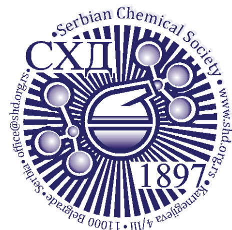

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Properties of double W/O/W emulsions containing Vitamin C and E stabilized with a gelatin/sodium caseinate complex

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Abstract: Double emulsions are complex liquid dispersion systems in which the droplets of one dispersed liquid are further dispersed in another liquid, producing W/O/W or O/W/O emulsions. W/O/W emulsions are the most studied systems because they have great potential application. However, despite all the advantages, that these systems offer, it is very difficult to obtain stable formulations, and this is the reason for their limited practical application. The use of biopolymers to stabilize double emulsions could give rise to pharmaceutical and food applications. Based on previous studies, appropriate concentrations of gelatin and sodium caseinate (NaCAS) were selected to investigate the possibility of stabilization of double W/O/W emulsions by this system, if they are present in the outer aqueous phase. The investigations showed that interactions between gelatin and NaCAS in the outer water phase, as well as the composition of the mixtures of lipohilic emulsifiers used for the primary W/O emulsions preparation, influences the droplets size and sedimentation stability of double emulsions. The most stable emulsions were obtained at a NaCAS concentration when an insoluble coacervate forms (0.5 mass %) and at concentrations higher then this, when soluble negatively charged complexes adsorb at the oil/water interface.

Keywords: double emulsions; biopolymers; proteins; interactions; coacervation.

INTRODUCTION

Double (or multiple) emulsions are complex liquid dispersion systems in which the droplets of one dispersed liquid (water in oil or oil in water) are further dispersed in another liquid (water or oil, respectively), producing W/O/W or O/W/O emulsions. W/O/W emulsions are the most studied systems because they have great potential application, first of all in the food, pharmaceutical and cosmetic industries. In the food industry, W/O/W multiple emulsions can improve

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the solubility of a certain active matter and the production of low calorie and reduced fat products, serve as protective liquid reservoirs for molecules sensitive to external environmental reactivity, such as oxidation, light or enzymes, and serve as entrapment reservoirs for masking undesired flavors and odorants.[1–3] Application in the cosmetic industry include aqueous preparations that provide a "good feel", slow release of active materials, and deposition of water soluble agents onto the skin from wash-off systems.[4,5] Most applications are related to the pharmaceutical industry, such as enhancement of the chemotherapeutic effect of anticancer drugs, drug immobilization and protection of insulin from enzymatic degradation.[6–8] However, despite all the advantages, that these systems offer, it is very difficult to obtain stable formulations, and for this reason, their practical application is limited. Compared with simple emulsions of only two phases and one interface, many more destabilization processes need to be taken into consideration for multiple emulsions. Four possible mechanisms lead to the instability of W/O/W emulsions:[9]

− coalescence of the internal aqueous droplets,

− coalescence of the oil droplets,

− rupture of the oil film resulting in the loss of the internal aqueous droplets, and

− passage of the water and water soluble substances through the oil layer between water phases. This can occur in two various ways: *via* reverse micellar transport by the lipophilic emulsifier and by simple diffusion across the oil phase connected with the osmotic difference between two water phases.

Two different emulsifiers are necessary for the stabilization of W/O/W emulsions: the first with a low hydrophilic–lipophilic balance (HLB) for the W/O interface and the second one with a high HLB for the O/W interface. Most of the current research in this field is about improving the stability of these systems, and most of the proposed techniques can be classified into three groups:

− techniques that include improving stability of the primary W/O emulsion,

− techniques that include changes of the oil phase properties, such as increasing viscosity and

− techniques that include improvement of the stability of the interfaces by using polymeric emulsifiers or natural biopolymers.

During years of investigations to improve the stability and to control sustained and prolonged release of active materials, monomeric surfactants have been progressively replaced by polymeric emulsifiers.[10] Polymeric amphiphilic molecules, synthetically tailor made and naturally occurring ones, are known to possess surface activity and to improve the interfacial coverage during emulsification. The use of biopolymers to stabilize double emulsions could give rise to pharmaceutical and food applications. The use of proteins has long been adopted by scientists exploring the stability of double emulsions. Gelatin,[11] milk pro-

teins,[12] bovine serum albumin,[13] caseins[14,15] and other proteins have been mentioned and evaluated. However, a single protein is far from an ideal emulsion stabilizer as the prepared emulsions are sensitive to temperature, salt and pH.[16,17] The method proposed to compensate the deficiency of single proteins is to form a multilayer on the surface of oil droplets through electrostatic interactions between biopolymers. There are many published scientific papers in which complexes, formed as results of interactions between proteins and polysaccharides were applied for emulsion stabilization.[18–20] However, to our best knowledge, there are only a few studies investigating the possibility of emulsion stabilization by mixtures of two proteins, which are simultaneously systems of two polyelectrolytes.[21,22]

Due to its unique hydrophilic character, gelatin is really the only protein that can be properly categorized as a hydrocolloid.[23] It does have some emulsifying ability, but its more characteristic roles in formulations are as a stabilizer, film former and gelling agent. According to this, gelatin has a wide range of applications, especially in the food and pharmaceutical industries.

Sodium caseinate (NaCAS) has been widely used as an ingredient in food systems due to its functional and nutritional properties. It is prepared from coagulated casein micelles and possesses considerable negative charge at around neutral pH.[24] NaCAS adsorbs readily at the oil/water interface, thus stabilizing emulsion droplets through a combination of both electrostatic and steric mechanisms[25] However, NaCAS stabilized emulsions are unstable at pH values close to its isoelectric point (IEP, ≈4.6) due to a reduction in electrostatic repulsion between the oil droplets.[26]

In some previous study, the mechanism of interactions between gelatin and sodium caseinate was investigated in detail and explained whereby it was concluded that at certain proteins mass ratio, complex coacervation occurs.[27] The main objective of the present paper was to investigate the possibility of the application of mixtures of gelatin and sodium caseinate for the stabilization of double W/O/W emulsions. Based on previous results, the appropriate concentrations of both proteins in the outer aqueous phase of emulsions were selected.

EXPERIMENTAL

Materials

The experiments were realized using two proteins, acid-processed gelatin, type A (300 Bloom), from bovine skin, with an IEP at pH 7.43, and casein sodium salt (NaCAS), from bovine milk, with an IEP at pH 4.6,[26] both product of Sigma, USA. For primary stabilization of 20 % W/O emulsions, the oil soluble surfactants polyglycerol polyricinoleate (PGPR) donated by Jaffa Crvenka, Serbia, and decaglycerol decaoleate (Caprol 10G10O) from Abitec, USA, were used. As the oil phase, medium chain triglycerides (MCT) of caprylic/capric fatty acids (Saboderm TCC) produced by Sabo Spa, Italy, were used. Vitamin C, ascorbic acid, and vitamin E, tocopherol acetate, both obtained from Alfa Aesar, Germany, were used as model substances for encapsulation. Distilled water was used as the aqueous phase.

Preparation of solutions

The stock solutions of lipophilic emulsifiers were prepared by dissolution of an appropriate amount of emulsifier in the oil phase. Binary mixtures of emulsifiers PGPR and Caprol 10G10O, at different mass ratios (1:0, 1:1 and 1:2), were prepared by mixing stock solutions and addition of desired amount of oil phase to obtain a concentration of the emulsifiers of 3 vol. %.

Stock solution of 1.7142 mass % gelatin was prepared by adding 1/5 of the total amount of water to a given mass of protein and then left for 15 min at the room temperature to swell. Then, the rest amount of water was added under mild stirring at 40 °C. Stock solution of 10 % NaCAS was prepared by dispersing a given mass of protein in water, left for 1 h at room temperature to allow hydration, then gently stirred and heated (up to 40 °C) until complete dissolution. Solutions of lower concentrations were obtained by dilution of the stock solution.

Preparation of double W/O/W emulsions

Double emulsions were prepared by a two-step procedure using a homogenizer, Ultra Turrax T25, Ika, Germany, and a magnetic stirrer, C-MAG HS7, Ika, Germany. In the first step, primary W/O emulsions were prepared at a water–oil mass ratio of 20:80. The aqueous phase was a 25 vol. % solution of vitamin C in deionized water, while the continuous phases were a 1.6 vol. % solution of vitamin E in 3 vol. % solutions of binary mixtures of lipophilic emulsifiers PGPR and Caprol 10G10O, at the announced mass ratios, in MCT. The emulsions were prepared by dispersing the water phase in the continuous phase at 40 °C by means of homogenizer, Ultra Turrax T25, at 20000 rpm during 10 min.

The second step involved the dispersion of the primary emulsion (30 g) into 1.7142 mass % gelatin solution (70 g), using the same homogenizer, at 5000 rpm during 10 min, at 30 °C. In this way, 30 % W/O/W emulsions were obtained. The final 20 % W/O/W emulsions were obtained by the slow addition of NaCAS solutions of appropriate concentrations, to the 30 % W/O/W emulsions, during 20 min under mild stirring on a magnetic stirrer at 40 °C. Thus, the final double emulsions represent 20 % emulsions of primary W/O emulsions in mixtures of 1 mass % gelatin and various NaCAS concentrations (0.01, 0.1, 0.5, 1 and 3 %).

Droplet size analysis

The droplet size of the double emulsions were determined by microphotography analysis using Qwin software. Microphotographs were taken on an optical microscope Bel 3000 Bioptica, Italy. Droplet mean diameter, expressed as volume–surface mean value d_{vs}/μm, was calculated from the experimental data, Eq. (1):

$$d_{\mathrm{vs}}/\mu\mathrm{m} = \frac{\sum n_{\mathrm{i}} d_{\mathrm{i}}^{3}}{\sum n_{\mathrm{i}} d_{\mathrm{i}}^{2}} \qquad (1)$$

where d_{i} is the droplet diameter and n_{i} is the number of droplets.

Emulsions stability test

For stability test, the double emulsions were transferred into 10 mL graduated cylinders and stored at room temperature for 14 days. The emulsions were observed for changes in homogeneity and phase separation during storage. Continuous phase separation was visually monitored at certain time intervals. The total height of the emulsion, H_{E}, and the height of the separated continuous phase, H_{C}, were measured. The extent of the phase separation was characterized by the creaming index, H (Eq. (2)). A higher value of the creaming index indicates a worse emulsion stability:

$$H\,/\,\% = 100\frac{H_{\rm C}}{H_{\rm E}} \qquad (2)$$

RESULTS AND DISCUSSION

The investigation of the interaction in mixtures of two oppositely charged proteins, gelatin and sodium caseinate,[27] showed the presence of complex coacervation resulting in soluble (charged) and insoluble (uncharged) complex formation. The formation of an insoluble, neutral complex coacervate always appeared at a gelatin:NaCAS mass ratio of 2:1. Since these interactions had an influence on the system properties, in the present work, the possibility of the stabilization of double emulsions by these mixtures was investigated. The gelatin concentration was 1 mass % while the NaCAS concentrations were chosen to cover the region before insoluble coacervate formation (0.01 and 0.1 %), concentration when insoluble coacervate forms (0.5 %), and region after insoluble coacervate formation (1 and 3 %).

In addition, based on some previous studies, mixtures of lipophilic emulsifiers PGPR and Caprol 10G10O (at the announced mass ratios) were selected for primary emulsions stabilization.[28]

Microscopic observation of the emulsion samples showed the presence of inner water droplets inside oil droplets, *i.e.*, the existence of double W/O/W emulsions. The microphotograph of the emulsion stabilized with mixtures of 1 mass % gelatin and 0.5 % NaCAS showed the presence of a coacervate layer around the oil droplets (Fig. 1). For better contrast, a solution of methylene blue was added before microscopy.

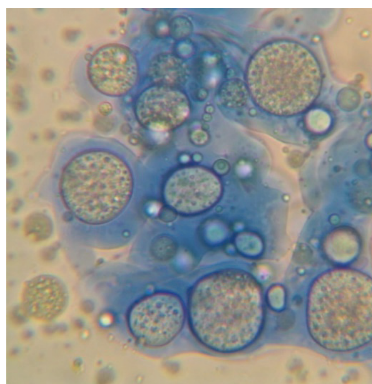

Fig. 1. Microphotograph of a 20 % W/O/W emulsion stabilized with a mixture of gelatin:NaCAS at a mass ratio 2:1, at a magnitude 40×.

Influence of the gelatin/NaCAS interaction on the mean diameter of the droplets in 20 % W/O/W emulsions

Since the formed complexes showed different behavior in the bulk, as well as at the interface,[27] it was expected that changes in emulsions properties would

occur during storage. Changes in the mean diameters of the droplets of 20 % W/O/W emulsions stabilized with 1 mass % of gelatin and various NaCAS concentrations during storage at room temperature are shown in Fig. 2.

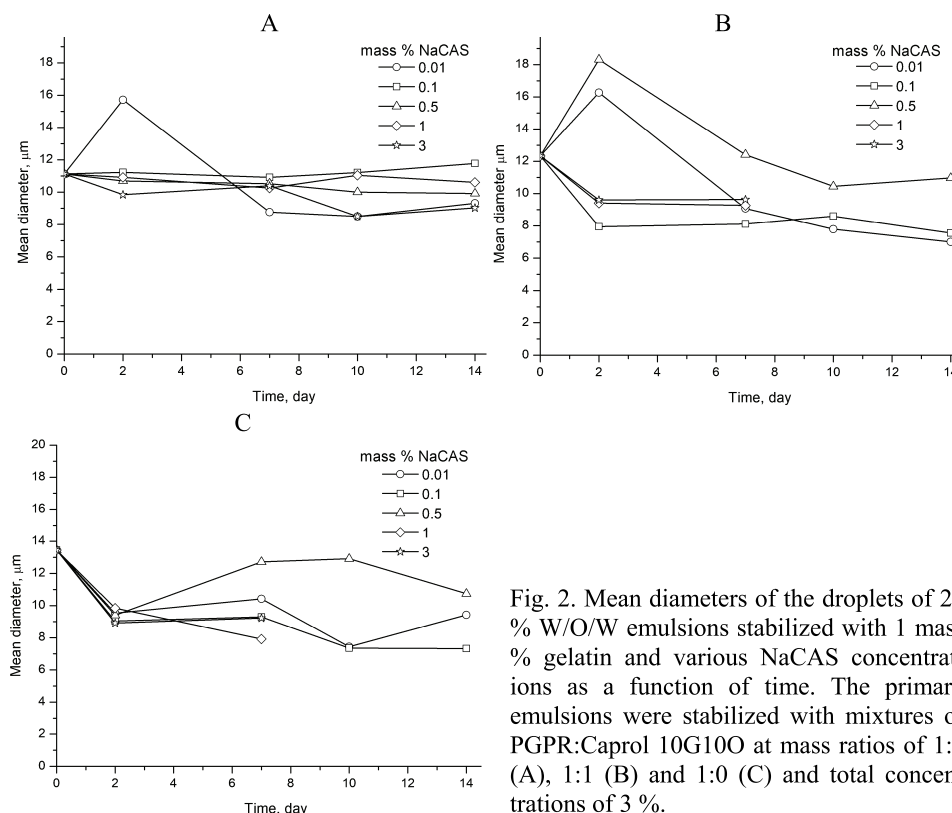

Fig. 2. Mean diameters of the droplets of 20 % W/O/W emulsions stabilized with 1 mass % gelatin and various NaCAS concentrations as a function of time. The primary emulsions were stabilized with mixtures of PGPR:Caprol 10G10O at mass ratios of 1:2 (A), 1:1 (B) and 1:0 (C) and total concentrations of 3 %.

It could be noticed that in freshly prepared emulsions, mean diameters of the droplets were in interval from 11.12 to 13.45 μm. The mass ratio of Caprol 10G10O in the mixture with PGPR, which has an influence on the droplet diameter of primary emulsions,[28] also affects the droplet diameter of the double emulsions. Namely, on dispersing the primary emulsion in the gelatin solution, its molecules adsorb at the interface forming a film around the oil droplets. At the same time, excess PGPR molecules present in the oil phase, also adsorb at the outer oil/water interface, additionally stabilizing the formed gelatin film. The increase in Caprol 10G10O mass ratio in the mixture reduces the concentration of PGPR molecules, thus inducing a slight increase in the mean diameter of the droplets.

During storage, the influence of gelatin/NaCAS interactions, as well as the concentration of PGPR molecules in the oil phase, on the properties of the emulsions was clearly noticeable. Namely, in single emulsions, the change in droplet

size is mostly associated with a coalescence process. On the other hand, in double emulsions, a number of different factors can affect such changes. Changes in the mean diameters of the droplet of double emulsions, in which primary emulsions were stabilized with PGPR:Caprol 10G10O mixtures at mass ratio 1:2, are presented in Fig. 2A. The decrease in droplets mean diameter was noticeable in all emulsion samples after two days of storage. One of the instability factors in such systems might be water diffusion between the two sides of oil layer due to the osmotic gradient, since the inner aqueous phase was hypertonic vitamin C solution. This induces flow of water in the direction of decreasing osmotic gradient, *i.e.*, from the outer to inner water phase, and swelling of the internal water drops. When the critical limit is reached, the oil layer breaks down and the inner water phase migrates into the outer water phase resulting in double emulsion breakdown and the size of the oil droplets decreases.[29,30] These changes were confirmed by microscopic observation (not shown) where the existence of double emulsions had not been observed. Low stability of this emulsions series is a consequence of bad properties of the adsorption layer on the oil drops, where protein complexes as well as lipophilic emulsifiers adsorb.[18] In the emulsion stabilized with the gelatin:NaCAS complex at a mass ratio of 2:1, a slight increase in the diameter of the droplets after seven days was noticed. This could be a consequence of the coalescence of the droplets since they were stuck by the coacervate layer.

Changes in the mean diameter of the droplets in a series of double emulsions with a primary emulsion containing PGPR: Caprol 10G10O at a 1:1 mass ratio are shown in Fig. 2B. These results show an increase in the mean diamater of the droplets in emulsions with NaCAS concentrations of 0.01 and 0.5 mass %, while in the others, the mean diameter of the droplets decreases. The addition of the lower amount of NaCAS (0.01 mass%) does not significantly destroy the gelatin layer around oil drops, thereby slowing the diffusion of water from the outer phase and swelling of the inner water drops, *i.e.*, the breakdown of the double emulsion is slower. Microscopy of this emulsion still showed the existence of a double emulsion after two days of storage. Compared to the previous series of emulsions, here the improved emulsifying properties of the mixture with a higher mass ratio of PGPR are expressed. On the other hand, the added amount of NaCAS is not sufficient to cover the entire surface of the oil drops and to form compact coacervate layer and hence, one protein molecule is adsorbed on two or more oil drops. For this reason, the emulsion droplets are interconnected through so-called macromolecular bridges, *i.e.*, bridging flocculation occur. As a consequence of flocculation, there is coalescence leading to an increase in the mean diameter of the droplets.[31] The bridging flocculation appears in systems in which the concentration of the added macromolecule is so low that the process of droplets collision is faster than adsorption of the macromolecules on to the droplets.[32] In the emulsion with a NaCAS concentration of 0.5 %, the presence of inner

water droplets after two days of storage is also noticeable, which could be attributed to a compact coacervate film on the oil droplets. This sticky coacervate increases the coalescence process inducing an increase in the mean diameter of the droplets. In all other emulsions, there was a decrease in the mean diameter oh the droplets and a breakdown of the double emulsions.

The results of the droplets mean diameter measurement in the double emulsions in which the primary W/O emulsions were stabilized with pure PGPR (Fig. 2C) show that only in the sample with an NaCN concentration of 0.01 % was an increase in the mean diameter of droplets evidenced as a consequence of bridging flocculation. In the other emulsions, there were no significant changes in this parameter, which indicates stability of the adsorbed layer and the synergistic effect of PGPR molecules and the gelatin/NaCN complexes formed at the interface. Namely, at an NaCN concentration of 0.1 %, its molecules bind to the gelatin molecules adsorbed on the oil droplets thereby forming a compact layer and a retardation of the migration of the water between the two water phases. At a NaCN concentration of 0.5 %, emulsion stability is a result of a compact coacervate layer around the oil droplets. Further increase in NaCN concentration induces dissolution of the gelatin/NaCN complexes, but these complexes are surface active[28] *i.e.*, have a tendency to adsorb at the oil/water interface, thereby stabilizing the dispersed system. In addition, in this area of NaCN concentrations, a significant increase in the system viscosity was noticed, which retards droplets movement, and therefore, their collision and coalescence.[33,34] In all emulsion samples, the existence of double emulsions after two days of storage at the room temperature was confirmed. After this period, in the emulsion with 0.01 % of NaCN, a decrease in the mean diameter of the droplets occurs, as a consequence of worse properties of the adsorption layer. In the other emulsion samples, there were no significant changes in the mean diameter of the droplets, which indicates that the synergistic action of the lipophilic emulsifier PGPR and gelatin/NaCN complex can produce double W/O/W emulsions stable for 7 days.

Influence of the gelatin/NaCN interaction on 20 % W/O/W emulsions sedimentation stability

Along with the dispersed analysis of the 20 % W/O/W emulsions, their sedimentation stability was observed. The emulsions were transferred into graduated cylinders immediately after preparation and their phase separation was visually monitored at the room temperature during 14 days. Changes of the creaming index (H) with time and cylinders filled with emulsions after 14 days of storage are shown in Fig. 3.

It can be noticed that in all emulsions phase separation occurs in the first 24 h after preparation and subsequently, there were no significant changes in the creaming index. The separation of the serum layer appears due to the difference in density between the continouos and dispersed phases. At NaCAS concentrations

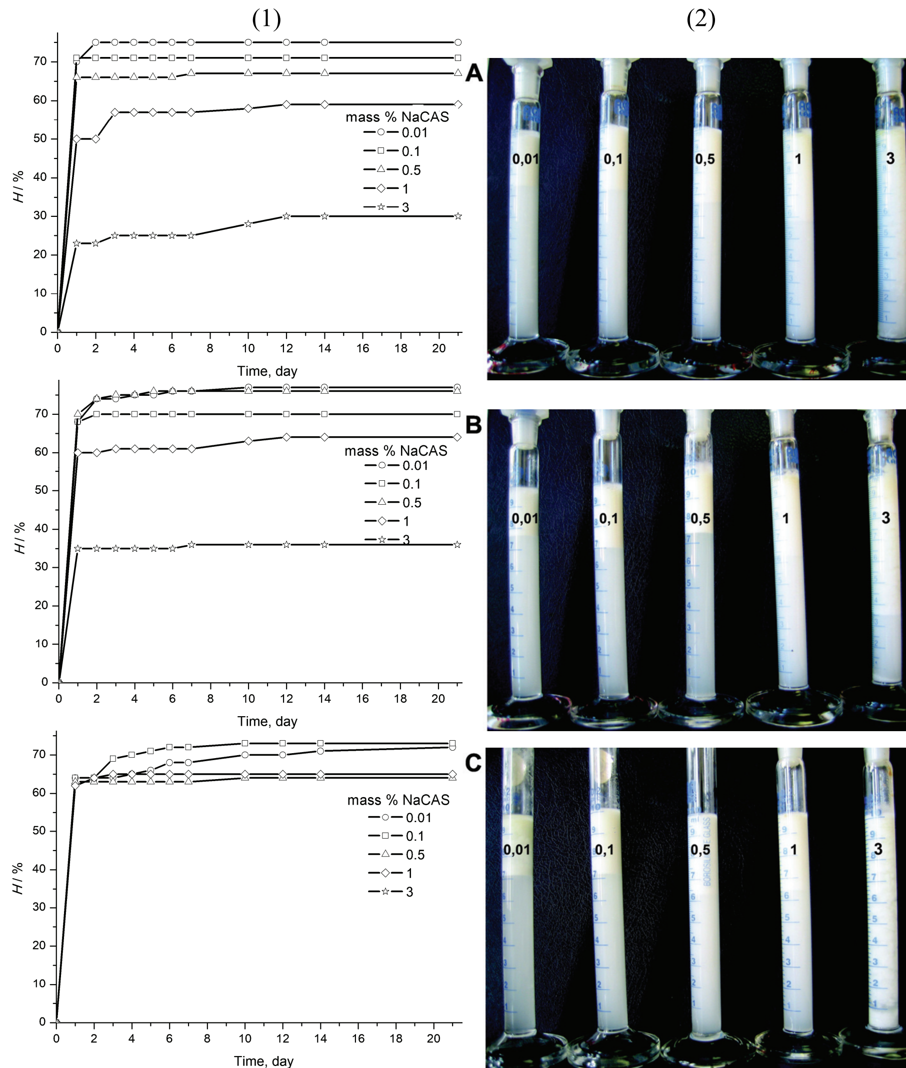

Fig. 3. Creaming index of 20 % W/O/W emulsions stabilized with mixtures of 1 mass % gelatin and various NaCAS concentrations as a function of time (1); cylinders filled with the emulsions after 14 days of storage at room temperature (2). The primary emulsions were stabilized with mixtures of PGPR:Caprol 10G10O at mass ratios of 1:2 (A), 1:1 (B) and 1:0 (C) at a total concentrations of 3 %.

of 0.01 and 0.1 %, a sharp boundary between the layers was clearly visible, which is mainly due to bridging flocculation, *i.e.*, flow of the flocculated droplets of the emulsion to the top of cylinder, while smaller and unflocculated ones remain in the serum layer.[31,35] In emulsions stabilized with insoluble gelatin/ /NaCAS coacervate (at NaCAS concentration of 0.5 %), there is also sharp

boundary between phases. Since the formed coacervate is electro neutral,[27] electrostatic repulsion between droplets is reduced, which enables their closer packing resulting in the separation of the floccules.

On further increase in NaCAS concentration, the creaming index is lower and the boundary between phases less sharp, which is a consequence of an increase in the viscosity of the continuous phase.[27] Namely, it is known that an increase in the viscosity of the continuous phase improves the sedimentation stability of emulsions due to retarded movement of the droplets.[33] In addition, the increase in the total negative charge on the surface of the droplets with increasing NaCAS concentration, also has a positive influence on emulsions stability by increasing the electrostatic repulsion between the droplets. It is also important to stress that in no emulsion was separation of the oil phase noticed.

CONCLUSIONS

Investigations of the properties of double 20 % W/O/W emulsions containing vitamin C and E showed that interactions between gelatin and NaCAS in the outer water phase, as well as the composition of the mixtures of lipohilic emulsifiers used for the preparation of the primary W/O emulsions, influences the droplet size and sedimentation stability of the emulsions. Increasing amount of PGPR in the mixtures with Caprol 10G10O had a positive effect on the stability of the emulsions with time. An investigation of the influence of gelatin and NaCAS interactions on properties of the emulsions showed that stable emulsions were obtained at the NaCAS concentration when an insoluble coacervate forms (0.5 %) and at concentrations higher then this, when soluble negatively charged complexes adsorb at the oil/water interface.

Further research will be focused on examining the possibilities of cross-linking the adsorbed proteins layer in order to obtain microcapsules in powder form suitable for the simultaneous encapsulation of hydrosoluble and liposoluble active substances.

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ИЗВОД

ОСОБИНЕ ДВОСТРУКИХ W/O/W ЕМУЛЗИЈА СА ВИТАМИНИМА ЦЕ И Е СТАБИЛИЗОВАНИХ ЖЕЛАТИН/НАТРИЈУМ-КАЗЕИНАТ КОМПЛЕКСИМА

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Двоструке емулзије су комплексни течни дисперзни системи, код којих је један дисперзни систем даље диспергован у другој течној фази, формирајући W/O/W или O/W/O емулзије. W/O/W емулзије су најчешће испитивани системи због њихове потенцијално веома широке примене. Ипак, и поред низа предности које ови системи имају, њихова примена је још увек доста ограничена, с обзиром на то да је тешко добити стабилан систем. Примена

биополимера за стабилизацију двоструких емулзија омогућава њихову ширу примену у прехрамбеној и фармацеутској индустрији. На основу наших претходних истраживања, изабрали смо одговарајуће концентрације желатина и натријум-казеината да испитамо могућност стабилизације двоструких W/O/W емулзија овим системом биополимера, када су они присутни у спољашњој воденој фази. Резултати ових истраживања су показали да интеракција између желатина и натријум-казеината у спољашњој воденој фази, као и састав смеше липофилних емулгатора коришћених за припрему примарних W/O емулзија, утичу на величину честица и седиментациону стабилност двоструких емулзија. Најстабилније емулзије су добијене при концентрацији натријум-казеината када долази до формирања нерастворног коацервата (0,5 %) и вишим, када долази до адсорпције негативно наелектрисаних комплекса на граници фаза.

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