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The Potential of Combined Emulsification and Spray Drying Techniques for Encapsulation of Polyphenols from Rosemary (*Rosmarinus officinalis* L.) Leaves

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SUMMARY

The present study evaluates the potential of encapsulation of polyphenolic antioxidants from rosemary (*Rosmarinus officinalis* L.) leaves by combining emulsification and spray drying techniques. To stabilize the emulsions and prepare samples suitable for use in dry products, double emulsions encapsulating rosemary polyphenolic extract and containing polyglycerol polyricinoleate (4 %), whey protein isolates (2 and 4 %) as emulsifiers, and maltodextrins (MDE 10 and 21) as enhancing coatings were subjected to spray drying. The obtained results show insignificant ($p>0.05$) effect of used maltodextrin type and protein content on mean particle size of double emulsions containing rosemary polyphenols. Morphology analyses showed that double emulsions were successfully prepared, spherical microcapsules were obtained after spray drying of double emulsions and double emulsion form was still preserved after rehydration of spray-dried microcapsules. Regardless of used maltodextrins, significantly ($p<0.05$) higher encapsulation efficiencies (EE) of total polyphenols (39.57 and 42.83 %) in rehydrated samples were achieved when higher protein content (4 % whey protein isolate) was used, indicating the major impact of protein content on EE of rosemary polyphenols. Also, using HPLC analysis, rosmarinic and caffeic acids, apigenin and luteolin derivatives were detected among specific polyphenols, where rosmarinic acid had notable encapsulation efficiency ranging from 62.15 to 67.43 %. In this way, the obtained microcapsules encapsulating rosemary polyphenols could be easily blended with various dry mixtures, and serve for delivery in different functional products.

Key words: double emulsions, encapsulation efficiency, maltodextrin, microcapsules, rosemary polyphenols, spray drying, whey protein isolates

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is a medicinal herb and culinary spice, which is widely used in European folk medicine to treat numerous diseases (*1*). Its extracts are used mainly to obtain essential oils and to prepare extracts that are increasingly utilized to provide natural alternatives to synthetic antioxidants and artificial preservatives for foodstuffs (*2*), or as components of cosmetics (*3*). Antioxidant activity of rosemary extracts derives mainly from polyphenolic compounds belonging to three groups: phenolic diterpenes of an abietic acid-related structure, flavonoids and phenolic acids (*4*).

Polyphenolic compounds possess a high spectrum of biological activities, and their concentration that appears effective *in vitro* is often much higher than the levels measured *in vivo*. Additionally, their direct use in food matrix is limited due to insufficient gastric residence time, low permeability and/or solubility within the gut and due to their instability under conditions encountered in food processing and storage, or in the gastrointestinal tract. Thus, only a small content of such molecules remains available following oral administration (*5*). Therefore, there is a big challenge for product formulators and manufacturers to find out and provide protective delivery systems that can maintain the nutraceutical molecular form until the time of consumption, deliver it to the physiological target within the organism, and control the rate of bioactive release (*6*). These challenges could be solved mainly by means of encapsulation, where several techniques for the encapsulation

of polyphenols, such as spray drying, spray cooling/chilling, extrusion, fluidized bed coating, coacervation, liposome entrapment, inclusion complexation, centrifugal suspension separation, lyophilization, cocrystallization, emulsions, processes using the supercritical fluids, *in situ* polymerization, interfacial polycondensation and interfacial cross-linking, *etc.*, have been reported (*7,8*).

Within food and beverage industries there is a growing interest in the production and utilization of colloidal delivery systems like emulsions (*9*). Emulsions are very often used as vehicles to encapsulate, deliver, and release lipophilic (ω-3 fatty acids, conjugated linoleic acids, plant sterols, carotenoids) or hydrophilic (minerals, vitamins, enzymes, proteins, bioactive peptides, fibre) compounds in the field of food, medical, and pharmaceutical industries (*10*). It is also possible to produce more complex emulsion structures, such as double emulsions, where water-in-oil-in-water ($W_1/O/W_2$) emulsions take special place of interest. Double $W_1/O/W_2$ emulsions are characterized as complex multiphase liquid systems that consist of water-in-oil (W_1/O) emulsion dispersed in a second continuous water phase (W_2) (*11*). $W_1/O/W_2$ emulsions are suitable for entrapping a hydrophilic bioactive compound in the inner water phase (W_1), which is isolated from the outer water phase (W_2) by the oil phase. In this way, diffusion of immobilized substance from W_1/O phase to W_2 phase could be aggravated (*12,13*). When using $W_1/O/W_2$ emulsions, the release of immobilized bioactive compound when the emulsion droplet is surrounded with outer aqueous environment could also be delayed (*14*), and encapsulated content could be released at a controlled rate (*10*). Moreover, undesirable sensory properties (bitterness, astringency or metallic flavour) of water-soluble bioactives could be masked in inner aqueous phase of $W_1/O/W_2$ emulsions (*15*).

Despite all these advantages that make $W_1/O/W_2$ emulsions as an appropriate system for encapsulation of polyphenols, there are only few studies using these systems for polyphenol entrapment. For example, resveratrol (*16,17*), procyanidin-rich extract (*18*), phenolic mango seed kernel extract (*19*), anthocyanin-rich bilberry extract (*20*), hydrophilic catechin and hydrophobic curcumin (*21*) were encapsulated using $W_1/O/W_2$ systems.

Emulsions encapsulating polyphenolic compounds, due to numerous health benefits of polyphenols, are often designed as potentially functional ingredients that could be incorporated in different food or pharmaceutical products to obtain value-added products. However, the addition of emulsions to such products is hindered by their instability. In general, $W_1/O/W_2$ emulsions are highly susceptible to break down over time through different physicochemical mechanisms, such as gravitational separation (creaming and sedimentation), droplet aggregation (flocculation and coalescence) and droplet growth (Ostwald ripening) (*22*). Therefore, the use of $W_1/O/W_2$ emulsions as carriers for bioactive compounds is still not widespread, and to provide their long-term shelf life, spray drying of emulsions has been proposed as an effective approach (*23,24*). In order to prepare stable spray-dried emulsions, the presence of carbohydrates, such as maltodextrin or gum Arabic, in the outer aqueous phase is essential (*23,25*). Maltodextrins are one of the most popular carriers used in the food field, since capacity to form amorphous glassy matrices during the encapsulation is the basis of their encapsulation strength (*26*).

$W_1/O/W_2$ emulsion containing a hydrophilic fluorescent marker in its inner aqueous phase was spray dried to produce microcapsules in a study of Adachi *et al.* (*23*). Authors used maltodextrin and gum Arabic as wall materials, and revealed that microcapsules prepared with maltodextrin were more stable than those prepared with gum Arabic. Brückner *et al.* (*27*) stabilized a volatile aroma compound, 3-methylbutyraldehyde, using a combined emulsification ($W_1/O/W_2$) and spray drying process, where maltodextrin, whey protein concentrate, sodium octenyl succinate, modified starch and gum Arabic were found to be the best tailor-made microencapsulation materials. Microcapsules containing water- and oil-soluble carotenoids were obtained by spray drying of $W_1/O/W_2$ multiple emulsions stabilized by blends of biopolymers (gellan gum, gum Arabic, mesquite gum, maltodextrin) in a work of Rodríguez-Huezo *et al.* (*28*). There is still a lack of studies regarding spray-dried double emulsions ($W_1/O/W_2$) containing polyphenolic compounds, since majority is related to the entrapment of carotenoids or aromatic compounds. However, in 2015, Berendsen *et al.* (*24*) spray dried double emulsions containing procyanidin-rich extracts produced by premix membrane emulsification and stabilized using different hydrophilic emulsifiers, made of whey protein isolate (WPI) and WPI-polysaccharide complexes.

The objective of this study is to prepare stable spray-dried double emulsions ($W_1/O/W_2$) encapsulating rosemary (*Rosmarinus officinalis* L.) polyphenols. Polyphenol-enriched double emulsions were produced by high-pressure microfluidization, stabilized using polyglycerol polyricinoleate (PGPR) as lipophilic emulsifier, whey protein isolates (WPI) as hydrophilic emulsifier, and maltodextrins as enhancing coating carriers. The effect of different maltodextrin type (MDE 10 and MDE 21) and WPI content (2 and 4 %) on particle size, stability, morphological properties and encapsulation efficiency of rosemary polyphenols was evaluated. In this way, spray-dried double emulsions could serve for delivery of functional ingredients, like polyphenols, to novel food or pharmaceutical products. Furthermore, this work could give certain insight to the research of spray-dried double emulsions encapsulating bioactive compounds like polyphenols, since there is a lack of studies dealing with this topic.

MATERIALS AND METHODS

Materials

Sunflower oil (Solesta, Aldi, Fermoy, Ireland), purchased in a local supermarket, was used as oil phase for preparation of double emulsions (DEs). The lipophilic emulsifier used to

stabilize the colloidal emulsions, polyglycerol polyricinoleate (PGPR), was supplied by Danisco (Copenhagen, Denmark), while whey protein isolates (WPI), used as hydrophilic emulsifiers in outer aqueous phase and consisted of 95 % β-lactoglobulin, were from Davisco Foods International (Le Sueur, MN, USA). Maltodextrins with average dextrose equivalent values of 10 and 21 (MDE 10 and MDE 21) were obtained from Grain Processing Corporation (Muscatine, IA, USA). Deionised water was used for preparation of solutions. Folin-Ciocalteu reagent, sodium carbonate and formic acid were supplied by Kemika (Zagreb, Croatia), while ethanol was from Carlo-Erba (Peypin, France); all chemicals were of analytical grade. Gallic acid and HPLC standards, ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt], DPPH (2,2-diphenyl-1-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), and Nile blue were obtained from Sigma-Aldrich (Steinheim, Germany). Acetonitrile and methanol (HPLC grade) were purchased from J.T.Baker (Deventer, the Netherlands).

Preparation of rosemary polyphenolic extracts

Dry rosemary leaves (*Rosmarinus officinalis* L.) were purchased in a local herbal store (Suban d.o.o., Zagreb, Croatia). Rosemary polyphenolic extract (RPE) was prepared by pouring 300 mL of boiled deionised water over 15 g of dried herb, and by continuous stirring for 30 min (during extraction the water temperature was not maintained). The obtained extract was filtered through tea strainer and filled up until desired volume, after which it was left to cool down before mixing with oil phase.

Preparation of rosemary polyphenolic extract/oil/water double emulsions

Preparation of rosemary polyphenolic extract/oil/water double emulsions (RPE/O/W DEs) was divided into two main emulsification steps. The first step was to produce primary RPE/O emulsion, containing aqueous RPE. Commercial sunflower oil, containing hydrophobic emulsifier PGPR (4 %, *m*/*V*), was used as the continuous phase. Previously prepared RPE was slowly dispersed to oil phase in ratio 50:50, using a high-shear mixer (model L5RT-A; Silverson, Waterside, UK) in a water bath (25 °C) at 5000 rpm for 10 min. High dispersed phase volume (50 % of extract) was chosen in order to deliver high concentration of rosemary polyphenols into emulsion system, but also, carefully taking into account the preservation of desired emulsion type, and avoiding possible phase conversions, which can include shifting of extract/oil to oil/extract phase. The production of preferred RPE/O emulsion was confirmed by dissolving the prepared emulsion in sunflower oil, indicating that high extract/oil ratio did not alter the emulsion type. To produce fine colloidal emulsion, obtained crude RPE/O emulsion just after preparation underwent high-pressure homogenization process using a Microfluidizer™ M-110S (Microfluidics International Corp., Newton, MA, USA), containing a 75-μm Y type ceramic interaction chamber, at 50 MPa and three passes. After preparation, fine RPE/O emulsion was left to immediately cool down to room temperature using tap water.

Second step in a two-step emulsification process was the production of RPE/O/W DEs. Before adding RPE/O emulsion to external water phase (W), WPI as hydrophilic emulsifier in mass per volume ratios of 2 and 4 % was added to water phase. The WPI solution was stirred for few hours on a magnetic stirrer (model C-Mag HS 7; IKA, Staufen, Germany) at 300 rpm and then left overnight at 4 °C to ensure a complete dissolution of proteins. Afterwards, 15 % of maltodextrin (MDE 10 or MDE 21) as enhancing carrier was added to protein solution, and then the solution was mixed on a magnetic stirrer at 300 rpm for one hour.

RPE/O/W DEs were prepared by adding 40 % RPE/O fine emulsion to 60 % WPI and maltodextrin solution. Firstly, coarse RPE/O/W DEs were pre-mixed in a water bath using a high-shear mixer (model L5RT-A; Silverson, Waterside, UK) at a milder condition of 3500 rpm for 5 min, and afterwards fine RPE/O/W DEs were obtained by homogenization through microfluidizer (model M-110S; Microfluidics International Corp., Newton, MA, USA), passing once at 20 MPa. Certain aliquot (50 mL) of RPE/O/W DEs was left for analysis, while remaining part (250 mL) of RPE/O/W DEs was subjected to spray drying.

Spray drying of double emulsions containing rosemary polyphenols

The obtained RPE/O/W DEs were spray dried in a laboratory scale Büchi minispray dryer B-191 (Büchi Labortechnik AG, Flawil, Switzerland). The dryer was operated at inlet air temperature of (175±2) °C and outlet temperature of 90 °C, with drying air flow of 600 L/h, feed flow rate around 18 %, and compressor air pressure around 700 kPa. Dried microcapsules were collected in air tight plastic containers and stored in desiccators until further analysis. Furthermore, spray-dried microcapsules containing rosemary polyphenols were rehydrated according to their total solid content, by dissolving them in deionised water at room temperature on a magnetic stirrer (model C-Mag HS 7; IKA, Staufen, Germany) at 300 rpm.

Characterization of RPE/O/W DEs, their spray-dried microcapsules and rehydrated microcapsule counterparts

Particle size analysis

The particle size distribution of RPE/O/W DEs, their spray-dried microcapsules and rehydrated microcapsule counterparts was measured using Mastersizer 2000, equipped with the Hydro 2000 S wet and Scirocco 2000 dry dispersion units (Malvern Panalytical, formerly Malvern Instruments, Malvern, UK). For emulsions, refractive index of dispersed phase (sunflower oil) was set to 1.45, while 1.33 was used as a refractive index for a dispersant liquid (water), with an obscuration rate from 5 to 12 %. For each sample, measurements were made in triplicate, and the results are expressed as mean value±standard deviation (*N*=3).

Emulsion stability

Creaming stability of initial RPE/O/W DEs and rehydrated microcapsules (spray-dried RPE/O/W DEs) was evaluated using a multisampling analytical centrifuge (LUMiFuge 116, LUM GmbH, Berlin, Germany), where the slope of the integral transmission-time curve was an indicator of creaming stability (creaming index). Non-diluted emulsions were transferred to rectangular (2 mm×8 mm) measurement cells, and creaming tendency was determined by running the centrifugation process at 286.5×*g* and 25 °C, at scanning rate of once every 90 s for 6.5 h. The results were presented as the integrated transmission percentage against time (%/h) and expressed as mean value±standard deviation (*N*=3), where the creaming stability of emulsions was higher when the slope of curve was lower.

Morphology

Confocal scanning laser microscopy. Confocal scanning laser microscopy served to observe initial RPE/O/W DEs and rehydrated microcapsules using Leica TCS SP5® microscope (Leica Microsystems GmbH, Wetzlar, Germany) according to Su *et al.* (*29*) with certain modifications. A volume of 1 mL of the emulsion was firstly transferred to a concave glass slide and stained with Nile blue (0.1 mL of 1 %, *m/V*, in distilled water). Slides were covered with cover slide and images of emulsions were captured with a 100× magnification lens at wavelength of 488 nm by using Ar and He/Ne lasers.

Scanning electron microscopy. The microstructure of spray-dried microcapsules containing rosemary polyphenols was captured by scanning electronic microscopy (SEM) analysis, and evaluated using a TESCAN Mira3 microscope (Brno, Czech Republic). Microcapsules were attached to SEM stubs using a two-sided adhesive tape, and specimens were coated with a gold layer (50 nm). Images were scanned using an acceleration voltage of 5 kV.

Encapsulation efficiency of total polyphenols

The content of encapsulated rosemary polyphenols entrapped in spray-dried microcapsules was evaluated spectrophotometrically (model Helios γ; ThermoSpectronic, Cambridge, UK) at 765 nm in rehydrated microcapsules, using Folin-Ciocalteu reagent, according to a modified method of Lachman *et al.* (*30*). To determine total polyphenolic content (TPC), rehydrated samples were centrifuged (model SL 8/8R; Thermo Scientific, Suzhou, China) at 26 271.8×*g* for 30 min to eliminate oil phase from water phase. After centrifugation, dense creamy layer was covering the surface and the subnatant, *i.e.* recovered aqueous phase (consisting of RPE and water phase containing proteins and maltodextrins), was collected using a syringe needle and filtered through a 0.45-μm filter (Nylon Membranes, Supelco, Bellefonte, PA, USA) to remove still potentially present oily phase. The obtained recovered aqueous phase was used for further analysis, since the aim was to determine polyphenolic content in final product, *i.e.* microcapsules. Blank samples for each formulation were prepared in the same way as RPE/O/W DEs, but containing deionised water instead of rosemary polyphenolic extract. The emulsification preparation conditions and formulations were kept the same as spray drying parameters. Blanks were run through the analysis in the same way as evaluated samples, and obtained blank values were subtracted from sample values to eliminate potential masking effect of proteins and maltodextrins on real TPC.

TPC was expressed in mg of gallic acid equivalents (GAE) per L of recovered aqueous phase after rehydration of spray-dried microcapsules. All measurements were carried out in triplicate. To examine the content of encapsulated rosemary polyphenols entrapped in spray-dried microcapsules, the percentage of encapsulation efficiency for total polyphenolic content was calculated as the ratio of TPC determined in the recovered aqueous phase after rehydration of microcapsules and TPC theoretically expected, and it is expressed as mean value±standard deviation (*N*=3).

Encapsulation efficiency of specific polyphenolic compounds

Additionally, recovered aqueous phase after rehydration of spray-dried microcapsules was evaluated by HPLC analysis in order to determine encapsulation efficiency of individual polyphenolic compounds of rosemary extract.

Before HPLC analysis, samples (recovered aqueous phase after rehydration of microcapsules) were filtered once again through a 0.45-μm filter (Nylon Membranes, Supelco). The system was equipped with Infinity Agilent 1100/1200 Series HPLC device (Agilent, Santa Clara, CA, USA) and a Photodiode Array Detector (Agilent), where 20 μL of sample were injected into system for HPLC analysis. The column used for the separation was a reversed-phase Zorbax extended C-18 column (250 mm×4.6 mm, 5 μm i.d.; Agilent). The mobile phase consisted of 2 % formic acid in water (solvent A) and 2 % formic acid in acetonitrile (solvent B) at a flow rate of 1 mL/min. The elution was performed with a gradient starting at 10 % B, 40 % B at 25 min, 70 % B at 45 min and becoming isocratic for 5 min. Chromatograms were recorded at 278 nm. Detection was performed by scanning between 200 and 400 nm, with a resolution of 1.2 nm. Specific polyphenolics were identified by comparing the retention times and spectral data with those of standards (rosmarinic acid, caffeic acid, apigenin and luteolin). All analyses were repeated three times and the results were expressed in mg of evaluated compound per L of recovered aqueous phase after rehydration of microcapsules. The encapsulation efficiency (in %) of specific polyphenolics was calculated as ratio between specific polyphenolic compound content determined in the recovered aqueous phase after rehydration of microcapsules and its theoretically expected content, and the results were expressed as mean value±standard deviation (*N*=3).

Determination of antioxidant capacity

Antioxidant capacity was determined by DPPH and ABTS free radical scavenging activity assays, and it was measured in recovered aqueous phase after rehydration of spray-dried microcapsules.

Antioxidant capacity was performed using the $DPPH^{\cdot}$ radical scavenging assay described by Brand-Williams *et al.* (*31*). The free radical scavenging capacity using the $DPPH^{\cdot}$ radical reaction was evaluated spectrophotometrically (model Helios γ; ThermoSpectronic) by measuring the absorbance at 515 nm after 30 min of reaction at room temperature. The results were expressed in mmol of Trolox equivalents (TE) per L of recovered aqueous phase after rehydration of microcapsules.

The Trolox equivalent antioxidant capacity (TEAC) was estimated by the ABTS radical cation decolourization assay (*32*). The results obtained from triplicate analyses were measured at 734 nm, and expressed in mmol of TE per L of recovered aqueous phase after rehydration of microcapsules. All measurements were performed in triplicate, and the results were presented as mean value±standard deviation (N=3).

Statistical analysis

The results were analysed statistically using the Statistica v. 7.0 software (*33*) to determine the average values and standard errors. One-way ANOVA, using the Tukey's *post hoc* test, with a significance level of p=0.05 %, was performed to determine the influence of delivery system formulations on evaluated parameters. The probability level of $p<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Particle size of RPE/O/W DEs, their spray-dried microcapsules and rehydrated microcapsule counterparts

A high-pressure emulsification process, performed using a Microfluidizer homogenization device (model M-110S; Microfluidics International Corp.), was used to prepare DEs enriched with rosemary polyphenolic extract (RPE/O/W DEs). The appliance of such homogenizer resulted in a small, desirable particle size of the obtained emulsions, with mean particle size d(0.5) ranging from 0.54 to 0.64 μm (**Table 1**).

Both maltodextrin type (MDE 10 and MDE 21) and protein content (2 and 4 %) had no significant effect ($p>0.05$) on mean particle size of RPE/O/W DEs samples. However, RPE/O/W DE prepared with MDE 10 and 4 % WPI had the smallest mean particle size (0.54 μm). When using higher content of biopolymers in W_2 phase of $W_1/O/W_2$ emulsions, Rodríguez-Huezo *et al.* (*28*) produced multiple emulsions with smaller emulsion droplet particle size, which correlates well with the results presented in this study, where smaller droplet particle size of DEs was also achieved when higher content of proteins (4 %) was used in the outer phase. The same pattern among all RPE/O/W DEs was observed with d(0.1) and d(0.9) values, and mean diameters (D[3,2] and D[4,3]). However, the obtained results implied no significant impact ($p>0.05$) of protein content or maltodextrin type on d(0.1), d(0.5), d(0.9), D[3,2], and D[4,3] values of RPE/O/W DEs. Additionally, compared to this study, Cofrades *et al.* (*34*) obtained higher D[4,3] average values (3.77–9.44 μm) of $W_1/O/W_2$ DEs stabilized with 6 % of PGPR, 0.5 % sodium caseinate or 6 % whey concentrate in external phase than ones in this study (0.75–0.90 μm). Mun *et al.* (*35*) reported the mean size of $W_1/O/W_2$ DEs ranging from 3.3 to 9.9 μm in diameter, depending on the content of PGPR (4–8 %) and WPI (2–6 %). When using 4 % PGPR, the authors also reported that increasing WPI content in the external aqueous phase led to the decrease in mean droplet size, again affirming results summarized in this study. However, different values obtained among studies could be ascribed to various parameters used during DEs preparation, from different constituents used to design stable DE systems ($W_1/O/W_2$ ratio, selection and concentration of emulsifiers, or oil used in preparation of W_1/O phase) to wide emulsifying parameters (homogenization devices, valve design, applied pressures, number of passes, or temperature). Therefore, according to the obtained results, selected parameters used for the preparation of RPE/O/W DEs represent a good choice, resulting in fabrication of DEs with desirable droplet particle size.

As it was expected, significant difference in particle size parameters after spray drying of RPE/O/W DEs was observed, since particle size of the obtained spray-dried microcapsules containing rosemary polyphenols differed significantly ($p<0.05$) from both initial RPE/O/W DEs, and afterwards rehydrated microcapsules. Contrary to RPE/O/W DEs, in spray-dried microcapsules the addition of maltodextrin with higher dextrose equivalent (MDE 21) and lower protein content (2 % WPI) mostly resulted in a smaller particle size. Mean particle size, d(0.5), of spray-dried microcapsules differed significantly ($p<0.05$), where the sample comprising MDE 21 and 2 % WPI had the smallest particle size, with 50 % particles finer than 97.11 μm. Results implied that here, the choice and concentration of hydrophilic emulsifiers and maltodextrin type had the major impact on the mean particle size of microcapsules. Significant difference ($p<0.05$) in d(0.1) values was reported when MDE 10 and MDE 21 were used as enhancing coatings, while in d(0.9) values insignificant difference ($p>0.05$) was observed only between samples containing MDE 10 and MDE 21 and prepared with 4 % WPI. Again, when using MDE 21, lower diameter values, D[3,2] and D[4,3], were reported. For D[3,2], no significant difference ($p>0.05$) was observed only between microcapsules prepared with MDE 21.

Spray-dried microcapsules were additionally rehydrated and particle size of the obtained rehydrated microcapsules was also evaluated. Significant difference ($p<0.05$) in mean particle size of rehydrated microcapsules was noted among samples prepared with MDE 21 and 4 % WPI and in both rehydrated samples composed of MDE 10. No significant difference ($p>0.05$) in d(0.1) and D[3,2] values was observed between rehydrated microcapsules. The opposite was

Table 1. Particle size parameters of rosemary polyphenolic extract/oil/water double emulsions (RPE/O/W DEs), their spray-dried microcapsules (MC) and rehydrated microcapsule (RMC) counterparts

MDE type	(*m*(WPI)/*V*(emulsion))/%	Sample	Particle size			*D*[3,2]/μm	*D*[4,3]/μm
			d(0.1)/μm	*d*(0.5)/μm	*d*(0.9)/μm		
10	2	RPE/O/W DE	0.27±0.00	0.55±0.00	1.53±0.00	0.49±0.00	0.76±0.00
		MC	22.88±1.07	123.4±1.1	336.1±2.8	59.4±1.8	154.4±1.5
		RMC	0.55±0.00	5.09±0.05	18.3±0.2	1.77±0.01	9.4±0.2
	4	RPE/O/W DE	0.27±0.00	0.54±0.00	1.48±0.01	0.48±0.00	0.75±0.00
		MC	24.5±0.7	127.8±1.8	345.7±8.4	65.2±0.8	169.9±4.8
		RMC	0.54±0.00	4.57±0.02	17.8±6.2	1.60±0.01	9.0±0.8
21	2	RPE/O/W DE	0.30±0.00	0.64±0.00	1.87±0.03	0.56±0.00	0.90±0.01
		MC	13.0±0.3	97.1±0.5	254.6±13.8	36.2±2.2	118.7±3.6
		RMC	0.39±0.00	2.15±0.04	29.7±4.4	1.02±0.01	10.2±0.6
	4	RPE/O/W DE	0.29±0.00	0.59±0.00	1.70±0.03	0.53±0.00	0.83±0.01
		MC	12.86±0.06	117.9±1.0	344.9±1.6	35.08±0.03	149.6±0.9
		RMC	0.35±0.00	1.95±0.02	11.6±0.2	0.92±0.00	5.6±0.2

RPE/O/W DEs were prepared with maltodextrins with different average dextrose equivalents (15 % (*m/V*) MDE 10 or 21), and different contents of whey protein isolates (2 or 4 % WPI, *m/V*). MDE=maltodextrin, *D*[3,2]/μm=surface weighted mean, *D*[4,3]/μm=volume weighted mean

observed in *d*(0.9) and *D*[4,3] values, where significant difference ($p<0.05$) in samples composed of MDE 21 was found.

Moreover, due to the introduced drying step, rehydrated microcapsules had larger particle size than initial RPE/O/W DEs. As it can be seen in **Table 1**, although smaller particle sizes of primary RPE/O/W DEs were obtained when MDE 10 was used, their rehydrated microcapsules provided larger particle size values than MDE 21. Compared to initial RPE/O/W DEs analogues, rehydrated microcapsules prepared with MDE 10 had approx. 8–9 times larger mean particle size, while the ones prepared with MDE 21 had approx. 3 times larger size. When observing changes in mean particle size of RPE/O/W DEs and afterwards their rehydrated microcapsules, significant difference ($p<0.05$) was found among samples prepared with MDE 10. Possible reason why rehydrated microcapsules prepared with MDE 10 had larger mean particle sizes than the ones prepared with MDE 21 could be a different solubility of MDE 10 compared to MDE 21. MDE 10 has a higher molecular mass and longer chain than MDE 21, and it is consequently more difficult to dissolve it. As a result, samples prepared with MDE 10 after rehydration were not totally dispersed (even particles were not visually evident), which resulted in more rough particle size of rehydrated microcapsules prepared with MDE 10. Also, the additional potential explanation is the ability of MDE 10 for the coalescence, which in the end affects the final droplet particle size.

Rodríguez-Huezo *et al.* (*28*) produced spray-dried $W_1/O/W_2$ multiple emulsions containing carotenoids, whose *D*[4,3] values ranged from 25.05 to 34.01 μm. Their emulsions were prepared with esters of monoglycerides and diglycerides of diacetyl tartaric acid as hydrophilic emulsifier, and MDE 10 as additional carrier, combined with different blends of biopolymers. When compared to present results, their combination of chosen materials seems to be more suitable for obtaining spray-dried microcapsules with smaller particle size, even their initial DEs had higher *D*[4,3] values (1.48–5.31 μm) than the ones produced in this study (0.75–0.90 μm). Moreover, particle size analysis of their rehydrated microcapsules was not done afterwards, thus it was impossible to compare the impact of spray drying on changes in particle size after microcapsule rehydration. Differences among results are also not so surprising if taking into account the complexity of the prepared matrix that underwent through spray drying process, since delivery systems in this study comprised polyphenolic extract combined with oil (RPE/O phase), instead of less complex W_1/O phase used in a study of Rodríguez-Huezo *et al.* (*28*). In a study of Berendsen *et al.* (*24*), *D*[4,3] values of DEs encapsulating a water-soluble commercial grape seed extract rich in procyanidins, and produced by premix membrane emulsification, ranged from 7.7 to 10.5 μm. After spray drying, authors produced microcapsules whose *D*[4,3] values were 18.7–50.5 μm, while after rehydration of microcapsules, their D[4,3] values were from 9.9 to 33.8 μm. They produced microcapsules with smaller particle size than in this study, while their *D*[4,3] values of DEs and afterwards rehydrated microcapsules were higher than ours. However, their rehydrated microcapsule samples were characterized with larger particle size than the initial DEs, as in this study.

Stability of RPE/O/W DEs and their rehydrated spray-dried microcapsule counterparts

Stability of DEs that will be subjected to spray drying step could be interpreted differently. Some authors considered DEs as stable if no oil separation took place within time needed to spray dry the emulsions (*27*). In the present study, stability of RPE/O/W DEs and afterwards rehydrated microcapsules containing rosemary polyphenols was evaluated using a multisample analytical centrifuge. The addition of MDE 10 proved to be a better choice for increasing emulsion stability, since their transmission light values were almost 2-fold lower than when MDE 21 was used (**Fig. 1**). Both samples with MDE 10

addition showed minor change in stability, since their creaming index (observed through the rate/slope of the curve) was slightly increasing with time, being mostly stable during analysis (**Fig. 1**). The DE sample with the highest stability (MDE 10 and 4 % WPI) was characterized with the smallest droplet particle size, putting here the stability of DEs in correlation with their particle size (the highest stability, the smallest particle size). However, the results showed that there was no significant effect ($p>0.05$) of maltodextrin type or emulsifier concentration on the stability of the investigated DEs. Therefore, produced RPE/O/W DEs after centrifugation did not show phase separation or any other destabilization property, implying their high stability.

As **Fig. 1** shows, rehydrated microcapsules had lower stability than the initial RPE/O/W DEs, since their transmission light signals were notably higher, and light signals were increasing with time. Here, the impact of protein content had more influence than maltodextrin type, since samples prepared with both MDE 21 and MDE 10, but with 4 % WPI exhibited lower creaming index. Bigger difference at the beginning of scanning between these two samples was observed, but with time their transmission light signals were kept the same. However, there was no significant difference ($p>0.05$) between rehydrated microcapsule samples. Creaming index for rehydrated microcapsules demonstrated the potential beginning of phase separation on top of the test cuvette. Higher difference in creaming index was reported among samples containing MDE 10 (around 3 times), than among those prepared with MDE 21 (less than 2 times). This could be due to the already mentioned characteristics of MDE 10 (lower solubility and ability to coalesce and aggregate). However, significant differences ($p<0.05$) in stability among RPE/O/W DEs and rehydrated microcapsules were observed only in samples produced with MDE 10 and 2 % WPI.

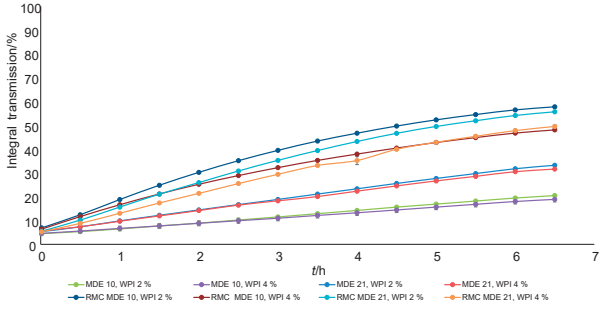

Fig. 1. Creaming stability of analyzed rosemary polyphenolic extract/oil/water double emulsions (RPE/O/W DEs) and their rehydrated spray-dried microcapsules (RMC) expressed through integral transmission profile-time curve. Samples were prepared with maltodextrins with different average dextrose equivalents (15 % MDE 10 or 21), and different contents of whey protein isolates (2 or 4 % WPI)

Morphology of RPE/O/W DEs, their spray-dried microcapsules and rehydrated microcapsule counterparts

Microscopic observation of RPE/O/W DEs and rehydrated microcapsules was performed by confocal scanning laser microscopy, and the results are presented in **Fig. 2**.

When comparing RPE/O/W DEs prepared with different maltodextrin type, it can be clearly seen that the ones prepared with MDE 10 (**Figs. 2a** and **2c**) had large number of small oil droplets, while in DEs containing MDE 21 (**Figs. 2e** and **2g**), more oil droplets of larger size were present than in the former ones. This is in consistence with particle size measurements, where DEs prepared with MDE 10 had in general smaller droplet particle size.

It is also obvious from **Fig. 2** that spray drying step influenced the particle size of rehydrated microcapsules. Rehydrated samples contained larger droplets (**Figs. 2b**, **2d**, **2f** and **2h**)

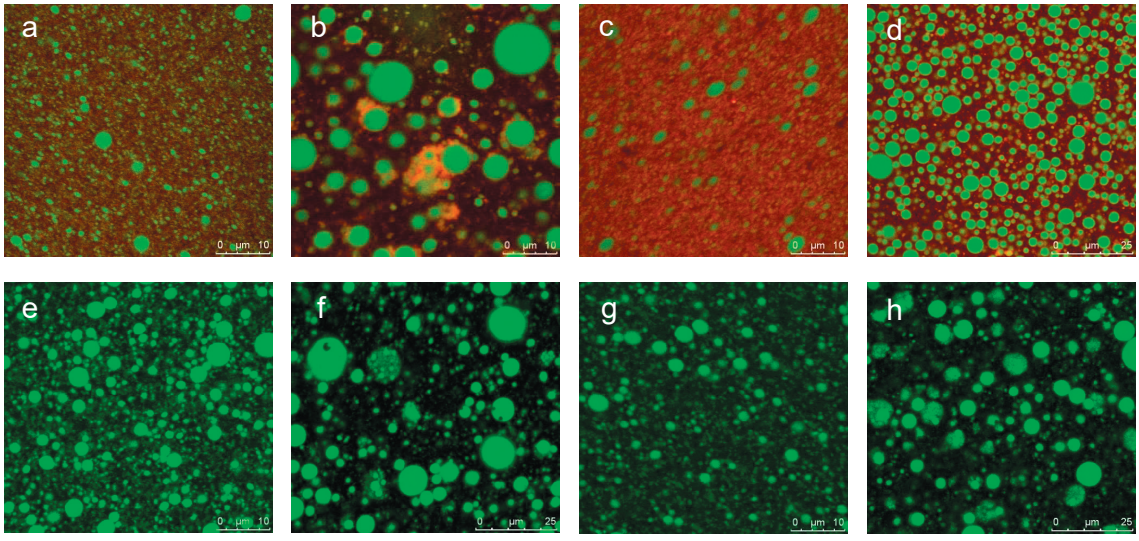

Fig. 2. Confocal micrographs of rosemary polyphenol extract/oil/water double emulsions (RPE/O/W DEs) and their rehydrated spray-dried microcapsules (RMC). RPE/O/W DEs were prepared with maltodextrins with different average dextrose equivalents (15 % (*m*/V) MDE 10 or 21), and different contents of whey protein isolates (2 or 4 % WPI, *m*/V): a and b) MDE 10 and WPI 2 %, and its RMC; c and d) MDE 10 and WPI 4 %, and its RMC; e and f) MDE 21 and WPI 2 %, and its RMC; g and h) MDE 21 and WPI 4 %, and its RMC. a-h: red=protein, green=lipid/fat

than initial RPE/O/W DEs, which again correlates well with particle size measurements, where rehydrated samples had larger particle size than DEs. Therefore, the micrographs of rehydrated samples consisted of more droplets of larger size and fewer droplets of smaller particle size, indicating the possible polydisperse structure, where certain flocculation and coalescence of oil droplets was observed. The same was also noted in RPE/O/W DEs, although in a smaller extent, but in the end the modifications did not influence the particle size of the prepared DEs, since particle size parameters (**Table 1**) showed that DEs with small, desirable particle size were produced. The images of both, RPE/O/W DEs and rehydrated microcapsules confirmed that there was no significant impact of protein content on droplet particle size distribution. However, images lead to a conclusion that samples prepared with 4 % WPI provided smaller droplets, accompanying thus values achieved by particle size measurements. Therefore, the fabrication of RPE/O/W DEs was confirmed by confocal microscopy; even small spots inside oil droplets representing RPE were hard to see in RPE/O/W DEs due to their very small particle size. Moreover, the micrographs depict preservation of DE shape in rehydrated microcapsule samples where spray drying step was involved, and due to the larger particle size of rehydrated samples, RPE phase inside oil droplets can be observed more easily.

Fig. 3 shows typical images of spray-dried microcapsules containing rosemary polyphenols. All microcapsules had spherical shape, with certain indentations on their surface. Such wrinkled surface could be attributed to the rapid shrinkage of the liquid emulsion droplets during the early stages of spray drying (*36*), and the consequent contraction of the particles (*37*). Similar capsule structure with dents on the surface was also observed in the study of Edris and Bergnståhl (*38*), where orange oil was encapsulated in DE system. Certain void centre and pores were observed in **Fig. 3d** (prepared with MDE 21 and 4 % WPI), where such formations could be related to the expansion of the particles during the latter stages of drying. Other microcapsules were free of cracks, but the appearance of the indentations was present. In the places with indentations, smaller particles were 'stuck' within the dents, and aggregation of small particles on the larger ones can be observed, indicating heterogeneous structure of the evaluated microcapsules.

Encapsulation efficiency of polyphenols

After rehydration of the obtained spray-dried microcapsules, encapsulation efficiency (EE) of total polyphenols was evaluated in recovered aqueous phase after rehydration of microcapsules. Obtained EE values for all samples ranged from 27.08 to 42.83 % (**Fig. 4a**), where sample prepared with MDE 21 and 2 % WPI had the lowest EE of rosemary polyphenols. It is reported that maltodextrin with higher dextrose equivalent is more sensitive to high temperatures that are involved in spray drying. Compounds with lower molecular mass contain shorter chains and oxidation reactions of aldehydes at the open sides of the molecules may lead to certain structural deformities during heating operations (*39*). On the other hand,

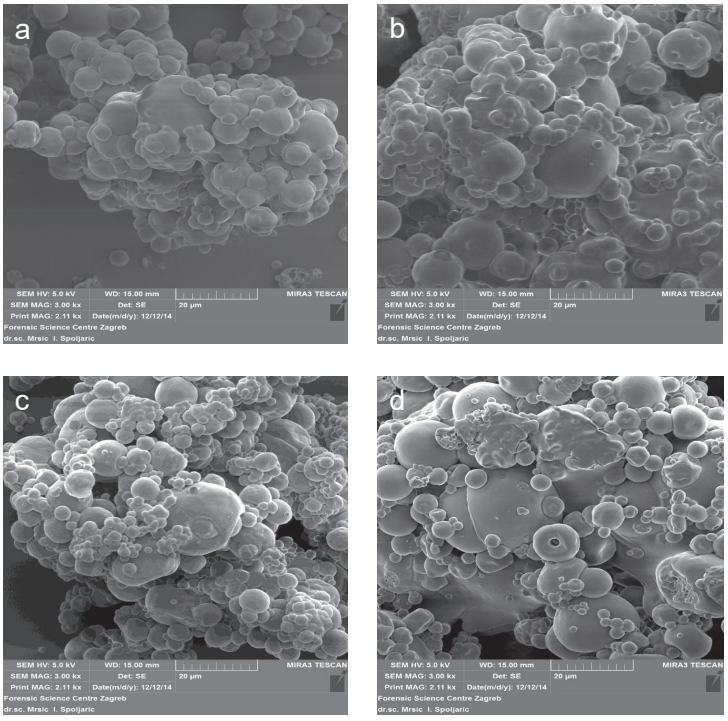

Fig. 3. Scanning electron micrographs of spray-dried microcapsules containing rosemary polyphenols. Rosemary polyphenolic extract/oil/water double emulsions were prepared with maltodextrins with different average dextrose equivalents (15 % (*m*/V) of MDE 10 and 21), and different contents of whey protein isolates (2 and 4 % WPI, *m*/V): a) MDE 10 and 2 % WPI, b) MDE 10 and 4 % WPI, c) MDE 21 and 2 % WPI, and d) MDE 21 and 4 % WPI

sample also comprising MDE 21 but 4 % WPI had the highest EE of polyphenols, indicating that here the presence of other materials during whole encapsulation process and their adequate concentrations were of essential importance. When using the same protein content but different maltodextrin type, there was no significant ($p>0.05$) difference among samples, implying that choice of maltodextrin did not affect EE. On the other hand, when using higher protein content (4 % WPI), significantly ($p<0.05$) higher EE of rosemary total polyphenols was observed. Therefore, the obtained results indicate the major impact of protein content on EE of rosemary polyphenols. Also, encapsulation efficiency exhibited inverse relation to the mean particle size, $d(0.5)$ of rehydrated microcapsules, since the sample with the smallest mean particle size provided the highest EE (sample consisted of MDE 21 and 4 % WPI).

When using maltodextrins with different dextrose equivalents as carriers for spray drying of black carrot anthocyanin pigments, Ersus and Yurdagel (*39*) concluded that maltodextrin with higher DE (DE 20-23) enabled higher anthocyanin content than powders produced with MDE 10. Furthermore, Rodríguez-Huezo *et al.* (*28*) achieved higher encapsulation efficiency of carotenoids when higher ratios of biopolymers blends were used, where EE ranged from 25.6 to 87.5 %, placing EE values obtained in the present study in that range. Also, maltodextrin was found to be one of the most effective encapsulation materials for stabilization of a volatile aroma compound using a combined emulsification and spray drying process in a study of Brückner *et al.* (*27*). However, as mentioned before, since various constituents and parameters used in the preparation of delivery systems in the end could significantly affect the achieved EE, certain discrepancies and contradictions among studies can be expected.

HPLC analysis was performed in order to determine individual, specific rosemary polyphenolic compounds remaining in the recovered aqueous phase after rehydration of microcapsules. Rosmarinic and caffeic acids as hydroxycinnamic acid representatives, and apigenin and luteolin derivatives (expressed as sum of each) as flavonoids were detected in recovered aqueous phase of evaluated samples. EE of rosmarinic acid ranged from 62.15 to 67.43 % (**Fig. 4b**), with no significant differences ($p>0.05$) among samples, revealing no impact of maltodextrin type or protein content. EE of caffeic acid varied between 20.28 and 63.30 %, where the addition of MDE 10 and 2 % WPI resulted in the highest EE, while sample prepared with MDE 10 but higher WPI content (4 %) exhibited again the lowest EE. Thus, significant difference ($p<0.05$) between these two samples was observed, while caffeic acid content of samples prepared with MDE 21 was not significantly ($p>0.05$) different.

Regarding detected flavonoids, sum of apigenin derivatives exhibited EE in range from 39.06 to 47.96 % (**Fig. 4b**). The highest EE was found in the sample produced with MDE 21 and 2 % WPI, which was significantly different ($p<0.05$) from both samples prepared with MDE 10. However, within each group of maltodextrins, the impact of WPI content was insignificant ($p>0.05$). Moreover, luteolin derivatives seem to

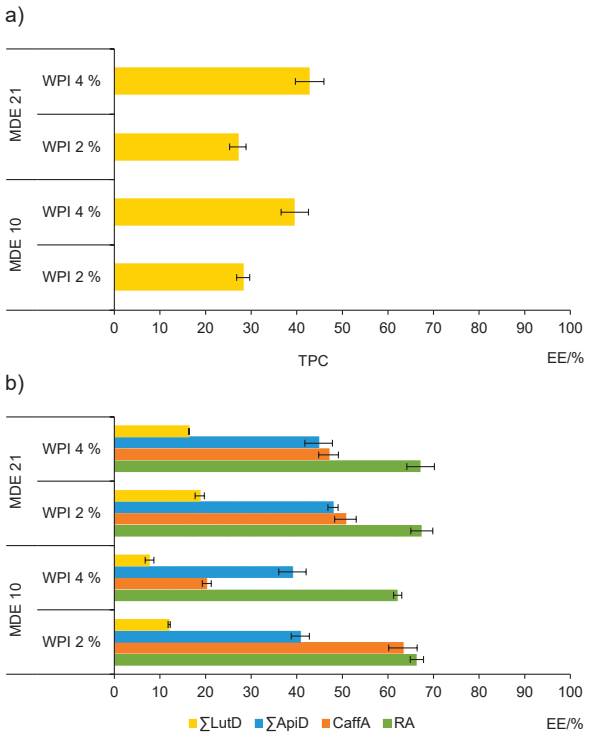

Fig. 4. Encapsulation efficiency (EE) of: a) total polyphenolic content (TPC) and b) specific polyphenolic compounds determined in recovered aqueous phase obtained after rehydration of spray-dried microcapsules containing rosemary polyphenols. Rosemary polyphenolic extract/oil/water double emulsions were prepared with maltodextrins with different average dextrose equivalents (15 % (*m*/V) MDE 10 or 21), and different contents of whey protein isolates (2 or 4 % WPI, *m*/V). ∑LutD=sum of luteolin derivatives, ∑ApiD=sum of apigenin derivatives, CaffA=caffeic acid, RA=rosmarinic acid

be the most sensitive polyphenolic compounds, since initially their content was low, and thus, EE of the sum of luteolin derivatives was very low, ranging from only 7.72 to18.72 %. Again, samples with MDE 21 had the highest EE, and they differed significantly ($p<0.05$) from the sample prepared with MDE 10 and 4 % WPI. Insignificant ($p>0.05$) impact of protein content within each group of maltodextrins on EE of luteolin derivatives was observed. Differences in EE among specific polyphenolic compounds were noticed, indicating different sensitivity of each compound to emulsification and drying processing, and thus resulting in wide range of EE values.

Antioxidant capacity of rehydrated spray-dried microcapsules

Antioxidant capacity of recovered aqueous phase after rehydration of spray-dried microcapsules containing rosemary polyphenols was evaluated using two radical scavenging assays, DPPH and ABTS. The highest antioxidant capacity in both assays was determined in the sample prepared with MDE 21 and 4 % WPI, implying high correlation between DPPH and ABTS assays. Also, when again MDE 21 but lower protein content (2 % WPI) was used, the lowest antioxidant capacity in both assays was achieved (**Fig. 5**). This correlates

well with the results obtained for TPC, where the same trend line was observed. Thus, the sample with the highest antioxidant capacity showed the highest EE, and accordingly the smallest mean particle size. Finally, no significant difference ($p>0.05$) in antioxidant capacity between samples was observed in both assays when different maltodextrin types and protein content were used.

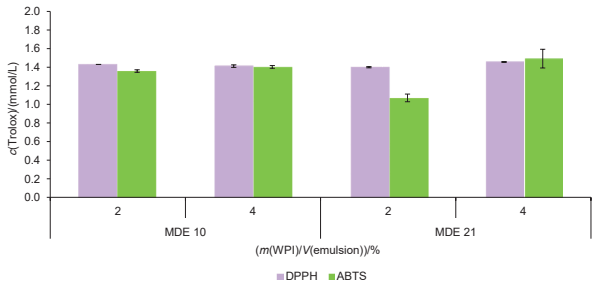

Fig. 5. Antioxidant capacity in recovered aqueous phase obtained after rehydration of spray-dried microcapsules containing rosemary polyphenols determined by DPPH and ABTS radical scavenging assays. Rosemary phenolic extract/oil/water double emulsions were prepared with maltodextrins with different average dextrose equivalents (15 % (*m*/V) MDE 10 or 21), and different contents of whey protein isolates (2 or 4 % WPI, *m*/V)

CONCLUSIONS

The present study demonstrates the potential of the combined emulsification and spray drying techniques to encapsulate polyphenolic compounds from rosemary (*Rosmarinus officinalis* L.) leaves. Different impact of varied carrier materials on the investigated parameters in obtained rosemary phenolic extract/oil/water double emulsions (RPE/O/W DEs), their spray-dried microcapsules and rehydrated microcapsule counterparts was observed. There was no significant impact of varied materials (maltodextrin type and protein content) on mean particle size of DEs, while microcapsules containing rosemary polyphenols differed significantly. Rehydrated microcapsules had larger droplet particle size parameters than their initial RPE/O/W DEs. Due to the higher molecular mass, longer chain and lower solubility, rehydrated microcapsules with MDE 10 had significantly higher mean particle size, up to 8–9 times higher, compared to initial RPE/O/W DEs. However, higher creaming index observed for rehydrated microcapsules implied the potential beginning of phase separation on top of the test cuvette. Morphology analyses showed that DEs were successfully formed, spherical microcapsules were produced after spray drying of DEs and DE form was still preserved after rehydration of microcapsules. Regardless of the used maltodextrin type, significant impact of protein content on encapsulation efficiency (EE) of rosemary total polyphenols, determined in the recovered aqueous phase after rehydration of microcapsules, was observed. Sample with the highest EE of total polyphenols was in an inverse relation to the mean particle size determined in rehydrated microcapsules; the highest EE, the smallest droplet particle size. HPLC analysis revealed the presence of specific polyphenols recovered in aqueous phase after rehydration of microcapsules, like phenolic acids (rosmarinic and caffeic) and flavonoids derivatives (apigenin and luteolin derivatives), where each polyphenolic compound was encapsulated at a different rate. The highest antioxidant capacity of the analysed samples was related to the highest EE, and thus the smallest particle size.

Therefore, the obtained spray-dried microcapsules containing rosemary polyphenols could be easily delivered to various dry mixtures, and thus serve in designing of novel pharmaceutical and food products. Since among studies there is a lack of surveys dealing with evaluation of combined encapsulation techniques for delivery of naturally derived polyphenols from traditional medicinal plants, these results could provide more clear insight into the potential of spray drying double emulsions for encapsulation of polyphenolic antioxidants. However, further study with emphasis on enhancing the encapsulation efficiency of natural polyphenolic compounds when using emulsification and drying techniques is needed.

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