DEVELOPMENT OF AUTHENTICITY METHODS FOR APPLE AND PEAR JUICES

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ABSTRACT

Due to its widespread global consumption and economic value, fruit juice is a common target for adulteration through debasing by unscrupulous producers. Two common methods of fruit juice adulteration are debasing with commercial sweeteners and juice-to-juice adulteration. The overarching goal of this research was to develop methods to detect the undeclared addition of less expensive commercial sweeteners to pear juice and the undeclared addition of apple to pear and pear to apple juice.

Methods to detect the undeclared addition of high fructose corn syrup (HFCS), hydrolyzed inulin syrup (HIS) and total invert sugar (TIS) to commercial pear juice were developed through oligosaccharide profiling employing high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) and capillary gas chromatography with flame ionization detection (CGC-FID). Based on the application of these methods to intentional pear juice debasing, these three commercial sweeteners could be detected at levels of 0.5-3.0% (v/v).

Coupled with the developed authenticity analysis for pear juice, the developed profiling methods were used to examine the carbohydrate/oligosaccharide profile of pear juice as a function of commercial processing. Chromatographic results showed that the majority of carbohydrate/oligosaccharide formation occurred during the mashing stage of juice production where enzymes (i.e. pectinases, hemicellulases and amylases) are employed. The remaining processing stages were found to have a minimal impact on the carbohydrate/oligosaccharide profile of commercial pear juice.

Methods for the detection of juice-to-juice debasing between apple and pear juices were developed using phenolic profiling. High performance liquid chromatography with photodiode array detection (HPLC-PDA) was used to determine the phenolic profiles of commercial apple and pear juice concentrates from major world production regions. The phenolic profiles were used to identify fingerprint compounds for use in juice-to-juice adulteration detection. One phenolic marker was identified in apple juice and two in pear juice (excluding arbutin). These compounds were analyzed by UV-vis and NMR spectroscopic methods, and high resolution MS and LC-MS/MS spectrometry. Results from these analyses identified the fingerprint compounds as 4-*O*-p-coumarylquinic acid in commercial apple juice, and isorhamnetin-3-*O*-rutinoside and abscisic acid in commercial pear juice.

The total phenolic content and antioxidant activities of the 27 apple and 32 pear juices used throughout this research were determined by the Folin-Ciocalteu (total phenolic content), HPLC-PDA (total phenolic chromatographic index), Trolox equivalent antioxidant activity (TEAC) and DPPH methods. The total phenolic content of apple and pear juices were found to be 294.7 ± 128.2 and 246.4 ± 45.1 ppm GAE and the total phenolic chromatographic indices were 128.8 ± 44.9 and 211.7 ± 57.2 ppm, respectively. The TEAC of apple and pear juices were found to be 130.8 ± 60.8 and 150.8 ± 63.9 mM Trolox/100 mL, while the DPPH radical scavenging abilities were 21.5 ± 12.1 and 13.6 ± 5.5 mL of DPPH/mL of juice, respectively.

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LIST OF SYMBOLS AND ABBREVIATIONS

ABA	Abscisic acid
ABTS	2,2′-azinobis-3-ethylbenzthiazoline-sulfonic acid
AJ	Apple juice
ANOVA	Analysis of variance
BMIS	Beet medium invert sugar
C_3	Calvin cycle photosynthetic pathway
C_4	Hatch-Slack photosynthetic pathway
CAM	Crassulacean acid metabolism photosynthetic pathway
CE	Collision energy
CGC	Capillary gas chromatography
CGC-FID	Capillary gas chromatography with flame ionization detection
CID	Collision induced dissociation
COI	Cytochrome oxidase I
CQA	Caffeoylquinic acid
CSIRA	Carbon stable isotope ratio analysis
DE	Dextrose equivalent
DNA	Deoxyribonucleic acid
DP	Degree of polymerization or declusturing potential
DPPH	2,2-diphenyl-1-picrylhydrazyl
DV	Dispersion voltage
EC	Enzyme Commission
EI	Electron impact
ESI	Electrospray ionization
FAIMS	High-field asymmetric waveform ion mobility spectrometry
F-C	Folin-Ciocalteu
F/G	Fructose/glucose ratio
FID	Flame ionization detection
FIR	Far-infrared
FOS	Fructooligosaccharide

FT-NIR	Fourier transformed near-infrared
GAE	Gallic acid equivalents
GC-MS	Gas chromatography-mass spectrometry
GRAS	Generally recognized as safe
HFCS	High fructose corn syrup
HG	Homogalacturonan
HIS	Hydrolyzed inulin syrup
HMF	Hydroxymethylfurfural
HPAE	High performance anion exchange chromatography
HPAE-PAD	High performance anion exchange chromatography with pulsed amperometric detection
HPLC	High performance liquid chromatography
HPLC-PDA	High performance liquid chromatography with photodiode array detection
HPLC-RI	High sperformance liquid chromatography with refractive index detection
HSD	Honest significant differences
IR	Infrared
IRMS	Isotope ratio mass spectrometry
IS2	O-β-D-fructofuranosyl-(2→6)-D-glucose
LC	Liquid chromatography
LIT	Linear ion trap
MIR	Mid-infrared
MIS	Medium invert sugar
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
ND	Not detected
NIR	Near-infrared
NIST	National Institute of Standards and Technology

NSERC	Natural Sciences and Engineering Research Council of Canada
PAD	Pulsed amperometric detection
PCR	Polymerase chain reaction
PDA	Photodiode array
PE	Pectinesterase
PG	Polygalacturonase
PGL	Polygalacturonate lyases
PJ	Pear juice
PMG	Polymethylgalacturonase
PMGL	Polymethylgalacturonate lyases
qPCR	Quantitative polymerase chain reaction
QqQ	Triple quadrupole
R^2	Coefficient of determination
RAPD	Random amplified polymorphic DNA
RFID	Radio frequency identification
RFLP	Restriction fragment length polymorphism
RGI	Rhamnogalacturonan I
RGII	Rhamnogalacturonan II
RI	Refractive index
RT	Retention time
SIRA	Stable isotope ratio analysis
s/n	Signal-to-noise ratio
SNIF-NMR	Site-specific natural isotope fractionation nuclear magnetic resonance
SPSS	Statistical Package for the Social Sciences
TEAC	Trolox equivalence antioxidant capacity
TIS	Total invert sugar
TMS	$-Si(CH_3)_3$
TMSI	1-(trimethylsilyl)imidazole
ToF	Time of flight
TPC	Total phenolic content
TPCI	Total phenolic chromatographic index

UHFS	Ultra-high fructose syrup
UV	Ultraviolet
UV-vis	Ultraviolet-visible

1. INTRODUCTION

1.1 Summary

Food adulteration is a serious worldwide issue that can have significant negative economic and health effects that impact both consumers and producers. Currently, food fraud takes on two main forms. The first being adulteration, such as the undeclared addition of a lesser value ingredient to a product (debasing), and the second being intentional mislabeling, which includes the use of misleading names, claims and descriptions, including concealment (Roberts, 1994).

Due to its extensive consumption, economic value and high carbohydrate content, fruit juice is a common target for adulteration through debasing by unscrupulous producers. One method of fruit juice adulteration is by simple dilution with water. This method of adulteration is relatively easy to detect (i.e. depending on the dilution level) by simple measurements of the total carbohydrate content (i.e., °Brix) coupled with industry/government standards for juice contents (i.e. 11.5 and 12.0 °Brix for single strength apple and pear juice, respectively). Therefore, a more sophisticated method of adulteration is the addition of lower economic value sweeteners such as high fructose corn syrup (HFCS), medium and total invert sugar (TIS), and hydrolyzed inulin syrup (HIS). These adulterants can be added alone or in combination to match the major fruit juice carbohydrate (fructose, glucose and sucrose) concentrations, so as to obfuscate their addition. An alternate method of fruit juice adulteration is the addition of a second juice of lesser economic value (i.e. juice to juice adulteration). This type of adulteration is possible when there are similarities in the major chemical composition, colour and flavour between juices as is observed with apple and pear (Thavarajah and Low, 2006a).

Adulteration detection in fruit juices can employ many different techniques, but the most prevalent include chromatographic profiling. Both high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) and capillary gas chromatography with flame ionization detection (CGC-FID) are commonly employed for the detection of debasing with less expensive sweeteners by oligosaccharide profiling (Stuckel and

Low, 1995; Low, 1998). Detection is carried out through the identification of fingerprint oligosaccharides, which are found in the adulterant but not in the pure juice. For example, isomaltose is a typical marker for the presence of HFCS in fruit juice (Swallow et al., 1991; Low, 1998; Thavarajah and Low, 2006b; Willems and Low, 2014). This oligosaccharide is formed during starch hydrolysis in the production of HFCS but not during juice processing. Similarly, juice-to-juice adulteration is typically carried out through phenolic profiling employing HPLC coupled with photodiode array detection (PDA) or mass spectrometry (MS). Unique phenolics are identified and used as markers for the presence/absence of that particular juice. For example, arbutin has been consistently reported to be present in pear juice and absent in other juices such as apple making it an effective marker for pear (Andrade et al., 1998; Thavarajah and Low, 2006a; Willems and Low, 2014).

1.2 Hypothesis

The central hypothesis of this research project was that authenticity methods based on modern analytical tools (CGC-FID, HPLC-PDA/PAD/MS) could be developed and implemented to detect the debasing of commercial apple and pear juices from the major world geographical production regions with commercial inexpensive sweeteners (i.e. HFCS, HIS and TIS), and juice to juice adulteration based on phenolic profiling.

1.3 Objectives

In addressing the hypothesis of this research project the following objectives were studied: (1) develop both CGC-FID and HPAE-PAD methods to analyze and establish (i.e. natural range) the oligosaccharide profiles of commercial pear juice from the five major geographical production regions; (2) study oligosaccharide formation and structure in pear juice concentrate by analyzing samples at each stage of commercial processing; (3) develop both CGC-FID and HPAE-PAD methods for oligosaccharide analysis of commercial debasing agents (i.e. HFCS, HIS and TIS); (4) develop a robust analytical method to detect the debasing of commercial pear juices with the aforementioned commercial sweeteners in a single chromatographic analysis; (5) develop a HPLC-PDA method for the analysis of the phenolic profiles of commercial apple and pear juices; (6) identify fingerprint phenolics/compounds as authenticity markers for each juice

chromatographically and determine their structure; and (7) confirm the efficacy of the developed juice to juice adulteration method via the intentional addition of pear to apple, and apple to pear.

2. LITERATURE REVIEW

2.1 Food Authenticity

The adulteration of food products with the purpose of financial gain has been a problem since the beginning of their buying and selling (Sumar and Ismail, 1995). This practice can lead to significant negative economic and health impacts for both producers and consumers. For example, in the early 1800's candy was often adulterated with red lead and copper to achieve the bright colours that were attractive to children (Accum, 1820). A more recent (2008) example occurred in China, where melamine was intentionally added to milk powder/infant formulations so as to provide false protein analysis results. This product adulteration resulted in six reported infant deaths and negatively affected the health of approximately 300,000 consumers worldwide (Pei et al., 2011). Therefore, the ability to detect and trace adulteration in the food chain is vitally important to consumers, companies and government regulatory agencies.

Food adulteration can take on many different forms including, but not limited to, geographical claims (e.g., mislabeling the country of origin), species/variety claims (e.g., a lower value fish such as escolar [*Lepidocybuim flavobrunneum*] being sold as a higher value fish such as tuna), processing/treatment (e.g., refined olive oil being sold as extra virgin), raw material production (e.g., non-organic vegetables being sold as organic) and debasing (i.e., the undeclared addition of a lower value ingredient to a higher value product).

Currently, there are two main pathways used for the detection of food adulteration; untargeted and targeted methods. Untargeted methods rely on knowledge of the natural composition of the product of interest. A sample is considered adulterated if its chemical composition is found to fall outside of the natural range for that product. This pathway for adulteration detection is beneficial as it can potentially identify any adulterated product without prior knowledge of what the adulterant may be. However, these methods do not indicate what the product was adulterated with. In addition, untargeted methods tend to be less sensitive when compared to targeted methods due to the wide variability in the composition of natural products.

For example, hydrolyzed inulin syrup (HIS), which is a common nutritive sweetener used in the food industry, could be added to pear juice as a debasing agent at concentrations of up to 30% without changing the major carbohydrate profile of this product, allowing this significant level of adulteration to go undetected (Willems and Low, 2014). Therefore, targeted methods for adulteration detection are highly valued.

Targeted adulteration detection techniques are based on the development and application of analytical methods designed to detect the presence of a specific adulterant or group of adulterants. These methods typically focus on the detection of specific marker or 'fingerprint' compounds that are unique to the adulterant(s) and are not found in the authentic product. Targeted methods not only identify adulterated samples but can also identify which adulterant(s) were used. In addition, targeted methods offer greater sensitivity (i.e., lower detection limits) when compared to untargeted methods, often being able to detect adulteration at levels of ≤5% (Stuckel and Low, 1995; Willems and Low, 2012; Wang et al., 2015). Depending upon the product and potential adulterant(s), targeted methods can make use of a variety of different analytical techniques (i.e., chromatography, spectroscopy, etc.) and are based on metabolomic, proteomic and genomic principles.

Currently, a wide variety of different analytical techniques have been applied to food authenticity analysis. However, in this review only a selection of these techniques will be covered including stable isotope ratio analysis, infrared spectroscopy, genomic based techniques and chromatographic profiling/fingerprinting. These techniques were chosen as they have been widely studied and applied to a variety of different food products for authenticity analysis.

2.2 Methods for Detecting Food Adulteration

2.2.1 Stable Isotope Ratio Analysis

It is well understood that elements found in nature do not occur as a single isotope but are found as isotopic mixtures. The ratio of these isotopes is not constant in nature and varies due to isotope fractionation, which can be caused by physical, chemical and biochemical processes (Rossmann, 2001; Benson et al., 2006). For example, the process of water evaporation enriches the ^{18}O content in the residue liquid water, with the amount of isotope fractionation depending upon a number of factors such as temperature and altitude (Krueger, 1998; Benson et al., 2006).

These differences in isotope ratios have been used for detecting product debasing as well as determining the geographical origin of foods.

While a variety of different elements have been used for stable isotope ratio analysis, including $^{15}N/^{14}N$ and $^{34}S/^{32}S$, the main elements/isotope ratios used for food adulteration detection are $^{13}C/^{12}C$, $^{2}H/^{1}H$ and $^{18}O/^{16}O$ (Krueger, 1998). Typically, for food authenticity analysis, isotope ratios are measured by one of two techniques, stable isotope ratio mass spectrometry (IRMS) or site-specific natural isotope fractionation by nuclear magnetic resonance spectroscopy (SNIF-NMR).

Isotope ratio mass spectrometers are specifically designed to measure variations in the isotopic ratios of chemical compounds (Benson et al., 2006). As such, an IRMS system contains a dual sample inlet device, which affords the introduction of both the sample (in a gaseous state) and a reference gas. These gases are alternately allowed into the system and data from these experiments are used to produce a ratio of the isotope abundance in the sample as compared to the reference (Krueger, 1998). While IRMS measures the isotopic ratio for an entire compound/sample, SNIF-NMR is based on the measurement of the deuterium/hydrogen ratio at a specific site in a molecule such as the methyl and methylene groups of ethanol (Ogrnic et al., 2003). Results are not reported as absolute values but rather as deviations of the ratios from a standard, known as δ-values and are reported as ‰ (Rossmann, 2001).

A common application of stable isotope analysis is in the detection of the debasing of high value carbohydrate products, such as honey and fruit juices, with less expensive commercially available nutritive sweeteners such high fructose corn syrup (HFCS) by carbon stable isotope ratio analysis (CSIRA). The detection of HFCS in a food product is linked to the biological pathway for carbon dioxide fixation by corn which follows the Hatch-Slack (C4) versus the Calvin cycle (C3) photosynthetic pathway, which is more common (i.e. in most fruits and honey nectar sources) (Krueger, 1998; Çinar et al., 2014). A third photosynthetic pathway, the Crassulacean acid metabolism (CAM) pathway is used by plants such as agave and pineapple (Krueger, 1998; Çinar et al., 2014). The C3 pathway results in greater carbon isotope fractionation with isotopic ratios ranging from -21 to -35‰ when compared to the C4 pathway with isotope ratios ranging from -9 to -20‰. The CAM pathway plants fall in between these two with variation depending on the proportion of carbon fixed in the dark by the C4 pathway or in the light by the C3 pathway (Badeck et al., 2005). Based on these differences in atmospheric carbon dioxide fixation during

photosynthesis, carbon stable isotope ratio analysis has been applied to the detection of HFCS and cane sucrose (sugar cane is also a C4 plant) to foods such as apple and orange juices, and honey which are produced from C3 plants (Krueger, 1998; Antolovich et al., 2001). For example, one study found that pure orange juice had a δ-value that ranged from -23.8 to -24.7‰ whereas a sample adulterated with ~30% carbohydrate from a C4 plant (i.e. cane sucrose) had a δ-value of -20.9‰ and an orange fruit drink product (which are legally allowed to contain added carbohydrates and water) had a δ-value of -14.1‰ indicating that >80% of this product originated from C4 plants (Antolovich et al., 2001).

However, not all potential debasing agents can be detected by CSIRA as beet sucrose/beet sucrose hydrolysates (e.g. beet invert sugar), are produced from a C3 plant. Also, plants which use the less common CAM pathway, such as agave and pineapple, have carbon stable isotope ratios that encompass the natural $^{13}CO_2/^{12}CO_2$ $\delta^{13}C$ values/ranges for C3 and C4 plants reducing and/or eliminating CSIRA for adulteration detection with any of the aforementioned commercially available nutritive sweetener debasing agents (Krueger, 1998; Willems and Low, 2012). Furthermore, adulteration detection limits based on CSIRA vary from 10 to 50% depending upon the natural stable isotope ratio distribution of the unadulterated product and the potential adulterant (Tremblay and Paquin, 2007).

In addition to carbon, oxygen isotope ratios ($^{18}O/^{16}O$) have been used to detect the addition of water to fruit juices and wines (Krueger, 1998; Jamin et al., 2003). Due to isotope fractionation during plant transpiration, $^{18}O/^{16}O$ ratios are usually higher in plants than in groundwater (Jamin et al., 2003; Calderone and Guillou, 2008). This allows for the detection of juices that have been concentrated and then reconstituted with ground water. For example, fresh orange juices from Brazil, Israel and Morocco all had positive $\delta^{18}O$ values (ranging from approximately 1.0 to 6.0‰) while local ground (i.e. tap) water had a negative $\delta^{18}O$ value of -10.0‰ (Calderone and Guillou, 2008). One of the major drawbacks of this method is that the oxygen isotope ratio of fruit and fruit products varies with growing location and harvest conditions, requiring the constant updating of databases of authentic values for comparison (Jamin et al., 2003). In addition, it is possible to obtain ^{18}O enriched water (i.e. discharge water) from food processing plants involved in the concentration or drying of foods (i.e. fruit juice producer) giving dishonest producers an economic way to circumvent detection by this method (Krueger, 1998).

In addition to IRMS, SNIF-NMR has been used to determine isotope ratios for food authenticity analysis. This method is based on deuterium/hydrogen (D/H) ratio measurements of the methyl $(D/H)_I$ and methylene $(D/H)_{II}$ sites in ethanol (Ogrnic et al., 2003). In this analysis, the sample is fermented under controlled conditions and the resulting ethanol distilled and collected (Ogrnic et al., 2003). Carbohydrate fermentation to ethanol allows for adulteration analysis of a wide variety of foods (e.g. fruits juices and wines) as their D/H ratios are generally different from those of commercial inexpensive sweeteners such as beet/cane sucrose. For example, apple fruit has a $(D/H)_I$ of 100.9 ppm, while beet, maize and cane have $(D/H)_I$ of 92.7, 110.7 and 112.0 ppm, respectively (Jamin and Martin, 2006).

Due to the large amount of natural variation in foods (e.g. orange juice), a large database of isotopic measurements from the same and different production regions is required, so as to reliably determine the authenticity of a product.

2.2.2 Infrared Spectroscopy

The infrared (IR) region of the electromagnetic spectrum that is used for the analysis of organic compounds ranges from 750 to 25,000 nm. Infrared spectroscopy has been extensively used to study the structural characteristics of organic molecules and as an important contributor to compound identification (Scafi and Pasquini, 2001). Organic molecules are able to absorb specific frequencies of IR radiation based upon their structures/functional groups, and the absorption of energy at these frequencies (i.e. the resonance frequency) causes the molecule to vibrate (i.e. vibrational excitation). If this vibration results in a change in a dipole moment of the molecule an IR signal is observed at that frequency and the collection of these vibrations gives rise to an IR spectrum, which can be used for sample identification (Yang et al., 2005).

Infrared spectroscopy is divided into three different regions based upon the wavelength of electromagnetic radiation used to produce the absorbance/transmission spectra, namely near-infrared (NIR), mid-infrared (MIR) and far-infrared (FIR) (Scotter and Wilson, 1998). Both NIR and MIR have been applied for food authentication as they provide diagnostic information, and are rapid and non-destructive techniques (Reid et al., 2006). Near-infrared is considered to be the region between 750 and 2,500 nm (13,400 to 4,000 cm^{-1}), whereas MIR has a spectral range of 2,500 to 25,000 nm (4,000 to 400 cm^{-1}) (Scotter and Wilson, 1998; Cozzolino, 2014). Infrared spectroscopy has been used to determine the authenticity of a variety of food samples, ranging

from beverages such as liquors (Chen et al., 2014) and milk (Santos et al., 2013), to solid foods such as butter (Nunes, 2014) and cereal flours (Cozzollino, 2014).

As an example, both Fourier transformed near-infrared (FT-NIR) and mid-infrared (MIR) spectroscopy have been applied to identify and discriminate between ten different edible oils. This was done by comparing the IR signals of the carbon-carbon double bond(s) in the unsaturated fatty acids of the oils, with FT-NIR being able to correctly classify 93% of the oil samples, while MIR had a higher success rate of 98% classification due to the stronger and narrower absorbance peaks/bands observed (Yang et al., 2005).

2.2.3 Genomic Based Techniques

Although genomic based authenticity methods have been applied to a wide variety of food products, such as onions and berries (Jaakola et al., 2010; Tedeschi et al., 2014), they are primarily used to identify the speciation of animal-based (i.e. meats and fish) products. A number of different genomic-based approaches have been employed for food adulteration detection with DNA as the marker, including DNA barcoding, restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD) and species-specific primers.

The DNA barcoding approach is based on the comparison of a known nucleotide region of the DNA of a species with the same DNA region of the sample. Typically, this method targets the gene that codes for cytochrome oxidase I (COI) which is found in the mitochondrial genome (i.e. mitochondrial DNA; Fajardo et al., 2010). However, other coding regions have been used for this purpose for plant speciation that include but are not limited to, *rpoB, rpoC1, rbcL* and *matK* (Galimberti et al., 2013). The use of a mitochondrial gene is reported to be beneficial as this genome is present in cells in higher numbers than nuclear DNA resulting in lower detection limits, and mitochondrial DNA tends to accumulate mutations at much faster rates than does nuclear DNA, making the differentiation of closely related species more facile (Fajardo et al., 2010; Lockley and Bardsley, 2010).

An example of the application of DNA barcoding for food authenticity determination based on COI analysis is the species identification of forty different fresh and frozen fish filets labelled as Europe's most commercially important flatfish species, European plaice (Pappalardpo and Ferrito, 2015). It was found that 37.5% of the analyzed samples were comprised of fish of lower value (e.g. Mediterranean scaldfish) despite being labelled as the more expensive flatfish species

(Pappalardpo and Ferrito, 2015). The DNA barcoding method was found to be highly effective for this authenticity application as the filets were comprised of a single fish species and had been minimally processed allowing 100% of the samples to be identified based upon species. However, as the primers used for DNA barcoding are universal, application of this method to mixed species products, such as sausages and mixed fruit beverages, results in the production of a number of different barcode sequences which complicates sequencing and analysis (Galimberti et al., 2013).

The RAPD genomic approach involves the amplification (polymerase chain reaction [PCR]) of DNA using a short primer(s). The primer(s) may be complementary to one or more regions of the sample genome resulting in the amplification of a variety of different fragments for a single species (Jhang and Shasany, 2012). The amplified fragments are then separated by gel/capillary electrophoresis and the resulting pattern is a 'fingerprint' that can be used to determine product adulteration by comparison to standards (Fajardo et al., 2010). One of the major benefits of this method is that it is rapid and relatively simple to perform as it does not require sequencing (Fajardo et al., 2010; Jhang and Shasany, 2012). Also, the use of arbitrary primers means that no prior knowledge of the sequence to be amplified is required, allowing for sample species identification where sequencing information is not available.

An example of the application of RAPD for food authenticity determination is the identification/differentiation of Tropea red onion, a special Italian variety, from other less commercially valuable onion species that are often unlawfully substituted for Tropea onions (Tedeschi et al., 2014). The authors found that with the use of 25 selected RAPD primers they could differentiate Tropea from three other common commercial onions (Tedeschi et al., 2014). However, while the authors used seven different Tropea onions as standards to establish genetic variability, they only analyzed one sample of each of the three commercial varieties, and as such did not take into account any variability in these onion species, which may have compromised the application of this method to this product.

The ability to obtain reproducible data using the RAPD approach is difficult because the fragments produced are effected by the reaction conditions employed, which must be carefully controlled (Fajardo et al., 2010; Jhang and Shasany, 2012). In addition, this method requires high quality DNA for amplification, which may not be obtainable from highly processed (e.g. heat processed) food products. Finally, as with DNA barcoding, this method has limited application to detect adulteration in mixed species food samples (Fajardo et al., 2010).

The RFLP genomic approach for food authentication is based on DNA extraction from a sample followed by its treatment with a selection of restriction enzymes (Bertea and Gnavi, 2012). These enzymes cut the DNA at specific nucleotide sequences resulting in DNA fragments that may be species specific. The fragments are then separated using gel electrophoresis and the resulting pattern is used to identify the species composition of a sample by comparison to standards (Bertea and Gnavi, 2012). Similar to RAPD, RFLP sample analysis is easy to perform and relatively inexpensive as it does not require the use of sequencing. A major limitation of this method is the requirement for a significant amount of sample DNA, which may be achieved by PCR amplification of isolated regions of the DNA under examination. The accuracy of this technique is also highly dependent on the reaction conditions, and as such results may vary between laboratories (Fajardo et al., 2010). Also, RFLP fragmentation patterns may not be readily distinguishable between closely related species, such as wild boar and domesticated pig (Wolf et al., 1999).

Species-specific primers offer the potential of overcoming some of the shortcomings of the aforementioned genetic approaches to food authenticity. In this method, primers are designed to amplify very specific DNA sequences that are indicative of a particular species (Fajardo et al., 2010; Rojas et al., 2011). The benefit of this approach is the targeted amplification of a region of DNA in a sample, allowing for the detection of a specific species in a mixed product (Rojas et al., 2011). Also, this method does not require sequencing or enzyme treatment after amplification thus simplifying sample analysis (Rojas et al., 2011). For example, this approach was used to detect the adulteration of mixed meat sausages claiming to contain traditional game meats, such as hare, rabbit and red deer, with less expensive pork or beef (Amaral et al., 2014). The use of species-specific primers coupled with real time PCR, may afford species quantitation in a sample (Rojas et al., 2011); however, this targeted method of adulteration detection requires species-specific primers.

2.2.4 Chromatographic Profiling/Fingerprinting

Chromatography is an analytical procedure designed for the separation of analytes in complex mixtures, such as foods. Separation is based on the partitioning of sample analytes between a stationary and mobile phase that in many instances results in the production of a unique chromatographic profile or fingerprint, which can be used to both differentiate (i.e. apple juice

from pear juice) and authenticate foods/food products (Danezis et al., 2016). The identification of pure and/or adulterated samples is based on differences in their chromatographic profiles based on the presence/absence of specific marker analytes. The analytes of interest often varies with food product and the type of adulteration being tested for. For example, amino acids, fatty acids, phenolics, oligosaccharides and organic acids have all been used for food authenticity purposes (Danezis et al., 2016). As oligosaccharide chromatographic fingerprinting is a major authenticity approach studied for use in fruit juices, the remainder of this section will focus on these analytes.

Oligosaccharide profiling/fingerprinting has been shown to be an effective method to detect the debasing of high value carbohydrate rich foods, such as honey, maple syrup and fruit juice (Stuckel and Low, 1995; Low, 1998). Due to their high carbohydrate content (often greater than 95% of the total soluble solids) these foods are susceptible to debasing with less expensive commercially available sweeteners including: high fructose corn syrup (HFCS), medium and total invert sugar (MIS/TIS) and hydrolyzed inulin syrup (HIS). The presence of these sweeteners in the aforementioned foods can be extremely difficult to detect due to similarities between their major carbohydrate profiles. Research has shown that trace levels of oligosaccharides are formed during the production of inexpensive commercial sweeteners due to transglycosylation and acid-reversion reactions, and these oligosaccharides can be used as markers for the undeclared presence of these sweeteners in carbohydrate rich foods (Thavarajah and Low, 2006b). For example, isomaltose (α-D-glucopyranosyl-(1→6)-D-glucopyranose) is both a byproduct of starch hydrolysis and transglycosylation reactions during HFCS production, and its presence in a selection of fruit juices (e.g. orange) is a clear indicator of their adulteration with this sweetener (Swallow et al., 1991; Low, 1998; Thavarajah and Low, 2006b; Willems and Low, 2014).

In addition to the ability to detect the undeclared addition of specific (i.e. targeted method) commercial sweeteners to foods/food products, oligosaccharide fingerprinting methodology has also been developed to examine the impacts that enzymatic and heating conditions have on oligosaccharide formation and structural changes during commercial fruit juice production. This knowledge is important as it can be used to ensure the legality of enzymes used, and to detect excessive heating to possibly mask the lack of good manufacturing practices employed in fruit juice production. As an example, during commercial conditions to ensure total fruit liquefaction, cellulase and excess pectinases are added to the fruit mash in order to break down cell walls so as to increase the total soluble solids content of the resultant juice (Will et al., 2000). However, this

practice is illegal in both Europe and North America and can be detected by the presence of the disaccharide cellobiose, which is not produced under normal (i.e. legal) juice processing conditions (Willems and Low, 2014; Willems and Low, 2016).

Oligosaccharide profiles can also be used by industry to monitor the quality of food products during processing. For example, the oligosaccharide profile of pear juice at different stages of processing can be used to detect potential issues during enzymatic treatments (Willems and Low, 2016). In addition, information on oligosaccharide profiling can be used to design enzyme treatments for juice production that lead to a final product higher in dietary fibre, allowing producers to offer a juice of greater nutritional value to the consumer (Willems and Low, 2016).

Oligosaccharide profiling/fingerprinting is most commonly conducted employing high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) and/or capillary gas chromatography with flame ionization detection (CGC-FID). The separation of carbohydrates/polyols by HPAE-PAD is based upon their weakly acidic nature with pKa's in the 12.0-13.6 range (Rohrer, 2012). At the high (>13.0) pH of the mobile phase, sample carbohydrates become negatively charged resulting in an affinity for the anion exchange stationary phase (Low, 1997). Typically, sodium hydroxide solutions are used as the mobile phase for HPAE-PAD, with concentrations of ~0.10 M being used for analysis of the major carbohydrates present in foods (i.e. fructose, glucose and sucrose). However, the analysis of oligosaccharides (i.e. degree of polymerization [DP] of 2-30) requires the addition of a stronger counterion (i.e. sodium acetate) to the mobile phase at concentrations up to 250 mM (Lee, 1996; Cataldi et al., 2000).

Pulsed amperometric detection (PAD) is highly beneficial for carbohydrate analysis as it is selective and highly sensitive without the need for derivatization (Cataldi et al., 2000; Dionex, 2004). The PAD system affords the detection of even minor carbohydrates as the limits of detection are in the low ppm (<10 ppm) range (Dionex, 2004; Willems and Low, 2016). Also, sample preparation is facile and non-carbohydrate components are not detected. Under the mobile phase alkaline conditions of HPAE, functional groups (i.e. carbonyl and/or hydroxyl groups on carbohydrates) are oxidized at the surface of a gold electrode resulting in the production of an electric current that is proportional to their concentration (Low, 1997; Dionex, 2004). In order to maintain detector sensitivity, the gold oxide formed during detection must be removed (Cataldi et al., 2000) and this is accomplished by applying a second electrode potential so as to desorb carbohydrate oxidation products from the gold surface followed by a third electrode potential

change to reduce the surface back to gold (Dionex, 2004). This entire process is called triple pulsed amperometry.

Carbohydrates require derivatization in order to be analyzed by CGC-FID as these compounds have no appreciable vapor pressure. While many different methods of derivatization have been used for carbohydrate analysis, the trimethylsilyl derivatives (e.g. trimethylsilylimidazole) are often employed so as to provide both volatility and stability by replacing active hydrogens (i.e. from hydroxyl groups) with trimethylsilyl groups (Little, 1999). Due to the requirement for analyte volatility, CGC analysis of oligosaccharides is typically limited to DP $\leq$ 3 because of molecular weight-volatility issues as the addition of a single trimethylsilyl group increases the molecular mass of the resulting carbohydrate by 72.19 g (Ruiz-Matute et al., 2011). As an example, the molecular mass of a disaccharide such as isomaltose increases from 342.30 to 919.80 g/mole upon trimethylsilylation. However, the increased analyte resolution capability of CGC (up to 150,000 theoretical plates for a 30 m column) under temperature programmed conditions is illustrated by the ability to separate α- and β-anomers of the same carbohydrate, and structurally similar di- and trisaccharides (e.g. maltose and isomaltose). These carbohydrate separation advantages make CGC-FID an extremely useful tool for oligosaccharide profiling.

Flame ionization detection (FID) works on the basis of organic analyte combustion via a mixed-gas (oxygen-hydrogen) flame to form ions and electrons, resulting in a current that is proportional to analyte concentration (Holm, 1999). Advantages of FID include but are not limited to, (a) universal detector for organic compounds; (b) wide linear response range (e.g. 10 to 200 ppm for carbohydrates); and (c) high sensitivity (e.g. 1 ppm for most carbohydrates) (Holm, 1999; Montilla et al., 2006).

Both HPAE-PAD and CGC-FID have been employed for adulteration detection by oligosaccharide profiling. Select examples include, CGC-FID for the undeclared addition of dextrose equivalents syrup (DE 42), HFCS 55 and 90, and sucrose to agave syrup with detection limits ranging from 0.5 to 1.0% (Willems and Low, 2012); and HPAE-PAD for the detection of HFCS and beet medium invert sugar (BMIS) in maple syrup with a detection limit of 5.0% for each (Stuckel and Low, 1995).

2.3 Oligosaccharide Formation in Pear Juice

2.3.1 Introduction

Pear and apple are both members of the Rosaceae family along with other major fruits such as apricot and peach, which are collectively known as pome fruits (Cornille et al., 2014). Due to similarities in their physical structure and chemical composition, apple and pear fruits undergo similar commercial processing steps for conversion into juices (Bates et al., 2001). The raw materials for commercial apple and pear juice production includes a number of different cultivars of the species *Malus domestica* and *Pyrus communis L.*, respectively. While apple juice production typically employs a much wider variety of apple cultivars that are often dependent on geographical location and availability, the three varieties commonly used for world pear juice production are Bartlett (also known as Williams), Beurre d'Anjou and Beurre Bosc (USDA, 2004). In 2013, an estimated 22.6 million metric tons of pears were produced worldwide (WAPA, 2016). In the United States of America 1.6 billion pounds of pears were grown in 2014 and its value was estimated to be $457.1 million (AgMRC, 2015). On average, 20% of total fruit production is used in processing, and of this just over a quarter is converted into juice (AIJN, 2014).

2.3.2 Pear Juice Production

During pear juice production, a number of commercial processing steps are employed. Initially, the fruit is washed with water to remove surface contaminants, and is then converted into a mash by milling (e.g. hammer mill). Carbohydrases, specifically amylases, hemicellulases and pectinases are added together to the mash so as to break down cell walls, which results in increased juice recovery (Ribeiro et al., 2010). The juice is then extracted from the enzyme treated mash by pressing (belt and/or hydraulic press) and the remaining pomace particles are removed by screening. The juice is transferred to clarification tanks where if required, additional carbohydrases are added. Juice cloud/haze primarily consists of protein particles coated with pectin, and at the pH of the raw pear juice (pH 3.5 to 4.0) pectin carries a negative charge that leads to protein-pectin particles repelling each other, resulting in an opaque juice. The second pectinase treatment degrades the negative pectin coat on these particles allowing them to aggregate and precipitate for removal through filtration (Sorrivas et al., 2006). The clarified juice is then pasteurized (70 to 100 °C for 6 to 40s) and packaged for sale or concentrated to 70-71 ^{o}Brix (multiple effect evaporator) for economical product transport and/or storage (room temperature for short term storage or <7 °C

for long term storage) (Bates et al., 2001; Ceci and Lozano, 2010; Horváth-Kerkai and Stéger-Maté, 2012).

2.3.3 Enzymes Employed in Pear Juice Production

Pectinases were one the first enzymes to be used for commercial fruit juice production purposes and were first used in the 1930's for wine and juice production (Kashyap et al., 2001; Ribeiro et al., 2010). While pectinases from a number of plants and microorganisms are commercially available, those from *Aspergillus niger* are the most widely used in the fruit (e.g. apple, cranberry, pear) juice industry due to their low cost and generally recognized as safe (GRAS) status (Gummadi and Panda, 2003).

Pectinases have been shown to degrade pectin by a variety of different mechanisms. As examples, polygalacturonase (PG) and polymethylgalacturonase (PMG) hydrolyze the α-1,4-glycosidic linkages of pectin resulting in the formation of pectin oligosaccharides and galacturonic acid/galacturonic acid methylester, while polygalacturonate lyases (PGLs) and polymethylgalacturonate lyases (PMGL) break the α-1,4-glycosidic linkages of pectin by a trans-elimination mechanism, resulting in a site of unsaturation at the non-reducing end (Kashyap et al., 2001). The PGs and PGLs preferentially act on low esterified pectin while PMGs and PMGLs work preferentially on highly esterified pectin (Pedrolli et al., 2009). Both of these classes of enzymes can hydrolyze in an endo or exo fashion, both of which are exploited in commercial juice production (Pedrolli et al., 2009). Polygalacturonase is the major pectin hydrolysis constituent in industrially produced enzyme cocktails for commercial pear juice processing/production, and its mechanism of substrate hydrolysis has been extensively studied (Lang and Dörnenburg, 2000). Based on research conducted on an endo-polygalacturonase from *Aspergillus aculeatus*, three aspartic acid residues were directly involved in pectin hydrolysis and the catalytic reaction was shown to follow an inverting mechanism (Cho et al., 2001). As illustrated in Figure 2.1, a pair of aspartic acid residues (i.e. carboxylic acid functional group) act as acids/bases and the reaction proceeds through an oxocarbenium-ion-like transition state resulting in the inversion of the anomeric configuration of the final product (Rye and Withers, 2000). In addition to three aspartic acid residues used during catalysis, arginine and lysine are involved in substrate binding and a histidine residue helps maintain the proper ionization state of the catalytic aspartic acid residue (Cho et al., 2001). These residues were found to be conserved in the polygalacturonase family

emphasizing their importance in catalyzing this hydrolysis reaction (Cho et al., 2001). Based on research conducted on four different endo-polygalacturonases isolated from *Aspergillus niger*, it was estimated that a minimum oligosaccharide degree of polymerization (DP) of four was required for hydrolysis (Benen et al., 1999). Similar results were found for an exo-polygalacturonase from *A. tubingensis* with an optimum oligosaccharide hydrolysis size of DP ≥ 4 (Kester et al., 1996).

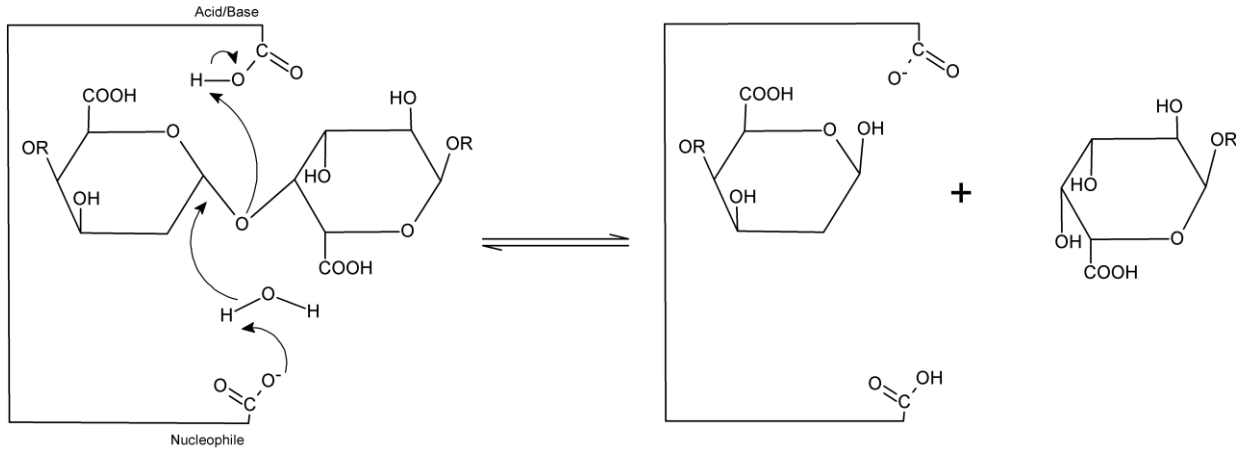

Figure 2.1 Mechanism of pectin hydrolysis by polygalacturonase.

Pectinesterases (PE) are also a common ingredient in industrially produced enzyme cocktails for commercial pear juice production. This enzyme hydrolyzes the methyl ester groups of pectin resulting in polygalacturonic acid and methanol formation (Horváth-Kerkai and Stéger-Maté, 2012; Kashyap et al., 2001). Pectinesterase acts preferentially on galacturonic acid methyl ester units that are adjacent to non-esterified galacturonic acid units (Pedrolli et al., 2009).

Although industrially produced and formulated for pome (e.g. apple and pear) juice production, enzyme cocktail preparations are proprietary as they contain a group of pectinases that include polygalacturonases, pectin lyases and pectinesterases at different concentrations, and often include enzymes with other carbohydrase activities (Novozymes, 2009). The use of a pectinase cocktail is required for commercial pear juice production as pectin is the major polysaccharide present in pear cell walls, and its hydrolysis is required to improve juice extraction and reduce product viscosity. In addition to pectin, starch and hemicellulose are also targeted by industrial enzyme manufacturers. The presence of colloidal starch and hemicelluloses in pome juices can cause cloudiness and sedimentation, which are undesirable (Carrín et al., 2004; Sinh, 2012).

Therefore, industrially produced enzyme cocktails for pear juice production also contain hemicellulases and amylases (primarily arabinase, amyloglucosidase and α-amylase) (Novozyme, 2009). In addition to juice clarification, hemicellulase activity increases fruit cell wall breakdown and improves juice extraction and filtration (Horváth-Kerkai and Stéger-Maté, 2012).

2.3.4 Pear Polysaccharides

Plant cell walls are comprised of a variety of polymers where, in dicotyledons such as pear, approximately 35% is pectin, 30% is cellulose, 30% is hemicellulose and 5% is protein (Fischer and Bennett, 1991; Schols, 1995; Dongowski and Sembries, 2001). In plant cell walls, cellulose exists as a complex with hemicellulose providing mechanical strength (Fischer and Bennett, 1991). The term hemicellulose encompasses a number of different polysaccharides including galactomannans, glucomannans, xyloglucan and xylans. The major hemicellulose in the cell walls of dicotyledon plants is xyloglucan, which accounts for ~20% of the cell wall (Darvill et al., 1985; Fischer and Bennett, 1991; Raffo et al., 2011).

As the major polysaccharide in the cell walls of dicotyledons, pectin is reported to be involved in plant growth and development, cell to cell adhesion and cell wall structure (Mohnen, 2008). Pectin is a polysaccharide primarily (~70%) comprised of galacturonic acid residues to which neutral carbohydrates, mainly L-arabinose, D-galactose, L-rhamnose and D-xylose are covalently bound (Kashyap et al., 2001; Mohnen, 2008). Pectin can be classified into three different categories based on structure. The simplest and most abundant of these is homogalacturonan (HG), which is defined as a linear polymer of α-1,4 glycosidically linked D-galacturonic acid residues. The second category is rhamnogalacturonan I (RGI) in which the disaccharide $[\rightarrow 4)$-α-D-GalA-$(1\rightarrow 2)$-α-L-Rha-$(1\rightarrow]_n$ repeats as the polysaccharide backbone, to which a variety of different oligosaccharides, primarily arabinans and galactans, are linked to the rhamnose residues at the O-4 or O-3 positions. It has been reported that RGI accounts for 20 to 35% of the pectin in plants (Mohnen, 2008). Rhamnogalacturonan II (RGII) consists of a HG backbone to which complex oligosaccharides are attached to the galacturonic acid residues through a variety of different glycosidic linkages (O'Neill, 2004; Willats et al., 2006; Round et al., 2010; Yapo, 2011). According to literature, the approximate content of RGII in the pectin of plants is 10% (O'Neill, 2004; Mohnen, 2008). In addition to the aforementioned structures, methyl esterification of galacturonic acid residues is also commonly observed in pectin. Plant pectin

structure has been shown to be dependent on plant species, location within the cell wall and plant age (Round et al., 2010).

2.4 Phenolics

The term phenolics encompasses a variety of structurally diverse compounds with the common feature of at least one hydroxyl group directly bonded to an aromatic ring. Phenolics are secondary metabolites that are derived from phenylalanine and to a lesser extent tyrosine with more than 8000 different compounds reported in plants (Manach et al., 2004; Vermerris and Nicholson, 2008; Terahara, 2015). Phenolic compounds serve a variety of purposes in plants such as imparting colour, attracting or repelling insects, exhibit both antimicrobial and antiviral activity, and provides UV protection (Manach et al., 2004).

Phenolics are broadly divided into two main classes, the phenolic acids and the flavonoids, however, they are subdivided further within these main classes. For example, phenolic acids are divided into the hydroxybenzoic and hydroxycinnamic acids subclasses, and the flavonoids are divided into five major subclasses, namely the anthocyanins, flavanols, flavanones, flavones and flavonols. In addition, other minor subclasses also exist within the two main classes (Manach et al., 2004; Green, 2007; Gharras, 2009).

2.4.1 Classification of Phenolics

2.4.1.1 Phenolic Acids

Phenolic acids are defined structurally by the presence of a carboxylic acid functional group. In the hydroxybenzoic acids subclass, the carboxylic acid group is directly substituted onto the phenolic ring (C_6-C_1 structure), while in the hydroxycinnamic acids subclass the carboxylic acid is attached to the phenolic ring via a three carbon chain resulting in a C_6-C_3 structure (Vermerris and Nicholson, 2008) (Figure 2.2). Compounds within each subclass differ based upon the number and location of hydroxyl and/or methoxyl groups on the aromatic ring (Mattila et al., 2006). The majority of phenolic acids found in nature are either glycosidated (e.g. with glucose) or esterified (e.g. with quinic acid) (Mattila et al., 2006; Green, 2007). The most common hydroxybenzoic acids are gallic, *p*-hydroxybenzoic and vanillic acids, which have been reported to be ubiquitous in plants (Robbins, 2003). The concentration of hydroxybenozic acids in plants is generally low, with a few exceptions such as onions and black radishes (Manach et al., 2004).

Conversely, hydroxycinnamic acids are abundant in food plants making them an important component in the human diet (Manach et al., 2004; Manach et al., 2005). Within the hydroxycinnamic acids subclass, chlorogenic acid (5-*O*-caffeoylquinic acid) is the most common in fruits, such as apple and pear, as well as being predominant in coffee and tea (Clifford, 1979; Spanos and Wrolstad, 1990; Spanos et al., 1990; Clifford 1999; Manach et al., 2005; Gharras, 2009).

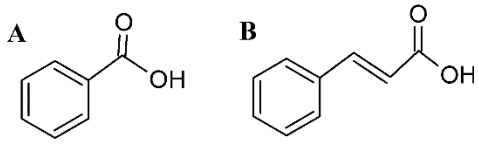

Figure 2.2 Basic structures of hydroxybenzoic acids (A) and hydroxycinnamic acids (B).

2.4.1.2 Flavonoids

Flavonoids are defined by their structure as having two aromatic rings which enclose a heterocyclic oxygen containing ring, labelled as the A-, B- and C-rings (Figure 2.3) (Heim et al., 2002; Terahara, 2015). Flavonoids are subdivided based upon variations in their degree of unsaturation and oxidation of the C ring and differ within subclasses based on the number and location of hydroxyl and methoxy groups on the A and B rings (Heim et al., 2002). Flavonoids are commonly found in nature in their glycosidated form with the majority as 3-*O*-glycosides, however, they can also exist as *C*-glycosides (Terahara, 2015). The most common flavonoid carbohydrates are D-glucose and L-rhamnose, but also include others such as D-galactose, D-xylose and D-arabinose (Heim et al., 2002; Manach et al., 2004; Terahara, 2015). Glycosylation improves the water solubility of flavonoids, which is essential for their storage in plant cell vacuoles (Aherne and O'Brien, 2002).

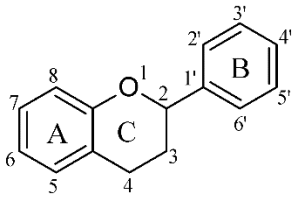

Figure 2.3 Basic structure of flavonoid aglycones.

Flavonols are structurally defined by the presence of a double bond at the 2-3 position and a hydroxyl group at the 3 position on the C-ring (Figure 2.4A) (Hollman and Arts, 2000). Of the flavonols, quercetin-3-*O*-glycosides, such as isoquercetin, quercitrin and rutin, are the most abundant in nature, but glycosides of isorhamnetin, kaempferol and myricetin are also common (Hollman and Arts, 2000; Gharras, 2009; Terahara, 2015). Flavonols are widely distributed in nature, being found in fruits, vegetables and especially in green leaves (Aherne and O'Brien, 2002; Terahara, 2015). As such, flavanols represent the predominant flavonoid subclass in a typical human diet.

Flavanones are characterized by the presence of a carbonyl group at C4 of the C-ring (Figure 2.4B). Approximately 350 different flavanone aglycones (i.e. compound without glycosylation) have been identified in nature, with naringenin and hesperetin being the most common and at their highest concentrations in citrus fruits, primarily in their peels (Iwashia, 2000; Nogata et al., 2006; Khan et al., 2014).

Similar to flavanones, flavones have a carbonyl group at C4 of the C-ring, however, they also contain a site of unsaturation between C2 and C3 of the C-ring (Figure 2.4C). Two of the most common flavone aglycones are apigenin and luteolin. While flavones have been found in a wide variety of plants, they tend to be less abundant than other flavonoids and are primarily found in citrus fruits (Iwashia, 2000; Green, 2007).

Flavanols (or flavan-3-ols) are defined by a saturated C-ring with a hydroxyl group attached at C3 (Figure 2.4D). Flavanols can be found in nature as monomeric units, but are also commonly found as oligomers or polymers, commonly referred to as proanthocyanidins or condensed tannins (Aron and Kennedy, 2008). The most common flavan-3-ols include the monomers catechin and epicatechin and the dimer procyanidin B2, which are abundant in a variety of food sources, including tea, red wine and plums (de Pascual-Teresa et al., 2000).

Anthocyanins are water soluble pigments responsible for the red, blue and purple colours in plant parts such as leaves, flowers and fruits (Jackman et al., 1987; Lee and Finn, 2007; Castañeda-Ovando et al., 2009). By definition, anthocyanins are glycosylated and their unstable aglycones are referred to as anthocyanidins (Jackman et al., 1987; Castañeda-Ovando et al., 2009). Anthocyanins/anthocyanidins are defined by the presence of two conjugated sites of unsaturation between the ring oxygen and C1 and between C3 and C4, resulting in a positive charge on the

oxygen of the C-ring (Figure 2.4E). The most common anthocyanidins include cyanidin, melvidin, delphinidin, pelargonidin, peonidin and petunidin (Iwashina, 2015).

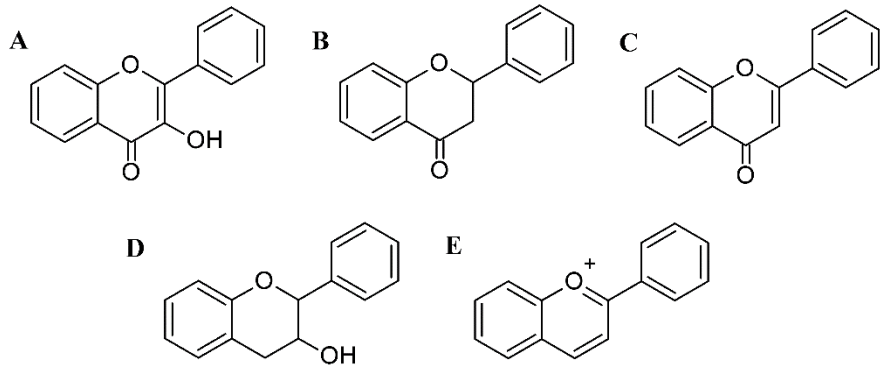

Figure 2.4 Basic aglycone structures of the major flavonoid subclasses: (A) flavonols, (B) flavanones, (C) flavones, (D) flavanols and (E) anthocyanidins.

2.4.2 Antioxidant Potential of Phenolics

Phenolics have been widely studied for their health promoting attributes in humans, including antioxidant, anti-inflammatory and antiviral properties as well as the ability to inhibit carcinogenesis (Mori et al., 1986; Rice-Evans et al., 1996; McDougall et al., 1998; Kweon et al., 2001; Dos Santos et al., 2006; Aron and Kennedy, 2008; Joseph et al., 2016). In addition to health benefits, phenolics as antioxidants are important ingredients in the food industry, aiding in extending product shelf-life by delaying undesirable free radical reactions such as lipid oxidation (Bonilla et al., 1999; Ahmad et al., 2015). The activity of an antioxidant is determined by a number of factors including their ability to donate hydrogens or electrons, the stability of the resulting radical, its reactivity with other antioxidants, and their transition metal-chelating potential (Rice-Evans et al., 1997).

There are a wide variety of analytical methods available to measure the antioxidant potential of compounds such as phenolics, but the most commonly used include the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the Trolox equivalence antioxidant capacity (TEAC) assay (Green, 2007). These *in vitro* methods are widely used due to their simplicity, short analysis time and low cost (Green, 2007). Both of these methods work by the generation of a synthetic free radical that is scavenged by the antioxidant resulting in a sample colour change that can be measured spectrophotometrically. For example, in the DPPH assay, the $DPPH^{\bullet}$ radical absorbs at 515 nm (A_{515}) and when it is scavenged by an antioxidant the absorbance decreases/disappears (Mishra et

al., 2012). Therefore, by monitoring the decrease in A_{515} over time (typically, 15 or 30 minutes) the effectiveness of an antioxidant can be measured (Sharma and Bhat, 2009; Mishra et al., 2012). Results from the DPPH assay are typically reported as the concentration of antioxidant required to scavenge 50% of the radicals in a set time period (Sharma and Bhat, 2009).

The TEAC assay works on a similar principle. In this method 2,2′-azinobis-3-ethylbenzthiazoline-sulfonic acid radical cations ($ABTS^{\bullet+}$) are generated by reaction with potassium persulfate. These radicals have an absorbance maximum at 734 nm and as they are scavenged by an antioxidant this absorbance is reduced. The effectiveness of an antioxidant is determined by comparison to Trolox, a vitamin E analog (Rice-Evans et al., 1996; Re et al., 1999).

With respect to their antioxidant activity, when all other factors (e.g. environmental) are kept constant, phenolic structure has the greatest impact on this function. As a compound class, phenolics can be potent antioxidants due to the presence of hydroxyl and aromatic functional groups, which aid in free radical stabilization (Heim et al., 2002). An example of the role of phenolic structure to antioxidant activity is illustrated by the lower TEAC values observed for phenolic acids when compared to flavonoids (Rice-Evans et al., 1996). The observed reduced antioxidant activity of phenolic acids was likely due to the presence of fewer hydroxyl groups and aromatic rings relative to flavonoids. Structure-activity studies showed that the antioxidant activity of phenolic acids is related to the number and location of the hydroxyl groups relative to the electron withdrawing carboxylic acid functional group (Rice-Evans et al., 1996).

While flavonoids are grouped into different subclasses based upon aglycone structure, it has been reported that hydroxyl group arrangement on the aglycone is a greater determinant of antioxidant activity than flavonoid subclass (Heim et al., 2002). Research has shown that the number of hydroxyl groups on the aglycone is positively correlated with free radical scavenging ability and glycosylation decreases free radical scavenging effectiveness (Cao et al., 1997; Burda and Oleszek, 2001; Pannala et al., 2001). The arrangement of the hydroxyl groups on the B-ring has also been found to have a significant effect on the antioxidant activity of flavonoids, while the arrangement of hydroxyl groups on the A-ring does not correlate well with antioxidant activity (Pannala et al., 2001; Heim, 2002). For example, having hydroxyl groups at both the 3′- and 4′-positions on the B-ring (i.e., a catechol structure) improves phenolic electron donating properties making it a target for free radicals (Pietta, 2000). However, the presence or absence of a free 3-OH (i.e. a hydroxyl group at the C3 position of the C-ring) substituent is one of the largest

determinants of the antioxidant activity of flavonoids (Burda and Oleszek, 2001). Flavonoids with a free 3-OH group show higher antioxidant activity when compared to flavonoids lacking a hydroxyl group at this position or those that are O-glycosylated at this position (Burda and Oleszek, 2001; Heim, 2002). It is postulated that the presence of the 3-OH group increases the stability of the flavonoid radical by forming an intramolecular hydrogen bond with hydroxyl group(s) on the B-ring, which result in an aligning of the B-ring with the A- and C-rings making the molecule more planar and improving electron delocalization (van Acker et al., 1996). When this hydrogen bond is absent, this planarity is lost between the B-ring and the rest of the flavonoid backbone hindering electron delocalization and thereby decreasing the stability of the flavonoid radical (van Acker et al., 1996).

2.4.3 Phenolic Compounds in Apple and Pear Fruit

Many different and often contradictory studies on the phenolic compound content of apples and pears and their juices exist in literature. These results may be explained by the roles that fruit variety, maturity and ripeness, growing conditions, storage and processing, and fruit product (e.g. juice) have on phenolic composition (Spanos and Wrolstad, 1992; Tanrıöven and Ekşi, 2005; Nayak et al., 2015).

The most commonly reported phenolics found in both apple and pear include: catechin, chlorogenic acid, epicatechin and various quercetin glycosides (Babsky et al., 1986; de Simón et al., 1992; Spanos and Wrolstad, 1992; Lu and Foo, 1997; Picinelli et al., 1997; Andrade et al., 1998; Lee et al., 2003; Sánchez et al., 2003; Tanrıöven and Ekşi, 2005; Díaz-García et al., 2013). In addition, numerous minor phenolics have been reported in apple and pear fruit and their products, based upon the analytical techniques and parameters employed and differences in the fruit/product itself (Escarpa and González, 1998; Kolniak-Ostek and Oszmiański, 2015).

Phenolics that are present solely in apple or in pear fruit are of major importance as they can act as marker compounds for the presence/absence of these fruits in products and therefore can be used for authenticity purposes. For example, arbutin is a major phenolic in pear that is not found in apple and has been used for the detection of the debasing of apple juice with pear juice (Andrade et al., 1998; Thavarajah and Low, 2006a; Willems and Low, 2014), while phloridzin has been reported to be a marker for apple (Andrade et al., 1998). Similarly, isorhamnetin-3-glucoside has also been proposed to be a potential pear marker for pear (de Simón et al., 1992; Schieber et al.,

2001). However, research has shown that phloridzin is not always present in apples/apple products, and isorhamnetin-3-glucoside was not detected in pear/pear products (Schieber et al., 2002; Alonso-Salces et al., 2004; Thavarajah and Low, 2006a), thus eliminating their use as authenticity markers.

2.4.4 Phenolic Analysis

The Folin-Ciocalteu (F-C) assay is a simple analytical method that is widely used to estimate the total phenolic content of food products. The F-C reagent consists of a mixture of sodium molybdate and sodium tungstate that react with phenols to produce a blue colour, which is measured at 765 nm (Everette et al., 2010). The absorbance at 765 nm is then related to the phenolic content by comparison to a standard, typically gallic acid (hydroxybenzoic acid class) and is reported as gallic acid equivalents or GAE per weight of sample. The major limitation of the F-C assay is that other reducing compounds in a sample such as vitamins and amino acids can artificially inflate the total phenolic content result (Everette et al., 2010).

Chromatographic methods are commonly employed for more accurate and detailed sample phenolic analysis. Reversed phase high performance liquid chromatography (RP-HPLC) coupled with either a photodiode array (PDA) detector or mass spectrometry (MS) is the preferred method used for accurate phenolic identification and quantitation. While there are numerous published HPLC methods for phenolic analysis, the stationary phase most commonly used is an end-capped C_{18}, typically ranging in column size from 100-300 mm in length and 4.6 mm in diameter with a particle size of 100 Å, coupled with gradient elution employing a polar acidified organic solvent (e.g. acetonitrile or methanol) as the mobile phase (Robbins, 2003; Thavarajah and Low, 2006a; Ignat et al., 2011; Khoddami et al., 2013). In some instances, samples can be directly analyzed by HPLC (i.e., liquids), but more commonly sample phenolics are extracted prior to analysis. The most common method for phenolic extraction is by organic-water solvent mixtures such as 70% aqueous ethanol or methanol. The choice of organic used in these solvent mixtures plays an important role in phenolic extraction efficiency as they are intermediate polarity compounds and solvent hydrophobicity can significantly impact their solubility. In addition, weak acids (i.e. formic acid) are often added to the solvent mixture, which results in phenolic protonation, which can increase phenolic solubility in the solvent mixture. However, if the acid concentration is too high, phenolic structure changes can occur due to hemiketal formation (e.g. anthocyanins) and/or

hydrolysis of glycosidic bonds, which impact their extraction properties and analysis (de Rijke et al., 2006; Garcia-Salas et al., 2010; Khoddami et al., 2013).

In addition to the use of solvents, solid phase systems have been used for phenolics extraction. As an example, phenolics are readily adsorbed onto Amberlite XAD-16 and Sepabeads SP70 resins, which are nylon-based hydrophobic materials and non-phenolics can be removed from the sample by washing with water, followed by phenolic elution with an organic solvent (e.g. ethanol or methanol) or fractionation with an organic-aqueous gradient solvent mixture (Li et al., 2001; Llorach et al., 2003).

2.5 Mass Spectrometry

2.5.1 General Background

Mass spectrometry (MS) is a widely applied analytical technique throughout the life sciences (Cuyckens and Claeys, 2004). It is commonly coupled with a separation technique such as high performance liquid chromatography (HPLC) or capillary gas chromatography (CGC) affording compound separation and increased structural information for the analytes of interest. Mass spectrometry involves the separation of analyte ions (positive or negative) in the gas phase based upon their mass-to-charge (*m/z*) ratio (Paré and Yaylayan, 1997).

Mass spectrometers are comprised of three major components, the ion source, mass analyzer and the detector. The ion source is responsible for converting the sample into a gas (unless it already is in the gas phase as is the case for CGC-MS) and imparting a charge on the analyte. A wide variety of ionization techniques are available and are used in commercial instruments and can be broadly divided into hard and soft ionization techniques. Hard ionization techniques, such as electron impact (EI), often result in the breakdown of complex biomolecules complicating analysis (Paré and Yaylayan, 1997). The introduction of soft ionization techniques in mass spectrometry minimize the amount of energy transferred to the analyte, which affords improved parent ion detection and the analysis of molecules with higher molecular masses (El-Aneed et al., 2009). Soft ionization techniques include plasma desorption (PD), fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). Among these, ESI is one of the most common as it is readily interfaced with liquid chromatography systems (Korfmacher, 2005).

In ESI, the sample is introduced as a solution through a thin needle to which an electric potential is applied resulting in the production of highly charged droplets (nebulization) (Cole, 2000; Kebrale, 2000). These droplets are then dried via a stream of inert gas, which results in the charges becoming closer to each other until the repulsive forces (or Coulombic forces) exceed that of the droplet surface tension causing the ions to desorb into the gas phase (Kebrale, 2000; El-Aneed et al., 2009). Additionally, for biomolecules (e.g. proteins), a second mechanism has been proposed where all of the solvent is evaporated by the gas leaving behind a single, multiply charged molecule (Kebrale, 2000; El-Aneed et al., 2009).

In addition to ion sources, a variety of different mass analyzers are available. In the mass analyzer, analyte ions are separated based upon their *m/z* value. Mass analyzers are often classified on their analyte resolution, with low resolution systems affording differentiation based on a single *m/z* value (i.e. unit resolution) and high resolution systems producing higher mass accuracy (e.g. four decimal places), which can be used to determine the exact chemical formula of an analyte. Common mass analyzers include the quadrupole (Q), which is a low resolution system, and time-of-flight (ToF), a high resolution system.

The quadrupole mass analyzer consists of four rods in parallel in a square arrangement. Ions are separated based upon their stability in oscillating electric fields applied to the rods. At a given ratio of voltages only ions with a specific *m/z* ratio will be able to reach the detector, other ions will be deflected into the rods. In this way, a quadrupole acts as an ion filter (El-Aneed et al., 2009; Clavete, 2013).

Time of flight (ToF) mass analyzers are used for high resolution analysis (Chernushevich et al., 2001). As the name suggests, this method is based upon the time an ion takes to reach the detector under the influence of an applied electric field (Clavete, 2013). Ions with higher *m/z* values take longer to reach the detector than those with lower *m/z* values under identical instrument conditions. In ToF, resolution can be affected by the kinetic energy of the ions entering the system, as not all ions of the same *m/z* will start with the same kinetic energy and as such they can reach the detector at different times (Clavete, 2013). This issue has been overcome through the use of a reflectron, which uses a constant electric field to deflect ions to the detector. Ions with higher kinetic energy will penetrate further into the reflectron and therefore, will take a longer path to the detector than those with low kinetic energy, improving resolution (El-Aneed et al., 2009). In

addition, the reflectron increases the total path length of a ToF system, which aids in analyte resolution.

2.5.2 Ion Mobility Mass Spectrometry

Ion mobility coupled with mass spectrometry (IMMS) is a rapidly growing research and application area due to its ability to separate mixtures of structurally similar compounds, including structural isomers and chiral compounds (Dwivedi et al., 2006; Kanu et al., 2008). Ion mobility is interfaced between the ion source and the mass analyzer(s) and separates gas-phase ions based upon their mobility in a gas under the influence of an electric field (Verbeck et al., 2002; Kanu et al., 2008). This technique affords the separation of ions based upon their shape and size in addition to their mass and charge (Armenta et al., 2011).

While there are a variety of ion mobility techniques available, one of the most common is high-field asymmetric waveform ion mobility spectrometry (FAIMS) (Purves et al., 2014). In this technique, ions are separated based upon differences in their mobility in a strong and weak electric field (Guevremont, 2004). Ions are introduced between two metal plates in conjunction with a carrier gas such as helium or nitrogen (Verbeck et al., 2002). The alternating high/low electric field results in sample ions moving through the system to deflect towards one of the plates (Purves and Guevremont, 1999; Purves et al., 2014). A compensation voltage (CV) must be applied in this system in order for the separated analyte ions to reach the detector. The magnitude of the CV required varies from ion to ion and changing the CV allows for select ions (i.e. analytes) to reach the detector (Purves and Guevremont, 1999; Guevremont, 2004; Purves et al., 2014).

2.5.3 Phenolic Analysis by Mass Spectrometry

Phenolics have been widely studied using mass spectrometry, most commonly through LC-MS with reversed phase HPLC. Based on the combined sensitivity and selectivity of MS, little to no sample preparation for sample phenolics analysis is required (Cuyckens and Claeys, 2004). For solid samples, LC-MS analysis is usually preceded by homogenization or solvent extraction, whereas liquid samples are often analyzed directly following filtration (Cuyckens and Claeys, 2004).

While phenolics have been analyzed by MS in both the positive and negative ion modes, the highest sensitivity has been reported to be in the negative mode (de Rijke et al., 2003). Analysis

in the negative ion mode also results in less overall analyte fragmentation when compared to the positive ion mode, making molecular weight determination and analysis of phenolics in low concentrations easier (de Rijke et al., 2003). However, the increased fragmentation of phenolics observed in the positive ion mode offers additional structural information (Abad-García et al., 2009). With MS fragmentation analysis, structural information on the nature of the aglycone, the presence or absence of a carbohydrate or other substituent and its nature (i.e., mono- or disaccharide, pentose or hexose) can be elucidated (Abad-García et al., 2009). As an example, the main fragmentation of *O*-glycosylated flavonoids in both the positive and negative ion modes is the neutral loss of the carbohydrate moiety, with the mass difference between the molecular ion and the aglycone fragment providing important information on the carbohydrate moiety (Abad-García et al., 2009). For instance, a loss of 162 is indicative of a hexose while 132 would indicate the presence of a pentose.

3. AUTHENTICITY ANALYSIS OF PEAR JUICE EMPLOYING CHROMATOGRAPHIC FINGERPRINTING[1]

3.1 Abstract

Pear juice is predominately comprised of carbohydrates/polyols (>95% of the total soluble solids) making it susceptible to adulteration by the addition of less expensive commercial sweeteners. In this research, the major carbohydrate and polyol (fructose, glucose, sucrose and sorbitol) content of thirty-two pure pear juices representing five world producing regions and three years of production was determined. Additionally, methods employing oligosaccharide profiling to detect the debasing of these samples with four commercial sweeteners (HFCS 55 and 90, TIS and HIS) were developed using capillary gas chromatography with flame ionization detection (CGC-FID) and high performance liquid chromatography with pulsed amperometric detection (HPAE-PAD). Detection limits for the four commercial sweeteners ranged from 0.5 to 5.0% (v/v). In addition, the developed CGC-FID method could be used to: (a) detect the addition of pear to apple juice via arbutin detection; and (b) determine if a pear juice was produced using enzymatic liquefaction via the presence of O-β-D-glucopyranosyl-(1→4)-D-glucopyranose (cellobiose); all within a single chromatographic analysis.

3.2 Introduction

In 2012, world pear production was approximately 23.5 million metric tons, of this approximately 20 to 30% is subsequently processed into juice/concentrate (Thavarajah, 2004; USDA, 2012; FAOSTAT, 2014). Pear fruit production in 2013-14 in North America and Asia was down significantly; for example, the overall crop season shortage was 50-60%, which has resulted in less fruit being available in cold storage (e.g. for use in juice production) and price increases of up to 35-40% (Gingsberg's Foods, 2014; Tree Top Fruit Ingredients, 2014).

[1]Reproduced with permission. Willems, J. L. and Low, N. H. (2014). Authenticity analysis of pear juice employing chromatographic fingerprinting. *Journal of Agricultural and Food Chemistry*, 62: 11737-11747. Copyright (2014) American Chemical Society.

Due to its high market value, the adulteration of fruit juice is a common problem, which can lead to unscrupulous producers succeeding in the market place at the expense of consumers and honest producers. The current and expected situation with low pear fruit production, high fruit prices and the fact that supplier contracts for juice are negotiated in advance, has the possibility of increasing the likelihood of current and future pear juice adulteration.

The simplest and least expensive method of juice adulteration is dilution with water. This form of adulteration is simple to detect using a refractometer to determine the °Brix value of the juice which should match the industry standard of 12.0 °Brix for pear juice (Bates et al., 2001).

Due to the high carbohydrate content of fruit juices (>95% of the total soluble solids), a common adulteration method is the addition of readily available less expensive nutritive sweeteners (e.g., high fructose corn syrup; HFCS). One of the earliest methods developed for detecting this type of adulteration is the matrix approach (Thavarajah and Low, 2006a). Adulterated samples are identified by comparing their chemical composition (e.g. major carbohydrates, organic acids, minerals) to the mean and range values of authentic samples. This method is limited by the addition of sweeteners or sweetener combinations whose major or combined carbohydrate profile closely resembles that of the pure juice, and by the large amount of natural variation found in authentic products (Wallrauch and Faethe, 1988). Also, adulterated product detection employing this method is not able to identify the adulterant(s) used and as such it is referred to as an untargeted method.

Because of these limitations, more advanced adulteration detection techniques have been developed. One method which has been applied for fruit juice authenticity determination is stable isotope ratio analysis (SIRA). This technique is based upon the principle that naturally occurring elements exist in different isotopic forms and the distribution of these isotopes in the chemical compound profile of a fruit juice depends upon a variety factors, such as growing region, temperature and photosynthetic pathway (Perez et al., 2006). Food authenticity analysis employing this technique focuses on the distribution of hydrogen, carbon and oxygen isotopes which can be measured by two techniques, namely site-specific natural isotope fractionation nuclear magnetic resonance (SNIF-NMR) and isotope ratio mass spectrometry (IRMS) (Krueger, 1988; Reid et al., 2006). For example, the natural variation in the $^{13}C/^{12}C$ ratio in plants which use different photosynthetic pathways for carbon dioxide fixation has been applied to detect the adulteration of fruit juices with certain sweeteners (Krueger, 1988). The two major photosynthetic routes for

plants are the Calvin cycle pathway (C_3) and the Hatch-Slack pathway (C_4) (Doner, 1988; Krueger, 1988). Because the difference in the $^{13}C/^{12}C$ ratio between the C_3 and C_4 pathways is statistically significant, analytical results derived from this assay can be used to detect the addition of a C_4 plant based product (e.g. cane sugar or HFCS) to a C_3 plant based product (e.g. apple juice) (Doner, 1988; Pupin et al., 1998). Although no publications exist on the direct application of this technique to pear, it is hypothesized that this method could be applied as pear is a C_3 plant and its $^{13}C/^{12}C$ ratio is very similar to that of apple (Krueger, 1998). This technique is limited by its detection limit of approximately 10-15% for C_4-based sweeteners (e.g. HFCS), and its inability to detect the addition of beet sugar or inulin derived syrups as these are produced from C_3 (Jerusalem artichoke and chicory) and Crassulacean acid metabolism (CAM) pathway plants (agave), respectively (Nobel and Hartsock, 1978; Krueger, 1988; Kelly and Downey, 2005; Yan et al., 2012). In addition, this technique requires the use of specialized and expensive equipment that limits its routine application.

Another method for the detection of fruit juice adulteration is by oligosaccharide profiling. This technique works by identifying oligosaccharides which are present in the adulterants but are not naturally occurring in the product of question (Low, 1998). One of the benefits of this method is that it is a targeted approach; that is, it can be used to not only determine whether a product has been adulterated but also to identify which adulterant was used. The two most common techniques used for oligosaccharide profiling are high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) and capillary gas chromatography with flame ionization detection (CGC-FID). Both HPAE-PAD and CGC-FID methods have been developed and successfully used to detect the debasing of foods rich in carbohydrates such as agave syrup, honey and maple syrup, as well as in select fruit juices such as apple and grapefruit (Low and Wudrich, 1993; Stuckel and Low, 1995; Low et al., 1999; Morales et al., 2008; Willems and Low, 2012).

To date, no literature reports on the application or development of analytical techniques to detect the debasing of pear juice with less expensive nutritive sweeteners exists. Therefore, the primary goal of this research was to develop an analytical method employing oligosaccharide profiling that would be able to detect the debasing of pear juice at low concentrations (5-10%) with a series of commercially available carbohydrate syrups including, HFCS, hydrolyzed inulin syrup (HIS) and total invert sugar (TIS) in a single chromatographic analysis. In addition, a

secondary research goal was to expand the developed analytical method to detect select juice-to-juice adulteration (i.e. pear to apple) and to detect juices produced using a total fruit liquefaction process.

3.3 Materials and Methods

3.3.1 Samples

Thirty-two pure commercial pear juice concentrates (~70 °Brix) representing three production years (2012-14) and five world production regions (Argentina; Chile; China; New Zealand; United States of America) were analyzed in this study. Commercial nutritive sweeteners used in this study as debasing agents (i.e., adulterants) included high fructose corn syrup (HFCS 55 and 90; CASCO, Canada), hydrolyzed inulin syrup (HIS; Raffinerie Tirlemontoise S. A., Belgium) and total invert sugar (TIS; Lantic Sugar Ltd., Canada).

3.3.2 Chemicals

Arbutin, D-fructose, D-glucose, β-glucosidase (from almond), isomaltose, maltoheptaose, maltohexaose, maltopentaose, maltose, maltotetraose, maltotriose, sodium hydroxide (NaOH) solution (50% w/w), D-sorbitol, D-sucrose and Sylon TP (TMSI + pyridine, 1:4) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Sodium acetate (NaOAc) was obtained from Fisher Scientific (Ottawa, ON, Canada). The water used throughout this study was obtained from a Millipore Milli-Q™ water system (Millipore Corporation, Milford, MA, USA).

3.3.3 Major Carbohydrate and Polyol Analysis by High-Performance Liquid Chromatography with Refractive Index Detection (HPLC-RI)

Major carbohydrate and polyol analysis was carried out employing an Agilent 1100 series HPLC system with a refractive index detector controlled by ChemStation LC-3D software (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Samples were initially diluted to 12.0 ± 0.1 °Brix (Auto Abbe Refractometer; Leica Inc., Buffalo, NY, USA) before being further diluted 1:1 with water. Samples were syringe filtered (nylon, 0.2 µm pore size; 13 mm diameter; Chromatographic Specialties, Brockville, ON, Canada) prior to analysis. Carbohydrate separation was accomplished on a Capcell-Pak-NH_2 column (250 x 4 mm, 5 µm; Phenomenex, Torrance, CA) in series with a guard column (4 x 3 mm) of the same stationary phase. The mobile phase

consisted of acetonitrile/water (75:25, v:v) run isocratically at a flow rate of 1.0 mL/min. The injection volume was 5.0 µL and the column and RI detector were maintained at 25 °C and 35 °C, respectively. Standard curves for fructose, glucose, sorbitol and sucrose were prepared in water at concentrations ranging from 0.06 to 5.00 g/100.00 mL. Standard curves had R^2 values of 0.999 or greater. All samples and standards were analyzed in triplicate.

3.3.4 Arbutin Analysis by High-Performance Liquid Chromatography with Photodiode Array Detection (HPLC-PDA)

Samples were initially diluted with water to 12.0 ± 0.1 °Brix before being further diluted 1:1. Arbutin (4-hydroxyphenyl-β-D-glucopyranoside) content in the pear juice samples was determined employing HPLC with photodiode array (PDA) detection (Agilent Technologies Canada Inc.). Samples were syringe filtered prior to analysis. Arbutin separation from other sample phenolics was carried out employing an ODS-3 (250 × 4.6 mm; 5 µm C_{18}, 100 Å) column (Phenomenex) in series with a guard column (4 x 3 mm) of the same stationary phase. A gradient program employing aqueous 10 mM formic acid (pH 3.5; mobile phase A) and 70% acetonitirile:30% solvent A (v:v; mobile phase B) was used for arbutin separation as follows: initial 100% A for 3.0 min, followed by a linear gradient to 4.0% B at 6.0 min, followed by a linear gradient to 7.0% B at 15.5 min, followed by column washing with 100% B for 10.0 min prior to re-equilibration with 100% A. The flow rate was 0.8 mL/min and the injection volume was 20.0 µL. Arbutin detection was afforded by PDA at a wavelength of 280 nm. A standard curve for arbutin prepared in water at concentrations ranging from 5.0 to 110.0 µg/mL was employed for quantification. Standard curves had R^2 values of 0.999 or greater. All samples and standards were analyzed in triplicate.

3.3.5 Pure and Intentionally Adulterated Pear Juice Oligosaccharide and Arbutin Analysis by Capillary Gas Chromatography with Flame Ionization Detection (CGC-FID)

Pure pear juice concentrates and debasing agents were individually prepared by dilution with water to 5.5 ± 0.1 °Brix. For pure pear juice samples a 150 µL aliquot of each sample was freeze dried (Heto Lab Equipment, Allerod, Denmark) in a 12 x 32 mm glass vial (Chromatographic Specialties) for one hour. To the resulting dried foam 500 µL of Sylon TP was added using a gas-tight glass syringe and the vials were capped and heated in a block heater

(Denville Scientific Inc., Metuchen, NJ, USA) at 70 °C for one hour with shaking every 10-15 minutes. Selected pear juice samples were intentionally adulterated with equivalent soluble solid content solutions of HFCS 55 and 90, HIS and TIS at levels of 15.0, 10.0, 5.0 and 2.0% (v:v) and prepared as outlined previously. Sample oligosaccharide analysis was carried out using an Agilent 6890 gas chromatograph equipped with an Agilent 6890 series injector autosampler (Agilent Technologies Canada Inc.). Samples were analyzed in the splitless mode using ultrapure hydrogen as the carrier gas at a constant flow rate of 1.5 mL/min. Ultrapure nitrogen was used as the makeup gas at a flow rate of 30.0 mL/min. The injection port temperature was maintained at 250 °C and the detector at 300 °C. Oligosaccharide separation was afforded using an Agilent J&W DB-5 (95% dimethyl-5% diphenyl polysiloxane; 30 m x 0.25 mm, 0.25 μm film thickness) open tubular fused-silica capillary column (Chromatographic Specialties). The injection volume was 3.0 μL. Data acquisition and processing was carried out using Agilent ChemStation Rev. A.06.03 software (Agilent Technologies Canada Inc.). The following temperature profile was used for oligosaccharide and arbutin analysis: initial temperature of 210 °C for 10.0 min; 0.55 °C/min from 210 to 235 °C; hold at 235 °C for 5.0 min; 30 °C/min from 235 to 295 °C; hold at 295 °C for 12.0 min. The total run time was 74.45 minutes. The minimum qualitative detection limit for arbutin was determined as the concentration that would give a peak height equivalent to 3x the signal-to-noise ratio (s/n).

3.3.6 Pure and Intentionally Adulterated Pear Juice Oligosaccharide and Arbutin Analysis by High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD)

Pure pear juice samples and debasing agents were individually prepared by dilution with water to 5.5 ± 0.1 °Brix followed by syringe filtration. Select pear juice samples were intentionally adulterated with equivalent soluble solid content HFCS 55 and 90, HIS and TIS solutions at levels of 20.0, 10.0 and 5.0% (v:v). For pear juice intentionally adulterated with HIS a further dilution (1:3) with water was required. Sample oligosaccharide analysis was carried out on a Dionex Bio LC 4000 gradient HPLC system (Dionex Corp., Sunnyvale, CA, USA) containing a 50.0 μL sample loop. Carbohydrate separation was performed using two Dionex CarboPac PA1 (Thermo Fisher Scientific) columns (4 × 250 mm) connected in series without a guard column. The flow rate was 0.7 mL/min and the carbohydrates were detected using a gold electrode employing triple

pulsed amperometry at a sensitivity of 10K. The electrode was maintained at the following potentials and durations: E_1 = 0.05 V, t_1 = 120 ms; E_2 = 0.80 V, t_2 = 120 ms; E_3 = -0.60 V, t_3 = 420 ms. The gradient mobile phase consisted of 160 mM NaOH (mobile phase A), 160 mM NaOH/1 M NaOAc (mobile phase B) and 1 M NaOH (mobile phase C) with the following linear gradient elution program: initial conditions of 96.0% A and 4.0% B for 15.0 min; gradient to 70.0% A and 30.0% B at 41.0 min; 100% B from 41.1 min to 44.0 min; 100% C from 44.1 min to 48.0 min. The system was re-equilibrated at the initial conditions for 12.0 minutes prior to the next injection. Data acquisition and analysis was carried using Chromatography Data Station software (Wei Ma Long Chromatographic Techniques Ltd., Nanning, China).

3.3.7 Arbutin Identification by Capillary Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatographic conditions were as described above for CGC-FID analysis, with the exception of the use of ultrapure helium as the carrier gas. Mass spectrometry was carried out on a VG Analytical 70SE mass analyzer (VG Instruments Canada Inc., St. Laurent, QC, Canada) operated in the electron impact (EI) mode at an ionization energy of 70.0 eV. The source temperature was set at 200.0 °C. Measurements were carried out in full scan mode with scanning from *m/z* 50.0 to *m/z* 650.0. A solvent delay of 10.0 minutes was used. Samples of arbutin and select pure pear juices were prepared as outlined previously for CGC-FID analysis.

3.3.8 Arbutin Hydrolysis with *β*-Glucosidase

Three pure pear juices representing low (<64.9 µg/mL; PJ 28), medium (65.0 to 79.9 µg/mL; PJ 15) and high (>80.0 µg/mL; PJ 27) concentrations of arbutin were selected for analysis. These juice concentrates were diluted to 5.5 ± 0.1 °Brix and approximately 10 U/mL of β-glucosidase was added to each sample. The solutions were stirred at ambient temperature and samples were taken after 16 and 24 hours. The enzyme reaction was quenched by placing the samples in boiling water for 5 minutes. Samples were syringe filtered and analyzed by CGC-FID and HPAE-PAD as outlined previously. Samples of the same juices without enzyme addition were also analyzed as controls.

3.3.9 Preparation of a Multiply Adulterated Pear Juice Sample with Cellobiose

A commercial pear juice concentrate (PJ 7) was diluted with water to 5.5 ± 0.1 °Brix and HFCS 55, HIS and TIS (at the same °Brix) were added at levels of 10.0, 3.0 and 10.0% (v:v), respectively, and cellobiose was added to yield a final concentration of 50.0 ppm. The sample was then freeze dried, derivatized and analyzed by CGC-FID as described previously.

3.4 Results and Discussion

3.4.1 Major Carbohydrate and Sorbitol Analysis

The major carbohydrates and polyol in pear juice have been reported to be fructose, glucose, sucrose and sorbitol, respectively (Wallrauch and Faethe, 1988; Thavarajah and Low, 2006a). Together, these compounds comprise greater than 95% of the total soluble solids found in pear juice. The major carbohydrate/polyol composition plays an important role in the nutritional and sensory attributes of fruit juices such as pear. In addition, their concentrations and ratios have been used to determine the authenticity of a variety of fruit juices (Low and Wudrich, 1993).

The concentrations of fructose, glucose, sucrose and sorbitol in the thirty-two commercial pear juice samples used in this study were determined by normal phase high performance liquid chromatography with refractive index detection (HPLC-RI). The mean, range, standard deviation and fructose/glucose (F/G) ratio results for each of these major pear juice components are reported in Table 3.1. Comparisons of these values for each of the major carbohydrates were within the literature reported range (adjusted to 12.0 °Brix) of: 4.59-8.89% (w/v) for fructose; 0.76-3.90% (w/v) for glucose; and 0.18-3.70% (w/v) for sucrose (Wallrauch and Faethe, 1988; Low and Wudrich, 1993; Thavarajah and Low, 2006a; Morales et al., 2008). The maximum concentration of 3.25% (w/v) observed for sorbitol in this study was greater than that reported in literature for this compound of 2.96% (w/v) (Thavarajah and Low, 2006a).

The observed high content, and the wide natural concentration ranges for each of these carbohydrates and sorbitol makes pear juice susceptible to adulteration through debasing with commercial sweeteners of lower economic value. Four commercial sweeteners, high fructose corn syrup (HFCS 55 and 90), hydrolyzed inulin syrup (HIS) and total invert sugar (TIS), were chosen as potential debasing agents for pear juice based on both their lower economic value and their natural carbohydrate content. The major carbohydrate concentrations and F/G results for each of the commercial sweeteners used as debasing agents in this study are shown in Table 3.2. Based on

these values, it is clear that the debasing of commercial pear juices analyzed in this study with any of the analyzed commercial sweeteners on their own or in combination at appreciable (15-30%) levels would not significantly change the major carbohydrate and sorbitol profiles present in the adulterated juices and would also maintain the industry standard final °Brix value (Table 3.3). As examples, (a) the addition of 30% HIS to a pear juice with average major carbohydrate and sorbitol concentrations (PJ 9); and (b) the addition of 10% TIS or 10% HFCS 90, or 15% TIS + 15% HFCS 90 to PJ 14, would all result in calculated final glucose, fructose, sucrose and sorbitol concentrations that would all fall within the natural ranges (Tables 3.1 and 3.3) for these compounds as determined in this study. These results show that the limiting factor in the amount of debasing agent that can be added without resulting in a value below the published minimum is the sorbitol concentration of the starting juice as none of the commonly used commercial sweeteners contain this compound. Therefore, unscrupulous producers may be able to disguise even higher amounts of debasing by the addition of exogenous sorbitol.

It has been suggested that the fructose-to-glucose ratio is a better indicator of pear juice authenticity as it is more constant than absolute concentrations (Wallrauch and Faethe, 1988). As such, a fructose-to-glucose ratio of >2.7 with a lower limit of 2.4 has been proposed as the indicator for pear juice authenticity (Thavarajah and Low, 2006a). In this study, 28 of the 32 commercial pear juices showed fructose-to-glucose ratios greater than 2.7; the remaining four samples, which had F/G ratios ranging from 2.1 to 2.4, originated from China. Of the four commercial sweeteners analyzed in this study, two (HFCS 90 and HIS) had fructose-to-glucose ratios greater than the lower limit for authentic pear juice of 2.4. As such, pear juice debasing with either of these commercial sweeteners would maintain the major carbohydrate and sorbitol levels within the natural range and would also result in F/G ratios >2.7. The same results would be observed if blends of debasing agents were used such as HIS:TIS, where a 7:1 ratio of these specific samples would result in a calculated F/G ratio of at least 2.7.

Table 3.1 Mean, range and standard deviation results for fructose, glucose, sucrose, sorbitol, and arbutin concentrations and fructose/glucose ratios in 32 commercial pear juice samples (n=3).

Sample	Country of Origin	Fructose (g/100mL)	Glucose (g/100mL)	Sucrose (g/100mL)	Sorbitol (g/100mL)	F/G Ratio[a]	Arbutin (µg/mL)
1	Argentina	5.48	1.78	0.64	2.56	3.1	68.6
2	Argentina	5.41	1.92	0.56	2.57	2.8	70.3
3	Argentina	6.04	1.83	0.33	2.34	3.3	69.0
4	Argentina	5.57	1.92	0.60	2.55	2.9	68.4
5	Argentina	5.69	1.92	0.58	2.59	3.0	67.0
6	Argentina	5.47	1.71	0.50	2.41	3.2	66.1
7	Argentina	5.52	1.93	0.54	2.72	2.9	67.5
8	Argentina	5.54	1.94	0.59	2.79	2.9	69.3
9	Argentina	5.44	1.83	0.66	2.58	3.0	73.3
10	Argentina	5.58	1.77	0.54	2.53	3.2	62.1
11	Argentina	5.61	1.96	0.52	2.70	2.9	64.7
12	Chile	5.85	2.03	0.23	2.62	2.9	72.3
13	Chile	5.71	1.98	0.21	2.59	2.9	77.5
14	Chile	5.79	2.04	0.20	2.53	2.8	77.0
15	Chile	5.98	1.97	0.19	2.58	3.0	73.3
16	Chile	5.84	2.03	0.77	2.38	2.9	83.1
17	Chile	5.97	1.99	0.24	2.52	3.0	74.5
18	Chile	5.72	1.99	0.26	2.60	2.9	75.0
19	China	5.26	2.32	0.62	2.98	2.3	128.7
20	China	5.97	2.50	0.74	1.73	2.4	286.9
21	China	4.88	2.35	0.80	3.25	2.1	114.0
22	China	4.97	2.28	0.37	3.19	2.2	223.7
23	N. Zealand	6.32	1.29	1.45	1.99	4.9	69.9
24	N. Zealand	6.19	1.08	1.35	2.72	5.7	64.9
25	N. Zealand	6.86	1.03	1.10	2.60	6.7	58.8
26	N. Zealand	6.48	1.37	0.47	2.41	4.7	134.0
27	N. Zealand	6.44	1.22	0.50	2.37	5.3	128.8
28	USA	5.80	1.80	0.68	2.51	3.2	53.7
29	USA	5.80	1.79	0.63	2.50	3.2	50.6
30	USA	5.79	1.76	0.55	2.44	3.3	60.7
31	USA	5.94	1.90	0.51	2.50	3.1	64.8
32	USA	5.92	1.83	0.52	2.54	3.2	57.1
Mean		5.78	1.85	0.58	2.56	3.3	86.7
SD		0.41	0.34	0.29	0.28	1.0	49.6
Range		4.88 - 6.86	1.03 - 2.50	0.19 - 1.45	1.73 - 3.25	2.1 - 6.7	50.6 - 286.9

[a]Fructose-to-glucose ratio.

Table 3.2 Mean, range and standard deviation results for fructose, glucose, sucrose and sorbitol concentrations and fructose/glucose ratios in the four commercial nutritive sweeteners used in this study (n=3).

	Fructose (g/100mL)	**Glucose (g/100mL)**	**Sucrose (g/100mL)**	**Sorbtiol (g/100mL)**	**F/G Ratio[a]**
HFCS[b] 55	7.62	4.07	ND[c]	ND	1.9
HFCS 90	10.99	0.81	ND	ND	13.5
HIS	8.21	2.54	ND	ND	3.2
TIS	5.16	5.58	1.29	ND	0.9

[a]Fructose-to-glucose ratio.
[b]HFCS, high fructose corn syrup; HIS, hydrolyzed inulin syrup; TIS, total invert sugar.
[c]Not detected, <0.02 g/100 mL.

Table 3.3 Calculated[a] fructose, glucose, sucrose and sorbitol concentrations, and fructose/glucose ratios of select pear juices (9 and 14) with the four debasing agents.

	Fructose (g/100 mL)	**Glucose** (g/100 mL)	**Sucrose** (g/100 mL)	**Sorbitol** (g/100 mL)	**F/G Ratio[b]**
PJ[c] 9	5.44	1.83	0.66	2.58	3.0
PJ 9 + 30% HIS	6.27	2.05	0.46	1.81	3.1
PJ 14	5.79	2.04	0.20	2.53	2.8
PJ 14 + 10% TIS	5.73	2.40	0.31	2.27	2.4
PJ 14 + 10% HFCS 90	6.31	1.92	0.18	2.27	3.3
PJ 14 + 15% TIS + 15% HFCS 90	6.48	2.39	0.33	1.77	2.7

[a]Calculated amounts based on the analytically determined concentrations at equivalent soluble solids.
[b]Fructose-to-glucose ratio.
[c]PJ: Pear juice.

It should also be noted that three of the four samples from China (PJ 19, 21 and 22) had sorbitol concentrations at the upper end of the natural sorbitol range as determined by this study (Table 3.1). This indicates that greater amounts of debasing can occur in these samples without their sorbitol concentration dropping below the lower natural sorbitol level. As well, all four of the Chinese pear juices had fructose-to-glucose ratios at or below the lower limit for authentic samples (2.4) due to high glucose concentrations and/or lower than average fructose concentrations (Table 3.1). This indicates that care must be taken to avoid false positives when applying the F/G ratio for pear juice authenticity analysis, especially to samples originating from China or to samples of unknown origin.

Based on the potential issues previously discussed and the data presented in Table 3.3, it is clear that the untargeted approach to pear juice adulteration detection based on major carbohydrate and sorbitol concentrations, ranges and select ratios (i.e. F/G) is not a reliable means for pear juice

authenticity analysis and that there is a significant need for a more targeted approach to detect the debasing of this juice with common commercial sweeteners.

A more targeted approach to detecting the adulteration of foods rich in carbohydrates is oligosaccharide fingerprinting (Low and Wudrich, 1993; Stuckel and Low, 1995; Low et al., 1999; Morales et al., 2008; Willems and Low, 2012). This method is based on the formation of oligosaccharides in commercial sweeteners by acid-reversion and/or transglycosylation reactions that are unique to each of the identified debasing agents. Based upon this premise, the natural oligosaccharide profiles of the thirty-two pure commercial pear juice concentrates and the four identified debasing agents used in this study were determined by capillary gas chromatography with flame ionization detection (CGC-FID) and high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD).

3.4.2 Pear Juice Adulteration with High Fructose Corn Syrup

High fructose syrups are produced from a number of different starting materials throughout the world, including a variety of starches isolated from rice, wheat, tapioca and potato (Hanover and White, 1993). However, the most common substrate used for the production of high fructose syrups is corn starch (Hanover and White, 1993). The commercial production of high fructose corn syrup (HFCS) from starch normally involves both acid and enzymatic hydrolysis. Starch is first treated with acid (typically hydrochloric acid) to produce a reduced viscosity syrup with low amounts of reducing sugars (low dextrose equivalent, DE). This syrup is then treated with α-amylase (EC 3.2.1.1), glucoamylase (EC 3.2.1.3) and glucose isomerase (xylose isomerase; EC 5.3.1.18) (Low, 1998). Alpha-amylase and glucoamylase are added first to break down the remaining starch into free glucose molecules forming a monosaccharide rich syrup. Some of the glucose in this syrup is then isomerized to fructose using glucose isomerase (Hanover and White, 1993; Low, 1997). Theoretically, the highest concentration of fructose possible from a typical glucose stream is 47% at equilibrium. However, most processing plants settle for yields around 42% (HFCS 42) due to the amount of enzyme and reaction time needed to produce higher fructose concentrations (Hanover and White, 1993). To achieve higher concentrations of fructose in the final product the HFCS 42 syrup is fractionated using a cation exchange resin to separate fructose from glucose and other carbohydrates (Hanover and White, 1993). The fructose and glucose

fractions are then blended in the appropriate proportions to obtain a product with the desired fructose concentration (e.g. HFCS 55 or 90).

During the production of high fructose corn syrup a number of oligosaccharides are formed including maltose and isomaltose along with a variety of higher molecular weight glucose polymers (Low, 1998). The concentration of these oligosaccharides can be significant. For example, HFCS 42 and 55 have been reported to contain 5 and 3% oligosaccharides, respectively (Hanover and White, 1993). This is due to the incomplete hydrolysis of starch, as well as the lack of a debranching enzyme (i.e. isoamylase/pullulanse) which can hydrolyze the α-(1→6) glycosidic bonds in starch (Low, 1997; Low, 1998). It is also possible that oligosaccharide formation can occur via the transglycosylation activity of the carbohydrases employed in HFCS production. One method to detect the adulteration of pear juice with high fructose corn syrup is to look for the presence of these oligosaccharides, which can act as a chemical fingerprint facilitating the identification of this sweetener. A CGC-FID method has been previously developed and successfully used for the detection of high fructose corn syrup in apple juice at low levels via the detection of isomaltose (Low et al., 2001). However, when the published protocol/temperature program was applied to pear juice it was found that this method either gave a false positive for HFCS due to the presence of a naturally occurring compounds in pure pear juice which eluted at retention times similar to that of isomaltose, or indicated that isomaltose was a natural constituent of pear juice. To test these hypotheses, a new CGC-FID carbohydrate temperature program was developed that separated the interfering compound(s) that co-eluted with β-isomaltose. This clearly showed that isomaltose was not a natural constituent of pear juice, and established that the debasing of pear juice with HFCS could be detected by oligosaccharide fingerprinting employing the developed temperature gradient program. These results are illustrated in Figure 3.1 where chromatogram A shows the CGC-FID carbohydrate profile of pure pear juice (PJ 7) and chromatogram B shows the same juice intentionally adulterated with 5.0% (v:v) HFCS 55. The *-symbol in Figure 3.1 corresponds to an unknown compound(s) that co-elutes with α-isomaltose, and the arrow identifies β-isomaltose with retention times (RT) of 51.6 and 57.5 minutes, respectively. As supported by the lack of both α- and β-isomaltose peaks, isomaltose was not found in any of the analyzed pure pear juice samples at a detection limit of 6.5 ppm, therefore its presence in pear juice would be indicative of adulteration with HFCS and/or a glucose/maltose syrup. Maltose is also formed during the production of HFCS and is a potential fingerprint compound for

adulteration detection. However, under the temperature program conditions employed in this study maltose eluted in a region of the chromatogram where pear juice is naturally rich in compounds which interfered with its detection. The detection limit for HFCS 90 in pear juice by CGC-FID was determined to be 2.0% (v/v) based on β-isomaltose detector response and the 3x the signal-to-noise ratio detection limit used throughout this study. The concentration of isomaltose in HFCS 55 is >1.5 times that found in HFCS 90 (Willems and Low, 2012), resulting in a lower detection limit (1.0%) for this commercial sweetener. The third sweetener in this class, HFCS 42, was not analyzed in this study due to the fact that its fructose to glucose ratio of approximately 0.7 is considerably lower than that observed in this study for pure pear juices at ≥2.1, and the other commercial sweeteners used in this research. In addition, HFCS 42 has been shown to contain higher concentrations of oligosaccharides such as isomaltose than either HFCS 90 or 55 (Hanover and White, 1993; White, 2008) making its undeclared addition to pear juice even easier to detect by the developed CGC-FID method.

The detection of intentionally added HFCS to pear juice by HPAE-PAD posed a significant challenge due to the high monosaccharide content of these samples, which obscured not only the isomaltose peak (RT = 8.5 minutes), but also that of maltose (RT = 15.2 minutes). However, the excellent resolving power and low detection limits of high molecular weight glucose polymers by HPAE-PAD could readily detect the adulteration of pear juice with HFCS. These results are shown in Figure 3.2 where chromatogram A is of pure pear juice (PJ 23) and chromatogram B is the same pear juice intentionally adulterated with 10.0% (v:v) HFCS 55. We employed the peak eluting at approximately 40.2 minutes for adulteration detection based on its relative intensity. This peak was tentatively identified as maltohexaose (DP 6) based on retention time comparison to glucose polymer standards (DP 2-7; maltose to maltoheptaose) analyzed under the same conditions. Under the experimental conditions used in this study the detection limit for HFCS 55 by HPAE-PAD was determined to be 3.0% (v:v). Due to the fractionation and blending involved in the production of HFCS 90, the concentration of the DP6 marker is approximately 40 to 50% lower than in HFCS 55 resulting in a higher detection limit of 5.0% (v:v).

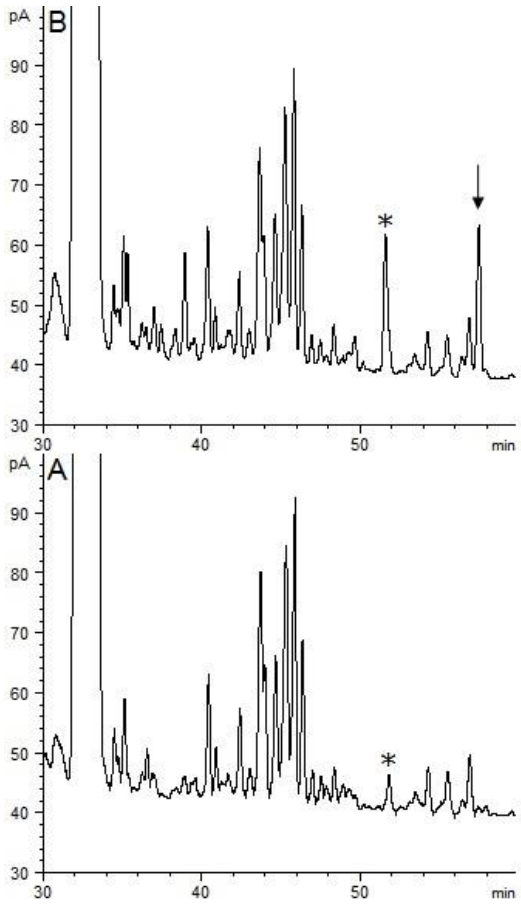

Figure 3.1 CGC-FID chromatograms from 30.0 to 60.0 min of (A) pure pear juice (PJ 7) and the same juice adulterated with (B) 5.0% (v/v) HFCS 90. The compound marked with * corresponds to a naturally occurring oligosaccharide that co-elutes with α-isomaltose and the arrow indicates β-isomaltose.

3.4.3 Pear Juice Adulteration with Total Invert Sugar

Invert sugar is produced from the treatment of beet or cane sucrose with either acid (typically hydrochloric acid) or invertase (EC 3.2.1.26), which hydrolyzes the glycosidic bond so as to yield equimolar amounts of glucose and fructose (White, 2008). In industry, invert sugar is mainly produced using acid hydrolysis. Invert sugar can be produced to different degrees of inversion by controlling the time and temperature conditions used in its production (Thavarajah and Low, 2006a). Total invert sugar is produced when this reaction is allowed to proceed until only small amounts of sucrose remain (~5-8%) resulting in a syrup containing a high concentration of glucose and fructose in a 1:1 ratio (Low, 1997). During the production of invert sugar, oligosaccharides may be formed via a process known as reversion (Swallow and Low, 1993). Three of these oligosaccharides have been previously identified as O-α-D-glucopyranosyl-(1→3)-

D-fructose, O-β-D-fructofuranosyl-(2→6)-D-glucose and O-α-D-glucopyranosyl-β-D-glucopyranoside (Thavarajah and Low, 2006b).

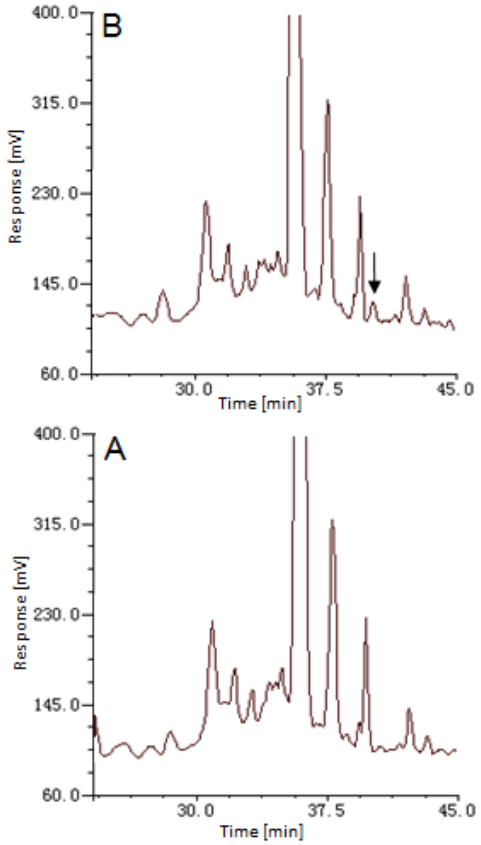

Figure 3.2 HPAE-PAD chromatograms from 22.0 to 45.0 minutes of (A) pure pear juice (PJ 23) at 5.5 °Brix and (B) the same pear juice intentionally adulterated with 10.0% (v/v) HFCS 55 at 5.5 °Brix. The fingerprint oligosaccharide tentatively identified as maltohexaose is indicated by an arrow.

Total invert sugar was selected as a potential adulterant in pear juice due to its low level of sucrose as compared to medium invert sugar which has a glucose to fructose to sucrose ratio of 1:1:2 (Low, 1997). This means that the addition of medium invert sugar to pear juice should be detectable by a noticeable change in the glucose:fructose:sucrose ratios of the juice. The debasing of pear juice with total invert sugar was readily detected employing developed CGC-FID method, and is illustrated in Figure 3.3 where chromatogram A shows the CGC-FID carbohydrate profile of pure pear juice (PJ 7) and chromatogram B shows the same juice intentionally adulterated with 10.0% (v:v) TIS. The fingerprint oligosaccharides for TIS detection have retention times of

approximately 41.9 and 49.7 minutes, respectively. The peak eluting at 49.7 minutes was tentatively identified as IS2 (O-β-D-fructofuranosyl-(2→6)-D-glucose) based on literature results (Thavarajah, 2004; Thavarajah and Low, 2006b) while the peak at 41.9 minutes is currently unidentified. The detection limit for TIS addition to pear juice by the developed CGC-FID method was found to be 3.0% (v/v).

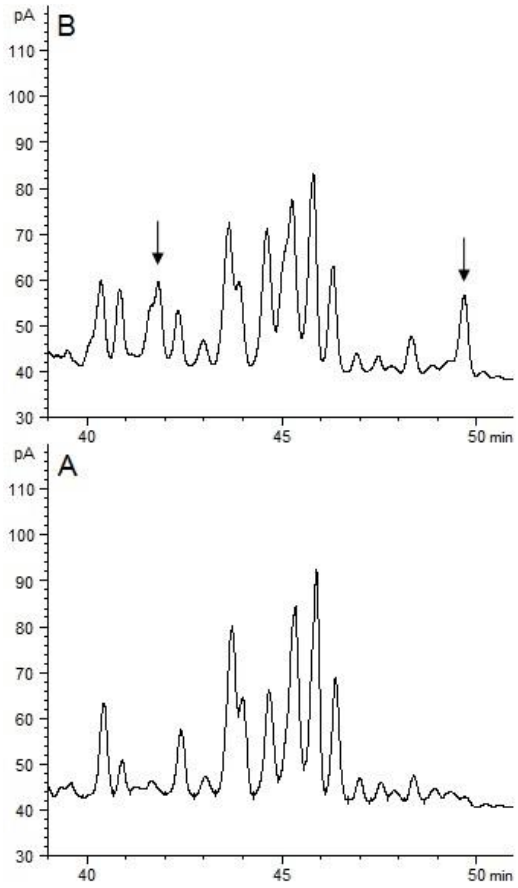

Figure 3.3 CGC-FID chromatograms from 39.0 to 51.0 min of (A) pure pear juice (PJ 7) and (B) the same pear juice intentionally adulterated with 10.0% (v/v) TIS. The fingerprint oligosaccharides tentatively identified as IS2 (O-β-D-fructofuranosyl-(2→6)-D-glucose) and an unknown compound are indicated by arrows.

Pear juice samples intentionally adulterated with TIS were also analyzed by the developed HPAE-PAD method. Unfortunately, the presence of naturally occurring oligosaccharides, and the high concentration of monosaccharides in pure pear juice samples precluded the use of this technique to detect pear juice adulterated with TIS.

3.4.4 Pear Juice Adulteration with Hydrolyzed Inulin Syrup

Inulin is a naturally occurring polysaccharide found in plants and some bacterial and fungal species (Franck, 2002). It is a mostly linear polysaccharide of varying chain length comprised of fructose units glycosidically linked by β-(2→1)-D-fructosyl-fructose units terminated by D-glucose linked via an α-(2→1) bond as in sucrose (Ronkart et al., 2007; Borromei et al., 2009). Hydrolyzed inulin syrup (HIS) is produced by the controlled enzymatic or acid (typically hydrochloric) hydrolysis of inulin. This results in the production of a syrup that is rich in fructose with minor amounts of glucose present (Ricca et al., 2007). It has been reported that the hydrolysis of inulin can result in the formation of a variety of different oligosaccharides, which consist either solely of fructose units (inulooligosaccharides) or of fructose units linked with a terminal glucose unit (fructooligosaccharides) (Ronkart et al., 2007).

CGC-FID oligosaccharide profiling of HIS shows the presence of two fingerprint oligosaccharides with retention times of approximately 21.5 and 34.9 minutes, respectively. When HIS is added to pear juice, the peak at 34.9 minutes was masked by the naturally occurring oligosaccharides, however the peak at 21.5 minutes eluted in a region devoid of interfering compounds. These results are illustrated in Figure 3.4 where chromatogram A shows the CGC-FID carbohydrate profile of pure pear juice (PJ 7) and chromatogram B shows the same juice intentionally adulterated with 2.0% (v:v) HIS. This fingerprint oligosaccharide for HIS is postulated to be a carbohydrate comprised of two fructose moieties (i.e. disaccharide) based on inulin structure and hydrolysis, and its elution time, which occurs in the typical range for disaccharides (Low and Sporns, 1988). This oligosaccharide has been tentatively identified as inulobiose (O-β-D-fructofuranosyl-(2→1)-D-fructose), a disaccharide commonly reported to be formed during the hydrolysis of inulin based on both literature results and HPAE-PAD analysis of standards (unpublished results). Supporting evidence for this identification is based on the inulin source used in this study which was chicory root, which has been reported to contain low amounts of branching (1-2%) when compared to other sources (e.g. bacterial; branching >15%) (Ricca et al., 2007). The detection limit for HIS addition to pear juice by the developed CGC-FID method was found to be 0.5% (v/v).

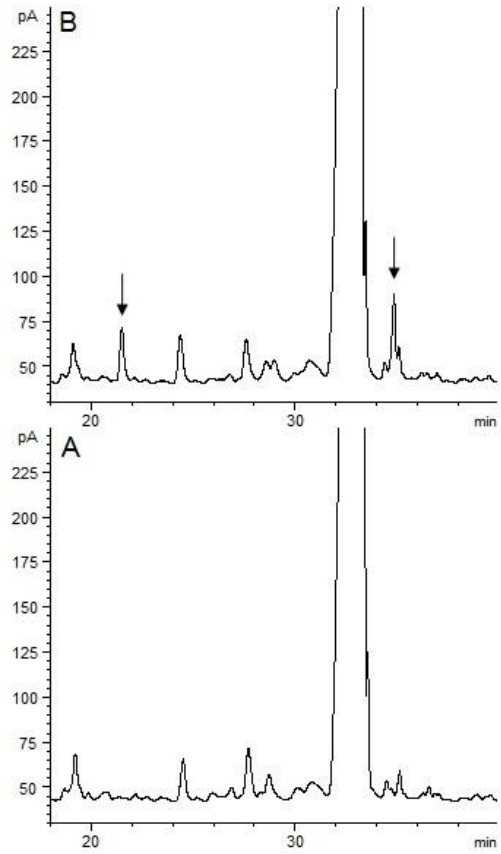

Figure 3.4 CGC-FID chromatograms from 18.0 to 40.0 min of (A) pure pear juice (PJ 7) and (B) the same pear juice intentionally adulterated with 2.0% (v/v) HIS. The fingerprint oligosaccharides tentatively identified as α- and β-inulobiose are indicated by arrows.

Pear juice samples intentionally adulterated with HIS were also analyzed by the developed HPAE-PAD method. A fingerprint oligosaccharide with a retention time of ~17.5 minutes was readily identified when HIS was intentionally added to pure pear juice. The application of this method to a pure pear juice (PJ 23; chromatogram A) and the same pear juice intentionally adulterated with 5.0 and 10.0% (v:v) HIS (chromatograms B and C) is shown in Figure 3.5. The detection limit for HIS addition to pear juice by the developed HPAE-PAD method was found to be 2.0% (v/v).

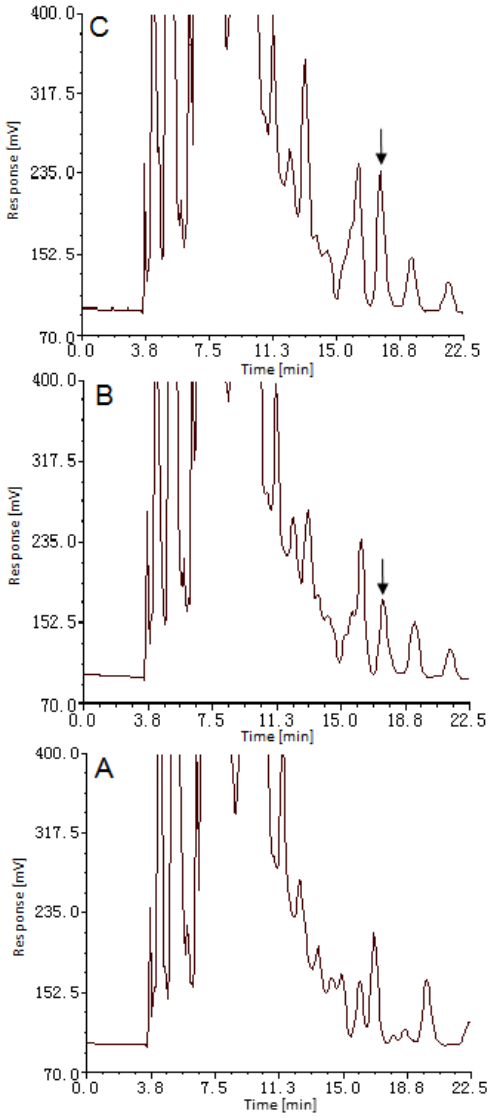

Figure 3.5 HPAE-PAD chromatograms from 0.0 to 22.5 minutes of (A) pure pear juice (PJ 23) and the same pear juice intentionally adulterated with (B) 5.0% and (C) 10.0% (v/v) HIS. The fingerprint oligosaccharide tentatively identified as inulobiose is indicated by an arrow.

3.4.5 Detection of Juice-to-Juice Adulteration by Arbutin Analysis

The developed chromatographic techniques were examined for their potential to detect types of adulteration besides debasing with less expensive commercial sweeteners. One common method of fruit juice adulteration is the addition of a juice of lesser value to one of higher value. This type of adulteration is referred to as juice-to-juice debasing. Due to the fact that apple and pear juices share similarities in properties such as major carbohydrate and polyol profiles, juice-to-juice adulteration between these two can be a significant problem as it is difficult to detect using traditional methods such as the matrix approach (Thavarajah and Low, 2006a). Arbutin (4-hydroxyphenyl-β-D-glucopyranoside) has been previously identified as a natural constituent of pear juice which is not found in apple juice (Thavarajah and Low, 2006a). It was found that arbutin could be detected by both of the developed chromatographic methods (i.e., CGC-FID and HPAE-PAD). The presence of arbutin was confirmed in all 32 pure pear juice concentrates by both CGC-FID and HPAE-PAD with retention times of 27.6 and 37.8 minutes, respectively.

GC-MS was carried out in order to confirm that the peak observed by CGC-FID corresponded to arbutin and not another compound with a similar retention time. The mass spectra of the silylated arbutin standard and the peak with the matching retention time in pear juice showed good agreement. The spectrum was also matched against those found in the National Institute of Standards and Technology (NIST) Mass Spectral Library as well as with literature data (Lamien-Meda et al., 2009; Chisvert et al., 2010). Figure 3.6 shows the mass spectra of silylated arbutin as found under these conditions. As can be seen no fragments greater than a mass-to-charge (*m/z*) ratio of 450 were observed even though silylated arbutin has a molecular weight of 632 g/mol. This indicates that the molecular ion readily fragments under the conditions used in this study. Currently, there is only one literature report where the identities of any of the observed fragments are suggested (Chisvert et al., 2010). However, the authors do not give detailed structures of the proposed fragments and fail to identify a major fragment (*m/z* 254). As well, the fragment at *m/z* 217 is clearly misidentified as the proposed structure has an *m/z* value of only 130. Therefore, more work in this area is needed. A number of the fragments were tentatively identified in this study and are shown in Figure 3.7.

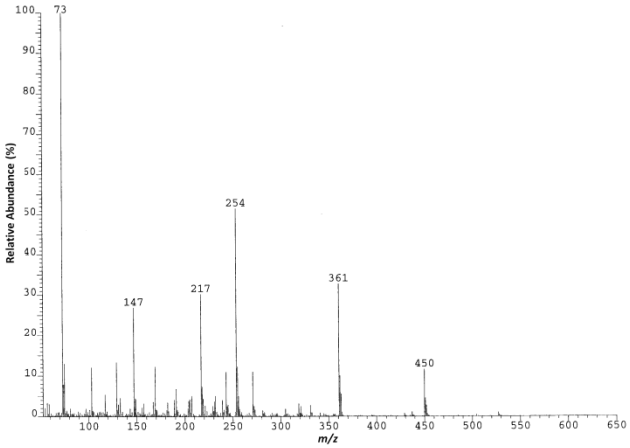

Figure 3.6 CG-MS spectra of silylated arbutin in pear juice.

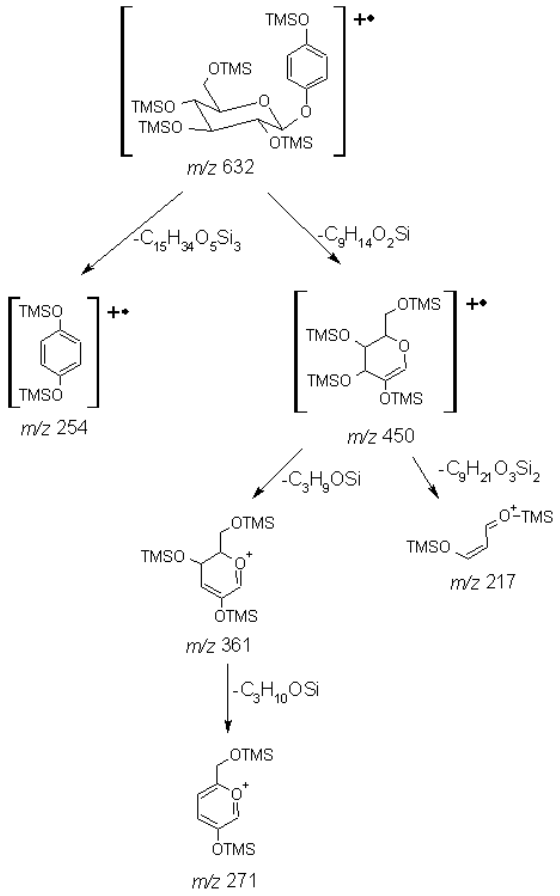

Figure 3.7 Proposed fragments of silylated arbutin under CG-MS condtions. TMS: -Si(CH_3)$_3$

Loss of the hydroquinone portion of arbutin as a neutral species results in the formation of the fragment at *m/z* 450 with further fragmentation by loss of trimethylsilyl (TMS) groups and by ring cleavage. This type of behavior has been previously reported for TMS derivatized glucose during analysis by CG-MS (DeJongh et al., 1969). As well, a structure for the fragment observed at *m/z* 254 has been suggested. The fragments at *m/z* 147 (TMS-O-DMS) and *m/z* 73 (TMS) have been previously identified and are characteristic of trimethylsilylated compounds (DeJongh et al., 1969; Chisvert et al., 2010).

A second method was also applied to confirm the presence of arbutin in the pear juice samples. In this method β-glucosidase (EC 3.2.1.21) was added to select pear juice samples which resulted in the hydrolysis of arbutin to free D-glucose and hydroquinone. As expected, this hydrolysis resulted in a disappearance of the arbutin peak as observed by both CGC-FID and HPAE-PAD. The use of β-glucosidase for arbutin identification is beneficial as it is simple to carry out and allows confirmation of the presence of arbutin by both CGC-FID and HPAE-PAD without the use of a mass spectrometer.

The concentration of arbutin in the pure samples was analyzed by HPLC-PDA in order to determine how effective this compound would be for adulteration detection. The average arbutin content of the 32 samples was found to be 86.7 ± 49.6 µg/mL with a range of 50.6 to 286.9 µg/mL (Table 3.1). The experimental average was found to be higher than the average arbutin content reported for a similar set of samples of 75.5 µg/mL (after adjustment to 12.0 °Brix) (Thavarajah and Low, 2006a). This is likely due to the presence of six samples with arbutin contents greater than 100.0 µg/mL in the present study. Of these six samples, four of were obtained from China and two from New Zealand. The arbutin content in all of the remaining 26 pear juice samples was less than 85.0 µg/mL.

A literature range for pear arbutin content has been reported as 42.3 to 157.7 µg/mL, which is in agreement with the arbutin values obtained in this study with the exception of PJ 20 and 22, which had concentrations of 223.7 and 286.9 µg/mL, respectively (Thavarajah and Low, 2006a). Based upon the pear juice sample in this study with the lowest concentration of arbutin and a detection level of 3x the signal-to-noise ratio the addition of pear juice to apple juice can be detected at levels down to 3.0% (v:v). This detection limit would be even lower if a pear juice rich in arbutin was used to adulterate a sample. As well, it may be possible to lower this detection limit

even further by the application of CG-MS for arbutin detection due to the increased sensitivity of this method.

Arbutin also shows the potential of acting as an indicator for pear juice authenticity. The absence of arbutin in a product claiming to contain pear would be a good indication that pear juice is not actually present in the product. As well, if a sample claiming to be a pure pear juice contains arbutin in concentrations considerably lower than those reported for authentic pear juice, it is likely that the sample is not pure and that further authenticity analysis needs to be undertaken.

3.4.6 Detection of Total Liquefaction by Cellobiose Detection

The production of pear and apple juice is aided by the use of enzymes, such as pectinases and amylases, which aid in cell wall breakdown, improving juice extraction and filtration (Kashyap et al., 2001). Total liquefaction involves the addition of cellulases and excess pectinases to the fruit mash in order to achieve a more complete breakdown of the cell walls resulting in an increase in the total soluble solids of the final juice (Mehrländer et al., 2002). This process is illegal in both North America and the European Union as there are no regulatory guidelines in place for cellulase use in fruit juice production and because little is known about the nutritional aspects of liquefaction juice (Mehrländer et al., 2002; Piatka et al., 2010). It should also be noted that total liquefaction leads to a lower quality juice with an increased propensity to browning and haze formation (Mehrländer et al., 2002). The undeclared substitution or blending of a premium juice with a juice of lower quality produced by liquefaction constitutes adulteration and would be considered illegal. Consequently, it is important to be able to distinguish between juices produced by these different methods.

Cellobiose, a disaccharide comprised of two glucose units linked via a β-(1→4) glycosidic bond, is formed by the breakdown of cellulose found in the cell wall of fruits when cellulases are used for juice production. In one study, cellobiose was not found in apple juices produced using normal enzymatic treatments (premium juice), but was detected in three out of four juices produced by total liquefaction at concentrations ranging from 16.6 to 55.4 ppm (Will et al., 2000). The presence of cellobiose, in combination with excess galacturonic acid, is considered to be indicative of pomace liquefaction by the fruit juice industry (Will et al., 2000). Currently, no official limits exist on the concentration of cellobiose in pear and apple juices (Will et al., 2000).

The developed CGC-FID method was shown to be able to effectively separate and detect cellobiose in pear juice. As it is a reducing carbohydrate, cellobiose has two chromatographic peaks at 36.2 and 45.8 minutes, corresponding to α- and β-cellobiose, respectively. While β-cellobiose overlaps with naturally occurring pear compounds, α-cellobiose is free of such interferences. When analyzed by HPAE-PAD, the high monosaccharide content of pear juice obscured the cellobiose peak (RT = 10.4 minutes) precluded the use of this method for total liquefaction detection.

Cellobiose was not detected by CGC-FID in any of the thirty-two pure pear juice concentrates analyzed in this study at a detection limit of 8.0 ppm in the 12.0 °Brix juice by the developed analytical method clearly indicating that these juices were not produced by total liquefaction.

In summary, these results show that the addition of less expensive nutritive sweeteners, namely HFCS, HIS and TIS, can be readily detected in pear juice by the application of the developed CGC-FID method at levels of 0.5 to 3.0% (v:v). In addition, HPAE-PAD profiling can also be used to confirm the presence of HFCS and HIS in pear juice. CGC-FID fingerprinting also affords the detection of pear to apple (and potentially apple to pear) juice debasing and process adulteration all within a single chromatographic analysis. Figure 3.8 shows the CGC-FID chromatogram of pear juice to which the studied pear juice adulterants (HFCS, HIS and TIS) and cellobiose have been added. The ability to detect each of these adulterants at low concentrations, arbutin (to confirm the presence or absence of pear), and cellobiose (indicator of liquefaction processing) in a single chromatographic run is beneficial as it is both a robust and accurate method, and reduces sample preparation and analysis time. In addition, the use of the developed method not only allows an adulterated sample to be identified, it also provides information on the adulterant used. It is envisaged that this method will result in a decrease in food fraud so as to maintain consumer confidence in the food industry.

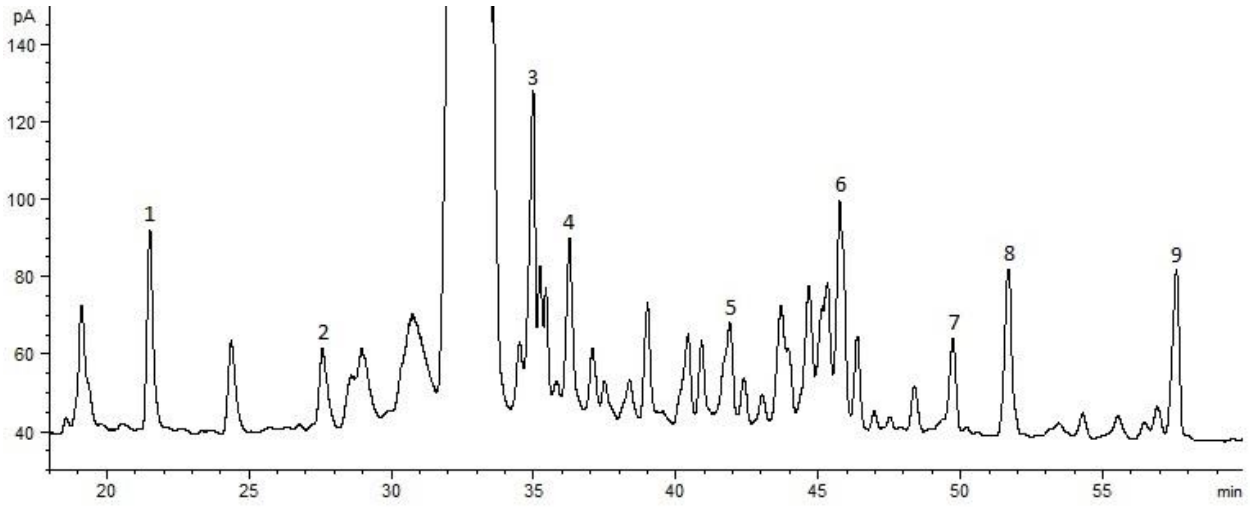

Figure 3.8 CGC-FID chromatogram of pear juice (PJ 7) to which HFCS 55, HIS, TIS and cellobiose have been added. Identified peaks are as follows: 1 = α-inulobiose (HIS marker); 2 = arbutin (pear marker); 3 = β-inulobiose (HIS marker); 4 = α-cellobiose (liquefaction marker); 5 = unidentified TIS marker; 6 = β-cellobiose; 7 = O-β-D-fructofuranosyl-(2→6)-D-glucose (TIS marker); 8 = α-isomaltose; 9 = β-isomaltose (HFCS marker).

3.5 Linkage Between Authenticity Analysis of Pear Juice and Oligosaccharide Formation during Pear Juice Processing

Oligosaccharide profiling was found to be an effective method to detect the undeclared addition of a series of less expensive nutritive sweeteners, namely HFCS 55 and 90, HIS and TIS, to pear juice. These sweeteners were identified by the presence of marker oligosaccharides in the sweeteners that were not natural constituents of pure pear juice samples. However, unadulterated pear juice samples were found to contain a number of oligosaccharides. It was hypothesized that many of these compounds were formed during pear processing. In order to determine the role(s) that processing played in the formation of these oligosaccharides as well as their identification, pear juice production under laboratory conditions employing a series of commercial juice enzymes was conducted. Samples from each stage of processing were analyzed by HPAE-PAD and CGC-FID to determine carbohydrate/oligosaccharide structures and concentrations (Chapter 4).

4. OLIGOSACCHARIDE FORMATION DURING COMMERCIAL PEAR JUICE PROCESSING[1]

4.1 Abstract

The effect of enzyme treatment and processing on the oligosaccharide profile of commercial pear juice samples was examined by high performance anion exchange chromatography with pulsed amperometric detection and capillary gas chromatography with flame ionization detection. Industrial samples representing the major stages of processing produced with various commercial enzyme preparations were studied. Through the use of commercially available standards and laboratory scale enzymatic hydrolysis of pectin, starch and xyloglucan; galacturonic acid oligomers, glucose oligomers (e.g., maltose and cellotriose) and isoprimeverose were identified as being formed during pear juice production. It was found that the majority of polysaccharide hydrolysis and oligosaccharide formation occurred during enzymatic treatment at the pear mashing stage and that the remaining processing steps had minimal impact on the carbohydrate-based chromatographic profile of pear juice. Also, all commercial enzyme preparations and conditions (time and temperature) studied produced similar carbohydrate-based chromatographic profiles.

4.2 Introduction

Commercial pear juice production in the United States and South America mainly consists of three cultivars of the species *Pyrus communis* L., commonly referred to as European pear, namely Bartlett (or Williams), Beurre d'Anjou and Beurre Bosc (USDA, 2004). Other pear cultivars, such as Packham's Triumph, may also be used in juice processing based upon location and availability.

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During pear juice production, the following common commercial processing steps are employed: (1) the fruit is washed to remove surface and chemical contaminants; (2) the washed fruit is converted into a mash by milling (e.g., hammer mill) and carbohydrases, including amylases, hemicellulases and pectinases are added; (3) the enzyme treated mash is pressed (belt and/or hydraulic press) and the remaining pomace particles are removed by screening; (4) the resulting juice is transferred to clarification tanks where, if required, additional carbohydrases are added followed by filtration; and (5) the resulting clarified juice is pasteurized (70 to 100 °C for 6 to 40s) and packaged for sale or concentrated (multiple effect evaporation ranging from 45 to 100 °C) for transport and/or future use (Bates et al., 2001; Ceci and Lozano, 2010; Horváth-Kerkai and Stéger-Maté, 2012).

Pear fruit cell walls are comprised of a variety of polymers, including polysaccharides, proteins and lignin (Schols, 1995; Dongowski and Sembries, 2001). Of these, pectin is the major target of enzymatic treatment during pear juice production. Pectin is a polysaccharide comprised of galacturonic acid residues to which neutral carbohydrates, mainly L-arabinose, D-galactose, L-rhamnose and D-xylose are covalently bound (Kashyap et al., 2001). Pectin can be classified into three different categories based on structure. The simplest of these is homogalacturonan (HG), which is defined as a linear polymer of α-1,4 linked D-galacturonic acid residues. The second category is rhamnogalacturonan I (RGI) in which the disaccharide [→4)-α-D-GalA-(1→2)-α-L-Rha-(1→]$_n$ repeats as the polysaccharide backbone, to which a variety of different oligosaccharides, primarily arabinans and galactans, are linked to the rhamnose residues at the O-4 or O-3 positions. Finally, rhamnogalacturonan II (RGII) consists of a HG backbone to which complex oligosaccharides may be attached to the galacturonic acid residues through a variety of different glycosidic linkages (Willats et al., 2006; Round et al., 2010; Yapo, 2011). Further pectin structure changes also commonly exist in nature where some of the galacturonic acid residues are methyl esterified. As such, pectin structure in plants varies depending upon plant species, location within the cell wall and plant age (Round et al., 2010).

Due to both its high concentration and structural complexity in pear fruit, a variety of different pectinases are required for pectin hydrolysis so as to improve juice extraction and reduce product viscosity. Pectinases (both exo and endo) commonly used for these purposes include: (1) polygalacturonase (PG) and polymethylgalacturonase (PMG), which hydrolyze the α-1,4-glycosidic linkages of pectin; and (2) polygalacturonate lyases (PGLs) and

polymethylgalacturonate lyases (PMGL), which break the α-(1→4)-glycosidic linkages of pectin by a *trans*-elimination mechanism, resulting in a site of unsaturation at the non-reducing end (Kashyap et al., 2001). In addition, pectinesterases (PE) may also be used, which hydrolyze the methyl-ester groups of pectin resulting in polygalacturonic acid and methanol formation (Kashyap et al., 2001; Horváth-Kerkai and Stéger-Maté, 2012). Typically, commercial pear/apple pectinase preparations contain many if not all of these activities so as to effectively hydrolyze pectin (Horváth-Kerkai and Stéger-Maté, 2012).

Along with pectin, starch and hemicellulose are also targeted by enzyme hydrolysis during juice production. The presence of colloidal starch and hemicelluloses can cause cloudiness and sedimentation, which is undesirable in pear juice (Carrín et al., 2004; Sinh, 2012). Therefore, commercial juice enzyme preparations often contain amylases and/or hemicellulases, in addition to pectinases, in order to help clarify the juice. Along with clarification, the addition of hemicellulases also helps to break down the fruit cell wall improving juice extraction and filtration (Horváth-Kerkai and Stéger-Maté, 2012).

Although published work exists on cell wall changes that occur in pear during its ripening and storage (Ahmed and Labavitch, 1980; Dick and Labavitch, 1989; Hiwasa et al., 2004; Raffo et al., 2011), no literature reports are available on the role of juice processing enzymes on cell wall polysaccharides under industrial and laboratory scale pear juice production. Therefore, the aim of this work was to examine the formation (i.e. presence of new compounds) and changes (i.e. loss and/or increase in concentration) in pear juice carbohydrates (polysaccharide, oligosaccharide and monosaccharide) during the processing stages commonly employed in commercial pear juice production through high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) and capillary gas chromatography with flame ionization detection (CGC-FID).

4.3 Materials and Methods

4.3.1 Samples

Samples collected at various stages of pear juice processing (mash to concentrate) were received from three different commercial producers representing the USA (Juice 1) and South America (Juices 2 and 3). Processing stage samples were immediately heated at 90 °C for 3 min in order to inactivate enzymes prior to shipping. Received commercial samples were stored at -30

°C until analyzed. The °Brix of each sample was measured using an Auto Abbe refractometer (Leica Inc., Buffalo, NY, USA).

Major commercial enzymes used for pear juice production were kindly provided by the same commercial companies along with dosage regimes used for pear juice production. These enzymes included: Pectinex Ultra Clear, Pectinex Ultra Mash, Amylase AG 300 L and Pectinex UF (Novozymes, Bagsvaerd, Denmark); SEBAmyl L and LiquiSEB APL (Specialty Enzymes, Chino, CA, USA); Pear Adex and Adex-d (DSM, Heerlen, Netherlands); and Natuzyme A and Natuzyme Extra (WeissBioTech, Ascheberg, Germany). All enzymes were stored 4 °C.

4.3.2 Chemicals

L-Arabinose, cellobiose (O-β-D-glucopyranosyl-(1→4)-O-D-glucopyranose), digalacturonic acid (O-α-D-galactopyranuronosyl-(1→4)-D-galacturonic acid), D-fructose, D-fucose, D-galacturonic acid, D-galactose, D-glucose, D-mannose, pectin from apple, L-rhamnose, sodium hydroxide (NaOH) solution (50% w/w), D-sorbitol, starch from wheat, D-sucrose, Sylon TP (TMSI + pyridine, 1:4), trigalacturonic acid (O-α-D-galactopyranuronosyl-(1→4)-O-α-D-galactopyranuronosyl-(1→4)-D-galacturonic acid) and D-xylose were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). D-galacturonic acid methyl ester was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Sodium acetate (NaOAc) was obtained from Fisher Scientific (Ottawa, ON, Canada). Cellotriose (O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→4)-O-D-glucopyranose), isoprimeverose (O-α-D-xylopyranosyl-(1→6)-O-D-glucopyranose) and xyloglucan from tamarind were obtained from Megazyme (Cedarlane, Burlington, ON, Canada). The water used throughout this study was obtained from a Millipore Milli-Q™ water system (Millipore Corp., Milford, MA, USA).

4.3.3 Laboratory Scale Polysaccharide Sample Preparation

A solution of 2.0% glucose, 6.0% fructose, 1.0% sucrose and 2.5% sorbitol (w/v) was prepared in water to approximate the major carbohydrate composition of pear juice (mock pear juice; Thavarajah and Low, 2006; Willems and Low, 2014). Samples containing 2.0% (w/w) pectin and 2.0% (w/w) pectin plus 0.5% (w/w) starch in water and in mock pear juice were prepared in order to approximate the average concentration of these polysaccharides in pear (Whitaker, 1984; Raffo et al., 2011). One set of the aforementioned polysaccharide samples was prepared at their

natural pH of 3.2 ± 0.1 and one set was adjusted to pH 4.0 ± 0.1 with sodium hydroxide. In addition, aqueous 1.0% (w/w) xyloglucan (pH 4.0 ± 0.1) samples were prepared.

4.3.4 Enzymatic Hydrolysis of Laboratory Scale Polysaccharide Samples

Each of the aforementioned samples was subjected to enzymatic treatment following four different dosage/time/temperature conditions as supplied by the commercial juice producers (Table 4.1). In brief, the samples were brought to 50.0 ± 2.0 °C in an AquaTherm shaking water bath (New Brunswick Scientific Co. Inc., Edison, NJ, USA) and pectinases were added at concentrations of 1.5 to 2.6 mg/g pectin (commercial producer dosages ranged from 30 to 50 ppm mash, which converts to 1.5 to 2.6 mg enzyme/g pectin [assuming a level of 2.0% pectin]) and amylases at concentrations of 1.9 to 3.5 mg/g pectin (commercial producer recommended dosages ranged from 40 to 70 ppm mash, which converts to 1.9 to 3.5 mg enzyme/g pectin [assuming a level of 2.0% pectin]) depending upon the enzyme set used by the commercial producer (Table 4.1). Enzyme treated sample solutions were maintained at temperature and time conditions recommended by the commercial juice producer with mild shaking (Table 4.1). Enzymatic reactions were terminated by sample heating at 90.0 °C for 10 minutes. Samples were diluted 1 in 4 with water prior to syringe filtration (nylon, 0.2 μm pore size; 13 mm diameter; Chromatographic Specialties Inc., Brockville, ON, Canada) and analyzed by HPAE-PAD and CGC-FID. All enzyme hydrolysis reactions were conducted in triplicate.

4.3.5 Laboratory Scale Pear Juice Production

Laboratory scale pear juice was prepared using a mixture of Bartlett and D'Anjou pears purchased at a local supermarket. The pears were washed, cut into small pieces removing the core and 300.0 g of pear fruit (equal parts by weight of each pear cultivar) was blended along with 20.0 mL of water in an Oster® blender (Jarden Consumer Solutions, Brampton, ON, Canada). The resulting mixture was treated with commercial enzyme set A under the same conditions employed industrially (Table 4.1). Samples were taken immediately after mashing, after enzyme treatment for 90 minutes at room temperature and after further enzyme treatment at 50 °C for 90 minutes. All samples were immediately heated for 10 minutes at 90.0 °C for enzyme inactivation. The juice was separated from the pulp by vacuum filtration with VWR 417 filter paper. The juice was then centrifuged at 6000 rpm (Clinical 200 centrifuge, VWR International, Edmonton, AB, Canada) for

15 minutes before being syringe filtered and diluted with water to 5.5 °Brix for chromatographic analysis. Laboratory scale pear juice production and sampling was done in triplicate.

Table 4.1 Enzyme dosage, times and temperatures used for laboratory scale hydrolysis experiments.

	mg Enzyme/ g Pectin	Time	Temperature
Enzyme Set A			
Pectinex Ultra Mash	2.3	90 min, 90 min	21-23 °C, 50 °C
Pectinex Ultra Clear	2.5		
Amylase AG 300 L	2.0		
Enzyme Set B			
LiquiSEB APL	2.6	90 min	50 °C
SEBAmyl L	1.9		
Enzyme Set C			
Natuzyme Extra	1.5	180 min	50 °C
Natuzyme A	3.5		
Enzyme Set D			
Pear Adex	1.5	180 min	50 °C
Adex-d	2.3		
Natuzyme A	3.5		

4.3.6 Commercial Samples

Commercial pear juice processing samples included: (1) a set consisting of pear mash (before carbohydrase addition), juice after first extraction and enzyme treatment, juice after preconcentration, juice after one and eight hours of enzyme treatment, juice after ultrafiltration and finished pear juice concentrate; (2) a set consisting of pear mash (before carbohydrase addition), juice after complete enzyme treatment, juice after preconcentration and ultrafiltration and finished pear juice concentrate; and (3) a set consisting of pear mash (before carbohydrase addition), juice from the decanter after enzyme treatment, the juice before and after ultrafiltration and the finished pear juice concentrate.

4.3.7 Commercial Pear Juice Processing Sample Preparation for Chromatographic Analysis

Commercial juice samples containing particulates (i.e., mash samples) were centrifuged at 6000 rpm for 15 minutes and the supernatant was collected and used for further analysis. All

commercial samples were prepared for chromatographic analysis by dilution to 5.5 ± 0.1 °Brix with water prior to syringe filtration. Samples were further diluted 10x with water for galacturonic acid quantitation by HPAE-PAD.

4.3.8 Galacturonic Acid and Oligosaccharide Analysis by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD)

Carbohydrate analysis was carried out using a Dionex ICS 5000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Dionex AS autosampler, and an ICS 5000 electrochemical cell with a disposable gold electrode. The potentials and durations of the gold electrode were as follows: E1 = 0.10 V, t1 = 0.00 s; E2 = −2.00 V, t2 = 0.41 s; E3 = 0.60 V, t3 = 0.43 s; E4 = −0.10 V, t4 = 0.44 s; E5 = −0.10 V, t5 = 0.50 s and data acquisition was carried out using Dionex Chromeleon 7.0 software. Carbohydrate separation was accomplished using a Dionex CarboPac PA1 column (4 x 250 mm) in series with a CarboPac PA1 guard column (4 x 50 mm) at room temperature (21-23 °C). A linear gradient elution program was used for oligosaccharide separation where solvent A was 160 mM NaOH, solvent B was 160 mM NaOH/1.0 M NaOAc and solvent C was 1.0 M NaOH. Initial conditions were 100.0% A for 1.5 min; linear gradient to 75.0% B at 75.0 min; 100.0% B at 75.1 min with a hold until 78.0 min; 100.0% C at 78.1 min with a hold until 80.0 min; 100.0% A at 80.1 min with a hold until 90.0 min. The flow rate was 0.7 mL/min. The injection volume was 25.0 µL for laboratory scale pear juice samples and 10.0 µL for commercial samples. A galacturonic acid standard curve was prepared in water at concentrations ranging from 10 to 100 ppm with a R^2 value of > 0.99. All samples were analyzed in triplicate.

4.3.9 Monosaccharide Analysis by HPAE-PAD

Monosaccharide (arabinose, galactose, glucose and xylose) analysis of the laboratory scale pear juice samples was carried out employing a Dionex ICS 5000 HPLC system as described in section 4.3.8. Separation was accomplished using a Dionex CarboPac PA1 column (4 x 250 mm) in series with a CarboPac PA1 guard column (4 x 50 mm) at room temperature in conjunction with an isocratic mobile phase of 30 mM NaOH. Sample injection volume was 25.0 µL.

4.3.10 Oligosaccharide Analysis by Capillary Gas Chromatography with Flame Ionization Detection (CGC-FID)

Oligosaccharide analysis by CGC-FID was carried out as previously described (Willems and Low, 2014). In brief, samples were analyzed on an Agilent 6890 gas chromatograph equipped with an Agilent 6890 series autosampler (Agilent Technologies Canada Inc., Mississauga, ON, Canada). The injection volume was 3.0 µL and samples were analyzed in the splitless mode. Ultrapure hydrogen was used as the carrier gas at a constant flow rate of 1.5 mL/min with ultrapure nitrogen as the makeup gas at a flow rate of 30 mL/min. The injection port temperature was maintained at 250 °C and the detector at 300 °C. Oligosaccharide separation was afforded using an Agilent J&W DB-5 (95% dimethyl-5% diphenyl polysiloxane; 30 m x 0.25 mm, 0.25 µm film thickness) open tubular fused-silica capillary column (Chromatographic Specialties Inc.). Data acquisition and processing was carried out using Agilent ChemStation Rev. A.06.03 software (Agilent Technologies Canada, Inc.). The following temperature program was used for oligosaccharide analysis: initial temperature of 210 ºC for 10.0 min; 0.55 ºC/min from 210 to 235 ºC; hold at 235 ºC for 1.0 min; 30 ºC/min from 235 to 295 ºC; hold at 295 ºC for 12.0 min. The total run time was 70.5 min.

Samples were prepared by freeze-drying (Heto Lab Equipment, Allerod, Denmark) 150 µL aliquots in individual 12 x 32 mm glass vials (Chromatographic Specialties Inc.). To the resulting dried foam 500 µL of Sylon TP was added and the vials were capped and heated in a block heater (Denville Scientific Inc., Metuchen, NJ, USA) at 70.0 °C for one hour with shaking every 10-15 min. Standards for maltose and isoprimeverose were prepared at concentrations ranging from 15 to 300 ppm. Standard curves had R^2 values ≥ 0.98. All samples were analyzed in triplicate.

4.3.11 Galacturonic Acid Analysis by CGC-FID

Galacturonic acid analysis by CGC-FID was carried out on an Agilent 6890 series gas chromatograph as outlined in section 4.3.10. The following temperature program was employed: initial temperature of 150 °C; 1.0 °C/min from 150 °C to 180 °C; 30 °C/min from 180 °C to 295 °C; hold at 295 °C for 5.9 min. The total run time was 38.83 min. Samples were prepared as in section 4.3.10, with a derivatization time of two hours.

4.3.12 Trisaccharide Analysis by CGC-FID

Trisaccharide (i.e., cellotriose and maltotriose) analysis by CGC-FID was carried out on an Agilent 6890 series gas chromatograph as outlined in section 4.3.12. The following temperature program was employed: initial temperature of 250 °C for 10.0 min; 1.0 °C/min to 290 °C for 3.0 min; 30 °C/min to 295 °C; hold at 295 °C for 12.0 min. The total run time was 65.17 min. Samples were prepared as outlined in section 4.3.10.

4.4 Results and Discussion

Pear juice samples from three commercial juice processors located in different geographical regions and representing each stage of processing were used in this study. With the exception of the finished concentrates, process stage samples were immediately heated (90 °C for 3 min) following collection so as to inactivate enzymes added during processing as well as any naturally occurring enzymes in the fruit. The samples were then frozen and shipped, and were received in undamaged condition at our laboratory for sample analysis by both HPAE-PAD and CGC-FID.

4.4.1 HPAE-PAD Analysis

4.4.1.1 Carbohydrate Profile Changes in Commercially Produced Pear Juice as a Function of Processing

Figure 4.1 shows the HPAE-PAD monosaccharide and oligosaccharide profiles of a representative set of commercial pear juice samples at each stage of processing. Figure 4.1A shows the carbohydrate profile of the mash (first stage) prior to the addition of exogenous carbohydrases. Under the chromatographic condition employed, the major carbohydrates and polyol found in pear juice, namely fructose, glucose, sucrose and sorbitol (Thavarajah and Low, 2006a; Willems and Low, 2014), eluted as a single off-scale peak with retention times ranging from approximately 2.5 to 8.0 minutes. A number of small peaks were also observed between 8.0 and 15.0 minutes, however, relatively few peaks with retention times greater than 15 minutes were observed in the mash, with the exception of a major peak at 24.2 minutes, the identity of which will be discussed in the next section. Under the mobile phase conditions employed (NaOAc gradient to 1.0 M), oligosaccharides (degree of polymerization [DP] of 1 to 20) and lower molecular weight polysaccharides (DP 21-50) would be detected (Lee, 1996). Therefore, these classes of

carbohydrates are not present naturally in pear and are not produced during the commercial mashing process prior to exogenous carbohydrase addition at appreciable (≥10 ppm) levels. It should also be noted that pulsed amperometric detection is selective for carbohydrates, therefore, other components in the mash, such as unglycosylated phenolics, would not contribute to the chromatographic profile.

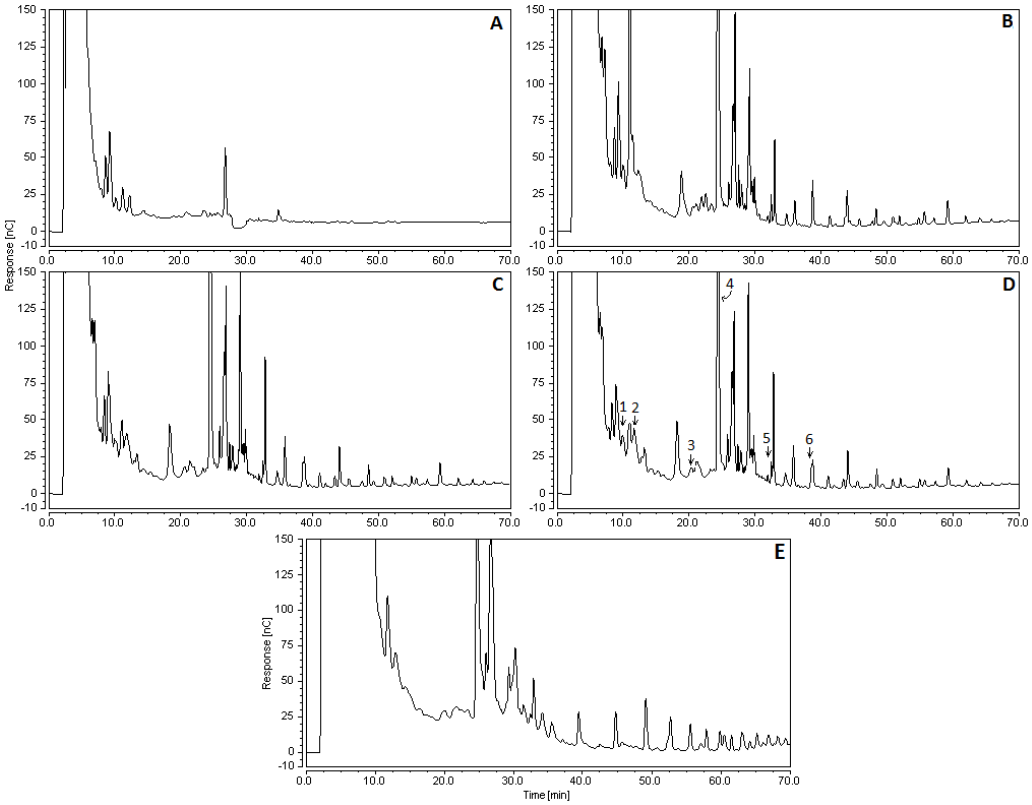

Figure 4.1 Representative HPAE-PAD chromatograms of pear juice at different stages of processing. Commercial pear mash before enzyme addition (A), pear juice after enzymatic treatment to hydrolyze pectin and starch, (B), pear juice after preconcentration, activated charcoal treatment and ultra-filtration (C), the final pear juice after pasteurization and concentration (D) and laboratory scale pear juice produced with Pectinex UltraMash, Pectinex UltraClear and Amylase AG 300 L as outlined in Table 4.1A (E). Identified peaks are as follows: 1, maltose; 2, isoprimeverose; 3, maltotriose; 4, D-galacturonic acid; 5, digalacturonic acid; 6, trigalacturonic acid.

Figure 4.1B shows the HPAE-PAD profile of the juice following exogenous carbohydrase treatment. The chromatogram shows the presence of a number of peaks with retention times >24 minutes indicating the production of oligosaccharides and low molecular weight polysaccharides.

Polysaccharide (i.e. pectin and starch) hydrolysis is an important step in the commercial production of pear juice, as pectinases and amylases are employed to degrade cell walls so as to improve juice yield (Kashyap et al., 2001; Horváth-Kerkai and Stéger-Maté, 2012), and this was clearly evident in all of the enzyme treated commercial juice samples. As well, additional peaks between 8.0 and 24.0 minutes, including a large peak at 11.0 minutes, were observed after the addition of carbohydrases to the mash (Figure 4.1B).

Following exogenous carbohydrase addition, further pear juice processing stages of preconcentration, activated charcoal treatment and ultra-filtration (Figure 4.1C) and pasteurization and concentration (Figure 4.1D) showed minimal impact on the HPAE-PAD chromatographic profile of the juice as no new peaks were identified. However, some changes in the concentration of oligosaccharides in the 8-15 and 25-35 minute regions of the chromatogram were observed as indicated by changes in peak heights/areas (samples were analyzed at the same °Brix values). For example, the peak with a retention time of 11.0 minutes decreased substantially in height after activated charcoal treatment and ultra-filtration (Figures 4.1B and C). The minimal change in the chromatographic profiles for these samples may be explained by enzyme inactivation during these processing steps due to heating, the lack of substrate (i.e., pectin and starch), and enzyme removal through ultra-filtration.

Experimental results for all commercial samples showed the same HPAE-PAD chromatographic profiles at each similar processing stage (e.g. the mash stage for all three commercial producers), indicating that no differences in compound formation was observed. The only differences observed in these samples were in their oligosaccharide/low molecular weight polysaccharide concentrations as determined by changes in their peak heights/areas. As the three processors used enzymes from different commercial sources and treated their juices for varying lengths of time (1.5 to 8 hours) and temperatures (22-23 to 50°C), the results from this study indicate that the choice of enzyme mixture, and processing time and temperature, did not play a major role in the final oligosaccharide profile (i.e. the formation of new peaks) of the juice as determined by HPAE-PAD.

4.4.1.2 Peak Identification from Laboratory Scale Pectin, Starch and Xyloglucan Hydrolysis

Based on the HPAE-PAD results from commercial pear processing samples (Figures 4.1B-D), it was hypothesized that the oligosaccharides observed in these juice samples were formed from enzymatic hydrolysis of pectin, starch and xyloglucan.

In order to confirm this hypothesis, laboratory scale reactions were conducted on commercially available pectin (apple), starch (wheat) and xyloglucan (tamarind) using enzyme preparations employed for commercial pear juice production (Table 4.1). Pectin and starch solutions were prepared at concentrations that were equivalent to the average levels (2.0% w/w for pectin, 0.5% w/w for starch and 1.0% w/w for xyloglucan) in pear and apple fruit based on literature reports (Whitaker, 1984; Raffo et al., 2011). Pectin from apple was chosen as the starting material based on purity (≥90%), the fact that apples and pears are closely related botanically, and commercial availability. Starch from wheat was used as its amylose content falls within literature values for apple at 26 to 29% (Bates et al., 2001; Stevenson et al., 2006) and commercial availability. Xyloglucan from tamarind was employed in this study because xyloglucan is a natural component of apple with a reported concentration of 1.0% (Raffo et al., 2011), the purity (approximately 95%) of this polysaccharide and its commercial availability. Representative HPAE-PAD chromatograms of pectin, pectin plus starch, and xyloglucan after laboratory scale enzymatic hydrolysis using a representative set (set B, Table 4.1) of commercial pear juice production enzymes at the average recommended supplier levels are shown in Figure 4.2.

The major peak observed in all of the commercial and laboratory scale pectin and pectin plus starch samples at a retention time of approximately 24.2 minutes was identified as D-galacturonic acid by comparison to an external standard and by spiking experiments. This carbohydrate was also observed in the pear mash (Figure 4.1A) before exogenous carbohydrase addition, indicating the presence of an exo-pectinase or an exo-pectin lyase naturally in the fruit. It has been reported that the level of endogenous pectinase activity increases with fruit maturity, however pectinases are compartmentalized within the cell to prevent cell wall breakdown during normal fruit growth and development (Pressey and Avants, 1976; Koch and Nevins, 1989). During the fruit mashing stage, cell walls are damaged resulting in enzyme and substrate interaction and the production of D-galacturonic acid.

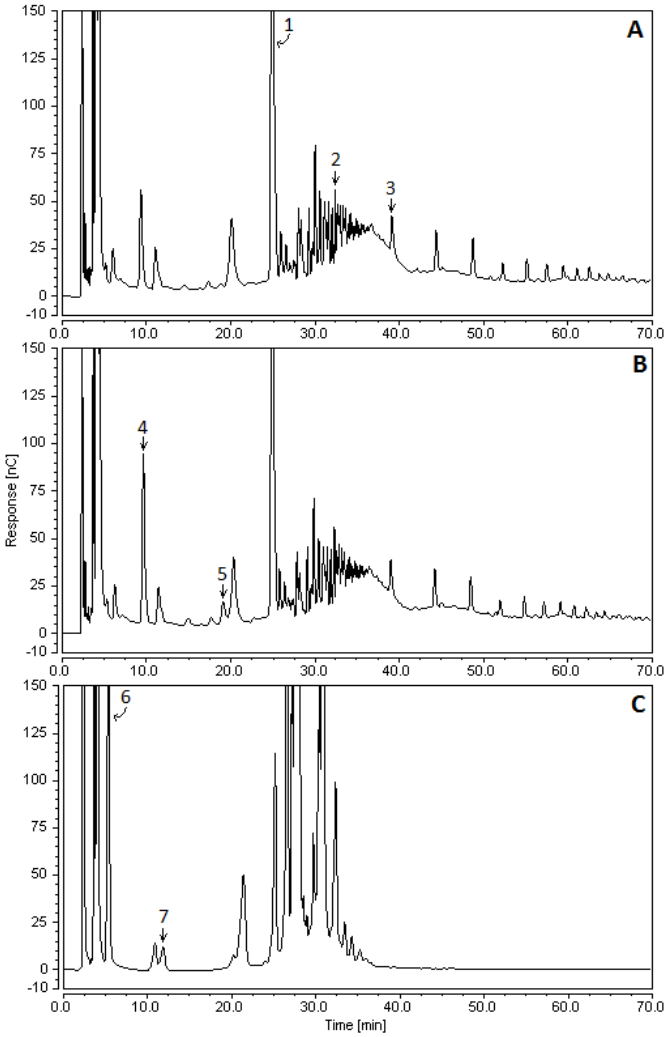

Figure 4.2 Representative HPAE-PAD chromatograms of pectin (A), pectin plus starch (B) and xyloglucan (C) in water (pH 3.2) after enzymatic hydrolysis with SEBAmyl L and LiquiSEB APL (Specialty Enzymes, Table 4.1B) at pH 4.0. Identified peaks are as follows: 1, D-galacturonic acid; 2, digalacturonic acid; 3, trigalacturonic acid; 4, maltose; 5, maltotriose; 6, isoprimeverose; 7, cellotriose.

In addition to D-galacturonic acid, both di- and trigalacturonic acids were identified in the laboratory scale and commercial samples with retention times of 32.6 and 39.5 minutes, respectively, by comparison with external standards and by spiking experiments. A number of peaks with retention times greater than 39.5 minutes were observed in all pectin containing samples (Figures 4.1B,C and Figures 4.2A,B). Due to the lack of commercially available standards, further D-galacturonic acid linear oligomers could not be identified based on retention time comparison to standards. However, a linear relationship between retention time and the degree of polymerization (R^2 value of 0.996) for D-galacturonic acid to trigalacturonic acid was observed. As the HPAE-PAD peak retention times increased in these samples, the linear response was lost resulting in a convex graph. These results indicated that many of the chromatographic peaks observed in these samples were due to branched oligosaccharides with an estimated maximum DP of 10 under the chromatographic conditions employed, based on their retention times. Similar linear and non-linear relationships have been observed for glucose, maltose and maltotriose (linear DP1-3) and isomaltose and isomaltotriose (branched DP2 and DP3), respectively (Lee, 1996). The presence of branched D-galacturonic acid containing oligosaccharides would be expected in these samples due to the complex structure of pectin (Willats et al., 2006; Round et al., 2010; Yapo, 2011) and the endo-hydrolytic activity of polygalacturonases and polygalacturonate lyases in the commercial enzyme preparations.

The lack of identity of D-galacturonic acid methyl esters in these samples is readily explained by the highly alkaline conditions used in HPAE-PAD, which readily hydrolyzes the ester to its corresponding carboxylic acid (Daas et al., 1998). This observation was confirmed as a D-galacturonic acid methyl ester standard eluted at the same retention time as D-galacturonic acid.

A large number of chromatographic peaks eluting between galacturonic acid and trigalacturonic were observed in the laboratory scale pectin hydrolysis samples (Figure 4.2A) and were not present in the commercial juice samples (Figure 4.1). The presence of these compounds in the laboratory scale pectin hydrolysis samples was shown to be due to incomplete pectin hydrolysis as they were absent in laboratory scale samples treated with additional pectinases (6 to 10 times the producer recommended dosages).

In addition to pectin oligosaccharides, a number of the peaks eluting before 5.0 minutes in the laboratory scale pectin hydrolysis samples were identified as the neutral monosaccharides arabinose, galactose and glucose by retention time comparison to standards and spiking

experiments (Section 4.3.9), which is in agreement with literature reports on the monosaccharide composition of pectin (Round et al., 2010).

Due to the presence of starch in pear and the inclusion of amylases during commercial fruit juice processing, laboratory scale samples containing starch (2.0% w/w pectin plus 0.5% w/w starch) were subjected to each of the commercial enzyme treatments shown in Table 4.1. Each of the enzyme sets resulted in similar chromatographic profiles, and a representative HPAE-PAD chromatogram showing the results from these experiments is shown in Figure 4.2B. The major change observed in the oligosaccharide pattern when compared to that of pectin hydrolysis only (Figure 4.2A) was the production of maltose, which overlapped with a compound found in pectin hydrolysis, and maltotriose at retention times of ~9.6 and ~19.0 minutes, respectively. The identity of these compounds was confirmed by retention time comparisons to standards and spiking experiments. Isomaltose (RT of 5.0 minutes under the experimental conditions employed), a related disaccharide, was not identified in the laboratory scale pectin plus starch samples. As enzymatic hydrolysis of wheat starch would be expected to show some isomaltose due to its high amylopectin content (70 to 80%) (Båga et al., 1999), the absence of this compound in these samples may be explained by the inability of the amylases in the commercial enzyme preparations to hydrolyze starch near the α-(1→6) branch points or through the activity of a debranching enzyme (e.g. pullulanase) in the commercial enzyme mixture. For example, it was shown that human salivary α-amylase was not able to hydrolyze amylopectin near branch points as illustrated by the production of only higher molecular weight oligosaccharides (i.e. DP >4) containing an α-(1→6) bond (Walker and Whelan, 1960). Also, it has been reported that commercial pectinase cocktails employed in juice processing may contain 20 or more enzymes with various activities (Horváth-Kerkai and Stéger-Maté, 2012). Isomaltose was not detected (<4 ppm) in any of the finished commercial pear concentrates, and these results were in agreement with a recent literature report on the oligosaccharide profile of 32 commercial pear juice concentrates representing five world production regions (Willems and Low, 2014). A series of glucose-oligosaccharide standards with degree of polymerization (DP) ranging from 4 to 7 (maltotetraose to maltoheptaose) were also analyzed using the same chromatographic conditions and had retention times ranging from 26.5 to 31.8 minutes. However, this region of the chromatogram contained a large number of peaks that were also present in the pectin hydrolysis samples and as such, no new peaks and/or significant changes in peak heights were observed. Based on these results, it was determined that with the

exception of maltose and maltotriose production, the presence of starch would not have a significant impact on the oligosaccharide profile of commercial pear juice as determined by HPAE-PAD.

Samples containing pectin and starch were run at both the natural pH of the pectin solution (pH ~ 3.2) as well as samples adjusted to pH 4.0 ± 0.1 to mimic the pH of pear fruit (FDA, 2007). It was found that changes in pH effected the overall rate of hydrolysis as determined by an increase in peak height/area of approximately 50% in the pH adjusted verses the unadjusted samples (chromatograms not shown), but did not otherwise impact the overall oligosaccharide profile (i.e. the formation of new peaks).

Laboratory prepared samples of pectin and pectin plus starch (2.0 and 0.5% w/w respectively) containing fructose (6.0%, w/w), glucose (2.0%, w/w), sucrose (1.0%, w/w) and sorbitol (2.5%, w/w), the major carbohydrates and polyol present in pear at their average concentrations in this fruit (Thavarajah and Low, 2006a; Willems and Low, 2014) were also subjected to each of the commercial processing enzyme sets (Table 4.1). Experimental results showed that the presence of these carbohydrates and sorbitol had no impact on oligosaccharide formation as determined by HPAE-PAD.

A series of experiments were conducted with mock pear juice (Materials and Methods Section 4.3.3) containing the average concentrations of pectin (2.0% w/w) and starch (0.5% w/w) in pear so as to determine the impact of a commercial enzyme set treatment (A; Table 4.1) on the °Brix of the finished juice. It was found that pectin hydrolysis resulted in a minimal (0.13) increase in mean °Brix, however starch hydrolysis resulted in a more significant increase of 0.58 (Table 4.2). The mean °Brix values for pectin (2.0%) alone and pectin (2.0%) plus starch (0.5%) in water at pH 4.0 were, 1.92 and 1.96, respectively, indicating that the presence of 0.5% starch had minimal impact on total °Brix (Table 4.2). However, when the same samples were subjected to enzyme set treatment (A; Table 4.1) the °Brix values increased by 0.19 (pectin only) and 0.57 (pectin plus starch). From these experimental results in can be concluded that pectin hydrolysis has a minimal impact on the °Brix value, however starch hydrolysis can lead to a significant increase in the total soluble solids as measured by °Brix.

Based on literature reports, an additional source of oligosaccharide formation during commercial pear processing is hemicellulose (Kashyap et al., 2001; Raffo et al., 2011). Although hemicellulose is not a primary target for hydrolysis during pear juice processing, many of the

commercially available pectinase preparations claim to contain hemicellulase activity. In order to investigate the role of hemicellulose hydrolysis on the carbohydrate profile of commercial pear juice, xyloglucan was studied as this polysaccharide has been reported to be the major constituent of hemicellulose in dicotyledons such as pear (Raffo et al., 2011). Structurally, the backbone of xyloglucan is identical to that of cellulose with repeating β-(1→4)-linked D-glucopyranose residues (Fry, 1989). In dicotyledons 60-75% of the glucose residues have an α-D-xlyopyranose residue attached at O-6, and while this residue may be present on its own, 30 to 50% are linked to D-galactopyranose by β-(1→2) linkages or, more rarely, to an L-arabinopyranose residue. Also, in some cases the galactose residue may be linked to L-fucose through an α-(1→2) linkage (Fry, 1989).

Table 4.2 Mean and standard deviation °Brix values for laboratory scale pectin (2.0% w/w) and pectin plus starch (2.0% w/w pectin and 0.5% w/w starch) hydrolysis experiments before and after treatment with Pectinex UltraMash, Pectinex UltraClear and Amylase AG 300 L (Enzyme Set A).

Sample	°Brix Before Enzyme Treatment	°Brix After Enzyme Treatment	Change in °Brix
Pectin in mock pear juice (pH 4.0)[a,b]	12.72 ± 0.03[c]	12.85 ± 0.04	0.13
Pectin plus starch in mock pear juice (pH 4.0)	12.78 ± 0.03	13.36 ± 0.05	0.58
Pectin in water (pH 4.0)	1.92 ± 0.03	2.10 ± 0.01	0.19
Pectin plus starch in water (pH 4.0)	1.96 ± 0.04	2.47 ± 0.01	0.51

[a]Pectin and starch concentrations were 2.0% and 0.5% (w/w) respectively.
[b]Mock pear juice: 2.0% glucose, 6.0% fructose, 1.0% sucrose and 2.5% (w/v) sorbitol in water.
[c]Average ± standard deviation, n=3.

Treatment of xyloglucan with each of the commercial pear juice processing enzyme sets (Table 4.1) resulted in the production of a series of peaks clustered around 30 minutes that ranged in retention times from 26.6 to 37.4 minutes (Figure 4.2C). Standards of cellobiose, cellotriose and isoprimeverose were analyzed in order to attempt to identify oligosaccharides produced during xyloglucan hydrolysis. Isoprimeverose was identified in all of the xyloglucan hydrolysis experiments with a retention time of 5.5 minutes, however its presence was obscured in the commercial juice processing stage samples by their major carbohydrate/polyol composition and concentration. Cellotriose was also observed in the xyloglucan hydrolysis samples at 11.8 minutes (Figure 4.2C), and this compound may also be present in commercial pear mash before and after

enzyme addition (Figures 4.1A and B), however this region of the HPAE-PAD chromatogram is complex.

Of significant interest is the lack of cellobiose (retention time of 7.0 minutes) production from xyloglucan employing commercial pear juice processing enzyme cocktails. This absence is supported by the analytical results from 32 commercial pear juice concentrates, which showed non-detectable levels (<4 ppm) of cellobiose (Willems and Low, 2014). The lack of cellobiose in commercial pear juice concentrates is important as its presence, coupled with excess concentrations of galacturonic acid, has been used as a marker for the total liquefaction of apples for juice production. During the total liquefaction process cellulases are added to the mash in order to completely degrade fruit cell walls, resulting in a higher yield of soluble solids in the final juice (Will et al., 2000). As this practice is illegal in both North America and the European Union, the absence of cellobiose in the samples analyzed in this study coupled with the oligosoaccharide profiles of finished concentrates previously reported by our group, increases the validity of using cellobiose as a marker for identifying juices produced via total liquefaction (Will et al., 2000; Willems and Low, 2014).

In addition to oligosaccharides, a number of other low molecular weight carbohydrates with retention times in the 4-5 minute region of the chromatogram were observed during xyloglucan hydrolysis and were tentatively identified by HPAE-PAD as arabinose, galactose, glucose and xylose. Based on these results, it reasonable to assume that pear xyloglucan would be hydrolyzed during commercial pear juice production.

Due to the complexity of the commercial pear juice chromatograms, which contained both linear and branched chain oligosaccharides from the hydrolysis of hemicellulose, pectin and starch, in addition to challenges with oligosaccharide (i.e. polysaccharide hydrolysis products) identification due to the lack of standards, the accurate identification and quantitation of the majority of these compounds was not possible. However, the concentrations of galacturonic acid, isoprimeverose and maltose in the commercial samples at each juice processing stage were determined by HPAE-PAD and CGC-FID. The concentration of galacturonic acid in the juice samples increased during processing with the largest increase (in general) occurring during enzymatic sample treatment (Table 4.3). Minimal changes in galacturonic acid levels were observed during juice concentration. Similar to galacturonic acid, the concentration of isoprimeverose in the commercial juice samples increased with processing stage. However, unlike

galacturonic acid, the final concentration step resulted in an increase in isoprimeverose concentration with the largest observed for juice 1 (USA product). Finally, maltose showed very consistent concentrations throughout processing and between all three commercial juices. While there was some variation in the concentration of the three carbohydrates analyzed in the three juices, commercial juice processing resulted in increasing galacturonic acid and isoprimeverose concentrations as a function of processing stage (Table 4.3).

Table 4.3 Galacturonic acid, isoprimeverose and maltose concentrations (ppm) in the three commercial pear juices at each processing stage, standardized to 12.0 °Brix.

	Galacturonic Acid (ppm)			Isoprimeverose (ppm)			Maltose (ppm)		
Processing Stage	**Juice 1**	**Juice 2**	**Juice 3**	**Juice 1**	**Juice 2**	**Juice 3**	**Juice 1**	**Juice 2**	**Juice 3**
Mash	404.1	292.5	56.9	178.0	145.1	130.5	61.4	70.2	57.2
After Enzyme Treatment	807.2	1479.4	1424.8	143.2	178.5	161.6	60.3	64.5	57.3
After Filtration	1383.8	1686.0	1472.6	196.3	175.1	200.2	58.3	63.5	58.0
Concentrate	1334.7	1712.7	1481.4	398.9	232.2	233.9	61.0	61.1	56.5

Chromatographic results from laboratory scale pear juice production employing Bartlett and D'Anjou pears (equal weight mixtures) and a representative set of commercial enzymes (set A, Table 4.1) is shown in Figure 4.1E. The resulting carbohydrate/oligosaccharide profile of the juice shows many similarities to the commercial juices with mono, di and trigalacturonic acid being identified in these samples. However, some peaks were absent in the laboratory scale sample, most notably the unidentified peak at approximately 19 minutes. These differences could be due to fruit maturity and/or processing differences (i.e. commercial vs. laboratory).

4.4.2 CGC-FID Analysis

4.4.2.1 Carbohydrate Profile Changes in Commercially Produced Pear Juice as a Function of Processing

Figure 4.3 (A-D) shows CGC-FID chromatograms of a representative commercial pear juice at each of the aforementioned (Section 4.4.1.1) stages of processing. As was the case with HPAE-PAD carbohydrate profiles, no significant differences were observed between the commercial samples obtained from the various producers, excluding some variations in compound concentration as indicated by peak height/area. Unlike HPAE-PAD, CGC-FID carbohydrate

analysis requires derivatization so as to afford compound volatility, and this typically limits detection to oligosaccharides of DP ≤ 3 (Lee, 1996; Montilla et al., 2006). However, the increased resolution of this method for structurally similar di- and trisaccharides makes CGC-FID a useful tool for oligosaccharide profiling (Low and Sporns, 1988; Ruiz-Matute et al., 2011). As was the case for HPAE-PAD, the derivatization method and separation conditions employed results in minimal interferences from non-carbohydrates (e.g. organic acids). However, it should be noted that some glycosylated phenolics, most notably arbutin, can be detected by this method (Willems and Low, 2014).

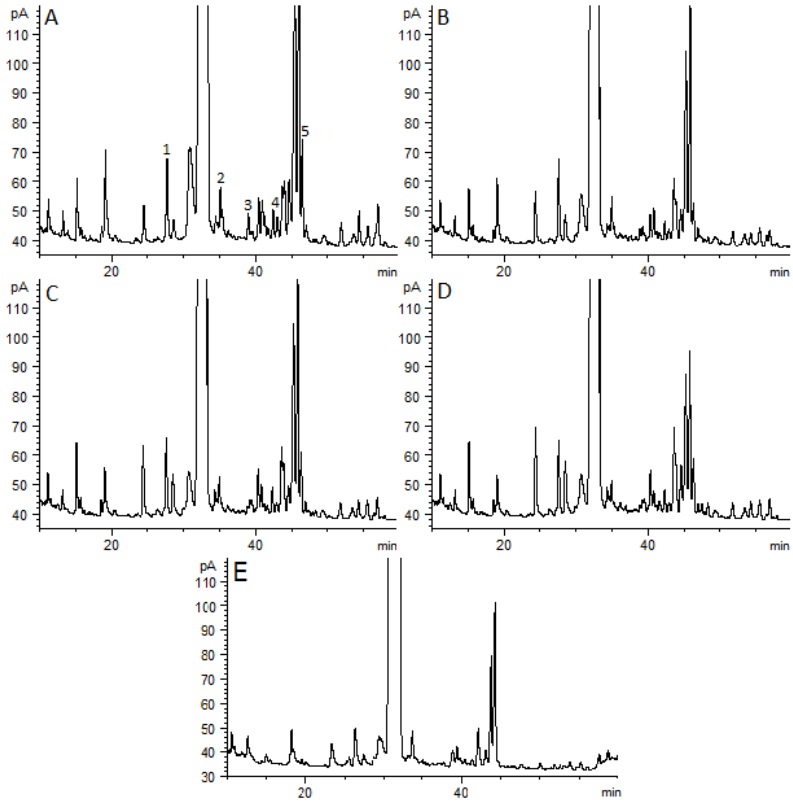

Figure 4.3 Representative CGC-FID chromatograms of pear juice at different stages of commercial processing. Commercial pear mash before enzyme addition (A), pear juice after enzymatic treatment to hydrolyze pectin and starch, (B), pear juice after preconcentration and ultra-filtration (C), the final pear juice after pasteurization and concentration (D) and laboratory scale pear juice produced with Pectinex UltraMash, Pectinex UltraClear and Amylase AG 300 L as outlined in Table 4.1A (E). Identified peaks are as follows: 1, arbutin; 2, α-maltose; 3, β-maltose; 4, α-isoprimeverose; 5, β-isoprimeverose.

Figure 4.3A represents the first stage of pear juice processing (mash) with glucose, fructose and sorbitol, as well as other monosaccharides (e.g. arabinose from hemicellulose hydrolysis) present, eluting as a large off-scale peak between ~4-8 minutes. The other major off-scale peak in this chromatogram at approximately 33 minutes was identified as sucrose. As well, arbutin was identified with a retention time of ~27.5 minutes (Willems and Low, 2014). The CGC-FID chromatogram contains a number of other peaks with retention times that correspond to disaccharides based upon the chromatographic conditions employed (Low and Sporns, 1988). The remaining three processing stage CGC-FID chromatograms (Figures 4.3B-D) were virtually identical in peak profile, however changes in the concentrations of the compounds present were observed as indicated by their peak heights. For example, there was a noticeable decrease in height in the peaks eluting between 43 and 47 minutes in the pasteurized and concentrated stage samples (Figure 4.3D).

4.4.2.2 Peak Identification from Laboratory Scale Pectin, Starch and Xyloglucan Hydrolysis

The CGC-FID chromatographic profiles for laboratory prepared pectin, pectin plus starch, and xyloglucan enzymatic hydrolysis samples were used to confirm some of the HPAE-PAD results.

Because Sylon TP has been shown to be effective for the derivatization of glucose polymers (e.g. DP1-3; Low and Sporns, 1988) prior to CGC-FID analysis, this derivatizing agent was employed for both the pectin plus starch, and the xyloglucan enzymatic hydrolysis samples. Galacturonic acid was identified in the pectin hydrolysis samples by CGC-FID (Section 4.3.11) as a group of four chromatographic peaks observed representing the α- and β- anomers of both the pyranose and furanose forms of galacturonic acid (Raymond and Nagel, 1969) with retention times of 19.6, 23.1, 24.9 and 28.6 minutes. Starch hydrolysis resulted in the production of maltose with retention times of 33.0 (α-anomer) and 37.2 (β-anomer), and xyloglucan hydrolysis produced α- and β-D-isoprimeverose with retention times of 43.2 and 46.7 minutes, respectively. Maltotriose and cellotriose were also identified by CGC-FID (Section 4.3.12) with retention times of 40.5 (α-anomer) and 41.1 minutes (β-anomer), and 41.3 (α-anomer) and 44.5 (β-anomer) minutes, respectively. The aforementioned CGC-FID results confirm those obtained by HPAE-PAD and also support the lack of isomaltose (retention times of 51.6 [α-anomer] and 57.5 [β-anomer]

minutes) and cellobiose formation (retention times of 36.2 [α-anomer] and 45.8 [β-anomer] minutes) in all commercial pear juice processing samples analyzed in this study. Also, no significant differences in the CGC-FID oligosaccharide profiles as a function of pH (i.e. 3.2 vs. 4.0) were observed in these samples.

The laboratory scale pear juice was also analyzed by CGC-FID (Figure 4.3E), and the resulting chromatogram showed noticeably fewer peaks when compared to the commercial samples (Figure 4.3D), which may be explained by fruit maturity and/or processing differences (i.e. commercial vs. laboratory).

Based on HPAE-PAD and CGC-FID results for commercial pear juice processing stage samples, a number of monosaccharides and oligosaccharides arising from pectin, starch and hemicellulose hydrolysis are produced during the mashing stage following exogenous carbohydrase treatment. Chromatographic results show minimal changes in this carbohydrate profile during the remaining commercial processing stages. Chromatographic results confirmed the presence of the following carbohydrates from pectin, starch and hemicellulose hydrolysis: D-galacturonic acid/D-galacturonic methyl ester; linear (DP2 and 3) polymers of D-galacturonic acid/D-galacturonic acid methyl ester; glucose, maltose and maltotriose; cellotriose; and isoprimeverose. Based on their retention times, it is hypothesized that the remaining chromatographic peaks observed in the commercial processing stage samples are due to branched oligomers/polymers of the aforementioned monomers. Due to the complexity of the HPAE-PAD and CGC-FID chromatograms of commercial pear juice processing stage samples, coupled with the lack of commercially available pectin and hemicellulose DP standards, complete compound identification was not possible.

The significance of these results positively impacts both industry and government regulatory agencies. From an industrial perspective, the ability to monitor changes and/or consistency in carbohydrate profiles chromatographically can be used for both quality assurance (i.e. so as to minimize mistakes and/or defects in the final product) and control (i.e. so as to meet the quality requirements of the manufacturer/industry) parameters. In addition, enzymatic treatment of juices could be tailored so as to increase its soluble fibre content, which could result in a final product with improved health properties, positively impacting both producers and consumers. For government regulatory agencies, the observed consistency in carbohydrate profiles of the finished product regardless of enzyme preparation and treatment, provides a baseline for

pure pear juice/concentrate. As such, observed changes in the carbohydrate profile would indicate the use of non-approved enzyme preparations (i.e. cellulases) and/or the undeclared addition of sweeteners (e.g. high fructose corn syrup), that is, product adulteration.

4.5 Linkage Between Oligosaccharide Formation in Pear Juice and Phenolic Content of Apple and Pear Juice

Results from this study proved that a number of monosaccharides and oligosaccharides arising from pectin, starch and hemicellulose hydrolysis were produced during pear juice production due to the activities of exogenous carbohydrases. It was proposed that this knowledge could be applied to the commercial processing of pear so as to produce juices with increased soluble fibre and therefore, improved health properties. As fruit juices are important sources of phenolics in the human diet, and these compounds are purported to have a number of beneficial health properties, the focus of the next study was the analysis and identification of phenolics in commercial apple and pear juices employing HPLC-PDA. In addition, these samples were analyzed for their antioxidant potentials employing two *in vitro* free radical scavenging assays.

5. PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITIES OF COMMERCIAL APPLE AND PEAR JUICES REPRESENTING MAJOR WORLD PRODUCING REGIONS[1]

5.1 Abstract

Due to their popularity, apple and pear juices are an important contributor of phenolics to the human diet. Many different and often contradictory studies on the phenolic content of apples and pears and their juices exist in literature. Therefore, the overarching goal of this work was to examine the phenolic profile and antioxidant capacity of a large (n = 56) database of commercially produced apple and pear juices representing the major world producing regions. The mean and standard deviation TPC of the commercial pear juice samples was 246.4 ± 45.1 ppm GAE. The mean and standard deviation TPC of the commercial apple juice samples was 294.7 ± 128.2 ppm GAE, and this mean value was not significantly different than that observed for pear juice (p-value > 0.05). In addition, changes in the phenolic profile of pear juice as a function of commercial processing conditions was examined employing TPCI.

5.2 Introduction

The term phenolics encompasses a variety of structurally diverse compounds with the common feature of at least one hydroxyl group directly attached to an aromatic ring. Phenolics are secondary metabolites that are derived from phenylalanine and to a lesser extent tyrosine, which are widely distributed in plants with more than 8,000 having been reported in literature (Manach et al., 2004; Vermerris and Nicholson, 2008; Tvermoes et al., 2014).

Phenolics are broadly divided into two main classes, the phenolic acids and the flavonoids, however, they are subdivided further within these main classes. For example, phenolic acids are

[1]Reproduced with permission. Willems, J. L. and Low, N. H. (2017). phenolic content and antioxidant activities of commercial apple and pear juices representing major world producing regions. Submitted to the Food & Nutrition Journal, June 2017.

divided into the hydroxybenzoic and hydroxycinnamic acids subclasses, and the flavonoids are divided into five major subclasses, namely the anthocyanins, flavanols, flavanones, flavones and flavonols. In addition, other minor subclasses also exist within the two main classes (Manach et al., 2004; Green, 2007; Gharras, 2009). Phenolics have a variety of functions in plants such as imparting colour, attracting or repelling insects, possessing both antimicrobial and antiviral activity, and providing UV protection (Manach et al., 2004).

Phenolics have been widely studied for their health promoting properties in humans, including antioxidant, anti-inflammatory and antiviral activities as well as the ability to inhibit carcinogenesis (Mori et al., 1986; Rice-Evans et al., 1996; McDougall et al., 1998; Kweon et al., 2001; Dos Santos et al., 2006; Aron and Kennedy, 2008; Joseph et al., 2016). In addition to health benefits, the presence of phenolics in foods as antioxidants are beneficial in the food industry as they extend product shelf-life by delaying undesirable free radical reactions such as lipid oxidation (Bonilla et al., 1999; Ahmad et al., 2015).

Apple and pear are both members of the Rosaceae family along with other major fruit producing plants such as apricot and peach, which are collectively known as the pome fruits (Cornille et al., 2014). Apples are grown in a variety of world geographical regions and are second in terms of fruit utilization for juice behind oranges (Bates et al., 2001), whereas pear juice is widely used in juice blends. While the concentrations of phenolics in apple and pear are lower than in other fruits/berries such as cranberry or blueberry, their high consumption levels (approximately 2.6 billion liters of apple juice was consumed by Americans in 2012) makes them an important contributor of phenolics to the human diet (Tanrıöven and Ekşi, 2005).

Many different and often contradictory studies on the phenolic content of apples and pears and their juices exist in literature. These results may be explained by the roles that variety, fruit maturity and ripeness, environmental growing conditions, storage, and processing have on both the fruit and fruit product (e.g. juice) phenolic composition and concentration (Spanos and Wrolstad, 1992; Tanrıöven and Ekşi, 2005; Nayak et al., 2015). The majority of published studies on apple and pear were conducted on whole fruit or on laboratory scale prepared juice and may not fully represent the phenolic/antioxidant profile of the commercial juices typically consumed. Therefore, the overarching goal of this work was to examine the phenolic profile and antioxidant capacity of a large (n = 56) database of commercially produced apple and pear juices representing the major world producing regions.

5.3 Materials and Methods

5.3.1 Samples

Thirty-two commercial pear juice and 24 commercial apple juice concentrates (~70 °Brix) representing three production years (2012-14) were analyzed in this study. Pear juice concentrates were obtained from Argentina, Chile, China, New Zealand and the United States of America. Apple juice samples were obtained from Argentina, Brazil, Chile, China and the United States of America.

Three sets of samples collected at various stages of pear juice processing (mash to concentrate) were received from two different commercial producers from South America. Processing stage samples were immediately heated at 90 °C for 3 min in order to inactivate enzymes prior to shipping. All samples were stored at -30 °C until analyzed.

5.3.2 Chemicals

Amberlite XAD16N resin, apigenin, ascorbic acid, arbutin, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), caffeic acid, 5-*O*-caffeoylquinic acid (chlorogenic acid), catechin, p-coumaric acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), epicatechin, ferulic acid, Folin and Ciocalteu's phenol reagent (2.0 N), formic acid, gallic acid, 4-hydroxybenzoic acid, 5-hydroxymethylfurfural (HMF), isorhamnetin-3-O-glucoside, isoquercetin, narigenin, phloridzin, potassium persulfate, quercetin, resveratrol, rutin, sodium carbonate and Trolox (6-hydroxy-2,5,6,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma Aldrich Canada Ltd. (Oakville, ON, Canada). Acetonitrile (HPLC grade) and methanol (ACS grade) were purchased from Fisher Scientific (Ottawa, ON, Canada). The water used throughout this study was produced using a Milli-Q™ water system (Millipore Corp., Milford, MA, USA).

5.3.3 Total Phenolic Chromatographic Index (TPCI)

The total phenolic chromatographic index (TPCI) of the apple and pear juice samples was determined employing an Agilent 1100 series HPLC system with a photodiode array (PDA) detector controlled by ChemStation LC-3D software (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Juice samples were prepared by dilution with water to 11.5 ± 0.1 and 12.0 ± 0.1 °Brix (Auto Abbe Refractometer; Lecia Inc., Buffalo, NY, USA) for apple and pear

juice, respectively. Samples containing particulates (i.e., mash samples) were centrifuged at 6000 rpm (Clinical 200 centrifuge, VWR International, Edmonton, AB, Canada) for 15 minutes and the supernatant was collected prior to dilution. Samples were syringe filtered (nylon, 0.2 µm pore size, 13 mm diameter, Chromatographic Specialties, Brockville, ON, Canada) into 2 mL amber HPLC vials (Chromatographic Specialties) prior to analysis. Phenolic separation was accomplished on an ODS-3 (250 x 4.6 mm; 5 µm, C_{18}, 100 Å) column (Phenomenex, Torrance, CA, USA) in series with a guard column (4 x 3 mm) of the same stationary phase. The sample injection volume was 60.0 µL. A linear gradient mobile phase system employing 10.0 mM aqueous formic acid (mobile phase A; pH 3.5) and 70% acetonitrile:30% mobile phase A (v:v; mobile phase B) was used for phenolic separation as follows: initial 100% A for 3.0 min, followed by a gradient to 4.0% B at 16.0 min, followed by a gradient to 10.0% B at 25.0 minutes, followed by a gradient to 15.0% B at 40.0 min, followed by a gradient to 20.0% B at 45.0 min, followed by a gradient to 23.0% B at 50.0 min, followed by a gradient to 25.0% B at 55.0 min, followed by a gradient to 30.0% B at 61.0 min, followed by a gradient to 50.0% B at 75.0 min, followed by a gradient to 80.0% B at 80.0 min, hold at 80.0% B for 5.0 min. The column was then re-equilibrated with 100% A for 7.0 minutes prior to the next injection. The total run time was 95.0 minutes.

Sample phenolic profiles were monitored at 254, 280 and 360 nm. Based upon their UV-vis spectra, chromatographic peaks were assigned to the following phenolic classes, simple phenols, hydroxybenzoic acids, hydroxycinnamic acids, flavanols, flavonols, dihydrochalcones, flavones and flavanones. Each phenolic class was quantified using a representative compound from that class as follows, simple phenols were represented by arbutin, hydroxybenzoic acids by gallic acid, hydroxycinnamic acids by chlorogenic acid, flavanols by epicatechin, flavonols by quercetin, dihydrochalcones by phloridzin, flavones by apigenin and flavanones by narigenin. Sample phenolics were quantified at 280 nm except for the flavonols, which were quantified at 360 nm. Standard curves were prepared and ranged from 0.1 to 100.0 ppm and had r^2 values $\geq$0.990. The concentration of each phenolic class was then summed to calculate sample TPCI. All samples were analyzed in duplicate.

5.3.4 Total Phenolic Content (TPC)

The total phenolic contents of whole and fractionated juices were determined by the Folin-Ciocalteu method. To 1.25 mL of 0.2 N Folin-Ciocalteu reagent (diluted from 2.0 N stock with

water), 250.0 μL of appropriately diluted sample (i.e., approximately 2.0 and 2.5 °Brix for apple and pear juice, respectively) was added. Samples were vortexed (Fisher Scientific) for 15 seconds followed by the addition of 1.0 mL of 15.0% (w:v) sodium carbonate with vortexing for an additional 15 seconds. Samples were then held static in the dark at room temperature (23 ± 2 °C) for 2.0 hours. Sample absorbance was measured at 765 nm employing a Genesys 10S UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Sample total phenolic content was reported as gallic acid equivalents by comparison to a concurrently analyzed standard curve of this compound in water at concentrations ranging from 10.0 to 50.0 ppm. Standard curves had r^2 values ≥0.999. All samples were analyzed in triplicate.

5.3.5 Juice Phenolic Fractionation

Six commercial juice concentrates (three each of apple and pear) were chosen as representatives of high (H), medium (M) and low (L) TPC (H: 250-311 ppm and 350-490 ppm; M: 205-249 ppm and 200-349 ppm; and L: 120-204 ppm and 80-199 ppm for pear and apple respectively). Sample phenolic fractions were produced using Amberlite XAD16N resin (Sigma-Aldrich) in conjunction with differing aqueous methanol concentrations (i.e. 0, 40 and 70%, v:v). Juice samples were prepared by water dilution of commercial concentrates to 23.0 ± 0.1 and 24.0 ± 0.1 °Brix for apple and pear juice, respectively. The resin was initially hydrated in 50% aqueous methanol (v:v) for 30 minutes before being transferred into a glass column (30 x 0.5 cm) to produce a resin bed of approximately 30 mL. The resin bed was conditioned by sequential washing with 60 mL each of water and 90% aqueous methanol (v:v), followed by an additional 60 mL of water. A 5.0 mL aliquot of juice sample was then loaded onto the resin bed for phenolic fractionation. Water soluble sample components (e.g. carbohydrates and organic acids) were eluted with 60 mL (two bed volumes) of water, followed by sequential elution with 60 mL of 40% methanol (v:v) and 60 mL of 70% methanol (v:v) to produce phenolic fractions. Individual fractions were concentrated to approximately 1 mL by rotary evaporation (Büchi, Switzerland) before being transferred to 2.0 mL HPLC vials and freeze-dried (Heto Lab Equipment, Allerod, Denmark). Samples were stored at -18 °C for further analysis. All samples were prepared in triplicate.

5.3.6 Trolox Equivalence Antioxidant Capacity (TEAC) Assay for ABTS Radical Scavenging Activity

A 7.0 mM ABTS stock solution was prepared by dissolving 38.5 ± 0.2 mg of ABTS in 10.0 mL of water. ABTS radical cations ($ABTS^{\bullet+}$) were produced by mixing 2.0 mL of stock solution with 1.0 mL of 7.0 mM potassium persulfate (18.9 mg/10.00 mL water) and this mixture was kept in the dark overnight at room temperature. The $ABTS^{\bullet+}$ solution was then diluted with 70% aqueous methanol (v:v) to give an absorbance reading of 0.75 ± 0.05 at 734 nm. Fresh $ABTS^{\bullet+}$ solution was prepared for each sample set.

Juice samples were diluted with water, and phenolic fractions with 70% aqueous methanol to produce data curves that had $ABTS^{\bullet+}$ radical scavenging activities ranging from ~10 to 80% (i.e. concentrations ranging from 1.5 to 24.0 °Brix). The synthetic vitamin E analogue Trolox was used for $ABTS^{\bullet+}$ radical scavenging comparison and was prepared at concentrations ranging from 0.4 to 2.0 mM in 70% aqueous methanol (v:v).

Sample radical scavenging ability was determined by adding 10.0 µL of the appropriately diluted juice, phenolic fraction or standard to 1.0 mL of diluted $ABTS^{\bullet+}$ solution. The absorbance at 734 nm was recorded after 6 minutes of incubation in the dark for each sample and the percent inhibition was calculated as follows, where 10.0 µL of 70% methanol was used as the blank:

$$\%\ ABTS^{\bullet+}\ \text{inhibition} = [1 - (A_{734}\ \text{sample}/A_{734}\ \text{blank})] * 100$$

The % $ABTS^{\bullet+}$ inhibition was plotted as a function of sample concentration and the linear regression equation for each sample was determined. The % $ABTS^{\bullet+}$ inhibition of 1.0 mM Trolox was determined from the slope of the linear regression line. Sample linear regression equations were then used to determine the concentration of sample required to inhibit the same percentage of $ABTS^{\bullet+}$ as 1.0 mM of Trolox (Y_{TE}). The Trolox equivalent antioxidant capacity (TEAC) was expressed as the equivalent activity of 1.0 mM Trolox in 100 mL of sample and was calculated as $100/Y_{TE}$. Sample analysis was performed in triplicate.

5.3.7 DPPH Radical Scavenging Assay

A 500 µM DPPH solution was prepared by dissolving 9.8 ± 0.2 mg of DPPH in 50 mL of 70% aqueous methanol (v:v). Solutions were sonicated (Branson 2510 Ultrasonic Cleaner,

Branson, MO, USA) for 20 minutes to ensure complete solubilisation and fresh solutions were prepared for each sample set. Juice samples were diluted with water, and phenolic fractions with 70% aqueous methanol to give final % DPPH radical scavenging activities from 10 to 80%. A 250.0 µL aliquot of appropriately diluted sample was added to 1.0 mL of DPPH solution and the mixture was vortexed for 10 to 15 seconds. The samples were kept static at room temperature in the dark for 15 minutes before their absorbance at 517 nm was determined. A control consisting of 250.0 µL of 70% methanol was also analyzed in conjunction with samples. Percent DPPH radical scavenging activity was calculated as follows:

$$\% \text{ DPPH radical scavenging} = [1 - (A_{517} \text{ sample}/A_{517} \text{ control})] * 100$$

A plot of the % DPPH radical scavenging activity versus sample concentration for each sample was prepared and the concentration at 50% radical scavenging activity (IC_{50}) was determined from linear regression equations. Sample antioxidant activity was reported as $1/IC_{50}$. All samples were analyzed in triplicate.

5.3.8 Statistics

An analysis of variance (one-way ANOVA) with Tukey's HSD (honest significant difference) *post hoc* test was used to determine significant differences ($p<0.05$) between experimental means. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software (SPSS Inc., ver. 24.0, 2016, Chicago, IL).

5.4 Results and Discussion

Apple is ranked second in juice consumption after orange, with an estimated value of $82.8 million in the United States alone (USDA, 2016). While pear juice consumption is lower than apple, it is often used as an ingredient in juice blends. Although apple and pear juices have lower concentrations of bioactive compounds when compared to cranberry or raspberry juice, their popularity and usage make them an important source of bioactive compounds such as phenolics in the North American diet (Chun et al., 2005). Goals of this research were, (1) to examine the total phenolic content (TPC) and antioxidant potentials of commercial apple and pear juice from the major world producing regions; (2) to relate TPC and total phenolic chromatographic index (TPCI)

data to world producing regions; (3) examine the relationship between apple and pear juice free radical scavenging and phenolic structure; and (4) investigate the relationship between the different stages of commercial pear processing and their phenolic content by TPCI.

5.4.1 Total Phenolic Content (TPC) and Total Phenolic Chromatographic Index (TPCI)

Currently, there are two major methods for measuring total phenolics in food products, one that is chemical and the other chromatographic, namely the total phenolic content (TPC) and the total phenolic chromatographic index (TPCI), respectively (Green, 2007; Khoddami et al., 2013). The TPC method is the older of the two and is also referred to as the Folin-Ciocalteu (F-C) assay.

Due to its simplicity, reproducibility and compatibility with a wide range of foods, the F-C method is often used to obtain an estimate of the total phenolic content of fruits and juices (Green, 2007; Shaghaghi et al., 2008). The F-C reagent consists of a mixture of sodium molybdate and sodium tungstate that react with phenols to produce a blue colour, which is measured at 765 nm (Shaghaghi et al., 2008; Everette et al., 2010). The absorbance at 765 nm is then related to sample phenolic content by comparison to a standard, typically gallic acid, and is reported as gallic acid equivalents or GAE. Unfortunately, the F-C assay is subject to interference from other compounds commonly found in food products, such as amino acids, inorganic ions (i.e., Fe^{2+} and Mn^{2+}) and vitamins (i.e., Vitamin C), and other less ubiquitous compounds such as copper complexes, which can artificially inflate the total phenolic content value obtained by this method (Everette et al., 2010).

The total phenolic chromatographic index (TPCI) avoids the issues of the interfering compounds that plague the F-C method, however, it requires sophisticated analytical equipment, more advanced personnel training, and is more time consuming than the F-C method (Green, 2007). The major advantages of the TPCI method is that it provides important information on the subclasses and specific phenolics present in a product, something the F-C method cannot do. Sample TPCI is measured by determining its phenolic profile by high performance liquid chromatography coupled with photodiode array detection (HPLC-PDA). Phenolics are then grouped into classes based upon their characteristic UV-vis spectra and each class is quantified using a standard from that class. In order to obtain the final TPCI value the concentration of all phenolic classes are summed (Escarpa and González, 2001a; Green, 2007). The phenolic classes assigned to samples in this study included, hydroxybenzoic acids, hydroxycinnamic acids and the

flavonoids, specifically, flavanols, flavanones/dihydrochalcones, flavones and flavonols. A representative chromatogram of the phenolic standards analyzed in this study is shown in Figure 5.1.

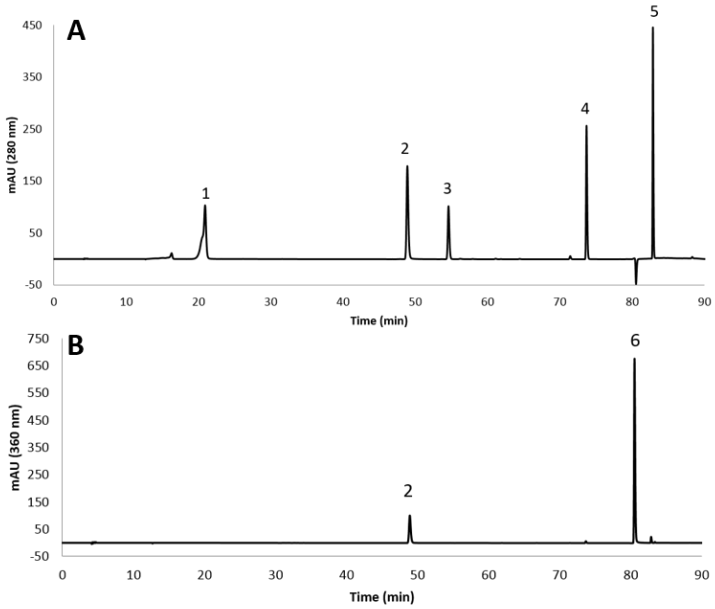

Figure 5.1 HPLC-PDA chromatograms of phenolic standards at 280 and 360 nm. Where: 1) gallic acid, 2) chlorogenic acid, 3) epicatechin, 4) phloridzin, 5) narigenin and 6) quercetin.

Commercial apple and pear juice concentrates were obtained from the major world production regions for these fruits, which were subsequently used in juice production. Pear juice was received from five different companies representing Argentina, Chile, China, New Zealand and the United States, while apple juices were obtained from five different companies representing Argentina, Brazil, Chile, China and the United States (Willems and Low, 2014).

The TPC values for the 24 commercial apple and 32 commercial pear juice samples representing each of the major world geographical regions for these fruits/juices were determined by the F-C method and are shown in Tables 5.1 and 5.2. The mean and standard deviation TPC of the commercial pear juice samples was 246.4 ± 45.1 ppm GAE with a range of 120.9 to 311.1 ppm GAE. The mean and standard deviation TPC of the commercial apple juice samples was 294.7 ± 128.2 ppm GAE, and this mean value was not significantly different than that observed for pear juice (p-value > 0.05). While the mean TPC did not differ significantly between commercial apple

and pear juice samples, apple showed a greater TPC range of 82.8 to 487.6 ppm GAE, and a higher maximum value (i.e. 487.6 vs. 311.1 ppm GAE). The observed larger range in TPC and greater standard deviation values for apple are most likely due to the number of different fruit varieties used in commercial apple juice production (i.e. greater than 14 varieties in a single blend in some cases) as compared to the two to three varieties normally used in pear juice (Willems and Low, 2014), as it has been shown that the total phenolic content can vary with fruit variety (Spanos and Wrolstad, 1990; Lee et al., 2003).

Comparison of TPC results for pear juice samples showed that there were no significant differences based on fruit production and processing regions (Table 5.3). However, apple juice concentrates from China showed significantly lower mean TPC values (147.0 ± 61.7 ppm GAE) when compared to those from Argentina (359.2 ± 150.9 ppm GAE), Chile (384.2 ± 28.1 ppm GAE) and the United States (369.1 ± 108.6 ppm GAE). However, the mean TPC values for China were not significantly different from those obtained for apple juices from Brazil (231.9 ± 56.4 ppm GAE) (Table 5.4). These differences were most likely due to differences in the apple varieties used in juice production from this geographical region and the environmental growing conditions during fruit production. The limitations of this study include the small selection of commercial samples analyzed from each world production region (ranging from three to five samples) and the lack of information on the specific apple varieties used in juice production.

The observed TPC mean (294.7 ppm GAE) and range (82.8 to 487.6 ppm GAE) values for commercial apple juice samples in this study were similar to the range of values (142 to 780 ppm GAE) previously reported for laboratory scale apple juices produced from different apple varieties (Spanos et al., 1990). The apple varieties used for laboratory juice production were Granny Smith, McIntosh, Red Delicious and Spartan which are all used in commercial apple juice production, however, these varieties were analyzed separately where commercial juices utilize a blend of varieties. The same authors analyzed four commercial apple juice concentrates and found the total phenolic content of these samples ranged from 49 to 224 ppm GAE (Spanos et al., 1990). Although these values were on the lower end of the concentration range observed in our study, only one (i.e., 49 ppm GAE) was outside the range reported for the 24 commercial apple juices analyzed. The lower TPC values observed in the commercial apple juice samples in this study when compared to the laboratory produced juices can be explained by the harsher processing conditions in the former, which can result in phenolic structure changes and degradation.

Table 5.1 Mean and standard deviation data for total phenolic content (TPC), total phenolic chromatographic index (TPCI), and Trolox equivalence antioxidant capacity (TEAC) and DPPH radical scavenging assays of 24 commercial apple juices.

Juice	Geographical Origin	Total Phenolic Content (TPC)[a]	Total Phenolic Chromatographic Index (TPCI)[b]	TEAC[c]	DPPH Radical Scavenging Activity[d]
1	Argentina	378.1	142.1	179.2	30.6
2	Argentina	101.6	108.3	45.5	6.1
3	Argentina	384.6	134.0	183.6	28.9
4	Argentina	487.6	178.1	216.9	37.8
5	Argentina	444.2	160.9	210.9	37.7
6	Brazil	183.3	88.0	88.6	11.0
7	Brazil	195.7	84.9	82.1	13.6
8	Brazil	193.5	83.5	89.9	12.9
9	Brazil	296.5	117.3	132.9	10.1
10	Brazil	290.4	119.6	134.6	10.5
11	Chile	393.9	132.5	190.2	30.5
12	Chile	350.7	131.1	196.1	29.6
13	Chile	375.4	159.1	149.8	24.9
14	Chile	417.0	150.0	160.9	27.0
15	China	83.0	33.6	33.3	4.9
16	China	214.3	203.2	79.1	12.5
17	China	82.8	41.5	33.8	6.5
18	China	160.3	102.8	59.1	10.1
19	China	194.8	118.4	88.6	12.9
20	USA	410.0	160.8	167.7	30.1
21	USA	175.5	88.9	79.1	13.5
22	USA	407.7	167.3	180.3	39.3
23	USA	419.3	194.9	222.0	38.2
24	USA	432.9	189.2	199.2	38.0
Mean		294.7 ± 128.2	128.8 ± 44.9	130.8 ± 60.8	21.5 ± 12.1
Min		82.8	33.6	33.2	4.9
Max		487.6	203.2	222.0	39.3

[a]Reported as ppm gallic acid equivalents (n = 3).
[b]Reported as mg phenolics/L of juice (n = 2).
[c]Expressed as mM Trolox/100 mL juice (n = 3).
[d]Expressed as $1/IC_{50}$ (1/mL of juice for 50% DPPH radical inhibition; n = 3).

Table 5.2 Mean and standard deviation data for total phenolic content (TPC), total phenolic chromatographic index (TPCI), and Trolox equivalence antioxidant capacity (TEAC) and DPPH radical scavenging assays of 32 commercial pear juices.

Juice	Geographical Origin	Total Phenolic Content (TPC)[a]	Total Phenolic Chromatographic Index (TPCI)[b]	TEAC[c]	DPPH Radical Scavenging Activity[d]
1	Argentina	247.6	193.1	99.8	14.1
2	Argentina	238.8	196.2	134.7	14.4
3	Argentina	238.7	185.5	122.9	15.2
4	Argentina	277.7	220.5	125.5	15.7
5	Argentina	264.4	215.8	133.8	17.5
6	Argentina	274.8	225.7	130.6	18.2
7	Argentina	281.7	229.1	159.6	18.8
8	Argentina	278.3	222.5	150.3	15.5
9	Argentina	295.7	224.9	147.4	15.6
10	Argentina	289.0	229.7	114.7	25.0
11	Argentina	298.5	244.4	148.7	15.9
12	Chile	248.1	222.4	148.9	12.5
13	Chile	265.9	235.9	140.2	14.6
14	Chile	260.0	222.2	144.5	12.9
15	Chile	269.7	227.5	143.4	12.5
16	Chile	257.0	224.8	125.6	11.9
17	Chile	256.7	223.7	123.5	12.8
18	Chile	273.5	232.5	157.0	13.8
19	China	187.7	172.4	167.3	6.6
20	China	311.1	401.4	375.2	14.4
21	China	181.6	178.4	172.2	10.3
22	China	250.9	305.6	360.4	10.0
23	New Zealand	203.6	147.0	149.8	11.7
24	New Zealand	120.9	120.1	76.9	5.5
25	New Zealand	167.5	108.8	92.6	5.5
26	New Zealand	279.9	257.9	188.0	17.3
27	New Zealand	288.8	287.9	225.5	13.5
28	USA	253.7	187.9	111.9	13.9
29	USA	159.4	100.6	93.8	7.5
30	USA	232.3	177.5	131.3	15.2
31	USA	191.4	156.2	110.7	11.0
32	USA	238.9	197.0	117.7	16.4
Mean		246.4 ± 45.1	211.7 ± 57.2	150.8 ± 63.9	13.6 ± 5.5
Min		120.9	100.6	76.9	5.5
Max		311.1	401.4	375.2	25.0

[a]Reported as ppm gallic acid equivalents (n=3).
[b]Reported as mg phenolics/L of juice (n=2).
[c]Expressed as mM Trolox/100 mL juice (n=3).
[d]Expressed as $1/IC_{50}$ (1/mL of juice for 50% DPPH radical inhibition; n=3).

Table 5.3 Mean and standard deviation data for total phenolic content (TPC), total phenolic chromatographic index (TPCI), DPPH and TEAC of commercial pear juices by country.

Geographical Origin[a]	Total Phenolic Content (ppm GAE)	Total Phenolic Chromatographic Index (ppm)	DPPH[b]	TEAC[c]
Argentina	359.2 ± 150.9[a]	144.7 ± 26.6[a]	28.2 ± 13.0[ab]	167.2 ± 70.0[a]
Brazil	231.9 ± 56.4[ab]	98.7 ± 18.1[a]	11.6 ± 1.5[ac]	105.6 ± 25.9[ab]
Chile	384.2 ± 28.1[a]	143.2 ± 13.7[a]	28.0 ± 2.6[ab]	174.2 ± 22.4[a]
China	147.0 ± 61.7[b]	99.9 ± 68.6[a]	9.4 ± 3.6[c]	58.8 ± 25.4[b]
United States	369.1 ± 108.6[a]	160.2 ± 42.4[a]	31.8 ± 10.9[b]	169.7 ± 54.6[a]

[a]Values marked with different letters within a column were statistically different ($p < 0.05$).
[b]Reported as mL of DPPH/mL of juice.
[c]Reported as mM Trolox equivalents/100 mL juice.

Table 5.4 Mean and standard deviation data for total phenolic content (TPC), total phenolic chromatographic index (TPCI), DPPH and TEAC of commercial apple juices by country.

Geographical Origin[a]	Total Phenolic Content (ppm GAE)	Total Phenolic Chromatographic Index (ppm)	DPPH[b]	TEAC[c]
Argentina	217.0 ± 18.0[a]	271.4 ± 21.4[a]	16.9 ± 3.1[a]	133.4 ± 17.4[a]
Chile	227.0 ± 5.3[a]	261.6 ± 8.7[a]	13.0 ± 0.9[ab]	140.4 ± 12.1[a]
China	264.5 ± 110.0[a]	232.8 ± 60.9[a]	10.3 ± 3.2[b]	268.8 ± 114.5[b]
New Zealand	184.4 ± 82.7[a]	212.2 ± 72.2[a]	10.7 ± 5.1[b]	146.5 ± 62.7[a]
United States	163.8 ± 38.5[a]	215.1 ± 38.8[a]	12.8 ± 3.6[ab]	113.1 ± 13.5[a]

[a]Values marked with different letters within a column were statistically different ($p < 0.05$).
[b]Reported as mL of DPPH/mL of juice
[c]Reported as mM Trolox equivalents/100 mL juice

Spanos and Wrolstadt (1990) reported that the mean TPC of laboratory scale pear juices produced separately from Bartlett, Comice and d'Anjou pears ranged from 134 to 542 ppm GAE depending upon pear variety and processing conditions. The observed mean (246.4 ppm GAE) and range (120.9 to 311.1 ppm GAE) TPC values for the 32 commercial pear juice samples in this study were on the lower end of the aforementioned results. These differences in TPC can be explained by the harsher processing conditions (e.g. time and temperature) employed in commercial processing than those used to produce the laboratory scale samples.

To the best of our knowledge, this work is the first to focus on the TPC values for an extensive sampling of commercial apple and pear juices, and the first to investigate the relationship between sample TPC value and world fruit geographical regions.

The 24 commercial apple juices analyzed in this study had a mean TPCI of 128.8 mg/L and ranged from 33.6 to 203.2 mg/L. The TPCI values of the 32 commercial pear juices had a mean value of 211.7 mg/L, and ranged from 100.6 to 401.4 mg/L. Representative chromatographic phenolic profiles as determined by HPLC-PDA for apple and pear juices are shown in Figure 5.2. The observed wide TPCI ranges for both commercial apple and pear juices are due to a number of factors including, but not limited to, fruit variety, environmental fruit growing conditions and ripeness, and juice processing and storage conditions (Klimczak et al., 2007; Sánchez-Rodríguez et al., 2012; Teixeira et al., 2013). The calculated TPCI mean values for apple and pear juices were found to be significantly different (p-value < 0.05). Previous research has shown that TPC values are not always correlated or may only show poor correlation with TPCI results, which is likely due to compounds which may interfere with the F-C method (Spanos et al., 1990).

Literature has focused on the TPCI of unprocessed fruit, often looking at the peel and pulp separately. In general, the peel of the fruit is reported to have a higher phenolic content than the pulp as shown by TPCI range values for apple pulp and peel of 176 to 891 mg/kg and 663 to 4265 mg/kg, respectively; and for pear pulp and peel of 73 to 149 mg/kg and 386 to 2951 mg/kg, respectively (Escarpa and González, 2001a; Escarpa and González, 2001b; Tsao and Yang, 2003). The experimental mean TPCI range values for the commercial pear juice samples analyzed were comparable to the literature midrange of the pulp, and on the low end of the peel. The TPCI range observed for the commercial apple juice samples analyzed in this study were much lower than those reported for both apple pulp and peel. These observed TPCI differences were most likely due to the phenolic extraction solvents used for the pulp and peel samples of methanol:water (70:30 to 100:0; v:v). The use of an alcohol like methanol with a dielectric constant (i.e. polarity measure, where the higher the number the more polar the solvent) of 32.7 at 25 °C and when mixed with water (dielectric constant of 78.5) gives 70:30 methanol:water a dielectric constant of 47.1 (Albright and Gosting, 1946); are specifically used to extract both hydrophilic (e.g., hydroxybenzoic acids) and hydrophobic (e.g. flavonols) phenolic compounds from fruits such as pear and apple. Whereas, in commercial juice processing the focus is on increasing overall juice yield (i.e., total carbohydrates) rather than phenolic extraction, and water is the solvent.

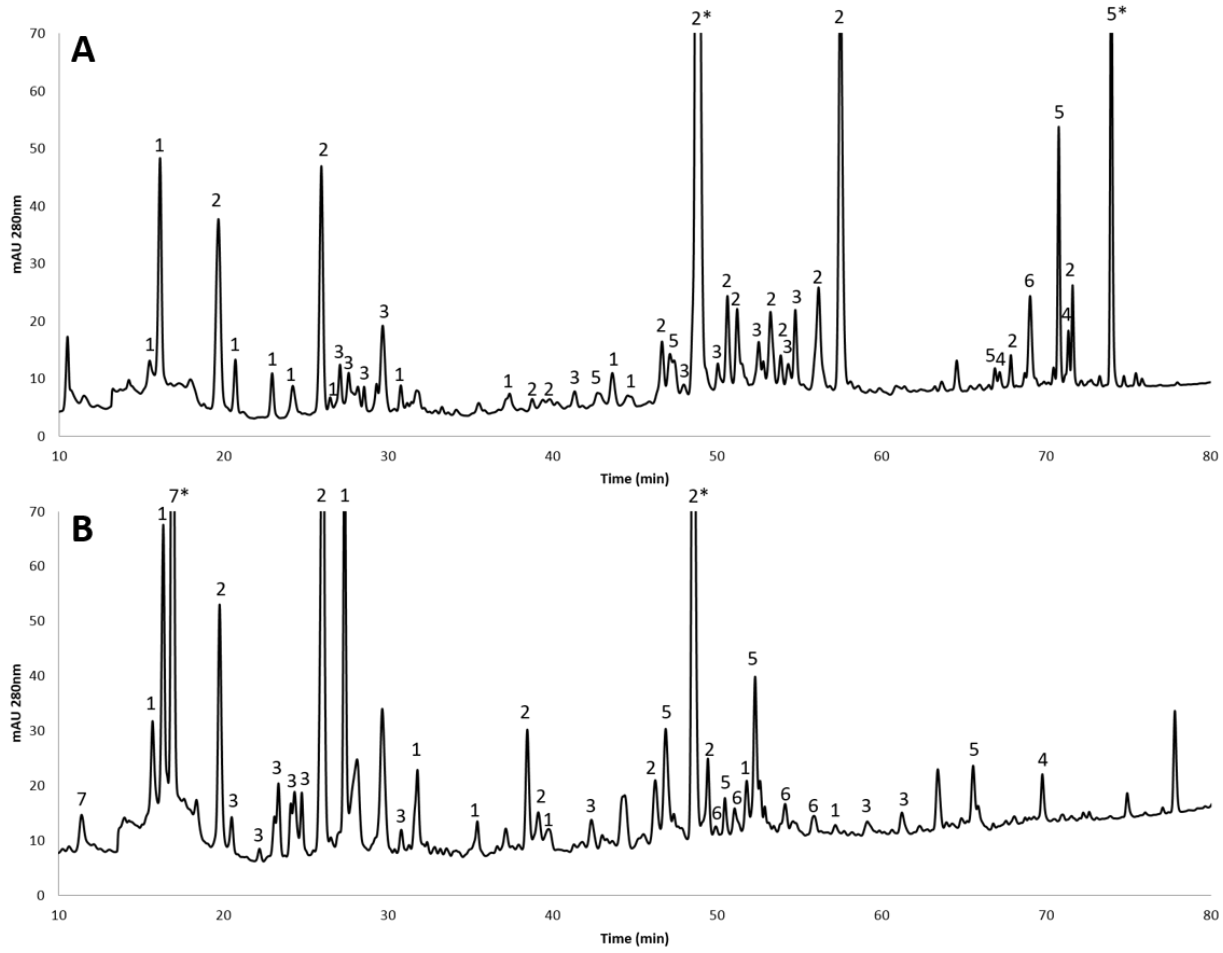

Figure 5.2 HPLC-PDA chromatograms of pear (A) and apple (B) juice. Where: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols; 5) dihydrochalcones/flavanones; 6) flavones; 7) simple phenolics. Peak assignments: 2*: 5-*O*-caffeoylquinic acid; 5*: phloridzin; and 7*: arbutin.

The hydroxybenzoic and hydroxycinnamic acids had the largest contribution to the overall TPCI of the commercial apple juice (Table 5.5) with concentrations ranging from 10.1 to 71.8 ppm and 14.0 to 165.2 ppm corresponding to 5.5 to 40.3% and 33.9 to 81.3% of the overall TPCI, respectively. As examples, when the hydroxybenzoic acid content was low (i.e., 5.5% for AJ 23) the hydroxycinnamic acid was higher (56.4%), whereas AJ 7 had a lower contribution from hydroxycinnamic acids (33.9%) and a higher contribution from hydroxybenzoic acids (40.3%). The most abundant phenolic in apple juice was the hydroxycinnamic acid 5-*O*-caffeoylquinnic acid (5-CQA; chlorogenic acid), which ranged in concentration from 7.0 to 86.0 ppm. Following these two classes, the flavanols were the next most abundant with concentrations ranging from 2.1 to 53.6 ppm (5.0 to 52.2% of the TPCI) in apple juice. The remaining phenolic classes (flavonols,

dihydrochalcones, flavones, flavanones) each individually accounted for less than 10% of apple juice TPCI.

For the commercial apple juice samples, it was found that there was no significant difference between geographical regions based on their TPCI (Table 5.3). However, differences were observed between the different phenolic subclasses. For example, samples from Argentina had the highest hydroxybenzoic acid content (43.3 ± 19.9 ppm), thus being significantly different from all other geographical regions except Chile.

Arbutin was a major contributor to pear juice TPCI with concentrations ranging from 50.6 to 286.0 ppm (Willems and Low, 2014), which corresponded to 28.4 to 75.9% of the overall TPCI. Additionally, the hydroxycinnamic acids were a significant contributor to pear juice TPCI in the majority of cases with concentrations ranging from 6.4 to 89.5 ppm, which corresponded to 4.2 to 36.6% of the overall TPCI. The most abundant hydroxycinnamic acid in pear was 5-*O*-caffeoylquinic acid with concentrations ranging from <0.1 to 45.7 ppm. Following arbutin and the hydroxycinnamic acids, the hydroxybenzoic acids and flavanols were the next major phenolic classes identified in pear juice with concentrations ranging from 7.0 to 43.7 ppm (5.1 to 20.0%) and 12.4 to 70.6 ppm (7.0 to 28.8%), respectively. The remaining phenolic classes each individually contributed <10% to total sample TPCI (Table 5.6).

As with the TPC, there was no statistically significant differences observed between the overall TPCI of samples from the different geographical regions for the commercial pear juices. However, phenolic subclasses were found to vary significantly between regions (Table 5.6). For example, the concentration of hydroxybenzoic acids were significantly higher in the pear juice samples from China (27.7 ± 14.2 ppm) as compared to those from Argentina (15.4 ± 1.9 ppm), New Zealand (15.9 ± 6.7 ppm) and the United States (12.7 ± 3.4 ppm). In addition, the hydroxycinnamic acid and dihydroxychalcone concentrations in the Chinese samples were significantly lower than all other geographical regions studied except for New Zealand (Table 5.6). The only other observed significant difference between regions was the high concentration of arbutin in the Chinese samples compared to all other regions. The high arbutin content (192.1 ± 80.3 ppm), coupled with the lower hydroxycinnamic acid (17.5 ± 14.8 ppm) and flavanol (25.9 ± 13.7 ppm) contents of the Chinese samples when compared to juices from the other geographical regions, is likely the reason why the overall TPCI of the samples studied were similar across all regions even though phenolic concentrations varied within specific classes.

Table 5.5 Mean and standard deviation of the different phenolic classes as identified by TPCI in commercial apple juices by country.

Geographical Origin[a]	HBA[b] (ppm)	HCA[c] (ppm)	Flavanols (ppm)	Dihydrochlacones (ppm)	Flavanones (ppm)
Argentina	43.3 ± 19.9[a]	63.0 ± 4.8[a]	31.5 ± 6.0[a]	4.8 ± 2.6[abc]	1.6 ± 1.3[a]
Brazil	24.3 ± 1.9[b]	53.4 ± 14.5[a]	17.6 ± 3.7[b]	2.0 ± 0.5[bc]	1.1 ± 0.9[a]
Chile	25.5 ± 4.4[ab]	74.1 ± 2.8[a]	33.3 ± 9.5[a]	7.3 ± 0.7[ab]	1.4 ± 0.2[a]
China	19.2 ± 4.9[b]	64.8 ± 60.5[a]	13.2 ± 9.4[b]	1.9 ± 0.9[bc]	1.4 ± 0.7[a]
United States	12.4 ± 2.1[b]	82.9 ± 31.0[a]	49.0 ± 2.8[c]	9.8 ± 5.2[a]	9.8 ± 5.2[a]

[a]Values marked with different letters within a column were statistically different ($p < 0.05$).
[b]HBA: hydroxybenzoic acids.
[c]HCA: hydroxycinnamic acids.

Table 5.6 Mean and standard deviation of the different phenolic classes as identified by TPCI in commercial pear juices by country.

Geographical Origin[a]	HBA[b] (ppm)	HCA[c] (ppm)	Flavanols (ppm)	Dihydrochlacones (ppm)	Flavanones (ppm)	Arbutin (ppm)
Argentina	15.4 ± 1.9[a]	71.4 ± 9.9[a]	38.4 ± 10.5[a]	7.3 ± 2.1[a]	2.7 ± 0.7[a]	69.4 ± 2.9[a]
Chile	18.6 ± 2.0[ab]	59.2 ± 7.7[a]	44.7 ± 3.6[a]	7.0 ± 1.4[ab]	4.4 ± 2.1[a]	80.9 ± 2.8[a]
China	27.7 ± 14.2[b]	17.5 ± 14.8[b]	25.9 ± 13.7[a]	2.1 ± 1.6[b]	1.3 ± 1.2[a]	192.1 ± 80.3[b]
New Zealand	15.9 ± 6.7[a]	26.2 ± 14.6[bc]	43.5 ± 23.1[a]	7.0 ± 6.4[ab]	3.0 ± 0.9[a]	85.2 ± 32.5[a]
United States	12.7 ± 3.4[a]	42.0 ± 16.9[ac]	30.7 ± 7.6[a]	7.7 ± 2.1[ab]	3.1 ± 2.0[a]	61.5 ± 7.3[a]

[a]Values marked with different letters within a column were statistically different ($p < 0.05$).
[b]HBA: hydroxybenzoic acids.
[c]HCA: hydroxycinnamic acids.

In order to determine the effect of processing on the final phenolic content of commercial pear juice, the TPCI of three commercial pear juices at various stages of production were determined. These stages included the initial mash stage before the addition of processing enzymes (i.e., the crushed whole fruit), the mash after enzymatic treatment with carbohydrases and the final juice concentrate (Willems and Low, 2016). It was found that the TPCI increased throughout processing from 140.1 ± 46.4 ppm for mash, to 217.6 ± 35.7 ppm following enzyme addition, to 276.2 ± 96.9 ppm in the final concentrate. In addition, all phenolic classes, excluding the flavonols, increased in concentration from the mash to the final concentrate. Of interest was the fact that sample flavonols showed a decrease in concentration during processing in two of the samples and were not detected in the third, indicating that this phenolic class underwent destruction during these processing stages (from an average of 10.9 ppm in the mash to a final average of 4.9 ppm in the concentrate) and as such had minimal impact on final concentrate TPCI values due to their low concentrations. Flavonols have been previously shown to be sensitive to heat treatment (Makris and Rossiter, 2001).

During commercial juice production, carbohydrases (primarily pectinases) are added to improve juice yield by breaking down cell walls so as to increase the total soluble carbohydrate content and reduce the viscosity of the juice (Willems and Low, 2016). The observed increase in TPCI during laboratory pear processing with commercial enzymes was likely due to breakdown of the fruit cell walls releasing phenolics during the mashing stage. Based on this result, it is postulated that an increase in phenolics release from the fruit to the juice would also occur in commercial apple/pear juice production.

5.4.2 DPPH and ABTS Radical Scavenging Activity of Commercial Apple and Pear Juice

Although there are numerous analytical methods available to measure the antioxidant potential of compounds such as phenolics, the most commonly used include the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the Trolox equivalence antioxidant capacity (TEAC) assays. These *in vitro* methods are widely used due to their simplicity, short analysis times and low cost (Koleva et al., 2002; Floegel et al., 2011). Both methods work by the generation of a synthetic free radical that is scavenged by the antioxidant resulting in a sample colour change that can be measured spectrophotometrically. For example, in the DPPH assay, the DPPH$^{\bullet}$ radical absorbs at 515 nm (A_{515}) and when it is scavenged by an antioxidant the absorbance decreases/disappears. Therefore,

by monitoring the decrease in A_{515} over time (typically, 15 or 30 minutes) the effectiveness of an antioxidant can be assessed (Sharma and Bhat, 2009; Mishra et al., 2012).

The TEAC assay works on a similar principle. In this method 2,2′-azinobis-3-ethylbenzthiazoline-sulfonic acid radical cations ($ABTS^{\bullet+}$) are generated by reaction with potassium persulfate. These radicals have an absorbance maximum at 734 nm and as they are scavenged by an antioxidant, this absorbance is reduced. The effectiveness of an antioxidant is assessed by its comparison to Trolox, a vitamin E analog (Rice-Evans et al., 1996; Re et al., 1999).

The average DPPH radical scavenging activity (Table 5.1) for commercial apple juice was found to be 21.5 ± 12.1 mL of DPPH/mL of juice with a range of 4.9 to 39.3 mL of DPPH/mL of juice. While the commercial pear juice samples showed an average DPPH radical scavenging activity of 13.6 ± 5.5 with a range of 5.5 to 25.0 mL of DPPH/mL of juice (Table 5.2). It was found that these results were significantly different with commercial apple juice having a greater DPPH radical scavenging ability that commercial pear juice.

The average TEAC values (Table 5.1) for the commercial apple juice samples was 130.8 ± 60.8 with a range of 33.2 to 260.5 mM Trolox/100 mL juice. While the average TEAC for the commercial pear juices was 150.8 ± 63.9 with a range of 76.9 to 375.2 mM Trolox/100 mL juice (Table 5.2). These results were not significantly different from each other (p-value > 0.05).

Due to a lack of published results on the free radical scavenging ability of commercial apple and pear juices, and the differences in the way free radical scavenging results are reported and variations in the method (i.e., concentration of DPPH/ABTS used, reaction time, etc.) direct comparisons of the results from this study to those reported from laboratory scale experiments would lack scientific rigor. Therefore, a selection of commercial fruit juices (blueberry, cranberry, strawberry and watermelon; n = 1) were analyzed under the same reaction conditions as the apple and pear juices analyzed in this study. The resulting DPPH scavenging activity results showed that cranberry showed the highest (447.0 mL of DPPH/mL of juice), followed by strawberry (379.5 mL of DPPH/mL of juice), blueberry (198.8 mL of DPPH/mL of juice), apple (25.6 mL of DPPH/mL of juice), pear (13.6 mL of DPPH/mL of juice) and watermelon juice (4.5 mL of DPPH/mL of juice). Free radical scavenging results employing the TEAC method for the same commercial juices showed that strawberry was the highest (1890.4 mM Trolox/100 mL juice), followed by cranberry (1423.2 mM Trolox/100 mL juice), blueberry (1029.4 mM Trolox/100 mL

juice), pear (150.8 mM Trolox/100 mL juice), apple (146.7 mM Trolox/100 mL juice) and watermelon juice (36.0 mM Trolox/100 mL juice).

These results agree well with literature reports, which show that red/blue fruits and berries (such as cranberry) that are rich in phenolics are better radical scavengers than fruits with less colour (such as apple and pear) that have lower phenolic contents and therefore lower free radical scavenging ability (Ryan and Prescott, 2010). Differences in the ordering between the TEAC and DPPH methods is likely due to differences in the structures of the radicals scavenged. The $ABTS^{\bullet+}$ radical involves the transfer of two electrons from one or two different antioxidants while the DPPH assay is based on hydrogen transfer and proceeds via a single electron transfer mechanism, as such TEAC and DPPH results for the same sample may not show strong correlation (Wootton-Beard et al., 2011).

Apple and pear showed significant differences in their DPPH and TEAC values based upon geographical region (Tables 5.3 and 5.4). It was found that the mean DPPH value for apple juice samples from Argentina (28.2 ± 13.0 mL of DPPH/mL of juice), Chile (28.0 ± 2.6 mL of DPPH/mL of juice) and the United States (31.8 ± 10.9 mL of DPPH/mL of juice) were not significantly different. While samples from Brazil (11.6 ± 1.5 mL of DPPH/mL of juice) were not significantly different from those from Argentina or Chile but were different from those of the United States. Finally, the Chinese samples showed the lowest overall mean DPPH radical scavenging activity (9.4 ± 3.6 mL of DPPH/mL of juice), which was significantly different from those found for all other geographical regions excluding Brazil.

The mean TEAC value for samples from Argentina (167.2 ± 70.0 mM Trolox/100 mL juice), Brazil (105.6 ± 25.9 mM Trolox/100 mL juice), Chile (174.2 ± 22.4 mM Trolox/100 mL juice) and the United States (169.7 ± 54.6 mM Trolox/100 mL juice) were found to be not significantly different. However, the mean TEAC value for Chinese samples was the lowest (58.8 ± 25.4 mM Trolox/100 mL juice) and was significantly different from all other geographical regions excluding Brazil.

For the pear juice samples, the mean DPPH radical scavenging activities of samples from Argentina (16.9 ± 3.1 mL of DPPH/mL of juice), Chile (13.0 ± 0.9 mL of DPPH/mL of juice) and the United States (12.8 ± 3.6 mL of DPPH/mL of juice) were not significantly different. Also, samples from Chile, China (10.3 ± 3.2 mL of DPPH/mL of juice), New Zealand (10.7 ± 5.1 mL of DPPH/mL of juice) and the United States did not show significant differences in mean DPPH

values. Fewer statistically-based differences in mean TEAC values were observed for pear juice samples from different geographical regions with the exception of the Chinese samples, which had both the highest value of 268.8 ± 114.5 mM Trolox/100 mL juice, and which was significantly higher than all other regions (113.1 to 146.5 mM Trolox/100 mL juice; Table 5.4).

To gain an understanding of the role that individual classes of phenolics play in the overall antioxidant potential of commercial apple and pear juices, three representative samples for both apple and pear were selected based on low (AJ 17 and PJ 23), medium (AJ 12 and PJ 13) and high (AJ 23 and PJ 7) TPC and radical scavenging ability. These juices were initially separated into three fractions using Amberlite XAD16N resin, a water fraction, a 40:60 methanol:water fraction and a 70:30 methanol:water fraction. Results from these experiments are shown in Table 5.7. These fractions were then individually analyzed for their TPC, DPPH and ABTS radical scavenging ability. The water fraction contains the water-soluble components, including the carbohydrates and ascorbic acid. The remaining mobile phases were found to elute the majority of the sample phenolics from the column with the most hydrophobic eluting in the 70% methanol fraction and those of intermediate polarity in the 40% methanol fraction.

Table 5.7 Mean data for total phenolic content (TPC), total phenolic chromatographic index (TPCI), DPPH and TEAC of fractionated apple and pear juices.

	Fraction	**Total Phenolic Content (ppm GAE)**	**DPPH[a]**	**TEAC[b]**
Pear Juice	Water	25.2	1.1	6.9
	40% methanol	117.4	5.5	76.2
	70% methanol	80.2	5.3	47.9
Apple Juice	Water	19.3	1.2	4.9
	40% methanol	77.9	5.4	46.4
	70% methanol	133.5	10.7	90.9

[a]Reported as mL of DPPH/mL of juice.
[b]Reported as mM Trolox equivalents/100 mL juice.

The water fractions of all samples (apple and pear) showed the lowest TPC and antioxidant activity for both the DPPH and TEAC methods. In general, for apple juice the highest TPC, DPPH and TEAC values were found in the 70% methanol fraction. For pear juice the reverse was true, on average the highest TPC, DPPH and TEAC values were found in the 40% methanol fraction.

The higher contribution of the 40% methanol fraction, as compared to the 70% methanol fraction, in the pear juices is likely due to the higher concentration of phenolics of intermediate

polarity in these samples (i.e., 5-*O*-caffeoylquinic acid) as can be observed in Figure 5.3. This is illustrated by the majority of phenolics eluting within 55 minutes where the acetonitrile content in the mobile phase was 0-17.5%, whereas less polar compounds eluted after 55 minutes, where the acetonitrile content increased to 56.0% (i.e. more hydrophobic mobile phase conditions). For apple juice samples, there were greater concentrations of hydrophobic phenolics (i.e., phloridzin) eluting later in chromatograms, which contributed to the higher observed TPC and antioxidant activity of the 70% methanol fraction.

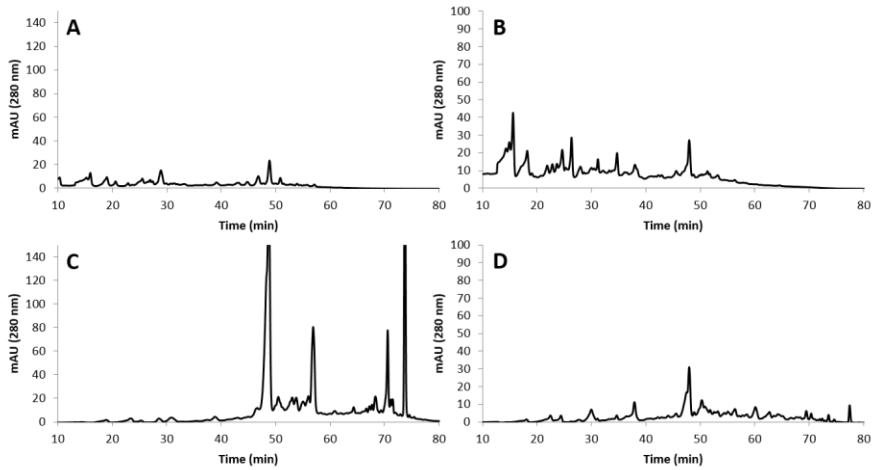

Figure 5.3 HPLC-PDA chromatograms of juice phenolic fractions. A) AJ 12, 40% methanol fraction; B) PJ 23, 40% methanol fraction; C) AJ 12, 70% methanol fraction; D) PJ 23, 70% methanol fraction.

5.5 Linkage Between Phenolic Content of Apple and Pear Juice and Analysis of Chlorogenic Acid Isomers using Ion Mobility and Tandem Mass Spectrometry

Results from this study showed that commercial apple and pear juices contained a variety of phenolics which contributed to their TPC and TPCI values, and antioxidant activities. In addition, apple and pear juice phenolics were assigned to their phenolic subclasses by HPLC-PDA based on their UV-vis spectra. Because phenolic subclasses share a large amount of structural similarities, compound identification is challenging. It was hypothesized that a series of naturally occurring (i.e. present in both apple and pear juices) structurally similar chlorogenic acid isomers (a subclass of hydroxycinnamic acids) could be identified using differential ion mobility and tandem mass spectrometry methods; which was the focus of Chapter 6.

6. ANALYSIS OF A SERIES OF CHLOROGENIC ACID ISOMERS USING DIFFERENTIAL ION MOBILITY AND TANDEM MASS SPECTROMETRY[1]

6.1 Abstract

Chlorogenic acids are among the most abundant phenolics found in the human diet. Of these, the mono-caffeoylquinic acids are the predominant phenolics found in fruits, such as apples and pears, and products derived from them. In this research, a comprehensive study of the electrospray ionization (ESI) tandem mass spectrometric (MS/MS) dissociation behavior of the three most common mono-caffeoylquinic acids, namely 5-O-caffeoylquinic acid (5-CQA), 3-O-caffeoylquinic acid (3-CQA) and 4-O-caffeoylquinic acid (4-CQA), were determined using both positive and negative ionization. All proposed structures of the observed product ions were confirmed with second-generation MS^3 experiments. Similarities and differences between the dissociation pathways in the positive and negative ion modes are discussed, confirming the proposed structures and the established MS/MS fingerprints. MS/MS dissociation was primarily driven via the cleavage of the ester bond linking the quinic acid moiety to the caffeic acid moiety within tested molecules. Despite being structural isomers with the same *m/z* values and dissociation behaviors, the MS/MS data in the negative ion mode was able to differentiate the three isomers based on ion intensity for the major product ions, observed at *m/z* 191, 178 and 171. This differentiation was consistent among various MS instruments. In addition, ESI coupled with high-field asymmetric waveform ion mobility spectrometry-mass spectrometry (ESI-FAIMS-MS) was employed for the separation of these compounds for the first time. By combining MS/MS data and differential ion mobility, a method for the separation and identification of mono-caffeoylquinic in apple/pear juice samples was developed with a run time of less than one minute. It is envisaged that this methodology could be used to identify pure juices based on their chlorogenic acid profile

[1]Reproduced with permission. Willems, J. L., Khamis, M. M., Saeid, W. M., Purves, R. W., Katselis, G., Low, N. H. and El-Aneed, A. (2016). Analysis of a series of chlorogenic acid isomers using differential ion mobility and tandem mass spectrometry. *Analytica Chimica Acta*, 933: 164-174. Copyright (2016) Elsevier.

(i.e., metabolomics), and could also be used to detect juice-to-juice adulteration (e.g., apple juice addition to pear juice).

6.2 Introduction

The term phenolics refers to a class of plant metabolites, which are defined by the presence of at least one phenol functional group (de Simon et al., 1992; Friedman and Jürgens, 2000; Shahidi and Naczk, 2003). Phenolics are ubiquitous in nature and have been reported to have a number of positive functions in plants that include, but are not limited to: protection against pathogens, parasites, predators, ultraviolet radiation and oxidants; cell signalling; attraction of pollinators and seed dispersing animals; and cell wall strengthening (Friedman and Jürgens, 2000; Dai and Mumper, 2010; Lattanzio, 2013; Nayak et al., 2015). In addition, phenolics contribute to the sensory properties of food; for example, phenolics affect the bitterness, astringency and colour of fruit juices (de Simon et al., 1992; Versari et al., 1997; Dai and Mumper, 2010).

Due to their structural diversity, phenolics are typically subdivided into a number of structurally related classes. One class is the chlorogenic acids, which are composed of quinic acid linked to *trans*-cinnamic acids, such as caffeic acid, via an ester bond (Clifford, 2000; Jaiswal et al., 2011; Upadhyay and Rao, 2013). The most common chlorogenic acid is 5-O-caffeoylquinic acid (5-CQA; chlorogenic acid) (IUPAC, 1968; Clifford, 2000; Jaiswal et al., 2011). However, many other structural isomers are present in plants, such as 3-O-caffeoylquinic acid (3-CQA; neochlorogenic acid) and 4-O-caffeoylquinic acid (4-CQA; cryptochlorogenic acid) (Figure 6.1) (Upadhyay and Rao, 2013). Along with these, diCQAs, triCQAs and a tetraCQA have been reported in literature (Jaiswal et al., 2011).

Chlorogenic acids are one of the most abundant classes of phenolics in the human diet (Feng et al., 2005; Jaiswal et al., 2011), and have been purported to have numerous health benefits. For instance, it was demonstrated that they possess antioxidant (Rice-Evans et al., 1996; Kono et al., 1997; Nakatani et al., 2000; Kweon et al., 2001), anti-inflammatory (Dos Santos et al., 2006) and anti-HIV properties (McDougall et al., 1998) as well as the ability to inhibit carcinogenesis (Mori et al., 1986; Feng et al., 2005). In addition, chlorogenic acids have been reported to play an important role in food quality. In coffee, high levels of mono-caffeoylquinic acids are indicators of lower coffee quality while higher levels of di-caffeoylquinic acids were positively correlated with higher quality (Clifford, 1979; Farah et al., 2006). Chlorogenic acids are also prominent in

fruit juices (Clifford, 1999). For example, 5-CQA is reported as the major phenolic found in both apple and pear juices (Spanos and Wrolstad, 1990; Spanos et al., 1990; Cui et al., 2005). However, the exact structural identity of the various chlorogenic isomers in these juices has only been partially determined (Spanos et al., 1990; Cui et al., 2005). In addition, chlorogenic acids are thermally labile, as such they can undergo structural changes under common food processing conditions such as canning, roasting and pasteurization (Jaiswal et al., 2012). Therefore, the ability to differentiate between various chlorogenic acids (Figure 6.1) and an understanding of their breakdown products is important for assessing both food quality and in estimating the health benefits of foods rich in these compounds.

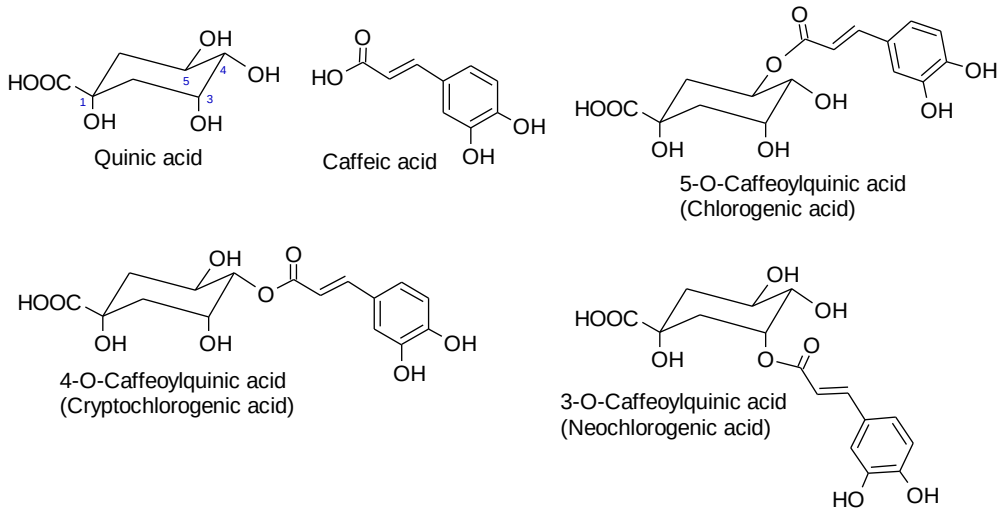

Figure 6.1 Structures and nomenclature of the common mono-caffeoylquinic acids and their building blocks, quinic acid and caffeic acid (trivial names in brackets).

Various analytical techniques have been developed for the separation and detection of chlorogenic acids, where the most widely employed method is reversed phase high performance liquid chromatography (RP-HPLC), typically coupled with photodiode array (PDA) detection (Shahidi and Naczk, 2003; Ignat et al., 2013). Another analytical method that has been used to confirm structural information about chlorogenic acids in a variety of food products, including apples, coffee beans and dried plums is HPLC coupled to mass spectrometry (MS) (Clifford et al., 2000; Fang et al., 2002; Marks et al., 2007). MS is a superior platform for the analysis of phenolics due to its high sensitivity and selectivity. It allows for the identification and quantification of minor chlorogenic acids in a complex phenolic mixture (Ignat et al., 2013).

To fully utilize MS, an understanding of analyte dissociation patterns during tandem (MS/MS) and multi-stage (MS^n) mass spectrometric analysis is required. The establishment of MS/MS fingerprints can be used for structural elucidations as well as for identifying unique product ions that can be used for targeted multiple reaction monitoring (MRM) quantitative analysis (Buse et al., 2011; Awad et al., 2015). The application of MS/MS analysis to a selection of chlorogenic acids and other closely related compounds employing ion trap mass spectrometers have been reported in the literature (Clifford et al., 2003; Clifford et al., 2005; Clifford et al., 2006; Jaiswal et al., 2010; Jaiswal et al., 2011). However, the reported work was primarily focused on the negative ion mode in which only few product ions were structurally identified. In addition, some of the reported work proposed the formation of multisite-radical product ions (Clifford et al., 2003; Jaiswal et al., 2010), which is not probable during collision induced dissociation (CID)-MS/MS conditions. As such, there is an absence of universal MS/MS fragmentation patterns with well-rationalized structures for chlorogenic acids. In addition, only two product ions have been reported for chlorogenic acids during positive ionization, which indicate that there is still much work to be done in the elucidation of the MS/MS fragmentation pathways of chlorogenic acids (Sakushima et al., 1985; Fang et al., 2002; Schram et al., 2004). Therefore, one of the goals of this research was to establish the MS/MS fragmentation pathways of three common mono-caffeoylquinic acid isomers (Figure 6.1) using both positive and negative ionization. The primary goal was to gain an understanding of how caffeoylquinic acids dissociate during CID-MS/MS analysis as well as to develop a method for the differentiation of chlorogenic acid isomers.

An additional goal was to separate these isomeric species within juice samples without the need for chromatographic separation. Due to the similarity in structures of the three isomers used in this study (Figure 6.1), we also employed differential ion mobility-MS/MS (Verbeck et al., 2002). Ion mobility has been used to effectively separate structurally similar compounds, including isomers and chiral mixtures (Dwivedi et al., 2006; Kanu et al., 2008). Differential mobility devices are ideal in this type of analysis as ion subsets (ideally only containing one isomer) are continuously delivered to the mass spectrometer for an in-depth analysis. More critically, ion mobility linked to MS/MS can allow for the identification and quantification of structurally similar compounds without the need for tedious chromatographic separation (Kanu et al., 2008). Therefore, a second major goal of this research was to employ high field asymmetric waveform

ion mobility spectrometry (FAIMS) to separate the three most common mono-caffeoylquinic acids (3-CQA, 4-CQA and 5-CQA) in commercial apple and pear juice samples.

6.3 Materials and Methods

6.3.1 Materials

Standards of 5-O-caffeoylquinic acid (chlorogenic acid; 5-CQA), 4-O-caffeoylquinic acid (cryptochlorogenic acid; 4-CQA) and 3-O-caffeoylquinic acid (neochlorogenic acid; 3-CQA) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All standards were at least 95% in purity. HPLC grade methanol was obtained from Fisher Scientific (Ottawa, ON, Canada). The water used throughout this study was from a Millipore Milli-Q water system (Millipore Corp., Milford, MA, USA). Commercial apple and pear juice concentrates were from China and the United States, respectively.

Stock solutions of the caffeoylquinic acids at a concentration of 1.0 mM were prepared by dissolving the standards in 50% (v:v) aqueous methanol. The standards were then syringe filtered (nylon, 0.2 μm pore size, 13 mm diameter; Chromatographic Specialties, Brockville, ON, Canada) and stored at -20°C until use.

6.3.2 Mass Spectrometric Analysis

Mass spectrometric analysis of the caffeoylquinic acids was performed on three different MS platforms, in order to confirm reproducibility and the applicability of the developed methods across a variety of instruments. These included a triple quadrupole linear ion trap mass spectrometer (QqQ-LIT MS) and two different quadrupole orthogonal time-of-flight mass spectrometers (QqToF MS). All instruments were equipped with an electrospray ionization source (ESI). The experimental conditions of the sample (i.e. analyte concentration and solvent makeup) was optimized for each instrument as discussed below.

6.3.3 Triple Quadrupole Linear Ion Trap Mass Spectrometry (QqQ-LIT MS)

First stage tandem MS/MS and second generation MS/MS/MS (MS^3) experiments were performed using both a QTRAP 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) and a hybrid triple quadrupole linear ion trap mass spectrometer (QqQ-LIT) equipped with a “Turbo V Ion Spray” ESI source.

Stock solutions of the individual caffeoylquinic acids were diluted 100x with 50% (v/v) aqueous methanol and infused directly into the ESI source using a model 11 Plus syringe pump (Harvard Apparatus, MA, USA) at a flow rate of 10 µL/min. The declustering potential (DP) and collision energy (CE) were ramped and optimized to obtain the greatest number of product ions while the precursor ion remained abundant. Optimum CE values employed were -20 eV and +18 eV for sample analysis in the negative and positive ion modes, respectively. To assess if the MS response was proportional to analyte concentration, three different concentrations were tested, namely 10 µM, 5 µM, and 2.5 µM.

6.3.4 Electrospray-Quadrupole Orthogonal Time-Of-Flight Mass Spectrometry (ESI-QqTOF MS)

The stock solutions of the three caffeoylquinic acids were diluted 10x with 50% (v/v) aqueous methanol containing 0.15% formic acid. Samples were analyzed using both the positive and negative ionization modes on an API QSTAR XL MS/MS hybrid QqToF tandem mass spectrometer equipped with an ESI source (Applied Biosystems Inc., CA, USA). The DP was set at 80 V and sample solutions were infused into the ESI chamber (Turbo Ionspray source) by an integrated Harvard syringe pump (Harvard Apparatus, MA, USA) at a rate of 10 µL/min with the following parameters: spray chamber temperature 100° C, needle voltage 5500 V. Nitrogen was used as both the drying and ESI nebulizing gas. External calibration employing caesium iodide (*m/z* 132.9054) and sex pheromone inhibitor iPD1 (*m/z* 829.5398) was used to ensure high mass accuracies and to minimize errors in mass measurements. Mass spectra were analyzed using the Analyst software. MS/MS analysis was performed in the negative and positive ion modes and the CEs were optimized to induce fragmentation while the precursor ion remained abundant; the final CEs were -19 eV and +22 eV in the negative and positive ion modes, respectively.

The same experiments were repeated using an Agilent 6550 iFunnel Q-TOF (Agilent Technologies, Mississauga, ON, Canada) system. Caffeoylquinic acid stock solutions were diluted 40x with 50% aqueous methanol. Standards were infused, using an infusion pump (KD Scientific, Holliston, MA, USA) for enhanced reference mass delivery, Agilent Jet Stream (AJS) ESI source at a flow rate of 50 µL/min. The ESI source was operated in both the negative and positive ion modes. Mass spectra were recorded in the range of 50-1,200 m/z at a rate of 2 spectra/s with an isolation width of 1.3 amu. Gas temperature, sheath gas flow and the nebulizer were set at 250 °C,

11 L/min and 20 psi, respectively. The source VCap was set at 4000 V and the nozzle voltage at 500 V while the fragmentor and Octopole RF peak were set at 365 V and 750 V, respectively. MS/MS spectra were acquired using a collision energy of 20 eV in both the positive and negative ion modes. The system continually corrected any mass drifts by passing Agilent reference solutions (+ve mode ion: HP-0921, 922.009798 m/z; -ve mode ion: HP-0921-TFA adduct, 1033.988109 m/z) through the reference nebulizer.

6.3.5 Phenolic Isolation from Commercial Apple and Pear Juice

Phenolics were isolated from commercial apple and pear juice concentrates employing Amberlite XAD-16N resin (Sigma-Aldrich Canada Ltd., Oakville, ON) (Green, 2007). The resin was hydrated in 50% (v/v) aqueous methanol for 30 minutes before being transferred to a glass column (8.0 cm x 3.0 cm) to produce a bed volume of approximately 55 mL. The resin bed was pre-conditioned with 110 mL of water followed by 110 mL 90% (v/v) aqueous methanol and 110 mL of water.

Juice samples were diluted to 11.5 ± 0.1 °Brix for apple and 12.0 ± 0.1 °Brix for pear juice (Auto Abbe Refractometer; Leica Inc., Buffalo, NY, USA) prior to phenolic isolation. Columns were loaded with 15 mL of appropriately diluted juice and then washed with 110 mL of water to elute water-soluble compounds. Fruit phenolics were eluted by washing with 110 mL of 70% (v/v) aqueous methanol. The phenolic fractions were concentrated using a Büchi rotary evaporator (Flawil, Switzerland) and samples were freeze dried (Hetro Lab Equipment, Allerod, Denmark) in 12 x 32 mm glass vials (Chromatographic Specialties, Brockville, ON, Canada) and stored at -18 °C until use. Separate Amberlite XAD-16N columns were used for each juice sample.

6.3.6 High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS)-Mass Spectrometric Analysis

The three caffeoylquinic acids were analyzed using ESI-FAIMS-MS on a FAIMS-equipped Thermo Fisher TSQ Vantage mass spectrometer (San Jose, CA). A modified FAIMS electrode set was employed, which enabled improved desolvation and higher electric field strengths to be achieved compared with the commercial FAIMS electrode set (Prasad et al., 2014). System setup was also modified to allow for the introduction of gas additives into the FAIMS gas flow as described previously (Purves et al., 2014). Briefly, gas additives were introduced by teeing

liquids into the gas line using an Agilent 1200 HPLC system (Mississauga, ON, CA). A mixing chamber was inserted between the tee and the FAIMS device to ensure a uniform gas composition and to prevent droplets from entering the FAIMS. The amount of gas additive present in the gas stream was calculated based on a molar ratio and expressed as a percentage (Purves et al., 2014). For the spectra shown in this study, methanol was used as the gas additive and it was delivered to the gas stream at a rate of 0.175 mL/min (into 5 L/min nitrogen) resulting in a molar ratio of 2.1%.

Caffeoylquinic acid standard solutions were prepared in 50% methanol at a concentration of 10 µM or in a mixture at a concentration of 3.3 µM. Phenolic isolates from apple and pear juices were prepared by adding 1.0 mL of 50% methanol to the freeze dried extracts with sonication for 10 minutes to ensure solubilisation. The solutions were then diluted 100 fold with 50% methanol. Samples, infused using a Harvard Apparatus syringe pump at a flow rate of 10 µL/min, were diluted by teeing in a solvent of 0.1% formic acid in 50% methanol (delivered using an Agilent 1100 HPLC system) to give a flow rate of 200 µL/min at the ESI needle tip. Ions were generated using negative ionization and MS detection in the multiple reaction monitoring (MRM) mode. The monitored precursor ion → product ion transitions for the acids were *m/z* 353→*m/z* 191, *m/z* 353→*m/z* 179 and *m/z* 353→*m/z* 173. The conditions were optimized as follows: capillary temperature of 275 °C, ion spray voltage of -3300 V, vaporizer temperature of 350 °C, dispersion voltage of -3500 V, inner/outer electrode temperatures of 90/90 °C, sheath gas of 60 and auxiliary gas of 10 and collision energy of 20 V.

6.4 Results and Discussion

6.4.1 Single-Stage MS Analysis

The ESI-MS full scan analysis of the mono-caffeoylquinic acid isomers gave the singly charged molecule for all three isomers in both the positive and negative ion modes, $[M+H]^+$ and $[M-H]^-$, respectively. The theoretical *m/z* values of the protonated and deprotonated species of the three isomers were 355.1024 and 353.0878, respectively. MS exact measurements were reported using the ESI-QqToF MS system (6550 iFunnel), which gave mass accuracies of less than 2 ppm for all three isomers. As these isomers, by definition, have the same molecular weights (and *m/z* values), these measurements could not be used for any sort of differentiation between these compounds.

6.4.2 Tandem MS Analysis

Chlorogenic acid (5-CQA) is the most common of the mono-caffeoylquinic acids found in nature and is also the most readily commercially available (Clifford, 2000; Upadhyay and Rao, 2013). Therefore, 5-CQA was used to optimize the MS conditions in this study (i.e., instrument parameters, collision energy and concentration). The MS/MS dissociation behaviour for all three isomers was studied using both negative and positive ionization on three different mass spectrometer systems. Generally, negative ionization has been reported to result in higher sensitivity for phenolics than positive ionization and therefore it is commonly used for caffeoylquinic acid MS/MS analysis (Cuyckens and Claeys, 2004). Since MS/MS dissociation behavior using positive ionization has not been thoroughly addressed previously (Sakushima et al., 1985; Fang et al., 2002), it was also evaluated in this study.

6.4.2.1 MS/MS Analysis in the Positive Ion Mode

To the best of our knowledge, a thorough comparison of MS/MS spectra of mono-caffeoylquinic acids produced using positive versus negative ionization has not been reported. The presence of the carboxylic acid functional group in each of these compounds makes them readily ionizable in negative ion mode resulting in good signal intensity. However, chlorogenic acids can also be readily ionized in positive ion mode due to the presence of multiple OH groups. Therefore, we performed dual MS/MS analysis giving insights into how these molecules dissociate under CID conditions in both ionization modes.

Figure 6.2 shows MS/MS spectra acquired with the QTRAP mass spectrometer and the proposed fragmentation pattern for the representative caffeoylquinic acid, 5-O-monocaffeoylquinic acid. All three mono-caffeoylquinic acid isomers followed the same MS/MS dissociation behavior as shown in Table 6.1, with a base peak observed at *m/z* 163.1 as well as a major product ion at *m/z* 181.0 which agrees well with published reports (Sakushima et al., 19856; Fang et al., 2002). However, no work has been presented that gives a detailed evaluation of the MS/MS dissociation behavior of chlorogenic acids using positive ionization, such as identifying the structures of the various observed product ions and rationalizing their fragmentation pathways.

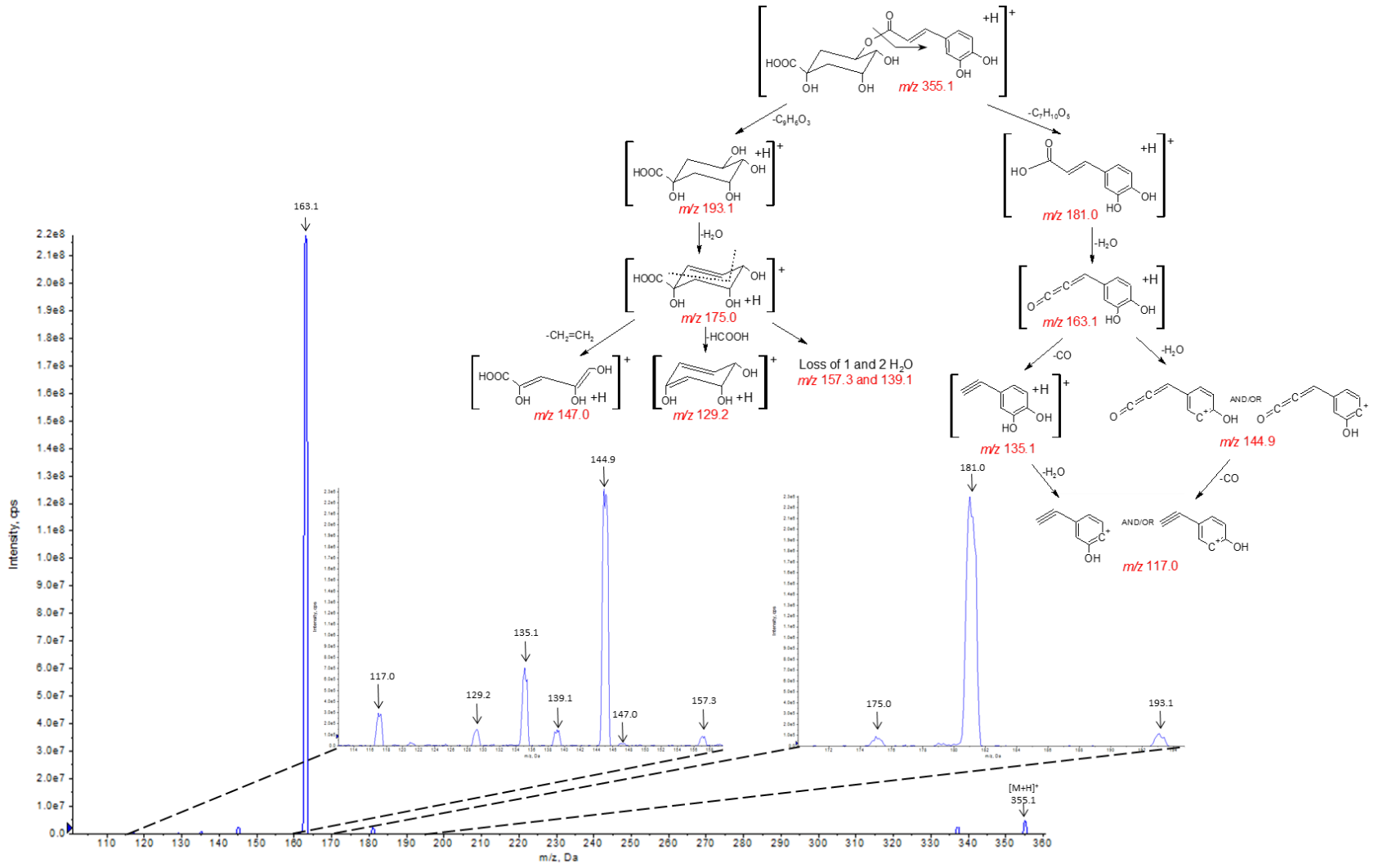

Figure 6.2 The proposed generalized positive ion mode MS/MS fragmentation pattern and ESI-QqQ-LIT MS/MS spectrum of 5-CQA as a representative spectrum.

Table 6.1 Product ions of mono-caffeoylquinic acid isomers observed in the positive ion mode during CID-MS/MS analysis.

Compound	Precursor Ion $[M+H]^+$	193	181	175	163	157	147	145	139	135	129	117
5-CQA	355	✓[a]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
4-CQA	355	N	✓	N	✓	N	N	✓	N	✓	N	✓
3-CQA	355	N	✓	N	✓	N	N	✓	N	✓	N	✓

[a]✓: Present, N: Not observed

The product ion at *m/z* 181.0 was identified as the caffeic acid portion of the molecule formed through the cleavage of the ester linkage between chlorogenic and quinic acid. It was anticipated that the ester linkage would be the primary location for dissociation in the gas phase as this bond is highly prone to hydrolysis. However, the product ion at *m/z* 181.0 was a minor ion as compared with the ion observed at *m/z* 163.1 in all three isomers. It is proposed that the production of the ion at *m/z* 163.1 was formed through water loss from caffeic acid with *m/z* 181.0. This is supported by a MS^3 experiment that showed that *m/z* 163.1 is formed from the ion at *m/z* 181.0 (Table 6.2A). The product ions observed at *m/z* 144.9, 135.1 and 117.0 were all identified as being formed from *m/z* 163.1 and are likely formed through the subsequent elimination of water and carbon monoxide moieties, as illustrated in Figure 6.2. The formation of the product ion at *m/z* 117.0 from both *m/z* 144.9 and 135.1 was confirmed through MS^3 experiments (Table 6.2A). As expected, conjugation within the structures of the observed product ions resulted in enhanced stability during CID-MS/MS analysis.

Similar to the formation of a product ion representing the caffeic acid portion, the product ion observed at *m/z* 193.1 was identified as being derived from the quinic acid portion of the molecule also formed through the cleavage of the ester linkage. When subjected to MS^3, the observed 193.1 product ion dissociated into additional ions, namely the product ions observed at *m/z* values of 175.0, 157.3, 147.0, 139.1 and 129.2. The ions at *m/z* values 175.0, 157.3 and 139.1 were formed through subsequent water losses from the ion observed at *m/z* 193.1. Whereas, the product ions observed at *m/z* values147.0 and 129.2 originated from the ion observed at *m/z* 175.0 through: a) inner ring fragmentation with the neutral loss of an ethylene molecule; and b) the loss of a formic acid molecule, respectively. While these product ions were readily detected in 5-CQA they were not identified in either 3-CQA or 4-CQA (Table 6.1). However, the product ions

observed for 5-CQA in the positive ion mode correlated well with those obtained in the negative ion mode, which will be discussed in the following section. Table 6.1 summarizes the product ions observed for each of the three mono-caffeoylquinic acid isomers.

Table 6.2 Tabulated MS^3 experiments in the positive ion mode (A) and the negative ion mode (B) for the monocaffeoylquinic acids.

(A) Positive Ion Mode MS^3 Analysis		
Precursor Ion	**First Generation Product Ions (MS/MS)**	**Second Generation Product Ions (MS^3)**
355.1	193.1	175.0, 157.3, 147.0, 129.2
	181.0	163.1, 135.1
	175.0	157.3, 147.0, 139.1, 129.2
	163.1	144.9, 135.1, 117.0
	144.9	117.0
	135.1	117.0
	117.0	
(B) Negative Ion Mode MS^3 Analysis		
Precursor Ion	**First Generation Product Ions (MS/MS)**	**Second Generation Product Ions (MS^3)**
353.2	190.7	172.6, 155.0, 137.0, 127.1, 110.6, 108.9
	178.8	134.8
	172.6	155.0, 137.0, 110.6
	161.0	
	155.0	137.0, 110.6
	134.8	
	127.1	
	110.6	

Due to the similarity in the MS/MS profiles of the three isomers (Table 6.1) and the low abundance of some of the observed product ions, it was not feasible to reliably differentiate between these compounds in positive ion mode. However, if combined with chromatographic separation, the produced product ions can be used for the development of targeted LC-MRM quantification methods (Andrade et al., 1998; Vrhovsek et al., 2012; Orčić et al., 2014).

6.4.2.2 MS/MS Analysis in the Negative Ion Mode

Figure 6.3 shows a negative ion mode MS/MS spectrum and the proposed fragmentation pattern for a representative mono-caffeoylquinic acid, 4-O-monocaffeoylquinic acid. Unlike the results from the positive ion mode, all three isomers produced the same ions under MS/MS

conditions in the negative ion mode. Structural assignment and the proposed MS/MS fragmentation pathway were confirmed by employing MS^3 experiments using ESI-QqQ-LIT (Table 6.2B).

All three mono-caffeoylquinic acid isomers analyzed showed the same product ions allowing for the determination of a generalized MS/MS fragmentation pathway. However, the relative abundances of the observed product ions varied among the different isomers as described in more detail below. In addition, all three of the MS systems showed the same general fragmentation patterns as well as relative abundances, with the only difference among them being sensitivity. That is, some of the minor product ions were only observable by ESI-QqQ-LIT. This was due to the trapping capability of the linear ion trap, allowing for the observation of minor product ions. However, all of the major product ions were observed in all three systems.

The three major product ions detected in the negative ion mode were observed at *m/z* 190.7, 178.8 and 172.6. The product ion at *m/z* 190.7 was identified as quinic acid, which is formed by the cleavage of the ester bond between the quinic and caffeic acid moieties. This product ion was a major peak for all three isomers. The proposed structure agreed well with literature that reported the presence of the quinic acid product ion with the only variations being in the location of the charge (Fang et al., 2002; Clifford et al., 2003; Clifford et al., 2006). The abundance of this ion was not surprising as the ester bond is prone to cleavage during CID-MS/MS. The abundance of the quinic acid ion is likely explained by the presence of a carboxylic acid functional group that can be readily ionized in the negative ion mode. It should be noted that similar behavior has been observed in analogous compounds. For example, it was observed that chlorogenic acid lactones, where a lactone is formed between the carboxylic acid and a hydroxyl group on the quinic acid moiety, showed a major product ion corresponding to the quinic acid lactone (quinide) (Jaiswal et al., 2011).

The quinic acid product ion (*m/z* 190.7) dissociated further under the CID conditions employed. The loss of a water molecule to form the product ion at *m/z* 172.6 is expected, as there are many potential locations for water loss due to the number of hydroxyl groups on the quinic acid moiety. As with the ion observed at *m/z* 190.7, this structure agreed with those previously reported in the literature for both mono-caffeoylquinic acids as well as other related chlorogenic acids (Fang et al., 2002; Clifford et al., 2003; Clifford et al., 2006).

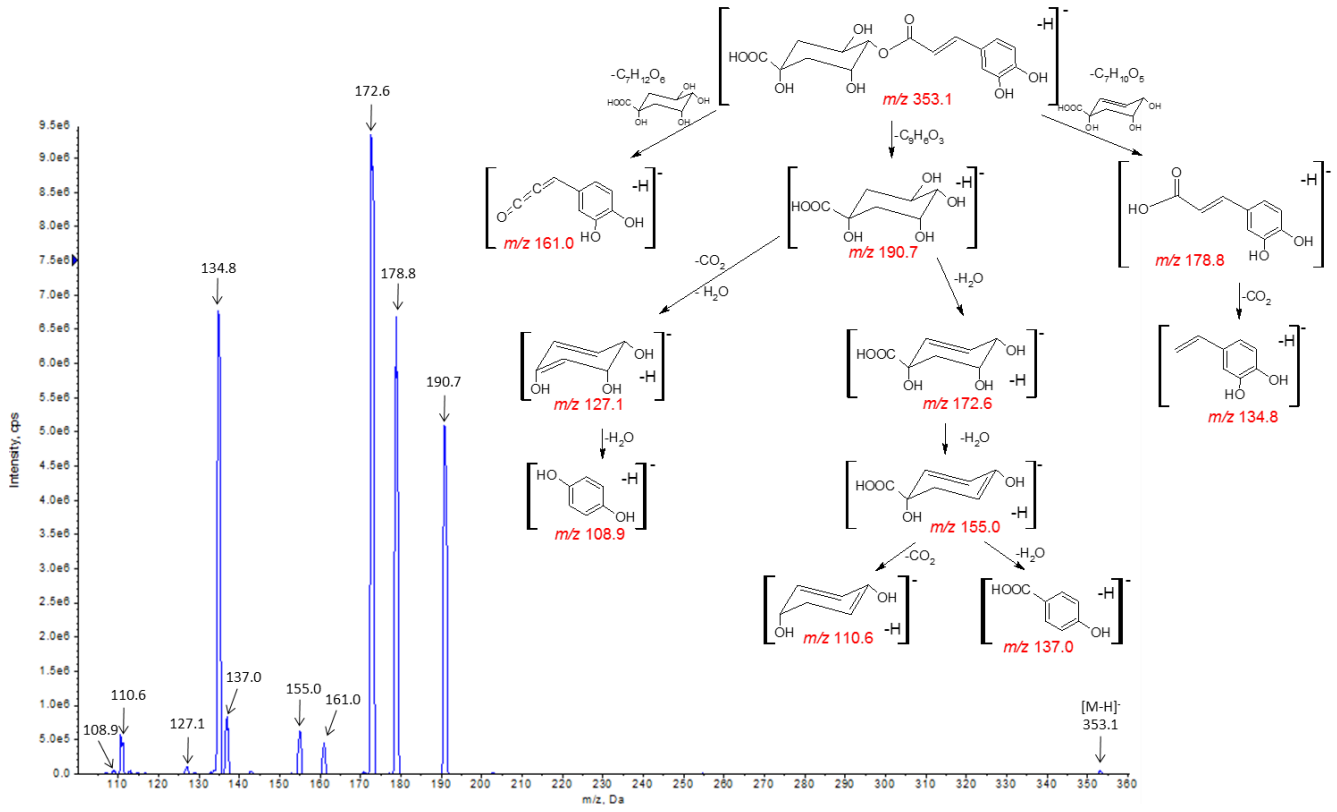

Figure 6.3 The proposed generalized negative ion mode MS/MS fragmentation pattern and ESI-QqQ-LIT MS/MS spectrum of 4-CQA.

The ion representing quinic acid (*m/z* 190.7) fragmented to yield the product ion at *m/z* 127.1 via the concurrent losses of water and carbon dioxide moieties. Theoretically, this product ion could be formed from the ion observed at *m/z* 172.6, via the loss of carbon dioxide, however no evidence of this mechanism was observed by MS^3 experiments (Table 6.2B). In addition, the product ion at *m/z* 127.1 dissociated further to form the ion observed at *m/z* 108.9 through the loss of an additional water molecule. The proposed structure for the ion at *m/z* 108.9 consists of an aromatic ring after the loss of three water molecules with the subsequent formation of three double bonds.

Similarly, the product ion at *m/z* 172.6 was shown to lose a water molecule forming the ion observed at *m/z* 155.0, which subsequently lost either water or carbon dioxide to form the product ions observed at *m/z* 137.0 and 110.6, respectively (Figure 6.3). Structures previously reported for the ions observed at *m/z* 127.1 and 110.6, were reported as those containing multiple radicals within the structure (Clifford et al., 2003). This is highly unlikely as a structure containing three radical sites on carbon within the same molecule would be unstable and would not be observed during ESI-CID-MS/MS (Cech and Enke, 2001).

The product ion at *m/z* 178.8 was identified at the caffeic acid portion of the molecule (Figure 6.1). This fragment has been previously reported in literature for 5-CQA (Fang et al., 2002). As well, other chlorogenic acids have been shown to undergo similar fragmentation behavior where the cinnamic acid is lost from the molecule under MS/MS analysis in negative ion mode (Jaiswal et al., 2010; Jaiswal et al., 2011). The ion at 178.8 loses a carbon dioxde moiety to produce the product ion observed at *m/z* 134.8.

Finally, a product ion at *m/z* 161.0 was observed in the negative ion mode for all three isomers. This product ion has been previously reported in literature (Jaiswal et al., 2010; Jaiswal et al., 2011). Interestingly, this ion was not observed in any of the MS^3 experiments, suggesting that it was derived directly from the deprotonated precursor ion and not from another product ion such as *m/z* 178.8, as might be speculated.

6.4.2.3 Comparison Between the Positive and Negative Ion Modes

Of interest are the similarities between the product ions observed in the positive and negative ion modes, with many of the ions differing by only two mass units (i.e., the two hydrogen difference between positive and negative ion modes). For example, both the positive and negative

ion modes show the quinic acid product ion and subsequent product ions derived from quinic acid at *m/z* 193/191, 175/173, 157/155, 137/135 and 129/127. In addition, the positive and negative ion modes also shared some of the caffeic acid-based product ions at *m/z* 181/179 and 163/161. This observation of complementary ions aids in the confirmation of the proposed structures, and are readily explained by the cleavage of the same bonds in these compounds in both the positive and negative ion modes. However, more product ions derived from the caffeic acid portion of the molecule were observed in positive ion mode (Figures 6.2 and 6.3), namely those at *m/z* 144.9, 135.1 and 117.0. This may be due to the caffeic acid portion of the molecule being more likely to retain a charge in the positive ion mode, whereas the quinic acid-related product ions would be more likely to retain a charge in negative ion mode, due to the presence of the carboxylic acid functional group. This was supported by the observation that quinic acid-related product ions showed higher abundances in negative ion mode when compared with the analogous ions observed in positive ion mode.

While some prior work has examined the fragmentation of mono-caffeoylquinic acids in negative ion mode, most of these focused only on the major product ions, such as *m/z* 190.7, 178.8 and 172.6. In addition, no comprehensive general MS/MS fingerprints showing the genesis and structures of all of the observed product ions of mono-caffeoylquinic acids has been published either in positive or negative ion modes.

Based on previous reports of the MS/MS dissociation behaviour of other chlorogenic acid compounds (e.g., chlorogenic acid lactones and diferuloylquinic acids) in negative ion mode (Clifford et al., 2006; Jaiswal et al., 2011) other chlorogenic acids may also have similar positive ion mode MS/MS fragmentation to the mono-caffeoylquinic acids shown here. The generalized dissociation pathways shown in Figures 6.2 and 6.3 may allow for the identification of a variety of different chlorogenic acids in both the positive and negative ion modes.

6.4.2.4 Differentiation of Mono-Caffeoylquinic Acids by MS/MS

While all three mono-caffeoylquinic acid isomers showed the same general MS/MS fragmentation behaviour, it is possible to differentiate among the three isomers in the negative ion mode. This statement is based on the relative abundances of the three major product ions, namely *m/z* 190.7, 178.8 and 172.6 (Figure 6.4; Table 6.3) as follows: 5-CQA was identified by a major base peak at *m/z* 190.7 with relatively minor product ions at *m/z* 178.8 and 172.6; 3-CQA's base

peak was also at *m/z* 190.7, but it was differentiated from 5-CQA through the presence of a relatively abundant peak at *m/z* 178.8; and 4-CQA was differentiated from the other two isomers by having a base peak at *m/*z 172.6 at collision energies ranging from -15 to -35 V (Figure 6.4). It was also found that there was concentration dependent MS/MS response for all isomers when three different analyte concentrations were tested using the Q-LIT instrument, namely 10 µM, 5 µM, and 2.5 µM. For example, the ion count for the diagnostic ion observed at *m/z* 190 for the 3-CQA isomer was 7.5^{e5}, 3.2^{e5}, and 1.4^{e5}, respectively (data not shown), indicating a concentration-dependent MS/MS response.

Table 6.3 Major MS/MS product ions at *m/z* 191, 179 and 173 (counts >5.0 x 10^4 by 6550 iFunnel QTOF) used for isomer identification.

Compound	**191**	**179**	**173**
5-CQA	✓		
4-CQA	✓	✓	✓
3-CQA	✓	✓	

The observed MS/MS patterns were confirmed by analyzing the three mono-caffeoylquinic acid isomers on three different mass spectrometers under a variety of collision energies on different days. These experiments confirmed that the relative abundances of the three product ions as shown in Table 6.2 could be reliably used to differentiate between these isomers regardless of the mass spectrometer and range of collision energies employed. Therefore, we utilized this feature to aid in the optimization of the differential mobility settings so that the all three mono-caffeoylquinic acids could be separated in commercial pure apple and pear juices.

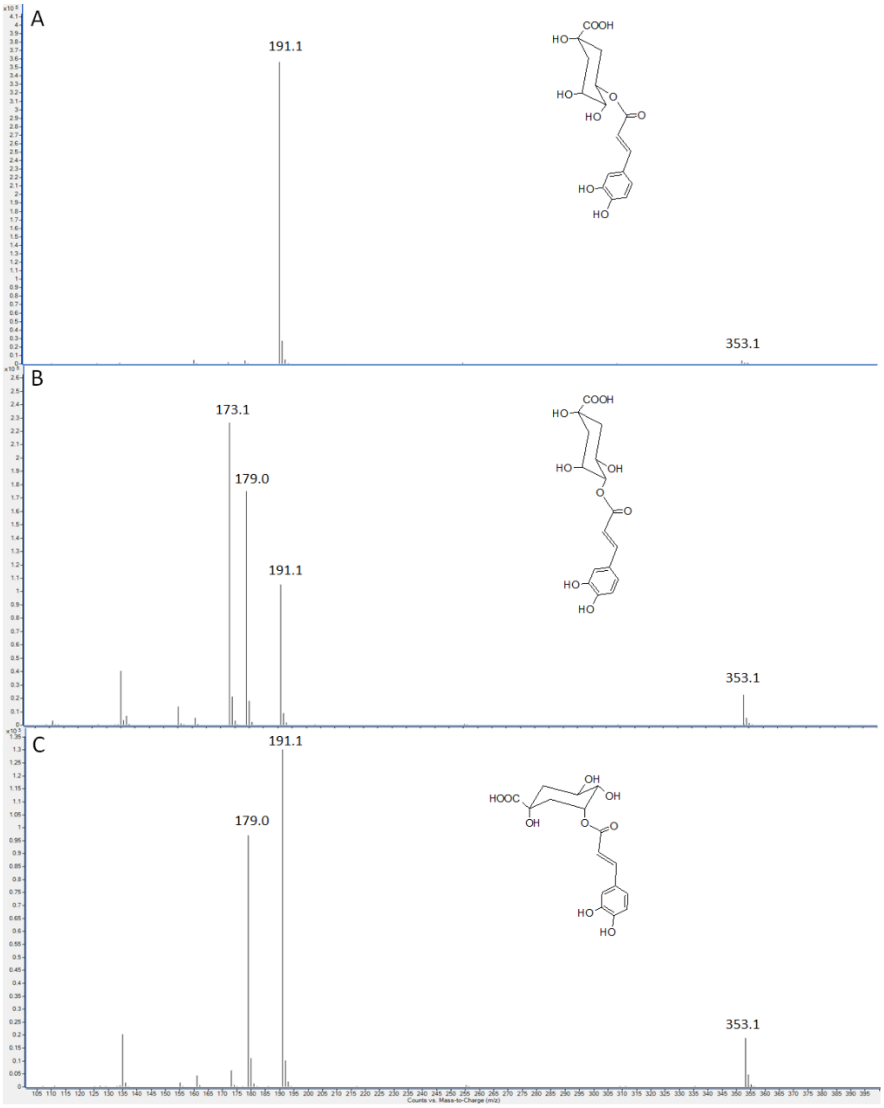

Figure 6.4 Negative mode mass spectra of 5-CQA (A), 4-CQA (B) and 3-CQA (C) obtained with the 6550 iFunnel QTOF.

6.4.2.5 Separation of the Mono-Caffeoylquinic Acid Isomers Using FAIMS-MS/MS

Previously, HPLC alone or in conjunction with MS was used to separate caffeoylquinic acid isomers (Andrade et al., 1998; Vrhovsek et al., 2012). However, HPLC-based methods are tedious requiring a long run time that can exceed an hour; therefore, they are impractical for high throughput screening of a large number of samples. As such, it would be highly advantageous to develop new analytical methods that can benefit from the selectivity and specificity of a mass spectrometer without the need for HPLC separation.

FAIMS is a rapidly growing separation technology that has been successfully interfaced with MS for various applications (Kanu et al., 2008). In principle, ions generated by an ESI source, for example, are filtered based on their differences in mobility employing oscillating high and low electric fields that act perpendicular to ion motion (Beach et al., 2015) prior to their transmission to the MS analyzer. FAIMS-MS has been shown to be an effective tool for the separation of isobaric compounds in short periods of time (Kanu et al., 2008; Purves et al., 2014; Beach et al., 2015). Therefore, we explored the possibility of employing FAIMS-MS/MS for the separation of mono-caffeoylquinic acids without the use of an HPLC. In combination with the isomer-specific MS/MS fragmentation pattern observed in the negative ion mode (Figure 6.4 and Table 6.3), the use of FAIMS can provide a method to identify and potentially quantify these compounds in complex samples containing a variety of phenolics.

Optimization of the FAIMS parameters was performed on commercial standards in order to obtain reproducible separation of these isomers. The effects of various FAIMS parameters on the resolution of the isomers were investigated sequentially including the magnitude of the dispersion voltage (DV), electrode temperature and carrier gas composition as well as its flow rate. Initially, standards were analyzed without the presence of a gas modifier (100% nitrogen as the carrier gas). Under such conditions, standards eluted from the smallest to the largest CV values in the order of 4-CQA, 5-CQA and finally 3-CQA, where all three isomers eluted at positive CV values. However, significant overlap between 4-CQA and 5-CQA was observed (data not shown), and variation of the DV from -3500 to -4000 V resulted in minimal improvement in their separation.

Previous research has shown that the addition of a gas modifier to the nitrogen stream can aid in analyte separation during FAIMS analysis, particularly for low molecular weight compounds (Purves et al., 2014). In addition, it was shown that acetonitrile provided the best separation, as

compared to other gas modifiers such as methanol, for a series of 15 compounds when analyzed by FAIMS-MS/MS (Purves et al., 2014). Therefore, acetonitrile was used initially for the analysis of the mono-caffeoylqinic acid isomers. The addition of acetonitrile afforded the separation of the three isomers without affecting the elution order. Increasing the flow rate of acetonitrile from 0.050 mL/min to 0.150 mL/min resulted in a gradual improvement in isomer resolution that eventually deteriorated at a flow rate of 0.200 mL/min. Therefore, 0.150 mL/min was chosen as the optimum flow rate. While the use of acetonitrile allowed for the separation of these mono-caffeoylquinic acids, it also resulted in the appearance of a small secondary peak for each standard (data not shown). These secondary peaks are likely dimers, multimers or adducts formed with acetonitrile that decompose to monomers upon their introduction to the mass spectrometer (Beach et al., 2015). Unfortunately, these secondary peaks overlapped with the main peaks of other isomers. Varying the DV values from -3300 to -3700 V did not improve the separation between the secondary peak of 4-CQA and the main peak of 5-CQA. Therefore, the gas modifier was switched from acetonitrile to methanol.

When methanol was used as the modifier gas, a single peak was observed for each standard (Figure 6.5A). In addition, the initial experiments exhibited significantly enhanced resolution in comparison with the optimized method using acetonitrile as the gas modifier. Near baseline resolution was achieved upon the optimization of the methanol flow rate at 0.175 ml/min and a DV value of -3500 V. Figure 6.5A shows the optimum separation of the three standards, where 4-CQA elutes at a CV value of 5.8 V, 5-CQA at 8.8 V and 3-CQA at 16.9 V under the optimized experimental conditions. Identification of each isomer was based on the established CV values combined with the diagnostic MS/MS behaviour discussed previously (Table 6.3). During ESI-FAIMS-MS analysis, the three diagnostic product ions were monitored (i.e., *m/z* 173, 179 and 191) and their abundances are represented as the solid, dotted and dashed lines in Figure 6.5, respectively. Each mono-caffeoylquinic acid can be identified by its unique product ion ratios as shown in Table 6.3 and Figure 6.4. For example, 5-CQA could be readily identified by its CV value of 8.8V along with the large abundance of the *m/z* 191 product ion (dashed line, Figure 5A) and minimal amounts of either the *m/z* 179 or 173 ions (dotted and solid lines, respectively). The other two isomers were identified using the same approach, combining their differential ion mobility data with MS/MS ion intensity. Each approach alone was not sufficient for the absolute

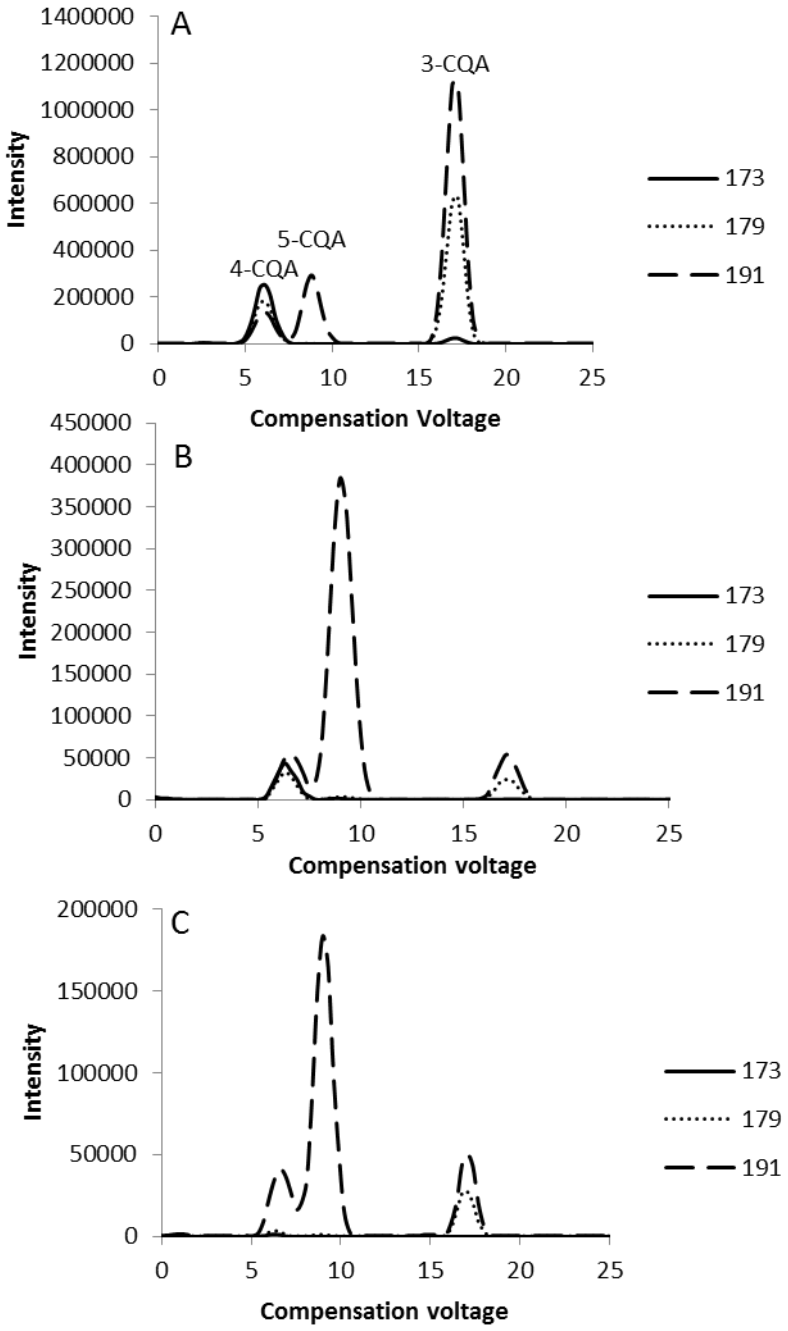

Figure 6.5 CV spectra for the mixed mono-caffeoylquinic acid isomer standards (A), and commercial apple (B) and pear juices (C).

identification of the target isomers within juices. However, the combination of FAIMS with MS/MS afforded the identification of the three mono-caffeoylquinic acids in complex mixtures.

In addition to the gas modifier and DV values, the influence of electrode temperature on the resolution of the isomers was also evaluated. In these experiments, the temperatures of the inner and outer electrodes were varied synchronously from 55 to 110°C, and the temperature of the outer electrode was held constant at 90°C and the temperature of the inner electrode was varied to produce gradients of -20 to +5 °C. It was found that increasing the temperature of both electrodes simultaneously resulted in significant improvement in the separation of the isomers. Similar improvement was noted when increasing the inner electrode temperature while maintaining the outer one at 90°C. However, increasing the temperature above 90°C resulted in a remarkable decrease in the signal intensity with no further separation benefits. Therefore, the electrodes' temperature was fixed at 90° C.

To demonstrate the use of FAIMS-MS/MS for the separation of mono-caffeoylquinic acids in real samples, commercial apple and pear juices were analyzed employing the optimized conditions. These samples were selected as they are known to contain a variety of phenolics (Spanos and Wrolstad, 1990; Spanos et al., 1990) that may interfere with the separation and detection of mono-caffeoylquinic acids. Initially, the juices were infused into the system without any prior sample preparation apart from dilution with water and filtration. However, the high carbohydrate content of these products (>95% of the total soluble solids) (Willems and Low, 2014) interfered with the analysis causing strong ion suppression. Therefore, each juice was extracted as described (Green, 2007) to separate the phenolics from carbohydrates. Once the carbohydrates were removed, clear CV spectra were obtained for each sample (Figures 6.5B and C).

The apple juice sample contained all three of the mono-caffeoylquinic acid isomers, where 5-CQA was the major peak (Figure 6.5B). This result agreed with literature as 5-CQA (chlorogenic acid) has been reported to be the major phenolic found in apple products and the presence of other chlorogenic acids have also been noted (Spanos et al., 1990). However, the peak corresponding to 4-CQA isomer did not follow the exact pattern as shown in the standards in terms of ion intensities. Major product ions at m/z 191 and 173 were observed while 179 was present at lower abundance. This deviates from the pattern established using the standards (Figure 6.5A). Most likely, the observed peak, corresponding to 4-CQA in Figure 6.5B is a mixture of 4-CQA and other mono-

caffeoylquinic acid isomers reported in the literature, such as 1-*O*-caffeoylquinic acid or one of the cis mono-caffeoylquinic acids (Kolniak-Ostek and Oszmiański, 2015).

In the case of pear, 5-CQA has also been reported to be a major phenolic (Kolniak-Ostek and Oszmiański, 2015) supporting our findings that showed a major 5-CQA peak as well as a smaller 3-CQA peak (Figure 6.5C) in this juice. In the case of pear, a compound was observed that eluted at the same CV value as 4-CQA with a $[M - H]^-$ *m/z* of 353. However, its fragmentation pattern did not match that of 4-CQA as it had a major product ion at *m/z* 191 (dashed line, Figure 6.5 C) with minor product ions at *m/z* 179 and *m/z* 173. Therefore, this compound is not 4-CQA. It is most probably another mono-caffeoylquinic acid isomer such as 1-caffeoylquinic acid or one of the *cis* mono-caffeoylquinic acids which have been recently reported to be found in various parts of pear fruit and leaves (Kolniak-Ostek and Oszmiański, 2015).

Previously, phenolic profiling has been used as a method to detect adulteration in fruit based products (de Simon et al., 1992; Versari et al., 1997). Therefore, the presence or absence of certain mono-caffeoylquinic acids, as determined by FAIMS-MS, may also be beneficial for authenticity analysis. For example, the presence of 4-CQA in juice claiming to be 100% pear may be indicative of the presence of undeclared apple juice. However, further work would need to be performed in order to determine the robustness of such methods for the detection of juice-to-juice adulteration.

From the aforementioned results, it can be deemed that ESI-FAIMS-MS/MS acts as a two-dimensional analytical platform that allows for the separation and identification of mono-caffeoylquinic acid isomers in apple and pear juice samples without the need for time consuming HPLC separation. The short acquisition time (less than 1 min) suggests the potential suitability of the method to handle high volumes of samples typically observed in a food quality control laboratory in a timely fashion.

6.5 Conclusions

Generalized MS/MS fragmentation behavior was developed for mono-caffeoylquinic acids in both the positive and negative ion modes and the similarities and differences between the two modes were evaluated. Unique diagnostic product ions were identified that can be used in any future targeted analysis for either identification or quantification purposes. Interestingly, the three positional isomers were differentiated based on the MS/MS data in negative ion modes allowing

for the development of a fast FAIMS-MS/MS identification method. A rapid (<1 min) method for the separation and identification of these compounds in complex juice samples employing ESI-FAIMS-MS/MS was developed. The method allowed for the separation of the three isomers without the need for HPLC separation. We will utilize FAIMS-MS/MS to develop targeted quantification methods so that we can determine the amounts of each isomer within juice samples. This research lends itself well to further developments, such as authenticity analysis of fruit juices.

6.6 Linkage Between Chlorogenic Acid Analysis by Ion Mobility and Tandem Mass Spectrometry and Structural Identification of Phenolic Compounds for Authenticity

Results from this research showed that FAIMS-MS/MS could be successfully used to rapidly differentiate structurally similar phenolics, specifically the naturally occurring chlorogenic acids in apple and pear juices. Based on the HPLC-PDA (Chapter 5) and mass spectral (Chapter 6) analysis of commercial apple and pear juice phenolics, it was hypothesized that a phenolic fingerprinting method could be developed to detect juice-to-juice debasing between these two genera and research on solving this important adulteration issue is reported in Chapter 7.

7. STRUCTURAL IDENTIFICATION OF PHENOLIC COMPOUNDS FOR USE IN THE DETECTION OF JUICE-TO-JUICE DEBASING BETWEEN APPLE AND PEAR JUICES[1]

7.1 Abstract

The ability to detect the undeclared addition of a juice of lesser economic value to one of higher value (juice-to-juice debasing) is a particular concern between apple and pear juices due to similarities in their major carbohydrate/polyol profiles. Fingerprint compounds for the detection of this type of adulteration were identified in both commercial apple and pear juices by HPLC-PDA, were isolated chromatographically, and structurally identified by LC-MS/MS. The apple juice fingerprint was identified as 4-*O*-p-coumarylquinic acid and two pear compounds as isorhamnetin-3-*O*-rutinoside and abscisic acid. Additionally, the HPLC-PDA profile of pear juices in combination with pear fingerprint compounds including arbutin could be used to identify samples originating from China versus those from other geographical locations.

7.2 Introduction

Food adulteration is a serious issue that can have negative impacts on both consumers and honest producers. In 2014, it was estimated that food fraud affected approximately 10% of all commercially sold food products costing the global food industry between $10 and $15 billion per year (Johnson, 2014). Fruit based products, such as juices and jams, are common targets for adulteration due to their high carbohydrate content and the availability of less expensive ingredients which can closely match the carbohydrate profile of the unadulterated product (Silva et al., 2000; Thavarajah and Low, 2006a; Willems and Low, 2014). For example, one method of fruit juice adulteration is the undeclared addition of a less expensive juice, which is dependent upon its current market value, to one of higher value, and is referred to as juice-to-juice

[1]Reproduced with permission. Willems, J. L. and Low, N. H. (2017). Structural identification of phenolic compounds for use in the detection of juice-to-juice debasing between apple and pear juices. Submitted to Food Chemistry, May 2017.

adulteration. This is a particular concern between apple and pear juices due to similarities in their organoleptic properties, such as colour, flavour and mouthfeel, coupled with their virtually indistinguishable major carbohydrate (glucose, fructose and sucrose) and polyol (sorbitol) profiles (Thavarajah and Low, 2006a). The current (2017) market value of apple and pear juice concentrates in the United States are $8.20 and $9.00/US gallon, making the addition of apple-to-pear juice financially viable (personal communication). However, the value of these products can vary such that apple juice may have a higher market value than pear. Also, pear juice concentrate is valued at $5.10/gallon while apple juice concentrate is worth $5.40/gallon in China and the large price differences between geographical regions makes adulteration using concentrates from these regions financially incentivized.

One method to detect the undeclared addition of a fruit of lesser economic value to another fruit product is through phenolic profiling (Silva et al., 2000). Phenolics are secondary plant metabolites that are derived from phenylalanine and to a lesser extent tyrosine with more than 8000 different compounds reported in nature (Manach et al., 2004; Vermerris and Nicholson, 2008; Terahara, 2015). Phenolics serve a variety of purposes in plants such as imparting colour, attracting or repelling insects, they exhibit both antimicrobial and antiviral activity, and they also provide UV protection (Manach et al., 2004). Many factors can affect the phenolic profile of a fruit including variety, maturity and ripeness, and growing, storage and processing conditions (Spanos and Wrolstad, 1992; Tanrıöven and Ekşi, 2005; Nayak et al., 2015). Consistent differences in the phenolic profiles between fruits can be used to identify which fruit or fruits are present in a product. This can be accomplished via the identification of a unique phenolic/group of phenolics that can act as a fingerprint(s)/marker(s) for the presence or absence of that fruit in a product. For example, the glycosylated phenolic arbutin has been consistently reported to be present in pear and absent in apple making it a marker for pear to apple juice adulteration (Andrade et al., 1998; Thavarajah and Low, 2006a; Willems and Low, 2014). However, there are many contradictory studies in literature where a phenolic has been identified as a specific apple or pear fruit marker. As examples, isorhamnetin-3-glucoside has been proposed as a potential pear marker, and phloridzin a potential marker for apple juice (de Simón et al., 1992; Versari et al., 1997; Andrade et al., 1998; Schieber et al., 2001). However, research has shown that phloridzin is not ubiquitous in apples/apple products nor isorhamnetin-3-glucoside in pear/pear products; in addition, isorhamnetin-3-glucoside has been reported in apple fruit (Schieber et al., 2002; Alonso-Salces et

al., 2004; Thavarajah and Low, 2006a), thus eliminating their use as phenolic-based authenticity markers. Finally, it is possible to enzymatically hydrolyze a marker such as arbutin with the possibility of subsequent masking of the undeclared addition of pear juice addition to another fruit/food product (Thavarajah and Low, 2006a)

Based on this literature information, the goal of this research was to identify unique phenolics in commercial apple and pear juice samples for use in juice-to-juice authenticity analysis. For this purpose, a group of commercial apple (n = 27) and pear (n = 31) juices were obtained from major world producing regions. Fingerprint compounds for each fruit juice species were identified and structurally characterized.

7.3 Materials and Methods

7.3.1 Materials

Thirty-one commercial pear juice and twenty-seven commercial apple juice concentrates (~70 °Brix) representing three production years (2012-14), and the major world producing regions for these fruits/juice concentrates were analyzed in this study. Pear juice concentrates were obtained from Argentina, Chile, China, New Zealand and the United States of America. Apple juice concentrates were obtained from Argentina, Brazil, Chile, China and the United States of America.

Abscisic acid, Amberlite XAD16N resin, apigenin, arbutin, caffeic acid, 5-*O*-caffeoylquinic acid (chlorogenic acid), catechin, p-coumaric acid, epicatechin, ferulic acid, formic acid, gallic acid, 4-hydroxybenzoic acid, isorhamnetin-3-O-glucoside, isoquercetin, narigenin, phloridzin, quercetin, resveratrol and rutin were purchased from Sigma Aldrich Canada Ltd. (Oakville, ON, Canada). Isorhamnetin-3-*O*-rutinoside was purchased from Extrasynthese (Lyon, France). Acetonitrile (HPLC grade) and methanol (ACS grade) were purchased from Fisher Scientific (Ottawa, ON, Canada). The water used throughout this study was produced using a Milli-Q™ water system (Millipore Corp., Milford, MA, USA).

7.3.2 High Performance Liquid Chromatography with Photodiode Array Detection (HPLC-PDA)

Fingerprint compound identification was conducted using an Agilent 1100 series HPLC system with a photodiode array (PDA) detector controlled by ChemStation LC-3D software

(Agilent Technologies Canada Inc., Mississauga, ON, Canada). Phenolic separation was accomplished on an ODS-3 (250 x 4.6 mm; 5 µm, C_{18}, 100 Å) column (Phenomenex, Torrance, CA, USA) in series with a guard column (4 x 3 mm) of the same stationary phase. A linear gradient mobile phase system employing 10.0 mM aqueous formic acid (mobile phase A; pH 3.5) and 70% acetonitrile:30% mobile phase A (v:v; mobile phase B) was used for phenolic separation as follows: initial 100% A for 3.0 min, followed by a gradient to 4.0% B at 16.0 min, followed by a gradient to 10.0% B at 25.0 minutes, followed by a gradient to 15.0% B at 40.0 min, followed by a gradient to 20.0% B at 45.0 min, followed by a gradient to 23.0% B at 50.0 min, followed by a gradient to 25.0% B at 55.0 min, followed by a gradient to 30.0% B at 61.0 min, followed by a gradient to 50.0% B at 75.0 min, followed by a gradient to 80.0% B at 80.0 min, hold at 80.0% B for 5.0 min. The column was then re-equilibrated with 100% A for 7.0 minutes prior to the next injection. The total run time was 95.0 minutes. Sample phenolic profiles were monitored at 254, 280 and 360 nm.

Juice samples were prepared by dilution with water to 11.5 ± 0.1 and 12.0 ± 0.1 °Brix (Auto Abbe Refractometer; Lecia Inc., Buffalo, NY, USA) for apple and pear, respectively. Samples were syringe filtered (nylon, 0.2 µm pore size, 13 mm diameter, Chromatographic Specialties, Brockville, ON, Canada) into 2 mL amber HPLC vials (Chromatographic Specialties) prior to analysis. The sample injection volume was 60.0 µL.

7.3.3 Isolation of Fingerprint Compounds

Identified fingerprint compounds (one for apple and two for pear) were isolated from commercial apple and pear juice samples for structural identification as follows: (a) for each species, a selected (based on fingerprint compound concentration) juice concentrate was diluted to 24.0 ± 0.1 °Brix and 15 mL was added to a 8.0 cm x 3.0 cm glass column packed with approximately 55 mL of solvent treated Amberlite XAD-16N resin. The resin was initially hydrated in 50% (v:v) aqueous methanol for 30 minutes before being transferred to the glass column followed by pre-conditioning with 110 mL of water then by 110 mL 90% (v:v) aqueous methanol and 110 mL of water. Once loaded on the resin, juice samples were fractionated with 110 mL water followed by 110 mL of 70% aqueous methanol (v:v). The 70% aqueous methanol fraction was collected and concentrated to approximately 5.0 mL using a Büchi rotary evaporator (Flawil, Switzerland) and (b) the concentrated 70% aqueous methanol fraction was syringe filtered

prior to HPLC-PDA fractionation to obtain purer fractions of the fingerprint compounds. The relative retention times of the fingerprint compounds were 57.7 minutes for apple and 70.6 and 77.1 minutes for pear. Chromatographically isolated fractions containing the identified fingerprint compounds were collected and combined prior to being concentrated employing rotary evaporation, followed by freeze drying (Hetro Lab Equipment, Allerod, Denmark) in 12 x 32 mm glass vials (Chromatographic Specialties, Brockville, ON, Canada). Isolated compounds were stored at -18 °C until structurally analyzed.

7.3.4 Mass Spectrometric Analysis

High resolution mass measurements of fingerprint compounds were obtained using an Agilent 1100 series HPLC as described above coupled with an API QSTAR XL MS/MS hybrid QqToF mass spectrometer equipped with an ESI source (Applied Biosystems Inc., CA, USA). Nitrogen was used as both the drying and ESI nebulizing gas. External calibration employing caesium iodide (*m/z* 132.9054) and sex pheromone inhibitor iPD1 (*m/z* 829.5398) were used to ensure high mass accuracies. Samples were analyzed in the negative mode and the injection volume was 20.0 µL. Mass spectra were analyzed using Analyst software (version 1.62).

Tandem MS was carried out for fingerprint compound structural identification using an Agilent 1200 series HPLC system with a photodiode array (PDA) detector coupled to a QTRAP 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA), which is a hybrid triple quadrupole linear ion trap mass spectrometer (QqQ-LIT) equipped with a Turbo V Ion Spray ESI source. Chromatography parameters were as described above; however, the injection volume was reduced to 7.5 µL and an analytical fixed flow post column splitter (split ratio of 3:1; ASI QuickSplit) was inserted after the PDA detector to reduce the amount of mobile phase entering the ESI source. All samples were analyzed in the negative ion mode.

7.3.5 Nuclear magnetic resonance spectroscopy (NMR)

^{1}H nuclear magnetic resonance spectroscopy of the fingerprint compounds was performed on a Brüker Avance 500 Mhz spectrometer (Brüker, Rheinstetten, Germany) using D_2O as the solvent.

7.4 Results and Discussion

The chromatographic profiles of the 27 commercial apple juices and 31 commercial pear juices were determined by HPLC-PDA. This information was used to identify potential fingerprint compounds for each juice species so as to be employed for the detection of juice-to-juice adulteration. Based on chromatographic profiling, one compound in the apple juice samples and two in the pear juice (excluding arbutin) samples were identified as fingerprint compounds based on their exclusive presence in either commercial apple or pear juice. In addition to exclusive presence, the identified fingerprint compounds had retention times that made them readily identifiable from other naturally occurring compounds detected under the chromatographic conditions employed. Also, these fingerprint compounds were present in sufficient concentrations for detection. Fingerprint/marker compounds were isolated from selected apple and pear juice samples based on their concentrations and were structurally identified using a combination of UV-visible spectroscopy, mass spectrometry, nuclear magnetic resonance spectroscopy (NMR) and comparison to commercial standards.

7.4.1 Identification of the Apple Juice Fingerprint Phenolic

Based on its detection under the HPLC-PDA conditions employed and UV-vis spectrum, the apple juice fingerprint was tentatively identified as a phenolic. To aid in its identification, a selection of commercial standards were analyzed by the developed HPLC-PDA method. These standards included arbutin, apigenin, caffeic acid, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, catechin, p-coumaric acid, epicatechin, ferulic acid, gallic acid, 4-hydroxybenzoic acid, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, isoquercetin, narigenin, phloridzin, quercetin, resveratrol and rutin. These standards were chosen to represent the major phenolic subclasses, and to cover a selection of phenolics previously identified in apple and pear fruit and juices (Spanos and Wrolstad, 1992; Pincinelli, 1997; Tanrıöven and Ekşi, 2005; Lee et al., 2017).

The apple juice fingerprint compound had a retention time of 57.7 minutes under the chromatographic conditions employed, and did not match any of the standards (Figure 7.1A). The UV-vis spectrum of this compound showed a distinct spectral pattern with λ_{max1} at 228 nm and λ_{max2} at 312 nm as shown in Figure 7.2A. Phenolics have been shown to have characteristic UV-vis absorbance profiles that are dependent upon structure, which affords their classification

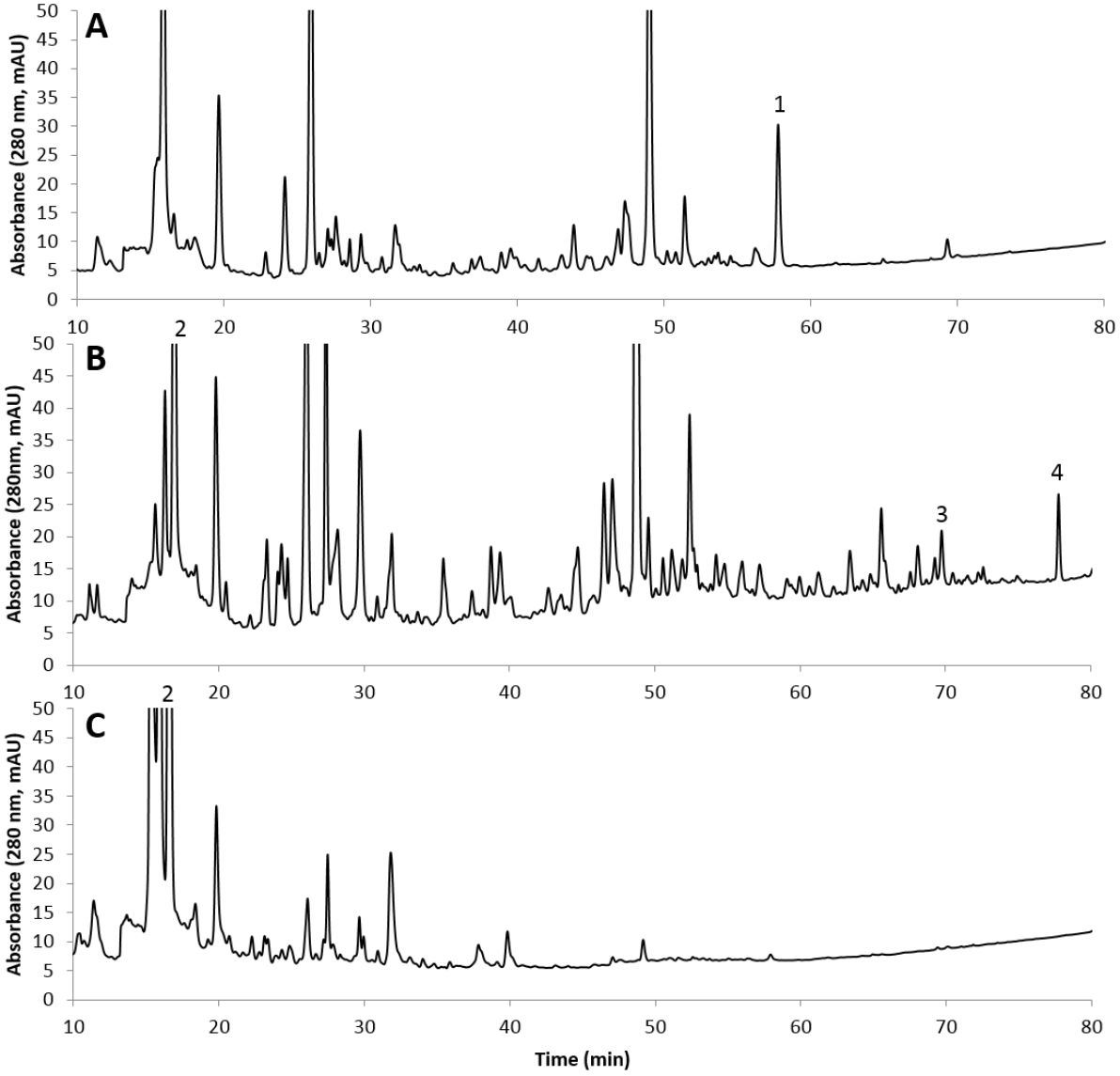

Figure 7.1 HPLC-PDA chromatograms (280 nm) of a representative apple juice (A), a representative pear juice (Argentina, B) and a Chinese pear juice (C). Fingerprint compounds are labelled as follows: 1) 4-*O*-p-coumarylquinic acid, 2) arbutin, 3) isorhamnetin-3-O-rutinoside and 4) abscisic acid.

(Escarpa and González, 2001a). Phenolic acids and flavonoids have been further differentiated into subclasses based on characteristic ultraviolet radiation absorbance patterns in the range of 190 to 380 nm (Green, 2007). Based on its UV-vis spectrum, the apple juice fingerprint compound was identified as a phenolic and as a member of the hydroxycinnamic acid class by its characteristic absorbance in the 200 to 290 nm and 270 to 360 nm ranges, and by comparison to 5-*O*-caffeoylquinic acid (Figure 7.2B) (Robbins, 2003; Abad-García et al., 2009).

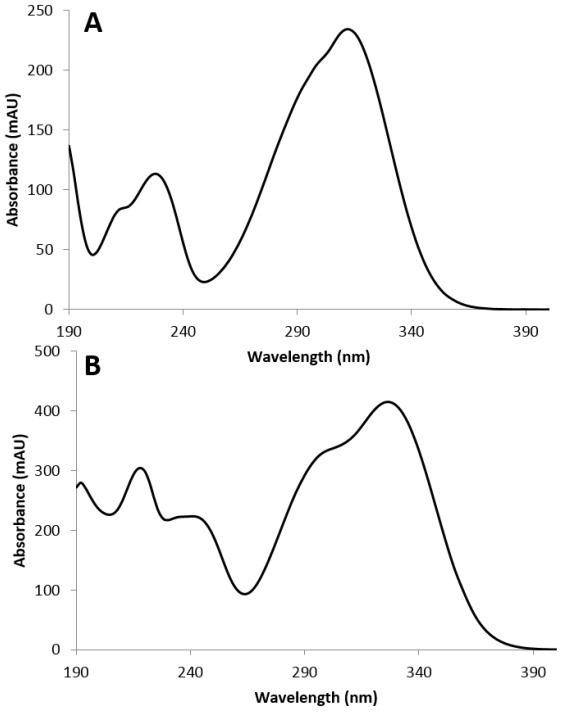

Figure 7.2 UV-visible spectra of the apple juice phenolic fingerprint (A) and 5-*O*-caffeoylquinic acid (B).

Analysis of the chromatographically isolated fingerprint compound by mass spectrometry in the negative ion mode gave a deprotonated ion at *m/z* 337.1 with MS/MS analysis giving a base peak at *m/z* 173.0 and major fragment ions at *m/z* values of 191.3 and 162.1 (Figure 7.3A). Additionally, high resolution mass spectral analysis of this compound in the negative ion mode produced a m/z value of 337.1002.

Based on both mass spectral and UV-vis spectroscopy results, the apple juice fingerprint phenolic was tentatively identified as a p-coumarylquinic acid, which has a monoisotopic mass of 338.1002 g/mole (mass accuracy of -22.0 ppm) and a molecular formula of $C_{16}H_{18}O_8$. This

compound is a phenolic and a member of the hydroxycinnamic acid class; it is also a chlorogenic acid which is a further subclass of hydroxycinnamic acids defined by the presence of one or more hydroxycinnamic acids linked to quinic acid through an ester bond.

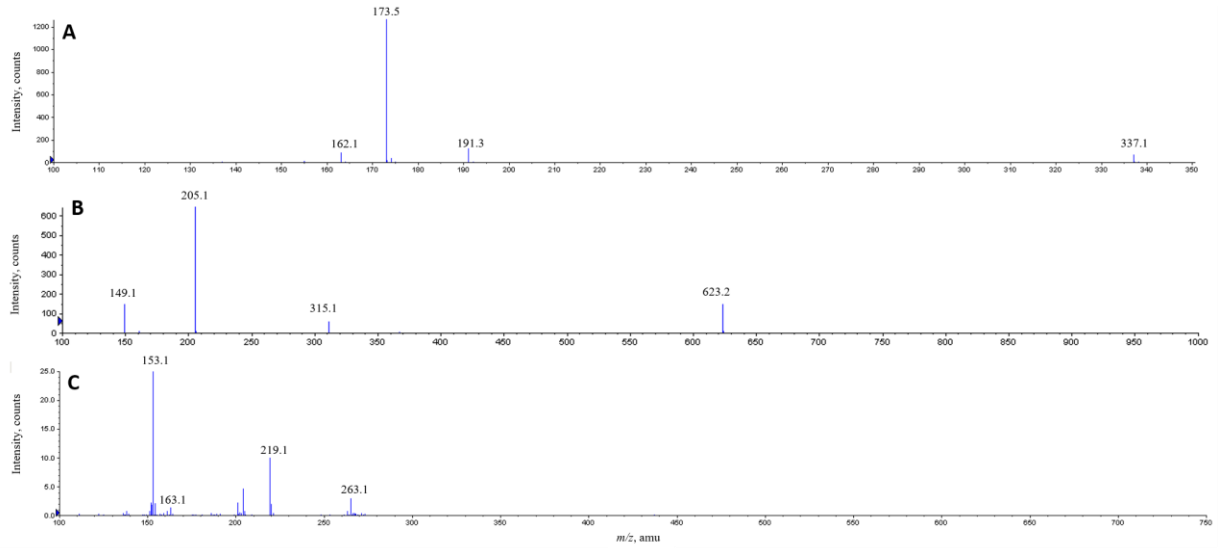

Figure 7.3 Negative ion mode LC-MS/MS spectra of the apple (A) and pear (B and C) juice fingerprint compounds.

Research results from our group has shown that chlorogenic acids fragment in a specific manner which is dependent upon the location of the ester bond linking hydroxycinnamic and quinic acids (Willems et al., 2016). Chlorogenic acids show three main fragments during MS/MS analysis in the negative ion mode corresponding to the hydroxycinnamic acid, quinic acid (*m/z* 191) and dehydrated quinic acid (*m/z* 173). The abundance of these three product ions in relation to each other has been shown to be a reliable indicator of the position of the ester linkage (i.e., 3, 4 or 5) for chlorogenic acids (Jaiswal et al., 2010; Jaiswal et al., 2011; Willems et al., 2016). For example, 4-*O*-caffeoylquinic acid can be distinguished from the other two isomers by having a base peak corresponding to the dehydrated quinic acid product ion (*m/z* 173) with significant amounts of the caffeic acid (*m/z* 179) and quinic acid (*m/z* 191) product ions (Willems et al., 2016). This pattern has been shown to be consistent with other chlorogenic acids such as feurloylquinic acids and p-coumarylquinic acids (Jaiswal et al., 2010).

The apple juice fingerprint compound showed a MS/MS fragmentation pattern consistent with an ester linkage at the 4-position of quinic acid (Figure 7.3) as the base peak corresponded to

the dehydrated quinic acid fragment (*m/z* 173), with the other major ions for the p-coumaric acid (*m/z* 162) and quinic acid (*m/z* 191) fragments. Based on this information the apple juice fingerprint phenolic was identified as 4-*O*-p-coumarylquinic acid (Figure 7.4A).

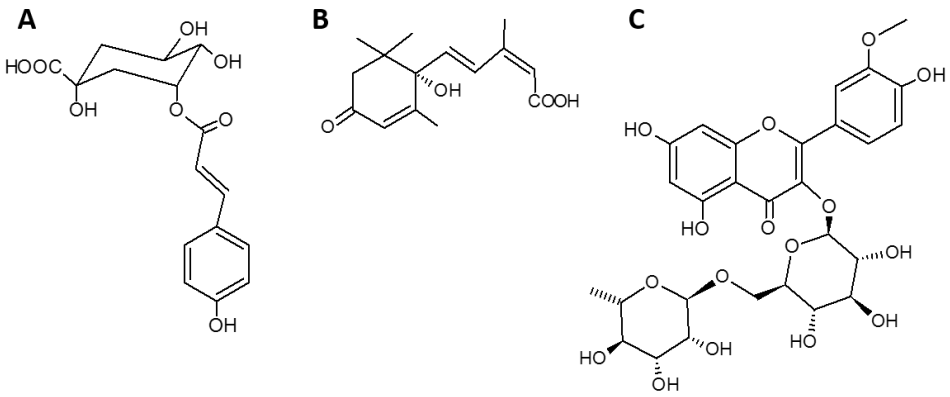

Figure 7.4 Proposed structure of the apple juice fingerprint phenolic, 4-*O*-p-coumarylquinic acid (A) and the pear juice fingerprint compounds, abscisic acid (B) and isorhamnetin 3-*O*-rutinoside (C).

Due to the lack of a commercial standard, additional evidence to support the proposed structure of this compound was gathered using ^{1}H-NMR. The presence of the p-coumaryl group was confirmed by examining the aromatic hydrogen region of the NMR spectrum from 6.3 to 7.8 ppm. Aromatic hydrogens were identified as doublets at 7.55 (integration = 2) and 6.89 ppm (integration = 2), which was confirmed by the coupling constant between these two of J = 8.65, which is consistent for the presence of ortho hydrogens. The vinyl hydrogens were identified as doublets at 7.73 (integration = 1) and 6.44 ppm (integration = 1). The *Z* configuration of the vinyl hydrogens was confirmed by the observed coupling constant of J = 16.01, which is consistent with the large coupling constants (i.e., 12-18 Hz) seen between *Z*-hydrogens rather than the smaller coupling constants (i.e., 6-12) seen with *E*-hydrogens (Silverstein and Webster, 1998). The identity of the p-coumaric acid moiety was also confirmed by comparison to the p-coumaric acid standard. The quinic acid moiety was confirmed by comparison to a quinic acid standard.

To determine the concentration of 4-*O*-p-couraylquinic acid in the 27 commercial apple juice samples, 5-*O*-caffeoylquinic acid (chlorogenic acid) was used as the standard based on structural similarities and the absence of a commercial standard. Calculated concentrations ranged from 2.3 to 20.2 ppm with an average of 9.2 ± 4.5 ppm. p-Coumarylquinic acid has been reported

in apple fruit as identified by both HPLC-PDA and HPLC-MS, being predominantly found in the flesh with other hydroxycinnamic acids (Khanizadeh et al., 2008; Lee et al., 2017), however, this structure was not confirmed by chromatographic isolation, high resolution mass spectrometry and NMR spectroscopy identification and the identity of which isomer(s) were present was not determined.

7.4.2 Identification of Pear Juice Fingerprint Compounds

Previous research has shown that arbutin can be used as an effective marker for the presence of pear juice in a product and arbutin was identified in all 31 of the analyzed pear juices with a retention time of 16.4 minutes and with concentrations ranging from 50.6 to 286.9 ppm (Willems and Low, 2014). The highest concentrations of arbutin were found in the Chinese samples, with two of the four samples containing concentrations >200 ppm (223.7 and 286.9 ppm). The other two Chinese samples showed arbutin concentrations between 100 and 200 ppm (114.0 and 128.7 ppm). Only two other samples, both from New Zealand, showed similarly high concentrations (128.8 and 134.0 ppm). The remaining samples had arbutin concentrations <85.0 ppm (Willems and Low, 2014).

Arbutin was not detected in any of the analyzed apple juices making it a fingerprint phenolic for the presence of pear-to-apple adulteration. However, it is possible to enzymatically hydrolyze a marker such as arbutin with the possibility of subsequent masking of the undeclared addition of pear juice addition to another fruit/food product (Thavarajah and Low, 2006a). Therefore, two additional fingerprint compounds were identified, isolated and their structures were determined.

The first pear juice fingerprint compound eluted at a retention time of 70.6 minutes under the chromatographic conditions employed. The UV-vis spectrum of this compound showed characteristic absorbances at, 202 (λ_{max1}), 253 (λ_{max2}) and 350 nm (λ_{max3}) (Figure 7.5A). Based on its UV-vis profile, this compound was classified as a flavonol, which is a subclass of the flavonoids. Flavonoids can be classified based on their absorbances in the 300 to 500 nm and 240 to 295 nm ranges corresponding to the B- (Band I) and A-rings (Band II) of the flavonoid backbone, respectively (Green, 2007). Flavonols typically have a Band I absorbance in the 352 to 385 nm range and a Band II absorbance between 250 to 275 nm (Green, 2007), which were observed for this fingerprint compound.

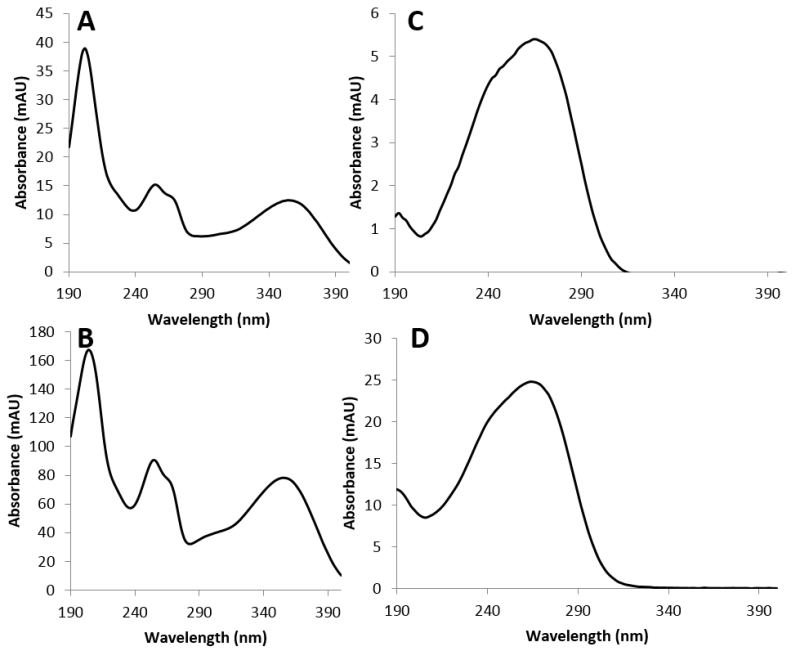

Figure 7.5 UV-visible spectra of the first pear juice fingerprint (A), isorhamnetin 3-*O*-rutinoside (B), the second pear juice fingerprint (C) and abscisic acid (D).

Analysis of the compound by high resolution MS in the negative ion mode gave a *m/z* value of 623.1610 which, when combined with the UV-vis data, suggested an identity of isorhamnetin-3-*O*-rutinoside which has a monoisotopic mass of 624.1690 g/mole and a molecular formula of $C_{28}H_{32}O_{16}$, which corresponds to a $[M-H]^-$ of 623.1618 (mass accuracy of -1.3 ppm). MS/MS analysis showed product ions at *m/z* 315.0, 205.1 and 149.1 (Figure 7.3B), where the fragment at 315.0 corresponds to the negatively charged isorhamnetin aglycone and the neutral loss of 308.1 corresponding to the carbohydrate moiety (Kolniak-Ostek and Oszmiański, 2015). The identification of isorhamnetin-3-O-rutinoside was confirmed by comparison to a commercial standard and its structure is shown in Figure 7.4B. Isorhamnetin-3-O-rutinoside has been previously reported in both the skin and flesh of pear fruit as identified by HPLC-MS/MS (Lin and Harnly, 2008; Kolniak-Ostek and Oszmiański, 2015).

The second pear juice fingerprint compound eluted at a retention time of 77.1 minutes and while this compound showed an absorbance in the typical range for phenolics (i.e. 280 nm) its UV-vis profile did not match that of any phenolic subclass (Figure 7.5C). However, its λ_{max} of 270 nm and overall UV-vis profile closely matched that of the plant hormone abscisic acid (ABA)

(Figure 7.5D; Jenkins and Shepherd, 1972). Structural identification of ABA, which has a monoisotopic mass of 264.3169 g/mole, a molecular formula of $C_{15}H_{20}O_4$ and a $[M-H]^-$ of 263.1289, was confirmed through high resolution MS which gave a *m/z* of 263.1301 in the negative ion mode (mass accuracy of 4.6 ppm). Additionally, MS/MS analysis showed product ions at *m/z* values of 219.1 and 153.1 (Figure 7.3C), which agrees with previous reports (Gómez-Cadenas et al., 2002). Finally, the identification of ABA was confirmed by comparison to a commercial standard and its structure is shown in Figure 7.4C.

ABA is a phytohormone that is ubiquitous in plants which helps to regulate development and metabolism and plays an important role in environmental stress responses (Gómez-Cadenas et al., 2002; Gao et al., 2004). ABA has been reported in the pear plant and while it was not detected in any of the commercial apple juice samples studied, it has been reported to be present in apple fruit (Berüter, 1983). Therefore, it is not clear why this compound was detected only in the pear juice samples. On its own, ABA may not be an effective authenticity marker for pear juice. However, when used in combination with arbutin and isorhamnetin-3-O-rutinoside, it can offer additional confirmation of the botanical origin of a product.

Isorhamnetin-3-O-rutinoside was detected in 28 out of 31 pear juice samples and ABA was detected in 27 out of 31 pear juice samples, with qualitative detection limits (i.e., 3xS/N) of 0.47 and 0.03 ppm, respectively. Pear juice isorhamnetin-3-O-rutinoside concentrations ranged from 2.20 to 11.10 ppm with a mean of 7.43 ± 2.53 ppm, whereas ABA concentrations ranged from 0.11 to 0.95 ppm, with a mean of 0.57 ± 0.22 ppm.

Of interest was the fact that the three samples in which isorhamnetin-3-O-rutinoside was not detected overlapped with the four samples in which ABA was not detected; all of which came from China (4 out of 4 of the Chinese samples). These results may be due to the species or variety of fruit used in pear juice production in this geographical region. Each of these four pear samples also showed a scientifically different 280 nm absorbance profile when compared to pear juices from the other geographical regions, particularly in the 50-80 minute range (Figure 7.1B and C).

7.4.3 Juice-to-Juice Debasing Detection Employing the Fingerprint Compounds

Based on this work, the presence of apple in pear or pear in apple juice can be detected through the presence/absence of the identified fingerprint compounds. For example, the addition of apple to pear juice could be determined by the presence of 4-*O*-p-coumarylquinic acid in the

product. Based upon the lowest concentration of 4-*O*-p-coumarylquinic acid in the analyzed apple juice samples and a qualitative detection limit of 0.2 ppm, the addition of apple-to-pear juice could be detected chromatographically at levels down to 7.0% (v:v), however lower detection limits could be obtained employing LC-MS/MS. As 4-*O*-p-coumarylquinic acid was detected in 100% of the apple juice samples and none of the pear juice samples no false positives or false negatives were identified.

The addition of pear to apple juice can be detected by the presence of arbutin and confirmed through the presence of isorhamnetin-3-*O*-rutinoside and ABA. Based upon the lowest concentration of arbutin in pear juice and a qualitative detection limit of 0.3 ppm, the addition of apple-to-pear can be detected at levels down to 0.6% (v:v); again, detection of this compound at lower levels is possible through LC-MS/MS analysis.

These fingerprint compounds could also be used to determine the absence of apple or pear juices in products claiming to contain them. As an example, a product claiming to be 100% pure apple juice in which 4-*O*-p-coumarylquinic acid was not detected or was present at a concentration $\leq$ 2.3 ppm could be deemed mislabeled.

The identified pear juice fingerprint compounds could possibly be used as an indicator for the country of origin of a product through the analysis of more samples from each geographical region. Specifically, samples obtained from China in this study showed high arbutin concentrations (>100 ppm) coupled with a low or non-detectable (by HPLC-PDA) concentration of isorhamnetin-3-*O*-rutinoside and non-detectable levels of ABA, which afforded their differentiation from juices obtained from other regions (Figures 7.1B and C). These differences are most likely due to fruit varieties used for juice production. Commercial pear juice produced in North and South America primarily uses Bartlett (also known as Williams), Beurre d'Anjou and Beurre Bosc pears, which are all members of the species *Pyrus communis* L., or European pear (Willems and Low, 2016). However, Asian pear (also known as Chinese pear) or *Pyrus pyrifolia,* cultivars are commonly grown in Asia and have been shown to have lower quantities of phenolics as compared to other *Pyrus* spp. (Lin and Harnly, 2008). The low number and concentrations of compounds eluting in the 50-80 minute region of the HPLC-PDA chromatogram detected at 280 nm could also be characteristic of a pear juice originating in China.

Finally, results from this study take into account the roles of commercial juice processing on the presence, absence and concentrations of fingerprint phenolics and abscisic acid in apple and pear juices from the major world producing regions.

8. GENERAL DISCUSSION

Fruit juices, such as apple and pear, are common targets for adulteration due to their vast consumption, economic value and high carbohydrate content. Typically, adulteration is carried out through the undeclared addition of an ingredient of lower value (i.e., debasing). In the case of fruit juices these adulterants are commonly less expensive commercial sweeteners (i.e., HFCS, TIS, HIS) or juices of a lower economic value. These adulterants can be added in such a manner that the major carbohydrate and polyol profiles of the juice remains within the natural range, obscuring their detection. Therefore, targeted methods are required for their detection.

The best method for targeted adulteration detection is through the identification of fingerprint compounds, which are unique to a potential adulterant and as such, can be used for its detection. While adulteration detection in fruit juices can employ many different techniques, the most prevalent are based on chromatographic profiling. In this research, targeted methods based on oligosaccharide and phenolic fingerprints were developed for the detection of pear juice debasing with less expensive commercial sweeteners, and apple to pear and pear to apple adulteration detection (i.e. juice-to-juice).

Oligosaccharide profiling as a method of adulteration detection is based upon the formation of oligosaccharides during the production of commercial sweeteners through acid reversion and/or transglycosylation reactions (Low, 1998; Thavarajah and Low, 2006). Oligosaccharides that are present in these sweeteners but not in the authentic product act as a fingerprint for their undeclared presence. In this research, HPAE-PAD and CGC-FID were used to determine the oligosaccharide profile of a selection of low cost commercial sweeteners and a database of pure commercial pear juices from the major world geographical regions. It was found that, isomaltose could act as fingerprint for the presence of HFCS, inulobiose as a fingerprint for HIS, and TIS showed fingerprint compounds which were previously identified to be glucose-glucose and glucose-fructose disaccharides (Thavarajah and Low, 2006).

Commercial pear juice was also shown to contain a number of naturally occurring monosaccharide/oligosaccharides. During juice production a variety of carbohydrases, namely pectinases, amylases and hemicellulases, are employed to help break down pear polysaccharides improving juice yield, reducing juice viscosity and aiding in juice clarification (Bates et al., 2001; Ceci and Lozano, 2010; Horváth-Kerkai and Stéger-Maté, 2012). In this research, it was found that the majority of polysaccharide hydrolysis and monosaccharide/oligosaccharide formation occurred during enzymatic treatment at the pear mashing stage and that the remaining processing steps had a minimal impact on the carbohydrate-based chromatographic profile of pear juice. Also, all commercial enzyme preparations and conditions (time and temperature) studied produced similar carbohydrate-based chromatographic profiles. The results from this work offer industry a method to increase the soluble fibre content of their juices, and government agencies a method to monitor pear juice concentrates for authenticity as changes to this profile may be indicative of the use of unapproved enzymes, such as cellulase(s) which are employed in fruit liquefaction which is an illegal practice in both Europe and North America (Mehrländer et al., 2002; Piatka et al., 2010).

In addition to debasing with less expensive commercial sweeteners, fruit juice is susceptible to adulteration by the addition of a juice to lesser value to one of higher value. This a concern between apple and pear juice due to similarities in their major carbohydrate/polyol profiles. The phenolic profiles of 27 pure apple and 31 pure pear juices were used to identify potential fingerprint compounds for use in detecting apple to pear and pear to apple adulteration. Published research on the presence of phenolics for this purpose have been contradictory. For example, both isorhamnetin-3-*O*-glucoside and phloridzin have been reported to be potential markers for pear and apple juices, respectively. However, other studies have reported that these compounds are not ubiquitous in their respective products or may be present in both apple and pear products (de Simón et al., 1992; Versari et al., 1997; Andrade et al., 1998; Schieber et al., 2001; Schieber et al., 2002; Alonso-Salces et al., 2004; Thavarajah & Low, 2006). Therefore, one new fingerprint compound in apple juice and two in pear juice were chromatographically detected, isolated and structurally identified as fingerprint compounds. The apple juice fingerprint was identified as 4-*O*-p-coumarylquinic acid and the pear juice fingerprints as isorhamnetin-3-*O*-rutinoside and abscisic acid employing UV-vis and NMR spectroscopic methods, and high resolution MS and LC-MS/MS spectrometry. In conjunction with this phenolic profiling research,

variations in the HPLC-PDA profile of commercial pear juices at 280 nm were found to be able to differentiate Chinese samples from those of other geographical regions.

Finally, the antioxidant properties and total phenolic content of commercial apple and pear juices were determined by the Folin-Ciocalteu method. The total phenolic content of apple and pear juices were found to be 294.7 ± 128.2 and 246.4 ± 45.1 ppm GAE, respectively, which were not significantly different ($p > 0.05$). Additionally, HPLC-PDA was used to determine the total phenolic chromatographic index (TPCI) of commercial apple and pear juices. Based on TPCI results, hydroxycinnamic acids were identified as a major contributor to the phenolic content of both juices. Within the hydroxycinnamic acid class, the chlorogenic acid subclass has been found to be a major contributor to the phenolic content of many fruits (Clifford, 1999; Jaiswal et al., 2011). However, due to structural similarities the HPLC-PDA analysis of fruit juice chlorogenic acids is challenging. To address this analytical problem, the dissociation behavior of the three most common chlorogenic acid isomers in fruits were studied by MS/MS and research results showed that they could be differentiated in the negative ion mode through the ratios of the three major product ions (*m/z* 191, 179 and 173). Specifically, 5-*O*-caffeoylquinic acid was identified by a base peak at *m/z* 191 with minimal to non-detectable amounts of the other product ions, while 4-*O*-caffeoylquinic acid had a base peak at *m/z* 173 with significant amounts of the 191 and 179 product ions. Finally, 3-*O*-caffeoylquinic acid was identified by a base peak of *m/z* 191 with significant amounts of the 179 product ion. Additionally, by combining MS/MS data and FAIMS, a method for the separation and identification of chlorogenic acids in apple/pear juice samples was developed with an analysis time of less than one minute.

9. GENERAL CONCLUSIONS

The overall aim of this research was to develop analytical methodology for the detection of apple and pear juice adulteration. Specifically, looking at the detection of pear juice debasing with less expensive commercial sweeteners and juice-to-juice debasing between apple and pear juices. In addition, this work examined the oligosaccharide profile of pear juice throughout processing and the phenolic content/antioxidant activity of apple and pear juices for their application to authenticity detection.

In the first segment of this research (Chapters 3 and 4) methods for oligosaccharide profiling employing HPAE-PAD and CGC-FID were developed and used to determine the natural oligosaccharide profile of 30 commercial pear juices for use in authenticity analysis. In addition to the 30 pure commercial pear juices, the oligosaccharide profiles of four commercial sweeteners (HFCS 55 and 90, HIS and TIS) were determined (Chapter 3). These profiles were then used to identify fingerprint oligosaccharides that could be used to detect the undeclared addition of these sweeteners to pear juice by CGC-FID at levels of 0.5-3.0% (v/v). Additionally, HPAE-PAD was shown to be an effective method to confirm the presence of HFCS and HIS in pear juice.

The methods developed in Chapter 3 were then employed to examine the natural oligosaccharide profile of commercial pear juice as a function of processing (Chapter 4). During pear juice processing a variety of enzymes, namely pectinases, amylases and hemicellulases, are added to break down the fruit cell walls improving the overall juice yield and quality. Using HPAE-PAD and CGC-FID a selection of monosaccharides and oligosaccharides were identified from the hydrolysis of pectin, starch and hemicellulose through the addition of exogenous carbohydrases. Chromatographic results showed that the majority of oligosaccharide formation occurs during the mashing stage of juice production. The remaining processing stages had minimal impact on the carbohydrate profile of commercial pear juice. The ability to monitor changes and/or consistency in the carbohydrate profile of pear juice during processing offers producers an additional option for monitoring quality assurance and control. Additionally, changes in the

observed carbohydrate profile can be used to detect the use of non-approved enzymes or other forms of pear juice adulteration (Chapter 3).

The second segment of this research (Chapters 5-7) focused on phenolic profiling of apple and pear juice for use in juice-to-juice adulteration detection. The mean and standard deviation TPC of the commercial pear juice samples was 246.4 ± 45.1 ppm GAE. The mean and standard deviation TPC of the commercial apple juice samples was 294.7 ± 128.2 ppm GAE, and this mean value was not significantly different than that observed for pear juice (p-value > 0.05). The mean and standard deviation of the TPCI for the commercial pear juice samples was 211.7 ± 57.2 ppm and was 128.8 ± 44.9 ppm for the commercial apple juices. The calculated TPCI mean values for apple and pear juices were found to be significantly different (p-value < 0.05) (Chapter 5).

Hydroxycinnamic acids were a major contributor to the TPCI in the commercial apple and pear juices. Within the hydroxycinnamic acids, 5-*O*-caffeoylquinic acid (5-CQA) was the major phenolic in the apple juice samples and was also one of the major phenolics in pear juice (Chapter 5). Along with 5-CQA, 3-*O*-caffeoyquinic acid (3-CQA) and 4-*O*-caffeoylquinic acid (4-CQA) are also common in fruits and are very similar structurally to 5-CQA. Therefore, a method to rapidly (<1 min) separate and identify these structural isomers using FAIMS-MS/MS was developed. Using MS/MS, isomers could be differentiated from each other based on the relative abundances of the three major product ions observed at *m/z* 191, 179 and 173. In addition, ESI-FAIMS-MS/MS was employed for the separation of these compounds for the first time (Chapter 6).

Apple and pear juice chromatographic phenolic profiles (TPCI) as determined by HPLC-PDA were used to identify fingerprint phenolics for use in the detection of juice-to-juice debasing (Chapter 7). One phenolic marker was identified in apple juice and two in pear juice. These compounds were structurally identified as 4-*O*-p-coumarylquinic acid in apple juice and isorhamnetin-3-*O*-rutinoside and abscisic acid in pear juice. Additionally, it was found that the presence of high concentrations of arbutin (>100 ppm) coupled with low or non-detectable amounts of isorhamnetin-3-*O*-rutinoside and non-detectable amounts of abscisic acid could be used to differentiate pear juices produced in China from those of other geographical regions (i.e., Argentina, Chile, New Zealand and the United States of America).

10. FUTURE STUDIES

This research examined the relationship between oligosaccharide formation and hydrolase enzymes in commercial pear juice production. The structural identities and polysaccharide source (i.e., pectin, starch or xyloglucan) of a selection of these oligosaccharides was determined, however, many of these compounds remain unidentified. Future research aimed at the identification of a larger number of these compounds would provide greater information on the chemical composition of pear juice as well as provide greater insight into the hydrolysis of polysaccharides during juice production, allowing greater tailoring of this process. Due to the lack of commercial standards for these compounds, their identification would require a more involved analytical approach. One possible approach would be through their isolation during chromatographic separation (i.e. analytical or preparatory columns) and structural identification employing analytical methods such as mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). In addition, experiments conducted with specific hydrolases (e.g. α-glucosidase) would aid in the elucidation of glycosidic linkage and oligosaccharide structure.

The aforementioned approach could also be applied to identifying the myriad of unidentified low molecular weight compounds detected by capillary gas chromatography coupled with flame ionization detection (CGC-FID) in pear juice. At this time, it has not been established if these compounds are produced during enzymatic processing or during the heating cycle for carbohydrate derivatization with Sylon TP (i.e. pyridine/70 °C/1 hour). During the caramelization reaction carbohydrates react to form a wide variety of both low and high molecular weight compounds. Caramelization is known to occur when carbohydrate solutions are heated in acidic (pH < 3) or basic (pH > 9) environments. As Sylon TP (trimethylsilyl imidazole + pyridine, 1:4) is a basic solution it is possible that some of the compounds observed in the CGC-FID chromatogram arise from caramelization-type reactions rather than from enzymatic treatment. In order to determine the identity of a selection of these compounds GC-MS could be used to obtain molecular weight and structural information. Additionally, individual compounds formed during

the treatment of carbohydrate solutions under defined derivatization (i.e. pH, time, temperature) conditions could be isolated and identified as described above.

In this research the phenolic composition and concentration, and *in vitro* antioxidant activities of commercial apple and pear juices were investigated. While a number of phenolics were identified in these commercial juice samples, and all phenolics were categorized into their appropriate subclass by high performance liquid chromatography (HPLC) coupled with photodiode array detection (HPLC-PDA), future work could look to further characterizing the structures of these compounds by HPLC-MS. This work would provide additional information on the roles that geographical region and species have on the phenolic profiles of both apple and pear juices and the role of specific phenolics on the antioxidant activity of these products. Also, further information on the effect(s) of processing on the phenolic profile of a juice could be obtained. In conjunction with the analysis of commercial apple and pear juices by HPLC-PDA, specific fingerprint compounds for each juice were identified. This work could be expanded to other juices (i.e. fruits and vegetables) so as to identify specific fingerprint compounds for individual ingredients (e.g. celery, orange). With this information, the authenticity of multi-juice products such as Five Alive® could be established based on the presence of fingerprint compounds. Also, it may also be possible to determine the percent of individual ingredients in these products.

A FAIMS-MS/MS method was developed for the rapid separation and identification of caffeoylquinic acids. Future work could focus on further developing FAIMS-MS/MS for applications with different phenolics for authenticity purposes as presented previously. For example, HPLC profiling could be used to identify fingerprint phenolics for which a FAIMS-MS/MS method could be developed. This would shorten analysis times (i.e., 60 to 90 minutes for HPLC vs. <1 minute for FAIMS) allowing for increased sample throughput as well as improved sensitivity allowing the detection of even low levels of adulterants.

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