Bioactive compounds and antioxidant activity of hot pepper fruits at different stages of growth and ripening

Mira Elena Ionica¹, Violeta Nour¹, Ion Trandafir²
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Summary
The evolution of some bioactive compounds and antioxidant activity has been investigated during fruit growth and ripening of five pepper cultivars: ‘Dracula’, ‘Pintea’, ‘Pepperone’, ‘Bulgarian carrot’ (C. annuum) and ‘Christmas bell’ (C. baccatum var. pendulum). High-performance liquid chromatography was used to quantify the content of capsaicin in the fruit in order to determine the pungency level of analyzed peppers. Pepper fruits were collected at five stages of growth and ripening. Dry matter, soluble solids, ascorbic acid, total phenolics, including total flavonoids, capsaicin content and antioxidant activity were determined at each stage. There were major differences among the cultivars in the accumulation of the bioactive compounds in the fruit during their growth and ripening, although the quantitative accumulation pathway of various components had a similar trend during phenophases. Antioxidant activity and ascorbic acid content increased during growth and ripening of hot peppers, the highest levels being found in the last stage of ripening. The pattern of variation of total flavonoid content was cultivar dependent. In most cultivars, an important increase of the total phenolic and total flavonoid content was observed in the last stage of ripening. Capsaicin content recorded a maximum level in F3 or F4 depending on cultivar, and decreased afterwards until the complete ripening of the pepper fruits. ‘Dracula’ cultivar was classified as “non-pungent” (fruits are not spicy) while ‘Pintea’ was classified as “highly pungent”, the other analyzed cultivars having an average level of pungency.

Keywords: hot pepper, antioxidant activity, phenolics, flavonoids, capsaicin

Introduction
Hot pepper was part of ancient human diet in the American continent since 7500 years BC. Its South American origin is supported for all species of Capsicum genus, which probably occurred in the region between southern Brazil and Bolivia. Hot pepper has a sweet-pungent flavor, with pungency ranging by thousands to tens of thousands of units. It is cultivated in various regions of the world, including the South of Romania. Capsicum annuum, the most common species, is used in a variety of cuisines.

Materials and methods
Plant material
Fruits of five pepper cultivars (Capsicum annuum and Capsicum baccatum): ‘Dracula’, ‘Pintea’, ‘Pepperone’, ‘Bulgarian carrot’ (C. annuum) and ‘Christmas bell’ (C. baccatum var. pendulum), were collected at five stages of development and ripening i.e. F1-14 days after flowering, F2-20 days after flowering, F3-30 days after flowering, F4-40 days after flowering, F5-50 days after flowering (physiological maturity) from Dabuleni, Dolj county, Romania and analyzed in terms of chemical characteristics and antioxidant activity. The selection of the fruits was based on the number of days after flowering.

Dabuleni is a sandy area in the South of Romania close to the Danube River. It has a strong continental climate with mild Mediterranean influence and an average temperature of 11 °C. The region gets severe drought from July to September and average rainfalls in May and June. The orchard management was consistent with cultural practice (10-12 watering sessions with 300-350 m³/ha, 100-100-50 NPK fertilization, breaking the tip of plant, pests and diseases control). The planting was carried out in sandy soil, with the experiment being set up as randomized block design in 3 replicates with 30 plants per cultivar. From each cultivar 10 fruits in 3 replicates were collected in order to perform chemical analysis.
Analytical methods
Several analyses have been performed: dry matter, soluble solids, ascorbic acid, total phenolic and total flavonoid content, capsaicin content and antioxidant activity. The dry matter content was determined by removing water from the sample in an oven at 105 °C and the result was expressed in percentages. The soluble solids were measured with a digital refractometer (Hanna Instruments, Woonsocket, USA) from the juice pressed from the fruit, the results being expressed in percentages.

Ascorbic acid content
Ascorbic acid was extracted and analyzed by reversed phase HPLC. Fresh pepper homogenate (5 g) was mixed and diluted with 2% HCl. After 20 minutes the solution was centrifuged at 4200 rpm for 10 min. The supernatant was filtered through 0.2 mm pore size filter. The ascorbic acid in the sample test solution was separated by reversed phase chromatography on a 250 mm × 4.6 mm i.d., 5 μm particle Hypersil Gold Aq Analytical Column, of which was detected by absorbance and quantified with external calibration graph. The detector was set at λ=254 nm. This setting was chosen since ascorbic acid has its maximum optical absorbance close to 254 nm. The HPLC analysis was performed with a Surveyor Thermo Electron system (Thermo Electron Corporation, San Jose, CA, USA) comprising a vacuum degasser, Surveyor Plus LCPMPP pump, Surveyor Plus ASP autosampler and diode array detector with 5 cm flow cell. Integration, data storage and processing were performed by Chrom Quest 4.2 software. The determinations were made in isocratic conditions, at 10 °C, using a mobile phase made of 50 mM phosphate solution filtered through a polyamide membrane (0.2 μm) and degassed in a vacuum. The flow rate of the mobile phase was 0.7 mL/min for all the chromatographic separations. The volume injected was 5 μL for either prepared sample or standard solution. The results were expressed in mg kg⁻¹ fresh weight (fw). All reagents were acquired from Sigma-Aldrich, Germany and ultrapure water was obtained from a Milli-Q water purification system (TGI Pure Water Systems, USA).

Total phenolic content
Total phenolic content was assessed by using the Folin-Ciocalteu phenol reagent method (Singleton and Rossi, 1965). Folin-Ciocalteu reagent (2 N, Merck), gallic acid (99% purity, Sigma-Aldrich), anhydrous sodium carbonate (99% Sigma-Aldrich) were used. One g dried pepper homogenate was extracted with 15 mL ethanol in an ultrasonic bath for 60 min at ambient temperature. After extraction, the samples were centrifuged for 5 min at 4200 rpm and supernatants were filtered through polyamide membranes with pore diameter of 0.45 μm and stored at a temperature of -20 °C. The absorbance at 765 nm of each mixture was measured on a Varian Cary 50 UV-VIS spectrophotometer (Varian Co., USA). The absorbance of the mixture was measured at 415 nm on a Varian Cary 50 UV-VIS spectrophotometer (Varian Co., USA). The quantification of flavonoids was carried out on the basis of a calibration curve using quercetin as standard reference in the range of 0-100 mg/L. The results were expressed as mg of quercetin equivalents kg⁻¹ dw.

Capsaicin content
Identification and quantification of capsaicin were carried out by HPLC following the method described by Zhuang et al. (2012), with some modifications. For sample preparation, fresh matter (pulp transformed into a paste by a disintegrator) was dried in an oven at 55 °C to a constant weight and the dried matter was ground finely in a blender (Polytron). An amount of 1 g of the dried matter was extracted with 20 mL of ethanol 95%. After 30 min, the extract was kept in an ultrasonic bath for 60 min at 60 °C and filtered through a 0.2 mm pore size filter. HPLC-DAD analysis was performed on a Finnigan Surveyor Plus system (Thermo Electron Corporation, San Jose, CA, USA) coupled with diode array detector (PDADP with cell flow in 5 cm). The separation was performed using a Hypersil Gold aQ column (250 × 4.6 mm) with a particle size of 5 μm. Capsaicin was detected at 278 nm and quantified (μg kg⁻¹ dw) using standard compound. The determinations were made in isocratic conditions at 25 °C, using a mobile phase made of 50 mM acetoniitrite solution (water:acetoniitrite 57:43) filtered through a polyamide membrane (0.2 μm) and degassed in a vacuum. The flow rate of the mobile phase was 1 mL min⁻¹ for all the chromatographic separations. The volume injected was 5 μL for either prepared sample or standard solution. For device control, data acquisition and processing, Chrom Quest 4.2 software was used.

Antioxidant activity
The antioxidant activity (AOX) was measured in the ethanolic extract using the DPPH (2,2-diphenyl-1-picylhydrazil) assay. Ethanol (Merck, Germany), DPPH (2,2-diphenyl-1-picylhydrazil) (Sigma-Aldrich, Germany) and Trolox (Merck, Germany) were employed. The extraction of samples was made according to the same protocol described for total phenolic content. The free radical scavenging ability of the extracts against DPPH free radical was evaluated as described by Oliveira et al. (2008), with some modifications. Each ethanol pepper extract (50 μL) was mixed with 3 mL of a 0.004% (v/v) DPPH methanolic solution. The mixture was incubated for 30 min at room temperature in the dark and the absorbance was measured at 517 nm on Varian Cary 50 UV-VIS spectrophotometer. The DPPH free radical scavenging ability was calculated in reference to Trolox (6-hydroxy-2,3,7,8-tetramethylchroman-2-carboxylic acid), which was used as standard reference to convert the inhibition capability of each extract solution to the mmol Trolox equivalent antioxidant activity/L. The radical was freshly prepared and protected from light. A blank control of methanol/water was used in each assay. All assays were conducted in triplicate and results were expressed in mmol Trolox kg⁻¹ dw.

Statistical analysis
Significance of differences between cultivars was determined by performing a one-way ANOVA test using Statgraphics Centurion XVI Software (StatPoint Technologies, Warenton, VA, USA).
Results and discussion
The data obtained concerning the dry matter, soluble solids and ascorbic acid content of the hot peppers are presented in Tab. 1. The dry matter content (%) in hot peppers followed an upward trend during growth and ripening except the F2 (20 days after flowering) where a small absolute decrease was noticed. However, the dry matter content varied little in immature stages but showed high increase in ripening stages with a maximum in the physiological maturity, corresponding to the ripening process of fruits, which was manifested through the synthesis of metabolites with complex molecular structure. The same changes were reported by Niklis et al. (2002). There were also differences among cultivars, the highest content of dry matter being found in the ‘Pepperone’ cultivar while the ‘Pintea’ cultivar registered the lowest content. The differences among cultivars were maintained during all the stages of growth and ripening. Regarding the soluble solids content (%), it was found that it had the same upward trend during growth and ripening except the ‘Pintea’ cultivar which registered a small decrease in F2 and ‘Bulgarian carrot’ cultivar which registered the same decrease in F3. The highest rate of accumulation of soluble substances was found in the last stages with similar values to those found by Dorni et al. (2005). Dorni et al. also found that the highest changes in soluble solids happened in the last stages of ripening and in the firm red stage, respectively. There were differences among cultivars: the highest content of soluble solids being found in the ‘Bulgarian carrot’ (9.82%) followed by ‘Christmas bell’ (7.40%).

In all cultivars the ascorbic acid content was positively correlated with the dry matter and the soluble solids content, which is in accordance with the data presented by Niklis et al. (2002).

Considering that the glucose is the precursor in the ascorbic acid synthesis (Davey et al., 2000; Niklis et al., 2002) it is understandable why there is a positive correlation between dry matter, soluble solids and ascorbic acid content. The highest accumulation of ascorbic acid was recorded between the first phenophases (F1-F2). In this period in all cultivars high plant growth rates were observed.

The data obtained are comparable to data presented in the literature, the upward trend being also described by Kumar and Subba Tata (2009) and Martinez et al. (2005). There were significant differences among cultivars, the highest content of ascorbic acid being found in the ‘Bulgarian carrot’ cultivar (1606.47 mg kg⁻¹ fw) and the lowest content in the ‘Pintea’ (1161.35 mg kg⁻¹ fw) cultivar.

According to the classification of hot pepper cultivars depending on the content of ascorbic acid described by Khadi et al. (1987) and Simonne et al. (1997), the analyzed cultivars fall into the category of an average content of ascorbic acid (1010-2000 mg kg⁻¹ fw) even if analyzing in terms of species, chilli is considered a species with high ascorbic acid content.

The results obtained on the content of phenolics, total flavonoids, and antioxidant activity (AOX) in hot peppers are shown in Tab. 2. Antioxidant activity is an important parameter to establish the health functionality of a food product and there are many methods used for its measurement (Kaur and Kapoor, 2001). AOX increased during growth and ripening of the hot peppers, the highest levels being found in the last stage (F5). There were differences among cultivars, the highest AOX level being found in the ‘Pintea’ cultivar (20.04 mmol Trolox kg⁻¹ dw) and the lowest in the ‘Dracula’ cultivar. The differences between cultivars were also reported by Zhuang et al. (2012), who mentioned some pepper extracts as effective electron donors. Hot peppers contain several flavonoids in different forms including glycosides (Baë et al., 2012). The data presented in Tab. 2 show important variations in the content of phenolics and total flavonoids in hot peppers. The total phenolic content increases during growth and ripening with the highest level in the last stage of ripening, the results being in accordance with the data presented by Zhuang et al. (2012) and Deepa et al. (2007).

Righetto et al. (2005) mentioned that the content of phenolics is

| Tab. 1: Dry matter, soluble solids and ascorbic acid content in hot peppers during growth and ripening. Different letters within the same row indicated significant differences (P < 0.05) among cultivars |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | Dracula         | Pintea          | Pepperone       | Bulgarian carrot | Christmas bell  |
| Dry matter (%)                 |                 |                 |                 |                 |                 |
| F1 10.11±1.41c                  | 11.38±1.09b     | 11.80±1.41ab    | 12.39±1.36a     | 12.53±1.62a     |
| F2 9.99±1.19c                   | 10.65±1.17bc    | 10.50±1.29b     | 10.12±1.11bc    | 12.13±1.49a     |
| F3 10.86±1.75c                  | 11.47±1.37b     | 13.31±1.73a     | 10.57±1.26c     | 12.73±1.52ab    |
| F4 15.96±2.01b                  | 14.17±1.73bc    | 15.42±2.15b     | 18.00±2.52a     | 13.66±1.77c     |
| F5 17.43±2.26b                  | 16.16±2.02c     | 18.71±2.6a      | 18.09±2.48a     | 17.53±2.38ab    |
| Soluble solids (%)              |                 |                 |                 |                 |                 |
| F1 4.45±0.53b                   | 5.45±0.65a      | 4.70±0.56ab     | 3.72±0.40c      | 3.86±0.41c      |
| F2 4.67±0.61ab                  | 4.37±0.48c      | 4.54±0.49b      | 5.10±0.58a      | 4.66±0.51ab     |
| F3 5.64±0.69bc                  | 5.82±0.62b      | 7.10±0.91a      | 4.57±0.54c      | 6.00±0.72ab     |
| F4 7.77±0.93ab                  | 4.80±0.52d      | 7.60±0.98b      | 8.32±1.05a      | 6.66±1.86c      |
| F5 8.40±1.09b                   | 8.10±1.03b      | 9.65±1.32ab     | 9.82±1.27a      | 7.40±1.96c      |
| Ascorbic acid (mg kg⁻¹ fw)      |                 |                 |                 |                 |                 |
| F1 170.32±15.40a                | 152.80±12.80b   | 169.85±14.20ab  | 173.62±13.60a   | 105.43±90.80c   |
| F2 1186.54±108.70a              | 1102.38±97.30d  | 1188.21±99.10a  | 1178.89±86.70b  | 1158.40±109.20c |
| F3 1104.62±100.40c              | 1123.19±104.10bc| 1191.05±110.50b| 1289.21±116.90a| 1259.47±114.90a|
| F4 1211.20±109.60c              | 1149.52±98.30dc | 1176.23±105.80d| 1297.58±113.70b| 1393.62±121.40a|
| F5 1383.09±118.70c              | 1161.35±106.10d| 1387.11±120.80bc| 1603.57±147.50a| 1446.47±137.30b|
affected by the type and cultivar of pepper, maturity and cultural conditions which are in accordance with the data presented in Tab. 2, the highest content in phenolics (F5) being found in the 'Pintea' cultivar (8331.25 mg GAE kg⁻¹ dw) and the lowest in the 'Bulgarian carrot' cultivar (2964.25 mg GAE kg⁻¹ dw). The total flavonoids content grew strongly in the last stage of ripening. There are major differences between cultivars regarding the content of capsaicin, the highest level being found in the 'Pintea' cultivar (2229.81 mg kg⁻¹ dw) with an equivalent pungency of 35676.96 SHU followed by the 'Bulgarian carrot' and 'Pepperone'. The lowest level of capsaicin content was found in the 'Dracula' cultivar (24.85 mg kg⁻¹ dw) with an equivalent pungency of 397.68 SHU. Similar differences between cultivars were reported by Estrada et al. (2000) and Kirschbaum-Titze et al. (2002). There are major differences between cultivars regarding the content of capsaicin, the highest level being found in the 'Pintea' cultivar (2229.81 mg kg⁻¹ dw) with an equivalent pungency of 35676.96 SHU followed by the 'Bulgarian carrot' and 'Pepperone'. The lowest level of capsaicin content was found in the 'Dracula' cultivar (24.85 mg kg⁻¹ dw) with an equivalent pungency of 397.68 SHU. Similar differences between cultivars were reported by Usman et al. (2014), Véra-Guzmán et al. (2011) and Gnyafeed et al. (2001). To express the pungency level of the studied cultivars, capsaicin content (grams of capsaicin per grams pepper dry weight) was converted to Scoville Heat Units by multiplying it by the coefficient of the heat value (1.6 × 10⁷) (Tilahun et al., 2013). The data obtained are shown in Tab. 3.

Scoville score analysis showed that the 'Dracula' cultivar is classified as 'non-pungent' (fruits are not spicy) while 'Pintea' is classified as 'highly pungent', the other analyzed cultivars being classified as 'moderately pungent'. Although the 'Pintea' cultivar registered the highest capsaicin content and the highest level of pungency, it is very far from the first places on the Scoville scale (Scoville units between 200,000 and 300,000 – Capsicum chinense, while very spicy peppers from Thailand only reach 100,000).
However we can say that the fruits of ‘Pintea’ cultivar are swifter than the Mexican Jalapeno or the Italian Peperoncino cultivars that barely reach a score of 500-5,000 Scoville Units (Mathur et al., 2000).

### Tab. 3: The pungency of the hot peppers

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Maximum content of capsaicin mg kg⁻¹ dw</th>
<th>Scoville Units</th>
<th>Pungency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dracula</td>
<td>24.85±2.89</td>
<td>397.68</td>
<td>Non-pungent (0-700 SHU)</td>
</tr>
<tr>
<td>Pintea</td>
<td>2229.81±220.37</td>
<td>35676.96</td>
<td>Highly pungent (25000-70000 SHU)</td>
</tr>
<tr>
<td>Pepperone</td>
<td>777.49±75.69</td>
<td>12439.92</td>
<td>Moderately pungent (3000-25000 SHU)</td>
</tr>
<tr>
<td>Bulgarian carrot</td>
<td>990.37±93.15</td>
<td>15846.00</td>
<td>Moderately pungent (3000-25000 SHU)</td>
</tr>
<tr>
<td>Christmas bell</td>
<td>187.90±17.73</td>
<td>3006.48</td>
<td>Moderately pungent (3000-25000 SHU)</td>
</tr>
</tbody>
</table>

### Conclusions

There are major differences among cultivars in the accumulation of the bioactive compounds in the fruit during their growth and ripening, although the quantitative accumulation pathway of various components had a similar trend during phenophases. The dry matter content varied little in immature stages but a large increase was observed at ripening stages with a maximum at the physiological maturity. The soluble solids content had the same upward trend during growth and ripening and the ascorbic acid content was positively correlated with the dry matter and the soluble solids content. Antioxidant activity increased during growth and ripening of hot peppers, the highest levels being found in the last stage of ripening. Although the pattern of variation of total flavonoid content was affected by the cultivar, a lower value was recorded 30 days after flowering (F3) as compared with F1 in all cultivars. Also, in most cultivars, an important increase of the total phenolic and total flavonoid content was observed in the last stage of ripening. Cultivar’s greatest influence on the accumulation of bioactive compounds was observed regarding the content of capsaicin that was found in peppers in very small amounts (undetectable in the ‘Bulgarian carrot’ and ‘Christmas bell’ cultivars) in F1. During growth and ripening, fruit content of capsaicin increased to a maximum level (F3 or F4), then declined until the full ripening of the pepper fruits.

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Address of the authors:
Mira Elena Ionica, Violeta Nour: University of Craiova, Faculty of Horticulture, Department of Horticulture and Food Science, 13 A.I.Cuza Street, 200585, Romania
E-mail: vionor@yahoo.com
Ion Trandafir: University of Craiova, Faculty of Sciences, Department of Chemistry, 107 Calea Bucuresti Street, 200529, Romania

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