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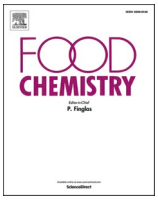

A robust method for quantifying 42 phenolic compounds by RP-HPLC/DAD: Columns performance and characterization of Brazilian *Citrus* peels

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ABSTRACT

Reliable analytical methods are the basis for the elucidation of phenolic compounds in foods. This study aimed to optimize and validate a method for determining 42 phenolics using reverse-phase (RP) high-performance liquid chromatography (HPLC) coupled to diode-array-detector-DAD. The performance of two RP columns was evaluated. The 150x4.6 mm 3-μm column showed superior separation quality, whereas 35 of the 42 phenolics showed a separation resolution ≥1.5. The method's linearity, precision (coefficient variation< 3.09%), recovery (87.5-103.2%), specificity, limits of detection (0.04-0.25 mg/L), and quantification (0.06-0.25 mg/L) had acceptable ranges. Thirty phenolics were quantified in *Citrus* peels, mainly flavanones, flavanols, flavonols, and phenolic acids, highlighting the high values of hesperidin (535-35070 mg/kg) and naringin (26-36466 mg/kg). Lemon peels named 'Lisboa,' 'Thaiti,' 'Thaiti-2000', and 'Thaiti-2001' presented the main phenolics associated with antioxidant capacity. The presented method was robust for determining 42 phenolic compounds, offering a new approach for bioactive compound quantification in food matrices.

1. Introduction

The increasing demand for quantifying compounds responsible for the bioactive potential and antioxidant capacity of foods and beverages has driven the search for suitable analytical methods for simultaneously determining different classes of phenolic compounds (Granato et al., 2018). The phenolic compounds are mainly determined using high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC), employing UV/Vis detectors by diode array (DAD), fluorescence detection (FD) and mass spectrometers (MS) (Padilha et al., 2017; Nicácio et al., 2021; Urbstaite et al., 2022; Karunarathna et al., 2023). In HPLC, the quality of the separation of compounds is a key factor in analytical reliability, and it depends on different factors, including the column type (Mizzi et al., 2020; USP, 2022).

The methods for the simultaneous determination of different classes of phenolics classically use 18-carbon silica (C18) columns in reverse phase (RP) for separation, with classic dimensions (250 x 4.6 mm) or rapid resolution (≤ 150 x 4.6 or 2.1 mm), and the type poroshell/coreshell (Barbosa et al., 2020; Manns & Mansfield, 2012; Padilha et al., 2017; Sanches et al., 2022). The correct choose of the appropriate column for the desired separation is the basis of the development and optimization of a trustable method (Blumberg, 2020).

Previously validated methods for quantification of phenolics in different matrices (by HPLC or UPLC) allowed the determination of 15 to 41 compounds among the classes of flavanols, flavonols, flavanones, phenolic acids, anthocyanins, and stilbenes (Manns & Mansfield, 2012; Natividade et al., 2013; Bae et al., 2015; Seraglio et al., 2016; Barbosa et al., 2020; Sanches et al., 2022; Dos Santos Lima et al., 2024). In most cases, columns of rapid resolution were used because they produce

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separation in shorter running times and use smaller solvent flows. However, they may present resolution limitations when involving many substances in the quantification (Manns & Mansfield, 2012; Mizzi et al., 2020; Urbstaite et al., 2022). In this way, comparative evaluation of the performance of both classic and rapid-resolution columns is important. The development and optimization of chromatographic methods requires, in addition to the classic validation parameters, the evaluation of the quality of the separation. Also, parameters such as resolution, efficiency (number of theoretical plates), and asymmetry should be determined (Ravisankar et al., 2019; Blumberg, 2020; USP, 2022).

Validation protocols must include complexes matrices to ensure the robustness in separation (Dos Santos Lima et al., 2024). *Citrus* peels, including peels of oranges, lemons, pomelos, and tangerines, are rich in several flavanones, flavonols, and phenolic acids, such as hesperidin, naringin, naringenin, and quercetin (Aznar et al., 2022; Czech et al., 2021; Gargouri et al., 2017; Hunlun et al., 2017). Therefore, they are a rich and complex raw material suitable for evaluating the robustness and separation capacity of a chromatographic method.

Brazil is one of the world's largest producers of *Citrus*, mainly for obtaining juices from a great diversity of cultivars of oranges, pomelos, tangelos (mandarin x pomelo hybrids), lemons, and tangerines (Coelho et al., 2021). This generates a large amount of waste, such as peels, which has not yet been characterized and may be a sustainable source of phenolic compounds.

The present study aimed to optimize a method for simultaneously determining 42 phenolic compounds by RP-HPLC/DAD by evaluating the performance of different columns (one classic and one rapid resolution). Parameters of linearity, precision, recovery, selectivity, and limits of detection, and quantification validated the optimized method. Peels from 20 new Brazilian *Citrus* cultivars were characterized for the first time to verify the method's robustness.

2. Material and methods

2.1. External standards for HPLC and reagents

Standards of malvidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucoside, pelargonidin 3-glucoside, peonidin 3-glucoside, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin-gallate, (-)-epicatechin gallate, procyanidin A_2, procyanidin B_1, procyanidin B_2, kaempferol 3-glucoside, quercetin 3-glucoside, rutin, myricetin, and isorhamnetin were from Extrasynthese (Genay, France).

Caffeic acid, 3,4-dihydroxybenzoic acid, vanillic acid, gallic acid, 4-hydroxybenzoic acid, vanillin, *O*-vanillin, (-)-epigallocatechin, ρ-coumaric acid, chlorogenic acid, caftaric acid, quercetin hydrate, syringic acid, ferulic acid, fumaric acid, hesperidin, naringenin, hesperitin, naringin, cyanidin-3,5-diglucoside, malvidin-3,5-diglucoside, and pelargonidin-3,5-diglucoside were from Sigma-Aldrich. *t*-resveratrol and *c*-resveratrol were obtained from Cayman Chemical Company (Michigan, USA).

HPLC grade methanol and acetonitrile, phosphoric acid, and ethanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). TPTZ (2,3,5-Triphenyltetrazolium chloride), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2-azino-bis (3-ethylbenzthiazoline-6 sulfonic acid) (ABTS), ferric chloride hexahydrate, and hydrogen peroxide, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide and potassium phosphate monobasic were purchased from Merck (Darmstadt, Germany).

2.2. Brazilian Citrus samples and preparation of peel extracts

Brazilian *Citrus* fruits, 20 new cultivars, were collected from the Experimental Station of Embrapa Semiárido, in the São Francisco Valley, Brazil, situated at latitude 09°08′S and longitude 40°18′W. All fruits were harvested in the commercial maturity stage based on size uniformity and peel color. Cultivars included 3 lemons of the varieties' Fino Cravo', 'Lisboa' and 'Siciliano' (*Citrus limon* B.); 3 lemons of the varieties' Thaiti 2000', 'Thaiti 2001' and 'Thaiti' (*Citrus latifolia*); 7 oranges cv. 'Pera D09', 'Pera D12', 'Pera C21', 'Natal 112', 'Bahia', 'Baianinha' and 'Cara-cara' (*Citrus sinensis* O.); 3 tangerines cv. 'Piemonte', 'Kinnow' and 'Murcott' (*Citrus reticulata*); 2 pomelos cv. 'Henderson' and ' Flame' (*Citrus maxima*); and 2 hybrids cv. 'Minneola' and 'Page' (*Citrus reticulata* x *Citrus maxima*). The morphometric characteristics, color, and composition of the fruits were previously published by Coelho et al. (2021).

The fruits were peeled manually (10 units per cultivar), separating the peel from the albedo. The peels were dried in an oven with forced air circulation at 55 ± 1 °C until constant weight. The dry material was crushed and sieved to obtain a powder with a particle size ≤ 20 mesh, packaged in vacuum-aluminized polyethylene bags, and kept in a freezer at -25 ± 1 °C until extraction. The extracts were obtained by macerating the peel powder 1:20 m/v in 80% ethanol in an ultrasound bath (sonicated at 40 kHz/60 min, 45 °C (UNIQUE model USC-1400A, SP, Brazil)), according to the procedures described by Dadwal et al. (2021). After maceration, centrifugation was carried out at 3000 g for 10 min, and the supernatant was collected and filtered through a 0.45 μm membrane for subsequent HPLC analysis (free phenolics) and antioxidant capacity assessment. Extractions were performed in triplicate.

2.3. Instrumentation and columns

The analyses were carried out using an Agilent 1260 Infinity LC System liquid chromatograph (Santa Clara – USA) equipped with a quaternary solvent pump and degassing system (model G1311C), thermostated compartment for columns model G1316A, automatic sampler model G1329B and diode array detector – DAD model G1315D. Data were collected and processed using the OpenLAB CDS ChemStation Edition software (Agilent Technologies, Santa Clara – USA). The columns used were Gemini NX RP-C18 150 x 4.6 mm with porosity of 3 μm (Phenomenex, Torrance, USA) and Zorbax Eclipse Plus RP-C18 250 x 4.6 mm with porosity of 5 μm (Zorbax, SC, USA), both classified as L1 (Octadecyl silane chemically bonded to porous or non-porous silica or ceramic microparticles) by the United States Pharmacopeia-USP (USP, 2022). In this study, they are called columns A and B, respectively.

2.4. Optimization, performance, and validation of the method

The optimization of the present study came from adaptations to the gradient of the method validated by Padilha et al. (2017) to determine 15 phenolic compounds by RP-HPLC/DAD, which used an RP-C18 100 x 4.6 mm 3.5 μm rapid-resolution column. The present work evaluated two columns: RP-C18 250 x 4.6 mm 5 μm (classic column) and RP-C18 150 x 4.6 mm 3 μm (rapid resolution). A new gradient was obtained, maintaining phases A (0.52% H_3PO_4 solution) and B (methanol + 0.52% H_3PO_4) of the original method. The study of gradient modification was based on the separation capacity of the mixture of the 42 available external standards based on the classic column (250 x 4.6 mm 5 μm). The new gradient was adapted to the rapid-resolution column (150 x 4.6 mm 3 μm). Separation quality parameters compared the two columns.

Validation consisted of studying the performance of the method, using the resolution parameters, determination of the number of theoretical plates, and asymmetry to evaluate the efficiency of the method (USP, 2022). In addition to the quality of the separation, recovery, linearity, precision, selectivity/specificity, and limits of detection and quantification were also evaluated, as established by the Guidelines for Standard Method Performance Requirements of the Association of Official Analytical Chemists (AOAC, 2016).

2.4.1. Separation efficiency: resolution, number of theoretical plates, and asymmetry

The methods used to obtain resolution, theoretical plates, and asymmetry followed the United States Pharmacopeia-USP protocol

(USP, 2022). All calculations of these parameters were obtained automatically with the OpenLAB CDS 3D UV software (Agilent Technologies, SC, USA) (Figure S1).

The number of theoretical plates (N) was obtained using Equation 1.

$$N = 5.54\left(\frac{tR}{Wh}\right)^2 \quad (1)$$

where: tR = retention time of the peak corresponding to the component and Wh = peak width at half-height (h/2).

The resolution (Rs) was obtained using Equation 2.

$$Rs = \frac{1.18(tR2 - tR1)}{Wh1 + Wh2} \quad (2)$$

where: $tR2$, $tR1$ = retention times of the peaks and $Wh1$, $Wh2$ = peak widths at half-height.

The asymmetry factor (As) was obtained using Equation 3.

$$As = \frac{W0.05}{2d} \quad (3)$$

where: $W_{0.05}$ = width of the peak at one-twentieth of the peak height, and d = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

2.4.2. Linearity, limits of detection (LOD), and limits of quantification (LOQ)

The linearity was obtained through the calibration with external standards (2.1 section) using five concentration points (n=5). The calibration curve was obtained using the least squares method by correlating the peak height with known concentrations of the standards. The LOD and LOQ values were obtained according to Hubaux and Vos (1970). Initially, three *Citrus* peel extracts were spiked with external phenolics standards in triplicate (n=9) and diluted to reach values close to the estimated LOD. All samples were analyzed, and an analytical curve was obtained by plotting the values generated from the analysis (Axis X) *vs.* the sample theoretical values (Axis Y). The slope values of the curve, intercept, and correlation coefficients were obtained. The residual standard deviation (RSD) was also calculated, and the LOD and LOQ limits were 3 and 10 times greater than the RSD value.

2.4.3. Recovery, precision, and specificity

Precision was evaluated by the coefficient of variation (CV%) obtained from six independent injections (n=6) in three different concentrations, using *Citrus* peel extract samples spiked with external phenolics standards. Recovery values were calculated by comparing the amount obtained for the spiked sample with the amount of the non-spiked samples. Specificity evaluation was performed according to the methodology by Padilha et al. (2017) using the spectral purity factor (*match factor*). Samples of *Citrus* extracts and phenolics external standards were injected to assess the method's specificity. The analyzed phenolic peaks were subjected to a threshold test, allowing for an acceptable factor $\geq$ 950.

2.5. Optimized method for determining phenolic compounds by RP HPLC/DAD

Column (A) method: The Gemini NX RP-C18 column (150 × 4.6 mm, 3 μm) guarded by a Zorbax C18 precolumn (12.6 × 4.6 mm, 5 μm) (Agilent Technologies, SC, USA) was used. The mobile phase consisted of a solution of 0.52% v/v phosphoric acid (phase A) and methanol acidified with 0.52 % v/v of phosphoric acid (phase B). The flow rate was 0.8 mL/min at 35 °C, and 20 μL of the sample/standard was injected. The gradient used was 0 min: 5 % B; 5 min: 23 % B; 14 min: 26 % B; 30 min: 50 % B; 33-34 min: 80% B; 34.1-36.6 min: 100% B; 36.7 min: 5% B; and 4.5 min post run.

Column (B) method: The Zorbax Eclipse Plus RP-C18 column (250 × 4.6 mm, 5 μm) guarded by a Zorbax C18 precolumn (12.6 × 4.6 mm, 5 μm) (Agilent Technologies, SC, USA) was used. The phases consisted of some solutions used in Column A. The Flow rate was 1.0 mL/min at 35 °C, and 20 μL was injected. The gradient used was 0 min: 5 % B; 5 min: 23 % B; 14 min: 26 % B; 30 min: 50 % B; 37-38 min: 80% B; 38.1-42.1 min: 100% B; 42.2 min: 5% B; and 5 min post run.

Compound detection was performed in DAD at 220, 280, 320, 360, and 520 nm. Phenolic identification and quantification were carried out by comparison with external standards (by retention time, spectrum similarity, and calibration curves). The UV spectra of the individual standards of the main phenolics used in identification/quantification in the present study are presented in Figure S2.

2.6. Determination of antioxidant capacity by ABTS, H_2O_2, and FRAP

The antioxidant capacity was evaluated by free radical scavenging with 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), ferric reducing antioxidant power (FRAP) and hydrogen peroxide scavenging activity (H_2O_2), following the adaptations described by Lima et al. (2022). Analytical standard Trolox was used to obtain the calibration curves for $ABTS^{\bullet+}$ and H_2O_2. For the FRAP method, ferrous sulfate was used. Results were expressed as Trolox equivalents per kg of peel (mM TE/kg) and millimole of Fe^{2+} per kg of peel (mM Fe^{2+}/kg). All absorbance lectures were performed using a UV-Vis 2000A spectrophotometer (Instrutherm, Brazil).

The $ABTS^{\bullet+}$ radical, formed by a 7 mmol ABTS solution + 140 mmol potassium persulfate reaction, was incubated at 25 °C without light incidence for 16 h. The radical was diluted in ethanol (0.70 ± 0.05 at 734 nm). A 300 μL aliquot of the extract was transferred to 2700 μL of the $ABTS^{\bullet+}$ radical, and 6 min after adding the extract, the readings were carried out (λ=734 nm).

The FRAP reactant was prepared in 300 mmol/L acetate buffer (pH=3.6), (2,4,6-tris (2-pyridyl)-s-triazine)-TPTZ 10 mmol/L in a solution of HCl 40 mmol/L and 20 mmol $FeCl_3$. Extract (90 μL) and 270 μL of water were mixed with 2.7 mL of FRAP reagent. They were subsequently mixed in a tube and incubated for 30 min in a thermoreactor block for tubes (Bioplus IT-2002 (Barueri, SP, Brazil)). The absorbance (λ=595 nm) was measured.

For the H_2O_2 method, a 40 mmol H_2O_2 solution was prepared in phosphate buffer pH=7.4. The extracts (400 μL) were mixed with 600 μL of H_2O_2 solution, and the final volume was adjusted to 3000 μL using the phosphate buffer pH=7.0. The UV absorbances were determined at 230 nm after 10 min of reaction. Phosphate buffer was taken as a blank sample.

2.7. Statistical analysis

The results of the characterization of *Citrus* peels were tabulated and expressed as average values. To differentiate cultivars and associate bioactive phenolics with antioxidant capacity, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were performed using the Past 4.03 program (University of Oslo, Oslo, Norway).

3. Results and discussion

3.1. Performance of the method with different columns

Table 1 presents the values obtained for the separation quality parameters. In the present work, resolution (Rs), number of theoretical plates (N), and peak asymmetry factor (As) were evaluated.

Resolution is the measurement of the distance between the peaks of two components, representing a total separation from the baseline, and according to the United States Pharmacopeia (USP, 2022), it must be $\geq$ 1.5. In other words, Rs values greater than 1.5 indicate a baseline between the peaks, and numbers less than 1.5 indicate some degree of co-

Table 1
Separation performance of the 42 phenolic compounds in the presented method using two different RP-C18 columns.

Phenolic compounds	**Peak number ID**	(A) RP-C18 150 x 4.6 mm, 3 μm				(B) RP-C18 250 x 4.6 mm, 5 μm			
		Retention time min.	Resolution	Plates number	Asymmetry factor	Retention time min.	Resolution	Plates number	Asymmetry factor
Phenolic acids									
Gallic acid	**2**	5.66	3.0	5098	1.45	6.43	3.7	14366	1.07
Syringic acid	**20**	13.94	1.1	58305	0.96	15.83	1.8	43950	1.07
p-Coumaric acid	**24**	18.28	6.0	29827	1.14	20.31	2.6	40813	1.06
Caffeic acid	**17**	12.78	1.7	37663	1.09	14.26	6.3	20573	1.10
trans-Caftaric acid	**6**	9.59	2.2	38890	1.03	10.24	3.6	29121	1.08
Chlorogenic acid	**12**	11.69	4.6	28768	1.01	12.29	7.9	18940	1.04
3,4-dihydroxybenzoic acid	**3**	7.97	2.9	27022	1.12	8.76	1.4	22451	0.86
Vanillic acid	**14**	12.42	2.2	48961	1.09	13.97	1.0	52243	0.80
4-hydroxybenzoic acid	**19**	13.65	2.7	55575	0.87	11.94	2.0	11114	0.80
trans-Cinnamic acid	**38**	30.07	4.3	193937	0.88	33.12	4.4	130666	1.06
Ferulic acid	**28**	20.36	2.9	56410	1.05	21.48	1.9	585031	0.94
Fumaric acid	**1**	4.75	4.5	5042	1.11	5.71	9.9	11736	1.10
Phenolic aldehydes									
orto-Vanillin	**25**	18.66	6.2	26217	1.03	21.7	1.4	103011	1.19
Vanillin	**21**	13.99	1.1	36950	1.06	16.17	6.5	11400	1.01
Stilbenes									
trans-Resveratrol	**35**	27.12	1.7	99064	0.95	28.10	1.7	55418	0.85
cis-Resveratrol	**37**	29.90	0.8	55210	0.98	31.71	1.2	239422	1.01
Flavanones									
Hesperidin	**32**	26.38	2.3	138766	1.04	25.26	3.1	88403	0.94
Hesperitin	**42**	34.07	2.9	291799	1.05	35.47	4.1	403393	0.97
Naringin	**31**	25.72	3.1	147654	1.02	27.49	6.8	145232	1.04
Naringenin	**40**	33.07	11.5	268442	1.07	34.49	4.5	210373	0.85
Flavanols									
(+)-Catechin	**8**	9.60	2.1	129986	0.86	10.37	1.0	119722	0.91
(-)-Epicatechin	**16**	12.81	1.5	36214	1.10	14.85	1.7	34985	1.05
(-)-Epigallocatechin	**5**	9.24	1.5	22532	1.16	10.02	2.0	73028	1.10
(-)-Epicatechin gallate	**27**	19.01	1.0	69377	0.91	20.1	1.3	15356	0.82
(-)-Epigallocatechin gallate	**15**	12.43	2.2	49409	1.09	13.09	1.7	48982	0.80
Procyanidin A2	**30**	24.77	1.0	28570	0.85	21.42	1.5	45753	0.81
Procyanidin B1	**4**	8.66	1.7	34358	1.00	8.76	1.4	31148	1.09
Procyanidin B2	**9**	10.52	1.9	27194	1.00	10.97	1.8	22118	1.10
Flavonols									
Quercetin 3-glucoside	**33**	26.91	1.4	98723	1.08	27.92	1.5	85806	1.03
Rutin	**34**	27.99	1.5	238790	1.09	28.49	1.5	101413	0.83
Kaempferol 3-glucoside	**36**	29.84	1.5	254789	1.04	30.93	1.9	168470	1.10
Isorhamnetin	**39**	30.24	1.7	301379	1.02	31.43	1.7	135680	1.07
Quercetin hydrate	**41**	33.45	2.1	128022	1.35	34.43	4.3	97064	1.28
Myricetin	**29**	23.27	2.8	87422	1.06	24.31	1.4	133046	0.97
Anthocyanins									
Cyanidin 3,5-diglucoside	**7**	9.85	19.9	8339	0.93	11.94	16.3	5971	0.98
Malvidin 3,5-diglucoside	**13**	12.86	1.3	7245	0.92	16.56	1.3	9216	1.08
Pelargonidin 3,5-diglucoside	**10**	11.10	2.5	9131	0.96	14.05	3.2	6584	0.97
Delphinidin 3-*O*-glucoside	**11**	12.12	2.1	12910	0.92	15.82	2.9	14321	1.10
Pelargonidin 3-*O*-glucoside	**22**	16.40	5.2	21437	0.99	21.55	6.1	46832	1.02
Cyanidin 3-*O*-glucoside	**18**	14.07	4.3	16095	0.98	18.99	3.1	24100	1.00
Peonidin 3-*O*-glucoside	**23**	18.59	4.5	13941	0.94	23.03	3.9	52271	0.98
Malvidin 3-*O*-glucoside	**26**	20.10	3.1	13339	0.96	24.01	2.2	35878	0.93

elution. However, the minimum acceptable value for quantification is Rs $\geq$ 1.0 (Ravisankar et al., 2019).

The chromatogram of the 42 phenolic compounds separating from column A (150 x 4.6 mm, 3 μm) and column B (250 x 4.6 mm, 5 μm) is shown in Figure 1. In the present optimized method, the resolutions obtained for column A were greater than 1.5 for most of the compounds studied (35 phenolics). The exceptions were for 7 compounds: cis-resveratrol (Rs = 0.8), epicatechin gallate and procyanidin A_2 (Rs = 1.0), syringic acid and vanillin (Rs = 1.1), malvidin 3,5-diglucoside (Rs = 1.3), and quercetin 3 -glucoside (Rs = 1.4). In column A, the only phenolic with critical separation (Rs < 1.0) was cis resveratrol, presenting a greater risk of coelution with trans-cinnamic acid in a matrix

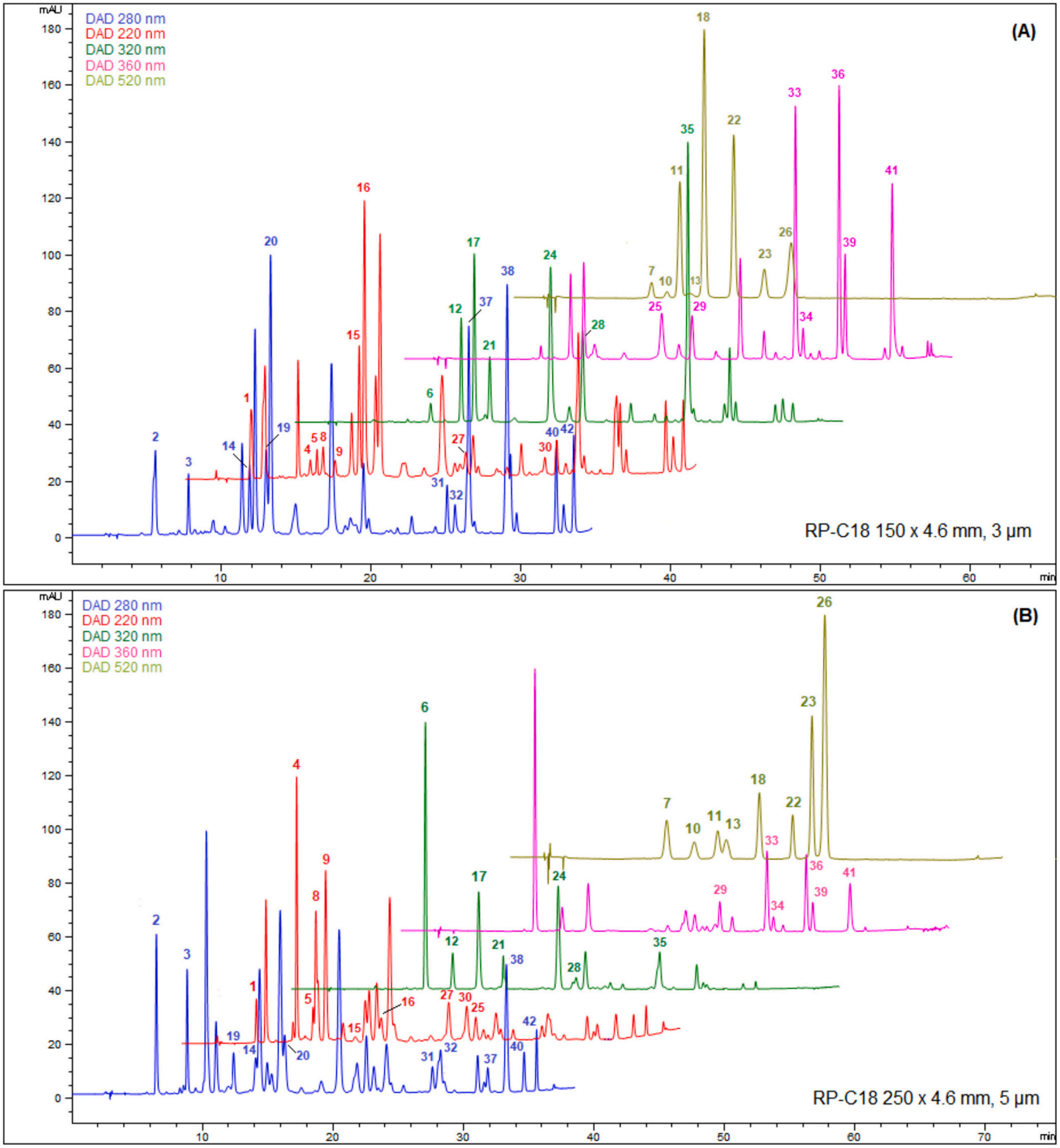

Figure 1. 3D chromatogram of the separation of the 42 phenolic compound external standards using different RP-C18 columns. **Legend:** 1= fumaric acid; 2=gallic acid; 3= 3,4-dihydroxybenzoic acid; 4= procyanidin B1; 5= epigallocatechin; 6= caftaric acid; 7= cyanidin 3,5-diglucoside; 8= catechin; 9= procyanidin B2; 10= pelargonidin-3,5 diglucoside; 11= delphinidin 3-glucoside; 12= chlorogenic acid; 13= malvidin 3,5-diglucoside; 14= vanillic acid; 15= epigallocatechin gallate; 16= epicatechin; 17= caffeic acid; 18= cyanidin 3-glucoside; 19= 4-hydroxybenzoic acid; 20= syringic acid; 21= vanillin; 22= pelargonidin 3-glucoside; 23= peonidin 3-glucoside; 24= p-coumaric acid; 25= orto-vanillin; 26= malvidin 3-glucoside; 27= epicatechin gallate; 28= ferulic acid; 29= myricetin; 30= procyanidin A2; 31= naringin; 32= hesperidin; 33=quercetin 3-glucoside; 34= rutin; 35= trans-resveratrol; 36= kaempferol 3-glucoside; 37= cis-resveratrol; 38= trans-cinnamic acid; 39= isorhamnetin; 40= naringenin; 41= quercetin hydrate; 42 hesperitin.

containing both substances.

For column B, resolutions lower than 1.5 were found for 9 compounds: catechin and vanillic acid (Rs =1.0), cis-resveratrol (Rs =1.2), epicatechin gallate and malvidin 3,5-diglucoside (Rs =1.3), and 3,4-dihydroxybenzoic acid, procyanidin B_1, O-vanillin and myricetin (Rs =1.4). However, column A's critical separation resolution of cis resveratrol improved from 0.8 to 1.2 with Column B.

Few studies validating methods for determining bioactive phenolic

compounds have used resolution as a quality parameter. In the work of Bae et al. (2015), in the validation of a method for the simultaneous determination of 15 phenolic compounds and caffeine in teas by RP-HPLC/UV using a column RP-C18 150 x 4.6 mm, 3 μm and acetonitrile as eluent, Rs values ranging from 1.5 to 17.25 were obtained. In the study by Manns and Mansfield (2012), for method validation using core-shell columns (RP-C18 4.6x100 mm, 2.6 μm, and RP-C18 2.1x100 mm 2.6 μm) in the separation of 16 phenolics; several critical resolutions were reported, and to obtain acceptable results, the column, type of solvent, gradient, and temperature were modified. These studies demonstrate that simultaneous determination methods for many compounds may present limitations in a single chromatographic condition. In the present study, a complex separation of 42 phenolics was performed, and we consider the resolutions obtained to be acceptable and with good robustness for most of the compounds used.

The number of theoretical plates (N) indicates efficiency and is used to compare the performance of different columns (Ravisankar et al., 2019). A column with a high N will have a narrower peak at a given retention time compared to a column with a lower N, helping to obtain better detection limits, a desired factor in method validation. In general, column A presented a greater quantity of phenolics with higher N values (27 compounds) when compared to column B, except for anthocyanins: malvidin 3,5-diglucoside, delphinidin 3-glucoside, pelargonidin 3-glucoside, cyanidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside. In addition to anthocyanins, column B also presented better N values for gallic acid, p-coumaric acid, vanillic acid, fumaric acid, O-vanillin, cis-resveratrol, hesperidin, epigallocatechin, and procyanidin A_2, totaling a better performance for 15 compounds.

According to the United States Pharmacopeia (USP, 2022), the asymmetry factor (tail factor) of the peak used in a quantification must be 0.8-1.8 in a test or assay. In the present study, Columns A and B presented satisfactory results, where all phenolics quantified presented *As* values ranging from 0.8 to 1.45.

The development of chromatographic methods is mainly focused on achieving optimal performance under experimental and instrumental conditions. Column selection is one of the most important aspects of this process (Godinho et al., 2020). The performance of the present method using two columns of different resolutions demonstrated that the optimized chromatographic condition was adequate to produce an acceptable separation for the determination of many phenolics in a single run. However, due to the resolution values and number of theoretical plates, in addition to using a lower solvent flow (0.8 mL/min) and total running time (40.1 min), it was decided to use column A (Gemini NX RP-C18 150 x 4.6 mm, 3 μm) to conduct the validation study and application in the characterization of the phenolic profile of Brazilian *Citrus* peels.

3.2. Method validation

3.2.1. LOD and LOQ

The results obtained for the validation parameters are presented in Table 2. A linear response between the maximum peak area or height, 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). The use of the area is more common but requires complete separation between peaks (resolution ≥ 1.5). When the complete separation do not occurs in all the peaks, height is the most suitable integration technique (Dos Santos Lima et al., 2024). On this basis, this method used the heights for quantification.

In the present study, due to some compounds not presenting complete separation resolution, calibration curves were obtained using the maximum height of the peaks (maximum absorbance) compared with the concentrations of external standards ($n = 5$ points). Linearity was assessed by r values ranging from 0.9988 to 1.0000 for all compounds evaluated. According to the AOAC Official Methods of Analysis analytical method validation guide (AOAC, 2016), *r* values must be ≥ 0.995 (or $R^2 > 0.99$) for calibration acceptance, and the results of the present study are in agreement with what is required.

Detection limits are important parameters of the quality of the method. They are mainly associated with the detector's sensitivity, the number of theoretical plates and the preparation technique used. The analytical method must be sensitive enough to quantify the analyte in the range of interest, and the lower the LOD and LOQ values, the greater the ability to quantify minority phenolics. In the present study, the values obtained for LOD ranged from 0.04 to 0.25 mg/L, and the LOQ values between 0.06 and 0.32 mg/L (Table 2). Other validated methods for determining phenolics with UV/Vis or DAD detection obtained: LOD 0.06-2.92 mg/L and LOQ 0.33-9.72 mg/L (Bae et al., 2015), LOD 0.04–0.85 mg/L and LOQ 0.04–1.41 mg/L (Padilha et al., 2017), LOD 0.01-0.14 mg/L and LOQ 0.05-0.71 mg/L (Herrera & Magariño, 2020), and LOD 0.18-1.01 and LOQ 0.54-3.06 (Urbstaite et al., 2022). In the study by Ahmed et al. (2023), a method for determining phenolics in *Citrus* juice was validated using various HPLC and UPLC configurations, with MS and DAD detections, and obtained LODs of 0.02 to 1.22 mg/L for DAD detection and 1.11 to 3.12 mg/L for MS detection. Based on these previous works, the LOD and LOQ of the present study can be considered adequate.

3.2.2. Recovery, precision, and specificity

According to the AOAC Official Methods of Analysis (AOAC, 2016), the maximum values for method precision depend on the analyte concentration range. According to the calibration ranges of the present study, the maximum should not exceed 7.3%. For recovery, values must be between 80 and 110% (AOAC, 2016).

Precision values in the present method ranged from 0.20 to 3.09% (Table 2). In the recovery parameter, the present method presented values ranging from 87.5% (gallic acid) to 103.2% (hesperidin), following what is recommended by the AOAC (2016), demonstrating that the present method has acceptable precision and recovery. For comparison purposes, other validated methods for determining phenolics in different matrices presented recovery values ranging from 85-118% (Herrera & Magariño, 2020), 95.3 to 103.8% (Reis et al., 2021), and 70 to 104.5% (Karunarathna et al., 2023).

The ideal condition in method development is for the quantified compounds to be confirmed in LC-MS. However, when a mass spectrometer is not available on the HPLC, confirmation of the compounds should be done by the retention time compared to the external standards by comparing the spectrum (UV/Vis) of the peak with the spectrum of the external standard, and by checking that the sample peak is pure, as co-elution is one of the main problems with liquid chromatography (Dos Santos Lima et al., 2024). In this study, in addition to retention times and spectral similarity for identification/confirmation (Figure S2), possible coelutions were assessed using the spectral purity factor (Figure S3).

Figure S3 presents the spectral purity test for the naringin peak quantified in a lemon peel extract. Among the 42 phenolic compounds evaluated in the present study, only 7 did not present a purity factor $\geq$ 950, indicating a possibility of co-elution during the analysis. They are syringic (863), O-vanillin (763), vanillin (714), cis-resveratrol (811), epicatechin (906), epigallocatechin gallate (853) and malvidin 3,5-diglucoside (840). In the method of Padilha et al. (2017), malvidin 3,5-diglucoside also showed a low spectral purity factor, indicating that it is an anthocyanin that is difficult to separate. The compounds that presented a low spectral purity value were generally the same ones that presented a resolution <1.5, and these results suggest that the spectral purity factor, together with the resolution parameter, complement each other in the study of specificity with detection in DAD.

The present method showed linearity, precision, recovery, and detection and quantification limits suitable for analytical purposes and good specificity for 35 of the 42 phenolics evaluated. This demonstrates that it is a robust method for quantifying these analytes.

Table 2
Validation parameters for determining phenolics in the present method using a RP-C18 150x4.6 mm column.

Phenolic compounds	Peak number ID	Detection λ (nm)	Range (mg/L)	Calibration curve	Correlation coefficient (*r*)	Recovery %	Spectral purity factor	Precision CV%	LOD (mg/L)	LOQ (mg/L)
Phenolic acids										
Gallic acid	2	280	0.55 - 17.5	Y= 10.44X + 0.68	0.9998	87.5	996	0.25	0.12	0.16
Syringic acid	20	280	0.55 - 17.5	Y= 13.30X – 0.15	0.9999	101.1	863	0.28	0.11	0.13
p-Coumaric acid	24	320	0.55 - 17.5	Y= 12.06X - 0.58	1.0000	99.1	956	0.24	0.07	0.10
Caffeic acid	17	320	0.55 - 17.5	Y= 12.19X - 0.29	1.0000	99.4	999	0.23	0.08	0.11
trans-Caftaric acid	6	320	0.55 - 17.5	Y= 4.76X + 0.253	0.9999	98.2	950	0.29	0.09	0.10
Chlorogenic acid	12	320	0.5 - 10	Y= 7.55X – 0.105	0.9999	95.6	999	0.21	0.08	0.09
3,4-dihydroxybenzoic acid	3	280	0.5 - 10	Y= 5.78X – 0.032	0.9999	98.1	998	0.22	0.08	0.10
Vanillic acid	14	280	0.5 - 10	Y= 5.08X – 0.08	0.9999	91.5	999	0.20	0.11	0.14
4-hydroxybenzoic acid	19	280	0.5 - 10	Y= 3.16X - 0.06	0.9999	96.7	981	0.21	0.10	0.11
trans-Cinnamic acid	38	320	05 - 10	Y= 16.34X - 0.31	1.0000	98.2	991	0.23	0.10	0.12
Ferulic acid	28	320	0.5 - 10	Y= 10.22X - 0.27	0.9999	96.7	977	0.29	0.09	0.10
Fumaric acid	1	220	0.5 - 10	Y= 17.52X + 3.31	0.9988	95.3	999	0.20	0.09	0.10
Phenolic aldehydes										
orto-Vanillin	25	360	0.5 - 10	Y = 1.39X – 0.15	0.9997	95.1	763	0.51	0.11	0.19
Vanillin	21	320	0.5 - 10	Y= 6.85X - 0.199	0.9999	93.5	714	0.22	0.11	0.12
Stilbenes										
trans-Resveratrol	35	320	0.55 – 17.5	Y= 18.12X - 0.24	0.9999	98.6	956	0.51	0.06	0.08
cis-Resveratrol	37	280	0.55 – 17.5	Y= 5.681X + 0.188	0.9998	99.2	811	0.48	0.12	0.25
Flavanones										
Hesperidin	32	280	0.5 - 20	Y= 2.15X + 0.09	0.9998	98.9	999	0.42	0.05	0.06
Hesperitin	42	280	0.5 - 10	Y= 7.33X + 0.41	0.9999	103.2	996	0.40	0.06	0.08
Naringin	31	280	0.5 - 10	Y= 3.45X - 0.05	0.9999	101.1	977	0.41	0.06	0.07
Naringenin	40	280	0.5 – 20	Y= 5.32X + 0.11	0.9999	99.6	996	0.36	0.09	0.10
Flavanols										
(+)-Catechin	8	220	0.55-17.5	Y= 17.28X - 0.64	1.0000	102.1	995	0.26	0.06	0.07
(-)-Epicatechin	16	220	0.5 - 20	Y= 17.273X + 0.593	0.9995	98.7	906	0.39	0.06	0.08
(-)-Epigallocatechin	5	220	0.5 - 10	Y= 40.64X + 1.12	0.9999	94.5	999	0.52	0.08	0.11
(-)-Epicatechin gallate	27	220	0.55 - 17.5	Y= 8.94X - 0.40	0.9999	99.8	853	0.40	0.06	0.07
(-)-Epigallocatechin gallate	15	220	0.55 - 17.5	Y= 41.44X + 1.02	0.9999	99.1	974	0.29	0.04	0.05
Procyanidin A2	30	220	0.5 - 20	Y= 7.97X – 1.71	0.9994	90.5	991	0.41	0.11	0.15
Procyanidin B1	4	220	0.31 - 10	Y= 37.953X - 1.980	0.9998	95.6	998	0.31	0.10	0.12
Procyanidin B2	9	220	0.31 - 10	Y= 18.57X – 1.252	0.9999	98.5	998	1.12	0.06	0.08
Flavonols										
Quercetin 3-glucoside	33	360	0.5 – 20	Y = 4.71X – 0.47	0.9996	95.4	977	0.55	0.09	0.14
Rutin	34	360	0.5 - 20	Y= 5.132X – 0.030	0.9992	97.5	985	0.74	0.08	0.17
Kaempferol 3-glucoside	36	360	0.5 – 20	Y= 2.23X - 0.036	0.9999	98.2	968	0.56	0.04	0.06
Isorhamnetin	39	360	0.5 – 20	Y= 4.73X - 0.53	0.9996	98.5	994	0.52	0.10	0.22

(*continued on next page*)

Table 2 (*continued*)

Phenolic compounds	Peak number ID	Detection λ (nm)	Range (mg/L)	Calibration curve	Correlation coefficient (*r*)	Recovery %	Spectral purity factor	Precision CV%	LOD (mg/L)	LOQ (mg/L)
Quercetin hydrate	41	360	0.5 – 10	Y = 4.81X – 0.68	0.9995	91.2	883	0.50	0.11	0.21
Myricetin	29	360	05 - 20	Y= 0.73X + 0.039	0.9997	95.8	959	0.48	0.10	0.25
Anthocyanins										
Cyanidin 3,5-diglucoside	7	520	0.55 - 17.5	Y= 2.539X - 0.007	1.0000	95.6	951	0.11	0.07	0.10
Malvidin 3,5-diglucoside	13	520	0.55 - 17.5	Y= 0.52X - 0.06	0.9985	95.8	840	3.09	0.16	0.24
Pelargonidin 3,5-diglucoside	10	520	0.55 - 17.5	Y= 1.195X - 0.03	0.9999	93.3	999	0.14	0.05	0.09
Delphinidin 3-*O*-glucoside	11	520	0.5 – 20	Y= 10.214X + 0.744	0.9995	98.5	920	1.12	0.12	0.15
Pelargonidin 3-*O*-glucoside	22	520	0.5 - 20	Y= 2.49X – 0.12	0.9999	98.2	974	0.96	0.09	0.11
Cyanidin 3-*O*-glucoside	18	520	0.5 – 20	Y= 3.74 – 0.257	0.9997	99.4	993	0.88	0.10	0.14
Peonidin 3-*O*-glucoside	23	520	0.55 - 17.5	Y= 5.57X - 0.001	1.0000	98.1	955	0.22	0.09	0.10
Malvidin 3-*O*-glucoside	26	520	2.74 - 87.5	Y= 2.11X - 0.05	1.0000	99.4	960	1.78	0.25	0.32

Legend: Y = peak height (mAU); X = Amount (mg/L). CV = coefficient of variation; LOD = limit of detection; LOQ = limit of quantification.

3.3. Determination of the phenolic profile of Brazilian Citrus peels and antioxidant capacity

The optimized method was used to characterize Brazilian *Citrus* peels, and the results obtained for oranges and tangerines are presented in Table 3. The results obtained for lemons, pomelos, and hybrids are presented in Table 4. All phenolic composition results are presented as mg per kg of peel powder (dry weight (dw)). Thirty phenolics were quantified in *Citrus* peels, including 11 phenolic acids, 8 flavanols, 5 flavonols, 4 flavanones, 1 stilbene (trans-resveratrol), and 1 phenolic aldehyde (vanillin). Supplementary Figure S4 shows a typical chromatogram of a citrus peel sample.

Among determined phenolics, the families with the highest values were flavanones> flavonols> flavanols> phenolic acids. *Citrus* samples did not detect the anthocyanin family, stilbene cis-resveratrol, and flavonol rutin. From the flavanone family, the main compounds, in terms of quantity, were hesperidin (535 to 35070 mg/kg) and naringin (26 to 36466 mg/kg). The main flavonols quantified were quercetin 3-glucoside (28.8 to 911.4 mg/kg), kaempferol 3-glucoside (95.5 to 1771.2 mg/kg) and myricetin (13 to 642.6 mg/kg). The main flavanol quantified was the procyanidin A_2 dimer (proanthocyanidin), in values ranging from 37.2 to 384.6 mg/kg. The main phenolic acids were trans-caftaric acid (18.6 to 208.2 mg/kg), caffeic acid (26.4 to 248.4 mg/kg), vanillic acid (19.2 to 157.8 mg/kg), ferulic acid (19.8 to 128.4 mg/kg) and chlorogenic acid (10.8 to 130.2 mg/kg). The total phenolics quantified by HPLC ranged from 4012.8 to 40873.6 mg/kg, corresponding to between 0.40 and 4.87% of the mass of the peel powder.

Antioxidant capacities were measured using ABTS, H_2O_2, and FRAP methods. The values obtained ranged from 35.58 to 66.48 mmol of Trolox per kg of powder (mmol TE/kg) for ABTS, from 2984.9 to 5377.3 mmol TE/kg for H_2O_2, and from 68.36 to 92.82 mmol of Fe^{2+} per kg of powder (mmol Fe^{2+}/kg) for FRAP. Techniques for measuring antioxidant capacity are essential for evaluating the antioxidant potential of foods, although methods with different mechanisms of action make data analysis difficult, requiring the evaluation to be carried out individually for each method (Lima et al., 2022). This is a common issue when using *in vitro* antioxidant methods based on different principles, analytical systems, reagent specificity, and sample interference in each system (Granato et al., 2018).

In the study by Aznar et al., 2022, the peels of Australian finger limes (*Citrus australasica* L.) were characterized using UPLC-MS, and 15 compounds were identified. The majority of compounds in the sample were pyrogallol, caffeic acid, coumarin, rutin, naringin, 2-coumaric acid, didymin, naringenin, and isorhamnetin, which were found in a range of 2.7 to 916.3 μg/g dw. Naringin was the main compound in quantity (916.3 μg/g). In this study, the antioxidant capacity measured by FRAP was 176.43 mg TE/g for the peel extract evaluated. In the study by Gargouri et al. (2017) peels of *Citrus limon* originating from Tunisia were characterized by RP-HPLC/MS and 38 flavonoids were quantified were the flavanones hesperetin 7-*O*-rutinoside (1234.7 mg/kg dw), eriodictiol 7-O-rutinoside (955.3 mg/kg dw), and hesperetin 7-*O*-neohesperidoside (950.8 mg/kg dw). In the study by Czech et al. (2021), 6 phenolic acids were analyzed in 8 Citrus cultivars from Turkey, including oranges, lemons, tangerines and pomelos, where the highest values were present in the peels, with emphasis on chlorogenic acid (20.9-75.7 mg/kg), p-coumaric acid (9.1-36.8 mg/kg) and ferulic acid (12.6-54.6 mg/kg). In the work of Barrales et al. (2018), the main flavanones were evaluated in orange peel extracts (cultivar not defined), where hesperidin values ranged from 7100 to 58000 mg/kg dw, followed by naringenin (118-1380 mg/kg dw), hesperetin (120-1370 mg/kg dw) and naringin (ND at 2500 mg/kg dw). In the study by Abad et al. (2012), an attempt was made to identify phenolic compounds (UPLC-MS) in Spanish Citrus (cultivars not defined), evaluating 9 orange cultivars, 7 tangerines, 4 lemons, and 5 pomelos, where the main families found were flavanones, flavonols, and phenolic acids. These studies on the characterization of *Citrus* peel extracts corroborate the phenolic profile obtained in the current study with the Brazilian *Citrus cultivars*. Therefore, the method has good specificity and is suitable for quantifying phenolics in complex matrices such as *Citrus* peels.

To differentiate *Citrus* cultivars in the present study, multivariate analyses of HCA and PCA were applied, as seen in Figure 2 and supplementary Figure S5, respectively. HCA formed 3 Clusters of *Citrus* cultivars, Cluster 1: the pomelos 'Flame,' 'Henderson,' the hybrid 'Mineolla,' the tangerine 'Piemonte,' and the orange 'Pera C21'; Cluster 2: 'the oranges' Pera D12', 'Pera D09', 'Natal 112', 'Bahia,' 'Baianinha' and 'Cara-Cara,' the tangerine 'Kinnow,' and the hybrid 'Page'; and Cluster 3: 'Thaiti', 'Thaiti 2000' and 'Thaiti 2001' lemons, and 'Murcott' mandarin (Figure S4). Therefore, the *Citrus* were grouped in different clusters according to the fruit type.

The PCA biplot explained 60% of the experiment's variance and presented details of the groups formed (Figure 2). Component 1 (PC1) explained 32% of the variance, and component 2 (PC2) explained 28%.

Table 3
Average values of phenolic compounds and antioxidant capacity in the peels of Brazilian oranges and tangerines cultivars.

Phenolic compounds mg/kg of powder	**Oranges**							**Tangerines**		
	'Pera D12'	'Pera C21'	'Pera D09'	'Natal 112'	'Baianinha'	'Bahia'	'Cara-Cara'	'Kinnow'	'Piemonte'	'Murcott'
Phenolic acids										
3,4-hydroxybenzoic acid	17.4	ND	24	17.4	13.2	ND	16.8	14.4	13.2	ND
Vannilic acid	33	33	41.4	158.4	35.4	21.6	60	133.2	43.8	90.6
4-hydroxibenzoic acid	67.2	ND	66	12	45	46.2	60	198	59.4	55.2
Syringic acid	ND	ND	ND	5.6	ND	ND	ND	ND	ND	ND
Fumaric acid	4.8	7.2	9.6	4	10.2	4.8	1.8	37.2	0	33.6
trans-Cinnamic acid	15.6	34.2	27.6	17.4	35.4	11.4	37.2	69.6	16.2	54
trans-Caftaric acid	61.2	58.8	70.2	45	79.2	69	54.6	208.2	42.6	191.4
Chlorogenic acid	10.8	16.8	48	53.4	10.2	31.2	54.6	45	44.4	36
Caffeic acid	182.4	51.6	191.4	38	140.4	78	108	117	41.4	45
p-Coumaric Acid	23.4	8.4	23.4	12.6	18.6	12.6	20.4	16.8	18	ND
Ferulic acid	46.2	22.8	51	26.4	51.6	45.6	66.6	82.8	37.8	47.4
∑ *Phenolic acids*	*262.8*	*99.6*	*313.8*	*377.6*	*220.8*	*167.4*	*249.6*	*261.6*	*141.6*	*128.4*
Stilbene										
trans-Resveratrol	32.4	24.6	78	10.2	58.2	12	41.4	42	ND	25.8
Phenolic aldehyde										
Vanillin	13.8	ND	19.2	11.4	ND	ND	13.8	21	16.8	ND
Flavanones										
Naringin	275.4	29.4	88.2	72	112.2	247.2	47.4	325.2	ND	76.2
Hesperidin	28026	22710	35070	28452	34260	26310	29004	25704	19554	2550
Naringenin	18	ND	16.8	134	31.8	55.2	11.4	21.6	40.8	10.2
Hesperitin	70.2	19.2	175.8	52	51.6	286.2	19.2	31.8	25.8	62.4
∑ *Flavanones*	*28389.6*	*22758.6*	*35350.8*	*28710*	*34455.6*	*26898.6*	*29082*	*26082.6*	*19620.6*	*2698.8*
Flavanols										
Catechin	44.4	40.8	22.2	42	22.2	73.2	66	81.6	66	94.2
Epicatechin	ND	16.2	ND	18	ND	ND	43.2	ND	7.2	40.2
Epicatechin gallate	ND	ND	10.2	8	10.8	16.2	ND	ND	ND	ND
Epigallocatechin	16.8	96	19.2	315	16.8	79.8	81.6	36.6	18.6	28.8
Epigallocatechin gallate	32.4	9	33.6	20	24.6	13.2	19.2	33.6	9.6	18.6
Procyanidin A2	236.4	272.4	315.6	232	319.2	214.2	281.4	384.6	43.2	271.8
Procyanidin B1	8.4	45.6	4.8	15	6.6	1.2	ND	34.8	ND	ND
Procyanidin B2	22.2	16.2	13.2	28	13.2	13.8	10.8	25.8	ND	24.6
∑ *Flavanols*	*360.6*	*496.2*	*418.8*	*678*	*413.4*	*411.6*	*502.2*	*597*	*144.6*	*478.2*
Flavonols										
Myricetin	201.6	439.2	735.6	620	642.6	618.6	583.8	348	122.4	212.4
Quercetin 3-Glucoside	803.4	666	29.4	80	26.4	120.6	911.4	21.6	555	77.4
Kaempferol 3-Glucoside	164.4	391.2	373.2	592	468.6	843	450	893.4	168.6	310.2
Isorhamnetin	19.8	21	ND	51	23.4	39.6	ND	21	ND	51.6
Quercetin hydrate	22.2	ND	ND	72	48.6	1150.2	42.6	69	ND	30
∑ *Flavonols*	*1211.4*	*1517.4*	*1138.2*	*1415*	*1209.6*	*2772*	*1987.8*	*1353*	*846*	*681.6*
Total Phenolics Quantified	**30270.6**	**24896.4**	**37318.8**	**30942.4**	**36357.6**	**30261.6**	**31876.8**	**28357.2**	**20769.6**	**4012.8**
Antioxidant capacity										
ABTS mmol Trolox/kg	51	66.48	51	46.26	47.28	66.18	43.8	55.26	36.06	36.36
H_2O_2 mmol Trolox/kg	3780.3	3493.9	4049.8	4215.4	1572.5	3881.4	3513	4303.9	3881.48	2984.9
FRAP mmol Fe^{2+}/kg	91.25	92.82	90.97	90.13	90.16	85.13	89.31	91.475	91.39	89.2

ND = Not detected or < LOD.

Defining the number of principal components does not depend exclusively on the variance explained. One of the criteria to be assessed is the eigenvalue for each component in the Scree Plot. Often, the insertion of the third component does not represent a considerable increase in eigenvalue for its use (as in this study: PC1+PC2 eigenvalue= 18.2 & PC3 eigenvalue= 3.68) (Sclove, 2021). The negative PC1 grouped the pomelos 'Flame' and 'Henderson,' the tangerine 'Piemonte,' the hybrid 'Mineolla,' and the oranges 'Natal 112', 'Pera D09' and 'Cara-Cara' for the highest values of the total of quantified phenolics, total flavanones, hesperidin, and 3,4-hydroxybenzoic acid, and strongly associated the total of quantified phenolics with hesperidin. The positive PC1 grouped the lemons 'Thaiti,' 'Thaiti 2000', 'Thaiti 2001', and 'Lisboa' by the highest values of naringenin, procyanidin B_1, procyanidin B_2, hesperetin, isorhamnetin, epicatechin gallate, vanillic acid, kaempferol 3-glucoside, ∑phenolic acids, ∑flavonols, ∑flavanols and antioxidant capacity by H_2O_2. Positive PC2 grouped the 'Henderson' and 'Flame' pomelos by the highest levels of syringic acid, epigallocatechin, and naringin.

Cultivars with higher quantified phenolic content, strongly influenced by hesperidin, were negatively correlated with antioxidant capacity (PC1<0). At the same time, the cultivars with higher levels of flavanols, flavonols, and phenolic acids (PC1>0), with highlights for procyanidins A_2, B_1 and B_2, kaempferol, isorhamnetin, trans-cinnamic acid, chlorogenic acid, and caffeic acid, were those with the highest antioxidant capacities. The inverse correlation between the highest values of total phenolics quantified (TFC) with antioxidant capacity can be explained by the high contribution of hesperidin in TFC, which is a compound with low antioxidant activity when compared to flavanols

Table 4
Average values of phenolic compounds and antioxidant capacity in the peels of Brazilian lemons, pomelos and hybrids.

Phenolic compounds mg/kg of powder	**Lemons**						**Pomelos**		**Hybrids**	
	'Fino Cravo'	'Lisboa'	'Siciliano'	'Thaiti'	'Thaiti 2000'	'Thaiti 2001'	'Flame'	'Henderson'	'Page'	'Minneola'
Phenolic acids										
3,4-hydroxybenzoic acid	16.2	ND	ND	12.6	ND	ND	17.4	17.4	22.8	26.4
Vannilic acid	62.4	114	87.6	126.6	146.4	19.2	82.2	157.8	31.8	45.6
4-hydroxibenzoic acid	42	44.4	28.8	73.8	31.2	29.4	39.6	ND	48.6	ND
Syringic acid	ND	ND	ND	ND	ND	ND	9	17.4	7.2	5.4
Fumaric acid	31.2	ND	ND	4.2	19.8	ND	18.6	ND	ND	ND
trans-Cinnamic acid	24	52.2	20.4	56.4	66.6	21	72	17.4	18.6	24
trans-Caftaric acid	41.4	52.8	18.6	113.4	67.8	28.8	47.4	24	108.6	53.4
Chlorogenic acid	55.2	130.2	79.8	41.4	45	72	55.2	53.4	102.6	49.2
Caffeic acid	120	108	43.2	248.4	261.6	26.4	64.8	97.8	73.2	37.2
p-Coumaric Acid	10.2	30.6	15.6	12.6	8.4	10.8	55.2	27.6	21	11.4
Ferulic acid	47.4	128.4	103.2	54	16.8	79.8	19.8	26.4	55.8	43.8
∑ *Phenolic acids*	*450*	*660.6*	*397.2*	*743.4*	*663.6*	*287.4*	*481.2*	*439.2*	*490.2*	*296.4*
Stilbene										
trans-Resveratrol	79.2	18	18.6	243.6	7.2	16.2	ND	10.2	9.6	8.4
Phenolic aldehyde										
Vanillin	12	ND	ND	13.2	12.6	ND	19.2	11.4	18	22.2
Flavanones										
Naringin	23.4	97.8	66.6	235.2	408	149.4	18714	38466	951	ND
Hesperidin	12756	3735	3918.6	1801.8	933	12768	7146	535.2	27873	21538.2
Naringenin	25.8	81	31.8	21.6	576	119.22	942	189.6	55.8	53.4
Hesperitin	54.6	91.2	34.8	777.6	948.6	379.2	13.2	21	22.8	30
∑ *Flavanones*	*12859.8*	*4005*	*4051.8*	*2836.2*	*2865.6*	*13415.82*	*26815.2*	*39211.8*	*28902.6*	*21621.6*
Flavanols										
Catechin	35.4	27	34.2	54	62.4	78	82.2	16.8	158.4	98.4
Epicatechin	22.8	ND	ND	ND	120.6	ND	53.4	55.2	ND	46.8
Epicatechin gallate	ND	12.6	ND	60.6	51	18	14.4	29.4	25.8	0
Epigallocatechin	42.6	57.6	42.6	62.4	81	54	186.6	315	18.6	25.8
Epigallocatechin gallate	20.4	18.6	7.8	42.6	ND	4.2	ND	36	13.8	12.6
Procyanidin A2	177	142.2	119.4	253.8	276	234.6	166.2	117.6	250.2	37.2
Procyanidin B1	ND	ND	ND	39.6	90	72.6	ND	10.2	7.2	ND
Procyanidin B2	ND	43.2	36.6	30	27	12	37.2	ND	16.2	ND
∑ *Flavanols*	*298.2*	*301.2*	*240.6*	*543*	*708*	*473.4*	*540*	*580.2*	*490.2*	*220.8*
Flavonols										
Myricetin	492.6	165	142.8	173.4	211.2	135	118.2	13	139.8	338.4
Quercetin 3-Glucoside	366.6	105.6	107.4	59.4	84	214.2	35.4	28.8	91.2	32.4
Kaempferol 3-Glucoside	282.6	1771.2	1095	261.6	729.6	1278	658.8	143.4	95.4	262.2
Isorhamnetin	ND	183	58.2	253.2	211.8	75.6	91.8	51	84	79.2
Quercetin hydrate	92.4	205.2	107.4	554.4	1140.6	1526.4	30	384.6	52.8	79.8
∑ *Flavonols*	*1234.2*	*2430*	*1510.8*	*1302*	*2377.2*	*3229.2*	*934.2*	*620.8*	*463.2*	*792*
Total Phenolics Quantified	**14933.4**	**7414.8**	**6219**	**5681.4**	**6634.2**	**17422.02**	**28789.8**	**40873.6**	**30373.8**	**22961.4**
Antioxidant capacity										
ABTS mmol Trolox/kg	35.58	54.18	37.2	48.3	53.46	45.24	42.24	43.92	41.58	44.40
H_2O_2 mmol Trolox/kg	3975.6	3955.6	3710.1	4683.6	5377.3	3804.3	3664	3433.1	4035.5	4118.3
FRAP mmol Fe^{2+}/kg	86.17	88.95	85.21	92.62	92.64	81.46	68.36	65.01	91.05	89.55

ND = Not detected or < LOD.

and flavonols (Tabart et al., 2009). The *Citrus* peels that presented the highest contents of flavanols, flavonols, and phenolic acids were those associated with the highest antioxidant capacities, such as the lemons 'Lisboa,' 'Thaiti,' 'Thaiti 2000', 'Thait 2001'.

4. Conclusions

The study of the performance of the columns demonstrated that the rapid resolution column RP-C18 150 x 4.6 mm and 3μm porosity was the most suitable for the proposed purpose, as it presented separation resolution ≥1.5 in 35 of the 42 phenolics, an acceptable asymmetry factor, and a greater number of theoretical plates for most of the compounds evaluated. In addition, it showed a shorter total running time (41.1 min) at lower solvent flow (0.8 mL/min). The optimized method presented linearity, precision, recovery, specificity, and detection and quantification limits suitable for the proposed purpose. The validated method allowed the quantification of 30 phenolics in the peels of 20 Brazilian Citrus cultivars, including 11 phenolic acids, 8 flavanols, 5 flavonols, 4 flavanones, 1 stilbene, and 1 phenolic aldehyde. The main compounds in *Citrus* peels, in terms of quantity, were the flavanones hesperidin and naringin, the flavonols quercetin 3-glucoside, kaempferol 3-glucoside and myricetin, the flavanol procyanidin A_2, and the phenolic acids trans-caftaric acid, caffeic acid, vanillic acid, ferulic acid, and chlorogenic acid. The lemons Lisboa', 'Thaiti,' 'Thaiti 2000', 'Thaiti 2001' presented the highest values of flavanols, flavonols, and phenolic acids and were associated with the highest antioxidant capacities by the ABTS,

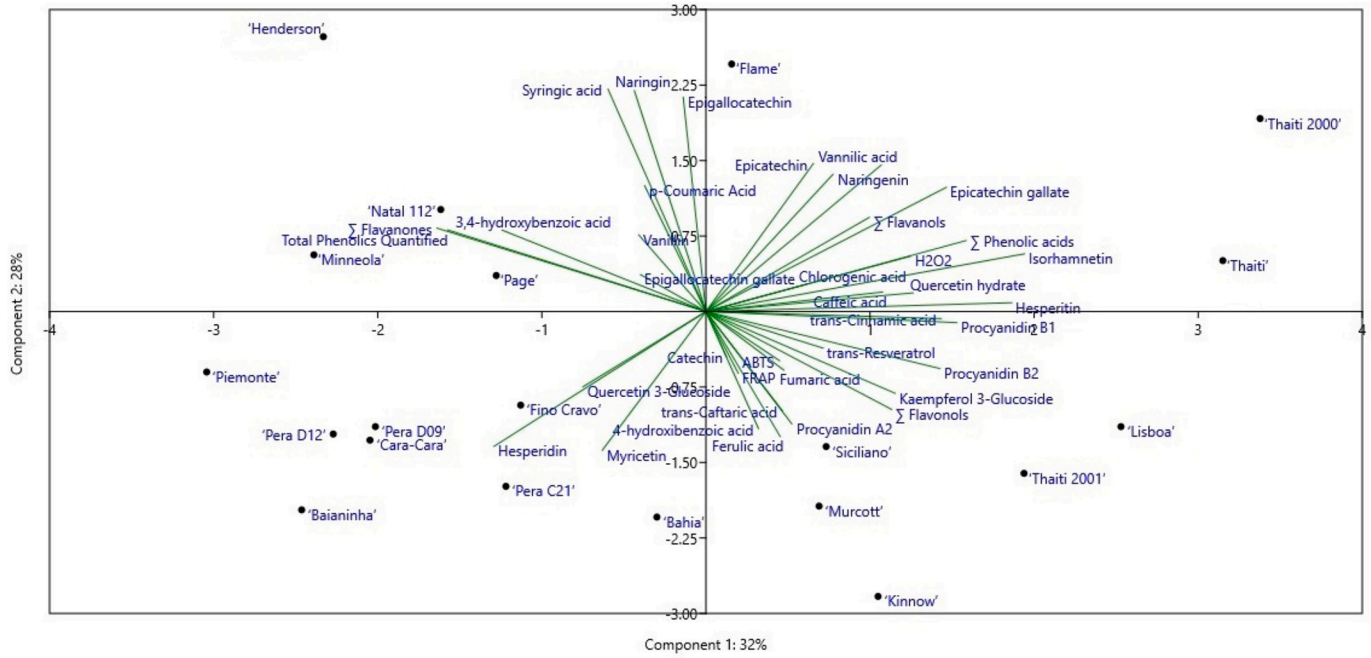

Figure 2. Principal component analysis of the phenolic compound's composition of *Citrus* peels.

H_2O_2 and FRAP methods. The presented method was robust for simultaneously determining 42 bioactive phenolic compounds in citrus peels. The characterization of bioactive phenolic compounds in the peels of 20 Brazilian citrus fruits was presented for the first time, making it possible to carry out future work on using this by-product in various applications.

CRediT authorship contribution statement

Marcos dos Santos Lima: Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Larissa Iris da Silva Monteiro:** Investigation, Formal analysis. **Ana Júlia de Brito Araújo Carvalho:** Visualization, Formal analysis. **Débora Costa Bastos:** Visualization, Resources. **Tatiana Colombo Pimentel:** Writing – original draft, Visualization, Investigation. **Marciane Magnani:** Writing – original draft, Methodology, Investigation.

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.2024.140807.

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