CI&CEQ
Chemical Industry & Chemical Engineering Quarterly

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Chem. Ind. Chem. Eng. Q. 23 (4) 457–471 (2017) CI&CEQ

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SCIENTIFIC PAPER

UDC 663.4:635.8:66:60:54

MODIFICATION OF FUNCTIONAL QUALITY OF BEER BY USING MICROENCAPSULATED GREEN TEA (*Camellia sinensis* L.) AND *GANODERMA* MUSHROOM (*Ganoderma lucidum* L.) BIOACTIVE COMPOUNDS

Article Highlights

- Alginate and pectin microbeads were produced by electrostatic extrusion and spray drying
- Chitosan reinforcement provided smaller beads but prolonged release of polyphenols
- Pilsner beer with Ganoderma and radler with green tea encapsulates were preferred
- The use of spray dried microbeads enabled the most pronounced augmentation of TPC of enriched beer
- Stability of enriched beer's phenolic content during one month-storage was established

Abstract

Increasing interest in production of frequently consumed functional food products has focused the present study on implementation of microencapsulated Ganoderma mushroom and green tea bioactive compounds in beer production. Electrostatic extrusion assisted microencapsulation of green tea and Ganoderma extracts enabled production of particles ranging from 490 to 1000 μm in size, with up to 75% of entrapped total polyphenols. Dried, powdered extracts, as well as microparticles encapsulating Ganoderma and green tea extracts that exhibited the best morphological properties and retarded release of polyphenols (alginate and alginate-chitosan coated, as well as chitosan coated pectin microbeads) were implemented in beer production. The addition of Ganoderma microbeads to pilsner beer did not augment its polyphenolic concentration (TPC), as opposed to the addition of green tea encapsulating microbeads to radler, while adding dried Ganoderma and spray dried green tea extracts enabled to increase the TPC for up to 3-fold higher values. Ganoderma dried extract-enriched pilsner beer and spray dried green tea extract-enriched radler were preferred in terms of sensory properties, due to the lowest bitterness intensity and most pronounced herbal aroma of the added adjuncts. Refrigerated storage of Ganoderma hydrogel microbeads-enriched pilsner beer revealed fluctuations of TPC, while green tea hydrogel microbeads-enriched radler exhibited better stability. The established methodology provides a procedure suitable for microencapsulate-enrichment of drink and food products, thus setting a reliable basis for future functional food production by microencapsulate implementation strategies.

Keywords: beer, Ganoderma, green tea, microencapsulation, polyphenolic antioxidants.

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Paper received: 22 July, 2016
Paper revised: 14 Novembar, 2016
Paper accepted: 16 December, 2016

https://doi.org/10.2298/CICEQ160722060B

Beer is the most popular alcoholic beverage worldwide and the second most consumed alcoholic beverage in Europe, accounting for 37% of the total EU alcohol consumption [1]. Beer contains numerous ingredients that exert physiological effects upon consumption, which can be attributed to its diverse composition (water, malt, hops, brewer's yeast, non-malted cereals, starch or starch syrups as adjuncts). Characterized by a high carbohydrate concentration and presence of proteins, amino acids, vitamins, organic acids, over 30 different micro and macro elements and a range of antioxidants, beer is considered as a nutritively valuable product [2]. However, in recent years, food industry efforts are focused on fitting the consumer trends towards more natural products, obtained by either using natural raw materials for food production or enriching conventional products with diverse natural substrates or extracts. With regard to the beer segment, the mixtures of high-quality beer and lemonade (popularly called radler) have become the largest volume innovation on beer markets globally, while interest has arisen in specialty types of beer enriched with other bioactive compounds. Various natural sources of bioactive compounds, such as medicinal plant extracts (*Melissa officinalis* and *Thymus vulgaris*) [3], medicinal mushrooms (*Ganoderma lucidum*) [4], grape must [5] and honey [6] have been used so far for enriching beer and formulating new taste and aroma attributes.

When consumed in the form of a functional food product, the effectiveness of naturally derived bioactive compounds in providing therapeutic or physiological benefits is usually relieved by their low concentration and bioavailability, or the effects of chemical, physical and physiological factors during food production and consumption [7]. Encapsulation enables to improve the bioactive effectiveness of beneficial compounds by protecting unstable substances of various external influences, as well as by enhancing their stability and activity during production and storage [8]. In recent years, encapsulation has been used extensively for immobilization of polyphenolic compounds from different plant substrates such as medicinal plant extracts [9], cocoa extract [10] or blueberry anthocyanins [11], etc. Encapsulation of polyphenolic compounds as highly potent antioxidants, but characterized with a pronounced instability and reactivity, contributes to their preservation, increasing their bioavailability, masking of unpleasant tastes, such as polyphenol-imparted bitterness and astringency and enabling the formation of new dosage forms for production of innovative food products [8,9].

Following the principle of using widely available, economically acceptable sources of functional ingredients for production of novel food products, instead of using plain, isolated and expensive bioactive compounds, green tea (*Camellia sinensis* L.) and *Ganoderma* (*Ganoderma lucidum*) powder were selected as the active ingredients in the present study. Green tea and medicinal mushrooms such as *Ganoderma* are today among the most well-known and popular functional ingredients, owing to their scientifically proven beneficial health effects attributed to a high concentration of polyphenols and polysaccharides [12,13]. Although polyphenolic compounds are not the main active ingredients of *Ganoderma* mushroom, in order to achieve innovative and sensory appealing beer, *Ganoderma* was chosen as the functional substrate to be implemented in different beer products. Namely, a previous study by Leskošek-Čukalović *et al.* [4] revealed that *Ganoderma*-extract enriched beer exhibited significantly improved sensory properties in comparison to plain beer, especially with regard to bitterness which was scored much higher and more harmonic and finer. The reason for this may be the combination of all specific bioactive compounds of *Ganoderma*, comprising terpens and polyphenols which contributed to that aspect. For the purpose of encapsulation of green tea polyphenols, different techniques, such as internal gelation [14], spray drying [15] or liposome entrapment [16] have been applied so far, while limited number of studies such as the ones from broken *Ganoderma lucidum* spores [17] or *Ganoderma* mushroom oil [18] reported the microencapsulation of *Ganoderma* bioactive compounds (polysaccharides and ganoderic acids). Therefore, in the present study, electrostatic extrusion and spray drying microencapsulation of polyphenolic compounds from *Ganoderma* mushroom and green tea extracts were employed, in alginate and pectin or chitosan-reinforced hydrogel microparticles, aiming to augment the retention performance of entrapped bioactives. The effect of encapsulates upon their incorporation into a real food product was examined by using microencapsulated polyphenolic compounds from *Ganoderma* mushroom and green tea extracts for the formulation of innovative beers with improved bioactive and sensory properties.

EXPERIMENTAL

Chemicals

All chemicals used for the analytical procedures were of analytical grade, while the natural biopoly-

mers used as carriers for encapsulation purposes were appropriate for food purposes. Sodium alginate was purchased from Sigma-Aldrich (St. Louis, MI, USA). Chitosan (molecular weight 100000–300000 $g \cdot mol^{-1}$) was obtained from Acros organic (Geel, Belgium) and pectin (low methoxyl pectin, molecular weight 70–140 kDa) from Cargill (Zagreb, Croatia). Green tea *Darjeeling* (Müller, Abtswind, Germany) in loose leaf form and powdered *Ganoderma* mushroom extract (Fujian Xianzhilou Biological Science & Technology, Fuzhou, China) were purchased at a local organic market in Croatia.

Preparation of green tea and Ganoderma mushroom extracts

Green tea (ground to a fine powder using a ball mill) was repeatedly extracted (2-fold extraction), by pouring 100 ml of distilled water heated to 80 °C over 8 g of plant material and stirring on a magnetic stirrer at 80 °C for 15 min. After each extraction, the extracts were centrifuged at 5310*g* for 5 min and the combined supernatants filled up to 200 ml.

In case of *Ganoderma* mushroom extract, 5 g of powdered extract was resuspended in 100 ml of distilled water on a magnetic stirrer during 24 h, centrifuged at 5310*g* for 5 min and filled up to 100 ml. In order to determine the content of soluble (extractable) solids present in the obtained extracts, dry matter content of prepared *Ganoderma* and green tea extracts was determined according to the AOAC method 920.151 [19] and exhibited 32.3 and 15.1 $g \cdot kg^{-1}$, respectively. The polyphenolic yield (total phenol concentration – TPC determined using the Folin-Ciocalteu assay [20]) of the extracts amounted to 820 $mg \cdot L^{-1}$ expressed as mg of gallic acid equivalents (GAE) per L and 3472 $mg \cdot L^{-1}$ GAE, respectively.

Microencapsulation by electrostatic extrusion in different carrier materials

The preparation of microbeads by electrostatic extrusion was carried out by ionic gelation technique following the method adopted by Belščak-Cvitanović *et al.* [9] Alginate (2 $g \cdot L^{-1}$) and pectin (2 $g \cdot L^{-1}$) solutions were prepared by dissolving sodium alginate or pectin in the previously prepared mushroom or green tea extracts. Dissolved solutions were homogenized and microencapsulated using the electrostatic droplet generation method [9], at a constant flow rate (25.2 $mL \cdot h^{-1}$) of encapsulant solution and varying the voltage between 4.6 and 5.2 kV, depending on the formulation (Table 1). The cross-linking solution consisted of 2 $g \cdot L^{-1}$ of calcium chloride prepared in the green tea or *Ganoderma* mushroom extracts. For chitosan coating of alginate and pectin microparticles, the encapsulant solutions were extruded into the collecting solution consisting of 1 $g \cdot L^{-1}$ of chitosan dissolved in the previously prepared mushroom or green tea extract containing 2 $g \cdot L^{-1}$ of citric acid (pH 2.35) and 2 $g \cdot L^{-1}$ of calcium chloride. All obtained microparticles were washed several times with the extract after production and stored in the original extracts containing 2 $g \cdot L^{-1}$ calcium chloride in the dark at 4 °C. By preparing and storing the formulated microbeads in the corresponding extracts, isotonic concentrations (the same con-

Table 1. Encapsulation parameters, loading efficiency and particle size distribution of microparticles encapsulating Ganoderma mushroom and green tea polyphenols; The values in each column superscripted with the same alphabeth letter (a-h) are not significant ($p > 0.05$); CH – chitosan coating; d(0.1), d(0.5), d(0.9) – particle diameters indicating that 10, 50 and 90% of the particle size distribution is below these values, respectively; encapsulation efficiency and retained antioxidant capacity are expressed as percentages of total polyphenols concentration and antioxidant capacity of formulated particles and initial encapsulant solution

Particle type	Material	Extrusion voltage, kV	Needle dimension mm	Encapsulation efficiency, %	Retained antioxidant capacity, %	Particle size, μm		
						d (0.1)	*d* (0.5)	*d* (0.9)
Hydrogel	*Ganoderma* mushroom							
	Alginate	4.8	0.60	71.8±4.3^{abc}	72.7±1.4^{a}	428.96±0.18^{a}	621.20±7.27^{a}	826.19±13.70
	Pectin	4.9	0.84	66.6±2.4^{adg}	67.2±2.3^{b}	147.53±2.36	682.79±6.81	907.94±12.47^{a}
	Alginate CH	5.0	0.60	75.2±3.8^{be}	70.2±3.5^{ab}	310.45±0.42	490.64±4.53	881.68±12.68^{a}
	Pectin CH	5.2	0.84	61.6±4.2^{df}	71.3±3.3^{ab}	393.71±1.47	616.42±5.35^{a}	955.04±15.78
	Green tea							
	Alginate	4.9	0.60	62.6±3.8^{d}	56.8±1.7^{cd}	791.20±39.61	987.27±40.48	1250.28±75.02^{b}
	Pectin	4.7	0.41	52.2±1.7^{h}	52.2±2.8^{c}	425.17±21.14^{a}	814.23±36.78	1237.15±35.75^{b}
	Alginate CH	5.2	0.41	51.4±2.6^{h}	56.0±3.4^{c}	725.87±14.52	905.55±54.33	1156.88±62.47
	Pectin CH	4.6	0.41	47.6±1.4^{h}	59.4±2.4^{d}	546.41±21.86	773.17±18.56	1047.30±29.32
Spray dried green tea	Plain extract	-	-	66.9±4.7^{cdf}	69.1±3.4^{ab}	1.45±0.12	3.71±0.15	7.80±0.06

centration of polyphenolic compounds in the formulated microbeads and outside) during all stages of microbeads preparation (carrier, cross-linking and storage solutions) were maintained. In that way, the concentration gradient between the beads and surrounding solution was maintained and the driving force for diffusion-related release of polyphenols was eliminated, ensuring equal polyphenol content of the beads at all times.

Green tea extract was also spray dried using a Büchi mini B-290 spray dryer (Büchi Labortechnik, Flawil, Switzerland) equipped with a 0.7 mm standard diameter nozzle. The inlet and outlet temperature were 130±3 and 56±2 °C, respectively. The spraying air flow rate, rate of liquid feed, atomisation pressure and pump speed were 536 $L \cdot h^{-1}$, 8 mL min^{-1}, 41 kPa and 30%, respectively. A free-flowing powder used for enriching beer was obtained.

Characterization of physico-chemical and bioactive properties of formulated particles

Randomly selected hydrogel beads were visualized using an optical microscope, whereas the appearance and surface morphology of spray dried beads were examined by scanning electronic microscopy (SEM) performed by using a Mira3 microscope (Tescan, Brno – Kohoutovice, Czech Republic). The samples were attached to stubs, coated with a layer of gold (50 nm) and examined using an acceleration voltage of 4-5 kV. Particle size of the obtained microparticles was determined using a Mastersizer 2000 (Malvern Instruments, Malvern, United Kingdom) equipped with a Hydro 2000S dispersion unit in case of hydrogel beads and a Scirocco 2000 unit in case of spray dried green tea extract.

The concentration of entrapped total polyphenols and antioxidant capacity of produced hydrogel microbeads was estimated by dissolving a known amount of microparticles in 2 $g \cdot L^{-1}$ of sodium citrate (or in distilled water in case of spray dried microbeads). The concentration of total polyphenols was determined using the Folin-Ciocalteu assay [20], and the antioxidant capacity using the DPPH radical scavenging assay [21]. The percentage of loading efficiency of hydrogel beads was calculated as the ratio between the total polyphenols concentration in the citrate solution of dissolved beads and their respective concentration in the initial solution.

The release kinetics of total polyphenols and antioxidant capacity profile was determined from the obtained hydrogel microbeads in distilled water. For the analysis a known amount of microparticles (of about 3 g) was suspended in 50 mL of the medium (distilled water). The samples were submitted to continuous agitation on an orbital shaker (New Brunswick Scientific, Edison, New Jersey, USA) operating at 1.67 Hz. At defined time intervals, an aliquot of the supernatant was taken for analysis and replaced by the same amount of fresh medium.

Formulation of beers enriched with microencapsulated Ganoderma or green tea extract polyphenols

In order to improve the functional properties of beer, pilsner beer and lemon radler were enriched with prepared liquid extracts, microencapsulated *Ganoderma* mushroom and green tea extracts in hydrogel and dried (powdered) form (according to the schematic display on Figure 1). With the purpose of neutralizing the interferences of naturally present antioxidants in lemon radler (ascorbic acid) which can cause interferences and overestimation of total polyphenols and antioxidant capacity during analytical measurements, the authors wanted to „equalize“ both types of beer samples (pilsner and radler), so lemon juice was added to pilsner beer. Preliminary sensory evaluations (data not shown) also revealed that in that way the taste attributes of pilsner beer were improved in comparison to plain beer without lemon juice. For the preparation of samples,1 $g \cdot L^{-1}$ of *Ganoderma* mushroom hydrogel microbeads or 1.5 $g \cdot L^{-1}$ of green tea extract hydrogel microbeads and 0.5 $g \cdot L^{-1}$ of lemon juice were added to pilsner beer, while 1.5 $g \cdot L^{-1}$ of green tea extract hydrogel microbeads or 0.5 g L^{-1} of spray dried green tea extract were added to radler. The described microencapsulates and plain non-encapsulated green tea or *Ganoderma* mushroom extracts (in concentration equal as the added microbeads, representing control sample) were added aseptically to commercially produced bottled beers or radler, and the bottles immediately closed to mature at 5 °C for one day.

In the formulated enriched beers, the concentration of total polyphenols and antioxidant capacity were determined as previously described and their stability was evaluated during one month of refrigerated storage (4 °C), directly upon production and each 10th day of storage. The basic chemical parameters of enriched beers such as: alcohol concentration, real extract, apparent extract and energy value, were also determined during storage using Alcolyzer Beer ME analyzing system (Anton Paar, Graz, Austria).

Sensory evaluation of enriched beers

The enriched beers were evaluated using quantitative descriptive analysis procedure excerpt from the literature data on sensory evaluation of beer [5]

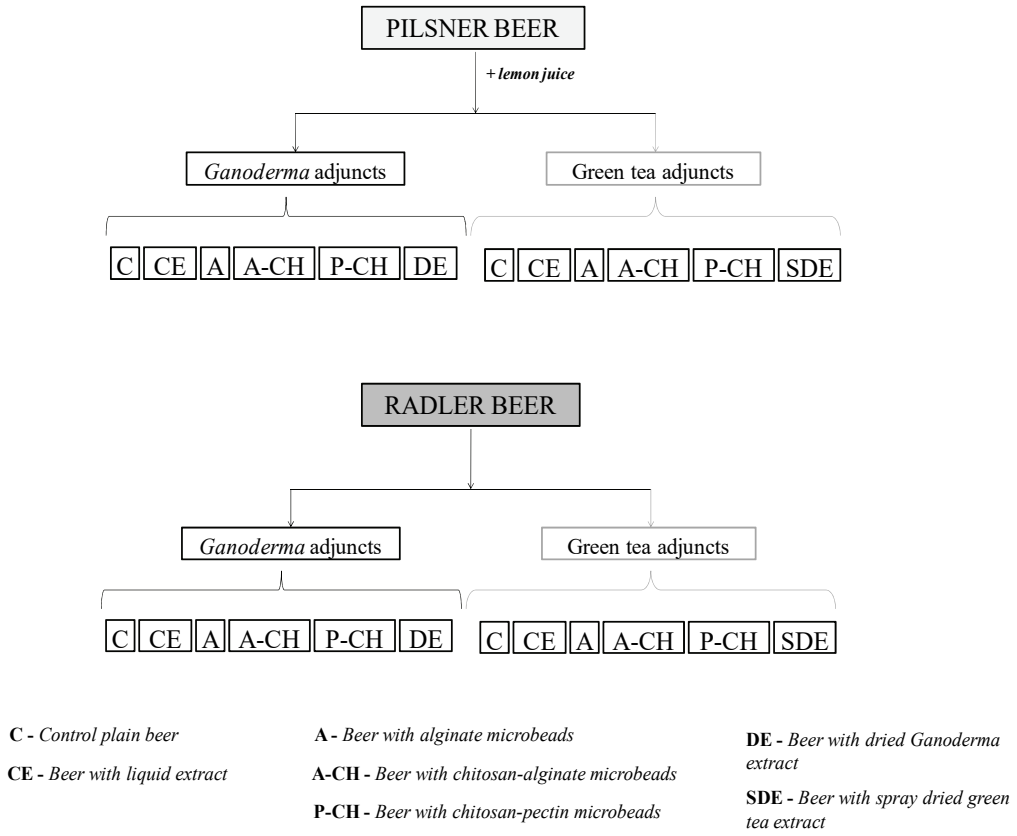

Figure 1. Schematic display of sample setup during preparation of enriched beers.

and according to ISO standard [22]. The internal sensory panel of the Faculty of Agriculture in Belgrade, Serbia comprised 25 people, 7 female and 18 male members, who had undergone extensive sensory training and had previous experience in the assessment of beer. The experimental samples were evaluated during two sessions (one during which only *Ganoderma*-enriched drinks were evaluated and the second one evaluating only green tea-enriched drinks), with conventional beer (or radler) produced with the addition of plain *Ganoderma* or green tea extract serving as the controls. For the sensory analysis, 50 mL of sample adjusted to 12 °C was served in transparent plastic cups encoded with random alphabet letters. Water was provided for rinsing between samples. The sensory properties were presented on a 5-point scale (according to which 5 meant extremely desired quality and 1 was for a defective product).

Eight attributes were evaluated for all beers (appearance, colour, transparency, odour, taste, mouthfeel, aftertaste, acidity, bitterness and overall acceptability). For each assessed attribute an importance factor ($F = 0.05$ to $F = 0.5$) was considered, based on the guidelines determined by MEBAK [23], where $F = 0.05$ was used for the less important attributes (colour, transparency), $F = 0.29$ was used for odour, aftertaste and acidity, $F = 0.41$ for taste and bitterness, while $F = 0.5$ for remaining attributes (appearance and overall acceptability). The number of points describing each of the attributes was multiplied by the importance factor, and the average point number was calculated.

Statistical analysis

The results were analyzed statistically using the Statistica AXA ver.12 ACADEMIC software (StatSoft, Tulsa, Oklahoma, USA) to determine the average value and standard error. Variance analysis, with a significance level of $\alpha = 0.05\%$, was performed to determine the influence of adding different types of substrates and microbeads on the evaluated beer parameters.

RESULTS AND DISCUSSION

Microencapsulation of green tea and Ganoderma mushroom polyphenolic extracts

Results of the microencapsulation experiments aimed at producing encapsulated particulates as the delivery systems of green tea and *Ganoderma* bioactives revealed that the particle size and morphology, encapsulation yields and release profiles as the main

attributes of formulated microparticles were in dependence on both the employed carrier material (or combination thereof) as the encapsulants and the type of substrate (green tea or *Ganoderma*).

Particle size and morphological properties of formulated microparticles

Marked differences can be observed in the particle size distribution parameters of microbeads encapsulating *Ganoderma* extract, which were significantly ($p < 0.05$) smaller in comparison to the ones encapsulating green tea extract (Table 1). According to the median diameter (d 0.5) alginate particles encapsulating *Ganoderma* were smaller than the pectin particles, but an opposite ranking can be observed in case of green tea encapsulating microbeads. Although pectin particles encapsulating *Ganoderma* exhibited higher d 0.5 in comparison to alginate microparticles, they were characterized with a very low d 0.1 = 147.53 μm (particle diameter at which 10% of the sample is comprised of smaller particles), indicating that a low number of very small microbeads (smaller than 147.53 μm) is formed during the microparticles production. If taking into account the high d 0.9 (particle diameter at which 90% of the sample is comprised of smaller particles) of pectin particles (907.94 μm), the results imply that this sample exhibits a very broad particle size distribution (PSD) width, suggesting uneven and non-homogenous microparticle formation, in comparison to alginate particles. The same situation was observed in case of pectin particles encapsulating green tea extract, indicating more non-homogenous particles than alginate ones. These results imply that alginate hydrogel particles may be preferred over pectin ones according to the PSD and size homogenicity (Figure 2), since for the purpose of food and beverage applications visual appearance is a crucial factor for the product development.

The differences observed in the particle size of alginate and pectin microparticles reveal the effect of pH influenced by the substrate (*Ganoderma* or green tea) composition that can mediate during ionic cross-linking of alginate and pectin hydrogels and result with possible modifications of the hydrogel properties. Namely, the gelation kinetics and the mechanism involved in the ionotropic gel formation are strictly dependent upon the pH of alginate and pectin solutions. pH regulates the dissociation and availability for ionic complexation of guluronic and carboxylic groups of alginate and pectin, affecting the final gel properties [24]. At lower pH, the ionization of galacturonate carboxylic (pectin) groups is repressed by the

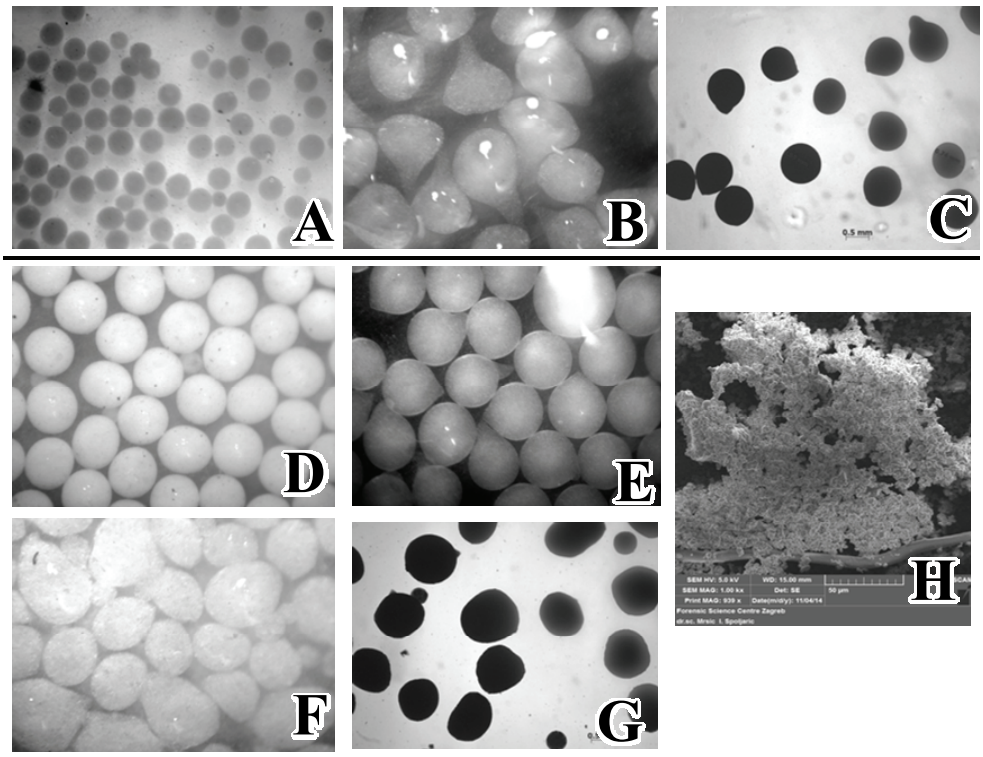

Figure 2. Optical micrographs of plain alginate (A, D), plain pectin (B, E), chitosan coated alginate (C, F) and chitosan coated pectin (G) hydrogel microbeads encapsulating Ganoderma (A, B, C) and green tea (D-G) extracts, as well as SEM micrograph of spray dried green tea extract (H); the enhancements of ~10× for optical micrographs and 3000× for SEM imaging was used.

higher concentration of hydrogen ions, thus producing less negatively charged groups (such as COO^-) that can form intermolecular junction zones, *via* Ca^{2+}-bridges [25]. Since the pH of *Ganoderma* extract (4.12) was lower than the one of green tea extract (5.15), less Ca-crosslinking could have occurred while encapsulating *Ganoderma* bioactive compounds, thus producing larger pectin particles with a very broad PSD width. Correspondingly, in case of green tea bioactives encapsulation in pectin hydrogel, higher pH favored more pronounced ionization of carboxylate groups that could form the cross-linked junction zones with Ca^{2+}, producing better cross-linked and smaller particles.

According to the d 0.5, chitosan coated microbeads were slightly smaller in comparison to the non-coated particles. Similar results were already previously observed in case of chitosan coating formation on alginate particles [9]. This may have occurred due to the hypertonic concentration of chitosan coating medium in relation to the hydrogel beads, which facilitated the hypertonic-driven diffusion of water from the beads, resulting with smaller particles. Another possible explanation is that since alginate and pectin as anionic polysaccharides form polyelectrolyte complex with the oppositely charged (cationic) chitosan, the interactions between the carrier materials and coating resulted with additional cross-linking of particle matrix, reducing the water concentration and shrinking of the formed particles [26]. Spray dried green tea extract was expectedly in micro-sized range (< 4 μm, according to d 0.5).

With regard to the morphological properties of produced microparticles (Figure 2), electrostatic extrusion of both *Ganoderma* and green tea extracts and plain alginate as the encapsulants provided spherically shaped particles.

Employing pectin as the carrier material, as well as chitosan as the coating revealed more irregularities and size-inhomogeneous particles with pronounced structural differences. Both plain pectin and chitosan coated alginate and pectin particles were characterized with more conically-shaped ("tear-like") structure, which has previously also been observed for similar delivery formulations [27]. SEM micrograph of spray dried green tea extract revealed a polydispersed system, and large aggregates formed by the particles. The obtained particles had a pronounced, irregular shape with morphologically dented and disturbed surfaces, which occurs as the consequence of water leakage and shrinking of particles during spray drying [15].

Encapsulation efficiencies and release profiles of polyphenolic compounds

The loading capacities of polyphenols as the target active ingredients revealed a fluctuating trend, depending on the encapsulated substrate and employed carrier material. According to the obtained results, the substrate and the composition of its active ingredients, rather than their concentration is the predominant factor influencing the encapsulation performance. This is confirmed by the fact that a much higher TPC of green tea extract (3472 $mg \cdot l^{-1}$ GAE) in comparison to low TPC of *Ganoderma* extract (820 $mg \cdot l^{-1}$ GAE), did not account for higher encapsulation efficiency of polyphenolic compounds from green tea. As can be seen in Table 1, hydrogel microparticles encapsulating green tea generally exhibited lower encapsulation efficiency of total polyphenols in comparison to the particles encapsulating *Ganoderma*. The same was also applied to the percentage of retained antioxidant capacity, which was evidenced by a high correlation (r = 0.79) established between the total polyphenolics loading capacity and percentage of retained antioxidant capacity. Lower total polyphenolics loading capacity and percentage of retained antioxidant capacity in case of green tea encapsulating microbeads can be attributed to the composition of green tea and its active constituents, which influence their retention in the hydrogel microbeads structure. Namely, at the pH of green tea extract (pH 5.1), flavan-3-ol catechin molecules as the representative active compounds of green tea [12] are dissociated to some extent (deprotonated) [28], and produce more electrostatic repulsion between both anionic alginate or pectin charged groups. This electrostatic repulsion of deprotonated catechins [28] and alginate or pectin functional groups may contribute to the poor retention of small molecular weight catechins in the porous matrix of alginate and pectin microbeads. Since the predominant active ingredients of *Ganoderma* are soluble polysaccharides [13], rather than polyphenolic compounds, the fair encapsulation efficiency of polyphenols achieved in this type of microbeads may be the consequence of the higher molecular weight and size of polysaccharides. Moreover, the neutral character of soluble *Ganoderma* polysaccharides [29] may have facilitated the retention of *Ganoderma* extract constituents in the hydrogel matrix and induced possible interactions with the carrier materials, resulting in better retention and loading yield of polyphenolic compounds. Chitosan reinforcement of alginate and pectin particles of both *Ganoderma* and green tea encapsulating microbeads produced no marked effect on the loading capacity of total polyphenols and anti-

oxidant capacity (Table 1). With regard to the spray dried green tea extract, employing lower spray drying temperatures (130 °C) in order to minimize the negative temperature-related degradation of green tea extract polyphenols, enabled to entrap a high proportion of these compounds in the produced powder. The spray dried green tea extract even exhibited significantly ($p < 0.05$) higher contents of total polyphenols loading capacity and retained antioxidant capacity in comparison to hydrogel microencapsulated green tea, implying that the use of this dosage form of bioactive compounds would be preferred in terms of augmenting the bioactive content of an enriched food product.

Although chitosan coating provided structurally poorer particles and no marked differences in the loading capacities of active compounds (in comparison to plain, uncoated particles), it enabled an improved, prolongued release pattern of total polyphenols (Figure 3A) and antioxidant capacity (Figure 3B) from the particles in water. Green tea encapsulating microparticles exhibited significantly higher polyphenolic concentration and antioxidant capacity in relation to the ones encapsulating *Ganoderma*. Due to the pronounced porosity of ionically cross-linked alginate gel, a rapid release (within the first 10 min) of encapsulated compounds occurs. Chitosan coating on both alginate and pectin microbeads enabled to retard the release even up to 60 min in case of total polyphenols released from chitosan coated alginate particles, or antioxidant capacity exhibited by chitosan coated pectin particles. In this way, longer stability and protection of the encapsulated bioactives and their functional properties may be achieved when incorporated in beer.

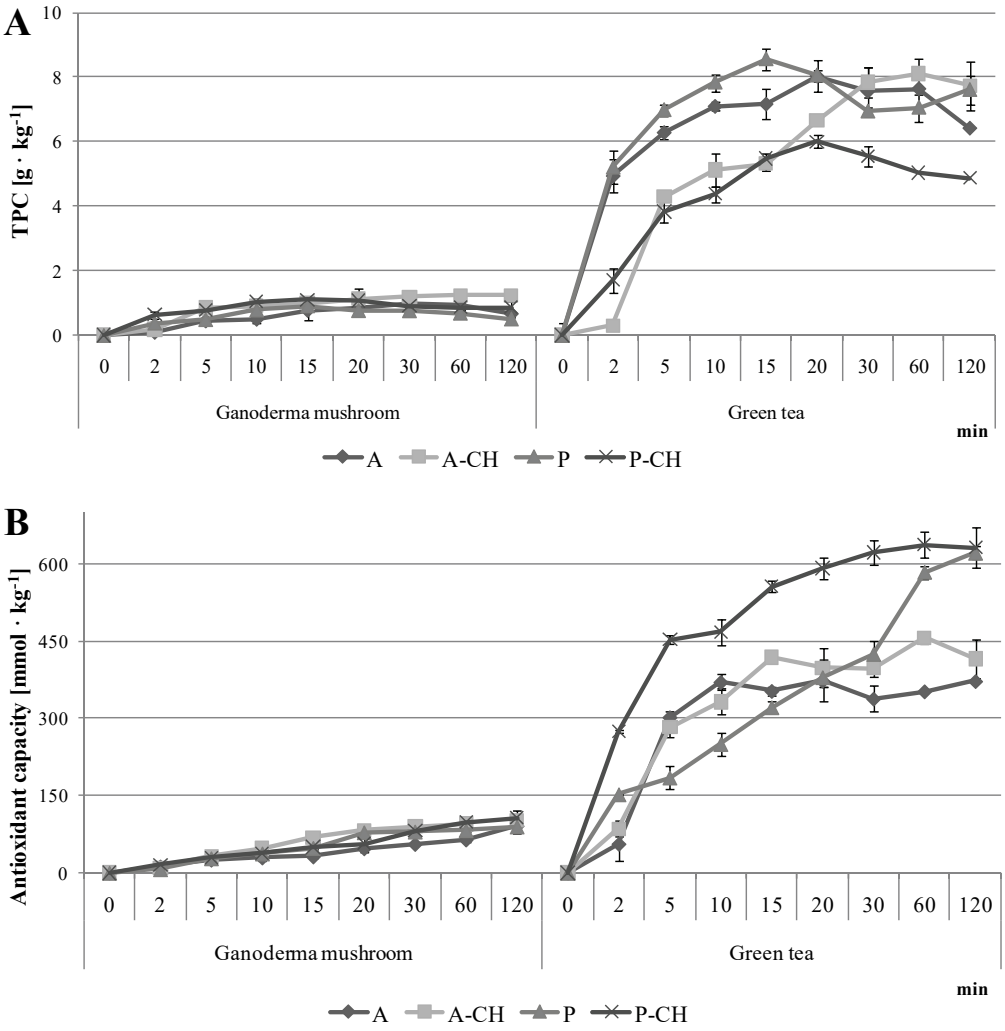

Figure 3. Release profiles of: A) total polyphenolic compounds (mg GAE/g beads) and B) antioxidant capacity (μmol trolox/g beads) from alginate, pectin and corresponding chitosan coated microbeads with encapsulated Ganoderma mushroom and green tea extracts in water. The total polyphenolic content is expressed as g of gallic acid equivalents per kg of beads ± standard deviation. Antioxidant capacity is expressed as mmol of trolox equivalents per kg of beads ± standard deviation. A - alginate microbeads; P - pectin microbeads; A-CH - chitosan-coated alginate microbeads; P-CH - chitosan-coated pectin microbeads.

A correspondence of the sample ranking according to the content of released total polyphenols and antioxidant capacity (Figure 3) and the total polyphenols loading capacity and retained antioxidant capacity was observed (Table 1). Namely, it can be observed that the samples exhibiting the highest loading capacity of total polyphenols and retained antioxidant capacity also released the highest contents of the corresponding compounds. The discrepancy of the sample ranking based on the released TPC and antioxidant capacity (lack of the same trend) may be the consequence of the non-selectivity and method mechanisms involved in the assays employed for the release determinations, since it is well known that the presence of some substances such as the citric acid in chitosan-coated particles may quench the ABTS radical thus providing smaller discrepancies between the sample ranking based on their TP and antioxidant capacity release profiles. The release of polyphenols from the spray dried green tea extract was not evaluated since thus obtained powders dissolve in water and do not display classic diffusion- or swell-mediated release.

Formulation of beers and radlers enriched with green tea and Ganoderma microencapsulates and dried powders

For the purpose of enriching beers with different types of microencapsulated bioactive compounds, only morphologically regular and visually acceptable microparticles enabling the most retarded release of polyphenols were implemented in the production of pilsner or radler beers; alginate and alginate-chitosan coated, as well as chitosan coated pectin microbeads encapsulating *Ganoderma* and green tea extract.

Polyphenolic content of microencapsulates-enriched beers

The addition of hydrogel microbeads encapsulating either *Ganoderma* (Figure 4A) or green tea extract (Figure 4B) to pilsner beer or radler, regardless of the employed carrier material, did not markedly modify the TPC of enriched drinks when compared to control beers, so the addition of dried *Ganoderma* extract and spray dried green tea extract were also evaluated.

Generally, there was no significant ($p > 0.05$) increase in TPC after implementation of *Ganoderma*

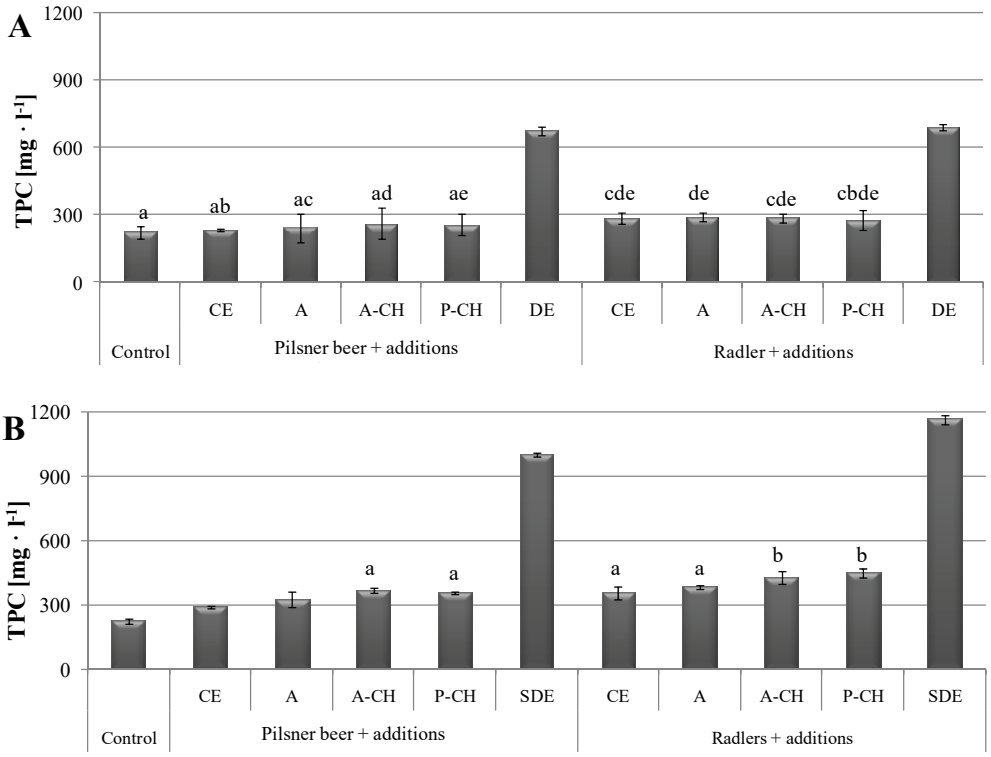

Figure 4. The content of total polyphenolic compounds (mg GAE/L) of beers and radler enriched with hydrogel encapsulates and dried extracts of: A) Ganoderma and B) green tea polyphenols. Control - plain pilsner beer; CE - control pilsner beer or radler + liquid (non--encapsulated) extract; A - alginate microbeads; A-CH - chitosan-coated alginate microbeads; P-CH - chitosan-coated pectin microbeads; DE -dried Ganoderma extract; SDE - spray dried green tea extract. The total polyphenolic concentration is expressed as mg of gallic acid equivalents per L of sample ± standard deviation. The values for each sample superscripted with the same alphabeth letter (a-e) are not significant ($p > 0.05$).

encapsulating hydrogel microbeads to either pilsner or radler beer. Only the addition of dried *Ganoderma* extract enabled to significantly ($p < 0.05$) augment the TPC of enriched beer and radler, even up to 3-fold in comparison to control drinks (Figure 3a). The addition of chitosan coated alginate and pectin hydrogel beads encapsulating green tea bioactives to pilsner beer enabled a marked increase of TPC (Figure 4B) in comparison to both plain control beer (sample C) and the control beer enriched only with liquid green tea extract (sample CE). In case of chitosan coated alginate particles encapsulating green tea bioactives, up to 40% increase in the TPC of formulated pilsner beer was achieved. Likewise, in case of radlers, a significant ($p < 0.05$) increase of 32% of TPC was obtained upon the addition of chitosan coated alginate and pectin particles to radler. Slightly higher TPC that can be observed for radler in comparison to pilsner beer may be attributed to the presence of lemon juice and thus ascorbic acid in radler drinks influencing the overall polyphenolic concentration. The addition of spray dried green tea extract significantly ($p < 0.05$) augmented the TPC of enriched samples, in comparison to plain pilsner beer or radler (~3.2-fold higher TPC than control radler). With regard to different carrier materials, chitosan coated particles enabled to deliver a higher TPC to pilsner and radler drinks, which may indicate that a higher amount of polyphenolics is released from those particles in comparison to alginate ones, when incorporated in beer as a medium. These findings point to the fact that the release of polyphenols may be triggered in beer due to its complex physicochemical properties. The release of catechins as the representative polyphenols of green tea is known to be more pronounced at lower pH [30], which corresponds to the lower pH of chitosan coated particles in beer. Insignificant differences ($p > 0.05$) of TPC between the alginate and pectin microencapsulate-enriched beers were established. The obtained results suggest that dried polyphenolic extracts and substrates are more appropriate for increasing the bioactive concentration of beers, rather than hydrogel beads. However, since this is only valid in case of compositional/bioactive purposes, other product properties, such as the sensory properties, especially visual appearance and the stability of the product, need to be taken into account.

Sensory properties of microencapsulates-enriched beers

Sensory properties of microencapsulate-enriched beers differed markedly depending on the type of extract (*Ganoderma* or green tea extract) and the type of beer. With regard to the type of beer, primarily the combinations of pilsner beer and *Ganoderma* additions, as well as radler and green tea were established as the preferred ones, which can be observed as the much lower sensory scores of all properties for radler with *Ganoderma* additions (Figure 5B) and pilsner beer with green tea (Figure 5C).

In the category of pilsner beers, *Ganoderma* enriched samples were preferred by the panelists over the green tea-enriched beers with respect to odour, taste, mouthfeel and overall acceptability. Although higher bitterness intensity was perceived in *Ganoderma*-enriched beers in comparison to green tea, this did not affect the overall preference of consumers towards the beer. Figure 5A reveals that *Ganoderma* hydrogel microbeads enriched pilsner beer exhibited equal or slightly higher bitterness intensity, which is attributed to the bitter-tasting triterpenoids characteristic for *Ganoderma* [31]. However, in case of *Ganoderma* enriched radler even lower bitterness intensity than the control beer was noted, which confirms the off-taste masking effect of microencapsulation approach. Namely it has been well established that microencapsulation in different polymers can mask the bitter taste of numerous pharmaceutically active compounds, as well as to retard the release of bitter tasting active compound [32]. Also, higher sweetness and sourness in radler drinks, which may have masked and covered the bitterness of *Ganoderma*, can be attributed for the lower bitterness intensity of those beverages.

The application of *Ganoderma* in beer production was already evaluated [4] and confirmed to provide sensory viable and attractive products, while according to our knowledge no scientific studies are available adressing the incorporation of green tea extract in beer and its functional effects. The taste attributes of green tea microencapsulate-enriched radlers did not differ or were slightly higher than the control indicating the favourable effect of enriching radler with green tea encapsulating microbeads (Figure 5D). Adding dried powders of *Ganoderma* or spray dried green tea was preferred only in case of taste attributes of pilsner beer with *Ganoderma* powder. The addition of spray dried green tea powder to both pilsner beer and radler markedly enhanced the aftertaste and bitterness attributes, providing generally lower overall acceptability of enriched drinks.

Regarding the visual appearance of enriched beers, no haze or turbidity development was noticed following the addition of hydrogel microencapsulated *Ganoderma* or green tea extracts to beer. The most common cause of haze formation in beer production

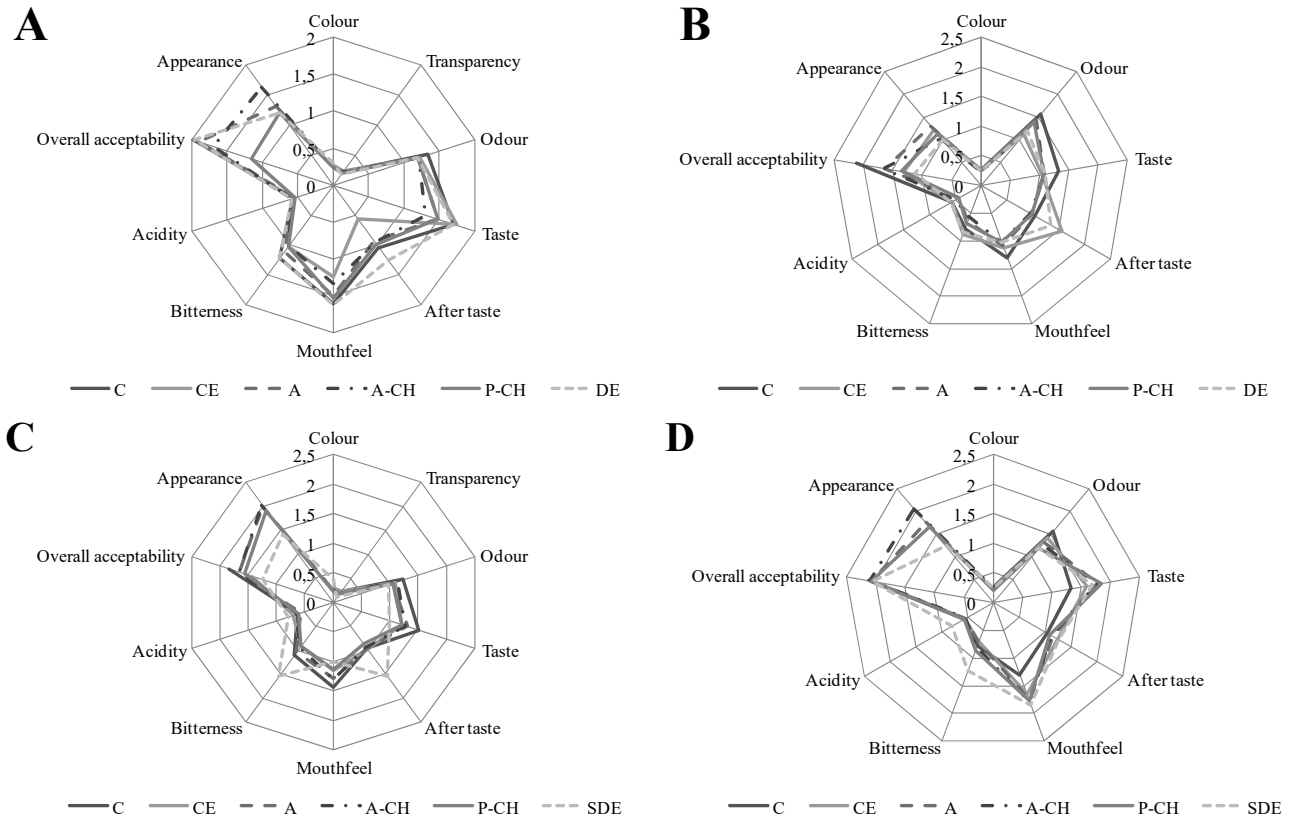

Figure 5. Average scores of sensory quality indicators for: A) pilsner beer with Ganoderma additions; B) radler with Ganoderma additions; C) pilsner beer with green tea additions; D) radler with green tea additions.

are the interactions between proteins and polyphenols, while polysaccharides do not participate in the haze formation mechanism, but are instead simply incorporated as haze particles [33]. Being composed of polysaccharide biopolymers (alginate and pectin), the microencapsulates in this study clearly did not affect the postproduction haze formation in beer, revealing their suitability for this purpose. The addition of hydrogel microbeads enabled to produce visually attractive products, in case of *Ganoderma* encapsulating microbeads due to their more pronounced, darker colour, while in case of green tea encapsulating microbeads due to their larger size (d 0.5 < 1000 μm) which evoked a specific consumer interest, especially in case of morphologically regular particles. The larger size of green tea encapsulating microbeads contributed to the sensory preference of radlers containing green tea microbeads exhibited by the panelists (in comparison to control radler), and formulation of an innovative, consumer-appealing product. With respect to the type of microparticles, chitosan coated alginate particles encapsulating *Ganoderma* dispersed in pilsner beer (Figure 5A) provided visually the most acceptable product, while in case of green tea encapsulating microbeads no significant differences in the visual appearance of microencapsulate enriched beers was observed.

Due to the natural turbidity of radler, the addition of hydrogel microbeads encapsulating green tea extract did not visually modify the appearance of enriched radler. Conversely, the addition of dried *Ganoderma* and spray dried green tea powders caused significant modifications of their visual appearance with a pronounced turbidity development (figure not shown), which may be due to the *Ganoderma* polysaccharides precipitation initiation by the presence of alcohol in the medium [34]. Namely, beers also contain a number of constituents, such as alcohol and the hydrogen ion (pH), which can influence the interactions between the main constituents and protein-polyphenol haze formation [35].

Since visual appearance can be regarded as one of the most important quality parameters of food, directly related to consumer perception and purchase decisions, which was in both pilsner beer and radlers achieved by using hydrogel microbeads, while the combinations of pilsner beer and *Ganoderma* additions, as well as radler plus green tea additions exhibited better taste properties, only those combinations with the highest overall acceptability were selected for additional beer quality control and stability analysis.

Stability of the polyphenolic content and quality parameters during refrigerated storage of beer

Beer quality is known to be affected by the storage conditions, such as temperature and light, which is in the brewing process often solved by the addition of antioxidants (*e.g.*, glutathione [36]), aiming to improve beer stability. The approach of using microencapsulated polyphenolic antioxidants, especially from green tea, may provide a natural alternative to the improvement of beer stability during storage. In the present study, fluctuations of TPC of hydrogel microbeads enriched beers during the storage period were revealed, since during storage period a slightly higher TPC was revealed in both green tea and *Ganoderma* enriched beers (Figure 6A and B), in comparison to initial control sample (upon production), while after one month of storage even a decrease of TPC occurred (Figure 6A).

The fluctuations were however not so obvious for the dried extracts enriched beer and radler (DE *Ganoderma* and spray dried green tea extract), since no significant differences between the TPC of those samples even after one month of storage were determined. The observed fluctuations may be an indicator of polyphenolic compositional changes that may occur during storage. Namely, it is well established that flavonoid polyphenols are very prone to polymerization, or formation of complexes with proteins or carbohydrates. In case of those compositional modifications, overestimation of total polyphenolic concentration may occur as already previously established in numerous studies [37]. Although previous studies on enriched beers [6] evidenced an achieved increase in the polyphenolic concentration in comparison to control (beer enriched with honey), the stability of enriched beers and its bioactive concentration was not evaluated. The results obtained in this study suggest that although no marked increase in the polyphenolic concentration of *Ganoderma* and green tea microbeads enriched beers and radler in comparison to plain, control beers was obtained, the stability of polyphenolic concentration in the enriched beers was achieved, pointing to a beneficial effect of functional ingredients added for production of enriched beers from the preservation aspect.

Quality parameters of finally selected enriched beers and radlers (Table 2) such as alcohol concentration, real extract, apparent extract and energy value revealed that the addition of either plain extracts or microencapsulates of *Ganoderma* and green tea (hydrogel and dried extracts) resulted with no sig-

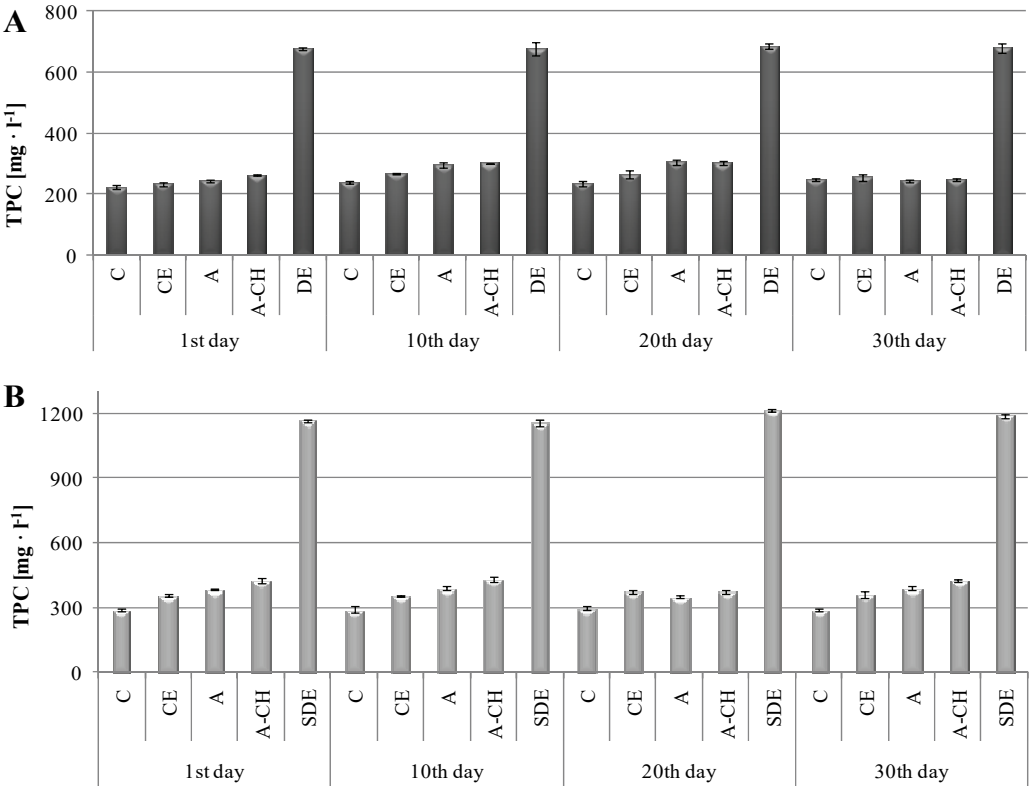

Figure 6. Storage stability of total polyphenolic compounds (mg GAE/L) in A) pilsner beer with Ganoderma mushroom additions and B) radler with green tea additions during one month. The total polyphenolic concentration is expressed as mg of gallic acid equivalents per L of product ± standard deviation

Table 2. The concentration of alcohol, real and apparent extract and energy value of enriched beers

Enriched beer samples	1st day				30th day			
	Alcohol concentration, $mg \cdot ml^{-1}$	Real extract %	Apparent extract, %	Energy, kJ	Alcohol concentration, $mg \cdot ml^{-1}$	Real extract %	Apparent extract, %	Energy, kJ
Pilsner beer enriched with *Ganoderma* additions								
C	4.6	3.9	2.2	165.6	4.6	4.1	2.5	164.3
CE	4.7	3.9	2.2	165.7	4.6	4.0	2.3	166.2
A	4.7	3.8	2.1	164.4	4.5	4.1	2.5	164.8
A-CH	4.6	4.0	2.3	165.6	4.3	4.1	2.5	161.3
DE	4.3	4.2	2.4	164.8	4.3	4.2	2.5	165.2
Radler enriched with green tea additions								
C	2.2	7.6	6.6	172.1	2.2	7.6	6.8	164.3
CE	2.4	7.8	6.9	173.3	2.2	7.7	6.9	169.8
A	1.9	7.8	7.1	164.1	1.9	7.9	7.2	164.5
A-CH	1.8	7.4	6.8	156.2	2.1	7.9	7.2	168.6
SDE	2.5	7.8	6.9	176.2	2.5	7.9	6.9	175.2

nificant differences in these parameters compared to the control drinks.

The values obtained for each of the parameters do not differ notably between the control beer enriched with plain *Ganoderma* extract and beers enriched with *Ganoderma* hydrogel microparticles, which is also applicable to beers enriched with plain green tea extract and beers containing green tea hydrogel microparticles. Although radlers were characterized by higher values of real and apparent extract (due to the higher saccharides concentration) no significant effect of enrichment with different microencapsulated green tea delivery systems was observed, except of a slight increase of real and apparent extract in the beer and radler with dried and spray dried extracts. The four-week refrigerated storage had no significant effect on the quality parameters of enriched beers, which supports the notion that the addition of microparticles with polyphenolic compounds does not affect the basic parameters of beer quality.

CONCLUSIONS

In this study, the potential and suitability of implementing microencapsulated bioactive compounds from *Ganoderma* mushroom and green tea extracts for the formulation of innovative, attractive beers with improved functional properties was revealed. Electrostatic extrusion assisted microencapsulation of polyphenolic compounds in alginate and pectin particles, mediated by ionic gelling, enabled to entrap up to ~70% of polyphenolic compounds from both examined substrates, with up to ~72% of their antioxidant capacity recovered after encapsulation. The incorporation of formulated hydrogel microparticles to beer or radler did not provide a significant enhancement of their TPC, which was instead achieved by adding dried or spray dried extracts. The combinations of pilsner beer enriched with *Ganoderma* and radler with green tea hydrogel microbeads and spray dried extract were preferred according to their sensory profiles, especially lower bitterness, astringency and more intense herbal flavour, respectively.

Acknowledgements

This work was performed within the framework of the research projects 46001, 46010 and 31020, supported by the Ministry of Education, Science and Technological Development, Republic of Serbia.

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NAUČNI RAD

MODIFIKACIJA FUNKCIONALNOG KVALITETA PIVA KORIŠĆENJEM BIOAKTIVNIH JEDINJENJA MIKROENCAPSULIRANOG ZELENOG ČAJA (*Camellia sinensis* L.) I HRASTOVE SJAJNICE (*Ganoderma lucidum* L.)

Sve veći interes za proizvodnju često konzumiranih funkcionalnih prehrambenih proizvoda usredsredilaa je istraživanja primeni bioaktivnih jedinjenja mikroencapsulisane gljive hrastova sjajnica i zelenog čaja u proizvodnji piva. Mikrokapsulacija zelenog čaja i ekstrakta gljive pomoću elektrostatičke ekstruzije daje čestice u rasponu od 490 mm do 1000 mm, sa sadržajem ukupnih polifenola do 75%. U proizvodnji piva su korišćeni osušeni ekstrakti u prahu, kao i mikročestice sa kapsulama ekstrakta gljive i zelenog čaja koje su pokazale najbolje morfološke osobine i odloženo oslobađanje polifenola (pektinske mikrosfere obložene alginatom, alginatom i hitozanom ili hitozanom). Dodavanje mikrosfera sa gljivom u pilsner pivo nije povećalo koncentraciju polifenola (TPC), za razliku od dodavanja mikrosfera sa zelenim čajem na radler pivo, dok je dodavanje suvog ekstrakta gljive ekstrakte zelenog čaja sušenog u spreju povećalo TPC do tri puta. Pilsner pivo obogaćeno suvim ekstraktom gljive i radler pivo obogaćeno ekstraktom zelenog čaja sušenog u spreju imaju poboljšana senzorna svojstva, zbog najnižeg intenziteta gorčine i najizraženije biljne arome dodatih ekstrakata. Kod pilsner piva obogaćenom hidrogelnim mikrosferama gljive zapažene su fluktuacije TPC, dok je radler pivo obogaćeno hidrogelnim mikrosferama zelenog čaja pokazao bolju stabilnost. Razvijena metodologija obezbeđuje proceduru pogodnu za mikroencapsulaciju - obogaćenje pića i prehrambenih proizvoda, čime se postavlja pouzdana osnova za buduću proizvodnju funkcionalne hrane primenom strategija mikroenkapsulacije.

Ključne reči: pivo, hrastova sjajnica. zeleni čaj. mikroenkapsulacija, polifenolni antioksidanti.