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Phenolic Profile and Hydrophilic Antioxidant Capacity as Chemotaxonomic Markers of Tomato Varieties

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ABSTRACT: Tomatoes (*Solanum lycopersicum* L.), the second most important vegetable crop worldwide, are a key component in the so-called "Mediterranean diet", which is strongly associated with a reduced risk of chronic degenerative diseases. In this work, we evaluate the differences in the total and individual polyphenol content and hydrophilic antioxidant capacity of seven varieties of tomato cultivated in Vegas Bajas del Guadiana, Badajoz (Spain), which were collected from two consecutive harvests (2008−2009). Hydrophilic antioxidant capacity was evaluated using the TEAC assay, while the Folin−Ciocalteau assay with a previous cleanup was used to establish total polyphenol content. The method was optimized and validated. Individual polyphenols were quantified using liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) on a triple quadrupole. All compounds were found to be significantly different when analysis of variance was performed. Results from the principal component analysis show that phenolic compounds and hydrophilic antioxidant capacity were responsible for the differences among tomato samples according to variety.

KEYWORDS: Tomato varieties, chemotaxonomic markers, hydrophilic antioxidant capacity, total polyphenols, LC-MS-MS, principal component analysis

INTRODUCTION

Epidemiological studies have shown that consumption of raw tomato and its tomato-based products (ketchup, tomato sauce, and tomato juice) is associated with a decrease in chronic degenerative diseases.[1] In addition to its micronutrient content, such as minerals (potassium), vitamin C, vitamin E, and folate,[2] tomato contains some valuable bioactive components, including antioxidants. The main tomato antioxidants are carotenoids, principally lycopene, which is largely responsible for the red color of the fruit. A number of epidemiologic studies have associated lycopene with a lower risk of prostate cancer.[3] In addition to carotenoids, other functional compounds such as phenolics also contribute to the beneficial effects of tomato products. Recently, the high content of phenolic compounds such as flavonoids and hydroxycinnamic acids in tomato has been gaining interest because of their apparent multiple biological effects, including free-radical scavenging, metal chelation, inhibition of cellular proliferation, and modulation of enzymatic activity and signal transduction pathways.[4] The presence of antioxidants in tomatoes has been the object of several studies[5−7] on the positive physiological properties attributed to these compounds. However, to our knowledge, no study has used the phenolic profile and hydrophilic antioxidant capacity as a varietal marker of tomatoes.

One of the important features of phenolic compounds is their usage as taxonomical markers.[8−12] Singleton et al.[8] showed that the patterns of phenolic substances in Chenin blanc, French Colombard, Semillon, and Thompson wines are influenced by the genetics of the grapevine. The polyphenolic profile determined by HPLC revealed a similarity within a grape variety and differences between varieties. Similarly, varieties of white musts,[11] wines,[10,13] and sparkling wines[12] have been shown to have different phenolic profiles.

Moreover, Russo et al.[9] studied the relationship between chemical composition and biotypes of 24 steam-distilled samples of essential oils from inflorescences of *Origanum vulgare* ssp. *hirtum*. Four chemotypes were identified on the basis of the phenolic content, i.e., thymol, carvacrol, thymol/carvacrol, and carvacrol/thymol chemotypes. Another investigation was carried out into the composition and variability of the essential oils contained in 63 individual plants taken from 21 populations of *Thymus zygis* L. in southern Spain.[14] A chemometric investigation of the infraspecific variability of the essential oils of these populations led to the distinction of seven main chemotypes: thymol, carvacrol, linalool, α-terpinyl acetate, thymol/*p*-eymene/γ-terpinene, 1,8-cineole/myrcene/spathulenol, and 1,8-cineole/α-terpineol. The phenolic compound thymol was the most common constituent in the majority of the samples studied.

An attempt was made, by means of principal component analysis (PCA) and linear discriminant analysis (LDA), to identify those parameters that could be useful to classify clones. Phenolic compounds such as anthocyanins, flavonols, and hydroxycinnamates

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were used as taxonomical markers to discriminate *Vitis vinifera* L. cv 'Barbera' clones.[15]

The distribution and expression of phenolic constituents and hydrophilic antioxidant capacity can differ significantly in tomato varieties. Our initial purpose was to evaluate total polyphenols (TP), hydrophilic antioxidant capacity, and the levels of flavonols (kaempferol-3-*O*-glucoside, rutin, and quercetin), flavanones (naringenin), hydroxycinnamic acids (chlorogenic, caffeic, caffeic-*O*-hexoside, and ferulic acids) and phenolic acids (protocatechuic and gallic acids) in seven varieties of tomato by liquid chromatography coupled to mass spectrometry in tandem mode (LC-ESI-MS/MS). We observed a different characteristic phenolic profile for each variety and, surprisingly, found that tomatoes could be grouped within varieties by their polyphenol content and hydrophilic antioxidant activity. In this article, 7 different varieties of tomato (H-9661, H-9997, H-9776, Albastro, Guadiva, Elegy, and Malva) harvested over two consecutive years, 2008 and 2009, were identified and classified by their phenolic profile and hydrophilic antioxidant activity. The analytical variables were subjected to analysis of variance (ANOVA) and principal component analysis (PCA).

MATERIALS AND METHODS

Standards and Reagents. All samples and standards were handled without exposure to light. Quercetin, rutin, caffeic, gallic, ferulic, protocatechuic and chlorogenic acids, and Folin−Ciocalteu (F−C) reagent, ABTS (2,2′azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), PBS (phosphate-buffered saline pH 7.4), Trolox ((±)-6-hydroxy- 2,5,7,8-tetramethylchromane-2-carboxylic acid 97%), and manganese dioxide were purchased from Sigma (Madrid, Spain); naringenin and kaempferol-3-*O*-glucoside from Extrasynthèse (Genay, France); hydrochloric acid 35% and acetic acid 99.8% from Panreac (Barcelona, Spain); and anhydrous sodium acetate (2 M) from Merck (Darmstadt, Germany). Methanol and formic acid were obtained from Scharlau (Barcelona, Spain) and ultrapure water (Milli-Q) from Millipore System (Bedford, USA).

Sampling and Processing Conditions. Tomato varieties (Malva, H-9661, H-9776, H-9997, Albastro, Guadiva, and Elegy) were cultivated in Vegas Bajas del Guadiana, Badajoz (Spain) over two consecutive years, 2008 and 2009. Each variety was independently sampled three times. All varieties were harvested at the same degree of ripeness (4.3−5°Brix) and were of normal size (75−80 g) to accurately compare polyphenol levels and hydrophilic antioxidant capacity.

The technique of crop management was transplantation with a root ball, with small plants grown in a greenhouse for a period of approximately 40−45 days. Planting was done on beds 1.5 m wide. The period for transplantation was between the first days of April and late May, and the cycles of these varieties ranged between 100 and 125 days. The mean maximum temperature ranged from 25 to 30 °C, and the variations in mean minimum temperature were 15−20 °C for the two years.

As a method of fertilization, fertigation (application of fertilizers, soil amendments, or other water-soluble products through an irrigation system) was applied.

Extraction of Hydrophilic Compounds. First, tomatoes were washed, sorted, and collected in a sterilized recipient. Tomato fruits were then beaten and homogenized over an ice bed; 0.5 g was weighed and homogenized with 4 mL of 80% ethanol in Milli-Q water; and they were sonicated for 5 min and centrifugated (4000 rpm at 4 °C) for 20 min. The supernatant was transferred into a flask, and extraction was repeated. Both supernatants were combined and evaporated under nitrogen flow; finally, the residue was reconstituted with Milli-Q water (0.1% of formic acid) up to 4 mL. Samples were frozen at −20 °C until analysis.

For the solid phase extraction (SPE) cleanup, Oasis MAX cartridges with 30 mg of mixed-mode anion-exchange and reversed-phase solvent from Waters (Milford, USA) were equilibrated with 1 mL of methanol 100% and 1 mL of 50 mM sodium acetate at pH 7, 1 mL of Milli-Q water, and 34 μL of hydrochloric acid at 35% were added to 1 mL of nitrogen evaporation extract before being loaded into the cartridges separately. These were rinsed with 50 mM sodium acetate at pH 7 (5% methanol), and finally, the polyphenols were eluted with 1800 μL of methanol (2% formic acid). The eluted fractions were evaporated under nitrogen, and the residue was reconstituted with water (0.1% formic acid) up to 250 μL and filtered through a 13 mm, 0.45 μm PTFE filter (Waters).

Analysis of Total Polyphenols. For the TP assay, each sample was analyzed in triplicate; 20 μL of the eluted fractions was mixed with 188 μL of Milli-Q water in a thermo microtiter 96-well plate (nunc, Roskilde, Denmark), 12 μL of F−C reagent and 30 μL of sodium carbonate (200 g/L) were added following the procedure described by Medina-Remón et al.[16] The mixtures were incubated for 1 h at room temperature in the dark. After the reaction period, 50 μL of Milli-Q water was added, and the absorbance was measured at 765 nm in a UV/vis Thermo Multiskan Spectrum spectrophotometer (Vantaa, Finland). This spectrophotometer allowed the absorbance of a 96-well plate to be read in 10 s. Results were expressed as mg of gallic acid equivalents (GAE)/100 g fresh weight (FW).

Folin−Ciocalteau Assay validation.]The method was validated with gallic acid. To evaluate the linearity, standards between 0.25 and 10 mg/L were studied. The sensitivity was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ). These were calculated by measuring the analytical background response by reading 10 blanks at the maximum sensitivity, and the standard deviation (SD) was calculated. The LOD was defined as 3 times the SD of the 10 blanks, whereas the LOQ was 10 times the SD. The intra- and interday accuracy and precision were determined by spiking the matrix with known levels of gallic acid at seven different concentrations. The precision was calculated as the relative standard deviation (RSD). The assay was validated according to the recommendations of AOAC International.[17]

The short-term temperature stability was evaluated by freezing three aliquots of each of the seven concentrations between 0.25 and 10 mg/L of gallic acid in Milli-Q water at −20 and −80 °C. The aliquots were thawed at room temperature for 6 h (the mean sample preparation time) and then analyzed. To evaluate the stability after successive freeze−thaw cycles, three aliquots of each concentration were stored at −20 and −80 °C for 24 h and then thawed at room temperature up to four times over a one-week period. Gallic acid was assessed in each aliquot in a single run at the end of the last freeze−thaw cycle. Aliquots of each concentration for long-term stability were prepared and immediately frozen at −20 and −80 °C until analysis (within 2 years). The concentrations of all the stability samples were compared with the mean back-calculated values for the standards at each concentration from the first day of long-term stability testing. The same procedures were followed for sample stability. We used HPLC-UV to calculate the recovery of seven representative polyphenols with different polarities after spiking the tomato samples with 5, 10, 25, and 50 mg/L of each standard.

We tested the possible interferences from vitamin C (ascorbic acid at 50 mg/L and 200 mg/L), sugar (glucose at 2 mg/L), iron (Fe(II) at 1 mg/L), and amino acids (phenylalanine, tyrosine, glutamine, and arginine at 1 mg/L) according to Roura et al.[18]

Hydrophilic Antioxidant Capacity. The antioxidant activity in the tomato samples was measured using $ABTS^+$ radical decolorization assay.[19,20] The $ABTS^+$ radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS (in PBS) through manganese dioxide powder. The manganese dioxide excess was removed by filtering the solution through a 13 mm, 0.45 μm PTFE filter (Waters). Prior to the assay, the solution was diluted in PBS at pH 7.4 to give an absorbance at

Table 1. Intra- and Interday Accuracy and Precision of the Assay

calibrator concentration (mg L^{-1})		means measured concentration (mg L^{-1})		precision, RSD[a] (%)		recovery, error (%)	
intraday	interday	intraday	interday	intraday	interday	intraday	interday
1.12	1.07	1.01	0.98	0.10	0.12	90.57	91.97
2.24	2.14	2.24	2.06	0.10	0.11	100.08	96.25
4.48	4.27	4.38	4.25	0.09	0.09	97.77	99.60
6.73	6.41	6.69	6.40	0.09	0.10	99.38	99.87
8.97	8.55	8.93	8.21	0.06	0.08	99.58	96.00
11.21	10.69	11.62	11.03	0.05	0.07	103.67	103.21
13.40	13.81	13.89	14.51	0.04	0.05	103.66	105.10

[a] RSD: relative standard deviation.

734 nm of 1.0 ± 0.01 and preincubated in ice. The $ABTS^+$ radical cation solution was prepared daily.

One millimolar Trolox in PBS at pH 7.4 was used as an antioxidant standard for the ABTS assay. Working standards were prepared each day by diluting 1 mM Trolox with PBS at pH 7.4.

Then, 245 μL of $ABTS^+$ solution was added to 5 μL of Trolox or to the tomato samples, and the solutions were stirred for 30 s. The absorbance was recorded continuously every 30 s with a UV/vis Thermo Multiskan Spectrum spectrophotometer for 1 h. PBS blanks were run in each assay.

The working range for Trolox (final concentration 0−750 μM) was based on triplicate determinations and consisted of plotting the absorbance as a percentage of the absorbance of the uninhibited radical cation (blank). The activities of the tomato samples were assessed at four different concentrations, which were within the range of the dose−response curve. Each sample was analyzed in triplicate at these four concentrations. Results of Trolox equivalent antioxidant capacity (TEAC) were expressed as (mmol Trolox equivalent (TE)/100 g FW).

LC-MS/MS Analysis. Tomato fruit polyphenols were identified in a previous study using LC-ESI-LTQ-Orbitrap-MS.[21] We selected the most predominant polyphenols of these tomatoes to evaluate the differences between the seven varieties of tomato. These polyphenols were quantified using LC-ESI-MS/MS. An API 3000 (PE Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer equipped with a Turbo Ionspray source in negative-ion mode was used to obtain MS/MS data. Turbo Ionspray source settings were as follows: capillary voltage, −3500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 4 (arbitrary units); focusing potential, −200 V; entrance potential, −10 V; and drying gas (N_2), heated to 400 °C and introduced to a flow rate of 8000 cm^3/min. The declustering potential and collision energy were optimized for each compound in infusion experiments: the individual standard solution (10 μg/mL) dissolved in 50:50 (v/v) mobile phase was infused at a constant flow rate of 5 μL/min using a model syringe pump (Harvard Apparatus, Holliston, MA, USA).

For quantification purposes, data was collected in the multiple reaction monitoring (MRM) mode, tracking the transition of the specific parent and product ions for each compound. In particular, we selected 10 transitions corresponding to the most abundant polyphenols in tomato fruit: ferulic acid m/z 193→134 (CE: −20 V); chlorogenic acid m/z 353→191 (CE: −20 V); caffeic acid m/z 179→135 (CE: −20 V); caffeic acid-*O*-hexoside m/z 341→179 (CE: −20 V); quercetin m/z 301→151 (CE: −30 V); rutin m/z 609→300 (CE: −50 V); protocatechuic acid m/z 153→109 (CE: −20 V); gallic acid m/z 169→125 (CE: −20 V); naringenin m/z 271→151 (CE: −30 V) and kaempferol-3-*O*-glucoside m/z 477→285 (−30 V); ethyl gallate was used as an internal standard m/z 197→169 (CE: −25 V). The standards of these polyphenols confirmed the previously reported fragmentation patterns. The compounds were deduced from the different transition signals and from comparing the observed accurate transitions and retention times with those of the standards.

Quantification of polyphenols was performed by the internal standard method. Polyphenols were quantified with respect to their corresponding standard. When standards were not available, as in the case of caffeic acid-*O*-hexoside, it was quantified with respect to the corresponding hydroxycinnamic acid (caffeic acid).

The liquid chromatograph was an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an autosampler, and a column oven set to 30 °C. A Luna C_{18} column 50 × 2.0 mm i.d., 5 μm (Phenomenex, Torrance, CA, USA) was used. The injection volume was 20 μL, and the flow rate was 0.4 mL/min. Mobile phases consisted of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B). Separation was carried out in 15 min under the following conditions: 0 min, 5% B; 10 min, 18% B; 13 min, 100% B; 14 min, 100% B; 15 min, 5% B. The column was equilibrated for 5 min prior to each analysis.

Statistical Analysis. The significance of the results was analyzed using the Statgraphics Plus v.5.1 Windows Package (Statistical Graphics Co., Rockville, MD). Analysis of variance (ANOVA) was used to compare the means of groups of measurement data, and principal component analysis (PCA) was carried out to obtain correlations among variables.

RESULTS

Validation of the Method for the Analysis of Total Polyphenols in Tomato Samples. The F−C method was linear over 0.25 to 10 mg/L for gallic acid. Least squares regression analysis gave the following result for the calibration curve: mean (SD); slope, 0.100 (0.001); y intercept, −0.003 (0.001); r^2 = 0.9993 (0.0004); and a standard deviation of residuals, 0.053. The LOD and LOQ were 0.006 mg/L and 0.019 mg/L, respectively. The intra- and interday precision and accuracy were evaluated at seven different concentrations (Table 1); for the values analyzed, recovery and precision were between the accepted values for AOAC International.[17]

Freeze and thaw cycles did not significantly modify the gallic acid concentration at either temperature tested; they were between 91.7% and 106.5% at −80 °C and between 94.9% and 106.2% at −20 °C. For short-term stability, the recoveries of gallic acid were between 90.1% and 104.0% at −20 °C and between 85.2% and 102.6% when the temperature was −80 °C. Long-term storage at −20 and −80 °C did not affect the gallic acid concentration. It decreased to 95% after 2 years, at both temperatures. We observed no statistically significant differences ($P > 0.05$) in TPs in the tomato samples after testing freeze and thaw cycles, and short-term and long-term stability. The storage and sample handling conditions used for the assays allowed the phenols to remain stable.

Table 2. Total Polyphenols (mg GAE/100g FW) and Hydrophilic Antioxidant Capacity (mmol TE/100g FW) of the Seven Varieties (Mean ± SD), Malva, H-9661, H-9776, H-9997, Albastro, Guadiva, and Elegy, Investigated in this Study over 2008 and 2009[a]

	total polyphenols (mg GAE/100 g FW)	hydrophilic antioxidant capacity (mmol TE/100 g FW)
Malva	10.04 ± 0.30 e	1.79 ± 0.10 e
H-9661	13.32 ± 0.70 g	2.80 ± 0.20 g
H-9776	8.64 ± 0.20 b	1.29 ± 0.10 b
H-9997	9.01 ± 0.40 c	1.40 ± 0.10 c
Albastro	8.60 ± 0.30 a	1.25 ± 0.09 a
Guadiva	9.06 ± 0.20 d	1.63 ± 0.08 d
Elegy	12.69 ± 0.40 f	1.98 ± 0.10 f

[a] Different letters in the columns represent statistically significant differences ($P < 0.05$). SD, standard deviation; GAE, gallic acid equivalent; FW, fresh weight; TE, Trolox equivalent

Table 3. Quantification of Individual Polyphenols (Mean ± SD) Described in the Literature[21,37] for Seven Varieties, Malva, H-9661, H-9776, H-9997, Albastro, Guadiva, and Elegy, Expressed as μg/g FW over 2008 and 2009[a]

	ferulic acid	chlorogenic acid	caffeic acid	caffeic acid-*O*-hexoside	quercetin	rutin	protocatechuic acid	gallic acid	naringenin	kaempferol-3-*O*-glucoside
Malva	BQL	0.36 ± 0.01 a	0.68 ± 0.01 c	0.26 ± 0.01 a	0.73 ± 0.02 f	19.79 ± 0.77 e	0.20 ± 0.02 a	BQL	3.03 ± 0.21 d	BQL
H-9661	0.35 ± 0.03 c	0.46 ± 0.01 c	1.02 ± 0.05 d	0.94 ± 0.04 f	0.76 ± 0.04 f	20.44 ± 0.47 e	0.46 ± 0.02 b	BQL	6.90 ± 0.19 e	BQL
H-9776	0.10 ± 0.05 a	0.40 ± 0.01 b	0.53 ± 0.01 a	0.32 ± 0.01 b	0.42 ± 0.01 a	2.68 ± 0.05 b	BQL	BQL	0.75 ± 0.04 b	BQL
H-9997	BQL	0.38 ± 0.03 b	0.53 ± 0.04 a	0.47 ± 0.08 d	0.47 ± 0.04 b	4.65 ± 0.04 c	BQL	BQL	0.50 ± 0.01 a	0.27 ± 0.03 a
Albastro	0.20 ± 0.01 b	0.39 ± 0.02 b	0.55 ± 0.02 a	0.38 ± 0.02 c	0.63 ± 0.04 c	0.79 ± 0.06 a	BQL	0.96 ± 0.04 a	1.59 ± 0.25 c	BQL
Guadiva	BQL	0.38 ± 0.03 b	1.25 ± 0.01 e	0.44 ± 0.06 d	0.69 ± 0.06 d	6.07 ± 0.51 d	BQL	BQL	1.71 ± 0.06 c	BQL
Elegy	0.21 ± 0.01 b	0.40 ± 0.02 b	0.63 ± 0.01 b	0.83 ± 0.02 e	0.69 ± 0.05 d	21.80 ± 0.68 f	BQL	0.93 ± 0.01 a	0.70 ± 0.01 b	0.59 ± 0.01 b

[a] Different letters in the columns represent statistically significant differences ($P < 0.05$). SD, standard deviation; FW, fresh weight; BQL, below quantification limit.

Absolute recoveries for the 7 polyphenol standards in the tomato samples using SPE were as follows: polyphenol, mean (SD); gallic acid, 92.5 (4.5); naringenin, 98.2 (3.4); caffeic acid, 96.7 (10.7); rutin, 87.3 (4.8); quercetin, 77.5 (5.4); protocatechuic acid, 108.8 (3.2); and chlorogenic acid, 101.1 (5.2). These recoveries are between the accepted values established by AOAC International.[17]

Total Polyphenols and Hydrophilic Antioxidant Capacity in Tomato Samples. SPE has been introduced to eliminate interfering nonphenolic reductants before the F−C assay. We tested for interferences from vitamin C (ascorbic acid at 50 mg/L and 200 mg/L), sugar (glucose at 2 mg/L), iron (Fe(II) at 1 mg/L), and amino acids (phenylalanine, tyrosine, glutamine, and arginine at 1 mg/L), according to Roura et al.[18] None of these substances reacted with the F−C reagent after SPE. After the SPE procedure, the phenolic levels were between 56.54% and 64.70% lower than the values obtained before the extraction procedure.

The content of TP evaluated by the F−C assay after SPE and the relative contribution of individual compounds to the hydrophilic antioxidant capacity of tomato samples from both seasons (2008 and 2009) were analyzed and expressed as the mean ± SD as shown in Table 2. The F−C assay showed that Albastro and H-9776 contained the lowest concentrations of phenolics, followed by H-9997 and Guadiva. The varieties that possessed the highest concentrations were H-9661, Elegy, and Malva for the F−C assay.

A similar trend was observed in the TEAC results. The lowest hydrophilic antioxidant capacities were determined for Albastro and H-9776, followed by H-9997 and Guadiva. H-9661 contained the highest antioxidant capacity with Elegy ranked second, followed by Malva. No significant changes ($P > 0.05$) in TP content and hydrophilic antioxidant capacity were observed over the two consecutive years, 2008 and 2009. This data indicates that the TP and hydrophilic antioxidant content of tomatoes was mainly influenced by varietal factors rather than environmental conditions or year changes.

Quantification of Individual Polyphenols in Tomato Samples. The most abundant polyphenols in tomato varieties are quantified in Table 3. The statistically significant differences found between the varieties for each compound analyzed were highlighted with different superindexes. An HPLC chromatogram of the crude extract of the H-9661 variety, including the identification of each peak, is shown in Figure 1.

The main polyphenol in all the varieties was rutin (m/z 609→300), present at levels ranging between 0.79 and 21.80 μg/g FW, followed by naringenin (m/z 271→151), which was found at levels between 0.50 and 6.90 μg/g FW. The variety with the highest levels of individual polyphenols was H-9661, followed by Elegy and Malva, and this was reflected in the analysis of total phenolics and hydrophilic antioxidant capacity as well.

Hydroxycinnamic acids were mainly represented by ferulic acid (m/z 193→134) in H-9661, H-9776, Albastro, and Elegy, with chlorogenic acid (m/z 353→191), caffeic acid (m/z 179→135), and caffeic acid-*O*-hexoside (m/z 341→179) present in all the varieties. Chlorogenic acid levels were similar in all the varieties, whereas the amounts of caffeic and caffeic acid-*O*-hexoside were higher in H-9661, Elegy, and Guadiva. Ferulic acid was particularly high in H-9661, whereas in other varieties, this hydroxycinnamic acid was below the limit of quantification.

Regarding the family of phenolic acids, the main compound was gallic acid (m/z 169→125) in Albastro and Elegy, followed by protocatechuic acid (m/z 153→109) in Malva and H-9661. In some varieties, gallic and protocatechuic acids were below the detection level.

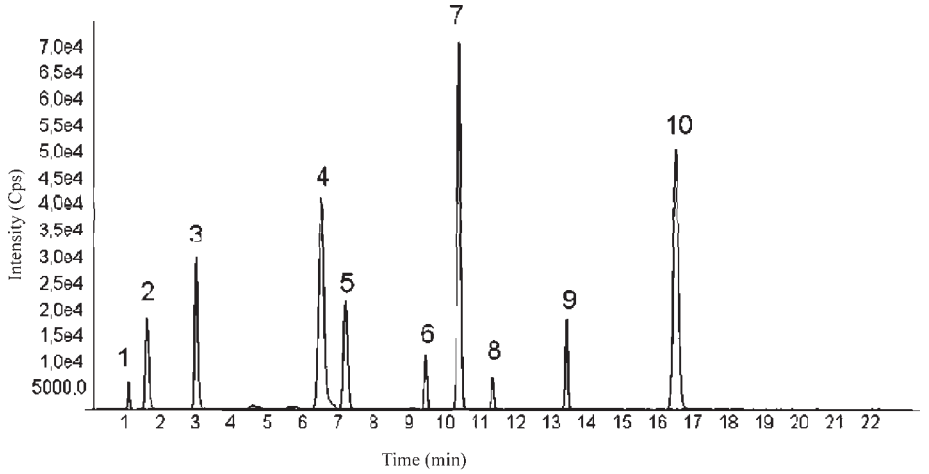

Figure 1. HPLC chromatogram of the crude extract of the H-9661 variety including the identification of each peak. 1:gallic acid; 2:protocatechuic acid; 3: caffeic-*O*-hexoside; 4: caffeic acid; 5: chlorogenic acid; 6: ferulic acid; 7: rutin; 8: kaempferol-3-*O*-glucoside; 9: naringenin; 10: quercetin.

Flavonol concentration varied from one variety to another. Flavonols were mainly characterized by quercetin (m/z 301→151) and rutin (m/z 609→300) in all the varieties, and kaempferol-3-*O*-glucoside (m/z 477→285) in H-9997 and Elegy. Quercetin levels were similar in all the varieties, whereas the amounts of rutin were higher in Malva, H-9661, and Elegy. Kaempferol-3-*O*-glucoside reached its maximum concentration in Elegy and H-9997.

Flavanones were mainly represented by naringenin (m/z 271→151) in all the varieties. As can be observed, the amounts of naringenin present in H-9661 and Malva were higher than those in other varieties.

Correlation between Tomato Compounds and Seven Different Varieties. The ANOVA of the polyphenolic data and hydrophilic antioxidant activity showed that there were significant differences ($P > 0.05$) among the varieties (Table 3). PCA was performed on all the samples and variables (total phenolics, hydrophilic antioxidant activity, ferulic, chlorogenic, caffeic, caffeic-*O*-hexoside, protocatechuic, and gallic acids, and quercetin, rutin, naringenin, and kaempferol 3-*O*-glucoside) and was able to separate the tomato samples according to variety.

Two principal components (PC1 and PC2) were obtained and accounted for 74.59% of the variability of the original data (Figure 2). The statistical analysis for the data showed a strong positive correlation between TP content, rutin, and ferulic and caffeic-*O*-hexoside acids. Moreover, a positive correlation was observed between protocatechuic acid and naringenin. In contrast, ferulic and caffeic-*O*-hexoside acids, rutin and TP content were weakly correlated with naringenin and caffeic and protocatechuic acids.

The score plot of PC1 versus PC2 from the full-data PCA model plotted in Figure 2 describes differences between the seven varieties of tomato. Tomato samples are gathered in seven different groups (Figure 3). Guadiva and Malva are situated close together in the bottom part of the plot; H-9997, Albastros and H-9776 are situated in the left part of the score plot; whereas the variety H-9661 appears on the right-hand side and Elegy in the upper part of the plot. The H-9661 variety correlated well with naringenin and protocatechuic acids, whereas the H-9997 and H-9776 varieties scored lower values of these parameters as they were located on the left-hand side. However, the Elegy variety correlated well with kaempferol-3-*O*-glucoside and gallic acid,

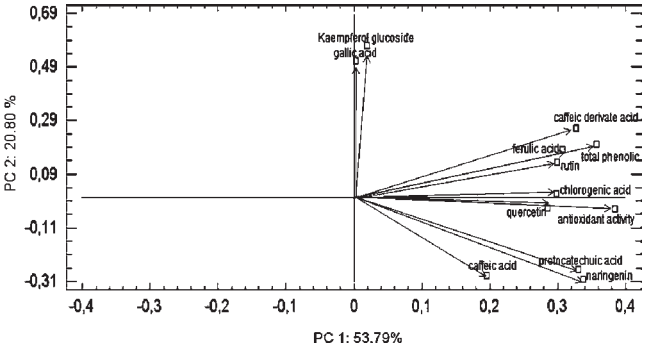

Figure 2. Principal components plot of the seven varieties of tomato.

although this sample scored lower values of naringenin and caffeic and protocatechuic acids. Moreover, H-9661 and Elegy correlated well with rutin, TP content, hydrophilic antioxidant capacity, and caffeic-*O*-hexoside and ferulic acids as these varieties scored the highest levels of these parameters.

DISCUSSION

The antioxidant capacity and polyphenol content of tomatoes are greatly affected by both the ripening stage and part of the fruit;[22,23] therefore, in our experiment tomato fruits were harvested at the same degree of ripeness (4.3−5°Brix). Since 98% of total flavonols occur in the skin, tomato types with different fruit size and thus different skin−volume ratios or different skin color are expected to have varying flavonol contents.[24] However, in our experiment, since all the varieties were normal-sized (75−80 g), the skin-volume ratio and fruit size were not expected to be a determining factor for the polyphenol content.

In addition, all the studied varieties were cultivated in Vegas Bajas del Guadiana, Badajoz (Spain), in order to attenuate the variability due to the country of origin. Analysis of cherry tomato cv. Favorita obtained from Spain, South Africa, England, and Scotland has shown widely varying flavonol contents of 21.5, 16.0, 3.4, and 6.6 μg/g FW, respectively, based on whole fruit samples.[25]

Stewart et al.[25] suggested that choice of cultivar is a major factor contributing to the total phenol content in tomatoes when grown under similar environmental conditions. Two normal-sized field

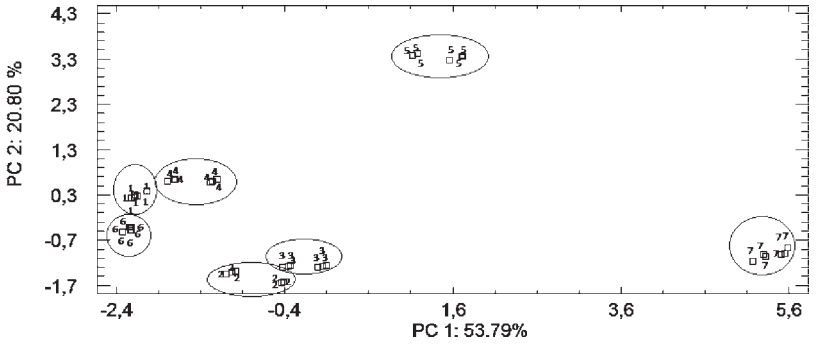

Figure 3. Score plot of PC1 vs PC2 of all the varieties analyzed. Varieties: 1, H-9997; 2, Guadiva; 3, Malva; 4, Albastros; 5, Elegy; 6, H-9776; 7, H-9661.

grown tomatoes, cv.'s Bond and Havanera, grown alongside each other in Spain, contained 10.9 and 6.6 μg/g FW of flavonols, respectively. Therefore, to evaluate the effects of the cultivar, we chose seven normal-sized varieties of tomato harvested at the same degree of ripeness (4.3−5°Brix) from Vegas Bajas del Guadiana, Badajoz (Spain): Malva, H-9661, H-9776, H-9997, Albastro, Guadiva, and Elegy.

Total phenolic content is indicative of the amount of polyphenols in vegetables. The F−C method is the most commonly used assay to analyze TP in tomatoes.[26,27] However, TP content is sometimes overvalued, as tomatoes contain reducing substances (ascorbic acid, sugars, and amino acids) that interfere with the assay.[16,28] In this work, we developed an optimized F−C method with SPE to accurately establish the real content of TP in tomato samples.

A prerequisite for the evaluation of the total antioxidant contents and antioxidative capacities of tomato products is the separation of hydrophilic and lipophilic fractions, which contain vitamin C and polyphenols or vitamin E and carotenoids, respectively. In this work, we took into account the hydrophilic antioxidant capacity due to polyphenols and vitamin C.

Curiously, our TP content results were comparable to the existing data. However, total polyphenol levels in tomatoes have been overestimated until now because previously cleanup SPE was not assessed. Gahler et al.[29] reported TPs in tomatoes, and their results varied from 9.86 to 22.32 mg of GAE/100 g FW. Minoggio et al.[19] analyzed different polyphenol fractions of tomato cultivars and found that the content of total phenolics varied from 4.43 to 25.84 mg GAE/100 g FW. Our data are in agreement with the results shown by these authors. Shen et al.[30] also analyzed tomato cultivars and determined a TP content from 1.8 to 2.3 mg GAE/100 g FW. Therefore, the tomatoes analyzed in this work from Vegas Bajas del Guadiana had a slightly higher total phenolic content than tomatoes analyzed in the other works.

When the hydrophilic antioxidant capacity was evaluated, our results were within the range described by other authors. Gahler et al.[29] determined hydrophilic antioxidant capacity in tomato fruit, and their results were 0.045 mmol TE/100 g FW, whereas Minoggio et al.[19] reported hydrophilic antioxidant capacities from 60 to 230 mmol TE/100 g FW in tomato lines and cultivars. These results show that the hydrophilic antioxidant capacity differs greatly between studies, probably due to the variety, stage of ripeness, light, temperature, climatic growing conditions, or soil characteristics of tomato fruits.[31] The antioxidative effects of the examined tomatoes correlated with the TP content, which suggests that these bioactive components contribute significantly to the hydrophilic antioxidant capacity of tomato fruits. Surprisingly, Minoggio et al.[19] found that almost all the tomato lines they tested with low carotenoid content produced high levels of phenolics and consequently had the strongest antioxidant capacity.

The qualitative and quantitative determination of individual polyphenols in foods is becoming extremely interesting in the field of nutrition and food technology. The highest concentrations of tomato polyphenols have been found in epidermal and placental tissues.[24] Stewart et al.[25] measured the distribution of flavonols in Spanish cherry tomatoes and found a flavonol content of 25.3 μg/g FW in the whole fruits, 143.3 μg/g FW in the peel or epidermis, and 0.12 μg/g FW in the flesh, whereas the seed had 1.5 μg/g FW.

According to the USDA flavonoid database, red tomatoes contain on a year average basis 15 μg/g FW of flavonoids determined as aglycones. Naringenin (45%) is reported to be the main flavonoid, followed by quercetin (39%), myricetin (10%), and kaempferol (5%).[24] Other studies report rutin to be the major flavonoid in several tomato cultivars.[32,33] In our investigation, rutin (quercetin-3-*O*-rutinoside) was the dominant flavonol in all the samples, followed by naringenin.

The naringenin content in these varieties fell within the range (4.50− 9.50 μg/g FW) reported by Martinez-Valverde et al.[34] The rutin content of Malva, H-9661, and Elegy was very similar to the value (19.4 μg/g FW) found by Proteggente et al.[35] Minoggio et al.[19] also reported levels of rutin between 0.7 and 23.5 μg/g FW, and our values are in accordance with these results.

Some studies have reported quercetin and kaempferol in tomato fruits.[36,37] In our study, quercetin achieved a content of 0.76 μg/g FW in the H-9661 variety, and kaempferol-3-*O*-glucoside was only detected in H-9997 and Elegy. The differing flavonol levels in tomato fruits found in different studies are probably due to the variety, state of ripeness, light, temperature, climatic growing conditions, or soil.

Tomato fruits also contain hydroxycinnamic acids, which are found as simple esters, chlorogenic acid being the most predominant.[38] The concentration of this hydroxycinnamic acid in pulp is nearly twice that in pericarp tissues.[39] The chlorogenic acid content in the varieties we studied fell within the range (0.3−5.8 μg/g FW) reported by Minoggio et al.[19] All the varieties contained caffeic acid, and our results are in accordance with the value (<1 μg/g FW) found by Minoggio et al.[19] Ferulic acid was also found in this work, but only in H-9661, H-9776, Albastro, and Elegy. The variations in the content of hydroxycinnamic acids depend on the degree of maturity of the tomato fruits concerned and on agricultural practices. Chlorogenic acid appears in young fruits and decreases during fruit maturity.[38]

Some of the varieties we analyzed also contained phenolic acids, the most predominant being gallic acid, as described in

other studies,[30] with a value lower than 1 μg/g FW in Albastro and Elegy. We also found protocatechuic acid, but only in Malva and H-9661, and at a level close to the limit of detection.

The variables studied enabled the differentiation of H-9661, H-9997, H-9661, Malva, Guadiva, Albastros, and Elegy. Significant differences among the varieties were observed after applying analysis of variance, and these were then confirmed by principal component analysis. H-9661 and Elegy were clearly distinct from all other varieties, while this distinction was not so clear for Guadiva and Malva.

In conclusion, phenolic and hydroxycinnamic acids and flavonoids, total polyphenols, and hydrophilic antioxidant capacity can be used as chemotaxonomic tomato markers to distinguish between tomatoes according to variety.

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ABBREVIATIONS USED

F−C, Folin−Ciocalteau;; SPE, solid phase extraction; TP, total polyphenols; LC-MS/MS, liquid chromatography coupled to mass spectrometry in tandem mode; ABTS, 2,2′azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); PBS, phosphate-buffered saline 5 mM; Trolox, (±)-6-hydroxy- 2,5,7,8-tetramethylchromane-2-carboxylic acid; GAE, mg of gallic acid equivalents; FW, fresh weight; LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation; HPLC, high pressure liquid chromatography; TEAC, Trolox equivalent antioxidant capacity; TE, Trolox equivalent; FS, full scan; CE, collision energy; PIS, product ion scan; Prec, precursor ion scan; NL,, Neutral loss;; MRM, multiple reaction monitoring; ANOVA, analysis of variance; PCA, principal component analysis; PC, principal component.

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