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RESEARCH

Dispersive Pipette Extraction and HPLC-DAD for the Determination of Polyphenols in Grape Juice

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Abstract

A simple and green sample preparation protocol using dispersive pipette extraction (DPX) followed by identification and quantification by high-performance liquid chromatography (HPLC) is proposed for the determination of 11 polyphenols, including phenolic acids and flavonoids, in grape juice. Sample preparation variables by DPX, including extraction phase, sample volume, pH, desorption solvent, salt concentration, time and cycles of extraction and desorption, were evaluated and optimized using univariate and multivariate designs. The analytical performance was satisfactory, with coefficient of determination greater than or equal to 0.9621, precisions with values lower than 20%, and recoveries ranging from 80 to 120%, demonstrating good method trueness. In addition, the method proved to be robust and showed no matrix effect. Finally, the method was applied to determine polyphenols in three grape juice samples. The major compounds determined in the samples were chlorogenic acid (70.84–120.70 mg L^{-1}) and gallic acid (69.62–96.08 mg L^{-1}). Finally, the green character of this method was evaluated using the tools Green Analytical Procedure Index (GAPI), Analytical Greenness Metric (AGREE), and Analytical Greenness Metric for Sample Preparation (AGREEprep). According to the metrics, an excellent green character was determined, emphasizing the minimization of sample (160 µL) and solvent (200 µL) volumes, as well as the short extraction time (< 5 min).

Keywords Polyphenols · Grape juice · Dispersive pipette extraction · High-performance liquid chromatography · Greenness evaluation

Introduction

Grape juice is an unfermented grape derivative widely consumed in the world that has unique sensory characteristics in terms of flavor, aroma, and color (Guler 2023). In addition to the sensory profile, grape juice has potential health benefits, largely related to the rich and natural composition of polyphenols, including flavonoids and non-flavonoids. The main grape polyphenols are flavan-3-ols, flavonols, phenolic acids, proanthocyanidins, anthocyanins, and stilbenes (Sabra et al. 2021). These compounds are secondary metabolites that affect the sensory and nutritional properties of plants, especially fruits and their derivatives (Lucci et al. 2017). The beneficial effects of polyphenols on health, when consumed regularly, are associated with antioxidant, anti-inflammatory, and cardioprotective actions (Tashakkori et al. 2021).

The phenolic profile of grape juice can be influenced by different factors, related to grape production, including the cultivation system (conventional or organic), agricultural practices, environmental conditions, maturity level, and grape variety (Granato et al. 2016; da Silva Haas et al. 2016). Factors related to beverage production, including processing conditions, techniques, heat treatments, and application of enzymes, are also involved. During the elaboration of grape juice, production factors can directly lead to the transfer of grape polyphenols to the juice and consequently affect the concentration and composition of these compounds in the product (Guler 2023). Therefore, it is understood that the phenolic composition of grape juice is complex and dependent on several factors. In this sense, effective analytical methods to extract and analyze polyphenols are useful for determining the quality of grape juice and potential health benefits.

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Polyphenol determinations are commonly performed using liquid chromatography (da Silva Haas et al. 2016; Pereira-Coelho et al. 2023) and gas chromatography (Robles et al. 2019) coupled to different detectors. Although grape-derived products have been extensively studied in recent years, there are still few studies dedicated to solving problems with analytical procedures for the determination of polyphenols in grape juice. In the determination of polyphenols in grape juice, several factors must be considered, including low concentration of analytes, and complex composition of the matrix of the sample (sugars, organic acids, and metals) (Kersh et al. 2023). In the literature, there are recurrent works that apply the dilute-and-shoot approach, which consists of diluting and injecting the sample into the analytical instrument (Granato et al. 2016; Seraglio et al. 2016; Toaldo et al. 2015). However, it is important to consider that dilution can prevent the detectability of polyphenols in the sample. In addition, even with dilution, a series of compounds are introduced into the analytical instrument, and the presence of compounds with retention times similar to those of the analytes can make it difficult to identify the analyte peaks and negatively influence the analytical parameters of the method, mainly linearity, precision, and accuracy (Mesquita et al. 2023). In addition to the parameters of merit, it is important to consider the care of the chromatographic column. A chromatographic analysis without previous sample preparation allows a series of compounds to be inserted into the chromatographic column, and then some of them can be strongly retained and accumulate along the column, resulting in deterioration of the column. There may be several other problems, including poor peak shape, loss of resolution, split peaks, decreased retention times, and high back pressure in the case of liquid chromatography analysis. Because of this practice, it is necessary to change the column frequently, increasing the analysis costs (Michael Dong 2019). For these reasons, sample treatment is a resource by which to enrich the analytes, allowing the detection of polyphenols even at low concentrations, and to eliminate interferents from the matrix before the chromatographic determination.

Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are techniques commonly used to prepare liquid samples, but it is common knowledge that both techniques are laborious and require moderate to high solvent consumption during extraction (Sajid and Płotka-Wasylka 2018). Therefore, sample treatment remains one of the main challenges in the development of analytical methods for the determination of polyphenols in grape juice. Aresta et al. (2018) and Tashakkori et al. (2021) presented alternative methods, applying microextraction techniques.

Dispersive pipette extraction (DPX) is a microextraction technique developed based on the principles of SPE. In DPX, the novelty is using the extraction phase freely dispersed in a tip, which differs from SPE, which accommodates the extraction phase packaged in a cartridge (Bordin et al. 2016). Briefly, the tip is attached to a micropipette and the extraction takes place by aspirating the liquid sample into the tip. In this step, the air is aspirated in sequence to promote a dispersive mixture between the sample and the extraction phase, which contributes to the fast and efficient extraction of analytes. Afterwards, the sample is discarded and the same procedure is repeated with a few microliters of organic solvent to desorb the analytes. If necessary, a cleaning step can be performed between the extraction and desorption steps (Carasek et al. 2022). The DPX procedure allows the enrichment to be performed, while also cleaning the sample, contributing to the success of the instrumental analysis. In addition, DPX stands out as an extraction technique due to its ease of operation and the reduction in extraction time and consumption of solvents and reagents (Morés et al. 2021).

This study aimed to develop a DPX sample preparation method for the extraction of polyphenols in grape juice using HPLC-DAD. This work is the first to apply the DPX technique to develop a multi-analyte method to determine phenolic acids and flavonoids in grape juice samples. The extraction conditions were optimized using univariate and multivariate designs. The method DPX/HPLC-DAD was validated and applied to three samples of grape juice. Green metrics were applied with the aim of providing relevant data on the greenness of the method, including Green Analytical Procedure Index (GAPI) (Płotka-Wasylka 2018) and Analytical Greenness Calculator (AGREE) (Pena-Pereira et al. 2020). Finally, the green aspect of the sample preparation step was evaluated using the tool AGREEprep (Wojnowski et al. 2022).

Materials and Methods

Chemicals and Materials

The analytical standards of polyphenols (gallic acid (≥98%), protocatechuic acid (≥98%), (+)-catechin (≥99%), chlorogenic acid (≥99%), vanillic acid (≥97%), (−)-epicatechin (≥97%), ferulic acid (≥99%), *p*-coumaric acid (≥98%), myricetin (≥98%), quercetin (≥95%), and kaempferol (≥99%)) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, ethanol, and acetonitrile with purity greater than 99.8% were purchased from Merck (Kenilworth, NJ, USA). The sodium chloride (ACS grade) used to assess the saline effect was purchased from Vetec (Rio de Janeiro, RJ, Brazil). Ultrapure water for aqueous solutions was prepared using Millipore Milli-Q-System (Billerica, MA, USA). For the disposable pipette extraction (DPX) procedure, tips with the capacity of 1 mL were purchased from DPX Technologies (Columbia, SC, USA), including two extraction phases: RP (Reverse Phase, styrene-divinylbenzene polymer,

mass: 20 mg, and particle size: 75 µm) and WAX (Weak Anion Exchange, secondary amine phase with a styrene-divinylbenzene, mass: 20 mg, and particle size ranges: 55–65 µm).

Instrumentation

An Agilent Technologies 1200 Series HPLC system (Waldbronn, Germany) equipped with Diode-Array Detection (DAD) was used to perform all chromatographic analyses. Separation was performed on a C-18 column (150 × 4.6 mm ID, 4-µm particle size; Phenomenex, USA) with the temperature set at 30°C. The mobile phase was adjusted to a flow rate of 0.8 mL/min and consisted of (A) 0.1% formic acid in water and (B) methanol. The gradient program was performed as follows: 20% B from 0 to 5 min, 20 to 80% B from 5 to 35 min, and 80 to 20% B from 35 to 40 min. The injection volume was 10 µL. The detection wavelength was set at 280 nm (gallic acid, protocatechuic acid, (+)-catechin, vanillic acid, (−)-epicatechin, and ferulic acid), 320 nm (chlorogenic acid and *p*-coumaric acid), and 360 nm (myricetin, quercetin, and kaempferol). The identification of the retention times of each compound was determined by injecting the individual standards. Chromatographic separation was evaluated using a mixture of standards (10 mg L^{-1}) containing the 11 polyphenols.

Samples and DPX Procedure

Samples of whole grape juice obtained from American grapes (*V. labrusca L.*) were commercially available in local supermarkets. In this study, three samples of grape juice were used, including red grape juice (Bordô varietal), organic red grape juice (Bordô and Isabel varietal), and white grape juice (Niágara varietal). All samples were stored at 4°C protected from light. The samples were filtered with a 0.45 µm PTFE membrane filter to discard all possible suspended material.

For the DPX procedure, a single channel micropipette (Basic Model, fixed volume 1000 µL, Kasvi, Brazil) using constant pressure for aspiration and manual distribution was used to perform the DPX procedure. 3200 µL of sample (pH 2.0) was required, fractionated into 4 aliquots of 800 µL, and a DPX tip with a capacity of 1 mL, containing 20 mg of the extractor phase. The DPX procedure is described below:

i. Conditioning: The conditioning of the extractor phase was performed with 800 µL of ultrapure water.
ii. Extraction: Extraction was performed with 5 cycles (aspiration and dispensing) of 10 s in each 800 µL aliquot of sample.
iii. Cleaning: Cleaning of the extractor phase was performed with 800 µL of ultrapure water in 1 cycle of 10 s.
iv. Desorption: Desorption was carried out with 200 µL of acetonitrile in 2 cycles of 10 s.

After the DPX procedure, 10 µL of the extract was analyzed by HPLC-DAD. A schematic representation of the extraction process is shown in Fig. 1.

Optimization of the DPX Procedure

The proposed method was optimized with solutions of red grape juice and ultrapure water enriched with 5 mg L^{-1} of polyphenols. Initially, the DPX procedure was evaluated in red grape juice diluted in three proportions, namely 1:1, 1:10, and 1:20 (red grape juice and ultrapure water). Assays were performed in triplicate (n=3), and the results were statistically evaluated by analysis of variance (ANOVA). The extraction phase was chosen through a univariate design. The extraction efficiencies

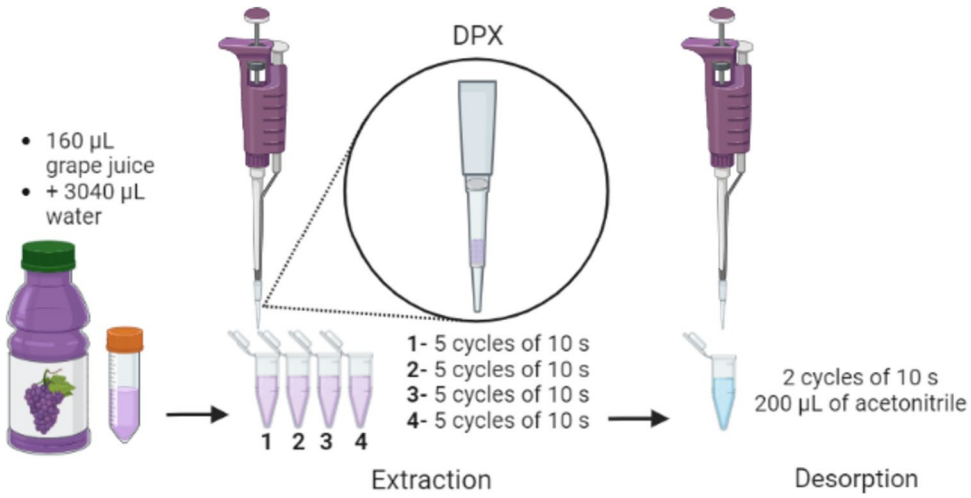

Fig. 1 Scheme of the DPX procedure for analyzing polyphenols in grape juice samples

of two extraction phases were evaluated, including DPX-RP (reverse phase-styrene-divinylbenzene polymer) and DPX-WAX (weak anion exchange-secondary amine phase with a styrene-divinylbenzene). Assays were performed in triplicate (n=3), and results were statistically evaluated by analysis of variance (ANOVA). Next, the desorption solvent was optimized using the *Simplex Lattice* design (Table S1). The evaluated solvents were acetonitrile, methanol, and ethanol. The variables sample volume, extraction and desorption time, extraction and desorption cycles, salt concentration, and pH were initially evaluated by a 2^{7-3} Fractional Factorial Design (FFD) (Tables S2 and S3) to select only the parameters that significantly affect the extraction process. Among these parameters, sample volume, pH, and extraction cycles were statistically significant and were optimized using a central composite design (CCD) (Table S4). Results were expressed as the geometric mean of the area of 11 analytes.

Method Performance

The following validation parameters were evaluated: linear working range, coefficient of determination (R^2), limit of quantification (LOQ), limit of detection (LOD), relative recovery (accuracy), intraday and interday precisions, and matrix effect. Calibration solutions were prepared in ultrapure water at eight concentration levels ranging from 0.1 to 10 mg L^{-1}. The extraction of 11 polyphenols was performed in triplicate (n=3), using the optimized conditions followed by HPLC-DAD analysis. The LOD was calculated using 3 times the standard deviation of the lowest concentration on the calibration curve divided by the slope of the calibration curve, and the LOQ was calculated as 3.3 times the LOD. Intraday and interday precision was determined by applying three replicates of grape juice samples enriched at three levels (0.5, 2.5, and 7.5 mg L^{-1}), which were also applied to estimate the relative recovery. Matrix effect was evaluated by comparing the slopes of the calibration curve in water and the calibration curve in the corresponding matrix.

The robustness of the method DPX/HPLC-DAD was evaluated using a fractional factorial design (Karageorgou and Samanidou 2014). The parameters were evaluated: sample dilution, pH and sample volume, extraction cycles, desorption solvent volume, percentage of formic acid in the mobile phase, and column temperature. The test was performed at two levels close to the established levels of each parameter, with values varying in levels slightly lower and higher than the established value. The matrix with the parameter combinations and their levels is shown in Table S5.

Data Analysis

OpenLab® software was used to process the chromatographic results. All statistical treatments were performed using Statsoft Statistica® 8.0 (Statsoft, USA) and Microsoft Excel® 365 (USA) software. Greenness assessment for the developed procedure was performed using freeware software, including ComplexGAPI (ComplexGAPI_V0.2_BETA), AGREE-Analytical Greenness Calculator (AGREE_sfx), and AGREEprep-Analytical Greenness Metric for Sample Preparation (AGREEprep 0.91).

Results and Discussion

Chromatographic Separation

The chromatographic separation of the analytes was evaluated using a solution containing the 11 working analytes at a concentration of 10 mg L^{-1}. The instrumental conditions are described in the "Instrumentation" section. The selection of the analytes was based on literature articles that address the characterization of different grape juices (da Conceição Prudêncio Dutra et al. 2023; dos Santos Lima et al. 2015; C. Padilha et al. 2019; Toaldo et al. 2015). Fig. 2 presents the chromatograms obtained in the monitored ranges, including 280, 320, and 360 nm. The separation condition was developed based on methods presented by Yang et al. (2017), Giusti et al. (2017), and da Silva Padilha et al. (2017) with modifications, varying the elution gradient, temperature, flow rate, and column length.

DPX Method Optimization

Sample Dilution

Grape juice has a heterogeneous composition characterized by the variety of classes of organic and inorganic compounds (Kersh et al. 2023) that can influence the extraction efficiency of target analytes. Therefore, the DPX procedure was initially applied to samples of grape juice diluted in three ratios, including 1:1, 1:10, and 1:20 (red grape juice and ultrapure water). Figure S1 shows the result in a bar graph with normalized geometric means. In agreement with the results, the highest extraction efficiency was achieved with the sample diluted by 20 times with statistically different results (p<0.05) from the other conditions, according to the analysis of variance (ANOVA). The result obtained indicates a greater availability of the analytes to be extracted in greater dilution. Therefore, the 20 times dilution was selected as a compromise condition for method development.

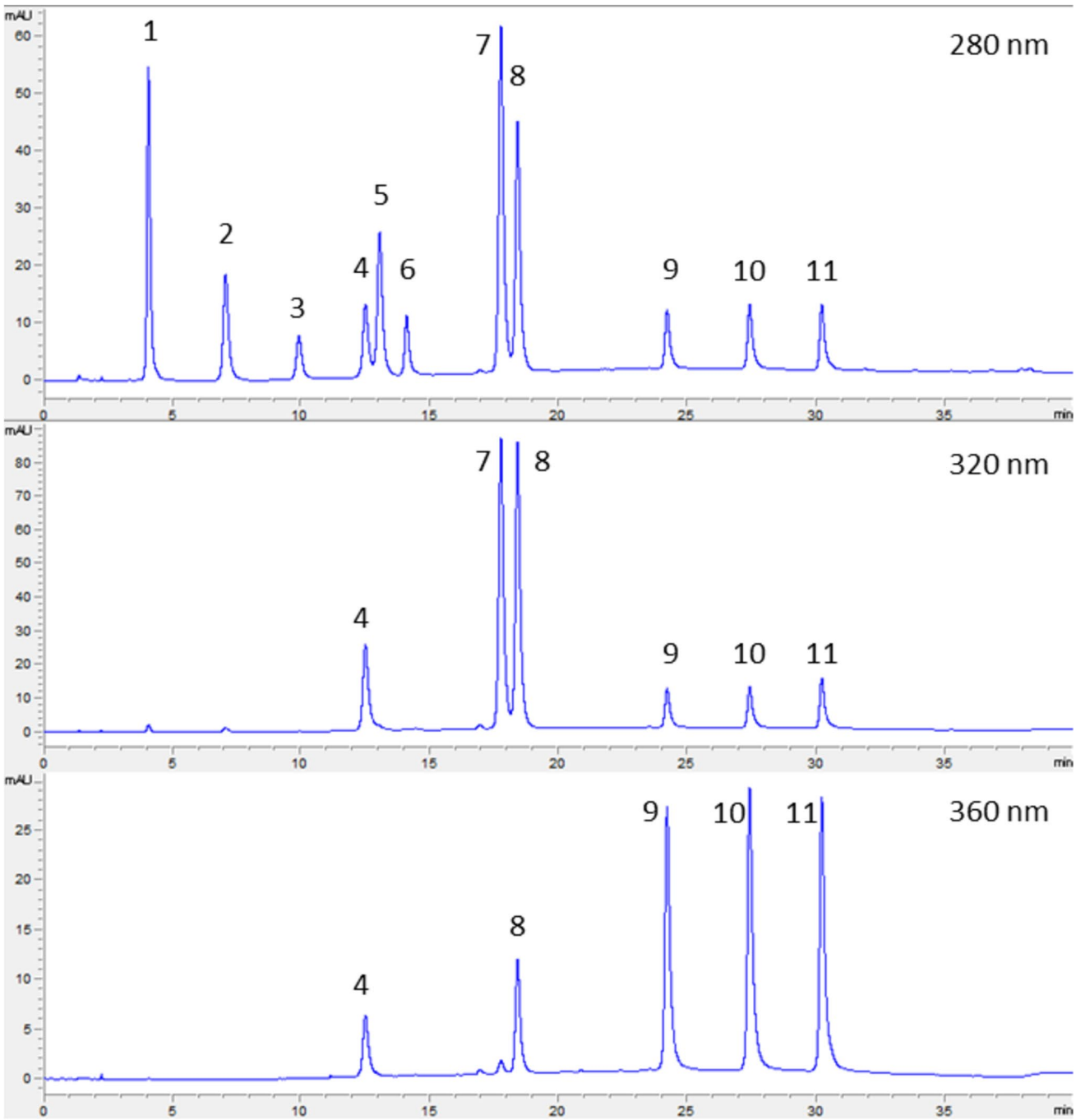

Fig. 2 Chromatographic separation of eleven polyphenols. 1: gallic acid; 2: protocatechuic acid; 3: (+)-catechin; 4: chlorogenic acid; 5: vanillic acid; 6: (−)-epicatechin; 7: ferulic acid; 8: *p*-coumaric acid; 9: myricetin; 10: quercetin; and 11: kaempferol

Extraction Phase

The performance of two commercial extraction phases, including DPX-RP and DPX-WAX, was evaluated using a univariate design. Figure S2 shows the result in a bar graph with normalized geometric means. According to the result, the highest extraction efficiency was achieved with the DPX-RP with statistically different results ($p<0.05$) according to the student's *t*-test. The performance in the extraction of analytes is linked to the ability of the extraction phase to selectively interact with the target compounds. In both evaluated extraction phases, the analytes can interact through π-π interactions. Additionally, DPX-WAX can interact via hydrogen bonds. However, the difference between phases did not promote an increase

in extraction efficiency (Carasek et al. 2022). Therefore, DPX-RP was chosen as the extraction phase to continue the development of the method.

Desorption Solvent

Acetonitrile, methanol, and ethanol solvents were evaluated as desorption solvents using a Simplex Lattice design. Figure 3 presents the ternary surface graph obtained from a quadratic mathematical model with an R^2 of 0.9757. The maximum response trend region is observed by applying acetonitrile in greater proportion. Given the best response, acetonitrile was chosen as the desorption solvent. In this step, the solvent must be able to break the analyte/extraction phase interaction. The observed results can be attributed to the less polar character of acetonitrile compared to methanol and ethanol. This characteristic corroborates the polarity of the extraction phase. This means that when using acetonitrile, the polarity of the solvent and the extraction phase are close, and therefore, the strength of the acetonitrile solvent is higher than other solvents.

Fractional Factorial Design

The main variables that can significantly influence the extraction efficiency in the DPX process are sample volume, extraction time, extraction cycles, salt concentration, desorption time, desorption cycles, and pH (Carasek et al. 2022). Considering the importance of these parameters, a fractional factorial design (2^{7-3}) was initially performed to assess the significant influence of these parameters on the extraction of polyphenols in grape juice. Figure S3 presents the Pareto chart obtained for this evaluation. According to the results, the volume and pH parameters of the sample and extraction cycles have a significant influence on the extraction of analytes. There is a tendency to increase the analytical response using sample volumes close to the high level evaluated (3200 µL) and sample pH close to the low level evaluated (pH 2). These results guided the next optimization, where we decided to optimize the sample volume, extraction cycles, and sample pH through a central composite design. The other parameters did not have a significant influence on the polyphenol extraction, so we decided to establish these values. Time and desorption cycles were fixed at 2 cycles of 10 s, and the time for extraction was fixed at 10 s. The result for the extraction time corroborates the

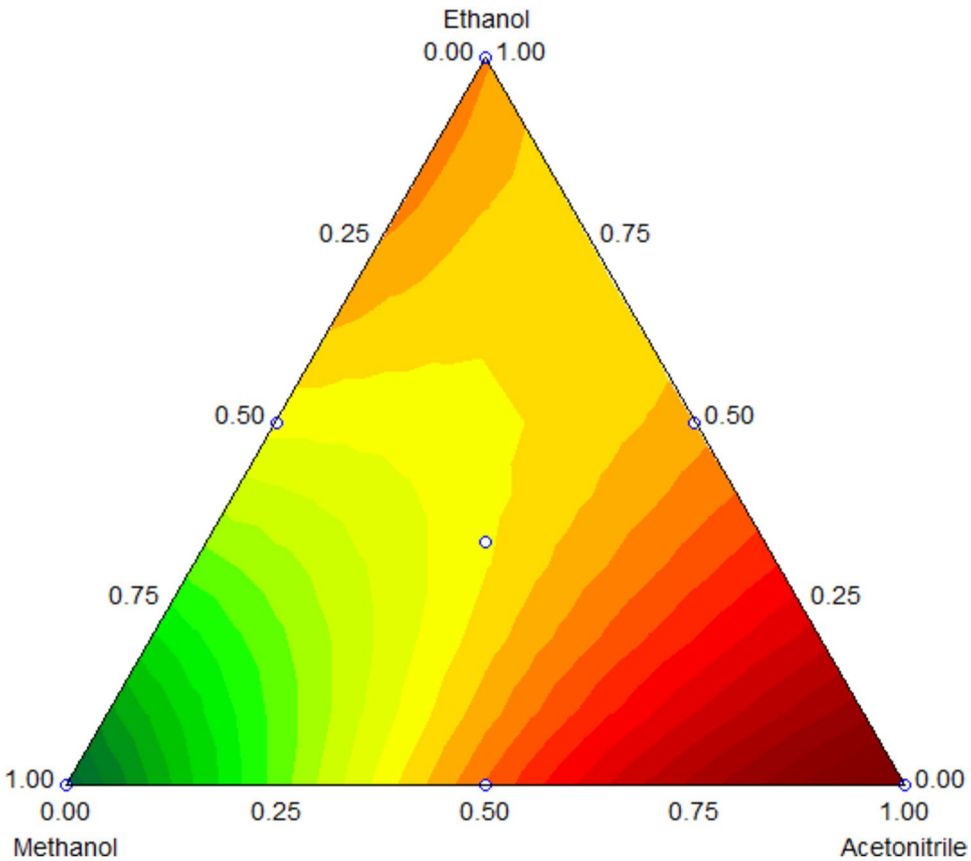

Fig. 3 Ternary surface for optimization of the desorption solvent. The analyses were performed in grape juice and ultrapure water solution (1:20). The sample volume was 800 µL at pH 3.0 and DPX-RP as the extraction phase. The cycles and extraction time were 5 cycles of 30 s. The cleaning was 1 cycle of 10 s with ultrapure water. The desorption cycles, time, and solvent were 2 cycles of 10 s with 200 µL of each solvent. The response is expressed as the geometric mean of the chromatographic peak area of 11 polyphenols

proposal for rapid extraction presented by the DPX technique. It should be noted that during the aspiration of the sample, the air is aspirated in sequence, and as a result, small bubbles with a large surface area form, and these promote a dispersive mixture between the sample and the extraction phase, making the extraction occur in a short period of time (Turazzi et al. 2019).

Sample Volume, Extraction Cycles, and pH

Sample volume, number of extraction cycles for each sample aliquot, and pH were optimized using central composite design. The response surfaces (Fig. 4) were obtained using a quadratic mathematical model with an R^2 of 0.9679, and according to ANOVA, all parameters had a significant influence on the extraction. According to the graphs, the sample volume and number of extraction cycles showed an increasing trend in the analytical response towards the maximum levels evaluated (volume, 4800 µL; extraction cycles, 6). In addition to the observed response, it is important to consider the analytical frequency of the developed method, so we chose to use a volume of 3200 µL of the sample, that is, 4 aliquots of 800 µL, and to perform 5 extraction cycles in each aliquot, totaling 20 extraction cycles (5 cycles multiplied by 4 sample aliquots). Regarding the pH, the results presented indicate a trend towards an increase in the analytical response at values close to the lower level evaluated (pH 2). Therefore, pH 2 was selected as the extraction condition. This result indicates that the analytes are preferably extracted in their neutral form, since all analytes have a pKa greater than 3.3.

Method Performance

Grape juice has a high complexity inherent to the diversity of compounds present. Therefore, the matrix effect (ME) on the extraction of polyphenols was evaluated by comparing the slopes of the analytical curves of the 11 polyphenols in solutions of grape juice and ultrapure water (1:20) (*S matrix*) and in ultrapure water (*S water*) according to Eq. 1 (Aguiar Jr. et al. 2020).

$$\%ME = \left(\frac{S\ matrix}{S\ water} - 1\right) \times 100 \quad (1)$$

The ME percentages obtained ranged from −18.3% for vanillic acid to 9.7% for kaempferol. The other values are presented in Table 1, where it is emphasized that the closer to 0%, the smaller the matrix effect. The results obtained indicate that the sample preparation by DPX was able to mitigate the effect of the matrix, since the variations were in the range of −20% to 20%. Therefore, the analytical curves were obtained through calibration curves with the addition of analytical standard solutions in ultrapure water.

Parameters of analytical merit are shown in Table 1. Coefficient of determination showed values greater than or equal to 0.9621, indicating good linear relationships for all analytes. The limits of detection and quantification ranged from 0.01 to 0.09 mg L^{-1} and 0.03 to 0.30 mg L^{-1}, respectively. Precision and accuracy values are presented in Table 2. Method precision was evaluated in intraday (n=3) and interday (n=9) assays at three concentration levels. The results were expressed as relative standard deviations (RSD). Intraday and interday precision values ranged between 0.2 and 17.5% and 0.7 and 19.6%, respectively. The accuracy of the method was evaluated using relative recovery with

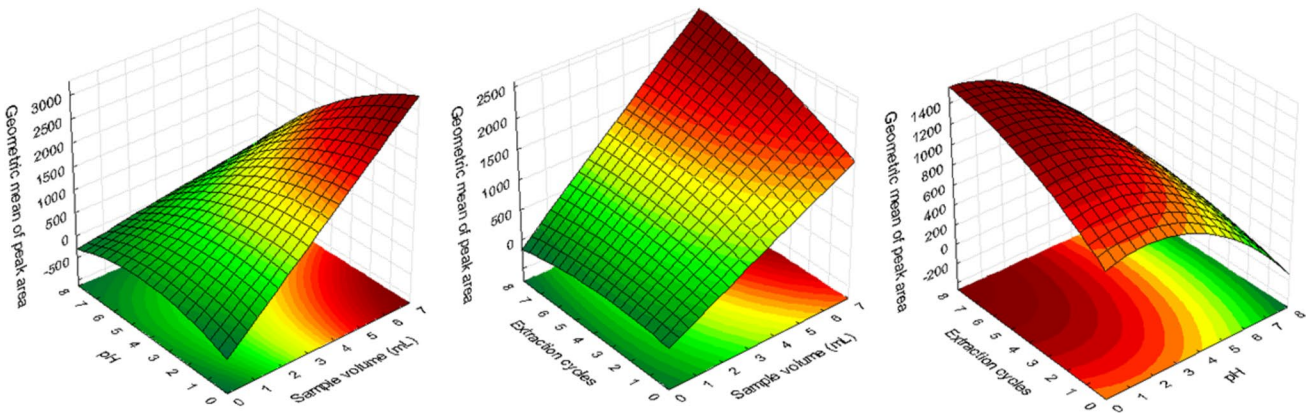

Fig. 4 Response surface obtained through the central composite design in optimizing sample volume, pH, and extraction cycles. The analyses were performed in grape juice and ultrapure water solution (1:20). DPX-RP as the extraction phase. The extraction time was 10 s per cycle. The cleaning was 1 cycle of 10 s with ultrapure water. The desorption cycles, time, and solvent were 2 cycles of 10 s with 200 µL of acetonitrile. The response is expressed as the geometric mean of the chromatographic peak area of 11 polyphenols

Table 1 Working range, linear equations, coefficient of determination, detection and quantification limits, polyphenol concentration, matrix effect, retention time, and mass spectrum parameters of the twelve polyphenols

Analyte	Retention time (min)	Linear working range (mg L^{-1})	Linear equation	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})	Matrix effect (%)
Gallic acid	4.37	0.25–7.5	y=48.7x − 1.1	0.9962	0.09	0.30	−4.4
Protocatechuic acid	7.78	0.25–7.5	y=210.8x − 32.8	0.9964	0.09	0.30	−11.6
(+)-Catechin	10.75	0.25–7.5	y=163.9x − 48.9	0.9908	0.09	0.30	−15.4
Chlorogenic acid	13.07	0.25–7.5	y=389.8x − 205.0	0.9870	0.01	0.03	13.0
Vanillic acid	13.74	0.25–7.5	y=525.8x − 178.2	0.9856	0.01	0.03	−18.3
(−)-Epicatechin	14.73	0.25–7.5	y=202.9x − 70.3	0.9621	0.09	0.30	14.7
Ferulic acid	18.38	0.10–10	y=1623.1x − 172.3	0.9956	0.02	0.06	2.2
p-Coumaric acid	18.98	0.10–10	y=1593.8x − 81.5	0.9912	0.03	0.10	0.9
Myricetin	24.69	0.10–10	y=671.1x − 80.1	0.9942	0.01	0.03	1.6
Quercetin	27.96	0.10–10	y=1593.8x − 81.5	0.9910	0.01	0.03	−5.4
Kaempferol	30.74	0.10–10	Y=547.0x − 61.2	0.9986	0.02	0.06	9.7

values ranging from 81 to 120%. These results are considered satisfactory by the AOAC guidelines (AOAC 2016).

The robustness of the DPX/HPLC-DAD method was evaluated through a fractional factorial design (Karageorgou and Samanidou 2014). Robustness provides an indication of the reliability of the analytical procedure; that is, it measures the ability of the method to remain unchanged by small variations during routine application (Leonardi et al. 2015). Figure S4 presents the Length Plot, where the effects of the seven variables are shown, relative to the margin of error (ME) and the simultaneous margin of error (SME). According to the Length Plot, no variable has a more significant effect than the margin of error. Thus, the analytical procedure proposed in this study can be considered robust.

Application of the DPX/HPLC-DAD Method

Grape juice is a natural source of polyphenols for the human diet, and these compounds play a key role in the quality of juices, mainly in color, aroma, astringency, and flavor. The concentrations of these biologically active substances in grape juice are determined by several factors, such as the grape variety, climatic conditions, cultivation techniques, and the production process (Guler 2023). The polyphenols present in grapes act as natural antioxidants and make an important contribution to the diet, especially in the prevention of numerous chronic diseases. In this study, it was proposed for the first time to use a simple and green sample preparation approach for grape juices employing the dispersive pipette extraction (DPX) technique to determine 11 polyphenols, including hydroxybenzoic acids, hydroxycinnamic acids, flavonols, and flavan-3-ols. Different classes of polyphenols were identified in red grape juice (Bordô varietal), organic red grape juice (Bordô and Isabel varietal), and white grape juice (Niagara varietal) (Table 3).

According to the results, organic red grape juice showed higher concentrations for all classes of polyphenols evaluated, except for quercetin, where the concentration in conventional grape juice was 44% higher compared to red organic grape juice. Chlorogenic acid (70.84–120.70 mg L^{-1}) and gallic acid (69.62–96.08 mg L^{-1}) were the major polyphenols in both red grape juices. We emphasize that the concentration of gallic acid and chlorogenic acid was 38 and 70% higher in organic red grape juice, respectively, compared to conventional red grape juice. Chlorogenic acid is associated with some health benefits, acting as a cardiovascular and antidiabetic protector, mainly associated with the anti-inflammatory and antioxidant role of this compound (Rashmi and Negi 2020; Singh et al. 2023; Tousch et al. 2008). Similarly, several beneficial effects are reported for gallic acid, also related to antioxidant and anti-inflammatory properties, such as neuroprotective (Obafemi et al. 2023), anti-obesity (Behera et al. 2023), and anti-tumor effects (Bai et al. 2021). Additionally, kaempferol was not identified in either sample of red grape juice, and protocatechuic acid was not identified in any of the evaluated samples.

White grape juice had the lowest concentrations for all polyphenols evaluated, and only five polyphenols were quantified in the sample (gallic acid > chlorogenic acid > catechin > quercetin > kaempferol). It is noteworthy that gallic acid (58.73 mg L^{-1}) and chlorogenic acid (34.71 mg L^{-1}) were also the major compounds in white grape juice. Protocatechuic acid, vanillic acid, epicatechin, ferulic acid, *p*-coumaric acid, and myricetin are below the quantification limits of the method for the white grape juice sample.

Table 2 Intraday and interday precision in water ultrapure and relative recoveries was carried out in a solution of grape juice and ultrapure water (1:20)

Analyte	Spiked concentration (mg L^{-1})	Intraday precision (%) n=3	Interday precision (%) n=9	Relative recovery (%) n=3
Gallic acid	0.5	7.0	19.0	88
	2.5	17.5	11.1	106
	7.5	6.9	8.8	111
Protocatechuic acid	0.5	7.4	17.1	120
	2.5	10.9	11.0	81
	7.5	6.0	13.3	92
(+)-Catechin	0.5	12.1	11.7	108
	2.5	15.6	3.4	98
	7.5	7.6	9.7	81
Chlorogenic acid	0.5	2.8	8.6	94
	2.5	9.2	15.5	112
	7.5	3.4	3.5	86
Vanillic acid	0.5	1.3	0.7	95
	2.5	7.7	10.1	80
	7.5	6.5	19.6	81
(−)-Epicatechin	0.5	3.6	3.4	118
	2.5	11.5	0.7	92
	7.5	7.1	18.3	86
Ferulic acid	0.5	0.4	6.0	116
	2.5	5.1	8.8	84
	7.5	5.2	9.8	90
p-Coumaric acid	0.5	0.3	19.9	99
	2.5	4.2	2.6	81
	7.5	5.1	12.0	87
Myricetin	0.5	0.2	5.5	114
	2.5	3.5	9.5	94
	7.5	4.4	9.8	84
Quercetin	0.5	0.2	4.0	119
	2.5	3.0	7.9	97
	7.5	0.7	14.1	93
Kaempferol	0.5	1.5	3.9	114
	2.5	1.4	9.0	98
	7.5	0.3	11.8	102

Table 3 Application of the proposed method for the determination of polyphenols in grape juice

Analyte	Red juice (mg L^{-1})	Organic red juice (mg L^{-1})	White juice (mg L^{-1})
Gallic acid	69.62 (± 2.9%)	96.08 (± 8.5%)	58.73 (± 17.9%)
Protocatechuic acid	<LOD	<LOD	<LOD
(+)-Catechin	17.05 (± 7.0%)	<LOD	13.50 (± 12.4%)
Chlorogenic acid	70.84 (± 8.8%)	120.70 (± 4.4%)	34.71 (± 0.7%)
Vanillic acid	11.92 (± 18.5%)	23.86 (± 19.0%)	<LOD
(−)-Epicatechin	34.38 (± 12.7%)	42.42 (± 0.6%)	<LOD
Ferulic acid	9.13 (± 13.9%)	16.28 (± 13.8%)	<LOD
p-Coumaric acid	<LOD	4.44 (± 13.5%)	<LOD
Myricetin	5.12 (± 6.9%)	5.58 (± 1.1%)	<LOD
Quercetin	3.86 (± 12.1%)	1.72 (± 16.80%)	1.88 (± 19.0%)
Kaempferol	<LOD	<LOD	0.86 (±1 5.4%)

Evaluation of Greenness and Comparison with Other Methods in the Literature

The use of metrics to evaluate analytical procedures in terms of "greenness" is a useful tool to define the green character of analytical methods and identify positive points presented by a new approach, as well as points to be improved. In this sense, the Green Analytical Procedure Index (GAPI) (Płotka-Wasylka 2018) and Analytical Greenness Calculator (AGREE) (Pena-Pereira et al. 2020) metrics were used to evaluate the proposed method for the determination of polyphenols in grape juice. Additionally, the AGREEprep metric (Wojnowski et al. 2022) was used to evaluate especially the sample preparation characteristics. In summary, GAPI presents a pictogram in colors (green, yellow, and red) and AGREE and AGRREprep additionally present a final score (from 0 to 1). In both, the assessment is based on the 12 principles of Green Analytical Chemistry (GAC) (Gałuszka et al. 2013).

The results are presented in Fig. 5. The evaluation of "greenness" indicates that the proposed methodology with DPX followed by HPLC-DAD provides an excellent green character, with emphasis on the miniaturization of the technique and zero energy consumption in the extraction, since no instrumentation is required as the DPX procedure only requires a micropipette to perform the extraction/desorption step. In addition, the minimization of the sample (160 µL) and solvent (200 µL) volumes and of the extraction phase (20 mg) mass stands out. Another important factor is the time required to prepare the sample: in the proposed method, an average of 5 min is required to prepare the sample, which reduces the time of the analytical procedure and the exposure of the analyst. Regarding the critical points, the main penalties are related to ex situ sample preparation, common to the analysis of polyphenols in grape juice by research laboratories, use of acetonitrile as desorption solvent; on the other hand, only 200 µL of solvent are used, contributing to the reduction of waste. And finally, the use of HPLC implies a negative weight in the evaluation since the technique requires solvents to compose the mobile phase. However, it should be noted that this technique is widely used for the analysis of polyphenols, since for the application of gas chromatography, it is necessary to include a derivatization step, which is not recommended by the guidelines for green analytical chemistry.

The developed method was juxtaposed with other methods based on solid phase extraction found in the literature for the determination of polyphenols in grape juice. The comparison is shown in Table 4. Aresta et al. (2018) proposed a method by SPME and HPLC-DAD for analysis of trans-resveratrol, using 500 µL of sample, and no solvent was required. The sample preparation time was 105 min, so that the sample preparation yield was 0.57 samples prepared per hour, while the method proposed in this study presents a yield of 20 samples prepared per hour. Tashakkori et al. (2021) developed two methods based on SPME, the first using HPLC-DAD and the second GC-MS for analysis of 16 polyphenols; both methods used 5 mL of sample. The SPME/HPLC-DAD method required 500 µL of solvent for the desorption of the analytes, and the SPME/GC-MS method did not use solvent, but it was necessary to perform a derivatization step with the derivatization reagent N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA). This reagent is widely used due to its high reactivity, but its high acquisition cost is a disadvantage. Sample preparation times were 55 min for SPME/HPLC-DAD and 65 min for SPME/GC-MS, and sample preparation yields were 1.09 and 0.92 samples prepared per hour, respectively. Therefore, DPX stands out for presenting fast and effective extraction, due to the unique characteristic of the technique in forming a dispersive mixture between the sample and the extraction phase, promoting rapid adsorption of the analytes.

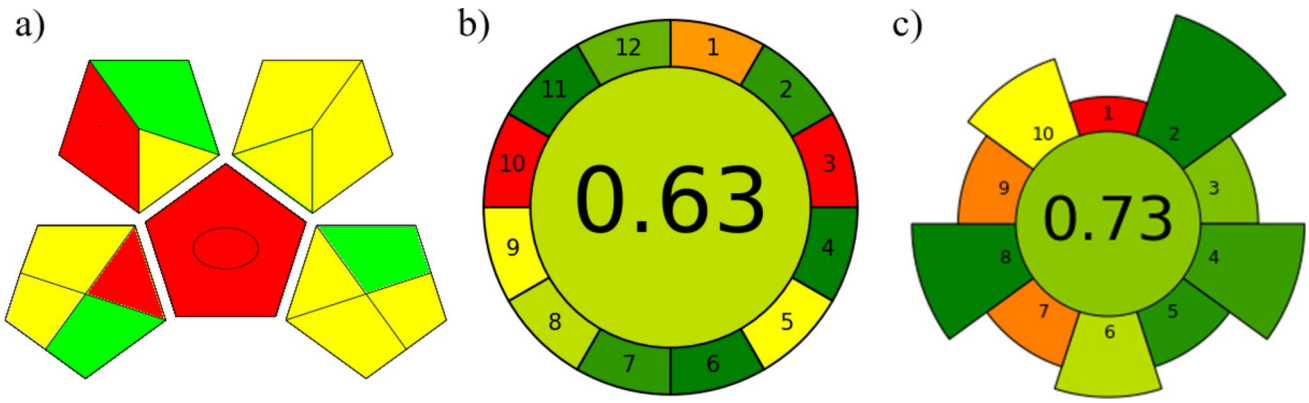

Fig. 5 Green evaluation of the proposed analytical methodology for the determination of polyphenols in grape juice samples: **a** GAPI, **b** AGREE, and **c** AGREEprep

Table 4 Comparison with reported methods in the literature for the determination of polyphenols in grape juices

Technique	Instrumentation	Analytes	Sample volume	Solvent volume	Sample preparation time	Sample throughput (number of samples that can be prepared in 1 h)	Sample preparation steps	Linear working range (mg L^{-1})	LOD (mg L^{-1})	Reference
DPX	HPLC-DAD	Gallic acid, protocatechuic acid, (+)-catechin, chlorogenic acid, vanillic acid, (−)-epicatechin, ferulic acid, *p*-coumaric acid, myricetin, quercetin, and kaempferol	160 µL	200 µL	<5 min	12	Conditioning of the extractor phase extraction and desorption	0.1 to 10	0.01 to 0.09	This work
SPME	HPLC-DAD	Trans-resveratrol	500 µL	–	105 min	0.57	Drying in a vacuum concentrator and extraction	0.0013 to 0.5	0.0013	(Aresta et al. 2018)
SPME	HPLC-DAD	Syringic acid, protocatechuic acid, cinnamic acid, *p*-coumaric acid, sinapinic acid, ferulic acid, caffeic acid, gallic acid, chlorogenic acid, quercetin, kaempferol, resveratrol, vanillic acid, rutin, catechin, and epicatechin	5000 µL	500 µL	55 min	1.09	Extraction and desorption	0.005 to 1	0.0025	(Tashakkori et al. 2021)

Table 4 (continued)

Technique	Instrumentation	Analytes	Sample volume	Solvent volume	Sample preparation time	Sample throughput (number of samples that can be prepared in 1 h)	Sample preparation steps	Linear working range (mg L^{-1})	LOD (mg L^{-1})	Reference
SPME	GC-MS	Syringic acid, protocatechuic acid, cinnamic acid, *p*-coumaric acid, sinapinic acid, ferulic acid, caffeic acid, gallic acid, chlorogenic acid, quercetin, kaempferol, resveratrol, vanillic acid, rutin, catechin, and epicatechin	5000 µL	–	65 min	0.92	Extraction, derivatization, and desorption	0.00002 to 1	0.00001 to 0.00005	(Tashakkori et al. 2021)

DPX dispersive pipette extraction, *SPME* solid phase microextraction. *HPLC-DAD* high-performance liquid chromatography-diode-array detection, *GC-MS* gas chromatography-mass spectrometry, *LOD* limit of detection

Regarding detection limits, both methods discussed presented lower limits than the method proposed by DPX, characteristic of the SPME technique, which presents a high enrichment factor, considered an advantage. On the other hand, one should consider the possibility of irreversible adsorption of compounds naturally present in grape juice, such as organic acids, minerals, and sugars (Cai et al. 2009), which would be considered a disadvantage.

Finally, the AGREEprep metric was applied to evaluate the greenness of the listed methods. The results are presented in Fig. S5. Both methods had a lower score than the method proposed by DPX. Although SPME is considered a green technique, the described procedures have some disadvantages that reduced the final score, including very low sample yield, energy use during extraction, and a procedure in several steps.

Conclusion

A new DPX/HPLC-DAD method to determine 11 polyphenols (phenolic acids and flavonoids) in grape juice was developed. The main advantages are the ease of preparing the sample in a short extraction time, less than 5 min, and with low consumption of organic solvent, only 200 µL. In addition, the method showed an excellent green character, according to three analytical metrics, GAPI, AGREE (score 0.63), and AGREEprep (score 0.73). The method was fully validated and showed satisfactory linearity, $R^2 \geq 0.9621$, LOQ (0.03-0.30 mg L^{-1}), precisions with values lower than 20%, and recoveries ranging from 81 to 120 %. Furthermore, the optimized and validated method showed no matrix effect. Therefore, the results obtained confirm that the DPX/HPLC-DAD method is appropriate for the determination of polyphenols in grape juice samples, making it a useful method to evaluate the quality of grape juices by means of phenolic composition, maintaining a balance between analytical results, environmental considerations, and analyst safety.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest Marina Pereira-Coelho declares that she has no conflict of interest. Isabel Cristina da Silva Haas declares that she has no conflict of interest. Luciano Vitali declares that he has no conflict of interest. Luiz Augusto dos Santos Madureira declares that he has no conflict of interest.

Competing Interests The authors declare no competing interests.

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