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A novel method for ultra-fast determination of phenolics with performance comparable to UPLC/DAD: Method development and validation on analysis of seedless table grapes

Marcos dos Santos Lima [a,b,*], Bruno Silva Dantas [b], Ana Júlia de Brito Araújo Carvalho [a], Giuliano Elias Pereira [c], Tatiana Colombo Pimentel [d], Marciane Magnani [e]

[a] Instituto Federal do Sertão Pernambucano, Campus Petrolina, Department of Food Technology - Beverages and Liquid Chromatography Laboratory, João de Deus, Petrolina, PE CEP 56316-686, Brazil
[b] Universidade Federal da Paraíba, Postgraduate Program in Food Science and Technology, Castelo Branco III, João Pessoa, PB CEP 58059-900, Brazil
[c] Empresa Brasileira de Pesquisa Agropecuária – Embrapa Uva e Vinho, Rua Livramento 515, PO Box 130, Bento Gonçalves, RS 95701-008, Brazil
[d] Instituto Federal do Paraná, Campus Paranavaí, Paranavaí, PR CEP 87703-536, Brazil
[e] Laboratory of Microbial Process in Foods, Department of Food Engineering, Universidade Federal da Paraíba, João Pessoa, PB, Brazil

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ABSTRACT

Grapes are well-known for the rich content of phenolics with benefits for human health. The reliable identification of phenolics in grapes is essential to explore the related bioactivity, highlighting the value of the grape source. In this work, an ultra-fast method for determining phenolic compounds in HPLC-DAD using a Rapid Resolution High Throughput-RRHT column (RP-C_{18} 4.6 ×50 mm, 1.8 μm) was developed and validated and nine seedless table grapes produced in the São Francisco Valley, Brazil were analyzed. The method showed good sensitivity (LOD≤ 0.65 mg/L and LOQ< 1.12 mg/L), high linearity (R2 > 0.998), selectivity, precision (CV%< 6.82). The recovery (81.5–105.6 %) was adequate for the desired purpose. A total of 41 phenolics were separated in 20 min, rending a resolution equivalent to UPLC-DAD. Thirty-four phenolics were quantified in grapes, including eight phenolic acids, two stilbenes, one phenolic aldehyde, three isoflavones, four flavanols, three proanthocyanidins, five flavonols, and eight anthocyanins. Patented grapes presented a high content of mono glucoside anthocyanins. Brazilian registered grapes presented high anthocyanin content and the presence of 3,5-diglucoside anthocyanins. The validated method is an alternative for rapid identification of phenolics and maybe useful to stablish procedures for identifying compounds in determining the markers in fruit.

1. Introduction

Grapes, renowned for their rich content of bioactive phenolic compounds, hold immense potential for human health. These compounds have demonstrated various pharmacological properties, including antimicrobial, antioxidant, anti-inflammatory, antitumor, vasodilator, and antihyperglycemic activities. Phenolics belong to various phenolic classes, such as flavonols, flavanols, isoflavones, anthocyanins, phenolic acids, and stilbenes (Machado et al., 2024). Exploring these compounds and their potential benefits *in vitro* and *in vivo* necessitates a comprehensive understanding of their molecular composition in the food or drug (Granato et al., 2018). This task demands robust analytical techniques, which is particularly challenging due to the complex matrices such as grapes.

The technique regularly used to analyze phenolic compounds is high-pressure liquid chromatography-HPLC or ultra-performance liquid chromatography-UPLC, primarily using UV-Vis detectors and mass spectrometers (MS) (Sik et al., 2022). In UPLC analysis, due to the availability of pumps with pressure ≥ 600 bar in the equipment, reverse-phase silica columns (RP-C_8 or RP-C_{18}) with a diameter of 2.1 mm and porous particles smaller than 2 μm are used, allowing ultra-fast separation/detection, high sensitivity, shorter run times and lower solvent consumption compared to HPLC (Sanches et al., 2022). However, by using RP-C_{18} core-shell columns (sub-3 μm particles), run

* Corresponding author at: Instituto Federal do Sertão Pernambucano, Campus Petrolina, Department of Food Technology - Beverages and Liquid Chromatography Laboratory, João de Deus, Petrolina, PE CEP 56316-686, Brazil.
E-mail addresses: marcos.santos@ifsertao-pe.edu.br (M. dos Santos Lima), ana.julia@ifsertao-pe.edu.br (A.J.B. Araújo Carvalho), tatiana.pimentel@ifpr.edu.br (T. Colombo Pimentel).

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times were drastically reduced when phenolics were analyzed on an HPLC equipped with a 400-bar pump, flow cell, and data processing software adequate for high-resolution acquisition (Manns and Mansfield, 2012). With the availability of Rapid Resolution High Throughput (RRHT) columns (4.6 mm diameter, sub 2 μm particles, ≤ 600 bar) for HPLC, there are new possibilities for rapid analysis (Luan and Wang, 2007; Hormaza et al., 2017).

Scientific advances in genetics have produced seedless table grape varieties that are highly appealing to consumers due to their nutritional value, attractive visual appearance, and ease of consumption (Izcara et al., 2021). Table grapes are one of the main Brazilian fruit exports, where the São Francisco Valley (SFV) region, located between the states of Pernambuco and Bahia, accounts for more than 90 % of the total grapes exported by the country (Nascimento et al., 2023). The grapes production in SFV has focused on seedless cultivars of table grapes, which are patented or registered by private companies and by the genetic improvement program of the Brazilian Agricultural Research Corporation (Embrapa) (Leão et al., 2017; Oliveira et al., 2023). Patented cultivars with exotic physical and chemical characteristics and flavors, such as Autumn Crisp™ (Sun World Corporation, 2024), Candy Dreams™, Candy Snaps™, Sugar Crisp™ (International Fruit Genetics-IFG, 2024), ARRA Sweeties™, and ARRA Cherry Crunsh™ (Grapa Varieties, 2024), among others, have been gaining ground even though they require producers to pay royalties. On the other hand, Brazilian hybrid cultivars (*V. vinifera* x *V. labrusca*) developed by Embrapa, such as 'BRS Vitória' and 'BRS Isis' have been freely disseminated among producers to make the Brazilian grape agribusiness more competitive (Ahmed et al., 2017; Colombo et al., 2020; Nascimento et al., 2023). 'BRS Melodia' is a new Brazilian seedless table grape cultivar developed specifically for the SFV, with a pink color and red fruit flavor, and originated from the cross {'BRS Linda' x 'CNPUV681–29 ['Arkansas 1976' X 'CNPUV 147–3′ ('Niágara Branca' x 'Vênus')]} (Ritschel et al., 2021).

In this context, the present aimed to develop an ultra-fast method for determination of phenolic compounds by HPLC-DAD using an RRHT RP-C_{18} column with sub-2 μm particles with performance comparable to UPLC-DAD. The performance of the column and method validation parameters were evaluated, and the validated methodology was used to analyze nine patented or registered seedless table grape cultivars produced in the São Francisco Valley, Brazil.

2. Material and methods

2.1. Reactants and external standards for HPLC

Standards of catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, procyanidin A_2, procyanidin B_1, procyanidin B_2, quercetin 3-glucoside, kaempferol 3-glucoside, myricetin, rutin, isorhamnetin, malvidin 3-glucoside, pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucoside, peonidin 3-glucoside, were from Extrasynthese (Genay, France). *T-r*esveratrol and *c*-resveratrol were obtained from Cayman Chemical Company (Michigan, US). Vanillic acid, caffeic acid, gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, syringic acid, ferulic acid, fumaric acid, *orto*-vanillin, vanillin, epigallocatechin, ρ-coumaric acid, chlorogenic acid, trans-caftaric acid, quercetin hydrate, hesperidin, naringin, naringenin, hesperitin, cyanidin-3,5-diglucoside, malvidin-3,5-diglucoside, and pelargonidin-3,5-diglucoside were from Sigma-Aldrich. Methanol degree HPLC and phosphoric acid were from Riedel-de HaënTM (Seelze, Germany).

2.2. Grapes and extracts preparations

Table grapes from the patented cultivars were provided by licensed farms in Petrolina, PE, Brazil, in the São Francisco Valley. All the grapes were harvested in May 2024 at a stage of commercial ripeness (Brix ≥ 15), where the sample corresponded to 200 berries picked randomly from 50 plants. Autumn Crisp™ white grape (Sun World International, CA, USA); Candy Dreams™ black grape (cv. 'IFG Twenty-two'), Candy Snaps™ red grape (cv. 'IFG Twenty-one'), and Sugar Crisp™ (cv. 'IFG eleven') white grape (International Fruit Genetics, CA, USA); ARRA Sweeties™ white grape (cv. 'ARRA 15') and ARRA Cherry Crunsh™ red grape (cv. 'ARD 36') (Grapa Varieties, CA, USA); 'BRS Vitória' black grape, 'BRS Isis' and 'BRS Melodia' red grapes (Embrapa, RS, Brasil) were analyzed. Supplementary Figure S1 shows the visual characteristics of the bunches of each cultivar analyzed.

The extraction (n=3) was carried out following the methodology of Aubert and Chalot (2018) with modifications. Fresh grape berries (30 g) were added to 30 mL of methanol/HCl (99.5:0.5 v/v), crushed/homogenized in a blade mill for 60 s, and centrifuged at 4000 *g* for 5 min. The supernatant (1 mL) was mixed with 1 mL of phase A, filtered through a 0.45-micron membrane, and injected into the HPLC.

2.3. Instrumentation and ultrafast method for determining phenolic compounds

In the present study, a liquid chromatograph-LC Agilent 1260 model (Santa Clara – USA) equipped with a quaternary solvent pump and in-line degassing (model G1311C), thermostated compartment for columns G1316A model, automatic sampler G1329B model, and Diode Array Detector (DAD) G1315D model, was used. OpenLAB CDS ChemStation Edition software (Agilent Technologies, Santa Clara – USA) was used for data collection and processing. The ultrafast column used was Eclipse Plus RRHT RP-C_{18} 50 ×4.6 mm with a particle size of 1.8 μm (Zorbax, SC, USA). The mobile phase consisted of a phosphoric acid solution at 0.5 % v/v (solvent A) and methanol acidified with 0.5 % v/v of phosphoric acid (solvent B). The flow rate was 1.0 mL/min at 40 ºC, and ten μL of the sample was injected. The gradient used was 0 min: 0 % B; 2 min: 10 % B; 13 min: 26 % B; 19 min: 50 % B; 21 min: 80 % B; 21.1–23.1 min: 100 % B; 23.2 min: 0 % B; and 3 min post run. Phenolic detections were performed in DAD at 280, 220, 320, 360, and 520 nm. Compounds were identified and quantified by comparison with external standards using retention time, calibration curves, and spectrum similarity.

2.4. Column performance and method validation

This method was developed based on the chromatographic conditions described by Manns & Manfield (2012) using core-shell columns (2.6 μm particles), with solvents A and B maintained. The separation condition was optimized from injections of a mixed standard containing the 41 phenolic compounds using the RP-C_{18} column (50 ×4.6 mm, 1.8 μm). Solvent flows of 0.5–1.2 mL/min, 30–45 ºC temperatures, and 3–10 μL injection volumes were evaluated. The best separation condition obtained (2.3 section) was used to study the column's performance and validate the method.

The column's performance was evaluated using the parameters resolution, symmetry factor, and number of theoretical plates established by the United States Pharmacopeia-USP (USP, 2022). All performance calculations were automatically performed using the OpenLAB CDS 3D UV software program (Agilent Technologies, SC, USA), as shown in supplementary data (Figure S2).

The method was validated using the Guidelines for Standard Method Performance Requirements of the Association of Official Analytical Chemists (AOAC, 2016).

2.4.1. Detection and quantification limits and linearity of the method

Linearity was obtained through the calibration with external standards (n = 5 points). The calibration curve was obtained using the peak height by correlating with known concentrations of the external standards for HPLC. The detection limit (LOD) and quantification limit (LOQ) values were obtained according to Hubaux and Vos (1970) method. Three grape extracts were spiked with external phenolic

standards in triplicate (n=9) and diluted to reach values close to the estimated LOD. The samples were analyzed, and an analytical curve was obtained by plotting the values generated (Axis-X) *vs.* the sample theoretical amounts (Axis-Y). Slope values of the curves, intercept, and correlation coefficients were generated. The residual standard deviation-RSD was also calculated, and limits (LOD and LOQ) were 3 and 10 times greater than the RSD value.

2.4.2. Specificity, recovery, and precision

The specificity analysis was performed according to the methodology by Padilha et al. (2017) using the *match factor* (spectral purity factor). To assess the method's specificity, samples of grape extracts and a mix of phenolic external standards were injected. The major analyzed phenolic peaks were subjected to a threshold test, allowing for an acceptable spectral purity factor $\geq$ 950. Precision was evaluated by the coefficient of variation (CV%) obtained from six independent injections (n=6) of different concentrations (low, intermediate, and high values), using grape extracts spiked with external phenolics standards. Percentual recovery values were calculated by comparing the values obtained for the spiked sample with those of the non-spiked samples.

2.5. Statistical analysis

The results of the characterization of seedless table grapes were tabulated and expressed as average values. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis were performed using the Past 4.03 program (University of Oslo, Oslo, Norway) to differentiate grape cultivars.

3. Results and discussion

3.1. Column performance

Table 1 shows the values obtained for the column's performance parameters. The United States Pharmacopeia (USP, 2020) establishes that the resolution measure (Rs) is the distance between two peaks, representing a total separation from the baseline. It states that it must be $\geq$ 1.5. However, according to Ravisankar et al. (2019), the minimum acceptable value for quantification is Rs $\geq$ 1.0.

Resolution is an important parameter for measuring the quality of the separation obtained. Of the 41 phenolics analyzed, 30 had a resolution $\geq$ 1.5, fully meeting the established analytical criteria. Ten compounds had Rs values between 1.0 and <1.5, which are considered acceptable for quantification; however, there is some risk of coelution depending on the complexity of the matrix analyzed. The ten compounds with resolution $\geq$ 1.0 and < 1.5 were procyanidin B2 (1.45), chlorogenic acid (1.40), trans-Resveratrol (1.34), cyanidin 3-glucoside (1.36), pelargonidin 3,5-diglucoside (1.27), malvidin 3,5-diglucoside (1.22), quercetin 3-glucoside (1.12), epicatechin gallate and rutin (1.10), and syringic acid (1.05). The only phenolic that showed Rs < 1.0 was *cis*-resveratrol (0.87), making it a compound with a high risk of coelution during the running. Fig. 1 shows the chromatograms representing this method's separation of the 41 phenolic compounds in 20 min of running. Fig. 2 shows the chromatogram obtained from the analysis of the Candy Snaps™ red grape by direct injection of the extract, where it is possible to see a good separation quality for the vast majority of the compounds (25 phenolics) and with a stable baseline.

In an analysis, the symmetry factor of the peak should be in the order of 0.8–1.8 (USP, 2021). In this study, all the compounds showed satisfactory results, with all the phenolics analyzed having symmetry values ranging from 0.82 to 1.46 (Table 1). The number of plates (*N*) indicates the column's efficiency. A column with many theoretical plates will have a narrower peak at a given retention time, favoring better detection limits. In this method, the *N* values were greater than 10000 for 38 of the phenolics analyzed, except fumaric acid (2131), gallic acid (3452) and cyanidin 3,5-diglucoside (4425).

Few studies have presented the quality parameters of the separation, especially about resolution. In the study by Sanches et al. (2022), an ultra-fast method was validated for the determination of phenolics by UPLC-DAD-MS and allowed the separation of 29 compounds in 26 min of running using an RP-C_{18} fused-core column (50 x 2.1 mm, 1.3 μm), where two compounds showed resolution < 1.5 (*p*-coumaric acid and limonin), N > 17477 for all compounds and symmetry factor ranging from 0.83 to 1.49. In the work of Manns and Mansfield (2012), in a fast method for the determination of phenolics in grape juices and wines by HPLC-DAD using RP-C_{18} core-shell columns (4.6x100 mm, 2.6 μm and 2.1x100 mm 2.6 μm) for the separation of 16 phenolics in 15 min, several critical resolutions were reported ranging from total coelution to < 1.5 for anthocyanins. In the study by Barbosa et al. (2020), a method was validated for phenolic determining in UPLC-MS using an RP-C_{18} fused-core column (100 x 2.1 mm, 2.7 μm) and allowed the separation of 36 compounds in 23 minutes of running. From the chromatogram presented, it was possible to observe separation resolution < 1.5 for several compounds such as chlorogenic acid, catechin, epicatechin, rutin, and procyanidin A_2, and total coelution for *p*-coumaric acid+epigallocatechin gallate and vanillic acid+syringic acid. Evidence that this should be one of the main parameters to be evaluated in ultra-fast determinations.

The present method separated 41 phenolics in 20 minutes using a sub-2 μm RRHT column in HPLC, in a total run time of 23.2 minutes. The performance obtained for 40 compounds was considered acceptable and similar to ultra-fast UPLC methods.

3.2. Validation parameters

3.2.1. Linearity and limits of detection-LOD and quantification-LOQ

A linear response between the peak area or maximum height (maximum absorbance), using standards of different concentrations, is a necessary condition for quantification, where the correlation coefficient (r) is a parameter indicator of the quality of the analytical curve (USP, 2022). In this study, the R^2 values ranged from 0.998 to 0.999 for all the compounds analyzed (Table 1). According to the AOAC (2016), R^2 values must be $\geq$ 0.99 for calibration acceptance, and the present method's results agree with this requirement.

In this method, the values obtained for the LOD ranged from 0.08 to 0.65 mg/L for trans-resveratrol and cyanidin 3,5-diglucoside, respectively. The LOQ ranged from 0.12 to 1.12 mg/L for trans-resveratrol and *o*-vanillin, respectively. High-resolution columns with sub-2 μm particles help obtain lower detection limits (Sanches et al., 2022). Factors such as sample injection volume, extraction techniques, and detector type influence the method's sensitivity. Other validated methods for determining phenolics by UPLC-DAD have reported LODs ranging from 0.18 to 0.99 mg/L and LOQs from 0.54 to 3.03 (Urbstaite et al., 2022). In UPLC-MS determinations, LOD values ranged from 0.52 to 0.56 and LOQ from 0.54 to 0.63 (Karunarathna et al., 2023). In a validated method for wine analysis by direct injection in HPLC-DAD using a core-shell column (C_{18} 100 x 4.6 mm, 2.7 μm), the limits of detection were 0.03–0.62 mg/L and LOQ 0.11–2.08 mg/L (Krstonošić et al., 2020). These results show that the detection limits obtained in this method are adequate and are in line with those obtained in other methods for the rapid determination of phenolics by UPLC.

3.2.2. Specificity, recovery, and precision

Compared to mass spectrometers, one of the disadvantages of DAD detection is the difficulty in evaluating possible coelutions of compounds during analysis in a complex matrix (selectivity/specificity) when only the retention time and spectral similarity of the peak compared to external standards are used. In this study, in addition to retention times and spectral similarity for identification, possible coelutions were assessed using the spectral purity factor (acceptable $\geq$ 950) for the main peaks ($\geq$ 10 mAU), which corresponds to possibly being a pure peak (Padilha et al., 2017), as can be seen in Fig. 3 for the flavonol

Table 1
Column performance and method validation parameters.

Phenolic compounds	**Peak ID number**	Retention time	λ (nm)	Resolution	Symmetry	Plates	Range mg/L (n=5)	Calibration curve	Linearity R^2	Precision CV %	Recovery %	RSD	LOD mg/L	LOQ mg/L
Fumaric acid	**1**	1.43	220	2.72	1.16	2131	0.5–10	Y= 14.808X – 0.066	0.999	1.48	87.1	0.044	0.23	0.52
Gallic acid	**2**	2.10	280	6.92	1.01	3452	0.5–10	Y= 5.404X – 0.032	0.999	1.44	87.6	0.033	0.27	0.53
3,4-dihydroxybenzoic acid	**3**	3.36	280	1.56	1.04	10255	0.5–10	Y= 4.196X + 0.033	0.999	1.23	96.7	0.027	0.18	0.27
trans-Caftaric acid	**4**	4.88	320	2.15	1.10	13660	0.5–10	Y= 6.45X – 0.031	0.999	6.28	88.8	0.052	0.16	0.45
Procyanidin B1	**5**	4.89	220	1.71	1.25	13016	0.5–15	Y= 28.721X – 6.581	0.998	1.13	103.8	0.049	0.11	0.23
Epigallocatechin gallate	**6**	5.22	220	1.67	1.04	16006	0.5–5.0	Y= 6.80X + 0.001	0.999	6.5	100.7	0.018	0.30	0.45
Catechin	**7**	5.50	220	1.50	1.12	17270	0.5–10	Y= 54.039X – 0.329	0.999	2.73	89.6	0.033	0.13	0.41
4-hydroxybenzoic acid	**8**	6.02	280	1.50	1.08	30381	0.5–10	Y= 2.40X – 0.001	0.999	6.35	87.5	0.023	0.10	0.35
Vanillic acid	**9**	6.32	280	1.78	1.02	26504	0.5–10	Y= 3.404X – 0.032	0.999	0.47	93.4	0.022	0.15	0.42
Caffeic acid	**10**	6.51	320	3.76	1.01	36726	0.5–10	Y= 7.478X – 0.038	0.999	2.82	93.2	0.066	0.22	0.56
Cholorogenic acid	**11**	6.64	320	1.40	1.33	44606	0.5–10	Y= 4.996X + 0.033	0.998	2.33	98.1	0.067	0.32	0.60
Procyanidin B2	**12**	6.82	220	1.45	1.38	30743	0.5–15	Y= 15.380X – 0.155	0.999	4.25	102.2	0.044	0.13	0.35
Vanillin	**13**	7.49	320	3.20	1.35	11199	0.5–10	Y= 4.80X + 0.002	0.999	0.45	89.9	0.021	0.15	0.34
Syringic acid	**14**	7.88	280	1.05	1.13	45583	0.5–10	Y= 4.40X + 0.001	0.999	3.32	105.6	0.088	0.41	0.86
Cyanidin 3,5-diglucoside	**15**	8.29	520	24.03	1.04	4425	0.5–15	Y= 0.996X + 0.033	0.999	4.78	92.1	0.112	0.65	0.95
Epicatechin	**16**	8.40	220	1.95	1.44	22280	0.5–15	Y= 8.60X – 0.002	0.999	3.01	85.9	0.038	0.12	0.38
p-Coumaric acid	**17**	9.00	320	1.94	1.08	18393	0.5–10	Y= 8.497X – 0.141	0.999	2.23	96.3	0.049	0.21	0.49
Pelargonidin 3,5-diglucoside	**18**	9.44	520	1.27	1.12	11856	0.5–10	Y= 0.596X + 0.033	0.999	6.42	90.5	0.062	0.55	0.86
Delphinidin 3-glucoside	**19**	9.64	520	3.29	0.90	14336	0.5–10	Y= 4.00X + 0.001	0.999	2.49	82.2	0.054	0.45	0.73
Orto-Vanillin	**20**	10.09	360	1.73	1.02	18510	0.5–10	Y= 1.20X + 0.001	0.999	2.92	89.9	0.086	0.58	1.12
Ferulic acid	**21**	10.99	320	3.02	1.00	22945	0.5–10	Y= 6.40X + 0.000	0.999	0.41	98.4	0.011	0.12	0.35
Cyanidin 3-glucoside	**22**	11.00	520	1.36	1.18	23064	0.5–15	Y= 1.401X + 0.002	0.999	6.13	86.1	0.024	0.48	0.54
Malvidin 3,5-diglucoside	**23**	11.21	520	1.22	1.21	12345	0.5–10	Y= 0.50X + 0.003	0.999	5.64	93.7	0.015	0.58	0.75
Epicatechin gallate	**24**	11.32	220	1.10	1.32	21807	0.5–5.0	Y= 4.103X + 0.94	0.998	4.47	103.5	0.066	0.29	0.55
Procyanidin A2	**25**	12.35	220	2.06	1.02	36801	0.5–15	Y= 2.80X + 0.001	0.999	5.97	84.9	0.044	0.23	0.45
Pelargonidin 3-glucoside	**26**	12.37	520	4.81	0.95	32136	0.5–15	Y= 1.004X + 0.001	0.999	2.84	87.4	0.055	0.52	0.92
Myricetin	**27**	13.44	360	4.46	0.97	47139	0.5–10	Y= 1.05X – 0.003	0.999	4.93	84.2	0.049	0.15	0.48
Peonidin 3-glucoside	**28**	13.73	520	4.59	1.10	29265	0.5–10	Y= 1.805X + 0.001	0.999	5.21	92.4	0.025	0.44	0.84
Malvidin 3-glucoside	**29**	14.80	520	4.37	1.08	14322	0.5–10	Y= 1.60X – 0.003	0.999	6.08	89.4	0.023	0.33	0.72
trans-Resveratrol	**30**	15.78	320	1.38	1.06	68249	0.5–10	Y= 6.05X + 0.005	0.999	3.13	102.9	0.005	0.08	0.12
Quercetin 3-glucoside	**31**	16.01	360	1.12	0.88	117275	0.5–10	Y= 4.40X – 0.001	0.999	5.22	100.8	0.038	0.12	0.35
Rutin	**32**	16.17	360	1.10	1.11	135134	0.5–10	Y= 3.991X – 0.048	0.999	4.84	98.5	0.032	0.13	0.34
Naringin	**33**	16.43	280	1.52	0.98	156171	0.5–10	Y= 2.80X – 0.004	0.999	6.82	97.7	0.035	0.15	0.52
Hesperidin	**34**	17.03	280	3.83	1.11	171051	0.5–10	Y= 0.80X + 0.001	0.999	3.98	91.5	0.034	0.15	0.49
trans-Cinnamic acid	**35**	17.50	280	2.10	1.44	53390	0.5–10	Y= 11.404X – 0.033	0.999	1.28	102.8	0.033	0.21	0.32
Kaempferol 3-glucoside	**36**	17.52	360	1.72	0.98	132593	0.5–10	Y= 1.004X – 0.032	0.999	4.86	95.1	0.025	0.15	0.32
cis-Resveratrol	**37**	17.71	280	0.87	0.96	138720	0.5–15	Y= 2.40X + 0.001	0.999	6.41	94.4	0.024	0.15	0.28
Isorhamnetin	**38**	17.91	360	1.54	1.00	225982	0.5–5.0	Y= 2.45X – 0.002	0.999	3.68	91.8	0.021	0.18	0.35
Quercetin Hydrate	**39**	18.90	360	4.79	1.46	33798	0.5–10	Y= 3.004 – 0.033	0.999	6.81	81.5	0.027	0.22	0.46
Naringenin	**40**	18.92	280	5.65	0.82	62627	0.5–10	Y= 1.21 – 0.003	0.999	6.19	93.4	0.110	0.31	0.88
Hesperitin	**41**	19.75	280	5.03	1.02	221710	0.5–10	Y= 6.207 – 0.063	0.999	6.15	101.5	0.088	0.26	0.82

Legend: Calibration curve (Y = peak height (mAU); X = Amount (mg/L)).

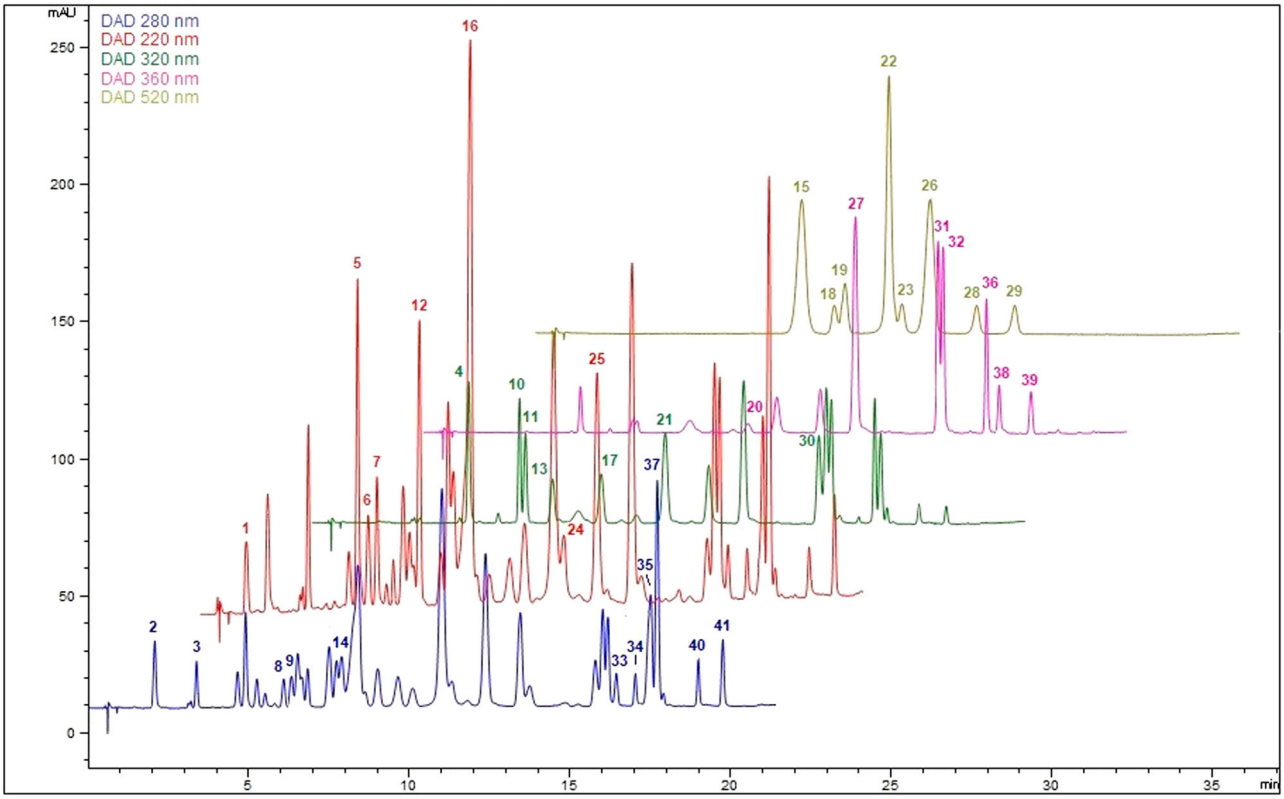

Fig. 1. Chromatogram of the separation of the 41 phenolic compounds by HPLC-DAD using an RRHT column (RP-C_{18} 50 ×4.6 mm, 1.8 μm). Legend: 1= fumaric acid; 2=gallic acid; 3= 3,4-dihydroxybenzoic acid; 4= caftaric acid; 5= procyanidin B1; 6= epigallocatechin gallate; 7= catechin; 8= 4-hydroxybenzoic acid; 9= vanillic acid; 10= caffeic acid; 11= chlorogenic acid; 12= procyanidin B2; 13= vanillin; 14= syringic acid; 15= cyanidin 3,5-diglucoside; 16= epicatechin; 17= p-coumaric acid; 18= pelargonidin 3,5-diglucoside; 19= delphinidin 3-glucoside; 20= ferulic acid; 21= cyanidin 3-glucoside; 22= malvidin 3,5-diglucoside; 23= epicatechin gallate; 24= procyanidin A2; 26= pelargonidin 3-glucoside; 27= myricetin; 28= peonidin 3-glucoside; 29= malvidin3-glucoside; 30= t-resveratrol; 31= quercetin 3-glucoside; 32= rutin; 33= naringin; 34= hesperidin; 35= t-cinnamic acid; 36= kaempferol 3-glucoside; 37= c-Resverarol; 38= isorhamnetin; 39= quercetin hydrate; 40= naringenin; 41= hesperitin.

quercetin 3-glucoside in the analysis of Candy Dreams™ grape. In this sample, only 3 of the 27 phenolics quantified showed a risk of coelution: caffeic acid, vanillic acid, and epicatechin. These results demonstrate the good specificity of this method.

The method's precision (CV%) ranged from 0.41 % to 6.82 %, which aligns with the acceptable values. The analyte ranges in this study should be a maximum of 7–11 % (AOAC, 2016). Recovery in this study ranged from 81.5 % to 105.6 % for quercetin hydrate and syringic acid, respectively, and is within acceptable values. According to the AOAC (2016) Official Methods of Analysis, values must be between 80 % and 110 % for recovery. In a method validated for wine analysis by direct injection in HPLC-DAD, the recovery varied from 97.9 % to 102.4 % (Krstonošić et al., 2020). Other methods for quantifying phenolics by UPLC with DAD and MS detection, the recovery values have ranged from 70.8 % to 104.8 % (Karunarathna et al., 2023), 94.3–109.4 (Setyaningsih et al., 2019), 94.1–108.1 % (Urbstaite et al., 2022). The method in this study showed good sensitivity, selectivity, precision, and recovery and is suitable for the proposed purpose.

3.3. Determination of the phenolic profile of seedless table grapes

Table 2 shows the phenolic compound profile of the seedless grapes obtained using the method validated in this study. The total number of compounds quantified by HPLC was highest in black grapes (763.5–1644.7 mg/kg), followed by red grapes (414.1–573.7 mg/kg) and white grapes (53.4–229.4 mg/kg). Of the 41 compounds available in this method, 34 were quantified in grapes. These included 8 phenolic acids, 2 stilbenes, 1 phenolic aldehyde, 3 isoflavones, 4 flavanols, 3 proanthocyanidins, 5 flavonols and 8 anthocyanins. Among the classes of phenolics, those present in the greatest quantity in grapes in general were phenolic acids (28.2–352.7 mg/kg) and in red and black grapes anthocyanins (216.3–1003.5 mg/kg). The main phenolics quantified in white grapes were *trans*-caftaric acid (12.4–85.2 mg/L) and procyanidin B_1 (5.0–25.1 mg/kg). In red grapes, the main phenolics were *trans*-caftaric acid (57.5–205.2 mg/kg), cyanidin 3-glucoside (23.6–196 mg/kg), and peonidin 3-glucoside (40.6–115.6 mg/kg). In black grapes, the anthocyanins cyanidin 3-glucoside (151–223.8 mg/kg), peonidin 3-glucoside (119.4–277.2 mg/kg), *trans*-caftaric acid (69.2–285.8 mg/kg) and malvidin 3-glucoside (78.6–120.2 mg/kg) stood out.

The differentiation of the grapes was assessed by multivariate hierarchical cluster analysis (HCA) and principal component analysis (PCA) (Fig. 4). The HCA analysis (Figure S3) formed three groups based on the phenolic profile quantified in the grapes in this study. Cluster 1 consisted of the white grapes Sugar Crisp™, ARRA Sweeties™ (cv. 'ARRA 15'), and Autumn Crisp™, which are associated with lower phenolic values. Cluster 2 was formed by the ARRA Cherry Crush™, Candy Snaps™, and Candy Dreams™ grapes associated with the profile of mono glucoside anthocyanins, and Cluster 3 by the Brazilian grapes 'BRS Vitória,' 'BRS Isis,' and 'BRS Melodia,' influenced by the exclusive presence of 3,5-diglucoside anthocyanins in Brazilian grapes because they are hybrids (*V. vinifera x V. labrusca*).

PCA explained 60.4 % of the variance in the experiment, with component 1 (PC1) accounting for 43.1 % and component 2 (PC2) for 17.3 %. In the positive part of PC1, the 'BRS Vitória' grape was separated because it had higher values for all the anthocyanins and the compounds ferulic acid, *p*-coumaric acid, isorhamnetin, myricetin, naringin, hesperitin and procyanidin A_2. The negative part of PC1 grouped the white grapes Sugar Crisp™, Autumn Crisp™, and ARRA

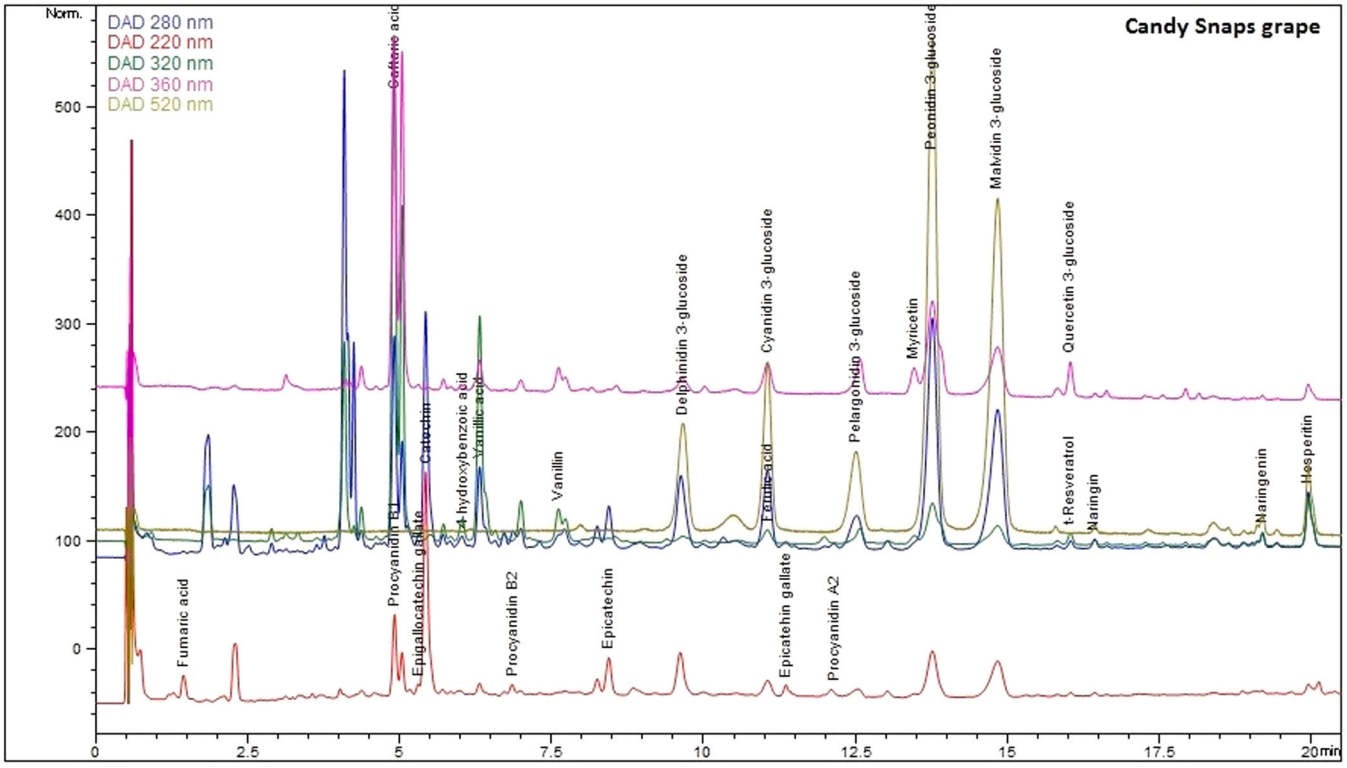

Fig. 2. HPLC-DAD chromatogram of the Candy Snaps™ grape sample using the method validated in this study.

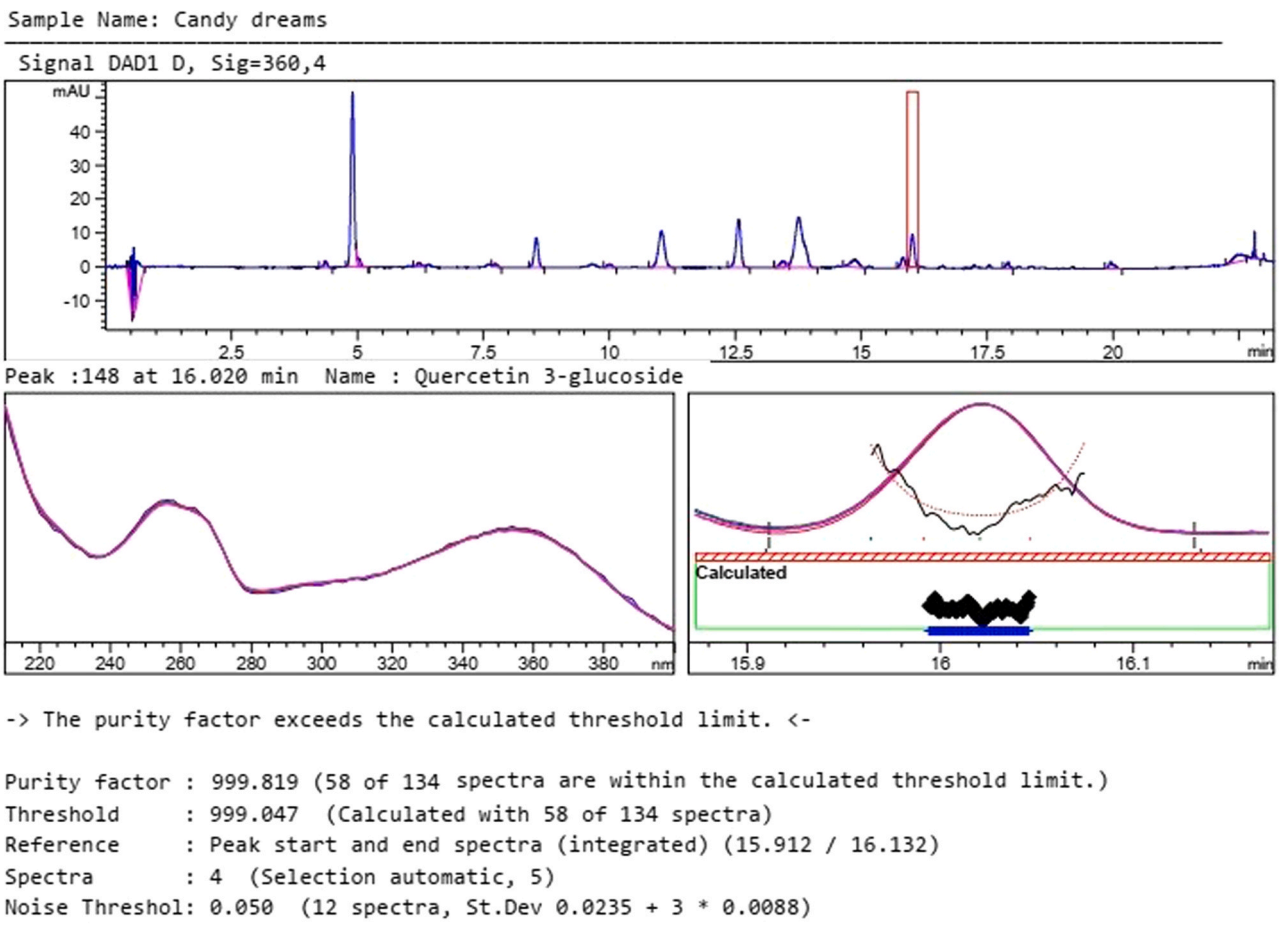

Fig. 3. A spectral peak purity test complemented the selectivity/specificity evaluation in the present method.

Table 2
Average ± standard deviation phenolic composition values of patented or registered seedless table grapes grown in the São Francisco Valley, Brazil.

Phenolic compounds mg/kg FW	**White grapes**			**Red grapes**				**Black grapes**	
	Autumn Crisp™	ARRA Sweeties™	Sugar Crisp™	Candy Snaps™	ARRA Cherry Crush™	'BRS Melodia'	'BRS Isis'	Candy Dreams™	'BRS Vitória'
Phenolic acids									
4-hydroxybenzoic acid	6.5 ± 1.1	8.4 ± 0.8	3.1 ± 0.5	2.3 ± 0.1	10.0 ± 0.6	4.2 ± 0.4	4.3 ± 0.1	3.7 ± 0.1	8.4 ± 0.4
Vanillic acid	3.0 ± 0.3	7.2 ± 1.4	0.7 ± 0.2	12.1 ± 0.4	9.1 ± 1.4	4.4 ± 0.5	6.3 ± 0.5	2.8 ± 0.1	15.8 ± 0.8
trans-Cinnamic acid	ND	ND	ND	ND	2.7 ± 1	0.6 ± 0.1	ND	0.5 ± 0.1	17.8 ± 0.2
trans-Caftaric acid	12.4 ± 2.9	82.5 ± 17	19.3 ± 8	83.8 ± 14	57.5 ± 12	130.9 ± 42	205.2 ± 43	69.2 ± 18	285.8 ± 40
Caffeic acid	2.8 ± 0.4	7.1 ± 2.3	1.1 ± 0.6	ND	8.8 ± 1.4	4.6 ± 2.2	6.1 ± 0.9	3.0 ± 1.9	15.5 ±4.7
Fumaric acid	9.4 ± 0.3	5.3 ± 0.1	4.0 ± 0.1	5.4 ± 0.1	13.5 ± 0.4	3.9 ± 0.1	6.2 ± 0.1	6.7	2.6 ± 0.1
p-coumaric	ND	ND	ND	ND	1.5 ± 0.8	ND	0.7 ± 0.4	ND	2.2 ± 0.8
Ferulic acid	ND	ND	ND	0.9 ± 0.5	3.5 ± 1.1	4.1 ± 2.3	1.6 ± 1	3.1 ± 0.5	4.5 ± 1.5
∑ *phenolic acids*	*34.2*	*110.5*	*28.2*	*104.5*	*106.7*	*152.9*	*230.4*	*89.0*	*352.7*
Stilbenes									
cis-Resveratrol	ND	ND	ND	ND	ND	ND	3.3 ± 1.1	ND	3.8 ± 0.4
trans-Resveratrol	1.6 ± 0.8	2.7 ± 0.7	2.4 ± 0.9	0.7 ± 0.4	4.0 ± 0.6	9.0 ± 3.7	1.7 ±0.7	1.9 ± 1	5.2 ± 1.9
∑ *stilbenes*	*1.6*	*2.7*	*2.4*	*0.7*	*4.0*	*9.0*	*5.0*	*1.9*	*9.1*
Phenolic aldehyde									
Vanillin	1.4 ± 0.6	70.7 ± 31	ND	2.8 ± 1.4	1.2 ± 0.6	5.5 ± 1.4	4.5 ± 0.5	1.7 ± 0.3	3.1 ± 0.7
Isoflavones									
Naringin	ND	ND	ND	1.6 ± 0.1	1.9 ± 0.2	ND	2.0 ± 0.2	ND	8.2 ± 0.1
Naringenin	ND	ND	ND	6.2 ± 0.3	24.8 ± 1.4	54.0 ± 5.7	13.6 ± 1	10.9 ± 4.5	4.5 ± 0.6
Hesperitin	ND	ND	ND	4.5 ± 0.2	7.6 ± 0.3	6.4 ± 1.8	6.1 ± 2.6	13.3 ± 1.1	48.4 ± 2.8
∑ *isoflavones*	ND	ND	ND	12.3	34.4	60.4	21.7	24.2	61.1
Flavan-3-ols									
Catechin	1.2 ± 0.2	2.3 ± 0.2	8.2 ± 1.8	13.0 ± 0.3	5.7 ± 0.4	6.2 ± 0.1	2.8 ± 0.3	3.8 ± 0.5	2.0 ± 0.1
Epicatechin	1.2 ± 0.2	5.4 ± 0.5	1.8 ± 0.3	13.2 ± 0.6	5.6 ± 0.2	3.8 ± 0.8	5.9 ± 0.2	3.8 ± 0.2	5.5 ± 0.2
Epicatechin gallate	2.3 ± 0.2	ND	ND	2.4 ± 0.3	ND	ND	ND	2.4 ± 1.2	6.5 ± 2.3
Epigallocatechin gallate	3.0 ± 0.8	7.3 ± 1.1	36.1 ± 8.2	4.0 ± 0.4	8.0 ± 0.1	8.9 ± 0.1	ND	4.6 ± 0.8	8.1 ± 0.2
∑ *flavan-3-ols*	*7.7*	*15.1*	*46.1*	*32.6*	*19.2*	*19.0*	*8.7*	*14.6*	*22.1*
Proanthocyanidins									
Procyanidin A2	ND	ND	ND	5.4 ± 2	6.1 ± 2.1	2.8 ± 0.8	10.3 ± 1.8	14.0 ± 2.1	63.1 ± 9
Procyanidin B1	5.0 ± 0.8	12.5 ± 2.1	25.1 ± 5.7	9.5 ± 0.4	11.6 ± 0.5	14.6 ± 1.1	14.8 ± 3.4	8.9 ± 0.8	23.4
Procyanidin B2	0.9 ± 0.2	1.6 ± 0.2	3.0 ± 1	2.1 ± 0.2	1.5 ± 0.3	1.4 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	2.0 ± 0.1
∑ *proanthocyanidins*	*5.9*	*14.1*	*28.1*	*17.0*	*19.2*	*18.8*	*25.8*	*24.0*	*88.5*
Flavonols									
Myricetin	ND	ND	ND	11.1 ± 2.1	19.4 ± 3	8.2 ±1	12.6 ± 1.8	51.5 ± 4	63.4 ± 6.1
Rutin	1.3 ± 0.2	6.2 ± 0.8	4.0 ± 0.5	1.4 ± 0.2	11.1 ± 1.2	20.6 ± 2.1	5.8 ± 1	4.3 ± 1	16.4 ± 2.2
Quercetin 3-glucoside	1.3 ± 0.1	4.9 ± 1.5	1.1 ± 0.4	ND	15.2 ± 2.9	12.8 ± 2.4	19.4 ± 1.8	5.8 ± 0.8	13.4 ± 1.6
Kaempferol 3-glucoside	ND	5.3 ± 1.7	2.0 ± 0.8	ND	5.7 ± 1.5	3.3 ± 0.3	8.4 ± 0.8	ND	4.7 ± 0.5
Isorhamnetin	ND	ND	ND	ND	4.6 ± 1.7	3.2 ± 1.3	6.7 ± 1.6	3.3 ± 0.3	6.9 ± 1
∑ *flavonols*	*2.6*	*16.4*	*7.1*	*12.5*	*56.1*	*48.0*	*52.9*	*64.8*	*104.8*
Anthocyanins									
Cyanidin 3,5-diglucoside	ND	ND	ND	ND	ND	6.6 ± 2.3	ND	ND	20.5 ± 3.6
Pelargonidin 3,5-diglucoside	ND	ND	ND	ND	ND	ND	ND	ND	101.6 ± 12
Delphinidin 3-glucoside	ND	ND	ND	9.5 ± 2.8	1.1 ± 0.2	1.3 ± 0.2	9.8 ± 1	6.6 ± 0.5	104.5 ± 8
Cyanidin 3-glucoside	ND	ND	ND	23.9 ± 5	92.6 ± 11	196.0 ± 23	37.8 ± 5	151 ± 25	223.8 ± 32
Malvidin 3,5-diglucoside	ND	ND	ND	ND	ND	ND	54.9 ± 11	ND	126.5 ± 12
Pelargonidin 3-glucoside	ND	ND	ND	28.1 ± 5	3.2 ± 1	1.3 ± 0.5	28.7 ± 4	29.9 ± 5.2	186.8 ± 15
Peonidin 3-glucoside	ND	ND	ND	96.3 ± 18	115.6 ± 21	54.8 ± 5.2	40.6 ± 4.5	277.2 ± 32	119.4 ± 13
Malvidin 3-glucoside	ND	ND	ND	73.9 ± 11	4.4 ± 1.2	ND	44.5 ± 8	78.6 ± 9	120.2 ± 11
∑ *anthocyanins*	*ND*	*ND*	*ND*	*231.7*	*216.9*	*260.1*	*216.3*	*543.3*	*1003.5*
Total phenolics quantified	***53.4***	***229.4***	***111.9***	***414.1***	***457.7***	***573.7***	***565.2***	***763.5***	***1644.7***

ND = Not detected or < LOD.

Sweeties™ due to their lower levels of phenolic compounds and lack of anthocyanins. The positive PC2 grouped the 'BRS Melodia,' ARRA Crush Cherry™, and 'BRS Isis' grapes by the highest values of Naringenin, trans-resveratrol, quercetin 3-glucoside, and rutin. In negative PC2, Candy Snaps was separated because it had high levels of malvidin 3-glucoside, epicatechin gallate, and procyanidin B2. The 'BRS Vitória' grape generally had the highest levels and diversity of bioactive phenolic compounds.

In the present study, several patented and registered seedless grape cultivars were characterized for the first time, with the exception of the Brazilian grape 'BRS Vitória,' which is already recognized for its high content of bioactive phenolic compounds, especially anthocyanins, including 3,5-diglucosides (Colombo et al., 2020), corroborating the results of the present study. In this study, mainly red and black grapes

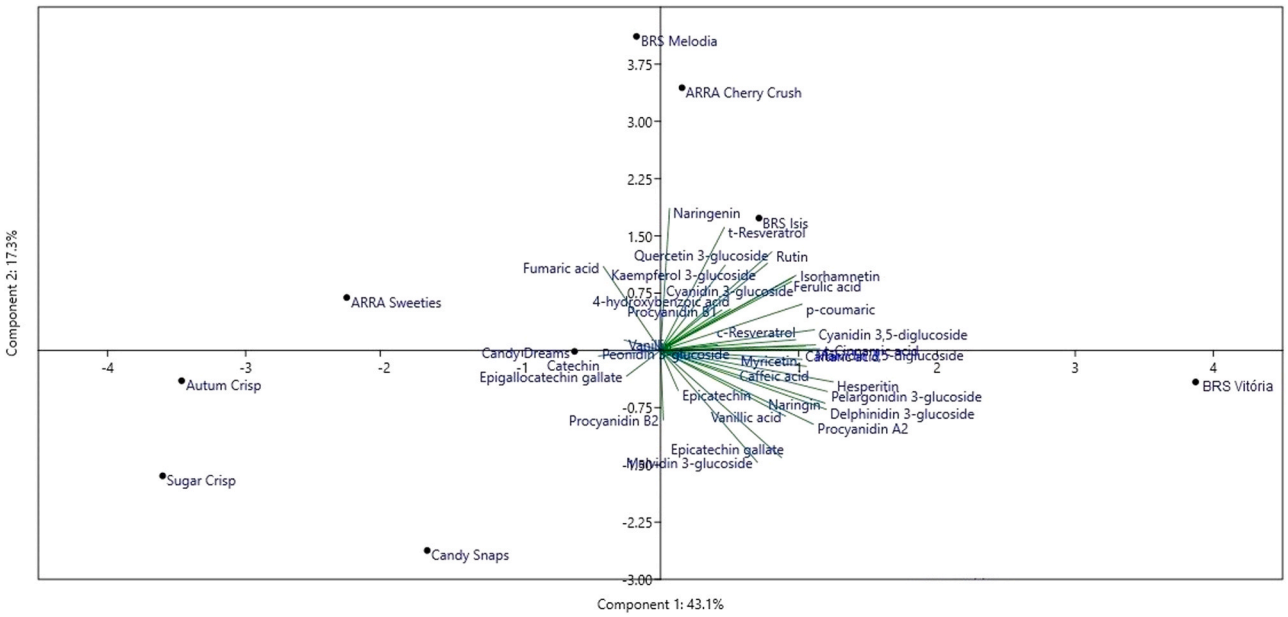

Fig. 4. Principal component analysis between patented seedless grape cultivars and the phenolic profile obtained using the validated method in this study.

had a high content of bioactive phenolics, reinforcing the role of grapes as potential functional foods. In the study by Pascoal et al. (2022) patented black seedless table grape cultivars Sweet Jubilee™ and Sweet Sapphire™, also originating from the São Francisco Valley-Brazil, were characterized and were noted for their high content of delphinidin 3-glucoside (12.2–19.8 mg/kg), cyanidin 3-glucoside (6.3–10.9 mg/kg), peonidin 3-glucoside (7.7–37.7 mg/kg), petunidin 3-glucoside (ND-22 mg/kg) and malvidin 3-glucoside (27.6–144.6 mg/kg). These same anthocyanins were highlighted in the patented Tinco™ and Krissy™ table grapes grown in Chile, where the main ones in terms of quantity were peonidin 3-glucoside (120–210 mg/kg) and malvidin 3-glucoside (39–88 mg/kg) (Neira et al., 2023). The phenolic richness of the grapes in this study corroborates that mentioned by Izcara et al. (2021) for table grapes grown in Spain such as Scarlet, Beauty seedless, Marroo seedless, Corinthe Noir, Flame seedless, and Blush seedless, where considerable levels of catechin, procyanidin B_2, epicatechin, quercetin 3-glucoside, 4-hydroxybenzoic acid, vanillic acid, caftaric acid and rutin were found.

The growth in the cultivation of new grape cultivars developed by genetic improvement programs, patented or registered by public and private companies, is a phenomenon that has grown not only in Brazil's São Francisco Valley. In Chile, one of the main exporters of table grapes, traditional cultivars such as Crimson Seedless, Red Globe, Flame, Sugraone, Thompson and Autumn Royal have been replaced by 30 patented cultivars such as Sweet Celebration™, Timco™, Magenta™, Scarlotta™, Arra15™, Allison™, Pristine™, Sable™, Maylen™ and Krissy™ (Neira et al., 2023). In addition to patented cultivars already being grown in Spain, there has also been investment in breeding programs to obtain new hybrid cultivars (Izcara et al., 2021).

4. Conclusions

An ultra-fast method for determining phenolic compounds in HPLC-DAD using a Rapid Resolution High Throughput column (RP-C_{18} 4.6 x 50 mm diameter, 1.8 μm particles) was developed and validated. The method can separate 41 phenolics in 20 min in a resolution equivalent to UPLC-DAD with high sensitivity, selectivity and precision. The validated method identified phenolics that characterize seedless table grape cultivars, eight of which were characterized for the first time. Cultivars such as ARRA Cherry Crush™, Candy Snaps™, and Candy Dreams™ stood out for their high content of mono glucoside anthocyanins. Brazilian grapes 'BRS Vitória,' 'BRS Isis,' and 'BRS Melodia' stood out for their high anthocyanin content and the exclusive presence of 3,5-diglucoside anthocyanins. Findings highlight the high content of phenolics in the red and black ones.

CRediT authorship contribution statement

Tatiana Colombo Pimentel: Writing – original draft, Visualization. **Marciane Magnani:** Writing – original draft, Investigation, Conceptualization. **Ana Júlia de Brito Araújo Carvalho:** Visualization, Formal analysis. **Giuliano Elias Pereira:** Resources. **Bruno Silva Dantas:** Investigation, Formal analysis. **Marcos dos Santos Lima:** Writing – original draft, Project administration, Methodology, Investigation, Conceptualization.

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 the paper: A novel method for ultra-fast determination of phenolics with performance comparable to UPLC/DAD: method development and validation on analysis of seedless table grapes, submitted to Journal of Food Composition and Analysis.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2024.106511.

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