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1 **Resveratrol proniosomes as a convenient nanoingredient for functional food**

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26

27 **Abstract**

28 Proniosomes are free-flowing powders composed of water-soluble carriers blended with surfactants, which
29 form niosomes upon hydration. In this work, proniosomal formulations containing the natural antioxidant
30 resveratrol (RSV) were prepared and fully characterized. A pre-formulation study on RSV-loaded niosomes
31 was carried out to determine the most promising ratio between the two surfactants, Tween 20 and Span 60, in
32 terms of entrapment efficiency and antioxidant activity. The optimized formulae were subsequently adapted
33 to be prepared as proniosomes by the slurry method, including lactose or maltodextrin as carriers. The impact
34 of surfactants and carriers properties on size, entrapment efficiency and release kinetics of proniosomes were
35 evaluated. In vitro release of RSV in simulated gastric and intestinal media was determined, as well as the
36 vesicular stability. Moreover, the biocompatibility of the formulations was determined on intestinal cells in
37 vitro. Overall, the developed proniosomes provide promising nanoingredient for functional food, improving
38 resveratrol stability and bioavailability.

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43 **Keywords:** resveratrol, niosomes, proniosomes, carrier, Span 60, Tween 20

44

45 **List of compounds:**

46 Resveratrol (PubChem CID: 445154)

47 Cholesterol (PubChem CID: 5997)

48 Lactose (PubChem CID: 440995)

49 Maltodextrin (PubChem CID: 107526)

50 Span 60 (PubChem CID: 16218600)

51 Tween 20 (PubChem CID: 443314)

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56 **1. Introduction**

57 Resveratrol (trans-3,5,4'-trihydroxystilbene, RSV) is a polyphenol compound, which is produced by several
58 plant species to defend them from injuries. It is naturally present in high levels in some fruits, which are part
59 of the human diet, such as blueberries (*Vaccinium* spp.), blackberries (*Morus* spp.), and peanuts
60 (*Arachis hypogaea*). Red wine is the main source of RSV in the Mediterranean diet, since *Vitis vinifera*
61 synthesize this polyphenol in response to environmental stress such as UV radiation, drought, parasitic or
62 fungal attack (Sinico et al., 2016). In addition to the well-known potent antioxidant and anti-microbial activity,
63 RSV exerts anti-inflammatory, neuro-protective, anti-aging, and anti-cancer effects (de Sá Coutinho, Pacheco,
64 Frozza, & Bernardi, 2018; Salehi et al., 2018; Smoliga, Baur, & Hausenblas, 2011; Zupančič, Lavrič, & Kristl,
65 2015). Food supplementation with RSV has proved to reduce oxidized LDL, as well as blood sugar levels and
66 oxidative stress, thus decreasing the risk for cardiovascular and metabolic disease. More recently, the
67 preventive effect of RSV on body fat accumulation has been demonstrated (Wang, Zhu, & Du, 2015).
68 Consequently, RSV could be a useful drug as well as ingredient for functional foods (Breuss et al., 2019).

69 Despite of the great beneficial potentials for human health, the use of RSV in both nutritional and therapeutic
70 context is strongly affected by the combination of several limiting factors including poor water solubility, low
71 chemical stability and high metabolization (Gambini et al., 2015; Zupančič et al., 2015). When RSV has been
72 used in its free form as solid dietary supplements in capsules, it presented a very low bioavailability as a
73 consequence of poor hydrosolubility. On the other hand, when RSV has been administrated in vegetable
74 matrices or in liquid vehicles promoting an high oral absorption, results from pharmacokinetic studies indicated
75 a limited bioavailability, since the majority of the absorbed RSV was rapidly conjugated and transformed into
76 metabolites (Gandolfi et al., 2017). Moreover, RSV is a photosensitive molecule that exists in cis and trans
77 structural isomers. Many studies have shown that the main antioxidant action is exerted by trans-resveratrol,
78 which is converted into its cis isomer, a less active form, upon light exposure (Amri, Chaumeil, Sfar, &
79 Charrueau, 2012).

80 In this context, an increasing number of recent studies have focused on novel formulation approaches to
81 overcome RSV weak oral bioavailability, including nanosizing technologies, complexation with
82 macromolecules and encapsulation in nanocarriers (Lai, Schlich, Pireddu, Fadda, & Sinico, 2018; Pandita,
83 Kumar, Poonia, & Lather, 2014; Sessa et al., 2014; Singh et al., 2017; Sinico et al., 2016).

84 Among various nanocarriers, vesicular systems based on a bilayer membrane such as liposomes and niosomes
85 have been proposed as convenient oral delivery systems for RSV (Bonechi et al., 2012; Pando, Beltrán, Gerone,
86 Matos, & Pazos, 2015). Indeed, these colloidal nanocarriers are able not only to increase the drug solubility,
87 but also to protect the encapsulated molecules from degradation and to provide a controlled release, which can
88 be tuned changing the vesicle composition and/or decorating their surface. Particularly, RSV-loaded niosomes
89 have been suggested as a food additive in enriched yogurts, since they did not involve changes in the textural
90 properties of this food (Pando et al., 2015).

91 Niosomes are lipid nanoparticles with an aqueous core delimited by close concentric bilayers composed of
92 hydrated non-ionic surfactants, with or without incorporation of cholesterol or its derivatives (Marianecci et
93 al., 2014). Non-ionic surfactants are amphiphilic molecules that have two distinct regions in their chemical
94 structure, one of which is hydrophilic and the other is hydrophobic. Their aggregation structures, namely
95 niosomes, are analogous to phospholipid vesicles and, like liposomes, they are capable of encapsulating both
96 hydrophilic and lipophilic molecules. The loading can be achieved by entrapping hydrophilic compounds in
97 the aqueous core while the lipophilic substances are incorporated into the lipophilic domain of the bilayers
98 (Uchegbu & Florence, 1995).

99 Niosomes were originally developed as an alternative drug delivery system to liposomes, in order to overcome
100 the problems associated with high cost and low stability of phospholipids. Indeed, there is a great variety of
101 suitable surfactants, which show high chemical stability and affordable cost.

102 Despite the reported advantages over liposomes, also niosomal dispersions present problems related to physical
103 instability including vesicle fusion, aggregation, sedimentation and drug leakage during storage. Moreover,
104 the traditional method for producing vesicles, such as niosomes or liposomes, involves drying the lipid to a
105 thin film from an organic solvent solution, and then hydrating it with an aqueous medium. The resulting
106 multilamellar lipid nanoparticles can be further processed by sonication, extrusion, or other treatments to
107 optimize size, morphology and drug entrapment. All other methods reported in the literature involve
108 specialized apparatus and are time consuming.

109 Proniosomes, which are the dry powder of the vesicular systems, have been developed by simple preparation
110 procedures for circumvent all of the above reported complications, while preserving composition and features
111 of niosomes. Indeed, they are dry and free flowing powders of a surfactant-coated carrier, which can be

112 hydrated in aqueous food matrices (such as milk, yogurt or functional beverages) to form niosomes
113 immediately before use (Hu & Rhodes, 1999). Moreover, proniosomal powders offer several further
114 advantages over their liquid counterparts, such as ease of dosing and transportation and reduced risk for
115 microbiological contamination (Ravaghi et al., 2017; Shehata, Abdallah, & Ibrahim, 2015).

116 In the present work, novel RSV formulations based on proniosomes technology were developed with the dual
117 aim of increasing solubility while keeping a solid form. Moreover, as RSV is particularly instable in aqueous
118 environment, its inclusion in lipid nanoparticles would increase its shelf life.

119 For this purpose, at first, six niosomal formulations have been prepared using Tween 20 or Span 60 or different
120 combinations of two surfactants. Then, the two best formulations in term of RVS encapsulation efficiency and
121 antioxidant activity, have been selected for developing proniosomal powder by using lactose or maltodextrin
122 as carrier.

123

124 **2. Material and methods**

125 **2.1. Materials**

126 Polysorbate 20 (Tween 20), sorbitan monostearate (Span 60) and cholesterol were purchased from Sigma-
127 Aldrich (St. Louis, MO, USA). *Trans-resveratrol* (RSV) and spray dried lactose were purchased from Galeno
128 (Prato, Italy). Maltodextrin (Glucidex 6-D) was a kind gift from Roquette (France). All other chemicals used
129 in the experiments were of analytical grade. Ultrapure water was obtained from a Millipore system and was
130 used throughout the experiments.

131

132 **2.2. Preparation of niosomes**

133 Niosomes were prepared by the thin film hydration method, combined with probe sonication (complete
134 compositions are reported in Table 1-A). Briefly, weighed amounts of surfactant, cholesterol and RSV were
135 dissolved in 10 mL ethanol in a round bottom flask. Ethanol was evaporated under reduced pressure and a thin
136 film was formed. The thin film was hydrated with 10 mL ultrapure water at 60°C under continuous mixing
137 triggered by an overhead stirrer and palette (Eurostar 40, IKA, Germany). After hydration, probe sonication
138 was applied to niosome dispersions (Soniprep 150 plus, MSE Crowley, UK) to reduce the diameter of lipid

139 nanoparticles. The obtained niosome dispersions were stored at room temperature and characterized within 24
140 hours from the preparation.

141

142 **2.3. Preparation of proniosome powders**

143 Proniosomal powders were prepared by the slurry method. The exact composition of each proniosomal
144 formulation is reported in Table 1-B. Briefly, weighted amounts of Tween 20, cholesterol, Span 60 (if present)
145 and resveratrol were mixed in a round bottom flask. After complete dissolution of the components in 10 ml
146 ethanol, the carrier (lactose or maltodextrin) was added to form a slurry. The organic solvent was removed
147 under vacuum to yield a free flowing powder. Just before the following experiments, proniosome powders
148 were hydrated with 10 mL deionized water, the mixture was vortexed and probe sonicated to obtain niosomes.
149 Unloaded proniosome powders (blank), without RSV, were prepared using the same method.

150

151 **2.4. Size analysis, size distribution and zeta potential measurement**

152 Mean diameter, size distribution (polydispersity index, PDI) and zeta potential of proniosomes-derived
153 niosomes were determined by dynamic light scattering (DLS) and Electrophoretic Light Scattering (ELS),
154 respectively, using a Malvern Nano ZS (Worcestershire, Malvern Instruments Ltd., UK) at 25 °C. For size and
155 PDI determination, the niosomal dispersion was diluted (1:100) with water, while for Z potential analysis a
156 1:10 dilution was employed to prepare the sample.

157

158 **2.5. Entrapment efficiency**

159 The entrapment efficiency (EE%) of proniosome-derived niosomes was evaluated on the same day of the
160 hydration. The unencapsulated RSV was separated from niosomes by exhaustive dialysis. Briefly, 1 mL of
161 niosomal suspension was loaded into a dialysis bag (Spectra/Por membranes: 12–14 kDa MW cut off, 3 nm
162 pore size; Spectrum Laboratories Incorporation, USA) and dialysed against 1000 ml of distilled water for 2
163 hours, at 4°C, replacing the water after 1 hour. Purified and unpurified lipid nanoparticles were disrupted by
164 the addition of 9 parts of ethanol to 1 part of niosomes. The obtained mix was vortexed and centrifuged at 8000
165 RPM for 5 minutes on a Mikro 200 Centrifuge (Hettich, Tuttlingen, Germany). The supernatant (1 mL) was
166 recovered and transferred in HPLC vials for subsequent analysis.

167 **2.6. DPPH assay**

168 The antioxidant activity of niosomal RSV was assessed by evaluating its ability to scavenge the stable radical
169 2,2-diphenyl-1-picrylhydrazyl (DPPH). A methanolic solution of DPPH (0.04 mg/mL) was mixed with
170 appropriate amount of niosomes to yield final RSV concentrations in the range 0.005-0.05 mg/mL. A negative
171 control was prepared by mixing 980 μ L of DPPH solution with 20 μ L of deionized water. After 30 minutes of
172 incubation in the dark at room temperature, the absorbance was read at 517 nm by a Synergy 4 multiplate
173 reader (BioTek, Winooski, USA). The percent antioxidant activity was calculated according to the following
174 equation: % antioxidant activity = $100 * (ABS_{control} - ABS_{sample}) / ABS_{control}$.

175

176 **2.7. In vitro release in SGF and SIF**

177 Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to United States
178 Pharmacopoeia (USP) XXIV (Cohen et al., 1990). To determine the RSV release from the formulations, 4 mL
179 of niosomal suspension was loaded into a dialysis bag and incubated in a dissolution apparatus type 2 (paddle),
180 filled with 800 ml of SGF or SIF and thermostated at 37°C. The dialysis bags were stirred at 180 rpm
181 throughout the experiment, and aliquots were withdrawn after 15, 30, 60, 90 and 120 minutes (release in gastric
182 medium) or 1, 2, 4, 6 and 8 hours (release in intestinal medium). The samples were prepared for HPLC analysis
183 as previously reported.

184

185 **2.8. HPLC method**

186 RSV content was quantified at 283 nm using a chromatograph Alliance 2690 (Waters, Italy). The column was
187 a Sunfire C18 (3.5 μ m, 4.6 \times 150 mm, Waters). The mobile phase was a mixture of 20 % water and 80%
188 acetonitrile (v/v), delivered at a flow rate of 0.5 ml/min. A standard calibration curve (R^2 of 0.999) was built
189 up by using working, standard solutions (1.0–0.01 mg/ml). The retention time of RSV was 4 min, and the
190 minimum detectable amount was 2 ng/ μ l.

191

192 **2.9. FTIR spectroscopy (FT-IR)**

193 Fourier-transform infrared measurement were performed using reflectance FT-IR spectroscopy and attenuated
194 total reflectance (ATR-FT-IR) spectroscopy. FT-IR analyses were collected using Perkin Elmer FTIR

195 Spectrometer Spectrum One (PerkinElmer Waltham, MA, USA) in a spectral region between 4000 and 600 or
196 450 cm^{-1} for solid or liquid compounds respectively and analysed by transmittance technique with 8 scansions
197 and 4 cm^{-1} resolutions. Solid samples were mixed in a mortar with KBr (1:100) and pressed in a hydraulic
198 press (14 tons) to small tablets, while for liquid samples one drop was placed between two widows of sodium
199 chloride. RSV, Lactose, Maltodextrin, Span 60, cholesterol, proniosomal powder and unloaded formulation of
200 the optimized proniosome powder were also analyzed in ATR-FT-IR using a Perkin Elmer Universal ATR
201 Sampling Accessory with a resolution of 4 cm^{-1} and 32 scans in the range 4000–650 cm^{-1} .

202

203 **2.10. X-ray powder diffractometry (XRPD)**

204 For further characterisation of the crystalline state XRPD diffractograms of RSV, Lactose, Maltodextrin, Span
205 60, cholesterol, and unloaded formulation of the optimized proniosome powder were recorded using a Rigaku
206 Miniflex diffractometer (Rigaku Corporation, Tokyo, Japan) with a Ni-filtered CuK α radiation detector ($\lambda =$
207 1.5405 Å) operating at a voltage of 30 kV and a current of 15 mA in the 2θ range from 3° to 60° with a scan
208 angular speed of 2°/min and a scan step time of 2.00s. The results were then obtained as peak height (intensity)
209 versus 2θ .

210

211 **2.11. Biocompatibility**

212 The biocompatibility of the formulations was tested on human epithelial intestinal cells (Caco-2) kindly gifted
213 by Prof. Caitriona O'Driscoll (School of Pharmacy, UCC, Ireland). The cells were cultured in Dulbecco
214 Modified Eagle Medium (DMEM, high glucose, Sigma Aldrich, Milan, Italy) supplemented with 50 units/mL
215 penicillin and 50 mg/mL streptomycin and 10% fetal bovine serum (FBS) and maintained in a humidified, 5%
216 CO $_2$ atmosphere tissue culture incubator at 37 °C. Caco-2 were seeded in 96-well plates 24 h prior to the
217 treatment with the formulations (10 4 cells/well). Proniosomes-derived niosomes were mixed with complete
218 medium to achieve different RSV concentrations (in the range 37-365 μM) and cells were exposed to the
219 formulations for 4 h. After this time the medium was discarded and replaced with 100 μL MTT in PBS (0.5
220 mg/ml). Cells were incubated for 4 h at 37°C after which the MTT was removed and 100 μL DMSO was added
221 to each well. Absorbance was measured at 517 nm using a Synergy 4 plate reader (BioTek, Winooski, USA).
222 The results are expressed as the percentage cell viability relative to untreated cells.

223

224 2.12. Statistical analysis

225 Data are expressed as mean \pm standard deviation of at least 3 independent determinations if not otherwise
226 stated. Data analysis was carried out using the software package R, version 2.10.1. Student's t-test was used to
227 substantiate statistical differences between two samples at $p < 0.05$. Multiple comparisons of means (Tukey
228 test) was used to carry out comparisons between groups at $p < 0.05$ as a minimal level of significance.

229

230 3. Results and discussion

231 At first, a preliminary formulation study of RSV niosomes was carried out to investigate the effect of surfactant
232 properties on RSV encapsulation and antioxidant activity. To this aim, Tween 20 and Span 60, a hydrophilic
233 and a hydrophobic surfactant, respectively, were used alone or blended in different ratios to prepare niosomes
234 (Table 1-A).

235

236 **Table 1.** Composition of niosome dispersions (mg for 10 ml of formulation) (A) and proniosomal powders (mg) (B)

237

A

Niosome Formulations	HL B	Components					
		Resveratrol	Tween 20	Span 60	Cholesterol	Lactose	Maltodextrin
N-HLB-16.7	16.7	5	184	-	58	-	-
N-HLB-13.5	13.5	5	135	17.2	58	-	-
N-HLB-11.0	11.0	5	96.6	30.6	58	-	-
N-HLB-8.5	8.5	5	53.5	44	58	-	-
N-HLB-4.5	4.5	5	-	64.5	58	-	-

B

Proniosomal Formulations							
PL16.7	16.7	5	184	-	58	736	-
PM16.7	16.7	5	184	-	58	-	736
PL13.5	13.5	5	135	17.2	58	736	-
PM13.5	13.5	5	135	17.2	58	-	736

238

239

240 Tween 20 and Span 60 have a hydrophilic-lipophilic balance (HLB) of 16.7 and 4.5, respectively, thus
241 providing the ideal starting material to obtain a range of intermediate HLB values through mixing in

242 appropriate ratios. In addition to surfactants, cholesterol represents an essential component to induce the
243 formation and stabilization of bilayers (Uchegbu & Florence, 1995). Indeed, when cholesterol is not included
244 in the formulation, a micelle or vesicle dispersion is obtained depending on the type and ratio between
245 surfactant mixture. Surfactants with a single alkyl tail normally form micelles in diluted aqueous solutions and
246 need additives such as cholesterol to achieve suitable molecular geometry and hydrophobicity for bilayer
247 vesicle formation. However, non-ionic surfactant with a single alkyl tail such as Span 60 can form vesicular
248 structure since it has relatively large hydrophobic moiety (HLB=4.7) with low water solubility. Some
249 surfactants cannot form vesicle without cholesterol, due to the higher HLB (hydrophilicity), such as Tween 20
250 that has a large hydrophilic head group and a HLB of 16.7.

251 In this study, the total surfactant concentration was kept constant at the value of 15 mM, fixing the
252 cholesterol/surfactant molar ratio at 1:1. The preparation of niosomes was carried out exploiting the thin film
253 hydration method followed by probe sonication, which yielded low dispersity (PDI < 0.2) populations of lipid
254 nanoparticles having mean diameters in the range 110-190 nm. The DLS analysis of niosomes did not show a
255 statistically significant correlation between size of nanoparticles and HLB (data not shown), thus these data
256 did not contribute to the choice of the formulations to be prepared as proniosomes. The entrapment efficiency
257 (EE%) of RSV in the formulated niosomes was assayed by HPLC, after removal of the un-encapsulated drug
258 by exhaustive dialysis. Also in this case it was not possible to detect any significant difference in term of EE%
259 between the analyzed formulations. However, niosomes prepared with the surfactant blends at HLB of 4.5, 8.5
260 and 11 showed a high variability in the RSV EE%, while niosomes having more hydrophilic HLB values were
261 characterized by highly reproducible data (Figure 1, A). Lower reproducibility of data were detected as the
262 amount of Span 60 in the formulation increased, thus suggesting that this highly hydrophobic surfactant may
263 be not suitable for the incorporation of RSV in stable niosomes in the applied conditions. Indeed, previous
264 published formulation studies on RSV-containing Span 60 niosomes revealed a wide range of entrapment
265 efficiencies (from 25 to 72%) when minor changes to the process parameters and/or components are applied
266 (Pando et al., 2015). Conversely, in addition to the high and reproducible EE%, RSV-niosomes having HLB
267 of 13.5 and 16.7 also displayed the highest antioxidant activity in vitro, assessed by the DPPH assay (Figure
268 1, B). Indeed, RSV loaded on HLB 4.5 and 8.5 niosomes showed a remarkably lower antioxidant activity as
269 compared to more hydrophilic niosomes and to a methanolic solution, used as control. Once again, Span 60 is

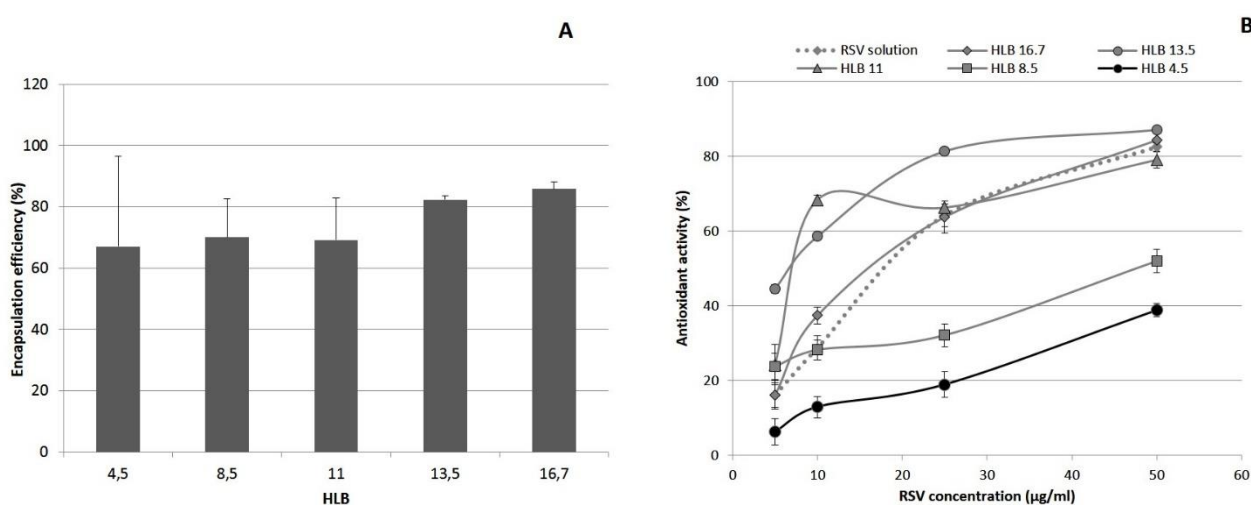
270 probably responsible of such results, since its highly hydrophobic character may induce a strong interaction
271 with lipophilic RSV, which is prevented from reaching and scavenging the DPPH radical. On the other hand,
272 niosomes prepared with surfactant blends with HLB higher than 11 resulted in an antioxidant activity of RSV
273 equivalent or superior to the control.

274

275

276

277



278

279 **Figure 1.** Characterization of niosomes. Entrapment efficiency (A) and antioxidant activity of RSV-loaded niosomes
280 prepared with surfactant blends having different HLB (B). Results are expressed as means of 4 independent measurements
281 \pm standard deviations

282

283 In light of these results, niosomes with an HLB of 13.5 and 16.7 were chosen to be formulated as proniosomes.

284 The niosomes formulae were adapted to the preparation of proniosomes by including a quantity of inert carrier
285 sufficient to obtain a free flowing powder (Table 1-B).

286 For the production of proniosomes, the slurry method was employed using environmentally friendly ethanol
287 as the only non-aqueous solvent. Moreover, the slurry method allows the preparation of proniosomal powders
288 through a faster and cheaper procedure, as compared to the freeze-drying method. Spray-dried lactose and
289 maltodextrin were selected as carriers, among several water-soluble excipients, for their convenient flow and
290 compressibility properties. In particular, maltodextrin due to high surface area and the porous structure was
291 chosen as carrier to prepare proniosomes with high surfactant to carrier mass ratios (Sahoo, Biswas, Guha, &
292 Kuotsu, 2014). A detailed physico-chemical analysis of the proniosomal powders was carried out to describe

293 the main features of the formulations, such as interactions between the components, chemical identity of the
294 active ingredient (including its isomeric form), and crystalline or amorphous state.

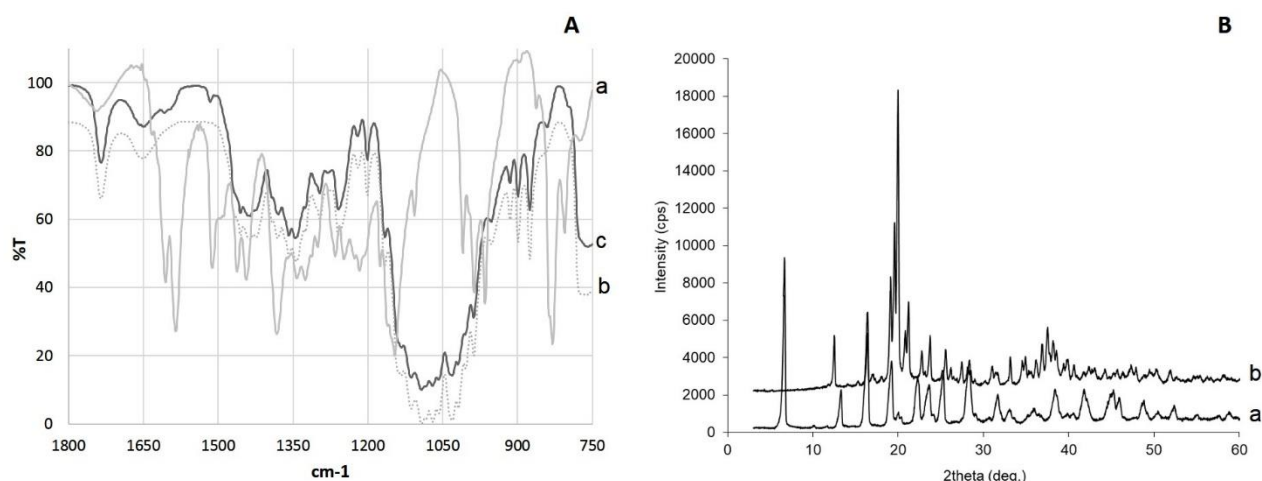
295 The ATR-FTIR method makes it possible to monitor the slightest changes in the state of the sample at
296 molecular level, since there is no sample preparation, unlike in the case of the KBr pellet method, when
297 grinding and pressing mostly unifies the samples with weak interactions.

298 In the spectra the main absorption band of resveratrol are at 3270 cm^{-1} (O-H stretching), at 3019 cm^{-1} (H-Csp3
299 stretching), at $1606, 1587, 1384\text{ cm}^{-1}$, the three characteristic strong bands corresponding to C-C aromatic
300 double bond stretching, C-C olefinic stretching and C-O stretching, respectively. Moreover, the band at 965 cm^{-1}
301 demonstrated the *trans* form of RSV. The characteristic peak corresponding to either excipients or drug
302 did not show any significant shift in the spectrum suggesting no interactions between them.

303 The XRPD pattern of RSV evidenced its crystalline pattern with intense reflection peaks at $6.52, 13.16, 16.26,$
304 $19.08, 20.84, 22.22, 28.20, 31.50\ 2\theta$. X-ray diffraction analyses of PL formulation showed a crystalline pattern
305 with reflection peaks attributable to RSV, even if some of them with lower intensities (Figure 2B). Otherwise,
306 PM has an amorphous profile due to maltodextrins.

307

308



309

310 **Figure 2.** ATR-FTIR spectra in the 1800-750 cm⁻¹ range: RSV (a), RSV loaded PL16.7 proniosome (b), empty PL16.7
311 proniosome (c) (A). Diffraction patterns of RSV (a) and RSV loaded PL16.7 proniosome (b) (B)

312

313

314 The physico-chemical and biopharmaceutical properties of niosomes generated by the hydration of
315 proniosomal powders were subsequently evaluated.

316 As reported in table 2, the hydration and sonication of proniosomes resulted in the formation of lipid
317 nanoparticles with a mean diameter in the range 180-300 nm and highly negative surface charge. Of note, the
318 different Span60/Tween20 ratio had an impact on the physico-chemical properties of proniosome-derived
319 niosomes. Indeed, formulations having HLB of 13.5 were significantly less polydisperse than PL16.7 and
320 PM16.7, which also showed higher batch to batch variability in terms of mean diameter, which resulted in
321 higher standard deviation. However, these differences in size and PDI did not have consequences on the
322 entrapment efficiency of RSV within the formed nanoparticles. Indeed, no statistically significant differences
323 on the amount of incorporated RSV were determined between the hydrated proniosomes, thus suggesting that
324 the polyphenol could be efficiently loaded on the nanoparticles regardless of their size. On the other hand, the
325 carrier used did not influence the mean size and polydispersity of niosomes. This can be especially noted by
326 comparing the physicochemical attributes of PL13.5 and PM13.5, where the latter only shows a slightly larger
327 mean diameter, which however becomes not significant if the PDI is considered. Apparently, the different
328 molecular weight and chemical structure of lactose and maltodextrin do not have an influence on lipid
329 nanoparticles properties. Indeed, it has been reported that the dissolution behavior of the carrier play a key role
330 in the efficient hydration of the surfactant and the consequent formation of niosomes from proniosomal
331 powders, and both lactose and maltodextrin show excellent dissolution properties (Ravaghi et al., 2017).

332 **Table 2.** Mean diameter, PDI, Z potential and entrapment efficiency of the proniosome-derived niosomes. Results are
333 expressed as means of 4 independent measurements \pm standard deviations

334

Formulations	Mean diameter (nm)	PDI	Z potential	Entrapment efficiency (EE%)
PL16.7	282 \pm 142	0.30 \pm 0.01	- 38.3 \pm 6.7	84 \pm 3
PM16.7	194 \pm 55	0.28 \pm 0.11	- 37.3 \pm 4.7	76 \pm 5
PL13.5	180 \pm 2	0.14 \pm 0.11	- 47.5 \pm 2.2	75 \pm 4
PM13.5	194 \pm 3	0.20 \pm 0.11	- 43.7 \pm 5.0	81 \pm 1

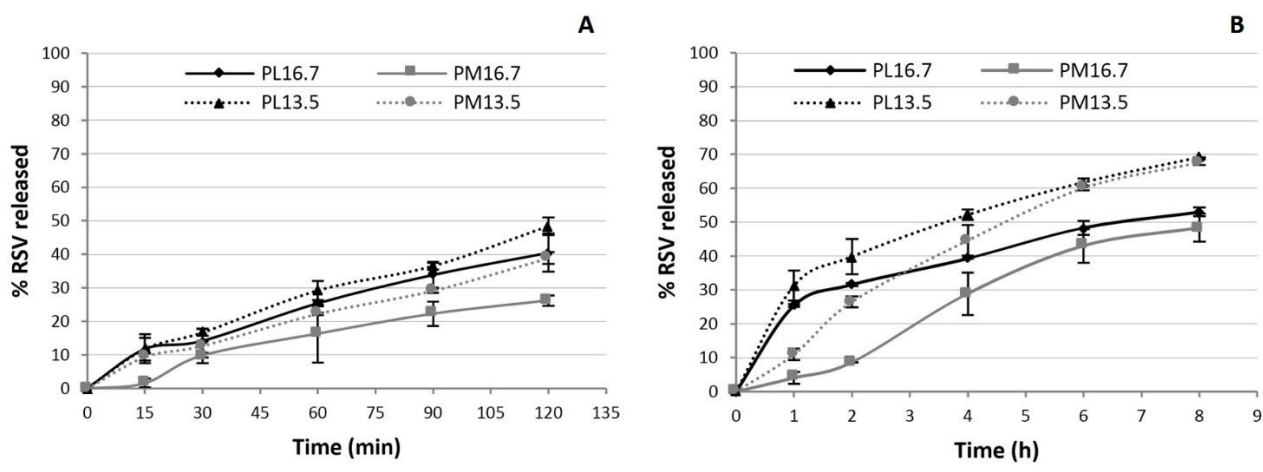
335

336 The RSV release from proniosome-derived niosomes was investigated in simulated gastrointestinal
337 environments. To this aim, the formulations were loaded into a dialysis tubing and incubated at 37°C in a type
338 2 dissolution apparatus, using either simulated gastric or intestinal fluids as release media. Since no regulatory

339 guideline exists regarding the release studies from nanoparticles, the dialysis bag method was used in
340 combination to the type 2 apparatus to adapt the official dissolution method reported by the US and EU
341 pharmacopoeias to the developed nanoformulations (Souza, 2014). Results reported in Figure 3A show a
342 slightly slower release rate of RSV from PM16.7 in gastric environment, as compared to all the other
343 formulations, which do not differ significantly from one another. Indeed, after two hours of incubation in
344 simulated gastric fluid PM16.7-derived niosomes released 26% of the initial RSV dose, while all the other
345 formulations triggered the release of 39%, 41% and 48% (PM13.5, PL13.5 and PL16.7, respectively) of the
346 loaded dose. A stronger interaction between the hydrophilic surfactant tween 20 and the bulky maltodextrin
347 may be responsible for this reduced release rate, which was also partially observed in simulated intestinal fluid
348 (Figure 3B). The behavior of lactose-based (PL) formulations in the simulated intestinal environment is
349 characterized by an initial burst release of 25-31% of the dose after 1 hour of incubation, followed by a decrease
350 in the release rate for the rest of the experiment. Interestingly, PL16.7 showed a markedly slower release rate
351 as compared to PL13.5, which ultimately resulted in 53% and 69% of RSV released at the end of the incubation
352 time, respectively. The faster release from PL13.5 may be attributed to the blend of surfactants, which is the
353 only difference between the cited formulations. Indeed, also the other formulation with HLB of 13.5 (PM13.5)
354 reached the same amount of total RSV released (68%) while the lipid nanoparticles with higher HLB (PM16.7)
355 liberated only 48% of RSV dose. It is interesting to note that a lower HLB value seem to trigger a faster release
356 in simulated intestinal fluid but not in the gastric one. Although none of the employed surfactants are charged
357 molecules, their behavior seems to be influenced by pH of the release medium, where more alkaline
358 environment probably loosen the interactions of Span 60 with the other nanoparticles components, ultimately
359 leading to a faster release compared to the HLB16.7 formulations.

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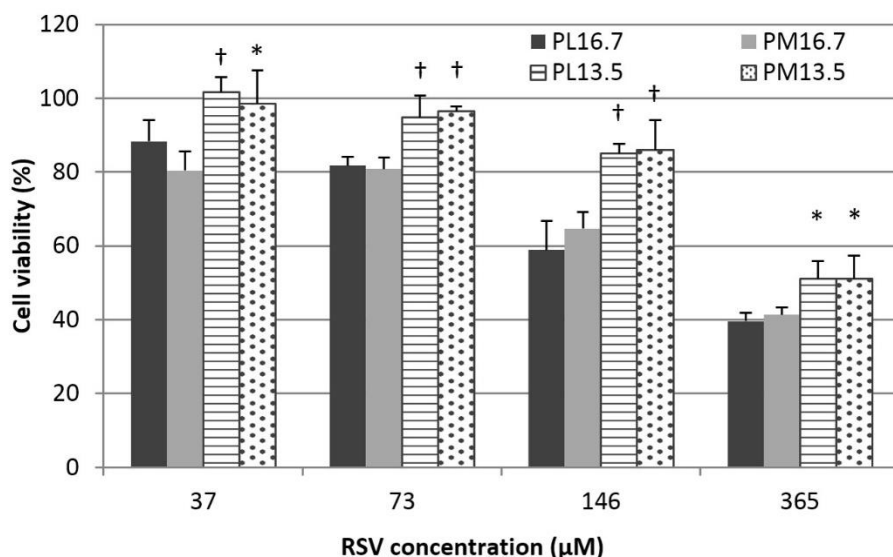
362

363 **Figure 3.** In vitro RSV release in simulated gastric fluid (SGF) (A), or simulated intestinal fluid (SIF) (B) from
 364 proniosome-derived niosomes. Values are expressed as % of the dose used for the experiments
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367 All the components of the proniosomal formulations developed in this work are GRAS listed and accepted for
 368 oral use in pharmaceutical products by FDA and EMA. However, to evaluate the safety of the
 369 nanoformulations, a biocompatibility assay was carried out on intestinal cells (Caco2). The histogram reported
 370 in Figure 4 represent the viability of cells after 4 hours of incubation with the proniosomal formulations,
 371 included in the medium in different amounts expressed as RSV concentration. Interestingly, proniosomes
 372 prepared with a HLB of 13.5 showed to be significantly less toxic than their HLB16.7 counterpart, at every
 373 concentration used. Indeed, given 80% viability as the lowest acceptable limit, PM13.5 and PL13.5 showed to
 374 be safe up to a RSV concentration of 146 μ M, while the highest non-toxic dose reported for PL16.7 and
 375 PM16.7 was 73 μ M. Of note, this experiment was carried out testing medium to high doses of formulations,
 376 as compared to other in vitro bioavailability testing of RSV containing nanoparticles (Caddeo et al.,
 377 2017)(Rocha et al., 2017). Thus, keeping in mind the higher risk for epithelial toxicity, we speculate that also
 378 the formulations having HLB of 16.7 could be employed safely at lower-but-therapeutically-relevant doses of
 379 RSV (Ramírez-Garza et al., 2018).

380



381

382 **Figure 4.** Biocompatibility of proniosome-derived niosomes on epithelial intestinal cells (Caco-2), compared to untreated
 383 cells (100%). Symbols represent level of significance between PL16.7 and PL13.5 or PM16.7 and PM13.5 formulations
 384 (* P<0.03; † P<0.01)

385

386

387 CONCLUSION

388 In this study RSV proniosomal powders containing Tween 20 and/or Span 60 as surfactants, and spray-dried
 389 lactose or maltodextrin as carriers, were successfully prepared by using the slurry method and characterised.
 390 The hydration and sonication of proniosome powders resulted in the formation of homogenous lipid
 391 nanoparticles dispersion with high entrapment efficiency values. *In vitro* studies indicated that the two
 392 surfactants ratio, and therefore the surfactant blend HLB, affected the release behaviour of RSV in both
 393 simulated gastric and intestinal fluid. Finally, biocompatibility assay carried out on intestinal cells clearly
 394 demonstrated that all the tested formulations could be employed safely at the doses commonly administrated
 395 by oral route. In conclusion, this study highlights the great potential of proniosomes as an effective strategy to
 396 improve bioavailability of poor water-soluble molecules, such as RSV, in enriched foods.

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