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Chloroplast-rich material from the physical fractionation of pea vine (*Pisum sativum*) postharvest field residue (Haulm)

Amelia Torcello-Gómez[1], Mohamed A. Gedi, Roger Ibbett, Khatija Nawaz Husain, Rhianna Briars, David Gray*

Division of Food Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, United Kingdom

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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.

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 acids (PUFA) (Dubacq, Drapier, & Tremolieres, 1983), which are associated

* Corresponding author.
E-mail addresses: M.A.TorcelloGomez@leeds.ac.uk (A. Torcello-Gómez), Mohamed.Gedi1@nottingham.ac.uk (M.A. Gedi), Roger.Ibbett@nottingham.ac.uk (R. Ibbett), Khatija.Nawazhusain@nottingham.ac.uk (K. Nawaz Husain), Rhianna.Briars@nottingham.ac.uk (R. Briars), David.Gray@nottingham.ac.uk (D. Gray).
[1] Present address: School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, United Kingdom.

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with decreasing risk of coronary heart disease (Willett, 2012).

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

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

Nevertheless, the nutritional content in plants starts decreasing after harvesting and dramatic losses occur when the biomass is subjected to undesired post-harvest fermentation (Ferreira, et al., 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 gastrointestinal 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, 1960). An aliquot from each biomass batch (fresh, blanched, wilted or fermented) was freeze-dried to determine the moisture content.

2.3. Isolation of chloroplast-rich fraction

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

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

The protein content in the CRF was quantified by means of the bicinchoninic acid method (Pierce® BCA Protein Assay Kit, Thermo