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Therapeutic compounds as an onion quality parameter

P Brown, et al
TIAR and IHD

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Final Report

Therapeutic Compounds as an Onion Quality Parameter

Project number: VG99054

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CONTENTS

Industry Summary

Onions are one of Australia's largest export vegetable crops, with 50,000 tonnes exported in 1997/98 from a total production of 225,000 tonnes (1). In order to remain competitive within export markets, and to increase domestic consumption, the onion industry must continue to improve product quality and seek new marketing opportunities based on product quality. Increasing consumer awareness of the health benefits that can result from eating vegetables represents an aspect of product quality that has received little attention to date but may provide a significant market advantage to industries which can prove the therapeutic value of their product. Such products are being described as functional foods (foods containing significant levels of biologically active components that impart health benefits beyond basic nutrition) and their therapeutic benefits may be an important selling point for consumers. The health benefits of eating onions and other related allium species are widely recognised, and have been attributed to two main groups of compounds in onions, the flavonoids and sulfur compounds. The objectives of this one year feasibility study were to assess the levels of sulfur compounds and flavonoids in Australian onions, and to compare these with levels in onions produced in other countries and in stored onions.

Onions from thirty commercial crops grown in Tasmania were used to assess the levels of therapeutic compounds and to determine variability within crops and between crops. These values were compared to published values for European and US onions, and also to values recorded for onions stored for six months. The first phase of the project involved development and validation of analytical methods for therapeutic compounds. Despite extensive method development work, procedures for determining the level of sulfur compounds (cysteine sulfoxides) were not completed and validated by the conclusion of the project. Methods for analysis of flavonoids were developed and used to determine the concentration of this group of compounds in onion samples.

Analysis of flavonoid composition revealed only quercetin to be present in significant concentrations. More thorough investigation of the extracts may have allowed identification of trace amounts of other flavonoids (eg. kaempferol and isorhamnetin, which have been previously described in onion extracts) but the quercetin concentration effectively represented total flavonoid concentration. Quercetin was present either linked to one or two sugar molecules (monoglycoside and diglycoside forms) or as a free molecule (aglycone). In addition, a previously unreported methylated quercetin glycoside was identified in the onion samples. Quercetin monoglycosides (including methylated monoglycosides) were the most prevalent form of flavonoid present in all onion samples examined. The monoglycosides represented 85 to 90% of total flavonoid concentration. Quercetin diglycosides were present in smaller concentrations, representing approximately 10% of total flavonoid concentration while the free or aglycone form of quercetin made up only 1 or 2% of total flavonoid levels. The form of quercetin present in bulbs is likely to be an important factor in determining the health benefits derived from consuming onions. The aglycone form is readily absorbed in the gut but the glycosides must be converted to the aglycone form to be absorbed. While the gut microflora may remove the sugar molecules from the glycoside forms to release the aglycone, the rate of conversion and uptake in the gut requires further study.

The concentration of flavonoids found in Australian onions was five to ten times higher than that reported in overseas onions. The data generated in this project represents the first

reported detailed analysis of flavonoid content in Australian onions. Direct examination of flavonoid content in onions from other countries was not possible in the project due to strict quarantine regulations which prevent the import of onion bulbs from most foreign countries. However, the flavonoid concentrations reported in this study can be compared with previously published levels reported in overseas studies. The highest concentration of quercetin in Australian onions in the present study is nearly 10 times higher than the highest previously published concentration. The concentration in all samples analysed was higher than previously reported data.

The conclusion drawn from the comparative analysis was that the climate and agronomic practices (the combination of high light levels, including UV light, field curing practices and varietal selection) used in Southern Australian onion production regions stimulates the accumulation of these compounds. Previous studies have shown that exposure to light stimulates flavonoid accumulation, and that field curing leads to increased flavonoid levels in comparison to artificially cured bulbs, while brown onion varieties are also richer sources of quercetin that many other varieties. Some caution must be observed in interpreting the results of this study as the comparison between Australian and overseas data is not a direct one. Sampling and analysis methods vary between the studies, and therefore the results may reflect differences in experimental methods rather than true differences between samples. In particular, the method of analysis in this study was state of the art, and allowed direct identification of glycoside forms of quercetin from extracts. Previous studies have relied on acid hydrolysis of glycosides to determine total quercetin levels. Acid hydrolysis results in some degradation of flavonoids, and despite efforts in previous studies to limit this loss, the concentrations determined may have underestimated the total concentration due to degradation of flavonoids during hydrolysis. While these factors must be considered when interpreting the comparative data, it is unlikely that differences in methodology could account for the magnitude of the difference between flavonoid concentrations in Australian and overseas bulbs. The conclusion from this preliminary study is therefore that in southern Australia results in significantly higher concentrations of quercetin than in onions grown in other countries.

The concentration of flavonoids in individual onion bulbs from a single crop varied significantly, while variation in average concentrations between crops was lower but still significant. Variation between bulbs may reflect differences in exposure to light during bulb development or, more likely, field curing where windrowing of bulbs may partially cover a proportion of the bulbs in the crop. Variation between crops may be due to different management practices (spacing uniformity, row alignments, fertilization, irrigation etc) or microclimate. Further examination of the sources of variation may reveal management strategies to maximise the flavonoid concentration in bulbs.

Examination of changes in flavonoid concentration during storage revealed a 20% decrease over a six month storage treatment. Previous studies have shown similar declines in bulb quercetin content and indicate that fresh product has more therapeutic value than stored product.

Technical Report

Introduction

A review of the onion industry in 1993 by the Horticultural Policy Council listed four key strategies required for the industry to maintain and improve its level of competitiveness (2). Two of these strategies, improved levels of domestic consumption and product innovation to expand marketing opportunities, have been addressed in this project.

In major markets such as Europe and Japan, Australian onions are competing against Southern hemisphere producers, particularly New Zealand, as well as stored domestic product. In both these markets, as well as the Australian domestic market, consumers are increasingly becoming health conscious. Previous work on garlic, a crop closely related to onions, has indicated that Australia is uniquely positioned to produce plants with high levels of sulfur compounds beneficial to human health (3). In addition, the levels of flavonoids, the second important group of therapeutic compounds in onions, has been shown to be increased by high light levels and UV light exposure (4) such as that experienced in onion production regions in southern Australia. The documented presence of high levels of therapeutic compounds in Australian onions would provide the industry with an opportunity to gain a marketing advantage in competitive export markets as well as in the domestic market. Diversification of existing markets to include supply to the pharmaceutical industry may also be possible if the concentration of therapeutic compounds is high.

Marketing campaigns based on health promoting attributes of food products, for example the promotion of tea based on anti-oxidant properties, have become popular over the past decade as consumers have become more health conscious. Onions and other edible allium species are widely considered to be 'healthy foods' and several epidemiological studies have shown that people who regularly eat garlic and onion have lower risk of cancers of the gastrointestinal tract (5). In addition, onions are commonly eaten raw, for example in salad preparations, or lightly sauteed in stir fries, ensuring that the cooking process does not reduce the levels of therapeutic compounds consumed. The relative contribution of onions and other vegetables and fruits to the dietary intake of anti-oxidants has been suggested to be significant, particularly amongst the younger generation who have a low intake of tea and red wine, the two main dietary sources of flavonoid anti-oxidants. Promotion of onion consumption based on health benefits is therefore valid and the assessment of levels of therapeutic compounds in onions represents a novel method of product quality differentiation.

Therapeutic compounds in onions

The two main groups of compounds in onions that have been associated with therapeutic properties are the flavonoids and sulfur compounds (cysteine sulfoxides). While the sulfur compounds represent a group of chemicals who's main dietary source is the allium vegetables, the flavonoids (anthocyanins, flavonols, flavones, catechins, and flavonones and their glycosides) are phenolic compounds present in all crop species. Current interest in the contribution of bioactive components of food in disease prevention has lead to numerous studies of flavonoid concentrations and activities in foodstuffs while less attention has been given to the sulfur compounds.

Flavonoids are a large family of over 4000 secondary plant metabolites, comprising anthocyanins, flavonols, flavones, catechins, and flavonones. Many flavonoids are present in plant tissues in relatively high concentrations as sugar conjugates or glycosides. Flavonols and flavones are flavonoids of particular importance as they have been found to possess antioxidant and free radical scavenging activity in foods, and epidemiological studies have indicated that their consumption is associated with reduced risk of cancer and cardiovascular disease (6). Vegetables, fruit and beverages are the main dietary sources of the flavonols and flavones. In addition to natural food sources, a number of therapeutic preparations containing flavonols and flavones have been promoted as treatment for a variety of circulatory disorders, hypertension and as a co-factor with vitamin C (7). Kaempferol, quercetin, myricetin, apigenin and luteolin are the major flavonols and flavones derived from fruit and vegetables. A list of important flavonoids, sources and antioxidant activity is shown in table 1.

Table 1 – Relative antioxidant activities and dietary sources of flavonoids.

Antioxidant	Sources	Antioxidant activity (mM)*
Anthocyanidins		
Oenin	Black grapes/red wine	1.8±0.02
Cyanidin	Grapes, raspberries, strawberries	4.4±0.12
Delphinidin	Aubergine skin	4.4±0.11
Flavon-3-ols		
Quercetin	Onion, apple skin, berries, black grapes, tea, broccoli	4.7±0.10
Kaempferol	Endive, leek, broccoli, grapefruit, tea	1.3±0.08
Flavones		
Rutin	Onion, apple skin, berries, black grapes, tea, broccoli	2.4±0.12
Luteolin	Lemon, olive, celery and red pepper	2.1±0.05
Chrysin	Fruit skin	1.4±0.07
Apigenin	Celery and parsley	1.5±0.08
Flavan-3-ols		
(Epi)catechin	Black grapes/red wine	2.4±0.02
Epigallocatechin	Teas	3.8±0.06
Epigallocatechin gallate	Teas	4.8±0.06
Epicatechin gallate	Teas	4.9±0.02
Flavanones		
Taxifolin	Citrus fruit	1.9±0.03
Narirutin	Citrus fruit	0.8±0.5
Narigenin	Citrus fruit	1.5±0.05
Hesperidin	Orange juice	1.0±0.03
Hesperetin	Orange juice	1.4±0.08
Theaflavins		
Theaflavin	Black tea	2.9±0.08
Theaflavin-3-gallate	Black tea	4.7±0.16
Theaflavin digallate	Black tea	6.2±0.43
Hydroxycinnamates		
Caffeic acid	White grapes, olive, cabbage, asparagus	1.3±0.01
Chlorogenic acid	Apple, pear, cherry, tomato, peach	1.3±0.02
Ferulic acid	Grains, tomato, cabbage, asparagus	1.9±0.02
p-Coumaric acid	White grapes, tomato, cabbage, asparagus	2.2±0.06

*Measured as the Trolox equivalent antioxidant activity (TEAC) – the concentration of Trolox with the equivalent antioxidant activity of a 1 mM concentration of the experimental substance

Onions and other allium vegetables have been reported to contain high levels of flavonoids. Quercetin and its glycosides are the major flavonoids in onions, with kaempferol and isorhamnetin also identified in trace amounts (8). Other studies have suggested that only quercetin and its glycosides are present in onions (6). The amount of quercetin glycosides in onions is much larger than that in other vegetables (9, 10) and the major glycosides are quercetin 3, 4′-diglucoside and quercetin 4′-monoglucoside (8).

While flavonoids have attracted significant interest across a range of food and beverage products, the sulfur compounds from onions have also been studied for their therapeutic properties. Flavour in onions is dominated by up to 80 unique sulfur compounds and several water soluble carbohydrates (11). Highly reactive sulphenic acids that are released from reaction of flavour precursors undergo spontaneous non-enzymic rearrangement and inter-reactions to produce a wide range of volatile products. In alliums, most of the sulfur is in the form of various non-protein amino acids which include the precursors of the volatile flavour compounds. These precursors are odourless, non-volatile amino acids of the general name S-alk(en)yl cysteine sulfoxides (12). For the brown onion cv. Pukekhoe Long Keeper (±)-S-1-propyl-L-cysteine sulfoxide is the predominant flavour precursor, with (±)-trans-S-1-propenyl-L-cysteine sulfoxide and (±)-S-1-methyl-L-cysteine sulfoxide in a minor role (13). These sulfur compounds have been shown to have antithrombotic (inhibitors of platelet aggregation), antibiotic and hypocholesterolaemic properties (11).

Factors influencing levels of therapeutic compounds

Recognition of the importance of therapeutic compounds in the diet has lead to investigation not only of major dietary sources of the compounds but also the sources of variability in concentration of therapeutic compounds within food products. As discussed above, onions and other vegetable alliums represent the only major source of cysteine sulfoxides while all plants contain flavonoid compounds. The amounts of flavonoids vary considerably between plant sources; in a study of 16 vegetables, total flavonoid content was shown to range from 0.13mg.kg^{-1} (fresh weight) in potatoes to 427mg.kg^{-1} in purple sweet potato leaves (14). Only purple and green sweet potato leaves (427 and 185mg.kg^{-1} respectively) and onion (264mg.kg^{-1}) had total flavonoid contents above 25mg.kg^{-1}. A second study reported quercetin contents ranging between 2 and 203mg.kg^{-1} in tomatoes and 11 to 911mg.kg^{-1} in lettuce and flavonoid content in celery from 24 to 226mg.kg^{-1} (6). Flavonoid contents in onion samples have been reported in the ranges 55.5 to 345mg.kg^{-1} (15), 185 to 634mg.kg^{-1} (6), 54 to 286mg.kg^{-1} (16), 28 to 82mg.kg^{-1} (4), 18 to 69mg.kg^{-1} (17), 544mg.kg^{-1} (18), 125 to 1495mg.kg^{-1} (19) and 284 to 486mg.kg^{-1} (9). Variation in flavonoid content in onions has been attributed to varietal differences, climate and soil factors, duration in storage, and position of the tissue sampled for analysis (skins, outer scales, inner scales).

Conflicting reports have been published on differences in flavonoid contents between different coloured onions. Hermann (19) reported higher concentrations of quercetin in red skinned than white skinned onions while Crozier et al (6) found no significant differences. Patil and Pike (15) reported slightly higher total quercetin concentration in 2 red onion cultivars than 4 yellow onion cultivars, with white onions having significantly lower contents than the coloured onions. Regardless of onion type, the highest flavonoid concentrations have always been found in the dried skins and outer scales of the bulb. This distribution in quercetin content has been attributed to light exposure (15). Sun exposure has been shown to be the primary factor determining quercetin content in wine grapes, and

the accumulation of flavonols in response to light is thought to be a screening response intended to protect the tissue from UV radiation damage (20). Exposure of plant tissue to UV radiation has been shown to induce the accumulation of flavonoids (21).

Site of growth of onions has been reported as the major factor in determining quercetin content, with soil type and stage of growth having only a small effect (4). Significant differences in quercetin contents in seven genotypes were recorded between two locations, with higher contents consistently recorded in one location. The difference was suggested to be due to higher light levels at that location. Large variations in quercetin content in onions sampled from different locations at different times of the year (6) are also consistent with climatic factors influencing flavonoid accumulation. Evidence has also been presented that field curing of onions for 3-4 days resulted in accumulation of higher concentrations of quercetin (4). The same study also presented limited evidence that nitrogen stress (low soil nitrogen levels) could stimulate increase quercetin accumulation.

Little data on the effects of storage duration and conditions on quercetin content has been published. Bulbs stored for five months at 5, 24 or 30 degrees C showed little change in flavonoid levels, but a trend of slightly increased concentrations over 2 to 3 months storage was observed (16). Controlled atmosphere storage had little effect on quercetin content. Cooking of onions decreases the concentration of quercetin, with frying, boiling and microwaving reducing the concentration by 21%, 75% and 64% respectively (6). It was suggested that quercetin may be extracted from onion tissue by hot water more efficiently than with hot oil.

As is the case with flavonoids, the concentration of cysteine sulfoxides in onions is influenced by genetic and environmental factors and also varies with developmental stage. The potential of the onion for flavour production is closely linked to variety although that potential may be modified by the environment (22). Concentration of cysteine sulfoxides in brown onions (cv. Pukekohe Longkeeper) has been reported in the range 5.6 to 11.7 $\mu mol.g^{-1}$ fresh weight (23). In comparison, the white onion cv. Dehyso, which has been bred for high flavour for the dehydration industry, has a concentration of 17.8$\mu mol.g^{-1}$ (23). The flavour precursors have been found in higher concentrations in the inner scales of the bulb, grading to negligible contents in the outermost scales (24).

Climatic and crop management practices have been shown to influence cysteine sulfoxide content. Excess irrigation and nitrogen may produce rapid growth and poor flavour, while onions grown with restricted water supply have a decreased yield but are richer in flavour (22). Sulfur nutrition is obviously important in the accumulation of the sulfur containing flavour precursors. Available sulfur is used preferentially for growth, and when growth requirements have been met, sulfur becomes available for incorporation into the biosynthetic pathway leading to cysteine sulfoxides (22). Experimental work has shown that flavour strength increases in response to sulfur applications (25). Application of elemental sulfur, calcium sulphate, and ammonium sulphate has been shown to increase the concentration of cysteine sulfoxides in onion bulbs.

Storage of bulbs for 3-4 months did not reduce the concentration of cysteine sulfoxides, but storage for longer duration may lead to reduced levels if sprouting and the consequent increase in respiration rate occurs. Preparation of onion tissue for consumption reduces the flavour precursor concentration by promoting the alliinase catalysed conversion of cysteine sulfoxides to produce the characteristic volatile products associated with cut onions.

Cooking is also likely to lead to a further decline in the concentration of cysteine sulfoxides in the tissue.

Methods and Results

The project aimed to develop and optimise methods for determining the concentration of flavonoids and cysteine sulfoxides in onions, and to apply the methods to commercial onion samples grown in Tasmania. Samples were selected to allow assessment of the level of variability between bulbs within crops and between crops, and to compare concentrations with previously published data for onions grown in other regions. It was originally intended that samples from overseas countries would be analysed, but quarantine restrictions on entry of onions from all major production regions precluded this analysis. Detailed analysis of changes occurring during storage of onions was undertaken in place of the analysis of overseas samples. This allowed conclusions to be drawn concerning relative concentrations expected in onions in Northern Hemisphere markets where fresh Australian onions compete with stored domestic product.

Crop and sample selection

All onions analysed in this project were sourced from Field Fresh Tasmania and were grown in North and North West Tasmania. The brown onion cultivar Creamgold was used in the study and selection of a single cultivar permitted evaluation of non-genetic factors influencing the concentration of flavonoids and cysteine sulfoxides. Onion crops were grown and harvested commercially and samples were collected at intact at the Field Fresh packing facility near Devonport, Tasmania. All crops were field cured (lifted from the soil and left to dry in the paddock until harvest) for approximately ten days. Crops were selected to cover the range of Tasmanian planting locations (Northern Midlands region to far North West Coast) and harvest dates (January until April).

Onion samples were collected from 30 crops. A minimum of 30 onion bulbs was collected from each crop and transferred to the Agricultural Science laboratories at the University of Tasmania. Bulb samples for flavonoid determination were freeze dried (lyophilised) within 2 days of arrival at the laboratory, while samples for cysteine sulfoxide analysis were couriered to the Knoxfield laboratories of DNRE. Skins were removed from all bulbs prior to lyophilisation, and the outer three scales were sampled. Remaining bulb tissue was discarded. Lyophilised tissue was stored at -18°C until extraction and analysis of flavonoids was undertaken.

Three of the onion crops were selected at random for examination of the variation in composition between bulbs within a crop. Eight individual bulbs from each crop were analysed for flavonoid concentration.

Variation between crops was assessed by determination of flavonoid concentration in pooled samples of bulbs from each crop. Each sample comprised bulb tissue from ten onions from the crop. The ten bulbs were quartered, with one quarter from each bulb selected, the three outermost fleshy scales removed then roughly chopped, mixed and a subsample of the tissue taken for analysis.

The influence of storage duration on bulb flavonoid composition was examined over a six month period using bulbs from a commercial onion crop. Bulbs were stored under ambient temperature conditions at the University of Tasmania and sampled at monthly intervals for flavonoid analysis. At each sample date six onions were taken from the storage conditions,

skins were removed and the onions roughly chopped. Triplicate samples were selected from the bulk tissue and were lyophilised prior to flavonoid analysis.

Flavonoid determination

Assessment of previously published methodology
Various conditions and times have been recommended in the literature as optimal for the hydrolysis of quercetin mono- and diglycosides in onion (*Allium cepa*) (eg 4, 6, 9). Hydrolysis of glycosides to quercetin aglycone is necessary for quantification due to the wide range of possible isomers, particularly of diglycosides, and the absence of readily available standards and relative molar absorbtivity data. Using ion-trap HPLC-electrospray ionization-mass spectrometry (HPLC-ESI-MS) quercetin-based compounds may be readily resolved and identified as such, however, quantification remains problematic.

This current preliminary study has found that for assessment of quercetin content based on hydrolysis of glycosides, some onion crop samples may require a more intensive sample preparation than what has been previously reported in the literature. In this preliminary study, rapid extraction and hydrolysis methods (above references) either failed to fully hydrolyse samples, or, when hydrolysis of glycosides was demonstrated, resulted in a loss of aglycone. 1 g lyophilised samples were extracted in 20 ml 80% MeOH (with 2.5 mg/L kaempferol as an internal standard), which was passed through filter paper, followed by a 0.45 µm Alltech filter. Separation and detection was performed using a Finnigan LCQ coupled to a Waters Alliance 2690 HPLC which was equipped with a Waters Nova –Pak 3.9 x 150 mm C18 column and an Alltech Econosphere C18 guard cartridge. For mass spectrometery, the heated capillary and voltage were maintained at 170°C and 20 kV respectively. The mass/ charge range monitored was 100-900. An isocratic mobile phase was used, consisting of 50% MeOH (2% acetic acid)/ 50% water (2% acetic acid). The flow rate was 0.8 ml/min, and injection was 10 µl. UV-vis analyses were performed using a Waters 996 photodiode array detector. Hydrolysis of glycocydes was performed by adjusting the extract to1.2 M with HCl, and incubating the solution at 80°C.

The above hydrolysis method proved unsuitable due to instability of the quercetin aglycone which became evident after hydrolysis periods greater than 60 min. Time intervals less than 60 min produced unsatisfactory hydrolysis. The kaempferol internal standard was substantially degraded after 60 min of hydrolysis.

Table: Relative ion quantities prior to, and following hydrolysis in 1.2 M HCl for various periods of time.

	Quercetin diglycosides	*Quercetin monoglycosides*	*Quercetin aglycone*	*Kaempferol standard*
Unhydrolsed	5.31×10^7	1.06×10^8	7.25×10^5	1.95×10^7
60 min	1.82×10^6	7.71×10^6	3.74×10^6	3.62×10^6
90 min	1.68×10^5	6.63×10^4	Negligible	Negligible
120 min	Negligible	Negligible	Negligible	Negligible

Extraction and analysis methodology
All analyses used the outer 3 scales (rings) from sampled onions, with all skin tissue removed. The onion tissue was lyophilised prior to extraction and analysis.

0.3 g lyophilised material was suspended in 7 ml 80% MeOH (HPLC grade) containing 5 µg/ml kaempferol (Sigma Chemical Co.) in new 21 ml scintillation vials. The sample was agitated for exactly 5 minutes and poured into a glass syringe with a round filter paper disc (Whatman 113) in the bottom of the syringe chamber and an Alltech 0.45 µm syringe filter attached to the base of syringe. Flow was started by depressing the syringe plunger. Eluent was collected in 2.5 ml GC vials.

The HPLC system used was a Waters Alliance 2690 equipped with a Waters Nova –Pak 3.9 x 150 mm C18 column and an Alltech Econosphere C18 guard cartridge. The mobile phase program used was 50% MeOH (2% acetic acid)/ 50% water (2% acetic acid) isocratic for 2 min, ramped to 80% by 6 min, followed by a rinse program after 8 min for 5 min. The flow rate was 0.8 ml/min, and the injection volume was 10 µl. UV-vis detection/ quantification was conducted using a Waters 996 photodiode array detector.

Quantification of flavonoids was by standard curve of 3 x 4 points containing both quercetin and rutin (quercetin rutinoside – quercetin +(rhamnose + glucose residues)). Pure standards were obtained from Sigma Chemicals. For the above extraction method, the recommended standard curve concentrations are 2 ng/µl, 4 ng/µl, 8 ng/µl, and 16 ng/µl. Quercetin monoglycoside standards were not availablein this study but can be obtained from Extrasynthese (France).

Cysteine sulfoxide determination

The analysis of cysteine sulfoxide compounds in onions is complex. The key issues are:

1. the compounds are volatile and therefore extraction and analysis times need to be minimised.
2. there are a wide range of other sulfur-based compounds present in onion tissues that may interfere with the analysis of the target compounds if not properly removed/deactivated prior to the analysis.

At the start of the project, there were two HPLC methods used widely in the scientific literature for analysis of cysteine sulfoxides in onions- one method was developed by the University of Georgia, USA; and the other was developed by Crop and Food Research, NZ. Prior to starting the current project, Sam Sterling had been in regular communication with scientists conducting similar analyses at the University of Georgia (UGA)- David Kopsell and Bill Randle, hence the Georgia method was initially chosen to conduct the onion analysis. A detailed method was obtained from UGA (see below). A C18 column, guard columns and other attachments were purchased and extraction optimised as described below.

The Georgia method (Bill Randle and David Kopsell, University of Georgia)

1. Sample Preparation

A wedge is cut from the onion and frozen (-20° C) in 80% methanol overnight. The onion tissue is then strained and fresh extract (80% methanol) is added, the sample and the initial extract are returned to the freezer for 4 hours. The tissue is again strained

and this time extracted in 80% ethanol for 2 hours in the freezer. The onion tissue is strained and discarded, the ethanol extract is added to the two methanol extracts and returned to the freezer until ready to use.

2. Internal standards

Three internal standards are added to the samples for HPLC quantification: L-glutamyl-L-glutamic acid (g-glu glu), S-methyl glutathione (S-MeG), and Butyl-L-cysteine sulfoxide (BCSO). These concentrations are added to each sample: 0.2 mg of g-glu glu per gfw (gram fresh weight of tissue), 0.5 mg of S-MeG per gfw, and 1.0 mg BCSO per gfw.

3. Concentration of extracts

15.0 ml of sample extract is placed into 40 ml test tubes. Internal standards are added to each sample and vortexed. The test tubes are placed in a drying rack in the fume hood and blown to dryness. Upon dryness, the samples are capped and placed into the -20° C freezer. Samples are rehydrated in 1.0 ml of Milli-Q water, vortexed, and transferred to a 1.5 ml capped centrifuge tube.

4. Ion exchange of samples

Acetic acid is prepared in two concentrations: 0.1 M and 5.0 M. Ion exchange columns are prepared using Bio-Rad anion exchange gel (AG 1-X8, 200-400 mesh, acetate form). 0.5 ml of the sample is loaded onto the column. 10ml of 0.1 M acetic acid is added. This is collected in a 20 ml scintillation vial and it contains the S-alk(en)yl-L-cysteine sulfoxides, or flavor precursors. 14.0 ml of 0.2 10.0 ml of 5.0 M acetic acid is loaded onto the column to remove any compounds still bound to it. After that has dripped through, flush the column with 30-40 ml of Milli-Q water to regenerate the column. It is now ready for the next sample. The eluted fractions are dried down, capped and stored in the freezer.

5. Derivitization of Samples

The samples are rehydrated with 1.0 ml of Milli-Q water, and vortexed. 100 μl of the sample is pipetted into a 1.5 ml plastic centrifuge tube and samples concentrated in a vacuum drier. (This process may take up to 5 hours). 250 μl of a 1:1:1 solution of ethanol:water:triethylamine is added to each sample to counter the acidity. Samples are again dried down in the vacuum drier. (3+ hours). 100 μl of a 7:1:1:1 solution of ethanol:water:triethylamine:phenylisothiocyanate is then added to each sample and the tubes flushed with nitrogen gas, capped immediately, and allowed to stand at room temperature for 20 minutes. After 20 minutes, the samples are uncapped and dried down again in the vacuum drier (2 hours). Once dry, the samples can be stored in the freezer for up to 30 days.

6. Running the samples on the HPLC

The samples are rehydrated with 1.0 ml of a 2:7 acetonitrile:water solution and vortexed. The sample is pipetted into the HPLC sample vials.

A Waters 2690 HPLC separations module with 996 Photodiode array detector and an autosampler is used for analysis. A RP-C18 column (5 micron, 250 x 4.6-mm) is used, with temperature maintained at 30°C. 40 μls of sample is injected onto the column and compounds are detected at 254 nm. Mobile phase consists of 0.14 M sodium acetate with 0.05% TEA buffered to pH 6.35 with acetic acid (A) and 60% acetonitrile (B). HPLC gradient begins with 85% A, 15% B and linearly decreases over 21 minutes to a

concentration of 45% A, 55% B. It continues downward to 0% A, 100% B over the next minute and remains there for 14 minutes before re-equilibrating to the original conditions.

Results

Onion samples from ten crops across Tasmania were collected and stored at 0°C prior to analysis. Initially, the Georgia method was followed as outlined above. The results however, were not optimal. Difficulty in separating the resultant peaks, and in observing the correct peaks were the initial problems. The method was further developed using several methods:

1. the inclusion of external standards (methyl cysteine sulfoxide and propyl cysteine sulfoxide)
2. the use of methanol as a mobile phase
3. the purchase and use of a vacuum dryer
4. optimisation of the HPLC running conditions

The level of variability obtained in the results continued to be very high (even between repeat assays of the same sample), hence it was difficult to obtain any meaningful information on the differences between what were essentially very similar onion crops. That is, variability between sub-samples was too large to allow any differences between samples (between crops) to be expressed (ie. error margins were high, therefore any real differences between onion types was masked). The sources of this variability might have been:

1. the long extraction process in the Georgia method (up to a week to prepare the samples) may have led to degradation of the samples prior to analysis
2. variable conditions in and around the HPLC equipment- temperature fluctuations in the lab. were significant as the analysis was conducted during the summer and this can affect the chemistry of the target compounds

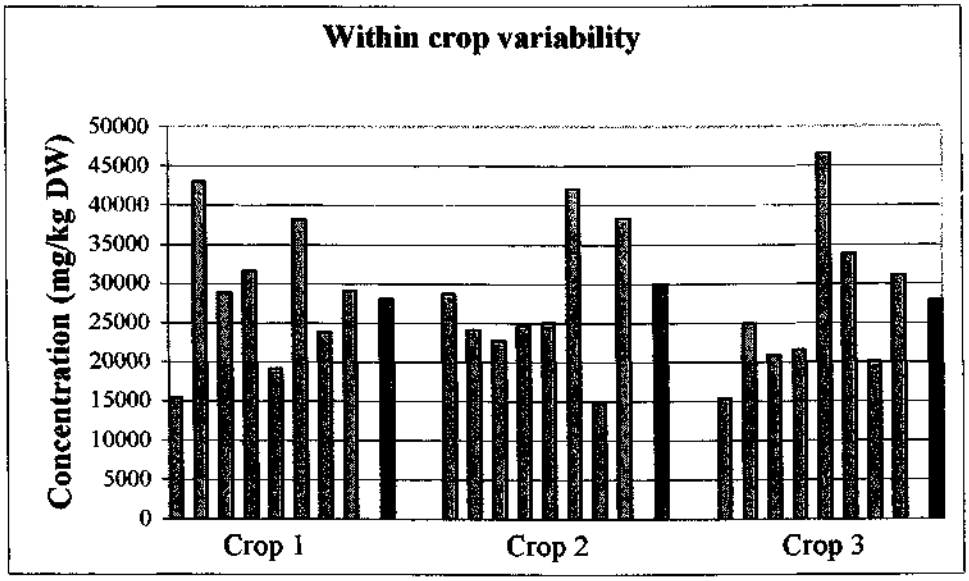
These difficulties have been partially overcome with the recent publication of a new method by Leonard Pike and Kil Sun Yoo from the Texas A&M University, USA, which takes an amino acid HPLC method and tailors it for cysteine sulfoxide analysis.
The extraction process is cut from a week, down to several hours and hence the method is very different to either of the methods previously in use (the Georgia and NZ methods) and has achieved much more consistent results in a variety of laboratories overseas. It is our intention to utilise this new method in the analysis of the onion compounds, but as this means going back to square one with the method development and purchasing different analysis equipment (a C8 column for example), it is not envisaged that these results will be available until after the end of April 2001.

Sources of Variation

Following the development and validation of the flavonoid analysis procedure, analysis of onion samples was undertaken to elucidate the level of variability between onions within crops and, using bulked onion samples, between crops.

Variation within crops

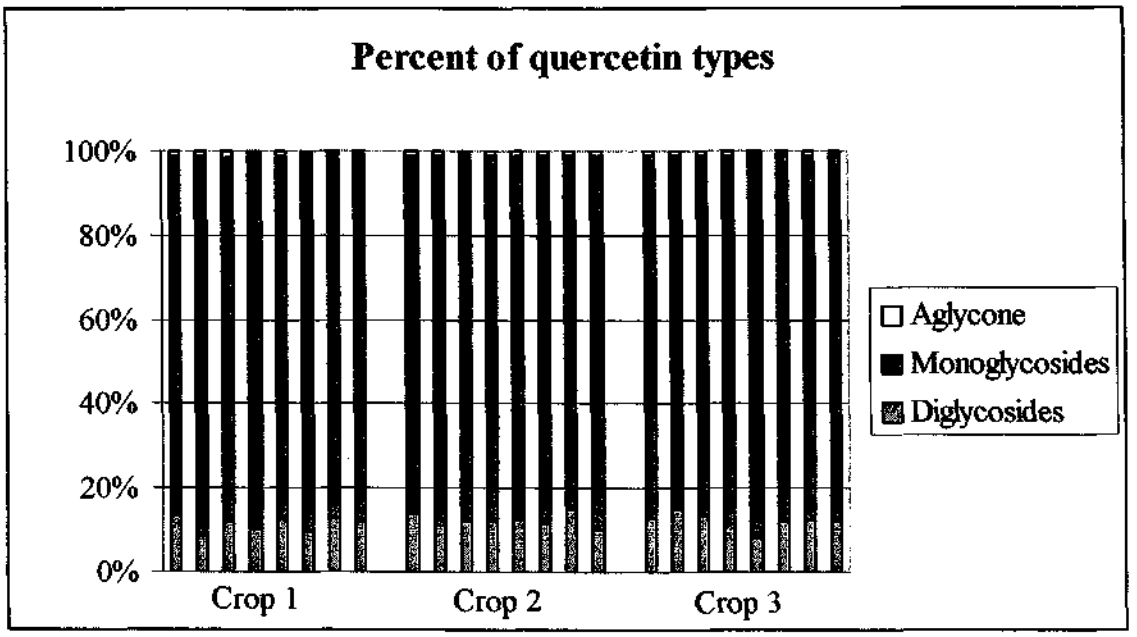
Determination of the degree of variability between individual bulbs within a crop is important for developing an appropriate sampling strategy for assessing differences in flavonoid concentrations between crops. Large variations between bulbs within a crop result in higher bulb numbers being required to determine the mean flavonoid concentration within a crop. Flavonoid concentrations were determined for eight individual bulbs (using only the outermost three fleshy scales from each bulb) from each of three crops. Results are expressed as milligrams of flavonoid per kilogram dry weight of bulb tissue (mg/kg DW).



Significant variation between bulbs was recorded in each crop. In all cases the total concentration of flavonoids (sum of aglycone, monoglycoside and diglycoside forms) ranged from a minimum of approximately 15000 mg/kg DW to a maximum between 42000 and 47000 mg/kg DW. The red bars in the above graph are the mean total flavonoid concentrations for the three crops. There was no significant difference in mean total flavonoid concentration between the three crops examined.

Analysis of flavonoid composition revealed only quercetin to be present in significant concentrations. More thorough investigation of the extracts may have allowed identification of trace amounts of other flavonoids (eg. kaempferol and isorhamnetin, which have been previously described in onion extracts) but the quercetin concentration effectively represented total flavonoid concentration. Quercetin was found in aglycone, monoglycoside and diglycoside forms. In addition, a previously unreported methylated quercetin glycoside was identified in the onion samples.

Quercetin monoglycosides (including methylated monoglycosides) were the most prevalent form of flavonoid present in all onion samples examined. The monoglycosides represented 85 to 90% of total flavonoid concentration. Quercetin diglycosides were present in smaller concentrations, representing approximately 10% of total flavonoid concentration while the free or aglycone form of quercetin made up only 1 or 2% of total flavonoid levels. The relative contributions of the various quercetin forms in each of the samples analysed are shown in the following graph.



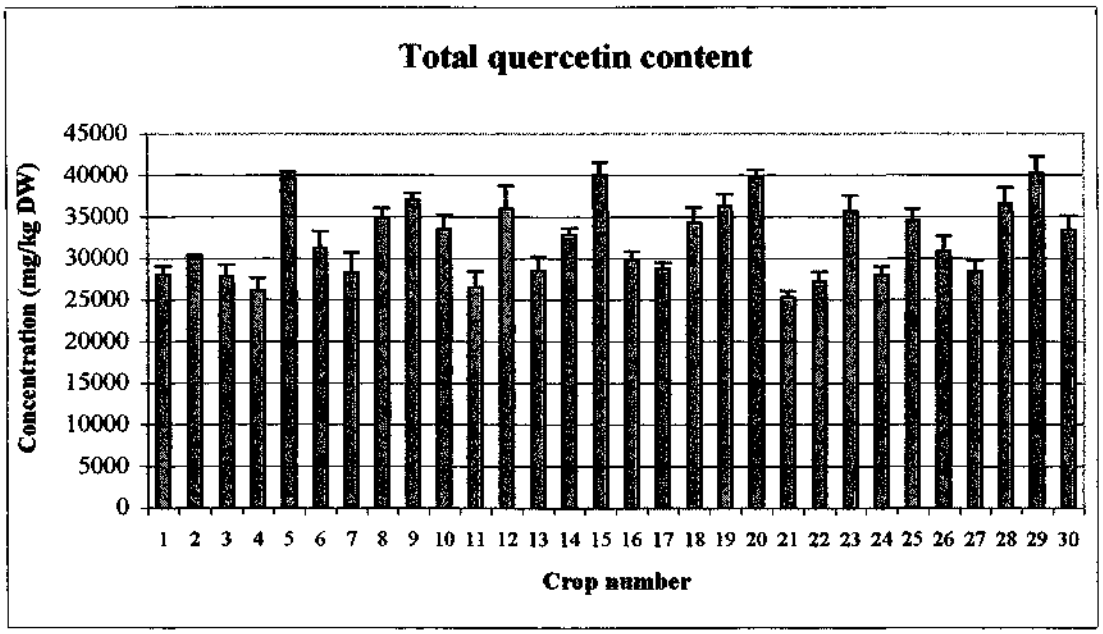
The concentration data for the quercetin forms in each crop is shown in the following table.

		Diglycosides	Monoglycosides	Aglycone	Total
Crop 1	1	2038	13219	149	15406
	2	3731	38876	353	42960
	3	3439	25069	354	28861
	4	3243	28110	216	31569
	5	2344	16547	174	19066
	6	3689	34253	248	38190
	7	3029	20549	166	23744
	8	3454	25418	207	29080
	mean	**3121**	**25255**	**233**	**28609**
Crop 2	1	3954	24527	207	28688
	2	2594	21288	204	24086
	3	2757	19788	149	22695
	4	2967	21405	239	24612
	5	3093	21683	184	24961
	6	4844	36806	409	42058
	7	2182	12265	156	14603
	8	3941	34126	284	38352
	mean	**3292**	**23986**	**229**	**27507**
Crop 3	1	1987	13219	151	15356
	2	3734	20866	264	24864
	3	2790	17859	202	20851
	4	2472	18777	180	21429
	5	3681	42651	227	46560
	6	3981	29634	235	33850
	7	2510	17327	160	19996
	8	3656	27160	215	31031
	mean	**3101**	**23437**	**204**	**26742**

Variation between crops

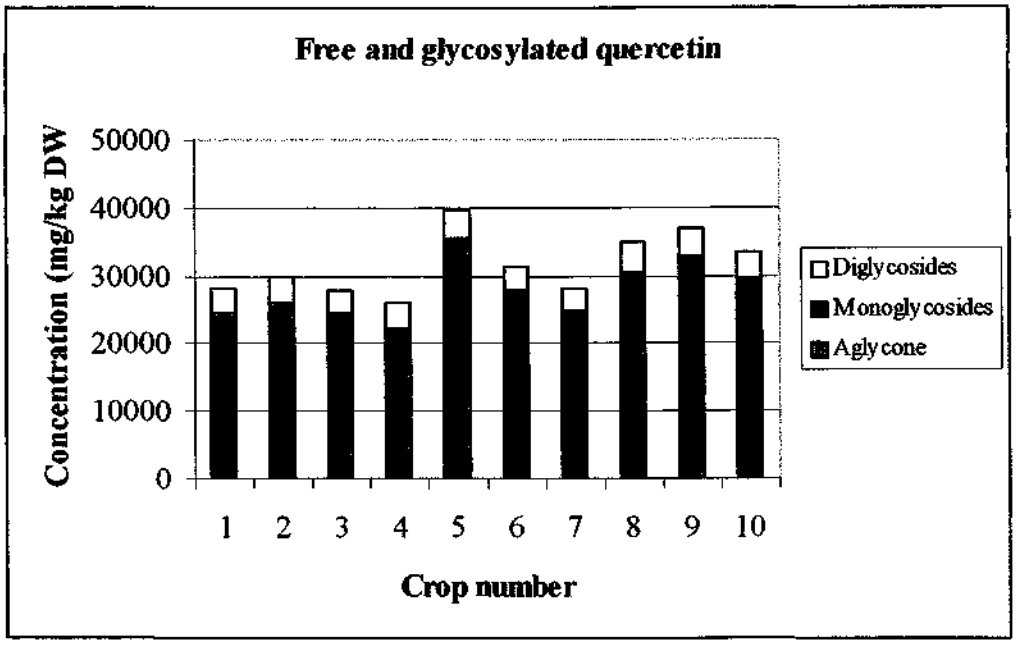
Following the assessment of variability in flavonoid concentrations within crops, a sample size of 10 bulbs per crop was selected to determine mean flavonoid concentrations in 30 crops. The sample size allowed accurate assessment of mean concentration and therefore comparisons between crops could be made. The sample preparation involved quartering on the 10 bulbs from each crop, removal of the outer three fleshy scales from one quarter of each bulb, pooling of the scales and roughly chopping into smaller pieces, freeze drying on the tissue, grinding and mixing of the pooled sample and finally taking triplicate samples of the tissue for flavonoid extraction and analysis. Variation around the mean was lower than would be expected from the analysis of individual onions within a crop due to the use of pooled samples.

Mean flavonoid concentration in Tasmanian onion crops ranged from approximately 25000 mg/kg DW to 40000 mg/kg DW.



Analysis of quercetin composition within crops revealed similar distributions to those reported in individual onions. Quercetin monoglycosides (including methylated monoglycosides) were the most prevalent form of flavonoid present in each crop. The monoglycosides represented 85 to 90% of total flavonoid concentration. Quercetin diglycosides were present in smaller concentrations, representing approximately 10% of total flavonoid concentration while the free or aglycone form of quercetin made up only 1 or 2% of total flavonoid levels. The form of quercetin present in bulbs is likely to be an important factor in determining the health benefits derived from consuming onions. The aglycone form is readily absorbed in the gut but the glycosides must be converted to the aglycone form to be absorbed. While the gut microflora may remove the sugar molecules from the glycoside forms to release the aglycone, the rate of conversion and uptake in the gut requires further study.

The concentrations of the quercetin fractions identified in ten representative crop samples are shown in the following graph.



Comparison between Australian and overseas onions

The data generated in this project represents the first reported detailed analysis of flavonoid content in Australian onions. Direct examination of flavonoid content in onions from other countries was not possible in the project due to strict quarantine regulations which prevent the import of onion bulbs from most foreign countries. However, the flavonoid concentrations reported in this study can be compared with previously published levels reported in overseas studies. Most of the published flavonoid concentrations have been expressed in milligrams flavonoid per kilogram (fresh weight) of bulb tissue. The results from the present study have been converted to a fresh weight basis using an average of 14% dry matter (86% moisture content) for the bulbs analysed.

Country	Onion type	Sample	Total	Authors
Australia	Brown	Outer scales (single bulbs)	2044 – 6014	
Australia	Brown	Outer scales (crop average)	3529 – 5622	
UK	Red	Skin removed, all scales	201	6
UK	White	Skin removed, all scales	185 – 634	6
US	Red	Skins	27320 – 30668	15
US	Red	Outer scales	324 – 584	15
US	Red	Inner scales	56 – 73	15
US	Yellow	Outer scales	121 – 324	15
US	Yellow	Inner scales	31 – 89	15
US	White	Outer scales	1.6	15
US	White	Inner scales	0.6	15
China	Not described	Outer scales	259	14
China	Not described	Inner scales	26	14
Netherlands	Not described	Skin removed, all scales	544	18
Germany	Not described	Skin removed, all scales	125 – 1495	19
US	Yellow	Skin removed, all scales	28 – 82	4

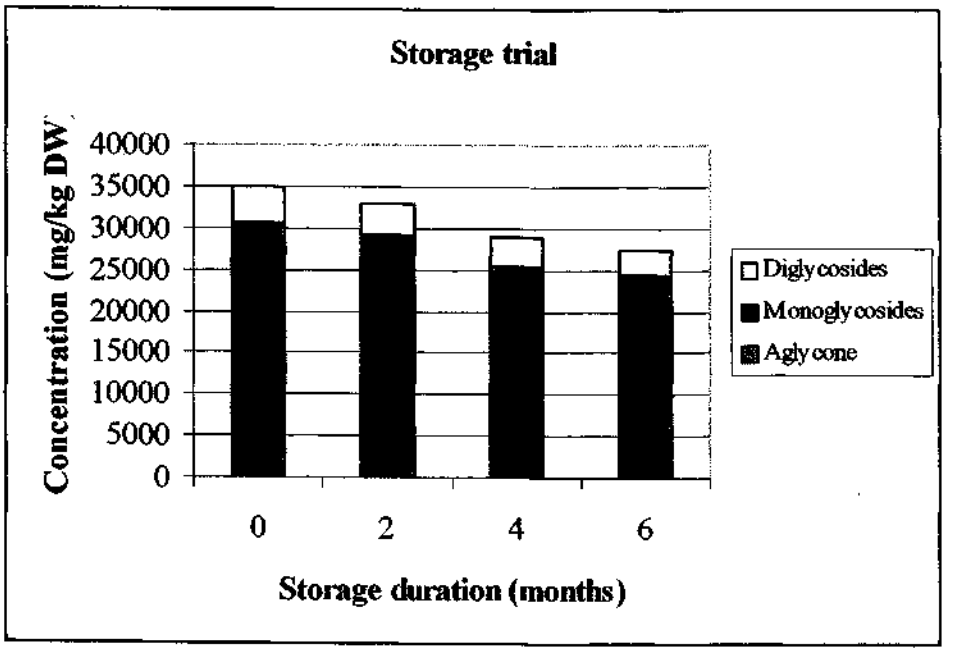
The highest concentration reported was in the skins of onions, but as this part of the bulb is generally not consumed the comparison between concentrations in scale tissue is a more revealing one. The highest concentration of quercetin in Australian onions in the present study is nearly 10 times higher than the highest previously published concentration. The concentration in all samples analysed was higher than previously reported data.

The comparison between data in this study and previous examinations of flavonoid concentrations in onions supports the hypothesis that the climate and agronomic practices used in Southern Australian onion production regions stimulates the accumulation of these compounds. The comparison is, however, not a direct one as sampling and analysis methods vary between the studies, and therefore some caution should be applied in interpreting the results. The sampling strategy used in this study involved collection of onions soon after harvest and assessing flavonoid concentrations in the outermost three scales. In contrast, previous studies have collected samples from the field or from commercial vegetable retailers (where presumable the time between harvest and sampling, and postharvest storage and handling practices, were not known) and different sections of the bulbs used in the analysis. The method of analysis in this study was state of the art, and allowed direct identification of glycoside forms of quercetin from extracts. Previous studies have relied on acid hydrolysis of glycosides to determine total quercetin levels. Acid hydrolysis results in some degradation of flavonoids, and despite efforts in previous studies to limit this loss, the concentrations determined may have underestimated the total concentration due to degradation of flavonoids during hydrolysis. While these two factors must be considered when interpreting the comparative data, it is unlikely that differences in methodology could account for the magnitude of the difference between flavonoid concentrations in Australian and overseas bulbs. The conclusion from this preliminary study is therefore that the combination of high light levels (including UV light), field curing practices and varietal selection in southern Australia results in significantly higher concentrations of quercetin than in onions grown in other countries.

Effect of storage

Previous studies (16) have suggested that flavonoid concentration does not change significantly in onion bulbs during storage. The effects of storage duration on quercetin concentration were examined in a six month trial using a sample of bulbs from a single onion crop. Bulbs were stored under ambient temperature conditions and sampled at 2 monthly intervals. A sample size of 10 bulbs was selected to determine mean flavonoid concentrations at each storage sample date. The sample preparation involved quartering on the 10 bulbs from each crop, removal of the outer three fleshy scales from one quarter of each bulb, pooling of the scales and roughly chopping into smaller pieces, freeze drying on the tissue, grinding and mixing of the pooled sample and finally taking triplicate samples of the tissue for flavonoid extraction and analysis.

The total quercetin concentration decreased during storage, with a 21% decrease recorded over a six month period. No significant change in the proportion of free and glycosylated quercetin was identified. The results are summarised in the graph below.



The size of the reduction in quercetin concentration is significant in two major respects. Firstly, it is not sufficient to explain the differences in flavonoid concentration reported in this study and those reported in previous studies, providing support for the conclusion that Australian onions are a superior source of flavonoids. Secondly, the reduction in quercetin concentration during storage demonstrates the superiority of fresh onions as a source of flavonoids in comparison to stored onions. The naturally high concentrations of flavonoids in fresh Australian onions thus would compare very favourably with low concentrations expected in stored northern hemisphere product in the major Australian export markets.

Conclusions

Australian onions appear to be a very rich source of the antioxidant flavonoid quercetin. Based on published epidemiological studies, consumption of moderate amounts of lightly sauted onions or raw onions in salads may reduce the risk of cancer and cardiovascular disease. The therapeutic benefits of onion consumption may be at least partially attributed to the concentration of quercetin in the bulbs, with sulfur compounds also likely to contribute to the health benefits derived from onion consumption. Vegetables, fruit and beverages are the main dietary sources of the flavonoids, and the high concentration of quercetin identified in Australian onions suggests that they would represent a significant percentage of flavonoid in the typical diet.

Variation in flavonoid content in onions has been attributed to varietal differences, climate and soil factors, duration in storage, and position of the tissue sampled for analysis (skins, outer scales, inner scales). Highest flavonoid concentrations have always been found in the dried skins and outer scales of the bulb and this distribution in quercetin content has been attributed to light exposure (15). Sun exposure has been shown to be the primary factor determining quercetin content, possibly as a screening response intended to protect the tissue from UV radiation damage (20). Exposure of plant tissue to UV radiation has been shown to induce the accumulation of flavonoids (21). Large variations in quercetin content in onions sampled from different locations at different times of the year (6) may be due to climatic factors, principally light levels, influencing flavonoid accumulation. Evidence has also been presented that field curing of onions for 3-4 days resulted in accumulation of higher concentrations of quercetin (4). The detection in this study of quercetin concentrations in onions five to ten times higher than previous reports may also reflect the effects of high light exposure (including UV light) and field curing in Tasmania.

Significant variability in quercetin concentrations between bulbs within a crop and between samples from different crops was recorded in this project. This suggests that crop management practices influence flavonoid accumulation in bulbs. Identification of effective strategies to maximise flavonoid accumulation would ensure that the natural advantages associated with the climate and varieties grown in southern Australia are fully realised.

The analytical methods developed in the study appear to offer significant advantages over previously published methods. The elimination of an acid hydrolysis step reduces degradation of flavonoids during sample preparation, thus increasing recovery and accuracy in the assay. Direct testing of quercetin concentrations in overseas onions using the new methodology would be advantageous to confirm that high quercetin levels detected in onions during this study reflect naturally high concentrations in Australian product rather than simply improved recovery and detection using the new methodology.

The identification of very high concentrations of an important group of therapeutic compounds in Australian onions may be a valuable marketing tool in an increasingly health conscious market. This preliminary study provides sufficient evidence to justify further work demonstrating advantages of consuming Australian onions based on composition of therapeutic compounds if the onion industry wishes to develop such a marketing strategy.

Communications:

This work, in conjunction with prior garlic research conducted by Agriculture Victoria, was presented to the International Society for Horticultural Science's "Alliums 2000" conference at the University of Georgia in October 2000 (see Appendix 1). Significant interest in the work was shown by a range of groups interested in onions internationally. These include the Pike/Yoo group at Texas A&M University who will host Ms. Sterling in the coming months to ensure any remaining problems with the analysis method are overcome, and scientists at the University of Wisconsin who have conducted medical tests to determine the efficacy of the cysteine sulfoxide compounds in the body, and who will likewise host Ms. Sterling in the next few months to promote discussion of the medical benefits and agronomic requirements for onions with high levels of cysteine sulfoxide compounds.

An article outlining the aims of the project and preliminary results was published in the industry journal Onions Australia in October 2000 (Appendix 2).

A short television segment on the project was filmed for the Channel 9/WIN TV program 'On The Land' and will be screened in the first half of 2001.

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Appendix 1:

Abstract from "Alliums 2000" conference

THE PRODUCTION OF HIGH HEALTH VALUE GARLIC AND ONIONS

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Abstract

The medicinal applications and health value of garlic and onions have been well documented, with scientific studies confirming long held beliefs as to the efficacy of a range of compounds found in the *Allium* family. However, the level of variability inherent in natural systems poses problems with respect to the economic extraction of such compounds for health products. Aspects of crop location, varietal impact, plant nutrition and post-harvest handling have been optimised for the production and retention of key health compounds in garlic and onions (thiosulfinates, cysteine sulfoxides (CSOs) and flavonoids). Our research in garlic suggests that the Australian climate promotes the production of sulfur compounds. Work on crop management issues is aimed at further enhancing the level of health compounds in these crops, and indicates that there is significant opportunity for the market diversification of both fresh and processed *Allium* products based on nutritional quality.

Appendix 2: Onions Australia article

AUSTRALIAN ONIONS: FOR YOUR HEALTH!

Phil Brown is with the Tasmanian Institute of Agricultural Research at the University of Tasmania. Sam Sterling and David Eagling are with the Institute for Horticultural Development, Agriculture Victoria.

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Onions are an important ingredient in many styles of cooking from around the world and have been a part of mankinds diet for centuries. As well as tasting good, onions and other members of the Allium family such as garlic and leeks have long been considered to be good for your health. Recent research has confirmed this long held belief and demonstrated that allium vegetables have powerful cancer-fighting properties. Several epidemiologic studies show that people who eat a lot of garlic and onion have lower risk of cancers of the gastrointestinal tract, such as stomach and colon cancer. In addition onions are known to be rich sources of a group of compounds with antioxidant properties, while other compounds have been linked to lowering of cholesterol and inhibition of blood platelet aggregation. Increasing consumer awareness of the health benefits that can result from eating allium vegetables represents an aspect of product quality that has received little attention to date but may provide a significant marketing tool for the onion industry. The success of the marketing campaign for tea based on its antioxidant properties demonstrates the potential of this marketing strategy.

The health benefits of eating onions and other alliums have been attributed to two main groups of compounds, the flavonoids and sulfur compounds. Previous work undertaken by Agriculture Victoria on garlic indicates that Australia is uniquely positioned to produce plants with high levels of these beneficial compounds. The environment and growing practices used in Australia appear to stimulate accumulation of higher concentrations of compounds beneficial to human health in alliums than have been reported in other parts of the world. A research project undertaken by the Tasmanian Institute of Agricultural Research and the Institute for Horticultural Development has now begun to document the levels of sulfur compounds and flavonoids in Australian onions, and compare these with levels from onions sourced from both European countries and other southern hemisphere production regions.

The flavonoids are a group of compounds with high antioxidant activity and are found in onions and many other plants. The potent antioxidant properties of tea are largely due to these compounds, while a number of flavonoids in red wine are under investigation as possible factors contibuting to protection from coronary heart disease. The major flavonoids in onions are quercetin (present in the free or aglycone form, or as a glycoside or diglycoside which means that the compound has either one or two sugar molecules attached to it), isohamnetin and kaempferol. Quercentin is present in the highest concentrations and has been shown to be present in a range of concentrations in European and US onions (Table 1).

Table 1
Quercetin concentrations (total of aglycone, glycoside and diglycoside) in onions (Crozier et al, 1997; Hertog et al, 1992; Bilyk et al, 1984)

Origin	Type	Content (mg/kg)
Netherlands	not reported	544
UK	red	201
UK	white	185 – 634
US	brown	15 – 62

Preliminary analysis of Tasmanian onions has revealed that the concentration of quercetin aglycone of 124 mg/kg fresh onion tissue. The aglycone form of quercetin represents between 1% and 50% of total quercetin, indicating that Tasmanian onions may have significantly higher concentrations than those reported from other countries. This is not surprising as flavonoid synthesis is stimulated by exposure to UV light, so the high light levels in Tasmania, combined with a thin ozone layer, may provide conditions which are ideal for production of high flavonoid onions (Figure 2).

The analytical methods used in this study are more advanced than those used in previous studies, allowing more accurate detection of the flavonoids present in the onions (see Figure 1). One interesting finding is that the proportion of aglycone to glycoside forms varies significantly between onion crops (Table 2). This has significant health implications as the aglycone form is readily absorbed by humans while the glycosides are not as readily absorbed, meaning that onions with a high aglycone to glycoside ratio are likely to have greater therapeutic benefits. It may be possible to manipulate crop development to maximise concentration of the quercetin aglycone and therefore the potential health benefits from the crop.

Table 2
Ratios of aglycone, glycoside and diglycoside forms of quercetin in Tasmanian onion crops

Type	Crop 1	Crop 2	Crop 3
Diglycoside	3.2	5.36	0.4
Glycoside	6.3	8.21	0.25
Aglycone	0.019	0.08	1

Onions are also an important source of sulfur containing compounds and in particular organosulfides in the diet. Organosulfides are the sulfur compounds that give onions their pungent taste and smell. These compounds have been shown to help prevent cancer as well as having antithrombotic (inhibitors of platelet aggregation), antibiotic and hypochlolesterolaemic properties. Heating can destroy the compounds but a quick saute will retain the majority of organosulfides. This strategy is likely to be more beneficial to your digestive system and social life than eating raw onions.

Our research suggests that the climate of Australian *Allium* production regions naturally favours the production of flavonoids and sulfur compounds and with crop management protocols aimed at further enhancement of the health value of these crops, there are significant opportunities for the market diversification of both fresh and processed product.

Acknowedgements
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Figure 1 UV Chromatogram of flavonoids in onion extract
Figure 2 Field curing under sunny conditions may contribute to high levels of flavonoids in Tasmanian onions