Monitoring natural phenolics and antioxidants in processing apple juice

Dr John Golding
Department of Primary Industries

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FINAL REPORT

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May 2012

NSW GOVERNMENT Department of Primary Industries

Horticulture Australia **Project Number AP10020**

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May 2012

This report provides data and describes the antioxidant potential and phenolic profile of locally produced apple juices.

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Media Summary

Apple fruit are nutritious and should be an integral part to a balanced diet. Besides their traditional nutritional benefits, such as vitamins, minerals, fibre etc, apples are a rich source of naturally-occurring phenolic compounds which significantly contribute to the antioxidants in the human diet. However, apples are not just eaten whole as fresh apples; approximately one third of apples are processed, with apple juice being the most popular form of processed apples. There are two types of apple juices on the market; cloudy and clarified. Cloudy apple juice is made from crushed and pressed apples and has rapidly grown in popularity. Clarified juice is clear and is made by processing the cloudy apple juice. This study measured the antioxidant activity level and the concentration of naturally-occurring phenolic compounds in a range of 17 different commercial apple juices (6 cloudy and 11 clarified apple juices) purchased from supermarkets.

The results showed that the levels of naturally-occurring phenolic compounds were 2.8 times higher in the cloudy than in the clarified apple juices. Similarly, the cloudy apple juices contained 2.5 times more antioxidant activity compared to the clarified apple juices. Therefore, it was shown that the clarification process decreases the phenolic compound content and the antioxidant activity of commercial apple juices. Further research in the 'healthiness' of Australian apple juice will increase the consumer appeal of Australian apple juice. This project is partly funded through a voluntary contribution from 'Appledale Co-op' in Orange, NSW.

Technical Summary

Apple fruit are nutritious and should be an integral part to a balanced diet. Besides their traditional nutritional benefits, such as vitamins, minerals, fibre etc, apples are a rich source of naturally-occurring phenolic compounds which significantly contribute to the antioxidants in the human diet. However, apples are not just eaten whole as fresh apples; approximately one third of apples are processed, with apple juice being the most popular form of processed apples. There are two types of apple juices on the market; cloudy and clarified. Cloudy juice has a hazy appearance, due to the presence of pectin substances and fibres; whilst clarified juice is a brighter colour and has a clearer appearance. This is achieved by subjecting the juice to a process called clarification which usually involves enzymatic treatment and filtration to remove the pectin substances and fibres. This study measured the antioxidant activity level and the concentration of naturally-occurring phenolic compounds in a range of 17 different commercial apple juices (6 cloudy and 11 clarified apple juices) purchased from supermarkets. This was accomplished by using high performance liquid chromatography (HPLC) and the Oxygen Radical Absorbance Capacity (ORAC) assay to measure the content of phenolic compounds and the antioxidant activity, respectively. In addition the physical properties of the different apple juices were measured. As expected, the results showed that cloudy apple juices had a much higher level of turbidity than the clarified juices and were often 37 times higher than that of the clarified juice. The levels of Total Soluble Solids (TSS) were similar in all juice samples (10.9 - 13.4 °Brix), regardless of the type of juice, clarified or cloudy. This was due to the standardisation procedure employed by the juice manufacturers.

The concentrations of total phenolics in cloudy apple juices were high and ranged from 276 µM to 2,510 µM. These levels were significantly higher (p = 0.049) than in clarified juices which were variable and ranged from 48 µM to 954 µM. On average across all the samples, the average total phenolic compounds were 2.8 times higher in the cloudy than in the clarified apple juices. This increase in phenolic content appeared to be due to higher concentrations of the individual phenolics, chlorogenic acid, rutin and quercetin glucoside. High levels of gallic acid, chlorogenic acid and caffeic acid were measured in both the clarified and cloudy apple juices. However the phenolics, coumaric acid, rutin, quercetin glucoside and phloridzin, were present in most of the cloudy juices.

The major contributors to the antioxidant activity in apples are vitamin C and the phenolic compounds. In order to calculate the contribution of the phenolic compounds to the total antioxidant activity, the antioxidant activity of vitamin C must be taken into account. However, the correlation between the total phenolic compounds and the vitamin C corrected antioxidant activity was not significant. This suggests that an increase or decrease in total phenolic compounds in apple juice does not necessarily mean an increase or decrease in the total antioxidant activity. However it was found that the six cloudy apple juices possessed significantly more phenolic antioxidant activity (as measured with the ORAC assay) compared to the eleven clarified apple juices (13,778 versus 5,533 µM Trolox Equivalents, respectively). This difference was also observed with the laboratory made clarified apple juices suggesting that clarification adversely affects the phenolic compound content in apple juice. Therefore, it was concluded that the clarification process decreases the phenolic compound content and the antioxidant activity of commercial apple juices.

Further research in the 'healthiness' of Australian apple juice will increase the consumer appeal of Australian apple juice. This project is partly funded through a voluntary contribution from 'Appledale Co-op' in Orange, NSW.

List of Abbreviations

°Brix	Degree(s) Brix
°C	Degree(s) Celcius
µg	Microgram(s)
µg/g	Microgram(s) per gram
µm	Micrometre(s)
µM	Micromolar
v/v	Volume per volume
AAPH	2,2'-azobis(2-amidino-propane) dihydrochloride
ABS	Australian Bureau of Statistics
Abs	Absorbance
ANOVA	Analysis of Variance
AUC	Area under the curve
CO2	Carbon dioxide
cP	centiPoise
DAD	Diode Array Detector
NH&MRC	National Health and Medical Research Council
FDA	Food and Drug Administration
FSANZ	Food Standards Australia and New Zealand
g	Gram(s)
h	Hour(s)
HMF	5-hydroxymethylfurfural
HPLC	High Performance Liquid Chromatography
IS	Internal Standard
kcal	Kilocalorie(s)
kg	Kilogram
kj	Kilojoule(s)
LSD	Least Significant Difference
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mM	Millimolar
nm	Nanometre(s)
ORAC	Oxygen Radical Absorbance Capacity
PET	Polyethylene terephthalate
ppm	Part per million
RFU	Relative Fluorescence Unit
RI	Refractive Index
ROO°	Alkyl peroxyl radical
ROS	Reactive Oxygen Species
S.E.	Standard Error
Sec	Second(s)
SPSS	Statistical Package for the Social Science
TE	Trolox Equivalents
TSS	Total soluble solids
UV	Ultraviolet
UV-VIS	Ultraviolet-visible
WHO	World Health Organization

Introduction

Apple fruit are nutritious and should be an integral part to a balanced diet. Besides their traditional nutritional benefits, such as vitamins, minerals, fibre etc, apples also contain many biologically active phytochemicals compounds such as antioxidants which have been shown to provide protective health benefits. Antioxidants are naturally-occurring compounds which neutralize free radicals and protect cells from oxidative damage and apple fruit are one of the best sources of antioxidant phenolic compounds in the Western diet.

The major contributors to the antioxidant activity in apples are vitamin C and the phenolic compounds. Vitamin C plays a major role in cellular functions by acting as an antioxidant. In addition, phenolic compounds also show important health benefits. These naturally-occurring compounds have been shown to help scavenge free radicals in the human body thereby reducing the risk of cancer and heart disease. There have been many recent reviews on apple phenolics which have linked the consumption of apples with reduced risk of some cancers, cardiovascular disease, asthma, and diabetes. In laboratory tests, apples have been found to have very strong antioxidant activity, inhibit cancer cell proliferation, decrease lipid oxidation, and lower cholesterol.

Apples contain a variety of naturally-occurring phenolic compounds, including anthocyanins, quercetin, catechin, and chlorogenic acid, all of which are strong antioxidants. The phenolic composition of apples varies greatly between different varieties of apples, and there are also small changes in phenolics during the maturation and ripening of the fruit. Despite being abundant in apples, vitamin C and the phenolic compounds are known to be lost when apples are processed into juice. The loss of vitamin C during processing is compensated for by adding ascorbic acid into the juice. However there is little information about the loss of phenolic compounds during processing.

Consumer interest in the nutritional value of food necessitates the examination of the impact of processing on the antioxidant and phenolic profile of apple juices. It is therefore the aim of this project was to examine the content of phenolic compounds and their contribution to the antioxidant activity in various types of apple juices (cloudy and clarified) made from imported concentrate or from Australian apples. This was achieved by (a) measuring the content of phenolic compounds and the total antioxidant activity of commercially available apple juices made from imported concentrate and from apple juices made from locally grown apples, and (b) by determining the effect of clarification on the concentration of phenolic compounds and total antioxidant activity by comparing clarified juices with cloudy juices.

An analysis involving high performance liquid chromatography (HPLC) and the oxygen radical absorbance capacity (ORAC) antioxidant assay was conducted to quantify the content of phenolic compounds in apple juice samples and their contribution to the total antioxidant activity.

In the following section, the literature was reviewed for information on apples, apple juice processing, phenolic compounds in apples, phenolic compounds in apple juice and antioxidants. The experimental rationale for the study is also described.

Background

Apple juice

Apple juice is an important outlet for Australian grown apples. There are two types of apple juice; cloudy, which has a hazy appearance, and fully clarified, which has brighter colour and clearer appearance. In Australia, Food Standards Australia and New Zealand (FSANZ) in Standard 2.6.1 defines that fruit juice may contain added ingredients such as sugar to a specific amount.

Cloudy apple juice is a colloidal system where water acts as the dispersing medium for particles which are formed from cellular tissue released by processing (milling and pressing) (Barret *et al.*, 2005). If required, this dispersed phase can be removed by filtration. The cloudiness of fruit juices can also be caused by the presence of pectin substances. In order to fully clear the juice, a clarification process is performed by adding enzymes, such as pectinases, to the juice; these enzymes will break down the cloud-forming pectin substances (Brown, 2004; Ashurst, 2005).

Apple juice processing

Although the majority of Australian grown apples are sold and consumed fresh, approximately 25%- 30% of all apples go into juice (Hassall & Associates Pty Ltd., 2001; Australia Bureau of Statistics (ABS), 2000). Apple juice is one of the most popular fruit juices in the US, second only to orange juice on market share (Index Mundi, 2005) and it is the most popular juice in Germany with a per capita consumption about 12 litres annually (VDF, 2000 as quoted from Schieber *et al.*, 2003). In the USA, 37% of all apples are consumed in the form of processed food, such as cider, apple sauce, canned apple and apple juice (Brown, 2004). In average in 2007, an American consumed an estimated 21.4 kg of apple and apple products (US Apple Association). This may be an important consideration for the Australian apple industry to increase consumption.

Apple juice processing involves several processes such as preparation, extraction, clarification, pasteurization, concentration, addition of food additives and packaging (Potter and Hotchkiss, 1998; Ashurst, 2005). A summary of the processing flowchart of various type of apple juice is outlined in Figure 1.

Preparation. The preparation step includes screening, sorting and cleaning of the raw materials (apples). This cleaning step is essential to remove external surface dirt, impurities and rotten fruit.

Extraction. The extraction can be performed mechanically using vacuum filtration or extraction tools on the apple mash, or may be done by manually by crushing the fruits (Cabalerro *et al.*, 2003; Belitz *et al.*, 2004). One of the leading mechanisms in juice extraction from fruits' mash, including apples, is the horizontal rotary press. The utilization of this extraction mechanism in apple juice production gives a considerably good yield with 85 – 95% juice by weight of initial mash (Ashurst, 2005).

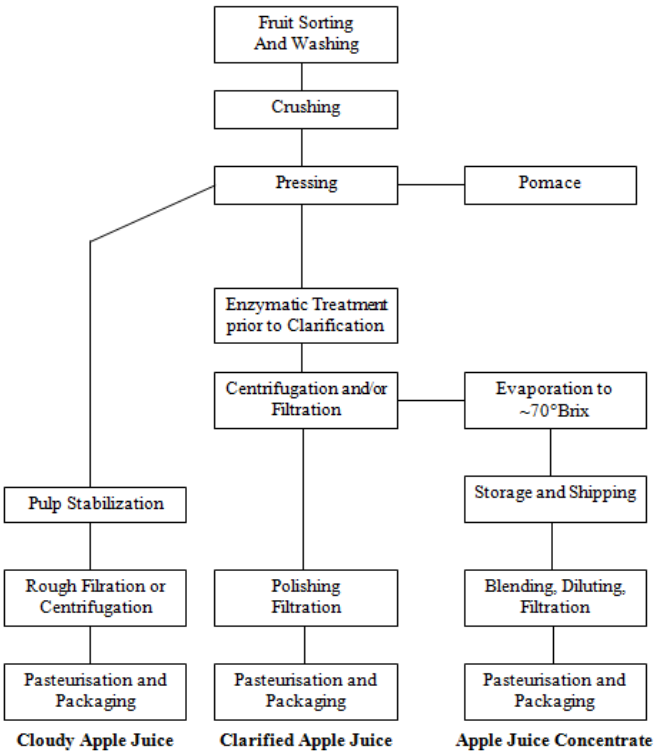

Figure 1. Processing flowcharts of various type of apple juice

Some processors may add vitamin C immediately after the extraction, in order to prevent oxidation at the early stage. Liquefaction of the apple pulp, which normally contains pectin, is done by adding pectinolytic and cellulolytic enzymes to break down the pectin and cellulose in order to increase production yield. During this enzymatic treatment, the pulp is usually aerated to increase the effectiveness of the enzymes (Fellows, 2000; Barret *et al.*, 2005; Ashurst, 2005). The implementation of this technique may increase the yield by up to 40% in comparison to the conventional extraction. The ready-to-drink juice which is sold to the customer is known as single strength juice.

Clarification and stabilization. For clarified juice production, clarification can be carried out by first adding pectolytic enzymes such as pectinase, into the product which digest the pectin substances. The pulp which is no longer suspended by the pectin can then settle and the process is completed by filtration and/or centrifugation (Potter and Hotchkiss, 1998; Brown, 2004; Ashurst, 2005).

For cloudy apple juice: In order to produce uniform products and maintain company specification, cloudy apple juice also requires some treatment. This includes the addition of polygalacturonase, which has low pectin esterase activity, in order to only partially degrade pectin and stabilize the turbidity of the juice (Potter and Hotchkiss, 1998; Bates *et al.*, 2001; Barret *et al.*, 2005). The manufacturer will often set the standard for their cloudy juice product based on the pulp percentage. It is also a common practice to mix juices with different total soluble solids content (°Brix) in order to achieve uniformity across the products (Potter and Hotchkiss, 1998; Ashurst, 2005).

Chemical preservatives are commonly added into juices to inhibit fermentation and microbiological deterioration, thus increasing the shelf life of the juice (Rahman, 2007). Sodium benzoate, sodium bisulphate, potassium sorbate and potassium metabisulphite are

examples of widely used preservatives. In apple juice, ascorbic acid is often added to prevent browning, by acting as an antioxidant, and to replace the vitamin C lost during processing (Fellows, 2000; Brown, 2004; Cabalerro *et al.*, 2003).

Pasteurisation. Apart from preservatives, several other ways are used to extend the shelf life of juices. Pasteurization is carried out to kill pathogenic microorganisms and eliminate spoilage microorganisms, such as yeasts and moulds in apple juice. This process also inactivates some of the natural enzymes, particularly polyphenol oxidases (Potter and Hotchkiss, 1998; Cabalerro *et al.*, 2003; Brown, 2004). Membrane filtration is also being used by some manufacturers. The membranes used in the sterile filtration usually provide a porosity value of 0.02µm to guarantee the removal of spoilage microorganisms (Ashurst, 2005).

Other processes to assure product quality uniformity

In cases where the juice is low in solids, manufacturer may concentrate the product by using low temperature vacuum evaporation. This technique is chosen to retain maximum flavour (Potter and Hotchkiss, 1998). However, along with the water removal some of the volatile compounds will also be lost. Therefore, the evaporated water is not discarded but is subjected to an additional process to recover the essence. The principle of this step is to distil the volatile compounds from the water and recondense it, which is then added back to the concentrated juice to enhance the flavour (Potter and Hotchkiss, 1998; Ashurst, 2005).

Apple juice concentrate

The majority of apple juice marketed in the world is in the form of concentrated slurry. This slurry is produced by evaporating most of the juice's water (approximately reaching 70°Brix). Stabilizing agents, such as gelatine and benzoate may be added into the concentrate and in this form, the apple juice could last up to five years (Barret *et al.*, 2005; Ashurst, 2005).

Local manufacturers are only required to reconstitute the concentrate by diluting it with water to achieve a certain °Brix value (soluble solids concentration) comparable to the fresh apple juice. The reconstituted apple juice generally has vitamin C added as a natural preservative agent as well as to make up for the loss during processing. Pasteurization and aseptic filling and packaging will result in shelf stable products, without any need for refrigeration (Brown, 2004; Ashurst, 2005). In line with FSANZ regulation, the product must contain at least 8% of Australian apple juice concentrate to be able to retain the claim as Product of Australia. Some manufacturers mix the concentrate with fresh apple juice, which enables them to achieve the cloudiness caused by the pectin compounds from the fresh apples (Potter and Hotchkiss, 1998; Ashurst, 2005).

Human nutrition

Fruit and vegetables are essential to the human diet and according to the Australian Dietary Guidelines (2003) issued by the National Health and Medical Research Council (NH&MRC), adults should consume at least two servings of fruits and five servings of vegetables and legumes on a daily basis.

The consumption of fruit is shown to have positive effects on improving health and reducing the risk of diseases such as cardiovascular disease and cancers (Hertog *et al*., 1993; Hertog *et al.*, 1994; Boyer and Liu, 2004; McCann *et al.*, 2007). Important for maintaining wellbeing, fruits contain vitamins and minerals, which are required by the body, as well as phytochemicals such as anthocyanins, catechins and flavonoids, which demonstrate anti-histamine, anti-inflammatory and antioxidant activities (Ignat *et al.*, 2011).

Composition and characteristics of apple fruit

Apples are a good source of vitamins and minerals in the human diet and are free from fat, cholesterol and sodium (Cabalerro *et al.*, 2003; Roupas and Noakes, 2010). Apples also contain high amount of soluble fibre which is useful in helping lowering the blood cholesterol levels. A typical nutritional content of apple can be seen in Table 1 from Cabalerro *et al.*, (2003).

Table 1. Nutritional content per 100 g of apple (Cabalerro *et al.*, 2003)

Water (g)	83.93
Food energy (kcal)	59
Food energy (kJ)	247
Protein (g)	0.19
Total lipid (g)	0.36
Cholesterol (mg)	0
Carbohydrate (g)	15.25
Fibre (total dietary) (g)	2.7
Calcium (mg)	7
Magnesium (mg)	5
Phosphorus (mg)	7
Potassium (mg)	115
Vitamin C (mg)	5.7
Folate (µg)	3
Vitamin A (µg retinol equivalent)	5

Apple phenolics in human health

In addition to the traditional health benefits of apples such as vitamins and minerals, apples are also good sources of phytochemicals such as phenolic compounds and flavonoids which have antioxidant activity (Ki *et al*., 2003; Vieira *et al.*, 2009). Phenolic compounds demonstrate a strong linkage with decreased mortality and apples are considered as one of the main sources of these compounds in western diets (Ki *et al.*, 2003).

In comparison with other popular fruits in the USA, apples are among the richest sources of antioxidants, second only to cranberries (Boyer and Liu, 2004). On a consumption basis, apples are the largest contributors of fruit phenolics to the diet (Wolfe *et al.*, 2008). Apples are also a significant source of flavonols for Australian adults (Somerset and Johannot, 2008). In terms of bioavailability of phenolics, apples rank first among fruits, due to the large proportion of these compounds which are absorbed into the bloodstream (Boyer and Liu, 2004). Therefore apples are a very important dietary source of phenolics such as flavonoids (Ki *et al.*, 2003).

The health benefits of apples and apple phenolics

There is increasing scientific studies showing strong relationship between the phenolic compounds, which have antioxidant activity, and reduced risk of various diseases (Boyer and Liu, 2004). Quercetin glycosides and procyanidin have been linked with strong antioxidant activity in *in vitro* observations (Knekt *et al.*, 2000). Therefore the consumption of apples and apple products may protect human cells from oxidative damage and have been associated with prevention of several diseases (Hollman *et al.*, 1996; Hollman and Katan, 1999; Femenias, 2005; Boyer and Liu, 2004).

Cardiovascular diseases

A reduced risk of cardiovascular diseases (CVD) has been linked with regular apple consumption, with flavonoids associated with a positive outcome (Hollman and Katan, 1999; Knekt *et al.*, 1996; Sesso *et al.*, 2003). Research shows that fewer incidents of thrombotic stroke occur in people who consume apples on a daily basis as opposed to those with minimum consumption (Knekt *et al.*, 1996). Oxidation of the major cholesterol carrying lipoprotein in the blood, low-density lipoprotein (LDL), is a major risk factor for CVD. In an *in vitro* study conducted by Pearson *et al.* (1999), apple consumption resulted in a 9-34% decrease in low-density lipoprotein (LDL) oxidation, so reducing the risk of CVD. Based on the study in rats conducted by Aprikian *et al.* (2001), the inclusion of moderate amount of apple in the diet will decrease intestinal cholesterol absorption. Furthermore, in a study in a group elderly people, apple flavonoids were associated with a decreased mortality from heart disease, while quercetin showed no such effect (Hertog *et al.*, 1993).

Cancers

Research also suggests that eating apples may reduce the risk of lung cancer (Boyer and Liu, 2004), especially among current and past smokers (Cutler *et al.*, 2008). In general, individual fruits and vegetables are not related with lung cancer risk in humans. However, apples are among the few examples of fruits which are strongly associated with a significantly reduced risk of lung cancer (Hertog *et al.*, 1994). Studies in Finland and Hawaii found quercetin reduces lung cancer risk and concluded that only apple consumption was inversely related to the risk of lung cancer (Boyer and Liu, 2004). According to Fiuza *et al.*, (2004), gallic acid derivatives are known to cause apoptosis in tumour cell lines as well as inhibiting lymphocyte proliferation. In addition, apple flavonoids were shown to have a synergistic effect with tea catechin in reducing cancer risk (Hertog *et al.*, 1994). Also, quercetin in apple demonstrates the ability to retard the growth of cancer cells in colon and liver (*in vitro*) (Ebenhardt *et al.*, 2000; Rossi *et al.*, 2006; McCann *et al.*, 2007).

Pulmonary and respiratory diseases

Regular consumption of apples has also been related to general wellbeing of the pulmonary and respiratory system. According to a study in Australia, apple and pear intake may reduce the risk of asthma and bronchial hypersensitivity (Woods *et al.*, 2003), while other known antioxidants such as vitamin E, retinol and vitamin C have no effect (Shaheen *et al.*, 2001). A similar inverse relationship between apple intake and asthma was found in studies conducted in Netherlands, Wales and Finland (Boyer and Liu, 2004). Importantly, it is suggested that a minimum of two apples per week will decrease the severity of asthma and its symptoms (Shaheen *et al.*, 2001).

Diabetes

The risk of developing type II diabetes may be lowered by consuming apples (Boyer and Liu, 2004). In a human observational study, it was suggested that consuming an apple per day may reduce the risk by 28%. In addition to lowering diabetes risk, apples are rich in dietary fibre

and have a low glycemic index which helps in lowering the blood glucose level and balancing the body's insulin response (Femenias, 2005).

The nature of apple phenolics

Phenolic compounds in apples

There are six major classes of phenolic compounds found in apples (Table 2) (Golding *et al*., 2001; Boyer and Liu, 2004):

- flavonol glycosides (flavonoids, quercetin and quercetin conjugates),
- catechins and epicatechins,
- anthocyanins,
- dihydrochalcones (phlorotin and phlorizin),
- phenolic acids (gallic acid and chlorogenic acid) and
- procyanidins.

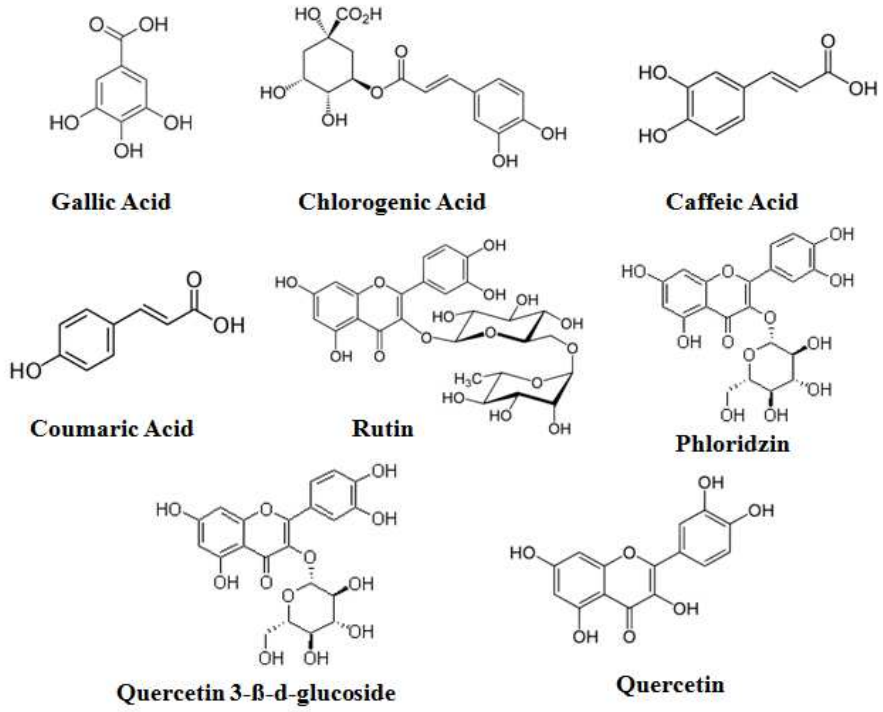

Figure 2. Chemical structures of selected apple phenolic compounds

Apart from quercetin conjugates which are exclusive to the peel (Boyer and Liu, 2004; Oszmianski *et al*., 2009), these compounds are found in apple peel, flesh and seeds (Schieber *et al.*, 2003). Nevertheless, their concentrations are much lower in the flesh compared to the peel, except for chlorogenic acid which tends to be higher in the flesh (Oszmianski *et al*., 2009). Due to this, it is suggested that apples be consumed with the skin, rather than just the flesh or in processed forms such as apple juice (Van der Sluis *et al.*, 2001; Van der Sluis *et al.*, 2002; Van der Sluis *et al.*, 2004; Roupas and Noakes, 2010). Recent studies suggested that apple seeds contain high amount of phenolic compounds (Schieber *et al.*, 2003).

Phenolic compounds in apple juice

By its nature, processing will often affect the physical properties of the food as well as its nutritional value (Brown, 2004). Nutritionally, apple juice has similar values to fresh apples, except for fibre, of which it contains almost none, especially the clarified type (Van der Sluis *et al.*, 2002). However, in conventional apple juice production, the antioxidant activity decreases by up to 97% in comparison with the fresh apple. Filtering and clarifying are the major reasons for the losses, as the phenolic compounds, the contributors to the antioxidant activity in apples, are mainly found in the pulp part of the peel and flesh which are excluded from the final clarified product. The more highly hydrophilic compounds, like chlorogenic acid are found in relatively higher concentration in apple juice, in comparison with the other more hydrophobic phenolic compounds (Van der Sluis *et al.*, 2002; Van der Sluis *et al.*, 2004; Bhushan *et al.*, 2008; Oszmianski *et al.*, 2009).

Apart from the filtration and clarification factors, if juice production involves liquefaction, then this additional step must also be taken into account. Liquefaction involves aeration which is exposing these compounds to oxygen thus allowing them to be oxidized. Juices produced with this supplementary process have lower level of catechins and epicatechins, phloridzin and chlorogenic acid (Van der Sluis *et al.*, 2002; Van der Sluis *et al.*, 2004; Bhushan *et al.*, 2008; Oszmianski *et al.*, 2009). Although delays during processing can result in browning, generally losses in phenolic compounds are not significant (Rocha and Morais, 2001).

In order to minimize the destruction of phenolic compounds and vitamin C, as well as other changes caused by oxygen, deaeration may be employed as an intermediate step to release the entrapped air after the apple mash is subjected to liquefaction and aeration process (Potter and Hotchkiss, 1998).

According to Markowski and Plocharski (2006), the total phenolic compounds in cloudy apple juice (average from Jonagold, Sampion, Idared and Topaz) was 462 mg/litre while clear apple juice only contained 160 mg/litre. While the initial content of polyphenols in the apples was 857 mg/kg. Based on Roupas and Noakes (2010), the levels of flavonoids and chlorogenic acid in the juice were reduced to between 50% (chlorogenic acid) and 3% (catechins) of whole apple levels.

The phenolic content of apple juice may change during storage, because of both oxidative and non-oxidative degradation (such as hydrolysis). After 11 months of storage at ambient temperature, apple juice packed in laminated carton will generally lose around 20% of the phenolic compounds, especially the phenolic acids and flavonoids components. However it is hard to determine the respective rate of oxidative and non-oxidative degradation (Van der Sluis *et al.*, 2005). The manufacturers can minimize these losses by controlling the temperature during factory storage and transportation and by utilizing packaging materials like aluminium foil laminated carton to protect phenolic compounds against exposure to light (Cabalerro *et al.*, 2003; Van der Sluis *et al.*, 2005; Ashurst, 2005).

Antioxidants

Antioxidants are very important compounds as they neutralize free radicals by donating an electron to unstable molecules and protect cells from oxidation by being oxidized themselves (Kennedy *et al.*, 1993; Sohal, 2002). The major contributors to the antioxidant activity in apples are vitamin C and the phenolic compounds. The human body antioxidant defence mechanism is able to utilize these phenolic compounds and vitamin C to prevent and reduce the propagation of oxidative damage caused by free radicals (Ki *et al.*, 2003, Mullen *et al.*, 2007; Sohal, 2002). We have shown that antioxidant activity in apple peel is up to eight times higher than in the flesh in Australian grown Pink Lady™ apples (Hoang *et al*., 2011).

Antioxidant activity of apple juice

The antioxidant activity of apple juice is determined by two major factors: the level of vitamin C and the content of phenolic compounds (Boyer and Liu, 2004; Ki *et al.*, 2003; Roupas and Noakes, 2010). As previously mentioned, vitamin C can be added into the juice and will contribute, to an extent, in increasing the antioxidant activity of the product. However, the antioxidant activity contributed by the phenolic compounds decreases dramatically, due to the low content of these compounds (Van der Sluis *et al.*, 2002; Van der Sluis *et al.*, 2004; Markowski and Plocharski, 2006; Bhushan *et al.*, 2008; Oszmianski *et al.*, 2009). Without the addition of vitamin C, cloudy apple juice obtained from Jonagold apples by straight pressing had an antioxidant activity that was only 10% of the activity of the fresh apples (Roupas and Noakes, 2010).

In light of the processing being the main reason for the loss of phenolic compounds and hence the lowering of the antioxidant activity, the application of more advanced production techniques to retain maximum phenolic compounds in the juice have been studied. It is possible by techniques such as by pulp and pomace extraction and diffusion extraction technique, but production costs are much higher (Schieber *et al.*, 2003; Van der Sluis *et al.*, 2004).

Measuring antioxidant capacity

One of the standardized methods for the determination of antioxidant capacity of a substance is oxygen radical absorbance capacity (ORAC) assay. The ORAC assay is often employed to measure the capacity of antioxidants in foods or food extracts or *in vivo* to neutralise oxygen free radicals, also known as reactive oxygen species (ROS) (Cao *et al.*, 1993). The ORAC-hydro assay reflects water-soluble antioxidant capacity, while the ORAC-lipo assay measures lipid-soluble antioxidant capacity. The values of these two assays are additive.

The ORAC assay is based on the on the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of the azo-compound, 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH). A peroxyl-radical is a ROS which quenches the signal from the fluorescent probe, fluorescein. The addition of an antioxidant produces a more stable fluorescence signal with the fluorescence signal depending on the antioxidant's capacity (more antioxidant capacity, more signal). The ORAC assay is a kinetic assay, ie measurements are taken repeatedly over a time period (usually 60 or 90 minutes after addition of AAPH). Over the course of the assay, the antioxidant capacity of the antioxidant is "used up" and the fluorescence decreases to reflect the decreasing antioxidant capacity of the substance. The antioxidant Trolox (a water-soluble analogue of vitamin E) is used as a standard against which all other antioxidants are compared. Hence, the ORAC result is expressed as micromole Trolox Equivalents (TE) per gram (Ganske and Orban, 2009).

The ORAC assays have their limitations as they do not directly measure the scavenging capacity against the ROS found in the body, because the activity and mechanism of multifunctional natural antioxidants are affected by many factors. Therefore, many analysts suggest that the influence of all these factors cannot be evaluated using only a single assay (Bank and Schauss, 2004). Biological activity of phenolic compounds is often assessed by using cultured cells as tissue models (Roupas and Noakes, 2010). However the ORAC assay has the advantage of being standardised, relatively simple and inexpensive, and in microtitre plate format allows for the measurement of antioxidant activity in a large number of samples simultaneously.

Aim and Objectives

The main aim of this study was to examine the content of phenolic compounds and their contribution to the antioxidant activity in various types of apple juices (cloudy and clarified) made from imported concentrate and from Australian apples. An HPLC technique was employed to measure the phenolics and the ORAC assay was used to measure antioxidant activity.

Therefore the objectives of this study were:

1. To determine the content of the phenolic compounds and the antioxidant activity in commercial apple juices made from imported concentrate and from Australian grown apples.

2. To determine the content of the phenolic compounds and the antioxidant activity in cloudy and clarified commercial apple juices.

3. To determine the content of the phenolic compounds and the antioxidant activity in apples (Royal Gala and Granny Smith) and to quantify the loss of these during the juicing process.

4. To determine the relationship between the content of the phenolic compounds and the antioxidant activity of apples and apple juice.

Materials and Methods

General Materials

Chemicals and reagents

The phenolic standards; gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, quercetin, quercetin glucoside, phloridzin and naringenin were purchased from Sigma Aldrich Laboratory Chemicals (Castle Hill, NSW). Ascorbic acid (HistoLabs) was purchased from Fronine Ltd., NSW. Extraction and mobile phase reagents for HPLC included ortho-phosphoric acid (APS Chemical, Seven Hills, NSW), tetrahydrofuran, HPLC grade acetonitrile and HPLC grade methanol (Lomb Scientific, Taren Point, NSW). Liquid nitrogen was provided by the University of Newcastle. The reagents for the ORAC assay were potassium phosphate, potassium hydroxide, sodium fluorescein, Trolox and gallic acid (Sigma Aldrich Laboratory Chemicals, Castle Hill, NSW) and 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) (Wako Pure Chem.Ind., VIC). Deionised water was prepared on the day of use with a Millipore Milli-Q water purification system (Millipore Australia, Pty, Ltd. North Ryde, NSW).

Apples and apple juices

Seventeen commercial apple juices (eleven clarified and six of cloudy appearance) were purchased from supermarkets in Sydney and the NSW Central Coast. A batch of each juice was purchased on three separated occasions (3 batches). Of these juices, ten were bought from the refrigerated section of the stores (chilled) and seven were obtained from the long life non-refrigerated shelves of the stores (shelf).

From the selected eleven clarified apple juices: nine were made from imported concentrate and two were made from Australian grown apples. Of the six cloudy juices, four were made from Australian apples. However, juice C7 was made from a combination of imported concentrate and Australian apple, while C8 was entirely made from concentrate produced in Australia.

The juices were randomly allocated a code between C1 and C17 with C standing for 'Commercial', as listed in Table 2.

Juice preparation from apples

A batch of Granny Smith and Royal Gala apples were also purchased from the supermarket. Cloudy juice was made from 250 g of each type of apple (Royal Gala and Granny Smith) using a Sunbeam domestic juice extractor. The yield of juice (ml) was recorded for calculation purposes. A sample of the cloudy juices for each type of apple was then clarified by centrifugation at 12,100 × *g* using a Beckman J2-AC centrifuge (Beckman Instruments Inc., California, USA) at 5°C. The resulting supernatant was vacuum filtered through a double layer of cheese cloth. Three different juice samples were prepared for each of the Granny Smith and Royal Gala apples.

Samples from one batch of clarified and one batch of cloudy apple juice made from a combination of Granny Smith and Royal Gala apples were also obtained from a commercial apple processor.

Table 2. Origins of the commercial apple juices used in study

Samples	Chilled / Shelf	Clarified/Cloudy	Apples* / Concentrate**	Source
C1	Shelf	Clarified	Concentrate	Imported
C2	Shelf	Clarified	Concentrate	Imported
C3	Shelf	Clarified	Concentrate	Imported
C4	Shelf	Clarified	Concentrate	Imported
C5	Shelf	Clarified	Concentrate	Imported
C6	Shelf	Clarified	Concentrate	Imported
C7	Chilled	Cloudy	Concentrate and Apples	Imported and Australia
C8	Chilled	Cloudy	Concentrate	Australia
C9	Chilled	Clarified	Concentrate	Imported
C10	Chilled	Cloudy	Apples	Australia
C11	Chilled	Cloudy	Apples	Australia
C12	Chilled	Clarified	Apples	Australia
C13	Chilled	Cloudy	Apples	Australia
C14	Chilled	Clarified	Apples	Australia
C15	Chilled	Cloudy	Apples	Australia
C16	Chilled	Clarified	Concentrate	Imported
C17	Shelf	Clarified	Concentrate	Imported

* 'Apples' refers to juice made directly by crushing apples.
** 'Concentrate' refers to juice made by reconstituting concentrated apple juice with water.

Measurement of the physical properties of apple juices

The physical properties of the apple juices were measured as parameters for analysing the relationship between the nature of the juices and their content of phenolic compounds and in turn, their antioxidant activity.

Turbidity

The commercial juices were subjected to turbidity measurement using a UV-VIS spectrophotometer CARY 50 BIO (Varian Australia Pty. Ltd. Oakleigh, VIC) with absorbance set at 600nm (Venolia *et al*., 1974). Each of the three batches of the commercial juices was analysed once and the three values averaged and expressed as mean±S.E. of the Abs at 600nm.

Viscosity

A Brookfield Viscosmeter Model DU-11+ (Brookfield Engineering Labs. Inc., Massachusetts, USA) was used to measure the viscosity of the commercial apple juices (Oszmianski *et al.*, 2009). For each of the three batches of the commercial juices, one 100mL sample was conditioned at room temperature prior to using spindle 3 at 50rpm to determine the viscosity. The three values were averaged and expressed as mean±S.E. in centiPoise (cP).

Total soluble solids

The measurement of the total soluble solids (TSS) in all juices was carried out using a Pocket Refractometer PAL-1 (ATAGO, Tokyo, Japan; supplied by Extech Equipment Pty. Ltd., Melbourne, VIC) (Golding *et al.*, 2001). Each of the three batches of the commercial juices was measured in triplicate with deionised water used to wash the optical lens in between each reading. The values for the triplicates were averaged to generate a value for each of the three batches. These values were then averaged to generate a value for the TSS in each commercial juice and expressed as mean±S.E. ^{o}Brix.

HPLC identification and quantification of phenolic compounds and vitamin C in apples and apple juices

Extraction of the phenolic compounds from apples

The method for extracting the phenolic compounds from apples was adapted from Golding *et al.* (2001). The Granny Smith and Royal Gala apples were sliced and weighed into three 20 g samples. Both peel and flesh were included. Each sample of the apple slices was then frozen with liquid nitrogen to optimize the crushing process. A WARING Commercial Blender set at low speed (John Morris Scientific Pty. Ltd., Chatswood, NSW) was used to crush the apple slices into a powder.

An extraction stock solution containing the internal standard (IS) (0.1mM naringenin in HPLC grade methanol) was prepared. In order to ensure an accurate concentration, this stock solution was made fresh on the day prior to analysis. Naringenin has been selected as an IS for the analysis of the apple phenolic compounds due to the similarity of its UV spectrum and retention time with those of the apple phenolics, and because it is not naturally present in apples (Hoang *et al.*, 2011).

The powder from the blender (being 20g apple) was mixed with 100mL of the extraction stock solution and sonicated for 20 min with an UltraSONIK 57X NEY sonicator (Extech Equipment Pty. Ltd., Melbourne, VIC) before it was vacuum filtered through a double layer of cheese cloth. The filtrate was finally filtered through a 0.45 µm Phenex syringe filter (Phenomenex, Lane Cove, NSW) to guarantee the removal of any residue. The filtrate was transferred into a clean and labelled amber HPLC vial and placed on the autosampler of the HPLC system.

Preparation of the apple juices for HPLC analysis

The apple juices were prepared for HPLC analysis by mixing one part of each juice with one part of the extraction stock solution (0.1mM IS in methanol) and kept at 4^{o}C in a refrigerator for 20 min. The concentration of the IS in these juice mixtures was 0.05mM.

The mixtures were then filtered through a 0.45 µm Phenex syringe filter to guarantee the removal of any residue. The filtrate was transferred into a clean and labelled amber HPLC vial and placed on the autosampler of the HPLC system.

The HPLC system

A Shimadzu (Shimadzu Scientific Instrument, Rydalmere, NSW) Ultraviolet-Visible High Performance Liquid Chromatography (UV-VIS HPLC) system was used to identify and quantify the phenolic compounds in the apple and apple juices samples with a method adapted from Golding *et al.* (2001) and Hoang *et al.* (2011).

The HPLC system consisted of a LC-10 AT liquid chromatography pump and sample runs were initiated via a SIL-10 A XL VP autoinjector with a 100µL sample loop. The phenolic compounds were separated using a reverse phase Prodigy ODS-3 Phenomenex Column (5µm, 100A, 250 x 4.6mm, cat no. 00F-4097-E0) which was protected by an analytical-size guard

column. The level of absorbance was determined at 280 and 254nm wavelengths using a SPD-10 A Dual 1/2 UV-VIS detector. A SCL-10 A VP control unit and the Class VP 5.03 software were used to control the system. The flow rate was set at 1mL/min and the injection volume was 20µl with the maximum pressure set at 3500psi. The column was kept at 40ºC and the total running time for each sample was 60 min/injection.

Mobile phase A consisted of 88.5% of 0.2% (v/v) ortho-phosphoric acid, 10% (v/v) acetonitrile and 1.5% (v/v) tetrahydrofuran. Mobile phase B was 48.5% of 0.2% (v/v) ortho-phosphoric acid, 50% (v/v) acetonitrile and 1.5% (v/v) tetrahydrofuran. The mobile phase sequence was 100% mobile phase A for the first 5 min, then a gradient from 100% mobile phase A to 100% mobile phase B from 5 to 50 min. From 50 to 55 min, 100% mobile phase B was maintained, then from 55 to 60 min, mobile phase B was decreased to 0 while mobile phase A was increased to 100%. There was a 5 min period allowed to re-equilibrate the column using 100% mobile phase A in between each injection (Golding *et al.*, 2001 and Hoang *et al.*, 2011).

The elapsed HPLC running time for each sample was 60 min and the time for each batch of samples was limited to 30 h to prevent possible oxidation of the phenolic compounds prior to injection. For each batch of samples on the HPLC, a sample of 0.1mM IS in methanol was placed at the start to prepare the whole system, in particular the UV-VIS detector which, according to the operation manual, requires approximately 60 min to perform properly. A blank containing deionised water was also placed after every ten samples.

Identification and quantification of phenolic compounds

Eight apple phenolic compounds from three major classes (phenolic acids, dihydrochalcones and flavonol glycosides) were used in this study. The selection was made based on Golding *et al.* (2001), whose methodology was adapted for this analysis. Each phenolic standard containing 3mM gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, quercetin, quercetin glucoside, phloridzin and 0.1mM IS in methanol (HPLC grade) was run through the HPLC one compound at a time in order to identify their retention times. The retention times were recorded for the subsequent identification of each of these phenolics in the HPLC chromatograms for the apple and apple juice samples.

To generate external standard curves, an external standard stock solution containing 3mM of each phenolic compound (gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, quercetin, quercetin glucoside, phloridzin) and 0.1mM IS was prepared in methanol (HPLC grade) and stored at 4ºC in the dark until used. The external standard curve solutions were prepared from the external standard stock solution by serial dilution with 100% methanol containing 0.1mM IS to give concentrations ranging from 0.003mM to 3mM. The concentration of the IS in all the external standard curves solutions was 0.1mM.

The standard curve for each phenolic compound was generated by plotting the peak area ratios (the peak area of each phenolic external standard divided by the peak area of the IS) on the y-axis against the known concentrations of the phenolic compounds. Microsoft Excel 2007 from Microsoft Office was used to obtain the regression line equations and the R^2 values for the external standard curves.

Identification of the phenolic compounds in the apples and the apple juices was done by comparing their retention times and UV-spectra with those of the external standards. Quantification was done by using the regression equations generated from the standard curves. For the apples, the concentration (in mM) of each phenolic compound was converted into mass (in µg) using the known volume of methanol used for the extraction (100mL) and the respective molecular weight of the phenolic compounds. The value (µg) generated was

then divided by the initial mass of apple used (20 g) and the final concentration expressed as µg of each phenolic compound per g of apple (µg/g).

In the quantification of the phenolic compounds in the apple juices (but not the fresh apples), there was a systematic error due to a 2-fold dilution which occurred during sample preparation; the naringenin concentration was diluted to 0.05mM from the intended 0.1mM. Therefore, to allow the use of the regression equations generated from the phenolic compounds standard curves the IS was first normalised from 0.05mM to 0.1mM by multiplying by a factor of two. This is supported by the results in Figure 3 which shows that a doubling of the naringenin concentration gives a doubling of the peak area.

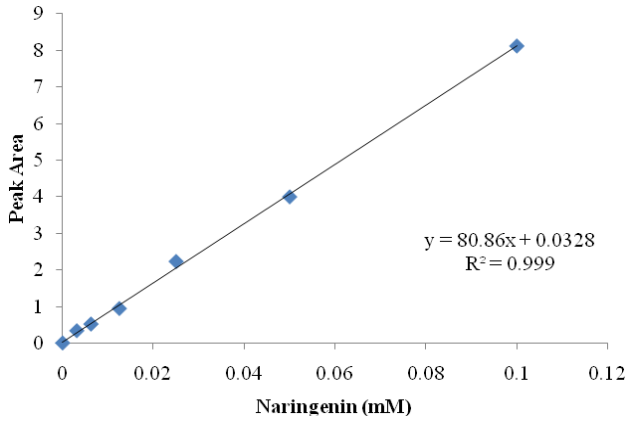

Figure 3. Naringenin standard curve
The area of the naringenin peak after HPLC and detection at 280nm is plotted against increasing naringenin concentrations

After normalizing the IS to 0.1mM, the concentrations of the phenolic compounds (in mM) were calculated using the regression equation for the external standard curves. The concentrations of the phenolic compounds in the apple juices were then converted from mM to µM for ease of use.

For the concentrations (in µM) of the phenolic compounds in the juices made in the laboratory from Granny Smith and Royal Gala apples, the values were also converted into µg of the phenolic compounds per g of apple to allow comparisons with the whole apples and the calculation of the % recovery in the juice compared to the initial apples. This was done by converting the concentration (in µM) of each phenolic compound using the known volume of the juice (for example 103.3ml for cloudy juice from Granny Smith and 104ml for cloudy juice from Royal Gala) and the respective molecular weight of the phenolic compounds. The value generated was then divided by the initial mass of apple juiced (250 g) and expressed as the µg of each phenolic compound per g of apple (µg/g). The % recovery was calculated by multiplication by 100 the ratio of the mass of phenolic compounds retained in the juice divided by the mass of the phenolic compounds in the initial apple by 100.

Quantification of vitamin C in apple juices

A solution of 64mM of ascorbic acid (vitamin C) was prepared in deionised water. This solution was then diluted one to one with a stock solution containing 0.2mM IS in methanol, yielding a solution of 32mM of vitamin C and 0.1mM of IS in 50% (v/v) methanol. Concentrations for the vitamin C standard curve ranging from 0.03 to 32mM, were then prepared by serial dilution with 0.1mM IS in methanol.

The standard curve for vitamin C was generated by plotting the peak area ratios (peak area of the vitamin C divided by the peak area of the IS) on the y-axis against the known concentrations of vitamin C. Microsoft Excel 2007 from Microsoft Office was used to obtain the regression line equation and the R^2 value for the vitamin C standard curve.

Quantification of the vitamin C in the apple juices was done by using the regression equation generated from the standard curve and the concentration of the vitamin C in the juices was expressed in µM. However, the vitamin C concentration could not be determined using the methanol extracts from the Granny Smith and Royal Gala apple because vitamin C is not very soluble in methanol.

Measurement of antioxidant activity in apples and apple juices

The ORAC assay system and Trolox Equivalents calculations

The antioxidant activity assay was carried out in a microtitre plate format using the ORAC method described previously by Cao *et al.* (1993), Ou *et al.* (2001) and Prior *et al.* (2003). The FLUOstar Omega microplate reader (BMG LABTECH Pty. Ltd., Mount Eliza, VIC) was used with excitation and emission wavelengths of 485nm and 520nm respectively. The antioxidant Trolox (a water-soluble analogue of vitamin E) is used as the standard against which all other antioxidants are compared, and the antioxidant activity is expressed as Trolox equivalents.

Stock solutions of 10nM fluorescein, 240mM AAPH and 500µM Trolox were prepared in 10mM potassium phosphate buffer, pH 7.4. The 500µM Trolox stock standard was then used to prepare the following Trolox working standard concentrations: 50µM, 37.5µM, 25µM, 12.5µM, 6.25µM and 3.125µM in 10mM potassium phosphate buffer, pH 7.4. Quality control solutions of 10µM and 20µM of gallic acid in 10mM potassium phosphate buffer, pH 7.4 were also used.

Prior to determining the antioxidant activity, suitable dilutions of the samples in 10mM potassium phosphate buffer, pH 7.4, were established to ensure that the readings would fall within those of the Trolox standard curve. Though there were some exceptions, 1 in 250 and 1in 500 dilutions gave the best results for the apple juice and apples respectively.

The antioxidant assay was done in a black 96-well flat-bottomed microtiter plate. The assay set-up is summarised in Table 3. For the assay, suitable dilutions of one of the three batches of commercial apple juices, the methanol extract from the Granny Smith apples, the methanol extract from the Royal Gala apples, the cloudy and clarified juices from the Granny Smith and from Royal Gala apples and the cloudy and clarified juices from the commercial apple processor were all analysed in triplicate.

The blank of 10mM potassium phosphate buffer (pH 7.4), the 6 Trolox standards and the 2 gallic acid control samples were also analysed in triplicate.

The assay ran for a total of 60 cycles, with the first 10 cycles used to equilibrate the system to 37°C. After the 10^{th} cycle was completed, 25µL of AAPH was automatically added by the injector in the instrument (Table 3).

Table 3. Setup for the microtiter plate ORAC assay

Reagents	Volume
10nM Fluorescein*	150 µL
Sample, standard, control or buffer*	25 µL
240mM AAPH**	25 µL

*Added into wells before the assay was started.
**Added into wells after the 10th cycle by the FLUOstar Omega's injector. A cycle was 75 sec long.

Using the FLUOstar Omega MARS Data Analysis Software (BMG LABTECH Pty. Ltd., Mount Eliza, VIC), the area under the curve (AUC) value for the fluorescence in Relative Fluorescence Units (RFU) measured over time minus the AUC for the blank (buffer only) was calculated for each Trolox concentration. This blank-adjusted AUC for each Trolox concentration was plotted against the Trolox concentrations (µM) to construct a standard curve. The regression equation for the standard curve was then used to determine a Trolox Equivalents (TE) for each of the samples after adjusting their AUC by subtracting the AUC for the blank. The antioxidant activity of the samples was then expresses in terms of µM TE.

Calculation of the non-vitamin C antioxidant activity of the apple juices

The ORAC antioxidant assay (Cao *et al.* 1993; Ou *et al.* 2001; Prior *et al.* 2003) was also done for a series of concentrations of vitamin C to construct a standard curve to determine the contribution of vitamin C to the total antioxidant activity of the apple juices. Following the same ORAC procedure as for the apple juice and apple extract samples, the antioxidant activity of the vitamin C standards, ranging from 100 to 1000 µM in deionised water, were plotted against the known concentrations of the vitamin C. Microsoft Excel 2007 from Microsoft Office was then used to obtain the regression line equation and the R^2 value for the vitamin C standard curve.

Using the vitamin C concentration measured by HPLC in each apple juice and the regression line equation for the vitamin C standard curve, the contribution of vitamin C to the total antioxidant activity of each juice was determined and expressed in µM TE. For each juice, this value was then subtracted from its total antioxidant activity to determine the non-vitamin C antioxidant activity (µM TE) of the apple juices. This calculated non-vitamin C antioxidant activity was taken to represent the antioxidant activity which could be due to the phenolic compounds. The contribution of the phenolic compounds towards the antioxidant activity (µM TE) of the juices was thus calculated by subtracting the contribution of vitamin C from the total antioxidant activity in the juices.

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Science (SPSS) (Licensed to the University of Newcastle) and Microsoft Excel 2007 Package with statistical significance for difference set at $p < 0.05$ for all statistical tests.

For comparing mean values between 2 samples, the Student T-Test was used and for comparing mean values between more than 2 samples, the one-way ANOVA and the LSD post-hoc test were used.

The SPSS software was also used to generate Pearson correlation coefficients and *p* values for correlations between parameters of the commercial apple juices.

Results

Physical properties of the commercial apple juices

The physical properties of the juices (turbidity, viscosity and total soluble solids) are presented in Table 4. The turbidity of the juices was clearly related to whether the juice was cloudy or clarified. The clarified apple juices had a much lower level of turbidity, with values ≤ 0.06 Abs at 600nm, than the cloudy juices (Table 4). The turbidity of the cloudy juices ranged from 2.22 to 5.35 Abs at 600nm; these values were at least 37 times higher than that of the clarified juice with the highest turbidity (C14).

Table 4. Physical properties of commercial apple juices

Samples	Clarified/Cloudy	Turbidity (Abs 600nm)*	Viscosity (cP)**	Total Soluble Solids (°Brix)*
C1†	Clarified	<0.01	8	11.1±0.0
C2†	Clarified	0.02±0.01	8	11.1±0.1
C3†	Clarified	0.01±0.00	8	11.2±0.0
C4†	Clarified	0.02±0.01	8	10.9±0.0
C5†	Clarified	0.03±0.01	8	11.1±0.0
C6†	Clarified	0.01±0.01	8	11.0±0.0
C7††	Cloudy	4.12±0.01	10	12.0±0.1
C8†	Cloudy	4.46±0.03	12	11.2±0.0
C9†	Clarified	0.01±0.06	8	11.1±0.0
C10‡	Cloudy	2.22±0.01	10	11.9±0.1
C11‡	Cloudy	2.30±0.04	10	13.1±0.0
C12‡	Clarified	<0.01	8	12.1±0.0
C13‡	Cloudy	5.35±0.05	10	11.0±0.1
C14‡	Clarified	0.06±0.01	8	13.4±0.1
C15‡	Cloudy	3.60±0.09	8	12.2±0.0
C16†	Clarified	0.02±0.01	8	12.4±0.1
C17†	Clarified	0.02±0.01	8	11.7±0.1

* Values are expressed as mean±S.E. for the three batches of each juice.

** Values for the three batches of each juice were exactly the same.

† Made from reconstitiuted juice concentrate; ‡ Made from crushed apples

†† Made from reconcentrated juice concentrate and crushed apples

The viscosity was also dependant on whether the juice was cloudy or clarified (Table 4). All the clarified juices showed the same level of viscosity (8 cP). Of the six cloudy juices, 5 juices had a higher viscosity (10 or 12 cP) than the clarified juices. However, the other cloudy juice (C15) had the same viscosity level as the clarified juices (8 cP). With a viscosity of 12 cP, cloudy juice C8 was the most viscous of all the juices.

In general, the measurement of the total soluble solids for the juices reconstituted from concentrate generated similar °Brix values (10.9 - 11.2°Brix), regardless of the type of juice, clarified or cloudy (Table 4). However, C16 and C17, although made from concentrate, had higher amounts of soluble solids (12.4 and 11.7°Brix, respectively). On the other hand, most of the juices made from crushed apples had higher values than those made from concentrate and the values had more variance, ranging from 11.9° - 13.4°Brix. However, C13, made from crushed apples had a °Brix value (11.0 °Brix) similar to most of the juices made from concentrate.

HPLC analysis of the apple phenolic compounds and vitamin C

The peaks indicating the elution time for each of the phenolic compounds of interest are presented in Figure 4 and the standard curve for each phenolic compound is presented in Figure 5. The R^2 values or correlation for all the phenolics standard curves were very close to 1, indicating near-perfect linear relationships. Therefore, the external standard curves were reliable for translating the peak areas generated by HPLC, for each of the monitored apple phenolic compounds in the analysed juices, into their concentrations.

Quercetin was not found in any of the samples analysed. This finding indicated that there was no free quercetin in the apples or apple juices. Therefore, the results presented in the subsequent tables and graphs, do not report on quercetin.

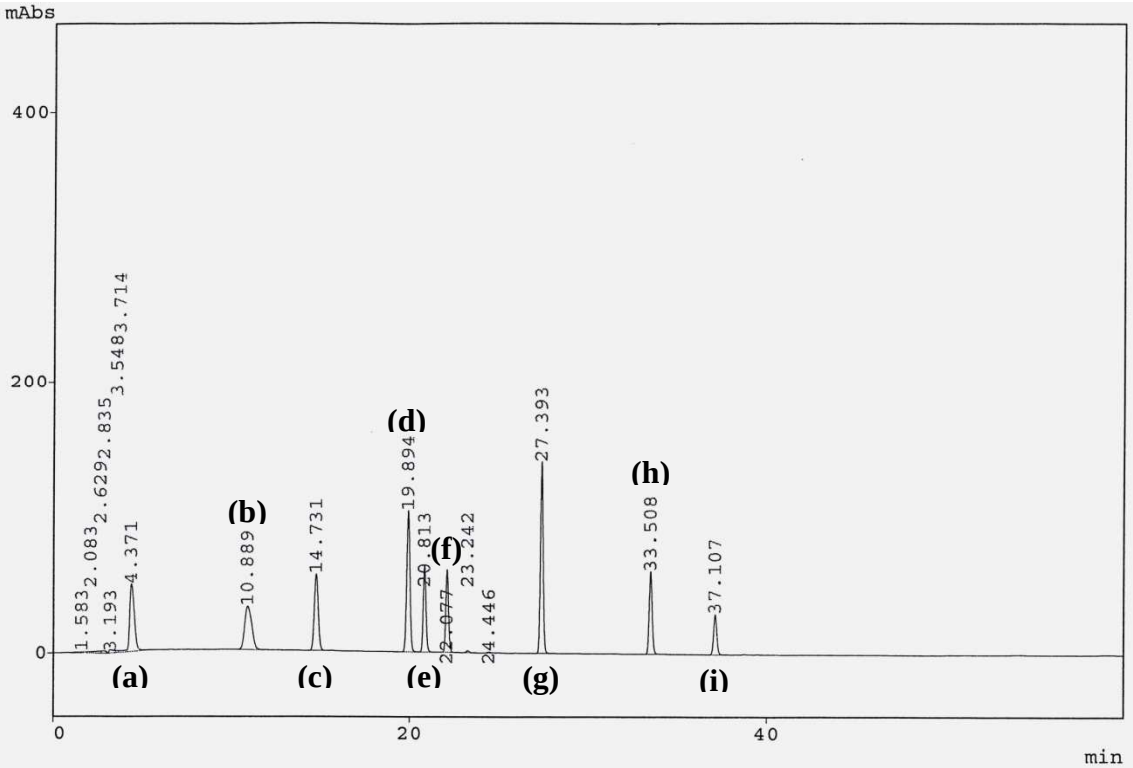

Figure 4. Typical HPLC chromatogram for apple phenolics with the internal standard (IS)

The peaks separated by HPLC and detected at 280nm are in order of elution **(a)** gallic acid, **(b)** chlorogenic acid, **(c)** caffeic acid, **(d)** coumaric acid, **(e)** rutin, **(f)** quercetin glucoside, **(g)** phloridzin, **(h)** quercetin, **(i)** naringenin (IS).
The concentration of the apple phenolic compounds was 0.375mM and the IS was 0.1mM.

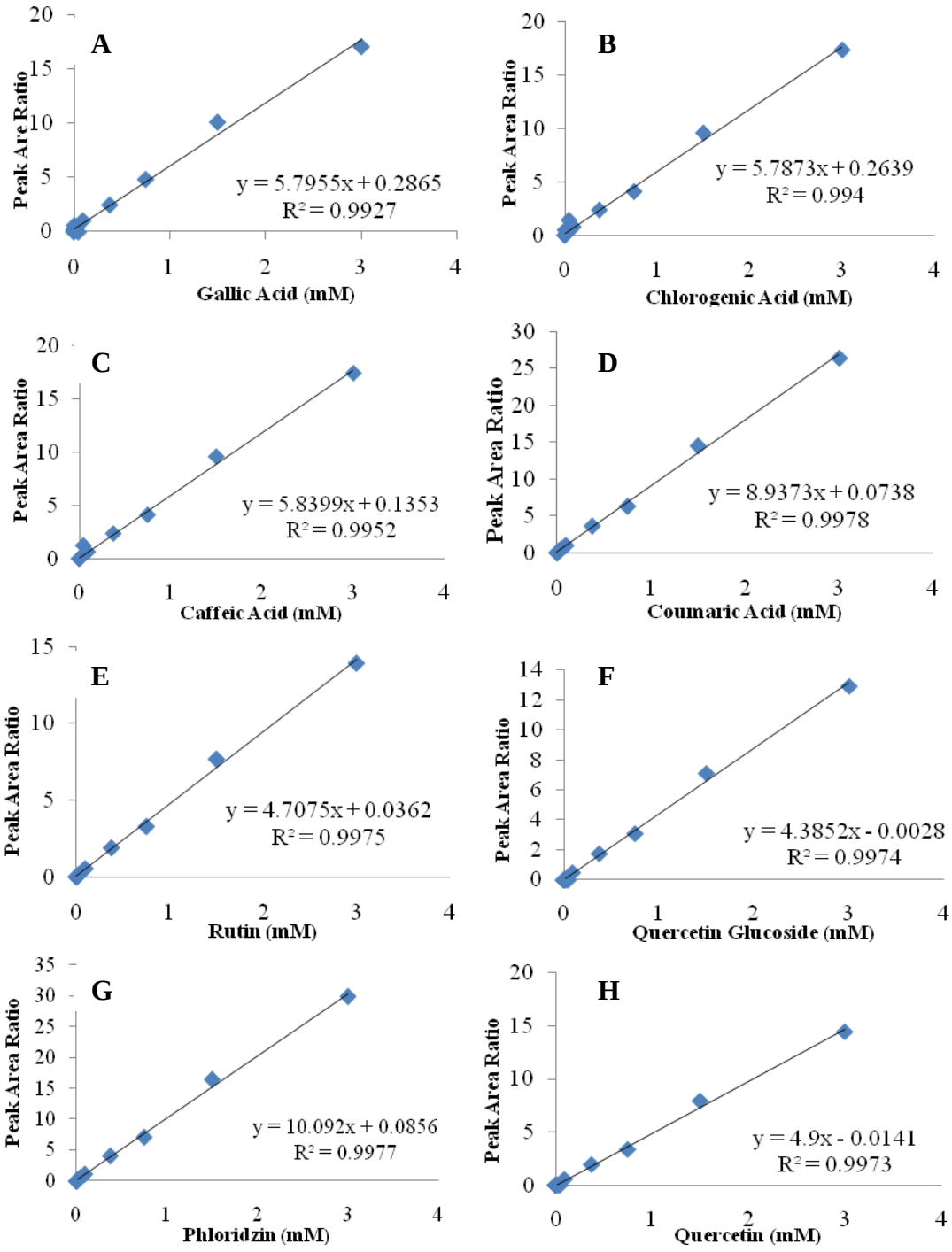

Figure 5. Apple phenolic compound standard curves
As detected at 280nm, the peak area of each phenolic compound was divided by the peak area of the IS (Peak Area Ratio)

In order of elution from the HPLC column, the standard curves are for: **A**. gallic acid, **B.** chlorogenic acid, **C.** caffeic acid, **D.** coumaric acid, **E.** rutin, **F.** quercetin glucoside, **G.** phloridzin and **H.** quercetin

In addition to phenolics, vitamin C content was also measured in all the apple juices as it significantly contributes to the total antioxidant activity. Figure 6 shows the identification of the vitamin C peak after HPLC and detection at 254nm.
The standard curve for vitamin C is also shown by Figure 7. The R^2 value was close to 1, ensuring the reliability of the linear regression equation for use in calculating the vitamin C concentration in the apple juices.

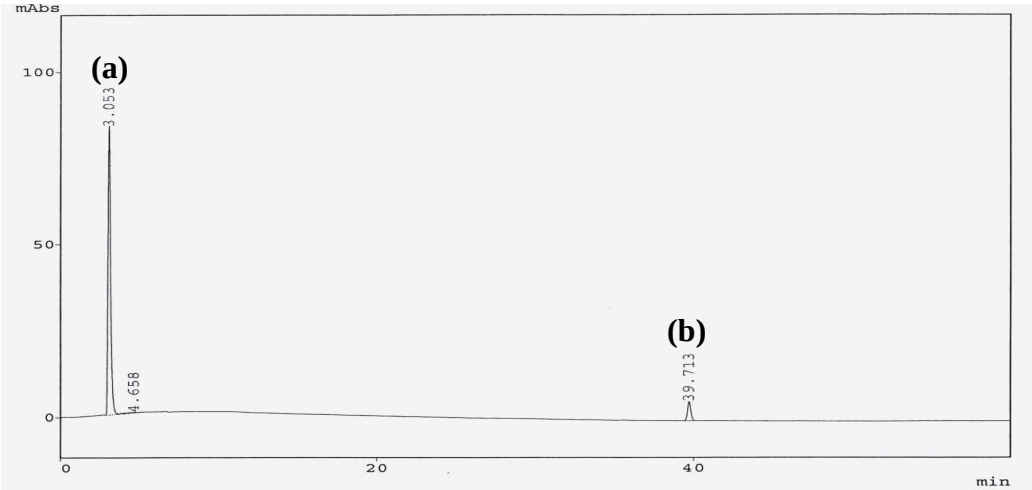

Figure 6. Typical HPLC chromatogram for Vitamin C
The peaks separated by HPLC and detected at 254nm are in order of elution:
(a) vitamin C and **(b)** naringenin (IS)
The concentration of the vitamin C is 4mM and IS is 1mM

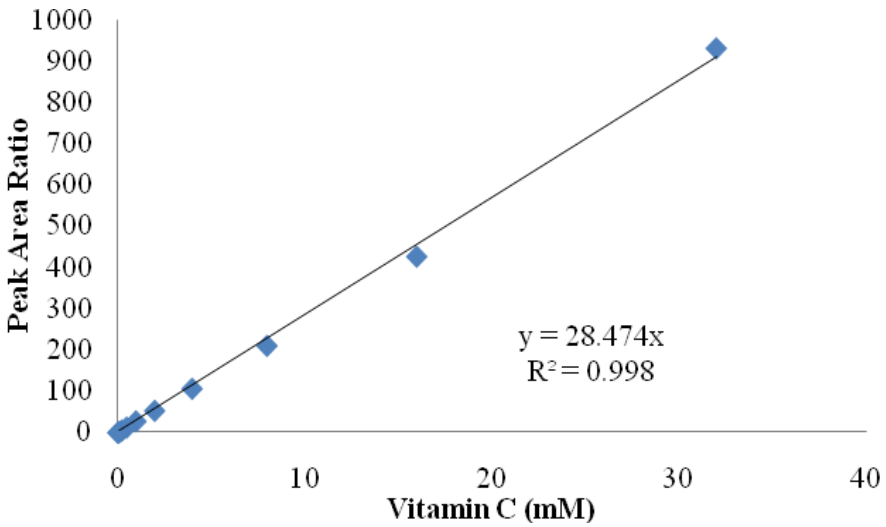

Figure 7. Vitamin C standard curve
As detected at 254nm, the peak area of vitamin C was divided by the peak area of the IS (Peak Area Ratio)

Phenolics and vitamin C in commercial apple juices

Table 5 shows the concentration of vitamin C and phenolics in the nine commercial apple juices made from imported concentrate as determined by HPLC analysis. Of these C5, C1 and C3 had the highest vitamin C concentrations (2,835µM, 2,447µM and 2,406µM, respectively). In the other six juices, the vitamin C concentrations ranged from 606 µM to 1,528µM.

Of the phenolic compounds, gallic acid and chlorogenic acid were predominant in all the commercial juice made from imported concentrate, except for C9, in which the caffeic acid was at a similar lower level to the gallic acid. The clarified juice C4 had the most gallic acid (665µM) and C3 had the highest concentration of chlorogenic acid (461µM). Caffeic acid was present in seven out of these nine commercial juices mostly at a lower concentration compared to gallic acid (except C9) and chlorogenic acid, and the highest concentration of caffeic acid was found in C3 (74µM). However C1, C16 and C17 had no detectable caffeic acid.

Coumaric acid, rutin, quercetin glucoside and phloridzin were all very low across the juices made from the imported concentrate and were absence in most of these juices. The total phenolic compound concentrations ranged from a very low 48µM in juice C16 to 954µM in juice C3.

Commercial apple juices from Australian apples

Table 6 shows the concentrations of vitamin C and phenolics in the eight commercial apple juices made from Australian apples. On average, the vitamin C content in the juices made from Australian apples (Table 6) did not differ (p = 0.228) from those made from imported concentrate (Table 5). The highest in vitamin C were C8, C10 and C13 (3237µM, 3,128µM and 2,751µM, respectively) and the lowest concentration was in juice C11 (829µM).

The phenolics were more widely distributed in this group of juices, although gallic acid, chlorogenic acid and caffeic acid were still the predominant compounds present in all the juices made from Australian apples. In contrast to the reconstituted juices, coumaric acid, rutin, quercetin glucoside and phloridzin were present in most of the juices made from Australian apples; of these compounds, only coumaric acid, rutin and phloridzin were absent in one juice for each. The total phenolic compounds ranged from 276µM (C15) to 2,510µM (C8).

Comparison of juices from Australian apples with juices from imported concentrates.

The comparison of the phenolic components of the juices made from Australian apples with the juices made from imported concentrate is presented in Figure 8. As depicted in the graph, the apple juices made from Australian apples contained higher concentrations of chlorogenic acid (p = 0.024), caffeic acid (p = 0.018), rutin (p = 0.007) and quercetin glucoside (p = 0.0009). However, the concentration of gallic acid, coumaric acid and phloridzin did not differ between the two types of juice. Furthermore, the total phenolic compounds did not significantly differ between the two types of juice either (p = 0.058).

Clarified juices – imported concentrates versus Australian apples

A comparison between the nine clarified apple juices made from imported concentrate (Table 5) and the 2 clarified apple juices made from Australian apples (C12 and C14, Table 6) was also done. Statistical analysis showed no significant difference in the total phenolic compounds between these two groups (p = 0.327), despite the Australian clarified juices being significantly higher in caffeic acid, rutin and quercetin glucoside (p = 0.012, <0.01 and = 0.014, respectively) than the clarified juices made from imported concentrate.

Table 5. Constituents in apple juices made from imported apple juice concentrate

Constituents	Commercial Apple Juices Made from Imported Concentrate								
	C1 Clarified	**C2 Clarified**	**C3 Clarified**	**C4 Clarified**	**C5 Clarified**	**C6 Clarified**	**C9 Clarified**	**C16 Clarified**	**C17 Clarified**
Vitamin C (µM)	2,447±116	1,378±58	2,406±92	1,528±188	2,835±91	606±38	1,067±36	1,133±47	951±43
Gallic Acid (µM)	214±8	456±61	370±94	665±111	236±24	177±19	40±9	33±10	89±61
Chlorogenic Acid (µM)	16±2.8	291±133	461±132	188±65	151±26	68±22	372±59	15±2	22±7
Caffeic Acid (µM)	0	40±21	74±62	45±34	27±14	50±50	44±20	0	1±1
Coumaric Acid (µM)	0	0	1±0.6	0	0	0	32±32	0	0
Rutin (µM)	0	0	1±1	0	0	4±5	19±10	0	0
Quercetin Glucoside (µM)	0	0	12±10	0	0	5±5	67±15	0	1±1
Phloridzin (µM)	0	0	35±10	0	3±2	13±13	1±1	0	7±3
Total Phenolics (µM)	231 ± 10	787±116	954±289	898±58	417±53	317±75	574±111	48±10	120±73

Values are expressed as mean±S.E. for the three batches of juice.

Table 6. Constituents in apple juices made from Australian apples

Constituents	Commercial Apple Juices Made from Australian Apples							
	C7* Cloudy	**C8** Cloudy**	**C10 Cloudy**	**C11 Cloudy**	**C12 Clarified**	**C13 Cloudy**	**C14 Clarified**	**C15 Cloudy**
Vitamin C (µM)	2,533±142	3,237±118	3,128±44	829±31	1,252±55	2,751±136	1,290±81	1,920±99
Gallic Acid (µM)	144±21	934±197	704±329	37±29	6±4	1 60±72	12±1	68±21
Chlorogenic Acid (µM)	459±93	1,131±204	793±89	869±337	109±4	736±45	231±18	165±35
Caffeic Acid (µM)	55±3	124±47	78±24	82±36	67±17	90± 14	156±46	3±2
Coumaric Acid (µM)	0	14±14	14±14	23±16	16±8	2±2	12± 2	2±2
Rutin (µM)	26±13	70±35	78±8	61±30	24±7	21±21	27±5	0
Quercetin Glucoside (µM)	83±8	183±31	101±42	103±29	45±20	144±59	78±6	39±8
Phloridzin (µM)	13±7	55±28	23±6	27±13	12±10	15±15	5 ±5	0
Total Phenolics (µM)	780±91	2510±394	1,791±329	1,202±467	279±43	1,167±170	522±50	276±21

Values are expressed as mean±S.E. for the three batches of juice.

*C7 was made from a combination of imported apple juice concentrate and Australian apples.
**C8 was made from concentrate originating from Australian apples.

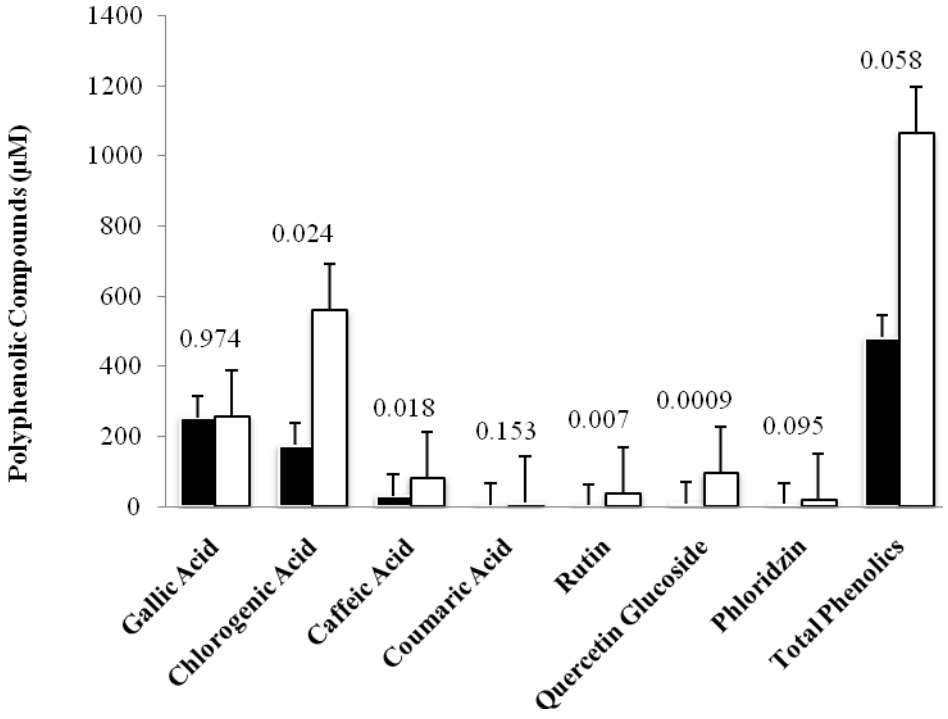

Figure 8. Comparison of phenolic compounds in the commercial apple juices made from imported concentrate (■) vs. from Australian apples (□)

The bar values are means±S.E. and the numbers over the bars indicate the *p* value for the comparisons between the two types of juices

Juices from Australian apples – cloudy versus clarified juices

A comparison was also made between the six cloudy juices and the two clarified juices within the commercial apple juices made from Australian apples (Table 6). Statistically, the cloudy apple juices contained higher total phenolic compounds than the clear juices (p = 0.041) which appeared to be due mainly to differences in chlorogenic acid (p = 0.013) as no other phenolic compound was significantly different in concentration between the cloudy and clarified juices made from Australian apples.

Clarified versus cloudy juices, irrespective of origin

Based on the difference in the total content of phenolic compounds between the cloudy apple juices and the clear juices (p = 0.041) made from Australian apples, the two Australian clear apple juices were grouped with the clarified apple juices made from imported concentrate as shown in Table 7. Similarly, although one of the other juices contained imported concentrate and another was made from Australian concentrate, the other six apple juices were all cloudy and were grouped together as shown in Table 8. This allowed for a comparison to be made between the commercial apple juices based on whether they were clarified or cloudy irrespective of whether they originate in Australia or not or from concentrate or not.

For vitamin C levels, there was no difference (p = 0.074) between the average content in the clarified apple juices (Table 7) versus the cloudy apple juices (Table 8). However, as seen in Figure 9, the total content of phenolic compounds in the cloudy juices was significantly higher in comparison with their clarified counterparts (p = 0.049) which appeared to be mainly due to a higher concentration of chlorogenic acid (p = 0.011), although rutin (p = 0.037) and quercetin glucoside (p = 0.004) were also higher in the cloudy juices compared to the clarified juices.

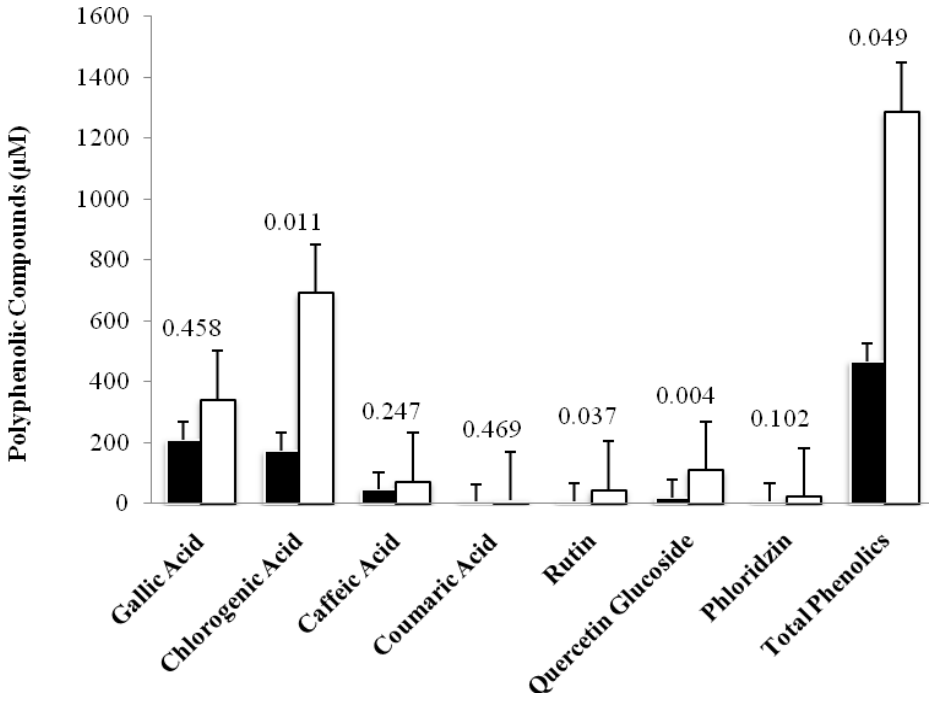

Figure 9. Comparison of phenolic compounds in the commercial clarified apple juices (■) vs the commercial cloudy apple juices (□)

The bar values are means±S.E. and the numbers over the bars indicate the p values for the comparisons between the two types of juices

Table 7. Constituents in commercial clarified apple juices

Constituents	Commercial Clarified Apple Juices										
	C1 Imported Conc.*	**C2 Imported Conc.***	**C3 Imported Conc.***	**C4 Imported Conc.***	**C5 Imported Conc.***	**C6 Imported Conc.***	**C9 Imported Conc.***	**C12 Aus** Apples**	**C14 Aus** Apples**	**C16 Imported Conc.***	**C17 Imported Conc.***
Vitamin C (µM)	2,447±116	1,378±58	2,406±92	1,528±188	2,835±91	606±38	1,067±36	1,252±55	1,290±81	1,133±47	951±43
Gallic Acid (µM)	214±8	456±61	370±94	665±111	236±24	177±19	40±9	6±4	12±1	33±10	89±61
Chlorogenic Acid (µM)	16±2.8	291±133	461±132	188±65	151±26	68±22	372±59	109±4	231±18	15±2	22±7
Caffeic Acid (µM)	0	40±21	74±62	45±34	27±14	50±50	4 4±20	67±17	156±46	0	1±1
Coumaric Acid (µM)	0	0	1±0.6	0	0	0	32±32	16±8	12±2	0	0
Rutin (µM)	0	0	1±1	0	0	4±5	19±10	24±7	27±5	0	0
Quercetin Glucoside (µM)	0	0	12±10	0	0	5±5	67±15	45±20	78±6	0	1±1
Phloridzin (µM)	0	0	35±10	0	3±2	13±13	1±1	12±10	5±5	0	7±3
Total Phenolics (µM)	231 ± 10	787±116	954±289	898±58	417±53	317±75	574±1 11	279±43	522±50	48±10	120±73

Values are expressed as mean±S.E. for the three batches of juice

* 'Imported Conc.' refers to juices made from imported concentrate
** 'Aus Apples' refers to juices made from Australian apples

Table 8. Constituents in commercial cloudy apple juices

Constituents	Commercial Cloudy Apple Juices					
	C7 *Imported Conc. & **Aus Apples	**C8 ***Aus Conc.**	**C10 **Aus Apples**	**C11 **Aus Apples**	**C13 **Aus Apples**	**C15 **Aus Apples**
Vitamin C (µM)	2,533±142	3,237±118	3,128±44	829±31	2,751±136	1,920±99
Gallic Acid (µM)	144±21	934±197	704±329	37±29	160±72	68±21
Chlorogenic Acid (µM)	459±93	1,131±204	793±89	869±337	736±45	165±35
Caffeic Acid (µM)	55±3	124±47	78±24	82±36	90±14	3±2
Coumaric Acid (µM)	0	14±14	14±14	23±16	2±2	2±2
Rutin (µM)	26±13	70±35	78±8	61±30	21±21	0
Quercetin Glucoside (µM)	83±8	183±31	101±42	103±29	144±59	39±8
Phloridzin (µM)	13±7	55±28	23±6	27±13	15±15	0
Total Phenolics (µM)	780±91	2,510±394	1,791±329	1,202±467	1,167±170	276±21

Values are expressed in mean±S.E. for the three batches of juice

* 'Imported Conc.' refers to juices made from imported concentrate
** 'Aus Apples' refers to juices made from Australian apples
** 'Aus Conc.' refers to juices made from Australian concentrate

Phenolics and vitamin C in Granny Smith and Royal Gala apples and their laboratory prepared juices

Granny Smith and Royal Gala apples

The content of the phenolic compounds in a batch of Granny Smith and a batch of Royal Gala apples is presented in Table 9. The Royal Gala apples contained over twice the total phenolic compounds (2,363 µg/g) compared to the Granny Smith apples (1,036 µg/g) mainly due to their almost four fold higher content of chlorogenic acid (2,125 µg/g) compared to Granny Smith apples (591 µg/g).

Compared to the commercial juices (Table 5, 6, 7 and 8), chlorogenic acid was still the largest contributor to the total phenolic compounds in the two apple cultivars (Table 9). Gallic acid, a major phenolic in commercial juices, was present at only low levels in these two cultivars. In fact, the phenolic with the second highest concentration was quercetin glucoside, followed by rutin, caffeic acid and phloridzin. However, the concentration of vitamin C was very low, due to the vitamin not being very soluble in methanol.

Table 9. Constituents in Granny Smith and Royal Gala apples

Constituents*	Granny Smith	Royal Gala
Vitamin C (µM)	4.3±0.01[a]	1.5±0.01[b]
Gallic Acid (µg/g)	9.7±1.2[a]	18.2±6.5[a]
Chlorogenic Acid (µg/g)	590.7±10.1[a]	2,124.8±9.3[b]
Caffeic Acid (µg/g)	44.4±1.6[a]	45.4±0.6[a]
Coumaric Acid (µg/g)	8.8±1.1[a]	10.7±0.5[a]
Rutin (µg/g)	58.8±2.5[a]	49.1±7.5[a]
Quercetin Glucoside (µg/g)	308.7±2.2[a]	97.2±4.0[b]
Phloridzin (µg/g)	15.0±0.6[a]	17.2±0.5[b]
Total Phenolics (µg/g)	**1,036.0±9.0[a]**	**2,362.6±2.7[b]**

*The values are the mean±S.E. for one batch of each apple type of apple measured in triplicate and are expressed as µg of constituent per g of apple (µg/g)
Values in a row not sharing a superscript letter are significantly different from each other (p<0.05)

Cloudy and clarified juices from Granny Smith and Royal Gala apples

The constituents measured in the cloudy and clarified juices made from the analysed batch of Granny Smith and of Royal Gala apples are presented in Table 10. Similar to the content in the apples used to make the juices, the chlorogenic acid levels in both the cloudy (39.8 ± 7.4µg/g) and the clarified (37.4 ± 1.6µg/g) juices from the Royal Gala apples were higher compared to their counterparts made from Granny Smith (cloudy, 20.5 ± 0.8µg/g and clarified, 8.6 ± 5.6µg/g). However, there were no differences in the total phenolic compounds between any of the four juices (Table 10). All the juices also had very similar values for total soluble solids and vitamin C.

Table 10. Constituents in juices made in the laboratory from Granny Smith and Royal Gala apples

Constituents	Granny Smith		Royal Gala	
	Cloudy	Clarified	Cloudy	Clarified
Total soluble solids (ºBrix)	11.8±0.0 [a]	11.6±0.1[a]	11.8±0.1[a]	11.7±0.1[a]
Vitamin C (µM)	125±3[a]	78±8[a]	88±17[a]	71±8[a]
Gallic Acid (µg/g)	3.8±0.3[a]	1.2±0.6[a]	0.4±0.4[a]	0[a]
Chlorogenic Acid (µg/g)	20.5±0.8[ac]	8.6±5.6[a]	39.8±7.4[b]	37.4±1.6[bc]
Caffeic Acid (µg/g)	6.2±1.2[a]	3.1±0.2[b]	3.4±0.4[b]	2.7±0.3[b]
Coumaric Acid (µg/g)	1.7±0.4[a]	0.7±0.1[b]	0[b]	0[b]
Rutin (µg/g)	4. 9±4[a]	1.2±0.1[a]	0[a]	0[a]
Quercetin Glucoside (µg/g)	9.0±0.7[ac]	3.0 ±0.2[b]	4.9±0.3[bc]	1.8±0.2[b]
Phloridzin (µg/g)	0.6±0.5[a]	0.5±0.1[a]	2.47±0.1[b]	2.6±0.2[b]
Total Phenolics (µg/g)	**46.6±4.4[a]**	**18.2±3.2[a]**	**50.9±6.0[a]**	**44.5±0.3[a]**

Values are expressed as mean±S.E. for three juice samples made from one batch of apples
Values in a row not sharing a superscript letter are significantly different from each other (p<0.05)

Total phenolic compounds

The values for the total polyphenolic compounds in the Granny Smith apples and in its cloudy and clarified juices and in the Royal Gala apples and its cloudy and clarified juices are presented in Figure 10. The total phenolic compounds in the Granny Smith apples was statistically different to the total phenolic compounds in the Royal Gala apples (p < 0.01) and the total phenolic compounds in all the juices were dramatically lower than in the apples (p < 0.001).

Recovery of phenolic compounds in juices

Table 11 shows the percentage recovery of the phenolic compounds in the juices (Table 10) compared to their content in the Granny Smith and Royal Gala apples used to make the juices (Table 9). Overall, the recovery of the total polyphenols was very low, ranging from 1.7 ± 0.6% for the Granny Smith clarified juice to 4.5 ± 0.4% for the Granny Smith cloudy juice. Apart from the recovery of phloridzin being higher in the juices made from the Royal Gala apples (14.4 ± 0.5% for the cloudy juice and 10.7 ± 1.1% for the clarified juice) compared to the Granny Smith apples (3.6 ± 2.6% for the cloudy juice and 2.3 ± 0.2% for the clarified juice), there were no major differences in the recovery of phenolic compounds between any of the juices.

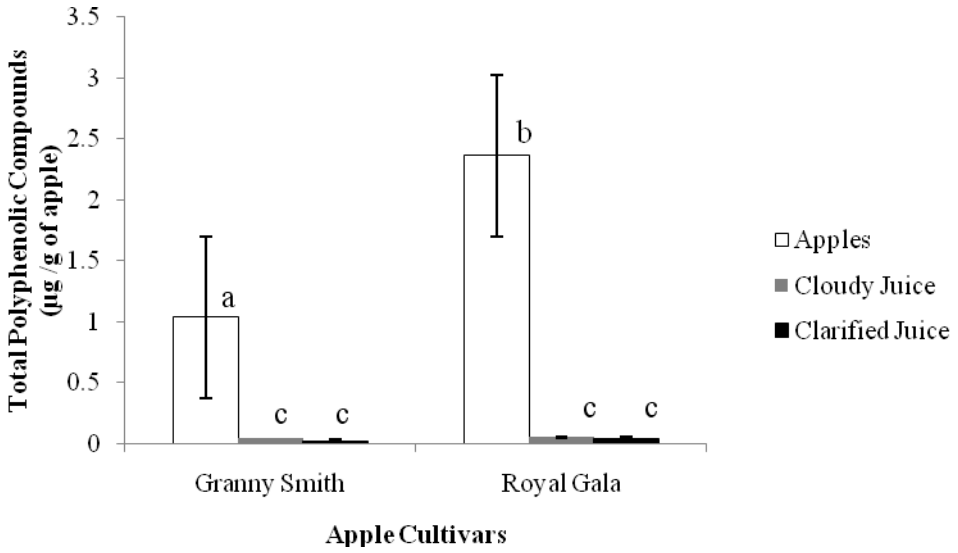

Figure 10. The total phenolic compounds in apples and their cloudy and clarified apple juices

The total phenolic compounds are expressed in µg/g of apple. Values not sharing a superscript letter are significantly different from each other (p<0.05).

Table 11. Recovery of apple phenolic compounds in juice

Phenolic Compounds	Granny Smith		Royal Gala	
	Cloudy	Clarified	Cloudy	Clarified
Gallic Acid (% Recovery)	38.9±3.5^{a}	12.3±0.4^{a}	1.8±1.8^{a}	0^{a}
Chlorogenic Acid (% Recovery)	3.5±0.1^{a}	1.5±0.1^{a}	1.9±0.3^{a}	1.8±0.3^{a}
Caffeic Acid (% Recovery)	13.9±1.6^{a}	6.9±0.5^{b}	7.5±1.3^{b}	6.0±0.2^{b}
Coumaric Acid (% Recovery)	19.6±0.9^{a}	7.2±0.03^{b}	0^{b}	0^{b}
Rutin (% Recovery)	8.3±6.9^{a}	2.0±0.1^{a}	0^{a}	0^{a}
Quercetin Glucoside (% Recovery)	2.9±0.2^{ab}	1.0±0.2^{a}	5.1±0.5^{b}	1.8±0.3^{ac}
Phloridzin (% Recovery)	3.6±2.6^{a}	2.3±0.2^{a}	14.4±0.5^{b}	10.7±1.1^{b}
Total Phenolics (% Recovery**)**	**4.5±0.4^{a}**	**1.7±0.6^{a}**	**2.2±0.4^{a}**	**1.9±0.3^{a}**

Values are expressed as mean±S.E. for three juice samples made from one batch of apples
Values in the row not sharing a superscript letter are significantly different from each other (p<0.05)

Cloudy and clarified juices obtained direct from a commercial apple processor

The values for the constituents measured in the cloudy and clarified apple juices obtained from a commercial apple processor are presented in Table 12. These juices were made from a combination of Granny Smith and Royal Gala apples. The total soluble solids values for the cloudy and clarified juices from the commercial apple processor (12.0 and 11.6 ºBrix, respectively) were within the range of the values from all other juices measured in this study (Tables 4 and 10). Both the cloudy and clarified apple juices from the commercial apple processor contained higher vitamin C ($p<0.01$) than the cloudy and clarified commercial apple juices. Chlorogenic acid was the predominant phenolic compound in both juices. This result was consistent with the measurement of the phenolic compounds in the Granny Smith and Royal Gala apples where chlorogenic acid was also the highest phenolic. Significant differences between the commercial apple processor cloudy and clarified juices were only found for caffeic acid and phloridzin (Table 12) with both being higher in the cloudy juice.

Table 12. Constituents in the commercial apple processor juice

Constituents	Cloudy	Clarified
Total soluble solids (ºBrix)	12.0±0.0[a]	11.6±0.0[b]
Vitamin C (µM)	5,919±90.8[a]	4,652±48.9[b]
Gallic Acid (µM)	68±7.8[a]	36±10.5[a]
Chlorogenic Acid (µM)	600±10.6[a]	412±79.6[a]
Caffeic Acid (µM)	129±3.1[a]	57±8.4[b]
Coumaric Acid (µM)	0[a]	1±1.4[a]
Rutin (µM)	88±1.1[a]	71±14.6[a]
Quercetin Glucoside (µM)	27±0.9[a]	19±3.3[a]
Phloridzin (µM)	27±0.4[a]	16±3.4[b]
Total Phenolics (µg/g)	939±12.43[a]	613±117.5[a]

Values are expressed as mean±S.E. for three juice samples obtained from commercial apple processor

Values in the row not sharing a superscript letter are significantly different from each other ($p<0.05$)

Comparison of commercial processor juices with market-sourced juices

A comparison of the total phenolic compounds in the commercial apple processor juices with the commercial apple juices (Figure 11) showed that there was no difference between the juices. The only significant difference in the comparison was that the cloudy market-sourced commercial juices had a higher content of phenolic compounds than the clarified market-sourced commercial juices, as previously observed in Figure 9.

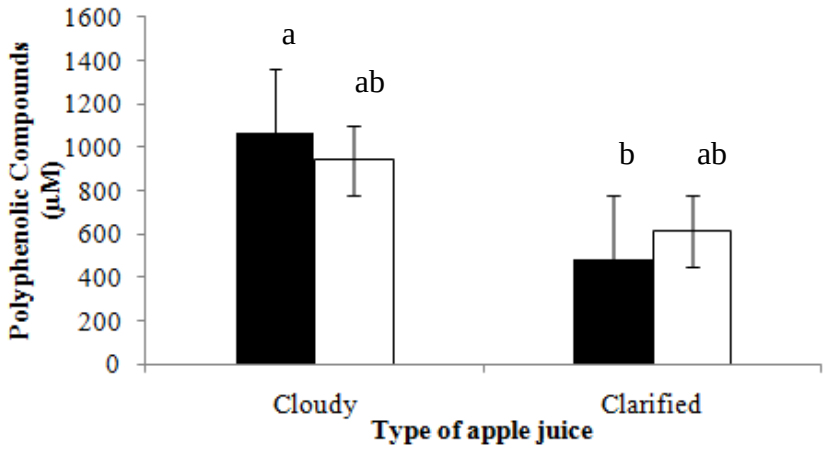

Figure 11. Comparison of phenolic compounds between the market apple juices (■) and the processor apple juices (□)

Values sharing a superscript letter are not significantly different from each other (p<0.05).

ORAC assay antioxidant activity of apples and apple juices

Trolox and Vitamin C standard curves

Figure 12 shows a Trolox standard curve, plotting the fluorescence AUC relative to Trolox concentration that was used to subsequently analyse samples and convert their antioxidant activity into µM TE. A standard curve for the antioxidant activity of vitamin C based on its concentration was also constructed. Increasing concentrations of vitamin C were measured for their antioxidant activity and then plotted as shown in Figure 13. Both relationships showed a very good linear response with R^2 values close to 1, thus allowing the generated linear equations to be used in subsequent analyses.

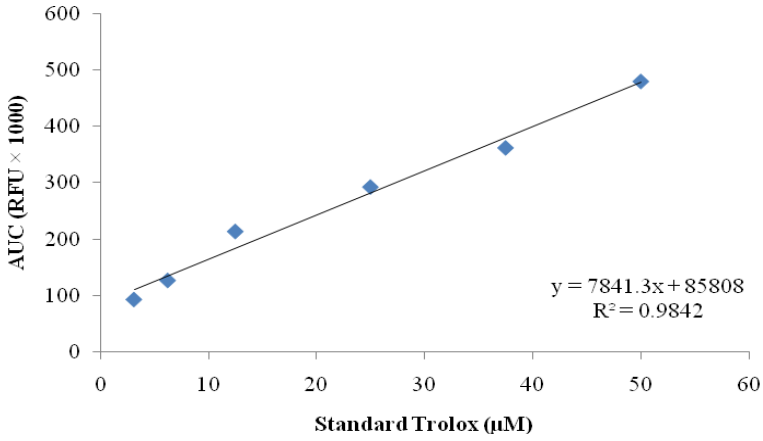

Figure 12. Trolox Standard curve for ORAC assay

The area under the curve (AUC) in relative fluorescence units (RFU) is plotted against increasing concentrations of Trolox

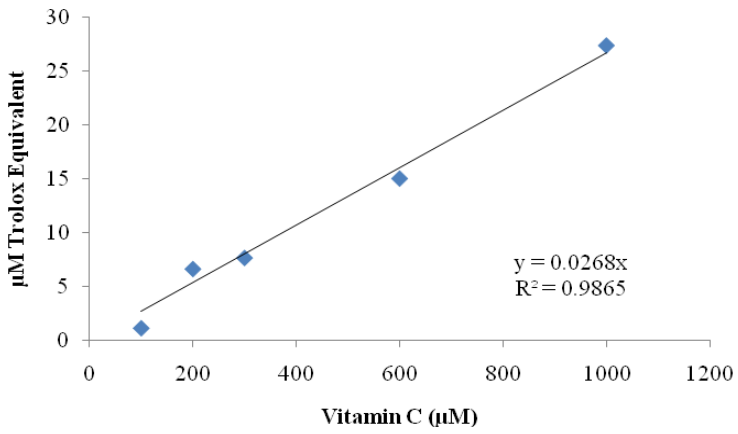

Figure 13. Vitamin C antioxidant activity standard curve

The area under the curve (AUC) in relative fluorescence units (RFU) for increasing concentrations of vitamin C were converted to Trolox Equivalents using the regression equation in Figure 11 and plotted against the vitamin C concentration

In Table 13, the measured total antioxidant activity, the vitamin C antioxidant activity and the non-vitamin C antioxidant activity (total minus vitamin C antioxidant activity) are presented for all the apples and apple juices analysed. The non-vitamin C antioxidant activity was taken to reflect the contribution of the phenolic compounds to the total antioxidant activity of the juices. Vitamin C was not the major source of the antioxidant activity in the juices, only contributing on average 0.62% of the total antioxidant activity in the juices. The highest antioxidant activity was exhibit by C15 (36,001 µM TE) and the lowest antioxidant activity belonged to C6 (93 µM TE).

Phenolic antioxidant activity

Using the µM TE values for the phenolic antioxidant activities in Table 13, the 8 commercial apple juices made from Australian apples (juices C7, C8, C10-15) were found to exhibit a significantly higher antioxidant activity (p = 0.033) in comparison with the 9 commercial apple juices made from imported concentrate (juices C1-C6, C9, C16, C17). It was also found that the 6 cloudy apple juices (juices C7, C8, C10, C11, C13, C15) possessed significantly more antioxidant activity (p = 0.036) compared to the 11 clarified apple juices (C1-C6, C9, C12, C14, C16, C17). There was no significant difference in the antioxidant activity from vitamin C across all the commercial juices.

Antioxidant activity in apples compared to cloudy and clarified juices

The antioxidant activity in the Granny Smith and Royal Gala apples compared to the antioxidant activity retained in the cloudy and clarified juices made from these apples is presented in Figure 14. Similar to the total phenolic compounds measured in the initial apples and in their juices (Figure 10), there was a substantial loss of antioxidant activity when the apples were processed into juices. The antioxidant activity of the Royal Gala apples was significantly (p = 0.031) higher than the Granny Smith apples, which mirrored the higher total phenolic compounds measured in the Royal Gala apples compared to the Granny Smith apples (Table 9).

Table 13. Antioxidant activity of apple juices

Apple and Apple Juices	Total Antioxidant Activity (μM TE)	Vitamin C		Phenolic** Antioxidant Activity (μM TE)
		Concentration (μM)	Antioxidant* Activity (μM TE)	
Commercial				
C1	5,873	2,447	66	5,807
C2	2,989	1,378	37	2,952
C3	3,213	2,406	64	3,138
C4	6,551	1,528	41	6,510
C5	5,201	2,835	76	5,125
C6	109	606	16	93
C7	11,414	2,533	68	11,346
C8	13,859	3,237	87	13,772
C9	9,863	1,067	29	9,835
C10	7148	3,128	84	7,064
C11	4,465	829	22	4,443
C12	10,931	1,252	34	10,898
C13	10,115	2,751	74	10,041
C14	7,908	1,290	35	7,873
C15	36,053	1,920	51	36,001
C16	4,237	1,133	30	4,206
C17	4,455	951	25	4,429
Lab made from Apples* **				
Cloudy GS	4,450	125	3	4,447
Clarified GS	4,073	78	2	4,071
Cloudy RG	4,544	88	2	4,542
Clarified RG	4,169	71	2	4,167
Whole apples				
Granny Smith	6,128	4.3	<1	6,128
Royal Gala	10,058	1.5	<1	10,058
Commercial processor				
Cloudy	14,519	5,919	125	14,394
Clarified	8,512	4,652	159	8,353

*Vitamin C antioxidant activity was calculated using the regression equation obtained from the standard curve shown in Figure 13 and the HPLC determined vitamin C levels

**Phenolic antioxidant activity was calculated by subtracting the antioxidant activity of vitamin C from the total antioxidant activity, except for apples where the vitamin C concentration was negligible as seen in Table 9

***GS means juice from Granny Smith apples and RG means Royal Gala apples

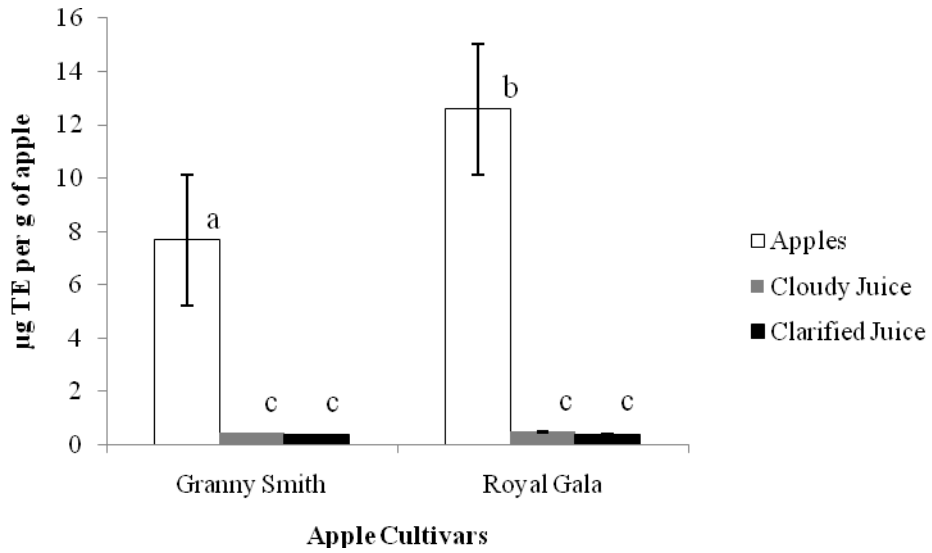

Figure 14. The antioxidant activity measure in apple, cloudy juice and clarified juice of two apple cultivars (Granny Smith and Royal Gala)
The antioxidant activities were expressed in µg Trolox Equivalents (TE) per g of apple

Correlations between parameters of the commercial apple juices

The correlation between the total phenolic compounds and the total antioxidant activity (Figure 15) and between the total phenolic compounds and the vitamin C corrected antioxidant activity (Figure 16) in the commercial samples did not show any significance with $p = 0.068$ and $p = 0.069$, respectively.

Figure 17 shows the relationship between the level of turbidity and the total phenolic compounds in the commercial apple juice samples. The R^2 value indicated that there was approximately a 48% chance that the increase in turbidity will lead to an increase in the total phenolic compound concentration ($p<0.01$). However, there was no significant correlation ($p = 0.28$) between the turbidity and the vitamin C corrected antioxidant activity of the commercial apple juices (Figure 18).

However, viscosity showed a significant correlation to both the total phenolic compounds (Figure 19) and the vitamin C corrected antioxidant activity (Figure 20) in the commercial apple juices ($p < 0.001$ and $p = 0.005$, respectively). Based on the R^2 values, there was approximately a 75% chance of an increase in the total phenolic compounds and a 45% chance of an increase in the antioxidant activity as the viscosity increased.

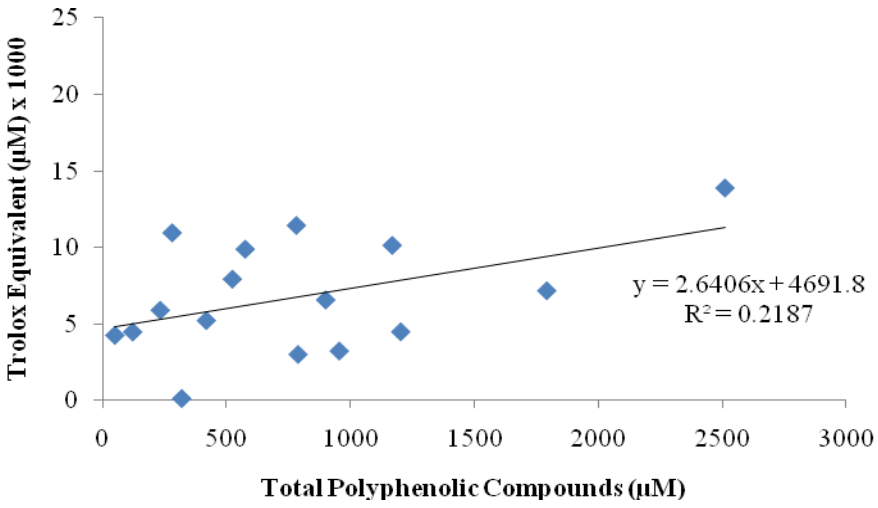

Figure 15. Correlation between the phenolic compounds and the total antioxidant activity in the commercial apple juices without correction for the vitamin C content (p = 0.068.)

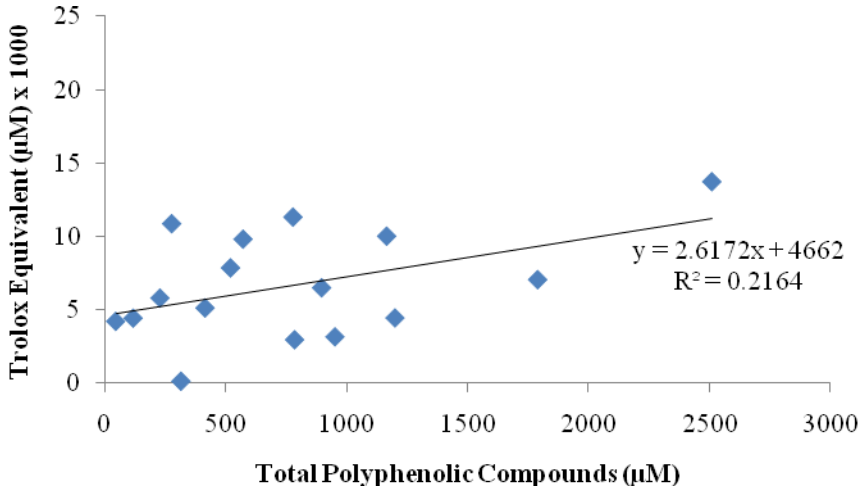

Figure 16. Correlation between the phenolic compounds and the total antioxidant activity in the commercial apple juices with correction for the vitamin C content (p = 0.069)

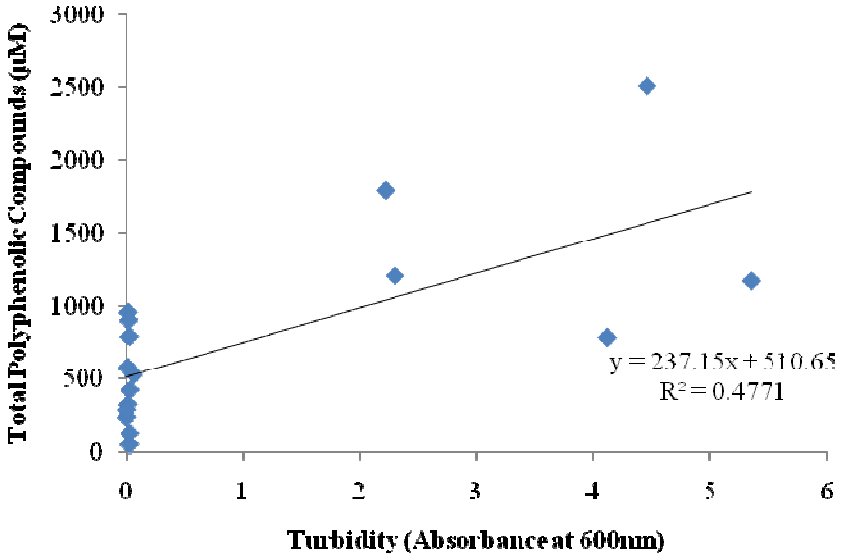

Figure 17. Correlation between the turbidity and the phenolic compounds in the commercial apple juices ($p < 0.001$)

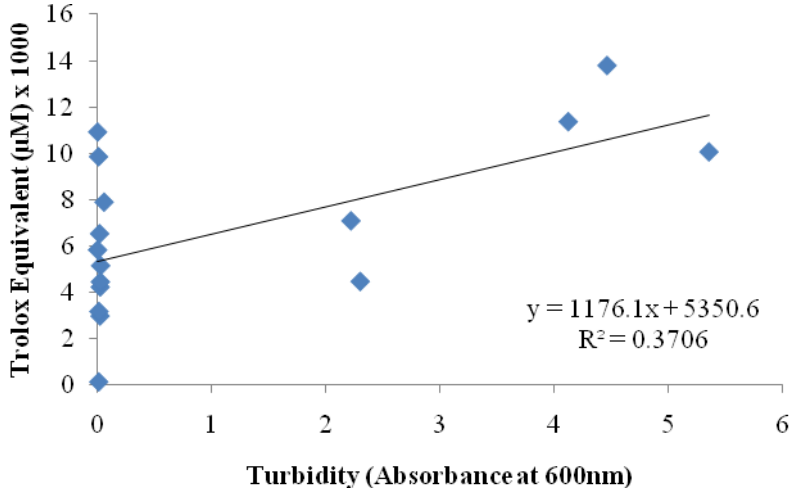

Figure 18. Correlation between the turbidity and the vitamin C corrected antioxidant activity in the commercial apple juices ($p = 0.28$)

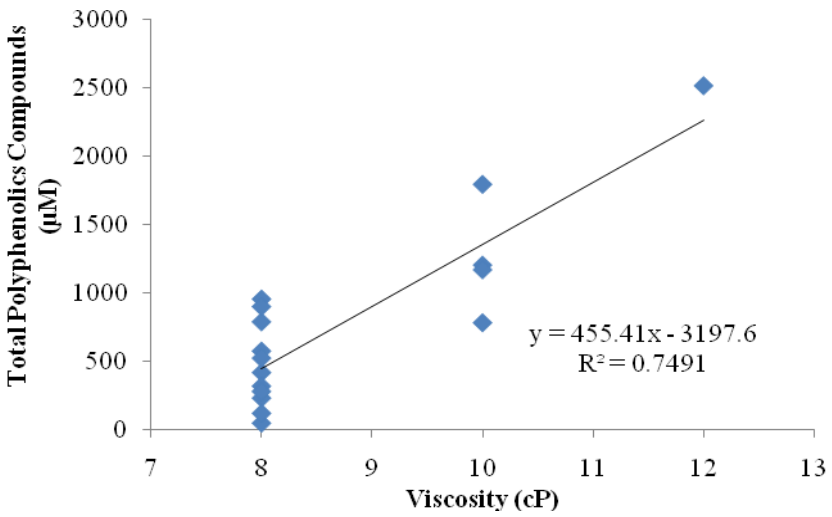

Figure 19. Correlation between the viscosity and the phenolic compounds in the commercial apple juices ($p < 0.001$)

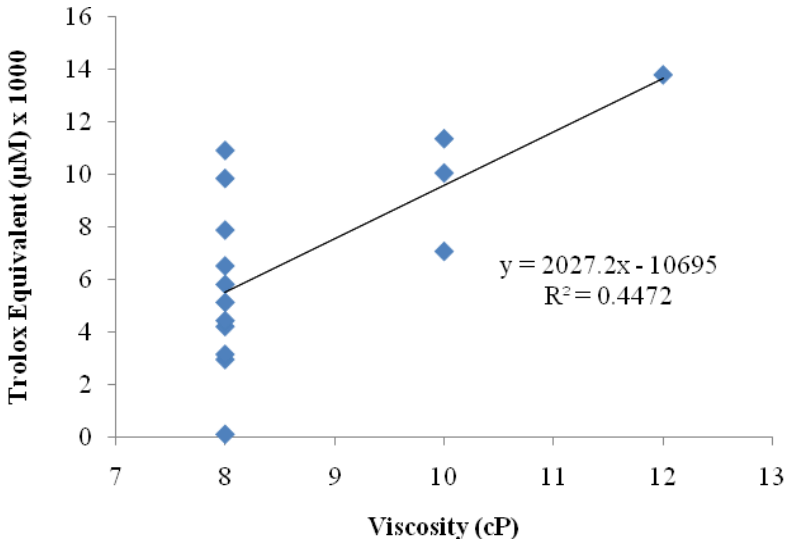

Figure 20. Correlation between the viscosity and the vitamin C corrected antioxidant activity in the commercial apple juices ($p = 0.005$)

Discussion

A range of 17 commercial apple juices (11 clarified juices and 6 cloudy juices) were purchased from the market and analysed for their physical and chemical properties.

The physical properties of the different apple juices were measured. As expected the results showed that cloudy apple juices had a much higher level of turbidity than the clarified juices and were often at least 37 times higher than that of the clarified juice. The levels of TSS were similar in all juice samples (10.9 - 13.4° Brix), regardless of the type of juice, clarified or cloudy. This was due to the a standardisation procedure employed by the juice manufacturers to determine the amount of water to be added to a concentrate based on calculation of the initial ºBrix of the concentrate and the targeted ºBrix in the final product (Ashurst, 2005).

The main aim of this study was to examine the content of phenolic compounds and their contribution to the antioxidant activity in various types of apple juices (cloudy and clarified). The first objective under this aim was to determine the content of phenolic compounds in the commercially available apple juices. Each juice was analysed by HPLC and a comparison made of the total phenolic compounds between the different juices.

Clarified juice phenolic content
The concentrations of total phenolics in clarified juices were variable and ranged from a very low 48 µM in juice C16 to 954 µM in juice C3.

High levels of gallic acid, chlorogenic acid and caffeic acid were measured in the clarified juices. These compounds were present in all 11 clarified juices with juice C4 having the most gallic acid (665 µM) and juice C3 having the highest concentration of chlorogenic acid (461 µM). Caffeic acid was detected in 8 out of the 11 clarified juices but mostly at a lower concentration compared to gallic acid (except juice C9) and chlorogenic acid. The highest concentration of caffeic acid was found in juice C14 (156 µM). However, juices C1, C16 and C17 contained no detectable caffeic acid.

The levels of coumaric acid, rutin, quercetin glucoside and phloridzin were all very low or absent in the clarified juices (C3, C9, C12 and C14).

Cloudy juice phenolic content
The concentrations of total phenolics in cloudy juices were high and ranged from 276 µM (juice C15) to 2510 µM (juice C8). The phenolic compounds were more widely distributed in the cloudy juices, although gallic acid, chlorogenic acid and caffeic acid were still the predominant phenolics in all cloudy juices. In contrast to the clarified juices, coumaric acid, rutin, quercetin glucoside and phloridzin were present in most of the cloudy juices. Of these compounds, only coumaric acid, rutin and phloridzin were absent in one juice for each.

In this study, the total content of phenolic compounds in the cloudy juices was significantly higher compared to the clarified juice ($p = 0.049$) (1,288 versus 468 µM, respectively). This increase in phenolic content appeared to be due to higher concentrations of chlorogenic acid, rutin and quercetin glucoside.

The large variance of the phenolic compound content across the different commercial juice samples may be due to different conditions and methods employed during the processing. According to Van der Sluis *et al.* (2002), the processing steps in the making of apple juice such as filtering and clarification were the major reasons for the

loss of phenolic compounds and different processing methods will result in different concentrations of the compounds in the final product. In order to reconfirm this observation, phenolic compounds in Granny Smith and Royal Gala apples were measured. These cultivars were chosen to mirror the apples used by industry. In this study, the percentage recovery of the phenolic compounds after the juicing process was determined by making cloudy and clarified apple juices from Granny Smith apples and from Royal Gala apples in the laboratory using a domestic juice extractor. The clarification was then achieved by conducting centrifugation followed by vacuum filtration on the cloudy juice. The results showed that Royal Gala apples contained higher total phenolic compounds compared to the Granny Smith apples. However, the recovery of the phenolic compounds and of antioxidant activity was extremely low (<5%) for both cloudy and clarified juices. This has also been observed in other studies which have also shown that the majority of the phenolics present in the apples were not present in the juice (Van der Sluis et al., 2002; Van der Sluis et al., 2004; Bhushan et al., 2008; Oszmianski et al., 2009).

Comparison of imported and local juices

The comparison of the imported juices with local juices is problematic as almost all apple juices in the local market that are made from imported concentrate are clarified apple juices, while the majority of the juices made from Australian apples are cloudy. According to Ashurst (2005), to avoid any impracticality, the majority of the juice manufacturers will market their reconstituted apple juices as clarified apple juice. Therefore, when comparison is made solely based on the source of ingredients regardless of the type of the juice, it is likely that apple juice made from Australian apples could contain higher levels of phenolic compounds because only the Australian juices will be cloudy. Therefore these comparisons indicate that the type of apple juice is likely to be the major factor influencing the total phenolics content in the product.

Antioxidant capacity

Antioxidants are very important compounds as they protect cells from oxidation by being oxidized themselves. The major contributors to the antioxidant activity in apples are vitamin C and the phenolic compounds. In order to calculate the contribution of the phenolic compounds to the total antioxidant activity, the antioxidant activity of vitamin C must be taken into account. However, the correlation between the total phenolic compounds and the vitamin C corrected antioxidant activity was not significant. This suggests that an increase or decrease in total phenolic compounds in apple juice does not necessarily mean a direct increase or decrease in the total antioxidant activity.

However it was found that, on average, the six cloudy apple juices possessed significantly more phenolic antioxidant activity (as measured with the ORAC assay) compared to the eleven clarified apple juices (13,778 versus 5,533 µM TE, respectively). This difference was also observed with the laboratory made clarified apple juices. suggesting that clarification adversely affects the phenolic compound content in apple juice.

Clarification was also shown to decrease the antioxidant activity of the juice, mainly due to the decrease of the phenolic compound concentration. According to the literature, the clarification of apple juice may significantly lower the total phenolic compounds as these compounds are mainly found in the pulp (Van der Sluis *et al*., 2001, Van der Sluis *et al*., 2002; Van der Sluis *et al*., 2004). However, phenolic

compounds with high solubility in water, such as gallic acid and chlorogenic acid, which were consistently observed in all juices analysed in this study (Bhushan *et al*.; 2008 Oszmianski *et al*., 2009).

The establishment of scientific evidence for any nutritional or functional claim
The term "claim", as defined by The Codex Alimentarius in the General Guidelines on Claims (1991) is "*Any representation which states, suggests or implies that a food has particular characteristics relating to its origin, nutritional properties, nature, production, processing, composition, or any other quality*". By the same guidelines, a "nutritional claim" is "*Any representation and any advertising message which states, suggests or implies that a foodstuff has particular nutrition properties*."

Although there is still a debate on whether or not phenolics / antioxidants are considered nutrients, under this definition, scientific evidence for a nutritional claim may be established based on this current study, with the specific condition of the type of apple juice, while ensuring strict quality control of the raw materials, the processing line and the degree of turbidity which indicates the amount of apple pulp in the juice. Based on the results from this study, there was a trend showing that cloudy apple juice contained more phenolic compounds and in turn antioxidant activity compared to clarified apple juices.

However, the functional claim must be supported by scientific evidence that the phenolic compounds may have beneficial functions relating to normal human body physiological conditions, while a health claim must be supported by scientific evidence on how these compounds may aid in improving the human body's physiological functions (The Codex Alimentarius, 2001; FSANZ, 2003). Therefore, unless studies are conducted to address these matters in relation to apple juices, there is no functional nor health claim which could be established based on this current study.

Conclusions

The results indicated that cloudy apple juice was associated with a higher concentration of phenolic compounds and a higher antioxidant activity in the analysed commercial apple juices. Therefore, the clarification process in apple juice production has a significant deleterious effect on the phenolic compound content and the antioxidant activity of the final juice product. However, due to the limited number of samples and geographical sampling area, a larger range of apple juice samples would be required to further validate these results.

Recommendations

Due to the large variation between juices any future studies should increase sampling numbers, so that the accuracy, precision and reproducibility of the data can be improved. Furthermore, expanding the study's coverage by increasing the variety of apple juices from a larger geographical area and the number of apple cultivars would also increase the validity of the study. A further analysis conducted on apple juice concentrate made from Australian apples versus imported apple juice concentrate may also allow for a better understanding of the nature of phenolic compounds in apple juice. Comparisons on Australian apple juices whether cloudy, clear or reconstituted concentrate, with a wider range of juices made from imported juice concentrate would

provide evidence on whether Australian apple juices are superior to imported juice concentrates as a source of phenolic compounds and hence antioxidants. If Australian apple juices did provide possibly greater nutritional value and provide additional health benefits, this would be of great use in the marketing of Australian apple juices.

Publications and presentations from this work:

Title **: Effect of clarification on the polyphenolic compound content and antioxidant activity of commercial apple juices**

International Food Research Journal.
Accepted for publication on 8th March 2012
MANUSCRIPT IFRJ-2011-275

Title : **The Content of Polyphenolic Compounds in Commercial Apple Juice**

44th Annual AIFST Convention, held by The Australian Institute of Food Science and Technology
10 -13 July 2011.
Sydney Convention and Exhibition Centre.

Title : **Effect of clarification on the polyphenolic compound content and antioxidant activity of commercial apple juices**

XVI ISA 2012 Symposium, held by The Australian Atherosclerosis Society
25 -29 March 2012.
Sydney Convention and Exhibition Centre.

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