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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 α -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.¹ 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.² 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,³ or it is ploughed back into the soil to provide nitrogen.⁴ 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.⁵⁻⁷ 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.⁸⁻⁹ 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 α -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**.¹⁰ 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.¹¹ 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).¹²⁻¹⁵ 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.¹⁶ Both human and guinea
78 pig PLRP2s were found to display a high activity on DGDG and MGDG from spinach leaves.¹⁰ 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.¹⁷⁻¹⁸ Many publications on *in-vitro* digestion use
93 pepsin as the single enzyme in the gastric phase.^{17,19,20-21} 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.²²⁻²³ 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 lipases²⁴⁻²⁶ can be chosen as alternative sources
99 instead of human gastric juice.²² Human Pancreatic Juice (HPJ) has been used as a source of
100 pancreatic lipase for the intestine phase of *in vitro* digestion.²⁷⁻²⁸ 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.^{17,19,20-21} 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 solution²⁹ and from PVH using a slow-screw twin gear
107 juicer without added water.³⁰ 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.³¹ 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 CaCl₂ 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)²⁸ 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.³²⁻³³ 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^{22,34}, 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.^{17,32} 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),³⁵ modified by Bligh and Dyer (1959).³⁶ 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).³⁷ 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.³⁸ 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.³⁹ 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.¹² 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.^{9,40-41} 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 ± 0.35 mg/g CRF or 5.08 ± 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 ± 0.72 mg/g CRF or 71.95 ± 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,^{27,33} 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).²³ 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).⁴² Both
321 PLRP2 and CEH/BSSL show galactolipase activity on MGDG and DGDG,^{11,14-15,43-45} as well as
322 phospholipase A1 activity on phospholipids.^{16,43} 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.¹⁷ It is commonly used for *in vitro* digestion studies as a substitute of human
325 pancreatic enzymes.^{17,22} Nevertheless, it was recently shown that PPE does not contain as much
326 galactolipase activity as HPJ.⁴⁶ 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)¹¹, 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.^{12,14} 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.⁴⁶ 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 lipids⁴⁷ 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 α -linolenic acid (ALA; 18:3) but its amounts in spinach CRF (35.56 ± 2.56
370 mg/g CRF DW) were 2.5-fold higher than in PVH CRF (14.29 ± 2.06 mg/g CRF DW). ALA

371 represented 55.38 ± 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.¹⁰ In pea vine CRF, ALA
373 represented only 37.82 ± 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 ± 0.24 mg/g
375 CRF; 20.77 ± 0.76 % w/w of total FA) and pea vine CRF (12.35 ± 1.86 mg/g CRF; 32.65 ± 0.12 % w/w
376 of total FA). Hexadecatrienoic acid (16:3) was found at significant levels in spinach CRF (5.98 ± 0.12
377 mg/g CRF; 9.34 ± 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.⁴⁸ 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.⁴⁹ 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.⁵⁰

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 MGMT (**Figure 4**) and DGMT (**Figure 3** and **S1**), in line with the preferential hydrolysis by PLRP2 of
398 the ester bond at the sn-1 position of galactolipids.¹⁰ 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.⁵¹ 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, α -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 α -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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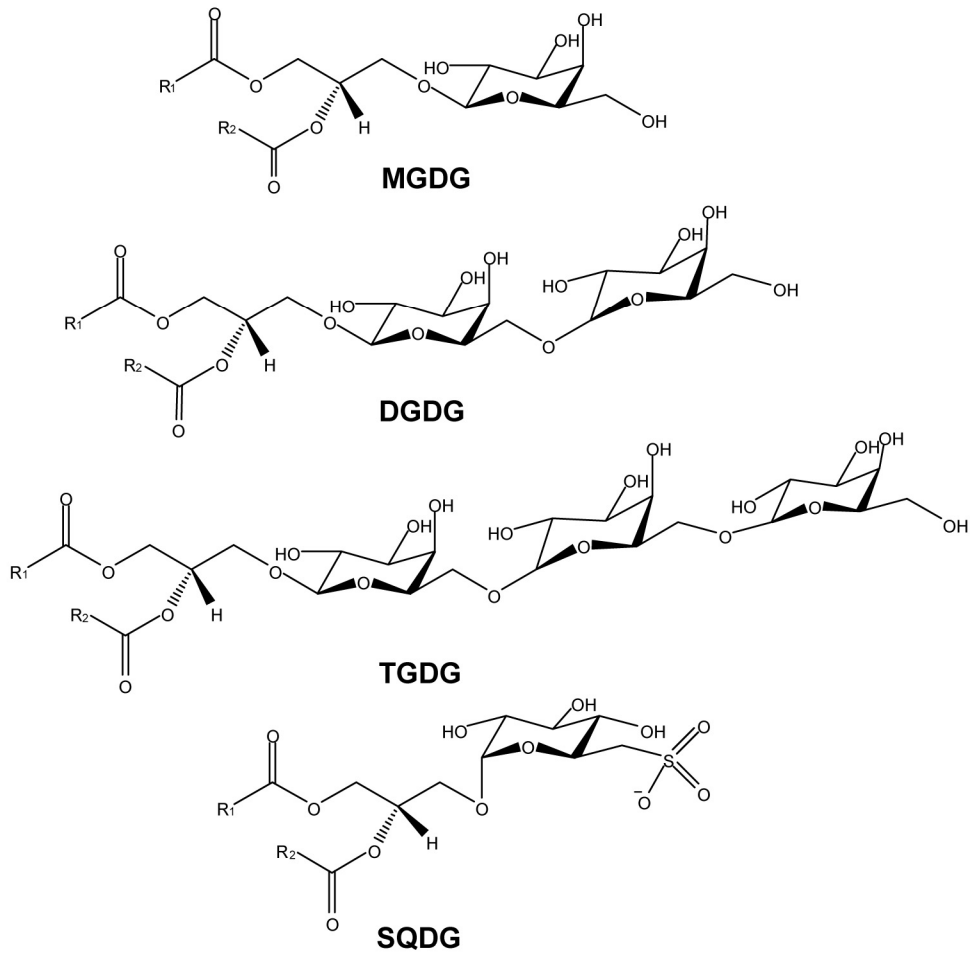
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625 **Figure legends**

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

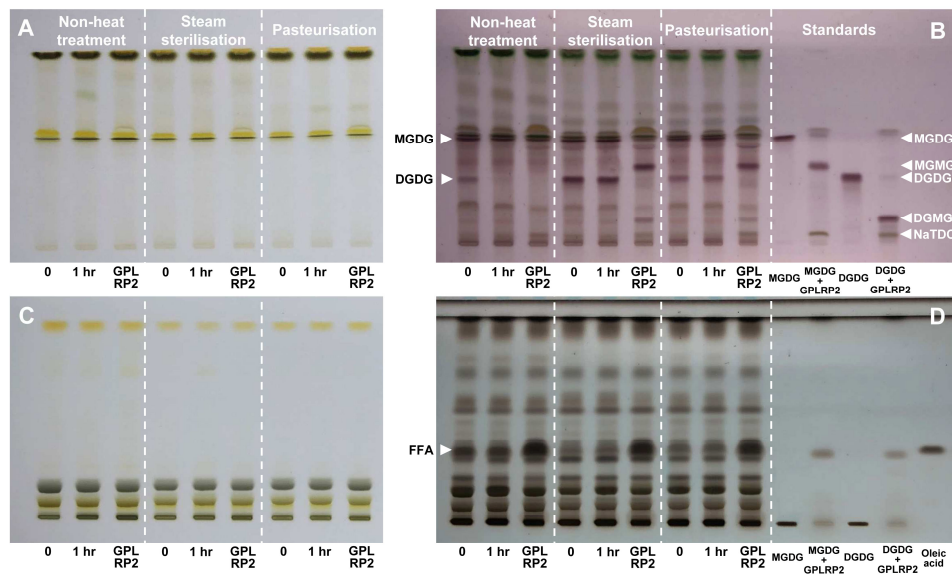
628 digalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol; SQDG, sulfoquinovosyl diacylglycerol.



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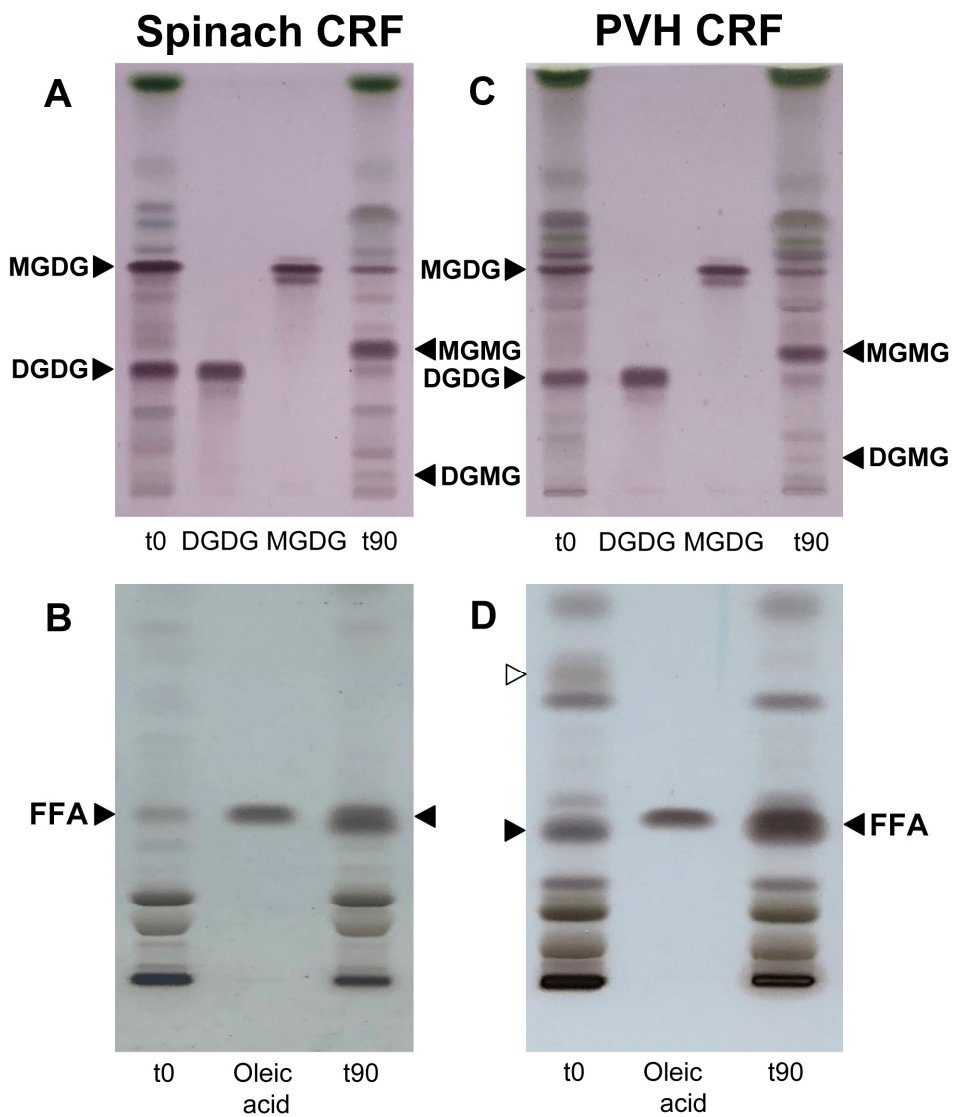
631 **Figure 2:** TLC analysis of lipid extracts from CRF of PVH showing the effects of endogenous and
 632 exogenous (GPLRP2) enzymes on the hydrolysis of galactolipids, depending on CRF pre-treatment.
 633 CRF were either non-treated or steam sterilised or pasteurised, and lipids were extracted after 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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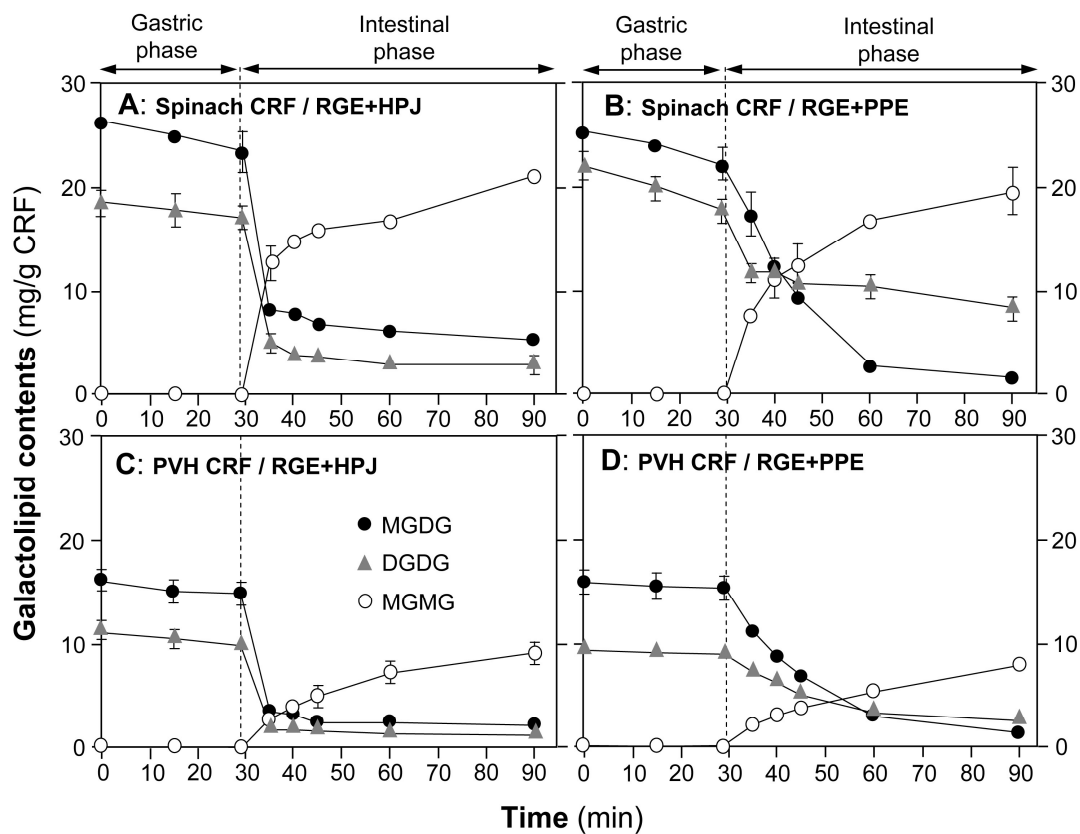
642 **Figure 3:** TLC analysis of galactolipids and free fatty acids initially present in CRFs (t0) and after 90-
 643 min digestion (t90) by a combination of RGE and HPJ. Panels A and B: CRF from blanched spinach
 644 leaves; Panels C and D: CRF from steam sterilised pea wine haulm (PVH). Polar lipids (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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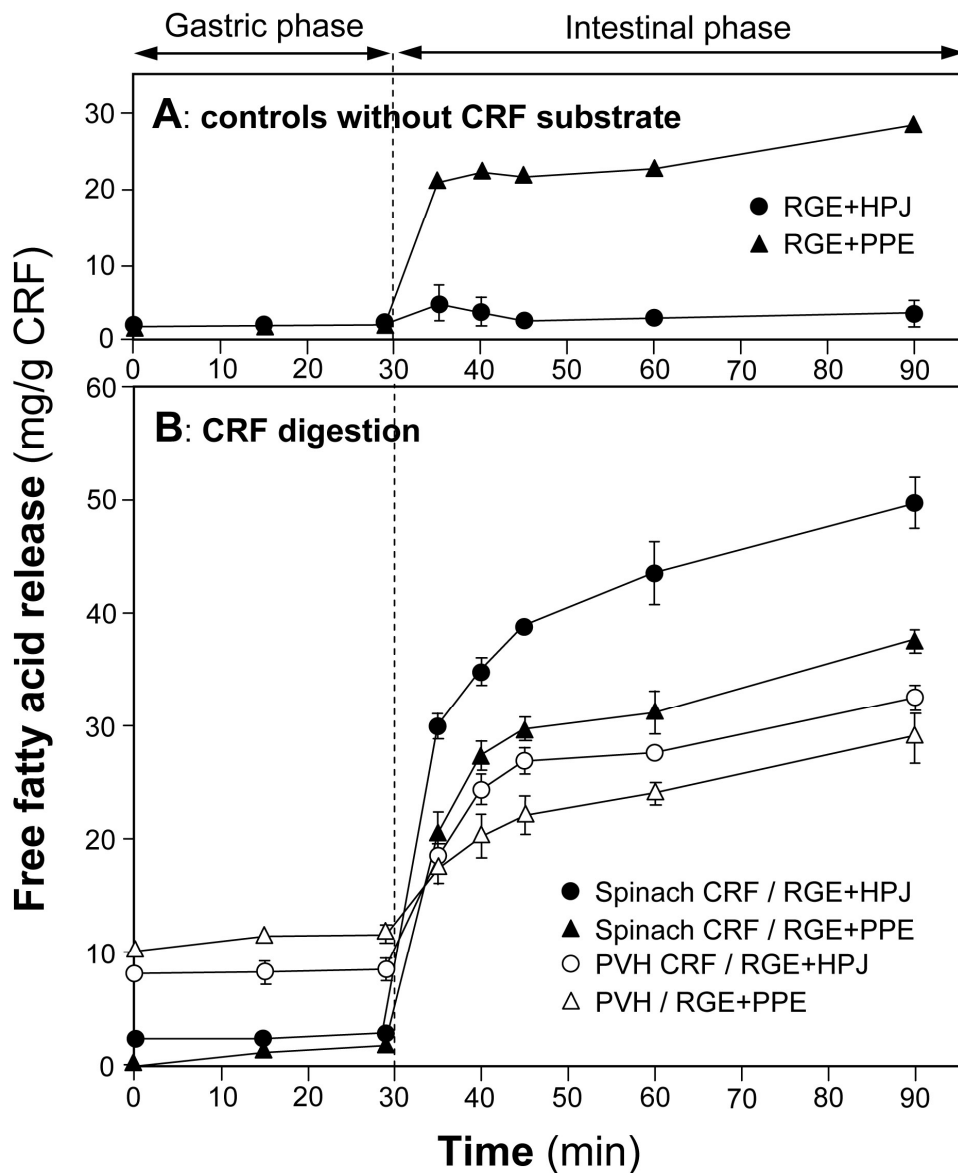
651 **Figure 4:** Hydrolysis of galactolipids in the course of two-step static digestion of CRFs. CRF from
 652 blanched spinach leaves were digested using a combination of RGE and HPJ as sources of gastric
 653 and pancreatic enzymes respectively (panel A) or a combination of RGE and PPE as an alternative
 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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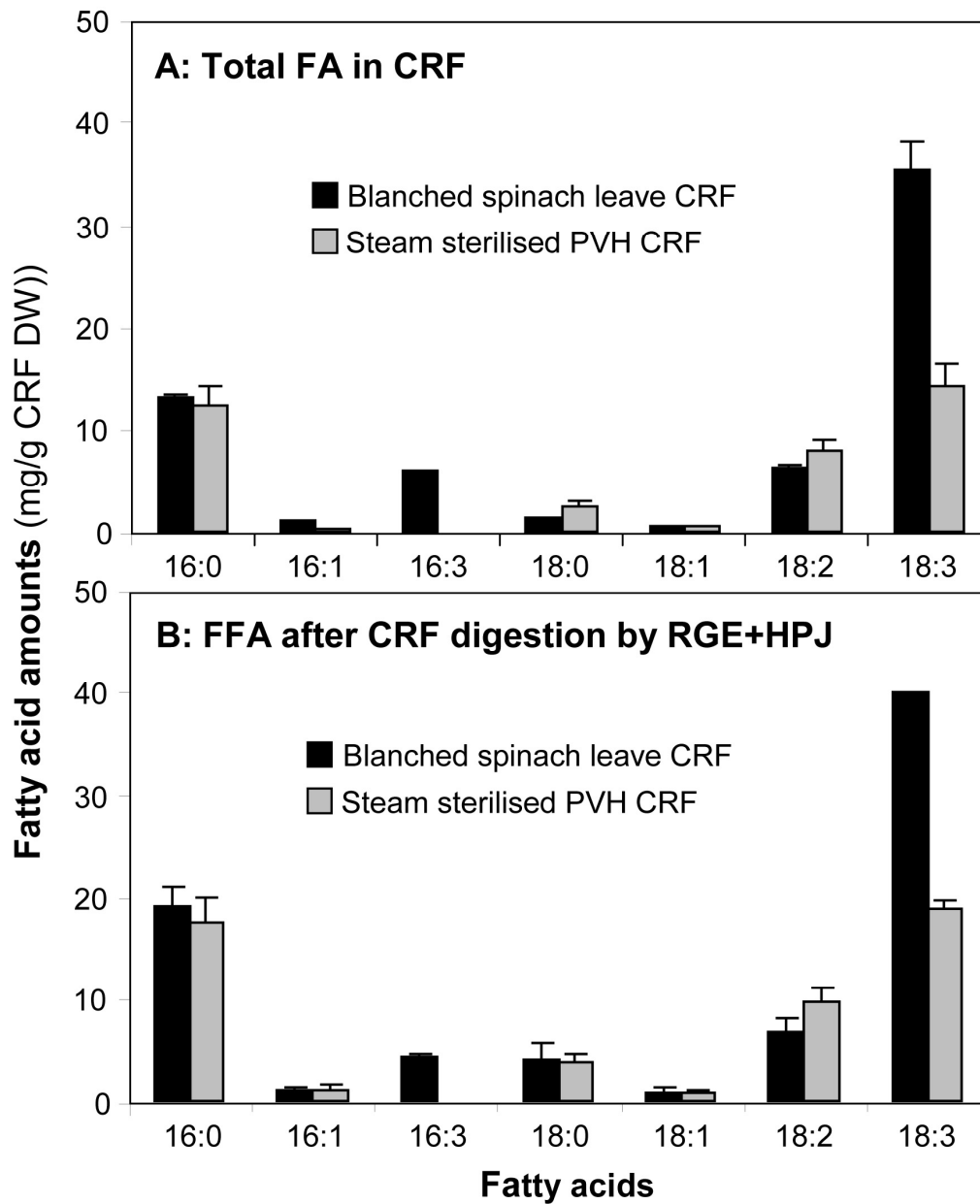
660 **Figure 5:** Free fatty acid release during the two-step static digestion of CRFs **A)** blank without CRF
 661 substrate showing that some FFA can be released from PPE. **B)** Digestion of CRFs from blanched
 662 spinach leaves and steam sterilised PVH, incubated with either a combination of RGE 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 ± 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 ± 0.22^c	7.29 ± 0.45^c	34.46 ± 1.64^a	47.25 ± 1.88^b
	mg/g total lipids	33.77 ± 1.36^b	44.81 ± 2.75^c	211.74 ± 10.07^a	290.31 ± 11.53^a
PVH CRF, steam sterilisation	mg/g CRF (DW)	18.89 ± 1.97^b	13.22 ± 0.99^b	11.71 ± 0.72^b	43.82 ± 2.72^c
	mg/g total lipids	116.04 ± 12.08^a	81.21 ± 6.08^b	71.95 ± 4.43^b	269.19 ± 16.71^b
Spinach leaves CRF, blanching	mg/g CRF (DW)	28.59 ± 1.12^a	25.81 ± 1.12^a	1.30 ± 0.35^c	55.70 ± 1.96^a
	mg/g total lipids	111.35 ± 4.35^a	100.50 ± 4.35^a	5.08 ± 1.35^c	216.93 ± 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 μ 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 μ 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 ± 5.67^b	32.96 ± 2.71^b	32.65 ± 0.88^b
	(DW)			
	μ mole/g CRF	139.35 ± 20.90^b	121.46 ± 9.98^b	120.35 ± 3.23^b
	(DW)		(87.2 %)	(86.4 %)
Blanched spinach leaves CRF	mg/g CRF	64.16 ± 3.11^a	36.83 ± 1.81^a	49.81 ± 2.23^a
	(DW)			
	μ mole/g CRF	237.18 ± 11.51^a	136.15 ± 6.68^a	184.13 ± 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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