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Final Report

Naturally Nutritious

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Summary

Australian consumers are becoming increasingly aware of the importance of healthy eating, and how eating well can lead to long-term health benefits. National programs such as the 'Go for 2 & 5 Fruit and Vegetable' campaign have educated the Australian population of the importance of fruit and vegetables to human health, and that in general Australians do not get this recommended amount of fruit and vegetables. Although the campaign was aimed at educating consumers with a subsequent aim to increase fruit and vegetable intake, education did not necessarily lead to consumers eating more fruit and vegetables. In order to address this issue, the Naturally Nutritious project investigated the potential to increase the nutrient density of a range of fruit and vegetables, such that increased nutritional benefit would be possible without necessarily having to increase intake. A range of phytonutrients were targeted, including anthocyanins, zeaxanthin, folate, and saturated fat, which have been linked to a range of readily measurable health markers such as hypertension, macular degeneration, and LDL cholesterol. Importantly, the targets were also linked to product differentiation where possible, so that a biofortified product was attractive and visually distinguishable from a standard product, ideally by colour, and where this was not possible, by permissible label health claims. Additionally, the Naturally Nutritious project was structured not just to investigate the technical feasibility of biofortification, but also consumer interest in the specific biofortified products, both from a visual and health benefit perspective. Finally, feedback was obtained from industry, to identify other aspects of importance (e.g., marketing, agronomic issues) potentially emanating from the production of a new product. The products selected for biofortification included sweetcorn, strawberry, macadamia, plum, capsicum and tomato. Biofortification was found to be technically feasible for most of the target commodities, with the degree of consumer acceptance varying, depending on the specific health benefit. The resulting combination of technical feasibility, consumer acceptance, and industry feedback identified at an early stage of investigation, what commodities were likely or not to succeed, the breadth of consumer interest, and any additional issues that would have to be accounted for, or addressed, as a product was developed towards commercial reality. In addition to fortifying specific nutrients in target crops, the Naturally Nutritious project investigated two of the other major mechanisms by which fruits, vegetables and nuts contribute to a healthy diet. These are (i) the ability to promote both short-term (mins; satiation) and longer-term (hours; satiety) feelings of fullness and (ii) having a positive healthy influence on the gut microbiota. These two nutritional benefits of horticultural products can be used to reinforce the '2 & 5' message as well as to identify specific opportunities for commodities to differentiate themselves in the marketplace. General rules of thumb for understanding the perceptions of fullness were derived with all horticultural products showing nutritional value but some being more suited to e.g., promoting short-term satiation and some more suited to promoting longer-term satiety. Similarly, all horticultural products showed positive effects on gut microbiota and their health-associated fermentation products, with apparent benefits over current refined prebiotics. Horticulture products can realistically be promoted as 'Nature's prebiotics'. For both fullness and microbiota, there are predicted to be marked differences between individuals based on perceptions of hunger and diversity of microbiota. This study opens the way towards more personalised healthy food choices from amongst horticultural products based on individual characteristics as a realistic future target.

Public summary

The Naturally Nutritious project was a 5-year journey into investigating the health benefits of a range of fruit, vegetables, and nuts. The project aimed to capitalise on the already existing knowledge that fruits, vegetables and nuts are good for you, and to explore the possibilities of further enhancing their health benefits, through increasing their nutrient density. A wide range of fruit, vegetables, and nuts presently exist, and within each of these, numerous varieties also exist, with a huge diversity in their nutrient content. Some of these nutrients are linked to the colour of the fruit or vegetable, while others are much less obvious. The project aimed to document the range of specific nutrients, and to determine if it is possible to further enhance these levels through targeted breeding programs. The Naturally Nutritious project also investigated which fruit, vegetables and nuts make you eat less by making you feel full, and those that keep you feeling full for longer. And of course (at the other end!), to find out how these different products affect our gut bacteria, which are so important to the health of our bodies.

Keywords

Biofortification, nutrient density, health markers, visual differentiation, phytonutrients, zeaxanthin, anthocyanin, folate, saturated fat, saffron, lycopene, sweetcorn, macadamia, strawberry, capsicum, tomato, blood-plum, satiety, satiation, gut health, microbiome, bioavailability, consumer evaluation, photosensitization.

Introduction

The Naturally Nutritious project is aimed at raising the health profile of Australian horticultural commodities. Globally, there is an ever-increasing movement towards more natural products that are healthy for you. Although there is common knowledge that fruits, nuts, and vegetables are 'good' for you, this has not necessarily increased consumption, partly due to having to increase personal intake to reach recommended levels, and partly due to confusion over what the actual benefits are. Underlying these considerations, is the fact that the food must also taste good and be appealing enough to encourage habitual repurchase.

To address these issues, one strand of the Naturally Nutritious project is to increase the nutrient density of a range of commodities, such that food intake does not necessarily have to be increased to reach recommended daily requirements of specific phytonutrients to achieve in-body concentrations that are metabolically active. In some cases, this is readily achievable within existing available material, whereas in others, the potential to increase phytonutrient density requires further breeding, combining characteristics from different varieties together in order to maximise the phytonutrient concentration. A previous example of this is high-zeaxanthin sweet-corn for eye-health, in which the concentration of the phytonutrient 'zeaxanthin' was increased a factor of 10 times, such that a supplemental dose could be obtained by eating a small cobette as part of a normal meal.

Increasing nutrient density is not sufficient on its own to achieve a successful product, as it must also have sensory properties that are appealing to consumers. Ideally, the product should be visually differentiated from the standard product, such that it can be readily identified. It is therefore important that nutrient-enhanced products should have visual appeal and taste as good, if not better, than standard fare. In this way, we capture both the 'Conscious Improvers' (22% of consumers who buy on health reasons), as well as the 'Eager Explorers' (30% of consumers who are attracted to new ideas and motivated by taste, colour and texture). Health and nutrition benefits that are easy to confirm by consumers are ideal targets, giving positive reinforcement for repurchase. Examples include decreases in blood pressure, glucose, triglycerides and LDL cholesterol, and weight reduction, increase in folate concentration, all of which are easily measured to observe a benefit firsthand. In this regard, we have chosen a number of representative commodities which we believe have the potential to address all of these factors.

The second strand of our project is to characterize and quantify nutritional benefits of fruit, vegetables and nuts focusing on areas likely to lead to messages for consumers and health professionals that support health advice. The two focus areas are appetite regulation and gut microbiome health. Consumers are familiar with and responsive to the general health benefits of fruit, vegetable, and nut consumption, but the evidence behind public health campaigns is based almost exclusively on epidemiology data (i.e., correlations between long term dietary intake and health outcomes). Although epidemiology can provide statistically robust correlations, it provides no information on the mechanisms underlying health benefits. Some of these benefits are due to specific phytonutrient components, whilst other benefits derive from the indigestibility of the plant cell walls that form the physical structure of intact produce. Two specific nutrition/health benefits of intact horticultural produce are (a) satiation (the feeling of fullness that terminates meal eating) and satiety (the delay in hunger onset following a meal), and (b) promotion of a healthy gut microbiome by providing carbohydrates (and phytonutrients) that are not digested in the stomach or small intestine but are fermented in the large intestine, providing a supportive environment for 'good' bacteria to out-compete 'bad' bacteria and counter-acting the potentially deleterious effects of protein fermentation.

In regard to satiety and satiation, the project aims to characterize the effects of a range of horticultural products (apple, avocado, banana, carrot, macadamia) consumed as a mid-morning snack. In a first pilot stage, the food factors responsible for different amounts of food required to feel comfortably full will be identified along with ratings of fullness during (satiation) and after (satiety) the meal, with a view to identifying routes to portion sizes for either immediate or long-lasting fullness. In a second pilot stage, the human factors (both physiological and psychological) contributing to satiation and satiety will be explored, to provide insights into the relevant importance of food and human (physiological and psychological) factors on perceived fullness (satiation/satiety). This information is needed to identify the potential for targeted messages to groups of people ('precision nutrition') or individuals ('personalised nutrition'). Based on the results of the two pilot trials a larger scale trial will be conducted with more participants to test the robustness of models. A challenge in measuring human factors is that they often involve specialist facilities (e.g., in clinics) and/or labour-intensive analysis. In order to reduce the need to rely on such invasive/expensive/time-consuming methods, we will investigate the potential of infrared spectroscopy of saliva or skin to provide a sufficient fingerprint of

physiological (and possibly psychological) factors to predict satiation/satiety responses. With further work this could provide a powerful approach to identify personalized nutrition requirements.
In regard to promoting a healthy microbiome, we will isolate in vitro digestion-resistant residues (i.e., the fraction of ingested food that is likely to reach the large intestine (colon) where microbial fermentation predominates) from a range of horticultural products (apple, banana, spinach, celery, carrot, almond, macadamia) and study their rate and extent of fermentation as well as nutritionally-beneficial fermentation end-products in a validated in vitro model for colonic fermentation. One important aspect of a healthy microbiome is that fermentation takes place throughout the colon i.e., not too quickly. We will test the hypothesis that the complex fibre structure of undigested fruit/veg/nuts result in controlled and steady fermentation to nourish the whole colon. In order to understand the reasons for different fermentation behavior, residual non-fermented material will be isolated after various fermentation times to characterize both the microscopic cellular structure and the molecular features that resist fermentation. We will also identify the opportunity to use juicing by-products to act as prebiotics ('nourish the gut microbiome') by studying the fermentation in our in vitro model of undigested fractions of juice waste. For this study, apple, carrot, pineapple, beetroot, celery, and orange will be used, and the potential for developing horticultural prebiotic products identified.

Methodology

(a) Screening, bioprospecting, and consumer evaluation

Potential innovations for vegetables, fruits and nut products will target increasing nutrient density in appealing backgrounds. Germplasm from commercial/non-commercial lines was analysed for specific phytonutrient concentration to identify variability and underlying biochemical reasons for why different lines are higher in nutrient density than standard produce. Consumer appeal will be assessed using a combination of visual images, taste, and health messages to assess potential products prior to development.

The commodities chosen for initial biofortification were based on a number of factors, including the availability and accessibility of germplasm (breeding programs etc.), the size of the industry, the ability to market or to make a label claim for a phytonutrient, the ability to differentiate the product from its standard equivalent, the immediacy/measurability of the health benefit (e.g., blood pressure), and preliminary estimates as to what products may be appealing to consumers. An underlying premise is that apart from biofortification, visual differentiation is preferable, and flavour equal to or better than the existing product is essential.

In concurrence with analysis to gauge existing range in phytonutrients and potential to reach targets, consumer testing was undertaken to assess the acceptability of potential products. Factors assessed included overall acceptability of a product with/without a health message, the degree to which visual change is desirable/unacceptable, and market segmentation (is this a niche product or broader consumer segment product).

Consumer and sensory evaluation were conducted alongside the development of new products and 'concepts' to ensure that outputs align closely with high consumer value and acceptance (Figure 1). Trained panel assessments were conducted to define and quantitatively profile the improvements made to product qualities from breeding efforts, postharvest studies, and shelf-life extension trials. Focus groups and consumer taste testing were also conducted to understand the underlying values that different consumer segments place on specific products, colours, flavours or other attributes, and to elicit new ideas to trial in terms of product packaging, presentation, and market placement to ensure product differentiation.

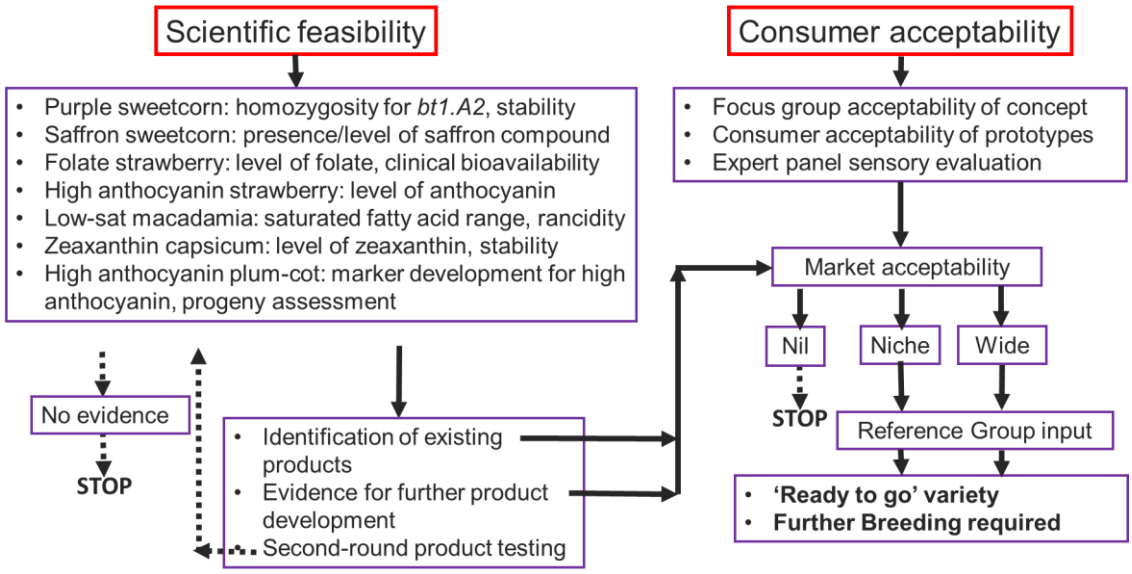


Figure 1: Product biofortification within the Naturally Nutritious project assessed both scientific feasibility to enhance a phytonutrient and consumer acceptability of such a change.

Analysis of phytonutrient content and other compounds in biosynthesis pathways was used to identify inter-relationships. Methods of analysis included GC-MS and HPLC for saffron compounds, zeaxanthin, and fatty

acids. Folate and folate vitamins in strawberries, human blood and urine were analysed by stable isotope dilution assays and ultra-high performance liquid chromatography (UHPLC) coupled with mass spectrometry. Anthocyanins, non-anthocyanin polyphenols and metabolites in a range of high anthocyanin fruit and vegetables including sweetcorn, strawberries, Queen Garnet plum and in human blood, urine and faeces were analysed by high performance liquid chromatography (HPLC) coupled with photodiode array detection and mass spectrometry. Methods for accelerating selection were based on phenotyping where applicable to improve analysis efficiency. Where applicable, new genetic markers will be developed for key attributes leading to specific phytonutrient biofortification.

(b) Preservation of nutritional content

Nutrient decline in the supply chain was identified via sub-sampling and under various cooking processes and storage conditions, where applicable. Natural preservation technologies to minimise nutrient decline/inhibit growth of spoilage microorganisms were developed based on photosensitization technology. Natural preservation agents (plant extracts) were used to protect fresh cut surfaces. The effect of cooking methods on nutrient content was investigated. Detailed plans include:
1. Photosensitization used to minimise fungal contamination in strawberries and extend shelf life by retaining nutritional value and quality. Different cultivars of strawberries with varying nutritional (folates) and phytochemical (anthocyanin) content were assessed for fungal contamination. Suitable photodyes (e.g., curcumin) using novel delivery methods were assessed against the relevant fungi in strawberries to extend chilled storage life. Pilot scale trials were undertaken to validate results.
2. Cooking methods such as microwave, pressure cooking and boil in the bag using thermoformable films were trialled for sweet corn to assess anthocyanin loss. Roasting as a heat treatment for different cultivars of macadamia nuts and effects on rancidity during storage was assessed by measuring fatty acid oxidation product, hexanal.
3. Different storage conditions and suitable packaging formats to assess nutritional losses in fresh-cut capsicum were determined. Natural plant extracts were assessed for antimicrobial activity and shelf-life extension in fresh-cut capsicum. Spoilage microorganisms were screened via micro-titre assay using a range of plant extracts. Extracts were combined with antimicrobials for effective synergistic combinations and trialled at bench and pilot levels.

(c) Building the evidence base

New consumer nutrition messages on the benefits of fresh produce focussed on:

(1) Satiation and satiety, using human subjects. Satiety and satiation properties of a range of horticultural products (apple, avocado, banana, carrot, macadamia), consumed as a mid-morning snack, were characterised. In a first pilot stage, the food factors responsible for different amounts of food required to feel comfortably full were identified. The ratings of fullness during (satiation) and after (satiety) the meal were recorded by participants and used to define the food factors that influence satiation and satiety with a view to identifying routes to portion size specification for either immediate or long-lasting fullness. In a second pilot stage, the human factors (both physiological and psychological) contributing to satiation and satiety were explored, providing a total of 28 variables that were modelled to provide insights into the relative importance of individual variables and grouped variables (food, physiology, psychology) on perceived fullness (satiation or satiety). This information is needed to identify the potential for targeted messages to groups of people (‘precision nutrition’) or individuals (‘personalised nutrition’) for either public health or commercial marketing purposes. Based on the results of the two pilot trials a larger scale trial was conducted with more participants to test the robustness of models. A challenge in measuring human factors is that they often involve specialist facilities (e.g., in clinics) and/or labour-intensive analysis (e.g., counting tongue papillae density). In order to reduce the need to rely on such invasive/expensive/time-consuming methods, in a third stage of this project we investigated the potential of infrared spectroscopy of saliva or skin to provide a sufficient fingerprint of physiological (and even in some cases psychological) factors to predict individualised satiation/satiety responses. With further work this could provide a powerful approach to identify personalized nutrition requirements.

(2) Microbiome effects were studied using an in-vitro fermentation system. Fruit, vegetable, and nut samples were subjected to human chewing (or a simulation of it) followed by in vitro gastric/small intestinal digestion.

Digesta were then incubated with faecal inocula from pigs (controlled diet) or humans. Gas production kinetics were analysed to determine rate of fermentation, and fermentation end-products used to identify indicators of a healthy microbiome.

Following in vivo chewing and in vitro digestion processing, residual material (a model for what passes to the large intestine) were fermented in the presence of faecal inocula from pigs fed a precisely controlled diet or from human volunteers. Comprehensive profiling of the fermentation process was carried out to determine the rate of fermentation and production of end-products. The aim was to demonstrate both the generic benefits of increased fruit/veg/nut consumption, and to identify any specific effects of individual produce types that could be used as a market differentiator. We aimed to provide data in support of communication messaging and potential product design through addressing three research questions:

- What is the relative fermentability of DF from a range of fruits, vegetables, and nuts, and why do these differ?
- What is the consequence of having entrapped macronutrients e.g., starch and lipid in banana and nuts respectively?
- Do fibre fractions recovered as waste from juicing processes have useful fermentation behaviour?

3) A clinical study with humans was conducted to assess the bioavailability of strawberry folate by analysis of blood/urine after consumption.

The bioavailability of folate from commercial strawberries was investigated in a single-centre, randomised, two treatment, two period, cross-over design. Four healthy women in childbearing age received a folate supplement (5-methyltetrahydrofolate) or a punnet of strawberries (250 g). Blood (plasma) samples was collected pre-dose as well as at different time points up to 24 hr after the consumption of the strawberries or supplement. In addition, the participants collected post-dose urine for 24 hr. Blood and urine samples are currently analysed for folate (vitamers and metabolites) by stable isotope dilution assays (SIDA) and bioavailability/pharmacokinetic parameters will be determined and published as soon as the folate concentrations of the analysed samples are calculated. However, a relatively high folate bioavailability from Australian grown strawberries, similar to that found in a human pilot study with German grown strawberries, is expected.

Case studies

Macadamia - Reduced saturated fat / Increased Omega 7 (palmitoleic acid)

Background

Health issues and label health claims

Macadamia nut kernels (*Macadamia integrifolia*, *M. tetraphylla*) are very high in oil, accounting for about three-quarters of their weight (Saleeb et al., 1973). The oil itself consists of approximately 77-80% monounsaturated fat, 1-7% polyunsaturated fat, and the remaining 14-21% saturated fat (Aquino-Bolaños et al., 2017; Beuchat and Worthington, 1978; Carrillo et al., 2017; Cavaletto et al., 1966; Saleeb et al., 1973). Although unsaturated fats are considered "good" for cardiovascular health, there is a general consensus that saturated fats are detrimental (Clifton and Keogh, 2017; Sacks et al., 2017; Wang and Hu, 2017). However, it is generally acknowledged that replacing saturated fat with mono-unsaturated fats or poly-unsaturated fats is the principal mode of benefit to cardiovascular health, rather than simply reducing saturated fat on its own.

Although the majority of fats in macadamia are believed to be 'good' for health, the level of saturated fat present (>8 g/100 g kernels, i.e., >8%) does restrict label health claims both in Australia (Front-of-Pack Labelling Secretariat, 2018) and in the United States (Food and Drug Administration, 2003, 2017). Along with cashews and Brazil nuts, macadamias are slightly higher than this threshold level. In fact, saturated fat content is the one factor that stops macadamia from receiving a perfect 5-star health rating in Australia. In the United States, macadamias, in contrast to almonds (and other tree-nuts not listed above), require an additional health statement suggesting that macadamias are healthy only as long as a commensurate amount of saturated fat is reduced elsewhere in a person's diet.

In addition to the health claims above, more general health claims are also permissible within Australia for foods with either low saturated fat (<1.5%) or reduced saturated fat (25% less than average). Generally speaking, the average saturated fat percentage is listed as 10.6% for macadamia kernels (and 14.2% in extracted macadamia oil) (FSANZ, 2021). The narrow difference between the 8% threshold listed above, and the 'average' 10.6% in macadamia kernels suggested that there was a strong potential to identify or develop lines with saturated fat either lower than the 8% threshold or a saturated fat content 25% lower than the 'average' macadamia nut (a reduced or 'lite' saturated fat macadamia).

Fatty acid profiles vary widely within a species (Aquino-Bolaños et al., 2017; Mereles et al., 2017), with 10.6% merely indicating a 'average' saturated fat content across a limited number of cultivars that were available at the time of assessment. Considering the large number of available cultivars, new breeding lines, and wild accessions, both within *M. integrifolia* and *M. tetraphylla*, and also in *M. jansenii* and *M. ternifolia*, the potential to identify existing cultivars that have a naturally reduced percentage or identify non-cultivated or wild accessions that could be utilised to reduce existing levels of saturated fat to 8% or lower, is potentially quite high.

From a commercial perspective, an improvement of health claims could be seen as the removal of a purchase barrier by health-conscious consumers, and also as a possible competitive advantage for Australia in the global export market, where increasing production of macadamia nuts is rapidly increasing in countries such as China and South Africa. Product differentiation in respect to improved dietary health may be sufficient to achieve a premium price, or alternatively to replace an existing supplier.

Although a reduction in saturated fat would enable a stronger health claim, the aim is not to reduce the total fat content of macadamia nuts, which could potentially change the texture of the kernel. Rather, in line with the current cardiovascular advice above, the preference was to replace saturated fat through an increase in unsaturated fat. One particular unsaturated fat of interest was the omega-7 fatty acid, palmitoleic acid. This mono-unsaturated fatty acid has been reported to be associated with other health benefits, particularly associated with diabetes (Hu et al.,2019). Palmitoleic acid is fairly rare in the diet, and macadamias are currently reported to be one of the highest dietary sources.

Currently, no label health claims are possible in Australia for palmitoleic acid, although extracts from Sea buckthorn are manufactured as nutraceuticals. From a chemical perspective, palmitoleic acid is formed from the principal saturated fat in macadamia, 'palmitic' acid. Increased conversion of palmitic to palmitoleic acid would therefore benefit twofold: firstly, by decreasing saturated fat, and secondly, by increasing omega-7 concentration, with additional health benefits other than for cardiovascular disease.

The following case study explores the variability in fatty acid profiles of a range of commercial cultivars, breeding lines, and wild accessions across the four Macadamia species. The aim of this analysis was to identify the existence of reduced-saturated fat varieties, or accessions that may have potential as prospective parents for breeding a low- or a reduced-saturated fat variety. In the case of the latter, the purpose was also to understand the possible mechanism by which reduced saturated fat could be accomplished, and other factors that could positively or negatively influence this.

It was also considered that alteration in the fatty acid profile could theoretically influence the flavour and shelf-life of macadamia nuts. Change in flavour, was not an aim of this project, but is an important consideration. Similarly, shortening of shelf-life through increased rates of rancidity is a possibility, although the small changes in fatty acid required are unlikely to have any significant effect, but were tested within the range of macadamia lines available.

As with other case studies in this project, a consumer evaluation of reduced saturated fat macadamias was conducted. Because of the unavailability of any still to be identified reduced saturated fat nuts, a concept evaluation of 200 macadamia nut eaters was conducted, identifying the main factors influencing or inhibiting purchase, and what proportion of consumers would be interested in purchasing reduced-saturated fat macadamias.

Industry feedback was also obtained in regard to the concept of reduced saturated fat or enhanced omega-7 macadamias.

Technical feasibility

Existing fatty acid profiles of cultivars and breeding lines

The fatty acid profiles of cultivars, breeding lines, wild accessions, and non-cultivated macadamia species (where available) were characterized over a series of trials, as nuts became available for assessment. This involved separation of the oil from the kernel for GC-MS analysis by either initially hydraulic pressure or with solvent extraction. Following characterization of the individual fatty acids present, the total saturated fat content was calculated, as was the omega-7 (palmitoleic acid) content of the oil. Total saturated fat was determined by the sum of saturated fatty acids of C12:0 to C24:0 carbon chain length.

Initially, 14 cultivars and 20 breeding accessions from the Australian macadamia breeding program were harvested at commercial maturity from Nambour and Bundaberg (Queensland) germplasm collection in 2016. Where possible, nuts were harvested from two different trees of each line, spaced as distantly as possible from each other within the germplasm collection. All trees were grown under the same fertiliser and irrigation regime on a uniform soil-type.

Nuts were dehusked, and then dried down to approximately 1-2% kernel moisture content over a three-day period. Nuts were hand-cracked, and five kernels randomly selected for oil extraction. Oil was extracted with a hydraulic press operated at 24°C. Approximately 3 ml of oil was collected as a composite sample.

Approximately 0.1 g oil was dissolved in 1.6 mL hexane before adding 100 µL 2M methanolic KOH. The mixture was shaken for 30 seconds and then centrifuged at 2000 rpm for 2 min. The hexane layer was collected and filtered for GC-MS analysis. One µL of the filtered hexane layer was injected into a gas chromatograph (GC-2010 Plus) coupled with mass spectrometer (MS-TQ8040) using an AOC 6000 autosampler. Fatty acid methyl esters (FAMES) were identified by comparison of retention time, molecular mass and fragment ions. Oil concentration was determined as a percentage of total oil, based on the ratio of individual peak area to total peak area (Shimadzu Labsolutions Insight).

The major fatty acids in all cultivars and breeding accessions were the monounsaturated fats, oleic acid (C18:1) and palmitoleic acid (C16:1). Concentration of oleic and palmitic acid ranged from 57-67% and 14-24%, respectively. The next highest concentrated fatty acids were the saturated fats, palmitic acid (C16:0; 7-10%), stearic acid (C18:0; 2-4%), and arachidic acid (C20:0, 2%). The remaining fatty acids (C18:3, C22:1) were each generally lower than 2% (Table 1). In addition to the major saturated fatty acids, other saturated fats detected in macadamia included, in order of concentration, myristic acid (C14:0), behenic acid (C22:0), and lauric acid (C12:0).

Table 1: Kernel oil fatty acid profiles of (A) cultivars, and (B) breeding accessions (the number following the cultivar or accession name is the tree number).

A.

Cultivar	Fatty acid (%)												Total sat-fat
	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0	C22:1	
A16-1	0.0	0.6	7.6	19.5	1.9	64.2	2.1	0.1	1.7	1.6	0.5	0.1	12.4
A376-2	0.0	0.6	7.6	24.0	2.2	59.5	2.1	0.1	1.7	1.8	0.3	0.1	12.5
A376-1	0.0	0.5	7.2	20.4	2.8	62.8	1.9	0.1	2.1	1.7	0.3	0.1	13.0
A447-2	0.1	0.6	8.1	21.8	2.6	61.5	1.4	0.1	1.9	1.7	0.2	0.1	13.5
A447-1	0.1	0.8	8.4	20.8	2.1	62.8	1.4	0.1	1.9	1.3	0.3	0.0	13.5
A403-1	0.1	0.7	8.2	23.0	2.4	59.2	1.8	0.1	2.0	2.2	0.3	0.1	13.6
HAES791	0.0	0.4	7.1	18.1	3.4	64.7	1.6	0.1	2.3	1.8	0.3	0.1	13.6
HAES344-2	0.2	1.3	7.6	19.7	2.3	63.3	1.5	0.1	1.7	1.6	0.5	0.1	13.6
A538-2	0.1	0.7	8.3	16.6	2.3	66.0	1.7	0.1	2.0	1.9	0.3	0.1	13.7
A403-2	0.1	0.6	8.0	19.4	2.7	63.9	1.4	0.1	1.9	1.5	0.4	0.1	13.7
HAES741-2	0.1	1.1	7.1	19.4	3.3	63.6	1.4	0.1	2.0	1.6	0.2	0.1	13.9
A422-2	0.1	0.7	8.0	17.1	2.6	64.5	1.7	0.1	2.2	2.5	0.5	0.2	14.0
A422-1	0.1	1.1	8.1	18.0	2.7	64.3	1.7	0.1	2.0	1.7	0.2	0.0	14.1
HAES741-1	0.1	1.2	7.9	21.8	3.1	61.0	1.4	0.1	1.9	1.4	0.2	0.0	14.3
Beaumont	0.1	0.6	9.3	22.6	2.5	58.6	2.4	0.1	1.7	1.7	0.3	0.1	14.4
HAES816-2	0.1	0.9	9.0	19.7	2.2	62.3	1.6	0.1	1.8	1.7	0.6	0.1	14.5
HAES816-1	0.1	0.8	8.4	16.7	2.9	64.6	2.0	0.1	2.3	1.8	0.3	0.0	14.8
A538-1	0.1	0.7	8.9	15.9	2.8	64.8	1.9	0.1	2.2	2.2	0.4	0.1	15.0
A16-2	0.1	1.3	8.1	19.9	3.2	61.6	1.4	0.1	2.2	1.7	0.3	0.1	15.3
HAES849	0.1	0.9	9.2	17.8	3.0	63.7	1.5	0.1	2.0	1.4	0.2	0.1	15.4
HAES246-2	0.1	1.0	9.5	18.0	2.5	62.2	2.0	0.1	2.1	2.0	0.3	0.1	15.6
HAES344-1	0.1	1.4	8.6	21.0	2.9	60.0	1.4	0.0	2.0	1.8	0.5	0.2	15.6
HAES842	0.0	0.6	9.0	18.2	3.5	61.5	1.6	0.1	2.6	2.0	0.6	0.1	16.3
HAES246-1	0.1	0.9	10.3	17.1	3.0	62.1	1.6	0.1	2.3	2.0	0.3	0.1	17.0

B.

Accession	Fatty acid (%)												Total sat-fat
	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0	C22:1	
S-1	0.1	0.7	7.6	17.8	2.1	66.0	1.8	0.1	1.7	2.0	0.2	0.1	12.3
T-2	0.1	0.7	7.5	22.1	2.2	62.0	1.5	0.1	1.8	1.7	0.2	0.0	12.6
E-1	0.1	0.6	7.8	18.0	1.8	65.7	1.6	0.1	1.7	1.9	0.5	0.1	12.6
L-1	0.1	0.8	7.9	16.8	1.8	64.5	2.8	0.1	1.9	2.9	0.3	0.1	12.7
H-2	0.1	1.4	7.2	21.3	2.1	63.6	1.0	0.1	1.6	1.2	0.2	0.0	12.7
D-2	0.1	0.8	7.7	16.4	2.1	66.0	2.1	0.1	1.7	2.2	0.6	0.2	13.1
B-1	0.1	0.9	7.5	18.5	2.4	64.8	1.6	0.1	1.7	1.8	0.5	0.1	13.1
F-2	0.0	0.6	8.4	16.5	2.2	66.4	2.0	0.1	1.8	1.6	0.4	0.1	13.4
O-2	0.0	0.3	7.5	18.8	3.2	65.2	1.2	0.1	2.0	1.3	0.4	0.1	13.4
T-1	0.1	0.8	8.8	24.5	1.9	58.9	1.3	0.1	1.6	1.6	0.3	0.0	13.6
I-1	0.1	1.1	8.0	19.7	2.2	63.1	1.7	0.1	1.9	1.7	0.3	0.0	13.6
Q-2	0.0	0.6	8.8	16.8	2.1	65.2	2.4	0.1	1.7	1.8	0.5	0.2	13.6
N-1	0.1	0.8	7.9	20.8	2.6	62.3	1.6	0.1	1.9	1.4	0.4	0.1	13.7
Q-1	0.1	0.9	8.4	18.0	2.1	63.3	2.7	0.1	1.8	2.2	0.3	0.1	13.7
G-1	0.1	1.1	8.7	22.3	1.9	61.5	1.0	0.1	1.5	1.3	0.4	0.1	13.7
R	0.1	1.0	8.0	18.7	2.3	63.3	1.8	0.1	2.0	2.3	0.3	0.1	13.8
C-1	0.0	0.7	7.7	19.3	3.1	63.2	2.0	0.1	2.0	1.6	0.3	0.0	13.8
O-1	0.0	0.5	7.8	21.3	3.0	61.2	1.7	0.1	2.3	1.8	0.2	0.1	13.9
G-2	0.1	0.6	8.7	24.0	2.5	58.7	1.7	0.1	1.7	1.5	0.4	0.1	14.0
B-2	0.1	1.3	7.6	21.0	2.8	61.8	1.4	0.1	1.9	1.7	0.2	0.1	14.0
F-1	0.1	0.6	8.2	14.5	2.9	67.3	2.3	0.1	2.0	1.6	0.2	0.1	14.1
C-2	0.1	0.8	7.6	20.2	3.1	61.9	1.8	0.1	2.3	1.8	0.3	0.1	14.1
P-1	0.1	0.8	7.5	18.7	3.2	63.4	1.7	0.1	2.2	1.9	0.4	0.1	14.1
D-1	0.1	0.9	8.0	17.2	2.7	64.9	1.5	0.1	2.0	1.9	0.7	0.2	14.3
H-1	0.1	0.8	7.5	17.6	3.2	63.5	2.6	0.1	2.4	1.8	0.3	0.0	14.3
E-2	0.1	0.6	8.5	18.2	2.7	63.2	2.3	0.1	2.2	1.8	0.2	0.0	14.3
L-2	0.1	0.7	8.4	24.2	2.6	57.5	1.7	0.1	2.2	2.1	0.4	0.1	14.4
P-2	0.1	0.7	7.5	19.1	3.5	63.0	1.6	0.1	2.5	1.8	0.2	0.0	14.4
I-2	0.1	1.0	7.5	17.4	3.1	64.9	2.2	0.1	2.2	0.8	0.6	0.2	14.4
J-1	0.0	0.5	8.7	15.5	2.6	65.3	2.4	0.1	2.1	2.1	0.6	0.1	14.5
A-2	0.1	0.8	8.4	16.4	2.6	64.5	2.4	0.1	2.0	1.9	0.7	0.2	14.5
J-2	0.0	0.6	8.3	19.9	3.3	62.1	1.3	0.1	2.2	1.7	0.3	0.1	14.8
M-2	0.1	1.0	8.0	21.7	3.3	60.3	1.3	0.1	2.1	1.5	0.6	0.1	15.1
N-2	0.1	1.2	8.0	17.0	2.8	63.5	2.3	0.1	2.3	1.9	0.7	0.1	15.1
A-1	0.1	0.8	8.7	13.8	3.1	67.4	2.3	0.1	2.2	1.1	0.2	0.0	15.3
M-1	0.1	1.0	8.9	18.1	3.2	62.7	1.2	0.1	2.2	1.8	0.6	0.1	16.0
K-1	0.0	0.9	9.7	20.3	2.9	60.1	1.7	0.0	2.0	1.8	0.5	0.1	16.0
K-2	0.1	1.0	9.3	20.6	3.3	59.3	1.9	0.1	2.3	1.8	0.3	0.1	16.2
S-2	0.1	1.0	7.8	19.1	4.3	60.6	2.0	0.1	2.8	1.6	0.5	0.2	16.4

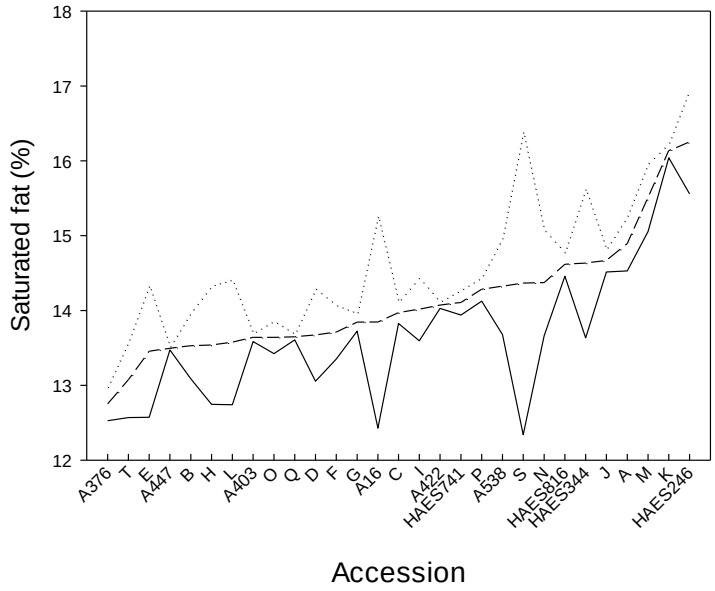


Figure. 1: Variation in total saturated fat content (%) between trees of the same cultivar/accession in different locations within the orchard. The mid-line (dashed) indicates the average of the two trees (upper and lower lines). Cultivars or accessions with only one tree are not shown.

Total saturated fatty acid concentration across the lines varied over a narrow range of 12.3-17.0% (Figure 1), with an average of 14.2%. Highest average saturated fat percentage was observed in HAES246, while the lowest was observed in A376. Different tree location of the same accession was also observed to have an impact on saturated fat concentration. While in some accessions (A447, A403, Q, A422) inter-tree variability was quite small at (e.g., 0.1%), in others (S, A16) it was as high as 4.1%.

The fatty acid profiles observed in the accession and cultivars assessed in the above trial indicated that total saturated fat varied in a narrow range from 12.3-17.0%. The average was 14.2%, which is similar to that published for macadamia oil in the Food Standards Australia and New Zealand nutrition tables (FSANZ, 2017). As the lowest saturated fat concentration (i.e., 12.3%) is only 13% lower than the FSANZ reference value of 14.2%, this oil is insufficient to qualify as a 'reduced' saturated fat product, which requires at least a 25% reduction relative to the reference value (Federal Register of Legislation, 2017).

Similarly, on a whole kernel value, which consists of 75% oil, the amount of saturated fat (4.6 g per 50 g kernel) exceeds that required (<4.0 g/50g) by the US-FDA for an unqualified nut health claim possible for many other commercial nuts (e.g., almond, pistachio, peanut) (FDA, 2003). Clearly, assessment of other macadamia accessions, including those outside the *M. integrifolia* and *M. tetraphylla* species, or targeted breeding, will be necessary to reach lower total saturated fat concentrations.

In agreement with previous reports (Beuchat and Worthington, 1978; Saleeb et al., 1973; Caveletto et al., 1966), palmitic (C16:0) and stearic acid (C18:0) were the major saturated fatty acids present in the breeding accessions/cultivars assessed. Palmitic acid always constituted the highest fatty acid, with approximately three times as much as stearic acid. From the data (Table 1), endogenous desaturation of palmitic to palmitoleic acid (C16:1) is considerably less efficient than stearic to oleic (C18:1).

Different saturated fatty acid profiles for trees of the same accession positioned at different locations within the orchard were also observed in the above trial (Figure 1). It is possible that these differences were due to different pollinators for each tree. Macadamia has a strong tendency to outcross (Sedgley et al., 1990), and the kernel tissue is made up of genetic material from both the maternal and paternal genome. It is therefore likely that the pollinizer would modify the fatty acid profile of the maternal accession, although a controlled pollination trial would be necessary to confirm this. Such a situation should be taken into account in the development of a reduced-saturated fat breeding line. In the current study, the pollinizers were not identified, although it is likely they may have been the adjacent flowering trees. A subsequent controlled-pollination trial was conducted (see below), and the significant effect of the pollen source on the fatty acid profile confirmed.

The above study indicated that saturated fat content in the extracted oil of cultivars and breeding accessions tested (predominantly *M. integrifolia*) varied within the range 12.3-17.0%. The predominant saturated fatty acids, in order of concentration, were palmitic, stearic and behenic acid. In general, desaturation of palmitic acid was less efficient that desaturation of stearic acid.

Although the majority of lines with lower saturated fat content had a combination of both higher C16- and higher C18-desaturation efficiency, some lines had a combination of higher C18-desaturation and higher C16-elongation efficiencies. Both combinations were able to achieve lower saturated fat content, due to the latter shifting fatty acid flux towards C18:0, which is more efficiently desaturated.

Existing fatty acid profiles of wild accessions and non-cultivated Macadamia species

Mature macadamia nuts of *M. integrifolia*, *M. tetraphylla*, *M. jansenii*, and *M. ternifolia* were harvested and dried to 1-2% moisture content before being crushed at 23-24°C with a hydraulic press to extract an oil sample (> 0.5 ml) for fatty acid analysis. Samples included 14 cultivars, 20 breeding accessions, 60 wild specimens of *M. integrifolia*, 5 wild specimens of *M. tetraphylla*, and 1 and 3 wild specimens of *M. ternifolia* and *M. jansenii*, respectively. Cultivars that may have included dual parentage from *M. integrifolia* and *M. tetraphylla*, and one cultivar ('HAES791') with partial parentage from *M. ternifolia* were listed under their principal parent background of *M. integrifolia* (Peace et al., 2005).

Similar to the trial above, the principle saturated fatty acid in oil extracts of all macadamia accessions and species tested was palmitic acid (C16:0), followed by stearic acid (C18:0) and arachidic acid (C20:0). Palmitic acid varied in concentration within a narrow range from 4.9 to 10.3%, stearic from 1.8 to 5.7%, and arachidic from 1.5 to 4.2%. Total saturated fatty acid concentration of the oil varied from 12.3 to 19.5%, which extended the upper range, but not the lower range, of saturated fat observed in the previous trial.

Although variation in saturated fat concentration existed within each macadamia species (Table 2), there was a tendency for *M. jansenii* to have lower total saturated fat (13-14%), and *M. tetraphylla* to have higher saturated fat (17-20%). The single accession of *M. ternifolia* had slightly higher saturated fat (14.2%) than *M. jansenii*. *M. integrifolia* exhibited the greatest range in saturated fat content, only being exceeded in saturated fat by *M. tetraphylla*.

The monounsaturated fatty acid, palmitoleic acid (C16:1), which is derived from palmitic acid via desaturation, varied in concentration from 9.9% (*M. jansenii*) to 24.5% (*M. integrifolia*) (Table 2). While this upper range was very similar to the previous trial, the lower range of palmitoleic acid was extended, predominantly due to the non-cultivated species, *M. jansenii* and *M. ternifolia*. The predominant monounsaturated fatty acid in all macadamia extracts was oleic acid (C18:1), which varied from 52.6 to 69.3%. The only other major monounsaturated fatty acid observed was gadoleic acid (C20:1), which varied from 0.8 to 3.4%.

Table 2: Concentration ranges of palmitic, palmitoleic, and total saturated fat of oil extracts from accessions of four Macadamia species.

Species	Palmitic acid C16:0 (%)	Palmitoleic acid C16:1 (%)	C16:1/C16:0 ratio	Total saturated fat (%)
M. jansenii	4.9-6.3	9.9-13.5	2.0-2.3	12.9-13.8
*M. ternifolia**	5.5	11.1	2.0	14.2
M. integrifolia	7.1-10.3	14.5-24.5	1.5-3.2	12.3-17.0
M. tetraphylla	6.7-8.1	15.3-20.1	2.1-2.5	16.7-19.5

*Single accession available only.

Concentration of the monounsaturated fatty acid palmitoleic acid (C16:1) was highest (24%) in four *M. integrifolia* accessions, A376-2, T1, G2 and L2, and lowest (10%) in an accession of *M. jansenii*. Although there was a slight trend for palmitoleic concentration to be inversely correlated with total saturated fat concentration, this relationship was quite weak (P=0.01, r2=0.04) (Figure 2a).

The high-palmitoleic accessions above varied in their saturated fat concentration from low to moderate, with A376-2 notably having one of the lowest total saturated fat contents (12.5%). Palmitoleic concentration, however, was found to be closely correlated to the ratio of palmitoleic to palmitic acid (Figure 2b). While high-palmitoleic accessions tended to have high palmitoleic: palmitic ratios of 3:1, low-palmitoleic accessions had ratios as low as 1.5:1.

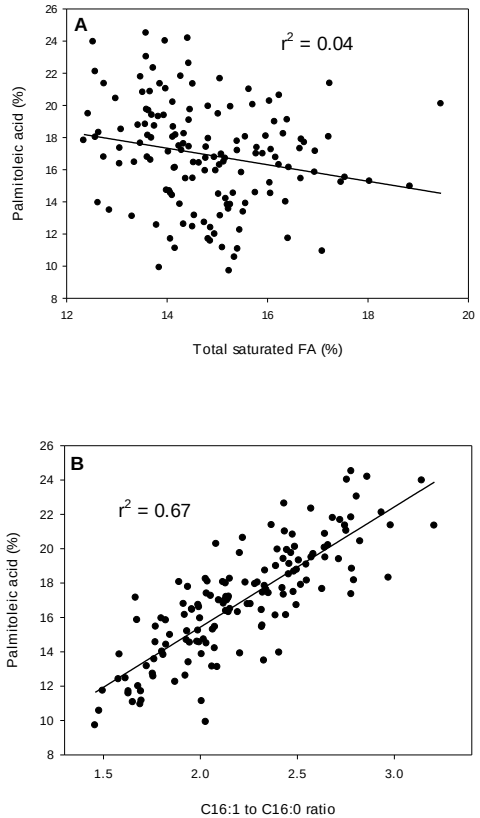


Figure 2: Linear relationship between palmitoleic acid and (A) total saturated fat, and (B) C16:1 to C16:0 ratio.

In the current study, saturated fat was found to vary within a small range of 12.3 to 19.5% of oil extracted. In a macadamia kernel, of which three quarters of its mass is oil, even accessions identified at the lower range of saturated fat concentration were still about 2% above that considered healthy. Despite evidence alluding to consumption of macadamias being beneficial to cardiovascular health (Stewart et al., 2015), from a health-claim perspective the development of a reduced-saturated fat macadamia may be advantageous in the marketplace, as it will allow stronger health claims.

The principal saturated fatty acid across all macadamia accessions in the current study, including non-commercial species, was palmitic acid. Palmitic acid (C16:0) is the immediate precursor of monounsaturated omega-7 fatty acid, palmitoleic acid (C16:1), in which the former is desaturated to form the latter by introduction of a double bond. Interestingly, those accessions with the highest percentage of palmitoleic acid, also had the greatest ratio of palmitoleic to palmitic acid (Figure 2b), indicating that variation in desaturation efficiency may exist across genotypes. Increasing the desaturation of palmitic acid would potentially have a twofold benefit: firstly, to reduce the total concentration of 'bad' saturated fat closer to that of the threshold for a label claim, and secondly, to further increase 'good' palmitoleic acid concentration.

Although there was a trend for higher-palmitoleic/lower palmitic accessions to be lower in total saturated fat, this correlation was not strong (Figure 2a). This can be explained by other saturated fats present in macadamia oil contributing to total saturated fat content. As with the variation in desaturation of palmitic to palmitoleic acid, variation in desaturation efficiency of stearic (C18:0) to oleic acid (C18:1) is also likely to exist. Furthermore, both this and elongation of palmitic acid to stearic acid (rather than desaturation to palmitoleic acid), will also be contributing factors towards determining final total saturated fat concentration.

The current study indicated that enhancing palmitoleic concentration and reducing total saturated fat content in macadamia are not mutually exclusive. Although an increase in monounsaturated palmitoleic acid may be achieved by selection of lines with greater C16-desaturation efficiency, it is probable that in order to achieve lines with an added characteristic of lower total saturated fat, selection will also be required for factors reducing other saturated fatty acids (e.g., stearic acid) in macadamia oil.

Further fatty acid analysis of available cultivars, breeding accessions and wild germplasm

To further explore the variability in the fatty acid profile of macadamia kernels, 197 macadamia accessions were collected from a wider range of germplasm, consisting of 82 cultivars, 60 breeding lines, and 55 wild accessions. Samples were collected from three different areas in Queensland, including Nambour (Maroochy Research Facility), Bundaberg (Bundaberg Research Facility), and Tiaro during April 2019. The nuts were all collected at a fully mature stage, and each variety was harvested from the same tree.

Nuts (three biological replicates for every variety) were dehusked, shelled, and the kernels immersed in liquid nitrogen and freeze-dried for 3 days (alpha 1-2 LD freeze drier John Morris Scientific Pty Ltd), followed by pulverising using a Breville coffee and spice grinder. Two hundred mg of the powdered macadamia kernel was weighed and mixed with 1.5 ml of chloroform: methanol (2:1, v/v) with 0.01% BHT. The samples were placed on a horizontal shaker (Ratek, Thermo Fisher Scientific) and extracted for 10 hours at 300 rpm at ambient temperature, followed by centrifugation at 18000g for 5 minutes (Heraeus Pico 17, Thermo Scientific). The supernatant was then transferred to 2 ml Eppendorf tubes and dried using a RVC 2-18 CD Plus vacuum concentrator (John Morris Scientific Pty Ltd) and 2 µL of the final oil product aliquoted and analysed, as in the earlier trials.

The percentage of the fatty acids present in the oil of 197 macadamia accessions are shown in Figure 3. As before, the main fatty acids present in macadamia were oleic acid (C18:1) (45.2-74.9%), followed by palmitoleic acid (C16:1) (7.8-33.2%), palmitic acid (C16:0) (6.4-12.5%), stearic acid (C18:0) (1.1-8.5%), and vaccenic acid (trans-C18:1) (1.2-4.7%). Other minor fatty acid components identified in this study included arachidonic acid (C20:0) (0.7-3.9%), eicosenoic acid (C20:1) (0.1-2.8%), linoleic acid (C18:2) (0.2-2.8%), and docosanoic acid (C22:0) (0.03-1.16%).

A wide range of variation was observed within the range of each fatty acid content, especially C18:1 and C16:1. Interestingly, the range of palmitoleic acid was significantly extended beyond that observed in the previous trials, reaching as high as 33.2% palmitoleic acid, and as low as 7.8%. The current study identified twelve high C18:1 accessions (>70%), with the three highest accessions being elite breeding line MIVI-E (unknown species, 73.66%) , elite breeding line MIVI- O (unknown species, 74.69%), and commercial cultivar 'mini macca' (unknown species, 74.86%) , as well as five high C16:1 accessions (>28%), with the three highest accessions being the wild accessions Wter107 (*M. ternifolia*, 29.01%) and Wtet194 (*M. tetraphylla*, 33.24%), and the

commercial cultivar A9/9 (*M. integrifolia*, 29.27%).

The saturated fat range of the macadamia accessions in this study was also extended beyond that observed previously. In the current study, saturated fat ranged from 10.8% to 22.2%, while that observed in the previous trial was 12.3% to 19.5%. In the current study, fifteen accessions with saturated fat below 12% were identified, with the three lowest accessions being the breeding lines UQM48 (unknown species, 10.80%) and MIVI-E (unknown species, 11.04%), and the wild accession WMInt75 (M. integrifolia, 11.34%).

Despite at least one accession reaching a saturated fat percentage of 10.8%, this is still marginally above that required to be considered having 'reduced-saturated fat', which is <10.65%, calculated as 25% lower than the 14.2% average saturated fat content for macadamia oil listed by the FSANZ food composition tables.

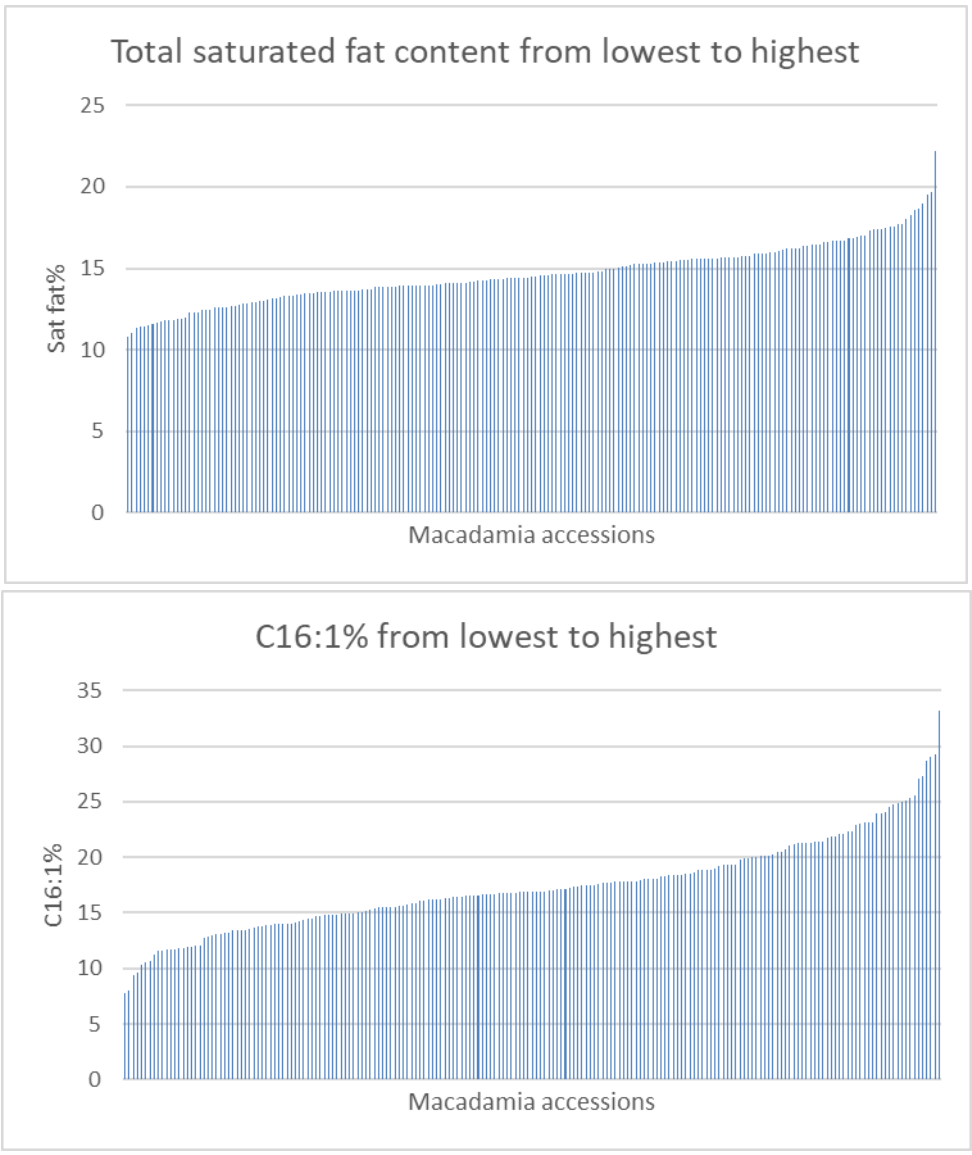


Figure 3: Percentage of saturated fat (top) and C16:1 palmitoleic acid (bottom) across 197 accessions, collated from lowest to highest.

An interesting observation, which seems consistent with macadamia kernel analysis, is that individual replicates can vary, some widely, in their fatty acid profile. In the current trial, it was noted that some individual replicates of certain macadamia breeding accessions (from the same maternal tree) did record saturated fat levels below the target level of 10.65%. The three individual kernels with saturated fat levels below 10.65% were:

1. 8.7%, belonging to BFA1, which had the lowest average saturated fat of 10.8%.

2. 9.7%, belonging to BFA2, which had an average saturated fat of 11.4%.

3. 9.8%, belonging to BFA3, which had an average saturated fat of 12.4%.

As macadamia is prone to out-crossing, and the kernel is formed from a maternal ovule and paternal pollen, it is probable that the fatty acid profile of an accession will be influenced by the pollinizer. In the current and previous trials, all nuts collected were open-pollinated, and as such, the fatty acid profiles of each kernel likely to vary according to the accession that was the pollen source. Consequently, the fatty acid values generated were either from a composite group of kernels, or from individual kernels averaged together. The above results are individual kernels would indicate though, that reduced saturated fat content (<10.65%) is quite achievable for at least some accessions but is likely to be dependent on the pollinizer for this to be achieved.

In summary, the average saturated fat content varies greatly (10.8-22.2%) between maternal accessions, and under uncontrolled pollination conditions, marginally exceeds the 10.65% threshold for a reduced saturated fat claim. It was observed, however, that this threshold can be reached in individual kernels, which may indicate that controlling the pollinizer (a reduced saturated-fat paternal parent) for a reduced saturated-fat maternal parent would enable consistent reduced saturated fat. Although requiring proof of concept, firstly to indicate the impact of paternal parent on the fatty acid profile, and secondly to identify matching pairs of accessions, this could be achieved with standard orchard practice, where two cultivars are planted in alternate rows to encourage cross-pollination.

Identification of non-genetic factors that may influence macadamia saturated fat content

Non-genetic factors (other than the genetics of the variety or breeding accession) potentially influence the fatty acid profile of macadamia nuts. Because of the potential size of such a study, an analysis was conducted on an earlier existing dataset that had collected fatty acid profiles across a number of varieties, across multiple sites, and over three seasons.

Within this dataset, it was generally found that the oil profile was largely controlled by tree genetics (>82%), rather than environmental effects. The very high heritability values for each environment indicates that most of the variation is due to the mean average effect of the cultivar across the environments, i.e., low GxE. Consequently, breeding for a particular fatty acid profile, such as reduced saturated fat or higher palmitoleic acid, was less likely to be influenced by the prevailing environment, and more by the genetics of the accession.

Impact of pollen parent on saturated fat and palmitoleic acid content in macadamia kernels

Over the course of the Naturally Nutritious project, it was considered possible that the male pollen source (macadamias are largely an out-crossing species) involved in the pollination of the resulting macadamia kernel on the mother tree could also have a strong potential influence on the resulting fatty acid profile. This ‘xenia’ effect has not been previously shown but considering the edible kernel of the macadamia is the diploid combination of the female ovule (haploid) and the male pollen (haploid), logic would indicate that both parents are likely to influence the resulting fatty acid profile.

Our earlier fatty acid profiling data of open-pollinated accessions (above) within a mixed germplasm orchard indicated that tree to tree fatty acid profile within the same variety could vary significantly, even between composite samples. We subsequently designed a trial involving controlled crosses between ‘low-saturated fat’ and ‘high-saturated fat’ varieties to see if the pollinizer did indeed affect the fatty acid profile of the kernels.

Three female parents (breeding accessions B, I, M) were hand pollinated using pollen from three male pollinisers (HAES 741,344,246). The parents were selected based on their previously measured saturated fat levels, based on open-pollinated kernels. Kernels were harvested when fully mature and prepared for analysis as previously described.

The results of this controlled pollination trial indicated that the pollinizer parent did indeed have a significant and strong effect on the fatty acid profile (Figure 4). For example, kernels from the accession ‘B’ could vary in saturated fat from 14.7% to 16.7%, depending on the pollinizer parent, with ‘741’ inducing a significantly lower saturated fat content than ‘246’. Similarly, other fatty acids such as palminoleic acid, were also significantly

affected by the pollinizer parent, with '246' inducing a significantly higher level in both the 'I' and 'M' maternal parents (Figure 4).

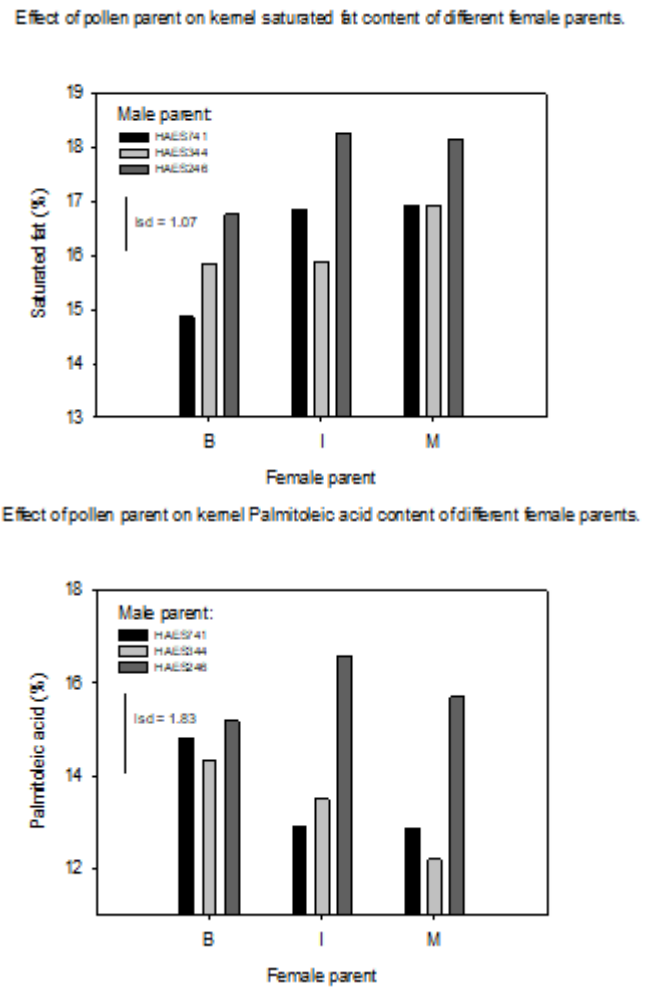


Figure 4: The impact of male parent (pollen source) on the saturated fat (top) and palmitoleic acid content in kernels produced on female breeding accessions (recipient of pollen).

These results indicate that future breeding or selection of a macadamia for reduced saturated fat or higher palmitoleic acid would require the breeding of two varieties that could pollinate each other to produce a desired fatty acid profile. As macadamias are largely an out-crossing species, the further identification of appropriate parent-pairs, to reciprocally pollinate each other, should be investigated in future trials.

It also puts in question previously published data in the scientific literature on the fatty acid profiles of individual macadamia cultivars, as the data would have been 'randomly' influenced by the identity of the male parent.

Impact of kernel maturity on fatty acid profile

A further comparison was made of the effect of kernel maturity on the fatty acid profiles of three macadamia accessions from *M. integrifolia*: R, P, and HAES791. For this comparison, mature and unmatured nuts from the three accessions were collected from Nambour (Maroochy Research Facility) during March-April 2018. Mature and unmatured nuts were identified according to their inner husk color (the inner husk color of mature nuts is brown, while unmatured nuts have a white inner husk) and density (unmatured nuts tend to sink in water). Both mature and unmatured nuts were harvested at different time periods from the same tree and were stored at-80°C prior to fatty acid analysis.

The results showed an interesting tendency where the percentage of both palmitoleic acid (C16:1) and saturated fat content tended to decline with increasing kernel maturity, while that of C18:1 tended to increase (Table 3). These findings are consistent with previous results reported by Hiroshi et al. (2012).

Table 3: Decline in percentage of saturated fat and palmitoleic acid (C16:1) and increase in oleic acid (C18:1) with increase in kernel maturity.

Accession	C16:1	C18:1	Saturated fat
R (unmatured)	27.18b	48.48b	16.19c
R (mature)	21.93c	57.40c	15.00a
791 (unmatured)	24.15bc	54.76bc	13.82b
791 (mature)	14.13d	68.86e	11.87e
P (unmatured)	18.62a	61.12a	15.66a
P (mature)	17.86a	63.42d	14.93d

An interesting observation in Table 3 was that the change in the percentages of C16:1, C18:1, and saturated fat in accession 'P' was significantly smaller than those in accessions '791' and 'R', suggesting that accession 'P' could have a different elongation upregulation mechanism.

Based on the above observations, it is possible that in the early stages of kernel development, the enzyme responsible for the elongation of C16:0 to C18:0 has not yet been upregulated, resulting in the main pathway of C16:0 metabolism being the desaturation of C16:0 to C16:1.

Two possibilities potentially exist for this desaturation process: (1) the C16:0 desaturation is catalyzed by a specific enzyme for C16:0 but it's efficiency is lower than the C18:0 desaturation process (2) the C18:0 desaturase is responsible for the desaturation of both C16:0 and C18;0 but shows poorer substrate specificity towards C16:0. However, either possibility results in the accumulation of C16:0 due to the apparent limited efficiency of the C16:0 desaturation. After the nut kernel reaches maturity, the C16:0 elongase appears to be upregulated, resulting in most of the accumulated C16:0 being converted to C18:0, where it subsequently undergoes desaturation to C18:1 by an efficient C18:0 desaturase.

Competition within an accession appears to exist between the accumulation of C16:1 and C18:1. Similarly, the elongation of C16:0 to C18:0 fatty acid is overall higher than C16:0 desaturation to C16:1. However, if the C16:0 elongase is upregulated at an earlier stage of kernel maturity, which might be the case for cultivars R and 791, an increased proportion of C18:0 will be available for C18:1 synthesis, resulting in less C16:0 and C16:1. This potentially explains the strong negative correlation between C16:1 and C18:1.

In macadamia, the accumulation of palmitoleic acid is likely to occur before the kernel reaches full maturity (Table 3). However, after the elongation pathway from C16:0 to C18:0 gets upregulated with increasing kernel maturity, most of the synthesized C16:0 gets converted to C18:0, followed by desaturation to C18:1, resulting in a decrease of C16:0 and C16:1 content.

Based on these observations, it could be safe to assume that the impact of the elongation pathway is far greater than the impact caused by the variation in the efficiency of the C16:0 desaturation on total saturated fat content or palmitoleic acid content. Therefore, a macadamia variety with a C16:0 elongase that is upregulated earlier during nut maturation would tend to have a lower C16:0 (the main saturated fat in macadamia) as well as palmitoleic acid (C16:1) content, and a higher monounsaturated C18:1 content. If such is the case, then it may be difficult to develop a macadamia accession that is low in saturated fat and high in palmitoleic acid at the same time. Considering that some accessions do display both reduced saturated fat and high palmitoleic acid (Figure 2a), this would indicate that more than one mechanism may exist in macadamia and could be incorporated/selected for in an accession with both properties in the future.

Impact of FA profile and roasting on rancidity and shelf-life

A potential issue that may arise in conjunction with altering the fatty acid profile of macadamia nuts is an alteration in their shelf-life. This is related to the relative stability, and likelihood to go rancid, of fats in regard

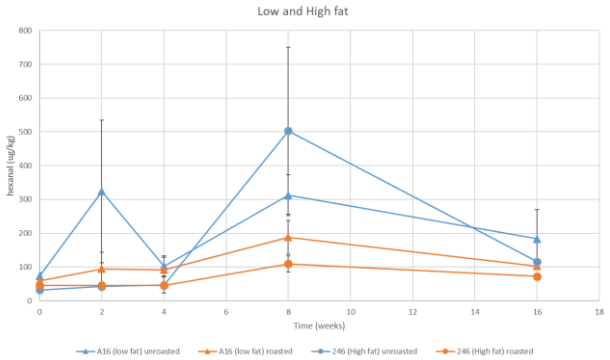
to their number of double bonds present. Poly-unsaturated fats (> one double bond) tend to be less stable than monounsaturated fats (single double bond), and these again are less stable than saturated fats (no double bonds). The main fat group in macadamia kernels is mono-unsaturated fat, comprised largely of oleic acid (C18:1) and palmitoleic acid (C16:1). Macadamias contain only a small amount of poly-unsaturated fat (<4%), but they do contain saturated fat (on average, 14.2% of the oil fraction). Consequently, reducing the saturated fat content, by simultaneously increasing the mono-unsaturated fat content could theoretically reduce fat stability and reduce shelf-life.

In the current trial, four macadamia accessions were selected based on their saturated fat content and previously found hexanal content, hexanal being an indicator of rancidity. At the same time, one half of the kernels were roasted, to observe what effect this may also have on shelf-life stability. Four accessions (A16, 246, I and 344) were investigated in the present study, one each from higher and lower saturated fat, high and low hexanal varieties. The nuts for these four cultivars were supplied by Dougal Russell, Principal Horticultural Experimentalist, Maroochy Research Facility, Queensland Department of Agriculture and Fisheries.

Macadamia kernels were separated from shells using a nutcracker. Only full and half kernels were used in the trial to have consistency in sample quality and roasting. Kernels without roasting were used as control samples. Roasting was done in a preheated (200°C) oven using a baking tray. Each of the samples were roasted for 7 minutes at 200°C with frequent shaking of the sample every 2 minutes. At the end of roasting the samples were taken out of the oven and transferred into another tray at room temperature to stop any further roasting.

Samples, both control and roasted, were vacuum packed into small Cryovac Foil vacuum bags with 5 nuts in each bag and stored in an incubator at 30°C to accelerate rancidity. Rancidity (hexanal) levels were be tested at regular intervals of time over a period of 24 weeks.

Hexanal readings of the four roasted or unroasted accessions indicated that neither roasting nor storage for up to 4 months at 30°C appeared to have any impact on the fatty acid profiles of macadamia kernels (Figure 5). The trial was subsequently extended to six months storage, to observe if any changes occur with a longer storage period. At this stage, roasting did not appear to have any impact on the fatty acid profile that was different from raw kernels.



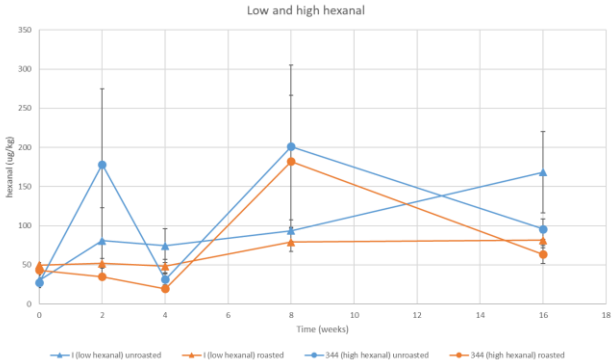


Figure 5: Effect of storage and roasting on hexanal production as impacted by saturated fat content (top) and previous putative identification of being a high or low hexanal variety (bottom).

Interestingly, the putative high-hexanal cultivar '344' (hexanal is an indicator of rancidity) had lower poly-unsaturated fat percentages than the putative low-hexanal cultivar, 'I'. Based on scientific observations previously reported with other nut species, high poly-unsaturated fat content is associated with shorter shelf-life, because of the presence of less stable double bonds in the fatty acid. This preliminary observation may indicate that the limiting factor to shelf-life in macadamia is not in fact the level of poly-unsaturated fat, but another factor moderating the rate of oxidation, such as Vitamin E-like antioxidant compounds. It is also possible that at 30°C, rancidity may take longer than 4 months to become apparent.

Following the 4-month analysis, a final withdrawal at 6 months storage was made (Figure 6). From the overall study (0-24 weeks), the following key observations from this study were:

- For all four varieties, there was minimal increase in hexanal;
- Roasted kernel hexanal levels were generally lower than in unroasted kernels;
- Roasted kernel hexanal levels were generally less variable across replicates than in the unroasted treatment.
- All unroasted samples showed evidence of hexanal conversion to hexanol. All roasted kernels exhibited no evidence of hexanal reduction to hexanol. Hexanol has a lower flavour activity than hexanal.

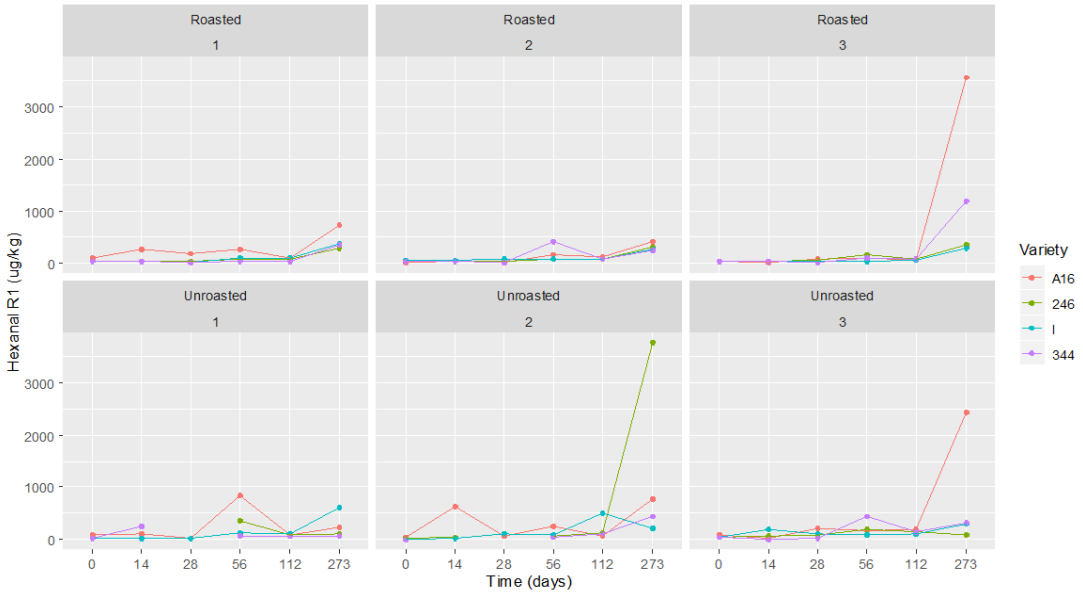


Figure 6: Effect of accelerated storage (30°C) and/or roasting on shelf-life as reflected by hexanal production. The unplanned final withdrawal time at 6 months was limited to two samples and showed very high variability.

From the results of the current storage trial, there was no indication or trend that kernels with lower saturated fat concentrations are more susceptible to rancidity than those with higher saturated fat concentrations. Similarly, the two cultivars that have been previously noted as producing high or low hexanal (by a different method), did not show any differences. Roasting however, was found to significantly reduce the level of rancidity during storage, which may impact on how nuts are stored, particularly those requiring roasting.

The absence of any clear-cut effect of fatty acid profile having an impact on shelf-life of macadamias in the present trial tends to indicate that within the range of fatty acid content assessed in the current trial, there was no significant effect. Where hexanal did increase, it tended to be random within a group of kernels, but did not appear to have any strong link to fatty acid profile.

Considering the above, it is probable that a slight reduction in saturated fat to 8-10%, is unlikely to impact on macadamia kernel shelf-life. It is possible that other factors such as vitamin E analogues may have anti-oxidant

activity, and greater impact on shelf-life. This however would require further investigation beyond the scope of the current project.

Consumer response

A nation-wide online survey was conducted of 211 nut consumers who purchase and eat macadamia nuts at least several times per year. The population consisted of a cross section of age and gender as well as constituting people from the majority of Australian States and Territories (no responses were obtained from those living in the Northern Territory) (Figure 7).

Drawing from the information gained in the preliminary literature review, a questionnaire was designed to gain insight into the purchase and consumption habits of macadamia consumers as well as their views on the health aspects (in particular, fat content) of macadamias. The questionnaire addressed the following topics: demographics, household information, nut consumption, macadamia consumption and purchase habits, importance of fats in the diet to the consumer and views and opinions on the proposed low saturated fat macadamia. The aim of this study was to identify whether current macadamia consumers would be open or averse to a new macadamia product on the market, one which could pose greater health benefits to the consumer and provide the Australian growers with a distinct point of difference on the future market (Figure 8).

In summary (Figure 9), macadamia consumers primarily purchase the macadamia nuts for their taste (83% of the population) however, other strong purchase motives include a convenient snack (45%) and the health benefits (43%). Despite fat content not being a primary concern of macadamia consumers (82% of the population not considering it of importance when make their purchase decision), a resounding 70% of the population would consider purchasing a macadamia with a lower saturated fat content. Of those consumers who would not consider purchasing lower saturated fat macadamias (n=66) the following reasons were of primary concern; happy with current product (64%), they might taste different (38%), not concerned with saturated fat content (29%) and they might be more expensive (20%). And for those consumers who would be willing to purchase lower saturated fat macadamias (n=145) the following reasons would prevent them from doing so; if they were more expensive (50%), if they tasted different (39%) and if their nutritional content was lower (20%).

On the whole current macadamia consumers would be open and willing to purchase macadamia nuts and nut products with lower saturated fat content. Health is of concern to a considerable proportion of the population indicating that a space may exist on the market with this consumer group as a primary target. However, it is important to note that taste is of utmost importance to a majority (83%) of the population and must therefore be paramount throughout the process.

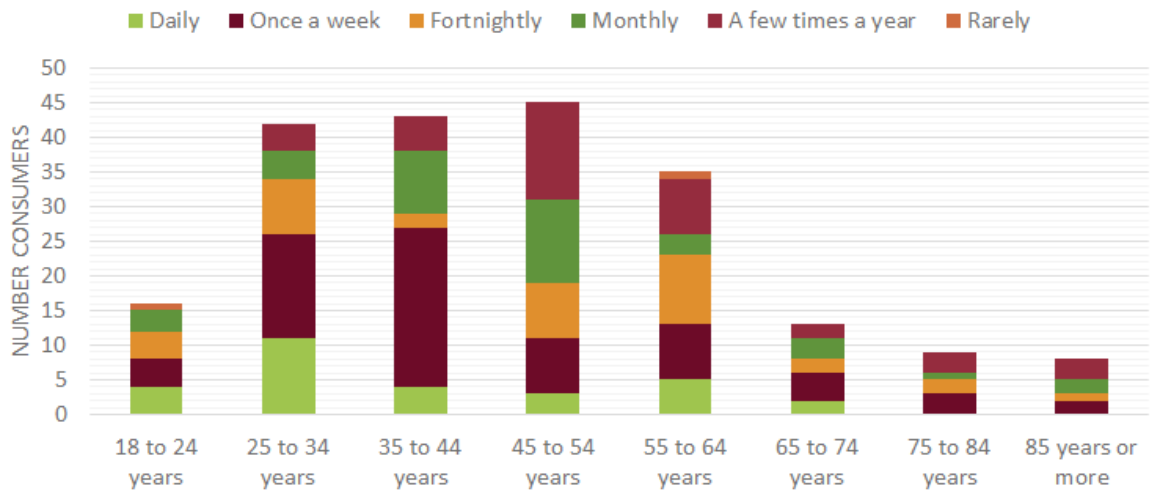


Figure 7: Macadamia consumers age versus frequency of consumption

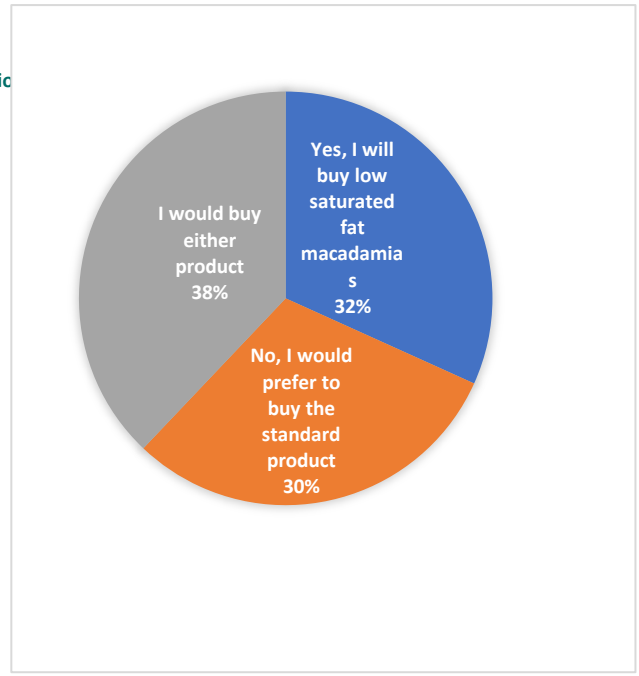


Figure 8: Low saturated fat macadamias purchase intention

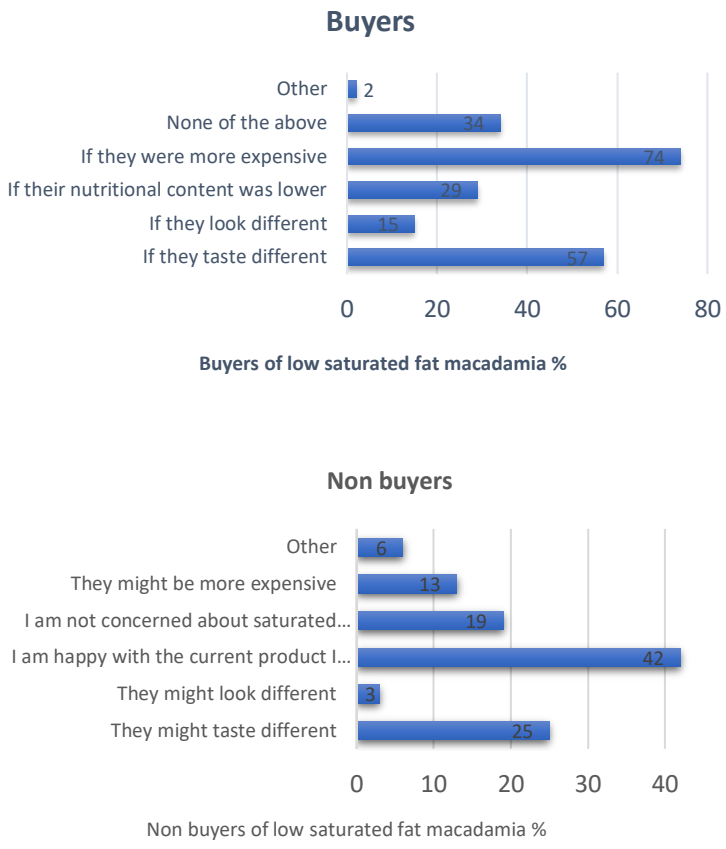


Figure 9: Barriers to purchase of low saturated fat macadamia nuts comparing those consumers who indicated they would buy versus those who would not.

Sensory profiling of promising lines of low saturated fat macadamia nuts

A range of whole macadamia nut samples (in-shell) were provided from Maroochy Research Station, Nambour consisting of a range of varieties known to express a range of high and low saturated fat content (12-17% - based on previous years data) (Table 4). An initial bench top tasting session (1 hr) was conducted (Figure 10) to determine if the sample of different fat content were different enough to warrant sensory profiling. Four experienced tasters were presented six of the nut cultivars for descriptive bench-top evaluation. The descriptors and comments provided by the tasters were taken into consideration in a subsequent discussion to determine if the samples warranted further sensory profiling using a trained panel.

Table 4: Macadamia nut samples collected (pooled) for potential sensory evaluation with a range of high and low saturated fat content

Variety	Saturated fat content (%)	Unsaturated fat content (%)	Monounsaturated fat content (%)	Polyunsaturated fat content (%)	Weight of nuts in shell	Estimated individual kernels
A16	**14.6**	85.4	83.7	1.7	insufficient	
A376	**13.9**	86.1	84.3	1.8	1050	143
A4	**14.6**	85.4	84.2	1.2	insufficient	
A447	**14.1**	85.9	84.4	1.6	1910	261
E	**15.0**	85.0	82.9	2.1	4650	635
HAES246	**16.3**	83.7	82.3	1.3	3872	202
HAES333	**15.2**	84.8	82.7	2.1	insufficient	
HAES791	**13.1**	86.9	85.2	1.8	insufficient	
HAES842	**16.4**	83.6	82.7	0.9	insufficient	
HAES849	**14.8**	85.2	84.2	1.0	insufficient	
K	**15.3**	84.7	82.9	1.8	2906	255
L	**14.1**	85.9	84.4	1.6	4885	667
M	**16.2**	83.8	82.7	1.2	1632	223
N	**15.0**	85.0	83.5	1.6	3416	466
O	**15.2**	84.8	83.8	1.1	insufficient	
R	**15.1**	84.9	83.2	1.7	3416	466
T	**14.4**	85.6	83.2	2.3	2798	382

While each variety was clearly distinctive in terms of sensory characteristics, it may be that a general difference between high and low saturated fat nuts can be detected in the nut texture. Higher saturated fat nuts were typically softer in texture and more buttery/oily in-mouth. Lower saturated fat nuts were typically dry, powdery and disintegrating in texture.

Samples HAES791 and HAES842 were clearly rancid and unpleasant and not suitable for further evaluation. Fortunately, these two varietals also had insufficient nut numbers to continue with further sensory testing.

Samples A447, HAES246, A376 and M were selected for further sensory evaluation with a trained panel.

Figure 10: Photograph of the six nut samples presented for bench-top sensory evaluation.

A trained sensory panel (13 assessors) were employed to rate sensory attributes for appearance, flavour and texture of the 4 varieties of macadamia nuts including A376 “low”, M “high”, A447 “medium” and HAES246 “high (Figure 11). Conventional descriptive analysis techniques were applied (Table 5).

Figure 11. Trained sensory panel assessing macadamia nut quality.

Table 5: Sensory attribute terms and definitions used during descriptive profiling of low saturated fat macadamia nuts

Aroma	
aroma intensity	The overall aroma intensity of the sample.
savoury	A savoury aroma, like sesame, dried onion, almost herby and like chilli flakes, capsicum.
raw nutty	Aroma of raw, unroasted nuts.
roasty / toasty	A roasted, toasted aroma.
cooked rice	Aroma of cooked rice
rancid	The pungent aroma of rancid oil or nuts.
'other' aroma	
Texture	
firmness	The firmness of the sample, its resistance to biting through.
crunchiness	The degree of crunchiness of the sample.
disintegrating	The disintegrating nature of the sample where it readily crushes into tiny powdery pieces.
dryness	The dry feeling of the sample on the surfaces of the mouth after chewing.
oily / fatty	The oily creamy sensation of the sample against the surfaces of the mouth when chewing down the sample.
sticky	The sticky or tooth stick of the sample in the molars.
Flavour	
sweetness	The sweet flavour of the sample.
savoury	A savoury flavour, like sesame, dried onion, almost herby and like chilli flakes, capsicum.
nutty	A nutty flavour, cashew, brazil nut, almond, chestnut.
nutskin / woody	A woody flavour like nutskin, hazelnut skin or walnuts, slightly bitter.
buttery / creamy	A fresh buttery and creamy flavour.
rancid	A pungent flavour like rancid oil or rancid nuts.
'other' flavour	
Aftertaste and mouth feel	
astringency / dryness	The dry, rough, astringent sensation remaining on the surfaces of the mouth.
oily mouth coating	An oily mouth coating sensation left in the mouth after swallowing, like that experienced after consuming dairy.
bitsy	The amount of bits remaining in the mouth after swallowing.

The results of the sensory evaluation are given in Figure 12 and Figure 13. The PCA explained 48% of variation in PC1 and PC2. Replication and panel performance was acceptable for the analysis. Significant differences were observed between the nut samples for aroma intensity, savoury aroma, firmness, crunchiness, disintegrating and oily/fatty texture and oily mouthcoating aftertaste.

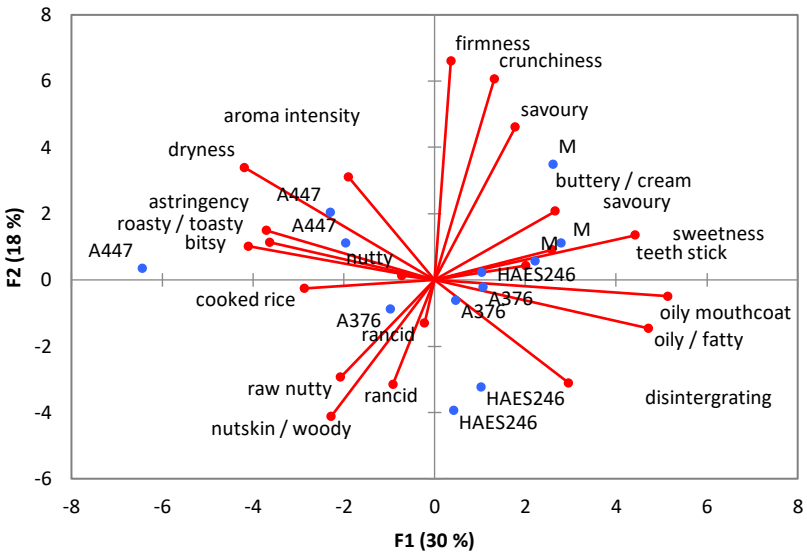


Figure 12: PCA of macadamia nuts evaluated (x 3 replicates x 13 panellists)

Bench-top evaluation indicated that "low" saturated fat nuts were dry and more brittle and "high" saturated fat nuts were more oily/creamy in texture, less dry and less brittle. However, during formal sensory evaluation

with the trained panel no important difference between high and low saturated fat nuts were observed. A major limitation to this study was the insufficient number and quality of suitable quality samples supplied, and the low number of representative varieties for each category.

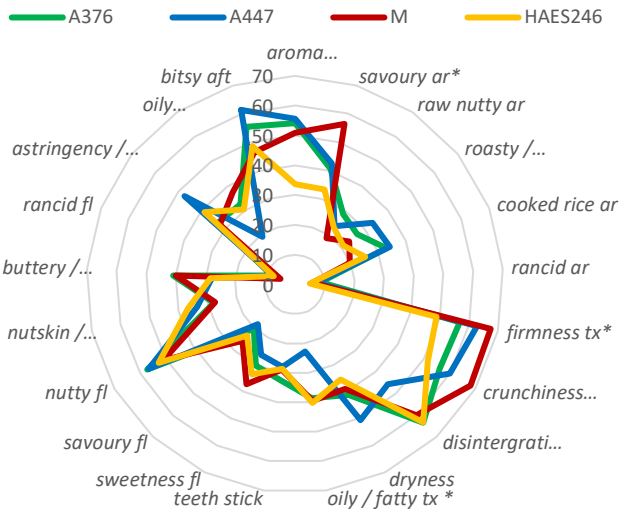


Figure 13: Cobweb plot of macadamia nuts evaluated (x 3 replicates x 13 panellists)

Macadamia consumer-sensory summary:

- Macadamia industry is generally positive about the concept but wouldn't want the low saturated fat nut to taste different.
- In Australia, younger consumers (25-45) are more likely to be regular consumers of macadamia nuts (daily or weekly consumption).
- 70% of consumers said they would buy a "low sat fat macadamia nut", but not if it was more expensive, or tasted different.
- Those consumers who would not buy reported they were concerned it would not taste the same.
- In terms of sensory properties, cultivar differences were more important in driving sensory differences than saturated fat content
- There was no clear difference in taste or texture, however, the sensory study was limited by sample availability and quality.

Industry feedback

The sensory and consumer scientists from the Naturally Nutritious project initially attended the 2016 Macadamia Industry conference, to discuss the concept of low-saturated fat macadamia with members of the macadamia industry from different areas of the macadamia production chain - growers, processing companies, marketing, researchers, etc.

In general, those industry members approached had a positive response towards the possibility of a low saturated fat macadamia product, as they were aware of current consumers' choices/trends towards healthier products. However, their views also supported the opinion of the surveyed macadamia consumers, as to a low-saturated fat macadamia product not tasting different to current macadamias available on the market.

Some sectors of the macadamia industry were concerned that the development of a reduced-saturated fat macadamia may reflect badly on the remainder of the 'non-improved' industry, especially prior to reduced-saturated fat orchards being established. This advice was taken on board by the project team. A larger meeting was held via Zoom in 2020 to get industry feedback, in which a wider range of the macadamia industry were invited. It was suggested that the consumers' perceived health benefit of reducing saturated fat was likely to

be much stronger than the benefits of increasing palmitoleic acid. In addition, although label health claims can be made for reduced saturated fat, it is not currently possible to do so for the omega-7, palmitoleic acid.

In addition, it was suggested that further work on Vitamin E analogues present in macadamia could be also pursued, due to their combined benefit of both health and extending the shelf-life of kernels due to the compounds antioxidant capacity. Finally, the recent novel results from the Naturally Nutritious project that macadamia nuts may actually give gut health benefits should be pursued in the future.

Outcomes and Recommendations

Technical feasibility would indicate that a reduced saturated fat macadamia is achievable using selected paired plantings of existing macadamia cultivars, owing to the partial influence of the paternal pollen parent on the fatty acid profile of the maternal nut kernel. Similarly, paired plantings are also possible for enhanced omega-7 concentrations. A combination of reduced saturated-fat with enhanced omega-7 is possible, but not essential to achieve either outcome. Reduced saturated fat did not appear to alter flavour or decrease kernel shelf-life within the range tested.

Consumer evaluation indicated that 70% of those evaluated were interested in purchasing reduced saturated fat macadamia nuts. Issues that would reduce purchase would be if the flavour was changed, or the price was higher. Although consumers know about saturated fat, little is probably known about health benefits of omega-7, so the latter may not necessarily aid in marketing until more information is available about the health benefits of omega-7.

Despite the technical feasibility and positive consumer response, some sectors of the macadamia industry were concerned that the development of a reduced-saturated fat macadamia may reflect badly on the remainder of the ‘non-improved’ industry, especially prior to reduced-saturated fat orchards being established. It is considered strategic, however, due to the potential competitive advantage of a reduced-saturated fat macadamia in the global marketplace, to continue development of a reduced-saturated fat macadamia, as a strategic development for the future global competitiveness of the Australian Industry.

The possibility exists for entrepreneurs or breeding programs to develop this area as a niche product, especially in regard to international competition in the future. The novel findings of the potential impact of macadamia having a positive impact on gut health should also be pursued.

Purple Sweetcorn – anthocyanin-pigmented supersweet sweetcorn

Background

Health issues and product visual differentiation

Although purple sweetcorn does not commercially exist, starchy purple maize has existed in parts of South America (Peru, Bolivia) for thousands of years, where it has been traditionally used as the purple colourant for the beverage, 'chicha morada' and the dessert, 'mazamorra morada'. The compound responsible for the purple colour of purple maize is anthocyanin, with the principal anthocyanins being cyanidin glycosides.

The effects of purple maize on blood pressure regulation have been studied in both animal models and humans. In an animal study, spontaneously hypertensive rats administrated purple corn colourant at a dose of 7.4 mg anthocyanin/kg body weight twice daily exhibited a significant inhibition of increase in systolic blood pressure after just 8 d (Toyoshi and Kohda, 2004). Similarly, another study feeding spontaneously hypertensive rats with purple corn rich in anthocyanins for 15 weeks reduced systolic blood pressure significantly lower than that of the control group (Shindo et al., 2007), suggesting that purple maize anthocyanin has an anti-hypertensive effect on hypertensive animals.

A subsequent clinical study assessing the impact of purple maize extract on blood pressure in mild-to-moderate hypertensive humans showed that over a three-week period, purple maize extract had a beneficial effect on reducing systolic and diastolic blood pressure (from139/88 to 132/81 mm Hg) in early-stage hypertension patients, regardless of age, gender, body mass index, or initial average blood pressure reading (Finkel et, al. 2013).

The majority of sweetcorn is yellow or white in colour, owing to a lack of anthocyanin biosynthesis in the pericarp or aleurone tissue of the kernel. This is primarily due to the very close genetic linkage between the supersweet mutations, *brittle1* and *shrunken2*, and non-functional alleles of the *A1* and *A2* (*Anthocyaninless1*, *Anthocyaninless2*) anthocyanin biosynthesis genes. Development of a supersweet purple sweetcorn therefore required breaking this genetic link, which has occurred in parallel with the Naturally Nutritious project.

Because purple sweetcorn is a novel product, consumer assessment was made initially made of the concept and using photographs of various forms of the *brittle1* purple sweetcorn, which was more advanced in its development. This was later followed by taste and appearance evaluation, once more product became available.

In conjunction with this, testing was conducted of published genetic markers to aid in future breeding of purple sweetcorn, as well as fundamental studies of the effect of harvest maturity, postharvest storage and cooking on the novel product.

At the same time, industry feedback was periodically obtained in regard to the concept, and physiological and agronomic issues that may impact on growing and marketing this product.

Technical feasibility

Germplasm & Introducing supersweet mutations into a high-anthocyanin background

The development of purple-pericarp sweetcorn was initiated in 2014 before the start of the Naturally Nutritious project, and development has continued in parallel with the current project.

Initial purple sweetcorn accessions were developed by combining the high-anthocyanin attributes of purple waxy maize with a white sweetcorn based on the *brittle1* supersweet genetic mutation. The challenge of developing a purple supersweet sweetcorn is high, because the brittle1 mutation is very closely positioned (7 cM apart) to an anthocyanin biosynthesis gene (*anthocyaninless2, a2*), and this linkage must be broken to produce anthocyanin pigment in a supersweet background. The original decision to use the *brittle1* mutation, rather than the more widely used *shrunken2* supersweet mutation was due to the knowledge that the former would be easier to accomplish than the latter, as the *shrunken2* gene is even more closely situated with another anthocyanin biosynthesis gene, *anthocyaninless1* (*a1*, 0.1 cM apart).

Despite this challenge, it was decided that the benefits to the Australian industry of using the *shrunken2* supersweet mutation (later confirmed by industry) were high enough to attempt breaking this extremely close genetic link. This was conducted by combining a purple-pericarp maize with a white sweetcorn based on the *shrunken2* supersweet genetic mutation. Subsequently, purple-pericarp sweetcorn accessions, based on both the *brittle1* and *shrunken2* supersweet mutations have been successfully developed.

The larger part of the study conducted on purple sweetcorn within the current Naturally Nutritious project is based on the *brittle1* purple sweetcorn, due to its earlier availability during the course of the project.

Anthocyanin profile and impact of harvest maturity on anthocyanin concentration in purple sweetcorn

Anthocyanin purple pigments are accumulated in the outer layer (pericarp) of the sweet-corn kernel, while the interior (endosperm) is white. In general, as the kernel increases in maturity, anthocyanin concentration also increases. This coincides with a gradual spreading of purple pigmentation from the tip to the base of the kernel. Colour zones are defined, so an immature kernel looks something like an eyeball with a purple iris. By contrast, a mature kernel is fully purple.

Depending on the extent of spread of anthocyanin across the kernel surface, it is possible to give the impression of purple and white rows at eating stage, especially if the underlying cob is also purple in colour (Figure 1).

The anthocyanin profile of purple and reddish-purple accession variants of 'supersweet' *brittle1* sweetcorn 'PB12.5–2-1' were found to contain twenty anthocyanins. These consisted primarily of cyanidin-, peonidin- and pelargonidin-based glucosides (Table 1). While the predominant anthocyanin compounds in the purple accession consisted of Cy3MG (33.6%) and Cy3DMG (25.7%), the compounds in the reddish-purple accession predominantly consisted of Pg3MG (29.3%), Cy3MG (16.7%) and Pg3DMG (15.3%).

Table 1: Individual anthocyanins and total anthocyanin content (TAC) in the purple-pericarp sweetcorn accession at different kernel maturities (DAP = days after pollination). All experiments were performed in triplicate (n = 3) for intra-cob variation (technical replicates), and in replicates of n = 3–6 for inter-cob variation biological replicates).

Total

Anthocyanins		Anthocyanin content from 20 to 36 DAP (mg/100g FW)						
		20	23	24	25	28	32	36
1	*Cy3G*	2.11a*	3.96b	5.73c	7.15cd	7.65de	8.65de	9.06e
2	*Cy3MG*	7.14a	9.92a	14.20b	14.68bc	16.99bc	17.33bc	18.38c
3	*Cy3DMG*	5.57a	9.57ab	12.14bc	14.22bcd	16.54cd	17.52d	18.58d
4	*Pg3G*	0.02a	0.14ab	0.21ab	0.21ab	0.24b	0.23b	0.32b
5	*Pg3MG*	0.45a	0.56ab	0.92bc	0.88abc	1.06c	1.23c	1.28c
6	*Pg3DMG*	0.05a	0.29ab	0.31ab	0.42b	0.45b	0.54b	0.54b
7	*Pn3G*	0.66a	0.71ab	1.16abc	1.20bc	1.43cd	1.42cd	1.80d
8	*Pn3MG*	1.75a	1.79a	2.91b	2.77b	3.45bc	3.42bc	4.07c
9	*Pn3DMG*	1.89a	2.08a	2.72a	2.74a	3.04ab	4.24bc	5.04c
Moisture (%)		81.20a	78.01b	75.42c	73.90c	70.61d	69.32de	67.03e
TAC (FW)		19.68a	28.83b	40.43c	44.27cd	50.85d	54.59e	59.07e
TAC (DW)		104.68a	131.1ab	164.48bc	169.62bc	173.02c	177.93c	179.16c

anthocyanin concentration (TAC) in both accessions was found to increase with increasing kernel maturity from 20 to 36 DAP, although optimum eating quality, as judged by moisture content, was between 24 and 28 days after pollination. Increase in anthocyanin concentration appeared to be at least partly related to increased expansion of anthocyanins across the surface of the kernel, which strongly impacts on the appearance of the cob. Total anthocyanin content of purple corn ranged from 20-60 mg cyanidin-3-glucoside equivalents/100 g fresh weight, depending on genotype, with darker cobs having higher anthocyanin levels. Most cobs contained the anthocyanins cyanidin-3-glucoside (purplish), pelargonidin-3-glucoside (reddish), peonidin-3-glucoside and their respective malonate derivatives.

Although the concentration of anthocyanin in purple and reddish-purple pericarp sweetcorn at optimum eating stage is less than fully mature kernels of purple maize, it was still higher than many anthocyanin-rich fruits and vegetables and could be considered a valuable addition to increasing anthocyanin intake in the western diet.

Figure 1: Increase in purple pigment coverage with increasing kernel maturity from 20 to 32 DAP.

Development of genetic markers for breeding purple sweet corn

The original parents of purple sweetcorn (purple-waxy, white-brittle), a fixed purple sweetcorn accession, and a non-related purple Peruvian maize accession were assessed using the candidate gene markers, purpleplant1 (*pl1*) and booster1 (*b1*). Both genes encode transcription factors that are required for the production of anthocyanin in vegetative tissue of corn. As anthocyanin in our purple sweetcorn is produced in the outer layer of the kernel (pericarp), this is maternal tissue, and therefore is under the same control as vegetative tissue such as the stem and leaves. Initial assessment indicated that the purple-waxy parent was heterozygous for *pl1*, but homozygous for *b1*. The white-brittle parent was homozygous for a non-functional *pl1*, but it was inconclusive if it was homozygous for a functional *b1*, as the band-size difference is very small. Subsequent assessment of a fixed purple sweetcorn accession and Peruvian purple maize indicated that both were homozygous for a functional *pl1*. The purple sweetcorn accession was also homozygous for a functional *b1* allele, although the Peruvian purple maize appeared to segregate for this gene, which would explain why a small number of plants produce red-tannin (phlobaphene) pigmented cobs. As this latter pigmentation has not been observed in any of the purple sweetcorn breeding lines, this may indicate that all purple sweetcorn breeding accessions are homozygous for functional *b1*.

Subsequent testing of two segregating purple/white sweetcorn lines confirmed that both had functional *pl1* and *b1* transcription factors. The reason for the presence of white cobs being produced in the purple sweetcorn program is to segregation of homozygous non-functional anthocyaninless2 (*a2*) which was originally closely linked with the super sweet *bt1* gene. A potential genetic marker for the *a2* gene has been identified

but did not produce a robust result.

Subsequent testing of the *pl1* and *b1* genetic markers, and a marker for *anthocyaninless1* (*a1*) has been made with our purple-pericarp sweetcorn based on the *shrunken2* supersweet mutation. It was confirmed that the purple sweetcorn accession was homozygous dominant for *a1* and *pl1* genes as of purple maize parent, but it remained unclear using this marker if it was heterozygous for the *b1* gene.

To further optimize these genetic markers for use in purple sweetcorn breeding (outside the scope of Naturally Nutritious), genetic sequencing has been conducted to identify the exact allele differences for the transcription factors, *pl1* and *b1*, and the biosynthesis alleles of *a1*. This will provide exact genetic markers for rapid identification of genotypes in subsequent purple sweetcorn breeding.

Impact of postharvest storage on anthocyanin content

Although there are no published reports on the impact of storage temperature on anthocyanin content in purple sweetcorn, storage temperature has been reported to variously affect the anthocyanin and total phenolic content of other red and purple fruit and vegetables. As the concentration of anthocyanin is likely to impact on the visual appearance of purple-pericarp sweetcorn, it was prudent to investigate how postharvest conditions may affect this concentration.

Despite postharvest storage temperature having been found to have little impact on the change of pigment levels in yellow sweetcorn (Calvo and O'Hare, 2020), no such evidence exists with purple-pericarp sweetcorn. This study investigated for the first time the effect that postharvest storage on anthocyanin concentration, and the subsequent visual appearance of purple-pericarp sweetcorn. The concurrent impact of storage temperature on changes in sugar content, an important organoleptic parameter in sweetcorn, was also investigated.

Cobs of purple-pericarp sweetcorn (PPS), based on the *brittle-1* (*bt1*) supersweet mutation, were harvested in autumn 2018 at the Gatton Research Facility, The University of Queensland, QLD, Australia. Individual plants grown under uniform field conditions were manually self-pollinated to exclude foreign pollen. Cobs of each accession were harvested at increasing physiological maturity at 23, 26 and 31 days after pollination (DAP), correlating to early, normal, and late stages of commercial sweetcorn harvest maturity.

Cobs at the three maturity stages above were randomly divided into two batches (three cobs per maturity stage) for storage at 4 °C and 23 °C, respectively. For an initial baseline sample (0 day), two rows of kernels from each cob from six biological replicates of PPS were removed and immediately snap frozen using liquid nitrogen in order to terminate further metabolic activity. The remaining cobs were placed in loosely-sealed plastic barrier bags (6 bags per maturity stage) and stored at 4 °C (batch-1) and 23 °C (batch-2), at a relative humidity of 90%.

Cobs were withdrawn after 1, 3, 7 and 14 days at 4 °C and 23 °C. A two-row sample of kernels was removed and cryo-milled. Aliquots of the frozen powdered sample were immediately stored at -80 °C for subsequent analysis of total and individual anthocyanins, total soluble solids and individual sugars, moisture content, and total phenolic compounds.

Representative photos taken from 26 DAP cobs at different storage periods show changes in colour coverage and colour intensity of PPS with increased storage period. For samples stored at 23 °C (Figure 2b), the coverage of purple pigment gradually spread from the stigma end of the kernel towards the base of the kernel, eventually covering the entire kernel surface. This observed change potentially indicated that anthocyanins were continuing to be biosynthesised/accumulated during storage at this temperature. In contrast, the visual appearance of PPS stored at 4 °C (Figure 2a) did not show any changes in colour coverage and intensity, which may indicate that storage at 4 °C may be sufficient to inhibit further anthocyanin biosynthesis.

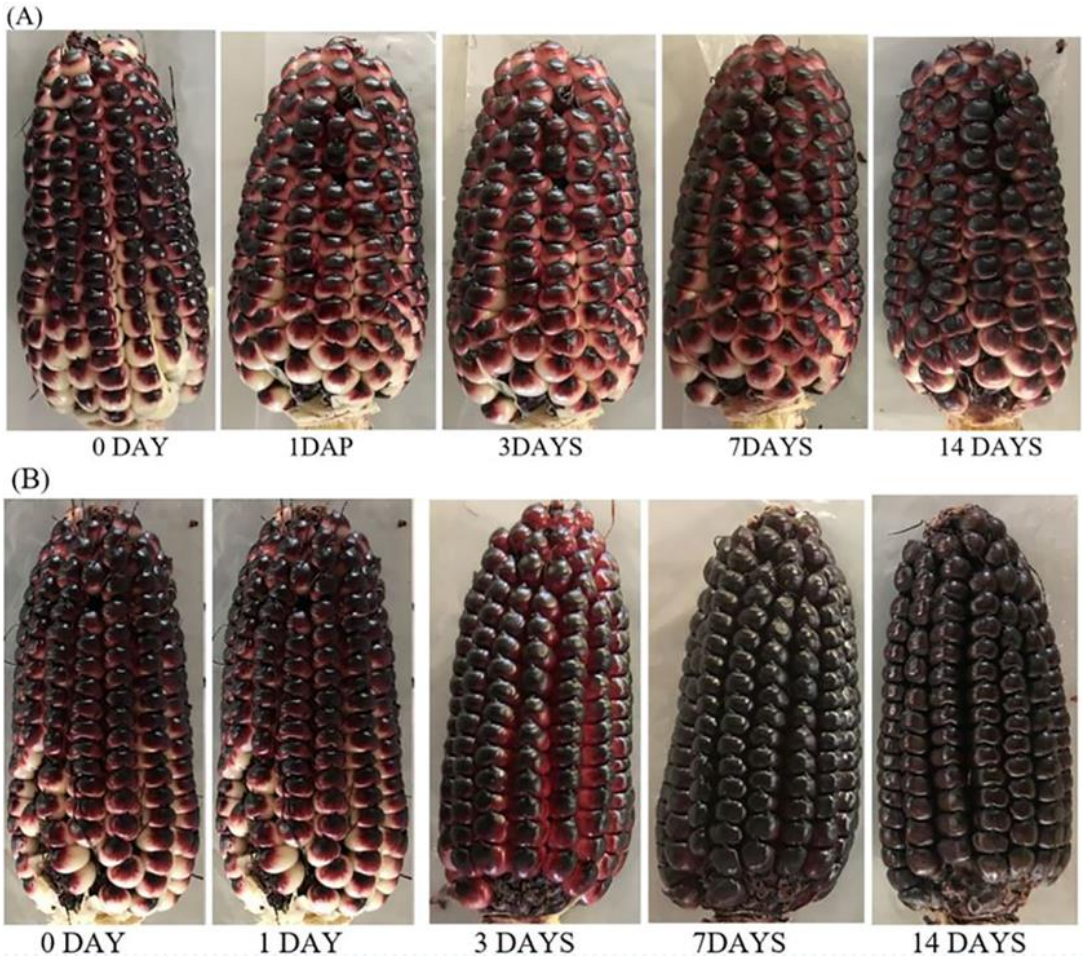


Figure 2: The effect of postharvest storage on visual appearance of PPS (26 DAP) stored at 4 °C (A) and 23 °C (B).

Total anthocyanin concentration (TAC) was observed to increase significantly ($P<0.05$) with increasing harvest maturity, with overall means of 53.4, 62.7, and 75.3 mg/100g FW, for 23, 26, and 31 DAP, respectively. The significant increase in TAC of the PPS kernels with maturity indicates that anthocyanins are continuing to be synthesised as kernel maturation progressed from 23 DAP to 31 DAP. These findings are in agreement with previous studies reporting an increase in anthocyanin concentration with increasing kernel maturity of purple maize. The lack of increase in anthocyanin accumulation during two-weeks storage at 4 °C indicated that tissue-specific accumulation of anthocyanins in kernel pericarp was largely inhibited at 4 °C, in comparison to 23 °C.

Although harvest maturity had an initial impact on TAC, during storage all three harvest maturities behaved similarly, with all increasing in parallel at 23 °C (Figure 3B) or remaining unchanged at 4 °C (Figure 3A). This would indicate that different kernel maturity (within the range studied) does not affect the further rate of accumulation of anthocyanin during storage.

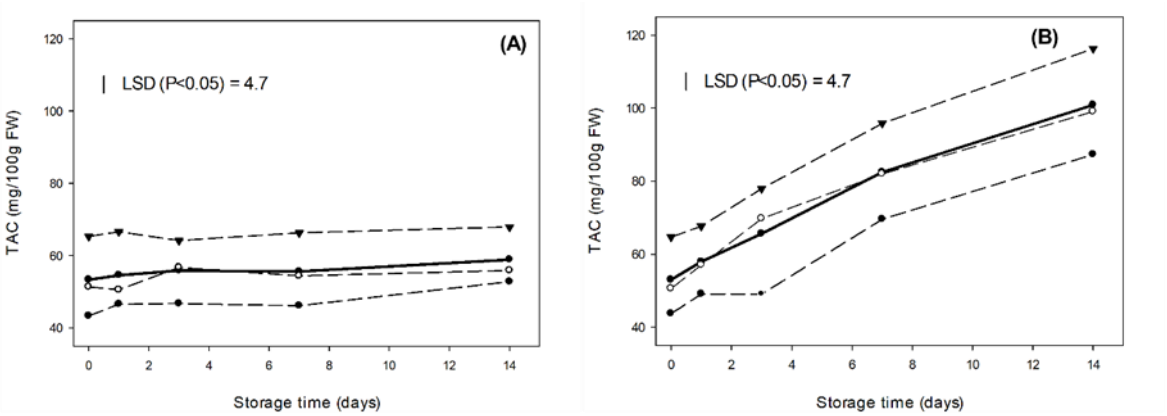


Figure 3: The effect of storage time on TAC at 4 °C (A), and at 23 °C (B). All maturities (23, ●; 26, ○; 31 DAP, ▼) behaved similarly, with the dashed plot-lines shown to indicate the parallel response of all cob maturities within each storage temperature. The bold plot-lines represent mean maturity, with the vertical bar representing the LSD (P<0.05) applicable to these means

In contrast to the continual accumulation of anthocyanin in PPS during storage at 23 °C, storage had a negative effect on sugar content of PPS (26 DAP) at both temperatures (Table 2 and Figure 4C). The concentration of all sugars of the PPS kernels decreased significantly ($p<0.05$) from approximately 125 mg/g FW at day 0 to below 30 mg/g FW at day 14 at 23 °C.

In contrast, total sugar concentration still remained at the same level (over 120 mg/g) for the first 7 days of storage, before decreasing significantly to 113.5 mg/g FW at day 14 at 4 °C. These findings support previous studies, which reported storage at low temperatures maintaining better sugar content in sweetcorn kernels (Shao and Li, 2011; Vigneault et al., 2007). The results also show that the summed sugar concentration of PPS (125 ± 6.9 mg/g FW) (Table 2) was initially significantly higher than other sweetcorn cultivars, including 'Aussie Gold 12' (su, 26 mg/g FW), 'Rosella 425' (su, 23 mg/g FW), and 'Sucro' (sh2, 78 mg/g FW).

Table 2: Individual sugars and total sugar content of PPS at 26 DAP during two-week storage at 4 °C and 23 °C (n=6).

Days of storages	Sugar concentrations (mg/g FW)							
	Fructose	Glucose	Sucrose	Maltose	Fructose	Glucose	Sucrose	Maltose
	23 °C				4 °C			
0	20.2 ± 1.4a	24.0 ± 1.7a	67.1 ± 6.8a	13.6 ± 1.4a	17.7 ± 2.2a	20.7 ± 2.3a	70.7 ± 8.1a	25.3 ± 1.8a
1	14.6 ± 2.1b	18.1 ± 2.1b	54.7 ± 6.3b	11.1 ± 1.6ab	17.3 ± 2.1a	22.5 ± 2.3ab	71.1 ± 2.8a	18.2 ± 1.5ab
3	11.9 ± 1.9c	16.5 ± 1.9b	34.2 ± 9.9c	9.7 ± 1.7b	20.0 ± 3.0ab	24.8 ± 4.0ab	66.9 ± 3.9a	13.8 ± 1.7b
7	6.0 ± 0.4d	13.0 ± 1.3c	8.1 ± 3.6d	10.1 ± 1.5b	22.0 ± 2.7ab	29.1 ± 3.4b	60.4 ± 6.0ab	14.0 ± 2.8b
14	5.4 ± 1.5d	13.9 ± 2.2bc	4.4 ± 1.8d	6.2 ± 2.3c	22.0 ± 1.7b	31.5 ± 4.8b	48.7 ± 7.6b	11.3 ± 3.0b

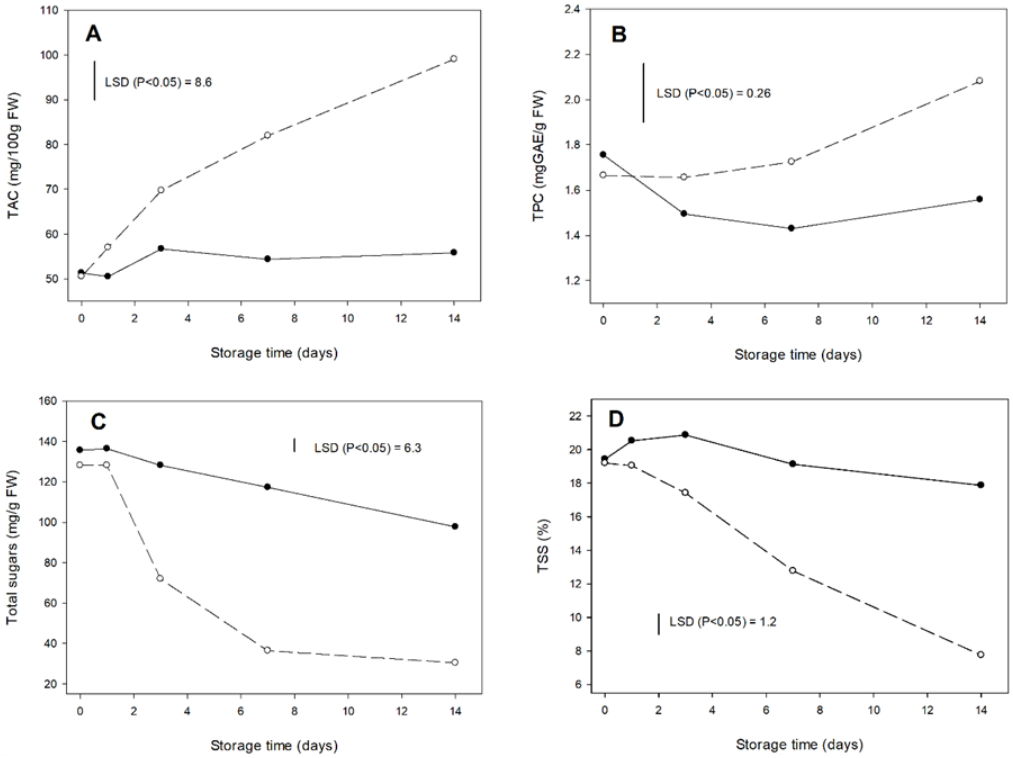


Figure 4: Effect of temperature (4 °C •, 23 °C ◦) and storage time on (A) total anthocyanin concentration (TAC), (B) total phenolic concentration (TPC), (C) total sugar concentration, (D) total soluble solids (TSS in purple sweetcorn at 26 DAP. Least significant difference (P<0.05) is indicated by a vertical bar.

In general, the present study indicated that the biosynthetic pathway for anthocyanin is still operative during post-harvest storage at 23 °C over the range of maturity stages tested. Although anthocyanin concentration continued to increase at 23 °C, it remained unchanged when stored at 4 °C. This increase of anthocyanin content was visually obvious by an increase in the spread of anthocyanin pigmentation across the kernel surface.

Storage at low temperatures (such as 4 °C, which is common in domestic refrigeration) is important for delaying a decrease/hydrolysis of sugar components and thereby maintaining the sweetness of PPS. Although storage at higher temperatures could potentially be used to increase the anthocyanin concentration in PPS, this would have to be balanced with a concurrent decline in sweetness. Decline in sweetness, due to the metabolisation of sugars during respiration, occurs in PPS when stored under ambient temperatures (23 °C), and is an equally important determinant of acceptability to sweetcorn consumers.

Effect of cooking on anthocyanin

Four common cooking methods were evaluated on purple sweetcorn (based on the *brittle1* supersweet mutation) following harvest at the Gatton Research Facility. Cooking methods included microwave cooking, steaming, boil in the bag, and pan-frying kernels.

Microwave cooking (1000w): Ten cobs were microwaved for 10 minutes.

Steaming: Ten cobs were steamed for 10 minutes in the Convotherm steam oven.

Boil in the bag: Ten cobs were vacuum-sealed individually in vacuum bags and boiled for 10 minutes.

Pan Frying kernels: Kernels were removed by hand as whole kernels from 10 cobs of corn. Kernels were mixed together into sub-samples. Two tablespoons of oil was heated,and the frypan covered with a single layer of kernels and fried for 5 minutes.

Immediately after cooking treatments, cobs or kernels were placed in ice water to stop any further cooking.

Whole intact kernels were subsequently removed by hand for subsequent analysis.

Total anthocyanin concentration (TAC) was affected differently by the four cooking methods, with microwave cooking causing the greatest decline (40%) in anthocyanin and boiling in bag and steaming causing less degradation at 20% (Table 3). Pan-frying caused least degradation, although the cooking time was one half that compared to the other treatments to avoid charring the kernels.

Table 3: Impact of cooking method on total anthocyanin concentration (mg/100g FW) in purple sweetcorn.

Treatment	TAC	Decline
Uncooked	42.1	
steamed	33.4	20.7%
pan-fried	36.1	14.3%
boil in bag	34.0	19.2%
microwave	25.3	39.9%

In a subsequent trial, microwave cooking time was reduced to 4-5 minutes, as the 10-minute microwave time was considered possibly excessive. In this trial, a purple sweetcorn accession and a reddish-purple sweetcorn accession, harvested at two different maturities (21 and 24 DAP) were assessed.

Analysis of the kernels before and after cooking indicated a variable loss in anthocyanin pigment concentration by microwave steaming, ranging from 16 to 28% (Table 4), which was less than that measured in the previous trial using longer microwave times. The initial anthocyanin concentration of the purple accession was higher than the reddish-purple accession, and anthocyanin concentration was initial higher in kernels of higher harvest maturity (24 DAP). The reduction in anthocyanin was not obvious from visual inspection.

Table 4: Impact of microwave cooking (4-5 minutes) on total anthocyanin concentration (mg/100g FW) of purple and reddish-purple sweetcorn of two different harvest maturities.

Sweetcorn sample	TAC	Decline
purple (uncooked, 24 DAP)	52.4	
purple (cooked, 24 DAP)	37.6	28.1%
purple (uncooked, 21 DAP)	40.6	
purple (cooked, 21 DAP)	33.9	16.4%
reddish-purple (uncooked, 24 DAP)	27.3	
reddish-purple (cooked, 24 DAP)	22.7	16.9%
reddish-purple (uncooked, 21 DAP)	23.3	
reddish-purple (cooked, 21 DAP)	17.2	26.1%

Pigment appeared to not ‘leak’ into adjacent non-pigmented areas of the kernel. This contrasts with other anthocyanin-containing vegetables (e.g., purple carrot) which lose significant amount of pigment due to contact of cut surfaces with cooking water. Some slight leakage of sweetcorn anthocyanin into the fluid in the base of the cooking vessel was observed, although whether this came from the cut ends of the cob has not been confirmed.

Consumer response

Purple sweetcorn was initially evaluated in 2017 using visual images of a number of visual variants of purple sweetcorn to assess consumer focus group (37 participants) response (Figure 5).

Figure 5: Purple sweetcorn visual variation assessed by the initial focus groups. Yellow sweetcorn on the extreme left (cob 1) was included as a control.

In response to the question 'What are your initial reactions/ first opinions regarding the corn cobs you see in the photo provided?', the following positive and negative reactions were recorded:

Positive comments:
- Juicy, like a mulberry (cob #8)
- Sweet, like a pomegranate
- Good visual appeal
- Uniform samples most appealing
- Darker colours more appealing
- Look like a vegetable with added nutritional value
- Look like pomegranate seeds (cobs #5, #6)

Negative comments:
- Looks like snakes skin/ googly eyes (cob #4)
- Non-uniform samples not visually appealing
- The white patches look like mould
- Are they genetically modified?
- Would look unsightly when stuck in teeth
- Would need to try before buying
- Do they have thick skin?
- Dark colours look almost rotten
- Those with white patches look unripe

When asked to rank the cobs in order, from least to most appealing, the following responses were recorded:

Most appealing:
- #1 (yellow), #7 and #8 most appealing
- #1 (yellow) is the most familiar sample and therefore people feel more comfortable eating and preparing it
- #7 looks like pomegranate seeds, juicy and sweet and is uniform in appearance
- #8 is consistent, looks ripe and juicy and the healthiest of them all due to darkest colour

Least appealing:
- #2 and #4 least appealing
- #2 looks raw and unripe. It doesn't look healthy and the kernel pattern is too obscure, like eyeballs.
- #4 looks like snakeskin, fish scales and eyeballs. Looks least like traditional corn kernels.

When asked about indicating how much you would be willing to pay for one cob of your preferred corn, 36 of the 37 consumers were willing to pay more (on average 35% more) for a cob of purple corn than for a cob of regular yellow sweetcorn. Consumers indicated that it is the norm for novelty products to carry a higher price tag. Of the one consumer that didn't, he would be willing to pay more should he consider the product to be premium after tasting it.

In regard to cooking at home, the majority of consumers would likely prepare purple corn in the same manner they currently prepare yellow sweetcorn, although concerns were raised over boiling due to the potential leaching of colour. Salads were positively considered to be enhanced by the purple kernels, as they would add a "sparkle". The appearance of the purple corn was highlighted as a positive when preparing food, in that the corn would enhance colours on the plate and make dishes "pop", "look healthier" and "add contrast" Participants also responded to the following knowledge statements, that would potentially accompany purple sweetcorn:

Origin: 'Purple sweet corn is nonGMO and has been naturally developed from combining the sweet flavour of sweet corn with Peruvian purple maize, a traditional food of Peru (and the Incan Empire). Purple sweet corn tastes exactly the same as standard yellow or white sweet corn, but has a very different appearance, being purple with a white centre':

- Interesting
- Naturally developed and non-GMO is a huge positive
- Advertising linked back to Peruvian roots would be beneficial to my purchase intent
- More likely to purchase with the knowledge it has the same taste
- Would prefer them to taste different, would be only willing to pay the same as yellow now
- Disappointed if the kernel centre is colourless

Health: 'The purple sweet corn contains a pigment called anthocyanin, which has been linked to lowering blood pressure and cholesterol levels. Normal yellow sweet corn does not contain this pigment, so has no additional health value':

- Many consumers would pay a higher price due to enhanced health aspects
- Very interesting
- Information very important to encourage the consumer
- Brilliant, especially as they will have the same great taste
- Need to be affordable so people can benefit from the added nutrition
- Not totally convinced by health benefits – how much do you need to consume?

Practical considerations: 'Purple sweet corn contains anthocyanin, which is water soluble, similar to the pigment in beetroot and blackberries. Unlike yellow corn, it can stain your fingertips purple if you pick it up with your hands (can be washed off, not permanent!). If you cook your sweet corn in water, it may also bleed a little into the water from any cut kernels (e.g. at the end), colouring the water':

- Pleased that colour doesn't leach unless damaged
- Colour could be used as a dye in sauces/soups
- Concerns over stains when eating off the cob with teeth

From the initial consumer focus group, purple sweetcorn was generally approved of as a novel product, with an overwhelmingly positive response from consumers. It is considered that education regarding the nutritional benefits of purple sweetcorn would be also important for consumers. From the feedback, purple sweetcorn could be sold at a premium price point, akin to broccolini.

Eating quality trial

In 2018, following the initial focus group evaluation above, a ten-person consumer panel was evaluated in regard to visual appearance and eating quality of purple and reddish-purple sweetcorn (based on the *brittle1* mutation) at two stages of kernel maturity (21 and 24 DAP), and a commercial yellow control (Figure 6).
It should be noted, that the timing of the focus group assessment necessitated cool storage of the purple sweetcorn samples for 11 days at 7-8°C, during which time kernel sugar levels, and subsequently sweetness, may have declined for these samples.

Consumers assessed the sweetcorn samples for visual appearance (uncooked and cooked), flavour, texture, and overall acceptability on a hedonic 1-9 scale (1 = lowest, 9 = highest). The mean ratings are shown in Table 5.

Table 5: Consumer quality assessment of yellow, purple and reddish-purple sweetcorn at two stages of harvest maturity.

Accession	Visual (uncooked)	Visual (cooked)	Flavour	Texture	Overall acceptability
Yellow (control)	7.7c	8.3b	7.9c	6.9a	7.6c
Purple (24 DAP)	6.2b	7.1a	5.9ab	5.9a	5.7a
Purple (21 DAP)	5.2ab	6.4a	5.8a	6.1a	6.2ab
Red-purple (24 DAP)	4.6a	6.6a	6.9bc	6.3a	6.3abc
Red-purple (21 DAP)	4.1a	6.2a	7.9c	6.6a	7.3bc

Visual (uncooked): Assessors tended to prefer the purple-coloured cobs over the reddish-purple-coloured cobs. Yellow cobs were the most preferred, potentially due to consumer familiarity. Although a trend existed for preference towards the more mature cob (24 DAP) in each purple accession, this was not significant ($P<0.05$).
Visual (microwaved cooked): Trend (not significant $P<0.05$)) for purple-cobs to higher scores than reddish-purple cobs. Darkest purple (24DAP) scored similarly to the yellow commercial for visual appearance when cooked. In general, cooking significantly improved scores for all lines (purple and yellow) compared to raw appearance by about 1.2 hedonic units.
Flavour: Reddish-purple cobs tasted better than purple-cobs. Yellow cobs scored highest, but similar to reddish-purple cobs.
Texture: No significant difference, although a trend similar to flavour. Yellow corn scored highest and purple cobs lowest.
Overall: Similar scores to flavour/texture scores. Assessment was made directly after tasting, so taste may have influenced the overall score the most. Yellow corn scored highest, but not significantly different to the reddish-purple cobs (especially the 21DAP maturity stage).

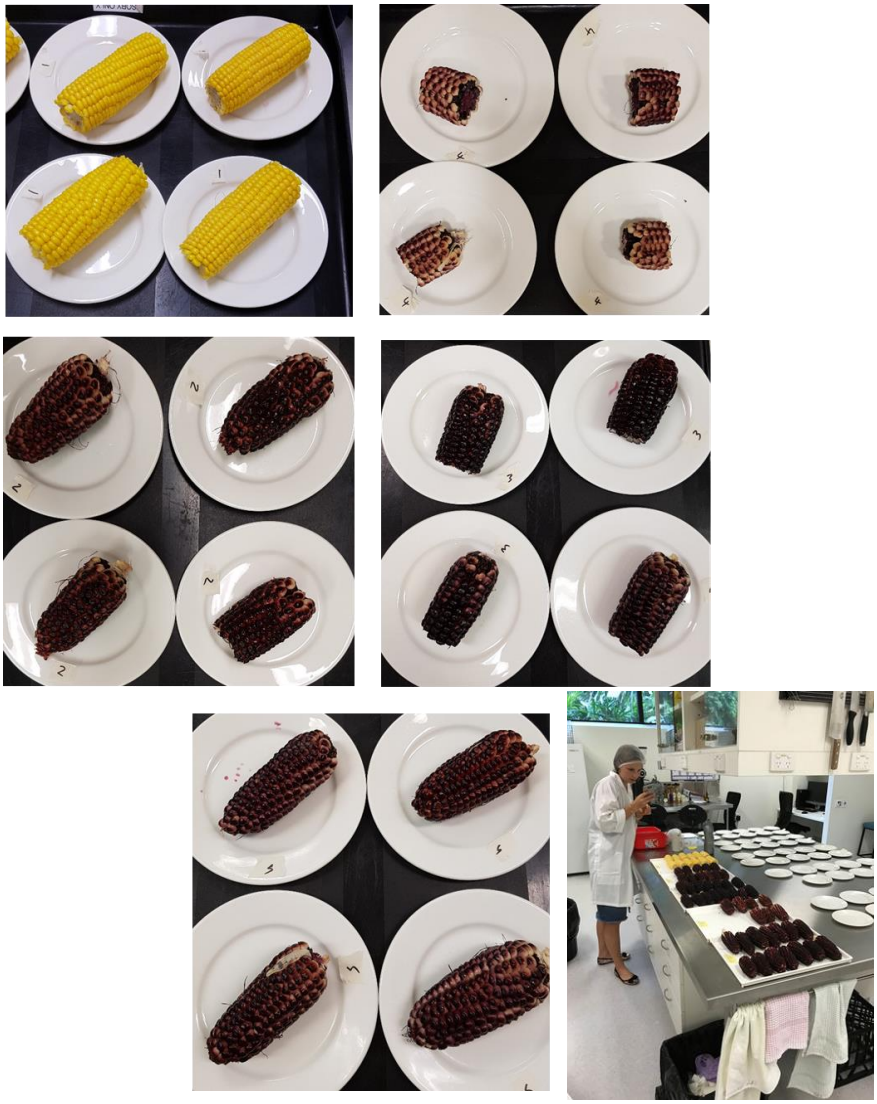


Figure 6: Samples as prepared for consumer evaluation. A, control; B, reddish-purple (21 DAP); C, reddish-purple (24 DAP); D, purple (21 DAP); E, purple (24 DAP). Inset: preparing cobs prior to cooking.

In general, consumers found the purple sweetcorn to be acceptable (scoring parity with a yellow commercial sweetcorn cultivar). Purple sweetcorn lines tested were liked especially for their 'vibrant colour' and "sweet, raspberry aroma". However, the texture of these samples was a little disappointing to consumers, being described as 'dry', 'tough' and 'fibrous' in some cases, mainly due to the presence of papery glumes at the base of the kernels.

The yellow control sample was preferred by consumers above all other samples for the attributes raw appearance and cooked (microwaved) appearance, and texture. The control sample and reddish-purple sample 21 DAP scored parity with one another for the attributes flavour and overall liking, and were the preferred samples for these two attributes.

In regard to appearance, purple cobs of both maturities were preferred over the reddish-purple cobs.

For both samples, however, the reverse was reported for the attributes flavour, texture and overall eating quality, with the mean liking data indicating that the reddish-purple sweetcorn sample (21 DAP) was the preferred sample for eating quality.

The consumers enjoyed the sweet, juicy characteristics of the control sample but disliked any woodiness present at the base of the kernels. This pattern was observed throughout all the comments provided, with consumers consistently providing negative comments pertaining to any fibrous, dry and woody characteristics. These comments can be attributed to the glumes, the papery structure at the base of kernels, which was more prominent in the experimental purple sweetcorn lines.

The presence of larger glumes appears to come from the purple maize parent, which historically is used for the crushed cob itself, including the papery glumes (but not the kernels), as a source of anthocyanin colourant for the Peruvian drink, chicha morada. The glumes more readily release pigment compared to the kernels, so the large papery glumes have been previously selected for but are a negative attribute for eating as a sweetcorn. In contrast, positive comments were made on glossy appearance, raspberry aromas, and sweet flavour. The presence of raspberry aromas was unexpected and was stronger as maturity increased. Although the purple sweetcorn tasted like sweetcorn, the presence of the raspberry undertone was seen as a strong positive, which further differentiated purple sweetcorn from standard yellow sweetcorn, in addition to the obvious colour difference.
It should be noted that subsequent to the current consumer assessment, the development of purple sweetcorn based on the more widely used *shrunken2* supersweet mutation has been instigated with the development of improved lines. From observation, the resulting *shrunken2* purple sweetcorn, which comes from a different purple maize background, lacks the larger papery glumes that were present in the *brittle1* purple sweetcorn, which were objectionable to a number of the panellists. Preliminary measurements also indicate that the new purple supersweet accessions are also sweeter than commercial yellow sweetcorn, which is a positive attribute for consumers.

Industry feedback

Industry is very supportive of purple sweetcorn as a new product in the Australian marketplace and agree that development of a sweetcorn based on the *shrunken2* supersweet mutation would greatly assist in any cob quality effects that would be induced by cross-contamination of pollen between existing yellow sweetcorn and purple sweetcorn. Similarly, the removal of the aleurone-based anthocyanin trait would also be very useful to avoid the development of purple spotting in nearby yellow sweetcorn.

At present, the future development of purple sheath-leaves is not an essential, although it would be a bonus to be able to visually differentiate for the sale of whole loose cobs, especially if the use of plastic overwrapping is curtailed or wound back by the supermarkets. Similarly, whether the underlying cob is purple or not would be a decision based on consumer assessment, but the presence of a purple underlying cob is not a priority, but not a negative either.

The observation that kernels will increase in anthocyanin concentration and colour coverage after harvest was of potential interest, and definitely not seen as a negative as consumers prefer well-coloured kernels. It is likely that some colouration will occur in the current supply chain, as low temperatures cannot always be assured.

It was suggested that the utilization of waste would be also of interest, in regard to high-anthocyanin powders developed from non-marketable purple sweetcorn or even purple-pigmented non-cob tissues.

Outcomes and Recommendations

The development of purple-pericarp supersweet sweetcorn was shown to be technically possible, with the development of purple sweetcorn based on two forms of the naturally-found supersweet genetic mutations, *brittle1* and *shrunken2*. During the course of the Naturally Nutritious project, it was considered to be in the industry's interest that a *shrunken2*-based sweetcorn (more difficult to attain) would be of particular benefit over that of a *brittle1*-based sweetcorn, and consequently future development of purple sweetcorn will be largely focused on *shrunken2*. The major benefits include removal of the possibility of pollen cross-contamination inducing starchiness in both purple and yellow sweetcorn grown in close proximity to each other. This will allow purple and yellow sweetcorn to be grown in close proximity without any impact on sweetness.

In addition, the move to a *shrunken2*-based sweetcorn aligns the purple sweetcorn with the Australian and global sweetcorn industry, which is largely producing sweetcorn based on this mutation. This will allow easier improvements in purple sweetcorn in the future, by allowing crossing with existing (non-purple) breeding material held elsewhere (e.g., for new disease resistance etc.) without the need to continue to select for the 'supersweet' phenotype.

The observation that anthocyanin could be produced in both the pericarp (maternal tissue) and the aleurone layer (filial tissue) still needs to be addressed. From a practical perspective, aleurone pigmentation is not essential, and if anything may potentially cause 'purple spotting' within any adjacent stands of yellow sweetcorn. By contrast, the purple-pericarp trait cannot be transferred to developing cobs by pollen. Consequently, it is recommended that the pigmented-aleurone trait should be removed (if possible) as part of the development of purple sweetcorn. This would allow purple and yellow sweetcorn to be grown alongside each other without any visual issues.'

In regard to kernel quality in purple sweetcorn, the rate of pigmentation development across the kernel surface in relation to kernel maturity is very important. It is essential that any future development of purple sweetcorn monitors rate of development, with early pericarp pigmentation during kernel development being essential. From a consumer perspective, even coverage of colour was preferred. The observation that anthocyanin can continue to increase in concentration and kernel coverage after harvest may be a useful tool for visual quality management, but its implementation as a tool will require monitoring of the sweetness, as this will gradually decline, as with yellow sweetcorn, after harvest, if higher temperatures are experienced.

In general, purple colouration was preferred by consumers over a purplish-red colouration, which is caused by a slight difference in the anthocyanin profile. It is therefore recommended that future focus, initially at least, be placed on purple, rather than purplish-red sweetcorn development.

Apart from the kernel colour, potential also exists to modify the colour of both the underlying cob and the overlying sheath-leaves surrounding the cob. In regard to underlying cob colour, it is not essential to have a purple cob for purple sweetcorn, but the preference for or against purple cob-colour should be consumer-assessed in the future, ideally early in any breeding program, to determine if this is a positive or negative attribute to consumers. One issue is that if a cob is eaten by hand, then a consumer's fingers could be stained by the cut ends of a purple cob.

Purple pigmentation of overlying sheath-leaves may be worthwhile in the future if cobs are to be marketed whole, rather than in plastic-overwrapped trays where the sheath-leaves have been largely or wholly removed to display the purple kernels. The continued increase in the drive to minimize plastic use in Australian supermarket products should be seriously considered. Removal of the ability to use plastic overwrapping, which is used to both display product and minimize moisture loss and kernel shriveling, will require an alternative. Any rules that do not allow purple kernels to be displayed, will ideally require other forms of product differentiation, and purple sheath-leaves is an obvious possibility for this. Consequently, it is recommended that future purple sweetcorn development include the option for purple sheath-leaves.

Although not investigated in the Naturally Nutritious project, the potential use of non-saleable purple cob waste, or from the plant itself, is worthy of future investigation.

High-anthocyanin strawberry & purple strawberry

Background

Health issues and product visual differentiation

Strawberry fruit are most commonly red in colour, which is largely due to the presence of the red-pigmented anthocyanin, pelargonidin-3-glucoside (Lukton et al., 1955; Lopes da Silva et al., 2007). The intensity of redness varies from a pale pink to a deep crimson and is usually correlated with the concentration of this anthocyanin (Yoshida et al., 2002; Fredericks et al., 2013).

A recent breeding addition to the Australian market is the strawberry cultivar 'Red Rhapsody'. The cultivar has a deeper red colour intensity than previously seen on the market, but its popularity in sales has proven that it is well accepted. Further increases in anthocyanin concentration are also possible, with the intensely coloured cultivar 'Nerina' (in Europe) being an example of a high-anthocyanin fruit, the colour of which is a deep crimson, owing to enhanced concentration of the red-coloured anthocyanin, pelargonidin (Katz et al., 2020).

Currently, there is no recommended daily intake of anthocyanins, although there is a growing body of scientific evidence that they are beneficial in a range of health issues, particularly lowering LDL cholesterol (Basu et al, 2010; Basu et al., 2014). The mode of action appears to be largely through their action on the gut microbiota, which metabolise them to produce active phenolic compounds that are readily taken up into the body. The concentration of anthocyanin required for efficacy has not been officially established, though based on the dosage rate (154 mg/day) used to lower LDL by Basu et al 2010 and 2014, an anthocyanin concentration of at least 62 mg/100g FW in fresh fruit would be required, if a single 250 g punnet were consumed. Commonly, red strawberries have an anthocyanin concentration of 20-40 mg/100gFW (Lopes da Silva, 2007), although an accession within the DAF-Qld breeding program (BL 2006-221) has previously been identified as having an anthocyanin concentration of approximately 100 mg/100gFW (Fredericks et al., 2013).

The fact that anthocyanins are both a visible pigment and an active phytonutrient make products containing them readily identifiable and simpler to market. A particular example that has been very successful in the Australian market has been the 'Queen Garnet' plum, a deeply-coloured high-anthocyanin blood-plum, that has been associated with anthocyanin-related health benefits in animal studies.

Although strawberry variants exist in which fruit may be coloured white (Hartl et al, 2017) or yellow (Hawkins et al, 2016), this is primarily due to an absence of anthocyanin biosynthesis, leading to the unmasking of non-anthocyanin pigmentation. Much less common however, are purple, or burgundy-coloured, strawberries. The colour of burgundy-coloured strawberry fruit is more reminiscent to that observed in blood-plums, in which a principal anthocyanin is cyanidin-3-glucoside (Fanning et al., 2014; Fredericks et al., 2013).

The following case study investigated anthocyanin composition in putative high-anthocyanin varieties and breeding-lines, evaluated how anthocyanin concentration increases during postharvest storage, and evaluated consumer responses to the concept of dark high-anthocyanin strawberries.

During the course of the Naturally Nutritious project, 'purple-coloured' or 'burgundy-coloured', as opposed to dark-red strawberries, were identified within the strawberry breeding program. This was a departure from dark-red strawberries, and the underlying cause of the purple colouration was investigated, together with the observation that different tissues could possess different colours (and anthocyanin-profiles) within the same fruit. 'Purple' coloured fruit potentially offer a further point of difference to 'deep-red' fruit, although this assessment was not conducted within the current project timeframe.

Technical feasibility

Germplasm, colour & anthocyanin content

Red strawberry varieties selected for testing were grown at DAF Maroochy Research Facility and harvested at two time points, with the least mature fruit at each harvest being 70% coloured, and maximum maturity being one- or two-days post first 'eating ripe'. All collected fruit was frozen and stored at -20°C for several days after each harvest.

Harvest date did not significantly influence the anthocyanin content, but the impact of accession/cultivar on

the content values was significant (Table 1).

Table 1: Mean total anthocyanin contents for all accessions tested. Means followed by the same letter are not significantly different from each other.

Variety	Mean anthocyanin content (mg P3G eq./100 g FW	
2018-098	13.50	a
Phenomenal	13.72	a
2018-184	14.19	a
2018-086	18.47	ab
2018-163	20.90	bc
2018-240	22.65	bcd
2018-114	24.73	cde
2018-150	24.97	cde
2018-182	25.87	cde
2018-241	27.17	cde
2018-152	27.18	de
2018-137	27.96	def
2018-234	29.16	ef
2018-157	29.81	ef
2018-178	34.07	fg
2018-094	37.08	gh
2018-199	39.03	gh
2018-242	41.59	h
lsd (5%)	6.35	

Total anthocyanin contents ranged from 13.50 mg P3G eq./100 g FW for accession 2018-098 to 41.59 mg P3G eq./100 g FW for accession 2018-242.

In a complementary study of available strawberry cultivars (Figure 1), the total anthocyanin contents for the latest harvest time point ranged from 17.97 mg P3G eq./100 g FW for cv. Phenomenal (not shown) to 54.45 mg P3G eq./100 g FW for cv. Rosalie.

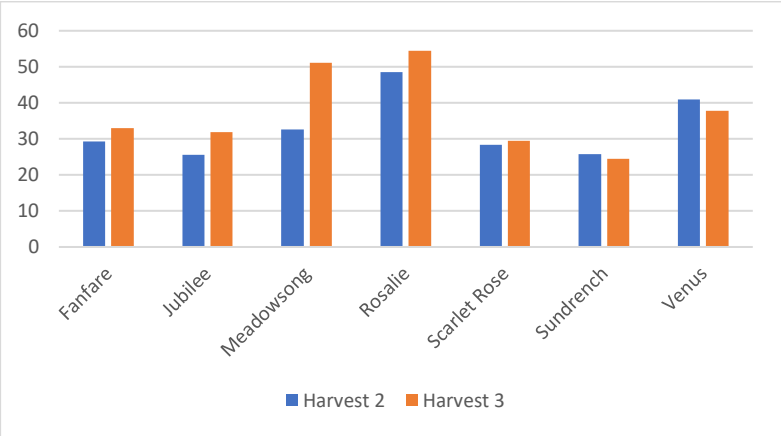


Figure 1: Anthocyanin content (mg P3G eq./100g FW) for the strawberry cultivars across harvests 2 and 3.

Previous testing of the two cultivars, Fortuna and Red Rhapsody (tested as part of a 2018 storage trial), displayed initial total anthocyanin contents of 36.22 and 40.51 mg P3G eq./100 g FW, respectively. However,

in a DAF report to the Queensland Strawberry Growers Association (contract: PS2015-3161) (Fanning et al., 2016), the day 0 total anthocyanin contents of the cultivars Fortuna and Red Rhapsody were significantly higher, with values of 86.94 and 93.22 mg P3G eq./100 g FW, respectively. It is uncertain why these latter anthocyanin results were much greater, and methodological differences in analysis may have been responsible, as the latter results were analysed using HPLC, and the former using a spectrophotometric technique. Differences between these two methodologies were confirmed during the Naturally Nutritious project, with HPLC being a more accurate measure on anthocyanin measurement. Other spectrophotometric studies with the high-anthocyanin Queen Garnet plum (Filmban, 2011) have also underestimated total anthocyanin, as much as 40% lower than the HPLC value.

The current results are in a similar range compared with those published by other researchers (Aaby et al. 2012; Ayala-Zavala et al. 2004; Buendia et al., 2010). For example, in a study of 27 strawberry cultivars, Aaby et al. (2012) reported samples contained between 9 and 66 mg P3G eq./100 g FW, with an average of 34 mg/100 g FW. This is similar to the anthocyanin concentration previously reported for other strawberry cultivars (Aaby et al., 2005; Buendia et al., 2010) data from the USDA (nutrient reference database for Standard Reference 27 Software v.2.2.4). In the Ayala-Zavala et al. (2004) storage experiment, an initial value for total anthocyanin content of 30.5 mg P3G eq./100 g FW was presented for samples of the strawberry cultivar, Chandler.

To summarise, in the three strawberry trials conducted by DAF Food Analytics on strawberries grown in south-east Queensland (covering 27 different strawberry varieties and a range of harvest dates), the highest anthocyanin content recorded was 54.45 mg P3G eq./100 g FW for late harvested cv. Rosalie.

The above anthocyanin concentrations all fall below 62 mg/100g FW, as estimated as an equivalent concentration for a 250 g punnet to supply an anthocyanin dosage equivalent to that used by Basu et al (2010) to reduce LDL cholesterol. It should be noted that these values were lower than those reported by Fredericks et al. (2013) for breeding accession 2006-221 (98.4 mg /100g FW), and Fanning (2016) for 2013-055 (101 mg/100gFW), Festival, Camarosa, Monterey (>90 mg/100gFW), Fortuna, Red Rhapsody, Suncoast Delight, St Andreas, 2013-027, 2011-192 (>80 mg/100g FW). Both of the latter studies utilised dual standards of C3G and P3G, while the Food Analytic studies used only P3G. It is possible that this may have also contributed to the differences in total anthocyanin (sum of cyanidin and pelargonidin derivatives) observed between the trials, although the small amount of cyanidin present in most fruit would tend to discount this effect.

It was also noted that the relationship between fruit colour parameters and total anthocyanin concentration was not as strong as previously reported. If anything, colour intensity (L value), rather than the colour itself (hue value), presented a stronger relationship. Generally speaking, deeper coloured fruit had higher total anthocyanin content. It is probable that the weakness in the relationship between colour (hue) and total anthocyanin concentration was directly due to the presence of other coloured anthocyanins, particularly ‘purple’ cyanidin, which would have altered the colour of the fruit independently of the total anthocyanin present. On the whole, though, darker colour intensity of strawberries indicates a higher total anthocyanin concentration.

Factors affecting anthocyanin content (postharvest storage)

It has been previously reported that at least some varieties of strawberries continue colour development following harvest, increasing in anthocyanin concentration (ref). In the current trial, two cultivars (cvs. Fortuna and Red Rhapsody) grown at DAF Maroochy Research Facility were stored at two temperatures (5ºC and 15ºC) and assessed over a storage period of 11 days. The traits measured were anthocyanin content, moisture, pH, and three colour parameters (L*, C* and hue angle).

In early September (2018), 140 fruit per cultivar of Fortuna and Red Rhapsody were collected from the commercial grower Taste n’ See, Bellmere, south-east Queensland. The fruit were immediately transported to the IFT-Laboratory, Coopers Plains. Fruit was randomly allocated to the above treatments, with fruit being stored in 250 g punnets (as used by industry) to avoid moisture loss. Punnets were subsequently placed inside loosely bound polyethylene bags, surrounded by wet towels to maintain uniform humidity inside a temperature incubator-maintained 15°C, or in a cold room set at 5°C. Fruit moisture content ranged within 89-91% for the course of the trial.

In the current trial, despite a slightly increasing trend (10-13%), anthocyanin concentration did not significantly increase from day 0 when stored at 5°C (Figure 2a). By contrast, a significant increase was observed for both

cultivars at 15°C, with the increase being significantly greater (73%) for cv. Red Rhapsody than for Fortuna (19%) (Figure 2b). It should be noted that the final day 11 assessment was not included due to the appearance of fungal rots by this time at 15°C.

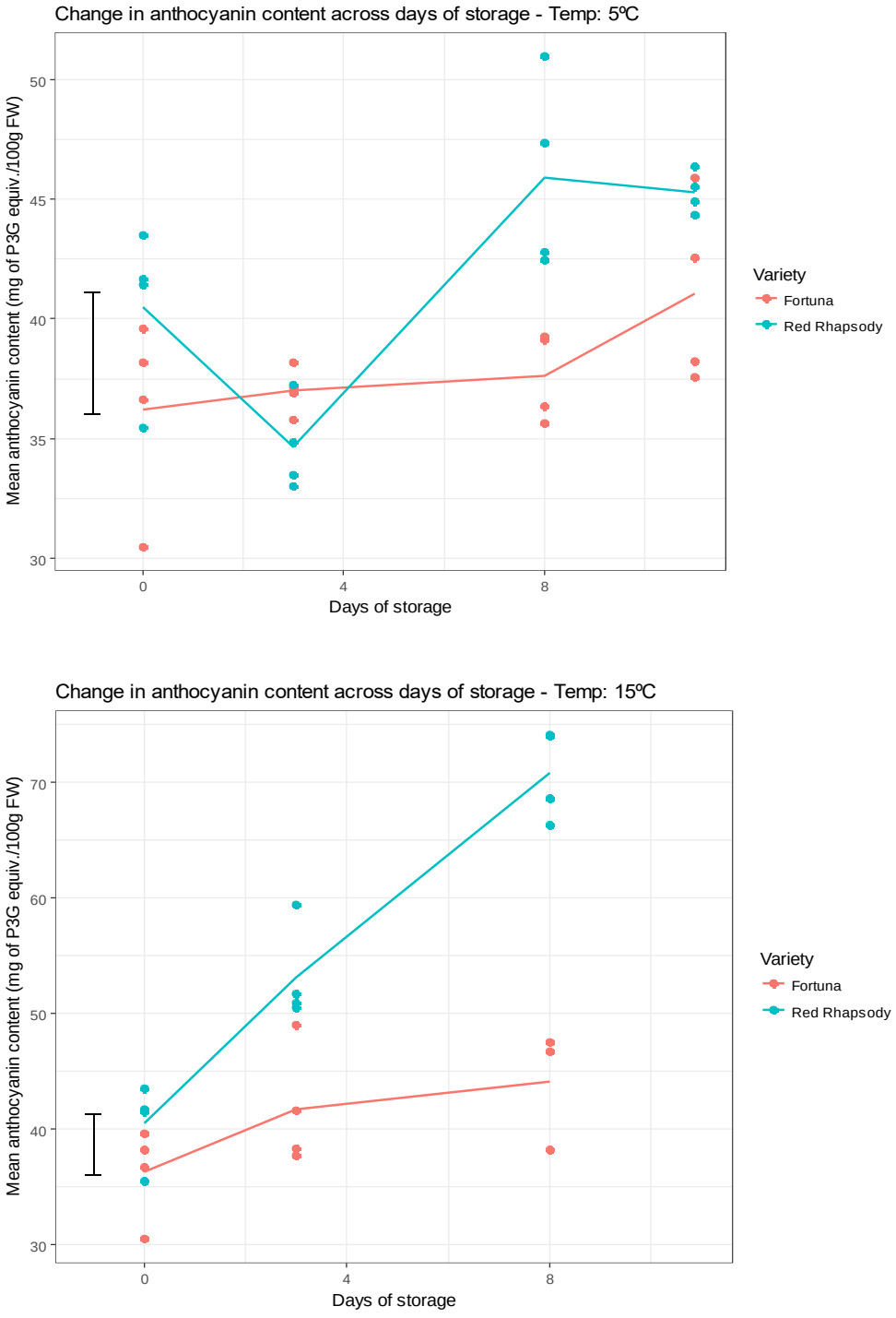


Figure 2: Changes in total anthocyanin content of cvs. Fortuna and Red Rhapsody stored at (a) 5C and (b) 15C. The vertical bars indicate least significant different at P<0.05. Note the different in the vertical scale for Figures 2a and 2b.

The large increases (up to 73%) in anthocyanin concentration observed during postharvest storage at 15C highlight the importance of postharvest handling temperature in subsequent anthocyanin assessment in strawberries. Although not assessed in the current trial, it is quite feasible that higher ambient temperatures (e.g., 25C) that may be commonly experienced during harvesting and packing may have an even greater impact on increase in anthocyanin content. Consequently, differences in temperature postharvest, rather than inherent cultivar differences that may exist may have an equal impact on measured anthocyanin content.

In the current trial, fruit of cv. Red Rhapsody reached anthocyanin concentrations of around 70 mg/100gFW, even when stored at 15C. What is of interest, however, is that different cultivars appear to have different potential to accumulate anthocyanin during postharvest storage, with Red Rhapsody being more efficient than Fortuna. It was observed that a significant correlation existed between anthocyanin content and hue angle for both external and internal flesh for both cultivars, apart from internal flesh of Fortuna at 15C. It is possible that the large increases in total anthocyanin content observed in cv. Red Rhapsody may have been due to increases in anthocyanin in the internal tissue, increasing the internal red colour of fruit. The current trial would tend to support this observation (Figure 3). Consequently, external fruit colour is not necessarily a reliable indicator of total anthocyanin content, as much of the anthocyanin continues to accumulate internally with storage in cultivars such as Red Rhapsody.

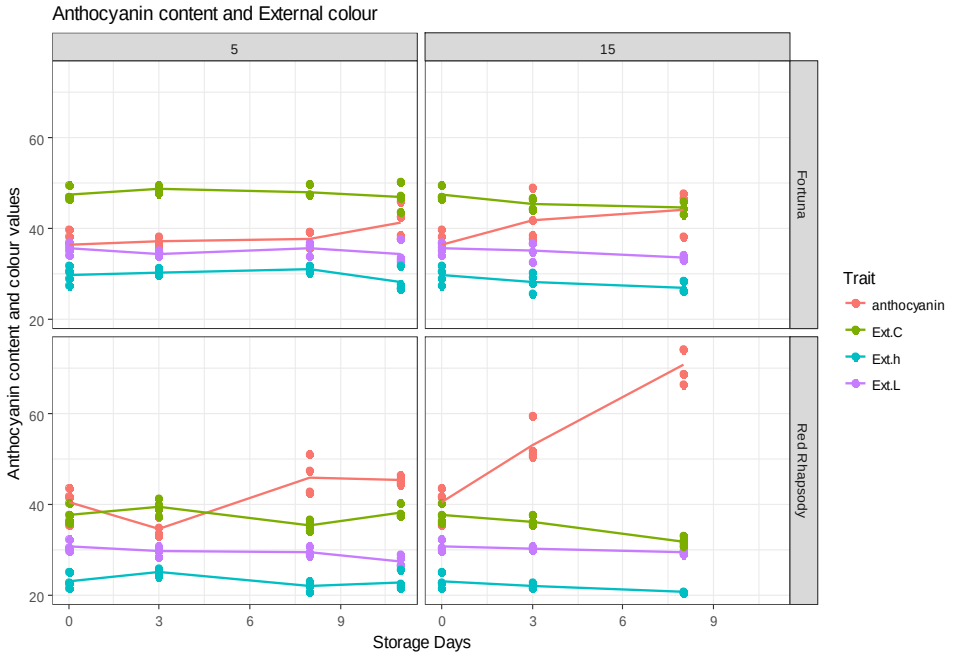


Figure 3: Changes in colour parameters in relation to total anthocyanin content in inner internal flesh (upper graph) and external skin (lower graph). Quantitative changes in colour parameters (L, C, Hue) were greatest in the inner internal flesh during storage at 15C, rather than the skin where these parameters tended to change only slightly.

An interesting result to arise from the current study involves the comparison of mean anthocyanin content values at day 0 for both Fortuna and Red Rhapsody with those reported by Fanning et al. (2016). The results reported in the Fanning study were approximately 2.4 times greater than the ones found in this experiment for the same cultivars. It is possible that apart from differences in methodology between the two trials (HPLC versus spectrophotometry), it is also possible that the fruit may have experienced different temperature regimes during harvest and postharvest before treatments were imposed.

Factors affecting anthocyanin profile and content (tissue location)

Strawberry fruits are most commonly red in colour, which is largely due to the presence of the red-pigmented anthocyanin, pelargonidin-3-glucoside (Lukton et al., 1955; Lopes da Silva et al., 2007). The intensity of redness varies from a pale pink to a deep crimson and is usually correlated with the concentration of this anthocyanin (Yoshida et al., 2002). Although strawberry variants exist in which fruit may be coloured white (Hartl et al, 2017) or yellow (Hawkins et al, 2016), this is primarily due to an absence of anthocyanin biosynthesis, leading to the unmasking of non-anthocyanin pigmentation. Much less common however, are red-purple, or burgundy-coloured, strawberries.

During the course of the Naturally Nutritious project, it was noticed that several variants in the strawberry breeding program existed with burgundy-coloured skin, rather than red or deep-red (Figure 4). This has been previously unreported, and it was considered important to clarify why this colouration is observed, and at the same time to look at the variation in colour in the different tissues of the same fruit, as it appeared that external colour and internal coloured did not necessarily match (e.g., purple outside and red inside, or purple outside and purple inside etc).

Figure 4: Variants of purple (burgundy) strawberry colour identified within the strawberry breeding program: normal red fruit (left); purple skin/red flesh fruit (centre); purple skin/purple flesh fruit (right).

The colour of burgundy-coloured strawberry fruit is more reminiscent to that observed in blood-plums, in which a principal anthocyanin is cyanidin-3-glucoside (Fanning et al., 2014; Fredericks et al., 2013). The purpose of the following study was to investigate if the burgundy colouration observed in outer or inner flesh of several strawberry breeding accessions was due to the presence of cyanidin-based anthocyanins, rather than pelargonidin, and if the presence of cyanidin may be more widespread in other tissues (leaf, achene, pigmented petal) of the strawberry plant.

Strawberry fruit were harvested at full-colour development from within the strawberry breeding program at Maroochy Research Facility, Nambour, Queensland, in winter 2019 (humid subtropical climate). Three strawberry accessions exhibiting epidermis/inner-flesh colour, respectively, of burgundy/burgundy (B/B, 2018-179), burgundy/red (B/R, 2018-214) and red/red (R/R) were selected from within the strawberry breeding program for examination. Three fruit of similar size and appearance were collected and combined as a composite sample for each accession.

Anthocyanins of strawberry fruit tissue consisted solely of pelargonidin-based and cyanidin-based pigments, with pelargonidin-3-glucoside being the principal anthocyanin found in both red and burgundy-coloured flesh of all strawberry accessions (Table 2). While pelargonidin-3-glucoside was the principal anthocyanin found in all strawberry fruit analysed, the proportion of cyanidin-3-glucoside was approximately 70-160% higher in the outer flesh of burgundy-coloured fruit (B/B, B/R) compared to red-coloured fruit (R/R), and approximately 380-1600% higher in the inner flesh of B/B fruit compared to both B/R and R/R fruit.

Achenes displayed a totally different anthocyanin profile to strawberry flesh. In contrast to strawberry flesh, the predominant anthocyanin in achenes was cyanidin-3-glucoside, comprising approximately 90% of the total anthocyanins, with the remaining 10% consisting of pelargonidin-3-glucoside (Table 2). Colour development in

the achenes appeared to be confined to the outer pericarp layer of the achene, overlaying a largely colourless seed. Cyanidin-3-glucoside appeared to be the only anthocyanin present in red leaves/petioles, and in pink petals (Table 2). No anthocyanin compounds were detected in green leaves/petioles or white petals.

Total anthocyanin concentration was found to be significantly higher in burgundy-coloured flesh tissue compared to red-fleshed tissue (Table 2). Higher anthocyanin concentration was universally observed in the outer flesh (5 mm depth) of fruit, with highest concentration (97 mg/100gFW) found in the outer flesh of B/B, and lowest (6 mg/100g FW) in the inner flesh of R/R. Total anthocyanin concentration in achenes (R/R) was approximately two-thirds that of the outer flesh of R/R fruit, while pink petals displayed a high total anthocyanin concentration similar to that of burgundy-coloured outer flesh. Anthocyanin concentrations in the red leaf lamina was consistently higher than that of the petiole, but was variable between leaves, depending on the extent of leaf redness in the sample.

Table 2: Individual anthocyanin profiles and total anthocyanin concentration (TAC) of different strawberry tissues (B: burgundy, R: red; Cy3G: cyanidin-3-glucoside, Cy3MG: cyanidin-3-malonylglucoside, Pg3G: pelargonidin-3-glucoside, Pg3MG: pelargonidin-3-malonylglucoside, Pg3R: pelargonidin-3-rutinoside; TCy: total cyanidin-based anthocyanins, TPg: total pelargonidin-based anthocyanins).

Tissue	Colour	Cy3G (%)	Cy3MG (%)	Pg3G (%)	Pg3MG (%)	Pg3R (%)	TCy (%)	TPg (%)	TAC (mg/100gFW)
Outer flesh	B/B	12.6	0.0	78.3	0.3	8.8	12.6	87.4	96.9
	B/R	18.8	0.0	79.3	0.5	1.4	18.8	81.2	69.7
	R/R	7.3	0.0	76.0	11.9	4.7	7.3	92.7	32.8
Inner-flesh	B/B	13.4	0.0	76.2	0.3	10.1	13.4	86.6	31.1
	B/R	2.8	0.0	93.7	0.1	3.4	2.8	97.2	30.7
	R/R	0.8	0.0	74.9	15.8	8.5	0.8	99.2	5.6
Achene	R/R	79.6	10.8	9.6	0.0	0.0	90.4	9.6	18.8
Leaf-lamina	red	100.0	0.0	0.0	0.0	0.0	100.0	0.0	3.0-63.3
	green	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Leaf-petiole	red	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.6-4.3
	green	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Petal	pink	100.0	0.0	0.0	0.0	0.0	100.0	0.0	80.2
	white	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Despite 'purple' strawberry fruit having a deep-burgundy colour typical of that produced by the anthocyanin, cyanidin, it would appear that a small increase in the proportion of cyanidin from 7% to 12-19% (as cyanidin-3-glucoside) is sufficient to induce a large change in visual appearance (Table 2). Previous reports (Goiffon et al, 1999; Lopes da Silva et al., 2007) have indicated that cyanidin-3-glucoside accounts for between 3-11% of the anthocyanin content of whole strawberry fruit extracts. In the current trial, red pelargonidin-based anthocyanin was still the predominant flesh pigment of all fruit tested, but it would appear that burgundy-coloured cyanidin effectively masks the brighter red colour of pelargonidin, at least once a critical percentage of cyanidin is reached. It is also probable that an increase in total anthocyanin concentration also contributes to increased colour intensity, although the burgundy-coloured inner-flesh of B/B had a similar total anthocyanin concentration to the red-coloured outer-flesh of R/R (Table 2).

In the current trial, it was of particular interest that cyanidin-3-glucoside was the predominant anthocyanin in

all strawberry tissues, except the fruit flesh itself. In both the leaves and the pink petals, it was the only anthocyanin detectable, and in the achenes, it accounted for 90% of the anthocyanin present (Table 2), although it is possible that the 10% pelargonidin present may have been due to adhering flesh tissue still attached after removing the achenes. Previous reports have also indicated that cyanidin-3-glucoside is the predominant anthocyanin in pink strawberry petals (Xue et al., 2016) and has a similar concentration to pelargonidin-3-glucoside in achenes (Aaby et al., 2005).

Both red-coloured pelargonidin and burgundy-coloured cyanidin are formed within the anthocyanin biosynthesis pathway, with the direction of biosynthesis towards cyanidin or pelargonidin depending on the activity of the enzyme, flavonoid-3-hyroxylase (F3'H). If F3'H is functional, it adds a hydroxyl group to dihydrokaempherol, the precursor of pelargonidin, to form dihydroquercetin, the precursor of cyanidin. If it is non-functional, or its action is blocked, then dihydrokaempherol will subsequently form pelargonidin.

It would appear that since cyanidin-3-glucoside is widely produced throughout the strawberry plant, then strawberry F3'H is functional, but not operating very efficiently in red strawberry flesh. In burgundy flesh, it appears to be operating slightly more efficiently, increasing the proportion of cyanidin-3-glucoside sufficiently enough to cause a colour change. Thill et al. (2013) have reported that F3'H expression in red strawberries significantly reduces as the fruit ripens, although expression remains high in the wild strawberry (F. vesca), where the concentration of cyanidin-3-glucoside in whole fruit (flesh, plus achenes) can reach 40-50% (Sondheimer and Karash, 1956; Thill et al., 2013).

The answer as to why F3'H is less active in strawberry flesh than in other tissues is not known. Considering that the progenitors of modern strawberry fruit originated in the sub-story of shaded forest conditions, it could be speculated that producing a darkly-coloured burgundy fruit would have been less visible to herbivores, and hence the spread of seed less likely (Nevo et al., 2018). Red fruit are more likely to be seen under these low-light conditions, and so there may have simply been an evolutionary advantage to increase red pelargonidin synthesis by down-regulating the action of F3'H in strawberry flesh tissue. How this down-regulation is controlled is also currently unknown.

Extension of shelf-life using photosensitisation methodology

Four different cultivars of strawberry named Festival (Fe), Ruby Gem (RG), Fortuna (FO) and Red Rhapsody (RR) were used to enumerate and identify the yeast and mould on the surface of the fruits (for loosely attached microbes) as well as on the whole fruit (for systemic microbes). Further, in-vitro studies of photosensitisation in the presence of photo-dye (PD) curcumin (50 ppm hydroethanolic solution; ca. 5% ethanol in the working solution) indicated that PD curcumin is very effective in inactivating the following microbes: *Mucor rudolphii, Pestalotiopsis theae, Penicillium raistrickii, Cladosporium bruhnei, Botrytis cinerea, Hanseniaspora uvarum* (yeast), *Saccharomycodes sp* (yeast), *Hanseniaspora sp* (yeast) and *Rhodosporidium diobovatum* (yeast). In continuation to the above, in-vivo studies were undertaken to see the effect of photosensitisation on actual fruit to extend shelf life of strawberries. The commercial cultivar Red Rhapsody was used due to least fungal spoilage in our previous in-vitro studies. Samples from ALDI store was used for these experiments due to seasonal limitation. Two in-vivo trials were done**.**

In trial 1, two controls with no treatment and two light-PD treated samples were tested for shelf life extension at room (25^{o}C) and refrigeration (4^{o}C) temperatures. There were 10 strawberries for each of the control and treated samples and the fruits were individually packed in non-perforated sterile bags and observed daily for visible microbial growth for 14 days. In control 1 stored at room temperature, fungal colonies appeared on the fruits from day 4 and fully covered the fruit surface by day 6-7 (*Mucor* sp., *Penicilium* sp, and *Cladosporium* sp). Control 2 stored at 4^{o}C did not show any growth however, heavy condensation of water was observed in the bag. In the light-PD treated samples kept at room temperature, no growth was observed, till day 8. The PD treated sample of strawberries had twice the shelf life in comparison to the control sample. This indicates the light treatment with curcumin as the photodye is having a significant effect in extending the shelf life of the strawberry stored at room temperature. Condensation was observed in the PD treated sample bag stored at 4^{o}C similar to control 2. The strawberries stored at 4^{o}C with the non-perforated packaging material showed condensation, to avoid this for the next trial perforated plastic containers were used.

In trial 2, perforated containers were used with 3 fruits packed in each container. The experiment was conducted with one control and one light-PD treated samples stored at 4^{o}C. No fungal growth was observed on both control and test sample until day 14. Packaging was good, and no condensation occurred.

To validate the above experiments, curcumin-based photosensitization of strawberries was conducted using strawberries purchased from a retail store (Brand: Sweetberries). Using an air spray gun, curcumin solution (50 ppm hydroethanolic solution; ca. 5% ethanol in the working solution) was finely sprayed on strawberries. This was followed by illumination using the pilot scale LED light unit (75% light intensity, 10 cm distance, 20 min). Then the strawberries were air-dried in the laminar flow cabinet for 30 min. Four strawberries were packed in each clamshell (which was wiped with ethanol 70%, before using), and stored at 4 °C. Twenty fruits were used per treatment (control 1 (no treatment), control 2 (5% ethanol), and photosensitization). After 15 days of storage at 4 °C, 19% of the photosensitized strawberries, 33% of the control fruits with no treatment and 64% of control fruits with 5% ethanol showed mould growth. However, the overall appearance of the photosensitized fruits was retained and was observed to be fresher than the untreated ones. It should also be mentioned that the untreated strawberries showed other types of spoilage (yeast and bacterial growth) in addition to mould growth. In line with our previous results, photosensitization leads to a better overall appearance and a substantial reduction in fungal spoilage in comparison to the control samples.

In a study done by Sarwar et al (2021) within this project, it was shown that the total anthocyanin content after photosensitization remained similar to the control. Pelargonidin-3-glucoside (Pg3G), cyanidin-3-glucoside (Cy3G) and pelargonidin-3-rutinoside (Pg3R) were unaffected by photosensitization. The same study reported that photosensitization had no impact on ascorbic acid for control (46.98 mg/100g FW) and photosensitized samples (46.60 mg/100 g FW).

Photosensitization, combination of light (LED – light emitting diode) and a photodye (photosensitiser) has proven to be an effective, environmentally friendly decontamination technique for reduction of microbial loads in strawberry. There has been a doubling of storage life at chilled temperature in comparison to the control without photosensitization treatment.

Concept model for full-scale photosensitization for strawberries developed

Photosensitization (Figure 5), a combination of light (LED – light emitting diode) and a photodye (photosensitiser) has proven to be an effective, environmentally friendly decontamination technique for reduction of microbial loads in strawberry. There has been a doubling of storage life at chilled temperature in comparison to the control without photosensitization treatment. For scaling up trials in pack houses the following concept model is proposed where curcumin is used a natural photosensitiser/photodye.

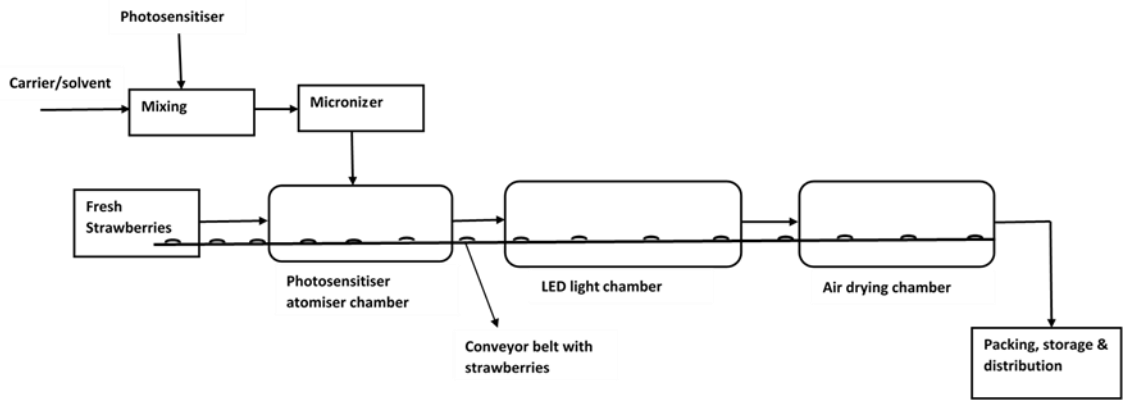


Figure 5: Photosensitisation Treatment of Strawberries – A Conceptual Model

Consumer response

The consumer testing for high-anthocyanin ('darker') strawberries. was conducted onsite at HFSP sensory facility. The focus groups were recruited by an external recruitment company and they were classified as: Start-up families – oldest child <6 years old (mix of male and female respondents), Small Scale Families – oldest child 6-11 years old (mix of male and female respondents, Bustling Families – oldest child 12-17 years old (mix of male and female respondents, Young Transitionals – under 35 years old with no children (mix of male and female respondents, Family planners – females only, those planning to have children within the next 18 months, Health/Nutrition Focused Shoppers – those that consider the nutritional aspects of food before purchasing (mixed age groups and gender), Vegetarians/Vegans/Flexitarians – People trying to reduce meat

consumption and increase fruit and vegetable consumption (mixed of male and female respondents), and Chefs/Café Owners (mixed age groups and gender).

Consumers were shown photographs of standard red strawberries and asked to give the first 3 words that came to mind. They were then shown photographs of the deep red variety and asked for the first 3 words that came to mind. This was then discussed further to find out what they think of this variety/whether they would purchase and what they would use it for.

Most respondents understood that eating a wide range of different fruit and vegetables is better for their health, with many speaking of "Eating the Rainbow" or eating lots of different colours. While most respondents had heard of antioxidants, they had not heard of, but not anthocyanins. This means that education is required in this area if wanting to market a product with a high anthocyanin level.

There was some awareness of purple coloured fruit and vegetables being "healthier" or "good for you. Overall, when purchasing strawberries, most consumers are presently consumers looking for a bright red colour with little/no white or green colouring on the outside and a firm, but not too hard texture. Paler colours were associated with the fruit being "under-ripe", "sour", "hard", "too crunchy" and "tart". Darker red colour was associated with the fruit being "very sweet", "soft", "mushy" and "overripe".

The main things that consumers are presently looking for when consuming strawberries are a sweet taste, strong strawberry aroma/flavour, and a firm & juicy texture. Some of the purchase barriers for consumers buying strawberries are condensation/moisture in the pack, mould, underripe berries, too large or too small-sized berries, price and bruising. Very large strawberries are associated with being flavorless and hollow in the centre.

The group of Chefs/Café Owners were more specific with things that they looked for in strawberries. Most of them shopped at wholesalers and they were concerned with the consistency of the product (both quality & appearance), shelf life, price, and flavour.

Based on the discussions with respondents, the deep red strawberries would require good marketing and the public would need to be provided with information about the ripeness and different colour to the standard strawberries before they would be willing to purchase them. Once informed about the higher anthocyanin content of the deep red strawberries, some of the respondents were more interested in trying/buying these strawberries. Again, educating the consumers about the health benefits of these products through marketing would make them more popular. Most respondents said that they would like to "try before you buy."

Industry feedback

Industry feedback included that standard red cultivars that are left too long can develop a flat, unattractive, dark appearance, and it would be important that a high-anthocyanin strawberry purple fruit was easily differentiated from such fruit. The development of a high-anthocyanin strawberry was also seen as a 'no-brainer', based on the success of other high-anthocyanin fruit, such as the Queen Garnet plum, but the lower total anthocyanin levels than products such as the Queen Garnet reduce the strength of any health claim.

Currently, the fact that high-anthocyanin health claims are unable to be made, and that the level of anthocyanin was below that of other high-anthocyanin products, such as the QG plum, makes pursuing this product less attractive to industry (at present) than further establishing the high-folate nutrient health claim. However, the national strawberry breeding program will continue to breed and improve 'dark' strawberries, purely for their visual attraction for potential marketing in the future.

Outcomes and Recommendations

The development of both a high-anthocyanin 'deep-red' strawberry and 'purple/burgundy' strawberries appears technically feasible. Existing breeding accessions already exist within the Qld Strawberry breeding program with these characteristics, plus combination of colours, such as purple exteriors and red inside, and purple exteriors with purple inside, or all deep-red.

Apart from changing the colour from red to purple, it appears that total anthocyanin levels can also be increased, which would increase the intensity of the red and/or purple colour. In addition to a genetic disposition for higher total anthocyanin in some accessions, a postharvest increase in anthocyanin has also been recorded, which could be further utilised for colour intensity optimisation. This appears to vary between cultivars and may be an underlying cause of variation in previous anthocyanin measurements. This would not be a disadvantage in the supply chain, as it tends to lead to an increase in internal colour, which is a favourable consumer trait.

Technical issues that still need to be addressed include the acceptability of the slight flavour difference of purple strawberries, and how acceptable this may be to consumers. Similar to purple sweetcorn, a slight flavour difference (that is palatable), over a general background strawberry flavour, may actually be beneficial to product differentiation, to further distinguish fruit from standard red strawberries. An additional potential issue observed by the breeder was the potential effect of postharvest moisture loss on the surface gloss of purple strawberries. This is a factor that also occurs with red strawberry fruit but might be less acceptable with purple fruit.

Consumer acceptance of the appearance was generally mixed, although the fruit assessed were less 'purple' than had been previously assessed in the laboratory. These deep-red (rather than purple) fruit were perceived as 'Overripe', 'Sweet', 'Dark', 'Rich', 'Juicy', 'Soft', compared to the control red fruit, which were perceived as 'Fresh', 'Ripe', 'Perfect', Juicy', 'Nice colour', 'Sweet'. Based on the deep-red fruit assessed by the consumer focus group, they would require good marketing and the public would need to be provided with information about the ripeness and different colour to the standard strawberries before they would be willing to purchase them.

Based on the above, assessing consumer preferences across a wider range of fruit across the colour spectrum, ranging from deep-red to purple, would be advisable.

As mentioned above, the national strawberry breeding program will continue to breed for improved 'dark' (i.e., purple/burgundy) strawberries, particularly for their visual attraction, for potential marketing in the future. At this stage, the potential health benefit of higher anthocyanin will be a secondary benefit.

Fresh strawberries are highly perishable and have a short storage life. Microbial contamination by fungi is one of the main causes and the industry uses fungicides to control infection in the field. Post- harvest disinfestation treatments such as methyl bromide as a fumigant are used in some states and for export to certain countries. However, this treatment is now being phased out from most countries. Consumer awareness on the use of chemical preservatives and detrimental effects on health has increased the demand for natural preservative technologies. Photosensitization, a combination of light and a natural photodye, has proven to be an effective, environmentally friendly decontamination technique for reduction of microbial loads in strawberry. We have achieved a doubling of storage life at chilled temperature in comparison to the control without photosensitization treatment in pilot scale trials. This treatment had no effect on the nutritional properties and appearance of the strawberry during the storage period in comparison to the untreated sample. In 2018/2019 the wholesale value of fresh strawberry was $434 million of which Queensland produced 42% of the total volume. Post-harvest losses in fresh produce can be between 10– 40%, if 10% of this loss can be prevented with this treatment, there will be a saving of about $40 million for the strawberry industry in Australia.

High-folate strawberry

Background

Health issues, analysis, and label health claims

The vitamins of the folate group play a crucial role as coenzymes in the metabolism of one-carbon groups, and are decisively involved in DNA synthesis, amino acid metabolism and methylations (Selhub, 2002). However, intake of folate from natural sources is considered to be below the human dietary recommendations. Low dietary intake of folate is associated with the risk of neural tube defects (Czeidal and Dudas, 1992) and is suspected to be associated with the development of certain forms of cancer (Caudill, 2004), Alzheimer's disease (Snowdon et al., 2000) and cardiovascular disease (Robinson, 2000). Over 50 countries have introduced mandatory folate fortification with pteroylmonoglutamic acid administration implemented in 1998 in the USA and Canada and in Australia in September 2009. The benefits of this fortification program with regard to neural tube defects have been obvious, as their incidence in Canada decreased by up to 3.8 cases per 1000 births from 1998 to 2002 (De Wals et al., 2007). However, discussions about the safety of this measure are still ongoing since reports on increased incidence of colon cancer in some countries with mandatory folate fortification (Mason et al., 2007) alternate with such on no significant effect on any kind of cancer (Vollset et al., 2013). The molecular cause is suggested to be a high plasma level of pteroylmonoglutamic acid that may lead to neoplastic transformations and formation of adenomas due to its effect on DNA synthesis (Cole et al., 2007) and DNA methylation (Coppede, 2014). Moreover, pteroylmonoglutamic acid supplementation in rats has stimulated the progression of aberrant crypt foci (ACF), the earliest precursor of colorectal cancer (Lindzon et al., 2009). In a human study, pteroylmonoglutamic acid supplementation decreased the cytotoxicity of circulating natural killer cells potentially affecting the destruction of neoplastic cells (Troen et al., 2006). Therefore, many countries in the EU have refused mandatory fortification and favour the consumption of foods endogenously high in folates or increasing endogenous folate content in foods. Strawberries (Fragaria x ananassa) are considered a tasty and healthy fruit consumed all over the world and may potentially be an important dietary source of natural folates. However, the relative importance of strawberry as a dietary source of folate will depend on the total folate concentration, vitamer profile, folate storage stability and bioavailability to humans. Reliable, accurate, and sensitive analytical methods are crucial for the determination of individual folate vitamers in strawberries and other folate containing food sources. A state-of-the-art analytical method, stable isotope dilution assays (SIDA), was used for the quantitation of folate and individual folate vitamers in strawberries (Striegel et al., 2018).

Technical feasibility

Germplasm & folate variation

Commercial strawberry cultivars and new breeding lines were sourced fresh from the Queensland Government, Department of Agriculture and Fisheries (DAF), Research Station Nambour, QLD, Australia and a commercial farm in Brisbane. In total, 130 samples (commercial cultivars and breeding lines) were harvested from 2016-2019 and analysed for total folate and individual folate vitamers by SIDA (Striegel et al., 2018). Individual folate vitamers quantified in the present study are: pteroylmonoglutamic acid/folic acid (PteGlu), tetrahydrofolate (H4folate), 5-methyltetrahydrofolate (5-CH3-H4folate), 5-formyltetrahydrofolate (5-CHO-H4folate), and 10-formyl-pteroylmonoglutamic acid (10-CHO-PteGlu).

The total folate content ranged from 59.1 µg/100 g fresh weight (FW) (breeding line CL03, harvested in July 2018) to 168.2 µg/100 g FW (breeding line 2011-174, harvested in June 2016). More than 90% of the studied strawberry samples had folate levels above 80 µg/100 g FW. These folate levels are considerably higher than the value in the Australian Food Composition Database (AFCD; https://www.foodstandards.gov.au/science/monitoringnutrients/afcd/pages/default.aspx) (39 µg/100 g FW). Figure 1 shows the folate content of the AFCD "standard strawberry", the best performing commercial cultivar and the "top" breeding line (highest value determined in the present study).

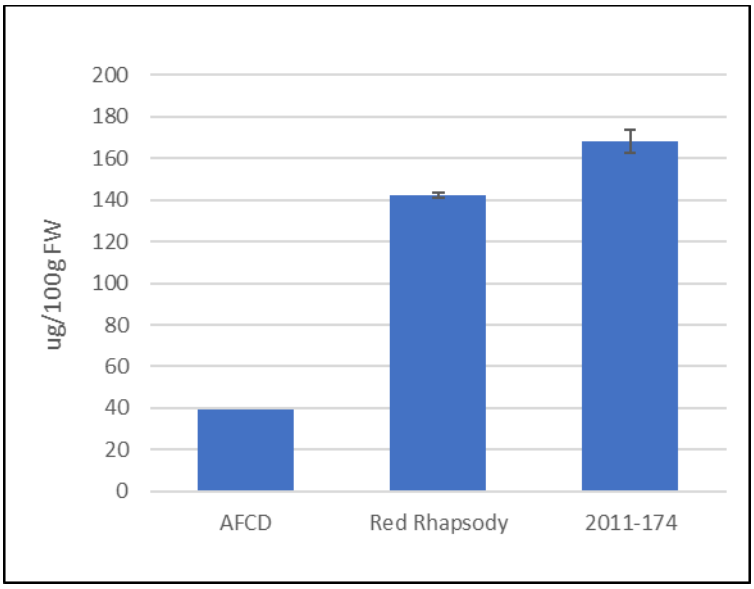


Figure 1: Total folate content of the best performing commercial strawberry cultivar (Red Rhapsody, harvested in June 2016) and the "top" breeding line (2011-174, harvested in June 2016) in comparison to the AFCD value; data are mean ± SD (n=3) for Red Rhapsody and breeding line 2011-174; FW: fresh weight.

Stralsjö and co-workers found folate concentrations between 30 and 69 µg/100 g FW (Stralsjo et al., 2003), Tulipani et al. of up to 96 µg/100 g FW (Tulipani et al., 2008), and Striegel et al. between 59 and 153 µg/100 g FW (Striegel et al., 2018). Our results are therefore in the same range as reported in the literature. The main vitamer in all samples was 5-CH3-H4folate, the biologically active form in humans, which contributed more than 90% to the total folate content (Figure 2).

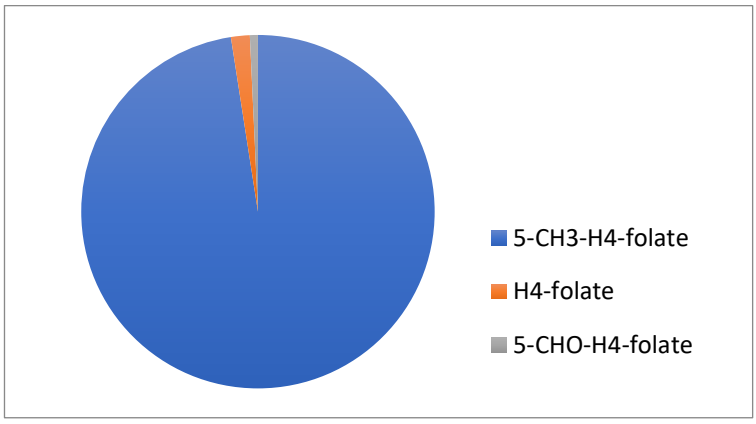


Figure 2: Distribution of folate vitamers in the analysed strawberry samples.

It should be noted that vitamin C in strawberry fruit can affect its folate content, as higher vitamin C levels can result in an increased stability of folates (Wilson and Horne, 1983). The stabilizing mechanism of vitamin C was confirmed by Ringling and Rychlik, who conducted *in vitro* studies to simulate the digestion of food folates. The addition of ascorbic acid in physiological amounts improved the stability of some folate vitamers, particularly of 5-CH3-H4folate, during the simulated digestion process (Ringling and Rychlik, 2017). Strawberries can contain a considerably amount of vitamin C, depending on the cultivar, growing conditions and postharvest treatment (Fredericks et al., 2013). However, the high-folate characteristic appears to be strongly genetic, based on the higher-folate breeding lines being related to each other (Table 1).

Table 1: Total folate content in the best performing strawberry breeding lines

Breeding line	Total folate content [µg/100 g FW]
2011-174	168.2 ± 5.41
2011-192	164 ± 6.32
2014-174	160.3 ± 2.91
2013-055	159.6 ± 1.06
2014-122	152.5 ± 0.92

A 250 g punnet of the high-folate strawberry breeding lines 2011-174, 2011-192 and 2014-174 would supply the Recommended Dietary Intake (RDI) of folate for adults which is 400 µg dietary folate equivalents/day (https://www.nrv.gov.au/nutrients/folate).

Figure 3 shows the total folate content in the commercial cultivar Red Rhapsody strawberry over three harvest seasons. The total folate content was relatively stable with a max. variation of only 26% (112 to 142 µg/100 g FW) and was still considerably higher than the AFCD value of 39 µg/100 g FW.

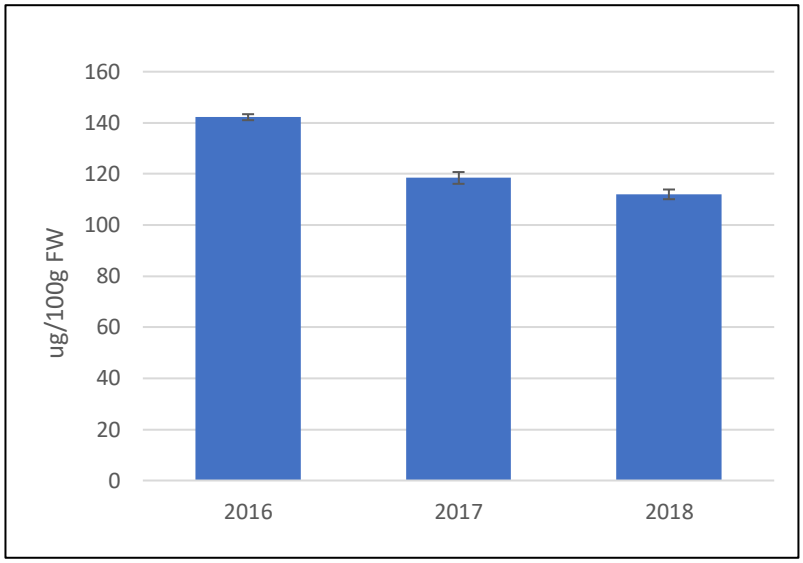


Figure 3: Total folate content in Red Rhapsody over three harvest seasons (2016-2018); data are mean ± SD (n=3); Red Rhapsody samples were sourced from the Research Station Nambour.

Overall, Queensland, and possibly Australian, strawberries were significantly higher in folate than previously listed in the Australian Food Composition Database. The higher level of folate would enable strawberries to a stronger label claim for folate than that allowed currently. Instead of simply 'a source of folate', this could be raised to 'a good source of folate' (25% of the RDI must be delivered in a serving [1 cup or 144 g]), which is currently the highest folate claim permitted.

Factors affecting folate

Early and late harvest

Several commercial strawberry cultivars and breeding lines (24 in total) were harvested in July 2018, respectively, August 2018 (same maturity stage), to determine the effect of different harvest dates on the total folate content (Figure 4). The average total folate content in the strawberries harvested in August was slightly higher than that in July (104 µg vs. 94.9 µg/100 g FW).

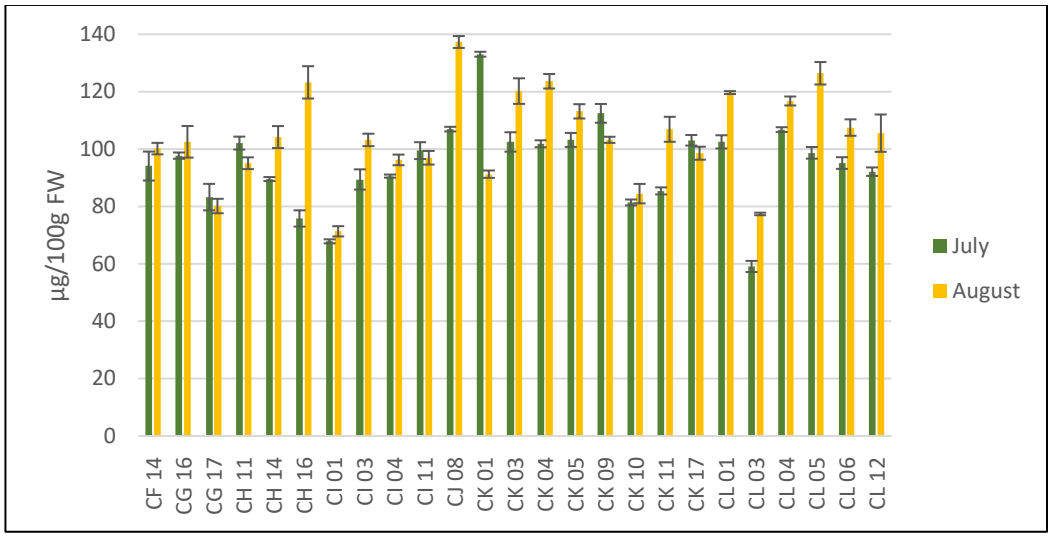


Figure 4: Total folate content in different commercial strawberry cultivars and breeding lines sourced from the Queensland Government's Research Station Nambour, QLD, Australia. CG16, CK09: Red Rhapsody; CG17, CK10: Parisienne Kiss; CH11: Sundrench; CK11: Scarlet Rose; all other are breeding lines; FW: fresh weight; data are mean ± SD (n=3).

A "slight trend" could be observed regarding increased folate levels at a later harvest date (August). However, CG17, CH11, CI11, CK09, CK17 and CK01had higher folate levels in July than August. Therefore, further more detailed and systematic testing should be conducted to get a better understanding of how the harvest date affects the folate content in strawberry.

Tissue distribution and fruit shape

The distribution of folate within the strawberry fruit is currently unknown and consequently fruit shape characteristics (e.g., surface-area to volume ratio) could potentially impact on folate concentration. Camarosa, Red Rhapsody and Fortuna, three commercial strawberry cultivars in Australia, were sourced fresh from a commercial farm in Brisbane, Queensland, Australia. Inner tissue, outer tissues and calyx were manually separated (Figure 5) and analysed by SIDA (Striegel et al., 2018).

Figure 5: Red Rhapsody (as an example) manually separated into inner and outer tissue as well as calyx.

For the three commercial cultivars, 'Camarosa', 'Red Rhapsody' and 'Fortuna', total folate levels were found to be 1.5 to 1.8-fold higher in the outer strawberry tissue compared to the inner tissue per 100 g fresh weight (Table 2).

Table 2: Folate content in inner and outer tissue of three commercial strawberry cultivars.

Sample	H_4folate [µg/100g]	5-CH_3-H4folate [µg/100g]	5-CHO-H_4folate [µg/100g]	Total folate DW [µg/100g]	moisture [%]	Total folate FW [µg/100g]
Camarosa inner tissue	47.6	874	55.6	977	89.6	**102** ± 0.56
Camarosa outer tissue	57.6	1440	67.0	1560	88.1	**177** ± 0.10*
Red Rhapsody inner tissue	32.0	1110	45.6	1190	91.1	**105** ± 1.86
Red Rhapsody outer tissue	39.7	1830	54.3	1930	90.0	**192** ± 1.23*
Fortuna inner tissue	31.6	786	47.6	865	90.1	**85.9** ±1.63
Fortuna outer tissue	43.4	1250	53.6	1350	90.2	**132** ± 0.40*

DW: dry weight; FW: fresh weight; data are mean ± SD (n=3); *p < 0.05 vs. inner tissue (t-test).

5-CH3-H4folate was the main folate vitamer in both tissues (>90% of total folate), whereas PteGlu was below the LOQ (and therefore not considered for the calculation of the total folate content), and 10-CHO-PteGlu was not detectable. 5-CH3-H4folate as the main folate vitamer in strawberry fruit is in agreement with the literature (Jastrebova et al., 2013; Striegel et al., 2018). Due to light induced degradation of folates, the obtained results of higher folate levels in the outer tissue compared to the inner tissue were unexpected. However, according to Rebéllé et al. (2006), the folate synthesis in leaves is increased by light. This light-induced accumulation of folates was related to an increased activity of 6-hydroxymethyl-7,8-dihydropterin pyrophoshokinase (HPPK), dihydropteroate synthase (DHPS) and dihydrofolate reductase-thymidylate synthetase (DHFR-TS). Although the strawberry fruit matrix is different to the leave matrix, a light induced folate synthesis might also happen in the strawberry matrix. Furthermore, strawberries belong to the Rosacea family and the actual fruit is not the red-white pulp, but it is the seeds or achene-likes on the surface trapped by the flesh (Davis et al., 2007). These achenes are accumulated on the outer tissue of the strawberry. Ariza et al. (2016) reported that the achenes had a significantly higher total phenolic and total flavonoid content compared to the pulp. To the best of our knowledge, quantitative folate data of strawberry achenes have not been reported yet. However, there might be a significant biosynthesis in the achenes, similar to that observed for phenolic compounds, contributing to the higher folate concentration in the outer tissue. Another reason for the higher accumulation of folate in the outer tissue could be the result of an antioxidative stabilisation of folate. According to Basset and co-workers, the folate accumulation is expected to increase by higher vitamin C levels (Basset et al., 2005). Besides vitamin C, strawberries contain polyphenols, and particularly anthocyanins, which are powerful antioxidants. Since anthocyanins are accumulated in the outer tissue (responsible for the characteristic red colour of strawberries), an additional stabilizing effect on folate resulting in a higher total folate content is likely.

Strawberry calyx is usually not consumed; therefore, only a qualitatively analyses of folate ("detectable or not detectable") was conducted. However, folate could be detected in all calyx samples and should therefore be quantified in future studies to explore the "value" of this by-product as a potential source of natural folate (e.g., extract, powder).

Overall, our results (folate in outer tissue > folate in inner tissue) suggest that flatter, longer strawberries may have greater potential to accumulate folate than fruit with a more spherical shape.

Storage stability of folate in strawberry fruit

The storage stability of folate in strawberry fruit is another important parameter for the evaluation of strawberry as a valuable dietary source of natural folate.

Red Rhapsody strawberries, an important commercial strawberry cultivar in Australia, were sourced fresh from a commercial farm in Brisbane. Total folate content and individual folate vitamers in the strawberry samples (fresh and during a 14-day storage trial at 4°C) were determined by SIDA (Striegel et al., 2018).

Total folate content was 102 ± 4.26 µg/100 g FW at day 0, which was well above the value in the AFCD of 39 µg/100 g FW. However, similar values have been reported by Tulipani et al. (2008) (up to 96 µg/100 g FW) and Striegel et al. (2018) (up to 153 µg/100 g fw), respectively. 5-CH3-H4folate was the main vitamer present.

Furthermore, folate concentration remained relatively stable during refrigerated (4°C) storage, with a loss of only 28% after 14 days of storage (Figure 6). No significant changes ($p > 0.05$) in the folate vitamer profile (ratio of vitamers) could be observed during the storage trial (Figure 7). The total folate content in Red Rhapsody after storage was still considerably higher than in the AFCD.

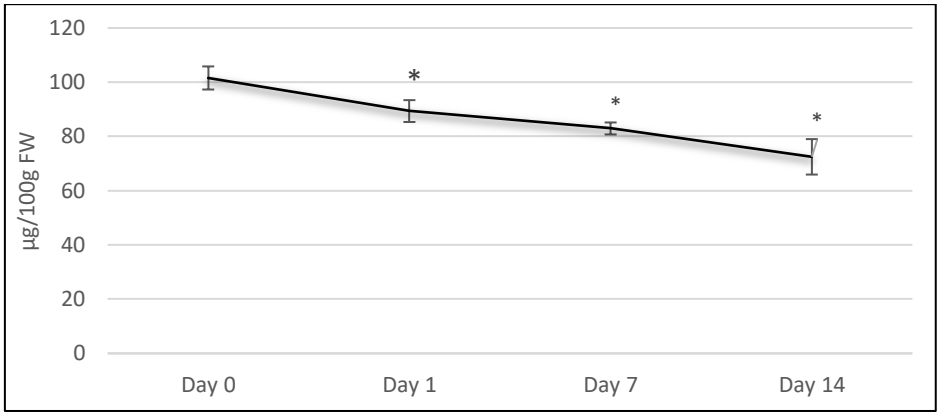


Figure 6. Total folate content in Red Rhapsody strawberries during a 14-day storage trial at 4°C. Total folate: sum of 10-CHO-PteGlu, PteGlu, H4folate, 5-CHO-H4folate and 5-CH3-H4folate (>90%); data are mean ± SD (n=5); *p<0.05 vs. day 0 (t-test).

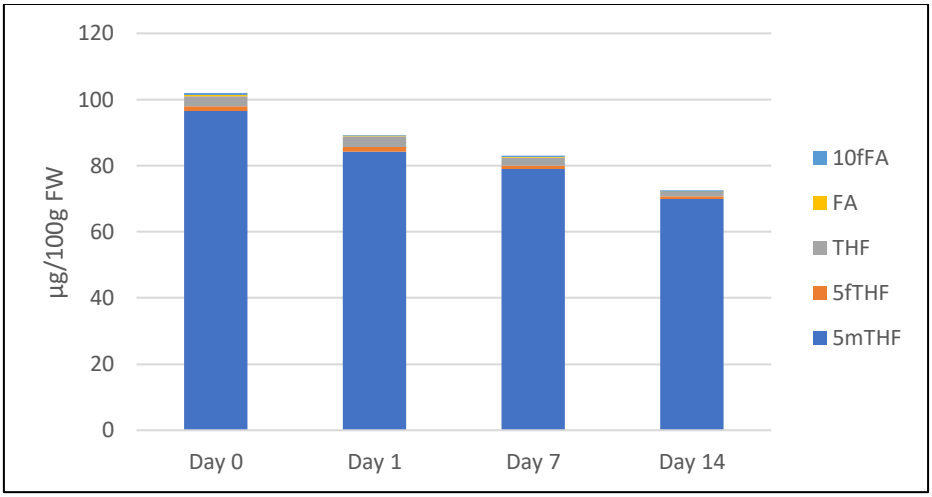


Figure 7: Folate vitamers in Red Rhapsody strawberries during a 14-day storage trial at 4°C; data are mean ± SD (n=5, error bars not shown); 10fFA: 10-formyl-pteroylmonoglutamic acid (10-CHO-PteGlu), FA: pteroylmonoglutamic acid/folic acid (PteGlu), THF: tetrahydrofolate (H4folate), 5fTHF: 5-formyltetrahydrofolate (5-CHO-H4folate) and 5mTHF: 5-methyltetrahydrofolate (5-CH3-H4folate).

The results of the storage trial are relevant for consumers since the inherent perishability of strawberry fruit makes refrigerated storage (approx. 4°C) common practice in Australian households. Furthermore, Red Rhapsody strawberries with a total folate content of up to 102 µg/100 g FW when fresh, can also be regarded as an important dietary source of natural folates even when stored at 4°C for 14 days.

Folate bioavailability study

The purpose of this study was to test the bioaccessibility and bioavailability of folate from the consumption of one punnet of strawberries in women of childbearing age to see if consumption of one punnet per day is a feasible option for improving folate intake in this group. It is important to determine whether the folate from strawberries is absorbed and whether it is better absorbed than a standard folic acid supplement (here 5-methyltetrahydrofolate (MTHF) as the biologically active form). The results will assist consumers to make a more informed decision about whether to utilise natural food sources of folate, such as strawberries, or take supplements for nutrition and will be of particular relevance to women in their childbearing years who are

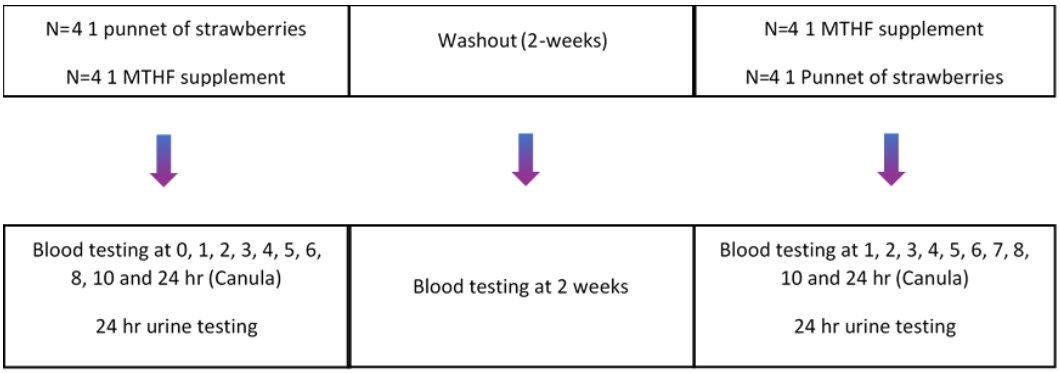
planning on becoming pregnant. Furthermore, benefits for strawberry growers are expected, as a broader range of consumers may begin to consume more strawberries more frequently.

Trial Design: A single-centre, randomised, two treatment, two period, cross-over trial was conducted with four healthy female volunteers, aged 18-40 years. Each arm of the trial lasted for 1 day, with a 2-week washout period in between. The order in which the treatments were given was randomised using a block randomisation design.

The study was run at the University of Queensland Clinical Research Facility at the Translational Research Institute (Princess Alexandra Hospital). This facility is fully equipped to run clinical trials with full clinical support, offering optimum conditions and safety.

The schematic for the study is outlined below:

Participants (n=4)



The four female participants adhered to their usual diet, but they received the MTHF vitamin supplement (500 µg) for 14 days before the first testing and between the testings (during the "washout"), which was discontinued two days prior to blood testing days. This "saturation" was done to improve uniformity among the four participants and subsequently the precision of bioavailability measurements. This methodology aligns with published folate studies previously completed (Mönch et al., 2010).

Intervention: Between 8 am-9 am, after an overnight fast, the participants consumed a punnet of commercial strawberries or the MTHF supplement, provided with some folate free food for breakfast (yoghurt, gluten-free bread [folic acid free], cheese). The experimental treatment period was 24 hours. The consumption of water was permitted ad libitum, and one further standardised and folate-free meal consisting of a milk drink and a cheese sandwich on gluten free bread [folic acid free] was offered for lunch and suggestions provided for dinner (e.g., frozen meals, lean cuisine with meat and pasta, scrambled egg on gluten free toast, meat with potato, carrot, corn). All food items were common brands and purchased at local supermarkets in Brisbane. Further study details are outlined in our Ethics proposal which was assessed and approved by the Human Research Ethics Committee of the University of Queensland (HREC Reference number: 2020002194). Blood and urine samples are currently being analysed for folate (vitamers and metabolites) and bioavailability/pharmacokinetic parameters will be determined and published as soon as the folate concentrations of the analysed samples are calculated. However, a relatively high folate bioavailability from Australian grown strawberries, similar to that found in a human pilot study with German grown strawberries (Striegel et al., 2018), is expected. The delay in conducting the human bioavailability study was caused by an unexpected long and severe impact of the pandemic on the operational capacity of UQ's clinical research facilities. It should be also mentioned that the relatively small number of participants (n=4) in the present study is not unusual for these kinds of human trials: Cao & Prior, 1999 (n=1), Cao et al, 2001 (n=4), Kay et al., 2004 (n=2) and Kay et al., 2005 (n=3).

Consumer response

A consumer focus group study was conducted to evaluate the consumer acceptance of new, novel bio-fortified fruit and vegetable products, including the high folate strawberry. The focus groups involved focus group discussions involving, separated into four demographic groups (~12 participants per group): young professionals (typically young people working full time or students who live in shared accommodation), couples with children, couples without children and empty nesters (seniors, living alone or with their partners).

The high folate strawberry concept was the least popular amongst the three concepts that were presented (cf orange capsicum, high lycopene tomato) as the strawberries were not visually different and had a very specific target market. Many participants agreed that the strawberries would appeal to women trying to be pregnant, however, they were not interested as they did not identify themselves as the target market. Participants loved the concept that a single punnet was equivalent to the Recommended Dietary Intake (RDI) of folate, as it was a logical, measurable dose that they could simply understand. The extra folate, however, was not enough to capture their attention, given that there are a number of other sources of folate available in other food products such as breakfast cereals and bread. Many of the participants were not very concerned about the health benefits of strawberries, as they thought of them more as a 'treat'. Although targeted towards women aiming to be pregnant, the majority of young female participants, including a few expectant mothers, indicated that they would rather take a supplement tablet, than to consume a punnet of strawberries a day, as a tablet was thought to be more economical and have a better controlled dosage of folate. Some also had concerns as to whether one could overdose on folate if they consumed both supplement tablets and the high folate strawberries. However, the high folate strawberries were thought to be a good gift to purchase for baby showers or for daughters/daughter in laws, when they are ready for pregnancy. Although the vast majority of the participants did not recognise themselves as the target market, it was agreed that other health benefits of folate that are applicable to everyone should be advertised, as it can appeal to more consumers. On average, consumers were willing to pay around $3.00-$3.50 per punnet to try, but not for regular purchase. This was around the same or slightly more compared to a regular punnet of strawberry. If they were priced the same as regular strawberries, participants indicated that they would purchase over regular strawberries.

Industry feedback

Satisfied with the folate strawberry outcome and satisfied to see this aspect of the project go further (the 'pregnancy berry'). Will the consumer differentiate between natural folate and an added folate compound?

Research needs to focus on marketability with the new product, and it must benefit both growers and the industry.

Sensory and consumer acceptance: problem with folate-rich cultivars and breeding lines since there is no visual difference as for the dark/anthocyanin-rich strawberry. A "smart/innovative" packaging would be a possibility to address this issue.

Strawberry waste is a serious concern for the industry and there is great interest in sustainable/cost-efficient ideas or concepts to utilise this waste material (e.g., isolation of folate from strawberry waste and its application in nutraceuticals/functional food products).

Awareness that strawberry cultivars only have a limited "market-life", usually a couple of years.

Outcomes and Recommendations

Overall, Queensland, and possibly Australian, strawberries were considerably higher in folate than listed in the Australian Food Composition Database (AFCD).

Three high-folate strawberry breeding lines have also been identified, such that a 250 g punnet of these accessions would supply the RDI of folate for adults which is 400 µg dietary folate equivalents/day.

Total folate content in the outer strawberry tissue was 1.5-1.8times higher than in the inner tissue suggesting that flatter, longer strawberries may have greater potential to accumulate folate than fruit with a more spherical shape.

Folate concentration in the commercial cultivar Red Rhapsody remained relatively stable during refrigerated (4°C) storage, with a loss of only 28% after 14 days of storage. These results are relevant for consumers since the inherent perishability of strawberry fruit makes refrigerated storage (approx. 4°C) common practice in Australian households.

Consumer interest in high-folate strawberry is low to moderate based on no visual distinction from other fruit, and that folate can be accessed from other sources.

A human bioavailability study was conducted with four healthy female volunteers at the University of Queensland Clinical Research Facility in Brisbane (a punnet of commercial strawberries (250 g) vs. a folate

supplement). Blood and urine samples are currently being analysed for folate and bioavailability/pharmacokinetic parameters will be determined and published as soon as the folate concentrations of the analysed samples are calculated. However, a relatively high folate bioavailability from Australian grown strawberries, similar to that found in a human pilot study with German grown strawberries, is expected.

A consistent finding within the Naturally Nutritious project was that the folate (Vitamin B9) level in Queensland-sourced strawberries was well above (at least double) the average folate level cited in the AFCD for this fruit. From a label claim perspective, this would potentially enable strawberries being listed as 'a good source of folate', rather than the present lower-level statement of 'source of folate'.

Technically, it was also found that at least three breeding accessions of strawberry had approximately four times the level of that listed in the AFCD, such that a 250 g punnet of these strawberries would actually supply the RDI of folate for adults. If increasing folate was to be pursued within a breeding program, it also appeared that those breeding accessions at the higher folate concentrations were also genetically related, so it is likely that this trait is highly heritable.

The finding that folate was consistently 1.7 times higher in outer tissue compared to inner tissue would tend to indicate that flatter longer fruit would have a higher folate level than more spherically shaped fruit, because of the greater surface area to volume ratio tending to favour a greater volume of outer tissue.

An intervention study over 4-6 weeks with more participants (≥30) should be conducted to determine the acute effects of strawberry consumption not only on the blood (plasma and erythrocyte) folate levels, but also on different health (bio)markers such as blood glucose, blood lipids (cholesterol, triglycerides), anti-inflammatory markers, and blood pressure. Such an evidence-based study would provide a useful marketing-tool for the Australian strawberry industry to promote the potential longer-term health benefits of domestic grown strawberries (similar to the American "Blueberry-Story").

From a consumer perspective, folate content was not seen as an important driver for strawberry purchase, despite its health benefit. Despite the importance of folate to females prior to pregnancy, in regard to preventing spina bifida in the developing foetus), the availability of folate as a supplement was considered sufficient, even to this section of the focus group. Males were even less interested in folate. Higher folate was not seen as a negative trait, but also not as a positive trait for purchase. In this regard, folate can be seen as a 'bonus'. Based on this, further breeding for enhanced folate in strawberries is not considered a priority.

However, industry was quite interested in establishing the higher folate content for Australian strawberries than what is officially in the nutritional tables for this fruit. The fact that label claims can be made, and that it potentially would apply to the whole Australian industry, was definitely worth pursuing. As the current assessments of folate only utilized Queensland-sourced strawberries, this would require an Australian-wide sub-sampling, potentially in a stand-alone project. Food Standards Australia New Zealand (FSANZ) were also supportive of this and would be quite happy to update their nutrition tables, accordingly.

High-zeaxanthin Orange capsicum

Background

Health issues and visual differentiation

Among all the reasons behind blindness in people in Australia, age-related macular degeneration (AMD) is the leading cause (Keel et al., 2017). The macula of the eye is the central region of the retina that we use for reading, driving, recognising faces, thus the degeneration of the macula leads to debilitating 'central' blindness. At present, about one in seven Australians, or 1.29 million people over the age 50, have some evidence of this disease. It is estimated that approximately 17% of these people (over 200,000) will experience vision impairment. Almost 15% of Australians over 80 years of age (around 160,000) have vision loss or blindness from AMD (MDF website, 2020).

Zeaxanthin and lutein are carotenoid pigments derived from plants, that we absorb from our food to form protective macular pigments (Arunkumar et al., 2020; Abdel-Aal et al., 2013). They play a protective role in shielding the retina from light damage and reducing the progression of AMD (Tosini et al., 2016)]. Lutein is mainly deposited towards the periphery of the macula, while zeaxanthin is deposited towards the centre, where our photo-receptors are at their most dense concentration.

A dietary intake of 10 mg and 2 mg per day of lutein and zeaxanthin, respectively, has been associated with reduced progression of AMD (Chew et al., 2014). People with AMD commonly have low macular pigment optical density. Studies have found that increased intake of zeaxanthin and lutein leads to a greater pigment density, within weeks of consumption. As both lutein and zeaxanthin cannot be synthesised by humans, their supply through dietary sources or supplements is vital (Perera and Yen, 2007). Although green leafy vegetables such as spinach and kale are good sources of lutein, dietary sources of zeaxanthin are much less common, limited to a few orange-coloured fruits and vegetables, such as orange capsicums and sweet corn (Perry et al., 2009). Lutein and zeaxanthin have now been added to artificial nutraceutical supplements around the world for treating macular degeneration, in line with the recent results of the Age-Related Eye Disease Study 2 (AREDS2), a large-scale clinical study in the United States.

As sweetcorn has been reported previously as a potential source of zeaxanthin, we successfully developed a zeaxanthin-biofortified sweetcorn with sufficient concentration of zeaxanthin to allow intake of 2 mg zeaxanthin as part of a normal meal. Orange capsicums, however, are potentially an additional source of zeaxanthin, and offer another horticultural product that may aid against the progression of AMD.

Some orange capsicums have been identified as containing a high percentage of zeaxanthin (Robman et al. 2007), but many varieties exist, and orange colour can potentially be induced by other non-macular pigments. Consequently, a scan of existing varieties for zeaxanthin concentration is required, firstly to identify varieties with a high percent zeaxanthin, and secondly to evaluate the actual zeaxanthin concentration to establish the amount that would have to be consumed to achieve an intake of 2 mg.

A particular advantage of zeaxanthin is that this phytonutrient gives a fruit or vegetable a bright orange or golden colour, which can differentiate it from products not containing zeaxanthin. This is particularly so in capsicum, where red, green, and sometimes yellow fruit, all low in zeaxanthin, predominate the Australian market. Orange capsicums are not totally new to the market, although their high-zeaxanthin status, and benefit to AMD, is largely unknown by consumers and the industry. In addition, the benefit of increasing macular pigment optical density can now be easily assessed non-invasively by ophthalmologists, allowing positive feedback to affected consumers.

Technical feasibility

Orange capsicum germplasm & zeaxanthin content

Eight accessions/varieties of orange capsicums were able to be sourced in Australia for the current assessment (Table 1). For further studies on the orange bell peppers following eight varieties, all belonging to *C. annuum* were investigated for their carotenoid profiles to identify high zeaxanthin varieties.

Table 1: Sourced accessions of orange capsicum and their subjective/objective colour.

Cultivar name	Seed source	Colour	Hue angle
Mini Sweet pepper	Coles (Supermarket)	Orange	63.50
SV3936PS (PS)	SEMINIS	Orange	60.35
Orange Bell	The Climbing Fig	Orange	59.91
Orandino	South Pacific Seeds	Orange	57.04
199-9	South Pacific Seeds	Orange	54.72
Boogie RZ	Rijk Zwaan	Orange	54.61
DSP 7054	De Ruiter	Orange	51.62
179-8	South Pacific Seeds	Dark-Orange	42.56

Zeaxanthin was observed to be the principal carotenoid present in seven of the eight accessions assessed (Figure 1), although its concentration varied from 15-fold from 1.8 mg/100gFW to 28.0 mg/100g FW (Table 2). Interestingly, the variety '179-8', which was a darker orange hue than the other orange varieties, had violaxanthin (33%) and capsanthin (39%) as its major carotenoids.

While the orange colour of the seven accessions can be explained by the presence of the orange pigment, zeaxanthin, the dark-orange colour of '179-8' appeared to be due to a mixture of the yellow and red carotenoids, violaxanthin and capsanthin, resulting in a dark-orange colour. Consequently, although it is likely that consumers purchasing an orange capsicum will end up with a fruit high in zeaxanthin, it is theoretically possible though unlikely (179-8 is not a commercial variety), that it could contain a mixture of non-macular carotenoids.

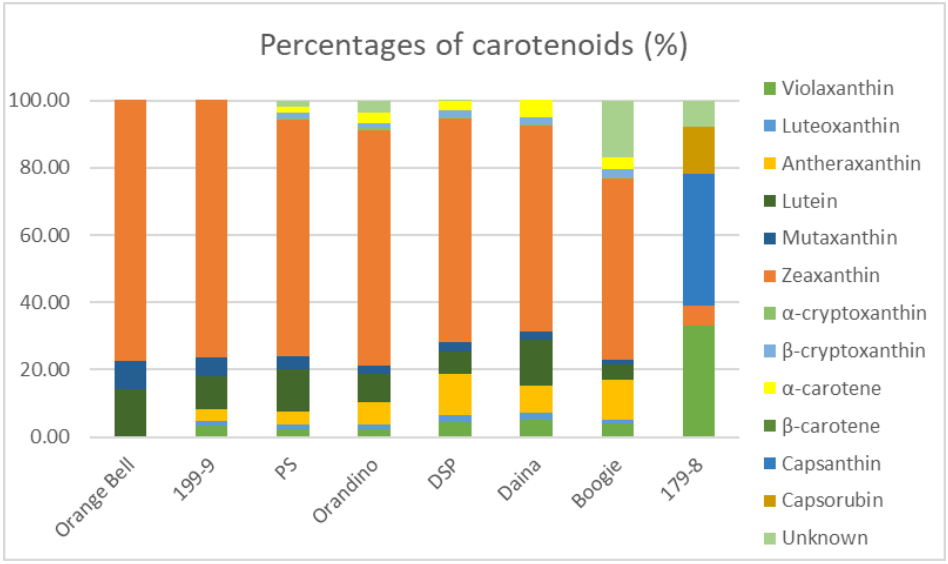


Figure 1: Carotenoid profiles of orange capsicums. Zeaxanthin was the principal carotenoid present in seven of the eight accessions.

Table 2: Zeaxanthin concentration and the tissue needed to meet the daily dietary intake of zeaxanthin of 2mg/person/day.

Variety	Total Carotenoid Conc.	Zeaxanthin conc. (mg/100gFW)	Tissue needed (g)
Orandino	40.0 ± 12.3	28.0 ± 8.5	7
Boogie	50.3 ± 22.5	27.0 ± 9.4	7
PS	29.1 ± 13.7	20.5 ± 10.3	10
Mini Small	22.0 ± 14.1	13.5 ± 8.4	15
199-9	10.4 ± 3.2	8.0 ± 2.7	25
DSP	9.3 ± 1.5	6.2 ± 1.0	30
BP	2.4 ± 0.1	1.9 ± 0.1	100
179-8	3.7 ± 1.2	0.2 ± 0.1	910

The proportion of zeaxanthin in the seven accessions above also varied in the proportion of zeaxanthin, ranging from 50% to 75% of the carotenoid pigments present (Figure 1). Although this has an impact on zeaxanthin flesh concentration, total carotenoid synthesis also varied substantially from 3.7 to 40.0 mg/100gFW (Table 2). Consequently, even though zeaxanthin may constitute a high percentage of carotenoids present, if the total carotenoid concentration is low, then final zeaxanthin concentration is also negatively affected.

The reason for lower total carotenoid concentration, and hence zeaxanthin concentration, can be for at least two reasons, one being a naturally lower rate in the carotenoid pathway of carotenoid biosynthesis, or secondly due to a lack of suitable storage compartments (chromoplasts) in which carotenoids are stored within the fruit tissue. It is likely that in accessions in which the pigmentation becomes significantly paler away from the skin surface, than chromoplasts are limiting, whereas accessions that are uniformly pale within the flesh are simply likely to have lower carotenoid synthesis.

From Table 2, it can be calculated that the accessions requiring the least amount of tissue to be ingested to meet the daily suggested intake of zeaxanthin of 2 mg/day, are 'Orandino' and 'Boogie' (7 g), whereas 25 g and 30 g of tissue would be required to meet the same suggested intake for the '199-9' and 'DSP' accessions. Despite its intense orange colour, the dark-orange accession '179-8' had the lowest zeaxanthin concentration, so that as much as 900 g would be required to meet the suggested zeaxanthin intake, which is clearly infeasible for most consumers.

In summary, orange capsicums are generally very high in zeaxanthin, with some varieties only requiring a small amount of fruit tissue (7 g) to satisfy the 2 mg/day recommendation for zeaxanthin uptake. The fact that the dark-orange capsicum contained very little zeaxanthin indicates that purchase purely on fruit colour is not a 100% guarantee.

Orange chilli germplasm & zeaxanthin content

Both hot chillies and capsicums are part of the same Capsicum genus, with some chillies also being the same species as capsicum (C. annuum). Chillies and capsicums can also be inter-crossed, although this varies in difficulty depending on the inter-species cross.

Because of the possibility that orange chillies may possess useful traits regarding zeaxanthin concentration that could be crossed into capsicum, or simply to supply a high-zeaxanthin chilli to the Australian market, several available orange chillies were assessed for their zeaxanthin concentration and carotenoid profiles.

Within the current trial, we were able to source examples of five orange chilli accessions from three Capsicum species (C. annuum, C. chinense, C. baccatum) and two orange capsicum accessions as controls (Table 3).

Table 3: Orange chilli and orange capsicum accessions sourced for analysis. Accessions were sourced from three species, varying slightly in orange colouration.

Cultivar/accession name	Species	Colour/ Pungency	Hue Angle	Shape	Source
PI 439420 01 SD (B58C)	*C. chinense*	Orange/Pungent	62.06	Pointed	USDA-derived accession
Diana	*C. annuum*	Orange/) Sweet	61.58	Mini small pepper	Coles (Supermarket)
Bulgarian	*C. annuum*	Orange/Pungent	61.08	Pointed	Chilliseedbank.com.au
Zara	*C. chinense*	Orange/Pungent	60.90	Pointed	USDA-derived accession
Orange Bell	*C. annuum*	Orange/Sweet	58.53	Blocky	The Climbing Fig
PI 439420 01 SD (B58B)	*C. chinense*	Orange/Pungent	56.41	Pointed	USDA-derived accession
Aji Amarillo	*C. baccatum*	Orange/Pungent	55.86	Pointed	Tim O'Hare

From Figure 2, it can be seen that despite all chilli and capsicum accessions being orange in colour (hue angle 55-62, Table 1), the carotenoid profiles were remarkedly different.

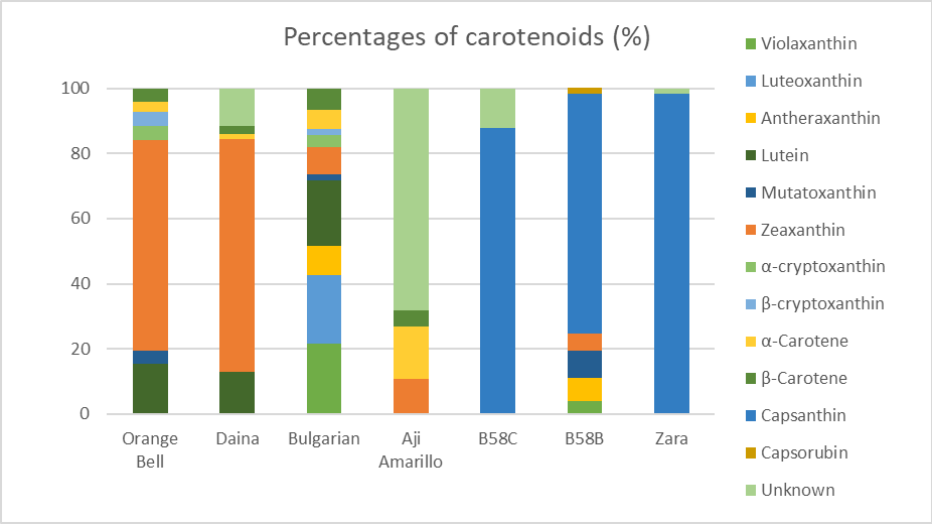


Figure 2: Carotenoid profiles of orange chillies.

It was observed that whereas the non-pungent orange capsicums (Orange Bell and Diana) had zeaxanthin as their principal carotenoid (65-70%), the remaining chilli accessions all had other carotenoids acting as principal carotenoids. Bulgarian chilli had no one major carotenoid but instead, consisted of multiple carotenoids such as violaxanthin (21%), luteoxanthin (21%) and lutein (20%) together comprising of 60% of total carotenoids. Aji Amarillo belonging to *C. baccatum* mostly consisted of a range of presently unidentified carotenoids (70%), while the other three accessions (B58B, B58C and Zara) had capsanthin as their principal carotenoid (70-90 %)

The current trial would indicate, at least for accessions available for analysis, that orange hot chillies are not a good source of zeaxanthin, despite being closely related to capsicums. In addition, because their orange colour is for reasons other than zeaxanthin, they are unlikely to offer in themselves an obvious benefit to further increasing zeaxanthin content in orange capsicums, and in themselves are not a source of zeaxanthin.

It is suggested that the mutations that resulted in the development of high-zeaxanthin capsicums occurred within the non-pungent capsicum and have since been bred across other non-pungent accessions. Consequently, if a high-zeaxanthin hot chilli was to be bred, it is suggested that superior red chilli accessions be crossed with a high-zeaxanthin orange capsicum, after which selection could be made after several generations for orange, pungent

segregants visually similar to the original red chilli. The identification of a pre-existing high-zeaxanthin orange chilli, or developing a pungent orange chilli through crossing, could be advantageous due to the potentially low weight of fruit tissue (e.g., 7 g) needed to achieve 2 mg zeaxanthin/day, and the low amount of hot chilli needed in recipes for condiment purposes.

Extension of shelf-life of fresh-cut capsicum

Fresh-cut capsicum shelf-life is generally limited by microbial growth. Aqueous extracts of three Australian native plants, namely Tasmanian pepper leaves, Boonjie tamarind, and Lilly pilly were observed to exhibit a significant antimicrobial activity against the isolated microorganisms from fresh-cut capsicums (*Pseudomonas viridiflava*, *Bacillus subtilis*, *Rhodotorula diobovata* and *Aleternaria alternata*). Therefore, the optimum combination of these extracts was selected as the natural and chemical-free preservative agents to be applied on the fresh-cut red capsicums.

Fresh red capsicums were purchased from local supermarket and disinfected for 2 min by dipping in a 2% sodium hypochlorite (v/v) solution, followed by a second 2-min dipping and rinsing in tap water. The washed capsicums were allowed to dry, and then the peduncle and seeds of the capsicums were removed, and the fruit cut lengthwise to produce 'capsicum sticks'.

Two treatments were conducted including (A) distilled water, and (B) 6% Tasmanian pepper leaves + 6% Boonjie tamarind + 10% Lilly pilly + 6% gum arabic. The fresh-cut capsicums were dipped in the working solutions for 2 min, and dried in a cabinet dryer for ca. 2 h. Three capsicum sticks were packed in polypropylene clamshells and stored at 4 °C for 18 days.

The microbial total plate count results demonstrated 3×10^7 growth on the control after 15 days, however, less than 100 was detected on the treated capsicums. The considerable efficiency of the optimum extract combination was established through these results and our previous experiments.

A pilot scale trial using red fresh cut capsicum prepared as described above treated with an improved optimized formulation was used. The trial comprised of an untreated control, potable water, 5% gum Arabic and 0.24% medium-chain triglyceride oil and dipped in 9% Tasmanian pepper leaves + 5% Boonjie tamarind + 5% Lilly pilly + 5% gum arabic + 0.24 %medium-chain triglyceride oil. All the fresh-cut capsicum were dipped in the given solutions for two minutes, dried and then packaged in polypropylene containers and stored for 4 °C for 16 days. There was a total of 144 packages, 6 sampling days, 3 replications, with each replication consisting of two packages with 5 sticks of fresh-cut capsicum.

After 16 days of storage the treated sample with antimicrobial plant extracts had a total plate count of 5 log CFU/g in comparison to the to the control (10.7 log CFU/g, potable water 8 log CFU/g and 9.5 log CFU/g gum arabic + medium-chain triglyceride oil). The yeast and mold counts were 1 -2 log CFU/g lower for the antimicrobial plant extract treatment at Day 13. The capsicum sample treated with plant extract during the two-week storage period had microbial counts that were within the acceptable levels for ready-to eat foods according to Food Standards Australia and New Zealand. The standard plate count results showed 5×10^{10} CFU/g growth on the control after 16 days, however, less than 1×10^5 CFU/g was detected on the treated capsicums.

According to the Australian New Zealand Food Standards Compendium of Microbiological Criteria in food, under Ready-To-Eat (RTE), in foods that contain components that are not cooked, a standard plate count ≥107 CFU/g is considered unsatisfactory. The untreated, potable water and gum arabic + medium-chain triglyceride oil samples in this study fell under this category and subsequently were not fit for consumption. This clearly shows that the native plant extract when applied as an edible coating in fresh-cut capsicum can very effectively extend the storage life to 16 days. Currently, fresh-cut capsicums have a shelf life of less than 7 days.

Consumer response

Focus group consumer study on product concepts

A consumer focus group study was conducted to evaluate the consumer acceptance of orange capsicums bio-fortified with zeaxanthin. The focus groups involved focus group discussions involving, separated into four demographic groups (~12 participants per group): young professionals (typically young people working full time or students who live in shared accommodation), couples with children, couples without children and empty nesters (seniors, living alone or with their partners).

Overall, the concept of orange capsicums (Figure 3), biofortified with zeaxanthin, was well received across the four demographic groups. All four demographic groups were attracted by the vibrant orange colour and indicated that they would purchase them to try, based on the visual appeal. The health benefit of zeaxanthin, however, was more

appealing to the empty nesters group, who were more concerned about macular degeneration, compared to the other groups with younger participants who were not very aware of this health problem.

Figure 3: A visual example of a large, block-shape, orange capsicum (specialtyproduce.com, 2021).

On average, consumers were willing to pay approximately $2.50 - $3.00 for an individual orange capsicum to try, slightly more or around the same price of a single yellow capsicum. The empty nesters were willing to pay the most out of the four demographic groups, while the young professionals were willing to pay the least. Repeated purchases at this price point, however, would be dependent on the taste.

Participants across the four demographic groups were interested in the dosage amount of the orange capsicums, in order to gain the health benefits, as that was also a contributing factor as to whether they would make continuous purchases. Another point that many consumers were interested in, was whether zeaxanthin is denatured during cooking, as that was an important factor that determined how they would consume the orange capsicums.

Across the four groups, most said that they would try to eat it raw more in a salad, or slightly cooked in a stir fry to preserve the nutrient and colour and would not use it in a casserole or stew, where the colour is lost. All four groups agreed that naming it simply as ‘orange capsicum’ was very suitable, as other capsicums are named for their colour. Other ideas included ‘sunset/sunrise capsicum’ and ‘golden capsicum’. However, a concern from the young professionals was that a weird name may be less appealing as it could be associated with GMO.

Industry feedback

Although orange capsicum cultivars are presently available to growers, the lack of orange capsicums on the market is largely a decision by larger supermarkets, in which the orange capsicum are just seen as another colour of capsicum. It is possible that yellow capsicums (do not contain zeaxanthin) may be currently preferred to orange capsicums, possibly due to a larger visual colour difference to red and green capsicums, although this has not been confirmed.

In glasshouse-grown environments, the decision of growers to grow or not grow orange capsicums is based largely on market demand. For field-grown capsicums, the price of seed becomes an important factor, with seed of red cultivars being significantly cheaper than orange capsicums.

Based on the discussions with industry there is a need for natural preservation solutions that are environmentally friendly for storage life extension of both whole and fresh-cut capsicum. A storage period of in excess of 1 week for fresh-cut capsicum is attractive for industry adoption.

Outcomes and Recommendations

The majority of orange capsicums available for assessment were very high in zeaxanthin, making them an ideal vegetable to be included in a diet aimed at reducing the progression of age-related macular degeneration. The level of zeaxanthin varied somewhat between varieties, although generally the concentration of zeaxanthin present in orange capsicum fruit was higher than other known dietary vegetables in the marketplace, including sweetcorn, which

is normally associated with high zeaxanthin concentrations. In some cases, the level of zeaxanthin was sufficiently high enough, that only 7 g of orange capsicum provided 2 mg zeaxanthin, or what is considered a daily dose in macular degeneration supplements.

In contrast to orange capsicum, orange varieties of chillies (closely related to capsicum) that were also assessed were not a good source of zeaxanthin. The orange colour appeared to be due to other orange-coloured carotenoid pigments, or alternatively, a mixture of red and yellow pigments. This, however, does not rule out the possibility of developing a high-zeaxanthin orange chilli, which would be fairly straight-forward from a breeding perspective. Considering the small amount of tissue required to provide 2 mg zeaxanthin, a high-zeaxanthin chilli may easily provide this, as part of a meal, where hot chilli is a spice ingredient.

Although orange capsicums appear to be an excellent dietary source of zeaxanthin, we are still unsure of the relative bioavailability of capsicum zeaxanthin for uptake in the human gut. We still need to evaluate this, although it is likely to be highly available. As with all carotenoids, the addition of oil (e.g., salad dressing) assists with uptake.

The next step, we consider, is to communicate with organisations such as the Macular Disease Foundation, and the Nutrition Society of Australia, to provide them with any necessary evidence to provide awareness that orange capsicum should be provided in any dietary advice.

From a consumer perspective, the vivid visual appearance of orange capsicum was enthusiastically accepted. This was further so with the provision of information that it was potentially helpful to slowing age-related macular degeneration, although this was of most interest to the older age-group, where it is obviously more relevant, age-wise.

From an industry perspective, the present lack of orange capsicums in the market seems to be primarily driven by lack of demand by the supermarkets, although field-grown capsicum can also be significantly influenced by the higher price of seed. Orange capsicums are currently seen as merely another coloured capsicum, amongst red, green and yellow (none of which are good sources of zeaxanthin). Increasing demand for orange capsicum is most likely to come from consumer-demand, which in turn is likely to come from nutritional dietary advice via organisations such as the Macular Disease Foundation. The possibility of label ‘endorsement’ by this foundation should also be investigated.

The pilot study for shelf life extension of fresh-cut capsicums using plant extracts indicated that the natural plant extracts used were very effective as an antimicrobial preservation solution and there was significant reduction of microbial counts during the two-week storage period at chilled temperature. Taking into full scale commercialization with an industry partner will need further trials for scale up and validation at factory premises.

Early season high-anthocyanin Queen Garnet blood plum

Background

Health issues and product visual differentiation

The 'Queen Garnet' plum has been highly successful on the Australian (and overseas) market. It is a high-anthocyanin Japanese blood-plum characterised by its high anthocyanin concentration of skin and flesh, but also its flavour and texture. Considerable marketing has been conducted to promote the sale of this fruit, which has commonly sold at 2-3 times the price of other plums on the market.

Anthocyanin has been linked to benefits for cardiovascular disease, cancer, and reducing weight, although the latter claim was derived from trials with obese rats, rather than humans. The putative health benefits of the Queen Garnet plum have undoubtedly helped the sale of this plum, together with its attractive dark appearance and flavour.

One of the issues with the Queen Garnet plum is its narrow harvest season, which in Australia is relatively late for plums, being from approximately mid-February to April. In an attempt to bring forward the season, the Queen Garnet plum was crossed with a plum-cot, cv. Rubycot, which is itself a cross between a blood-plum (red-anthocyanin flesh) and an apricot (no anthocyanin in flesh). Rubycot was selected as a crossing parent because of its apricot-derived very early harvest time (mid-November) and its red flesh characteristics.

The purpose of this cross was initially to develop a large segregating population of progeny (~400 seedlings) that could then be assessed (phenotyped) for physical characteristics (high anthocyanin development/flesh colour) and their harvest time. This population would then be used to identify genetic markers for the high-anthocyanin characteristic, that could then be used in a breeding program for future selection of high-anthocyanin fruit.

In conjunction with the genetic marker development for a high-anthocyanin plum breeding, consumer focus groups were also assessed on the concept of the cross between the Queen Garnet plum and the Rubycot, and what they would like to see as an outcome, as well as what they would not like to see. Because the progeny was still to be produced, the consumers were able to examine and taste the fruit of the two parents, rather than the progeny themselves.

Industry feedback was also obtained from the current licence-holder of the Queen Garnet plum.

Technical feasibility

Germplasm & Developing an early-season Queen Garnet blood plum

Crosses between adjacent rows of trees of the Queen Garnet and Rubycot parents were conducted at Applethorpe Research Facility in 2016 (uncontrolled pollination) and 2017 (controlled pollination). The number of successful crosses from the controlled pollination trial was lower than predicted, due to rain during the pollination period in 2017. The progeny of the uncontrolled and controlled pollination crosses between 'Queen Garnet plum' and 'Rubycot' were stratified in a cool-room and then planted in seedling trays, prior to transfer to the field at Applethorpe Research Station in 2017 and 2018, respectively.

A total of 432 seedling plum, apricot and hybrid trees were planted at Applethorpe Research Facility as part of the project (Table 1). This population included open-pollinated seed collected from adjacent rows of 'Queen Garnet' and 'Rubycot' which are expected to include hybrids of the two parents as both are self-infertile and obligate outcrossing. Additionally, controlled cross-pollinations were performed to produce plumcot hybrids.

Table 1: Numbers of seedling trees field planted at Applethorpe Research Facility from 2016-2019 as part of Naturally Nutritious project.

Seed parent	Pollen parent	Number
Queen Garnet	OP	85
	Rubycot	40
Rubycot	OP	304
	Queen Garnet	3
		432

The trees were badly affected by drought, wallaby damage and lack of irrigation water. As supplies of irrigation water dwindled at ARF the remaining water became salty and this imposed a further restriction of tree growth. Although the first fruit from these seedlings were expected to be available for phenotyping in the 2019-2020 season, the fruit were

not available due to the unusually severe drought in 2019. Apart from generally poor growth due to a shortage of, and poor quality (high salinity) of irrigation water at the DAF Applethorpe Research Facility, a number of progeny seedling trees subsequently died, or were diminished by herbivory (starving wallabies). It was therefore not possible for the fruit from the crosses to be able to be phenotyped within the lifespan of the Naturally Nutritious project, which ends May 31st, 2021.

Despite these deprivations, twelve trees produced fruit that were evaluated in the 2020-2021 season. The following illustrate the combinations of plum and apricot we observed. Q715-6 was a controlled-cross of 'Queen Garnet' and 'Rubycot'. The fruit showed predominately the characteristics of the 'Queen Garnet' parent except for an earlier harvest-date inherited from the 'Rubycot'. Fruit was harvested at eating ripe, on 8th January 2021 (Figure 1). The fruit were small-medium size (mean weight = 61 g), oblong with a red skin and red flesh. The flesh was firm, melting, sweet (TSS = 12.5%) and acidic, with a balanced plum flavour (rated 7 out of 9) and semi-clingstone. The crop was light. The tree has been propagated for testing under less stressful conditions.

Figure 1: Red-skinned, red-fleshed fruit of 'Q715-6' from the hybrid cross between QG plum and Rubycot plumcot.

A second selection from the 2020-21 season was Q711-40 (Figure 2). It was an open pollination of 'Rubycot' and the small tree produced a moderate crop with an earlier harvest date of 10th December 2020. Fruit resembled the 'Rubycot' parent, more than the QG plum parent. Fruit was small (33 g), with a slight fuzz similar to an apricot on the skin and round in shape. The fruit had a balanced flavour (more plum-like than apricot) and a yellow skin over a pale red flesh, which gave a mottled appearance. The fruit are probably too small to be of commercial value.

Figure 2: Yellow-skinned, pale red fleshed fruit of 'Q711-40' hybrid plumcot

Genomic sequencing of Queen Garnet and Rubycot

Genomic sequencing of the parent cultivars, Queen Garnet and Rubycot, was conducted by QUT, using both younger tissues and older stem growth harvested by UQ from the DAF Applethorpe Research Facility. The older stems were initially held under cool-room conditions to accelerate the production of new growth if DNA extraction needed to be repeated, but this was unnecessary. DNA was successfully extracted from the younger tissues and quality checked with Illumina, prior to NextSeq 500 sequencing. Genome sequencing of the two parent cultivars generated approximately 40 GB of data. The data-files are currently held at QUT.

In parallel with the genome sequencing, a literature review was completed with the identification of 23 relevant gene sequences. The biosynthesis and regulation of anthocyanin has been well described in reference models and crop plants, including a number of commercially important rosaceous crops that are related to plums and apricots. Although there are examples where genes involved in anthocyanin biosynthesis have been associated with elevated levels pigments, the genes responsible for anthocyanin regulation are most commonly associated with expression differences in segregating populations. Of the anthocyanin transcription regulators, the MYB class of transcription factors is most commonly associated with anthocyanin expression. This includes the MYB10 genes of apple, pear, strawberry, raspberry and other related rosaceous fruit crops. In addition, there are examples where the bHLH transcription factor is associated with anthocyanin accumulation in segregating populations. Most notably in pea, petunia and maize. Minor candidates for anthocyanin regulation also include WD-40 and NAC transcription factors.

Postharvest storage and tissue factors affecting anthocyanin content in Queen Garnet plum

Mature ripe Queen Garnet plums were obtained from a commercial grower in Queensland, Australia in the 2018/2019 season. Mature fruits were harvested and preliminarily stored at 2 °C for 3 weeks (as a pre-storage simulation). After 3 weeks, approximately 200 plums were transported to the laboratory at Coopers Plains (QLD) and 90 homogeneous fruits (size, colour and absence of any defect) were subsequently selected. The fruits were randomly divided into 9 groups of 10 fruits. Four groups were stored in a cold room at 4 °C, another 4 groups at room temperature (23 °C) and one group were used as reference for day 0.

On 0, 4, 7, 10 and 14 days of storage, a group of fruits was taken randomly from each storage temperature. Each fruit was weighed and cut along a suture into two halves and the peel was manually separated from the flesh. The flesh was further separated into outer flesh (OF; 7mm of flesh from the outer edge), and the inner flesh (IF) (Figure 3). Weight of each fruit tissue were recorded. IF and OF were separately pureed (MM400 Retsch Mixer Mill, Haan, Germany), and a portion was used to measure chroma, hue angle, total soluble solids (TSS) and titratable acidity (TA). The separated tissues were freeze-dried and ground into powder and stored at –20 °C for further analysis.

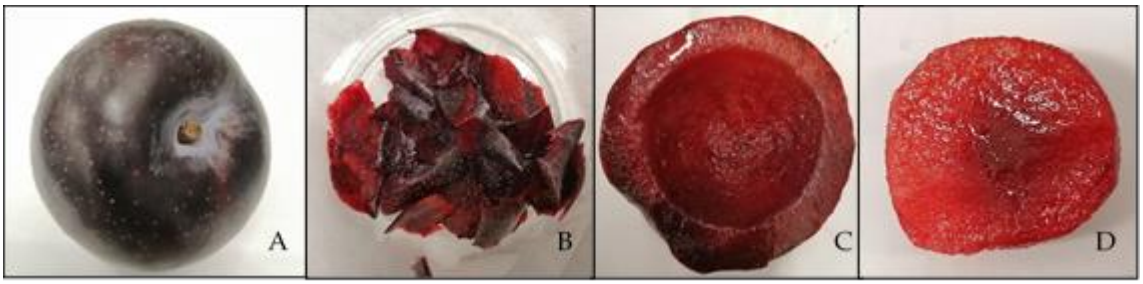


Figure 3: (A) Mature QGP; (B) Peel; (C) Outer Flesh – OF; (D) Inner Flesh – IF

Anthocyanins are the main pigments in Japanese plums having higher anthocyanin concentration in the peel than in the flesh (Fanning et al., 2014). The anthocyanin content of the plums varies noticeably among cultivars and continues to increase during ripening (Usenik et al., 2009; Jaakola, 2013; Usenik et al., 2008) and postharvest storage (Kumar et al., 2017). Queen Garnet plum is a dark red-fleshed and dark red-peeled ("blood") plum and anthocyanins are accumulated in both peel and flesh, and the accumulation varies between different tissues (Figure 4).

The main anthocyanins found in Japanese plums, including Queen Garnet, are cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside (C3R). TAC of the peel was highest ($p<0.05$) after 10 days of storage at 23 °C (3-fold increase), whilst at 4 °C, the highest anthocyanin content was recorded after 14 days of storage (1.2-fold increase) (Figure 4). Of the three tissues analyzed, peel had the highest anthocyanin content (1251 mg/100 g FW, day 10) followed by OF and IF (75 and 21 mg/100 g FW, respectively, on day 14).

The peel content in the present study is considerably higher than the previously reported highest peel anthocyanin concentration of 538 mg anthocyanins/100g FW (Bobrich et al., 2014; Fanning et al., 2013). This can be caused by differences in the initial maturity of the fruits and/or different storage conditions. Highest OF-TAC was observed after 14 days of storage at 4 and 23 °C and highest IF-TAC after 10 days of storage at 4 and 23 °C. There was a significant ($p<0.05$) difference between the TAC of the peel at 4 and 23 °C at each storage day. C3G was the predominant anthocyanin found in all the tissues. There was an increase ($p<0.05$) of C3G towards day 14 in peel (+20%), OF (+16%) and IF (+10%) at 23 °C, and peel (+4%) and IF (+17%) at 4 °C.

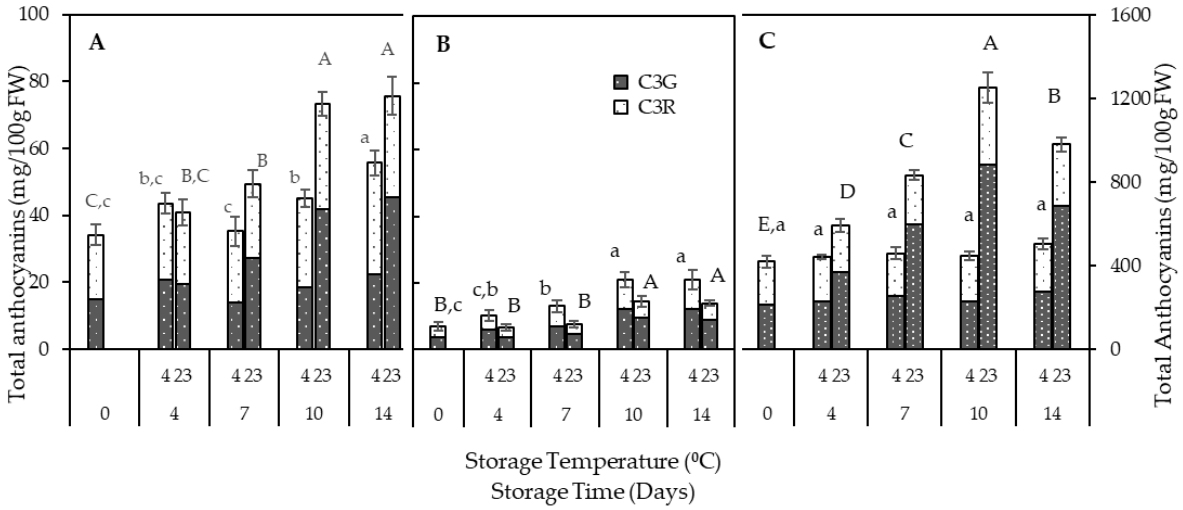


Figure 4: Anthocyanins in (A) Outer Flesh; (B) Inner Flesh; (C) Peel of Queen Garnet plum during storage at either 4°C or 23°C. Data are presented as mean ± SE (n=6-10). Different upper-case and lower-case letters indicate significant ($p<0.05$) differences between storage days.

The effect of storage temperature (4 and 23 °C) and storage time (up to 14 days) on the anthocyanin content in the different tissues of Queen Garnet plum were demonstrated in the present study. The increase in anthocyanins in flesh and peel was more prominent at ambient temperature (23 °C) than at refrigerated (4 °C) storage, indicating the importance of postharvest storage temperature to final anthocyanin concentration in this cultivar.

Assessment of an early-season Queen Garnet plum sibling '401-43'

In addition to baseline assessment of the Queen Garnet plum cultivar, assessment was also conducted of a Queen Garnet plum relative '401-43' (sibling), from the same breeding program from which the commercially successful Queen Garnet was originally derived. The sibling has an earlier harvest date (late December to early January) than the Queen Garnet plum (mid-February), and is roughly in the vicinity of what we were aiming for in the cross progeny.

Fruits were harvested from four '401-43' plum-bearing trees on 3rd January 2020 and from four Queen Garnet trees on the 22nd of January. These '401-43' fruit were harvested, based on subjective assessment of taste. This harvest date for '401-43' was quite close to the earlier harvest date aimed for. After cold storage at 5-6°C for 5 days, the '401-43' plums were then packed into large plastic bags (100/bag) and stored at -20°C until extraction and analysis.

The '401-43' sibling was observed to have a lower anthocyanin concentration (but still high compared to plums) compared to the Queen Garnet plum stored under similar postharvest conditions (Table 2). The anthocyanin concentration, using either HPLC or the AOAC method, was about 25% lower. The relatively low values of anthocyanin for both the Queen Garnet and the '401-43' than have been reported previously may be partly explained by the fruit not experiencing higher postharvest ripening temperatures, as the previous trial showed. Nevertheless, the trial enabled a comparison between fruit held under similar postharvest regimes.

Subjective tasting by the analysts prior to preparation also found that the skin of '401-43' was slightly bitter compared to the Queen Garnet, and in general the fruit-size smaller. Although the sibling may not be commercially viable in its own right due to these attributes, it could be potentially be crossed with the Queen Garnet plum to transfer the early harvest characteristic into the Queen Garnet genetic background. Because of their close genetic relationship (siblings), this is more likely to produce a fruit similar to the Queen Garnet plum, which was what consumer focus group testing indicated was preferred by the Australian market.

Table 2: Anthocyanin contents (mg C3G eq./100 g FW) and AOX capacities (mg AA eq./100 g FW) of 401-43 and QG plums grown at Applethorpe Research Facility during the 2019/2020 growing season as measured by two anthocyanin methods (HPLC-based method; the pH differential method) and a recently optimised AOX capacity assay.

Sample ID	Anthocyanin Content [mg of C3G eq./100g FW] by HPLC	Anthocyanin Content [mg of C3G eq./100g FW] by AOAC	AOX Capacity [mg of AA eq./100g FW] by optimised method
2020 QGP Tree 1	87.0	88.6	501.9
2020 QGP Tree 3	111.6	110.7	444.6
2020 QGP Tree 4	109.1	110.4	515.4
2020 QGP Tree 5	73.8	76.0	496.0
2020 QGP Average ± SD	**95 ± 16**	**96 ± 16**	**489 ± 31**
2020 401-43 Tree 1	80.2	80.2	518.5
2020 401-43 Tree 2	69.0	66.7	420.4
2020 401-43 Tree 3	69.5	67.0	428.8
2020 401-43 Tree 4	69.8	69.0	428.5
2020 401-43 Average ± SD	**72 ± 6**	**71 ± 7**	**449 ± 47**

Consumer response

The focus group assessment trial indicated that there was consumer enthusiasm for an earlier season version of the Queen Garnet plum, but also enthusiasm for a high-anthocyanin apricot. There was no interest in another type of plum on the market, as the plum market was already considered to have ample choice. In this regard, we believe it is important that a high-anthocyanin plum-cot be as similar to its Queen Garnet parent in every way, except for being harvested earlier in the season, ideally around the festive Christmas season. This way, the early season plum-cot will be able to capitalise on the existing Queen Garnet plum marketing, rather than further confuse the market with yet another plum variety.

Interestingly, there was enthusiasm for a high-anthocyanin apricot, as it was felt that there was presently only one type of apricot on the market. The flavour of the Rubycot plum-cot was unlike either a plum or an apricot and was generally very well received. Since our crosses are between a plum-cot and a plum, the progeny is more likely to resemble a plum than an apricot due to the genetic shift towards plum-like characteristics. It is possible however, that some high-anthocyanin progeny may exhibit apricot characteristics (e.g., freestone seed, apricot flavour), so this possibility should not be ignored. Keeping in mind that the focus group was mainly interested in an earlier season fruit similar to Queen Garnet plum, this would lend support for considering future crossing of the Queen Garnet with an early-harvest accession more similar in these flavour characteristics, such as the '401-43' sibling of Queen Garnet, to satisfy this requirement.

Industry feedback

An early-season (lower chill required to flower) Queen Garnet plum has commercial value to Nutrafruit and growers. Any fruit that can be exported into China before Chinese New Year will receive double the value of that arriving later.

According to Nutrafruit, a high anthocyanin plum-cot (with the flavour of a plum-cot, rather than a Queen Garnet plum) is nice to have, but not a 'need to have'. This is in contrast to an early-season Queen Garnet plum. No massive commercial outcome will be gained from plum-cot research, as plum-cot is such a minor crop.

Optimising the anthocyanin content in the Queen Garnet plum was also of particular interest, as it can cause the internal fruit colour to vary considerably.

Outcomes and Recommendations

Owing to the weather conditions (bushfire, drought-related low-quality water supply, wildlife herbivores), a significant

proportion of the seedling population failed to survive or failed to set fruit within the duration of the Naturally Nutritious project. Despite this, 12 crosses were able to be evaluated by the breeder. Of these, two possessed an earlier harvest date, one of which displayed red-flesh and red-skin characteristics similar to the Queen Garnet plum, while the second displayed some apricot-like characteristics, but with a plum-flavour.

The consumer focus group response was highly in favour of an earlier-season plum, similar to the Queen Garnet in characteristics, rather than a plum with different visual or flavour characteristics. This was primarily due to the feeling that there were already too many types of plums available, and that it was confusing to have yet another type. The ideal harvest time was considered to be within the Christmas festive period, rather than in the new year.

In this respect, the discovery of a sibling of the Queen Garnet plum, '401-43', which had still survived from a previous breeding program was found to have both moderate, but 25% lower levels of flesh-anthocyanin than Queen Garnet, a similar appearance to Queen Garnet, a slightly bitter flavour in the skin, but a Christmas festive period harvest time.

The closer genetic relationship to the Queen Garnet plum makes the '401-43' a potentially better cross candidate with Queen Garnet plum than the Rubycot plumcot, if an early-season Queen Garnet plum is required, as suggested by the consumer focus groups. A segregating population would potentially have both flavour and appearance characteristics closer to the Queen Garnet plum, and not introduce flavour factors from the apricot background of the Rubycot.

Unexpected consumer feedback also suggested the potential market acceptance of a dark anthocyanin-fleshed apricot, owing to the perception of there only being a single orange apricot currently on the market, in contrast to the wide variety of plums.

Industry feedback from the Queen Garnet licence holder was also in favour of an earlier-season Queen Garnet plum, stating that any fruit harvested before Chinese New Year was likely to receive twice the price of fruit harvested later.

Saffron Sweetcorn

Background

Health issues and product differentiation

Saffron is a spice that is harvested from the stigmas and styles of the flowers of the Crocus plant (Crocus sativus) and is considered the most expensive spice by weight globally, often described as being worth three times that of gold. The principal compound in saffron is crocin, or crocetin, an orange-coloured water-soluble pigment, that is derived from the enzymatic cleavage (CCD2) of the carotenoid pigment, zeaxanthin (Figure 1).

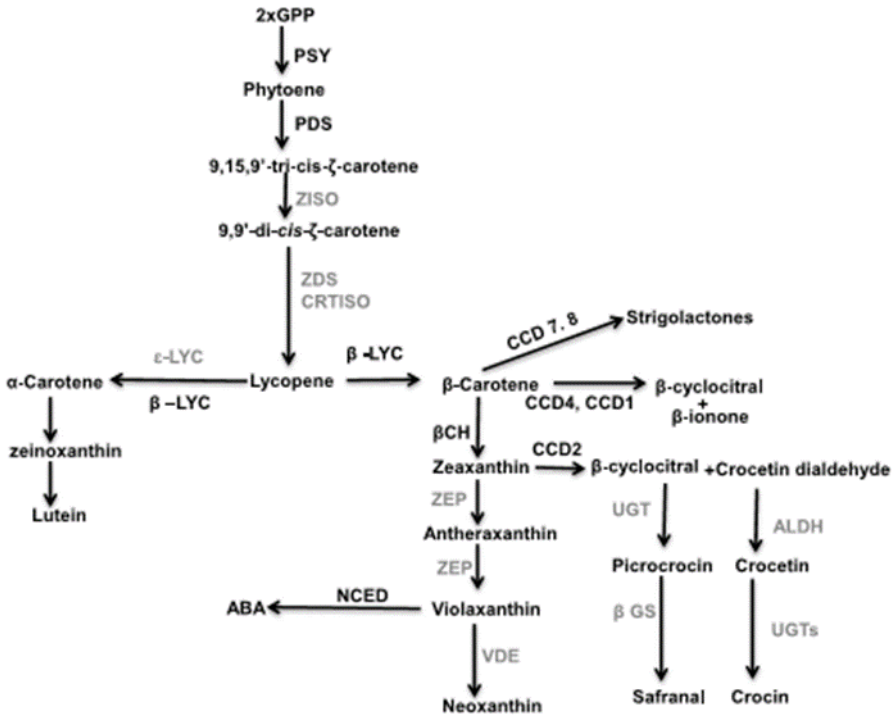


Figure 1: Pigmented saffron compounds, crocin and crocetin, are derived through enzymatic cleavage of the orange carotenoid, zeaxanthin.

Previously, we have developed high-zeaxanthin sweetcorn (Project VT07081, developing 'Super yellow' enhanced pigment sweet-corn for eye health), in which zeaxanthin has been enhanced by approximately tenfold in the kernels. During laboratory extractions for zeaxanthin analysis, it was observed that in some sweetcorn accessions, a water-soluble orange-yellow pigment was left behind once the zeaxanthin (and other non-water-soluble carotenoids) were separated. Preliminary comparison against a known chemical standard of crocetin indicated that the compounds may have been crocetin or a crocetin derivative.

Due to the market value of the spice, saffron, it was considered worthwhile to confirm the presence of saffron in these sweetcorn accessions, as a potential by-product, or primary product, that could be capitalised on by industry as a cheaper form of this very expensive spice.

From a health perspective, saffron has been reported to relieve certain forms of mild to moderate depression (Hausenblas et al., 2013, Siddequi et al., 2018). The studies reported that saffron was more effective than placebo and at least equivalent to the therapeutic doses of imipramine and fluoxetine. Saffron may act as an antidepressant effect by regulating mood-related chemicals in the brain such as serotonin. Although it has been proposed that saffron increases serotonin levels in the brain, the exact mechanism of action is unknown. More specifically, saffron extract might maintain serotonin in the brain longer.

The purpose of the current case study was to identify and confirm the pigmented aqueous compound observed previously in some zeaxanthin-biofortified sweetcorn accessions.

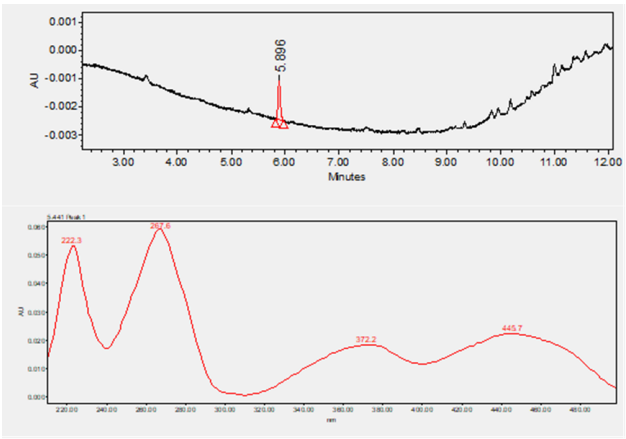
Technical feasibility

Pigment identification and quantification

This study looked at identifying possible saffron apocarotenoids in the kernels of 12 high zeaxanthin sweet corn inbreds which had been previously identified as having pigmented aqueous layers during analysis of zeaxanthin. The water-soluble compounds were extracted from the kernels using a 50% methanol: water (v: v) extraction procedure before the samples were analysed using UHPLC (ultra-high performance liquid chromatography) and LC-MS (liquid chromatography and mass spectrometry) to identify and quantify the polar yellow compound present.

The UPLC results showed there was no match between the sweet corn extract and saffron apocarotenoids. Figure 2A shows the chromatogram of the saffron extract with crocin compounds with retention times of 10.01 min and 10.89 min and absorbance profiles showing peak absorbance at 442 nm and 465 nm. The chromatogram for the sweet corn extract that identified a major peak at 5.8 min (Figure 2B) indicated that the peaks did not match, and therefore were not crocetin or its derivatives. The absorbance profile of the compound was conducive with a yellow pigment showing absorbance at 372 nm and 445 nm, which does not match the absorbance profile obtained from saffron.

A.



B.

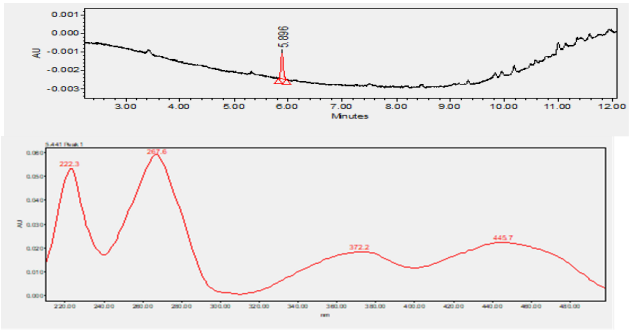


Figure 2: UPLC and LC-MS chromatograms for (a) saffron and (b) high-zeaxanthin inbred 6-1 indicated that they were not the same compound.

Figure 3 shows the major peaks of a known riboflavin standard at 5.9 min with an absorbance profile with peak absorbance at 372 nm and 445 nm, which is the same as the unknown compound observed in Figure 2b. The LC-MS therefore confirmed the identification of the pigmented aqueous compound as riboflavin, and not crocetin. The concentrations of riboflavin in the different sweetcorn lines are shown in Table 1.

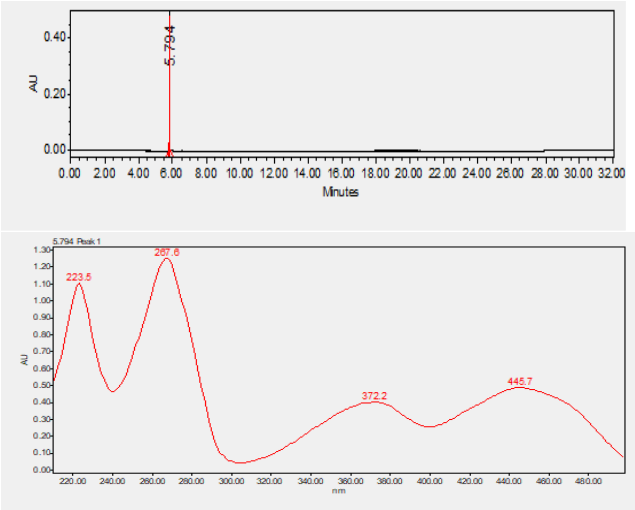


Figure 3: UPLC and LC-MS chromatograms for a known riboflavin standard, indicating that the identity of the unknown aqueous pigment in sweetcorn was riboflavin and not crocetin.

Table 1: Riboflavin concentrations and objective visual scores in sweetcorn accessions.

Inbred	Visual score	Chroma	Hue	Concentration (mg/kg)
6-1	2	7.91	103.12	2.49*
15-2	2.5	8.50	106.91	2.56*
15-6	2.5	8.88*	106.54	2.38*
23-6	1	5.77	107.96	2.15*
23-7	1	5.95	107.44	2.01*
23-1	3	8.70	107.41	2.23*
11-7	1	5.55	108.02	2.61*
13-2	1	5.78	106.92	2.50*
1-1	1	6.78	107.40	1.70
2-9	3	9.83*	107.25	2.52*
14-6	2	6.28	106.16	2.19*
10-3	1	5.26	108.19	1.97*
saffron	4	19.75	105.54	

*(P<0.05)

Consumer response

Not conducted, as not technically feasible without recombinant genetic modification.

Industry feedback

Not conducted, as not technically feasible without recombinant genetic modification.

Outcomes and Recommendations

Development of saffron sweetcorn appears only possible only through the introduction of the *Crocus* CCD2 gene necessary for cleaving zeaxanthin into crocetin. This would require genetic engineering methodology, such as CRISP-CAS9, which currently is not permitted in Australia for this type of genetic modification, without being categorised as genetically-modified.

As crocetin or crocetin derivatives were not identified within the available sweetcorn germplasm, the study was terminated, and no consumer evaluation or industry feedback was obtained.

The project identified that the compound responsible for the yellow-orange pigment was actually riboflavin (Vitamin B2), which is unrelated to crocetin. The possibility to develop a high-Vitamin B2 sweetcorn is a future possibility, but was not pursued in the current Naturally Nutritious project. It is possible that enhanced riboflavin may have been 'co-

selected' for during the previous development of high-zeaxanthin sweetcorn, as both zeaxanthin and riboflavin produce a similar colour, with the latter potentially contributing to kernel colour.

‘Deep-pink’ high-lycopene tomato

Background

Health issues and product visual differentiation

High-lycopene tomatoes with a deep-pink pigmented epidermis were developed previously in Hort Innovation project VT13002 ‘Optimisation of high-lycopene tomatoes for tropical conditions’.

Initially, they were not included in the Naturally Nutritious project, but were included following the termination of further examination of Saffron sweetcorn, which was found not to be technically feasible without genetic modification. Although deep-pink high-lycopene tomatoes satisfied the prerequisite of Naturally Nutritious as being visually differentiated and having a putative health benefit (prostate cancer), the health marker is not easily measured and may take years to develop, and consequently, the health benefits of consumption alone are difficult to promote re-purchase. Despite this, as this product had been developed, it was decided to further assess its consumer attributes.

High-lycopene tomatoes are difficult to differentiate from standard tomatoes, without being cut open to show their deep-red internal pigmentation, which is due to the enhanced levels of the red pigment, lycopene. Consequently, we previously added a natural skin mutation to the high-lycopene fruit, which allowed the underlying flesh colour to be visible through the tomato skin. The colour was more akin to a deep-pink colour, and visually differentiated from either normal high-lycopene tomatoes or normal red tomatoes (Figure 1).

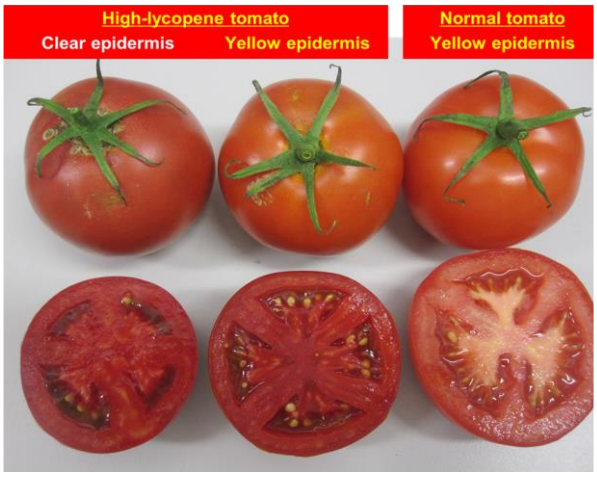


Figure 1: Deep pink high-lycopene tomato experimental hybrid ‘1-5x7-6’ with colourless epidermis (left), ‘3-4x8-1’ with normal epidermis (middle), and standard tomato control (right). Note the deeper skin colour on the Deep pink high-lycopene fruit at the left.

High-lycopene tomatoes have been previously developed partly for their enhanced red appearance when cut open, and the perceived health benefit of tomato/lycopene consumption to lowering the incidence of prostate cancer.

There is sufficient evidence for a protective relationship between tomato (Rowles et al., 2018), dietary lycopene (Rowles, et al., 2017) or serum lycopene (Rowles et al., 2017) and prostate cancer with dose–response relationships. Prostate cancer risk decreased by 13% at 200 g/week, 28% at 500 g/week, 46% at 1000 g/week, and 56% for 1350 g/week of tomato consumption (Rowles et al., 2018). Prostate cancer risk decreased by 1% for every additional 2 mg of dietary lycopene intake (Rowles et al., 2017). Additionally, prostate cancer risk decreased by 3.5%–3.6% for each additional 10 µg/dL of serum lycopene (Rowles et al., 2017). Lycopene, which is a powerful antioxidant in its own right, has been thought to be the active phytonutrient in tomato associated with health benefit (Agarwal and Rao, 2000; Fraser et al., 2005; Giovannucci, 2002), although health claims are still unable to be made.

Technical feasibility

High-lycopene experimental hybrid tomatoes with either deep-pink or standard red skin colour were previously developed in project VT13002.

During the Naturally Nutritious project, the inbred parent lines for each hybrid were re-propagated. During this time, it was noted by the propagator (Mr Ian Walker, DAF) that fruit of three of the four inbred parent lines were susceptible to displaying variable degrees of the physiological disorder ‘fruit pox’ (Figure 2), a recessive genetic

disorder portrayed as small occasional scattered blemishes (<1 mm), that only appear under certain environmental conditions (Crill et al., 1973). Their development is thought to be more severe when the fruit is exposed to high temperatures and the plant and fruit are growing rapidly. However, more work is still needed to fully understand the cause (Seminis, 2021). The first symptom on the green fruit is small, clear, or tan coloured. slightly elongated or oval lesions. As the fruit matures. these lesions enlarge and cause the fruit epidermis to rupture, imparting a necrotic corky appearance.

Figure 2: An example of the genetic disorder 'fruit pox' in ripe and unripe fruit (University of Florida, 2021).

Hybrid seed of the deep-pink and red hybrids were subsequently produced in 2019, with 1800 seed of the pink hybrid and 2000 of the red hybrid available for commercial assessment. Germination rate for both hybrids was approximately 95%.

The inbred parents of these two crosses were grown through for seed and tomato fruit pox (a recessive gene) was found in three of the four inbred parents. 1-5-1-1 (pink female), 8-1-1-1 (red female) and 3-4-1-1 (red male) had fruit pox, 7-6-7-1 (pink male) had no fruit pox, but only four fruit were available for assessment.

Tomato fruit pox assessment is problematic as it is a recessive gene and can be very variable in its expression. Even within a single bush one leader can be severely 'poxed' and the other free. The tomato lines are possibly segregating for the gene and it should be possible to select clean lines from them, but accurate genotyping is essential for this, as previous selections based on phenotypes have failed to rid the lines of this character.

Consumer response

Focus group consumer study on product concepts

A consumer focus group study was conducted to evaluate the consumer acceptance of new, novel bio-fortified fruit and vegetable products. The focus groups involved focus group discussions involving, separated into four demographic groups (~12 participants per group): young professionals (typically young people working full time or students who live in shared accommodation), couples with children, couples without children and empty nesters (seniors, living alone or with their partners).

The participants were a bit divided with the high lycopene tomato idea. While the majority of the participants across the four demographic groups agreed that the dark colour was appealing and associated it with better flavour, quite a few participants thought that, based on the image, the tomato looked over-ripe and assumed it would be mushy. However, if the tomato was firm in reality, they would consider purchasing it.

The health benefits of lycopene were again, more appealing to the older male participants, while the younger participants expressed that they were a bit sceptical about the health claim. However, if justified with more research, they would consider purchasing it to try, but repeated purchases, again would depend more on flavour, especially if sold for a premium price. Although female participants did not identify themselves as the target consumers, many still expressed interest in this product and would purchase it, provided that the flavour is good.

On average, consumers were willing to pay around $4.50 per 300 gram pack, more than the market price of existing premium tomato varieties such as Kumatoes ($4.00 per 300g pack). However, at this price point, many participants indicated that this would not be their regular tomatoes and would only be used occasionally for special events such as

dinner parties.

As many associated the darker colour with more flavour, quite a few participants indicated that they would use it in a sauce, while others would rather eat it raw more in a salad or sandwich to better appreciate the flavour and preserve the nutrients. Similar to the orange capsicums, participants were again interested as to whether lycopene is lost during cooking.

It was agreed that this tomato needed a name to differentiate it from other tomato varieties. Popular names included 'ruby tomato' and 'blood tomato'. Participants also suggested that having a blurb such as "eat _ tomatoes a week can reduces risks of prostate cancer by _%" would appeal more, as it gave a dosage indication.

Industry feedback

Limited industry assessment of fruit of the deep-pink hybrid was made during the initial VT13002 project, with a favourable outcome in regard to appearance, flavour and texture. The potential issue of 'fruit pox' has been a stumbling block, however, and needs to be overcome for commercial adoption of the deep-pink high lycopene tomato.

Outcomes and Recommendations

The issue of removing 'fruit pox' from the tomato inbred parent lines has been recommended to Queensland DAF Horticulture and Forestry Science, the owners of the germplasm. The process takes two generations (Crill et al., 1973), although the variability of its expression makes it difficult to phenotype. It was suggested that it would be firstly worthwhile investigating if a public domain genetic marker for 'fruit pox' was available.

It was further suggested that while this was happening, it would be of commercial value to grow the hybrid seed under commercial conditions (by a commercial tomato grower) both under glasshouse and field conditions to assess the agronomic characteristics of the hybrids. This would also allow fruit to be harvested for consumer assessment, which has been limited within the project to concept analysis by focus groups, based on photographs of the fruit.

Satiety & Satiation

Background

Satiation and satiety are complex perceived feelings, and major mechanisms controlling food intake in humans. Satiation is the feeling of fullness immediately after consuming food, whereas satiety is the postprandial feeling that determines the transition from fullness to hunger and can therefore influence subsequent meal initiation. These feelings are affected by many factors such as environment, human metabolism, psychological attributes, and, specifically, food features.

Previous work has focussed on the role of food composition, particularly the amounts of major nutrients such as fat, protein, carbohydrate, as well as dietary fiber. For example, increasing the protein content of the diet with the same amount of energy can enhance satiety. It has also been reported that dietary fiber influences satiation and satiety, possibly through changing the viscosity and bulk volume of food in the gastrointestinal (GI) tract, particularly in the stomach. Increasing energy density can also slow down gastric emptying and enhance satiety. However, by focussing on molecular composition, these previous approaches typically ignore the physical structure of the foods.

Plant-based foods are recommended to be the cornerstone of a healthy diet and include fruits, vegetables, and nuts, which all have a cellular structure. It is widely accepted that plant cellular structures affect nutritional functionality of food via their effects on digestion and fermentation in the GI tract. As plant-derived foods originate from a wide range of plant species and different parts of plants, their microstructure especially cell wall characteristics can show large variations with major effects on the texture of foods as well as nutrient release in the GI tract.

Plant cellular structures can also have effects on satiety, with more intact (solid tissue) structures having a greater effect than e.g., blended (liquid dispersion) versions of the same tissue. These studies typically focus on satiety of plant-based foods after a constant energy pre-load (meal). Whilst this is a well-controlled iso-energetic approach, it ignores the potential differences between different types of plant food structure resulting in satiation at different energy contents.

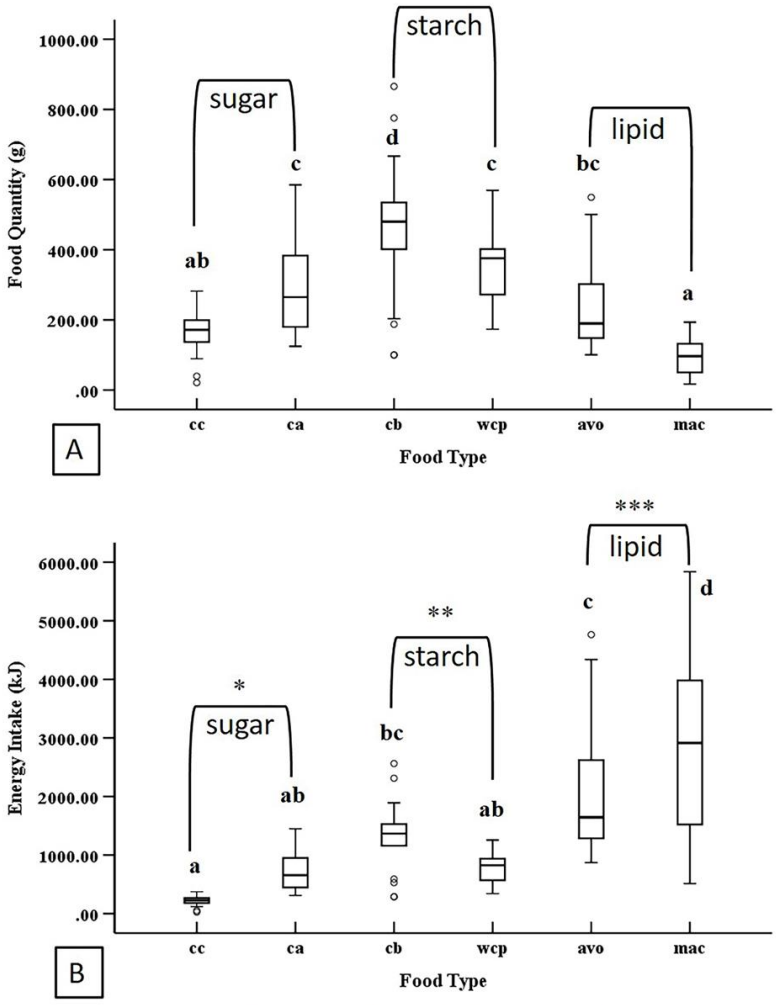
In the Naturally Nutritious project, the satiety and satiation properties of a range of horticultural products (apple, avocado, banana, carrot, macadamia), consumed as a mid-morning snack, were characterised. In a first pilot stage, the food factors responsible for different amounts of food required to feel comfortably full were identified. The ratings of fullness during (satiation) and after (satiety) the meal were recorded by participants and used to define the food factors that influence satiation and satiety with a view to identifying routes to portion size specification for either immediate or long-lasting fullness. In a second pilot stage, the human factors (both physiological and psychological) contributing to satiation and satiety were explored, providing a total of 28 variables that were modelled to provide insights into the relative importance of individual variables and grouped variables (food, physiology, psychology) on perceived fullness (satiation or satiety). This information is needed to identify the potential for targeted messages to groups of people (‘precision nutrition’) or individuals (‘personalised nutrition’) for either public health or commercial marketing purposes. Based on the results of the two pilot trials a larger scale trial was conducted with more participants to test the robustness of models. A challenge in measuring human factors is that they often involve specialist facilities (e.g., in clinics) and/or labour-intensive analysis (e.g., counting tongue papillae density). In order to reduce the need to rely on such invasive/expensive/time-consuming methods, in a third stage of this project we have investigated the potential of infrared spectroscopy of saliva or skin to provide a sufficient fingerprint of physiological (and even in some cases psychological) factors to predict individualised satiation/satiety responses. With further work this could provide a powerful approach to identify personalized nutrition requirements.

Technical insights

In a first study, we assessed the effects of plant-foods (apple, avocado, banana, carrot, and macadamia nuts compared with protein-rich chickpeas) on the amounts needed for satiation at a mid-morning snack, as well as the subsequent resulting time course of fullness (satiety). As a pilot study, we chose to compare a wide range of foods with a limited number of participants (10) but with a complete randomized and replicated crossover design. We aimed to identify potential relationships between satiation, subsequent perceived satiety, and either structural or compositional features of plant-based foods, which could be used to select a small number of samples for subsequent trials with a greater number of participants. The foods tested were selected to encompass starch-rich (banana), lipid-rich (macadamia, avocado), and sugar-rich (carrot, apple) horticulture-based foods compared with a protein- and starch-rich food (chickpeas), all of which can be the core of a snacking occasion. We hypothesized that (i) multiple factors underpin each of satiation and satiety across the samples examined, and (ii) the plant food factors that drive satiation and satiety are different.

Participants were asked to eat the snack food provided until they felt 'comfortably full'. The amounts of food eaten are presented in Figure 1 in terms of either grams or energy consumed, grouped according to the major energy source within the food (sugar, starch, or lipid). For each major energy source, there was an example of a relatively hard tissue structure (carrot, chickpea, macadamia) and a softer tissue structure (apple, banana, avocado). It was noteworthy that the amount consumed was greater for each of the softer compared with harder tissues for the same macronutrient (Figure 1A). There was greater consumption of starch-rich foods compared with sugar or lipid foods. The two most effective foods in terms of achieving comfortable fullness were the hardest of the structures consumed (carrot, macadamia). As discussed later, this may be related to the number of chews required prior to swallowing.

When expressed in terms of energy consumed, the different energy densities of the foods led to a different order of perceived satiation, with carrot the most effective and lipid-rich foods the least effective. Thus, there is an opportunity to market appropriate snack foods for portion size or energy intake differently.



*The mean difference is significant at the 0.05 level. The lower case words mean the significant difference in food types and * mean the significant different in nutrients types.*

Figure 1: Food eaten until 'comfortably full' expressed as weight of food (A) or energy intake (B). cc = cut carrot; ca = cut apple; cb = cut banana; wcp = whole chickpea; avo = avocado; mac = whole macadamia nuts

Despite the fact that participants were asked to eat until 'comfortably full', their perceptions of fullness differed at the end of the snacking time (20 mins; Figure 2A), with sweeter and softer foods (apple, banana) giving a higher fullness rating. Over the whole timescale of fullness rating, the results show a range of satiation (Figure 2A) and satiety (Figure 2B) responses covering the 20 minutes allocated for snack eating (Figure 2A) and the subsequent 3 hours (Figure 2B). This way of plotting the data shows that there are major differences in responses to the test foods, prompting further analysis of the underlying causes. Despite the popular concept that protein helps you feel full, low-protein horticultural products were superior to protein-rich chickpeas in both satiation and satiety.

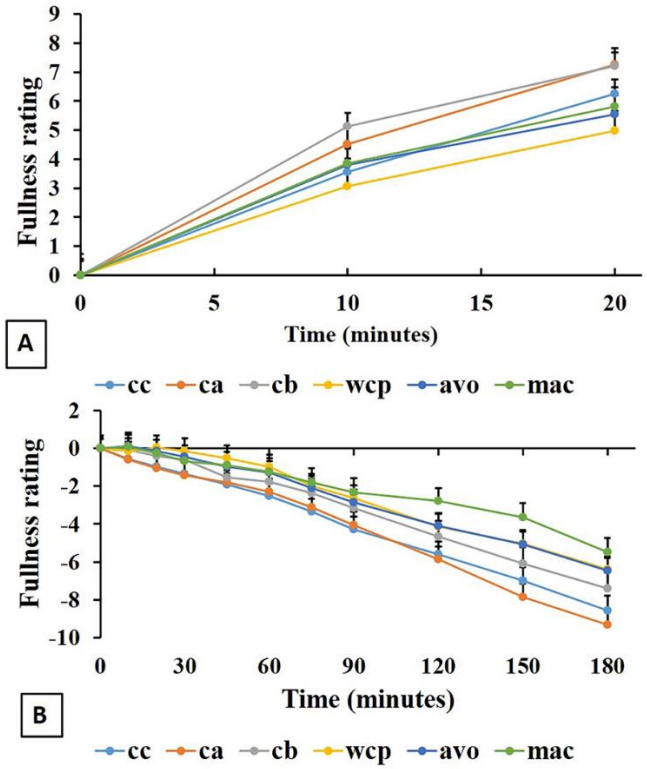


Figure 2: Fullness ratings (mean ± se) in food types (A. the fullness during the meal or satiation stage; B. the postprandial fullness or satiety stage). cc = cut carrot; ca = cut apple; cb = cut banana; wcp = whole chickpea; avo = avocado; mac = whole macadamia nuts

In order to quantify satiation and satiety, the modulus of the average slope over time (Figure 2) was compared between food samples as in Figure 1 i.e., per gram of food and per energy consumed (Figures 3 and 4). This analysis emphasised the greater perception of fullness (satiation) from hard tissue structures per gram (Figure 3A) and the dominant role of lipid energy density in changing this profile when expressed per kJ (Figure 3B).

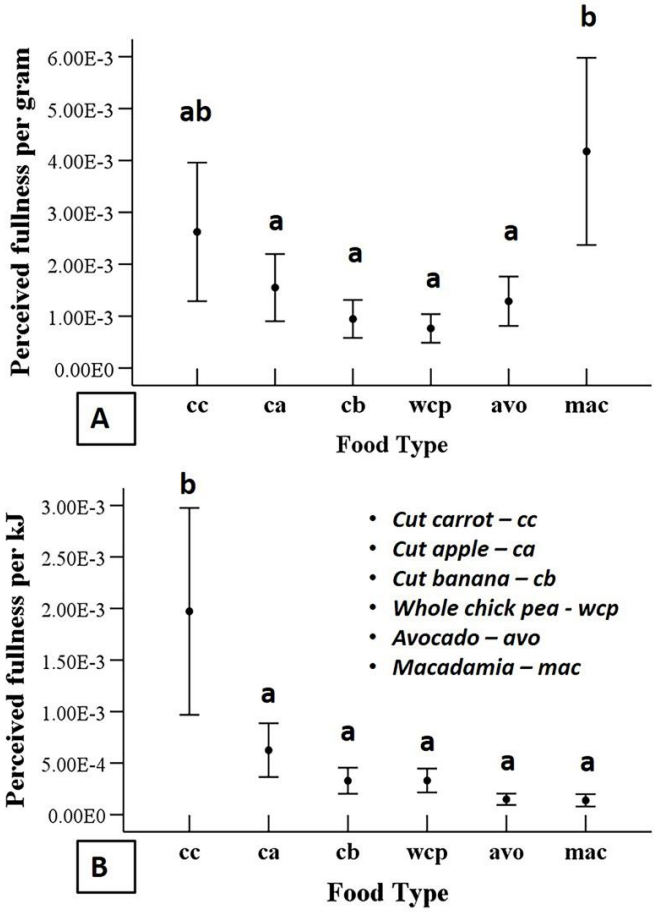


Figure 3: The distribution of perceived fullness or satiation (mean ± sd) in food types (A. the perceived fullness divided by food intake quantity; B. the perceived fullness divided by energy intake quantity).

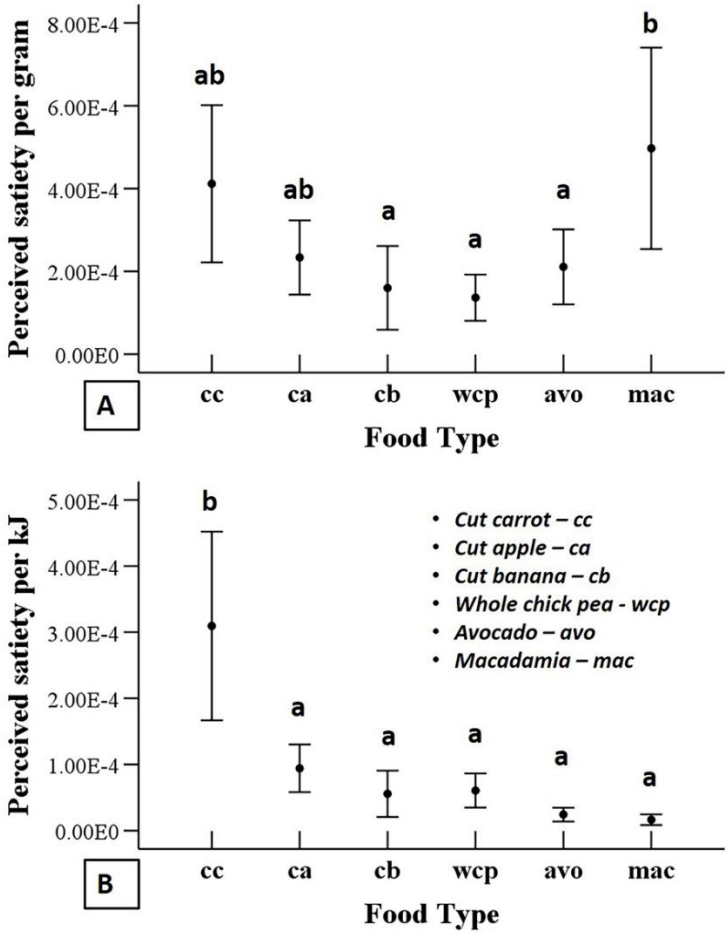


Figure 4: The distribution of perceived satiety in food types (A. the perceived satiety divided by food intake quantity; B. the perceived satiety divided by energy intake quantity).

Perceived satiety effects (Figure 4) were qualitatively similar to satiation effects with carrot having the greatest effect per kJ, but macadamia the greatest effect per gram.

Food liking and the number of chews required before swallowing (mastication no.) were also measured and all data from all individuals subjected to Principal Components analysis. This gave a complex picture (consistent with human factors as well as food factors being important) with the first four dimensions accounting for 34%, 23%, 16% and 9% of variation respectively (Figure 5). However, there were some consistent messages from this analysis. Firstly, the factors affecting perceived fullness (satiation) are different to those affecting perceived satiety (arrows in different directions). Secondly, mastication number was inversely related to perceived fullness (satiation) consistent with the number of chews being a key determinant of short-term fullness. Thirdly, satiety was associated with food energy (particularly in the first two dimensions – Figure 5) and not particularly related with mastication number.

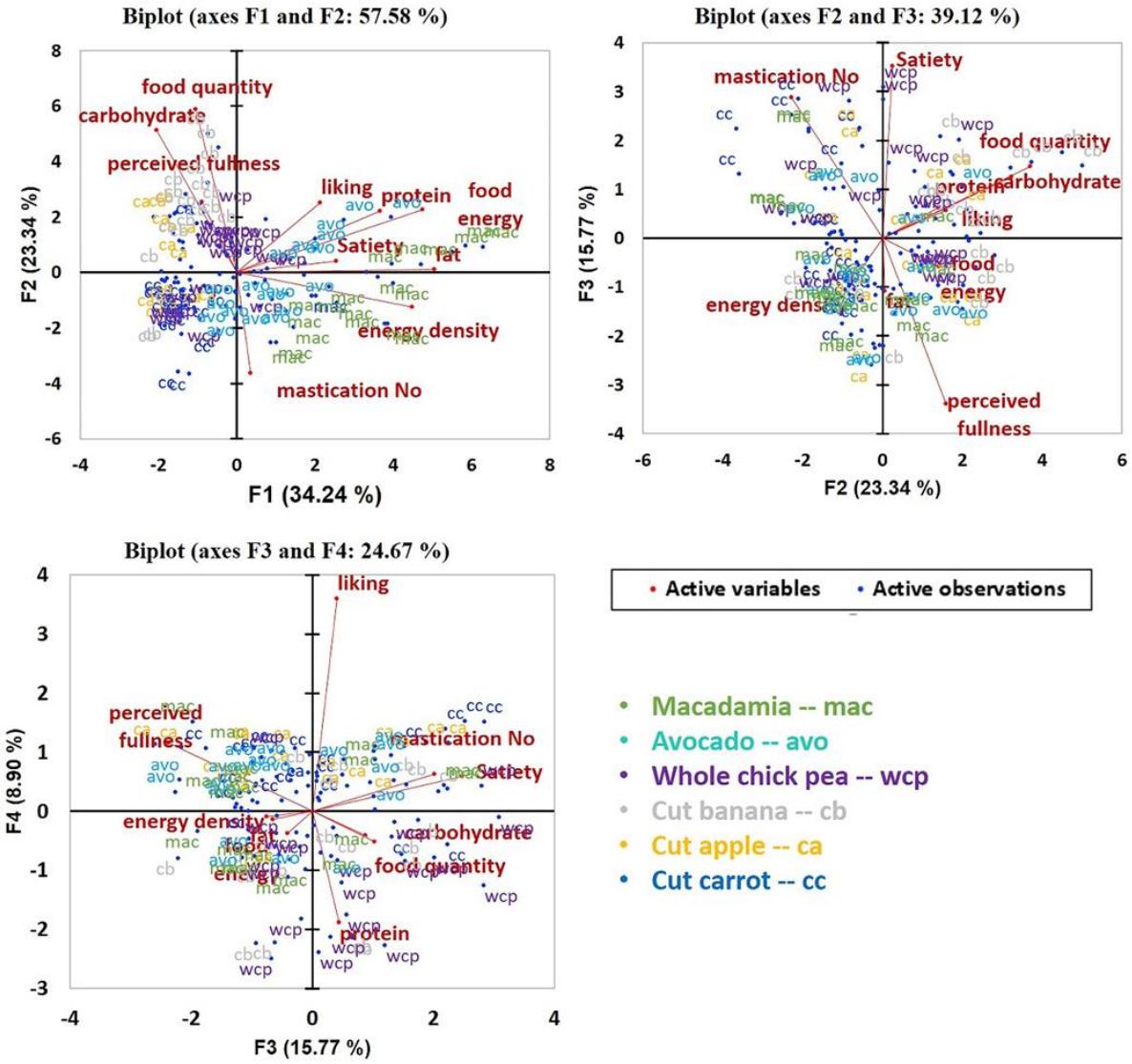


Figure 5: PCA biplots of the relationships among perceived fullness, perceived satiety, and food features in food types

Based on the evidence that human factors as well as food factors contributed to perceived satiation and satiety, a second study undertook a further set of analyses involving 28 variables in total (Table 1) to assess the relative importance of both human (physiological as well as psychological) and food factors in determining fullness responses and food intake. Metabolic factors were measured in a clinic, oral physiology factors were assessed as part of panel sessions, and psychological factors were assessed by questionnaire (see methods section)

Table 1: Variables included in the study.

Food factors (7)	Metabolic factors (7)	Oral Physiology factors (7)	Psychological factors (7)
1. energy intake	1. age	1. rested saliva flow	1. liking
2. energy density	2. respiratory quotient (RQ)	2. saliva α-amylase activity	2. food neophobic score
3. food intake	3. resting energy expenditure (REE)	3. papillae density of tongue	3. extraversion
4. protein content	4. resting metabolic rate (RMR)	4. tactile letter mean of tongue	4. neuroticism
5. fat content	5. body mass index (BMI)	5. mouth temperature	5. openness
6. carbohydrate content	6. heart rate (HR)	6. mouth volume	6. agreeableness
7. dietary fibre content	7. body fat rate (BFR)	7. chew rate	7.conscientiousness

Overall, the striking result (Figure 6) was that food factors dominated food intake quantity, but human factors dominated the fullness perception. Although similar types of factor importance were seen for satiation and satiety (Figure 6), the individual variables contributing to these factors were quite different for satiation and satiety, as well as for food intake (Table 2). This is consistent with the lack of correlation between satiation and satiety measures (Figure 5), and further emphasises the qualitatively different factors.

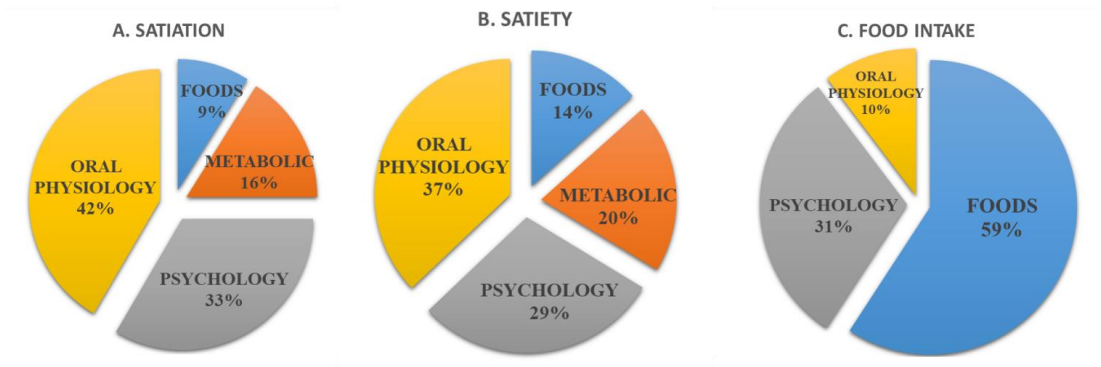


Figure 6: Distributions of variable importance to perceived satiation, satiety and food intake, based on projections (VIP) of a PLS-R model with uncertainty tested variables.

underlying short-term vs long-term feelings of fullness and hunger. Based on Figure 6, it is not surprising that human factor variables dominate satiation and satiety responses, whereas food factors dominate intake determinants (Table 2). Further work is needed to determine how consistent these human factor hierarchies are before creating hypotheses for testing underlying mechanisms.

Table 2: Hierarchy of key attributes (Table 1) contributing to satiation, satiety, and food intake with uncertainty test selected variables.

Satiety	Satiation	Food intake
RQ	Tongue papillae density	Carbohydrate percentage
Tongue papillae density	Conscientiousness	Dietary fibre percentage
Protein quantity	Tactile letter mean	Fat percentage
Neuroticism	Carbohydrate quantity	Protein percentage
Chew rate	RMR	Liking
Extraversion	Neuroticism	Extraversion
HR	Mouth temperature	Mouth volume
α amylase activity	Rested saliva flow	Conscientiousness
Neophobic score	Liking	
Mouth temperature	Openness	
Tactile letter mean	BFR	
Carbohydrate quantity	RQ	
Openness	α amylase rested activity	
Rested saliva flow		
Agreeableness		
REE		

In these three models, the independent variables only include those selected by uncertainty testing. The ranking of variables is based on the Variable Importance in the Projection (VIP) of the PLS regression models. High ranking means high importance and high contribution to the dependent variable in the model. VIP value of variables above the dashed lines were larger than 1, representing more important dependent variables.

Based on the promising results from the first two studies, a larger study was set up with 52 participants and the same 28 food and human variables. Because of the large number of participants and the requirement for a controlled sensory laboratory environment, only three foods could be tested. Apple, avocado and banana were selected as examples of sugar, lipid and starch-rich foods. Analysis of this experiment is ongoing, but results are broadly in line with the smaller scale (10 participants) first two studies. An example is shown in Table 3, which lists the most important variables contributing to perceived satiety. With the larger number of participants, more robust correlations could be determined, as shown in Table 3.

Table 3: Attributes contributing to perceived satiety (52 participants) based on the important factor's loadings of PLS regression models. The rank order of loadings shows the importance of that variable to satiety, for both positive and negative effects.

	Variable	R^2
1	HR	0.528
2	Carbohydrate amount	0.467
3	RQ	0.417
4	Number of chew	0.272
5	Oral exposure time	0.200
6	Papillae density	0.159
7	BMI	0.094
8	Chew rate	0.052
6	Saliva flow	-0.008
5	Height	-0.023
4	Conscientiousness	-0.230
3	Fat amount	-0.230
2	Age	-0.350
1	Extraversion	-0.383

This table 3 shows similarities to table 2 but the order of variables is not identical. This is to be expected as these reflect the nature of the individual participants. This has two implications. One is that, in order to achieve a population-average hierarchy of variables contributing to perceived satiation and satiety, a large cohort of people will be required. The second is that there are very likely to be demographic groups (based on physiology and psychology) who respond differently in terms of fullness perception to the same foods.

The comprehensive suite of variables studied (Table 1) is experimentally challenging to obtain. Metabolic factors are typically derived from clinical measurements, using specialist facilities and requiring individuals to change clothes and spend 1-2 hours at the clinic. Oral physiology factors are obtained from a range of tests carried out on participants in the sensory laboratory, followed by extensive analytical work-up (e.g., counting of tongue papillae on micrograph images of tongues). Psychological factors involve participants completing a detailed questionnaire followed by researcher analysis. While this study has shown the importance of collecting this data for understanding the variation in individual perceptions of fullness, and it is important to continue doing so to further develop the research findings, longer-term application would be helped greatly if there was a convenient and non-invasive approach to obtaining a fingerprint that reflected the physiological and psychological factors of interest. To this end, we have investigated the application of infrared spectroscopy, as a well-established high-information-content approach. We reasoned that oral physiology factors may have a 'fingerprint' in saliva composition, so collected saliva samples from participants and analysed their infrared spectra. We further reasoned that body metabolism factors may have a 'fingerprint' in accessible body tissues that could be probed with a hand-held near-infrared instrument.

For saliva (52 participants), drying of saliva samples improved mid-infrared spectral variations sufficiently to generate a wide range of spectral fingerprints (Figure 7 - Ni, D., et al., 2021).

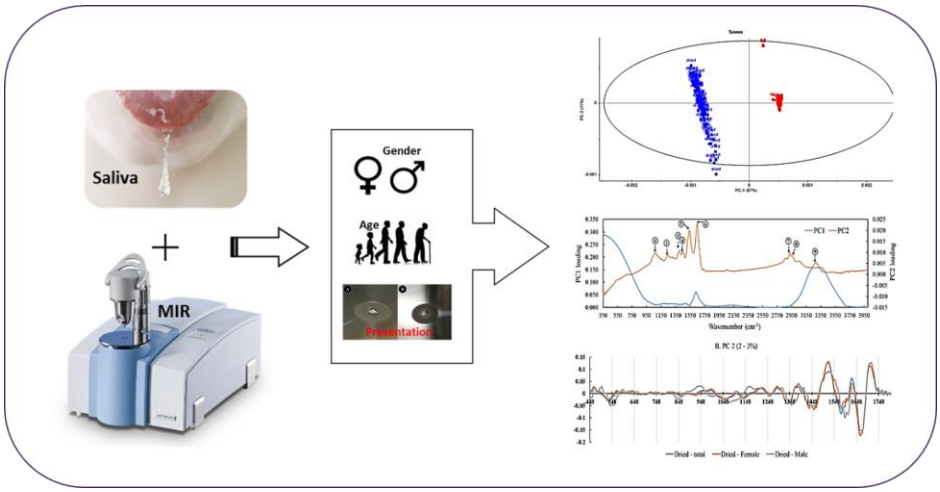


Figure 7: Saliva mid-infrared spectra are similar in wet state (dominated by water – red points) but well-dispersed when dried (blue points). Specific infrared bands associated with proteins, lipids and carbohydrates are the main determinants of differences.

Of particular interest is that these variations between individual saliva spectra fingerprints were correlated not only with oral sensitivity factors (tactile perception, papillae density etc) and oral processing (number of chews, amylase activity etc), but also with food intake, satiation and (to a lesser extent) subsequent satiety (Figure 8). The approach here was to construct a partial least squares regression model of fingerprint features, with the results showing that there are indeed features in the infrared spectra of saliva that correlate with measures of oral processing as well as food intake and satiation. Other analysis showed that saliva fingerprints were less successful in predicting whole body metabolic factors such as resting metabolic rate or fat content. However, NIR spectra from tissues (that sample to depths of several cm) such as wrist, arm, ear, jaw, and cheek (Figure 9) show promising correlations with at least some of the metabolic factors that underpin satiety and satiation perceptions. The best correlations were found for spectra from the upper arm (Table 4) and show promise for further use as predictors of satiation and satiety responses. These methods could, in the long term, be deployed by GPs or dietitians to advise individuals of the horticultural produce to select for their fullness needs. In addition, they may form the basis for consultancy businesses in the way that individual microbiome analysis is starting to do.

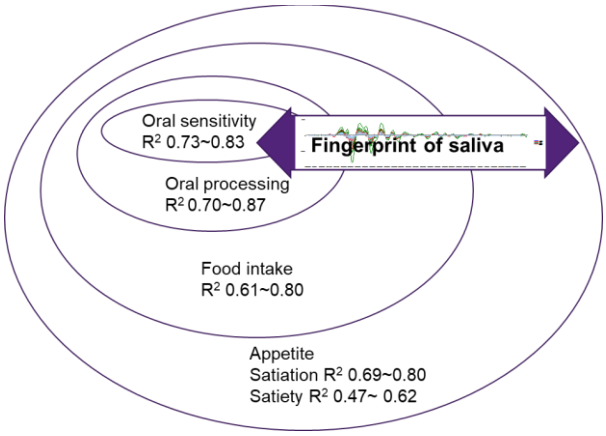


Figure 8: Relationships between saliva mid-infrared spectral fingerprints and human factors. R^2: coefficient of determination in cross validation of PLS regression model

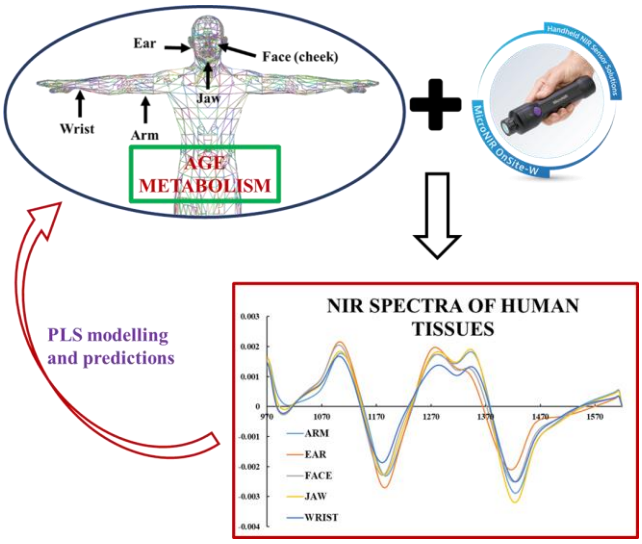


Figure 9: A hand-held NIR instrument can provide spectral information to compare with metabolic (and age) factors.

Table 4: Relationships between NIR spectra of arms and metabolic factors

Groups	n	LV	R^2	SECV	Bias	SLOPE
Age (year)	131	9	**0.904**	3.86	0.026	0.91
RMR (kcal/day)	132	11	**0.82**	85.17	-1.33	0.86
TEE (kcal/day)	132	12	**0.78**	234.32	0.17	0.82
RQ	127	14	**0.49**	0.019	-5E-05	0.58
Fat rate (%)	129	10	**0.8**	3.58	-0.019	0.83
Fat mass (kg)	126	12	**0.88**	2.55	-0.0077	0.89
Fat free mass (kg)	132	13	**0.83**	3.8	0.034	0.86
BMI	130	13	**0.82**	1.46	-0.0002	0.85

Implications for consumers

Taken together, the large body of data obtained can be summarised into a number of key messages that could be used to promote the nutritional value of horticulture products in general or specific crops in particular:

- fruits, vegetables, and nuts can deliver short-term and long-term fullness comparable with protein-rich foods
- for immediate fullness, a hard tissue structure, requiring more chews prior to swallowing, will lead to more efficient satiation than softer tissue structures
- from previous data, juiced or pureed fruits and vegetables are less efficient in providing both satiation and satiety compared with intact plant tissues
- the factors determining effective satiation are different to those for satiety, so marketing messages can be tailored accordingly e.g., cut carrots emphasising calorie-efficient fullness or macadamia nuts emphasising long-lasting fullness
- food factors mostly determine the portion size for comfortable fullness, so there is the opportunity to define relevant pre-packed serving sizes to effectively achieve satiation
- however, the subjective perception of fullness as satiation or satiety is mostly determined by characteristics of the individual (physiological and psychological)

Industry opportunities

- Identification of horticulture product portion sizes for efficient satiation or satiety
- Marketing messages on the effective short- and longer-term fullness provided by intact horticultural products, comparable with protein foods
- Raw vegetables such as carrots for energy efficient short-term alleviation of hunger
- Nuts for effective longer-term satiety
- Information for health professionals to reinforce and bring to life dietary guideline recommendations that most of the diet should be whole foods from plants
- Psychological (and physiological) effects on perceived fullness could be incorporated into targeted marketing messages

Recommendations

1. Working up opportunities for consumer messaging at whole of sector level and/or individual commodities

2. Capitalising on the finding that precision (group) and personalised (individual) differences in satiation and satiety responses have been identified, allowing conversion of the current whole-of-population-average message about fruit and vegetable consumption to more tailored messages for specific consumer groups, either through direct marketing or via information for dietitians and other nutrition advisors.

3. NIR or alternative non-invasive categorisation of individuals that allow targeting of effective satiation/satiety products to individuals. Work needed to define a business model that would provide benefit to producers.

Overall, the general principles involved in satiation and satiety from fruits, vegetables, and nuts (using mid-morning snacks as a model) have been established. Each of food factors, human physiology and psychology contribute in different ways to satiation (short term fullness during the eating process) and satiety (subsequent return to hunger after a meal). This has provided information to support targeted marketing platforms for e.g., portion control / satisfying hunger (satiation) or sustained energy release / preventing snacking between meals (satiety) based on the different characteristics of fruits, vegetables, and nuts.

Opportunities requiring further research include:

- Identification of the effects of different human physiology and psychology features on perceptions of satiation and satiety

- Rapid screening (e.g., using NIR screening of saliva, skin) to define human physiology (and possibly psychology) characteristics related to satiation/satiety perception

- Definition of interactions between food factors and human factors (physiology, psychology) in determining perceived satiation/satiety

This would lead to:

- Precision (group) and personalised (individual) differences in satiation and satiety responses being identified, allowing conversion of the current whole-of-population-average message about fruit and vegetable consumption to more tailored messages for specific consumer groups, either through direct horticulture sector/product marketing or via information for dietitians. GPs and other nutrition advisors.

Microbiome benefits

Background

There is intense current interest in dietary fibre (DF), both in terms of its nutritional functionality and its potential effects on human health worldwide. When consumed in appropriate amounts on a daily basis, DF has been associated with health benefits such as lowering blood pressure, reducing plasma cholesterol, attenuating blood glucose, and promoting beneficial gut microbial populations and fermentation end products. Modern diets, which often contain inadequate DF, have been linked with the development of coronary heart diseases (CHD), stroke, and colonic cancer. A major component of these systemic health benefits derives from the fermentation of DF (primarily) in the large intestine with end-products such as short chain fatty acids (SCFA).

There has been a common misconception that DF fermentability is correlated with its solubility, and therefore that only soluble DF (SDF) can have beneficial health effects for humans However, this view is changing. For example, cellulose, which is considered quite recalcitrant, can be fermented in both the human and animal large intestine (LI), to variable extents.

Insoluble dietary fibre (IDF) constitutes the major portion of total DF (TDF) in plant-based foods due to the complex structures of the plant cell walls which are an intrinsic part of all plant tissues. In fruits and vegetables, apple DF contains 56% IDF while carrot contains up to 92% IDF. In nuts, almond contains 89.9% IDF, and macadamia 79.2% IDF, of their TDF. In the context of effects of dietary fruits, vegetables, and nuts on fermentation by the gut microbiota, the concept of IDF needs to be extended to include any macronutrients encapsulated by cellular structures in undigested food.

Although consumers are generally aware that fruits, vegetables, and nuts are part of a healthy diet and contain dietary fibre, those seeking to increase their DF intake may choose refined oligo- or polysaccharides such as inulin or fructo-oligosaccharides. The same components are typically used by processed food sectors as a 'white powder' ingredient approach to boosting the DF content on food labels. However, these traditional prebiotic (SDF) components have simple structures, are usually fermented rapidly, and may produce too much gas for some consumers. On the other hand, it is expected that fruit, vegetable, or nut fibre would have a more complex structure, be fermented over a long time period and would not lead to rapid gas production. In addition, if the food material contained intact plant cells, then it is possible that these cells survive the mouth, stomach, and small intestinal processing to deliver not only fibre but also their cell contents (e.g., starch from banana or triglycerides from nuts). A further aspect to consider is that fibre fractions from horticulture produce could be recovered from waste streams (e.g., from juicing) providing a potentially attractive waste recycling benefit as well.

In the Naturally Nutritious project, we aimed to provide data in support of communication messaging and potential product design through addressing three research questions:

1. What is the relative fermentability of DF from a range of fruits, vegetables, and nuts, and why do these differ?

2. What is the consequence of having entrapped macronutrients e.g., starch and lipid in banana and nuts respectively?

3. Do fibre fractions recovered as waste from juicing processes have useful fermentation behaviours?

Technical feasibility

All the fibres isolated after simulated chewing and digestion of fruits and vegetables showed substantial beneficial end-products (SCFA – primarily acetate, propionate, and butyrate) of fermentation (Figure 1). Apple, celery, and carrot were fermented more extensively than wheat bran (a well-known DF), but spinach and banana were fermented less extensively. All fibres continued to be fermented over extended time periods, consistent with steady fermentation throughout the large intestine – a potential key nutrition message.

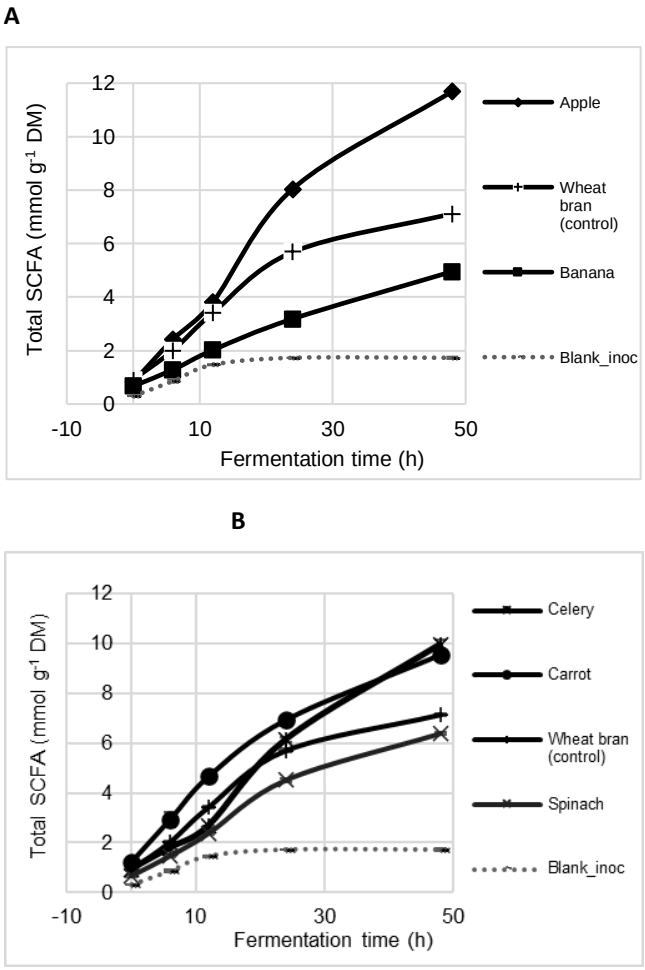


Figure 1: Gas production profiles from IDF fermentation isolated from fruits (A) and vegetables (B) with wheat bran as control (each line graph shows a representative profile) and a blank without added fibre.

The extended fermentation process involved initial fermentation (first 24h) of more soluble components such as pectin (Figure 2), leaving less soluble (IDF) components that can be visualised by microscopy and characterised by NMR spectroscopy (Widaningrum et al., 2020).

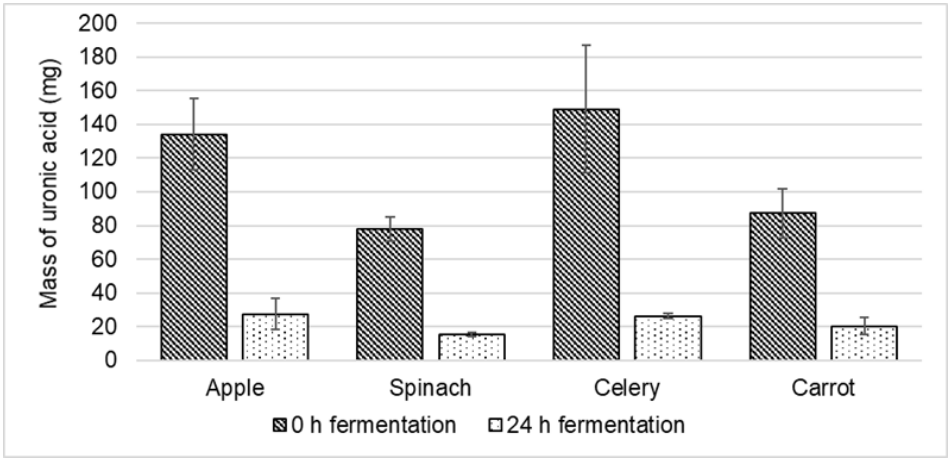


Figure 2: Extensive degradation of pectin (uronic acid content) during 24h of fermentation

The reason why banana was fermented more slowly was probably because of the 'resistant' starch trapped inside the fibre. Analysis of starch content through the fermentation, compared with the only other starchy substrate (wheat bran), shows that relatively little banana starch was fermented in the first 24h whereas essentially all of the wheat bran starch was (Figure 3).

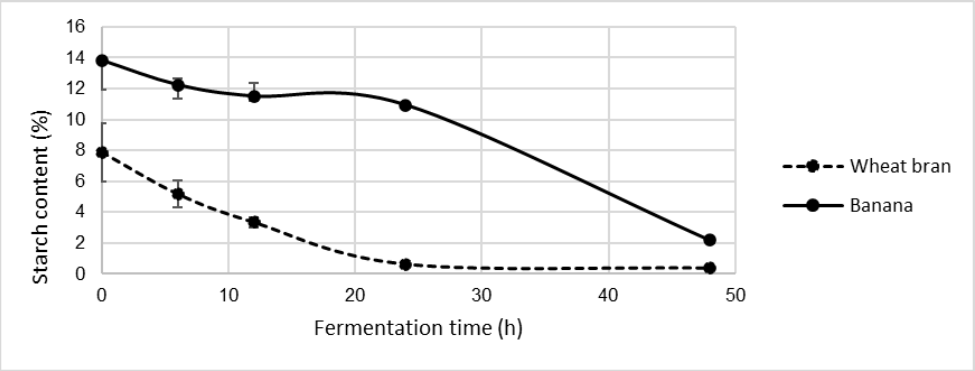


Figure 3: Starch disappearance from banana-IDF and wheat bran during fermentation as determined by solution state ^{1}H NMR.

The reason why spinach was less fermentable was probably because of the fermentation-resistant vascular tissue that makes up a substantial weight fraction of leaf DF. Thus, after fermentation, spinach IDF still contains tightly attached, dense and compact structures (Figure 4B) with the thinner (most likely pectin-containing) cell wall material present at the beginning (Figure 4A) being lost after 48h of fermentation.

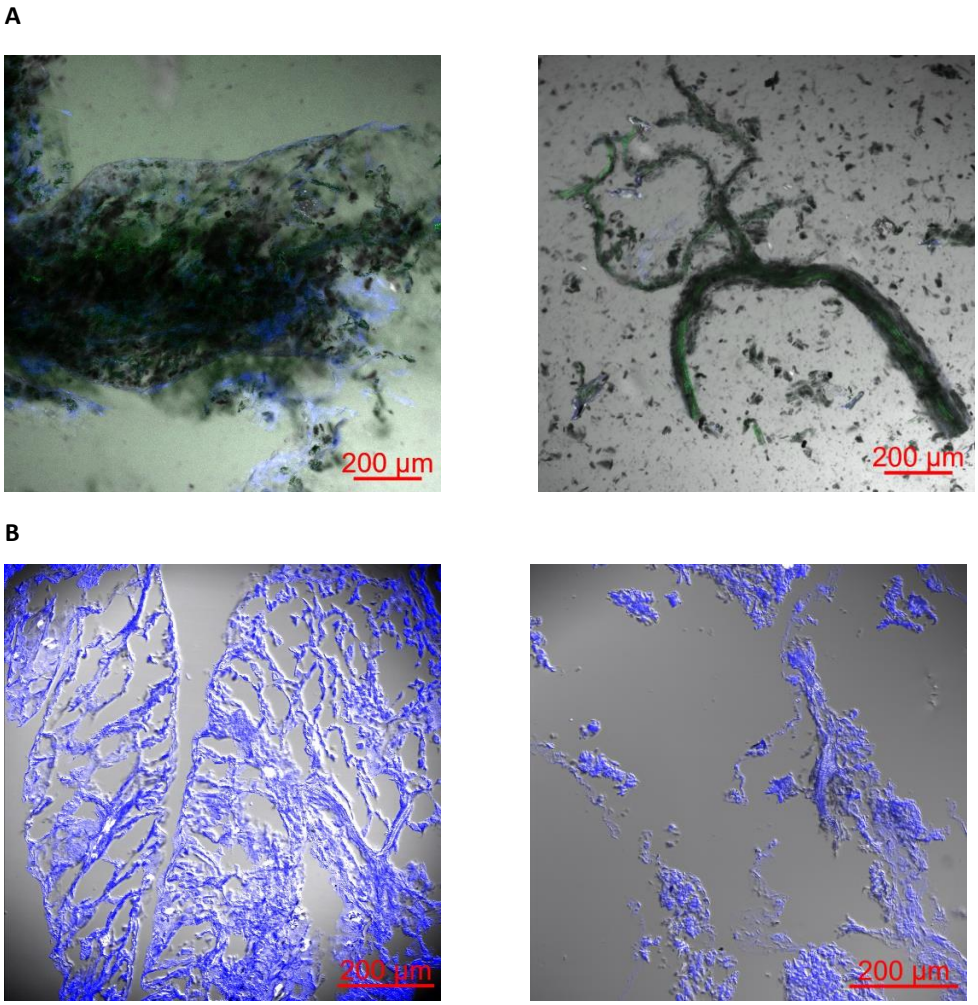


Figure 4: Microstructure of spinach leaf IDF: (A) before fermentation (0 h), (B) after fermentation (48 h). Top images (1) = stained with FITC and Calcofluor White, bottom images (2) = sectioned by microtome, 20 µM thickness, autofluorescence.

For nuts (almond, macadamia), an extra variable was used – the particle size, to represent potential differences between coarse (> 700 µM; labelled 'CC') and fine (250 – 700 µM; labelled 'F') chewing/grinding. CC samples were typically clusters of cells, whereas the F fraction contained more single or broken cells.

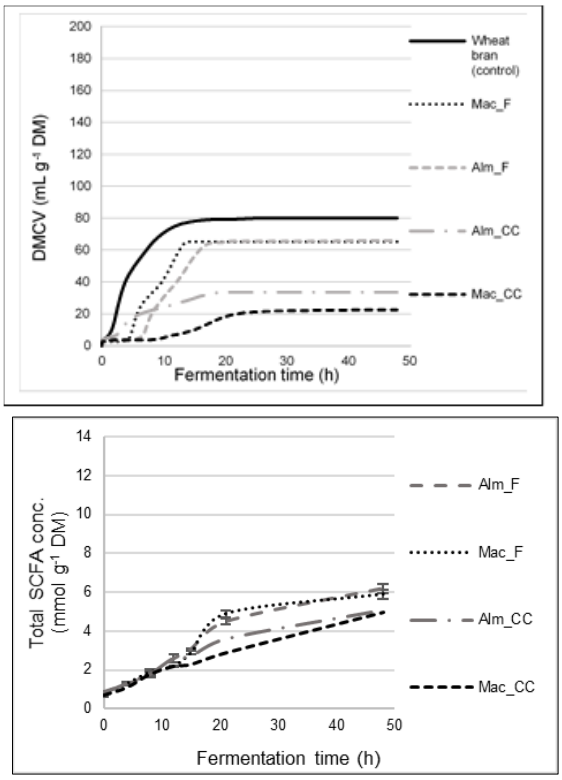


Figure 5: Gas (top) and SCFA (bottom) production from almond and macadamia in both fine (F; 250 – 700 µM) and coarse (CC; >700 µM) forms.

Fermentation of digestion residues from nuts was comparable to the less extensively / slower fermented fruits and vegetables (banana, spinach), with smaller particle sizes showing greater fermentability than larger particles (Figure 5). Chemical and NMR spectroscopy analysis of nut digestion residues (i.e., before fermentation) showed that they contained high levels of lipid (27% for almond F, 67% for almond CC, 55% for macadamia F, and 86% for macadamia CC). Microscopy analysis showed that this was due to lipids encapsulated within some residual cellular material (Figure 6).

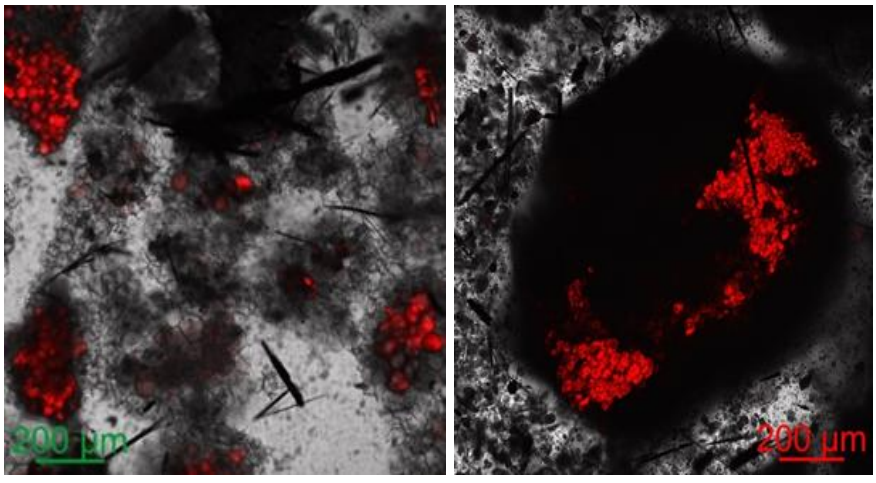


Figure 6: Digestion-resistant (pre-fermentation) particles of (left) macadamia F and (right) almond CC, stained with Nile red, which is specific for lipids.

After fermentation, these lipid-rich structures had largely disappeared (Figure 7) consistent with microbial breakdown of cell walls and release of encapsulated lipid. Thus, it is predicted that digestion-resistant residues of nuts would release their substantial lipid payload (27 – 86% for the samples studied here). Chemical analysis of fermentation

vessels showed (Figure 8) that this lipid was nearly (for CC samples) and essentially completely (for F samples) lost after fermentation for 48h in vitro. Taken together, the high lipid content of pre-fermentation digestion-resistant residues, the loss of lipid during fermentation and the substantial SCFA amounts produced (comparable to banana or spinach IDF) suggests that the encapsulated lipids were fermented to beneficial SCFAs. More work is needed to confirm this, but the indication is that the high lipid content of nuts contributes positive gut microbiota benefits, provided the nuts are not ground too small before consumption.

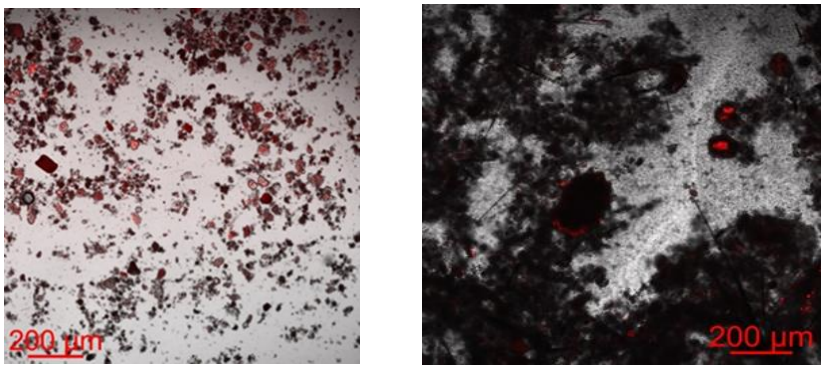


Figure 7: Remaining particles after 48h fermentation of (left) macadamia F and (right) almond CC, stained with Nile red.

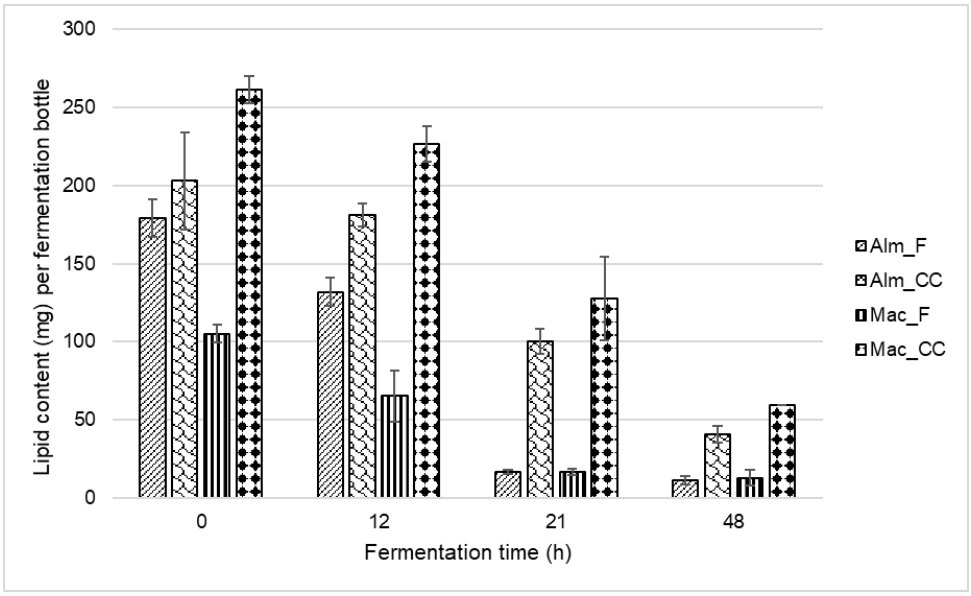


Figure 8: Gradual disappearance of lipid content from nuts during in vitro fermentation

In a third aspect of this work, fibrous waste material from small-scale juicing of pineapple, apple, orange, beetroot, and celery were compared in terms of their fermentation behaviour. As shown in Figures 9 and 10, both gas and SCFA production were extensive for apple, orange, and carrot fibre, intermediate for beetroot and celery fibre and least for pineapple fibre. This correlated with microscopy observations, which showed higher levels of vascular fibres (cf spinach – Figure 4) in the more recalcitrant fibres. This is illustrated in Figure 11 for pineapple waste, showing survival of vascular fibre structures after 48h fermentation. Nevertheless, all fibres showed clear potential for beneficial gut microbiota benefits. Compared with wheat bran, all fruit and vegetable fibres had higher water-holding capacity (9 – 12 g water / g dry matter) compared with wheat bran (3.7 g water / g dry matter), suggesting that they would make good food structuring agents, as well as being a source of fermentable fibre.

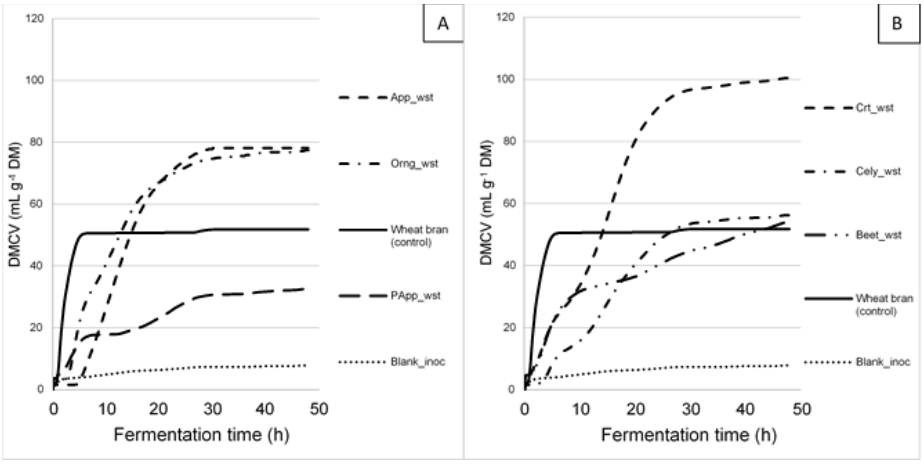


Figure 9: Gas production profiles from fermentation of FVJW isolated from: fruits (A) and vegetables (B) with wheat bran as control (each line graph shows a representative profile).

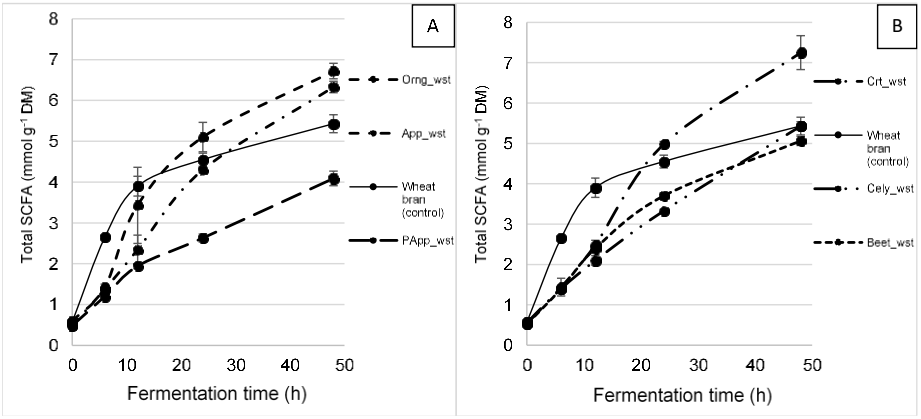


Figure 10: Total short-chain fatty acids (SCFA) concentrations as fermentation end-products from IDF fermentation isolated from fruit wastes (A) and vegetable wastes (B).

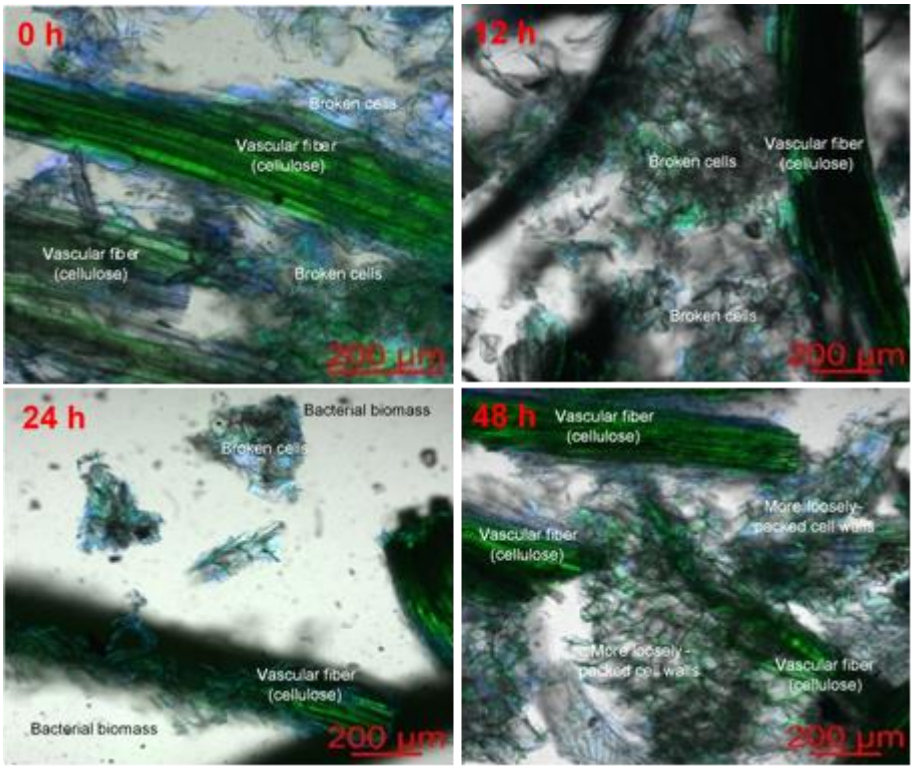


Figure 11: Micrograph profile of fermented pineapple juice waste fibre during in vitro fermentation.

Overall, addressing the three research questions,

1. What is the relative fermentability of DF from a range of fruits, vegetables, and nuts, and why do these differ?

2. What is the consequence of having entrapped macronutrients e.g., starch and lipid in banana and nuts respectively?

3. Do fibre fractions recovered as waste from juicing processes have useful fermentation behaviours?

It can be concluded that,

- All digestion-resistant materials from fruits, vegetables and nuts studied were fermentable to beneficial short-chain fatty acids. The rate and extent were dependent on the tissue type with secondary-thickened (e.g., vascular) tissues more resistant than primary (pectin-rich) cell walls.
- Entrapped macronutrients (starch in banana or lipid in almond/macadamia) are slowly but essentially completely fermented. This means that these macronutrients behave similarly to dietary fibre in terms of gut fermentation.
- Fibre fractions from juicing wastes have useful fermentation behaviours, with relative effectiveness depending on the level of fermentation-recalcitrant vascular tissue.

Implications for consumers

This study has identified a number of key messages that could be used to promote the nutritional value of horticulture products in general or specific crops in particular:

- All horticultural products tested had slow and steady, but eventually complete fermentation. This is an ideal combination for foods to nourish microbiota throughout the large intestine, without rapid production of gas, and with long-lasting production of beneficial end-products.
- The benefit of complex tissue structures in horticulture products is what leads to the extended fermentation timescale with diverse cell wall types having characteristic rates of fermentation. Thin/primary walls are fermented more rapidly than thickened/secondary cell walls, in contrast to conventional prebiotics such as inulin that are simple in structure and fast to ferment.
- The benefits of encapsulated starch in e.g., banana has long been recognised by the term ‘resistant starch’ and plays a role in extending the timeframe of fermentation of banana fibre. However, the implication that lipids

in intact nut cells are fermented to short chain fatty acids is novel and opens a new marketing angle for the health benefits associated with (intact) nuts.

Industry opportunities

The groundwork has been laid for horticultural products to be recognised as Nature's prebiotics. In multiple laboratory trials, we have demonstrated that the complex fibres present in fruits, vegetables and nuts are fermented extensively but slowly. This is a major advantage over many current prebiotics, which are fermented rapidly and therefore do not survive to the end of the colon where protection against colon cancer is most needed. A second benefit of horticulture products as prebiotics is that the intrinsic 'fibre' also includes bound and trapped phytonutrients (e.g., anthocyanins) that can be beneficial for the microbiota and are metabolised to end-products that are known health markers in humans. This 'fibre +' concept provides a further point of differentiation from current prebiotic products.
The finding of lipid fermentation to beneficial end products needs to be followed up to provide the nut sector with a new nutrition story.

Horticultural waste streams can be considered as viable sources of nutrition ingredients, based on their slow and steady fermentation to beneficial end-products (SCFAs).

Recommendations

Opportunities requiring further input include:

- Individual people have characteristic gut microbiome types that can now be characterised by DNA sequencing. Over the next few years, this will open up the field of precision and personalised nourishing of an individual's microbiota, holding out the prospect of tailored selection of horticultural products for optimising individual microbiomes either through self-diagnosis or under nutritional/dietetic advice.
- In collaboration with the leading gut microbiome screening company in Australia (Microba) we could work with their database of individual microbiomes, in order to identify beneficial effects of different horticultural products on diverse human microbiotypes. As precision and personalised nutrition of the microbiome becomes more common, this will provide the tools needed for dietitians and other to optimise individual microbiota using fruit/veg/nut products as prebiotics.
- Testing of the effect of phytonutrients trapped in horticultural fibre on gut microbiota fermentation outcomes including metabolism of phytonutrients.
- Validation of the beneficial effects of lipids entrapped within nut cells as gut microbiome modulators through feeding trials followed by faecal sampling to identify changes as a result of nut interventions.
- Building new ingredient industries from horticulture waste streams as 'fibre+' prebiotics, in tune with public sentiment and government policy to upcycle waste to foods.
- Identification of opportunities for precision nourishing of gut microbiota ('Nature's prebiotics') from selected fruits, vegetables and nuts to build market segment messaging in 3 – 5 years.

'Simply Red'

Background

Originally, a standalone one-year project, 'Simply Red', was proposed as a project concept by Prof. Roger Hellens, QUT, focussing on anthocyanin pigments in fruit and vegetables, and using enhanced anthocyanin levels as a potential marketing tool based on the anthocyanin pigment's visual pigmentation and associated health benefits. This project was subsequently embedded within the Naturally Nutritious project as a stand-alone sub-project and is reported here.

The development of a 'high anthocyanin' brand

All work associated with the 'High-Anthocyanin' branding via the 'Simply Red' sub-project was funded for the first year of the project only. Adoption would require further extension or work to engage the retail or consumer communities to generate traction or interest. There is an opportunity for this 'brand' concept to be picked up by retailers or marketing. It is ready for the next stage, e.g., marketing and retail linkages, potentially through support of the identified high-anthocyanin products with scientific and clinical evidence to support a product range.

We have developed a database of research articles that report nutrition benefits from anthocyanins. The database includes the source of the anthocyanin, a compositional breakdown of the anthocyanin compounds tested, the nature of the assay used to test the effects on nutrition and the potential health effects of these compounds. The database currently has 78 curated entries and then next phase of this database (beyond the scope of Naturally Nutritious) will be to make the datable publically available and enable remote data entry. The initial development of the database has highlighted the key fields and information appropriate to this resource.

Anthocyanin analysis of 20 high anthocyanin fruit and vegetable cultivars and germplasm accessions

The Simply Red group at QUT have extracted and analysed the anthocyanin composition of 29 high-anthocyanin foods. These include well know red and purple fruit and veg such as 'Pink lady' apples, blackcurrants, blood oranges, red cabbage and red onion, black olives. We have also analysed some novel red and purple fruit and veg such as: 'Bravo' apples, purple wombok, purple basil, purple sprouts, and purple asparagus. Finally, we have analysed some Australian native fruit, including Davidson plum and Australian black plum (*Diospyros australis*).

Cross-referencing of anthocyanin-nutrition data and metabolite profiles (Simply Red)

Following the Hortconnections conference in Adelaide 2017, ten growers were interviewed in regard to high anthocyanin fruit and vegetables. A database of literature describing nutritional effects of anthocyanin was developed using the 'Confluence' Wiki database. The key anthocyanin compounds were detailed from the publication and linked to our database.

A database of common and emerging high anthocyanin fruit and vegetables was subsequently developed, and the key anthocyanin compounds linked to the literature database. Each anthocyanin compound has been linked, through a report card, showing the compound structure and the composition in our fruit and vegetable collections.

Together these data can now be used to develop a prototype consumer interface that enables users to compare the level and type of anthocyanin of common red and purple fruit and vegetable with wild accessions or novel new cultivars.

The database inspired two entrepreneurs from QUT to establish a company Van&Villa (http://vanandvilla.com/) through QUT's Foundry (https://www.qutbluebox.com.au/news-events/news/Acccelerator-finalists-have-been-announced). They secured a license with Nutrafruit to take 'Queen Garnet' high anthocyanin plum waste from juice extraction, to develop their first product, 'Powda'.

Literature review of anthocyanin-nutrition data

The Simply Red group at QUT developed a database of research articles that report nutrition benefits from anthocyanins. Our database included the source of the anthocyanin, a compositional breakdown of the anthocyanin compounds tested, the nature of the assay used to test the effects on nutrition and the potential health effects of these compounds. The database currently has 78 curated entries and the next phase of this database (beyond the scope of Simply Red) will be to make the database publicly available and

to enable remote data entry. The initial development of the database has highlighted the key fields and information appropriate to this resource. Together with the anthocyanin levels tested in "Metabolite analysis of 20 high anthocyanin fruit and vegetable cultivars and germplasm accessions, this data can now be used to develop a prototype consumer interface enabling users to compare the level and type of anthocyanin of common red and purple fruit and vegetables with wild accessions or novel new cultivars.

Communications & Extension

Media releases / media stories

1. $10m research project seeks 'superfoods' to keep us healthy, https://www.uq.edu.au/news/article/2016/11/10m-research-project-seeks-superfoods-keep-us-healthy, (23 November 2016)
2. http://statements.qld.gov.au/Statement/2016/11/23/10m-project-to-focus-on-next-generation-superfoods
3. Brisbane Times, Search for new superfoods given $10m boost http://www.brisbanetimes.com.au/queensland/search-for-new-superfoods-given-10m-boost-20161127-gsykfv.html, (27 November 2016)
4. The Sydney Morning Herald, Search for new superfoods given $10m boost, http://www.smh.com.au/queensland/search-for-new-superfoods-given-10m-boost-20161127-gsykfv.html, (27 November 2016)
5. http://mogaznews.com/en/collection/262860.html
6. Queensland Country Life, Nutrient-rich 'superfoods' to drive Qld agriculture http://www.queenslandcountrylife.com.au/story/4311002/agfutures-qld-targets-superfoods/ (23 November 2016)
7. 'New research for super foods combat diseases', Gatton Lockyer Brisbane Valley Star, Gatton QLD, General News, (30 Nov 2016)
8. 'Food Research Investigating 'superfoods', Rural Weekly - Southern QLD, Toowoomba QLD, General News, (02 Dec 2016)
9. 'Naturally Boosted', The Australian, Australia, General News, Sean Parnell 17 Feb 2017
10. Hort Innovation | New high folate strawberry a sweet find. http://horticulture.com.au/new-high-folate-strawberry-a-sweet-find/ (26 September 2017)
11. New Folate strawberry a sweet find for researchers. Fresh Plaza, (26 September 2017) http://www.freshplaza.com/article/182070/New-high-folate-strawberry-a-sweet-find-for-researchers
12. HIA Naturally Nutritious Video in preparation with Aaron Darc. https://vimeo.com/241867084 (Draft video, password: natural)
13. Courier Mail (Qld), 'Superfoods on menu', http://www.couriermail.com.au/news/queensland/scientists-work-on-frankenstein-foods-to-keep-queenslanders-healthy/news-story/1ac1b23b6f76613bf16a2c0971ab8475, (15 Dec 2017)
14. Sunraisia Daily, Purple corn set for taste test. http://www.sunraysiadaily.com.au/story/5146181/purple-corn-set-for-taste-test/, (2 Jan 2018)
15. Hort-daily-Orange capsicums on the menu for long-term eye health https://www.hortidaily.com/article/9273181/orange-capsicums-on-the-menu-for-long-term-eye-health/ (1 December 2020)
16. ABC online news - ABC Far North by Renee Cluff. Orange capsicums help combat blindness, but you won't find them easily in shops. https://www.abc.net.au/news/2021-01-12/orange-capsicums-help-prevent-blindness-but-not-widely-available/13049208, (12 January 2021)
17. Could research into eye health lead to more commercial production for orange capsicums? https://www.freshplaza.com/article/9288646/could-research-into-eye-health-lead-to-more-commercial-production-for-orange-capsicums/, (28 January 2021)
18. O'Hare, T., 2021. AMD: The gift of fruit and veggies. Mivision: The Ophthalmic Journal, The Macular Disease Issue 167, https://www.mivision.com.au/2021/05/amd-the-gift-of-fruit-and-veggies/, May 2021.

Grower/consumer newsletters/presentations

1. Fanning, K., Simply Redder – Significant opportunities to boost strawberry health credentials. Simply Red-Strawberry Industry Newsletter- p.3, No. 43, September 2016
2. Striegel, L., Netzel, M., Netzel, G., Rychlik, M., 2016. Validation of stable isotope dilution assays for the quantitation of folates in strawberries. 1st Queensland Annual Chemistry Symposium, St. Lucia, QLD, Australia, 25 November 2016, Programme and Abstracts: 84.
3. AIFST NUTRITION FOR OPTIMAL HEALTH SESSION Food Australia, National, General News 01 Apr 2017
4. Women of Queensland Strawberry Growers Association presentation (Coopers Plains, Tour of Coopers Plains laboratory and pilot plant facilities and summary of research findings to date in relation to strawberry

anthocyanin and strawberry folate. Feedback survey of participants on high-anthocyanin strawberry and high-folate strawberry

5. O'Hare, T., Souza, B., S., Fanning, K., J., 2017. Changes in carotenoid concentrations in zeaxanthin-biofortified sweetcorn and popcorn following microwave cooking, 18th International Carotenoid symposium, Lucerne, Switzerland, July 9–14, 2017 (abstract)
6. Low-saturated fat macadamia nuts – Is it possible?' International Macadamia Research Symposium, Hilo, Hawaii, 12-15 Sept 2017 (Oral presentation)
7. 'High-folate strawberries – finally something tasty! International Tropical Agriculture Conference-TropAg, 20-22 November 2017, Brisbane Convention and Exhibition Centre, Brisbane (Oral presentation)
8. 'Lessons from temperate crops for tropical crop biofortification', International Tropical Agriculture Conference-TropAg, 20-22 November 2017, Brisbane Convention and Exhibition Centre, Brisbane (Oral presentation)
9. 'Isolation and identification of fungi from four different strawberry cultivars', International Tropical Agriculture Conference-TropAg, 20-22 November 2017, Brisbane Convention and Exhibition Centre, Brisbane (Poster presented)
10. 'The effect of physiological maturity on the anthocyanin profile of purple sweet corn', International Tropical Agriculture Conference-TropAg, 20-22 November 2017, Brisbane Convention and Exhibition Centre, Brisbane (Poster presented)
11. 'Healthy reds and purples - developing an anthocyanin database to create certified standards for new food commodities', International Tropical Agriculture Conference-TropAg, 20-22 November 2017, Brisbane Convention and Exhibition Centre, Brisbane (Poster presented)
12. Widaningrum, W., Mikkelsen, M., Flanagan, B., Williams, B., A., Gidley, M., J., 2017. Isolation of Dietary Fibres from Selected Fruit and Vegetables for In Vitro Fermentation', International Tropical Agriculture Conference-TropAg, 20-22 November 2017, Brisbane Convention and Exhibition Centre, Brisbane
13. Netzel, M., E., O'Hare, T., Striegel, L., Rychlik, M., 2017. 'High-folate strawberries' Simply Red Newsletter. Australian Strawberry Industry Newsletter. SimplyRed 48, December 2017
14. 'Deep-pigmented strawberries – a potential niche market for the strawberry industry?' M. Netzel & T. O'Hare was submitted to 'Simply Red' (the Australian Strawberry Industry Newsletter) to be published in the December Issue. This follows on from 'High-folate strawberries' publication, which was published in the December issue for 2017
15. Hong, H., T., Netzel, M., E., Netzel, G., Giles, C., O'Hare, T., 2017. Identification of purple anthocyanin pigments from purple sweetcorn using LC-PDA-MS/MS. 2nd Queensland Mass Spectrometry Symposium, Brisbane, QLD, Australia, 2-3 November 2017.
16. Hong, H., T., Netzel, M., E., Netzel, G., O'Hare, T., 2017. Determination of pigments of purple sweetcorn using LC-PDA-MS/MS. 2nd Queensland Annual Chemistry Symposium QACS 2017, Brisbane, QLD, Australia, 27 November 2017
17. A presentation was given to the Macadamia's Handlers Association in Brisbane in July 2018. A presentation was made of work being conducted within the NN project in relation to macadamia and biofortification. The meeting included 7 members of the association, including Jolyon Burnett, Lynne Ziehlke, Richard Genest, and Steve Lee. The presentation was followed by a discussion, including the positive/negative health benefits of saturated fat, and potential issues of making potential health issues public regarding impact on macadamia sales.
18. Striegel, L., Netzel, M., Rychlik, M., 2018. Strawberries as a source of highly bioavailable folates. 5th International Vitamin Conference 2018, Sydney, NSW, Australia, 8-10 August 2018, Abstracts: 68 (1st Prize for best Poster Presentation)
19. Hong, H., T., Netzel, M., E., O'Hare, T., 2018. The effect of physiological maturity and different cooking methods on anthocyanin accumulation in purple sweetcorn kernels. 3rd Queensland Annual Chemistry Symposium QACS 2018, Brisbane, QLD, Australia, 23 November 2018
20. Sarwar, S., Netzel, M., N., Hong, H., T., Netzel, G., Mereddy, R., Sultanbawa, Y., 2018. Physicochemical characteristics of commercial strawberry cultivars grown in Australia. 3rd Queensland Annual Chemistry Symposium QACS 2018, Brisbane, QLD, Australia, 23 November 2018, QACS 2018
21. Hong, H., T., Netzel, M., E., O'Hare, T., 2018. Extraction efficiency and stability of anthocyanins using acidified extraction solvents and UHPLC-PDA-MS/MS. 3rd Queensland Mass Spectrometry Symposium, Brisbane, QLD, Australia, 6-7 December 2018
22. A brief discussion was held with Matt Hood (CEO of Rugby Farms) about purple sweetcorn. The issue was raised as to the importance of growing the purple sweetcorn in isolation from yellow sweetcorn. It was

suggested that it should be grown in a similar way to white sweetcorn, to avoid foreign pollen causing xenia effects, particularly inducing yellow endosperms (internal) in the purple sweetcorn (which have white endosperm)

23. Netzel, M., Weber, N., Dumler, C., Striegel, L., Phan, A, D, T., Trieu, H, H., Rychlik, M., O'Hare, T., 2018. Strawberries – an underestimated dietary source of folate? Nutrition Society of Australia, 42nd Annual Scientific Meeting, Canberra, Australia, 27-30 November 2018 (abstract)
24. Netzel, M., Wright, O., Netzel, G., 2018. Understanding the nutritional value of anthocyanins, Nutrition Society of Australia, 42nd Annual Scientific Meeting, Canberra, Australia, 27-30 November 2018 (abstract)
25. Netzel, M, E., Weber, N., Dumler, C., Striegel, L., Phan, A, D, T., Trieu, H, H., Rychlik, M., O'Hare, T., 2018. Strawberries – Higher in folate than previously thought! QACS 2018-Queensland Annual Chemistry Symposium, Brisbane, Australia, 23 November 2018 (accepted abstract)
26. Netzel, M., O'Hare, T., Rychlik, M., 2018. Naturally Nutritious – Development of the Alpha Strawberry Packed with Folate. Launch of the Australia-Germany Research Network at the Australian Embassy in Berlin, 20 November 2018, Appendix–Poster Exhibition.
27. Netzel, M.E., 2019. Strawberries - delicious and a 'valuable' dietary source of folate. Joint DAF and QAAFI Food Science Seminar Series, 26 March 2019, Coopers Plains, QLD, Australia.
28. Purple sweetcorn and high-zeaxanthin capsicum field and laboratory visit: A field and laboratory presentation on purple sweetcorn and high-zeaxanthin capsicums was held with representatives (Ms Genevieve Windley, Mr Ed Windley) from KalFresh Pty Ltd on the 8 th May 2019. Discussions were held regarding the present progress and areas of research we were investigating, as well as a field-walk through the purple sweetcorn nursery.
29. Damyeh, M., S., Mereddy, R., Netzel, M., E., Sultanbawa, Y. 2019. Curcumin-based photosensitization: a novel and green technology to decontaminate food systems. Proceedings Volume 11070, 17th International Photodynamic Association World Congress, 28 June – 4 July 2019, Cambridge, Massachusetts, United States, https://doi.org/10.1117/12.2530661
30. O'Hare, T., Trieu, H., Alam, M., Russell, D., Pun, S., Torrisi, C., Liu, L., Williams, D., Topp, B., 2018. Macadamia nuts 'Good fats, bad fats and biofortification'. 30th International Horticultural Congress, 12-16th August 2018, Istanbul, Turkey. OS 4-3 (oral abstract)
31. Netzel, M.E., O'Hare, T., 2019. Deep-pigmented strawberries-a potential niche market for the strawberry industry? SimplyRed, Strawberry Industry Newsletter, 52, 8-9
32. Netzel, M.E., O'Hare, T., Rychlik, M., 2019. Australian grown strawberries on show in Germany. SimplyRed, Strawberry Industry Newsletter, 53, 1
33. Agarwal, R., O'Hare, T., Hong, H, T., Cave R., Harper, S., 2019. Orange capsicums and chillies as a potential source of dietary zeaxanthin, an important macular carotenoid for eye health. International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane, 285 (Oral abstract)
34. Damyeh, S, M., Sultanbawa, Y., 2019. Optimising the antibacterial activity of Tasmanian pepper leaf oil emulsion as a natural preservative for capsicum. International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane, 649 (Poster abstract)
35. Hong, H., T., O'Hare, T., 2019. Differences in the anthocyanin profile of different tissues in strawberry fruit. International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane, 671 (Poster abstract)
36. Hu, W., O'Hare, T., Fitzgerald, M., Topp, B., Alam, A., 2019. Investigating the effects of pollen and environment on palmitoleic acid and saturated fat content of macadamia nuts. International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane, 436 (Poster abstract)
37. Kodagoda, G., M, Netzel., T, O'Hare, T., 2019. The effect of post-harvest storage on the physicochemical properties and physiochemical content of Queen Garnet plum. International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane, 654 (Poster abstract).
38. Netzel, M., Dumler C., Weber, N., Striegel, L., Rychlik, M., Hong H, T., O'Hare, T., 2019. The inside and out of folate in strawberries and avocados. International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane. 180 (Oral abstract)
39. Olarte, S., Smyth, H., 2019.Purple sweetcorn- An innovative horticultural product-consumer views. International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane 663 (Poster abstract)

40. Ray, A., L., 2019. Super-sweet purple sweetcorn: Breaking the genetic link. International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane, 183 (oral abstract)
41. Dumler, C., Weber, N., Striegel L., Rychlik M., Hung, T, H., O'Hare T., Netzel M., 2019. B6. Folate in strawberry and avocado – profile, distribution, and total content. 4th Queensland Annual Chemistry Symposium QACS 2019, Brisbane, QLD, Australia, 29 November 2019, QACS 2019 Programme 34
42. Hong, H.T., Agarwal, R., Netzel M., O'Hare T., 2019. Optimisation of extraction and saponification for the determination of free- and bound-carotenoids in orange capsicum and avocado. Queensland Annual Chemistry Symposium QACS 2019, Brisbane, QLD, Australia, 29 November 2019, QACS 2019 Programme 31
43. Kodagoda, G., Hung, T, H., Topp B., Sultanbawa Y., O'Hare T., Netzel M., 2019. The effect of storage temperature on the accumulation of anthocyanins in different fruit tissues of Queen Garnet plum. Queensland Annual Chemistry Symposium QACS 2019, Brisbane, QLD, Australia, 29 November 2019, QACS 2019 Programme 18
44. Sarwar, S., Mereddy, R., Netzel, M, N., Netzel, G., Sultanbawa, Y., 2019. Effect of curcumin-based photodynamic treatment on nutritional properties of strawberry fruit. Queensland Annual Chemistry Symposium QACS 2019, Brisbane, QLD, Australia, 29 November 2019, QACS 2019 Programme 12
45. Damyeh, M., S., Netzel, M., E., Mereddy, R., Sultanbawa, Y. 2020. Curcumin-loaded emulsion as a delivery vehicle for the photosensitization of fresh foods. 15th International Hydrocolloids Conference, 2-5 March 2020, Melbourne, Australia
46. Sarwar, S., Netzel, G., Netzel, M., E., Mereddy, R., Sultanbawa, Y., 2020. Light-emitting Diode based Photosensitization and its Effect on the Quality of Australian grown Strawberry. Australian Institute of Food Science and Technology (AIFST) Virtual Convention (virtual Poster and Abstract), 23 June-2 July 2020. https://www.aifst.asn.au/2020-AIFST-Convention
47. Damyeh, M., S., Netzel, M., E., Mereddy, R., Sultanbawa, Y. 2020. Developing an edible coating as a preservative, incorporating Australian native plants with antimicrobial properties. 15th International Hydrocolloids Conference, 2-5 March 2020, Melbourne, Australia
48. Hong, H., T., O'Hare, T., 2020. Optimisation of extraction procedure and LC-DAD-MS methodology for anthocyanin analysis in pigmented corn kernels. Queensland Annual Chemistry Symposium, Brisbane, Australia, 27 November 2020
49. Kodagoda, K., 2020. Increasing bioactive anthocyanins in plumcots. QAAFI 3MT, 13 July 2020 (Top 8 presentation)
50. Kodagoda, G., O'Hare, T., Sultanbawa Y., Topp, B., Hong, H., T., Netzel, M., E., 2020. Impact of storage on phytochemicals and sugars in Queen Garnet plum. 2020 Australian Institute of Food Science and Technology (AIFST) Virtual Convention, 23/25/30 June & 2 July 2020 (virtual Poster and Abstract; https://www.aifst.asn.au/2020-AIFST-Convention). [2020 Research Poster Competition – 1st Place/Winner]
51. Kodagoda, G., Topp, B., Sultanbawa, Y., Hong, H., T., O'Hare, T., Netzel, M., 2020. Assessment of the nutritional quality of Australian grown Plumcot. NSA Brisbane Group's Virtual Research Showcase Events, Online, 25-26 August 2020. [oral presentation by zoom]
52. Netzel, M., Wright, O., Hong, H., T., Rychlik, M., O'Hare, T., 2020. How do delicious strawberries help us reach our folate goals? In: Science Week 2020, Brisbane, QLD Australia, 17-20 August 2020. [oral]
53. Netzel, M., Dumler, C., Weber, N., Striegel, L., Rychlik, M., Hong, H., T., O'Hare, T., 2021. Does shape matter? Folate in strawberry and avocados. VIII International Symposium on Human Health Effects of Fruits and Vegetables, 9-11 March 2021, Book of Abstracts: 5 [oral/zoom]
54. Kodagoda, G., Hong, H., T., Topp, B., Sultanbawa, Y., O'Hare, T., Netzel, M., E., 2021. Do blood-plums (Prunus salicina) have carotenoids? The 1st Virtual International Conference on Carotenoids, 22-24th June 2021, (abstract)
55. Hong, T., H., Agarwal R., Michael E., N., O'Hare, T., 2021. Free- and esterified-carotenoids in high oil content matrices: an optimised procedure of saponification for carotenoid quantification. The 1st Virtual International Conference on Carotenoids, 22-24th June 2021, (abstract)
56. Agarwal, R., Hung, T., H., Cave, R., Hayward, A., Harper, S., O'Hare, T., 2021. Chillies as potential candidates in breeding for high zeaxanthin capsicum varieties. The 1st Virtual International Conference on Carotenoids, 22-24th June 2021, (abstract)
57. O'Hare, T., Hong, H., T., 2021. The hunt for the purple strawberry: Change in anthocyanin profile leads to a colour change from red to burgundy. IX International Strawberry Symposium, Rimini (Italy), 1-5 May 2021

Refereed scientific publications

Journal articles

1. Damyeh, M., S., Fernando, W. C., Anh, D., T., P., Hung, T., H., Mereddy, R., Netzel, M., E., Chaliha, M., Sultanbawa, Y., 2021. Post-harvest fungal occurrence on commercial strawberry cultivars grown in Australia–impact of phytochemical composition" Journal of Food Measurement and Characterization
2. Ni, D. Gunness, P., Smyth, H., E., Gidley, M., J., 2021. Exploring relationships between satiation, perceived satiety, and plant-based snack food features. International Journal of Food Science and Technology; in press doi:10.1111/ijfs.15102
3. Hong, H., T., Netzel, M., E., O'Hare, T., J., 2020. Anthocyanin composition and changes during kernel development in purple-pericarp super sweet sweetcorn. Food Chemistry, 315, https://doi.org/10.1016/j.foodchem.2020.126284
4. Hong, H., T., Netzel, M., E., O'Hare, T., J., 2020. Optimisation of extraction procedure and development of LC–DAD–MS methodology for anthocyanin analysis in anthocyanin-pigmented corn kernels. Food Chemistry, 319, https://doi.org/10.1016/j.foodchem.2020.126515
5. Hong, H., T., Netzel, M., E., O'Hare, T., J., 2020. Purple sweetcorn – something tasty and healthy? Acta Horticulturae 1292, 393-398, https://doi.org/10.17660/actahortic.2020.1292.52
6. Hong, H., T., Netzel, M., E., O'Hare, T., J., 2020. A dataset for anthocyanin analysis in purple-pericarp sweetcorn kernels by LC-DAD-MS. Data in Brief, 30 105495, 105495. doi: 10.1016/j.dib.2020.105495
7. Hong, H., T., O'Hare, T., J. 2021. Postharvest storage of reddish-purple sweetcorn: changes in anthocyanin, starch, and sugar content during ambient and refrigerated storage. Proceedings, 70 (1) 13, 13. doi: 10.3390/foods_2020-07728
8. Hong, H., T., Phan, A., D., T., O'Hare, T., J., 2021. Temperature and maturity stages affect anthocyanin development and phenolic and sugar content of purple-pericarp supersweet sweetcorn during storage. Journal of Agricultural and Food Chemistry, 69 (3) acs. jafc.0c06153, 922-931. doi: 10.1021/acs.jafc.0c06153
9. Hu, W., Fitzgerald, M., Topp. B., Alam, M., O'Hare, T., J. 2019. A review of biological functions, health benefits, and possible de novo biosynthetic pathway of palmitoleic acid in macadamia nuts. Journal of Functional Foods, 62, https://doi.org/10.1016/j.jff.2019.103520
10. Kodagoda, G., Hong, H., T., O'Hare, T., Sultanbawa, Y., Topp, B., Netzel, M., E., 2021. Effect of storage on the nutritional quality of Queen Garnet plum. Foods, 10 (2) 352, 352. doi: 10.3390/foods10020352
11. Kodagoda, G., Netzel, M., 2021 Does storage improve the nutritional quality of plums? Food Australia 73(1): 7
12. O'Hare, T., J., Hong, H., T., Pun, S., Liu, D., Torrisi, C., Mai, T., Alam, M., Russell, D., Topp, B. 2020. Macadamia nuts – good fats, bad fats, and biofortification. Acta Horticulturae, 1292, 377-382, https://doi.org/10.17660/actahortic.2020.1292.50
13. O'Hare, T. J., Hong, H., T., Topp, B., Russell, D., Pun, S., Torrisi, C. and Liu, D. (2019). Assessing Fatty Acid Profiles of Macadamia Nuts. HortScience, 54 (4), 633-637. https://doi.org/10.21273/HORTSCI13418-18
14. Sarwar, S., Netzel, G., Netzel, M., E., Mereddy, R., Phan, A., D., T., Hong, H., T., Cozzolino, D., Sultanbawa, Y., 2020. Impact of curcumin-mediated photosensitization on fungal growth, physicochemical properties, and nutritional composition in Australian grown strawberry. Food Analytical Methods, 14 (3), 465-472, https://doi.org/10.1007/s12161-020-01896-z
15. Seidi, D., M., Mereddy, R., Netzel, M., E., Sultanbawa, Y., 2020. An insight into curcumin-based photosensitization as a promising and green food preservation technology. Comprehensive Reviews in Food Science and Food Safety, 19 (4), 1727-1759, https://doi.org/10.1111/1541-4337.12583
16. Striegel, L., Chebib, S., Netzel, M, E., Rychlik, M., 2018. Improved stable isotope dilution assay for dietary folates using LC-MS/MS and its application to strawberries. Frontiers in Chemistry, 6 (11), https://doi.org/10.3389/fchem.2018.00011
17. Widaningrum, W., Flanagan, B., M., Williams, B., A., Sonni, F., Mikkelsen, D, Gidley, M., J., 2020. Fruit and vegetable insoluble dietary fibre in vitro fermentation characteristics depend on cell wall type. Bioactive Carbohydrates and Dietary Fibre, 23, https://doi.org/10.1016/j.bcdf.2020.100223
18. Wright, O., R., L., Phan, Anh D., T., Hong, H., T., Netzel, G., Sultanbawa, Y., Netzel, M., E., 2020. Anthocyanins: "berry" much more than common plant pigments. Australian Berry Journal, Summer (5), 22-23
19. O'Hare, T., Hong, H., T., 2021. The hunt for the purple strawberry: Change in anthocyanin profile leads to a colour change from red to burgundy. Acta Horticulture (accepted).

Chapter in a book or Paper in conference proceedings

1. Agarwal, R., O'Hare, T., Hong, H., T., Cave R., Harper, S., 2020. Orange capsicums and chillies as a potential source of dietary zeaxanthin, an important macular carotenoid for eye health. Third International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane. 161, doi: 10.3390/proceedings2019036161
2. Agarwal, R., Hong, H., T., Hayward, A., Harper, H., Mitter, N., O' Hare, T., 2020. Carotenoid profiling of orange-coloured capsicums in search of high zeaxanthin varieties. The 1st International Electronic Conference on Food Science and Functional Foods: Nutraceuticals and Functional Foods https://foods_2020.sciforum.net/. DOI: 10.3390/foods_2020-07717, proceedings036183
3. Hong, H., T., Chen, A., Netzel, M., E., O'Hare, T., J., 2020. Differences in the anthocyanin profile of different tissues of the strawberry fruit. Third International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane. 74, doi: 10.3390/proceedings2019036074
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5. Kodagoda, G., Netzel., M., O'Hare, T., 2020. The effect of post-harvest storage on the physicochemical properties and physiochemical content of Queen Garnet plum. Third International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane. 1-1, doi: 10.3390/proceedings2019036099 (Best Scientific Poster – Nutrition and Food)
6. Netzel, M., Dumler, C., Weber, N., Rami, J., Rychlik, M., Netzel, G., Wright, O., Hong, H, T., O' Hare, T, J., 2020. Folate in Red Rhapsody Strawberry – Content and Storage Stability. The 1st International Electronic Conference on Food Science and Functional Foods: Nutrition and Health Claims https://foods_2020.sciforum.net/. DOI: 10.3390/foods_2020-07670, proceedings036958
7. Netzel, M., Dumler C., Weber, N., Striegel, L., Rychlik, M., Hong H, T., O'Hare, T., 2020. The inside and out of folate in strawberries and avocados. Third International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane. 86, doi: 10.3390/proceedings2019036086
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10. Olarte, S., Lyons, P., O'Hare, T., Smyth, H., 2020. Purple —an innovative horticultural product—consumer views. Third International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane. 102, doi: 10.3390/proceedings201903610
11. Ray, A., L., O'Hare, T., 2020. Super-sweet purple sweetcorn: breaking the genetic link. Third International Tropical Agriculture Conference-TropAg, 11-13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane. 6134, doi: 10.3390/proceedings2019036134
12. Sarwar, S., Netzel, M, N., Netzel, G., Sultanbawa, Y., 2019. Impact of Photosensitization on Physicochemical Properties in Strawberries. International Tropical Agriculture Conference-TropAg, 11–13 th November 2019, Brisbane Convention and Exhibition Centre, Brisbane QLD, Australia. 36(1), 80, doi: 10.3390/proceedings2019036080
13. Huang, Li., Yong, K., W., L., Fernando, W., C., Carpinelli, D., J., Matheus, D., V., James, J., Sultanbawa, Y., Fletcher, M., T., 2021. The inactivation by curcumin-mediated photosensitization of Botrytis cinerea spores isolated from strawberry fruits. Toxins, 13 (3), 196. doi: 10.3390/toxins13030196.

Recommendations

Increasing nutrient density (biofortification):
The Naturally Nutritious project evaluated a range of potential products for biofortification, based on their technical feasibility and consumer acceptability, together with industry feedback.

Macadamia (reduced-saturated fat)
Technically, the development of a reduced-saturated fat macadamia nut was shown to be possible but will require the pairing of reduced-saturated varieties/accessions, as the level of saturated fat was not only influenced by the maternal tree, but also by the paternal pollen parent (macadamias are strong out-crossers). This discovery was novel and requires further exploration to identify variety/accession combinations that can constantly yield kernels with 8-10% saturated fat within their oil profile. Reduced-saturated fat is a potential label claim for such a product, as well as potentially increasing the health star rating to 5 stars.

Importantly, as the macadamia kernel is the immediate result of two parents, it is possible that breeding for this characteristic is not necessary, which would significantly reduce the time required to achieve reduced-saturated fat nuts. Having said that, it would still require paired-planting within an orchard once appropriate parent have been identified through controlled-pollination experiments.

From a consumer-perspective, the concept of low-saturated fat macadamia was well received by 70% of those assessed, indicating that such a trait could be viewed as a marketing bonus, to elite orchards, or potentially over standard macadamia nuts produced and exported by other countries. The wide community understanding of saturated fat being associated with increased risk of cardiovascular disease helped support this concept. The proviso being that the flavour of the macadamia remained the same, and ideally cost no more than standard nuts. Note, that preliminary evaluation of the flavour of macadamia nuts with varying saturated fat levels did not indicate any flavour difference. Similarly, no reduction in shelf-life was observed.

Despite the technical feasibility and positive consumer response, some sectors of the macadamia industry were concerned that the development of a reduced-saturated fat macadamia may reflect badly on the remainder of the 'non-improved' industry, especially prior to reduced-saturated fat orchards being established. It is recommended that development of a reduced-saturated fat nut be considered as a strategic tool for future global competitiveness.

A secondary benefit identified during the project, was the potential to replace excess saturated fat with the mono-unsaturated fat, palmitoleic acid, which is already higher in macadamia from most other products. Although the benefits of palmitoleic acid have been reported previously, the fact that no label claim can be made for palmitoleic acid, and the low knowledge base of this with consumers, would make it a bonus, rather than a driver for marketing and consumption. Reduction of saturated fat through increased oleic acid, however, was also observed within the project, and oleic acid, which is also the main fatty acid in olive oil, is better known to the public. Consequently, the production of reduced-saturated fat macadamias in conjunction with either oleic or palmitoleic acid could be pursued, as the knowledge of the latter may increase with time.

Purple sweetcorn (high-anthocyanin)
Technically, purple sweetcorn based on both the supersweet genetic mutations, *shrunken2* or *brittle1*, were shown to be possible within the Naturally Nutritious project. The purple pigmentation, due to the phytonutrient anthocyanin was introgressed from non-sweet starchy purple maize, a traditional purple corn from South America. The development of the purple colour is limited to the outer kernel (pericarp), with the inside being white. Colour development on the pericarp increases with kernel maturity, but over-maturity is also related to a reduction in sugars, which are important for the palatability of sweetcorn. Unexpectedly, and unlike yellow sweetcorn, pigment development could continue after harvest if held for periods of non-refrigeration (> 4°C).

Although no label claim can currently be made for anthocyanin, currently a body of supporting data for the benefits of anthocyanin to cardiovascular disease exists, and 'purple' is generally seen as healthier by consumers. Consumers were generally acceptive of purple sweetcorn, especially with the information that it was developed naturally from traditional Peruvian purple maize. The flavour of purple sweetcorn was generally that of normal sweetcorn, but a slight raspberry flavour was also evident, which pleased consumers, and offered another point of product differentiation. One negative that was reported by consumers was the dislike of papery glume tissue at the base of

kernels. It should be noted that this negative was observed with the *brittle1* mutation and has been largely removed in the more recently developed purple sweetcorn based on the *shrunken2* mutation. Other consumer preferences that also should be included in further development of purple sweetcorn, were (1) good colour coverage across the kernel surface, and (2) preference for purple rather than a reddish-purple colour.

From an industry perspective, the development of purple sweetcorn was fully supported, and seen as an additional sweetcorn for the consumer, offering a uniquely different product. From a practical perspective, the development of purple sweetcorn based on the *shrunken2* mutation, which is what the rest of the Australian yellow sweetcorn industry is based on, was seen as a strong positive, due to (1) not inducing 'starchiness' in adjacent yellow sweetcorn plantings and therefore being able to be planted closer without isolation, and (2) future introgression of positive traits (e.g. disease resistance etc.) from yellow sweetcorn germplasm, without the need for re-selection for the (recessive) *shrunken2* mutation.

It is also suggested that future development ideally remove any aleurone-based pigmentation, as this can be transferred by pollen to yellow sweetcorn, and cause spotting, as opposed to pericarp pigmentation which cannot be transferred by pollen, being a maternal plant trait. Other recommendations include exploring the advantages of a white-coloured underlying cob, as opposed to a purple cob, which could potentially leach pigment during processing into cobettes, or during cooking and eating (purple fingers). Finally, it is recommended that the inclusion of purple sheath leaves (which cover the cob) should also be included as a strategic trait against the possibility of plastic film withdrawal (overwrapped cobettes) such that cobs need to be sold as fully sheathed cobs. Purple-coloured sheath leaves would be a visual differentiation against yellow sweetcorn, which has green sheath leaves.

Orange-capsicum (high-zeaxanthin)

The Naturally Nutritious project identified that most current orange capsicums available in Australia are a very good, and in some cases, extremely good, source of zeaxanthin, which plays a role in eye-health, specifically age-related macular degeneration (AMD). For at least one cultivar of orange capsicum, 7 g of fruit tissue contained the same amount of zeaxanthin (2 mg) as a supplement tablet, such that a single 400 g fruit could contain the equivalent of over 50 tablets.

In a single case, a darker-orange coloured capsicum was found to not contain high zeaxanthin, but rather a mixture of yellow and red pigments, which when mixed gave an orange-like colour. This could potentially lead to some confusion to consumers who are seeking orange capsicums with high-zeaxanthin, although this particular variety was not grown commercially, as far as we are aware.

From a consumer perspective, the focus group assessed indicated that they loved the vivid orange colour of the orange capsicum and were very supportive of this product. In fact, we received a large number of requests of where consumers could obtain orange capsicums in stores, as they were generally difficult to locate, in contrast to red, green or yellow fruit (all of which are not a good source of zeaxanthin). As might have been expected, the information that the orange pigment, zeaxanthin, was associated with slowing the progress of macular degeneration was well received, especially by the older age-group, who are more prone to AMD.

From an industry perspective, the cost of orange capsicum seed relative to red capsicum seed was only an issue to field growers, whereas, for glasshouse growers, this was not an issue. In general, the low number of orange fruit in stores is based primarily on existing consumer demand, in that the orange capsicum is currently just perceived as another coloured capsicum, with no specific health benefit. Consequently, availability of orange capsicums tends to be variable, with growers generally growing the mainstream red and green (which are actually immature red fruit) based on what they are told by the supermarkets or agents.

We would consider that an increase in consumer demand, based on the knowledge of their benefit to AMD, would eventually increase orange capsicum availability to consumers. This, however, would require an information program, potentially using conduits such as the Macular Disease Foundation including orange capsicums in their dietary information, which does not currently list them. Additional studies showing the high bioavailability of zeaxanthin from orange capsicums would also add supporting information, specifically for this fruit.

Strawberry (high-folate)

A consistent finding within the Naturally Nutritious project was that the folate (Vitamin B9) level in Queensland-sourced strawberries was well above (at least double) the average folate level cited in the FSANZ nutritional content tables for this fruit. From a label claim perspective, this would potentially enable strawberries being listed as 'a good source of folate', rather than the present lower-level statement of 'source of folate'.
Technically, it was also found that at least one breeding accession of strawberry had approximately four times the level of that listed in the FSANZ nutritional tables, such that a 250 g punnet of these strawberries would actually supply the recommended daily intake of folate. If increasing folate was to be pursued within a breeding program, it also appeared that those breeding accessions at the higher folate concentrations were also genetically related, so it is likely that this trait is highly heritable.
The finding that folate was consistently 1.7 times higher in outer flesh compared to inner flesh would tend to indicate that flatter longer fruit would have a higher folate level than more spherically-shaped fruit, because of the greater surface area to volume ratio tending to favour a greater volume of outer flesh.

The human folate bioavailability study conducted on four healthy female volunteers at the University of Queensland Clinical Research Facility in Brisbane (a punnet of commercial strawberries (250 g) vs. a folate supplement) is still underway at the writing of this report. Blood and urine samples are currently being analysed for folate and bioavailability/pharmacokinetic parameters will be determined and published as soon as the folate concentrations of the analysed samples are calculated. However, a relatively high folate bioavailability from Australian grown strawberries, similar to that found in a human pilot study with German grown strawberries, is expected.

From a consumer perspective, folate content was not seen as an important driver for strawberry purchase, despite its health benefit. Despite the importance of folate to females prior to pregnancy, in regard to preventing spina bifida in the developing foetus), the availability of folate as a supplement was considered sufficient, even to this section of the focus group. Males were even less interested in folate. Higher folate was not seen as a negative trait, but also not as a positive trait for purchase. In this regard, folate can be seen as a 'bonus'. Based on this, further breeding for enhanced folate in strawberries is not considered a priority.
However, industry was quite interested in establishing the higher folate content for Australian strawberries than what is officially in the nutritional tables for this fruit. The fact that label claims can be made, and that it potentially would apply to the whole Australian industry, was definitely worth pursuing. As the current assessments of folate only utilized Queensland-sourced strawberries, this would require an Australian-wide sub-sampling, potentially in a stand-alone project. Food Standards (FSANZ) were also supportive of this and would be quite happy to update their nutrition table, accordingly.

Strawberry (high anthocyanin / purple strawberry)
The anthocyanin concentration in strawberry was found to be high in some genotypes, although the amount of anthocyanin was considered to be less than other high-anthocyanin commodities (such as Queen Garnet plum). In addition, anthocyanin was observed to increase in some cultivars after harvest, so the reliability of previously published data is variable, depending on temperatures experienced between harvest and preparation for analysis.

The Naturally nutritious project established that apart from dark red strawberries, breeding accessions also existed that were a burgundy/purple colour, with variation in regard to the inner and outer flesh/skin colour. Consequently, it was possible to have a strawberry with purple skin with red flesh, or purple skin with purple flesh. The main difference in colour was less to do with total anthocyanin content, and more to do with a change in the type of anthocyanins present, with dark red fruit simply having mainly red pelargonidin present, while the purple fruit had a higher proportion of cyanidin, which is a burgundy/purple-colour. These purple-coloured fruit were very unique, and attracted much interest when presented at the online International Strawberry Symposium in 2021.

From a consumer perspective, dark-red strawberries were associated with the fruit being "very sweet", "soft", "mushy" and "overripe". Based on the discussions with respondents, the deep red strawberries would require good marketing and the public would need to be provided with information about the ripeness and different colour to the standard strawberries before they would be willing to purchase them. Once informed about the higher anthocyanin content of the deep red strawberries, some of the respondents were more interested in trying/buying these strawberries. Again, educating the consumers about the health benefits of these products through marketing would make them more popular. Most respondents said that they would like to "try before you buy."

Unfortunately, purple-coloured strawberries were not able to be assessed. From the strawberry breeder's experience, a slight flavour difference exists for purple strawberries, and it remains to be assessed how acceptable this may be to

consumers. Similar to purple sweetcorn, a slight flavour difference (that is palatable), over a general background strawberry flavour, may actually be beneficial to product differentiation, to further distinguish fruit from standard red strawberries. An additional potential issue observed by the breeder was the potential effect of postharvest moisture loss on the surface gloss of purple strawberries. This is a factor that also occurs with red strawberry fruit but might be less acceptable with purple fruit.

The strawberry industry feedback included that standard red cultivars that are left too long can develop a flat, unattractive, dark appearance, and it would be important that a high-anthocyanin strawberry or purple fruit was easily differentiated from such fruit. However, the development of a high-anthocyanin strawberry was also seen as a good idea, based on the success of other high-anthocyanin fruit, such as the Queen Garnet plum, but the lower total anthocyanin levels than products such as the Queen Garnet reduce the strength of any health claim.

Currently, the fact that high-anthocyanin health claims are unable to be made, and that the level of anthocyanin was below that of other high-anthocyanin products, such as the QG plum, makes pursuing this product less attractive to industry (at present) than further establishing the high-folate nutrient health claim (above). However, the national strawberry breeding program will continue to breed and improve 'dark' strawberries, purely for their visual attraction for potential marketing in the future.

It is also suggested that a larger human intervention study should be conducted to better understand the molecular mechanisms of the health-promoting effects of strawberry consumption, for example, the reduction in inflammation associated with the anthocyanin pigments. Such an evidence-based study would provide a useful marketing-tool for the Australian strawberry industry to promote the health benefits of domestic grown strawberries (similar to the American "Blueberry-Story").

Early season high-anthocyanin plum

Within the Naturally Nutritious project, crosses of an existing high-anthocyanin plum (Queen Garnet) were crossed with an early season anthocyanin-containing plumcot (Rubycot), in order to transfer the early-season characteristic of the Rubycot into a high-anthocyanin background. A major drawback that occurred within the project timeline was the severe drought resulting in the loss of seedling trees, and the slowing of growth of those that survived. Consequently, only about 10 trees were able to be assessed, and the development of a phenotype population to aid in the development of genetic markers for selection of high-anthocyanin was not possible. Despite this, at least one of the progeny trees expressed an earlier harvest date, and some of the characteristics of the Queen Garnet plum parent, though to a lesser extent.

From consumer feedback, it was clear that there was enthusiasm for an earlier season version of the Queen Garnet plum, but also enthusiasm for a high-anthocyanin apricot. There was no interest in another type of plum on the market, as the plum market was already considered to have ample choice. In this regard, we believe it is important that a high-anthocyanin plum-cot be as similar to its Queen Garnet parent in every way, except for being harvested earlier in the season, ideally around the festive Christmas season. This way, the early season plum-cot will be able to capitalise on the existing Queen Garnet plum marketing, rather than further confuse the market with yet another plum variety.

In addition to the above trial, a pre-existing sibling (401-43) from the original Queen Garnet plum breeding population was 'discovered', which had the early-harvest characteristics being aimed for. This is considered a good alternative cross parent to Queen Garnet, as it is less likely to display non-QG characteristics that were present in the Rubycot parent. Keeping in mind that the consumer focus group was mainly interested in an earlier season fruit similar to Queen Garnet plum, this would lend support for considering future crossing of the Queen Garnet with an early-harvest accession more similar in these flavour characteristics, such as the '401-43' sibling of Queen Garnet, to satisfy this requirement.

From industry feedback, an earlier-season Queen Garnet plum has commercial value to Nutrafruit and growers. It was suggested that any fruit that can be exported into China before Chinese New Year will receive double the value of that arriving later. Optimising the anthocyanin content in the Queen Garnet plum was also of particular interest, as it can cause the internal fruit colour to vary considerably.

Further development of an early-season Queen Garnet plum definitely has merit for industry but would require further funding.

Saffron sweetcorn

Saffron sweetcorn was found not to be technically feasible without a transgenic intervention by introducing the gene for the specific enzyme from *Crocus* plant, responsible for cleaving zeaxanthin into crocetin, the principle saffron compound. Although use of such technologies such as CRISP-CAS9 are permitted in some cases (e.g., knocking out the activity of a gene), the introduction of a novel gene is not currently permitted without being labelled a transgenic genetically modified product. Consequently, it is not currently recommended to pursue this aim.

High-lycopene tomato ('deep-pink' clear epidermis)

Seed and fruit of high-lycopene gourmet tomatoes that are able to be differentiated externally from standard tomatoes were re-generated. During this procedure, however, it was identified that at least some of the parent lines had the genetic disorder 'fruit-pox', characterized by the generation of small scattered brown marks on the surface of the skin under certain environmental conditions.

It is recommended that prior to any commercialisation of the deep-pink high-lycopene tomatoes, removal of this genetic disorder from both inbred parents would be necessary. The use of a genetic marker would be of assistance in this regard, as the phenotype does not always present itself.

Strawberry shelf-life extension (photosensitization)

Promising results of photosensitization as a clean, green technology and doubling in shelf life with no change in the nutritional quality in comparison to untreated samples, demonstrates the potential of this innovative technology for the strawberry industry. Research on photosensitization in strawberry and other fresh horticultural produce will continue and scaling up to a mobile photosensitization unit to use in different farms is planned as future research.

Capsicum shelf-life extension (natural products)

Storage life extension of fresh cut capsicums using plant extracts indicated they were very effective as an antimicrobial preservation solution and there was a significant extension of storage life to 16 days in comparison to the untreated sample with 7 days. Research will continue in taking this process to full scale commercialization with an interested industry partner with scale up and validation trials at factory premises.

Satiety & satiation

Taken together, the large body of data obtained can be summarised into a number of key messages that could be used to promote the nutritional value of horticulture products in general or specific crops in particular:

- Fruits, vegetables and nuts can deliver short-term and long-term fullness comparable with protein-rich foods
- For immediate fullness, a hard tissue structure, requiring more chews prior to swallowing, will lead to more efficient satiation than softer tissue structures
- From previous data, juiced or pureed fruits and vegetables are less efficient in providing both satiation and satiety compared with intact plant tissues
- The factors determining effective satiation are different to those for satiety, so marketing messages can be tailored accordingly e.g., cut carrots emphasising calorie-efficient fullness or macadamia nuts emphasising long-lasting fullness
- Food factors mostly determine the portion size for comfortable fullness, so there is the opportunity to define relevant pre-packed serving sizes to effectively achieve satiation
- However, the subjective perception of fullness as satiation or satiety is mostly determined by characteristics of the individual (physiological and psychological)

These findings led to a number of identifiable industry opportunities:

- Identification of horticulture product portion sizes for efficient satiation or satiety
- Marketing messages on the effective short- and longer-term fullness provided by intact horticultural products, comparable with protein foods
- Raw vegetables such as carrots for energy efficient short-term alleviation of hunger
- Nuts for effective longer-term satiety

- Information for health professionals to reinforce and bring to life dietary guideline recommendations that most of the diet should be whole foods from plants
- Psychological (and physiological) effects on perceived fullness could be incorporated into targeted marketing messages.

Gut health (microbiota)

This study has identified a number of key messages that could be used to promote the nutritional value of horticulture products in general or specific crops in particular:

- All horticultural products tested had slow and steady, but eventually complete fermentation. This is an ideal combination for foods to nourish microbiota throughout the large intestine, without rapid production of gas, and with long-lasting production of beneficial end-products.
- The benefit of complex tissue structures in horticulture products is what leads to the extended fermentation timescale with diverse cell wall types having characteristic rates of fermentation. Thin/primary walls are fermented more rapidly than thickened/secondary cell walls, in contrast to conventional prebiotics such as inulin that are simple in structure and fast to ferment.
- The benefits of encapsulated starch in e.g., banana has long been recognised by the term 'resistant starch' and plays a role in extending the timeframe of fermentation of banana fibre. However, the implication that lipids in intact nut cells are fermented to short chain fatty acids is novel and opens a new marketing angle for the health benefits associated with (intact) nuts.

Specific industry opportunities were also highlighted:

- The groundwork has been laid for horticultural products to be recognised as Nature's prebiotics. In multiple laboratory trials, we have demonstrated that the complex fibres present in fruits, vegetables and nuts are fermented extensively but slowly. This is a major advantage over many current prebiotics, which are fermented rapidly and therefore do not survive to the end of the colon where protection against colon cancer is most needed. A second benefit of horticulture products as prebiotics is that the intrinsic 'fibre' also includes bound and trapped phytonutrients (e.g., anthocyanins) that can be beneficial for the microbiota and are metabolised to end-products that are known health markers in humans. This 'fibre +' concept provides a further point of differentiation from current prebiotic products.
- The finding of lipid fermentation to beneficial end products needs to be followed up to provide the nut sector with a new nutrition story.
- Horticultural waste streams can be considered as viable sources of nutrition ingredients, based on their slow and steady fermentation to beneficial end-products (SCFAs).

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