Production of water soluble quercetin formulations by pressurized ethyl acetate-in-water emulsion technique using natural origin surfactants

V.S.S. Gonçalvesabc, S. Rodríguez-Rojo*, E. de Pazc, C. Matac, Á. Martínc, M.J. Coceroa

a Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da Republica, 2780-157 Oeiras, Portugal
b Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2781-901 Oeiras, Portugal
c Escuela de Ingenierías Industriales, Universidad de Valladolid, C/Dr. Mergelina s/n 47011 Valladolid, Spain

*Corresponding author: E-mail address: sorayarr@iq.uva.es (S. Rodríguez-Rojo)

Abstract

Quercetin is a strong antioxidant flavonoid with several bioactive properties such as anti-inflammatory and anticarcinogenic activities, becoming an interesting compound to be incorporated into pharmaceutical, cosmetic or food products. However, these applications are limited «by the low bioavailability of this flavonoid. Quercetin is poorly soluble in aqueous media, such as gastrointestinal fluids, being also degraded by gut flora. Thus, it is necessary the development of quercetin’s formulations capable of improving its water solubility resulting in increased bioavailability and thus higher biological activity of this compound.

The aim of the present work was the formulation of quercetin using three distinct natural origin surfactants, namely OSA-starch, Lecithin and β-glucan, by precipitation from a pressurized ethyl acetate-in-water emulsion. Formulations of quercetin with encapsulation efficiencies up to near 76% and a micellar particle size in the range of nanometers were obtained using lecithin. An improved antioxidant activity (3-fold higher per unit mass of quercetin) was also observed in these formulations, demonstrating that lecithin is a good emulsifier for the encapsulation of quercetin. Furthermore, the addition of glycerol as co-
solvent increased the colloidal stability of the suspension and the encapsulation efficiency
of the flavonoid.

**Keywords:** Quercetin, OSA-starch, Lecithin, β-glucan, Encapsulation, High Pressure Emulsion Technique, Nanosuspension

1. Introduction

Quercetin (3,3',4',5,7-Pentahydroxyflavone), one of the most representative member of the flavonoid family with high antioxidant activity, is commonly found in several fruits and vegetables like onions, apples, grapes or strawberries, as well as in red wine or green/black tea (Priprem, Watanatorn, Sutthiparinyanont, Phachonpai, & Muchimapura, 2008; Souza et al., 2013). This compound has attracted the interest of the pharmaceutical and nutraceutical industries due to its bioactive properties, such as anti-inflammatory, anti-proliferative and neuroprotective effects (Boots, Haenen, & Bast, 2008; Priprem et al., 2008). In order to achieve quercetin plasma's concentration above 10 mM required for obtaining pharmacological activity, the ingestion of quercetin-enriched foods or supplements could not be enough due to the low bioavailability of this flavonoid (Russo, Spagnuolo, Tedesco, Bilotto, & Russo, 2012). The low water solubility (2ppm at 25°C to 60 ppm at 100°C) allied with gastrointestinal degradation limits quercetin's biological effects in vivo (Souza et al., 2013; Srinivas, King, Howard, & Monrad, 2010). There are two main approaches to increase the bioavailability of this compound, whether by chemical modification or by the development of colloidal quercetin delivery systems (Barras et al., 2009).

Formulations of quercetin using different methods and distinct carrier materials have been developed by several authors. Regarding polymers, Kumari and co-authors achieved a controlled release of quercetin by its encapsulation into poly-D,L-lactide (PLA) nanoparticles
through solvent evaporation technique (Kumari, Yadav, Pakade, Singh, & Yadav, 2010). Wu et al. have produced quercetin-loaded nanoparticles by a nanoprecipitation method using Eudragit® E and polyvinyl alcohol (PVA) as carriers, obtaining a quercetin’s release 74-fold higher in comparison with the pure compound (Wu et al., 2008). Quercetin was also encapsulated in Pluronic F127 through supercritical antisolvent method by Fraile and co-authors, enabling an improved dissolution behaviour of the compound in simulated physiological fluids (Fraile, Buratto, Gómez, Martín, & Cocero, 2014). Cyclodextrins (CD), such as α-CD, β-CD or HP-β-CD, have also been used for the encapsulation of quercetin using the freeze-drying or co-evaporation method (Calabrò et al., 2004; Pralhad & Rajendrakumar, 2004). Barras and co-authors used lipids to encapsulate quercetin, being able to increase its apparent aqueous solubility by a factor of 100 (Barras et al., 2009). Besides solid lipid nanoparticles, nanostructured lipid carriers and lipid nanoemulsions were produced using a high pressure homogenizer for the encapsulation of quercetin by Aditya et al., achieving encapsulation efficiencies above 90% (Aditya et al., 2014). Quercetin-loaded liposomes have also been produced and are reported in the literature (Mignet, Seguin, & Chabot, 2013; Priprem et al., 2008). Inorganic materials can also be an option as carriers for the encapsulation of quercetin and, recently, quercetin-loaded silica microspheres were developed by Kim and co-authors using polyol-in-oil-in-water (P/O/W) emulsion and sol-gel methods to improve the flavonoid’s stability as well as its properties (Kim et al., 2014). In 2012, de Paz and co-authors (de Paz, Martín, Estrella, et al., 2012) developed a novel method for the encapsulation of hydrophobic compounds, based on the production and processing of ethyl acetate-water emulsions at high pressure and temperature. The authors achieved stable aqueous suspensions of β-carotene with micellar particle sizes down to 400 nm and encapsulation efficiencies up to 80%. Moreover, the type of emulsifier used had been shown to affect the final properties of the suspension (de Paz, Martín, Bartolomé, Largo, & Cocero, 2014). This process is an attractive alternative to the conventional emulsion
evaporation process, since it enables the acceleration of the mass transfer kinetics to the time scales of the precipitation processes. This intensification of the process allows an improved control over the precipitation, at the same time that the exposition of the product to degrading high-temperature conditions is decreased.

This work presents the development of water soluble formulations of quercetin through pressurized ethyl acetate-water emulsion technique. Ethyl acetate has been chosen as organic solvent because it is a Generally Recognized as Safe (GRAS) solvent with low toxicity (Lethal Dose LD50 in rats: 11.3 g/kg) and it can be safely used as a flavouring agent (Iemenschneider, Ag, & Republic, 2000). Three natural origin surfactants, namely modified n-octenyl succinate anhydride (OSA) starch, soybean lecithin and barley β-glucan, were used in this work for the encapsulation of quercetin. Starch is the second most abundant biomass material present in nature being the most abundant storage polysaccharide in plants (Fathi, Martín, & McClements, 2014; Nitta & Numata, 2013). However, natural starch is mainly hydrophilic, which could limit its application in the encapsulation of hydrophobic compounds. Nevertheless, OSA (modified amphiphilic starch) is capable to overcome this drawback, and was already used for the encapsulation and delivery of compounds with distinct polarities (Fathi et al., 2014). Lecithin is a mixture of naturally occurring phospholipids, mainly phosphatidylcholine, which is usually available from sources such as soybeans or eggs. Phospholipids are amphiphilic molecules composed by hydrophobic tails and hydrophilic heads, being capable to rearrange themselves as liposomes, spherical and closed structures composed of lipid bilayers (Bouarab et al., 2014; Suriyakala, N, D, & Prabakaran, 2014). Liposomes are interesting carrier materials for the delivery of hydrophobic/hydrophilic compounds, and since they have affinity to cellular membranes, they are capable to increase the absorption of several drugs (Hoeller, Sperger, & Valenta, 2009). β-glucans are soluble fibers present in cereal grains, especially in barley, constituted by linear polysaccharides of glucose units, connected by (1 → 3) or (1 → 4)-beta linkages. These carbohydrates are known
for their therapeutic effects on coronary heart disease, diabetes and hypercholesterolemia, and have been used as encapsulating agents (Charalampopoulos, Wang, Pandiella, & Webb, 2002; Raemdonck, Martens, Braeckmans, Demeester, & De Smedt, 2013; Wang, Liu, Chen, & Zhao, 2013; Wood, 2007). The influence of the main process parameters has been studied, namely the effect of quercetin and emulsifier's concentration, the effect of the flows of organic solvent, suspension of quercetin and dissolution of emulsifier and also the organic to water ratio. By comparing the results obtained with the three different emulsifiers, their roles on the emulsion formation and quercetin's encapsulation can be established. Product analysis included particle size, encapsulation efficiency, antioxidant activity and structural characterisation.

2. Materials and methods

2.1. Materials

Quercetin hydrate (Q) with a (purity ≥ 95%) was purchased from Acros Organicos. Ethyl Acetate with a purity of 99.5% and glycerol were purchased from Panreac Química (Barcelona, Spain). Modified OSA-starch refined from waxy maize was kindly provided by Ingredion Germany GmbH. Soybean lecithin (97% phospholipids) was obtained from Glama-Sot (SOTYA, Madrid, Spain). Glucagel™ (barley β-Glucan) was kindly supplied by DKSH France (purity 78%, MW: 125-140 kDa).

2.2. Precipitation from pressurized ethyl acetate-on-water emulsions

The equipment used in this work, already described by De Paz et al. (de Paz, Martín, Estrella, et al., 2012; de Paz et al., 2014), is represented in Fig. 1 with a schematic flow diagram.

(FIGURE 1)
Briefly, it consists of three small storages at ambient pressure, corresponding to the feed of
pure ethyl acetate (V-1), quercetin suspension in the same organic solvent (V-2) and the
aqueous solution of the emulsifier (V-3). The installation also counts with two piston pumps
GILSON 305 (maximum flow rate: 25 mL/min; flow rate control with an a accuracy of 0.1
mL/min) used to feed the aqueous dissolution of the emulsifier and the quercetin suspension
(pumps P-3 and P-2, respectively) and a piston pump JASCO PU-2080 plus (maximum flow rate:
10 mL/min; flow control with an a accuracy of 0.1 mL/min) used to feed the pure organic
solvent (pump P-1). An oven (KNK-2000-C series GAS CHROMATOGRAPH) is used to preheat
the organic solvent stream.

This process starts with the total dissolution of quercetin in hot and pressurized ethyl acetate,
where by increasing temperature, it is possible to increase the solubility of quercetin in this
solvent, which is around 1g/L at ambient conditions. In this work, a temperature, typically,
between 125-140°C was used, keeping a constant pressure between 6.0 and 6.5 MPa in order
to maintain the solvent in liquid state. The dissolution is achieved by mixing a flavonoid’s
suspension in pressurized ethyl acetate at ambient temperature, with a stream of preheated
and pressurized ethyl acetate, using a T- mixer (M-1 in Fig. 1). In order to reduce the exposure
of quercetin to high temperatures, this hot and pressurized solution is mixed with the
ambient-temperature aqueous solution of emulsifier using the second T-mixer of the
equipment (M2). Therefore, the contact time between quercetin and the hot solvent is
reduced to less than two seconds, avoiding the degradation of the product. The contact of the
hot solution of quercetin with the aqueous solution in mixer M-2 causes the emulsification of
the organic solvent and the precipitation of quercetin by a combined antisolvent and cooling
effect. The last step is the removal of the organic solvent from the formed emulsion using a
rotary evaporator, in order to obtain an aqueous dispersion of quercetin encapsulated by the
surfactant.
A typical experiment started with the preparation of an aqueous solution of emulsifier and a suspension of quercetin in ethyl acetate, which were pre-heated and stirred in order to obtain homogeneous mixtures. Afterwards, pumps were switched on and the pressure in the system was fixed between 6.0 and 6.5 MPa. The oven was switched on to heat the organic solvent until the desired temperature (140-150°C). When the required temperature in mixer M-1 was reached, the pure solvents used until this moment were replaced with the quercetin suspension, in continuous agitation, and with the aqueous solution of the emulsifier. In all experiments, a volume of approximately 25 mL of suspension containing quercetin was processed. Afterwards, the organic solvent of the obtained emulsion was removed from the sample using a rotary evaporator (BÜCHI 011-BÜCHI 461 Water Bath) under vacuum (-0.06, 60°C and 110min), to eliminate the organic solvent.

2.3. Product characterization

2.3.1. Microscopy

An automated upright microscope system for life science research (Leica DM4000 B, Wetzlar, Germany) was used to obtain microscopic images of the emulsions and suspensions.

2.3.2. Physical stability of emulsion

The physical stability of prepared emulsions was studied through the optical analyser Turbiscan™ Classic on the production day, by measuring the variations in backscattering during 4h.

2.3.3. Particle size

The particle size of the micelles were measured by a Laser Diffraction (LD) equipment model Malvern Mastersizer 2000 following a centrifugation step at 7800rpm, 5min, in order to precipitate the crystals of quercetin that were not encapsulated. Suspensions were dispersed in distilled water and measurement was carried out after a gentle rotation of the suspension
container in order to obtain a better dispersion of the micelles. In this work, particle size measurements are reported as volume distribution and defined as the average diameter ($d_{0.5}$).

2.3.4. Encapsulation efficiency (EE)

For the quercetin’s water suspensions stabilized with starch the Encapsulation Efficiency (EE) was determined through Ultraviolet-visible spectrophotometry, while in the suspensions stabilized with lecithin or β-glucan, due to the interference of these carrier materials on UV assays, this parameter was determined by HPLC.

For UV-Vis measurements, the suspensions were previously centrifuged at 7800 rpm (5 min) and diluted in water. The concentration of quercetin was determined using a UV-Visible Spectrophotometer (Shimadzu UV-2550, $\lambda = 375$ nm). Calibration was obtained by using standard samples with concentrations between 3 and 12 μg/mL, using ethanol as solvent.

HPLC analysis of quercetin was performed in accordance with a method previously reported (Gao et al., 2011). A Waters 515 HPLC pump, equipped with In-Line Degasser AF (Waters), 717 plus Autosampler (Waters) and 2487 Dual λ Absorbance detector (Waters) were used. Chromatographic separation was carried out with a Symmetry C18 Column (5 μm, 4.6 mm X 150 mm, Waters) coupled with a Bio-Sil C18 precolumn (5 μm, 4.6 mm X 30 mm, Bio-Rad) with mobile phase of acetonitrile/5% acetic acid solution (27:73, v/v) at 30 °C. The detection wavelength was set at 373 nm and the flow rate was 1.0 mL/min. The calibration curve was linear within the range of 25–200 μg/mL ($R^2 = 0.9997$) using methanol:water (70:30) solution as solvent. The quercetin suspension samples were detected by HPLC following centrifugation at 7800 rpm (5 min) and dilution in methanol. The run time for the assay was 13 min and the retention time for quercetin was 8.32 min.

The absorbance is proportional to the amount of quercetin dispersed in solution which corresponds to the encapsulated quercetin. Non-encapsulated quercetin can correspond either to quercetin precipitated as big crystals and not stabilized in the suspension, or to
quercetin that has undergone degradation during the process. The encapsulation efficiency is
reported as the ratio between the amount of flavonoid dispersed in solution and total
quercetin amount in the initial feed.

2.3.5. Structural characterisation

Infrared spectra of the suspensions and pure materials were recorded on a Bruker ALPHA FT-IR
apparatus equipped with a Platinum ATR sampling module including a diamond crystal. The
suspensions were centrifuged at 13300rpm during 30min. The supernatant was removed and
the sample pellet’s was submitted to further evaporation of water at -0.09MPa and 35ºC for
48h. The pellet was then used to perform FTIR. The spectra in the range from 4000 to 400 cm⁻¹
were the average of 64 scans at a resolution of 2cm⁻¹. The ATR signal was transformed to
Transmittance and the obtained spectra was normalised after the baseline correction.

2.3.6. Cryo-TEM

Cryo-TEM analysis were performed through a method already described (Melanson, 2009).
Briefly, 4μl of sample was deposited on a rack C-Flat 1.2/1.3, which was previously
hydrophilized by a plasma cleaner. A blotting is performed on either side of the filter paper
grid during 4s where, afterwards, liquid ethane is introduced in order to freeze the samples,
avoiding the formation of crystals (Gatan Cryoplunge 3). Samples are maintained in liquid
nitrogen until their transfer to the holder (Gatan Cryotransfer 626). JEOL JEM-FS2200 HRP 200
kV TEM equipment with electron filtering was used to perform the TEM images.

2.3.7. Stability of suspensions

One of the aqueous suspensions with the best relation between EE and final quercetin’s
concentration was chosen for stability examination. Some authors demonstrated that it’s
possible to increase the stability of lecithin, reducing the appearance of its agglomerates and
increase drug’s solubility by adding glycerol (G), as co-solvent, to aqueous lecithin dispersions
(Płaczkę, Sznitowska, & Jacyna, 2014; Watrobska-Swietlikowska, Przybyło, Procek, & Sznitowska, 2014). Thus, an equal suspension with 2.5% (v/v) of glycerol as co-solvent in the water phase was prepared in order to compare the physicochemical properties of the suspension without it. The stability examination was performed through the assessment of micellar particle size, presence of crystals, quercetin’s concentration and antioxidant activity of the suspensions after 75 days of storage in a refrigerated dark room (10°C).

The assessment of antioxidant activity was performed through oxygen radical absorption capacity (ORAC) assay, which was carried out by a method previously described (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002). This assay measures the ability of the antioxidant species present in the sample to inhibit the oxidation of disodium fluorescein (FL) catalysed by peroxyl radicals generated from AAPH.

Briefly, in a 96-well microplate, 25 µL of diluted sample and 150 µL of disodium fluorescein (2×10⁻⁷ mM) are added. Then, 25 µL of AAPH (153 mM) are added to start the reaction. The microplate is put in a fluorescent reader (FLUOstar OPTIMA, BMG Labtech) at 37°C. Fluorescence emitted by the reduced form of fluorescein is measured and recorded every 1 min at the emission wavelength of 530±25 nm and excitation wavelength of 485±20 nm for a period of 90 min. Phosphate buffer (75 mM, pH=7.4) is used to prepare AAPH and fluorescein solutions and is used as blank. Solutions of 13.5, 25, 50, 100, and 200 µM/L of Trolox are used as standards. Standards and blank are analyzed as triplicates, while the samples and one control of 50 µM/L ofTrolox are analysed 6 times. Final ORAC values are calculated by a regression equation between the Trolox concentration and the net area under the FL decay curve and are expressed as µM Trolox Equivalents (TE) per L of quercetin’s suspension (µM TE/L).

3. Results and discussion

3.1. Starch
As already mentioned, De Paz et al (de Paz, Martín, Estrella, et al., 2012) studied β-carotene formulation using modified OSA-starch as the emulsifier, showing that it is possible to obtain micelle’s particle size in the range of 300-600nm with a high percentage of encapsulated pigment (over 70%). This emulsifier has a critical micelle concentration around 5g/L (Varona, Martín, & Cocero, 2009), and thus equal or higher concentration of starch should be used in order to guarantee the formation of micelles. In the first experiments performed in this work, the encapsulation of quercetin in modified OSA-starch was studied. Table 1 presents the operating conditions of the experiments performed and the results obtained.

Regarding experiments E1-E8, and according to results obtained in the work of De Paz et al. (de Paz et al., 2014), a constant organic-water volume ratio (calculated as flow of organic solvent + flow of quercetin suspension/flow of the dissolution of modified starch) of 0.7 was used, and the concentration of surfactant and quercetin were changed. In experiments E9-E11, a constant concentration of quercetin (5 g/L) and modified starch (20g/L) was used, and the organic-water volume ratio was changed from 0.6 to 0.9.

Overall, the encapsulation efficiency was not higher than 10%, while the particle size was in the range of micrometer, showing that modified starch is not an appropriate emulsifier to encapsulate quercetin.

The highest percentage of encapsulated quercetin was reached when low starch concentrations were used, while particle size increased when the concentration of the emulsifier increased. In general, in a successful encapsulation the opposite trends of variation with the concentration of carrier are expected, as observed by de Paz et al in the encapsulation of β-carotene in starch (de Paz et al., 2014). Therefore, this result confirms that the OSA-modified starch is not a suitable carrier for quercetin, as it does not favor the encapsulation or reduction of particle size of the compound, and on the contrary it even
precludes it, probably due to the higher viscosity and increased mass transfer resistances in
the samples with higher starch concentrations.

Regarding the influence of the concentration of quercetin, in general high concentrations of
quercetin implied lower encapsulation efficiency and higher particle size. This could be
explained owing to the fact that at high concentrations of quercetin it gets more difficult for
the flavonoid to dissolve after M-1, leading to lower encapsulation efficiency and higher
particle size due to the presence of quercetin crystals.

An increase of the organic-water ratio led to an increase of both particle size and
encapsulation efficiency. Probably the higher initial volume of ethyl acetate used led to the
formation of an emulsion with larger droplet size and thus the formation of bigger particles
inside the micelles. A higher percentage of the organic solvent may also lead to increased
dissolution of quercetin, resulting in higher final EE.

3.2. Lecithin

The encapsulation of quercetin by using lecithin as the emulsifier was also studied in this work.
Since this emulsifier has a critical micelle concentration around 20g/L (experimentally
determined, data not shown), equal or higher concentration of lecithin was used in order to
guarantee the formation of micelles (de Paz, Martín, & Cocero, 2012; Varona, Martín, &
Cocero, 2011). In Table 2 the operating conditions of the experiments performed and the
results obtained are displayed.

(TABLE 2)

The experiments started with the determination of the organic-water ratio and lecithin’s
concentration most suitable to produce stable emulsions (E12-E15), using a concentration of
quercetin soluble in ethyl acetate at ambient temperature (1g/L). In fact, it was found that the
experiment performed with organic-water ratio and lecithin’s concentration at higher values
(E13) led to the formation of an unstable emulsion, with separation of phases immediately after its production. Overall, the emulsions produced proved to be more stable at lower lecithin concentration and lower organic solvent:water ratio tested. Moreover, in experiments E12-E14, aggregates of lecithin precipitated during the evaporation step, perhaps due to the higher concentration of the emulsifier in these experiments. Finally, by analysing the results obtained in experiments E12 – E13, it is possible to see that an increase of the lecithin’s concentration led to an increase of particle size and decrease of EE. The same occurred while comparing the results from E14 –E15, despite the organic-water volume ratio being lower in these experiments.

Thus, experiments from E16-E19 were performed using a concentration of lecithin fixed at 25g/L, further reduced organic solvent:water ratio fixed at 0.35 and variable concentrations of quercetin. The emulsion’s internal phase diameter was determined by turbiscan, revealing that the diameter of the discontinuous phase was in general below 5μm. Moreover, none of the emulsions showed the presence of quercetin’s crystals, revealing a good encapsulation of this flavonoid. Nevertheless, crystals of quercetin precipitated in the flask during the evaporation of ethyl acetate from the emulsions E18 and E19, revealing that quercetin’s concentration of 7.5 and 10g/L, respectively, may be too high for efficient encapsulation. The presence of crystals in these experiments after solvent removal was confirmed by optical microscopy. Microscope pictures of the emulsions and suspensions can be seen in figure 2.

(Figure 2)

The final particle of the quercetin-loaded micelles was in the nanometer range. Regarding encapsulation efficiency, almost 76% was achieved for experiment E16, where the final quercetin’s concentration in the aqueous suspension was 260ppm. Experiments E17, E18 and E19 show lower EE, probably due to the higher initial concentration of quercetin used, yet showing higher final quercetin’s concentration (E19 shows a concentration of 630ppm). In
Figure 3 it is possible to verify that the EE decreased as the initial concentration of quercetin used increased. However, final quercetin’s concentration in the suspension increased as the initial concentration of quercetin used increased, though this increase was first proportional, yet reaching a plateau at 630ppm. This value could represent a saturation limit point, where no more quercetin could be solubilised in the lecithin-aqueous suspension.

(Figure 3)

Lecithin showed to be a good emulsifier for the encapsulation of quercetin. In fact, the ability of these two compounds to form chain-like structures linked by hydrogen bonds has been already reported and demonstrated with nuclear magnetic resonance spectroscopy, being these structures called phenolipids (Nasibullin, Nikitina, Afanas’eva, Nasibullin, & Spirikhin, 2002). Furthermore, there seems to exist a synergism between lecithin and quercetin due to the formation of these bonds, resulting in an increased antioxidant potential (Ramadan, 2012, 2014).

In Fig.4 it is possible to see the Cryo-TEM pictures taken to the suspension resulting from experiment E16.

(Figure 4)

By observing the Cryo-TEM pictures of lecithin’s water suspension (Fig 4. a) it is possible to see multivesicular and multilamellar vesicular formulations of the emulsifier with particle size around 100nm (Varona et al., 2011). Nevertheless, regarding the quercetin aqueous suspensions, it is possible to verify the presence of small vesicles around 100nm with dark double layers (Fig. 4 b) and c)) probably due to presence of quercetin in this area. Moreover, in Fig.4 d), it is possible to observe some larger vesicles with beehive-like structure, probably resulting from the formation of quercetin-enriched lecithin structures linked by hydrogen bonds.
Fourier transform infrared spectroscopy measurements were performed in order to study the possible formation of a complex or chemical association between quercetin and lecithin, since such interaction bonds can be detected by this technique (Fraile et al., 2014). In figure 5, the FTIR spectrum of unprocessed quercetin hydrate presents its characteristic bands, like the broad band at 3500–3000 cm$^{-1}$, assigned to a free -OH bond vibration, bands at 1660 and 1600 cm$^{-1}$, assigned to the stretching vibration of the C=O group, the band at 1515 cm$^{-1}$, assigned to aromatic groups, the bands at 1310 and 1160 cm$^{-1}$, assigned to the C-O-C vibration, and finally the band at 1010 cm$^{-1}$, assigned to aromatic C-H groups (Fraile et al., 2014; Li, Yang, Bai, & Zhu, 2010).

(FIGURE 5)

By analyzing the FTIR spectra of the encapsulated quercetin (E16) it is possible to see some complexation between lecithin and quercetin, since it presents different band shapes observed between 1,500 and 1,660 cm$^{-1}$ when compared with unprocessed quercetin and physical mixture spectra. It must be noticed that quercetin’s crystals show a characteristic carbonyl absorption band around 1600 cm$^{-1}$, assigned to aromatic ketonic carbonyl stretching (Pralhad & Rajendrakumar, 2004; Zheng & Chow, 2009), which is almost no detectable in the spectrum of encapsulated quercetin, revealing a good encapsulation of this flavonoid.

3.3. $\beta$-Glucan

The encapsulation of quercetin through the use of $\beta$-Glucan as emulsifier was also performed in this work.

Since this emulsifier has a critical micelle concentration around 2g/L (experimentally determined, data not shown), higher concentration of $\beta$-Glucan (15g/L) was used in order to guarantee the formation of micelles. Moreover, $\beta$-Glucan has low oral bioavailability, due to its poor absorption, and its complexation with lecithin was suggested to overcome this undesirable characteristic (Veverka et al., 2014). For that purpose, lecithin was also used in
some experiments, with a mass ratio between β-Glucan and Lecithin varied according to Veverka et al (Veverka et al., 2014). Quercetin’s concentration of 1g/L was chosen for these experiments. In Table 3 it is displayed the operating conditions of the experiments performed and the results obtained.

(TABLE 3)

Emulsions from experiments performed with mixtures of surfactants, E21-E23, presented some carrier’s agglomerates. Nevertheless, emulsion from experiment E20 and stabilized just with β-glucan, did not show these aggregates. It seems that mixtures of β-glucan and lecithin form complexes with tendency to precipitate without improving the stability of the emulsions.

In fact, in experiments performed with both emulsifiers, the precipitation of the lecithin:β-glucans increased during the evaporation step. Nevertheless, none of the emulsions showed the presence of quercetin’s crystals, being the diameter of the discontinuous phase in general below 4μm. According to turbiscan measurements, the sample that contained higher concentration of lecithin, E23, showed higher diameter of organic solvent droplets. Still, it is important to remember that turbiscan only provides mean particle size value but not the dispersion of this value, hence the differences between turbiscan’s values and optical microscope images.

Regarding experiment E20, crystals of quercetin precipitated in the flask during the evaporation of the emulsions, which was confirmed by optical microscopy. β-Glucan did not seem to be a good carrier/emulsifier to encapsulate this flavonoid, and so, higher concentrations of quercetin were not tested. The final particle of the quercetin-loaded micelles was in the micrometer range. In the case of E20, the presence of quercetin’s crystals contributed for the micrometer size, whereas in the samples E21-E23 the presence of carrier’s agglomerates contributed to this particle size. Comparing E20 and E22, it is possible to see that the addition of lecithin increased the particle size due to the formation of lecithin:β-glucan
complexes (Veverka et al., 2014). Nevertheless, the addition of more lecithin (E21 and E23) led to a reduction of particle size, probably due to the formation of higher amount of small sized quercetin encapsulated particles.

Regarding the encapsulation efficiency, it is possible to verify that comparing E20 and E22, the addition of lecithin to the system led to a decrease of EE. The addition of lecithin led to the complexation of the carriers, and their further precipitation. Perhaps there is a competition between quercetin and lecithin for the complexation with β-glucan, resulting in less EE in E22 comparing with E20.

Nevertheless, as the amount of the lecithin increased in β-glucan:lecithin mixtures (E22<E21<E23), so as increased the EE of the system. These values are in agreement with the reduction of particle size in these samples, as discussed previously. Perhaps in experiment E23 there is a higher portion of free lecithin, not complexed with β-glucan, to interact with quercetin, increasing its encapsulation. This fact reinforces the capacity of lecithin to encapsulate quercetin.

3.4. Stability

Experiment E17 was repeated with and without the inclusion of glycerol in order to evaluate and compare their stability. For that purpose, the micellar particle size, presence of crystals, quercetin’s concentration and antioxidant activity of the resulting suspensions were measured after 75 days of storage. During this period, the aqueous solutions were stored in a refrigerated room at temperatures around 10°C and protected from light. The refrigeration condition was chosen for the stability study since it is recommended as the storage condition for the phospholipids (Date et al., 2011). Results are reported in Table 4.

(TABLE 4)

As presented in this table, the sample prepared with glycerol as co-solvent maintained colloidal stability at the end of one month and a half, with no significant change in its particle
size, indicating good stability during this period. In contrast, the sample without glycerol nearly
tripled its size after the same period, being this value even higher after two months and a half
after its preparation.

Evaluating the shape of the size distribution, shown in Fig. 6, the distribution of the sample
freshly prepared have main peak in the nanometers range, corresponding to quercetin
particles that were successfully encapsulated, and other peaks at sizes above 1 µm possibly
corresponding to quercetin’s crystals and lecithin’s agglomerates. During storage, the peaks
above 1 µm are displaced to considerably higher particle sizes, indicating that these bigger
particles are becoming more agglomerated and further destabilizing the suspension due to
their higher size. The addition of a co-solvent is essential for an adequate stability of the
formulation, avoiding not only the agglomeration of lecithin, but also delaying the appearance
of quercetin’s crystals. In fact, in the sample prepared without glycerol, the appearance of
flavonoid’s crystals was observed shortly after fifteen days of preparation. Regarding
encapsulation efficiency, the addition of a co-solvent appeared to slightly enhance the
solubility of quercetin in aqueous media. Nevertheless, the stability of quercetin over storage
was similar in the two tested samples.

Table 4 also shows the results achieved by ORAC antioxidant assay for the two suspensions
over storage. The results are expressed as micromoles of Trolox equivalents per gram of
quercetin. Antioxidant activity of pure quercetin are lecithin was also measured in this work,
revealing values of 6586 and 2.5 µmol of TE/g of product, respectively. The probable formation
of hydrogen bonds between lecithin and quercetin resulted in a 3-fold increased antioxidant
activity per unit mass of quercetin for both suspensions, being these values nearly preserved
after 75 days of storage.

4. Conclusion
The formulation of quercetin using three distinct emulsifiers, namely n-octenyl succinate anhydride starch, soybean lecithin and barley β-glucan, was investigated in this work through pressurized ethyl acetate-in-water emulsions. Results showed that OSA-starch was not suitable for the encapsulation of quercetin due to the low encapsulating efficiencies achieved (below 10%) and micellar particle sizes in the range of micrometers. Experiments carried out with lecithin yielded the best results, with encapsulation efficiencies up to near 76% and particle sizes in the sub-micrometer range. Moreover, the aqueous suspensions of quercetin stabilized with this surfactant presented concentrations of the flavonoid up to 630ppm, being 315-fold higher compared with solubility of pure quercetin in water. The formation of hydrogen bonds between lecithin and quercetin could explain the good results obtained with this emulsifier, further comprising improved antioxidant activity (3-fold higher per unit mass of quercetin) due to a synergistic effect. Furthermore, the addition of lecithin to the emulsions stabilized with β-glucan improved the encapsulation efficiency and particle size of the micelles, reinforcing the capacity of lecithin to encapsulate quercetin.

The results obtained in this work showed that high pressure emulsion technique is a versatile method for the production of water soluble formulations of quercetin, avoiding quercetin’s degradation and increasing its solubility. Furthermore, the addition of glycerol as co-solvent can increase the colloidal stability of the suspension and the encapsulation efficiency of the flavonoid.

Acknowledgements

This work was supported by Marie Curie Industry-Academia Partnerships and Pathways (European Commission) through the WineSense project (FP7-PEOPLE-2012- IAPP-612608). V.S.S. Gonçalves is grateful for the doctoral grant SFRH/BD/77350/2011 from Fundação para a Ciência e Tecnologia. S. Rodríguez-Rojo acknowledges the Ministerio de Economía y
Competitividad and Universidad de Valladolid for her Juan de la Cierva fellowship (JCI-2012-14992). A. Martín thanks the MINISTERIO DE CIENCIA E INNOVACIÓN for his Ramón y Cajal contract (RYC-2010-06063).

References


FIGURE CAPTIONS

Figure 1. Schematic flow diagram of the experimental apparatus employed for quercetin formulation.

Figure 2. Microscopy images of quercetin’s emulsions (E16-17) and suspensions (E18-19) stabilized with lecithin.

Figure 3. Influence of initial concentration of quercetin used in a) encapsulation efficiency and b) final concentration of quercetin in the suspensions stabilized with lecithin.

Figure 4. Cryo-TEM images of a) lecithin’s water suspension and b), c) and d) E16 suspension; the orange arrows show the double dark layers and the black arrow shows the beehive structure.

Figure 5. FTIR spectra of encapsulated quercetin (E16), pure quercetin and lecithin, and a physical mixture of quercetin:lecithin (mass ratio 1:75). Black lines represent the spectrum range characteristic of crystalline quercetin.

Figure 6. Particle size distributions of experiments E17 measured directly after preparation of the suspension and after 45 and 75 days of storage.
FIGURES

Figure 1. Schematic flow diagram of the experimental apparatus employed for quercetin formulation
Figure 2. Microscopy images of quercetin’s emulsions (E16-17) and suspensions (E18-19) stabilized with lecithin
Figure 3. Influence of initial concentration of quercetin used in a) encapsulation efficiency and b) final concentration of quercetin in the suspensions stabilized with lecithin
Figure 4. Cryo-TEM images of a) lecithin’s water suspension and b), c) and d) E16 suspension; the orange arrows show the double dark layers and the black arrow shows the beehive structure.
Figure 5. FTIR spectra of encapsulated quercetin (E16), pure quercetin and lecithin, and a physical mixture of quercetin:lecithin (mass ratio 1:75). Black lines represent the spectrum range characteristic of crystalline quercetin.
Figure 6. Particle size distributions of experiments E17 measured directly after preparation of the suspension and after 45 and 75 days of storage.
### Table 1. Summary of operating conditions and experimental results obtained with OSA modified-starch

<table>
<thead>
<tr>
<th>Exp</th>
<th>C_{quercetin} (g/L)</th>
<th>C_{starch} (g/L)</th>
<th>F_{organic} (mL/min)</th>
<th>F_{quercetin} (mL/min)</th>
<th>F_{starch} (mL/min)</th>
<th>ratio_{org-water}</th>
<th>ratio_{starch/quercetin}</th>
<th>Particle size d_{0.5} (µm)</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>2.5</td>
<td>5</td>
<td>9</td>
<td>6</td>
<td>20.6</td>
<td>0.7</td>
<td>7</td>
<td>27.4</td>
<td>7.9</td>
</tr>
<tr>
<td>E2</td>
<td>2.5</td>
<td>10</td>
<td>9</td>
<td>6</td>
<td>20.6</td>
<td>0.7</td>
<td>14</td>
<td>32.0</td>
<td>4.8</td>
</tr>
<tr>
<td>E3</td>
<td>2.5</td>
<td>15</td>
<td>9</td>
<td>6</td>
<td>20.6</td>
<td>0.7</td>
<td>21</td>
<td>38.8</td>
<td>8.7</td>
</tr>
<tr>
<td>E4</td>
<td>5</td>
<td>10</td>
<td>9</td>
<td>6</td>
<td>20.6</td>
<td>0.7</td>
<td>7</td>
<td>31.7</td>
<td>10.0</td>
</tr>
<tr>
<td>E5</td>
<td>5</td>
<td>15</td>
<td>9</td>
<td>6</td>
<td>20.6</td>
<td>0.7</td>
<td>10</td>
<td>38.9</td>
<td>5.8</td>
</tr>
<tr>
<td>E6</td>
<td>20</td>
<td>15</td>
<td>9</td>
<td>6</td>
<td>20.6</td>
<td>0.7</td>
<td>3</td>
<td>39.1</td>
<td>5.7</td>
</tr>
<tr>
<td>E7</td>
<td>20</td>
<td>60</td>
<td>9</td>
<td>6</td>
<td>20.6</td>
<td>0.7</td>
<td>10</td>
<td>61.6</td>
<td>2.4</td>
</tr>
<tr>
<td>E8</td>
<td>20</td>
<td>100</td>
<td>9</td>
<td>6</td>
<td>20.6</td>
<td>0.7</td>
<td>17</td>
<td>71.3</td>
<td>3.9</td>
</tr>
<tr>
<td>E9</td>
<td>5</td>
<td>20</td>
<td>9</td>
<td>6</td>
<td>25.0</td>
<td>0.6</td>
<td>17</td>
<td>29.7</td>
<td>5.8</td>
</tr>
<tr>
<td>E10</td>
<td>5</td>
<td>20</td>
<td>9</td>
<td>6</td>
<td>23.0</td>
<td>0.7</td>
<td>15</td>
<td>39.5</td>
<td>6.9</td>
</tr>
<tr>
<td>E11</td>
<td>5</td>
<td>20</td>
<td>8</td>
<td>7</td>
<td>16.5</td>
<td>0.9</td>
<td>9</td>
<td>52.1</td>
<td>6.5</td>
</tr>
</tbody>
</table>
Table 2. Summary of operating conditions and experimental results obtained with lecithin

<table>
<thead>
<tr>
<th>Exp</th>
<th>$C_{\text{quercetin}}$ (g/L)</th>
<th>$C_{\text{lecithin}}$ (g/L)</th>
<th>$F_{\text{organic}}$ (mL/min)</th>
<th>$F_{\text{quercetin}}$ (mL/min)</th>
<th>$F_{\text{lecithin}}$ (mL/min)</th>
<th>ratio$_{\text{org-water}}$</th>
<th>ratio$_{\text{lecithin:quercetin}}$</th>
<th>Particle size $d_{0.5}$ (nm)</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E12</td>
<td>1</td>
<td>60</td>
<td>9</td>
<td>6</td>
<td>20</td>
<td>0.75</td>
<td>200</td>
<td>530</td>
<td>59.1</td>
</tr>
<tr>
<td>E13</td>
<td>1</td>
<td>95</td>
<td>9</td>
<td>6</td>
<td>20</td>
<td>0.75</td>
<td>317</td>
<td>1101</td>
<td>6.2</td>
</tr>
<tr>
<td>E14</td>
<td>1</td>
<td>60</td>
<td>4.5</td>
<td>3</td>
<td>20</td>
<td>0.38</td>
<td>400</td>
<td>458</td>
<td>37.2</td>
</tr>
<tr>
<td>E15</td>
<td>1</td>
<td>25</td>
<td>4.5</td>
<td>3</td>
<td>20</td>
<td>0.38</td>
<td>167</td>
<td>160</td>
<td>66.7</td>
</tr>
<tr>
<td>E16</td>
<td>2.5</td>
<td>25</td>
<td>4.25</td>
<td>2.75</td>
<td>20</td>
<td>0.35</td>
<td>73</td>
<td>165</td>
<td>75.9</td>
</tr>
<tr>
<td>E17</td>
<td>5</td>
<td>25</td>
<td>4.25</td>
<td>2.75</td>
<td>20</td>
<td>0.35</td>
<td>36</td>
<td>167</td>
<td>69.3</td>
</tr>
<tr>
<td>E18</td>
<td>7.5</td>
<td>25</td>
<td>4.25</td>
<td>2.75</td>
<td>20</td>
<td>0.35</td>
<td>24</td>
<td>757</td>
<td>60.7</td>
</tr>
<tr>
<td>E19</td>
<td>10</td>
<td>25</td>
<td>4.25</td>
<td>2.75</td>
<td>20</td>
<td>0.35</td>
<td>18</td>
<td>213</td>
<td>44.7</td>
</tr>
</tbody>
</table>
Table 3. Summary of operating conditions and experimental results obtained with β-glucan and β-glucan:lecithin

<table>
<thead>
<tr>
<th>Exp</th>
<th>C_{quercetin} (g/L)</th>
<th>C_{emulsifier} (g/L)</th>
<th>ratio_{β-Glucan:lecithin}</th>
<th>F_{organic} (mL/min)</th>
<th>F_{quercetin} (mL/min)</th>
<th>F_{emulsifier} (mL/min)</th>
<th>ratio_{org-water}</th>
<th>ratio_{emulsifier:quercetin}</th>
<th>Particle size d_{0.5} (µm)</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E20</td>
<td>1</td>
<td>15</td>
<td>1:0</td>
<td>4.25</td>
<td>2.75</td>
<td>20</td>
<td>0.35</td>
<td>109</td>
<td>3.5</td>
<td>49.9</td>
</tr>
<tr>
<td>E21</td>
<td>1</td>
<td>15</td>
<td>2:1</td>
<td>4.25</td>
<td>2.75</td>
<td>20</td>
<td>0.35</td>
<td>109</td>
<td>4.9</td>
<td>33.2</td>
</tr>
<tr>
<td>E22</td>
<td>1</td>
<td>15</td>
<td>4:1</td>
<td>4.25</td>
<td>2.75</td>
<td>20</td>
<td>0.35</td>
<td>109</td>
<td>7.1</td>
<td>31.6</td>
</tr>
<tr>
<td>E23</td>
<td>1</td>
<td>15</td>
<td>1:1</td>
<td>4.25</td>
<td>2.75</td>
<td>20</td>
<td>0.35</td>
<td>109</td>
<td>1.3</td>
<td>56.2</td>
</tr>
</tbody>
</table>
Table 4. Physical and Chemical Stability of E17 suspension prepared with/without glycerol stored in a refrigerated dark room (10ºC).

<table>
<thead>
<tr>
<th>Day</th>
<th>Particle size d_{0.5} (nm)</th>
<th>Encapsulation Efficiency (%)</th>
<th>ORAC μM TE/g quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>E17</td>
<td>E17 + G</td>
<td>E17</td>
<td>E17</td>
</tr>
<tr>
<td>0</td>
<td>270 ± 2</td>
<td>384 ± 75</td>
<td>61.8 ± 0.13</td>
</tr>
<tr>
<td>45</td>
<td>697 ± 67</td>
<td>390 ± 12</td>
<td>51.9 ± 0.22</td>
</tr>
<tr>
<td>75</td>
<td>3626 ± 1069</td>
<td>1307 ± 224</td>
<td>39.6 ± 0.18</td>
</tr>
</tbody>
</table>