

CAPSAICIN, PHENOLS AND FLAVONOIDS QUANTIFICATION IN LESKOVAC AND HABANERO HOT PEPPERS

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Both as food and spice, pepper is unavoidable on the tables in Serbia and many other countries. Because of its popularity, the contents of capsaicin, total phenols and total flavonoids, as well as the antioxidant activity of hot peppers extracts (Leskovac hot pepper, so called “džinka” and chili Habanero pepper) were examined in this paper. The extracts were obtained by Soxhlet extraction with 96% v/v ethanol. The content of capsaicin in dry extracts of the studied hot peppers was calculated by the HPLC method (13.038 mg/g d.e. for “džinka” and 76.516 mg/g d.e. for Habanero pepper, respectively). Total phenol and total flavonoids contents were examined by the UV-VIS method. Leskovac “džinka” was characterized by both higher contents (47.17 mgGAE/g d.e. and 14.64 mgGAE/g d.e., respectively) in comparison to chili Habanero pepper (29.43 mgGAE/g d.e. and 0.25 mgGAE/g d.e., respectively). DPPH test was used for the antioxidant activity determination. The EC₅₀ values obtained indicate a better antioxidant activity of Leskovac “džinka” (1.760 mg/ml) compared to chili Habanero pepper (6.016 mg/ml).

Keywords: *Capsicum annum* L., *Capsicum chinense* Jacq., Capsaicin, Extraction

Introduction

Pepper fruit (*Capsicum* spp.) is a very well-known and useful crop worldwide in different cultures and gastronomies [1]. The genus *Capsicum* comprises more than 200 varieties and five main species: *Capsicum annum* (comprising the NuMex, Jalapenõ and Bell varieties), *Capsicum chinense* (Habanero and Scotch Bonnet varieties), *Capsicum frutescens* (Tabasco variety), *Capsicum baccatum* (Aji varieties) and *Capsicum pubescens* (Rocoto and Manzano varieties) [2]. The fruits vary widely in the size, shape, flavour and sensory heat, and total capsaicinoids, colour and volatile compounds were determined [3]. Some of them are a rich source of alkaloid capsaicin (*trans*-8-methyl-N-vanillyl-6-nonenamide) [4]. The total content of capsaicinoids is usually expressed in pungency. This sum of the concentrations of capsaicinoids is calculated in the Scoville scale [5]. Pure capsaicin has a Scoville heat value (SHV) rating of approximately 16000000 compared to 150000 SHV in habanero peppers [6].

The excessive exposure to capsaicin can cause irritation or respiratory problems, as well as some types of cancers due to high-quantity ingestion [7-9]. Some studies have shown that capsaicin possesses positive effects in the treatment of rheumatoid arthritis and headaches [10]. However, some other studies confirmed its irritative and analgesic properties. Also, it has a slimming effect affecting thermoregulation and the adipose tissue metabolism which

is characterized by hypotensive and anticancer effects [11]. Anticancer effects of capsaicin are related to the induction of cancer cell apoptosis [12, 13]. The results of some studies have shown that dietary capsaicin may have a potential use in the prevention of Alzheimer's disease [14] and also in the protection of cardiometabolic organs from dysfunction [15]. Capsaicin may have the influence on hypothermia by the dilatation of blood vessels in the skin and increase the heat exchange. On the other hand, this alkaloid may cause the increase in the metabolic activity [16]. Capsaicin may be used as an ingredient in insect repellents, as well as a biochemical pesticide [17]. Some experiments on cats have shown that capsaicin induced pulmonary chemoreflex consisting of prompt apnea, bradycardia and hypotension [18]. On the other hand, capsaicin applied into the stomach of rats or cats inhibited gastric acid secretion [19]. Capsaicin has been used to treat foot pain, laminar ischemia and arthritis [20] in the therapy of animals. In beef cattle studies, capsaicin oleoresin was applied as a feed additive [21].

Capsaicin also possesses antimicrobial effects (against *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Helicobacter pylori* and *Streptococcus pyogenes*). Because of that, it could be used as a natural inhibitor of pathogenic microorganisms in food [22-25]. The antioxidant effect of capsaicin has been reflected on the neutralization of free radicals [26].

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The extraction techniques for capsaicin from peppers include maceration, magnetic stirring, enzymatic extraction, microwave-extraction, ultrasound-assisted extraction, Soxhlet extraction, supercritical fluid and pressured liquids extraction [27]. Solid-liquid extraction with solvents such as hexane, chloroform, and ethanol is the most commonly employed method for the capsaicin recovery [28,29]. At-tuquayefio and Buckle [30] examined capsaicin extraction yields from *Capsicum annum* (cayenne pepper) using acetone (the highest yield), chloroform, methanol, acidified methanol, and acetonitrile. Kurian and Starks [31] extracted capsaicin from orange habanero peppers (*C. chinense*) using methanol and reported yields of 1.25 mg/g pepper (8840 ppm).

Leskovac chili pepper called "džinka" is a domestic product present in our region since ancient times, unlike Habanero chili pepper which has been found in our market lately. There are no data available in literature for Leskovac "džinka" described in this paper, except for its sensory characteristics that have been examined [32]. Therefore, the aim of the paper was to compare the total phenol and flavonoids content, the antioxidant activity and the capsaicin content of those two hot peppers as frequently consumed products. Further work will be focused on optimizing the extraction process in order to obtain an extract suitable for incorporation into some products (food, pharmaceutical industries, etc.).

Experimental

Plant material

Hot pepper "džinka", a type of rosehip (*Capsicum annum* L.), was grown on the farm "Stamenković" (Trnjane village, Leskovac municipality). Hot red chili pepper type Habanero (*Capsicum chinense* Jacq.) was from the farm "Milošević" (Bogojevce village, Leskovac municipality). The whole pepper fruits were air-dried in a shady site for 15 days during the summer and ground in the electric mill.

Extraction procedure

Hot pepper extracts were obtained by Soxhlet extraction with 96% v/v ethanol as a solvent. Fifty grams of the plant material and 320 ml of the solvent were used, with 7 presiphons. The final volumes of the extracts were 240 ml (Leskovac "džinka") and 305 ml (Habanero).

The obtained extracts (2 ml of the samples) were dried in the dryer at 105 °C until constant mass and the concentrations of the extracts were calculated on the basis of the dry residue content.

Total phenols content

Total phenols content was determined according to the Folin-Ciocalteu procedure [33,34] with specified modifications: 0.5 ml methanolic solution of the plant extract (0.25 mg/ml), 4.5 ml of water and 0.5 ml of Folin-Ciocalteu reagent were mixed. After 5 minutes, 5 ml of 7% Na₂CO₃ was added. The absorbance was read after 90 minutes at 765 nm (Cole-Parmer spectrophotometer) against blank con-

taining distilled water instead of the extract. Total phenols content was calculated from the calibration curved constructed under the same experimental conditions using gallic acid solutions in the range from 0.00625 mg/ml to 0.2 mg/ml as a standard substance, and expressed as gallic acid equivalents per dry extract (mgGAE/g of the dry extract).

Total flavonoids content

The total flavonoids content was determined according to the aluminium chloride colorimetric method [34,35]. Ethanolic solutions of plant extracts (2 ml, 0.25 mg/ml) were mixed with 0.1 ml of 10% aluminium chloride hexahydrate solution, 0.1 ml of 1 M potassium acetate solution and 2.8 ml of distilled water. After 40 minutes of incubation at the room temperature, the absorbance of the reaction mixture was determined spectrophotometrically at 415 nm (Cole-Parmer spectrophotometer). Rutin (0.005-0.100 mg/ml) was used as a standard and the total flavonoids content was expressed as rutin equivalents per dry extract (mgRE/g of the dry extract).

Antioxidant activity

DPPH test was used to determine the capacity of the ethanol extracts to scavenge free 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Dry extracts obtained according to the procedure described above were dissolved in ethanol and the ethanolic extract solutions with different concentrations were prepared. One milliliter of DPPH ethanolic solution (300 µmol/l) and 2.5 ml of the extract ethanolic solution were mixed and after 20 min the absorbance value at 517 nm was read (Cole-Parmer spectrophotometer). The scavenging capacity was calculated from the following equation [36]:

$$\text{DPPH radicals scavenging capacity} = 100 - \frac{100 \cdot (A_s - A_b)}{A_c} \dots\dots\dots(1)$$

where A_s , A_b and A_c represent absorbance values read at 517 nm for the ethanolic solution of the extract treated with DPPH radical solution; for the non-treated ethanolic solution of the extract and for the pure, ethanolic solution of DPPH radical, respectively. From the curve of dependence of calculated DPPH scavenging capacities on the concentrations of the extract ethanolic solution, the concentration of the extract needed for neutralization of 50% of DPPH radical - EC₅₀ (mg/ml) was determined.

All reagents were of analytical grade. The reagents and standards of gallic acid and rutin were purchased from Sigma-Aldrich (Darmstadt, Germany). All experiments were performed in triplicate.

Capsaicin content in the extracts (HPLC chromatography)

The samples were filtered through 0.45 µm filter (Thermo Scientific, Germany) and analyzed with Agilent 1100 Series system (Waldbron, Germany), equipped with a binary pump (Agilent 1100 Series), an autosampler (Agilent 1200 Series) and a DAD detector (Agilent 1200 Series, wavelength range 190-800 nm). The sample volume, in-

jected in the mobile phase under the flow rate of 1.0 ml/min was 10 μ L. The samples were separated into individual components on Zorbax Eclipse Plus C18 column (4.6 \times 250 mm, 5 μ m), thermostated at 25 $^{\circ}$ C. The mobile phase consisted of 0.1% v/v formic acid in water (A) and acetonitrile (B). The samples were eluted using the following gradient: the linear gradient from 0% B to 50% B during the first 30 minutes, followed by the linear gradient to 100% B from 30th to 40th minute, then an isocratic run with 100% B for the next three minutes, and finally a linear gradient to 0% B from 43th to 50th minute. The isocratic run of 0% B for the next 5 minutes was used to re-establish the initial conditions before the injection of another sample. All separated components present in the studied extracts arrived to a DAD detector. The capsaicin content was determined from the signal obtained at 280 nm corresponding to its absorption maximum wavelength. Ten concentrations of standard capsaicin ranging from 0.006 mg/ml to 1 mg/ml were used for the calibration curve construction by plotting the peak area against the concentration of analyte.

Results and discussion

The content of the dry matter, total phenols and total flavonoids in the extracts are shown in Table 1. The content of the dry matter in the liquid extracts of Leskovac "džinka" (L) and Habanero chili pepper (H) were approximately the same. However, the content of total phenols and total flavonoids was significantly different. The content of total phenols was 1.6 times and of total flavonoids about 60 times higher in Leskovac "džinka" than in Habanero pepper.

Table 1. The content of the dry matter, total phenols and total flavonoids in the extracts

Sample	Dry matter, mg/ml	Total phenols, mgGAE/g d.e.	Total flavonoid, mgGAE/g d.e.
Leskovac "džinka"	32.00 \pm 1.35	47.17 \pm 0.24	14.64 \pm 0.06
Habanero chili pepper	33.45 \pm 0.89	29.43 \pm 0.81	0.25 \pm 0.00

Pérez-Ambrocio et al. [37] examined the content of bioactive compounds in *C. chinense*. Their results for total phenols (8.3 mgGAE/kg) and total flavonoids (9.0 mg/kg) did not match our results. They proved that blue and UV-C light may stimulate the synthesis of chlorophylls and total carotenoids (the first days of storage), total flavonoids and phenolic compounds.

The results of the antioxidant activity test are shown in Figure 1 and Table 2. Leskovac "džinka" had a higher antioxidant activity than Habanero for about 3.5 times. According to the total phenols and total flavonoids content shown in Table 1, it could be concluded that these compounds are probably responsible for the antioxidant activity observed. Namely, in the Leskovac "džinka" extract, showing a better activity (lower EC₅₀ value, Table 2), a significantly higher content of total phenols and total flavonoids was determined.

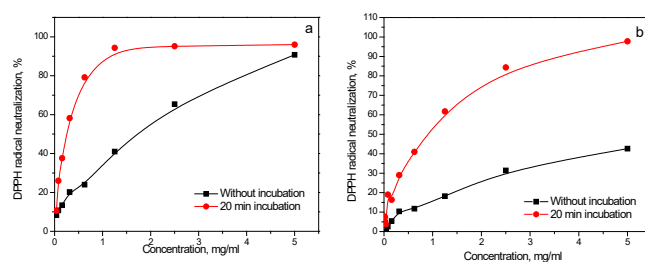


Figure 1. DPPH antioxidant activity (a - Leskovac "džinka"; b - Habanero chili pepper)

Table 2. EC₅₀ values of the extracts from Leskovac "džinka" and Habanero chili pepper

Sample	EC ₅₀ , mg/ml	
	Without incubation	20 min incubation
Leskovac "džinka"	1.760	0.267
Habanero chili pepper	6.016	1.008

EC₅₀ - extract concentration required for neutralization of 50% of the starting concentration of DPPH radicals

Habanero pepper treated with 3 and 0.5 min of blue and UV-C light, respectively, shows higher values of bioactive compounds and the antioxidant capacity during the storage [37]. The antioxidant capacity could be increased depending on the maturity process [38].

The capsaicin content was about 6 times higher in the Habanero chili pepper than in the Leskovac "džinka" (Table 3). HPLC chromatograms of the extracts examined, and the UV/Vis spectrum of capsaicin, as well as the calibration curve of capsaicin are shown in Figures 2-4.

Table 3. Capsaicin content in the extracts

Sample	Capsaicin content	
	mg/ml	mg/g d.e.
Leskovac "džinka"	0.417	13.038
Habanero chili pepper	2.559	76.516

d.e. - dry extract

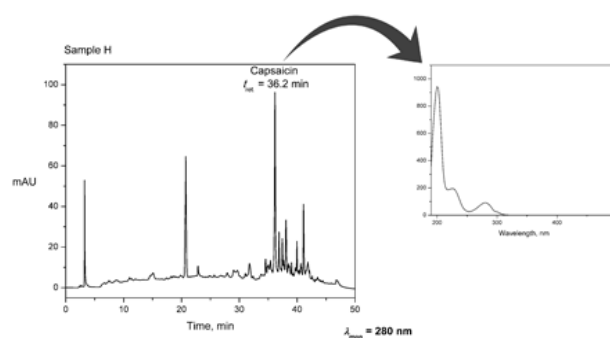


Figure 2. HPLC chromatogram of the extract and UV/Vis spectrum of capsaicin from Leskovac "džinka"

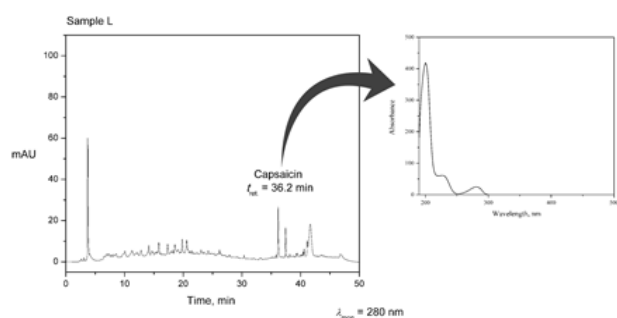


Figure 3. HPLC chromatogram of the extract and UV/Vis spectrum of capsaicin from Habanero chili pepper

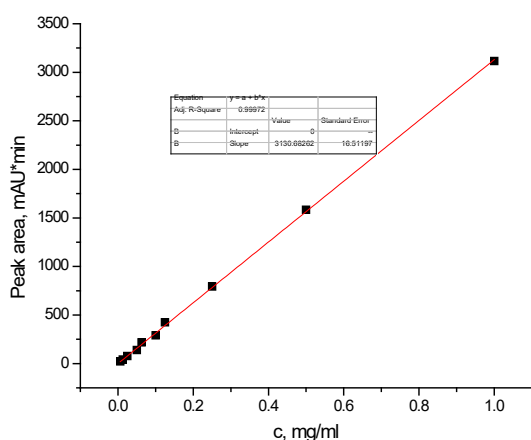


Figure 4. Calibration curve of capsaicin ($y = 3130.68262x$, $R^2 = 0.99972$)

An insight into the scientific literature reveals that the content of capsaicin (as well as other compounds) depends on the plant species, variety, habitat, temperature, humidity and other conditions. Because of this, there are differences in the content of total phenols and total flavonoids between our hot peppers and the hot peppers grown in other geographical areas. The same refers to the capsaicin content.

During storage, a total capsaicin content increased the reaching values of 39.4 mg of capsaicin/kg being responsible for the maturity process [38]. Anatol Schmidt et al. [39] were investigating the relation between capsaicin and dihydrocapsaicin in chilies. The ratio ranged from 1.3 to 4.7 in favor of capsaicin. In Habanero chocolate (placenta and seeds), capsaicin (12396 $\mu\text{g/g}$ of the dried weight) and dihydrocapsaicin (3715 $\mu\text{g/g}$ of the dried weight) were found to be the highest. In Habanero orange (whole fruit) the content of the two compounds was slightly lower (11841 $\mu\text{g/g}$ of the dried weight and 6022 $\mu\text{g/g}$ of the dried weight, respectively).

Total capsaicinoids, color and volatile compounds of 10 Habanero chili peppers (*Capsicum chinense* Jack.), grouped by their colors: four red, five orange and one brown were determined. The content of capsaicinoids, responsible for the pungency of chili peppers varied be-

tween 41.8 and 65.9 mg/g dry fruit [3].

Lu, Ho and Huang [27] were investigating the extraction process, bioavailability and bio-efficacy of capsaicinoids from different species of peppers. The capsaicin quantity of 0.54 g/kg and dihydrocapsaicin of 0.41 g/kg of the dried ground sample were found in yellow habanero fruit.

Cisneros-Pineda et al. [40] were examining the content of capsaicin and dihydrocapsaicin in different dry parts of chili peppers (pericarp, placenta, seeds). The capsaicin quantity of 62886 $\mu\text{g/g}$ and dihydrocapsaicin of 1607 $\mu\text{g/g}$ were found in the dry placenta of Habanero orange. The Habanero white placenta contains less capsaicin 29483 $\mu\text{g/g}$ and more dihydrocapsaicin 2349 $\mu\text{g/g}$. Less content was found in pericarp and seeds.

The capsaicinoid extraction from peppers is typically performed using different kinds of solvents (ethanol, acetone and acetonitrile). However, the extraction efficiencies can vary with peppers, their parts (seeds, shells) and pre-extraction processing (freeze and oven drying material) [41]. Capsaicin appears to be a substance with many beneficial effects. It can be cardio-protective, analgesic have an antiinflammatory effect, thermogenic influence, beneficial effects on gastrointestinal system and potential clinical value for pain relief, cancer prevention and weight loss [42]. Therefore, it is proposed to continue studies of the effects of capsaicin with a particular reference to new applications of this alkaloid.

Conclusion

Unlike Leskovac "džinka", Habanero chili pepper has been extensively studied. The results shown in this paper were expected with respect to the capsaicin content. Leskovac "džinka" has lower capsaicin content than Habanero. However, other results are in favor of our hot pepper. Leskovac "džinka" has more total phenols and total flavonoids, as well as a more pronounced antioxidant activity. Although it is widely used now, it opens the possibility of being used in the production of various types of food products with a certain amount of spiciness.

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Izvod

ODREĐIVANJE SADRŽAJA KAPSAICINA, FENOLA I FLAVONOIDA U LJUTIM PAPRIKAMA LESKOVAČKA DŽINKA I HABANERO

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U ljutim paprikama leskovačka džinka i čili habanero određen je sadržaj kapsaicina, sadržaj ukupnih fenola i flavonoida, i antioksidantna aktivnost. Ekstrakti su dobijeni Soxhlet ekstrakcijom sa 96% V/V etanolom. Sadržaj kapsaicina u suvim ekstraktima ljutih paprika (13,038 mg/g s.e. i 76,516 mg/g s.e., respektivno) izračunat je HPLC metodom. Sadržaj ukupnih fenola i ukupnih flavonoida određen je UV-VIS metodom. Leskovačku džinku odlikuje veći sadržaj ukupnih fenola i ukupnih flavonoida (47,17 mgGAE/g s.e. i 14,64 mgGAE/g s.e., respektivno) u odnosu na čili habanero (29,43 mgGAE/g s.e. i 0,25 mgGAE/g s.e., respektivno). Za određivanje antioksidantne aktivnosti korišćen je DPPH test. Ekstrakt leskovačke džinke pokazao je bolju antioksidantnu aktivnost (EC₅₀ vrednost: 1,760 mg/ml) od ekstrakta čili habanero (EC₅₀ vrednost: 6,016 mg/ml).

Ključne reči: *Capsicum annum* L., *Capsicum chinense* Jacq., Kapsaicin, Ekstrakcija