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

Jutarat Wattanakul[a,b] (jutarat.wattanakul@nottingham.ac.uk), Moulay Sahaka[c] (msahaka@imm.cnrs.fr), Sawsan Amara[c,d] (sawsan.amara@lipolytech.com), Mansor Syamila[a,e] (syamilamansor@gmail.com), Brigitte Gontero[c] (bmeunier@imm.cnrs.fr), Frédéric Carrière[c] (carriere@imm.cnrs.fr), David Gray[a*] (david.gray@nottingham.ac.uk)

[a] Division of Food, Nutrition and Dietetics, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, United Kingdom.

[b] Department of Food Sciences and Technology, Faculty of Home Economics Technology, Rajamangala University of Technology Krungthep, Bangkok, 10120, Thailand.

[c] Aix Marseille Univ, CNRS, UMR7281 Bioénergétique et lngénierie des Protéines, 31 Chemin Joseph Aiguier, 13402 Marseille, Cedex 09, France.

[d] Lipolytech, Zone Luminy Biotech, 163 avenue de Luminy, 13288 Marseille Cedex 09, France

[e] Universiti Sains Islam Malaysia, Bandar Baru Nilai, 78100 Nilai, Negeri Sembilan, Malaysia

*Corresponding Author

Abstract

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

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

1. Introduction

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

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

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

2. Materials and methods

2.1. Materials

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

2.2. Chemicals

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

2.3. Enzymes

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

2.4. Post-harvest treatment of the pea vine haulm

The fresh biomass was washed with tap water to remove soil and rocks, and then the excess of water removed using an industrial salad spinner (Sammic ES-200). The haulm was split into two batches for different treatments. One batch (13 kg) was packed into a vacuum sealed bag for steam condition (section 2.4.1). Another batch of haulm (5 kg) was juiced immediately using a twin gear juicer

(Angel 7500) which separated the fibrous pulp from the nutrient rich juice. The juice was filtered through a 75 μm stainless steel mesh sieve and processed (with or without pasteurisation) to isolate the CRF (section 2.6).

2.4.1. Steam sterilisation of pea vine haulm

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

2.4.2. Pasteurisation of juice extracted from pea vine haulm

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

2.5. Hot-water blanching of spinach leaves

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

2.6. Isolation of CRF

CRF was isolated according to slightly modified method described in Gedi et al. (2017)[29] and Torcello-Gómez et al. (2019).[30] The PVH juice was centrifuged at 17,700 RCF or 10,000 rpm (Beckman Coulter JS-21M with JA-10 rotor) for 10 min at 4°C. The CRF-containing pellet was retained while the supernatant was centrifuged again under the same conditions to obtain a further pellet. The CRF

fractions were pooled, weighed and frozen at -80°C prior to freeze drying (Edwards Freeze Dryer Super Modulyo) for 3-5 days. Freeze-dried CRF was then ground using a pestle and mortar, and stored in a vacuum-sealed foil pouch at -20°C for further analysis.

2.7. Digestion of CRF galactolipid by GPLRP2

CRF (25 mg) was suspended in 1 mL of buffer solution at pH 8 containing 0.3 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$ and 4 mM sodium taurodeoxycholate (NaTDC). GPLRP2 was further added at a final concentration of 20 μg/mL and the reaction mixture was incubated at 37°C for 1 hr and compared with a control without GPLRP2. The reaction was stopped by adding 200 μL of 1 N HCl and further extracted lipid (section 2.9).

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

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

2.9. Lipid extraction

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, Jounan 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.

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

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 were dried again in the fume hood for 10 min and then placed in an oven at 110°C for 10 min. The plate for FFA analysis was dipped in a copper acetate-phosphoric acid solution prepared by mixing a saturated copper acetate solution with 85% phosphoric acid in a 1 to 1 volume ratio. The plates were dried for 10 min in the fume hood

and then placed in an oven at 180°C for 15 min. Densitometry analysis of the stained lipids on the TLC plate was carried out using a Camag TLC scanner II and a D2000+ chromato-Integrator (Merck). Lipid bands were scanned at 366 nm for thymol staining and at 500 nm for copper acetate-phosphoric acid staining, with a 0.5 x 7 mm slit and a speed of 2.5 cm/min. Slit conditions were selected accordingly to band size. The slit should always cover the whole band size. The densitograms of all tracks were integrated using D2000+Chromator-Integrator. Quantities of the lipids on the TLC plates were estimated from the linear standard curves established with the pure lipid standards (MGDG, DGDG, and oleic acid (18:1)).

2.11. Fatty acid analysis by GC-MS

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

2.12. Statistical analysis

All experiments were performed in triplicate. The statistical analysis was carried out using IBM SPSS statistic 25 using post hoc analysis of variance (ANOVA) and according to the Tukey test with statistically significant at $p<0.05$ or an independent-sample t-test with statistically significant at $p<0.05$. The data were expressed as mean ± standard deviation. Differences of means at $p<0.05$ were considered significant.

3. Results and Discussion

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

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

279 blanched in hot water at 85°C for 3 min to knock out the endogenous enzymes before preparing CRF.
280 It was checked that this treatment allowed the inactivation of the endogenous galactolipase activity of
281 CRF from spinach leaves.

282 **3.2. Galactolipid and free fatty acid content in CRF before digestion**

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

305 **3.3. *In-vitro* digestion of galactolipids in CRF**

306 The experimental conditions to simulate lipid digestion were based on *in vivo* studies and
307 parameters measured at 50% meal gastric emptying,[27,33] such as the lipase concentrations (17 µg/mL

of gastric lipase in the stomach and 250 µg/mL of pancreatic lipase in the small intestine) and the pH values (5 for the gastric phase and 6 for the intestinal phase). In these experiments, rabbit gastric extract (RGE) was chosen as the source of gastric enzymes because it is composed of pepsin and gastric lipase, and the activity of rabbit gastric lipase has a similar range of activity as the human gastric lipase (HGL).[23] Porcine Pancreatic Extract (PPE) or Human Pancreatic Juice (HPJ) were used as the sources of pancreatic enzymes for the intestinal phase of *in vitro* digestion. HPJ contains various lipolytic enzymes, including pancreatic lipase, pancreatic lipase related protein 1 and 2 (PLRP1 and PLRP2) and carboxyl ester hydrolase/ bile salts stimulated lipase (CEH/BSSL).[42] Both PLRP2 and CEH/BSSL show galactolipase activity on MGDG and DGDG,[11,14-15,43-45] as well as phospholipase A1 activity on phospholipids.[16,43] PPE consists of a mixture of digestive enzymes produced by the exocrine cell of the porcine pancreas and contains trypsin, chymotrypsin, α-amylase, lipase and colipase.[17] It is commonly used for *in vitro* digestion studies as a substitute of human pancreatic enzymes.[17,22] Nevertheless, it was recently shown that PPE does not contain as much galactolipase activity as HPJ.[46] Therefore, it was important to compare here both sources of pancreatic enzymes.

According to the TLC analysis of galactolipid digestion (**Figures 3**, **4, S1** and **S2**), both the MGDG and DGDG of CRF from blanched spinach leaves and steam sterilised PVH were hydrolysed and converted to lysogalactolipid (monogalactosylmonoglycerol, MGMG, and digalactosylmono glycerol, DGMG) during the whole digestion process. During the 30-min gastric phase, galactolipids decreased slightly from 25-26 to 22-23 mg MGDG /g CRF and 18-22 to 17-18 mg DGDG /g CRF for spinach leaves CRF (**Figure 4A** and **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 (**Figure 4C** and **4D**). The weak increase in FFA during the gastric phase (**Figure 5B**) confirms that galactolipid hydrolysis is not very important under gastric conditions, in line with the fact that gastric lipase has no demonstrated galactolipase activity. Nevertheless, RGE might contain some traces of other enzymes with galactolipase activity. After adding HPJ and bile salts to the system to initiate the intestinal phase of digestion, both MGDG and DGDG of spinach and PVH CRF were immediately hydrolysed to MGMG and DGMG, respectively (**Figures 4A, 4C** and **S1**), and high amounts of free fatty acids were released (**Figure 5** and **S2**), especially during the first 5 min of the intestinal phase. Because the monogalactosyl galactolipids (MGDG and MGMG) are revealed similarly upon thymol staining (**Figures 2B**), and in the absence of a pure MGMG reference standard, the generation of MGMG was tentatively quantitated by TLC using the calibration curve established

with MGDG as reference standard. A good correlation was observed between the appearance of MGMG and the disappearance of MGDG (**Figure 4**). The band corresponding to DGMG on TLC plates (see **Figure 3** and **Figure S1** in Supplementary Data) was however too faint and not enough resolved to apply the same method. These results are supported by those of Andersson et al. (1995)[11], who demonstrated that pure galactolipids were hydrolysed by Human Pancreatic Juice. This previous study had led to the characterisation of the galactolipase activity of HPJ and its association with PLRP2 and to a lower extent to CEH/BSSL.[12,14] Galactolipids from both CRF preparations were hydrolysed at a slower rate when RGE and PPE were combined for *in vitro* digestion (**Figures 4B** and **4D**). It confirms that PPE contains a lower galactolipase activity.[46] Additionally, it was observed that PPE prefers to hydrolyse MGDG over DGDG, which is not the case for HPJ.

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

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

The compositions of total fatty acids in spinach leaves and pea vine CRF were analysed using GC-MS and the results are shown in the **Figure 6**. The main fatty acid in both spinach leave and pea vine CRF was α-linolenic acid (ALA; 18:3) but its amounts in spinach CRF (35.56 ± 2.56 mg/g CRF DW) were 2.5-fold higher than in PVH CRF (14.29 ± 2.06 mg/g CRF DW). ALA represented 55.38 ± 1.33 % w/w of the total fatty acids in spinach leave CRF, which is in agreement with the fatty acid composition of spinach leaves and isolated galactolipids.[10] In pea vine CRF, ALA represented only 37.82 ± 0.31 % w/w of the total fatty acids. The second most abundant fatty acid was palmitic acid (PA;

16:0), which was found at similar levels in spinach CRF (13.31 ± 0.24 mg/g 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 of total FA). Hexadecatrienoic acid (16:3) was found at significant levels in spinach CRF (5.98 ± 0.12 mg/g CRF; 9.34 ± 0.29 % w/w of total FA) but was not found in pea vine CRF. Therefore, the levels of polyunsaturated omega 3 fatty acids were globally reduced in PVH CRF compared to spinach CRF. This may results from a preferential oxidation of these fatty acids by endogenous lipoxygenases from harvesting to steam sterilisation of PVH. Moreover, this oxidation is known to be coupled to and favoured by the release of FFA by endogenous galactolipase activity as often seen in plant response to stress.[48] The absence of 16:3 fatty acid in PVH CRF may also be explained by the classification of pea among “eukaryotic plants”, i.e. plants which preferentially have C18 fatty acids at sn-2 position of the glycerol backbone in galactolipids, while “prokaryotic” plants like spinach preferentially have C16 fatty acids at sn-2 position.[49] Indeed, the predominant galactolipids in “prokaryotic” plants are MGDG (18:3/16:3) and DGDG (18:3/16:0), while MGDG (18:3/18:3) and DGDG (18:3/18:3) are the predominant ones in “eukaryotic” plant.[50]

After 90 min of digestion by RGE and HPJ, the composition of FFA generated from CRF hydrolysis was similar to the composition of total FA in both spinach and PVH CRF (**Figure 6**), with ALA representing the most abundant FFA, followed by PA. With the combination of RGE and PPE, some changes were observed in the relative distribution of fatty acids, which certainly reflects the contribution of fatty acids present in PPE (see **Figure S3** and **Table S1** in Supplementary data). Therefore, we only discuss here the data obtained with RGE and HPJ. We observed that the amounts of total FFA released during the CRF digestion were higher than those expected from the complete lipolysis of MGDG and DGDG, both for spinach leave and PVH CRF (**Table 2** and **Figure 5B**). Moreover, we have seen that galactolipid lipolysis was not complete, with some accumulation of MGMG (**Figure 4**) and DGMG (**Figure 3** and **S1**), in line with the preferential hydrolysis by PLRP2 of the ester bond at the sn-1 position of galactolipids.[10] Therefore, some FFA were certainly released from other acyl lipids present in CRF preparations. This hypothesis is supported by the levels of total fatty acids present in CRF and quantified by GC-MS (**Table 2**). **Figure 3D** on TLC separation of neutral lipids shows that some apolar lipids present in PVH CRF at time 0 and migrating like triglycerides or sterol esters are no more present at time 90 min. Phospholipids, mainly PG, are also present in chloroplast membranes and can be degraded by the phospholipase activity of HPJ. During stress or senescence, the degradation of

chlorophyll and galactolipid from thylakoid membranes in chloroplasts can also lead to the conversion of a large proportion of phytol and fatty acids into fatty acid phytyl esters (PFAE) and triacylglycerol.[51] We did not search nor analyse these various lipids here but it would be worth identifying these other sources of fatty acids in future studies in order to better describe the lipid composition of CRF. At this stage, it is important to keep in mind that 87.2 % of the total fatty acids of PVH CRF are present in galactolipids and FFA, and that 86.4 % of total fatty acids are released upon *in vitro* digestion, while the corresponding values for spinach CRF are 57.4 % and 77.6 %, respectively (**Table 2**). The proportion of fatty acid sources other than galactolipids is therefore higher in spinach CRF than in PVH CRF. In both cases, a large proportion of the total fatty acids can be converted to FFA upon digestion, including ALA as the main FFA.

4. Conclusion

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

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6. Conflict of interest statement.

There are no conflicts to declare.

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Figure legends

Figure 1. Chemical structures of galactolipids in plants. MGDG, mongalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

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

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

Figure 4: Hydrolysis of galactolipids in the course of two-step static digestion of CRFs. CRF from blanched spinach leaves were digested using a combination of RGE and HPJ as sources of gastric and pancreatic enzymes respectively (panel A) or a combination of RGE and PPE as an alternative source of pancreatic enzymes (panel B). Similarly, CRF from steam sterilised PVH were digested using a combination of RGE and HPJ (panel C) or a combination of RGE and PPE (panel D). Symbols: full

black circles, MGDG; open circles, MGMG; grey triangles, DGDG. Values (mg of galactolipid per g (DW) of CRF) are means ± SD (n=3).

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

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

Table 1: Galactolipids and free fatty acid contents in different samples of CRF from PVH and spinach leaves. The effect of steam sterilisation versus non-heat treatment before CRF preparation was studied with PVH, while spinach CRF were obtained from blanched leaves. For each lipid class (MGDG, DGDG or FFA), concentration was expressed either in mg per g of CRF (DW) or mg per g of total lipid extract from CRF. On average, 1 g of dried CRF from spinach and PVH contains 256.77 ± 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}

Data were presented as a mean ± SD of 3 separated *in-vitro* digestion and analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistically significance at $p<0.0.5$, a>b.

Table 2: Total fatty acids in PVH and spinach leave CRF and their release upon digestion. Total fatty acids (FA) in CRF were estimated from GC-MS analysis. FA presents in galactolipids (MGDG and DGDG) and free fatty acids (FFA) initially present in CRF were estimated from TLC analysis and data in Table 1. Mass amounts (mg/g CRF, DW) of MGDG, DGDG and FFA were converted in µmoles per g of CRF (DW) using average molar masses of 760, 922 and 271 g/mole, respectively, which were estimated from the fatty acid composition of CRF (Figure 6 and Table S1). FA mole equivalents present in MGDG and DGDG were then estimated. FFA released after 90-min digestion by the combination of RGE and HPJ were estimated from TLC analysis. Values, expressed either in mg/g CRF (DW) or µmole/g CRF (DW), are means ± SD (n=3). Values into brackets are the percentage of 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 (DW)	37.81 ± 5.67[b]	32.96 ± 2.71[b]	32.65 ± 0.88[b]
	µmole/g CRF (DW)	139.35 ± 20.90[b]	121.46 ± 9.98[b] (87.2 %)	120.35 ± 3.23[b] (86.4 %)
Blanched spinach leaves CRF	mg/g CRF (DW)	64.16 ± 3.11[a]	36.83 ± 1.81[a]	49.81 ± 2.23[a]
	µmole/g CRF (DW)	237.18 ± 11.51[a]	136.15 ± 6.68[a] (57.4 %)	184.13 ± 8.24[a] (77.6 %)

Data were presented as a mean ± SD of 3 separated *in-vitro* digestion and analysed using an independent-sample t-test with statistically significance at $p<0.0.5$, a>b.