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# 1 **Controlled release of riboflavin encapsulated in pea protein** 2 **microparticles prepared by emulsion-enzymatic gelation process**

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## 9 **Abstract**

10 Riboflavin was encapsulated in pea protein microparticles crosslinked by transglutaminase  
11 and the release properties of this system was studied in simulated gastric (pH=1.2) and  
12 intestinal (pH=7.4) fluids. The microparticles were obtained from a water-in-oil emulsion **and**  
13 **subsequent** enzymatic gelation. In presence of polyglycerol polyricinoleate as hydrophobic  
14 surfactant, magnetic agitation was preferred to high-speed homogenization in order to form  
15 spherical microparticles of ~150 µm average size. The encapsulation **efficiency** of crystallized  
16 riboflavin (RF) varied from 74% to 84% depending on the amount of loaded RF (0.1-1.0  
17 wt%). According to the kinetic power law model, the release mechanisms of riboflavin **under**  
18 **simulated** gastrointestinal conditions were governed by diffusion in absence of digestive  
19 enzymes and by a support degradation phenomenon in **their** presence. The designed  
20 microparticles **represented** a potential system for the delivery of riboflavin **up to** the intestinal  
21 digestion phase.

22

23 **Keywords** : riboflavin, pea proteins, emulsion, transglutaminase, encapsulation, release

24

## 25 **1. Introduction**

26 The encapsulation of active substances is one of the major innovation challenges in the  
27 pharmaceutical, cosmetic and food industries. Encapsulation is a technology that can be  
28 applied to a large number of bioactive molecules (antioxidants, vitamins, probiotics, minerals)  
29 and **aims** to protect or isolate them from adverse environments, or **allows to** control the  
30 release of the encapsulated substances in the gastrointestinal tract ([Đorđević et al, 2015](#)).

31 In food systems, many of these molecules have low solubility, are sensitive to environmental  
32 factors (such as light, temperature and oxygen), or are degraded by the conditions encountered  
33 in the gastrointestinal tract (digestive enzymes, pH). **These properties can thus affect**  
34 **significantly** their bioavailability. The **basis** of encapsulation is to create a biocompatible  
35 barrier between the environment and the substance **entrapped** in the protective matrix  
36 ([McClements, 2015](#)). Various protection and delivery systems have been proposed to form  
37 (micro-)capsules which can release their contents under specific conditions. One well-known  
38 strategy is that of trapping bioactive molecules in hydrogel matrices ([McClements, 2017b](#)).  
39 Hydrogels are three-dimensional cross-linked networks of natural macromolecules adapted to  
40 the controlled release of bioactive compounds due to their sensitivity to environmental  
41 stimuli, such as pH and digestive enzymes in the gastrointestinal tract. Proteins, such as dairy  
42 or plant proteins, have been **stated as** attractive choices for designing hydrogels **thanks** to their  
43 high nutritional value, **their** excellent functional properties and **their** amphiphilic nature  
44 **compatible with** many active substances ([Shewan & Stokes, 2013](#)). **Furthermore these**  
45 **hydrogels are particularly suitable to entrap hydrophilic compounds thanks to their capacity to**  
46 **retain water** ([Aditya et al, 2017 ; McClements, 2017a](#)).

47 Among hydrophilic bioactive molecules, Riboflavin (RF), also known as vitamin B2, is a  
48 water-soluble vitamin indispensable for living beings. **It** plays an essential role in energy

49 metabolism of cells and acts as coenzyme factor in various oxidation-reduction processes in  
50 cell (Suwannasom et al. 2020). Sufficient dietary or supplemental intake in RF appears to  
51 have biological protective effects encompassing anti-oxidant, anti-aging, anti-inflammatory,  
52 anti-nociceptive and anti-cancer properties (Suwannasom et al. 2020). However, this molecule  
53 of low molar mass (376,36 g/mole) is highly photosensitive and slightly soluble in water  
54 (~0.08 g/L at 25 °C) (Morrison et al, 2013 ; Sheraz et al, 2014). Many encapsulation strategies  
55 have been proposed in the past 10 years to overcome these drawbacks and to offer  
56 nutraceutical solutions. In addition, RF has been chosen as a model molecule in several  
57 studies on encapsulation and controlled release of hydrophilic bioactive compounds. Thus, in  
58 the recent period authors have investigated the encapsulation of RF in different biopolymeric  
59 systems. For instance, particulate systems were proposed such as hydrogel microspheres  
60 based on proteins (Chen & Subirade, 2009 ; O'Neill et al, 2014) or sodium alginate (El-  
61 Ghaffar et al, 2012 ; Messaoud et al, 2016), dried polysaccharide carriers (de Farias et al,  
62 2018 ; Prosapio et al, 2020), or water-in-water emulsions (Chen et al, 2019). Different  
63 macromolecular complexes such as inclusion complexes in cyclodextrins (Terekhova et al,  
64 2011), protein-polysaccharide electrostatic complexes (Kurukji et al, 2016),  $\beta$ -lactoglobulin-  
65 based nanocomplexes (Madalena et al, 2016) or molecularly imprinted polymers (Mokhtari &  
66 Ghaedi, 2019), were also investigated. Finally, lipid-based systems such as double emulsions  
67 (Bou et al, 2014), liposomes (Ahmad et al, 2015), or solid lipid nanoparticles (Couto et al,  
68 2017), were designed. Recently, some authors proposed the use of enzymatically crosslinked  
69 plant protein-based hydrogels by using laccase (Yan et al, 2020) or transglutaminase (Wen et  
70 al, 2018) for RF encapsulation. In these latter works, encapsulation systems were designed as  
71 macrogels, divided in small pieces for release properties evaluation. Assuming that delivery  
72 systems usually gain advantages in the form of microparticles, the present paper proposes to  
73 design microparticulated hydrogels from crosslinked pea proteins for RF encapsulation.

74 Among plant proteins, pea proteins are considered as good candidates to design encapsulation  
75 carrier because of their sustainable production, non-allergenic status and tunable functional  
76 properties (Quintero et al, 2018). Furthermore, they have demonstrated good susceptibility to  
77 enzymatic crosslinking with transglutaminase, due to their high content in reactive amino acid  
78 residues (glutamine and lysine) (Djoullah et al, 2015).

79 In the present work, we evaluated the encapsulation of RF in microparticulated pea protein  
80 hydrogels obtained from a double stage process based on the formation of water-in-oil  
81 emulsion and subsequent enzymatic gelation of the internal protein phase by using  
82 transglutaminase. The emulsification process was first developed to form spherical  
83 microparticles of a few tens of micrometers. In a second step, we investigated the in vitro  
84 kinetics and release mechanisms of RF in simulated gastric and intestinal fluids.

## 85 2. Material and methods

### 86 2.1. Materials

87 Pea flour from yellow peas (*P. sativum* L.) was supplied by Roquette SA (Lestrem, France). A  
88 Ca<sup>2+</sup>-independent microbial transglutaminase (MTGase) derived from *Streptoverticillium*  
89 *mobarraense* was obtained from Ajinomoto Foods Europe SAS, France. The commercial  
90 enzyme preparation (Activia<sup>®</sup> WM) contains 1% active enzyme and 99% maltodextrin. The  
91 powdered preparation was assessed to have an activity of 100 U/g. The oily phase (Miglyol  
92 812 N) was supplied by Sasol Germany GmbH (Germany). Miglyol is a neutral, colorless,  
93 odorless and tasteless edible oil containing medium chain triglycerides. It has a high oxidation  
94 stability and remains liquid at 0 °C. Polyglycerol polyricinoleate (PGPR, Grinsted<sup>®</sup> PGPR  
95 90, E 476) was obtained from Danisco (Denmark). PGPR is a hydrophobic emulsifier  
96 (HLB=1.5±0.5) used for the stabilization of water-in-oil emulsions. Riboflavin, vitamin B2,

97 was supplied by Sigma Aldrich® (France). It is a water-soluble (67-333 mg/L) orange  
98 powder. All other chemicals of analytical grade were purchased to VWR International SAS  
99 (France) and Sigma–Aldrich® (France).

## 100 **2.2. Methods**

### 101 **2.2.1. Pea protein extraction**

102 The extraction of the total pea protein fraction (TPP) was carried out by dispersing 100 g of  
103 pea flour in 1 liter of phosphate buffer (0.1 M, pH=8) in the presence of K<sub>2</sub>SO<sub>4</sub> 1% for 1 h at  
104 4 °C. The protein extract (supernatant) was recovered by centrifugation (12,000g, 10 min)  
105 then concentrated (x5) by using an ultrafiltration device (Millipore Pellicon 2®) equipped  
106 with a Kwick™ cassette (0.11 m<sup>2</sup>; 10 kDa cutoff) and operating at 2.2 bar at ambient  
107 temperature. The liquid sample was then purified by diafiltration against 10 volumes of a 5  
108 mM solution of ammonium carbonate by using the same ultrafiltration device. The pea  
109 protein extract obtained was consecutively frozen and lyophilized. The dried protein extract  
110 was collected, grinded and stored at -20 °C. The freeze-dried protein isolate had a protein  
111 content of 87.2% on a dry basis and a 3.6% water content. The polypeptide profile determined  
112 by SDS-PAGE indicated that the protein isolate contained ~20% albumins and ~80%  
113 globulins (data not shown).

### 114 **2.2.2. Microparticle preparation**

115 The pea protein microparticles were prepared by using a microencapsulation process similar  
116 to that proposed by Heidebach et al (2009). The procedure consisted in forming a water-in-oil  
117 (W/O) emulsion and subsequently applying enzymatic crosslinking for gelling the dispersed  
118 protein phase. First, a 15 wt% TPP dispersion was prepared in phosphate buffer (50 mM,  
119 pH=7). After 2 h stirring, the MTgase preparation was quickly dispersed in the protein

120 solution at the rate of 20 MTGase U/g protein and the mixture (20 g) was slowly introduced  
121 under magnetic stirring (~ 900 rpm) in vials containing 100 g of oil (Miglyol® 812 N)  
122 previously heated to 45 °C with or without PGPR. The samples were maintained under  
123 stirring during 2 h at 45 °C. After incubation, the dispersed TPP globules were gelled **in the**  
124 **form of** microparticles under the action of MTGase. The microparticles were separated from  
125 the oil by gentle centrifugation (500g, 5 min), washed twice with 2% Tween 80 solution, then  
126 collected on a filter paper and freeze-dried. Alternatively, in order to reduce the size of the  
127 microparticles, a thinner emulsion was formed by 5 min homogenization with an Ultra-Turrax  
128 (IKA T25) at 10,000 rpm before incubation in the same conditions.

### 129 **2.2.3. Microparticle size**

130 The size distribution of the microparticles was determined by a Mastersizer 2000 laser  
131 granulometer (Malvern Instruments, Southborough, MA). The average size of protein  
132 microparticles were calculated as the Sauter mean diameter ( $d_{32}$ ).

### 133 **2.2.4. Microscopy**

134 The morphology and external structure of the microparticles were visualized by an optical  
135 microscope (Nikon TE2000) equipped with a camera (EMCCO Andor iXon +885).

### 136 **2.2.5. Microparticle dissolution rate**

137 The dissolution of the microparticles was evaluated in synthetic gastric (HCl solution at  
138 pH=1.2) **and intestinal (phosphate buffer at pH=7.4) juices with or without 0.1% pepsin and**  
139 **1% pancreatin respectively (Chen and Subirade, 2009)**. For that, **200** mg of freeze-dried  
140 microparticles were dispersed in the four digestive fluids (30 ml) with continuous stirring  
141 (~100 rpm) at 37 °C. Two ml of supernatant excluding any particles were taken at regular



142 time intervals and the amount of protein was assayed by Kjeldahl method. After each  
143 sampling, an equivalent volume of fresh digestive juice was added to maintain a constant  
144 volume. The percentage of solubilized microparticles was calculated from Eq.1.

$$145 \quad \% \text{ solubilized microparticles} = (Q_{Pi}/Q_{PT}) * 100 \quad (1)$$

146 where  $Q_{Pi}$  is the cumulative amount of dissolved proteins at time  $i$ ;  $Q_{PT}$  is the amount of  
147 proteins after complete microparticle dissolution (10 h).

### 148 2.2.6. Riboflavin encapsulation

149 The RF encapsulation process was performed as previously described for the preparation of  
150 microparticles. RF was introduced into the protein solution at 0.1, 0.5 or 1% concentration  
151 before the addition of MTGase. Considering the low solubility of RF powder, the loaded RF  
152 in microparticles remained mainly in a crystal form.

153 To evaluate the encapsulation rate of RF in TPP microparticles, encapsulation efficiency (EE)  
154 and loading efficiency (LE) were calculated according to the method of Chen and Subirade  
155 (2009) with some adaptations. For that, 200 mg of RF-loaded microparticles were dissolved in  
156 25 ml of intestinal juice (pH=7.4) with 1% pancreatin at 37 °C under vigorous stirring for 6 h.  
157 The mixture was then centrifuged (10,000g, 20 min) and RF was assayed in the supernatant  
158 with an UV spectrophotometer (Spectronic BioMate 3) by measuring absorbance at 445 nm  
159 and using a calibration curve. EE and LE were calculated by Eqs. 2 and 3 respectively.

$$160 \quad EE = A / B * 100 \quad (2)$$

$$161 \quad LE = A / C * 100 \quad (3)$$

162 where  $A$  is the measured amount of RF encapsulated in the sample (mg);  $B$  is the theoretical  
163 amount of RF corresponding of the initial load in the sample (mg);  $C$  is the weight of  
164 microparticle sample (mg).

### 165 **2.2.7. Release kinetics in simulated gastro-intestinal fluids**

166 The RF release kinetics were studied in the gastric and intestinal juices with or without  
167 digestive enzymes as proposed by Chen & Subirade (2009) with some modifications. 200 mg  
168 of lyophilized microparticles were suspended in 30 ml of each digestive fluid with gentle  
169 stirring (~100 rpm) at 37 °C. Two milliliters of supernatant excluding any particles were  
170 removed at regular time intervals and then centrifuged (12,000g, 10 min) and the amount of  
171 RF in the supernatant was assayed by absorbance measurement at 445 nm. After each  
172 sampling, an equivalent volume of fresh digestive juice was added to maintain a constant  
173 volume. The percentage of RF released at each time was calculated from Eq. 4.

$$174 \quad \% \text{ of released RF} = (QR_i/QRT) * 100 \quad (4)$$

175 where  $QR_i$  is the amount of RF released at time  $i$  (mg);  $QRT$  is the amount of total RF  
176 encapsulated in sample estimated after total dissolution of microparticles (6 h) in the fluid  
177 containing digestive enzymes (mg).

### 178 **2.2.8. Release kinetics modelling**

179 A semi-empirical model, establishing the exponential relationship between the release rate  
180 and the time, was used to evaluate the main controlling phenomena. This model known as the  
181 Peppas equation (Ritger & Peppas, 1987) represents the release rate as a power law function  
182 of time as follows:

$$183 \quad Mt/M_{\infty} = K * t^n \quad (5)$$

184 *where  $M_{\infty}$  is the amount of active molecule released at the equilibrium (it was assumed that*  
185 *total RF release was achieved at an infinite time);  $M_t$  is the amount of active molecule*  
186 *released over time  $t$ ;  $K$  is the the release velocity constant, and  $n$  is the exponent of release.*

### 187 **2.2.9. Statistic analysis**

188 All measurements were performed with at least three replicates. The statistical analyses were  
189 performed with XLSTAT Pro v2013 software by analysis of variance (ANOVA). A Tukey  
190 post-hoc test was used to determine significant differences between group means with a  
191 confidence interval of 95% ( $p < 0.05$ ). *Linear regressions and related statistical parameters*  
192 *were obtained by using Excel® software (2016, 16.0.5017.1000).*

### 193 3. Results and discussion

#### 194 3.1. Elaboration and characteristics of microcapsules

195 In order to produce individualized gelled microparticles, a reverse emulsion was first formed  
196 by dispersing a 15 wt% TPP suspension containing MTGase in Miglyol oil and incubating the  
197 mixture for 2 h at 45 °C. The protein concentration, incubation temperature and time were  
198 chosen from preliminary gelation experiments in order to form strong crosslinked protein gels  
199 (data not shown). In order to improve the formation of the W/O emulsion, a hydrophobic  
200 emulsifying agent (PGPR) was used. Fig. 1 shows light microscopy images of the W/O  
201 emulsions after gelation in the absence (Fig. 1a) and in the presence of 1% (Fig. 1b) or 2%  
202 (Fig. 1c) PGPR. Without PGPR, the dispersed TPP was gelled in the form of a semi-  
203 continuous network composed of interconnected blocks of irregular shape. This could be  
204 explained by the poor ability of protein to stabilize reverse emulsion. Proteins are hydrophilic  
205 emulsifiers that do not generate W/O interfaces stable enough to prevent the association of  
206 TPP globules with each other (Lam & Nickerson, 2013).

207 With 1% PGPR addition (Fig. 1b), the gels were in the form of aggregated microparticles of  
208 spherical shape. PGPR is a lipophilic emulsifying agent that facilitate the formation of the  
209 W/O interfaces and dispersed globules with regular shape (Zhu et al., 2019). The formation of  
210 W/O emulsions generally needs high emulsifying agent concentrations. The 1% PGPR  
211 concentration was insufficient to prevent flocculation among microparticles. A higher  
212 quantity of PGPR was thus necessary to separate the clustered microparticles. Indeed, the use  
213 of 2% of PGPR (Fig. 1c) led to the formation of individual microparticles of spherical shape.  
214 Synergistic effect may occur between PGPR and the proteins from the internal phase, that  
215 would enhance the efficiency of the lipophilic emulsifying agent (Zhu et al., 2019).

216 The size distribution of the microparticles produced under different conditions were evaluated  
217 and typical size profiles are presented in Fig. 2. The microparticles produced in the presence  
218 of 1% of PGPR under 900 rpm magnetic agitation had a non-Gaussian and relatively wide  
219 distribution with an average diameter of ~320  $\mu\text{m}$ , reflecting a multimodal distribution. This  
220 result was consistent with the microscopic observations (Figure 1b) showing that the  
221 microparticles gathered in small groups. The size distribution of the systems with 2% and 3%  
222 PGPR addition had Gaussian and narrow profiles centered to ~153 and ~149  $\mu\text{m}$  respectively.  
223 These microparticle size values were close to those obtained by Heidebach et al. (2009) who  
224 used a similar process for encapsulating probiotic cells in MTGase-induced caseinate gels.  
225 These sizes cover the main food applications (Heidebach et al., 2009). To produce smaller  
226 microparticles, the emulsion was prepared using an Ultra-Turrax homogenizer (5 min, 10,000  
227 rpm) with 2% PGPR before incubation. The high-speed homogenization step led to a  
228 monomodal size distribution with a ~28  $\mu\text{m}$  average diameter.

229 In the case of food applications, the size of the microcapsules is a determining factor. While  
230 large microparticles can change sensory perception, too small microparticles are sometimes  
231 unable to offer satisfactory encapsulation capacity. Indeed, the release of the encapsulated  
232 molecules is controlled by diffusion phenomena and/or degradation of the matrix, which are  
233 generally inversely proportional to the size of the microparticles (Berkland et al., 2002).

234 The degradation of crosslinked TPP-based microparticles was studied in **synthetic** gastric and  
235 intestinal fluids **with or without** digestive enzymes at 37 °C. For that, freeze-dried  
236 microparticles **resulting** from emulsions prepared using gentle agitation and **with** 2% PGPR,  
237 were **assessed**. The dissolution profiles of the microparticles **were** recorded for 10 hours **and**  
238 **are reported in** Fig. 3.

239 In the absence of digestive enzymes, the microparticles **were slightly solubilized** (<10%)  
240 whatever the pH of the fluid (pH=1.2 and pH=7.4) even up 48 h dissolution (results not  
241 shown). **This observation is in agreement with previous works showing the stability of**  
242 **MTGase-induced protein microparticles in gastric juice without pepsin (Heidebach et al.,**  
243 **2009). Limited particle dissolution** is a desirable feature to control the release of the  
244 encapsulated molecules. **Cho et al (2003)** showed **that thermal gels of soy proteins** were 5  
245 times more **solubilized** than gels prepared **by enzymatic crosslinking**. This was due to the  
246 nature of the bonds stabilizing the protein network, which are covalent in the case of  
247 enzymatic crosslinking and mainly non-covalent in the case of thermal gelation.

248 On the other hand, the **addition** of digestive enzymes accelerated the degradation of  
249 microparticles (**Fig.3**). The degradation was faster in the intestinal conditions than in the  
250 gastric ones. **In the intestinal fluid, the dissolution rate reached 80% and 100% after 90 min**  
251 **and 4 h incubation respectively. Under** gastric conditions, the dissolution rate was limited to  
252 32% after 90 min and complete dissolution was achieved **after** 10 h. **Smaller degradation of**  
253 **microparticles in gastric juice with pepsin in comparison to intestinal conditions was also**  
254 **recently reported for similar enzymatically crosslinked protein hydrogels (Wen et al, 2018;**  
255 **Yan et al, 2020).** The **observed dissolution** behavior seemed **adapted** to protect the active  
256 molecules from the gastric environment and **significantly** release them **during** the intestinal  
257 **phase**, as the gastric transit time is typically not longer than 1-2 h (**Gonçalves et al, 2018**).

### 258 3.2. Riboflavin encapsulation

259 In order to encapsulate RF in the microparticles, RF was dispersed in the TPP solution before  
260 emulsification. Three RF contents (0.1, 0.5 and 1.0 wt%) in the protein solution were studied  
261 to assess the loading capacity of the microparticles. The microcapsules were prepared from  
262 W/O emulsions containing 2% PGPR using magnetic agitation only or with pre-  
263 homogenization by Ultra-Turrax. The encapsulation efficiency (EE) as well as the loading  
264 efficiency (LE) of the microparticles are reported in Table 1. The size of the loaded  
265 microparticles resulting from mild agitation condition was close to 150  $\mu\text{m}$  whatever the RF  
266 concentration. The size of the microparticles prepared with high-speed homogenization was  
267 significantly lower ( $\sim 32 \mu\text{m}$ ) in agreement with the previous observations (section 3.1). For  
268 the largest microparticles, the EE values decreased significantly from  $\sim 84\%$  to  $\sim 74\%$  when  
269 increasing RF concentration, while the smallest microparticles had the lowest EE ( $\sim 56\%$ ).  
270 The same trends were logically observed for LE values. The microparticle size variation from  
271  $\sim 150$  to  $\sim 30 \mu\text{m}$ , following the use of high-speed homogenization, resulted in one-third  
272 decrease of LE and EE values. The EE values were in the same order of magnitude as those  
273 found by Chen et al. (2007) for RF encapsulation in alginate-whey protein microparticles  
274 generated from an emulsification and internal gelation process. The decrease in EE was  
275 mainly explained by the size of the RF crystals relative to the microparticle size. This  
276 behavior was confirmed by microscopic observations of loaded microparticles (Fig. 4). It was  
277 clearly observed that the larger microparticles (Fig. 4a) are significantly better suited for  
278 incorporating large RF crystals than the smaller microparticles (Fig. 4b). These observations  
279 were again consistent with the findings of Chen et al (2007) for alginate-whey protein  
280 microspheres ( $<100 \mu\text{m}$ ) encapsulating RF crystals. Typically, RF solubility vary between a  
281 few mg/L and a few tens of mg/L depending on the crystal form, the solvent temperature and  
282 pH (Rivlin, 2012). Owing to its low solubility, RF remained mainly crystallized in the form of

283 “short thin needles” (5 to 10  $\mu\text{m}$ ) in the TPP microparticles after encapsulation. Because of  
284 these particular shape and size, the RF crystals were not totally incorporated in 30  $\mu\text{m}$   
285 microparticles. Part of the RF flakes remaining outside the microparticles would be then  
286 dissolved and/or removed by the washing step after enzymatic incubation.

### 287 3.3. Release kinetics and mechanism

288 The microparticles produced from W/O emulsion by applying magnetic stirring at 1% RF  
289 concentration were chosen for further release experiments. The release kinetics of RF from  
290 the loaded microparticles was carried out at 37 °C in four different fluids: pH=1.2; pH=1.2 +  
291 pepsin; pH=7.4 and pH=7.4 + pancreatin for 6 hours. The corresponding results are presented  
292 in Fig. 5.

293 The release of RF was enhanced by the addition of digestive enzymes in both gastric and  
294 intestinal juices. RF release evolved faster under intestinal conditions (pH=7.4 + pancreatin)  
295 than under gastric ones (pH=1.2 + pepsin). Complete release of RF (100%) was achieved after  
296 4 and 6 h respectively. These release profiles were very similar to those observed for the  
297 dissolution experiments (Fig. 3) in intestinal conditions but slightly different in the case of  
298 gastric conditions since the degradation was longer in the latter case. The present release  
299 behaviours reflected probably different phenomena such as mainly matrix degradation,  
300 solubilization of crystallized RF and/or its diffusion. As shown in Fig. 4C at a microscopic  
301 scale, the surface of TPP microparticles were early degraded in the intestinal juice with  
302 pancreatin, whereas the peripheral RF crystals became exposed. The gradual release of RF  
303 from the outer layers to the inner layers due to the proteolytic action of pancreatin could be  
304 thus hypothesized.



305 In the absence of digestive enzymes, RF release rates evolved **much** more slowly  
306 **independently** of digestive conditions (pH=1.2; pH=7.4), reaching only a 40% value after 6 h.  
307 The release profiles differed in intensity from those observed under the same conditions for  
308 microparticle dissolution since the degradation rate **remained below** 10% in the latter case  
309 (Fig. 3). This could indicate that diffusion phenomena predominated in the case of limited  
310 microparticle degradation.

311 The release mechanisms of active molecules from polymeric matrices are generally based on  
312 two main phenomena: degradation (dissolution **or** erosion) of the encapsulating support and  
313 diffusion of the active molecule through the support; but other phenomena have to be taken  
314 into account such as swelling, glass transition, chemical degradation, osmotic effect or  
315 reaction. (Peppas & Narasimhan, 2014). Many mathematical models have been proposed to  
316 simulate the release kinetics of encapsulated agents from hydrogel-**based** systems (Lin &  
317 Metters, 2006, Peppas & Narasimhan, 2014). In the present paper, the Peppas equation (Eq. 5)  
318 was used in order to distinguish between the main controlling phenomena. This **power law**  
319 model is generally applicable for the first ~60% of the release profile (Lin & Metters, 2006),  
320 so that it was applied to release rate values below 70% in a logarithmic representation for all  
321 the **experimental** release kinetics (Fig. 6). Thus, the n value corresponded to the slope of the  
322 interpolating straight line for each release condition. The experimental values of n as well as  
323 the regression coefficients ( $R^2$ ) were reported Table 2.

324 All the regression coefficients were found higher than ~0.96 indicating the appropriateness of  
325 the model to fit the data. The n values **varied** between 0.48 and 0.81 **and were** compared to **the**  
326 **theoretical values corresponding to** Fickian and non-Fickian transport behaviours (Ritger &  
327 Peppas, 1987). In a pure Fickian model (Case I),  $n = 0.45$  for a spherical geometry and the  
328 active molecule release is governed by diffusion. The exponent values calculated for the

329 simulated gastric and intestinal fluids without digestive enzyme were 0.48 and 0.51  
330 respectively, close to the theoretical value for diffusion behaviour. The discrepancy  
331 observed between experimental and theoretical values could be explained by a limited  
332 dissolution effect (<10% degradation rate) and other phenomena like swelling or  
333 heterogeneous particle size distribution, that may influence diffusion kinetics (Ritger &  
334 Peppas, 1987). In the present case, progressive solubilization of RF crystals inside  
335 microparticles accompanied also diffusion of RF molecules through the crosslinked protein  
336 network.

337 A pure erodible system corresponds generally to a zero-order release kinetics (Lin et al.,  
338 2006). In this case, the amount of active substance released is proportional to time ( $n=1$ ) for a  
339 system with a constant planar surface as a slab-shaped geometry. It could be assimilated to the  
340 Case II condition of the power law model for which  $n=0.85$  for a spherical geometry even if  
341 this case is generally related to relaxation of the polymeric matrix (Ritger & Peppas, 1987).  
342 The  $n$  value determined for intestinal fluid with added pancreatin ( $n = 0.81$ ) was close to this  
343 value, indicating the RF release was mainly controlled by the enzymatic degradation of the  
344 protein microparticles. The release exponent calculated for the gastric fluid with pepsin was  
345 0.6 indicating an intermediary behaviour as the dissolution of TPP microparticles was  
346 significantly slower compared to intestinal conditions. In agreement with these results,  
347 comparable systems based on enzymatically crosslinked protein hydrogels (Wen et al, 2018;  
348 Yan et al, 2020) have shown non-Newtonian RF release behaviour under gastric conditions  
349 and erosion-controlled behaviour under intestinal conditions. The choice to test the  
350 microparticles in the gastric and intestinal artificial juices separately, made it possible to  
351 clearly identify the behavior of our system for each digestive condition. Extrapolated to real  
352 digestive conditions, the present results suggested that RF could be regularly released up to  
353 ~40-50% during the gastric phase (<90 min), likely followed by a very fast release of the

354 remaining RF from the pre-degraded protein microparticles **under** intestinal conditions.  
355 **Sequential and standardized in vitro digestion protocol such as the INFOGEST method**  
356 **(Minekus et al., 2014) would be necessary to validate these synergistic release effects.**

#### 357 **4. Conclusion.**

358 Pea protein microparticles encapsulating **RF** were successfully prepared by W/O emulsion  
359 and enzymatic gelation. The size of the microparticles (~150 µm) was controlled by mild  
360 agitation during emulsion preparation and adequate concentration of the hydrophobic  
361 emulsifying agent. This size was adapted to entrap **RF** crystals with a significant yield  
362 (>74%). The release of **RF** from the enzymatically crosslinked microparticles seemed  
363 controlled by diffusion in digestive juices when no proteolytic enzyme was added. In the  
364 presence of digestive enzymes, the release kinetics could correspond to erosion-like behaviour  
365 **under** intestinal conditions and intermediary situation **under** gastric **conditions** where diffusion  
366 and matrix degradation occurred simultaneously. The protein microparticles would thus  
367 ensure a sufficient protection of **RF** to allow significant release during intestinal phase. This  
368 **conclusion needs further validation** by applying successive digestive steps or **using a**  
369 **continuous-type digester.**

#### 370 **Acknowledgement**

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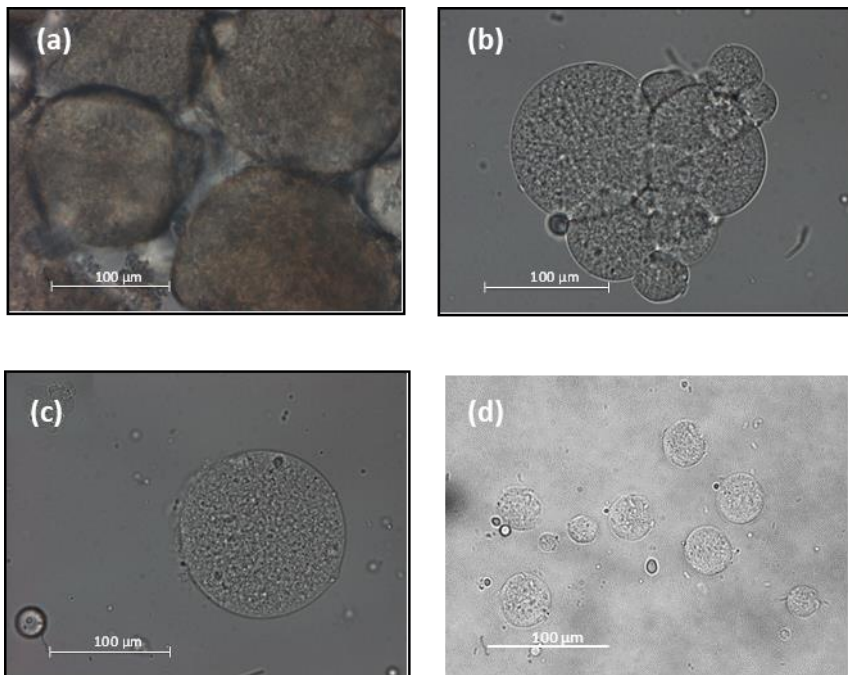
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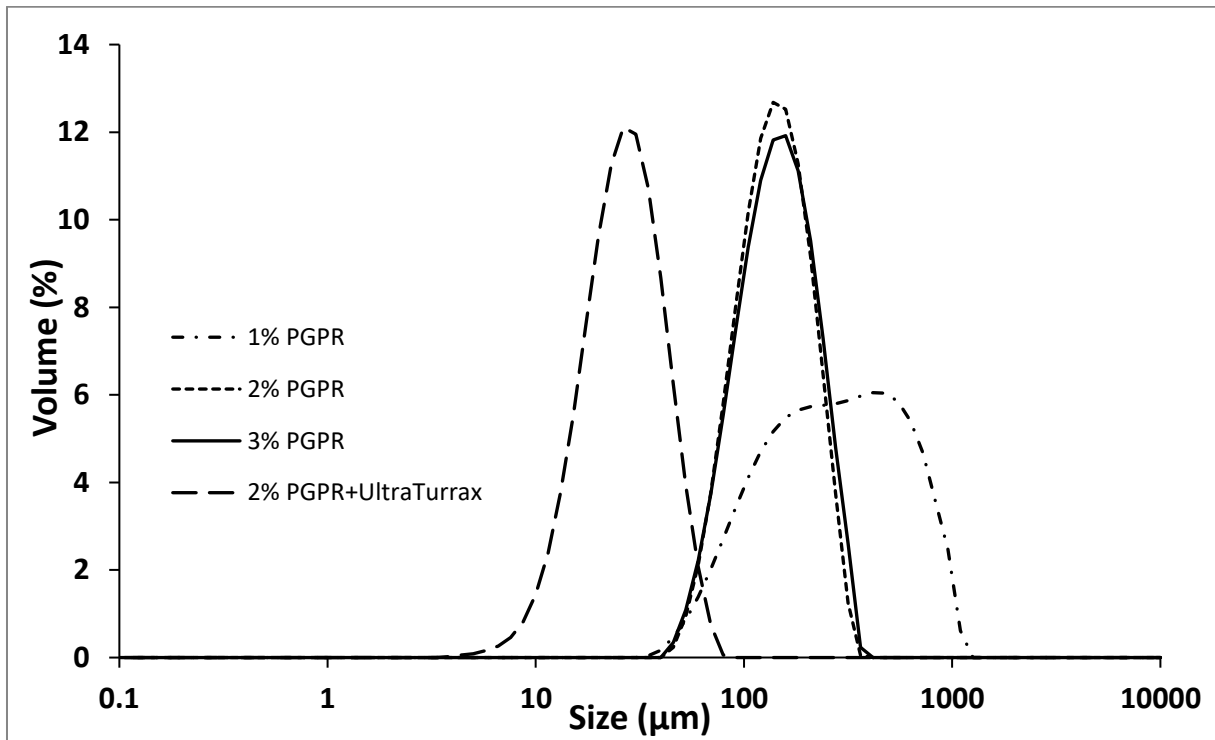
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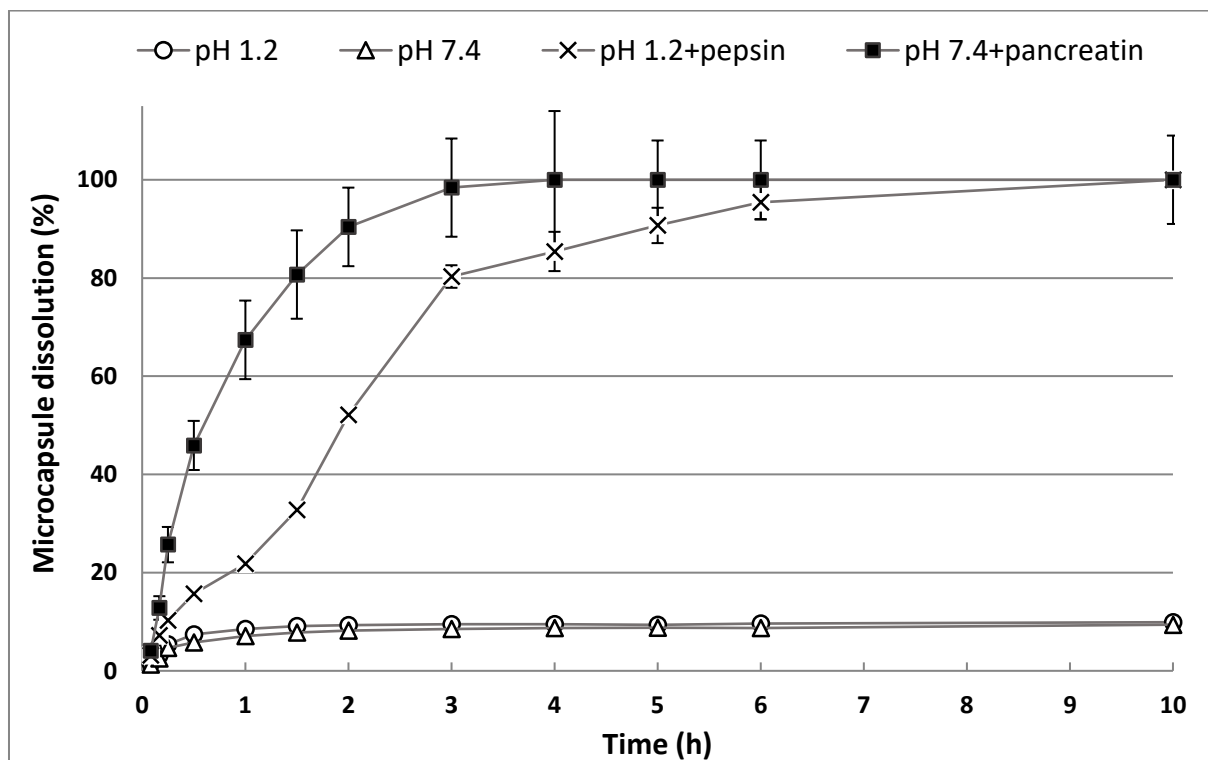
**Figure 1 :** Light microscopy images of the TPP microparticles in absence or presence of emulsifying agent : (a) without PGPR; (b) with 1% PGPR; (c) with 2% PGPR (d) with 2% PGPR and applying Ultra-Turrax homogenization.



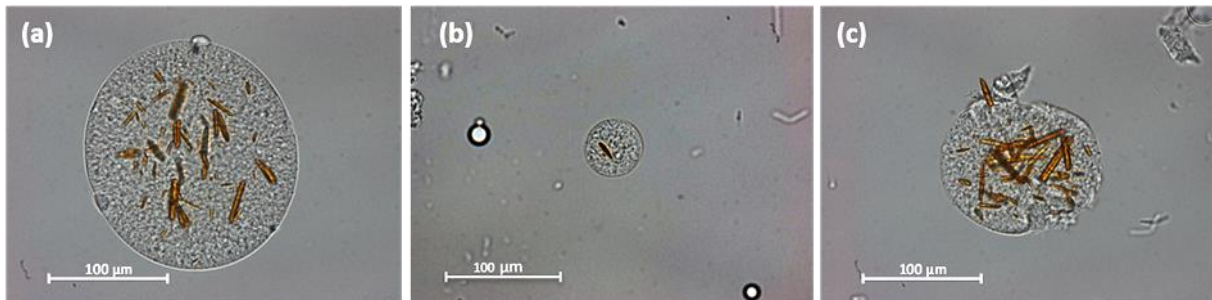
**Figure 2:** Size distribution of crosslinked TPP microparticles prepared with different emulsifying agent (PGPR) concentrations and different emulsifying conditions.



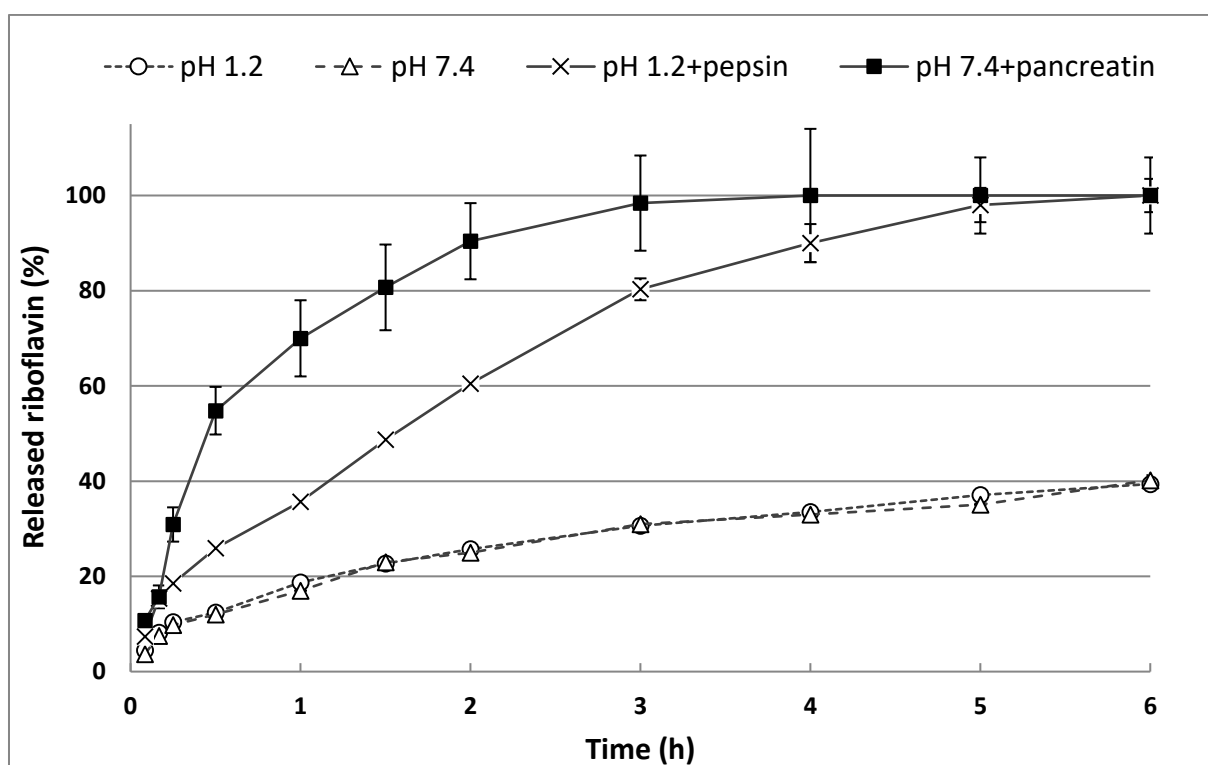
**Figure 3 :** Dissolution rate of TPP microparticules under gastric (pH 1.2 ; pH 1.2 + pepsin) and intestinal conditions (pH 7.4 ; pH 7.4 + pancreatin) at 37 °C.



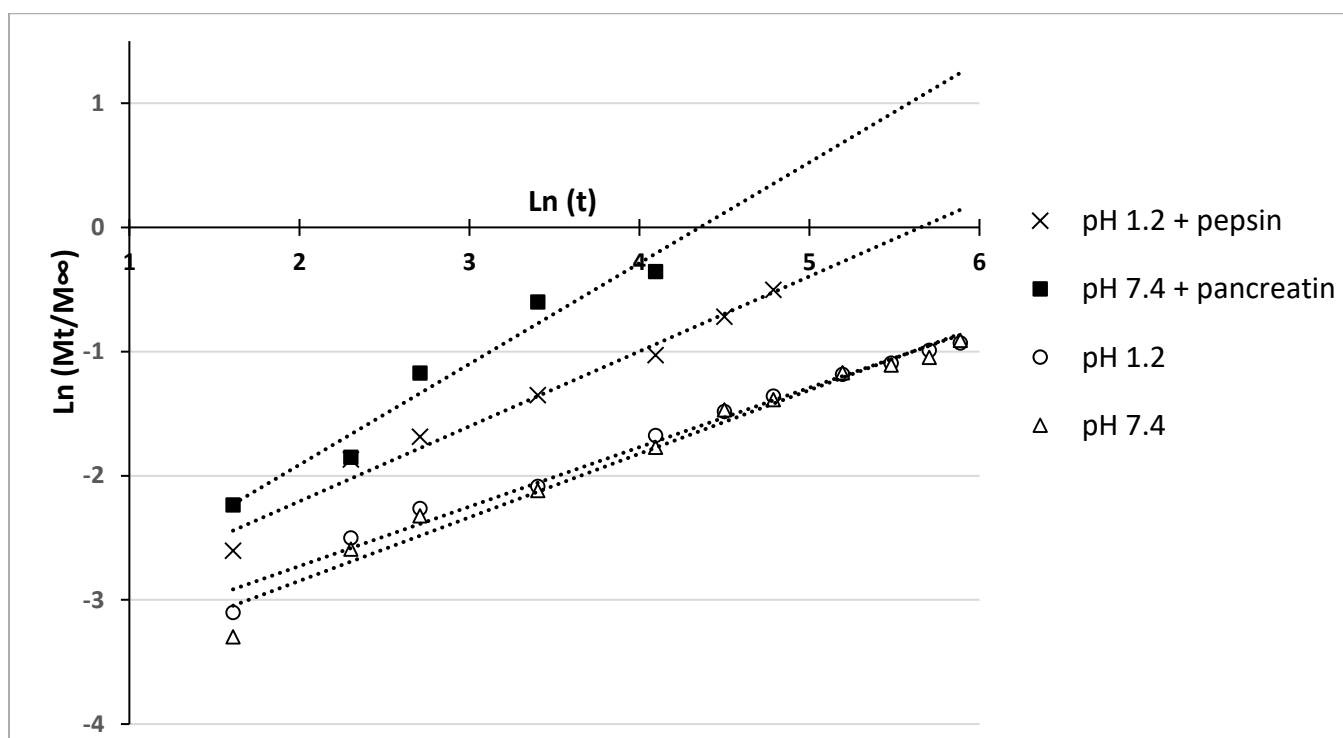
**Figure 4 :** Light microscopy images of the RF-loaded microparticles prepared by magnetic agitation (a) or by Ultra-Turrax homogenization (b); microparticle after 5 minutes in the intestinal fluid with pancreatin (c). **RF crystal appeared in yellow-orange.**



**Figure 5 :** Release kinetics of riboflavin from microparticles in different simulated digestive fluids at 37 °C.



**Figure 6 :** Logarithmic representation of the release kinetics according to the power law model (Ritger & Peppas, 1987). Points : experimental data. Dashed straight lines : adjusted linear models.



**Table 1 :** Mean diameter ( $d_{32}$ ), **encapsulation efficiency (EE)** and **loading efficiency (LE)** of microparticules prepared with different emulsification methods and at different riboflavin contents ( $C_{\text{Ribo}}$ ).

$C_{\text{Ribo}}$ (%)	Agitation (rpm)	$d_{32}$ ( $\mu\text{m}$ )	EE (%)	LE (%)
0.1	900	$149.2 \pm 1.8^a$	$84.1 \pm 2.4^a$	$0.56 \pm 0.02^a$
0.5	900	$148.4 \pm 2.9^a$	$81.7 \pm 2.6^a$	$2.72 \pm 0.09^b$
1.0	900	$151.6 \pm 3.1^a$	$74.5 \pm 3.4^b$	$4.97 \pm 0.23^c$
0.1	+10000 (Ultra-Turrax)	$31.7 \pm 1.2^b$	$56.5 \pm 3.4^c$	$0.38 \pm 0.02^d$

Different superscripts in the same column represent significant differences ( $p < 0.05$ ) among samples

**Table 2 :** Release exponent values ( $n$ ) and regression coefficient ( $R^2$ ) of the power law model from different release kinetics.

Release fluids	$n$	$R^2$
pH 1.2	$0.48 \pm 0.07^a$	0.986
pH 1.2 + pepsin	$0.60 \pm 0.03^b$	0.956
pH 7.4	$0.51 \pm 0.02^a$	0.978
pH 7.4 + pancreatin	$0.81 \pm 0.02^c$	0.979

Different superscripts in the same column represent significant differences ( $p < 0.05$ ) among samples