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

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Abstract: Apples are a rich source of polyphenolic compounds and significantly contribute to the antioxidants in the human diet. However, apples are not just consumed fresh; approximately one third of apples produced are processed into numerous products, apple juice being the most popular. Clarification, one of the processes involved in apple juice production, removes pectin substances and fibres, resulting in clear juice. However, some juices are not clarified and are sold as cloudy apple juice. The aim of this study was to determine the effect of clarification on the polyphenolic compound content and antioxidant activity of commercial apple juices. The aim was accomplished by analysing 6 cloudy and 11 clarified apple juices. High performance liquid chromatography (HPLC) and the Oxygen Radical Absorbance Capacity (ORAC) assay were used to measure the content of polyphenolic compounds and the antioxidant activity, respectively. The results generated from the analyses indicated that the total polyphenolic compounds were 2.8 times higher in the cloudy than in the clarified apple juices ($p = 0.049$). Similarly, the cloudy apple juices possessed significantly more (2.5 times) antioxidant activity ($p = 0.036$) compared to the clarified apple juices. Therefore, it was concluded that the clarification process decreases the polyphenolic compound content and the antioxidant activity of commercial apple juices.

Keywords: Clarification, commercial apple juice, polyphenolic compound, antioxidant activity

Introduction

Fruits and vegetables are the main sources of antioxidants in the human diet (Boyer and Liu, 2004), with apples among the top sources based on the high popularity of the fruit (Vinson *et al*., 2001). Apples belong to the genus Malus and family Rosaceae. They are among the most widely cultivated and consumed fruits, with *Malus domestica* being the most common cultivar (Roupas and Noakes, 2010; Bates *et al*., 2011). Australia is among countries like China, Germany, Italy, Japan, the Netherlands, New Zealand, Poland and the United States who grow apples for both their own markets as well as for export (Ashurst, 2005). Apples are the second most consumed fruit after bananas in the United States (Ki *et al*., 2003) and ranked as the fourth most important fruit worldwide (Roupas and Noakes, 2010). There are many varieties of apples, such as Red Delicious, Royal Gala, Fuji and Granny Smith. Different varieties of apples have different sizes, shapes, sugar content, levels of acidity and firmness (Cabalerro *et al*., 2003). Apples are categorised as climacteric fruit, indicating the occurrence of an upsurge in metabolic rate during the maturation stage after harvest. During the climacteric stage, the starch reservoirs in apples are converted into sugar by starch degrading enzymes (Wills *et al.,* 2007).

Nutritionally, apples contain many important vitamins and minerals, such as vitamin C and potassium. Apples are free from fat, cholesterol and sodium (Cabalerro *et al.*, 2003; Roupas and Noakes, 2010) and contain high amounts of soluble fibre, which is useful in lowering blood cholesterol levels. Furthermore, apples can help to maintain the health of the digestive system because they contain enzymes which assist in the breakdown of foods and insoluble fibre, which is beneficial for the digestive tract (Riboli and Norat, 2003; Femenias, 2005).

The major contributors to the antioxidant activity in apples are vitamin C and the polyphenolic compounds (Boyer and Liu, 2004; Roupas and Noakes, 2010). Vitamin C is an antioxidant which plays a major role in cellular functions by i) acting as a nonspecific electron donor, ii) promoting wound healing through aiding in collagen synthesis and iii)

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being involved in cellular protection during immune function (Wardlaw and Hampl, 2006).

There are six major classes of polyphenolic compounds found in apples: flavonol glycosides (flavonoids, quercetin and quercetin conjugates), catechins and epicatechins, anthocyanins, dihydrochalcones (phlorotin and phlorizin), phenolic acids (gallic acid and chlorogenic acid) and procyanidins (Boyer and Liu, 2004; Van der Sluis *et al.,* 2005). 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 (Escarpa and Gonzalez, 1998; Oszmianski *et al*., 2009). Due to this, it is recommended 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 have also suggested that apple seeds contain high amounts of polyphenolic compounds (Schieber *et al.,* 2003).

Studies reviewed by Boyer and Liu (2004), showed that the polyphenolic compounds have numerous beneficial effects on human health. In fact, Ki *et al*. (2003) demonstrated that these compounds were strongly linked with decreased mortality mainly by reducing the risk of terminal diseases such as cardiovascular diseases and cancers. Furthermore, in vitro studies have shown they can retard the growth of cancer cells (Hertog *et al.,* 1993; Hertog *et al.*, 1994; McCann *et al.*, 2007).

In Australia, although the majority of apples are sold and consumed fresh, approximately 25% - 30% of all apples are processed into juice (ABS, 2000; Hassall & Associates Pty Ltd., 2001). Juice is the most common fruit based product and is defined as the liquid extract from any kind of fruit. In general, there are two types of apple juice, cloudy, which has a hazy appearance, due to the presence of pectin substances and fibres; and fully clarified, which has a brighter colour and a clearer appearance (Potter and Hotchkiss, 1998; Belitz *et al.*, 2004; Ashurst, 2005). Due to its characteristics and consumers' preference, apple juice is often processed into clear (fully clarified) juice. 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 (Fellows, 2000; Vaillant *et al.*, 2001).

Despite being abundant in apples, the polyphenolic compounds are known to be lost when apples are processed into juice. In conventional apple juice, the antioxidant activity decreases by up to 97% in comparison with fresh apples, mainly due to the much lower concentration of polyphenolic compounds (Van der Sluis *et al.*, 2001; Van der Sluis *et al*., 2002).

The polyphenolic compounds, the main contributors to the antioxidant activity in apples, are mostly found in the peel and flesh. In apple juice production, these components are excluded from the final clarified product. Based on this observation, clarification was likely to be an important contributing factor to the losses. Therefore, the aim of this study was to determine the effect of clarification on the polyphenolic compound content and antioxidant activity of commercial apple juices.

Materials and Methods

Materials

Polyphenolic standards, namely gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, quercetin, quercetin glucoside, phloridzin and naringenin, and ascorbic acid were purchased from Sigma Aldrich Laboratory Chemicals (Castle Hill, NSW, Australia). Extraction and mobile phase reagents for HPLC included ortho-phosphoric acid (APS Chemical, Seven Hills, NSW, Australia), tetrahydrofuran, HPLC grade acetonitrile and HPLC grade methanol (Lomb Scientific, Taren Point, NSW, Australia). Liquid nitrogen was purchased from BOC Ltd. (Sydney, NSW, Australia). The reagents for the Oxygen Radical Absorbance Capacity (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-methyl-propanimidamide, dihydrochloride (AAPH) (Wako Pure Chem., VIC, Australia). Deionised water was prepared on the day of use with a Millipore Milli-Q water purification system (Millipore Australia, North Ryde, NSW, Australia). Seventeen commercial apple juices (11 clarified and 6 of cloudy appearance) were purchased from local retailers at locations on the NSW Central Coast and Sydney.

Measurement of the physical properties

Turbidity

The commercial juices were subjected to turbidity measurement using a UV-VIS spectrophotometer CARY 50 BIO (Varian Australia, Oakleigh, VIC, Australia) with absorbance set at 600 nm (Venolia *et al*., 1974; Tochi *et al*., 2009). Each of the three batches of the commercial juices was analysed once and the three values averaged and expressed as mean ± S.D. of the absorbance at 600 nm.

Total soluble solids

The measurement of the total soluble solids in all juices was carried out using a Pocket Refractometer PAL-1 (ATAGO, Tokyo, Japan; supplied by Extech Equipment, Melbourne, VIC, Australia) (Tochi *et al.,* 2009). 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 measurements for the triplicates were averaged to generate a value for each of the three batches. The average of those values was generated for each commercial juice and expressed as mean ± S.E.º Brix.

Measurement of the polyphenolic compounds and vitamin C in apple juices by HPLC

The analysis of the polyphenolic compounds in the apple juices was conducted according to Golding *et al.* (2001) and Hoang *et al.* (2011), with some minor modifications. Briefly, the extraction was performed with a 1:1 dilution of apple juice with HPLC grade methanol containing 0.1 mM of naringenin as internal standard (IS) and filtration was done using a syringe filter (0.45 µm).

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 100 µL sample loop. The polyphenolic compounds were separated using a reverse phase C18 (Prodigy 50-00S3-100A) Phenomenex Column (5 µL, 250 x 4.6 mm) which was protected by an analytical-size guard column (Phenomenex, Pennants Hills, NSW). The level of absorbance was determined at 280 nm (for polyphenolics) and 254 nm (for vitamin C) 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 was used to control the system.

The identification of the components in the sample was done by comparing their retention time and UV spectra with those of 8 apple polyphenolic compound external standards from three major classes (phenolic acids, dihydrichalcones and flavonol glycosides). The selection of the standards was made based on Golding *et al*. (2001). The external/internal standard method was employed to quantify the components. The range of the external standard concentrations for polyphenolic compounds and for vitamin C was from 0.003 to 3 mM and from 0.03 to 32 mM, respectively.

Measurement of antioxidant activity in apple juices

The ORAC assay system and trolox equivalence (TE) calculations

The antioxidant assay was done using the FLUOstar Omega microplate reader (BMG LABTECH, Mount Eliza, VIC) and the method was based on the ORAC method described previously by Cao *et al*. (1993), Ou *et al.* (2001), Prior *et al*. (2003) and Jimenez-Alvarez *et al*. (2008) with some modifications, using 10 nM fluorescein and 240 nM AAPH in 10 mM potassium phosphate buffer, pH 7.4. From a 500 µM trolox stock standard solution the following trolox working standard concentrations: 50 µM, 37.5 µM, 25 µM, 12.5 µM, 6.25 µM and 3.125 µM were prepared, in 10 mM potassium phosphate buffer, pH 7.4. Solutions of 10 µM and 20 µM gallic acid in 10 mM potassium phosphate buffer, pH 7.4 were used as quality controls, and the 10 mM PPB pH 7.4 was used as a blank.

The apple juice samples were diluted 1:250 with 10 mM PPB pH 7.4 to give values within the trolox standard curve.

Only one batch of the three batches of the commercial apple juices was used for antioxidant activity analysis. The antioxidant activity of the samples was calculated and expressed in µM TE based on Jimenez-Alvarez *et al*. (2008).

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; Jimenez-Alvarez *et al.,* 2008) was also done for a series of concentrations of vitamin C as a reference to determine the contribution of vitamin C to the total antioxidant activity of the apple juices. Using the vitamin C concentration measured by HPLC in each apple juice, the contribution of vitamin C to the total antioxidant activity of each juice was determined and expressed in µM TE.

The contribution of the polyphenolic compounds towards the antioxidant activity (µM TE) of the juices was then calculated by subtracting the antioxidant activity 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) 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 Least Significant Difference (LSD) post-hoc test were used.

Results and Discussion

The physical properties of the juices (turbidity and total soluble solids) are listed in Table 1. 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 Absorbance at 600 nm, than the cloudy juices (Table 1). The turbidity of the cloudy juices ranged from 2.22 to 5.35 Absorbance at 600nm; these values were at least 37 times higher than that of the clarified juice with the highest turbidity (C14). In general, the measurement of the total soluble solids (TSS) for the juices generated similar °Brix values (10.9-13.4° Brix), regardless of the type of juice, clarified or cloudy (Table 1).

The peaks indicating the elution time for each of the polyphenolic compounds of interest are presented in Figure 1. Although the peaks were routinely observed at both 280 nm and 254 nm, it was decided that the peaks generated from the detection at 280 nm would be used for the analysis of the apple polyphenolic compounds because they were more prominent, stable and reproducible. However, vitamin C was detected and quantified only at 254 nm (elution time 3.05 min, data not shown). Free quercetin was not found in any of the samples analysed. Therefore, the subsequent results do not report on quercetin.

Table 2 shows the major polyphenolic compounds and the concentration of vitamin C in the 17 commercial apple juices (11 clarified juices and 6 cloudy juices). Of the polyphenolic compounds, gallic acid, chlorogenic acid and caffeic acid were the most prominent in the clarified juices. They were present in all 11 juices with C4 having the most gallic acid (665 µM) and C3 having the highest concentration of chlorogenic acid (461 µM), among the clarified juices. Caffeic acid was present in 8 out of the 11 clarified juices but mostly at a lower concentration compared to gallic acid (except C9) and chlorogenic acid. The highest concentration of caffeic acid was found in C14 (156 µM). However, C1, C16 and C17 contained no detectable caffeic acid.

Coumaric acid, rutin, quercetin glucoside and phloridzin were all very low or absent in the clarified juices (C3, C9, C12 and C14). The total polyphenolic compound concentrations ranged from a very low 48 µM in juice C16 to 954 µM in juice C3.

Table 1. Physical properties of selected commercial apple juices

Sample Type	Sample Number	Turbidity (Abs 600nm)*	Soluble Solids (°Brix)**
Cloudy	C7	4.12±0.02	12.0±0.1
Cloudy	C8	4.46±0.05	11.2±0.0
Cloudy	C10	2.22±0.03	11.9±0.1
Cloudy	C11	2.30±0.07	13.1±0.0
Cloudy	C13	5.35±0.09	11.0±0.1
Cloudy	C15	3.60±0.16	12.2±0.0
Clarified	C1	<0.01	11.1±0.0
Clarified	C2	0.02±0.02	11.1±0.1
Clarified	C3	0.01±0.00	11.2±0.0
Clarified	C4	0.02±0.02	10.9±0.0
Clarified	C5	0.03±0.02	11.1±0.0
Clarified	C6	0.01±0.02	11.0±0.0
Clarified	C9	0.01±0.13	11.1±0.0
Clarified	C12	<0.01	12.1±0.0
Clarified	C14	0.06±0.02	13.4±0.1
Clarified	C16	0.02±0.02	12.4±0.1
Clarified	C17	0.02±0.02	11.7±0.1

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

Table 2. The polyphenolic compound content and polyphenolic antioxidant activity of individual juices

Apple Juice Sample	Constituents (µM)*								Total Polyphenolics (µM)	Polyphenolic** Antioxidant Activity (µM TE)
	Vitamin C	Gallic Acid	Chlorogenic Acid	Caffeic Acid	Coumaric Acid	Rutin	Quercetin Glucoside	Phloridzin		
C7	2533±142	144±21	459±93	55±3	0	26±13	83±8	13±7	780±91	11346
C8	3237±118	934±197	1131±204	124±47	14±14	70±35	183±31	55±28	2510±384	13772
C10	3128±44	704±329	793±89	78±24	14±14	78±8	101±42	23±6	1791±329	7064
C11	829±31	37±29	869±337	82±36	23±16	61±30	103±29	27±13	1202±467	4443
C13	2751±136	160±72	736±45	90±14	2±2	21±21	144±59	15±15	1167±170	10041
C15	1920±99	58±21	165±35	3±2	2±2	0	39±8	0	276±21	36001
C1	2447±116	214±6	16±2.8	0	0	0	0	0	231 ± 10	5807
C2	1378±56	456±61	291±133	40±21	0	0	0	0	787±116	2952
C3	2406±92	370±84	461±132	74±62	1±0.6	1±1	12±10	35±10	954±289	3138
C4	1528±188	665±111	189±65	45±24	0	0	0	0	899±59	6510
C5	2635±91	236±24	151±26	27±14	0	0	0	3±2	417±53	5125
C6	606±38	177±19	68±22	50±50	0	4±6	6±6	13±13	317±76	93
C9	1057±36	40±9	372±59	44±20	32±32	19±10	67±15	1±1	574±111	9935
C12	1252±55	6±4	105±4	67±17	16±8	24±7	45±20	12±10	279±43	10898
C14	1290±81	12±1	231±18	156±46	12±2	27±5	78±6	5±5	522±50	7873
C16	1133±47	33±10	15±2	0	0	0	0	0	48±10	4206
C17	951±43	89±61	22±7	1±1	0	0	1±1	7±3	120±73	4429

*Values are expressed as mean±S.E. for the three batches of each juice
**Polyphenolic antioxidant activity was calculated by subtracting the antioxidant activity of vitamin C from the total antioxidant activity. The values are from one batch of juices only.

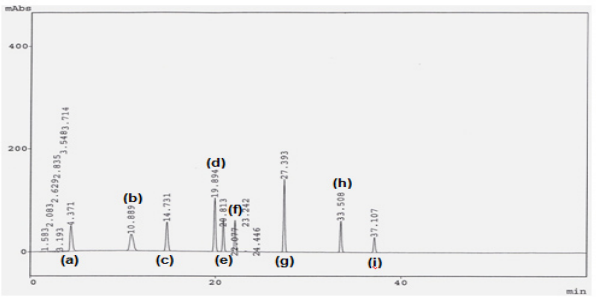

Figure 1. Typical HPLC chromatogram for apple polyphenolic compounds and internal standard (IS). The peaks separated by HPLC and detected at 280nm are in order of elution: (a) gallic acid (b) chlorogenic acid (c) caffeic acid (d) coumaric acid (e) rutin (f) quercetin glucoside (g) phloridzin (h) quercetin (i) naringenin (IS). The concentration of the apple polyphenolic compounds was 0.375mM and the IS was 0.1mM.

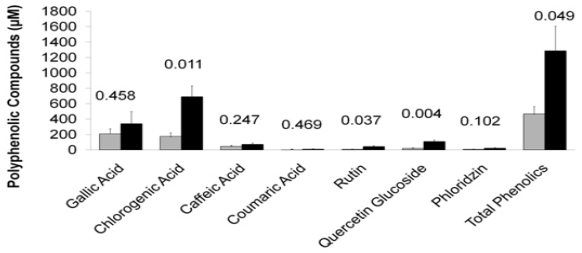

Figure 2. Comparison of polyphenolic compounds in the clarified ( ) vs the cloudy apple juices (). The bar values are means±S.E. and the numbers over the bars indicate the p values for the comparisons between the two types of juices.

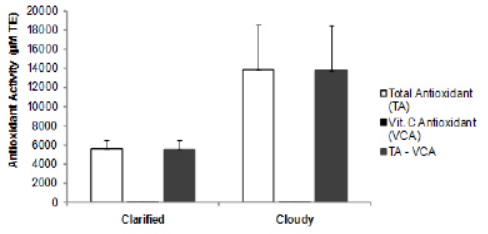

Figure 3. Antioxidant activity of the apple juices. Averages of eleven clarified juices and 6 cloudy juices measured by the ORAC assay. Polyphenolic antioxidant activity was calculated by subtracting the antioxidant activity of vitamin C from the total antioxidant activity.

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

The large variance of the polyphenolic compound content across the commercial juice samples may be due to different conditions and methods employed during the processing. According to Van der Sluis *et al.* (2001) and Van der Sluis *et al.* (2004), processing is the major aspect contributes to the lost of the phenolic compounds in the apple juice and different processing method will result in different concentration of the compounds in the final product.

As seen in Figure 2, the total content of polyphenolic compounds in the cloudy juices was significantly higher in comparison with their clarified counterparts ($p = 0.049$) (1287.7 versus 467.9 µM, respectively) which appeared to be mainly due to a higher concentration of chlorogenic acid ($p = 0.011$) (692.1 versus 174.9 µM, although rutin ($p = 0.037$) and quercetin glucoside ($p = 0.004$) were also higher in the cloudy juices compared to the clarified juices (42.6 and 108.7 µM versus 6.9 and 18.8 µM, respectively).

Of all the juices, C8, C10 and C13 (3237 µM, 3128 µM and 2751 µM, respectively) contained the highest vitamin C content. However, there was no significant difference in the antioxidant activity from vitamin C across all the juices (Figure 3).

Using the µM TE values for the polyphenolic antioxidant activities in Table 2, it was found that the 6 cloudy apple juices (samples 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) (13778 versus 5533 TE, respectively). This comparison is illustrated by the bar graph in Figure 3.

The results generated from the analyses showed that clarification adversely affects the polyphenolic compound content in apple juice. Clarification was also shown to decrease the antioxidant activity of the juice ($p = 0.036$), mainly due to the decrease of the polyphenolic compound concentration ($p = 0.049$). According to the literature, the clarification of apple juice may significantly lower the total polyphenolic 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, polyphenolic 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).

Conclusion

The results indicated that the presence of cloudiness was significantly associated with a higher concentration of polyphenolic 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 polyphenolic 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.

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