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Title: Preparation of water-in-oil-in-water (W1/O/W2) double emulsions containing trans-resveratrol

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Keywords: trans-resveratrol, encapsulation, double emulsions, RV-HPLC, stability

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Abstract: Trans-resveratrol (3,5,4´-trihydroxystilbene) is a naturally occurring polyphenol phytoalexin easily oxidizable and extremely photosensitive with a short biological half-life. The goal of this work was to prepare W1/O/W2 double emulsions of food-grade formulation to encapsulate trans-resveratrol. Mechanical agitation and membrane emulsification (ME) were the techniques used for emulsion preparation. A technique based on RV-HPLC to determine trans-resveratrol concentration in the external aqueous phase with VIS/UV and fluorescence detectors was developed. Several inner emulsifiers were tested to produce stable water-in-oil W1/O emulsions containing 20% (v/v) of ethanol. Polyglycerol polyricinoleate (PGPR) was the only emulsifier with good stabilizing properties. Non-ionic surfactants (Tween 20 and Tween 80) were used as outer emulsifiers. Other food bioemulsifiers, as sodium caseinate (NaCn), sodium carboxymethylcellulose (CMCNa) or gelatin were also added as stabilizers to improve W1/O/W2 double emulsions stability. Initial encapsulation efficiency (EE) and encapsulation stability (ES) were measured. The combination of Tween 20 and CMCNa in the external aqueous phase seemed to have a synergetic effect leading to better initial EE values. More stable emulsions were obtained with mechanical agitation. An increase in PGPR content yielded a slight increase in initial EE values.

RESPONSE TO REVIEWERS` COMMENTS

Journal: Colloids and Surfaces A: Physicochemical and Engineering Aspects
Ms. Ref. No.: COLSUA-D-12-01589
Title: Preparation of water-in-oil-in-water ($W_1/O/W_2$) double emulsions containing trans-resveratrol
Authors: María Matos, Gemma Gutiérrez, José Coca, Carmen Pazos

We would like to thank the reviewers for their comments on our manuscript entitled *Preparation of water-in-oil-in-water ($W_1/O/W_2$) double emulsions containing trans-resveratrol* (Ref.: COLSUA-D-12-01589). They pose practical and stimulating questions. After careful revision and taking into consideration those comments, some changes have been made which have been highlighted in blue in the revised manuscript. These changes are discussed in the following paragraphs.

Reviewer #1: *The article given to me for review is dedicated to the preparation and characterization of double water-in-oil-in-water (W/O/W) emulsions containing trans-resveratol (TR). The authors have shown that W/O/W emulsion is the appropriate means to deliver TR, which has positive bio effects (anti-oxidant, anti-inflammatory) but is very photosensitive and easily oxidizable. The study is very systematic and includes different various aspects of the formulation development: emulsification method, appropriate choice of emulsifiers, characterization of the stability of the emulsion etc.*

I have two minor comments and one question:

1. The quantity "span" defined by the authors via expression (6) should be referred to as polydispersity.

Span is given according to the definition in the *Mastersizer User´s Manual* and it is used to measure the width of the distribution. The narrower the distribution, the smaller the span. However, the term polydispersity is now mentioned in the revised manuscript.

2. The results would be easier to read and understand if Tables 2-5 are presented as figures.

Table 2 shows the mean droplet diameter, span, maximum backscattering variation (ΔBS_{max}), interfacial tension (γ) and viscosity (μ) values of W_1/O emulsions obtained with different PGPR concentrations. These data were included in the manuscript to supplement the results shown in Figures 3 and 4. Figure 3 shows the droplet size distributions of W_1/O emulsions obtained with different PGPR concentration while in Figure 4 are shown the corresponding Kinetic ΔBS profiles of these W_1/O emulsions.

Accepted. Experimental results from Table 3 are now depicted in Figure 6.

The data from Table 4 (now Table 3) are the viscosities of the external aqueous phases at different CMCNa concentrations. We consider appropriate to keep them as a table.

Table 5 (now Table 4) shows the mean droplet sizes of $W_1/O/W_2$ emulsions with 5% and 10% (w/v) of PGPR in Miglyol 812 as oily phase and different CMCNa concentrations in the W_2 phase. The same trend was obtained in both cases as it is noticed in the manuscript. For a better understanding of these experimental results, the droplet size distributions of $W_1/O/W_2$

emulsions prepared with 5% (w/v) of PGPR at different CMCNa concentrations are also shown in Figure 7.

3. The Du Nouy ring is unreliable method to measure surface tension because the measured values depend very much on the contact angled of the bulk phase on the ring material. This angle depends very much on the particular material, and is not a parameter which the experimenter can control. Could the authors explain why they chose this method instead of using other, more reliable methods, like capillaru pressure tensiometry or spinning drop tensiometry?

In conclusion I think the study is sound, innovative and worthy of publishing.

Interfacial tensions were measured using a KSV Sigma 700 equipment. This device allows calculating the interfacial tension from measurements of the interaction of a probe at the boundary between the two liquids. Although this method might not be the most reliable to measure interfacial tensions, we consider that it is accurate enough to compare the systems formulated in this work.

Reviewer #2: *Authors present various methods of preparing w/o/w emulsions using different surfactants. Manuscript is acceptable after addressing following issues:*

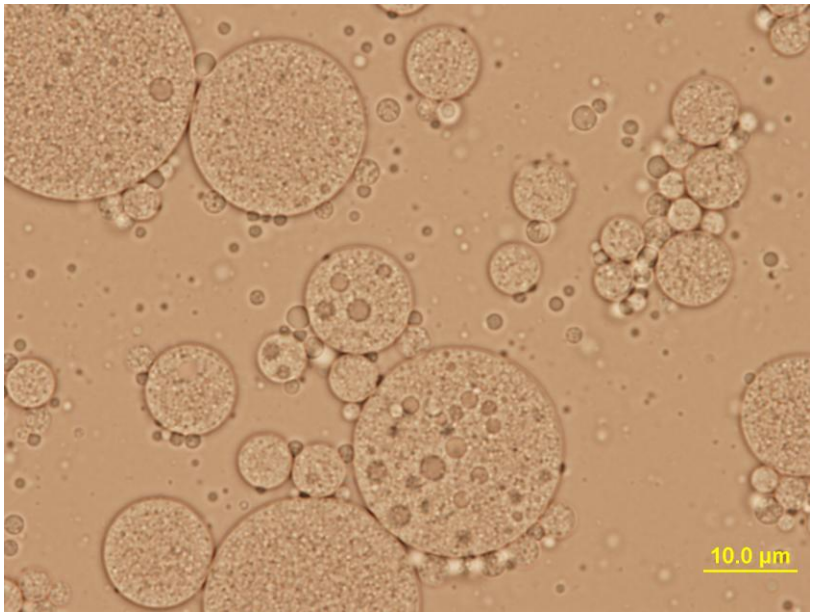
1. Though the emulsions were prepared by two methods- stirring and membrane, it is not clear from the manuscript how the emulsions produced by two methods differed and why stirring method was chosen?

(W_1/O/W_2) double emulsions were prepared by both methods, *i.e.* mechanical agitation and membrane emulsification (ME), in order to study the effect of the emulsification process (in the second step of preparation) on their encapsulation ability. Current industrial emulsification processes, such as rotor stator devices can produce small droplets but with high shear stress on the liquids, causing a loss of activity of the encapsulated compounds. Furthermore, the droplet size is difficult to control and, therefore, usually polydisperse emulsions are obtained. Using the membrane emulsification (ME) technique the strain on the liquid phases is reduced, and the droplet size is narrowly distributed with less shear stress and energy consumption. Thus, (W_1/O/W_2) double emulsions containing 5% and 10% (w/v) PGPR were prepared by both techniques in order to compare the initial encapsulation efficiency as well as the encapsulation stability with time.

2. The abstract should be rewritten to better reflect the conclusions obtained in this study.

Accepted. The abstract has been rewritten tacking into account the main conclusions of the study.

***Graphical Abstract (for review)**



Highlights

- Resveratrol was encapsulated in water-in-oil-in-water double emulsions
- These emulsions were prepared by mechanical agitation and membrane emulsification
- Resveratrol encapsulation efficiency was determined by RV-HPLC method
- An appropriate selection of emulsion stabilizers increased resveratrol encapsulation
- Encapsulation and release of resveratrol were influenced by PGPR content

Preparation of water-in-oil-in-water ($W_1/O/W_2$) double emulsions containing *trans*-resveratrol

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Abstract

Trans-resveratrol (3,5,4´-trihydroxystilbene) is a naturally occurring polyphenol phytoalexin easily oxidizable and extremely photosensitive with a short biological half-life. The goal of this work was to prepare $W_1/O/W_2$ double emulsions of food-grade formulation to encapsulate *trans*-resveratrol. Mechanical agitation and membrane emulsification (ME) were the techniques used for emulsion preparation. A technique based on RV-HPLC to determine *trans*-resveratrol concentration in the external aqueous phase with VIS/UV and fluorescence detectors was developed. Several inner emulsifiers were tested to produce stable water-in-oil W_1/O emulsions containing 20% (v/v) of ethanol. Polyglycerol polyricinoleate (PGPR) was the only emulsifier with good stabilizing properties. Non-ionic surfactants (Tween 20 and Tween 80) were used as outer emulsifiers. Other food bioemulsifiers, as sodium caseinate (NaCn), sodium carboxymethylcellulose (CMCNa) or gelatin were also added as stabilizers to improve $W_1/O/W_2$ double emulsions stability. Initial encapsulation efficiency (EE) and encapsulation stability (ES) were measured. The combination of Tween 20 and CMCNa in the external aqueous phase seemed to have a synergetic effect leading to better initial EE values. More stable emulsions were obtained with mechanical agitation. An increase in PGPR content yielded a slight increase in initial EE values.

Keywords: *trans*-resveratrol, encapsulation, double emulsions, RV-HPLC, stability

1. INTRODUCTION

Trans-resveratrol (3,5,4´-trihydroxystilbene) is a natural occurring polyphenol found in a wide variety of plants. It has beneficial effects for human health, such as anti-oxidant, anti-inflammatory, cardioprotective and anti-tumour properties. However, the applications of *trans*-resveratrol are limited because it is an easily oxidizable and extremely photosensitive compound, with low water solubility, short biological half-life, and rapid metabolism and elimination [1-3]. The level of *trans*-resveratrol in wines depends on the production technology and is usually lower in white wines [4]. The average content in red wines reported by Gürbüz *et al.* was 1.089 mg/L ± 0.002 [5].

Encapsulation of polyphenols can effectively mitigate these limitations [1, 6]. Encapsulation studies have been carried out to protect *trans*-resveratrol from

degradation, increasing its solubility in water and targeting it to specific locations via multiparticulate forms and colloidal carriers [1, 3, 7-14].

Several methods for the encapsulation of polyphenols have been reported, such as spray drying, coacervation, liposome entrapment [11-12, 14-15], inclusion complexation [13], cocrystallization, nanoencapsulation [7, 10], freeze drying and emulsification [8-9].

Multiple emulsions were first reported in 1925 by Seifriz. The simplest multiple emulsions are called double emulsions and they are ternary systems, having either a water-in-oil-in-water ($W_1/O/W_2$) or an oil-in-water-in-oil ($O_1/W/O_2$) structure, whereby the dispersed droplets contain smaller droplets of a different phase [16]. The structural properties of this kind of multiple emulsions permit controlled release of a component from the inner to the outer phase. This leads to a number of potential applications in the fields of medicine, pharmacy, cosmetics and separation processes [16-23].

$W_1/O/W_2$ double emulsions have potential applications in food, cosmetic and pharmaceutical industries as vehicles for encapsulation and delivery of nutrients during food digestion or for drug release [24-31]. This type of emulsions may also be used for the encapsulation of sensitive food materials and flavours and in the formulation of low calorie food products [26]. The main problem in the production of double emulsions is their instability, due to the excess of free energy associated with the surface of the emulsion droplets [16-17].

The suitability of $W_1/O/W_2$ double emulsions to encapsulate *trans*-resveratrol has been reported by Hemar *et al.* [32]. Its concentration in the external aqueous phase was measured by a simple UV method. However, other techniques have been developed for determining *trans*-resveratrol content, such as gas chromatography with mass selective detection (GC-MS) and high-performance liquid chromatography (HPLC) with UV or fluorescence detection [4-5, 33-34].

The aim of this work was to prepare $W_1/O/W_2$ emulsions containing *trans*-resveratrol, either by mechanical agitation or membrane emulsification (ME). Encapsulation stability (ES) was determined by HPLC-RV using VIS/UV and fluorescence detectors.

2. MATERIALS AND METHODS

2.1. MATERIALS

Trans-resveratrol, absolute ethanol, Tween 20, Tween 80, Span 80, sodium carboximethylcellulose (CMCNa), gelatin and sodium caseinate salt from bovine milk (NaCn) were purchased from Sigma Aldrich (USA). Miglyol 812 (density 945 kg/m^3 at 20 ^{o}C) was supplied by Sasol GmbH (Germany). Polyglycerol polyricinoleate (PGPR) was supplied by Brenntag AG (Germany). Sodium chloride was obtained from Panreac (Spain). Plurol oleique (polyglyceril-6-dioleate) and Peceol (glycerylmonoleate, type 40) were purchased from Gattefossé SAS (France). Methanol, acetonitrile, 2-propanol and acetic acid of HPLC-grade were obtained from Sigma Aldrich (USA).

2.2. METHODS

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

Primary W_1/O single emulsions were prepared using 20% (v/v) of the inner aqueous phase (W_1) and 80% (v/v) of the continuous oily phase (O). *Trans*-resveratrol is barely soluble in water and its solubility in alcohols decreases as the carbon number of the alcohol increases [34]. Thus, a 20% ethanol (v/v) solution was used as the dispersed phase containing 50 mg/L of *trans*-resveratrol.

Miglyol 812 was used as the continuous phase containing the corresponding hydrophobic emulsifier previously dissolved by stirring at 50°C for 30 min. The hydrophilic-lipophilic balance (HLB) of an emulsifier is an adequate parameter to predict the resulting emulsion type (W/O or O/W) [20, 27]. The HLB values of the inner emulsifiers tested in this study are as follows: Span 80 = 4.3, PGPR = 3.0, Peceol= 3.0, Plurol oleique = 6.0. PGPR is commonly used in food formulation and it has been demonstrated to be highly effective for stabilizing W_1/O emulsions [35, 24].

It has been reported that the addition of electrolytes to the aqueous phase increases the W_1/O emulsion stability. It has been suggested that the presence of electrolytes lowers the attractive force between water droplets, decreasing the dielectric constant of the aqueous phase and therefore reducing collision frequency [24, 29-30]. Consequently, 0.1M NaCl was added to the internal aqueous phase to ensure inner droplets stability.

Both continuous and dispersed phase were emulsified in glass vessels by high shear mixing (Miccra D-9 mixer, ART, Germany) using a 6 mm dispersing tool at 20,000 rpm for 2 min.

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

W_1/O/W_2 double emulsions were prepared either by mechanical agitation or ME by dispersion of 20% (v/v) of W_1/O emulsions in an external continuous phase (W_2) containing 0.1M NaCl in order to match the osmotic pressure between the two aqueous phases.

When Tween 20, Tween 80 and NaCn were used as outer stabilizers they were previously dissolved by stirring for 30 min. Nevertheless, when CMCNa and gelatin were used they needed to be agitated overnight.

For mechanical agitation, the continuous and dispersed phases were emulsified using the aforementioned Miccra D-9 mixer at 11,000 rpm for 2 min.

A 200 mL Amicon model 8200 stirred batch ultrafiltration cell (Amicon Inc., USA) was fitted to the membrane emulsification experiments. The dispersed phase was injected from the bottom side of the cell by a syringe pump KDS-100-CE (Kd Scientific, USA) at a rate of 20 mL/min. The continuous phase was placed on the upper part of the cell being continuously stirred at 600 rpm, to enhance droplet detachment. Operating conditions were selected based on results obtained from previous studies (results not shown).

The membrane used was a hydrophilic metallic membrane with 5 μm pore size, supplied by Micropore Ltd. (UK). When using this membrane emulsification technique, the diameter of the droplets produced is approximately between 2 and 10 times the diameter of the membrane pore [25, 36].

After each experiment membranes were cleaned with a dishwashing detergent and rinsed with deionized water and acetone in an ultrasound bath for 15 min. Finally, they were dried using compressed air and pre-soaked in the continuous phase.

2.2.3. Emulsion characterization

Droplet size distributions were obtained by the laser light scattering technique in a Mastersizer S long bench apparatus (Malvern Instruments, Ltd. UK). For double emulsions a refractive index of 1.54 was used.

Samples were first diluted with deionized water to prevent multiple scattering effects. They were then circulated through the measuring zone using a Hydro SM small volume sample dispersion unit, following the manufacturer recommendations for this type of emulsions. For the single W_1/O emulsion, the water refractive index was used and the samples were dispersed in paraffin oil (VWR Int., Barcelona).

Several measurements were made for each emulsion changing the dilution ratio. No significant differences were observed in the mean droplet diameters, ranging from 1:10 to 1:100 dilution ratios. Three replicates were obtained for each emulsion and results were reported as the typical droplet size distribution in μm. The mean diameters, $D_{[4,3]}$ and $D_{[3,2]}$, were calculated by equations (1) and (2):

$$D_{[4,3]} = \frac{\sum n_i d_i^{\,4}}{\sum n_i d_i^{\,3}} \quad (1)$$

$$D_{[3,2]} = \frac{\sum n_i d_i^{\,3}}{\sum n_i d_i^{\,2}} \quad (2)$$

where d_i is the droplet diameter and n_i the number of droplets with diameter d_i. $D_{[4,3]}$ is the volume weighted mean diameter and $D_{[3,2]}$ is the surface weighted mean diameter or Sauter mean.

A Zetasizer NanoZS (Malvern Instruments Ltd., UK) was utilized for zeta potential (ζ) measurements of the $W_1/O/W_2$ double emulsions. Three replicates were conducted for each sample at constant temperature of 25 °C.

Micrographs of the emulsions were obtained with a light microscope Olympus BX50 (Olympus, Japan) with 10-100x magnification using UV/VIS and fluorescence lamps.

Emulsion stability was analyzed by measuring backscattering (BS) and transmission (TS) profiles in a Turbiscan apparatus (Formulaction, France). Emulsions samples were placed without dilution in the test cells and transmitted and backscattered light was monitored as a function of time and cell height for 7 days at 30 °C. The optical reading head scans the sample in the cell, providing TS and BS data every 40 μm in % relative

to standards (suspension of monodisperse spheres and silicone oil) as a function of the sample height (in mm). These profiles build up a macroscopic fingerprint of the emulsion at a given time, providing useful information about changes in droplet size distribution or appearance of a creaming layer or a clarification front with time [37-38].

Emulsions viscosity measurements were performed with a Haake RS50 rheometer (Haake, Germany) using plate-plate configuration at 25 °C. Viscosity was measured at 20 s^{-1} constant shear rate for 180 s.

The viscosities of external aqueous phases were measured by an Ubbelohde type viscometer PSL-Rheotek (Poulten Selfe & Lee Ltd., United Kingdom) at 25 °C.

Interfacial tension (γ) was determined following the Du Noüy's platinum ring method at 20 °C using a Sigma 700 tensiometer (KSV Instruments Ltd., Finland).

2.2.4. Determination of the initial encapsulation efficiency (EE) and encapsulation stability (ES) by RV-HPLC analysis

Trans-resveratrol content in the external aqueous phase was determined by chromatography (HP series 1100 chromatograph, Hewlett Packard, US). The system was equipped with a VIS/UV absorbance detector HP G1315A and a fluorescence detector 1260 Infinity A (Agilent Technologies, US).

The column used for the separation was a reversed phase column Zorbax Eclipse Plus C_{18} of 5 μm particle size, 4.6 mm × 150 mm (Agilent Technologies, US). 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 min 5 for 10 min. The mobile phase (B) was run for 2 min after each injection to prepare the column for the next run. The separation was carried out at room temperature.

A wavelength of 305 nm was used for UV/VIS detector while fluorescence detector was used at $\lambda_{excitation}/\lambda_{emission}$ at 310/410 nm. The column was cleaned after each analysis by running first 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 mobile phase (A) and 50% of mobile phase (B) for another 20 min.

The external aqueous phase injected in the HPLC system was previously recovered by centrifugation at low speed (1,000 rpm for 20 min) and filtration with a 0.22 μm polyvinylidene difluoride (PVDF) syringe filter, to eliminate all the cream oily phase still present.

Other filters as polyethersulfone (PES) or nylon were also tested although considerably high resveratrol retention values were obtained: 29 and 100%, respectively.

The recovery yield (R_y) was determined to measure the amount of *trans*-resveratrol retained during the centrifugation and filtration processes. A standard emulsion, where 100% of the W_1 is present in W_2, was required. For this purpose, an oil-in-water

emulsion (O/W_2) was prepared using the same formulation as in the experiments. This O/W_2 emulsion was then diluted at the same ratio with W_1, which contained the appropriate amount of *trans*-resveratrol. Then, the concentration of *trans*-resveratrol in the recovered aqueous phases ($C_{recovered}$) was determined by HPLC-RV, using the absorbance and fluorescence calibration curves previously obtained. For this analysis, a blank reference was used. It consisted of an O/W_2 emulsion diluted with W_1, in which *trans-resveratrol* was not present. Finally, the R_y was calculated as:

$$R_y(\%) = \frac{C_{recovered} \times 100}{C_0} \quad (3)$$

where C_0 is the maximum concentration of *trans*-resveratrol expected in the external aqueous phase.

The encapsulation efficiency (EE) of these double emulsions was defined as the percentage of *trans*-resveratrol in W_1 that remained in the primary emulsion (W_1/O) after the second emulsification step [26, 39]. It was calculated by equation (4):

$$EE(\%) = 100 - \frac{C_{recovered} \times 100}{C_0 R_y} \quad (4)$$

The encapsulation stability (ES) was defined as the amount of *trans*-resveratrol that remained entrapped in the inner aqueous phase (W_1) during storage or after double emulsion exposure to environmental stresses [39]. It was calculated by equation (5):

$$ES(\%) = 100 - \frac{C_{recovered} \times 100}{C_0 R_y} \quad (5)$$

Several samples were prepared and stored at room temperature to measure the ES weekly along a month. Three replicates of each sample were determined.

3. RESULTS AND DISCUSSION

3.1. Water-in-oil (W_1/O) emulsions

To obtain a stable double emulsion, the stability of the single W_1/O emulsion must be ensured. This stability depends on droplet size (normally around 1 μm), amounts of dispersed and continuous phase (water is usually in the range 20-30% v/v), and emulsifier affinity for both phases (HLB) [17-19, 20, 27].

Several W_1/O emulsions were prepared at the same concentration, 5% (w/v), varying the type of inner emulsifier present in the oily phase. The droplet size distributions of the resulting emulsions were measured and their stability was determined and compared by laser light scattering.

The mean diameters obtained with the Malvern Mastersizer S are shown in Table 1. The $D_{[3,2]}$ values are in the 0.3-1.7 μm range, except for Peceol, which leads to sizes considerably higher. The polydispersity of the droplet size distribution was expressed in terms of span, which is a measure of the width of the droplet size distribution. It is defined as:

$$span = \frac{D(v,0.9) - D(v,0.1)}{D(v,0.5)} \quad (6)$$

where D(v,0.5), D(v,0.1) and D(v,0.9) are standard percentile readings from the analysis. D(v,0.5) is the size in microns at which 50% of the sample is smaller and 50% is larger. D(v,0.1) and D(v,0.9) are the size of the droplets below 10% and 90% respectively of the sample lies. It can be observed in Figure 1 that droplet size distributions are highly polydisperse. When PGPR was used lower span values were obtained.

Table 1

Figure 1

Figure 2A shows the kinetic BS profiles obtained in the middle zone of the cell (from 10 to 30 mm). The corresponding TS profiles obtained at the bottom of the cell (from 0 to 10 mm) are also shown for a better understanding of emulsions behavior. Samples were monitored for a week. A photograph of the glass cells, containing the W_1/O emulsions after being measured, is shown in Figure 2B.

Figure 2

The stability of W_1/O emulsions prepared with PGPR and Spans as emulsifiers had been studied by Márquez *et al.* using a similar vertical scan analyzer [24, 40]. It was reported that a decrease of the BS values, along the height of the cell, implies an increase of the water droplets size due to a coalescence process. It was also confirmed that an increase of BS at the bottom corresponds to sedimentation of the water droplets [24, 40]. This trend was observed for Peceol and Span 80 (Figure 2A).

In addition, a simultaneous increase in TS values was also observed at the bottom what indicates the formation of the water layer. This behavior was confirmed regarding Figure 2B where the water layer appears in the cells containing these emulsions.

However, the opposite behavior in the TS profiles was observed when Plurol oleique and PGPR were used. This indicates the presence of an emulsion in this area, as shown in Figure 2B. In the case of Plurol oleique, an oil-in-water O/W_1 emulsion was formed and settled at the bottom. This may be explained because this emulsifier has the largest HLB value (6.0) and shows higher affinity for the aqueous phase. The emulsion prepared with PGPR offers higher stability, giving the lowest variation of BS with time (2.5%). This indicates that there are no changes in droplet size, remaining the emulsion stable.

Therefore, all the emulsifiers studied offered poor stabilizing properties, except PGPR, as was previously reported by J.S de los Reyes *et al.* [19]. They determined that more stable ethanol-in-water (E/O) emulsions were obtained using polyglycerol esters of oleic acid.

Several W_1/O emulsions were prepared at different concentrations of PGPR: 2.5, 5, 10, 20 and 30% (w/v) to determine the optimum value. The resulting droplet size distributions are shown in Figure 3.

Figure 3

All the emulsions prepared with PGPR had a mean diameter $D_{[3,2]}$ lower than 1 μm (Table 2). Comparing emulsions prepared with 2.5 to 10% (w/v) of PGPR, it was observed that the mean droplet size decreased as the amount of PGPR increased, as it might be expected.

Otherwise, for emulsions prepared with 20 and 30% (w/v) of PGPR an increase in $D_{[4,3]}$ was observed, with values up to 2 μm. This effect of PGPR on the stability of W_1/O emulsions had been previously reported [24]. A lower droplet size at higher surfactant content reduces coalescence, as a result of lower collision efficiency due to the higher emulsion viscosity.

Márquez *et al.* determined that the presence of salts may also interfere on the adsorption density of PGPR at the interfacial film and concluded that the increase of stability produced by increasing salt or PGPR concentration (0.2-0.5 and 1.0% (w/w)) could be attributed to the reduction of the interfacial tension, rather than to the viscoelastic properties of the film [24].

In Table 2 are shown the interfacial tension (γ) and viscosity (μ) values of the emulsions as a function of PGPR content. There were no considerable differences between the interfacial tensions obtained, probably due to the presence of NaCl and the large amount of PGPR added. Furthermore, an increase in viscosity values as PGPR content rises was observed, as predicted.

For better comparison of these emulsions stability, the kinetic BS profiles were obtained plotting incremental values of BS (ΔBS) versus time for a week. Table 2 also shows maximum ΔBS (ΔBS_{max}) values obtained applying equation (7):

$$\Delta BS_{max}=\Delta BS_{max10\text{-}30}-\Delta BS_{min10\text{-}30} \quad (7)$$

Table 2

Figure 4

No significant backscattering variation with time was observed for emulsions prepared with 2.5, 5 and 10% (w/v), as shown in Figure 4. The values of ΔBS_{max} obtained with these emulsions were quite low (< 2.5%) indicating high stability, probably due to 0.1M NaCl addition. Emulsions prepared with 20 and 30% (w/v) of PGPR showed BS variations slightly higher with values of 8-9%.

Márquez *et al.* also compared the stability of W_1/O emulsions prepared with PGPR and studied the influence of $CaCl_2$ addition at several concentrations [24]. Using 1% of PGPR they obtained BS variations from 15%, with no addition of $CaCl_2$, to 1% when 1,000 mg/100 g of $CaCl_2$ was added. The low ΔBS values obtained indicate that there was no considerable change in droplet size, remaining the emulsions stable after one week.

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

$W_1/O/W_2$ emulsions were prepared with several outer emulsifiers to choose the best formulation, i.e. which provides the higher initial EE. The addition of stabilizing agents, such as CMCNa or gelatin, was also studied.

In $W_1/O/W_2$ double emulsions preparation, Tween 20, Tween 80 and NaCn at the same concentration, 2% (w/v), were selected as outer emulsifiers. The resulting droplet size distributions and their stability in terms of initial EE were compared. The oily phase used in this set of experiments was Miglyol 812 containing 5% (w/v) of PGPR.

Figure 5

The bimodal droplet size distributions shown in Figure 5 were highly polydisperse. Droplet sizes were in the 1-30 μm range with two well-defined peaks at 4 and 10 μm when Tween 20 and Tween 80 were used. With NaCn small droplets (0.1-0.6 μm range) and large droplets from 1.5 to 56 μm were obtained.

Hemar *et al.* prepared double emulsions containing *trans*-resveratrol using NaCn 0.5% (w/w) and also obtained a bimodal droplet size distribution, with small particles in the 0.1-1 μm range and large particles in the 1-100 μm range [32].

When Tween 20 was used as outer emulsifier, the R_y value for *trans*-resveratrol was 97.84% ± 2.96 using the UV/VIS detector, and 97.78% ±2.74 with the fluorescence detector. For Tween 80 similar values were obtained, being 95.14% ± 3.37 and 97.42% ± 1.01, respectively.

Otherwise, for NaCn neither signal was obtained being 0% the corresponding R_y value. In conclusion, it was not possible to determine the EE by this method for double emulsions prepared with this emulsifier. However, if *trans*-resveratrol concentration was measured in aqueous samples prepared only in presence of NaCn, the expected signals appeared. Taking into account that *trans*-resveratrol binds to diary proteins [32, 42], it may be located at the interface between the oil and the external aqueous phase containing the NaCn layer.

A slightly higher *trans*-resveratrol concentration (2% w/v) was obtained in W_2 for emulsions prepared with Tween 20. Consequently, it was selected as the most appropriate emulsifier for the subsequent experiments.

Several $W_1/O/W_2$ emulsions with three different concentration values, 2, 5, and 10% (w/v) were prepared to determine the optimal concentration of Tween 20. Two set of experiments were performed containing 5 and 10% (w/v) of PGPR in the oily phase. In both cases it was observed that increasing Tween 20 concentration led to a decrease in the mean droplet diameter $D_{[4,3]}$ (Figure 6). No significant variations in zeta potential were appreciated by increasing emulsifier content, being negative values in all cases in the range of (-1.29mV)-(-2.98mV).

Figure 6

An increase in Tween 20 concentration led to a systematic decrease in the initial EE values (Figure 7), presumably because of the mean droplet size reduction.

Kawashima *et al*. [42] also found that high concentrations of hydrophilic surfactant gave emulsions with lower entrapment capacity. It had been previously reported that a high concentration of hydrophilic surfactant led to the oil film rupture and facilitated the release of inner water droplets [19]. Therefore, a 2% (w/v) value was the concentration selected for the rest of the experiments.

Figure 7

The use of soluble polysaccharides acting as thickening/gelling agents, to stabilize the outer droplets of double emulsions preventing creaming and coalescence phenomena, has been previously reported [26, 44]. One of the advantages of polysaccharides solutions is their low plastic viscosity at low concentrations, which prevents the breakdown of multiple droplets during double emulsion manufacturing [44].

Several $W_1/O/W_2$ emulsions were prepared adding CMCNa, in the range 0-0.5% (w/v), to the W_2 phase, which consisted of 2% (w/v) of Tween 20 and 0.1M NaCl. Two set of experiments were performed using 5% and 10% (w/v) of PGPR, respectively, in the oily phase. Table 3 presents the external aqueous phase viscosity values measured at 25°C.

Table 3

The bimodal droplet size distributions obtained with 5% PGPR (w/v) are shown in Figure 8, although the same trend was observed in both cases. As CMCNa concentration increased, a gradual and slight increase in the number of droplets of 4 μm was detected, while the number of droplets of 10 μm size decreased. As a result, the mean diameter decreased as CMCNa concentration rose (Table 4).

Figure 8

Table 4

It was also observed a decrease in the interfacial tension (γ) of the outer interface when CMCNa was added. Values of 3.43 ± 0.03 and 2.57 ± 0.16 mN/m were obtained without CMCNa, when 5 and 10% (w/v) of PGPR were respectively used. Values of 2.84 ± 0.03 and 2.34 ± 0.24 mN/m were obtained with 0.5% (w/v) CMCNa addition. This reduction of interfacial tension would also explain the decrease of the mean diameters values when CMCNa was added to W_2.

Apart from reducing the interfacial tension and increase the viscosity of W_2, CMCNa could also affect the interactions between Tween 20 molecules adsorbed at the interface. As Figure 9 shows, the increase of CMCNa concentration resulted in a systematic enhancement of initial encapsulation efficiency value. These results suggested that CMCNa played a significant role in the stabilization of the outer interface. The combination of Tween 20 and CMCNa seemed to have a synergetic effect. Thus, a cumulative adsorption at the interface, instead of competitive, was suggested. Similar behavior was observed when $W_1/O/W_2$ double emulsions were prepared in presence of Bovine serum albumin (BSA) and Tween 20 in the external aqueous phase [28].

Hence, the subsequent experiments were carried out by adding 0.5% (w/v) of CMCNa to the external aqueous phase.

Figure 9

A strategy to improve the initial EE value consists of incorporating various food biopolymers in the internal aqueous phase, to provide long-term stability to the first W_1/O emulsion by converting it into soft solid-like particles [45].

The use of compounds such as gelatin or NaCn has been reported showing that 1% (wt) gelatin content produced a considerable encapsulation increase, improving the stability against coalescence occurred during the emulsification process [26].

It was also found that $W_1/O/W_2$ emulsions prepared with 0.5% (w/v) of NaCn resulted in stable emulsions, requiring lower PGPR concentration [46].

Therefore, W_1/O emulsions were prepared by dispersing 0.5% (w/v) of gelatin in W_1 in an oily phase containing 5% (w/v) of PGPR. The resulting emulsion was immediately cooled at 4°C to allow the sol-gel transition of the gelatin. On the contrary, the second stage of homogenization was developed at room temperature as previous studies showed that emulsification at low temperatures could be inefficient [45].

The same procedure was also applied adding CMCNa 0.5% (w/v) to the internal aqueous phase. The corresponding $W_1/O/W_2$ double emulsions were prepared and *trans*-resveratrol content was determined. No improvement was observed obtaining similar initial encapsulation values, around 33% in both cases.

3.3. Encapsulation stability

Several $W_1/O/W_2$ emulsions were prepared by the aforementioned methods, varying the PGPR content, to study the encapsulation capacity of these emulsions. The resulting droplet size distributions were measured and their structures were also analyzed by confocal laser microscopy (Figures 10-11).

Emulsions prepared by mechanical agitation contained 5%, 10%, 20% and 30% (w/v) of PGPR. $W_1/O/W_2$ double emulsions with 5% and 10% (w/v) PGPR were also prepared by ME yielding $D_{[4,3]}$ values of 56.1 and 49.2 μm, respectively. These values correspond to 10 times the pore size used, as expected.

Two peaks were clearly appreciated at 4 μm and 10 μm comparing the emulsions prepared with 5% and 10% (w/v) of PGPR by mechanical agitation. Furthermore, emulsions prepared with 5% (w/v) of PGPR yielded more droplets with 10 μm size. However, more droplets of 4 μm size appeared for 10% (w/v) of PGPR.

In addition, emulsions prepared with 20% and 30% (w/v) of PGPR showed small droplets in the range of 0.1-0.9 μm and large droplets in the range 2-4 μm. Consequently, the mean droplet sizes of these double emulsions decreased as PGPR concentration increased.

Zeta potentials for these emulsions were also measured, obtaining negative values very close to zero, range (-0.15mV)-(-2.02mV), in all cases.

Figure 10

In Figures 11A-B, oil fat globules containing small droplets inside (inner phase) can be clearly identified. Smaller droplets (around 4 μm) were observed for 10% (w/v) of PGPR, according to the droplet size distributions for this emulsion. Smaller oil fat globules were detected in the case of emulsions with 20% and 30% (w/v) of PGPR.

Figures 11E-F shows the bigger droplet size (50-60 μm range) for emulsions prepared by ME. In Figure 11E the oil fat globules have a blue color, due to the fluorescence of entrapped *trans*-resveratrol, while in Figure 11F the blue color is also observed in the external aqueous phase.

Figure 11

These emulsions were stored at room temperature in darkness and ES was monitored weekly for one month. The calibration curves obtained by HPLC-RV using VIS/UV absorbance (signal DAD) and fluorescence (signal FLD) detectors are shown in Figure 12. A linear trend was obtained with high correlation coefficients.

Figure 12

Figure 13 shows a typical chromatographic profile where DAD and FLD signals are plotted versus time. It was observed that *trans*-resveratrol peaks were eluted in both cases at a retention time of 6 min, when 100% methanol was run as mobile phase.

Figure 13

It has been reported that release of encapsulated compounds in $W_1/O/W_2$ double emulsions can mainly occur by two permeation mechanisms through the oil phase: (a) reverse micellar transport and (b) diffusion across a very thin lamellae of surfactant formed in areas where the oil layer is very thin [28]. Hemar *et al*. confirmed the difficulty demonstrating unequivocally the *trans*-resveratrol release mechanism [33].

Figure 14 shows encapsulation stability (ES) versus time for all the emulsions studied. As it can be appreciate, ES values obtained with FLD were slightly higher than those obtained with DAD, although both signals showed the same trend. Comparing emulsions formulated with the same PGPR content, higher values were obtained for emulsions prepared by mechanical agitation. It has been previously reported that relatively high concentrations of hydrophilic surfactant in the outer aqueous phase are required for the production of stable emulsions by ME [19-21].

Emulsions prepared both by mechanical agitation or ME processes yielded slightly higher initial EE values with higher PGPR content, up to 40% when 30% (w/v) of PGPR was added. The same influence with PGPR concentration in the oil phase had been obtained by Su *et al*. [46].

Hemar *et al*. reported that the amount of *trans*-resveratrol released after two weeks under storage was lower than 10% of the total *trans*-resveratrol initially encapsulated. Initial EE values were not mentioned.

For double emulsions prepared by mechanical agitation with 10, 20 and 30% (w/v) of PGPR, *trans*-resveratrol release after two weeks was 10%, while for emulsions prepared with 5% the release was 15%. Thereby, the stability of double emulsions was also influenced by PGPR concentration. Similar results were reported by Su *et al.* [46].

Emulsions prepared by ME showed higher release probably due to the bigger size of the oil fat globules obtained by this technique.

Figure 14

4. CONCLUSIONS

A procedure to determine the encapsulation efficiency (EE) of $W_1/O/W_2$ double emulsions containing *trans*-resveratrol using RV-HPLC with VIS/UV absorbance and fluorescence detectors was described.

For the formulation of water-in-oil-in-water ($W_1/O/W_2$) double emulsions to encapsulate *trans*-resveratrol, polyglycerol polyricinoleate (PGPR) was the best inner emulsifier. The combination of Tween 20 and CMCNa in the external aqueous phase seemed to have a synergetic effect leading to better initial EE values.

More stable emulsions were obtained when they were prepared by mechanical agitation. An increase in PGPR content yielded a slight increase in initial EE values.

$W_1/O/W_2$ double emulsions formulated to encapsulate *trans*-resveratrol are complex systems due to ethanol present in W_1, which was required to dissolve *trans*-resveratrol in water. Further formulations tests should be undertaken to improve the encapsulation ability of these emulsions.

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Table 1

Table 1. Mean droplet diameters and span values of W_1/O emulsions with different inner emulsifiers at 5% (w/v) (SD = standard deviation)

Inner emulsifier	$D_{[4,3]}$ (μm)	SD	$D_{[3,2]}$ (μm)	SD	span	SD
PGPR	0.74	0.16	0.27	0.18	1.68	0.47
Span 80	3.73	0.05	0.64	0.04	2.32	0.32
Plurol Oleique	2.81	0.08	1.73	0.01	3.81	0.21
Peceol	10.34	1.31	4.39	5.11	2.05	0.70

Table 2

Table 2. Mean droplet diameters, span, maximum backscattering variation (ΔBS_{max}), interfacial tensions (γ) and viscosity (μ) values of W_1/O emulsions with different PGPR concentrations (SD = standard deviation)

PGPR % (w/v)	$D_{[4,3]}$ (μm)	SD	$D_{[3,2]}$ (μm)	SD	ΔBS_{max}	γ (mN/m)	SD	μ (mPas)	SD
2.5	0.57	0.06	0.25	0.02	2.33	0.56	0.09	3,788	44
5	0.74	0.16	0.27	0.18	2.20	0.41	0.11	4,075	74
10	1.02	0.18	0.29	0.17	2.03	0.39	0.10	4,801	659
20	1.29	0.28	0.35	0.16	7.78	0.28	0.09	8,660	495
30	2.33	0.20	0.65	0.23	8.87	0.20	0.08	14,832	974

Table 3

Table 3. Viscosity values of external aqueous phases at different CMCNa concentrations

CMCNa % (w/v)	Viscosity mPas
0	1.14
0.1	1.75
0.2	2.37
0.3	3.64
0.4	5.34
0.5	6.85

Table 4

Table 4. Mean droplet diameters of $W_1/O/W_2$ emulsions with 5% and 10% (w/v) of PGPR in Miglyol 812 as oily phase and different CMCNa concentrations in W_2 phase (SD = standard deviation)

CMCNa % (w/v)	PGPR 5% (w/v)				PGPR 10% (w/v)			
	$D_{[4,3]}$ (µm)	SD	$D_{[3,2]}$ (µm)	SD	$D_{[4,3]}$ (µm)	SD	$D_{[3,2]}$ (µm)	SD
0.5	5.05	0.16	4.31	0.12	4.24	0.38	3.68	0.37
0.4	5.61	0.19	4.71	0.15	4.56	0.30	3.91	0.15
0.3	6.42	0.38	5.21	0.22	4.76	0.18	4.03	0.05
0.2	6.77	0.04	5.39	0.04	5.16	0.03	4.30	0.01
0.1	7.65	0.12	5.90	0.09	6.01	0.37	4.81	0.42
0	8.28	0.33	6.27	0.13	6.63	0.31	5.20	0.30

Figure captions

Figure 1 color (web)

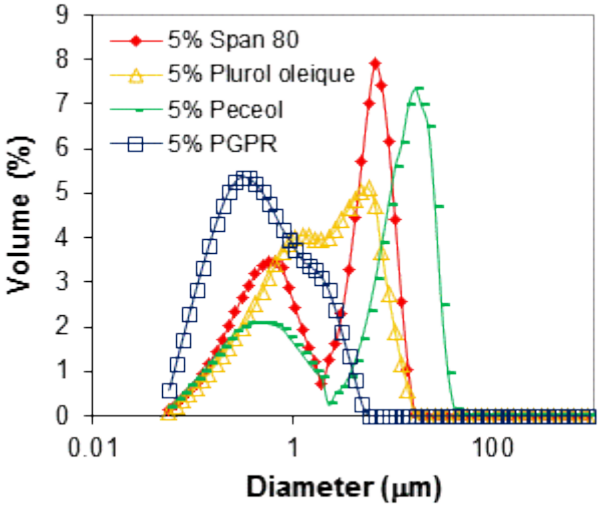

Figure 1

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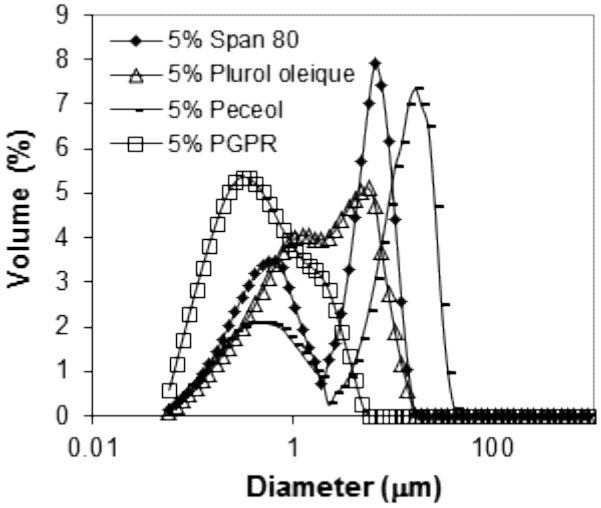
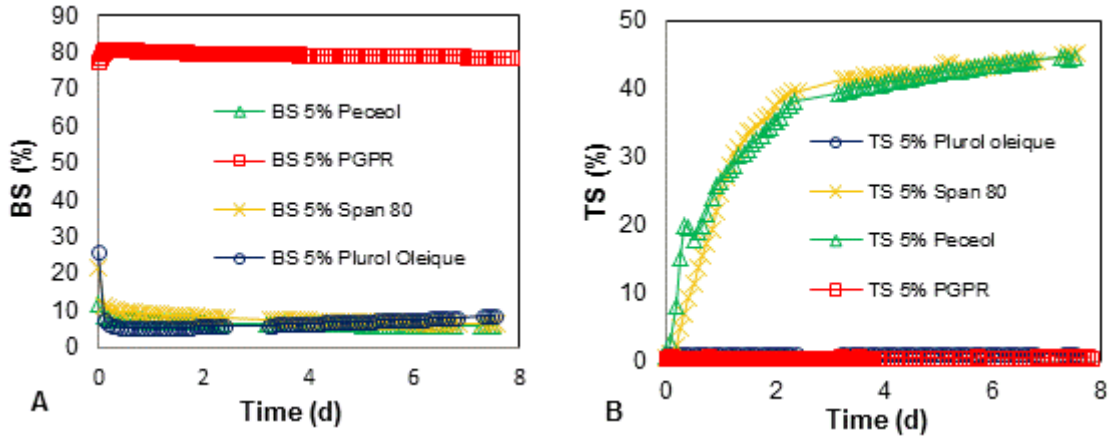


Figure 1

Figure 2 color



C Peceol Plurol oleique Span 80 PGPR

Figure 2

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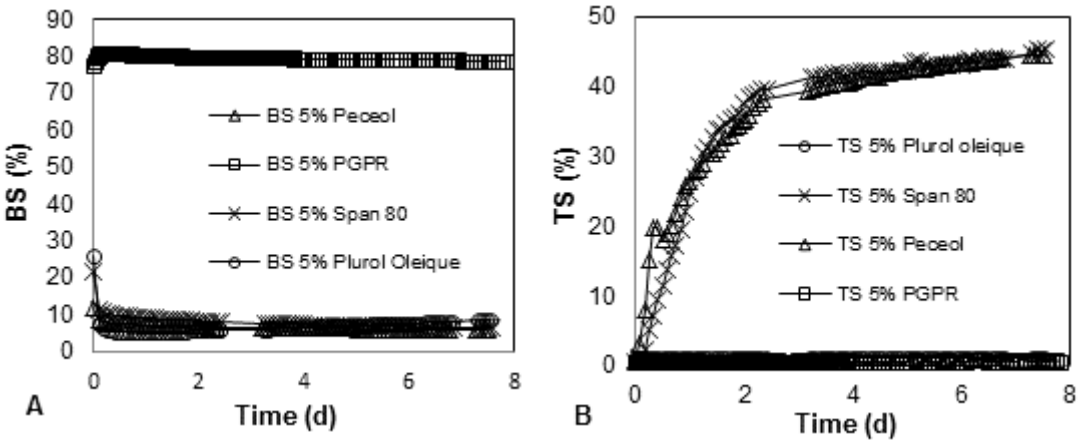

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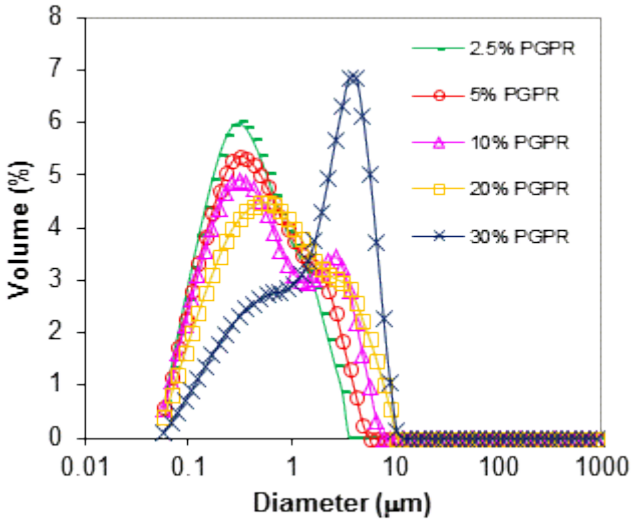

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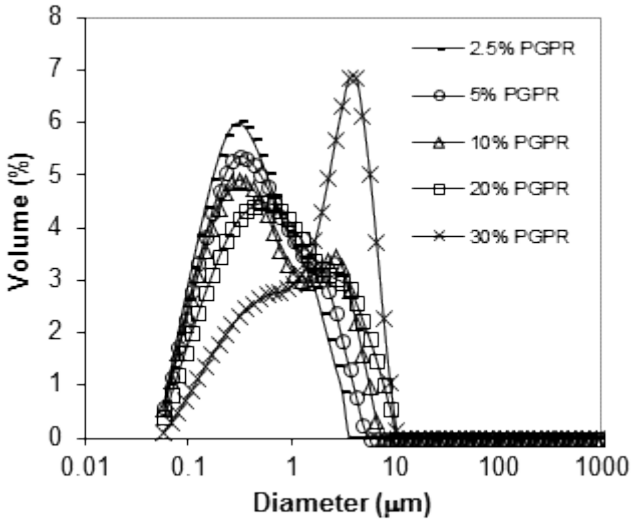

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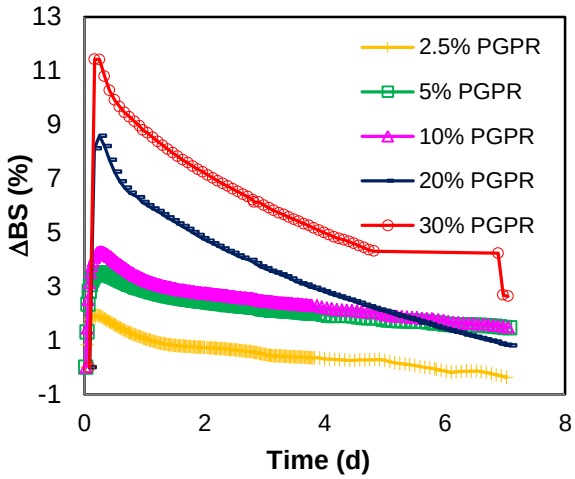

Figure 4

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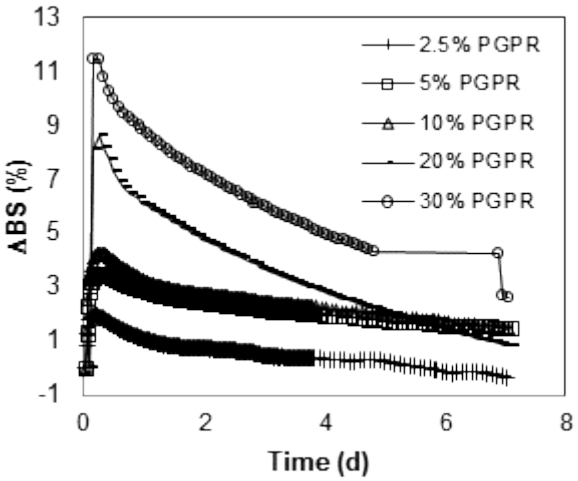

Figure 4

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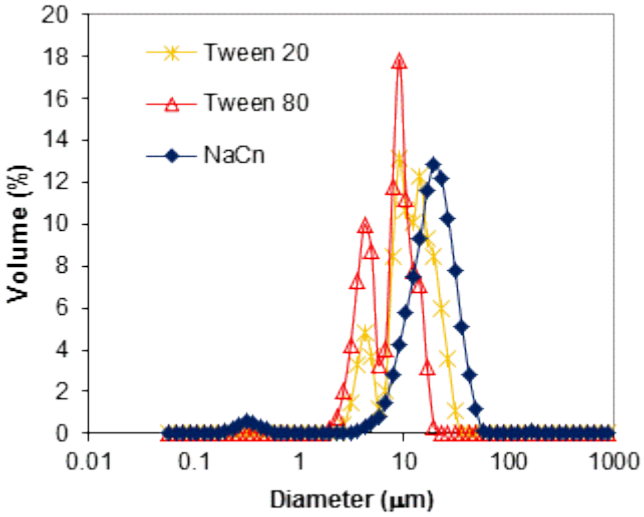

Figure 5

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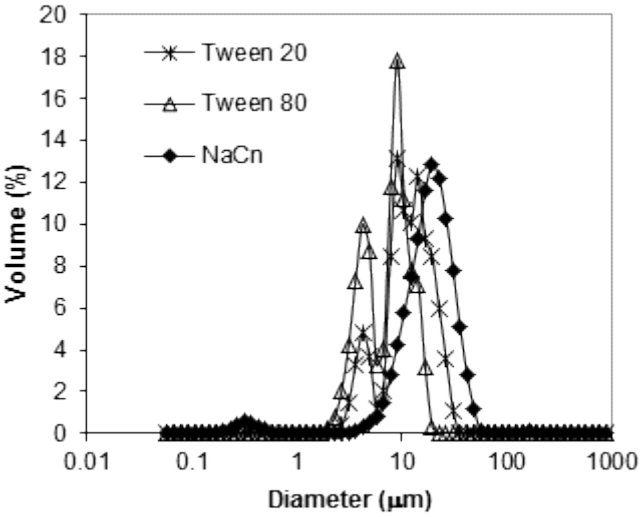

Figure 5

Figure 6 color (web)

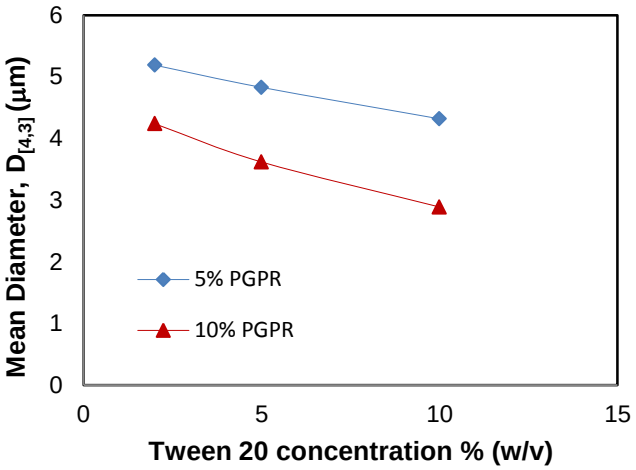

Figure 6

Figure 6 black and white

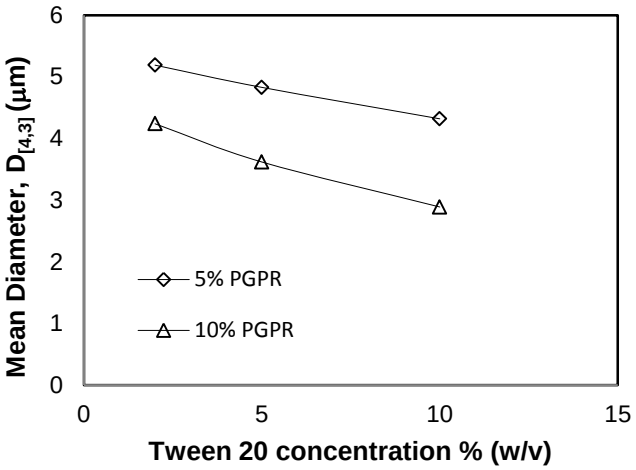

Figure 6

Figure 7 color (web)

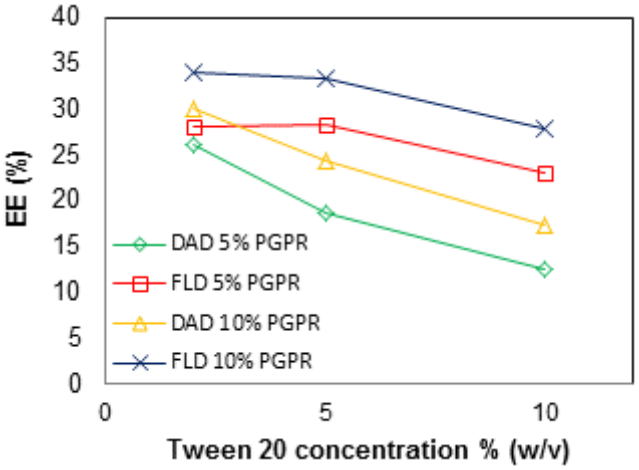

Figure 7

Figure 7 black and white

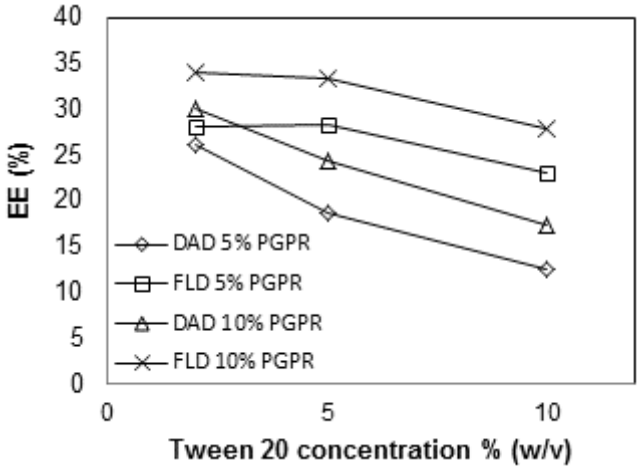

Figure 7

Figure 8 color (web)

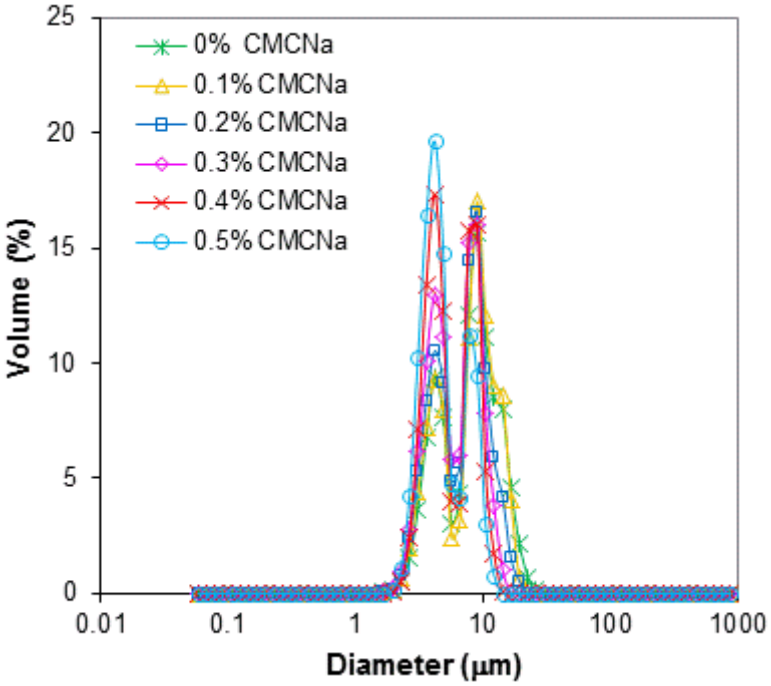

Figure 8

Figure 8 black and white

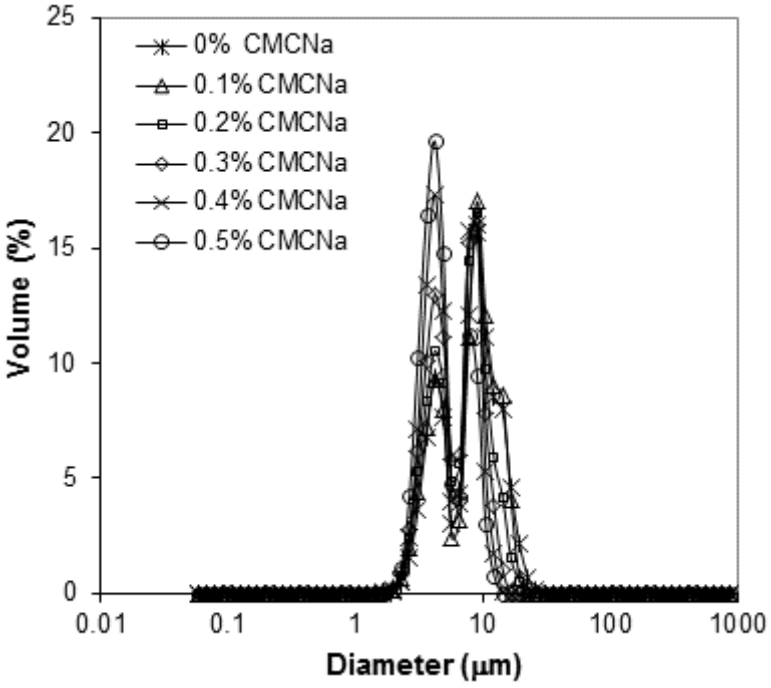

Figure 8

Figure 9 color (web)

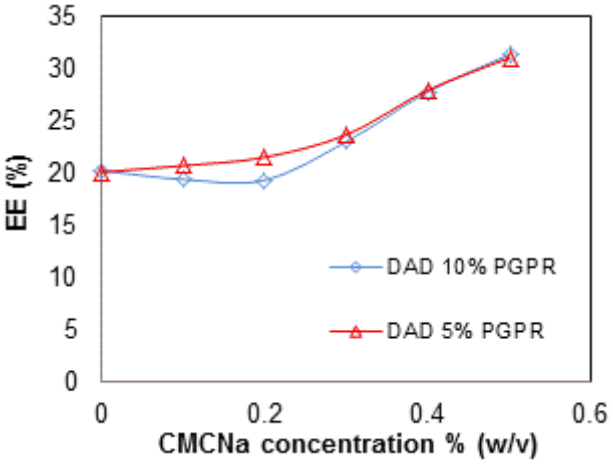

Figure 9

Figure 9 black and white

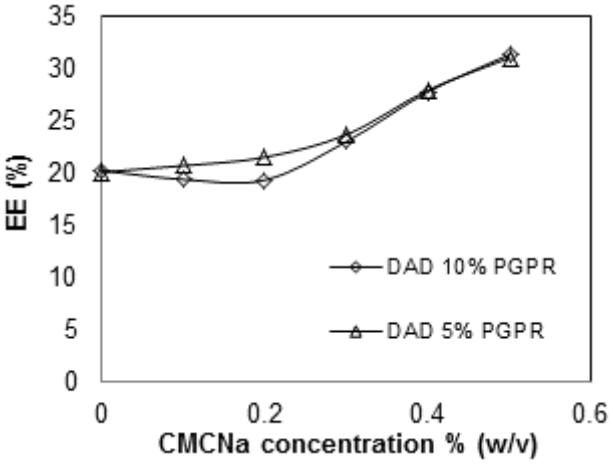

Figure 9

Figure 10 color (web)

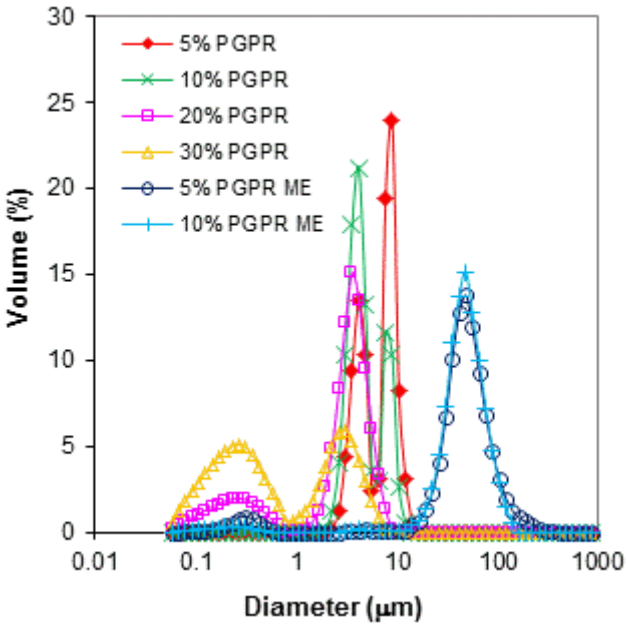

Figure 10

Figure 10 black and white

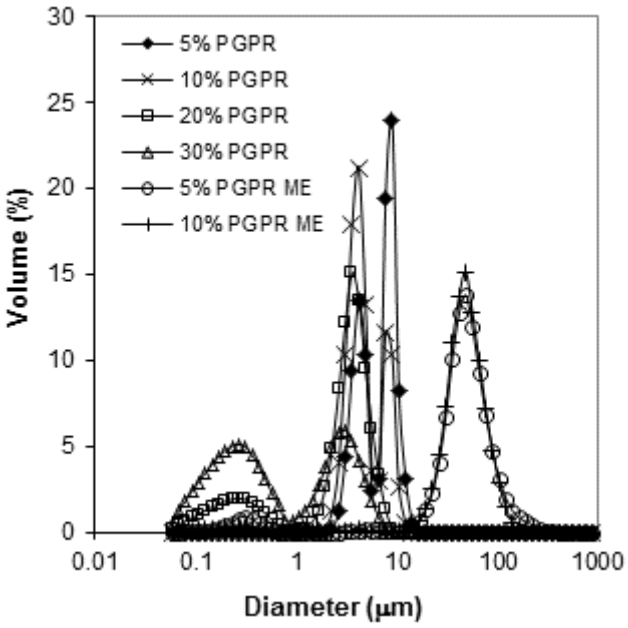

Figure 10

Figure 11 color (web)

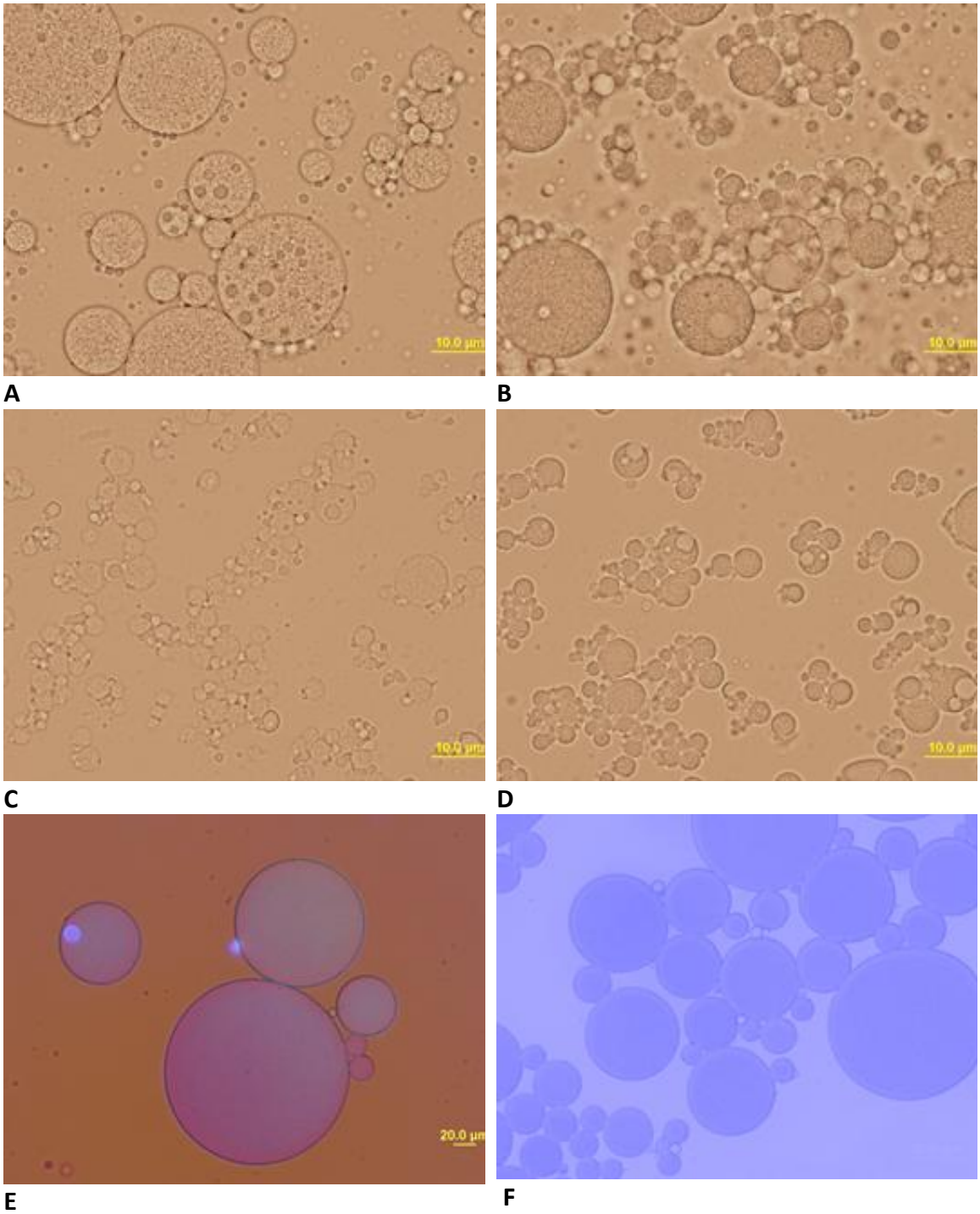

Figure 11

Figure 11 black and white

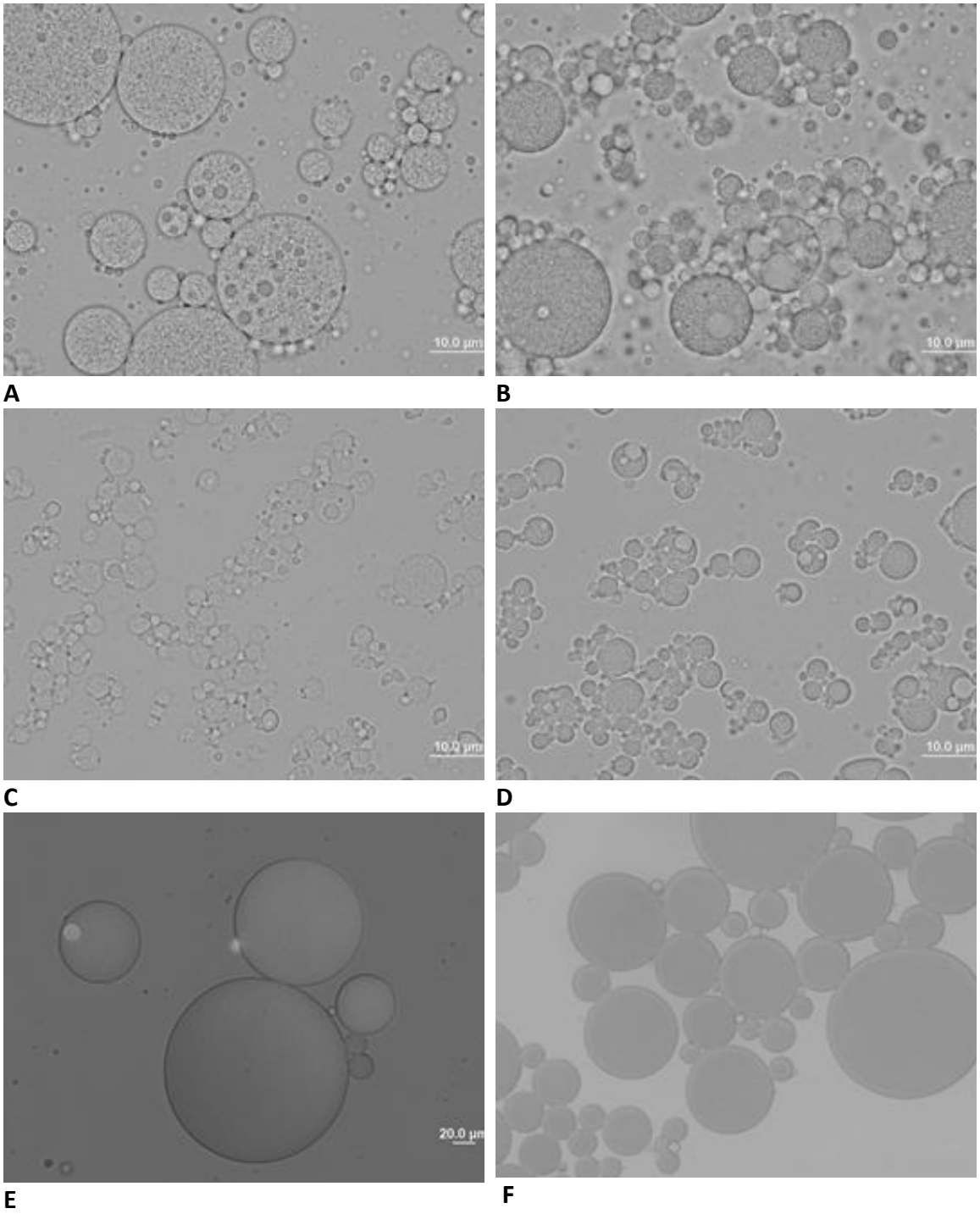

Figure 11

Figure 12 color (web)

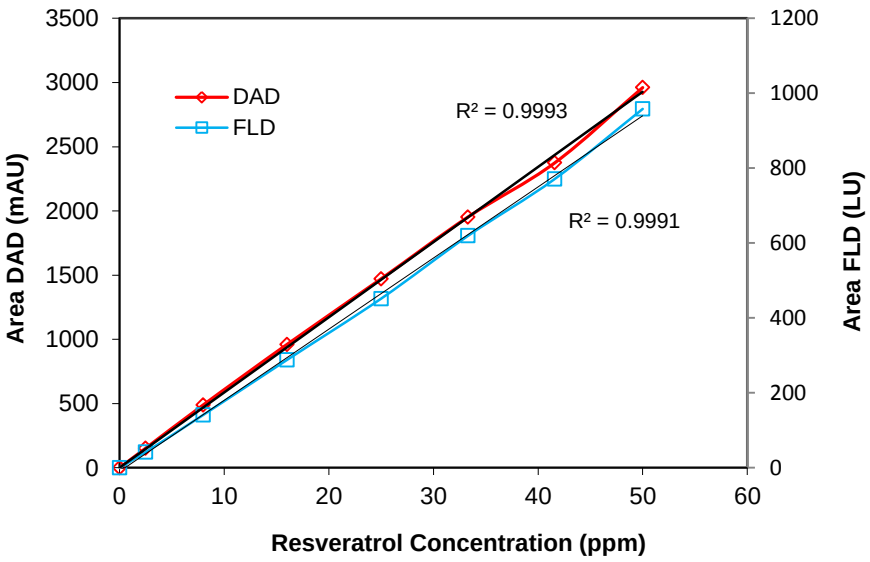

Figure 12

Figure 12 black and white

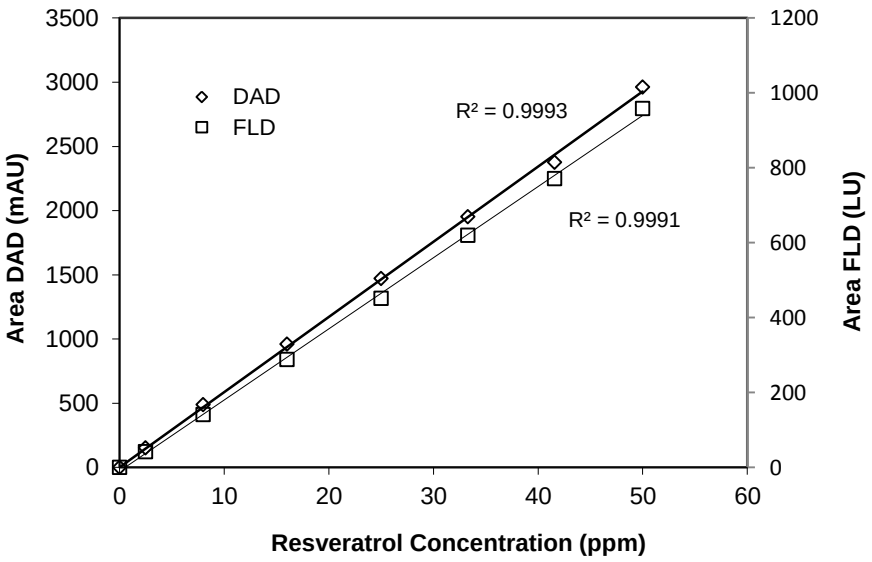

Figure 12

Figure 13 color (web)

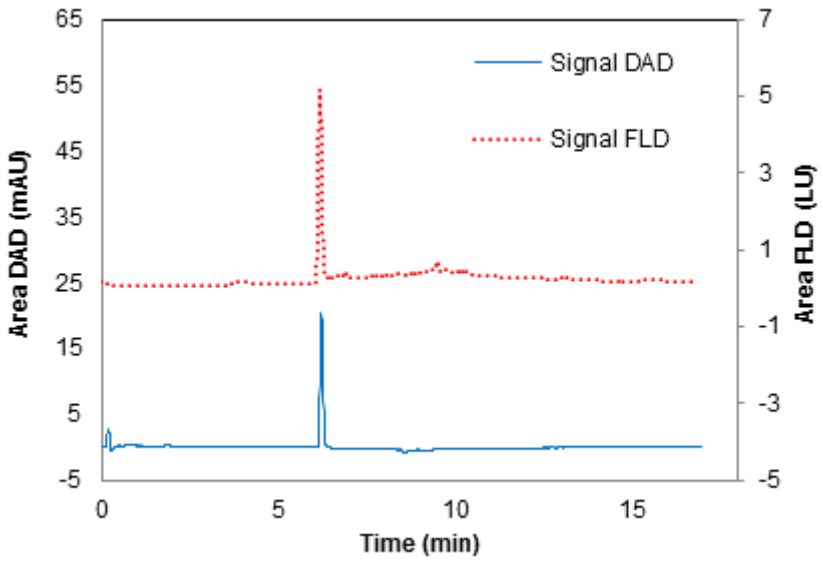

Figure 13

Figure 13 black and white

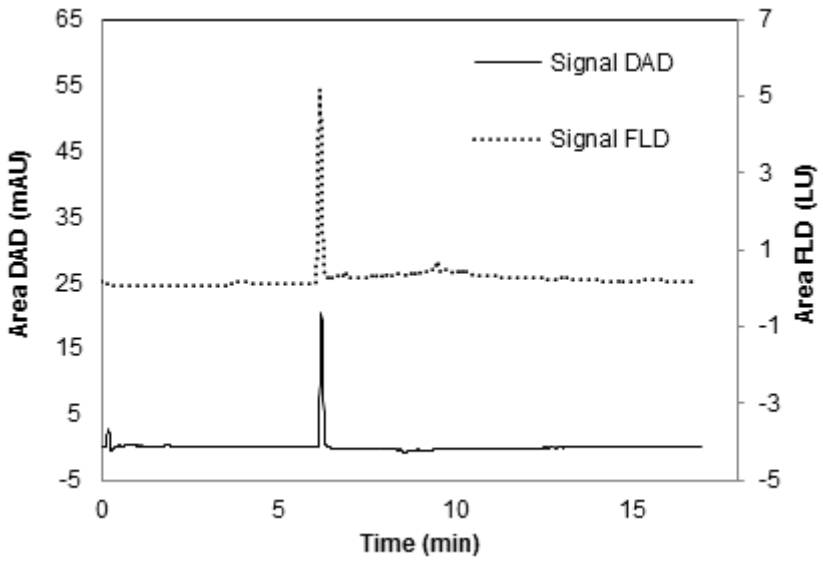

Figure 13

Figure 14 color (web)

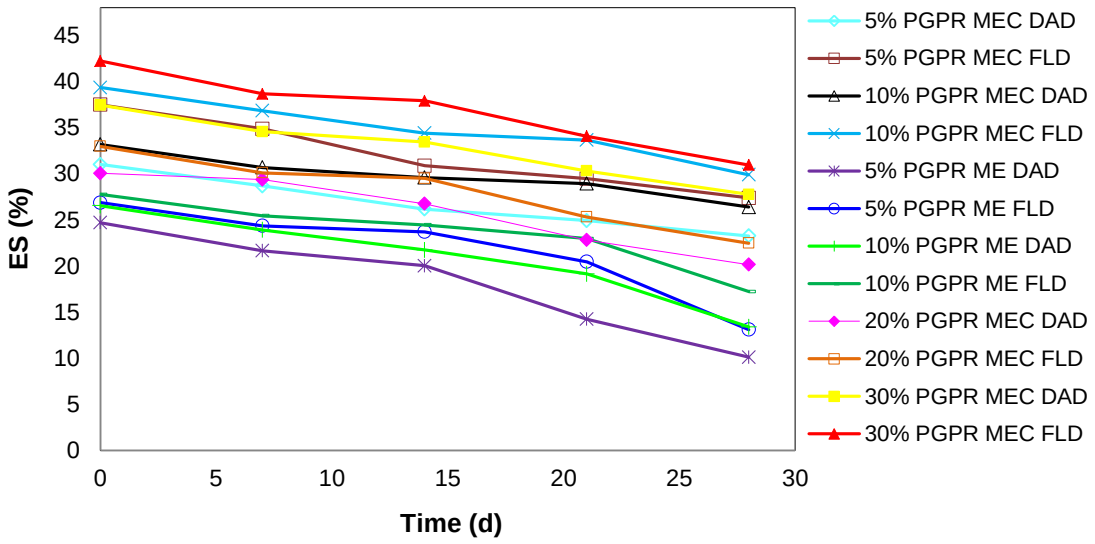

Figure 14

Figure 14 black and white

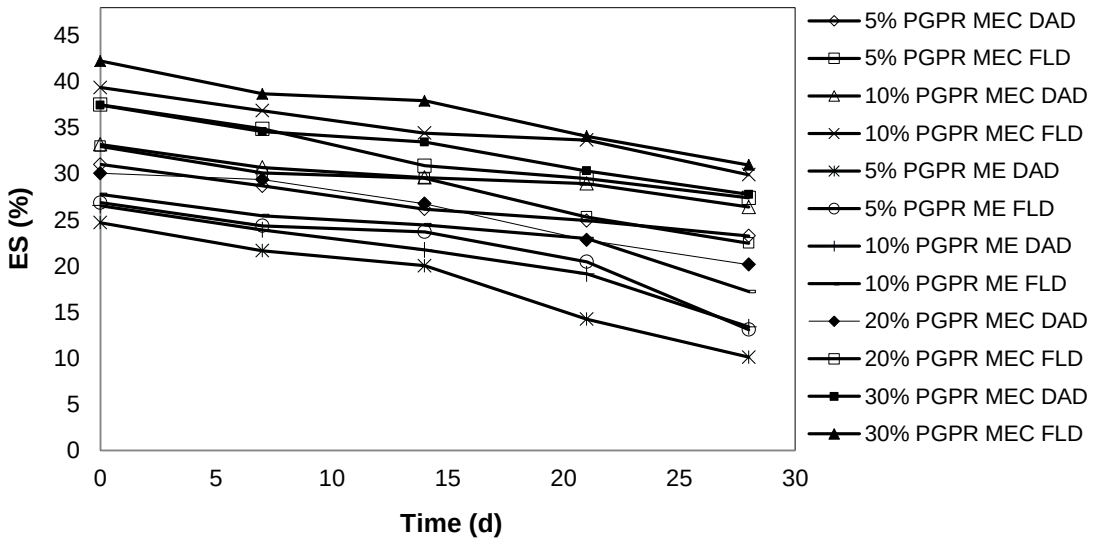

Figure 14