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Research Article

Encapsulation of EGCG and esterified EGCG derivatives in double emulsions containing Whey Protein Isolate, Bacterial Cellulose and salt

Vasiliki Evageliou*, Elli Panagopoulou, Ioanna Mandala

Department of Food Science and Human Nutrition, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece

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ABSTRACT

Double (W1/O/W2) emulsions of 0.8 %wt PGPR-0.25 %wt BC-1 %wt WPI and NaCl (1.6–8 %wt) were characterized regarding their microstructure, droplet size, stability and viscosity. Droplet size increased with salt concentration. Salt addition at 4–5% wt limited the instability rate. Viscosity increased with NaCl concentration up to 4.8 %wt, above which it decreased. Eventually, the emulsion with 4.3 %wt salt was selected for catechin incorporation. In order to increase its antioxidant activity, Epigallocatechin Gallate (EGCG) was esterified with stearic acid. Esterification was partial and a mixture of esters was obtained. Both EGCG and its esterified derivatives were incorporated in the double emulsion. Storage reduced encapsulation. Encapsulation efficiency was reduced in the order "EGCG in the W1 phase" emulsion > "EGCG in the O phase" emulsion > "esterified EGCG in the W1 phase" emulsion. The presence of both catechins did not have a great effect on retarding emulsion oxidation.

1. Introduction

Epigallocatechin gallate (EGCG) is the predominant catechin of tea as it accounts for about 50% of its total polyphenols (Chaturvedula & Prakash, 2011). It has a molecular formula of $C_{22}H_{18}O_{11}$ and a four-ring structure with eight hydroxyl groups. Thus, it is highly hydrophilic and exhibits poor solubility in lipid medium. EGCG has exhibited significant health benefits like antiaging activity (e.g. Li, Chan, Huang, & Chen, 2007), in the prevention and treatment of various diseases like tumor (e.g. Larsen & Dashwood, 2010) or cardiovascular diseases (McKay & Blumberg, 2002).

According to literature, EGCG has the highest antioxidant activity among catechins (Dai, Chen, & Zhou, 2008; Devika & Stanely, 2008; Li, Taylor, Ferruzzi, & Mauer, 2013; Saffari & Hossein, 2004). Although catechins are the most powerful antioxidants among the known plant phenols (Bancirova, 2010), pro-antioxidant activity is also reported when they are added to lipid dispersions. Their concentration and the matrix pH were the factors affecting this behaviour. For example, Zhou and Elias (2013) reported that EGCC had a pro-oxidant effect when added in o/w emulsions at concentrations of 1–500 μM and for pH < 4. Literature also reports that structural modification of EGCG via esterification with long chain fatty acids resulted in derivatives with greater antioxidant activity than EGCG itself (e.g. Zhong & Shahidi, 2011). Furthermore, EGCG's presence in foods enhances food safety as it shows antimicrobial activity (Nikoo, Regenstein, & Ahmadi Gavlighi, 2018).

In order to exploit these advantages, the incorporation of EGCG in food products would be of great interest especially due to the consumers' growing need for healthier food. However, EGCG in solution is unstable and degrades faster with an increase in pH or temperature. Thus, encapsulation seems a good solution to overcome this obstacle. In the present work, double emulsions will be used as matrices.

Double emulsions are "emulsions of emulsions" and they can be characterized either as water droplets–in-oil droplets–in–water (W/O/W) or oil droplets–in-water droplets–in–oil (O/W/O). In W/O/W systems, oil droplets containing small water droplets are dispersed in an aqueous continuous phase. Double emulsions are not thermodynamically stable. Critical parameters affecting their stability are the emulsion composition, the emulsification conditions and the osmotic pressure of the inner droplets. However, they present several advantages over single emulsions. For example, double emulsions can trap and protect various substances (e.g. hormones, vitamins, minerals, carotenoids, microorganisms, phenolic compounds, amino acids) and then control their release from inside one phase to another. As a result,

Abbreviations: EGCG, Epigallocatechin gallate; SA, stearoyl chloride; BC, Bacterial Cellulose; ΔBS, variation of backscattering; EE%, encapsulation efficiency; W1, internal aqueous phase; O, oil phase; estEGCG, esterified EGCG derivatives; PGPR, Polyglycerol polyricinoleate; WPI, Whey Protein Isolate; SI%, serum index; ES%, encapsulation stability; W2, external aqueous phase

* Corresponding author.
E-mail addresses: evageliou@aua.gr (V. Evageliou), imandala@aua.gr (I. Mandala).

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double emulsions are used as means of micro-encapsulation for various industries (e.g. pharmaceutical, cosmetics etc). Regarding food industry, they can incorporate nutritional and bioactive compounds and as such they can be used as food ingredients. Other uses include the formation of products with low calories and reduced fat and salt, the masking of flavours, the prevention of oxidation and the improvement of sensory characteristics of foods (Muschiolik, 2007).

The present study comprised three steps. Initially, modification of EGCG via esterification with stearic acid was carried out. The reaction and its products were evaluated and identified. Then, double emulsions were formed in the presence of Bacterial Cellulose (BC), Whey Protein Isolate (WPI) and Polyglycerol polyricinoleate (PGPR). Both WPI and BC are green biopolymers coming from renewable resources and waste streams, respectively. PGPR, due to the excellent water-binding capacity of the long hydrophilic polyglycerol chain, is probably the most effective lipophilic emulsifier. However, it is not a natural product and at concentrations greater than 5% its presence results in an unpleasant off-taste (Márquez, Medrano, Panizzolo, & Wagner, 2010). In the present study, the used PGPR concentration is well below that limit. Based on the findings of a recent work of our team (Panagopoulou et al., 2017) a double emulsion with 0.8 %wt PGPR-0.25 %wt BC-1 %wt WPI was used in the present work but as a further step salt was also incorporated. Our aim was these emulsions to simulate real food emulsion systems like dressings and fillings for batter applications that include salt in their recipe. All produced emulsions were characterized in terms of microstructure, droplet size, stability and viscosity. Finally, EGCG and its esterified derivatives were incorporated in the double emulsion which had the best overall performance in the previous step. The effect of catechins on emulsion oxidation was determined.

As mentioned earlier, both EGCG and its esterified derivatives are great antioxidants. Thus, our first aim was to investigate the encapsulation of both catechins in double emulsions and furthermore, to check whether or not their incorporation affected oil oxidation.

2. Materials and methods

2.1. Materials

(−)-Epigallocatechin gallate hydrate (EGCG) was from TCI (TCI, Tokyo, Japan). Whey Protein Isolate (WPI), Lacprodan DI-9224, was kindly donated by Arla Foods Ingredients (Amba-Denmark). PGPR (Grindsted PGPR 90 Kosher) was a product of Danisco (Copenhagen, Denmark) and was kindly donated by Alteco Ltd (Athens, Greece). Bacterial cellulose (BC) was produced according to the method of Tsouko et al. (2015) and had a water content of 99 %wt. Extra virgin olive oil Altis Klasiko (Elais, Unilever S.A., Athens, Greece) was purchased from a local supermarket and used without further purification. Sodium chloride (NaCl) was purchased from Panreac Quimica S.A. (Barcelona, Spain) and all the remaining reagents from Sigma-Aldrich (Steinheim, Germany). Distilled water was used throughout.

2.2. Methods

2.2.1. EGCG esterification

2.2.1.1. Preparation of esterified EGCG (est EGCG). EGCG was esterified by stearoyl chloride (SA) at a molar ratio of 1:1. Initially, EGCG was dissolved in ethyl acetate at 40 °C. Then, the stearoyl chloride was added dropwise, in the presence of pyridine which removed the released HCl from the medium. The mixture remained at 40 °C for 3 h under constant stirring. The mixture was then cooled to ambient temperature and filtrated. The filtrate was washed three times with distilled water (60 °C). The upper organic layer was collected, passed through anhydrous sodium sulphate and evaporated in vacuum at 40 °C to yield a light yellow dry powder. The powder was stored in desiccators at −20 °C until further use.

2.2.1.2. Identification of esterified EGCG derivatives. As the dry powder was a mixture of EGCG esters, its composition was determined by reversed phase LC-MS, using a Shimadzu LC/MS-2010A (Tokyo, Japan) equipped with a LC-10ADvp binary pump, a DGU-14A degasser, a SIL-10ADvp autosampler, a FCV-10ALvp Solvent Mixer, a SCL-10Avp System Controller, a MS-2010A Detector and a SPD-M10Avp Photo Diode Array Detector. A C-18 column (Phenomenex Luna C18 column, 4.6 mm × 250 mm, 5 μm) was used for the separation and a gradient of methanol:water as the mobile phase (from 95:5 to 100:0 in 30 min at a flow rate of 1 mL/min). Chromatograms were recorded at 280 nm and peaks were detected by the MS detector. The compounds present in the samples were tentatively identified according to their UV and mass spectra and retention times compared to those of the parent EGCG molecule.

2.2.2. Double emulsions

2.2.2.1. Preparation of double (W1/O/W2) emulsions. Double (W1/O/W2) emulsions at a specific PGPR-BC-WPI combination (0.8 %wt-0.25 %wt-1 %wt) in the presence of NaCl were prepared at ambient temperature (25 °C) and at pH 3.6, as described by Panagopoulou et al. (2017). Briefly, the water in oil W1/O emulsion was prepared by dispersing the W1 internal aqueous phase (20%) in the O phase (80%). Then, 40 %wt of the W1/O emulsion was dispersed into 60 %wt W2 external aqueous phase. Mixing for both steps was performed by Ultra Turrax T25, (IKA, Werke Staufen, Germany). PGPR was added in the O phase (extra virgin olive oil) of the W1/O emulsion whereas BC and WPI were the external surfactants in W2 phase. Elevated amounts of NaCl (1.6, 3.2, 4.3, 4.8, 5.3, 6.4 and 8 %wt) were added in the W1 phase.

2.2.2.2. Light microscopy. In order to evaluate microstructure, images of the emulsions were taken using an optical trinocular microscope MBL 2100 (A. Krüss Optronic, Hamburg, Germany), as described by Panagopoulou et al. (2017).

2.2.2.3. Apparent viscosity. A Discovery Hybrid Rheometer HR3 (TA Instruments, New Castle, DE, USA) equipped with a concentric cylinder jacket assembly (30 mm cup diameter, 28 mm bob diameter) was used for measuring the viscosity of the emulsions. Measurements were performed at a constant temperature (25 °C) via a thermoelectric chiller ThermoCube (Solid State Cooling systems, NY, U.S.A.) Steady flow curves were obtained from shear stress versus shear rate (1–1000 s^{-1}) data.

2.2.2.4. Determination of average particle size and droplet size distribution. The particle size distribution and the mean particle diameter were investigated by measuring the droplet size of the droplets via the Image Pro Plus 7.0 analysis software (Media Cybernetics, Inc., Rockville, USA). Approximately 300 droplets were measured for each sample and the arithmetic mean diameter was calculated. Measurements were carried out twice per formulation. Prior to measurement, the emulsions were diluted with citrate buffer solution (pH 3.6) at a 1:1000 ratio.

2.2.2.5. Emulsion stability. A Turbiscan Classic MA 2000 and the Turbisoft software (Formulaction SA, L' Union, Toulouse, France) were used for the evaluation of emulsion stability under storage for 7 days at ambient temperature. Backscattering (BS) intensity along the height of a transparent tube was measured and variation of backscattering with time (ΔBS) as well as serum index (SI%) were calculated following the equations reported by Panagopoulou et al. (2017). Measurements were performed twice per formulation.

2.2.3. EGCG incorporation

2.2.3.1. EGCG incorporation. Based on the results of § 2.2.2, the double emulsion with the best overall performance was selected. Both EGCG

and esterified EGCG derivatives (estEGCG) were dissolved both at the internal dispersed phase W1 and the oil phase O of the selected emulsion at a (final) concentration of 0.23 mg/mL. This concentration was selected following preliminary experiments on EGCG and estEGCG solutions (Panagopoulou, Mandala, & Evageliou, 2015). Several properties of both solutions like total phenolics content, antioxidant activity (FRAP), solubility and stability, were studied under varying pH, temperature, concentration and shear parameters. According to our findings, among the concentrations studied, 0.23 mg/mL had the greatest antioxidant activity and stability in the acidic conditions that the emulsions were made.

When present in the aqueous phase, catechins were dissolved under continuous stirring. For the oil phase, catechins were dissolved in ethanol. Oil was then added and the mixture was placed in a rotary evaporator for the evaporation of ethanol. The catechins' encapsulation efficiency as well as the oxidation of the produced emulsions were evaluated.

2.2.3.2. Encapsulation efficiency and stability. Encapsulation efficiency (EE%) was calculated as the ratio of EGCG or estEGCG concentration entrapped in the inner aqueous phase (W1) or the oil phase (O) upon preparation to their concentration initially added to the emulsions. Encapsulation stability (ES%) was calculated as the ratio of EGCG or estEGCG concentration which remained entrapped in the inner aqueous phase (W1) or the oil phase (O) following storage to their concentration initially added to the emulsions. When encapsulated in the inner aqueous phase (W1), both catechins were extracted from the emulsions following the procedure described by O'Regan and Mulvihill (2009, 2010). In this procedure, centrifugation is used to separate the cream phase (W1/O) and the aqueous phase (outer aqueous phase, W2). The absorbance of the recovered outer aqueous phase at 274 nm was measured. The concentration of the EGCG or estEGCG in the outer aqueous phase (W2) of the emulsion was determined by reference to a standard curve.

EE% and ES% were calculated from the following equations:

$$EE\% = \frac{\textit{initial concentration of catechin in the emulsion} - \textit{concentration of catechin day 0}}{\textit{initial concentration of catechin in the emulsion}} \times 100\% \quad (1)$$

$$ES\% = \frac{\textit{initial concentration of catechin in the emulsion} - \textit{concentration of catechin day 30}}{\textit{initial concentration of catechin in the emulsion}} \times 100\% \quad (2)$$

where "concentration of catechin day 0" is the concentration of EGCG or estEGCG in the outer aqueous phase W2 upon preparation. "Concentration of catechin day 30" is the concentration of EGCG or estEGCG in the outer aqueous phase W2 after storage at room temperature for 30 days.

When EGCG was dissolved in the oily phase, its extraction was achieved by the procedure described by Paximada, Echegoyen, Koutinas, Mandala, and Lagaron (2017). Briefly, ethanol was added to the emulsion for releasing the entrapped EGCG followed by hexane needed for EGCG to be extracted. A 2-phase system was produced. The organic (upper) phase was separated, and the EGCG concentration was determined spectrophotometrically, as previously described.

EE% and ES% were calculated from the following equations:

$$EE\% = \frac{\textit{concentration of catechin day 0}}{\textit{initial concentration of catechin in the emulsion}} \times 100\% \quad (3)$$

$$ES\% = \frac{\textit{concentration of catechin day 30}}{\textit{initial concentration of catechin in the emulsion}} \times 100\% \quad (4)$$

where "concentration of catechin day 0" is the concentration of EGCG recovered from the oil phase upon preparation and "concentration of catechin day 30" is the concentration of EGCG recovered from the oil phase after storage at room temperature for 30 days.

2.2.3.3. Emulsion oxidation. Two markers of lipid oxidation were used: the conjugated dienes (CD) and the p-anisidine value (AV). CD formation is an index of intermediate products of the first stage of lipid oxidation and it was determined by absorbance measurements at 234 nm, following the procedure described by Jayasinghe, Gotoh, and Wada (2013).The secondary products of the oxidation process were evaluated with the p-anisidine method, as described by the official AOCS method (AOCS CD 18-90, 2006). For comparison reasons, an emulsion with no catechins present was tested as control. Measurements were performed in a double-beam UV–Vis spectrophotometer (Shimadzu UV-1800, Tokyo, Japan).

2.2.4. Statistical analysis

Data were analyzed with the one way ANOVA program. The level of confidence was 95%. Significant differences between means were identified by the LSD procedure with the statistical software package Statistica v.8.0 for Windows.

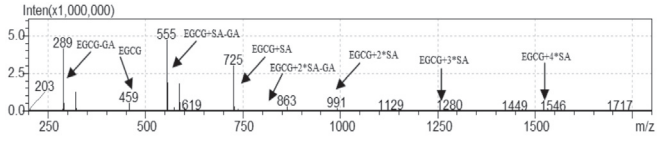
3. Results & discussion

3.1. EGCG esterification

Esterification was achieved via a reaction of EGCG with an acylating agent, namely the stearoyl chloride (SA). The result of the esterification was a mixture of EGCG-stearic acid polyesters (tetraester and lower) as well as unreacted EGCG which was not calculated as a component of the crude products. The yield of the esterification was calculated as the ratio of total amount of esters obtained to amount of esters expected for a full reaction and it was 55.8%. Fig. 1 presents the composition of the crude products. The percentage of each ester was calculated as the ratio of its amount to the total amount of all esters. It is clear that under the selected experimental condition, the esterification was partial and that the tetraester was the product with the lower yield.

The products were identified with HPLC-MS. The mass spectrum of the esterified EGCG is presented in Fig. 1. The molecular ion peak detected showed an m/z at 1524 representing EGCG-tetrastearate (EGCG + 4*SA). The peaks at m/z 1258, 991, 725 and 459 showed one or multiple mass loss of the stearoyl moiety, thus corresponding to EGCG + 3*SA, EGCG + 2*SA, EGCG + 1*SA and EGCG, respectively. In addition, the peaks at m/z 821, 555 and 289 resulted from additional cleavage of a gallic acid moiety (GA). As a result, they correspond to EGCG + 2*SA-GA, EGCG + SA-GA and EGCG-GA, respectively.

Zhong and Shahidi (2011) esterified EGCG with selected long-chain saturated and unsaturated fatty acids (i.e. stearic, eicosapentaenoic and docosahexaenoic acid). Greater esterification yield was reported for



Esters	m/z	%
EGCG+4*SA	1524	0.016
EGCG+3*SA	1258	0.205
EGCG+2*SA	991	7.170
EGCG+1*SA	725	92.609

Fig. 1. Mass spectra of EGCG derivatives and composition of the crude products.

stearic acid esters (56.9%), which is in good agreement with the yield reported in the present study (55.8%). The lower yields of the unsaturated fatty acids were attributed to steric hindrance due to their non linear structure. In the present study, preparation of EGCG esters using linoleoyl chloride also took place. However, due to the low, as expected, yield and handling problems, the stearic esters were chosen for further experiments.

Regarding the composition of the esters, Zhong and Shahidi (2011) found that the EGCG esterification with stearic acid resulted predominantly in tetraesters, as well as pentaesters. In our case, tetraesters were also formed but at very low yields, whereas the monoester was the predominant form. This is in good agreement with the findings of Chen, Wang, Du, and Ito (2002). They reported a monoester as the main derivative of the esterification of EGCG with hexadecanoyl chloride. These discrepancies can be attributed to the fact that esterification is a complicated procedure and the conditions applied (e.g. EGCG: fatty acid ratio) are very critical and affect the composition of the products significantly.

3.2. Double emulsions

The phase behaviour of double emulsions with a PGPR-BC-WPI combination of 0.8 %wt-0.25 %wt-1 %wt, respectively, and elevated amounts of NaCl (1.6, 3.2, 4.3, 4.8, 5.3, 6.4 and 8 %wt) is shown in Fig. 2a. For emulsions with 1.6 %wt and 3.2 %wt NaCl, clarification was obvious. At salt concentrations of 4.3–5.3 %wt the emulsions were homogeneous whereas at 6.4 %wt clarification was apparent again. Fig. 2b is a micrograph showing the expected microstructure of a double emulsion as well as the formation of flocs.

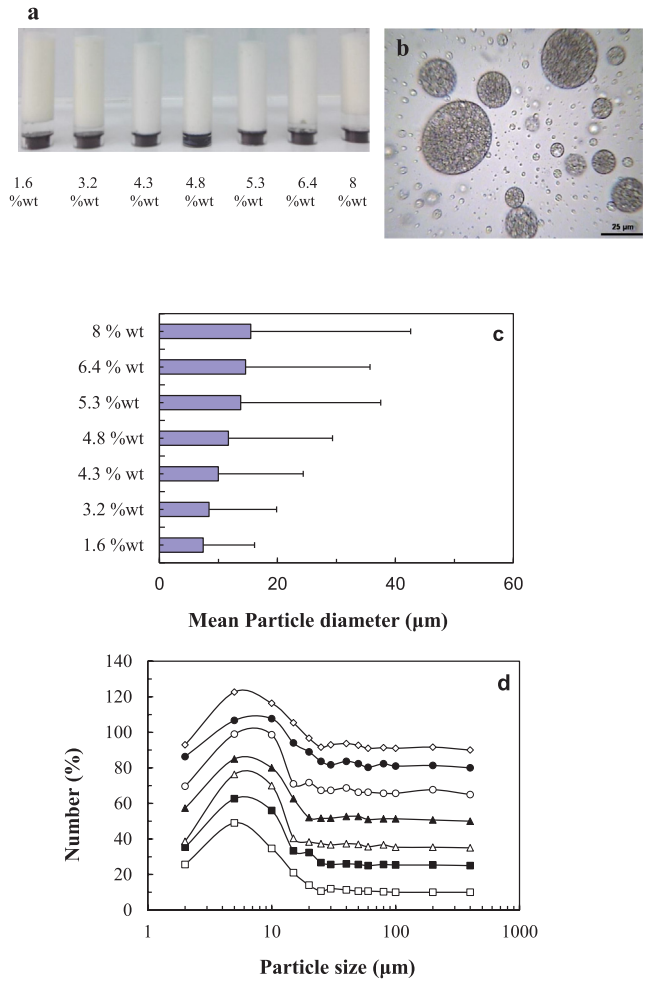


Fig. 2. Double W1/O/W2 emulsions (0.8%wt PGPR, 0.25%wt BC and 1%wt WPI) in the presence of 1.6–8 %wt NaCl. (a) Photographs taken on the seventh day of storage, (b) Micrographs after preparation (Scale bar represents 25 μm), (c) Mean particle size and (d) particle size distribution [1.6 %wt (□), 3.2 %wt (■), 4.3 %wt (Δ), 4.8 %wt (▲), 5.3 %wt (○), 6.4 %wt(●) and 8 %wt (◊)].

Fig. 2 also presents the mean particle size and the particle size distribution of the emulsions formed at various salt concentrations (Fig. 2c and d, respectively). The clear trend was that increasing amounts of salt led to increased particle size (Fig. 2c), with the mean droplet size ranging from 7.5 to 15.5 μm. Moreover, the diameter for the vast majority of the particles ranged from 5 to 15 μm. The corresponding primary emulsion (W1/O) had a mean droplet size of 0.46 μm.

According to literature, the addition of electrolytes to the internal aqueous phase results in a modification of several stabilising parameters like Ostwald ripening, coalescence etc (e.g. Park, Cho, & Lee, 2003). This is achieved by several ways. Electrolytes lower the attractive forces between the water droplets and thus, enhance stability, decrease the dielectric constant of the aqueous phase and reduce the collision frequency (Paunovic & Schlesinger, 2006).

Regarding stabilisation of double emulsions in the presence of electrolytes, Kanouni, Rosano, and Naouli (2002) reported that the presence and concentration of salt in the W1 phase plays an important role in balancing the Laplace and the osmotic pressures (between W1 droplets in oil and between W1 droplets and the external aqueous phase W2). It is also suggested that electrolytes can increase the emulsifier adsorption density at the oil- water interface and reduce interfacial tension in W/O emulsions (Foudazi, Qavi, Masalova, & Malkin, 2015).

When PGPR is used as an emulsifier, Scherze, Knoth, and Muschiolik (2006) showed that addition of NaCl to the dispersed phase of PGPR was essential for preventing the dispersed water droplet coalescence. Additionally, NaCl was found to increase the adsorption density of PGPR at the interfacial film (Márquez et al., 2010). Furthermore, a synergistic effect between NaCl and PGPR on stabilising W/O emulsion was also reported (Sapei, Naqvi, & Rousseau, 2012).

At the same time, the same researchers (Sapei et al., 2012) reported an increase in average droplet size with an increase in salt concentration, which is in good agreement with our findings. Literature (Lutz & Garti, 2006; Mezzenga, Folmer, & Hughes, 2004) attributed the increase in droplet size to higher osmotic pressure gradient experienced between the two aqueous phases, implying greater water infusion from the external to the internal aqueous phase. As a result, oil droplets swelled and expanded. Additionally, their packing density was increased because the polydispersed aqueous droplets effectively filled the available space between the oil droplets (Chanamai & McClements, 2000).

The viscosity of the produced double emulsions was measured and presented in Fig. 3. All emulsions exhibited shear thinning behaviour. However, the major observation is that initially viscosity increased with NaCl content up to a certain salt concentration, above which it decreased. The critical salt concentration was 4.8 %wt.

The viscosity of double emulsions depends on the nature and behaviour of the biopolymers in the continuous phase solution, the arrangement of the biopolymers at the surface and the water concentration of the outer aqueous phase (Bonnet et al., 2009). Water can migrate either from the internal to the external phase or vice versa, depending on the relative difference in osmotic pressure between the two phases and by Laplace Pressure (Mezzenga et al., 2004). Its transport rate is affected by various parameters among them the magnitude of the osmotic pressure gradient between the aqueous phases, the nature and concentration of the surfactant as well as the nature and the viscosity of the oil used (Leal-Calderon, Bibette, & Schmitt, 2007). In our case, the emulsions prepared differed only on their salt concentration. Thus, this is the critical parameter in our attempt to explain our findings. The addition of salt in the inner phase induces an osmotic gradient that, as mentioned earlier, leads to the penetration of the outer aqueous phase into the inner phase. Thus, the concentration of the

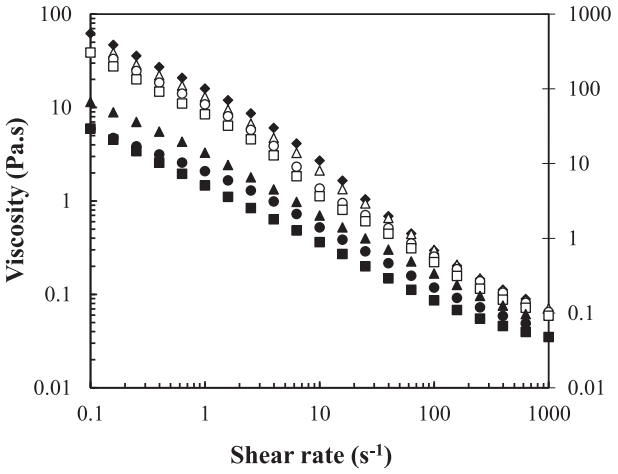


Fig. 3. Flow curves for double emulsions containing 0.8%wt PGPR, 0.25%wt BC-1%wt WPI and varying concentrations of NaCl [1.6 %wt (■), 3.2 %wt (●), 4.3 %wt (▲),4.8 %wt (♦), 5.3 %wt (Δ), 6.4 %wt (○) and 8 %wt (□)]. For clarity reasons the three lower salt concentrations are plotted in the secondary Y-axis.

outer aqueous phase decreases. In order to equalize the osmotic pressure, droplets pack closer to each other and as a result entanglement and viscosity increase.

In the present study, viscosity increased for salt concentrations up to 4.8 %wt, in good agreement with the results from Lutz, Aserin, Wicker, and Garti (2009). However, as the salt concentration increased further a small drop in viscosity was seen. Torres, Iturbe, Snowdden, Chowdhry, and Lehane (2007) also reported similar behaviour in O/W Pickering emulsions. According to literature, emulsions containing either macromolecules (like WPI) or particles (e.g. BC) are highly affected by the presence of salt. Initially, the addition of free ions produces a screening of the repulsive DVLO potential, the attractive interactions between the droplets become stronger and thus, viscosity is increased. The viscosity decrease can be attributed to the salt's release from the inner phase. According to Sapei et al. (2012), the NaCl release in double emulsions increased with the increase in its concentration presumably due to loss of NaCl from the internal aqueous phase during the second stage of homogenisation. In agreement to this suggestion, Lakkis (2007) suggested that low molecular weight compounds can be released rapidly as a result of high osmotic and/or increased concentration gradient.

Based on the flocculated droplets seen in the micrographs of Fig. 2b the shear thinning behaviour observed in the flow curves was expected. Flocculated droplets exhibit shear-thinning behaviour because flocs are deformed and disrupted as shear rate increases (Panagopoulou et al., 2017).

Stability is a major concern of emulsions. Table 1 demonstrates the variation of backscattering (ΔBS) and the serum index (SI%) of the emulsions prepared following their storage at ambient temperature for 7 days. Salt concentration was an important parameter for the emulsion's stability. Among the emulsions, those containing the two lower and the two higher salt concentrations exhibited phase separation and thus, their Serum Index (SI%) was calculated. SI% values ranged from 7.5 to 14 % with the higher salt concentration (8 %wt) exhibiting the higher SI% value. The remaining emulsions, which did not exhibit phase clarification, were examined for their flocculation rate according to the variation of backscattering with time (ΔBS). For emulsions containing NaCl at concentrations 4.3, 4.8 and 5.8 %wt, ΔBS was statistically the same.

As already mentioned, the addition of salt causes interfacial conformational changes which would increase the stability of emulsions by stiffening the interface (Aronson & Petko, 1993). However, it seems that there is an optimal concentration range for greater stability. In our systems, better results were achieved at 4.3–5.3 %wt NaCl. Combining all our findings from particle size, viscosity, stability and phase behaviour tests, the emulsion with 4.3 %wt salt was selected for the incorporation of both catechins.

Table 1
Backscattering variation (ΔBS) and Serum Index (SI%) of double emulsions formed in the presence of salt.

NaCl (%wt)	ΔBS	SI%
1.6		11.3^{a} ± 0.5
3.2		7.6^{b} ± 0.5
4.3	1.8^{a} ± 0.5	
4.8	2.9^{a} ± 0.4	
5.3	3.5^{a} ± 0.9	
6.4		9.6^{a} ± 0.6
8		14.3^{c} ± 0.9

*Mean values followed by the same letters within the same parameter are not significantly different (P > 0.05).

3.3. EGCG and estEGCG incorporation in double emulsions

A standard amount of both EGCG and estEGCG in either W1 or O phase was added in a 0.8 %wt PGPR – 0.25 %wt BC – 1 %wt WPI – 4.3% wt NaCl double emulsion. Thus, that resulted in 4 combinations, i.e. EGCG in W1, EGCG in O, estEGCG in W1 and estEGCG in O. The latter was highly unstable, possibly due to the difficulty of a macromolecule like the catechins to be dissolved, and thus, was not studied any further. The encapsulation was measured upon preparation and after 30 days of storage at ambient temperature and encapsulation efficiency (EE%) and encapsulation stability (ES%) were calculated, respectively. According to our findings (Table 2), encapsulation ranged from 84–91% for day 0 and 63–84% for day 30. Among the emulsions and for both storage days, the "EGCG in W1" one showed the greater encapsulation whereas the "estEGCG in W1" the lower. Moreover, the concentration of encapsulated catechin over storage showed a reduction that varied from 4 to 13% for the various emulsions. The known degradation of EGCG solutions over time can explain the reduction in encapsulation over storage. The hydrophilic nature of EGCG can explain its better encapsulation. As estEGCG is less hydrophilic than EGCG (Zhong & Shahidi, 2011), it presented reduced encapsulation.

Emulsion oxidation upon preparation and on the 30th day of storage was then determined by the conjugated dienes (CD) and the p-anisidine value (AV) methods. The corresponding data are presented in Table 3. The emulsion in the absence of catechins is also measured ("control"). As expected the values for both parameters are greater after storage. Regarding CD results, the "EGCG in W1" emulsion had the best performance. The "control", despite the fact that upon preparation had the second best performance, over storage showed the greatest CD value. Regarding AV values, all the emulsions apart from the "EGCG in W1" emulsion exhibited the same level of oxidation. Overall, upon preparation, the oxidation process is going faster for the control sample and slower for the "EGCG in W1" emulsion. The exact opposite is reported for storage.

The behaviour of the control emulsion was expected as the O phase

Table 2
Encapsulation efficiency (EE%) and encapsulation stability (ES%) of the studied emulsions. Three emulsions per formulation were measured.

	Encapsulation Efficiency (EE %)	Encapsulation Stability (ES%)
EGCG in W1	90.8^{a} ± 0.1	83.8^{a} ± 0.1
EGCG in O	86.3^{b} ± 0.1	81.5^{b} ± 0.1
estEGCG in W1	76.3^{c} ± 0.4	63.2^{c} ± 0.2

*Mean values followed by the same letters within the same storage time are not significantly different (P > 0.05).

Table 3
Conjugated dienes (CD) values and p-anisidine (AV) values of the studied emulsions upon preparation (t = 0) and after 30 days of storage (t = 30 days). Three emulsions per formulation were measured.

	Conjugated dienes (CD) values		p-anisidine (AV) values	
	t = 0	t = 30 days	t = 0	t = 30 days
EGCG in W1	$0.66^{a} \pm 0.01$	$1.25^{a} \pm 0.02$	$0.36^{a} \pm 0.09$	$1.84^{a} \pm 0.08$
EGCG in O	$1.00^{b} \pm 0.02$	$1.65^{b} \pm 0.01$	$0.89^{b} \pm 0.2$	$1.67^{b,c} \pm 0.01$
estEGCG in W1	$1.18^{c} \pm 0.01$	$1.54^{c} \pm 0.01$	$1.00^{b} \pm 0.04$	$1.57^{b} \pm 0.01$
Control	$0.96^{d} \pm 0.01$	$1.76^{d} \pm 0.01$	$1.15^{b} \pm 0.09$	$1.70^{c} \pm 0.03$

*Mean values followed by the same letters within the same storage time are not significantly different (P > 0.05).

is extra virgin olive oil. Extra virgin olive oil apart from its nutritional properties contains significant amounts of phenolic compounds. Phenolics contribute to the stability of olive oil against oxidation as they are powerful antioxidants (e.g. Berzas Nevado, Rodriquez Robledo, & Sanchez-Carnerero Callado, 2012). Apart from phenolics, other natural antioxidants are also present e.g. α-tocopherol.

Given that, someone would expect that the presence of catechins will act additionally and enhance the resistance of the emulsion to oxidation. However, our observations did not support this expectation. The "EGCG in O" emulsion presented similar behaviour as the control, which is rather anticipated considering the hydrophilic nature of EGCG. Due to that, in the "EGCG in W1" emulsion, the presence of EGCG delayed oxidation upon preparation. The "estEGCG in W1" emulsion performed worse than the "EGCG in W1" emulsion which can be attributed to its lower encapsulation efficiency (Table 2).

Seeing oxidation as an overall and after storage, the incorporation of either form of EGCG did not make the emulsion more resistant. It is known that EGCG in solution is unstable. Its degradation is affected by a number of factors like temperature, pH, concentration and ionic strength. Regarding the concentration, pH and temperature of the present experiment, both catechins in solution are stable (Panagopoulou et al., 2015). Thus, the presence of salt seems to be the dominant factor. Aditya et al. (2015) studied the stability of EGCG solutions in the presence of sodium chloride. According to their findings, the presence of salt increased the rate of EGCG degradation. Moreover, storage also accelerated degradation. As such, although the selected salt concentration was the minimum in order to achieve emulsion stability, it creates an unfavourable environment for the catechins. A possible solution to this problem can be the addition of extra antioxidants like ascorbic acid, which have a protective effect on catechin degradation by inhibiting procyanidin formation (Dube, Ng, Nicolazzo, & Larson, 2010; Mahmoud, Chedea, Detsi, & Kefalas, 2013).

4. Conclusions

Epigallocatechin gallate (EGCG) was esterified with stearic acid. The esterification was partial and a mixture of esters was obtained and identified. As both EGCG and its esterified derivatives are great antioxidants, they were encapsulated in a 0.8 %wt PGPR – 0.25 %wt BC – 1 %wt WPI – 4.3% wt NaCl double emulsion. Double emulsions can be used as food ingredients acting as delivery systems for various nutritional and bioactive compounds. Better encapsulation was achieved for the "EGCG in the W1 phase" emulsion and worst for the "estEGCG in the W1 phase" emulsion. Storage reduced encapsulation. Furthermore, the effect of both catechins on emulsion oxidation was tested. However, their presence did not make the emulsion more resistant to oxidation. This was attributed to EGCG degradation which was accelerated by both the presence of salt and storage.

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Conflict of interest

The authors declare no conflict of interest.

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