Simultaneous encapsulation of *trans*-resveratrol and vitamin D_3 in highly concentrated double emulsions

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Abstract

Background

Biocompounds encapsulation is essential in order to protect them from environmental factors that could enhance their oxidation or make them loss their beneficial properties due to extreme photosensitivity, among others. The main goal of this work was to study the feasibility of preparing concentrated double emulsions with a high loading capacity containing simultaneously *trans-Resveratrol (*RSV) and vitamin D_3 ($VitD_3$). Such emulsions could be used for food fortification or pharmaceutical formulations or as vehicles for targeted controlled release.

Results

In order to achieve large concentrations of the encapsulated compounds all the double emulsions were formulated using a W_1/O in W_2 ratio of 80/20, while the ratios tested for W_1 in O where 20/80 and 30/70. All the emulsions were characterized by droplet size, morphology, colloidal stability and encapsulation efficiency (EE) during six weeks. $VitD_3$ and RSV concentration were determined by a

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technique based on RP-HPLC (Reverse Phase High Performance Liquid Chromatography). The viability of preparing concentrated $W_1/O/W_2$ emulsions containing both biocompounds has been demonstrated with satisfactory results. Initial RSV concentrations in the concentrated double emulsions formulated varied from 5.0 to 8.3 mg/L while for $VitD_3$ values of 28-32 mg/L were obtained.

The presence of Vit D3 retarded RSV release in the formulated emulsions. It was observed that during the first week of storage it varied considerably between 10-50%, depending on the W_1/O ratio used. However, after two weeks of storage RSV release has been slightly retarded by 1-5 % by the presence of Vit D3.

Conclusion

Simultaneous encapsulation of RSV and $VitD_3$ was possible in high internal phase emulsions. The emulsions presented high colloidal stability being suitable for food fortification applications.

Keywords

Trans-Resveratrol, Vitamin D_3, Simultaneously encapsulation, High concentrated double emulsions, Biocompounds release, Double emulsion stability.

1. Introduction

A functional product consists on a food matrix enriched with any potential micronutrients, such as vitamins or antioxidant compounds, which provides health benefits reducing risk disease. However, frequently protection of these micronutrients is essential in order to avoid their oxidation or lost of their beneficial properties. For these purpose, several colloidal systems, such as emulsions, polymeric capsules or vesicles, had been used among others. However, in the food industry emulsion-based systems are commonly used to encapsulate hydrophobic bioactive compounds due to their relative ease of manufacturing using existing food processing operations[1,2].

Double emulsions are a potential vehicle for both molecules at the same time since W/O/W emulsions present a hydrophobic oil compartment/layer between two hydrophilic aqueous phases. Therefore, this colloidal delivery system would allow to encapsulate and protect both hydrophilic

RSV[3,4] and $VitD_3$ simultaneously[5–7]. Emulsions could also produce a controlled release of the encapsulated compounds. Moreover, the use of high concentrated emulsions, that is, emulsions with a high proportion of the internal phase, provides greater stability allowing at the same time to contain larger amount of entrapped active biocompound[8,9].

Resveratrol (RSV) is a polyphenol with a slightly hydrophilic[10] character that has interesting and beneficial health properties. It produces beneficial effects on human health due to its antioxidant activity and anti-aging effect, which has greatly increased its applications in pharmaceutical or cosmetics industries[11–15]. RSV has a great number of potential health benefits against several diseases such as cancer[16], diabetes, neurodegeneration, cardiovascular disorders, inflammation, and other age-related pathologies[17–22].In previous studies, with in vitro and in vivo experiments, it has been reported that RSV presents more evident effects when administrated in combination with Vitamin D_3 ($VitD_3$) caused by a biphasic cooperative effect in ovarian cells[23]. It was also stated that supplementation of RSV and $VitD_3$ could reduce colon cancer risk[24]. RSV has high instability (cis-isomerization[25] and very photosensitive character[26]) so its encapsulation is necessary before administration[26–28].

Vitamin D is a hydrophobic micronutrient that is required in the human diet to maintain good health and well-being[29].Calcitriol (25-dihydroxy vitamin D_3) is the biological form of $VitD_3$, which plays a critical role in osteoporosis prevention[30,31].$VitD_3$ deficiency often occurs in people who are not exposed to sufficient sunlight and with metabolic or gastrointestinal disorders.

$VitD_3$ is highly sensitive to environmental factors, such as exposure to light, heat and oxygen. It can therefore be easily oxidized, leading to loss of functionality and physiological benefits[29]. In addition, it is a molecule that generally has low water solubility and low oral bioavailability. For these reasons, $VitD_3$ is often encapsulated within colloidal delivery systems, such as nanoparticles or microparticles assembled from food grade biopolymers[5,30] or lipids[1,32].

For these exposed reasons, it would be interesting to formulate a system that allows the incorporation of both active biocompounds (RSV and $VitD_3$), while protecting them could also control their release[33,34].

Highly internal phase concentration are referred to those emulsions which its external phase do not overpass the critical Oswald number (26 % v/v), those emulsions describe a clear advantage versus creaming instability respect more diluted emulsions since the high presence of droplets surrounding each individual droplet neighbour creates hindrance droplets movements[8]. Hence internal fraction is considered for the droplet movement models prediction[35]. However, in the case of W/O/W double emulsions two internal fractions are combined simultaneously. Taking into account the density difference between phases, the presence of highly concentration of W/O droplets in the external aqueous phase will retard droplets creaming, since the lower density of the oil respect to the water is the main responsible for this phenomena, as it was reported in previous works[36]. However, it is also important to take into account that the inner water droplets will increase oil droplets viscosity producing a retard on oil droplets movement.

In previous works RSV has been satisfactory encapsulated in high concentrated double emulsions[36] with high stability. Moreover, it has also been reported the effect of concentrating both the primary W/O (increase of the content of water in the primary emulsion) and secondary WO/W emulsions (increase of the content of W/O droplets in the double emulsion) simultaneously in order to increase the amount of RSV encapsulated using concentrated double water-in-oil-in-water emulsions (W/O/W) optimizing the resulting colloidal stability and encapsulation efficiency (EE)[37].

Therefore, the aim of this work was to study the viability of encapsulating simultaneously RSV and $VitD_3$ in concentrated double emulsions with high loading capacity, regarding the synergistic effect of both encapsulated compounds on final emulsion properties. For this purpose, emulsions containing individual and combined biocompounds were synthesized at two internal emulsion (W/O) ratios: 20/80 and 30/70. All formulated emulsions were characterized in terms of droplet size distribution, zeta potential, rheology, EE and colloidal stability.

2. Materials and methods

2.1. Materials

RSV ($C_{14}H_{12}O_3$), $VitD_3$, absolute ethanol and Tween 20 were purchased from Sigma–Aldrich (USA). Miglyol® 812 (density 945 kg/m^3 at 20°C), which is a neutral oil formed by esters of caprylic and capric acids with glycerol, was supplied by Sasol GmbH (Germany). Polyglycerol Polyricineoleate

(PGPR, $C_{21}H_{42}O_6$) was supplied by Brenntag AG (Germany). Sodium chloride was purchased from Panreac (Spain). Distilled water was used for the preparation of both aqueous phases. HPLC-grade methanol, acetonitrile, 2-propanol, and acetic acid were purchased from Sigma Aldrich (USA).

2.2. Methods

Four types of emulsions were prepared: (i) blank (no encapsulated compounds), (ii) only RSV, (iii) only $VitD_3$ (iv) both RSV and $VitD_3$. Emulsions containing separate compounds (types (ii) and (iii)) were prepared in order to study the effect produced by RSV and $VitD_3$ and the possible interactions between both biocompounds.

For the preparation of the primary W_1/O emulsions two different internal ratios of W_1 in O (also represented as W_1/O) were used being 20/80 and 30/70. All the concentrated double $W_1/O/W_2$ emulsions were formulated with the same external ratio of W_1/O in W_2 (also represented as W_1O/W_2) of 80/20.

RSV was added to the internal aqueous phase W_1, in contrast, $VitD_3$for its hydrophobic nature was added to the oily phase O.

2.2.1. Water-in-oil (W_1/O) emulsion preparation

RSV is barely soluble in water, so an alcohol was added to water as solubilizing agent. The solubility of RSV in alcohol decreases as the carbon number of the alcohol increases[38]. Thus, a 20% ethanol (v/v) solution was used as the dispersed phase containing 50 mg/l of RSV. 0.1 M NaCl was added to the inner aqueous phase in all double emulsions to ensure W_1 droplet stability balancing the osmotic pressure in the system[39–41].

Miglyol 812 was used as the oil phase containing the hydrophobic emulsifier (PGPR) previously dissolved by magnetic stirring for 5 min. PGPR is commonly used in food formulation and has been demonstrated to be highly effective at stabilizing W_1/O emulsions[40,42,43].

The amount of PGPR to be dissolved is very important for the preparation of the organic phase in order to make the initial emulsion as stable as possible. Since twoW_1/O ratios were used for the preparation of the primary W_1/O emulsion, the surfactant/internal aqueous phase ratio was kept constant, to ensure that the surface of the dispersed droplets was always covered by the stabilizer,

maintaining the amount of mass per unit area constant. The concentration used in previous studies[4,36] for a simple W_1/O emulsion ratio of 20/80 was 20% (w/w) of PGPR in oil phase dispersed in the emulsion. However, previous experiments show considerable instability in maintaining the same concentration of surfactant for higher internal phase proportions, so the proportion used was 40% (w/w) for simple W_1/O emulsions preparation using the ratio of 30/70[36].

In the case of the emulsions that contained $VitD_3$, the vitamin was added to Miglyol 812 to a concentration of 50 mg/l.

Two W_1/O ratios were used 20/80 and 30/70. One hundred grams of emulsion were prepared for each test. Both phases were emulsified in glass vessels by high shear mixing (SilentCruser M Homogenizer, Heidolph, Germany) using a 6 mm dispersing tool at 2012 g for 5 min at room temperature.

2.2.2. Water-in-oil-in-water ($W_1/O/W_2$) double emulsions preparation

Thirty grams of the $W_1/O/W_2$ double emulsions were prepared by dispersing the W_1/O primary emulsion into the external aqueous phase (W_2) at a volumetric ratio of 80/20, with the intention of obtaining more concentrated double emulsions taking into account articles previously published[36,37].

The external aqueous phase W_2 was formulated using a 2% (w/v) Tween 20 solution and 0.1 M NaCl in order to match the osmotic pressure between W_1 and W_2, in all emulsions.

Emulsification was carried out by mixing the continuous and dispersed phases with the before mentioned Homogenizer at 224 g for 2 min.

The conditions of agitation are considerably milder than in the case of simple emulsion because applying too much energy could break the initial emulsion and, thus, the internal aqueous phase would migrate towards the external one, obtaining a simple O/W emulsion.

Emulsions were stored at controlled temperature of 20°C for six weeks for further characterization.

2.2.3. Emulsion characterization

2.2.3.1. Droplet size distribution

156 Emulsion droplet size distributions were obtained by the laser light scattering in a Mastersizer S long
157 bench apparatus (Malvern Instruments, Ltd., UK).

158 For single W_1/O emulsion measurements, the samples were dispersed in paraffin oil, whereas
159 $W_1/O/W_2$ double emulsion samples were diluted with deionized water. The refractive index used for
160 measuring W_1/O emulsions droplet size were 1.3300 for the aqueous internal phase and 1.4500 for
161 the oil external phase, while for measuring $W_1/O/W_2$ emulsions droplet size 1.4500 was used for the
162 W_1/O drops and 1.3300 for the aqueous external phase.

163 Three replicates were performed for each emulsion and the results were reported as droplet size
164 distribution in μm.

165 The size results are expressed in terms of equivalent spherical diameter (the diameter of a sphere
166 of the same volume as the measured particle) although for the emulsions the spherical shape can
167 be assumed.

168 2.2.3.2. Visual inspection

169 Micrographs of the emulsions were obtained with an Olympus BX50 light microscope (Olympus,
170 Japan) with 10–100× magnification using UV–vis and fluorescence lamps. Micrographs were used
171 for emulsions visual inspection and, with the proper scale, to confirm the droplet size obtained by
172 laser light scattering.

173 2.2.3.3. Colloidal stability

174 Emulsion stability at 30°C was analysed by measuring backscattering (BS) and transmission (TS)
175 profiles in a Turbiscan apparatus (Formulaction, France). Twenty ml were placed on the equipment
176 cells, transmitted and backscattered light was monitored as a function of time and cell height for six
177 weeks. The optical reading head scans the sample in the cell, providing TS and BS data every 40
178 μm in percentage relative to the standard (suspension of monodisperse spheres and silicone oil) as
179 a function of the sample height (in mm), being the total height occupied by the sample 40 mm. These
180 profiles provide useful information about changes in droplet size distribution, appearance of a
181 creaming layer or a clarification front with time[44–46].

The Turbiscan Stability Index (TSI) is the sum of all the variations detected in the samples in terms of size and/or concentration, and is defined by the following equation, where H is the total height of the cell at i interval time and is defined by equation 1.

$$TSI = \sum_i \frac{\sum_i |scan_i - scan_{i-1}|}{H} \qquad \textit{Equation 1}$$

2.2.3.4. Zeta potential

A ZetasizerNanoZS (Malvern Instruments Ltd., UK) was utilized for zeta potential (ς) measurements of the $W_1/O/W_2$ double emulsions. Two replicates were conducted for each sample at room temperature.

2.2.3.5. Interfacial tension

Surface and Interfacial tension (γ) were measured at room temperature following the Du Noüy's platinum ring method using a Sigma 700 tensiometer (KSV Instruments Ltd., Finland).

2.2.3.5. Rheology

The rheological tests were carried out with a MARS II rotational rheometer (Haake). All the analyses were carried out at room temperature and a plate/plate measuring system (PP35) with a gap of 1 mm was employed. Samples rested for at least 5 min previous to any measurement, allowing the stresses induced during sample load to relax. All measurements were replicated twice and the measured data were processed by the HaakeRheowin 4.0 Software.

Steady-state flow measurements were carried out from 0.01 to 500 s^{-1} Pa in 500 s at 25°C, shear stress versus shear rate data were recorded.

Oscillatory measurements were carried out from 0.1 to 10 Hz at a constant shear stress of 1 Pa and at 25°C. The storage modulus (G') and loss modulus (G'') were recorded versus frequency (rad/s).

2.2.4. Determination of the initial encapsulation efficiency (EE) by RP-HPLC analysis

The biocompounds (RSV and $VitD_3$) content in the external aqueous phases was determined by chromatography (HP series 1100 chromatograph, Hewlett Packard, USA). The system was

equipped with a UV-vis absorbance detector HP G1315A or a fluorescence detector 1260 Infinity A (Agilent Technologies, USA).

The separation was performed with a Zorbax Eclipse Plus C18 reversed phase column, with a particle size of 5 μm and 4.6 mm × 150 mm (Agilent Technologies, USA).

The mobile phase consisted of a mixture of (A) 100% milliQ-water and (B) 100% methanol with gradient elution at a flow rate of 0.8 mL/min. The step gradient started with 80% mobile phase (A) running 100% of mobile phase (B) in minute 5 for 10 min. The mobile phase (B) was run for 2 min after each injection to prepare the column for the next run. Separation was carried out at room temperature. A wavelength of 305 nm was used by the UV-vis detector while the fluorescence detector was used at $\lambda_{excitation}/\lambda_{emission}$ of 310/410 nm. The column was cleaned after each analysis by first running the mobile phase (A) for 20 min and a mobile phase (C) consisting of 50% acetonitrile, 25% milliQ-water, 25% 2-propanol, and 0.01% acid acetic for 40 min at a flow rate of 0.25 mL/min. Finally, the column was rinsed with 50% of the mobile phase (A) and 50% of the mobile phase (B) for another 20 min.

The emulsions external aqueous phases injected in the RP-HPLC were previously recovered by centrifugation of the double emulsion at 8944 g for 10 min. The recovered samples was diluted with methanol in volumetric ration 1:1 in order to increase RSV solubility in the phase, then the mixture was filtrated with a 0.22 μm polyvinylidene difluoride syringe filter to remove all the oil phase that could be still present.

The recovery yield (R_y) of RSV after the centrifugation and filtration stages was used to determine the amount of biocompound lost during separation processes, since other authors reported on the importance of taking into account to determine the EE values[4,47]. For this purpose, a standard emulsion, where all W_1 has migrated to W_2 (with a 0 % EE) was prepared. Therefore, an oil-in-water (O/W_2) emulsion was prepared following the procedure previously described, and then W_1 phase was added, simulating that all RSV transferred to W_2. The theoretical RSV concentration for this case is denominated the maximum concentration expected (C_0) in equations 2 and 3. The RSV concentration measured in the separated aqueous phase from these simple emulsions prepared and

232 determined by RP-HPLC was denominated blanch concentration (C_{Blanch}). The recovery yield, Ry, of
233 the process was calculated by equation 2:

$$.R_y(\%) = \frac{C_{blanch}}{C_0} \cdot 100 \qquad \textit{Equation 2}$$

234 The encapsulation efficiency (EE) of these double emulsions was defined as the percentage of
235 encapsulated biocompound that remained in the W_1/O primary emulsion after the second
236 emulsification step. It was calculated by equation 3.

$$.EE(\%) = 100 - \frac{C_{recovered} \cdot 100}{C_0 \cdot R_y} \qquad \textit{Equation 3}$$

237 where $C_{recoverd}$ is the RSV concentration measured in W2 by RP-HPLC after centrifugation of each
238 individual double emulsion.

239 **3. Results and discussion**

240 The emulsions were characterized in terms of droplet size distribution, morphology, stability and
241 encapsulation efficiency. These parameters were evaluated weekly for six weeks.

242 **3.1. Droplet size distribution**

243 First of all, the droplet size distribution of the primary (W_1/O) emulsions was analysed (Figure 1).

244 It can be seen that there is a clear difference in the size distribution due to the different W_1/O ratios
245 used. The emulsion prepared using a 20/80 ratio had a narrower droplet size distribution, with a
246 single peak near the value of 3µm, while the emulsion prepared using a 30/70 ratio showed wider
247 size distribution that goes from 1.5 to 13 µm, what could be to for the larger internal aqueous volume
248 what could produce less efficient agitation.

249 **Figure1.Droplet size distribution of W_1/O emulsions.**

250 The results of the droplet size distribution for the concentrated $W_1/O/W_2$ double emulsions prepared
251 using each type of the primary emulsions are shown in Figure 2A (internal W_1/O ratio of 20/80) and
252 Figure 2B (internal ratio of 30/70).

Figure 2. Droplet size distribution of the double $W_1/O/W_2$ emulsions formulated with an internal phase of W_1/O emulsion in fixed ratio 20/80 (A) or 30/70 (B) varying the encapsulated biocompound.

It has been clearly observed that the presence of an encapsulated biocompound did not affect the droplet size of the final concentrated double emulsion. Zeta potential of all formulated emulsions with and without encapsulated biocompound is presented in Table 1. It was observed that the presence of biocompound does not affect the emulsion zeta potential and hence its stability, according to electrostatic repulsion. For emulsions formulated with both internal ratios containing only RSV encapsulated presented the lower zeta potential values. In all cases zeta potential had negative values and lower than -30 mV what is an indication that the formulated emulsions presented electrostatic stability despite the use of non-ionic surfactant in the formulations.

Table 1. Zeta potential and Span values of formulated double emulsions

However, there was an evident difference in their droplet size distributions regarding the different internal phase ratios. When the 20/80 W_1/O internal phase ratio was used two clearly differentiated peaks were observed in Figure 2A, one (between 0.1 and 1 μm) probably due to the formation of excess surfactant agglomerates in the external aqueous phase W_2 and another due to the droplets present in the double emulsion around 13 μm. This bimodal distribution has been observed in previous studies were double $W_1/O/W_2$ emulsions were prepared using the same ratio for the primary emulsion[3,4,36,47]. However, when the concentrated double emulsions were prepared with the 30/70 W_1/O ratio, two peaks were also observed in Figure 2B but the proximity of the smaller peak to the larger one, indicated that both of them correspond to a wider oil drops size distribution. However, the larger peak has slightly lower main value (9 μm) than the one obtained with emulsions 20/80 W_1/O ratio. Span of all formulated emulsions is presented in Table 1, smaller values registered for emulsions prepared with internal ratio of 30/70 corroborate the proximity of the two peaks observed on the droplet size distributions of these emulsions. Span values varied from 1.5 to 1.8 for fresh emulsions prepared using an internal ratio of 20/80 while values from 0.9 to 1.0 were obtained for fresh emulsions formulated with an internal ratio of 30/70.

Figures 3 and 4 present the optical images of the fresh concentrated $W_1/O/W_2$ emulsions prepared with the two W_1/O ratios used (20/80 and 30/70). Optical microscope corroborated the presence of double emulsions. Both Figures showed very small water droplets (W_1) inside the bigger oil droplets (O) dispersed in the external aqueous phase (W_2). It has also been observed that when the 30/70 internal emulsion ratio was used the double emulsions had smaller droplet size in all cases.

Figure 3. Influence on morphology according to the type of emulsion prepared with 20/80 ratio of internal phase at 40x: (A) Double emulsion without encapsulated compound; (B) Double emulsion containing RSV; (C) Double emulsion containing $VitD_3$; (D) Double emulsion with RSV and $VitD_3$.

Figure 4. Influence on morphology according to the type of emulsion prepared with 30/70 ratio of internal phase at 40x: (A) Double emulsion without encapsulated compound; (B) Double emulsion containing RSV, (C) Double emulsion containing $VitD_3$; (D) Double emulsion with RSV and $VitD_3$.

Values lower than 2 mN/m were registered for all interfacial tension measurements, indicating a low tension and hence easy emulsified capacity for all systems. Moreover, surface tension measurements of all systems were measured and any influence was observed by the presence of biocompounds on them. Values between 40 and 42 were obtained for the internal aqueous phases (W_1), while values between 29-30 mN/m were registered for the oil phases used being 35.5 mN/m the value observed for the external aqueous phase (W_2). The fact that the presence of biocompound do not affect surface tension of the different phases indicate that its presence did not affect the emulsification properties of the systems.

Weekly evolution of the droplet size distribution for all the double emulsion formulated is shown in **Figure 5**.

Size distribution of the concentrated double emulsions formulated without any compound encapsulated did present significant changes during the 6 weeks studied for emulsions prepared with an internal W_1/O ratio of 20/80 (Figure 5A). However, when the 30/70 ratio was used, slightly wider size distributions with time were observed for the final $W_1/O/W_2$ emulsions, indicating faintly coalescence (Figure 5B). Span values (Table 1) remained constant with time for all emulsions

formulated with an internal phase ratio of 20/80 while for emulsions prepared with an internal phase ratio of 30/70 an increase of span value with time was observed for samples formulated with and without biocompounds encapsulated, span values increase from 0.9-1.0 to 1.5-1.7, indicating the presence of a wider droplet size distribution and hence instability.

Figure 5. Influence of time (weekly evolution) on droplet size distribution of double emulsions with internal phase of 20/80 ratio (left column) or 30/70 ratio (right column) without encapsulated biocompound (A and B), with encapsulated RSV (C and D), with encapsulated VitD$_3$ (E and F) and with encapsulated RSV and VitD$_3$ (G and H).

No changes on droplet size distribution were appreciated when double emulsions were prepared loaded with RSV and VitD$_3$ respectively using a 20/80 W$_1$/O internal ratio for primary emulsions (Figure 5C and 5E). However, a 30/70 W$_1$/O ratio was used a substantial variation was also observed (Figure 5D and 5F) regarding a wider size distribution indicating oil droplets coalesce.

For W$_1$/O/W$_2$ emulsions encapsulating both compounds simultaneously (Figure 5G and 5H) droplet size distribution was appreciated to be constant when using a W$_1$/O internal ratio of 20/80 as was the case when individual biocompounds were encapsulated. Surprisingly, for the double emulsion prepared using 30/70 W$_1$/O ratio the droplet size distribution seemed to remain constant what was not the case for individual biocompounds encapsulations.

Size distribution for double emulsions encapsulating only VitD$_3$ (Figure 5E and 5F) showed slight variations with time for both W$_1$/O ratios studied. However, the main droplet size was not affected.

Figure1S of Supplementary material present an optical image of all double emulsions formulated after 6 weeks, for both internal phase ratios. There was no clear evidence of the influence of time on morphology for all emulsion prepared. The images did not show significant changes in distribution or droplet size after 6 weeks. However, it was evidence that the average oil droplet size was clearly smaller for emulsions with a W$_1$/O ratio of 30/70 versus those of 20/80.

3.2. Colloidal stability

Stability was evaluated through the TSI value evolution during six weeks (Figure 6). The lower the values of TSI the higher the emulsion stability, i.e. it reflects minor changes in the emulsion during ageing[48].

Figure 6. Evolution of TSI values during 45 days for double emulsions formulated with a fixed volumetric ratios of W_1/O dispersed in W_2 (80/20) with different proportion of W_1 dispersed in O: 20/80 (A) or 30/70 (B) and different encapsulated biocompounds.

Figure 6 showed that concentrated double emulsions formulated using a W_1/O ratio of 20/80 had a greater stability, with TSI values varying from 5 to 10 after 6 weeks (Figure 6A), than those prepared using a 30/70 ratio that showed variations from 40 to 55 after 6 weeks (Figure 6B). This indicates that the W_1/O ratio used for the preparation of the primary emulsion had larger effect on emulsion stability than the encapsulated biocompound. This effect was also observed when the span value was evaluated, since an increase on span value with time was observed for emulsions formulated with an internal emulsion ratio of 30/70 and it was not the case for emulsions prepared with 20/80.

The presence of biocompounds in the primary emulsions formulated with a 20/80 internal ratio gave higher stability to the final concentrated double emulsions, up to 20 days. After this point, emulsions with only $VitD_3$ encapsulated presented the lowest stability (larger TSI values)

The presence of biocompounds in the W_1/O emulsions formulated with a 30/70 internal ratio increased also the stability of the double emulsions up to 20 days, although the TSI values were 10 times higher than those prepared with a 20/80 internal ratio, indicating significantly higher instability for the ones formulated with 30/70 W_1/O ratio.

Measurement of the clarification height for all the formulated double emulsions was carried out using Turbisoft software, results are presented in Figure 7.

Figure 7. Clarification height observed during 45 days for samples with internal phase ratio of 20/80 (A) and internal phase ratio of 30/70 (B)

The clarification of all the concentrated double emulsions formulated using a 20/80 internal ratio is negligible (lower than 1 mm). Only the very last days a sudden increase up to 1.2 mm of the clarification height is observed for emulsion containing only $VitD_3$ encapsulated. For the W_1/O/W_2

emulsions prepared with a 30/70 internal ratio the clarification height varied with time stabilising at 9-10 mm after 20 days. This indicated that TSI values cannot be attributed to the migration of oil droplets to the surface, hence any other destabilization phenomena takes place at the same time.

Droplets of the dispersed phase tend to migrate to the top of the cell, separating from the continuous phase in which they are present. This process is known as creaming. Larger particles will tend to migrate to the surface more quickly (according to Stoke's law). On the other hand, a higher density difference between the droplets and the continuous phase would increase the droplets migration velocity.

According to the theoretical behaviour described, double emulsions formulated with a 30/70 internal ratio would be more stable against creaming than the ones prepared with 20/80. However, experiments showed the opposite trend regarding a higher clarification front observed at the bottom part of the cell for emulsions prepared with a W_1/O ratio of 30/70, which corroborates that the observed creaming phenomenon is not only due to migration of the drops and there is another instability phenomenon that is taking place simultaneously.

Backscattering profiles (Figure 2S and 3S of Supplementary Material) indicate droplet coalesce for emulsions prepared with a 30/70 internal phase ratio, especially for those without biocompound encapsulated (indicated by the backscattering difference observed at the medium part of the cell along time). In addition, in the upper part of emulsions with internal phase ration of 30/70 a decrease in backscattering along time, determined as a second clarification layer. This upper clarification layer was assumed to be to the presence of a small layer of free oil as a consequence of oil drops coalescence and migration.

Moreover, clarification height observed at the bottom part of the cell registered for 30/70 internal phase ratio emulsions indicated that part of inner water phase (W_1) has migrated to the external water phase (W_2), since the bottom clarification height was larger than expected if just all W_2 will be at the bottom of the cell. The formation of this water layer at the bottom of the cell was reported in previous works where the stability of W_1/O emulsions was studied using different types of emulsifiers at 5% (w/v) for the formulation of double W_1/O/W_2 emulsions containing RSV[4].

On the other hand, double emulsions formulated with a 20/80 W_1/O ratio did not present any significant coalescence phenomenon, since backscattering profiles did not present significant changes on the middle part of the cell and the thickness of the top clarification layer is nearly negligible and any free oil was observed at naked eye.

Recently the swelling and deswelling of inner droplets of double emulsions was modelled by other authors by the use of osmotic pressure[49–51]. The use of different salinity in both aqueous phases of double emulsions verified the effect of the sweeling and deswelling phenomena in order to equal salinities of both phases[49]. However, in the present study deswelling will be expected when a biocompound is encapsulated while for emulsions with non biocompound encapsulated any swelling or deswelling behaviour will be estimated since salinity was the same in both aqueous phases.

Moreover, the same authors validated a model to evaluate inner droplets deswelling rate (W_1 migrate to W_2), using two main forces: Separation force governed by the interfacial tension between phases and resistance force governed by oil viscosity.

These two models indicate that for big oil drops the effect of the W_1 drop size had a minor importance. However, for small oil droplets (a few microns) the effect of inner water drop diameter had a major influence. The W_1 deswelling rates sharply increases in double emulsions with small oil droplets, with large W_1 droplets[49].

According to he mentioned models, it can be assumed that in the present study, the W_1 droplets from emulsions prepared with an internal ratio of 30/70 will leave easily oil drops to escape into the external W_2 phase than those emulsions formulated with a W_1/O ratio of 20/80 and this phenomena can be attributed to the highest bottom clarification layer observed on emulsions prepared with internal phase ratio of 30/70 respect the ones with 20/80. Moreover, it is important to point out that the similar droplet size obtained between W_1/O emulsions (range between 1.5 and 13 µm) and O/W_2 emulsions (range between 2-22 µm) when an internal ratio of 30/70 was used what could enhance W_1 escape from oil droplets

3.3 Rheology behavior

Emulsions were characterized in terms of rheology. Curves flows for emulsions with and without encapsulated biocompounds with W_1/O ratio of 20/80 and 30/70 are presented in Figure 8. It was observed that all emulsions presented a pseudoplastic behavior. Emulsions with W_1/O ratio of 20/80 presented lower viscosity than emulsions with W_1/O ratio of 30/70 in all cases. The presence of the biocompounds had minor influence on emulsion viscosity, without a clear trend observed for emulsions with W_1/O ratio of 20/80, but regarding a decrease for emulsions with W_1/O ratio of 30/70.

Figure 8. Flow curves of emulsion with W_1/O ratio of 20/80 (A) and 30/70 (B)

Storage modulus or elastic modulus (G') and loss or viscous modulus (G'') were recorded for a frequency range of 0-63 rad/s, for emulsions with W_1/O ratio of 20/80 and 30/70 and presented in Figure 9. For the range studied all emulsions showed larger elastic than viscous behaviour. Similar viscous modulus values were observed for all emulsions formulated but large difference was observed regarding elastic modulus. Emulsions with W_1/O ratio of 20/80 presented values more than twice the registered for emulsions with W_1/O ratio of 30/70. Moreover, for both type of emulsions the presence of biocompound increased viscous modulus values, but it was not found a clear trend between regarding the effect produced by the biocompound encapsulated.

Figure 9. Elastic modulus (G ') and viscous modulus (G ") versus frequency for double emulsions $W_1/O/W_2$ formulated with an internal phase of W_1/O of 20/80 (A) and 30/70 (B)

3.4 Encapsulation efficiency (EE)

The double emulsions that contained $VitD_3$ did not show any evidence of the compound recovery, implying EE values of 100%. This was the expected result considering the low solubility of $VitD_3$ in water[52].

The EE values obtained of all the concentrated double emulsions formulated were calculated and are shown in Figure 10 (A) and the RSV encapsulated concentration on the emulsions with time is presented in Figure 10 (B).

Figure 10. (A) Encapsulation efficiency and (B) Resveratrol loaded concentration for emulsions with an internal phase concentration W_1/O of 20/80 or 30/70.

RSV EE decreased with time in all cases. Initial RSV EE varies from 62.9 to 69.2 % without a clear influence of the internal phase ratio used (20/80 or 30/70). Moreover, the presence of $VitD_3$ does not offer a clear influence on the initial RSV EE of the formulated emulsions. However, the encapsulation of $VitD_3$ presents an advantage on the RSV release with time. RSV release was retarded when $VitD_3$ was simultaneously encapsulated in double emulsions, especially for the case of the emulsions formulated with W_1/O ratio of 20/80 for the six weeks studied. Significant retarded release was observed after one week of storage, from them on differences on RSV EE between double emulsions with only RSV and double emulsions with RSV and $VitD_3$ were less significant. After one week of storage, RSV encapsulated increased from 4.0 to 4.4 mg RSV/L for emulsions formulated with a W_1/O ratio of 20/80, while an increase from 4.6 to 8.7 mg/L was obtained for the emulsions formulated with W_1/O ratio of 30/70 when $VitD_3$ and RSV were encapsulated simultaneously respect when just RSV was used. From them on, the presence of $VitD_3$ on the emulsion increases RSV emulsion loading capacity, the differences observed are lower (between 0.3-2.0 mg/L)

Despite the similar initial EE values obtained for all formulations, the emulsions formulated with a W_1/O ratio of 30/70 presented higher loading capacity (Figure 10B) due to the higher amount of W_1, with values in the range 7.5-8.4 mg/L, while for emulsion with W_1/O ratio of 20/70 the loading capacity obtained varied from 5.0 to 5.5 mg/L, being in good agreement with concentrations obtained in previous studies[36,37]. After six weeks of storage emulsions presented a loading capacity of 2.6-3.2 mg/L for emulsions with W_1/O ratio of 20/80 and 4.1-4.6 mg/L for emulsions formulated with a W_1/O ratio of 30/70.

There was no release of $VitD_3$ observed during all the period studied as expected due to its high hydrophobicity. As consequence, the concentration was constant with values of 28 and 32 mg/L $VitD_3$ for emulsions formulated with a W_1/O ratio of 20/80 and 30/70, respectively.

4. Conclusions

The viability of preparing concentrated $W_1/O/W_2$ double emulsions containing either RSV or $VitD_3$ and both simultaneously with an external W_1O in W_2 ratio of 80/20 has been demonstrated when using primary W_1/O emulsions formulated with internal ratios of W_1 in O of 20/80 and 30/70.

The colloidal stability of the formulated double emulsions was higher for those prepared using an internal W_1/O ratio of concentration 20/80 than for those with primary emulsion W_1/O 30/70.

Initial RSV EE of the concentrated double emulsions increases with increasing the internalW_1/O ratio. However, these emulsions with higher internal phase showed higher instability and therefore led to lower RSV EE values after one week. The initial RSV EE values were between 62.9 and 69.2% and it decreased asymptotically down to 32-40%. Initial RSV concentrations in the concentrated double emulsions formulated varied from 4.6 to 8.2 mg/L while for $VitD_3$ values of 32 and 28 mg/L were obtained.

The presence of $VitD_3$ in emulsions do not significantly increased the initial RSV EE values but offers a clear advantage on the retarded RSV release, especially from one to three weeks of storage. Moreover, according to the stability results of the concentrated double emulsions formulated, it was evidenced that the presence of $VitD_3$ in the oily phase could significantly improve the colloidal stability in comparison to those emulsions that only contained RSV. Therefore, the $W_1/O/W_2$ emulsions formulated in this work could be suitable to be incorporated into food or cosmetic products in order to supplement in a control manner a considerable amount of both RSV and $VitD_3$increasing the health benefits on the human body.

5. Acknowledgments

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Figure Legends

Figure 1.Droplet size distribution of W_1/O emulsions.

Figure 2. Droplet size distribution of the double $W_1/O/W_2$ emulsions formulated with an internal phase of W_1/O emulsion in fixed ratio 20/80 (A) or 30/70 (B) varying the encapsulated biocompound.

Figure 3. Influence on morphology according to the type of emulsion prepared with 20/80 ratio of internal phase at 40x: (A) Double emulsion without encapsulated compound; (B) Double emulsion containing RSV; (C) Double emulsion containing $VitD_3$; (D) Double emulsion with RSV and $VitD_3$.

Figure 4.Influence on morphology according to the type of emulsion prepared with 30/70 ratio of internal phase at 40x: (A) Double emulsion without encapsulated compound; (B) Double emulsion containing RSV, (C) Double emulsion containing $VitD_3$; (D) Double emulsion with RSV and $VitD_3$.

Figure 5. Influence of time (weekly evolution) on droplet size distribution of double emulsions with internal phase of 20/80 ratio (left column) or 30/70 ratio (right column) without encapsulated biocompound (A and B), with encapsulated RSV (C and D), with encapsulated $VitD_3$ (E and F) and with encapsulated RSV and $VitD_3$ (G and H).

Figure 6. Evolution of TSI values during 45 days for double emulsions formulated with a fixed volumetric ratios of W_1/O dispersed in W_2 (80/20) with different proportion of W_1 dispersed in O: 20/80 (A) or 30/70 (B) and different encapsulated biocompounds.

Figure 7. Clarification height observed during 45 days for samples with internal phase ratio of 20/80 (A) and internal phase ratio of 30/70 (B)

Figure 8. Flow curves of emulsion with W_1/O ratio of 20/80 (A) and 30/70 (B)

Figure 9. Elastic modulus (G ') and viscous modulus (G '') versus frequency for double emulsions $W_1/O/W_2$ formulated with an internal phase of W_1/O of 20/80 (A) and 30/70 (B)

Figure 10. (A) Encapsulation efficiency and (B) resveratrol loaded concentration for emulsions with an internal phase concentration W_1/O of 20/80 or 30/70.

Table 1. Zeta potential and Span values of formulated double emulsions.

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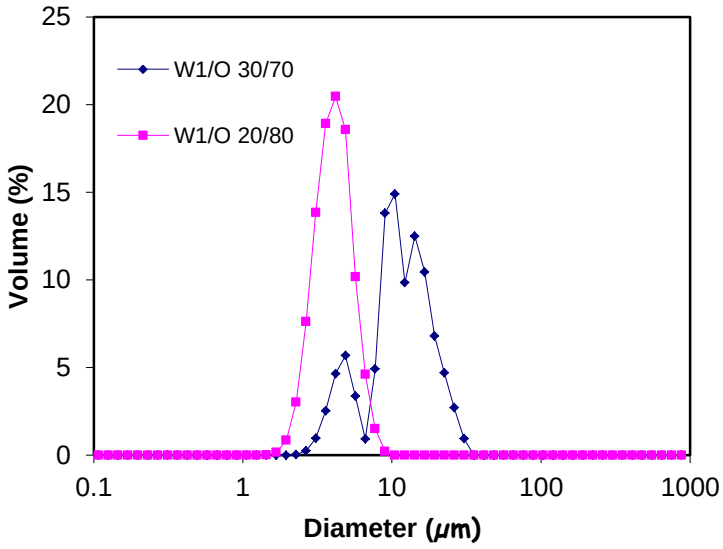

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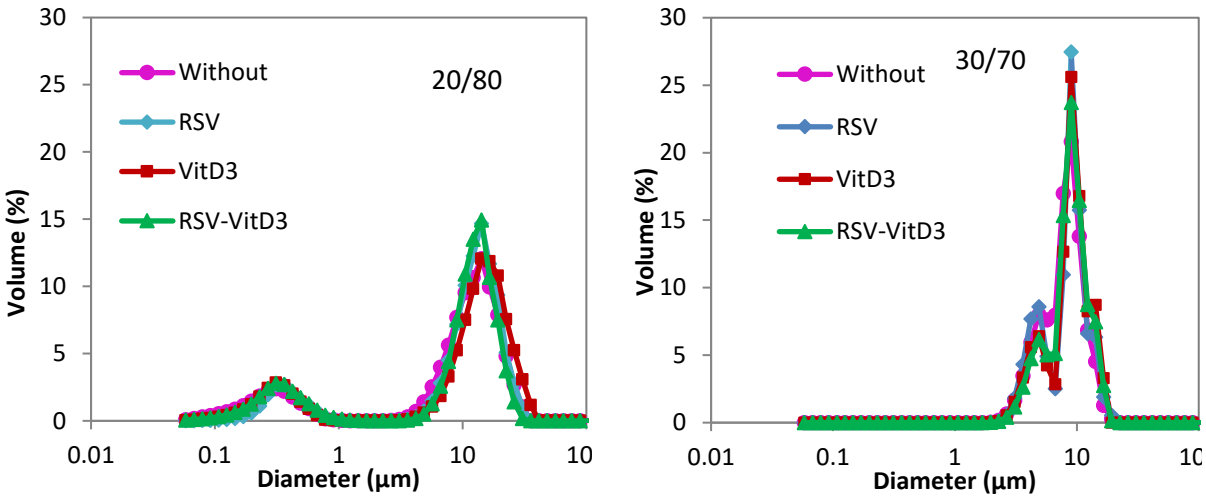
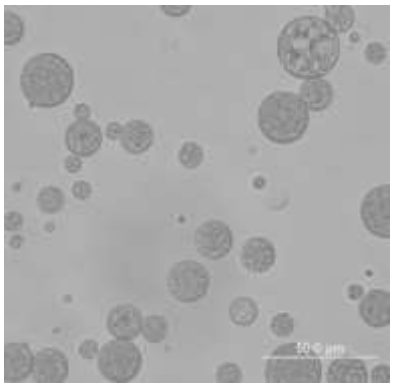


(A) (B)

Figure 2



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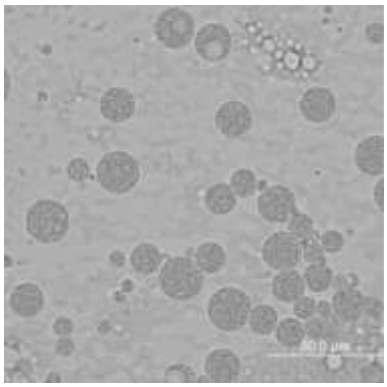

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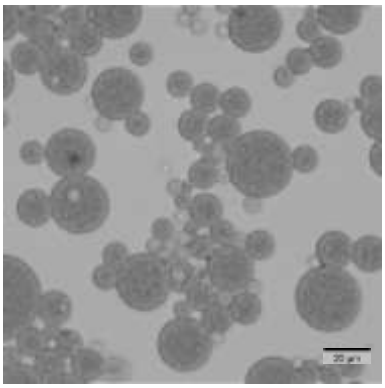

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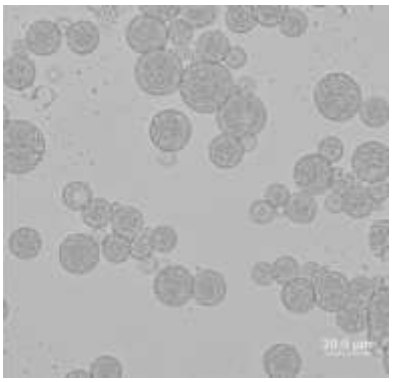
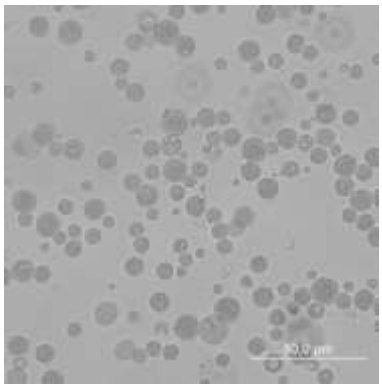


(D)

Figure 3



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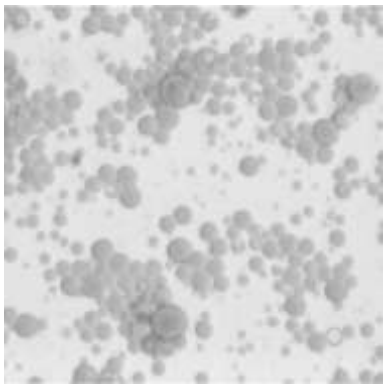

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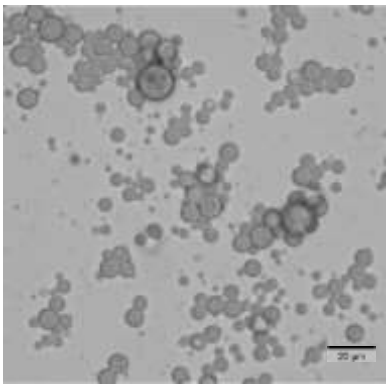

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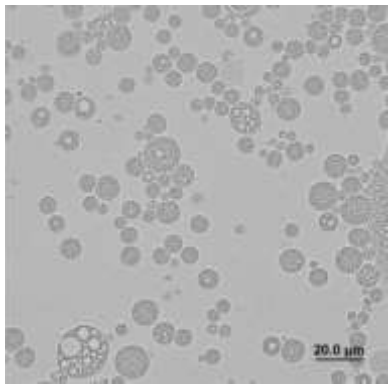

(D)

Figure 4

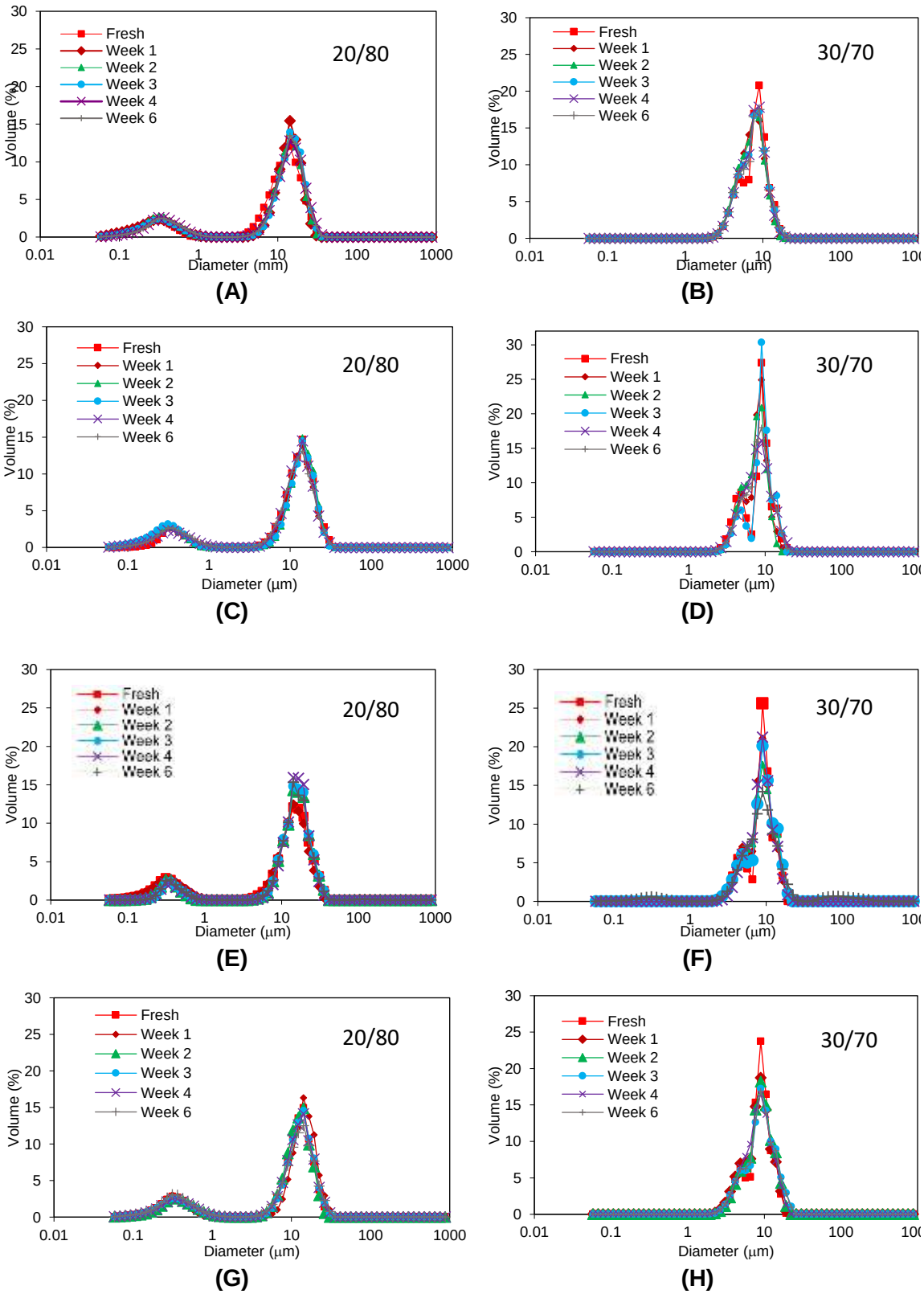
Fresh
Week 1
Week 2
Week 3
Week 4
Week 6
20/80
30/70
Volume (%)
Diameter (mm)
Diameter (µm)
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)

Figure 5

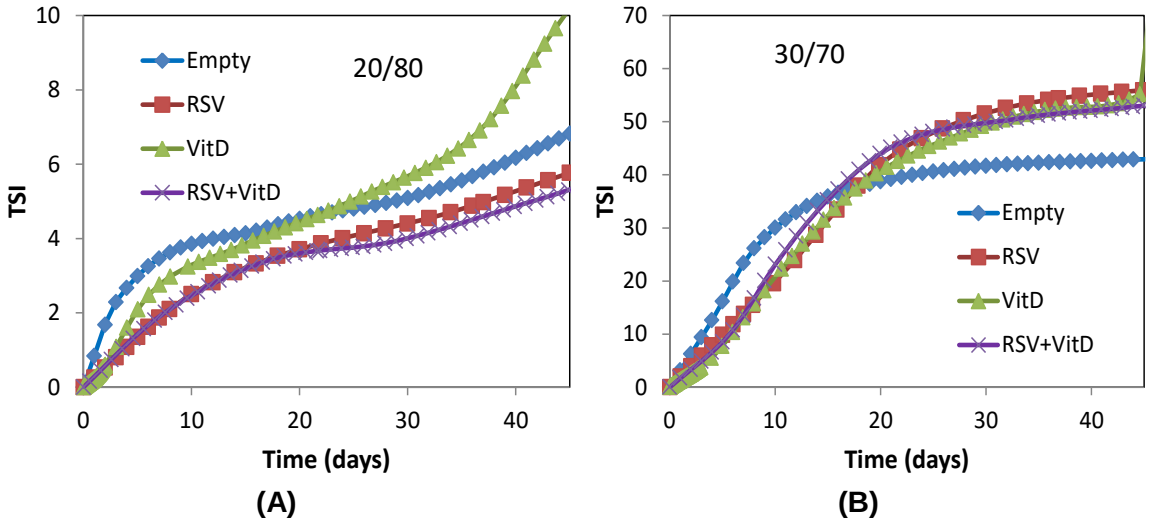

Figure 6

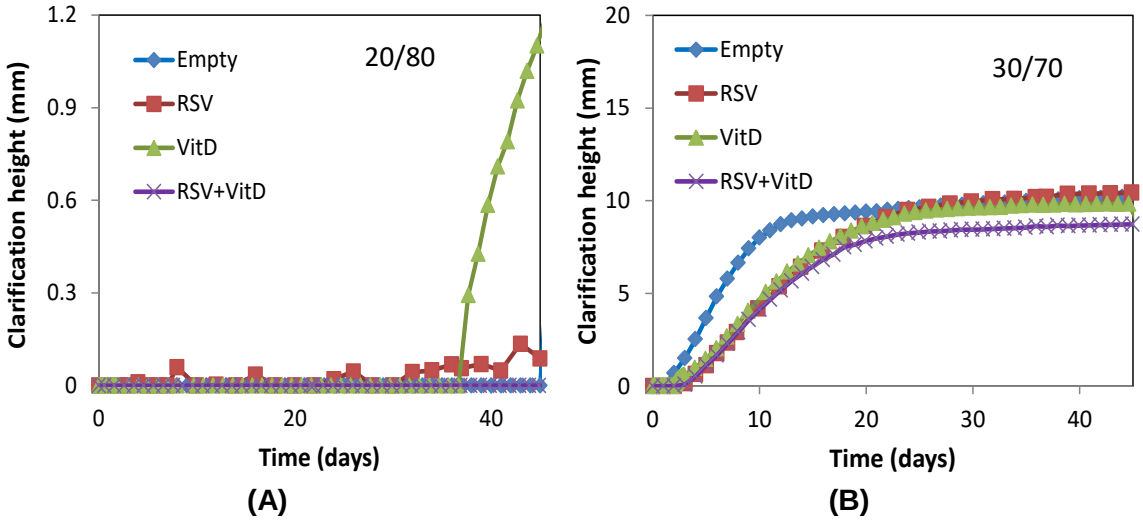

Figure 7

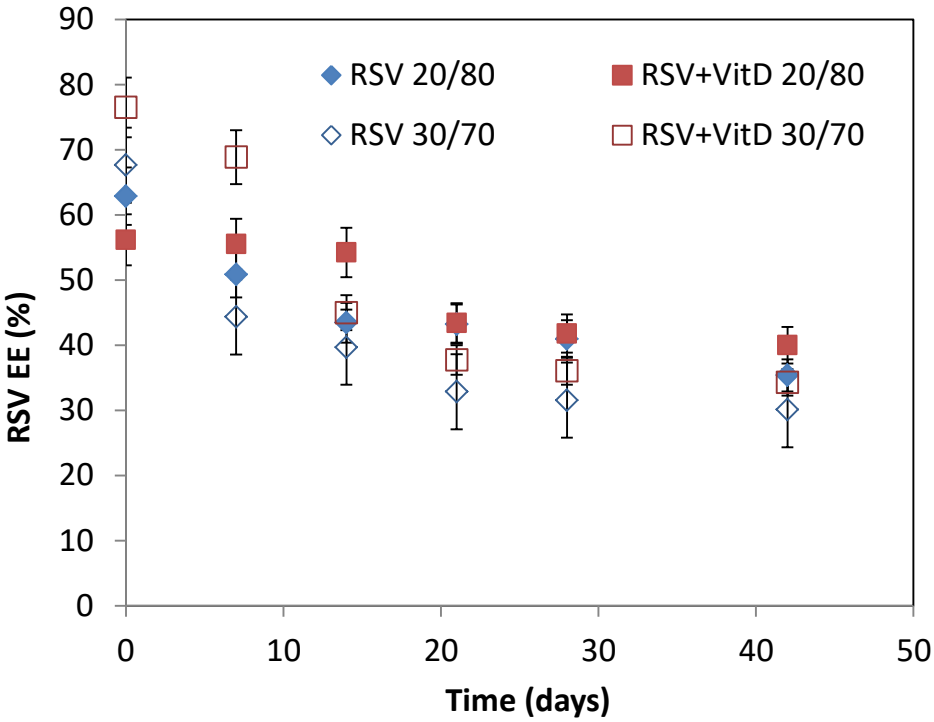
RSV 20/80
RSV+VitD 20/80
RSV 30/70
RSV+VitD 30/70
RSV EE (%)
Time (days)
0
10
20
30
40
50
60
70
80
90

Figure 8

Preparation of water-in-oil-in-water ($W_1/O/W_2$) concentrated double emulsions containing biocompounds of different nature: trans-resveratrol and vitamin D_3

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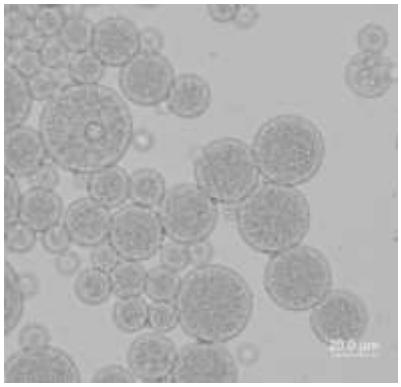
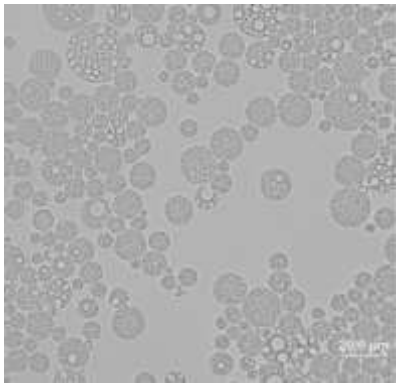


(A)



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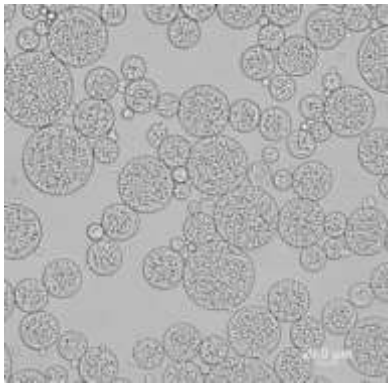
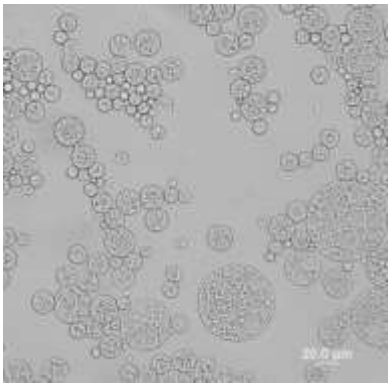


(C)



(D)

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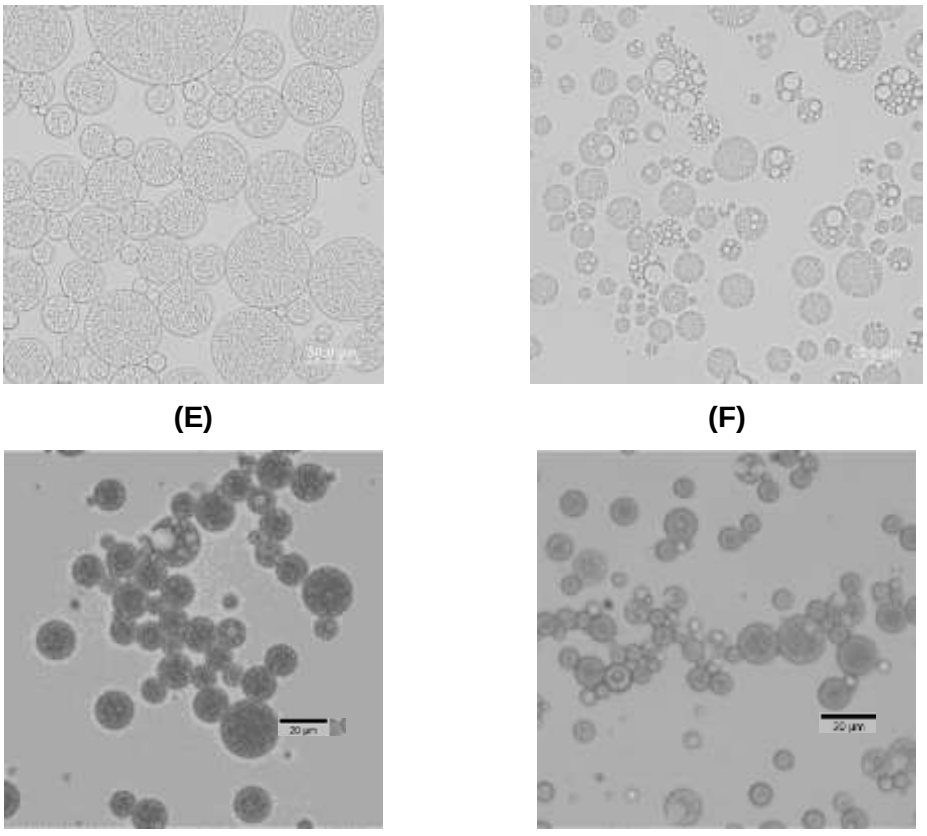
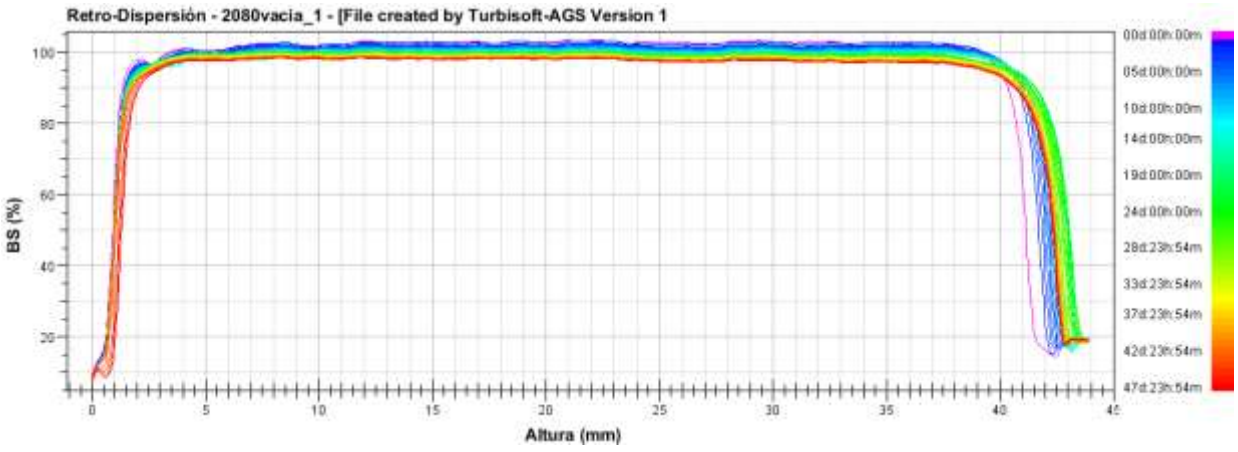


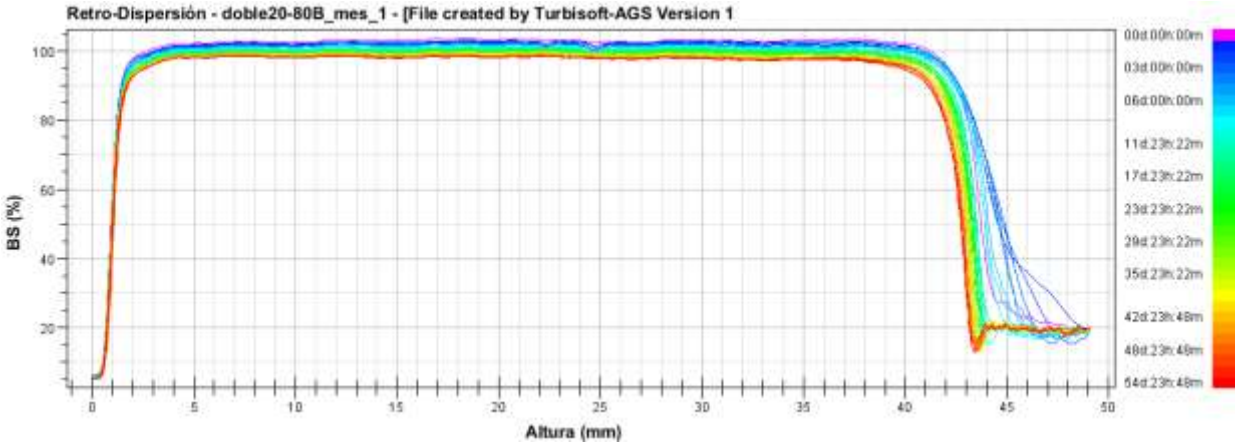
(E) **(F)**

(G) **(H)**

Figure 1S. Optical image of emulsions formulated after 6 weeks, for internal phase ratio 20/80 (left column) and 30/70 (right column) without encapsulated biocompound (A and B), with encapsulated RSV (C and D), with encapsulated VitD$_3$ (E and F) and with encapsulated RSV and VitD$_3$ (G and H).



(A)



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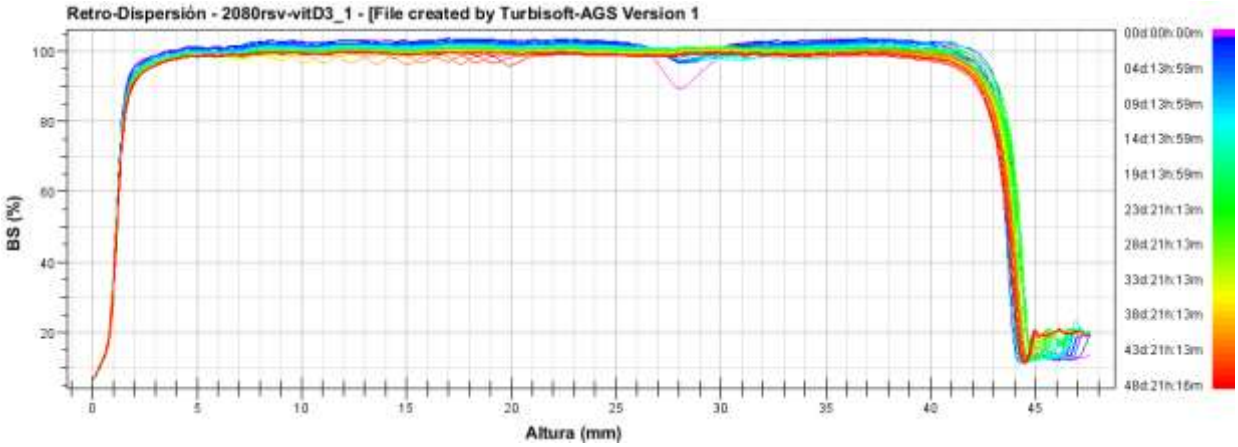

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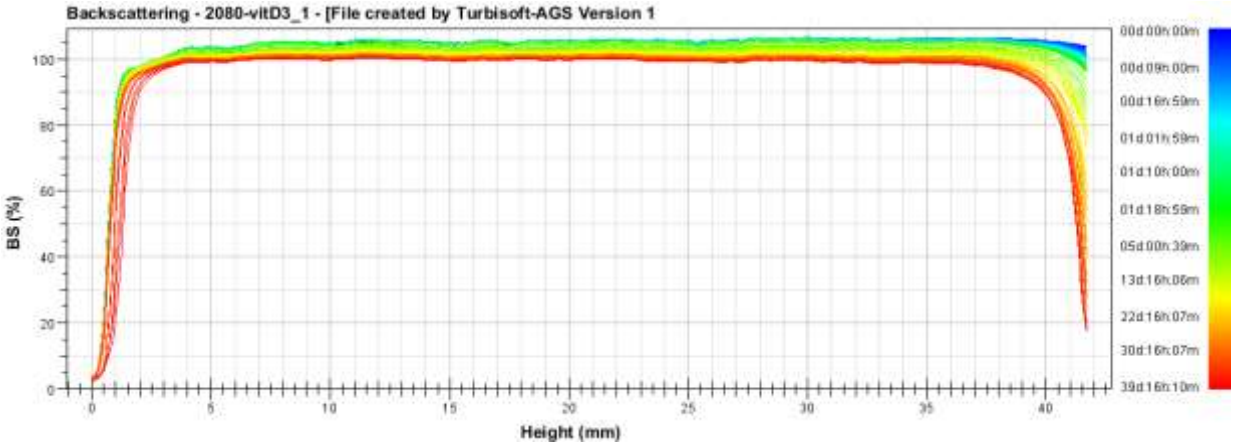
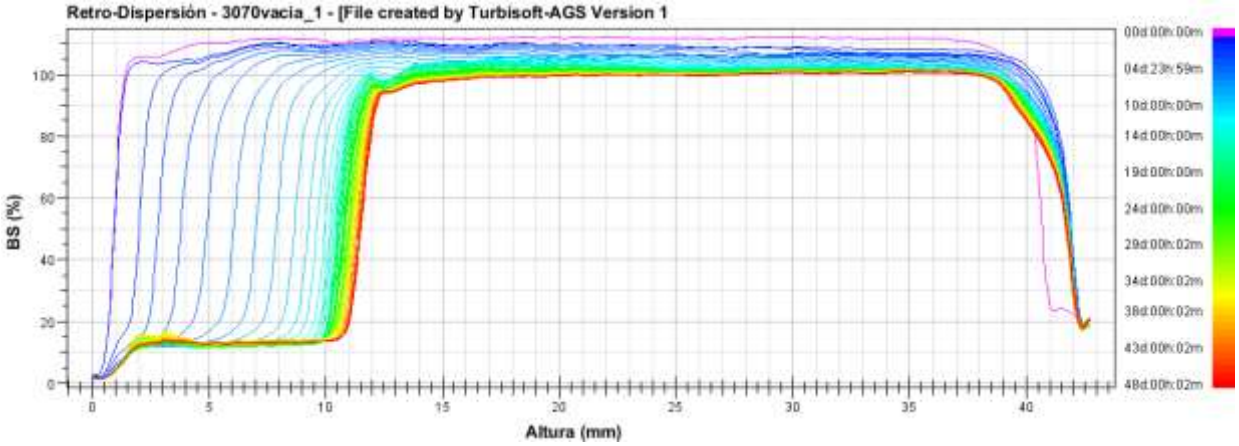


(D)

Figure 2S. Stability of the 20/80 W_1 to O ratio emulsions: (A) Double emulsion without encapsulated biocompound; (B) Double emulsion with resveratrol; (C) Double emulsion with resveratrol and vitamin D_3; (D) Double emulsion with vitamin D_3.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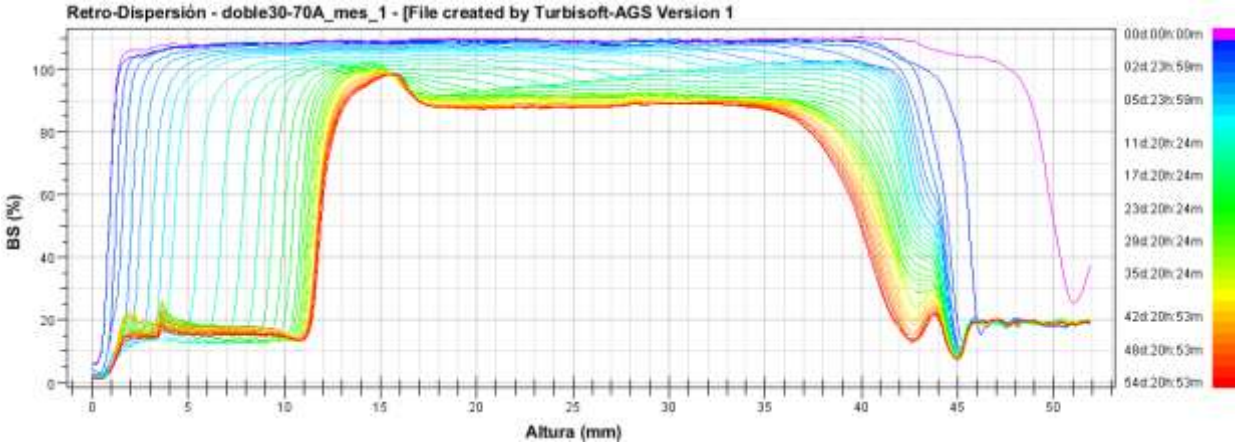
BS (%)
Altura (mm)

(A)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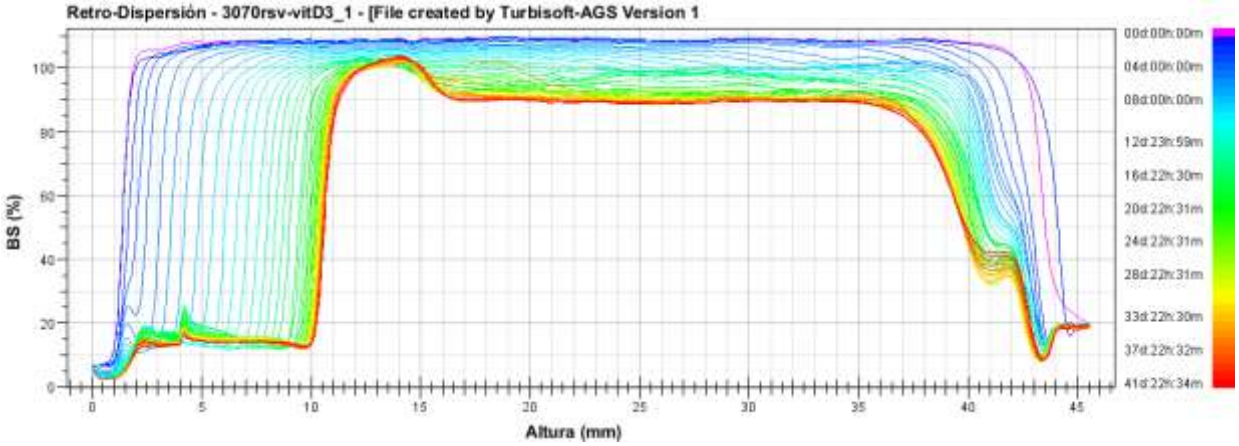
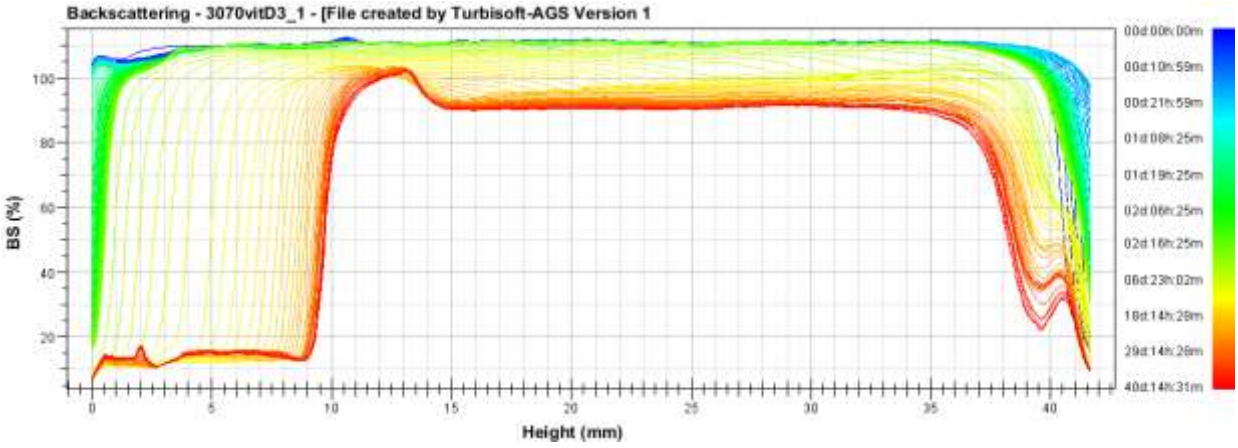
Altura (mm)

(B)


Retro-Dispersión - 3070rsv-vitD3_1 - [File created by Turbisoft-AGS Version 1
BS (%)
Altura (mm)

(C)



(D)

Figure 3S. Stability of the 30/70 W_1 to O ratio emulsions: (A) Double emulsion without encapsulated biocompound; (B) Double emulsion with resveratrol; (C) Double emulsion with resveratrol and vitamin D_3; (D) Double emulsion with vitamin D_3.