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Characterization of zein-based core-shell microcapsules and matrix nanocapsules with encapsulated carvacrol

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Abstract: Composite micro- and nanocapsules made from natural proteins are increasingly used for various bioactive compound delivery applications due to their versatile nature and ability to carry a wide variety of therapeutic drug molecules. Capsules, both, core-shell and matrix types, have gained prominence in diverse sectors, from pharmaceuticals to food technology. Their widespread use is due to their encapsulation efficiency and controlled release properties. This study provides a physical-morphological characterization and a presentation of the preparation of particles derived from the corn protein-zein, with emphasis on core-shell and matrix configurations. Due to its strong antimicrobial effect against many different strains of bacteria and fungi, carvacrol was considered in this work. It was found that Pickering emulsions prepared as 10% O/W emulsion with 15% GA with the ratio Z:O=1:1.5 and the ratio Z:OSA starch=1:1 and 20 % of carvacrol on dry matter were the most suitable for the preparation of dry capsules. Analysis of matrix-type nanocapsules with 10% encapsulated carvacrol showed that the particles were spherical in shape with a smooth surface and could be successfully dried and prepared in this formulation.

INTRODUCTION

Nanoparticle synthesis is a complex process, and hence there is a wide range of techniques available to produce different types of nanoparticles. The encapsulation process forms a protective barrier (a matrix or polymer coating), preventing chemical interactions and shielding it from environmental factors (such as temperature, pH, enzymes, and oxygen). The primary goals of encapsulation are to enhance the stability of bioactive compounds against unfavorable environmental factors, facilitate their incorporation into food matrices, thus imparting functional properties to the products, and ensure their controlled release at targeted sites (Corrêa-Filho et al., 2019; De Jong and Borm, 2008). One of the main challenges is the increased production costs associated with advanced techniques (Misra et al., 2021; Tarone et al., 2020). Therefore, it is very important to examine different types of particles and their preparation methods in order to obtain clear formulations with minimal economic investment. Depending on the physicochemical characteristics of the bioactive compound, it is now possible to choose the best preparation method and the best polymer to achieve an efficient entrapment of the bioactive compound (Pinto Reis et al., 2006). Core-shell particles consist of a core coated with an outer shell layer, which reduces the reactivity of the core and increases the dispersibility of the modified particle. These classes of particles are known as “smart particles” and have a wide range of applications in various fields, such as drug delivery, biosensors, chemical separation, biomaterials, catalysis and similar suitable materials for semiconductors (Tiwari et al., 2021; Yadav et al., 2023; Kumar et al., 2013). In core-shell capsules, there are clearly defined internal phase boundaries that represent the container system (Baghaban-Eslaminejad et al., 2017). Matrix-type capsules are solid colloidal particles in which encapsulated substances are dissolved, entrapped, chemically bound, or uniformly adsorbed within a homogeneous polymer matrix component. In addition to providing mechanical strength and ensuring protection for their encapsulated guest molecules, nanospheres offer a relatively simple way to adjust the degree of porosity, allowing for fine-tuning of the cargo release profile (Muttaquien et al. 2023; Brannon-Peppas, 1995; Gummustas et al. 2017; Soppimath et al., 2001). The carrier plays an important role in the formulation of

colloidal particles, so there is an increasing trend to use natural biodegradable materials that are readily available. Compared to carbohydrates or metals, protein-based particles are considered ideal for medical, nutritional, and cosmetic applications. Proteins offer the possibility of surface modifications and binding of drugs and other biomolecules through covalent, ionic, hydrogen, and other bonding (Reddy, 2021; Weber, 2000; Rahimnejad, 2009). As a natural protein, zein has good mechanical properties that allow it to be mixed with other substances, as well as modified to obtain the desired product. However, biodegradability and biocompatibility are key parameters enabling new uses of zein and zein-based materials in biotechnological fields (Corradini et al., 2014). The tertiary structure of globular-type zein provides the possibility of creating nanoparticles. Side chains of polar amino acids are located on the surface of the zein molecule, interact with polar solvents (water, ethanol) and allow the protein to remain in solution (Guo et al., 2005).

Essential oils as active substances obtained from different parts of plant species are notable for their excellent activity against bacteria, viruses, fungi, parasites and insects. As a monoterpenoid phenol, carvacrol is a component of many essential oils and is usually found in plants together with its isomer, thymol. Carvacrol, either alone or in combination with other compounds, has a strong antimicrobial effect on many different strains of bacteria and fungi that are dangerous to humans, which is why it was taken into consideration in this paper. Carvacrol also exerts strong anti-inflammatory properties by preventing the peroxidation of polyunsaturated fatty acids (Aprotosoaie, 2019; Mączka et al., 2023). The aim of this work is to present the two preparation methods for obtaining core-shell type and matrix type capsules shown side by side, where in both cases zein was used as a carrier and carvacrol as an active component. The emphasis was placed on finding the best formulation with the given ingredients in order to obtain a stable product with a sufficient amount of encapsulated active substance. This work also aims to perform separated physical and morphological characterization of both type particles obtained with natural polymers.

EXPERIMENTAL

Materials

Zein (Zein purified, CAS 9010-66-6) was purchased from Acros Organics (USA), and carvacrol (Carvacrol 5-isopropyl-2-methylphenol, 98% CAS 499-75-2) was purchased from Sigma Aldrich (Germany). Ethanol (96%) was purchased from Reahem (Serbia). Gum Arabic (GA) was purchased from Sigma Aldrich (Germany). OSA starch (OS) was purchased from National Starch & Chemical (UK) and sunflower oil was purchased from Dijamant (Serbia). All chemicals were of analytical grade unless stated otherwise.

Preparation of zein nanoparticles

2% wt. zein stock solutions were prepared in 100 mL sealed flasks by dissolving zein powder in 90% v/v aqueous ethanol by constant stirring using a magnetic stirrer for 1 hour. The solutions were left overnight in a sealed container at room temperature to ensure complete dissolution of zein. The solutions were subsequently filtered through quantitative filter paper to remove any undissolved particles. 20 mL of 2% wt. zein stock solution was added in a continuous stream to 80 mL of water, in a flask with the ground joint, with constant stirring on a magnetic stirrer to prepare zein nanoparticles. The stirring was continued for 30 min after all of the stock solution was added to water to ensure complete precipitation of zein. In this way, 0.4% wt. nanoparticle suspensions were prepared. Rotary evaporation was used to prepare concentrated zein suspensions. 500 mL of 0.4% wt. suspension was evaporated to 200 mL of 1% wt. zein suspension. Two batches of 1% wt. suspensions were combined and evaporated to 100 mL of 4% wt. zein suspension. Furthermore, an 8% wt. zein suspension was obtained by evaporating 200 mL of 4% wt. zein suspension to 100 mL

Preparation of O/W stock emulsions without and with carvacrol

20 g emulsion of sunflower oil in water of 5%, 10%, 20% and 50% wt. (S1-S4 respectively, Table 1) was prepared by dispersing sunflower oil in the continuous phase using Ultra Turrax IKA T25 (IKA, Germany) homogenizer at 15000 rpm for 15 min at 25^{o}C. The continuous phase emulsions were 3%, 5%, 10% and 15% wt. gumarabic (GA) solution in water, based on mass of sunflower oil for each emulsion (S11-S14; S21-S24; S31-S34; S41-S44, Table 1). GA solutions were prepared by dissolving GA powder in water at T=80^{o}C. In this way, sunflower oil droplets in emulsions were stabilized with GA molecules adsorbed at the O/W interface. Some of the GA molecules were not adsorbed, but remained dissolved in the continuous phase of the emulsions. To remove unadsorbed GA molecules, the emulsion was centrifuged for 10 minutes at 4000 rpm, resulting in an upper, creamy layer rich in oil and a lower aqueous phase depleted of oil droplets. The creamy layer was carefully collected, while the lower aqueous phase was discarded. The cream was then diluted with demineralized water to obtain the initial emulsion mass. The centrifugation–dilution cycle was repeated three times to ensure that all GA not adsorbed on the surface of the oil droplets was removed from the continuous phase of the emulsions. In this way, GA stabilized stock emulsions were obtained. Different stock emulsion compositions were prepared to obtain the most stable one for further research as shown in Table 1.

The droplet size distribution of the 10% O/W stock emulsion with 15% gum arabic was determined by analyzing microphotographs of the emulsions obtained with a Biooptica BEL-3000 microscope (BEL-photonics, Germany) using Belview software. After the most stable formulation was determined, the preparation of emulsions with the addition of carvacrol began. Similarly, 10% stock emulsions were prepared with 8.33%, 25%, and 50% of carvacrol in the oil phase (S5, S6, S7, Table 1), where appropriate mixtures of sunflower oil and carvacrol were used as the oil phase. The continuous phase of these emulsions was a 15% wt. solution of gum arabic (GA) in water, based on the weight of sunflower oil.

Table 1: Specification of prepared stock O/W emulsions without and with carvacrol

Sample ID	Sunflower Oil (g)	Carvacrol (g)					
				S11	S12	S13	S14
S1-5% O/W	1.00	0.0	Gum Arabic (g)	0.03	0.05	0.10	0.15
			Water (g)	18.97	18.95	18.90	18.85
			Stability	U	U	U	U
				S21	S22	S23	**S24**
S2-10% O/W	2.00	0.0	Gum Arabic (g)	0.06	0.10	0.20	**0.30**
			Water (g)	17.94	17.90	17.80	**17.70**
			Stability	U	U	U	**S**
				S31	S32	S33	S34
S3-20% O/W	4.00	0.0	Gum Arabic (g)	0.12	0.20	0.40	0.60
			Water (g)	15.88	15.80	15.60	15.40
			Stability	U	U	U	U
				S41	S42	S43	S44
S4-50% O/W	10.00	0.0	Gum Arabic (g)	0.30	0.50	1.00	1.50
			Water (g)	9.70	9.50	9.00	8.50
			Stability	U	U	U	U
			Gum Arabic (g)		Water (g)		
S5-10% O/W	1.84	0.16	0.28		17.72		
S6-10% O/W	1.50	0.50	0.23		17.77		
S7 10% O/W	1.00	1.00	0.15		17.85		

*U- Unstable after 24h; *S-Stable after 24h

Preparation of Pickering emulsions with and without carvacrol

Pickering emulsions were prepared by mixing the selected stock emulsion with a previously prepared suspension of zein nanoparticles. First, a 10% stock emulsion was added to a 0.4% suspension of zein nanoparticle to obtain 30 g of Pickering emulsion with a zein:oil (Z:O) ratio of 1:1, where three different homogenization methods i.e. Ultra Turrax T25 (3000 rpm), magnetic stirring, and propeller mixer (both 600 rpm, for 5 min), were applied in order to determine the optimal homogenization method (PE1, PE2, PE3, Table 2). The 0.4% zein nanoparticle suspension was used only in a preliminary screening step to identify a technically feasible homogenization device (Ultra-Turax, magnetic stirrer, or propeller mixer) under conditions that avoid excessive viscosity and clogging. Once the propeller mixer was selected, all Pickering emulsions discussed in this paper were prepared with a 1.0% zein nanoparticle suspension and targeted Z:O ratios. 10% stock emulsion was added to a 1% suspension of zein nanoparticles to facilitate Pickering emulsions 1:2, 1:1.5, 1:1, 1.5:1, 2:1 and 4:1 (PE4-PE9,, Table 2), with Z:O ratios using propeller stirring at 600 rpm for 5 min. These emulsions were then analyzed by light microscopy. In addition, immediately after the emulsion preparation, the emulsions were transferred into sealed 10 mL graduated glass cylinders and left for 24 hours at room temperature to visually observe their colloidal stability. Furthermore, the flocculation of Pickering emulsions during mild stirring was investigated. Pickering emulsions with Z:O ratios of 1:2, 1:1.5, 1:1, 1.5:1, 2:1 and 4:1 were allowed to stir on a magnetic stirrer for 30 min and their stability towards flocculation was visually observed.

Table 2: Specification of prepared stock O/W emulsions without and with carvacrol

Sample ID	Zein Conc. (%)	Z:O Ratio	Homogenization Method	Carvacrol in Oil Phase	Stability Test	Flocculation Test
Choosing the appropriate method of homogenization						
PE1	0.4	1:1	Ultra Turrax (3000 rpm)	No	Yes	No
PE2	0.4	1:1	Magnetic stirrer (600 rpm)	No	Yes	No
PE3	**0.4**	**1:1**	**Propeller stirrer (600 rpm)**	**No**	**Yes**	**No**
Choosing the most stable Z:O ratio						
PE4	1.0	1:2	Propeller stirrer (600 rpm)	No	Yes	Yes
PE5	**1.0**	**1:1.5**	**Propeller stirrer (600 rpm)**	**No**	**Yes**	**Yes**
PE6	1.0	1:1	Propeller stirrer (600 rpm)	No	Yes	Yes
PE7	1.0	1.5:1	Propeller stirrer (600 rpm)	No	Yes	Yes
PE8	1.0	2:1	Propeller stirrer (600 rpm)	No	Yes	Yes
PE9	1.0	4:1	Propeller stirrer (600 rpm)	No	Yes	Yes

Preparation of Pickering emulsions with OSA starch

OSA starch solutions of 6%, 8%, 12%, and 21% wt. were added to the zein suspension of 8% wt., to obtain a 4% wt. zein suspension, with mass ratios of zein to OSA starch of 1:0.75, 1:1, 1:1.5, and 1:3.5, (PE-OS1, PE-OS2, PE-OS3, PE-OS4, Table 3). Furthermore, 40 g of a 10% O/W stock emulsion was added to 100 g of a suspension mixture of zein and OSA starch to obtain a Z:O mass ratio of 1:1.5, and homogenized using a propeller mixer at 600 rpm, for 5 min. Pickering emulsions containing 3.35%, 10%, and 20% carvacrol were prepared by dispersing 40 g of stock O/W emulsions with 8.33%, 25%, and 50% of carvacrol, in 100 g of a zein and OSA starch suspension mixture, with the most preferred mass ratio of zein and OSA starch being1:1. The emulsion was homogenized using a propeller mixer at 600 rpm, for 5 min.

Drying of Pickering emulsions

Prepared Pickering emulsions with ratio Z:O=1:1.5 and added OSA starch (zein:OSA starch ratio=1:0.75; 1:1; 1:1.5) were spray-dried using Mini Spray Drier (Buchi 190, Switzerland). Two different inlet temperatures were applied, 105ºC and 130ºC, and the outlet temperature was around 60ºC. The sample flow rate varied between 3.2–6.0 mL/min, and the air flow rate was maintained at 0.6 m^3/min. In each batch, 140–240 g of emulsion was dried with or without OSA starch. OSA starch was used to prevent agglomeration and improve drying efficiency. The number mean diameter (d_{10}) and Sauter mean diameter (d_{32}) of dry powder particles, with a ratio of Z:OS=1:1, dried at 105°C and 130°C, were determined.

Table 3: Summary table of data on Pickering emulsions with OSA starch

Sample ID	Zein suspension conc. (wt. %)	OSA starch concentration (wt. %)	Zein:OSA starch ratio	Carvacrol conc. (%)	Z:O ratio	Drying applied	Drying temp. (°C)
PE-OS1	4.00	6.00	1:0.75	-	1:1.5	Yes	105, 130
PE-OS2	**4.00**	**8.00**	**1:1**	**-**	**1:1.5**	**Yes**	**105, 130**
PE-OS3	4.00	12.00	1:1.5	-	1:1.5	Yes	105, 130
PE-OS4	4.00	21.00	1:3.5	-	1:1.5	Yes	105, 130
PE-OS-C1	4.00	8.00	1:1	3.35	1:1.5	Yes	130
PE-OS-C2	4.00	8.00	1:1	10	1:1.5	Yes	130
PE-OS-C3	4.00	8.00	1:1	20	1:1.5	Yes	130

HPLC determination of carvacrol

The amount of encapsulated carvacrol from dried samples of Pickering emulsions with 3.35%, 10%, and 20% carvacrol, on a dry matter basis, with a mass ratio of Z:O 1:1.5 and zein:OSA starch ratio of 1:1, was analyzed by HPLC using a method adapted from Rajić et al. (2021). 10 mg of dry carvacrol nanocapsule was dispersed in 2 ml plastic vials and vortexed (Eppendorf Mix Mate, USA) for 5 minutes. After that, the vials were transferred to a centrifuge (Rotina 380 R, Hettichzentrifugen, Germany), and centrifuged at 13000 rpm for 20 minutes. The supernatant was collected, filtered through 0.45 µm PVDF syringe filters, and transferred to 2 ml vials for HPLC analysis. The sample was analyzed using a HPLC 1260 Series device (Agilent Technologies, Germany) equipped with an Agilent ZORBAX Eclipse Plus C18 column (4.5 µm, 100×3.5 mm, Agilent, USA). Chromatograms were recorded and integrated by the 1260 LC Chromatography Data System. A binary mixture of methanol and water was used as the mobile phase, and the flow rate was 5 µL/min at 25°C. The chromatograms were recorded at 275 nm, and each sample was analyzed in triplicate. Encapsulation efficiency was calculated using HPLC data and oil concentration as input parameters.

Preparation of matrix type nanocapsule

Matrix-type nanocapsule were prepared according to the method given in a previous study of Rajić et al. (2021). Carvacrol was added to the previously prepared 2% zein solutions at a concentration of 10% wt. based on the mass of zein. 20 ml of the stock solution of zein with carvacrol was injected using a syringe into 80 ml of water to obtain a 0.4% wt. suspension, which was further stirred for 30 min on a magnetic stirrer. The resulting suspension was lyophilized and zein nanoparticles with encapsulated carvacrol were obtained. The prepared nanoparticles were stored in sealed containers at 4°C and used for further experiments.

Characterization of matrix-type nanocapsule

The zeta potential and particle size of carvacrol-encapsulated zein nanocapsule were determined in suspension by electrokinetic measurements and dynamic light scattering (DLS) respectively, using a Zetasizer Nano ZS (Malvern Instruments, UK), at pH 3–10. Samples were diluted to 0.1 wt% with demineralized water, and the pH of the samples was adjusted using 0.1 M HCl and 0.1 M NaOH. All samples were equilibrated for 60 s at 25°C inside the instrument before analysis. Size measurement data were collected over 12 consecutive readings for 10s. Each sample was analyzed in triplicate, and the results were collected as the cumulative mean intensity diameter. Zeta potential measurements for all nanoparticle samples were performed in triplicate, and average values were reported.

FESEM analysis of matrix type nanocapsule

A suspension of nanocapsules of 0.4% wt. with 10% wt. encapsulated carvacrol was pipetted onto glass and after drying, imaged with a scanning electron microscope. FESEM of the obtained samples was performed on a Tescan Mira 3XMU (Tescan, Czech Republic) for morphological characterization. Before analysis, the samples were coated with a layer of gold using a Polaron SC502 sputter coater.

RESULTS AND DISCUSSION

Properties of sunflower O/W emulsions

Oil-in-water emulsions in concentrations 5%, 10%, 20% and 50% were prepared by emulsifying sunflower oil in an aqueous solution of gum arabic. Gum arabic as a stabilizer was added to each emulsion in amounts of 3%, 5%, 10% and 15% based on the oil mass. After 1 hour of observation, it was found that only the 10% O/W emulsion (S24, Table 1) with the addition of 15% gum arabic to the mass of oil remained stable. All other emulsions were unstable, and the oil phase separated on the surface within 30 min after the preparation of the emulsions. The selected 10% O/W emulsion with 15% gum arabic also remained stable for 24h after preparation, as shown at Table 1. These results imply that emulsion S24 can be used for further experiments as the most stable.

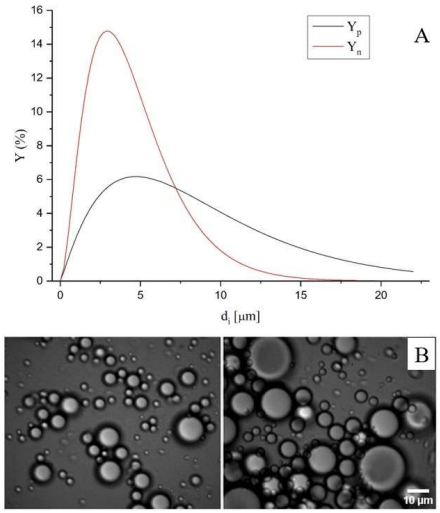

Fig 1: Emulsion with 10% sunflower oil and 15% gumarabic (S24): A- Droplet size distribution by number (Yn) and by surface area of droplets (Yp); B- Microphotographs of emulsion (40x)

Figure 1A shows the droplet size distribution by number (Yn) and by surface area of droplets (Yp) for an emulsion with 10% wt. sunflower oil and 15% wt. gum arabic (S24), without carvacrol. The obtained particle size distributions are monomodal where Yp is quite broad compared to Yn, indicating that a small number of droplets with large diameter have a large contribution to the total surface area of droplets in the O/W emulsion. The average number diameter (d_{10}) of this emulsion was 3.64 μm, and the Sauter ,diameter (d_{32}) was 13.54 μm. Figure 1B shows microphotographs of the previously described emulsion. Microscopic analysis showed spherical oil droplets, with no coalescence observed, which confirmed the good stability of the selected emulsion and its suitability for further analyses.

Preparation of Pickering emulsions

Three different homogenization procedures of zein NP suspension and O/W emulsion were investigated in order to prepare a stable Pickering emulsion, based on the interaction of positively charged zein NP and negatively charged GA molecules on the surface of oil droplets in the stock O/W emulsion. During the procedure involving ultraturax homogenization, the turbine became clogged due to the formation of agglomerates of zein nanoparticles, so this procedure was discarded. Furthermore, the procedure using a magnetic stirrer did not provide sufficient mixing, so the nanoparticle suspension and the stock emulsion eventually separated, leading to the abandonment of this procedure. Finally, stable Pickering emulsions with the following zein to oil ratio (Z:O) 1:2; 1:1.5; 1:1; 1.5:1; 2:1 and 4:1 were formed using a propeller stirrer for 5 minutes at 600 rpm and room temperature. Therefore, this method of homogenization was used in further experiments.

Subsequently, the stability of prepared Pickering emulsions was investigated under the mild stirring conditions for 24 h at room temperature. During the stability testing, in PE4, Z:O=1:2 and PE6, Z:O=1:1 emulsion, aggregates formation and phase separation occurred after 5-10 minutes. Pickering emulsions PE7, PE8 and PE9 with the ratio Z:O=1.5:1; Z:O=2:1 and Z:O=4:1 were stable for 4 hours, after which aggregates formed. Pickering emulsion PE5 with a Z:O=1:1.5 ratio proved to be the most stable during 24 hours of observation and was used for further analyses.

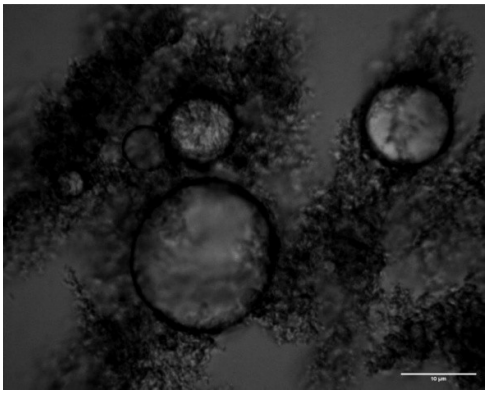

Fig 2: Microphotograph of Pickering emulsion PE5 with Z:O=1:1.5 ratio (40x)

Figure 2 shows a microphotograph of the most stable Pickering emulsion with a zein:oil ratio Z:O=1:1.5 at 40x magnification. The picture shows oil drops from the stock O/W emulsion. The droplets kept their spherical shape, while at the same time they were surrounded by aggregates of zein nanoparticles. Zein nanoparticles were bound to the surface of the oil droplets due to the electrostatic interaction of negatively charged gum arabic molecules adsorbed on the drop's surface and positively charged zein nanoparticles (Spasojević et al., 2020).

Spray-drying of Pickering emulsions

Pickering emulsions with 2.36% wt. and 6.81% wt. dry matter (zein + sunflower oil), without OSA starch, were dried at 105°C and a sample flow rate 6 mL/min. It was observed that under these drying conditions the efficiency of the drying process was very low. Most of the particles were not fully dried and remained stuck to the drying chamber walls. The dry particle yield was very low. This was, most probably, due to the low temperature and high sample flow rate.

Pickering emulsion, with 6.81% wt. dry matter (zein + sunflower oil) was dried at 130ºC and a sample flow rate 3.2 mL/min. The higher inlet temperature and lower flow rate improved the drying efficiency. Dry powder of particles was obtained after drying 240 g of the Pickering emulsion and the yield of drying process was 18.36%. OSA starch was added to Pickering emulsions to improve the efficiency of the drying process. OSA starch was added in four different zein:OSA starch mass ratios (1:0.75, 1:1, 1:1.5, and 1:3.5). Four Pickering emulsions (PE-OS1, PE-OS2, PE-OS3, PE-OS4) were spray–dried as shown in Table 3. The effect of increased temperature on the mean particle diameter is shown in Table 4A. It can be seen that the increased temperature had a slight influence on both d_{10} and d_{32}.

Table 4. A-Influence of drying temperature on the number mean diameter (d_{10}) and Sauter mean diameter (d_{32}) of the particles obtained by Pickering emulsion spray drying; B-Influence of zein:OSA starch ratio on particle size of obtained nanocapsules shown as (number mean diameter (d_{10}) and Sauter mean diameter (d_{32}); C-Influence of carvacrol concentration on the number mean diameter (d_{10}) and Sauter mean diameter (d_{32}) of redispersed powder of spray-dried Pickering emulsions with encapsulated carvacrol

A				
T (°C) drying temperature	**Number mean diameter (d_{10}) (μm)**	**Y err (d_{10})**	**Sauter mean diameter (d_{32}) (μm)**	**Y err (d_{32})**
105	16.05	0.372	22.97	0.940
130	11.84	0.392	27.02	0.155
B				
zein:OSA starch ratio	**Number mean diameter (d_{10}) (μm)**	**Y err (d_{10})**	**Sauter mean diameter (d_{32}) (μm)**	**Y err (d_{32})**
1:0	11.84	0.086	27.02	0.093
1:0.75	10.54	0.586	25.91	0.271
1:1	13.39	0.056	16.61	0.370
1:1.5	9.17	0.100	21.4	0.377
C				
C carvacrol (%)	**Number mean diameter (d_{10}) (μm)**	**Y err (d_{10})**	**Sauter mean diameter(d_{32}) (μm)**	**Y err (d_{32})**
3.35%	6.37	0.293	8.56	0.266
10%	11.69	0.240	16.8	0.472
20%	11.58	0.355	13.83	0.091

Table 4B shows the effect of the mass ratio of zein to OSA starch on d_{10} and d_{32} of the redispersed spray-dried particles. It can be observed that increasing the OSA starch ratio leads to a decrease in both d_{10} and d_{32}. The decrease is much more pronounced for d_{32}, compared to d_{10}, indicating that fewer aggregates are formed when the OSA starch ratio is higher. However, as more OSA starch is added, the total oil concentration in the dried particles decreases, and the optimal (minimal) zein:OSA starch mass ratio needs to be determined. Spray drying of emulsions with 1:0 and 1:0.75 zein:OSA starch mass ratios was difficult, and the spray–drier nozzle clogged several times, so the drying process was stopped. On the contrary, at 1:1.5 zein:OSA starch mass ratio there was no nozzle cloggage and drying was easily performed, but at this ratio the theoretical total oil concentration is only 2.5% wt. based on the dry matter of the emulsion, which was too low for application. The drying efficiency at 1:1 and 1:1.5 zein:OSA starch mass ratios was comparable. Given that the proportion of OSA starch should be as low as possible, 1:1 zein:OSA starch mass ratio was determined to be optimal and help balance the spray drying formulation for encapsulation of carvacrol.

Encapsulation of carvacrol in Pickering emulsions

Three different Pickering emulsions with 3.35%, 10%, and 20% carvacrol were prepared and dried, on a dry matter basis, with a Z:O mass ratio 1:1.5 and zein:OSA starch 1:1 ratio. Table 4C shows the effect of carvacrol concentration on the d_{10} and d_{32} of spray-dried particles encapsulating carvacrol, after redispersion in water. It can be seen that the largest particles were obtained at a carvacrol concentration of 10%. With the addition of 3.35% carvacrol, the d_{10} was 6.37 μm and d_{32} was 8.56 μm. With the addition of 10% carvacrol, the d_{10} was 11.69 μm and d_{32} was 16.8 μm. The particles obtained as the final product with the addition of 20% carvacrol by dry matter mass had a d_{10}, 11.58 μm and a d_{32} 13.83 μm. Figure 3 shows microphotographs of dry powder of particles encapsulating 3.35% (A), 10% (B) and 20% (C) of carvacrol, redispersed in water. Non-spherical, irregular shape of the redispersed particles indicate that the solid shell of the capsules remained intact after particle dispersion in water.

Determination of carvacrol encapsulation efficiency

Table 5 shows the effect of carvacrol concentration on encapsulation efficiency. It can be seen that for all three carvacrol concentrations studied, the encapsulation efficiency was higher than 85%. Particles with encapsulated 3.35% carvacrol by weight of dry matter had an average encapsulation efficiency 94.39%. With 10% encapsulated carvacrol, the efficiency was 88.18%, and with 20% encapsulated carvacrol, the encapsulation efficiency was 90.14%.

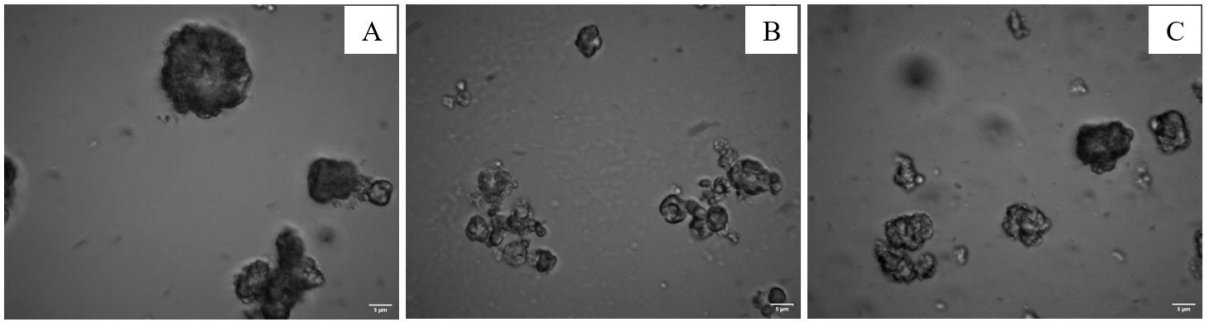

Fig 3: Microphotographs of dry powder of particles encapsulating carvacrol, redispersed in water in following concentrations: (A) 3.35%; (B) 10%; (C) 20%

Table 5. Influence of carvacrol concentration on encapsulation efficiency (EF)

C carvacrol (%)	EF (%)	Yerr EF
3.35	94.396	0.099
10	88.180	0.375
20	90.148	0.762

Development and characterization of matrix-type nanocapsules

The zeta potential of fresh dispersions with 10% carvacrol was determined by electrokinetic measurements at pH 3–9, Figure 4A. Figure 4A shows that the particles were positively charged at pH 3-5 and negatively charged at pH 6–9. The most negative value was -42.83 mV at pH=8, while the highest positive value was measured at pH=5, i.e. 31.27 mV. At pH=4, which is the value of freshly prepared samples, the zeta potential was 29.37 mV. With increasing pH, the zeta potential decreases and an isoelectric point is reached, which is at pH=5.42. The obtained values are in accordance with previously published results (Patel and Velikov, 2014). With a further increase in pH, the nanocapsules become negatively charged.

Figure 4B shows the effect of pH on the mean intensity diameter of particles with 10% encapsulated carvacrol. It can be observed that the zein nanoparticles were smallest at pH 3–4, while the largest diameters were at pH=6, which is consistent with the results of zeta potential measurements. Namely, the largest particles were obtained at pI (isoelectric point), where the nanospheres were effectively uncharged, were the least electrostatically stable and therefore prone to particle aggregation. A decrease in particle size, Figure 4B, was observed as the pH moved away from pI

Figure 5 shows FESEM micrographs of prepared samples with 10% encapsulated carvacrol. FESEM analysis was consistent with the determination of particle size by the method of dynamic light scattering. The largest particles recorded have a diameter of 150-200 nm when individual particles are involved, but range up to 1.5-15µm when aggregates of nanospheres are formed. At a magnification of 10,000x (Figure 5A), agglomerates of the resulting particles are presented, while at a magnification of 100,000 times (Figure 5B), individual particles are shown.

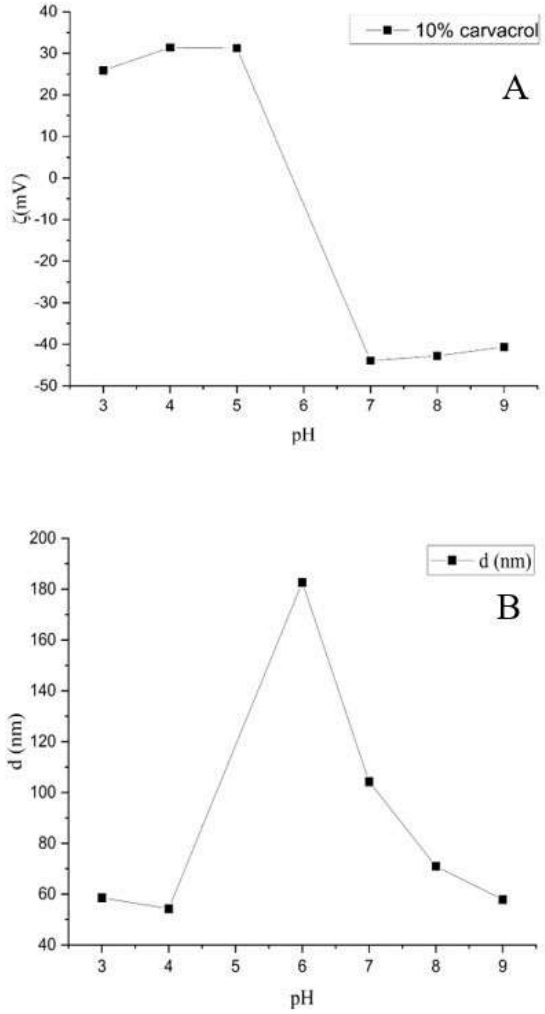

Fig 4: Nanocapsules with 10% carvacrol: A- Effect of pH on the zeta potential; B-Effect of pH on the size of nanocapsules

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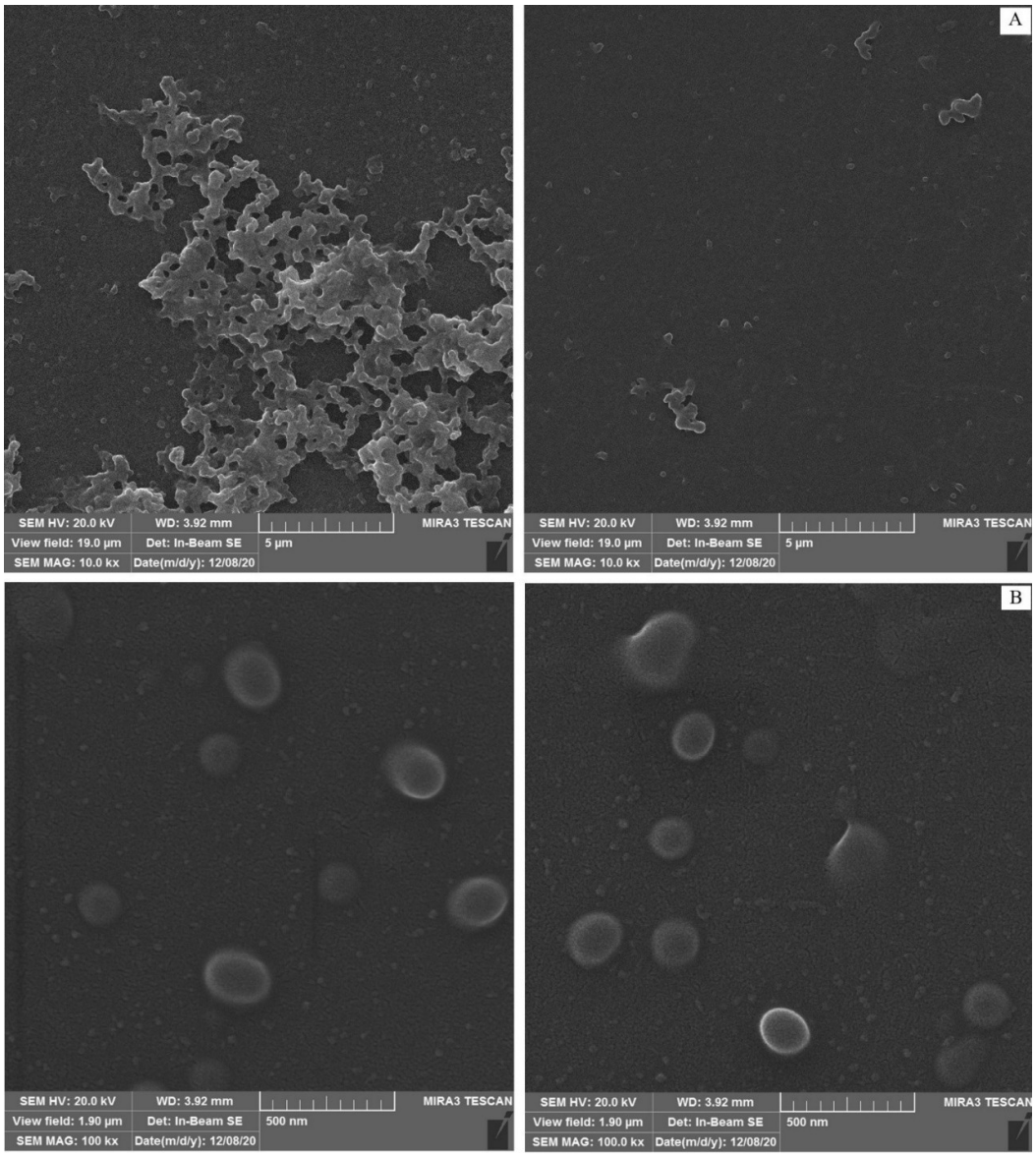

Fig 5: FESEM microphotographs of zein nanoparticle samples with 10% encapsulated carvacrol (magnification 10kx-A, 100kx-B)

CONCLUSION

This work has shown that zein can be successfully used to prepare , core-shell microcapsules, where zein nanoparticles are used to stabilize Pickering O/W emulsions with the active component i.e. carvacrol, and matrix nanocapsules, where zein nanoparticles are used as a matrix carrier for carvacrol. In both cases, carvacrol was used as the encapsulated bioactive compound. The size of the nanocapsules was strongly influenced by the pH of the suspension, where the largest size was obtained at pI. The encapsulation efficiency of the core-shell microcapsules was influenced by the carvacrol content in the formulation, where the encapsulation efficiency of capsules with 3.35% carvacrol by weight of dry matter was 94.39%, with 10% carvacrol was 88.18% and with 20% carvacrol encapsulation efficiency was 90.14%. The ratio of components used to prepare Pickering emulsions significantly affected the spray drying process and the preparation of dried capsules. It was found that Pickering emulsions prepared as 10% O/W emulsion with 15% GA with the ratio Z:O=1:1.5 and the ratio Z:OSA starch=1:1 and 20% carvacrol on dry matter were the most suitable for the preparation of dried capsules. Different Z:O ratios were taken to test a wider range of proportions of both components and to find the one that is the most stable over the longest period of time and is homogeneous enough to be dried in a spray drier, while at the same time having the largest possible amount of emulsion, i.e. oil, to be later determined by HPLC. FESEM analysis of matrix-type nanocapsules with 10% encapsulated carvacrol showed that the particles were spherical in shape with a smooth surface and could be successfully dried and prepared in this formulation. Finally, it was shown that the preparation procedure and formulation significantly affect the properties of core-shell microcapsules and matrix nanocapsules in terms of particle size, encapsulation efficiency, charge and stability.

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Summary/Sažetak

Kompozitne mikro- i nanokapsule napravljene od prirodnih proteina se sve više koriste za razne primjene isporuke bioaktivnih jedinjenja zbog svoje svestrane prirode i sposobnosti da nose širok spektar molekula. Kapsule jezgro-ljuska i matričnog tipa imaju značaj u različitim sektorima, od farmaceutske do prehrambene tehnologije. Široka upotreba je posljedica njihove efikasnosti inkapsulacije i svojstava kontrolisanog oslobađanja. Ovaj rad pruža fizičko-morfološku karakterizaciju i prezentaciju pripreme čestica dobijenih od kukuruznog proteina-zeina kao nosača, sa akcentom na stabilnost dobijenog proizvoda. Karvakrol je uzet kao bioaktivno jedinjenje u ovom radu zbog svog snažnog antimikrobnog dejstva na mnoge različite sojeve bakterija i gljivica. Utvrđeno je da su Pikering emulzije pripremljene kao 10% U/V emulzija sa 15% GA sa odnosom Z:O=1:1,5 i odnosom Z:OSA skroba=1:1 i 20% karvakrola na suvu materiju bile najpogodnije za pripremu suvih kapsula. Analiza nanokapsula matričnog tipa sa 10% enkapsuliranog karvakrola pokazala je da su čestice sfernog oblika sa glatkom površinom i da se mogu uspešno osušiti i pripremiti u toj formulaciji.