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EVALUATION OF METHODS TO INCREASE FREE PROCYANIDIN CONTENT IN THE CIDER FERMENTATION PROCESS OF JONATHAN APPLES

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Department of Food Science and Human Nutrition

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EVALUATION OF METHODS TO INCREASE FREE PROCYANIDIN CONTENT IN THE CIDER FERMENTATION PROCESS OF JONATHAN APPLES

By

Mavis Tan

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ABSTRACT

EVALUATION OF METHODS TO INCREASE FREE PROCYANIDIN CONTENT IN THE CIDER FERMENTATION PROCESS OF JONATHAN APPLES

By

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Procyanidins impart the desired astringent mouthfeel and bitterness flavor in fermented cider. Methods were investigated to enhance free procyanidin levels in fermented cider made from Jonathan dessert apples during and prior to fermentation. Colorimetric assays (i.e. BuOH/HCl and Vanillin in methanol) were found to be more effective in analyzing procyanidins than HPLC acid – phloroglucinol method.

Cider samples were fermented using DV-10 yeast for 20 days at temperatures 13-14 °C at 0, 10, and 30% apple solids (crushed apples with no peel and core). No significant effect of alcohol production was observed on the procyanidin content, chain length and consequently, mean degree of polymerization (mDP). mDP of procyanidins increased in samples containing 30% apple solids between Day 0 and 5. Procyanidin content was higher in samples containing 0% apple solids on Day 14 vs Day 5. Overall, alcohol production, apple solids and time did not impact procyanidin content in hard cider during yeast fermentation.

Unfermented cider samples heated at treatments of 30, 40, and 50 °C had no significant differences in free procyanidin content. Unfermented cider samples treated with tenfold pectolytic enzyme concentrations of industrial dosages (~1000 ppm) significantly increased procyanidin concentrations without affecting mDP. Procyanidin levels in cider were enhanced using high concentration pectolytic enzyme treatments.

DEDICATION

To my parents, Tan Soo Jin and Seow Ah Choo for their unconditional love and words of encouragement which will always live in me.

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INTRODUCTION

Fermented apple juice or hard cider was widely consumed during the early history of the United States (U.S.). Hard cider was regarded as a safe alternative to water because alcohol prevented bacterial contamination and it remained a popular beverage until the late 19th century. At that time, beer began to dominate in the American market with the influx of German immigrants into the U.S. Beer made from grain was cheaper and easier to produce since it did not require the development of mature fruit orchards to provide the raw material. Beer also fermented more quickly and consistently and thus, was the more popular option for commercial producers (Rowles 2000). The Prohibition Act dealt the final blow to hard cider's popularity in the 1920s.

Until about 1990, there were few domestic ciders commercially available and most imported ciders were from the United Kingdom and France (Proulx and Nichols 2003). In 1990, only 115,000 cases of hard cider were sold in the U.S. while by 1997, the total has risen to 2.7 million cases (Fabricant 1997; Koeppel 1998). While the hard cider accounts for less than 0.2% of the total U.S. beer market in 1999, the hard cider industry is expected to grow rapidly (Rowles 2000).

Unlike the common sweet beverage cider known as cider in the U.S., 'hard cider' contains alcohol and unique tastes that are produced during fermentation. The distinction between hard cider and apple wine is usually made based on alcohol content, but there is much overlap between the two products. Apple wine is typically above 7% alcohol content and not carbonated while hard cider is usually below 7% and carbonated. In the U.S., commercial hard ciders contain about 5.5% alcohol while apple wine generally has 10-12% alcohol.

Apples specifically grown and harvested for traditional European cider are selected for their sugar, acidity and tannin contents and are not commonly available in the U.S. These bittersweet and bittersharp apples, as they are called, produce a cider with an astringent mouthfeel and somewhat bitter taste. Astringent is described as a puckery mouth-drying attribute while bitterness is a harsh taste sensation usually detected at the back of the tongue. These astringency and bitterness has been attributed to the polyphenolic compounds, especially procyanidins (Lea and Arnold 1978). Procyanidins have been found to bind with polysaccharides in the skins and structure of apples, specifically in the cell wall (Renard and others 2001; Le Bourvellec and others 2004). Unbound procyanidins in fermented cider impart the desired mouth feel and dry taste through their capacity to interact strongly with salivary proteins (Murray and others 1994). This binding ability increases with the degree of polymerization (number of flavan-3-ols units) of procyanidins (Da Silva and others 1991) while the monomeric catechins are responsible for bitterness (Lea 1990; Peleg and others 1999). Procyanidins measured in this research refer exclusively to unbound procyanidins ('free').

Various studies have documented that total polyphenol content and specifically, procyanidin content of dessert apples are significantly lower as compared to cider apple varieties (Guyot and others 1998). Such studies confirm that unlike the imported cider, American hard ciders are made from sweeter, less tannic apples commonly grown in the U. S., produces a fruity, somewhat sweeter, less tannic flavor than the European ciders.

In addition, procyanidins in plant-based beverages such as red wine, tea, and cider are increasingly being recognized as important in long-term health and reduction in the risk of chronic diseases (Santos-Buelga and Scalbert 2000).

Standard cider fermentation procedures usually involve the fermentation of apple juice or juice concentrates. The extraction and solubilization of procyanidins during processing play an important role in regulating the phenolic pool in apple juice and cider. Little work has been done on measuring free procyanidin levels in hard cider made from dessert apples and investigating methods of extracting procyanidins from the flesh of the apples into the juice.

The apples used in the present research were peeled and cored before juicing because compounds in the peel and seeds can interfere with the analytical method used. The apple material left after juice extraction is described as apple solids and contains approximately 61-68% moisture content as measured in this study.

The enhancement of free procyanidin levels in hard cider made from local Michigan dessert apples with the aim to improve its sensory characteristics may provide an alternative method for local cider producers making quality hard cider, without utilizing difficult to obtain rare and more expensive cider apple varieties. The goal of this research was to develop methods to enhance free procyanidin levels in hard cider through fermentation and various juice treatments using Michigan dessert apple varieties.

We hypothesized that mash fermentations would release procyanidins bound in apple solids into the cider, and thus, increase the levels of free procyanidins in fermented cider. The effects of 'mash' fermentations (i.e. cider fermentation with apple solids) are investigated by determining whether alcohol production, apple solids percentage and fermentation time influence free procyanidin levels in apple cider made from Michigan dessert apple varieties.

1. LITERATURE REVIEW

1.1 Polymeric flavan-3-ols (Procyanidins)

Polymeric polyphenols, commonly called tannins, are found in plants and foods of plant origin, particularly in fruits, legume seeds, cereal grains and beverages such as wine, tea, cocoa and apple cider. Bate-Smith and Swain (1962) described tannins as water soluble phenolic compounds having molecular weights between 500 and 3000. Besides undergoing the usual phenolic reactions, these compounds are also reported to have special properties such as the ability to precipitate alkaloids, gelatin and other proteins.

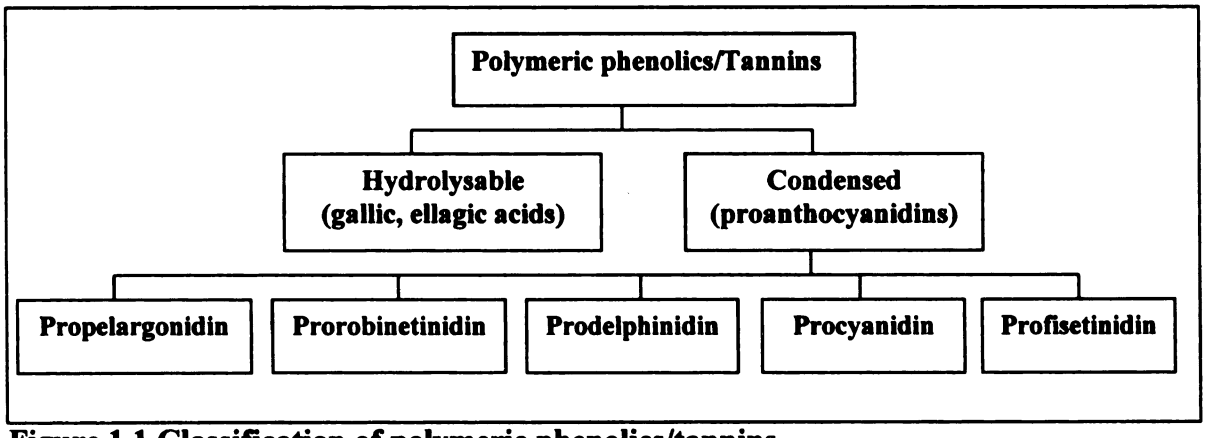

Figure 1.1 Classification of polymeric phenolics/tannins

Tannins are classically divided in two groups, hydrolysable and condensed as showed in Figure 1.1. Proanthocyanidins are oligomers or polymers of flavans (2-phenylchromanes) that are linked by C-C and occasionally C-O-C bonds and have the typical C6-C3-C6 flavonoid skeleton (Figure 1.2).

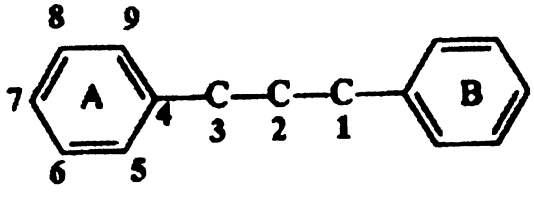

Figure 1.2 Basic $C_6 - C_3 - C_6$ structure (Haard 1985)

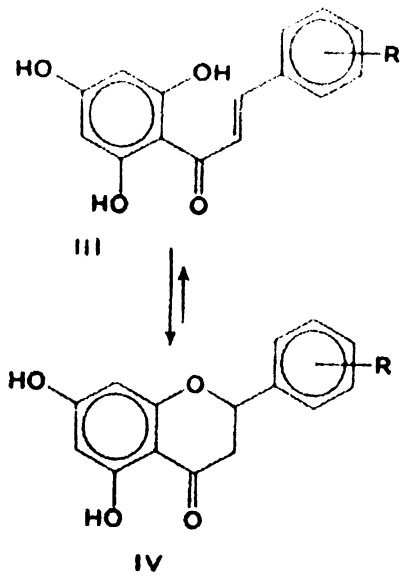

Figure 1.3 Flavonoid biosynthesis (Belitz and Grosch 1987)

Table 1.1 Substitution patterns within procyanidin classes in reference to Figure 1.2 (Cheynier and Fulcrand 2003)

		Substitution pattern					
Proanthocyanidin class		3	5	7	3'	4'	5'
Propelargonidin	Flavan-3-ols	OH	OH	OH	H	OH	H
Procyanidin		OH	OH	OH	OH	OH	H
Prodelphinidin		OH	OH	OH	OH	OH	OH
Profisetinidin	5-Deoxyflavan-3-ols	OH	H	OH	OH	OH	H
Prorobinetinidin		OH	H	OH	OH	OH	OH

The formation of the flavonoid structure is a result of a series of condensations from hyroxycinnamic acid to a chalcone (III) and finally, a flavanone (IV) (Figure 1.3). These compounds are further divided into subgroups, depending on the substitution pattern of their constitutive flavan units (Table 1.1) and named after the corresponding red anthocyanidin (2-phenylchromenium) pigments released under acidic conditions

(Cheynier and Fulcrand 2003). These compounds have molecular weights to 20,000 (around 70 units) (Halsam and Lilley 1988).

The main proanthocyanidins encountered in foods are procyanidins, consisting of (epi) catechin units (2-(3, 4-dihydroxylphenyl)chromane-3,5,7-triol) and prodelphinidins, in the form of (epi)gallocatechin [2-(3,4,5-trihydroxyphenyl)chromane-3,5,7-triol]. However, the major industrial proanthocyanidins, isolated from mimosa and quebracho, are predominantly composed of their 5-deoxy analogues, namely profisetinidins consisting of fisetinidol [2-(3,4-dihydroxyphenyl)chromane-3,7-diol] and prorobinetinidins, of robinetinidol [2-(3,4,5-trihydroxylphenyl)chromane-3,7-diol] (Cheynier and Fulcrand 2003).

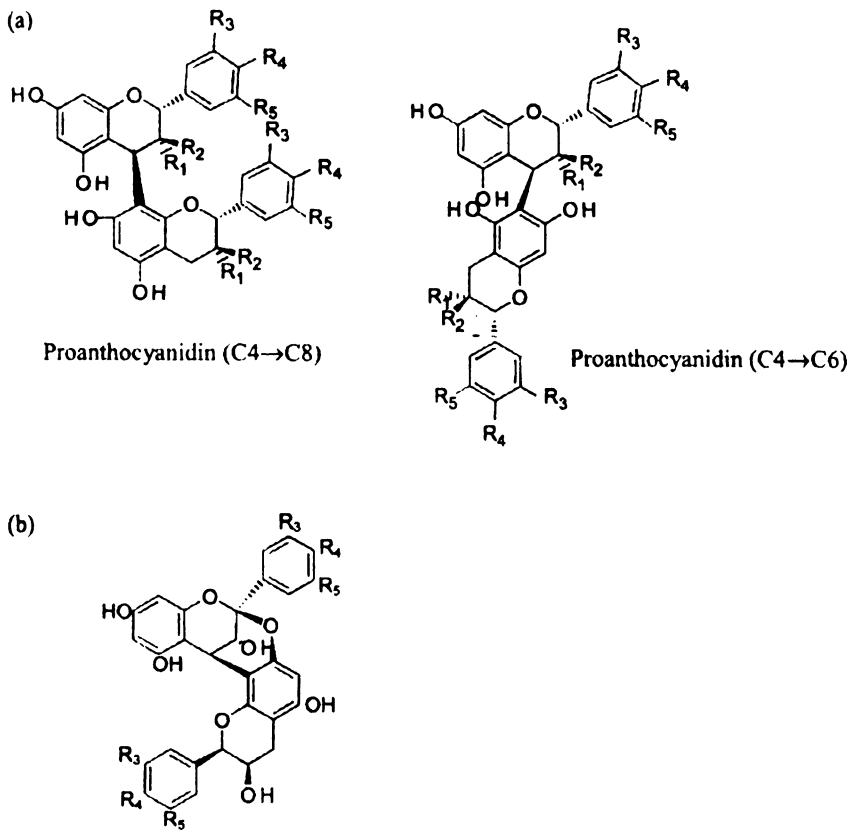

Figure 1.4 Structure of proanthocyanidins dimers (Lazarus 2003)

While several types of units often occur within a single proanthocyanidin chain, they can be linked by C-4–C-6 and/or C-4–C-8 bonds (a) or doubly linked, with an additional C-2–O-C-7 or C-2–O–C-5 linkage (b) and eventually substituted (e.g. glycosylated, galloylated) (Figure 1.4). Some plants only synthesize proanthocyanidins based on a single constitutive unit such as epicatechin-based procyanidins in apples. Polymeric fractions with molecular weight of approximately 55,000 (an average of 190 units) have been isolated from cider apple varieties by Guyot and others (2001).

1.1.1 Procyanidins in apples/apple products

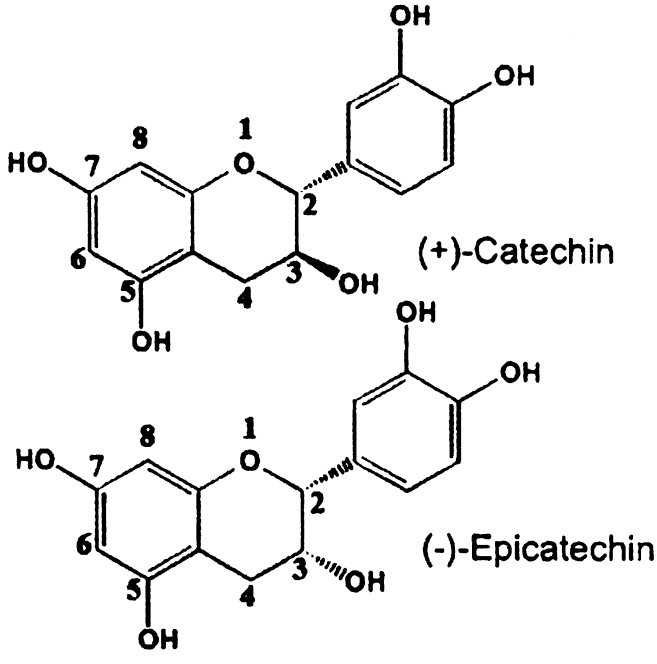

Figure 1.5 Monomeric units of procyanidins (Tsao and Yang 2003)

HPLC-MS analysis of an apple extract demonstrated that a complex series of proanthocyanidin oligomers was present in whole Red Delicious apples (Lazarus and others 2003). Oligomers through dodecamers were present and consisted entirely of the

procyanidin monomers (-) epicatechin and (+) catechin (Figure 1.5). Guyot and others (1998) concluded that the most frequently found unit in apple or apple products was procyanidin B2, a two-epicatechin unit with a C4-C8 interflavanic linkage (Figure 1.6).

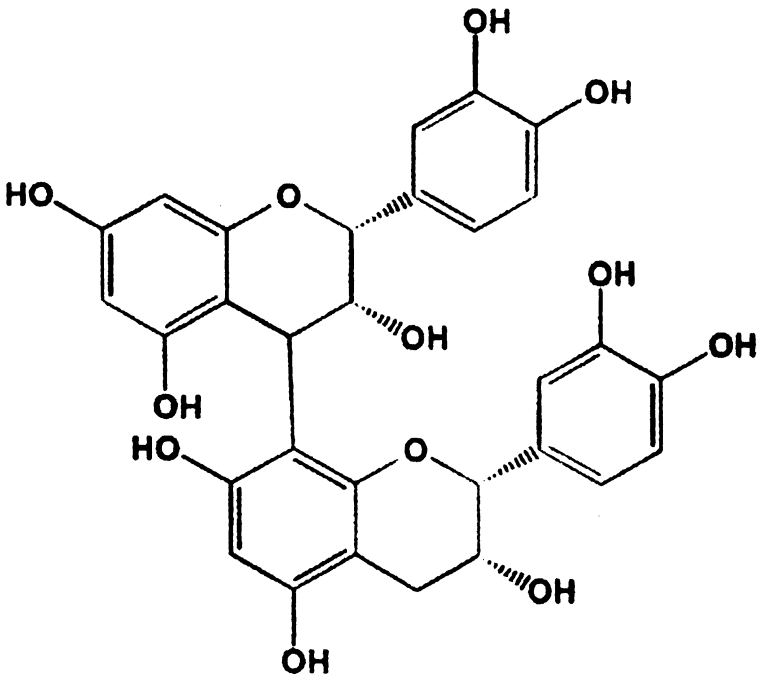

Figure 1.6 Procyanidin B_2 (Tsao and Yang 2003)

Research efforts have concentrated on the procyanidin content of apples (Lees and others 1995; Guyot and others 1997; Sanoner and others 1999; Price and others 1999), crushed apples (Foo and Lu 1999) and apple juice (Will and others 2002; Guyot and others 2003; Van der Sluis and others 2002, 2004) rather than hard cider. Suarez and others (1996) investigated the polyphenol content in hard cider but focused on optimizing the analytical methodology (i.e. solid phase extraction and HPLC). To date, procyanidin levels of hard cider made from dessert apples have not been reported in research literature. Most studies investigate procyanidin concentrations in cider apple varieties and utilized HPLC as their chemical analytical method.

The levels of general polyphenolic content in cider apple varieties are assumed to be tenfold higher than in dessert apples (Lea and Drilleau 2003). The same trend was observed specifically to procyanidins when Sanoner and others (1999) reported that cider apples varieties (e.g. Kermerrien and Jeanne Renard) contained 1000 to 3000 mg procyanidins per kg of fresh matter while there was 761 mg procyanidins per kg of fresh matter in Golden Delicious apples.

The highest concentration of tannins may be found in the peel with a continual presence of these compounds throughout the various apple tissues. Lees and others (1995) reported the amount of 19.2 mg, 2.7 mg and 1.2 mg condensed tannin content/g dry weight, in Golden Delicious apple peel, pulp and seed, respectively. The same trend in McIntosh apples was observed. Specifically, Guyot and others (1998) found 4964 mg procyanidins/kg fresh tissue in apple peel (epidermis zone) as opposed to 3379 mg procyanidins/kg fresh tissue of apple flesh (parenchyma zone) by HPLC analysis. Even when the BuOH/HCl assay resulted in lower readings as compared to HPLC (Guyot and others 1998), the same trend was observed 1231 mg procyanidins/kg fresh tissue in apple peel as opposed to 805 mg procyanidins/kg fresh tissue in apple flesh.

The final procyanidin content in the cider product is highly dependent on the procyanidin content of the raw material, fresh apples. Various researchers have studied the effect of agricultural factors such as cultivar, harvest years, weather and soil type (Van der Sluis and others 2001) on the general polyphenol content of dessert apple varieties while Burda and others (1990) investigated factors such as maturation and cold storage.

1.1.2 Importance to industry

Polyphenols in the diet are becoming increasingly recognized as important in long-term health and risk reduction of chronic disease. Various experimental studies have investigated the effects of the consumption of food products rich in polymeric polyphenol content such as tea, onions, apples and wine on cardiovascular diseases (CVDS) and cancer (Santos-Buelga and Scalbert 2000). These polymeric compounds are also thought to have antioxidant properties and are still under investigation (Santos-Buelga and Scalbert 2000).

Five classes of phenolic compounds are present in apple fruit: (1) hydroxycinnamic acid derivatives, (2) monomeric and oligomeric/polymeric flavan-3-ols, (3) flavonols (quercetin derivatives), (4) dihydrochalcones (phloretin glycosides) and (5) anthocyanidins (cyanidin glycosides) (Alonso – Salces and others 2001). Each of these polyphenol groups plays an important role in color, aroma, formation of hazes and flavor in cider (Alonso 2001). Polymeric flavan-3-ols (procyanidins) are major phenolic constituents in juices and fermented beverages as they are involved in many quality criteria such as bitterness, astringency and shelf life (Lea 1990).

In apple cider, procyanidins are recognized as principal separate contributors to bitterness and astringency which impart the desired mouth feel and dry taste in fermented cider (Lea and Arnold 1978). This ability is attributed to the capacity of the compound to interact strongly with proteins and, in this case, salivary proteins (Murray and others 1994). The ability to associate increases with the degree of polymerization (number of flavan-3-ols units) of procyanidins (Da Silva and others 1991). Lea and Arnold (1978) also established that monomeric catechins are responsible for bitterness while the longer

polymeric compounds contribute to astringency. Vidal and others (2003) further supported this theory through formal sensory descriptive analysis study on isolated cider apple and grape proanthocyanidin fractions.

Traditional ciders made from bittersweet cider apple varieties have been distinguished by relatively high levels of bitterness and astringency caused by the procyanidins (Lea and Drilleau 2003). Although cidermakers may be developing orchards with bittersweet apple cultivars, a deficit still remains. Hard cider making in the U.S. currently relies on the use of surplus dessert fruit, which has less procyanidin content and is less suitable for cidermaking. Since processing conditions play a part in determining the final procyanidin content and the final flavor profiles of the hard cider product, this study aimed to investigate methods of processing the juice of dessert apples varieties to increase the procyanidin concentration.

1.2 Analysis of procyanidins

Qualitative and quantitative information on the procyanidin profiles in food products are especially lacking, due in large part to the unavailability of appropriate analytical methodology and commercially available standards for such complex structures (Lazarus and others 2003). While formal identification of proanthocyanidins can be achieved by bidimensional NMR techniques, this is restricted to pure compounds, which are increasingly difficult to isolate as their degree of polymerization (DP) increases. This difficulty is due to the larger number of possible isomers and smaller amounts of individual compounds (Cheynier and Fulcrand 2003). However, with the potential for

health claims attached to these compounds, methods for accurate and sensitive analysis will increasingly become more important.

1.2.1 Colorimetric assays

Historically, the analytical methods estimate procyanidins by spectrophotometric means. Methods based on oxidizing properties such as the Folin-Ciocalteu assay have been proposed to estimate flavanol content, but these methods lack specificity and can only be applied to isolate tannins (Lazarus and others 2003).

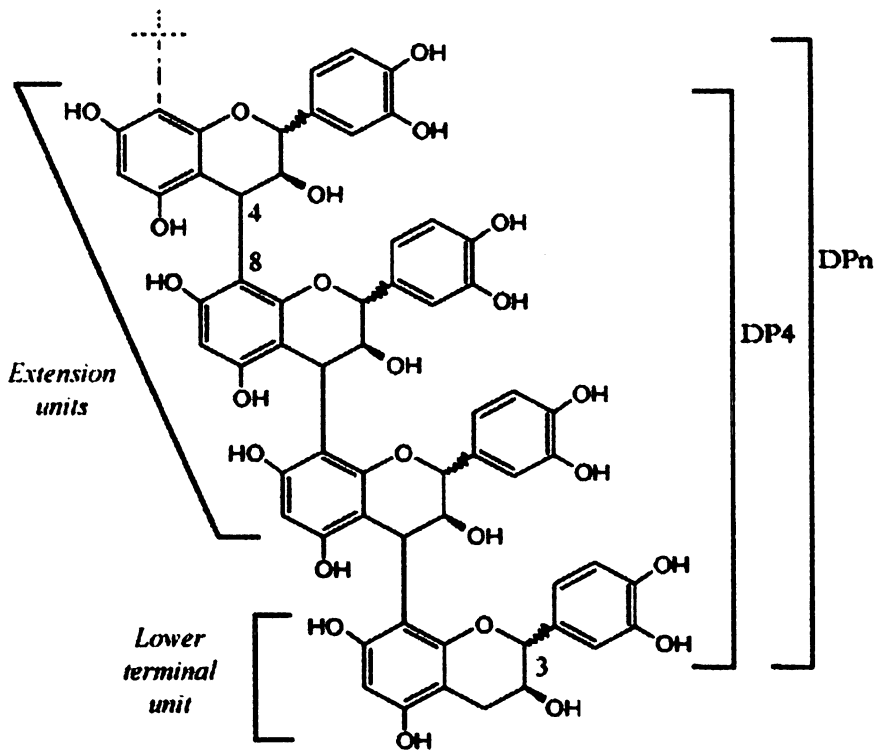

Figure 1.7 Extension and lower terminal units of procyanidins (Kennedy and Jones 2001)

The BuOH/HCl assay was originally created by Bate- Smith and Swain (1962) and further developed by Porter and others (1986). This method relies on the acid-catalyzed oxidative cleavage of interflavanic linkages between extension units (Figure 1.7) of polymeric procyanidins to produce cyanidins (Figure 1.8), which are then measured spectrophotometrically. While this method is specific, disadvantages to this

method include lower reaction yield (Guyot and others 1998) and sensitivity to interference from water (Porter and others 1986). However, Cheynier and others (2003) have found the reaction yield to be approximately 48% and adjustments can be made accordingly while water can be removed during solid phase extraction where extracts that have been eluted through the cartridges are dried prior to being collected by methanol. While the butanol-HCl assay is definitive for the polymer, it is only recommended for use in determining polymeric polyphenols in the peel of gold or green varieties because tissues which contain anthocyanins interfere with the colorimetric assay (Lees and others 1995).

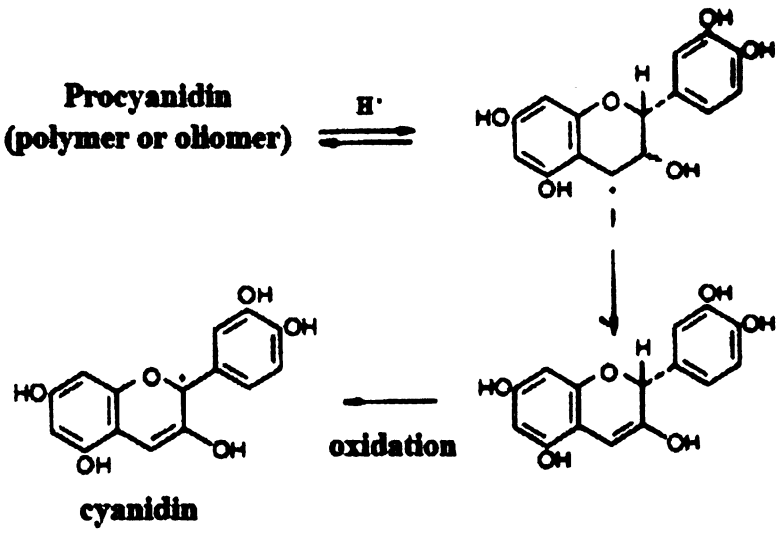

Figure 1.8 Acid-catalyzed cleavage of procyanidins to cyanidins (Porter and others 1986)

The vanillin-HCl or dimethylaminocinnamaldehyde (DMCA) reactions rely on the coupling of chemical reactions enabling them to specifically measure lower end units of flavanol chains as seen in Figure 1.7 (Lazarus and others 2003). The vanillin assay involves the reaction of an aromatic aldehyde, vanillin with the metasubstituted ring of

flavanols to yield a red adduct (Price and others 1978). Although the vanillin reaction has been widely used to estimate condensed tannins (proanthocyanidin), there have been disadvantages to its application. The reaction is not specific for condensed tannins, and the monomeric unit catechin reacts to yield a red colored adduct (Price and others 1978). A modified version of the vanillin assay using methanol (Butler and others 1982) results in monomers present in crude plant extracts yielding color at a slower reaction rate in the assay than polymers. Thus, less interference in the procyanidin measurement occurs.

1.2.2 Chromatography

Other alternative methods such as electrospray ionization mass spectrometry are used to analyze a complete series of polymeric procyanidins with degrees of polymerization up to 17 (Guyot and others 1997). The structural characterization of proanthocyanidin fractions is performed by ^{13}C or ^{1}H NMR (Nuclear Magnetic Resonance) methods. These methods provide precise information on the average degree of polymerization, the stereochemistry of the heterocycles of the constitutive units and on the hydroxylation pattern of the B nuclei (Guyot and others 1997). However, polymers with molecular weight above 8000 cannot be precisely characterized according to this procedure.

In the early 1990s, various chromatographic techniques such as reversed or normal phase liquid chromatography were developed to allow separation of oligomeric proanthocyanidins, and acidic phenolic compounds (hydroxycinnamic acid derivatives) from neutral compounds (flavonoids) (Jaworski and Lee 1987; Delage and others 1991; Suarez-Valles and others 1994). While better separations have been achieved, problems

with procyanidins exhibiting different degrees of polymerizations of different constitutive units remain a problem, resulting in an unresolved clump of peaks in chromatograms and leading to underestimations (Guyot and others 1997).

Reversed phase HPLC followed by thiolysis has been developed to address this problem in peak resolution. Concentrations of both extension and terminal units, and consequently *m*DP are determined in a single reaction based on acid catalyzed degradation in the presence of a nucleophilic agent (Figure 1.9). This is followed by HPLC or NMR analysis of the resulting solution (Cheynier and Fulcrand 2003). Breakage of the interflavanic C-C bond under mild acidic conditions releases the terminal units as the corresponding flavanols and the upper and intermediate units as carbocations. These carbocations react with the nucleophile reagent (usually phenyl-methanethiol or phloroglucinol) to form stable adducts (e.g. benzyl thioethers in the presence of phenyl methanethiol) (Cheynier and Fulcrand 2003).

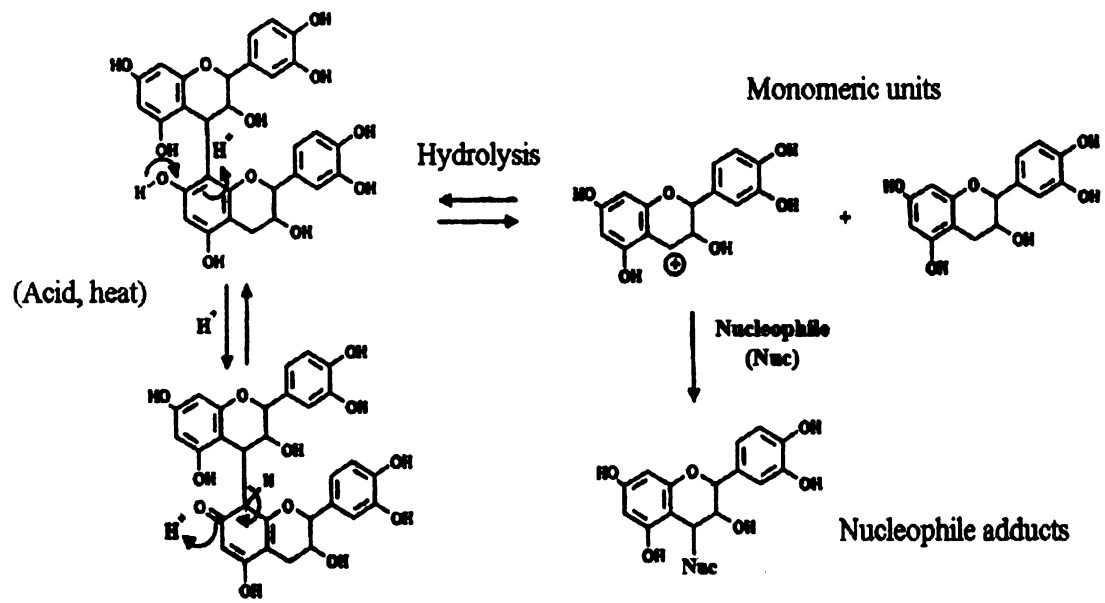

Figure 1.9 Proposed mechanism of acid cleavage of procyanidins and formation of nucleophile adducts (Kennedy and Jones 2001)

Guyot and others (1997) investigated the concentrations of both extension and terminal units, and consequently *m*DP in cider apple varieties through thiolysis, which utilized benzyl thioether. Matthews and others (1997) and Gupta and Haslam (1978) observed that benzyl mercaptan served as a better trapping agent since it resulted in significantly higher nucleophilic adducts yields than those degraded by phloroglucinol. However, Kennedy and Jones (2001) utilized phloroglucinol to analyze procyanidins in grapes and found that results obtained compared favorably to those that used benzyl mercaptan, thus, identifying a safer and less toxic alternative.

1.3 Hard cider production

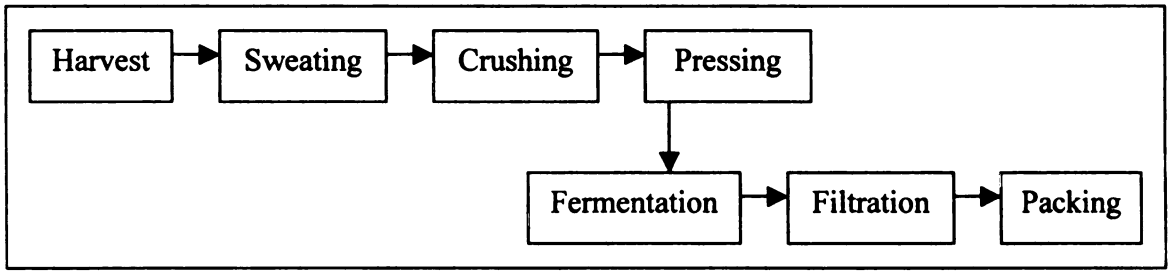

Figure 1.10 Basic hard cider making steps

The traditional steps in hard cider making are seen in Figure 1.10: harvest and fruit selection (on the basis of cultivar and quality), sweating, crushing, pressing (or extraction), fermentation, filtration/racking and bottling/packing (Proulx and Nichols 2003). After harvesting, the apples are left to mature for a week (i.e. sweating). In modern plants, the apples are crushed in a grater type mill made of stainless steel. Next, the pulp is crushed to extract the juice using a cider press. During pressing, the juice is typically exposed to air and oxidation occurs. The polyphenol oxidase (PPO) enzyme reacts with apple fruit tannin in the presence of air to develop soluble color. If this

oxidation continues further, the oxidized polyphenols (particularly procyanidins) are tanned back into the pulp and the level of soluble polyphenols and color may be diminished (Lea and Drilleau 2003). Mechano-hydraulically operated plate presses are used in modern manufacturing facilities to extract the fruit juice. The freshly pressed juice may be fermented straight away or concentrated and stored for later conversion to cider, in which case it is extensively treated to pasteurize and remove pectin.

Apple juice concentrate (AJC) is widely used in English cidermaking and to a limited extent in France cidermaking. The advantage of a 70°Brix concentrate to the cidermaker is that it may be stored for months or years with relatively little deterioration compared to fresh juice, which complements just-in-time (JIT) business practices. Hard cider production in Europe generally utilizes AJC in a controlled fermentation process using deliberate addition of selected yeast and malolactic bacteria, which both help to control fermentation rates and ensure uniform product quality (Lea and Drilleau 2003).

1.3.1 Fermentation

Hard cider is a product of apple juice that has undergone two different kinds of fermentation. The first fermentation is carried out by yeasts in anaerobic conditions, which converts fermentable sugars to alcohol (Figure 1.11).

$C_6H_{12}O_6$	$\rightarrow$	$2C_2H_5OH$	+	$2CO_2$
Sugar (Glucose, Fructose)		Alcohol (Ethyl Alcohol)		Carbon Dioxide (Fermentation Gas)

Figure 1.11 Conversion of fermentable sugars to ethanol

Refractive Index (RI) is a measurement useful for analyzing the density of a substance (e.g. sugar dissolved in water), based on the principle of light refraction. It is a

simple method of measuring the reduction of fermentable sugar. Most UK cidermakers take the view that a complete 'dry' fermentation 10-12% alcohol in as little as two weeks is a desirable objective (Lea and Drilleau 2003). Incomplete fermentation can be obtained by removing the yeast halfway throughout the process, thus retaining less alcohol and more fermentable sugars than 'dry' hard ciders. In the U.S, commercial hard ciders usually contain about 5.5% alcohol and most are carbonated (Proulx and Nichols 2003).

The second fermentation (*malo-lactic fermentation*) converts L(-)-malic acid to L(+)-lactic acid and carbon dioxide and is carried out by lactic acid bacteria present in the apple juice. The malo-lactic fermentation can occur concurrently with the yeast fermentation but more often it is delayed until a few months later. Most modern commercial cidermakers regard this fermentation as a nuisance and do not encourage it (Lea and Drilleau 2003).

Since the 1980s, specific cultured yeasts have been used in cider making. Inoculum mixes of active dried wine yeasts of S. *uvarum* and S. *bayanus* are widely used as the former provides a speedy start while the latter copes better with fermentations to dryness since some yeast types are not tolerant to high alcohol concentrations (Lea and Drilleau 2003). The vitality and viability of cultured cider yeasts under high stress conditions (i.e. higher alcohol concentrations and different types of alcohol) have recently been investigated (Seward and others 1996).

Prior to fermentation, SO_2 is added to the juice to suppress or kill wild yeasts and most bacteria. This step allows the added yeast to multiply and dominate the fermentation (Lea and Drilleau 2003). The effectiveness of SO_2 is pH dependent since it is only the dissociated form that has antimicrobial properties (Lea and others 2003). Therefore, cider

fermentation is best executed at pH <3.8. The amount of added sulfite (e.g. potassium metabisulfite) ranges from 50-70 ppm for most commercial wines.

Fermenting cider in the presence of fruit solids (mash) is common within the wine-making industry. The intentions are to release polyphenolic compounds such as anthocyanins to enhance wine flavor and color from the crushed skins of grapes. Mash fermentation of apple fruit was informally investigated by Padilla-Zakour and others (2003) who reported that polyphenol content doubled in mash fermentation as compared to juice fermentations.

1.3.1.1 Complexation of apple polyphenols with polysaccharides

Studies have reported that the transfer of procyanidins from apple fruit to juice during processing is hindered by the complexation of apple polyphenols to polysaccharides. Haslam and Lilley (1988) were among the first investigators to suggest that higher molecular weight procyanidins become bound to soluble polysaccarides such as pectin (which dramatically increases as fruit ripens) and therefore, are unavailable to exert the astringent mouthfeel desired in hard cider. Tsao and Yang (2003) report significant differences in the concentration of procyanidins in Red Delicious apple tissues and juice as follows: 1655, 342, 6 μg flavan-3-ols per g fresh weight, in apple peel, fresh and juice, respectively.

Plant cell walls consist of complex, porous polysaccharidic material. In fruit and vegetables, the cell wall consists of three interpenetrating but not interconnected networks: a cellulose/xyloglucan framework (>50% dry weight) embedded in a pectin matrix (24 - 40% dw) and locked into shape by glycoproteins (~1% dw) (Carpita and

Gibeaut 1993). In intact plant tissues, cell walls, polyphenols and polyphenoloxidase are present and react with each other during processing when the cell walls are ruptured. In apples, this results in formation of apple solids, where cell walls and (oxidized) polyphenols form a single material (Renard and others 2001). Two mechanisms are thought to be jointly responsible: adsorption of native and oxidized polyphenols to the cell wall matrix and formation of covalent bonds between quinines and cell wall polymers.

The adsorption was thought to be mediated by H-bonding and hydrophobic interactions, the latter being favored by the existence of hydrophobic cavities and crevasses such as the internal cavity of cyclodextrins (Renard and others 2001). Interactions between suspended cell walls and polyphenol extracts were investigated by quantitating residue polyphenols by HPLC. It was observed that hydroxycinnamic acids and (-) epicatechin did not bind to cell walls and that binding between apple cell walls and procyanidins depended on the concentration and the molecular weight of the procyanidins. Le Bourvellec and others (2004) conducted a similar experiment by bringing into contact a solution of procyanidins and a suspension of apple cell wall material. The amount of procyanidins bound to the cell wall increased with the degree of polymerization (DP), the percentage of galloylation (% gall), the proportion of (+)catechin and increasing ionic strength. Since the bindings decreased with increasing temperature, the bonds between procyanidins and apple cell wall material were concluded to be weak energy bonds such as hydrogen bonds and hydrophobic interactions.

The possibility of improving the flavonoid content of apple juice by adjustments in production and processing methods is easier and faster to implement than changes in

the raw material itself (i.e. cultivar, storage, harvest time, maturity) (Van der Sluis and others 2004). Since the processing steps of apple juice production are similar to hard cider production, methods of improving procyanidin content in apple juice may be applicable to cider making production.

Van der Sluis and others (2004) suggested that flavonoid content in juice made from Elstar, Golden Delicious and Jonagold apple varieties and its antioxidant activity may be improved by extracting flavonoids from the pulp using alcohol. Complex formation between procyanidins and apple cell wall material was decreased by ethanol production (Le Bourvellec and others 2004). However, there are no studies relating the effect of natural alcohol production (sugar fermentation) on the release of procyanidins from apple solids.

1.3.2 Heat treatments

A study by Lea and Timberlake (1978) found that warm anaerobic incubation of apple [solids] for four hours before extraction increases the level of organoleptically significant procyanidins in juice by 50%. The corresponding increase in bitterness and astringency were highly significant. This study utilized total phenolic colorimetric assays and did not offer specific information on procyanidins.

High temperatures are known to inactivate the enzyme polyphenol oxidase and therefore, help to retain polyphenolic content. Will and others (2002) also evaluated the influence of mash temperature on total polyphenol content using another colorimetric method, the Folin Ciocalteu reagent assay (FCR) and HPLC. A nearly linear increase of phenolic substance was found between ambient and 50 °C heating after 1.5 hr (Will and

others 2002). (-) Epicatechin and procyanidin B2 did not appear on the chromatograms until after incubation of dessert apple solids at temperatures of 30 and 40 °C, respectively. Diffusion extraction methods employed at HTST (High Temperature Short Time) conditions at 55, 63, 67, and 73 °C at 90 seconds increased the procyanidin content of Red Delicious, McIntosh and Spartan apple juice two to three times higher (Spanos and others 1996)

1.3.3 Pectolytic enzymatic treatments

Approximately 75% of the solid matter of plants is carbohydrate and generally consists of simple sugars, polysaccharides, pectic substances and lignin (Haard 1985). In plants, carbohydrates are localized in the cell wall and intracellularly in plastic, vacuoles, or the cytoplasm. The cell wall is primarily consists of cellulose, hemicellulose, pectins and lignin. The relative proportion and contents of these constituents vary considerably among species, with maturity at harvest, and with elapsed time after harvest.

Cellulose is largely insoluble and indigestible by human beings while hemicelluloses are a heterogeneous group of polysaccharides that contain numerous kinds of hexose and pentose sugars and in some cases, residues of uronic acids (Haard 1985). Pectin consists of α1,4-linked galacturonic acid residues esterified to varying degrees with methanol.

Industrial enzymatic treatment of the apple solids before pressing is often applied to increase juice yields. Pectolytic enzymes are used to increase the pressability by degrading pectins in the cell walls where the polysaccharides are solubilized and depolymerized (Dongowski and others 2002). Pectolytic, cellulolytic and hemicelluloytic

enzymes (Table 1.2) present in commercial enzyme preparation are used in fruit and vegetable technology.

Table 1.2 Enzymes typically used in fruits and vegetables processing (Richardson and Hyslop 1985)

Enzyme	Sources	Conversions catalyzed
Pectinase (polygalacturonase, pectin methylesterase and pectate lyase)	1. *Aspergillus niger,* var. 2. *Rhizopus oryzae,* var.	Pectin methylesterase demethylates pectin, polygalacturonase hydrolyzes β-D-1,4-galacturonide.
Cellulose	1. *Aspergillus nige, var.* 2. *Trichoderma reesei, var.*	Cellulose + H_2O → β-dextrins (β-1, 4-glucan bonds)
Hemicellulase	*Aspergillus niger,* var.	Hemicellulase + H_2O → β-dextrins (β-q, 4-glucan bonds of hum – locust bean, guar, etc).

Lea and Timberlake (1978) recommended that warm anaerobic incubation in the presence of a pectolytic enzyme may help increase the yield of procyanidins from apple solids. Enzymatic mash maceration of the Brettacher apple variety (European) carried out under periodic stirring at 20 ± 3 °C for 1 hr resulted in reduced concentrations of procyanidin B2 and (-) epicatechin without the addition of ascorbic acid (Mihalev and others 2004). This reduction was attributed to oxidative browning reactions. Spanos and others (1990) report that enzymatic clarification caused some procyanidin degradation. However, Will and others (2000) reported that apple solids liquefaction with pectolytic enzymes increased (-) epicatechin and procyanidin B2 concentration in apple juice containing apple solids as temperatures rise to 30 and 40 °C, respectively. More recently, Renard and others (2001) found that procyanidins may inhibit pectolytic enzymatic degradation of apple cell walls.

2. MATERIALS AND METHODS

2.1 Plant material

Apples fruits (*Malus domestica*) from the Jonathan variety were harvested from the MSU Horticultural Farm or obtained from commercial stores. The Jonathan variety was used as it is one of the dessert apple varieties that is abundant in the State of Michigan, thus making the variety an appropriate choice for the development of value-added apple products. Additionally, the Jonathan apple was selected for the sweet tart flavor profile produced in the fermented product after preliminary fermentation tests involving various dessert apples such as McIntosh, Northern Spy and Winesap. The Jonathan apples were stored in the MSU Food Science Pilot Plant cooler in the fall season (September to November) at 8 °C until further analysis within 3 months. Apples of similar sizes were pooled together and bruised sections of the apples were discarded during preparation.

Since the anthocyanidins in the red skin of the Jonathan variety can interfere with the BuOH/HCl assay (Lees and others 1995), the apples were skinned and cored before the juice was extracted. Apples which had anthocyanidin 'veins' extending from the epidermis up to the apple core within the flesh (Figure 2.1) were rejected and not included in the experiments.

2.2 Juice Preparation

The apple juice samples were prepared using Acme 5001 White Supreme Juicerator™ Juice Extractor (Waring Products, Torrington, CT, USA). The apple pieces were cored, peeled by hand and juice was collected as a single batch. The apple solids were added into the juice samples at 0, 10 and 30% (w/w).

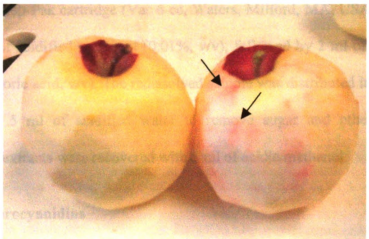

Figure 2.1 Apple samples with anthocyanins imbedded in flesh

2.3 Extraction of procyanidins

2.3.1 Centrifugation

The apple juice samples were centrifuged using an Allegra 6R Centrifuge Rotor Model GH3.8 with swinging buckets (Beckman, Fullerton, CA, USA) to separate the juice from the solids at 3000 rpm (~ 1411 g) for 15 min at 10 °C. The supernatants were decanted.

2.3.2 Rotary Evaporation

For the fermented cider samples, alcohol was removed by evaporation by vacuum (Escribano-Bailón and others 1992) to minimize procyanidin loss during extraction since procyanidins dissolve in ethanol. The method of evaporation was adapted from Suarez and others (2003). Rotary evaporation was achieved by using Laborota 4002 digital (Heidolph Instruments, Cinnaminson, NJ, USA) at 60 rpm. Ten ml of supernatant was transferred into 250 ml round flask Pyrex bottles and evaporated to dryness at 35 °C for 20 minutes. Ten ml of purified MilliQ water was reintroduced into the flask to dissolve the dried residue.

2.3.3 Solid Phase Extraction

Each C_{18} Sep-Pak cartridge (Vac 6 cc, Waters, Milford, MA, USA) was activated by eluting 2 ml of acidified methanol (0.01%, v/v), followed by 2 ml of acidified water (0.01 % hydrochloric acid, v/v). The redissolved sample was distributed into the cartridge and eluted with 5 ml of acidified water to remove sugar and other high polarity compounds. The extracts were recovered with 5 ml of acidic methanol.

2.4 Analysis of procyanidins

2.4.1 High liquid performance chromatography

2.4.1.1 Sample preparation

25 ml of cider samples were concentrated into 5 ml using Sep Pak C_{18} cartridges by eluting 5 ml of water and 5 ml of extract was recovered with 2 ml of methanol. 1 ml of extract was added to the reaction.

Grape seed extract preparation was adapted from Escribano-Bailón and others (2003) by homogenizing 5 g of grape seeds in 15 ml of methanol. The sample was centrifuged at 12000 rpm for 15 min. 4 ml of extract was dried and used in the reaction.

Both samples were treated with acid-cleavage in the presence of phloroglucinol using the method as adapted from Kennedy and others (2001). Each sample was reacted with 5 ml of 0.1 N HCl in methanol containing 50 g/L phloroglucinol and 10 g/L ascorbic acid for 20 min at 50 °C in a water bath shaker. The reaction was stopped by addition of five volumes of 40 mM sodium acetate and cooled at 4°C for 10 minutes. The samples were filtered using 0.45 μm Millipore syringe filters prior to analysis by HPLC.

2.4.1.2 Instrumental Settings

The HPLC apparatus consist of a 717 plus autosampler, 996 Photodiode Array Detector and Millenium 32 Manager System (Waters, Milford, MA, USA). The column used was a *Waters* Atlantis dC18 RP column (particle size 5 μm, 150 × 4.6 mm), protected by a *Waters* Guard Sentry column. The method utilized a binary gradient with mobile phase containing 1% aqueous formic acid (Mobile Phase A) and pure MeOH (Mobile Phase B).

The sample injection volume was 20 µL while the elution conditions were as follows: 1.0 mL/min, a linear gradient from 20 to 30% B in 10 min, 30 to 60% B in 10 min. The column was then washed and reequilibrated with 20% B for 40 mins before the next injection. Standard peaks of (+) catechin and (-) epicatechin obtained from Sigma-Aldrich (St. Louis, MO, USA) were monitored at 280 nm at ambient column temperature.

2.4.2 Colorimetric assays

Calculations of concentrations were based on the Beer Lambert's Law (Equation 1), where concentrations units were expressed on a molar basis.

(1) $A_\lambda = b\,(\varepsilon)_\lambda\,c$

where:

A = absorbance at wavelength λ *(measured)*

ε = molar absorptivity at wavelength λ ($M^{-1}.cm^{-1}$) *(obtained from literature)*

b = path length of the cell (cm)

c = concentration (M or moles/liter) *(calculated)*

2.4.2.1 Depolymerization in Butanol-HCl (Porter's reagent)

Each of the extract samples of 0.5 ml were dissolved into 2.5 ml of a solution of butanol-12 *N* HCl (95:5), v/v, and 0.2 ml of $(NH_4)Fe(SO_4)_2$, 12 H_2O (2%, w/v in 2 N HCl). The glass test tubes were sealed with a Teflon-lined screw cap, mixed thoroughly and heated for 30 min in a water bath at 95 °C. The solutions were cooled immediately in a water bath at ambient temperature and the absorbance was measured at 550 nm using a DU 520 General Purpose UV/Vis spectrophotometer (Beckman, Fullerton, CA, USA).

The molar concentration of released cyanidins (equal to procyanidin upper and extension units) was calculated using the molar absorptivity of cyanidin (35,000) estimated by Cheynier and others (2003). Calibration with known solutions of procyanidin standards as reported showed that the reaction yield as 48% (Scalbert 1992). Taking this reaction yield into account, adjustments to the raw measured values obtained in this research were made to reflect 100% yields in the final estimated values.

2.4.2.2 Vanillin in Methanol assay

The vanillin method used in this study was adapted by Butler and others (1982). Extract samples of 0.5 ml were dissolved in 2.5 ml of a 1:1 solution, (8% HCl in methanol: 1% vanillin in methanol) (w/vl) prepared immediately before use. The absorbance is measured at 510 nm after 20 min of incubation.

The reaction in the vanillin assay theoretically reacts specifically with proanthocyanidin end groups. The molar absorptivity (ε) was determined to be 25,200 (Cheynier and others 2001).

2.4.2.3 Mean Degree of Polymerization (mDP)

The mDP of procyanidins was calculated from Equation 2 as obtained from Cheynier and others (2001).

(2) $$mDP = ([\text{upper and extension units}]_M + [\text{end units}]_M)/[\text{end units}]_M$$

2.5 Fermentation

The juice samples were prepared as described in Section 2.2 and distributed into 200 ml French-capped bottles and treated with 0.053g potassium metabisulfite per L of sample for 24 hr. The addition of SO_2 to cider was intended to suppress or kill non-*Saccharomyces* yeast and most bacteria to allow the fermentation to proceed with a more homogenous and benign microflora and to reduce the chances of a secondary infection (Lea and Drilleau 2003). The amount of potassium metabisulfite was used at 53 ppm, which is within the 50-70 ppm range typical for most commercial wines.

The commercial yeast DV10 (Lallemand Inc, Rexdale, Ontario) is a *Saccharomyces cerevisiae bayanus* strain. It was chosen for this study because it is a clean rapid fermenter that retains flavor, is tolerant to alcohol levels (15%) and has the ability to ferment under stressful conditions (i.e. low pH, high total SO_2, relatively low nitrogen content, and low temperature). The yeast was added to the samples at 0.3 g/L and the samples were stored at 12-14 °C. Day 0 indicates the day yeast was first added.

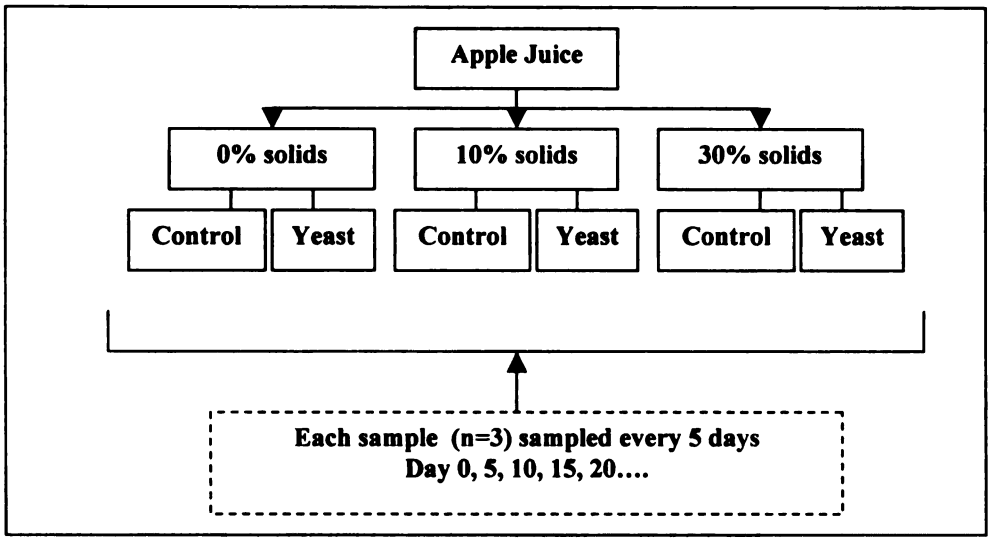

Figure 2.2 Experiment design of fermentation setup

Refractive index (RI), pH and procyanidin levels in the hard cider samples were measured every three days (Figure 2.2). The RI values were measured using a S-28 Hand Refractometer (Atago, Japan) while the pH values were obtained using a pH meter 440 with "3 in 1" Combo W/RJ electrode (Corning, NY, USA). The analysis was complete once most of the fermentable sugar in the samples had been converted into alcohol or until 'dry' when RI values were relatively consistent.

Alcohol content could not be analyzed using the alcohol dehydrogenase enzymatic assay kit (Sigma-Aldrich, St. Louis, MO, USA), which was no longer commercially available. Alcohol % values obtained by HPLC means resulted in values that were unreasonably higher than expected. Therefore, the potential alcohol could only be roughly estimated by calculating RI differences from the beginning to the end of fermentation. The RI values obtained from the cider fermentations were converted to sugar content by making a standard curve between RI and glucose concentrations (g/ml) (Appendix 2). After adjusting the values for residual solids, the amount of potential alcohol was estimated to be 4.0%.

Procyanidin concentrations were measured using the colorimetric methods described in Section 2.4.2. There were three variables in the fermentation data. 'v' referred to the concentration values calculated from the Vanillin in methanol assay, 'p' referred to concentration values calculated from the BuOH assay while 't' referred to the calculated mean degree of polymerization calculated from Equation 2.

2.6 Statistical Analysis

The three variables (v, p and t) were analyzed using a model corresponding to a split-plot design in blocks with repeated measures, where mash was the main plot factor and treatment was the sub plot factor and the blocks were represented by batches. For the repeated measures (times within mash and treatments) several covariance structures were compared and a heterogeneous compound symmetry model was selected as the best fit. After analysis of variance, single or main effect contrasts were obtained. The model was fitted using Proc Mixed of SAS (Littell and others 1996). The $p \leq 0.05$ was used as the level of significance.

Images in this thesis are presented in color.

3. RESULTS AND DISCUSSIONS

3.1 Evaluation of analytical methods on fermented hard cider

Guyot and others (2001) reported that HPLC analysis of polyphenol extracts result in a single large clump (marked by 'X'). The proposed heat-acid treatments in the presence of phloroglucinol on the samples extracts prior to HPLC are expected to resolve the clump into single peaks consisting of (-) epicatechin and phloroglucinol adducts.

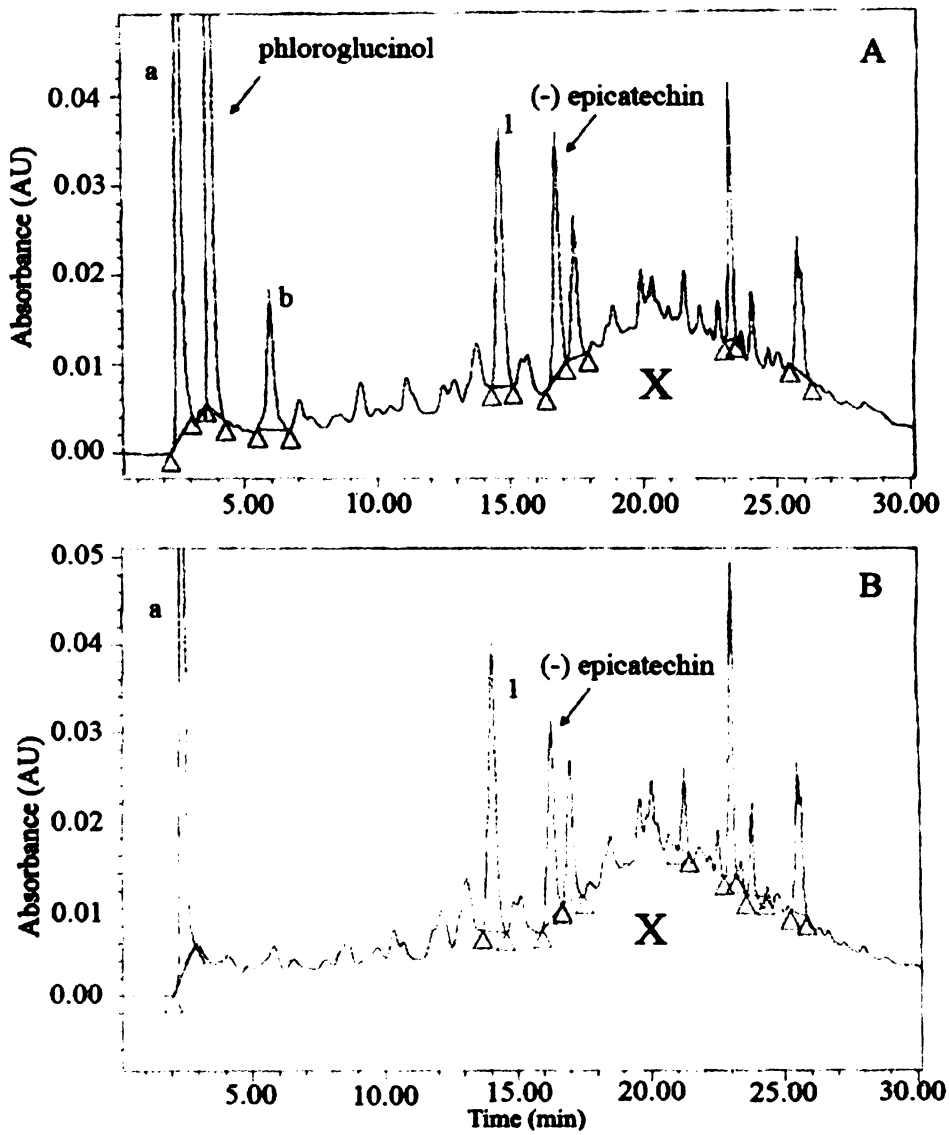

Figure 3.1.1 HPLC chromatogram of procyanidin cleavage product from Jonathan apple extracts following acid-catalysis (A) in the presence of phloroglucinol (B) without phloroglucinol

In this study, phloroglucinol and (-)-epicatechin standards eluted in peaks at retention times 2.42 and 16.4 min respectively. However, the 'X' polyphenol clump as seen in literature did not resolve into individual peaks when the reaction was carried out with and without phloroglucinol (Figure 3.1.1). Peaks 'a' and '1' were expected to be ascorbic acid and (+)-catechin, respectively, based on literature (Kennedy and Jones 2001). Peak 'b' was not identified as a phloroglucinol adduct based on the order of elution observed in the findings of Kennedy and Jones (2001) and remained an unidentified product of the acid cleavage reaction.

The same method was repeated to a known high source of procyanidins as described in Appendix 1. HPLC chromatograms of crushed grape seeds extracted subjected to heat-acid treatments in the presence of phloroglucinol showed that the clump successfully resolved into individual peaks, where phloroglucinol and (-) epicatechin peaks were identified at 3.38 and 16.34 min, respectively (Figure 3.1.2).

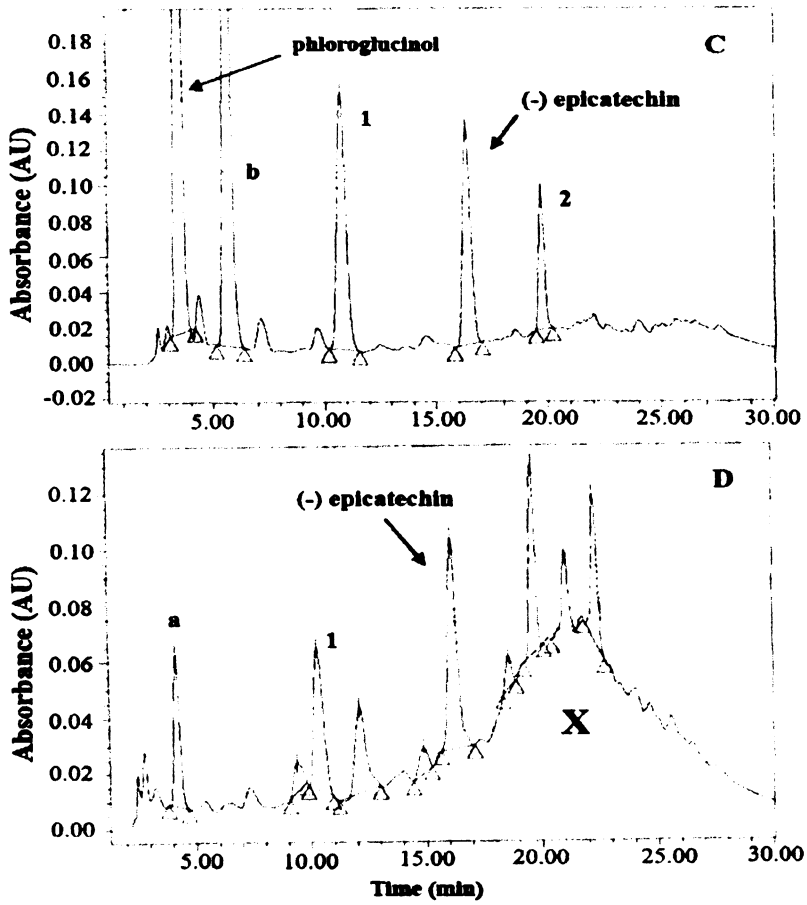

Figure 3.1.2 HPLC chromatogram of procyanidin cleavage products from grape seed extracts following acid-catalysis (C) with the presence of phloroglucinol and (D) with phloroglucinol

Peaks (a) and (b) may be traces of phloroglucinol or unidentified products of the acid cleavage while peaks (1) are identified as (+) catechin. Peak (2) should be the phloroglucinol adduct, which agrees with results found by Kennedy and others (2001). The unresolved clump and peak heights in Figure 3.1.2 are relatively higher than that found in Figure 3.1.1, indicating that grape seed extracts contain significantly higher procyanidin content than apple cider extracts made from dessert varieties.

When unsatisfactory results were obtained with the HPLC methodology, colorimetric assays were investigated for alternative methods of estimating procyanidin content in cider samples made from Jonathan dessert apple varieties. Colorimetric means

such as BuOH/HCl and Vanillin (methanol) assays resulted in sufficient color reaction when tested on polyphenol extracts of Jonathan hard cider. Using (-) epicatechin as a standard on the Vanillin assay, the concentrations of procyanidins were estimated to range from 0.093 to 4.28 g procyanidin/ml juice (Appendix 1). While overestimations might have occurred (the Vanillin assay was subjected to interferences with procyanidin monomeric units), the concentration values obtained from the cider samples of this study were comparable to values obtained from juices measured in literature, which ranged from 1.2 to 2.4 g/L (Guyot and others 2001; 2002).

It was concluded that despite multiple attempts, the heat-acid treatment prior to HPLC analysis failed to cleave procyanidins in sample extracts into monomeric units, thus resulting in absence of phloroglucinol adducts as seen in the HPLC chromatograms. Among possible explanations of this outcome include the sensitivity of this reaction to the presence of water, where the addition of water reduced the formation of phologlucinol adducts (Kennedy and Jones 2001) and the unstability of phloroglucinol adducts. Some factors which could have reduced the degradation yields might also be the presence of phenolic or nonphenolic impurities and the existence of degradation resistance bonds within the polymer units (Matthews and others 1997).

Nevertheless, using colorimetric means, the calculation of mean degree of polymerization using the concentrations obtained from molar absorptivity of cyanidin and procyanidins resulted in mDP ranges of 2-7, which agreed with the results of Guyot and others (2001). Therefore, the colorimetric assays were selected for procyanidin analysis in this study.

3.2 Juice sample preparation

The addition of apple solids to juice samples posed some sample preparation challenges. First, the apple solids hinder mixing, which was necessary for ensuring homogeneity of fermentation and collecting samples for analysis. Preliminary experiments resulted in airlocks being blown off the bottle tops when apple material was pushed up by the CO_2 gas pressure generated from the fermenting yeast. The size reduction of fermentation bottle from 1 L to 200 ml helped to increase sample size (n) and allowed the whole bottles to be mixed by hand without opening.

Secondly, microbial contamination in the samples was increased as apple solids were increased. In this study, the following microbial contaminations were observed: 1) microbial growth on control samples within 10-14 days at 13-14 °C, and 2) microbial growth in samples with yeast added after 20 – 25 days of fermentation. The first observation was expected since there was an insufficient amount of yeast to dominate growth in the controls and introduction of microbes from the outside environment during sampling allowed undesirable microbial growth. The second observation was made when an acidic aroma similar to vinegar was detected in the hard cider samples. This aroma was considered a defect and an undesirable flavor in hard cider production that was thought to be produced by the growth of acetic acid bacteria. Such contamination most likely occurred when sampling was performed after the completion of the yeast fermentation when the yeast population declines and allows other microbes to proliferate. The equipment, whole apples and bottles used in sample preparation were sanitized using domestic bleach (Ultra Clorox) at 200 ppm for 1 to 2 min to reduce microbial contamination.

Additionally, the separation of apple solids from the juice was difficult to achieve despite centrifugation efforts, and juice samples tended to be cloudy. Therefore, samples of 0% solids were not completely devoid of any apple solid particles. The percentage of apple solids of samples was defined as the weight percentage of apple solids deliberately added back, rather than the total apple solid content of the juice.

In this study, enzymatic oxidation was a factor that was difficult to control. While the enzyme polyphenol oxidase was responsible for producing the yellow and browning pigments that impart the coloring effect welcomed in apple cider, oxidation played a role in reducing procyanidin levels in cider (Guyot and others 1999, 2003). While the addition of potassium metabisulfite at 53 ppm may help to minimize this reaction, oxidation may have reduced the procyanidin concentration values obtained in this study due to the lack of practical options in controlling this reaction.

3.3 Fermentation

3.3.1 Sugar content

The refractive index value (RI) was used to monitor the fermentation rate as the concentration of fermentable sugars decrease during fermentation. RI values may vary between cider batches. This variation was expected as the measurement was non-specific and any dissolved solute such as soluble pectin within the cider sample matrix may produce interferences. A sample that had higher RI value does not necessarily mean that it contains more sugars.

The RI values obtained in hard cider made from Jonathan apples, which were chosen for this study, ranged from 10.8 to 11.0. This value was converted to sugar percentages (glucose) and adjusted according to a standard curve (Appendix 1).

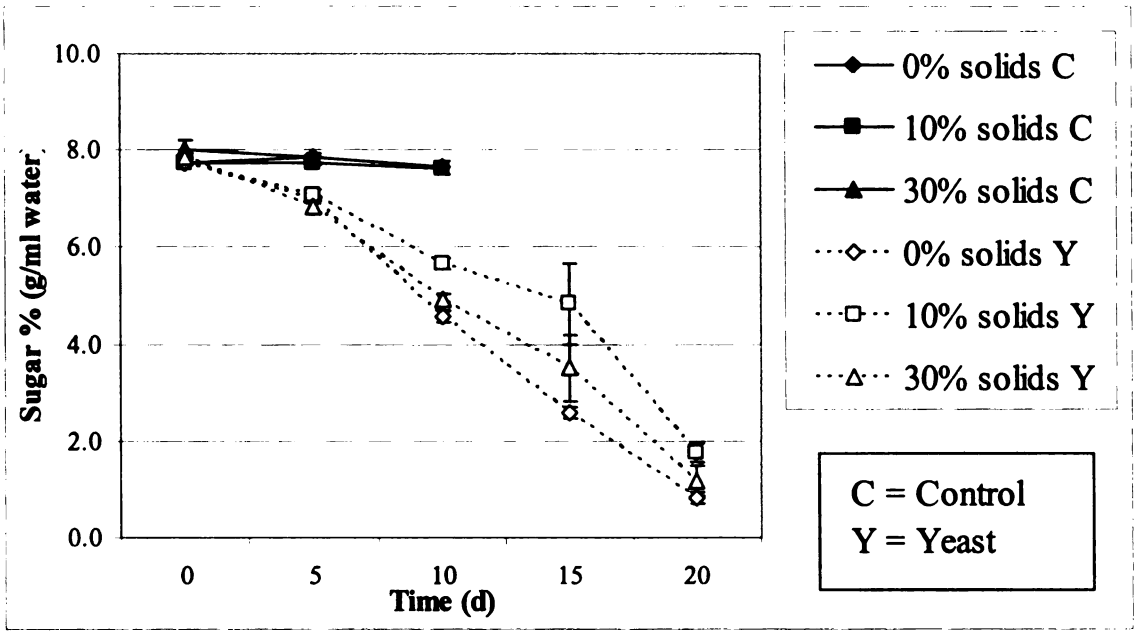

Figure 3.3.1 Sugar content of Jonathan hard cider during fermentation (n = 3, p <0.001)

In this study, yeast fermentation for hard cider production was achieved within 20 days based in the reduction of RI values (Figure 3.3.1). Most large UK cidermakers prefer fermentations to be completed within 14 days (Lea and Drilleau 2003) while Johansen (2000) reported that English cider-making generally lasts for 1 to 4 weeks. Some variations in the fermentation rates were observed between batches, in which one batch fermented a few days more slowly than the other. The rate of fermentation varied according to external parameters (yeast type, yeast concentration and temperature) and internal parameters (pH, nitrogen content). Cider contains significantly less (approximately 1/8th) free amino nitrogen than do grape musts and beer worts (Lea and Drilleau 2003). Variations in fermentation rates between batches may be due to external

factors such as yeast viability. The capability of yeast to live, develop, or germinate (viability) influences fermentation rates. The lower the viable yeast cells, the slower the fermentation rates become.

3.3.2 pH

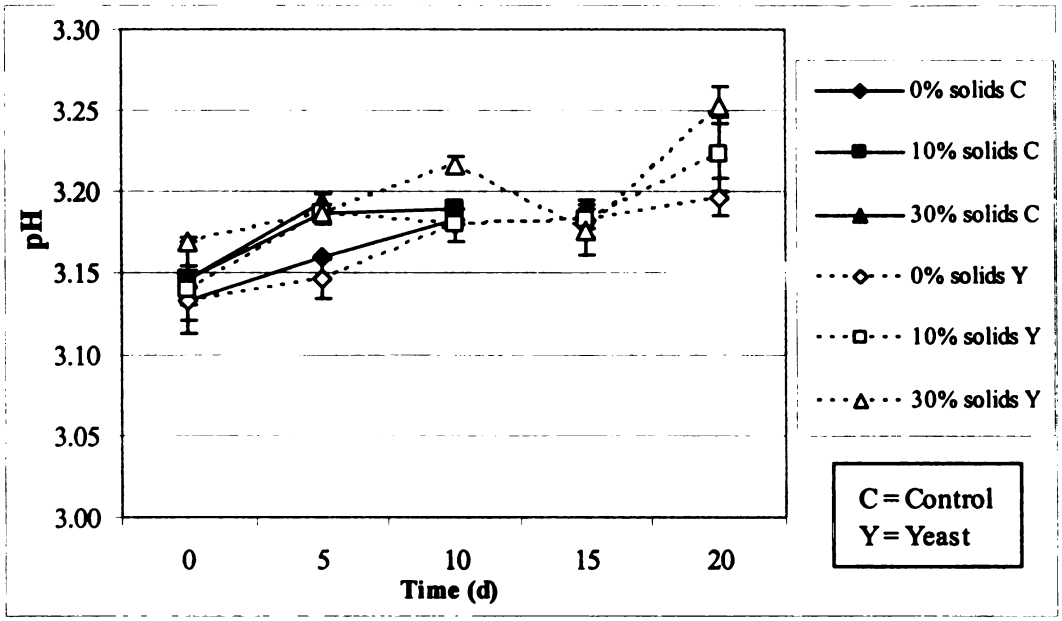

Figure 3.3.2 pH of Jonathan hard cider during fermentation (n = 3, p <0.001)

Throughout the fermentation studies, the pH range of hard cider made from Jonathan apples ranged from 3.13 to 3.25 ± 0.01 to 0.03 (Figure 3.3.2). The pH levels of the Jonathan hard cider increased throughout yeast fermentation, which agreed with Campo and others (2003) who observed the same trend in large batch fermentations of cider made from cider cultivars within 15 fermentation days. While the slight rise in pH could be contributed to malo-lactic fermentations (which decreases the acidity), this fermentation would usually occur after the yeast fermentation had completed and had been inhibited by low pH (Lea and Drilleau 2003). One factor that could have influenced

this pH rise may include biochemical reactions within the cider that have yet to be identified and investigated.

3.4 Procyanidin estimation

3.4.1 The effect of fermentation on the concentration of procyanidins in cider

Each effect was evaluated on the following dependent variables: 1) the concentration of procyanidin terminal end units 2) the concentration of procyanidin extension units and 3) degree of polymerization.

There were three factors that were evaluated during fermentation; alcohol production, percentage of apple solids and time. Two batches (each n =3) were combined for statistical analysis and samples contained apple solids ranging from 0 to 30%. There were missing data points among the control samples due to microbial contamination, which were denoted with an asterisk (*). The missing samples included the following:

i) Samples containing 0% apple solids: control, day 20

ii) Samples containing 10 and 30% apple solids: control, day 15 and 20

Data points marked with '◊ ○ Δ' indicate mean values (n = 3) calculated only from samples with yeast.

Due to the complications of the experiment design and time constraints, replications for the fermentation experiments were limited to two and are referred as batches. Data sets from preliminary batches were combined to reduce errors by increasing sample size.

3.4.1.1 The effect of alcohol production by yeast fermentation on the concentration of procyanidins in cider fermentation made from Jonathan apples

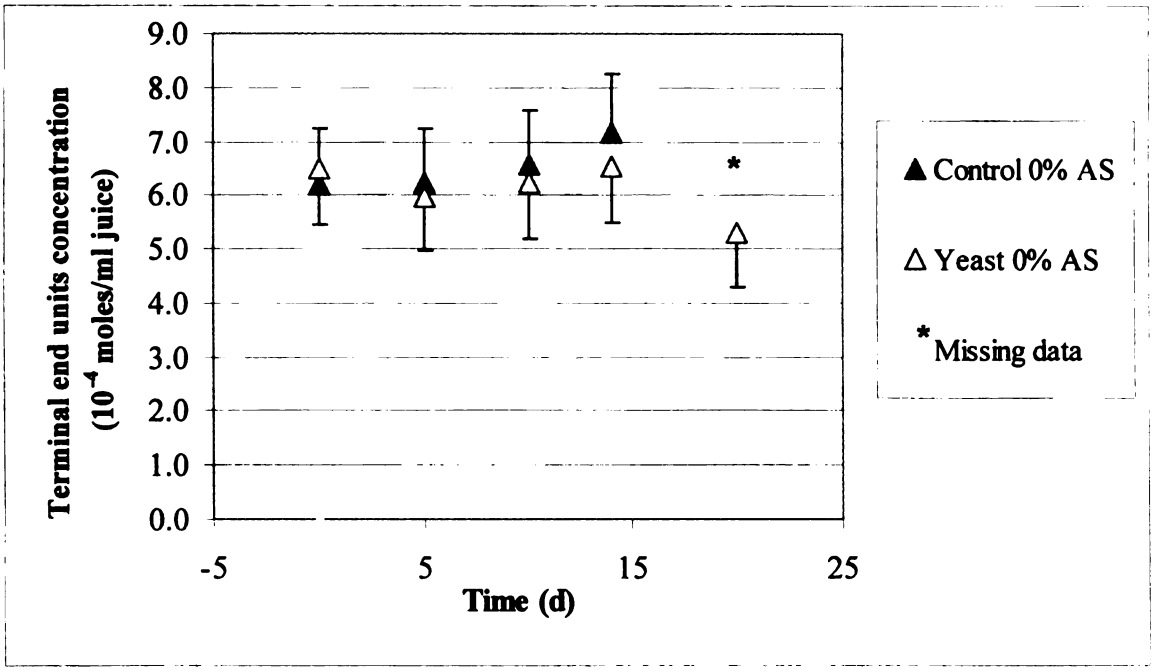

Figure 3.4.1 The effect of alcohol production on procyanidin terminal end units concentrations in Jonathan cider fermentation containing 0% apple solids on fermentation days 0, 5, 10, 14 and 20 (n = 3, p <0.001, half error bars)

There were no significant differences in the concentrations of procyanidins (as measured by terminal end units) between control and yeast samples containing 0% apple solids (Figure 3.4.1). Both control and yeast samples had similar concentration trends between Day 0 and Day 14. No comparisons were made on Day 20 since control samples were discarded due to microbial contamination after Day 14.

Similarly, no significant differences between procyanidin concentrations (measured as terminal end units) of control and yeast samples containing 10 and 30% apple solids were observed (Table 3.4.1). The amounts of alcohol produced at the end of yeast fermentation may not be sufficient enough to either, 1) reduce the binding effect between procyanidins and apple solids, or 2) release significant amounts of procyanidins from apple solids through alcohol extraction.

Table 3.4.1 Procyanidin terminal end unit concentrations in control and yeast samples of Jonathan cider fermentation containing 0, 10 and 30% apple solids on fermentation days 0, 5, 10, 14 and 20 (n = 3, p <0.001)

	Concentration (10^{-4} moles/ml juice)					
Apple solids%	0		10		30	
Time (d)	C	Y	C	Y	C	Y
0	6.204 (±1.04)	6.500 (±1.04)	4.410 (±1.04)	4.497 (±1.04)	0.941 (±1.04)	0.809 (±1.04)
5	6.227 (±1.00)	5.973 (±1.00)	4.272 (±1.00)	4.263 (±1.00)	0.387 (±1.00)	0.613 (±1.00)
10	6.563 (±1.03)	6.228 (±1.03)	4.513 (±1.04)	3.549 (±1.03)	0.384 (±1.06)	0.556 (±1.03)
14	7.177 (±1.07)	6.528 (±1.03)	N/A	3.910 (±1.03)	N/A	0.549 (±1.03)
20	N/A	5.311 (±1.01)	N/A	3.449 (±1.01)	N/A	0.455 (±1.01)

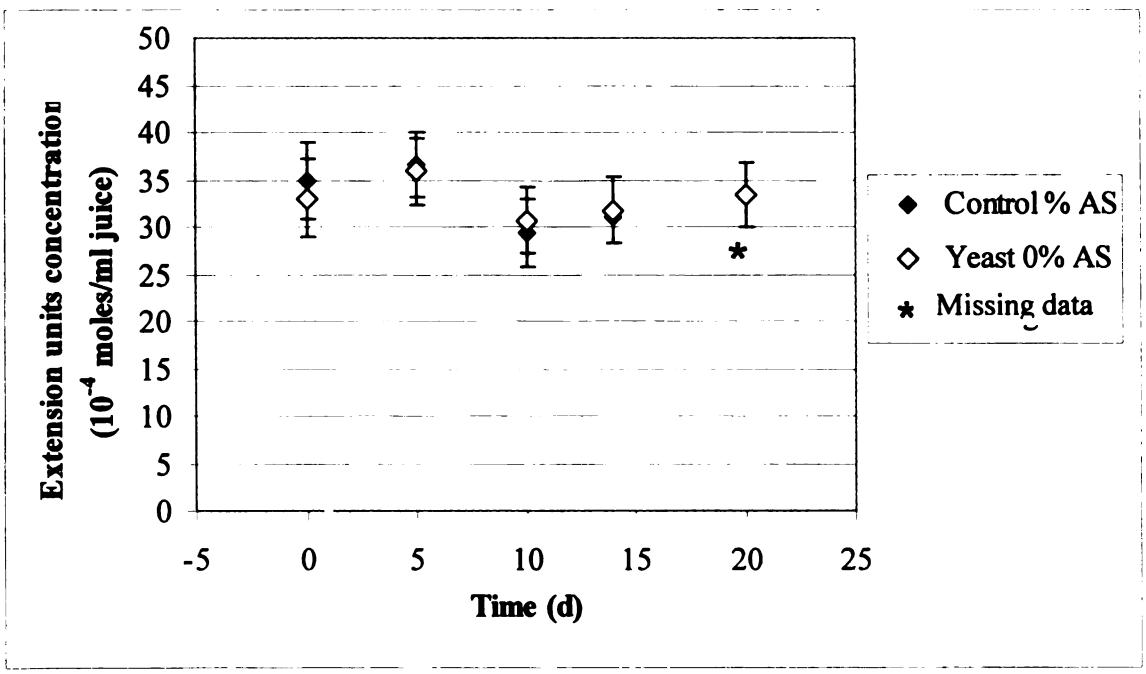

Figure 3.4.2 The effect of alcohol production on the procyanidin extension unit concentrations in Jonathan cider fermentation containing 0% apple solids on fermentation days 0, 5, 10, 14 and 20 (n = 3, p <0.001)

Similarly, no significant effect of alcohol production on the concentration of procyanidin extension units was observed (Figure 3.4.2). A similar pattern was also observed in samples containing 10% and 30% apple solids.

Table 3.4.2 Procyanidin extension unit concentrations in control and yeast samples of Jonathan cider fermentation containing 0, 10 and 30% apple solids on fermentation days 0, 5, 10, 14 and 20 (n = 3, p <0.001)

	Concentration (10^{-4} moles/ml juice)					
Apple solids%	**0**		**10**		**30**	
Time (d)	**C**	**Y**	**C**	**Y**	**C**	**Y**
0	34.96 (±7.04)	33.07 (±7.04)	30.47 (±7.04)	24.45 (±7.04)	9.36 (±7.04)	8.05 (±7.04)
5	36.68 (±5.93)	35.85 (±5.93)	24.73 (±5.93)	23.25 (±5.93)	4.39 (±5.93)	6.57 (±5.93)
10	29.32 (±6.21)	30.72 (±6.21)	18.79 (±6.28)	20.28 (±6.21)	1.88 (±6.55)	4.06 (±6.21)
14	31.13 (±6.10)	31.78 (±5.83)	N/A	19.27 (±5.99)	N/A	4.10 (±5.99)
20	N/A	33.38 (±6.12)	N/A	18.22 (±5.99)	N/A	4.39 (±6.00)

The results indicated that alcohol produced during fermentation may not be sufficient to release procyanidin units from the apple solids. Additionally, it was most likely that there were no significant structural changes i.e. oligomer to polymer and vice versa occurring within the procyanidin units (Lea and Timberlake 1978).

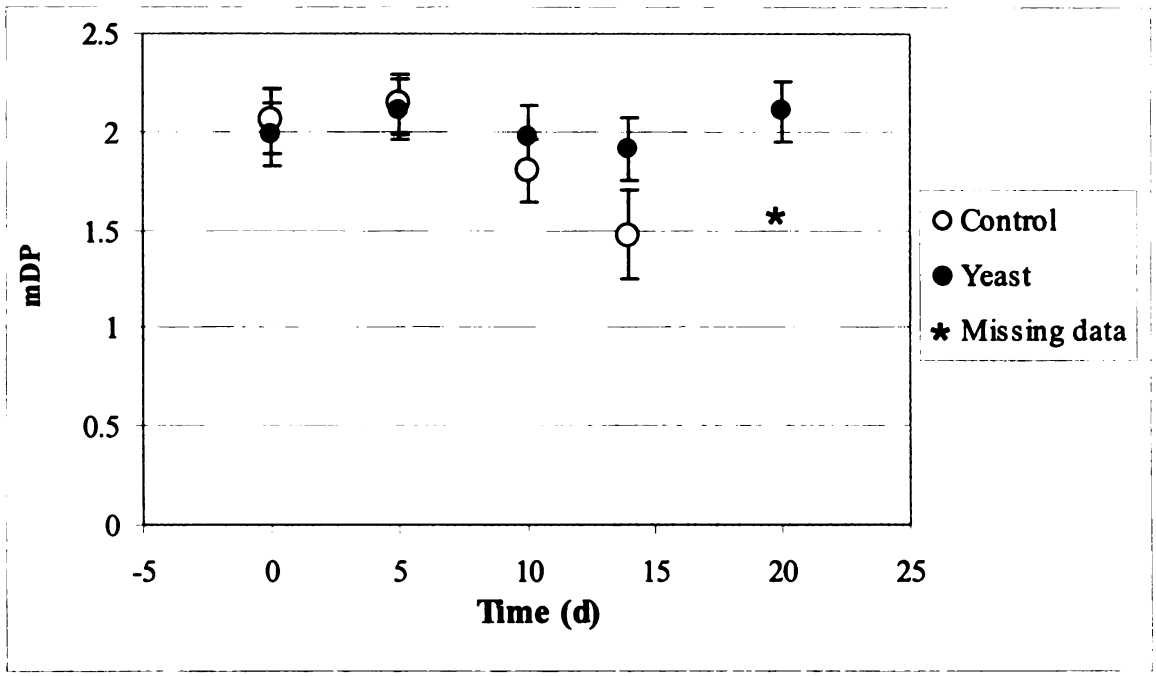

Figure 3.4.3 The effect of alcohol production on the mean degree of polymerization of procyanidins in Jonathan cider fermentation on fermentation days 0, 5, 10, 14 and 20 (n = 3, p <0.001)

There was no significant effect of alcohol production on the mDP of procyanidins of the control and yeast samples (Figure 3.4.3). The same trend was observed in all samples of varying apple solid percentages (0-30%). This observation was expected since the mDP value was calculated from the concentration of terminal end units and extension units.

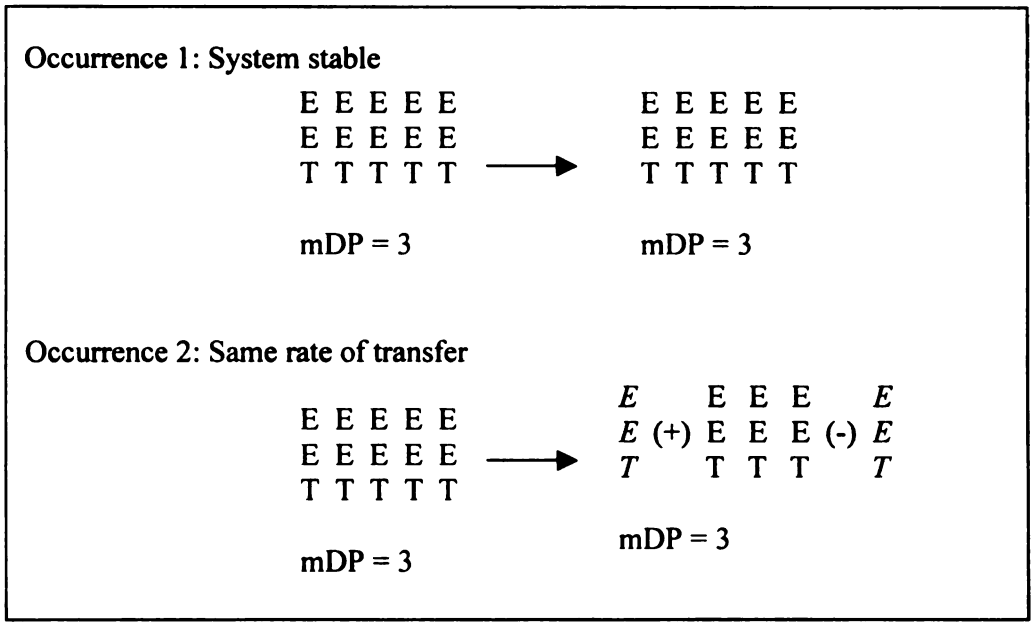

Figure 3.4.4 Proposed behavior of procyanidins in Jonathan cider during fermentation (T= terminal end units, E = Extension units): Stable or same transfer rate

No alteration in the concentration of procyanidin units was detected during fermentation. If there were any changes in procyanidins being released from or absorbed in to the solids, the transfer rate of procyanidins into and out of the juice system remained consistent and didn't affect the mDP of the samples (Figure 3.4.4). Therefore, it was concluded that yeast fermentation has the potential to retain the mDP of procyanidins during fermentation and does not increase mDP values.

3.4.1.2 The effect of apple solids on the concentration of procyanidins in cider fermentation made from Jonathan apples

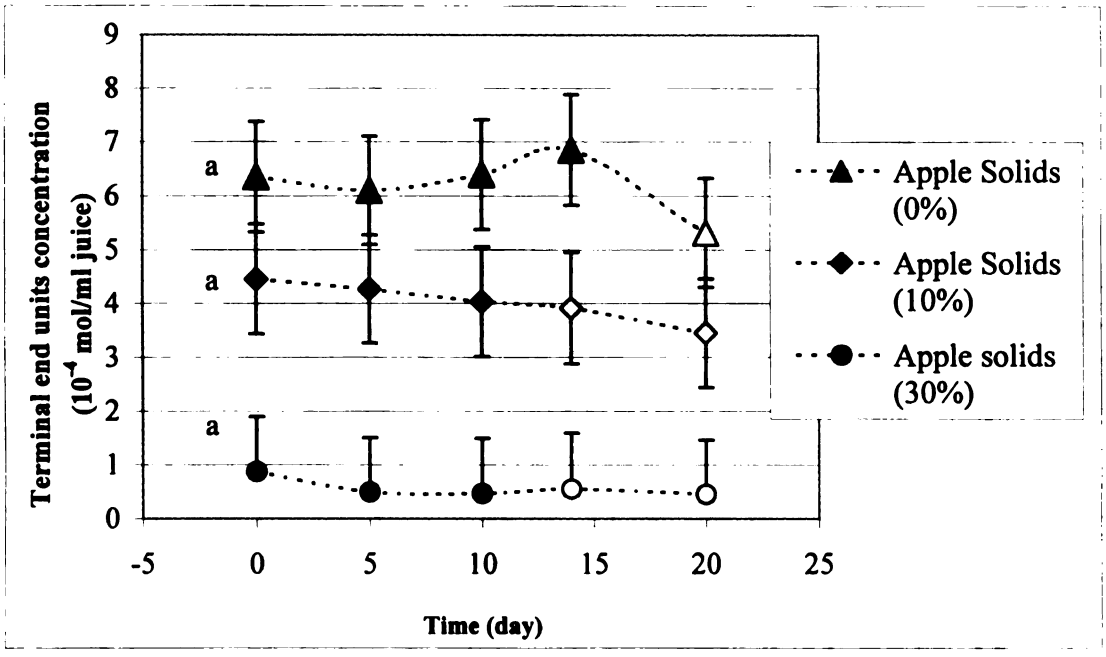

Figure 3.4.5 The effect of apple solids on procyanidin terminal end units of Jonathan cider fermentation (n = 3, p <0.001, half error bars for 30% apple solids samples)

Statistically, there were no significant differences in the concentrations of procyanidins between samples of varying apple solids % (Figure 3.4.5). However, since the statistical model was conservative, significant differences were not detected, such as in this case, where p = 0.085 when mash was compared over time. Samples of 0% and 10% apple solids had higher levels of procyanidin concentrations than samples of 30% apple solids, but it was not significantly high enough. This would have agreed with Renard and others (2003) who reported that the more apple solids were added, more binding of procyanidins with apple flesh occurred. However, it was possible that the procyanidin - cell walls interactions that occurred in samples consisting of 0% apple solids were sufficiently established and the addition of more apple solids did not make a significant difference in the procyanidin content starting from Day 0 onwards.

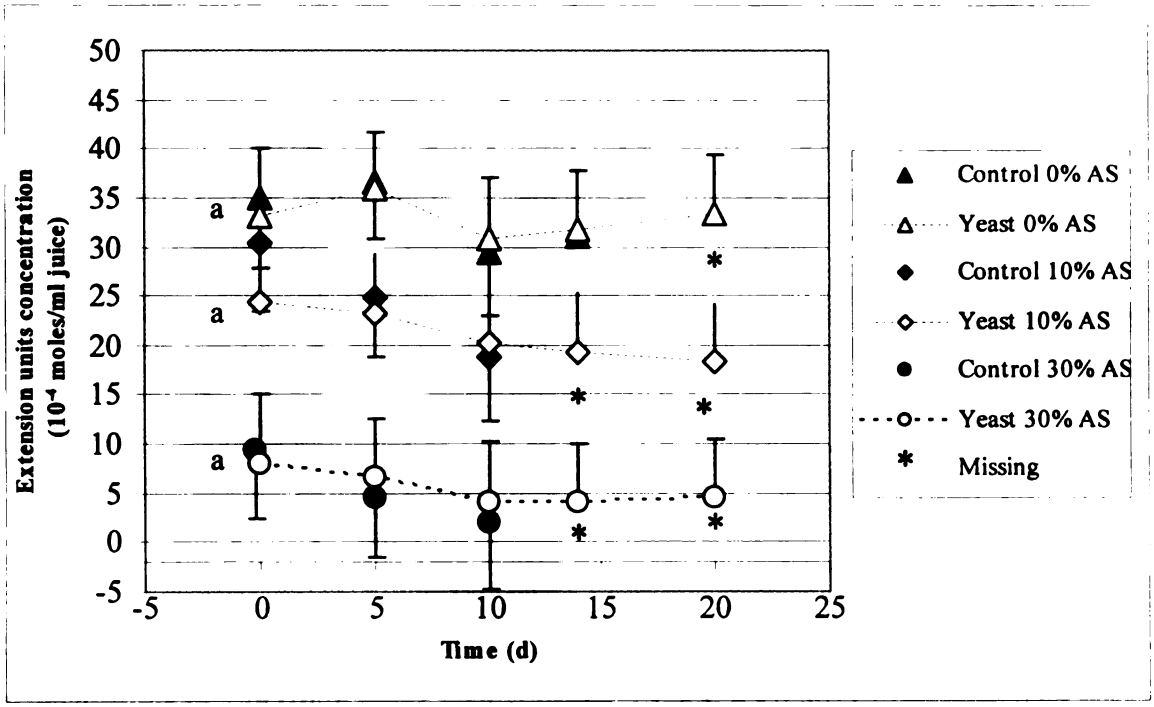

Figure 3.4.6 The effect of apple solids on procyanidin extension units of Jonathan cider fermentation (n = 3, p <0.001, half error bars)

Similarly, there were no significant differences observed between the concentrations of extension units in apple solid percentages (Figure 3.4.6).

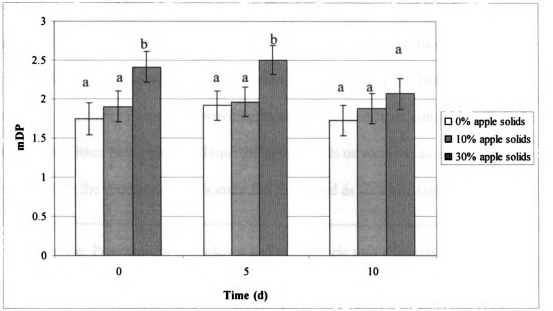

Figure 3.4.7 The effect of apple solids on mean degree of polymerization of procyanidins in Jonathan cider fermentation ($n = 3$, $p < 0.001$)

Samples containing 30% apple solids had significantly higher mDP values on Day 0 and 5 compared to samples with 0 and 10% apple solids (Figure 3.4.7). This finding was unexpected since the concentration of terminal end units and extension units demonstrated no significant differences on an individual basis. However, the combination of the concentration values to calculate mDP may result in some significant differences especially if the extension unit concentration increased while the terminal end unit concentration decreased or remained constant.

The presence of additional apple solids may have contributed to the presence of longer chained procyanidins for the first two sampling days. On Day 10, the differences were no longer observed as the mDP of samples containing 30% apple solids dropped. This observation may be due to the decrease in procyanidin extension unit concentrations as seen on Day 10 (Figure 3.4.6) while procyanidin terminal end units remained relatively constant. It could be possible that procyanidin units broke down into

monomeric units during fermentation on Day 10, when the cider samples were undergoing yeast fermentation, possibly at its optimum. Investigations to determine the potential role of yeast breaking down procyanidin units have not been reported in research literature. These monomeric units may have then been eliminated from the juice system by either being absorbed into the apple solids or were oxidized, thus reducing or maintaining the procyanidin concentration measured as terminal end units (Figure 3.4.8).

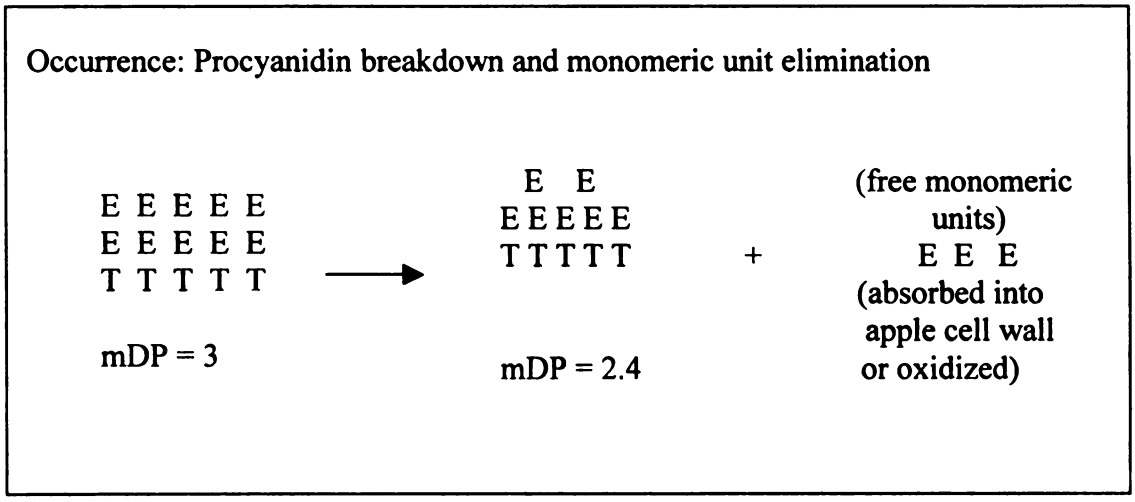

Figure 3.4.8 Proposed behavior of procyanidins in Jonathan cider during fermentation: Polymeric breakdown and monomeric unit elimination

3.4.1.3 The effect of fermentation time on the concentration of procyanidins in cider fermentation made from Jonathan apples

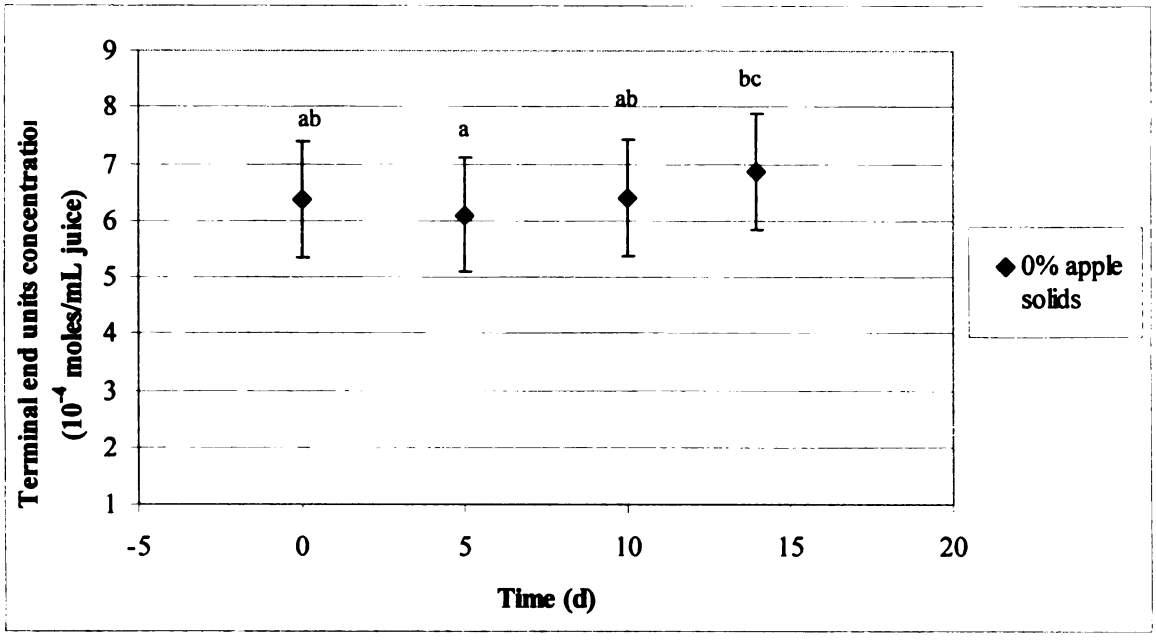

Figure 3.4.9 The effect of fermentation time on procyanidin terminal end unit concentration in Jonathan cider fermentation containing 0% apple solids (n = 3, p <0.001)

Significant differences in the concentrations of procyanidin terminal end units were only observed in samples containing 0% apple solids between Day 5 and Day 14, where Day 14 was significantly higher (Figure 3.4.9). The presence of apple solids may have hindered the effects of fermentation on procyanidins and therefore, no significant differences throughout time were observed in samples of higher apple solids content (10% and 30%) when treatment was held constant.

The concentration of procyanidin terminal end units may have increased due to the degradation of apple cell wall materials over time or other side reactions of yeast fermentation (apart from alcohol production), which released procyanidin units into the juice system.

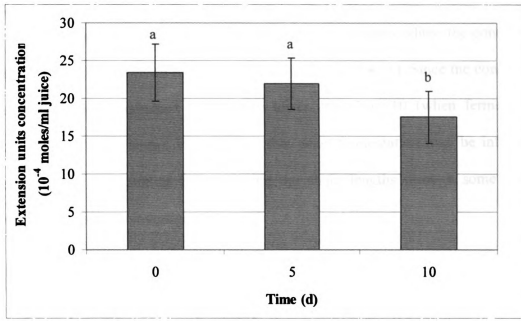

Figure 3.4.10 The effect of fermentation time on the concentration of extension units in Jonathan cider fermentation (n = 3, p <0.001)

Procyanidin extension unit concentrations were significantly lower on Day 10 when the mash and treatment factor remained constant (Figure 3.4.10). Since data from the control samples was limited due to microbial contamination after Day 10, the effect of time was further investigated for the yeast samples.

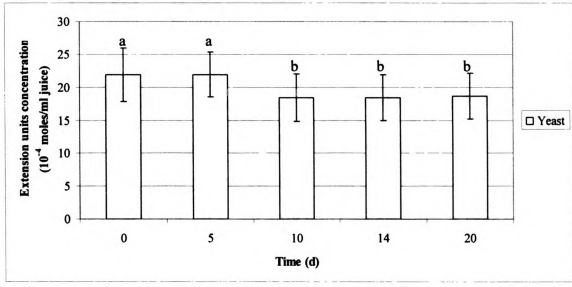

Figure 3.4.11 The effect of fermentation time on the concentration of extension units in Jonathan cider fermentation: Yeast samples (n = 3, p <0.001)

A similar pattern was observed in the yeast samples where the concentration of extension units decreased on Day 10 onwards (Figure 3.4.11). Since the concentration of extensions units remained consistently lower after Day 10 (when fermentation was ongoing at its optimum), it is possible that yeast fermentation may be influencing the structure of procyanidins by shortening the chain lengths through some mechanism, which has yet to be investigated.

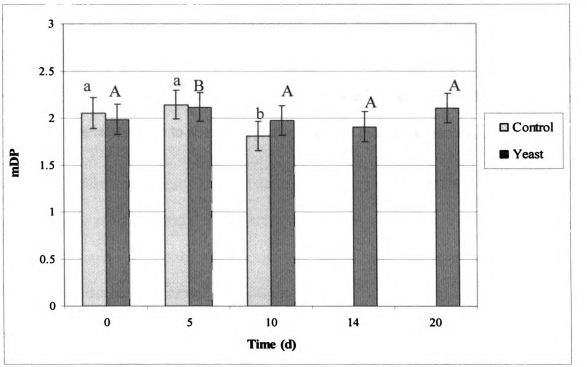

Figure 3.4.12 The effect of fermentation time on mean degree of polymerization of procyanidins in Jonathan cider fermentation (n = 3, p <0.001)

There were significant differences in mDP values between both control and yeast samples over time (Figure 3.4.12). Control samples at Day 10 had significantly lower mDP than those at Day 0 and 5. The mDP may have decreased as more procyanidins were bound to apple solids as time increased which agreed with the observation made by Le Bourvellec and others (2004) regarding the complexation of procyanidins with apple

cell walls. For the fermented yeast samples, there were variations in the mDP levels between days and no consistent trend was observed. It may be due to a combination of factors that both increase and decrease procyanidin content (whether terminal end or extension unit-wise) simultaneously during fermentation.

Overall, it was observed that alcohol production, apple solids and time did not increase procyanidin content, chain length and consequently, mDP during yeast fermentation. These results indicated that 'mash fermentations' practiced in the wine industry may not yield the same procyanidin boost expectations in hard cider mash fermentations made from dessert apple varieties. The oxidation effect on procyanidins was a major factor that served as an obstacle towards the success of the project. For example, sample exposure to air during preparation and analysis were unavoidable.

3.4.2 The effect of heat on the concentration of procyanidins in cider made from Jonathan apples

Since fermentation did not significantly increase free procyanidin content in hard cider, heat and pectolytic enzymatic treatments were considered as alternative options to support the research hypothesis.

The plant material used was similar to Methods and Material (Section 2.1). Similarly, the apple juice samples were prepared using Acme 5001 White Supreme Juicerator™ Juice Extractor (Waring Products, Torrington, CT, USA). The apple pieces were cored, peeled by hand, and juice was collected as a single batch. All samples had 10% apple solids (w/w) added back to the cider and distributed into 50 ml disposable centrifuge bottles in triplicates.

The apple juice samples were centrifuged using an Allegra 6R Centrifuge Rotor Model GH3.8 with swinging buckets (Beckman, Fullerton, CA, USA) to separate the juice from the solids at 3000 rpm (~ 1411 g) for 15 min at 10 °C. The supernatants were decanted. Procyanidin concentrations were measured using the colorimetric methods described in Methods and Material (Section 2.4.2).

The samples were held in water baths of the following temperature profiles, 40, 50, and 60 °C for 90 min (Figure 3.5.1).

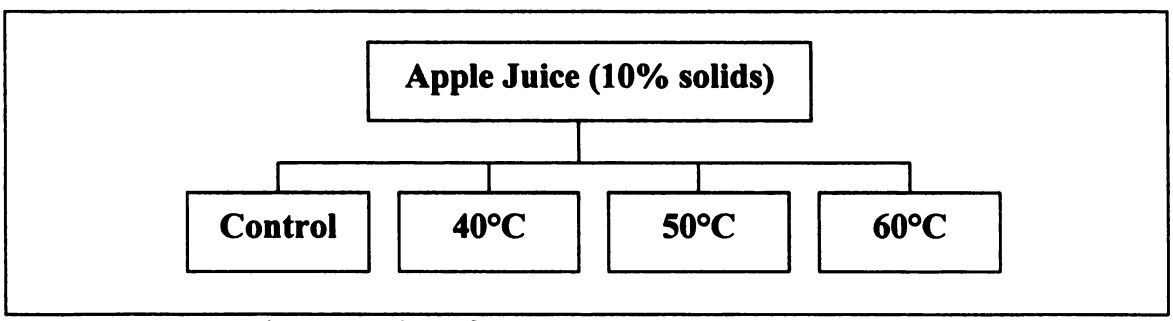

Figure 3.5.1 Experiment design of heat treatment

The data was analyzed using a one-way factorial analysis and Tukey's HSD. The $p \leq 0.001$ was used as the level of significance.

Elevated temperatures of 40°C and above are known to inactivate polyphenol oxidase, the enzyme responsible for browning. Procyanidins have been reported to be heat stable up to boiling temperatures (Renard and others 2004).

Significant differences observed between experiment batches were thought to be due to the variances between batches of raw apple material since the apples used in this objective were not obtained from the same source (local grocery store vs MSU Horticulture farm). While the samples were harvested in the same Fall season, variations in procyanidin content were potentially present in each batch or bag since growing conditions (i.e. climate, soil type, and orchard location) may differ. Additionally, the batches of apples may further differ in their cold storage conditions since each batch was studied on different days. While there was no literature on the behavior of phenolic compounds in Jonathan apples, studies reported that (-) epicatechin concentrations in dessert apple varieties remained constant during storage (Burda and others 1990) and the concentration of (+) catechins in apples had a tendency to decrease during storage (Kolesnik and others 1977).

Since the phenolic content of apples influences the oxidation rate in cider samples, it is possible that batches of apples with significantly lower procyanidin concentrations either had reduced procyanidin content within the apples or experienced different levels of oxidation during sample preparation for each batch.

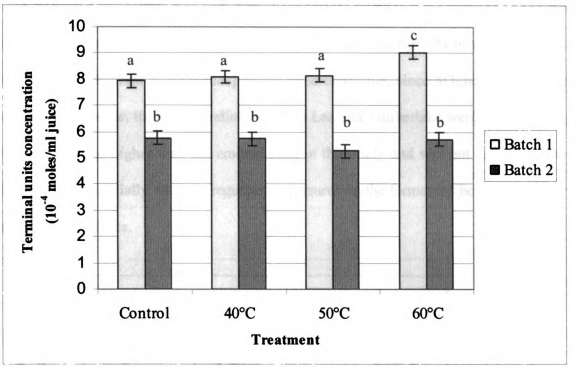

Figure 3.5.2 The effect of heat treatments on the procyanidin terminal end unit concentration in Jonathan cider containing 10% apple solids (n = 3, p <0.001)

There were no significant differences observed between all samples in Batch 2 while in Batch 1, samples treated at 60°C were significantly higher in procyanidin concentrations than the other samples of lower temperature profiles as measured by terminal end units (Figure 3.5.2). Heat may have weakened the hydrogen bonds that bound procyanidins with polysaccharides, and increased diffusion of procyanidin from the apple solids into the cider significantly in Sample 60°C of Batch 2.

However, many problems were encountered in this experiment. In preliminary tests (Appendix 3, Table A.3.3 and A.3.4), error values in the data obtained were as high as 30% and no conclusions could be made. Additionally, repeats of this experiment with other batches yielded varying results and no definite trend could be concluded by comparing significant differences obtained between Batch 1, 2, and 3 (Appendix 3, Table A.3.1 and A.3.2). This inconsistency may be due to the variances between batches that

were discussed previously. Lea and Timberlake (1978) found that the addition of SO_2 was vital in successfully increasing organoleptically procyanidins by 50% in warm anaerobic incubation of Dabinett apple [solids] before juice extraction, since SO_2 helped to control oxidation. However, the concentrations used by Lea and Timberlake were 200 ppm SO_2, which is 4 times higher than the amount used in this study and was not feasible for the industry use, especially with the regulatory pressures on the fermented beverage industry in regards to sulfites.

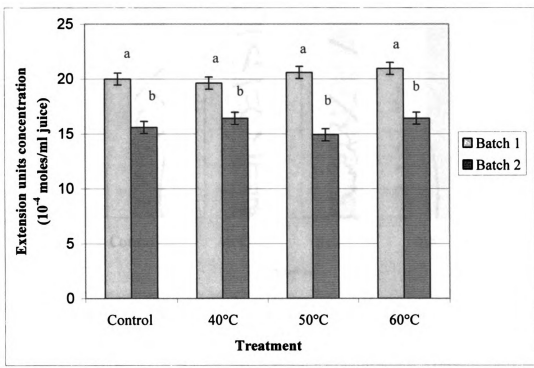

Figure 3.5.3 The effect of heat treatments on the procyanidin extension unit concentration in Jonathan cider containing 10% apple solids (n = 3, p <0.001)

No significant differences were observed between the concentrations of procyanidin extension units based on different temperature profiles (Figure 3.5.3), which was consistent among the two batches. This observation indicated that procyanidin chain length was not affected by temperature. An explanation may be that the concentration of whole procyanidin units released from apple solids did not significantly change. This

agreed with the previous observation of terminal end concentration. Another explanation may be that the phenomenon of oligomers changing to polymers did not occur during heat treatment. Procyanidins had been reported to be heat stable up to boiling temperatures (Renard 2004) and this may be why procyanidin content did not decrease after heat treatments.

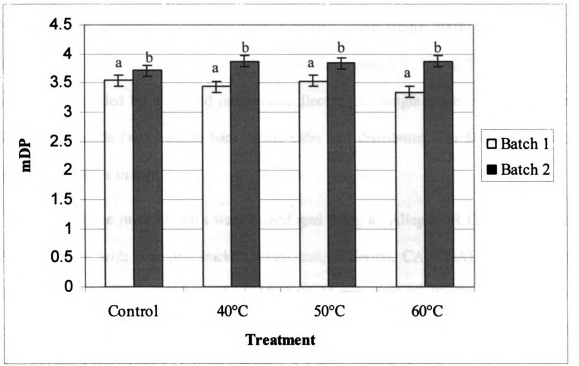

Figure 3.5.4 The effect of heat on the mean degree of polymerization of procyanidins in Jonathan cider containing 10% apple solids (n = 3, p <0.001)

No significant differences were observed in the mDP of procyanidins between treatments of varying temperature profiles (Figure 3.5.4). This observation was expected since the concentration of procyanidins (measured as terminal end and extension units) influence the mDP and both concentrations demonstrated the lack of or limited significant changes as affected by heat treatments.

The lack of trend was most likely because higher temperatures did not provide sufficient disintegration of the apple cell walls to release procyanidin constituents since

the samples contain 10% apple solids. The behavior of procyanidins was thought to be similar to what was seen from the data in Figure 3.4.4.

3.4.3 The effect of pectolytic enzymes on the concentration of procyanidins in cider made from Jonathan apples

The plant material used was similar to Methods and Material (Section 2.1). Similarly, the apple juice samples were prepared using Acme 5001 White Supreme Juicerator™ Juice Extractor (Waring Products, Torrington, CT, USA). The apple pieces were cored, peeled by hand and juice was collected as a single batch. All samples had 10% apple solids (w/w) added back to the cider and distributed into 50 ml disposable centrifuge bottles in triplicates.

The apple juice samples were centrifuged using an Allegra 6R Centrifuge Rotor Model GH3.8 with swinging buckets (Beckman, Fullerton, CA, USA) to separate the juice from the solids at 3000 rpm (~ 1411 g) for 15 min at 10 °C. The supernatants were decanted. Procyanidin concentrations were measured using the colorimetric methods described in Methods and Materials (Section 2.4.2).

Commercial pectolytic enzymes, Pectinex® Ultra SP-L and Crystalzyme® 200XL were used at concentrations of 0.02 ml/ml juice (Figure 3.6.1). The samples treated with Pectinex® Ultra SP-L were placed in a water bath at 35 °C while samples treated with Crystalzyme® 200XL were heated at 50 °C. Both samples were held in water baths for 90 min.

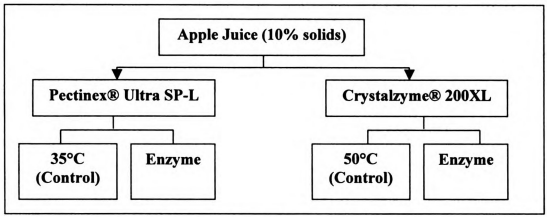

Figure 3.6.1 Experiment design of pectolytic enzyme treatments

The data was analyzed using a one-way factorial analysis. The $p \leq 0.001$ was used as the level of significance.

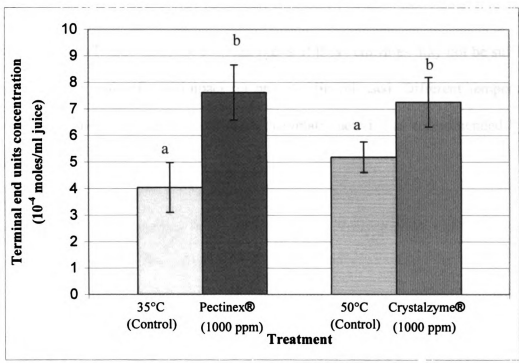

Figure 3.6.2 The effect of pectolytic enzyme treatments on procyanidin terminal end unit concentration in Jonathan cider containing 10% apple solids (n = 6, p <0.001)

Samples which contained 10% apple solids were treated with commercial pectolytic enzymes, Pectinex® and Crystalzyme® had higher concentration of procyanidins as measured by terminal end units (Figure 3.6.2). This result agreed with results of Will and

others (2002) who found that the concentration of procyanidins increased with enzymatic treatments at 40, 50 and 60°C. Enhanced cell wall degradation by the pectolytic enzymes may have led to a higher procyanidin release.

There were no significant differences between the concentrations of procyanidin, measured as terminal end units between samples treated with the two pectolytic enzymes. Crystalzyme 200XL contains arabinase and cellulose while both Crystalzyme 200XL and Pectinex Ultra Sp-L contain pectolytic and a range of hemicellolulytic activities. First, the composition of both enzyme systems may not differ significantly from each other. Specific information on the composition of enzyme mixes is not available from the manufacturer. If Crystalzyme 200XL does contain arabinase and cellulase, which Pectinex Ultra Sp-L may lack, the concentrations of these enzymes may not be sufficient enough to cause a significant impact on procyanidin released. Different temperatures were used in order to obtain the optimum enzymatic activity as recommended by the manufacturers.

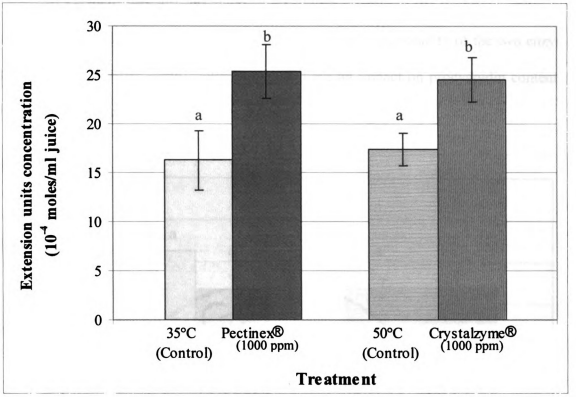

Figure 3.6.3 The effect of pectolytic enzyme treatments on the procyanidin concentration of extension units in Jonathan cider containing 10% apple solids (n = 6, p <0.001)

Samples treated with pectolytic enzymes increased concentrations of procyanidin extension end units (Figure 3.6.3). While there is no literature to explain the effect of pectolytic enzymatic treatments on the concentration of procyanidin extension units in apple cider, the increase is mostly likely due to the release of procyanidins due to the degradation of apple solids. The released procyanidins trap these compounds within their polysaccharide matrix as observed by Le Bourvellec and others (2004). In this observation, it is possible that the release of procyanidins is proportional to the measured concentration of procyanidin extension units in apple cider. Under this assumption, it may be further proposed that procyanidins do not undergo major changes in chain length under the conditions of this study.

Since there was also no significant difference between the pectolytic enzymatic systems, it was also concluded that the composition and functionality of the two enzyme systems did not differ significantly enough to have an impact on procyanidin content in Jonathan apple cider.

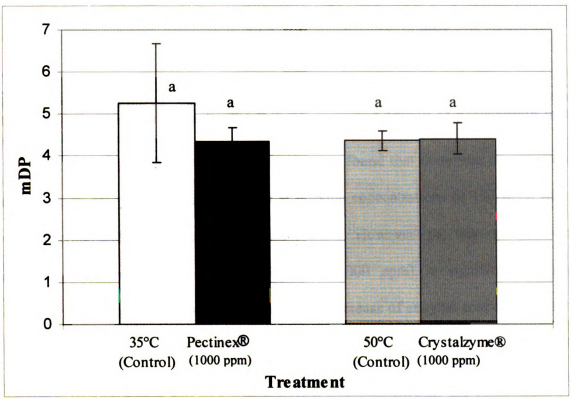

Figure 3.6.4 The effect of pectolytic enzyme treatments on the mean degree of polymerization of procyanidins in Jonathan cider containing 10% apple solids (n = 6, p <0.001)

Pectolytic enzyme treatments did not have a significant impact on the mDP values (Figure 3.6.4) and this observation further supports the proposed theory that the chain length of procyanidins was not affected by pectolytic enzymes. The behavior of procyanidins in this experiment is thought to be similar to the proposed scheme illustrated in Figure 3.6.5.

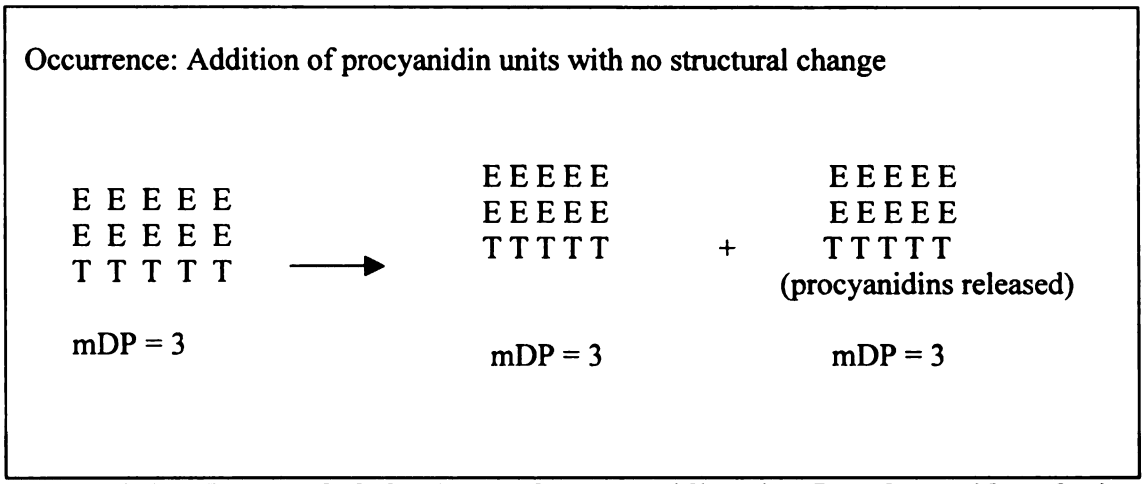

Figure 3.6.5 Proposed behaviour of procyanidins in Jonathan cider during treatment (T= terminal end units, E = Extension units): Addition of procyanidin units with no structural change

Preliminary studies with pectolytic enzymes found that there was no significant difference in procyanidin extension units at enzyme concentrations of 100 ppm, which were recommended by enzyme manufacturers. However in this study, when concentrations were increased to tenfold (i.e. 1000 ppm), a significantly higher concentration of procyanidins were obtained. The increase of enzyme concentration may have increased the enzymatic effect on the breakdown of apple solids, thus releasing more procyanidins into the cider.

Based on these observations, it was concluded that pectolytic enzyme treatments were not feasible for increasing procyanidin content in apple cider made from Jonathan apples and possibly other dessert apple varieties. The enzyme concentrations necessary to have a significant impact are higher than the typical industry dosages and enzymatic treatments, which utilized enzymes listed in Table 1.2 would not be economical.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1 Summary and conclusions

The original goal of the hard cider research grant was to produce a hard cider product that Michigan consumers would accept positively and provide an alternative option of convert excess supply of Michigan apple varieties into value-added products. When this research effort was initiated, it was conducted under the assumption that the average U.S. consumer desires bitter and astringency sensory characteristics found in traditional hard cider made in Europe. While the effort to increase procyanidin consumption in fermentation processing methods was carried out, extensive marketing research on hard cider including consumer taste panels were conducted at the same time.

As part of the effort to increase procyanidin content by investigating fermentation methods, it was found that the methodology consisting of HPLC followed by acid hydrolysis cleavage in the presence of phloroglucinol did not successfully estimate procyanidin in polyphenolic extracts obtained from cider samples in this research. While possible explanations of this outcome point towards problems encountered during the reaction treatment, these conclusions have yet to be confirmed due to time and facility constraints. Therefore, the analysis of procyanidins in hard cider made from Jonathan apples was achieved using classical colorimetric methods.

Alcohol production from yeast fermentations did not increase procyanidin concentrations and mean degree of polymerization (mDP). Yeast fermentation may not have produced sufficient alcohol (4%) to release procyanidins from apple cell wall materials in apple solids since Renard and others (2003) indicated a minimum 15%

alcohol was necessary to reduce the binding effect between procyanidins and apple solids.

Statistical analysis of measured procyanidin concentrations indicated that the percentage of apple solids did not have a major impact on the procyanidin concentrations and mean degree of polymerization. This observation was mostly likely due to the procyanidin-apple cell wall interactions, which formed quickly within cider upon contact (Renard and others 2003).

The duration of fermentation resulted in varying significant differences of procyanidin concentration between days in samples of 0% apple solids. It was found that mDP levels decreased after Day 10 and it was proposed that yeast fermentation breaks down the linkages within the procyanidin chain through unknown mechanisms. Free monomeric units were then quickly eliminated from the juice system by either absorption by cell wall material or oxidation.

When it was found that fermentation did not affect procyanidin content in hard cider, the study was extended to alternative methods such as heat and pectolytic treatments. Inconclusive data obtained from heat treatments indicated that procyanidin concentrations in dessert apple varieties may not contain significant amounts of procyanidins and did not influence mDP of procyanidins. Pectolytic enzyme treatments increased the procyanidins concentrations but did not influence the procyanidin mDP at enzyme concentrations of industry standard dosages. The usage of high enzyme concentrations is not considered feasible for the industry especially if the procyanidin increase is not substantially increased.

While the lack of success in increasing procyanidin content in hard cider made from dessert apple varieties did not provide scientific support for the original hypothesis of this study, the application of this information ultimately depends on how the U.S. consumer prefers their hard cider product. Michigan consumers may not necessarily desire a hard cider product that is as astringent or bitter as the traditional European product. Behe and others (unpublished) have found that consumer panelists rated hard cider made from Jonathan and McIntosh dessert varieties positively and acceptable overall.

Additionally, the concentrations of procyanidins in hard cider made from fresh Michigan dessert apples were comparably higher than commercially available imported hard cider brands.

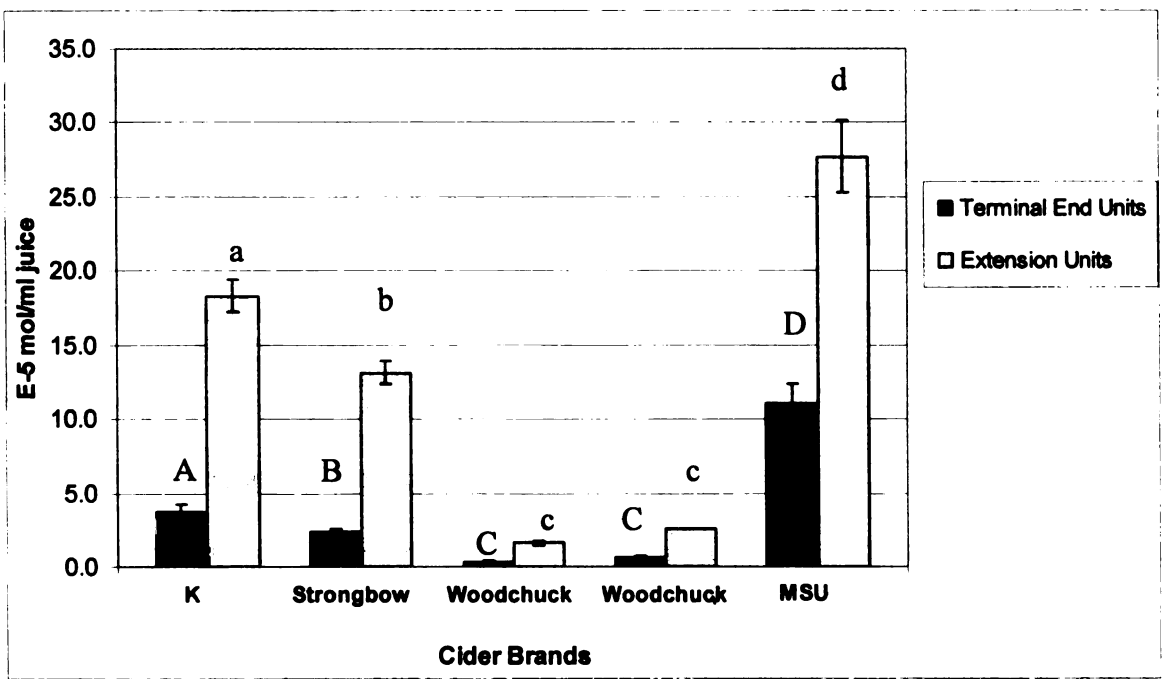

Figure 4.1 Estimated procyanidin concentrations of extension and terminal end units in K, Strongbow, Woodpecker, Woodchuck and MSU hard cider brands (n = 6, p >0.001)

In Figure 4.1, MSU cider (i.e. hard cider made from a blend of Jonathan: McIntosh, 1:1) recorded higher procyanidin readings than K, Strongbow, Woodchuck and Woodpecker. This observation may be due to the following explanation. While procyanidin concentrations may be higher in apple varieties used in Europe, these high concentrations may have been drastically reduced during the process of converting fresh apple juice into apple juice concentrate (AJC). This process mostly likely utilizes high temperatures and allows more oxidation to occur, thus reducing procyanidin concentrations. While AJC is commonly used in U.K. cidermaking, MSU cider utilized freshly pressed apple juice, which undergoes less processing and most likely retains more procyanidins.

As a conclusion, hard cider made from fresh Michigan dessert apple varieties has the potential of retaining satisfactory levels of procyanidins, even if the dessert apple varieties have lower levels of procyanidins than cider apple varieties within the fruit itself without the use of alternative 'mash' fermentation, heat and pectolytic enzymatic treatments. The findings of this research will provide useful information to any future investigations on the cider making process using dessert apples varieties.

4.2 Recommendations for future research

The following topics are recommended for future research:

1. Investigate the concentration of procyanidins for longer time durations (e.g. 3 months) during fermentation and storage to determine whether flavor development in hard cider maturity is due to increased procyanidin content.
2. Investigate the effect of extended heat treatments (higher temperatures, longer heating times) on the concentrations of procyanidins during cider fermentation

APPENDIX 1: Reagents

1) High performance liquid chromatography

Cider extract: 25 ml of cider samples was concentrated into 5 ml using Sep Pak C_{18} cartridges by eluting 5 ml of water and 5 ml of extract was recovered with 2 ml of methanol. 1 ml of extract was added to the reaction.

Grape seed extract: The sample preparation was adapted from Escribano-Bailón and others (2003) was prepared by homogenizing 5 g of grape seeds in 15 ml of methanol. The sample was centrifuged at 12000 rpm for 15 min. 4 ml of extract was dried and used in the reaction.

Phloroglucinol Solution: Dilute 1 ml of 10M HCl and 99 ml methanol. Dissolve 5 g phloroglucinol and 1 g ascorbic acid into 100 ml of the acidic solution.

2) Colorimetric Assays

Extraction solutions
1% HCl in methanol: Dilute 0.01 ml of 10 N HCl into 100 ml of methanol
1% HCl in H_20: Dilute 0.01 ml of 10 N HCl into 100 ml of H_2O

Vanillin in methanol assay
Dissolve 1 g of vanillin into 50 ml of methanol and 50 ml of 8% HCl in methanol
Add 0.5 ml of sample extract into 2.5 ml of solution.

Porter Reagents
i) 2% ferric ammonium sulphate in 2 N HCl in methanol
Dilute 20 ml of 10 M hydrochloric acid into 80 ml of methanol. Dissolve 2 g of ferric ammonium sulphate in 100 ml of the acidic solution

ii) 95:5, butanol: hydrochloric acid
Dilute 5 ml of 10 M hydrochloric acid into 95 ml of butanol.

APPENDIX 2: Raw data for fermentation tests

Table A.2.1 RI values of Jonathan apple cider fermentation: Batch 1

Treatment	% apple solids	Time (d)				
		0	5	10	15	20
Control	0	10.8	10.8	10.8	10.8	N/A
	0	10.8	11.0	10.8	10.8	N/A
	0	10.8	10.8	11.0	10.8	N/A
	10	10.8	10.8	11.0	N/A	N/A
	10	11.0	10.8	11.0	N/A	N/A
	10	10.8	11.0	10.6	N/A	N/A
	30	11.0	10.8	10.8	N/A	N/A
	30	10.8	10.8	10.8	N/A	N/A
	30	10.8	11.0	10.8	N/A	N/A
Yeast	0	10.8	9.6	8.4	5.2	N/A
	0	11.0	9.6	8.2	5.4	N/A
	0	11.0	9.6	8.2	5.2	N/A
	10	11.0	10.2	6.6	5.2	N/A
	10	10.8	10.2	6.6	5.2	N/A
	10	10.8	10.2	6.6	5.0	N/A
	30	11.0	9.0	5.2	4.8	N/A
	30	10.8	9.4	5.4	5.2	N/A
	30	10.8	9.2	5.2	5.0	N/A

Table A.2.2 RI values of Jonathan apple cider fermentation: Batch 2

Treatment	% apple solids	Time (d)				
		0	5	10	15	20
Control	0	10.8	10.8	10.8	N/A	N/A
	0	10.8	11.0	10.8	N/A	N/A
	0	10.8	10.8	10.6	N/A	N/A
	10	10.8	10.8	10.6	N/A	N/A
	10	10.8	10.8	10.8	N/A	N/A
	10	10.8	10.8	N/A	N/A	N/A
	30	11.0	11.0	N/A	N/A	N/A
	30	10.8	10.8	N/A	N/A	N/A
	30	11.2	10.8	N/A	N/A	N/A
Yeast	0	10.8	9.6	8.2	6.6	5.2
	0	10.8	9.6	8.4	6.8	5.4
	0	10.8	9.6	8.2	6.6	5.2
	10	10.8	10.2	9.2	8.2	6.0
	10	10.8	10.4	9.2	9.4	6.2
	10	10.8	10.2	9.0	7.8	5.8
	30	11.0	10.2	8.6	6.6	5.6
	30	10.8	10.4	8.4	7.8	5.2
	30	10.8	10.2	8.6	7.8	5.8

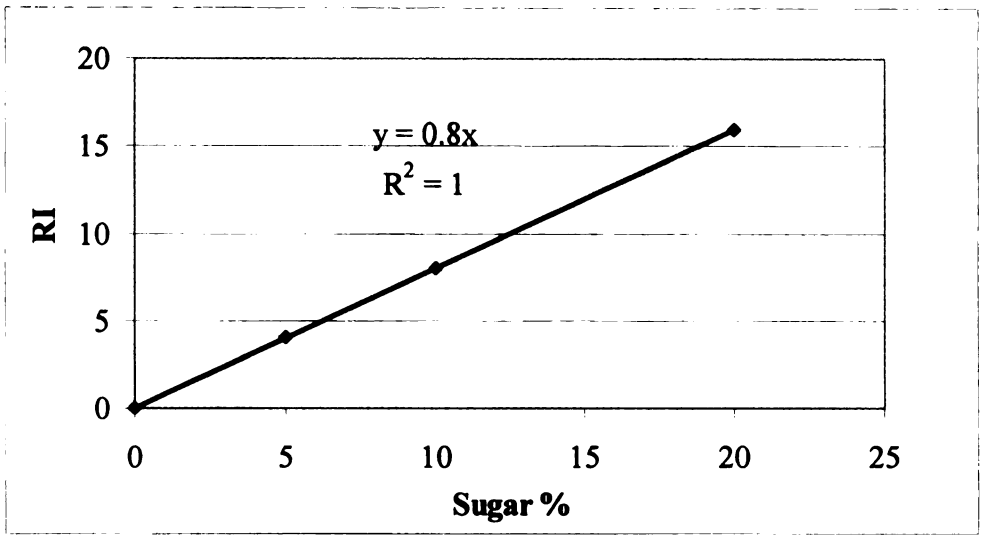

Figure A.2.1 Standard Curve: RI vs Sugar %

Table A.2.3 pH values of Jonathan apple cider fermentation: Batch 1

Treatment	% apple solids	Time (d)				
		0	5	10	15	20
Control	0	3.14	3.11	3.13	3.16	N/A
	0	3.15	3.10	3.15	3.17	N/A
	0	3.14	3.12	3.15	3.16	N/A
	10	3.11	3.12	3.16	N/A	N/A
	10	3.14	3.13	3.17	N/A	N/A
	10	3.11	3.09	3.15	N/A	N/A
	30	3.13	3.16	3.16	N/A	N/A
	30	3.13	3.16	3.17	N/A	N/A
	30	3.14	3.15	3.15	N/A	N/A
Yeast	0	3.14	3.08	3.15	3.20	N/A
	0	3.14	3.07	3.14	3.21	N/A
	0	3.14	3.07	3.17	3.23	N/A
	10	3.11	3.11	3.14	3.24	N/A
	10	3.11	3.10	3.14	3.22	N/A
	10	3.11	3.11	3.15	3.23	N/A
	30	3.14	3.11	3.14	3.24	N/A
	30	3.15	3.10	3.16	3.23	N/A
	30	3.15	3.08	3.17	3.26	N/A

Table A.2.4. pH values of Jonathan apple cider fermentation: Batch 2

Treatment	% apple solids	Day				
		0	5	10	15	20
Control	0	3.14	3.17	3.18	N/A	N/A
	0	3.14	3.15	3.19	N/A	N/A
	0	3.12	3.16	3.18	N/A	N/A
	10	3.17	3.19	3.19	N/A	N/A
	10	3.12	3.19	3.19	N/A	N/A
	10	3.15	3.19	N/A	N/A	N/A
	30	3.15	3.19	N/A	N/A	N/A
	30	3.15	3.20	N/A	N/A	N/A
	30	3.14	3.19	N/A	N/A	N/A
Yeast	0	3.11	3.16	3.18	3.18	3.21
	0	3.14	3.14	3.17	3.19	3.19
	0	3.15	3.14	3.19	3.18	3.19
	10	3.14	3.19	3.18	3.19	3.21
	10	3.15	3.19	3.19	3.17	3.21
	10	3.13	3.18	3.17	3.19	3.25
	30	3.17	3.19	3.21	3.18	3.26
	30	3.17	3.18	3.22	3.16	3.26
	30	3.17	3.19	3.22	3.19	3.24

Table A.2.5 Mean absorbance values of Jonathan apple cider fermentation using Vanillin in methanol assay

Batch	Treatment	% apple solids	Time (d)				
			0	5	10	15	20
1	Control	0	0.948 (3.4%)	0.855 (6.2%)	0.925 (6.5%)	1.051 (8.8%)	N/A
		10	0.824 (4.1%)	0.994 (5.6%)	1.085 (2.7%)	N/A	N/A
		30	0.173 (14.3%)	0.079 (6.3%)	0.088 (11.8%)	N/A	N/A
	Yeast	0	0.935 (2.1%)	0.837 (6.7%)	1.005 (7.7%)	1.118 (8.3%)	N/A
		10	0.826 (5.3%)	0.975 (10.4%)	0.819 (23.4%)	0.881 (4.3%)	N/A
		30	0.166 (34.2%)	0.145 (4.4%)	0.128 (14.1%)	0.113 (14.3%)	N/A
2	Control	0	0.569 (10.4%)	0.618 (6.2%)	0.639 (7.3%)	N/A	N/A
		10	0.329 (12.8%)	0.221 (9.2%)	0.180 (13.0%)	N/A	N/A
		30	0.072 (28.7%)	0.025 (9.9%)	N/A	N/A	N/A
	Yeast	0	0.624 (4.2%)	0.585 (6.5%)	0.544 (11.7%)	0.538 (5.3%)	0.518 (6.5%)
		10	0.343 (7.5%)	0.228 (3.8%)	0.184 (9.12%)	0.216 (9.8%)	0.169 (3.9%)
		30	0.053 (45.1%)	0.031 (10.4%)	0.029 (3.4%)	0.036 (4.3%)	0.024 (31.1%)

Mean based on three measurements. % CV given in parenthesis

Table A.2.6 Mean absorbance values of Jonathan apple cider fermentation using BuOH/HCl assay

Batch	Treatment	% apple solids	Time (d)				
			0	5	10	15	20
1	Control	0*	0.465 (6.7%)	0.754 (5.9%)	0.729 (11.2%)	0.578 (18.2%)	N/A
		10*	0.451 (6.4%)	0.719 (9.3%)	0.580 (4.6%)	N/A	N/A
		30	0.567 (13.4%)	0.298 (6.4%)	0.172 (13.4%)	N/A	N/A
	Yeast	0*	0.458 (2.4%)	0.702 (6.7%)	0.624 (7.1%)	0.573 (2.9%)	N/A
		10*	0.428 (4.4%)	0.670 (5.1%)	0.593 (15.8%)	0.480 (6.4%)	N/A
		30	0.511 (13.0%)	0.494 (4.6%)	0.246 (6.1%)	0.188 (13.2%)	N/A
2	Control	0*	0.741 (7.1%)	0.650 (2.4%)	0.456 (31.3%)	N/A	N/A
		10*	0.455 (8.8%)	0.333 (2.0%)	0.220 (8.0%)	N/A	N/A
		30	0.241 (30.8.%)	0.097 (11.3%)	N/A	N/A	N/A
	Yeast	0*	0.697 (7.6%)	0.652 (8.5%)	0.548 (3.33%)	0.603 (9.0%)	0.608 (7.2%)
		10*	0.471 (7.7%)	0.315 (2.3%)	0.271 (9.2%)	0.299 (5.1%)	0.252 (2.3%)
		30	0.195 (36.3%)	0.121 (8.2%)	0.105 (8.1%)	0.139 (2.9%)	0.088 (18.2%)

Mean based on three measurements. % CV given in parenthesis

*** Samples subjected to 1:1 dilutions prior to measurement**

APPENDIX 3: Raw data for heat and pectolytic enzymatic tests

Table A.3.1 Mean absorbance values of Jonathan apple cider heat treatments using Vanillin in methanol assay

Batch	Rep	Temperature (°C)			
		C	40	50	60
1	1	0.492	0.450	0.420	0.568
	2	0.469	0.486	0.431	0.461
	3	0.445	0.456	0.448	0.473
	4	0.482	0.540	0.415	0.484
	5	0.506	0.475	0.466	0.453
	6	0.506	0.486	0.471	0.443
	Mean	0.483 (4.8%)[a]	0.482 (6.6%)[a]	0.442 (5.3%)[b]	0.480 (9.4%)[ab]
2	1	0.529	0.519	0.497	0.435
	2	0.524	0.509	0.535	0.489
	3	0.462	0.503	0.525	0.505
	4	0.450	0.527	0.476	0.522
	5	0.448	0.481	0.485	0.471
	6	0.458	0.514	0.439	0.437
	Mean	0.478 (7.8%)[a]	0.508 (3.1%)[a]	0.492 (7.1%)[a]	0.476 (7.5%)[a]
3	1	0.671	0.651	0.633	0.755
	2	0.684	0.641	0.662	0.896
	3	0.717	0.704	0.759	0.713
	4	0.532	0.652	0.636	0.831
	5	0.725	0.76	0.687	0.664
	6	0.671	0.666	0.733	0.698
	Mean	0.667 (10.4%)[a]	0.679 (6.6%)[a]	0.685 (7.5%)[ab]	0.760 (11.6%)[b]

Mean based on six measurements. % CV given in parenthesis

Table A.3.2 Mean absorbance values of Jonathan apple cider heat treatments using BuOH/HCl assay

Batch	Rep	Temperature (°C)			
		C	40	50	60
1	1	0.836	0.837	0.736	1.104
	2	0.883	0.944	0.839	0.870
	3	0.842	0.960	0.804	0.913
	4	0.873	0.864	0.873	0.861
	5	0.916	0.954	0.884	0.866
	6	0.885	0.955	0.873	0.903
	Mean	0.872 (3.4%)[a]	0.919 (5.8%)[a]	0.834 (6.7%)[a]	0.919 (10.0%)[a]
2	1	0.929	0.896	0.874	0.828
	2	0.877	0.834	0.847	0.863
	3	0.903	0.834	0.858	0.778
	4	0.955	0.849	0.811	0.897
	5	0.846	0.855	0.866	0.874

	6	0.726	0.835	0.816	0.897
	Mean	0.872 (9.3%)[a]	0.860 (3.2%)[a]	0.845 (3.1%)[a]	0.856 (5.3%)[a]
3*	1	0.479	0.472	0.556	0.278
	2	0.609	0.545	0.578	0.289
	3	0.559	0.543	0.561	0.280
	4	0.546	0.621	0.542	0.271
	5	0.581	0.531	0.620	0.310
	6	0.584	0.586	0.603	0.301
	Mean	0.560 (8.0%)[ab]	0.549 (9.2%)[a]	0.576 (5.1%)[b]	0.586 (7.8%)[a]

Mean based on six measurements. % CV given in parenthesis
*** Samples subjected to 1:1 dilutions prior to measurement**

Table A.3.3 Mean absorbance values of Jonathan apple cider heat treatments using Vanillin in methanol assay: Preliminary

Batch	Rep	Treatment (°C)			
		C	40	50	60
1	1	0.088	0.133	0.095	0.222
	2	0.092	0.119	0.174	0.225
	3	0.092	0.179	0.119	0.231
	Mean	0.091 (2.6%)	0.144 (21.8%)	0.129 (31.3%)	0.226 (2.0%)
2	1	0.142	0.130	0.220	0.214
	2	0.143	0.141	0.196	0.254
	3	0.091	0.157	0.155	0.348
	Mean	0.125 (23.7%)	0.143 (9.5%)	0.190 (17.2%)	0.272 (25.3%)

Mean based on three measurements. % CV given in parenthesis

Table A.3.4 Mean absorbance values of Jonathan apple cider heat treatments using BuOH/HCl assay: Preliminary

Batch	Rep	Treatment (°C)			
		C	40	50	60
1	1	0.274	0.369	0.296	0.523
	2	0.286	0.344	0.510	0.560
	3	0.317	0.480	0.326	0.564
	Mean	0.292 (7.6%)	0.398 (18.2%)	0.377 (30.7%)	0.549 (4.1%)
2	1	0.394	0.388	0.581	0.865
	2	0.305	0.413	0.518	0.684
	3	0.421	0.399	0.435	0.613
	Mean	0.373 (16.2%)	0.400 (3.1%)	0.511(14.3%)	0.721 (18.0%)

Mean based on three measurements. % CV given in parenthesis

Table A.3.5 Mean absorbance values of Jonathan pectolytic enzyme treatments using Vanillin in methanol assay

Batch	Rep	Treatment			
		30°C	Pectinex®	50°C	Crystalzyme®
1	1	0.378	0.546	0.489	0.535
	2	0.416	0.553	0.483	0.531
	3	0.251	0.595	0.409	0.627
	Mean	0.348 (24.8%)	0.565 (4.6%)	0.460 (9.6%)	0.564 (9.6%)
2	1	0.235	0.678	0.408	0.737
	2	0.409	0.733	0.457	0.650
	3	0.348	0.737	0.368	0.579
	Mean	0.331 (26.6%)	0.716 (4.6%)	0.411 (10.8%)	0.655 (12.0%)

Mean based on three measurements. % CV given in parenthesis

Table A.3.6 Mean absorbance values of Jonathan pectolytic enzyme treatments using BuOH/HCl assay

Batch	Rep	Treatment			
		35°C*	Pectinex®*	50°C*	Crystalzyme®*
1	1	0.534	0.669	0.549	0.274
	2	0.536	0.636	0.531	0.265
	3	0.402	0.678	0.501	0.250
	Mean	0.490 (15.6%)	0.661 (3.3%)	0.527 (4.6%)	0.659 (3.5%)
2	1	0.525	0.783	0.458	0.229
	2	0.386	0.829	0.452	0.226
	3	0.353	0.665	0.43	0.215
	Mean	0.421(21.6%)	0.759 (11.1%)	0.419 (3.3%)	0.714 (12.1%)

Mean based on three measurements. % CV given in parenthesis
*** Samples subjected to 1:1 dilutions prior to measurement**

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