Characterization and quantification of phenolic compounds in four tomato (Lycopersicon esculentum L.) farmer’ varieties in Northeastern Portugal homegardens

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Abstract Tomato (*Lycopersicon esculentum* L.) is one of the most widely consumed fresh and processed vegetables in the world, and contains bioactive key components. Phenolic compounds are one of those components and, according to the present study, farmer’ varieties of tomato cultivated in homegardens from the northeastern Portuguese region are a source of phenolic compounds, mainly phenolic acid derivatives. Using HPLC-DAD-ESI/MS, it was concluded that a *cis* *p*-coumaric acid derivative was the most abundant compound in yellow (“Amarelo”) and round (“Batateiro”) tomato varieties, while 4-*O*-caffeolyquinic acid was the most abundant one in long (“Comprido”) and heart (“Coração”) varieties. The most abundant flavonoid was quercetin pentosylrutinoside in the four tomato varieties. Yellow tomato presented the highest levels of phenolic compounds (54.23 µg/g fw), including phenolic acids (43.30 µg/g fw) and flavonoids (10.93 µg/g fw). The phenolic compounds profile obtained for the studied varieties is different from other tomato varieties available in different countries, which is certainly related to genetic features, cultivation conditions, and handling and storage methods associated to each sample.

Keywords Tomato; *Lycopersicon esculentum*; Farmers’ varieties; Phenolic compounds; HPLC-DAD-ESI/MS

Abbreviations

**DAD** Diode Array Detector  
**ESI** Electron Spray Ionization  
**HPLC** High-Performance Liquid Chromatography  
**MS** Mass Spectrometry
**Introduction**

Phenolic compounds are one of the main groups of dietary phytochemicals found in fruits, vegetables and grains. They include a range of plant secondary metabolites that can be divided in different groups, i.e., flavonoids (e.g. anthocyanins, flavanols, flavones, or isoflavones), phenolic acids, tannins, stilbenes and lignans. Several of these compounds are found in nature as glycosides and/or as esters and/or methyl ethers [1].

In plants, they tend to accumulate in dermal tissues where they play a potential role in protection against UV radiation, as attractants in fruit dispersal or as defense chemicals against pathogens and predators [2]. They also exhibit a wide-range of physiological properties in animals, such as anti-allergenic, anti-atherogenic, anti-inflammatory, antimicrobial, anti-thrombotic, cardioprotective, and vasodilatory effects [3]. In recent years, dietary phenolics have attracted considerable attention for their putative effects on human health, which have been associated to their antioxidant and free-radical-scavenging activities [4, 5].

Tomato (*Lycopersicon esculentum* L.) is one of the most widely consumed fresh and processed vegetables in the world and contains bioactive components such as phenolics, carotenoids and vitamins C and E. Carotenoids consumption has been associated with a lower risk of several types of cancer [6, 7] and a lower incidence of coronary heart disease [8]. Lycopene is the major carotenoid present in tomato and shows strong antioxidant activity [9, 10]. However, lycopene alone does not account for tomato’s health benefits. Phenolics have been found to act synergistically with lycopene in preventing cell damage [11].

Phenolic compounds have been extensively characterized in tomato varieties from different countries [12-16], including genetically modified tomatoes [17, 18]. However,
the chemical composition of tomatoes can vary among tissues of a single fruit [19, 20] and type of tomatoes, according to the cultivar, cultivation conditions, and handling and storage methods [21, 22].

There are a large number of tomato cultivars with a wide range of morphological and sensory characteristics that determine their use. In Trás-os-Montes, Northeastern Portugal, local population’s lifestyles have highlighted the importance of local tomato farmers’ varieties, which are grown using extensive farming techniques and considered very tasty and healthy food [23]. We had previously reported the nutritional composition and antioxidant activity of four farmers’ varieties [24], but their phenolic composition was not studied. Therefore, the present work aims to characterize the phenolic profiles of these tomato farmer’ varieties from Trás-os-Montes.

**Materials and Methods**

**Samples**

Four common farmer’ varieties of tomato widely cultivated in rural communities from Miranda do Douro, Trás-os-Montes, Northeastern Portugal, were chosen according to morphological, sensory and usage characteristics such as size and exterior colour of mature fruits [24]: “tomate amarelo” (yellow tomato; Royal Horticultural Society Colour Chart (RHS), yellow-orange group 14), “tomate redondo or batateiro” (round tomato; RHS, Red group 42), “tomate comprido” (long tomato; RHS, Red group 34) and “tomate coração” (heart tomato; RHS, Red group 47). Tomato fruits at the ripe stage were hand harvested randomly in September 2010 from the middle of six plants of
each of the four varieties, in selected homegardens of two villages in the studied area. The seeds were selected and kept by local farmers. The ripening stage for all samples was selected according to local consumers’ criteria. The edible portion (pericarps without jointed pedicels) of six fruits of each variety was prepared and used for analysis. The samples were lyophilised (4.5 model 7750031, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and kept at -20 ºC until analysis.

Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid was purchased from Prolabo (VWR International, France). The phenolic compound standards were from Extrasynthese (Genay, France). All the other chemicals were of analytical grade and purchased from chemical suppliers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Phenolic compounds extraction procedure

Each sample (1 g) was extracted with 30 mL of methanol:water 80:20 (v/v) at room temperature, with agitation (150 rpm) for 1h. The extract was filtered through Whatman nº 4 paper. The residue was re-extracted twice with additional 30 mL portions of the same solvent. The combined extracts were evaporated at 35 ºC (rotary evaporator Büchi R-210) to remove methanol. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3 cc cartridge (Phenomenex), wetted and activated with methanol followed by water; sugars and more polar substances were removed with 10 mL of
water, and phenolic compounds were further eluted with 5 mL of methanol. The methanolic extract was concentrated under vacuum, re-dissolved in 1 mL of water:methanol 80:20 (v/v) and filtered through a 0.22-µm disposable LC filter disk for HPLC analysis.

HPLC-DAD-ESI/MS analysis

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by the authors [25]. Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, a calibration curve was obtained by injection of known concentrations (2.5-100 µg/mL) of different standard compounds: caffeic acid (y=617.91x-691.51; $R^2=0.9991$); chlorogenic acid (y=600.27x-763.62; $R^2=0.9998$); p-coumaric acid (y=447.12x-1580.7; $R^2=0.9962$); ferulic acid (y=779.11x-869.22; $R^2=0.9987$); kaempferol-3-O-rutinoside (y=175.02x-43.877; $R^2=0.9999$); quercetin 3-O-glucoside (y=316.48x-2.9142; $R^2=1$); quercetin-3-O-rutinoside (y=222.79x-243.11; $R^2=0.9998$); and syringic acid (y=641.76x+246.82; $R^2=0.9988$). The results were expressed in µg per g of fresh weight (fw).

Statistical analysis
For each sample three extracts were obtained and all the assays were carried out in triplicate. The results are expressed as mean values with standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program.

Results and Discussion

Fig. 1a shows the phenolic profile of one of the studied tomato farmer’s varieties: yellow tomato (Amarelo). Phenolic acid derivatives, mainly hydroxycinnamoyl derivatives, were the most abundant compounds in all tomato varieties (Table 1).

Compounds 12 and 17 corresponded to caffeic acid and trans-p-coumaric acid respectively, identified by comparison of their UV and mass characteristics and retention time with those of commercial standards. Compounds 6 and 7 showed the same pseudomolecular ion $[M-H]^-$ at $m/z$ 353 consistent with caffeoylquinic acid isomers. Compound 7 was positively identified as 5-O-caffeoylquinic acid by comparison with an authentic standard, and also its MS fragmentation pattern [26, 27]. Compound 6 was identified as 4-O-caffeoylquinic acid based on the fragmentation pattern described by Clifford et al. [26, 27] for these compounds, with a base peak at $m/z$ 173 ([$\text{quinic acid-H-H}_2\text{O}$]) accompanied by a secondary fragment ion at $m/z$ 179 with approximately 65% abundance of base peak. Similar reasoning was applied for the identification of compounds 11 and 13 as 4-O-p-coumaroylquinic acid and 5-O-p-coumaroylquinic acid, respectively.
Compounds 3 and 5 presented the same pseudomolecular ion [M-H]- at m/z 341 and similar fragmentation pattern with the loss of 162 mu (hexosyl moiety) yielding a base peak at m/z 179 mu ([caffeic acid-H]-) and other two fragments at m/z 161 ([caffeic acid-H-H$_2$O]) and 135 ([caffeic acid-H-CO$_2$]), which allowed assigning them as caffeoyl hexosides I and II, respectively. Similarly, compounds 8 and 9 with MS$^2$ fragments at m/z 145 (base peak; [coumaric acid-H-H$_2$O]) and 163 (-162 mu; [coumaric acid-H]) could identified as p-coumaroyl hexosides. To confirm the existence of cis and trans isomers, a commercial standard of (trans) p-coumaric acid was submitted to UV irradiation (366 nm, 24h). Partial transformation was observed with the appearance of a new peak at earlier retention time in the HPLC chromatogram and a different UV spectrum with $\lambda_{max}$ at 300 nm, which was attributed to the corresponding cis isomer (Fig. 1b). Therefore, compound 9 could be assigned as cis p-coumaroyl hexoside based on its UV spectrum with $\lambda_{max}$ at 300 nm. Compound 8 might be the corresponding trans isomer, although it could be expected to elute later than the cis isomer if the pattern observed for trans and cis p-coumaric acid was maintained. The fact that both compounds eluted close to each other might explain the interchange in their elution order, although we cannot discard that a different hexosyl substituent could exist in each compound, either. Thus, the compound was tentatively identified as trans p-coumaroyl hexoside. Furthermore, peak 10 with MS$^2$ fragments at m/z 193 (-162 mu; [ferulic acid-H]) and 176 ([ferulic acid-H-H$_2$O]) was tentatively assigned as ferulic acid glucoside. The MS$^2$ fragmentation of compound 2 presented a base peak corresponding to the ion at m/z 163, corresponding to p-coumaric acid. The observation of a loss of 162 mu (hexosyl moiety), the base peak at m/z 163 ([coumaric acid-H]) and the presence of the ion at m/z 325 (coumaroyl hexose) in the MS$^2$ fragmentation of the compound pointed
to that it could be a derivative of a \( p \)-coumaroyl hexose. Furthermore, the UV spectra showing \( \lambda_{\text{max}} \) at 300 nm, as mentioned above, suggested it as a possible \( cis \) isomer.

Compound 15 presented a pseudomolecular ion \([M-H]^-\) at \( m/z \) 359, yielding MS\(^2\) fragments at \( m/z \) 197 (loss of a hexosyl moiety; \([\text{syringic acid-H}]^-\)) and 153 (base peak; \([\text{syringic acid-H-CO}_2\]^-\)), suggesting that it could be a syringic acid hexoside. The UV spectrum with \( \lambda_{\text{max}} \) at 274 nm was also coherent with a syringic acid derivative. Compound 14 presented similar UV spectrum and more 44 mu (CO\(_2\), carboxyl moiety) than compound 15. The observation of an MS\(^2\) base peak at \( m/z \) 197 (\([\text{syringic acid-H}]^-\)) and another fragment at \( m/z \) 241 from the loss of a hexose pointed to it was a syringic acid hexoside derivative, although no final structure could be assigned.

Peaks 1 and 4 presented pseudomolecular ions identical to two non-phenolic compounds reported by Gomez-Romero et al. [13] to occur in tomato samples, i.e., benzyl alcohol dihexose and (iso)pentyl dihexose. Those authors did not present a fragmentation pattern for the first one, but the fragmentation pattern reported for (iso)pentyl dihexose was similar to the one obtained in our study. Furthermore, the early retention time and the elution order of both compounds was coherent with the proposed identities. Therefore, compounds 1 and 4 were tentatively assigned as benzyl alcohol dihexose and (iso)pentyl dihexose, respectively.

The rest of detected compounds (peaks 16, 18, 19 and 20) were identified as flavonol derivatives derived from kaempferol and quercetin. Compounds 18 and 20 were positively identified as quetcetin-3-\( O \)-rutinoside and kaempferol-3-\( O \)-rutinoside, respectively, by comparison of MS fragmentation pattern and UV spectra with authentic standards. Compound 16 and 19 showed a pseudomolecular ion \([M-H]^-\) at \( m/z \) 741 and 725, and similar MS\(^2\) fragmentation patterns releasing two fragments from the
successive losses of pentosyl ([M-H-132]; m/z at 609 and 593, respectively) and rutinosyl moieties ([M-H-132-308]; m/z at 301 and 285). Thus, these compounds were tentatively identified as quercetin pentosylrutinoside and kaempferol pentosylrutinoside, respectively.

Yellow tomato (Amarelo) was the variety that presented the highest levels of phenolic compounds (54.23 µg/g fw) followed by round tomato (Batateiro, 29.42 µg/g), long tomato (Comprido, 8.50 µg/g) and heart tomato (Coração, 3.72 µg/g) (Table 1). Phenolic acids were the most abundant group, being compound 2 (cis p-coumaric acid derivative) predominating in Amarelo and Batateiro tomato varieties, and 4-O-caffeoylquinic acid the most abundant compound in Comprido and Coração varieties. The non-phenolic compound, benzyl alcohol dihexose, was also predominant in all tomato varieties. The most abundant flavonoid was quercetin pentosylrutinoside in all the studied tomato varieties.

According to literature, chlorogenic acid (i.e., 5-O-caffeoylquinic acid) was the main phenolic compound in tomato and the most extensively studied [14-16, 18], whereas flavonoids are represented by flavanones (naringenin glycosylated derivatives) and flavonols (quercetin, rutin and kaempferol glycosylated derivatives) [12, 13, 15, 17]. In the samples studied herein, main phenolics also corresponded to hydroxycinnamoyl derivatives, although 5-O-caffeoylquinic acid was not the majority compound; furthermore, neither naringenin nor naringenin glycosylated derivatives were found; which can be interpreted as composition characteristics of the studied tomato samples, as related to genetic features, cultivation conditions, and/or handling and storage methods associated to each sample [21, 22]. In fact, phenolic compounds have been reported as
cultivar- and variety-distinguishing factors in some plant products [28], being dependent on genotype and environmental factors [29].

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References


Fig. 1. Individual chromatogram of yellow tomato variety (Amarelo) recorded at 280 nm (a) and UV spectrum of compounds 2 and 9 (b).
Table 1 Retention time (Rt), wavelengths of maximum absorption in the visible region (λ<sub>max</sub>), mass spectral data, tentative identification and concentration of phenolic acids and flavonoids in four different tomato Portuguese farmer’ varieties.

<table>
<thead>
<tr>
<th>Compounds identification</th>
<th>Concentration of the identified compounds (µg/g fw)</th>
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<tbody>
<tr>
<td></td>
<td>Amarelo</td>
</tr>
<tr>
<td><strong>Benzyl alcohol dihexose</strong></td>
<td></td>
</tr>
<tr>
<td><strong>cis p-Coumaric acid derivative</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Caffeic acid hexoside</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Caffeic acid hexoside II</strong></td>
<td></td>
</tr>
<tr>
<td><strong>5-O-Caffeoylquinic acid</strong></td>
<td></td>
</tr>
<tr>
<td><strong>trans p-Coumaric acid hexoside</strong></td>
<td></td>
</tr>
<tr>
<td><strong>cis p-Coumaric acid hexoside</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Ferulic acid hexoside</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Coumaroylquinic acid</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Coumaric acid hexoside</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Kaesmeprol pentosylrutinoside</strong></td>
<td></td>
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<tr>
<td>Total phenolic acids</td>
<td>43.30 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total flavonoids</td>
<td>10.93 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total phenolic compounds</td>
<td>54.23 ± 2.55&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total non-phenolic compounds</td>
<td>14.00 ± 0.90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Figures in brackets after MS<sup>2</sup> fragment ions refer to their relative abundances. *Relative abundance < 2%. nd- not detected; tr- traces. **Concentrations of compound 1 and 4 were expressed as equivalents of caffeic acid and syringic acid, respectively. In each row different letters mean significant differences (p<0.05).