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Research Article

Capsaicinoids content in some Bulgarian varieties of *Capsicum annuum* L. obtained by RP-HPLC

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Abstract

Capsaicinoids are amides, a type of secondary metabolites in hot peppers, responsible for their hot taste also known as pungency. They possess many pharmacological properties with great potential for pharmacy like analgesic, blood glucose level reduction, insulin level improvement, reduction of triglycerides and cholesterol levels, etc. For the needs of this study a reliable and easy applicable RP-HPLC method with UV detection for determination of capsaicinoids was developed. Two traditional and two newly selected Bulgarian hot pepper varieties were studied. The concentrations of capsaicin and dihydrocapsaicin in the pericarp and the seeds were determined respectively. According to the Scoville heat unit (SHU) equivalence of the pericarps, the varieties Dzhulyunska shipka 1021, Zlatna shipka and Kehlibar show moderate pungency and present a potential value for the pharmaceutical and food industries. The current study contributes to the scientific database with regard to the pungency of *Capsicum annuum* L. varieties.

Keywords

capsaicine, dihydrocapsaicine, HPLC determination, *Capsicum annuum*

Introduction

Chili peppers are world famous for their distinctive aroma, color and spiciness. They belong to the genus *Capsicum*, which comprises of some 30 species and more than 200 varieties. However, only five species are domesticated and these are *Capsicum annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L. and *C. pubescens* Ruiz &Pav. So far *C. annuum* appears to be the most economically valuable species with the largest distribution and demand worldwide (González-Zamora et al. 2013; Panayotov et al. 2017; Batiha et al. 2020; Hernandez-Pérez et al. 2020). The chili peppers fruits are an important source of many health beneficial compounds such as ascorbic acid (vitamin C), carotenoids (provitamin A), tocopherols (vitamin E), phenolics, minerals, essential oils, etc. (González-Zamora et al. 2013; Hernandez-Pérez et al. 2020). Among these compounds the capsaicinoids particularly stand out for their unique distribution bound only to the *Capsicum* genus. They are synthesised and mainly accumulated in the placental tissue, but a substantial amount passes into the pericarp as well (Barbero et al. 2014). Capsaicinoids are

alkaloids, which result from enzymatic condensation of vanillylamine produced by the phenylpropanoid pathway and a branched-chain fatty acid produced by the catabolism of amino acids. Several capsaicinoids are known although capsaicin (C) and dihydrocapsaicin (DHC) (Fig. 1) are the major and generally they comprise some 80–90% of the total capsaicinoid content of the individual varieties (Hamed et al. 2019). They possess many remarkable pharmacological properties such as fat energy metabolism enhancement, antiobesity and overweight control, cardiovascular protective, antiplatelet, anticarcinogenic, antioxidant, anti-diabetic, anti-inflammatory, analgesic, gastrointestinal protective, and antimicrobial effect, potential in urinary and dermatological disorders (Kwon et al. 2006; Chaiyasit et al. 2009; Luo et al. 2011; Barbero et al. 2014; Khan et al. 2014; Srinivasan 2015; Bacon et al. 2016; Clark and Seong-Ho 2016; Qin et al. 2017; Zsiboras et al. 2018; Friedman et al. 2019; Batiha et al. 2020; Hernandez-Pérez et al. 2020; Jang et al. 2020).

The aim of the presented study is to investigate the pungency levels of four Bulgarian *Capsicum annuum* L. varieties as a preliminary step in the direction of their potential utilisation for the needs of the pharmaceutical or food industries.

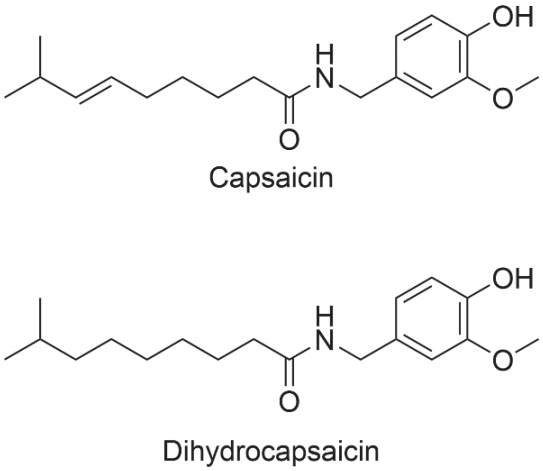

Figure 1. Chemical structure of capsaicin (C) and dihydrocapsaicin (DHC).

Materials and methods

Plant material

The experiments were carried out with four Bulgarian varieties of *Capsicum annuum* L.: Dzhulyunska shipka 1021, Zlatna shipka, Osmarsko kambe and Kehlibar. Dzhulyunska shipka 1021 and Osmarsko kambe are well known and traditional for Bulgaria, while Zlatna shipka and Kehlibar are newly selected at Agricultural University-Plovdiv. The plants were grown in the Experimental field at the Agricultural University-Plovdiv by conventional and traditional for middle early field production technology. All necessary agricultural practices were applied according to the technology and plant development. At the stage of full botanical maturity, randomly from different plants of each variety were harvested a total amount of 500 g fruits. In this phenological phase the fruits of three of the varieties were red, whereas those of Kehlibar were orange in colour. The whole fruits were air dried under shade at room temperature and the pericarps with the attached placenta were separated from the seeds afterwards.

The analytical method described below is based on the method used by Topuz and Ozdemir (2007) but with major changes.

Chemicals and reagents

Gradient grade methanol and acetonitrile were obtained from Honeywell. Glacial acetic acid was purchased from Merck. Capsaicin and dihydrocapsaicin primary standards were obtained from Merck, Sigma-Aldrich.

Sample preparation

About 0.5 g minced dried fruits were sieved through test sieve with 500 μm mesh size and then transferred into 20 ml screw tube. The content of the tube was diluted with 20 ml methanol and then sonicated in ultrasonic bath at 75 °C for 15 min. The resulting extract was cooled to room temperature and filtered through 0.45 μm syringe filter before injecting 20 μl in to the HPLC system.

Instruments

All analyses were performed on an HPLC Thermo Scientific Ultimate 3000 equipped with Chromeleon software for data acquisition, analysis and reporting.

Preparation of the solutions

1% acetic acid was filtered through membrane filter with 0.45 μm pore size.

Standard solution

20.0 mg capsaicin and 20 mg dihydrocapsaicin primary standards were dissolved in methanol into a volumetric flask of 50.0 ml. Then 1.0 ml of the solution was diluted with methanol to the full volume of 20.0 ml volumetric flask ($C_{Capsaicin}$ = 0.020 mg/ml, $C_{Dihydrocapsaicin}$ = 0.020 mg/ml).

Chromatographic conditions

The chromatographic separation was conducted using isocratic elution at ambient temperature (25 °C) on Zodiac C18 column (100 mm × 4,6 mm, 3 μm) with UV detection at 280 nm. The mobile phase was 1% acetic acid and acetonitrile at the ratio of 50:50 (v/v). The flow rate was set at 1.0 ml/min. The injection volume was 20 μl. The chromatography time was set at 10 min. The retention times of Capsaicin and Dihydrocapsaicin were about 5,5 min and 8,1 min respectively.

Conversion of capsaicinoids concentration into Scoville Pungency Scale

The pungency in Scoville heat units was calculated by multiplying the capsaicinoid concentrations in ppm units (1 ppm=1 μg/g dry weight) with the pungency coefficient of the pure compounds as given by Todd et al. (1977), so as 1 ppm C/DHC= 16.1 SHU (Scoville heat units).

Results

The presented method was here originally developed and was validated according to the following parameters: selectivity, linearity, repeatability, recovery and limit of quantitation (LOQ).

Selectivity

The resolution between the peaks of C and DHC is more than 5, and there are no other peaks from the matrix to affect the resolution between the investigated peaks (Fig. 2).

Linearity

The linearity of the method for the assay of capsaicin and dihydrocapsaicin in hot peppers dried fruits is set from 1.30 μg/ml for C and 1.55 μg/ml for DHC to 52 μg/ml and 62 μg/ml respectively. The calculated correlation coefficient is very close to the ideal value 1. The excellent correlation expressed as R^2 is 0.9998 for C and 0.9999 for DHC (Fig. 3). As a first point of the linearity is set the limit of qantitation (LOQ) where the relative standard deviation is less than 3%, and the ratio signal to noise (S/N) is not less than 10.

Repeatability

The repeatability was calculated at concentrations 2.4 μg/ml for capsaicin and 2.9 μg/ml for dihydrocapsaicin (Table 1).

Recovery

The recovery of the extraction procedure was proven by analysis of double extracted samples.

At the first extraction step more than 99% of the capsaicin and dihydrocapsaicin content was found in the investigated samples. The results of capsaicin and dihydrocapsaicin from the second extraction step were several times lower than LOQ so accurate results could not be calculated (Fig. 4).

Limit of quantitation – LOQ

The limit of quantitation is the lowest point where the concentration of the investigated compounds (capsaicin and dihydrocapsaicin) can be determined with relative standard deviation less than 3 per cent (Fig. 5, Table 2).

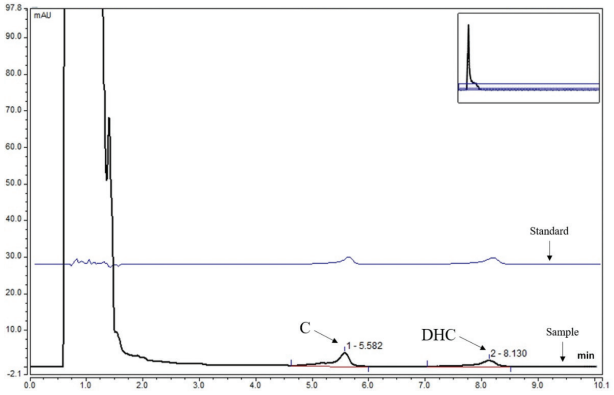

Figure 2. Sample and standard.

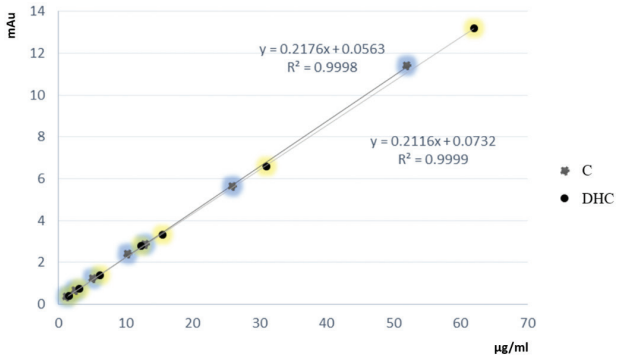

Figure 3. Linearity of capsaicin (C) and dihydrocapsaicin (DHC).

Table 1. Repeatability of the areas of capsaicin and dihydrocapsaicin.

№ of injection	Area (mAU) Capsaicin	Area (mAU) Dihydrocapsaicin
1.	0,5856	0,7012
2.	0,5872	0,6926
3.	0,5834	0,6793
4.	0,5794	0,6919
5.	0,5973	0,6966
6.	0,5992	0,7022
SD*	0,0079	0,0083
Average	0,5887	0,6940
RSD%**	1.3419	1.1960
	1,3419	1,1960

* SD – Standard deviation; **RSD% - Relative standard deviation in percents.

The separation coefficient between the two measured capsaicinoids was conducted via isocratic elution. Its value, which was more than 5, showed excellent baseline separation, which other authors had also observed (Duelund and Mouritsen 2017).

The separate concentrations of C and DHC in the fruit and seeds of the four hot pepper varieties under investigation are given in Table 3.

Based on the results presented in Table 3, the individual varieties can be successfully rated according to their C and DHC content. Dzhulyunska shipka 1021 has the most pungent fruit with their summed up C and DHC

Table 2. Limit of Quantitation (LOQ).

№ of injection	Area (mAU) Capsaicin 1.30 µg/ml	Area (mAU) Dihydrocapsaicin 1.55 µg/ml
1.	0,3161	0,3760
2.	0,3352	0,3863
3.	0,3125	0,3587
4.	0,3204	0,3698
5.	0,3306	0,3785
6.	0,3214	0,3789
SD*	0,0086	0,0095
Average	0,3227	0,3747
RSD%**	2,6650	2,5354

* SD – Standard deviation; **RSD% - Relative standard deviation in percents.

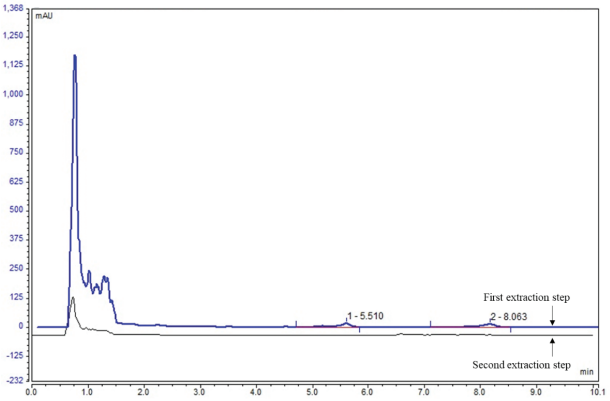

Figure 4. Recovery of capsaicin (C) and dihydrocapsaicin (DHC).

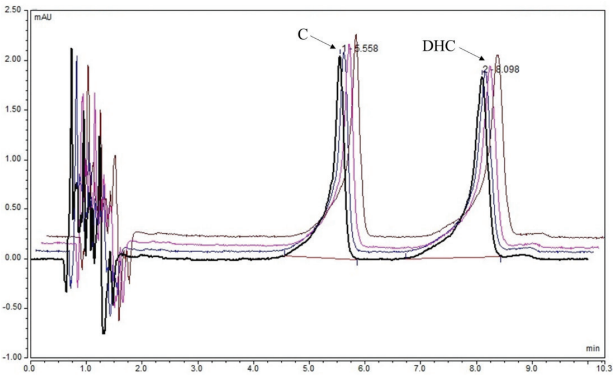

Figure 5. Limit of quantitation of capsaicin (C) and dihydrocapsaicin (DHC).

content 3 times larger than that of the runner-up in the list – Kehlibar (Fig. 6). Interestingly, the seeds of Kehlibar, which was the only one yellow-orange coloured among the samples, proved to be the hottest of all investigated

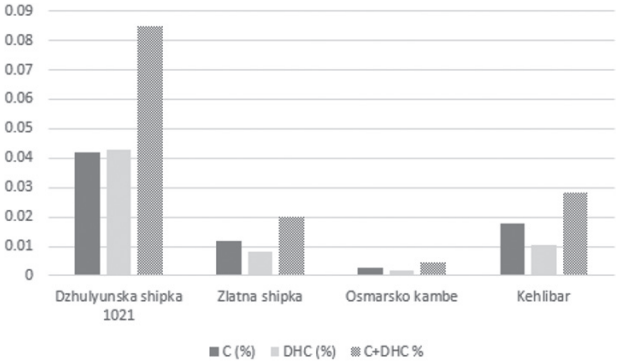

Figure 6. Capsaicin (C) and dihydrocapsaicin (DHC) concentrations in the fruits of four varieties of *Capsicum annuum* L.

seeds. They showed 1.4 times the pungency of Dzhulyunska shipka 1021 seeds and appeared to be even hotter than Osmarsko kambe fruit. The sequence in which the fruit/seed summed up C and DHC content ratio by variety decreases is as follows: Dzhulyunska shipka 1021 (12.1:1) => Zlatna shipka (8.7:1) => Osmarsko kambe (3.9:1) => Kehlibar (2.9:1). Across the different varieties the individual fruit concentrations of C and DHC showed to be positively correlated (Pearson Correlation Test, r=0.98125, N=4, T-statistic=7.20003, df=2, p=0,01875). However, regarding the seeds such a dependence was not observed (Pearson Correlation Test, r=0,84063, N=4, T-statistic=2,19502, df=2, p=0,15937).

In regard to the chili peppers processing, most often the seeds are removed prior to this process. Therefore here is given the SHU equivalent only for fruit without seeds (Table 3). Dzhulyunska shipka 1021, Zlatna shipka and Kehlibar fell into the same category – moderately pungent, although the significant difference in the pungency between Dzhulyunska shipka 1021 and the other two. Dzhulyunska shipka 1021 was 4.25 times hotter than Zlatna shipka and 3 times hotter than Kehlibar. In strict terms, with its 692.3 SHU Osmarsko kambe came under the first category "non-pungent" of the Scoville scale. However due to its proximity to the upper limit of this category and the organoleptically perceptible mild hotness we referred to this sort as "mildly pungent".

Discussion

The studied in this work varieties were chosen in order to be compared two new hot pepper varieties selected in Agricultural University – Plovdiv (Bulgaria) to two

Table 3. Capsaicin (C) and dihydrocapsaicin (DHC) concentrations in the fruit and seeds of four varieties of *Capsicum annuum* L. and SHU for the fruits.

Sort	C(%)		DHC(%)		C+DHC total amount (%)		C+DHC total amount (SHU)
	Fruit	Seed	Fruit	Seed	Fruit	Seed	Fruit
Dzhulyunska shipka 1021	0.0420	0.0034	0.0430	0.0036	0.0850	0.0070	**13685 (moderately pungent)**
Zlatna shipka	0.0120	0.0013	0.0080	0.0010	0.0200	0.0023	**3220 (moderately pungent)**
Osmarsko kambe	0.0025	0.0005	0.0018	0.0006	0.0043	0.0011	**692.3 (mildly pungent)**
Kehlibar	0.0179	0.0065	0.0103	0.0033	0.0282	0.0098	**4540.2 (moderately pungent)**

traditional and well-known Bulgarian varieties in regard to their pungency. Many authors have asserted that capsaicin and dihydrocapsaicin equal together around 80–90% of the total capsaicinoid content in different hot varieties (Zewdie and Bosland 2001; Ishikawa 2003; Eich 2008; Reyes-Escogido et al. 2011; Wahyuni et al. 2013; Barbero et al. 2014), although few altering cases have also been reported (Topuz and Ozdemir 2007; Duelund and Mouritsen 2017). It is also important that capsaicin and dihydrocapsaicin are significantly more pungent compounds in terms of SHU than other capsaicinoids as homocapsaicin, homodihydrocapsaicin and nordihydrocapsaicin (Topuz and Ozdemir 2007; Duelund and Mouritsen 2017). This has resulted in the presented in many publications on the same topic general view of the summed-up capsaicin and dihydrocapsaicin content as a main attribute to the level of pungency, regardless of its approximation. Therefore, in the presented study these two substances were chosen for the analysis of the herein investigated varieties as well.

Bearing in mind that the capsaicinoids content in hot peppers is a genetically controlled trait strongly influenced by the environment and the fruit developmental stage (Burgos-Valencia et al. 2020; Hernandez-Pérez et al. 2020), Table 4 provides a provisional comparison between capsaicin and dihydrocapsaicin content of the analysed Bulgarian varieties and some other varieties, grown in different geographical locations according to the available reference sources. In this sense the Bulgarian varieties with the exception of Osmarsko kambe have similar content of capsaicin and dihydrocapsaicin to the Mexican variety Ancho cv. Don Matias and some varieties reported from Turkey. However, their similarity to Yellow Habanero and Chiltepin grown in Czech Republic according to the underpinned publication should be assigned as misleading since the capsaicinoids content of the latter varieties was strongly influenced by the Soxhlet extraction method (SOX) used. There are evidences for considerable capsaicinoids yield reduction due to this methods in comparison to others, such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and pressurized liquids extraction (PLE) (Lu et al. 2017). For comparison, González-Zamora et al. (2013) established via UAE C and DHC concentrations of 15.36 mg. g^{-1} dry weight and 13.39 mg. g^{-1} dry weight respectively for Chiltepin variety, which corresponds to pungency of 462 875 SHU based on these two compounds solely. Canto-Flick et al. (2008) reported capsaicinoid content of 59.51 mg. g^{-1} fresh weight for Yellow Habanero (Accession NP1EG) whole fruits, which equals to 892 719 SHU.

According to Table 4 the C : DHC ratio varies a lot and there cannot be observed any dependence between this ratio and the pungency level across the indicated varieties. Generally, the prevalence of C or DHC is a matter of the plant developmental stage. For some varieties of *C. annuum* like Cayenne and Padrón the dynamics in capsaicin and dihydrocapsaicin fruit concentrations were studies in strict terms of days after the flowering stage (postanthesis) (Estrada et al. 2002; Kirschbaum-Titze et al. 2002; Barbero et al. 2014).

Table 4. Capsaicin (C) and dihydrocapsaicin (DHC) content of hot pepper varieties with different origin.

Origin	Species	Variety	C	DHC	C:DHC ratio	Extraction method*	Source
			mg g^{-1} dry weight	mg g^{-1} dry weight			
Bulgaria	*C. annuum*	Dzhulyunska shipka 1021	0.420	0.430	1:1.02	UAE	original data
	C. annuum	Zlatna shipka	0.120	0.080	1:0.67		
	C. annuum	Osmarskokambe	0.025	0.018	1:0.72		
	C. annuum	Kehlibar	0.179	0.103	1:0.58		
Mexico	*C. annuum*	Ancho cv. Don Matias	0.290	0.770	1:2.66	UAE	González-Zamora et al. 2013
	C. annuum	De árbol	5.220	6.250	1:1.20		
	C. annuum	Chiltepín	15.360	13.390	1:0.87		
	C. annuum	Guajillo cv. San Luis	0.170	0.610	1:3.59		
	C. annuum	Jalapeño cv. Don Julio	8.030	9.390	1:1.17		
	C. annuum	Puya	1.180	2.320	1:1.97		
	C. annuum	Serrano cv. Don Diego	1.520	3.540	1:2.33		
Czech Republic	*C. chinense*	Trinidad Scorpion Moruga	42.880	18.090	1:0.42	SOX	Bajer et al. 2015
	C. annuum	Yellow Bedder	2.490	2.530	1:1.02		
	C. annuum	Ring of Fire	1.740	1.730	1:0.99		
	C. chinense	Jamaican Hot Red	2.080	1.170	1:0.56		
	C. frutescens	Tabasco	3.190	2.500	1:0.78		
	C. annuum	Chiltepin	0.285	0.220	1:0.77		
	C. chinense	Yellow Habanero	0.540	0.413	1:0.76		
	C. chinense	Yellow Habanero	0.739	0.506	1:0.68	PHWE	Bajer et al. 2015
Turkey	*C. annuum*	730 F1	0.308	0.208	1:0.68	heat	Topuz and Ozdemir 2007
	C. annuum	1245 F1	0.271	0.123	1:0.46		
	C. annuum	Amazon F1	0.016	0.000	1:0.01		
	C. annuum	Serademre 8	0.149	0.073	1:0.49		
	C. annuum	Kusak 295 F1	0.011	0.002	1:0.15		

* SOX – soxhlet extraction method; UAE – ultrasound-assisted extraction; PHWE – pressurised hot water extraction.

Conclusions

The current study contributed to the expanding of the scientific database in regard to *Capsicum annuum* pungency through special attention on two traditional and two newly selected Bulgarian hot pepper varieties. The varieties Dzhulyunska shipka 1021, Zlatna shipka and Kehlibar possess moderate level of pungency based on their capsaicinoid content and could be of potential value for the pharmaceutical and food industries.

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