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

Original

Availability:

This version is available <http://hdl.handle.net/11390/1070317> since 2020-07-16T16:16:32Z

Publisher:

Published

DOI:10.1016/j.lwt.2015.03.091

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

Manuscript Number:

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

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

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13 **Abstract**

14 The purpose of this study was to investigate the potential of nanoemulsion delivery systems
15 to carry silybin from silymarin. To this purpose, different carrier oils (i.e. sunflower oil, extra
16 virgin olive oil and castor oil) were used to prepare silymarin loaded nanoemulsions. The
17 effect of oil type on the silybin solubility and *in vitro* bioaccessibility was evaluated.
18 Moreover, the physical and chemical stability of nanoemulsions was studied by measuring
19 particle size, silybin concentration, oxygen consumption and hydroperoxide formation during
20 storage at 20 °C.
21 Results showed that silybin can be successfully incorporated into physically stable
22 nanoemulsions prepared with the different oils. The oil type used slightly affected the silybin
23 *in vitro* bioaccessibility. On the contrary, the oil nature influenced the nanoemulsion particle
24 size as well as silybin stability during storage: silybin underwent degradation, showing lower
25 stability in extra virgin and sunflower oil than in castor oil. Results also showed that
26 silymarin did not affect the oxidation kinetics of the carrier oils.

27

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

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30 **1. Introduction**

31 Silymarin is a flavolignans extract of *Silybum marianum*. The flavolignan silybin or silybinin
32 is the most abundant biologically active compound of silymarin. The use of silymarin to treat
33 liver diseases, such as cirrhosis, hepatitis, alcoholic liver disease and toxin exposure has been
34 well documented (Flora, Hahn, Rosen, & Benner, 1998; Frascini, Demartini, & Esposti,
35 2002). These biological effects are attributed to the antioxidant, antifibrotic, anti-
36 inflammatory, anti-lipid-peroxidative and anti-carcinogenic activity of silymarin components
37 (Basaga, Poli, Tekkaya, & Ara, 1997; Luper, 1998; Yang, Liu, & Liu, 2004).

38 Although results can be hardly summarized, studies on the liver-protective capacity of
39 silymarin (Loguercio & Festi, 2011) and cell oxidation mechanisms (Dehmlow, Murawski, &
40 de Groot, 1996; Zielinska-Przyjemska & Wiktorowicz, 2006) evidenced an important
41 inhibitory effect of silymarin flavonoids on cell enzymes (e.g. lipoxygenase) involved in
42 inflammatory reactions, whereas reaction with O_2^- occurred to a lesser extent (Dehmlow et
43 al., 1996). Despite this, clinical application and therapeutic efficiency of silymarin
44 flavolignans are limited due to their poor bioavailability. As known, molecule physical
45 properties may greatly affect its bioavailability. At ambient temperature, silybin is in
46 crystalline state and has low water solubility (Gazak et al., 2004). In recent years, attempts
47 have been made to develop pharmaceutical preparations with increased silybin bioavailability
48 (Jia et al., 2010; Li, Yuan, Huang, Zhou, & Liu, 2010; Javed, Kohli, & Ali, 2011; Parveen,
49 Baboota, Ali, Ahuja, Vasudev, & Ahmad, 2011), while no information is available on ways to
50 improve its bioaccessibility in foods. According to Rao and McClements (2012), lipid-based
51 delivery systems for food application, such as microemulsions, nanoemulsions, liposomes,
52 solid lipid nanoparticles, polymeric nanoparticles, filled hydrogel particles, can be effectively
53 used to incorporate poorly water-soluble nutraceuticals in functional foods. As known,
54 nanoemulsions are thermodynamically unstable colloidal systems containing small lipid
55 droplets dispersed in an aqueous medium (Rao & McClements, 2012). Generally, they show

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56 good stability to gravitational separation and particle aggregation becoming a good
57 component to be added to foods that have to be processed and stored under different
58 conditions. Nanoemulsions have been actually proposed to increase the solubility and stability
59 of bioactive molecules, such as quercetin (Pool, Mendoza, Xiao, & McClements, 2013),
60 polymethoxyflavone (Li, Zheng, Xiao, & McClements, 2012) and curcumin (Ahmed, Li,
61 McClements, & Xiao, 2012), to be incorporated into foods. The choice of the lipid medium
62 appears particularly critical, since the chemical and physical characteristics of the lipid carrier
63 greatly affect the solubility of the compound to be delivered. Also, the presence of bioactive
64 molecule crystals may negatively affect nanoemulsion physical stability, leading to possible
65 undesired phase separation during food processing and storage, as well as reduction of the
66 bioavailability of the selected component that may not be adsorbed in this form into the
67 gastrointestinal tract (Giacomelli et al., 2002; Kawabata, Wada, Nakatani, Yamada, & Onoue,
68 2011).

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

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3 **77 2. Materials and methods**

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5 **78 2.1. Materials**

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7 **79** Silymarin extract containing 210 mg/g of silybin, 2,2-diphenyl-1-picrylhydrazyl free radical
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9 **80** (DPPH·), lipase from porcine pancreas, porcine bile extract, di-hydrated calcium chloride,
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11 **81** Tween 80, sodium azide, ethyl acetate, HPLC grade methanol, isooctane, 2-propanol, 1-
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13 **82** butanol, ammonium thiocyanate, cumene hydroperoxide, analytical grade hydrochloric acid
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15 **83** and sodium hydroxide were from Sigma Aldrich (St. Louis, MO, USA). Sodium chloride,
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17 **84** barium chloride, ferrous sulphate, monosodium dihydrogen phosphate, disodium hydrogen
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19 **85** phosphate and 85% w/v phosphoric acid were from Carlo Erba Reagents (Milano, Italy).
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21 **86** Analytical standard grade silybin and naringenin-7-*O*-glucoside were from Extrasynthese
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23 **87** (Genay, France). Sunflower oil, extra virgin olive oil and castor oil were purchased in a local
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25 **88** market.
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32 **90 2.2. Nanoemulsion preparation**

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34 **91** The oil phase was prepared by mixing silymarin powder (2.5 mg/g) and Tween 80 (10 mg/g)
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36 **92** in sunflower oil, extra virgin olive oil or castor oil. The systems were stirred in the dark until
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38 **93** the silymarin was completely dissolved. No recrystallization events were observed before
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40 **94** emulsion preparation. The aqueous phase consisted of deionised water added with 0.1 mg/g of
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42 **95** sodium azide, to avoid microbial spoilage during the storage experiments. The stock
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44 **96** emulsions were prepared by mixing 20% (w/w) oil phase with 80% (w/w) aqueous phase with
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46 **97** a high speed blender for 1 min at 9000 rpm (Polytron, PT 3000, Cinematica, Littau, Swiss).
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48 **98** Aliquots of 250 mL of the stock emulsions were homogenised at 10 L/h flow rate by two
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50 **99** passes at 150 MPa through a two stage high pressure homogeniser provided with cylindrical
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52 **100** tungsten carbide homogenising valves (Panda PLUS 2000, Gea Niro Soavi, Parma, Italy).
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54 **101** Aliquots of 18 mL of the nanoemulsions were inserted into 20 mL colourless glass vials,
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3 102 sealed with butyl septa and metallic caps and stored at 20 °C in a thermostatic cell for up to
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5 103 50 days.
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7 104 *2.3. Analytical determinations*
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9 105 *2.3.1. Particle size*
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11 106 The mean diameter of emulsion droplets was measured by using the dynamic light scattering
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13 107 instrument Particle Sizer NICOMP™ 380 ZLS (PSS NICOMP Particle Sizing System, Santa
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15 108 Barbara, California, USA). Samples were diluted 1:1000 (v/v) with deionised water prior to
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17 109 the analysis to avoid multiple scattering effects. The angle of observation was 90°. Solution
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19 110 refractive index and viscosity were set at 1.333 and 1.0 cP, respectively, corresponding to the
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21 111 values of pure water at 20 °C. Particle mean diameter corresponding to volume distribution
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23 112 was calculated by NICOMP Distribution Analysis.
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30 114 *2.3.2. Silybin solubility*
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32 115 Aliquots of 3 mL of sunflower oil, castor oil, extra virgin olive oil, Tween 80, deionized water
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34 116 or an oil and Tween 80 mixture (1:1 v/v) were introduced in 5.0 mL capacity vials and excess
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36 117 amount of silymarin was added (2% w/w). Samples were kept at a constant temperature
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38 118 (25±1.0 °C) under shaking for 72 h to reach equilibrium (Parveen et al., 2011). The samples
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40 119 were centrifuged at 13100 g for 10 min (MiniSpin, Eppendorf, Hamburg, Germany) and the
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42 120 solubilised silybin in the supernatant was then recovered and quantified by HPLC analysis.
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48 122 *2.3.3. Silybin concentration*
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50 123 Silybin extraction was performed by introducing 1 g supernatant or nanoemulsion into 10 mL
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52 124 Pyrex tubes, added with 5 mL water:methanol mixture (20:80 v/v), and manually shaken for 2
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54 125 min. The tubes were then treated for 15 min in an ultrasonic bath (25 °C) and finally
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56 126 centrifuged at 1000 g for 10 min (Labofuge I, Heraeus Christ GmbH, Osterode am Harz,
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58 127 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, Ševčík, Voldřich, and
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9 131 Krátkà (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 C₁₈ 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
28
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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144 2.3.4. Chain breaking activity

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

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3 154 $\frac{1}{A^3} - \frac{1}{A_0^3} = 3kt$ (eq. 1)
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6 155 where k is the DPPH· bleaching rate, A_0 is the initial absorbance value, and A is the
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8 156 absorbance at increasing time, t . The chain-breaking activity was expressed as the slope (k)
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10 157 obtained from eq. 1 per milligram of dry matter ($A^{-3}/\text{min} \cdot \text{g}_{\text{dm}}$), assuming that all of the sample
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12 158 dry matter possessed antioxidant capacity.
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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 mol/L BaCl₂ and 0.144 mol/L FeSO₄). After 20 min incubation
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3 180 at room temperature, absorbance was measured at 510 nm with a spectrophotometer
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5 181 (Shimadzu, UV-2501PC, Japan). Hydroperoxide concentration was determined using cumene
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7 182 hydroperoxide standard curve.
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10 11 184 2.3.7. *Silybin in vitro bioaccessibility*

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14 185 *In vitro digestion.* A dynamic *in vitro* digestion model was used to study the influence of
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16 186 emulsion composition on silybin bioaccessibility. The methods of Zangerberg, Mullertz,
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18 187 Kristensen, and Hovgard (2001a and 2001b) and Mun, Decker, Park, Weiss, and McClements
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20 188 (2006) were followed with some modifications. 0.75 mL nanoemulsion was mixed with
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23 189 phosphate buffer (pH 7) and heated at 37 °C for 10 min in a water bath. Then, the pH of the
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25 190 sample was adjusted to 7.00 with 2 M NaOH and 4 mL bile extract (46.9 g/L in phosphate
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27 191 buffer at pH 7) and 1 mL calcium chloride (110 g/L in deionized water) were added. The pH
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29 192 was adjusted to 7.00 if necessary. Finally, 2.5 mL of freshly prepared lipase suspension (24
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31 193 g/L in phosphate buffer at pH 7) was added to the mixture. The pH of the mixture was
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33 194 monitored and maintained at 7.00 by adding 0.1 M NaOH. The volume of NaOH added to the
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35 195 sample was recorded and used to calculate the concentration of free fatty acids (FFA)
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37 196 generated during lipolysis. The extent of lipolysis was determined as follows:
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$$44 \quad 198 \quad FFA \text{ released } (\%) = \frac{NaOH \text{ amount consumed}}{\text{theoretical NaOH amount for complete lipolysis}} \cdot 100 \quad (\text{eq. 2})$$

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49 200 To calculate the amount of NaOH required for complete lipolysis, it was assumed that 1
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51 201 molecule of sunflower oil, extra virgin olive oil or castor oil consumed 2 molecule of NaOH
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53 202 (Yu & Huang, 2012), each oil molecule being hydrolysed by pancreatic lipase into two free
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55 203 fatty acids and one monoacylglycerol molecule.
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58 204 *In vitro bioaccessibility determination.* The *in vitro* bioaccessibility of silybin was evaluated
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60 205 after the *in vitro* digestion was completed. The digest was immediately centrifuged (XL-70
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3 206 Ultracentrifuge, Beckman, Palo Alto, CA, USA) at 165000 g at 4 °C for about 70 min. After
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5 207 centrifugation, the sample was separated into an opaque sediment phase (pellet) and a clear
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7 208 phase containing the mixed micelles (supernatant). Silybin was extracted from both the digest
8
9 209 and the micelles by liquid-liquid extraction. Briefly, 20 mL digest was introduced in 50 mL
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11 210 Pyrex tubes and mixed with 200 µL of 218.8 g/L hydrochloric acid and 100 µL of naringenin-
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13 211 7-*O*-glucoside methanol solution (0.73 g/L). 10 mL ethyl acetate was then added and the
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15 212 tubes were subjected to manual shaking (5 min) followed by immersion in ultrasonic bath (1
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17 213 h, 40 °C). The sample was then centrifuged (1000 g, 10 min) and the organic phase separated.
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19 214 Manual extraction and ultrasound treatment were repeated twice and the ethyl acetate extracts
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21 215 concentrated to a final volume of approximately 1 mL, in a vacuum centrifuge (Univapo 100
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23 216 H, UniEquip GmbH, Freital, Dresden, Germany). Silybin quantification in the concentrated
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25 217 extracts was carried out by HPLC as reported above (Paragraph 2.3.3.). The bioaccessibility
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27 218 (%) was defined as the percentage ratio between silybin concentration in the mixed micelles
28
29 219 and the silybin concentration in the digest. In addition, to allow a better traceability of the
30
31 220 repartition of the active compound between the pellet and micelles, the pellet was also
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33 221 analysed for silybin content. To this purpose, the sediment was re-suspended in 5 mL
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35 222 methanol and 100 µL 218.8 g/L hydrochloric acid. Extraction was performed by manual
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37 223 shaking (5 min) followed by immersion in ultrasonic bath (40 °C for 1 h). Samples were
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39 224 finally filtered on 0.20 µm pore size nylon membranes (Albet-Hahnemühle, Barcelona,
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41 225 Spain), and silybin content was determined by HPLC as reported above (Paragraph 2.3.3.).
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43 226 Mass balance of silybin in pellet and micelles evidenced the complete recovery of the
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45 227 bioactive compound.
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54 55 229 *2.4. Statistical analysis*

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57 230 The results are averages of at least three measurements taken from different samples and are
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59 231 reported as means ± SD. Analyses of variance (ANOVA) was performed with significance
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232 level set at $p < 0.05$ (Statistica for Windows, ver. 5.1, Statsoft Inc. Tulsa, USA, 1997). The
233 Tukey procedure was used to test for differences between means.

234 **3. Results and discussion**

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236 *3.1. Silybin solubility*

237 The solubility of silybin in water, selected oils and Tween 80 is shown in Table 1. In
238 accordance with the literature, the solubility of silybin in water was negligible (Gazak et al.,
239 2004; Yang et al., 2013). Silybin presented the highest solubility in castor oil followed by
240 extra virgin olive oil and sunflower oil. As known, among the selected oils, castor oil is the
241 most polar oil due to its high content of ricinoleic acid. This result is consistent with the data
242 reported by Yang et al. (2013) and seems to indicate that a certain degree of polarity of the oil
243 phase might favour silybin solubility. Data on the surfactant Tween 80 support this hypothesis
244 showing silybin solubility one order of magnitude higher than in the oils. As reported in the
245 literature, Tween 80 is actually used as surfactant in different nanoemulsions for drug delivery
246 (Parveen et al., 2011; Yang et al., 2013). Furthermore, silybin solubility was determined in
247 mixes of sunflower oil, extra virgin olive oil or castor oil, and Tween 80 in 1:1 (v/v) ratio.
248 Results showed that in all mixtures the silybin solubility had a value comparable to that found
249 in the Tween 80 alone.

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

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255 *3.2. Physical properties of silymarin enriched nanoemulsions*

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

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3 258 showed results not significantly different from the bioactive enriched counterparts (data not
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5 259 shown). Although the samples had relatively small mean particle diameters, appreciable
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7 260 differences in the values among the three types of nanoemulsions can be observed, the one
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9 261 prepared with castor oil showing higher values. As emulsion properties are greatly affected by
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11 262 the nature of the oil used (McClements, 2005), these results can be attributed to differences in
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13 263 chemical properties among the oils considered. In particular, the higher particle size of
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15 264 nanoemulsion with castor oil can be due to higher viscosity and polarity of castor oil in
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17 265 comparison with extra virgin olive oil and sunflower oil. Neither significant increase in
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19 266 nanoemulsions particle size nor visible sediments at the bottom of the test vials were found
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21 267 during storage at 20 °C for up to 50 days. These results suggest that silymarin did not undergo
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23 268 separation and re-crystallisation phenomena during storage.
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30 270 3.3. *Silybin in vitro bioaccessibility of silymarin enriched nanoemulsions*

32 271 The effect of carrier oil on the silybin *in vitro* bioaccessibility in nanoemulsions was studied.
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34 272 As well known, silymarin carrying triacylglycerols have to be decomposed into free fatty
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36 273 acids and monoglycerides to allow the bioactive molecule to be released and subsequently
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38 274 incorporated into the mixed micelles, i.e. made of bile salts and lipolytic products, to be
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40 275 transferred to the epithelium cells (Hofmann & Borgstrom, 1964). To monitor the rate and
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42 276 extent of lipid digestion, the formation of free fatty acids from the nanoemulsions during
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44 277 simulated small intestine digestion was measured (Figure 1). The free fatty acids release
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46 278 steadily increased in the first minutes of the digestion, suggesting that the lipase promptly
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48 279 attached to the oil droplets surface due to an efficient displacement of the surfactant layer by
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50 280 the bile salts (Qian, Derek, Xiao, & McClements, 2012). In our experimental conditions, the
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52 281 free fatty acids release during the *in vitro* digestion was not affected by the carrier oil type,
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54 282 which is consistent with previous results (Hur, Joo, Lim, Decker, & McClements, 2011). The
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56 283 oils used were actually all composed of long chain fatty acids, the chain length being an
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3 284 influencing factor of the extent and rate of *in vitro* lipolysis. Moreover, our results clearly
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5 285 show that in all cases the free fatty acids release was almost complete (around 80-90%
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7 286 digestion). The silybin concentration in the mixed micelles (i.e. silybin bioaccessibility) and
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9 287 precipitated pellets obtained from the digest was then measured (Table 3). Results showed
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11 288 that the carrier oil only slightly influenced the silybin *in vitro* bioaccessibility. In fact,
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13 289 independetly of oil type, silybin concentration in the micelles was approximately 25-30%,
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15 290 indicating that most of the bioactive compound incorporated into the nanoemulsions was not
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17 291 available for absorption.
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22 23 293 *3.4. Chemical properties of silymarin enriched nanoemulsions*

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25 294 Despite a huge number of papers deal with the mechanisms of silymarin biological activity, to
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27 295 our knowledge the effect of flavonoids from silymarin on food lipid oxidation has not been
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29 296 well investigated. Figure 2 shows the changes in oxygen concentration of sunflower oil, extra
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31 297 virgin olive oil and castor oil containing nanoemulsions enriched with silymarin as a function
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33 298 of storage time at 20 °C. Nanoemulsions not containing silymarin were used as controls.
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35 299 Oxygen concentration decreased faster in nanoemulsions containing sunflower oil, followed
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37 300 by nanoemulsions with extra virgin olive oil and castor oil. Such a reactivity rank is consistent
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39 301 with the unsaturation degree of the incorporated oils, sunflower oil having a higher value than
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41 302 extra virgin olive oil, which in turn has a greater number of carbon-carbon double bonds than
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43 303 castor oil. No significant differences in oxygen concentration were found between
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45 304 nanoemulsions with and without silymarin. This result suggests that silymarin did not act as
46
47 305 an oxygen scavenger, in agreement with the pionering data reported by Dehmlow et al.
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49 306 (1996), who found a negligible capacity of silybin to react with $O_2^{\cdot-}$ species. Figure 3 shows
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51 307 the changes in hydroperoxide concentration of nanoemulsion with or without silymarin during
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53 308 storage. It can be observed that the hydroperoxide concentration changed during storage with
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55 309 different kinetics depending on the nature of the carrier oil, the sunflower oil and castor oil
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3 310 containing emulsions being the most and the least susceptible to oxidation, respectively.
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5 311 However, also in this case, the evolution of hydroperoxides in the nanoemulsions enriched
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7 312 with silymarin did not significantly differ from those of the respective nanoemulsions without
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9 313 the bioactive molecule. These results further indicate that silymarin incorporation into
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11 314 nanoemulsions did not influence the pathway of the oxidative reactions occurring in oils.
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13 315 However, they contrast with data on the liver protective effect of silymarin, that is generally
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15 316 attributed to the antioxidant activity of the bioactive molecule (Shaker, Mahmoud, & Mnaa,
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17 317 2010). To have insights on the chain breaking activity of silymarin extract used in this study,
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19 318 the DPPH· assay was performed. Only a weak ability of silymarin to scavenge the DPPH·
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21 319 radical was measured (i.e. $0.14 \pm 0.06 \text{ A}^{-3}/\text{min}\cdot\text{g}_{\text{dm}}$). Such a value was much lower than the
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23 320 chain breaking activity of α -tocopherol and Trolox, that were 0.94 ± 0.24 and $8.47\pm 0.45 \text{ A}^{-3}/\text{min}\cdot\text{g}_{\text{dm}}$,
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25 321 respectively. These results are in agreement with the data of the literature (Gazak et
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27 322 al., 2004; Henning et al., 2014). In particular, Henning et al. (2014) reported that the *Silybum*
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29 323 *marianum* extracts had antioxidant properties lower than other dietary supplements, such as
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31 324 pomegranate, resveratrol and green tea. It is noteworthy that other authors found that silymarin
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33 325 had high *in vitro* radical scavenging activity (Koksal, Gulcin, Beyza, Sarikaya, & Bursal,
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35 326 2009).
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37 327 Despite the results described above, silybin concentration in nanoemulsions greatly decreased
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39 328 during storage (Figure 4). Such a decrease was greater in sunflower oil based nanoemulsions
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41 329 followed by extra virgin olive oil and castor oil containing samples. In particular, at 50 days
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43 330 storage, silybin losses were 60% and 55% in nanoemulsions containing sunflower oil and
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45 331 extra virgin olive oil, respectively, whereas 25% reduction of silybin content was found in the
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47 332 nanoemulsion with castor oil. These discrepancies in silybin degradation kinetics might be
48
49 333 brought back to differences in the bioactive reactivity in the media considered. It can be
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51 334 inferred that in sunflower oil and extra virgin olive oil containing nanoemulsions, silybin
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53 335 could be preferably located at the interface, near the emulsifier layer, where a higher polarity

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3 336 would favour its degradation. On the contrary, when castor oil was used as a carrier, silybin
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5 337 would be preferably located inside the oil droplets, being thus less prone to degradation.
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7 338 Possible silybin degradation by reactive species generated from oil oxidative reactions may
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9 339 not be ruled out. Gazak et al. (2004) actually described a number of oxidized derivatives of
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11 340 silybin. These compounds were obtained under strong oxidative conditions (e.g., H₂O₂ in
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13 341 NaHCO₃, iodine in glacial acetic acid, high temperature). In our experimental conditions, the
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15 342 greater the oil susceptibility to oxidation, and thus the formation of oxidation products, the
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17 343 greater the silybin degradation. It is a matter of fact that due to the scarcity of information
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19 344 available in the literature, further experiments are needed to understand the fate of silybin in
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21 345 food related environments.
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29 347 **Conclusions**

30 348 The present study is a first attempt to develop delivery systems for incorporating silymarin
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32 349 into functional foods. Results showed that silymarin can be successfully incorporated into
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34 350 physically stable nanoemulsions prepared with different carrier oils. The oil type used slightly
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36 351 affected the *in vitro* bioaccessibility of the main bioactive compound of silymarin, i.e. silybin.
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38 352 Although silymarin incorporation resulted not to affect oil oxidative kinetics, silybin
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40 353 concentration decreased during storage. Such a decrease was greater in extra virgin and
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42 354 sunflower oil than in castor oil. This instability rank is consistent with that relevant to the
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44 355 susceptibility to oxidation of the carrier oils considered. Although additional studies should be
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46 356 accomplished to fully elucidate the mechanism of silybin degradation in lipid carriers, the
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48 357 information acquired represents an important contribution for the design and fabrication of
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50 358 silymarin delivering nanoemulsions.
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56 360 **Acknowledgements**

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

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471 **Figure captions**

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

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

476 Figure 3. Changes in hydroperoxide concentration in sunflower oil, extra virgin olive oil and
477 castor oil based nanoemulsions with and without silymarin, as a function of storage time at 20
478 °C.

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

481 **Table captions**

482 Table 1. Silybin solubility in water, sunflower oil, extra virgin olive oil, castor oil and Tween
483 80.

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

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

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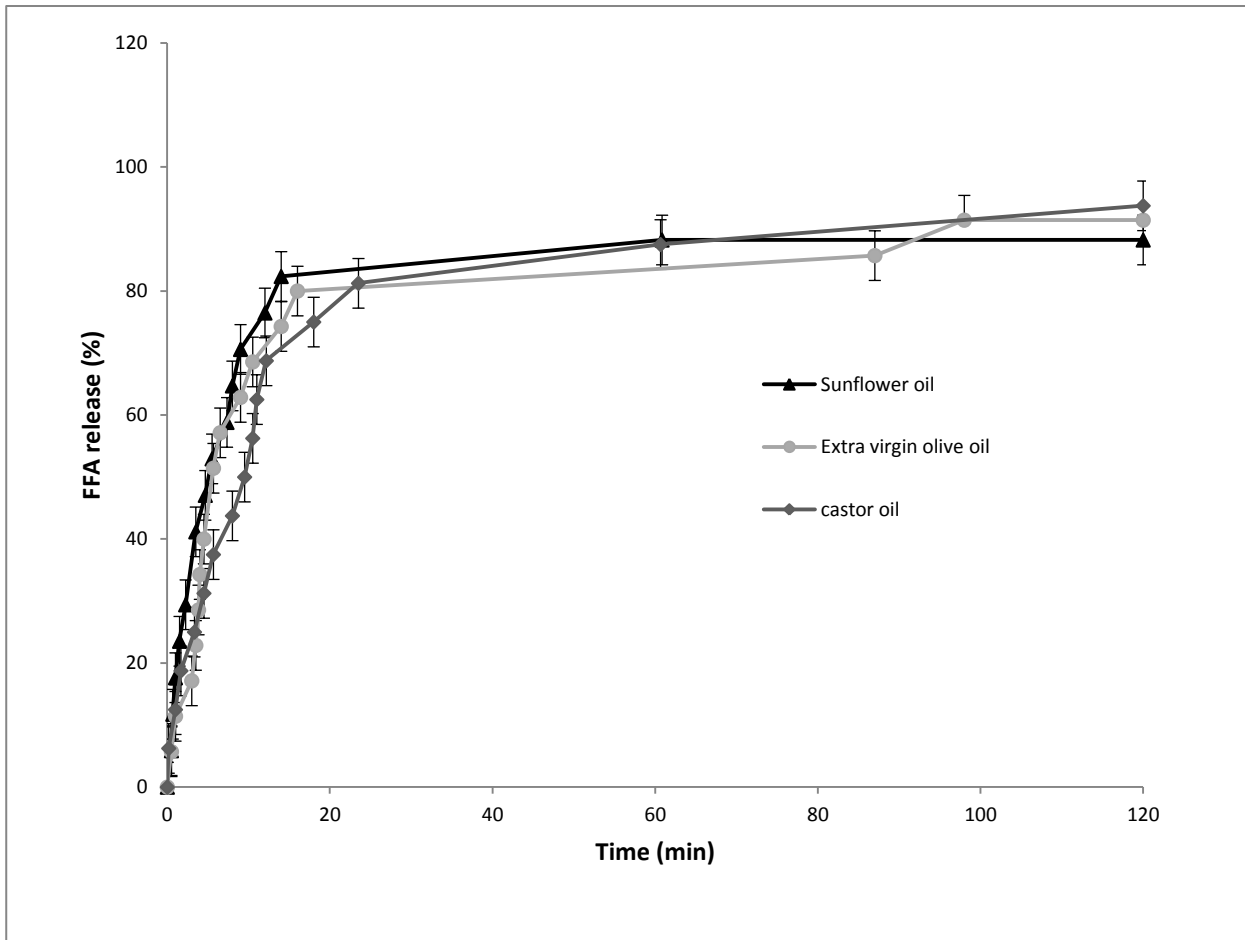


Figure 1. Free fatty acids (FFA) release from sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin.

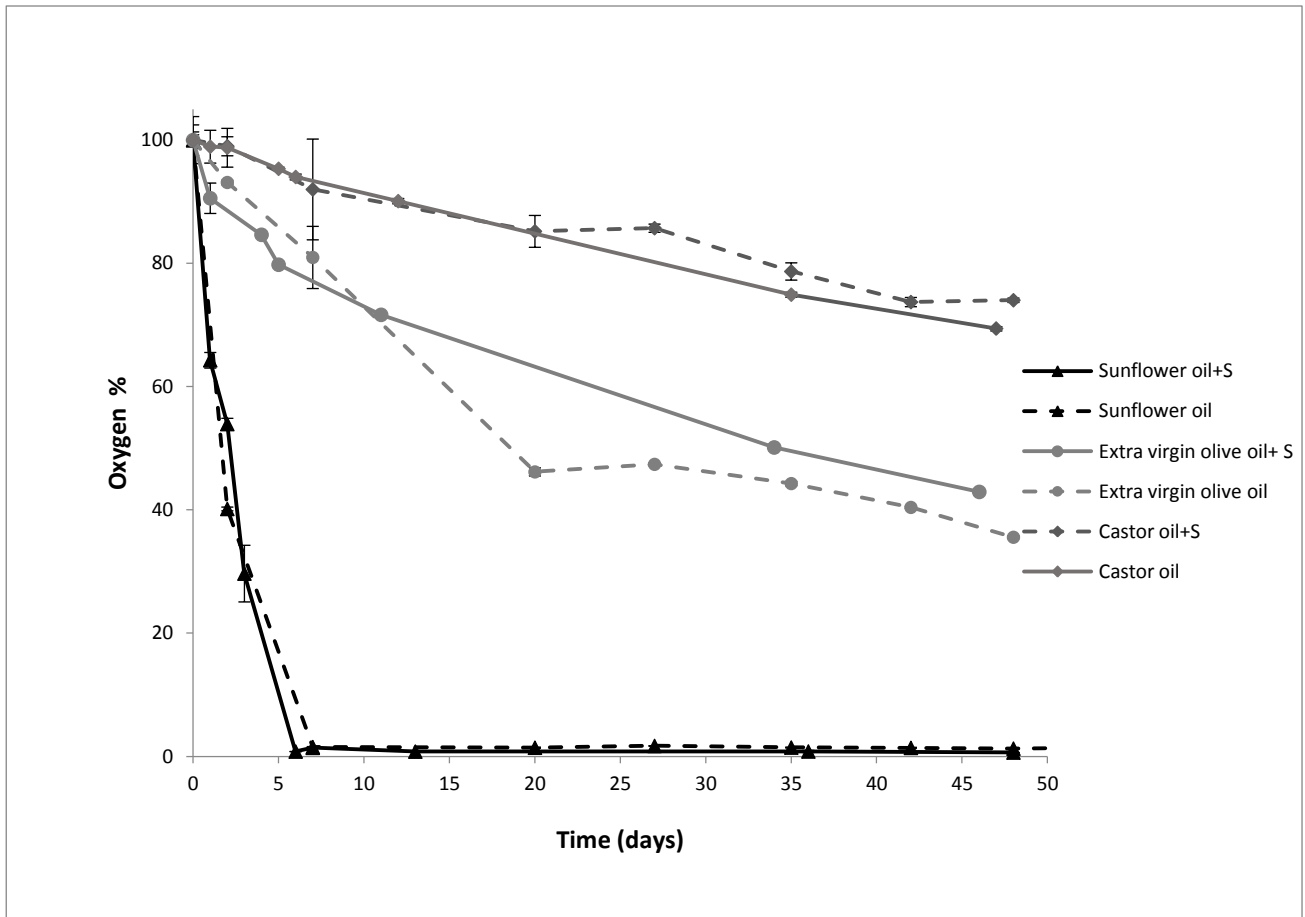


Figure 2. Percentage of residual oxygen in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions with and without silymarin, as a function of storage time at 20 °C.

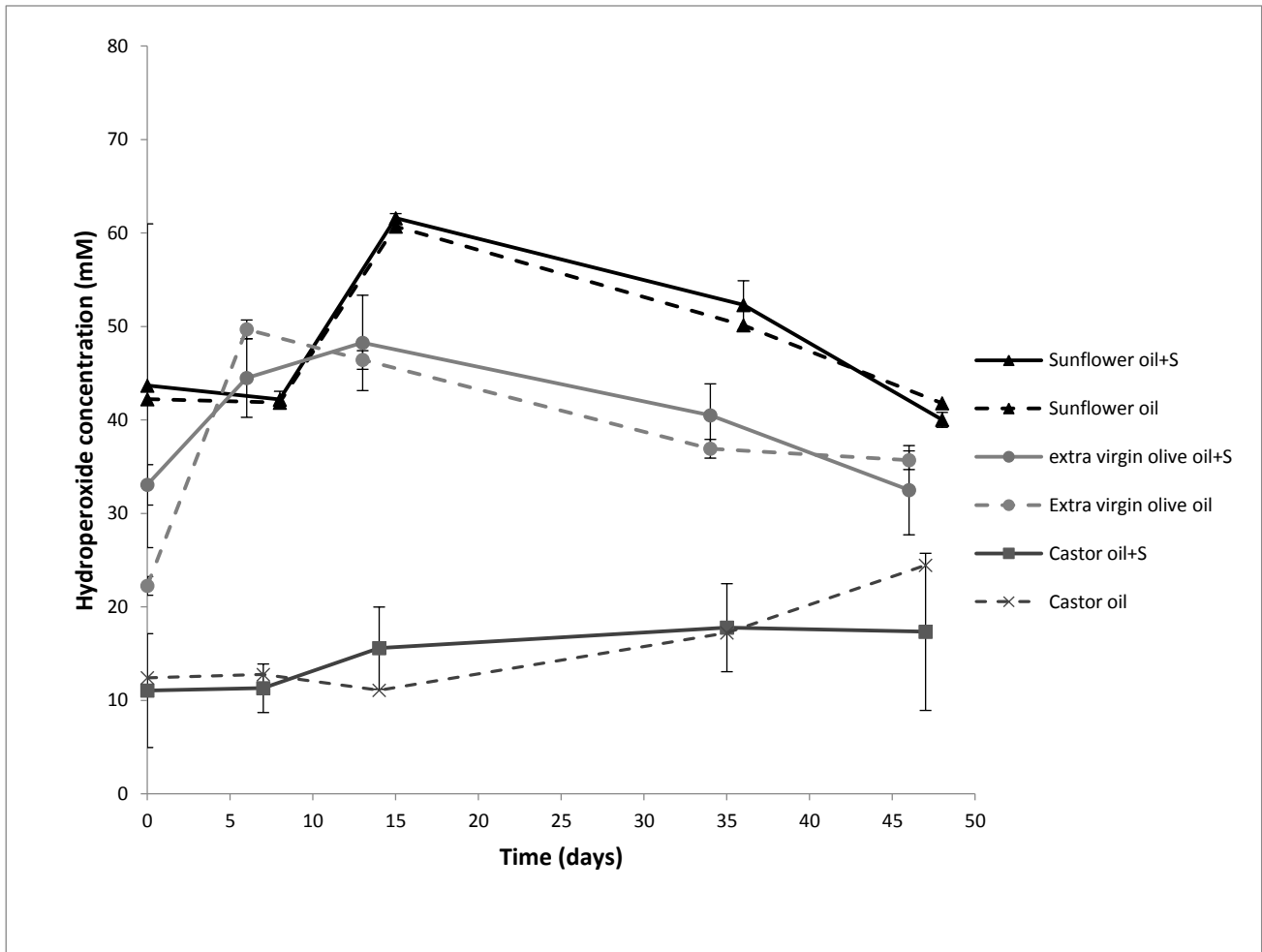


Figure 3. Changes in hydroperoxide concentration in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions with and without silymarin, as a function of storage time at 20 °C.

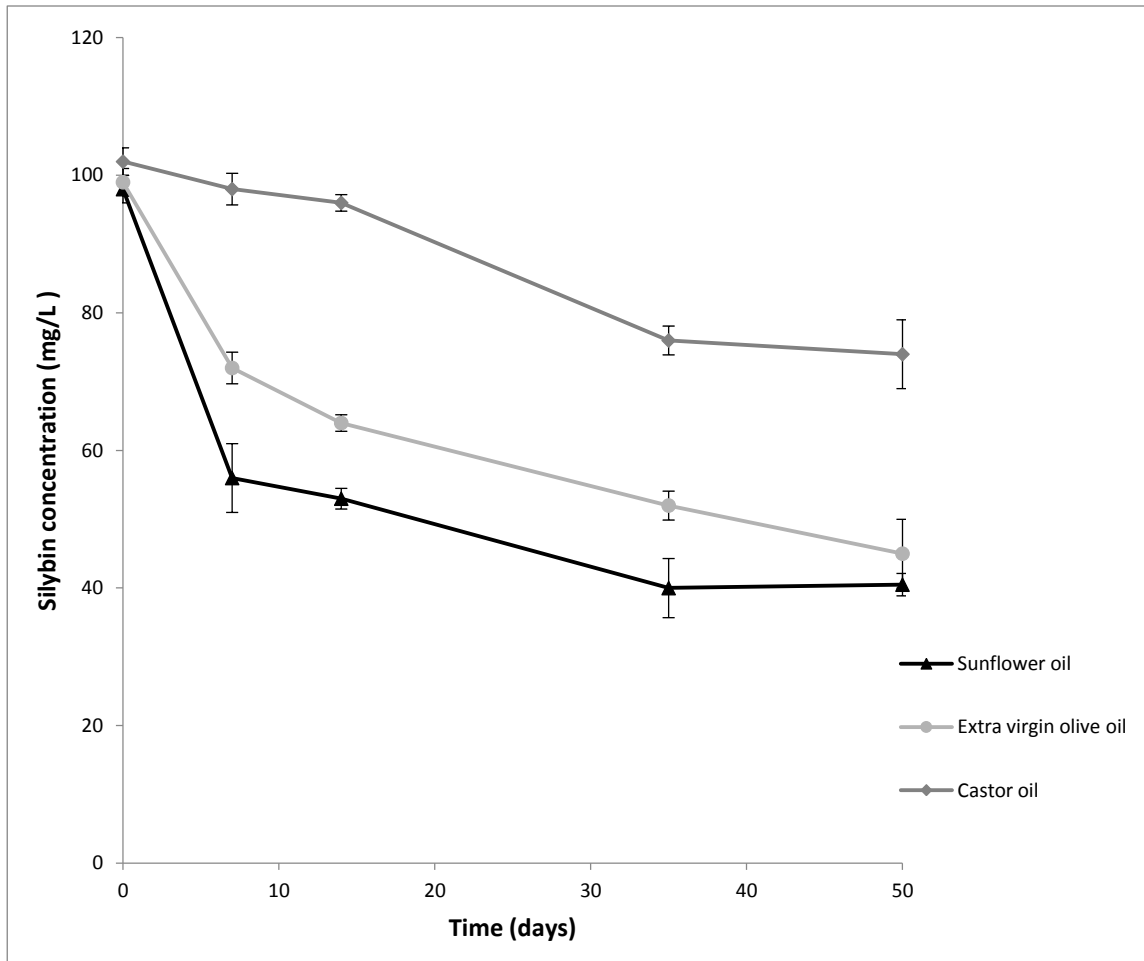


Figure 4. Silybin concentration in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin, as a function of storage time at 20 °C.

Table 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 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 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 ($p < 0.05$)