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Developing ‘superyellow’ enhanced pigment sweetcorn for eye-health.

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The Department of Agriculture and Fisheries (DAF)

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Developing 'Superyellow' enhanced pigment sweet-corn for eye health

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Summary

When you think of sweet-corn, you don't necessarily think of macular degeneration. Macular degeneration is the leading cause of blindness in Australia, and the rest of the developed world. The total cost of vision loss associated with macular degeneration in Australia was estimated at $5 billion dollars in 2010. The science is increasingly stacking up that the yellow pigments, zeaxanthin and lutein, in the macula protect the eye against the progress of macular degeneration, and that people with low levels of these pigments are more likely to suffer. As the human body cannot synthesize these pigments, they have to be obtained through our diet or supplements. While lutein can be sourced readily from green leafy vegetables, zeaxanthin is much rarer in the diet. Sweet-corn is one of the best sources of zeaxanthin, but the levels present in a normal cob of corn would require you to consume somewhere between 4-11 cobs per day to achieve a zeaxanthin-intake equivalent to that used in clinical studies with supplements. Consequently, the aim of this project was to develop a sweet-corn which could supply a supplemental dose of zeaxanthin (2 mg/person) as part of a normal meal (100 g kernels, or a small cob of corn), potentially minimising the need for an artificial supplement.

Inspiration for this research originally came from the knowledge that zeaxanthin had been linked to a lower incidence of macular degeneration, and that zeaxanthin was one of the main pigments in sweet-corn. We also had access to the DAF sweet-corn breeding program, and a wide range of sub-tropical germplasm to work with. As Australian consumers wanted a natural (non-GMO) product, we used a combination of selection, colour and carotenoid analysis, and cross-breeding to develop a range of high-zeaxanthin sweet-corns. This process was gradual, and required us to select for lines producing a lot of carotenoids (zeaxanthin is a carotenoid) and lines that favoured the synthesis of zeaxanthin over lutein, which competes with the building blocks for zeaxanthin. We then had to combine these attributes together, to give us sweet-corn lines with a zeaxanthin-concentration approximately 10 times higher (1000%) than standard sweet-corn. To complicate matters further, we then had to develop hybrids (matching 2 parents together) that produced commercially-sized cobs from vigorous, healthy plants. We now have the first cobettes of sweet-corn in the world that supply as much zeaxanthin as a supplement, with our hybrids producing 7-10 times the concentration of zeaxanthin concentration of a regular yellow sweet-corn.

An additional benefit of increasing the zeaxanthin concentration is that it gave the sweet-corn a deeper golden colour, so it can be easily differentiated in the marketplace from regular sweet-corn. This was seen as a major advantage, as the compound that changed the colour of the corn was also the active compound for ameliorating the effects of macular degeneration (what you see is what you get). Consumer assessment was carried out to determine how orange is too orange, and a working colour-range for hybrids was established, to which we fitted our hybrids. Equally important, flavour and texture was found to be as good as, if not better than, regular yellow sweet-corn. Other factors were also investigated, including the effect of seasonality on zeaxanthin concentration, refrigerated storage on zeaxanthin stability, cooking on changing colour intensity, the transient effect of freezing on kernel colour (if you were to freeze your corn), as well as whether an increase in zeaxanthin causes a change in

flavour, through carotenoid-derived volatile compounds.

Keywords

sweet-corn; zeaxanthin; macular degeneration; breeding; biofortification

Introduction

Sweet-corn is a popular vegetable in Australian households, with consumption increasing over the last decade. Australian cultivars have been developed to optimise eating quality and pest/disease resistance, although sweet-corn has potential health benefits. Sweet-corn is one of the highest natural dietary sources of the yellow pigment zeaxanthin (USDA database, 1998; Holden et al, 1999), a strong antioxidant that is selectively accumulated in the macula (Humphries & Khachik, 2003), the centre of the retina used for central detailed vision (e.g. reading). Macular Degeneration (MD) is the leading cause of blindness in Australia, affecting central vision (Australian MD Foundation, 2007). Zeaxanthin acts like 'sunglasses' by protecting critically important central sight from blue-light oxidation (Plate & Gallaher, 2005).

MD is primarily age related and most frequently affects people over the age of 50. One in seven people over the age of 50 are affected by the disease, which increases to one in three for people over the age of 75 (Australian MD Foundation, 2007; Stanley, 2002). It is sometimes referred to as Age Related Macular Degeneration or 'AMD'. Macular Degeneration (MD) is associated with a progressive, painless loss of central vision, affecting the ability to see fine detail, drive, read and recognise faces. You are reading this text using your macula. Macular Degeneration (MD) or Age-Related Macular Degeneration (AMD) is the leading cause of visual loss in people over the age of 65 years in the Western world (Stanley, 2002). Low levels of zeaxanthin and lutein in the retina have been found to be associated with an increased risk of macular degeneration (EDCC Study Group, 1993).

The importance of zeaxanthin to macular degeneration has become increasingly realised over the last five years. Zeaxanthin is selectively accumulated in the central macula from dietary sources such as sweet-corn (Humphries & Khachik, 2003). The central macula is the region used for central detailed vision (eg. reading). Lutein, a similar carotenoid in dark leafy vegetables appears to be accumulated towards the outer edge of the macula (Plate & Gallaher, 2005). It has been suggested that zeaxanthin may protect the cones in the inner macula (Bone *et al*, 1988) and lutein may protect the rods in the outer region (Sommerburg *et al*, 1999). Sweet-corn is the highest natural source of zeaxanthin amongst horticultural crops (more than 40% higher than spinach), as well as being a significant source of lutein (USDA database, 1998; Holden et al, 1999). Zeaxanthin and lutein cannot be synthesised in the body and must be obtained from the diet (Stanley, 2002). Their concentrations in the macula can be manipulated by choice of dietary source (Hammond *et al*, 1997). Increased dietary intake of both dark green leafy vegetables and sweet-corn are recommended as one means of reducing the incidence of MD due to the high levels of lutein (a yellow pigment similar to zeaxanthin) found in dark green leafy vegetables and Zeaxanthin in sweet-corn (Australian MD Foundation, 2007).

Lutein, however appears to be accumulated towards the outer edge of the macula, a less critical area for central vision. The selective uptake of dietary zeaxanthin in the central macula (Humphries & Khachik, 2003) occurs even though lutein is considerably more abundant in the diet by a 20:1 ratio (Rodriguez-Amaya, 2001). Gale et al (2003) reported that the risk of AMD was significantly higher in people with low levels of zeaxanthin, but not in those with low

levels of lutein. It could be argued that good dietary sources of zeaxanthin such as sweet-corn may be more beneficial for eye health than dark green leafy vegetables.

Sweet-corn also is a good source of lutein (Humphries & Khachik, 2003). Both lutein and zeaxanthin are xanthophylls, oxygenated derivatives of carotenes, which contain alternating double and single bonds (Moros *et* al, 2002). This structure imparts light-absorbing properties, producing coloured compounds of various shades of yellow, orange and red, and also enables them to act as strong anti-oxidants (Moros *et* al, 2002), through singlet oxygen quenching and free radical deactivation. Although both lutein and zeaxanthin protect cell membranes from free radical attack, zeaxanthin has been shown to be a better photoprotector during prolonged exposure to ultraviolet radiation (Sujak et al., 1999), which may explain its special position in the macula and its preferential uptake in the diet.

These compounds exert their protective effects by filtering short-wave blue light and by acting as anti-oxidants to remove harmful reactive oxygen species (Plate & Gallaher, 2005). People with high macular density have been shown to retain visual sensitivity at older ages (Hammond et al, 1998), and results of case-control studies suggest that such persons have a reduced risk of age-related macular degeneration (Bone et al, 2001; Beatty et al., 2001). A Cochrane review of health studies (Evans, 2006) indicated zeaxanthin and lutein have a strong link to delaying the progression of early to late macular degeneration (the leading cause of blindness in Australia). This was later confirmed in AREDS2 (Chew et al, 2013), a large clinical study in the United States involving over 4000 participants over 5 years, which showed that participants with low initial macular pigment levels had significantly slower progression of macular degeneration, when supplemented with zeaxanthin and lutein.

The incidence of macular degeneration is continuing to increase as the Australian population ages. Zeaxanthin and lutein cannot be synthesised in the body, however, their levels in macular tissue can be manipulated by dietary intake. In an Australian consumer survey in 2001, 49% of participants bought sweet-corn because it was a healthy food. This, together with the high levels of zeaxanthin (and lutein) in sweet-corn make it an ideal candidate to undertake a breeding program for enhanced eye health.

The aim of this project was to breed sweet-corn for enhanced zeaxanthin concentration, or, in essence, to develop a 'super-gold' sweet-corn variety that can be visibly recognised by consumers and associated with good eye-health. The variety would be fundamentally similar to normal supersweet corn in almost every way, but with an enhanced level of zeaxanthin, making it a more intense golden colour.

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Methodology

Initial screening of existing population lines

Initial development of 'superyellow' varieties involved screening of existing supersweet breeding lines (approximately 300 supersweet inbred lines) for the carotenoids, zeaxanthin and lutein. Our existing lines vary significantly in yellow intensity and hue. Ears were harvested at normal harvest stage (21 days after anthesis), kernels objectively graded for yellow colour (Minolta chromameter) and assessed for carotenoid/xanthophyll profile (zeaxanthin, lutein, β-carotene) and concentration using the HPLC method developed specifically for sweet-corn by Kurilich and Juvic (1999) in the United States. Initial screening was for identification of optimum inbred parents for preparation of hybrids. These inbreds were derived from our mainstream breeding program and therefore have desirable commercial traits.

A recent survey of the Australian sweet-corn industry revealed that the majority of sweet-corn production for the domestic market is based on temperate or subtropical cultivars. The tropical cultivar, Hybrix5, was estimated to be used in 28% of production. Given this dominant contribution from temperate and subtropical hybrids we decided to examine carotenoid values on sub-tropical germplasm as well as tropical germplasm in the quest to determine the basic material for raising pigment levels. After this initial comparison a decision was made on the potential benefits of each group for genetic advance. Two subtropical populations (PRO1 and PRO2) have been under development in the Kairi program for 5 cycles of selection using recurrent selection aimed at enhancing eating quality and resistance to the major diseases of sweet-corn (viz. Common rust and Turcicum leaf blight) as well as resistance to Heliothis corn ear worm. These populations also have a high frequency of resistance to Johnson grass mosaic virus. The recurrent or cyclic selection breeding procedure to be employed involved selecting amongst a large number of S0 self-pollinated plants for endosperm colour and pigment level. At the same time we evaluated the individual plants for flavour, tenderness and Heliothis resistance. Inter-crossing the elite inbreds was used to generate the next cycle of selection. This method enabled a cycle of improvement per year.

Rapid identification of high-zeaxanthin lines

The ability to select lines without carotenoid analysis was facilitated by development of a correlation between external kernel colour (hue) (identified using a Minolta chromameter) and high-performance liquid chromatography (HPLC) carotenoid profiles for zeaxanthin and lutein. This enable for only promising lines to be subjected to the more laborious task of HPLC carotenoid analysis, which increased the rate and efficiency of line selection.

Effects of kernel development, cooking and storage on pigmentation

It is important that zeaxanthin accumulation occurs in the early stages of kernel development as sweet-corn is harvested approximately 21 days after anthesis. Late accumulation is not useful from a commercial viewpoint. It is expected that carotenoid accumulation will occur in a similar early manner to existing sweet-corn cultivars. It is equally important that cooking or refrigerated storage does not adversely affect kernel colour. Cooking normally induces

membrane disruption which can lead to a perception of deeper yellow colour. We conducted trials to test the level of colour changes and if this was beneficial or not from a visual perspective. Refrigerated storage is a normal procedure for consumers. We will test effects of refrigeration on colour change, moisture loss and changes in carotenoid profile.

Consumer evaluation of 'superyellow'

The development of 'superyellow' sweet-corn was assessed initially in the concept phase using digital imagery. A digital image of a cob was increasingly coloured artificially (by computer) and assessed for consumer reaction and preference (e.g. How yellow is too yellow, how orange is too orange?) based on appearance and perceived health benefit for eye health. Breeding was subsequently directed accordingly to consumer preferences.

Consumer assessment of 'superyellow' lines as they were developed (experimental hybrids) was carried out for flavour, texture and general acceptability. This occurred towards the end of the project as hybrid lines became available.

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Population screening for carotenoid content and inbreeding to enhance zeaxanthin concentration

Summary
Age-related macular degeneration (AMD) is the leading form of blindness in Australia. There is growing scientific evidence that the dietary carotenoids, zeaxanthin and lutein, may be effective in slowing the progression of this disorder. Zeaxanthin is less common in the diet than lutein, but may play a greater protective role than lutein. Sweet-corn is one of the greatest sources of dietary zeaxanthin. The potential to further increase zeaxanthin concentrations in two tropical sweet-corn populations (PRO1 and PRO2) was investigated in the present study. Through selection and in-breeding of lines high in zeaxanthin, zeaxanthin concentrations were able to be increased from 1.1 µg/gFW to 5.2 and 11.9 µg/gFW, respectively. This was achieved partly by increasing total carotenoid synthesis, and partly by increased partitioning of carotenoid flux towards the zeaxanthin-arm of the carotenoid synthesis pathway. Further increases were due to an increased conversion of β-carotene and β-cryptoxanthin to zeaxanthin.

Introduction
Age-related macular degeneration (AMD) is the leading form of blindness in Australia (AMD Foundation, 2009). It is associated with cell death in the macula, the part of the retina used for central vision. There is mounting scientific evidence that the carotenoids, zeaxanthin and lutein, which are actively accumulated in the macula from dietary sources (Hammond et al., 1997), operate as a protective agent against oxidative damage from blue light (Krinsky et al., 2003; Plate and Gallaher, 2005; Whitehead et al., 2007), and slow the progression of AMD (Evans, 2006). Zeaxanthin may actually play a more important role than lutein (Bone et al., 1988; Sommerburg et al., 1999; Gale et al., 2003), but is much less common in the diet (Rodriguez-Amaya, 2001). Sweet-corn is one of the highest sources of dietary zeaxanthin (USDA Database, 1998; Holden et al., 1999), but the concentration of zeaxanthin in current commercial varieties is still much lower than levels suggested for supplementary use in the clinical Age-Related Eye Disease Study (AREDS2), which is presently being conducted in the USA (AREDS2, 2009).

The major carotenoids present in sweet-corn are lutein and zeaxanthin, and to a lesser degree β-cryptoxanthin and β-carotene (Kurilich and Juvic, 1999). While lutein and zeaxanthin are very similar molecules (isomers), they are formed on different arms of the carotenoid pathway (Harjes et al., 2008). Theoretically, zeaxanthin levels can be increased by increasing total carotenoid synthesis, and/or by increasing the proportion of zeaxanthin relative to other carotenoids. The present study details the range of zeaxanthin and total-carotenoid levels present in two tropical sweet-corn populations (PRO1 and PRO2), and the gains that can be made in zeaxanthin concentration by selection and inbreeding.

Materials and Methods

Tropical sweet-corn populations

Fresh kernels of the tropical sweet-corn breeding populations 'PRO1' and 'PRO2' were analysed for total carotenoid concentration and individual concentrations of zeaxanthin, lutein, β-cryptoxanthin and β-carotene. From approximately 300 plants, those showing high zeaxanthin levels were selected and self-pollinated to produce a segregating population, which was reanalysed for carotenoid concentrations. Selection and self-pollination of high-zeaxanthin segregants from the self-pollinated progenies was repeated and subsequent segregants analysed for carotenoid concentration. Kernels from the commercial hybrid Hybrix5, the parents of which were originally derived from PRO1 and PRO2, were used as a control.

Sample preparation

Sweet-corn cobs were harvested at commercial eating stage (21 days after pollination) and approximately 2 g of kernels removed, frozen in liquid nitrogen and ground using a ball-mill. The resultant powder was weighed, extracted for carotenoids under orange light, and analysed using a modified method of Howe and Tanumihardjo (2006). Six millilitres of ethanol (containing 0.1% butylated hydroxy-toluene) was added to 0.6 g of ground kernel. Samples were vortexed, and the solution extracted with hexane, using centrifugation to separate layers. Combined hexane fractions were dried and then reconstituted in 2 ml methanol/dichloromethane (50/50, v/v) and stored under nitrogen at -80˚C prior to HPLC analysis.

HPLC analysis

The HPLC system consisted of a SIL-10AD VP auto injector (Shimadzu), SCL-10A VP system controller (Shimadzu), LC-10AT VP liquid chromatograph (Shimadzu) and a SPD-M10 A VP diode array detector (Shimadzu). Forty microlitres of each extract was injected into a Waters YMC C30 Carotenoid Column (3 µm, 4.6 x 250 mm), with a mobile phase consisting of 92% methanol/8% 10mM ammonium acetate (phase A), and 100% methyl tert butyl ether (phase B). The following 40 minute gradient was used (1): 0 min, 80% phase A; 32 min, 40% phase A; 34 min, 80 % phase A; 40 min, 80% phase A. Lutein, zeaxanthin, β-cryptoxanthin and β-carotene were identified and quantified using commercially obtained standards.

Results

Zeaxanthin and total-carotenoid concentration

Carotenoid ranges of the two tropical breeding populations and the commercial cultivar Hybrix5 are shown in Table 1. The initial populations for both PRO1 and PRO2 generally contained lines with carotenoid concentrations outside of the range (both higher and lower) than that observed for the hybrid Hybrix5. Zeaxanthin levels were observed to be up to 4.5 times higher in individuals of PRO2 (4.9 µg/gFW), and 3.2 times higher in PRO1 (3.5 µg/gFW) than Hybrix5 (1.1 µg/gFW). Similarly, total-carotenoid concentration was up to 2.2 times higher in individuals of PRO2 (10.7 µg/gFW), and 2.0 times higher in PRO1 (9.4 µg/gFW) than Hybrix5 (4.8 (µg/gFW).

Table 1. Carotenoid ranges (μg/gFW) for the commercial hybrid Hybrix5 and two tropical sweet-corn breeding populations, PRO1 and PRO2. Average carotenoid concentrations are shown for Hybrix5 in parentheses.

	Hybrix5	PRO1	PRO2
zeaxanthin	0.9-1.6 (1.1)	0.4-3.5	0.8-4.9
lutein	2.4-3.7 (2.9)	0.9-6.1	1.0-3.7
β-cryptoxanthin	0.2-0.8 (0.5)	0.2-2.2	0.4-2.0
β-carotene	0.3-0.5 (0.3)	0.1-0.8	0.3-0.8
Total	4.1-5.7 (4.8)	2.0-9.4	2.4-10.7

Self-pollination of S1 individuals exhibiting high zeaxanthin concentration in the initial PRO1 and PRO2 populations resulted in further changes in both zeaxanthin and total-carotenoid levels in the segregating progeny (Table 2). In the PRO2 population, zeaxanthin concentration was further increased to up to 5.6 times that of Hybrix5 (reaching a maximum of 6.2 μg/gFW), and up to 3.1 times total-carotenoid concentration (14.5 μg/gFW). By contrast, increases in the PRO1 population were not as great, with no increase in zeaxanthin, and only a relatively slight increase in total-carotenoid concentration from 2.0 to 2.6 times that of Hybrix5.

Table 2. Carotenoid ranges (μg/gFW) for progeny of self-pollinated (S1) high zeaxanthin lines identified in PRO1 and PRO2 sweet-corn breeding populations.

	PRO1	PRO2
zeaxanthin	0.7-5.2	0.8-6.2
lutein	1.3-4.5	1.3-6.0
β-cryptoxanthin	0.4-2.1	0.5-4.7
β-carotene	0.1-0.6	0.2-0.7
Total	2.8-12.4	3.5-14.5

Further self-pollination of S2 individuals (S2 self-pollinated ears) from the PRO1 and PRO2 populations again resulted in increases in zeaxanthin and total-carotenoid concentration for the PRO2 population, but not for the PRO1 population (Table 3). Zeaxanthin concentration in the PRO2 population was increased to up to 10.8 times that of Hybrix5 (reaching a maximum of 11.9 μg/gFW), and up to 6.2 times total-carotenoid concentration (29.6 μg/gFW). As with the previous self-pollination, the PRO1 population failed to gain any further increase in zeaxanthin concentration, and this time made no further gain in total carotenoid concentration.

Table 3. Carotenoid ranges (μg/gFW) for self-pollinated (S2) high zeaxanthin lines identified from self-pollinated PRO1 and PRO2 sweet-corn breeding populations.

	PRO1	PRO2
zeaxanthin	1.6-3.0	4.7-11.9
lutein	1.9-5.5	1.9-6.3
β-cryptoxanthin	1.3-2.6	2.3-9.2
β-carotene	0.6-1.1	0.7-4.7
Total	7.2-11.3	12.0-29.6

Changes in carotenoid ratios

The ratios of carotenoids on the zeaxanthin-arm of the carotenoid-synthesis pathway (i.e. zeaxanthin, β-cryptoxanthin, and β-carotene) to lutein on the other arm are shown in Table 4. For Hybrix5, the ratio (0.5:1) indicated that lutein concentration either exceeded, or was the same as the concentration of the sum of zeaxanthin-arm carotenoids. This ratio was found to vary in the initial PRO1 and PRO2 populations, although no ratio lower than Hybrix5 was observed.

Self-pollination and a second self-pollination of high zeaxanthin lines from these populations resulted in little change in the upper ratio range within the PRO1 population, but resulted in a large increase in the upper ratio range for the PRO2 population (Table 4). Ratios of up to 8.7:1 were observed, indicating the concentration of zeaxanthin-arm carotenoids to be almost 9 times higher than lutein. This is equivalent to zeaxanthin-arm carotenoids constituting up to 90% of total carotenoid concentration.

Table 4. Range of (zeaxanthin + β-cryptoxanthin + β-carotene):lutein ratios for the commercial hybrid Hybrix5, initial PRO1 and PRO2 populations, 'selfed' high zeaxanthin (Z) lines, and 're-selfed' high zeaxanthin lines. The average ratio for Hybrix5 is shown in parenthesis.

	Hybrix5	PRO1	PRO2
initial population	0.5-1.0 (0.7)	0.5-3.2	0.7-3.6
'selfed' high-Z lines		0.4-2.9	0.4-5.7
're-selfed' high-Z lines		1.0-2.8	1.8-8.7

Discussion

The current trial indicated that significant variability in carotenoid levels exists within the PRO1, and particularly the PRO2 sweet-corn populations. Total carotenoids were able to be increased from an average of 4.8 μg/gFW for Hybrix5 to 12.4 μg/gFW and 29.9 μg/gFW, respectively, for PRO1 and PRO2 by selection during inbreeding. Increasing the total synthesis of carotenoids was accompanied by an increase in zeaxanthin concentration in the inbred

progenies, increasing zeaxanthin from an average of 1.1 µg/gFW for Hybrix5 to 5.2 µg/gFW and 11.9 µg/gFW, respectively for PRO1 and PRO2.

This concurrent increase in zeaxanthin concentration along with total carotenoid concentration was not solely due to an increase in total carotenoid synthesis, but partly due to a change in carotenoid ratio. In Hybrix5, zeaxanthin accounts for 23% of the total carotenoids present, while in the PRO1 and PRO2 populations it was able to be increased to approximately 40% (Table 5). This indicates that increasing zeaxanthin concentration in sweet-corn is possible through both increasing total carotenoid concentration, and increasing the ratio of zeaxanthin to other carotenoids.

Table 5. Concentration of zeaxanthin and zeaxanthin-arm carotenoids (zeaxanthin + β-cryptoxanthin + β-carotene) within segregants of the PRO1 and PRO2 populations exhibiting highest total carotenoids (high). The percentage of zeaxanthin (Z) within the zeaxanthin-arm carotenoids is shown. The concentrations for the PRO segregants (low) showing the highest Z percentage are shown.

	Zeaxanthin (µg/gFW)	Total zeaxanthin-arm carotenoids (µg/gFW)	Total carotenoids (µg/gFW)	Z (%) of Z-arm carotenoids
PRO1 (high)	5.2	7.9	12.4	66
PRO2 (high)	10.3	23.3	29.6	44
PRO1 (low)	2.4	3.1	6.6	77
PRO2 (low)	3.6	4.7	7.9	77

In maize (field corn), it has been shown that key genes responsible for variation in carotenoid concentration are phytoene synthase (PSY), zeta carotene desaturase (ZDS) (Wong et al., 2004), and lycopene epsilon cyclase (LCYE) (Harjes et al., 2008). The first two of these genes (PSY and ZDS) are upstream in the synthesis pathway and regulate total carotenoid synthesis. The third (LCYE) however, is only involved in the lutein arm of the carotenoid pathway, forming an epsilon ring on the lycopene molecule. It has been shown that allelic differences in the LCYE enzyme are responsible for the different ratios of carotenoids in the lutein arm of the pathway to the zeaxanthin arm of the pathway (Harjes et al, 2008).

The current trial indicates that significant allelic variation is likely to exist in our sweet-corn populations not only for PSY or ZDS (or both) regulating total carotenoid synthesis, but also for LCYE within the PRO1 and PRO2 populations. Comparing the two populations, it would seem that PRO2 may contain 'stronger' alleles for total carotenoid synthesis than PRO1, which would account for the higher level of total-carotenoids observed in the former. PRO2 would also appear to contain 'stronger' alleles for LCYE than the PRO1 population, as the ratio between carotenoids of the zeaxanthin and lutein arms of the pathway was increased from 0.7:1 in Hybrix5 to 8.7:1 in PRO2, but only to 3.2:1 in PRO1 (Table 4).

Considering that zeaxanthin levels of the PRO1 and PRO2 were able to be increased up to 49% and 51% of total carotenoids, respectively (data not shown), but the ratio of zeaxanthin-arm carotenoids to lutein was much less in PRO1 (Table 4), it is likely that another gene was involved in increasing zeaxanthin concentration. It is possible that the gene responsible is beta-hydroxylase (B1Hyd), which is involved in the conversion of β-carotene to β-cryptoxanthin, and its subsequent conversion to zeaxanthin (Jaradat et al., 2005). For example, in the two highest total-carotenoid segregants of the PRO1 and PRO2 populations, zeaxanthin comprised 66% of carotenoids of the zeaxanthin-arm for the PRO1 segregant, but only 44% for the PRO2 segregant (Table 5). From Table 5, it is evident that much of the carotenoid flux in the PRO2 segregant is reaching the zeaxanthin-arm of the pathway, but is not being converted from β-carotene and β-cryptoxanthin into zeaxanthin. In the current trial, the highest zeaxanthin percentage observed was 77% (Table 5).

Theoretically, it is possible that zeaxanthin concentration could be further increased if the LCYE alleles most favouring zeaxanthin-arm carotenoid synthesis (ie. 90% of total carotenoids), and the B1Hyd alleles maximising conversion of β-cryptoxanthin and β-carotene to zeaxanthin (ie. 77% of zeaxanthin-arm carotenoids) were selected. If these were applied to the PRO2 (high) carotenoid segregant in Table 5, zeaxanthin concentration could reach as high as 20 µg/gFW. Within the PRO1 population, there appears to be less potential however, with a more moderate increase in zeaxanthin concentration of 7.3 µg/gFW. This is primarily due to lower total-carotenoid synthesis occurring in the PRO1 population, and partly to a reduced partitioning of carotenoids to the zeaxanthin arm of the carotenoid pathway.

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Effect of carotenoid profile changes on kernel colour

Summary
To assist the development of high-zeaxanthin sweet-corn, an analytical screening method was developed, including chromameter measurement of kernel colour (hue angle) and optimized extraction for HPLC. From 385 lines of a breeding population and six commercial varieties, carotenoid levels were observed to have a wide range, with the highest levels of zeaxanthin being 11.9 mg/kg fresh weight, which was at least six times greater than the tested commercial varieties. Kernel colour (hue angle) was measured objectively with a Chromameter, and the regression of hue angle versus zeaxanthin was described by, Hue angle = $76.16 + 4.50e^{(-0.24*\text{zeaxanthin})} + 11.73e^{(-0.24*\text{zeaxanthin})}$, r^2 of 0.59. The top 6% of lines, in regards to concentration of zeaxanthin, (zeaxanthin+β-cryptoxanthin+β-carotene), and total carotenoid all had hue angles ≤ 84.1˚. The use of a hue angle of 85˚ as a maximum cut off for kernel selection was subsequently established for HPLC analysis. This subsequently allowed for much increased efficiency in screening germplasm for high zeaxanthin lines.

Introduction
Carotenoids are a key class of phytochemicals found in fruits and vegetables, which may exhibit protective actions against certain cancers, eye conditions, cardiovascular diseases and improve the function of the immune system (1-4). In recent years there has been much interest in improving the carotenoid content of various crops to increase the derived health benefit from dietary consumption (5, 6). For example, new varieties of high beta-carotene maize have been bred to combat vitamin A deficiency in African countries (7).

Screening studies, utilizing both analytical chemistry and molecular biology, have investigated the variability in breeding populations for individual carotenoids to provide the baseline data to support the development of new varieties (8). Knowledge of the carotenoid synthesis pathway (Figure 1) has been utilized to breed new lines with increased concentrations of specific carotenoids, with the activities of certain enzymes shown to play key roles in regulating the proportions of different carotenoids (7).

There is much potential value in developing high zeaxanthin sweet-corn varieties as sweet-corn is a major dietary source of zeaxanthin (9), the carotenoid zeaxanthin may have an important role in ameliorating the progression of age-related macular degeneration (10, 11), the leading cause of blindness in developed countries (12), and usual zeaxanthin intake is low (13).

In this study the genetic variability of individual and total carotenoid levels, and the various ratios of these carotenoids within broad-based heterogeneous composites constructed from tropical supersweet synthetics and hybrids developed by James Brewbaker at the University of Hawaii was examined. Optimal extraction conditions were developed with regard to heating and saponification, and then used to assay six commercial varieties of sweet-corn grown in Australia. As carotenoids can vary in colour, the relationship between carotenoid content and kernel colour was examined.

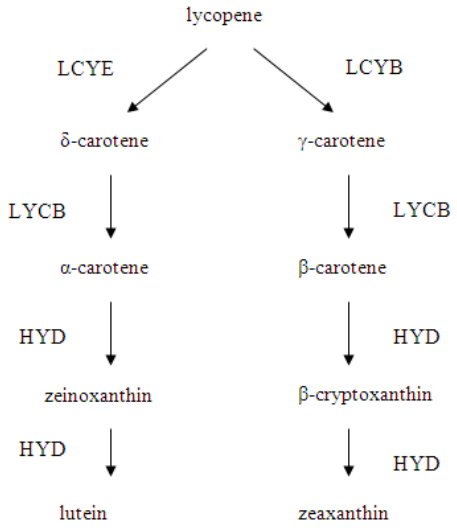

Figure 1. General overview of part of the carotenoid synthesis pathway in plants, adapted from Harjes et al 2008 (7). Enzymes: LCYE, lycopene epsilon cyclase; LCYB, lycopene beta cyclase; HYD, carotene hydroxylase enzymes, which include ε and β-ring hydroxylases. Structures of carotenoids are shown in Kopsell & Kopsell 2006 (5).

Materials and Methods

Chemicals

Hexane (Ajax, Taren Point, New South Wales, Australia) and ethanol (LabServ, Clayton, Victoria, Australia) used in the current trial were analytical grade, and methyl tert butyl ether (Burdick & Jackson, Muskegon, MI), methanol (Mallinckrodt, Phillipsburg, NJ) and dichloromethane (Merck, Darmstadt, Germany) were HPLC grade. Lutein, zeaxanthin and β-cryptoxanthin were purchased from Extrasynthase (Genay, France), and β-carotene and β-Apo-8'-carotenal purchased from Sigma Aldrich (St Louis, MO).

Corn Samples

All sweet-corn lines from the breeding population and the commercial hybrid Hybrix5 were sourced from Kairi Research Station (Department of Agriculture, Forestry and Fisheries). Commercial varieties (Golden Sweet Improved, Thunderbolt, Sentinel, Obsession, Everest) were sourced from Mulgowie Farming Company. Once harvested, all sweet-corn samples were stored at -20˚C. Light, Chroma and Hue angle values were recorded for intact kernels using a Minolta Chromameter using a 6 mm aperture and C light source. Triplicate measurements were made randomly over the surface of the cob, when the kernels were fully hydrated, and averaged.

Extraction

The extraction and analytical methods described by Moros et al (2002) and Howe and Tanumihardjo (2006) were used as base methods (14, 15). The basic outline of the extraction

procedure from their work on sweet-corn and maize was to solubilize the sample in heated-ethanol, prior to saponification with KOH and heat. Carotenoids were subsequently extracted with hexane. The effect of heating and the addition of KOH on carotenoid extraction were examined. Sweet-corn samples were cryogenically milled using a Retsch MM301 mill (Haan, Germany). Three extraction conditions were compared following addition of ethanol to milled samples and vortexing. These included (i) extraction with hexane; (ii) heat for 5 min at 85˚C prior to extraction with hexane, and; (iii) heat for 5 min at 85˚C, add 14.3M KOH (120, 240 or 500 µL), heat for 10 min at 85˚C, followed by extraction with hexane.

The detailed procedure following the initial milling was as follows. Samples were cooled on ice, and 3 mL of cold water and 250 µL of β-Apo-8'-carotenal (7.2 mg/L in isopropanol) as an internal standard, were added. Samples were then extracted 5 times with 3 mL hexane, with centrifugation used to separate layers (5000 x g for 3 min, at 4˚C). Combined hexane fractions were dried in a centrifugal evaporator at 30˚C and then reconstituted in 2 mL of methanol/dichloromethane (50/50, v/v), containing 0.1% butylated hydroxy-toluene (BHT). Samples were then filtered (0.22 µm syringe filter, Grace, Sydney, Australia) and placed into HPLC vials, before storing under nitrogen at -80˚C prior to HPLC analysis. Standard solutions were prepared similarly in methanol/dichloromethane (50/50, v/v), containing 0.1% BHT.

HPLC Analysis

The HPLC system consisted of a SIL-10AD VP auto injector (Shimadzu), SCL-10A VP system controller (Shimadzu), LC-10AT VP liquid chromatograph (Shimadzu) and a SPD-M10 A VP diode array detector (Shimadzu). 40 µL of each extract was injected onto a YMC C30 Carotenoid Column, 3 µm, 4.6 x 250mm (Waters), with a mobile phase consisting of 92% methanol/8% 10mM ammonium acetate (phase A), and 100% methyl tert butyl ether (phase B). The following 40 min gradient was used (15): 0 min, 80% phase A; 32 min, 40% phase A; 34 min, 80 % phase A; 40 min, 80% phase A.

Identification and Quantitation

Lutein, zeaxanthin, β-cryptoxanthin and β-carotene were identified by comparison with the retention times and absorption spectra of the standards. Solutions of approximately 2 µg/mL of the standards were made up in ethanol (lutein) or hexane (zeaxanthin, β-cryptoxanthin, and β-carotene), and absorbance measured at 445 nm for lutein and 450 nm for zeaxanthin, β-cryptoxanthin, and β-carotene (16). The concentration of solution was determined by the following formula, using the values in Table 1:

$$\text{Concentration } (\mu g/mL) = \text{Absorbance} \times 10000 / A^{1\%}$$

Table 1. λ_{max} and extinction coefficients for carotenoids (16)

Carotenoid	$A^{1\%}$	λ (nm)	Solvent
β-carotene	2592	450	hexane
β-cryptoxanthin	2460	450	hexane
lutein	2550	445	ethanol
zeaxanthin	2480	450	hexane

Each carotenoid standard was run by HPLC to determine peak purity. The actual concentrations of the standard solutions were then calculated by multiplying the concentration determined spectrophotometrically by the % peak area of the standard peak as determined by HPLC. Standard curves were linear over the range 0.03 to 10 μg/mL with r^2 values of < 0.999. Carotenoid concentrations were expressed as mg/kg fresh weight of corn kernels (FW), where FW was the fresh weight of the wet kernels. The range of moisture content of analysed cobs was 66-75%.

Data and Statistical Analysis

Various ratios of the carotenoids were examined because the carotenoid synthesis pathway has two branches from lycopene, with lutein on one side and zeaxanthin, β-cryptoxanthin and β-carotene on the other (see Figure 1). Differences in carotenoid concentration in the various extraction conditions were evaluated using one-way analysis of variance (ANOVA) and the Tukey HSD procedure using JMP software (Version 7). Mean ± standard deviation (SD) carotenoid values for the commercial varieties of sweet-corn were calculated from single measurements of three different samples of each variety. Regression analysis of carotenoid concentration versus Hue angle was performed using SigmaPlot (Version 10.0).

Results

Figure 2 shows a typical chromatogram of the carotenoids extracted from sweet-corn in the present investigation. The major peaks identified were lutein and zeaxanthin, although β-cryptoxanthin and β-carotene were also present. The peaks at 11.9 and 21.3 min could not be identified but had similar visible spectra to lutein and zeaxanthin, respectively. Heating or saponification was found to have no significant effect on carotenoid profile with respect to either elution time or peak area.

Figure 3A shows the extensive variation in the levels of individual and total carotenoid concentration within the 385 breeding lines that were examined. The differences were 19-fold for total carotenoids (1.57-29.59 mg/kg FW), 14-fold for lutein (0.44-6.31 mg/kg FW), 39-fold for zeaxanthin (0.31-11.93 mg/kg FW), 45-fold for β-cryptoxanthin (0.21-9.43 FW), and 43-fold for β-carotene (0.11-4.72 mg/kg FW). As shown in Figure 3B, there was also significant variation in the ratios of carotenoids, with a 33 (0.10-3.33) and 24-fold (0.42-10.16) difference, across the lines, for zeaxanthin/(β-cryptoxanthin+β-carotene) and (zeaxanthin+β-cryptoxanthin+β-carotene)/lutein, respectively.

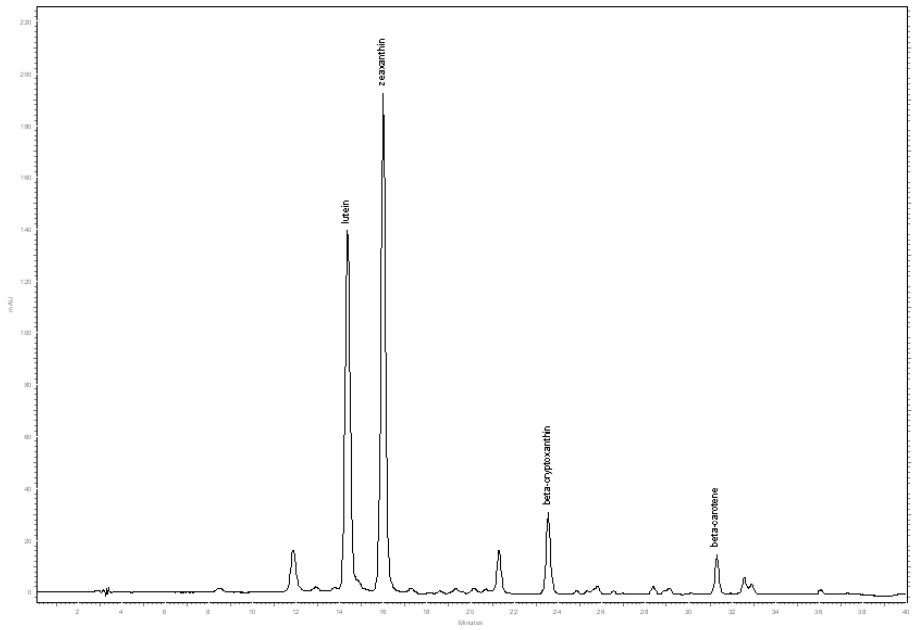

Figure 2. Representative chromatogram of carotenoids extracted from sweet-corn.

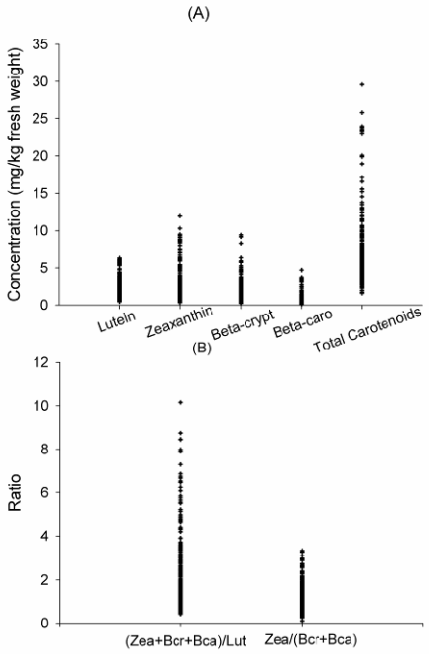

Figure 3. Range of carotenoid levels (A) and carotenoid ratios (B) in the screened breeding population.

Figure 4 shows regressions of Hue angle versus carotenoid concentration, fitted using an exponential decay. Hue angle was most strongly correlated to the sum of zeaxanthin + β-cryptoxanthin + β-carotene (adjusted r^2 = 0.70). As zeaxanthin, β–cryptoxanthin and β–carotene are orange pigments, and lutein yellow, the ratio of orange to yellow pigments was also tested. However, this caused significant decreases in the adjusted r^2 values in all cases. Of the 385 cobs analysed as part of the germplasm screening, only 23 had zeaxanthin levels >5 mg/kg FW. Of these 23, 16 had a hue angle less than 80, and the remaining 7 had a hue angle equal to or less than 84.1˚. In regards to total carotenoids and the sum of (zeaxanthin + β–cryptoxanthin + β-carotene), the top 6.5% and 6.2% of lines, respectively, all had hue angles equal to or below 84.1˚.

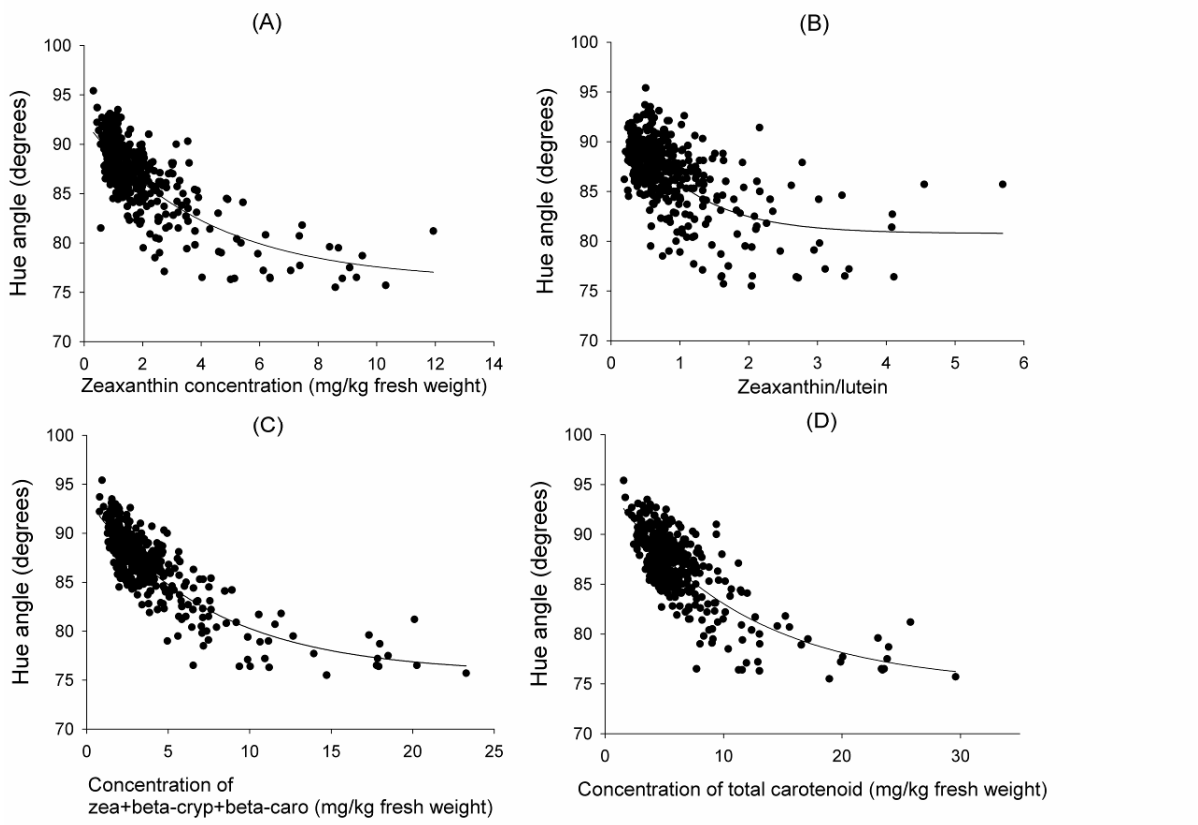

Figure 4. Regressions of Hue angle versus zeaxanthin (A), zeaxanthin/lutein (B), (zeaxanthin+β-cryptoxanthin+β-carotene) (C), and total carotenoids (D), fitted using an exponential decay. Regression equations were (A) Hue angle = $76.16 + 4.50e^{(-0.24*\text{zeaxanthin})} + 11.73e^{(-0.24*\text{zeaxanthin})}$, adjusted r^2 = 0.59; (B) Hue angle = $80.76 - 15.57e^{(-6.24*(\text{zeaxanthin/lutein}))} + 14.77e^{(-1.08*(\text{zeaxanthin/lutein}))}$, adjusted r^2 = 0.38; (C) Hue angle = $75.69 + 10.22e^{(-0.14*(\text{zeaxanthin+β-cryptoxanthin+β-carotene}))} + 7.66e^{(-0.14*(\text{zeaxanthin+β-cryptoxanthin+β-carotene}))}$, adjusted r^2 = 0.70; (D) Hue angle = $74.89 + 11.50e^{(-0.09*\text{total}}$

Table 2 shows the carotenoid content of six commercial sweet-corn cultivars grown in Australia. Goldensweet Improved had the highest concentration for all four carotenoids.

However it had less than 25% of the zeaxanthin concentration of the best lines of the breeding line population. The white cultivar Everest had the lowest concentrations of lutein, zeaxanthin, and total carotenoids of both the commercial cultivars and the breeding population lines.

Table 2. Carotenoid levels and hue angles of six commercial sweet-corn varieties (mean ± SD)

Cultivar (n=3)	Lutein (mg/kg FW)	Zeaxanthin (mg/kg FW)	β-cryptoxanthin (mg/kg FW)	β-carotene (mg/kg FW)	Hue Angle (˚)
GS-I*	4.9 ± 0.5	1.9 ± 0.2	0.9 ± 0.1	0.5 ± 0.02	85.7
Thunderbolt	3.2 ± 0.3	1.5 ± 0.04	0.6 ± 0.01	0.3 ± 0.003	88.7
Sentinel	0.7 ± 0.1	1.4 ± 0.2	0.8 ± 0.004	0.3 ± 0.005	88.0
Hybrix5	2.9 ± 0.5	1.1 ± 0.3	0.4 ± 0.1	0.3 ± 0.06	90.5
Obsession	0.8 ± 0.1	0.5 ± 0.08	0.4 ± 0.02	0.2 ± 0.007	92.7
White	0.3 ± 0.03	0.3 ± 0.01	0.3 ± 0.02	0.2 ± 0.01	95.8

FW=fresh weight
*Goldensweet improved

Discussion and Conclusions

To screen large numbers of germplasm, relatively fast extraction methods are required. The simplest extraction method, without heating or saponification, used in the present study was therefore deemed to be most appropriate for carotenoid extractions to support sweet-corn breeding. Although several published methods have reported the need for saponification (14, 17, 18), this was not the case in the present study. Saponification is used to hydrolyse esterified xanthophylls, which are the predominant form of xanthophylls in certain plants (eg., orange capsicum, sea buckthorn), to allow for suitable analysis (19). However the carotenoids in sweet-corn are not esterified and thus saponification was not needed. In support of the present findings, a recent paper describing a diastereomeric dilution assay for quantifying zeaxanthin did not use saponification as part of the extraction process for sweet-corn but did use saponification for orange capsicum and sea buckthorn (19). Carotenoid extraction without saponification has also been successfully used for maize and mango (20, 21). In the current study, the use of a centrifugal evaporator to dry down samples prior to HPLC was a significant improvement on the previously used method of drying under nitrogen, being faster and easier (14).

The wide variation of carotenoid concentrations in the current breeding population were similar to that reported previously. Literature values for sweet-corn range from 0.02-14.68 mg/kg FW (lutein), 0.02-6.83 mg/kg FW (zeaxanthin), 0.02-0.36 mg/kg FW (β-cryptoxanthin), and 0.01-1.6 mg/kg FW (β-carotene) (14, 17, 18). Wide variation in individual carotenoid content in a maize breeding population was also recently described (7). In maize, as in sweet-corn, the levels of individual carotenoids are determined by the activity of the various enzymes of the carotenoid pathway (see Figure 1) with Harjes et al (2008) recently detailing a strong association between lycopene ε cylase expression and the ratio of carotenoids present in maize kernels (7).

High correlations between carotenoid content and flesh colour have been shown in other fruits and vegetables (22-24). For example, correlation coefficients (r^2) of 0.67-0.88 have been shown for the relationship between Hue angle and the concentration of violaxanthin and β -carotene in mangoes (23). Interestingly, in maize, Harjes et al saw no correlation between grain colour of maize kernels and total carotenoids (r^2=0.119), β-carotene (r^2=0.045), and β-cryptoxanthin (r^2=0.130) (7). As the grain colour was simply scaled from 1-6 according to shade of yellow, these values may have been increased if a chromameter had been used for objective colour measurement. In the present study, we achieved correlations of 0.59-0.70 for zeaxanthin, β-cryptoxanthin, β-carotene and total carotenoids.

From the current screening study, an arbitrary hue angle of 85˚ would appear to be a useful maximum angle for rapidly identifying sweet-corn kernels with potentially high levels of zeaxanthin and other orange carotenoids for subsequent HPLC carotenoid analysis. In the present trial, for instance, this screening would remove more than 90% of lines requiring milling, extraction, and analysis.

The results in the present study suggest current commercial sweet-corn varieties in Queensland have zeaxanthin levels of nearly 2 mg/kg FW or less. However the best performing line from our current breeding population had a zeaxanthin concentration of 11.9 mg/kg FW, approximately 6 times higher than the highest commercial cultivar tested (Goldensweet improved). As this and other lines within the breeding population have total carotenoid levels approaching 30 mg/kg FW, there appears to be significant opportunity to further increase zeaxanthin concentration through channeling carotenoid synthesis towards the zeaxanthin side of the carotenoid pathway, rather than the lutein side, (as has been suggested by Harjes et al to enhance β-carotene in maize). Such a strategy would also apply to increasing zeaxanthin in sweet-corn, although selecting lines that more fully convert β-carotene and β-cryptoxanthin to zeaxanthin would also lead to higher zeaxanthin concentrations.

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Preliminary consumer assessment using colour-altered images

Summary
In the absence of actual high-zeaxanthin samples, digitally-altered images of sweet-corn of varying colour (white, yellow, gold, deep-gold, orange) were shown to sweet-corn consumers, with and without knowledge that more-deeply coloured cobs contained more zeaxanthin, which has been linked to a lower incidence of macular degeneration. The purpose of this assessment was to provide a guide to breeders as to what kernel colour is acceptable when zeaxanthin concentration is increased (how orange is too orange). Gold sweet-corn was as acceptable as regular yellow sweet-corn, with both significantly preferred over the 3 other corn colours. Increasing the orange colour further tended to negatively impact on consumer acceptability of the sweet-corn. Purchase intention relied on the corn colour. Knowledge of the zeaxanthin health-benefit however, increased the acceptability of deep-gold to the level of that of yellow and gold sweet-corn. According to this preliminary study, a hue angle between 72° and 90° would be considered as acceptable for consumers with an optimal value around 81° (i.e. gold). This value would enable differentiation from the colour of regular corn and would still not be too distant to keep its natural appearance. In order for the golden colour of kernels and the related health benefit to have impact on consumers, preliminary quality requirements (an appearance which is not dry, kernels tender, juicy and sweet) must first be met. If these preliminary requirements are not fulfilled, then consumers will not give credit and not be influenced by the golden colour of the kernel and the health advantage of zeaxanthin-biofortified sweet-corn.

Introduction
The overall aim of this project is to deliver sweet-corn enhanced in zeaxanthin to the domestic market. Zeaxanthin is a compound found in corn which protects against blindness (macular degeneration). One in seven Australian people over the age of 50 are affected by macular degeneration and the incidence increases with age. Enhancing the content of zeaxanthin will be achieved by increasing total carotenoid synthesis, as well as directing synthesis away from yellow lutein synthesis and towards the beta-arm of the carotenoid pathway, which contains more orange-coloured carotenoids, such as zeaxanthin. Both pigments (zeaxanthin and lutein) naturally contribute to the yellow colour of the kernel. Elevated levels of zeaxanthin naturally give corn a golden-orange colour. Changing the colour of the cob might affect consumer acceptability of this new corn variety.

A consumer study 'A sweet-corn consumer survey' led in 2001 by NCS Pearson on 200 consumers from the Brisbane area provided quantitative data to identify consumer preferences for sweet corn traits and product attributes. The study revealed that respondents saw the main benefit of eating sweet corn was for enjoyment and taste (68%), followed by healthy food to eat (49%), good nutritional balance (18%), vitamins (9%) and fibre (8%). Based on the conclusions of this study, sweet-corn was perceived as naturally healthy, and therefore sweet-corn is an ideal candidate for functional food.

This preliminary investigation will provide information on consumer perception of different gradations of yellow and orange colourings of sweet-corn, based on digitally-altered images.
The principal objectives of this study were to determine (1) the importance of the colour on consumer appreciation and purchase intention of fresh super sweet corn cob; (2) if knowledge of the health benefit of zeaxanthin can affect consumer willingness to pay and purchase intention, and; (3) if the age of consumer has an effect on consumer purchase intention.

This study also measured the importance of health in comparison with other criteria when participants of this study purchase sweet-corn, and to collect information to anticipate corn tasting preferences (next consumer study of the project) in regard to consumer purchase and preparation habits.

Materials and Methods

Sweet-corn pictures

Hue is one of the main properties of a colour described with names such as "red", "yellow", etc. The two other main properties are lightness and colour intensity. Hue spectrum is defined by a scale 0—360°. Yellow to orange values are comprised between 90° (yellow) and 30° (orange).

Four different levels of hue values (Table 1) were selected to cover the range of hue angles between a standard yellow sweet-corn (90°) and a hypothetical orange super sweet-corn, equivalent to the value observed for orange capsicums (57-60°), containing mainly zeaxanthin.

A picture of the white sweet-corn (cv. Everest) was also included to cover with the regular super sweet corn the range of super sweet corns available on the market place and which would be direct competitors of this new variety of sweet-corn.

A total of 5 digitally-altered pictures were presented to consumers (Figure 1). Pictures were coded with a 3 digit number. They were assessed by consumers according to a balanced design to avoid any presentation effect. Pictures were displayed in envelopes to ensure that pictures are assessed individually and not compared with other pictures.

Table 1. Sweet-corn picture descriptions.

Name	Corn description	Code	Hue angle
White	white sweet-corn	487	96°
Yellow	Regular sweet-corn	227	90°
Gold	Orange 5	135	81°
Deep-gold	Orange 8	694	72°
Orange	Orange 15	826	57-60°

Figure 1. Digitally-altered images of supersweet-corn with increasing orange colour.

Consumer profile

Consumers were recruited by phone based on their frequency of consumption of fresh super sweet corn (not frozen or canned). They had to consume super sweet corn at least once a month.

As much as possible, they were recruited to represent the preselected following groups of age:

- consumers 60 +: direct target
- consumers between 50-59: the new target
- consumers between 20 and 49: future target

They also declared to be the main grocery shopper of the household in which they reside or that they at least share grocery shopping.

Sessions took place on the 4th, 5th and 9th of August 2008 at DAFF Hamilton. Assessments were conducted in individual booths. Consumers received $30 as an incentive at completion of the assessment.

Questionnaire

The questionnaire was divided in 4 sections:

Section 1: Background questionnaire
Questions were designed to obtain demographic and market information.

Section 2: Identification of consumers' major criteria when they select corn cobs in the shop.
Eighteen items have been formulated on a functional side (driver) and on a dysfunctional side (barrier). A total of thirty-six statements have been assessed by the consumers to check the relative importance of health in comparison with other criteria. This approach is derived from the Kano model as described by Matzler *et al*., 1996.

Consumers answered the following question: *'If one of the pre-packed fresh super sweet corn you are presented with is* [statement]. *How will it affect your choice?*' by using a 5 points scale from 1: '*I will definitely not choose this variety*' to 5: '*I will definitely choose this variety*'.

Section 3: Consumer primary perception of the 5 different corns in appearance.
The consumers assessed the appearance of 5 corn pictures through an acceptance test. Corn pictures were presented individually in accordance with a balanced statistical design. Consumers marked directly on the screen their responses on line scales anchored from dislike extremely (0) to like extremely (100). They were also given the opportunity to comment on anything they liked or disliked regarding the corn.
Consumers were then asked to tell which corn they would purchase based on their individual appearance.
They considered the 5 corn pictures together. They were asked to rank them from the one which appears the most healthy to eat to the one which appears to be the least healthy to eat based on their own appearance judgement.

Section 4: Influence of the knowledge of health benefit
Consumers depending on their session attendance were divided in two groups:

- the control group: consumers who did not receive information regarding the healthy potential of the target corn.
- the test group: consumers who have been explained the healthy potential of the target corn before giving their maximal price.

The following information was delivered to the test group: '***Zeaxanthin*** *is a compound found in corn which protects against blindness (macular degeneration). One in seven people over the age of 50 are affected by macular degeneration and the incidence increases with age. At elevated levels zeaxanthin naturally gives corn a golden-orange colour*.'

Consumers were asked to provide the maximal price they would pay for the corn target with (test group) and without (control group) prior knowledge. All consumers were given the market price of the 2 commercial corns:

- $2.50 for the regular corn (227),
- $2.98 for the white corn (487).

At the end, all consumers received the information of the health benefit of eating a corn with high level in zeaxanthin. They were then asked as in section 3 to indicate which corn they would purchase based on their individual appearance and knowing the impact of zeaxanthin on their health.

Data analyses

The first three sections of the questionnaire were designed under Compusense software. It enabled direct acquisition of consumer data. Section 4 was delivered on a paper ballot at completion of the previous sections.

Section 1: Background questionnaire
Percentages (%) for individual selected items were calculated

Section 2: Identification of consumer major criteria when they select corn cobs in the shop.
Consumer major criteria when they purchase corn have been analysed based on the Kano model as described by Matzler *et al.,* 1996.
This model classified the eighteen items into categories according to their level of fulfilment:

- A: attractive: attributes which increase consumer satisfaction or drivers but not barriers,
- M: must-be: attributes which decrease consumer dissatisfaction or barriers but not drivers,
- R: reverse: attributes which have a reverse impact on consumer: drivers and/or barriers
- O: one-dimensional: attributes which decrease consumer dissatisfaction and increase consumer satisfaction: barriers and drivers
- I: indifferent: attributes with no impact on consumer: not a driver and not a barrier.

The frequency of occurrence of each category indicates the classification of each attribute. Two coefficients are calculated:

1. the extent of satisfaction: satisfaction coefficient = (A+O)/(A+O+M+I),
2. the extent of dissatisfaction: dissatisfaction coefficient = (O+M)/(A+O+M+I)

A coefficient equal to zero means that this criterion does not cause satisfaction/dissatisfaction even if the criterion is not filled. This criterion is not a driver or a barrier for consumers when they purchase corn.

Section 3: Consumer primary perception of the 5 different corns in appearance.
Analysis of variance (ANOVA) was performed on consumer acceptability of the 5 corn appearances and on ranking tasks using the software GenStat® 9th edition, Lawes Agricultural Trust. Where a significant ($p < 0.05$) sample F-ratio was found using ANOVA, pair-wise comparisons using Fishers least significant difference procedure were completed.

Section 4: Influence of the knowledge of health benefit
Price estimation was collected using paper questionnaires. Consumer scores were measured by hand using a standard ruler, recorded in a Microsoft Excel spreadsheet and converted, linearly, to a score out of $8. An ANOVA was performed to assess if a significant price difference existed between consumers who did not know that target corn was high in zeaxanthin (e.g. control group) and consumers who received this information (e.g. test group).
An ANOVA was run on consumer reported purchase intention before and after knowing the health property linked with a more orange colour on the corn. Results indicated the potential effect of the corn colour, the effect of knowledge and the effect of age group.

Results and Discussion

Consumer profile (questionnaire - section 1)

Fifty-two consumers (13% male and 87% female) took part in the study. Consumers were classified according to their age group: (>60: 35%; 50-59: 35%; 20-49: 30%). They were generally the main grocery shopper (88% of respondents) of the household in which they resided, or they at least shared grocery shopping (12% of respondents of the study). The majority of participants consumed fresh corn (not frozen or canned) at least once a month (94%, including 63% per fortnight).

Consumers from the study also indicated:

- they preferably buy corn: loose fresh corn (67%), pre-pack fresh corn (17%), frozen (10%) and canned (6%)
- their usual method of preparing corn is to boil (62%), microwave (31%) or barbecue (6%).

According to this information, it is recommended for the subsequent consumer acceptability tasting to present uncooked loose fresh corn to be assessed in appearance. Boiled preferably or microwaved corn could represent cooked corn and be assessed in appearance, taste and texture.

Identification of consumer drivers and barriers (questionnaire - section 2).

Using the Kano model, 18 items were assessed in a 'positive' (driver) and 'negative' (barrier) perception. To facilitate results presentation and discussion, items have been grouped:

- sensory attributes (Table 2, Figure 2),
- health and environment (Table 3, Figure 3),
- production (Table 4, Figure 4).

Sensory attributes classification indicated three different levels to satisfy consumer (Table 2):

Level 1 of requirement: surveyed consumers will certainly not purchase a sweet-corn with a dry appearance. The dryer super sweet corn cob looks, the less it is likely to be purchased. On the contrary, a moist appearance will not affect their decision.

Level 2 of requirement and expectation: the tougher and the less juicy and sweet a super sweet corn tastes, the less it is likely to be purchased. On the contrary, the sweeter, juicier, tenderer a sweet-corn is, the more it satisfies consumers and the more they are likely to repeat their purchase.

Level 3 of expectation: if consumer preliminary requirements are met, e.g. an appearance which is not dry, pre required levels of perceived tenderness, juiciness and sweetness, then having kernels with a golden colour will positively influence consumer in their choice. If these preliminary requirements are not met then the golden colour will not attract consumer.

Table 2. Coefficients of satisfaction, dissatisfaction and classification of the sensory attributes.

Sensory attribute	Satisfaction	Dissatisfaction	Classification
Gold/light colour	0.51	0.14	A
Moist/dry appearance	0.51	0.98	M
Tender/tough	0.82	0.74	O
Juicy/not juicy	0.67	0.67	O
Large/small kernels	0.06	0.02	I
Sweet/not sweet	0.75	0.54	0

I: Indifferent, O: One-dimension, A: Attractive, R: Reverse, M: Must-be

When considering the sensory aspects evaluated, the most important positive criteria to increase consumer satisfaction were identified as tender, sweet and juicy corn (Figure 2). The deep golden colour of the corn cob and a moist appearance were considered less important sensory characteristics. The sensory criterion considered to be a highest barrier towards purchase was a dry appearance. Other strong sensory related barriers were tough kernels and lack of juiciness and sweetness. Whether the corn has small or large kernels was not an important consumer driver.

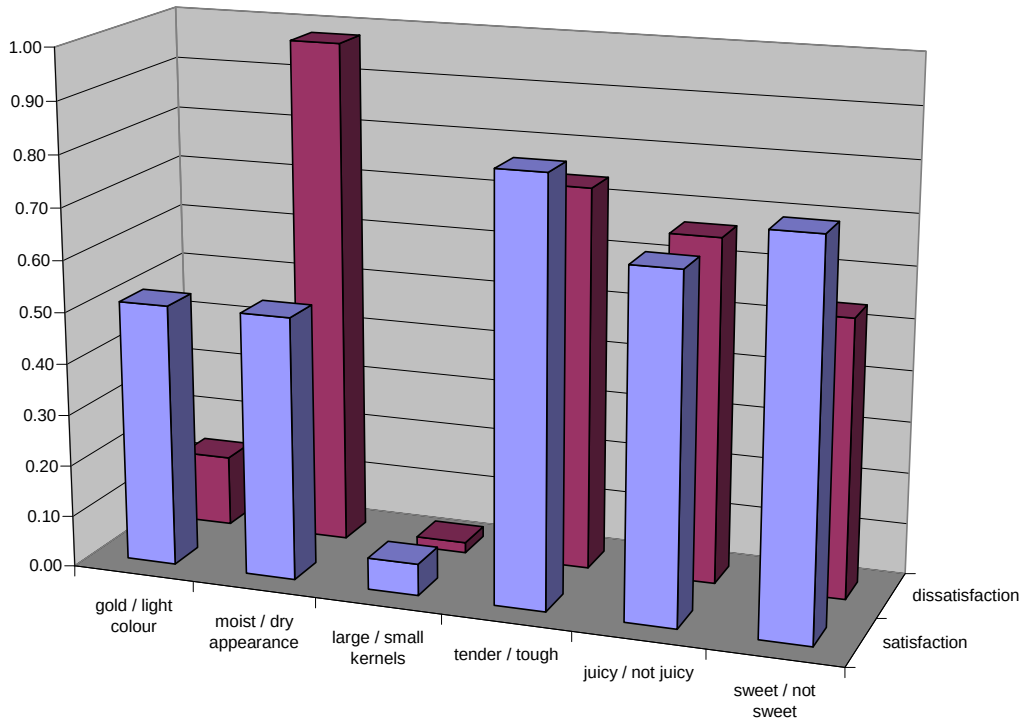

Figure 2. Relative importance of sensory attributes based on consumer coefficient of satisfaction and dissatisfaction

Coefficients of satisfaction, dissatisfaction and classification of environmental & health criteria (Table 3) are not as important in comparison with sensory criteria. Consumers were predominantly indifferent to these attributes. Nevertheless, two levels of consumer expectation could be identified:

Level 1: surveyed consumers will tend to avoid GMO super sweet corn. On the contrary, it's not because a corn is not a GMO that they will buy it, e.g. labelling a corn variety as being a GMO will decrease consumer decision for this variety, labelling a corn variety as not a GMO will not affect consumer's choice.

Level 2: knowing that a variety is grown without the use of pesticide and herbicide or that a variety provides a health benefit will attract consumers. On the contrary, knowing that a variety has been grown with the use of pesticide/ herbicide or that a variety has not an enhanced health benefit in comparison with standard super sweet-corn, will not influence consumer choice decision.

Table 3. Coefficients of satisfaction, dissatisfaction and classification of the environmental & health attributes.

Environmental/health aspect	Satisfaction	Dissatisfaction	Classification
No chemical/pesticide	0.52	0.20	I A
Organic/conventional	0.27	0.02	I
Non-GMO/GMO	0.26	0.40	I M
Respect/no environmental respect	0.40	0.36	I
Antioxidants/no health benefit	0.51	0.02	A

I: Indifferent, O: One-dimension, A: Attractive, R: Reverse, M: Must-be

For the environmental and health aspects of sweet-corn, the most important drivers were that the variety was 'grown without using pesticides or any chemicals' and that the variety has a 'high level of antioxidants and presents enhanced health benefits' (Figure 3). Criteria considered to be barriers were transgenic (Genetically Modified Organism) corn and if the corn was 'not grown with respect for the environment'. Consumers did not consider non-organic corn or if the corn had 'no specific impact on your health' to be barriers.

Consumers were identified as having two levels of requirement for attributes in relation with production (Table 4). Level 1: consumers will preferably avoid imported super sweet corn and preferably choose Australian grown super sweet corn. Level 2: knowing that a specific variety has just been harvested and is fresh, is the major criteria which will attract consumers, even if knowing that a variety has been cold stored will not affect them. A price of $0.50 a cob or

knowing that it has been grown on a small plantation or has a constant quality will also positively influence consumers.

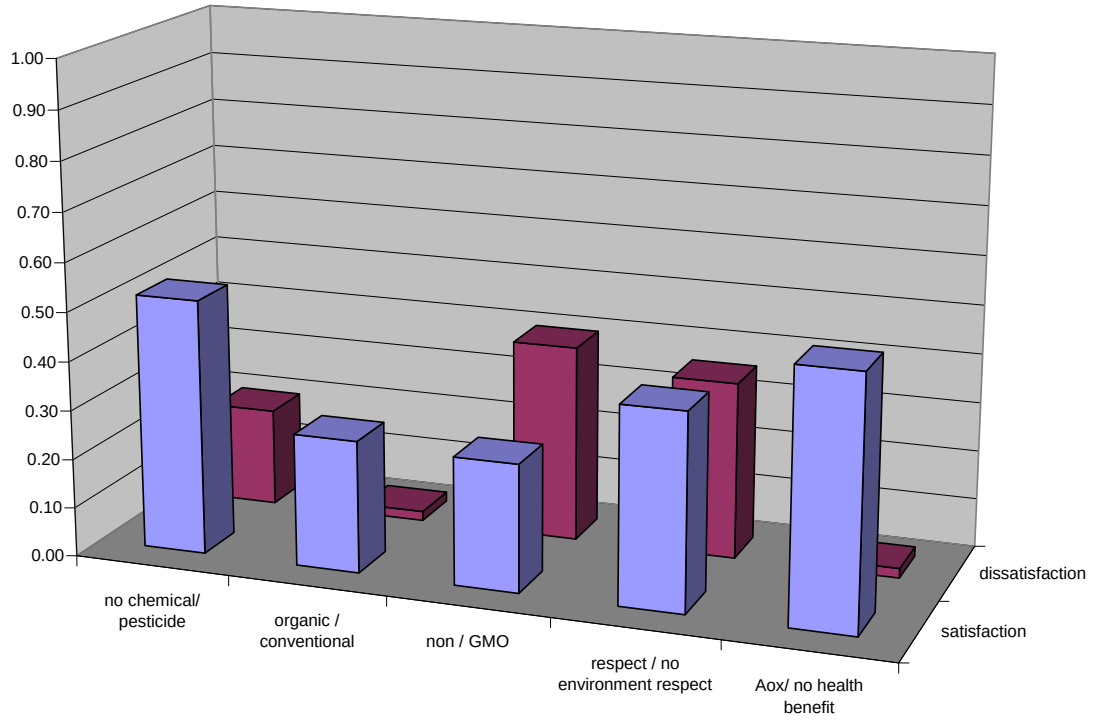

Figure 3. Relative importance of environmental & health attributes based on consumer coefficient of satisfaction and dissatisfaction.

Table 4. Coefficients of satisfaction, dissatisfaction and classification of the production attributes.

Production aspects	Satisfaction	Dissatisfaction	Classification
Australian/imported	0.75	0.43	O
Constant/variable quality	0.44	0.08	I A
All year/seasonal	0.21	0.03	I
Fresh/cold-stored	0.86	0.20	A
Small/large plantations	0.56	0.04	A
All shops/speciality shops	0.36	0.06	I
$0.50/$1.50	0.67	0.02	A

I: Indifferent, O: One-dimension, A: Attractive, R: Reverse, M: Must-be

Several production-related criteria were assessed by the consumers. The most important driver was that the corn was 'fresh and came directly from the producer' (Figure 4). Other important drivers were that the corn was grown in Australia and that it was $0.50 per cob. The highest barrier identified from the production aspect was that the corn was imported. Other criteria (variable quality, corn availability during the year and depending on the shopping place, cold stored, large plantations, or a price of $1.50 per cob) did not seem to affect consumer dissatisfaction and/ or satisfaction.

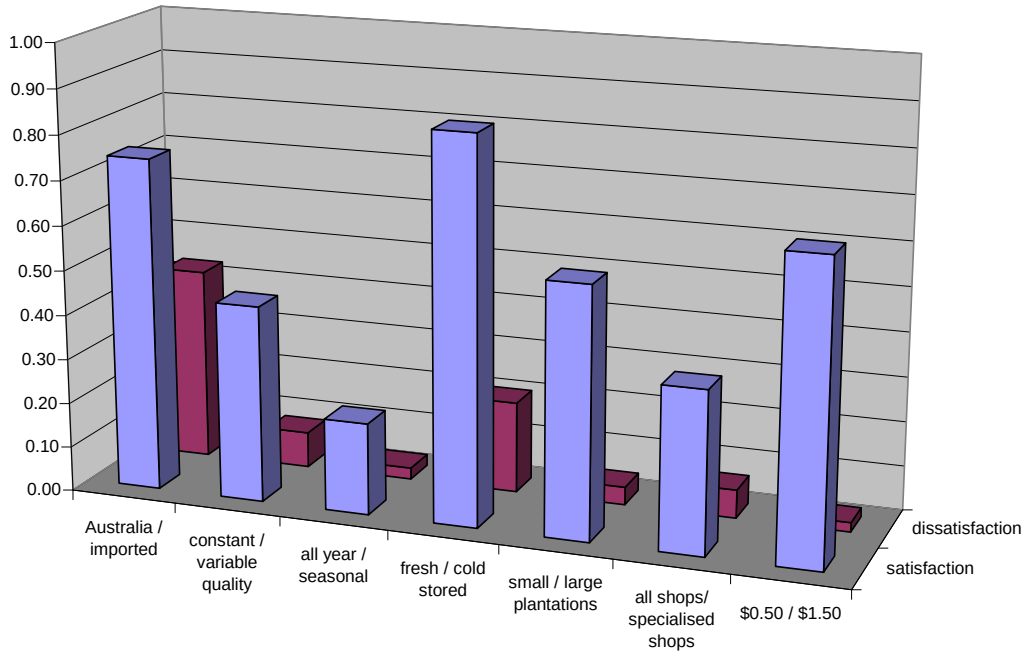

Figure 4. Relative importance of production attributes based on consumer coefficient of satisfaction and dissatisfaction.

In summary, the most important criteria, with the highest coefficient of satisfaction, were that the corn was/had (in decreasing order of importance):

- fresh and direct from the producer
- tender to eat
- grown in Australia
- very sweet to taste
- $0.50 per cob
- very juicy in the mouth
- grown by a small grower
- grown without using pesticide or any chemicals
- deep golden yellow kernels
- high level of antioxidants and presents enhanced health benefits
- moist kernels in appearance

The criteria with the highest coefficients of dissatisfaction and therefore considered barriers when choosing corn were corn with/which were (in decreasing order):

- dried or shrivelled kernels in appearance
- tough to eat
- not juicy at all in the mouth
- a taste not sweet at all
- a GMO

Consumer primary perception of the 5 different corn images (section 3).

The consumers rated the appearance acceptability of each of the 5 corn cob pictures individually (Table 5).

Table 5. Mean sensory scores for appearance acceptability of five corn cobs (scale: dislike extremely, 0; neither like nor dislike, 50; like extremely, 100).

Corn cob	White	Regular	Gold	Deep-gold	Orange	LSD
Mean	18.9 d	81.4 a	78.0 ab	69.2 b	51.7 c	9.47

Means within a row followed by the same letter are not significantly different (P<0.05).

The regular corn and gold corn had the highest appearance acceptability score. The deep-gold was scored significantly lower than the regular corn. The orange sample had a mean sensory score equating to 'neither like nor dislike' whereas the white super sweet corn appearance was rated towards 'dislike extremely' on the line scale. These results indicate that although the regular corn and gold corn appearances were rated very highly in terms of likeability, the deep-gold corn was still rated high in terms of appearance acceptability. Surprisingly, the white corn which is currently available in stores was disliked in terms of appearance.

The consumers were given the opportunity to make comments on what they liked (Table 6) and disliked (Table 7) about the 5 corn samples. For all 5 samples, consumers commented that they liked the shape/plumpness/roundness and size of the kernels. The appearance of the samples was also described as juicy, moist and fresh. For the regular, the target and the double super sweet corns, the consumers most frequently commented that they liked the colour.

Table 6. Like Comments – Frequency.

	White	Regular	Gold	Deep-gold	Orange
Colour, yellow kernels	6	34	32	27	17
Fresh	7	10	12	12	6
Juicy/Moist	6	17	20	15	11
Plump, full kernels, well presented, full cob, consistent kernels, tight together, well formed, rounded kernels, good shape	11	16	11	16	11
Size of kernels	8	14	8	18	12
Appealing/good/nice/clean/appetising/quality	2	3	2	4	4
Healthy/natural/ripe	1	5	5	2	7
Other (uneven kernels, crunchy, shiny, tasty, firm, texture, tender texture, everything, delicious)	1	1	6	5	1
Nothing	17	-	-	-	-

Table 7. Dislike Comments – Frequency.

	White	Regular	Gold	Deep-gold	Orange
Colour (pale, artificial, too golden, too orange, too bright, fake, unnatural, strange)	44	2	6	24	37
Not fresh, old	1	2	3	54	5
Dry/less juicy	4	1	5	4	2
Uneven/shape of kernels	4	4	2	1	
Size of kernels	3	-	1		2
Tough/texture/ starchy	1	2	1	1	1
No husk	1	4	-	1	-
Not appetising/tasty	8	-	-	-	-
Not ripe/overripe	1	-	-	2	1
Decreased nutrition/GMO	3	-	-	-	-
Nothing	2	25	20	12	9

When asked what they disliked about the regular corn, a large number of consumers indicated 'nothing' or left a blank comment. This was also the case for the gold and the deep-gold but to a lesser extent. A proportion of the consumers indicated that they disliked the colour of these samples with words like too golden, too orange, artificial, too bright, fake and artificially

enhanced used to describe these 2 samples especially for the deep-gold. For the orange and the white corns, a small number of consumers commented that they liked the colour of these samples. However, more consumers commented that they disliked the colour of these samples. The consumers selected words like unnatural, fake, artificial and strange to describe the capsicum sample and indicated that it was too bright/yellow/orange.

Table 8. Ranking of five corn cob pictures for perceived greatest health benefit when eaten (scale: 1 = greatest health benefit; 5 = least health benefit).

Corn cob	White	Regular	Gold	Deep-gold	Orange	LSD
Mean	4.8 d	2.2 a	2.0 a	2.7 b	3.4 c	0.39

Means within a row followed by the same letter are not significantly different (P<0.05).

Directly comparing the 5 super sweet corn cob pictures, the consumers were asked to rank the samples according to which they perceived would provide the greatest health benefit. The regular and gold samples were ranked the highest and therefore associated with the greatest health benefit. Both the deep-gold and the orange samples were classified as healthier in appearance than the white super sweet corn. These results are encouraging towards the perception of orange corn by consumers and its link with a healthy perception.

Influence of the knowledge of health benefit on consumer judgement (section 4)

Without any information, consumers gave a price equivalent to white super sweet corn to the gold corn (highest market price). With the information, consumers gave spontaneously a higher price for this healthy corn in comparison of the product category (Table 9).

Table 9. Price spontaneously given by two independent groups of consumers for gold supersweet-corn.

Consumer group	Control group	Test group	LSD
Number of consumers	28	24	
Mean price	$2.80 b	$3.65 a	0.44

Means within a row followed by the same letter are not significantly different (P<0.05).

Knowing that the gold corn is high in zeaxanthin and knowing the health benefit of this molecule increases spontaneously the maximal price given by the surveyed consumers by 30%. This information needs to be considered as a trend, it can not predict the final price of the target on the market place. This result indicates that consumers spontaneously add value to this specific health benefit carried by super sweet corn.

Table 10. Effect of knowledge of purchase intention.

Corn		White	Regular	Gold	Deep-gold	Orange	LSD
before	% Yes	8	83	71	63	29	
knowing	% Maybe	17	13	23	17	46	
	% No	73	2	4	17	23	
	Mean	1.3 d	2.8 a	2.7 ab	2.5 b	2.1 c	0.2
after	% Yes	4	50	65	69	48	
knowing	% Maybe	12	38	23	19	27	
	% No	75	2	2	4	15	
	Mean	1.2 c	2.5 ab	2.7 a	2.7 a	2.4 b	0.2

Means within a row followed by the same letter are not significantly different (P<0.05)
Scale used to code and analyse data from the ranking tests: 1: No, 2: Maybe, 3: Yes.

Without any knowledge of potential health benefit related to the colour of the sweet-corn cob, purchasing intention matched appearance acceptability. Regular corn and gold presented the highest purchase intention, followed by deep-gold, orange, and finally, white. White super sweet corn presented the lower significant purchase intention.

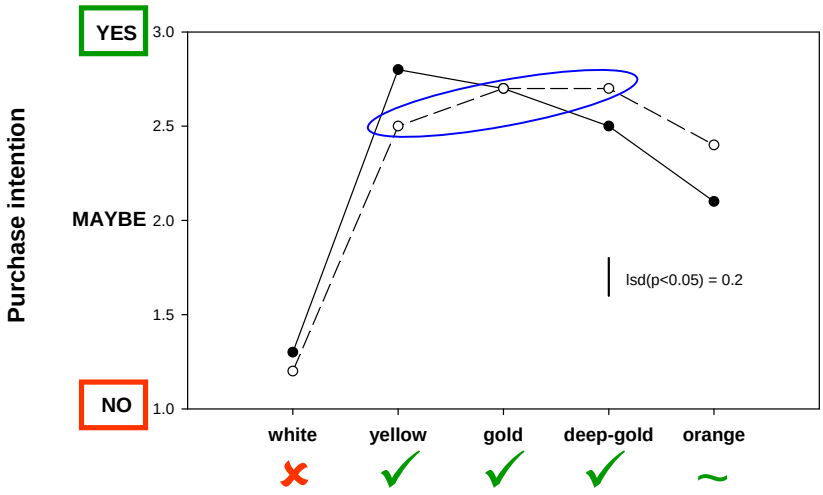

Figure 5. Purchase intention with (solid line) and without knowledge (broken line) of zeaxanthin health-benefit. In the latter, yellow, gold, and deep-gold (circled) were not significantly different in regard to purchase intention. A green tick indicates positive purchase intention.

Knowing orange colour can be related to the level of zeaxanthin and that zeaxanthin presents healthy benefits, changed the order of purchase intention (Figure 5). Consumers reported a similar purchase intention for regular, gold and deep-gold sweet-corn. Orange purchase intention was not significantly differently lower than regular corn purchase intention, but it was lower than gold and deep-gold. The observation that orange was lower than both gold and deep-gold, despite the knowledge that it might have highest zeaxanthin concentration, infers that the colour was too extreme for consumers. White sweet-corn still presented the lowest purchase intention.

Conclusions

Gold sweet-corn presented a similar level of spontaneous acceptability as regular sweet-corn. Both of them were significantly preferred over the 3 other corn colours. Purchase intention relied on the corn colour and no age effect was observed. Knowledge of the health benefit however, increased the acceptability of deep-gold (orange 8) to that of the regular and gold sweet-corn. The orange sweet-corn and snow white sweet-corn scored significantly lower, indicating that breeding a cob too orange would be detrimental to consumer acceptance.

According to this preliminary study, a hue angle between 72° and 90° would be considered as acceptable for consumers with an optimal value around 81° (i.e. gold). This value would enable differentiation from the colour of regular corn and would still not be too different from its regular yellow colour.

Knowing that the gold sweet-corn had a higher level of zeaxanthin and knowing the health benefit of this carotenoid also raised the spontaneous price given for this corn (by 30%). Even if this result may not be representative of the Australian population, it was in accordance with the 30% price increase for Vital Vegies in comparison with the corresponding vegetable category.

While colour and communication of health benefits can influence purchase of sweet-corn, preliminary requirements must first be met. The study found that sweet-corn must have an appearance which is not dry, and have tender, juicy and sweet kernels. If these preliminary requirements are not fulfilled, then consumers will not appreciate or be influenced by the golden colour and the health advantage of zeaxanthin-biofortified sweet-corn.

This study was limited to consumer appreciation of the appearance of uncooked super sweet corn cobs and can not predict consumer acceptability of the corresponding cooked variety. For future acceptability tasting and assessment of the appearance of cooked sweet-corn, it is recommended to boil or microwave the sweet-corn cobs based on the responses in the present study.

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Effect of increase in zeaxanthin and other carotenoids on carotenoid-derived flavour volatiles

Summary
Carotenoids are responsible for the yellow colour of sweet-corn, but are also potentially the source of flavour compounds from the cleavage of carotenoid molecules. Increasing zeaxanthin may therefore increase the concentration of carotenoid-derived volatiles which may ultimately influence flavour. The carotenoid-derived volatile, β-ionone was identified in both standard yellow sweet-corn (Hybrix5) and a zeaxanthin-enhanced experimental variety (HZ). As β-ionone is highly-perceivable at extremely low concentration by humans, it was important to confirm if alterations in carotenoid profile may also affect flavour volatiles. The concentration of β-ionone was most strongly correlated ($r^2 > 0.94$) with the β-arm carotenoids, β-carotene, β-cryptoxanthin and zeaxanthin, and to a lesser degree ($r^2 = 0.90$) with the α-arm carotenoid, zeinoxanthin. No correlation existed with either lutein ($r^2 = 0.06$) or antheraxanthin ($r^2 = 0.10$). Delaying harvest of cobs resulted in a significant increase of both carotenoid and β-ionone concentrations, producing a six-fold increase of β-ionone in HZ, and a two-fold increase in Hybrix5, reaching a maximum of 62 μg/kg FW and 24 μg/kg FW, respectively. In the present trial, there was no obvious taste difference between Everest (white sweet-corn with no detectable β-ionone) and either uncooked Hybrix5 or HZ sweet-corn when tasted by the authors. It is likely that any flavour differences brought about by an increased β-ionone concentration are either subtle, or are masked by other volatile compounds not derived from carotenoids.

Introduction
The major carotenoids present in sweet-corn are lutein and zeaxanthin, together with smaller amounts of β-cryptoxanthin, β-carotene, zeinoxanthin, α-carotene and antheraxanthin (*1-2*). Apart from providing the characteristic pigmentation of yellow corn, carotenoids may also contribute to flavor through the synthesis of carotenoid-derived volatiles formed by the action of carotenoid-cleavage enzymes (Figure 1). In tomato (*Solanum lycopersicum*), for example, lycopene and β-carotene are thought to be cleaved at the 9-10 and 9'-10' double bonds (*17*) to form the volatiles 6-methyl-5-hepten-2-one and β-ionone, respectively, both of which contribute strongly to the characteristic tomato flavor (*3*). In corn, Buttery and Ling (*4*) reported the presence of the volatiles, β-ionone and α-ionone in corn tortillas, but these were seen as minor constituents relative to non-carotenoid derived volatile compounds present, and not believed to contribute significantly to flavor.

Recently, there has been considerable research into the development of high-carotenoid corn varieties, either to enhance production of the pro-vitamin A carotenoid, β-carotene (*5-6*), or the production of zeaxanthin, a carotenoid associated with protection against age-related macular degeneration (AMD) (*7-8*). Zeaxanthin, and its isomer lutein, are actively accumulated in the human macula from dietary sources (*9*), and are thought to protect against the degradative effects of blue light oxidation on photoreceptor cells (*10-12*). Zeaxanthin, which is relatively rare in the diet, tends to be located towards the center of the macular, while lutein, its more commonly found isomer, is located towards the periphery (*13*). Although sweet-corn

is considered a good source of zeaxanthin relative to many other food sources (*14*), the amount of zeaxanthin present in a cob of corn is well below what is considered a supplementary dosage-rate (*15*), which would require consumption of between 4 and 11 cobs of standard yellow sweet-corn per day to be achieved.

Part of the strategy to enhance zeaxanthin concentration in sweet-corn has been to increase total carotenoid production and to shift the synthesis of carotenoids towards the β-arm of the carotenoid-synthesis pathway (Figure 1) where zeaxanthin is located (*7*). Although such a change will alter the color of the corn kernels from yellow to an orange hue (*8*), it is also possible that this change may affect the profile and synthesis of carotenoid-derived volatiles. As volatiles such as β-ionone can be perceived by humans at a very low concentration (7 ng/L) (*4, 16*), increasing their concentration may have a significant effect on sweet-corn flavor. The aim of the following investigation was to quantify changes in carotenoid-derived volatiles of a zeaxanthin-enhanced sweet-corn line HZ relative to the standard yellow sweet-corn cultivar Hybrix5 over a range of harvest maturities, and to determine if these changes in volatiles are correlated to changes in carotenoid concentration and profile.

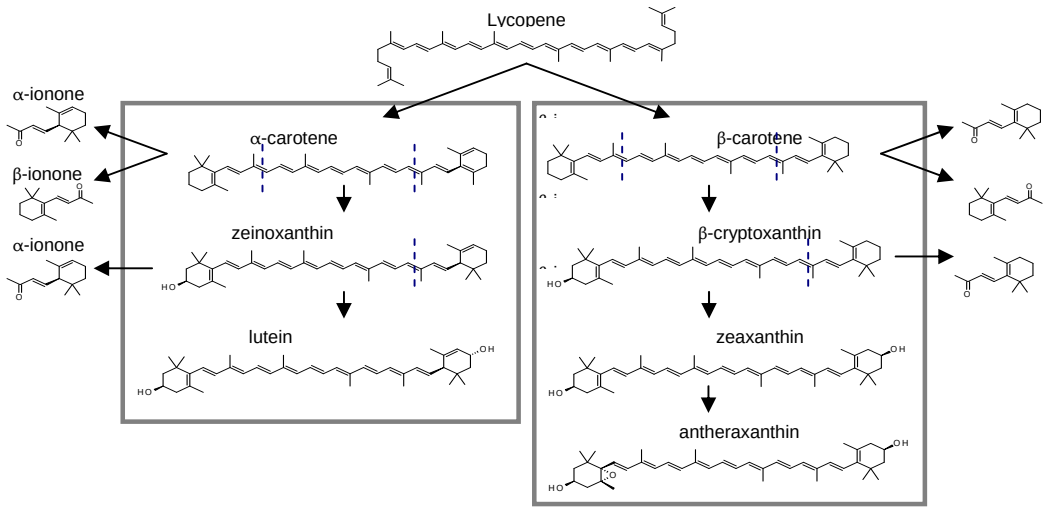

Figure 1. Structures of the principal carotenoids (boxed) of the carotenoid synthesis pathway found in sweet-corn kernels and their known carotenoid-derived volatiles formed by cleavage (dotted lines). (left box: α-arm carotenoids; right box: β-arm carotenoids).

Material and Methods

Corn Samples

Sweet-corn of a standard yellow cultivar, Hybrix5, and an enhanced-zeaxanthin experimental line HZ were grown at Gatton Research Facility, Queensland, Australia. Both varieties have similar total carotenoid concentration, but vary in their proportion of α-arm and β-arm carotenoids. Twenty-five plants of each variety were grown in parallel rows immediately beside each other. Plants were manually self-pollinated and three cobs harvested randomly each at 16, 20 and 24 days after pollination (DAP), representing early, normal, and late

harvests for sweet-corn. Harvested cobs were immediately stored at -20°C for up to 15 days subsequent to carotenoid and volatile analysis. Previous analysis in our laboratory showed no significant change in carotenoids in freshly harvested kernels stored for up to 4 weeks at 4°C. Commercially-harvested cobs of a white cultivar Everest were purchased from a local supermarket and stored similarly at -20°C to serve as a non-pigmented control.

Carotenoid Extraction and HPLC Analysis

Carotenoid extraction and HPLC analysis were conducted according to the method of Fanning et al. (*8*). Fifteen kernels were randomly selected from each of the three cobs per harvest date. Sweet-corn samples were cryogenically milled using a Restch MM301 ball-mill (Haan, Germany) and approximately 0.6 g of sample was weighed and 6 ml ethanol and 250 µL of β-apo-8'carotenal (7.2 mg/L in isopropanol) as an internal standard added. Samples were vortexed, and 3 ml deionized water and 5 ml hexane added, and revortexed for 20 sec before placing capped tubes on ice. Samples were centrifuged for 2 min at 83 Hz (4°C) to separate layers. The upper hexane layer was removed and transferred to a second tube. Fresh hexane (5 ml) was added to the non-hexane fraction containing the pellet, vortexed, and the procedure repeated as above 2 to 3 times until the pellet became a white color. The combined hexane fractions were dried in a centrifugal evaporator at 30°C and the extracted carotenoids reconstituted in 2 ml methanol/dichloromethane (50:50, v/v), containing 0.1% butylated hydroxy-toluene (BHT). Samples were filtered (0.22 µm syringe filter; Grace, Sydney, Australia) and placed into HPLC vials, and stored at -80°C prior to HPLC analysis. CV was less than 5%. Authentic standards of lutein, zeaxanthin, β-carotene, and β-cryptoxanthin (Sigma-Aldrich) were prepared similarly in methanol/dichloromethane (50:50, v/v), containing 0.1% BHT. Each carotenoid standard was run by HPLC to determine peak purity. The actual concentrations of the standard solutions were then calculated by multiplying the concentration determined spectrophotometrically by the % peak area of the standard peak as determined by HPLC. Standard curves were linear over the range 0.03-10 µg/mL with r^2 values of >0.999.

The HPLC system consisted of a SIL-10AD VP auto-injector, SCL-10A VP system controller, LC-10AT VC liquid chromatograph and a SPD-M10 A VP diode array detector (Shimadzu, Kyoto, Japan). Forty microlitres of each extract was injected onto a YMC C30 Carotenoid Column, 3 µm, 3.6 x 250 mm (Waters, Milford, MA, USA), with a mobile phase consisting of 92 % methanol/8 % 10 mmol/L ammonium acetate (phase A), and 100 % methyl *tert*-butyl ether (phase B). The 10mM ammonium acetate solution was made up in water and mixed with methanol in a ratio of 92:8, methanol:10mM ammonium acetate solution (v:v). The following 40 min gradient was used (*5*): 0 min, 80 % phase A; 32 min, 40 % phase A; 34 min, 80 % phase A; 40 min, 80 % phase A.

Carotenoid Identification and Quantification

Lutein, zeaxanthin, β-cryptoxanthin, and β-carotene were identified by comparison with the retention times and absorption spectra of the standards and quantified as described previously (*8*). Antheraxanthin and zeinoxanthin were identified by comparison of retention time, absorption spectra and mass data, obtained by LC-MS (*10*). The standard curve of lutein was used to quantify antheraxanthin and the standard curve of β-cryptoxanthin was used to

quantify zeinoxanthin. Carotenoid concentration was expressed as $\mu g.g^{-1}$ fresh weight (FW) of corn kernels.

Preparation of Kernels for Volatile Extraction

Approximately 5 g of randomly selected sweet-corn kernels from the same cobs as those used above for carotenoid analysis (n = 3) were accurately weighed (+/- 0.01 g) directly into a Restch MM301 ball-mill stainless steel vessel (Haan, Germany). Five mL of saturated sodium chloride solution containing 2-nonanone (0.06362 µg/L, Sigma-Aldrich) as an internal standard was added. The sample and extraction solution were then homogenised for 30 sec at 30 cycles/sec. A 10 g aliquot of the resulting homogenate was then added to a 20 mL headspace vial which was immediately sealed using a crimp seal cap and septum. The prepared samples were stored at 4°C prior to analysis.

Headspace GC-MS Analysis.

Analysis of carotenoid-derived volatiles was performed using a Shimadzu GC-2010 gas chromatograph coupled with a Shimadzu GCMS-QP2010S mass selective detector (MSD). Headspace sampling was undertaken by solid-phase microextraction (SPME) using a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) controlled by Cycle Composer software (CTC Analytics, version 1.5.2). A 50/30 μm carboxen/divinylbenzene/polydimethylsiloxane (Car-DVB-PDMS StableFlex, Supelco, Bellefonte, PA) SPME fibre was used for all analyses. Prior to headspace sampling, the vials were equilibrated at 60°C for 15 min. During extraction, the SPME fiber was exposed to the sample headspace for 30 min at 60°C, then inserted into the heated GC inlet, and desorbed at 250°C in splitless mode. After 2 min, a 1:50 split ratio was programmed and maintained for the duration of the analysis. The GC column oven was fitted with a DB-1 capillary column (50 m × 0.22 mm i.d., 1 μm phase; SGE, Australia). The carrier gas was helium set to a flow rate of 1.2 mL/min, linear velocity 30.6 cm/sec. The initial oven temperature was 40°C for 2 min, then ramped at 5°C/min to 100 °C, then ramped at 20°C/min to 220°C and held for 8 minutes. The interface temperature was set to 280°C.

A scan of volatile compounds using an *m/z* range of 35 to 350 was conducted. Spectra were examined and compounds identified using Shimadzu GC-MS solutions software (version 2.53). Compounds with ionone-ring structures were tentatively identified. In addition, a search was conducted for the specific direct and indirect potential carotenoid-cleavage products, β-ionone, α-ionone, 3-hydroxy-β-ionone, 3-oxo-α-ionone, 3-oxo-β-ionone, β-damascenone, blumenol C and metastigm-4-ene-3,9-dione.

Quantification of β-ionone was further achieved using 2-nononone (Sigma-Aldrich) as an internal standard and with the MSD in Selective Ion Monitoring (SIM) mode. The ion source was set at 70 eV and electron multiplier at 1350 V. The target ions monitored were *m/z* 58 and *m/z* 177 for 2-nonanone and β-ionone respectively and the ratios of their integrated area counts were used for quantification. The qualifier ions monitored for 2-nononone were *m/z* 57 (23.2), 71 (22.6) and 59 (22.3) and those monitored for β-ionone were *m/z* 91 (20.8), 93 (17.6), 135 (17.1) and 178 (13.8). Positive identification was confirmed by the presence of both target and qualifier ions at the correct retention time and with the correct ion ratios. A 6 point

internal standard calibration was made by the addition of β-ionone (Sigma-Aldrich) to the white corn cultivar Everest known to contain no endogenous β-ionone.

Statistical Analysis

The trial was conducted as a completely randomized design. Differences in carotenoid and β-ionone concentrations were evaluated using one-way analysis of variance (ANOVA) and means separated using least significant difference (LSD) at $P<0.05$. Carotenoid and β-ionone concentrations were correlated using linear regression analysis and coefficient of determination (R^2) determined. Both ANOVA and linear regression analyses were performed using Genstat software (version 11.1).

Results

Effect of Variety and Harvest Maturity on Carotenoid Accumulation

The principal carotenoids identified in both Hybrix5 and HZ cultivars were the α-arm carotenoids, lutein and zeinoxanthin, and the β-arm carotenoids, zeaxanthin, β-cryptoxanthin, β-carotene, and antheraxanthin (Figure 2; Table 1). α-carotene was detected in only trace amounts. Zeaxanthin was the predominant carotenoid in HZ, and lutein the predominant carotenoid in Hybrix5. Zeaxanthin and lutein reached maximum concentrations of 8.3 and 4.0 µg/g FW in HZ, and 2.9 and 6.2 µg/g FW, respectively, in Hybrix5 (Table 1). Carotenoid levels in the white cultivar Everest were extremely low (data not presented).

Table 1. Carotenoid concentrations (± SE) of standard yellow hybrid, Hybrix5, and enhanced β:α ratio hybrid HZ sweet-corn at increasing harvest maturities (DAP).

Hybrid		Carotenoid ($\mu g.g^{-1}$ FW)	Days after pollination (DAP)		
			16	20	24
Hybrix5	β-arm	β-carotene[A]	0.24±0.01 a	0.41±0.05 ab	0.51±0.02 b
		β-cryptoxanthin	0.31±0.04 a	0.34±0.03 a	0.41±0.03 a
		zeaxanthin	2.09±0.14 a	2.80±0.23 a	2.92±0.10 a
		antheraxanthin	1.30±0.02 a	1.64±0.09 b	1.74±0.29 b
		Total β-arm	3.95±0.21 a	5.21±0.39 b	5.60±0.16 b
	α-arm	zeinoxanthin	1.54±0.29 a	1.81±0.19 a	2.68±0.16 a
		lutein	2.38±0.09 a	5.52±0.38 b	6.21±0.23 b
		Total α-arm	3.39±0.35 a	7.34±0.54 b	8.90±0.23 c
HZ	β-arm	β-carotene	0.28±0.01 a	0.49±0.06 a	1.07±0.12 b
		β-cryptoxanthin	0.19±0.02 a	0.45±0.03 b	0.87±0.06 c
		zeaxanthin	2.62±0.05 a	4.90±0.27 b	8.27±0.97 c
		antheraxanthin	0.94±0.01 a	1.18±0.04 ab	1.49±0.15 b
		Total β-arm	4.05±0.06 a	7.04±0.38 b	11.72±0.95 c
	α-arm	zeinoxanthin	1.20±0.08 a	2.17±0.54 a	2.68±0.55 b
		lutein	1.60±0.05 a	2.75±0.05 b	3.97±0.24 c
		Total α-arm	2.81±0.09 a	4.93±0.50 b	7.67±0.79 c

[A]Data (n = 3) within rows followed by different letters are significantly different (P<0.05)

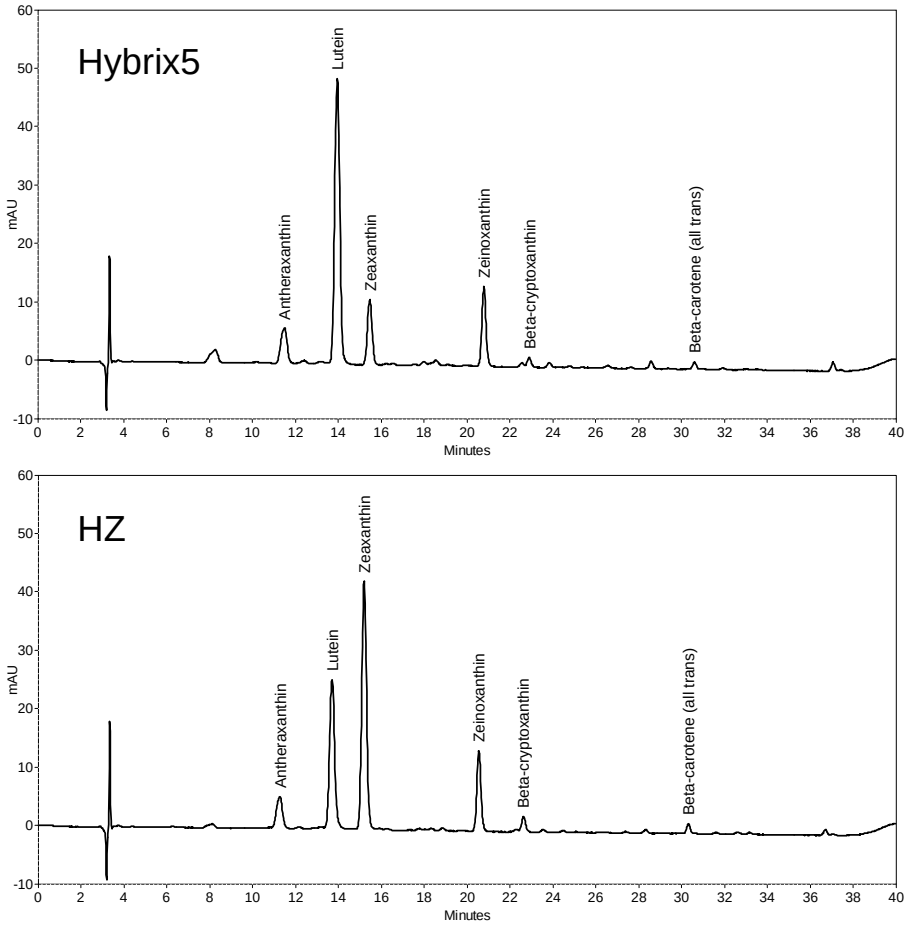

Figure 2. Principle carotenoid peaks identified in Hybrix5 (top) and HZ sweet-corn (*Zea mays* var. *saccharata*) kernels (bottom).

As expected, the ratio of β-arm carotenoids to α-arm carotenoids differed markedly between the two yellow cultivars, with Hybrix5 accumulating more α-arm carotenoids than HZ, and HZ accumulating more β-arm carotenoids than Hybrix5 (Table 1). This was reflected by Hybrix5 averaging a β:α ratio of 0.78 and HZ a β:α ratio of 1.48 (Table 2). Generally, carotenoid concentrations increased with increasing harvest maturity (Table 1), although in Hybrix5, zeaxanthin, β-cryptoxanthin and zeinoxanthin did not significantly increase ($P<0.05$) after the initial harvest time at 16 DAP. Consequently, the β:α ratio of Hybrix5 was observed to decline with increasing harvest maturity, while that for HZ remained relatively constant (Table 2).

Table 2. Ratio of β-arm to α-arm carotenoids (± SE) in Hybrix5 and HZ sweet-corn hybrids (*Zea mays* var. *saccharata*) at different harvest maturities (DAP).

Hybrid	Days after pollination (DAP)		
	16[A]	20	24
Hybrix5[B]	1.01±0.05 Aa	0.71±0.01 Ba	0.63±0.02 Ba
HZ	1.45±0.05 Ab	1.44±0.06 Ab	1.54±0.07 Ab

[A]Data (n = 3) within columns followed by different lowercase letters are significantly different (P<0.05)
[B]Data (n = 3) within rows followed by different upercase letters are significantly different (P<0.05)

Identification and Quantification of Carotenoid-Derived Volatile Compounds

Potential carotenoid-derived volatiles identified with an ionone-like ring structure eluted at 24.56 min and 25.53 min (Figure 3). These peaks were tentatively identified as either α-ionone, 6-methyl-α-ionone, or γ-ionone at 24.56 min, and β-ionone at 25.53 min. There was no spectral evidence for the presence of the potential primary and secondary cleavage products, 3-hydroxy-β-ionone, β-damascenone, 3-oxo-α-ionone, 3-oxo-β-ionone, blumenol C or metastigm-4-ene-3,9-dione. The presence of β-ionone was confirmed by selected ion monitoring mode (SIM), and although common ions (eg. *m/z* 177) were found at a retention time expected for α-ionone, the identity of α-ionone was not confirmed.

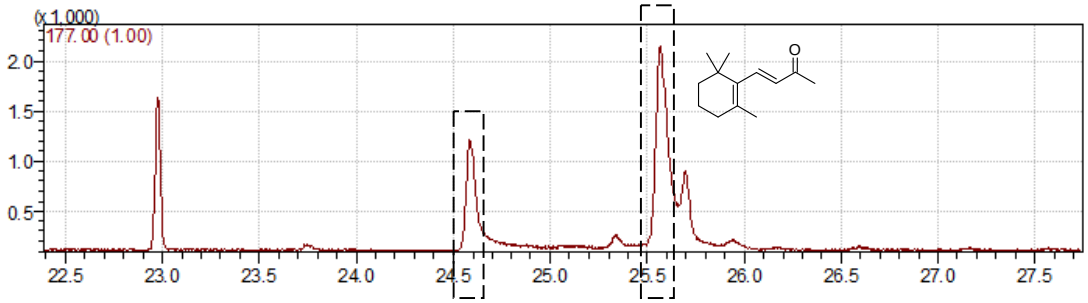

Figure 3. Boxed peaks detected in Hybrix5 and HZ sweet-corn with an ionone-like ring structure. The boxed peak at right was identified as β-ionone.

β-ionone increased with increasing harvest maturity in both Hybrix5 and HZ, but was absent in the white cultivar Everest (Figure 4a). β-ionone reached a maximum concentration of 0.063 µg/g FW in HZ at 24 DAP, approximately 2.5 times higher than that observed in Hybrix5 (0.024 µg/g FW).

Correlation between β-ionone and Carotenoid Concentration

β-ionone was found to be strongly correlated with the β-arm carotenoids, β-carotene (r^2 = 0.94), β-cryptoxanthin (r^2 = 0.95) and zeaxanthin (r^2 = 0.95), but not antheraxanthin (r^2 = 0.10). β-ionone was also positively correlated with the α-arm carotenoid, zeinoxanthin (r^2 = 0.90), but not lutein (r^2 = 0.06) (Table 3).

The carotenoids that correlated well with β-ionone also correlated highly with each other. For example, β-carotene was highly correlated with the β-arm carotenoids, β-cryptoxanthin (r^2 = 0.95) and zeaxanthin (r^2 = 0.90), as well as the α-arm carotenoid, zeinoxanthin (r^2 = 0.90) (Table 3). However, as with β-ionone, β-carotene was poorly correlated with lutein (r^2 = 0.09) and antheraxanthin (r^2 = 0.13).

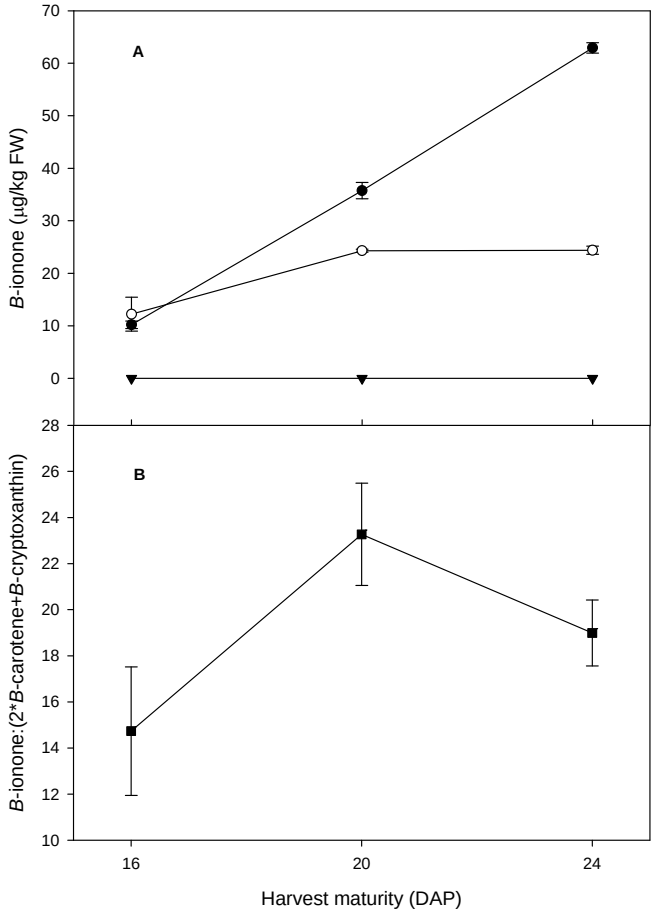

Figure 4. (A) Accumulation of β-ionone in HZ(●), Hybrix5 (○), and Everest (▼) sweet-corn kernels with increasing harvest maturity (n = 3). (B) Ratio of β-ionone to its precursor carotenoids at different harvest maturity stages (n = 6; data for HZ and Hybrix5 were pooled due to nil significant varietal difference at each harvest point).

Table 3. Coefficient of determination (R^2) of linear correlations between the volatile, β-ionone, and β-arm and α-arm carotenoids, and; between β-carotene (a known β-ionone precursor) and other carotenoids present in sweet-corn.

Compound	β-arm carotenoids	α-arm carotenoids	R^2
β-ionone	β-carotene		0.94
	β-cryptoxanthin		0.95
	zeaxanthin		0.95
	antheraxanthin		0.10
		zeinoxanthin	0.84
		lutein	0.06
β-carotene	β-cryptoxanthin		0.95
	zeaxanthin		0.90
	antheraxanthin		0.13
		zeinoxanthin	0.90
		lutein	0.09

Change in β-ionone Concentration Relative to Potential Precursor Carotenoids

The ratio of β-ionone produced relative to the concentration of its potential precursor carotenoids, β-carotene and β-cryptoxanthin, was observed to change with harvest maturity in the present trial (Figure 4b). Both varieties behaved similarly, with no significant difference ($P<0.05$) detected between Hybrix5 and HZ at each harvest maturity. The ratio of β-ionone to β-carotene(x2)+β-cryptoxanthin significantly increased from 16 to 20 DAP, and then declined slightly from 20 to 24 DAP (Figure 4b).

Discussion

The current study indicated that altering the carotenoid profile of sweet-corn, specifically the ratio of the β-arm to the α-arm of the carotenoid synthesis pathway, can significantly impact on the production of carotenoid-derived volatile compounds. The underlying reason for increasing zeaxanthin concentration in sweet-corn was to provide a more concentrated source of zeaxanthin in the diet, as a means of potentially ameliorating the progression of AMD. However, increasing this compound by increasing the ratio of β-arm carotenoids may potentially influence the synthesis of carotenoid-derived volatiles, some of which are strongly perceived at extremely low concentrations, and may therefore affect the flavor of the kernels, either favorably or otherwise. In the current study, β-ionone and a second compound with an ionone-like ring structure (potentially α-ionone) were the only carotenoid-derived volatiles detected by GC-MS. Although α-ionone, 6-methyl-α-ionone and γ-ionone were potential candidates for the unknown second compound, the latter two compounds were considered unlikely, as both are relatively rare or formed via a different pathway (*18*). Both β-ionone and α-ionone have been previously detected in corn-tortillas (*4*), but have not been reported in fresh or cooked corn (*19*). The presence of β-ionone was confirmed in the present trial, but the presence of α-ionone remains to be confirmed. In the variety HZ, increasing the proportion

of β-arm carotenoids was found to increase β-ionone concentration up to 2.5 times relative to the standard yellow variety 'H5', with the effect becoming more pronounced with increased harvest maturity ((Figure 4A). Whether this increase impacts significantly on flavor remains to be assessed, but is potentially possible, as β-ionone can be perceived by humans at extremely low concentrations (*4, 16*).

β-ionone is thought to be a direct cleavage product from β-carotene and potentially the non-hydroxylated end of β-cryptoxanthin (*20-21*), while α-ionone is a cleavage product of α-carotene and zeinoxanthin (*22-23*) (Figure 1). Other potential carotenoid cleavage volatiles such as 3-hydroxy-β-ionone, which has shown *in vitro* to be a cleavage product of zeaxanthin in bacterial cultures (*24*), were not detected, despite large concentrations of zeaxanthin being present in samples. Interestingly, α-carotene was detected in only trace amounts in Hybrix5 and HZ, while zeinoxanthin was detected in significant amounts. Li et al. (*2*) reported a similar observation recently with 'B73' field-corn.

Increased carotenoid concentration, particularly of the β-arm carotenoids, zeaxanthin, β-cryptoxanthin and β-carotene, was significantly correlated with an increased production of β-ionone. As would be expected, the white sweet-corn Everest which had minimal carotenoids present, had no detectable β-ionone, owing to a lack of suitable precursors. β-ionone can be directly formed following enzymatic cleavage of β-carotene and β-cryptoxanthin (non-hydroxylated end) by carotenoid cleavage dioxygenase enzymes (CCDs) (*25*). Although β-ionone is not reported to be formed directly from zeaxanthin, β-ionone was observed to be strongly correlated with zeaxanthin concentration (r^2 = 0.95). Zeaxanthin is not an obvious precursor of β-ionone, and would be theoretically cleaved to form 3-hydroxy-β-ionone, which was not detected in the present trial. Whether secondary conversion of 3-hydroxy-β-ionone to β-ionone is possible is not currently known, although secondary conversion of initial cleavage elements is not unusual in carotenoid-derived volatile formation, and accounts for the majority of carotenoid-derived volatiles found in plants (*26*). However, considering that 3-hydroxy-β-ionone is also a potential cleavage product of lutein and antheraxanthin, and yet there was no correlation between these compounds and β-ionone, this would make conversion of 3-hydroxy-β-ionone to β-ionone appear unlikely. If zeaxanthin is subject to similar cleavage mechanisms (ie CCD1) as β-carotene, it is possible that a cleavage product such as 3-hydroxy-β-ionone may undergo rapid conversion to other compounds such as C13 cyclohexenone, a carotenoid-cleavage product that has been detected in maize roots (*27*).

Although zeaxanthin, β-cryptoxanthin and β-carotene concentrations were highly correlated with β-ionone production (and zeinoxanthin to a lesser degree), it was interesting that the carotenoids, lutein and antheraxanthin were poorly correlated with β-ionone (Table 3). In *in vitro* systems, CCD1 will accept a wide range of C-40 carotenoid substrates (*28*), cleaving at the 9-10 and 9'-10' double bonds (*24*). Amongst the carotenoids present, this would result in an initial cleavage product of β-ionone for only β-carotene, β-cryptoxanthin and α-carotene (Figure 1). By contrast, lutein would be cleaved to form 3-hydroxy-β-ionone and 3-hydroxy-α-ionone, while antheraxanthin would form 3-hydroxy-β-ionone and 3-hydroxy-5,6-epoxy-β-ionone. As 3-hydroxy-β-ionone is a potential cleavage product of both lutein and

antheraxanthin, but also of zeaxanthin (which was highly correlated to β-ionone), this would support the proposal that conversion of 3-hydroxy-β-ionone to β-ionone is unlikely in sweet-corn, and that the strong correlation between zeaxanthin and β-ionone is simply because zeaxanthin is strongly correlated (r^2 = 0.90) with its precursor carotenoids, β-carotene and β-cryptoxanthin (Table 3).

Both cultivar and harvest maturity were observed to impact β-ionone production. Within the current trial, β-ionone concentration reached a maximum of 63 µg/kg FW in HZ at the late harvest maturity (24 DAP), relative to 24 µg/kg FW in the standard yellow hybrid, Hybrix5. Later harvest also corresponded to maximum concentrations of the potential β-ionone precursor carotenoids, β-carotene and β-cryptoxanthin, in both varieties, which is likely to explain the differences observed. Interestingly, the ratio of β-ionone to these carotenoids increased between 16 and 20 DAP, before declining again at 24 DAP. This may indicate that activity of the carotenoid-cleavage enzyme, CCD1 changes with harvest maturity, first increasing and then decreasing again as sweet-corn kernels become increasingly mature.

Although β-ionone dissolved in water has been reported to be perceptible by humans at concentrations as low as 7 ng/L (*4, 16*), there was no obvious taste difference between Everest (white sweet-corn with no detectable β-ionone) and either uncooked Hybrix5 or HZ sweet-corn when tasted by the authors. It is consequently possible that any flavor differences brought about by an increased β-ionone concentration are either subtle, or may be masked by other volatile compounds not derived from carotenoids, such as dimethyl sulphide or 2-acetyl-1-pyrroline, which have been identified as major contributors to sweet-corn aroma (*4, 19*). Despite the potential advantages of a high-zeaxanthin sweet-corn for sufferers of AMD, further study using a trained taste panel would be required to determine if any significant difference in flavour exists in either cooked or uncooked zeaxanthin-biofortified sweet-corn, in order to provide evidence that an increase in carotenoid-derived volatiles would not make the product unpalatable.

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Effect of harvest maturity on zeaxanthin and kernel colour

Summary
Sweet-corn is harvested at an immature stage of development, when it is both sweet and tender to eat, and has developed a yellow kernel colour. Delaying harvest of both normal yellow sweet-corn and a zeaxanthin-biofortified sweet-corn line was found to cause an increase in both colour and total carotenoid concentration, including zeaxanthin. Kernel colour was closely correlated to zeaxanthin concentration (r^2 = 0.95). Although, delaying harvest is a means of increasing zeaxanthin concentration, reduction in moisture content and other parameters is likely to result in lower eating quality due to a 'chewier' texture, and is therefore not recommended. Similarly, harvesting sweet-corn too early is likely to result in lower zeaxanthin concentrations, negating the production of a high-zeaxanthin product.

Introduction
Sweet-corn kernels are accumulators principally of the xanthophyll carotenoids, lutein and zeaxanthin, which are responsible for the characteristic yellow colour of sweet-corn. Generally, most varieties of sweet-corn are higher in lutein than zeaxanthin, with smaller concentrations of β-carotene, β-cryptoxanthin, zeinoxanthin and antheraxanthin (Kopsell et al., 2009; Li et al., 2010). Within the carotenoid synthesis pathway, lutein and zeinoxanthin are formed on the alpha-arm of the pathway, while the remainder are formed on the beta-arm. The relative carotenoid flux to either arm has been reported to be due to the enzyme, lycopene epsilon cyclase, which can vary in activity depending on the particular allele present (Harjes et al, 2008). Sweet-corn is harvested in an immature state, approximately 18-24 days after pollination, depending on temperature conditions. Harvest at this time optimises sweetness and tenderness, as well as providing the typical yellow colour. Little is known on the effect of harvest maturity on carotenoid accumulation in sweet-corn, although there is some evidence that the carotenoids, lutein and β-cryptoxanthin may increase as kernels mature, while no consistent change was observed for zeaxanthin, β-carotene or α-carotene (Kurilich and Juvic, 1999).

The purpose of the following investigation was to determine the impact of harvest maturity on changes in individual sweet-corn carotenoids in both a regular yellow sweet-corn variety and two varieties that had been selected for enhanced zeaxanthin concentration. Harvest maturity was assessed for impact on total and individual carotenoid accumulation, and changes in the ratio of beta-arm to alpha-arm carotenoids. The impact of change in carotenoid profile on kernel colour was also assessed.

Materials and Methods
Plant material and harvest maturity
Seed from the yellow sweet-corn hybrid cultivar Hybrix5 and two experimental high-zeaxanthin hybrids (HZ9 and HZ12) were sown in three blocks at Kairi Research Station, Queensland, in September, 2010. Plants were self-pollinated and cobs from six plants per

variety (2 per block) were harvested at 20, 24 and 28 days after pollination (DAP). All cobs were harvested between 9:00-11:00am in the morning, and subsequently frozen at -20°C prior to carotenoid and colour analysis.

Colour and carotenoid analysis
Prior to carotenoid analysis, colour (hue angle) of the kernel surface was determined using a Minolta chromameter (aperture 6 mm diameter, D65 light source). Measurement consisted of an average of three random measurements across the cob surface while the sample was still frozen. Following measurement, approximately 2 g of frozen kernels were removed from the cob and pounded into a fine powder using a refrigerated ball-mill for two minutes. Exactly 1.5 g of powder was removed and extracted and analysed by HPLC according to the method of Fanning et al (2010).

Dry matter content of kernels was analysed by taking 5 g of material and drying it in a vacuum oven (0.1 MPa) at 70°C for 48 hours. Samples were re-weighed, and dry matter percentage calculated by subtraction. This data was used to calculate carotenoid levels expressed on both a fresh-weight and dry-weight basis.

Results
Effect of harvest maturity on total and individual carotenoid concentration
Increase in harvest maturity from 20 to 28 DAP was associated with an almost linear increase in total carotenoid concentration, both on a fresh and dry weight basis (Figure 1). There was no significant difference between cultivars at each harvest point, with total carotenoid concentration for both the yellow cultivar and the high-zeaxanthin experimental hybrids statistically the same.

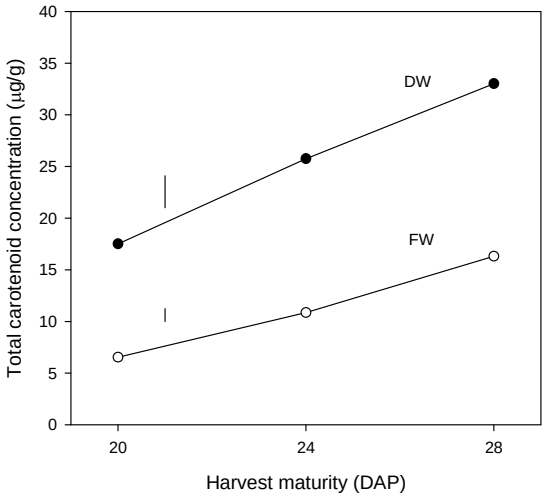

Figure 1. Total carotenoid content increased with later harvest maturity on both a fresh weight (FW) and dry weight (DW) basis. All three hybrids had similar total carotenoid concentration at

each harvest maturity, and have been subsequently pooled together. Vertical bars indicate LSD ($P<0.05$).

Lutein and zeaxanthin, which were the principal carotenoids recorded, also increased with increasing harvest maturity for each cultivar (Figures 2A, 2B). There were however, contrasting differences between the yellow and high-zeaxanthin hybrids. Firstly in the yellow cultivar, lutein levels were significantly higher than in the high-zeaxanthin hybrids, and increased at a faster rate with increasing maturity. This was in direct contrast to zeaxanthin, which from 24 days onwards was significantly higher in the high-zeaxanthin hybrids, and increased at a faster rate than in the yellow cultivar. The high-zeaxanthin hybrids (HZ9 and HZ12) had similar zeaxanthin and lutein kernel concentrations at all harvest points.

β-carotene and β-cryptoxanthin, which are the immediate precursors of zeaxanthin in the carotenoid pathway, behaved similarly to zeaxanthin in the high-zeaxanthin hybrids. Both β-carotenoids increased at a faster rate than in the yellow cultivar, although differences in concentration only became significant at 28 days maturity (Figures 2C, 2D). Interestingly, β-cryptoxanthin concentration for the yellow cultivar did not change over the harvest period, whereas β-carotene showed a significant increase after 24 days.

Atheraxanthin, which is synthesised from zeaxanthin, was generally higher in concentration in the yellow cultivar than in the high-zeaxanthin hybrids (Figure 2e). Antheraxanthin concentration increased more quickly in the yellow cultivar, but plateaued after 24 days. Antheraxanthin increased more slowly with harvest maturity in the high-zeaxanthin hybrids, with one of the hybrids (HZ9) approaching the same concentration as the yellow cultivar at 28 days.

Zeinoxanthin, which is the immediate precursor of lutein, was generally highest in the yellow cultivar (Figure 2f). Whereas zeinoxanthin concentration did not change with increasing harvest maturity in the yellow cultivar, it gradually increased in the high-zeaxanthin hybrids, with one of the hybrids (HZ9) reaching the same concentration as the yellow cultivar at 28 days.

Effect of harvest maturity on kernel colour

Both harvest maturity and cultivar significantly affected kernel colour. Hue angle for both the yellow cultivar and the high-zeaxanthin hybrids was similar (~89°) at 20 days harvest maturity, but diverged from this point onwards (Figure 3). While all lines significantly decreased in hue angle with increasing harvest maturity, the high-zeaxanthin hybrids declined more rapidly, reaching a hue angle of 82° at 28 days, in comparison to a hue angle of 86° for the yellow cultivar.

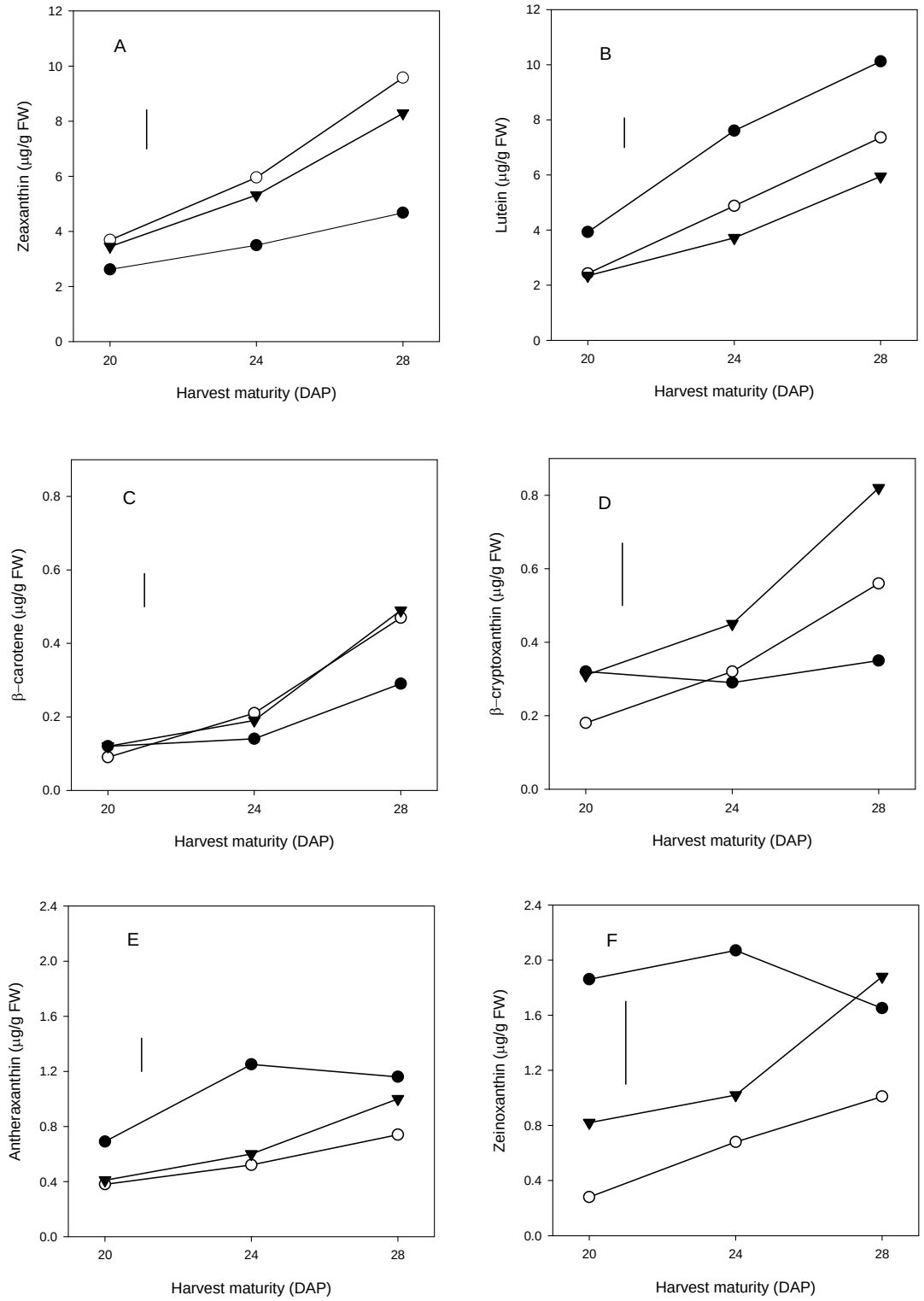

Figure 2. Individual carotenoid concentrations (zeaxanthin, A; lutein, B; β-carotene, C; β-cryptoxanthin, D; antheraxanthin, E; and zeinoxanthin, F) for Hybrix5 (●) and the high-zeaxanthin hybrids (○, ▼) with increasing harvest maturity. Vertical bars indicate LSD(P<0.05).

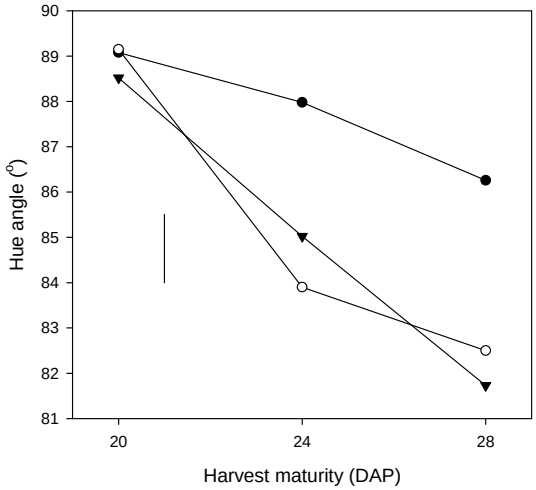

Figure 3. Change in kernel hue angle (frozen) with increasing harvest maturity of Hybrix5 (●) and the high-zeaxanthin hybrids (○, ▼). Vertical bar indicates LSD(P<0.05).

Kernel hue angle (across all harvest maturities) was strongly correlated with zeaxanthin concentration (r^2 = 0.95), and slightly more strongly correlated (r^2 = 0.96) with the sum of the orange-coloured beta-carotenoids (zeaxanthin + β-carotene and β-cryptoxanthin) (Figure 4). In general, as zeaxanthin concentration increased, hue angle decreased, such that kernels became more golden-orange in colour.

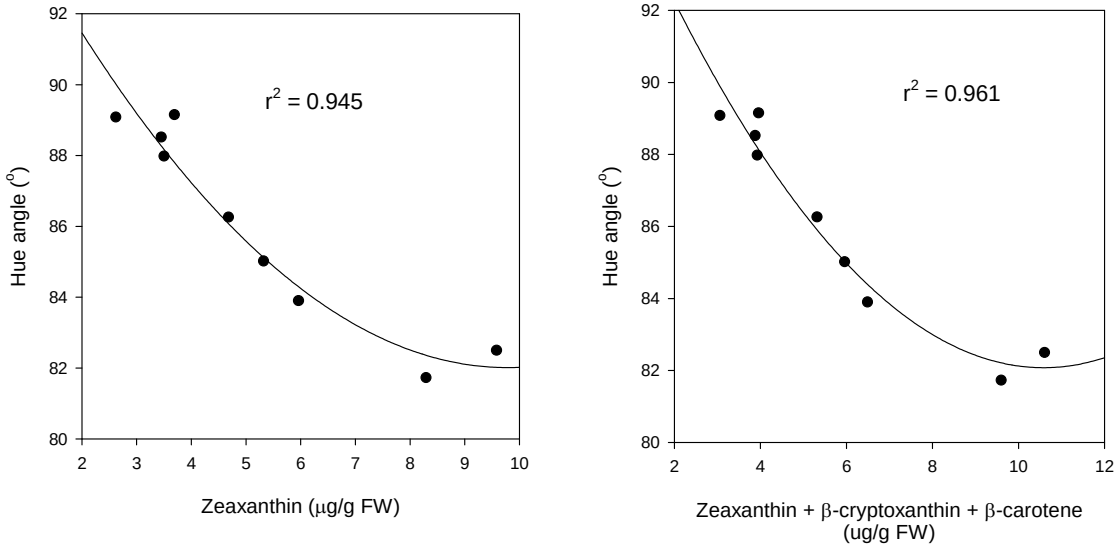

Figure 4. Curvilinear relationship of kernel hue angle to zeaxanthin (left) and the sum of the orange carotenoids, zeaxanthin, β-cryptoxanthin, and β-carotene (right).

Discussion

The early development high-zeaxanthin hybrids used in the current trial had similar total carotenoid levels to that of Hybrix5, but a higher proportion of zeaxanthin relative to lutein. This difference in the carotenoid profile is likely to be due to the high-zeaxanthin hybrids containing a weaker form of the enzyme, lycopene epsilon cyclase, such that carotenoid synthesis is directed towards the beta-arm of the pathway (Harjes et al., 2008).

An increase in total carotenoid concentration was observed equally in all hybrids as harvest maturity was delayed. It is likely therefore, that all three hybrids have similar genetic backgrounds in regards to genes affecting the early stages of synthesis. These genes, such as phytoene synthase and zeta-carotene desaturase, affect total carotenoid production (Wong et al., 2004) prior to the split into either the alpha-arm or beta-arm of the pathway.

Importantly, the current trial indicated that, apart from genetic background, harvest maturity has a significant effect on zeaxanthin concentration. It is therefore of paramount importance that sweet-corn cobs are harvested at a similar maturity, especially within a trial. Although it is normal practise to count days from pollination to compare cobs, the number of days can vary under different seasonal conditions so that it can be difficult to compare zeaxanthin concentrations between trials. We consequently make a judgement of when to harvest based on temperature and a visual inspection of kernel development. This is further confirmed by a measurement of kernel moisture content as a means of judging kernel maturity. Including a control line, such as Hybrix5, is also useful as a means of comparing both within and between trials.

As has been shown earlier when comparing genotypes varying genetically in zeaxanthin concentration, kernel colour becomes an increasing golden-orange colour with delayed harvest maturity, as zeaxanthin and other orange carotenoids increase in concentration at the same time. The relationship between colour and zeaxanthin concentration therefore appears independent of whether this effect is caused by genotype or physiological maturity. However, although it is tempting to delay harvest to increase zeaxanthin concentration, kernels lose their moisture content making them less turgid and 'chewier' and therefore less attractive to consumers. Consequently, it is important to select sweet-corn lines with high zeaxanthin concentration at the optimum eating-stage.

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Freezing induces reversible colour-change in both zeaxanthin-biofortified and yellow sweet-corn

Summary

Zeaxanthin-biofortified sweet-corn is a deeper golden-orange colour than regular yellow sweet-corn due to a greater ratio of orange carotenoids (zeaxanthin, β-cryptoxanthin, and β-carotene) to yellow carotenoids (lutein, zeinoxanthin). Freshly-harvested zeaxanthin-biofortified and yellow corn-cobs were subjected to freezing (-20°C) and assessed for colour-change (hue angle). Freezing kernels at -20°C was observed to significantly reduce hue angle by approximately 4-6°, with decline slightly greater in zeaxanthin-biofortified kernels than in yellow kernels. Frozen kernel hue angle could be predicted by linear correlation with fresh kernel hue angle (r^2 = 0.90). Carotenoid-analysis of zeaxanthin-biofortified and yellow corn confirmed no significant change in carotenoid profile due to freezing, apart from slight changes to antheraxanthin, β-cryptoxanthin and β-carotene. This indicates that colour changes may be due to physical, rather than chemical changes. In order to investigate if cell membrane disruption was the cause of colour change, fresh kernels of zeaxanthin-biofortified and yellow sweet-corn were blended into a slurry, and hue angle measured after freezing to -20°C. Decline in hue angle was similar to that of intact kernels, indicating membrane disruption was unlikely to be responsible. Fresh cobs were subsequently subjected to freezing and subsequent thawing. Thawing to room-temperature resulted in a reversal of hue angle to the original values of fresh kernels. The results indicate that colour change due to freezing may be due to the formation of ice-crystals, possibly interfering with light absorption, as the effect disappeared once the ice-crystals had melted. Rapid freezing with liquid nitrogen had less effect on hue angle than freezing at -20°C, indicating that ice-crystal size may also play a role. The observation of why orange cobs are affected more than yellow cobs remains to be elucidated.

Introduction

One of the side-effects of biofortifying sweet-corn with zeaxanthin is the increase in kernel colour towards the orange part of the spectrum (Fanning et al, 2010). This not only enables consumers to easily differentiate this corn from regular yellow sweet-corn, but also provides a simple means of pre-sorting cobs prior to laboratory carotenoid extraction and analysis, which can be time-consuming and expensive.

During the breeding process in far north Queensland, it was common practise to freeze cobs during the harvest and trans-ship these in a frozen state to the laboratory in south-east Queensland. It was soon observed, however, that frozen cobs appeared a more orange colour than freshly harvested cobs of the same genotype. This seemed to be more apparent with zeaxanthin-biofortified cobs than with regular yellow cobs. The reason for this colour change was unknown, although it was initially suspected that it may have been due to membrane leakage during the freezing process.

The purpose of the following investigation was to quantify the change in colour of a wide range of zeaxanthin-biofortified kernel colours, and to provide a preliminary hypothesis as to the mode of action of freezing on kernel colour change.

Materials and Methods

Plant Materials

Cobs were harvested from the commercial yellow sweet-corn cultivar Hybrix5 and numerous genotypes produced within the zeaxanthin-biofortification breeding program, ranging in hue angles from 72-91°. All cobs were self-pollinated by hand and harvested 25 days after pollination.

Correlation of fresh and frozen kernel colour

Colour (hue angle) was measured with a Minolta Chromameter of the outer surface of intact kernels attached to the cob. Colour measurement was the average of three measurements taken around the central circumference of a cob.

Kernel colour was measured firstly in 70 fresh cobs, and subsequently in the same cobs after freezing at -20°C for 24 hours. The correlation relationship between fresh and frozen hue angle was determined by linear regression using Genstat 8.0.

Membrane integrity and freezing

To investigate whether colour change may be due to a loss of membrane integrity during the process of freezing, six cobs of Hybrix5 and six of a zeaxanthin-biofortified line HZ7 were measured for hue angle and then blended into a fine, uniform slurry using an Ultraturrex blender. The surface of the slurry was measured for hue angle and subsequently frozen at -20°C for 24 hours before being re-measured.

Freezing, thawing, and colour and carotenoid measurement

Six fresh cobs of Hybrix5 and HZ7 were measured for hue angle and 10 kernels removed for subsequent carotenoid analysis using the method of Fanning et al (2010). The same cobs were then frozen at -20°C for 24 hours, measured, and sub-sampled again. The cobs were then thawed at 4 hours at 25°C and re-measured for colour.

Rate of freezing and colour change

Twelve fresh cobs of Hybrix5 and HZ25 were measured for hue angle and one half frozen at -20°C for 24 hours, and the other half frozen by immersion in liquid nitrogen. Once frozen, cobs were re-measured for hue angle. Cobs immersed in liquid nitrogen were wiped with a cloth immediately prior to measurement to remove white frost condensation from the surface of the kernels.

Analysis of variance was performed using Genstat 8.0. Data means were separated using least significance difference ($P<0.05$).

Results

Freezing at -20°C for 24 hours resulted in a decline in hue angle for both yellow and zeaxanthin-biofortified orange sweet-corn. This relationship was linear and reasonably strong, with a coefficient of determination (r^2) of 0.90 (Figure 1). Decline in hue angle was slightly greater for the orange biofortified kernels (6° decline) than for the yellow kernels (4° decline).

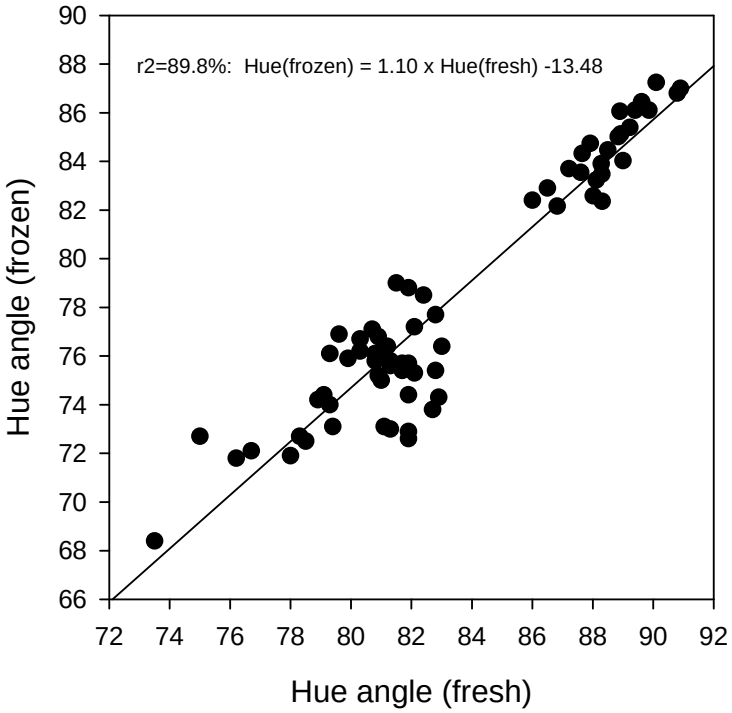

Figure 1. Relationship between hue angle of fresh and frozen sweet-corn kernels following freezing at -20°C.

Blending of kernels into a fine but uniform slurry had a similar general response in hue angle decline to intact kernels upon freezing. Although blending itself caused a significant increase in hue angle for both yellow and orange kernels relative to intact kernels (Table 1), freezing of the blended kernels caused a subsequent drop in hue angle of 6° for yellow kernels and 8° for orange kernels.

Table 1. Hue angle of intact, blended, and frozen blended sweet-corn kernels.

	Hue angle (°)	
Treatment	Hybrix5	HZ7
Intact kernels	91.6a*	85.8a
Blended kernels	95.1b	91.8b
Frozen blended kernels	88.4c	83.2c

*Means within columns followed by the same letter are not significantly different (P<0.05).

Carotenoid concentrations of both yellow and orange kernels were largely unaffected by freezing (Table 2), apart from a decline in antheraxanthin (both varieties) and a slight, but significant, increase in β-cryptoxanthin and β-carotene in Hybrix5. Similar to the previous observation, freezing caused a decline in hue angle of 3° and 5°, for Hybrix5 and HZ7, respectively. In contrast, thawing to room temperature caused a reversal in hue angle, such that hue angles were not significantly different to fresh unfrozen kernels (Table 3).

Table 2. Carotenoid profile of fresh kernels and kernels frozen at -20°C for 24 hours. Yellow carotenoids include lutein, zeinoxanthin and antheraxanthin; orange carotenoids include zeaxanthin, β-cryptoxanthin and β-carotene.

	Carotenoid concentration (µg/g FW)			
	Hybrix5		HZ7	
	Fresh	Frozen	Fresh	Frozen
lutein	4.8a*	4.8a	4.9a	3.7a
zeinoxanthin	3.6a	4.8a	4.1a	3.7a
antheraxanthin	2.1a	1.8b	1.8a	1.4b
(total yellow)	10.6a	11.4a	10.8a	8.8a
zeaxanthin	3.0a	3.4a	10.8a	10.3a
β-cryptoxanthin	0.54a	0.66b	1.67a	1.85a
β-carotene	0.51a	0.72b	1.47a	1.40a
(total orange)	4.1a	4.8b	14.0a	13.6a
total carotenoids	14.7a	16.2a	24.7a	22.4a

*Carotenoid means for fresh and frozen kernels (within each variety) followed by different letters are significantly different (P<0.05).

Table 3. Hue angle of fresh, frozen, and thawed intact kernels.

	Hue angle (°)	
Treatment	Hybrix5	HZ7
Intact kernels	92.0a*	88.7a
Frozen kernels	89.2b	83.4b
Thawed kernels	92.5a	88.0a

*Means within columns followed by the same letter are not significantly different (P<0.05).

Freezing at -20°C for 24 hours had a greater effect on colour change compared to rapidly freezing cobs in liquid nitrogen (Table 4). As before, freezing at -20°C caused a decline in hue angle of 3° and 5°, respectively, for yellow and orange kernels. Freezing in liquid nitrogen, however, resulted in a lesser decline of about 1.5° in both varieties.

Table 4. Hue angle of kernels following freezing at -20°C or with liquid nitrogen (-200°C). Data is shown for individual varieties and pooled together (Hybrix5+HZ25).

	Hue angle (°)		
Treatment	Hybrix5	HZ25	Hybrix5+HZ25
Fresh kernels	90.4a*	86.9a	88.7a
-20°C freezing	86.8b	81.6b	84.1c
Liquid nitrogen freezing	88.8a	85.5a	87.1b

*Means within columns followed by the same letter are not significantly different (P<0.05).

Discussion

The present investigation confirmed that freezing cobs at -20°C over a 24 hour period altered the external colour of kernels by a reduction in hue angle of up to 6°. This effect appeared to be linearly correlated to the initial hue angle of the fresh kernel, such that the frozen hue angle could be subsequently predicted with a high degree of certainty (r^2 = 0.90). The decline in hue angle due to freezing appeared to slightly increase with lower initial hue angle, such that the effect on zeaxanthin-biofortified sweet-corn was greater than that on regular yellow corn. At this stage, we are uncertain what the mechanism for this difference is.

Initially, it was hypothesised that change in the appearance of the frozen kernels may have been due to cell membrane damage during the formation of ice crystals. Blending of kernels into a fine slurry to minimise this potential effect of membrane integrity however, showed that freezing had a similar effect on the slurry as it did on intact kernels. Membrane integrity, therefore was eliminated as a reason for colour change. Interestingly, blending of kernels initially increased hue angle in comparison to intact kernels. It is possible that this may have been due to removing the spatial distribution of orange and yellow carotenoids within the corn kernel.

Most importantly, thawing of frozen material was observed to return the reduced hue angle of both intact kernels and slurries back to that of the fresh unfrozen material. Analysis of the fresh and frozen kernels indicated that there was no significant change in carotenoid concentration during this process, apart from minor changes in antheraxanthin, β-carotene and β-cryptoxanthin. It would appear that colour change was a physical effect related to ice-crystal formation, which was reversible when ice-crystals melted.

Similarly, during an evaluation of different freezing temperatures (-15, -20, -40, -80ºC and liquid nitrogen) on the colour of salmon fillets during freezing, samples were reported to appear paler and less red with decreasing freezing temperature, however following thawing, all samples recovered their original colour. Microscopic examination of the fillets concluded that lower freezing temperature produces smaller ice crystals than higher freezing temperatures, which subsequently led to higher light scattering in frozen salmon fillets (Ottestad et al., 2011).

In sweet-corn, the present results also indicated that the way ice-crystals are formed and potentially their size, could influence hue angle. Crystals formed by freezing rapidly with liquid nitrogen (approximately -200°C) had less effect on hue angle reduction than slower

cooling at -20°C. In the case of sweet-corn, however, hue angle was significantly reduced, rather than tissues appearing paler.

The above results would tend to support the supposition that the physical formation of ice crystals, particularly larger ice crystals, is the likely cause of hue angle reduction in the present study, although the mode of action on hue angle reduction remains unknown.

Conclusions

From the current investigation, it would appear that decline in hue angle due to freezing is associated with the formation of ice crystals, rather than with a disruption in cell membrane integrity. The observation that this effect could be reversed by thawing, that the rate of freezing impacts on the degree of colour change, and the lack of change in carotenoid profile with freezing, lends support to the supposition that the effect on colour is due to a physical rather than chemical effect. The question of why freezing has more impact on colour change of orange zeaxanthin-biofortified kernels rather than yellow kernels remains to be elucidated.

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Effect of cooking and refrigerated storage on kernel colour

Summary
Most consumers cook sweet-corn prior to eating it, and prior to cooking it is not unusual to store cobs in a refrigerator set at approximately 4°C. The current trial investigated the effect of microwave cooking and refrigerated storage on the colour and carotenoid profile on sweet-corn. Microwaving sweet-corn for 4 minutes resulted in a significant decline in all β-arm carotenoids (zeaxanthin, β-cryptoxanthin, β-carotene, antheraxanthin) in the zeaxanthin-biofortified hybrid (HZ), and in all β-arm carotenoids excepting zeaxanthin in Hybrix5. Cooking had no significant effect on α-arm carotenoids (lutein, zeinoxanthin) in either of the hybrids. Greatest decline in both hybrids was for β-carotene, followed by β-cryptoxanthin, antheraxanthin, and finally zeaxanthin. Absolute decline in zeaxanthin concentration in the HZ hybrid was 12.7%, reducing zeaxanthin from 10.6 to 9.2 mg/kg FW. Cooking was observed to increase the intensity (chroma) of kernel colour, with HZ cobs appearing more orange, and Hybrix5 cobs more yellow. Brightness (L-value) however was reduced, while hue angle remained relatively unchanged. Cool storage (4°C) had little significant effect on carotenoid concentration when stored up to 3 weeks. The only significant changes were in the minor carotenoids, antheraxanthin and β-cryptoxanthin. Antheraxanthin was observed to increase 15-20%, while β-cryptoxanthin slowly decreased during storage to about 50% of its original concentration. No significant change in kernel colour was observed during the storage period.

Introduction
Sweet-corn is normally eaten by consumers after it is cooked, either by microwaving, boiling, or baking. This process can potentially lead to colour change, and potentially changes in carotenoid composition. Furthermore, sweet-corn is often stored in a refrigerator (4-6°C) prior to consumption, sometimes up to a month before the product becomes inedible, due to bacterial or fungal diseases, or dehydration, making the product 'chewier' to eat.

The current investigation explored the effect of microwave cooking on both kernel colour and carotenoid profile, as well as changes in carotenoid composition up to 4 weeks storage under domestic refrigerator conditions.

Materials and Methods
Sweet-corn cobs of an early-development high-zeaxanthin hybrid (HZ) and a standard yellow hybrid (Hybrix5) were harvested at optimum maturity (21 DAP) for sweet-corn consumption.

Effect of cooking
Five cobs from each hybrid were cut into 4 segments (4-5cm long) and randomised before dividing 5 segments of each hybrid to a cooked and uncooked (control) treatment. Cooking was conducted using a 1000w microwave oven (Sharp). Sweet-corn segments were placed in a lidded plastic microwave container with a small amount of water in the base. The corn was

separated from the water by a plastic perforated insert. Samples were cooked for 4 minutes on the 'high' setting.

Corn segments of both cooked and uncooked corn were analysed for colour using a Minolta Chromameter (CR200). Kernels were then removed with a sharp knife. All kernels were removed and a subsample used for carotenoid analysis as described previously.

Effect of storage
Cobs of an intermediate-zeaxanthin experimental line were stored in unsealed plastic bags at 4°C for up to 3 weeks. Six cobs were withdrawn weekly (0, 1, 2, 3 weeks) and assessed for carotenoid concentration as described previously.

Results
Effect of cooking
Microwaving sweet-corn for 4 minutes resulted in a significant decline in all β-arm carotenoids (zeaxanthin, β-cryptoxanthin, β-carotene, antheraxanthin) in the HZ hybrid, and in all β-arm carotenoids excepting zeaxanthin in Hybrix5 (β-cryptoxanthin, β-carotene, antheraxanthin) (Table 1). Cooking had no significant effect on α-arm carotenoids (lutein, zeinoxanthin) in either of the hybrids. Greatest decline in individual carotenoid concentration for both hybrids was for β-carotene, followed by β-cryptoxanthin, antheraxanthin, and finally zeaxanthin. Total carotenoid concentration was only significantly affected (10.8%) in the HZ hybrid, specifically due to the large decline (16.4%) in β-carotenoids, which constituted the majority of carotenoids in HZ. Absolute decline in zeaxanthin concentration in the HZ hybrid (10.57 mg/kgFW) was 1.34 mg/kgDW (12.7% decline), reducing the final concentration to 9.23 mg/kgFW.

The main effect of cooking on kernel colour was to increase the intensity of colour (Figure 1). HZ cobs appeared more orange, and Hybrix5 cobs appeared more yellow. This increase in intensity was reflected by an increase in chroma value. At the same time, brightness (L-value) was reduced, while hue angle remained relatively unchanged.

Figure 1. A comparison between cooked (upper four cobettes) and uncooked (lower single cobette) for a yellow and zeaxanthin-biofortified hybrid. Note that this photo is for illustrative purposes only, and the hybrids shown (Garrison and 2-9x23-6) were not used in this trial.

Table 1. Effect of microwave cooking on the carotenoid profile of HZ and Hybrix5.

Hybrid	Carotenoid	Fresh (mg/kg FW)	Cooked (mg/kg FW)	Change (%)
HZ	zeaxanthin	10.57a*	9.23b	-12.7
	β-cryptoxanthin	1.76a	1.34b	-23.9
	β-carotene	1.44a	0.96b	-33.3
	antheraxanthin	1.57a	1.29b	-17.8
	Total β-arm	15.34a	12.82b	-16.4
	lutein	4.30a	4.42a	N.S.
	zeinoxanthin	3.92a	3.77a	N.S.
	Total α-arm	8.21a	8.19	N.S.
	Total	23.55a	21.01b	-10.8
Hybrix5	zeaxanthin	3.20a	3.03a	N.S.
	β-cryptoxanthin	0.60a	0.48b	-20.0
	β-carotene	0.62a	0.45b	-27.4
	antheraxanthin	1.96a	1.72b	-12.2
	Total β-arm	6.38a	5.67b	-11.1
	lutein	4.83a	5.04a	N.S.
	zeinoxanthin	4.20a	3.54a	N.S.
	Total α-arm	9.03a	8.58a	N.S.
	Total	15.41a	14.25a	N.S.

*Means followed by the same letter (within rows) are not significantly different ($p<0.05$).

Table 2. Effect of microwave cooking on the kernel colour of HZ and Hybrix5.

Hybrid	Colour parameter	Fresh	Cooked
HZ	Hue	88.9a*	88.8a
	L	75.5a	64.9b
	Chroma	54.4a	68.2b
Hybrix5	Hue	92.3a	93.6a
	L	75.8a	66.2b
	Chroma	52.1a	68.5b

*Means followed by the same letter (within rows) are not significantly different ($p<0.05$).

Effect of cool storage

Cool storage (4°C) had little significant effect on carotenoid concentration when stored up to 3 weeks (Table 3). The only significant changes were in the minor carotenoids, antheraxanthin and β-cryptoxanthin. Antheraxanthin was observed to increase 15-20% from 1 week storage onwards, while β-cryptoxanthin slowly decreased during storage to about 50% of its original

concentration. No significant change in kernel colour was observed during the course of the trial (data not shown).

Table 3. Effect of cool storage on the carotenoid profile of an intermediate-zeaxanthin experimental line.

Carotenoid	Storage time (weeks)				
(mg/kg FW)	0	1	2	3	LSD(P<0.05)
zeaxanthin	3.89a*	4.21a	4.13a	3.83a	N.S.
β-cryptoxanthin	1.45a	1.28a	0.87b	0.71b	0.41
β-carotene	0.38a	0.46a	0.37a	0.38a	N.S.
antheraxanthin	0.40a	0.49b	0.47b	0.46b	0.04
lutein	4.87a	5.10a	4.60a	4.46a	N.S.
zeinoxanthin	0.21a	0.19a	0.20a	0.19a	N.S.

*Means followed by the same letter (within rows) are not significantly different (p<0.05).

Discussion

Cooking sweet-corn by microwave was found to increase colour intensity of both the high-zeaxanthin and standard yellow hybrid to the same degree, making HZ appear more orange, and Hybrix5 more yellow. Despite the very visual differences, the hue angle remained significantly unchanged in both hybrids. At the same time, brightness (L) declined in both hybrids by a similar degree. Consequently, cooking cobs of either hybrid resulted in a more intense, less bright cob, but essentially of the same colour spectrum as the respective uncooked cob.

This change in colour parameters can possibly be explained by the effect of cooking on intercellular air-spaces and light reflection. In both HZ and Hybrix5 kernels, the outer pericarp surrounding the inner yellow/orange endosperm is essentially devoid of pigment. The kernels appear yellow/orange due to a certain amount of light passing through the pericarp and hitting the endosperm, at which point the yellow/orange part of the spectrum is reflected out again. However, a proportion of the incident light entering the pericarp does not reach the endosperm, but is scattered by the intercellular airspaces and reflected as white light. Consequently, uncooked kernels reflect a mixture of yellow/orange light and white light.

When cooking occurs, the cell membranes of the pericarp become leaky and the intercellular airspaces become flooded with cellular fluid. With less airspaces to scatter the incident light, more light reaches the underlying endosperm, which results in an increase in yellow/orange light. This increase in yellow/orange reflection from the endosperm together with a decrease in white light reflection by the pericarp is likely to account for an increase in chroma (more yellow/orange being reflected), decrease in brightness (less white light being reflected), and the hue angle essentially remaining unchanged.

Microwave cooking appeared to cause a significant decline in B-arm carotenoids, except for zeaxanthin in the Hybrix5 hybrid. Despite zeaxanthin, β-cryptoxanthin and β-carotene being orange carotenoids, and the remaining carotenoids yellow, this did not significantly affect kernel hue angle (Table 2). This may be because the major β-arm carotenoid was zeaxanthin in both HZ and Hybrix5, and was affected the least (or not significantly in Hybrix5). Overall, total β-arm carotenoid decline was 16 and 11%, respectively.

The observation that the β-arm carotenoids were affected adversely by cooking, while the a-arm carotenoids were not, is interesting. Lutein is an isomer of zeaxanthin, and zeinoxanthin is an isomer of β-cryptoxanthin, but this difference appears to be enough to make them more stable with cooking. Increased hydroxylation of the beta-rings also appears to increase stability, in that B-carotene (no hydroxyl groups) was the least stable, followed by b-cryptoxanthin (1 hydroxyl group), and zeaxanthin (2 hydroxyl groups).

Importantly, although microwave cooking did decrease zeaxanthin concentration significantly in the HZ hybrid, absolute decrease in zeaxanthin concentration was only 1.34 mg/kg FW. This however, should be taken into account when estimating available zeaxanthin following cooking, as zeaxanthin in raw kernels would slightly overestimate final zeaxanthin concentration. It is also unknown how increasing/decreasing microwave cooking times may influence zeaxanthin degradation. This, together with the effect of different cooking techniques (e.g. boiling), should be investigated in the future.

It was promising that cool storage (4°C) for up to 3 weeks had no significant effect on either lutein and zeaxanthin (the macular carotenoids), or β-carotene or zeinoxanthin. The reason why antheraxanthin concentration increased is uncertain, as is why β-cryptoxanthin was the only carotenoid to decrease in concentration. Importantly, β-cryptoxanthin is not a macular carotenoid, and therefore its decline has no direct impact on sweet-corn as a dietary source of zeaxanthin and lutein.

Creation of enhanced zeaxanthin lines by combining increased β-arm flux with increased total carotenoid synthesis

Summary

In the breeding program, two lines of zeaxanthin-biofortified sweet-corn have been developed. The enhanced zeaxanthin in one of these lines was due largely to an increase in total overall carotenoid synthesis, while in the other it was due to a shift in carotenoid flux towards the β-arm of the carotenoid synthesis pathway, in which zeaxanthin resides. Combining high total carotenoid production with an increased flux towards the β-arm of the pathway could potentially double zeaxanthin (and other β-carotenoids such as β-carotene) concentration in our high-zeaxanthin germplasm. The current series of crosses demonstrated that zeaxanthin concentration could be increased from approximately 12-14 μg/g FW in the donor lines to 23-25 μg/g FW in the progeny. The aim of our program was to produce sweet-corn with a zeaxanthin concentration of 20 μg/g FW or above, such that consumption of 100 g kernels (small cob of sweet-corn) would supply 20 μg zeaxanthin. The current crosses reached this threshold, and formed the basis for the development of inbred parents to produce high-zeaxanthin experimental hybrids.

Introduction

Yellow sweet-corn kernels are a significant source of zeaxanthin, which is important nutritionally for the amelioration of age-related macular degeneration. Zeaxanthin is formed on the β-arm of the carotenoid synthesis pathway, with zeaxanthin being synthesised by hydrolysis of β-carotene via a β-cryptoxanthin intermediate. Consequently, increasing carotenoid flux towards the β-arm (and away from the α-arm) increases the relative concentration of zeaxanthin, concomitant with a change to a golden-orange colour (Fanning et al, 2010). An increased shift towards the β-arm of the pathway appears to be due to the presence of less-efficient alleles of the gene for the enzyme, lycopene epsilon cyclase (Harjes et al, 2008), which is required to form α-carotene, the first carotenoid of the α-arm pathway.

In addition to shifting synthesis towards the β-arm, concentration of zeaxanthin can also be increased by increasing total carotenoid synthesis in sweet-corn (O'Hare et al., 2010). Although this results in an increase of both α-arm and β-arm carotenoids, similar or greater levels of zeaxanthin can potentially be achieved, in comparison to simply shifting the direction of flux towards the β-arm. The present trial aimed to potentially double the concentration of zeaxanthin in our existing high-zeaxanthin lines by combining high total carotenoid synthesis with an increased percentage of β-arm carotenoids relative to α-arm carotenoids. Each characteristic is present in separate high-zeaxanthin parent lines, but needs to be combined to get a further major increase in zeaxanthin.

Materials and methods

Plant material

Six experimental lines derived from tropical sweet-corn populations, PRO1 and PRO2 were selected for crossing. Three experimental lines (A1, A2, A3) from the PRO1 population, known to have a high β:α ratio and moderate total carotenoid production, and three experimental lines (B8, B9, B10) from PRO2 known to have a low β:α ratio and high total carotenoid production were established by HPLC carotenoid analysis based on the method of Fanning et al (2010). The standard yellow-coloured tropical hybrid, Hybrix5, with parents originating from the same PRO1 and PRO2 populations was selected as a control.

Crossing

Crossing between A1, A2 and A3 with B8, B9 and B10 was performed by hand, using A1, A2 and A3 as the pollen donors, and B8, B9 and B10 as the female parents. The resulting seed (uniformly heterozygous) was collected, re-sown and self-pollinated, along with Hybrix5 as a control. The distal half of the cob was collected at sweet-corn eating stage (24 DAP) and stored at -20°C immediately before analysis. Kernels were analysed for colour (hue angle) and carotenoid profile (HPLC). Analysis was conducted for both strongly-orange and yellow-orange kernels, as well as for composites of randomly removed kernels (i.e. an average). The lower half of the cob was subsequently allowed to mature on the plant and seed collected. Seed were sorted by colour and strongly-orange seed separated for re-sowing.

Orange seed were sown and plants self-pollinated. The distal half of the cob was again collected at sweet-corn eating stage (24 DAP) and stored at 20°C. Cobs were analysed for uniformity (mottling), colour (hue angle) and carotenoid profile (HPLC).

Uniformity, colour and carotenoid analysis

Cob colour uniformity was analysed subjectively according to variation of kernel colour within a cob at sweet-corn-eating stage. Cobs were scored as either having nil (uniform colour), slight, or moderate mottling. Kernel colour was assessed by measuring the hue angle of intact attached kernels randomly three times across the cob surface using a Minolta Chromameter (CR-200, D65 light source, 6 mm aperture). Carotenoid analysis of kernels was conducted by HPLC according to the method of Fanning et al (2010). Data was transformed into total carotenoid production, β:α arm ratio, zeaxanthin, and β-carotene concentrations.

Results

Sweet-corn lines with low hue angle (<82°) were selected from the PRO1 and PRO2 tropical sweet-corn background populations. From the PRO1 background, three lines (A1, A2, A3) were identified as having a high proportion of beta-arm carotenoids (87-89% of total) and a low to moderate total carotenoid concentration (17-23 μg/g FW) (Table 1). From the PRO2 background, three lines (B8, B9, B10) were identified as having a low proportion of beta-arm carotenoids (60-63% of total) and a high total carotenoid concentration (26-28 μg/g FW) (Table 1). All lines had similar zeaxanthin and β-carotene concentrations within the ranges of 11-14 μg/g FW and 1.6-2.0 μg/g FW, respectively. By contrast, kernels of the standard uniformly-yellow tropical hybrid Hybrix5 had a hue angle of 87° and a lower proportion of

beta-arm carotenoids (38%) than either the 'A' or 'B' lines (Table 1). Total carotenoid concentration of Hybrix5 was 16.9 µg/g FW, which was similar to the lowest concentration of the 'B' lines. Zeaxanthin and β-carotene concentrations of Hybrix5 were 3.6 and 0.6 µg/g FW, respectively.

Table 1. Kernel colour and carotenoid profile characteristics of initial parental lines (A and B) and a yellow control (Hybrix5).

Parental line	Kernel colour	Hue angle (°)	Zeaxanthin (µg/g FW)	β-carotene (µg/g FW)	Total carotenoids (µg/g FW)	β-arm carotenoids (%)
A1	orange	78.6	14.3	2.0	23.3	87
A2	orange	79.0	12.8	2.0	20.1	87
A3	orange	80.7	10.9	1.8	16.5	89
B8	orange	78.5	12.8	2.0	28.3	63
B9	orange	79.4	12.7	1.8	28.3	60
B10	orange	81.4	12.4	1.6	26.0	63
Hybrix5	yellow	87.0	3.6	0.6	16.9	38

As would be expected, crossing the A-lines with the B-lines resulted in uniformly coloured kernels (data not shown). This hybrid seed was subsequently planted, producing approximately uniformly sized plants which were self-pollinated. The resulting cobs, both at fresh-eating stage and at kernel maturity were mottled in colour, with kernels ranging from yellow to orange. From a random sampling of kernels, zeaxanthin and β-carotene concentrations were approximately 10 µg/g FW and 1.2-1.5 µg/g FW, respectively (Table 2), generally lower than either of the parental lines (Table 1).

When the kernels from each cob were separated into yellow and orange kernels and analysed separately, the orange and yellow kernels had higher (81%) and lower (47%) proportion of beta-arm carotenoid concentrations, relative to the random sampling above (Table 2). Similarly, the concentrations of zeaxanthin and beta-carotene were also higher in orange kernels (14-17 and 2.3-3.2 µg/g FW, respectively) compared to yellow kernels (8-9 µg/g FW and 0.9-1.2 µg/g FW, respectively). Total carotenoid concentration was variable however, ranging from 20 to 29 µg/g FW (Table 2), similar to the range observed across the original 'A' and 'B' parent lines.

Table 2. Colour and carotenoid profile characteristics of differently-coloured segregating F2 kernels. All kernels were taken from the same cob. 'Average' values were taken from a random selection of kernels within each cob.

Original parental cross	Kernel colour	Hue angle (°)	Zeaxanthin (µg/g FW)	β-carotene (µg/g FW)	Total carotenoids (µg/g FW)	β-arm carotenoids (%)
A2 x B10	average	82.4	9.9	1.2	20.5	60
	orange	78.6	16.7	3.2	28.7	81
	yellow	87.0	7.8	0.9	20.2	47
A3 x B8	average	83.2	9.6	1.5	22.8	56
	orange	79.6	13.8	2.3	24.1	81
	yellow	87.0	8.9	1.2	24.9	47
Hybrix5	yellow	87.3	3.0	0.6	18.5	39

Orange seed collected from a selection of the A x B crosses above was selected by eye and subsequently sown and self-pollinated. This resulted in a large number of progeny ranging in hue angle from 68.4 to 83.8°. Cobs with hue angles greater than 79° were discarded, as they were unlikely to yield higher zeaxanthin and beta-carotene concentrations than their 'A' or 'B' parents based on previous correlation studies between beta-carotenoid concentration and hue angle (Fanning et al, 2010).

Of the cobs selected with hue angles less than 79°, 39 out of 47 had a proportion of β-arm carotenoids making up more than 87% of total carotenoids, similar to that of the original 'A' parents (Table 1). In contrast to this, only 22 lines out of 47 had a total carotenoid concentration above 28 µg/g FW, the concentration of the original 'B' parents.

Combining high total carotenoid synthesis with a high β-carotenoid percentage resulted in an enhanced production of both zeaxanthin and β-carotene (Figure 1). In several lines, zeaxanthin concentration was approximately doubled, from 12-14 µg/g FW in the parental lines to 23-25 µg/g FW in the progeny (Table 3). Similarly, β-carotene was doubled from 1.6-2.0 µg/g FW in the parents to 3.5-3.9 µg/g FW in the progeny. In comparison to the standard commercial hybrid Hybrix5 with zeaxanthin and beta-carotene concentrations of 3.6 and 0.6 µg/g FW, this was equivalent to an increase of 700% and 600%, respectively, over the standard yellow hybrid.

Table 3. Typical kernel colour and carotenoid profile characteristics of F3 progeny derived from plants grown from orange F2 kernels.

Original parental cross	Kernel colour	Hue angle (°)	Zeaxanthin (µg/g FW)	β-carotene (µg/g FW)	Total carotenoids (µg/g FW)	β-arm carotenoids (%)
A1 x B8	orange	72.9	23.8	2.9	34.0	93
A2 x B8	orange	76.8	23.9	2.7	34.5	90
A1 x B9	orange	74.4	24.9	2.5	33.2	94
A2 x B9	orange	72.1	25.1	3.9	36.5	91
Hybrix5	yellow	86.9	3.6	0.6	16.9	37

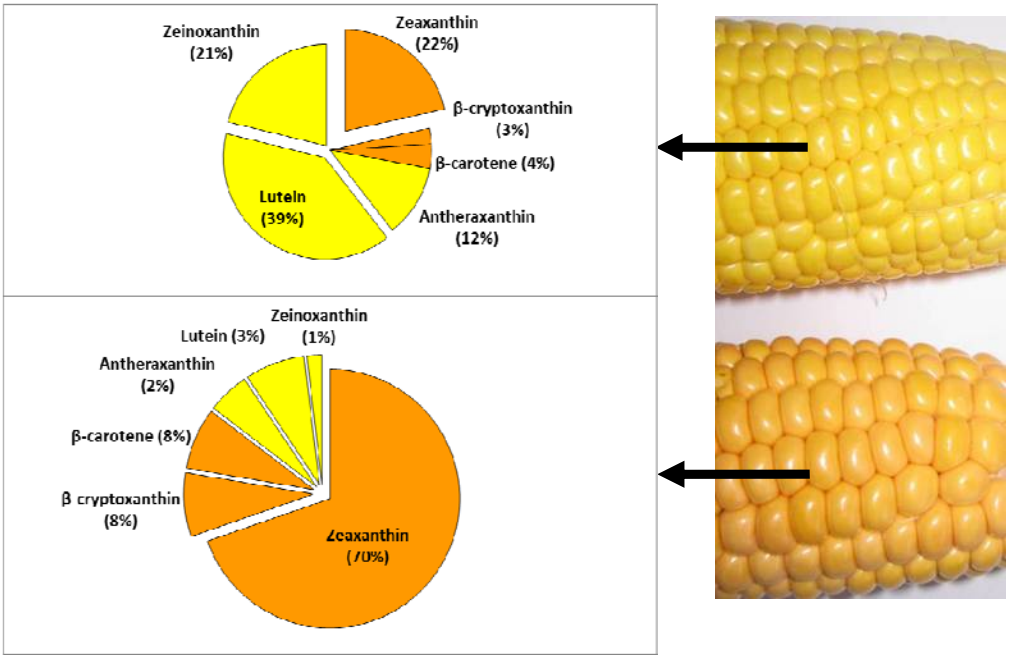

Figure 1. Colour change in high-zeaxanthin sweet-corn was associated with an increase in the proportion of zeaxanthin and other orange carotenoids relative to yellow carotenoids such as lutein. Zeaxanthin percentage was increased from 22% in Hybrix5 (yellow cob, top) to close to 70% in high-zeaxanthin cobs (orange cob, bottom).

Discussion

The present investigation demonstrated that the concentration of zeaxanthin and β-carotene can be significantly increased by combining high total carotenoid synthesis with a shift in carotenoid flux towards the β-arm of the pathway. This resulted in an approximate doubling of zeaxanthin and β-carotene in the parent lines (12-14 µg/g FW and 1.6-2.0 µg/g FW, respectively) to that in the progeny (23-25 µg/g FW and 3.5-3.9 µg/g FW, respectively) (Figure 2). To the best of our knowledge, the present zeaxanthin concentration of 25 µg/g FW and β-carotene concentration of 3.9 µg/g FW are the highest concentrations for these carotenoids to date for sweet-corn in the scientific literature.

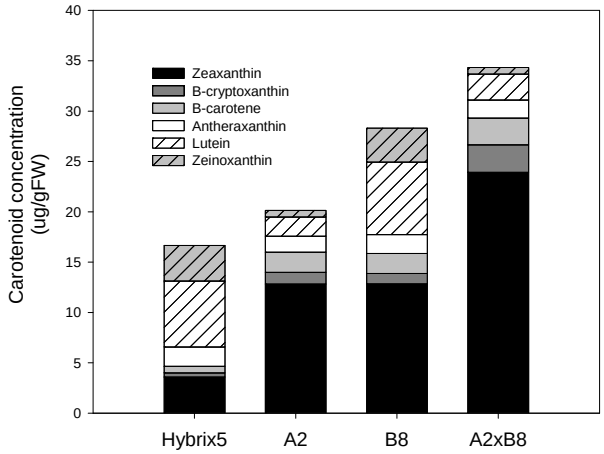

Figure 2. Comparison of carotenoid profiles in Hybrix5 (a commercial yellow hybrid), A2 (high ratio of β-carotenoids), B8 (high total carotenoids) and, A2xB8 (combination of high β-ratio and high total carotenoids). Combining these factors led to a further doubling of zeaxanthin concentration.

Variation in total carotenoid synthesis in *Zea mays* has been attributed to differences in the activity of phytoene synthase and zeta-carotene desaturase (Wong et al., 2004), both enzymes acting early in the carotenoid synthesis pathway. Subsequent to this, the pathway splits into the α-arm and β-arm, the latter of which produces β-carotene and zeaxanthin. It has previously been reported that the amount of carotenoid flux going towards the β-arm of the pathway is controlled by the activity of the enzyme, lycopene epsilon cyclase (Harjes et al., 2008).

The concentration of zeaxanthin and β-carotene in the current trial were reported on fresh weight samples, as this is how sweet-corn is consumed, in contrast to field corn which is allowed to mature and dry on the cob prior to harvest and milling. Although a comparison of carotenoid concentration can be made between sweet-corn and field-corn on a dry weight basis, the comparison is not truly valid, as the sweet-corn kernel accumulates significantly less starch than the latter, hence further concentrating carotenoid concentrations well above that

of biofortified field-corn. For instance, based on a dry matter content of 25%, a concentration of 23-25 µg/g FW zeaxanthin in fresh sweet-corn equates to approximately 92-100 µg/g DW zeaxanthin in fresh kernels, well above that of biofortified field-corn kernels. Similarly, 4-6 µg/g FW β-carotene equates to 16-24 µg/g DW on a dry weight basis, above the target (15 µg/g DW) of the HarvestPlus program aimed at biofortifying field-corn with β-carotene. The highest β-carotene concentration we have achieved in sweet-corn at fresh eating stage is 10.3 µg/g FW, still below the HarvestPlus target.

The current trial utilized kernel colour to identify genotypes likely to be high in β-arm carotenoids. Previously, Fanning et al (2010) observed hue angle to be moderately correlated with the sum of the β-carotenoids, β-carotene, β-cryptoxanthin and zeaxanthin, all of which have a similar orange-yellow colour (Melendez-Martinez et al., 2007). Following the cross between the high total carotenoid parent and the high β:α ratio parent, the segregating kernels on the F2 generation were able to be selected for those with the deepest orange colour (lowest hue angle). Based on this correlation, these were considered likeliest to have the highest concentration of beta-carotenoids. Subsequent cobs produced from these seed were scanned, and those with hue angles above 83 discarded, while those with hue angles below 83 analysed by HPLC. Although hue angle can identify putative genotypes high the beta-carotenoids, it is unable to distinguish between β-carotene, zeaxanthin and β-cryptoxanthin, and consequently must be measured directly, such as by HPLC. In this way, hue angle determination is useful to rapidly screen out the majority of lines with low β-carotenoid concentration, prior to the more laborious analysis required by HPLC.

In conclusion, the resulting progeny of the F2 segregants produced total carotenoid concentrations and β-arm carotenoid percentages similar or slightly better than that of the parents they were derived from. These high-zeaxanthin lines provided the basis for the development of high-zeaxanthin parental inbreds, and subsequently high-zeaxanthin hybrids.

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Development and assessment of high-zeaxanthin inbred parents and hybrids

Development of high-zeaxanthin inbred parents

From the F2 progeny of crosses B8xA1, B8xA2, B9xA1, B9xA2, a total of 24 lines were selected based on colour (hue angle <77°) and uniformity (unmottled) and analysed for carotenoids (Table 1). The most orange seed from these lines was subsequently sown, and the progeny from these lines re-analysed for zeaxanthin (Table 2).

Table 1. Initial selection of high-zeaxanthin lines from AxB F2 progeny.

Original cross	Line	Hue(fresh)	Hue(frozen)	Zeaxanthin (mg/kg FW)	β-arm (%)
Hybrix5		90.9	86.9	3.6	39
B8xA1	1	81.9	72.9	23.8	93
	2	79.1	73.1	13.5	91
	3	81.3	73.0	20.8	93
B8xA2	4	81.3	75.8	19.8	91
	5	80.9	76.8	23.9	90
	6	78.9	74.2	18.4	93
	7	82.8	75.4	20.6	90
B9xA1	8	76.2	71.8	18.4	91
	9	78.0	71.9	16.0	90
	10	81.9	74.4	24.9	94
	11	80.8	75.8	22.2	92
	12	80.9	75.2	21.9	90
B9xA2	13	78.3	72.7	20.2	93
	14	79.9	75.9	21.1	94
	15	78.5	72.5	20.7	90
	16	81.9	78.8	19.7	93
	17	81.1	73.1	19.9	90
	18	80.9	75.9	18.2	89
	19	79.3	74.0	18.3	90
	20	79.3	76.1	19.9	86
	21	81.7	75.6	21.7	91
	22	73.5	68.4	21.0	90
	23	76.7	72.1	25.1	91
	24	82.1	75.3	16.3	70

Table 2. High-zeaxanthin selections made from Lines 1 to 24.

Original cross	Line	Hue(fresh)	Zeaxanthin (mg/kg FW)	%Beta-arm	Inbred cob length (cm)
Hybrix5		90.9	2.9	31	-
B8xA1	1-1	86.2	-	-	12.1
	2-9	78.7	14.4	85	14.7
B8xA2	5-8	81.3	-	-	9.0
	6-1	82.7	15.9	89	11.6
B9xA1	8-6	80.1	-	-	9.8
	10-3	84.5	-	-	11.6
	10-6	83.0	16.5	81	12.7
	11-7	84.1	14.2	80	13.7
B9xA2	13-2	76.9	12.8	80	9.1
	14-6	83.3	15.5	85	11.2
	15-2	83.6	-	-	9.4
	15-6	83.0	-	-	10.4
	19-5	88.5	-	-	8.7
	21-8	83.7	-	-	9.9
	23-1	82.0	14.7	79	10.9
	23-6	80.1	17.4	80	10.0
	23-7	75.9	14.9	71	9.3

The above lines were subsequently inbred by self-pollination for use as potential test-parent for experimental hybrids.

Development of experimental hybrids

Inbred lines from the four crosses above were combined in factorial combination to produce a total of 92 combinations of test hybrid seed at Gatton. Hybrid seed was subsequently harvested, planted and hybrid cobs assessed at Bowen Research Station.

Bowen trial: (April-July 2013)

Hybrids were grown in Bowen and selected (in bold) for further evaluation at Gatton based on the following criteria:

Criteria 1: >15 mg/kg FW zeaxanthin (7 hybrids)
Criteria 2: hue <84 & >10 mg/kg FW zeaxanthin (15 hybrids <84) (7 hybrids <82)
Criteria 3: good agronomy & >10 mg/kg FW zeaxanthin (7 hybrids)

Table 3. Experimental high zeaxanthin hybrid zeaxanthin concentrations and kernel colour (winter harvest). Of these, 36 Hybrids were chosen for further evaluation (in bold). Zeaxanthin concentrations: dark-orange = <15 mg/kg FW; mid-orange = 10-15 mg/kg FW; yellow-orange = <10 mg/kg. Hue angle: dark-orange = <82°; mid-orange = 82-84°; yellow = >84°.

Hybrid	Zeaxanthin (mg/kg FW)	Hue (fresh)	Mottling
Garrison (control)	3.3	92.9	
Goldensweet-improved (control)	3.2	90.2	
Hybrix5 (control)	2.7	90.2	
10.3 x 14.6	**18.8**	**83.5**	
1.1 x 23.7	**16.6**	**80.7**	
11.7 x 2.9	**16.3**	**83.7**	
6.1 x 13.2	**15.6**	**83.6**	
10.3 x 15.6	**15.5**	**83.1**	
1.1 x 19.5	**15.5**	**84.8**	
10.6 x 5.8 (no seed)	15.2	82.5	
10.6 x 10.3	**15.0**	**84.5**	
8.6 x 15.6	14.8	82.5	mottled
10.3 x 13.2	**14.4**	**84.1**	
6.1 x 1.1	**14.4**	**83.5**	
2.9 x 23.6	**14.3**	**81.2**	
10.3 x 1.1	**14.3**	**83**	
10.3 x 6.1	**13.9**	**85.8**	
8.6 x 2.9	13.9	85.7	
10.3 x 23.1	**13.9**	**84**	
1.1 x 13.2	**13.7**	**81.8**	
6.1 x 15.2	**13.6**	**83.4**	
11.7 x 1.1	**13.6**	**86.3**	
10.6 x 6.1	13.5	83.5	
10.3 x 2.9	**13.5**	**83.3**	
8.6 x 19.5	**13.2**	**84.5**	
10.3 x 15.2	**13.1**	**85.8**	
11.7 x 14.6	**12.9**	**83.7**	
8.6 x 14.6	**12.8**	**82.9**	
6.1 x 23.1	**12.5**	**85.1**	
13-1/HZSC232	**12.3**	**84.7**	
8.6 x 1.1	**12.3**	**84.3**	
10.3 x 23.7	**12.3**	**83.2**	
8.6 x 6.1	12.1	85.2	mottled
2.9 x 23.7	**12.1**	**78.8**	
6.1 x 15.6 (no seed)	11.8	79.3	
10.6 x 1.1	11.8	83.2	

11.7 x 23.7	**11.6**	**83.7**	
8.6 x 15.2	11.6	83.9	
1.1x 21.8	11.6	84.8	
6.1 x 14.6	**11.5**	**85**	
1.1 x 15.6	**11.5**	**83.4**	
8.6 x 13.2	**11.4**	**80.9**	
8.6 x 21.8	**11.4**	**83.8**	
10.6 x 15.6	11.4	83.5	
5.8 x 23.1	**11.4**	**81.2**	
10.3 x 21.8	11.3	86.1	
6.1 x 2.9	11.3	86.1	
10.3 x 19.5	11.2	85	mottled
6.1 x 23.6	**11.2**	**84.4**	
10.3 x 23.6	11.1	85.9	
2.9 x 23.1	**11.1**	**79.4**	
8.6 x 23.6	**11.0**	**82**	
5.8 x 1.1	**10.9**	**84.1**	
13-1/HZSC188	**10.9**	**86.4**	
1.1 x 23.6	10.7	82.2	mottled
11.7 x 21.8	10.7	83.8	mottled
10.3 x 5.8	**10.7**	**84.6**	
1.1 x 14.6	**10.6**	**84.4**	
2.9 x 14.6	**10.5**	**83.1**	
11.7 x 15.6	10.4	85.9	
5.8 x 23.6	**10.4**	**84.3**	
13-1/HZSC182	**10.4**	**84.6**	
11.7 x 13.2	**10.3**	**83.9**	
13-1/HZSC193	10.2	86.3	
11.7 x 23.6	10.1	84.5	
11.7 x 19.5	10.1	86.4	
2.9 x 21.8	**10.1**	**82.6**	
13-1/HZSC183	**10.1**	**85.9**	
10.6 x 15.2	**10.1**	**83.3**	
13-1/HZSC192	**10.0**	**85.5**	mottled
2.9 x 19.5	9.9	85.2	
13-1/HZSC200	**9.8**	**83.6**	
2.9 x 15.6	9.8	84.1	
13.2 x 23.7	**9.8**	**77.7**	
11.7 x 6.1	9.7	86.4	
5.8 x 15.6	9.1	83.6	mottled
5.8 x 23-7	**8.5**	**81.6**	
5.8 x 2.9	**8.4**	**82**	
11.7 x 5.8	8.2	86.1	

Gatton trial (September-December 2013):
From the hybrids selected from the Bowen trial, the 8 best based on cob appearance were selected. In general, the high-zeaxanthin lines were sweeter (TSS) or as sweet as the yellow control hybrids (Table 4). Cobs from this trial were evaluated by industry, and the lines 10-3x14-6 and 11-9x2-9 selected as preferred hybrids. Unfortunately, zeaxanthin concentration was not analysed due to a cool-room failure.

Table 4. Cob characteristics of selected high-zeaxanthin experimental hybrids (summer harvest). All lines, including controls, were hand-pollinated.

	Av. Length (cm)	Av. Width (mm)	Av. Kernel (cm)	Av. fill (%)
Garrison	18.4	47.8	18.4	100.0
8-6x1-1	14.5	47.9	10.6	72.8
2-9x11-7	20.1	44.7	16.4	81.5
11-7x2-9	20.3	45.7	16.2	80.0
2-9x23-6	17.6	44.0	11.8	67.2
10-3x14-6	16.4	43.5	13.4	81.8
2-9x23-7	18.4	44.9	13.0	71.0
2-9x23-1	19.8	44.2	14.1	71.3
1-1x23-7	16.6	46.6	10.9	65.6
Hybrix5	19.7	46.8	18.8	95.4

Gatton trial (February-May 2014):
This later trial again produced high zeaxanthin concentrations for the selected hybrids, although there was some variability with the Bowen trial the previous year. For instance, the hybrid '8-6x1-1' produced a marginally higher zeaxanthin concentration this season, but was only about two-thirds this value in the Bowen trial (Tables 3, 5). In contrast, the hybrid '10-3x14-6' scored consistently highly in both the Gatton and Bowen trials.

The hybrid '11-7x2-9' produced the longest cobs, although tip-fill of the cob was not complete (80%) (Figure 1). Cut cobs are shown in Figure 2. Colour (hue) varied from yellow (90-92°), 'upper-gold' (82-84°), and 'lower-gold' (79-81°).

Interestingly, the sweetness levels (TSS) were all reduced compared to the Gatton summer harvest. This may be a seasonal effect, or more likely because the cobs were measured for TSS after 2 weeks storage at 4°C in the present trial, but immediately after harvest for the summer harvest. This highlights the effect of postharvest storage on kernel sweetness, but not on zeaxanthin content (see chapter on cooking and refrigerated storage).

Table 5. Cob characteristics of selected high-zeaxanthin experimental hybrids (autumn harvest).

Line	Zeaxanthin (mg/kg FW)	Hue (fresh)	TSS (%)	Cob length (cm)	Tip-fill (%)	Cob diam. (mm)
Hybrix5	2.2	90.4	12.3	19.7	95.4	46.8
Garrison	5.0	92.2	12.4	18.4	100.0	47.8
'lower-gold'						
2.9x23-7	15.0	78.8	11.9	18.4	71.0	44.9
2-9x23-6	14.9	79.3	11.3	17.6	67.2	44.0
1-1x23-7	18.5	80.1	12.5	16.6	65.6	46.6
2-9x23-1	15.5	80.5	13.3	14.1	71.3	44.2
'upper-gold'						
10-3x14-6	19.6	83.8	12.5	16.4	81.8	43.5
11-7x2-9	17.0	83.2	11.7	20.3	80.0	45.7
2-9x11-7	18.2	82.5	10.6	20.1	81.5	44.7
8-6x1-1	19.8	83.1	11.1	14.5	72.8	47.9

Figure 1. Composite image of whole uncooked cobs of controls (Garrison, Hybrix5) and selected high-zeaxanthin hybrids. Note that the image of 10-3x14-6 is sub-standard due to a lack of uncut cobs when this photograph was taken.

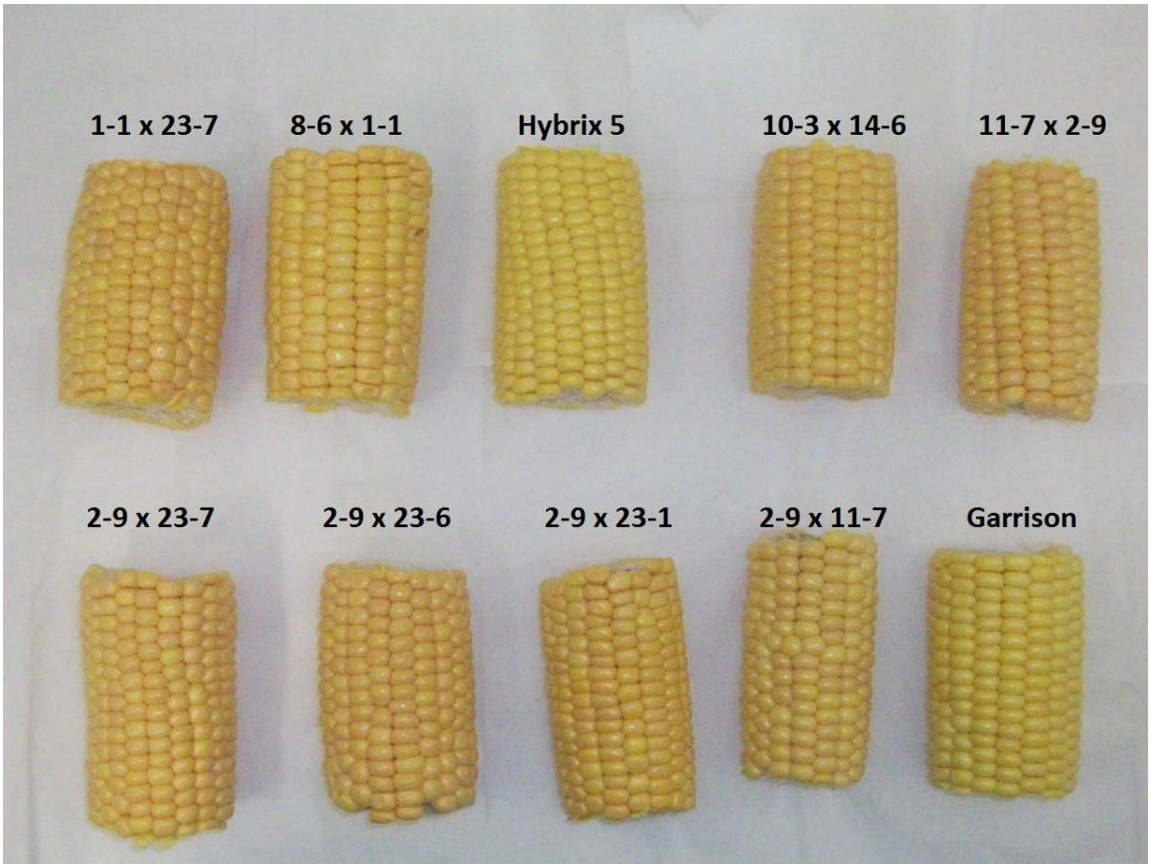


Figure 2. Cut cob kernel detail of controls (Garrison, Hybrix5) and selected high-zeaxanthin hybrids.

Gatton trial (October-December 2014):
This trial was confined to 11-7x2-9, 10-3x14-6, 2-9x23-6, and 23-7x1-1, and Hybrix5 (control). The zeaxanthin data showed that zeaxanthin concentration was considerably higher in summer-harvested cobs (Table 6) than in autumn-harvested (Table 5) or winter-harvested cobs (Table 3). Zeaxanthin concentrations reached 30 mg/g FW and 27.4 mg/g FW for 10-3x14-6 and 11-7x2-9, respectively. This equated to approximately a 50% increase relative to the autumn and winter harvest.

These values would indicate a seasonal effect on zeaxanthin accumulation, with higher temperatures encouraging higher zeaxanthin synthesis. Kernel colour was also affected, such that the cobs were 3-4° lower in hue angle.

Table 6. Cob characteristics of selected high-zeaxanthin experimental hybrids (summer harvest).

Line	Zeaxanthin (mg/kg FW)	Hue (°) (fresh)	TSS (%)	Moisture (%)
Hybrix5	4.1	86.9	12.7	75.8
10-3x14-6	30.7	78.8	13.2	74.8
11-7x2-9	27.4	80.1	11.7	n.a.
2-9x23-6	22.4	80.4	12.8	74.7
23-7x1-1	23.1	78.4	11.5	75.6

Consumer assessment of selected high-zeaxanthin hybrids

Summary
An evaluation of four experimental hybrids (10-3x14-6, 11-7x2-9, 2-9x23-6, 8-6x1-1) and a commercial control (Garrison) were evaluated for both overall raw and cooked appearance, as well as mottling and overall flavour. High-zeaxanthin selections were based on zeaxanthin concentration, colour, cob-length, and flavour variation. Twenty of the 39 panellists taking part were informed of the health benefits of high-zeaxanthin sweet-corn, and 19 remained uninformed. The four experimental hybrids included 10-3x14-6 (highest in zeaxanthin), 11-7x2-9 (high in zeaxanthin, and long cob good for processing in pre-cut packs), 2-9x23-6 (a deeper-orange coloured hybrid), and 8-6x1-1 (a different tasting hybrid).

Results indicated that overall, consumers preferred hybrid 10-3x14-6, regardless of whether or not they had been informed of the associated health benefits. 10-3x14-6 was the hybrid with highest zeaxanthin concentration. Flavour preference varied dependant on whether or not the panel was informed, with informed panellists showing greatest preference for 2-9x23-6, in comparison to the uninformed panel who scored this sample as the least preferred. Flavour preferences tended to vary only slightly however, with none of the hybrids scoring statistically significantly lower than the commercial cultivar, Garrison. From a visual point of view, informed consumers showed greater tolerance toward the deeper-coloured hybrid (2-9x23-6), scoring it within parity of Garrison. Uninformed consumers, however, showed dislike for this deeper colour, in both cooked and raw states.

Introduction
The objective of this work was to provide an overview of consumer acceptance from both a visual and eating quality perspective. Earlier in the project, consumers were assessed with colour-altered images due to the absence of high-zeaxanthin corn towards the beginning of the breeding program. The development of high-zeaxanthin experimental hybrids now allows for testing of actual samples. The current trial evaluated consumer acceptability of four high-zeaxanthin hybrids and one commercially-available sweet-corn variety (Garrison), both in their raw and cooked states. The trial also aimed to gain further insight into consumer purchase intent for the five sweet-corn samples evaluated, identify the specific consumer likes and dislikes of the five sweet-corn samples evaluated, and to identify whether informing consumers of the health benefits associated with the zeaxanthin-biofortified hybrids would influences overall acceptability.

Method
Sample selection and preparation methodology
To avoid consumer fatigue, this trial was limited to five sweet-corn lines. Lines were chosen based on extremes of elevated zeaxanthin concentration (10-3x14-6), cob-length (11-7x2-9), deeper kernel colour (2-9x23-6), and different flavour (8-6x1-1). Garrison was chosen as the control.

Cobs were harvested over as short a time-period as possible (12 days), such that lines could be assessed side by side. Harvest times were as follows:

Sample ID	Harvest date(s)
10-3x14-6	12th-17th May 2014
11-7x2-9	9th-14th May 2014
2-9x23-6	9th-14th May 2014
8-6x1-1	5th May 2014
Garrison	5th May 2014

Sweet-corn cobs (for all five samples) were prepared for consumer evaluation on the morning of the 19th May 2014. Cobs were cut into cobettes, measuring approximately 25 mm in length, and placed into zip-lock bags. Cobettes were held in a cold room at 4°C until they were required for assessment.

On the day of assessment (approximately 30 minutes prior to assessment), cobettes were removed from the cold room and placed onto their individual aluminium foil trays at room temperature (24°C) (see image below). Consumer assessments took place on the following dates:

Date and time	Panel type
19th May 2014 1300hrs	n=11 informed consumers
19th May 2014 1700hrs	n=9 informed consumers
20th May 2014 1000hrs	n=9 informed consumers
20th May 2014 1300hrs	n=10 uninformed consumers

Cooking was conducted in a Unox LineMiss™ steamer oven (Model XF135 Padova, Italy). All samples were cooked for 15 minutes at 95°C and 40% RH. Samples were prepared immediately prior to commencement of the evaluation sessions.

Participants

Consumer evaluation was carried out on site at the Coopers Plains facility, Brisbane. There were a total of 39 participants (20 belonging to the informed panel and 19 to the uninformed panel) who were recruited through a specialist recruitment agency (I-View) based on the following specific criteria:

- Regular (weekly-fortnightly) consumers of fresh sweet-corn (not tinned or frozen)
- Had no known food allergies
- Non smokers
- Aged between 18-75 years on the day of testing
- A cross section of age and gender was taken from all successful applicants

Table 1. Summary of participant demographics and eating habits.

	Informed	Uninformed
Gender	30% Male 70% Female	57% Male 42% Female
Age	10% 24-34 years 15% 35-44 years 10% 45-54 years 30% 55-64 years	16% 24-34 years 5% 35-44 years 32% 45-54 years 21% 55-64 years 26% 65-75 years
Sweet-corn purchase	35% once per week 50% once per fortnight 15% once per month	11% more than once per week 47% once per week 26% once per fortnight 16% once per month
	45% within husks 55% without husks	58% within husks 42% without husks
Sweet-corn consumption	20% more than once per week 45% once per week 25% once per fortnight 10% once per month	32% more than once per week 42% once per week 16% once per fortnight 10% once per month
Preparation techniques	45% microwave 40% boil 10% BBQ 5% raw	32% microwave 42% boil 26% BBQ

Consumer testing

Consumers were separated into two groups – informed and uninformed. The informed panel received the following statement prior to sample evaluations:

"Zeaxanthin is a compound found in sweet-corn which protects against macular degeneration (an age-related blindness). One in seven Australian people over the age of 50 are affected by macular degeneration and the incidence increases with age. Zeaxanthin naturally contributes to the strength of 'orange/gold' colour of a kernel. The greater the amount of zeaxanthin the more orange/gold a kernel appears."

Those consumers in the uninformed group did not receive the above statement. The following methodology was standard for both the informed and uninformed groups.

Each consumer (from both groups) was presented with five samples. For each sample, the consumers were given a raw cob (presented on a blind coded white plate) and cobette of cooked sweet-corn (presented on a blind coded alfoil tray (Figure 1). The samples were presented in a sequential monadic order to prevent bias. Palette cleansers (water and water biscuits) were also provided.

Figure 1. Presentation of raw (left) and cooked sample (right).

Assessments took place in individual booths of the sensory lab at Coopers Plains, equipped with day-light equivalent lighting, temperature and humidity control. The questionnaire was split into sections relating to the following: demographics, acceptability, willingness to purchase, and overall likes and dislikes.

The consumer acceptability parameters assessed, using a 9-point hedonic scale, were: appearance (raw), overall liking (cooked), appearance (cooked), flavour (cooked) and texture (cooked). The scale was structured as shown in Figure 2, consumers were asked to tick one box.

Please indicate on the scale below how much you <u>like</u> the sample OVERALL (please tick one):

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

Figure 2. Example of 9-point hedonic scale used in consumer testing.

The consumer acceptability parameters, colour and mottling, were assessed using a Just About Right (JAR) scale. The scales as shown in Figure 3, consumers were asked to tick one.

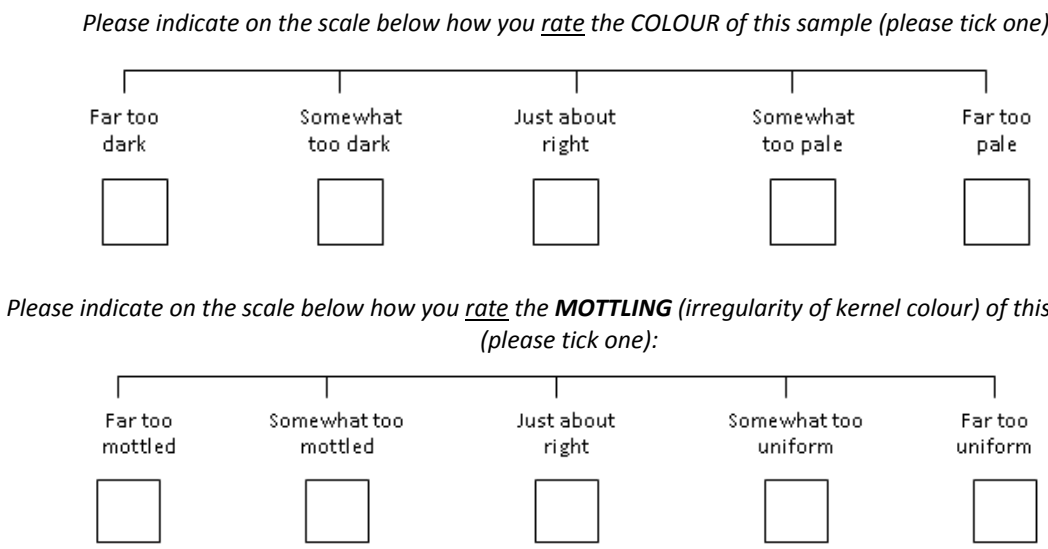


Figure 3. Example of JAR scales used in consumer testing.

Willingness to purchase (WTP) was included in the consumer evaluation in order to gain insight into potential market acceptability for the high zeaxanthin variety of sweet-corn in comparison to the current market sample, Garrison. Consumers were asked whether or not they would consider buying each of the samples based on its raw appearance (as it would be purchased in the supermarket).

Consumers were given the opportunity to detail any particular likes and dislikes they had of each sample. Comments were then collated for evaluation.

Data Analysis

Consumers were asked to rate each sample for the following; appearance (raw), overall liking (cooked), appearance (cooked), flavour (cooked) and texture (cooked), colour and mottling. All responses were analysed using XLStat Sensory Software. A repeated measure analysis of variance (ANOVA) was performed on the consumer data where a 1-9 hedonic scale was utilised, where a significant ($p<0.1$) sample F-ratio was found. Graphical scaling was used to analyse responses given on Just About Right (JAR) scales.

Consumer open-ended comments relating to sample like and dislikes were collated. This method involved identifying key statements/categories of statements given by consumers. Individual comments were then assigned to each of these categories. For example, the

following comment ‘dislike the flavour of the sample’ would be categorised into Dislikes > Flavour, for that particular sample.

Results

Visual appearance of four of the five hybrids (10-3x14-6 image unavailable), cooked and uncooked are shown in Figure 4. Note that cooked cobettes for flavour evaluation were smaller than in the image below.

Figure 4. Visual appearance of 4 of the 5 hybrids tested. Cooked samples are shown at the top, and an uncooked sample at the bottom of each photograph. The hybrid 10-3x14-6 is not shown due to sample unavailability at the time of photographing.

The consumer acceptability results for the informed and uninformed panels are shown in Tables 2 and 3, respectively.

Table 2. Consumer acceptability results – informed panel.

Sample ID	Attribute	Means*
10-3x14-6	Raw appearance	7.30a
11-7x2-9		6.85ab
2-9x23-6		6.45b
8-6x1-1		6.65ab
Garrison		6.75ab
10-3x14-6	Overall liking (cooked)	7.10a
11-7x2-9		6.45b
2-9x23-6		7.20a
8-6x1-1		7.30a
Garrison		6.85ab
10-3x14-6	Appearance (cooked)	7.30a
11-7x2-9		6.85a
2-9x23-6		6.95a
8-6x1-1		7.10a
Garrison		6.95a
10-3x14-6	Flavour (cooked)	6.80ab
11-7x2-9		6.35b
2-9x23-6		7.30a
8-6x1-1		7.25a
Garrison		6.40b
10-3x14-6	Texture (cooked)	7.25ab
11-7x2-9		6.20c
2-9x23-6		6.70bc
8-6x1-1		7.55a
Garrison		7.15ab

* P<0.10; 1=dislike extremely, 5=neither like nor dislike, 9=like extremely

Table 3. Consumer acceptability results – uninformed panel.

Sample ID	Attribute	Means*
10-3x14-6	Raw appearance	6.95a
11-7x2-9		6.16a
2-9x23-6		4.95b
8-6x1-1		6.32a
Garrison		6.26a
10-3x14-6	Overall liking (cooked)	7.26a
11-7x2-9		6.16bc
2-9x23-6		5.53c
8-6x1-1		6.95ab
Garrison		6.42abc
10-3x14-6	Appearance (cooked)	7.47a
11-7x2-9		6.74a
2-9x23-6		5.11b
8-6x1-1		7.26a
Garrison		6.63a
10-3x14-6	Flavour (cooked)	7.32a
11-7x2-9		6.05b
2-9x23-6		6.11b
8-6x1-1		6.79ab
Garrison		6.47ab
10-3x14-6	Texture (cooked)	7.47a
11-7x2-9		5.74c
2-9x23-6		5.90bc
8-6x1-1		6.68ab
Garrison		6.32bc

* P<0.10; 1=dislike extremely, 5=neither like nor dislike, 9=like extremely

For the informed panel, acceptability was as follows:

Raw Appearance:
Difference between the means of the five samples was significant. 2-9x23-6 was significantly less liked than sample 10-3x14-6 for the attribute Raw Appearance. No difference was found between samples 11-7x2-9, 2-9x23-6, 8-6x1-1 and Garrison.

Appearance (cooked)
Difference between the means of the five samples was not significant.

Flavour (cooked)
Difference between the means of the five samples was significant. 11-7x2-9 and Garrison were perceived as significantly less liked than samples 2-9x23-6 and 8-6x1-1, for the attribute

Flavour (cooked). No difference was found between samples 11-7x2-9, Garrison and 10-3x14-6.

Texture (cooked)
Difference between the means of the five samples was significant. 11-7x2-9 was significantly less liked than samples 10-3x14-6, Garrison and 8-6x1-1, for the attribute Texture (cooked). No difference was found between samples 11-7x2-9 and 2-9x23-6, nor between 10-3x14-6, 2-9x23-6 and Garrison.

Overall Liking (cooked):
Difference between the means of the five samples was significant. 11-7x2-9 was perceived as significantly less liked than samples 10-3x14-6, 2-9x23-6 and 8-6x1-1, but no difference was found between 11-7x2-9 and Garrison.

For the uninformed panel, acceptability was as follows:

Raw Appearance:
Difference between the means of the five samples was significant. 2-9x23-6 was significantly less liked than all other samples, for the attribute Raw Appearance. No difference was found between samples 11-7x2-9, 10-3x14-6, 8-6x1-1 and Garrison.

Appearance (cooked)
Difference between the means of the five samples was significant. 2-9x23-6 was significantly less liked than all other samples, for the attribute Appearance (cooked). No difference was found between samples 11-7x2-9, 10-3x14-6, 8-6x1-1 and Garrison.

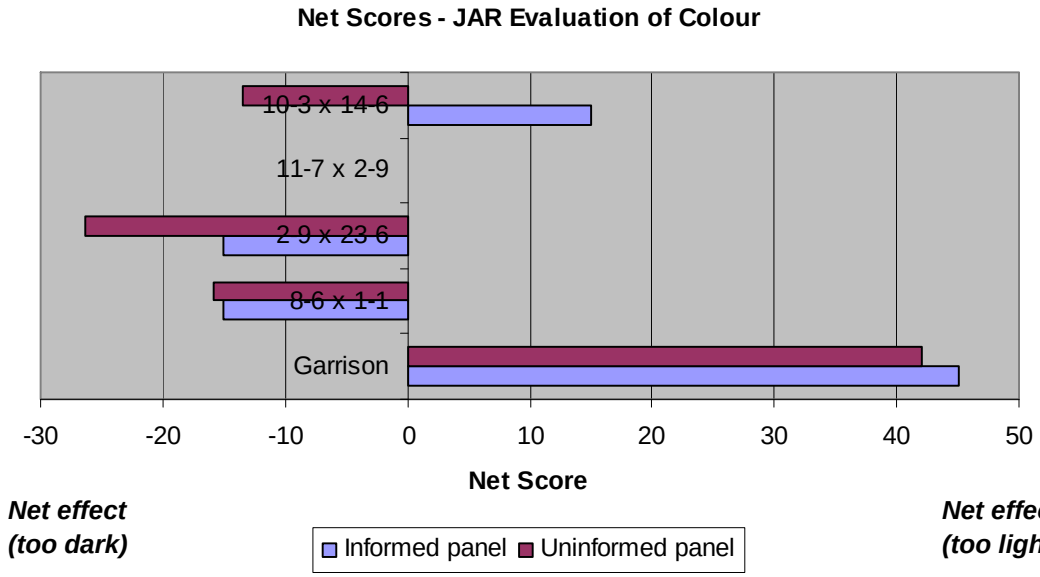
Flavour (cooked)
Difference between the means of the five samples was significant. 11-7x2-9 and 2-9x23-6 were significantly less liked than 10-3x14-6 for the attribute Flavour (cooked). No difference was found between samples 11-7x2-9, Garrison, 8-6x1-1 and 2-9x23-6.

Texture (cooked):
Difference between the means of the five samples was significant. 11-7x2-9 was perceived as significantly less liked than samples 10-3x14-6 and 8-6x1-1, for the attribute Texture (cooked). No difference was found between samples 11-7x2-9, garrison and 2-9x23-6, nor between 10-3x14-6 and 8-6x1-1.

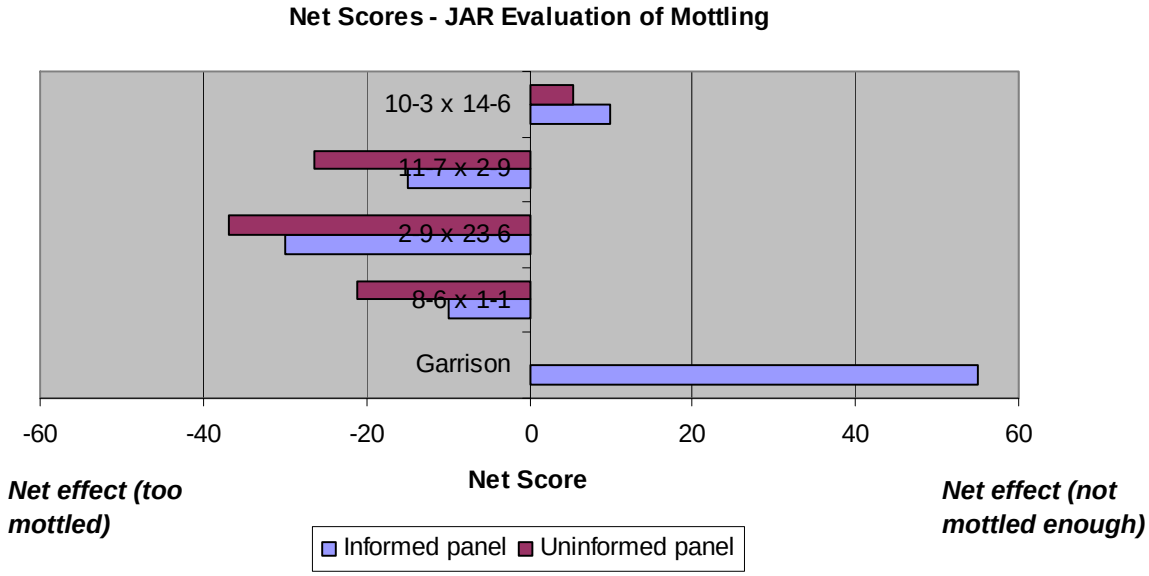
Overall liking (cooked):
Difference between the means of the five samples was significant. 2-9x23-6 was significantly less liked than sample 10-3x14-6 and 8-6x1-1, in addition, sample 11-7x2-9 was significantly less liked than sample 10-3x14-6, for Overall Liking. No difference was found between samples 11-7x2-9, 8-6x1-1 and Garrison.

Jar Analysis – colour and mottling.

Further analysis of colour and mottling (Figure 4) was performed with consumers to further elaborate on their acceptability results reported above.



Net score = percent of respondents rating products "too light" minus percent of respondents rating product "too dark".



Net score = percent of respondents rating product "not mottled enough" minus percent of respondents rating product "too mottled".

Figure 4. Net Scores – Consumer JAR evaluation of cooked samples for colour and mottling.

Individual comments for each cooked hybrid sample were also collated in Figure 5 for both informed and uninformed consumers.

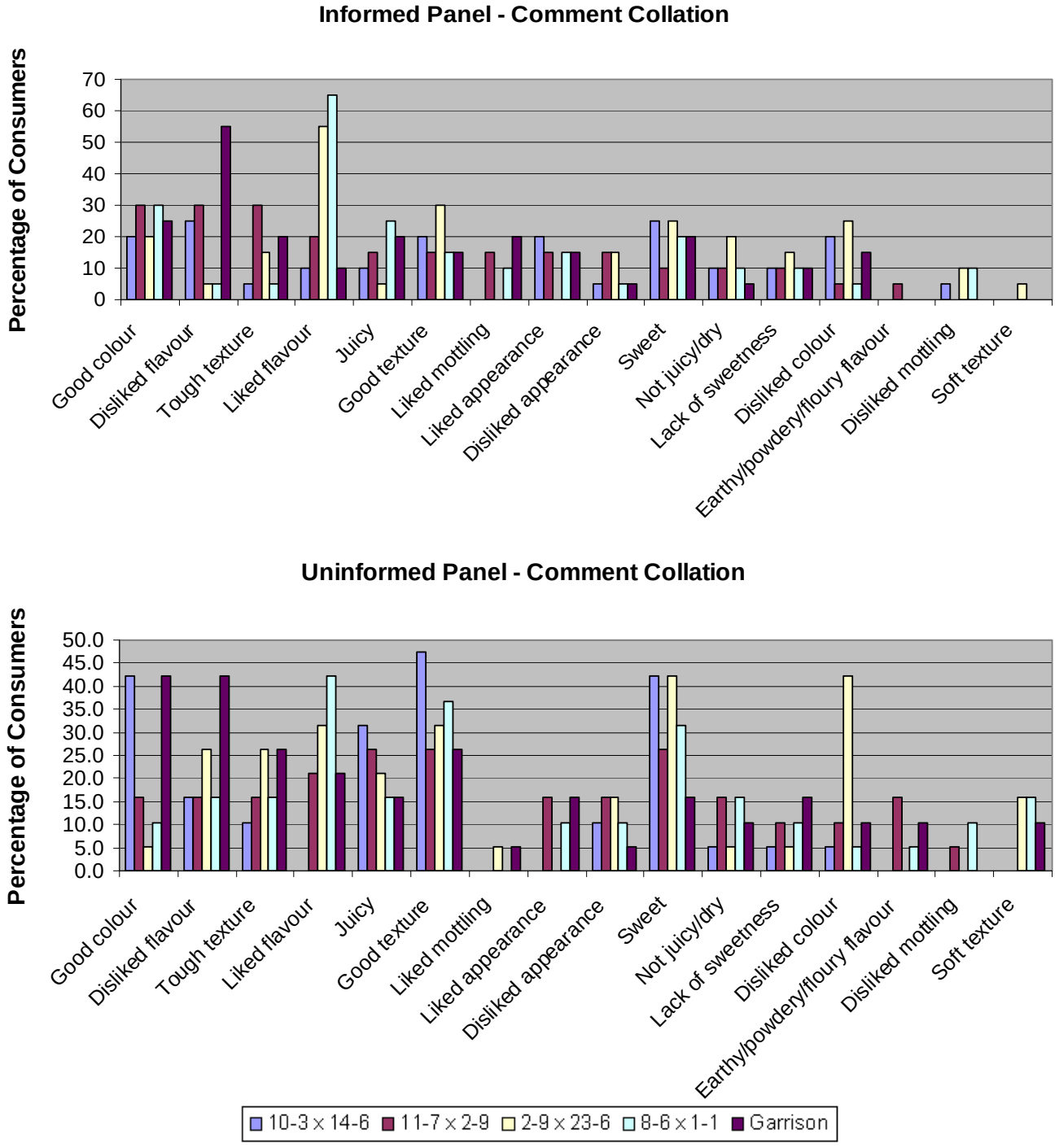


Figure 5. Informed and uninformed consumer comments of cooked samples of each hybrid.

Comment collation provided further correlation with the consumer acceptability scores. Over 40% of uninformed consumers commented on their dislike of the colour of sample 2-9x23-6, as well as 25% of informed consumers; greater, in both groups, than the number of comments regarding disliking of the colour, than all other samples.

Willingness to Purchase (WTP)

The willingness to purchase each hybrid for informed and uninformed consumers is shown in Figures 6 and 7, respectively.

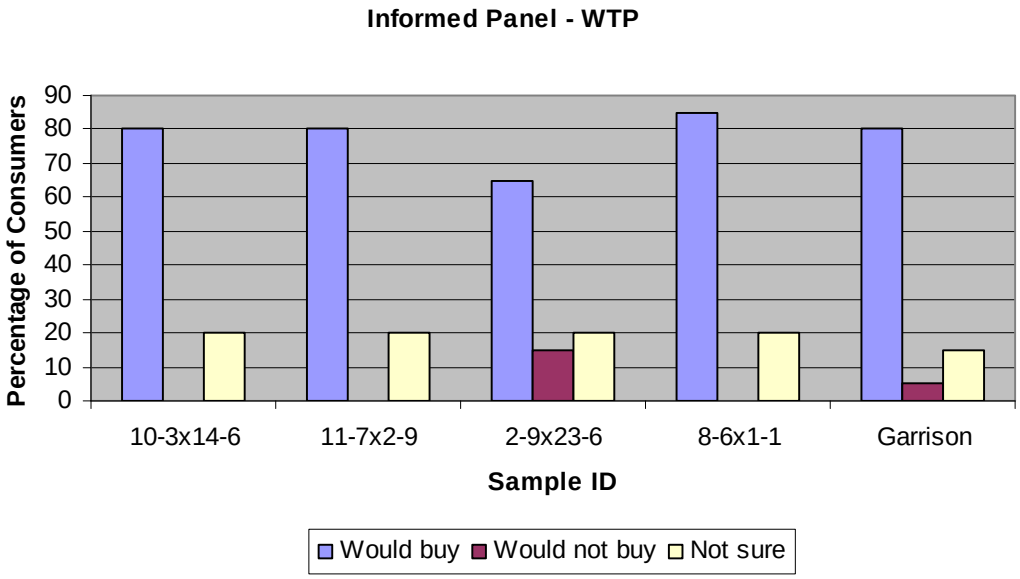


Figure 6. Willingness to purchase (raw sample) - Informed panel

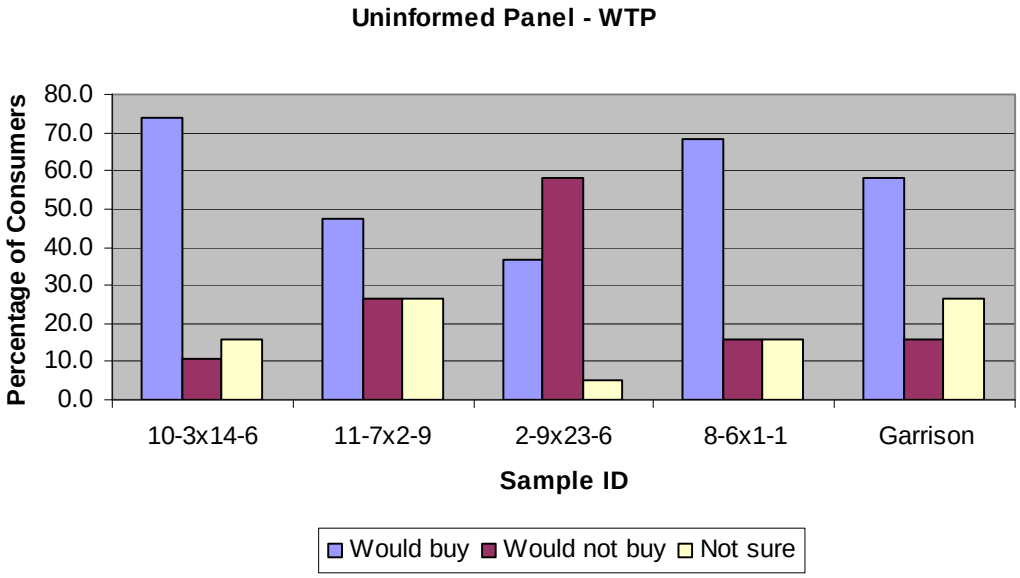


Figure 7. Willingness to Purchase (raw sample) - Uninformed panel.

Overall, there was a strong correlation with regards to providing consumers with product information and their willingness to purchase. For example, 80% of informed consumers were willing and 0% of informed consumers were unwilling to purchase sample 11-7x2-7, in comparison to 47% and 26% of the uninformed panel, respectively. In addition, although sample 2-9x23-6 performed poorly in a number of attribute evaluations, there was a very steep decline in the number of consumers unwilling to purchase the product between in the uninformed and informed panellists, 58% down to 15%, respectively.

Discussion

Both informed and uninformed panellists favoured sample 10-3x14-6 for appearance (raw) and appearance (cooked). Sample 2-9x23-6 was statistically the most disliked of all samples for both appearance (raw) and appearance (cooked) (uninformed panel) and appearance (raw) (informed panel). With a hue angle of 79.3° (the lowest of all samples evaluated), it can be assumed that this deeper orange colour was responsible for the dislike in the appearance.

Consumer acceptability scores of the highly coloured high-zeaxanthin samples was lower for all attributes for the uninformed group than the informed group. This suggests a decrease in tolerance for the high zeaxanthin hybrids, when consumers were uneducated as to the reasons behind the change in colour of corn kernels. This conclusion is supported by results of JAR colour analysis, where sample 2-9x23-6 was given a net score of -26.3 by the uniformed panel and -15 by the informed panel. Referring to the net scores for JAR mottling analysis, again, sample 2-9x23-6 had the most negative net score of all samples, a confounding factor likely to be partly responsible for lower acceptability of appearance.

With a net score of zero for JAR colour analysis, it can be deduced that the factor impacting acceptance of sample 11-7x2-9 for the attribute appearance (raw and cooked) was the level of mottling throughout the cob. JAR mottling analysis indicates that this sample has a less than acceptable degree of mottling, with net scores of -15 and -26.3 for the informed and uninformed panels, respectively.

It is important to note the opinions of the informed panel relating to the sample Garrison. The informed panel showed a clear preference for a greater degree of mottling (noting their JAR mottling analysis of Garrison, where net score = +55). Garrison scored parity with all samples (excluding 2-9x23-6) for both appearance (raw) and appearance (cooked). It can therefore be concluded that providing consumers with information relating to the health benefits and reasons behind the greater depth of colour in the corn kernels increases their acceptability of such characteristics.

Both informed and uninformed panellists found the texture (cooked) of sample 11-7x2-9 to be the most unacceptable of all samples, scoring a mean value of 5.7 and 6.2, respectively (where 6=like slightly). Referring to the overall likes and dislikes, consumers described sample 11-7x2-9 as having a tough and dry texture, with a floury/earthy flavour. These characteristics are those likely to be owing to the lower scores seen for the texture of this sample. Interestingly,

the authors of the study did not find this to be so (separate evaluation), which may indicate some variation between individual cobs exists within this line.

Both informed and uninformed panellists scored sample 11-7x2-9 as having flavour acceptability within parity of the sample Garrison. Although scores for 11-7x2-9 were statistically lowest for flavour, it should be noted that to be within parity of an already commercially acceptable sample is of importance. As is, samples 10-3x14-6, 2-9x23-6 and 8-6x1-1 scored either within parity or statistically higher than Garrison for flavour (informed panel). Again, the authors of the study had previously found 8-6x1-1 to have an earthy flavour, which is why it was included in the current study. Again, this may indicate some variability in flavour between cobs.

A disagreement to be noted between the informed and uninformed panel was the acceptability of flavour for sample 2-9x23-6. The informed panel rated this sample as statistically one of the most acceptable; however, the uninformed panel rated it as one of the most statistically disliked samples. These scores were reflected in the comments, with over 25% of uninformed consumers indicating dislike in their comments, in comparison to 5% of informed consumers (and over 55% commenting on how they like the sample). Indicating, that not only may tolerance to appearance increase with education, but appears also to be the case with flavour.

Samples 10-3x14-6 and 8-6x1-1 were the two most consistent samples across the set evaluated. Both samples scored, if not statistically highest, within parity of the highest scoring sample for each attribute across both groups. Net scores for both mottling and colour only exceeded +/- 20 on one occasion (sample 8-6x1-1 JAR mottling analysis uninformed panel), which indicates little desire for change. Sample 8-6x1-1 showed a peak for comments on liked flavour amongst the informed panel, and liked flavour, good texture and sweetness amongst the uninformed panel. Sample 10-3x14-6 showed peaks for sweetness amongst the informed panel and good colour, good texture and sweetness amongst the uninformed panel.

Recommendations

The zeaxanthin-biofortified hybrids developed in this project varied in zeaxanthin concentration, kernel colour, cob-length and tip-fill, and consumer acceptance. Experimental hybrids 10-3x14-6, 11-7x2-9, and 1-1x23-7 exhibited consistently high zeaxanthin concentrations (16-20 µg/g FW) at eating maturity, which is a primary prerequisite for use as a 'high-zeaxanthin' sweet-corn product. While a number of other hybrids such as 2-9x23-1, 2-9x23-6, 2-9x23-7 exhibited a deeper golden colour than these hybrids (except 1-1x23-7), zeaxanthin concentrations tended to be slightly lower (11-16 µg/g FW), but still significantly greater than commercial yellow hybrids such as Hybrix5, Garrison, or Goldensweet improved (2-5 µg/g FW).

Seasonality was also recently confirmed to have a profound impact on zeaxanthin levels, with summer-harvested sweet-corn producing approximately 50% higher zeaxanthin concentration (29-31 mg/g FW) than corn harvested in autumn or winter (16-20 mg/g FW). This would indicate an advantage to growing high-zeaxanthin hybrids under tropical conditions, or at least in areas experiencing high temperatures during cob development. Consequently, we would recommend that the high-zeaxanthin experimental hybrids be tested under a range of seasons not only in south-east Queensland and north Queensland, but in other sweet-corn-growing areas.

Hybrids, such as 8-6x1-1, were less consistently high in their zeaxanthin concentration across seasons, and therefore are not recommended. We would also tend to not recommend this hybrid based on its shorter cob-length and poor tip-fill, yielding less marketable cobettes during processing. Hybrid 1-1x23-7 also exhibited this drawback, despite having the positive characteristics of high-zeaxanthin and a deep golden colour, although too deep a golden colour was not seen as favourable by consumers at this stage of market development. In regard to cob-length, hybrid 11-7x2-9 had the longest cob, and reasonable tip-fill. None of the high-zeaxanthin hybrids displayed perfect tip-fill, as is often seen in the commercial yellow hybrids, Garrison and Hybrix5. Tip-fill however, if not extremely poor, is potentially less of an issue where cobs are 'topped and tailed' during processing into packaged over-wrapped display packages. In this regard, 11-7x2-9 would potentially produce a similar yield to the commercial hybrids.

All of the high-zeaxanthin hybrids produced cobs that were significantly different in colour to standard yellow commercial hybrids such as Garrison, Hybrix5 or Goldensweet improved. A gold colour was seen by consumers as quite acceptable (10-3x14-6, 11-7x2-9), although too deep a gold colour (2-9x23-6) reduced acceptability. We would strongly recommend for the gold colour difference to be used as a point of differentiation in the marketplace. To do this, high-zeaxanthin cobs would be best presented with all or most sheath-leaves removed, as is common practice with overwrapped cobettes in major Australian supermarkets. In this regard, we would recommend that supermarket lighting is optimized, as fluorescent lighting, which has more blue light than sunlight) can alter the colour of the cobs at the purchase point.

In addition, cooking will increase the intensity of colour, and this was seen as a positive attribute to consumers in the case of more moderately-coloured hybrids such as 10-3x14-6

and 11-7x2-9. This attribute could be further exploited through the development of pre-cooked, shrink-packaged corn-cobs, which have an extended shelf-life and are common in parts of Asia. The high-zeaxanthin hybrids do exhibit a small degree of kernel colour variation within the cob, and this becomes more apparent after cooking. This however was not seen as a negative attribute by Australian consumers, but could potentially be removed with further hybrid development.

At this stage of development where high-zeaxanthin sweet-corn is still a novel product to consumers, and the provision of health information was found to increase acceptability of the more deeply-coloured hybrids. Even so, 'gold' coloured cobs were still seen to be generally preferred to 'deeper-gold' cobs, and consequently we would recommend 'gold' coloured hybrids such as 10-3x14-6 and 11-7x2-9 for maximum market acceptance, regardless of the provision of any accompanying health information. Although there is an ever increasing body of scientific evidence to support the role of zeaxanthin (and lutein) in slowing the progression of macular degeneration, health claims on labels are still not permitted in Australia. However, eye health and product information can be released via general media, and recommendations by dieticians and nutritionists, general practitioners and eye-health specialists is fully permitted.

Harvest maturity of cobs was found to strongly influence zeaxanthin concentration, with later harvest significantly increasing zeaxanthin concentration and kernel colour. We would recommend however, for cobs to be harvested at normal eating stage (72-76% moisture content), as sweet-corn is less tender with later harvesting, whether it is high-zeaxanthin or a yellow commercial hybrid.

Freezing cobs was found to have a transient effect on kernel colour, with frozen kernels appearing a deeper, orange colour. This potentially has application to frozen corn, although thawing or cooking cobs returned the kernels to their original colour. Rapid-freezing had less effect on colour change, but domestic freezing of cobs by consumers will experience this phenomenon. This effect could potentially enhance the appearance of commercial frozen sweet-corn, if high-zeaxanthin sweet-corn was utilised as a frozen product.

We have produced a range of high-zeaxanthin experimental hybrids. At this stage of development, based on zeaxanthin concentration, cob-length, colour, and consumer preference, we would particularly recommend further evaluation of 10-3x14-6 and 11-7x2-9. From a human-nutrition perspective, we would also recommend further trials investigating the bioavailability of zeaxanthin from biofortified sweet-corn in comparison to artificial supplements.

Scientific Refereed Publications

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O'Hare, T.J., Calvo-Brenes, P., Fanning, K. and Pun, S. (2015). Freezing induces reversible colour-change in both zeaxanthin-biofortified and yellow sweet-corn. International Horticultural Congress, Brisbane, Qld, 17-22nd August 2014, *Acta Horticulturae* (in press).

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Zeilmann, T., K.J. Fanning, M. Rychlik, M.E. Netzel and T.J. O'Hare (2015). Impact of zeaxanthin concentration on its bioaccessibility in biofortified sweet-corn. Macular Carotenoid Conference, Cambridge, UK, 7-10th July 2015 (*European Journal of Ophthalmology,* Abstract submitted).

Intellectual Property/Commercialisation

Intellectual property generated by this project includes the zeaxanthin-biofortified germplasm developed in this project (inbred parents and experimental hybrids).

It is expected that this material will be commercialized via an open-tender process by DAF Queensland.

DAF Queensland has registered the trademark 'Supergold' as a potential marketing name for zeaxanthin-biofortified hybrids. The name reflects a combination of the term 'supersweet' describing the *shrunken2* genetic background, and the deeper golden colour brought about by elevated zeaxanthin concentration.

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