Chloroplast-Rich Material from The Physical Fractionation Of Pea Vine (*Pisum sativum*) Postharvest Field Residue (Haulm)

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ABSTRACT

An innovative procedure for plant chloroplasts isolation has been proposed, which consists of juice extraction by physical fractionation from plant material and recovery of its chloroplast-rich fraction (CRF) by centrifugation. This simple method has been applied to pea vine haulm subjected to different post-harvest treatments: blanching, storage at different relative humidity values and fermentation. Additionally, freeze storage of the extracted juice was carried out. The macronutrient (total lipids, proteins, ash and carbohydrates) and micronutrient (fatty acids, chlorophylls, β-carotene, α-tocopherol and ascorbic acid) content and composition of the CRF have been determined. The CRF isolated from fresh pea vine haulm is a potential source of essential micronutrients (α-linolenic acid, β-carotene, α-tocopherol) and carbohydrates, whereas the post-harvest treatments trialled have a detrimental effect on the nutritional content. Industrial applications for the recovered nutritionally rich fraction, such as food supplement ingredient or animal feeding, are likely envisaged, while optimising the use of green haulm.

Keywords: pea vine; chloroplast; α-linolenic acid; β-carotene; α-tocopherol

1. INTRODUCTION

Millions of tonnes of green haulm are generated from agricultural production every year in the United Kingdom, part of which is usually recycled as animal feed (forage or silage) or as a soil improver (compost), while large amounts still remain unused. From a dietary perspective, this biomass may have nutritional value. Chloroplasts, abundant in green plant material, have been studied extensively to elucidate the elegant process of photosynthesis; what is less well recognised is that separate researchers have identified this organelle as the location of biosynthesis for a number of molecules that have nutritional credentials as well as functional roles *in vivo*. For example, all of the plant's fatty acids and most of its vitamins are synthesised in and remain in the chloroplast (Block, Douce, Joyard & Rolland, 2007). The main lipids that constitute the thylakoid membranes within the chloroplasts are galactolipids, rich in the ω-3 fatty acid, α-linolenic acid; these membranes are also a major source of pigments, such as chlorophylls and carotenoids (Block, Dorne, Joyard & Douce, 1983). The lipids from the chloroplast envelope membranes have a larger percentage of prenylquinones, like tocopherols (Lichtenthaler, Prenzel, Douce & Joyard, 1981).

β-carotene is an inactive form of vitamin A, also known as provitamin A, which is converted to vitamin A once absorbed in the duodenum. It needs to be provided through dietary sources since it cannot be synthesised by humans and animals. It is essential for the maintenance of normal epithelial cellular differentiation. α-tocopherol is one of eight forms of active vitamin E, but most importantly one of the main forms of vitamin E in chloroplasts of higher plants and the one preferentially absorbed by humans (Rigotti, 2007). It is also essential for normal growth and development of human body. Being both antioxidants, the health benefits linked with their intake are numerous, such as reduction of potential initiators of cell death and carcinogenesis (Abuajah, Ogbonna & Osuji, 2015). Additionally, ascorbic acid (vitamin C) is another antioxidant present at relatively high concentrations in plant chloroplasts, although its synthesis and storage is not restricted to this organelle (Hancock, McRae, Haupt & Viola, 2003). On the other hand, chloroplasts are also rich in polyunsaturated fatty

71 acids (PUFA) (Dubacq, Drapier & Tremolieres, 1983), which are associated with decreasing risk of
72 coronary heart disease (Willett, 2012).

73 Current and emerging technologies of green recovery of valuable nutrients from green plants involve
74 solvent consumption, high temperature and time-consuming extraction (Koubaa et al., 2015).
75 Nonetheless, chloroplasts recovery from their cellular confines can be achieved by means of physical
76 fractionation via tissue disruption without applying heat treatments or using toxic solvents that may
77 degrade the nutritional value. However, conventional recovery procedures of chloroplasts for
78 biochemical analysis involve the addition of iso-osmotic solutions to the biomass before grinding
79 (Joly & Carpentier, 2011), in order to prevent them from either cytolysis or plasmolysis. Recent work
80 in our laboratory demonstrated the nutritional value of a chloroplast-rich fraction (CRF) obtained by
81 osmoticum-assisted recovery of chloroplasts from spinach leaves (Gedi et al., 2017). A more
82 sustainable method is used in the current study in which the green biomass is squeezed by passing
83 through a screw-press juicer and the extracted juice preserves the chloroplast integrity. Consequently,
84 the use of additional water is saved when scaling at industrial levels. Therefore, this isolation method
85 for chloroplast recovery might constitute a novel physical procedure to concentrate a wide range of
86 essential micronutrients recommended for daily intake in human beings and hence present the isolated
87 chloroplast-rich material as a food or food-supplement ingredient. In addition, the plant cell wall
88 fraction collected in the pulp can be exploited as a feedstock of fibre/carbohydrates for cellulose
89 processing.

90 Our focus for this study was to apply this more sustainable, physical method of chloroplast recovery
91 to pea vine haulm, and to establish the nutritional value of this material. To the best of our knowledge,
92 a complete biochemical composition of chloroplast from pea plants (*Pisum sativum* L.) is scarcely
93 studied in the literature (Ladygin, 2004; Rantfors, Evertsson, Kjellberg & Sandelius, 2000).

94 Nevertheless, the nutritional content in plants starts decreasing after harvesting and dramatic losses
95 occur when the biomass is subjected to undesired post-harvest fermentation (Ferreira, Lana, Zanine,

Santos, Veloso & Ribeiro, 2013) due to enzymatic activity. Plant cell death after harvest results in the loss of chloroplast protective mechanisms and nutrients (Makoni, Shelford, Nakai & Fisher, 1993). Hence, efficient biomass management needs to be performed to tackle this issue. Thus, the impact of possible post-harvest storage conditions of pea vine haulm on the nutritional content and composition of the isolated CRF was studied. Different batches of pea vine haulm from 2015 harvest were exposed to blanching (i.e. steaming), wilting (i.e. aging at different relative humidity values) or fermentation (i.e. storage under anaerobic conditions) before extracting the chloroplast-containing juice with a screw-press juicer, to compare the nutritional quality with that from fresh pea vine haulm (un-pretreated control batch). In addition, a batch of juice from fresh and blanched pea vine haulm was frozen before CRF extraction by centrifugation to test the effect of freeze-storage on nutritional content of the CRF. Blanching of fruit and vegetables is a well-known process which inactivates enzymes and microorganisms in order to preserve, not only the colour and flavour, but also the nutritional value, during freeze or canning storage (Reyes de Corcuera, Cavalieri & Powers, 2004). However, the high temperature reached in either steam blanching or in water blanching degrades, to a certain extent, the nutrients, which can additionally leach if they are soluble in water. For that reason, the nutritional value of isolated CRF from fresh and blanched pea vine haulm before and after freezing the juice was compared.

An added benefit of chloroplast isolation resides in a likely improved micronutrient bioaccessibility. Recent *in-vitro* studies have shown that the plant cell wall is a natural limiting factor for nutrient bioaccessibility (Palmero et al., 2013). During the digestion of fruit and vegetables, the plant cell wall material needs to be disrupted before nutrients are released for subsequent absorption. Nonetheless, mastication and other mechanical forces within the gastroinstestinal tract are not efficient to overcome the turgor pressure placed on plant cell tissues for this disruption to happen. Thus, the intake of already isolated chloroplasts may boost an optimised micronutrient absorption and hence bioavailability.

2. MATERIALS AND METHODS

2.1. Materials

The pea vine (*Pisum sativum* L.) haulm, comprising a complex mixture of leaves, vines, stems and peas, was kindly provided by The Green Pea Company (Yorkshire, United Kingdom). The biomass was freshly collected from the side of the harvesters during pea harvest (August, 2015) and immediately brought to our laboratory facilities to be processed.

All chemicals used were of analytical grade, high-performance liquid chromatography (HPLC)-grade in the case of solvents, and purchased from Sigma-Aldrich, unless otherwise stated. Ultrapure water purified in a Pur1te Select system was used for aqueous solutions preparation.

2.2. Post-harvest treatment

The fresh biomass brought from harvest was promptly washed with tap water and drained using a salad spinner, before distributing into different batches of at least 1 Kg. One of the batches (fresh un-pretreated control batch) was immediately juiced for chloroplast recovery by centrifugation and further analysis. A second one was steam-blanched, in a conventional kitchen steamer, for 7 min, followed by 5 min cooling with running tap water before recovering the chloroplast-containing juice. Another batch was fermented in a polyethylene food bag, which was sealed after carefully removing trapped air by gentle pressure application, in the dark, at room temperature, for one week, before chloroplast recovery. Three last batches were subjected to wilting, at several relative humidity (RH) values in dessicators, in the dark, at room temperature, for one week, before chloroplast recovery. To achieve the desired humidity conditions in the dessicators, these contained different saturated salt solutions. Namely, $MgCl_2 \cdot 6H_2O$, NH_4NO_3 (Scientific Laboratories Supplies) and KNO_3 (Fisher Scientific) were used to provide 33, 65.5 and 93.5% RH values, respectively (Winston & Bates,

145 1960). An aliquot from each biomass batch (fresh, blanched, wilted or fermented) was freeze-dried
146 to determine the moisture content.

147

148 *2.3. Isolation of chloroplast-rich fraction*

149 After each treatment, the biomass batches (fresh, blanched, wilted or fermented) were mechanically
150 juiced with a screw-press juicer (OSCAR Neo DA-1000, Hurom Co.) and the chloroplast-rich juice
151 collected was rapidly analysed. Specifically the pH was measured and the microstructure was
152 visualised by optical microscope (Leitz Diaplan, Germany). In the case of the fresh and blanched
153 batches, an aliquot of the extracted juice was also frozen at -80 ºC. The juice from fresh, blanched,
154 wilted or fermented batches was next centrifuged at 4420 × g (Beckman J2-21M induction drive
155 centrifuge) for 10 min at 4 ºC and the clear supernatant discarded. The pellets were then frozen at -
156 80 ºC prior to freeze-drying (Edwards Freeze Dryer Super Modulyo) and finally ground with a mortar
157 and stored in a dark, dry and cool atmosphere for chemical analysis. The freeze-dried material
158 constitutes the final CRF. The aliquots of frozen juice from fresh and blanched pea vine haulm was
159 defrosted after two months of storage and subsequently analysed and centrifuged for isolation of the
160 CRF with the above procedure.

161

162 *2.4. Macronutrient composition: total proteins, lipids, ash, carbohydrates*

163 The protein content in the CRF was quantified by means of the bicinchoninic acid method (Pierce®
164 BCA Protein Assay Kit, Thermo Scientific, No. 23225) after protein extraction with 2 wt.% sodium
165 dodecyl sulfate solution at 60 ºC for 30 min.

166 Lipid extraction from the CRF was carried out as follows. A mixture of chloroform and methanol
167 (2:1, v/v) in a volume of 1.2 mL containing 0.1 wt.% butylated hydroxytoluene (BHT, MP
168 Biomedicals) was added to 0.1 g of CRF and vortexed for 1 min. Next 0.5 mL of 0.9 wt.% NaCl

169 solution was added to this mixture, vortexed for 1 min and centrifuged at 1750 × g (Rotina 380R,
170 Hettich Zentrifugen) for 10 min at 4 ºC. The bottom phase, containing the lipids, was collected with
171 a glass Pasteur pipette. Two more successive steps of chloroform-methanol addition, vortex and
172 centrifugation were carried out to collect the maximum amount of total lipids. The lipid extracts were
173 passed through 0.45 μm PTFE filters (Whatman™ GE Healthcare) and dried under nitrogen. After
174 weighing, the dried lipids were dispersed in the corresponding solvent for micronutrient analysis as
175 specified below.

176 The ash content was determined by incineration in silica crucibles in a muffle furnace (Carbolite,
177 AAF 1100) at 550 ºC for 4 h.

178 Total carbohydrates were estimated by difference.

179 The content of native starch in the CRF from fresh pea vine haulm was quantified by means of
180 enzymatic hydrolysis method (SA20 Starch Assay Kit, Sigma-Aldrich), where the hydrolysis of
181 starch to glucose is catalysed by amyloglucosidase. Further details can be found in the Kit product
182 information provided by the supplier.

183 Further details of the methods for the determination of the macronutrients can be found in the
184 Supplementary Data.

185

186 *2.5. Micronutrient composition: fatty acids, chlorophylls, β-carotene, α-tocopherol, ascorbic acid*

187 Further details of the methods for the determination of the micronutrients can be found in the
188 Supplementary Data.

189

190 *2.5.1. Fatty Acids*

191 The dried lipids extracted from 0.1 g of dried CRF were dispersed in 2 mL chloroform and methyl
192 pentadecanoate was added as internal standard to a concentration of 0.91 mg/mL. Next, 0.4 mL of
193 trimethylsulfonium hydroxide was added for methylation and left standing for at least 10 min to
194 ensure complete conversion of fatty acids to fatty acids methyl esters (FAMEs) (Gedi et al., 2017).
195 Quantification of FAMEs was carried out by gas chromatography-mass spectrometry (GC-MS
196 Thermo Scientific, DSQII) with a Phenomenex Zebron ZB-FFAP (30 m × 0.22 mm) column using a
197 vaporising injector with a split flow of 50 mL/min of the carrier gas (He). The starting oven
198 temperature was 120 °C held for 1 min and then increased at 5 °C/min up to 250 °C and held for 2
199 min. Identification of individual fatty acids was achieved through mass spectrum library by
200 comparison of retention times of FAMEs. Individual fatty acid concentrations were calculated from
201 the ratio of the peak area of the FAME to the peak area of the internal standard.

202

203 *2.5.2. Chlorophyll*

204 The dried lipids extracted from 0.1 g of dried CRF were dispersed in 1 mL acetone followed by a
205 1000-fold dilution. Then the absorbance of the diluted lipid extracts was read in a spectrophotometer
206 (LKB Biochrom 4050 Ultrospec) blanked with acetone at 662 and 645 nm. The pigments
207 concentrations were calculated according to the equations used by Lichtenthaler and Buschmann
208 (Lichtenthaler & Buschmann, 2001).

209

210 *2.5.3. β-Carotene*

211 The dried lipids extracted from 0.1 g of dried CRF were dispersed in 10 mL acetone containing 0.1
212 wt% BHT. A sample aliquot (10 μL) was injected into the HPLC (Agilent 1100) equipped with UV-
213 VIS photodiode array (PDA) detection system and the mobile phase (acetonitrile/methanol/ethyl
214 acetate) running at 0.5 mL/min. The initial mobile phase proportion was 95:5:0, which was changed

linearly to 60:20:20 in 20 min, held for 20 min, returned back to the initial proportion in 0.5 min and held for 15 min. The sample separated on a Waters Spherisorb S3ODS2 monomeric C_{18} 3 µm 4.6 × 150 mm column with a security guard-column at 22 °C. β-carotene was detected at 454 nm after 33-36 min.

2.5.4. α-Tocopherol

Methanol (0.8 mL) containing 1 wt% BHT was added to 0.1 g of dried CRF, stirred for 1 min and centrifuged at 16200 × g (Thermo Electron Corporation, Heraeus Fresco 21 centrifuge) for 5 min at 4 ºC. The supernatant containing the lipids was collected. Two more successive steps of methanol addition, stirring and centrifugation were carried out to collect the maximum amount of lipids. A sample aliquot (20 µL) was injected into the HPLC equipped with fluorescent detection system (Jasco intelligent fluorescent FP-920) and the mobile phase (acetonitrile/methanol/isopropanol/1% acetic acid solution) running at 0.8 mL/min. The initial mobile phase proportion was 45:45:5:5 for 6 min, then changed linearly to 25:70:5:0 in 10 min, held for 12 min and returned back to the initial proportion in 1 min holding for 6 min. The sample separated on an Agilent Zorbax RX-C8 5 µm 250 × 4.6 mm column with a security guard-column at 20 °C. α-tocopherol was detected at excitation and emission wavelengths of 298 and 328 nm, respectively, after 11-13 min.

2.5.5. Ascorbic Acid

A volume of 5 mL of 1.5 wt% HPO_3 (Fisher) solution was added to 0.1 g of dried CRF, vortexed for 1 min and centrifuged at 4863 × g (Rotina 380R, Hettich Zentrifugen) for 5 min at 4 ºC. The supernatant was filtered through a Sep-pak Plus C18 cartridge (Waters), previously conditioned with a mixture of 10 mL methanol and 5 mL water. The first 4 mL of each sample (supernatant) being filtered were discarded, and the remainder was collected for analysis. Aliquots of 0.2 mL were then

transferred to HPLC vials and diluted with 0.8 mL acetonitrile. Next, 20 μL of sample from each HPLC vial were injected into the HPLC equipped with UV PDA detector and the mobile phase (100 mM ammonium acetate at pH 5.8/acetonitrile) running at 2 mL/min. The mobile phase was run isocratically at a proportion of 11:89 for 10 min. The sample separated on a Phenomenex® Luna Hilic 5 μm 4.6 × 150 mm column with a security guard-column at 20 °C. Ascorbic acid was detected at 265 nm after 5 min.

The concentrations were quantified from a linear calibration curve built with β-carotene (Fluka), α-tocopherol and L-ascorbic acid external standards at each day of analysis.

2.6. Statistical analysis

Biomass management and sample extractions were made from three pea vine haulm collections at different days of harvesting and averaged. On each collection, all the analyses were performed in triplicate and measurements were reported as the average and standard deviation values. Data were analysed with one-way analysis of variance (ANOVA) with Tukey's pairwise comparison post hoc test to determine significant differences ($p \leq 0.05$) between mean values of different postharvest treatments.

3. RESULTS AND DISCUSSION

3.1. Yield of juice and chloroplast-rich fraction

The pea plant biomass experienced changes in colour and texture after subjecting to different post-harvest conditions. After blanching, the biomass became darker and some tissue softening was observed. After wilting, at either RH value, or fermenting, the biomass lost its green colour

262 ('browned'); some material at high RH became mouldy. When the juice was extracted from the
263 treated or untreated (fresh) pea vine haulm, the volume collected was measured and in all cases a
264 similar yield was obtained: 0.6 mL per gram of wet biomass. The reason for this is the comparable
265 moisture content determined in all batches of treated and untreated biomass, ranging from 87.5 to 89
266 %. Only in the case of biomass subjected to the highest value of RH (93%) was there a slightly higher
267 moisture content, but still similar within the margin of error. The pH of the juice collected was also
268 measured. The juice extracted from the fresh pea vine haulm had a pH value of 5.0-5.8, which was
269 slightly increased up to pH 6.0 after blanching. Interestingly, the juice from wilted material
270 experienced, in all cases, an increase in pH, up to 7.8-8.0, which might be due to the release of NH_3
271 from the breakdown of plant cell membranes (Kung, Tung & Maciorowski, 1991). On the other hand,
272 the juice pH from fermented pea vine haulm decreased to pH 4.0, as compared to that from fresh
273 biomass. This is likely due to lactic acid production, which is an indicator of fermentation (Ferreira
274 et al., 2013).

275 Light-microscopy was used to observe the microstructure of the juice extracted from the fresh and
276 treated biomass batches (Fig. 1). In all cases, small clusters of green organelles, corresponding to
277 chloroplasts were observed, along with clusters of larger and non-coloured granules (Fig. 1a).
278 Polarised light allowed confirmation of the latter being starch grains with a size larger than 10 μm,
279 on average (Fig. 1b). Some individual chloroplasts were visualised in the juice of the fresh pea vine
280 haulm, which are within the range of 5 μm, in agreement with reported diameter values of higher-
281 plants' chloroplasts (Block et al., 2007). This confirms that the observed starch granules are
282 exogenous to chloroplasts and were released from plant cells during the physical tissue disruption
283 caused by the screw press juicer. It is worth mentioning that micrographs of the juice after a freeze-
284 thaw cycle, regardless whether the biomass was fresh or blanched, also showed intact chloroplasts
285 and starch granules (Fig. S1 of Supplementary Data).

After juice centrifugation, the bottom solid phase or pellet is characterised by a white layer, presumably starch-rich, intercalated between green layers of chloroplast-rich material (not shown). Indeed, the starch granules may promote fast sedimentation, which was observed over time before centrifuging, also dragging the chloroplasts to the bottom. Once this pellet was freeze-dried, its weight was measured to quantify the amount of solids, corresponding to the final CRF that was collected from each batch of biomass. The results are presented in Fig. 2 per gram of un-/treated initial wet biomass as for the rest of the study, since the moisture content was similar in all cases. It can be seen how the dry mass of the CRF decreases 50% or more after processing the pea plant biomass (Fig. 2a). Interestingly, the largest extent of solids reduction occurs after fermentation. Hydrolysis by plant enzymes and solubilisation of certain components that are discarded in the supernatant may have occurred. This is supported by the change of colour and turbidity in the supernatant which gradually ranged from transparent clear to turbid brown when the biomass was either fresh, blanched, fermented or wilted, in that order. It is also interesting to note in Fig. 2b the slight decrease (23%) in dry mass of chloroplast-rich material during freeze storage of the juice extracted from fresh pea vine haulm. However, when the biomass was previously blanched, no significant changes ($p \leq 0.05$) in the dry mass are observed. As previously observed, intact entities of chloroplasts and starch grains were still present in the extracted juice after a freeze-thaw cycle although freeze storage may have caused slight damage, as reflected in the minor decrease of dried chloroplast-rich material collected. The proximate composition of this CRF is analysed in next section, so the reduction in solids after each treatment can be further elucidated. There may be differences in pellet composition since the colour and layered pattern is altered due to post-harvest treatment. This may be a preliminary indication of chloroplast degradation.

3.2. Macronutrient composition of the chloroplast-rich fraction

The results of macronutrient content and composition of the CRF are displayed in Fig. 3 per gram of un-/treated initial wet biomass. It can be observed that the largest contribution of the isolated material from fresh pea vine haulm comes from the estimated carbohydrates (42% of the CRF on a dry-weight basis), such as starch, as previously observed in the juice micrographs, and sugars which could settle with the pellet during centrifugation. In particular, the starch content measured in the CRF on a dry-weight basis is 22%, which constitutes 52% of the estimated carbohydrates. Next, in decreasing order of content it is found 24% of soluble proteins, 22% of total lipids and 11.5% of ash, accounting for total minerals. The relatively high proportion of total lipids contained in the chloroplast-rich material (on a dry-weight basis) as compared to total lipids from pea plants or other vegetables, which usually is no more than 5% on a dry-weight basis (Murcia, Vera & Garcia-Carmona, 1992; Oulai, Zoue & Niamke, 2015; Santos et al., 2014), is attributed to the synthesis and concentration of thylakoid membranes, rich in galactolipids, and lipid soluble pigments and vitamins in the chloroplasts. The most remarkable observation is that after processing, there is a significant decrease ($p \leq 0.05$) in all macronutrients in the CRF as compared to the macronutrient content from CRF extracted from fresh biomass (Fig. 3a), which is linked to the loss of mass of CRF isolated from pea vine haulm after postharvest treatment.

In general, blanching decreases the nutritional content in vegetables by leaching (Reyes de Corcuera et al., 2004). This is reflected in the decrease in soluble protein content (74%), due to denaturation by heat and solubilisation when washing (Murcia et al., 1992), as well as in total minerals (64%) and carbohydrates (27%) (Oulai et al., 2015; Svanberg, Nyman, Andersson & Nilsson, 1997). Minerals are not destroyed by light, heat and oxygen and losses are only due to leaching or physical separation. The decrease in lipid content after blanching (58%) might be due to the loss of membrane lipids such as phospholipids caused by high temperature (Murcia, Lopez-Ayerra & Garcia-Carmona, 1999). On the other hand, the decrease in lipids (35%) and carbohydrates (27%) in chloroplast-rich material isolated from frozen juice of fresh pea vine haulm (Fig. 3b), unlike from blanched pea vine haulm,

might be due to the activity of cold stress activated enzymes that are otherwise inactive after blanching (Murcia et al., 1999), as also reflected in the decrease of total solids (Fig. 2b). As suggested above, the hydrolysis and solubilisation of some nutrients, such as sugars and low-molecular weight carbohydrates, may contribute to this feature.

After fermenting (Fig. 3a), carbohydrates decrease to the largest extent (71%) likely due to decomposition into acetic, butyric and lactic acids. There is also a decrease in carbohydrates content to a lesser extent (39%) after wilting at all RH values, which might be due to starch breakdown and subsequent metabolism of free sugars (Stewart, 1971). On the other hand, the decrease in protein content after wilting/fermenting (81%) might be due to proteolysis by proteolytic enzymes or enterobacteria as part of the microbial populations (Ferreira et al., 2013), and in the case of lipid content (decreased by 76%) due to hydrolysis and peroxidation by lipolytic acyl hydrolase and lipoxygenase, respectively, which are also involved in the degradation of chlorophylls (Yamauchi, Iida, Minamide & Iwata, 1986). Makoni et al. (1993) concluded that wilting and fermenting in alfalfa silage affect chloroplasts firstly through the loss of cellular compartments via hydrolysis of membrane lipids, such as glycolipids and phospholipids, resulting in subsequent oxidation reactions, proteolysis and pigment bleaching (Makoni et al., 1993).

3.3. Micronutrient composition of chloroplast-rich fraction

Regarding the fatty acids composition, the most abundant fatty acids in the chloroplast-rich material from fresh pea vine haulm (Fig. 4) are the polyunsaturated α-linolenic acid (C18:3n-3), being 40% of the total content, followed by palmitic (C16:0) and linoleic acid (C18:2n-6) which represent 23% and 20%, respectively. These fatty acids have also been previously reported as the most abundant in pea leaves' chloroplasts (Dorne & Heinz, 1989). In addition, other fatty acids have been detected in minor proportions, such as oleic (C18:1n-9) at 7%, stearic (C18:0) at 6%, palmitoleic (C16:1n-7) and

myristic (C14:0) at 2%. In general, these are the principal fatty acids of legumes' fruits, but the distribution varies according to species and geographical conditions. Figure 4 displays the fatty acid content after biomass processing, where a marked decrease is observed when comparing with CRF from fresh biomass, which is related to the decrease in the lipids content observed in previous section.

The fatty acid content decreases considerably after blanching and to a larger extent after either wilting or fermenting (Fig. 4a). Freezing the juice extracted from fresh biomass also causes a decrease in fatty acids, however, blanching the pea plant before juicing and freezing the juice before isolating the chloroplast-rich material (Fig. 4b), has little effect on the fatty acid composition, suggesting the inactivation of cold stress activated enzymes during blanching. This agrees with a previous report on blanching and freezing broccoli without affecting the fatty acid profile (Murcia et al., 1999). Namely, α-linolenic acid is still the most predominant (37% of the total content), followed by palmitic (24%) and linoleic acid (21%). However, the fatty acid profile dramatically changes after wilting/fermenting the biomass (Fig. 4a). Palmitoleic and oleic acid relative content increases up to 5% and 10% after fermentation, and up to 17% and 18% after wilting, respectively, in detriment of α-linolenic acid which decreases up to 22% after fermentation and up to 14% after wilting. In addition, saturated palmitic and stearic acid relative content increases up to 29% and 7%, respectively, after fermentation, whereas no changes are recorded after wilting. A major decrease in α-linolenic acid was also observed in wilted ryegrass (Khan, Cone, Fievez & Hendriks, 2011) and ryegrass silage (Van Ranst, Fievez, De Riek & Van Bockstaele, 2009) if anaerobic conditions are not reached quickly, where "oxidation" of α-linolenic acid largely contributes to the decrease of fatty acids content.

Chlorophyll *a* and *b* are the two major structural forms of chlorophyll present in plant chloroplasts. The structure *a* is the most abundant, as it is found in the pigment antenna system and the reaction centres of photosystem I and II, while chlorophyll *b* is only present in the pigment antenna (Lichtenthaler et al., 2001). Indeed, it can be seen in the CRF from fresh pea plant biomass (Fig. 5a) that chlorophyll *a* content (225 μg/g of wet biomass) is twice that of chlorophyll *b* content (100 μg/g

384 of wet biomass). The ratio chlorophyll *a*:*b* is an indicator of the light adaptation of the photosynthetic
385 system. Higher values of this ratio (3.0-3.8) are linked with plants exposed to sun whereas lower
386 values (2.0-2.8) are associated with shade plants that need to further develop the antenna system
387 where the light energy is first collected before being transferred to the reaction centres (Lichtenthaler,
388 Kuhn, Prenzel, Buschmann & Meier, 1982). Therefore, the relatively low ratio found here (*a*:*b* ~ 2.2)
389 indicates that leaves were grown in low light conditions (Lichtenthaler et al., 2001). In addition to
390 this, the quantification of total chlorophylls is a good indicator of the amount of intact chloroplasts.
391 Hence the comparison of chlorophyll content before and after processing will provide an estimate of
392 the damaged chloroplasts due to blanching, wilting or fermentation.

393 There is a considerable decrease in chlorophyll concentration after blanching (by 75% of total
394 chlorophylls), which is known to destroy pigments by heat. This decrease has been previously
395 attributed to conversion of chlorophylls to phaeophytin and to its leaching during blanching (Schwartz
396 & Vonelbe, 1983). In addition, degradation by photooxidation is also possible, since blanching was
397 performed in the presence of light and chlorophylls are photosensitive. An even greater decrease in
398 chlorophyll content is found after wilting or fermenting (by 93% of total chlorophylls), in which case
399 the chlorophyll may have been degraded by hydroperoxides of free fatty acids which are formed by
400 the degradation of polar lipids from the chloroplasts such as glycolipids (monogalactosyldiglyceride,
401 digalactosyldiglyceride) and phospholipids (phosphatidylglycerol) (Yamauchi et al., 1986). The ratio
402 *a*:*b* slightly increases after blanching, wilting or fermentation to ~ 2.5. This indicates a slightly faster
403 degradation of chlorophyll *b*. Conversely, a faster degradation of chlorophyll *a* has been previously
404 noted by Schwartz and Lorenzo (1991) in a study on the stability of chlorophylls during processing
405 and storage of spinach leaves (Schwartz & Lorenzo, 1991), although the degradation upon storage
406 was not related to predominant chlorophyll oxidation, but conversion to phaeophytin. Our findings
407 could be attributed to the close absorbance wavelengths of chlorophyll *a* and its degradation product
408 phaeophytin *a* (Makoni et al., 1993). Finally, an interesting observation is that chlorophyll content is

409 reduced by 25% when the CRF was isolated from frozen juice extracted from fresh pea vine haulm,
410 but remains unchanged when the biomass was previously blanched before freezing the juice (Fig.
411 5b). This suggests that some enzymatic activity was still occurring in the frozen juice extracted from
412 the un-blanched biomass, leading to chlorophyll degradation (Lopez-Ayerra, Murcia & Garcia-
413 Carmona, 1998).

414 β-carotene is another pigment, which is of nutritional interest as it is also known as pro-vitamin A,
415 and its content in the CRF isolated from pea vine haulm is shown in Fig. 6. Similarly to chlorophyll,
416 content decreases after processing can be observed for this lipid-soluble nutrient. Since all lipids of
417 isolated pea chloroplasts were affected after processing, a decrease in β-carotene content is expected.
418 Carotenoids are heat-labile in the presence of oxygen (Simpson & Chichester, 1981). Therefore, the
419 decrease in β-carotene content during blanching might be partially due to the antioxidant effect of β-
420 carotene on lipid oxidation and its isomerization (Simpson et al., 1981). The extent of β-carotene loss
421 after blanching (70%) contrasts with the dramatic loss after wilting or fermentation (98%) (Fig. 6a),
422 which correlates with the trend of loss of lipids and chlorophylls, as well as the changes in fatty acids
423 composition. Indeed the degradation of carotenoids is similar to oxidative degradation of unsaturated
424 fatty acids (Gregory, 1996). It was suggested above that the PUFA α-linolenic acid was oxidized to
425 the largest extent after wilting or fermentation of the pea vine haulm. It has been reported that PUFA
426 peroxidation destroys carotenoids and chlorophylls with the production of hydrogen peroxides (Lea
427 & Parr, 1961). This may explain the similar trend in the decrease of chlorophylls and β-carotene after
428 both of these processes. On the other hand, the relatively lower loss (30%) caused when freezing the
429 juice extracted from un-blanched pea plant biomass (Fig. 6b) suggests that β-carotene oxidation and
430 isomerization is not stopped during freeze storage unless the biomass is previously blanched. Losses
431 of β-carotene were also reported elsewhere during frozen storage of peas and spinach (Bouzari,
432 Holstege & Barrett, 2015), which was attributed to oxidation.

433 The losses of α-tocopherol (another fat soluble vitamin commonly referred to as vitamin E) after
434 blanching (77%) or wilting/fermenting (99%) follow the same trend as for β-carotene (Fig. 6a). A
435 previous study suggested that similarities in the localisation of β-carotene and α-tocopherol may lead
436 to similar losses during processing (Bernhardt & Schlich, 2006). Tocopherols are also susceptible to
437 heat treatment and their degradation can be additionally accelerated by the presence of oxygen and
438 exposure to light during processing. However, the initial α-tocopherol content in the chloroplast-rich
439 material from fresh pea vine haulm is lower than for β-carotene. This agrees with the lower content
440 of α-tocopherol as compared to β-carotene reported in peas (Bouzari et al., 2015), and in the CRF
441 isolated from spinach, kale, nettle and grass leaves (Gedi et al., 2017). The freeze storage of the juice
442 prior to chloroplast isolation leads to α-tocopherol loss of 54%, suggesting oxidation of α-tocopherol.
443 It is worth noting the contrast with previous findings where no significant changes in tocopherols
444 levels or even an increase were found between raw and frozen green vegetables (Bouzari et al., 2015).
445 The difference in the current study might be explained by liberated chloroplasts being more
446 susceptible to oxidation than if they are still cell-bound.

447 Ascorbic acid is present in even lower concentrations as compared to α-tocopherol or β-carotene in
448 the CRF from fresh biomass (Fig. 6a). The reason for this might be that ascorbic acid is non-
449 exclusively present in plant chloroplasts and because it is one of the most sensitive vitamins. In fresh
450 produce, ascorbic acid begins to degrade quickly, soon after harvest. During processing and storage
451 of vegetables, ascorbic acid oxidizes to dehydroascorbic acid, which is irreversibly hydrolysed to 2,3-
452 diketogulonic acid losing the vitamin C activity. The considerable loss of ascorbic acid after blanching
453 (72%) can be explained by its heat-susceptibility and high water-solubility. Previous studies on
454 blanched leafy vegetables have also reported comparable losses of vitamin C (Oulai et al., 2015).
455 More dramatic results are observed after wilting or fermentation as there is no retention of ascorbic
456 acid, at least detectable by HPLC. Freeze storage of the juice prior to chloroplast isolation led to
457 almost complete loss of ascorbic acid (91%), even in pre-blanched material (90%). These losses after

458 freeze storage of the juice are greater than those for β-carotene, which might be due to the instability
459 of ascorbic acid as compared to carotenoids (Buescher, Howard & Dexter, 1999).

460

461 *3.4. Comparison with nutritional reference values*

462 Spinach (*Spinacia oleracea* L.) is traditionally considered to have high nutritional quality. For that
463 reason, the nutritional content found in the CRF of pea vine haulm is going to be related in this section
464 to that of CRF isolated from spinach leaves (Gedi et al., 2017) and with the recommended nutritional
465 intake (RNI) for adults as stated by the FAO. Calculations related to nutritional content in fresh
466 spinach leaves were based on the USDA National Nutrient database. Table S1 of Supplementary Data
467 shows the grams of CRF needed to cover the RNI of macro- and micronutrients on dry-weight basis
468 since the freeze-dried material is considered the final product. The amount of CRF isolated from both
469 sources, pea vine haulm and spinach, in order to meet the RNI of micronutrients such as α-linolenic
470 acid, β-carotene and α-tocopherol, is comparable. In turn, these values are related to the wet amount
471 of initial fresh pea vine haulm needed to extract the equivalent CRF and, at the same time, compared
472 with the amount of fresh spinach leaves needed to achieve the RNI. In principle, 16 kg of fresh pea
473 vine haulm is enough to extract CRF to supply the recommended daily intake of the macronutrients
474 and micronutrients measured in this study. Three kg of fresh pea vine haulm will produce enough
475 CRF, using the screw press juicing and centrifugation procedure employed in this study, to satisfy the
476 RNI for the PUFA α-linolenic acid, β-carotene and α-tocopherol. In some cases, the biomass of pea
477 vine haulm needed, to extract CRF, is comparable to the biomass of raw spinach leaves required to
478 cover the same nutritional content; namely lipids, α-linolenic acid, β-carotene and α-tocopherol.
479 Considering the large amount of green haulm generated every year its use as a source of nutritionally
480 rich material seems reasonable.

481

4. CONCLUSIONS

CRF from pea vine haulm is a promising source of lipid-soluble micronutrients: the PUFA α-linolenic acid, pro-vitamin A and vitamin E, since these are mostly synthesised and stored within the chloroplasts, and carbohydrates. In addition, a sustainable physical fractionation has been used to extract the CRF, based on sole mechanical juicing. However, post-harvest conditions are crucial to preserve the nutrients in the green haulm prior to CRF isolation. The largest decrease in the nutritional content of CRF was caused by fermentation or wilting, regardless of the RH value, followed by a moderate loss during blanching or freeze storage. Optimisation of blanching parameters, e.g. time, and/or exploring alternative technologies, e.g. microwave, is still necessary to minimise the nutrient loss.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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FIGURE CAPTIONS

Fig. 1: a) Microstructure of the juice from fresh, blanched or wilted/fermented pea vine haulm; b) polarised light microscopy of juice from fresh pea vine haulm. (I): cluster of chloroplasts, (II): individual chloroplast, (III): starch grains.

Fig. 2: a) Dry mass of the chloroplast-rich fraction isolated from fresh, blanched, wilted (at 33, 65 or 93% RH) or fermented pea vine haulm; b) effect of freezing the juice from fresh and blanched pea vine haulm on the dry mass in CRF. Different letters mean significant differences ($p \leq 0.05$) between dry mass values.

Fig. 3: a) Macronutrients within the chloroplast-rich fraction isolated from fresh, blanched, wilted (at 33, 65 or 93% RH) or fermented pea vine haulm; b) effect of freezing the juice from fresh and blanched pea vine haulm on the macronutrients in CRF. Different letters mean significant differences ($p \leq 0.05$) within each type of macronutrient.

Fig. 4: a) Fatty acids within the chloroplast-rich fraction isolated from fresh, blanched, wilted (at 33, 65 or 93% RH) or fermented pea vine haulm; b) effect of freezing the juice from fresh and blanched

pea vine haulm on the fatty acids in CRF. Different letters mean significant differences ($p \leq 0.05$) within each type of fatty acid.

Fig. 5: a) Chlorophyll *a*, *b* and total chlorophylls (*a* + *b*) within the chloroplast-rich fraction isolated from fresh, blanched, wilted (at 33, 65 or 93% RH) or fermented pea vine haulm; b) effect of freezing the juice from fresh and blanched pea vine haulm on the chlorophylls in CRF. Different letters mean significant differences ($p \leq 0.05$) within each type of chlorophyll.

Fig. 6: a) Micronutrients within the chloroplast-rich fraction isolated from fresh, blanched, wilted (at 33, 65 or 93% RH) or fermented pea vine haulm; b) effect of freezing the juice from fresh and blanched pea vine haulm on the micronutrients in CRF. Different letters mean significant differences ($p \leq 0.05$) within each type of micronutrient.

FIGURES

Figure 1

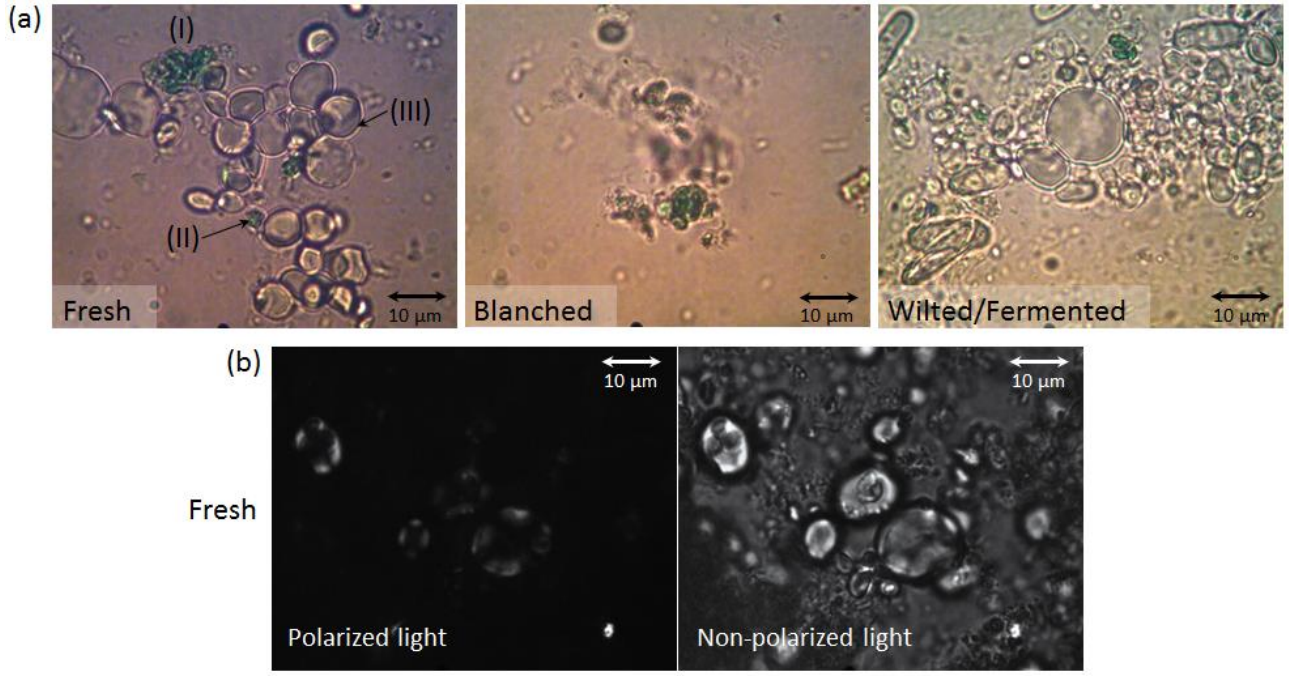


Figure 2

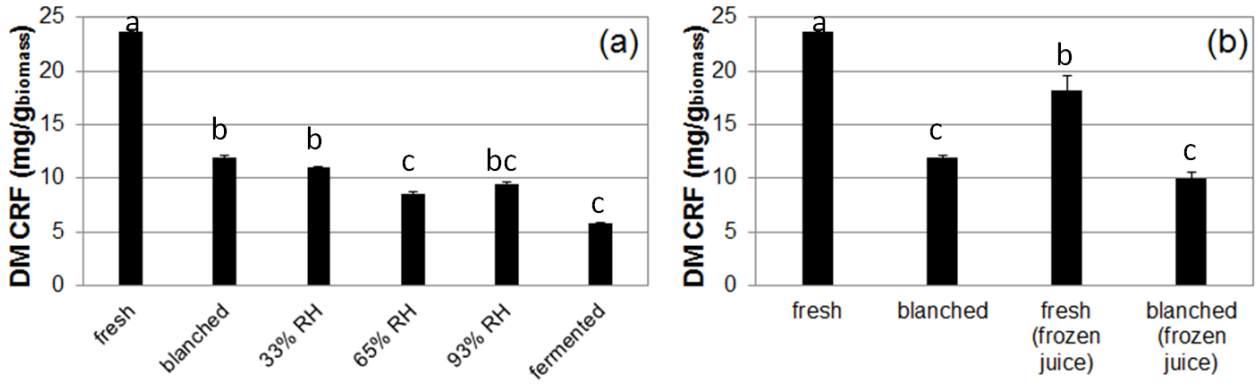

DM CRF (mg/g biomass)
25
20
15
10
5
0
(a)
a
b
b
c
bc
c
fresh
blanched
33% RH
65% RH
93% RH
fermented
(b)
a
c
b
c
fresh
blanched
fresh (frozen juice)
blanched (frozen juice)

Figure 3

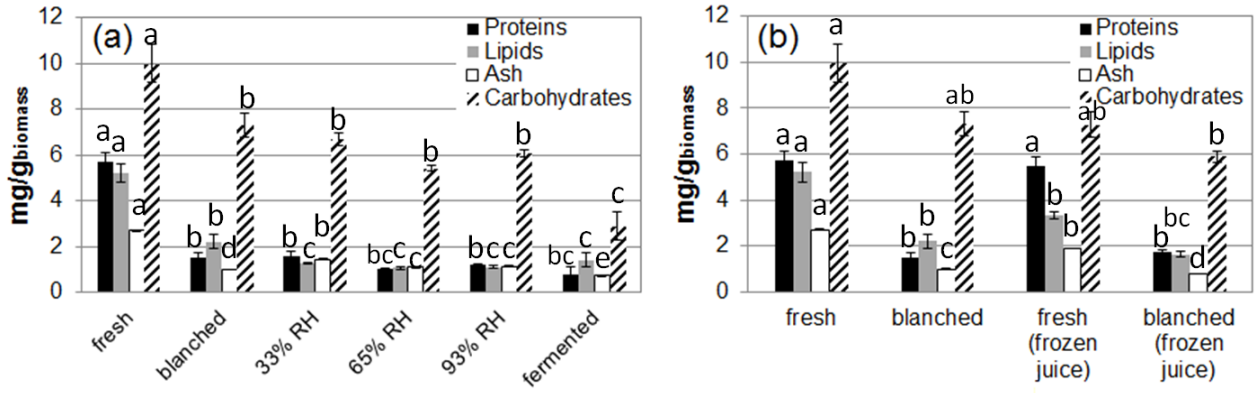

(a)
(b)
Proteins
Lipids
Ash
Carbohydrates
mg/gbiomass
fresh
blanched
33% RH
65% RH
93% RH
fermented
fresh (frozen juice)
blanched (frozen juice)

Figure 4

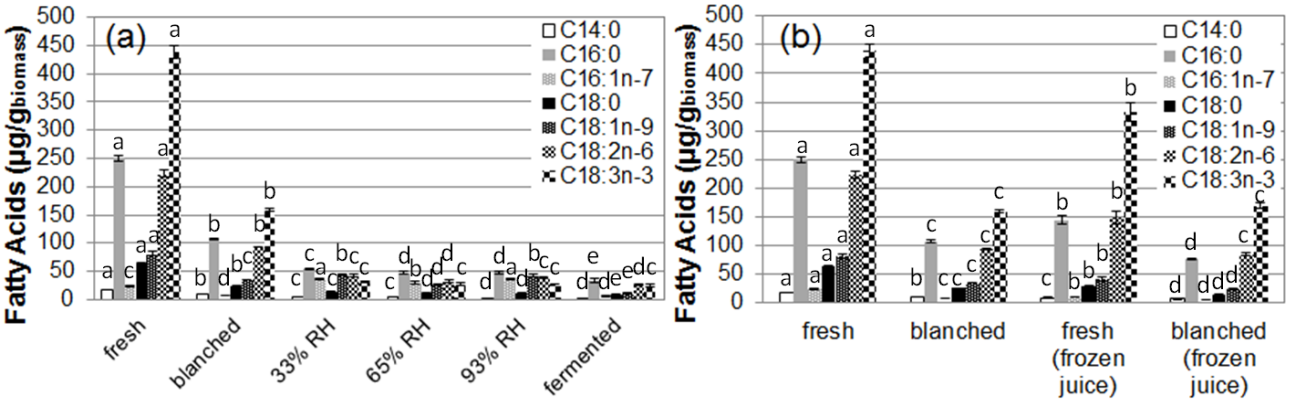

(a)
(b)
Fatty Acids (µg/g biomass)
C14:0
C16:0
C16:1n-7
C18:0
C18:1n-9
C18:2n-6
C18:3n-3
fresh
blanched
33% RH
65% RH
93% RH
fermented
fresh (frozen juice)
blanched (frozen juice)

Figure 5

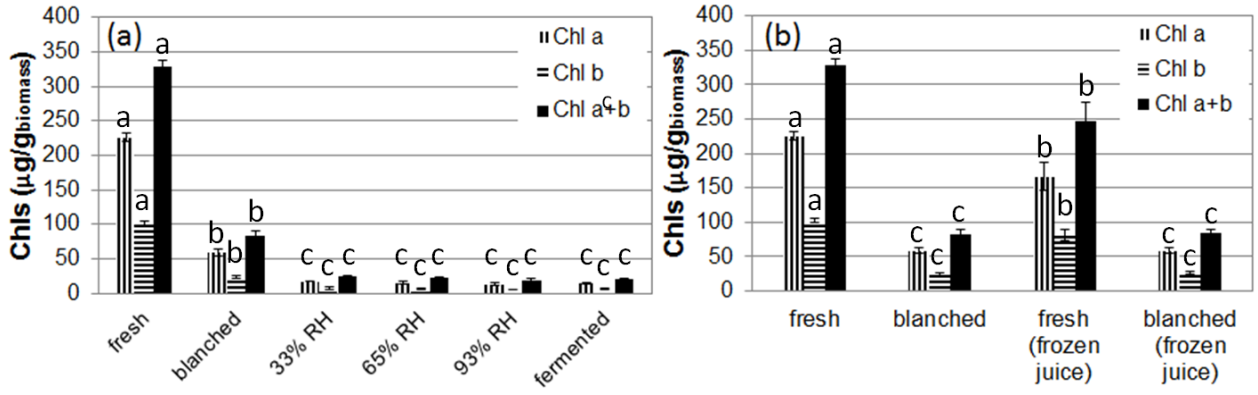
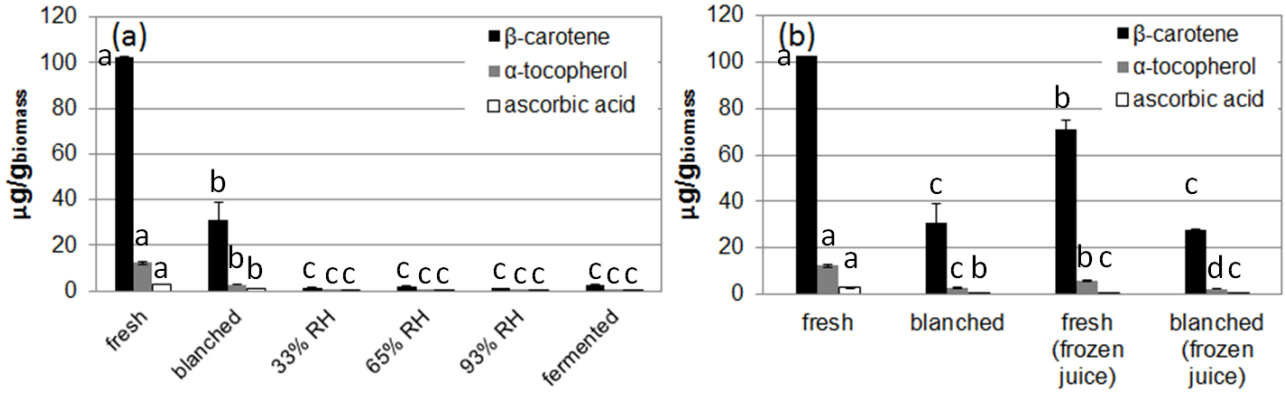

(a)
Chl a
Chl b
Chl a+b
Chls (µg/gbiomass)
400
350
300
250
200
150
100
50
0
fresh
blanched
33% RH
65% RH
93% RH
fermented
(b)
Chl a
Chl b
Chl a+b
Chls (µg/gbiomass)
fresh
blanched
fresh (frozen juice)
blanched (frozen juice)

Figure 6


(a)
β-carotene
α-tocopherol
ascorbic acid
µg/gbiomass
120
100
80
60
40
20
0
a
a
a
b
b
b
c c c
c c c
c c c
c c c
fresh
blanched
33% RH
65% RH
93% RH
fermented
(b)
β-carotene
α-tocopherol
ascorbic acid
µg/gbiomass
a
a
a
c
c b
b
b c
c
d c
fresh
blanched
fresh (frozen juice)
blanched (frozen juice)