glucoside	Red cabbage	(Fang, Lin, Qu, Liang, & Wang, 2018)
24	2.94	479	317	Petunidin	Petunidin-3-O-galactoside	Blueberry	(Li et al., 2016)
25	2.97	493	331	Malvidin	Malvidin-3-O-glucoside	Grape	(de Souza Mesquita et al., 2023b)
26	2.99	419	287	Cyanidin	Cyanidin-3-O-arabinoside	Blueberry	(Li et al., 2016)
27	3.01	579	433, 271	Pelargonidin	Pelargonidin-3-O-rutinoside	Strawberry	(Lopes-da-Silva et al., 2002)
28	3.02	1111	949, 449, 287	Cyanidin	Cyanidin-3-O-(feruloyl)- triglucosides-5-O-glucoside	Red cabbage	(Fang et al., 2018)
29	3.05	1141	979, 449, 287	Cyanidin	Cyanidin-3-O-(sinapoyl)- triglucoside-5-O-glucoside	Red cabbage	(Fang et al., 2018)
30	3.13	479	317	Petunidin	Petunidin-3-O-glucoside	Blueberry	(Li et al., 2016)
31	3.33	463	301	Peonidin	Peonidin-3-O-galactoside	Blueberry	(Li et al., 2016)
32	3.33	419	287	Cyanidin	Cyanidin-3-O-xyloside	Blackberry	(Ștefănuț, Căta, Pop, Moșoarcă, & Zamfir, 2011)
33	3.34	535	287	Cyanidin	Cyanidin-3- <i>O</i> -(6"-malonyl- glucoside)	Blackberry, Red onion	(Wu & Prior, 2005)
34	3.39	449	317	Petunidin	Petunidin-3-O-arabinoside	Blueberry	(Li et al., 2016)
35	3.57	697	655, 535, 493, 331	Malvidin	Malvidin-3-O-(6-O-acetyl)- glucoside-5-O-glucoside	Grape	(de Souza Mesquita et al., 2023b)
36	3.61	697	287	Cyanidin	Cyanidin-3-O-(6"-malonoyl- laminaribioside)	Red onion	(Wu & Prior, 2005)
37	3.65	1287	1125, 449, 287	Cyanidin	Cyanidin-3-O-(feruloyl) (feruloyl)-triglucoside-5-O- glucoside	Red cabbage	(Fang et al., 2018)
38	3.75	539	287	Cyanidin	Cyanidin-3-O-malonyl- glucoside	Blackberry	(Ștefănuț et al., 2011)
39	3.78	1317	1155, 449, 287	Cyanidin	Cyanidin-3-O-(feruloyl) (sinapoyl)-triglucoside-5-O- glucoside	Red cabbage	(Fang et al., 2018)
40	3.86	935	773, 449, 287	Cyanidin	Cyanidin-3-O-(caffeoyl)- diglucoside-5-O-glucoside	Red cabbage	(Fang et al., 2018)
41	3.91	463	301	Peonidin	Peonidin-3-O-glucoside	Blueberry	(Li et al., 2016)
42	4.45	433	301	Peonidin	Peonidin-3-O-arabinoside	Blueberry	(Li et al., 2016)
							(continued on next page)

Table 1 (continued)

Peak ID	Retention time (RT -min)	Molecular ion $[M]^+$ ( $m/z$ )	MS/MS	Aglycone	Putative identification	Food matrix	Reference
43	4.58	919	757, 449, 287	Cyanidin	Cyanidin-3- <i>O</i> -( <i>p</i> -coumaroyl)- diglucoside-5- <i>O</i> -glucoside	Red cabbage	(Murador et al., 2016)
44	4.67	801	639, 493, 331	Malvidin	Malvidin-3-O-( <i>cis</i> -6-O- coumaryl)-glucoside-5-O- glucoside	Grape	(de Souza Mesquita et al., 2023b)
45	4.71	949	487, 449, 287	Cyanidin	Cyanidin-3-O-(feruloyl)- diglucoside-5-O-glucoside	Red cabbage	(Fang et al., 2018)
46	4.81	787	625, 479, 317	Petunidin	Petunidin-3-O-(6-O- coumaryl)-glucoside-5-O- glucoside	Grape	(de Souza Mesquita et al., 2023b)
47	4.83	463	331	Malvidin	Malvidin-3-O-arabinoside	Blueberry	(Li et al., 2016)
48	4.93	1155	993, 449, 287	Cyanidin	Cyanidin-3-O-(feruloyl) (sinapoyl)-diglucoside-5-O- glucoside	Red cabbage	(Fang et al., 2018)
49	4.98	505	301	Peonidin	Peonidin-3-O-(6-O-acetyl)- glucoside	Grape	(de Souza Mesquita, Sosa, et al., 2023b)
50	5.05	1185	1023, 449, 287	Cyanidin	Cyanidin-3-O-(sinapoyl) (sinapoyl)-diglucoside-5-O- glucoside	Red cabbage	(Fang et al., 2018)
51	5.18	801	639, 493, 331	Malvidin	Malvidin-3-O-( <i>trans-6-O-</i> coumaryl)-glucoside-5- <i>O-</i> glucoside	Grape	(de Souza Mesquita et al., 2023b)
52	5.54	625	463, 317	Petunidin	Petunidin-3-O-(6-O- coumaryl)-glucoside	Grape	(de Souza Mesquita et al., 2023b)
53	5.84	639	331	Malvidin	Malvidin-3-O-(6-O-p- coumaryl)-glucoside	Grape	(de Souza Mesquita et al., 2023b)

material). Besides, the LOD and LOQ were, respectively,  $4.35 \times 10^{-3}$  ppm, and  $1.45 \times 10^{-2}$  ppm for Cy3G; and  $2.03 \times 10^{-3}$  ppm, and  $6.76 \times 10^{-3}$  ppm for Cy3R, highlighting excellent results for quantifying low-concentrated samples (Table S1 – supplementary material).

The accuracy of the developed method was assessed using Cy3G and

CyrR as standards in three concentrations: high – 150 ppm, medium – 50 ppm, and low – 10 ppm. Table S1 (supplementary material) shows that an acceptable range (80–110%) was achieved independently of the concentration, reinforcing the method's efficiency. Besides, the repeatability (intra-day –  $%_{RSD}$  0.53% for Cy3G and 0.41% for Cy3R) and

#### Table 2

System suitability parameters obtained from each major anthocyanin analyzed by the optimized chromatographic method (Fig. 1A - I). The repeatability of the retention time was expressed in %RSD (variation coefficient), calculated concerning the mean of the triplicate performed on two different days.

Food	Peak ID	Major anthocyanin <sup>a</sup>	RT (min)	Repeatability (%RSD) <sup>b</sup>	Retention Factor (K- prime) <sup>c</sup>	USP Resolution <sup>d</sup>	Selectivity <sup>e</sup>	Symmetry factor <sup>f</sup>	USP Tailing <sup>g</sup>	USP Plate Count <sup>h</sup>
Black grape	51	Malvidin-3-O-( <i>trans-</i> 6- O-coumaryl)-glucoside- 5-O-glucoside	5.18 ±0.012	0.23	1.49	1.99	1.08	1.17	1.17	39871.92
Blueberry	6	Delphinidin-3-O- galactoside	$\begin{array}{c} 2.19 \\ \pm 0.002 \end{array}$	0.09	-	-	-	1.07	1.07	60994.40
Blackberry	16	Cyanidin-3-O-glucoside	$\begin{array}{c} 2.51 \\ \pm 0.003 \end{array}$	0.12	-	-	-	1.07	1.07	51029.85
Strawberry	21	Pelagonidin-3-O- glucoside	$\begin{array}{c} 2.83 \\ \pm 0.002 \end{array}$	0.07	2.01	7.54	3.29	1.01	1.01	42272.95
Pomegranate	16	Cyanidin-3-O-glucoside	$\begin{array}{c} 2.51 \\ \pm 0.011 \end{array}$	0.44	1.51	3.14	0.69	1.00	1.00	29023.02
Brazilian berry	16	Cyanidin-3-O-glucoside	$\begin{array}{c} 2.51 \\ \pm 0.003 \end{array}$	0.12	1.51	5.71	-1.52	1.20	1.45	34079.78
Eggplant	13	Delphinidin-3- <i>O-</i> rutinoside	$\begin{array}{c} 2.34 \\ \pm 0.001 \end{array}$	0.04	1.34	10.81	-0.05	1.04	1.04	42810.54
Red onion	33	Cyanidin-3-O-(6"- malonyl-glucoside)	$\begin{array}{c} 3.36 \\ \pm 0.002 \end{array}$	0.06	2.36	10.95	2.17	1.00	1.00	53397.01
Red cabbage	48	Cyanidin-3-O-(feruloyl) (sinapoyl)-diglucoside- 5-O-glucoside	4.93 ±0.015	0.30	3.93	-	1.02	1.32	1.32	157007.70

<sup>a</sup> anthocyanins identified in the Table 1.

<sup>b</sup> Ideal repeatability <2%.

<sup>c</sup> ideal k-prime >1.

<sup>d</sup> ideal USP resolution >1.5.

<sup>e</sup> ideal selectivity between 0.9 and 1.1.

f ideal symmetry around 1.

<sup>g</sup> ideal USP tailing <2.5.

 $^{\rm h}\,$  ideal plate count >500; RSD: relative standard deviation (%).



**Fig. 2.** Non-metric multidimensional scaling of anthocyanins extracted from black grape, blueberry, blackberry, strawberry, pomegranate, Brazilian berry, eggplant, red onion, and red cabbage. (A) similarity index between the identified chromatographic peaks (peak ID 1 - 53). (B) similarity index between the identified aglycones (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Anthocyanins Aglycones similarity



intermediate precision (inter-day –  $%_{RSD}$  0.61% for Cy3G and 0.78 for Cy3R) corroborate those findings once a low  $%_{RSD}$  was achieved, which meets the demands of specialized guidelines, including United States Pharmacopeia (USP).

# 3.2. Applying the developed method to different anthocyanins rich extracts

The UPLC-PDA optimized method successfully analyzed 53 anthocyanins extracted from black grape, blueberry, blackberry, strawberry, pomegranate, Brazilian berry (*jaboticaba*), eggplant, red cabbage, and red onion. The technique was coupled with MS detection (UPLC-PDA- MS/MS) to identify each extracted anthocyanin. Table 1 presents the identification of each chromatographic peak and their respective retention times (RT),  $[M]^+ - m/z$ , and MS/MS data. Also, Fig. 1 A-I shows the chromatograms of each aqueous extract rich in anthocyanins. Furthermore, an nmMDS analysis was performed to illustrate the similarities between the anthocyanin extracts (Fig. 2A and B). The UPLC-PDA method developed to analyze anthocyanin extracts successfully identified a large plethora of anthocyanins, as indicated by the dispersion of dots across the field (Fig. 2A), demonstrating its comprehensiveness and effectiveness. This observation is further supported by Fig. 2B, where the most common anthocyanin aglycones were grouped, confirming the method's ability to distinguish the most common

#### Table 3

Representative chromatographic methods reported in the literature for anthocyanins analysis.

Sample	Stationary phase	Analysis time (min)	Column temperature (°C)	Flow rate (mL. min <sup>-1</sup> )	Mobile phase composition	Anthocyanins identified	Reference
Black grape, blueberry, blackberry, strawberry, pomegranate, Brazilian berry, eggplant, red onion, red cabbage	C18 solid-core particle (2.6 $\mu$ m, 150 mm $\times$ 4.6 mm)	6	55	1.0	(A) water with 0.25 moL <sub>citric acid</sub> . $L_{solvent}^{-1}$ (B) etOH	53 anthocyanins, as depicted in Table 1	Curent method
Strawberry, Blueberry	Synergi Polar–C18 (4 μm, 250 mm × 4.6 mm)	32	30	0.8	<ul><li>(A) water; (B) MeOH, both with formic acid 0.1% v/v</li></ul>	Pelargonidin-3-O-glucoside; Pelargonidin-3-O-rutinoside; Malvidin-3-O-galactoside	(Mustafa et al., 2022)
Eggplant	Synergi Fusion- C18 80 Å (150×4.6 mm, 4 μm)	60	25	1.0	(A) water with formic acid 10% v/v; (B) meOH	Delphinidin-3-O-rutinoside-5-O- glucoside; delphinidin-3-O- glucoside; delphinidin-3-O- rutinoside; cyanidin-3-O- rutinoside; petunidin-3-O- rutinoside	(Condurache (Lazăr) et al., 2021)
Blackberry	ZORBAX Eclipse Plus C18 (100 mm × 4.5 mm, 3.5 µm)	6	35	1.0	(A) water with 0.25 moLalpha-hydroxy acid- L <sup>-1</sup> <sub>solvent</sub> ; (B) etOH	Cyanidin-3-O-glucoside; cyanidin- 3-O-rutinoside; cyanidin-3-O- arabinoside; cyanidin-3-O- xyloside; cyanidin-3-O- malonylglucoside; cyanidin-3-O- dioxalyglucoside	(Sang et al., 2017)
Brazilian berry (jaboticaba)	Eclipse XDB-C18 (2.1 X 150 mm; 3.5 μm	45	40	0.19	<ul> <li>(A) ACN/water/ formic acid</li> <li>(3:88.5:8.5,v/v/v);</li> <li>(B) acetonitrle/ water/formic acid</li> <li>(50:41.5:8.5, v/v/v)</li> </ul>	Delphinidin-3-O-glucoside; cyanidin-3-O-glucoside; Pelargonidin-3-O-glucoside; Penidin-3-O-glucoside; cyanidin-3- O-cumaroyl-glucoside	(de Andrade Neves et al., 2021)
Grape, blackberry	Zorbax 300 Extended-C18, 4.6×150 mm, 5 μm)	47	35	1.0	(A) water with formic acid 1% v/v; (B) ACN: (A) (80:20 v/v)	Delphinidin-3-O-glucoside; cyanidin-3-O-glucoside; cyanidin- 3-O-galactoside; cyanidin-3-O- rutinoside; delphinidin-3-O- xyloside; cyanidin-3-O- arabinoside; acylated cyanidin and malvidin derivatives	(Paun, Botoran, & Niculescu, 2022)
Pomegranate	LiChroCART 100 C18 (25 cm $\times$ 0.4 cm, 5 $\mu m)$	20	35	0.3	(A) water with <i>T</i> - fluoro acetyl acid (0.001% v/v); (B) meOH	Cyanidin-3-O-glucoside; cyanidin- 3,5-O-diglucoside; pelargonidin-3- O-glucoside; pelargonidin-3,5-O- diglucoside	(Norhaslinda, Noratiqah, Amin, & Khalili, 2018)
Red Onion	C18 (2.1 mm × 100 mm, 2.6 μm)	7	35	0.4	(A) water with formic acid (2% v/v); (B) meOH	Cyanidin-3-O-glucoside; cyanidin- 3-O-laminaribioside; cyanidin-3-O- (3"-malonylglucoside; peonidin-3- O-glucoside; delphinidin-3,5-O- diglucoside; cyanidin-3-O-(6"- malonylglucoside); cyanidin-3-O- (6"-malonyl-laminaribioside); peonidin 3-O-(6"- malonylglucoside); delphinidin-3- O-glucoside	(González-de-Peredo, Vázquez-Espinosa, Espada-Bellido, Ferreiro-González, Carrera, Barbero, & Palma, 2021)
Red cabbage	Ultimate LP-C18 (4.6×250 mm, 5 μm)	25	35	1.0	(A) water with formic acid (5% v/v); (B) meOH	Glucosilated, acylated cyanidin derivatives	(Fang et al., 2018)

ACN: acetonitrile; etOH: ethanol; meOH: methanol.

anthocyanins. Therefore, the UPLC method can be considered a powerful translational tool for universally analyzing anthocyanins from other food sources.

chromatographic method is reliable for detecting and quantifying anthocyanins from several foods.

#### 3.3. Validation of the optimized method by system suitability

Each chromatogram obtained from the anthocyanin extracts was assessed according to the United States Pharmacopeia (USP), including peak capacity – k-prime, selectivity, symmetry factors, resolution of the markers, and the number of plates. The evaluation was based on the major peak for each anthocyanin extract. The results are presented in Table 2, indicating that the parameters studied comply with the USP recommendations (Chorilli, Bonfilio, da Silva Chicarelli, & Nunes Salgado, 2011). Thus, the present data suggest that the developed

## 3.4. Comparison to the existing methods and their evaluation concerning a green analytical metric

Although several efforts have been made to develop green analytical methods, the scientific community still performs chromatographic analysis using non-ecofriendly approaches (Kannaiah, Sugumaran, Chanduluru, & Rathinam, 2021). Typically, chromatographic techniques hyphenated with photodiode (PDA) array and mass spectrometry (MS) detection are used to characterize anthocyanins. Still, most methods are based on traditional high-volatile and toxic solvents, generating several negative impacts on the environment and the health



Fig 3. AGREE-scores of recent representative chromatographic analysis developed to characterize anthocyanins from grape, blueberry, blackberry, strawberry, pomegranate, Brazilian berry, eggplant, red onion, and red cabbage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of those handling them. That's why there is a current trend toward green liquid chromatography of analysis of several natural and pharmaceutically active compounds (Shaaban & Górecki, 2015). Table 3 summarizes the most recent chromatographic methods used to analyze the same anthocyanins studied in this work (i.e. from the same food samples). It is possible to conclude that the C18 stationary phase is the preferred choice to separate anthocyanins-rich extracts. However, many particle sizes, varying between 2.6 and 5.0 µm, are used, impairing standardization proposals and developing universal analysis protocols. Indeed, this reflects various analysis times (between 6 and 60 min), even if long running times are unnecessary. Fig. 3 shows how green each chromatographic method is according to an analytical green chemistry method - AGREE score (Pena-Pereira et al., 2020). The AGREE score could be a powerful tool to guide specific improvements in developing analytical techniques, such as those based on chromatography. For example, all the analytical methods evaluated have a red score in principle 3 (In situ measurements should be performed), demonstrating that online (or even at-line) analysis must be encouraged. However, it is comprehensible that this challenge is not still overcome once a precise online quantification is a considerable challenge, as discussed by the report of Viganó et al. (2021).

Another critical point is regarding the use of LC-MS devices instead UPLC-MS. Overall, the combination of smaller particle sizes of the stationary phases (columns) and higher operating pressures in UPLC results in a more efficient separation process, which requires less solvent *per* time and has a lower inherent environmental impact than those performed by HPLC. This aspect is emphasized in principle 9 (AGREE – *the use of energy should be minimized*), which is considered one of the main essential objectives of developing a genuine green analytical approach.

Using ethanol (etOH) in the mobile phase instead of acetonitrile (ACN) or methanol (MeOH) has led to significant improvements in sustainability and safety, as evidenced by high scores in principles 10, 11, and 12. Principle 10 emphasizes the importance of using reagents obtained from renewable sources, which etOH fulfills as it can be derived from various renewable sources such as sugarcane, corn, or molasses. Similarly, principle 11 promotes eliminating or replacing toxic reagents, which etOH meets the credential "generally recognized as safe -GRAS", with a lower toxicity profile than ACN and meOH. Moreover, principle 12 highlights the importance of prioritizing operator safety, which etOH could also be a good candidate for, being non-explosive and non-corrosive, mitigating potential hazards associated with the handling and disposal (Ogden & Dorsey, 2019). Therefore, in the current method described in this article, replacing toxic, explosive, and corrosive solvents and buffers with etOH in the mobile phase has led to an overall safer and more sustainable approach. This factor became even more evident once the second-highest score was achieved by a chromatographic method using etOH in the mobile phase -blackberry method-(Sang, Ma, & Li, 2017). In summary, the chromatographic method

presented in this article has been awarded the highest AGREE score, signifying that it represents the most environmentally friendly analytical approach reported for this specific purpose (anthocyanin characterization). This achievement indicates the significant strides towards developing a sustainable and ecologically conscious method that aligns with modern-day values and priorities.

#### 4. Conclusions

As more sustainable practices are needed, researchers focus on developing greener chromatographic separation methods. This shift in focus aims to reduce the amount of solvent used and minimize the environmental impact of organic solvents, mainly acetonitrile and meOH (the most used solvents in mobile phases of chromatographic analysis), which are known to pose significant risks to the environment and human health. This work optimized and validated an eco-friendly chromatographic method (UPLC-PDA) by specific international guidelines. Here, 53 anthocyanins (glucosylated and acylated), composed of the main aglycones, were successfully analyzed. The method achieved all internationally requested analytical parameters and could be a valuable tool to characterize anthocyanins extracts universally.

#### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136814.

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