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Phenolic composition and antioxidant activity of Chinese red-fleshed apples (*Malus pumila* Niedzwetzkyana (Dieck) and effect of different pasteurization treatments on the cloudy juice

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Abstract

Consumers are interested in the health benefits of food and also attracted by novelty. This study investigated the phenolic composition and antioxidant activity of a wild Chinese red-fleshed apple, *Malus pumila* Niedzwetzkyana (Dieck). The impact of pasteurization on the juice was also assessed. Substantial amounts of total polyphenols were present in the peel (5429.92 mg/kg), flesh (3087.37 mg/kg) and whole apple (3467.47 mg/kg). The whole apple contained high levels of total flavonoids and anthocyanins (1266.86 and 101.73 mg/kg, respectively), with significantly higher amounts in the peel ($p<0.05$). HPLC-DAD analysis showed that the anthocyanin, cyanidin-3-galactoside, was present throughout the apple, including the flesh and cloudy juice. Pasteurization of the cloudy juice by Pulsed electric fields (PEF) at 35 kV/cm for 258 µs combined with heating at 50ºC resulted in significantly lower ($p<0.01$) degradation of total polyphenols (3.74%), flavonoids (10.05%) and anthocyanins (14.75%), in comparison with conventional thermal treatments. A strong ($r>0.85$) and significant ($p<0.01$) correlation was found between the bioactive compounds and antioxidant activity of the apple and juice. Red-fleshed apples represent a substantial source of phenolic compounds, especially anthocyanins, which are not commonly present in the flesh of white-fleshed cultivars. Evidence of the presence of bioactive compounds together with the novelty and attractive red flesh color is a combination that is predicted would be appealing to consumers.

Introduction

Nutrition and medical research is continuously providing evidence of the health enhancing role of food. In contrast with the past, consumers have increased knowledge about the interaction between diet and health, and are more aware of food quality characteristics. Consequently, there is increased demand for high quality foods with improved nutritional, functional and sensory attributes (Fotopoulos and Krystallis, 2003).

Epidemiological studies highlight a high negative correlation between fruit intake and incidence of degenerative diseases (Tiwari *et al*., 2009). The protective effects of diets high in fruits is due to their high contents of phenolic phytochemicals. Phenolic phytochemicals including flavonoids and phenolic acids are secondary metabolites of plant origin. They function to protect plants against biological and environmental stress and therefore are synthesized in response to pathogenic infection, temperature changes, water stress, nutrient deficiency or high energy radiation exposure (Steyn *et al*., 2002). When consumed, phenolic phytochemicals exhibit antioxidant activity in the human body thereby protecting vital biomolecules such as DNA, proteins, carbohydrates and lipids from oxidation caused by free radicals produced under both normal metabolism and conditions of stress (Jaros *et al*., 2009). Research indicates that fruits with high concentrations of phenolics may help to protect against cancer (Faria *et al*., 2010; Stoner *et al*., 2010), diabetes (Wedick *et al*., 2012; Jennings *et al*., 2014), cardiovascular diseases (Mink *et al*., 2007; Jennings *et al*., 2014) and age-related neurodegenerative disorders (Persson *et al*., 2009).

Apples are a rich source of phenolic phytochemicals and as a result, are widely consumed as fresh or juice products (Boyer and Liu, 2004). The distribution of phenolics is different both in the cultivars and apple tissues. A wide variety of white-fleshed apple cultivars are available on the market worldwide. Red-fleshed apples are increasingly becoming a subject of interest due to the presence of high levels of anthocyanins, which are not commonly found in the flesh of commercial cultivars

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Figure 1. Variety of red-fleshed apple studied.

(Khanizadeh *et al*., 2008). Anthocyanins are a group of phenolic compounds primarily responsible for the red, blue or purple pigmentation in plants. There is ongoing research toward the breeding of red-fleshed apples to develop novel commercial cultivars with elevated concentrations of anthocyanins. The breeding programs involve crossing modern white-fleshed commercial varieties with red-fleshed wild apples, some originating from Central Asia (Volz *et al*., 2009; Sekido *et al*., 2010). *Malus pumila* Niedzwetzkyana (Dieck) is a fast growing wild red-fleshed apple variety found in the Tian Shan mountains, Xinjiang Province, northwest China. They are commonly known as 'red meat apples' by the Chinese living in this region. The fruits are small, with an average diameter of 4 cm, and have dark red skin and flesh (Figure 1). The tree has strong tolerance to very low temperatures. Such species that adapt to stress conditions are potentially rich in secondary metabolites and hence possess functional properties of interest (Jacques *et al*., 2009).

Mere identification or development of unique fruits is however insufficient for the fruit industry to cater for consumer demand. There is need to combine this with processing technologies that ensure preservation of the desired quality attributes. Pulsed electric fields (PEF) processing is a non-thermal technology that has drawn the attention of the food industry. The process is based on pulsed electrical currents delivered to a food product placed between a set of electrodes with the intent of inactivating pathogenic and spoilage microorganisms, modifying enzymes or intensifying extraction processes. The electrical currents are responsible for the irreversible electroporation of microbial cell membranes, resulting in increased permeability and electrical conductivity of the cell. This then leads to the dielectric breakdown of the cell membranes (Martin-Belloso and Elez-Martinez, 2005). The mechanism of enzyme inactivation by PEF processing is still unclear. However, some observations have led to the suggestion that conformational changes in the enzyme native structure maybe responsible for loss of activity (Martin-Belloso and Elez-Martinez, 2005). Hence, PEF processing has been suggested for the pasteurization of liquid foods such as juices, milk, yoghurt, soups and liquid eggs. PEF technology has been presented as advantageous in comparison to, for instance heat treatments, because it kills microorganisms whilst better maintaining the original color, flavor, texture and nutritional value of the food. However, during PEF processing, it is vital to balance the electrical energy input to prevent dielectric breakdown of the food. In view of this, recommendations that PEF be applied in combination with moderate temperatures have been made (Martin-Belloso and Sobrino-Lopez, 2011).

There is a dearth of literature on the composition of bioactive components in wild red-fleshed apples. Therefore, the purpose of this study was to analyze the total polyphenol, anthocyanin and flavonoid contents and antioxidant capacity of Chinese red-fleshed apples (*Malus pumila* Niedzwetzkyana (Dieck)). Stability of the bioactive components in the cloudy juice after pasteurization by combined PEF and mild temperature and conventional thermal treatments was also evaluated.

Materials and Methods

Materials

Red-fleshed apples (*Malus pumila* Niedzwetzkyana Dieck) were collected from Xinjiang Province, China during the autumn season. Gallic acid, Folin-Ciocalteu reagent, L-ascorbic acid, D-galacturonic acid, m-hydroxydiphenyl, 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2-hydroxy-3, 5-dinitrobenzoic acid, formic acid, cyanidin-3-galactoside, acetonitrile, HPLC grade methanol and D-glucose were purchased from Sigma-Aldrich (Shanghai, China). All the other chemicals used in this study were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All chemicals and solvents were of analytical grade.

Sample preparation and extraction procedure

The peel, flesh, whole apple and cloudy juice samples were obtained from randomly selected red, ripe apples. Phenolic compounds were extracted according to Maisarah *et al.* (2013). The samples were firstly homogenized separately, kept overnight in a freezer at −80ºC and lyophilized for 3 days. Each powdered sample (5 g) was extracted with 50 mL of 80% methanol acidified with 1% HCl, in an orbital shaker at 200 rpm for 2hr at room temperature. Flasks

covered with aluminium foil were used. The mixture was then centrifuged at 3000 rpm for 15 mins at 4ºC and the supernatant was concentrated under vacuum at 40ºC until about 10% of the initial volume was left. It was then diluted with distilled water to a total volume of 50 mL. For cloudy juice samples, a juice extractor (model AMR508, Zhongshan Kuaite Electrical Appliances Co. Ltd., Guangdong, China) was used for extraction and then filtration with eight layers of cheese cloth to remove coarse particles.

Determination of total polyphenols

Total polyphenolic content was determined by the Folin-Ciocalteu method according to Lachman *et al*. (2006). Diluted samples (1 mL) were mixed with 5 mL of 10% (v/v) Folin-Ciocalteu reagent and then left to stand for 5 mins in the dark. Dilute Na_2CO_3, 7.5% (w/v), was added making the total volume of the mixtures 10 mL. They were then allowed to stand for 1hr in the dark, at room temperature. Absorbance was measured at 765 nm, against a blank prepared by mixing all the reagents except the test sample, using a UV-1100 spectrophotometer (Mapada Instruments Co., Ltd, Shanghai). Gallic acid was used for the standard calibration curve.

Determination of total flavonoids

Total flavonoid content was measured according to Zhishen *et al.* (1999). Diluted samples (1 mL) were added to 4 mL water and immediately 0.3 mL of 5% (w/v) $NaNO_2$ was added and mixed well. After 5 mins, 0.3 mL of 10% (w/v) AlCl3 was added and the mixture was allowed to stand for 6min, followed by the addition of 2 mL of 1M NaOH. The mixture was diluted with 2.4 mL water and absorbance was determined at 510 nm against a blank consisting of all the reagents, except the test sample. Quercetin was used for the standard calibration curve.

Determination of total anthocyanins

The total monomeric anthocyanin content was determined by the pH differential method (AOAC, 2005). Two buffer systems, 0.025 M potassium chloride at pH 1 and 0.4 M sodium acetate at pH 4.5, were used. Samples were diluted, 1:4, with each buffer in a 50 mL volumetric flask. The absorbance of the dilutions at pH 1 and 4.5 were measured against distilled water blanks at 520 and 700 nm, respectively. Absorbance was measured within 20-50 mins of preparation and samples were always kept in the dark. Anthocyanin content, expressed as cyanidin-3-glucoside equivalents, was calculated as follows:

$$TA = \frac{A_0 \times MW \times DF \times 1000}{\varepsilon \times L} \quad (1)$$

where A_0 is $(A_{520nm} - A_{700nm})$ pH 1.0 – $(A_{520nm} - A_{700nm})$ pH 4.5, MW is molecular weight for cyanidin-3-glucoside (449.2 g/mol), DF is dilution factor, L is path length in cm, ε is molar extinction coefficient for cyanidin-3-glucoside (26 900 L/mol.cm) and 1000 is the conversion factor from g to mg.

HPLC-DAD analysis of anthocyanin profiles

The HPLC analyses were performed on a Hitachi series L-2000 HPLC instrument (Hitachi, Japan) equipped with a quaternary solvent delivery system, a diode-array detector, and a column compartment. Samples were separated on an Agilent Zorbax Sb-C18 column (5 μm, 4.6 mm × 250 mm). The mobile phase consisted of acetonitrile (A) and water containing 0.1% (v/v) formic acid (B). A gradient elution program was used as follows: 0−5 mins, 15−20% A; 5−10 mins, 20% A; 10−15 mins, 20−15% A; 15−30 mins, 15−25% A; 30−35 mins, 25−30% A; 35−50 mins, 30−35% A; 50−60 mins, 35−40% A; 60−70 mins, 40−100% A. The mobile phase flow rate was 1.0 mL/min. The column temperature was set at 30°C. Spectral data were recorded using a diode-array detector over the wavelength range 200−600 nm, and the anthocyanin chromatogram was extracted at 520 nm. Cyanidin-3-galactoside was tentatively identified by comparison of the retention time with that of an authentic standard.

Determination of DPPH radical scavenging activity

The scavenging activity of DPPH free radical was performed according to Thaipong *et al.* (2006). A DPPH methanolic solution was freshly prepared by dissolving 0.024 g DPPH and 100 mL methanol. The stock solution was then diluted with methanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm. Diluted samples, 300 μL, were mixed with 5700 μL of the DPPH solution and kept in the dark at room temperature for 3hr. Absorbance was measured at 515 nm against a blank of absolute methanol, without DPPH. Results were expressed as vitamin C equivalents.

Determination of ABTS radical scavenging activity

ABTS radical scavenging measurements were performed following the procedure by Re et al. (1999), with some modifications. ABTS radical cation was produced by reacting 8mM ABTS and 3mM K2S2O8 in equal volumes, for 16hr in the dark at room temperature. A working solution was then prepared by mixing $ABTS^+$ and phosphate saline buffer at pH 7.4, to an absorbance of 0.7 ± 0.01 at 734 nm. Dilute sample, 100 μL, was mixed with 2900 μL $ABTS^+$/buffer solution and kept for 6 mins

in the dark. Absorbance was taken at 734 nm against a buffer blank. Results were expressed as vitamin C equivalents.

Pasteurization by combined PEF and mild temperature

A bench scale continuous PEF system (OSU-4L, The Ohio State University, Columbus, Ohio, USA), as described and illustrated by Zhao *et al.* (2008), was used to pasteurize cloudy red apple juice. A trigger generator (model 9310, Quantum Composer Inc., Bozeman, MT, USA) was used to control the square wave, bipolar pulse width, pulse delay time and frequency, which were kept at 2 µs, 20 µs and 200 Hz, respectively. Signals of voltage, current, frequency and waveform were monitored by a digital oscilloscope (1GS/s, 60MHz bandwidth, model TDS 210, Tektronix Inc., Wilsonville, OR, USA). A pump (model 020-000-010, Micropump, Inc., Vancouver, WA) kept a steady flow of red apple juice at a rate of 20 mL/min. The juice was treated in six co-field flow tubular chambers, grouped in three pairs, each with an inner diameter and electrode gap distance of 0.23 cm and 0.292 cm, respectively. The chambers were connected by stainless steel tubing with the same inner diameter, inserted in an iced water bath to cool the treated sample.

Cloudy red apple juice, at 20ºC, was pasteurized at an electric field of 35kV/cm for 258µs. The inlet and outlet temperature of each pair of chambers was monitored by thermocouples (Fisher Scientific, Pittsburgh, PA). Temperature increase for all treatments did not exceed 5ºC. Immediately after treatment, the juice was heated in a water bath at 50ºC for 10 s and then cooled to room temperature. It was stored at 4ºC until analysis of total polyphenols, flavonoids, anthocyanins and antioxidant activity. In a previous study, we showed that the selected conditions for combined PEF and mild temperature result in 5.21, 6.02 and 5.49 log cycles reduction in *Escherichia coli* (ATCC 8739), *Salmonella enteritidis* (ATCC 6538) and *Saccharomyces cerevisiae* BY4742, respectively (Katiyo *et al.,* 2017). These treatment conditions meet the U. S. Food and Drug Administration (FDA) regulation for fruit juice pasteurization (HHS and FDA, 2001).

Conventional thermal pasteurization

Thermal pasteurization of cloudy red apple juice was carried out using a mineral oil bath, in a laboratory setup. Temperatures were monitored by K type thermocouples. Samples were heated at 80 and 90ºC for 10 and 5 mins, respectively, and then cooled immediately to room temperature before storage at 4ºC until analysis of total polyphenols, flavonoids, anthocyanins and antioxidant activity. The selected temperature-time combinations are commonly applied to ensure inactivation of enzymes, such as polyphenol oxidase (PPO), peroxidase (POD) and pectin methylesterase (PME), which cause browning and undesirable rheological effects during cloudy juice storage. In a previous study, we showed that pasteurization of cloudy red apple juice at 80ºC for 10 mins results in PPO and POD residual activity of 0.02 and 0.12, respectively. Heating the juice at 90ºC for 5 mins results in complete inactivation of PPO and POD residual activity of 0.10 (Katiyo *et al*., 2014).

Statistical analysis

All experiments were performed in triplicate and results were expressed as mean ± standard deviation. Data were analyzed using one-way analysis of variance (ANOVA) combined with Tukey's post hoc test at 95% confidence level, to differentiate sample means. A Pearson product-moment correlation was also run at 99% confidence level, to determine the relationship between antioxidant activity and bioactive components. Statistical software, Version 19.0 IBM SPSS Statistics Inc., New York, USA, was used for all analyses.

Results and discussion

Total polyphenol, anthocyanin and flavonoid content of red-fleshed apples

Diets high in antioxidants are encouraged as being beneficial to long-term health by minimizing oxidative stress within the body. As a result, interest in analyzing bioactive components in fruits has increased (Wargovich *et al.*, 2012). The total phenolic content of *Malus pumila* Niedzwetzkyana (Dieck) peel, flesh and whole apples was determined. The total polyphenol content ranged from 3087.37 ± 114.67 to 5429.92 ± 293.05 mg GAE/kg (Figure 2a). The apple peel had the highest total phenolic content (5429.92 ± 293.05 mg GAE/kg), followed by whole apples (3467.47 ± 63.72 mg GAE/kg) and flesh (3087.37 ± 114.67 mg GAE/kg). Wang *et al.* (2015) reported that red-fleshed apple 'Roberts Crab' contained 4727.8 mg GAE/kg total phenolic content in the peel, a value comparable to the observation in this study. However, they observed lower total phenolic content for other three varieties under study namely 'Xiahongrou' (2062.3 mg GAE/kg), 'No.1 Hongxun' (2476.2 mg GAE/kg) and 'Hongrouguo' (2814.5 mg GAE/kg). They measured lower values for the total phenolic content in the flesh of the four

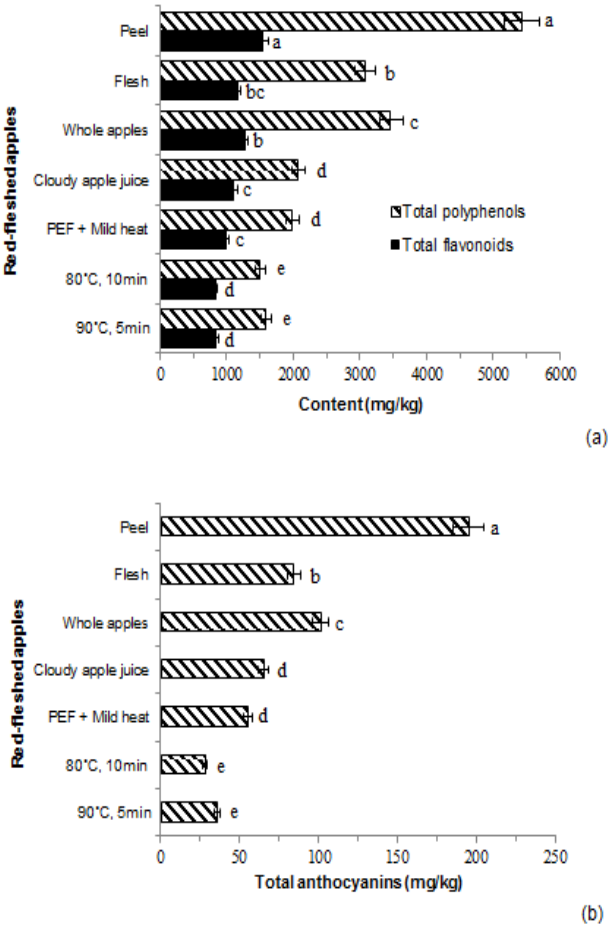

Figure 2. Total polyphenol (gallic acid equivalents) and flavonoid (quercetin equivalents) content (a), and anthocyanin content (cyd-3-glu equivalents) (b) of edible fractions and pasteurized cloudy juice of red-fleshed apples. Means with different letters were significantly different ($p<0.05$).

apple varieties. Interestingly, the four varieties are also within *Malus* Niedzwetzkyana. However, the researchers picked their samples from plants that were grafted and cultured in an orchard in Yangling, Shaanxi province. The samples in this study were obtained directly from Tian Shan mountain range, Xinjiang province. This could explain the differences in the total phenolic contents. Sadilova *et al*. (2006) also observed lower total phenolics in the peel, flesh and whole of 'Weirouge' red-fleshed apples, a German cultivar. On the other hand, Balazs *et al*. (2012) reported higher total polyphenol content in three Hungarian red-fleshed apple breeds ('GFV-3', 'GFV-4' and 'GFV-5'). It is generally accepted that in fruits, the range and abundance of phenolic compounds can vary according to the genetic variation, geographic location, growth period, year of harvest and storage conditions (Kevers *et al*., 2011).

The total phenolic content in the peel was significantly higher ($p<0.05$) than that in the flesh and whole apples (Figure 2a). This observation is in agreement with studies by Wolfe *et al.* (2003), Vieira *et al.* (2009) and Kevers *et al.* (2011). This was attributed to the color pigment distribution in the red-fleshed apples, with the skin being intensely red than the rest of the fruit (Figure 1). In addition, the flesh and whole apples also contained significantly different ($p<0.05$) amounts of total polyphenols. According to Chaovanalikit and Wrolstad (2004), polyphenols are generally not uniformly distributed in fruit tissue, with high concentrations present in the skin. The skin's function as a protective agent to environmental stress and attractant for seed dispersal initiates generation of these phenolic phytochemicals. Unfortunately, during apple juice production, most of the polyphenols in the skin are retained in the apple pomace. Research has demonstrated that polyphenols from apple pomace could be extracted and utilized to enrich other food products thereby significantly increasing the antioxidant activity (Savatovic *et al.*, 2009).

Total flavonoids were measured as quercetin equivalents (Figure 2a). Flavonoid content ranged from 1156.62 ± 5.12 to 1544.50 ± 45.44 mg/kg. The highest flavonoid content was measured in the peels (1544.40 ± 45.45 mg/kg), followed by whole apples (1266.86 ± 45.44 mg/kg) and flesh (1156.62 ± 5.12 mg/kg). In comparison with the study by Balazs *et al.* (2012), the apple peel had double the total flavonoid content than measured in the present study. The findings on apple peel were consistent with those reported for 'Xiahongrou', 'No.1 Hongxun' and 'Hongouguo' but lower than those for 'Roberts Crab' (Wang *et al*., 2015). However, the apple flesh in both previous studies contained lower total amounts of flavonoids. This could be due to the lower anthocyanin content in the flesh of these varieties. As expected, there was a significant difference ($p<0.05$) between the peel and the other apple fractions. No significant difference ($p>0.05$) was found between the flesh and whole apples. This was also observed by Wolfe *et al.* (2003). In their study, only one apple variety (Rome Beauty) showed significant difference between the flesh and whole apples. According to our knowledge, there is no report demonstrating if this trend is according to cultivar or environmental differences. In addition, the flavonoid content in the flesh was also similar ($p>0.05$) to that in the cloudy juice. This presents the possibility of relatively high total flavonoid content in juices from red-fleshed apples.

The total anthocyanin content of red apple fractions was also measured. The pigments were analyzed as monomeric, by the pH differential method. This method is often preferred because it

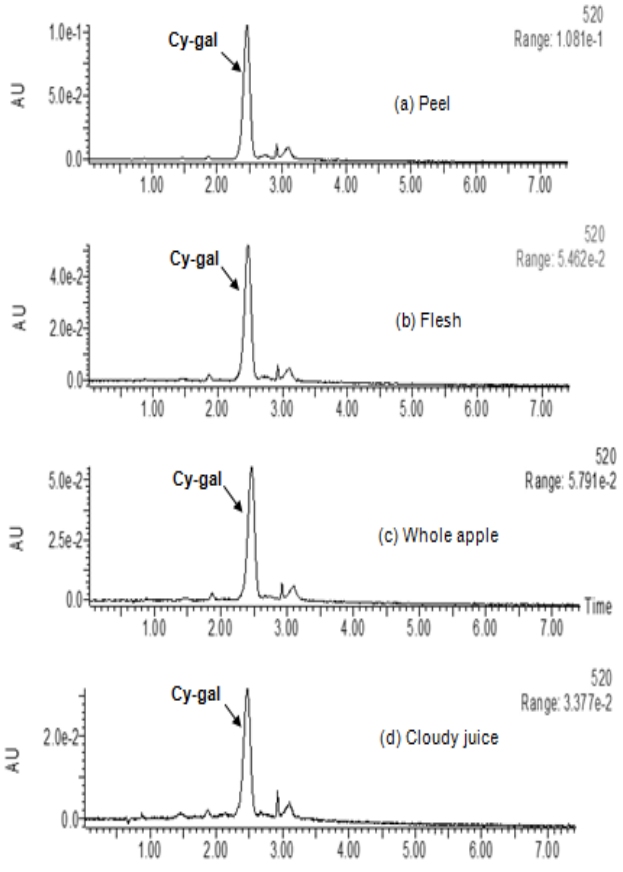

Figure 3. HPLC chromatograms at 520nm of anthocyanins from (a) peel (b) flesh (c) whole apple and (d) cloudy juice of *Malus pumila* Niedzwetzkyana (Dieck) red-fleshed apples.

ensures the exclusion of degraded anthocyanins during measurement (AOAC, 2005). Figure 2b shows the total anthocyanin content. Anthocyanin distribution in red-fleshed apples was similar to that of total polyphenols. The peel had the highest content (195.45 ±12.36 mg/kg), followed by whole apples (101.73 ± 7.09 mg/kg) and flesh (84.28 ± 4.57 mg/kg). The apple fractions had significantly different ($p<0.05$) anthocyanin content. The total anthocyanin yield of the apple fractions was in the same range (80.1 to 200.6 mg/kg) as reported previously by Mazza and Velioglu (1992) and Sadilova *et al.* (2006) for red-fleshed 'Scugog' and 'Weirouge' apples, respectively. Traditionally, colored fruits and vegetables have been recognized as having notable health benefits to the human body and there has been growing interest in their pigment components. The utilization of particular colored fruits such as berries in the food industry (e.g. in yoghurts, dairy juice blends and in bakery) has therefore increased considerably.

The chromatographic anthocyanin profiles of the apple fractions are shown in Figure 3. These results indicate cyanidin-3-galactoside as the only predominant anthocyanin present in *Malus pumila* Niedzwetzkyana (Dieck) apple peel, flesh, whole and cloudy juice. As expected, this anthocyanin was more concentrated in the peel followed by flesh, whole and juice of the apple. Mazza and Velioglu (1992), Sadilova *et al.* (2006) and Wang *et al.* (2015) also reported that quantitatively, cyanidin-3-galactoside was the most abundant anthocyanin present in red-fleshed cultivars and qualitatively, it was uniformly distributed throughout the apples. Studies on white-fleshed commercial cultivars, such as Jonagold, Red delicious and Elstar, with red or partially red skin have shown that cyanidin-3-galactoside is unique to and only found in the peels (Tsao *et al*., 2003; Khanizadeh *et al.*, 2008). Red apple skin color has been attributed to the expression of MYB1 and MYBA genes, whilst red flesh color is regulated by MYB10. The difference in the levels of expression of these genes correlates with anthocyanin biosynthesis and accumulation in red and white-fleshed apples (Espley *et al.,* 2007). Cyanidin-3-galactoside, in combination with arcabose, is an alpha-glucosidase inhibitor and hence could be used for the treatment of diabetes (Adisakwattana *et al.*, 2009; Akkarachiyasit *et al*., 2010). The present findings suggest and further substantiate the proposal that red-fleshed apples could be superior to white-fleshed ones. The development and utilization of red-fleshed apples could result in products with enhanced anthocyanin levels, particularly cyanidin-3-galactoside.

Effect of pasteurization on cloudy red-fleshed apple juice phenolic composition

Savatovic *et al.* (2009) noted that clarification of apple juice results in a product poor in polyphenols, with only 3-10% of the antioxidant activity of fresh apples. Health benefits are mainly expected from the consumption of cloudy apple juice. Hence, in this study, the red-fleshed apples were processed into cloudy juice and the impact of pasteurization on phenolic components was measured. According to our knowledge, there is no data available on the pasteurization of cloudy juice from red-fleshed apples. Preservation of the functional qualities of red-fleshed apples is possible through application of novel technologies for processing. Figure 2 (a and b) presents the total polyphenol, flavonoid and anthocyanin contents of cloudy red apple juice pasteurized by a hurdle technique (combined PEF and mild heat) and conventional thermal treatments (80ºC for 10 mins and 90ºC for 5 mins). Total polyphenol content ranged from 1510.68 ± 40.00 to 2076.00 ± 120.00mg/L. Pasteurization of fresh

apple juice resulted in a decrease in total phenolic content. Juice samples treated by combined PEF and mild heat were not significantly affected ($p>0.05$), in comparison with heat treated juices ($p<0.05$). There was a loss of 3.74, 27.23 and 23.38% in total polyphenols after treatment by PEF, 80ºC for 10 mins and 90ºC for 5 mins, respectively. Aguilar-Rosas *et al.* (2007) reported that heat treatment performed on juices from white-fleshed apples at 90°C for 30s caused 32.20% loss of phenols, while PEF treatment resulted in 14.49% reduction. When yeasts, molds and mesophilic bacteria were completely inactivated by PEF conditions of 40 kV/cm for 100 pulses, the loss of total polyphenol content in juice from white-fleshed apples was 34% (Ertugay *et al*., 2013). Spanos and Wrolstad (1992) reported that total phenol concentration is reduced up to 50% in juice from white-fleshed apples pasteurized at 80ºC for 15 mins. These reports agree with the results obtained in this study that combined PEF and mild temperature treatment better maintains total polyphenol content of apple juice than heat. With PEF processing, the diversity of experimental conditions (electrode gap distance, pulse width, pulse delay time, frequency, electric field strength) makes it difficult to compare research findings though.

The total anthocyanin content ranged from 65.29 ± 3.71 to 27.84 ± 2.70mg/L, with the lowest amounts present in thermally treated juices ($p<0.05$) (Figure 2b). Combined PEF and mild heat treated juice lost 14.75% of the anthocyanins whilst heat treatments resulted in 57.36% (80ºC, 10 mins) and 45.26% (90ºC, 5 mins) degradation. Degradation of anthocyanins was comparatively higher in all the samples. However, there were significantly more anthocyanins ($p<0.05$) in PEF juice than in thermally treated juice ($p>0.05$). Treatment of strawberry juice at an electric field strength of 20kV/cm for 2000µs resulted in degradation of anthocyanins by 4% (Odriozola-Serrano *et al.*, 2008). At 35kV/cm for 1000µs, the strawberry juice lost 14.3% of the total anthocyanins, similar to what was obtained in this study (Odriozola-Serrano *et al*., 2009). Shaheer *et al.* (2014) reported that pasteurization at 80ºC for 10 mins and 90ºC for 5 mins led to 36.79 and 42.12% decrease in jamun fruit juice *(Eugenia jambolana)* anthocyanins, respectively. Little is known about the degradation mechanisms of anthocyanins. Their chemical structure has however been reported to have strong influence. Anthocyanins are glycosylated anthocyanidins, that is, sugars are attached to the hydroxyl of the anthocyanidin. Degradation is primarily caused by oxidation, cleavage of covalent bonds or enhanced oxidation reactions due to thermal processing, producing colorless products (Patras *et al.*, 2010). It was observed that at 100ºC for pH range 2.0 to 4.0, cyanidin glycosides undergo glycosidic hydrolysis producing a colorless chalcone or α-diketone (Adams,1973).

A similar trend was observed for total flavonoids. Total flavonoid content ranged from 840.57 ± 42.03 to 1100.65 ± 91.26mg/L, with the lowest amounts present in thermally treated juices ($p<0.05$). For all the measured bioactive components, conventional thermally treated juices differed significantly ($p<0.05$) from PEF juice, indicating that this hurdle technique could better maintain the bioactive status of fresh juice. Thermal processing has been the most popular method for preservation of food. During thermal pasteurization, there is the inactivation of microorganisms and enzymes in food products. Unfortunately, this process reduces the nutritional quality of food (Lasekan *et al*., 2017). Non-thermal technologies are designed to eliminate the use of high temperatures during processing. During PEF pasteurization, high voltage electrical pulses are applied to food for a very short time (µs). The food product then experiences an electric field, which is responsible for the inactivation of microorganisms and enzymes. During treatment, the increase in product temperature is minimal and hence the fresh-like characteristics of the food are maintained, including nutritional and sensory quality (Martin-Belloso and Elez-Martinez, 2005). In a previous study, we demonstrated the effectiveness of PEF pasteurization against various pathogenic microorganisms and enzymes without appreciable changes in color, particle size, pH, acidity and soluble solids in cloudy juice from red-fleshed apples (Katiyo *et al*., 2014; Katiyo *et al*., 2017). The present finding gives an insight into the stability of red-fleshed apple polyphenols after cloudy juice pasteurization, and further substantiates the potential of novel technologies in the fruit industry.

Antioxidant activity of red-fleshed apples and pasteurized cloudy juice

Two standard antioxidant activity assays were performed. In the present study, the DPPH method was more consistent for repeated measurements, with the highest error being 10.11% and 14.74% for ABTS. Table 1 presents the antioxidant activity of *Malus pumila* Niedzwetzkyana (Dieck) apple fractions and pasteurized cloudy juice. For the DPPH assay, the highest activity was observed in the peel (2966.28 ± 62.29 mg/kg), followed by whole apples (1098.62 ± 111.14 mg/kg), flesh (883.60 ± 55.34 mg/kg and juices (less than 850mg/L). This observation is

Table 1. Antioxidant capacities of edible fractions and pasteurized cloudy juice from *Malus pumila* Niedzwetzkyana (Dieck) red-fleshed apples

Sample	mg/kg	
	DPPH	ABTS
Peel	2966.28 ± 62.29^a	5461.80 ± 461.90^a
Flesh	883.60 ± 55.34^b	1892.36 ± 91.39^b
Whole apples	1098.62 ± 111.14^c	2122.89 ± 122.11^b
Unpasteurized juice	813.94 ± 26.17^b	1365.98 ± 67.63^c
PEF + Mild heat	747.81 ± 22.59^b	1298.21 ± 38.39^c
80°C, 10min	547.23 ± 13.73^d	991.63 ± 146.21^d
90°C, 5min	503.76 ± 49.47^d	944.01 ± 54.07^d

Values are expressed as mean ± standard deviation (n=3). Means with different letters within a column were significantly different ($p<0.05$). Vitamin C was used as reference.

consistent with the total polyphenol, anthocyanin and flavonoid contents of the samples. However, analysis of variance showed that there was no significant difference ($p>0.05$) between the antioxidant activity of the flesh, unpasteurized juice and juice treated by combined PEF and mild heat. The antioxidant activity of the two conventional thermally treated juices was lower ($p<0.05$). Similar results were observed with the ABTS radical assay, except that there was no significant difference ($p>0.05$) between the activity of whole apples and the flesh. The variation in results could be due to the different reaction mechanisms of the DPPH and ABTS assays. It is widely accepted that various methods used to measure antioxidant activity of food can give varying results depending on the specific free radical being used as a reactant. The descending order of activity was peel > whole apples > flesh > unpasteurized cloudy juice > PEF + mild heat > 80ºC, 10 mins > 90ºC, 5 mins. Overall, for peel, flesh and whole apples, the order of activity exhibited is in agreement with studies on white-fleshed apples described by Wolfe *et al*. (2003), Vieira *et al.* (2009) and Kevers *et al.* (2011). Comparison of juices with whole apples indicates significant processing losses of antioxidant activity. Consumption of the fresh fruit, including the skin, would therefore be more beneficial.

Correlation analyses

Linear correlation analysis was run to determine the relationship between antioxidant components and antioxidant activity (Table 2). A strong ($r>0.85$) and significant ($p<0.01$), positive correlation exists between all the analyzed components and antioxidant assays. Total anthocyanin content was shown to provide the highest correlation with both assays, followed by polyphenols and flavonoids. This could suggest that anthocyanin compounds in red-fleshed apples contribute significantly to their antioxidant activity and that of the cloudy juice. Red-fleshed apples could therefore have a great health benefit. Several studies on white-fleshed apples found that the highest correlation existed between total polyphenols and antioxidant activity (D'Abrosca *et al.*, 2007; Matthes and Schmitz-Eiberger, 2008; Wojdylo *et al*., 2008).

Table 2. Correlation analysis between antioxidant components and antioxidant capacity of red-fleshed apple fractions and pasteurized cloudy juice

Antioxidant components	DPPH		ABTS	
	r	*P*	*r*	*P*
Total polyphenols	0.943	0.0001	0.964	0.0001
Total flavonoids	0.883	0.004	0.901	0.002
Total anthocyanins	0.968	0.0001	0.979	0.0001

$P<0.01$ indicates that correlation was statistically significant.

Conclusion

This study revealed that *Malus pumila* Niedzwetzkyana (Dieck) red-fleshed apples are rich in total polyphenols, anthocyanins and flavonoids, and have strong antioxidant activity. Phenolic content and antioxidant activity differs in the peel, flesh and whole apple, with significantly higher values in the peel. The apples are also a significant source of the anthocyanin, cyanidin-3-galactoside which is present in the peel, flesh, whole apple and cloudy juice. Pasteurization of the cloudy juice by combined PEF and mild heat does not significantly affect phenolic content and antioxidant activity, in comparison with convectional thermal treatments. A strong correlation exists between total polyphenols, anthocyanins, flavonoids and antioxidant activity indicating that these compounds are mainly responsible for the antioxidant capacity of the red-fleshed apples and juice. Wild red-fleshed apples have the potential for development into novel functional fruits with commercial success. The application of novel technologies during processing, such as PEF, is recommended for the preservation of health enhancing properties.

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