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**In vitro digestion of galactolipids from chloroplast-rich fraction (CRF) of postharvest, pea vine field residue (haulm) and spinach leaves**

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26 **Abstract**

27           The removal of intact chloroplasts from their cell wall confinement offers a novel way to obtain  
28 lipophilic nutrients from green biomass, especially carotenoids and galactolipids. These latter are the  
29 main membrane lipids in plants and they represent a major source of the essential  $\alpha$ -linolenic acid  
30 (18:3; ALA). Nevertheless, knowledge on their digestion is still limited. We have developed a physical  
31 method of recovering a chloroplast-rich fraction (CRF) from green biomass and tested its digestibility  
32 *in vitro* under simulated gastrointestinal conditions. Using a two-step static model, CRF from both  
33 spinach leaves and postharvest, pea vine field residue (haulm) were first exposed to enzymes from  
34 rabbit gastric extracts and then either to pancreatic enzymes from human pancreatic juice (HPJ) or to  
35 porcine pancreatic extracts (PPE). The lipolysis of monogalactosyldiacylglycerol (MGDG) and  
36 digalactosyl diacylglycerol (DGDG) was monitored by thin layer chromatography and gas  
37 chromatography of fatty acid methyl esters. For both CRF preparations, MGDG and DGDG were  
38 converted to monogalactosylmonoacylglycerol (MGMG) and digalactosylmonoacylglycerol (DGMG),  
39 respectively, during the intestinal phase and ALA was the main fatty acid released. Galactolipids were  
40 more effectively hydrolysed by HPJ than by PPE, and PPE showed a higher activity on MGDG than  
41 on DGDG. These findings may be explained by the higher levels of galactolipase activity in HPJ  
42 compared to PPE, which mainly results from pancreatic lipase-related protein 2. Thus, we showed  
43 that CRF galactolipids are well digested by pancreatic enzymes and represent an interesting vehicle  
44 for ALA supplementation in human diet.

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46 **Key words:** chloroplast, galactolipase, galactolipid, human pancreatic juice, porcine pancreatic  
47 extract

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## 50 1. Introduction

51 The United Kingdom is the largest producer of frozen peas in Europe with large areas given  
52 over for pea growing; at present, around 31,707 hectares. In 2015, the yield of pea production was  
53 4.69 tonnes per hectare.<sup>1</sup> After 8-10 weeks of pea growing, the peas are harvested, leaving the rest of  
54 the plant (stems, leaves, stalks, vines and pods -collectively called haulm) on the field.<sup>2</sup> The haulm  
55 can be used to feed livestock, such as cows, sheep, swine and poultry because it contains a high  
56 amount of carbohydrates and proteins,<sup>3</sup> or it is ploughed back into the soil to provide nitrogen.<sup>4</sup> Post-  
57 harvest, pea vine haulm (PVH) is a green waste material that is rich in chloroplasts, organelles in the  
58 plant where photosynthesis occurs, converting light energy into chemical energy. The chloroplast is  
59 an important reservoir of lipophilic nutrients, including carotenoids, vitamin E, and galactolipids  
60 enriched in omega-3 fatty acids.<sup>5-7</sup> Galactolipids are mainly found in the photosynthetic membranes of  
61 algae and plants, especially in the thylakoid and the envelope membranes of the chloroplast, and they  
62 represent more than 70% of the total membrane lipids.<sup>8-9</sup> Galactolipids represent the most plentiful  
63 lipid class and thus the main sources of fatty acids in the biosphere. They also represent the largest  
64 storage form of the essential  $\alpha$ -linolenic acid (18:3 n-3; ALA), which represents up to 60% of their total  
65 fatty acids. They also contain a large proportion of the shorter omega-3 hexadecatrienoic acid (16:3 n-  
66 3). The chemical structures of these diacylglycerolipids are characterised by one, two or more  
67 galactose moieties attached to the sn-3 position of the glycerol backbone. The major galactolipids in  
68 algae and plants are monogalactosyl diacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG).  
69 However, there are other galactolipids which are present in lower amounts such as  
70 trigalactosyldiacylglycerol (TGDG) as well as sulfoquinovosyldiacylglycerol (SQDG), a sulfolipid with a  
71 sulfoquinovose residue instead of galactose, as shown in the **Figure 1**.<sup>10</sup> It is therefore worth  
72 investigating the digestion of galactolipids in humans and other animal species eating green plant  
73 tissues. It was ignored for a long time before a galactolipase activity was discovered in human  
74 pancreatic juice and duodenal contents.<sup>11</sup> This activity was further associated with pancreatic lipase-  
75 related protein 2 (PLRP2) and to a lower extent to carboxyl ester hydrolase/bile salt-stimulated lipase  
76 (CEH/BSSL).<sup>12-15</sup> Since then, PLRP2 has been found to be present at high levels in monogastric  
77 herbivores, which supports its contribution to the digestion of plant lipids.<sup>16</sup> Both human and guinea  
78 pig PLRP2s were found to display a high activity on DGDG and MGDG from spinach leaves.<sup>10</sup> These  
79 previous studies were mainly dealing with the identification of pancreatic lipase-related protein 2 as a

80 galactolipase and its contribution to the galactolipase activity of pancreatic juice, as well as with the  
81 establishment of assay conditions to measure galactolipase activity with both synthetic (radiolabeled  
82 and medium chain galactolipids) and natural substrates. Nevertheless, the digestion of galactolipids  
83 has been rarely addressed under gastrointestinal (GI) conditions. The novelty of the present study is  
84 that we investigated the galactolipase activity of pancreatic juice, pancreatic extracts and a purified  
85 PLRP2 (GPLRP2) on chloroplastic fractions from whole plant materials under *in vitro* conditions  
86 mimicking those found in the GI tract which further support a physiological function for this digestive  
87 process and highlights the role of galactolipids in our diet.

88 *In-vitro* digestion models are widely used today for studying the digestibility and release of  
89 nutrient components under simulated gastrointestinal conditions with either fixed parameters (pH and  
90 enzyme concentration in static models) or variable parameters (dynamic models). The advantage of  
91 this technique is that it is inexpensive, rapid, consistent and does not have ethical restrictions  
92 compared to studies involving human or animal.<sup>17-18</sup> Many publications on *in-vitro* digestion use  
93 pepsin as the single enzyme in the gastric phase.<sup>17,19,20-21</sup> However, lipid digestion begins in the  
94 stomach with gastric lipase acting on triacylglycerides. Thus, it is now recommended to add gastric  
95 lipase during the stomach phase of *in-vitro* digestion.<sup>22-23</sup> Human gastric juice has been used as a  
96 source of pepsin and gastric lipase, but its use is limited by the ethical issue. Therefore, native gastric  
97 lipases from other mammalian species, such as dogs and rabbits, or from recombinant origin such as  
98 recombinant human (rHGL) and dog (rDGL) gastric lipases<sup>24-26</sup> can be chosen as alternative sources  
99 instead of human gastric juice.<sup>22</sup> Human Pancreatic Juice (HPJ) has been used as a source of  
100 pancreatic lipase for the intestine phase of *in vitro* digestion.<sup>27-28</sup> However, as for human gastric juice,  
101 the use of HPJ has been restricted by ethical constraints. Hence, pancreatin, an extract from porcine  
102 pancreas, is widely used for *in vitro* digestion in intestinal phase.<sup>17,19,20-21</sup> So far, there is no published  
103 work on the digestion of galactolipids in intact chloroplasts. Therefore, the aim of this study was to test  
104 the *in vitro* digestibility of the chloroplast-rich fraction (CRF) from green materials, including PVH and  
105 spinach leaves under simulated GI conditions. Our group has recovered intact chloroplasts from  
106 spinach leaves using grinding in 0.3 M sucrose solution<sup>29</sup> and from PVH using a slow-screw twin gear  
107 juicer without added water.<sup>30</sup> Both were exposed first to gastric enzymes from rabbit gastric extract  
108 (RGE) and then either to pancreatic enzymes from HPJ or to Porcine Pancreatic Extract (PPE).

## 109 **2. Materials and methods**

### 110 **2.1. Materials**

111 Pea vine haulm (*Pisum sativum* L.), composed of a mixture of vines, stems, leaves, peas,  
112 and pods was kindly donated by the Green Pea Company (Yorkshire, United Kingdom). The biomass  
113 was collected from the side of the fields during the pea harvest (July, 2017) and immediately brought  
114 to our laboratory facilities in Leicestershire, UK to be processed. Spinach leaves were brought from a  
115 local supermarket (Casino, Marseille, France).

### 116 **2.2. Chemicals**

117 Thin layer silica gel 60 plates (10 x 20 cm) from Merck were used to perform the separation  
118 of the lipids. Lipid standards, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol  
119 (DGDG) were purchased from Avanti Polar lipid (840523P and 840524P). Oleic acid,  
120 methylpentadecanoate, trimethylsulfonium hydroxide (TMSH), sodium taurodeoxycholate (NaTDC)  
121 were purchased from Sigma Aldrich. All solvents for lipid extraction, TLC and GC-MS analysis were  
122 HPLC grade and purchased from Fisher Scientific. The solvents for lipid extraction were bought from  
123 Carlo Erba.

### 124 **2.3. Enzymes**

125 Recombinant guinea pig pancreatic lipase-related protein 2 (GPLRP2) was produced in  
126 *Aspergillus oryzae* and purified as previously described.<sup>31</sup> Human Pancreatic Juice (HPJ) was  
127 provided by Prof. R. Laugier, MD (La Timone University Hospital, Marseille, France) and was  
128 obtained from a patient devoid of pancreatic disease by performing endoscopic retrograde  
129 catheterisation on the main pancreatic duct. It was collected on ice, freeze-dried and the resulting  
130 powder was stored at -20°C before use. Rabbit gastric extract (RGE) was provided by Lipolytech,  
131 Marseille, France. Porcine Pancreatic Extract (PPE) was purchased from Sigma Aldrich (P7545: 8 ×  
132 USP specifications activity).

### 133 **2.4. Post-harvest treatment of the pea vine haulm**

134 The fresh biomass was washed with tap water to remove soil and rocks, and then the  
135 excess of water removed using an industrial salad spinner (Sammic ES-200). The haulm was split  
136 into two batches for different treatments. One batch (13 kg) was packed into a vacuum sealed bag for

137 steam condition (section 2.4.1). Another batch of haulm (5 kg) was juiced immediately using a twin  
138 gear juicer (Angel 7500) which separated the fibrous pulp from the nutrient rich juice. The juice was  
139 filtered through a 75 µm stainless steel mesh sieve and processed (with or without pasteurisation) to  
140 isolate the CRF (section 2.6).

#### 141 2.4.1. Steam sterilisation of pea vine haulm

142 The pea vine biomass was packed into a vacuum sealed, clear, perforated bag (500 g of  
143 pea vine per bag). These bags were placed in the rack of a Retort (Lagarde RP362). The chamber  
144 was sealed, vented and heated over 5.30 min to reach a temperature of 100°C and 1 bar. These  
145 sterilisation conditions were held for 4 min before cooling and depressurising for 5.45 min. The sealed  
146 bag of pea vine was plunged into ice-water bath to rapidly cool. The steam treated haulm was  
147 immediately juiced using a twin gear juicer (Angel 7500) and the juice was filtered through a 75 µm  
148 stainless steel mesh sieve and processed to isolate the CRF (section 2.6).

#### 149 2.4.2. Pasteurisation of juice extracted from pea vine haulm

150 Pea vine juice (500 mL) was placed in a capped amber Duran bottle in a heated water bath  
151 with a magnetic stirrer (800 rpm). The temperature of the juice was raised from room temperature  
152 (20°C) to 85°C in 15 min. It was then held at this temperature for 1 min. The pasteurised juice was  
153 immediately immersed in an ice-water bath to rapidly cool the juice down to room temperature before  
154 further processing to isolate the CRF (section 2.6).

### 155 **2.5. Hot-water blanching of spinach leaves**

156 Spinach leaves (100 g) were blanched in hot water at 85°C for 3 min and then immediately  
157 immersed in an ice-water bath to rapidly cool to room temperature. Blanched spinach leaves were  
158 homogenised in a blender (Waring™) for 30 s with 0.3 M sucrose solution 1:6 (w/v). The homogenate  
159 was then filtered through a double-layered cheese cloth and processed to isolate the CRF (section  
160 2.6).

### 161 **2.6. Isolation of CRF**

162 The PVH juice was centrifuged at 17,700 RCF or 10,000 rpm (Beckman Coulter JS-21M with  
163 JA-10 rotor) for 10 min at 4°C. The CRF-containing pellet was retained while the supernatant was

164 centrifuged again under the same conditions to obtain a further pellet. The CRF fractions were  
165 pooled, weighed and frozen at -80°C prior to freeze drying (Edwards Freeze Dryer Super Modulyo) for  
166 3-5 days. Freeze-dried CRF was then ground using a pestle and mortar, and stored in a vacuum-  
167 sealed foil pouch at -20°C for further analysis.

## 168 **2.7. Digestion of CRF galactolipid by GPLRP2**

169 CRF (25 mg) was suspended in 1 mL of buffer solution at pH 8 containing 0.3 mM Tris-HCl,  
170 100 mM NaCl, 5 mM CaCl<sub>2</sub> and 4 mM sodium taurodeoxycholate (NaTDC). GPLRP2 was further  
171 added at a final concentration of 20 µg/mL and the reaction mixture was incubated at 37°C for 1 hr  
172 and compared with a control without GPLRP2. The reaction was stopped by adding 200 µL of 1 N HCl  
173 and further extracted lipid (section 2.9).

## 174 **2.8. Two-step static *in vitro* digestion of CRF**

175 A two-step static *in vitro* digestion model was slightly modified from the procedure of Carrière  
176 *et al.* (2001)<sup>28</sup> and set up at 37°C, pH 5 for the stomach phase and pH 6 for the small intestine phase  
177 in order to mimic the GI conditions at half gastric emptying time during a meal.<sup>32-33</sup> For the gastric  
178 phase, CRF (250 mg) was suspended in 10 mL of ultrapure water in a thermo regulated glass vessel  
179 (37°C) equipped with a pH electrode. Rabbit gastric extract (RGE; 25 gastric lipase U per mg of  
180 powder (tributylin as substrate<sup>22,34</sup>, equivalent to 21 µg lipase/mg) was added so that the final  
181 concentration of gastric lipase was 17 µg /mL (20 U/mL). The pH was adjusted at 5 and the solution  
182 was then incubated for 30 mins under gentle magnetic stirring. For the intestinal phase, the solution  
183 from the gastric phase was diluted by half using a pancreatic enzyme-bile salts solution, pH was  
184 adjusted at 6 and the incubation was continued for 60 min. The final concentration of pancreatic  
185 lipase was set at 250 µg/mL or 2000 lipase U/mL and that of bile salt (NaTDC) was 4 mM. Two  
186 sources of pancreatic enzymes were tested: (1) freeze-dried HPJ, that contained 228 lipase U per mg  
187 of powder or (2) Sigma PPE, that contained 67 lipase U per mg of powder. In both cases, lipase units  
188 (U) for pancreatic lipase refer to the assay using tributyrin as substrate.<sup>17,32</sup> Samples (1 mL) from the  
189 digestion mixture were collected at various time points (0, 15, 29, 35, 40, 45, 60 and 90 mins) and  
190 immediately mixed with 10 µL of protease inhibitor cocktail (Complete™ from Roche). Each digestion  
191 sample was also acidified with 200 µL of 1 N HCl to stop the enzymatic reaction and the lipids were  
192 extracted according to section 2.9.

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## **2.9. Lipid extraction**

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Lipid extraction was performed using the method of Folch *et al.* (1957),<sup>35</sup> modified by Bligh and Dyer (1959).<sup>36</sup> Sample (25 mg CRF or 1 mL of digestion sample) was mixed with 1 mL of 150 mM NaCl solution and 1.5 mL of a 2:1 v/v mixture of chloroform and methanol, then vortexed for 1 min. The mixture was then centrifuged (using Thermo Electron Corporation, Jouan CR3i multifunction) at 3,000 rpm or 1,750 RCF for 10 min at 4°C, which allowed the phase separation. The lowest organic phase, which contains lipids, was collected using a Pasteur pipette and transferred into a fresh tube. A further 1.5 mL of 2:1 v/v chloroform: methanol was added to the remaining aqueous phase and the mixture was vortexed and centrifuged again according to the same procedure. The lipid extracts were pooled and their volume was measured before the organic phase was dried using magnesium sulphate. After centrifugation, the lipid extract was kept at -20°C until analysis.

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## **2.10. Quantitative analysis of galactolipids and their lipolysis products by thin layer chromatography**

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In order to separate and quantify galactolipids and lipolysis products, 10 to 50 µL of lipid extracts and known amounts (2, 4, 6, 8, and 10 µg) of lipid standards (MGDG, DGDG and oleic acid) were spotted as a 5 mm band onto a thin-layer silica plate using a Limonat IV (Camag) equipped with a 100 µL Hamilton syringe. The separation of polar lipids was performed with a chloroform/methanol/water (47.5:10:1.25, v/v/v) elution mixture. The separation of free fatty acids (FFA) on a second silica plate was performed with a mobile phase consisting of heptane: diethyl ether: formic acid (55:45:1, v/v/v) solvent mixture. Following chromatography, the plates for polar lipid/galactolipid analysis were dried at room temperature under a fume hood for 15 min and then dipped in a thymol solution prepared by dissolving 1g of thymol in 190 mL ethanol and then addition of 10 mL of 96% sulphuric acid. Since the mixing reaction is highly exothermic, the ethanolic solution has to be placed first in a cold water bath before sulphuric acid is added dropwise. The thymol solution allows the staining of galactolipids while avoiding the interference of pigments, especially chlorophylls, during the densitometric analysis of the plates. After staining with thymol, the plates

221 were dried again in the fume hood for 10 min and then placed in an oven at 110°C for 10 min. The  
222 plate for FFA analysis was dipped in a copper acetate-phosphoric acid solution prepared by mixing a  
223 saturated copper acetate solution with 85% phosphoric acid in a 1 to 1 volume ratio. The plates were  
224 dried for 10 min in the fume hood and then placed in an oven at 180°C for 15 min. Densitometry  
225 analysis of the stained lipids on the TLC plate was carried out using a Camag TLC scanner II and a  
226 D2000+ chromato-Integrator (Merck). Lipid bands were scanned at 366 nm for thymol staining and at  
227 500 nm for copper acetate-phosphoric acid staining, with a 0.5 x 7 mm slit and a speed of 2.5  
228 cm/min. Slit conditions were selected accordingly to band size. The slit should always cover the whole  
229 band size. The densitograms of all tracks were integrated using D2000+Chromator-Integrator.  
230 Quantities of the lipids on the TLC plates were estimated from the linear standard curves established  
231 with the pure lipid standards (MGDG, DGDG, and oleic acid (18:1)).

### 232 **2.11. Fatty acid analysis by GC-MS**

233 The fatty acids contained within the lipid extracts were esterified to fatty acid methyl esters  
234 (FAMES) and analysed using gas chromatography coupled to mass spectrometry detection (GC-MS)  
235 (Thermo Scientific, DSQ) using a modified method based on Bahrami *et al.* (2014).<sup>37</sup> The solvent from  
236 lipid extracts (2.1 mL) was first evaporated under nitrogen and the resulting dry material was re-  
237 dissolved in 1 mL of chloroform. Methylpentadecanoate (internal standard) and trimethyl  
238 sulfoniumhydroxide (TMSH) were added to lipid extract in chloroform to convert both the FFA and  
239 esterified fatty acids into FAMES. The reaction was performed for at least 10 min to ensure a  
240 completed conversion. The mixture (1 mL) was then filtered through a 0.45 µm PTFE filter membrane  
241 into an amber glass vial. 10 µL of the sample was injected into a Phenomenex Zebron ZB-FFAP (30  
242 m x 0.25 mm) column using a vaporising injector with a split flow of 50 mL/min of helium. The oven  
243 temperature was maintained at 120°C for 1 min and then increased to 250°C at ramp 5°C/min and  
244 held for 2 min. Detection was conducted using a mass spectrophotometer and the identification of  
245 individual fatty acids was achieved using a mass spectrum library by means of comparison of  
246 retention time and molecular mass to FAME standards.

### 247 **2.12. Statistical analysis**

248 All experiment were performed in triplicate. The statistical analysis was carried out using IBM  
249 SPSS statistic 25 using post hoc analysis of variance (ANOVA) and according to the Tukey test with

250 statistically significant at  $p < 0.05$ . The data were expressed as mean  $\pm$  standard deviation. Differences  
251 of means at  $p < 0.05$  were considered significant.

252

253 **3. Results and Discussion**

254 **3.1 Effect of endogenous enzymes and heat-treatment on the CRF galactolipids**

255 It is well known that nutrient concentrations in the plants start to decrease after harvesting  
256 due to their degradation by endogenous enzymes.<sup>38</sup> Thermal processing can be used to inactivate  
257 enzyme reactions after harvesting, extending the shelf life of the nutrients and the stabilisation of  
258 texture, flavour and nutrients.<sup>39</sup> The action of endogenous enzymes on the lipids from PVH CRF was  
259 studied (**Figure 2**). TLC separations of polar and neutral lipids were analysed first without staining  
260 which allowed a qualitative visualisation of carotenoids and pigments, including chlorophylls (**Figures**  
261 **2A** and **2C**). No major changes were observed whatever the treatment of CRF. After lipid staining and  
262 from the comparison of non-heat treated and steam sterilised or pasteurised CRF, it was clear that  
263 galactolipid levels, especially those of DGDG, were lower in non-heat treated samples than in steam  
264 sterilised samples and after 1-hr incubation of these samples at 37°C, the band of DGDG has  
265 disappeared (**Figure 2B**). Without heat treatment, and in contrast to DGDG, the band of MGDG was  
266 apparent both before and after incubation. The low levels of galactolipids were associated with high  
267 levels of free fatty acids (FFA), which suggests the action of endogenous galactolipases being  
268 present in the material (**Figure 2D**). Thermal treatments did inactivate endogenous enzymes as  
269 shown in the **Figure 2B** where the bands of MGDG and DGDG are visible and remain at similar levels  
270 both before and after 1-h incubation at 37°C, for each treatment. The higher levels of galactolipids  
271 were associated with reduced levels of FFA, which indicates that endogenous galactolipases can be  
272 heat-inactivated (**Figure 2D**). In addition, CRF were also incubated for 1 hr at 37°C after addition of  
273 GPLRP2, an enzyme known to display galactolipase activity.<sup>12</sup> The galactolipids of both steam  
274 sterilised and pasteurised CRF from PVH were hydrolysed to lysogalactolipids (MGMG and DGMG;  
275 **Figure 2B**), while FFA levels increased (**Figure 2D**). No MGMG and DGMG could be observed  
276 however with CRF from non-heat treated PVH (**Figure 2B**), which suggests that endogenous  
277 enzymes are also able to hydrolyse MGMG and DGMG. It was established that steam sterilisation at  
278 100°C for 4 min knocks out the endogenous enzymes more thoroughly than pasteurisation at 85°C for  
279 1 min as indicated by a higher intensity of the band of DGDG and a lower amount of free fatty acids  
280 (**Figure 2B-2D**). Our results indicated that endogenous enzymes had an effect on the pea chloroplast  
281 galactolipids and this phenomenon was also observed with spinach galactolipids (data not shown).  
282 Therefore, heat treatment was applied to all CRF preparations used for *in vitro* digestion experiments.

283 While CRF from PVH was steam sterilised, the spinach leaves were first blanched in hot water at  
284 85°C for 3 min to knock out the endogenous enzymes before preparing CRF. It was checked that this  
285 treatment allowed the inactivation of the endogenous galactolipase activity of CRF from spinach  
286 leaves.

### 287 **3.2. Galactolipid and free fatty acid content in CRF before digestion**

288 The amounts of galactolipids and free fatty acids in the CRF of blanched spinach leaves and  
289 steam sterilised or non-heat treated PVH were measured (**Table 1**). The CRF prepared from heat-  
290 treated materials showed a higher MGDG content than DGDG. This is in agreement with what has  
291 been reported in photosynthetic tissues, especially in the inner envelope membrane and thylakoid  
292 membrane of chloroplast where the amount of MGDG is higher than DGDG, while DGDG amounts  
293 exceed MGDG in the non-photosynthetic tissues.<sup>9,40-41</sup> The CRF of spinach leaves had more  
294 galactolipids per dry mass of CRF than PVH CRF due to a higher lipid content in CRF of spinach  
295 leaves, but galactolipids were represented at similar levels in total lipids from both CRF (around 100  
296 mg/g of total lipid extracts). The other lipids, including carotenoids, tocopherols, chlorophyll esters,  
297 sterols and phospholipids were not quantified. The lower relative levels of lipids in pea vine CRF may  
298 be due to some dilution by components like starch. The level of free fatty acids in CRF spinach was  
299 quite low  $1.30 \pm 0.35$  mg/g CRF or  $5.08 \pm 1.35$  mg/g lipid extract, in line with the fact that spinach  
300 leaves were blanched to avoid lipolysis by endogenous enzymes. The CRF of steam sterilised pea  
301 vine showed a greater amount of FFA ( $11.71 \pm 0.72$  mg/g CRF or  $71.95 \pm 4.43$  mg/g lipid extract),  
302 which indicates some significant lipolysis occurring from harvesting and before heat treatment of PVH.  
303 However, the determination of the amount of FFA in haulm directly after harvesting was not possible  
304 due to logistical limitation. Nevertheless, the amounts of FFA in the CRF from steam sterilised pea  
305 vine are 3-fold lower than those found in CRF from non-heat treated pea vine as shown in the **Table**  
306 **1**. In agreement, MGDG and DGDG levels in CRF from steam sterilised PHV are 3.4-fold and 1.8-fold  
307 higher than in CRF from non-heat treated PVH. It is worth noting that the total masses of MGDG,  
308 DGDG and FFA are similar in both CRF preparations, which confirms that most FFA are generated by  
309 the endogenous hydrolysis of galactolipids.

### 310 **3.3. *In-vitro* digestion of galactolipids in CRF**

311 The experimental conditions to simulate lipid digestion were based on *in vivo* studies and  
312 parameters measured at 50% meal gastric emptying,<sup>27,33</sup> such as the lipase concentrations (17 µg/mL  
313 of gastric lipase in the stomach and 250 µg/mL of pancreatic lipase in the small intestine) and the pH  
314 values (5 for the gastric phase and 6 for the intestinal phase). In these experiments, rabbit gastric  
315 extract (RGE) was chosen as the source of gastric enzymes because it is composed of pepsin and  
316 gastric lipase, and the activity of rabbit gastric lipase has a similar range of activity as the human  
317 gastric lipase (HGL).<sup>23</sup> Porcine Pancreatic Extract (PPE) or Human Pancreatic Juice (HPJ) were used  
318 as the sources of pancreatic enzymes for the intestinal phase of *in vitro* digestion. HPJ contains  
319 various lipolytic enzymes, including pancreatic lipase, pancreatic lipase related protein 1 and 2  
320 (PLRP1 and PLRP2) and carboxyl ester hydrolase/ bile salts stimulated lipase (CEH/BSSL).<sup>42</sup> Both  
321 PLRP2 and CEH/BSSL show galactolipase activity on MGDG and DGDG,<sup>11,14-15,43-45</sup> as well as  
322 phospholipase A1 activity on phospholipids.<sup>16,43</sup> PPE consists of a mixture of digestive enzymes  
323 produced by the exocrine cell of the porcine pancreas and contains trypsin, chymotrypsin, α-amylase,  
324 lipase and colipase.<sup>17</sup> It is commonly used for *in vitro* digestion studies as a substitute of human  
325 pancreatic enzymes.<sup>17,22</sup> Nevertheless, it was recently shown that PPE does not contain as much  
326 galactolipase activity as HPJ.<sup>46</sup> Therefore, it was important to compare here both sources of  
327 pancreatic enzymes. According to the TLC analysis of  
328 galactolipid digestion (**Figures 3, 4, S1 and S2**), both the MGDG and DGDG of CRF from blanched  
329 spinach leaves and steam sterilised PVH were hydrolysed and converted to lysogalactolipid  
330 (monogalactosylmonoglycerol, MGMG, and digalactosylmono glycerol, DGMG) during the whole  
331 digestion process. During the 30-min gastric phase, galactolipids decreased slightly from 25-26 to 22-  
332 23 mg MGDG /g CRF and 18-22 to 17-18 mg DGDG /g CRF for spinach leaves CRF (**Figure 4A** and  
333 **4B**) and from 15-16 to 14-15 mg MGDG /g CRF and 9-11 to 8-9 mg DGDG /g CRF for PVH CRF  
334 (**Figure 4C** and **4D**). The weak increase in FFA during the gastric phase (**Figure 5B**) confirms that  
335 galactolipid hydrolysis is not very important under gastric conditions, in line with the fact that gastric  
336 lipase has no demonstrated galactolipase activity. Nevertheless, RGE might contain some traces of  
337 other enzymes with galactolipase activity. After adding HPJ and bile salts to the system to initiate the  
338 intestinal phase of digestion, both MGDG and DGDG of spinach and PVH CRF were immediately  
339 hydrolysed to MGMG and DGMG, respectively (**Figures 4A, 4C and S1**), and high amounts of free  
340 fatty acids were released (**Figure 5 and S2**), especially during the first 5 min of the intestinal phase.

341 Because the monogalactosyl galactolipids (MGDG and MGMG) are revealed similarly upon thymol  
342 staining (**Figures 2B**), and in the absence of a pure MGMG reference standard, the generation of  
343 MGMG was tentatively quantitated by TLC using the calibration curve established with MGDG as  
344 reference standard. A good correlation was observed between the appearance of MGMG and the  
345 disappearance of MGDG (**Figure 4**). **The band corresponding to DGMG on TLC plates (see Figure 3**  
346 **and Figure S1 in Supplementary Data) was however too faint and not enough resolved to apply the**  
347 **same method.** These results are supported by those of Andersson *et al.* (1995)<sup>11</sup>, who demonstrated  
348 that pure galactolipids were hydrolysed by Human Pancreatic Juice. This previous study had led to  
349 the characterisation of the galactolipase activity of HPJ and its association with PLRP2 and to a lower  
350 extent to CEH/BSSL.<sup>12,14</sup> Galactolipids from both CRF preparations were  
351 hydrolysed at a slower rate when RGE and PPE were combined for *in vitro* digestion (**Figures 4B** and  
352 **4D**). It confirms that PPE contains a lower galactolipase activity.<sup>46</sup> Additionally, it was observed that  
353 PPE prefers to hydrolyse MGDG over DGDG, which is not the case for HPJ.

354 TLC analysis of the fatty acids released during  
355 digestion of both CRF from spinach leaves and pea vine (See **Figures 5** and **S2**, in Supplementary  
356 Data) showed that the FFA bands obtained using the mixture of RGE and PPE, had a higher intensity  
357 than those obtained with the mixture of RGE and HPJ. This was not consistent with the lower  
358 hydrolysis of galactolipids by PPE. We then performed control experiments without the CRF substrate  
359 and found that FFA could be released during the intestinal phase when PPE was used but not HPJ  
360 (**Figure 5A**). This finding is probably due to the fact that PPE contains some lipids<sup>47</sup> and these lipids  
361 can be hydrolysed during the digestion experiment. The FFA analysis by TLC were therefore  
362 corrected accordingly by subtracting the FFA levels measured in the controls. This point was never  
363 raised however in previous *in vitro* digestion studies, probably because the levels of FFA released  
364 from PPE are much lower than those released from dietary triglycerides. But one has to be cautious  
365 when low levels of lipids are concerned, as here with CRF galactolipids.

### 366 **3.4. Composition and release upon digestion of the CRF fatty acids**

367 The compositions of total fatty acids in spinach leaves and pea vine CRF were analysed  
368 using GC-MS and the results are shown in the **Figure 6**. The main fatty acid in both spinach leave  
369 and pea vine CRF was  $\alpha$ -linolenic acid (ALA; 18:3) but its amounts in spinach CRF ( $35.56 \pm 2.56$   
370 mg/g CRF DW) were 2.5-fold higher than in PVH CRF ( $14.29 \pm 2.06$  mg/g CRF DW). ALA

371 represented  $55.38 \pm 1.33$  % w/w of the total fatty acids in spinach leave CRF, which is in agreement  
372 with the fatty acid composition of spinach leaves and isolated galactolipids.<sup>10</sup> In pea vine CRF, ALA  
373 represented only  $37.82 \pm 0.31$  % w/w of the total fatty acids. The second most abundant fatty acid  
374 was palmitic acid (PA; 16:0), which was found at similar levels in spinach CRF ( $13.31 \pm 0.24$  mg/g  
375 CRF;  $20.77 \pm 0.76$  % w/w of total FA) and pea vine CRF ( $12.35 \pm 1.86$  mg/g CRF;  $32.65 \pm 0.12$  % w/w  
376 of total FA). Hexadecatrienoic acid (16:3) was found at significant levels in spinach CRF ( $5.98 \pm 0.12$   
377 mg/g CRF;  $9.34 \pm 0.29$  % w/w of total FA) but was not found in pea vine CRF. Therefore, the levels of  
378 polyunsaturated omega 3 fatty acids were globally reduced in PVH CRF compared to spinach CRF.  
379 This may results from a preferential oxidation of these fatty acids by endogenous lipoxygenases from  
380 harvesting to steam sterilisation of PVH. Moreover, this oxidation is known to be coupled to and  
381 favoured by the release of FFA by endogenous galactolipase activity as often seen in plant response  
382 to stress.<sup>48</sup> The absence of 16:3 fatty acid in PVH CRF may also be explained by the classification of  
383 pea among “eukaryotic plants”, i.e. plants which preferentially have C18 fatty acids at sn-2 position of  
384 the glycerol backbone in galactolipids, while “prokaryotic” plants like spinach preferentially have C16  
385 fatty acids at sn-2 position.<sup>49</sup> Indeed, the predominant galactolipids in “prokaryotic” plants are MGDG  
386 (18:3/16:3) and DGDG (18:3/16:0), while MGDG (18:3/18:3) and DGDG (18:3/18:3) are the  
387 predominant ones in “eukaryotic” plant.<sup>50</sup>

388           After 90 min of digestion by RGE and HPJ, the composition of FFA generated from CRF  
389 hydrolysis was similar to the composition of total FA in both spinach and PVH CRF (**Figure 6**), with  
390 ALA representing the most abundant FFA, followed by PA. With the combination of RGE and PPE,  
391 some changes were observed in the relative distribution of fatty acids, which certainly reflects the  
392 contribution of fatty acids present in PPE (see **Figure S3** and **Table S1** in Supplementary data).  
393 Therefore, we only discuss here the data obtained with RGE and HPJ. We observed that the amounts  
394 of total FFA released during the CRF digestion were higher than those expected from the complete  
395 lipolysis of MGDG and DGDG, both for spinach leave and PVH CRF (**Table 2** and **Figure 5B**).  
396 Moreover, we have seen that galactolipid lipolysis was not complete, with some accumulation of  
397 MGG (Figure 4) and DGG (Figure 3 and S1), in line with the preferential hydrolysis by PLRP2 of  
398 the ester bond at the sn-1 position of galactolipids.<sup>10</sup> Therefore, some FFA were certainly released  
399 from other acyl lipids present in CRF preparations. This hypothesis is supported by the levels of total  
400 fatty acids present in CRF and quantified by GC-MS (**Table 2**). **Figure 3D** on TLC separation of

401 neutral lipids shows that some apolar lipids present in PVH CRF at time 0 and migrating like  
402 triglycerides or sterol esters are no more present at time 90 min. Phospholipids, mainly PG, are also  
403 present in chloroplast membranes and can be degraded by the phospholipase activity of HPJ. During  
404 stress or senescence, the degradation of chlorophyll and galactolipid from thylakoid membranes in  
405 chloroplasts can also lead to the conversion of a large proportion of phytol and fatty acids into fatty  
406 acid phytol esters (PFAE) and triacylglycerol.<sup>51</sup> We did not search nor analyse these various lipids  
407 here but it would be worth identifying these other sources of fatty acids in future studies in order to  
408 better describe the lipid composition of CRF. At this stage, it is important to keep in mind that 87.2 %  
409 of the total fatty acids of PVH CRF are present in galactolipids and FFA, and that 86.4 % of total fatty  
410 acids are released upon *in vitro* digestion, while the corresponding values for spinach CRF are 57.4 %  
411 and 77.6 %, respectively (**Table 2**). The proportion of fatty acid sources other than galactolipids is  
412 therefore higher in spinach CRF than in PVH CRF. In both cases, a large proportion of the total fatty  
413 acids can be converted to FFA upon digestion, including ALA as the main FFA.

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#### 428 **4. Conclusion**

429 We have shown that heat treatments by both steam sterilisation (at 100°C for 4 min) of  
430 postharvest, pea vine field residue (haulm) and hot water blanching (at 85°C for 3 min) of spinach  
431 leaves knock out endogenous galactolipase activity inside the plant materials. Based on our results, it  
432 is recommended that samples should be treated in this way to avoid the loss of galactolipid content,  
433 the release of FFA and further oxidation of polyunsaturated fatty acids. We have also shown that the  
434 galactolipids from both PVH and spinach leave CRF could be digested *in vitro*, mostly during the  
435 intestinal phase of digestion by pancreatic enzymes. PLRP2 and CEH/BSSL, are the main enzymes  
436 found in pancreatic secretion that can digest galactolipids and indeed, we showed that CRF  
437 galactolipids, as well as some other acyl lipids, are digested by the enzymes present in human  
438 pancreatic juice. Pancreatic extracts can also be used to show this digestion but lipolysis rates are  
439 slower due to a lower galactolipase activity compared to HPJ. In addition, it can be seen that the  
440 enzyme(s) with galactolipase activity in PPE hydrolyses MGDG more extensively than DGDG  
441 compared to HPJ. After digestion,  $\alpha$ -linolenic acid (18:3) is the main fatty acid from CRF of both  
442 spinach leaves and in post-harvest, pea vine field residue. Overall this work shows that chloroplasts  
443 liberated from their cell wall-bound environment act as substrates for digestive enzymes with  
444 galactolipase activity. Spinach CRF prepared under controlled laboratory conditions are the most  
445 enriched in the essential  $\alpha$ -linolenic acid, but it is worth noting that PVH CRF produced from a waste  
446 from agriculture still contain a large amounts of ALA and their post-harvesting heat treatment paves  
447 the way to their use as dietary supplements.

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#### 452 **6. Conflict of interest statement.**

453 There are no conflicts to declare.

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456 **7. References**

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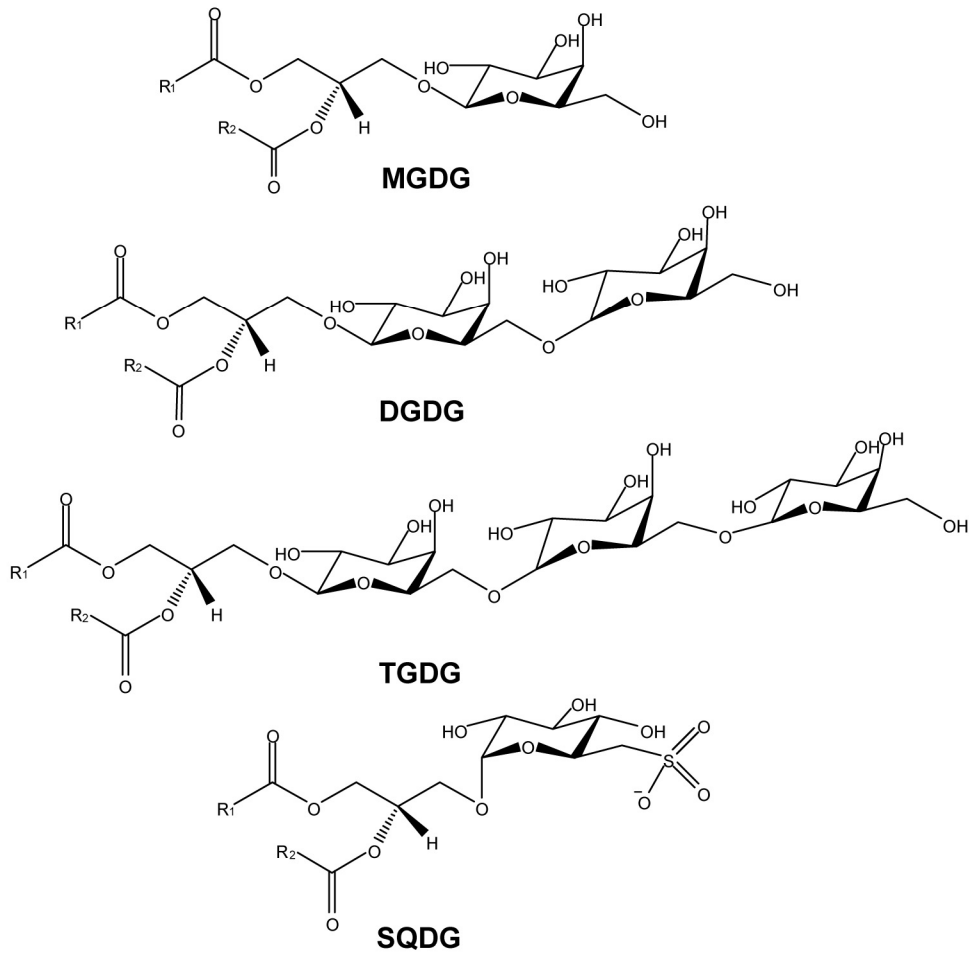
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625 **Figure legends**

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627 **Figure 1.** Chemical structures of galactolipids in plants. MGDG, mongalactosyldiacylglycerol; DGDG,

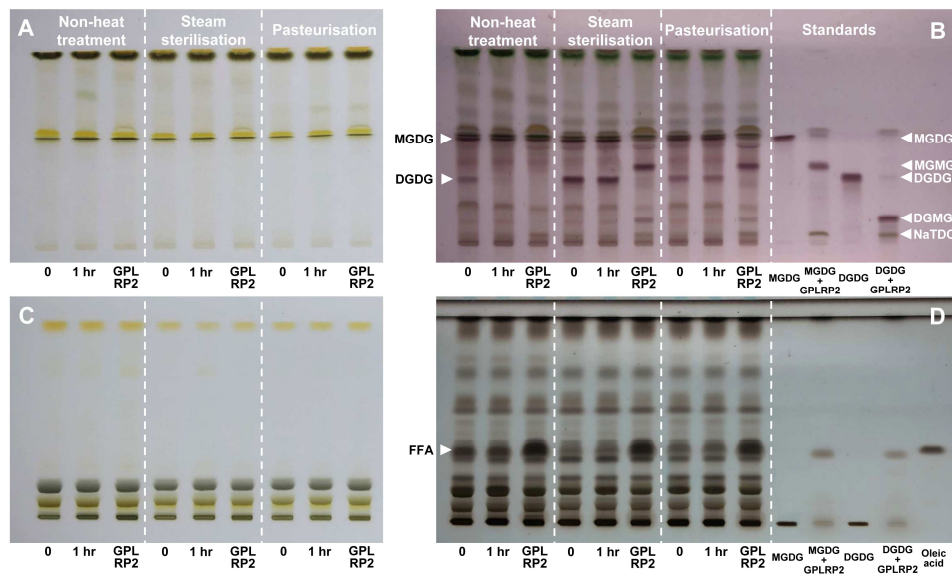
628 digalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol; SQDG, sulfoquinovosyl diacylglycerol.



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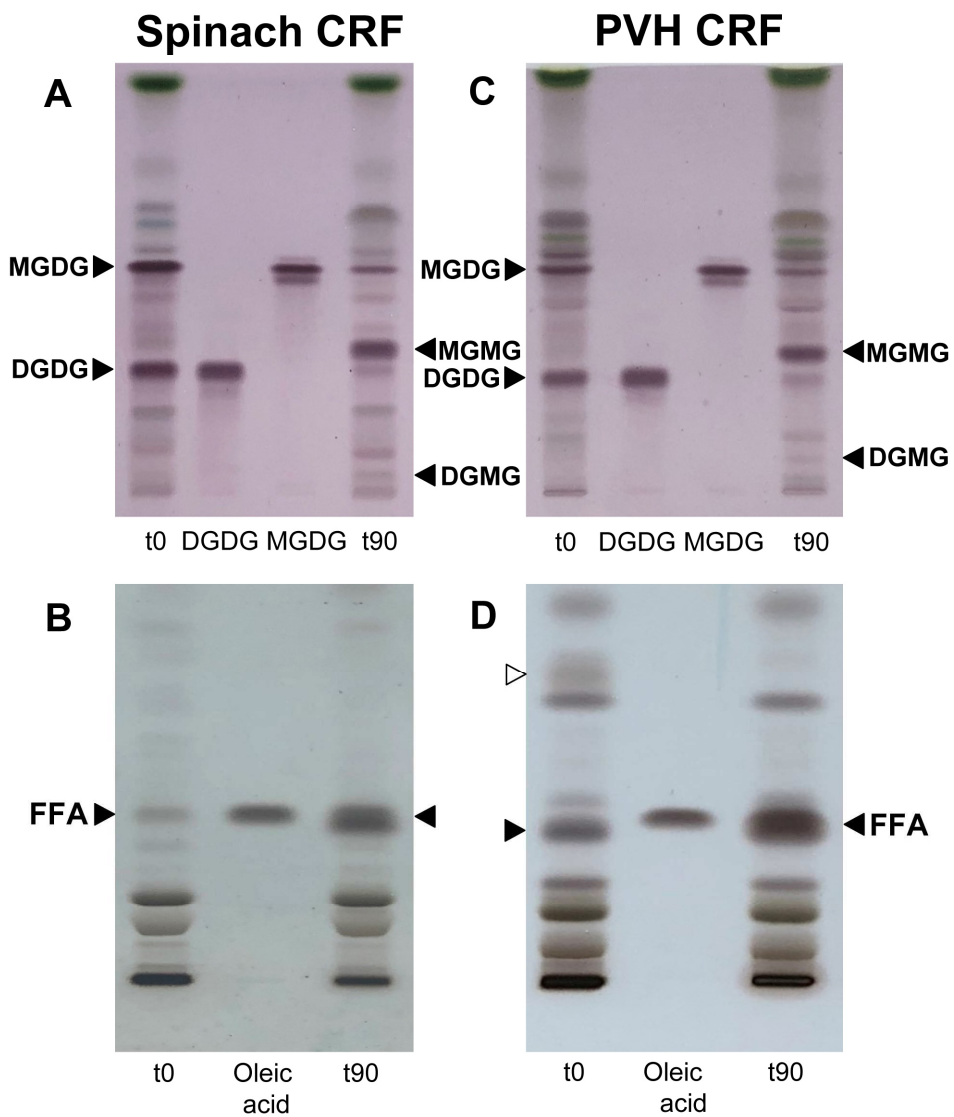
631 **Figure 2:** TLC analysis of lipid extracts from CRF of PVH showing the effects of endogenous and  
 632 exogenous (GPLRP2) enzymes on the hydrolysis of galactolipids, depending on CRF pre-treatment.  
 633 CRF were either non-treated or steam sterilised or pasteurised, and lipids were extracted after 1 hour  
 634 of incubation with or without GPLRP2. Polar lipid separation was visualised first without any staining  
 635 (panel A) and then revealed with a thymol solution (panel B). Neutral lipid separation was also  
 636 visualised first without any staining (panel C) and then revealed with copper acetate-phosphoric acid  
 637 solution (panel D). As reference standards, pure MGDG, DGDG and oleic acid were used, as well  
 638 MGDG and DGDG incubated with GPLRP2 in the presence of bile salts (NaTDC), which allowed  
 639 generating MGMG and DGMG.



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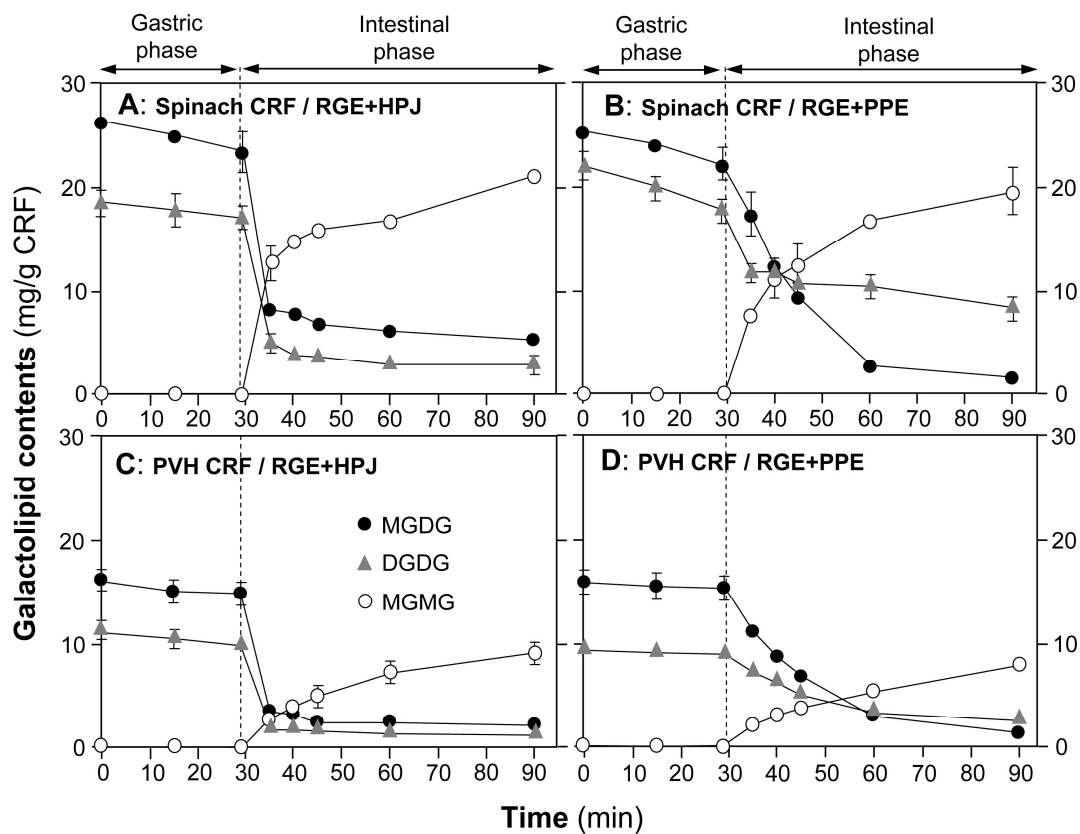
642 **Figure 3:** TLC analysis of galactolipids and free fatty acids initially present in CRFs (t0) and after 90-  
 643 min digestion (t90) by a combination of RGE and HPJ. Panels A and B: CRF from blanched spinach  
 644 leaves; Panels C and D: CRF from steam sterilised pea wine haulm (PVH). Polar lipids (panels A and  
 645 C) and neutral lipids (panels B and D) were revealed with a thymol solution and a copper acetate-  
 646 phosphoric acid solution, respectively. Pure MGDG, DGDG and oleic acid were used as reference  
 647 standards. The white arrow in panel C indicates a band of apolar lipids present in PVH CRF, that  
 648 disappears after digestion.



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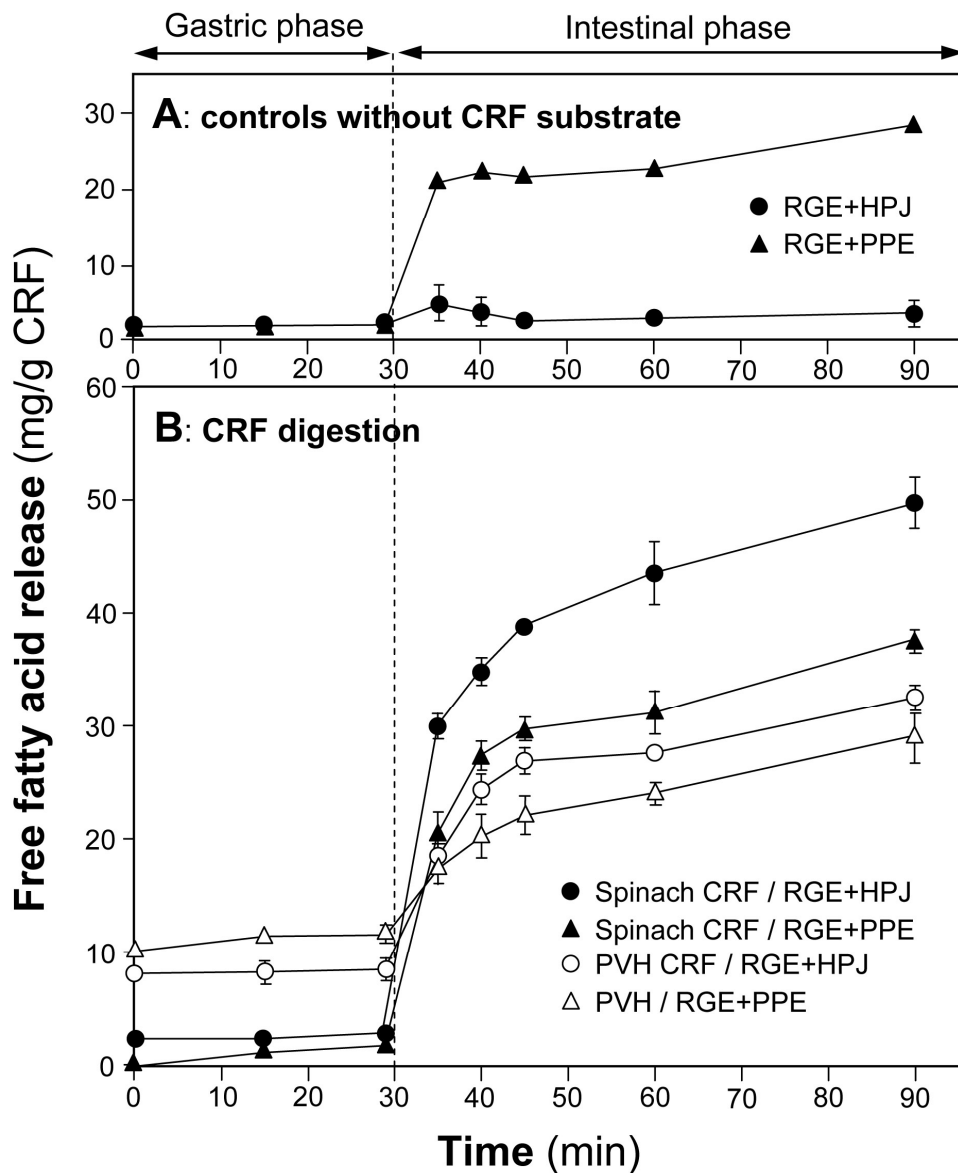
651 **Figure 4:** Hydrolysis of galactolipids in the course of two-step static digestion of CRFs. CRF from  
 652 blanched spinach leaves were digested using a combination of RGE and HPJ as sources of gastric  
 653 and pancreatic enzymes respectively (panel A) or a combination of RGE and PPE as an alternative  
 654 source of pancreatic enzymes (panel B). Similarly, CRF from steam sterilised PVH were digested  
 655 using a combination of RGE and HPJ (panel C) or a combination of RGE and PPE (panel D).  
 656 Symbols: full black circles, MGDG; open circles, MGMG; grey triangles, DGDG. Values (mg of  
 657 galactolipid per g (DW) of CRF) are means  $\pm$  SD (n=3).



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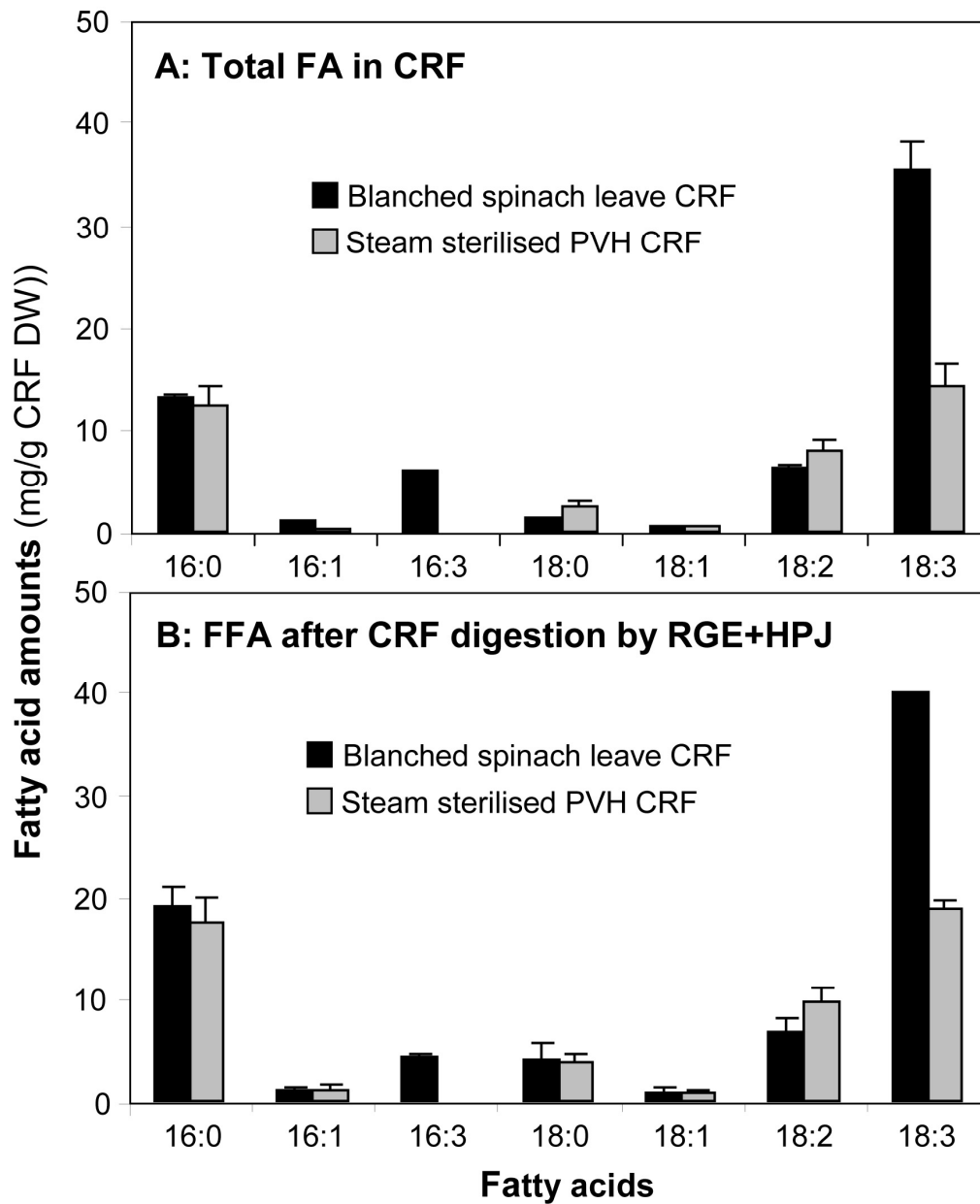
660 **Figure 5:** Free fatty acid release during the two-step static digestion of CRFs **A)** blank without CRF  
 661 substrate showing that some FFA can be released from PPE. **B)** Digestion of CRFs from blanched  
 662 spinach leaves and steam sterilised PVH, incubated with either a combination of RGE and HPJ as  
 663 sources of gastric and pancreatic enzymes or a combination of RGE and PPE as an alternative  
 664 source of pancreatic enzymes. Values (mg of FFA released per g (DW) of CRF) are means  $\pm$  SD  
 665 (n=3).



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668 **Figure 6:** Composition of total fatty acids initially present in blanched spinach and steam sterilised  
 669 pea wine haulm CRFs (panel A) and corresponding free fatty acids released after 90-min digestion by  
 670 RGE and HPJ (panel B). Data are expressed in mg per g of CRF dry weight and presented as mean  $\pm$   
 671 SD (n=3).



673 **Table 1:** Galactolipids and free fatty acid contents in different samples of CRF from PVH and spinach  
674 leaves. The effect of steam sterilisation versus non-heat treatment before CRF preparation was  
675 studied with PVH, while spinach CRF were obtained from blanched leaves. For each lipid class  
676 (MGDG, DGDG or FFA), concentration was expressed either in mg per g of CRF (DW) or mg per g of  
677 total lipid extract from CRF. On average, 1 g of dried CRF from spinach and PVH contains  $256.77 \pm$   
678  $9.15$  and  $162.77 \pm 4.63$  mg total lipid, respectively. DW, dry weight.

Sample, treatment		MGDG	DGDG	FFA	Total galactolipids + FFA
PVH CRF, non-heat treatment	mg/g CRF (DW)	$5.50 \pm 0.22^c$	$7.29 \pm 0.45^c$	$34.46 \pm 1.64^a$	$47.25 \pm 1.88^b$
	mg/g total lipids	$33.77 \pm 1.36^b$	$44.81 \pm 2.75^c$	$211.74 \pm 10.07^a$	$290.31 \pm 11.53^a$
PVH CRF, steam sterilisation	mg/g CRF (DW)	$18.89 \pm 1.97^b$	$13.22 \pm 0.99^b$	$11.71 \pm 0.72^b$	$43.82 \pm 2.72^c$
	mg/g total lipids	$116.04 \pm 12.08^a$	$81.21 \pm 6.08^b$	$71.95 \pm 4.43^b$	$269.19 \pm 16.71^b$
Spinach leaves CRF, blanching	mg/g CRF (DW)	$28.59 \pm 1.12^a$	$25.81 \pm 1.12^a$	$1.30 \pm 0.35^c$	$55.70 \pm 1.96^a$
	mg/g total lipids	$111.35 \pm 4.35^a$	$100.50 \pm 4.35^a$	$5.08 \pm 1.35^c$	$216.93 \pm 7.63^c$

679 Data were presented as a mean  $\pm$  SD of 3 separated *in-vitro* digestion and analysed using post-hoc analysis of  
680 variance (ANOVA) and according to a Tukey test with statistically significance at  $p < 0.05$ , a>b.

681 **Table 2:** Total fatty acids in PVH and spinach leave CRF and their release upon digestion. Total fatty  
682 acids (FA) in CRF were estimated from GC-MS analysis. FA presents in galactolipids (MGDG and  
683 DGDG) and free fatty acids (FFA) initially present in CRF were estimated from TLC analysis and data  
684 in Table 1. Mass amounts (mg/g CRF, DW) of MGDG, DGDG and FFA were converted in  $\mu$ moles per  
685 g of CRF (DW) using average molar masses of 760, 922 and 271 g/mole, respectively, which were  
686 estimated from the fatty acid composition of CRF (Figure 6 and Table S1). FA mole equivalents  
687 present in MGDG and DGDG were then estimated. FFA released after 90-min digestion by the  
688 combination of RGE and HPJ were estimated from TLC analysis. Values, expressed either in mg/g  
689 CRF (DW) or  $\mu$ mole/g CRF (DW), are means  $\pm$  SD (n=3). Values into brackets are the percentage of  
690 total FA in CRF. DW, dry weight.

Sample		Total FA in CRF	FA in galactolipids and FFA	FFA after 90- min digestion by RGE+HPJ
Steam sterilised PVH CRF	mg/g CRF	$37.81 \pm 5.67^b$	$32.96 \pm 2.71^b$	$32.65 \pm 0.88^b$
	(DW)			
	$\mu$ mole/g CRF	$139.35 \pm 20.90^b$	$121.46 \pm 9.98^b$	$120.35 \pm 3.23^b$
	(DW)		(87.2 %)	(86.4 %)
Blanched spinach leaves CRF	mg/g CRF	$64.16 \pm 3.11^a$	$36.83 \pm 1.81^a$	$49.81 \pm 2.23^a$
	(DW)			
	$\mu$ mole/g CRF	$237.18 \pm 11.51^a$	$136.15 \pm 6.68^a$	$184.13 \pm 8.24^a$
	(DW)		(57.4 %)	(77.6 %)

691 Data were presented as a mean  $\pm$  SD of 3 separated *in-vitro* digestion and analysed using an independent-  
692 sample t-test with statistically significance at  $p < 0.05$ , a>b.

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