Research Article

Design of flavonoid microparticles with channel forming properties to improve oxidative stability of sunflower oil

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Quercetin (Q) and epicatechin (E) microparticles were designed using an oil-insoluble polymer (inulin [In]) as encapsulating agent and with or without an oil-soluble polymer (soy protein isolate [SPI]) by spray-drying. Encapsulation efficiencies were significantly higher for the E systems than for Q systems, suggesting that the spatial arrangement may affect the hydroxyl groups availability to form hydrogen bonds. The microencapsulated flavonoids were added to sunflower oil (SO) in order to evaluate its oxidative stability. The induction period (IP) of SO, determined in Rancimat at 60°C, significantly increased when Q-microparticles with or without SPI were added, showing those with SPI the highest IP value. In the case of E systems, the IP of SO increased only when E–In microparticles with SPI were added. These results suggest that SPI may favor the diffusion of flavonoids to the lipid medium by the formation of channels into the microparticles. The channels formation was observed for Q–In–SPI and E–In–SPI by a confocal laser scanning microscopy study. Additional oxidation studies under conditions of lower oxygen availability resulted in overall more retarded oxidation and no clear effect of SPI incorporation was observed.

Practical application: The results show that it is possible to design flavonoid microparticles with antioxidant activity in bulk oils. The inclusion of a lipid-soluble polymer such as soy protein isolate in the microparticles favors the flavonoid release from the microparticles to bulk oil by channel formation.

Keywords: Epicatechin / Microencapsulation / Oxidation / Quercetin / Sunflower oil

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1 Introduction

Lipid oxidation is one of the the most relevant alterations occurring in foods, giving rise to the development of off-flavors, loss of nutrients and bioactives, and even formation of potentially toxic compounds [1]. Synthetic antioxidants such as BHT, BHA or n-propyl gallate exhibit strong antioxidant activity but their use is strictly regulated because of possible adverse effects on human health [2]. Therefore, there is a growing interest in using antioxidants from natural sources, such as polyphenols. Flavonoids are phenolic compounds widely distributed in the vegetable

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Abbreviations: AT, alpha-tocopherol; **CLSM,** confocal laser scanning microscopy; **E,** epicatechin; **EE,** encapsulation efficiency; **In,** inulin; **IP,** induction period; **ML,** methyl linoleate; **PC,** polar compounds; **Q,** quercetin; **R,** recovery; **RSM,** response surface methodology; **SEM,** scanning electron microscopy; **SO,** sunflower oil; **SPI,** soy protein isolate

kingdom, with a common phenylbenzopyrone structure that consists of two aromatic rings (A and B) linked through a pyrane ring [3]. Phenolic compounds have shown a wide range of biological activities, including antioxidant properties. The main mechanisms by which flavonoids act against lipid oxidation consist on transferring a hydrogen atom or electrons to a peroxyl radical and metal chelation [4].

Although the structure-antioxidant activity relationship of flavonoids has been well defined in hydrophilic systems [5], studies dealing with this in hydrophobic systems are scarce. Free flavonoids, mainly quercetin, myricetin, morin, (+) catechin, (−)epigallocatechin, (−)epicatechin, and kaempferol, have been incorporated in different lipid systems with varying degrees of success in terms of oxidative stability, such as methyl linoleate [6, 7], canola oil [8, 9], cottonseed oil [10], corn oil [11], or fish oil [12–14]. The antioxidant activity found in methyl linoleate and purified sunflower oil was related to the number of phenolic hydroxyl groups, 2,3 double bond in the C-ring and a glycoside moiety in the molecule [6, 15].

The incorporation of free flavonoids in lipid systems may have some drawbacks since these phenolic compounds have limited solubility and stability [16], together with unpleasant flavors in some cases [17]. Microencapsulation of flavonoids is a technology which could enhance the stability of flavonoids. However, there are few studies focused on the effect of the incorporation of microencapsulated flavonoids on oxidative stability of lipid systems [18, 19]. Furthermore, design of flavonoid microparticles for controlled release in lipid systems is still a challenge. In this context, flavonoid microparticles based on a lipid-insoluble polymer (encapsulating agent) and a lipid-soluble polymer (in low percentage), could allow the release of flavonoids, together with the improvement of oxidative stability in a lipid system. In a previous study undertaken by our research group, quercetin, naringenin, and epicatechin microparticles based on inulin-Capsul matrix, were incorporated in methyl linoleate [20, 21]. These microparticles were able to release the encapsulated flavonoids in methyl linoleate and improve the oxidative stability, suggesting that the incorporation of Capsul led to the formation of channels inside the microparticles, thus favoring the diffusion of flavonoids to methyl linoleate. However, the effect of flavonoid microparticles in real matrices such as vegetable oils has not been addressed yet. The objective of this research was to design microencapsulated flavonoids (epicatechin and quercetin) with inulin and soy protein isolate by spray-drying and evaluate the effect of their incorporation on the oxidative stability of sunflower oil.

2 Materials and methods

2.1 Materials

Inulin (In) Raftilina® HP (DP ≥ 23) was obtained from Alfa-Chilena (Chile). Soy protein isolate (SPI) was obtained from Prinal (Chile), Quercetin (Q) ≥ 90%, epicatechin (E) ≥ 90%, methyl linoleate (ML) >99%, alpha-tocopherol ≥ 90%, and Nile Red were obtained from Sigma-Aldrich (Chile). Sunflower oil (SO) (Natura, AGD Natural Food, Argentina) without synthetic antioxidants was acquired from local market. The major fatty acids were 51.3 ± 0.1% (C18:2 ω6), 37.3 ± 0.1% (C18:1 ω9 cis), 5.73 ± 0.01% (C16:0), and 3.27 ± 0.04% (C18:0). The alpha-tocopherol (AT) content was 548 ± 9 mg/kg oil. The initial values of polar compounds (PC) and peroxide value were 4.20 ± 0.04% and 0.37 ± 0.10 mEq O_2/kg oil, respectively.

2.2 Methods

2.2.1 Preparation of the flavonoids microparticles

Flavonoid microparticle systems with SPI (E–In–SPI and Q–In–SPI) were prepared using a Box–Behnken design with 15 runs each one (12 experimental points and 3 central points). The flavonoid/In ratio (1:20–1:50), inlet air temperature (120–160°C), and SPI content (0–1.7 g) were evaluated as independent variables, whereas encapsulation efficiency (EE) and flavonoid release in hexane at day 14 of storage (t_{14}) were the dependent variables. In this study, response surface methodology (RSM) and desirability function were used to determine the optimal conditions of flavonoid encapsulation for each system.

Inulin (6.8–17 g) in water (65–55 g) and SPI (0–1.7 g) in water (10 g) were heated at 70 and 40°C, respectively. Afterwards, they were cooled at room temperature and stirred. Q and E (0.34 g) were dissolved in ethanol (18–16 g), added to the polymer dispersion and stirred for 30 min. The resultant infeed was sonicated for 20 min (230 W, 50 H, room temperature, FS-30H Fisher Scientific, USA), and feed into a spray-dryer (mini Spray-Dryer B-290, Büchi, Switzerland). The inlet air temperature ranged from 120 to 160°C. The airflow, rate of feeding and atomization pressure were 600 L/h, 1 mL/min, and 5 bars, respectively. The powders obtained were stored in the dark at −20°C for subsequent analysis.

Furthermore, E–In and Q–In microparticles were prepared according to optimal conditions reported by Palma et al. [20]. These microparticle systems were used as control to evaluate the effect of the incorporation of SPI.

2.2.2 Characterization of flavonoid microparticles

2.2.2.1 Encapsulation efficiency and recovery of flavonoids

Total flavonoids were determined as follow: microparticles (E–In, Q–In, E–In–SPI, and Q–In–SPI) (100 mg) were treated with 4 mL of water:ethanol:acetone (50:25:25 v/v/v), on a vortex mixer for 1 min, ultra-sonicated for 20 min and centrifuged at 452.8*g* for 3 min. The supernatant was removed and the process was performed twice. The supernatants were transferred to a

volumetric flask. Flavonoid contents (E and Q) of each system were quantified by HPLC according to Palma et al. [20].

Surface flavonoids were determined as following: mcroparticles (E–In and Q–In, Q–In–SPI, and E–In–SPI) (100 mg) were dispersed in methanol (3 mL) and softly stirred. The supernatant was transferred to a volumetric flask, filled up with water:methanol:acetonitrile (45:40:15 v/v/v) containing glacial acetic acid (1%), and an aliquot was injected into the HPLC according to Palma et al. [20].

Encapsulation efficiency (EE) and recovery (R) were calculated according to Eqs. (1) and (2), respectively:

$$\mathrm{EE}\,(\%) = \frac{\text{Total flavonoid(Q or E)} - \text{Surface flavonoid(Q and E)}}{\text{Total flavonoid(Q or E)}} \times 100 \quad (1)$$

$$R\,(\%) = \frac{\text{Total flavonoids (Q or E) in the powder}(\mathrm{mg\,g^{-1}})}{\text{Total flavonoids (Q or E) in the feed solution}(\mathrm{mg\,g^{-1}})} \times 100. \quad (2)$$

2.2.2.2 Flavonoid release in hexane

Flavonoid release from Q–In–SPI and E–In–SPI microparticles was evaluated in hexane. Microparticle powders (0.5 g) in cellulose filter bags were placed into glass bottles containing hexane (100 mL) with 0.5% of Span, stoppered with teflon caps and stored at 30 ± 2°C in an oven (Memmert model UFE 500, Germany) in the dark. Aliquots of 2 mL were removed after 14 days of storage (t_{14}). The samples were treated with 3 mL of water:methanol:acetonitrile (45:40:15 v/v/v) containing 1% glacial acetic acid, stirred with a vortex for 1 min and centrifuged at 453*g* for 3 min. The aqueous-methanolic phase was removed and transferred to a volumetric flask. The process was repeated with 2 mL of the same extracting solvent. The aqueous-methanolic phases were pooled and an aliquot was injected into the HPLC. Flavonoid analysis (Q or E) was performed by HPLC according to Palma et al. [20].

2.2.2.3 Moisture content, hygroscopicity, and water activity (a_w)

Water activity (a_w) (AquaLAB, P. R. China) at 20 ± 0.3°C and moisture content were determined according to AOAC methods [22]. Hygroscopicity was determined according to the procedure described by Cai and Corke [23].

2.2.2.4 Morphology and particle size of the microparticles

The morphology and outer structures of flavonoid microparticles (E–In, Q–In, E–In–SPI, and Q–In–SPI) were examined using scanning electron microscopy (SEM). Microparticles were coated with gold/palladium, using a Varian Vacuum Evaporator PS 10E and analyzed using a LEO 142 OVP (LEO Electron Microscopy Ltd., Cambridge, UK) operated at 20 kV. The scanned images were collected digitally using EDS 7424 software (Oxford Instruments, Oxford, UK).

The particle size was determined for all systems by laser light scattering using a Mastersizer X (Malvern Instruments, Worcestershire, UK) with a lens of 300 mm. Microparticles were dispersed in propylene glycol prior to analysis [24].

2.2.3 Channels formation study

Confocal laser scanning microscopy (LSM 700, Carl Zeiss, Germany) was used to confirm the formation of channels within the microparticle systems with SPI. Nile Red (0.1%) was dissolved in SO (3 g), and flavonoid microparticles (E–In, Q–In, E–In–SPI, and Q–In–SPI) were dispersed in amount equivalent to 200 mg flavonoid/kg oil. The dispersions were stirred for 14 days at 300 rpm in a heating block at 60°C (the same conditions as the accelerated storage stability assay). Two excitation wavelengths were used, 488 nm for Nile Red and 405 nm for DAPI (4′, 6-diamidino-2-pehynilindole), while fluorescence signals were collected at 580 and 456 nm for Nile Red and DAPI, respectively.

The software used for the CLSM imaging was ZEN 2012 (Blue Edition, Carl Zeiss, Germany).

2.2.4 Oxidation assays

2.2.4.1 Rancimat

The induction period (h) was determined in samples of SO and ML (3 g), with and without the addition of flavonoid microparticles (E–In, Q–In, E–In–SPI, and Q–In–SPI) (amount equivalent to 200 mg flavonoid/kg. A Rancimat Oxidative Stability Instrument (Metrohm Ltd., Herisau, Switzerland) was used, at 60°C and air flow of 20 L/h [25]. ML was used as lipid model system devoid of antioxidants. Effluent air containing volatile organic acids from the oxidized samples was collected in a vessel with distilled water and conductivity measured automatically. The assays were carried out in triplicate. Protection factor (PF) was calculated according to Eq. (3):

$$\mathrm{PF} = \frac{\text{Induction period of ML or SO with flavonoid microparticle added}}{\text{Induction period of ML or SO}}. \quad (3)$$

2.2.4.2 Long-term assays

Open glass tubes ($10 \times 1\,\mathrm{cm^2}$) with 3 g of samples (SO, SO + E–In, SO + Q–In, SO + E–In–SPI, and SO + Q–In–SPI), were placed in tubes and heated at 60 ± 1°C in a heating block (Merck, Darmstadt, Germany), with constant

stirring (350 rpm) for 35 days. Tubes (in triplicate) were removed at specific time intervals (every 7 days) to determine the formation of polar compounds and loss of alpha-tocopherol. Polar compounds were determined by adsorption column chromatography [26].

Tocopherols were determined by HPLC according to AOCS [27]. The HPLC equipment consisted of a Merck–Hitachi L-7110 pump with a 20 μL injection loop, a Hitachi 5440 fluorescence detector, and a LiChroCART Superspher Si-60 column (5 μm particle size, 4 mm id × 250 mm; Merck). Propan-2-ol:n-hexane (1:99 v/v) was used as mobile phase at a flow rate of 1 mL/min. Detection was at 290 and 330 nm of excitation and emission wavelengths, respectively. Alpha-tocopherol was quantified using an external standard.

2.2.5 Statistical analysis

ANOVA one-way was applied to determine the statistical differences among systems, using Statgraphics, Version 7.0 (Manugistic Inc., Statistical Graphics Corporation, Rockville, MD).

3 Results and discussion

3.1 Optimization of flavonoid microparticles using response surface methodology

A Box–Behnken design for each system studied (E–In–SPI and Q–In–SPI) was applied to evaluate the effect of the formulation (flavonoid/In ratio and SPI content) and process (inlet air temperature) variables, whereas EE and flavonoid release in hexane at 14th day of storage in hexane were the response variables.

The E and Q EEs ranged from 36.7 to 77.0% (E–In–SPI) and 26.7 to 54.1% (Q–In–SPI), respectively. For E–In–SPI and Q–In–SPI microparticles, the release of flavonoids in hexane at day 14 of storage was 12.0–56.0 and 13.4–57.4%, respectively.

The response surface methodology (RSM) was applied to optimize the response variables considering the linear, quadratic, and cross-product forms of the independent variables studied (air inlet temperature, SPI content and flavonoid/inulin ratio) at $p \leq 0.05$ for each system. The variance analysis for EE showed that in the E–In–SPI system, the linear form of the E/In ratio and SPI content, the quadratic form of SPI content, and the interaction between E/In and SPI content were significant. In the case of the Q–In–SPI system all the variables were significant except the linear form of temperature. The R^2 adj values were 88.6 and 95.4% for E–In–SPI and Q–In–SPI, respectively.

The analysis of variance for flavonoid release in hexane showed that the SPI content both in its linear and quadratic form, the E/In ratio and inlet temperature in their quadratic forms, as well as the interaction between E/In and inlet temperature were significant in the E–In–SPI system. In the case of Q–In–SPI, the SPI in its linear and quadratic forms, and the Q/In ratio and inlet temperature in their quadratic forms were significant on the flavonoid release. The SPI content showed a significant effect on the flavonoid release in hexane for both flavonoid microparticle systems, suggesting a positive effect of this independent variable on the flavonoid release behavior in hexane. The R^2 adj values were 96.6 and 79.4% for E–In–SPI and Q–In–SPI, respectively. All these results showed that the structural features of each flavonoid determine the operational variables of the spray-drying process.

For the multiple optimization (Desirability Function) of the E–In–SPI and Q–In–SPI microparticle systems, the EE was maximized and the flavonoid release in hexane was minimized. Surface response graphics of the Box-Behnken for the E–In–SPI and Q–In–SPI (Supporting Information), where the optimal conditions were 1:50 and 1:20 for the F/In ratio, 0.4 and 0% for SPI content, and 160 and 132°C for inlet temperature, respectively, showed that the optimal conditions for spray-drying are specific for each flavonoid. As the SPI contents were different for each system, a 5% of SPI content was established in both systems for comparative purposes.

3.2 Characterization of flavonoid microparticles

Table 1 shows the characterization of flavonoid microparticles with SPI (E–In–SPI and Q–In–SPI) obtained under optimal conditions and without SPI (E–In and Q–In).

Despite high air inlet temperature has been associated with flavonoid degradation reactions induced by heat during spray-drying [3], the recovery of all the flavonoids from microparticles was high (above 70%). This high recovery could be attributed to short drying times or to the rapid formation of a dry crust [28] that allows the diffusion of water outside but retains the flavonoids within the microparticle. The EE is related to the interaction flavonoid-polymer mainly by hydrogen bonds. The EE values were significantly higher ($p \leq 0.05$) for the E than Q systems. Although both epicatechin and quercetin have five hydroxyl groups, different values for EE were found in this study, suggesting that the spatial arrangement may affect the hydroxyl groups availability. The same behavior was observed in E and Q microparticles without SPI (Table 1). The flavonoid EE values obtained in this study were comparable to the results reported by Palma et al. [20] for flavonoid–inulin–Capsul microparticles, with EEs values of 61.8 and 69.9% for Q–In–Capsul and E–In–Capsul, respectively. Higher EE values have been reported for Q (62–81%) with cellulose acetate phthalate (CAP) and a combination of carboxymethylcellulose with sodium dodecylbenzensulfonate [29], cellulose acetate trimellitate (CAT) or CAP (61.4–76%, respectively) [30]. All the systems had low moisture content and water activity, due to the high inlet air temperature of the

Table 1. Physical and chemical characteristics of flavonoid microparticles obtained under optimal conditions

	E–In–SPI	Q–In–SPI	E–In*	Q–In*
F/In ratio	1:50	1:20	1:41	1:43
SPI (%)	5	5	0	0
Inlet air temperature (°C)	160	132	145	160
Total flavonoids (mg/g)	14.9 ± 0.47^{a}	18.7 ± 0.29^{c}	18.6 ± 0.1^{c}	15.91 ± 0.11^{b}
Flavonoids EE (%)	71.4 ± 3.2^{d}	50.6 ± 0.68^{a}	68.7 ± 0.6^{c}	59.63 ± 0.57^{b}
F recovery (%)	79.4 ± 2.4^{a}	98.4 ± 1.84^{b}	82.0 ± 0.6^{a}	70.1 ± 0.52^{a}
Moisture content (%)	3.6 ± 0.47^{b}	5.7 ± 0.01^{c}	3.66 ± 0.07^{b}	2.63 ± 0.22^{a}
Water activity (a_w)	0.13 ± 0.06^{a}	0.24 ± 0.01^{c}	0.21 ± 0.01^{b}	0.14 ± 0.01^{a}
Hygroscopicity (g/100 g)	46.9 ± 0.46^{ab}	48.4 ± 0.50^{c}	46.6 ± 0.0^{a}	47.8 ± 0.70^{bc}

F, flavonoid; Q, quercetin; E, epicatechin; In, inulin; SPI, soy protein isolate.
Values are expressed as mean ± SD ($n = 3$), different letters mean significant differences ($p \leq 0.05$).
*Prepared according to optimal conditions reported by Palma et al. [20].

spray-drying process, thus assuring low microbial risk and long shelf life [31].

Figure 1(A and B) shows the SEM microphotographs of E–In–SPI and Q–In–SPI microparticles obtained under optimal conditions, respectively. The E–In–SPI and Q–In–SPI microparticles showed spherical and irregular shape with some shrinkage. It is usual that, at high inlet temperatures (as in this study), the rapid evaporation and high pressure inside of the particles produce shrinkage. Besides microparticles showed low agglomeration tendency, similar to flavonoid microparticles of E and Q with inulin–Capsul [20]. The microparticle size (D_{32}) was 2.0 and 2.5 μm for Q–IN and E–IN, respectively; whereas Q–IN–SPI and E–IN–SPI microparticles showed 2.5 and 2.8 μm, respectively.

3.3 Channels formation study

Confocal photographs are shown in Fig. 2(A and B) for Q–In–SPI and Q–In at day 14th of storage. Two fluorescence channels, red (Fig. 2I A and B) and blue (Fig. 2II A and B) lasers, were used to excite the SO and the SPI, respectively. Fig. 2III (A and B) is the superposition of the images from the red and blue lasers, which allows distinguishing between encapsulated oil and base structure of the microparticles.

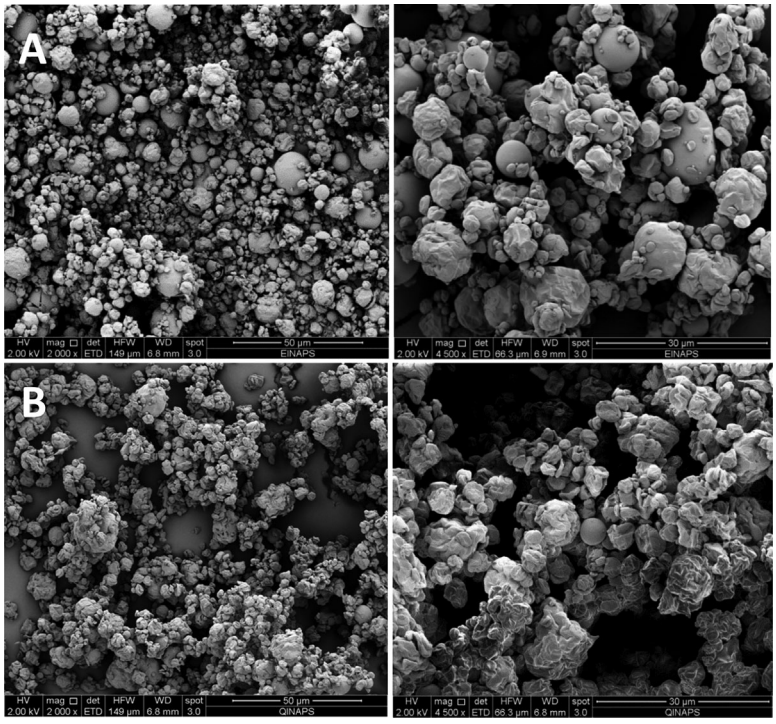

Figure 1. Scanning electron microscopic photographs for E–In–SPI (A) and Q–In–SPI (B) microparticles to 2000 and 4500 magnification.

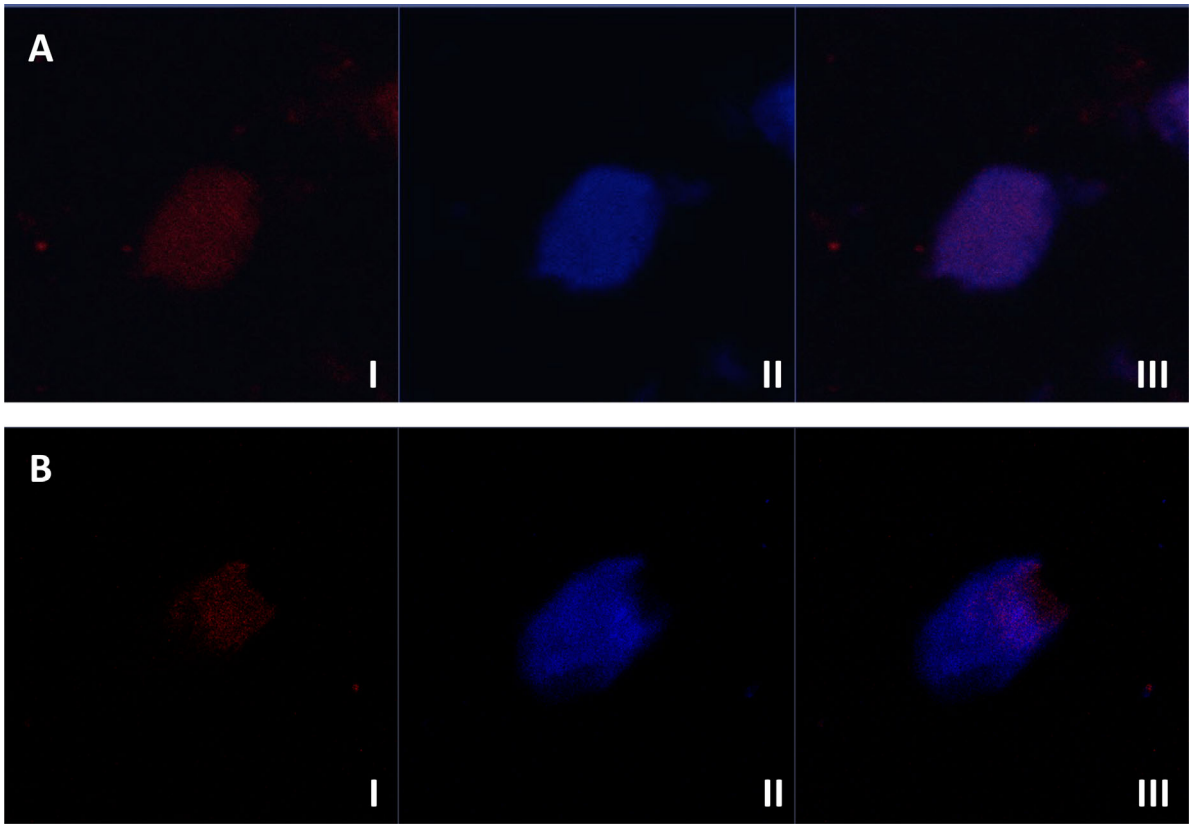

Figure 2. Confocal laser microscopic images for Q–In–SPI (A) and Q–In (B). (I) red laser (A and B), (II) blue laser (A and B), (III) superposition of red and blue laser (A and B).

Q–In–SPI image shows that the SO is located inside the microparticle (Fig. 2A). This result could be explained by dissolution of SPI molecules by oil, forming channels inside the network, favoring the oil-flavonoid encapsulated interaction and so its diffusion toward bulk oil where it exerts its antioxidant action. Besides, the inulin swelling in lipid medium could also take place. In contrast, the oil is mainly located on the surface of the Q–In microparticles (Fig. 2B). These results suggested that SPI is able to form channels within the microparticle, allowing the flavonoid release to the lipid medium.

3.4 Oxidation studies in rancimat

Table 2 shows the induction period (IP) values of ML and SO with and without flavonoid microparticles (equivalent to 200 mg flavonoid/kg ML or SO) determined in Rancimat at 60°C. IP values of SO were higher than ML, both with and without flavonoid microparticles incorporated at the same concentration, which can be expected due to the antioxidant action of tocopherol in SO. It is well-known that vegetable oils are natural sources of antioxidants such as tocopherols,

Table 2. Induction period of methyl linoleate (ML) and sunflower oil with and without flavonoid microparticles (200 mg flavonoid/kg)

	Induction period (IP) $X \pm SD$ (h)	Protection factor (PF)
ML	10.9 ± 0.7^{a}	–
ML + E–In	13.7 ± 1.1^{b}	1.25
ML + Q–In	90.7 ± 2.9^{c}	8.32
ML + E–In–SPI	15.7 ± 0.8^{b}	1.44
ML + Q–In–SPI	99.3 ± 0.9^{d}	9.10
SO	180 ± 5.1^{a}	1.00
SO + E–In	182 ± 6.2^{a}	1.01
SO + Q–In	205 ± 4.4^{b}	1.10
SO + E–In–SPI	223 ± 9.2^{c}	1.23
SO + Q–In–SPI	279 ± 8.7^{d}	1.55

Q, quercetin; E, epicatechin; In, inulin; SPI, soy protein isolate; SO, sunflower oil.
Values are expressed as mean $\pm$ SD ($n = 3$), different letters mean significant differences ($p \leq 0.05$).

which protect PUFA from oxidation and improve the oxidative stability of oils [32], being alpha-tocopherol the most abundant isomer in SO [32, 33].

Results showed that the IP of ML significantly increased when flavonoid microparticles were added, being the highest values obtained for quercetin-microparticles (Q–In and Q–In–SPI) The IP of Q–In–SPI was higher than Q–In, suggesting that ML would be able to dissolve SPI, forming channels within the microparticles that thus favor the diffusion of Q to lipid medium. However, there were not significant differences in IP between E–In and E–In–SPI, which may be attributed to a high interaction between E and In and/or higher polarity of E that may impair its diffusion to lipid medium.

In samples of SO, IP significantly increased when Q-microparticles with or without SPI were added, and those with SPI gave the highest IP value. As to E-microparticles, the incorporation of E–In did not have any significant effect on IP, while it increased when E-In microparticles with SPI were added, further supporting that SPI may favor the diffusion of flavonoids to the lipid medium by the formation of channels into the microparticles. Such a difference was not observed between ML samples and this is attributable to the higher polarity of SO versus ML, which facilitates the E diffusion.

In order to obtain a complete picture of the oxidation process in Rancimat, samples were withdrawn periodically from initial to advanced stages of oxidation in the case of SO, So + E–In–SPI and SO + Q–In–SPI, and analyzed for PC and alpha-tocopherol. Measurement of the PC formation is considered to be amongst the best methods to evaluate total oil oxidation [34]. The increase of PC during the oil oxidation progress is due to the rise of oxidized triacylglycerols, triacylglycerol dimers, and polymers, which are all more polar than the unoxidized triacylglycerols and globally constitute the total non-volatile oxidation compounds formed. Figure 3 shows the time course of formation of PC and loss of tocopherols together with conductivity values for SO (Fig. 3A), SO + E–In–SPI (Fig. 3B) and SO + Q–In–SPI (Fig. 3C), and results correspond to triplicate samples in different Rancimat tubes. As can be observed in all samples, the end of the IP was detected by the sudden increase in conductivity and was concurrent with the initiation of accelerated formation of PC.

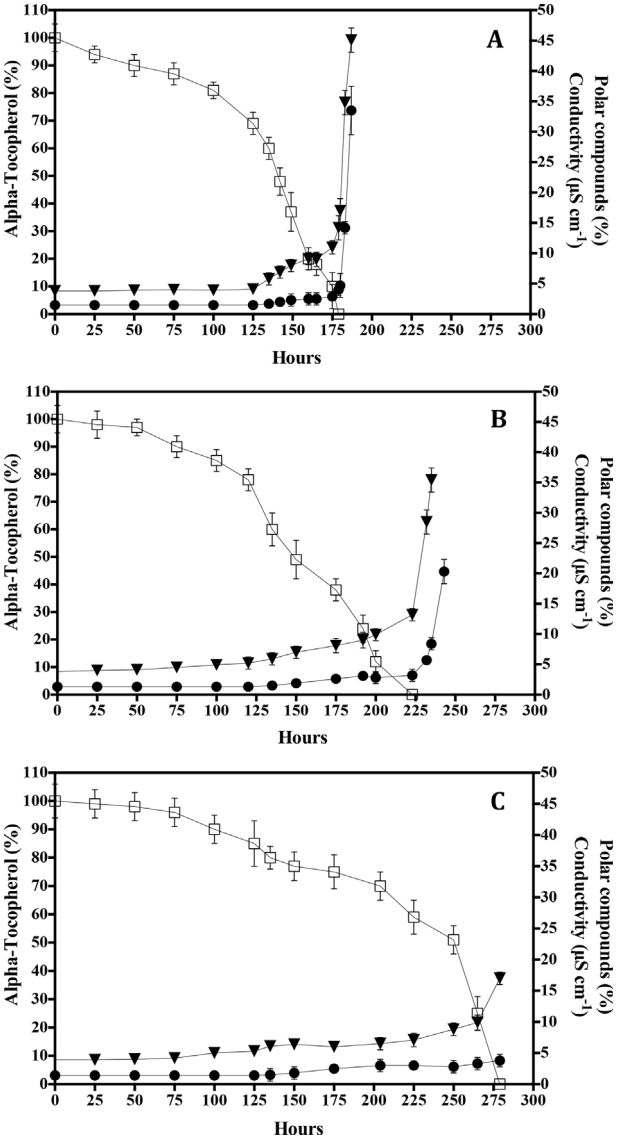

Figure 3. Formation of polar compounds (▼), loss of alpha-tocopherol (□), and increase of conductivity values (●) for SO (3A), SO + E–In–SPI (3B), and SO + Q–In–SPI (3C) in Rancimat.

3.5 Long-term oxidation studies

Figure 4 shows PC formation and alpha-tocopherol loss for SO and SO added with flavonoids microparticles with and without SPI: E-microparticles (SO + E–In, SO + E–In–SPI, Fig. 4A) and Q-microparticles (SO + Q–In SO + Q–In–SPI, Fig. 4B), under the oxidation conditions described in section 2.2.4.2.

First of all, it is important to remark that standard deviations were rather high as compared to results obtained for IP at Rancimat. In general, at 14–21 days, oxidation accelerated in all samples, being tocopherol loss earlier in the case of SO without flavonoid microparticles added. Regarding SO with E–In–SPI and Q–In–SPI microparticles, the PC values were always the lowest from 14 to 21 days, but only significantly lower when compared to SO (control). The fact that the differences among SO microparticles due to the incorporation of SPI were not significant in these assays, in contrast with the great differences found in Rancimat assays, can be attributed to the different conditions applied, i.e., in the Rancimat method, oxidation is accelerated as a result of air bubbling. In the same line, the influence of flavonoid structure features was not noted in long-term assays, since

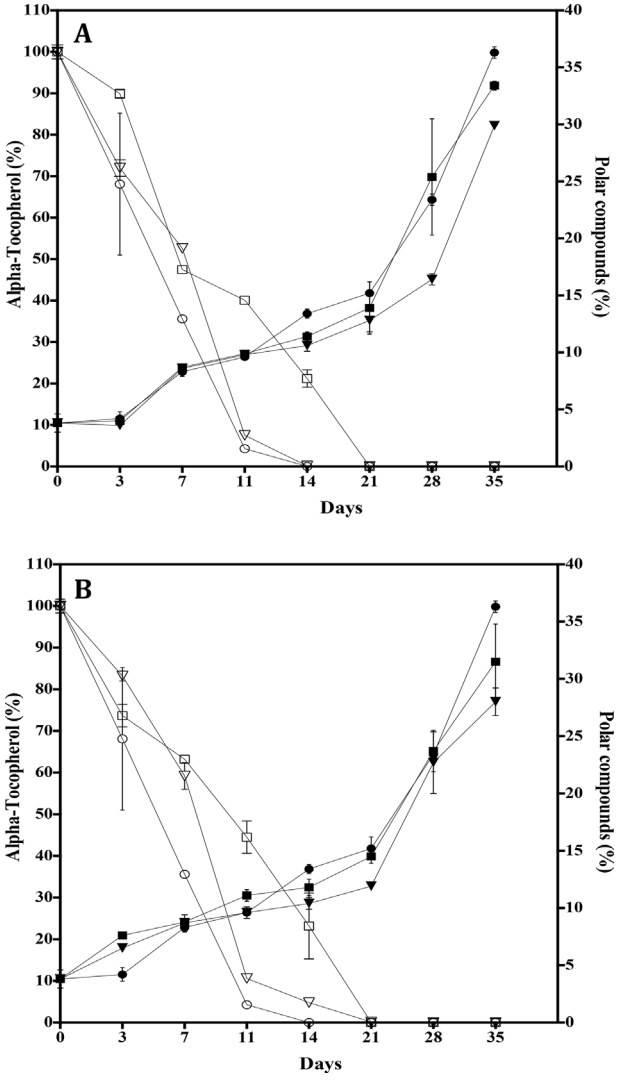

Figure 4. Formation of polar compounds (solid symbols) and loss of alpha-tocopherol (open symbols) for SO (○, ●), SO + E–In (□, ■) and SO + E–In–SPI (▽, ▼) (4A) and SO (○, ●), SO + Q–In (□, ■), and SO + Q–In–SPI (▽, ▼) (4B) during long-term oxidation assays.

either SO + E–In–SPI versus SO + Q–In–SPI, or SO + E–In versus SO + Q–In followed similar evolution.

As described before, IP value in Rancimat denotes the oxidation rate change by the inflection point of the curve of conductivity measurements, concomitant with the rate change of formation of PC. Also, results clearly showed that PC levels at the end of the induction period were similar in all samples, between 13 and 17%, as already reported for oils oxidizing at different rates [35]. In the long-term assays, the rate change of formation of PC occurred at about 10–15% but only low or no significant differences between samples were found in the time periods required to reach this inflection point. As has been already discussed [36], the combined effect of the variables involved in oil oxidation, among which oxygen may or not be a limiting factor, may have an important influence on the different comparative behavior between samples when assayed during long-term storage.

4 Conclusions

The channel forming properties of SPI in Q–In–SPI and E–In–SPI, which favors the flavonoid release from the microparticles to bulk oil, was confirmed by confocal laser scanning microscopy. The effect of channel formation resulted in an improvement of oxidative stability as assayed in Rancimat but only slight differences were observed in longer term assays under conditions of much lower oxygen availability. Overall results showed that Q microparticles designed with In and SPI have a potential application as oil antioxidants.

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References

[1] Min, D. B., Boff, J. M., in: Akoh, C. C., Min, D. B. (Eds.), *Food Lipids: Chemistry, Nutrition, and Biotechnology*, Marcel Dekker, New York, NY (USA) 2002, pp. 335–364.

[2] Ito, N., Hiroze, M., Fukushima, G., Tauda, H., et al., Studies on antioxidant; their carcinogenic and modifying effects on chemical carcinogenesis. *Food Chem. Toxicol.* 1986, *24*, 1071–1082.

[3] Bravo, L., Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance. *Nutr. Rev.* 1998, *56*, 317–333.

[4] Pietta, P. G., Flavonoids as antioxidants. *J. Nat. Prod.* 2000, *63*, 1035–1042.

[5] Rice-Evans, C., Miller, N. J., Paganga, G., Structure antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Bio. Med.* 1996, *20*, 933–956.

[6] Pedrielli, P., Skibstead, L., Antioxidant synergy and regeneration effect quercetin, (−)-epichatequin, and (+)-catechin on α-tocopherol in homogeneous solutions of peroxidating methyl linoleate. *J. Agric. Food Chem.* 2002, *50*, 7138–7144.

[7] Pekkarinen, S., Heinonen, M., Hopia, A., Flavonoids quercetin, myricetin, kaemferol and (+)-catechin as antioxidant in methyl linoleate. *J Sci. Food Agric.* 1999, *79*, 499–506.

[8] Su, Y., Xu, J., Ng, C., Leung, L., et al., Antioxidant activity of tea theaflavins and methylated catechins in canola oil. *J. Am. Oil Chem. Soc.* 2004, *81*, 269–274.

[9] Wanasundara, U., Shahidi, F., Stabilization of canola oil with flavonoids. *Food Chem.* 1994, *50*, 393–396.

[10] Tsimogiannis, D., Oreopoulou, V., Defining the role of flavonoid structure on cottonseed oil stabilization: Study of A- and C-ring substitution. *J. Am. Oil Chem. Soc.* 2007, *84*, 129–136.

[11] Naz, S., Siddiqi, R., Sayeed, S., Effect of flavonoids on the oxidative stability of corn oil during deep frying. *Int. J. Food Sci. Technol.* 2008, *43*, 1850–1854.

[12] Huber, G., Rupasinghe, H., Shahidi, F., Inhibition of oxidation of omega-3 polyunsaturated fatty acid and fish oil by quercetin glycosides. *Food Chem*. 2009, *117*, 290–295.

[13] Nieto, S., Garrido, A., Sanhueza, J., Loyola, L., et al., Flavonoids as stabilizer in fish oil: An alternative to synthetic antioxidants. *J. Am. Oil Chem. Soc*. 1993, *70*, 773–778.

[14] Wanasundara, U., Shahidi, F., Stabilization of marine oils with flavonoids. *J. Food Lipids* 1998, *5*, 183–186.

[15] Marinova, E., Toneva, A., Yanishlieva, N., Synergistic antioxidant effect of α-tocopherol and myricetin on the autoxidation of triacylglycerols of sunflower oil. *Food Chem*. 2008, *106*, 628–633.

[16] Wu, T., Yen, F., Lin, L., Tsai, T., et al., Preparation, physicochemical characterization, and antioxidant effects of quercetin nanoparticles. *Int. J. Pharm*. 2008, *346*, 160–168.

[17] Kosaraju, S., Labbett, D., Emin, M., Konczak, I., Lundin, L., Delivering polyphenols for healthy ageing. *Nutr. Diet*. 2008, *65*, S48.

[18] Deng, Z. Y., Yu, Y., Process for producing oil-soluble microencapsulated tea polyphenols. *Fine Specialty Chem*. 2004, *16*, 8–15.

[19] Wu, C., Chen, L., Li, W., Preparation of microencapsulation quercetin and its antioxidative properties in oils. *Food Indus*. 2005, *3*, 27–29.

[20] Palma, M., García, P., Márquez-Ruiz, G., Vergara, C., Robert, P., Release kinetics of flavonoids in methyl linoleate from microparticles designed with inulin and channelizing agent. *Food Res. Int*. 2014, *64*, 99–105.

[21] Palma, M., Márquez-Ruiz, G., García, P., Holgado, F., et al., Effect of spray-dried flavonoid microparticles on oxidative stability of methyl linoleate as lipid model system. *J. Am. Oil Chem. Soc*. 2017, *94*, 99–105.

[22] AOAC, Official methods of analysis of AOAC International, 16th Edn., Gaythersburg, MD, USA 1996.

[23] Cai, Y. Z., Corke, H., Production and Properties of Spray-dried Amaranthus Betacyanin Pigments. *J. Food Sci*. 2000, *65*, 1245–1252.

[24] Fernández, R. V., Borgesa, S., Botrela, D., Botrela, D., Gum arabic/starch/maltodextrin/inulin as wall materials on the microencapsulation of rosemary essential oil. *Carbohyd. Polym*. 2014, *101*, 524–532.

[25] AOCS, Official Methods and Recommended Practices of the American Oil Chemists' Society, 4th Edn., AOCS Press, Champaign, IL, USA 1994. Method Cd 12b-92.

[26] IUPAC, *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th Edn., International Union of Pure and Applied Chemistry, Pergamon Press, Oxford, UK 1992. Method 2.507.

[27] AOCS, *Official Methods and Recommended Practices of American Oil Chemists' Society*, 4th Edn., AOCS Press, Champaign, IL, USA 1993. Method Ce 8–89.

[28] Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A., Saurel, R., Applications of spray-drying in microencapsulation of food ingredients: An overview. *Food Res. Int*. 2007, *40*, 1107–1121.

[29] Sansone, F., Picerno, P., Mencherini, T., Villeco, F., et al., Flavonoid microparticles by spray-drying: Influence of enhancers of the dissolution rate on properties and stability. *J. Food Eng*. 2011, *103*, 188–196.

[30] Lauro, M. R., Maggi, L., Conte, U., De Simone, F., Aquino, R. P., Rutin and quercetin gastro-resistant microparticles obtained by spray-drying technique. *J. Drug Deliv. Sci. Technol*. 2005, *15*, 363–369.

[31] Sun-Waterhouse, D., Wadhwa, S., Waterhouse, G., Spray-drying microencapsulation of polyphenol bioactives: A comparative study using different natural fibre polymers as encapsulant. *Food Bioprocess Tech*. 2013, *6*, 2376–2388.

[32] Kiralan, M., Ulas, M., Özaydin, A., Özdemır, N., et al., Blends of cold pressed black cumin oil and sunflower oil with improved stability: A study based on changes in the levels of volatiles, tocopherols and thymoquinone during accelerated oxidation conditions. *J. Food Biochem*. 2017, *41*, e12272.

[33] Castelo-Branco, V. N., Santana, I., Oliveira Di-Sarli, V., Pereira Freitas, S., Guedes Torres, A., Antioxidant capacity is a surrogate measure of the quality and stability of vegetable oils. *Eur. J. Lipid Sci. Tech*. 2016, *118*, 224–235.

[34] Márquez-Ruiz, G., Velasco, J., Dobarganes, C., Evaluation of oxidation in dried microencapsulated fish oils by a combination of adsorption and size exclusion chromatography. *Eur. Food Res. Tech*. 2000, *211*, 13–18.

[35] Márquez-Ruiz, G., Martín-Polvillo, M., Velasco, J., Dobarganes, C., Formation of oxidation compounds in olive and sunflower oils under oxidative stability index (OSI) conditions. *Eur. J. Lipid Sci. Tech*. 2008, *110*, 465–471.

[36] Velasco, J., Holgado, F., Dobarganes, M. C., Márquez-Ruiz, G., Effect of relative humidity on the free and encapsulated oil fractions in freeze-dried microencapsulated oils. *Food Res. Int*. 2009, *42*, 1492–1500.

