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

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Original
Nanoemulsions as delivery systems of hydrophobic silybin from silymarin extract: Effect of oil type on silybin solubility, invitro bioaccessibility and stability / Calligaris, Sonia; Comuzzo, Piergiorgio; Bot, Francesca; Lippe, Giovanna; Zironi, Roberto; Anese, Monica; Nicoli, Maria Cristina. - In: LEBENSMITTEL-WISSENSCHAFT + TECHNOLOGIE. - ISSN 0023-6438. - 63:1(2015), pp. 77-84. [10.1016/j.lwt.2015.03.091]

Availability:
This version is available at: 11381/2919419 since: 2022-03-19T11:15:26Z

Publisher:

Published
DOI:10.1016/j.lwt.2015.03.091

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(Article begins on next page)

21 July 2024

Elsevier Editorial System(tm) for Food Research International
Manuscript Draft

Manuscript Number:

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Article Type: Research Article

Keywords: silybin, silymarin, delivery, nanoemulsion, stability, bioaccessibility

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Abstract: The purpose of this study was to investigate the potential of nanoemulsion delivery systems to carry silybin from silymarin. To this purpose, different carrier oils (i.e. 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 in vitro bioaccessibility was evaluated. Moreover, the physical and chemical stability of nanoemulsions was studied by measuring particle size, silybin concentration, oxygen consumption and hydroperoxide formation 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 used slightly affected the silybin in vitro bioaccessibility. On the contrary, the oil nature influenced the nanoemulsion particle size as well as silybin stability during storage: silybin underwent degradation, showing lower stability in extra virgin and sunflower oil than in castor oil. Results also showed that silymarin did not affect the oxidation kinetics of the carrier oils.

Dear Editor,

I would like to submit to your attention the manuscript entitled "Nanoemulsions as delivery systems of hydrophobic silybin from silymarin in foods: effect of oil type on silybin solubility, in vitro bioaccessibility and stability " (Authors: Sonia Calligaris, Piergiorgio Comuzzo, Francesca Bot, Giovanna Lippe, Roberto Zironi, Monica Anese, Maria Cristina Nicoli) for consideration for publication on Food Research International.

The use of the flavolignan silybin from silymarin to treat liver diseases has been well documented. Indeed, in recent years attempts have been made to develop pharmaceutical preparations with increased silybin bioavailability. At the same time no information is available on ways to improve its bioaccessibility in foods. Due to silybin chemical and physical properties, lipid-based delivery systems, such as nanoemulsions, could be effectively used to incorporate this nutraceuticals in functional foods. The choice of the lipid medium appears particularly critical, since the chemical and physical characteristics of the lipid carrier could greatly affect the solubility and the stability of the compound to be delivered.

The aim of this research was to study the potential for nanoemulsion delivery systems to carry silybin from silymarin into foods. 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.

Results showed that silybin can be successfully incorporated into physically stable nanoemulsions prepared with the different oils. The oil type used slightly affected the silybin in vitro bioaccessibility. On the contrary, the oil nature influenced the nanoemulsion particle size as well as silybin stability during storage: silybin underwent degradation, showing lower stability in extra virgin and sunflower oil than in castor oil. Results also showed that silymarin did not affect the oxidation kinetics of the carrier oils.

We would greatly appreciate your comments on the paper.

Best regards

Prof. Monica Anese

Highlights

Silybin was successfully incorporated into nanoemulsions containing different oils

The oil type slightly affected the silybin in vitro bioaccessibility

The oil nature influenced the nanoemulsion particle size and silybin stability

Silymarin did not affect the oxidation kinetics of the carrier oils.

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

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Abstract

The purpose of this study was to investigate the potential of nanoemulsion delivery systems to carry silybin from silymarin. To this purpose, different carrier oils (i.e. 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 *in vitro* bioaccessibility was evaluated. Moreover, the physical and chemical stability of nanoemulsions was studied by measuring particle size, silybin concentration, oxygen consumption and hydroperoxide formation 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 used slightly affected the silybin *in vitro* bioaccessibility. On the contrary, the oil nature influenced the nanoemulsion particle size as well as silybin stability during storage: silybin underwent degradation, showing lower stability in extra virgin and sunflower oil than in castor oil. Results also showed that silymarin did not affect the oxidation kinetics of the carrier oils.

Keywords: silybin, silymarin, delivery, nanoemulsion, stability, bioaccessibility

1. Introduction

Silymarin is a flavolignans extract of *Silybum marianum*. The flavolignan 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 $O_2^{-}\cdot$ occurred to a lesser extent (Dehmlow et al., 1996). Despite this, clinical application and therapeutic efficiency of sylimarin flavolignans are limited due to their poor bioavailability. As known, molecule physical properties may greatly affect its bioavailability. At ambient temperature, silybin is in crystalline state and has low water solubility (Gazak et al., 2004). In recent years, attempts have been made to develop pharmaceutical preparations with increased silybin bioavailability (Jia et al., 2010; Li, Yuan, Huang, Zhou, & Liu, 2010; Javed, Kohli, & Ali, 2011; Parveen, Baboota, Ali, Ahuja, Vasudev, & Ahmad, 2011), while no information is available on ways to improve its bioaccessibility in foods. 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, & 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 silymarin, and thus silybin, into foods. 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 i*n 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 85% w/v 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. Nanoemulsion preparation

The oil phase was prepared by mixing silymarin powder (2.5 mg/g) and Tween 80 (10 mg/g) in sunflower oil, extra virgin olive oil or castor oil. 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 20% (w/w) oil phase with 80% (w/w) 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 up to 50 days.

2.3. Analytical determinations

2.3.1. 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.

2.3.2. Silybin solubility

Aliquots of 3 mL of sunflower oil, castor oil, extra virgin olive oil, Tween 80, deionized water or an oil and Tween 80 mixture (1:1 v/v) were introduced in 5.0 mL capacity vials and excess amount of silymarin was added (2% w/w). 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 13100 *g* for 10 min (MiniSpin, Eppendorf, Hamburg, Germany) and the solubilised silybin in the supernatant was then recovered and quantified by HPLC analysis.

2.3.3. 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 (20:80 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.

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3 128 The upper water-methanol phase was filtered on 0.20 μm pore size nylon membranes (Albet-
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5 129 Hahnemühle, Barcelona, Spain), and analysed for silybin concentration by reverse-phase
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7 130 HPLC according to the slightly modified method of Kvasnička, Bìba, Ševčìk, Voldřich, and
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9 131 Kràtkà (2003). Analyses were performed by a LC-2010 AHT liquid chromatographic system
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11 132 (Shimadzu, Kyoto, Japan) equipped with an integrated UV-visible detector. A 4 μm packed
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13 133 150 x 4.6 mm C_{18} column (Synergi Polar, Phenomenex, Torrance, CA), thermostated at 35
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15 134 °C, was used. The elution was in gradient mode using a mixture of 0.5% (v/v) aqueous
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17 135 phosphoric acid (solvent A) and methanol (solvent B) as mobile phase at a flow rate of 1
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19 136 mL/min. Gradient was set as follows: solvent B was held at 36% for the first 5 min, increased
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21 137 to 45% in 1 min and held at this level for 25 min; then 100% solvent B was reached in 2 min
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23 138 and held for 5 min, before to be lowered in 2 min to the initial level (36%). The sample
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25 139 injection volume and the detection wavelength were 10 μL and 288 nm, respectively.
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27 140 Quantitative analysis was carried out by comparing the silybin peak area with the results of a
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29 141 calibration line, obtained by injecting silybin standard solutions (in water:methanol, 20:80
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31 142 v/v). Calibration line was linear (R^2 = 0.999) in the 0.5 to 18.0 mg/L concentration interval.
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33 143
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35 144 *2.3.4. Chain breaking activity*
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37 145 The chain-breaking activity was measured following the methodology of Brand-Williams,
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39 146 Cuvelier, and Berset (1995). The bleaching rate of the stable free radical 2,2-diphenyl-1-
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41 147 picrylhydrazyl (DPPH·) was monitored at 515 nm. A volume of 1.85 mL of 6.1×10^{-5} M
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43 148 DPPH· methanol solution was used. The reaction was started by the addition of 150 μL of
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45 149 sample, previously solubilised with methanol. The DPPH· bleaching was followed at 515 nm
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47 150 (Uvikon 860, Kontron Instruments, Milano, Italy) at 25 °C for at least 10 min. In all cases the
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49 151 DPPH· bleaching rate was proportional to the sample concentration added to the medium. The
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51 152 reaction rate of DPPH· bleaching was computed according to the following equation
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53 153 (Manzocco, Anese, & Nicoli, 1998):
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154 $$\frac{1}{A^3} - \frac{1}{A_0^3} = 3kt \quad \text{(eq. 1)}$$

155 where k is the DPPH· bleaching rate, A_0 is the initial absorbance value, and A is the
156 absorbance at increasing time, t. The chain-breaking activity was expressed as the slope (k)
157 obtained from eq. 1 per milligram of dry matter (A^{-3}/min·g_{dm}), assuming that all of the sample
158 dry matter possessed antioxidant capacity.

159

160 *2.3.5. Oxygen concentration*

161 Oxygen concentration was measured by an OxySense® fluorimeter (OxySense Inc., Dallas,
162 TX, USA). Aliquots of 18 mL of the nanoemulsions were introduced into 20 mL colourless
163 glass vials. Preliminarily, an oxygen sensitive sensor (O2xyDot®, OxySense Inc., Dallas, TX,
164 USA) was pasted on approximately 1 cm from the bottom edge of the internal surface of the
165 vials, by using an oxygen permeable glue (OxySense Inc.). When the sensor is illuminated by
166 a pulsed blue light, a red fluorescent light is emitted, that is measured by the fluorimeter. The
167 decrease of the O2xyDot® fluorescence lifetime, due to dynamic oxygen quenching, is
168 proportional to the oxygen concentration in the sample. Sample temperature was measured
169 simultaneously, by a sensor located in the reader pen of the fluorimeter (Li, Ashcraft,
170 Freeman, Stewart, Jank, & Clark, 2008). Results were expressed as mg/L of oxygen.

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172 *2.3.6. Lipid hydroperoxide concentration*

173 Lipid hydroperoxide concentration was determined following the methods of Shantha and
174 Decker (1994) and Katsuda, McClements, Migioranza, and Decker (2008). In particular, 3 mL
175 nanoemulsion were mixed three times with 15 mL isooctane:2-propanol (3:1 v/v) solution and
176 vortexed for 10 s. After centrifugation for 2 min at 2000 *g*, the clear upper layer was collected
177 (0.20 mL) and mixed with 2.8 mL methanol:1-butanol (2:1 v/v) solution, 15 µL of 3.94 mol/L
178 ammonium thiocyanate solution and 15 µL of 0.0072 mol/L ferrous ion solution (prepared
179 through the mixture of 0.132 mil/L $BaCl_2$ and 0.144 mol/L $FeSO_4$). After 20 min incubation

at room temperature, absorbance was measured at 510 nm with a spectrophotometer (Shimadzu, UV-2501PC, Japan). Hydroperoxide concentration was determined using cumene hydroperoxide standard curve.

2.3.7. Silybin in vitro *bioaccessibility*

In vitro digestion. A dynamic *in vitro* digestion model was used to study the influence of emulsion composition on silybin bioaccessibility. The methods of Zangerberg, Mullertz, Kristensen, and Hovgard (2001a and 2001b) and Mun, Decker, Park, Weiss, and McClements (2006) were followed with some modifications. 0.75 mL nanoemulsion was mixed with phosphate buffer (pH 7) and heated at 37 °C for 10 min in a water bath. Then, the pH of the sample was adjusted to 7.00 with 2 M NaOH and 4 mL bile extract (46.9 g/L in phosphate buffer at pH 7) and 1 mL calcium chloride (110 g/L in deionized water) were added. The pH was adjusted to 7.00 if necessary. Finally, 2.5 mL of freshly prepared lipase suspension (24 g/L in phosphate buffer at pH 7) was added to the mixture. The pH of the mixture was monitored and maintained at 7.00 by adding 0.1 M NaOH. The volume of NaOH added to the sample was recorded and used to calculate the concentration of free fatty acids (FFA) generated during lipolysis. The extent of lipolysis was determined as follows:

$$FFA\ released\ (\%) = \frac{NaOH\ amount\ consumed}{theoretical\ NaOH\ amount\ for\ complete\ lipolysis} \cdot 100 \quad \text{(eq. 2)}$$

To calculate the amount of NaOH required for complete lipolysis, it was assumed that 1 molecule of sunflower oil, extra virgin olive oil or castor oil consumed 2 molecule of NaOH (Yu & Huang, 2012), each oil molecule being hydrolysed by pancreatic lipase into two free fatty acids and one monoacylglycerol molecule.

In vitro *bioaccessibility determination.* The *in vitro* bioaccessibility of silybin was evaluated after the *in vitro* digestion was completed. The digest was immediately centrifuged (XL-70

Ultracentrifuge, Beckman, Palo Alto, CA, USA) at 165000 *g* at 4 °C for about 70 min. After centrifugation, the sample was separated into an opaque sediment phase (pellet) and a clear phase containing the mixed micelles (supernatant). Silybin was extracted from both the digest and the micelles by liquid-liquid extraction. Briefly, 20 mL digest was introduced in 50 mL Pyrex tubes and mixed with 200 μL of 218.8 g/L hydrochloric acid and 100 μL of naringenin-7-*O*-glucoside methanol solution (0.73 g/L). 10 mL ethyl acetate was then added and the tubes were subjected to manual shaking (5 min) followed by immersion in ultrasonic bath (1 h, 40 °C). The sample was then centrifuged (1000 *g*, 10 min) and the organic phase separated. Manual extraction and ultrasound treatment were repeated twice and the ethyl acetate extracts concentrated to a final volume of approximately 1 mL, in a vacuum centrifuge (Univapo 100 H, UniEquip GmbH, Freital, Dresden, Germany). Silybin quantification in the concentrated extracts was carried out by HPLC as reported above (Paragraph 2.3.3.). The bioaccessibility (%) was defined as the percentage ratio between silybin concentration in the mixed micelles and the silybin concentration in the digest. In addition, to allow a better traceability of the repartition of the active compound between the pellet and micelles, the pellet was also analysed for silybin content. To this purpose, the sediment was re-suspended in 5 mL methanol and 100 μL 218.8 g/L hydrochloric acid. Extraction was performed by manual shaking (5 min) followed by immersion in ultrasonic bath (40 °C for 1 h). Samples were finally filtered on 0.20 μm pore size nylon membranes (Albet-Hahnemühle, Barcelona, Spain), and silybin content was determined by HPLC as reported above (Paragraph 2.3.3.). Mass balance of silybin in pellet and micelles evidenced the complete recovery of the bioactive compound.

2.4. Statistical analysis

The results are averages of at least three measurements taken from different samples and are reported as means ± SD. Analyses of variance (ANOVA) was performed with significance

level set at $p<0.05$ (Statistica for Windows, ver. 5.1, Statsoft Inc. Tulsa, USA, 1997). The Tukey procedure was used to test for differences between means.

3. Results and discussion

3.1. Silybin solubility

The solubility of silybin in water, selected oils and Tween 80 is shown in Table 1. In accordance with the literature, the solubility of silybin in water was negligible (Gazak et al., 2004; Yang et al., 2013). Silybin presented the highest solubility in castor oil followed by extra virgin olive oil and sunflower oil. As known, among the selected oils, castor oil is the most polar oil due to its high content of ricinoleic acid. This result is consistent with the data reported by Yang et al. (2013) and seems to indicate that a certain degree of polarity of the oil phase might favour silybin solubility. Data on the surfactant Tween 80 support this hypothesis showing silybin solubility one order of magnitude higher than in the oils. As reported in the literature, Tween 80 is actually used as surfactant in different nanoemulsions for drug delivery (Parveen et al., 2011; Yang et al., 2013). Furthermore, silybin solubility was determined in mixes of sunflower oil, extra virgin olive oil or castor oil, and Tween 80 in 1:1 (v/v) ratio. Results showed that in all mixtures the silybin solubility had a value comparable to that found in the Tween 80 alone.

To compare the performances of different nanoemulsions to carrying silybin, before emulsion preparation 2.50 mg/g silymarin, corresponding to 0.525 mg/g silybin, was added to sunflower oil, extra virgin olive oil or castor oil containing 10 mg/g Tween 80. At this level, silybin was completely solubilized in the mixture.

3.2. Physical properties of silymarin enriched nanoemulsions

Table 2 shows the mean particle diameter of sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin. Control samples prepared without silymarin

showed results not significantly different from the bioactive enriched counterparts (data not shown). Although the samples had relatively small mean particle diameters, appreciable differences in the values among the three types of nanoemulsions can be observed, the one prepared with castor oil showing higher values. As emulsion properties are greatly affected by the nature of the oil used (McClements, 2005), these results can be attributed to differences in chemical properties among the oils considered. In particular, the higher particle size of nanoemulsion with castor oil can be due to higher viscosity and polarity of castor oil in comparison with extra virgin olive oil and sunflower oil. Neither significant increase in nanoemulsions particle size nor visible sediments at the bottom of the test vials were found during storage at 20 °C for up to 50 days. These results suggest that silymarin did not undergo separation and re-crystallisation phenomena during storage.

3.3. Silybin in vitro *bioaccessibility of silymarin enriched nanoemulsions*

The effect of carrier oil on the silybin *in vitro* bioaccessibility in nanoemulsions was studied. As well known, silymarin carrying triacylglycerols have to be decomposed into free fatty acids and monoglycerides to allow the bioactive molecule to be released and subsequently incorporated into the mixed micelles, i.e. made of bile salts and lipolytic products, to be transferred to the epithelium cells (Hofmann & Borgstrom, 1964). To monitor the rate and extent of lipid digestion, the formation of free fatty acids from the nanoemulsions during simulated small intestine digestion was measured (Figure 1). The free fatty acids release steadily increased in the first minutes of the digestion, suggesting that the lipase promptly attached to the oil droplets surface due to an efficient displacement of the surfactant layer by the bile salts (Qian, Derek, Xiao, & McClements, 2012). In our experimental conditions, the free fatty acids release during the *in vitro* digestion was not affected by the carrier oil type, which is consistent with previous results (Hur, Joo, Lim, Decker, & McClements, 2011). The oils used were actually all composed of long chain fatty acids, the chain length being an

284 influencing factor of the extent and rate of *in vitro* lipolysis. Moreover, our results clearly
285 show that in all cases the free fatty acids release was almost complete (around 80-90%
286 digestion). The silybin concentration in the mixed micelles (i.e. silybin bioaccessibility) and
287 precipitated pellets obtained from the digest was then measured (Table 3). Results showed
288 that the carrier oil only slightly influenced the silybin *in vitro* bioaccessibility. In fact,
289 independetly of oil type, silybin concentration in the micelles was approximately 25-30%,
290 indicating that most of the bioactive compound incorporated into the nanoemulsions was not
291 available for absorption.
292

293 *3.4. Chemical properties of silymarin enriched nanoemulsions*

294 Despite a huge number of papers deal with the mechanisms of silymarin biological activity, to
295 our knowledge the effect of flavonoids from silymarin on food lipid oxidation has not been
296 well investigated. Figure 2 shows the changes in oxygen concentration of sunflower oil, extra
297 virgin olive oil and castor oil containing nanoemulsions enriched with silymarin as a function
298 of storage time at 20 °C. Nanoemulsions not containing silymarin were used as controls.
299 Oxygen concentration decreased faster in nanoemulsions containing sunflower oil, followed
300 by nanoemulsions with extra virgin olive oil and castor oil. Such a reactivity rank is consistent
301 with the unsaturation degree of the incorporated oils, sunflower oil having a higher value than
302 extra virgin olive oil, which in turn has a greater number of carbon-carbon double bonds than
303 castor oil. No significant differences in oxygen concentration were found between
304 nanoemulsions with and without silymarin. This result suggests that silymarin did not act as
305 an oxygen scavenger, in agreement with the pionering data reported by Dehmlow et al.
306 (1996), who found a negligible capacity of silybin to react with $O_2^{-}\cdot$ species. Figure 3 shows
307 the changes in hydroperoxide concentration of nanoemulsion with or without silymarin during
308 storage. It can be observed that the hydroperoxide concentration changed during storage with
309 different kinetics depending on the nature of the carrier oil, the sunflower oil and castor oil

containing emulsions being the most and the least susceptible to oxidation, respectively. However, also in this case, the evolution of hydroperoxides in the nanoemulsions enriched with silymarin did not significantly differ from those of the respective nanoemulsions without the bioactive molecule. These results further indicate that silymarin incorporation into nanoemulsions did not influence the pathway of the oxidative reactions occuring in oils. However, they contrast with data on the liver protective effect of silymarin, that is generally attributed to the antioxidant activity of the bioactive molecule (Shaker, Mahmoud, & Mnaa, 2010). To have insights on the chain breaking activity of silymarin extract used in this study, the DPPH· assay was performed. Only a weak ability of silymarin to scavenge the DPPH· radical was measured (i.e. 0.14 ± 0.06 $A^{-3}/min \cdot g_{dm}$). Such a value was much lower than the chain breaking activity of α-tocopherol and Trolox, that were 0.94 ± 0.24 and 8.47 ± 0.45 $A^{-3}/min \cdot g_{dm}$, respectively. These results are in agreement with the data of the literature (Gazak et al., 2004; Henning et al., 2014). In particular, Henning et al. (2014) reported that the *Silybum marianum* extracts had antioxidant properties lower than other dietary supplements, such as pomegranate, resveratol and green tea. It is noteworthy that other authors found that silymarin had high *in vitro* radical scavenging activity (Koksal, Gulcin, Beyza, Sarikaya, & Bursal, 2009).

Despite the results described above, silybin concentration in nanoemulsions greatly decreased during storage (Figure 4). Such a decrease was greater in sunflower oil based nanoemulsions followed by extra virgin olive oil and castor oil containing samples. In particular, at 50 days storage, silybin losses were 60% and 55% in nanoemulsions containing sunflower oil and extra virgin olive oil, respectively, whereas 25% reduction of silybin content was found in the nanoemulsion with castor oil. These discrepancies in silybin degradation kinetics might be brought back to differences in the bioactive reactivity in the media considered. It can be inferred that in sunflower oil and extra virgin olive oil containing nanoemulsions, silybin could be preferably located at the interface, near the emulsifier layer, where a higher polarity

would favour its degradation. On the contrary, when castor oil was used as a carrier, silybin would be preferably located inside the oil droplets, being thus less prone to degradation. Possible silybin degradation by reactive species generated from oil oxidative reactions may not be ruled out. Gazak et al. (2004) actually described a number of oxidized derivatives of silybin. These compounds were obtained under strong oxidative conditions (e.g., H_2O_2 in $NaHCO_3$, iodine in glacial acetic acid, high temperature). In our experimental conditions, the greater the oil susceptibility to oxidation, and thus the formation of oxidation products, the greater the silybin degradation. It is a matter of fact that due to the scarcity of information available in the literature, further experiments are needed to understand the fate of silybin in food related environments.

Conclusions

The present study is a first attempt to develop delivery systems for incorporating silymarin into functional foods. Results showed that silymarin can be successfully incorporated into physically stable nanoemulsions prepared with different carrier oils. The oil type used slightly affected the *in vitro* bioaccessibility of the main bioactive compound of silymarin, i.e. silybin. Although silymarin incorporation resulted not to affect oil oxidative kinetics, silybin concentration decreased during storage. Such a decrease was greater in extra virgin and sunflower oil than in castor oil. This instability rank is consistent with that relevant to the susceptibility to oxidation of the carrier oils considered. Although additional studies should be accomplished to fully elucidate the mechanism of silybin degradation in lipid carriers, the information acquired represents an important contribution for the design and fabrication of silymarin delivering nanoemulsions.

Acknowledgements

Research was supported by the project "From nutrigenetics to nutraceutics: development of synergic and integrated actions for the development of tests, diets and foods able to improve the public well-being and prevent food related diseases. Art. 13 - D.M. 593, 8 August 2000.

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Figure captions

Figure 1. Free fatty acids (FFA) release from sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin.

Figure 2. Percentage of residual oxygen in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions with and without silymarin, as a function of storage time at 20 °C.

Figure 3. Changes in hydroperoxide concentration in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions with and without silymarin, as a function of storage time at 20 °C.

Figure 4. Silybin concentration in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin, as a function of storage time at 20 °C.

Table captions

Table 1. Silybin solubility in water, sunflower oil, extra virgin olive oil, castor oil and Tween 80.

Table 2. Mean particle diameter of sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin.

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2
3 486 Table 3. Silybin concentration (%) in mixed micelle (i.e. *in vitro* bioaccessibility) and pellet
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Figure

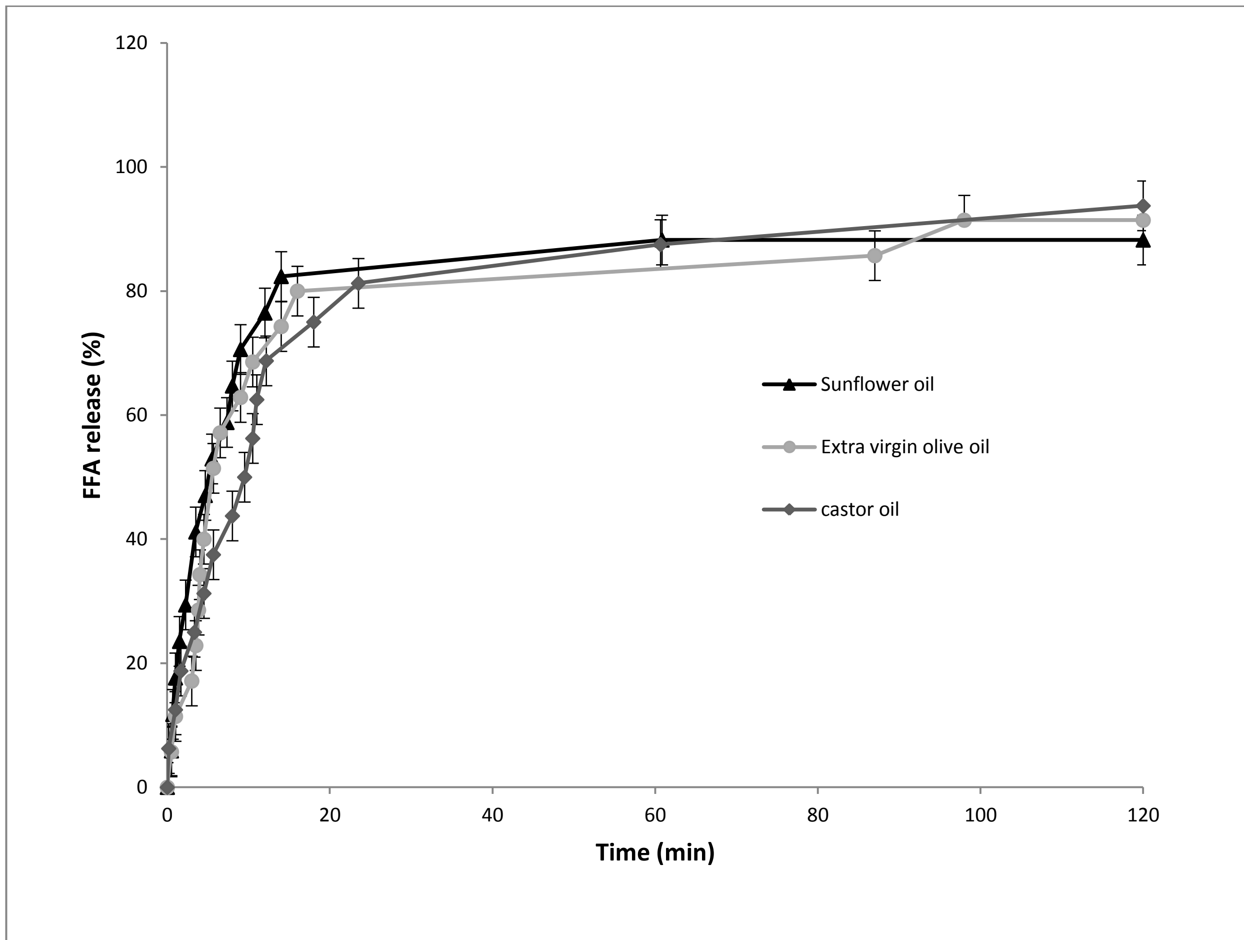

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Figure

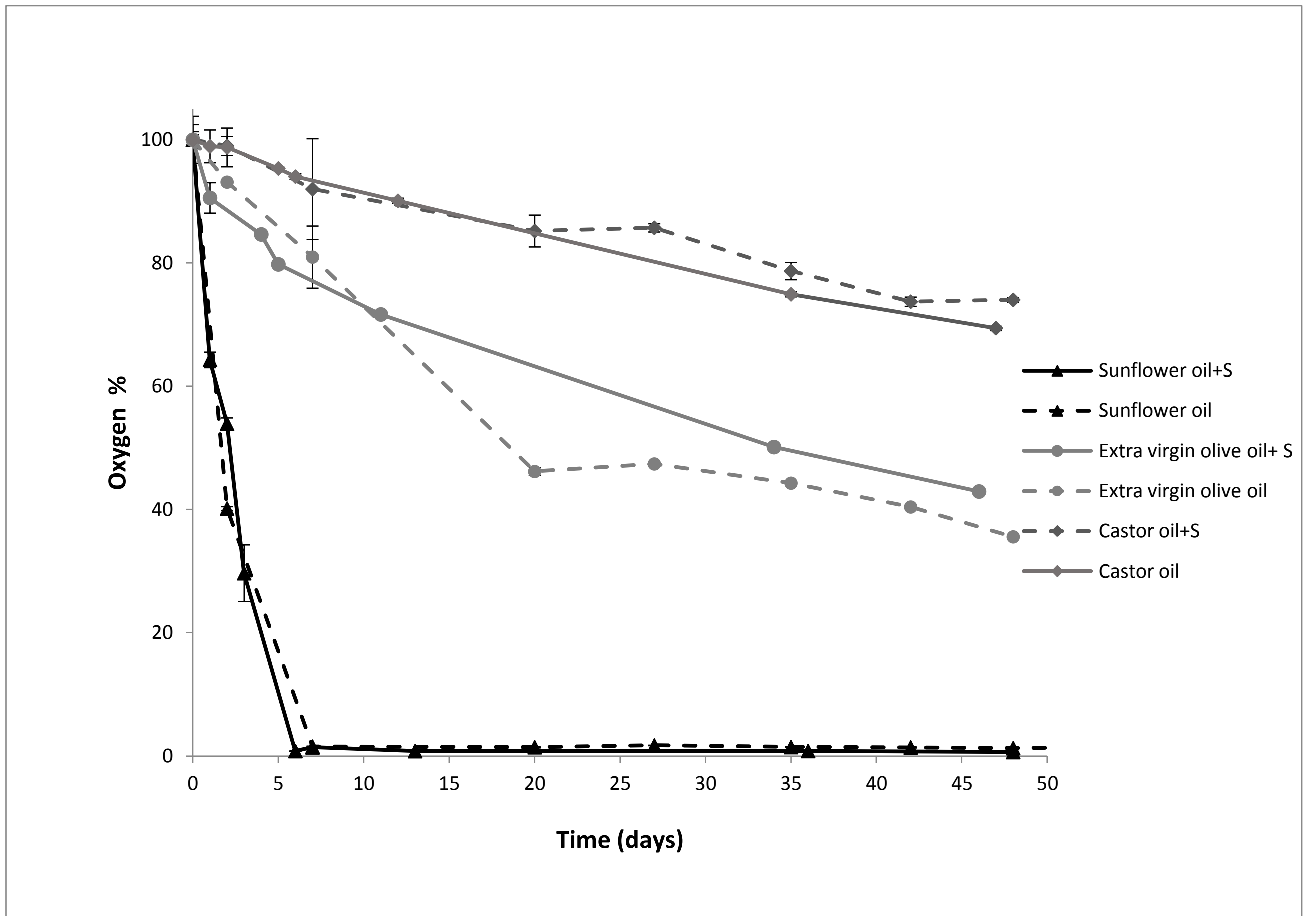
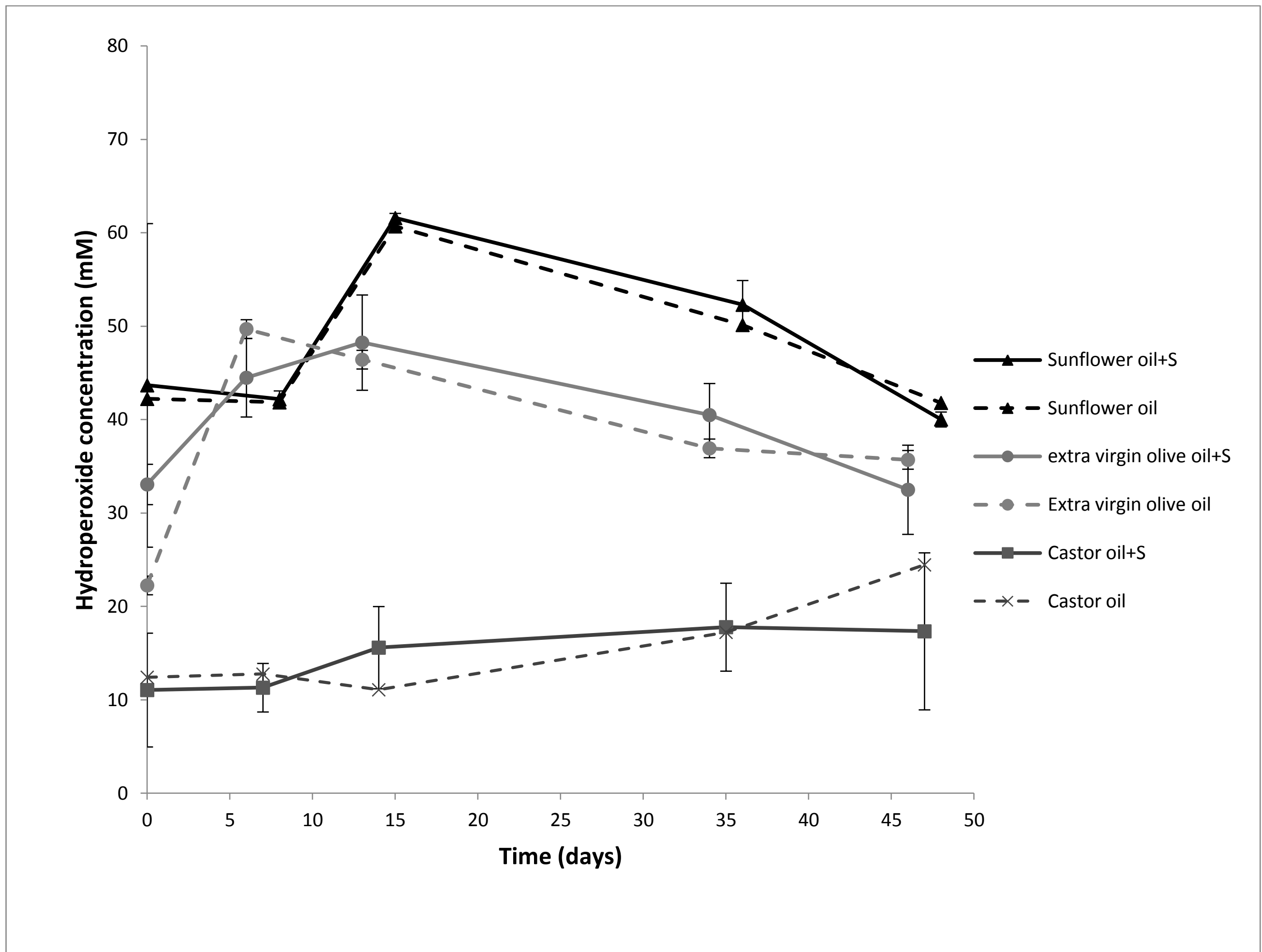


Figure 2. Percentage of residual oxygen in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions with and without silymarin, as a function of storage time at 20 °C.

Figure



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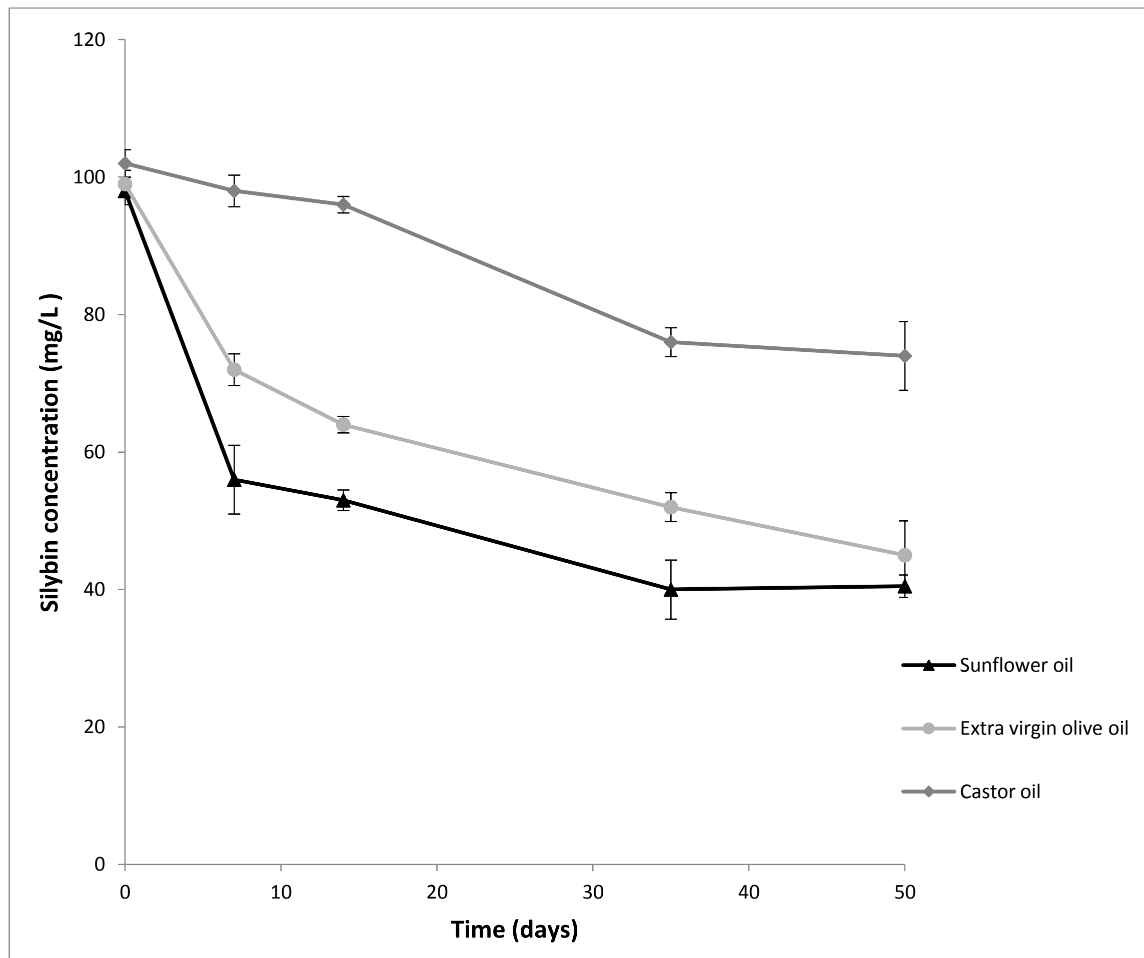

Figure 4. Silybin concentration in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin, as a function of storage time at 20 °C.

Table

Table 1. Silybin solubility in water, sunflower oil, extra virgin olive oil, castor oil and Tween 80.

Medium	Silybin (mg/g)
Water	nd
Sunflower oil	0.028 ± 0.006^{b}
Extra virgin olive oil	0.009 ± 0.002^{a}
Castor oil	0.668 ± 0.072^{c}
Tween 80	2.061 ± 0.110^{d}

[a-d]: means with different letters are significantly different ($p < 0.05$)

nd: not detectable

Table

Table 2. Mean particle diameter of sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin.

Storage time (days)	Mean particle diameter (nm)		
	Sunflower oil	Extra virgin olive oil	Castor oil
0	208±39[a]	241±46[a]	307±52[b]
15	208±37[a]	229±39 [a]	309±53[b]
36	208±38[a]	235±48[a]	329±6[b]
50	232±49[a]	235±47[a]	323±62[b]

[a-b] means with different letters in the same row are significantly different ($p<0.05$)

Table

Table 3. Silybin concentration (%) in mixed micelle (i.e. *in vitro* bioaccessibility) and pellet after *in vitro* digestion of sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin.

Carrier oil	Silybin (%)	
	Micelle	Pellet
Sunflower oil	25.3±2.1[b]	78.1±6.5[a]
Extra virgin olive oil	29.1±0.7[a]	71.1±4.5[ab]
Castor oil	29.6±1.6[a]	68.0±0.6[b]

[a-b] *means with different letters are significantly different ($p < 0.05$)*