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CAPSAICIN AND DIHYDROCAPSAICIN VARIABILITY IN *CAPSICUM* SP. CULTIVARS FROM REPUBLIC OF MACEDONIA REVEALED BY VALIDATED HPLC METHOD

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Abstract

Capsaicinoids are large group of analogues synthesized in hot peppers, *Capsicum annuum* L. as secondary metabolites. Hot peppers are widely used in nutrition but their exploitation could be increased because of capsaicin's pharmacological properties, as analgesic, antidiabetic, hypolipidemic and antitumor agent. Therefore, the aim of this study was to determine capsaicinoids (capsaicin and dihydrocapsaicin) in ethanolic extracts obtained from the fruits of 15 different genotypes and evaluate their variability. Quantification of capsaicinoids extracted from peppers cultivated in Republic of Macedonia has been performed by a validated simple and sensitive HPLC method. Although capsaicin has been known as the highest represent in the group of capsaicinoids, it was found in this study that genotypes that contain higher amount of total capsaicinoids has even higher content of dihydrocapsaicin than capsaicin. The ratio of capsaicinoids in the extracts obtained by Soxlet method was similar to that of extracts obtained by maceration. In the extract obtained from *vezena dolga,* capsaicin has been represented with 42.80% and 45.99% dihydrocapsaicin. Their content in the extract from *dzinki* was 31.44% and 45.41% for capsaicin and dihydrocapsaicin, respectively, and in the extract from *vezena kusa* capsaicin has been represented by 28.85% and dihydrocapsaicin by 48.82%. Since, the biological activity of dihydrocapsaicin has not been clearly reported; these data can be very useful for breeders of hot peppers aimed in further extraction of capsaicin for medicinal purposes.

Key words: *capsaicinoids, hot peppers, liquid chromatography, validation parameters*

INTRODUCTION

Hot peppers belong to the genus Capsicum, which is comprised of more than 200 varieties grouped into more than 30 species, out of which five are domesticated: *C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L. and *C. pubescens* (Hernandez et al., 1999). There are many different genotypes of hot pepper, *Capsicum annuum* L., (Solanace), cultivated in Republic of Macedonia, which present an important vegetable in food and agriculture for a long time. They have been grown mainly because of their fruits, which are widely used in nutrition and appreciated for their pungency, color and flavor aroma (Aranoff et al., 2008). Even more, hot peppers have shown many significant biological activities such as anti-inflammatory (Demirbilek et al., 2004), antioxidant (Maksimova et al., 2014), and hypocholesterolemic (Alvarez-Parrilla et al., 2012) or cytotoxic properties (Maksimova et al., 2016). Hot peppers have been characterized by the presence of vanillyl amide conjugates, known as capsaicinoids, which are absent or replaced by their non-pungent esters, isosteres (capsinoids) in the mild types of peppers (Kobata et al., 1998).

Capsaicinoids occur in the placental tissue of pepper fruits (Iwai et al., 1979) and their

biosynthesis depends on a complex and still not fully characterized enzymatic pathway. The two major capsaicinoids, responsible for up to 90% of pungency, are capsaicin and dihydrocapsaicin (Govindarajan et al., 1987), with at least nine more minor capsaicinoids occurring in pepper fruits (Suzuki et al., 1980; Kozukue et al., 2005). The type and amount of each capsaicinoid affect both the degree and the characteristics of pungency (Todd et al., 1977; Krajewska et al., 1988). Capsaicinoid levels depend on the genotype and also change during fruit development (Perucka and Materska, 2007). Moreover, environmental and nutritional conditions which occurred during the cultivation of peppers can affect the capsaicinoid content. For instance, significant differences in pungency were found in double-haploid chili plants grown in five different plots of the same field (Estrada et al., 2000), and the total capsaicinoid content in those pepper fruits developed in summer was found to be larger than in those fruits developed in autumn (Estrada et al., 2002). Also, the production of five capsaicinoids in four pepper genotypes was found to depend both on the field location and on the year (Harvell et al., 1997).

Hot peppers are usually consumed in our country in nutrition because of their pungency. Pungency or "hottness" that capsaicin causes when it is consumed *per os* can be measured as Scoville heat units (Scoville Heat Unit, SHU), according to a graduated scale that was set up for the first time by the American pharmacist Wilbur Scoville. SHU is a value that gives information about how many times the pepper extract should be diluted in water to lose the pungency or not to be sensed organoleptic. Pure capsaicin has pungency, which has been measured as 16 million SHU.

Reviewing the first scientific papers on the discovery of capsaicin from Micko et al. (1898) and characterization of its structure and nomenclature of Nelson, (1910), to the more recent data in which some of the methods for its extraction and quantification of capsaicin were proposed (Perucka and Materska, 2007), it was perceived that the content of capsaicin can be different depending on the genotypes used for its extraction and several factors used through the cultivation of the plants.

Thus, the present study aimed to evaluate the content of capsaicin and dihydrocapsaicin in 15 genotypes of cultivated peppers, applying HPLC method, because of their common use in nutritional aims and the possibility of capsaicin use in medical purposes. Through this analysis the variability of these two capsaicinoids could be assumed. The data obtained in this study could give further direction to the breeders of this culture about the genotype that is most appropriate to be used in nutrition or in medicinal purposes.

MATERIALS AND METHODS

Plant material

Fifteen different genotypes of *Capsicum annuum* L. with their local names: *vezena dolga, feferona, bombona, zlaten medal, fortense, dzinki, sivrija, kurtovska kapija, piran, vezena kusa, gambi, aiseff f1, hybrid 13514, hybrid 13515; hybrid 14530*, were cultivated on two different locations of Stip (41,746° N, 22,199° E) and Strumica (41,437° N, 22,643° E) in 2012 and 2013. Their fruits have been collected at the end of August in phenological phase of botanical maturity. They have been dried and grounded and then used as a plant material. Two of these genotypes (*zlaten medal* and *kurtovska kapija*) were not pungent and they have been taken as negative control.

Chemicals

All eluents, buffers, and standard solutions were prepared with analytical grade type I water (Milli-Q Synthesis, Millipore). Capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide, ≥ 97%), dihydrocapsaicin (8-methyl-N-vanillylnonamide, ≥ 90%), ethanol (≥ 96.0%), acetonitrile (LC-MS grade), were purchased from Sigma-Aldrich.

Extraction methods

The extraction method chosen must be fast, inexpensive, versatile and efficient and should have an easy performance and no toxicity. The most widely used solvent for extracting capsaicin is hexane, which is very toxic and produces residual solvent (Gao et al., 1996, Martins et al., 2014, 2015.)

Therefore, in this study we have chosen ethanol, as a non-toxic polar solvent, which is efficiently extractive agent with the lowest side toxic effects on the human organism. Many studies have examined the effectiveness of various methods of extraction and confirmed that the efficiency of extraction by conventional methods (maceration, percolation, Soxlet-extraction) and some novel methods (ultrasound, microwave extraction and extraction with supercritical fluids) is similar (Collins et al., 1995; Goci et al., 2013). Considering this data, two conventional extraction procedures have been taken in the experimental work: maceration followed by vacuum filtration and Soxlet extraction. For the maceration process 0.200 g of plant material were measured and mixed with 25 mL of ethanol (96% *v/v*). The extraction procedure has been performed on a water bath at a temperature of 50°C for 5 hours. The obtained extracts were filtrated then by vacuum pump and Gouch filter N#4. The extraction with Soxlet apparatus have been performed by use of 0.800 g plant material in 100 mL ethanol, for 5 hours at 80 ± 2°C. The obtained extracts were used for further analysis.

Method for quantitative determination of capsaicinoids by using a High Pressure Liquid Chromatography (HPLC)

Reverse-phase liquid chromatography has been used as a method for quantitative determination of capsaicinoids in ethanolic pepper extracts. According to the literature, many authors confirmed that C-18 column can be used for effectively partition and quantification of capsaicinoids, and so this column was also used in this research (Othman et al., 2011, Perucka and Oleszek, 2000).

Fruits, dried and grounded, were used as a plant material for Soxlet extraction by using a 96 % (*v/v*) ethanol as a solvent (70°C, for 5 hours) and capsaicinoids have been quantified by use of RP-HPLC (reverse-phase high performance liquid chromatography) system, on Zorbax SB-C18 column (5µm, 250 x 4.6 mm), mobile phase: H_2O/CH_3CN, 50:50 (*v/v*), flow rate: 1.5 mL/min. A suitable DAD (diode array detector) detector followed progress of chromatographic separation at 220 nm.

Equipment: chromatographic analyses were conducted on Agilent 1200 HPLC system, (Agilent Technologies Palo Alto, CA, USA), which contained: binary pump (Model Agilent 1100 series Pump), autosampler (Model Agilent 1100 series G-1329 ALS), DAD detector (Model Agilent series G-13158 Diode Array Detector), connected to Agilent ChemStation software.

The analytical method was validated by using the protocols set out in the International Conference on Harmonization (ICH) guidelines. The required validation parameters, specificity, linearity, accuracy, precision, limit of detection, and limit of quantification, were studied for capsaicin and dihydrocapsaicin.

RESULTS AND DISCUSSION

Quantitative determination of capsaicinoids has been performed by using an isocratic, reverse-phase high performance liquid chromatography, according to a method described by Othman et al., (2011).

UV spectra of capsaicin and dihydrocapsaicin in the wavelength range from 200 to 400 nm, were recorded by a Diode Array Detector (DAD), whereby they have shown two peaks characteristic for capsaicin and dihydrocapsaicin, on 228 and 280 nm. The wavelength of 228 nm, where the absorption maximum was measured for capsaicin and dihydrocapsaicin, was chosen to designate these two compounds in their standard solutions as well as in the extracts obtained from various genotypes of hot peppers.

A typical chromatogram (Fig.1) of the standard solution of capsaicin and dihydrocapsaicin (in equimolar concentration of 10 µg/mL) showed that the time required for elution of capsaicin was 7.65 minutes, while for dihydrocapsaicin was 10.82 minutes. Identification of capsaicin and dihydrocapsaicin in the extracts was based on comparison of their retention times with those obtained for the standard solutions.

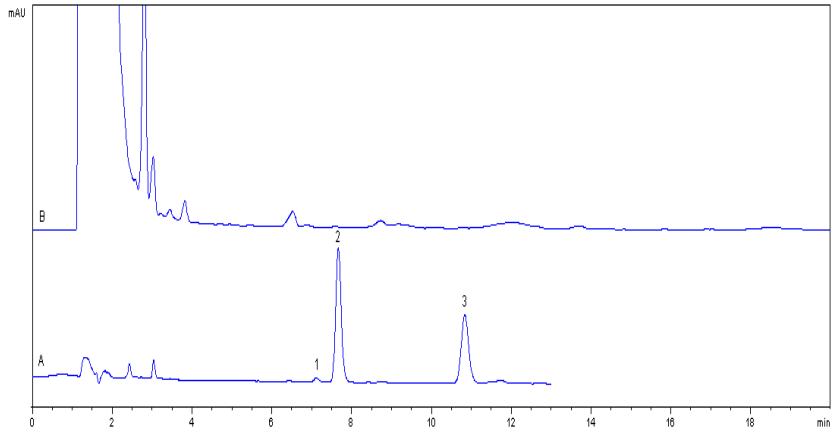

Figure 1. Chromatogram of A) standard solution, a mixture of capsaicin and dihydrocapsaicin (at equimolar concentrations 10 µg/mL), B) ethanolic extract of negative control, pepper genotype gold medal obtained by Soxlet extraction. Assignation: 1. nordihydrocapsaicin, 2. capsaicin, 3. dihydrocapsaicin.

The obtained results referring to the validation of the method for quantitative determination of capsaicinoids indicate that this method has been characterized with sufficient linearity, accuracy and precision. Correlation coefficient (R^2 = 0.999) indicated that there is a good linearity for tested concentration range for capsaicin (1.52 – 380.00 µg/mL) and for dihydrocapsaicin (1.12 – 279.00 µg/mL). Limits of detection (LOD) were 0.075 and 0.109 µg/mL, and limits of quantification (LOQ) were 0.230 and 0.331 µg/mL for capsaicin and dihydrocapsaicin, respectively. The high levels of analytical yield of 98.88 ± 2.87% for capsaicin and 98.62 ± 2.46% for dihydrocapsaicin indicated that the method is accurate. Values obtained from the examination of the repeatability of the method (RSD ≤ 2.0%) indicate that the method is precise (RSD ≤ 2.0%) and suitable for determination of the concentration of capsaicin and dihydrocapsaicin in extracts of chili peppers.

The results for the concentration of capsaicinoids (Fig.2) in the examined extracts have shown that the content of total capsaicinoids ranged from 23.27 to 6516.20 µg/g dry weight of pepper, which corresponds to the prescribed content in the literature (Othman et al., 2011, Gnayfeed et al., 2001). Genotype *feferona* presented the highest content of capsaicin, 2708.091 ± 48.75 µg/g dry weight, followed by genotype *dzinki* and *bombona* with 1725.625 ± 31.06 and 1040.431 ± 18.73 µg / g dry weight, respectively. Lowest capsaicin content has been measured in genotype *gambi*, 8.700 ± 0.16 µg/g dry weight, while in two genotypes that were not pungent and were used as controls (*gold medal* and *kurotvska kapija*), capsaicin have not been detected.

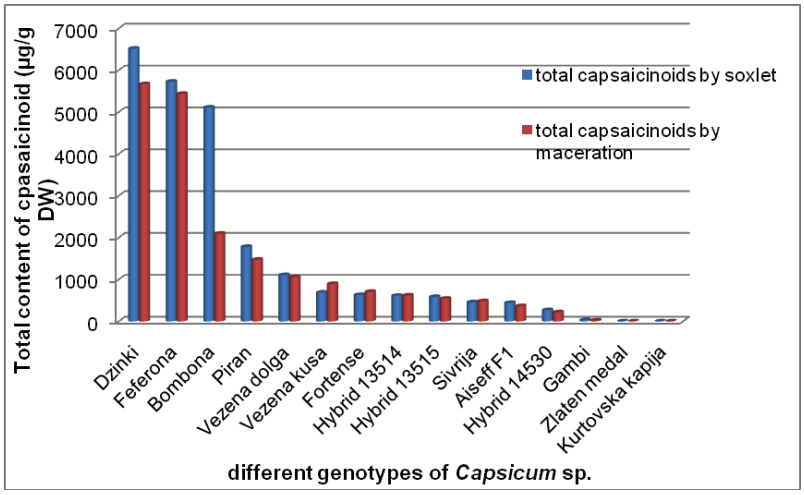

Figure 2. Total capsaicinoid content in two different extraction methods.

The results obtained from the analysis of ethanolic extracts by Soxlet extraction have shown that capsaicin is present again with the highest concentration in the extract obtained from the genotype *fefrona* (2835.190 ± 51.03 µg/g dry weight), and followed by *bombona* and *dzinki* with concentration of capsaicin 2437.991 ± 43.88 µg/g dry weight and 2048.533 ± 36.87 µg/g dry weight, respectively. The ratio of capsaicinoids in the extracts obtained by maceration (Fig.3) was similar to that of the extracts obtained by Soxlet method (Fig.4). In the extract obtained from *vezena dolga,* capsaicin has been represented with 42.80% and 45.99% dihydrocapsaicin. Their content in the extract from *dzinki* was 31.44 and 45.41% for capsaicin and dihydrocapsaicin, respectively, and in the extract from *vezena kusa* capsaicin has been represented by 28.85% and dihydrocapsaicin by 48.82%.

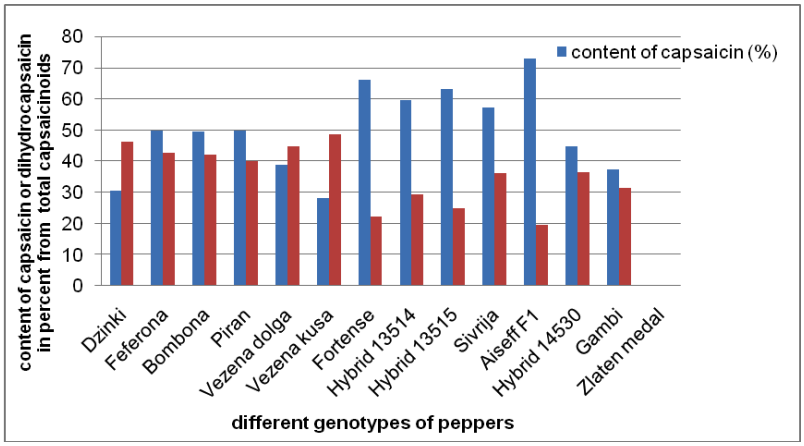

Figure 3. Variability of capsaicin and dihydrocapsaicin (% from total capsiaicinoids) in maceration extracts.

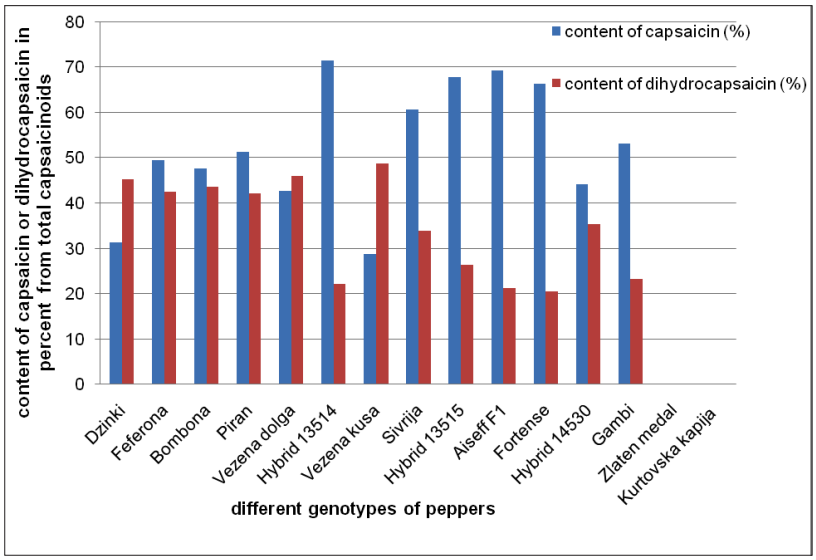

Figure 4. Variability of capsaicin and dihydrocapsaicin (% from total capsiaicinoids) in Soxlet extracts.

According to Suzuki and Iwai, (1984) capsaicin is usually represented by 69%, and dihydrocapsaicin with 30% of total capsaicinoids. Other analogues are represented in such a small amounts.

The results given for the quantitative content of capsaicinoids in hot pepper ethanolic extracts give a good basis for further investigation of the biological/pharmacological properties of capsaicin and pepper extracts.

CONCLUDING REMARKS

The analyzed Macedonian genotypes of hot peppers have characteristic high level of pungency capsaicin, which makes it promising for use, not only in food, but also in medicines. However, the development and efficient capsaicin extraction method for pharmaceutical and alimentary industry depends on the method, herbal material and optimization technique.

All the genotypes, except *gambi, zlaten* medal and *kurtovska kapija* can be used in extraction of capsaicin and dihidrocapsaicin, but the most appropriate are: *feferona, vezena dolga* and *dzinki*. From the genotypes that were taken for analysis, it was concluded that the genotype *feferona* contains the highest percentage of capsaicin.

This study has shown that genotypes which are characterized by higher content of total capsaicinoids may contain more dihydrocapsaicin than capsaicin. So, in the case of *vezena dolga,* capsaicin has been represented with 42.80% and 45.99% dihydrocapsaicin. Their content in the extract from *dzinki* was 31.44% and 45.41% for capsaicin and dihydrocapsaicin, respectively, and in the extract from *vezena kusa* capsaicin has been represented by 28.85% and dihydrocapsaicin by 48.82%. Since, the biological activity of dihydrocapsaicin has not been clearly reported; impermanence of capsaicinoids content can be very useful data. The variability of capsaicin and dihydrocapsaicin presented in these pepper genotypes can be exploited from breeders of these cultivars in order to improve content of capsaicin, which can be further used in medicinal purposes.

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ВАРИЈАБИЛНОСТ НА КАПСАИЦИН И ДИХИДРОКАПСАИЦИН ВО *CAPSICUM* SP. ОД РЕПУБЛИКА МАКЕДОНИЈА ОДРЕДУВАНИ СО ВАЛИДИРАН HPLC МЕТОД

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Резиме

Капсаициноидите се голема група на аналози кои се синтетизираат во лутите пиперки Capsicum annuum L. како секундарни метаболити. Лутите пиперки се користат во исхраната, но нивната експлоатација може да се зголеми поради фармаколошките својства на капсаицинот, како аналгетик, антидијабетичен, хиполипидемичен и антитуморен агенс. Затоа, целта на оваа студија беше да се одредат капсаициноидите, и тоа капсаицин и дихидрокапсаицин, во етанолни екстракти добиени од плодовите на 15 различни генотипови на лути пиперки и да се процени нивната варијабилност. Квантификацијата на капсаициноидите екстрахирани од пиперки одгледувани во Република Македонија се извршени со потврдениот HPLC метод. Иако капсаицинот е познат како највисок во групата на капсаициноиди, во оваа студија беше утврдено дека генотипите кои содржат повисоки количини на вкупни капсаициноиди имаат уште повисока содржина на дихидрокапсаицин отколку капсаицин. Односот на капсаициноидите во екстрактите добиени со Soxlet методот е сличен на оној на екстракти добиени со мацерација. Во екстрактот добиен од генотипот везена долга капсаицинот е застапен со 42,80% и 45,99% дихидрокапсаицин. Нивната содржина во екстрактот од џинки изнесувала 31,44 и 45,41% за капсаицин и дихидрокапсаицин соодветно, а во екстрактот од везена куса капсаицинот е претставен со 28,85%, а дихидрокапсаицин со 48,82%. Бидејќи биолошката активност на дихидрокапсаицинот не е сосема јасна, податоците добиени од овие истражувања можат да бидат многу корисни за одгледувачите на лути пиперки со цел за понатамошно искористување на капсаицинот во медицински цели.

Клучни зборови: *капсаициноиди, лути пиперки, течна хроматографија, валидациски параметри*