

# *Whey–pectin microcapsules improve the stability of grape marc phenolics during digestion*

Article

Accepted Version

De La Cruz Molina, A. V., Gonçalves, C., Neto, M. D., Pastrana, L. ORCID: <https://orcid.org/0000-0002-0852-826X>, Jauregi, P. ORCID: <https://orcid.org/0000-0003-4438-191X> and Amado, I. R. (2023) Whey–pectin microcapsules improve the stability of grape marc phenolics during digestion. *Journal of Food Science*, 88 (12). pp. 4892-4906. ISSN 1750-3841 doi: 10.1111/1750-3841.16806 Available at <https://centaur.reading.ac.uk/113940/>

It is advisable to refer to the publisher's version if you intend to cite from the work. See [Guidance on citing](#).

To link to this article DOI: <http://dx.doi.org/10.1111/1750-3841.16806>

Publisher: Wiley

All outputs in CentAUR are protected by Intellectual Property Rights law, including copyright law. Copyright and IPR is retained by the creators or other copyright holders. Terms and conditions for use of this material are defined in the [End User Agreement](#).

[www.reading.ac.uk/centaur](http://www.reading.ac.uk/centaur)

**CentAUR**

Central Archive at the University of Reading

Reading's research outputs online

1 **Whey-pectin microcapsules improve the stability of grape marc**  
2 **phenolics during digestion**

3 Aimara V. De La Cruz-Molina<sup>1</sup>, Catarina Gonçalves<sup>2</sup>, Mafalda D. Neto<sup>2</sup>, Lorenzo  
4 Pastrana<sup>2</sup>, Paula Jauregi <sup>1,3,4</sup>, Isabel R Amado<sup>2\*</sup>

5 <sup>1</sup>Department of Food and Nutritional Sciences, University of Reading, Whiteknights, Reading, UK,  
6 RG6 6A

7 <sup>2</sup>INL- International Iberian Nanotechnology Laboratory, Av. Mestre José Veiga s/n, 4715-330  
8 Braga, Portugal

9 <sup>3</sup> Current address: AZTI, Food Research, Basque Research and Technology Alliance (BRTA),  
10 Parque Tecnológico de Bizkaia, Astondo Bidea, Edificio 609, Derio, Bizkaia 48160, Spain

11 <sup>4</sup> Current address: Ikerbasque, Basque Foundation for Science, Bilbao 48013, Spain

12 **\* Correspondence:** Isabel R. Amado: isabel.rodriguez@inl.int

13 **Abstract**

14 Grape marc is an agri-food residue from the wine industry valuable for its high content  
15 of phenolic compounds. This study aimed to develop an encapsulation system for grape  
16 marc extract (GME) using food-grade biopolymers resistant to gastric conditions for its  
17 potential use as a nutraceutical. For this purpose, a hydroalcoholic GME was prepared  
18 with total phenolics content of  $219.62 \pm 11.50$  mg gallic acid equivalents (GAE)/ g dry  
19 extract and  $1389.71 \pm 97.33$   $\mu$ mol Trolox equivalents (TE)/ g dry extract antioxidant  
20 capacity, assessed through ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic  
21 acid) assay. Moreover, the extract effectively neutralised reactive oxygen species (ROS)  
22 in Caco-2 cells, demonstrating an intracellular antioxidant capacity comparable to Trolox.  
23 The GME was encapsulated using whey protein isolate and pectin through nano-spray  
24 drying (73% yield), resulting in spherical microparticles with an average size of  $1 \pm 0.5$

25  $\mu\text{m}$  and a polydispersity of 0.717. The encapsulation system protected the microcapsules  
26 from simulated gastrointestinal digestion, where at the end of the intestinal phase, 82%  
27 of the initial phenolics were bioaccessible compared to 54% in the free GME. Besides, the  
28 encapsulated GME displayed a higher antioxidant activity by the ferric reducing  
29 antioxidant power (FRAP) assay than the free extract after gastrointestinal digestion  
30 (GID). These results show the potential of this encapsulation system for applying GME  
31 as a nutraceutical with a high antioxidant capacity and protective effect against cellular  
32 oxidation.

33 **Keywords: grape marc phenolics, biopolymer, nano-spray drying, *in vitro* digestion,**  
34 **encapsulation.**

35

36

37        **1. Introduction**

38        Grape marc is a food by-product composed of the skins, seeds and stems recovered at the  
39        end of the winemaking process. This by-product has attracted significant attention due to  
40        its high phenolic content (Lavelli et al., 2016; Peixoto et al., 2018). Phenolics are a family  
41        of molecules with antioxidant properties, including phenolic acids and polyphenols such  
42        as flavonols and flavan-3-ols (Cao et al., 2021; Tsao, 2010). It has been found that they  
43        can play a significant role in the management and prevention of several diseases,  
44        especially cardiovascular and type 2 diabetes (Dias et al., 2022; Fraga et al., 2019)

45        Phenolics are extensively researched for their properties but are challenging molecules.  
46        They are susceptible to temperature changes, moisture, oxygen, and high/low pH values.  
47        In addition, once ingested, they present low stability and bioavailability in the human  
48        body due to their low solubility and low membrane permeability (Ludwig et al., 2015;  
49        Scalbert & Williamson, 2000; Stalmach et al., 2009; Teng & Chen, 2019). For these  
50        reasons, phenolics are unlikely to be used in their pure form and encapsulation is foreseen  
51        as an alternative to improve their stability and preserve their properties within food  
52        products and bioavailability after consumption (Brezoiu et al., 2019; Sessa et al., 2013;  
53        Spigno et al., 2013). The encapsulation process involves using materials to embed,  
54        complex, or create a protective wall around bioactives, and by carefully selecting these  
55        materials, a targeted release of the bioactives can be achieved.

56        Polysaccharides and proteins are vastly used biopolymers for encapsulation, and  
57        interestingly, many of these materials can be obtained from by-products, like whey  
58        protein isolate (WPI). WPI is a by-product of the cheese-making process, which contains  
59        proteins with high nutritional quality (de Wit, 1998; Jauregi & Welderufael, 2010; Yalçin,  
60        2006). Furthermore, WPI forms complexes with polyphenols, stabilising them by

61 improving their solubilisation and protecting their antioxidant activity from heat-induced  
62 loss (Guo & Jauregi, 2018). On the other hand, polysaccharides like pectin are found in  
63 the peel of citrus, apple, and other fruits. Pectin, as insoluble fibre, is poorly absorbed in  
64 the upper gastrointestinal tract (GIT), but pectinolytic enzymes produced by colonic  
65 microflora degrade the polysaccharide (Dongowski & Anger, 1996; Rehman et al., 2019).  
66 Pectin biodegradability is an interesting property to take advantage of as an effective  
67 carrier for the targeted release of bioactive compounds absorbed in the colon. Polyphenols  
68 can be absorbed in different parts of the GIT, and those reaching the colon are known to  
69 be metabolised by the microbiota into additional low molecular weight phenolic acids  
70 (Scalbert et al., 2002). Besides, pectin has other interesting technological properties like  
71 emulsifying, gelling and complexation properties (Rehman et al., 2019). In particular,  
72 pectin is known for its interaction with WPI through covalent/non-covalent interactions,  
73 and their complexes have been studied for their application in food colloidal systems (Du  
74 et al., 2022). All these properties of pectin and WPI, together with their known interaction  
75 with polyphenols, are expected to protect these labile compounds from processing and  
76 digestive conditions, providing their selective release in the lower intestine where they  
77 can be absorbed.

78 Among the most used encapsulation methods is spray drying, an efficient, fast, cost-  
79 effective, and protective method to obtain dry particles (Annunziata et al., 2020; De La  
80 Cruz-Molina et al., 2021; Fang & Bhandari, 2012). This encapsulation technique involves  
81 the formation of microcapsules by producing a mixture of bioactive compounds with  
82 carriers in solution or suspension and then atomising this mixture in a hot air stream to  
83 obtain a dry powder (Dias et al., 2022). Nano spray drying (NSD) has emerged as a  
84 technology to reduce particle size. With smaller particles, physiological fate is  
85 significantly enhanced due to the higher surface: volume ratio offering a higher

86 penetration rate into the cells, stability, target release and bioavailability (Chopde et al.,  
87 2020; Jafari et al., 2021)

88 Several studies have been carried out to study the use of these protein-polysaccharide  
89 interactions for spray drying of grape by-products and further *in vitro* digestion due to the  
90 excellent source of phenolics they represent (Brown Da Rocha & Zapata Noreña, 2020;  
91 Constantin et al., 2021; Du et al., 2022). However, few studies have investigated nano  
92 spray drying for raw extracts and their behaviour during gastrointestinal digestion. Desai  
93 et al. (2020) used nano spray drying to encapsulate a raw green coffee extract with  
94 maltodextrin; their findings showed that maltodextrin protected the chlorogenic acid and  
95 its antioxidant activity from digestion conditions and storage. Other works have used the  
96 nano spray dryer for the encapsulation of saffron and soy extracts; however, in these  
97 works, a purification of specific compounds was carried out before the encapsulation (Del  
98 Gaudio et al., 2016; Kyriakoudi & Tsimidou, 2018). Moreover, these mentioned studies  
99 investigate only the use of maltodextrin even though nano spray drying has been used for  
100 encapsulation of specific whey proteins such as bovine serum albumin and lactoferrin  
101 (Bourbon et al., 2020; Lee et al., 2011).

102 This work aims to produce nano spray dried microcapsules with whey protein-pectin as  
103 encapsulants for the encapsulation of a raw grape marc extract and to study the effect on  
104 the stability and bioaccessibility of the polyphenols. Moreover, the biocompatibility and  
105 antioxidant capacity of the extract are assessed using a Caco-2 cell line and compared  
106 against the commercial antioxidant compound Trolox.

107

108 **2. Materials and methods**

109

110 Casa Emma Winery (Firenze, Italy) kindly supplied commercial grape marc flour from  
111 Sangiovese grapes. The grape marc flour is obtained by drying the grape marc at 42 °C  
112 for three days to preserve the phenolics. The grape marc is constantly mixed to avoid  
113 mould growth, and after the drying process, it is pulverised to a 250-micron particle size.  
114 The final product has the following specifications (supplied by the manufacturer): 8.53%  
115 moisture, 8% carbohydrates (from which sugars are 0.56%), 58.6% fibre, and 11.8%  
116 protein. Whey protein isolate was purchased from Volac International Ltd (Hertfordshire,  
117 UK) with the following specifications (supplied by the manufacturer): protein: 92% min,  
118 lactose: 0.9% max, fat: 0.8% max, pH: 5.8 min (10% sol). Pectin from citrus peel with  
119 ≥74.0% of galacturonic acid and ≥ 6.7% of methoxy groups; pepsin from porcine gastric  
120 mucosa ≥ 250 units/mg solid, pancreatin from porcine pancreas 8 x USP, bile, 2,4,6-  
121 Tris(2-pyridyl)-s-triazine (TPTZ), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic  
122 acid) diammonium salt (ABTS), fetal bovine serum (FBS) Superior, Hanks' balanced salt  
123 solution (HBSS) Resazurin sodium salt, 2',7'-Dichlorofluorescin diacetate (DCFH-DA),  
124 3-Morpholinosydnonimine (Sin-1), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-  
125 carboxylic acid (Trolox), and tert-butyl hydroperoxide (tBOOH) were purchased from  
126 Sigma-Aldrich. Minimum essential medium Eagle (MEM) (with 2 mM L-Glutamine, 1  
127 mM Sodium pyruvate, non-essential amino acids (NEAA)) and Penicillin-Streptomycin  
128 (10,000 U/mL-10 mg/mL, respectively) were from PAN-Biotech GmbH.

129

### 130 **2.1 Extraction of phenolics from grape marc**

131

132 A hydroalcoholic extraction was applied following the methodology previously  
133 developed in our group (MohdMaidin et al., 2018) to extract phenolics from grape marc.  
134 The extraction was carried out in an 8:1 ratio (solvent: solid) using a solution of 60%

135 ethanol under magnetic stirring for 2 h at 60°C. After the extraction, the solids were  
136 separated through vacuum filtration using No. #1 Whatman paper. Later, the ethanol was  
137 removed from the extract using a rotavapor (RV 10 auto pro-V-C Complete, IKA,  
138 Staufen, Germany). Then, the grape marc extract (GME) was freeze-dried and stored at -  
139 18 °C for further analysis, described in sections 2.6 and 2.7.

140

141 **2.2 *In vitro* cell culture studies**

142 **Cell culture**

143 Caco-2 cell line (ATCC, HTB-37) from human colon epithelial carcinoma was routinely  
144 expanded in MEM, supplemented with 20% FBS, and 1% Penicillin/Streptomycin (final  
145 concentration of 100 U/mL and 100 µg/mL, respectively). The cells were kept in a  
146 humidified atmosphere of 5% CO<sub>2</sub>, at 37 °C, in 75 cm<sup>2</sup> flasks. Cells were used in passages  
147 33–52, being the cell culture media replaced every other day. Upon reaching confluence,  
148 cells were detached using 0.25% trypsin- ethylenediaminetetraacetic acid (EDTA)  
149 solution, then pelleted by centrifugation at 300 ×g for 5 min and resuspended in fresh  
150 MEM at a concentration of 1 × 10<sup>5</sup> cells·mL<sup>-1</sup>. Cells were seeded onto 96-well plates at  
151 a density of 1 × 10<sup>4</sup> cells (100 µL of cellular suspension) per well and left to adhere for  
152 over 24 h.

153

154 **Cell viability assay**

155 The cytotoxicity of GME was determined indirectly by the resazurin conversion assay.  
156 After adhesion, the culture medium was removed, cells were washed twice with pre-

157 warmed phosphate buffered saline (PBS) solution, and 200  $\mu$ L of samples or controls  
158 were applied and incubated for 24 h. GME was prepared as described in section 2.1, then  
159 further diluted with culture medium (10%, v/v) and tested at 33, 67 and 100 GAE  $\mu$ g/mL  
160 final concentrations based on total phenolic content (TPC) in GME. These concentrations  
161 were chosen based on preliminary studies using concentrations reported by Freitas et al.  
162 (2020). Negative control was performed using cells growing in MEM (considered 100%  
163 cell viability), and 40% (v/v) dimethyl sulfoxide (DMSO) was used as a positive control.  
164 After incubation, samples or controls were removed and washed twice with pre-warmed  
165 PBS. After this, 100  $\mu$ L of 10% (v/v) resazurin in the culture medium (0.01 mg/mL final  
166 concentration) was added. The fluorescence intensity, proportional to the number of  
167 viable cells, was measured after 5 h of incubation using a microplate fluorescence reader  
168 (Synergy H1, BioTek, Vermont, USA) at an excitation wavelength of 560 nm and an  
169 emission wavelength of 590 nm. The % cell viability was expressed as the fluorescence  
170 of treated cells compared to that of cells growing in the culture medium.

#### 171 **Intracellular reactive oxygen species (ROS) quantification**

172 The antioxidant activity of GME was determined in an *in vitro* cell assay using DCFH-  
173 DA as a cell-permeable probe to detect intracellular ROS. After cell adhesion, the culture  
174 medium was removed, and 100  $\mu$ L of 10  $\mu$ M DCFH-DA solution was added to each well  
175 and incubated for 1 h. Afterwards, the solution was removed, and 100  $\mu$ L of GME  
176 solubilised in HBSS was added to each well at a final concentration of 33 and 67 GAE  
177  $\mu$ g/mL, based on TPC content in GME, and incubated for 4 h. The fluorescence intensity  
178 was measured using a microplate fluorescence reader (Synergy H1, BioTek, Vermont,  
179 USA) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

180 Cells exposed to HBSS, Sin-1 (5  $\mu$ M) and Trolox (50  $\mu$ g/mL) were used as basal, positive,  
181 and negative controls, respectively.

182 Then, the protective effect of GME against oxidative stress was investigated using Sin-1  
183 as an oxidative stress inducer. First, Caco-2 cells were exposed to GME at a 33 and 67  
184 GAE  $\mu$ g/mL concentration based on TPC content in GME for 4 h. Then, Sin-1 was added  
185 to the cells at a final concentration of 5  $\mu$ M and incubated for 1 h. The fluorescence  
186 intensity was measured every 15 min using a microplate fluorescence reader (Synergy  
187 H1, BioteK) at an excitation wavelength of 495 nm and an emission wavelength of 525  
188 nm. Cells exposed to HBSS, Sin-1 (5  $\mu$ M) and Trolox (50  $\mu$ g/mL) were used as basal,  
189 positive, and negative controls, respectively.

190

### 191 **2.3 Nano-spray drying (NSD)**

192 First, 50 mL of 4% WPI and 0.4% pectin solutions were prepared separately and  
193 solubilised overnight at room temperature to ensure complete hydration. Then, 550 mg  
194 of GME was resuspended in the pectin solution (50 mL) and mixed with a magnetic stirrer  
195 for 5 min. This solution (pectin-GME) was mixed with the WPI solution (50 ml) and  
196 stirred for 10 min (magnetic stirring). Then the WPI-pectin-GME solution was  
197 centrifuged to remove any large undissolved particles and filtrated through a 0.45  $\mu$ m  
198 PVDF filter before passing it through the NSD. The final solution had a final  
199 concentration of 2% WPI, 0.2% pectin and 0.55% GME. A solution containing the same  
200 proportion of WPI and pectin, but no GME was prepared to compare physical  
201 characteristics. The encapsulation was performed using a Nano-spray Dryer B-90  
202 (BÜCHI Labortechnik AG, Flawil, Switzerland). Compressed air was used as the drying  
203 gas, and the flow rate was set to about 100 or 110 L/min. The inlet temperature was set

204 to 90°C, the spray rate to 65%, and the pump to 30%. WPI-pectin-GME (W-P-GME) and  
205 WPI-pectin (W-P) particles were stored at 4 °C.

206 **2.4 Characterisation of the microparticles**

207

208 **Scanning electron microscopy (SEM)**

209

210 The samples' surface morphology was evaluated through SEM using a Quanta FEG 650  
211 (FEI, Oregon, USA). Dried samples were affixed on aluminium stubs covered by carbon  
212 ribbon and coated with gold, and samples were observed using an accelerating voltage of  
213 5 kV under vacuum conditions.

214 **Size and polydispersity index**

215 The size of the particles was determined by analysing SEM images with the program  
216 ImageJ (National Institutes of Health, Maryland, USA). The scale was adjusted according  
217 to the parameters from SEM images, and the size of 175 particles was determined. After  
218 this, the mean and standard deviation was calculated, and from those values, the  
219 polydispersity index (PDI) was calculated with the following formula:

220 **Equation 1**

221 
$$PDI = \sqrt{\frac{\text{size } \sigma}{\text{size } \bar{x}}}$$

222 Where  $\sigma$  is the standard deviation of the particle size and  $\bar{x}$  is the mean size of the  
223 particles.

224

225

226 **Yield**

227 The drying yield was calculated from the ratio of total solids out (microcapsules) to total  
228 solids in (solids in extracts + encapsulants).

229 **Equation 2**

230 
$$EY\% = \frac{\text{Total solids out}}{\text{Total solids in}} \times 100$$

231

232 **Z-potential**

233 The particles' surface charge (Z- potential) was measured by dynamic light scattering  
234 using an SZ-100 particle analyser (Horiba Scientific, Kyoto, Japan). Microparticles (1  
235 mg/mL) were measured at 25 °C using a He-Ne laser (633 nm) in folded capillary cells.  
236 Five independent measurements of each sample were done, and data were expressed as  
237 mean  $\pm$ SD.

238 **Fourier Transform Infrared Spectroscopy**

239 Fourier Transform Infrared (FTIR) Spectroscopy determined functional groups and the  
240 bonding arrangement of sample constituents. FTIR analyses were carried out with an  
241 ALPHA II (Bruker, Ettlingen, Germany) spectrometer with a diamond composite in the  
242 400–4000 cm<sup>-1</sup> wavenumber region.

243 **2.5 In vitro digestion**

244 Particles were tested under simulated digestive conditions to evaluate the protective effect  
245 of polymeric particles on GME's activity and polyphenol content. First, the activity of  
246 the digestive enzymes (pepsin and trypsin in pancreatin) was quantified. Then, the  
247 experimental conditions were applied according to the *in vitro* static INFOGEST method  
248 (Brodkorb et al., 2019). The addition of gastric lipase was omitted due to the limited

249 access to the commercially available enzyme, and amylase was not used in the oral phase  
250 since there was no starch in the sample.

251 W-P-GME particles (200 mg) or free GME (100 mg) were resuspended in 1 mL of  
252 distilled water and digested. The sample was diluted 1:1 (v/v) in oral digestion with  
253 simulated salivary fluid,  $\text{CaCl}_2$  0.3 M and water. The tubes were incubated in an orbital  
254 incubator (Fisher Scientific) for 2 min at 37 °C and 150 rpm. For gastric digestion (GD),  
255 a pepsin solution (2000 U/mL) in water was prepared based on the previously determined  
256 activity. The 2 mL of oral phase were diluted 1:1 (v/v) with simulated gastric fluid, pepsin  
257 solution,  $\text{CaCl}_2$  0.3 M, HCl 1 M (to pH 3.0) and water. The samples were incubated for 2  
258 h at 37 °C and 150 rpm. A 1.8 mL sample was collected after the 2 h of GD. For intestinal  
259 digestion (ID), bile solution and pancreatin were prepared in simulated intestinal fluid.  
260 The 2.2 mL of gastric phase were diluted 1:1 (v/v) with simulated intestinal fluid,  
261 pancreatin solution, bile,  $\text{CaCl}_2$  0.3 M, NaOH 1 M (to pH 7.0) and water. The samples  
262 were incubated for 2 h at 37 °C and 150 rpm. Then the samples were put in an ice water  
263 bath for 30 min to stop the enzyme's activity.

264 After digestion, each digested sample was centrifugated in a Ministar blueline  
265 microcentrifuge (fixed speed 2,000  $\times g$ ) at room temperature for 5 min. The supernatants  
266 were collected and stored for analysis. Digestion of polyphenols was evaluated according  
267 to the analytical determinations described in sections 2.6 and 2.7 after GD and after  
268 gastrointestinal digestion (GID).

269 The residual values of polyphenols were calculated as a percentage of the total mass of  
270 TPC (mg) remaining after GD and after the overall GID in relation to the initial mass. In  
271 the case of the antioxidant capacity, the values correspond to the trolox equivalents (TE)

272 (mg) for ABTS and ascorbic acid equivalents (AAE) (mg) for FRAP remaining after each  
273 phase of the digestion in relation to the initial ones.

274

275 **2.6 Analytical determinations**

276

277 **Total Phenolic Content**

278 The total phenolic content (TPC) was determined by the Folin-Ciocalteu method  
279 (Singleton & Rossi, 1965). For the assay, 75  $\mu$ L de Folin-Ciocalteu reagent (1:10) was  
280 added in a 96-well microplate, with 15  $\mu$ L of the sample and 60  $\mu$ L of 7.5%  $\text{Na}_2\text{CO}_3$ . The  
281 samples were incubated in the dark for 30 min. After this time, the microplate was read  
282 at 765 nm in a microplate reader (Synergy H, BioTek, Vermont, USA). The results were  
283 quantified from a Gallic acid calibration curve ranging from 0.1 to 1.0 mg/ml and  
284 expressed as milligrams of gallic acid equivalents (GAE) per gram of dried extract (mg  
285 GAE / g de).

286

287 **Total Monomeric Anthocyanin Content**

288 Total monomeric anthocyanins content (TMAC) levels were quantified by the AOAC  
289 Official Method 2005.02 pH differential method (Lee et al., 2005). A sample of GME  
290 was combined in a 1:20 ratio (v:v) with potassium chloride and sodium acetate buffers  
291 (pH 1.0 and 4.5, respectively) separately. After an equilibration period of 15 min, the  
292 absorbance of each solution was measured at 520 and 700 nm in a microplate reader  
293 (Synergy H, BioTek, Vermont, USA). The values were calculated with the following  
294 formula.

295 **Equation 3**

296 Monomeric Anthocyanins = 
$$\frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

297 Where:

298 - A= corrected absorbance value calculated as  $[(A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5}]$

300 - MW= molecular weight of malvidin 3-O-glucoside (493.43 g/mol)

301 - DF= dilution factor

302 -  $\varepsilon$ = molar absorption: 28,000 L/mol · cm

303 The results were expressed as milligrams of malvidin 3-O-glucoside equivalents per litre  
304 (mg M3GE/L)

305

306 **Total Flavonoid Content**

307 The total flavonoid content (TFC) was measured using the aluminium method (Zhishen  
308 et al., 1999) with some modifications. Briefly, 100  $\mu$ L of the sample were added to an  
309 Eppendorf tube, and 430  $\mu$ L of solution A (1.8 mL of 5% NaNO<sub>2</sub> mixed with 24 mL of  
310 distilled water) was added to the sample and incubated for 5 min. Later 30  $\mu$ L of 10%  
311 AlCl<sub>3</sub> were added and left to rest for 1 min. Finally, 440  $\mu$ L of solution B (12 mL of NaOH  
312 1M mixed with 14.4 mL of distilled water) was added without further incubation. From  
313 this reaction, 150  $\mu$ L were transferred to a 96-well microplate in triplicate. The samples  
314 were read at 496 nm in a microplate reader (Synergy H1, BioTek, Vermont, USA). The  
315 absorbance was compared with a Catechin standard curve ranging from 0.1 to 1 mg/ml.  
316 The results were expressed as milligrams of Catechin equivalents (CE) per gram of dried  
317 extract (mg CE/ g de).

318 **2.7 Antioxidant Capacity assessment by ABTS and FRAP methods**

319 The total antioxidant activity of all samples was measured by ABTS (2,2'-Azino-bis(3-  
320 ethylbenzothiazoline-6-sulfonic acid)) assay (Re et al., 1999) with some modifications.  
321 The ABTS•+ stock solution was prepared by mixing 5 ml of 7 mM ABTS solution and  
322 88 µl of 140 mM potassium persulfate ( $K_2S_2O_8$ ) solution. Then, the mixture was kept in  
323 the dark and at room temperature for at least 16 h before use. The working solution of  
324 ABTS•+ was obtained by diluting the ABTS•+ stock solution with distilled water to an  
325 absorbance of  $0.70 \pm 0.02$  at 734 nm. Then, 5 µl of the sample was added to 245 µl of  
326 ABTS•+ working solution, and the mixture was homogenised and then incubated in the  
327 dark for 5 min. The absorbance of the control and the samples were recorded at 734 nm  
328 using a microplate reader (Synergy H1, BioTek, Vermont, USA). The scavenging activity  
329 of each sample on ABTS•+ was calculated from a Trolox standard curve at concentrations  
330 of 0.04 to 0.4 mg/mL. Results were expressed as micromole Trolox equivalents (TE) per  
331 gram of dry extract.

332 For the Ferric Reducing Antioxidant Power (FRAP) assay (Benzie & Strain, 1996), 10 µl  
333 of the sample was added to 300 µl of FRAP reagent in a microcentrifuge tube and  
334 vortexed for 10s. Then, in triplicate, 100 µl of this mixture was transferred into a 96-well  
335 microplate, and absorbance was measured at 595 nm in a microplate reader (Synergy H1,  
336 BioTek, Vermont, USA). An ascorbic acid standard curve from 0.01 to 0.2 mg/mL was  
337 used for the quantification. Results were expressed as micromole ascorbic acid  
338 equivalents (AAE) per gram of dry extract.

339

340

341 **2.8 Statistical Analysis**

342 The data were subjected to a One-Way ANOVA using IBM® SPSS® Statistics 27  
343 software, where statistical differences were noted. Differences among different  
344 treatments were determined using independent samples t-test for particle size and  
345 gastrointestinal results. For the metabolic activity, differences were determined by  
346 Dunnett's multiple comparison test, as this is more suitable for the mean comparison of  
347 different experimental groups against a control group. The significance level was defined  
348 at  $p < 0.05$ , and the results are reported as means  $\pm$  SD.

349

350 **3. Results and discussion**

351 **3.1 Characterization of grape marc extract**

352 Hydroalcoholic extractions have proven to be efficient for extracting phenolics from  
353 grape by-products (MohdMadin et al., 2018, 2019; Spigno et al., 2007, 2017). Indeed,  
354 we obtained a phenolics-rich extract with high total phenolic content (TPC), total  
355 flavonoid content (TFC) content, and antioxidant capacity (Table 1). The phenolics  
356 content was higher than those reported by Pintać et al. (2018) and Aresta et al. (2020).  
357 They obtained 69 and 70 mg gallic acid equivalents (GAE)/ g de, respectively, when  
358 conventional extraction of polyphenols from grape marc. However, we obtained a lower  
359 content of total monomeric anthocyanin content (TMAC), which might be explained by  
360 a combination of factors such as extraction method, grape variety, growing region, and  
361 processing, these conditions play a significant role since not all grapes bear the same  
362 TMAC (Rinaldi et al., 2020; Spigno et al., 2015).

363

364 **3.2 Biocompatibility of grape marc extract**

365 Studying the potentially toxic effects of bioactive compounds is essential to determine  
366 whether they are safe to consume without harming the host. The grape marc extract  
367 (GME) showed a dose-responsive effect after 24 h of incubation with Caco-2 cells (Fig.  
368 1). We observed cellular compatibility, *i.e.*, more than 70% of cell viability, for 33 and  
369 67 µg/mL TPC based on GAE. However, cell viability below 70% was observed at the  
370 highest concentration tested (100 GAE µg/mL), which is considered toxic. Studies in the  
371 grape phenolic extract have shown that concentrations between 0.1 to 10 µg/mL present  
372 no toxicity in Caco-2 cells with up to 93% viability (Wang et al., 2016). Another study  
373 by Costa et al. (2019) showed that concentrations of up to 2% of GME were non-toxic  
374 for Caco-2 cells before and after simulated *in vitro* digestion. Also, Wolfe et al. (2008)  
375 observed that concentrations below 60 mg/mL of different extracts, e.g., wild blueberry,  
376 red grape, and strawberry, showed no cytotoxicity in HepG2 cells. However, in a  
377 preliminary assay, we observed that concentrations of 5 mg/mL GME, in the  
378 concentration range of some reports, were highly toxic (0% viability) for Caco-2 cells  
379 (data not shown), highlighting the importance of assessing each extract for its safe  
380 application.

381

382 **3.3 Cellular antioxidant activity (CAA) of grape marc extract**

383 Reactive oxygen species (ROS) are natural by-products of cell activity and essential  
384 signaling molecules (Zhang et al., 2016). However, an imbalance between oxidant-  
385 producing systems and antioxidant defense mechanisms can trigger cell damage and  
386 cause cell death (Alfadda & Sallam, 2012). Cell-based assays have been used to assess  
387 the effectiveness of dietary antioxidant compounds (Kellett et al., 2018).

388 Studies of intracellular oxidant production in Caco-2 cells were evaluated using 2'-7'-  
389 Dichlorodihydrofluorescein (DCFH) fluorescence, testing GME at non-toxic  
390 concentrations (33 and 67 GAE  $\mu$ g/mL based on TPC). As shown in Fig. 2A, both GME  
391 concentrations decreased the intracellular ROS basal levels, comparing with the control  
392 (cells treated with Hanks' balanced salt solution (HBSS)) to a similar level to the one  
393 observed for ( $\pm$ )-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 50  
394  $\mu$ g/mL). This result suggests that GME can reduce ROS naturally produced by the Caco-  
395 2 cells, demonstrating a possible antioxidant effect (intracellular) against ROS.

396 To evaluate the potential protective effect of GME against intracellular oxidation, Caco-  
397 2 cells were pre-treated with GME at the non-toxic concentrations of 33 and 67  $\mu$ g  
398 GAE/mL based on TPC for 4 h. Then, cells were stimulated with 5  $\mu$ M of the oxidising  
399 agent 3-Morpholinosydnonimine (Sin-1), selected according to the literature (PD ISO/TS  
400 19006:2016). Cells treated with HBSS and stressed with Sin-1 were used as a positive  
401 control. As shown in Fig. 2B, cells pre-treated with non-toxic concentrations of GME  
402 significantly reduced intracellular ROS level produced after stimulation with Sin-1  
403 compared to cells pre-treated with HBSS (control). This reduction was similar to that  
404 observed for treated cells with 50  $\mu$ g/mL Trolox which was used as a potent antioxidant  
405 model compound.

406 GME showed a similar antioxidant effect to a well-known compound at similar  
407 concentrations, suggesting that GME polyphenols can effectively neutralise ROS-  
408 induced production (protective effect) in Caco-2 cells, demonstrating intracellular  
409 antioxidant capacity. The results of the CAA also corroborate the high antioxidant  
410 capacity of the GME observed by 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid  
411 (ABTS) and ferric reducing antioxidant power (FRAP) methods. Wang et al. (2016)

412 induced ROS production using t-BOOH (tert-butyl hydroperoxide) in Caco-2 cells treated  
413 with grape phenolic extract for 1 h, and their results showed that concentrations of 0.1 to  
414 10 µg/mL exert an antioxidant effect over ROS. Other studies have reported that  
415 concentrations of 100µ/mL, 200µ/mL and 500µ/mL reduced ROS production in Caco-2  
416 cells treated with grape pomace extract for 5 h (Martins et al., 2017, 2020). However, at  
417 500 µg/mL, the production of ROS was significantly reduced due to the pro-oxidant effect  
418 of polyphenols (Martins et al., 2020). Milinčić et al. (2021) observed an EC50 of ABAP  
419 (2,2'-azobis(2-amidopropane)) radical at a 54 mg TPC/mL concentration of grape pomace  
420 skin extract on the same cell line. The concentrations used in the previously mentioned  
421 studies are considerably higher than the ones we reported, indicating that while grape  
422 pomace is an excellent source of antioxidants, the analyses of cell biocompatibility and  
423 antioxidant capacity need to be carried out before their formulation as nutraceuticals or  
424 functional food ingredients.

425

#### 426 **3.4 Encapsulated GME morphology, size, and Z-potential**

427 The morphology and size of the encapsulated GME were studied through scanning  
428 electron microscopy (SEM) analysis. Fig. 3A shows the formation of large crystals with  
429 a wide distribution of submicron and micron particles during freeze-drying of GME  
430 (Table 2). For the nano spray dried particles, different morphologies were observed for  
431 the W-P particles with and without GME. Blank microparticles (W-P) had a spherical  
432 shape and smooth surface (Fig. 3B), while microparticles loaded with GME (W-P-GME)  
433 (Fig. 3C) kept their spherical shape but presented some wrinkles in their surface.  
434 Moreover, no breakage was seen in W-P and W-P-GME. Regarding the size, W-P-GME  
435 particles showed a smaller and narrower size distribution than W-P particles (Table 2).

436 Studies on the encapsulation of raw grape marc extract by conventional spray drying have  
437 reported sizes of 9.8  $\mu\text{m}$  when using pectin and casein, and 15  $\mu\text{m}$  when using whey  
438 protein isolate (WPI) alone. (Carra et al., 2022; Moreno et al., 2018). The results obtained  
439 here (1  $\mu\text{m}$ ) demonstrate that nano spray drying significantly affects the particles'  
440 reduction size. Moreover, the particles we obtained displayed a more homogeneous and  
441 well-defined particle shape than those in previously mentioned studies, where irregular  
442 and dented surfaces were obtained, and in the case of WPI, holes were seen in the  
443 microparticles (Moreno et al., 2018). The zeta potential of W-P-GME (Table 2) showed  
444 a medium to high particle surface charge, which confers the particles' colloidal stability.

445

### 446 **3.5 Fourier Transform Infrared (FTIR) analysis**

447 FTIR analysis was used to examine interactions between the biopolymers and GME. The  
448 infrared spectra of the carriers, GME and microparticles are shown in Fig. 4. For WPI,  
449 characteristic amide I and II bands can provide information about protein secondary  
450 structures, and their change in vibration frequencies is related to the interaction between  
451 their functional groups. Amide I, represents the C=O carbonyl stretching vibration of the  
452 peptide backbone (1600-1700  $\text{cm}^{-1}$ ), and the amide II band (<1550  $\text{cm}^{-1}$ ) represents the  
453 C-N stretching and N-H bending (López-Rubio & Lagaron, 2012; Meng et al., 2021). As  
454 for the GME, the characteristic bands of grape phenolic compounds were observed  
455 between 1700 and 900  $\text{cm}^{-1}$ . The band at 1710  $\text{cm}^{-1}$  was attributed to the stretching in the  
456 carbonyl group (C=O) band, 1600 and 1510  $\text{cm}^{-1}$  bands correspond to the C=C stretching,  
457 characteristic of aromatic systems. The peak around 1440  $\text{cm}^{-1}$  corresponds to the  
458 antisymmetric in-plane bending of -CH<sub>3</sub> related to aromatic rings and flavonoids (Moreno  
459 et al., 2018; Zhao et al., 2015). Characteristic peaks of pectin can be observed at 2920,

460 1740, 1610 and 900-1250  $\text{cm}^{-1}$  corresponding to the C-H stretching of the CH, CH<sub>2</sub> and  
461 CH<sub>3</sub> groups, C=O stretching vibration of the ester carbonyl, C=O stretching of the  
462 vibration the carbonyl group, C-O-C and O-H of pyranose rings respectively (Khodaiyan  
463 & Parastouei, 2020).

464 Looking at the infrared spectra of W-P and W-P-GME, slight shifts in the amide I and  
465 amide II regions were observed compared to WPI (1517 to 1535  $\text{cm}^{-1}$ ). These shifts can  
466 be attributed to the interaction between carboxyl groups of pectin and the charged amino  
467 groups of the main WPI proteins' composition (beta-lactoglobulin, alpha-lactalbumin,  
468 and serum albumin) (Raei et al., 2018). An increase in the intensity was observed for the  
469 W-P particles, which can be attributed to the rise in random coils and the previously  
470 mentioned interaction between WPI and pectin (El-Messery et al., 2020; He et al., 2016).  
471 However, when GME is added, a decrease in intensity is observed. This result is  
472 consistent with those obtained by Meng and Li (2021), where Gallic acid, chlorogenic  
473 acid, and epigallocatechin gallate-WPI complexes showed decreased intensity in the  
474 amide I band. This change can be attributed to the reduction of  $\alpha$ -helical structures as a  
475 result of protein conformational modifications upon phenolics complexation by hydrogen  
476 bonding and hydrophobic interactions between the phenolic compounds and hydrophobic  
477 groups of the protein, so there are not only interactions but also changes in the secondary  
478 structure of the proteins (Bourassa et al., 2013; He et al., 2016). According to previous  
479 reports, W-P-GME did not show any characteristic band from GME, indicating that  
480 phenolics distinct peaks can be hidden when in contact with other biopolymers like WPI.  
481 This change could mean the formation of complexes that reduce the bending and  
482 stretching of the bonds in GME polyphenols.

483 **3.6 *In vitro* digestion of free and encapsulated GME**

484 The results of the residual TPC and antioxidant activity for both free and encapsulated  
485 GME are shown in Fig. 5. These results represent the fraction of TPC (or activity which,  
486 is quantified as Trolox equivalents (TE) or ascorbic acid equivalents (AAE)) remaining  
487 after gastric digestion (GD) or gastrointestinal digestion (GID), the latter indicating the  
488 bioaccessible fraction. Therefore, these values show the fraction of TPC (or activity) that  
489 resisted the simulated gastrointestinal conditions in free GME. In contrast, for W-P-GME,  
490 these values account for the fraction of TPC that resisted the conditions and/or was  
491 encapsulated and effectively released from the microcapsules during digestion.

492 A different behaviour was observed for free and encapsulated GME, suggesting the  
493 microcapsules play an essential role in the phenolic content and their activity during  
494 digestion. For free GME, we observed that the TPC underwent some degradation due to  
495 the gastric conditions (acidic pH), as shown by a 76% residual TPC content (24%  
496 unaccounted for; Fig. 5A). The moderate stability of GME polyphenols to gastric  
497 digestion agrees with previous studies (Li et al., 2023). The free GME suffered further  
498 degradation after intestinal conditions, resulting in a further 30% TPC loss in relation to  
499 that remaining after GD; low stability of polyphenols has been reported at neutral pH  
500 conditions (Li et al., 2023). So, after GID, the overall bioaccessible TPC was 54%. In the  
501 case of encapsulated GME, about 30% of TPC was unaccounted for after GD (Fig. 5A),  
502 which may represent the fraction not released from the microparticles. Indeed, high  
503 preservation of the TPC was expected during GD since strong electrostatic interactions  
504 stabilise the WPI- pectin complex at acidic pHs (3.6-4.5) (Raei et al., 2017), which should  
505 protect phenolics from degradation. However, some release of phenolics will still occur  
506 as WPI is susceptible to enzymatic hydrolysis, but pectin should have a stabilising effect  
507 in the system (Reichembach & Lúcia de Oliveira Petkowicz, 2021; Wusigale et al., 2020).  
508 Yet, the released fraction can also undergo similar degradation as that observed for the

509 free extract (GME). Therefore, assuming the residual 70% TPC content in W-P-GME  
510 will undergo similar degradation as that of the free extract during GID, values close to 54  
511 % of residual TPC (as in GME) would be expected however, it was found that 83% of the  
512 TPC remained after GID. This indicates a protective effect of the microcapsules, which  
513 resulted in about 30% of the TPC in the gastric phase and their release at intestinal  
514 conditions, with an overall increase in the remaining TPC compared to free GME.

515 The behaviour of antioxidant activity during GID for both free and encapsulated GME  
516 showed a similar trend to TPC. Thus, the free GME showed a slight loss of activity after  
517 GD followed by a more pronounced decrease after GID, while for W-P-GME, the activity  
518 was slightly increased after GID compared to GD (Fig. 5B & 5C). Besides, free GME's  
519 bioactivity directly correlates with residual TPC values after GD and GID, achieving  
520 values of 73% and 57% of the initial activity, as assessed by the ABTS method. Although  
521 a similar trend was observed in both phases, lower values were recorded using the FRAP  
522 method.

523 For encapsulated GME, although a positive correlation was observed between residual  
524 TPC and antioxidant activity, the latter showed lower values than the residual TPC. For  
525 instance, 29 and 61% of the activity was observed using the ABTS method after GD and  
526 GID in W-P-GME. The reduced activity compared to the residual TPC might be due to  
527 released polyphenols from the capsules bearing lower antioxidant activity than those that  
528 were still encapsulated or that they might be complexed with the capsule components  
529 since they are known to interact with whey proteins and their peptides (Guo & Jauregi,  
530 2018), which has been confirmed by the FTIR spectra.

531 Overall, the results of GID showed that the encapsulation succeeded in preserving the  
532 TPC and increasing their bioaccessibility. For the antioxidant activity, similar results to

533 free GME were observed according to the ABTS method, and slightly higher activity  
534 according to the FRAP method.

535

536 **4. Conclusions**

537 A raw ethanolic extract of a winery by-product (grape marc) with antioxidant capacity  
538 was successfully encapsulated using whey protein isolate (WPI) and pectin and nano  
539 spray drying (73% yield), resulting in spherical smoothed-surface microparticles with an  
540 average size of 1  $\mu$ m, polydispersity index (PDI) of 0.717, and a surface charge (Z-  
541 potential) close to -30 mV. The Fourier Transform Infrared (FTIR) analysis of  
542 the microparticles confirmed the complexations between WPI, pectin and the phenolics  
543 in grape marc extract (GME) through non-covalent interactions. The developed  
544 encapsulation system protected the GME phenolics and the antioxidant activity during  
545 gastrointestinal digestion (GID), improving bioaccessibility. The potent antioxidant  
546 intracellular protective effect of GME observed, and its improved resistance to GID when  
547 encapsulated compared to the free form suggest this encapsulation system could be a  
548 promising strategy towards preserving the antioxidant activity of this high-value-added  
549 by-product of the wine industry. The selected wall materials proved that the  
550 microcapsules resisted gastric conditions and could provide a targeted release in the lower  
551 intestine, where phenolic compounds are absorbed and can be metabolised by the  
552 microbiota. Although further studies are needed to test the stability, biocompatibility, and  
553 *in vivo* bioactivity of the WPI-pectin-GME microcapsules, the presented results are  
554 promising towards using encapsulated GME as a nutraceutical.

555

556 **Conflict of Interest**

557 *The authors declare that the research was conducted without any commercial or*  
558 *financial relationships that could be construed as a potential conflict of interest.*

559

560 **Author Contributions**

561 *Data curation; Formal analysis; Methodology; Writing-original draft:* Aimara V. De La  
562 *Cruz Molina. Conceptualisation:* Isabel Rodriguez, & Lorenzo Pastrana. *Funding*  
563 *acquisition:* Lorenzo Pastrana. *Resources:* Lorenzo Pastrana. *Writing-review & editing:*  
564 *Isabel Rodriguez, Aimara V. De La Cruz Molina, Catarina Gonçalves, Mafalda D. Neto*  
565 *& Paula Jauregi. Cell assays:* Catarina Gonçalves, Mafalda D. Neto. *Supervision:* Isabel  
566 *Rodriguez, Lorenzo Pastrana, &Paula Jauregi.*

567 **Funding**

568 The research leading to these results received funding from the European Union's H2020.  
569 Research and Innovation Programme under the Maria Skłodowska-Curie grant agreement  
570 no. 778388 (H2020 MSCA-RISE-2017 project Food for Diabetes and Cognition  
571 (FODIAC), and the SbDtoolBox - Nanotechnology-based tools and tests for Safer-by-  
572 Design nanomaterials, with the reference NORTE-01-0145-FEDER-000047, funded  
573 by Norte 2020 – North-Regional Operational Programme under the PORTUGAL 2020  
574 Partnership Agreement, through the European Regional Development Fund (ERDF).

575

576 **Acknowledgements**

577 The authors would like to thank the Food Processing group at INL, especially Arlete

578 Marques and Isabel Bourbon.

579

580 **References**

581

582 Alfadda, A. A., & Sallam, R. M. (2012). Reactive oxygen species in health and disease.  
583 *Journal of Biomedicine and Biotechnology*, 2012.  
584 <https://doi.org/10.1155/2012/936486>

585 Annunziata, G., Jiménez-García, M., Capó, X., Moranta, D., Arnone, A., Tenore, G. C.,  
586 Sureda, A., & Tejada, S. (2020). Microencapsulation is a tool to counteract the  
587 typical low bioavailability of polyphenols in the management of diabetes. *Food*  
588 and *Chemical Toxicology*, 139. <https://doi.org/10.1016/j.fct.2020.111248>

589 Aresta, A., Cotugno, P., De Vistro, N., Massari, F., & Zambonin, C. (2020).  
590 Determination of Polyphenols and Vitamins in Wine-Making by-Products by  
591 Supercritical Fluid Extraction (SFE). *Analytical Letters*, 53(16), 2585–2595.  
592 <https://doi.org/10.1080/00032719.2020.1749846>

593 Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a  
594 measure of “antioxidant power”: the FRAP assay. *Analytical Biochemistry*, 239(1),  
595 70–76. <https://doi.org/10.1006/abio.1996.0292>

596 Bourassa, P., Bariyanga, J., & Tajmir-Riahi, H. A. (2013). Binding sites of resveratrol,  
597 genistein, and curcumin with milk  $\alpha$ - And  $\beta$ -caseins. *Journal of Physical*  
598 *Chemistry B*, 117(5), 1287–1295. <https://doi.org/10.1021/jp3114557>

599 Bourbon, A. I., Barbosa-Pereira, L., Vicente, A. A., Cerqueira, M. A., & Pastrana, L.  
600 (2020). Dehydration of protein lactoferrin-glycomacropeptide nanohydrogels.  
601 *Food Hydrocolloids*, 101, 105550. <https://doi.org/10.1016/j.foodhyd.2019.105550>

602 Brezoiu, A.-M., Matei, C., Deaconu, M., Stanciu, A.-M., Trifan, A., Gaspar-  
603 Pintilieescu, A., & Berger, D. (2019). Polyphenols extract from grape pomace.  
604 Characterisation and valorisation through encapsulation into mesoporous silica-  
605 type matrices. *Food and Chemical Toxicology*, 133, 110787.  
606 <https://doi.org/10.1016/j.fct.2019.110787>

607 Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T.,  
608 Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M.,  
609 Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le  
610 Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A. R., Martins, C., Marze, S.,  
611 McClements, D. J., Ménard, O., Minekus, M., Portmann, R., Santos, C. N.,  
612 Souchon, I., Singh, R. P., Vegarud, G. E., Wickham, M. S. J., Weitschies, W., &  
613 Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food  
614 digestion. *Nature Protocols*, 14(4), 991–1014. [https://doi.org/10.1038/s41596-018-0119-1](https://doi.org/10.1038/s41596-018-<br/>615 0119-1)

616 Brown Da Rocha, C., & Zapata Noreña, C. P. (2020). *Drying Technology*  
617 *Microencapsulation and controlled release of bioactive compounds from grape*  
618 *pomace Microencapsulation and controlled release of bioactive compounds from*  
619 *grape pomace*. <https://doi.org/10.1080/07373937.2020.1741004>

620 Cao, H., Saroglu, O., Karadag, A., Diaconeasa, ta, Zoccatelli, G., Adam Conte-Junior,  
621 C., Gonzalez-Aguilar, G. A., Ou, J., Bai, W., Mara Zamarioli, C., Alexandre Pedro  
622 de Freitas, L., Shpigelman, A., Campelo, P. H., Capanoglu, E., Lik Hii, C., Mahdi  
623 Jafari, S., Qi, Y., Liao, P., Wang, M., Zou, L., Bourke, P., Simal-Gandara, J., Xiao,  
624 J., & Jesus Simal-Gandara, C. (2021). Available technologies on improving the  
625 stability of polyphenols in food processing. *Food Frontiers*, 2, 109–139.  
626 <https://doi.org/10.1002/fft2.65>

627 Carra, J. B., Matos, R. L. N. de, Novelli, A. P., Couto, R. O. do, Yamashita, F., Ribeiro,  
628 M. A. dos S., Meurer, E. C., Verri, W. A., Casagrande, R., Georgetti, S. R.,  
629 Arakawa, N. S., & Baracat, M. M. (2022). Spray-drying of casein/pectin  
630 bioconjugate microcapsules containing grape (*Vitis labrusca*) by-product extract.  
631 *Food Chemistry*, 368(February 2021).  
632 <https://doi.org/10.1016/j.foodchem.2021.130817>

633 Chopde, S., Datir, R., Deshmukh, G., Dhotre, A., & Patil, M. (2020). Nanoparticle  
634 formation by nanospray drying & its application in nanoencapsulation of food  
635 bioactive ingredients. *Journal of Agriculture and Food Research*, 2(November),  
636 100085. <https://doi.org/10.1016/j.jafr.2020.100085>

637 Constantin, O. E., Stănciuc, N., Yan, Y., Ghinea, I. O., Ungureanu, C., Cîrciumaru, A.,  
638 Wang, D., Poklar Ulrich, N., & Râpeanu, G. (2021). Polymers and protein-  
639 associated vesicles for the microencapsulation of anthocyanins from grape skins  
640 used for food applications. *Journal of the Science of Food and Agriculture*, 101(7),  
641 2676–2686. <https://doi.org/10.1002/jsfa.10892>

642 Costa, J. R., Amorim, M., Vilas-Boas, A., Tonon, R. V., Cabral, L. M. C., Pastrana, L.,  
643 & Pintado, M. (2019). Impact of: In vitro gastrointestinal digestion on the chemical  
644 composition, bioactive properties, and cytotoxicity of *Vitis vinifera* L. cv. Syrah  
645 grape pomace extract. *Food and Function*, 10(4), 1856–1869.  
646 <https://doi.org/10.1039/c8fo02534g>

647 De La Cruz-Molina, A. v., Ayala Zavala, J. F., Bernal Mercado, A. T., Cruz Valenzuela,  
648 M. R., González-Aguilar, G. A., Lizardi-Mendoza, J., Brown-Bojorquez, F., &  
649 Silva-Espinoza, B. A. (2021). Maltodextrin encapsulation improves thermal and  
650 pH stability of green tea extract catechins. *Journal of Food Processing and  
651 Preservation*, 45(9), 1–13. <https://doi.org/10.1111/jfpp.15729>

652 de Wit, J. N. (1998). Nutritional and Functional Characteristics of Whey Proteins in  
653 Food Products. *Journal of Dairy Science*, 81(3), 597–608.  
654 [https://doi.org/10.3168/jds.S0022-0302\(98\)75613-9](https://doi.org/10.3168/jds.S0022-0302(98)75613-9)

655 Del Gaudio, P., Sansone, F., Mencherini, T., De Cicco, F., Russo, P., & Aquino, R.  
656 (2016). Nanospray Drying as a Novel Tool to Improve Technological Properties of  
657 Soy Isoflavone Extracts. *Planta Medica*, 83(05), 426–433.  
658 <https://doi.org/10.1055/s-0042-110179>

659 Desai, N. M., Gilbert Stanley, J., & Murthy, P. S. (2020). Green coffee nanoparticles:  
660 optimisation, *in vitro* bioactivity and bio-release property. *Journal of  
661 Microencapsulation*, 37(1), 52–64.  
662 <https://doi.org/10.1080/02652048.2019.1692946>

663 Dias, M., Romaní-Pérez, M., Romaní, A., de la Cruz, A., Pastrana, L., Fuciños, P., &  
664 Amado, I. R. (2022). Recent Technological Advances in Phenolic Compounds  
665 Recovery and Applications: Source of Nutraceuticals for the Management of  
666 Diabetes. In *Applied Sciences (Switzerland)* (Vol. 12, Issue 18). MDPI.  
667 <https://doi.org/10.3390/app12189271>

668 Dongowski, G., & Anger, H. (1996). Metabolism of pectin in the gastrointestinal tract.  
669 In *Progress in Biotechnology* (Vol. 14, Issue C, pp. 659–666). Elsevier.  
670 [https://doi.org/10.1016/S0921-0423\(96\)80300-5](https://doi.org/10.1016/S0921-0423(96)80300-5)

671 Du, Q., Zhou, L., Lyu, F., Liu, J., & Ding, Y. (2022). The complex of whey protein and  
672 pectin: Interactions, functional properties and applications in food colloidal  
673 systems – A review. *Colloids and Surfaces B: Biointerfaces*, 210(November 2021).  
674 <https://doi.org/10.1016/j.colsurfb.2021.112253>

675 El-Messery, T. M., Mwafy, E. A., Mostafa, A. M., El-Din, H. M. F., Mwafy, A.,  
676 Amarowicz, R., & Ozçelik, B. (2020). Spectroscopic studies of the interaction  
677 between isolated polyphenols from coffee and the milk proteins. *Surfaces and  
678 Interfaces*, 20, 100558. <https://doi.org/https://doi.org/10.1016/j.surfin.2020.100558>

679 Fang, Z., & Bhandari, B. (2012). Encapsulation Techniques for Food Ingredient  
680 Systems. *Food Materials Science and Engineering*, 320–348.  
681 <https://doi.org/10.1002/9781118373903.ch12>

682 Fraga, C. G., Croft, K. D., Kennedy, D. O., & Tomás-Barberán, F. A. (2019). The  
683 effects of polyphenols and other bioactives on human health. *Food and Function*,  
684 10(2), 514–528. <https://doi.org/10.1039/c8fo01997e>

685 Freitas, F. M. C., Cerqueira, M. A., Gonçalves, C., Azinheiro, S., Garrido-Maestu, A.,  
686 Vicente, A. A., Pastrana, L. M., Teixeira, J. A., & Michelin, M. (2020). Green  
687 synthesis of lignin nano- and micro-particles: Physicochemical characterization,  
688 bioactive properties and cytotoxicity assessment. *International Journal of  
689 Biological Macromolecules*, 163, 1798–1809.  
690 <https://doi.org/10.1016/j.ijbiomac.2020.09.110>

691 Guo, Y., & Jauregi, P. (2018). Protective effect of  $\beta$ -lactoglobulin against heat induced  
692 loss of antioxidant activity of resveratrol. *Food Chemistry*, 266(January), 101–109.  
693 <https://doi.org/10.1016/j.foodchem.2018.05.108>

694 He, Z., Zhu, H., Xu, M., Zeng, M., Qin, F., & Chen, J. (2016). Complexation of bovine  
695  $\beta$ -lactoglobulin with malvidin-3-O-glucoside and its effect on the stability of grape  
696 skin anthocyanin extracts. *Food Chemistry*, 209, 234–240.  
697 <https://doi.org/10.1016/j.foodchem.2016.04.048>

698 Jafari, S. M., Arpagaus, C., Cerqueira, M. A., & Samborska, K. (2021). Nano spray  
699 drying of food ingredients; materials, processing and applications. *Trends in Food  
700 Science and Technology*, 109(July 2020), 632–646.  
701 <https://doi.org/10.1016/j.tifs.2021.01.061>

702 Jauregi, P., & Welderufael, F. T. (2010). Added-value protein products from whey.  
703 *Nutrafoods*, 9(4), 13–23. <https://doi.org/10.1007/bf03223344>

704 Kellett, M. E., Greenspan, P., & Pegg, R. B. (2018). Modification of the cellular  
705 antioxidant activity (CAA) assay to study phenolic antioxidants in a Caco-2 cell  
706 line. *Food Chemistry*, 244(August 2017), 359–363.  
707 <https://doi.org/10.1016/j.foodchem.2017.10.035>

708 Khodaiyan, F., & Parastouei, K. (2020). Co-optimization of pectin and polyphenols  
709 extraction from black mulberry pomace using an eco-friendly technique:  
710 Simultaneous recovery and characterization of products. *International Journal of  
711 Biological Macromolecules*, 164, 1025–1036.  
712 <https://doi.org/10.1016/j.ijbiomac.2020.07.107>

713 Kyriakoudi, A., & Tsimidou, M. Z. (2018). Properties of encapsulated saffron extracts  
714 in maltodextrin using the Büchi B-90 nano spray-dryer. *Food Chemistry*, 266,  
715 458–465. <https://doi.org/10.1016/j.foodchem.2018.06.038>

716 Lavelli, V., Torri, L., Zeppa, G., Fiori, L., & Spigno, G. (2016). Recovery of  
717 Winemaking By-Products. *Italian Journal of Food Science*, 28(4), 542–564.  
718 <https://doi.org/10.14674/1120-1770/ijfs.v507>

719 Lee, J., Durst, R. W., & Wrolstad, R. E. (2005). Determination of total monomeric  
720 anthocyanin pigment content of fruit juices, beverages, natural colorants, and  
721 wines by the pH differential method: Collaborative study. *Journal of AOAC  
722 International*, 88(5), 1269–1278. <https://doi.org/10.1093/jaoac/88.5.1269>

723 Lee, S. H., Heng, D., Ng, W. K., Chan, H. K., & Tan, R. B. H. (2011). Nano spray  
724 drying: A novel method for preparing protein nanoparticles for protein therapy.  
725 *International Journal of Pharmaceutics*, 403(1–2), 192–200.  
726 <https://doi.org/10.1016/J.IJPHARM.2010.10.012>

727 Li, C. X., Wang, F. R., Zhang, B., Deng, Z. Y., & Li, H. Y. (2023). Stability and  
728 antioxidant activity of phenolic compounds during in vitro digestion. *Journal of  
729 Food Science*, 88(2), 696–716. <https://doi.org/10.1111/1750-3841.16440>

730 López-Rubio, A., & Lagaron, J. M. (2012). Whey protein capsules obtained through  
731 electrospraying for the encapsulation of bioactives. *Innovative Food Science and  
732 Emerging Technologies*, 13(JANUARY), 200–206.  
733 <https://doi.org/10.1016/j.ifset.2011.10.012>

734 Ludwig, I. A., Mena, P., Calani, L., Borges, G., Pereira-Caro, G., Bresciani, L., Del Rio,  
735 D., Lean, M. E. J., & Crozier, A. (2015). New insights into the bioavailability of  
736 red raspberry anthocyanins and ellagitannins. *Free Radical Biology and Medicine*,  
737 89, 758–769. <https://doi.org/10.1016/J.FREERADBIOMED.2015.10.400>

738 Martins, I. M., Macedo, G. A., & Macedo, J. A. (2020). Biotransformed grape pomace  
739 as a potential source of anti-inflammatory polyphenolics: Effects in Caco-2 cells.  
740 *Food Bioscience*, 35. <https://doi.org/10.1016/j.fbio.2020.100607>

741 Martins, I. M., Macedo, G. A., Macedo, J. A., Roberto, B. S., Chen, Q., Blumberg, J. B.,  
742 & Chen, C. Y. O. (2017). Tannase enhances the anti-inflammatory effect of grape  
743 pomace in Caco-2 cells treated with IL-1 $\beta$ . *Journal of Functional Foods*, 29, 69–  
744 76. <https://doi.org/10.1016/j.jff.2016.12.011>

745 Meng, Y., & Li, C. (2021). Conformational changes and functional properties of whey  
746 protein isolate-polyphenol complexes formed by non-covalent interaction. *Food*  
747 *Chemistry*, 364(February), 129622.  
748 <https://doi.org/10.1016/j.foodchem.2021.129622>

749 Meng, Y., Liang, Z., Zhang, C., Hao, S., Han, H., Du, P., Li, A., Shao, H., Li, C., & Liu,  
750 L. (2021). Ultrasonic modification of whey protein isolate: Implications for the  
751 structural and functional properties. *Lwt*, 152(March), 112272.  
752 <https://doi.org/10.1016/j.lwt.2021.112272>

753 Milinčić, D. D., Stanisavljević, N. S., Kostić, A., Soković Bajić, S., Kojić, M. O., Gašić,  
754 U. M., Barać, M. B., Stanojević, S. P., Lj Tešić, Ž., & Pešić, M. B. (2021).  
755 Phenolic compounds and biopotential of grape pomace extracts from Prokupac red  
756 grape variety. *LWT*, 138, 110739. <https://doi.org/10.1016/J.LWT.2020.110739>

757 MohdMadin, N., Michael, N., Oruna-Concha, M. J., & Jauregi, P. (2018). Polyphenols  
758 extracted from red grape pomace by a surfactant based method show enhanced  
759 collagenase and elastase inhibitory activity. *Journal of Chemical Technology and*  
760 *Biotechnology*, 93(7), 1916–1924. <https://doi.org/10.1002/jctb.5459>

761 MohdMadin, N., Oruna-Concha, M. J., & Jauregi, P. (2019). Surfactant TWEEN20  
762 provides stabilisation effect on anthocyanins extracted from red grape pomace.  
763 *Food Chemistry*, 271, 224–231. <https://doi.org/10.1016/j.foodchem.2018.07.083>

764 Moreno, T., Cocero, M. J., & Rodríguez-Rojo, S. (2018). Storage stability and  
765 simulated gastrointestinal release of spray dried grape marc phenolics. *Food and*  
766 *Bioproducts Processing*, 112, 96–107. <https://doi.org/10.1016/J.FBP.2018.08.011>

767 Peixoto, C. M., Dias, M. I., Alves, M. J., Calhelha, R. C., Barros, L., Pinho, S. P., &  
768 Ferreira, I. C. F. R. (2018). Grape pomace as a source of phenolic compounds and  
769 diverse bioactive properties. *Food Chemistry*, 253(January), 132–138.  
770 <https://doi.org/10.1016/j.foodchem.2018.01.163>

771 Pintać, D., Majkić, T., Torović, L., Orčić, D., Beara, I., Simin, N., Mimica-Dukić, N.,  
772 & Lesjak, M. (2018). Solvent selection for efficient extraction of bioactive  
773 compounds from grape pomace. *Industrial Crops and Products*, 111(October  
774 2017), 379–390. <https://doi.org/10.1016/j.indcrop.2017.10.038>

775 Raei, M., Rafe, A., & Shahidi, F. (2018). Rheological and structural characteristics of  
776 whey protein-pectin complex coacervates. *Journal of Food Engineering*, 228, 25–  
777 31. <https://doi.org/10.1016/J.JFOODENG.2018.02.007>

778 Raei, M., Shahidi, F., Farhoodi, M., Jafari, S. M., & Rafe, A. (2017). Application of  
779 whey protein-pectin nano-complex carriers for loading of lactoferrin. *International*  
780 *Journal of Biological Macromolecules*, 105, 281–291.  
781 <https://doi.org/10.1016/j.ijbiomac.2017.07.037>

782 Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999).  
783 Antioxidant activity applying an improved ABTS radical cation decolorization  
784 assay. *Free Radical Biology and Medicine*, 26(9–10), 1231–1237.  
785 [https://doi.org/10.1016/S0891-5849\(98\)00315-3](https://doi.org/10.1016/S0891-5849(98)00315-3)

786 Rehman, A., Ahmad, T., Aadil, R. M., Spotti, M. J., Bakry, A. M., Khan, I. M., Zhao,  
787 L., Riaz, T., & Tong, Q. (2019). Pectin polymers as wall materials for the nano-  
788 encapsulation of bioactive compounds. *Trends in Food Science and Technology*,  
789 90(March), 35–46. <https://doi.org/10.1016/j.tifs.2019.05.015>

790 Reichembach, L. H., & Lúcia de Oliveira Petkowicz, C. (2021). Pectins from alternative  
791 sources and uses beyond sweets and jellies: An overview. *Food Hydrocolloids*,  
792 118(April), 106824. <https://doi.org/10.1016/j.foodhyd.2021.106824>

793 Rinaldi, A., Louazil, P., Iturmendi, N., Moine, V., & Moio, L. (2020). Effect of marc  
794 pressing and geographical area on Sangiovese wine quality. *Lwt*, 118(October  
795 2019), 108728. <https://doi.org/10.1016/j.lwt.2019.108728>

796 Scalbert, A., Morand, C., Manach, C., & Rémesy, C. (2002). Absorption and  
797 metabolism of polyphenols in the gut and impact on health. *Biomedicine &*  
798 *Pharmacotherapy*, 56(6), 276–282. [https://doi.org/10.1016/S0753-3322\(02\)00205-6](https://doi.org/10.1016/S0753-3322(02)00205-6)

800 Scalbert, A., & Williamson, G. (2000). Dietary Intake and Bioavailability of  
801 Polyphenols. *The Journal of Nutrition*, 130(8), 2073S-2085S.  
802 <https://doi.org/10.1093/JN/130.8.2073S>

803 Sessa, M., Casazza, A. A., Perego, P., Tsao, R., Ferrari, G., & Donsì, F. (2013).  
804 Exploitation of Polyphenolic Extracts from Grape Marc as Natural Antioxidants by  
805 Encapsulation in Lipid-Based Nanodelivery Systems. *Food and Bioprocess  
806 Technology*, 6(10), 2609–2620. <https://doi.org/10.1007/s11947-012-0911-9>

807 Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of Total Phenolics with  
808 Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology  
809 and Viticulture*, 16, 144–158.  
810 <http://www.ajevonline.org/content/16/3/144.full.pdf+html>

811 Spigno, G., Amendola, D., Dahmoune, F., & Jauregi, P. (2015). Colloidal gas aphrons  
812 based separation process for the purification and fractionation of natural phenolic  
813 extracts. *Food and Bioproducts Processing*, 94, 434–442.  
814 <https://doi.org/10.1016/J.FBP.2014.06.002>

815 Spigno, G., Donsì, F., Amendola, D., Sessa, M., Ferrari, G., & De Faveri, D. M. (2013).  
816 Nanoencapsulation systems to improve solubility and antioxidant efficiency of a  
817 grape marc extract into hazelnut paste. *Journal of Food Engineering*, 114(2), 207–  
818 214. <https://doi.org/10.1016/j.jfoodeng.2012.08.014>

819 Spigno, G., Marinoni, L., & Garrido, G. D. (2017). State of the Art in Grape Processing  
820 By-Products. *Handbook of Grape Processing By-Products: Sustainable Solutions*,  
821 1–27. <https://doi.org/10.1016/B978-0-12-809870-7.00001-6>

822 Spigno, G., Tramelli, L., & De Faveri, D. M. (2007). Effects of extraction time,  
823 temperature and solvent on concentration and antioxidant activity of grape marc  
824 phenolics. *Journal of Food Engineering*, 81(1), 200–208.  
825 <https://doi.org/10.1016/j.jfoodeng.2006.10.021>

826 Stalmach, A., Troufflard, S., Serafini, M., & Crozier, A. (2009). Absorption,  
827 metabolism and excretion of Choladi green tea flavan-3-ols by humans. *Molecular*  
828 *Nutrition and Food Research*, 53(SUPPL. 1).  
829 <https://doi.org/10.1002/MNFR.200800169>

830 Teng, H., & Chen, L. (2019). *Critical Reviews in Food Science and Nutrition*  
831 *Polyphenols and bioavailability: an update.*  
832 <https://doi.org/10.1080/10408398.2018.1437023>

833 Tsao, R. (2010). Chemistry and Biochemistry of Dietary Polyphenols. *Nutrients*, 2,  
834 1231–1246. <https://doi.org/10.3390/nu2121231>

835 Wang, S., Mateos, R., Goya, L., Amigo-Benavent, M., Sarriá, B., & Bravo, L. (2016). A  
836 phenolic extract from grape by-products and its main hydroxybenzoic acids protect  
837 Caco-2 cells against pro-oxidant induced toxicity. *Food and Chemical Toxicology*,  
838 88, 65–74. <https://doi.org/10.1016/j.fct.2015.12.005>

839 Wolfe, K. L., Kang, X., He, X., Dong, M., Zhang, Q., & Liu, R. H. (2008). Cellular  
840 antioxidant activity of common fruits. *Journal of Agricultural and Food*  
841 *Chemistry*, 56(18), 8418–8426. <https://doi.org/10.1021/jf801381y>

842 Wusigale, Liang, L., & Luo, Y. (2020). Casein and pectin: Structures, interactions, and  
843 applications. *Trends in Food Science and Technology*, 97(January), 391–403.  
844 <https://doi.org/10.1016/j.tifs.2020.01.027>

845 Yalçın, A. S. (2006). Emerging therapeutic potential of whey proteins and peptides.  
846 *Current Pharmaceutical Design*, 12(13), 1637–1643.  
847 <https://doi.org/10.2174/138161206776843296>

848 Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., & Dong, W. (2016). ROS and  
849 ROS-Mediated Cellular Signaling. *Oxidative Medicine and Cellular Longevity*,  
850 2016. <https://doi.org/10.1155/2016/4350965>

851 Zhao, X., Zhu, H., Zhang, G., & Tang, W. (2015). Effect of superfine grinding on the  
852 physicochemical properties and antioxidant activity of red grape pomace powders.  
853 *Powder Technology*, 286, 838–844. <https://doi.org/10.1016/j.powtec.2015.09.025>

854 Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid  
855 contents in mulberry and their scavenging effects on superoxide radicals. *Food*  
856 *Chemistry*, 64(4), 555–559. [https://doi.org/10.1016/S0308-8146\(98\)00102-2](https://doi.org/10.1016/S0308-8146(98)00102-2)

857