Medicinal properties of *Triticum aestivum*.L

Effects of freezing on chlorophyll and antioxidant content of aqueous wheatgrass extract

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9th March 2012

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Abstract

Triticum aestivum when harvested as young green shoots provides the dietary supplement wheat grass juice, known as a “functional food”. This paper establishes the medicinal quality of some of the chemical constituents in this plant material. Chlorophyll is said to contribute to its medicinal properties. It is also said to have high antioxidant capabilities. The total chlorophyll content was extracted from frozen and fresh samples using 80% acetone and measured using a spectrophotometer at wavelengths of 663nm and 645nm. The general antioxidants are measured using the Ferric Reducing Ability of Plasma assay and absorptivity measured at a wavelength of 595nm. The quantities are then compared between samples that have been frozen for a varying lengths of time, up to 2.3 years, and a sample that is unfrozen and freshly pressed before undergoing assays.

Results show no significant reductions in chlorophyll content and no significant differences in antioxidant capacity between samples frozen for varying lengths of time. It was concluded that there was no difference in chlorophyll content or antioxidant capacity in samples frozen for up to 2.3 years in comparison to fresh, unfrozen juice.

The trial also briefly compares the difference between samples grown organically outdoors to those grown hydroponically in a greenhouse. A significant reduction in both chlorophyll content (48% less) and antioxidant capacity (28% less) was found in frozen greenhouse grown wheatgrass compared to frozen outdoor grown.

Key Words: *Triticum aestivum,* L, WGJ (wheat grass juice), medicinal properties, chlorophyll extraction, antioxidants, FRAP assay, freezing, pharmacology.

Introduction

Wheatgrass juice (WGJ) is the aqueous form of the young shoots of common wheatgrass *Triticum aestivum* .L belonging to the Poaceae family. It is sometimes referred to as 'Green blood' due to its high chlorophyll content (Reddy, 1996).

WGJ is most commonly used as a dietary supplement and has been exploited as such since the 1950's and became a popular trend in the 1970s. The juice has been shown to contain a vast array of compounds that may contribute to its medicinal capacity. For example, studies show WGJ to be effective in reducing symptoms associated with ulcerative colitis (Ben-Ayre, 2002), reduction in transfusion requirements in patients with the blood disease *Thalassemia major*, a chemopreventive (Arya, 2011; Bar-Sela, 2007) and prevention of DNA oxidative damage (Falcioni, 2002). Plant-based diets are regarded one of the potential chemopreventive agents. (Boivin *et al*, 2007)

These medicinal effects have been attributed to, amongst other compounds, its high chlorophyll content (Mishra, 2011). This has been referred to as 'green blood' and features in much anecdotal literature as one of the key constituents responsible for its health giving benefits.

It is also said to contain potent antioxidants due to its high content of bioflavonoids including Apigenin, Luteolin and Quercetin (Padalia, 2010). It also contains the enzyme Super oxide dismutase which is gaining much interest due to its ability to inhibit cell mutation and the potential applications of this activity.

Research into the individual chemical constituents show natural chlorophyll to be an effective inhibitor of cytotoxic cell proliferation (Simonich *et al*, 2007). Chlorophyll is also suggested to form part of the antioxidant properties observed in studies on Leukemia cell lines. (Aydos *et al*, 2011)

This paper aims to establish the content of chlorophyll and antioxidants in samples of wheatgrass juice (WGJ) that are commercially available in order to quantify its health giving properties. Since most available literature on WGJ is anecdotal it has been observed that properly conducted and validated scientific research is required to substantiate many of the claims surrounding this "functional food". Herbal or alternative medicine is gaining popularity and scientific research about wheatgrass as a functional food is becoming more available and popular as a research avenue.

The WGJ used is from LiveWheatgrass Ltd, commercially available, outdoor grown in organic soil and received by the customer in frozen form. Consumers then store the product in a home freezer and defrost as and when required. This paper aims to investigate potential effects that long term freezing may have on the constituents mentioned previously and to suggest implications this may have on its medicinal and health properties. The study also aims to measure and discuss differences that may occur between field grown and hydroponically, greenhouse grown samples with reference to its health giving properties.

The application of the compounds shown to be present in *T.aestivum* ranges from a dietary supplement, a preventative and a treatment for minor and major illnesses and disorders.

In a recent survey, more than half of Australian cancer patients reported using herbal medicine (MacLennan, Wilson & Taylor 2002) In view of increasing risk factors of humans to various deadly diseases, there has been a global trend toward the use of natural substances present in medicinal plants and dietary plants as therapeutic antioxidants. Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers (Lobo *et al,* 2010)

Current literature surrounding the subject is extensively anecdotal and has been the driving force behind its enormous contribution to the food supplement industry. Whilst there have been some validated trials, not enough in-depth, controlled clinical trials have been conducted to study the therapeutic effect of wheatgrass. (Iyer *et al*, 2010)

Medical trials are ceaselessly aiming to produce efficacious and sometimes novel approaches to treatment of the vast array of diseases and disorders that are current threats to humans worldwide. Many conventional methods of treatment are often accompanied by unwanted and sometimes harmful side effects. Crude drugs may be less efficient than modern medicines, but they are relatively free from side effects, which is a desirable quality. Often the limitation of the application of modern medicine is their adverse side effects. Therefore, there is an ever increasing need for efficacious, economic, safer medicinal agents producing permanent cure in recent times (Ashok, 2011)

Current gaps in knowledge occur mainly in the lack of scientific evidence based on controlled, in-vivo trials. This type of study would help to provide insight into the bioaccesibility of wheatgrass and its chemical constituents.

Since *T.aestivum* contains chlorophyll and an abundance of antioxidants its associated health benefits suggest it can have far reaching applications. It shows the preliminary potential to contribute to the treatment of some serious health issues. The World Health Organization expert committee on diabetes has listed as one of its recommendations that traditional methods of treatment for diabetes should be further investigated. (WHO, 1980)

The wheatgrass juice at LiveWheatGrass Ltd. is pressed and blast frozen within 10 minutes of its harvest. It is then stored at -30°C and transported commercially to consumers frozen and stored by them in home freezers. The focus of this work is to compare samples of *T.aestivum* juice from LiveWheatgrass Ltd for the content of some of its chemical components that can be attributed to its medicinal value. It does not give a value of all of the components that may be responsible for its medicinal qualities. Ingredients chosen to measure are chlorophyll and antioxidant capacity which have been shown to be of health giving value. Once content is quantified the study aims to observe any differences between quantities in samples that have been frozen for varying periods of time and those which have not been frozen at all. In addition, also to observe any differences in chlorophyll and antioxidant capacity in indoor/greenhouse, hydroponically grown samples (purchased from another company) compared to outdoor field grown. The tests are of importance commercially since freezing is the method by which WGJ from LiveWheatGrass Ltd is stored and it is relevant to observe whether there is any loss to the medicinal quality of this product once it has undergone these normal procedures.

Methods and Materials

Samples were received in frozen form by courier from LiveWheatGrass Ltd. The last sample was unfrozen and freshly pressed immediately before undergoing assays for both chlorophyll content and antioxidant content.

All samples are harvested with a leaf length of 15-20cm long, juiced and blast frozen. Frozen juice is then kept at a temperature of -30°C

Chlorophyll content was based on the procedure by Arnon, 1949. The chlorophyll was extracted in 80% acetone and optical density measured using a Unicam Heliose Spectrophotometer at wavelengths of 663nm and 645 nm. Antioxidant potential was measured based on the ferric reducing ability of plasma or FRAP assay (Benzie & Strain 1996). This was also measured using Unicam spectrophotometer at a wavelength of 595nm. This method was chosen as it is simple to use, gives a linear response over a large concentration range and can be made applicable to both water and lipid-soluble components. This serves as a general antioxidant content measure.

Chlorophyll extraction

For an exact quantitative determination of chlorophyll in the plant material the extract must be fully transparent. In most cases, the homogenized plant extract contains un-dissolved, very fine, solid material eg; fibre and cell wall debris. These materials make the extract turbid and scatter the light, rather than absorbing it. The scattered light increases from longer to shorter wavelengths (from red region to blue). Thus, the presumed absorbance signal measured in turbid solutions by the spectrophotometer is increased differentially for individual wavelengths. Hence, the concentration of pigments, calculated from these incorrect absorbance values, are too high. In order to have a transparent extract without turbidity, the homogenized extract was centrifuged. Samples were protected from light at all times. During analysis, samples were covered with aluminium foil.

Three replicates of each sample were obtained by the following method.

Each sample was placed into a glass beaker and labelled in numerical order. Defrosting took place in a plastic tray with tepid water. Once entire contents were thawed and no longer contained solid frozen material the samples were removed from the water.

Samples were stirred to prevent heavier particles falling to bottom. Using a 1ml pipette and digital scales up to and not exceeding 0.3g of extract was placed into 15ml polymer tubes with screw on lids. This was then made up to 10ml with 80% acetone.

Samples were then placed in balanced centrifuge tubes in an MSE Centrifuge and spun for 2 minutes at 2000rpm. The tubes were then removed from the centrifuge and the clear, green liquid was carefully removed using a pipette, making sure not to disturb the plant debris resting at the bottom of the tube. Using a pipette, each sample was placed into 1ml spectrophotometer cuvette. UV/VIS Spectrophotometer by Unicam was used to read absorbance of each sample at wavelengths of 645nm (Chlorophyll *b*) and 663nm (Chlorophyll *a*).

Zero was set on the spectrophotometer using a blank cuvette containing 80% acetone on its own. Once zero is set readings were taken for each sample at required wavelength. Readings were recorded. Total chlorophyll content in mg/g fresh weight was calculated using equations from (Arnon 1996) as follows:

Chlorophyll in 80% acetone. Absorbance for 10ml

i) *Chl a* = 12.7 x A663nm – 2.69 x A645nm (mg/g)

ii) *Chl b* = 22.9 x A645nm – 4.68 x A663nm (mg/g)

iii) Total Chlorophyll (*a*+*b*) = 20.2 x A645nm + 8.02 x A663nm

iv) For fresh weight (mg/g) = $\frac{\text{Total Chlorophyll figure}}{\text{Fresh weight x 100}}$

This gives required units of mg/g fresh weight for materials dispersed in 10ml, with 80% acetone. (Arnon, 1949)

Antioxidant potential of Wheat Grass Juice

The 'ferric reducing ability of plasma' or FRAP assay (Benzie & Strain, 1996) is one of several methods available that aim to measure 'antioxidant capacity'. At low pH the complex between the chelator TPTZ (2,4,6-tri-pyridyl-s-triazine) and ferric iron (Fe^{3+}) and can be reduced by most antioxidants. The resulting ferrous iron (Fe^{2+}) TPTZ complex is blue in colour and absorbs light strongly at a wavelength of 595nm.

Determination of a measure of the antioxidant capacity of Wheat Grass Juice extracted from *Triticum aestivum* : the FRAP assay.

Standards of known Fe (II) concentrations ($FeSO_4$ $7H_2O$) were run in triplicate using several concentrations between 200 and 1000 umol/ltr. A standard curve was then prepared (*Fig.3*) by plotting the average FRAP value for each standard versus its concentration. The FRAP values for the samples were then determined using this standard curve.

To construct the graph 30µl of each concentration of ferrous sulphate was added to 0.8ml of FRAP reagent in 1ml cuvettes. A reagent blank was made using 30µl of distilled water with 0.8ml FRAP reagent. Solutions were mixed with a glass rod. Unicam Spectrophotometer was set at 595nm (Absorbance) and zero was set using the reagent blank. Each concentration was read and the calibration graph was constructed using final readings which were not off scale.

To test wheatgrass extracts serial dilutions of each sample were mixed in three tenfold steps. 1:10, 1:100, 1:1000 ml. 30µl of each dilution was added to 0.8ml of FRAP reagent in 1ml spectrophotometer cuvettes and left at room temperature (around 20°C) for 1 minute. Zero was set on spectrophotometer using blank cuvette. Readings for each sample were then taken at 595nm. Regular readings of blank cuvette were taken before samples to ensure correct reading before absorbance was recorded. A dilution was selected at 1:10ml

for each sample as this was the first not off scale at this point. Readings were taken every 5 minutes to see when they stabilised. At 45 minutes the solutions stabilised.

Using a pipette 0.8ml of FRAP reagent was placed into 1ml cuvette. 30µl of each sample at 1:10 dilution was then added to FRAP reagent and mixed well before reading at 595nm. Initial reading was taken for each sample and measurement recorded. Samples were left at room temperature for 1 hour before second reading was taken and recorded. These values were plotted on the calibration graph. These were then multiplied by ten to give final values of µmol/ltr of Fe^{2}+ equivalence.

Results

Table 1. Chlorophyll *a* and *b* content of WGJ extracted in 80% acetone. Total values mg/g after calculation based on method by Arnon, 1949. Mean values are of three replicates of each sample

Sample	State	Month	Year	Origin	Weight grams	A663nm Chl *a*	A645nm Chl *b*	Total Chl mg/g	Mean
1	Frozen	Oct	2009	Field	0.27	0.97	0.36	6.14	7.28
1	Frozen	Oct	2009	Field	0.28	1.37	0.51	8.38	
1	Frozen	Oct	2009	Field	0.27	1.16	0.43	7.34	
2	Frozen	Nov	2010	Field	0.27	0.84	0.32	5.44	9.77
2	Frozen	Nov	2010	Field	0.30	1.52	0.58	12.63	
2	Frozen	Nov	2010	Field	0.29	1.83	0.71	11.25	
3	Frozen	May	2011	Field	0.28	1.22	0.45	7.15	7.57
3	Frozen	May	2011	Field	0.29	1.53	0.56	8.90	
3	Frozen	May	2011	Field	0.29	1.14	0.42	6.67	
4	Fresh	Jan	2012	Field	0.29	0.72	0.59	9.15	7.35
4	Fresh	Jan	2012	Field	0.29	1.50	0.25	4.18	
4	Fresh	Jan	2012	Field	0.28	1.46	0.53	8.72	
5	Frozen	Dec	2011	Green house	0.27	0.67	0.30	5.07	4.31
5	Frozen	Dec	2011	Green house	0.29	0.62	0.27	4.25	
5	Frozen	Dec	2011	Green house	0.27	0.25	0.12	3.63	

Table 2.

Antioxidant equivalence of WGJ extract using FRAP assay. Values of A (using Absorbance) of samples in spectrophotometer Absorbance at wavelength of 595nm. Total antioxidant capacity as umol/litre of Fe^{2+} equivalence calculated from calibration curve based on method by Benzie & Strain, 1996

Sample	Year	State	Month	Origin	1st reading A595nm	2nd reading A595nm	µmoles/ltr	µmoles Fe^{2+} equivalence	Mean of 3 replicas
1	2009	Frozen	Oct	Field	0.144	0.291	390	3900	3633
1	2009	Frozen	Oct	Field	0.137	0.269	360	3600	
1	2009	Frozen	Oct	Field	0.133	0.263	340	3400	
2	2010	Frozen	Nov	Field	0.075	0.259	350	3500	3533
2	2010	Frozen	Nov	Field	0.065	0.246	340	3400	
2	2010	Frozen	Nov	Field	0.089	0.271	370	3700	
3	2011	Frozen	May	Field	0.108	0.250	340	3400	3433
3	2011	Frozen	May	Field	0.094	0.255	345	3450	
3	2011	Frozen	May	Field	0.097	0.254	345	3450	
4	2012	Fresh	Jan	Field	0.112	0.356	480	4800	4500
4	2012	Fresh	Jan	Field	0.087	0.248	340	3400	
4	2012	Fresh	Jan	Field	0.109	0.405	530	5300	
5	2011	Frozen	Dec	Green house	0.042	0.177	245	2450	2533
5	2011	Frozen	Dec	Green house	0.042	0.172	240	2400	
5	2011	Frozen	Dec	Green house	0.065	0.199	275	2750	

Chlorophyll Content

Fig.1 Effects of length of freezing time on chlorophyll content mg/g in frozen and fresh samples measured in 80% acetone. Values are means of three measurements and 2xSD bars are shown. Time refers to the length of time the wheatgrass juice was frozen at -30°C.

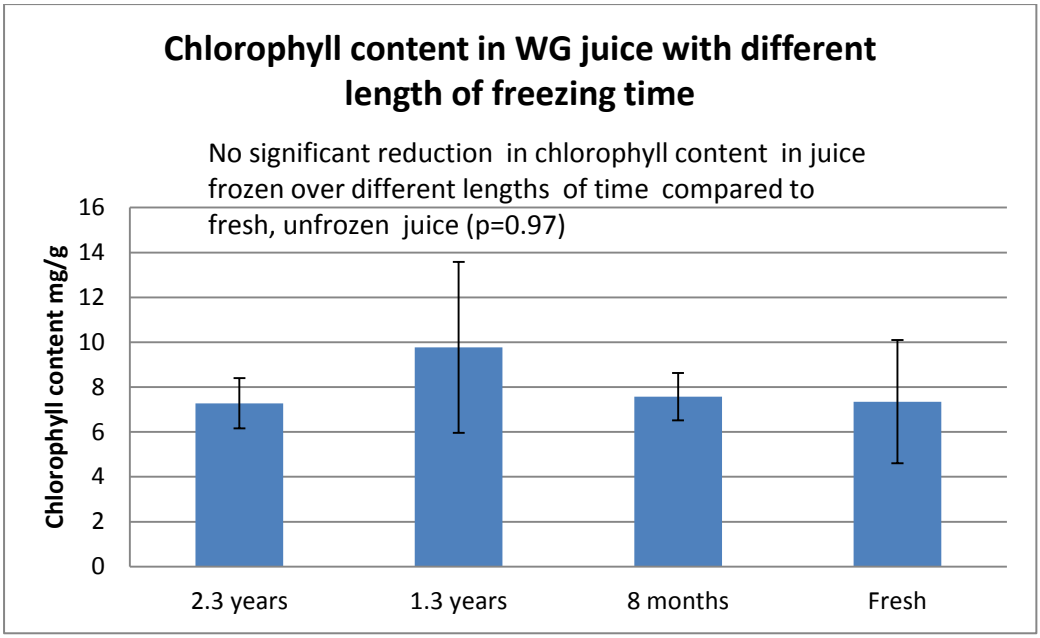
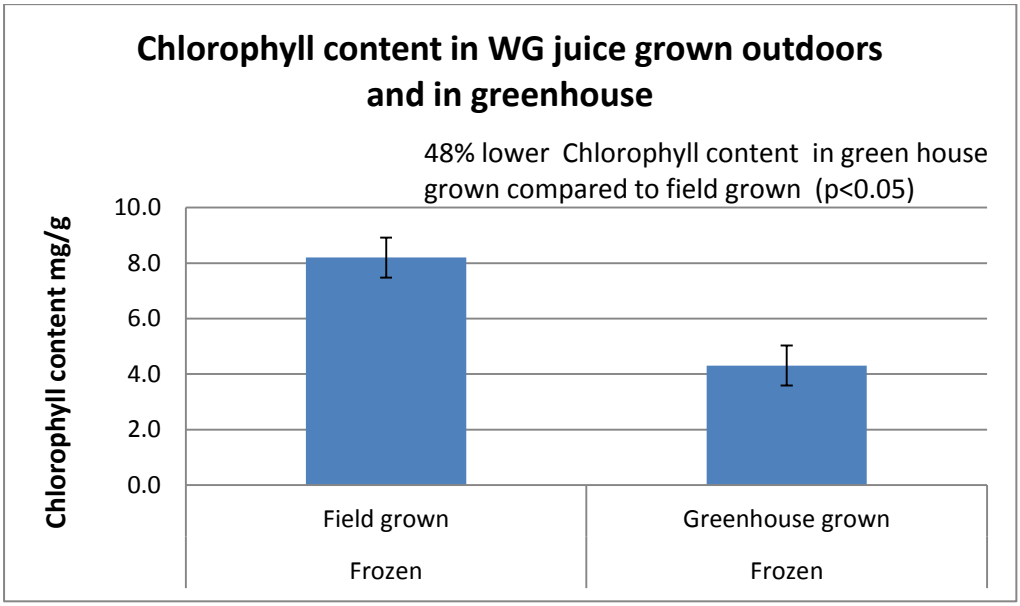


Fig.2 Chlorophyll content mg/g in samples grown organically in a field and hydroponically in a greenhouse. Values for field grown are means over 4 different seasons and greenhouse grown are means of three measurements (Table 1).



Data Analysis

Chlorophyll

Samples were subject to one way analysis of variance (ANOVA). Selected samples were subject to two sample T-Tests. Significance was determined using a $P \leq 0.05$.

Figure 1: **Results from the chlorophyll extraction assay show that length of freezing time on chlorophyll content over 2.3 years frozen yields a similar content at 7.28mg/g to that which was freshly pressed and unfrozen with a value of 7.35mg/g. No significant difference was observed between these two samples, P=*0.97.***

Figure 2: 48% lower chlorophyll content was observed in the greenhouse grown sample compared to the field grown samples, p<0.05.

Antioxidant capacity: FRAP

Fig.3 Effects on antioxidant capacity using FRAP on samples of varying freezing times and fresh, non-frozen sample. All samples are organic and field grown. Values are means of three measurements. 2 Standard Deviation values (2xSD) in each bar. There is no significant difference between the different freezing times and the fresh non-frozen sample based on Tukey HSD method, p=0.27

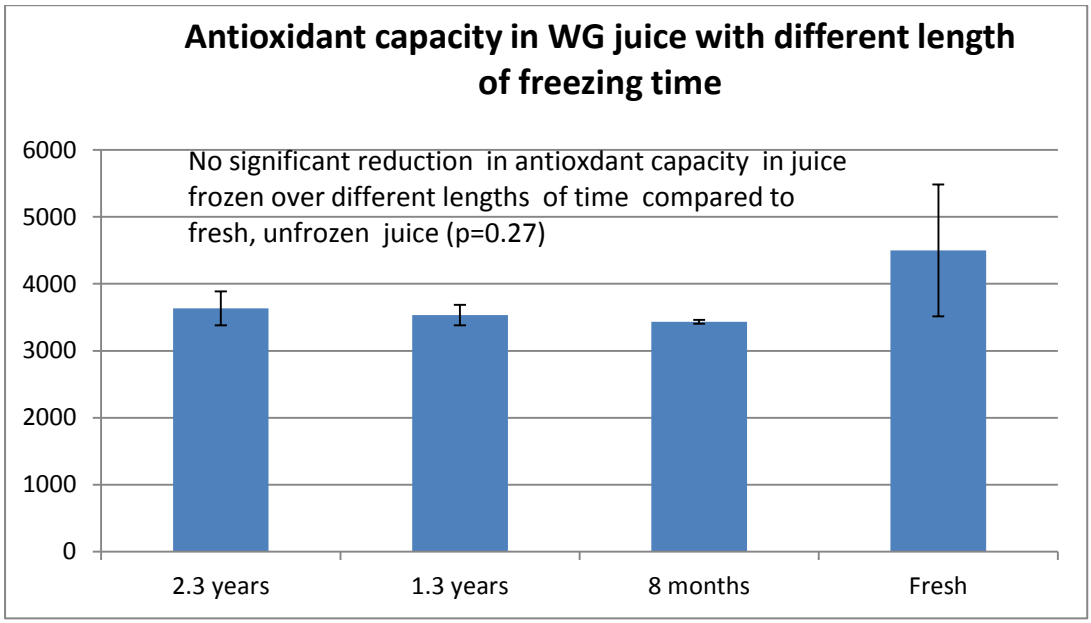
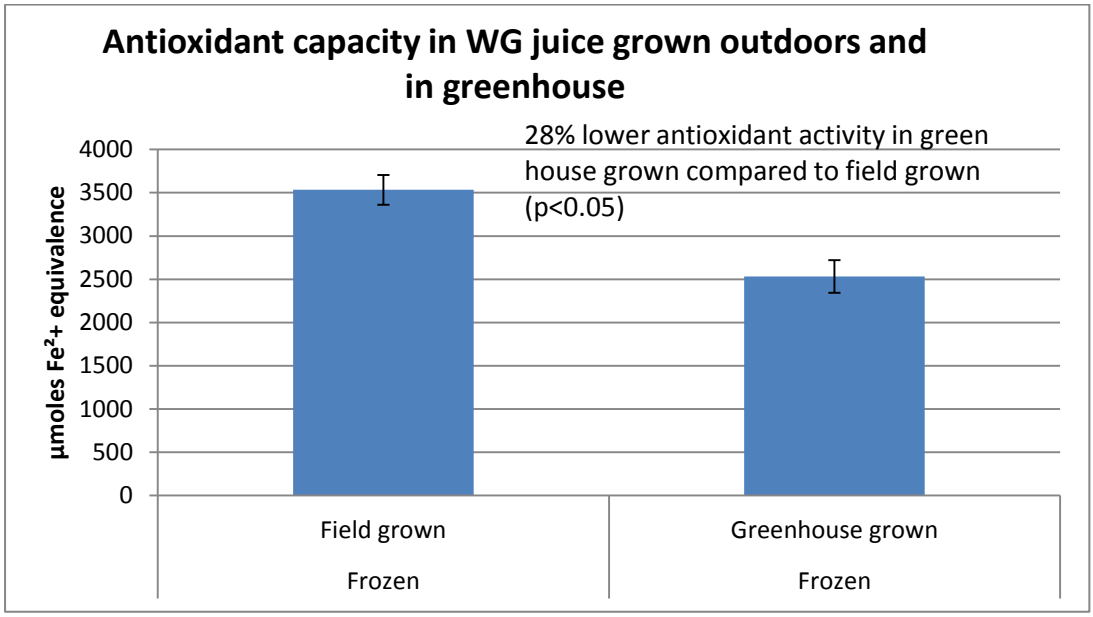


Fig.4 Effects of growing conditions (outdoor field grown and greenhouse grown) on antioxidant capacity of WGJ extract using FRAP. Value of frozen is calculated as a mean of measurements across the 2 year period and greenhouse grown are means of three measurements (Table 2).



Data Analysis – Antioxidant capacity

Figure 4: The samples from 2009, 2010 and 2011 are all of similar quantities which suggest that freezing does not affect antioxidant values over at least a 2 year period. Although freshly pressed WGJ appeared to show a higher antioxidant capacity than the frozen sample, this was not a significant difference *P=0.27*, due to the natural variation between samples. This suggests no significant loss in antioxidant capacity over time.

Figure 5: All field grown samples had higher Fe2+ equivalence values than the greenhouse grown sample. Field grown samples on average had a significant 28% higher Fe2+ equivalence value compared to greenhouse grown (p<0.05).

Discussion

With the increasing interest in functional foods it is surprising that only a limited amount of information exists regarding the total antioxidative properties of wheat grass and the impact of post-harvesting technologies. Results indicate that freezing does not cause deterioration of chlorophyll content in WGJ. The post-harvest processing procedure followed therefore does not affect the medicinal potential of this chemical component (chlorophyll) and therefore part of the medicinal quality of this product.

The results show that freshly pressed WGJ has a higher antioxidant content although this was not statistically significant over the natural variation between samples. There does not appear to be a correlation between length of freezing time and lower antioxidant values. This suggests that antioxidants in WGJ remain stable after blast freezing and storing at -30ºC.

Similar results are found in a study on antioxidant activity in fresh, dried and frozen blueberries in which no significant difference was found between all three storage methods (Lohachoompol, Srzednicki, Craske, 2004). Further studies include comparisons between antioxidant capacity in fresh, frozen, jarred and canned vegetables. Results showed samples of frozen peas and spinach to have much higher antioxidant content than canned or jarred samples.

When Davey *et al*, 2000, compared processing techniques most relevant to vegetables, i.e: canning, freezing and dehydration, they found that losses are greatest during dehydration and lowest during freezing. Storage of unripe canola seed in a freezer for up to one month prior to measuring the chlorophyll content did not alter the chlorophyll level in the seed. Seeds were frozen while still in the pods as well as after removal with no change in chlorophyll content over time. (Ward,Scarth & Daun, 1992)

The variation in different levels of chlorophyll and antioxidants could be due to external growing and harvesting conditions such as weather and temperature as opposed to those factors encountered prior to these events such as freezing. Freezing is considered to actively stop biological processes and therefore preserve the condition and activity of the chemical constituents contained within. This study resonates with this theory as well as others performed on fruit that verify freezing as a conservation method to maintain the physico-chemical stability and antioxidant potential of blackberry nectar (De Araujo, 2009). Further studies on storage of frozen foods show frozen products stored at -30ºC contained 6-29% more chlorophyll than those stored at -20ºC (Lisiewska, 2011).

The significantly lower levels of chlorophyll (48% lower) and antioxidant capacity (28% lower) in the greenhouse grown samples show that outdoor grown wheatgrass juice has a higher medicinal value in this context (chlorophyll and antioxidant content). The lower chlorophyll content in indoor grown wheatgrass juice is likely to be due to the lack in natural un-obscured sun light and the shorter growth time before harvesting (growth time for indoor grown WG is 2 weeks compared to 2-3 months for field grown). The lower antioxidant capacity in indoor, hydroponically grown WG is likely to be due to the lower levels of nutrients available to the plants when cultivated under these artificial circumstances compared to outdoor WG which grows in organic soil for a much longer time period.

This study presents results from a small component part of the potential medicinal capacity of WGJ. Further research into the behaviour of a wider sample of chemical components and their behaviour once frozen and indeed in field conditions would provide valuable insight into the physiology of this plant with reference to its medicinal quality.

A database compiled shows the antioxidant capacity of 3100 foods (Carlsen, Halvorsen,Willett,Phillips, 2010). It is the most comprehensive of its type, but unfortunately, units are in mmol/100g which makes a comparison difficult. It would be useful to potentially add these findings to this database in the future.

Acknowledgements

This work was greatly facilitated by my project advisor Dr. Maria Donkin and great thanks is extended to her for her time and expertise. Further thanks to Dr.Britt Cordi who provided samples from LiveWheatGrass Ltd and spent a great deal of time and effort in helping to bring this research to fruition. Laboratory work was greatly helped by Angela Harrop and the

University of Plymouth who provided all the necessary equipment and research material that made this project possible. Many thanks to all those involved.

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