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Article

Determination of Phenolic Compounds in Red Sweet Pepper (*Capsicum annuum* L.) Using a Modified QuEChERS Method and UHPLC-MS/MS Analysis and Its Relation to Antioxidant Activity

Carina A. Rodrigues,[a] *Antonio E. Nicácio,*[a] *Isabel C. S. F. Jardim,*[b] *Jesuí V. Visentainer**,[a] and Liane Maldaner*[a]

[a]*Departamento de Química, Universidade Estadual de Maringá (UEM), Avenida Colombo, 5790, 87020-900 Maringá-PR, Brazil*

[b]*Instituto de Química, Universidade Estadual de Campinas (UNICAMP), CP 6154, 13083-970 Campinas-SP, Brazil*

In the present work, a fast, easy and efficient analytical method was developed and validated for the determination of phenolic compounds (4-hydroxybenzoic, vanillic, caffeic, *p*-coumaric, sinapic, ferulic and ellagic acids, and naringenin) in red sweet pepper. Extraction of phenolic compounds was carried out using the quick, easy, cheap, effective, rugged and safe (QuEChERS) method, followed by separation and detection using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). When the acetate version of the QuEChERS method was used, higher amounts of the phenolic compounds were extracted. In the dispersive solid phase extraction (d-SPE) clean-up step, combination of 50 mg of octadecylsilane (C18) and 7.5 mg of graphitized carbon black (GCB), resulted in the greatest removal of interferents, especially carotenoids, without significant retention of phenolic compounds. The recoveries for the proposed method were 82-103%, the limits of quantification were 2-150 μg kg^{-1}, and the precision values expressed in terms of relative standard deviation (RSD) were ≤ 15%. The method developed was successfully applied to the analysis of different red sweet pepper cultivars.

Keywords: QuEChERS method, sorbents, antioxidant activity, UHPLC-MS/MS, validation

Introduction

Fruits and vegetables are natural sources with a high content of antioxidant compounds, including vitamins, carotenoids, minerals and phenolic compounds, which have been correlated with beneficial health effects with regular dietary intakes.[1-3] Antioxidant compounds can prevent or delay the effects of free radicals (reactive oxygen and nitrogen species), which are constantly generated from cell metabolism, and can cause damage to proteins, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), sugars and lipids.[4-6]

Phenolic compounds are the largest group of antioxidant compounds, with more than 8,000 molecules already isolated and described in plants, including flavonoids and phenolic acids.[1,7,8] These compounds are associated with the prevention of numerous diseases, such as cancers, cardiovascular diseases, autoimmune diseases, brain dysfunction, cataracts, obesity, type 2 diabetes and age related oxidative problems.[1,3,7,9]

The Solanaceae plant family is one of the largest and most diverse plant families found throughout the world, but it is the most abundant and widely distributed in tropical and temperate regions.[10,11] The most economically important crops of this family include potato (*Solanum tuberosum*), tomato (*S. lycopersicum*), eggplant (*S. melongena*), tobacco (*Nicotiana tabacumn* and *N. rustica*) and pepper (*Capsicum* species).[10,12-14]

The genus *Capsicum* includes numerous species of sweet and hot peppers, with *Capsicum annuum* L. the world's most widely grown specie of the five domesticated species, which also includes *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L. and *C. pubescens*.[15,16] The different species in this genus are used as spices, vegetables, and/or as medicines, and recent studies have shown that they are also a rich source of bioactive compounds.[3,16,17] Several antioxidant compounds have been characterized in both sweet and hot peppers, such as carotenoids,[18,19]

*e-mail: jesuiv@gmail.com

vitamin C,[18,20] capsaicinoids,[20-22] fatty acids (linoleic acid, palmitic acid and α-linolenic acid),[23] tocopherols,[23] and especially phenolic compounds (such as flavonoids and phenolic acids).[19,20,22,24-26]

Over the years, different methods have been used to determine phenolic compounds in plant matrices. Several works[25,27-29] reported the quantification of these compounds as total phenolic, flavonoid and anthocyanin content, or as total antioxidant capacity. However, for individual identification and quantification, chromatographic techniques based on liquid chromatography coupled to ultraviolet-visible spectrophotometry (UV-Vis) and mass spectrometry detectors have been widely used.[25,30-32]

The extraction step also plays a crucial role in the individual identification and quantification of phenolic compounds. Currently, there is a trend in the use of sample preparation techniques that are easier to perform, faster, inexpensive and that promote clean-up of the sample extract.[33] The quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction method is a promising alternative for the extraction of phenolic compounds in complex matrices such as plant matrices. This method was first proposed by Anastassiades *et al*.[34] for the analysis of multi-residues of pesticides in fruits and vegetables. The QuEChERS method remains extensively used for this purpose since modifications can be introduced according to the characteristics of the analyte / matrix to be analyzed, i.e., addition of acetate or citrate in the extraction / partitioning step and the use of alternative sorbents such as octadecylsilane (C18) and graphitized carbon black (GCB) in the dispersive solid phase extraction (d-SPE) clean-up step. Furthermore, the QuEChERS extraction method has been shown to be effective for the analysis of other groups of compounds, including mycotoxins,[35] tocopherols, and sitosterols[36] in pomegranate, seed, and nuts, respectively. However, for the analysis of phenolic compounds in fruits and vegetables, the QuEChERS extraction method has not been widely applied.[26,37]

Thus, the aim of this study was to determine the levels of 4-hydroxybenzoic, caffeic, *p*-coumaric, ferulic, ellagic, sinapic and vanillic acids, and naringenin from four different cultivars of *Capsicum annuum* L. ("gaston", "pampa", "rialto" and "Italian sweet"). A sample preparation step based on a QuEChERS extraction method, followed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis, was optimized and validated. As a complementary study, the phenolic content previously determined during the clean-up step optimization was compared to the total phenolic content (TPC) and antioxidant activity measured by spectrophotometric and spectrofluorimetric methods (2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS$^{\bullet+}$) and oxygen radical absorbance capacity (ORAC)).

Experimental

Standards, solvents and reagents

Phenolic compound standards, 4-hydroxybenzoic acid (HBA), caffeic acid (CA), *p*-coumaric acid (PCA), ferulic acid (FA), ellagic acid (EA), and naringenin (NAR) were purchased from Sigma-Aldrich (Saint Louis, USA), and sinapic acid (SA) and vanillic acid (VA) were purchased from Fluka (Saint Louis, USA). All reference standards had purities greater than 95%. Stock standard solutions of the phenolic compounds were prepared in methanol at concentration levels of 1000 mg L^{-1} and stored at −18 °C. Working standard mixture solutions were prepared by appropriate dilution of the stock solutions in methanol.

The reagents used in the antioxidant methods and the total phenolic content analysis, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), DPPH$^{\bullet}$, ABTS$^{\bullet+}$, 2,2-azobis(2-methylpropanimidamide) dihydrochloride (AAPH), and Folin-Ciocalteu reagent, were supplied from Sigma-Aldrich (Saint Louis, USA). Fluorescein was obtained from Fluka (Saint Louis, USA).

High performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from J. T. Baker (Edo. de Mexico, Mexico). Ultrapure water was obtained from a Milli-Q ultrapure water purification system (Millipore, USA). Formic acid, used in the mobile phase, and acetic acid, used in the extraction procedure, were purchased from Sigma-Aldrich (Saint Louis, USA) and Fluka (Saint Louis, USA), respectively.

The salts used in this study were of analytical reagent grade. Disodium hydrogencitrate sesquihydrate ($C_6H_6Na_2O_7 \cdot 1.5H_2O$) was purchased from Alfa Aesar (Ward Hill, USA). Sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), anhydrous magnesium sulfate ($MgSO_4$) and anhydrous sodium acetate (CH_3COONa) were purchased from J. T. Baker (Edo. de Mexico, Mexico). Sodium chloride (NaCl) was purchased from Merck (Darmstadt, Germany). The sorbents, Bondesil primary secondary amine (PSA) (40 μm) and GCB, were purchased from Agilent Technologies (Santa Clara, USA). Octadecylsilane (C18) was purchased from Supelco (Bellefonte, USA). The fluorinated sorbent was produced and characterized by the Institute of Chemistry, from the University of Campinas (São Paulo, Brazil).

Samples

For this study, four different cultivars of red sweet peppers called "gaston", "pampa", "rialto" and "Italian sweet", all belonging to the species *C. annuum* L., were purchased from a local market in Maringá (23°25'S, 51°57'W), Paraná, Brazil. The QuEChERS method optimization and validation study was carried out with "gaston" cultivar, and the other cultivars were used in the method applicability.

Before analysis, the samples were washed in tap water, and the placenta along with the seeds was manually removed from the fruit. After separation of the parts, the fruits were crushed to form a paste, packed under vacuum and stored in a freezer at −18 ºC until analysis.

Optimization of the QuEChERS method

Optimization of the QuEChERS method for the extraction of phenolic compounds from red sweet pepper was based on the different versions of the QuEChERS method, including the original method described by Anastassiades *et al.*,[34] the acetate buffer method studied by Lehotay *et al.*[38] and the citrate buffer modification described by Anastassiades *et al.*[39] The extraction, partitioning and clean-up steps were evaluated. For all evaluated methods, 10.0 g of the sample were transferred to a 50 mL polypropylene centrifuge tube and extracted following the proposed methodologies (QuEChERS original, QuEChERS acetate and QuEChERS citrate). After addition of the partition salts, the tubes were shaken by vortex AP 56 (Phoenix, Araraquara, Brazil) for 1 min and immediately centrifuged for 10 min at 4529 × g in a Harrier 18/80R centrifuge (Sanyo MSE, London, UK). For optimization of the extraction/partition step, the different QuEChERS methods were evaluated using the same d-SPE clean-up step (25 mg of PSA).

After optimizing the extraction/partition step, the d-SPE clean-up step was also optimized. For this, different amounts of sorbent used individually or in combination were evaluated, as shown in Table 1.

For all clean-up procedures, the mixture was shaken for 1 min and centrifuged for 10 min at 4529 × g. Then, the supernatant was filtered through a polytetrafluoroethylene (PTFE) syringe filter (13 mm diameter and 0.22 µm pore) before UHPLC-MS/MS analysis. The same aliquot of the extract was used to determine the TPC and antioxidant activity (DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and ORAC methods) and to evaluate the clean-up efficiency from the spectrophotometric analysis.

Finally, the validated QuEChERS method for the extraction of phenolic compounds from red sweet pepper was as follows: 10.0 g of the sample was transferred to a 50 mL polypropylene centrifuge tube. Then 10.0 mL of 1% (v/v) acetic acid in acetonitrile was added, and the tubes were shaken for 1 min. Next, anhydrous $MgSO_4$ (4.0 g) and CH_3COONa (1.0 g) were added, and the tubes were shaken for 1 min and immediately centrifuged for 10 min at 4529 × g. For the clean-up step, 1.0 mL of the supernatant was transferred to a 15 mL polypropylene centrifuge tube containing 150 mg of anhydrous $MgSO_4$, 50 mg of C18 and 7.5 mg of GCB. The mixture was shaken for 1 min and centrifuged for 10 min at 4529 × g. Then, the supernatant was filtered through a PTFE syringe filter (13 mm diameter and 0.22 µm pore) before UHPLC-MS/MS analysis.

Table 1. Sorbents used in the d-SPE clean-up step

	Test	Sorbent / mg
QuEChERS acetate	T1	25 PSA
	T2	50 PSA
	T3	25 C18
	T4	50 C18
	T5	50 C18 + 7.5 GCB
	T6	50 C18 + 2.5 GCB
	T7	50 C18 + 10 F

QuEChERS: quick, easy, cheap, effective, rugged and safe; PSA: primary secondary amine; C18: octadecylsilane; GCB: graphitized carbon black; F: fluorinated sorbent.

Antioxidant activity methods and total phenolic content

DPPH$^{\bullet}$ scavenging method

The DPPH radical scavenging method was carried out according to Ma *et al.*[40] The extracts obtained after optimization of the clean-up step (25 µL) were added to 2 mL of 6.25×10^{-5} mol L^{-1} DPPH radical methanolic solution. The solutions were incubated in the dark for 30 min, and then the absorbance was measured at a wavelength of 517 nm in a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Madison, USA). Trolox standards (ranging from 10-2000 µmol L^{-1}) were used to prepare the analytical curve ($y = -0.0003x + 0.6656$, coefficient of determination (r^2) = 0.990), and the results were expressed as micromoles of Trolox equivalents *per* 100 g of fresh weight (µmol TE 100 g^{-1} FW).

ABTS$^{\bullet+}$ scavenging method

The ABTS radical scavenging method was carried out according to Rufino *et al.*[41] The ABTS cation radical solution was prepared by reacting 7 mmol L^{-1} of ABTS with 2.45 mmol L^{-1} of potassium persulfate at room temperature in the dark for 16 h. This solution was then diluted with

ethanol to obtain an absorbance of 0.700 ± 0.020 at 734 nm. Approximately 30 μL aliquots of the extracts obtained after the clean-up optimization step were added to 3 mL of the diluted $ABTS^{\bullet+}$ solution. The solutions were incubated in the dark for 6 min, and then the absorbance was measured in a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Madison, USA). Trolox standards (ranging from 10-1500 μmol L^{-1}) were used to prepare the analytical curve ($y = -0.0004x + 0.7671$, $r^2 = 0.998$), and the results were expressed as μmol TE 100 g^{-1} FW.

ORAC method (oxygen radical absorbance capacity)

Determination of the antioxidant activity from the ORAC method was performed according to Ou *et al.*[42] A fluorescein stock solution (1.03 mmol L^{-1}) was prepared in a phosphate buffer solution (dibasic potassium phosphate and monobasic potassium phosphate, 75 mmol L^{-1}, pH 7.4) and diluted to obtain the working solution (40 nmol L^{-1}). The AAPH working solution was prepared daily by adding 434 mg of AAPH to 10 mL of phosphate buffer (75 mmol L^{-1}) to obtain a final concentration of 161 mmol L^{-1}. Adequate dilution of extracts obtained after the clean-up step optimization were performed in the phosphate buffer solution and 25 μL of these solutions were transferred to a microplate with 96 wells and 150 μL of the fluorescein solution (40 nmol L^{-1}) was added. The microplate was heated at 37 °C for 5 min. Then, 25 μL of the AAPH working solution (161 mmol L^{-1}) was added and the fluorescence was recorded immediately, with 485 nm for the excitation wavelength and 535 nm for the emission wavelength. The fluorescence was monitored kinetically and recorded in 1 min for 30 min in a Victor™ X4 multimode plate reader (PerkinElmer, Waltham, USA). Trolox standard solutions (ranging from 0.5-10.0 μmol L^{-1}) were used to prepare the analytical curve ($y = 0.6059x + 2.4954$, $r^2 = 0.997$), and the concentration calculations were based on the area under the fluorescence curve. The results were expressed as μmol TE 100 g^{-1} FW.

Total phenolic content (TPC)

The total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent[43] as described by Shahidi and Naczk.[44] For this, the extracts obtained after optimization of the clean-up step (250 μL) were mixed with 250 μL of the Folin-Ciocalteu reagent (diluted in distilled water, 1:1 v:v). Then, 500 μL of saturated sodium carbonate solution and 4 mL of distilled water were added. The solutions were incubated in the dark for 25 min, centrifuged for 10 min at 4529 × g, and the absorbance was measured at 725 nm in a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Madison, USA). Gallic acid standard solutions (ranging from 10-200 mg L^{-1}) were used to prepare the analytical curve ($y = 0.0059x + 0.1806$, $r^2 = 0.991$). The results were expressed as milligrams of gallic acid equivalents *per* 100 g of fresh weight (mg GAE 100 g^{-1} FW).

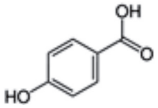
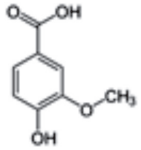
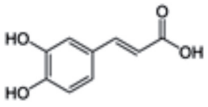
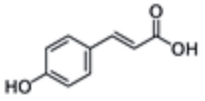
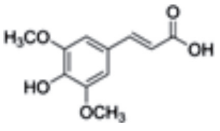
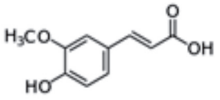
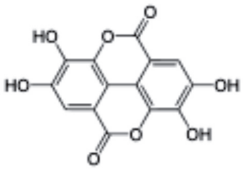
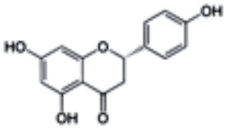
Clean-up efficiency evaluation by spectrophotometric analysis

Spectrophotometric analysis of the extracts obtained after each sorbent evaluation in d-SPE clean-up for the QuEChERS acetate method was carried out using a UV-Vis Cary 60 spectrophotometer (Agilent Technologies, Santa Clara, USA). The extracts were scanned in the wavelength range of 200-800 nm. Before scanning, 100 μL of each extract was diluted in 2 mL of acetonitrile and added to a quartz cuvette. Blank samples were recorded with acetonitrile.

UHPLC-MS/MS analysis

A UPLC Acquity H-CLASS coupled to a Xevo TQD triple-quadrupole mass spectrometer equipped with a Z spray™ ESI interface operating in both positive and negative mode (Waters, Milford, USA) was used to carry out the chromatographic analysis. Chromatographic separation was performed using a 1.7 μm Acquity UPLC® BEH C18 column (50 × 2.1 mm i.d.) from Waters at a flow rate of 0.150 mL min^{-1}. The column was kept at 30 ± 1 °C and the sample injection volume was 1.5 μL. The mobile phase was (A) H_2O (acidified with 0.1% formic acid) and (B) MeOH. Gradient elution was used and the organic solvent (MeOH) percentage was changed linearly as follows: 0-0.01 min (10% B), 1 min (30% B), 1.5 min (40% B), 2 min (50% B), 4-7 min (60% B), 7.5 min (50% B), 8 min (30% B), and 8.5-13 min (10% B). The mass spectrometer was operated using an electrospray (ESI) source in negative mode. ESI conditions were as follows: capillary voltage, 3.0 kV; extractor voltage, 3.0 V; source temperature, 130 °C; desolvation gas temperature, 550 °C; cone gas (nitrogen) flow of 50 L h^{-1} and desolvation gas (also nitrogen) flow of 700 L h^{-1}. Argon (99.9%) from White Martins (Rio de Janeiro, Brazil) was used as the collision gas at a constant pressure of 3.00×10^{-3} mbar. The mass spectrometer was operated in MS/MS mode using selected reaction monitoring (SRM). The most intense ion transition was selected for quantification and the second one for qualification. Specific MS/MS parameters for each phenolic compound are shown in Table 2. MassLynx and QuanLynx software version 4.1 (Waters) were used for instrument control and data processing.

Table 2. Chemical information, selected ion transitions and instrumental parameters for the phenolic compounds under study

Phenolic compound	Chemical structure	Chemical class	pK_a	t_R / min	ESI mode	SRM transition (m/z)[a]	Cone voltage / V	Collision energy / eV	Dwell time / s
4-Hydroxybenzoic acid		phenolic acid	4.6 9.5	4.04	–	136.8 > 92.9	25	15	0.05
Vanilic acid		phenolic acid	4.1 8.9	4.46	–	166.9 > 151.9 166.9 > 107.9	35 35	17 13	0.05
Caffeic acid		phenolic acid	4.4 8.3	4.46	–	179 > 135	38	20	0.05
p-Coumaric acid		phenolic acid	4.3 8.8	5.05	–	162.9 > 118.9 162.9 > 92.9	28 28	14 28	0.07
Sinapic acid		phenolic acid	4.5	5.10	–	222.9 > 193 222.9 > 148.9	36 36	21 24	0.07
Ferulic acid		phenolic acid	4.6 8.6	5.14	–	192.9 > 133.9 192.9 > 177.9	30 30	13 17	0.07
Ellagic acid		phenolic acid	6.3 11.2	5.50	–	301 > 229 301 > 284	60 60	31 28	0.07
Naringenin		flavonoid	6.7 9.1	6.70	–	271 > 150.9 271 > 118.9	36 36	17 32	0.07

[a]The first transition of each compound was used for quantification and the second one for qualification purposes. t_R: retention time; ESI: electrospray; SRM: selected reaction monitoring.

Method validation

Linearity, accuracy, inter and intra-day precision, limit of detection (LOD), limit of quantification (LOQ) and matrix effect (ME) were evaluated during the validation study according to the European SANTE/11945/2015 guidelines.[45] Linearity was evaluated using the standard addition method, analyzing red sweet pepper extracts at six levels of concentration (6-5400 μg kg^{-1}). In the same way, the endogenous amounts of the target phenolic compounds were also estimated. To evaluate the method accuracy, recovery studies were carried out at two concentration levels (determined for each phenolic compound after the estimation of the endogenous amount) by spiking five sample extracts at each concentration level. The quantification was performed using the standard addition method. The precision, intra-day and inter-day (evaluated on two consecutive days) were carried out at the same concentration levels of the recovery studies, and expressed in terms of relative standard deviation (RSD). LOD and LOQ were calculated as the quantity of analyte able to produce a chromatographic peak three and ten

times higher, respectively, than the noise of the baseline in a chromatogram of a non-fortified sample, after to estimate the endogenous amount. The ME values (%) for each phenolic compound were calculated (equation 1) by comparing the slopes of the solvent and standard addition (matrix) analytical curves obtained at the same concentration levels, as described by Kaczynski:[46]

$$\% \text{ ME} = \left(\frac{\text{slope matrix}}{\text{slope solvent}} - 1 \right) \times 100 \quad (1)$$

The matrix effect may be present as positive or negative values, indicating enhancement or suppression of the signals, respectively. Values of ME ≤ ±20% indicated no matrix effect, while ME values ≥ ±50% indicate a strong matrix effect.[47]

Application

Four different cultivars of red sweet peppers ("gaston", "pampa", "rialto" and "Italian sweet") were analyzed with the proposed method.

Statistical analysis

Data is presented as the mean and standard deviation (SD). Assistat® v. 7.7 software[48] was used for analysis of the Tukey's test ($p < 0.05$).

Results and Discussion

Optimization of the QuEChERS method for the extraction of phenolic compounds in red sweet pepper

Optimization of the extraction/partitioning steps

The optimization of the extraction/partitioning steps was carried out by comparison of extraction performance obtained with the three versions of the QuEChERS method (original, acetate and citrate). The original QuEChERS method consisted of the addition of the partitioning salts, anhydrous magnesium sulfate, and sodium chloride, followed by extraction of compounds with no pH correction (i.e., pH of the extraction medium varies according to the pH of the sample).[34] On the other hand, for the citrate and acetate QuEChERS methods, in addition to the partition salts, the pH of the extraction medium was adjusted to approximately 5.0-5.5 and 4.8, respectively.[38,39] For the citrate QuEChERS method, disodium hydrogencitrate sesquihydrate and sodium citrate tribasic dehydrate salts were added, and for the acetate QuEChERS method, the extraction solvent was acidified with acetic acid and sodium acetate salt was added.[38,39] Figure 1 presents the relation of the phenolic compounds extracted with the respective amounts (in means of peak area) for each QuEChERS method evaluated, keeping 25 mg of PSA as the d-SPE sorbent for all extractions.

In Figure 1, it can be observed that 4-hydroxybenzoic, vanillic, caffeic and ellagic acids were extracted only when the QuEChERS acetate method was employed. Also, naringenin, *p*-coumaric, ferulic and sinapic acids were extracted in higher amounts compared to those obtained with the original and citrate QuEChERS methods.

The best results obtained with the QuEChERS acetate method can be explained by the lower pH with this version (4.8) when compared to the QuEChERS citrate method (5.0-5.5) and to the QuEChERS original method in which the measured sample pH was approximately 5.15. Assuming that phenolic compounds are more stable at lower pH values since the phenol-phenolate equilibrium shifts toward the less polar phenol form, from the values of pK_a (Table 2), the acidification of the sample during extraction/partitioning steps promotes the extraction of the compounds to the organic phase (acetonitrile).[49,50]

Optimization of the d-SPE clean-up step

To obtain a final extract free of undesirable interfering compounds, the d-SPE clean-up step was optimized evaluating different sorbents used individually or in combination, as presented in Table 1.

Several sorbents have been reported as d-SPE clean-up sorbents in plant matrices, especially PSA, C18 and GCB.[51-57] Alternatively, sorbents such as oasis HLB®, chitosan and fluorinated sorbent (F) have already been used.[58-60] Among the commonly used sorbents cited above, PSA is used for removing sugars, fatty acids, organic acids, lipids and some polar pigments,[34,61] while C18 sorbent is used for removing nonpolar compounds, such as lipids and fatty acids, from fatty matrices (lipid content > 2%).[62] GCB, on the other hand, has a strong affinity for planar molecules and is used for removing some pigments (e.g., chlorophyll, carotenoids) and sterols.[34,63] The alternative sorbent, oasis HLB® polymeric sorbent, has a modified surface with divinylbenzene (nonpolar) and an *N*-vinylpyrrolidone (polar), which is used for the extraction of acidic, basic and neutral compounds with medium to high polarity.[64] Chitosan is a cationic polysaccharide that contains chemical reactive groups (hydroxyl, acetamido or amino functions) in polymer chains, which provides excellent selectivity with aromatic compounds and metals,[65] and the fluorinated sorbent displays unique selectivity of the specific properties of the C–F bonds that increase the interaction with polar compounds.[66]

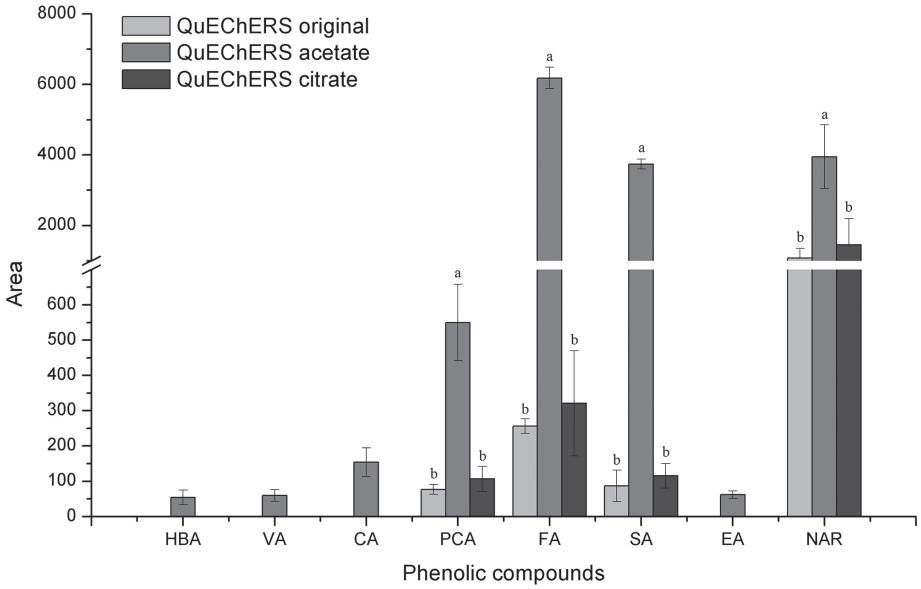


Figure 1. Evaluation of the extraction efficiency for each QuEChERS method evaluated (original, acetate and citrate), keeping 25 mg of PSA as the d-SPE sorbent for all extractions. Data given as the mean peak area ± the standard deviation (n = 3). HBA: 4-hydroxybenzoic acid; VA: vanillic acid; CA: caffeic acid; PCA: *p*-coumaric acid; FA: ferulic acid; SA: sinapic acid; EA: ellagic acid; NAR: naringenin. The QuEChERS methods followed by the same letter do not differ statistically from each other by the Tukey's test ($p < 0.05$).

Efficiency results for the d-SPE clean-up step (Table 1) for the QuEChERS acetate method are present in Figure 2. For most of the compounds, significantly lower amounts of the phenolic compounds were obtained when PSA was used as the clean-up sorbent (T1 and T2), especially when compared with results obtained with the C18 sorbent (T3 and T4). Based on the numerically higher amounts for all phenolic compounds when C18 sorbent (T4) was used, subsequent experiments were carried out by combining 50 mg of C18 with different amounts of GCB (T5 and T6) and F sorbent (T7). It can be noted that when compared to the T4 test, the amounts for all phenolic compounds were numerically lower, but with no significant difference for most of the compounds.

However, these results showed that both C18 sorbent alone (T4) and combined with GCB (T5 and T6) and F (T7) sorbents were more adequate as clean-up sorbents compared to PSA, but did not show the effectiveness

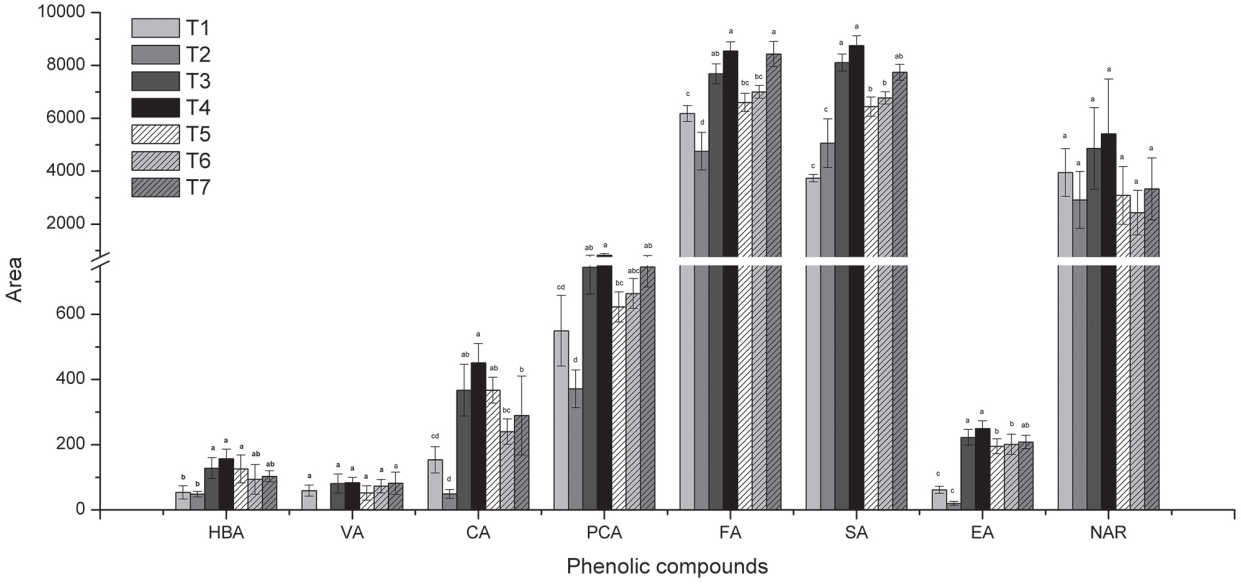


Figure 2. Evaluation of different sorbents used individually or in combination in the d-SPE clean-up step for the QuEChERS acetate method. Data given as the mean peak area ± the standard deviation (n = 3). HBA: 4-hydroxybenzoic acid; VA: vanillic acid; CA: caffeic acid; PCA: *p*-coumaric acid; FA: ferulic acid; SA: sinapic acid; EA: ellagic acid; NAR: naringenin. The clean-up followed by the same letter did not differ statistically from each other by the Tukey's test ($p < 0.05$).

on interferent removal. In this sense, the extracts of the red sweet pepper obtained after the following the clean-up steps (T4, T5, T6 and T7) were submitted to spectrophotometric analysis by scanning each extract in a wavelength range of 200-800 nm as presented in Figure 3. The analysis was carried out in this region because it includes the region of maximum absorption of the carotenoids,[67] which are the main interferents present in extracts of red sweet pepper.[68]

Comparing results obtained for extracts submitted to the clean-up step (T4, T5, T6 and T7) with the extract without a clean-up step (Figure 3), a decrease in extract absorption can be noted, especially in the region of maximum carotenoid absorption (410-510 nm).

Among the clean-up sorbents evaluated, C18 combined with GCB (T5 and T6) presented the best results since a significant decrease in extract absorption can be observed. The efficiency of C18 sorbent combined with GCB in the removal of interferences in red sweet pepper extracts can be explained by the power of GCB in the removal of pigments, such as carotenoids, and the power of C18 in the removal of interferences with nonpolar characteristics.

Thus, 50 mg of C18 with 7.5 mg GCB (T5) was chosen as the sorbent combination for the d-SPE clean-up step.

Evaluation of antioxidant activity and TPC before and after the clean-up step

As a complementary study, the composition of the phenolic compounds previously determined by the QuEChERS acetate method following the several clean-up sorbents evaluated and UHPLC-MS/MS analysis (Figure 2) was compared to the TPC and antioxidant activity of these extracts obtained by traditional methods (DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and ORAC).

According to Figure 4, it can be observed that in all clean-up tests evaluated, the extracts showed similar antioxidant activity values and TPC values, with an exception for clean-up tests in which PSA was used as the clean-up sorbent (T1 and T2) and the analysis was carried out by ABTS$^{\bullet+}$, ORAC and TPC methods. The lower antioxidant activity and TPC values presented by T1 and T2 extracts when analyzed by ABTS$^{\bullet+}$, ORAC and TPC methods, respectively, can be strongly correlated with the results showed in Figure 2 (i.e., the compounds were retained by the sorbent during the clean-up step). On the other hand, T1 and T2 extracts did not present the same behavior for the DPPH$^{\bullet}$ method. This fact can be associated with the presence of carotenoids that also absorb in the wavelength of maximum absorption of the DPPH radical (517 nm), causing interferences in the results.[69,70] For the other clean-up tests (T3, T4, T5, T6, T7), the same correlation was found between antioxidant activity values and the phenolic composition determined by the chromatographic analysis (i.e., higher numerical values of antioxidant activities were found, especially for extracts (T4 and T5), which also presented higher numerical values for phenolic composition). However, for the TPC analysis, no significant difference was found between the evaluated extracts.

In addition, the antioxidant activity and TPC analysis were carried out for an extract without a clean-up step as a way to verify if interferent removal in the clean-up step is occurring and, in the same way, if any removal of phenolic compounds also occurs. It can be observed that slightly higher values were obtained for the extract without

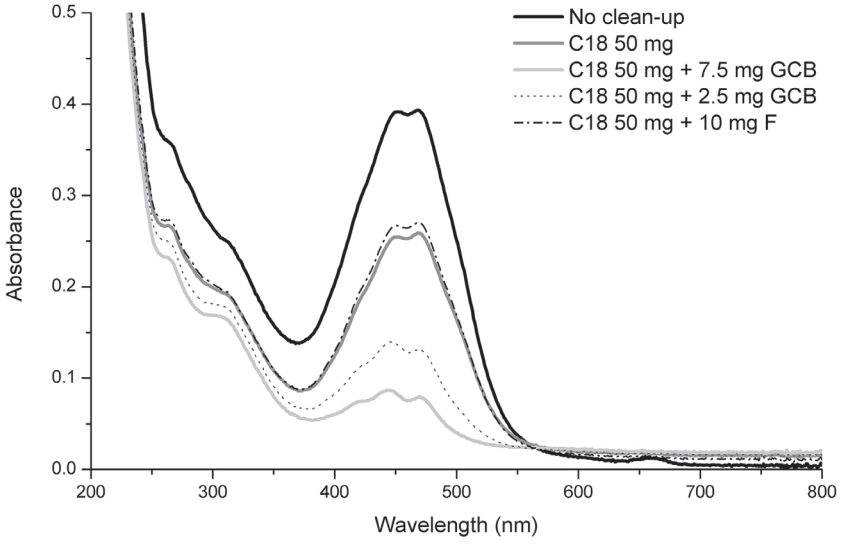


Figure 3. Spectrophotometric analysis of extracts obtained before and after each sorbent evaluated in the d-SPE clean-up for the QuEChERS acetate method.

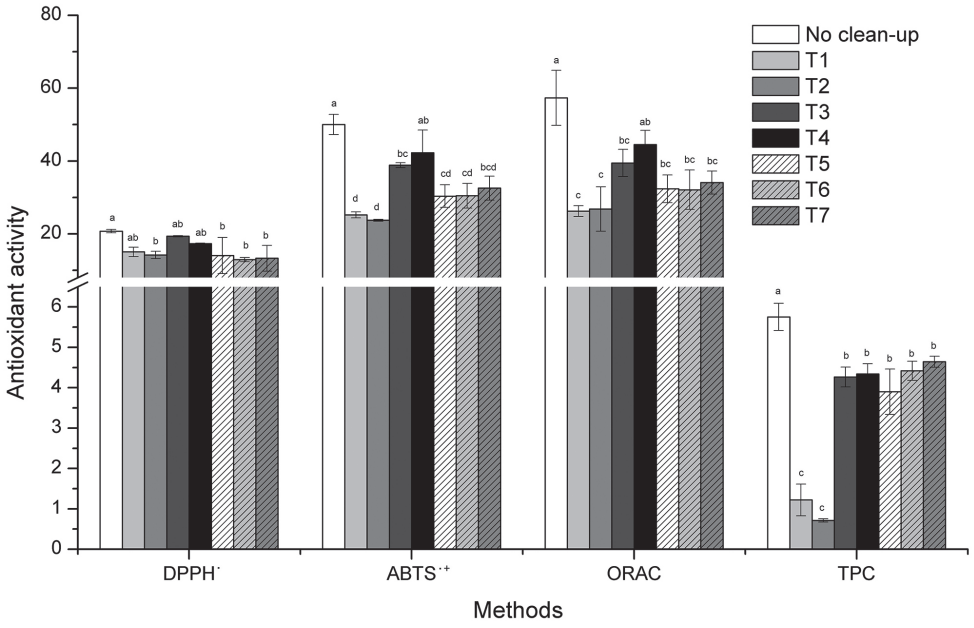


Figure 4. Antioxidant activity and TPC for extracts before and after the d-SPE clean-up step for the QuEChERS acetate method. Data given as the mean values ± the instrumental deviation (n = 3). Different letters in the same method represent statistical difference according to the Tukey's test ($p < 0.05$). TPC is expressed in mg AG 100 g^{-1} fresh weight. The radical DPPH, the cationic radical ABTS, and the ORAC method are expressed in μmol Trolox 100 g^{-1} of fresh weight.

a clean-up step when compared to T3, T4, T5, T6 and T7 extracts, suggesting that small quantities of the phenolic compounds may be retained in the clean-up sorbents and/or that other compounds (interferents) present in the extracts before the clean-up step may also contribute to the antioxidant activities and TPC values. These results suggest that the clean-up step did not compromise the determination of phenolic compounds in this study.

Method validation

The method developed was evaluated in terms of linearity, LOD, LOQ, accuracy, precision and matrix effects. Table 3 summarizes all data.

The standard addition method at six concentration levels (0.1-6.0 times the previously estimated endogenous amount) in a range of 6-5400 μg kg^{-1} was used to determine method linearity. As shown in Table 3, the method developed presented good linearity for all selected phenolic compounds with correlation coefficients higher than 0.99, and deviations of the individual points from the calibration curve lower than ± 20%, with exception for 4-hydroxybenzoic and caffeic acids which presented residue values slightly higher. The LOD and LOQ values ranged from 0.6-45.0 and 2-150 μg kg^{-1}, respectively, which is satisfactory for the quantification of all phenolic compounds found in the red sweet pepper samples analyzed.

Accuracy was determined by means of recovery studies obtained at two concentration levels (as described in the Experimental section) with five replicates at each of the levels. From Table 3, it can be verified that the method presented satisfactory recoveries for all phenolic compounds studied, which ranges from 82-103% at both concentration levels evaluated.

The precision of the method was calculated in terms of intra-day and inter-day precision and expressed in terms of RSD. The results are presented in Table 3 and show that RSD values were lower than 15% for all phenolic compounds in the study.

Medium to low matrix effect values were obtained for the phenolic compounds (Table 3). With an exception for *p*-coumaric acid, all the other phenolic compounds presented signal enhancement.

Application of the method

The method developed was applied to the determination of 4-hydroxybenzoic, vanillic, caffeic, *p*-coumaric, sinapic, ferulic, and ellagic acids, and naringenin in different red sweet pepper cultivars ("gaston", "rialto", "pampa" and "Italian sweet"). As shown in Table 4, all proposed phenolic compounds were found in the different cultivars of red sweet pepper at concentration levels above the LOQ; however, the amount found for each phenolic compound was significantly different between the cultivars.

Sinapic and ferulic acids (2025 and 1420 μg kg^{-1}) were found in higher concentrations for "gaston" cultivar, ellagic acid was found in higher concentration levels for "rialto"

Table 3. Analytical performance of the developed method

Phenolic compound	Linear range / (μg kg^{-1})	Linear regression			LOD[b] / (μg kg^{-1})	LOQ[c] / (μg kg^{-1})	Recovery[d] / %		Inter-day precision[e] (RSD) / %		Matrix effect / %
		$y = ax + b$	r[a]	Residual / %			F1	F2	F1	F2	
4-Hydroxybenzoic acid	8-50	$y = 16.911x + 296.49$	0.998	−10 to +22	2	8	100 (9)[d,e]	100 (7)[d,e]	10	2	+1
Vanillic acid	25-150	$y = 1.9676x + 83.048$	0.996	−18 to +17	6	20	99 (7)[d,e]	97 (12)[d,e]	9	10	+26
Caffeic acid	7-70	$y = 4.7552x + 176.4$	0.993	−8 to +22	0.9	3	99 (10)[d,e]	98 (3)[d,e]	10	12	+36
p-Coumaric acid	6-96	$y = 25.883x + 757.64$	0.999	−16 to +17	0.6	2	101 (6)[d,e]	100 (4)[d,e]	8	4	−10
Sinapic acid	180-5400	$y = 3.2934x + 4361.2$	0.999	−15 to +10	3	11	102 (6)[d,e]	96 (7)[d,e]	6	6	+18
Ferulic acid	120-3600	$y = 6.1806x + 5806.1$	0.999	−6 to +20	2	7	99 (5)[d,e]	95 (6)[d,e]	6	5	+1
Ellagic acid	190-3040	$y = 0.5656x + 340.03$	0.997	−18 to +5	45	150	97 (4)[d,e]	82 (3)[d,e]	6	4	+5
Naringenin	20-320	$y = 25.224x + 1781$	0.999	−11 to +6	1.5	5	98 (10)[d,e]	103 (9)[d,e]	12	15	+4

[a]Correlation coefficient; [b]LOD: limit of detection; [c]LOQ: limit of quantification; [d]recovery and precision values (intra-and inter-day) obtained analyzing five replicates at each concentration (n = 5); [e]intra-day values expressed as relative standard deviation (RSD) are given in parentheses. F1: 4-hydroxybenzoic acid (10 μg kg^{-1}), vanillic acid (25 μg kg^{-1}), caffeic acid (14 μg kg^{-1}), *p*-coumaric acid (12 μg kg^{-1}), sinapic acid (1800 μg kg^{-1}), ferulic acid (1200 μg kg^{-1}), ellagic acid (380 μg kg^{-1}) and naringenin (40 μg kg^{-1}); F2: 4-hydroxybenzoic acid (20 μg kg^{-1}), vanillic acid (50 μg kg^{-1}), caffeic acid (28 μg kg^{-1}), *p*-coumaric acid (24 μg kg^{-1}), sinapic acid (3600 μg kg^{-1}), ferulic acid (2400 μg kg^{-1}), ellagic acid (760 μg kg^{-1}) and naringenin (80 μg kg^{-1}).

Table 4. Phenolic composition of the different red sweet pepper cultivars

Phenolic compound	Phenolic composition / (μg kg^{-1})			
	"Gaston"	"Rialto"	"Pampa"	"Italian sweet"
4-Hydroxybenzoic acid	21 ± 2	37 ± 3	33 ± 8	18 ± 2
Vanillic acid	67 ± 8	45 ± 2	47 ± 3	149 ± 9
Caffeic acid	38 ± 5	54 ± 2	56 ± 8	63 ± 9
p-Coumaric acid	41 ± 7	63 ± 1	69 ± 6	14 ± 2
Sinapic acid	2025 ± 137	181 ± 6	554 ± 77	662 ± 48
Ferulic acid	1420 ± 89	404 ± 26	802 ± 33	2661 ± 252
Ellagic acid	896 ± 82	2434 ± 129	1566 ± 54	1256 ± 122
Naringenin	73 ± 17	526 ± 100	590 ± 157	968 ± 97

Data given as mean peak area ± standard deviation (n = 3).

(2434 μg kg^{-1}) and "pampa" (1566 μg kg^{-1}) cultivars, and ferulic and ellagic acids (2661 and 1256 μg kg^{-1}) were found in higher concentrations for "Italian sweet" cultivar. The quantitative differences between the cultivars analyzed may be associated with genetic, climatic, and pre- and post-harvest conditions.[68]

The quantitative results for the phenolic composition from different cultivars of red sweet peppers complement the data found with respect to the phenolic composition of the *C. annuum* L. species. Several published works,[19,20,22,26,71] indicate high concentrations of flavonoids, and the present study also highlights the presence of high concentrations of phenolic acids.

Conclusions

The QuEChERS acetate method extracted the highest amounts of phenolic compounds when compared to the other QuEChERS versions evaluated (original and citrate). These results can be associated with the lower pH presented by this version (4.8), in which phenolic compounds are more stable. The d-SPE clean-up step was also optimized, evaluating several sorbents (PSA, C18, GCB and fluorinated) individually or in combination. Based on the chromatographic analysis and spectrophotometric analysis of the extracts obtained before and after the d-SPE clean-up step, the condition that resulted in the greatest

removal of interferents without significant retention of the phenolic compounds was obtained with the use of 50 mg of C18 combined with 7.5 mg of GCB.

Antioxidant activity and TPC results obtained for the extracts before and after the d-SPE clean-up step showed a correlation with the chromatographic analysis, also suggesting that the clean-up step did not compromise the determination of the phenolic compounds in this study.

The method developed allowed for the determination of eight phenolic compounds in red sweet pepper at concentration levels of 2-150 μg kg^{-1}. Recoveries (in the range of 82-103%) and precision values (≤ 15%) were obtained for all phenolic compounds. All of the target phenolic compounds were found in the four cultivars of the sweet red pepper evaluated, but in significantly different concentration levels. For all cultivars, sinapic, ferulic, and ellagic acids, and naringenin were found at higher concentrations.

Based on the results, the QuEChERS method was fast, easy, and efficient for the determination of phenolic compounds in red sweet peppers, being a promising method for the analysis of these compounds in fruits and vegetables.

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