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Nanoemulsions as delivery systems of hydrophobic silybin from silymarin extract: Effect of oil type on silybin solubility, *in vitro* bioaccessibility and stability

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ABSTRACT

The potential of nanoemulsion delivery systems to carry silybin from silymarin extract was studied. To this purpose, sunflower oil, extra virgin olive oil and castor oil were used to prepare silymarin loaded nanoemulsions. The effect of oil type on the silybin solubility and i*n vitro* bioaccessibility was evaluated. Moreover, the changes in particle size, silybin concentration, oxygen consumption and hydroperoxide concentration were studied in nanoemulsions during storage at 20 °C. Results showed that silybin can be successfully incorporated into physically stable nanoemulsions prepared with the different oils. The oil type slightly influenced the silybin *in vitro* bioaccessibility, while it affected the nanoemulsion particle size as well as silybin stability during storage. In particular, silybin underwent degradation, showing lower stability in extra virgin oil and sunflower oil than in castor oil. Results also showed that the presence of the silymarin extract containing silybin did not affect the oxidation kinetics of the carrier oils.

1. Introduction

Silymarin is a mixture of flavolignans (i.e. silybin, silydianin and silychristin) extracted from *Silybum marianum*. Among the flavolignans, silybin or silybinin is the most abundant biologically active compound of silymarin. The use of silymarin to treat liver diseases, such as cirrhosis, hepatitis, alcoholic liver disease and toxin exposure has been well documented (Flora, Hahn, Rosen, & Benner, 1998; Fraschini, Demartini, & Esposti, 2002). These biological effects are attributed to the antioxidant, antifibrotic, anti-inflammatory, anti-lipid-peroxidative and anti-carcinogenic activity of silymarin components (Basaga, Poli, Tekkaya, & Ara, 1997; Luper, 1998; Yang, Liu, & Liu, 2004).

Although results can be hardly summarized, studies on the liver-protective capacity of silymarin (Loguercio & Festi, 2011) and cell oxidation mechanisms (Dehmlow, Murawski, & de Groot, 1996; Zielinska-Przyjemska & Wiktorowicz, 2006) evidenced an important inhibitory effect of silymarin flavonoids on cell enzymes (e.g. lipoxygenase) involved in inflammatory reactions, whereas reaction with O_2^{-}. occurred to a lesser extent (Dehmlow et al., 1996). Despite this, clinical application and therapeutic efficiency of silymarin flavolignans are limited due to their poor bioavailability. The latter is mainly due to the crystalline state and low water solubility of silymarin flavonolignans at ambient temperature, as well as to their poor enteral absorption (Gazak et al., 2004). These limitations have been overcome by developing pharmaceutical preparations consisting of lipid-based delivery systems with increased silybin bioavailability (Javed, Kohli, & Ali, 2011; Jia et al., 2010; Li, Yuan, Huang, Zhou, & Liu, 2010; Parveen et al., 2011). By contrast, ways to improve silybin bioaccessibility in foods have received little attention. According to Rao and McClements (2012), lipid-based delivery systems for food application, such as microemulsions, nanoemulsions, liposomes, solid lipid nanoparticles, polymeric nanoparticles, filled hydrogel particles, can be effectively used to incorporate poorly water-soluble nutraceuticals in functional foods. As known, nanoemulsions are thermodynamically unstable colloidal systems containing small lipid droplets dispersed in an aqueous medium (Rao & McClements, 2012). Generally, they show good stability to gravitational separation and particle aggregation becoming a good component to be added to foods that have to be processed and stored under different conditions. Nanoemulsions have been actually proposed to increase the solubility and stability of bioactive molecules, such as quercitin (Pool, Mendoza, Xiao, &

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McClements, 2013), polymethoxyflavone (Li, Zheng, Xiao, & McClements, 2012) and curcumin (Ahmed, Li, McClements, & Xiao, 2012), to be incorporated into foods. The choice of the lipid medium appears particularly critical, since the chemical and physical characteristics of the lipid carrier greatly affect the solubility of the compound to be delivered. Also, the presence of bioactive molecule crystals may negatively affect nanoemulsion physical stability, leading to possible undesired phase separation during food processing and storage, as well as reduction of the bioavailability of the selected component that may not be adsorbed in this form into the gastrointestinal tract (Giacomelli et al., 2002; Kawabata, Wada, Nakatani, Yamada, & Onoue, 2011).

The aim of this research was to study the potential for nanoemulsion delivery systems to carry silybin from silymarin extract. To this purpose, different carrier oils (sunflower oil, extra virgin olive oil and castor oil) were used to prepare silymarin loaded nanoemulsions. The physical and chemical stability of nanoemulsions was studied during storage at 20 °C. Also, the effect of oil type on the silybin *in vitro* bioaccessibility was evaluated.

2. Materials and methods

2.1. Materials

Silymarin extract containing 210 mg/g of silybin, 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH·), lipase from porcine pancreas, porcine bile extract, di-hydrated calcium chloride, Tween 80, sodium azide, ethyl acetate, HPLC grade methanol, isooctane, 2-propanol, 1-butanol, ammonium thiocyanate, cumene hydroperoxide, analytical grade hydrochloric acid and sodium hydroxide were from Sigma Aldrich (St. Luois, MO, USA). Sodium chloride, barium chloride, ferrous sulphate, monosodium dihydrogen phosphate, disodium hydrogen phosphate and 850 g/L phosphoric acid were from Carlo Erba Reagents (Milano, Italy). Analytical standard grade silybin and naringenin-7-*O*-glucoside were from Extrasynthese (Genay, France). Sunflower oil, extra virgin olive oil and castor oil were purchased in a local market.

2.2. Silybin solubility

Aliquots of 3 mL of sunflower oil, castor oil, extra virgin olive oil, Tween 80 or deionized water were introduced in 5.0 mL capacity vials and excess amount of silymarin extract was added. Similarly, 1.5 g of each oil and 1.5 g of Tween 80 were added with excess amount of silymarin extract in 5.0 mL vials. Samples were kept at a constant temperature (25 ± 1.0 °C) under shaking for 72 h to reach equilibrium (Parveen et al., 2011). The samples were centrifuged at 13,100 *g* for 10 min (MiniSpin, Eppendorf, Hamburg, Germany) and the solubilised silybin in the supernatant was then recovered and quantified by HPLC analysis (Paragraph 2.4.2).

2.3. Nanoemulsion preparation

The oil phase was prepared by mixing silymarin powder (2.5 mg/g) and the surfactant Tween 80 (10 mg/g) in sunflower oil, extra virgin olive oil or castor oil. Preliminary trials evidenced that this surfactant concentration allowed a stable emulsion to be obtained. The systems were stirred in the dark until the silymarin was completely dissolved. No recrystallization events were observed before emulsion preparation. The aqueous phase consisted of deionised water added with 0.1 mg/g of sodium azide, to avoid microbial spoilage during the storage experiments. The stock emulsions were prepared by mixing 200 g/L oil phase with the aqueous phase with a high speed blender for 1 min at 9000 rpm (Polytron, PT 3000, Cinematica, Littau, Swiss). Aliquots of 250 mL of the stock emulsions were homogenised at 10 L/h flow rate by two passes at 150 MPa through a two stage high pressure homogeniser provided with cylindrical tungsten carbide homogenising valves (Panda PLUS 2000, Gea Niro Soavi, Parma, Italy). Aliquots of 18 mL of the nanoemulsions were inserted into 20 mL colourless glass vials, sealed with butyl septa and metallic caps and stored at 20 °C in a thermostatic cell for approximatively 50 days.

2.4. Analytical determinations

2.4.1. Particle size

The mean diameter of emulsion droplets was measured by using the dynamic light scattering instrument Particle Sizer NICOMP™ 380 ZLS (PSS NICOMP Particle Sizing System, Santa Barbara, California, USA). Samples were diluted 1:1000 (v/v) with deionised water prior to the analysis to avoid multiple scattering effects. The angle of observation was 90°. Solution refractive index and viscosity were set at 1.333 and 1.0 cP, respectively, corresponding to the values of pure water at 20 °C. Particle mean diameter corresponding to volume distribution was calculated by NICOMP Distribution Analysis.

2.4.2. Silybin concentration

Silybin extraction was performed by introducing 1 g supernatant or nanoemulsion into 10 mL Pyrex tubes, added with 5 mL water:methanol mixture (1:4 v/v), and manually shaken for 2 min. The tubes were then treated for 15 min in an ultrasonic bath (25 °C) and finally centrifuged at 1000 *g* for 10 min (Labofuge I, Heraeus Christ GmbH, Osterode am Harz, Germany). Samples were then stored overnight at −20 °C, to improve the phase separation. The upper water-methanol phase was filtered on 0.20 μm pore size nylon membranes (Albet-Hahnemühle, Barcelona, Spain), and analysed for silybin concentration by reverse-phase HPLC according to the slightly modified method of Kvasnička, Bîba, Ševčìk, Voldřich, and Kràtkà (2003). Analyses were performed by an LC-2010 AHT liquid chromatographic system (Shimadzu, Kyoto, Japan) equipped with an integrated UV–visible detector. A 4 μm packed 150 × 4.6 mm C_{18} column (Synergi Polar, Phenomenex, Torrance, CA), thermostated at 35 °C, was used. The elution was in gradient mode using a mixture of 5 mL/L aqueous phosphoric acid (solvent A) and methanol (solvent B) as mobile phase at a flow rate of 1 mL/min. Gradient was set as follows: solvent B was held at 36% for the first 5 min, increased to 45% in 1 min and held at this level for 25 min; then 100% solvent B was reached in 2 min and held for 5 min, before to be lowered in 2 min to the initial level (36%). The sample injection volume and the detection wavelength were 10 μL and 288 nm, respectively. Quantitative analysis was carried out by comparing the silybin peak area with the results of a calibration line, obtained by injecting silybin standard solutions (methanol:-water 4:1 v/v). Calibration line was linear (R^2 = 0.999) in the 0.5–18.0 mg/L concentration interval.

2.4.3. Chain breaking activity

The chain-breaking activity was measured following the methodology of Brand-Williams, Cuvelier, and Berset (1995). The bleaching rate of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH·) was monitored at 515 nm. A volume of 1.85 mL of 6.1×10^{-5} mol/L DPPH· methanol solution was used. The reaction was started by the addition of 150 μL of sample, previously solubilised with methanol. The DPPH· bleaching was followed at 515 nm (Uvikon 860, Kontron Instruments, Milano, Italy) at 25 °C for at least 10 min. In all cases the DPPH· bleaching rate was proportional to the sample concentration added to the medium. The reaction rate of DPPH· bleaching was computed according to the following equation (Manzocco, Anese, & Nicoli, 1998):