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FOOD IN HEALTH AND DISEASE

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ELECTROCHEMICAL DETECTION OF VITAMIN C IN REAL SAMPLES

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Summary

Electrochemical properties of vitamin C (also known as L-ascorbic acid) have been studied by cyclic and differential pulse voltammetry in the model systems in order to develop simple and suitable method for vitamin C detection in real samples. The results indicated that vitamin C oxidation is a quasi-reversible and diffusion-controlled process, as well that the oxidation product of ascorbic acid, dehydroascorbic acid is adsorbed on the glassy carbon electrode surface. Calibration curve was constructed and the linear response was obtained in a concentration range from 0.0025 mol dm^{-3} to 1.0 mol dm^{-3}. Vitamin C was successfully determined in real samples (fruit juices and food supplement) with cyclic voltammetry within concentration range from 0.034 mol dm^{-3} to 0.340 mol dm^{-3}. In addition, antioxidant activity of vitamin C in real samples was determined using DPPH assay with a good linear correlation obtained between the cyclic voltammetry results and the results evaluated by DPPH assay of the samples.

Keywords: vitamin C, detection, cyclic voltammetry, DPPH assay, fruit juices

Introduction

Vitamin C (L-ascorbic acid) is water-soluble vitamin exhibiting many beneficial nutritional and medical properties. The biological activity of vitamin C is manifested through antioxidative capacity and ability to inhibit or quench free radical reactions, at the same time preventing cellular damage. Moreover, vitamin C is considered as biologically active compound and essential micronutrient widely present in fruits and vegetables, playing an important role in antioxidative capacity, especially in citrus fruits (Igual et al., 2011; Sdiri et al., 2019). Beyond the many beneficial effects, vitamin C is also participating in biological functions and metabolic pathways, such as a cofactor for enzymes involved in collagen synthesis and norepinephrine and adrenal hormones balancing, or as a reducing agent in the cellular metabolism (Skrovankova et al., 2015). According to the Recommended Dietary Allowances (RDA), the average and recommended daily intake of vitamin C is estimated to be 75 mg/day for adult women and 90 mg/day for men in order to maintain optimal health conditions (Chalmers et al., 1986). Therefore, deficiency or imbalance in vitamin C could cause severe health issues such as scurvy (lat. *scorbutus*), however the disease is not so widespread and rarely appears due to development of modern scientific medicine (Banan et al., 2013; Ly et al., 2004). The consequences of the scurvy disease include changes in the skin, mucous membranes, connective tissue and joints (Padayatty et al., 2003; Ravindran et al. 2018; Varvara et al., 2016).

Vitamin C is one of the most studied compounds among the all vitamins. However, due to its low stability vitamin C undergoes rapid oxidation process, forming one (ascorbyl radical) or two-electron oxidized product, known as dehydroascorbic acid (DHA). The oxidation processes of vitamin C can be enhanced by increasing temperature or pH value, while exposure to air, overcooking and storage temperature conditions showed significant effects on the compound stability (Oyetade et al., 2012). Ascorbic acid consists of highly reactive hydroxyl groups which are very sensitive to light, heating and the presence of oxidizing agents (Tadesse and Sirgawie, 2017). Above the pH 5.0, vitamin C exists predominantly in the form of ascorbate monoanin ($AscH^-$), and in the alkaline environment above pH 12.0, fully dissociated form ascorbate dianion ($AscH^{2-}$) is present. Therefore, the predominant product of vitamin C at physiological pH is undoubtely in the form of monoanion $AscH^-$, confering the main antioxidative activity of the molecule in living organisms (García-Rodríguez et al., 2017). Due to its donating-electron or reducing ability (Ngai et al., 2013), antioxidant vitamin C is capable of chelating metal ions or acting directly by scavenging reactive oxygen species (ROS) and preventing oxidative damage electrons (Yuswan et al., 2015).

Hence, simple and rapid methods for vitamin C determination could be study of interest. Nowdays, several analytical approaches have been established for vitamin C investigation in different samples: titrimetric, spectrophotometric, fluorimetric,

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chromatographic and electrochemical. Chromatographic and electrochemical techniques are considered as more sophisticated methods for chemical analysis providing selective and sensitive detection (Tadesse and Sirgawie, 2017). Chromatographic separation techniques most commonly include liquid chromatography coupled to mass spectrometry (LC-MS) and high-performance liquid chromatography (HPLC). However, these methods are more sensitive but quite expensive, therefore there is a constant search for cost-effective and reliable techniques for detection of certain analytes.

Electrochemical techniques can be used as an efficient alternative, providing an affordable and accurate approach for detection of compounds. Usually, electrochemical properties of electroactive molecules could be investigated by voltammetric techniques (cyclic voltammetry, differential pulse voltammetry, square wave voltammetry, linear sweep voltammetry, chronocoulometry, chronoamperometry), UV-spectroelectrochemistry and by biosensors. Antioxidative capacity can be also evaluated by electrochemical techniques, as well by employing UV-Vis spectrophotometric methods using ABTS, FRAP, and DPPH (Medvidović-Kosanović et al., 2010).

In this study, cyclic and differential pulse voltammetry have been employed in order to study electrochemical properties of vitamin C in model systems. Developed method was applied for qualitative and quantitative investigation of vitamin C in real samples including commercial juices and food supplements, while antioxidative activity was assessed using DPPH radical scavenging assay.

Materials and methods

Materials

All chemicals were of reagent grade and were used without further purification. Standard solutions of L-ascorbic acid were prepared by diluting stock solution in water from MiliQ Millipore system (conductivity ≤ 0.055 μS/cm) and are further used for electrochemical measurements. Commercial fruit juices were analyzed as purchased, while instant orange powder drink was dissolved in pure water (γ = 5 g of powder/100 mL of water) and vitamin C supplement was also dissolved in pure water (1 tablet/100 mL of water). Ascorbic acid ($C_6H_8O_6$) was purchased from Gram-mol (Zagreb, Croatia) and disodium hydrogen phosphate (Na_2HPO_4) was obtained from BDH Prolabo (Leuven, Belgium). Five samples were analyzed, sample 1 (home-made elderberry juice), sample 2 (instant orange powder drink), sample 3 (commercial orange juice), sample 4 (vitamin C supplement) and sample 5 (commercial plum juice).

Cyclic and Differential Pulse Voltammetry Measurements

Electrochemical experiments were performed in a three electrode cell on a PalmSens potentiostat/galvanostat (PalmSens BV, Utrecht, The Netherlands). Glassy carbon (geometrical area 0.018 cm^2) was used as a working electrode, a platinum wire as a counter electrode and Ag/AgCl as a reference electrode. Before each measurement, the glassy carbon electrode was polished with α-Al_2O_3 (0.05 μm, ALS, Japan) and the system was purged with high purity argon, Ar5 (ϕ_{Ar} = 99.999%). Natrium dihydrogen phosphate (NaH_2PO_4), c = 0.1 mol dm^{-3} was used as electrolyte. Stock solution of vitamin C (c = 0.01 mol dm^{-3}) was prepared daily and diluted to specific concentrations for measurement purposes. Cyclic voltammetry scan rate varied from 25 mV/s to 300 mV/s. Conditions used for differential pulse voltammetry were: pulse amplitude 25 mV, scan increment 5 mV, scan rate 5 mV/s and pulse width 70 ms.

DPPH Method (Brand-Williams Method)

Fresh DPPH (c(2,2-diphenyl-1-picrylhydrazyl) = $9.4x10^{-5}$ mol dm^{-3}) solution was prepared in ethanol according to the Brand-Williams method (Brand-Williams et al., 1995). The reaction of radical scavenging is carried out by mixing the 20 μL of vitamin C and 980 μL of DPPH solution and samples were kept in dark covered with foil for 15 minutes. The UV-Vis measurements were performed using Shimadzu UV-2600 Spectrophotometer at λ_{max} = 515 nm. The antioxidant activity of samples is evaluated by changes in colors from dark purple to pale yellow (Molyneux, 2004; Sharma and Bhat, 2009; Brand-Williams et al., 1995). The measurements were performed in triplicate and expressed as % scavenging activity (% DPPH).

Results and discussion

Cyclic voltammetry

Electrochemical properties of L-ascorbic acid were first studied with cyclic voltammetry in a potential range from -0.1 V to 1.0 V vs. Ag/AgCl reference electrode. In Fig. 1 cyclic voltammogram of L-ascorbic acid is shown.

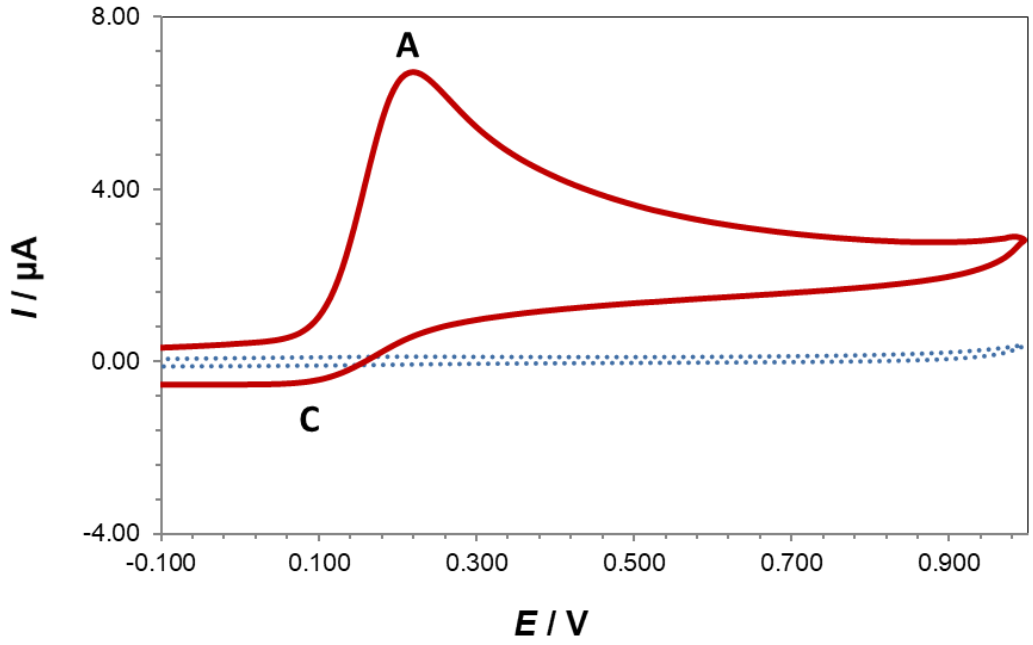

Fig. 1. Cyclic voltammogram of blank solution (…) and vitamin C (—) (c = 5x10^{-4} mol dm^{-3}) in NaH_2PO_4 (I_c= 0.1 mol dm^{-3}) at scan rate 150 mV/s

One anodic peak (A) at the potential, $E_{p,a}$ = 0.22 V, which corresponds to the oxidation of ascorbic acid to dehydroascorbic acid and one cathodic peak (C) at the potential, $E_{p,c}$ = 0.10 V which correspond to reduction of dehydroascorbic acid to ascorbic acid can be observed. Obtained ΔE_p value was 120 mV which indicates quasi-reversible oxidation reaction. Oxidation mechanism of ascorbic acid, which includes transfer of 2 electrons and 2 H^+ ions, is shown in Fig. 2 (Ruiz et al., 1977; Phong et al., 2018).

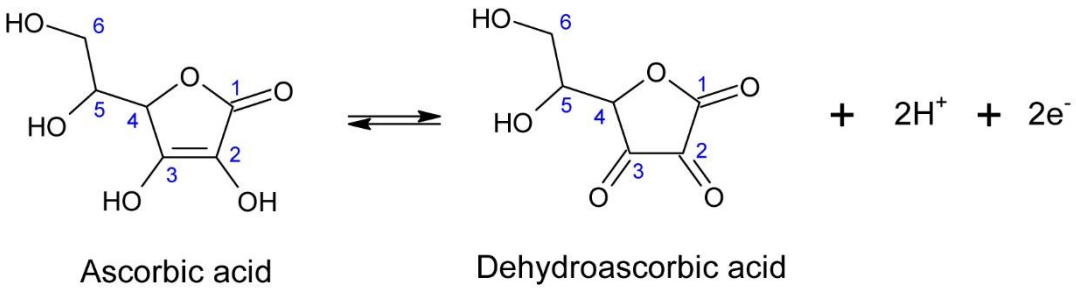

Fig. 2. Oxidation mechanism of vitamin C (L-ascorbic acid)

The effect of scan rate on L-ascorbic acid oxidation was also studied. Cyclic voltammograms of L-ascorbic acid (c = 5x10^{-4} mol dm^{-3}, I_c = 0.1 mol dm^{-3} NaH_2PO_4) recorded at different scan rates (v = 25 - 300 mV/s) are shown (Fig. 3A). As shown, both anodic and cathodic peak currents increase with the increase of scan rate. Vitamin C oxidation is diffusion-controlled process since linear correlation between anodic peak current and square root of scan rate was obtained (Fig. 3B). Linear correlation can be expressed with equation: $I_{p,a}$ (µA) = 0.3894 v (mV/s) + 2.3659 with R^2 = 0.942, where $I_{p,a}$ is oxidation peak current and v scan rate.

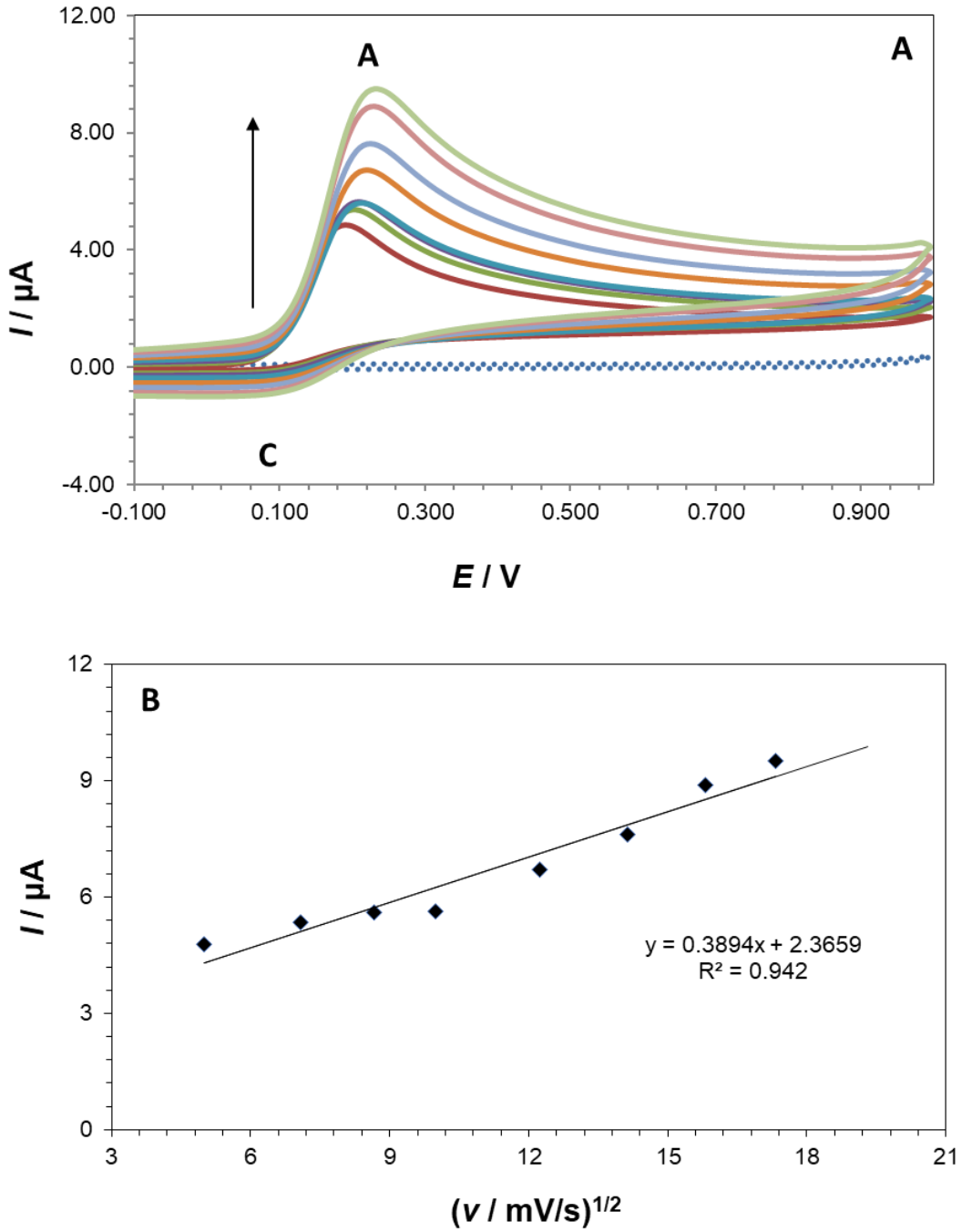

Fig. 3. A) Cyclic voltammograms of blank solution (…) and vitamin C (c = 5x10^{-4} mol dm^{-3}) in NaH_2PO_4 (I_c= 0.1 mol dm^{-3}) recorded at different scan rates (v = 25 - 300 mV/s). B) Oxidation peak current, $I_{p,a}$ as a function of square root of scan rate, $v^{1/2}$

The effect of L-ascorbic acid concentration was also studied and it was determined that anodic peak current increases linearly with the increase of ascorbic acid concentration (Fig. 4A). Based on data obtained from cyclic voltammograms, calibration curve was constructed and the linear equation: $I_{p,a}$ (µA) = 0.0097 c (mol dm^{-3}) + 0.1315 with R^2 = 0.9939 was obtained, where $I_{p,a}$ is oxidation peak current and c designates L-ascorbic acid concentration (Fig. 4B). Linear response was obtained in a concentration range from 0.0025 mol dm^{-3} to 1.0 mol dm^{-3}.

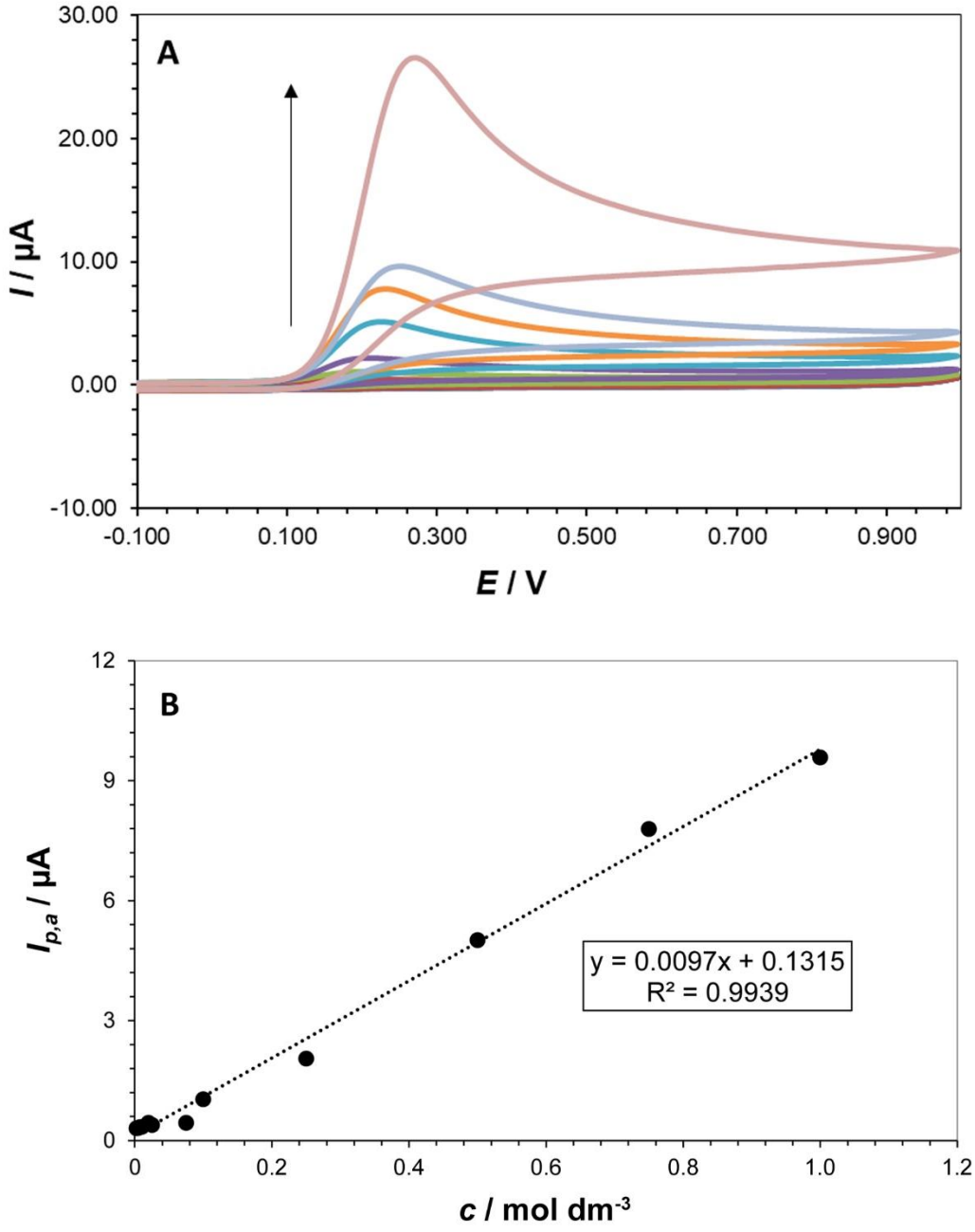

Fig. 4. A) Cyclic voltammograms of vitamin C (c = 0.0025 – 1.0 mol dm^{-3}) in NaH_2PO_4 (I_c= 0.1 mol dm^{-3}) recorded at scan rate 150 mV/s. B) Calibration curve for determination of vitamin C

Differential Pulse Voltammetry

Differential pulse voltammograms of L-ascorbic acid (c = 5x10^{-4} mol dm^{-3}) recorded are shown in Fig. 5. One oxidation peak at the potential, $E_{p,a}$, = 0.17 V, corresponding to the oxidation of L-ascorbic acid can be observed. It is indicative that the oxidation peak current decreases with succesive scans which shows that the oxidation product of L-ascorbic acid, dehydroascorbic acid is adsorbed on the glassy carbon electrode surface.

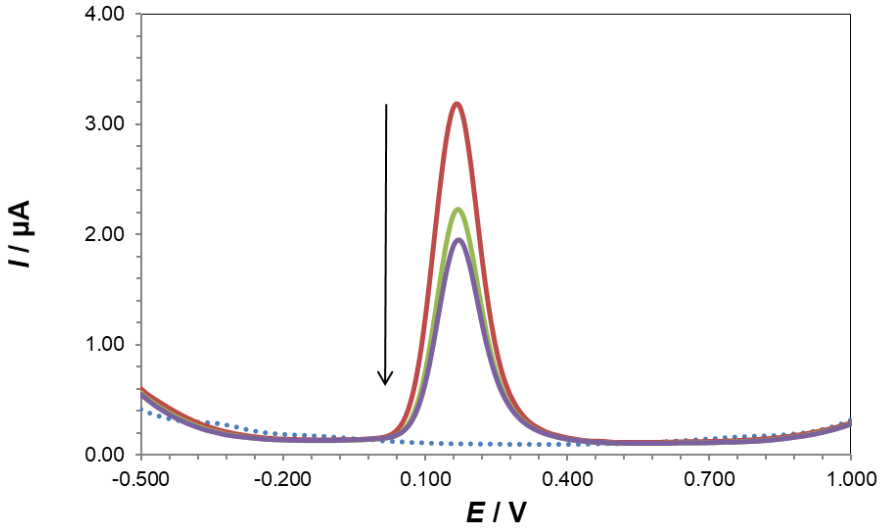

Fig. 5. Differential pulse voltammograms of blank solution (…) and vitamin C (c = 5x10^{-4} mol dm^{-3}) in NaH_2PO_4 (I_c = 0.1 mol dm^{-3}), scan rate 5 mV/s. 1st scan (—), 2nd scan (—) and 3rd scan (—)

Analysis of real samples
Cyclic voltammetry

In Fig. 6 cyclic voltammograms of five investigated real samples are shown. In all investigated samples one oxidation peak (A) around the potential $E_{p,a}$ = 0.36 V and one reduction peak (C) around the potential, $E_{p,c}$ = 0.22 V, which corresponds to oxido-reduction processes of vitamin C, were observed. Addition of 0.15 µL of 0.01 mol dm^{-3} L-ascorbic acid in each sample has confirmed that the vitamin C can be detected in each sample. It was determined that sample 4 (vitamin C supplement) has the highest (0.340 ± 0.028 mol dm^{-3}) and sample 1 (home-made elderberry juice) the lowest (0.034 ± 0.0020 mol dm^{-3}) vitamin C content among the investigated samples.

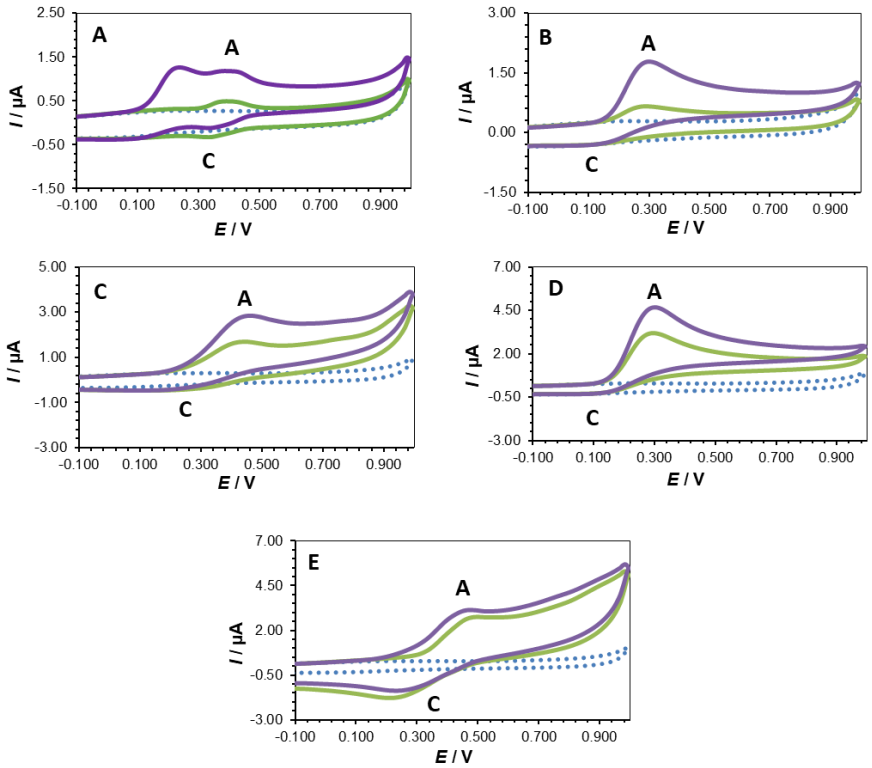

Fig. 6. Cyclic voltammograms of blank solution (…), vitamin C in real samples (—). Sample 1 (A), sample 2 (B), sample 3 (C), sample 4 (D), sample 5 (E) and with added 0.15 µL of vitamin C standard solution (c = 0.01 mol dm^{-3}) (—) in NaH_2PO_4 (I_c = 0.1 mol dm^{-3}) recorded at scan rate 150 mV/s

In Fig. 7 concentrations of vitamin C in all investigated real samples detected with cyclic voltammetry are shown.

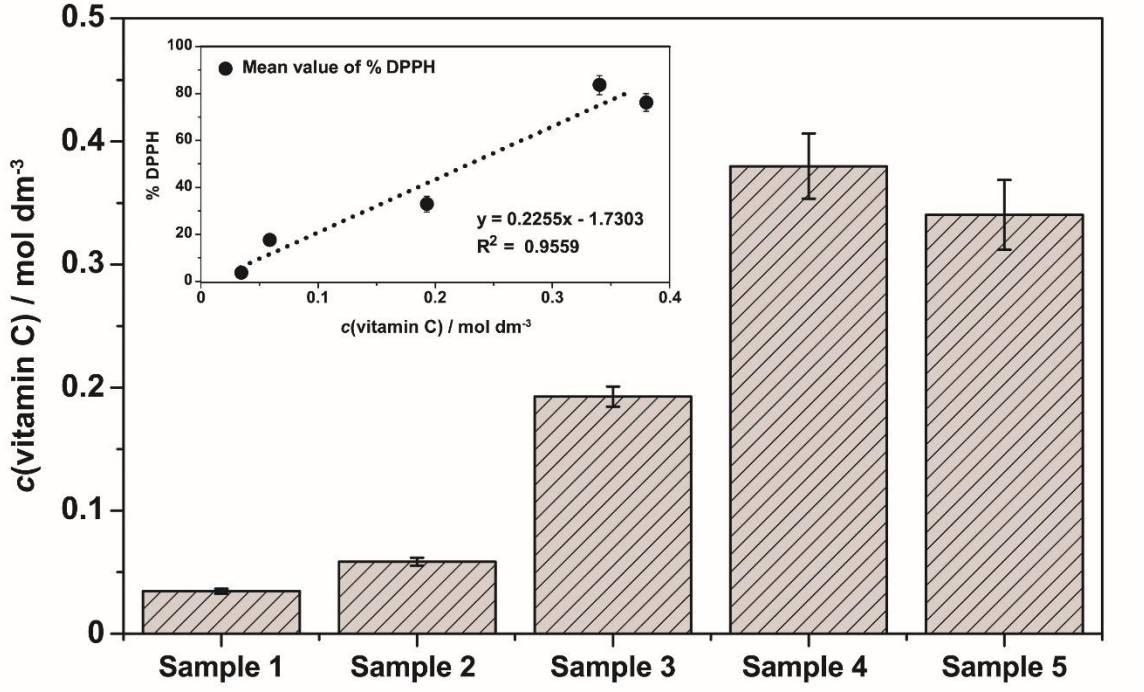

Fig. 7. Column graphs showing concentration of vitamin C in real samples determined with cyclic voltammetry (inset: correlation between % DPPH inhibition percentages and determined vitamin C concentrations)

Spectrophotometric Measurement – Radical Scavenging (DPPH$^{\bullet}$) Assay

Antioxidant activity of vitamin C in real samples was evaluated by DPPH assay. The results are shown in Fig. 7, and compared with the cyclic voltammetry results. At the lowest concentration of vitamin C determined by cyclic voltammetry (0.034 ± 0.0020 mol dm^{-3}), the inhibition percentage of DPPH radical scavenging activity of vitamin C was the lowest as well as the standard deviation value (3.56 ± 0.29 %). The DPPH activity follows this linear trend, until reaches the highest concentration of vitamin C (0.340 ± 0.028 mol dm^{-3}), where higher standard deviations were obtained. This variation in standard deviation values could be explained that due to high concentrations of vitamin C, the adsorption of vitamin C oxidation product to the electrode surface was more enhanced compared to samples with lower vitamin C concentration (Fig. 7). In addition, increased electrochemical signal in samples with higher vitamin C concentration, could be produced due to the presence of interfering substances from complex mixture consisting of multiple polyphenols, as it is the case in real samples. Moreover, the highest % DPPH activity obtained was at determined concentration of 0.340 ± 0.028 mol dm^{-3} and it was estimated as 83.54 ± 4.03 %. A good correlation with the results of two methods was observed with the obtained coefficient of correlation of R = 0.9770 and correspoding coefficient of determination of R^2 = 0.9559 and evident linear dependance of vitamin C content in samples with measured % DPPH activity.

Conclusions

The results of this study demonstrated that in all investigated systems reversible oxidation step of L-ascorbic acid to dehydroascorbic acid occurs, with high tendency of vitamin C adsorption to a glassy carbon electrode surface at higher concentrations. Oxidation was determined to be diffusion-controlled process as linear relation between anodic peak current and the square root of scan rate was observed. Adsorption of the ascorbic acid oxidation product (dehydroascorbic acid) on the glassy carbon electrode was confirmed with differential pulse voltammetry. Vitamin C was detected in model systems where linear response was obtained in a concentration range from 0.0025 mol dm^{-3} to 1.0 mol dm^{-3}. It was also successfully detected in real samples within concentration range from 0.034 ± 0.0020 mol dm^{-3} to 0.340 ± 0.028 mol dm^{-3}. DPPH assay results showed good correlation with the results obtained with cyclic

voltammetry, as % DPPH inhibition values were dependent on the detected concentrations of analyzed vitamin C.

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References

Banan, D., Tan, W.T., Sulaiman, Y., Yusri, M.F., Zidan, M., Ab Ghani, S. (2013): Electrochemical Oxidation of Ascorbic Acid Using MgB_2-MWCNT Modified Glassy Carbon Electrode, *Int. J. Electrochem. Sci*. 8 (12), 12519-12530.

Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995): Use of a Free Radical Method to Evaluate Antioxidant Activity, *LWT - Food Sci Technol* 28 (1), 25–30.

Chalmers, A., Cowley, D., Brown, J. (1986): A possible etiological role for ascorbate in calculi formation, *Clin Chem* 32 (2), 333-336.

García-Rodríguez, M., Gordillo-García, A., Altamirano-Lozano, M. (2017): The Role of Vitamin C in the Protection and Modulation of Genotoxic Damage Induced by Metals Associated with Oxidative Stress. In: Vitamin C, IntechOpen, Amal H. Hamza (ed.), pp. 99-112.

Igual, M., García-Martínez, E., Camacho, M.M., Martínez-Navarrete, N. (2011): Vitamin Content and Antioxidant Capacity of Grapefruit Jams and Candies Obtained by Different Dehydration Methods. In: European Drying Conference - EuroDrying'2011, Palma, Spain.

Ly, S.Y., Chae, J.I., Jung, Y.S., Jung, W.W., Lee, H.J., Lee, S. H. (2004): Electrochemical Detection of Ascorbic Acid (vitamin C) using a Glassy Carbon Electrode, *Food/Nahrung* 48 (3), 201-204.

Medvidović-Kosanović, M., Šeruga, M, Jakobek, L., Novak, I. (2010): Electrochemical and Antioxidant Properties of (+)-Catechin, Quercetin and Rutin, *Croat. Chem. Acta* 83 (2), 197–207.

Molyneux, P. (2004): The Use of the Stable Free Radical Diphenylpicrylhydrazyl (DPPH) for Estimating Antioxidant Activity, *Songklanakarin J. Sci. Technol*. 26 (2), 211-219.

Ngai, K.S., Tan, W.T., Zainal, Z., Mohd Zawawi, R., Zidan, M. (2013): Voltammetry Detection of Ascorbic Acid at Glassy Carbon Electrode Modified by Single-Walled Carbon Nanotube/Zinc Oxide, *Int. J. Electrochem. Sci.* 8 (8), 10557-10567.

Oyetade, O.A., Oyeleke, G.O., Adegoke, B.M., Akintunde, A.O. (2012): Stability Studies on Ascorbic Acid (Vitamin C) From Different Sources, *IOSR-JAC* 2 (4), 20-24.

Padayatty, S.J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J.H., Chen, S., Corpe, C., Dutta, A., Dutta, S.K., Levine, M. (2003): Vitamin C as an Antioxidant: Evaluation of its Role in Disease Prevention, *J Am Coll Nutr*. 2 (1), 18-35.

Phong, N.H., Tam Toan, T.T., Tinh, M.X., Tuyen, T.N., Mau, T.X., Khieu, D.Q. (2018): Simultaneous Voltammetric Determination of Ascorbic Acid, Paracetamol, and Caffeine Using Electrochemically Reduced Graphene-Oxide-Modified Electrode, *J. Nanomater.*, 1-15.

Ravindran, P., Wiltshire, S., Das, K., Wilson, R. B. (2018): Vitamin C Deficiency in an Australian Cohort of Metropolitan Surgical Patients, *Pathology* 50 (6), 654-658.

Ruiz, J.J., Aldaz, A., Dominguez, M. (2011): Mechanism of L-ascorbic Acid Oxidation and dehydro-L-ascorbic Acid Reduction on a Mercury Electrode. I. Acid Medium, *Can. J. Chem*. 55 (15), 2799-2806.

Sdiri, S., Cuenca, J., Navarro, P., Salvador, A., Bermejo, A. (2019): New Triploids Late-maturing Mandarins as a Rich Source of Antioxidant Compounds, *Eur Food Res Technol.* 1-13.

Sha, T., Liu, J., Sun, M., Li, L., Bai, J., Hu, Z., Zhou, M. (2019): Green and Low-cost Synthesis of Nitrogen-doped Graphene-like Mesoporous Nanosheets from the Biomass Waste of Okara for the Amperometric Detection of Vitamin C in Real Samples, *Talanta* 200, 300-306.

Sharma, O.P., Bhat, T.K. (2009): DPPH Antioxidant Assay Revisited, *Food Chem* 113 (4), 1202-1205.

Skrovankova, S., Sumczynski, D., Mlcek, J., Jurikova, T., Sochor, J. (2015): Bioactive compounds and antioxidant activity in different types of berries, *Int. J. Mol. Sci.* 16 (10), 246.

Tadesse, T., Sirgawie, A. (2017): A Comparative Study on Electrochemical Determination of Vitamin C in Liver and Tomato Using Platinum and Glassy Carbon Electrodes, *Biochemistry and Molecular Biology* 2 (3), 25-36.

Varvara, M., Bozzo, G., Celano, G., Disanto, C., Pagliarone, C.N., Celano, G.V. (2016): The Use of Ascorbic Acid as a Food Additive: Technical-Legal Issues, *Ital J Food Saf*. 5 (1), 7-10.

Yuswan, M.H.M.Y., Al-Obaidi, J.R., Rahayu, A., Sahidan, S., Shazrul, F., Fauzi, D. (2015): New Bioactive Molecules with Potential Antioxidant Activity from Various Extracts of Wild Edible Gelam Mushroom (*Boletus* spp.), *Adv Biosci Biotechnol* 6 (4), 320.

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PRODUCTION OF FETA CHEESE WITH A REDUCED SALT CONTENT

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Summary

Sodium chloride (NaCl) is crucial for proper functioning of the organism and plays a key role in many physiological processes. However, excessive sodium intake causes health disorders like elevated blood pressure, heart and cardiovascular diseases. Within the strategy based on lowering the NaCl intake in the Republic of Croatia, thus food production with the lower salt content is encouraged. Cheese is one of the foodstuffs that is widely consumed and has a high ratio of salt especially cheese in brine. This study aimed to investigate whether the replacement of 50% of NaCl with micronized salt in brine influences the physicochemical and sensory properties of feta cheese during maturation. Because of its larger surface area, micronized salt increases the salinity and, thus smaller amounts can be added into the foodstuff compared to the classic NaCl. Analyses of texture, salt content, physicochemical and sensory analyses were performed after 7, 14, 21 and 28 days of cold storage. Based on the results it can be concluded that micronized NaCl may serve as a replacement for NaCl up to 50% without significant change in the physicochemical and sensory properties of the cheese compared to the control sample.

Keywords: Feta cheese, micronized salt, NaCl, texture, sensory analysis

Introduction

Salt (NaCl) is after sugar the second most-used food additive in the food industry. Primary, it has been used as preservative but functional properties and nutritional considerations are now becoming more important in the use of such ingredients in food processing (Dötsch et al., 2009.; Katsiari and Voutsinas, 1998). Excessive sodium intake has become a public health problem since it can lead to the occurrence of hypertension, osteoporosis, and chronic heart diseases (Ferrão et al., 2016). World Health Organization (WHO, 2013) recommends 5 g of salt (equivalent to 2 g of sodium) intake per day. Salt intake in the Republic of Croatia is more than double of the recommendation and it amounts around 11.6 g of salt per day. Due to the pour knowledge, a bad eating habits, the ignorance of the recommendations and to the modern way of life (fast food, processed food, restaurant food), salt causes more harm than good for the human body. WHO estimates that chronic non-communicable diseases, including arterial hypertension, diabetes, obesity, heart, kidney and lung diseases and some types of cancer, are responsible for 86% of premature deaths in Europe. The occurrence of all these diseases as one of the most important risk factors is excessive intake of table salt (He et al., 2011; Blaustein et al., 2012). However, salt is very important in the cheese manufacture, since it controls the water activity and microbial growth, enzyme activity during ripening, curd syneresis, as well as influencing the flavour and texture of cheeses (Cruz et al., 2011). Cheese is consumed in large quantities worldwide, both directly as table cheese and, increasingly, as an ingredient in recipes (Cruz et al., 2011; Bord et al., 2015). Besides its interesting nutritional properties (particularly as a strong contributor to calcium and protein supplies), cheese (also bakery and meat products) is one of the foodstuff with greatest salt content. In fact, only 40 g of cheese which contain 2.00% of salt is equivalent to the 16% of recommended daily salt intake (WHO, 2013). NaCl contributes directly to saltiness in cheese, a flavour that is generally highly appreciated. It contributes indirectly to flavour of cheese by its controlling influence on microbial and enzymatic activities which, in turn, influence lactose metabolism, cheese pH, degradation of fats and casein, and the formation of flavour compounds, such as peptides, free amino acids, and free fatty acids (McSweeney, 2007). The traditional Greek feta cheese that matures in brine and contains 3.50-7.00 % of salt, stands out as a cheese with the greatest salt amount. Since feta cheese is relatively common one in the nutrition, due to the amount of present salt, frequent consumption can result or/and increase the risk of the digestive system diseases and cardiovascular diseases. Due to its frequent usage, efforts are being made to find new solutions in feta cheese production, in order to obtain cheese with sensory and technological characteristics as much as

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same as traditional feta cheese, but with the reduced salt content.

Various substitutions such as potassium chloride, magnesium chloride, calcium lactate or calcium citrate have already begun to be introduced as an alternative to sodium chloride in numerous food industries (Doyle and Glass, 2009; Gimeno et al., 1999). In the production of feta cheese, the tendency is to partially or completely replace the salt (NaCl) from the brine, thereby obtaining a product that will not cause a cost increase in production or change the production process and that will satisfy the needs of the consumers. The major lack of replacing NaCl is appearance of the aftertaste mostly bitterness at concentrations higher than 50% (Gore et al., 2019). Consequently, food industries started to use micronized NaCl. Micronized salt presents microparticles of NaCl crystals and thus increases the salinity due to the larger surface area and therefore, a smaller quantity of micronized salt creates the same salinity taste as higher quantity of classical salt. Compared to the standard salt, which is approximately 500 μm in diameter, the size of the micronized salt is from 10 to 20 μm. The main disadvantage of reducing the salt size is the sticking of fine particles, but solubility remains excellent and it can be easily added to the foodstuff during processing. Micronized salt can reduce salt content from 25 to 50 % in processed food while maintaining salinity and functional properties same as classical salt.

The aim of the present study was to produce feta cheese in brine prepared with micronized salt in amount 50% less salt compared to the control feta cheese. In addition, this work is aimed to observe the influence of the micronized salt in brine toward the ripening, production technology, physico-chemical properties, texture and sensory properties of feta cheese and compare it to the traditionally produced feta cheese with classical NaCl.

Materials and methods

Feta cheese production

The standardized milk (3% milk fat) was pasteurized at 63 °C for 30 min, cooled to 35 °C inoculated with 1% of mesophilic starter culture (Probat 222, Danisco, France) and ripen to obtain the pH around 6.0 units. Then, $CaC1_2$ (10-20 g/100 L milk), KNO_3 (0.01%) and rennet (according to the manufacturer instructions, Medimon, Croatia) were added and mixed well. Coagulation was achieved in about 40 min at 35 °C. After coagulation, the coagulum was cut into the 2 cm^3 cubes, rest for 10 min, and then the cutted curd was left to drain in cheesecloth, transferred into perforated round moulds for pressing. The moulds were inverted three times during the first 3 hours, and two more times until the next day (totally 24 hours at 16 °C). After pressing, cheese was portioned into slices of around 100 g and placed into the brine. The ratio between cheese and brine was 1:4. Control brine was prepared as a 10% solution of NaCl. For production of feta cheese with lower NaCl concentration, micronized salt SODA-LO™ (Tate and Lyle, IL, USA) was used. Since producer of SODA-LO™ recommends 20-50 % less salt use, brine was prepared as a 5% solution, meaning that amount of NaCl was reduced for 50%. Cheese was analysed before brining and after 7, 14, 21 and 28 days of cold storage (6 °C ± 2 °C). Performed analyses were: conductivity, total dissolved matter, acidity, salt content, protein amount, texture and sensory analysis. Experiments were repeated twice and results are shown as mean values.

Physico – chemical and sensory analyses

Conductivity (ms) and Total Dissolved Matter (g/L) were determined by TDS/Conductivity/˚C meter (RS-232 CON 200 series, Oacton, Singapur). They were determined in brine before brining and after 7, 14, 21 and 28 days of cheese brining. Active acidity of brine and cheese was determined by pH meter (WTW Instruments, pH 3110, Germany) and titratable acidity of cheese by Soxlet-Heenkel method (Božanić et al., 2010). The method is based on a titration of 100 g of cheese with 0.1 M NaOH with phenolphthalein as an indicator. Salt was determined according to the previously described method (Božanić et al., 2000). Protein amount in cheese was determined according to the Kjeldal method (Božanić et al., 2010).

Texture measurements in the form of texture profile analysis (TPA) of the samples were performed at room temperature using a texture analyzer (Ametek Lloyd Instruments Ltd., UK) with a 50 kg load cell supported by the software NexygenPlus. Duplicate cubes 10 x 10 x 10 mm were analyzed. The sample was compressed twice to 50% deformation at a crosshead speed of 1 mm/s (resting time between cycles was 5 s), and the following parameters were obtained from force–distance curves: hardness (N), adhesive force (N), cohesiveness, adhesiveness (Nmm), gumminess (N), springiness (mm), chewiness (Nmm), resilience, fracture (N) and stringiness (mm).

The sensory evaluation (weighted points method) of the cheese samples was performed after 7, 14, 21 and 28 days of cold storage. Ten trained analysts have

performed sensory evaluation. Properties that were determined were: appearance, colour, consistency, cut, odour and taste. Score for each evaluated property is given in Table 1. Analysts have scored each property with grades from 1 to 5 which were multiplied with the coefficients of significance (Fv). Coefficients of significance were for appearance 0.4, colour 0.2, consistency 0.4, cut 0.6, odour 0.4 and for taste 2. All the experiments were performed twice, and results are shown as average values.

Table 1. Evaluated properties of feta cheese and maximum scores

Properties and its description	**Score**
Appearance (snow white, homogenous cheese, minor cheese cracks)	2
Colour (porcelain, snow white)	1
Consistency (soft to semi hard creamy texture)	2
Cut (homogenous, possible slight cheese cracks)	3
Odour (sourly and milky, slightly picante)	2
Taste (sour and salt taste, slightly picante)	10
Total	**20**

Results and discussion

Brine for the control sample was prepared as a 10% solution of NaCl (control brine, CS) and brine with micronized salt was prepared as a 5% solution (micronized salt brine, MS) meaning that amount of NaCl was reduced for 50%. Results of electrical conductivity are shown in Fig. 1. Electrical conductivity depends on ions present in the solution and primary depends of the composition of the solution (Norberg et al., 2004). From the Fig. 1 it can be seen that before brining KS had conductivity 104.8 ms while MS brine had 51.2 ms, what was expected since MS had twice lower amount of NaCl. Furthermore, over the cold storage period, electrical conductivity of KS and MS decreases. At the end of cold storage, the drop of electrical conductivity was the highest and it can be explained by migration of NaCl into the cheese.

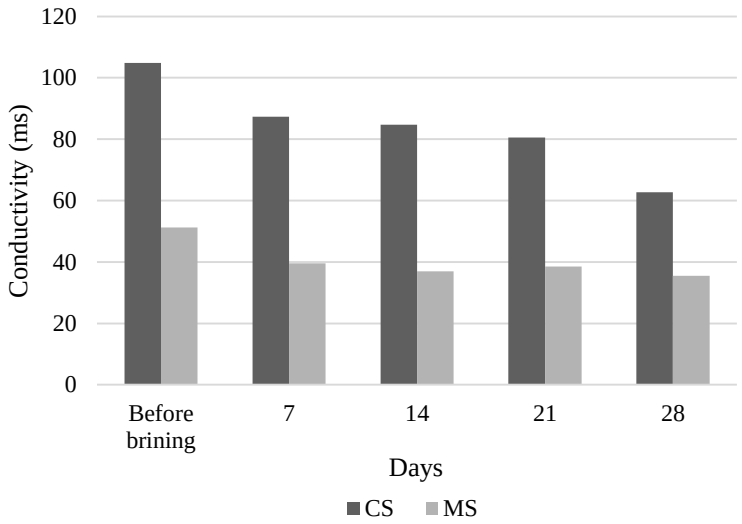

Fig. 1. Conductivity (ms) of control brine prepared with NaCl (CS) and brine prepared with micronized salt (MS) before feta brining and after 7, 14, 21 and 28 days of cold storage

The total dissolved solids (TDS, g/L) determined in brine are shown in Fig. 2 Total dissolved solids are in the correspondence to the electrical conductivity. Before brining KS has 52.7 g/L of dissolved solids and MS had 27.3 g/L of dissolved solids. As well as electrical conductivity TDS decreases during the cold storage period while the major drop was observed at the end of the storage (28th day).

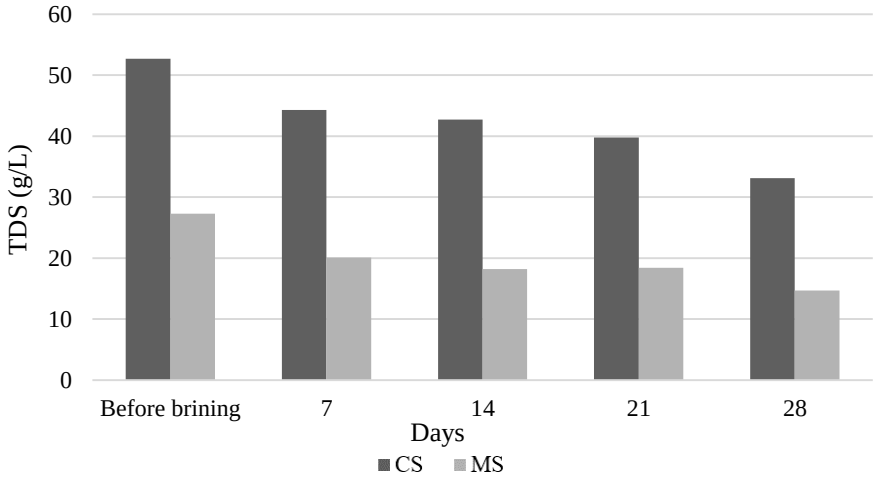

Fig. 2. Total dissolved solids (TDS, g/L) of control brine (CS) and brine prepared with micronized salt (MS) before brining and after 7, 14, 21, 28 days of cold storage.

Table 2 shows the active (pH) and titratable (SH°) acidity of control feta cheese (CF) brined in control brine and feta cheese brined in micronized salt (MF) and active acidity (pH) of CS and MS. From the obtained results it can be seen that both brines before cheese brining had the same pH value and it was 4.70 pH units. During the storage time pH value of control brine increased gradually over storage period for 0.30 pH units while in micronized salt brine the major pH increase was after 7 days of cold storage, also around 0.30 pH units and rest of the storage period pH was constant. Generally, the higher amount of sodium ions brings the lower pH value of the brine (Tratnik and Božanić, 2012). pH value of the control cheese increases over time from 4.85 to 5.23, while pH value of the cheese brined in micronized salt brine was more or less constant and it amounted from 4.85 to 4.89. In control cheese pH was more variable and it can be associate with the higher amount of NaCl in the brine. Titratable acidity expressed as degree per Soxhlet-Heenkel (°SH) decreases over storage period both in control cheese and in cheese brined in the micronized salt where the decrease was greater in the control sample and at the end of storage time control sample had 35.9 °SH while cheese brined in micronized salt had 68.8 °SH. The difference between samples is because of the different amount of the NaCl in the brine and in the cheese.

Table 2. Acidity (pH) of control brine (CS) and brine prepared with micronized salt (MS) and acidity (pH, °SH) of control cheese (CF) and cheese brined in micronized salt brine (MF) during 28 days of cold storage

		Before brining	7	14	21	28
CS	**pH**	4.70	4.77	4.88	4.91	4.99
MS	**pH**	4.70	5.03	5.10	5.05	5.01
CF	**pH**	4.85	4.87	5.00	5.13	5.23
	°SH	83.2	46.5	34.8	30.0	35.9
MF	**pH**	4.85	4.85	4.62	4.82	4.89
	°SH	83.2	60.8	69.6	57.6	68.8

Fig. 3 shows the changes in the salt content of the control cheese (CF) and the cheese brined in the micronized salt brine (MF) during 28 days of cold storage. In the control cheese, the amount of salt increases over storage time from 5.28% to 6.20%, while in the MF cheese amount of the NaCl was slightly decreased from 2.65% at the 7th day to 1.95% at the 28th day of cold storage. From the salt amount analyses, it can be seen that the amount of NaCl in the MF cheese 7th day is more than twice lower compared to the CF cheese, and at the end of storage period it is three times lower. According to the sensory score (Table 4), MF

cheese got the maximum score for the taste with a comment that there is no difference between CF and MF cheese. Micronized salt due to its smaller particles increases the surface area and thus increases the salinity of the cheese and smaller amount of NaCl is present in the final product.

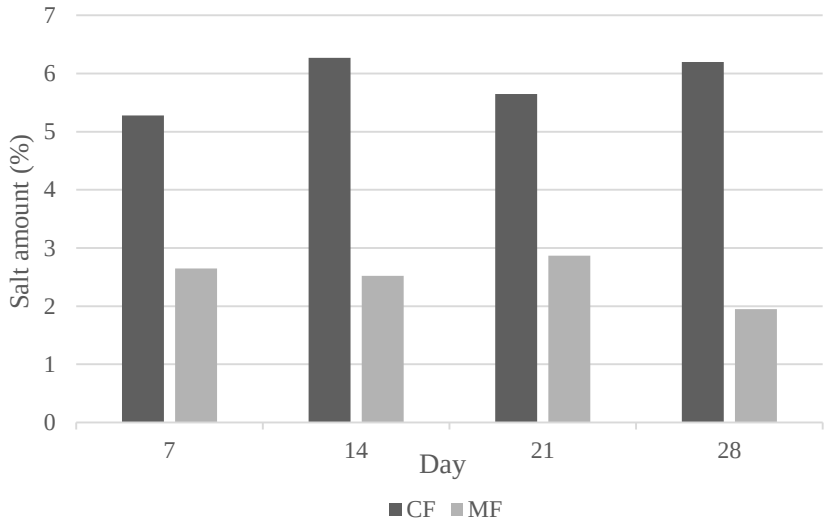

Fig. 3. Salt amount (%) in control cheese (CF) and cheese brined in micronized salt brine (MF) after 7, 14, 21 and 28 days of cold storage

Fig. 4 shows the protein amount before brining and after 28th days of cold brining in control cheese (CF) and cheese brined in micronized salt brine (MF). Before bringing the amount of proteins in the cheese was 21.80 %. In both cheese samples, the protein content decreases significantly after 28 days of brining, and it was 10.56% for CF cheese and 8.90% for MF cheese. Protein drop is a consequence of cheese ripening regarding to the biochemical processes such as glycolysis, proteolysis, lipolysis resulting with numerous secondary degradation products (amino acids and fatty acids). During the secondary degradation taste and aroma of the cheese is formed (Tratnik and Božanić, 2012).

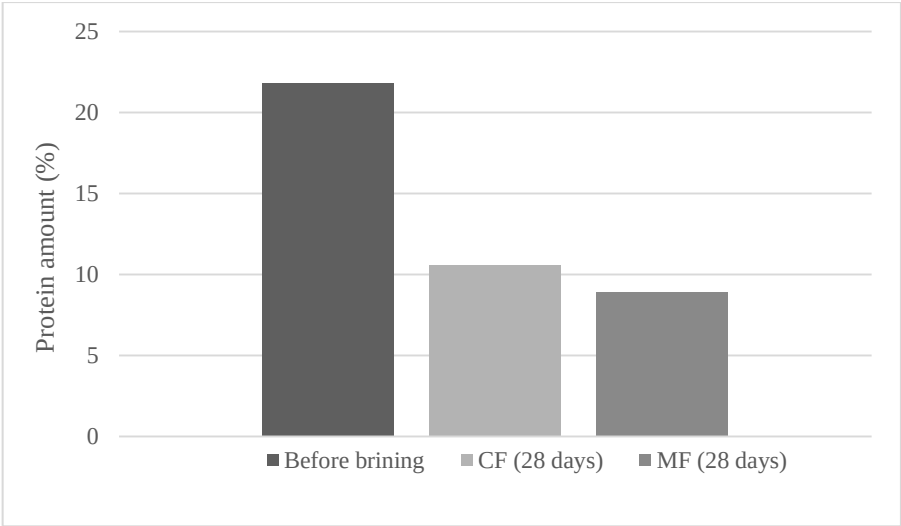

Fig. 4. Protein amount in cheese before brining and after 28 days of cold storage in control cheese (CF) and in cheese brined in micronized salt brine (MF)

Table 3 shows the results of textural analysis of control cheese (CF) and cheese brined in micronized salt brine (MF). Textural properties that were determined are: hardness (N), adhesive force (N), cohesiveness, adhesives (Nmm), gumminess (N), springiness (mm), chewiness (Nmm), resilience, fracture (N) and stringiness (mm).

The main difference between samples was among hardness, gumminess, adhesives and chewiness. After 7 days of brining, hardness of CF cheese was 10.35 N while hardness of MF cheese was more than twice higher and it amounted 21.95 N. Hardness of the cheese is directly related to the NaCl content in the cheese. Literature indicated that higher percentages of salt in brine developed a harder cheese with higher salt content (Ayyash and Shah, 2011). From obtained results in this research, cheese with micronized salt had the higher hardness. Reason for that could be that micronized salt, due to its smaller particles and bigger surface area, increases the aggregation or hydration of casein and cause an increase in the hardness (Akan and Kinnk, 2018; Guinee and O'Kennedy, 2007; Pastorino et al., 2003). Hardness was over the storage period decreased and it was 8.74 N for CF cheese and 5.21 N for MF cheese.

The adhesives was opposite among the CF and MF cheese. In the control cheese adhesives at the 7th day of storage was 0.51 Nmm and at the end of storage period was 1.01 Nmm, meanwhile MF cheese had 1.04 Nmm after seven days and 0.46 Nmm after 28 days of cold storage. Furthermore, gumminess and chewiness of the samples show the same trend as adhesives.

Table 4 presents the sensory score for the control cheese (CF) and the cheese brined in the micronized salt brine (MF). Generally, from the results it can be seen that total score for the MF cheese is higher during the storage period compared to the CF cheese. The main difference between cheese samples were in the colour and consistency. Control cheese got the lower score for colour at 21st and 28th day with a comment that it was too yellow for feta cheese. MF cheese got the lower score for consistency with a comment that it is too hard. This comment is in correspondence with the textural analyses (Table 3) where is obtained that MF cheese had the higher hardness compared to CF cheese.

Table 3. Textural properties of control feta (CF) and feta brined in micronized salt brine (MF) after 7, 14, 21 and 28 days of cold storage

	CF				**MF**			
Day	**7**	**14**	**21**	**28**	**7**	**14**	**21**	**28**
Hardness (N)	10.35	10.44	11.09	8.74	21.95	8.18	8.64	5.21
Adhesive force (N)	-0.15	-0.18	-0.51	-0.31	-0.46	-0.11	-0.16	-0.13
Cohesiveness	0.28	0.31	0.29	0.35	0.32	0.24	0.25	0.22
Adhesives (Nmm)	0.51	0.63	0.59	1.01	1.04	0.44	0.53	0.46
Gumminess (N)	3.00	3.45	3.31	8.21	6.96	1.98	2.14	1.17
Springiness (mm)	-3.01	-2.61	-3.89	-1.72	-1.75	-3.70	-0.99	-5.69
Chewiness (Nmm)	15.92	13.29	8.16	39.34	32.37	7.89	10.73	3.00
Resilience	0.27	0.28	0.19	0.28	0.26	0.24	0.38	0.18
Fracture (N)	10.05	8.91	9.75	19.64	6.95	7.37	8.46	4.58
Stringiness(mm)	8.04	4.35	2.91	4.32	4.82	7.43	5.61	8.69

Table 4. Sensory analysis (appearance, colour, consistency, cut, odour, taste) of control feta (CF) and feta brined in micronized salt brine (MF) after 7, 14, 21 and 28 days of cold storage

	Day	**Appearance (max 2)**	**Colour (max 1)**	**Consistency (max 2)**	**Cut (max 3)**	**Odour (max 2)**	**Taste (max 10)**	**Total (max 20)**
CF	7	1.9	1.0	1.9	3.0	2.0	9.0	18.9
	14	1.9	1.0	2.0	3.0	1.9	9.5	19.3
	21	1.9	0.4	1.9	3.0	2.0	8.9	19.1
	28	1.9	0.9	1.9	2.8	2.0	9.2	18.7
MF	7	2.0	1.0	2.0	3.0	2.0	10.0	20.0
	14	2.0	1.0	1.9	3.0	2.0	10.0	19.9
	21	2.0	1.0	1.8	3.0	1.9	10.0	19.7
	28	2.0	1.0	1.8	3.0	1.8	10.0	19.6

Conclusions

Excessive sodium intake has become a public health problem since it can lead to the occurrence of hypertension, osteoporosis and chronic heart diseases. Besides its nutritional properties (particularly as a strong contributor to calcium and protein supplies), cheese (also bakery and meat products) is one of the foodstuff with greatest salt content. The traditional Greek feta cheese that matures in brine and contains 3.50-7.00 % of salt, stands out as a cheese with the greatest salt amount. Usage of micronized salt due to its larger surface area can reduce salt content from 25 to 50 % in processed food while maintaining salinity and functional properties same as classical salt. According to the obtained results the main differences between control cheese and cheese brined in micronized salt brine was in its texture. Cheese brined in micronized salt brine had increased hardness, gumminess, fracture and adhesive force over the storage period compared to control cheese. Also, the colour of micronized salt brine was turbid over the storage period and control brine was transparent. Total sensory score of cheese brined in micronized salt brine was generally higher compared to the control cheese and consequently it can be concluded that micronized salt can be used in the production of feta cheese and thus amount of NaCl can be reduced for 50%. Obtained results are promising in the production of feta cheese with lower amount of NaCl (1.95%) where physico-chemical and technological parameters not differ from the traditional feta cheese production.

References

Akan, E., Kinik, O. (2018): Effect of mineral salt replacement on properties of Turkish White cheese *Mljekarstvo* 68, 46-56. https://doi.org/10.15567/mljekarstvo.2018.0106

Ayyash, M.M., Shah, P.H. (2011): The effect of substitution of NaCl with KCl on chemical composition and functional properties of low-moisture Mozzarella cheese, *J of Dairy Sci.* 94, 3761-3768. https://doi.org/10.3168/jds.2010-4103

Blaustein, M. P., F. H. H. Leenen, L. Chen, V. A. Golovina, J. M. Hamlyn, T. L. Pallone, J. W. V. Huysse, J. Zhang, and W. G. Wier (2012): How NaCl raises blood pressure: a new paradigm for the pathogenesis of salt-dependent hypertension, *Am. J. Physiol. Heart Circ. Physiol*. 302, 1031-1049. https://doi.org/10.1152/ajpheart.00899.2011

Bord, C., Guerinon, D., Lebecque, A. (2015): Impact of heating on sensory properties of French Protected Designation of Origin (PDO) blue cheeses. Relationships with physicochemical parameters, *Food Sci. Technol. Int.* 22, 377–388. https://doi.org/10.1177/1082013215605201

Božanić, R., Jeličić, I., Bilušić, T. (2010): Analiza mlijeka i mliječnih proizvoda, Zagreb, Hrvatska: Plejada, pp 23-61.

Cruz, A.G., Faria, J.A.F., Pollonio, M.A.R., Celeghini, R.M.S., Granato, D., Shah, N.P. (2011): Cheeses with reduced sodium content: Effects on functionality, public health benefits and sensory properties, *Trends Food Sci. Tech.* 22, 276-291. https://doi.org/10.1016/j.tifs.2011.02.003

Dötsch, M., Busch, J., Batenburg, M., Liem, G., Tareilus, E., Mueller, R., Meijer, G. (2009): Strategies to Reduce Sodium Consumption, *Crit. Rev. Food Sci*. 49, 841-851. https://doi.org/10.1080/10408390903044297

Doyle, M.E., Glass, K.A. (2009): Sodium reduction and its effect on food safety, food quality, and human health, *Compr. Rev. Food Sci. Food Saf.* 9, 44-56. https://doi.org/10.1111/j.1541-4337.2009.00096.x

Ferrão, L.L., Silva, E.B., Silva, H.L.A. , Silva, R., Mollakhalili, N., Granato, D., Freitas M.Q., Silva, M.C., Raices, R.S.L., Padilha, M.C., Zacarchenco, P.B., Barbosa, M.I.M.J., Mortazavian, A.M., Cruz, A.G. (2016): Strategies to develop healthier processed cheeses: Reduction of sodium and fat contents and use of prebiotics, *Food Res. Int.* 86, 93-102. https://doi.org/10.1016/j.foodres.2016.04.034

Gimeno, O., Astiasaran, I., Bello, J. (1999): Influence of partial replacement of nacl with KCl and CaCl2 on texture and color of dry fermented sausages, *J. Agric. Food. Chem.* 47, 873-877. https://doi.org/10.1021/jf980597q

Gore, E., Mardon, J., Bord, C., Lebecque, A. (2019): Calcium lactate as an attractive compound to partly replace salt in blue-veined cheese, *J. Dairy Sci.* 102, 1-13. https://doi.org/10.3168/jds.2018-15008

Guinee, T.P., O'Kennedy, B.T. (2007): Reducing Salt in Cheese and Dairy Spreads. In: Reducing Salt in Food, Kilcast, D., Angus, F. (ed.), Sawston, UK: Woodhead Publishing, pp. 316-357.

He, F. J., Burnier, M., MacGregor, G.A. (2011): Nutrition in cardiovascular disease: salt in hypertension and heart failure, *Eur. Heart J.* 32, 3073–3080. https://doi.org/10.1093/eurheartj/ehr194

Katsiari, M., Voutsinas, L.P. (1998): Manufacture of Kefalograviera cheese with less sodium by partial replacement of NaCl with KCl, *Food Chem*. 61, 63–70. https://doi.org/101016/S0308-8146(97)00113-1

McSweeney, P.L.H. (2007): Salt in cheese, Sawston, UK: Woodhead Publishing, pp. 80-99. https://doi.org/10.1533/9781845693534.80

Norberg, E., Rogers, G.W., Goodling, R.C., Cooper, J.B., Madsen, P. (2004): Genetic Parameters for Test-Day Electrical Conductivity of Milk for First-Lactation Cows from Random Regression Models, *J. Dairy Sci.* 87, 1917–1924. https://doi.org/10.3168/jds.S0022-0302(04)73350-0

Pastorino, A. J., C. L. Hansen, C.L., D. J. McMahon. (2003): Effect of pH on the chemical composition and structure-function relationships of cheese, *J. Dairy Sci.* 86, 2751–2760. https://doi.org/10.3168/jds.S0022-0302(03)73871-5

Tratnik, Lj., Božanić, R. (2012): Mlijeko i mliječni proizvodi, Zagreb, Hrvatska: Hrvatska mljekarska udruga.

WHO (2013): Salt reduction and iodine fortification strategies in public health. World Health Organization, Geneva, Switzerland. http://apps.who.int/iris/bitstream/handle/10665/101509/9789241506694eng.pdf?ua=1.

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THE INFLUENCE OF PROPOLIS SUPPLEMENTATION ON THE TECHNOLOGICAL PROPERTIES AND MACRONUTRIENT CONTENT OF SKINLESS CHICKEN BREASTS

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original scientific paper

Summary

The aim of this study was to determine the influence of dietary supplementation with propolis on the technological properties of skinless chicken breasts evaluated through breast muscle pH value measured 45 minutes (pH_1) and 24 hours post mortem (pH_2), water-holding capacity of breast muscle, consistency of breast muscle and its color (L*, a*, b*) and to determine its macronutrient content (protein and fat content). The study was conducted on 180 Ross 308 chickens equally distributed by sex and divided into three groups: the control group of chickens (C) fed with a basal diet and two experimental groups of chickens (E) fed with the same diet supplemented with propolis (E1 2g/kg and E2 4g/kg). There was no statistically significant difference between C and E considering pH_1 (p=0.260) but there was statistically significant difference between them considering pH_2 (p=0.037). There was statistically significant difference in L* breast muscle color (p=0.039) between C and E while there were no statistically significant differences in a* and b* breast muscle color between them (p=0.167 and p=0.637, respectively). There were no statistically significant differences between the C and E considering water-holding capacity (p=0.767) and consistency (p=0.505) of breast muscle. There were no statistically significant differences in protein and fat content between C and E (p=0.368 and p=0.244, respectively). The obtained results confirm the benefits of the tested supplementation.

Keywords: propolis, chicken breasts, chicken feeding, technological properties, macronutrient content

Introduction

Chronic non-communicable diseases are the leading cause of death globally (Dumic et al., 2017). The unbalanced or poor nutrition is the major risk factor for such diseases (Dumic et al., 2017; Dumic et al., 2018). Bearing in mind that many of chronic non-communicable diseases are directly linked to the human nutrition it is quite clear that many challenges in health care could be proactively improved by producing a healthier food supply as a preventive health care strategy (Decker and Park, 2010). Until now there has been several attempts to produce such foods but because of the complexity of this issue and many stakeholders who have interest in the subject matter the final solution has not yet been found. The one of the main challenges is to find the foodstuff that is necessary for human health and development that contains essential elements which one cannot substitute easily and to make it even more healthier and tempting for human nutrition. This is especially true for functional foods as they must be efficacious while also tasting good, being convenient and reasonably priced so that consumers will regularly purchase the products (Decker and Park, 2010).

Meat continues to supply nutrients and plays a vital role in human life because of its high biological value protein, iron, zinc, selenium and vitamin B12 contents being a crucial component of a well-balanced diet (Perreira and Vicente, 2013). Following the fact that red met has been connected with the onset of some chronic diseases such as colon cancer and cardiovascular diseases the popularity of poultry meat is growing throughout the world including Croatia (Park et al., 2017). Within the poultry meat the chicken meat is especially popular. The popularity of chicken meat and its growing consumption is contributed by a number of factors, most notably its low prices, the long tradition of poultry farming in almost all parts of the world, the indisputable dietary and nutritional value of chicken meat, the lack of cultural and religious barriers to consumption of this type of meat, but also of the crisis in the area of food safety in the late 90s of the last century due to bovine spongiform encephalopathy (Klarić., 2014; Klarić et al., 2016).

Propolis belongs to a group of natural substances of animal and vegetable origin with intense antioxidant and antimicrobial properties (Prakatur et al., 2019a). The bioactive components of propolis include polyphenolic constituents such as flavonoids, phenolic acids and their derivatives (Wang et al., 2016; Prakatur et al., 2019a). Polyphenolic constituents of propolis are responsible for its well-

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documented pharmacological activities, including antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, and cardioprotective effects (Wang et al., 2016; Prakatur et al., 2019a). Just because of these properties propolis is today widely used as a health/functional food worldwide (Wang et al., 2016).

Meat has great potential for introducing important nutrients into the human diet. The nutritional composition of meat products can be altered by the direct addition of bioactive food ingredients or the inclusion of bioactive compounds in animal nutrition. This latter technique has the advantage that bioactive compounds are biologically introduced into the food and thus would not have to be declared as a food additive. This is important because food additives are often not allowed in meat products as they may violate the product identity standard (Decker and Park, 2010).

Recent study had showed that propolis supplementation of chicken feed is a promising method to improve the quality of chicken meat since this supplementation elicited the best amino acids profile of the chicken meat (Haščík et al., 2020).

The aim of this study was to determine the influence of dietary supplementation with propolis on the technological properties of skinless chicken breasts (pH1 and pH2; water-holding capacity of breast muscle; consistency of breast muscle and its color) and to determine its macronutrient content (protein and fat content).

Materials and methods

Animals, diet, experimental design. The study was conducted on total 180 chickens of Ross 308 provenance, divided into 3 groups (60 chickens in each group with equally distributed sexes): one control group (C) and two experimental groups (E1 and E2). All chickens were placed on wooden sawdust under the same conditions throughout the experimental period (42 days) according to the manufacturer's recommendations for the Ross 308 hybrid (Aviagen, 2014). From day 1 to 21 of the study, chickens were fed with a starter mixture. From day 22 to 42 of the study, chickens were fed with a finisher mixture. During the whole study, feed and water were offered *ad libitum*. Throughout the study the control group (C) was fed a basal diet without additives, while the experimental groups (E1 and E2) were fed the same diet supplemented with propolis (E1 2g/kg and E2 4g/kg). The used amounts of propolis were chosen based on results of several previous studies (Klarić et al., 2018; Klarić et al., 2018a; Prakatur et al., 2019). The experimental protocol was approved by the Committee for Animal Welfare of the Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek (Approval code: 602-04/19-01/04; 2158-94-02-19-05). Samples of raw propolis used in this study were obtained from apiaries located in naturally preserved areas of continental Croatia (around the city of Osijek, Eastern Croatia). Inclusion of propolis into the feed mixture was performed using a vertical mixer (Briketstroj Ltd., Valpovo, Croatia).

Sample collection and measurements. On day 42, after 10-hour feed withdrawal, 14 chickens from each group was slaughtered by cervical dislocation and exsanguinated for 2 minutes. The carcasses were then manually de-feathered and eviscerated. Immediately after slaughtering and de-feathering, and without cooling, the carcasses were processed. Chicken carcasses were processed according to the principle "Prepared for barbecue" (Regulation European Commission No. 543/2008).

Carcass body weight was measured by using an electronic scale Avery Berkel FX 220 (Avery Berkel, Smethwick, UK). The carcass yield was calculated as the difference between the live weight (g) and carcass body weight (g) and expressed as a percentage of live weight.

Technological characteristics of chicken meat quality were described by analyzing the average pH_1 and pH_2 of breast muscle, water-holding capacity of breast muscle, consistency of breast muscle and breast muscle color expressed as L* (lightness), a* (redness), and b* (yellowness).

Chickens' breast muscle pH values were measured in the internal section of pectoral major muscle. The pH_1 value was determined 45 minutes' *post mortem* and pH_2 value was determined 24 hours *post mortem* by a contact pH meter (MP120-B, Mettler Toledo, Giessen, Germany).

Assessment of water holding capacity was determined by the method of Grau and Hamm (1953). A sample of 300 mg meat was applied to Whatman 1 paper, placed between two glass plates and subjected to an even loading of 2 kg for 5 min. From the size of the outflow area, the percentage of free water in the meat was calculated, assuming that 1 cm2 of the outflow corresponded to 10 mg of water. A smaller area of the outflow (the amount of free water) indicated the greater water holding capacity of the meat. Along with the water holding capacity the consistency of breast muscle was determined.

The color of breast muscle was determined on the cooled section of muscle after 24 hours of cooling at 4 °C by using the Minolta Chroma Meter CR- 410 (Minolta Camera Co. Ltd., Osaka, Japan). The

calibration of the device was done using a standard white plate (Reference No. 21633047, C Y = 94.3, x = .3135 and y = .3197; D Y = 94.3, x = .3160, y = .3232). Before the measurement, a fresh vertical incision was made in the middle of the breast muscle. The sample was left for 10 minutes at room temperature to "stabilize" the color, after which the color of the muscle was read by the Chroma meter. The color of chicken meat was expressed as CIE-L*a*b* (Commission Internationale de l'Eclairage, 1976) i.e. values of L* (lightness), a* (redness), and b* (yellowness).

Chemical composition of meat. Fat content of meat was determined by Soxhlet extraction method and Protein content by AOAC official method 928.08 (Kjeldahl method) (AOAC, 2000). All analyses were performed in duplicates. Energy content of samples was calculated using the Atwater general energy conversion factors where 4.0 kcal/g of protein and 9.0 kcal/g of fats (FAO, 2003).

Statistical analysis. The statistical analysis was carried out using statistical package Statistica for Windows 2010 (version 10.0, Stat Soft Inc., Tulsa, OK). Normality of data distribution was tested with the Kolmogorov-Smirnov test. The numerical variables were described as mean ± standard deviation (SD). ANOVA was used for the comparison of numerical variables among the groups. On all statistical analyses, two-sided P-values of 0.05 and lesser ones were considered significant.

Results and discussion

This study showed that there was no statistically significant difference between C and E considering pH1 (p=0.260) but there was statistically significant difference between them considering pH2 (p=0.037) (Table 1). The results of this study are opposite to the results of the study done by Šulcerová et al. (2011) who showed how pH2 values of experimental groups were lower than those from control group while in this study those values were higher than in control group. When observing all measured chickens' breast muscle pH values, it can be said that they indicate good quality of chicken meat of all groups since the pH values were not below 5.4 and not above 7.0 when autolysis of meat appears (Haščík et al., 2012). The results of this study clearly indicate that pH value drops after slaughter and therefore the meat pH2 values are lower than the pH1 values. The lowering of the chickens' breast muscle pH values is due to the fact that glycogen from the slaughtered animals is degraded in glucose. Glucose then passes the glycolysis process, but due to lack of oxygen, the formation of lactic acid leads to decrease of muscle tissue pH (Šulcerová et al., 2011). The described drop in pH value helps to convert muscle to meat.

Table 1. pH values (pH_1, pH_2) of the chickens' breast muscle

Parameters	Group of chickens $\bar{x} \pm s$			p*
	C	E1	E2	
pH_1	5.76±0.13	5.82±0.10	5.82±0.10	0.260
pH_2	5.63±0.10	5.70±0.08	5.71±0.06	0.037

**ANOVA; $\bar{x}$ = mean; s = standard deviation; C = control group; E1 = feed mixture + 2.00 g of propolis/kg of feed mixture; E2 = feed mixture + 4.00 g of propolis/kg of feed mixture; pH_1 - pH value measured 45 minutes post mortem; pH_2 - pH value measured 24 hours post mortem*

The study revealed that there was statistically significant difference in L* breast muscle color (p=0.039) between C and E while there were no statistically significant differences in a* and b* breast muscle color between them (p=0.167 and p=0.637, respectively) (Table 2). These results are slightly opposite to the results of study by Haščík et al. (2012) who did not find statistically significant differences in breast muscle color between control and experimental groups of chickens. However, our results are in concordance with the results of the study done by Šulcerová et al. (2011) who also showed how L* breast muscle color was statistically significant higher in experimental groups of chicken in comparison to control group. Meat color is a characteristic that significantly determines meat quality, as it is the first visual criterion by which consumers judge the appearance and appeal of a meat. Following that, fresh chicken breast muscle should be pink in color, and any deviation from this shade is considered unacceptable to the consumers (Garcia et al., 2010; Kralik G. et al., 2011). The results of our study clearly confirm that the type of chicken feeding significantly influences the color of meat, as has been shown previously in other studies (Karaoglu et al., 2006; Saláková et al., 2009).

Table 2. Average color values of chickens' breast muscle expressed as CIE-L*a*b* according to the groups of chickens

Parameters	Group of chickens $\bar{x} \pm s$			p*
	C	E1	E2	
L*	64.46±2.71	66.26±1.60	66.19±1.55	0.039
a*	11.32±1.27	10.86±1.26	11.83±1.46	0.167
b*	12.08±2.09	11.65±2.81	11.27±1.68	0.637

**ANOVA; $\bar{x}$ = mean; s = standard deviation; C = control group; E1 = feed mixture + 2.00 g of propolis/kg of feed mixture; E2 = feed mixture + 4.00 g of propolis/kg of feed mixture; L* - lightness; a* - redness; b* - yellowness*

The study further showed that there were no statistically significant differences between the C and E considering water-holding capacity (p=0.767) and consistency (p=0.505) of breast muscle (Table 3 and Table 4). These results are in concordance with the results of the study done by Klarić (2014) who also did not find statistically significant differences in mentioned parameters between control and experimental groups of chicken. The water-holding capacity is a very important parameter of meat quality since the color, juiciness and tenderness of the meat depend partially on the ability of the meat to retain moisture during normal storage conditions and during its heat treatment, making this parameter important for both fresh meat quality and for the quality of meat products (Mehaffey et al., 2006; Wang et al., 2009).

Table 3. Water-holding capacity of chickens' breast muscle (%) according to the groups of chickens

Parameter	Group of chickens $\bar{x} \pm s$			p*
	C	E1	E2	
Water-holding capacity	2.62±0.45	2.53±0.58	2.66±0.34	0.767

**ANOVA; $\bar{x}$ = mean; s = standard deviation; C = control group; E1 = feed mixture + 2.00 g of propolis/kg of feed mixture; E2 = feed mixture + 4.00 g of propolis/kg of feed mixture*

Table 4. Consistency of chickens' breast muscle according to the groups of chickens

Parameter	Group of chickens $\bar{x} \pm s$			p*
	C	E1	E2	
Consistency	2.15±0.25	2.15±0.25	2.07±0.15	0.505

**ANOVA; $\bar{x}$ = mean; s = standard deviation; C = control group; E1 = feed mixture + 2.00 g of propolis/kg of feed mixture; E2 = feed mixture + 4.00 g of propolis/kg of feed mixture*

Both, protein and fat content were lower in both experimental groups 20.26±1.61 g of proteins/100 g in E1 and 20.50±1.00 g of proteins/100 g in E2 in comparison to average of 21.16±2.28 g of proteins/100 g in control group; 2.03±0.58 g of fat/100 g in E1 and 1.78±0.50 g of fat/100 g in E2 in comparison to average of 2.12±0.56 g of fat/100 g in control group. Obtained values result in lower caloric value of skinless chicken breast (99.3±7.1 kcal/100g in E1 and 98.0±6.1 kcal/100g in E2 in comparison to 103.7±10.0 kcal/100g in C). Reduction in protein, fat content and energy value was not statistically significant (p=0.368; p=0.244; p=0.149, respectively) (Table 5) which confirms the plausibility of selected feeding profile from the aspect of macronutrient content.

Table 5. Protein and fat content of chickens' breast muscle according to the groups of chickens

Parameter	Group of chickens $\bar{x} \pm s$			p*
	C	E1	E2	
Proteins (%)	21.16±2.28	20.26±1.61	20.50±1.00	0.368
Total fat (%)	2.12±0.56	2.03±0.58	1.78±0.50	0.244
Energy (kcal/100 g)	103.7±10.0	99.3±7.1	98.0±6.1	0.149

**ANOVA; $\bar{x}$ = mean; s = standard deviation; C = control group; E1 = feed mixture + 2.00 g of propolis/kg of feed mixture; E2 = feed mixture + 4.00 g of propolis/kg of feed mixture; protein content determined by Kjeldahl method; Fat content determined by Soxhlet extraction*

Conclusions

The results of this study had justified the usage of propolis as a feed supplement in chickens feeding. This type of feeding opens up the possibility of the production of enriched chicken meat, which is of utmost importance in the context of the prevention of chronic non-communicable diseases, especially cardiovascular diseases, and the general improvement of the health of the population. Further studies are needed to determine the most optimal amounts of propolis for chickens feeding.

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References

AOAC, Official methods of Analysis. 17th ED. Association of Official Analytical Chemists. Washington DC, 2000.

Aviagen (2014): Ross 308 Broiler: Performance Objectives; Aviagen Group: Huntsville, AL, USA, 2014.

Decker, E.A., Park, Y. (2010): Healthier meat products as functional foods, *Meat. Sci.* 86 (1), 49-55.

Dumic, A., Miskulin, I., Matic Licanin, M., Mujkic, A., Cacic Kenjeric, D., Miskulin, M. (2017): Nutrition Counselling Practices among General Practitioners in Croatia, *Int. J. Environ. Res. Public Health* 14 (12), 1499.

Dumic, A., Miskulin, I., Pavlovic, N., Cacic Kenjeric, D., Orkic, Z., Miskulin, M. (2018): Attitudes toward Nutrition Care among General Practitioners in Croatia, *J. Clin. Med*. 7 (4), 60.

FAO, Food and Agriculture Organization of the United Nations: Food energy – methods of analysis and conversion factors. FAO Food and Nutrition Paper 77, 2002. http://www.fao.org/3/Y5022E/y5022e00.htm#Contents [December 10, 2019]

Garcia, R.G., Freitas, L.W. de, Schwingel, A.W., Farias, R.M., Caldara, F.R., Gabriel, A.M.A., Graciano, J.D., Komiyama, C.M., Almeida Paz, I.C.L. (2010): Incidence and physical properties of PSE chicken meat in a commercial processing plant, *Braz. J. Poultry. Sci.* 12, 233-237.

Grau, R., Hamm, R. (1953): Eine einfache Method zur Bestrimming der Wasserbindung im Muskel, *Naturwissenschaften*. 40, 29-30.

Haščík, P., Garlík, J., Kňazovická, V., Kačániová, M., Omer, I., Elimam, E., Pochop, J., Benczová, E., Vavrišinová, K. (2012): Technological properties of chickens' meat after application of propolis extract in their diet, *J. Microbiol. Biotechnol. Food Sci*. 1, 1295-1304.

Haščík, P., Pavelková, A., Tkáčová, J., Čuboň, J., Kačániová, M., Habánová, M., Mlyneková, E. (2020): The amino acid profile of broiler chicken meat after dietary administration of bee products and probiotics, *Biologia (2020).* https://doi.org/10.2478/s11756-020-00451-9

Karaoglu, M., Aksu, M.İ., Esenbuga, N., Macit, M., Durdag, H. (2006): pH and colour characteristics of carcasses of broilers fed with dietary probiotics and slaughtered at different ages, *Asian-australas. J. Anim. Sci*. 19, 605-610.

Klarić, I. (2014): Production and health effects of propolis and bee pollen as food additives in broilers feeding (in Croatian). PhD Thesis, Faculty of Agriculture in Osijek, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia.

Klarić, I., Domaćinović, M., Pavić, M., Miškulin, I., Miškulin, M., Steiner, Z. (2016): The use of propolis and bee pollen in chickens feeding – a natural way to protect the health of consumers (in Croatian), *Hrana u zdravlju i bolesti* 5, 37-43.

Klarić, I., Domaćinović, M., Šerić, V., Miškulin, I., Pavić, M., Paradinović, K. (2018): Effects of bee pollen and propolis on performance, mortality, and some haematological blood parameters in broiler chickens, *Slov Vet. Res*. 55, 23-34.

Klarić, I., Miškulin, I., Šerić, V., Dumić, A., Jonjić, J., Miškulin, M. (2018a): The effects of propolis and bee pollen supplementation on biochemical blood parameters of broilers, *Acta Vet-Beograd* 68, 190-200.

Kralik, G., Škrtić, Z., Kralik, Z., Đurkin, I., Grčević, M. (2011): Kvaliteta trupova i mesa Cobb 500 i Hubbard classic brojlerskih pilića, *Krmiva* 53, 179-186.

Mehaffey, J.M., Pradhan, S.P., Meullenet, J.F., Emmert, J.L., McKee, S.R., Owens, C.M. (2006): Meat quality evaluation of minimally aged broiler breast fillets from five commercial genetic strains, *Poult. Sci*. 85, 902-908.

Park, K.; Son, J.; Jang, J.; Kang, R.; Chung, H.-K.; Lee, K.W.; Lee, S.-M.; Lim, H.; Shin, M.-J. (2017): Unprocessed Meat Consumption and Incident Cardiovascular Diseases in Korean Adults: The Korean Genome and Epidemiology Study (KoGES), *Nutrients* 9, 498.

Pereira, P.M., Vicente, A.F. (2013): Meat nutritional composition and nutritive role in the human diet, *Meat. Sci.* 93 (3), 586-592.

Prakatur, I., Miškulin, I., Galović, D., Steiner, Z., Lachner, B., Domaćinović, M. (2019): Performance indicators of broilers fed propolis and bee pollen additive, *Poljoprivreda* 25 (1), 69-75.

Prakatur, I., Miskulin, M., Pavic, M., Marjanovic, K., Blazicevic, V., Miskulin, I., Domacinovic, M. (2019a): Intestinal Morphology in Broiler Chickens Supplemented with Propolis and Bee Pollen, *Animals* 9 (6), 301.

Saláková, A., Straková, E., Válková, V., Buchtová, H., Steinhauserová, I. (2009): Quality indicators of chicken broiler raw and cooked meat depending on their seks, *Acta. Vet. Brno* 78, 497-504.

StatSoft, Inc. (2010):Statistica for Windows 2010 (inačica 10.0). StatSoft Inc., Tulsa, OK, SAD.

Šulcerová, H., Mihok, M., Jůzl, M., Haščík, P. (2011): Effect of addition of pollen and propolis to feeding mixtures during the production of broiler chickens Ross 308 to the colour of thigh and breast muscle and pH determination, *Acta Univ. Agric. et Silvic. Mendel. Brun*. 59, 359-366.

Wang, K., Jin, X., Chen, Y., Song, Z., Jiang, X., Hu, F., Conlon, M.A., Topping, D.L. (2016): Polyphenol-Rich Propolis Extracts Strengthen Intestinal Barrier Function by Activating AMPK and ERK Signaling, *Nutrients* 8, 272.

Wang, K.H., Shi, S.R., Dou, T.C., Sun, H.J. (2009): Effect of a free-range raising system on growth performance, carcass yield, and meat quality of slow-growing chicken, *Poult Sci*. 88, 2219-2223.

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DIETARY HABITS AND USE OF DIETARY SUPPLEMENTS AMONG FEMALE CANCER PATIENTS

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original scientific paper

Summary

Cancer is one of the leading causes of death globally. Breast cancer has the highest incidence and mortality rate among female population in the world and in Croatia. There are many factors believed to affect cancer prevention and development, one of important is lifestyle including diet and dietary habits. The aim of this work was to determine dietary habits and dietary supplements intake among female cancer patients. For this purpose, dietary questionnaire was designed and conducted among 190 women, cancer patients, members of the Association of women affected by cancer EVERYTHING for HER. Most women surveyed had breast cancer (86%), average age 52.9 ± 0.9 years. According to body mass index, 40.5% of the patients were overweight while 8.4% were obese, and 2.1% undernourished. Dietary habits improvement after cancer diagnosis reported 85.3% of surveyed patients what was self-assessed by the patients and specially referred to fruit, vegetable and red meat intake. After the diagnosis, 77.9% patients consumed fruits, with 41.9% consuming more than 2 servings, and 98.4% consumed vegetables, with 72.1% consuming more than 2 servings, every day. Red meat was consumed by 22.6% of patients consuming meat while only 4.7% of patients stopped eating meat after diagnosis. Dietary supplements were used by 76.3% of patients, mainly probiotics, vitamin D and C, with boosting of the immune system as the main reason for use.

Keywords: carcinoma, breast cancer, dietary habits, dietary supplements, diet improvement

Introduction

Cancer is one of the leading cause of mortality in the world and number of cancer patients is increasing globally. Breast cancer has the highest incidence and mortality rate among female population in the world and in Croatia (WHO, 2018; HZJZ, 2019).

There are many factors believed to affect cancer prevention and development, with lifestyle factors, including diet, assumed to have the major impact. Factors affecting the incidence of cancer often interact and together can decrease or increase risk of cancer development (WCRF, 2007).

Diet plays an important role in the development of malignancies. High intake of fruits and vegetables, high consumption of soy products, reduced intake of processed and red meat as well as reduced intake of alcohol showed a positive impact (reducing) on the cancer incidence and reduced risk of cancer recurrence. The Mediterranean diet, characterised by the high intake of fruit, vegetables, cereals, grains and nuts, dairy products, fish and olive oil as the principal source of fat has been associated with reduced incidence or mortality from cancer (Escrich et al., 2011).

Patients with cancer should consume 5 to 9 servings of fruits and vegetables every day, where one serving represents 150 g of fruit and 75 g of vegetables. Adequate consumption of fruits, vegetables and whole grains provides the required amount of fibres (21 - 38 g per day) (Ruiz et al., 2014).

Red meat contains high levels of hem iron that can initiate the process of carcinogenesis by generating genotoxic free radicals and N-nitroso compounds (Diallo et al., 2018). Available studies suggest that for cancer prevention consumption of red and processed meat should be limited to less than 300 g per week (Lippi et al., 2016). Alcohol consumption is positively associated with the risk of upper gastrointestinal cancer (oral cavity, pharynx, larynx, oesophagus) as well as colon, liver, and breast cancer (LoConte et al., 2018).

Dietary supplements are considered to be food intendent to enrich the usual diet with the purpose of maintaining health, made from concentrated sources of nutrients or other substances with a nutritional or physiological effect (Regulation, 2013). Cancer patients and survivors often consume dietary supplements to control development of cancer (Patterson et al., 2003). American Cancer Society, World Cancer Research Fund and American Institute for Cancer do not support usage of dietary supplements for cancer prevention and suggest that all necessary nutrients should be taken from food (Harvie, 2014).

Aim of this study was to determine dietary habits and dietary supplements intake among female cancer patients.

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Materials and methods

Participans

190 female cancer patients, members of the Association of women affected by cancer EVERYTHING for HER, aged 26 to 82 participated into study. Recruitment was done from March to the May 2019 and the data were collected during 35 days (from 20 May to 30 June 2019) in accordance with the Helsinki Declaration and General Data Protection Regulation (GDPR).

Questionnaire design

The questionnaire was composed from 87 questions designed as combination of open, closed and multiple-choice questions. The dietary questionnaire consisted of three parts: general information, dietary habits and dietary supplementation. The general part of the questionnaire had 43 questions collecting data on age, working status, income, body weight and height, physical activity, place of residence, menopause and lifestyle. The second part of the questionnaire had 33 questions that concerns the dietary habits of respondents. Data about consummation of food that can have positive or negative impact on cancer risk such as intake of fruits and vegetables, nuts, red meat and food preparation were collected. The third part of the questionnaire had 11 questions regarding dietary supplements consumption where data about beginning of taking, reasons of using, sources of information, risk researching as well as type of dietary supplements used were collected.
Questionnaire was filled out anonymously in paper-based form or in electronic form for those that were unable to came into the association.

Statistical analysis

The collected data were statistically analysed in Microsoft Excel 2013 and SPSS StatisticV.22 (SPSS Inc., Chicago, Il, USA). Standard methods of descriptive statistics (mean, standard deviation, standard error, minimum, maximum) were used. Distribution normality was examined by the Shapiro-Wilk test.

Results

General informations

Average age of subjects was 52.9 ± 0.9 years. To assess the degree of malnutrition, body mass index (BMI) was used, where BMI under 18.5 kg/m^2 indicates underweight, BMI between 18.5 – 24.9 kg/m^2 indicates adequate weight, BMI between 25.0 – 29.9 kg/m^2 indicates overweight and BMI over 30 kg/m^2 shows obesity (WHO, 2000). According to the BMI adequate body mass had 48.9% of patients, 40.5% were overweight, 8.4% obese and 2.1% undernourished. 85.3% of surveyed patients stated that they improved their dietary habits after cancer diagnosis. In this study, most patients (68%) had breast cancer, and the remaining had ovarian cancer, cervical cancer, lung cancer, leukemia, gastric cancer and others, what was expected according to the trends in Western Europe and in Croatia (Ferlay et al., 2018; HZJZ, 2019).

Dietary habits

The questions about dietary habits were designed to get information about dietary consumption after cancer diagnosis what represents their actual food consumption. In this study, 26.3% of women stated that they did not changed, while 73.7% of them changed their dietary habits after cancer diagnosis which includes increased intake of fruits and vegetables, olive oil and reduced intake of alcohol, caffeine and caffeinated beverages. One earlier research showed that 30-48 % of women had changed their dietary habits after cancer diagnosis, what included increased consumption of fruits and vegetables (Limon-Miro et al., 2017).
To the question "Do you pay more attention to your diet and what do you eat since you have been diagnosed with the disease?", 82.31% of women responded positively.
According to the American Cancer Guidelines, it is recommended daily consumption of 5 servings from fruit and vegetable food groups (Doyle et al., 2006). The UK guidelines gives similar recommendations that include a minimum intake of 400 g of fruits and vegetables per day (BNF, 2018). Canada also has its own guidelines for women with cancer with focus on consuming 7 to 8 servings of fruits and vegetables every day, where ½ cup of certain fruit represent one serving (BCCANCER, 2012). About 65.2% of all surveyed women consume one serving of fruit per day, while according to the collected data, 55.3% of the respondents consume up to 2 servings of vegetables a day (Fig. 1). Dietary habits of surveyed patients do not comply with the recommendations. Most studies conclude that 5-9 servings of fruits and vegetables, where one serving represents 150 g of fruit and/or 75 g of vegetables, are needed daily to ensure adequate intake of antioxidants and fibres, with suggestion that vegetables included into diet should be rich in beta-carotene and vitamins A, C and E (Limon-Miro et al., 2017).

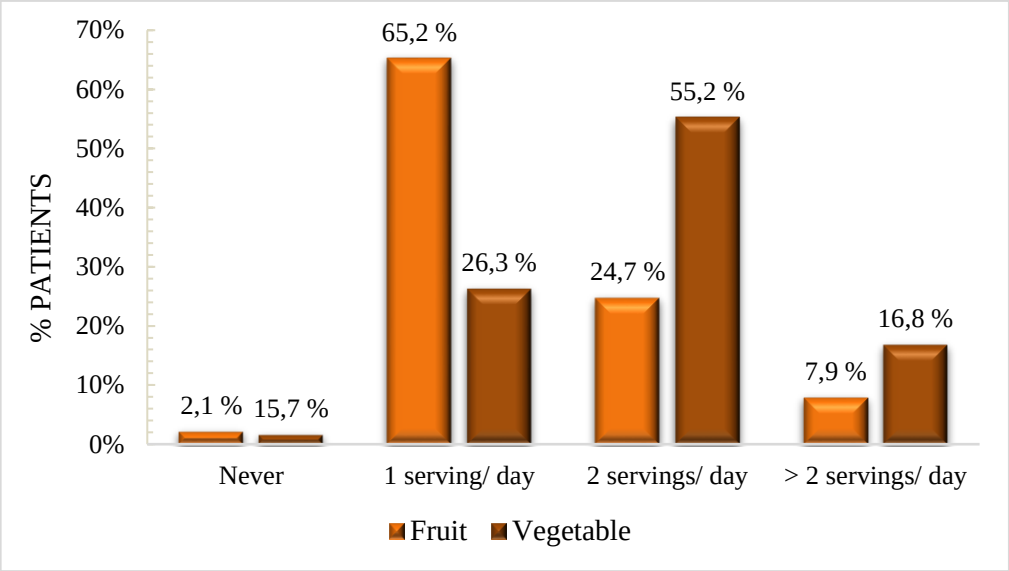

Fig. 1. Intake of fruits and vegetables among female cancer patients (n=190)

About 39% of patients surveyed consumed mostly leafy green vegetables, and 19.8% consume cabbage. Li et al. (2017) suggest a higher intake of cabbage because it is rich in isothiocyanates such as benzyl isothiocyanate, phenethylisothiocyanate and sulforafan which can inhibit tumor cell growth. Citrus fruits and apple fruits were the most commonly consumed fruits among women in this research. Apple fruits were consumed by 24.6% of women, while 19.8% of women consume citrus fruits (Table 1). Consumption of citrus fruits is recommended during treatment and recovery for certain cancers, including breast cancer (Li et al., 2017).

Table 1. Groups of consumed fruits and vegetables among female cancer patients (n=190)

Fruit food group	Respondents (%)
Citrus	19.8%
Berries	18.7%
Apple fruits	24.6%
South tropical fruits	16.6%
Nuts	11.2%
Vegetable food group	**Respondents (%)**
Cabbages	26.2%
Dark green leafy vegetables	39%
Beans and peas	9.1 %

Poultry meat was the most common type of meat consumed in this study. Most of women (87.3%) consume poultry meat and only 12.7% consumed red meat. Limon-Maroet al. (2017) propose that meat such as chicken and turkey meat should be prioritized for women with cancer (1-2 times per week) because is good source of animal protein and have a low fat content. According to the US recommendations, cancer patients should not consume more than 3 servings of red meat (350-500 g) per week (Doyle et al., 2006).

The consumption of processed meat is often one of controversy when it comes to patients with some type of cancer. The Fig. 2 shows that an equal number of women in this research (up to 30.5%) consumed processed meat several times a week and 1-3 times per month. About 19.5% of women never consumed processed meat, and 16.3% reported that they consume processed meat several times in a few months. Consumption of red and processed meat should be limited less than 300 g per week (Lippi et al., 2016). According to the results obtained in this study, it is evident that the intake of processed meat among surveyed patients is in accordance with the recommendations.

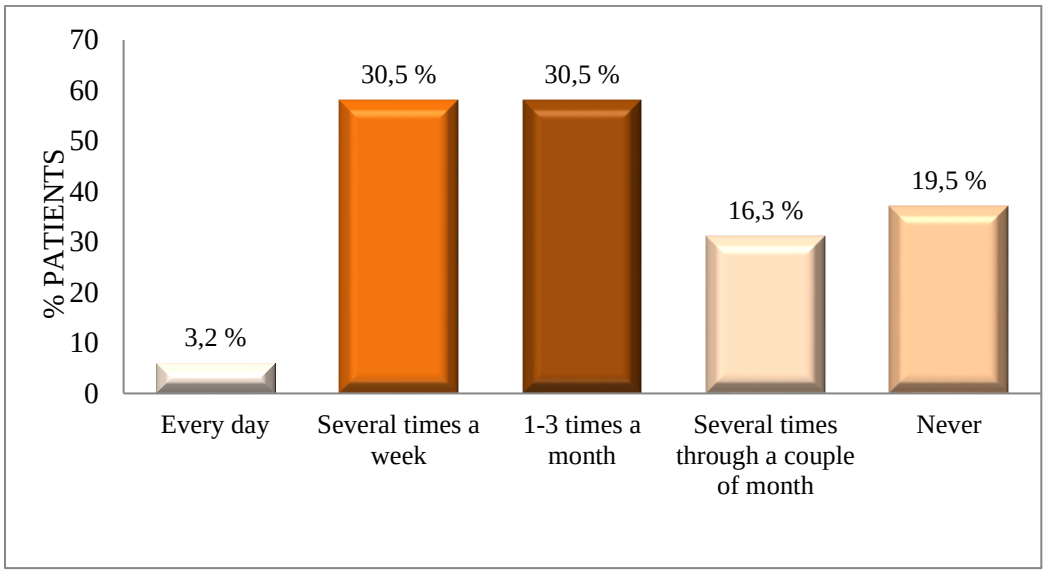

Fig. 2. Intake of processed meat among female cancer patients (n=190)

The Table 2 shows that blue fish was the most common choice among 56.9% of respondents, while white fish was more commonly consumed by 34% of women. Most of women (36.8%) eat fish once a week, while 30.5% of women eat fish several times a month. The UK recommendations focus on fish intake, recommending fish intake at least 2 times per week (BNF, 2018) and when compared to the results obtained in this study, it is clear that the intake does not comply with recommendations (Fig. 3). Consumption of fish is particularly important because it is a good source of omega-3 fatty acids especially during chemotherapy (Kotepui et al., 2016), among that fish intake is important because it is a source of high quality protein and fat-soluble vitamins such as A and D, as well as trace elements such as selenium and iodine.

Table 2. Consumed fish species in research (n=190)

Fish	Respondents (%)
Blue fish	56.9%
White fish	34%
Freshwater fish	9%

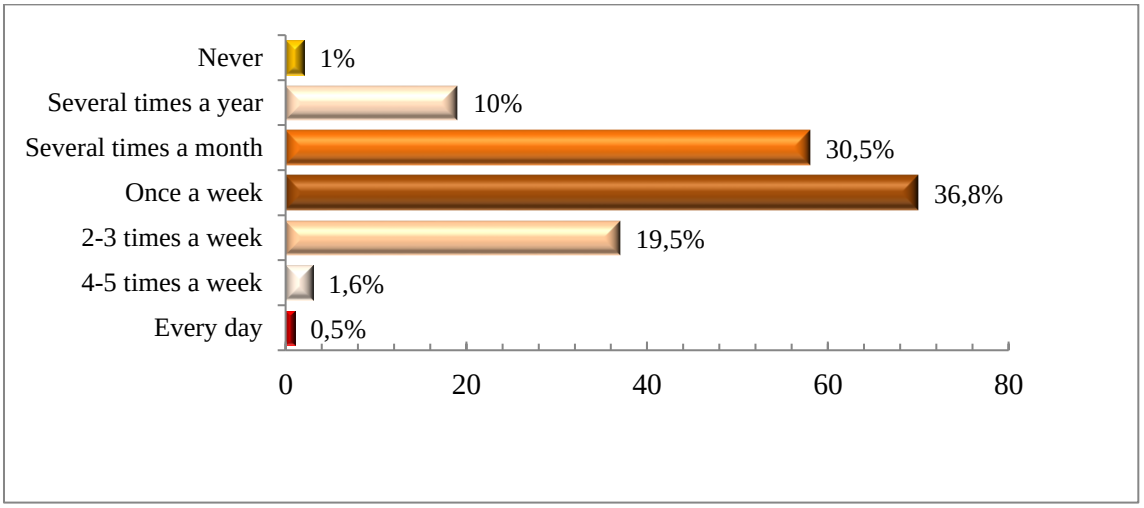

Fig. 3. Intake of fish among female cancer patients (n=190)

There was no significant difference between women according to the alcohol consumption. About 60.5% of respondents consume alcohol, while 39.5% do not. If we focus only on women who consume alcohol, 52% of them consume alcohol less than once a month. In this research, despite the fact that almost 60% of the respondents consumed alcohol, the frequency of consumption was rare. In particular, the effect of alcohol on women after diagnosed cancer is not clear. Some researchers showed that alcohol consumption which include ≥3 alcoholic drinks per week after a breast cancer diagnosis may increase risk of breast cancer recurrence, particularly among postmenopausal and overweight/obese women (Kwan et al., 2010).

Alcohol avoidance is preferred and consumption should be limited to one drink a day, what means a glass (145 mL) of wine, or bottle of beer (359 mL), or a glass of strong alcoholic beverage (45 mL) such as whiskey and gin (BCCANCER, 2012).

Dietary supplements

According to the obtained result about dietary supplements intake, there were differences in level of education between respondents with respect to dietary supplements intake. A lower education level implied elementary school and high school (n = 75), while higher education level included college, bachelor´s, master´s or higher degree. There was observed nominal, but not a significant difference (*p=0.472*) in consumption of dietary supplements regarding education level. Among patients with lower education level there is higher proportion of those who do not use dietary supplements, while among women with higher education level situation is the opposite. Song et al. (2017) found similar relationship between education level and use of dietary supplements, where patients with higher education level were more likely to use dietary supplements compared to those with lower education level.

In this study, 147 patients (77%) were using dietary supplements what is in accordance to previous where was assumed that 65% of the European cancer survivors use other methods which are not part of the conventional cancer treatment and as most commonly stated is the use of dietary supplements (Lopes et al., 2017).

Velicer and Urlich (2008) found the difference in the use of dietary supplements between breast cancer survivors compared to other types of cancer, where breast cancer survivors are more likely to use dietary supplements than patients suffering from other types of cancer. In this study, there was no difference (*p=0.875*) in consumption of dietary supplements among breast cancer patients (77.7%) compared to patients suffering from other types of cancer (76.7%). In earlier research there was estimated that 75% breast cancer survivors use dietary supplements (Astin et al., 2006).

Main reason for using dietary supplements in this study was boosting the immune system (Fig. 4). That can be explain by the fact that most of the patients start using dietary supplements during therapy because of decline of immunity and general weakness so they wanted to boost the immune system to help withstand the therapy.

In the literature, the main reasons for using dietary supplements is also boosting immune system, following by: curing the disease, decreasing side effects of chemotherapy, better quality of life, controlling disease, enhancing recovery and prevention of cancer recurrence (Vidal et al., 2013; Wong et al., 2010). Same results were obtained in this study (Fig. 4).

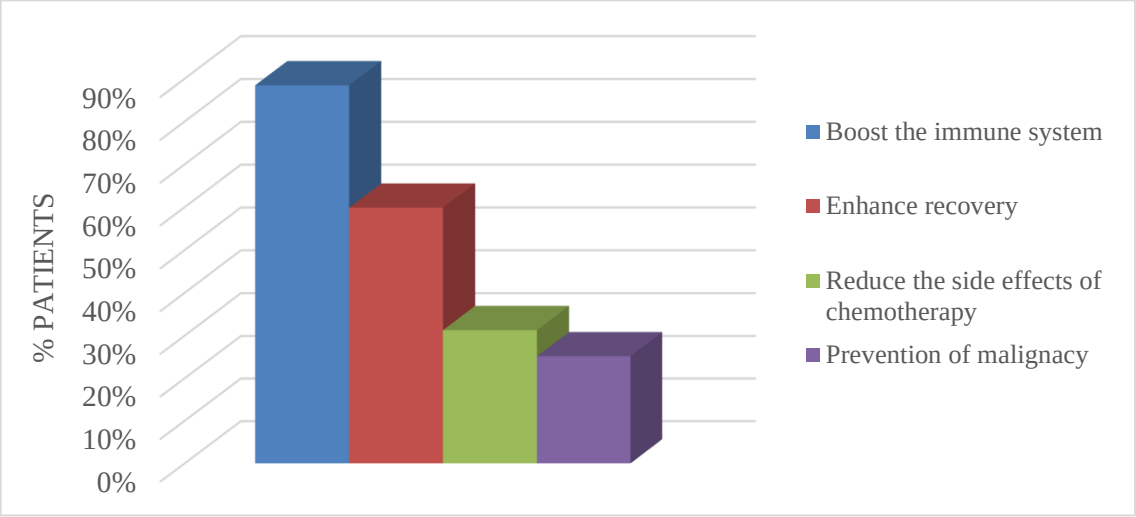

Fig. 4. Main reasons for using dietary supplements

Probiotics (41.5%) were the most commonly used dietary supplement in this study, followed by vitamin D (40.1%) and vitamin C (36.1%). Equal number of respondents (33.3%) used multivitamins, antioxidants and omega-3 fatty acid supplements. The similar results were obtained for consumption of curcumin and calcium supplements, used by 32.7% respondents. From all respondents who use dietary supplements, only 20 (13.6%) used one supplement exclusively, while others used two or more dietary supplements. Song et al. (2017) compared the consumption of dietary supplements between general population and cancer survivors, where cancer survivors were mostly using multivitamins and minerals, after that vitamin C, omega-3 fatty acid, red ginseng and calcium. In *Intergroup phase III Breast Cancer Chemotherapy trial (S0221)* most patients were using multivitamins, followed by calcium, vitamin C, vitamin D and omega-3 fatty acids (Harvie, 2014). Similar results were obtained in *WHEL study* (*Women´s Healthy Eating and Living study*), where the most commonly used dietary supplements were multivitamins, vitamin E and vitamin C (Rock et al., 2004). The main difference between results from literature and results obtained in this study are probiotics, with no differences in consumption of other supplements. Probiotics help reducing digestive disorders, which are often reported as side effects of chemotherapy (Serna-Thome, 2018). Also, probiotics were the most prevalent during therapy among respondents in this study. So it is possible that probiotics may have helped to reduce side effects of chemotherapy among patients, partially facilitated toleration of the therapy and improved quality of life. Of all consumed supplements in this study(Fig. 5), only probiotics and omega-3 fatty acids shows potential positive effects on cancer survivors (Serna-Thome, 2018; Fabian et al., 2015).

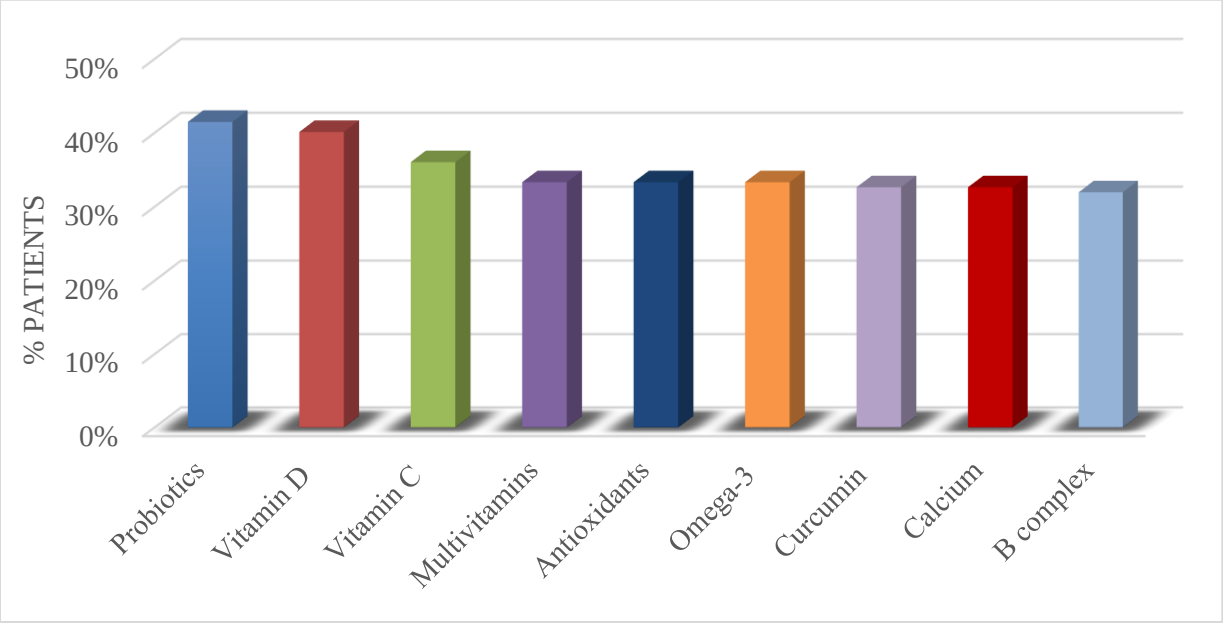

Fig. 5. The most commonly used dietary supplements

The majority of respondents (61%) began to use dietary supplements during therapy, 21% respondents started with supplementation before cancer was diagnosed and 18 % respondents started with supplements after the therapy. Only 28 respondents (19%) used dietary supplements only during therapy and did not continue using it after finishing therapy.

It is estimated that, in the US population, 45 – 80 % breast cancer survivors use antioxidants as dietary supplements, including a period during therapy (Greenlee et al., 2009). During therapy, 55 respondents used antioxidants as dietary supplement, including mix of antioxidants, vitamin C and vitamin E. That means that 63.21% respondents who were using supplements during therapy used some kind of antioxidants as dietary supplements. Possible problems with usage of antioxidants during therapy, is their possible pro-oxidative effect of weakening the effect of chemotherapy (Lawenda et al., 2008).

Among all respondents who used dietary supplements, 64% investigated the possible risks and side effects of using dietary supplements before staring using them.

Respondents were divided into three categories according to timing when started using dietary supplements - respondents who were using supplements before cancer diagnose, respondents who started using supplements during therapy and respondents who started with supplementation after therapy (Fig. 6). In the group of respondents who were using dietary supplements before cancer diagnose, multivitamins and probiotics were the most commonly used supplements. Similar results were given by *Pathway study*, where the most commonly used were multivitamins, vitamin C, calcium, vitamin D (Greenlee et al., 2014), the main difference between *Pathway study* and this study was probiotics usage. During the therapy respondents in this study mostly used probiotics, omega-3 fatty acids, and antioxidants, while vitamin D was the most commonly used supplement after therapy. In the study from Zirpoli et al. (2013), multivitamins were the most commonly used supplement before cancer diagnose and during therapy, also after therapy there was increase in vitamin D supplementation.

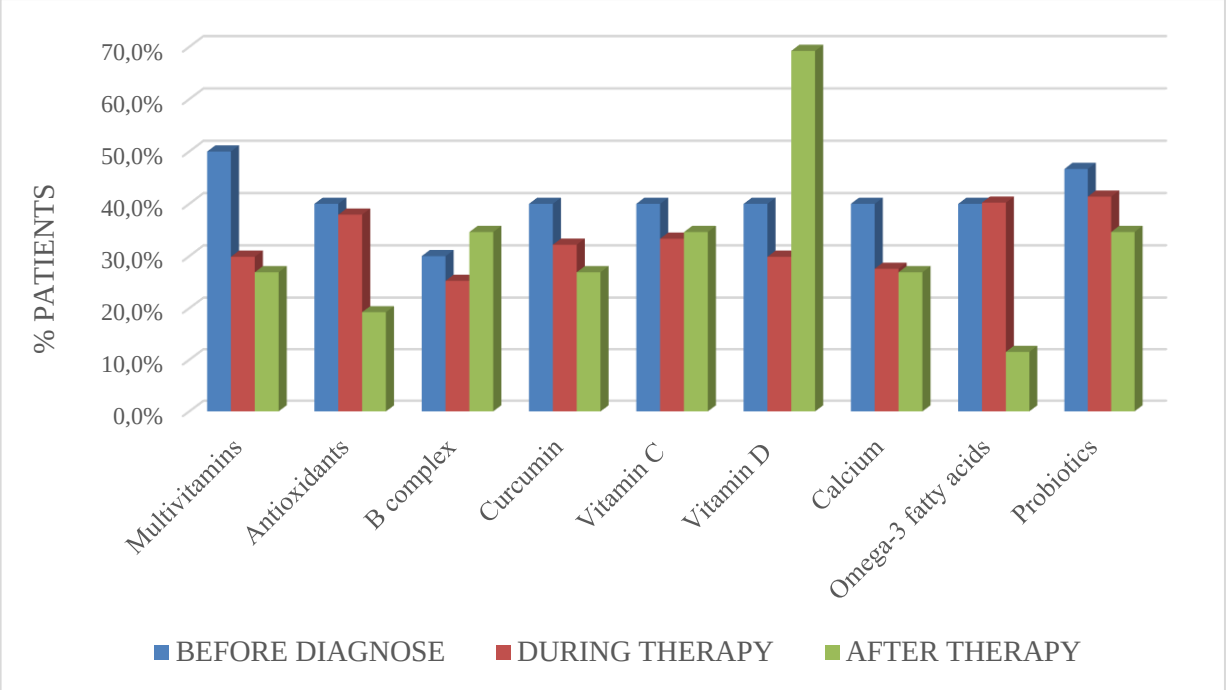

Fig. 6. Comparison of dietary supplements used before diagnose, during therapy and after therapy (n = 147)

The respondents in this study used more dietary supplements compare to herbal (alternative) supplements. Consumption ratio was 8:1 on behalf of dietary supplements. The most commonly used herbal supplement was beta glucan (41.5% of respondents who used herbal supplements). According to the frequency of use, glucosamine (24.6%) was at second place, and green tea extract and *Echinacea* were at the third place (21.5%).

Conclusions

In this study, about 7.9% of women, cancer patients, consumed an adequate amount of fruit, while 16.8% consumed adequate amount of vegetables. White meat was the choice of most women in this study. Only 19.5% of women met the recommendations for fish intake. In general, women cancer patients should increase their intake of fruits and vegetables (at least 400 g per day), fish (twice a week), and reduce intake of processed and red meat (less than 300 g per week). Alcohol consumption should be moderate (1 drink per day).

Regarding dietary supplements, 77.3% of respondents used dietary supplements at some stage of treatment with more educated respondents more likely to used dietary supplements. Dietary supplements were more likely used than herbal supplements. The main reasons for using dietary supplements were boosting the immune system and enhancing recovery. In this study, most commonly used supplements were probiotics, vitamin D, vitamin C, multivitamins, calcium and omega-3 fatty acids. Before diagnosis, most frequently consumed were multivitamins and probiotics, during the treatment probiotics, omega-3 fatty acids and antioxidants, while after therapy vitamin D was the most commonly used.

References

Abid, Z., Cross, A. J., Sinha, R. (2014): Meat, dairy, and cancer. *Am. Clin. Nutr.* 100, 386-393.

Astin, J. A., Reilly, C., Perkins, C., Child, W. L. (2006): Breast cancer patients' perspectives on and use of complementary and alternative medicine: a study by the Susan G. Komen Breast Cancer Foundation. *J. Soc. Integr. Oncol*. 4 (4), 157–169.

BCCANCER (2012) A nutrition Guide for Women with Breast Cancer. BCCANCER- Breast Cancer Agency, <http://www.bccancer.bc.ca/nutrition-site/Documents/Patient%20Education/a_nutrition_guide_for_women_with_breast_cancer.pdf>. Pristupljeno 10. kolovoza 2019.

BNF (2018) The recommendation in the World Cancer Research Fund 2018 Third expert report. BNF- British Nutrition Foundation, <https://www.nutrition.org.uk/nutritioninthenews/new-reports/new-report-from-the-world-cancer-research-fund-diet-nutrition-physical-activity-and-cancer-a-global-perspective.html>. Pristupljeno 15. kolovoza 2019.

Diallo, A., Deschasaux, M., Latino-Martel, P., Hercberg, S., Galan, P., Fassier, P., Touvier, M. (2018): Red and processed meat intake and cancer risk: results from the prospective NutriNet-Santé cohort study. *Int. J. Cancer* 142, 230-237.

Doyle, C., Kushi, L. H., Byers, T., Courneya, K. S., Demark-Wahnefried, W., Grant, B., Andrews, K. S. (2006): Nutrition and physical activity during and after cancer treatment: an American Cancer Society guide for informed choices. *CA-Cancer J. Clin.* 56, 323-353.

Escrich, E., Moral, R., Solanas, M. (2011): Olive oil, an essential component of theMediterranean diet, and breast cancer. *Public Health Nutr.* 14, 2323-2332.

Fabian, C. J., Kimler, B. F., Hursting, S. D. (2015): Omega-3 fatty acid for breast cancer prevention and survivorship. *Breast Cancer Res.* 17, 62-73.

Ferlay, J., Colombet, M., Soerjomataram, I., Dyba, T., Randi, G., Bettio, M., Gavin, A., Visser, O., Bray, F. (2018): Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancer in 2018. *Eur. J. Cancer* 103, 356-387.

Greenlee, H., Hershman D. L., Jacobson, J. S. (2009): Use of antioxidant supplements during breast cancer treatment: a comprehensive review. *Breast Cancer Res. Treat.* 115, 437–452.

Greenlee, H., Kwan, M. L., Ergas, I. J., Strizich, G., Roh, J. M., Wilson, A. T., Lee, M., Sherman, K. J., Ambrosone, C. B., Hershman, D. L., Neugut, A. I., Kushi, L. H. (2014): Changes in vitamin and mineral supplement use after breast cancer diagnosis in the Pathways Study: A prospective cohort study. *BMC Cancer* 14 (382), 1-16.

Harvie, M. (2014) Nutritional supplements and cancer: Potential benefits and proven harms. *Asco Educational Book* 478-486.

HZJZ (2019): Incidencija raka u Hrvatskoj. HZJZ – Hrvatski zavod za javno zdravstvo, <https://www.hzjz.hr/periodicne-publikacije/bilten-incidencija-raka-u-hrvatskoj-2016-godine/>Pristupljeno 18.lipnja 2019.

Kotepui, M. (2016): Diet and risk of breast cancer. *Contem.Oncol.* 20, 13-19.

Kunzmann, A. T., Coleman, H. G., Huang, W. Y., Cantwell, M. M., Kitahara, C. M., Berndt, S. I. (2016): Fruit and vegetable intakes and risk of colorectal cancer and incident and recurrent adenomas in the PLCO cancer screening trial. *Int. J. Cancer* 138, 1851-1861.

Kwan, M. L., Kushi, L. H., Weltzien, E., Tam, E. K., Castillo, A., Sweeney, C., Caan, B.J. (2010): Alcohol consumption and breast cancer recurrence and survival among women with early-stage breast cancer: the life after cancer epidemiology study. *J. Clin. Oncol.* 28, 4410.

Lawenda, B. D., Kelly, K. M., Ladas, E. J., Sagar, S. M., Vickers, A., Blumberg, J. B. (2008): Should supplemental antioxidant administration be avoided during chemotherapy and radiation therapy?. *J. Natl. Cancer Inst*. 100, 773–783.

Li, Y., Li, S., Meng, X., Gan, R. Y., Zhang, J. J.,Li, H. B. (2017): Dietary natural products for prevention and treatment of breast cancer. *Nutrients* 9, 728-766.

Limon-Miro, A. T., Lopez-Teros, V., Astiazaran-Garcia, H. (2017): Dietary guidelines for breast cancer patients: a critical review. *Adv. Nutr.* 8, 613-623.

Lippi, G., Mattiuzzi, C., Cervellin, G. (2016): Meat consumption and cancer risk: a critical review of published meta-analyses. *Crit. Rev. Oncol. Hemat.* 97, 1-14.

Liu, Y., Nguyen, N., Colditz, G. A. (2015): Links between alcohol consumption and breast cancer: a look at the evidence. *Ann. Ny. Acad. Sci.* 11, 65-77.

LoConte, N. K., Brewster, A. M., Kaur, J. S., Merrill, J. K., Alberg, A. J. (2018): Alcohol and cancer: a statement of the American Society of Clinical Oncology. *J. Clin. Oncol.* 36, 83-93.

Lopes, C. M., Dourado, A., Oliveira, R. (2017): Phytotherapy and nutritional supplements on breast cancer. *BioMed. Res. Int.* 1-42.

Patterson, R. E., Neuhouser, M. L., Hedderson, M. M., Schwartz, S. M., Standish, L. J., Bowen, D. J. (2003): Changes in diet, physical activity, and supplement use among adults diagnosed with cancer. *J. Am. Diet. Assoc*. 103 (3), 323–328.

Regulation on Dietary Supplements (2013): *Official Gazette* 126, Zagreb.

Rock, C. L., Newman, V. A., Neuhouser, M. L., Major, J., Barnett, M. J. (2004) Antioxidant supplement use in cancer survivors and the general population.*J. Nutr*. 134: 3194–3195.

Romieu, I. (2011): Diet and breast cancer. *Salud.Publica. Mex.* 53, 430-439.

Ruiz, R. B., Hernández, P. S. (2014): Diet and cancer: risk factors and epidemiological evidence. *Maturitas* 77, 202-208.

Sergentanis, T. N., Psaltopoulou, T., Ntanasis-Stathopoulos, I., Liaskas, A., Tzanninis, I. G., Dimopoulos, M. A. (2018): Consumption of fruits, vegetables, and risk of hematological malignancies: a systematic review and meta-analysis of prospective studies. *Leukemialymphoma* 59, 434-447.

Serna-Thome, G., Castro-Eguiluz, D., Fuchs-Tarlovsky, V., Sanchez-Lopez, M., Delgado-Olivares, L., Coronel-Martinez, J., Molina-Trinidad, E. M., De la Torre, M., Cetina-Perez, L. (2018): Use of functional foods and oral supplements as adjuvants in cancer treatment. *Rev. Inves. Clin*. 70, 136-146.

Song, S., Youn, J., Lee, Y. L., Kamng, M., Hyun, T., Song, Y., Lee, J. E. (2017): Dietary supplement use among cancer survivors and the general population: a nation-wide cross-sectional study. *BMC Cancer* 17, 891-903.

Velicer, C. M., Ulrich, C. M. (2008): Vitamin and mineral supplement use among US adults after cancer diagnosis: a systematic review. *J. Clin. Oncol.* 26 (4), 665–673.

Vidal, M., Carvalho, C., Bispo, R. (2013): Use of complementary and alternative medicine in a sample of women with breast cancer. *SAGE Open*. 1–4.

WHO (2018): Latest global cancer data: Cancer burden rises to 18.1 million new cases and 9.6 million cancer deaths in 2018. WHO- World Health Organization, Geneva <https://www.who.int/cancer/PRGlobocanFinal.pdf>Pristupljeno 4. svibnja 2019.

WHO (2000) Obesity: preventing and managing the global epidemic: report of a WHO consultation. WHO-World Health Organization, Geneva. *World Health Organ. Tech. Rep. Ser. 894, 1-253.*

Wong, K. K., Engelman, J. A., Cantley, L. C. (2010) Targeting the PI3K signaling pathway in cancer. *Curr.Opin.Genet. Dev*. 20, 87–90.

Zirpoli, G. R., Brennan, P. M., Hong, C., McCann, S. E., Ciupak, G., Davis., W., Unger, J. M., Budd, G. T., Hershman, D. L., Moore, H. C. F., Stewart, J., Isaacs, C., Hobday, T., Salim, M., Hortobagyi, N., Gralow, J. R., Albain, K. S., Ambrosome, C. B. (2013): Supplement use during an intergroup clinical trial for breast cancer (S0221). *Breast Cancer Res. Treat*. 137, 903-913.

WCRF (2007): Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Prospective. WCRF - World Cancer Research Fund, < https://www.wcrf.org/ >. Pristupljeno 6. travnja 2019.

Hrana u zdravlju i bolesti, znanstveno-stručni časopis za nutricionizam i dijetetiku (2020) 9 (1) 30-39
Food in Health and Disease, scientific-professional journal of nutrition and dietetics (2020) 9 (1) 30-39

OPTIMIZATION OF ETHANOL/WATER SOLVENT EXTRACTION OF BIOACTIVE COMPONENTS ORIGINATING FROM INDUSTRIAL HEMP (*Cannabis sativa* L.)

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original scientific paper

Summary

Hemp (*Cannabis sativa* L.) contains a wide range of biocompounds with different beneficial properties such as anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic and antioxidative. Response Surface Methodology (RSM) coupled with Box-Behnken design (BBD) was applied to determine the influence of extraction temperature, liquid to solid ratio, extraction time, rotational speed and ethanol/water solvent ratios at three levels on the solid-liquid extraction of the bioactives from the hemp (flowers, leaves, seeds, stems). Based on the obtained results, liquid to solid ratio, temperature and ethanol/water solvent ratio had statistically significant effects on the total polyphenolic content (TPC), while extraction time and rotational speed had no influence on the TPC extraction. Regarding antioxidant activity (AOX) determined by the DPPH method, only liquid to solid ratio had a statistically significant effect. Liquid to solid ratio, ethanol/water solvent ratio, temperature and rotational speed significantly influenced AOX determined by the FRAP method. According to BBD, the optimum extraction conditions were as follows: extraction temperature 45 °C, liquid to solid ratio 30 mL/g, extraction time 25 min, rotational speed 500 rpm, ethanol/water solvent ratio 25%. RSM coupled with a BBD model was shown to be effective for optimization the solid-liquid extraction of hemp.

Keywords: hemp (*Cannabis sativa* L.), solid-liquid extraction, optimization, bioactives

Introduction

Hemp (*Cannabis sativa* L.) is a herbaceous plant of the *Cannabaceae* family that grows in diverse climates all over the world (Kostić et al., 2013). It has been cultivated for a long time for medicinal and food purposes, together with its use as a source of textile fibre. The genus *Cannabis* has been divided into three main species: (i) *Cannabis sativa* L., a fibre-type one, rich of cannabidiol (CBD), (ii) *Cannabis indica* Lam., a drug-type one, characterised by high levels of the psychoactive compound Δ^9-tetrahydrocannabinol (Δ^9-THC) and (iii) *Cannabis ruderalis* Janisch with intermediate properties (Appendino et al., 2011; Thomas and ElSohly, 2015; Hartsel et al., 2016; Pellati et al., 2018).

Several chemical classes that have been identified in *C. sativa* are: terpenes, carbohydrates, fatty acids and their esters, amides, amines, phytosterols, phenolic compounds and cannabinoids. Among them, cannabidiol represents the most valuable one from the pharmaceutical point of view, since it has been found to possess a high antioxidant and anti-inflammatory activity, together with antibiotic, neuroprotective, anxiolytic, and anticonvulsant properties (Pellati et al., 2018). Regarding phenolic compounds present in *C. sativa*, several flavonoids have been identified, belonging mainly to flavones and flavonols (Pollastro et al., 2018). Most of these compounds have a wide range of different properties such as anti-inflammatory, neuroprotective, anticancer and antioxidative (Andre et al., 2016).

Extraction procedure is a primary step for identification and quantification of chemical compounds from plant materials. Many conventional methods can be used to extract these compounds, such as solid-liquid extraction and heated reflux extraction. In addition, a number of advanced methods, such as ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, accelerated solvent extraction, or high hydrostatic pressure extraction are also applied in the extraction of different compounds (Xu et al., 2017). Among them, solid-liquid extraction represents the most commonly used method for extracting phenolics from various plants. Many parameters, such as solvent concentration, extraction time, temperature, pH, liquid/solid ratio and particle size, may significantly influence the solid-liquid extraction (Radojković et al., 2012; Xu et al., 2017; Tchabo et al., 2018). These parameters should be taken into consideration in order to attain the optimal/highest extraction efficiency (Sifaoui et al., 2016). In order to optimize parameters of extraction process, Response Surface Methodology (RSM) has been widely used. RSM is a statistical method for evaluating multiple

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process parameters and their interactions using quantitative data, optimizing complex extraction procedures, thus reducing the number of experimental trials required. RSM methodology combines mathematical and statistical techniques for designing experiments, building models, evaluating effects of experimental parameters and searching optimum condition of parameters for desirable responses (Liu et al., 2015). Box-Behnken design (BBD), as a part of RSM, has been generally used in various experiments to investigate the impact of more factors at three levels with the ability of effective evaluation of the coefficients of first- and second-order mathematical models (Bezerra et al., 2008).

Bioactive compounds present in industrial hemp have received more and more attention by the biochemical and nutritional researchers due to their biological activities and health function in health-care food or medicine, especially their antioxidant, anti-ultraviolet radiation, and antibacterial effects (Wang et al., 2014; Cao et al., 2017). These antioxidant activities make this plant important for oil, food, pharmaceutical, cosmetics and fiber industries (Kolodziejczyk et al., 2012; Shahid-ul-Islam et al., 2013; Sarasini and Fiore, 2018). These industries are dependent on various extraction processes. Since many factors can influence the efficiency of antioxidants extractions from the plant matrices, an individual approach for extraction and optimization is required. There is no universal extraction and optimization technique, due to the diversity of bioactive compounds (Silva et al., 2007; Majeed et al., 2016).

Having in mind the above mentioned, the aim of this study was to apply the Response Surface Methodology (RSM) approach, coupled with Box-Behnken design (BBD), to optimize the ethanol/water solvent extraction of bioactive components from industrial hemp considering the following variable extraction parameters: extraction temperature, liquid to solid ratio, extraction time, rotational speed and ethanol/water solvent ratios. Analysis of Variance (ANOVA) was used to evaluate the significance of the extraction parameters on the total polyphenolic content (TPC) and antioxidant activity (AOX) of the obtained hemp extracts.

Materials and methods

Materials

Plant material

Dried industrial hemp (*Cannabis sativa* L.) (flowers, leaves, seeds, stems) was purchased from local producer (OPG Levačić, Prelog, Croatia). The plant material was collected in the north-western part of Croatia (Međimurje), 2018, dried naturally, and the final dry matter content of the lot was 91.03%.

Chemicals and reagents

Folin-Ciocalteu reagent and sodium carbonate were purchased from Kemika (Zagreb, Croatia). Gallic acid (3,4,5-trihydroxybenzoic acid) and iron(II) sulfate heptahydrate were obtained from Aldrich (Sigma–Aldrich, Chemie, Steinheim, Germany). DPPH (2,2-diphenyl-1-picrylhydrazyl) and Trolox (6-hydroxy-2,5,7,8-tetra methylchromane-2-carboxylic acid) was obtained from Fluka (Buchs, Switzerland). Methanol was obtained from J.T. Baker (Deventer, The Netherlands). Hydrochloric acid was obtained from Carlo Erba Reagents (Val de Reuil, France). Acetic acid was purchased from T.T.T. d.o.o. (Novaki, Sv. Nedelja, Croatia). Sodium acetate trihydrate, iron(III) chloride hexahydrate and ethanol (96%) were purchased from Gram-Mol (Zagreb, Croatia). TPTZ (2,4,6-Tris(2-pyridyl)-1,3,5-triazine) was obtained from Sigma (Buchs, Switzerland). Chemicals were of analytical reagent grade.

Methods

Design of experiments

Response Surface Methodology (RSM) was used for investigating the influence of five independent variables (extraction parameters) on the chemical characteristics (total polyphenolic content and antioxidant activity) of the hemp extracts. The experiment was performed using Box-Behnken experimental design. The main parameters affecting solid-liquid extraction including liquid to solid ratio (10, 30, 50 mL/g), ethanol content (0, 25, 50 %), temperature (30, 45, 60 °C), extraction time (5, 25, 45 min) and rotational speed (250, 500, 750 rpm) were selected as independent variables that should be optimized. The resulting experimental design comprised of 46 experiments as shown in Table 1.

Table 1. Response Surface Methodology, coupled with Box-Behnken experimental design

Experiment No.	Liquid to solid ratio (mL/g)	Ethanol content (%)	Temperature (°C)	Time (min)	Rotational speed (rpm)
1	10 (-1)	0 (-1)	45 (0)	25 (0)	500 (0)
2	50 (+1)	0 (-1)	45 (0)	25 (0)	500 (0)
3	10 (-1)	50 (+1)	45 (0)	25 (0)	500 (0)
4	50 (+1)	50 (+1)	45 (0)	25 (0)	500 (0)
5	30 (0)	25 (0)	30 (-1)	5 (-1)	500 (0)
6	30 (0)	25 (0)	60 (+1)	5 (-1)	500 (0)
7	30 (0)	25 (0)	30 (-1)	45 (+1)	500 (0)
8	30 (0)	25 (0)	60 (+1)	45 (+1)	500 (0)
9	30 (0)	0 (-1)	45 (0)	25 (0)	250 (-1)
10	30 (0)	50 (+1)	45 (0)	25 (0)	250 (-1)
11	30 (0)	0 (-1)	45 (0)	25 (0)	750 (+1)
12	30 (0)	50 (+1)	45 (0)	25 (0)	750 (+1)
13	10 (-1)	25 (0)	30 (-1)	25 (0)	500 (0)
14	50 (+1)	25 (0)	30 (-1)	25 (0)	500 (0)
15	10 (-1)	25 (0)	60 (+1)	25 (0)	500 (0)
16	50 (+1)	25 (0)	60 (+1)	25 (0)	500 (0)
17	30 (0)	25 (0)	45 (0)	5 (-1)	250 (-1)
18	30 (0)	25 (0)	45 (0)	45 (+1)	250 (-1)
19	30 (0)	25 (0)	45 (0)	5 (-1)	750 (+1)
20	30 (0)	25 (0)	45 (0)	45 (+1)	750 (+1)
21	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)
22	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)
23	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)
24	30 (0)	0 (-1)	30 (-1)	25 (0)	500 (0)
25	30 (0)	50 (+1)	30 (-1)	25 (0)	500 (0)
26	30 (0)	0 (-1)	60 (+1)	25 (0)	500 (0)
27	30 (0)	50 (+1)	60 (+1)	25 (0)	500 (0)
28	10 (-1)	25 (0)	45 (0)	5 (-1)	500 (0)
29	50 (+1)	25 (0)	45 (0)	5 (-1)	500 (0)
30	10 (-1)	25 (0)	45 (0)	45 (+1)	500 (0)
31	50 (+1)	25 (0)	45 (0)	45 (+1)	500 (0)
32	30 (0)	25 (0)	30 (-1)	25 (0)	250 (-1)
33	30 (0)	25 (0)	60 (+1)	25 (0)	250 (-1)
34	30 (0)	25 (0)	30 (-1)	25 (0)	750 (+1)
35	30 (0)	25 (0)	60 (+1)	25 (0)	750 (+1)
36	10 (-1)	25 (0)	45 (0)	25 (0)	250 (-1)
37	50 (+1)	25 (0)	45 (0)	25 (0)	250 (-1)
38	10 (-1)	25 (0)	45 (0)	25 (0)	750 (+1)
39	50 (+1)	25 (0)	45 (0)	25 (0)	750 (+1)
40	30 (0)	0 (-1)	45 (0)	5 (-1)	500 (0)
41	30 (0)	50 (+1)	45 (0)	5 (-1)	500 (0)
42	30 (0)	0 (-1)	45 (0)	45 (+1)	500 (0)
43	30 (0)	50 (+1)	45 (0)	45 (+1)	500 (0)
44	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)
45	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)
46	30 (0)	25 (0)	45 (0)	25 (0)	500 (0)

Solid-liquid extraction of bioactive components from the hemp

Solid-liquid extraction was performed according to the conditions defined using Box-Behnken experimental design (Table 1). Certain mass of dried hemp material was placed in a 50 mL glass beaker with certain volume of ethanol/water solvent. Extraction experiments were performed using IkaHBR4 digital oil-bath (IKA-WerkGmbH & Co.KG, Staufen, Germany) at defined temperatures, at a specified rotational speed, for a given time. After extraction, samples were filtered through a 100% cellulose filter paper (LLG Labware, Meckenheim, Germany) with 20–25 μm pore size and stored at 4 °C until analyzed (Jurinjak Tušek et al., 2018).

Determination of total polyphenolic content and antioxidant activity

Total polyphenolic content (TPC) of the hemp extracts was determined spectrophotometrically by the Folin-Ciocalteu reagent, according to Singleton and Rossi (1965). All analyses were performed in duplicate and the results were expressed as mg gallic acid equivalents (GAE) per gram of dry matter (DM) of plant material (Jurinjak Tušek et al., 2016; Benković et al., 2017; Valinger et al., 2017).
Antioxidant activity (AOX) was determined using two methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method (Brand-Williams et al., 1995) and Ferric Reducing Antioxidant Power (FRAP) method (Benzie and Strain, 1996). All analyses were performed in duplicate and the results were expressed as mmol Trolox equivalents per gram of dry matter (DM) of plant material and as mmol $FeSO_4 \cdot 7H_2O$ equivalents per gram of dry matter (DM) of plant material, respectively (Benković et al., 2017).

Statistical analysis

Analysis of Variance (ANOVA) was applied to determine the influence of individual variables (five extraction parameters) simultaneously on output variables (total polyphenolic content, antioxidant activity) in the ethanol/water solvent extraction of bioactives originated from the industrial hemp, with significance level of $p<0.05$. The adequacy of the second-order polynomial model was evaluated by the coefficient of determination (R^2). Experimental design and statistical analysis were performed using software Statistica v.10.0 (StatSoft Inc., Tulsa, USA).

Results and discussion

Chemical characteristics of the prepared hemp extracts

In this work, parameters of the solid-liquid extraction were defined in order to ensure maximum values of the total polyphenolic content and antioxidant activity (determined by the DPPH and the FRAP methods) of the hemp extracts.

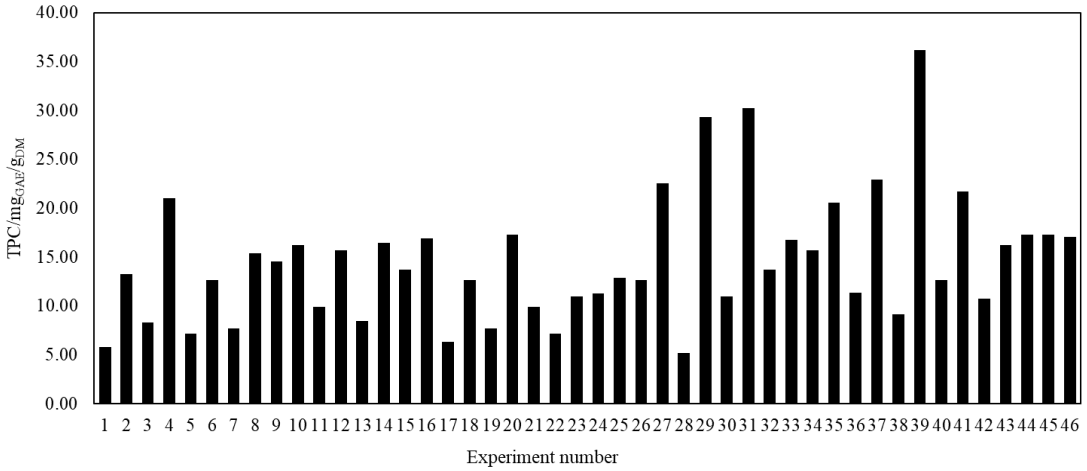

Fig. 1. The total polyphenolic content (TPC) at different experimental conditions

According to the results presented in Fig. 1, maximum TPC was extracted in exp. no. 39 (t = 25 min, T = 45 °C, 750 rpm, ethanol content = 25% and liquid to solid ratio = 50 mL/g); TPC = 36.160 mg_{GAE}/g_{DM} which is 4 fold higher compared to exp. no. 38 (t = 25 min, T = 45 °C, 750 rpm, ethanol content = 25% and liquid to solid ratio = 10 mL/g); TPC = 9.154 mg_{GAE}/g_{DM}. Experiments no. 38 and no. 39 differ only in the liquid to solid ratio and it can be concluded that higher liquid to solid ratio, the greater TPC amount will be extracted (Sifaoui et al., 2016). Minimum TPC value was obtained in exp. no. 28 (t = 5 min, T = 45 °C, 500 rpm, ethanol content = 25% and liquid to solid ratio = 10 mL/g); TPC = 5.218 mg_{GAE}/g_{DM}.
In the study of Drinić et al. (2018), water and ethanol content (30%, 50%, 70% and 90%) were used as a solvent in the extraction of two different samples of hemp (*Cannabis sativa* L.). It was determined that ethanol/water mixture (50%) was the best solvent for the extraction of phenolic compounds from both hemp samples (TPC = 17.05 mg GAE/g_{dw} and 9.25 mg GAE/g_{dw} for young and mature hemp, respectively). Regarding this work, higher TPC values were obtained in the experiments no. 4, 10, 12, 27, 41, 43 with ethanol content = 50%.

Cao et al. (2016) investigated optimum conditions of microwave-assisted extraction of total flavonoids (TF) from powders of hemp leaves. The influence of four factors (ethanol concentration: 40, 60, 80 %; liquid to solid ratio: 30:1, 35:1, 40:1 mL/g; extraction time: 10, 20, 30 min; temperature: 50, 60, 70 °C) for maximum recovery of TF yield was determined, using Box-Behnken design. The optimum microwave extraction conditions for obtain maximal yield of TF was as follows: solvent-to-solid ratio of 31.69 mL/g, extraction time of 25.14 min and extraction temperature of 69.96 °C. The predicted extraction yield of TF was 3.06%, which was in accordance with the experimental yield of 3.04 ± 0.62 %. Our study, together with the study of Cao et al. (2016), shows successful application of RSM methodology for modeling and optimization of extraction process biocompounds originating from hemp material.

Mkpenie et al. (2012) used organic solvents such as methanol, acetone and their 50% aqueous solutions for the extraction of *C. sativa* leaves. Extraction was performed on a shaker at room temperature for 2, 8 and 18 hours. According to the obtained results, different solvent extraction systems showed a wide range of polyphenols concentration: from 0.09 to 0.556 mg (GAE)/g_{dw}, depending on the solvent used. The highest TPC levels were achieved using methanol, followed by acetone, 50% methanol and 50% acetone. Compared to our work, higher TPC values were obtained using ethanol/water solvent, with shorter extraction time and the whole plant material was used for the extraction experiments.

Jokić et al. (2010) investigated the influence of the solvent, temperature and extraction time on the extraction yield of total polyphenols from milled soybeans variety IKA. The best extraction yield of total polyphenols was obtained using 50% aqueous ethanol solution at 50 °C after 60 min (TPC = 3.045 mg GAE/g_{DM}), compared to the extraction yield of total polyphenols obtained using water as a solvent (1.119 mg GAE/g_{DM}). The similar results were obtained in this work, in the experiments no. 26 (t = 25 min, T = 60 °C, 500 rpm, ethanol content = 0% and liquid to solid ratio = 30 mL/g); TPC = 12.633 mg_{GAE}/g_{DM} and no. 27 (t = 25 min, T = 60 °C, 500 rpm, ethanol content = 50% and liquid to solid ratio = 30 mL/g); TPC = 22.520 mg_{GAE}/g_{DM}. It can be concluded that the combination of water with organic solvent will ensure optimal conditions for polyphenols extraction from different plant materials (Rafiee et al., 2011; Dent et al., 2013).

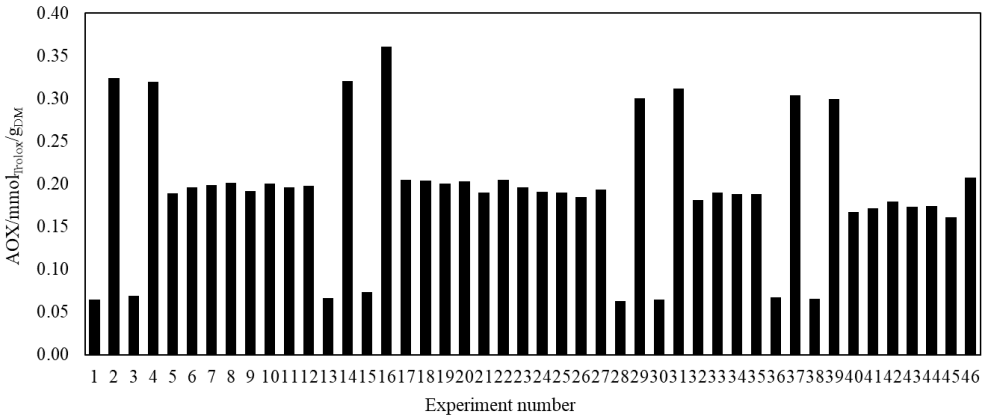

Fig. 2. The antioxidant activity (AOX) determined by the DPPH method, at different experimental conditions

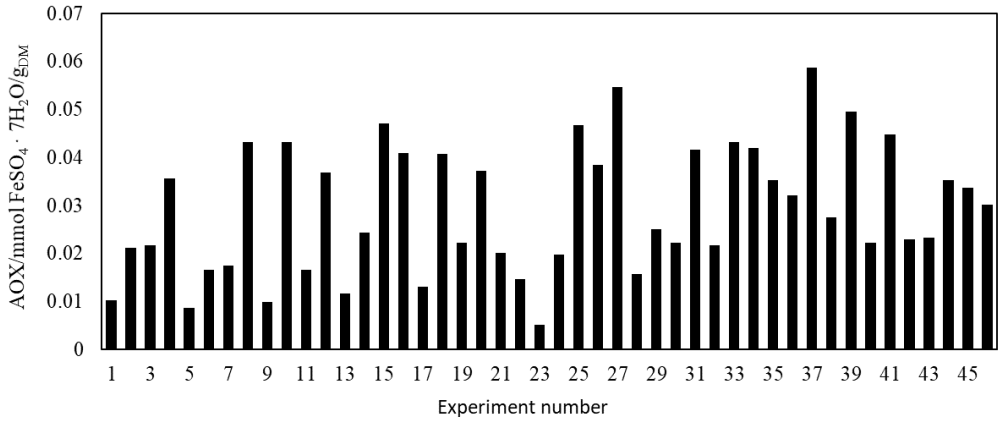

Fig. 3. The antioxidant activity (AOX) determined by the FRAP method, at different experimental conditions

Regarding AOX determined by the DPPH method (Fig. 2), the highest value was obtained in exp. no. 16 (t = 25 min, T = 60 °C, 500 rpm, ethanol content = 25% and liquid to solid ratio = 50 mL/g); DPPH = 0.361 $mmol_{Trolox}/g_{DM}$ while the lowest value was determined in experiment no. 28 (t = 5 min, T = 45 °C, 500 rpm, ethanol content = 25% and liquid to solid ratio = 10 mL/g); DPPH = 0.062 $mmol_{Trolox}/g_{DM}$. Regarding AOX determined by the FRAP method (Fig. 3) the highest value was obtained in exp. no. 37 (t = 25 min, T = 45 °C, 250 rpm, ethanol content = 25% and liquid to solid ratio = 50 mL/g); FRAP = 0.059 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$ while the lowest value was obtained in exp. no. 9 (t = 25 min, T = 45 °C, 250 rpm, ethanol content = 0% and liquid to solid ratio = 30 mL/g); FRAP = 0.001 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$.

In the study of Mkpenie et al. (2012), antioxidant activity was determined by reducing power assay and it was in the range from 0.202 to 0.866 mg/mL. Authors used methanol, acetone and their 50% aqueous solutions to perform polyphenols extraction from hemp leaves.

Drinić et al. (2018) expressed antioxidant activity in terms of EC_{50} values. These values were in the range from 0.1321 to 0.4353 mg/mL for young hemp and from 0.2055 to 0.7563 mg/mL for mature hemp. The value of EC_{50} is inversely related to its antioxidant activity and it presents the concentration of the test sample which neutralizes 50% of DPPH radicals. The lowest value of EC_{50} means the highest antioxidant activity. The highest antioxidant activity was obtained with 30% ethanol for young hemp (EC_{50} = 0.1321 mg/mL) and 50% ethanol for mature hemp (EC_{50} = 0.2055 mg/mL).

Compared to our results regarding the antioxidant activity with the results of the above mentioned authors, it can be concluded that different ethanol/water mixtures are better solvents for the extraction of polyphenols from industrial hemp.

Optimization of extraction conditions considering chemical characteristics of the hemp extracts

In this work, the effects of five factors (extraction temperature, liquid to solid ratio, extraction time, rotational speed and ethanol/water solvent ratio) on the total polyphenolic content (TPC) and antioxidant activity (AOX) (determined by the DPPH and the FRAP methods) of the hemp extracts were analysed. The results of optimization are presented in Fig. 4. Based on the obtained results, increment of the liquid to solid ratio up to 50 mL/g had significant effect on increase in TPC content and AOX determined by the DPPH and the FRAP methods. The higher the solvent to solid ratio, the higher the total amount of solids obtained, which is in accordance with literature data (Radojković et al., 2012; Sifaoui et al., 2016). In our work, maximum values of TPC, AOX (DPPH) and AOX (FRAP) were determined in the experiments with liquid to solid ratio 50 mL/g (exp. no. 39, 16 and 37, respectively) (Figs. 1-3). The extraction temperature is an important parameter in process optimization since high temperatures may lead to degradation of antioxidant compounds (Spigno et al., 2007; Radojković et al., 2012). In this work, the impact of temperature on the TPC, AOX (DPPH) and AOX (FRAP) was investigated in the range of 30 °C – 60 °C. TPC increased with the increase of temperature up to 45 °C after which further temperature increase did not cause significant changes in the TPC. Maximum TPC value was obtained at 45 °C (exp. no. 39) (Fig. 1). Temperature increase did not affect AOX determined by the DPPH method, but it influenced the AOX determined by the FRAP method. Similar values for AOX (FRAP) were obtained in exp. no. 27 at T = 60 °C (FRAP = 0.055 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$), and exp. no. 37 at T = 45 °C (FRAP = 0.059 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$), although these two experiments differed considering temperature, liquid to solid ratio, ethanol content and rotational speed (Fig. 3, Table 1). Regarding ethanol/water solvent ratio, increase in ethanol content from 0% to 50% had a significant effect on AOX (FRAP) while it did not influence AOX (DPPH) and it had a small influence on the TPC content. According to Rostango et al. (2004), it is necessary to add a certain amount of water to the extraction solvent to improve extraction of phenolics, although water content higher than 60% can result in reduction of the extraction yield of phenolic compounds. The impact of rotational speed on TPC and AOX was investigated in the range of 250 – 750 rpm. The increment of rotational speed did not influence on TPC and AOX (DPPH) while it influenced AOX (FRAP): in the range of 250 – 500 rpm values of AOX (FRAP) decreased, but in the range of 500 – 750 rpm AOX (FRAP) values increased. Extraction time did not affect AOX (DPPH) and TPC while it had a minor influence on AOX (FRAP): similar AOX determined by the FRAP method were obtained for 25 and 45 minutes (Fig. 3, Table 1).

Based on the results from Fig. 4, the optimum process conditions for the ethanol/water extraction of bioactives from the hemp, with regard to chemical properties were: t = 25 min, T = 45 °C, 500 rpm, ethanol content = 25% and liquid to solid ratio =

30 mL/g with values of the TPC = 13.27 mg_{GAE}/g_{DM}, AOX (DPPH) = 0.19 $mmol_{Trolox}/g_{DM}$, AOX (FRAP) = 0.02 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$. Optimum experimental values for AOX (DPPH) and AOX (FRAP) were close to the RSM predicted values while optimum experimental value for TPC somehow differed from the RSM predicted TPC value. Model predicted values were: TPC = 20.69 mg_{GAE}/g_{DM}, AOX (DPPH) = 0.21 $mmol_{Trolox}/g_{DM}$, AOX (FRAP) = 0.03 mmol $FeSO_4 \cdot 7H_2O/g_{DM}$.

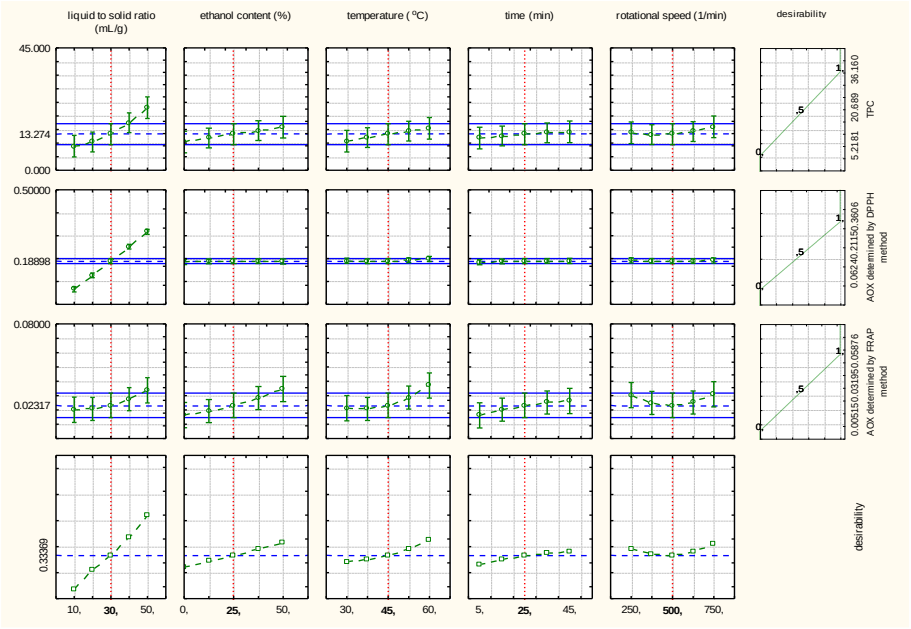

Fig. 4. Results of the optimization of the solid-liquid extraction of bioactives from industrial hemp with respect to chemical properties (TPC and AOX)

Analysis of variance (ANOVA)

Analysis of variance (ANOVA) was used to investigate the effect of process variables on the chemical characteristics of the hemp extracts. Regression parameters, together with determination coefficients, are presented in Table 2. The results indicated that linear parameters were positive for four investigated process variables, while the quadratic effects of independent variables demonstrated both positive and negative effects. The ANOVA for independent variables indicated that liquid to solid ratio was the most significant factor (p<0.05) affecting TPC, AOX (DPPH) and AOX (FRAP). The model indicated that liquid to solid ratio had positive linear effects on TPC and both antioxidant activities. The largest positive linear regression coefficient of the liquid to solid ratio was obtained for the TPC while the smallest value was obtained for AOX (FRAP). Ethanol content and temperature contributed significantly to the TPC and AOX (FRAP). On the other hand, these two parameters had no significant effect on AOX (DPPH). The extraction time did not significantly influence the chemical characteristics of the hemp extracts (p>0.05). The negative value of quadratic regression coefficient of rotational speed significantly influenced AOX (FRAP) (p<0.05) while this parameter had no effect on the TPC and AOX (DPPH). Considering the values of the linear regression coefficients, obtained for independent and dependent variables, liquid to solid ratio, ethanol content and extraction temperature were perhaps the most important factors contributing TPC extraction from the hemp using RSM methodology.

Based on the calculated determination coefficients it can be concluded that influence of process variables on AOX (FRAP) cannot be described well by the developed prediction models (R^2 = 0.540). On the other hand, determination coefficient calculated for AOX (DPPH) was higher than 0.9 (R^2 = 0.976), indicating a strong relationship between DPPH values and investigated process variables while in the case of TPC (R^2 = 0.603), there is a good relationship between the TPC and process variables.

Table 2. Regression coefficients and determination coefficients obtained by Box-Behnken experimental design for chemical properties of hemp extracts (bold values: $p<0.05$)

Analysed property	Parameter	Intercept ± standard error	Regression coefficients (L-linear, Q- quadratic) ± standard error	*P*	R^2	R^2adj.
TPC (mg_{GAE}/g_{DM})	Liquid to solid ratio (mL/g)	15.719 ± 1.763	**7.083 ± 1.164(L)** -1.342±0.788(Q)	**0.000(L)** 0.097(Q)	0.603	0.490
	Ethanol content (%)		**2.741±1.164(L)** 0.074±0.788(Q)	**0.024(L)** 0.926(Q)		
	Temperature (°C)		**2.3687±1.164(L)** 0.108±0.788(Q)	**0.049(L)** 0.892(Q)		
	Extraction time (min)		1.156±1.164(L) 0.138±0.788(Q)	0.327(L) 0.862(Q)		
	Rotational speed (rpm)		1.104±1.164(L) -0.812±0.788(Q)	0.349172(L) 0.310083(Q)		
DPPH ($mmol_{Trolox}$/gDM)	Liquid to solid ratio (mL/g)	0.193 ± 0.005	**0.125±0.003(L)** -0.001±0.002(Q)	**0.000(L)** 0.634(Q)	0.976	0.970
	Ethanol content (%)		0.001±0.003(L) 0.001±0.002(Q)	0.750(L) 0.525(Q)		
	Temperature (°C)		0.004±0.003(L) -0.003±0.002(Q)	0.247(L) 0.233(Q)		
	Extraction time (min)		0.003±0.003(L) 0.001±0.002(Q)	0.419(L) 0.707(Q)		
	Rotational speed (rpm)		-0.000±0.003(L) -0.001±0.002(Q)	0.899(L) 0.526(Q)		
FRAP ($mmol_{FeSO4\cdot 7H2O}/g_{DM}$)	Liquid to solid ratio (mL/g)	0.035 ± 0.004	**0.007±0.003(L)** -0.002±0.002(Q)	**0.012(L)** 0.293(Q)	0.540	0.409
	Ethanol content (%)		**0.009±0.003(L)** -0.001±002(Q)	**0.001(L)** 0.514(Q)		
	Temperature (°C)		**0.008±0.003(L)** -0.003±0.002(Q)	**0.004(L)** 0.098(Q)		
	Extraction time (min)		0.0050±0.003(L) 0.0010±0.002(Q)	0.059(L) 0.578(Q)		
	Rotational speed (rpm)		0.000±0.003(L) **-0.004±0.002(Q)**	0.914(L) **0.040(Q)**		

Conclusions

Optimization represents an essential tool in food engineering and biotechnology with the aim to yield a highly acceptable product. In this work, Response Surface Methodology, coupled with Box-Behnken design, was applied to determine the optimal conditions of the ethanol/water solvent extraction of bioactive components from industrial hemp investigating some influential parameters such as liquid to solid ratio, temperature, ethanol content, extraction time and rotational speed. The optimum TPC content and antioxidant activity (DPPH and FRAP) in the hemp extracts were achieved with ethanol/water ratio 25% at 45 °C for 25 min, with solvent to plant ratio 30 mL/g and rotational speed 500 rpm. Under optimized conditions, the obtained experimental values agreed with the predicted values. According to the ANOVA results, liquid to solid ratio significantly influenced TPC, AOX (DPPH) and AOX (FRAP), rotational speed had significant influence only on AOX (FRAP) while ethanol content and temperature contributed significantly to the TPC and AOX (FRAP). This research serves as the basis for further investigations on the optimization of extraction procedure of bioactive components and antioxidant activity from industrial hemp using response surface methodology approach.

Literature

Andre, C.M., Hausman, J., Guerriero, G. (2016): *Cannabis sativa*: The plant of the thousand and one molecules, *Front. Plant Sci.* 7, 1–17. https://doi.org/10.3389/fpls.2016.00019

Appendino, G., Chianese, G., Taglialatela-Scafati, O. (2011): Cannabinoids: Occurrence and medicinal chemistry,*Curr. Med. Chem.* 18, 1085–1099. https://doi.org/10.2174/092986711794940888

Benković, M., Belščak-Cvitanović, A., Jurina, T., Valinger, D., Jurinjak Tušek, A., Jakelić, M., Gajdoš Kljusurić, J. (2017): Effect of extraction conditions on extraction yield, total polyphenolic content and antioxidant capacity of yarrow (*Achilea millefolium* L.). In: Proceedings of the 16th Ružička days, Jukić, A. (ed.), Osijek, HR, pp. 155–162. https://doi.org/10.1016/j.indcrop.2016.07.015

Benzie, I.F., Strain, J.J. (1996): The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, *Anal. Biochem*. 239, 70–76. https://doi.org/10.1006/abio.1996.0292

Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., Escaleira, L.A. (2008): Response surface methodology (RSM) as a tool for optimization in analytical chemistry, *Talanta* 76, 965–977. https://doi.org/10.1016/j.talanta.2008.05.019

Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995): Use of free radical method to evaluate antioxidant activity, *Lebensm. Wiss. Technol*. 28, 25–30. https://doi.org/10.1016/s0023-6438(95)80008-5

Cao, J., Hao, L., Zhang, L., Xu, M., Ge, H., Kang, C., Yu, J., Wang, Z. (2016): Optimization of microwave-assisted extraction of total flavonoids from China-hemp leaves and evaluation of its antioxidant activities. In: International Conference on Applied Biotechnology, Liu, H., Song, C., Ram, A., (eds.), Springer, Singapore, pp. 555–567. https://doi.org/10.1007/978-981-10-4801-2_57

Dent, M., Dragović-Uzelac, V., Penić, M., Bosiljkov, T., Levaj, B. (2013): The effect of extraction solvents, temperature and time on the composition and mass fraction of polyphenols in dalmatian wild sage (*Salvia officinalis* L.) extracts, *Food Technol. Biotech.* 51, 84–91. https://doi.org/10.5562/cca3231

Drinić, Z., Vidović, S., Vladić, J., Koren, A., Kiprovski, B., Sikora V. (2018): Effect of extraction solvent on total polyphenols content and antioxidant activity of *Cannabis sativa* L. *Lekovite Sirovine* 38, 17–21. https://doi.org/10.5937/leksir1838017D

Hartsel, J.A., Eades, J., Hickory, B., Makriyannis, A. (2016): *Cannabis sativa* and Hemp. In: Nutraceuticals: Efficacy, Safety and Toxicity, Gupta, R.C. (ed.), Academic press, Elsevier, pp. 735–754. https://doi.org/10.1016/b978-0-12-802147-7.00053-x

Jokić, S., Velić, D., Bilić, M., Bucić-Kojić, A., Planinić, M., Tomas, S. (2010): Modelling of the process of solid-liquid extraction of total polyphenols from soybeans, *Czech J. Food Sci*. 28, 206–212. https://doi.org/10.1016/b978-0-12-802147-7.00053-x

Jurinjak Tušek, A., Benković, M., Belščak-Cvitanović, A., Valinger, D., Jurina, T., Gajdoš Kljusurić, J. (2016): Kinetics and thermodynamics of the solid-liquid extraction process of total polyphenols, antioxidants and extraction yield from *Asteraceae* plants, *Ind. Crop. Prod.* 91, 205–214. https://doi.org/10.1016/j.indcrop.2016.07.015

Jurinjak Tušek, A., Benković, M., Valinger D., Jurina, T., Belščak-Cvitanović, A., Gajdoš Kljusurić, J. (2018): Optimizing bioactive compounds extraction from different medicinal plants and prediction through nonlinear and linear models, *Ind. Crops Prod.* 126, 449–458. https://doi.org/10.1016/j.indcrop.2018.10.040

Kolodziejczyk, P., Ozimek, L., Kozłowska, J. (2012): The application of flax and hemp seeds in food, animal feed and cosmetics production, *Wood. Publish. Lim.* 329–366. https://doi.org/10.1533/9780857095510.2.329

Kostić, M.D., Joković, N.M., Stamenković, O.S., Rajković, K.M., Milić, P.S., Veljković, V.B. (2013): Optimization of hempseed oil extraction by n-hexane, *Ind. Crops Prod.* 48, 133–143. https://doi.org/10.1016/j.indcrop.2013.04.028

Liu, L., Shen, B.-J., Xie, D.-H., Cai, B.-C., Qin, K.-M., Cai, H. (2015): Optimization of ultrasound-assisted extraction of phenolic compounds from *Cimicifugae rhizoma* with response surface methodology, *Phcog. Mag.* 11, 682–689. https://doi.org/10.4103/0973-1296.165545

Majeed, M., Hussain, A.I., Chatha, S.A.S., Khosa, M.K.K., Kamal, M., Kamal, M.A., Liu, M. (2016): Optimization protocol for the extraction of antioxidant components from *Origanum vulgare* leaves using response surface methodology, Saudi J. Biol. Sci. 23, 389–396. https://doi.org/10.1016/j.sjbs.2015.04.010

Mkpenie, V.N., Essien, E.E., Udoh, I.I. (2012): Effect of extraction conditions on total polyphenol contents, antioxidant and antimicrobial activities of *Cannabis sativa* L., *EJEAFChe* 11, 300–907.

Pellati, F., Borgonetti, V., Brighenti, V., Biagi, M., Benvenuti, S., Corsi, L. (2018): *Cannabis sativa* L. and nonpsychoactive cannabinoids: their chemistry and role against oxidative stress, inflammation, and cancer, *Biomed. Res. Int.* 2018, 1–15. https://doi.org/10.1155/2018/1691428

Pollastro, F., Minassi, A., Fresu, L.G. (2018): Cannabis phenolics and their bioactivities, *Curr. Med. Chem.* 25, 1160–1185. https://doi.org/10.2174/0929867324666170810164636

Radojković, M., Zeković, Z., Jokić, S., Vidović, S., Lepojević, Ž., Milošević, S. (2012): Optimization of solid-liquid extraction of antioxidants from black mulberry leaves by response surface methodology, *Food Technol. Biotechnol.* 50, 167–176.

Rafiee, Z., Jafari, S.M., Alami, M., Khomeiri, M. (2011): Microwave-assisted extraction of phenolic compounds from olive leaves; a comparison with maceration, *J. Anim. Plant Sci*. 21, 738–745.

Rostango, M.A., Palma, M., Barroso, C.G. (2004): Pressurized liquid extraction of isoflavones from soybeans, *Anal. Chim. Acta* 522, 169–177. https://doi.org/10.1016/j.aca.2004.05.078

Sarasini, F., Fiore, V. (2018): A systematic literature review on less common natural fibres and their biocomposites. *J. Clean. Prod.* 195, 240 – 267.

Shahid-ul-Islam, Shahid, M., Mohammad, F. (2013): Perspectives for natural product based agents derived from industrial plants in textile applications - a review, *J. Clean. Prod.* 57, 2–18. https://doi.org/10.1016/j.jclepro.2013.06.004

Sifaoui, I., Chammem, N., Abderrabba, M., Mejri, M. (2016): Optimization of phenolic compounds extraction from olive leaves using experimental design methodology. *J. Mater. Environ. Sci.* 7, 1119–1127.

Silva, E.M., Rogez, H., Larondelle, Y. (2007): Optimization of extraction of phenolics from *Inga edulis* leaves using response surface methodology. *Sep. Purif. Technol.* 55, 381–387. https://doi.org/10.1016/j.seppur.2007.01.008

Singleton, V.L., Rossi, J.A. (1965): Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, *Am. J. Enol. Viticult.* 16, 144–158.

Spigno, G., Tramelli, L., De Faveri, D.M. (2007): Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics, *J. Food Eng.* 81, 200–208. https://doi.org/10.1016/j.jfoodeng.2006.10.021

Tchabo, W., Ma, Y., Kwaw, E., Xiao, L., Wu, M., Apaliya, M.T. (2018): Impact of extraction parameters and their optimization on the nutraceuticals and antioxidant properties of aqueous extract mulberry leaf, *Int. J. Food Prop.* 21, 717–732. https://doi.org/10.1080/10942912.2018.1446025

Thomas, B. F., ElSohly, M. A. (2015): The Analytical Chemistry of *Cannabis*: Quality Assessment, Assurance and Regulation of Medicinal Marijuana and Cannabinoid Preparations, Elsevier, Amsterdam, pp. 1–132.

Valinger, D., Jurina, T., Benković, M., Jurinjak Tušek, A., Belščak-Cvitanović, A., Jakelić, M., Gajdoš Kljusurić, J. (2017): Determination of correlations between electrical conductivity, dry matter, total polyphenolic content and antioxidant capacity of marigold extracts (*Calendula officinalis*). In: Proceedings of the 16th Ružička days, Jukić, A. (ed.), Osijek, HR, pp. 189–197. https://doi.org/10.1016/j.indcrop.2016.07.015

Wang, W.Y., Zhou, Z.H., Huang, N.J., Yun-lan, Tang, S.Y. (2014): Study on the extraction of active ingredients in bamboo leaves by enzymatic coupling high pulse electric field, *Food Ferment. Tech.* 4, 42–45.

Xu, C.-C., Wang, B., Pu, Y.-Q., Tao, J.-S., Zhang, T. (2017): Advances in extraction and analysis of phenolic compounds from plant materials, *Chin. J. Nat. Medicines* 15, 0721–0731. https://doi.org/10.1016/S1875-5364(17)30103-6

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RECOMMENDED AMOUNTS OF MACRONUTRIENTS BEFORE AND AFTER TENNIS MATCHES

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review paper

Summary

Tennis is one of the most popular sports played year-round. It is estimated that almost 60 million people are included in some sort of tennis matches and competitions. A tennis match can last longer than five hours at the professional level, so in tennis, similar to other sports that may last for extended periods of time, nutrition is considered an extremely important factor for ensuring the necessary energy during matches, as well as for the recovery period after the mentioned activities. The purpose of this paper is to investigate the area of the required intake of carbohydrates, protein, and fat before and after matches, to identify the desirable values that would ensure the necessary energy and expedite the recovery of tennis players after a match. Considering that tennis players use both the aerobic and the anaerobic energy systems as they play, carbohydrates are considered to be the main source of energy before and after matches, while proteins are considered to be extremely important factors for recovery after the activities. Unlike the mentioned food ingredients, the intake of fat has not been explored to that extent, from the aspect of the necessary intake before and after a match, but the daily recommended intake of 1.0 do 1.5 g/kg is very well known. Research has shown that the recommended values for the intake of carbohydrates before a match are 6 g/kg to 12 g/kg and 8 to 10 g/kg after a match. The desirable intake of protein just before a match is 0.3 g/kg and the same amount after a match (within 2 hours after the activity). Literature search has shown that the intakes are not uniform and that they depend on numerous factors, like sex, age, body mass, time of the match, length of the match, temperature, surface, tennis balls, and similar.

Keywords: tennis, carbohydrates, protein, fat, recovery

Introduction

Tennis, as it is played today, attracts millions of players and fans all over the world. Tournaments and many other tennis-related events are held year-round. Competitive tennis is played according to the rules of the International Tennis Federation (ITF) and its competitions range from high-level professional events, e.g. Grand Slams and the Olympic Games, to ITF male and female tournaments for beginners and players in wheelchairs. The Association of Tennis Professionals (ATP) and the Women's Tennis Association (WTA) encompass more than 60 (WTA) to 80 (ATP) tournaments in about 40 countries all around the world, which are organised in various categories, accompanied by reward funds and ranking points. Due to the large number of tournaments, there are many options for players at all levels who wish to compete during any week throughout the year. Apart from that, tennis is also a recreational sport, enjoyed by people of all age groups. It is the only sport played on various courts, with various types of tennis balls, and the matches are played in the best-of-three or best-of-five sets format. Changes in the scoring system, the duration of the matches, various surfaces, and the type of tennis balls may affect the physical and the physiological requirements for playing tennis matches (Fernandez et al., 2006). It is played year-round and it is one of the most popular sports today. It is estimated that almost 60 million players are included in some type of tennis competitions at the local, national, or international levels (Kondric at al., 2013). It is oriented around tournament play, which can result in a busy competition calendar, frequent travel, and unpredictable amounts of time spent playing competitive matches (Ranchordas et al., 2013). Tennis is characterised by short periods of active play (4-10 s), active recovery (10-20 s), and a longer passive recovery (60-90 s) (Fernandez et al., 2006). A tennis match often lasts longer than one hour, and in some cases longer than five hours, while the effective play time, i.e. the percentage of total play time in a match, is 20-30 % on clay courts and 10-15 % on fast courts. During that period, a player runs 3 m per stroke and 8 to 12 m total per point, which is 300 to 500 m of high-intensity activity total in a match that lasts for three sets. The number of exchanged strokes in an average point is four, and the exchanges in a match usually last less than eight seconds (five to seven) (Fernandez et al., 2006). A tennis match is characterised by fast and explosive movements, including accelerations, decelerations, jumps, and strokes from various positions and situations on the court. The length of a match from one hour to more than five hours, as well as severe mechanical and physiological stress, has significant consequences for the human body, so the

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speed of the strokes, running speed, maximum muscle strength, and the precision of strokes are significantly reduced during a tennis match (Kovacs, 2007).

Considering that an appropriate diet has a direct effect on the optimisation of energy reserves in the human body, some dietary regimes may reduce tiredness, and adequate nutrition and hydration are considered to be some of the most important factors in the prevention of injuries (Kondric et al., 2013). Some types of diets may facilitate recovery from injuries, while some forms of nutrition have direct effects on the health status of athletes. It is not surprising that nutrition is considered to be one of the key optimisation factors in the total development of an athlete in modern sports. There are various indicators that show that nutrition knowledge is a protective factor against potential doping. This obviously increases the need to initiate a wide-ranging educational programme on sport nutrition in tennis, as well as in other sports (Kondric et al., 2013).

The economic effect of tennis is significant, regarding financial rewards, tournament sponsorships, as well as coverage from various forms of media. All of this sets significant demands for the physical and psychological preparation of the participating players, as well as for nutrition advice as it pertains to training regimes and matches (Ranchordas et al., 2013).

Based on previous research, our aim is to provide guidelines on the appropriate amounts of carbohydrates, protein, and fat that should be consumed before (to improve performance) and after (for quicker recovery) a tennis match, in order to help the current and future tennis players, as well as all those who take care of their development.

Importance of nutrition in tennis

The physiological requirements of a tennis match are complex and dependant on highly variable interactions between technical, tactical, physical, and weather conditions. The surface, style of play, duration of the match, stage of play, temperature, and humidity all effect energy requirements, which means that the dietary habits are one of the main challenges in the situation. Furthermore, factors like the surface and the tennis balls have an effect on the speed and the bounce of the tennis balls, which ultimately affects the duration of the match, and consequently, the expenditure of energy (Ranchordas et al., 2013).

Table 1. Estimated energy expenditure according to sex and match duration. Calculated from: Christmass et al. (1998), O'Donoghue et al. (2001), Smekal et al. (2001), Girard et al. (2006), Fernandez-Fernandez et al. (2007), Hornery et al. (2007), Mendez-Villanueva et al. (2007), Murias et al. (2007), Fernandez-Fernandez et al. (2008) and Martin et al. (2011)

	Energy expenditure	
	Women AS±SD	**Men** AS±SD
kJ/min	30.9±5.5	45.3±7.3
kcal/min	7.4 ±1.3	10.8±1.8
kcal/kg/hour	7.5±0.5	8.4± 0.5
60 min match (kcal)	443±79	649±105
90 min match (kcal)	664 ±118	973±157
150 min match (kcal)	1107±196	1622±262
300 min match (kcal)		3244±524

The research conducted by Ranchordas et al. (2013) states that the nutrition challenges facing highest-level tennis player are unique, competitions that last year-round, extensive travel, and unpredictable amount of time spent playing a match require a complex nutrition strategy. It is important to match the training with the diet for a competitive programme, if tournaments are dominant throughout the year. Top tennis players must maintain optimal body mass throughout the year so they can adjust their energy intake during the short periods of rest or travel. The research conducted by Kovacs and Baker (2014) included four areas of recovery techniques which are usually used in tennis, proposing that interventions may enhance the recovery of athletes and improve performance in the future. One of those four recovery techniques is dietary intervention. Tennis players use the combination of anaerobic and aerobic energy systems, both of which rely on carbohydrates as the primary source of energy, so the main components of nutrition related to recovery include the intake of water and electrolytes for rehydration, replenishment of stored

carbohydrates, and the intake of protein for the recovery of muscles. They state that tennis matches, especially longer and more intensive ones, probably reduce the levels of glycogen. Therefore, the intake of carbohydrates before the following match is important for the recovery of tennis players.

Fleming et al. (2018) conducted a study on the diet and the recovery of tennis players before, during, and after matches, in which they state that 51% of the tennis players said that they consume unbalanced meals consisting of carbohydrates, protein, and fat the day before the match. Among the players, 27% said that they choose carbohydrates as dominant meals, and 13% said that they choose carbohydrates and protein only so they would meet the dietary regimes for the match. The analysis emphasizes the significant intake of carbohydrates as the dominant meal before matches among top players, when compared to lower-level players, which indicates that top players are more aware of the importance of carbohydrates. A second analysis was used to determine that junior players choose carbohydrates as the dominant meal before matches more often than adult players.

The results on dietary habits right after matches state that 34% of the players said that carbohydrates are dominant in meals, 26% said protein, 19% said that they eat balanced meals, 9% use sports drinks, and 9% said that they do not consume anything. 61% of the players said that they consume balanced meals in the period of 3 h after the match, and 44% of the players do so before they go to sleep.

On the day after the match, 39% of the players stated that they consume "nothing specific". This analysis indicates that some of the players are not adapting their diet for the days designated for rest and recovery. This is concerning due to the strong evidence that supports the use of nutrition strategies after training as key tools for supporting recovery. Technical and body conditioning performance reduces during tennis tournaments, and this is connected to tiredness (Ojala and Häkkinen, 2013), it is therefore important to implement effective strategies for improving performance, as shown in other sports (Rossi et al., 2017).

Vitale and Getzin (2019) made an overview and dietary recommendations for sports where endurance is the dominant factor. They state that there has been significant progress in understanding the dietary needs of athletes, and that the literature stills contains many differences. They also state that nutrition science is a complex and constantly developing subject, and that sport nutrition includes the areas of sport medicine, sport science, dietetics, culture, and even media.

Recommendations for macronutrients intake

Carbohydrates

In his survey research "*Carbohydrate Intake and Tennis: Are there benefits?"* Kovacs (2006) states that the intake of carbohydrates in extended aerobic training has shown to be effective for improving performance and delaying tiredness. He further states that it is necessary to maintain adequate concentrations of glucose and glycogen in order to perform optimal strokes and court movements. In general, athletes should consume the amount of carbohydrates recommended by the American College of Sports Medicine and the National Association of Sports Trainers, 30-60 g/h of carbohydrates during training. Carbohydrates may be in the form of glucose, saccharose, maltodextrins, or a highly glycaemic starch. Fructose should be limited due to the possibility of gastrointestinal discomfort. This amount of carbohydrates may be acquired by drinking 600-1200 ml/h of a solution containing 4-8 % of carbohydrates (4-8 g/100 ml) (Convertino et al., 1996). Athletes must maintain an appropriate daily energy intake, so Burke et al. (2001), in their review, stated the required amounts of carbohydrates for athletes, and reached the conclusion that the intake should be increased to 7-10 g/kg per day (tournament week) in order to maintain the sufficient supply of energy for efficiency and to encourage recovery (30-60 g/h). Galloway and Maughan (2010) stated that the period for taking carbohydrates during training or during matches should have the goal of creating a regular flow of carbohydrates from the intestines to the bloodstream. Considering that carbohydrates could be counterproductive when ingested in excessive amounts (>60-90 g/h) or concentrations (>7-8 %), it is advisable to take lower quantities regularly, e.g. during every switch, instead of taking large quantities during a single switch. Jung et al. (2005) stated another additional benefit of consuming carbohydrates / beverages with electrolytes during extended periods of training, which proves that it delays the start of muscle cramps caused by training. Regarding carbohydrates, it is said that many years have shown that a diet with a high share of carbohydrates leads to an increased level of glycogen in the muscles (Bergstrom, 1967), which contributes to optimum performance, especially in endurance-type activities (Hargreaves, 2004). It is also known that a diet with a low share of carbohydrates (<15% of the total energy intake) can have adverse effects on training and high-intensity endurance, and both of those are key aspects of a tennis match (Ranchordas et al., 2013). Outside of competition, top tennis players train between 4 and 6

hours per day and only take short rests, which maintains the energy requirements at a high level year-round. As a general guideline, top tennis players need to maintain a regularly high level of carbohydrates, in the range of 6-10 g/kg per day, in order to ensure the required levels of glycogen, and women usually require somewhat lower amounts than men (Ranchordas et al., 2013). These recommendations should be adapted to the daily energy expenditure. Top tennis players should make sure to be adequately charged and hydrated before every match, however, that can be a challenge considering they do not know how long a match may last. Furthermore, a delayed start of a tournament can disrupt the trained routines practiced before matches, which can lead to an insufficient amount of ingested food or hunger, or playing a match with a stomach full of undigested food. Other issues may arise if the tennis players are playing more than one match per day, e.g. if they are participating in both the singles and the doubles competitions at tournaments. This situation may result in inadequate or sometimes unknown recovery times for players, which makes any subsequent energy charging more difficulty. In that case, recovery may only include the intake of carbohydrates, because they can be digested more easily, and proteins are left out, which jeopardises optimal recovery. Apart from that, an unexpected result or an early drop from a tournament could mean that the athletes had been eating excessively during the preparation period. Tennis players should seek guidelines from a qualified sport nutrition expert in order to resolve these issues and to increase efficiency (Ranchordas et al., 2013). According to the research by Kovacs and Baker (2014) and the proposal of other researchers, the recommendation for the intake of carbohydrates during recovery depend on the demands of training and competitions. When the period between training sessions or matches is less than 8 hours of recovery, they recommend the intake of 1.0-1.2 g/kg of carbohydrates immediately after the first training session or match. This percentage of carbohydrate intake should be repeated every hour during 4 h. The carbohydrate intake time is especially important if the athlete has two training sessions or matches in a single day. If there is one or more days between intensive training sessions, the time for the replenishment of glycogen is less urgent, under the condition that enough carbohydrates are consumed during the 24 hours after a training session or a match. The daily requirement for carbohydrates in the process of supporting recovery, muscle recuperation, and the replenishment of glycogen in the liver (i.e. within 24 h between tennis matches) is 5-7 g/kg of carbohydrates per day for moderate training (1 h per day) or 6-10 g/kg of carbohydrates per day for moderate to highly intensive training (1-3 h per day). The types of meals consumed in short periods of recovery should contain easily digestible sources of carbohydrates. The players should avoid ingredients rich in fat, protein, and fibre to avoid the risk of gastrointestinal problems. Vitale and Getzin (2019) stated that carbohydrates (as blood glucose and muscle glycogen) have the advantage of creating more ATP per oxygen (O2) volume when compared to fat, but the depletion of the carbohydrate supply in the liver and muscles is connected to tiredness, reduced activity, and reduced concentration. The research by Jäger et al. (2017) states that the common position of the Academy of Nutrition and Dietetics (AND), Dietitians of Canada (DC), and the American College of Sports Medicine (ACSM) is that moderate training (1 h per day) requires 5-7 g of carbohydrates per kilogram of body weight per day, while moderate to high intensity training (1-3 h per day) requires 6-10 g/kg per day. Athletes in endurance sports who are extremely dedicated to daily activity (4-5 hours of moderate to high intensity training every day) may require up to 8-12 g/kg per day. Before a competition, if the event lasts <90 min, it is usually recommended to simply “top up” the glycogen levels to replenish the muscle and liver glycogen lost during the previous day by consuming meals rich in carbohydrates, with at least 6 g/kg (Getzin et al., 2017) and up to 7-12 g/kg (Jäger et al., 2017), in the 24-hour period before the event. Jäger et al. (2017) state that for competitions lasting less than 60 minutes there is no need to ingest exogenous carbohydrates, but if the activities last more than 60 minutes, they recommend active strategies for maintaining the availability of carbohydrates. For events lasting 1-2.5 h, it is usually recommended taking 30-60 g per hour in a 6–8 % carbohydrate solution (concentrations usually found in commercial sport drinks), and in an ideal situation, they would be taken every 10-15 minutes. For events lasting more than 2.5 h, higher intake of carbohydrates of 60-70 g/h and up to 90 g/h are connected to increased performance. In the last several years, some athletes have been manipulating their carbohydrate level by using a strategy that includes a lower intake of carbohydrates and a higher intake of fat. Periodic training sessions under the conditions of availability with lower glycogen content / low glucose can encourage the regulation of fatty oxidation routes, conserve glycogen levels, and extend the time before exhaustion (Getzin et al., 2017).

Proteins

Ranchordas et al. (2013) stated that there is limited data on the dietary intakes and requirements of protein

for racket sports, and most of the published guidelines are directed toward athletes who are training in strength of endurance sports, while tennis requires some aspects of both strength and endurance and cannot be compared directly. That is why it is more appropriate to evaluate the protein requirements for tennis players based on the volume and the intensity of training or competitions. Further in their overview, they compared the daily intake of protein by 4 university female tennis players during season and post-season, and it was from 1.3 g/kg per day and 1.2 g/kg per day (Nutter, 1991), and for 7 female tennis players aged 19 who trained 4 hours per day, 6 days per week, the protein intake was low and it was from 0.8 g/kg per day (Gropper et al., 2003). The protein intake guidelines for top level athletes, high-intensity and long training sessions on a daily basis, state that he recommended quantity is 1.6 g/kg/day, and usually lower for female athletes due to the lower energy input. Due to the deficiencies of research related to nutrition in top-level tennis, it is important to consider the time, type, and quantity of the consumed protein, in combination with other nutritious substances. The research by Kovacs and Baker (2014) states that proteins are another important factor for the recovery of tennis players. The recommended period for taking protein is as soon as possible after training or a match. In order to meet the daily requirements for protein, it is recommended that tennis players engaged in high-intensity and long training sessions ingest 1.6 g/kg of protein per day (Ranchordas et al. 2013). This is similar to 1.2-1.7 g/kg of protein per day recommended for endurance athletes (Rodriguez et al. 2009). It is important to reiterate that tennis trainers and players should consider the individual needs and inclinations of athletes. However, further research is required to determine the optimal quantity and time for taking liquids, carbohydrates, and protein for recovery after training, for athletes of various levels of maturity, especially regarding physiological and practical challenges related to tournament play. Tennis competitions are not only unique because of their regime of stops and movements, they are also characterised by the short time available for dietary replenishment between matches during tournaments. Quick recovery is especially important in this situation, that is why this area requires further extensive research. In their research, Phillips and Van Loon (2011) have stated that athletes training in sports where endurance is the dominant factor favour protein less when compared to carbohydrates. The adequate intake and the time of intake are crucial for every athlete, regardless whether they are training in a sport that requires more endurance or strength. Athletes require a higher intake of protein than what is currently recommended as the daily intake, 0.8 g/kg per day, so they could adapt to their training and improve their activity. The Academy of Nutrition and Dietetics (AND), Dietitians of Canada (DC), and the American College of Sports Medicine recommend the intake of protein for athletes in the range of 1.2 to 2.0 g/kg per day. Athletes may believe that "more is better" and increase their intake of protein without recommendations. However, a daily intake of protein above the recommended level (1.2-2.0 g/kg/day and/or individual meals with doses above 0.3 g/kg) have not been proven to provide any additional benefits. Temporary intake of more than 2.0 g/kg per day may be useful during short periods of increased training intensity that exceed an athlete's regular regime, but a higher total protein intake after that does not provide additional endurance for athletes (Jäger et al., 2017). For sports where strength is dominant, it is usually recommended to consume higher quantities, and lower quantities are recommended for endurance sports, depending on individual requirements, so Thomas et al. (2016) recommend 1.4-2.0 g/kg per day. During endurance training (if it is particularly intensive), the recommendation is to take approximately 0.25 g/kg of protein per hour, to reduce any possible muscle damage.

Fat

Ranchordas et at. (2013) stated that fat contributes to energy replenishment, especially during matches or training, but carbohydrates are the dominant source of energy in tennis. Similar to protein requirements, no study was conducted to investigate the daily requirement of fatty foods for top-level tennis players. One of only a few studies was conducted to investigate the dietary profiles of male tennis players. Fat intake was reported as the percentage of total energy intake, where 70% of the athletes spent >30% of their total energy from fat (Juzwiak, 2008). The suggested daily fat intake required to ensure the adequate supply for endurance training during >2 hours per day is 2 g/kg (Stellingwerff, 2011). This recommendation should not be applied to tennis directly, because matches include significant high intensity strain and carbohydrates are the main source of energy. Even though it is understood that moderately low body fat facilitates speed and agility on the court and increases heat tolerance, there is no scientific evidence that suggests low body fat is required for becoming a successful tennis player. Instead, the success of players with low fat and high muscle mass provide evidence that there are advantages of having low body fat. Regardless of the dietary requirements of athletes, some sources of fat must be included in the diet to

enable the absorption of vitamins which are soluble in fats, hormone synthesis, and support the efficient function of cellular membranes. Fats are the basic components of cellular membranes, they have a role in signalling and transport, as well as nerve function, they provide isolation and vital protection to the organs, and they are a source of essential nutritious fatty acids. Athletes who limit fat to <20% of total energy are susceptible to low intakes of vitamins and carotenoids soluble in fat, essential fatty acids, including n-3 (omega-3) fatty acids (Thomas et al., 2016). Some athletes competing in endurance sports have recently become interested in keto-adaptation ("fat adapted" or "low training") diets with high fat contents and low carbohydrate contents (Volek et al., 2014). This resurgence of interest in fat is based on higher oxidation of fat and glucose under the conditions of lower intensity training (<70% VO2max) (Getzin et al., 2011).

According to Tipton and Wolfe (2004), Table 2 shows the dietary guidelines for the stages of training: general preparation, specific preparation, competition (in season), and the transitional or transfer period. The table also contains: goals and characteristics of training, dietary goals and specificities, and the intake of carbohydrates, protein, and fat (g/kg/day).

Table 2. Dietary guidelines adapted according to Tipton and Wolfe, (2004)

Stages of training				
	General preparation	**Specific preparation**	**Competition / in season**	**Transition**
Goals and characteristics of training:	Basic strength and basic aerobic development of low and high intensity activities	Tennis-specific energy system and development of strength/maximum intensity training. Higher intensity, lower volume	Maintaining/ stabilising the technique, strength, and speed, "wavy" high and low intensity activities	Physiological and psychological recovery and renewal Lowest volume and intensity
Dietary goals and specificities:	Ensure sufficient amounts of energy and macro micro-nutrients to support high-volume training and muscle adaptations.	Energy intake can be reduced considering that the volume is reducing, but still provide sufficient nutrients and liquids to support the adaptation.	Ensure a sufficient diet for hydration and optimisation of recovery and performance. Energy could be further reduced.	Reduce the intake of energy and carbohydrates to the lowest level, approaching the levels of inactive/ sedentary individuals.
Carbohydrates (g/kg/day)	6-7	7-8	8-10	4-5
Protein (g/kg/day)	1.5 - 1.7	1.5 - 1.7	1.5 - 1.7	1.5 - 1.7
Fat (g/kg/day)	1.1 - 1.5	1.1 - 1.5	1.0	1.0

Considering that the daily requirements for carbohydrates, protein, and fat before training, during training, and after training depend on the level of training and can become confusing for an athlete, Table 3 contains the necessary lowest and highest limits that should be consumed in meals.

Table 3. Dietary guidelines according to Vitale and Getzin, (2019)

Nutrients	**Daily requirements**	**Before training**	**During training**	**After training**
Carbohydrates	5-7 g/kg/day (1 h/day) 6-10 g/kg/day (1-3 h/day) 8-12 g/kg/day (4≥ h/day)	6 g/kg/day (<90 min) 10-12 g/kg/day (> 90 min) + 1-4 g/kg (1-4 h before events)	30-60 g/h (<2.5 h) 60-70 g/h (>2.5 h) 90 g/h (>2.5 h, if tolerable)	8-10 g/kg/day (first 24 h) 1.0-1.2 g/kg/h (first 3-5 h) or 0.8 g/kg/h + protein (0.3 mg/kg/h) or caffeine (3 mg/kg)
Protein	1.4 g/kg/day 0.3 g/kg every 3-5 h	0.3 g/kg just before (or after training)	0.25 g/kg/h (in case of high intensity training/ eccentric)	0.3 g/kg within 0-2 h (or before training)
Fat	Do not limit to <20% of total caloric energy Unclear role of CLA, omega-3, MCT supplements Consider limiting the intake of fat only during carbohydrate charging or before a match because of GI			

Conclusion

In the overview of the studies we made for the purpose of evaluating the intake of carbohydrates protein, and fat before and after training or a tennis match, we have determined that there are not many studies that covered this topic. Due to the specificities of the sport (time the match starts, length of the match, temperature, surface, tennis balls…), it is very important to determine the optimal time and quantity for taking nutrients. Considering energy expenditure, a balanced intake of carbohydrates, protein, and fat can play a key role in the final result. However, more studies are required to determine the optimal quantity and time for taking carbohydrates, protein, and fat before a match, and for recovery after a match.

References

Bergström, J., Hermansen, L., Hultman, E., Saltin, B. (1967): Diet, muscle glycogen and physical performance. *Acta Physiologica Scandinavica* 71 (2), 140-150.

Burke, L. M., Cox, G. R., Cummings, N. K., Desbrow, B. (2001): Guidelines for Daily Carbohydrate Intake. *Sports Medicine* 31 (4), 267-299.

Christmass, M. A., Richmond, S. E., Cable, N. T., Arthur, P. G., Hartmann P. E. (1998): Exercise intensity and metabolic response in singles tennis. *Journal of Sports Sciences* 16 (8), 739-747.

Convertino, V, Armstrong, L. E., Coyle, E. F., Mack, G. W., Sawka, M. N., Senay, L. C. Jr., Sherman, W. M. (1996): American College of Sports Medicine position stand: exercise and fluid replacement. *Medicine and Science in Sports and Exercise* 28 (1), i-vii.

Fernandez, J., Mendez-Villanueva, A., Pluim, B. M. (2006): Intensity of tennis match play, *British Journal of Sports Medicine* 40 (5), 387-391.

Fernandez-Fernandez J., Mendez-Villanueva A., Fernandez-Garcia B., Terrados N. (2007): Match activity and physiological re-sponses during a junior female singles tennis tournament. *British Journal of Sports Medicine* 41 (11), 711-716.

Fernandez-Fernandez, J., Sanz-Rivas, D., Fernandez-Garcia B., Mendez-Villanueva A. (2008): Match activity and physiological load during a clay-court tennis tournament in elite female players. *Journal of Sports Sciences* 26 (14), 1589-1595.

Fleming, J. A., Naughton, R. J., Harper, L. D. (2018): Investigating the Nutritional and Recovery Habits of Tennis Players. *Nutrients* 10 (4), 443.

Galloway, S. D. R., Maughan, R. J. (2010): The effects of substrate and fluid provision on thermoregulatory and metabolic responses to prolonged exercise in a hot environment. *Journal of Sports Sciences* 18 (5), 339-351.

Getzin, A. R., Milner, C., Harkins, M. (2017): Fueling the Triathlete: Evidence-Based Practical Advice for Athletes of All Levels. *Current Sports Medicine Reports* 16 (4), 240-246.

Getzin, A. R., Milner, C., LaFace K. M. (2011): Nutrition update for the ultraendurance athlete. *Current Sports Medicine Reports* 10 (6), 330-339.

Girard, O., Lattier, G., Micallef, J. P., Millet, G. P. (2006): Changes in exercise characteristics, maximal voluntary contraction, and explosive strength during prolonged tennis playing. *British Journal of Sports Medicine* 40 (6), 521-526.

Gropper, S. S., Sorrels, L. M., Blessing, D. (2003): Copper status of collegiate female athletes involved in different sports. *International Journal of Sports Nutrition and Exercise Metabolism* 13 (3), 343-357.

Hargreaves, M., Hawley, J. A., Jeukendrup, A. (2004): Pre-exercise carbohydrate and fat ingestion: effects on metabolism and performance. *Journal of Sports Scinces* 22 (1), 31-38.

Hornery, D. J., Farrow, D., Mujika, I., Young, W. B. (2007): Caffeine, carbohydrate, and cooling use during prolonged simulated tennis. *International Journal of Sports Physiology and Performance* 2 (4), 423-438.

Jäger, R., Kerksick, C. M., Campbell, B. I., Cribb, P. J., Wells, S. D., Skwiat, T. M., Purpura, M., Ziegenfuss, T. N., Ferrando, A. A., Arent, S. M., Smith-Ryan, A. E., Stout, J. R., Arciero, P. J., Ormsbee, M. J., Taylor, L. W., Wilborn, C. D., Kalman, D. S., Kreider, R. B., Willoughby, D. S., Hoffman, J. R., Krzykowski, J. L., Antonio, J. (2017): International Society of Sports Nutrition Position Stand: protein and exercise. *Journal of the International Society of Sports Nutrition* 14 (33).

Jeukendrup, A. E., Jentjens, R. L. P. G., Moseley, L. (2005): Nutritional Considerations in Triathlon. *Sports Medicine* 35 (2), 163-181.

Jung, A. P.,Bishop, P. A., Al-Nawwas, A., Dale, R. B. (2005): Influence of Hydration and Electrolyte Supplementation on Incidence and Time to Onset of Exercise-Associated Muscle Cramps. *Jorunal of Athletic Training* 40 (2), 71-75.

Juzwiak, C. R., Amancio, O. M., Vitalle, M. S., Pinheiro, M. M., Szejnfeld, V. L. (2008): Body composition and nutritional profile of male adolescent tennis players. *Journal of Sports Sciences* 26 (11), 1209-1217.

Kondric, M., Sekulic, D., Uljevic, O., Gabrilo, G., Zvan, M. (2013): Sport Nutrition and Doping in Tennis: An Analysis of Athletes' Attitudes and Knowledge. *Journal of Sports Science & Medicine* 12 (2), 290–297.

Kovacs, M. S. (2006): Carbohydrate intake and tennis: are there benefits? *British Journal of Sports Medicine* 40 (5), e13.

Kovacs, M. S. (2007): Tennis physiology: Training the competitive athlete. *Sports Medicine* 37 (3), 189-198.

Kovacs, M. S., Baker, L. B. (2014): Recovery interventions and strategies for improved tennis performance. *British Journal of Sports Medicine* 48 (1), i18-i21.

Martin, C., Thevenet, D., Zouhal, H., Mornet, Y., Deles, R., Crestel, T., Ben Abderrahman, A., Prioux J. (2011): Effects of playing surface (hard and clay courts) on heart rate and blood lactate during tennis matches played by high-level players. *Journal of Strength and Conditioning Research* 25 (1), 163-170.

Mendez-Villanueva, A., Fernandez-Fernandez, J., Bishop, D., Fernan-dez-Garcia, B., Terrados N. (2007): Activity patterns, blood lactate concentrations and ratings of perceived exertion during a professional singles tennis tournament. *British Journal of Sports Medicine* 41 (5), 296-300.

Murias, J. M., Lanatta, D., Arcuri, C. R., Laino, F. A. (2007): Metabolic and functional responses playing tennis on different surfaces. *Journal of strength and conditioning research* 21 (1), 112-117.

Nutter, J. (1991): Seasonal changes in female athletes' diets. *International Journal of Sports Nutrition* 1 (4), 395-407.

Ojala, T., Häkkinen, K. (2013): Effects of the tennis tournament on players' physical performance, hormonal responses, muscle damage and recovery. *Journal of Sports Science & Medicine* 12 (2), 240-248.

Phillips, S.M., Van Loon, L. J. C. (2011): Dietary Protein for Athletes: From Requirements to Optimum Adaptation. *Journal of Sports Sciences* 29 (1), S29-S38.

Ranchordas, M. K., Rogersion, D., Ruddock, A, Killer, S. C., Winter, E. M. (2013): Nutrition for Tennis: Practical Recommendations. *Journal of Sports Science & Medicine* 12 (2), 211-224.

Rodriguez, N. R., DiMarco, N. M., Langley, S. (2009): Position of the American Dietetic Association, Dietitians of Canada, and the American College of Sports Medicine: Nutrition and Athletic Performance. *Journal of the American Dietetic Association* 109 (3), 509-527.

Rossi, F. E., Landreth, A., Beam, S., Jones, T., Norton, L., Cholewa, J. M. (2017): The Effects of a Sports Nutrition Education Intervention on Nutritional Status, Sport Nutrition Knowledge, Body Composition, and Performance during Off Season Training in NCAA Division I Baseball Players. *Journal of Sports Science & Medicine* 16 (1), 60-68.

Smekal, G., von Duvillard, S. P., Rihacek, C., Pokan, R., Hofmann, P., Baron R., Tschan, H., Bachl, N. (2001): A physiological profile of tennis match play. *Medicine and Science in Sports and Exercise* 33 (6), 999-1005.

Stellingwerff, T., Maughan, R. J., Burke, L. M. (2011): Nutrition for power sports: middle-distance running, track cycling, rowing, canoeing/kayaking, and swimming. *Journal of Sports Sciences* 29 (1), S79-S89.

Thomas, D. T., Erdman, K. A., Burke, L. M. (2016): Position of the Academy of Nutrition and Dietetics, Dietitians of Canada, and the American College of Sports Medicine: Nutrition and Athletic Performance. *Journal of the Academy of Nutrition and Dietetics* 116 (3), 501-528.

Tipton, K. D., Wolfe, R. R. (2004): Protein and amino acids for athletes. *Journal of Sports Sciences* 22 (1), 65-79.

Vitale, K., Getzin, A. (2019): Nutrition and Supplement Update for the Endurance Athlete: Review and Recommendations. *Nutrients* 11 (6), 1289.

Volek, J. S., Noakes, T., Phinney, S. D. (2014): Rethinking Fat as a Fuel for Endurance Exercise. *European Journal of Sport Science* 15 (1), 13-20.

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