Journal of Research in Pharmacy

Research Article

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The microencapsulation of oregano extract by using different techniques: Spray-drying and freeze-drying techniques and their *in vitro* characterization

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Received: 28 June 2021 / Revised: 10 December 2021 / Accepted: 12 December 2021

ABSTRACT: The potential antimicrobial and antioxidant benefit of high levels of ursolic, rosmarinic acids and carvacrol in oregano extract has been limited until now by poor bioavailability arising from the low aqueous-phase solubility and slow dissolution behavior of the freeze-dried extract. To address this issue, microencapsulation technique had been used to solve above mentioned problems and to protect active substances from deteriorating due to environmental conditions, such as moisture, oxidation. The experimental settings were performed by statistical mixture experimental design. Moreover, microcapsules and freeze-dried extract were compared to ascertain the merit of microcapsules as a carriers for these poorly aqueous-soluble compounds. When the wall material solution contained 1.43 g of maltodextrin, 4.17 g of gum arabic, 14.39 g of modified starch, and 20 g of ethanol oregano extract, the yield of oregano's active compounds, ursolic, rosmarinic acids and carvacrol, were 0.98 mg/g, 3.38 mg/g and 3.38 mg/g respectively. In particular, the release profile of ursolic, rosmarinic acids and carvacrol had levelled off after 15 minutes, depicting an impressive 2.7- 3.0 fold increase compared to the freeze-dried control extract. The results imply that all actives can be conveniently delivered via oral and mucosal routes by first internalizing oregano extracts into appropriately engineered microcapsules.

KEYWORDS: Microencapsulation; spray-drying; freeze-drying; Oregano; *in vitro* dissolution data.

1. INTRODUCTION

Oregano (*Origanum onites* L.) (Further referred to as oregano) is an aromatic plant widely cultivated in Mediterranean countries and mainly used as a spice herb. From a phytochemical point of view, oregano active compounds possess wide different in biological activity [1-4]. Oregano phenolic compounds (rosmarinic acid, caffeic acid) have been shown to act as antibacterial and antifungal agents [2-3]. According to literature, the antispasmodic, analgesic, diaphoretic, carminative, as well as antioxidant, antibacterial, antifungal activities of oregano essential oils have been extensively studied. Carvacrol has been identified as the main active compound responsible for these properties [1]. Moreover, the active ingredients of liquid oregano extract have stability and solubility problems so the extract has to be converted to powder form to avoid the problems. Microencapsulation is a process of enclosing the substances within an inert material, which protects them from the environment and sealed capsules release their contents at controlled rates under specific conditions [5].

The spray drying technique is the most common method that generates solid (dry) extract for pharmaceutical applications. Spray-dried technique a single-step process may provide dried particles with the required size and morphology. Spray dried technology comprises atomization, drying, and separation of particles. Oregano extract solution is sprayed through nozzles into a drying chamber [6,7].

Freeze drying is one of the most suitable techniques for dehydration of all sensitive materials and also for microencapsulation [8]. It is a multistage operation stabilizing materials through four main operations such as freezing, sublimation, desorption, and finally storage. The efficiency of protection or controlled release mainly depends on the composition and structure of wall material [9].

The choice of the wall material is an important step for the success of the microencapsulation process because it protects the bioactive compounds [10]. The ideal wall material should have film-forming and emulsifying properties, be biodegradable, resistant to the harsh gastrointestinal environment conditions,

How to cite this article: Ortasöz-Baranauskaite J, Üner B., Duman G., Taş Ç, The microencapsulation of oregano extract by using different techniques: Spray-drying and freeze-drying techniques and their *in vitro* characterization. J Res Pharm. 2022; 26(1): 102-111.

https://dx.doi.org/10.29228/jrp.107

ISSN: 2630-6344

exhibit low hygroscopicity, have low viscosity at high solid contents [11]. However, it is impossible that one single wall material will present all these properties. Therefore, a mixture of two or more components is often used [12]. Maltodextrin offers advantages, such as relatively low cost, neutral aroma, and taste, low viscosity at high solids concentrations, and good protection of flavors against oxidation [13,14]. Therefore, it is desirable to use maltodextrin in combination with other surface active biopolymers, such as gum arabic [15,16] modified starches [17] in order to obtain effective microencapsulation by spray-drying. According to Vaidya et al., the best mixture of encapsulating agents for encapsulation of cinnamon oleoresin consisted of gum arabic, maltodextrin, and modified starch (4:1:1), resulting in higher active compound yields than using gum arabic alone [15].

The objectives of the present work were to optimize the wall material composition (modified starch supplemented with gum arabic and maltodextrin) of oregano microcapsules prepared by spray-drying technology, the active compound yield and the narrower particle size distribution. The microscopic morphology of the prepared microcapsules and freeze-dried extract was also carried out and the *in vitro* release behavior of main oregano active compounds ursolic and rosmarinic acids, and carvacrol from the prepared microcapsules and freeze-dried extract was evaluated.

2. RESULTS AND DISCUSSION

2.1. Influence of wall material component amounts on the yield of ursolic, rosmarinic acids and carvacrol

In this study, D-optimal experimental design using Design-Expert® was applied to determine the optimal wall material composition. The effects of three independent variables: amounts of maltodextrin (0–20 g), gum arabic (0–20 g) and modified starch (0–20 g) on three response variables: yield of ursolic acid, yield of rosmarinic acid and yield of carvacrol were evaluated. The variables and their levels used in the design are presented in Table 1. The fitting models and statistical parameters are shown in Table 2.

Table 1. D-optimal design with independent and response variables for the preparation of Turkish oregano-loaded microcapsules

Run	A: Maltodextrin, g	B: Gum Arabic, g	C: Modified starch, g	UA[a] mg/g	CA[b] mg/g	RA[c] mg/g
1	9.91	10.08	0.00	0.76	1.24	1.71
2	9.81	0.14	10.04	0.13	3.33	3.2
3	0.00	20.00	0.00	0.23	1.62	1.48
4	1.58	13.48	4.92	0.25	0.99	0.84
5	6.58	6.75	6.66	0.45	2.00	1.38
6	9.91	10.08	0.00	0.85	1.05	1.89
7	4.92	15.07	0.00	0.75	0.75	0.61
8	0.00	10.05	9.94	0.89	2.83	2.15
9	0.00	0.00	20.00	0.34	2.65	2.25
10	0.00	10.05	9.94	0.95	2.79	2.15
11	13.25	3.35	3.38	0.87	3.14	3.14
12	20.00	0.00	0.00	0.28	2.78	2.54
13	9.81	0.14	10.04	0.67	3.56	3.10
14	9.81	0.14	10.04	0.69	3.33	2.95
15	20.00	0.00	0.00	0.36	2.85	3.00
16	1.43	4.17	14.39	0.98	3.38	3.38

[a] yield of ursolic acid.
[b] yield of rosmarinic acid.
[c] yield efficiency of carvacrol.

For this purpose basically, the oregano ethanol extract was encapsulated by using the spray drying method. The wall material mixture (variable amount of gum arabic, maltodextrin, modified starch) consisted of two parts of the solution. One of them is ethanol oregano extract, the other one is a different ratio of the wall materials. The spray drying process parameters: the feed flow rate was 40 mL/min, and the air inlet temperature was 170 °C [10].

Table 2. The fitting models, equations and statistical parameters of experimental design.

Response (mg/g)	Min value (g)	Max value (g)	Model	p value	r^2	r^2 adjusted	r^2 predicted
UA yield	0	20	Cubic	0.0001	0.8422	0.8422	0.8412
RA yield	0	20	Cubic	0.0001	0.9687	0.9687	0.9677
CA yield	0	20	Cubic	0.0001	0.9893	0.9893	0.9863

Figure 1 illustrates tridimensional plots of the different responses obtained by MSD using the above-mentioned models. The highest yield of ursolic, rosmarinic acids, and carvacrol are achieved when the wall material matrix contained 4.17 g of gum arabic, 1.43 g of maltodextrin and 14.39 g of modified starch.

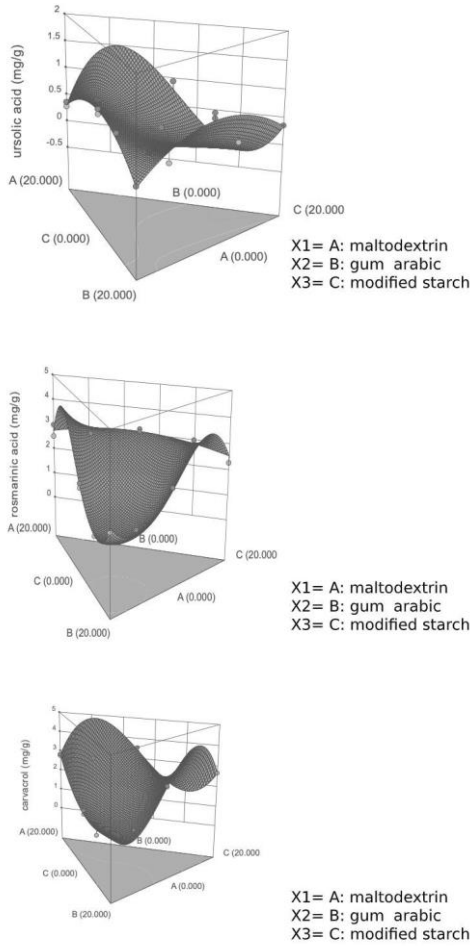

Figure 1. Response surfaces for the composition of the gum arabic, maltodextrin and capsul mixtures of spray-drying of ethanol oregano extract on the yields of the ursolic acid (a), rosmarinic acid (b), and carvacrol (c).

Moreover, numerical optimization of the wall material matrix using the desirability function has been performed. The optimization parameters are presented in Table 3. These results are similar to the other authors [12,18]. According to results, it was found that the optimal composition of Oregano microcapsules has been determined as follows: 4.17 g of gum arabic, 1.43 g of maltodextrin, and 14.39 g of modified starch, 20 g of ethanol oregano extract. The results showed that the experimental values did not significantly differ from the predicted values ($p > 0.05$).

2.2. Physicochemical properties of optimal formulation microcapsules and freeze-dried extract

The Scanning Electron Microscopy (SEM) is the most convenient visual technique to prove the mean size and the surface morphology of particles [5]. The morphology of Oregano microcapsules and freeze-dried extract are shown in Figure 2. According to SEM graph, the optimal formulation of microcapsules had a spherical shape. All microcapsules had uniform and slightly dented surfaces. Some fragments of broken microcapsules or cracks have also been observed. The irregularities arising on the surface of the microcapsules could be formed because of the shrinkage during drying and cooling processes [5]. Similar results have been observed by other authors [5] and are characteristics of the spray-drying techniques. Morphological analysis of the Oregano freeze-dried extract powder revealed the absence of small spherical shape of particles, which could imply dispersion properties poorly and hence low bioavailability [11].

Moreover, the results demonstrate that the size of the majority of freeze-dried oregano particles (75 %) was less than 70 ± 3.8 μm. The second smaller part of prepared particles fell into the group around the 1000 μm and the calculated relative span factor value was 11.96 in prepared freeze-dried oregano particles.

According to the literature, the relative span factor less than 2 is considered as a narrow distribution in prepared particles [20]. The determined value in freeze-dried extract particles indicated a wide size distribution and had high polydispersity index. According to literature the obtained results might indicate the freeze-drying process generates various stresses during processes. So these stresses significantly increase the particle size of material due to agglomeration [21].

Table 3. Numerical optimization of wall material amounts using desirability function.

Independent variables			Response Variables			
	Amount levels (g)	**Predicted optimal amount (g)**	**Yield**	**Criteria**	**Predicted mean value (mg/g)**	**Obtained mean value (mg/g)**
Maltodextrin	0 – 1.43	1.43	Ursolic acid	Maximize	0.96	0.93
Gum Arabic	0 – 4.17	4.17	Rosmarinic acid	Maximize	3.39	3.34
Modified starch	0 – 14.39	14.39	Carvacrol	Maximize	3.38	3.38

Desirability 0.991

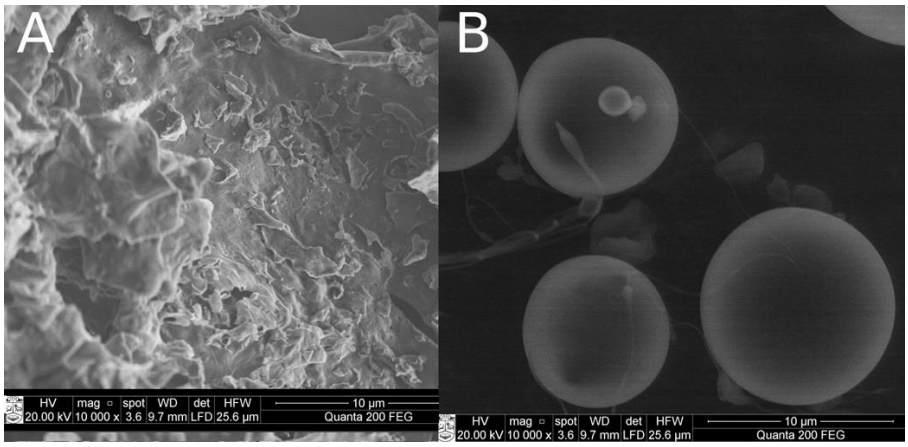

Figure 2. Scanning electron microscopy (SEM) of oregano freeze-dried extract (A) and an optimal formulation of oregano microcapsules (B).

The Laser Light Diffraction (LD) is the predominantly used technique for the particle size analysis of the spray-dried oregano microcapsules and the freeze-dried oregano extract and shown in Figure 3 [5,19]. During the microencapsulation process, the particle size distribution and their uniformity index are very important technological characteristics [5,19]. In our study, the surface-weighted mean diameter was 40.35 ± 2.5 μm, and the calculated relative span factor value was 1.35 in Oregano microcapsules.

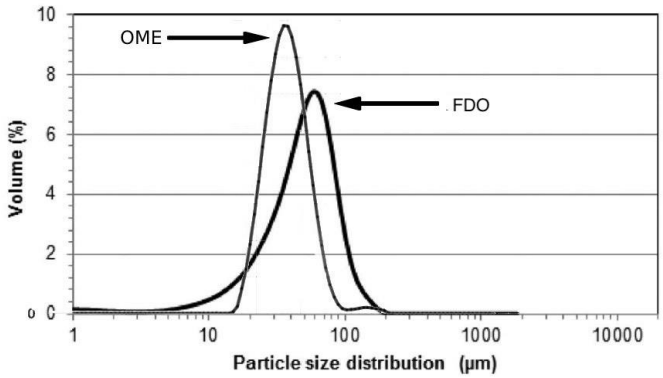

Figure 3. Particle size distribution of oregano microcapsules (OME) and freeze-dried extract (FDO).

2.3. *In vitro* dissolution study

According to the literature, the wall material composition in the microcapsules and the powder preparation method could influence the bioavailability of active compounds in capsules or tablets [22-24]. Therefore, we investigated the *in vitro* release of ursolic and rosmarinic acids and carvacrol from the optimal microcapsules formulation and freeze-dried extract in the artificial gastric medium. Figures 4 (a), 4 (b) and 4 (c) illustrate the *in vitro* dissolution profiles of the optimal formulation of microcapsules and Oregano freeze-dried extract. Firstly, the dissolution rate of ursolic acid, rosmarinic acid and carvacrol was obtained 2.7–3.0 fold more when compared to freeze-dried Oregano extract (Figure 4). Due to dissolution data from the optimal microcapsules formulation, ten minutes later the percentage release amount of ursolic rosmarinic acids and carvacrol was obtained around 75, 85 and 90 respectively.

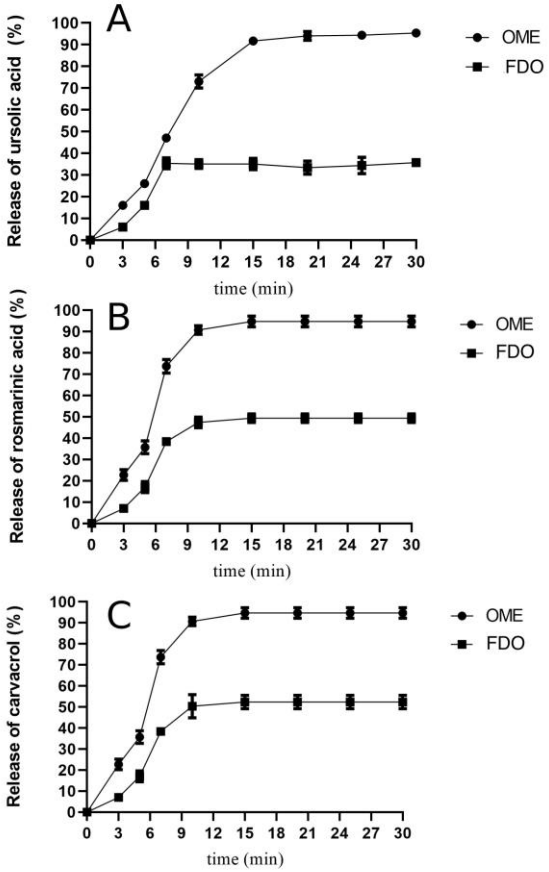

Figure 4. Release of ursolic acid, rosmarinic acid and carvacrol *in vitro* from oregano microcapsules (OME) and oregano freeze-dried (FDO) capsules.

In summary, the optimal formulation of Oregano extract microcapsules displayed a significantly better *in vitro* dissolution profile and dissolution rate compared to the oregano freeze-dried extract. Generally, the dissolution data influenced the choice of wall materials as a carrier, which plays an important role in encapsulated drug delivery systems [20,24]. In this study, the composition the optimal microcapsules was contained 1.43 g of maltodextrin, 4.17 g of gum arabic, 14.39 g of modified starch. Moreover, the modified starch, gum arabic and maltodextrin have low viscosity, high pigment binding capacity, good adhesion and low polymer weight gain, which are able to enhance the bioavailability of active compounds [10,25,26]. Also noteworthy is that the release of ursolic acid, rosmarinic acid and carvacrol from the optimal formulation of microcapsules had approached almost 95% in a 15 minutes. The improved release rate can be explained by the microcapsules' ability to enhance the solubility of encapsulated Oregano extract with proper wall material mixture. It has already been noted that the higher apparent dissolution rate of ursolic, rosmarinic acids and carvacrol achieved with spray drying process shows a promise for enhancing the in vivo bioavailability of Oregano extract microcapsules.

The use of natural substances from plant sources has gained increasing popularity in alternative medicine mainly due to their potential to target oxidative and inflammatory processes at the root of many chronic disorders. However, herbal extract-based preparations are not stable due to oxidation and photolysis processes. Also, the low solubility of plant-based active compounds could impair their desired bioavailability in vivo [10]. Therefore, there is a need for a formulation concept to find a way out of stability problems of herbal extracts as well as their poor absorption issue. According to literature nowadays, the microencapsulation formulation approach remedy to this challenge with the commonly used techniques, such as spray drying or freeze-drying. Each drying method offers advantages and disadvantages and so does each natural coating material like gum arabic, maltodextrin and modified starch, etc. [4,13].

The object of our study was to optimize *oregano* extract microencapsulation by spray-drying to achieve a high yield of oregano main active compounds ursolic, rosmarinic acids and carvacrol and preferable its narrower particle size distribution. Moreover, microcapsules and freeze-dried extract were compared to ascertain the merit of microcapsules as a carrier for these poorly aqueous-soluble compounds.

3. CONCLUSION

In this study, it was optimized the wall material composition (maltodextrin, gum arabic, modified starch) of oregano microcapsules prepared by spray-drying technology to increase the active compounds' yield value. When the wall material solution 1.43 g of maltodextrin, 4.17 g of gum arabic, 14.39 g of modified starch, the yield of oregano's active compounds, ursolic, rosmarinic acids and carvacrol, were 0.98 mg/g, 3.38 mg/g and 3.38 mg/g respectively. Moreover, microcapsules and freeze-dried extract were compared to ascertain the merit of microcapsules as a carrier for these poorly aqueous-soluble compounds. It was observed that the encapsulated Oregano extract dissolution rate is found dramatically higher than oregano freeze-dried extract alone. The obtained results confirmed that microcapsules as a carrier system for the herbal based active compounds are the most suitable, because the wall material complex protects sensitive biologically active compounds from the light, oxygen and degradation. Moreover, the use of modified starch as a main coating material, in supplementation with gum Arabic and maltodextrin not only improved the encapsulation efficiency, but also increased the *in vitro* release of the active compounds.

These results represent the basis for future trials that will validate the efficiency of microencapsulated Oregano extract to be delivered via oral and mucosal routes.

4. MATERIALS AND METHODS

4.1. Materials

Dried *Origanum onites* L. herb was obtained from "InanTarım ECO DAB'', Turkey. Modified starch was purchased from The National Starch lot: MFY-212, USA. HPLC eluent methanol was from Carl Roth GmbH (Karlsruhe, Germany) and rosmarinic acid (>98%) – from ChromaDex (Santa Ana, TX, USA). Carvacrol (>98%), acetic acid (99.8%), gum arabic and maltodextrin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (96%) was purchased from Vilniaus degtine (Vilnius, Lithuania). The purified water used in HPLC and for sample preparation was produced with a Super Purity Water System (Millipore, USA).

4.2. Experimental design

For wall material components: the experimental settings were performed by statistical mixture experimental design (MED). The experimental design of three components system is conducted by using Design Expert (version 9.0.4.01, Stat- Easy Inc., Minneapolis, USA). A set of candidate points in the design space is selected using the D – optimal criterion. In the D-optimal criterion, there are restrictions on the component proportions X_1 that take the form of lower L_1 and upper U_1 constraints, to keep the experimenter from exploring the entire simplex region. The mixture of the components was composed of gum arabic, maltodextrin, and modified starch. The constraints of the component proportions were from 0 to 20%. These lower and upper limits of Xj are chosen to describe the behavior of the formulations, which have compositions close to that of the best experiment obtained from preliminary work. In D-optimal design, there are restrictions on the component proportions X_1 that take the form of lower (Lj) and upper (Uj) constraints, to keep the experimenter from exploring the entire simplex region. The general form of constraints in D-optimal design is as follows: $\sum Xj = 1$ and $Lj \leq Xj \leq Uj$.

The design-led to 16 combinations (see more information in table 1). The analysis of variance (ANOVA) tables was generated and the effect and regression coefficients of the individual cubic model and the relationships between the variables were determined. The significance of all terms in the polynomial was judged statistically by computing the F value at the probability $p<0.05$. Optimization of the fitted polynomials was done using numerical optimization and desirability function. The optimum conditions were verified by conducting experiments at the conditions determined. Responses were monitored and the results were compared with model prediction.

4.3. Preparation of *oregano* ethanol extract

Prior to the extract preparation, the *oregano* herb was grinded in a cross beater mill IKA A11 Basic Grinder (IKA Works, Guanghou, China) and sieved using a vibratory sieve shaker AS 200 basic (Retch, UK) equipped with a 125 µm sieve. Powdered material (100 g) was then extracted with 1000 mL of 90% (V/V) ethanol in a round bottom flask by heat-reflux extraction performed in a water bath Memmert WNB7 (Memmert GmbH & Co. KG, Schwabach, Germany) at 95 °C for 4 hours. These conditions were determined as the best for the extraction of the main active compounds of Turkish oregano in our previous study [27]. The prepared extract was filtered using a vacuum filter.

4.4. Preparation of emulsion for spray-drying

The emulsion for the spray-drying consisted of the solution of maltodextrin, gum arabic and modified starch (wall material) and oregano ethanol extract. The wall material solution was prepared by hydrating required amounts of maltodextrin, gum Arabic and modified starch in purified water dissolving them at 25°C using magnetic stirrer hotplates (Heidolph MR, Germany) for 24 h. The emulsion for spray-drying (mixture of wall material solution and oregano ethanol extract) was homogenized using a magnetic stirrer for 1 h at 25°C. Further, the selection of an optimal ratio of wall material solution and ethanolic extract (wall material solution: oregano ethanol extract) was carried by previous studies in all experiments 20 g of oregano ethanol extract was used [10]. The wall material concentration in the emulsion was optimized by experimental design.

4.5. Preparation of microcapsules

The powders were obtained using a Buchi B-291 Mini Spray-Dryer (Flawil, Switzerland) operating with counter-current airflow. The equipment operated under the vacuum of 6 mBar and aspiration 100% in all experiments. The spray drying conditions depended on the previous studies' optimal results conditions [10]. The inlet temperature was 170 °C and the feed flow rate was 40 mL/min, which was equivalent to 6% of the equipment's full pumping capacity. The outlet temperatures depended on the inlet ones and were changing during the spray-drying process, when the inlet temperature was 170 °C the outlet ones ranged between 55-65 °C, outlet temperature has no significant data changes according to the process. Outlet temperatures were the lowest at the beginning of spray-drying and gradually increased as the process continued.

4.6. Oregano freeze-dried extract preparation method

Ethanol from oregano herb extract was removed under reduced pressure (15 min, 25°C) using a rotary evaporator (Heidolph, Schwabach, Germany) and freeze-dried overnight (-80°C) using a Martin Christ brand Alpha 2-4 LD Plus model freeze-dryer (Germany).

4.7. Microcapsules preparation for HPLC analysis

For determination of active ingredients (RA, UA and CA), 100 mg of the prepared microcapsules were accurately weighed and dispersed in 10 mL methanol in a volumetric flask and extracted for 10 min in an ultrasound bath (Memmert WNB7 water bath, Memmert GmbH & Co. KG, Schwabach, Germany). The extract has been passed through a 0.45 µm membrane filter for HPLC analysis.

HPLC analysis was carried out using a Waters brand 2695 model chromatography system (Milford, USA) equipped with a Waters brand 996 model PDA detector. Data were collected and analyzed using Empower 2 Chromatographic Manager System software (Waters Corporation, Milford, USA). Samples were chromatographed along ACE 5 C18 250×4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland) was used.

4.7.1. HPLC conditions for determination of UA

The mobile phase was composed of methanol and water (80/20, v/v). The flow rate was 0.6 mL/min and the injection volume was 1 µL. The absorption was measured at 203 nm. The quantification has been carried out by the external standard method. The calibration curves were made (UA R^2=0.9998) [28].

4.7.2. HPLC conditions for determination of RA

The mobile phase was composed of solvent A (methanol) and solvent B (0.5 % (v/v) acetic acid in water). The following linear gradient elution profile was used: 95 % A/5 % B – 0 min, 40 % A/60 % B – 40 min, 10 % A/90 % B – 41 - 55 min, 95 % A/5% B – 56 min. The flow rate was 1 mL/min and the injection volume was 10 µL. The effluent was determined at a wavelength of 329 nm. The quantification has been carried out by the external standard method. The linear calibration curve was made (R^2=0.9999), and the peak areas were used for quantification [28].

4.7.3. HPLC conditions for determination of CA

The mobile phase was composed of methanol and water (60/40, v/v). The flow rate was 0.6 mL/min and the injection volume was 10 µL. The absorption was measured at 275 nm. The quantification has been carried out by the external standard method. The calibration curve was made (CA R^2=0.9998) [28].

4.8. Particle morphology and particle size distribution

Particle morphology was evaluated by scanning electron microscopy (SEM) FEI Quanta 200 FEG (FEI, Oregon, USA). The SEM was operated at 10 kV with a magnification of 5000 - 10000 times.

Particle size distribution was measured using laser light diffraction equipment Mastersizer 2000z, model Hydro 2000 MU (Malvern Instruments, Malvern, UK). A small sample of powder was suspended in water under agitation, and the particle size distribution was monitored during each measurement until successive readings became constant. The surface-weighted and volume-weighted mean diameters were also measured.

4.9. *In vitro* release studies

Dissolution profiles of the active compounds ursolic acid, rosmarinic acid and carvacrol in optimized formutation of microcapsules and freeze-dried extract capsules were determined using a SOTAX brand AT7 smart model semi-automated dissolution tester (Switzerland). The basket method was applied using an artificial gastric medium without pepsin (50 rpm, 500 mL). The pH value was maintained at 1.7 (37 ± 0.5 °C). Aliquots (5 mL) were manually collected from parallel dissolution vessels at 1, 3, 5, 7, 10, 15, 20, 25, 30 minute time points, filtered through a nitrocellulose membrane (0.45 µm) and quantified via HPLC. The dissolution media in each vessel was replenished with a fresh dissolution medium (5 mL) to restore the original volume. The mean value of six trial runs and a standard deviation were calculated. The evaluation of dissolution profiles was carried out in triplicate.

4.10. Preparation of capsule fillings from prepared microcapsules and oregano freeze-dried extract

For the preparation of free-flowing powders from oregano freeze-dried extract, the material was crushed by a PM-100 planetary mills (Retsch Co., Ltd., Germany) by using a rotation speed of 300 rpm. The powder was then filled into number five gelatin capsules. The capsules were filled using the manual capsule-filling machine (Capsuline, Pompano Beach, FL, USA).

4.11. Statistical Analysis

Data are presented as mean ± SD. The software package Prism v. 5.04 (GraphPad Software Inc., La Jolla, CA, USA) was used to perform the analysis of variance (ANOVA) to detect differences among the mean values of responses and for the curve fitting. A value of $p < 0.05$ was taken as the level of significance.

Author contributions: Concept – J.O.; Design – J.O.; Supervision – G.D., Ç.T.; Resources – J.O., B.Ü.; Materials-– J.Ö., B.Ü; Data Collection and/or Processing-J.O.; Analysis and/or Interpretation - J.Ö., B.Ü., G.D., Ç.T.; Interpretation - J.Ö., B.Ü., G.D., Ç.T; Literature Search – J.Ö., B.Ü.; Writing – J.Ö., B.Ü.; Critical Reviews – - J.Ö., B.Ü., G.D., Ç.T

Conflict of interest statement: The authors declared no conflict of interest.

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