AN ASSESSMENT OF THE UTILITY OF SUBCRITICAL WATER TO RECOVER BIOACTIVE COMPOUNDS FROM CIDER LEES

by

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ABSTRACT

By-products from food and drink industries represent a natural source of bioactive compounds. Cider lees, in particular, is a by-product of cider production composed of spent yeast cells and residual cider. Cider apples are known to contain significant amounts of phenolics with well documented high antioxidant capacity therefore the overall goal of the research was to assess the potential of adding value to cider lees by recovering phenolics with application as antioxidants.

Sub and supercritical fluids have been widely studied as solvents with the potential to replace organic solvent for the extraction of bioactive compounds from biomass as they are "green" and environmentally benign but also have shown lower extraction times and higher selectivity. The objective of the present work was to assess the use of subcritical water to recover bioactive compounds, with antioxidant properties, from cider lees.

Subcritical water conditions were used to hydrolyse cider lees, and thereby solubilise phenolics in a batch mode, at a constant pressure of 100bar with temperatures ranging 100ºC to 250ºC with extraction times up to 60 minutes. The degrees of hydrolysis under subcritical water operating conditions were assessed and the extracts were analysed in terms of total phenolic content, phenolic composition and overall antioxidant.

Under subcritical water at 150ºC for 30 minutes, the highest solubilisation of cider lees was obtained, equal to 65.2±11.0 g/l. At higher temperatures, a decrease in solubility was observed, with concentration levels lower than the initial cider lees supernatant, indicating that under such conditions precipitation is favoured.

Cider lees had an initial total phenolic content (TPC) of 12.9±0.4 mg GAE/g Cider Lees DW which increased to a maximum of 38.1±0.5 mg GAE/g Cider Lees DW when subcritical water extraction was performed at 220ºC for 10 minutes. Analysis of the phenolic composition of the

extract revealed that chlorogenic acid was the major phenolic identified in cider lees with a concentration of 1.309±0.4 mg/g Cider Lees DW. However with the increasing temperature of subcritical water extraction the concentration of chlorogenic acid decreased to trace levels and same behaviour was mirrored by other phenolic compounds in the extracts. Therefore the results of the total phenolic assay could not be explained in terms of phenolic composition based on the HPLC conditions used, suggesting that the enhanced antioxidant capacity observed at elevated temperatures could not be directly apportioned to phenolic compounds. It was hypothesised that the under batch mode subcritical water conditions could promote interactions with other macromolecules or catalysis decomposition/transformation of natural phenolics there by generating new molecules.

To investigate the hypothesis that under batch conditions subcritical water generated a complex 'soup' of molecules that in turn provided the opportunity for interactions/reaction that generated extracts with enhanced antioxidant activity, a sequential subcritical water extraction from cider lees was performed from temperatures of 100ºC up to 220ºC, as a way of reducing the complexity of the extract and thereby selectively remove lipid, proteins soluble carbohydrates and phenolic thus enabling to begin to dissect out the factors contributing to the observed bioactivity. The highest TPC and antioxidant capacity was obtained at two different temperatures, 120ºC and 220ºC, with 98.0±9.6 mg GAE/g Extract DW and 90.7±7.2 mg GAE/g Extract DW, respectively, in contrast to 63.9±9.6 mg GAE/g Extract DW from extracts obtained by standard subcritical water extraction at 220ºC. Antioxidant capacity results from oxygen radical absorption capacity (ORAC) correlated with the results obtained from Folin Ciocalteu's method where extracts obtained at 120ºC with an antioxidant capacity of 2414.5±283.6 μmol Trolox/g Extract DW and extracts obtained at 220ºC with an antioxidant capacity of 2391.9±177.0 μmol Trolox/g Extract DW, while extracts obtained through standard

subcritical water extraction have shown an antioxidant capacity of 691.4±177.0 μmol Trolox/g Extract DW. Most natural phenolics from apple were extracted at temperatures below 170ºC, which show that while the antioxidant capacity of the extracts obtained at 120ºC is due to the natural phenolics from apple, the antioxidant capacity of the extracts obtained at 220ºC would appear to be of a different origin and given the sequential extraction would have removed all but yeast cell wall carbohydrates and chitin it is suggested that the hydrolysis of these polymers may result in derivatives that exhibit the antioxidant capacity.

The impact of encapsulating the extracts to enable the incorporation of the antioxidant activity into a skin care formulation was also assessed, using β-cyclodextrin. Interestingly the results showed that low temperature extracts, containing apple phenolics could be stabilised and protected from oxidation but also showed that the bioactive compounds in the extracts at higher temperatures does not form any inclusions, reinforcing the fact the bioactive molecules are of a different origin.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

3-pCoQA	3-*O*-*p*-coumaroylquinic acid
4-pCoQA	4-*O*-*p*-coumaroylquinic acid
4-CQA	4-*O*-caffeoylquinic acid
5-pCoQA	5-*O*-*p*-coumaroylquinic acid
5-HMF	5-(hydroxymethyl)-2-furaldehyde
AAPH	2,2'-azobis(2-methylpropionamidine) dihydrochloride
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AUC	Area under the curve
CQAE	Chlorogenic acid equivalent
CL	Cider lees
DMF	Dimethylformamide
DPPH	2,2-diphenyl-1-pierylhydrazyl
DW	Dry weight
ET	Electron transfer
FCR	Folin-Ciocalteu's reagent
FRAP	Ferric ion reducing antioxidant power
GAE	Gallic acid equivalent
HAT	Hydrogen atom transfer
HPLC	High performance liquid chromatography
LD50	Median lethal dosage
mAU	mili absorbance units
nd	not detected
ORAC	Oxygen radical absorbance capacity
p-CoAE	*p*-coumaric acid equivalent
PHLE	Phloridzin equivalent
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
R_t	Retention time
RW	Refrigeration water
SEM	Scanning electron microscope

SOD	Superoxide dismutase
SQ	Sequential
STD	Standard
SubCW	Subcritical water
TCA	2,2,2-trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity
TRAP	Total radical-trapping antioxidant parameter
TPC	Total phenolic content
Trolox	(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
v/v	volume/volume
w/w	weight/weight
β-CD	β-cyclodextrin
ε	Dielectric constant

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THESIS LAYOUT

The thesis is divided into six chapters. Chapter 1 provides literature review of the organic waste streams from the food and drink industries, with particular reference to the cider industry and the potential routes of adding value using sustainable green technology.

Chapter 2 includes the description and procedures for the main analytical methods used throughout the present work.

Chapter 3 provides an assessment of subjecting cider lees to subcritical water treatment and its impact on the overall phenolic content and antioxidant capacity of the bioactive compounds found in the cider lees extracts.

Chapter 4 provides an assessment of a sequential subcritical water treatment of cider lees with a view to reducing the extract complexity and therefore providing an opportunity to understand its impact on total phenolic and antioxidant capacity of bioactive compounds found in cider lees.

Chapter 5 illustrates the potential utility of β-cyclodextrin as a route to encapsulate the bioactive compounds found in cider lees extracts.

Lastly, the main conclusions of the present work and future recommendations are discussed in chapter 6.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The agri-food and drink industry in Europe produces annually an estimated 2.5×10^8 tonne of by-products, which can contain organic compounds of great nutritional interest as proteins, oils, sugars, vitamins, colorants and antioxidants (AWARENET, 2004). By-products which can represent a significant amount of the initial raw material as it can be seen in in Figure 1-1.

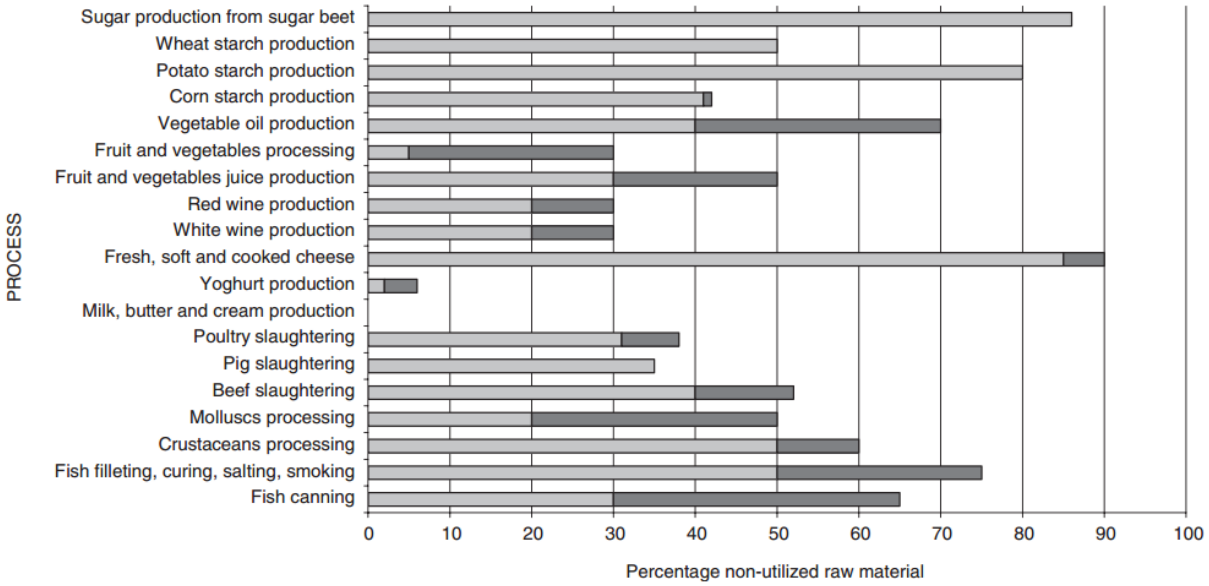


Figure 1-1 – Indication of the quantity of non-utilised raw material (light grey, minimum amount; dark grey, maximum amount) (AWARENET, 2004).

Historically, organic by-products have found application as low value feed and compost derived fertilizer, while a large proportion went to land fill (Lowe and Buckmaster, 1995). However, with increasing environmental legislation restricting the use of land fill management and disposal of large volumes of organic matter which is often wet and prone to microbial degradation is becoming a significant cost (A. Schieber et al., 2001b). Therefore the agri-food and drink sector is increasingly looking to find alternative routes of by-product disposal which at the same time can reduce cost and add overall value.

It is well established that biomass is comprised of a range of potential bioactive compounds and more basic chemical building blocks that can serve to support the production of platform chemical bioenergy and materials. In addition the transition to a bio-based economy is driving the legislative and consumer demand for "green" sustainable industrial feedstocks and ingredients. Therefore in the context of bio based products, there is increased interest from the pharmaceutical, cosmetic, chemical, material and biofuels sectors in exploiting the potential of agri-food by-products thereby adding value and at the same addressing waste management issues (Makris et al., 2007). However the challenge remains to develop suitable technology which enables full utilisation of the biomass creating multiple products.

1.2 Cider industry and its by-products

Fermented beverages such as wine, beer and cider have an annual production worldwide of more than 2 billion hectolitres (Karlsson, 2013, Statista, 2014) from which by-products and waste can be up to 30% of the initial raw material (Lowe and Buckmaster, 1995, Makris et al., 2007). In the UK fermented beverages account for 52 million hectolitres (Eurostat, 2012) where cider represents around 8.7 million hectolitres per year (Eurostat, 2012).

In the last 10-15 years cider has gained popularity in the UK, especially among younger generations, and as consequence the volume of cider sold in the UK has increased approximately 41% from 2001 to 2012 (Eurostat, 2001, 2012). The increase in cider consumption contrasts with beer consumption in the UK, which has decreased by around 24% during the same period of time, although beer still accounts for approximately 43.6 million hectolitres consumed in 2012 (Eurostat, 2001, 2012).

The United Kingdom is also the main cider producer in the world accounting for 40% of worldwide production (Eurostat, 2012, McKay et al., 2011). Bulmers, a subsidiary of Heineken, is the largest UK producer with a production capacity of 3 million hectolitres of cider annually (NACM, 2010b). The growth of the worldwide cider market and with UK as the main producer and consumer has increased the interest to add value to the by-products from this industrial sector.

1.2.1 Origins of cider production

Alcoholic beverages have been produced and consumed since ancient times where wine and beer are likely to have been the first fermented products produced. It has been established that wine making had its origins in Persepolis, present-day Iran, around 5400-5000BC (Hudson and Buglass, 2011), however it is believed that while beer may have the same origin it may pre-date wine (Hudson and Buglass, 2011). Cider was first documented in Greek literature, around 900BC, although there is circumstantial evidence which would suggest cider production evolved at the same time as wine and beer (McKay et al., 2011).

Cider was introduced to the United Kingdom after the Norman conquests in the 11th century, although apple orchards already existed in the United Kingdom as there were introduced by the Romans (Hudson and Buglass, 2011). Until the industrial revolution (1760) brewing expertise

laid within monastic community although by this time large brewing companies were starting to prosper (Hudson and Buglass, 2011).

The fermentation as we know it today was first described in 1857 by Pasteur who demonstrated that fermentation was a result of the metabolic activity of yeast which up until this point was believed to be a dead organism (Hudson and Buglass, 2011). Pasteur also identified different types of fermentation (alcoholic, lactic, acetic, butyric, etc.) that were causing problems in wine industry due the contamination of foreign organisms. The development of pasteurisation technology allowed brewing companies to start to employ chemists to control and optimise their brewing processes (Hudson and Buglass, 2011).

1.2.2 Cider production process

The total cider production in the UK is distributed between the following large producers, Bulmers (owned by Heineken), Magners and Gaymers from C&C Group, Sommersby (owned by Carlsberg) and Thatchers, and an increasing number of microbreweries. Cider fermentation is made from apple juice which can be fresh or diluted from apple juice concentrate. As large companies produce cider all year round, they mainly use apple juice concentrate while small producers use primarily fresh apple juice.

Table 1-1 – Classification of Cider Apples (McKay et al., 2011).

	Acid %(w/v)	Tannin %(w/v)
Sharp	>0.45	<0.2
Bittersharp	>0.45	>0.2
Bittersweet	<0.45	>0.2
Sweet	<0.45	<0.2

The UK has a long history in developing cider apple varieties which can be divided in four different classes: sharp, bittersharp, bittersweet and sweet (Table 1-1). The latter is often supplemented with imports from China (Nogueira and Wosiacki, 2012).

Therefore in the UK the industrial overall process to produce cider can be summarised in two steps, 1) apple juice concentrate production followed by 2) apple juice/concentrate fermentation.

1.2.2.1 Apple juice concentration

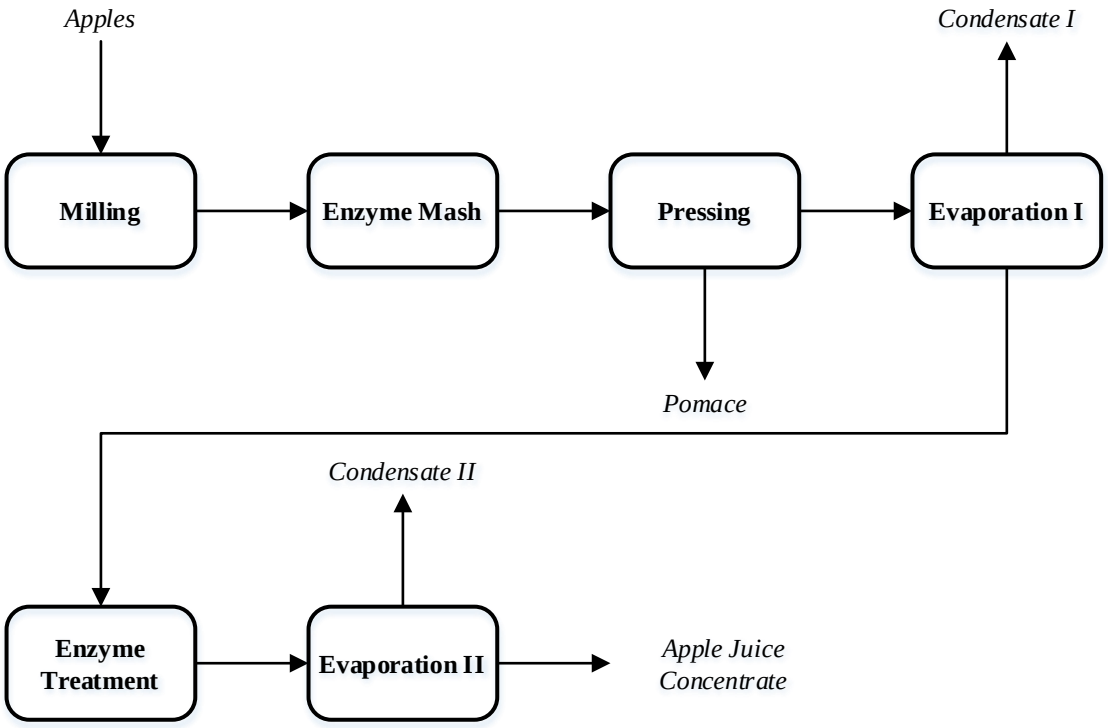


Figure 1-2 – Flow diagram of main stages in apple juice concentrate production process.

The apple juice concentrate process is illustrated in Figure 1-2. The apple juice concentrate process starts with the reception of apples, where they are washed and sorted to remove impurities such as leaves, bacteria, or chemical residues, before being crushed and milled to a fine pulp.

Traditional apple juice extraction processes suffer from low yields due to the presence of pectin in the cell wall which is bound to the apple's cellulose fibres. Pectin is a complex colloidal acid polysaccharide that is insoluble in water (Kashyap et al., 2001). Also, the bond between pectin and cellulose fibres improves cell wall water retention, consequently apple juice extraction is more difficult as it is retained alongside with water in the cellulose fibres (Kashyap et al., 2001).

The presence of pectin in apple juice increases its viscosity and it gives a particular cloudiness to it, as a consequence the apple juice has a degree of turbidity that requires filtration, concentration and clarification (Kashyap et al., 2001, Ribeiro et al., 2010). Apple juice extraction yields can be improved by treatment with pectinase an enzyme that degrades pectin molecules. Pectin molecular degradation decreases the capacity of apple pulp to retain water therefore higher yields of apple juice are obtained. In addition enzymes such as cellulase and xylanase are also used to assist the efficient extraction of apple juice (Ribeiro et al., 2010).

Modern large-scale production of apple juice consists of two stages, the juice extraction and the pomace discharge. Several presses are used in parallel to ensure a continuous process.

The moment apples start to be processed, enzymes are released into solution and their presence can result in the formation of atypical components, mostly volatiles that might have a negative effect in the final flavour. Apple juice is concentrated to a certain degree in an evaporation stage to remove these initial volatile components (Philip, 2005).

Once concentrated the juice obtained is a cloudy solution which contains suspended particles. To improve juice quality a second enzyme treatment, with the same enzymes used in the first enzyme treatment, is used, where long and complex molecules are decomposed to smaller molecules. Consequently viscosity decreases and suspended particles precipitate. The resultant apple juice is subjected to filtration to remove precipitated particles (Kashyap et al., 2001, Ribeiro et al., 2010).

At the final stage of the process, it is further concentrated with the objective of reducing costs in storage and transportation. Also, by concentrating apple juice, its resistance to microbial and chemical deterioration increases (Onsekizoglu et al., 2010).

1.2.2.2 Apple juice fermentation

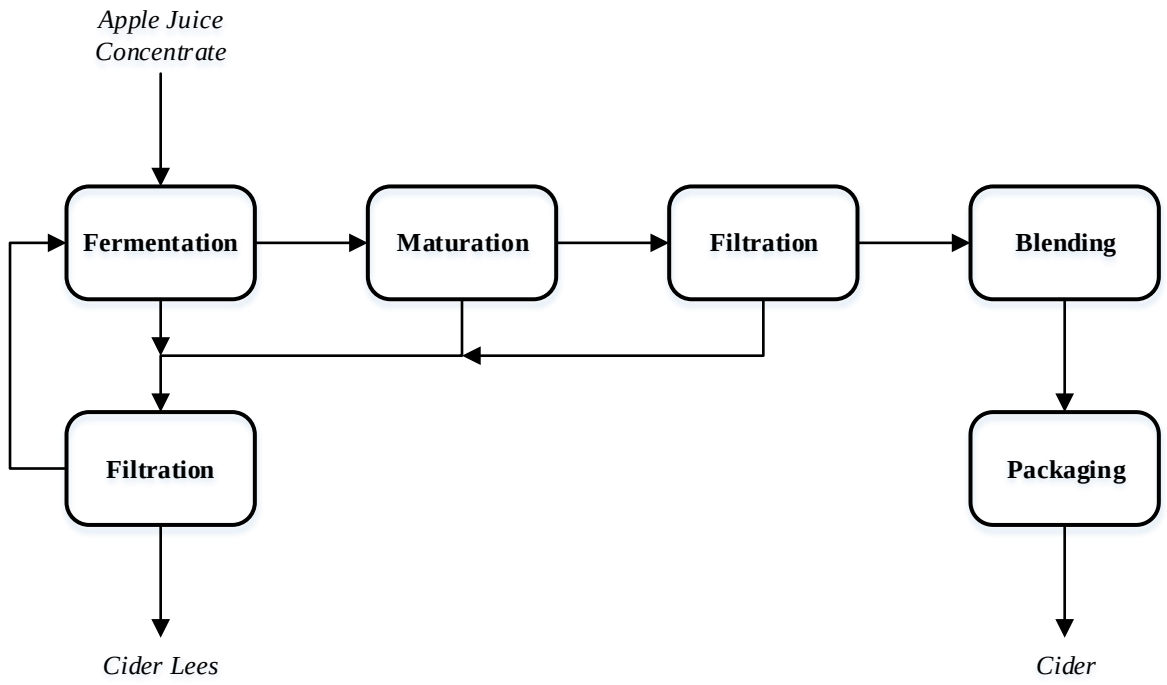


Figure 1-3 – Flow diagram of cider production process.

Cider production process is summarised in Figure 1-3 and consists of two fermentation stages followed by filtration and blending. When apple juice concentrate is used, it is important the apple juice concentrate is diluted (Nogueira and Wosiacki, 2012).

Cider fermentation is divided into two anaerobic fermentation stages: 1) alcoholic fermentation and 2) malolactic fermentation. The yeast used for cider fermentation is the same as that used for wine production where Saccharomyces cerevisiae is the most common strain used but unlike beer, where yeasts are re-used, new yeast cultures are used for every new fermentation batch (Nogueira and Wosiacki, 2012).

In alcoholic fermentation (commonly designated as fermentation) apple sugars are converted into ethanol and carbon dioxide in the presence of yeasts. Temperature and alcohol content are main variables controlled during fermentation. When fermentation solution reaches the desired properties, fermentation is stopped by controlled cooling. Yeasts and some by-products will be

precipitated and they are removed from solution (Durieux et al., 2005). Cider obtained by alcoholic fermentation has a high level of acidity due to the consumption of the sugars and the characteristics of cider apples.

The malolactic fermentation (commonly designated as maturation) is the transformation of malic acid to lactic acid in the presence of lactic acid bacteria, which reduces the overall acidity (Herrero et al., 1999b). Also, the by-products of this conversion will improve the final quality of the product, with respect to aromas and taste (Cabranes et al., 1998, Herrero et al., 1999a). As in the fermentation stage, yeasts and some by-products precipitate in the bottom of the maturation vat and are subsequently removed.

After maturation, the cider produced is filtered and the residue stream is collected and combined with the residue streams from fermentation and maturation. The combined residues are filtered and the resultant supernatant and slurry solution are separated. The supernatant solution is recycled to the fermentation stage while the slurry solution, referred as cider lees, is collected and removed from the process as a by-product.

Cider can be made from one type of apple or from a mixture of different types of apples. Apples are classified into four different types: sweet, bittersweet, sharp and bittersharp (Table 1-1), and cider apples fall in the bittersweet type. Apples used for cider production are classified by their acidity and tannin content (flavonoids) which are responsible for cider final flavour. After maturation it is common to blend ciders from different types of apples to obtain a characteristic final flavour (NACM, 2010a).

The last stage of cider production before packaging is blending. Blending has the objective of correcting cider acidity, sweetness and colour. Acidity is adjusted with pasteurised sharp apple juice, citric acid or malic acid, sweetness is adjusted with sugar syrup or artificial sweeteners

and colour is adjusted by adding caramel (Nogueira and Wosiacki, 2012). The cider is filtered again and finally it is carbonated before being packaged.

Apple pomace (Figure 1-2) and cider lees (Figure 1-3) are the main by-products from cider manufacture and the cider lees is used predominantly in animal feed, otherwise it has to be disposed of with additional costs (Chae et al., 2001, Ferreira et al., 2010). Alternative routes to the use of these by-products are necessary, as its disposal has legal restriction in addition to the additional costs. Its use as animal feed depends on its demand which vary differently from cider demand (Lowe and Buckmaster, 1995).

Apple pomace is the solid residue resulting from extraction of the apple juice, and has been extensively studied with a view to adding value, i.e. pectin recovery, citric acid production, ethanol production, enzyme production and polyphenol recovery (H. Wijngaard and Brunton, 2009a). Cider lees are the bottom residue from the fermentation stages and it is composed by yeast cells and residual cider. Cider apples and cider are recognised for their high phenolic content (Rosa M. Alonso-Salces et al., 2004b, Guyot et al., 1998, Marks et al., 2007a, Marks et al., 2007b, Sanoner et al., 1999), and similar high phenolic content in cider apple pomace has been confirmed by Diñeiro García et al. (2009) and H. Wijngaard and Brunton (2009a).

1.3 Cider lees and phenolics adsorption

Cider lees, as mentioned, is a slurry composed of residual cider and spent yeast (~10% solids), which is collected from the fermentation, maturation and filtration stages of cider production (Figure 1-3). As opposed to apple pomace, cider lees have not been widely studied.

The work of Bahari (2010) demonstrated for the first time that phenolics are adsorbed by yeast cells wall during fermentation, as it has previously been demonstrated to occur during wine fermentation (Mazauric and Salmon, 2006, Morata et al., 2003, Razmkhab et al., 2002). The

adsorption of phenolics by yeast cells makes cider lees interesting from point of view of added value through compounds recovery, as phenolic compounds are known for their antioxidant capacity (Manach et al., 2005).

1.3.1 Yeast cells composition

The yeast strain commonly used in the cider fermentation is *Saccharomyces cerevisiae* (Santiago and Mori, 1993). Yeast cells can be divided into intracellular compounds and cell wall. Among intracellular yeast cell compounds, there are two types of antioxidants: enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD), cytosolic SOD, catalase, glutathione reductase and cytochrome *c* peroxidase provide the enzymatic mediated antioxidant activity. While the non-enzymatic antioxidants include glutathione ubiquinone, hydroquinone, sulfhydryl amino acids and mineral ions (Mn, Zn) (Santiago and Mori, 1993).

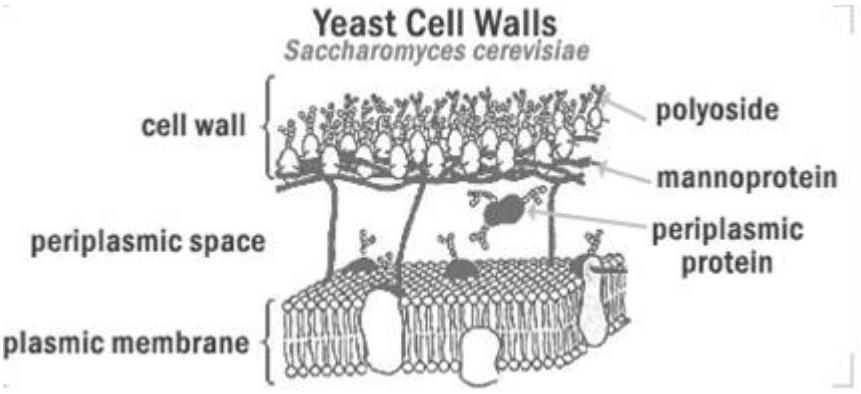


Figure 1-4 – Diagram of yeast cell wall structure.

The yeast cell wall is a complex of macromolecules which envelopes the cell protoplast, protecting against degradation (Fleet, 1991). The cell wall represents 26%-32% of cell dry weight (Fleet, 1991), and is composed of 15-30% β-glucan, 25-30% mannan, 5-15% proteins, 2-14% lipids and 1-2% chitin (Bacon et al., 1969, Beran et al., 1972, Cabib and Bowers, 1971,

Fleet, 1991, Fleet and Manners, 1976). The amount of cell wall and its composition varies according to strains and growth conditions.

β-Glucan is a branched polysaccharide where β(1→6)-linked D-glucose residues form a back bone to which linear chains of β(1→3)-linked D-glucose residues are attached (Manners and Masson, 1969). β-glucan can be further sub categorised according to solubility: 1 – alkali--insoluble acetic acid-insoluble β(1→3)-linked glucan (~60%); 2 – β(1→6)-linked glucan (~10%); 3 – alkali soluble β(1→3)-linked glucan (~30%) (Fleet and Manners, 1976).

Cell wall mannan is covalently linked to proteins. Four different classes of mannan have been identified: 1 – intracellular mannoproteins (enzymes); 2 – structural mannoproteins; 3 – mannoproteins involved in cell-mating reactions; 4 – extracellular enzymatic activities (Fleet and Manners, 1976). Protein content of mannoproteins is around 5 to 10% and mannoproteins also contain 0.1 to 1% of phosphorus (Fleet, 1991). Yeast cell wall mannan structure was proposed by Peat et al. (1961) and it is composed of α-(1→6)-linked mannose as a backbone connected to side chains of α-(1→2)-linked mannose and to a lesser extent of α-(1→3)-linked mannose. Mannan has been identified as the main antigenic compound in yeast cells and this feature is attributed to α-(1→3)-linked mannose side chains are the main responsible (Fleet, 1991). Mannan residues are attached to proteins through *O*-glycosyl bonds with hydroxyl groups of serine, threonine and N-acetylglucosamine.

Chitin is a linear polysaccharide β(1→4)-linked N-acetylglucosamine. Chitin is mainly found in bud scars zones after new cells are detached. It is believed that chitin are involved in cell division and it might provide a supportive channel between both cells (Fleet, 1991).

1.3.2 Yeast cell wall phenolics adsorption

During wine ageing techniques where spent wine yeast lees are kept with the wine, it has been verified that wine phenolics are rapidly adsorbed by the yeast lees, where condensed tannins are preferentially adsorbed rather than hydroxycinnamic acids (Mazauric and Salmon, 2005).

The capacity of the yeast lees, more specifically yeast cells, to retain or adsorb different molecules present in wine is defined by the different polarity, hydrophilic and hydrophobic nature of yeast cell wall polymers (Morata et al., 2003).

For the specific case of phenolics, particularly condensed tannins or procyanidins, its adsorption by yeast cell wall is believed to be through weak energy bonds (hydrogen) and hydrophobic interaction (Le Bourvellec et al., 2004) which leads to the formation of non-covalent complexes between phenolics and cell wall components. Polysaccharides present in the cell wall can form hydrophobic cavities that are able to encapsulate phenolics. All evidence suggests that it is these hydrophobic cavities that are the main cause of phenolics adsorption (Le Bourvellec et al., 2005). Phenolics molecular weight and conformational flexibility are the factors which determine the degree of interaction with cell wall compounds (Renard et al., 2001).

1.4 Phenolics added value: antioxidant activity

Phenolic compounds are not only known for their immense variety and wide distribution in fruit and vegetables but also for their antioxidant properties (Prior and Cao, 2000). The interest in phenolic compounds has been increasing in recent years, as it has been suggested they could have an important role in the prevention of degenerative diseases (Manach et al., 2005).

Degenerative diseases are linked to the formation of reactive oxygen species (ROS) which are responsible for oxidative stress in human cells (Carocho and Ferreira, 2013, Wu and Cederbaum, 2003). Oxidative stress is characterised by an excessive production ROS in human cells, which

are highly reactive, and can react with cellular macromolecules as proteins, lipids, saccharides and DNA causing damage on the cells and that can lead to the death of the cell (Wu and Cederbaum, 2003). These interactions can lead to chain reactions where free radicals of the target molecule are created which then will continue oxidative damage to other molecules.

Reactive oxygen species such as superoxide anion ($O_2^{\bullet-}$) or hydrogen peroxide (H_2O_2) are produced during the normal metabolic reaction of cells (Mittler, 2002) (Benzie and Strain, 1996), however external factors can enhance the formation ROS. Air pollutants, tobacco smoke, UV-light radiation, ozone, pesticides or industrial solvents are among the external factors that can contribute to an increase of ROS production in cells (Carocho and Ferreira, 2013).

Cells have an endogenous defence system that enables the control of ROS concentration. This system is composed of enzymatic and non-enzymatic antioxidants (Carocho and Ferreira, 2013). Antioxidants are substances that can delay, prevent or remove oxidative damage to a target molecule, after which they have the ability to form stable radicals (Carocho and Ferreira, 2013, Wu and Cederbaum, 2003). Also, antioxidants do not necessarily need to react with free radicals, as they can act indirectly by regenerating other antioxidant substances.

As mentioned before, in cells there are two types of antioxidants, enzymatic and non-enzymatic and the enzymatic antioxidants can be primary or secondary. Among the primary enzymatic antioxidants there are glutathione peroxidase, catalase, which is responsible for the removal of hydrogen peroxide, and superoxide dismutase, which is responsible for the removal of superoxide anion (Wu and Cederbaum, 2003). Secondary enzymatic antioxidants are responsible for the regeneration of the primary enzymatic antioxidants. As an example, glutathione reductase is responsible to regenerate glutathione peroxidise from its oxidised state (Carocho and Ferreira, 2013). Non-enzymatic antioxidants that can be found in cells are vitamin A (retinol), coenzyme Q10, uric acid and glutathione (Carocho and Ferreira, 2013).

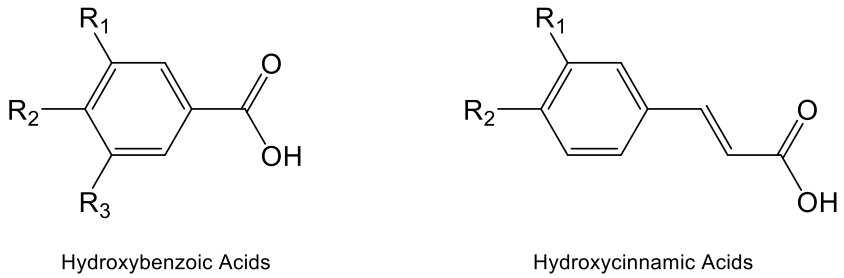
Despite the efficiency of the cell endogenous defence system, it is not enough and the cell depends on more antioxidants that are absorbed through human diet. Vitamin C, E and K, flavonoids, phenolic acids, carotenoids and minerals are the most important antioxidants that are present in the human diet and their intake is extremely important to reduce the oxidative stress in human cells (Carocho and Ferreira, 2013). Phenolic acids and flavonoids in particular can act as metal chelators and as free radical scavengers, reducing agents, hydrogen donators and as singlet oxygen quenchers (Carocho and Ferreira, 2013).

1.5 Phenolic compounds classification

Phenolic compounds are defined as a molecule composed by one or more aromatic rings with one or more attached hydroxyl groups (Crozier et al., 2006, Manach et al., 2004). Phenolic compounds can be of low molecular weight with one aromatic ring but it can also be large and complex tannins and derived polyphenols (Crozier et al., 2006). Normally, phenolics are not found in free state but in combined forms, for example as in glycosides or as esters with quinic acid (Fleuriet and Macheix, 2003). Phenolics are classified according to the number of aromatic rings and the structural groups attached to them. Phenolics can be divided into four main classes according to their chemical structures: phenolic acids, flavonoids, stilbenes and lignans (Manach et al., 2004).

1.5.1.1 Phenolic acids

Phenolic acids form two sub-classes: hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids are characterised by a C_6-C_1 structure while hydroxycinnamic acids are characterised by a C_6-C_3 structure (Manach et al., 2004) (see Figure 1-5).



Hydroxybenzoic Acids Hydroxycinnamic Acids

Figure 1-5 – Molecule structures of different phenolic acids (Manach et al., 2004).

1.5.1.1.1 Hydroxybenzoic acids

Hydroxybenzoic acid are present in fruits and vegetables most commonly in the *O*-glycosides form (see Figure 1-6), although it can be found in a free form as well (e.g. blackberries and strawberries). Hydroxybenzoic acid in glycoside form is normally classified as hydrolysable tannins. Tannins are phenolics that have the ability to bind to and precipitate alkaloids and proteins (Santos-Buelga and Scalbert, 2000). Tannins are classified between hydrolysable, condensed tannins and phlorotannins. Condensed tannins are polymers of flavanols, a class of flavonoids (see section 1.5.1.2.5). Phlorotannins are the less common type of tannins and it is a result of phloroglucinol polymerisation (Waterman and Mole, 1994).

Hydrolysable tannins are characteristic to decompose into hydroxybenzoic acid monomers when treated with a weak acid (Crozier et al., 2006). Hydrolysable tannins are found in onion peel or horseradish leaf (Fleuriet and Macheix, 2003). Gallic acid is the most common hydroxybenzoic acid present in plants, and for example is found in tea leaves (Manach et al., 2004).

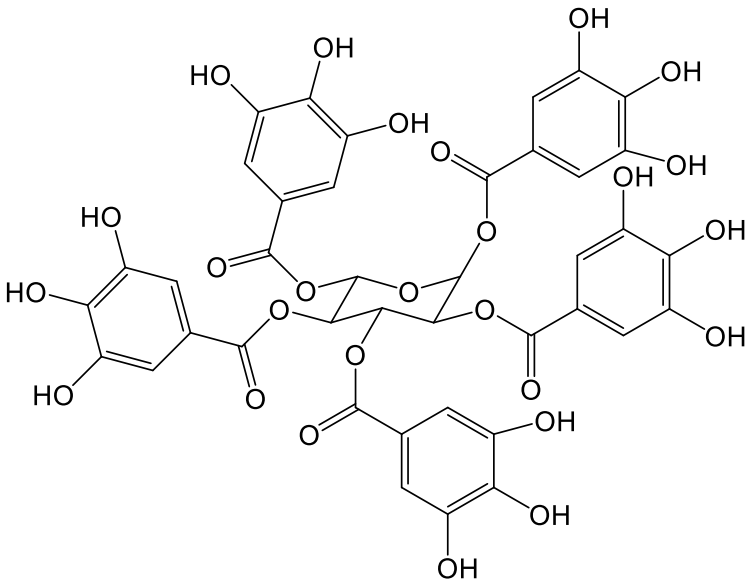


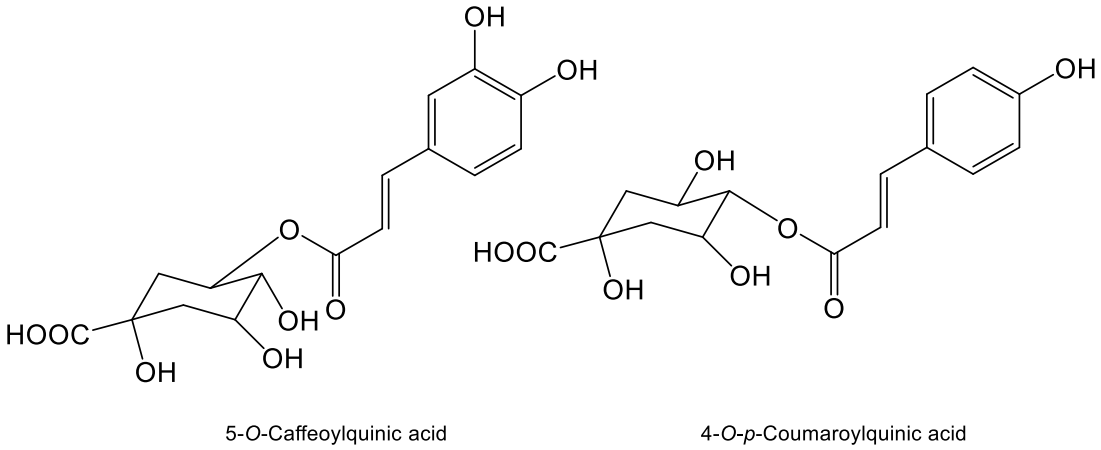
Figure 1-6 – Hydroxybenzoic acid in a *O*-glycoside form, pentagalloyglucose (Waterman and Mole, 1994).

In addition to the three types of tannins mentioned above there is another type of worth mentioning that is referred as tannin-like (condensed and hydrolysed) compounds, which share tannin common structural features such as high molecular weight and similar number of phenolic rings per mass unit. In food processing, the presence of enzymes promotes polyphenol oxidation, from which tannin-like compounds can be formed. Products such as wine and tea are rich in tannin-like compounds. Therefore, derived food products will have tendency to have higher concentration of this type of polyphenols, as it is observed in wine and tea (Santos-Buelga and Scalbert, 2000).

1.5.1.1.2 Hydroxycinnamic acids

Hydroxycinnamic acids are found in high concentrations in a wide range of fruit and vegetables. Hydroxycinnamic acids are normally found as a combined form of one of four different molecules: coumaric, caffeic, ferulic and sinapic acids (Fleuriet and Macheix, 2003). Hydroxycinnamic acids are rarely in a free form instead hydroxycinnamic acids are found as glycosylated derivatives or esters of quinic acid, shikimic acid or tartaric acid (Manach et al.,

2004). Chlorogenic acid (5-*O*-caffeoylquinic acid) and 4-*O*-*p*-coumaroylquinic acid (see Figure 1-7) were the first quinic esters identified that were found in apples and they are a combination of caffeic acid and *p*-coumaric acid with quinic acid, respectively (Fleuriet and Macheix, 2003). Chlorogenic Acid and *p*-coumaroylquinic acid was later found in high concentrations in several other types of fruit. Hydroxycinnamic acids are found in high concentrations in fruits such as blueberries, kiwis, plums, cherries and apples. Caffeic acid is the most abundant hydroxycinnamic acid (Manach et al., 2004).



5-*O*-Caffeoylquinic acid 4-*O*-*p*-Coumaroylquinic acid

Figure 1-7 – Molecular structures of two phenolic quinic esters found in cider apples (Marks et al., 2007a).

1.5.1.2 Flavonoids

Flavonoids are the largest group of phenolic compounds found in plants. Due to its structural diversity from hydroxylation, methoxylation, glycosylation and acylation reactions more than 8000 flavonoid compounds have been identified so far (Pietta et al., 2003). Flavonoids basic molecular structure is a diphenylpropane, C_6-C_3-C_6, that consists in two aromatic rings connected through three carbons that form an oxygenated heterocycle (Prior and Cao, 2000, Shahidi and Naczk, 2004). Flavonoids are found in high concentrations in the epidermis of leaves and in fruits skin. Flavonoids are responsible for UV protection, pigmentation, stimulation of nitrogen-fixing nodules and disease resistance(Crozier et al., 2006). Flavonoids

can be divided into six sub-classes: flavonols, flavones, isoflavones, anthocyanidins, flavanones and, flavanols (Figure 1-8).

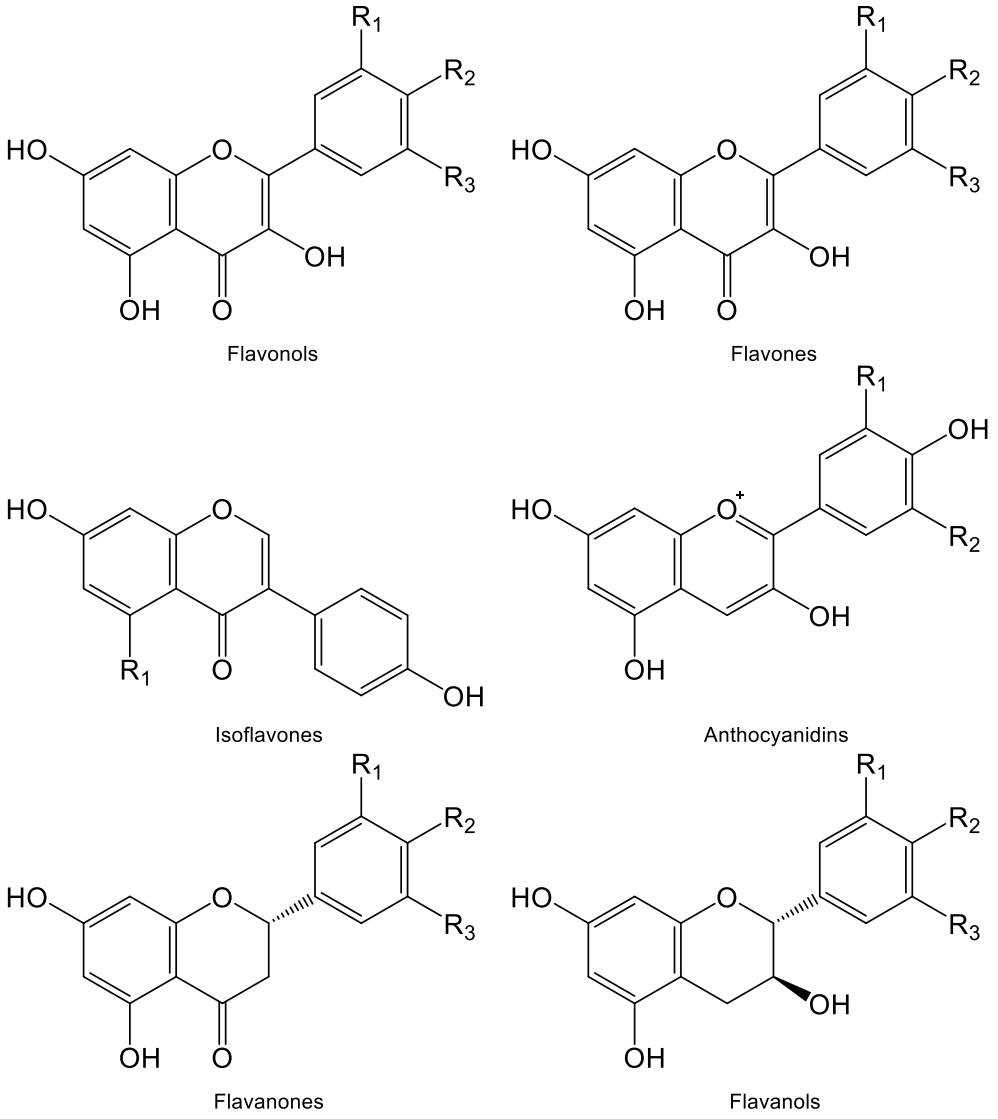


Figure 1-8 – Molecular Structures of sub-classes within the flavanoids (Manach et al., 2004).

1.5.1.2.1 Flavonols and flavones

Flavonols are the dominate sub-class of flavonoids found in the plant kingdom where quercetin, myricetin, isorhamnetin and kaempferol are the main known flavonols found (Crozier et al., 2006). Flavonols most commonly occur as *O*-glycosides (as seen in Figure 1-6 for phenolic acids) and are present in high concentration in onions, curly kale, leeks, broccoli and blueberries, especially in its skin and leaves (Manach et al., 2004). Although flavones are structurally similar

to flavonols, they are not as widespread as the latter. Flavones also occur as *O*-glycosides but are only found in significant concentrations in celery and parsley (Crozier et al., 2006). However, in citrus skin, flavones are present as polymethoxylated forms (Manach et al., 2004).

1.5.1.2.2 Isoflavones

Isoflavones are classified as phytoestrogen as they have several features in common with the mammalian oestrogen (oestradiol-17β). Isoflavones are the only sub-class of flavonoid where the structure is different from the standard flavonoid structure (C_6-C_3-C_6). Instead of three carbons between the two aromatic rings, isoflavones have only two carbons (C_6-C_2-C_6). Although isoflavones are present in several leguminous plants, soya and its derived products are a primary source of isoflavones in human diet (Manach et al., 2004) and they can be found either as aglycone or glucoside structure (Cassidy et al., 2000). Populations that have a diet rich in soya and soya products have shown low incidences of cardiovascular disease, osteoporosis, menopausal symptoms, breast and prostate cancer. The positive effects of soya and soya products have been linked to flavonoids although its precise contribution mechanism remains to be determined (Cassidy et al., 2000).

1.5.1.2.3 Anthocyanidins

Anthocyanidins are widely distributed in fruit and flower tissue and they normally occur as glycosides, also known as anthocyanin (Crozier et al., 2006). Anthocyanindins are most commonly found in fruit, especially red grapes, blackcurrants, blackberries, cherries and strawberries (Manach et al., 2004). Anthocyanidins are water-soluble pigments responsible for red, blue and purple colours present in fruit (Shahidi and Naczk, 2004). In plants, anthocyanins are responsible for UV protection and they have also an important role in attracting pollinating insects (Crozier et al., 2006). When extracted anthocyanidins are considered unstable as it is

observed that when foods containing anthocyanidins are processed or cooked they will produced yellowish or brownish pigments which are largely uncharacterised (Michael N. Clifford, 2000).

1.5.1.2.4 Flavanones

Flavanones are similar to flavanols in that they are the only two classes of flavonoids with a chiral centre in the carbon that links the oxygenated heterocycle to the aromatic ring (Crozier et al., 2006).

Flavanones are often classified as minor flavonoids due to their restricted occurrence in the plant kingdom. Chalcones and dihydrochalcones are also classified as minor flavonoids and they are most commonly included in the flavanones as chalcones present in plants are converted into flavanones in acidic media (Veitch and Grayer, 2006).

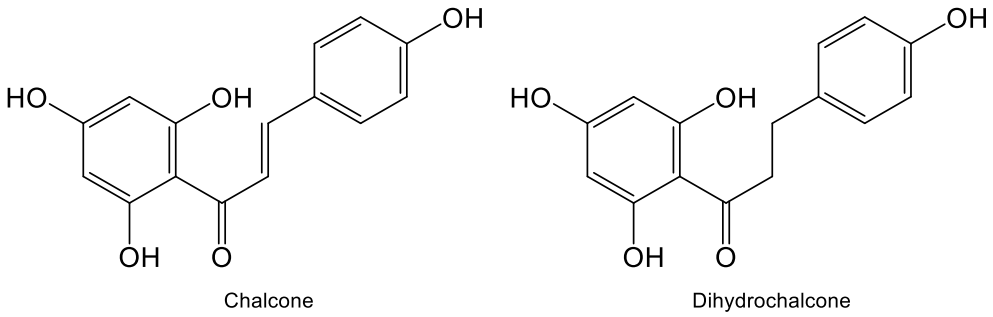


Figure 1-9 – Molecular structure of a chalcone and a dihydrochalcone (Tomás-Barberán and Clifford, 2000).

Chalcones and dihydrochalcones distinguish themselves from the rest of the flavonoids by not having the oxygenated heterocycle (see Figure 1-9), although they share the same flavonoid characteristic structure C_6-C_3-C_6 (Veitch and Grayer, 2006).

Flavanones are highly reactive and they are normally involved in hydroxylation, glycosylation and *O*-methylation reactions (Crozier et al., 2006). Flavanones occur as glycosides and can be

found in tomato, mint (Manach et al., 2004), citrus peel and orange and grapefruit bitterness is due to the presence of flavanones, (Tomás-Barberán and Clifford, 2000)

Chalcones occur as glycoside like flavanones and tomato skin is a rich source, but are also present at low concentrations in citrus peel, although it is its flavanone form the most common in citrus peel (Tomás-Barberán and Clifford, 2000).

Dihydrochalcones have only been found in apple and products derived from apples such as apple juice or cider. Dihydrochalcone concentration varies greatly with the variety of apple. Cider apples tend to have higher dihydrochalcone concentration than dessert apples while apple skin and seeds have higher dihydrochalcone content than apple flesh. Apple processing highly influences the dihydrochalcone concentration in the resultant products, for example treatment with pectinase will significantly decrease the dihydrochalcone concentration (Tomás-Barberán and Clifford, 2000).

1.5.1.2.5 Flavanols

Flavanols are found in both monomeric and polymeric forms, the latter are known as proanthocyanidins,. In contrast to other classes of flavonoids, flavanols are not glycosylated in foods (Manach et al., 2004). Flavanols are present in fruits, legume seeds and cereal grains and fruit derived products such as wine and cider (Santos-Buelga and Scalbert, 2000). Catechin and epicathechin are the principle flavanols and green tea and chocolate are their richest sources (Manach et al., 2004, Parr and Bolwell, 2000). Characteristics such as astringency, bitterness, sourness, sweetness, salivary viscosity, aroma and colour formation are affected by the presence of flavan-3-ols in food (Jaganath and Crozier, 2010).

Proanthocyanidins also known as condensed tannins, are found in fruits such as grapes, peaches or apples and they are responsible for its astringency flavour, which is a result of protein precipitation. Furthermore, cocoa bitterness is influenced by the presence of proanthocyanidins

(Manach et al., 2004). In plants proanthocyanidins are thought to play a role in protection against pathogens. Proanthocyanidins structure is made up of polymeric flavanols, normally linked by C-C bonds. Occasionally the flavanols monomers are linked by C-O-C bonds (Santos-Buelga and Scalbert, 2000). When proanthocyanidins are only composed by catechins they are called procyanidins and these are the most abundant type of proanthocyanidins in plants (Crozier et al., 2006).

1.5.1.3 Stilbenes and lignans

Stilbenes and lignans are classified as phytoestrogens (Cassidy et al., 2000). Stilbenes have a C_6-C_2-C_6 structure, named as 1,2-diarylethenes. Stilbenes originate from hydroxycinnamic acids (Cassidy et al., 2000) and are produced by plants in response to fungal, bacterial and viral pathogens attacks (Crozier et al., 2006). Stilbenes are commonly found in roots, barks, rhizomes and leaves. Grapes and peanuts are the dietary sources with higher stilbene content(Cassidy et al., 2000). Stilbenes are found in monomeric and polymeric forms, but also as glycoside (Crozier et al., 2006). Resveratrol is the most common stilbene and can be found in high levels in Red wine, which have been associated with lower incidence of coronary heart disease when consumed in moderation(Cassidy et al., 2000). Lignans are 1,4-diarylbutane (C_6-C_4-C_6) and are distributed as a minor constituent in plant species and are commonly found in fibre-rich foods (Cassidy et al., 2000). Linseed has been identified as the richest source of lignans (Manach et al., 2004).

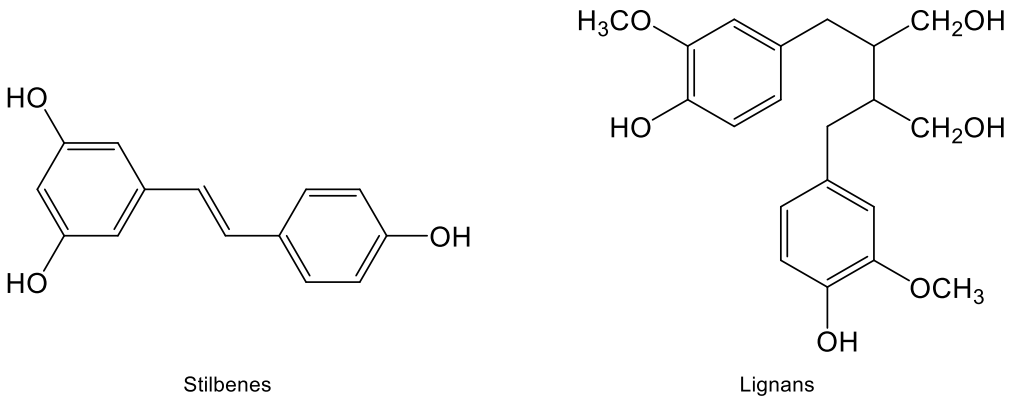


Figure 1-10 – Example of stilbenes and lignans molecular structures (Manach et al., 2004).

1.6 Phenolic compounds present in apple and derived products

1.6.1 Phenolics in apples

There are several studies on phenolic compounds present in different types of apple especially of British (Marks et al., 2007a), French (Guyot et al., 1998, Sanoner et al., 1999) and Basque (Rosa M. Alonso-Salces et al., 2004a, del Campo et al., 2006) origin. While the concentration of phenolics in apple is greatly influenced by fruit maturity, fruit season, light exposure, harvest year and storage conditions, apple variety has been shown to be the factor with most impact on apple phenolics content (Boyer and Liu, 2004, Marks et al., 2007a). Phenolic content observed in different varieties of apples can differ in an order of magnitude of 10 to 15 times, especially when dessert apples are compared with cider apples (Marks et al., 2007a).

The present work relates to UK cider production therefore, the phenolic composition of UK cider apples obtained by Marks et al. (2007a) was used as reference, where different hydroxycinnamic acids and four different types of flavonoids (anthocyanidins, flavanols, flavonols and dihydrochalcones) were identified in apple peel and/or apple flesh. In total 15 different phenolic compounds were detected: flavonols were represented by (–)-epicatechin, (+)-catechin and procyanidin B2 while the hydroxycinnamic acids identified were 5-*O*-caffeoylquinic acid (chlorogenic acid) and 4-*O*-*p*-coumaroylquinic acid. Dihydrochalcones

were represented by phloretin-2'-*O*-glucoside (phloridzin) and phloretin-2'-*O*-(2''-*O*-xylosyl)glucoside and cyanidin-3-*O*-galactoside was the only anthocyanidin identified. As for the flavonols, seven different quercetin glycosides were found: glucoside, galactoside, rutinoside, rhamnoside, xyloside, arabinopyranoside and arabinofuranoside (Marks et al., 2007a).

Chlorogenic acid and procyanidins (flavanols) are the predominant phenolics in the apple flesh and peel (Rosa M. Alonso-Salces et al., 2004a, Guyot et al., 1998, Marks et al., 2007a, Sanoner et al., 1999, Tsao et al., 2003), while quercetin glycosides and anthocyanidin are predominately in apple peel (Boyer and Liu, 2004, Guyot et al., 1998, Marks et al., 2007a, Tsao et al., 2003). The concentration of phenolics in apple peel can be two to six times higher than in the flesh and while chlorogenic acid is the only phenolic compound which showed higher concentration in the flesh than in the peel (Boyer and Liu, 2004).

1.6.2 Apple derived products

The conditions used to process apples lead to changes to the phenolic content as a result of incomplete extraction, enzymatic treatments and oxidation (Marks et al., 2007b).

Although apple juice and cider phenolic content can differ depending on the apple varieties used, chlorogenic acid and procyanidins are still the major phenolics, while quercetin glycosides are detected in trace amounts (Kahle et al., 2005, Marks et al., 2007b, A. Schieber et al., 2001a). Compounds such as caffeic acid, *p*-coumaric acid, phloretin and quercetin not previously detected in any apple variety, have been identified in apple juices and cider varieties and are thought to be the result of the decomposition of chlorogenic acid, 4-*O*-coumaroylquinic acid, phloridzin and querceting glycosides, respectively. In cider from Spain, other compounds not detected in apple were also identified, such as catechol, hydrocaffeic acid and protocatechuic acid (Lobo et al., 2009, Suarez et al., 2005).

In apple pomace compounds such as caffeic acid, *p*-coumaric acid, protocatechuic acid and quercetin were found (Diñeiro García et al., 2009, A. Schieber et al., 2001a). The main difference between apple pomace and apple juices and cider is the high content in phloridzin, that can be higher that the chlorogenic concentration for some of the apple pomace samples analysed (Lu and Foo, 1997)(A. Schieber et al., 2001a). The higher amount of phloridzin in apple pomace is the result of phloridzin been found in higher amounts in apple peel, from which extraction yield is lower than from apple flesh (A. Schieber et al., 2001a). Quercetin glycosides content in apple pomace is significant higher than in apple juice or cider, as it is also predominantly found in apple peel, although its amount is considerably lower than the amount of phloridzin found in apple peel (Marks et al., 2007a).

1.6.3 Apple phenolics antioxidant capacity

Quercetin glycosides and procyanidins in apple are the phenolics with higher antioxidant capacity, which is higher than vitamin C (Lee et al., 2003). Chlorogenic acid and phloridzin also exhibit significant antioxidant capacity, although it is lower than vitamin C (Lee et al., 2003). Antioxidant capacity of procyanidins can be affected by steric hindrance, especially for higher degrees of polymerisation (Lu and Yeap Foo, 2000).

1.7 Interaction between phenolics and other macromolecules

The first scientific account of non-enzymatic reactions involving the browning of sugar was published by the French biochemist Louis Camille Maillard in 1912 who reported that aqueous solutions of amino acids and reducing sugars, turned progressively yellow-brown when heated or stored under physiological conditions, the network of reactions have become known as the Maillard reactions .The specific reaction of a sugar, such as glucose or fructose, with the amino group of proteins is called protein glycation (Singh et al., 2001).

It has long been realised that solutions of amino acids and sugar should not be heated or mixed together. Therefore while thermal processes are used in the food industry to improve texture, colour, and flavour, and to sterilise and pasteurise, enabling longer shelf-life and improving product safety, it is understood that nutritional value can be lost due to undesirable reactions. These reactions occur involving phenolics, carbohydrates, proteins and lipids and their degradation can lead to the development of adverse flavours, colours and the destruction of essential nutrients (Rawel and Rohn, 2010).

Phenolics are highly reactive molecules which are normally found in conjugate forms as glycosides and esters (Fleuriet and Macheix, 2003). At the same time, during extraction, processing and storage phenolics can undergo modification and interaction with other molecules such as proteins and polysaccharides. These interactions can be covalent or non-covalent.

Phenolics can undergo enzymatic or non-enzymatic oxidation in which they are converted into their respective semi-quinones or quinones (Kroll et al., 2003). Semi-quinones and quinones on the other hand can take part in dimerisation reactions by nucleophilic addition. However, nucleophilic addition is not exclusive between semi-quinones and quinones and it can also occur with proteins and amino-acids to create new complex molecules. The new molecules formed can again be oxidised and take part of new nucleophilic addition reactions creating more new complex molecules (Namiki et al., 2001). The complete mechanism of these reactions is still not fully understood although it is generally accepted that these reactions start with the oxidation of diphenols into quinones (Kroll et al., 2003).

Phenolics can also interact with proteins and polysaccharides via non-covalent interactions. In 1.3.2 the phenolic adsorption by the yeast cell wall was briefly discussed and it is the result of non-covalent interaction between phenolics and cell wall polysaccharides. Same kind of

interactions can occur between phenolics and proteins. An example of that interaction is the formation of haze in beer or cider which is the result of the formation of a complex between proteins and phenolics that can grow to a colloidal size thus creating haze (Kroll et al., 2003). The formation of complexes between phenolics with proteins and polysaccharides are the result of hydrophobic interaction and hydrogen bonding (Hagerman and Butler, 1981, Oh et al., 1980). The development between protein and phenolic conjugates either covalently bound or non-covalently have an effect in its antioxidant capacity, especially if the proteins and phenolics involved have antioxidant capacity individually. Kwak et al. (2009) has demonstrated that the formation of conjugates between hydroxycinnamic acids and antioxidative peptides such as glutathione analogue and carnosine, increases the antioxidant capacity of the initial phenolics. There are also reports that show antioxidant capacity resultant from the formation of Maillard reaction products, as in coffee roasting (Moreira et al., 2012). It is important to emphasise that the complete mechanism of the formation of conjugates and the conjugates structure itself is not fully understood to the present moment. However it is worth stating that extraction, preparation and the type of analysis has the potential to influence what is measured and that our assessment of the bioactive properties of naturally occurring products is a ‘snap-shot’ in time and our desire to apportion the efficacy to one molecule maybe not be a reflection of in-vivo activity, Indeed the synergy derived from interaction of molecules and macromolecules may be a more relevant feature of bioactive compounds.

1.8 Critical fluids as green solvents

With increased consumer demand and legislation restricting the use of synthetic compounds there is greater emphasis placed on sourcing naturally occurring bioactive compounds which find application in food, feed, nutraceutical, cosmetic and pharmaceutical sectors. Similarly

there is an increased desire to extract and recover bioactive compounds with 'green' environmentally benign solvents to avoid potentially toxic solvent residues within the extract and the same time avoid waste management issues associated with disposal of organic solvents. Super and sub critical fluids are collectively referred to as critical fluids which are seen as potentially 'green' and environmentally benign solvents and therefore have attracted considerable research interest as alternative to organic solvents.

1.8.1 Critical fluid definition

Baron Charles Cagniard de la Tour in 1822 was the first to demonstrate that a substance has a critical point where it is not possible to distinguish liquid phase from gas phase and was referred to as a fluid (Alberty, 1987). The critical point of a substance arises due to molecular organisation similarities between liquid and gas phase. With the increase of temperature and pressure the liquid density decreases due to thermal expansion while gas density increases due to the increase in pressure. At specific temperature and pressure conditions, gas and liquid have the same density and consequently it is not possible to distinguish the difference between phases (T. Clifford, 1999).

A substance above its critical temperature and pressure is referred to as a supercritical fluid and its physio-chemical properties, such as density, viscosity, diffusivity, thermal conductivity, dielectric constant, ionic product and hydrogen bonding, will be different from its liquid and gas phase (Arai et al., 2002).

1.8.2 Supercritical carbon dioxide

The properties and utility of supercritical carbon dioxide (CO_2) has been extensively studied as it is characterised by a low critical temperature (~31ºC), which is a great advantage when extracting substances prone to thermal degradation. Other advantages of CO_2 are its chemical

stability, non-flammability and non-toxicity (Arai et al., 2002, T. Clifford, 1999). Also CO_2 is inexpensive as it is produced in large quantities as a by-product from processes as fermentation or combustion (T. Clifford, 1999).

1.8.3 Supercritical water

Supercritical water, as with CO_2, has the advantage of being environmentally friendly although it is critical at high pressures and temperatures (374ºC, 218atm) and if the water has a significant ion content it can create corrosion problems with equipment (Akiya and Savage, 2002, T. Clifford, 1999).

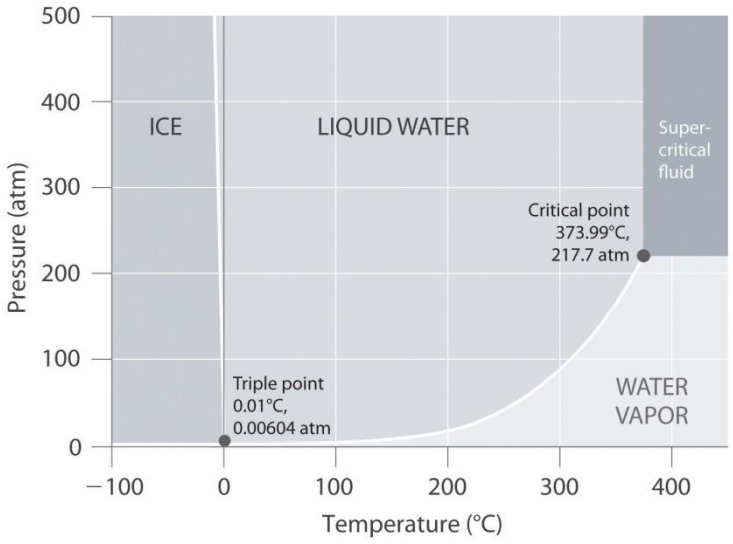


Figure 1-11 – Pressure-Temperature phase diagram of water.

Water at ambient temperature is characterised by an extended molecular structure due to intermolecular hydrogen bonding. The high degree of hydrogen bonding produces a high dielectric constant (ε) (T. Clifford, 1999), which results in water being poorly miscible with hydrocarbons and gases, however it is a good solvent for salts (Kruse and Dinjus, 2007). Under elevated temperature, hydrogen bonding is reduced between water molecules resulting an increase translation and rotational motions of water molecules (Kruse and Dinjus, 2007), and

as a consequence properties such as dielectric constant decreases to values similar to polar organic solvents (see Table 1-2), therefore water at these conditions will behave more like a polar organic solvent rather than water at ambient conditions (Akiya and Savage, 2002).

Table 1-2 – Dielectric constant of water at different temperature and pressure conditions and of some organic solvents.

	T (ºC)	P (bar)	ε	Reference
Water	20	0.913	79.99	(Mohsen-Nia et al., 2010)
Subcritical water	200	300.0	35.90	(T. Clifford, 1999)
Supercritical water	500	300.0	1.70	(T. Clifford, 1999)
Methanol	20	0.913	33.30	(Mohsen-Nia et al., 2010)
Ethanol	20	0.913	25.02	(Mohsen-Nia et al., 2010)
Butanol	20	0.913	17.68	(Mohsen-Nia et al., 2010)
Acetone	20	0.913	21.30	(Mohsen-Nia et al., 2010)

In addition as the fluid density decreases the ion product decreases by many orders of magnitude, which means that supercritical water is a less ionizing medium (T. Clifford, 1999). Supercritical water has properties of a non-polar solvent from the macroscopic point of view, although water molecule structure is still intact and it still is a polar molecule that can interact with ions. At supercritical conditions, as dielectric constant and ion product are low, free radical reactions are favoured (Arai et al., 2002, Kruse and Dinjus, 2007). As such, oxidation reactions are promoted by supercritical water. High temperature promotes faster reactions and as it is a single phase where water and organic compounds are miscible leads to the enhancement of the overall efficiency of the degradation process, in contrast to conventional oxidation which needs two phases and it is limited by mass transport between phases (Arai et al., 2002).

The utility of supercritical water has been successfully been demonstrated in a wide range of applications from oxidation as applied to decontamination of toxic wastes through to hydrothermal gasification and the production of hydrogen (T. Clifford, 1999).

1.8.4 Subcritical water

Subcritical fluids are those at pressures and temperatures below their corresponding critical point. The ion product of water at critical temperature and pressure decreases by several orders of magnitude. However between ambient and the critical temperature of water the ion product increases by a factor of 3, such a phenomenon is the result of an increase in self-dissociation of water molecules, which occurs when hydrogen bonds between water molecules decreases and it allows an increase in translation and rotation motions (Kruse and Dinjus, 2007). The sudden change in the ion product close to the critical point confers subcritical water with different properties from water at ambient temperature or at supercritical state.

Subcritical water has a higher ion product than water at ambient temperature thus hydrogen (H^+) and hydroxide ions (OH^-) exist in higher concentration, which favours acid/base reactions and act as a catalyst precursor (Arai et al., 2002, Kruse and Dinjus, 2007). As water can act as an acid/base catalyst or as a catalyst precursor means that the addition of a catalyst is not necessary or it is required in a lower amount when compared to water at ambient temperature. This means that the reaction's product at subcritical conditions is cleaner and less expensive (Kruse and Dinjus, 2007).

As a result of hydrogen-bonds breakdown dielectric constant and viscosity decreases while diffusion rates increases, similarly to supercritical water. A low dielectric constant makes solubility in subcritical water behave similar to solubility in polar organic solvents (see Table 1-2). In consequence small organic compounds are highly soluble in subcritical water whereas inorganic salts solubility is reduced (Akiya and Savage, 2002). Lower viscosity means that pumping requires less effort and higher diffusion rates allow rapid extraction (T. Clifford, 1999). Subcritical water extractions has been widely studied as an alternative to organic solvent in a wide range of applications with similar yield results but with the advantage of having lower

extraction times, higher selectivity, lower costs associated with extracting agent (Herrero et al., 2006, Smith, 2002). Also, contrary to supercritical fluids, pressure has minimal effect on solubility results (Smith, 2002). Subcritical water has been studied to extract of valuable compounds such as proteins, monosaccharides and antioxidants from natural sources or by-products streams such as pesticide residues from soils and plants (Smith, 2002).

1.9 Oxidative stabilisation: encapsulation with cyclodextrin

Naturally occurring bioactive compounds have and continue to attract considerable interest and find a wide range of applications, in particular bioactive compounds that exhibit antioxidant properties (Pinho et al., 2014, Zhao et al., 2010). However, the reactive nature of antioxidant compounds means that external factors such as temperature, pH and light can promote loss or change of the desired properties and when combined with limited water solubility and poor bioavailability it is clear that there is need to protect, improve presentation and overall bioavailability (Pinho et al., 2014). One way of protecting the bioactivity of molecules is to encapsulate the molecule to maximise the opportunity of presenting the desired activity to the target site which of course varies according to application.

Research into cyclodextrin complexation as a means of encapsulation has been widely studied and shown to protect easily oxidised substances against atmospheric oxidation, and the same could enhance solubility of poorly soluble drugs (Szejtli, 1998) and that it could also control volatility and sublimation properties (Pinho et al., 2014). As a result the recognised benefit and extensive research cyclodextrin is widely used in food, cosmetic and pharmaceutical products (Szejtli, 1998).

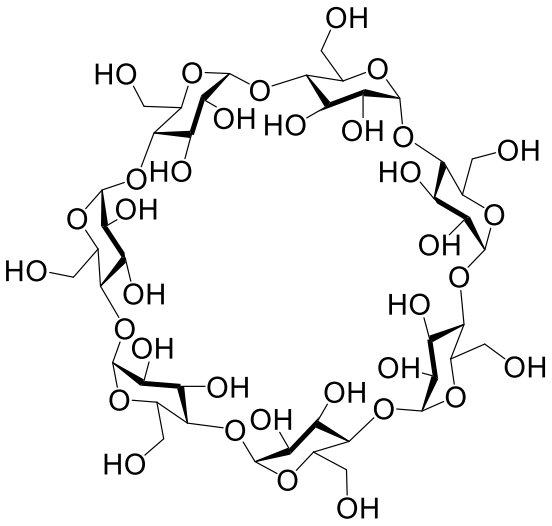


Figure 1-12 – β-cyclodextrin molecular structure.

Cyclodextrins are cyclic oligosaccharides with six or more glucose residues and the result of enzymatic modification of starch (Szejtli, 1998). Cyclodextrin molecules characteristically form a cone shape where the outside surface is hydrophilic while the inner area is hydrophobic (Figure 1-12). In aqueous solutions, cyclodextrin cavity is occupied by water molecules that can rapidly be replaced by "guest" molecules less polar than water. This inclusion complex process is characterised by the repulsive forces between the water molecules, the hydrophobic cyclodextrin cavity and the repulsive forces between the bulk water and the "guest" molecules (Szejtli, 1998). During the inclusion complex formation no covalent bonds are broken or formed between the "guest" compounds (Pinho et al., 2014).

Once the inclusion complex has formed the physicochemical characteristics of both cyclodextrin and "guest" molecule might improve oxidative stability as cyclodextrin cavity is a finite space, once occupied, other molecules are excluded, therefore prevents interaction among guest molecules and other molecules present. In addition the steric hindrance conferred by the cyclodextrin cavity prevents the exposed portion of the "guest" molecule form interacting with the "exterior" molecules (Hedges, 1998). The hydrophilic nature of the exterior

surface of cyclodextrin confers advantage as it will increase the solubility of the included "guest" molecule, the increase in solubility is extremely important for drug molecules as they are generally poorly soluble in water, therefore their absorption is slow (Szejtli, 1998).

The formation of inclusion complex can only be verified by NMR, where shifts in the peaks for cyclodextrin and the "guest" molecule are observed. These shifts are the result of the alteration of the surrounding environment of hydrogen atoms from both cyclodextrin and "guest" molecule, where the new environment is created by the cavity to the "guest" molecule, and from the presence of the "guest" molecule in the cyclodextrin cavity (Hedges, 1998). However, other methods can be employed, that although do not detect the formation of an inclusion complex, they give indication of its presence. Solvent extraction, differential scanning calorimetry (DSC) or Fourier transform infrared spectroscopy (FTIR) and functional assays are also viable ways to assess the encapsulation, e.g. accelerated stability tests where decomposition can be observed if it is prevented by the possible inclusion complex formed (Hedges, 1998).

The most common types of cyclodextrin are composed by six (α), seven (β) or eight (γ) glucose residues. Cyclodextrin with more than eight glucose residues do not have a regular cylinder shaped structure, therefore its cavity becomes smaller and consequently is capacity to form inclusion complex is lower (Szejtli, 1998). β-cyclodextrin has a considerable lower solubility when compared to α-cyclodextrin and γ-cyclodextrin, due to its structure with higher rigidity that is given by hydrogen bonds formed that are not formed in the other cyclodextrins. The lower solubility of β-cyclodextrin makes its production cheaper and therefore a more preferential choice for industrial applications (Szejtli, 1998).

Recently, the use of derivatives of cyclodextrin became a practice as cyclodextrin molecules can be enhanced for different purposes such as carriers, enzyme models, separating agents and

additives (Szejtli, 1998). The cyclodextrin derivatives are a result of the chemical modification cyclodextrin hydroxyl groups and the derivatives can be hydrophilic, hydrophobic and ionisable (Pinho et al., 2014). Hydroxypropyl-β-cyclodextrin is an example of a cyclodextrin derivative that is used as a drug carriers, as it as satisfactory toxicological documentation (LD50 in rats between1.008g/kg (intravenous) to 18.8g/kg (oral). For drug carriers it is important its solubility in water, price, availability in high purity, toxicity in high concentrations and solubility power for various drugs and its stability during heat sterilisation (Szejtli, 1998).

The majority of cyclodextrin produced is applied in food, cosmetic and toiletry industries, as the approval process in these fields is simpler and faster than for drug purposes (Szejtli, 1998). Nonetheless it is also used till some extent in pharmaceutical industry as drug carriers to enhance solubility, stability and bioavailability of the bioactive molecules (Pinho et al., 2014).

1.10 Objectives

The preceding literature review illustrated the need to develop routes of adding value to by-products of the agri-food and drink sector in general with the focus on cider lees a by-product of cider manufacture. Moreover the opportunity to recover bioactive compounds in particular phenolics which possess antioxidant capacity and find potential application in a range of sectors has driven the overall aim of the project which was to assess the utility of subcritical water, an environmentally benign solvent, to support the recovery of phenolics with bioactivity from cider lees.

To achieve the aforementioned aim, the present work was divided into the following main objectives:

- Evaluate the impact of subcritical water conditions within in a batch configuration on hydrolysis of cider lees and in turn the effect on the phenolic content, composition and overall antioxidant activity of the cider lee extracts.
- Study the opportunity to apply sequential SubCW extraction to enable selective recovery of phenolics from cider lees and in turn assess the influence of the process conditions on total phenolic content, composition and antioxidant activity
- Assess the oxidative stability of the cider lees extract and evaluate the potential benefits of encapsulation of the bioactive compounds using cyclodextrin as first step toward developing a skin care ingredient with associated antioxidant activity form the cider lees extract.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

In this chapter the protocol for the analytical methods used throughout the present research work are described.

2.2 Total solids method

The dry weight content of the cider lees and of extracts derived from cider lees was determined according a modified method described by (Sluiter et al., 2008) as follows.

2.2.1 Materials and equipment

A Mettler Toledo 4 Decimal Balance AE200 and a Thermo Labsystems Finnpipette Single Channel Manual 200-1000 μl Pipette, Model 4500 were used to measure mass and volume, respectively. A GallenKamp Vacuum oven was used for the drying step and 2 ml Eppendorf® safe-lock tubes were used for the samples. A desiccator was used for the cooling step.

2.2.2 Procedure

The cider lees slurry was thoroughly mixed before 1ml of sample was transferred to pre-weighed 2ml Eppendorf® tube (w_1). The weight of the Eppendorf® tube plus sample was recorded (w_2). The Eppendorf® tubes with open lid were placed at 100ºC and left to dry for 24 hours. After this period the Eppendorf® tubes were transferred to a desiccator to cool down to room temperature before reweighing (w_3). The dry weight (DW) was calculated according the following equation:

$$\%(w/w) = \frac{(w_3 - w_1)}{(w_2 - w_1)} \times 100 \quad (2\text{-}1)$$

2.3 Total phenolics content

Methods now known to determine total phenolic content (TPC) were originally developed to determine tannins content in wine and spirits. Later it was observed that these methods not only were able to quantify tannins but also other compounds such as monohydric phenols, polyphenols, flavonoids or any other readily oxidised substances such as ascorbic acid (Singleton and Rossi Jr., 1965).

2.3.1 Volumetric potassium permanganate titration

The volumetric potassium permanganate ($KMnO_4$) titration developed for the determination of tannins was originally developed by Monier (1858) and later optimised by Löwenthal (1877). The Volumetric Potassium Permanganate Titration consists of using the phenolics oxidation by the slow addition of $KMnO_4$ in the presence of an indigo carmine (pH indicator). The complete oxidation of phenols is determined visually when the extract solution turns golden yellow (Smit et al., 1955). The main disadvantage of the Volumetric Potassium Permanganate Titration is

that it must utilise visual determination which makes its reproducibility harder to achieve and demonstrate.

2.3.2 Folin-Ciocalteu's reagent method

The Folin Ciocalteu's reagent (FCR) method was originally developed by Folin and Denis (1912) as a colorimetric method for the determination of uric acid. The initial method, known as Folin-Denis method, had some problems such as the formation of sodium precipitates (Singleton and Rossi Jr., 1965) which led to further optimisation developed by Folin and Ciocalteu (1927) with the purpose to determine tyrosine and tryptophan, phenolic proteins. However the Folin Ciocalteu's reagent (FCR) method has become widely used with its last updated developed by Singleton et al. (1999).

The FCR contains a mixture of complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids. The FCR is reduced by phenolates when in alkaline solution, pH 10 (Singleton et al., 1999), producing a molybdenum-tungsten blue complex (Folin and Ciocalteu, 1927).

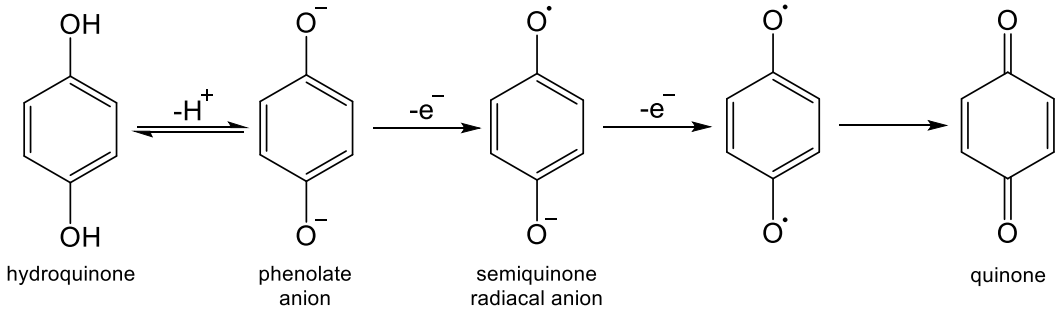


Figure 2-1 – Example of the oxidation path of a phenolic into a quinone (Singleton et al., 1999).

The FCR and the molybdenum-tungsten blue complex produced are unstable in alkaline conditions while these alkaline conditions are needed to ensure the presence of the phenolate ion that will reduce the FCR. Also, as seen in Figure 2-1, in a mixture of phenol (hydroquinone)

and quinone, intermediate semiquinone will be present in the equilibrium. As free radicals are highly reactive, dimerisation can occur, regenerating phenolates (Figure 2-2). The regenerated phenolates will be able to be oxidised once more what will increase the apparent TPC content(Singleton et al., 1999).

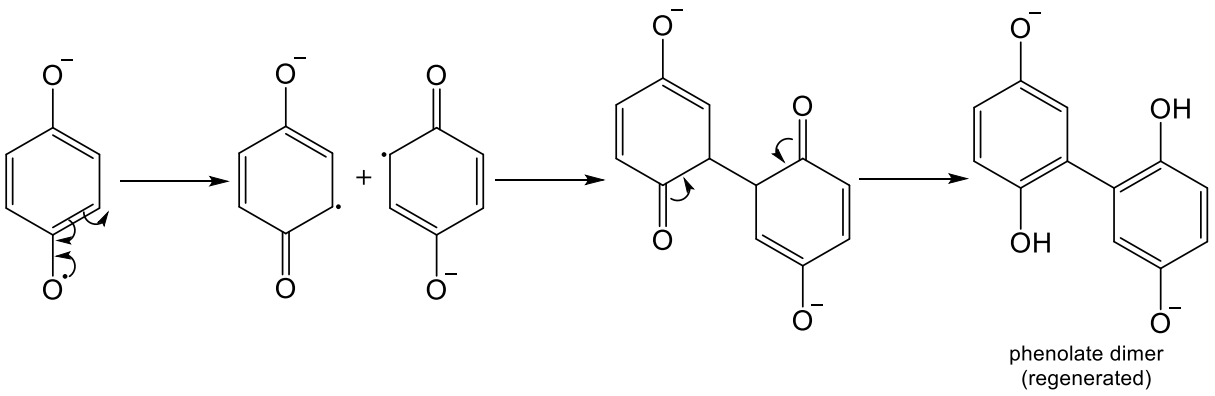


Figure 2-2 – Dimerisation of intermediate semiquinones (Singleton et al., 1999).

A great excess of FCR was the solution found to overcome these side reactions (Folin and Ciocalteu, 1927, Singleton et al., 1999), i.e. the FCR decomposition by the alkaline condition will still occur, however the quantity of FCR will be sufficient to oxidise the phenolate ion present to completion. It is extremely important that the FCR is in sufficient quantity to quickly oxidise all the phenolate present in solution to avoid regenerative polymerisation (Figure 2-2). As mentioned before, the FCR is unstable in alkaline solution. To avoid premature decompositon of FCR, the addition of an alkaline solution should be the last step of the method. Sodium Carbonate solution has been shown to be the optimal source of alkali. Alternatives such as Potassium Carbonate and Ammonia produce a precipitate when mixed with the phosphomolybdic and phosphotungstic complex (Folin and Denis, 1912). Sodium hydroxide and sodium cyanide were also taken into account, but the first promotes an higher precipitate formation and the second produces a blue complex that loses its color quicker than with sodium carbonate (Singleton and Rossi Jr., 1965). Sodium Cyanide, used in Folin and Looney (1922),

also produces a blue color complex when a blank solutions is being measured (Folin and Ciocalteu, 1927).

The time and temperature of reaction and also the alkaline solution concentration were determined by Singleton and Rossi Jr. (1965) taking into account not only the maximum absorbance of the blue color complex produced but also its stability. It was concluded that after 2 hours at room temperature (~24ºC) the color development and stability was satisfactory. A water bath at constant temperature is recommended to ensure a constant temperature throughout the reaction time. The reaction time can be decreased by increasing the temperature of reaction. Waterhouse (2001) verified that the same results could be achieved when the samples were kept at 40ºC for 30 minutes.

The blue complex produced from the FCR (using gallic acid as a phenolic standard) has maximum absorbance at a wavelength of 765nm (Singleton and Rossi Jr., 1965).

Initially Tannic Acid was the preferred choice for standard in total phenolic methods (Singleton and Rossi Jr., 1965) although any normal reactive phenol could be used as a standard for the FCR method. Tannic acid contains ten potential gallic acid molecules, which it is also observed in its molecular weight as tannic acid 1701.2 g/mol have a molecular weight 10 times that of gallic acid molecular weight 170.12 g/mol. The relation between tannic acid and gallic acid molecular weight allows an easy comparison to previous data to be obtained using tannic acid as a standard. Gallic acid is a preferred choice for a standard mainly because it is easier to obtain with higher purity. Although gallic acid at high concentrations does not follow the Beer-Lambert law strictly, it still shows an acceptable deviation (Singleton and Rossi Jr., 1965). The deviations observed are due to a need of higher time of incubation when higher concentrations are found.

Singleton and Rossi Jr. (1965) verified that if the FCR is mixed with an extract before the diluting the extract, reproducible results were not achieved. Also, when sodium carbonate was added in less than one minute after FCR being mixed with extract, the results were also not repeatable. As a result the sodium carbonate should be added 1 to 8 minutes after FCR is added to the extract, otherwise the results will not be reproducible.

2.3.2.1 Interferences

The FCR does not target phenolates exclusively, it targets any oxidisable subtrates, so depending on the composition of the sample being analysed, other compounds besides phenolics might interfere in the results achieved by using this method. Compounds such as proteins, sugars, ascorbic acid, sulfites and sulphur dioxide can interfere in the final result of the FCR method when present.

The FCR was initially developed not only to measure polyphenols but to determine tyrosine in wine samples (Folin and Ciocalteu, 1927). Tyrosine is a amino acid with a phenol group. Later, FCR was used for protein determination by Lowry et al. (1951), although in this method the proteins do not react directly with the FCR. The proteins need to undergo a treatment with copper ions what will transform a amino acid into a enolic compound. The amino acid within the protein in its enolic form will be able to reduce the FCR (Singleton et al., 1999). So only proteins with phenolic groups will directly interfere in the determination of TPC using FCR. The protein interference can be corrected by removing the proteins using 2,2,2-Trichloroacetic Acid (TCA) (Sivaraman et al., 1997). TCA addition will induce protein precipitation under centrifugation.

Like proteins, sugars by themselves do not react directly with FCR. But in contrast to proteins, alkaline conditions are sufficient to promote the formation of sugar enolic compounds (Singleton et al., 1999). In this form, sugars are able to reduce FCR but it will only have

significant interference if sugars are in concentrations higher than 2.5%(w/w). Slinkard and Singleton (1977) determined correction factors to total phenolic content when sugar concentration is above 2.5g/100g.

Ascorbic acid (Vitamin C) is a well-known antioxidant present in fruits. Ascorbic acid has the ability to react with the FCR under acidic conditions (Singleton et al., 1999). Its presence or the presence of any substance that can be oxidised in the same conditions, it is detected by the formation of the blue complex before the alkaline solution addition. The timing before the addition of the alkaline solution allows ascorbic acid or other similar compound detection beforehand. For example ascorbic acid under acidic conditions (pH 3) reacts with polyphosphotungate to form a blue color which allows the quantification of ascorbic acid. Therefore it can be subtracted from the apparent TPC (Singleton et al., 1999). Also, Johnson and Schaal (1957) showed that natural ascorbate (anion of ascorbic acid) in extracts is negligible when determined alongside a TPC method.

Sulphur dioxide and sulfites are often used for its antioxidant and antimicrobial effect in alcoholic beverages, especially in wines (Singleton et al., 1999). Somers and Ziemelis (1980) verified that sulphur dioxide addition to wine showed an increase in the apparent total phenolic content. It was found that sulfites were responsible for the regeneration of phenols, therefore an increase in the apparent total phenolic content. Later studies (Saucier and Waterhouse, 1999) showed that the sulphur dioxide on its own does not contribute to the apparent total phenolic content and therefore supports its direct interaction with phenols. The mechanism of the phenol regeneration by sulfites is still unknown (Saucier and Waterhouse, 1999).

Despite the interferences mentioned above, FCR method is the TPC method more accepted today due to its reproducibility and the possibility to easily correct most of its known interferences.

2.3.3 Price and Butler method

The Price and Butler method also known as Prussian blue assay was initially developed by Price and Butler (1977). The initial method had two issues: formation of a precipitate and an increase in colour density over time. These two problems were addressed and solved by Graham (1992). Like FCR method, the Price and Butler method is also a colorimetric assay, however with a different mechanism. Price and Butler method involves the phenol reduction of the ferricyanide ion ($Fe(CN)_6^{3+}$) to $Fe(CN)_6^{4-}$ by phenols. The $Fe(CN)_6^{4-}$is subsequently oxidised by ferric ion producing a ferric ferrocyanide ($Fe_4[Fe(CN)_6]_3$) known as Prussian blue complex (Graham, 1992). The Prussian blue complex has maximum absorbance at a wavelength of 720nm.

The Price and Butler method has the advantage of being a simple and quick method, redox reaction is complete within seconds and the colours developed can be distinguished visually (Price and Butler, 1977). However phenolic proteins and ascorbic acid interferences were also observed in the Price and Butler method (Price and Butler, 1977). Despites its advantages, the Price and Butler method still need to be tested repeatedly in a wider range of samples to be established as a reliable and reproducible method.

2.3.4 Estimation of total phenolic content

Total phenolic content (TPC) of cider lees and extracts of cider lees was determined by using a micro-scale Folin Ciocalteu's Reagent (FCR) method described by (Waterhouse, 2001). To reduce interference in this assay proteins were removed using TCA driven precipitation (Sivaraman et al., 1997).

For an accurate determination of TPC, the Beer-Lambert law needs to be followed, which means that the extracts to be analysed cannot have a concentration higher than 500mg/l (higher concentration of gallic acid on the standard curve). It was observed that the extracts in the

present work have to be diluted 1:10. Dilution plus the TCA addition are corrected for to determine the final total phenolic content.

2.3.4.1 Materials and equipment

Gallic acid 97.5%-102.5%, Folin-Ciocalteu's reagent (FCR) and sodium carbonate anhydrous 99.95-100.05% dry basis were purchased from Sigma-Aldrich (Dorset, UK). Trichloroacetic Acid 99+% (TCA) and ethanol absolute were purchased from Fisher Scientific (Loughborough, UK).

The protein was precipitate using a Sigma 3K30 refrigerated centrifuge. Clear 96 well flat bottom plates (Sterilin) were used in conjunction with a microplate spectrophotometer (Promega) to measure absorbance. The FCR method requires absorbance to be measured at a wavelength of 765nm, however due to a limitation with the available lens, all absorbance measurements were performed at 750nm. A vortex mixer MixiMatic – Jencons [Scientific] Limited was used for stirring mixtures. The water bath was prepared using a Clifton Unstirred Bath.

2.3.4.2 Solution preparation

2.3.4.2.1 2,2,2-Trichloroacetic acid

TCA 100% (w/v) solution was prepared by adding 30g of TCA to 11.64ml of distilled water. The solution was mixed till TCA was completely dissolved, before it was stored at 4ºC.

2.3.4.2.2 Sodium carbonate saturated solution

A saturated solution of sodium carbonate was created by first dissolving 20g of sodium carbonate in 80ml of distilled water using a heated stirrer plate to bring the solution to the boil. When dissolved the solution was cooled down to room temperature, where upon additional

sodium carbonate crystals were added before leaving the solution at room temperature for 24 hours. After 24 hours, the solution was filtered (Whatman qualitative paper filter grade 1: 11µm) to remove any sodium carbonate crystals excess. The solution was then adjusted to 100ml using a volumetric flask.

2.3.4.2.3 Gallic acid standard solutions

A gallic acid stock solution of 5g/l was prepared by adding 0.500g of Gallic Acid to 10 ml of ethanol to improve its solubility in water. Once completely dissolved the solution was made up to 100ml with distilled water using a volumetric flask. The Gallic acid stock solution was kept at 4ºC for a maximum of two weeks.

The Gallic Acid calibration curve was composed by five different concentrations: 50, 100, 150, 250 and 500 mg/l plus a blank which correspond to no gallic acid in solution. The blank was prepared directly in the microplate. The Gallic acid dilutions were prepared using 1, 2, 3, 5 and 10 ml of the stock solution and made up to 100ml using a volumetric flask.

2.3.4.3 Procedure

Prior to conducting the FCR method, proteins were precipitated from the sample as follows. A 120µl aliquot of TCA 100% (w/v) was added to 800µl of sample extract. The mixture was placed at -20ºC for 5 minutes and then for a further 15 minutes at 4ºC. The mixture was then centrifuged for 15 minutes at 15000g at 4ºC using the refrigerated centrifuge. The supernatant was removed and used for the assay.

The extract supernatant was diluted 1:10 with distilled water due to the high total phenolic content. In a 3ml glass test tube, 20µl of sample was diluted with 1.58ml of water to which 100µl of FCR was added and slightly stirred. After 1 to 8 minutes 300µl of Sodium Carbonate 20% (w/v) was added to the mixture. The same procedure was applied to the blank and the

Gallic acid solutions. The final mixture was stirred using the vortex mixer. The mixture was incubated at 40ºC for 30 minutes in the water bath. When incubation was concluded, 300µl of each sample was transferred to a clear flat bottom microplate and its absorbance was measured using the microplate spectrophotometer at 750nm. Each sample, blank and Gallic acid solutions were prepared in triplicate.

2.3.4.4 Gallic acid equivalent concentration

Total phenol content of the sample was determined by referencing a Gallic Acid standard curve therefore the total phenolics was initially expressed in mg GAE/l. For each total phenolic measurement, a Gallic acid calibration curve was determined.

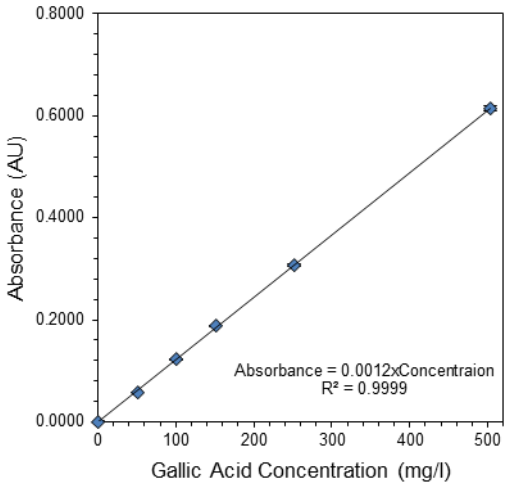


Figure 2-3 – Example of a gallic acid calibration curve.

The gallic acid standard curve describes a linear correlation between absorbance at 750nm and gallic acid concentration, the linear correlation follows the Beer-Lambert Law where Absorbance is directly proportional to concentration.

$$Absorbance = \varepsilon \times b \times Concentration \quad (2\text{-}2)$$

Where ε and b are constant and correspond to the slope obtained in Figure 2-3.

For extract samples with unknown concentration, the absorbance reading was used in the equation mentioned in above to determine the respective concentration (mg GAE/l). The Total Phenolic content estimated from the assay was then corrected for extract samples TCA dilution, resulting in an overall dilution factor of 11.5.

2.4 High performance liquid chromatography

2.4.1 Background

High performance liquid chromatography (HPLC) is widely used to resolve low molecular weight analytes (Neue, 1997). HPLC achieves the physical separation of the analytes in a sample by their distribution between two phases, one stationary and one mobile (Dong, 2006). The main advantages of HPLC are the ability to provide direct quantitative and qualitative information (Meyer, 2004), its versatility to use different separation methods (adsorption, partition, etc.) using the same instrumentation except for the stationary phase (Dong, 2006, Heftmann, 2004). Gas chromatography (GC) is another common analytical separation method and although GC allows higher diffusion rates when compared to HPLC, which increase its speed of separation, GC can only be used for substances that are volatile and can be evaporated without degradation, which corresponds to about 20% of known organic substances (Meyer, 2004).

As mentioned before, HPLC builds on a number of separation technologies. Partition is the most common separation method, which can be performed in normal phase or reverse phase. Normal phase chromatography is a polar stationary phase with a non-polar mobile phase while reverse-phase chromatography is the opposite, i.e. a non-polar stationary phase with a polar mobile phase.

Other HPLC separation methods are ion-exchange, size-exclusion and affinity Chromatography. Ion exchange is used for the separation of ionic or ionisable compounds, mainly proteins, carbohydrates and nucleic acids (Neue, 1997), and it consists in the interaction between ionic analytes and counter-ions from ionic groups bonded to the stationary phase (Dong, 2006). Size-exclusion chromatography is the separation of molecules by their molecular size. Stationary phases made from a porous material are used where small molecules are retained longer than larger molecules, as they can diffused into the pores of the stationary phase, this technique is used for the determination of molecular weight and molecular weight distribution of polymers (Heftmann, 2004). Affinity chromatography is the use of a specific binding agent for the isolation of sample components. Examples of binding agents can be enzymes with a substrate or a hormone with its receptor. An affinity ligand is bonded to the stationary phase and it works as one of a pair of interacting molecules. The separation is done in two stages: application buffer and elution buffer. The first stage uses a weak mobile phase of an affinity column that will have the right conditions to promote the solute-ligand binding. The second stage is initiated after all non-binding components are eluted from the column. The second stage is a solvent that promotes the dissociation of the solute-ligand complex (Corradini, 2010), i.e. that has a greater affinity to the ligand than the solute (Meyer, 2004). Affinity chromatography is used for purification and isolation of enzymes, antibodies and antigens. It can also be used for the isolation of DNA and RNA (Corradini, 2010).

In the present work, the HPLC separating method chosen was adsorption using reverse phase. Reverse phase has the ability to separate neutral and ionic analytes (Neue, 1997), and it is preferable to separate analytes that are water insoluble or with a low solubility. This corresponds to most of the existent analytes, polyphenols included, and it makes reverse phase HPLC method the first choice to separate and identify these kind of analytes (Heftmann, 2004).

Also, an equilibrium between mobile and stationary phase is easier to achieve in reverse phase (Heftmann, 2004). For instance, normal phase has a polar stationary phase which makes it susceptible to moisture and it eventually deactivate the stationary phase surface.

Regardless of which type of phase is used for the HPLC methods, there are five other elements that are chosen according to the type of separation desired, they are stationary phase, mobile phase, elution mode, temperature of operation and detector.

Silica is the most common stationary phase used in HPLC. Alumina or polymer stationary phases are also used. Silica is an amorphous substance composed by silicon atoms bridged by oxygen atoms. At the surface of these structure functional groups are present known as silanols (Meyer, 2004).

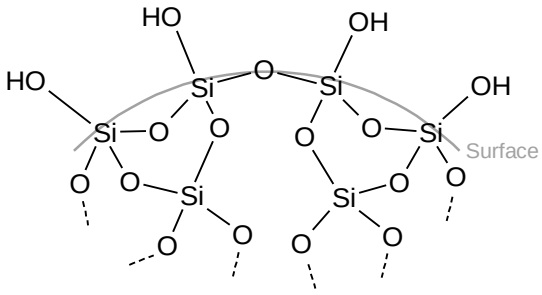


Figure 2-4 – Silica particle with free silanols in the surface.

Adsorption occurs at the silica surface. In Figure 2-4 an example of a silica particle can be seen. The silanol groups (Si-OH) are responsible for silica particles polarity and is used in normal phase chromatography. Silica has the advantages of incompressibility at high pressures and does not shrink or swell when mixed with different solvents, however silica can be dissolved in alkaline solution (Neue, 1997). Another important aspect of silica is the ability to be chemically transformed into a surface with a wider adsorptive range (Neue, 1997). This ability is known as silica bonding and it allows the preparation of silica stationary phases with different

surface properties. Silica bonding is the replacement of silanol groups by silicon-carbon groups (Neue, 1997). Silica-bonded phases are more stable when they have longer chain groups attached and less residual silanol groups (Meyer, 2004). The common silica-bonded phases are octyl (C8), octadecylsilane (C18), phenyl and cyanopropyl. Octadecylsilane (ODS) is the most widely used and it has demonstrated higher stability, better efficiency and reproducibility when compared to other silica-bonds (Heftmann, 2004).

The most common alternative stationary phases to silica are alumina and polymeric. Zirconia and porous graphitic carbon are less used alternatives. Alumina is rarely used, because it is less reactive which makes alkyl bonding harder to achieve. Polymeric stationary phases are made of polystyrene-divinilbenzene and it is possible to work at any pH. It also has a large surface area and it is stable at high temperatures (200ºC), however the mobile phase needs to be chosen carefully. For example, a mobile phase composed of water and methanol achieves poor yields of separation when used in a polymeric stationary phase (Meyer, 2004).

The common mobile phases used in reverse phase HPLC are a mixture of water and methanol or acetonitrile, a mixture that improves the selectivity of separation. Ethanol and isopropanol are not used due to their high viscosity when in an aqueous solution (Corradini, 2010). Also, it has been demonstrated that acetonitrile is more efficient than methanol, as it can achieve similar separations with lower retention times. Also, a significant increase in methanol viscosity is observed when mixed with water, although not as high as the viscosity of ethanol and isopropanol. The viscosity of acetonitrile, contrary to methanol, decreases when mixed with water, which makes acetonitrile the preferable solvent (Corradini, 2010). Buffers are commonly used when ionic compounds are present in solution to improve separation. Acetic acid and formic acid are among common buffers used in HPLC methods.

HPLC elution mode can be isocratic or gradient. Isocratic elution mode consists of a constant mobile phase composition during separation (Heftmann, 2004). However, when samples containing complex compounds that differ widely in retention in the stationary phase, low yields of separation are achieved (Corradini, 2010). Gradient eluent mode consists in a gradual composition change of the mobile phase throughout the separation. The elution time starts with a weak mobile phase, that enhances the retention time of weak retained compounds, and then the composition of the mobile phase gradually evolves to a stronger mobile phase to elute all compounds retained in the stationary phase. This gradual increase in the strength of the mobile phase results in shorter retention times for strongly retained compounds (Corradini, 2010). In reverse phase, elution starts with a mobile phase with higher polarity, i.e. with higher concentration in water, and elution finishes with a mobile phase less polar, i.e. with higher concentration of acetonitrile or methanol.

Temperature of stationary phase has an important influence on the separation. Higher temperatures decrease mobile phase viscosity, therefore diffusion coefficient increases and consequently efficiency of separation increases (Corradini, 2010). However, if temperatures are too high, decomposition of the sample or solvent can occur and silica solubility increases while adsorption equilibrium can change (Meyer, 2004).

HPLC can be coupled to in-line detectors that allow the detection of the separated compounds. Detectors are divided in four main types: ultraviolet/visible absorbance detector (UV/Vis), refractive index detector (RI), fluorescence detector, conductivity detector and mass spectrometry detector (MS) (Corradini, 2010) . UV/Vis detectors are the standard detector for HPLC (Heftmann, 2004) and it determines the presence of chromophore in samples. Despite UV/Vis detector not having the highest sensitivity of all detectors, it provides a simple answer to the majority of applications which makes it the commonly used detector. Also, modern

UV/Vis detectors, known as DAD (Diode Array Detector) allows the measurement of absorbance in the full absorption spectrum of each analyte peak, which improves identification of separated compounds (Corradini, 2010).

RI detectors have low sensitivity as they work bydistinguishing the small change in the refractive index of the mobile phase containing the separated compounds. These detectors are normally used as an alternative to UV detectors for non-chromogenic compounds and sugars, lipids and high molecular polymers are the types of compounds for which RI detectors are used (Heftmann, 2004).

Fluorescence detectors have a higher sensitivity and selectivity than UV detectors, however fluorescence detectors are limited to analytes with fluorescent properties (Heftmann, 2004). These analytes also have chromophore which means that UV/Vis detectors can also be used to detect compound with fluorescent properties. Fluorescence detectors are normally used when higher sensitivity and selectivity is needed, for example, to detect compounds in trace levels (Heftmann, 2004). Conductivity detectors are used for ionised analytes as they detect the changes in the conductivity of the mobile phase. There is another detector that recently is gaining more attention: light-scattering detector. This detector promotes the evaporation of the mobile phase solvent while non-volatile particles fall through a light beam as their scattered radiation is being measured. This method is highly sensitive and it has been used instead of RI detector in the determination of lipids and sugars (Heftmann, 2004).

Reverse-phase chromatography has been the first choice to identify and quantify polyphenols in fruits (Marks et al., 2007a, A. Schieber et al., 2001a, Yeap Foo and Lu, 1999), plants and beverages (Marks et al., 2007b). Normal phase has been employed for the determination of specific type of polyphenols, proanthocyanidins (Lazarus et al., 1999).

In the present work a reverse phase HPLC method was adapted from A. Schieber et al. (2001a), where a gradient elution mode was used with a mobile phase composed of water and acetonitrile. Acetic acid was used as a buffer and UV/Vis DAD detector was used. The column (stationary phase) used in the present work corresponds to the new model of the column used in A. Schieber et al. (2001a), which was specifically designed for the detection of polyphenols.

2.4.2 Analysis of polyphenolic composition: reverse phase HPLC

2.4.2.1 Materials and equipment

Acetonitrile (Chromasolv®Plus), HPLC quality water, Acetic Acid, 99.8-100.5%, chlorogenic acid, caffeic acid, *p*-coumaric acid, phloridzin dihydrate, 5-(hydroxymethyl)furfural (5-HMF) and furfural were purchased from Sigma-Aldrich (Dorset, UK).

HPLC analysis was performed using an Agilent HPLC system equipped with an autosampler and a quaternary pump from 1100 series, and a UV-DAD detector and a Vacuum degasser from 1200 series. Also, a Jones Chromatography Oven was used. The column used was a Phenomenex Prodigy 5µ ODS3 100A.

2.4.2.2 Procedure

The HPLC analytical method to resolve phenolics was adapted from A. Schieber et al. (2001a). The injection volume per sample was 10µl. Three solvents were used as the mobile phase: Solvent A (water with 2% (v/v) acetic acid), Solvent B (Acetonitrile and water with a ratio of 50:50 with 0.5% (v/v) acetic acid), Solvent C (100% Acetonitrile). A gradient solvent profile was used starting with 10%B to 55%B, 0-50 min; 55%B to 100%B, 50-60 min at a flow rate of 1ml/min. The column was re-conditioned under isocratic flow using 100%C isocratic 60-70min, 10%B isocratic 70-80min. The temperature of the column was kept constant at 40ºC and absorbance recorded at two different wavelengths: 280nm and 320nm.

2.4.2.3 Standard Curves

In previous work (Ali, 2010) several compounds were identified such as the polyphenols chlorogenic acid, *p*-coumaric acid, phloridzin but also 5-HMF and furfural as the result of monosaccharides dehydration. Standard curve to each standard compound need to be determine to quantify these compounds in the extract samples. Each standard curve was composed by six different concentrations: 0.01, 0.1, 0.2, 0.3, 0.5 and 1mg/ml. All standard stock solutions were prepared with a concentration equal to 1mg/ml. The stock solutions were prepared by diluting 50mg in distilled water and then made up to 50ml in a volumetric flask.

Table 2-1 – Standard calibration curves for HPLC.

(g/l)	**Slope**	**R^2**	**$\lambda_{measured}$** (nm)	**λ_{max}** (nm)
5-HMF	40715	0.97	280	284
Furfural	42138	0.98	280	278
Chlorogenic Acid	25806	1.00	320	324
***p*-Coumaric Acid**	58654	0.99	320	310
Phloridzin	18755	1.00	280	284

The standard curve describes the linear correlation between the chromatographic peak area and its concentration. Table 2-1 shows the standard curves determined.

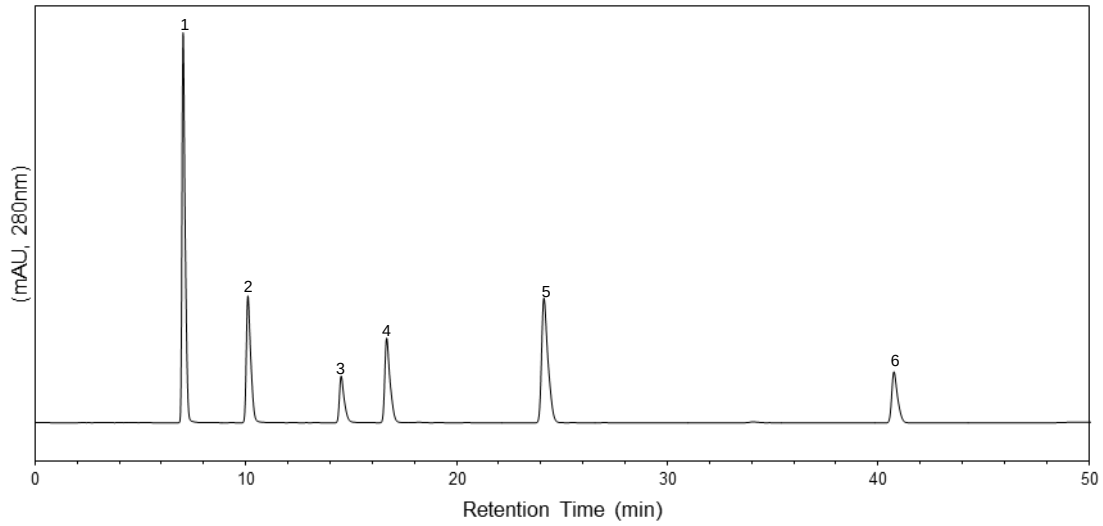


Figure 2-5 – Standard mixture separation chromatogram by HPLC.
1) 5-HMF, 2 - furfural, 3 - chlorogenic acid, 4 - caffeic acid, 5 - *p*-coumaric Acid, 6 - phloridzin.

To verify how the standards were separated when in the same sample, a mixture of the six standard compounds mention before was measure in HPLC and the resultant chromatogram is seen in Figure 2-5 .

2.5 Determining antioxidant capacity of extract

Antioxidant capacity assays are based in chemical reactions *in vitro* that are initiated by the addition of artificial oxidants or radical precursors, therefore the assays do not measure bioavailability, *in vivo* stability, retention of antioxidants by tissue or reactivity *in situ* (D. Huang et al., 2005). Consequently it is important to acknowledge that antioxidant capacity results cannot be translated directly to biological systems.

Antioxidant capacity assays are classified according to two reaction mechanism: electron transfer (ET) and hydrogen atom transfer (HAT). ET assays measure the capacity of an antioxidant as a reducing agent, where the reduction of an oxidant substance is the indicator for the reaction end-point. HAT is a competitive reaction scheme where the antioxidant and

substrate compete to react with peroxyl radicals, where the hydrogen atom donating capacity of an antioxidant is quantified (D. Huang et al., 2005). Both ET and HAT assays measure sacrificial capacity of antioxidants.

The review of antioxidant capacity assays developed by D. Huang et al. (2005) recommends the use of FCR method and ORAC assay for the determination of antioxidant capacity of a substance.

2.5.1 Electron transfer assays

Antioxidant capacity assays with electron transfer (ET) mechanism consist of a redox reaction between an antioxidant and an oxidant, which also works as an indicator of the end of reaction. In these assays it is assumed that antioxidant capacity is equal to reducing capacity. No reactive oxygen species (ROS) are used in ET antioxidant capacity assays. The most common ET assays are Folin-Ciocalteu's reagent (FCR), trolox equivalent antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAC) and DPPH. Because all these assays have the same or similar redox reactions, their results have a correlation above 0.99 (D. Huang et al., 2005).

2.5.1.1 Folin-Ciocalteu's reagent

Folin-Ciocalteu's Reagent (FCR) method is known as an assay to determine total phenolics content (as described in 2.3) through the measurement of the reducing capacity of a sample, therefore it can also be classified as an ET antioxidant capacity assay. The FCR method is standardised and because has been widely used, a considerable amount of comparable data is available, therefore makes it a reliable ET assay. Also, the use of a long wavelength minimises interferences from other compounds present in the sample (D. Huang et al., 2005). However correlations with HAT assays need care, as the FCR is conducted at high pH (10), where simple

phenols react and contribute to the measured reducing capacity, although they do not act as radical scavenging antioxidants (D. Huang et al., 2005).

2.5.1.2 Trolox equivalent antioxidant capacity

Trolox equivalent antioxidant capacity (TEAC) assay was developed by Miller et al. (1993) and improved by Re et al. (1999). It uses a pre-formed radical cation ($ABTS^{\bullet+}$) generated from 2,2'-azinobis-(3-ethylbenzothiazoline-6--sulfonic acid) (ABTS) by the addition of potassium persulfate. Cation $ABTS^{\bullet+}$ is characterised by a blue-green colour with maximum absorbance at 415nm, but also with significant absorbance at 734nm.

The TEAC method involves the decolourisation of the $ABTS^{\bullet+}$ cation when it is mixed with a sample with reducing capacity, the $ABTS^{\bullet+}$ is reduced to ABTS and its characteristic colour is lost. After mixing the $ABTS^{\bullet+}$ with a sample at 30ºC, absorbance is measured after 1, 4 and 6 minutes (Re et al., 1999). pH is kept constant at 7.4 by the use of a phosphate buffered saline. The percentage of inhibition from the sample is measured based in the initial and final absorbance of $ABTS^{\bullet+}$. Trolox is used as standard solution and TEAC values can be determined in two different ways. One way is by using the inhibition percentage of absorbance at different concentrations. The TEAC value is estimated from the ratio between the slope generated by the sample and the slope of the trolox. Another way is by using the absorbance decay curves of sample and trolox. TEAC value is the ratio between the area under the curve (AUC) of the sample and AUC of trolox of absorbance after 6 minutes. The use of AUC allow to compare the scavenging free radical ability with trolox (Re et al., 1999).

2.5.1.3 Ferric ion reducing antioxidant power

Ferric ion reducing antioxidant power (FRAP) assay is similar to TEAC but it is performed at a lower pH (3.6) and it uses ferric salt as an antioxidant standard. Absorbance is measured at 593nm every 15 seconds for 4 minutes (D. Huang et al., 2005).

The mechanism underpinning the FRAP assay involves the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) at low pH which produces a coloured ferrous-tripyridultriazine complex (Benzie and Strain, 1996). Samples are measured at different concentrations and, for each concentration, the change in absorbance from the starting point to 4 minutes is determined. The change in absorbance has a linear correlation over a wide concentration range and for each sample a correspondent slope is determined. FRAP results are derived from the ratio between sample's slope and standard's slope, which is ferric salt (Benzie and Strain, 1996).

The FRAP assay has the advantage of being inexpensive, its reagents are simple to prepare and results are reproducible with a straightforward and quick procedure. However compounds such as caffeic acid are not measured accurately in the default 4 minutes of assay (D. Huang et al., 2005).

2.5.1.4 DPPH assay

The molecule 2,2-diphenyl-1-pierylhydrazyl (DPPH) is one of the few commercially nitrogen stable radicals. The DPPH assay is simple and consists of mixing DPPH with the antioxidant sample. Absorbance of the mixture is measured at 515nm over a period of 30 minutes or untill absorbance becomes constant. DPPH loses its characteristic colour when reduced. For each sample concentration, a percentage of absorbance loss is determined (D. Huang et al., 2005). To compare different antioxidants a parameter called antiradical efficiency (AE) is determined. AE is a function of the concentration where 50% of absorbance is loss and the time needed for 50% of absorbance to be achieved at the same concentration (Jiménez-Escrig et al., 2000).

However, DPPH is less reactive than peroxyl radicals which may result in no reaction with some antioxidant compounds. Also, DPPH reaction kinetics is not linear with concentration (D. Huang et al., 2005).

2.5.2 Hydrogen atom transfer assays

The oxygen radical absorbance capacity (ORAC) and the total radical trapping antioxidant parameter (TRAP) are hydrogen atom transfer (HAT) antioxidant capacity assays consist of a molecular probe and an antioxidant to which a free radical is added to. The antioxidant substance and the molecular probe compete to react with the free radical added. Peroxyl radical is the most frequently used free radical because it is a key radical in autoxidation, it has the added advantage of being easily generated from thermal decomposition of azo compounds (D. Huang et al., 2005).

2.5.2.1 Total radical-trapping antioxidant parameter

Total radical-trapping antioxidant parameter (TRAP) is the use of a fluorescein as molecular probe and AAPH as free radical (Ghiselli et al., 1995, Ghiselli et al., 2000). The fluorescein solution is mixed with the substance containing the antioxidant, AAPH is added and the resultant fluorescence is measured. When solution fluorescence has decreased to about half of its initial value, an antioxidant standard, such as (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), is added. The fluorescence decay curve is obtained as a result where the sample and trolox will have a lag time, i.e. a period of time where fluorescein decreased at a different rate than what is observed after it. The antioxidant capacity is determined based in the concentration of trolox, its lag time and the sample lag time.

Main disadvantage of TRAP is that not all compounds have lag time and it usually overestimates the antioxidant activity for weaker antioxidants (D. Huang et al., 2005).

2.5.2.2 Oxygen radical absorbance capacity

The oxygen radical absorbance capacity (ORAC) assay is the method used to assess hydrophilic and lipophilic chain-breaking antioxidant capacity against peroxyl radicals.

The ORAC assay was initially developed by Cao et al. (1993) and consists of a molecular probe, B-phycoerythin, to which the peroxyl radical, AAPH, is added and complete oxidation of B-phycoerythin monitored. The assay allows the degree of inhibition and inhibition time to be measured unlike other antioxidant capacity assays. However the fluorescein probe used, B-phycoerythin was not photostable and it could also interact with polyphenols trough nonspecific protein biding. To overcome stability limitation of B-phycoerythin B. X. Ou et al. (2001) suggested the use of fluorescein as a replacement has the advantage of not interacting with any antioxidant and it is photostable.

It is important to recognise that ORAC assay is not a "total antioxidant capacity" assay, as it only measures the reactivity of an antioxidant with the peroxyl radical. To have a total antioxidant capacity value, the capacity of the antioxidant in presence of different radicals needs to be measured. Besides the peroxide radical, five other ROS are listed as causing oxidative stress to human cells. Superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$). In fact studies to adapt ORAC assay to other ROS species have been developed: SORAC for $O_2^{\bullet-}$ (Zhang et al., 2009), HORAC for $OH^{\bullet}$ (B. Ou et al., 2002). Other antioxidant capacity assays have been developed to determine the antioxidant capacity of a sample against $ONOO^-$ (Chung et al., 2001), H_2O_2 (Martínez-Tomé et al., 2001) and $^1O_2$ (Fu et al., 1997).

ORAC is widely accepted as a tool to measure antioxidant capacity in the nutraceutical, pharmaceutical and food industries (D. J. Huang et al., 2002) and it was the antioxidant capacity chosen, along with FCR, to be used in the present work.

2.5.3 ORAC assay

The assay used to determine antioxidant capacity of the extract *in vitro* was adapted from the Oxygen Radical Absorbance Capacity assay developed by D. J. Huang et al. (2002) and modified by Jumbu (2012).

2.5.3.1 Materials and equipment

Phosphate buffer solution (Na_2HPO_4, pH 7.4), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), fluorescein sodium salt were purchased from Sigma-Aldrich (Dorset, UK). Fluorescence was measured on a Microplate Spectrophotometer (Promega Glomax). A digital microplate shaker (IKA® MS3) was used to mix the samples directly in the microplate. A Px2 Thermal Cycler (Thermo Electron Corporation) was used to pre-heat the microplates.

2.5.3.2 Solution preparation

2.5.3.2.1 Phosphate buffer solution

Fluorescein and trolox solutions were prepared in 75mM phosphate buffer. Phosphate buffer solution was reconstituted to a 0.1M concentration with distilled water. One litre of 75mM phosphate buffer solution was prepared using 750ml of 0.1M Phosphate Buffer and made up to 1 litre in volumetric flask with distilled water.

2.5.3.2.2 Fluorescein solution

A 6.64µM stock solution of fluorescein (D. J. Huang et al., 2002) was prepared by dissolving 249.9µg of fluorescein in approximate 50ml of 75mM phosphate buffer solution and made up to 100ml with the same 75mM phosphate buffer solution using a volumetric flask. The solution was stored at 4ºC and covered with foil to protect from any contact with light.

A 300nM working solution of fluorescein was prepared by diluting 900µl of fluorescein stock solution in 20 ml of 75mM phosphate buffer solution. The working solution was also stored at 4ºC protected from any contact with light (D. J. Huang et al., 2002).

2.5.3.2.3 Trolox solution

As previously done with gallic acid, ethanol was also used to improve trolox solubility in water. Trolox stock solution with a concentration of 20mM (D. J. Huang et al., 2002) was prepared by dissolving 250mg of trolox in 10ml of ethanol. The mixture was then made up to 50ml in a volumetric flask with 75mM phosphate buffer.

A 25µM trolox working solution was prepared by diluting 62.5µl of trolox stock solution in 75mM phosphate buffer and made up to 50ml in a volumetric flask. Due to trolox instability at room temperature, the working solution was divided into 1ml aliquots and stored at -20ºC.

A trolox standard curve was determined in each ORAC assay measurement. The standard curve was prepared with three different concentrations 25µM, 12.5µM and 6.25 µM. Trolox concentrations of 12.5µM and 6.25 µM were directly prepared in the microplate with 75mM phosphate buffer. A microplate shaker was used to ensure a homogeneous mixture.

2.5.3.2.4 AAPH

AAPH is an unstable solution and it was freshly prepared for every ORAC assay measurements. The AAPH solution was prepared according to Jumbu (2012) by dissolving 0.414g of AAPH in 5ml of 75mM phosphate buffer. The AAPH solution was incubated at 37ºC in a water bath prior to use.

2.5.3.3 Procedure

Blank, trolox and sample solutions were prepared in quadruplicate in a 96 well black microplate. 150µl of Fluorescein are mixed with 25µL of blank/trolox/sample solution in a microplate well while for the control sample, 50µl of 75mM phosphate buffer was added instead. An example of a microplate prepared can be seen in Table 2-2. The solutions were stirred for three minutes using the microplate shaker.

Before AAPH addition, the samples previously prepared need to be at the same temperature as AAPH, 37ºC. The microplate was pre-heated to 37ºC in the thermal cycler. The thermal cycler was pre-heated to 37ºC for two minutes and a half after which the microplate was placed inside it. After 2 minutes the microplate was removed from the thermal cycler.

20µl of AAPH was added to each well except for the control samples. The microplate was stirred for 20s in the microplate shaker and then it was placed inside the microplate spectrophotometer for 45 minutes, where fluorescence was measured every one minute.

For every measurement a control and a blank sample was needed. Control sample has the objective to verify that fluorescein solution is only decomposed when in the presence of AAPH. Control is prepared by adding 50µl of 75mM phosphate buffer where 25µl accounts for sample solution and other 25µl accounts for AAPH. A blank solution has the objective to determine how fluorescein decomposes in the presence of AAPH without the addition of an antioxidant solution.

Table 2-2 – An example of a microplate layout.

	1	2	3	4
A[1]	150µl Fluorescein 50µl Phosphate Buffer	150µl Fluorescein 50µl Phosphate Buffer	150µl Fluorescein 50µl Phosphate Buffer	150µl Fluorescein 50µl Phosphate Buffer
B[2]	150µl Fluorescein 25µl Phosphate Buffer	150µl Fluorescein 25µl Phosphate Buffer	150µl Fluorescein 25µl Phosphate Buffer	150µl Fluorescein 25µl Phosphate Buffer
C	150µl Fluorescein 25µl Trolox 25µM	150µl Fluorescein 25µl Trolox 25µM	150µl Fluorescein 25µl Trolox 25µM	150µl Fluorescein 25µl Trolox 25µM
D	150µl Fluorescein 25µl Trolox 12.5µM	150µl Fluorescein 25µl Trolox 12.5µM	150µl Fluorescein 25µl Trolox 12.5µM	150µl Fluorescein 25µl Trolox 12.5µM
E	150µl Fluorescein 25µl Trolox 6.25µM	150µl Fluorescein 25µl Trolox 6.25µM	150µl Fluorescein 25µl Trolox 6.25µM	150µl Fluorescein 25µl Trolox 6.25µM
F	150µl Fluorescein 25µl Sample Conc.1	150µl Fluorescein 25µl Sample Conc.1	150µl Fluorescein 25µl Sample Conc.1	150µl Fluorescein 25µl Sample Conc.1
G	150µl Fluorescein 25µl Sample Conc.2	150µl Fluorescein 25µl Sample Conc.2	150µl Fluorescein 25µl Sample Conc.2	150µl Fluorescein 25µl Sample Conc.2
H	150µl Fluorescein 25µl Sample Conc.3	150µl Fluorescein 25µl Sample Conc.3	150µl Fluorescein 25µl Sample Conc.3	150µl Fluorescein 25µl Sample Conc.3

[1]Control, [2]Blank

2.5.3.4 Relative ORAC value

The fluorescence values measured every minute over 45 minutes provides the decay curves for every control/blank/trolox/samples (Figure 2-6). To determine the relative ORAC value the area under the curve (AUC) of the blank, control, trolox and samples needs to be determined first. The AUC was determined using the following equation:

$$AUC = 0.5 + f_1/f_0 + \cdots f_i/f_0 + \cdots f_{44}/f_0 + 0.5(f_{45}/f_0) \quad (2\text{-}3)$$

Where f_0 is the initial fluorescence (minute 0) and the f_i is the fluorescence at minute i.

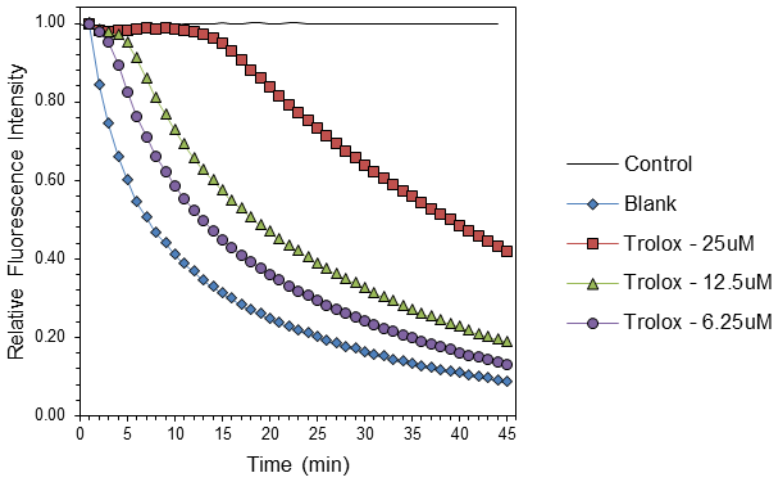


Figure 2-6 – Trolox decay curve.

All measurements were normalised with the blank that is designated as Net AUC, i.e. the difference between the AUC of trolox and sample to the blank. The relative ORAC value was determined by using the linear correlation between net AUC and concentration. D. J. Huang et al. (2002) showed that net AUC of trolox will have a linear correlation to its concentration for concentrations between 50μM and 6.25μM. For other samples, this concentration range needs to be determined. D. J. Huang et al. (2002) suggest starting by diluting samples to 1% (v/v). Lower concentrations were prepared by diluting by half the previous concentration, i.e. 0.5% (v/v), 0.25% (v/v), 0.125% (v/v), etc.

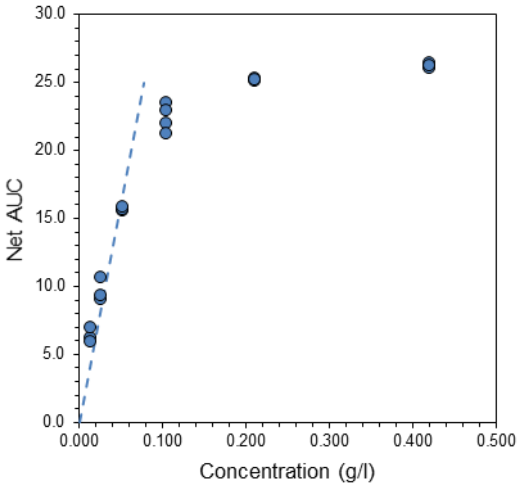


Figure 2-7 – Net AUC plot of cider lees supernatant at different concentrations.

Figure 2-7 shows an example of a cider lees sample. A linear correlation between net AUC and concentration was verified only for the lowest three concentrations prepared in this example. These three concentrations will be used in a run along with trolox to determine its relative ORAC value. Relative ORAC value was determined by the following equation:

$$Relative\ ORAC = \frac{slope(sample)}{slope(Trolox)} \quad (2\text{-}4)$$

The relative ORAC value is expressed as μM of trolox/g of dry weight (DW). If molar concentration of the samples is available, the relative ORAC value can be also expressed as M trolox/M sample.

2.5.3.5 Limitations

The spectrophotometer used could only record 32 readings per minute and as each sample was prepared in quadruplicate only eight different samples can be measured at one time. As seen in Table 2-2 the first five rows are reserved for control, blank and three different concentrations

of trolox. As each sample needs to be measured with three different concentrations, only one sample can be measured by plate.

The spectrophotometer used does not have the capacity to be at specific constant temperature, so it operates at room temperature. AAPH needs to be at 37ºC to act as a peroxyl free radical. So for every measurement AAPH and the control/blank/trolox/samples need to be pre-heated to 37ºC prior to be mixed. However, as the spectrophotometer does not have the ability to keep temperature constant, over time the temperature will decrease and it will not be possible to achieve a complete degradation of the fluorescein.

CHAPTER 3

AN ASSESSMENT OF THE UTILITY OF SUBCRITICAL WATER AND ITS IMPACT ON THE EXTRACTION OF POLYPHENOLS FROM CIDER LEES

3.1 Introduction

By-products produced by food and drinks industries represent an environmental problem due to their disposal costs and the limited options to be valorised (Chae et al., 2001, Ferreira et al., 2010). However there is potential routes to adding value to agri-food and drink by-products as they are composed by high amounts of proteins, polysaccharides, lipids and aliphatic and aromatic compounds, which can exhibit antioxidant activity that it is of great interest for pharmaceutical, cosmetic and food industries (Federici et al., 2009).

Cider production has increased significantly in the UK in the past 10 years and consequently its by-products production also has increased (NACM, 2010b). Apples, especially cider apples, are widely recognised to contain high amounts of bioactive compounds, more specifically, phenolics (Rosa M. Alonso-Salces et al., 2004b, Guyot et al., 1998, Marks et al., 2007a, Marks et al., 2007b, Sanoner et al., 1999). By-products from cider industries can be divided into apple pomace and cider lees. While apple pomace is the solid residue from juice extraction, cider lees

a slurry solution contains the spent yeasts cells from the fermentation stages. The latter has not been studied as extensively as apple pomace (R. M. Alonso-Salces et al., 2001, Bai et al., 2010, Pingret et al., 2012, Plaza et al., 2013, Andreas Schieber et al., 2003, H. Wijngaard and Brunton, 2009a), in terms of its composition and potential value. Cider lees have the potential to contain apple cider phenolics but also to contain proteins, lipids and polysaccharides from yeast cells.

Phenolics have been recognised as antioxidants, which take part in the prevention of free radical induced diseases, i.e. degenerative diseases such as cancer and cardiovascular diseases (Boyer and Liu, 2004, Cooper et al., 2004, Crozier et al., 2009, Nichenametla et al., 2006, Sies, 2010),(Del Rio et al., 2010), therefore phenolics can be used in products like cosmetics, pharmaceuticals or functional foods (Valls et al., 2009).

Subcritical water has the advantage of being non-toxic and at the same time behave similarly to organic solvents, as water dielectric constant decreases at higher temperatures and becomes closer to organic solvents such as acetone or methanol (Smith, 2002), it also has showed high selectivity and shorter extractions times (Basile et al., 1998, Herrero et al., 2006).

Subcritical water applied with elevated temperatures to a mixture like cider lees, where it is expected to have phenolics, proteins, polysaccharides and lipids, and because its catalytic nature it could induce interactions, reactions and transformations among these compounds which would create novel compounds. Proteins and phenolics can interact to form complexes that are responsible for haze in fermented beverages such as beer (Kroll et al., 2003). Proteins and polysaccharides at high temperatures can take part of glycation reactions to form high molecular weight brown pigments, known as melanoidins (Moreira et al., 2012, Singh et al., 2001). In catalytic conditions at high temperatures, reactions as oxidation, hydrolysis and polymerisation are favoured. For example, phenolics during coffee roast go through oxidation and dimerisation reactions (Mullen et al., 2011).

Bahari (2010) have previously used subcritical water to recover bioactive compounds from cider lees with temperatures ranging from 100ºC to 250ºC with extraction times from 0 to 30 minutes. It was observed that highest protein and carbohydrate recovery was obtained at 150ºC while total phenolic content and antioxidant activity was higher at 200ºC and 225ºC, respectively. It was also observed the formation of a by-product, 5-HMF, from dehydration of monosaccharides.

The present chapter had the objective to assess the impact of subcritical water conditions, under batch mode, on the recovery of phenolic compounds from cider lees and the associated antioxidant properties of the extracts derived from the cider lees at different temperatures.

3.2 Materials and experimental procedures

3.2.1 Cider lees

All the cider lees samples used during the course of this work were collected from Bulmers® (Hereford, UK). Three batches of cider lees were collected at different dates. The first cider lees sample was collected on the 28th of August of 2007 and it has been stored at -20ºC, while cider lees samples collected on the 14th of September of 2011 and on the 18th of June of 2012 were stored at 4ºC for immediate use.

Cider lees are normally collected after a filtration stage, however the sample collected on the 14th of September of 2011 was collected at a stage before filtration, and as consequence the sample was expected to have a lower total solids concentration.

3.2.2 Subcritical water (SubCW) extraction

Subcritical water mediated extraction of phenolics from cider lees was done using a 300ml stainless steel pressure vessel (Parr Instrument Company, model 5521) equipped with an

automatic stirrer and a heating jacket. The vessel incorporated a cooling system which consists in a U-shape ¼' tube and a water/ice bath is used as source for this cooling system and also for the cooling of the sample collection tube. A controller (Parr Instrument Company, model 4836) is used to set temperature and stirrer speed. Nitrogen gas at 200bar was supplied by BOC Limited (UK) and used to purge the system and partially pressurise the system. Figure 3-1 describes the experimental apparatus.

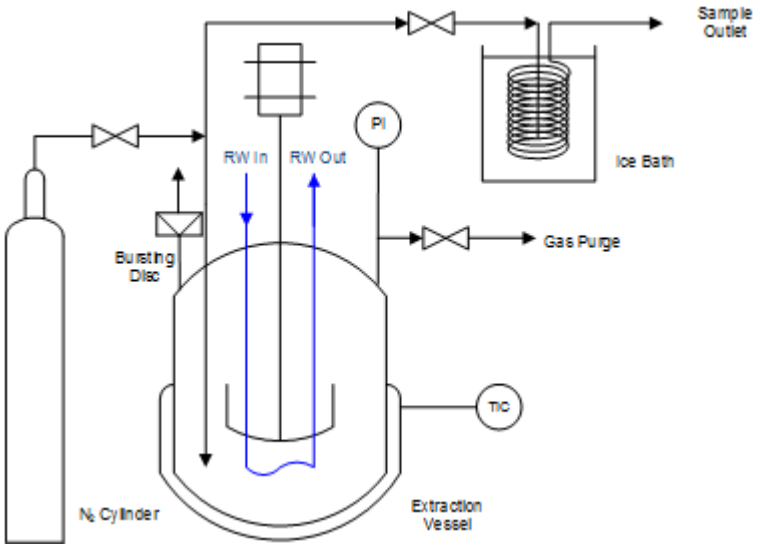


Figure 3-1 – Subcritical water extraction experimental apparatus.
RW – refrigeration water; PI – pressure indicator; TIC – temperature indicator and controller.

For large volume extraction, a 5 litres stainless steel vessel (Parr Instrument Company, model 4582) has been used. The 5 litres vessel experimental apparatus was similar to the one described in Figure 3-1 except it was equipped with chiller set at 0ºC instead of a water bath for cooling the sample collection tube and it does not have vessel cooling system incorporated.

For safety reasons, the pressure vessel was loaded with no more than 2/3 of its total volume. Prior to loading the cider lees slurry was vigorously stirred to ensure uniform crude sample was added to the reactor. The reactor was closed and the cooling system was turned on. The stirrer speed used was different for each vessel apparatus. For the 300ml vessel apparatus the stirrer

was set to 1000rpm, however due to stirrer limitation, the 5 litres vessel apparatus stirrer was set to only 80rpm. Before pre-heating the reactor, air inside the reactor was purged using nitrogen gas. The reactor was initially pressurised to between 50 and 70 bar, depending on the final temperature of extraction. All extractions were carried at a constant pressure of 100bar. For each different extraction temperature, different pre-heating times were needed as illustrated in Table 3-1. Time 0 of extraction was set when the desired temperature was reached.

Table 3-1 – Average heating time on the 300ml and the 5l vessel.

Pre-Heating *(min)*	*100ºC*	*120 ºC*	*150 ºC*	*170 ºC*	*200 ºC*	*220 ºC*	*250 ºC*
300 ml vessel	12	15	18	22	28	31	34
5l vessel	27	-	33	-	38	-	-

Collected extracts were centrifuged at 10000rpm for 10 min prior to analysis. The extract samples from the 300ml reactor were centrifuged using 50ml centrifuge tubes in a Sigma 3K30 refrigerated Centrifuge. For larger volumes of extracts 500ml centrifuge bottles were used with a Beckman J2-21 Centrifuge was used (500ml centrifuge bottles). Extract samples were stored at 4ºC and all extract precipitates were discarded.

3.2.3 Acetone polyphenol extraction

Acetone polyphenol solvent extraction method was adapted from (Lazarus et al., 1999). Cider lees were centrifuged for 10 minutes at 4000rpm using a Jouan C422 Centrifuge. The precipitated solid was separated from the supernatant and dried using an Edwards Freeze Dryer (Model EF 03, Edwards High Vacuum). Acetone polyphenol extraction was carried out in triplicate and for each extraction 500mg of the precipitated dried cider lees was mixed with 5ml of acidified aqueous acetone (70:29.5:0.5 by volume of acetone:water:acetic acid). The mixture was vortexed for 1 minute using a Jencons MixiMatic and centrifuged for 10 min at 4000rpm

using a Jouan C422 Centrifuge. The supernatant and precipitated solids were freeze dried separately, re-solubilised in distilled water and stored at 4ºC before analysis.

Cider lees extracts produced at 100ºC, 150ºC and 200ºC with extraction times of 0, 10, 20 and 30 minutes were dried using an Edwards Freeze Dryer (Model EF 03, Edwards High Vacuum). All dried extracts were subjected to acetone polyphenol extraction (triplicate) as described above with few differences. Instead of 500mg of dried extract, 1g was used and acetone polyphenol extraction was performed with pure acetone instead of aqueous acetone. Supernatant and precipitate from subcritical water hydrolysis of cider lees were both dried using a freeze drier. Dried supernatant and dried precipitate were solubilised in distilled water to a known concentration and stored at 4ºC prior to analysis.

3.2.4 Mass spectrometry

3.2.4.1 Material and equipment

Mass spectrometry (MS) was performed in a MS Time-of-flight electron spray from Waters LCT Systems using negative mode and within a range of 100 and 1000m/z.

3.2.4.2 Procedure

The MS used was not connected to an HPLC system, so samples had to be separated beforehand. The HPLC method described in 2.4 was used with a minor change. Injection volume of 100µl was used instead of the original 10µl, to ensure a higher concentration of the peak being collected. Collection was done in duplicates and the total volume of each peak was between 0.5 and 1 ml. The peaks collected were stored at 4ºC before being measured in the MS.

Total solids method, Folin Ciocalteu's method and HPLC analysis are described in 2.2.1, 2.3.4.1 and 2.5.3.1 respectively.

3.3 Results and discussion

3.3.1 The variability in total phenolic and dry weight content of cider lees

Cider lees is made up of residues from fermentation, maturation and filtration stages of cider manufacture and therefore it was a slurry solution broadly composed of spent yeast and residual cider (Figure 1-3). However cider lees collected on 14th of September of 2011 were collected prior final filtration (see process diagram in Figure 1-3) which results in a lower total dry weight as observed in Table 3-2.

Table 3-2 – The comparisons of total dry weights and the total phenolic content of the supernatant of three cider lees samples collected at three different dates from the same cider manufacturing facility.

Date of Collection	Total Dry Weight (g/l)	Supernatant Dry Weight (g/l)	Supernatant Total Phenolic Content (mg/l GAE)	Supernatant Total Phenolic Content (mg GAE/g Cider Lees DW)
28/08/2007	102.5±0.4	-	547±17	5.3±0.6
18/06/2012	90.3±1.4	45.2±1.4	1180±17	12.9±0.4
14/09/2011	51.5±2.3	-	1034±33	20.4±0.9

When allowed to settle or when subjected to centrifugation, the cider lees slurry will partition into two fractions, 1) an aqueous/supernatant fraction which contains dissolved and/or suspended solids and 2) a settled/precipitated fraction which is dominated by spent yeast as solid biomass.

3.3.2 Total phenolic and dry weight content of the supernatant fraction from cider lees slurry

The total dry weight (DW) of cider lees slurry and total phenolic content (TPC) of the supernatant of the corresponding slurry was analysed for three cider lees samples, collected at different dates from the same Bulmers cider manufacturing plant (Table 3-2). Total phenolic content of cider is dependent of factors such as apple variety, maturity, fruit season, light exposure, harvest year and storage conditions and processing are responsible for the TPC

differences observed in different types of cider (Boyer and Liu, 2004, Marks et al., 2007a). Therefore the potential phenolic content of cider lees and in turn cider lees will be dependent on the type of cider being manufactured during the period of cider lees collection and is thought to account for the variation observed. The works of Marks et al. (2007b) illustrated that the phenolic composition of 23 different ciders produced in the UK, analysed using HPLC, varied from 44 mg/l (ABV 3.5%) to 1559mg/l (ABV 7.4%). Therefore despite sample variation of the material collected the total phenolics content falls within the expected range observed by Marks et al. (2007b).

3.3.2.1 Total phenolic and dry weight content of the precipitated/spent yeast fraction from the cider lees

The total TPC of cider lees was evaluated using aqueous acetone polyphenol extraction. Aqueous acetone polyphenol extraction was performed on the sediment from cider lees. In Table 3-3 it is observed that using aqueous acetone 4.3% of the total cider lees dry weight was extracted and TPC extracted accounts to 11.6% of the total phenolic measured. So it can be said that the total total phenolic content to be extracted from cider lees was 14.6±0.4 *mg GAE/g Cider Lees DW.*

Table 3-3 – Cider lees potential total phenolic content (Cider Lees sample of 18/06/2012).

	Yield	**Total phenolic content**	
	%(w/w)	*(mg GAE/g Extract DW)*	*(mg GAE/g Cider Lees DW)*
Supernatant	50.1±1.1	25.7±0.7	12.9±0.4
Sediment	4.3±0.3	40.2±0.1	1.7±0.0
Total	54.4±1.4	-	14.6±0.4

3.3.3 Evaluation of the impact of subcritical water extraction conditions on the phenolic and dry weight content of extracts from cider lees

The cider lees sample collected on the 18th June 2012 was used for the present experimental work. Subcritical water extraction from cider lees was performed for temperatures equal to 100, 120, 150, 170, 200, 220 and 250ºC with extractions times of 0, 5, 10, 20, 30 and 60 minutes, at a constant pressure of 100bar.

It is important to note that in order to perform subcritical treatment of any biomass, water was first added at a specific biomass:water ratio. Performing such experiments Bahari (2010) showed that subcritical water could de-absorb phenolics from the cell walls of spent yeast from cider lees. However, due to the fact that cider lees was a slurry with a biomass:water ratio of ~10%, the entire slurry can be subjected to subcritical water conditions without the need to provide additional water. The results described in this chapter (Chapter 3) were derived from subjecting the entire cider lees slurry to subcritical water conditions.

3.3.3.1 Impact of temperature and time on the dry weight content of subcritical water extracts of cider lees

As illustrated in section 3.3.1 the supernatant/liquid fraction of cider lees had around 45g/l of dry matter (Table 3-2). Therefore when assessing the impact of subcritical water operating parameters, such as temperature and time, on the hydrolysis/solubilisation of spent yeast within cider lees and the resulting dry weight of the extracts, it was necessary to first normalise the estimates against the initial dry weight of the cider lees.

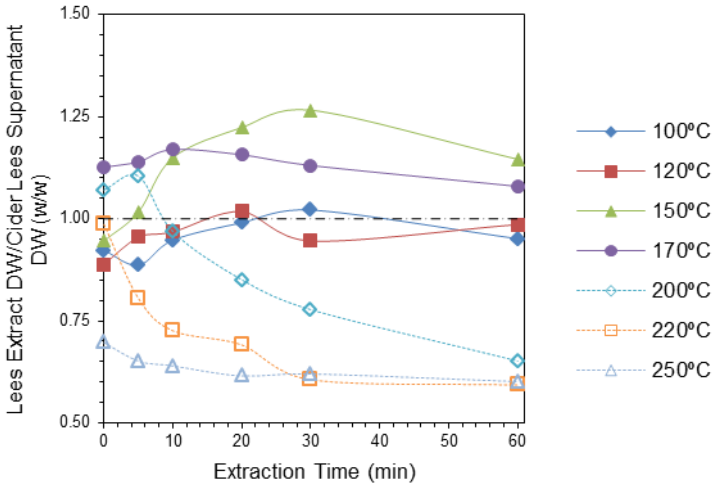


Figure 3-2 – Yeast extract DW as a function of the initial cider lees supernatant DW (See Table A-1 on Appendix A for errors associated with these results).

Figure 3-2 illustrates the interaction between temperature and residence time on the soluble dry weight content of cider lees extracts. Broadly three temperature regimes were identified, these were 1) 100-120ºC: where the dry weight content over the residence period was lower than the initial dry weight of the supernatant; 2) 150-170ºC: under these conditions the initial dry weight of the supernatant was augmented and remained above the initial weight at each time point; 3) 200-250ºC: where the soluble dry weight content of the extracts was below that of the initial dry weight of the supernatant.

Before analysing the dry weight data in more detail it is important to provide the context in which samples were collected. All experiments were conducted in batch and post subcritical water treatment, the entire cider lees fraction was subjected to low speed centrifugation to precipitate non soluble biomass, thereby creating an extract which supported soluble and suspended biomass. Furthermore, it is widely understood that naturally occurring chemical building blocks present in biomass can undergo conjugation, polymerisation and overall transformation under subcritical water conditions.

The highest yield of extraction was obtained at 150ºC after 30 minutes. Temperature regime of 100ºC to 120ºC shows a supernatant dry weight lower than the initial cider lees supernatant which could indicate that less water soluble compounds and/or high molecular weight compounds are being precipitated during the centrifugation step.

When temperatures between 200 and 250ºC were applied the cider lees extract dry weight was lower than the initial dry weight, which indicates that the initial compounds could have taken part in reactions where the resultant compounds were not soluble in water and again, centrifugation might had enhanced precipitation of high molecular weight molecules such as polymers formed during extraction.

3.3.3.2 Impact of temperature and time on total phenolics content (TPC) of subcritical water extracts of cider lees

Previous work by Bahari (2010) determined that the subcritical water conditions of 200ºC for 10 minutes resulted in the highest total phenolic content in a temperature range of 100ºC to 250ºC with extraction times up to 30 minutes. However, in the present work by using subcritical water with temperatures between 100 and 220ºCwith extraction times up to 30 minutes, maximum TPC (38.1±0.5 mg GAE/g Cider Lees DW) was achieved at the slightly elevated temperature of 220ºC for 10 minutes (Figure 3-3).

It is observed in Figure 3-3 that using SubCW to extract polyphenols from cider lees results in a yield 2 times higher than that observed when using aqueous acetone (Table 3-3). It is also observed that a similar TPC to the one obtained by aqueous acetone extraction (Table 3-3) can be obtained with subcritical water at 150ºC for 10 minutes (Figure 3-3).

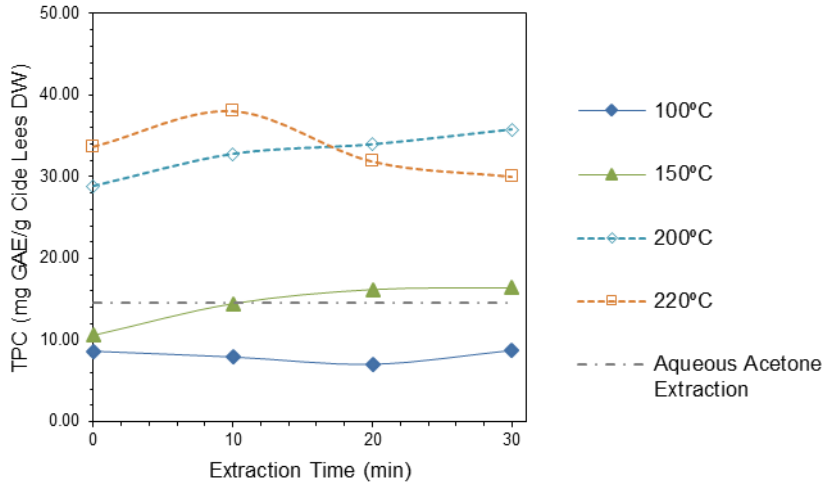


Figure 3-3 – TPC results for SubCW extraction and aqueous acetone extraction from cider lees (See Table B-1 on Appendix B for errors associated with these results).

Figure 3-3 also illustrates that when cider lees were subjected to SubCW conditions at 200 and 220ºC the TPC increased to more than double of the TPC recorded at 150ºC. Also the pre-heating stage showed to be an important factor as TPC increased around 3 times more when temperatures of 200 and 220ºC were used compared to 100 and 150ºC. Pre-heating to 200 and 220ºC has taken 20 to 26 more minutes than 150ºC and it was in total 28 to 34 minutes (Table 3-1). After temperatures of 200 and 220ºC were established, although an increase in TPC was verified, this increase was not as high as observed during pre-heating.

To date there are no reports of polyphenol extraction from cider lees beside the work developed within University of Birmingham (Bahari, 2010, Jumbu, 2012). On the other hand, apple pomace has been the target of several studies, not only on its chemical characterisation but also in the extraction of polyphenols. Although apple pomace, as a by-product, cannot be completely comparable to cider lees, its polyphenols are from the same source as cider lees, which makes apple pomace an acceptable comparison. In the past years different techniques have been employed to extract phenolics from apple pomace.

Conventional extraction methods to extract polyphenols from apple pomace have been used by Reis et al. (2012), Suarez et al. (2010) and H. H. Wijngaard and Brunton (2009b) using organic solvents such as acetone and methanol. H. H. Wijngaard and Brunton (2009b) used 65% aqueous acetone at 25ºC for 60 minutes to produce an apple pomace extract with a polyphenol concentration of 14.15 mg GAE/g DW, while Suarez et al. (2010) produced an extract with a TPC of 6.48 mg GAE/g DW using 70% aqueous acetone at 20ºC. Reis et al. (2012) employed a sequential extraction of apple pomace, using water as the first solvent, followed by 40% methanol and finally by 40% acetone. It was observed that 67% of the polyphenols extracted were extracted with water. A total of 2.56 mg GAE/g DW accumulated polyphenols was extracted.

Pingret et al. (2012) used ultrasound-assisted water extraction to recover polyphenols from cider apple pomace. Extraction was performed at 40ºC for 40 minutes and a total of *5.517* mg GAE/g DW were extracted, while using conventional extraction, by simple mixing apple pomace in water, a total of 4.905 mg GAE/g DW were obtained.

SubCW as a way to recover polyphenols from apple pomace has been used by H. Wijngaard and Brunton (2009a) and Plaza et al. (2013). While H. Wijngaard and Brunton (2009a) used ethanol as co-solvent, Plaza et al. (2013) only used water as a solvent for the SubCW extraction. H. Wijngaard and Brunton (2009a) obtained a maximum TPC of 57.82 mg GAE/g DW using 50% of ethanol with a temperature of 200ºC for 5 minutes. However, unwanted products were detected, in particularly 5-HMF. As a result, to avoid formation of unwanted products, H. Wijngaard and Brunton (2009a) suggested to use 60% Ethanol at 102ºC for 5 minutes, which resulted in a concentration of phenolics equal to 14.42 mg GAE/g DW. Plaza et al. (2013) without the use of any organic solvent obtained maximum TPC of 13.66 mg GAE/g DW at 200ºC after 10 minute. However 5-HMF formation was also detected.

Different results of polyphenol extraction were obtained in the different studies, even when similar techniques were applied, which shows that the final polyphenol content will strongly depend on the type and origin of apples used. Nonetheless, these results give an indication of the range expected of polyphenols extraction from apple pomace, which ranges from 2.56 up to 57.82 mg GAE/g DW.

Cider lees with an initial TPC equal to 12.9 mg GAE/g DW and a total of 14.6 mg GAE/g DW (Table 3-3) had a TPC comparable to the maximum TPC observed by solvent extraction of apple pomace H. H. Wijngaard and Brunton (2009b) which makes cider lees a by-product as interesting as apple pomace.

As seen in Figure 3-3, SubCW extraction of cider lees produced a maximum TPC of 38.1 mg GAE/g DW, which although was lower than the TPC produced by H. Wijngaard and Brunton (2009a) when compared to SubCW without the use of co-solvents, its TPC was double then the amount produced by Plaza et al. (2013).

H. Wijngaard and Brunton (2009a) and Plaza et al. (2013) have obtained maximum TPC using SubCW at 200ºC after 5 and 10 minutes of extraction, respectively. Although in the present work maximum TPC was achieved at a higher temperature of 220ºC, in the works mentioned higher temperatures were not tested and a decrease in TPC with the increase of temperature was not observed, which means that temperature and extraction temperature were not tested to the limit.

In concordance with H. Wijngaard and Brunton (2009a) and Plaza et al. (2013), previous work on SubCW extraction from cider lees (Bahari, 2010) also verified the presence of 5-HMF when higher temperatures were used for phenolic extraction. A chemical characterisation of the cider lees extract produced would give indication if undesirable products are being produced and also which compounds might be responsible for the high TPC.

3.3.4 Chemical characterisation of phenolic content of cider lees supernatant and cider lees extracts produced using SubCW

In 3.3.3.2 it was observed an increase in total phenolic content from cider lees by using subcritical water at high temperatures. However the phenolics that contribute to that increase in total phenolic content are not known as the phenolic content of cider lees. To improve the understanding of phenolic composition in cider lees and respective subcritical water extracts, a chemical characterisation using HPLC and targeting simple phenolics was used.

HPLC method and calibration curves are described in 2.4.

3.3.4.1 Analysis of the phenolic content of cider lees supernatant

3.3.4.1.1 Qualitative analysis

Cider lees were a slurry solution composed by solids (yeast cell) (~5% DW) and supernatant (~5% DW) (Table 3-2). Cider lees supernatant is expected to have a similar phenolic composition to cider as it is should be mostly residual cider while the solid should be mostly composed of spent yeast cells.

Marks et al. (2007b) has studied 23 commercial ciders produced in England. Four different classes of phenolics were identified: flavanols, hydroxycinnamic acids, flavonols and dihydrochalcones. A total of 18 different compounds were identified where hydroxycinnamic acids were the predominant polyphenol identified while flavonols were found in lower amounts.

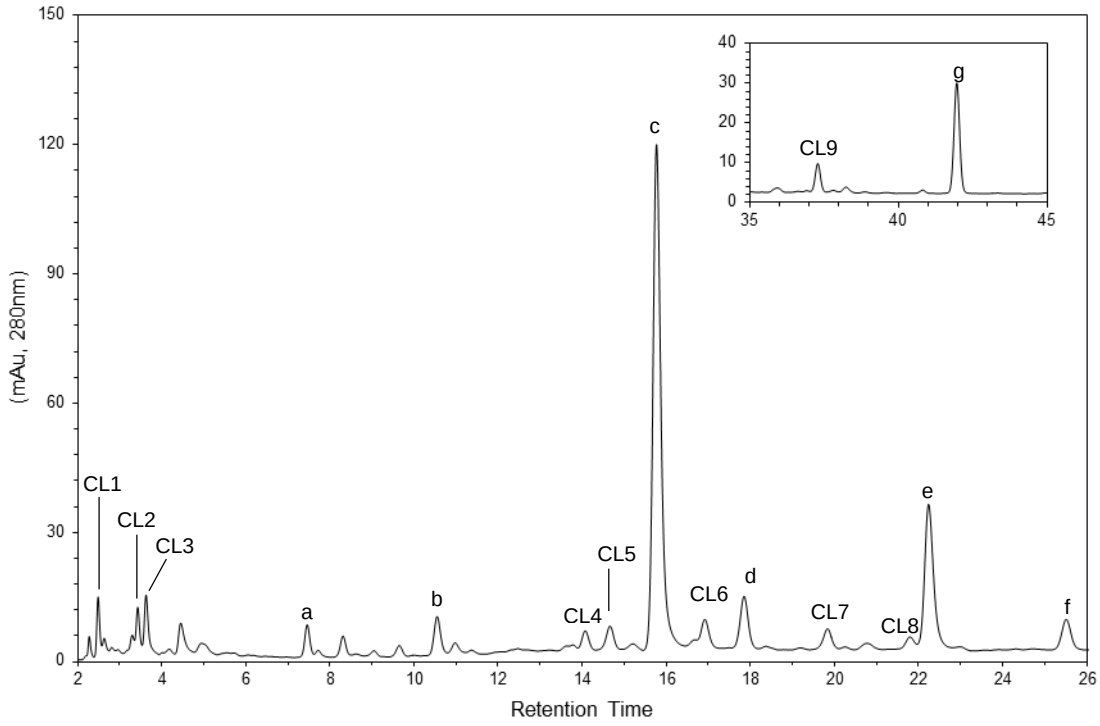


Figure 3-4 – Cider lees supernatant HPLC characterisation.
a – 5-HMF, b – Furfural, c – Chlorogenic Acid, d – Caffeic Acid, e – 4-*O*-*p*-coumaroylquinic acid, f – *p*-Coumaric Acid, g – Phloridzin, CL1-9 – unknown compounds.

The phenolic content of the cider lees supernatant was analysed as described in section 2.4.2. As illustrated in Figure 3-4 sixteen major compounds were detected, seven of which were identified: *a* (R_t=7.3 min, λ_{max}=284nm), *b* (R_t=10.4 min, λ_{max}=278nm), *c* (R_t=15.4 min, λ_{max}=326nm), *d* (R_t=17.7 min, λ_{max}=324nm), *e* (R_t=22.2 min, λ_{max}=312nm), *f* (R_t=25.5 min, λ_{max}=312nm) and *g* (R_t=41.9 min, λ_{max}=284nm). Compound *a* (R_t=7.3 min, λ_{max}=284nm) and compound *b* (R_t=10.4 min, λ_{max}=278nm) were identified as 5-HMF and Furfural, respectively, with the use of standards.

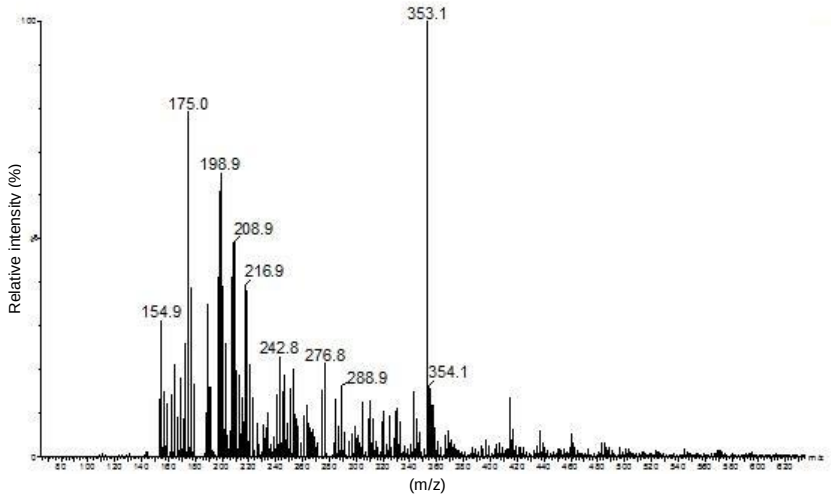


Figure 3-5 – Mass Spectrometry-Time of Flight (MS-TOF) of peak *c*, chlorogenic acid.

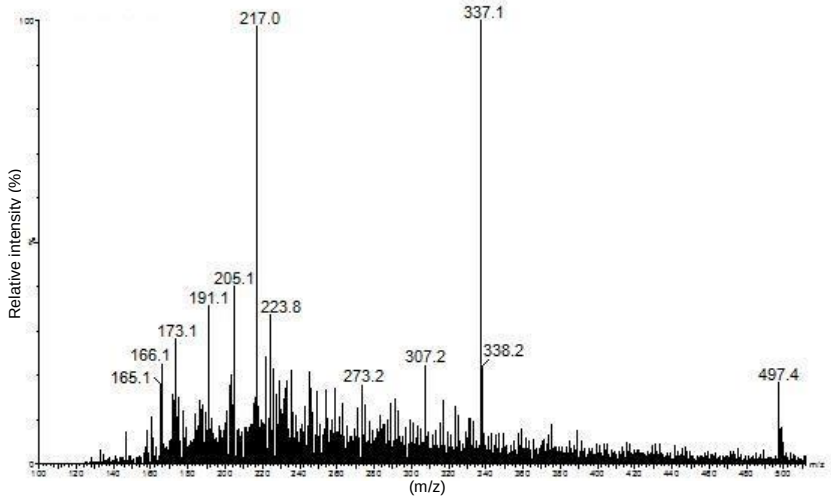


Figure 3-6 – MS-TOF of peak *e*, 4-*O*-*p*-coumaroylquinic acid.

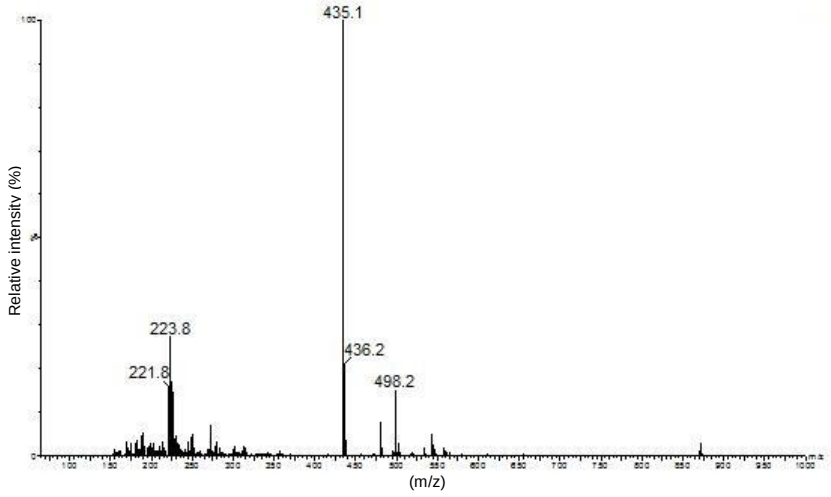


Figure 3-7 – MS-TOF of peak *g*, phloridzin.

Compound *c* with a fragmentation of the negatively charged molecular ion ($[M-H]^-$) at 353m/z (Figure 3-5), compound *e* with a $[M-H]^-$ at 337m/z (Figure 3-6) and compound *g* with a $[M-H]^-$ at 435m/z (Figure 3-7) were identified as 5-*O*-caffeoylquinic acid (chlorogenic acid), 4-*O*-*p*-Coumaroylquinic Acid and phloretin-2'-*O*-glucoside (phloridzin), respectively. With the use of standards chlorogenic acid and phloridzin were re-confirmed while compound *d* was identified as caffeic acid and compound *f* was identified as *p*-coumaric acid. With the exception of 5-HMF and furfural, all the other compounds have been previously detected in different cider samples produced in England (Marks et al., 2007b). HPLC information of compounds *a* to *g* is summarised Table 3-4.

Table 3-4 – Compounds identified on cider lees supernatant.

Compound	Compound no.	R_t (min)	UV Spectra	
			Main (nm)	Secondary (nm)
5-HMF	a	7.3	284	
Furfural	b	10.4	278	
Chlorogenic Acid	c	15.4	326	298
Caffeic Acid	d	17.7	324	298
4-O-p-Coumaroylquinic Acid	e	22.2	312	
p-Coumaric Acid	f	25.5	312	
Phloridzin	g	41.9	284	230

When cider lees sediment was subjected to aqueous acetone extraction, it was verified that the chromatographic profile was similar to cider lees supernant with one difference, it had one extract compound. This extra compound with R_t=57.2 min and λ_{max}=284nm according to compounds retention times from the HPLC method adopted from A. Schieber et al. (2001a) could be identified as phloretin, identification that was supported by phloretin standard UV spectra (Figure 3-8).

Phloretin is not found in apples, but it is a product from phloridzin decomposition that can occur during apple juice processing and cider fermentation and it has been identified in cider samples

by Marks et al. (2007b). As phloretin was not detected in cider lees supernatant it might indicate that it has been adsorbed onto the cell wall of yeast.

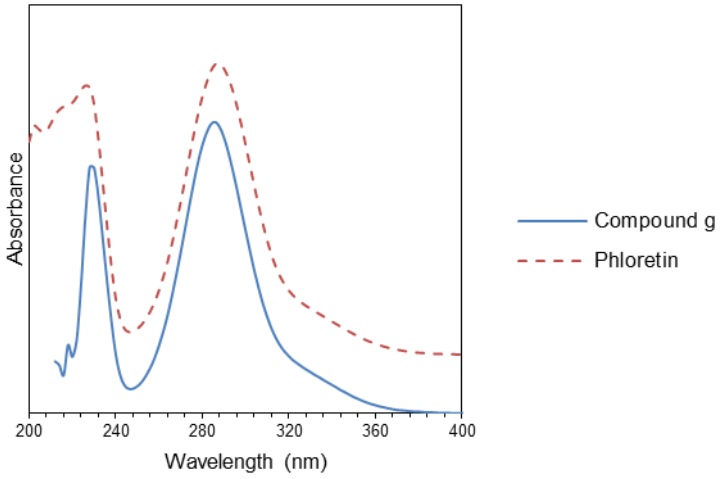


Figure 3-8 – UV spectra of compound *g* (Phloridzin) and phloretin standard[1].

The remaining major compounds detected, named as compounds CL1 to CL9 (Table 3-5), could not be accurately identified, however, suggestions of what compounds or class of compounds they could be will be discussed next.

Table 3-5 – Complete list of unknown compounds present in cider lees supernatant.

Compound no.	**R_t** *(min)*	**UV Spectra**	
		Main *(nm)*	Secondary *(nm)*
CL1	2.4	264	
CL2	3.3	258	
CL3	3.5	262	
CL4	14.0	280	230
CL5	14.4	310	230
CL6	16.8	326	232, 288
CL7	19.7	280	230
CL8	21.6	312	230
CL9	37.0	284	230

[1] water:methanol mobile phase

3.3.4.1.1.1 Compound CL1, CL2 and CL3

Mass spectrometry analysis of compound CL1 indicated molecular mass of $[M-H]^-$ at 179.1m/z $[M-H]^-$ and at 191.1m/z (Figure 3-9). The results suggest the presence of caffeic acid (180.1g/mol) and quinic acid (192.1g/mol) however these compounds were not expected to have retention time of 2 minutes. Therefore it could mean that compound CL1 is a complex of caffeic acid and quinic acid forming a structure which the column does not have the capacity to resolve.

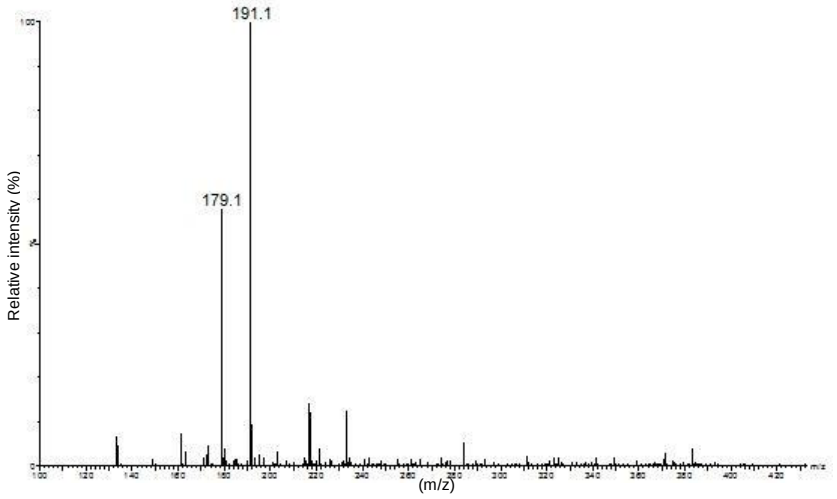


Figure 3-9 – MS-TOF of compound CL1.

Mass spectrometry analysis of compound CL2 was characterised by $[M-H]^-$ at 257.1m/z and 372.1 m/z (Figure 3-10). It also indicated $[M-H]^-$ at 128.0m/z and 185.m/z, however it was not possible to make any identification with these molecular weights to suggest which type compound CL2 could be.

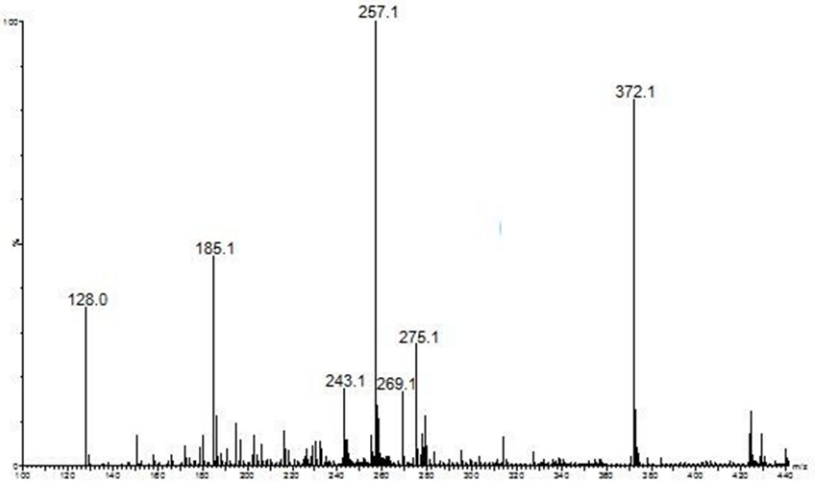


Figure 3-10 - MS-TOF of compound CL2.

HPLC results also have shown that compound CL1 and CL3 have similar UV spectra and low retention time in a reverse phase HPLC analysis, which means that these compounds should have higher polarity than all the other compounds that were identified and separated at higher retention times.

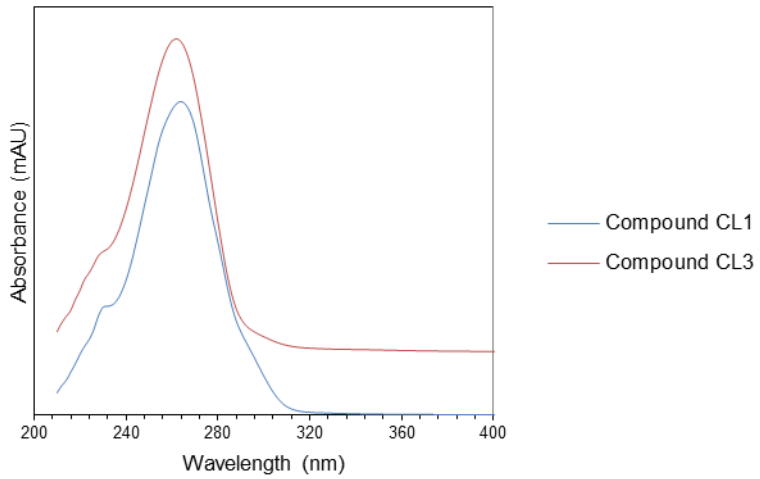


Figure 3-11 – UV spectra for compounds CL1 and CL3.

3.3.4.1.1.2 Compound CL4 and CL7

Compounds CL4 (R_t=14.0 min, λ_{max}=280nm) and CL7 (R_t=19.7min, λ_{max}=280nm) have similar UV spectra (Figure 3-12) to that of the flavanols (+)-catechin and (−)-epicatechin (Delage et al., 1991) and have previously been identified in cider by Marks et al. (2007b).

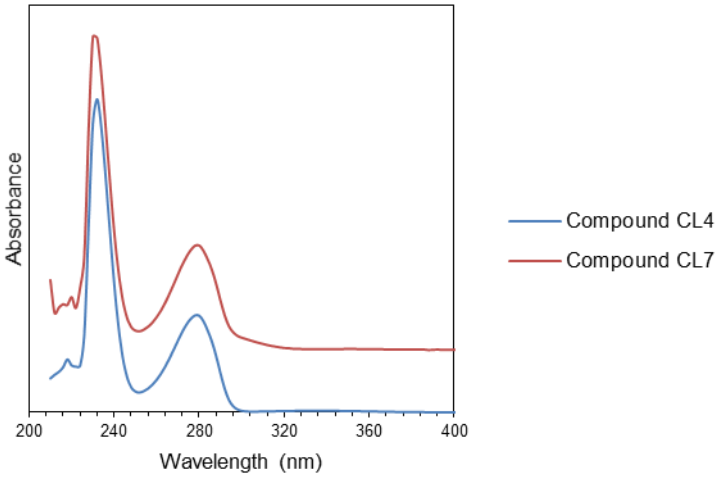


Figure 3-12 – UV spectra for compounds CL4 and CL7.

Combining the results of the UV spectra with the relative retention times of (+)-catechin and (−)-epicatechin reported by A. Schieber et al. (2001a) and Marks et al. (2007b) it was suggested that CL4 was (+)-catechin and CL7 was (−)-epicatechin.

3.3.4.1.1.3 Compound CL5, CL6, CL8 and CL9

Compounds CL5, CL6, CL8 and CL9 have UV spectra similar to compounds identified previously. Compounds CL5 and CL8 have similar UV spectra to *p*-coumaric acid (Figure 3-13), compound CL6 have similar UV spectra to caffeic acid and chlorogenic acid (Figure 3-14) and compound CL9 has similar UV spectra to phloridzin (Figure 3-16).

Besides 4-*O*-*p*-coumaroylquinic acid (4-pCoQA), Marks et al. (2007b) identified two isomers namely 3-*O*-*p*-coumaroylquinic acid and 5-*O*-*p*-coumaroylquinic acid. Isomers have the same

UV spectra but exhibit different retention times under chromatographic conditions. 4-*O*-*p*-coumaroylquinic acid has similar UV spectra to *p*-coumaric acid as compounds CL5 and CL8 have (Figure 3-13). Also these isomers were not present in apple samples, which suggest they were formed during cider processing.

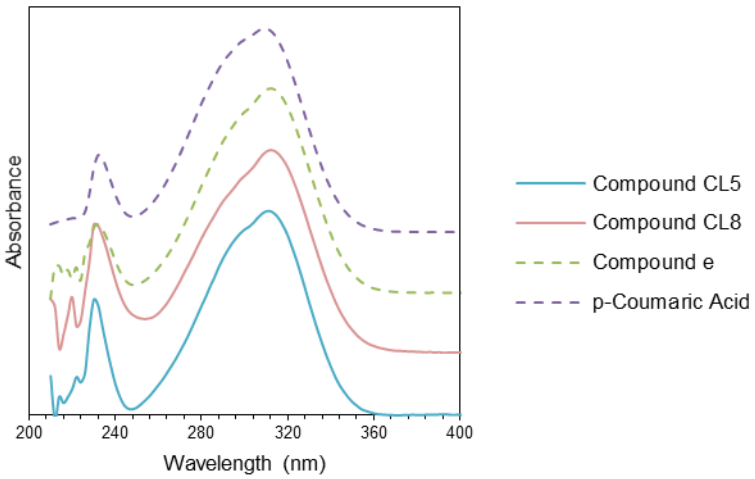


Figure 3-13 –UV spectra of Compound CL5, CL8, Compound *e* (4-*O*-*p*-coumaroylquinic acid) and *p*-Coumaric acid.

Using the relative retention times reported for the two isomers by Marks et al. (2007b) leads to suggest that compound CL5 is 3-*O*-*p*-coumaroylquinic acid and compound CL8 is 5-*O*-*p*-coumaroylquinic acid.

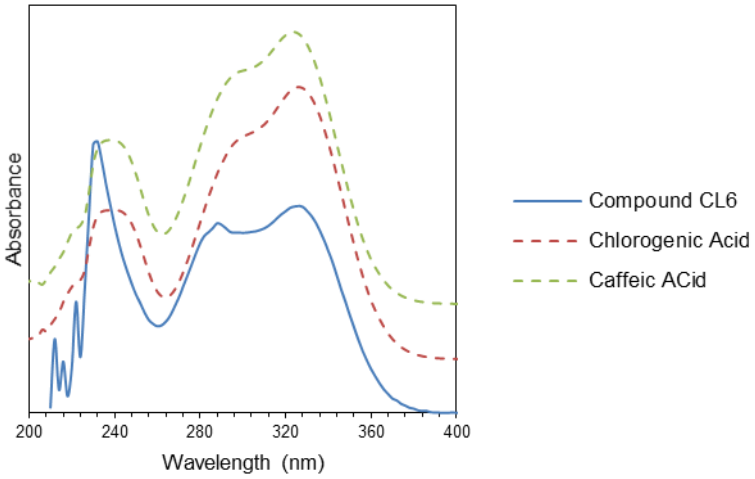


Figure 3-14 – UV spectra of compound CL6, chlorogenic acid and caffeic acid.

Compound CL6 (R_t=16.8min, λ_{max}=326nm, 288nm) shares some similarities with chlorogenic acid (R_t=15.4 min, λ_{max}=326nm, 298nm) and caffeic acid (R_t=17.7 min, λ_{max}=324nm, 298nm) as it is seen in Figure 3-14. Although compound CL6 secondary peak (λ_{max}=288nm) is different from secondary peak of caffeic acid and chlorogenic acid, they all have similar main wavelength of maximum absorption , 324-326nm. Cilliers and Singleton (1991) has observed the formation of compounds with similar UV spectra to compound CL6 from non-enzymatic autoxidation of caffeic acid. These compounds were designated as caffeicins, which are dimers from caffeic acid and are classified as neolignans.

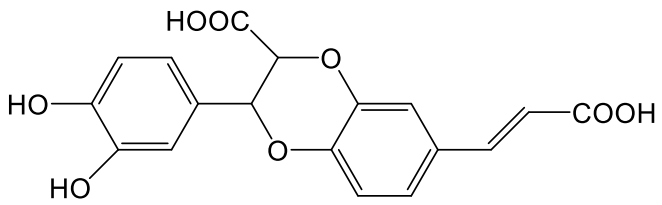


Figure 3-15 – Example of the molecular structure of a caffeicin.

There are no studies that suggest the presence of caffeicin in cider or apple derived products, however the results obtained at this stage suggest that compound CL6 will be a polyphenol derived from caffeic acid.

Chlorogenic acid and caffeic acid have the same UV spectra (Figure 3-14) as *p*-coumaric acid and 4-pCoQA, which enforces that quinic acid group in these esters does not contribute to UV absorption. Also, phloridzin (Figure 3-16) and phloretin (Figure 3-8) have similar UV spectra which once again supports the suggestion that the quinic acid group does not contribute to UV absorption.

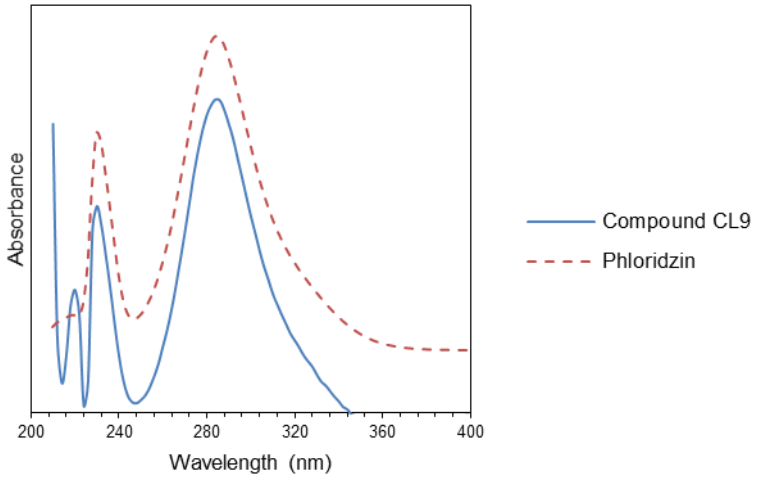


Figure 3-16 – UV spectra of compound CL9 and phloridzin.

Compound CL9 and phloridzin share the same UV spectra as seen in Figure 3-16 a compound that was also previously detected in different types of cider (Marks et al., 2007b). According to Marks et al. (2007b) and its relative retention times compound CL9 can be suggested as Phloretin-2'-*O*-(2''-*O*-xylosylglucoside).

3.3.4.1.2 Quantitative analysis

The compounds previously identified were quantified with the use of their respective calibration curves (Table 2-1). As 4-*O*-p-coumaroylquinic acid standard was not available, it was quantified as *p*-coumaric acid equivalents (p-CoE). Although the use equivalents will not give its accurate concentration, it will give the indication of which range its concentration should be.

Table 3-6 – Quantification of identified phenolics in cider lees.

Compound	Supernatant		Sediment	Total
	(mg/l)	*(mg/g Cider Lees DW)*	*(mg/g Cider Lees DW)*	*(mg/g Cider Lees DW)*
5-HMF	1.3±0.4	0.015±0.004	nd	0.015±0.004
Furfural	1.9±0.3	0.021±0.003	nd	0.021±0.003
Chlorogenic Acid	119.5±2.3	1.309±0.032	0.368±0.010	1.677±0.034
Caffeic Acid	6.2±0.9	0.061±0.014	0.021±0.001	0.083±0.014
4-O-p-Coumarylquinic Acid[2]	15.5±0.3	0.166±0.008	0.049±0.001	0.214±0.008
p-Coumaric Acid	2.6±0.2	0.029±0.002	0.007±0.000	0.036±0.002
Phloridzin	15.9±2.8	0.222±0.005	0.017±0.000	0.239±0.005

Throughout the apple juice manufacture, dehydration of monosaccharides was reported to happen where glucose and xylose was converted into 5-HMF and furfural, respectively (Spanos et al., 1990). Such reaction was favoured when monosaccharides are present in an acidic environment subjected to high temperatures (Kermasha et al., 1995). During apple juice manufacture, evaporations stages might enhance the formation of by-products as 5-HMF and furfural. It was also reported that during apple juice storage for more than 2 months, an increase of 5-HMF and furfural content was observed (Burdurlu and Karadeniz, 2003). So it was expected to detect 5-HMF and furfural in cider lees samples (Table 3-6), in amounts lower than what is recommended in fruit juices, 5-10ppm (Gokmen and Acar, 1999).

As observed by Marks et al. (2007b) in cider, chlorogenic acid was the main polyphenol present in cider lees, while caffeic acid and *p*-coumaric acid were found in low amounts (Table 3-6). In cider, chlorogenic acid ranged between 4 and 437mg/l (Marks et al., 2007b) where in cider lees chlorogenic acid was 119.5±2.3 mg/l (Table 3-6). The range of variation of chlorogenic acid in cider elucidates the variation of not only chlorogenic acid but also polyphenol content that can be expected in cider lees, as cider lees will be a combination of different types of cider produced.

[2] *p*-coumaric acid equivalent

As for the other hydroxycinnamic acids identified and phloridzin, their concentration in cider lees were also in the concentration range found in cider (Marks et al., 2007b).

p-Coumaric acid and phloridzin equivalents have been used to quantify the suggested *p*-coumaric acid isomers (CL5 and CL8) and phloretin-2'-*O*-(2''-*O*-xylosylglucoside) (CL9), respectively.

Table 3-7 – Quantification of unknown compounds (mg equivalent).

Compound no.	Supernatant (mg/g Cider Lees DW)	Sediment (mg/g Cider Lees DW)	Total (mg/g Cider Lees DW)
CL5[3]	0.021±0.002	0.003±0.000	0.024±0.002
CL8[3]	0.006±0.002	0.003±0.000	0.009±0.002
CL9[4]	0.051±0.001	0.017±0.004	0.069±0.004

Compound CL5, CL8 and CL9 were previously suggested as 3-*O*-*p*-Coumaroylquinic acid, 5-*O*-*p*-Coumaroylquinic acid and phloretin-2'-*O*-(2''-*O*-xylosylglucoside) and as their standards were not available, compound CL5 and CL8 were quantified as *p*-coumaric acid equivalents (p-CoE) while compound CL9 was quantified as phloridzin equivalents (PHLE). From Table 3-7 it can be observed that compound CL5 and CL8 were found in lower amount than its isomer 4-*O*-*p*-Coumaroylquinic acid (Table 3-6) while compound CL9 was also found in lower amount than phloridzin (Table 3-6).

It is interesting to observe that compound CL5 was found in cider lees in amounts three times higher than compound CL8 (Table 3-7) while in different ciders analysed by (Marks et al., 2007b), 3-*O*-*p*-Coumaroylquinic acid and 5-*O*-*p*-Coumaroylquinic acid were normally found with similar concentrations.

The remaining compounds (CL1 to CL4, CL6 and CL7) were quantified in terms of their area per the initial DW (Table 3-8).

[3] mg p-CoQAE/g Cider Lees DW
[4] mg PHL/g Cider Lees DW

Table 3-8 – Quantification of unknown compounds (area).

Compound no.	Supernatant (area/g Cider Lees DW)	Sediment (mg/g Cider Lees DW)	Total (mg/g Cider Lees DW)
CL1	1.355±0.169	0.089±0.005	1.444±0.169
CL2	1.792±0.157	0.058±0.001	1.850±0.157
CL3	1.957±0.114	0.136±0.004	2.093±0.114
CL4	0.687±0.057	nd	0.687±0.057
CL6	1.792±0.076	0.386±0.024	2.178±0.080
CL7	0.369±0.078	0.133±0.004	0.501±0.078

Compound CL4 and CL7 that were previously suggested as (+)-catechin and (–)-epicatechin, respectively. In Table 3-8 it is observed, in terms of area, CL4 was present in a higher concentration than CL7, while in different cider varieties, Marks et al. (2007b) has reported (–)-epicatechin (CL7) as the flavanol present in higher concentration in cider samples. This difference and also the difference previously verified with CL5 and CL8 could be the result of cider lees being the mixture of different types of ciders which could lead up to this concentration changes. Polymeric flavanols, known as proanthocyanidins, were detected by Marks et al. (2007b) in cider samples, although their detection and determination was not achieved by using a standard HPLC method. In the present work proanthocyanidins were not analysed but it is important to acknowledge that they can exist in cider and they can account for 2 to 40% of the total phenolics in cider (Marks et al., 2007b).

Quercetins were detected by Marks et al. (2007b) in low amounts in cider but it was not possible to detect them not in cider lees. Quercetins in cider determined by Marks et al. (2007b) can represent 0.2% up to 4.3% of total phenolics.

3.3.4.1.2.1 Comparison between FCR method and HPLC characterisation results

Previously only hydroxycinnamic acids and dihydrochalcones were quantified in cider lees samples, which already give an indication of the range of total phenolics in cider lees. It is

important to verify the results obtained using HPLC method against the results obtained by the FCR's method. FCR method total phenolic content was compared in Table 3-9 with the sum of all phenolics quantified in cider lees, i.e. hydroxycinnamic acids and dihydrochalcones.

Table 3-9 – Comparison between the total phenolic content by FCR's method and by HPLC analysis of cider lees supernatant.

	Concentration *(mg/g Cider Lees DW)*
FCR method	12.884±0.388
HPLC method	1.846±0.046

Comparing the results for total phenolics in cider lees determined by FCR's method and by HPLC method, a substantial difference was observed (Table 3-9). The difference could be the monomeric, dimeric and polymeric forms of flavanols not detected and quantified by HPLC method. However, according to the results obtained by Marks et al. (2007b), flavanols can be found in cider in amounts up to double of the amounts of hydroxycinnamic acids. Therefore, although flavanols should account for part of the difference between methods verified it does not account for the majority of that difference.

In 2.3.2.1 interferences in FCR's method was discussed. FCR's method does not target exclusively phenolic compounds but it targets any compounds that can act as reducing agents, therefore the results obtained for cider lees might be the result of the interference of other compounds than phenolics which can also react as reducing agents. Also there is the possibility that during FCR method where phenolics were oxidised to their respective semi-quinones, which can take part of dimerisation reaction resulting in a regeneration of their reducing ability and in consequence altering the results from FCR's method. Lastly, due to FCR's method mechanism it can also be used as an antioxidant capacity assay, as discussed in 2.5, so the results obtained seems to indicate the antioxidant capacity of the cider lees supernatant rather than the total phenolic content.

3.3.4.2 Analysis of the phenolic content of cider lees subjected to subcritical water mediated extraction

In 3.3.3 it was verified that using subcritical water, the total phenolic concentration from cider lees was increased and its highest concentration was obtained with a temperature of 220ºC after 10 minutes of extraction. Chemical characterisation of the lees extracts will allow understanding if this increase is due to the release of apple phenolics from yeast cells into solution and/or the result of the formation of new compounds under subcritical water conditions.

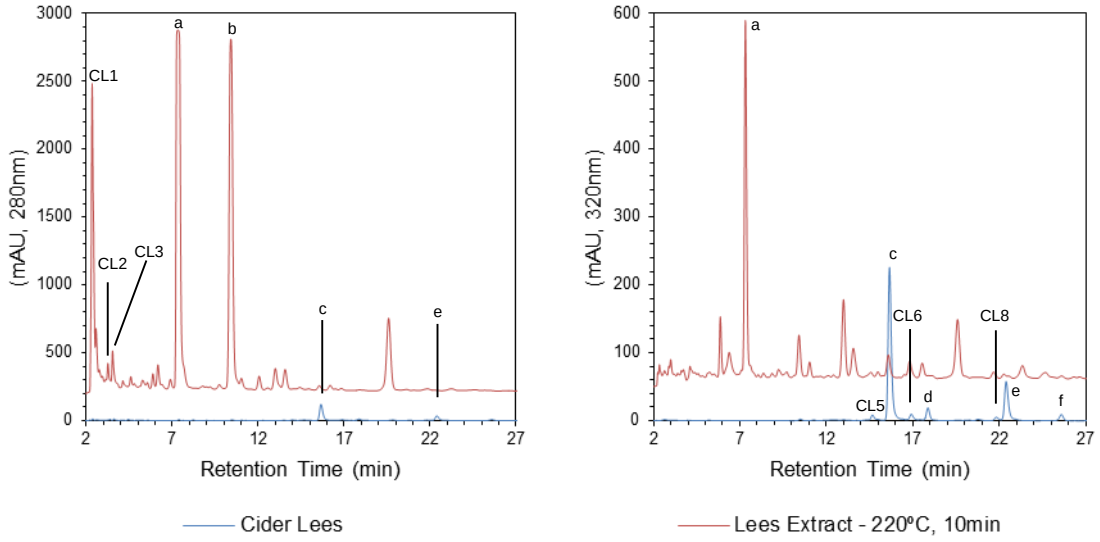


Figure 3-17 – Partial chromatogram of cider lees supernatant and of lees extract at 220ºC after 10 min.
a – 5-HMF; b – furfural; c – chlorogenic acid; d – caffeic acid; e – 4-*O*-*p*-coumaroylquinic acid; f – *p*-coumaric acid; CL-1 to CL3, CL5, CL6 and CL8 – unknown compounds.

A comparison between the chromatographic profile of cider lees and the cider lees extract produced at 220ºC after 10 minutes is illustrated in Figure 3-17. Cider lees extract chromatographic profile was significantly different from cider lees profile, where more compound peaks were observed and the main phenolic in cider lees, chlorogenic acid, was a minor compound. It is interesting to verify that main compounds, CL1, 5-HMF and furfural, were already detected in cider lees, although in low amounts.

3.3.4.2.1 Qualitative analysis

Figure 3-18 shows the chromatogram of the cider lees extract produced at 170ºC after pre-heating stage (0 minutes of extraction). This chromatogram has the particularity to have all the main compounds detected in cider lees and in the cider lees extracts which makes it ideal to use to illustrate all the compounds detected across the extracts obtained at different temperatures.

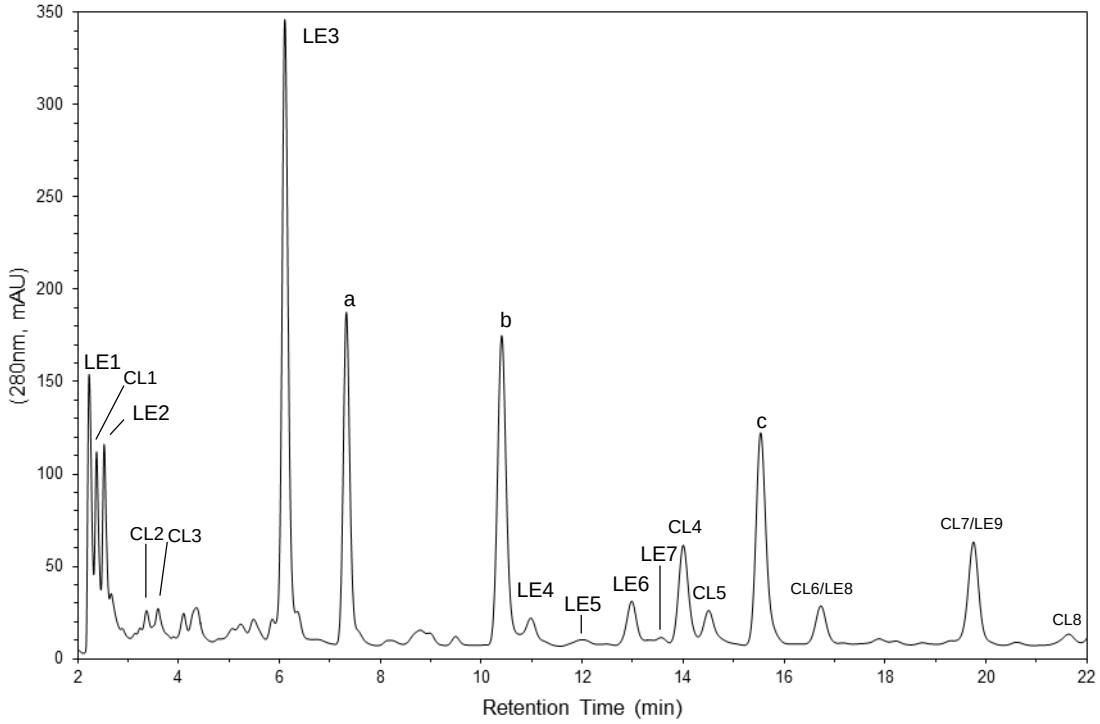


Figure 3-18 – Unknown compounds found in cider lees extracts (SubCW at 170ºC).
a – 5-HMF; b – Furfural; c – Chlorogenic Acid; CL1 to CL8 and LE1 to LE9 – unknown compounds.

In the cider lees extracts nine new compounds were detected, compounds LE1 to LE9. Exact identification of the compounds LE1 to LE9 was not possible with the information gather at this stage, however with the data retrieved from HPLC-DAD analysis (Table 3-10) and with relevant literature it was possible to suggest possible compounds match.

Table 3-10 – Complete list of unknown compounds present in the cider lees extract.

Compound no.	R_t (*min*)	UV Spectra	
		Main (*nm*)	Secondary (*nm*)
LE1	2.2	284	230
LE2	2.6	248	274
LE3	6.1	284	
LE4	11.0	252	226
LE5	12.1	256	224
LE6	12.9	280	310, 230
LE7	13.6	294	
LE8	16.7	280	230, 328
LE9	19.6	292	

3.3.4.2.1.1 Compounds LE1 and LE2

Compounds LE1, LE2 and also compound CL1 had retention times under three minutes which indicates that these compounds were barely retained in the HPLC column which could be due to its high polarity or high molecular weight. Also, as it can be seen in Figure 3-18, the resolution of these three compounds was weak.

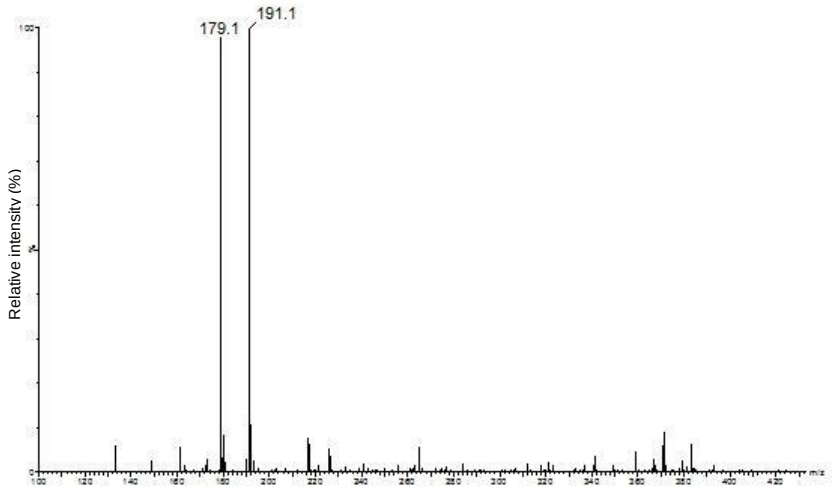


Figure 3-19 – MS-TOF of compound LE1.

As mentioned before, compound CL1 (R_t=2.4, λ_{max}=264nm) was characterised by mass spectrometry (MS) with $[M-H]^-$ at 179.1m/z and $[M-H]^-$ at 191.1m/z (Figure 3-19), which it wass exactly the same result for compound LE1 (Figure 3-19). Compound LE2 also had $[M-H]^-$ at 179.1m/z and $[M-H]^-$ at 191.1m/z (Figure 3-20), but it was also characterised with $[M-H]^-$ at 185.1m/z and $[M-H]^-$ at 312.1m/z (Figure 3-20) as well, to which there were no suggestion to which it could be. So as it was suggested for compound CL1, compound LE1 and LE2 could be complex compounds which were constituted by molecules of caffeic acid and quinic acid. However, LE1, LE2 and CL1 have different retention times and wavelength of maximum absorbance (Table 3-5 and Table 3-10), which suggests that although there were similarities in mass spectrometry results, these compounds should be different.

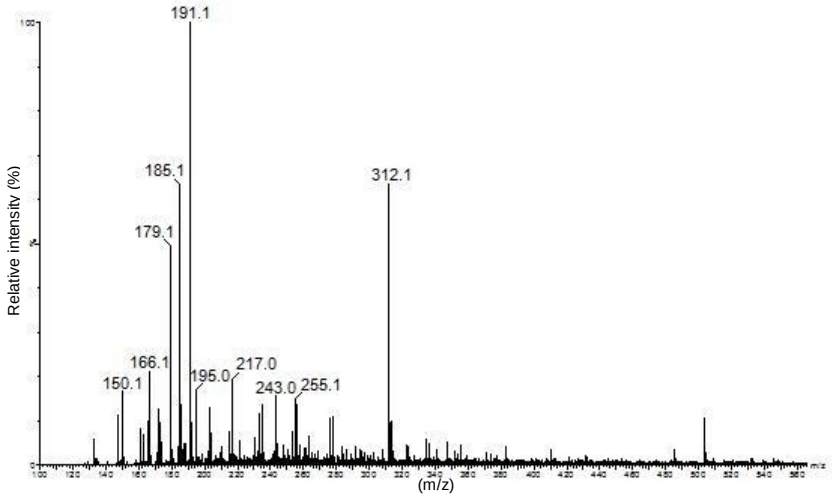


Figure 3-20 – MS-TOF of compound LE2.

3.3.4.2.1.2 Compound LE3

Compound LE3 was characterised by R_t=6.1 and λ_{max}=284nm and its UV spectra is illustrated in Figure 3-21. However, with these characteristics and with the literature available it was not possible to suggest which class of compounds that compound LE3 could make part of.

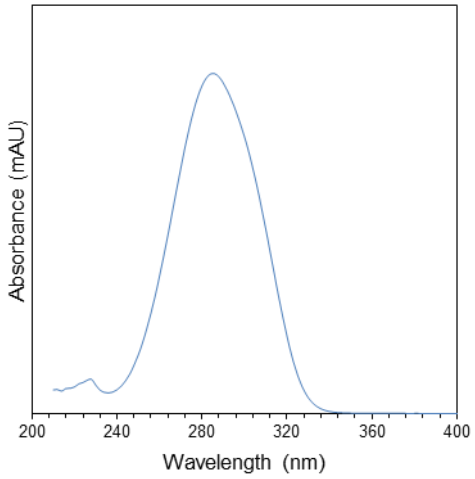


Figure 3-21 – UV spectra of compound LE3.

3.3.4.2.1.3 Compound LE4 and LE5

Compound LE4 and LE5 have similar UV spectra although their wavelength of maximum absorption differs in 4 nm, as it can be seen in Figure 3-22.

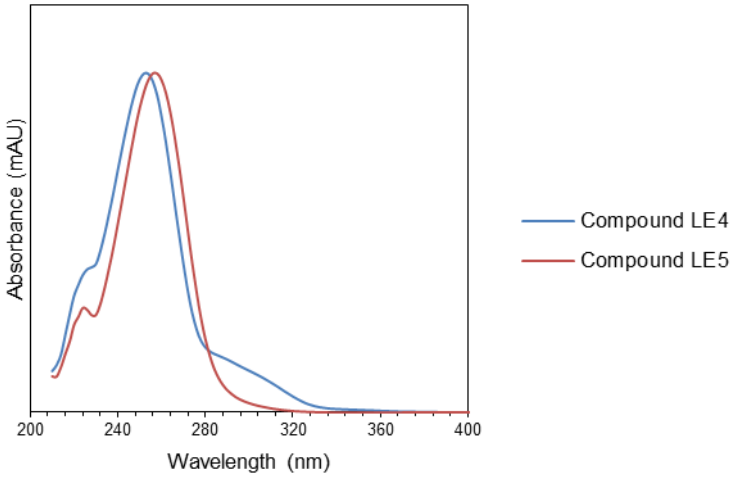


Figure 3-22 – UV spectra of compound LE4 and LE5.

A study of the impact of oxidation of the phenolics caffeic acid and chlorogenic acid, by Antolovich et al. (2004) identified that quinones of caffeic and chlorogenic acid. Moreover it appears that the relative retention times of the quinones of caffeic and chlorogenic acid were similar to the compounds LE4 and LE5 with λ_{max} equal to 249nm and also exhibited similarities

in terms of UV spectra to that of compounds LE4 and LE5. Thermal decomposition of caffeic acid and chlorogenic acid that could occur during SubCW extraction from cider lees, could also enhance the formation of quinones, so LE4 can be suggested as quinone of chlorogenic acid while LE5 as quinone of caffeic acid, by taking in consideration the retention time order observed by Antolovich et al. (2004).

3.3.4.2.1.4 Compound LE6

Compound LE6 was characterised by R_t=12.9 min, λ_{max}=230nm, 280nm, 328nm and MS $[M-H]^-$ at 137m/z (Figure 3-24). The compound 3,4-dihydroxybenzaldehyde (Figure 3-23), also known as protocatechuic aldehyde, has similar characteristics and has been identified as a hydrothermal degradation product of caffeic acid under subcritical water conditions (Khuwijitjaru et al., 2014) and also as a result of oxidation of caffeic acid and chlorogenic acid (Antolovich et al., 2004).

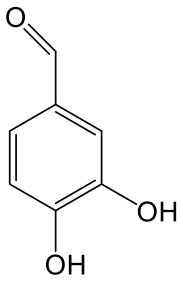


Figure 3-23 – Protocatechuic aldehyde molecular structure.

Davalos et al. (2004) has demonstrated that protocatechuic aldehyde has a similar antioxidant activity to that of caffeic acid but is higher than chlorogenic acid (ORAC-FL).

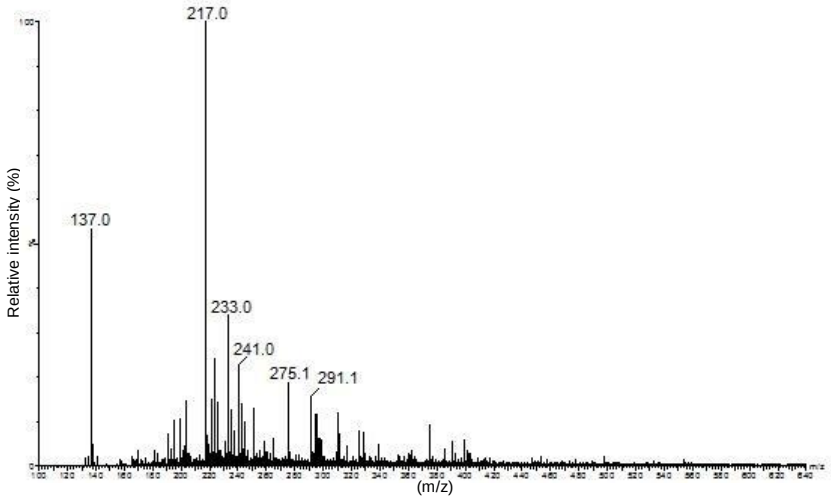


Figure 3-24 – MS-TOF of compound LE6.

3.3.4.2.1.5 Compound LE7 and LE9

Caffeic acid had a characteristic UV spectra with a maximum absorbance at 326nm and a absorbance shoulder at 298nm (Figure 3-25). Thermal degradation of caffeic acid produces mainly decarboxylation products such as 1,2-dihydroxybenzene, also known as catechol or pyrocatechol (Stadler et al., 1996). Catechol UV-spectra is characterised by maximum absorbance at 298nm (Stadler et al., 1996), which differs from caffeic acid by not having significant absorbance at 326nm. This results leads to suggest that the caffeic acid carboxyl group, that catechol has lost, is responsible for the absorbance at 326nm.

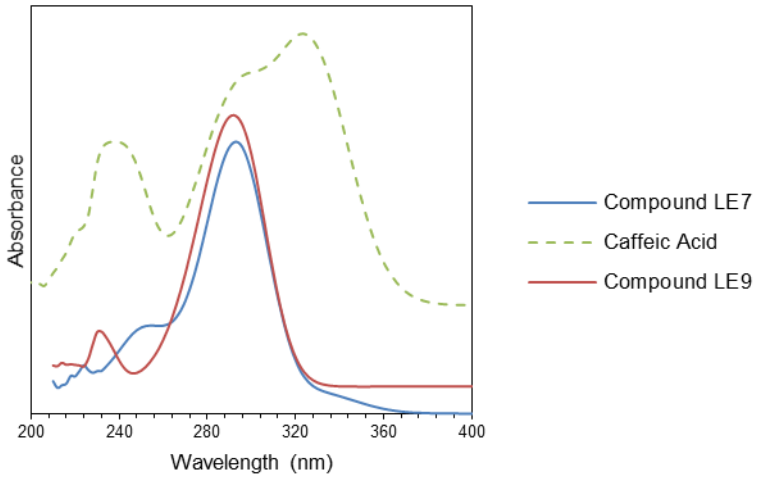


Figure 3-25 – UV spectra of compound LE7, LE9 and caffeic acid.

Compound LE7 and LE9 exhibited a maximum absorbance at 294nm and 292nm, respectively (Figure 3-25), therefore they might be decarboxylation products of caffeic acid. From retention times and elution order reported by Stadler et al. (1996) and Cilliers and Singleton (1991) compound LE7 could be catechol while compound LE9 could be 4-vinylcatechol. However, both compounds are susceptible of polymerisation, especially 4-vinylcatechol (Andueza et al., 2009), which could also occur under subcritical water conditions, therefore compound LE7 and compound LE9 could also be dimers or polymers of catechol and 4-vinylcatechol.

Catechol 4-Vinylcatechol

Figure 3-26 – molecular structure of catechol and 4-vinylcatechol.

3.3.4.2.1.6 Compound LE8 and CL6

Compound LE8 had the particularity of having a retention time close to compound CL6, however it is possible to distinguish them as compound LE8 have low absorbance at 320nm when compared to compound CL6 (Figure 3-27).

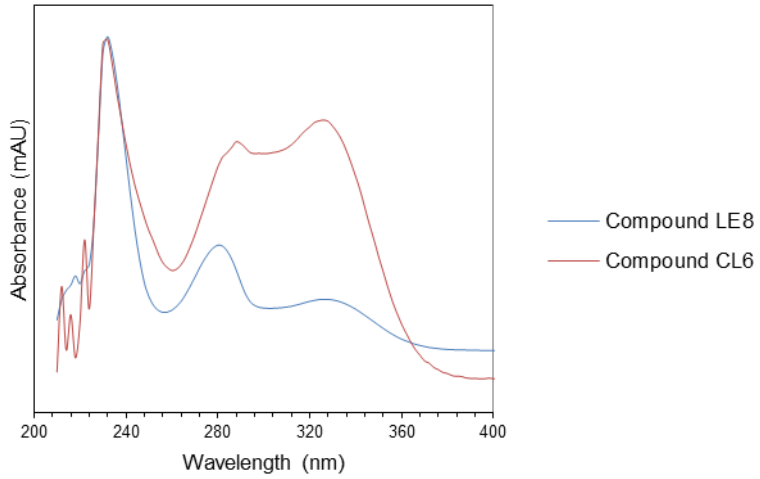


Figure 3-27 – UV spectra of compound LE8 and CL6.

Compound LE8, as it can be seen in Figure 3-28, had a similar UV spectra to compounds CL4 and CL7, compounds that were previously suggested as flavanols (+)-catechin and (-)-epicatechin, respectively. Compound LE8 could be suggested as procyanidin B2 taking in account its elution order in HPLC chromatogram (A. Schieber et al., 2001a). As (+)-catechin and (-)-epicatechin, procyanidin B2 has been previously identified in different kinds of cider (Marks et al., 2007b).

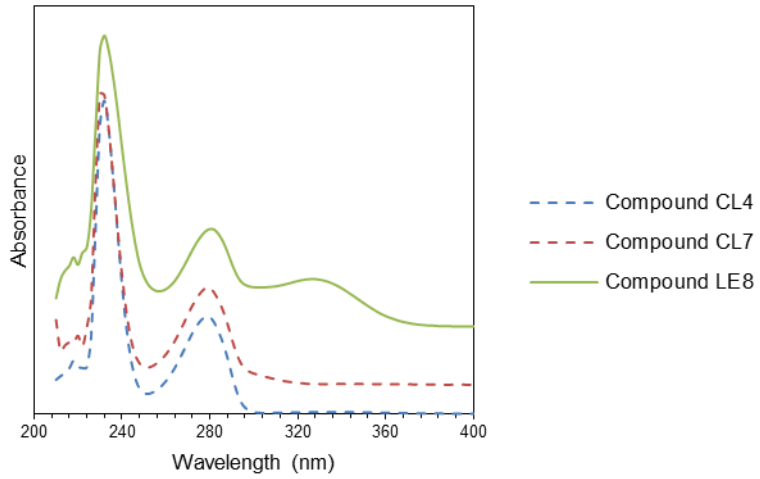


Figure 3-28 – UV spectra of compound CL4, CL7 and LE8.

The HPLC analysis of the cider lees extracts produced at different temperatures have given a change on UV spectra of compound CL6, as it can be observed in Figure 3-29. When cider lees extracts are obtained at temperatures higher than 120ºC, compound CL6 UV spectra becomes similar to the UV spectra observed for caffeic acid and chlorogenic acid (Figure 3-14).

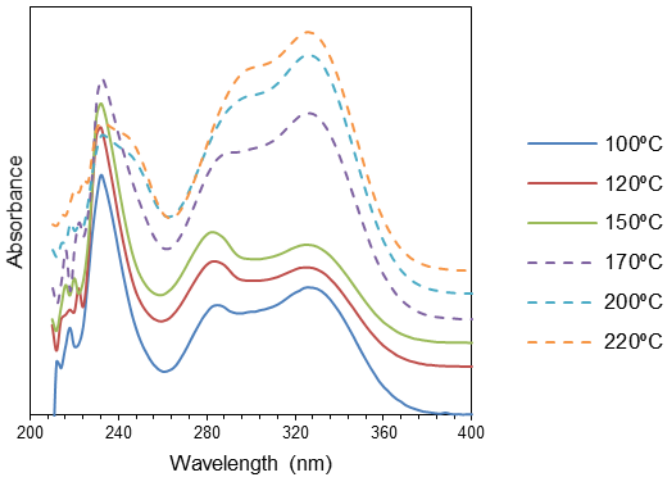


Figure 3-29 – Compound CL6 UV-spectra at different temperatures after 10 minutes of extraction.

Compound CL6 UV spectra change could imply that the compound observed at temperatures higher than 120ºC is in fact a different compound from the compound detected in cider lees. However compound CL6 was previously suggested as caffeicins (Figure 3-15), that are the result of caffeic acid oxidation where two molecules of partially oxidised caffeic acid react to form a new molecule (Cilliers and Singleton, 1991). So, if it is assumed that compound CL6 is in fact a caffeicin, when subjected to subcritical water at high temperatures it could be hydrolysed back into two molecules of caffeic acid. Compound CL6 elution order and its UV spectra above 150ºC suggest it could be an ester of caffeic acid, more specifically a isomer from chlorogenic acid, but because compound CL shares the same retention time as compound LE8 and its UV spectra seems to change with the increase of temperature, an improvement of the HPLC method is required to have a better understanding of what compound it could be.

A complete summary of all unknown compounds detected on cider lees supernatant (Figure 3-4) and subcritical water cider lees extracts (Figure 3-18) is listed on Table 3-11.

Table 3-11 – Complete list of unknown compounds present in cider lees supernatant.

Comp. no.	R_t (min)	λ_{max} (nm)	Tentative identification	References	$[M-H]^-$ (m/z)
CL1	2.4	264	na	na	179.1, 191.1
CL2	3.3	258	na	na	257.1, 372.1, 128.0, 1855.1
CL3	3.5	262	na	na	na
CL4	14.0	280, 230	(+)-catechin	(Delage et al., 1991, A. Schieber et al., 2001a)	na
CL5	14.4	310, 230	3-*O*-*p*-coumaorylquinic acid	(Marks et al., 2007b)	na
CL6	16.8	326, 232, 288	Caffeicins	(Cilliers and Singleton, 1991)	na
CL7	19.7	280, 230	(-)-epicatechin	(Delage et al., 1991, A. Schieber et al., 2001a)	na
CL8	21.6	312, 230	5-*O*-*p*-coumaorylquinic acid	(Marks et al., 2007b)	na
CL9	37.0	284, 230	Phloretin-2'-*O*-(2''-*O*-xylosylglucoside)	(Marks et al., 2007b)	na
LE1	2.2	284, 230	na	na	179.1, 191.1
LE2	2.6	248, 274	na	na	179.1, 185.1, 191.1, 312.1
LE3	6.1	284	na	na	na
LE4	11.0	252, 226	Quinone of chlorogenic acid	(Antolovich et al., 2004)	na
LE5	12.1	256, 224	Quinone of caffeic acid	(Antolovich et al., 2004)	na
LE6	12.9	280, 310, 230	Protocatechuic Aldehyde	(Antolovich et al., 2004, Khuwijitjaru et al., 2014)	137
LE7	13.6	294	Catechol	(Cilliers and Singleton, 1991, Stadler et al., 1996)	na
LE8	16.7	280, 230, 328	Procyanidin B2	(Marks et al., 2007b, A. Schieber et al., 2001a)	na
LE9	19.6	292	4-Vinylcatechol	Cilliers and Singleton, 1991, Stadler et al., 1996)	na

3.3.4.2.2 Quantitative analysis

In Figure 3-17 it was verified that the phenolics detected in cider lees were no longer the main compounds detected using HPLC. To understand the impact of subcritical water in their initial concentration it was used the calibration curves (Table 3-4), previously used in 3.3.4.1.2, for the polyphenols confirmed in cider lees, except for 4-*O*-*p*-coumaroylquinic acid which is not available commercially. For quantification purposes Compound CL6, which was suggested as a chlorogenic acid isomer, was quantified in equivalents of chlorogenic acid, with the assumption that its molar absorptivity should not differ from chlorogenic acid.

3.3.4.2.2.1 Hydroxycinnamic acids

3.3.4.2.2.1.1 Caffeic acid, chlorogenic acid and degradation products

Analysis of the phenolic content of the supernatant from cider lees indicates chlorogenic acid as the main phenolic (Figure 3-4). The result agrees with the findings of Marks et al. (2007b), where chlorogenic acid was similarly identified as the principle phenolic in 23 different ciders produced in England.

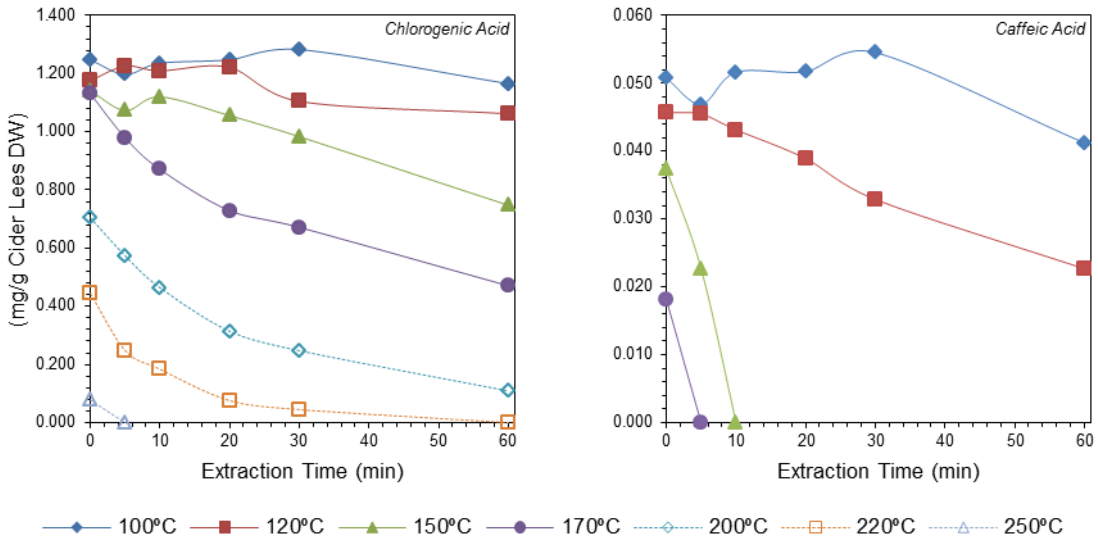


Figure 3-30 – Chlorogenic acid and caffeic acid concentration in the yeast extracts (See Table D-1 and Table D-2 on Appendix D for errors associated with these results).

Maximum TPC in SubCW cider lees extracts was achieved at 220ºC after 10 minutes, as seen in Figure 3-3, which could suggest higher content of the major phenolic. However chlorogenic acid maximum concentration (1.283±0.010 mg/g Cider Lees DW) is achieved at 100ºC with 30 min instead (Figure 3-30). Also its maximum concentration was slightly lower than its concentration in cider lees supernatant (1.309±0.032 mg/g Cider Lees DW). Chlorogenic acid concentration did not change markedly between 100ºC and 120ºC, however at 150ºC there was a decrease in chlorogenic acid concentration after 30 minutes of extraction. At 170ºC and temperatures above, chlorogenic acid concentration decrease was clear since the starting time and at 250ºC it was only identified at starting point (0 min). Therefore it can be suggested that thermal transformation of chlorogenic acid occurred significantly at temperatures of 170ºC and above. It was also verified that chlorogenic acid concentration decreased significantly during pre-heating stage (time 0) to temperatures of 200ºC and higher (ranging from 0.705±0.038 mg/g Cider Lees DW to 0.234±0.000 mg/g Cider Lees DW) when compared to chlorogenic acid

concentration at lower temperature between 100 and 170ºC (range between 1.247±0.048 mg/g Cider Lees DW and 1.132±0.053 mg/g Cider Lees DW) (Figure 3-30).

Caffeic acid concentration, as chlorogenic acid, also decreases with the increase of temperature (Figure 3-30). In fact caffeic acid seems to be even more sensitive to temperature than chlorogenic acid, as at 120ºC it was already verified a decrease in its concentration of 0.051 to 0.046 mg/g Cider Lees DW, and at 150 and 170ºC it was only identified up to 5 and 0 minutes of extraction with concentrations of 0.023 and 0.018 mg/g Cider Lees, respectively. So it can be suggested that above 170ºC caffeic acid degradation was complete.

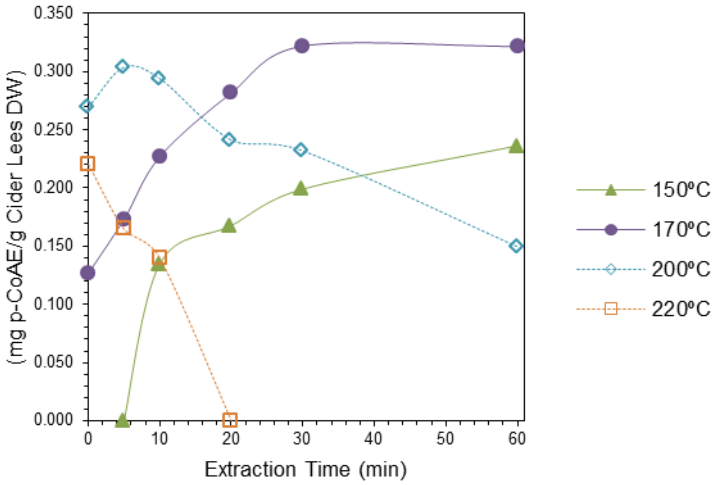


Figure 3-31 – Compound CL6 concentration in the yeast extracts (See Table D-3 on Appendix D for errors associated with these results).

In contrast to chlorogenic acid and caffeic acid, the concentration of compound CL6, a possible chlorogenic acid isomer, increased significantly for extraction times up to 30 minutes at 150ºC and 170ºC, while between 30 and 60 minutes its concentration was almost constant (Figure 3-31). Compound CL6 maximum concentration was achieved at 170ºC with 30 minutes of extraction (0.322±0.022mg/g Cider Lees DW). SubCW extraction at 220ºC only showed decrease in its composition after starting extraction time. Previously it was suggested that compound CL6 could be the result of the hydrolysis of caffeicins that were formed during cider

production, due to have the same retention time. However the detection of compound CL6 at 150ºC and temperatures above could also means that it was being desorbed from the yeast cells, as it was detected in cider (Marks et al., 2007b). Also, the isomer that was suggested as compound CL6 was not naturally found in apples (Marks et al., 2007a), therefore it is likely that it was being produced by isomerisation through the cider process, as it was observed in roasted coffee by Schrader et al. (1996) where isomerisation of chlorogenic acid occurs when its subjected to higher temperatures.

Compounds LE4 and LE5 were previously suggested as possible quinones from chlorogenic acid and caffeic acid, respectively. While compound LE4 was detected in cider lees extract obtained at 100ºC, compound LE5 was only detected when extraction took place at temperatures of 170ºC or higher (Figure 3-32). Although compound LE4 was detected at lower temperatures as 100ºC, its area only increased significantly at 170ºC and above, as the same for compound LE5. Their area increase was in agreement with the decrease verified for chlorogenic acid, however, caffeic acid starts to decompose at temperatures as low as 120ºC, which suggests that caffeic acid decomposition products at those temperatures are not related to the formation of its quinone.

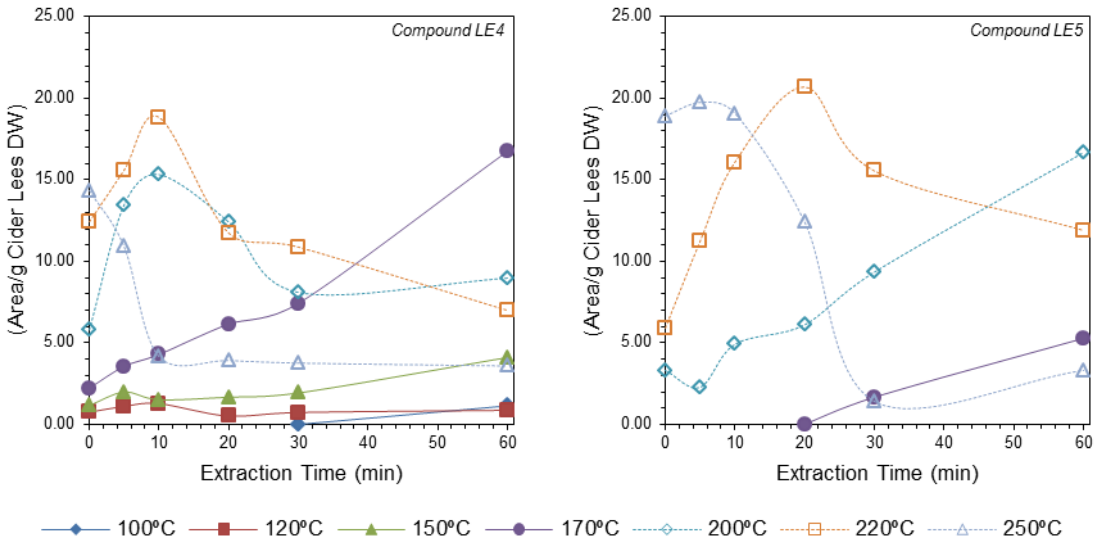


Figure 3-32 – Compound LE4 and compound LE5 area in the cider lees extracts (See Table D-23 and Table D-24 on Appendix D for errors associated with these results).

Compounds LE4 and LE5 had maximum area at the same temperature 220ºC, although they differed in the extraction time. Compound LE4 maximum area was obtained after 10 minutes of extraction (18.8±0.2 Area/g Cider Lees DW) while maximum area for compound LE5 took 20 minutes of extraction (20.7±4.3 Area/g Cider Lees DW). Maximum area of LE4 and LE5 occurred at similar operating conditions as the ones used for maximum TPC in cider lees extracts, however quinones that lack on hydroxyl group are unlikely to contribute to the total phenolic content in cider lees extracts.

Compound LE6 was previously identified as protocatechuic aldehyde and Khuwijitjaru et al. (2014) has identified it as a product of caffeic acid degradation when subcritical water is applied. Protocatechuic Aldehyde was not present in cider lees and it is only detected at temperatures of 120ºC and above. Significant increase of its area are observed at extraction temperatures of 150ºC and 170ºC but its maximum area was obtained at 250ºC after 5 minutes of extraction (28.1±4.2 Area/g Cider Lees DW) (Figure 3-33). Also, if extraction temperature of 250ºC is not taken in account, the maximum area of protocatechuic aldehyde was obtained at 220ºC after

10 minutes (27.1±3.3 Area/g Cider Lees DW) which is the same conditions for maximum TPC of the cider lees extracts.

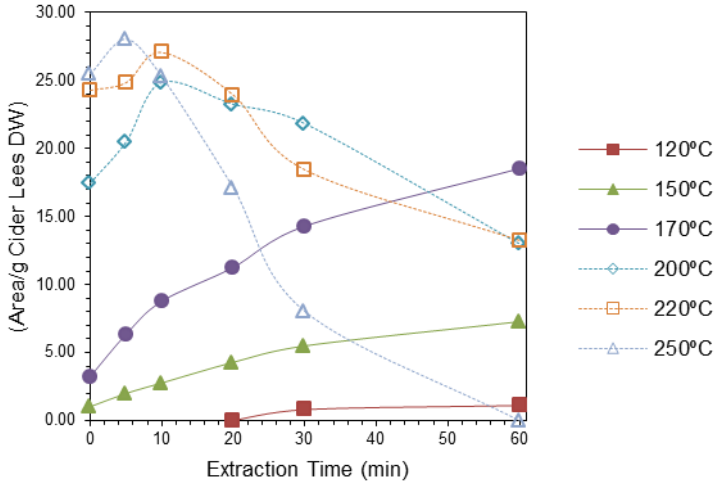


Figure 3-33 – Compound LE6 area in the cider lees extracts (See Table D-4 on Appendix D for errors associated with these results).

Protocatechuic aldehyde had the particularity of having a similar antioxidant activity to caffeic acid and higher antioxidant activity than chlorogenic acid (Davalos et al., 2004). In case protocatechuic aldehyde has its origin in chlorogenic acid degradation, it could be one of the compounds that contribute to the increase of TPC at higher temperatures of extraction, however as it was not one of the major peaks found in cider lees extracts, it is unlikely that it will be the only compound which contributes to the maximum TPC observed at 220ºC after 10 minutes of extraction.

Compound LE7 and compound LE9 were previously (3.3.4.2.1.5) identified as possibly caffeic acid and/or chlorogenic acid decarboxylation products. Compound LE7 was only identified in cider lees extracts from 120ºC and temperatures above while compound LE9 was only found in cider lees extracts from 150ºC and above (Figure 3-34). However, only when extraction was performed at 170ºC and temperatures above their area increased significantly. This increase occurred at the same temperature chlorogenic acid concentration started to decrease markedly.

At 250ºC a decrease in both decarboxylation products was evident. Such decrease could be further degradation of these decarboxylation products into phenol, benzene and benzoic acid (Sharma et al., 2002) or they could be taking part in polymerisation reactions (Stadler et al., 1996, Terpinc et al., 2011).

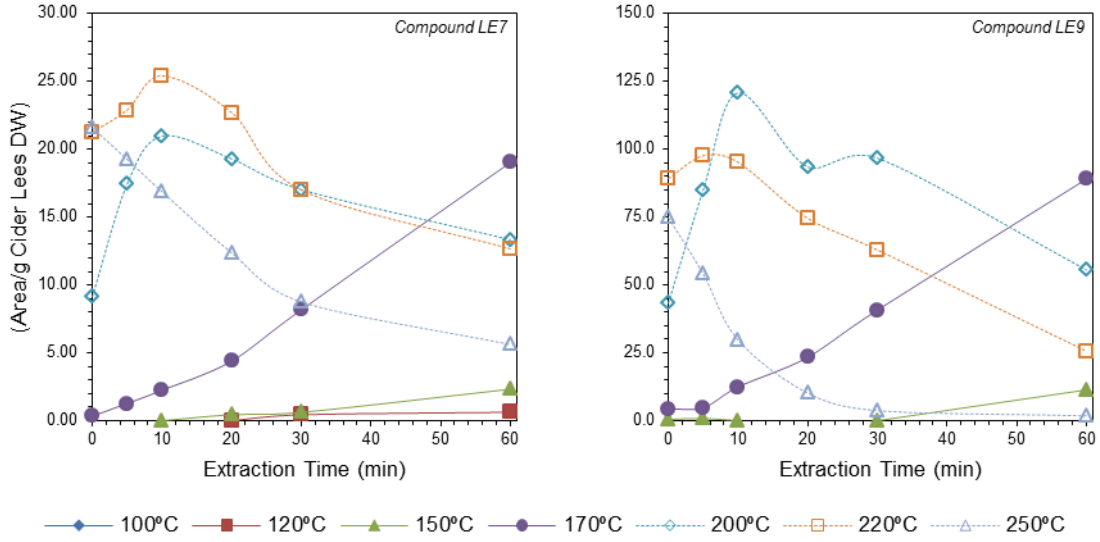


Figure 3-34 – Compound LE7 and LE9 area in the yeast extracts (See Table D-9 and D-10 on Appendix D for errors associated with these results).

Compound LE7 and LE9 had maximum area at 220ºC and 200ºC after 10 minutes, respectively. Compound LE7 and LE9 maximum area correspond to similar operating conditions which produced higher TPC, however Terpinc et al. (2011) and Guillot et al. (1996) have verified that caffeic acid and chlorogenic acid decarboxylation products although they exhibit antioxidant activity, it was lower than its initial compounds antioxidant activity, which mean that if compound LE7 and LE9 are confirmed as catechol and 4-vinylcatechol they will not contribute to the significant increase of polyphenol content observed at 220ºC after 10 minutes of extraction.

3.3.4.2.2.1.2 *p*-coumaric acid and its esters

As chlorogenic acid and caffeic acid, *p*-coumaric acid and 4-*O*-*p*-coumaroylquinic acid (Figure 3-35) concentration also decreased with the increase of temperature. Significant decrease in concentration of 4-*O*-*p*-coumaroylquinic acid iwas observed at 120ºC after 20 minutes and higher temperatures *p*-coumaric acid was found in low amounts and its concentration decreased markedly at 170ºC and it was not detected at higher temperatures.

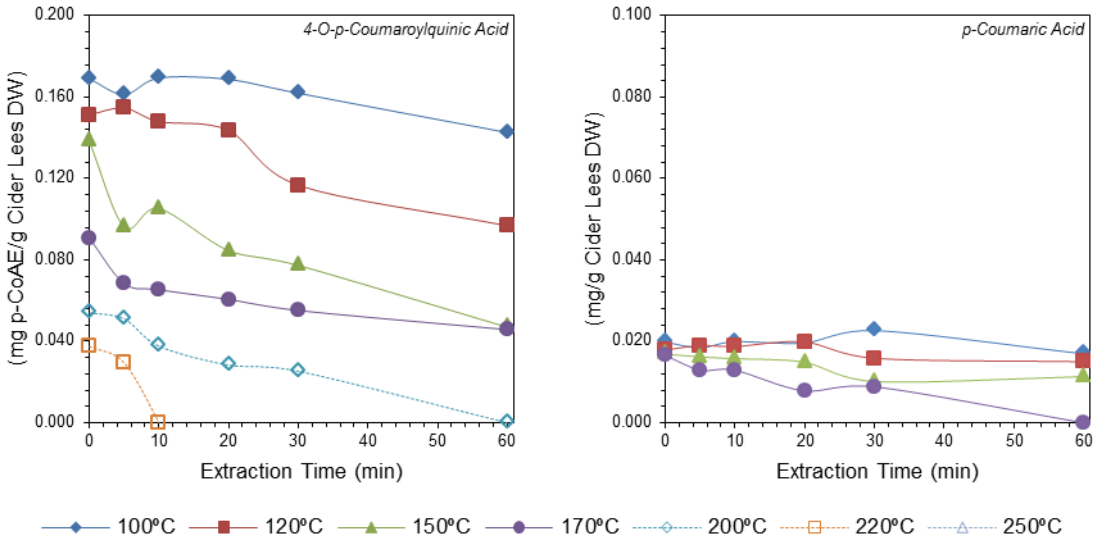


Figure 3-35 – 4-*O*-*p*-coumaroylquinic acid and *p*-coumaric acid concentration in yeast extract (See Table D-5 and Table D-6 on Appendix D for errors associated with these results).

Compound CL5 and CL8 that were previously identified as possible 4-*O*-*p*-coumaroylquinic acid isomers (3.3.4.1.1.3) exhibited an increase in concentration when temperatures lower than 170º were applied. Contrary to 4-*O*-*p*-coumaroylquinic acid their concentration increased when extraction was performed at temperatures under 170ºC and only at higher temperatures their concentration started to decrease (Figure 3-36), however their concentration was always lower than the 4-*O*-*p*-coumaroylquinic acid initial concentration (0.166±0.008 mg/g Cider Lees DW).

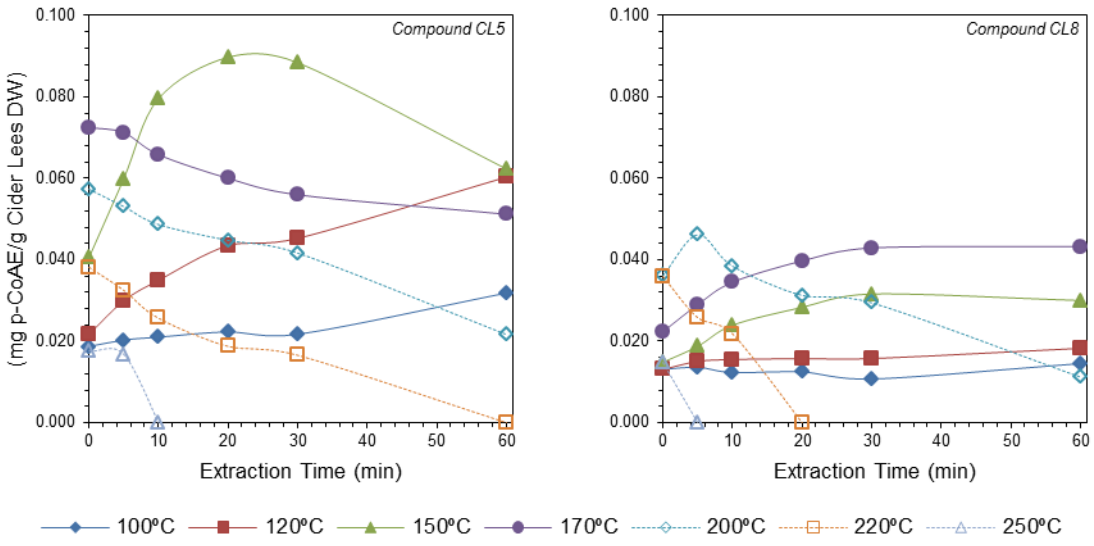


Figure 3-36 – Compound CL5 and CL8 concentration in the yeast extracts (See Table D-7 and Table D-8 on Appendix D for errors associated with these results).

Compound CL5 and CL8 concentration varied with temperature and extraction time in different manners. Compound CL5 concentration at 100ºC only increased when extraction time was higher than 30 minutes. At 120ºC a significant increase was observed with longer extraction times and at 150ºC after 20 minutes of extraction, maximum concentration of compound CL5 was produced (0.090±0.004 mg p-CoAE/g Cider Lees DW). While compound CL8 concentration remained almost constant when extraction was performed at 100ºC and 120ºC while at 150ºC and 170ºC significant increase in concentration was verified with longer extraction times, although after 30 minutes, extraction time does not had an impact in the final concentration. Compound CL8 had maximum concentration at 200ºC after 5 minutes (0.046±0.002 mg p-CoAE/g Cider Lees DW).

3.3.4.2.2.2 Dihydrochalcones

Phloridzin was one of three major polyphenols identified in cider lees supernatant. However, contrary to chlorogenic acid and 4-O-p-coumaroylquinic acid, phloridzin had an increase in

concentration with temperatures up to 170ºC (see Figure 3-37) and its maximum concentration was obtained at 150ºC after 5 minutes (0.587±0.032 mg/g Cider Lees DW), though similar results were obtained at 120ºC after 20 minutes (0.568±0.007 mg/g Cider Lees DW) and also at the start of the extraction at 170ºC (0.572±0.024 mg/g Cider Lees DW). Although its concentration already increased with longer extraction times at 100ºC, it was at 120ºC that a major increase was firstly observed while above 170ºC a major decrease was observed and at temperatures of 200ºC and 220ºC it was only found in the early minutes of extraction and at 250ºC was not even present. As chlorogenic acid its decomposition was mainly observed at extraction above 170ºC.

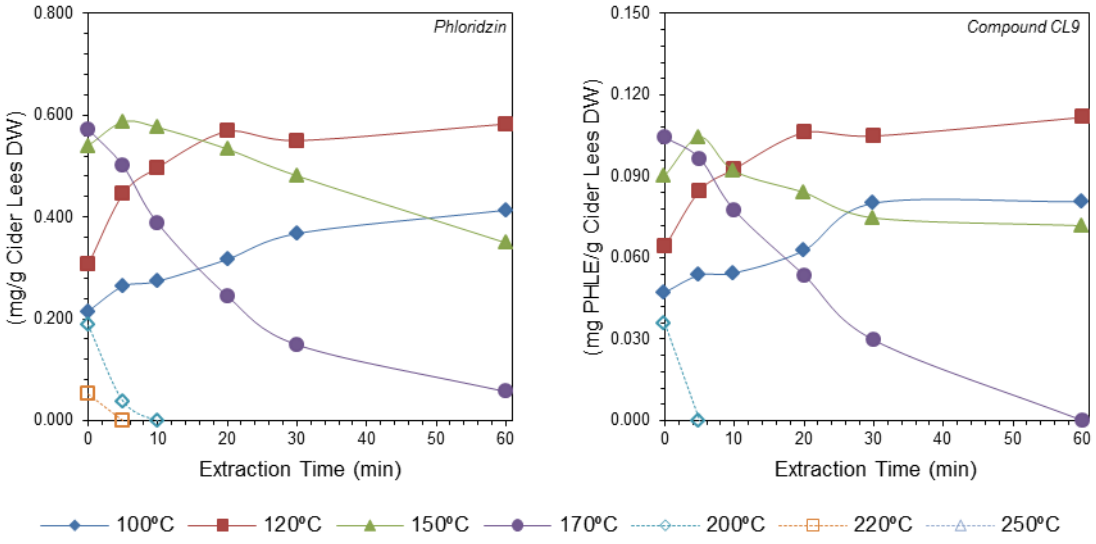


Figure 3-37 – Phloridzin and compound CL9 concentration in the yeast extracts (See Table D-11 and Table D-12 on Appendix D for errors associated with these results).

Compound CL9 was previously suggested as a phloretin diglycoside. Maximum concentration was obtained at 150ºC after 20 minutes (0.104±0.000 mg PHLE/g Cider Lees DW) however like phloridzin, similar results could also be achieved at 150ºC after 5 minutes (0.090±0.010 mg PHLE/g Cider Lees DW) and at the start of 170ºC extraction (0.104±0.005 mg PHLE/g Cider Lees DW). As seen in Figure 3-37 compound CL9 concentration variation with

temperature and extraction time was similar to the variation observed of phloridzin, however compound CL9 concentration showed faster degradation at higher temperatures and above 200ºC it was not present in cider lees extracts.

3.3.4.2.2.3 Flavanols

Compound CL4, CL7 and LE8 were suggested as flavanols, more precisely as possibly (+)-catechin, (–)-epicatechin and procyanidin B2, respectively. None of these compounds was detected in cider lees extracts obtained above 170ºC.

Compound CL4 had maximum area when extraction was performed at 170ºC after 5 minutes (7.7±0.1 area/g Cider Lees DW, see Figure 3-38). Compound CL4 was already present in cider lees supernatant and at 100ºC and 120ºC its area did not change markedly. At 150ºC it was visible an increase in its concentration, although after 20 minutes it decreased.

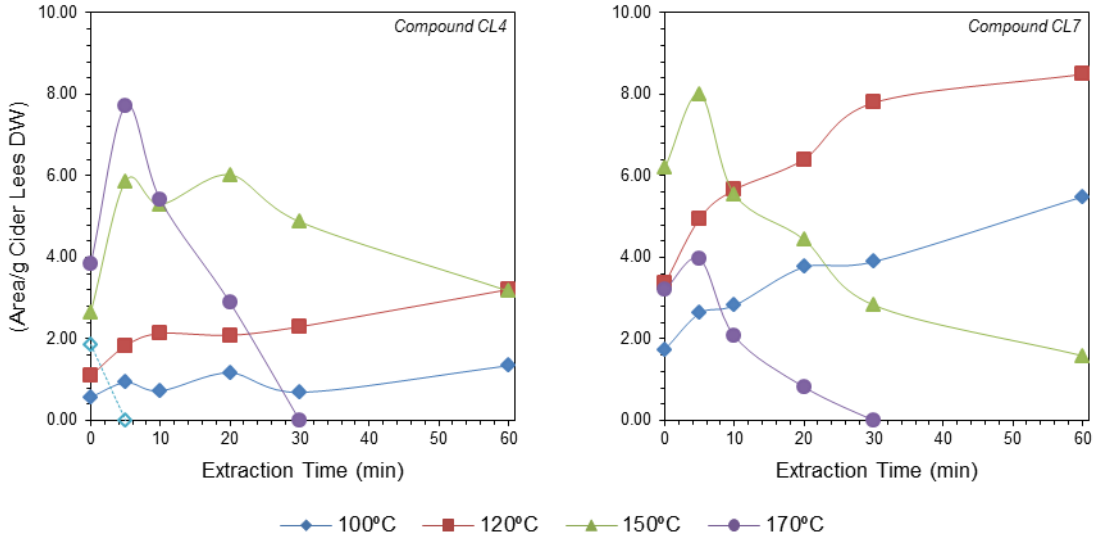


Figure 3-38 – Compound CL4 and compound CL7 area in the yeast extracts (See Table D-13 and Table D-15 on Appendix D for errors associated with these results).

Figure 3-38 also showed that extractions at 100ºC and 120ºC already produce a significant increase in compound CL7 area. And it was at 120ºC after 60 minutes of extraction that

maximum area of compound CL7 was achieved (8.5±0.1 area/g Cider Lees DW). When temperatures of 150ºC and 170ºC were applied there was an increase on their area in the first 5 minutes of extraction to then start to decrease with longer extraction times. The increase in area verified for compound CL4 and CL7 could be the result of desorption from yeast cells.

Flavonols, more specifically (+)-catechin and (-)-epicatechin are known to be among the polyphenols with higher antioxidant activity. Compound CL4 and CL7 area decrease occurs between 150ºC and 170ºC which correspond to the temperature range where a significant increase in TPC was observed (Figure 3-3). It can be suggested that the result from compound CL4 and CL7 degradation could be associated to the increase of TPC, in this case, of antioxidant activity. Previous reports (Nicoli et al., 2000) have shown that oxidation of (+)-catechin promotes the formation of dimers and polymers of (+)-catechin molecules which exhibit higher antioxidant activity. Such reactions are likely to happen when (+)-catechin is exposed to higher temperatures. However, as molecular weight increases with the degree of polymerisation, the possible number of regio- and stereoisomers becomes large making reverse-phase HPLC separation difficult (Monagas et al., 2010).

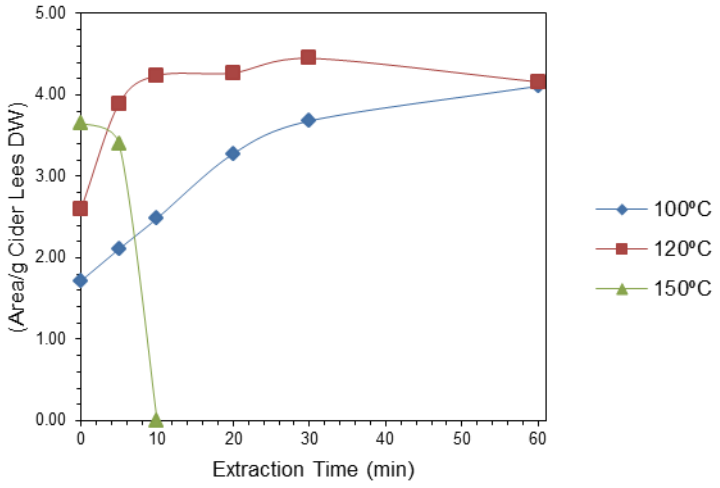


Figure 3-39 – Compound LE8 area in the yeast extracts (See Table D-14 on Appendix D for errors associated with these results).

Compound LE8, previously suggested as procyanidin B2, had an increase in area to more than double when temperatures of 100ºC and 120ºC were applied (Figure 3-39). At 150ºC compound LE8 concentration seems to start to decrease after 5 minutes of extraction, however after the initial 5 minutes, compound LE8 was not detected as it was not detected at higher temperatures. Compound LE8 had the particularity to have similar retention time to compound CL6, which UV spectra changes at 150ºC and also its area increased with higher temperatures. While compound LE8 was only observed at 280nm, compound CL6 was observed at 280nm and 320nm and it is believed that after the 5 minutes of extraction at 150ºC compound CL6 overlaid compound LE8 and it was not possible to detect it.

3.3.4.2.2.4 5-HMF and furfural

Cider fermentation is based on the principle of conversion of monosaccharides into ethanol, however a complete conversion of monosaccharides is not observed and cider lees will still have a certain amount of it in its composition. Higher amounts of monosaccharides in cider are especially detected when cider is produced from apple juice concentrate. As seen in Figure 1-2 two enzymatic treatments are performed in which a range of different monosaccharides are expected to be released into solution. Among these monosaccharides, glucose, rhamnose, arabinose or xylose were detected in the final cider (Gomis et al., 2001),(Knee, 1973).

So it will be expected to have these compounds in cider lees supernatant. Besides, during SubCW extraction is also expected to increase the monosaccharides content in cider extract by hydrolysis of polysaccharides present in the cell wall.

Previous work (Bahari, 2010) and work developed with similar operation conditions (Plaza et al., 2013)(H. Wijngaard and Brunton, 2009a) identified 5-HMF and furfural in yeast and apple extracts. 5-HMF and furfural are the result of hexoses and pentoses dehydration, respectively,

and they can be further dehydrated into levulinic acid. Also, 5-HMF is already present in apple juices (Kermasha et al., 1995).

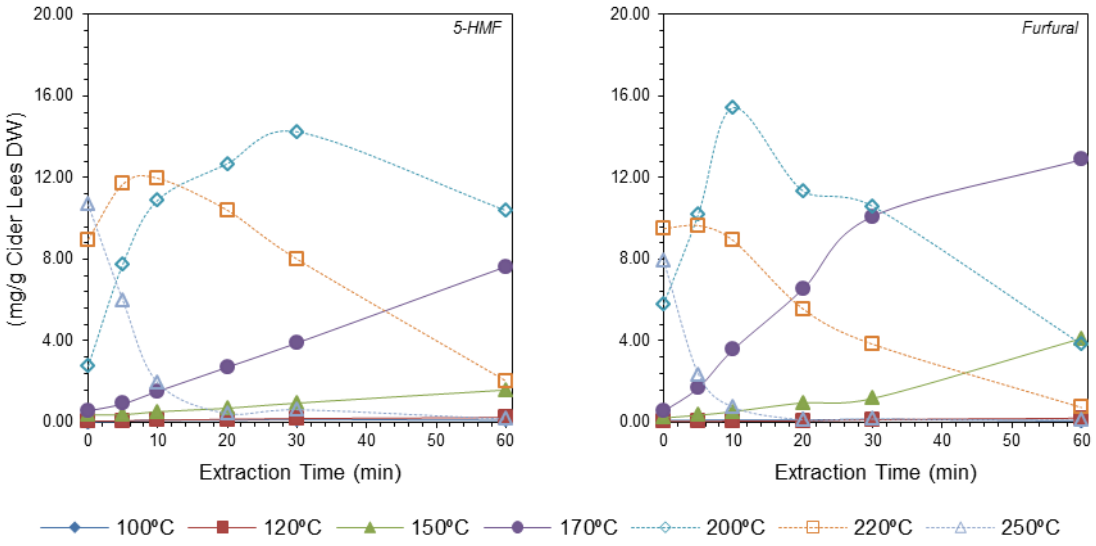


Figure 3-40 – 5-HMF and furfural concentration in the yeast extracts (See Table D-16 and Table D-17 on Appendix D for errors associated with these results).

As seen in Figure 3-4 and Figure 3-18 5-HMF and furfural were detected in cider lees and cider lees extracts and in Figure 3-40 can be seen their concentration profile at different operating conditions. While 5-HMF was found in spent yeast cider supernatant in low amounts, furfural was not detected, however yeast extract obtained at 100ºC had already small amounts of furfural. Although 5-HMF and furfural were detected in yeast extracts obtained at any temperature between 100ºC and 250ºC, only when extraction temperatures of 150ºC or higher were used that an increase in their concentration with longer extraction times was observed. SubCW extraction at 170ºC was when extraction time had a major impact on 5-HMF and furfural concentration. For extractions at 200ºC and above, a decrease in their concentration was verified which indicates further dehydration was occurring. 5-HMF and furfural maximum concentration was obtained at the same extraction temperature, 200ºC, but different extraction time, 30 minutes (12.688±1.392 mg/g Cider Lees DW) and 10 minutes (15.440±1.309 mg/g

Cider Lees DW), respectively. Even though 5-HMF and furfural maximum concentration was obtained at a temperature below 220ºC, at this temperature it was stiil present in yeast extracts in significant amount.

3.3.4.2.2.5 Other compounds

Information and data available at the moment did not allow to associate compounds CL1 to CL3 and LE1 to LE3 with possible known compounds. Compounds CL1, CL2, LE1 and LE2 had the particularity of not suffer any degradation even at temperatures high as 250ºC.

3.3.4.2.2.5.1 Compounds CL1, CL2, LE1 and LE2

Cider lees extracts obtained at 170ºC after 60 minutes and at 200ºC (except at initial extraction time) and above had the peaks correspondent to compound CL1 and LE1 merged into only one peak. The combined peak had a retention time of 2.3 minutes and it had a similar UV spectra to compound LE1 (Figure 3-41), also compound LE1 from cider lees extract obtained at 170ºC after 30 minutes had an area almost 4-fold bigger than compound CL1 peak area. So it can be suggested that compound LE1 was the predominant compound in the merged peak.

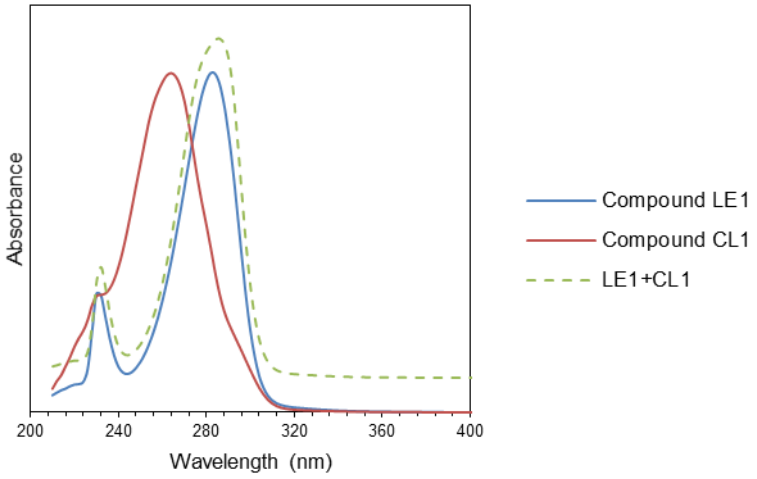


Figure 3-41 – UV Spectra of compound LE1, CL1 and the overlaid peak of LE1 and CL1.

Figure 3-42 shows that the combined peak area of LE1 and CL1 tended to stabilise at higher temperatures and after 20 to 30 minutes of extraction. Its maximum area was achieved at the extreme conditions used, 250ºC and 60 minutes of extraction ((256.3±10.5 area/g Cider Lees DW), which suggests that these compounds were stable up to the highest temperature used (250ºC). The combined area of LE1 and CL1 for extractions had minimum area change at 100ºC and 120ºC (see Figure 3-42). At 150ºC after 5 minutes a small increase in its area was observed but at 170ºC its concentration increased with the increase of extraction time. Nonetheless, when temperature was raised to 200ºC or higher, area increased to double of the maximum area obtained with 170ºC.

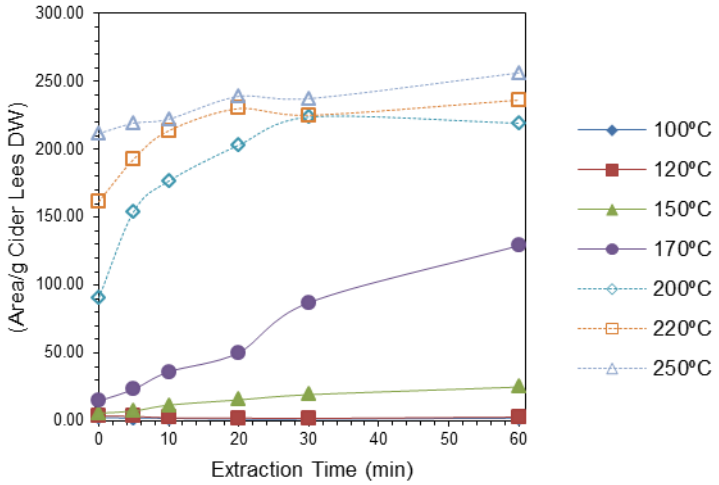


Figure 3-42 – Combined area of compound CL1 and LE1 in the yeast extracts (See Table D-18 on Appendix D for errors associated with these results).

In Figure 3-42 can also be observed that the pre-heating to temperatures of 200ºC and higher had a significant contribution to the amount extracted. Pre-heating to 220ºC and 250ºC alone achieved higher area of compound LE1 and CL1 than the maximum area obtained with temperatures up to 170ºC.

Compound LE2 and CL2 area variation with extraction temperature and extraction time is shown in Figure 3-43. Compound CL2 had a similar area variation to compound LE1 and CL1

(Figure 3-42), where at temperatures of 200ºC or higher a significant area increase was observed, which became almost constant after 5 to 10 minutes.

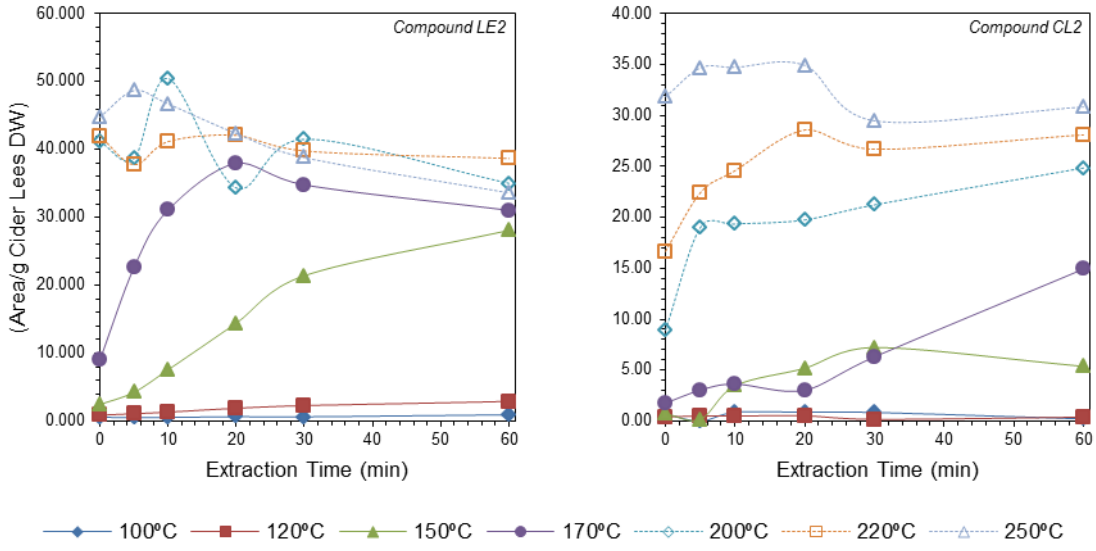


Figure 3-43 – Compound LE2 and CL2 area in the yeast extracts (See Table D-19 and Table D-20 on Appendix C for errors associated with these results).

Compound LE2 area variation differs from compounds LE1+CL1 and CL2 mainly when extraction temperatures of 150ºC and 170ºC were used. Compound LE2 at 150ºC had a significant increase of area with extraction time while at 170ºC it rapidly equalised areas achieved with the same extraction times at higher temperatures. Interestingly, the area evolution at 150ºC of compound LE2 was similar to the area evolution of compound LE1+CL1 and CL2 at 170ºC, just as the area evolution at 170ºC of compound LE2 was similar to the area evolution of compounds LE1+CL1 and CL2 at 200ºC. Another between compound LE2 and compounds LE1+CL1 and CL2 was a small area decrease with longer extraction time at 170ºC and higher temperatures which did not happened for compounds LE1+CL1 and CL2.

In general compound CL1, CL2, LE1 and LE2 showed that extraction at 150 to 170ºC had a great impact in its extraction into solution while at higher temperatures, area stayed almost constant showing resistance to thermal degradation. At temperatures higher than 170ºC, pre-

heating time was almost enough to achieve maximum area extracted and as their area did not change with extraction time at temperatures above 200ºC, these compounds should not had a great contribution to the overall TPC.

3.3.4.2.2.5.2 Compound CL3

Compound CL3 was detected in cider lees although in low amounts (2.0±0.1 area/g Cider Lees DW) when compared to the maximum area of CL3 obtained through SubCW extraction, which was obtained at 200ºC after 20 minutes of extraction (42.5±3.0 area/g Cider Lees DW). At extractions temperatures of 100ºC and 120ºC compound CL3 area remained almost constant, while at 150ºC and 170ºC significant concentration increase was observed. At temperatures above 200ºC degradation was observed, however after the initial minutes of extraction, compound CL3 area remained constant.

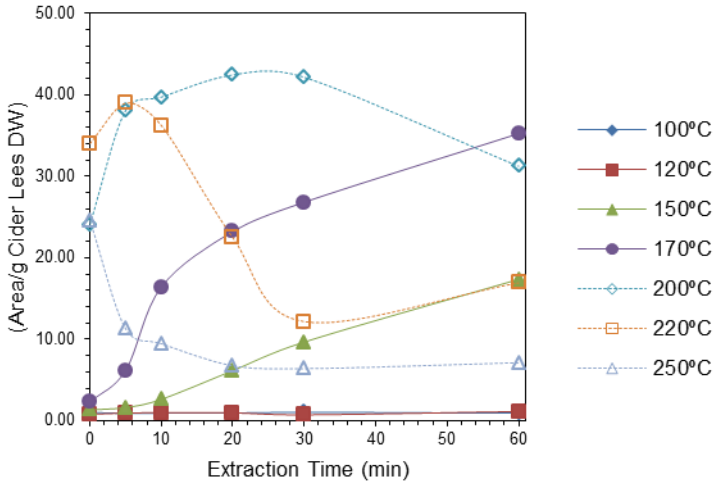


Figure 3-44 – Compound CL3 area in the cider lees extracts (See Table D-21 on Appendix D for errors associated with these results).

3.3.4.2.2.5.3 Compound LE3

Compound LE3 was not present on cider lees supernatant and while its area does not change when SubCW extraction was performed at 100ºC, at 120ºC and higher temperatures significant

changes in its concentration were observed (Figure 3-45). And although an increase in area was verified at 120ºC, this increase was low when compared with the increase on area at higher temperatures, especially at 150ºC after 60 minutes (44.0±0.6 area/g Cider Lees DW) and 170ºC after 5 minutes (43.1±2.9 area/g Cider Lees DW), where maximum area was achieved.

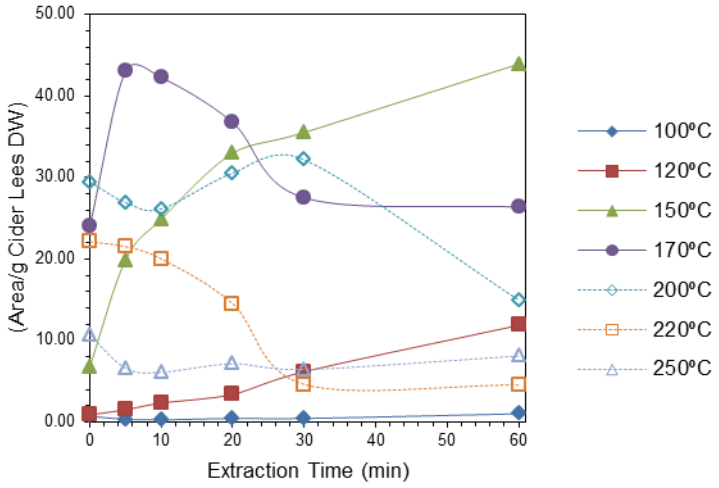


Figure 3-45 – Compound LE3 concentration in the yeast extracts (See Table D-22 on Appendix D for errors associated with these results).

A decrease in compound LE3 concentration was observed at 170ºC and higher temperatures, however its area stabilised at 220ºC after 30 minutes and at 250ºC after 5 minutes (Figure 3-45).

3.3.4.2.2.6 Comparison of total phenolics obtained by FCR's method and by HPLC

Previously in 3.3.4.1.2.1, total phenolic content of cider lees were compared when determined by FCR's method and HPLC method. A significant difference was verified which accounts for the flavanols that were not quantified in HPLC method, but also due to the interferences on FCR's method.

In the present chapter it was verified that the phenolics from cider lees were either not present or were detected in low amounts in the cider lees extract obtained by subcritical water at elevated temperatures. At elevated temperature, new compounds were detected and suggested

as the result of caffeic and chlorogenic acid thermal degradation. Although these compounds were not quantified with their respective standards, for comparison effects, compounds LE4 to LE9 were quantified in chlorogenic acid equivalents. In table Table 3-12, total phenolics content of the cider lees extract from 220ºC for 10 minutes is compared through FCR's method and HPLC method. Two total phenolics content values for HPLC method were determined: HPLC_sum_1 which accounts for all the hydroxycinnamic acids and dihydrochalcones; and HPLC_sum_2 which also accounts for all hydroxycinnamic acids and dihydrochalcones but also for compounds LE4 to LE9.

Table 3-12 – Comparison between the total phenolic content by FCR's method and by HPLC analysis at 220ºC with 10 minutes of extraction.

	Concentration *(mg/g Cider Lees DW)*
FCR method	38.095±0.544
HPLC_sum_1	0.371±0.043
HPLC_sum_2	12.282±0.847

In Table 3-12 it is clear that hydroxycinnamic acids and dihydrochalcones from cider lees had a minimum contribution to the total phenolics content determined by FCR method. When compounds LE4 to LE9 were taken in account, FCR method total phenolics was little more than 3 times the amount detected in the HPLC method. Again, the phenolics and derived products from the phenolics present in apple did not account for all the concentration determined by FCR's method.

Compounds LE4 to LE9 were previously associated with the thermal decomposition of caffeic acid and chlorogenic acid, and they could be either the result of decarboxylation, oxidation and/or dimerisation reactions. During such conditions not only reactions between phenolics are enhanced but also reaction of other compounds and reactions between different types of compounds. Before it was already mentioned the dehydration of monosaccharides to produce

5-HMF and furfural, but there are other reaction that can occur, especially at higher temperatures. As in solution amino acids and monosaccharides are present and in combination with high temperatures, glycation reaction can occur with the production of Maillard reaction products. There is evidence that Maillard reaction products exhibit antioxidant capacity (Plaza et al., 2010), which could explain part of the antioxidant capacity verified at higher temperatures. Also there is a suggestion that phenolics and Maillard reaction products can react creating more complex molecules which again exhibit antioxidant capacity (Moreira et al., 2012, Nunes and Coimbra, 2010). Although there is evidence for antioxidant capacity of Maillard reaction products, the mechanism of reaction and the structure of this products is not possible yet to fully characterise.

3.3.4.3 Acetone polyphenol extraction

Chlorogenic acid has been identified as the main polyphenol present in cider lees supernatant, however it was previously verified that chlorogenic acid decreases with the increase of temperature in SubCW extraction, although total phenolic concentration increases. This result suggests that chlorogenic acid might undertake thermal decomposition at higher temperatures (>170ºC) or its presence might not be fully detected due to interaction with other compounds present in solution, especially proteins (Haslam, 1974). When polyphenols and proteins are in solution, the formation of a phenol-protein complex is likely to be formed by cross-linking, an interaction similar to binding agent and ligand interactions(Hagerman and Butler, 1981). The phenol-protein complex can be dissociated when in the presence of acetone (Saravanan and Rose, 2004), also acetone extraction is used to quantify polyphenols content in fruits or other similar materials, so it was chosen as method to evaluate if chlorogenic acid was not detected on the HPLC analysis due to its interaction with other compounds. Acetone extraction was performed to yeast extracts obtained by SubCW extraction at 100, 150 and 200ºC with

extraction times of 0, 10, 20 and 30 minutes. HPLC was used to evaluate the chlorogenic acid concentration after the yeast extract was subjected to acetone extraction.

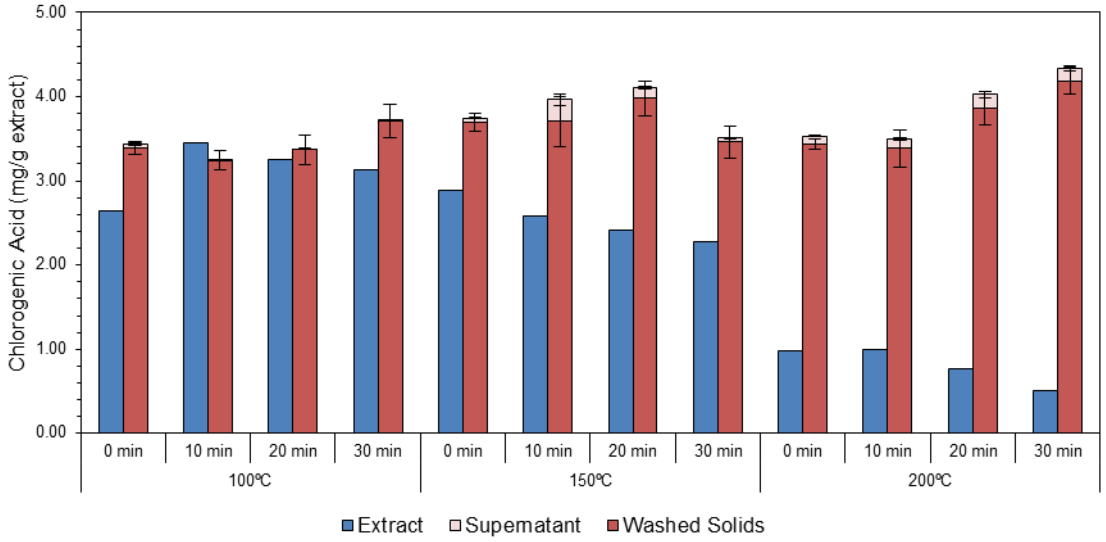


Figure 3-46 – Chlorogenic acid in the initial and the acetone washed extract.

Chlorogenic acid concentration in yeast extracts before and after acetone extraction can be seen in Figure 3-46. It was verified that acetone extraction yield was low, which confirms that aqueous acetone have higher affinity with polyphenols rather than pure acetone. Nonetheless, the washed solids, i.e., yeast extract non-soluble in acetone showed higher chlorogenic acid concentration than the initial yeast extract, especially at 200ºC. Results also showed that extraction time had a positive effect in chlorogenic acid concentration at any temperature (except at 150ºC after 30 minutes).

Results suggest that not only chlorogenic acid was taking part in interactions with other compounds but also that it concentration increased at higher temperatures, which could be the result of being desorbed from cider yeast cells. Although the fact that chlorogenic acid concentration increased it does not necessarily mean that it was not undertaking thermal decomposition reactions but it means that the amount of chlorogenic acid being released into

solution was higher than the amount of chlorogenic acid involved in thermal degradation reactions.

3.4 Conclusion

Subcritical water as a method to recover bioactive compounds has shown different results for the solubilisation of total solids from the cider lees slurry solution and for the total phenolic content. While solubilisation was highest at 150ºC after 30 minutes of extraction the total phenolic content was highest when it was used a temperature of 220ºC with 10 minutes of extraction. Also at the operating conditions of 220ºC for 10 minutes of extraction, the dry weight of the extract supernatant was lower than the initial dry weight in cider lees. These results indicated the selectivity of subcritical water at different temperature, as although solubilisation was higher at 150ºC, compounds which were quantified by the FCR's method were preferential solubilised at elevated temperatures, especially at 220ºC. Also, the decrease of solubility at elevated temperatures to levels lower than the initial cider lees indicated that subcritical water promoted reactions and transformation of the compounds present in solution, which resulted in the formation of compounds which would precipitate or could be easily precipitated under centrifugation conditions.

All the phenolic compounds from cider lees were detected in trace levels or not detected when subcritical water was used at temperatures above 170ºC. The results from chemical characterisation of the cider lees extracts suggested that phenolics undergo thermal decomposition, and it was suggested that compounds LE4 and LE5 were the result of chlorogenic acid oxidation while compounds LE7 and LE9 are suggested to be the result of decarboxylation of caffeic acid and chlorogenic acid. According to the nature of these compounds, they would exhibit similar or lower antioxidant capacity than the corresponding

phenolic (Andueza et al., 2009, Terpinc et al., 2011), therefore they should not contribute to the increase of antioxidant capacity observed. However dimerisation and polymerisation reaction could also occur between these compounds and compound CL6 was suggested to be the result of dimerisation of chlorogenic acid. Such compounds would enhance antioxidant capacity of the extract (Guillot et al., 1996). If in addition to dimerisation, polymerisation could also occur, the resultant polymers would contribute to an increase in antioxidant capacity, although the HPLC method in the present work did not have the capacity to separate such compounds.

Also, the increase of antioxidant capacity verified could also be the result of a synergistic effect between the different types of compounds in solution. Besides phenolics, proteins and monosaccharides were also expected to be in solution and they can react at high temperatures to create Maillard products which has been suggested to exhibit antioxidant capacity (Herrero et al., 2010).

Furfural and 5-HMF were found in high quantities in the cider lees extracts obtained at higher temperatures. Furfural and 5-HMF formation indicates the presence of monosaccharides in the cider lees extracts, which can be either from the release of monosaccharides from yeast cell wall and monosaccharides that remain in cider lees supernatant, from the cider process. 5-HMF and furfural do not contribute to the overall TPC (antioxidant capacity) therefore it will be important in the future to avoid or limit the production of these compounds.

Phenolics can interact non-covalently with macromolecules creating cross-linking complexes which can offer protection to the phenolics from the external environment conditions. Acetone extraction of the cider lees extract had the objective to disrupt possible these possible complex molecules between phenolics and macromolecules. Using chlorogenic acid as an indicator it was verified that these interactions are actually occurring and the chlorogenic acid reappears in

concentrations in the range of its concentration in cider lees, for all range of temperatures and extraction times applied.

The use of subcritical water to recover bioactive compounds from cider lees at elevated temperatures has promoted the transformation and reaction of the compounds existing either in the cider lees supernatant or compounds released from yeast cells. The novel compounds were interfering in the FCR's method to determine the total phenolic content, however this interference meant that the novel compounds obtained contributed to the increase of the reducing capacity of the initial cider lees sample which can be translated in the increase of antioxidant capacity.

CHAPTER 4

SEQUENTIAL SUBCRITICAL WATER EXTRACTION FROM CIDER LEES TO MINIMISE THERMAL DECOMPOSITION

4.1 Introduction

In Chapter 3 it was observed that temperature of subcritical water promotes the increase of total phenolic content determined by FCR's method, which also indicates an increase in antioxidant capacity that it is not necessarily from apple phenolic compounds, as HPLC results have shown. The increase of temperature in subcritical water shown to promote not only an increase in antioxidant capacity but also transformation and reaction of the compounds present in solution, in particular phenolics and monosaccharides. These reactions are not necessarily beneficial, as natural phenolics and monosaccharides were lost at elevated temperatures, although antioxidant capacity has increased. Also, the transformations observed at different temperatures, as the conversion of monosaccharides into furfural and 5-HMF was firstly detected at 170ºC and it was highest at 200ºC, cider phenolics had maximum concentration at 150ºC and highest antioxidant capacity was obtained at 220ºC.

It was hypothesised that by adopting sequential SubCW extraction the complexity of each extract would be reduced and therefore the potential for molecular interaction and

transformation might also be reduced thereby enable dissecting out the bioactive compound conferring the observed antioxidant activity. Sequential subcritical water extraction consists of performing an extraction at one determined temperature in which the residual biomass resultant from this extraction is used again in an extraction at a higher temperature.

Previously it was suggested that total phenolics by FCR's method results were influenced by the presence of other compounds than phenolics that could also act as a reducing agent, therefore as an antioxidant, which means that the results from FCR's method should be regarded as an antioxidant capacity assay rather than a total phenolic assay (D. Huang et al., 2005).

The present chapter had the main objective to evaluate the effect of sequential supercritical water extraction on the compounds recovered from cider lees at different temperatures and the effect on the transformation observed in Chapter 3. Ultimately the results obtained by sequential subcritical water extraction were compared to standard subcritical water extraction at the same operating conditions for a better understanding of the impact of sequential extraction.

4.2 Materials and experimental procedures

4.2.1 Sequential subcritical water extraction from cider lees

Sequential subcritical water extraction involved re-extracting the non-extractable material with incremental increases in temperature. In the present set of experiments the cider lees sample collected on the 18/06/2012 was used. As seen in Table 3-2, this cider lees sample was characterised with a DW of 90.3±1.4 g/l and a TPC of 12.9±0.4 mg GAE/g Cider Lees DW.

Sequential subcritical water extraction was performed in triplicate at a constant pressure of 100 bar and six different temperatures were applied: 100ºC, 120ºC, 150ºC, 170ºC, 200ºC and 220ºC. The first SubCW extraction was performed at 100ºC and it does not differed from the standard SubCW described in chapter 3. For higher temperatures the procedure was different. The cider

lees extracts were centrifuged at 10,000rpm for 10 minutes using Beckman J2-21 Centrifuge (500ml centrifuge bottles). Supernatant was stored at -20ºC for further analysis while the non-extractable solids were freeze dried using an Edwards Freeze Dryer (Model EF 03, Edwards High Vacuum). Once it was dried, cider lees sample was reconstituted using distilled water to a total solids concentration equivalent to the initial cider lees sample (~90g/l). Following the cider lees sample reconstitution, a new extraction was performed at the next higher temperature. Extractions at all temperatures, with the exception of 220ºC, were stopped as soon as the desired temperature was achieved. The extraction at 220ºC was performed for 10 minutes at constant temperature.

The experimental apparatus used is described in Figure 3-1. Differently from the standard SubCW extraction, a stainless steel vessel of 5 litres (Parr Instrument Company, model 4582) was used instead of the 300 ml vessel. As six sequential extractions were made from the same starting material, a larger vessel was used to ensure sufficient amount of non-extractable material for the last extraction. Also the extraction at 100ºC was performed in duplicate for each one of the three repetitions, as around half of the initial solids present in solution are soluble in water.

Table 4-1 – Comparison of the heating time for 300ml and 5 litres vessel.

Heating Time *(min)*	*100ºC*	*120 ºC*	*150 ºC*	*170 ºC*	*200 ºC*	*220 ºC*
300 ml vessel	12	15	18	22	28	31
5 litres vessel	27	29	33	36	38	41

As a larger vessel was being used, the heating time was different than heating times observed with the 300ml vessel. Table 4-1 compares the heating times needed for the vessels of 300ml and of 5 litres. It can be seen that differences in the heating time are higher at lower temperatures and in overall the differences ranges from 10 minutes to 15 minutes. Such differences imply that cider lees were exposed for longer times to high temperatures while using a larger vessel,

so decomposition transformations were likelier to occur in a faster rate when using a larger extraction vessel.

4.2.2 Acetone polyphenol extraction

As described previously in 3.2.3, acetone polyphenol solvent extraction method was adapted from Lazarus et al. (1999). In the present chapter acetone extraction was only employed to the cider lees extract obtained by sequential SubCW extraction at 220ºC after 10 minutes of extraction.

Acetone extraction was done in triplicate, using 0.1g of freeze dried cider lees extract sample. As before, freeze drying was done using an Edwards Freeze Dryer (Model EF 03, Edwards High Vacuum). For each extraction 1ml of acetone from Fisher Scientific (Loughborough, UK) was used. The mixture was mixed for 1 minute using a vortex Jencons MixiMatic and centrifuged for 10 min at 4000rpm using a Jouan C422 Centrifuge. The resultant supernatant and precipitate were freeze dried. While the precipitate was re-solubilised to the initial concentration of the cider lees extract, ~22g/l, the supernatant, due to its low amount, was re-solubilised in 600µl. All samples were stored at 4ºC prior to analysis.

4.3 Results and Discussion

4.3.1 Cider lees hydrolysis: Extract total solid content and yield

Sequential SubCW extraction consisted on six extraction steps at increasing temperatures from the same cider lees sample. There yield of extraction was determined in two different ways: *Overall yield* – amount of extract obtained at one of the temperature steps in comparison to the initial cider lees sample solids; *Yield of extraction* - amount of extract obtained at one of the temperature steps in comparison to the solids recovered from its previous step and used for that

specific step. It is important to note that the *Overall yield corresponds to the yield determined for th standard SubCW extraction of cider lees.*

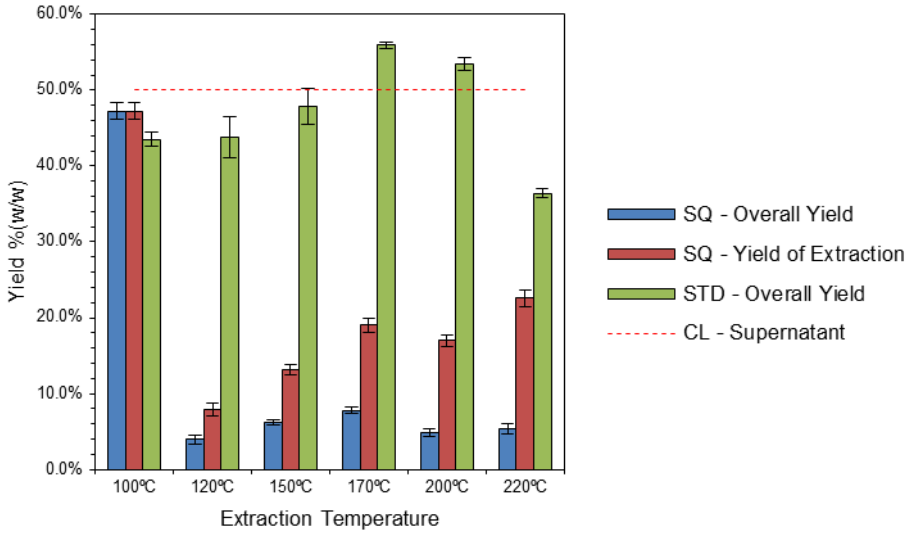


Figure 4-1 – Cider lees extract yield produced by standard (STD) and sequential (SQ) SubCW extraction, (CL-Cider Lees).

In the sequential SubCW extraction, the temperature of 100ºC corresponds to the first of six sequential steps of extraction from the same starting cider lees and as it is the first extraction, it was done in the same conditions as for the standard SubCW extraction at 100ºC. Contrary to the cider lees sequential extract produced at 120ºC and higher temperatures, the cider lees sequential extract produced at 100ºC contained the initial cider lees supernatant which gave it a significant higher yield than for higher temperatures.

Figure 4-1 compares the yields for sequential and standard SubCW extraction where it can be seen that although sequential and standard extraction at 100ºC had similar yields, standard extraction yield was lower. Such difference could be the result of using vessels of different volumes for the sequential and standard extraction. The use of different vessel volume ha produced different heating times especially at lower temperatures as 100ºC. The vessel used for the sequential extraction needed 27 minutes to achieve 100ºC while the vessel used for the standard extraction could reach the 100ºC in 12 minutes (Table 4-1). An increase in the heating

time was likely to have a positive effect in the extraction yield, as it is verified in Figure 3-2, where the yield of extraction increased with longer extraction times for temperatures up to 150ºC.

In chapter 3 it was observed that standard SubCW extraction produces maximum *overall yield* after 30 minutes of extraction at 150ºC (Figure 3-2). At higher temperatures a decrease in the yield was observed, which was associated to the thermal decomposition of the existent soluble compounds into non-soluble compounds. One of the aims of the sequential extraction was to minimise thermal decomposition and consequent precipitation of the compounds released into solution. It is important to note that when only the subcritical water operation conditions are considered, standard SubCW had highest *overall yield* at 170ºC and at temperatures above, a decrease was verified (Figure 4-1) as it was previously observed in chapter 3. Sequential subcritical water showed a similar trend to standard SubCW of cider lees for the *overall yield*, with maximum solubility at 170ºC. Sequential subcritical water from cider lees confirmed that at all the temperatures used, solubilisation of cider lees occured, as in standard SubCW such statement was not possible to affirm, as at high temperatures the amount solubilised was lower than the initial soluble material.

Due to the sequential SubCW from cider lees mechanism of operation, it was possible to determine the yield of extraction, which determined the extraction based on the concentration of the cider lees extract produced rather than the cider lees used in the first step. The *yield of extraction* differs from the *overall yield* by having its maximum at 220ºC, which demonstrated that at the same temperature standard subcritical water extraction exhibited the highest antioxidant capacity (Figure 3-3) it was also when a richer extract was produced exclusively from the non-soluble material from cider lees.

Further results were always represented per grams of the initial cider lees, to be able to be compared with the standard subcritical water extraction.

4.3.2 Compositional characterisation of sequential subcritical water extracts

In chapter 3 it was hypothesised that by adopting sequential SubCW extraction the complexity of each extract would be reduced and therefore the potential for molecular interaction and transformation might also be reduced thereby enable dissecting out the bioactive compound conferring the observed antioxidant activity . A comparison between the HPLC chromatogram of the standard and sequential SubCW extraction at 220ºC (Figure 4-2) showed that most of the compounds present in the standard extract were in reduced amounts or non-existent in the cider lees extracted sequentially.

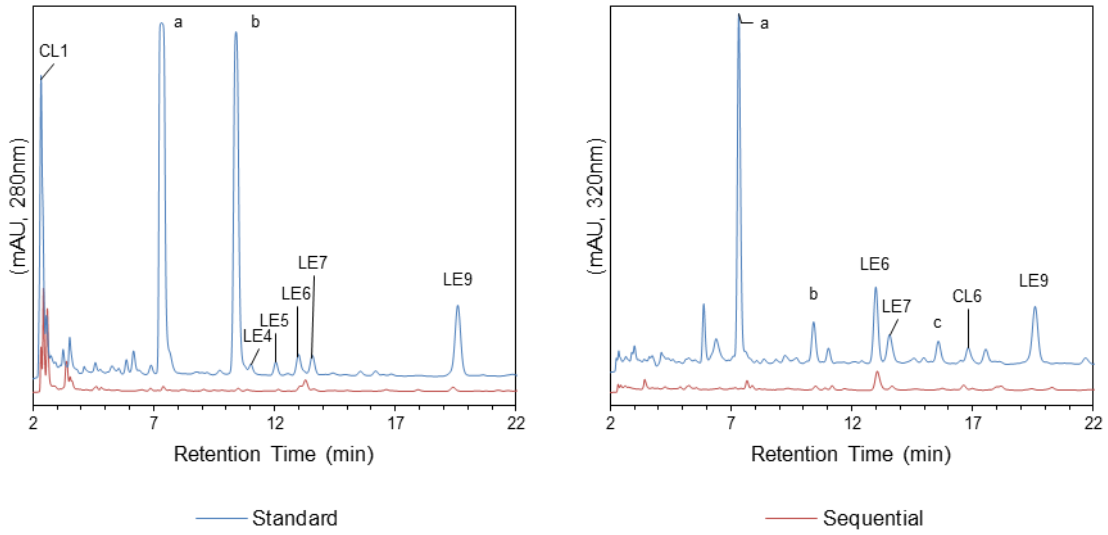


Figure 4-2 – HPLC chromatogram comparison between standard and sequential SubCW extraction at 220ºC after 10 minutes. a – 5-HMF; b – furfural; c – chlorogenic acid.

The major differences between the standard and sequential extracts at 220ºC were the absence of furfural (R_t=10.4min) and the much reduced amounts of 5-HMF (R_t=7.3 min) in the sequential extracts. Also, the compounds that were detected around R_t of 3 minutes in the

standard extract were again significantly reduced in sequential extract These results clearly indicated that the sequential extraction reduced the extract concentration and complexity.

For the standard extraction the amount of number of compounds identified increased almost exponentially with the increase of the extraction temperature (Figure 4-3) while the amount of compounds detected for the sequential extraction (*per g Cider Lees DW*) not only were significantly lower than the standard extraction but also it seemed to increase up to 170ºC, from which started to decrease. The exponential increase observed in the standard extraction was attributed to the increase in the amount of 5-HMF, furfural and compounds CL1+LE1. The sequential extraction result were in line with the results obtained from the total solids analysis, where the *overall yield* of the sequential extraction increased towards 170ºC and then decreased at higher temperatures.

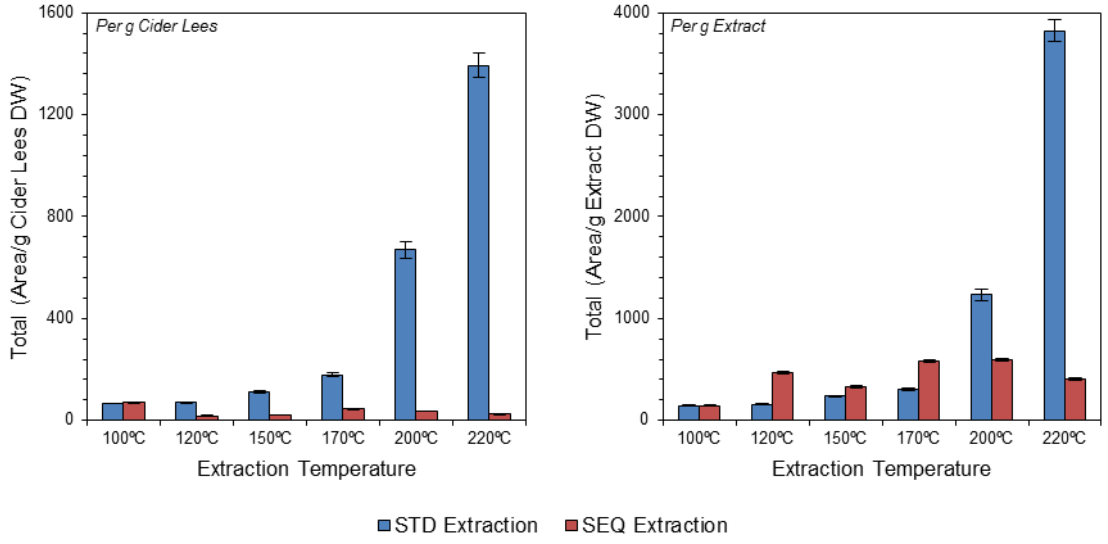


Figure 4-3 – Area sum of all compounds detected by HPLC in the standard (STD) and sequential (SEQ) extraction.

The total compounds area for sequential extraction followed the same trend verified for the total solids analysis (Figure 4-1), where the *overall yield* of the sequential extraction increased towards 170ºC to decrease at higher temperatures.

However, when the cider lees extracts were analysed from the point of view of the extract produced (yield of extraction) and not the starting material, the cider lees extracts from the sequential had a total compounds area higher than the extract from standard up to 170ºC, i.e. the extracts obtained at temperature lower than 170ºC from sequential had a higher concentration on the compounds being quantified by the HPLC method adopted.

Table 4-2 – List of new compounds detected with sequential SubCW extraction.

Compound no.	R_t (min)	Spectra	
		Main (nm)	Secondary (nm)
SQ1	7.6	262	
SQ2	8.5	260	294
SQ3	9.8	324	232, 288
SQ4	13.3	276	234, 316
SQ5	27.6	334	

From all new compounds previously detected in the cider lees extract, compounds LE3, LE7 and LE9 were not found in the cider lees extract from sequential extraction. The absence of compounds LE7 and LE9 indicated a decrease in polyphenol thermal decomposition, as compound LE7 and LE9 had been suggested as the result of thermal decomposition of caffeic acid and chlorogenic acid. At the same time, five new compounds were detected in cider lees extract from sequential extraction (Table 4-2).

4.3.2.1 UV-spectra of sequential SubCW extraction new compounds

While compounds SQ1, SQ3 and SQ4 have UV-spectra similar to compounds detected either in cider lees supernatant or in cider lees extract, compound SQ2 and SQ5 had a unique UV-spectra in the context of the present work (Figure 4-4). Particularly compound SQ5, as it had

maximum absorbance at 334 nm which no other compound exhibited in the present work. Due to compound SQ5 relative retention time, it could be suggested as a quercetin (A. Schieber et al., 2001a), however its maximum absorbance at 334nm was different from the maximum absorbance wavelength expected for quercetins which is 370nm (A. Schieber et al., 2001a) and 350nm to quercetin glycosides (Marks et al., 2007b). Also, quercetins are a minor polyphenol in cider (Marks et al., 2007b) and no quercetins were initially detected in cider lees supernatant (3.3.4.1).

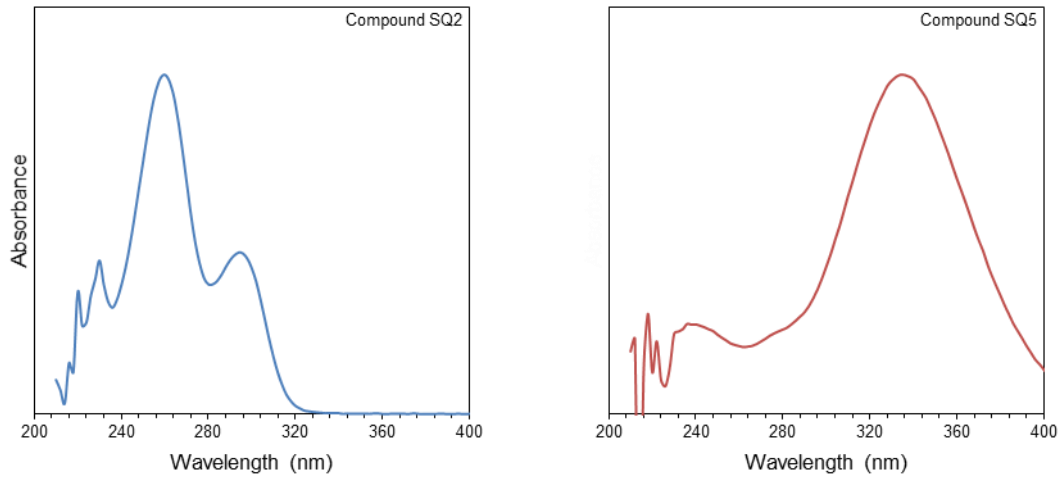


Figure 4-4 – UV-spectra of compound SQ2 and SQ5.

Compound SQ2 was characterised by having two maximum absorbance wavelengths, 260nm as the main wavelength and 292nm. Although no other compound detected has a similar UV-spectra, compounds CL1, CL2 and CL3 had maximum absorbance around 260nm while compound LE7 and LE9 had maximum absorbance around 290nm. The similarities however do not allowed reliable identification of the compound and therefore the class SQ2 falls into. Compound SQ1 had a retention time close to 5-HMF however compound SQ1 UV-spectra was similar to compound CL1 and CL3 (Figure 4-5).

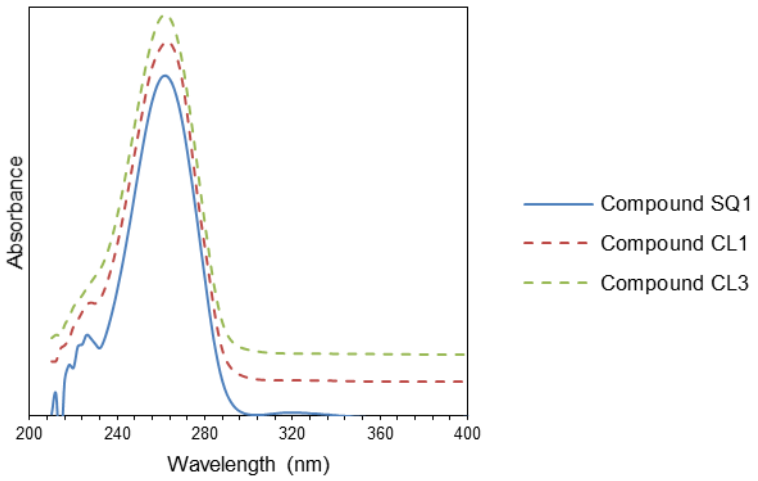


Figure 4-5 – UV-spectra comparison between compound SQ1 and compounds CL1 and CL3.

Compound SQ3 had a similar UV-spectra to caffeic acid and consequently to chlorogenic acid and its esters (Figure 4-6). However compound relative retention time did not permit to make any suggestion of which compound could it be other than it should be a hydroxycinnamic acid.

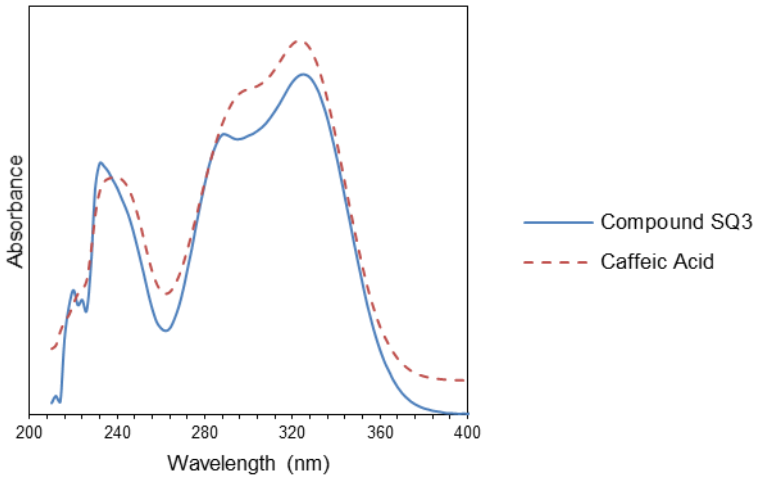


Figure 4-6 – UV-spectra comparison between compound SQ3 and caffeic acid.

Compound SQ4 had maximum absorbance at similar wavelengths to compound LE8. Compound LE8 has been previously suggested as a flavanol, more specifically as procyanidin B2. According to Marks et al. (2007b) besides the flavanols found in cider apples, catechin,

epicatechin and procyanidin B2, proanthocyanidins were also detected with different degrees of polymerisation. However, the detection of proanthocyanidins requires a different method. From the data collected in A. Schieber et al. (2001a), from where the HPLC method was adapted, it was observed that procyanidin B1 has lower retention time that any flavanol detected in cider apple, apple cider and in the cider lees supernatant. Although it was not possible to confirm compound SQ4 as procyanidin B1, it could be suggested that compound SQ4 was likely to be a dimer of catechin or epicatechin.

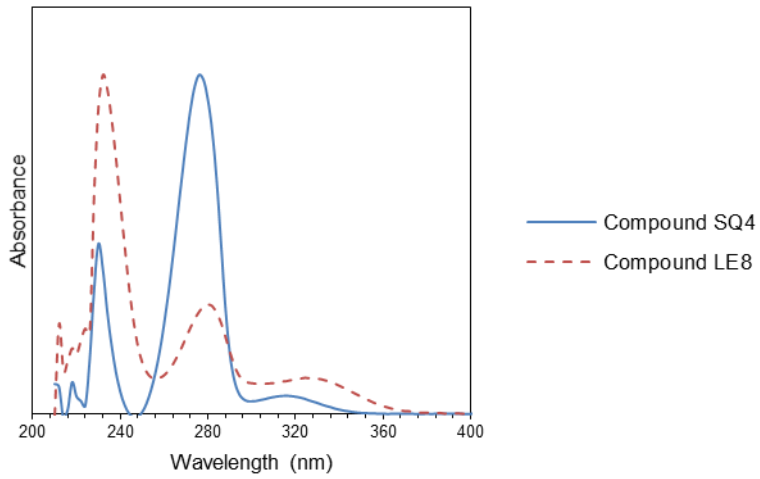


Figure 4-7 – UV-spectra comparison between compound SQ4 and LE6.

However there was one particular difference between compound SQ4 and the compounds previously identified as flavanols. Compound SQ4 overall maximum wavelength of absorbance was 280nm while for the flavanols previously suggested was at 230nm.

4.3.2.2 Quantitative analysis

Sequential SubCW extraction from cider lees had produced five new compounds, SQ1-SQ5, while compounds LE3, LE7 and LE9 were not detected.

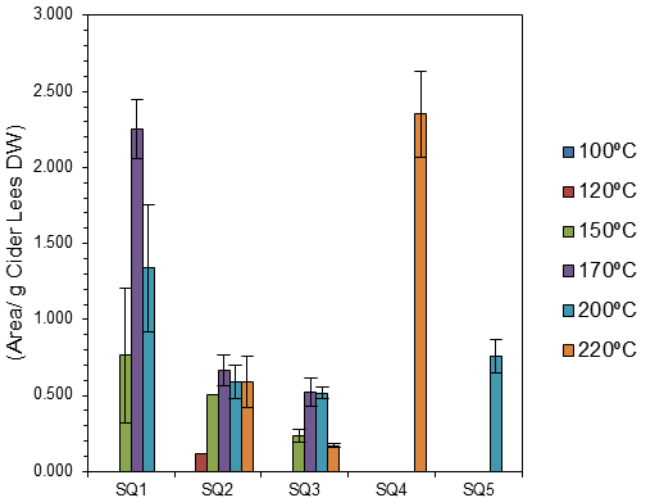


Figure 4-8 – Sequential SubCW extraction new compounds area in the cider lees extract.

Figure 4-8 shows the area of the new compounds produced under sequential SubCW conditions. Compounds SQ1 and SQ3 were first detected at 150ºC and their maximum area are at 170ºC. Compound SQ1 was still found in the 200ºC extract but not at 220ºC, while compound SQ3 was found at comparable levels at 170º and 200ºC, and at 220ºC was still observed in the extract, although it was less than half of what was observed at lower temperatures.

Compound SQ2 was first observed at 120ºC but it was from 150ºC that is amount increases with similarly area at 170ºC, 200ºC and 220ºC which indicated was being extracted/produced at a same rate when temperature was above 170ºC.

Compound SQ4 and SQ5 had the particularity of only being produced at a single temperature. Compound SQ4 was observed at 220ºC while compound SQ5 at 200ºC. Compound SQ4 and compound SQ1 were the compounds extracted in higher amounts during the sequential SubCW extraction from cider lees.

4.3.2.2.1 Hydroxycinnamic acids

During SubCW extraction at higher temperatures, thermal decomposition of polyphenols was expected, and as it was observed in the previous chapter, all polyphenols concentration decreased with the increase of extraction temperature, especially when above 170ºC.

One of objective of studying sequential SubCW extraction was to decrease the thermal decomposition of the polyphenols. The ability to detect compounds LE7 and LE9, previously suggested as decarboxylation products from caffeic acid and chlorogenic acid, indicated that the strategy of using sequential SubCW did reduce the degradation of polyphenol. Also, as it can be seen in Figure 4-9, chlorogenic acid was detected at all extraction temperatures which indicate that it was being released from the yeast cells as it had been observed by Bahari (2010). However chlorogenic acid release into solution seemed to occur at lower temperatures, as its extraction decreased with the increase of temperature.

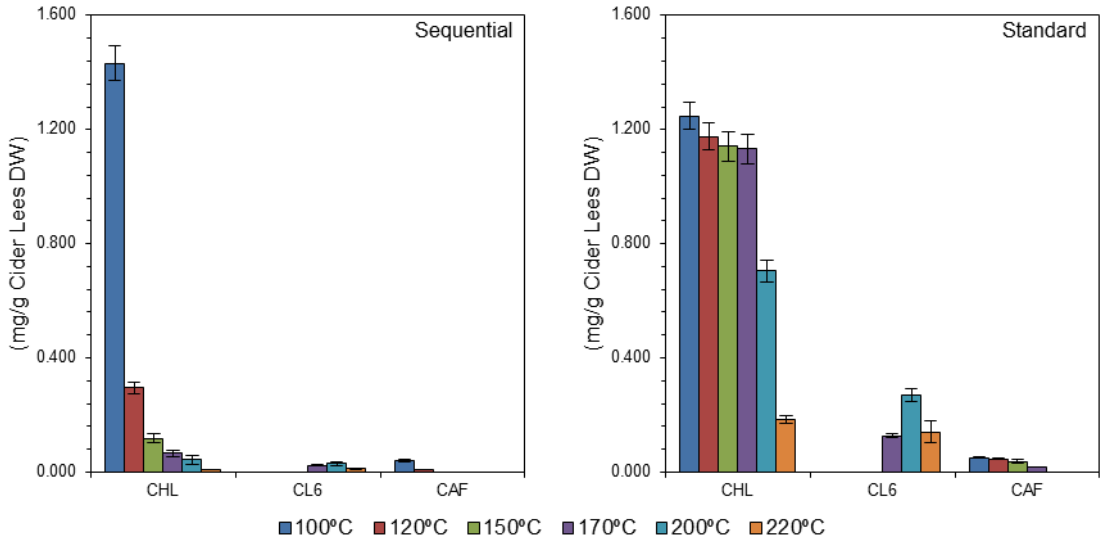


Figure 4-9 – Chlorogenic Acid (CHL), Caffeic Acid (CAF) and compound CL6 concentration in sequential and standard SubCW extraction.

Caffeic acid on the other hand, was not detected above 120ºC, and at 120ºC it was found at trace levels which led to suggest that there was no release of caffeic acid from yeast cells.

Compound CL6 as in the standard subcritical water extract, was detected between 170 and 220ºC, with a maximum concentration at 200ºC. Compound CL6 was suggested as a chlorogenic acid isomer that could be the result of chlorogenic acid undertaking isomerisation reaction. As chlorogenic acid was continually extracted at higher temperatures it was still possible that compound CL6 was the result of its isomerisation, which suggests that this reaction occured rapidly when temperatures of 170ºC or higher were used.

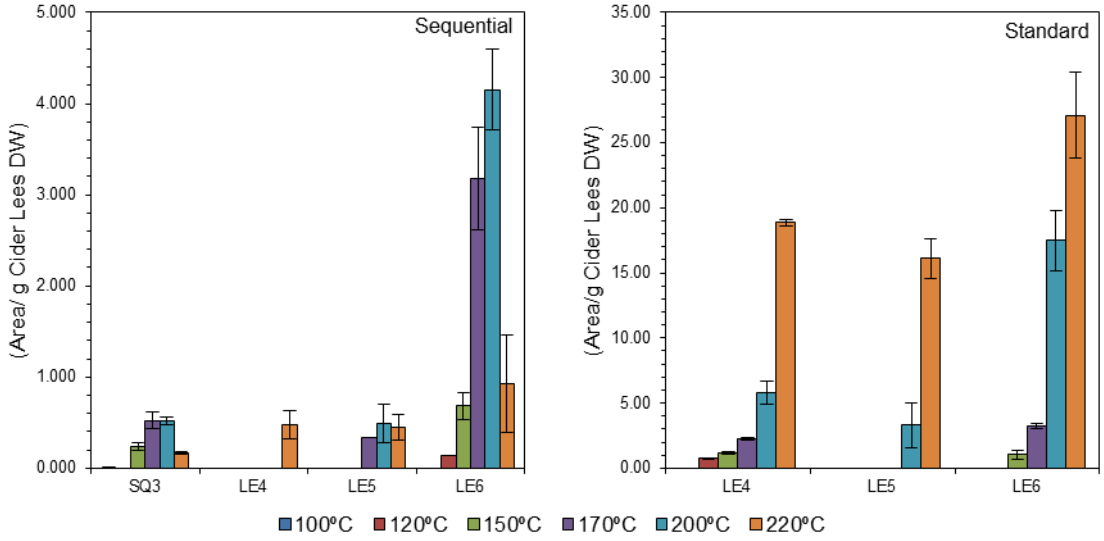


Figure 4-10 – Compounds LE4 to LE6 and compounds SQ3 area in standard and sequential SubCW extraction.

As mentioned before, compounds suggested as the product of chlorogenic acid thermal transformation were not detected, however other compounds associated with its transformation were detected, besides compound CL6. Compounds LE4 and LE5, possible caffeic acid and chlorogenic acid quinones, respectively and compound LE6, suggested as protocatechuic aldehyde. Although these compounds were detected, they were detected in amounts considerably lower than the amounts detected with standard extraction.

Compound LE4 had the parcularity of only being detected at 220ºC and as caffeic acid was not detected at temperatures above 220ºC, it is unlikely that it was a caffeic acid quinone. Nonetheless, it was still possible to be the result of polyphenol oxidation. Compound LE5 using

standard SubCW extraction at time 0 was only detected at 200ºC, although it was also detected at 170ºC after 30 minutes of extraction. With sequential SubCW extraction, compound LE5 was detected between 170ºC and 220ºC, with its maximum at 200ºC, however differences in extraction are minimal among the three different temperatures used. The temperature range above 170ºC was the temperature range associated with chlorogenic acid degradation and as it was observed in Figure 4-9 was where compound CL6, also associated with chlorogenic acid degradation, was detected. Also, chlorogenic acid was still detected in this temperature range but at lower amounts.

Compound LE6, indicated as protocatechuic aldehyde, that could be the result of oxidation of caffeic acid or chlorogenic acid, was the compound associated with chlorogenic acid degradation detected in higher amounts and in a wider range of temperatures. Protocatecuhic aldehyde suggested as compound LE6 possess a higher antioxidant activity than chlorogenic acid, contrary to quinones and decarboxylation products (Davalos et al., 2004). Compound LE6 extraction increased significantly at 170ºC, but it was at 200ºC that maximum extraction was observed. Again this observation correlates the fact that chlorogenic acid degradation occured at temperature of 170ºC and above.

Lastly, compound SQ3 which had similar UV-spectra to chlorogenic acid was detected in the same area range of compounds LE4 and LE5 and it was also detected at higher amounts when extraction was performed at 170ºC and 200ºC. Although compound SQ3 was not formally identified, results suggest that it could also be a degradation product from chlorogenic acid.

The results observed in Figure 4-8 and Figure 4-9 suggest that chlorogenic acid was being released from cider yeast cells at every temperature used during the sequential extraction and when that temperature is 170ºC or higher its degradation occured rapidly into isomers, quinones

and aldehydes. However, decarboxylation was not observed which suggest that was a transformation that requires longer extraction times to occur.

Besides chlorogenic acid and caffeic acid, other hydroxycinnamic acid were detected in cider lees and its extracts, more specifically *p*-coumaric acid and related esters.

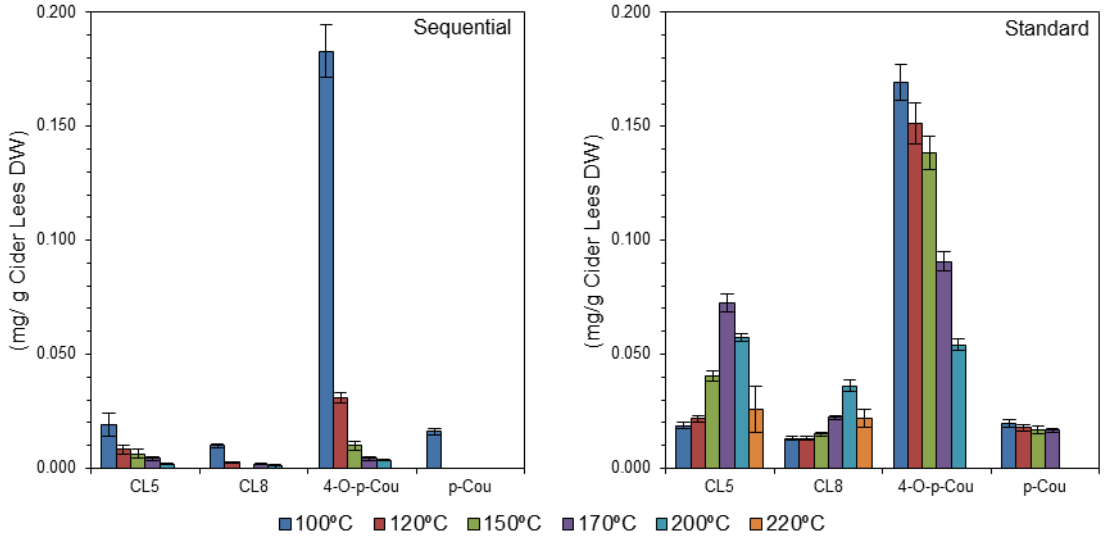


Figure 4-11 – Compound CL5, CL8, *p*-Coumaric acid (p-Cou) and 4-*O*-*p*-coumaroylquinic acid (4-O-p-Cou) concentration in standard and sequential SubCW extraction.

p-Coumaric acid and 4-*O*-*p*-coumaroylqunic acid results from sequential SubCW extraction (Figure 4-11) were similar to caffeic acid and chlorogenic acid results (Figure 4-9), respectively. *p*-Coumaric acid as caffeic acid was only detected at 100ºC while 4-*O*-*p*-coumaroylqunic acid was detected at all extraction temperatures except at 220ºC, and as chlorogenic acid results suggest that it was being released into solution from yeast cells, especially at 120ºC. Compound CL5 and CL8 were suggested as isomers from 4-*O*-*p*-coumaroylqunic acid, but contrary to chlorogenic acid and its isomer, compound CL6, these two isomers were found in higher amounts at 100ºC and although they were detected at higher temperatures, their concentrations decrease with the increase of temperature. These results differed from what was observed with standard extraction, where compounds CL5 and CL8 were found at higher amounts when

extraction temperatures were above 170ºC. Sequential extraction showed that compound CL5 and CL8 were being released into solution, but coupled to the increase in their concentration during standard extraction, release from yeast cells cannot be the only explanation for such increase. As it was suggested before that the increase of compound CL5 and CL8 was the result of isomerisation reaction of 4-*O*-*p*-coumaroylqunic acid, it can be concluded that such reactions did not occured during the sequential extraction.

Overall, hydroxycinnamic acids desorption from yeast cells was observed and it can be concluded that high temperatures were not needed for such desorption, as its highest desorption occurs at 120ºC.

4.3.2.2.2 Dihydrocalchones

Phloridzin and compound CL9 during standard extraction have shown that they were being released into solution when extraction was performed with temperatures up to 170ºC, above which their concentration started to decrease (Figure 4-12).

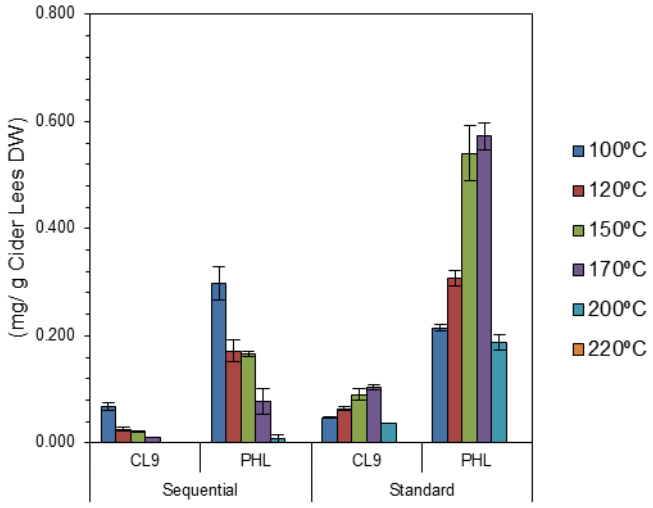


Figure 4-12 – Compound CL9 and Phloridzin concentration in standard and sequential SubCW extraction.

Sequential extraction confirms that phloridzin and compound CL9 were being released into solution when extraction was performed with temperatures up to 170ºC, above which these

compounds were not detected or were detected at trace levels. Their release into solution seems to be continuous and it should be complete by the time 170ºC was applied in sequential extraction. Above 170ºC the degradation of phloridzin and CL9 was expected as standard extraction results showed in Figure 4-12 however under sequential was not observed, which means that desorption of these compounds from yeast cells was complete at 170ºC or a complete degradation of the amount released into solution occured.

4.3.2.2.3 Flavanols

Compounds CL4, CL7 and LE8 previously identified as (+)-catechin, (–)-epicatechin and procyanidin B2, have shown during the standard extraction to increase in concentration when extraction temperature was not higher than 170ºC, after which the concentrations of these compounds started to decrease. The decrease in area of CL4, CL7 and LE8 was coincident with the decrease in concentration of chlorogenic acid but also with a significant increase in total phenolic content by FCR method. It was suggested that dimerisation and polymerisation reaction of these compounds could be occurring, and therefore account for their decrease in concentration and but yet support the increase in FCR results, as these also measure the antioxidant activity. Also polymers from catechin and epicatechin were not possible to be detected with the present reverse phase HPLC method used.

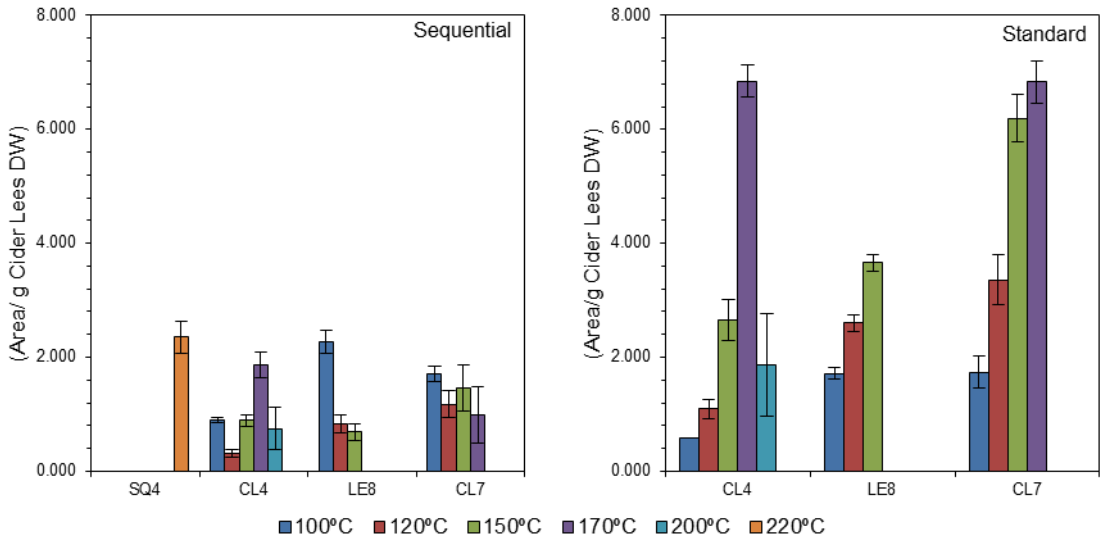


Figure 4-13 – Compound CL4, LE8 and CL7 area in standard and sequential SubCW extraction.

Sequential extraction of CL4, CL7 and LE8 compounds showed different results for the different compounds (Figure 4-13). Compound LE8 decreased in area and as in standard extraction it was also not detected above 150ºC, fact that was attributed to the presence of another compound with the same retention time (CL6) which increased in area in this temperature range. Compound CL7 had a slight decrease in extraction between 100ºC and 120ºC, and although extraction was maximum at 150ºC, it can be said that it did not vary significantly between 120ºC and 170ºC. Compound CL4 had a significant increase in area as observed for the standard extraction, with maximum at 170ºC.

The results corroborated the standard extraction, where a continuously release into solution was observed up to 170ºC, from which then a decrease or not detection was observed. A different conclusion from what was made for standard extraction was not possible to achieve. The theory of possible polymerisation is still plausible, especially due to compound CL4 being observed at 200ºC at a lower area, after removing the extracted before. Such result might indicate a higher desorption followed by a rapid polymerisation.

4.3.2.2.4 5-HMF and furfural

Standard SubCW extraction of cider lees resulted in 5-HMF and furfural when temperatures above 170ºC were applied. 5-HMF and furfural were the result of monosaccharide hexoses and pentoses dehydration, respectively. While hexoses as glucose and mannose were present in the yeast cell wall, pentoses were not expected to be found in cider yeast cells, therefore the pentoses in solution were expected to be the result of the enzyme treatments during apple juice production. Furfural (lower limit of detection 0.01 mg/ml) was not produced during sequential SubCW extraction which corroborates the existence of pentoses as the result of apple juice production.

As with furfural, 5-HMF production was expected to decrease, as hexoses that can be found in cider were removed before temperatures above 170ºC were employed, as it can be seen in Figure 4-14 where the amount of 5-HMF produced during sequential extraction at 170ºC were considerably lower than the amount produced with standard extraction, 0.269±0.006 mg/g Cider Lees DW against 0.566±0.097 mg/g Cider Lees DW. Also, it was considerably lower than the maximum amount of 5-HMF produced during standard extraction at 220ºC, which was 11.968±0.690 mg/g Cider Lees DW.

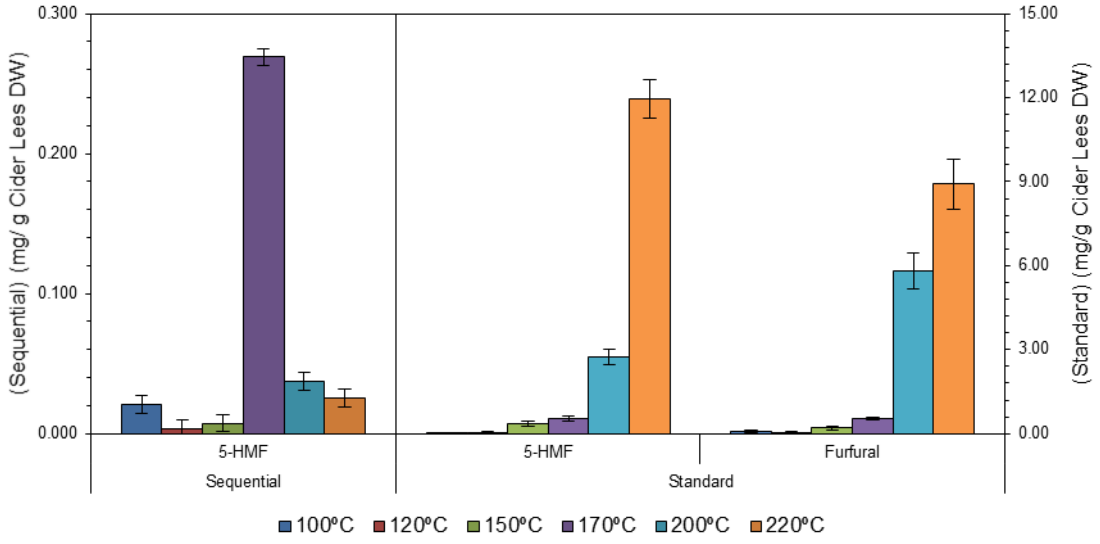


Figure 4-14 – 5-HMF and Furfural concentration using standard and sequential SubCW extraction.

The amount of 5-HMF produced in sequential hydrolysis was exclusively from glucose and mannose that was being released from the cider yeast wall, as it was demonstrated by (Bahari, 2010). Also the results indicated that was at 170ºC that the release of mannose from the yeast cell wall occurs, although at 200ºC and 220ºC this release continued to occur (Gomis et al., 2001).

4.3.2.2.5 Other compounds

It was observed that there was a significant decrease in all the compounds with retention time lower than 10 minutes under sequential Sub SCW as illustrated in Figure 4-15.

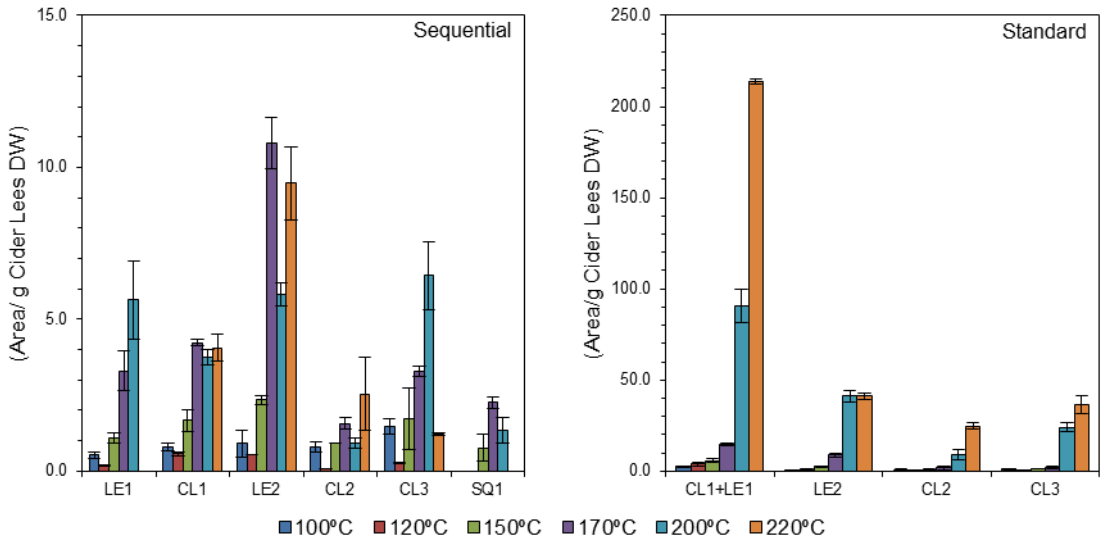


Figure 4-15 – Compounds CL1 to CL3, LE1, LE2 and SQ1 area using standard and sequential SubCW extraction.

While the compound CL1 to CL3, LE1, LE2 were significantly reduced during sequential SCW treatment most of these compounds did not decrease in area with higher temperatures and higher extraction times. Results from sequential work suggest that between 170-200ºC these compounds area increased and compounds CL1 and LE2 still had significant concentration at 220ºC.

4.3.3 Antioxidant capacity

In the previous chapter FCR method was used to determine the total polyphenol content in the cider lees extract sample obtained after SubCW extraction, although interferences from non-polyphenol compounds making value of the result obtained not entirely clear. Nonetheless, due to FCR method mechanism it can be used as an antioxidant capacity method and ORAC assay was used to assess this possibility in line with reports in the literature (D. Huang et al., 2005).

As seen in previous results, SubCW extraction can be assessed by the antioxidant capacity from the starting cider lees sample (per g Cider Lees DW) but also by the antioxidant activity of the

extract obtained (per g Extract DW). Cider lees extracts obtained from standard SubCW extraction showed similar trend for antioxidant capacity when measured by FCR method and ORAC assay (Figure 4-16), which allowed to conclude that FCR can be safely used as an indicator of antioxidant activity, as it was previously reviewed by D. Huang et al. (2005) and applied by Plaza et al. (2013).

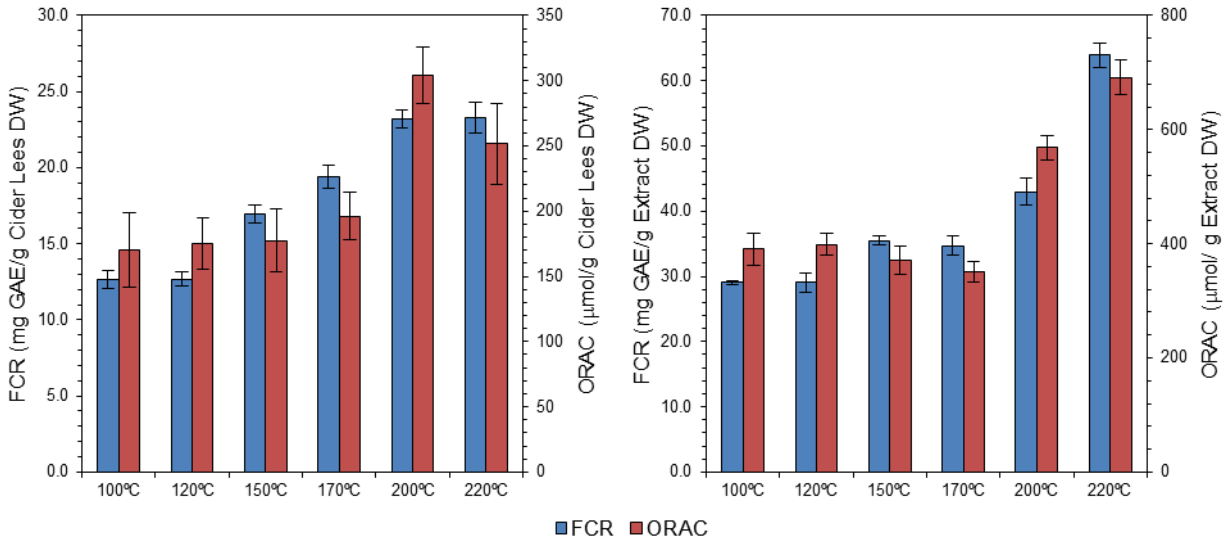


Figure 4-16 - Comparison between FCR, ORAC results for standard SubCW extraction.

As antioxidant capacity can be represented relative to the starting material and to the material extracted, it was interesting to observe that while there was an increase in the antioxidant capacity extracted from the starting material when extraction temperature was above 120ºC, the extract itself remained with similar antioxidant capacity independently of being extracted at 100ºC or 170ºC, which was a consequent result of the increase of the *overall yield* of extraction up to 170ºC (Figure 4-1), i.e. not only more antioxidants were being extracted into solution but also other non-antioxidant compounds which could mean that the relative composition of the extract should not differ for extracts obtained between 100ºC and 170ºC. Above 170ºC there was an increase of antioxidant capacity of the extract while *overall yield* of extraction was

decreasing (Figure 4-1), which indicates that extraction above 170ºC was more selective towards antioxidants, as it was observed in the previous chapter.

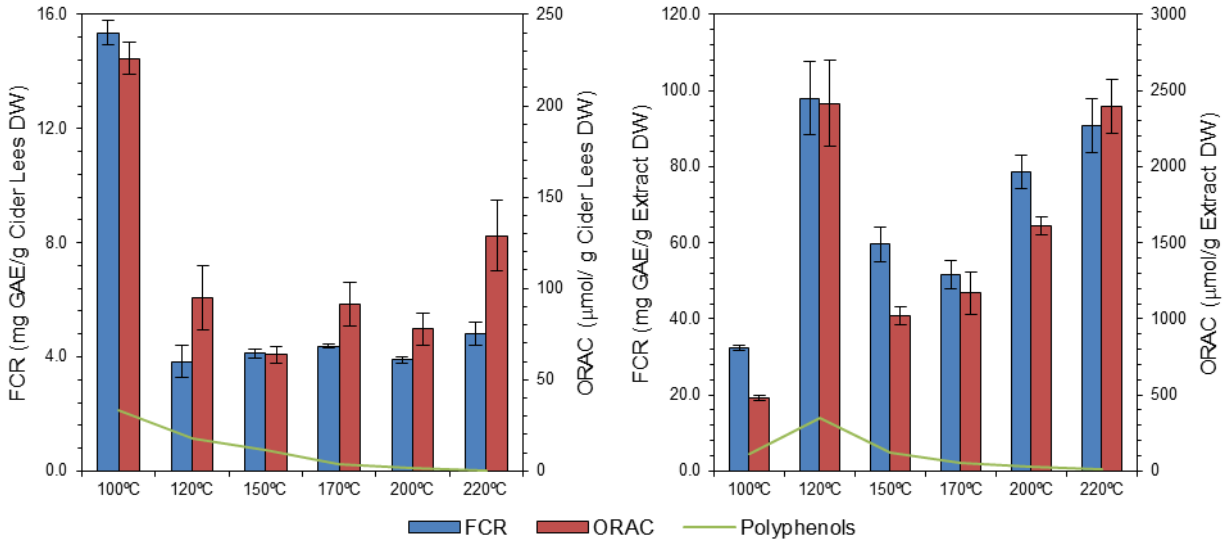


Figure 4-17 – Comparison between FCR, ORAC and HPLC results for sequential SubCW extraction.

As for sequential SubCW extraction, the antioxidant capacity results differed from the standard SubCW extraction. When antioxidant capacity by FCR method was determined from the starting cider lees, it seemed to not vary significantly for the extracts obtained between 120ºC and 220ºC, however it was at 220ºC that higher antioxidant activity from the starting cider lees was obtained (Figure 4-17). ORAC results showed higher differences between the cider lees extracts obtained at extraction temperatures between 120ºC and 220ºC but with similar result trend to the FCR results (except for 120ºC). Such variability was likely to be linked to the limitations of how this method was applied, as it can be seen for its higher associated error.

As for the antioxidant capacity of the cider lees extract produced from sequential extraction, the results showed two different highest values for antioxidant capacity, at 120ºC and at 220ºC (Figure 4-17). It was important here to verify that antioxidant capacity at 120ºC was coincident with the highest concentration of polyphenols detected by HPLC, which led to suggest that in this cider lees extract sample the polyphenols detected contribute to the antioxidant capacity

verified. However, the concentration of polyphenols in the cider lees extract at 220ºC was minimal which meant that antioxidant capacity of the 220ºC extract sample was from a different group of compounds, a group of compounds that were exclusively extracted above 200ºC. Previous work from (Bahari, 2010) had observed that cider yeast cell lose their shape after extractions at 180ºC and at higher temperatures was verified the disruption of the cell wall (Figure 4-18). When temperatures of 225ºC were employed the non-soluble compounds remaining had similar structure to β-glucan (Zechner-Krpan et al., 2010). So the antioxidant capacity verified for extraction at 220ºC could be the result of solubilisation of cell wall constituents, such as mannoproteins, glucan, chitin and lipids but also intracellular compounds (Santiago and Mori, 1993) and compounds that were still absorbed by the yeast cells.

(Bahari, 2010) (Zechner-Krpan et al., 2010)

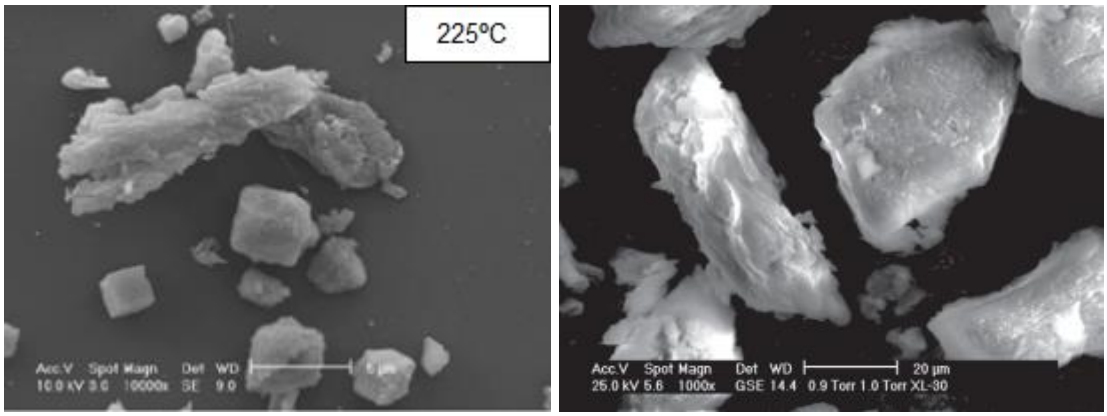


Figure 4-18 – Comparison between SEM images of beta-glucan (Zechner-Krpan et al., 2010) and cider yeast cells after treatment with subcritical water at 225ºC (Bahari, 2010).

4.3.4 Acetone polyphenol extraction

In the previous chapter it was suggested that chlorogenic acid was being desorbed from yeast cells and it was also taking part of complex interaction with proteins. In the present chapter the desorption of chlorogenic acid from yeast cells was already verified, now it will be important to verify if any complex interactions were occurring at 220ºC, as a higher antioxidant capacity was observed without being able to link it to apple natural polyphenols.

Table 4-3 – Acetone extraction yield and FCR results.

	Yield %(w/w)	**FCR (mg GAE/g extract)**
Supernatant	3.05±0.14	4.26±0.20
Washed Solids	96.95±0.14	69.15±2.35
Total	-	*73.41±2.36*
Seq-SubCW 220ºC	-	76.77±1.23

In the previous chapter it was suggested that chlorogenic acid was being desorbed from yeast cells and it was also taking part of complex interaction with proteins. In the present chapter the desorption of chlorogenic acid from yeast cells was already verified, now it will be important to verify if any complex interactions were occurring at 220ºC, as a higher antioxidant capacity was observed without being able to link it to apple natural polyphenols.

Table 4-3 shows that the yield of acetone extraction was low as verified previously and also that there was no significant increase or decrease in the FCR result before and after acetone extractions.

In the previous chapter it was verified that at temperatures of 200ºC, chlorogenic acid concentration was higher after the acetone extraction, and therefore allowed to suggest that chlorogenic acid was taking part of complex interactions which the HPLC method used was unable to detect.

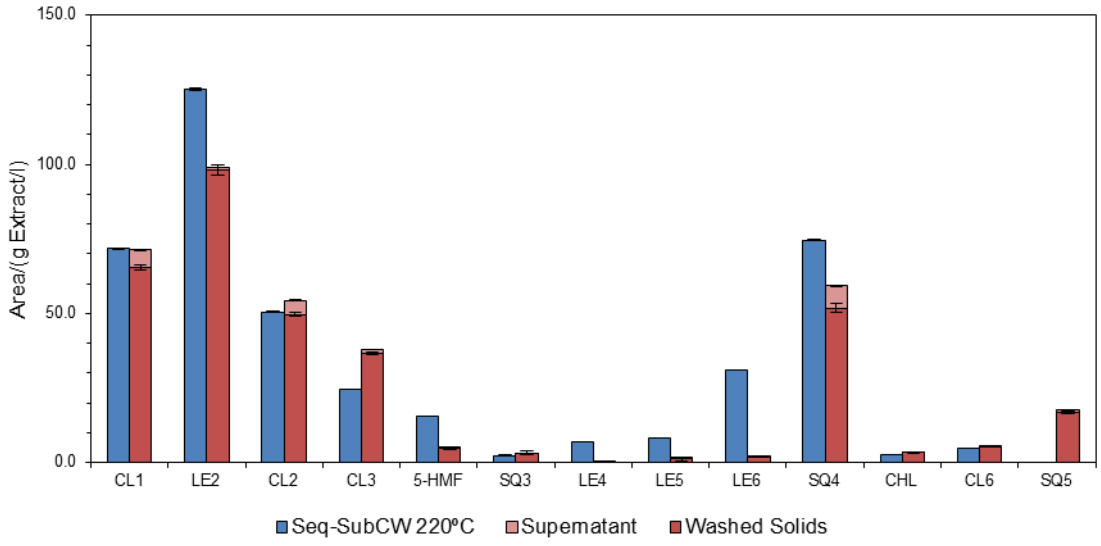


Figure 4-19 – Comparison of chemical characterisation of sequential cider lees extract and its respective acetone extract.

In Figure 4-19 can be seen the concentration of all compounds detected in the cider lees extract obtained from SubCW extraction at 220ºC before and after acetone extraction. An increase of more than 20% of chlorogenic acid was verified, however this increase was low compared to the increase of more than 8 fold verified in the previous chapter. Such difference can be mainly explained by the fact that in sequential extraction chlorogenic acid had been removed at lower temperatures, so less chlorogenic acid will be available to take part of complex interactions. Nonetheless it is interesting that the formation of complex still occured after the removal of most of the chlorogenic acid present in the cider lees sample.

Compounds CL6 and SQ3 that were suggested as polyphenols, also increased in concentration after acetone extraction, with increases between 10 and 30%. On the other side compounds as LE4, LE5 and LE6 all decreased significantly in concentration. But the most interesting observation was the reappearance of compound SQ5 in the washed solids. Compound SQ5 was only observed in the sequential SubCW extract obtained at 200ºC, with areas of 11.68±2.32 area/(g extract/l) and 0.76±0.11 area/(g cider lees DW/l). After acetone extraction, compound

SQ5 was found in higher concentrations which can also suggests that compound SQ5 interaction happened at 200ºC. From these changes it seemed that compounds that were being released between 200 and 220ºC were interacting.

Although there were small increases in some compounds and the reappearance of others, the difference in concentration was not markedly higher as verified in the previous chapter. Once again, it seemed that interaction with some of the compounds detected was occurring, mainly compound SQ5, however the amounts in question did not seem to be enough to had an impact in the final antioxidant capacity.

4.4 Conclusion

The present chapter had as main objective to recover the compounds released at different extraction temperatures and to minimise thermal degradation of compounds released at lower temperatures. By doing so it was observed that was with extraction temperatures of 150 to 170ºC that the highest amount of the starting cider lees was released into solution, however it was at higher temperatures, 200 to 220ºC that the SubCW mediated hydrolysis was more efficient, i.e. more of the solids for that particular extraction were solubilised. However, the total amount of compounds detected by HPLC at 200ºC and 220ºC was less than the amount detected at 170ºC, which indicated the inability of the HPLC method, designed to detect phenolic compounds, to identify the compounds present in solution and reinforced the fact that the compounds present in this solution were not polyphenols or were complex compounds which cannot be detected at the selected wavelengths with the column chosen.

As the cider lees extract obtained at different temperatures was being recovered, some transformations were expected to be avoided. From chemical characterisation with HPLC it was observed that furfural and compounds LE7 and LE9 were not produced. While furfural was

a result of monosaccharides from juice production that were present in cider lees supernatant, the lack of compounds LE7 and LE9 indicate that thermal degradation of chlorogenic acid or similar compounds was prevented/reduced. Indeed it was verified that chlorogenic acid and other polyphenols were desorbed from cider yeast cells, however it seemed that they were desorbed at lower extraction temperatures rather than higher. The exception to such behaviour was compound CL4, suggested as catechin, which area increased up to 170ºC, indicating that its desorption occured at higher temperatures. In summary, no cider polyphenols were detected in significant concentrations in the extract obtained at 220ºC. New compounds were detected from which compound SQ4 and SQ5 were detected at higher temperatures. While compound SQ5 have a distinct UV-spectra, compound SQ4 could be compared to flavanols (procyanidins), but again none of these new compounds were found in amounts that could suggest their fundamental contribution to the antioxidant capacity observed.

Sequential SubCW extraction from cider lees as with standard extraction resulted in maximum antioxidant capacity when the extraction temperature is 220ºC, and again the amount of polyphenols in that extract sample did not corroborate such capacity. However, when 120ºC was applied an extract with similar antioxidant capacity to the extract produced at 220ºC was produced and to which it was possible to link to simple polyphenols detected in apple cider. Nonetheless, the yield of extraction for at 120ºC was rather low compared to 220ºC, 7.9%±0.9% against 22.6%±1.2%. Also it was important to note that at 220ºC the extract from sequential had a higher concentration in antioxidants than the extract obtained from standard extraction (Figure 4-16, Figure 4-17), which was a direct result of the significant decrease in 5-HMF, the prevention of furfural formation and to some extent polyphenol thermal degradation but also the collection of compounds released at lower temperatures which might not had antioxidant properties.

At the same time, as mentioned previously, HPLC method used was not able to detect one or more groups of compounds that could be entirely associated with the highest antioxidant capacity obtained at 220ºC. Furthermore sequential extracts provided evidence that the high antioxidant capacity for extractions at 220ºC was exclusively from compounds that were being extracted or released into solution above 200ºC and not from compounds previously released or from their thermal transformations. Also, acetone extraction of the cider lees extract obtained at 220ºC gave indication of possible complex interaction present in solution, especially due to the detection of compound SQ5 which was not detected prior to acetone extraction.

In summary, the antioxidant capacity obtained for extraction at 220ºC is likely to be the result of cell disruption and the release into solution of cell wall constituents and intracellular compounds, which occurs at extraction temperatures above 170ºC to which the HPLC method used was not able to detect as it was designed for simple polyphenols present in fruit samples such as apple.

CHAPTER 5

EVALUATION OF β-CYCLODEXTRIN ENCAPSULATION TO IMPROVE OXIDATIVE STABILITY OF CIDER LEES EXTRACT

5.1 Introduction

Plant extracts with phenolics similar to active agents found in cider lees extracts have been employed in food industry but they also have been adapted to serve as major ingredients in both cosmetic and health products. However the effectiveness of these bioactive compounds relies on preservation of their stability and bioavailability. Most of these active agents, for example chlorogenic acid are sensitive to oxidation, high pH, temperature and light and also have poor water solubility (Pinho et al., 2014), which can restrict their use and their efficiency as bioactive agents.

Cyclodextrins have been widely used to prevent the degradation of active compounds and increase their water solubility thereby improve their bioavailability, in industries such as food, cosmetics or pharmaceuticals by forming inclusion complexes (Szejtli, 1998). It has also been used to protect polyphenols such as chlorogenic acid (Zhao et al., 2010).

The objective of the present chapter was to assess the use of β-cyclodextrin to improve the oxidative stability of cider lees extract produced by SubCW extraction from cider lees. FCR

method was used as an antioxidant capacity assay to evaluate if the addition of cyclodextrin had an impact on the initial antioxidant capacity. A heat stress method was used to evaluate the formation of an inclusion complex and also its capacity to prevent its decomposition. Lastly, the cider lees extract from 200ºC was applied in a skin care formulation, one of the possible future applications for these extracts. Its oxidative stability was evaluated over a period of six months, at three different storage conditions, 4ºC, room temperature and 37ºC. The difference in colour was measured to assess its stability when applied in a skin care cream formulation. Also, cider lees extract with cyclodextrin were used in skin care cream formulation to evaluate the formation of an inclusion complex and how it can prevent the colour decomposition of the skin care cream formulation.

5.2 Materials and methods

5.2.1 Cider lees extract

Three different SubCW cider lees extract were produced at three different temperatures to be used for β-cyclodextrin encapsulation: 150ºC, 200ºC and 220ºC. A 300ml vessel was used to produce cider lees extract at 150ºC after 30 minutes of extraction and at 220ºC after 10 minutes of extraction while a 5 litres vessel was used to obtain cider lees extract at 200ºC after 10 minutes of extraction (both procedures are described in 3.2.2).

Table 5-1 – Total solids for cider lees extract produced at different temperatures.

Temperature *(ºC)*	**Extraction Time** *(min)*	**Total Solids** *(g/l)*
150	30	84.53±0.59
200	10	59.63±0.10
220	10	50.00±0.40

The cider lees sample used for this experiment was collected on March 2011 and it is characterised by total solids of 146.28±0.79 *g/l*.

5.2.2 Cyclodextrin encapsulation

Two different derivatives of β-cyclodextrins were used: (2-Hydroxy)propyl-β-cyclodextrin (DS~4.5) and Methylated β-cyclodextrin (DS~12), which were supplied by CycloLab Ltd. (Budapest, Hungary).

The use of cyclodextrin derivatives improves their encapsulation ability of the β-cyclodextrin depending on the purpose of it. The two derivatives used are hydrophilic derivatives which means as they are more soluble in water, they are more suitable to molecules with low solubility in water (Pinho et al., 2014).

Encapsulation of cider lees extract by β-cyclodextrin was done using four different ratios of cider lees extract dry weight and b-cyclodextrin weight: 1:2.5, 1:5, 1:10 and 1:20. A volume 10 ml of cider lees extract was used for all encapsulations. As the dry weight of the cider lees extract was known (Table 5-1), the total cider lees extract solids in 10 ml can be determined and the amount of β-cyclodextrin estimated. Distilled water was then added up to the total volume of 50ml. A fixed total volume is necessary to ensure that all β-cyclodextrin encapsulation have the same concentration of the cider lees extract. The mixture was then stirred for 3 hours using a magnetic stirrer (Clifton® Hotplate Stirrer MSH-3) at approximately 1000rpm. When finished, inclusion complex samples were stored at 4ºC.

5.2.3 Heat stress treatment

The method was adapted from (Cheng et al., 2006). The heat stress treatment consisted of exposing the cider lees extract and the different inclusion complexes to a constant temperature of 60ºC for 28 days. A drying cabinet was used for this purpose, where temperature had a

variation in temperature of ±10ºC. Two different cider lees extract were used to have an understanding of the efficiency of the method applied. Cider lees extract obtained at 150ºC after 30 minutes and cider lees extract obtained at 220ºC after 10 minutes were used. Both extracts were encapsulated with the two types of β-cyclodextrin, DS~4.5 and DS~12, with four different ratios: 1:2.5, 1:5, 1:10 and 1:20.

5.2.4 Formulation

To assess the extract and respective encapsulation in a final product, a skin care base formulation was used. According to Boots (Industrial Partner) the extract should represent 5% (w/w) of the final product. The sample added to the final formulation will have 3% (w/w) of the cider lees extract solids independent from the amount of β-cyclodextrin used. In the final formulation the solid extract was 0.14% (w/w).

The cider lees extract used was obtained with subcritical water at 200ºC after 10 minutes. Formulations were prepared in triplicate and each formulation was kept at three different temperature conditions: 4ºC, room temperature and 37ºC. Encapsulation in the formulation was assessed by the change of colour based in the CIELab colour system. To measure the formulation colour a Colour i7 Benchtop Spectrophotometer was used with a reflectance aperture of 25mm. A Leneta 5DX brushout card with a formulation layer of 200μm was used for every colour measurement. All samples were measure in duplicates.

5.2.5 Colour-difference analysis CIELAB

The colour difference of the skin care base formulation was quantified the colour system CIELAB and the formula to obtain DE2000, which is a single number that measures the difference between two colours. DE200 formula was developed by the International Commission on Illumination (CIE) (Luo et al., 2001):

$$DE2000 = \sqrt{\left(\frac{\Delta L}{k_L S_L}\right)^2 + \left(\frac{\Delta C}{k_C S_C}\right)^2 \left(\frac{\Delta H}{k_H S_H}\right)^2 + R_T \left(\frac{\Delta C}{k_C S_C}\right)\left(\frac{\Delta H}{k_H S_H}\right)} \quad (5\text{-}1)$$

CIELAB colour space consists in the measurement of three coordinates: lightness of the colour (L), the position of the colour between green and red (a) and the position of the colour between blue and yellow (b). The colour difference is determined by measuring the difference of these three parameters against a standard colour, or as in this work, against the initial colour of the skin care formulations. To determine DE2000 two colour properties are necessary to be determined, these two properties are chroma (C) and hue (h) which are a function of a and b. The full list of equation needed to determine DE2000 using the formula in (2-3) are described in Luo et al. (2001).

5.3 Results and discussion

Previously it was verified that the compounds responsible for antioxidant capacity of cider lees extract when SubCW was performed at temperatures higher than 170ºC were different from the compounds present in cider lees supernatant. This meant that the inclusion complex of the compounds obtained above and below 170ºC and higher could produce different results as the molecules to be encapsulated were expected to be different.

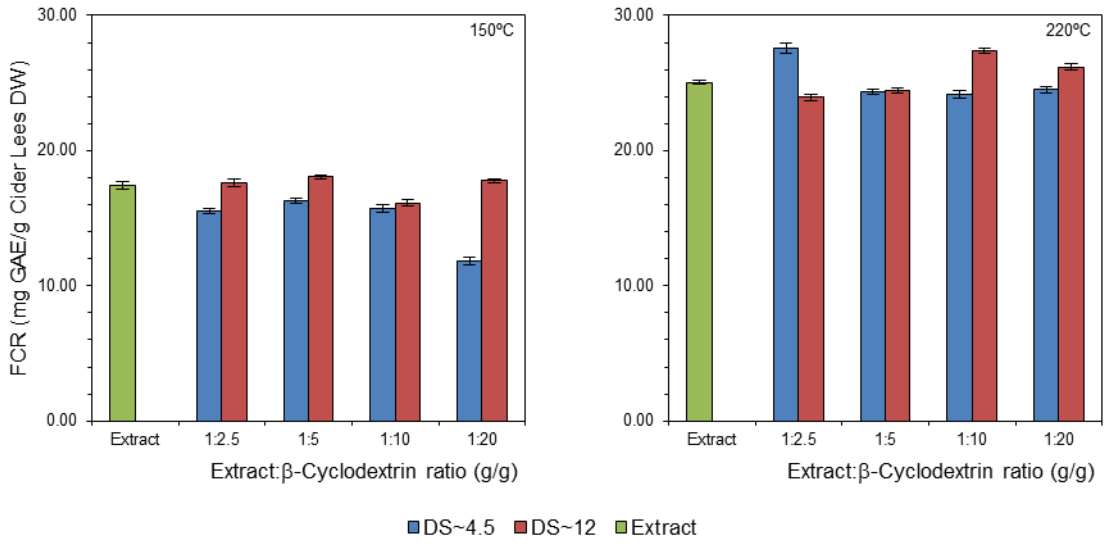


Figure 5-1 – Cider lees extract antioxidant activity after encapsulation.

Zhao et al. (2010) had verified that properties such as antioxidant capacity or antimicrobial activity were kept constant after the formation of an inclusion complex, which meant that although the inclusion complex prevented the active molecule from potential decomposition under adverse environmental condition it did not had an influence in its main properties. Figure 5-1 illustrates that the antioxidant capacity of inclusion complexes with cider lees extract from 150ºC and 220ºC did not differ significantly from the antioxidant capacity of the respective cider lees extract, with the exception for DS~4.5 when used in a ratio of 1:20. The decrease in antioxidant capacity when complexed 1:20 could be related to the high amount of β-cyclodextrin compared to cider lees extract.

5.3.1 Verification of the formation of an inclusion complex

As discussed in the sub-chapter 1.8, the formation of an inclusion complex can be verified by a functional assay, where the stability of the possible complex formed is put to test. As polyphenols decompose at high temperatures and have a gradual decomposition at room temperature, a heat-stress method was chosen to assess the formation of the inclusion complex.

Also, the cider lees extract from 220ºC was tested in a skin care formulation over three different storage temperatures to assess not only the formation of an inclusion complex but also the oxidative stability of the cider lees extract.

5.3.1.1 Heat stress stability

Figure 5-2 shows the antioxidant capacity (FCR method) lost after 28 days at 60ºC. It was observed that the cider lees extract from 220ºC had a lower decrease in antioxidant capacity than the cider lees extract from 150ºC, result that suggested that the compounds which contribute to the antioxidant capacity of the extract were not the same class of compounds.

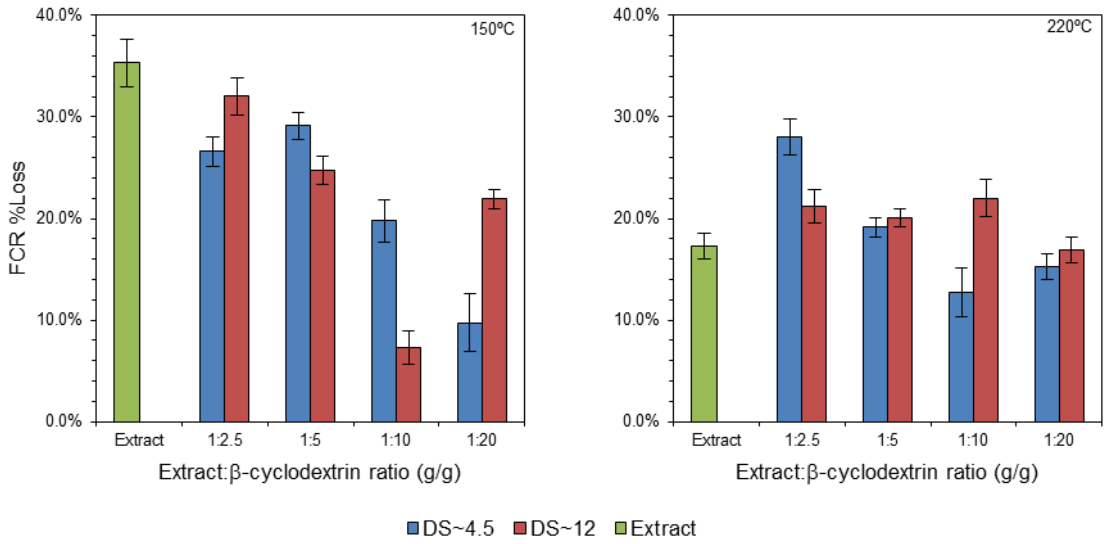


Figure 5-2 – Decrease in FCR value after subjecting to heat stress cider lees extract and encapsulated cider lees extracted for 28 days.

The results from Figure 5-2 show that cyclodextrin had a positive impact in the prevention of the antioxidant capacity loss of the cider lees extract from 150ºC, where cyclodextrin DS~12 with a ratio of 1:10 and the DS~4.5 with a ratio of 1:20 showed the best results. These results suggest the formation of an inclusion complex, although a complete prevention of degradation was not achieved. However the results for the cider lees extract from 220ºC complexation were not clear, and it suggested that a inclusion complex was not formed as the loss of antioxidant

activity did not change significantly when compared to the original extract. Also the use of DS~4.5 with a ratio of 1:2.5 seemed to have a negative effect, where the antioxidant capacity lost was higher than in the original extract. The lack of an inclusion complex formation could be due to the different nature of the compounds which contributed to the antioxidant capacity. Molecular size and its affinity to water are the characteristics of these compounds that can influence the not inclusion in the cyclodextrin cavity. At this stage these compounds were not fully characterised.

5.3.1.2 Skin care cream formulation

The colour change over time was chosen as the characteristic to evaluate not only the oxidative stability of the extract in formulation, but also the possibility of a prevention of oxidation by an inclusion complex with cyclodextrin.

According to Boots (Nottingham, UK) to test the colour stability of a skin care cream formulation, it should be stored over six months at three different storage temperatures. First conditionwas at 4ºC, second is at room temperature, where fluctuations on temperature occured and at 37ºC, which simulated three years of storage time where changes till a certain limit can be tolerated.

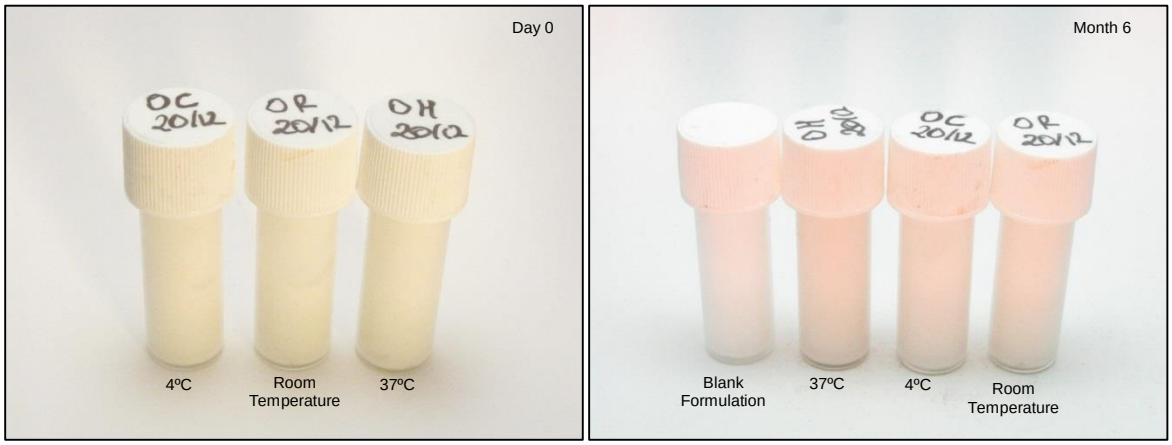


Figure 5-3 – Formulation on day 0 and after 6 months.

Figure 5-3 shows the appearance of the formulation of cider lees extract (200ºC) at day one and after 6 months. While in day one all formulation were visually equal, after 6 months it can be observed that the formulation kept at 37ºC were darker than the other samples, by naked eye.

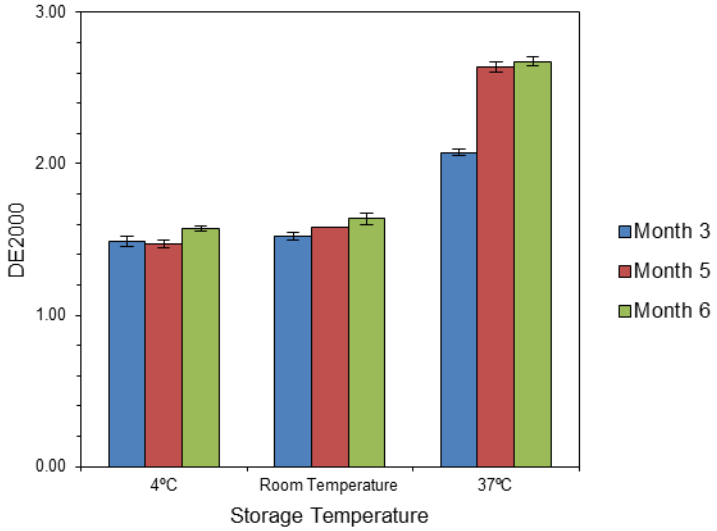


Figure 5-4 – Colour evolution of formulation with original cider lees extract through six months at three different storage temperatures.

In Figure 5-4 can be seen that at 4ºC only in the last month an increase in colour difference was observed, while at room temperature a small increase was verified over the last two months, but not in significant amounts. However, at 37ºC an increase in colour difference was observed, especially after 5 months. As it was expected it was at 37ºC that a major change in colour was observed, so this storage temperature was used to compare with the formulations with extract and cyclodextrin.

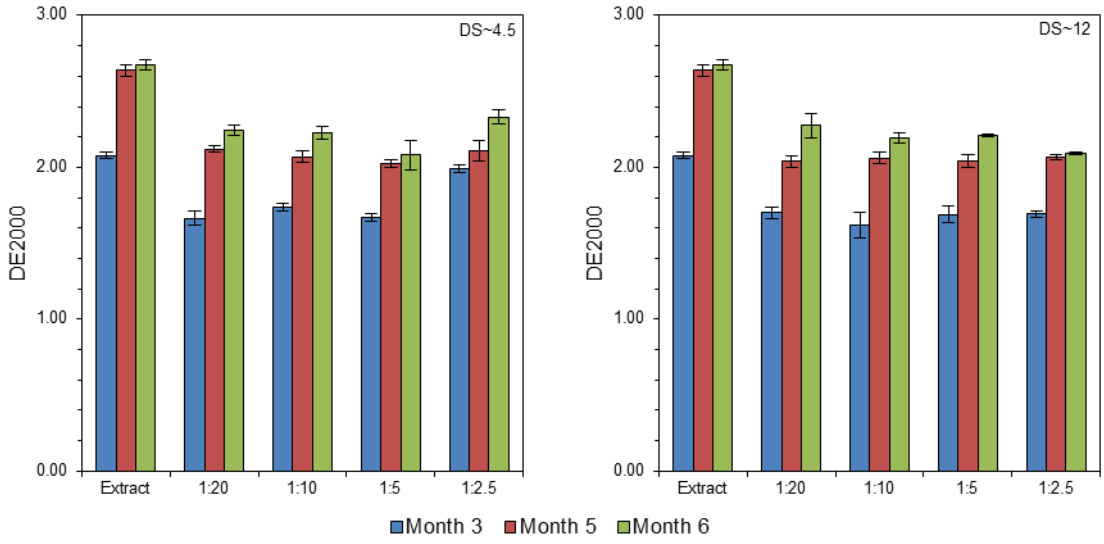


Figure 5-5 – DE comparison at 37ºC between extract and different encapsulations.

When comparing the colour difference of the formulation with the original cider lees extract and formulation with encapsulated extracts (Figure 5-5), it can be seen that at 37ºC all the encapsulated extracts had a smaller colour difference than the original cider lees extract. Encapsulated formulations with DS~4.5 with 1:5 and DS~12 with 1:2.5 showed better results as not only they had the lower colour difference but also they had almost no change in colour difference in the last month, which meant that colour change was stabilised from that point. Interestingly a lower colour difference was observed when a lower amount of cyclodextrin was used which was opposite to that observed for the 150ºC cider lees extract during the heat stress tests, where a higher amount of cyclodextrin had a greater impact in preventing the decrease of antioxidant capacity.

Although a decrease in the colour difference was observed when cyclodextrin was used, the difference between the formulation with original extract and with cyclodextrin it was not currently thought to justify the addition of cyclodextrin to improve oxidative stability.

5.4 Conclusion

Inclusion complexes have been formed with the use of cyclodextrins to protect bioactive agents that can be easily oxidised. One of the advantages of β-cyclodextrin was that it did not had a significant impact in the main characteristics of the compound to be protected, as it was verified for the antioxidant capacity of two different cider lees extract, which stayed constant after the addition of cyclodextrin.

The use of cyclodextrin at this stage was a preliminary study to evaluate how its effect on the cider lees extract obtained by SubCW from cider lees. Heat-stress results suggested that an inclusion complex and consequent protection of the active agents was obtained for the cider lees extract at 150ºC, although the level of protection produced was not 100%. Also, the results suggested that an inclusion complex was not formed when the cider lees extract from 220ºC was used, which leads to suggest that different active agents are present in the cider lees extract from 150ºC and from 220ºC.. At the same time, such results could also indicate that molecular size and water solubility are the responsible for the not formation of an inclusion complex. Heat stress results also suggested that the active agent in the cider lees extract from 220ºC exhibits a higher stability to decomposition than the cider lees extract from 150ºC, as it had a loss of 17.3%±1.3% of the initial antioxidant capacity against a loss of 35.3%±2.4% of the initial antioxidant capacity, respectively. Formulation also showed little protection to browning when using β-cyclodextrin.

The use of the cider lees extract in skin care formulations showed that there was benefit from the formation of an inclusion complex, especially with lower amounts of cyclodextrin, however the inclusion complex should be formed in low amounts, as the decrease in colour difference is not major when compared to the formulation with the original extract.

CHAPTER 6

CONCLUSION AND FUTURE WORK

6.1 Conclusions

Under standard subcritical water extraction conditions the temperature of 170ºC appeared to be a turning point. Below this temperature the cider lees extract composition profile was similar to the cider lees supernatant, which indicated that no significant changes occured. However above 170ºC it was observed the formation of 5-HMF, which indicated the dehydration of monosaccharides present in cider lees supernatant but also indicated the hydrolysis of monosaccharides from the yeast cell wall. It wsa also at this temperature that in previous work developed by Bahari (2010) the start of the disruption of cell wall. Also products from the thermal decomposition of polyphenols, more specifically hydroxycinnamic acids, were detected. From HPLC analysis it was detected an increase of compounds with low retention time, which could mean that either these compounds had higher polarity or that they had a high molecular weight which decreased their retention by the stationary phase of the column used. It is important to state that with the increase of temperature, water polarity decreased which indicate that compounds less polar should be released into solution rather than compounds with higher polarity, therefore these compounds detected at low retention times are likely to be compounds with high molecular weight.

Sequential subcritical water extraction was in accordance with the results obtained by standard extraction, where it was verified that the majority of the polyphenols are released into solution below 170ºC while the compounds released above this temperature appeared to be responsible for the higher antioxidant capacity observed. Which meant that the compounds responsible for the high antioxidant activity produced should come from yeast cell wall or be intracellular compounds that are likely to had undergone possible chemical reactions under SubCW. At this stage, SubCW can promote hydrolysis, but also acid/base catalysis.

With the encapsulation with cyclodextrin it was demonstrated that the compounds responsible for the antioxidant activity from cider lees extracts at 150ºC and 220ºC are from a different kind of compounds. Cider lees extracts at 150ºC appeared to form inclusion complexes to a certain extent while that did not occurred with cider lees extract from 220ºC. Also the fact that the cider lees extract suffered a higher relative decrease in antioxidant capacity than the cider lees extract at 220ºC showed that the compounds from 220ºC were different and had a higher antioxidant capacity than the compounds present in the cider lees extract at 150ºC. The fact that was not possible to verify the formation of inclusion complex with the cider lees extract at 220ºC lead to suggest that the molecules in question either have a molecular size that did not allowed to be encapsulated by cyclodextrin or the molecules in question were hydrophilic and therefore they did not had the tendency to be encapsulated.

In summary there were two types of compounds with antioxidant capacity that can be recovered from cider lees. The first group was the polyphenols that are characteristic from cider and that could be recovered using SubCW below 170ºC to avoid its decomposition. The second was the group of molecules being released or synthesised from the yeast cells. Also, as the residual cider contains a significant amount of monosaccharides, it was important that its removal be done before any subcritical water extraction at 170ºC, as these compounds in SubCW can be easily

used in other applications. From literature there were suggestions that reactions as Maillard reaction are the answer for the antioxidant capacity observed (Atrooz, 2008, Plaza et al., 2010, Yanagimoto et al., 2002).

6.2 Recommendations for future work

Future work should be focused to understand the chemical reactions that occur with subcritical water above 170ºC from cider lees. It was seen that neither simple polyphenols nor their degradation products were key to the observed antioxidant capacity. So, all results indicated that the resultant antioxidant capacity were the result of cider yeast cells, wall and its constituents, or of complex polymers formed with polyphenols. Hydrolysis was likely to be the reaction that promoted the disruption of cider yeast cell wall and consequent release of cell wall compounds into solution. So it is important to widen the range of analysis performed. Proteins and monosaccharides analysis will give indication how these compounds might be contributing to the overall antioxidant capacity. Also there is the possibility of the formation of molecules with high molecular weight. LC-MS should be used to understand the range of molecular weight of the molecules which will allow to narrow the list of possible compounds. At the same time, analysis of proanthocyanidins should be developed, as these polyphenols are present in cider samples at high percentages and to understand what contribution do they had to the overall antioxidant capacity.

Also models of SubCW extraction for yeast cells, washed yeast cells and a mixture of the main polyphenols should be developed to improve the understanding of how these compounds that are present in the cider lees sample evolve under different subcritical water conditions. These models should not only be analysed in terms of antioxidant capacity, as it had been done in previous works, but also in the characterisation of their constitution using HPLC and LC-MS.

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APPENDIX A Dry weight content of cider lees extracts

The following tables contain the errors associated with the dry weight measurements of the cider lees extracts using standard subcritical water extraction, which are represented in Figure 3-2, where to simplify the graph, error bars were not added.

Table A-1 - Yeast extract supernatant dry weight from subcritical water extraction.*g Extract/l*	**0 min**	**5 min**	**10 min**	**20 min**	**30 min**	**60 min**
100ºC	42.7±0.8	44.3±0.1	43.8±0.0	45.9±1.3	45.0±0.4	47.3±0.6
120ºC	44.4±0.7	45.7±0.7	46.3±1.1	48.7±0.2	49.5±0.6	51.7±0.8
150ºC	49.2±1.6	53.5±0.4	60.5±7.9	65.2±11.0	67.7±12.3	60.4±0.7
170ºC	54.8±1.7	59.4±1.3	61.0±1.8	60.3±2.0	52.7±3.0	50.5±4.1
200ºC	52.8±2.5	54.8±2.2	45.6±4.0	35.5±4.2	37.5±2.9	35.1±1.5
220ºC	49.7±2.7	39.7±1.1	35.8±1.1	34.1±1.0	31.2±4.3	30.5±4.0
250ºC	35.5±0.8	33.1±0.4	32.5±0.6	31.2±0.4	31.7±1.6	30.7±2.4

Table A-2 - Yeast extract dry weight as a function of the initial cider lees supernatant dry weight (Figure 3-2).

g Extract/g CL DW (%)	**0 min**	**5 min**	**10 min**	**20 min**	**30 min**	**60 min**
100ºC	46.3±1.8	44.4±0.7	47.5±2.4	49.7±1.0	51.2±0.5	47.7±0.7
120ºC	44.5±2.0	47.9±0.8	48.5±0.4	51.0±1.4	47.4±4.0	49.4±2.8
150ºC	47.4±2.7	51.0±2.8	57.6±1.9	61.3±0.3	63.5±1.0	57.5±2.1
170ºC	56.5±1.4	57.1±2.6	58.7±2.2	58.0±1.9	56.7±1.1	54.1±0.4
200ºC	53.7±0.8	55.4±2.2	48.6±1.3	42.6±0.0	39.0±0.3	32.7±1.2
220ºC	49.5±1.4	40.4±3.0	36.4±0.6	34.7±0.6	30.4±0.1	29.7±0.3
250ºC	34.9±1.0	32.7±0.7	32.1±0.4	30.8±1.4	31.1±0.6	30.1±1.4

APPENDIX B Total Phenolics content of cider lees extract

The following table contain the errors associated with the total phenolics content measurements of the cider lees extracts using standard subcritical water extraction, which are represented in Figure 3-3, where to simplify the graph, error bars were not added.

Table B-1 – The total phenolic content of subcritical water extract of cider lees according temperature and residence time.

Total Phenolics (mg GAE/g Cider Lees DW)	**Extraction Time**			
	0 min	**10 min**	**20 min**	**30 min**
100ºC	8.6±0.1	7.9±0.1	7.0±0.1	8.7±0.1
150ºC	10.6±0.4	14.5±0.5	16.2±0.4	16.4±0.3
200ºC	28.8±0.4	32.8±0.5	33.9±0.7	35.8±1.1
220ºC	33.8±0.4	**38.1±0.5**	31.9±0.3	30.1±0.5

APPENDIX C HPLC-DAD UV Spectra Data

Appendix C.1 UV-spectra of selected standard compounds

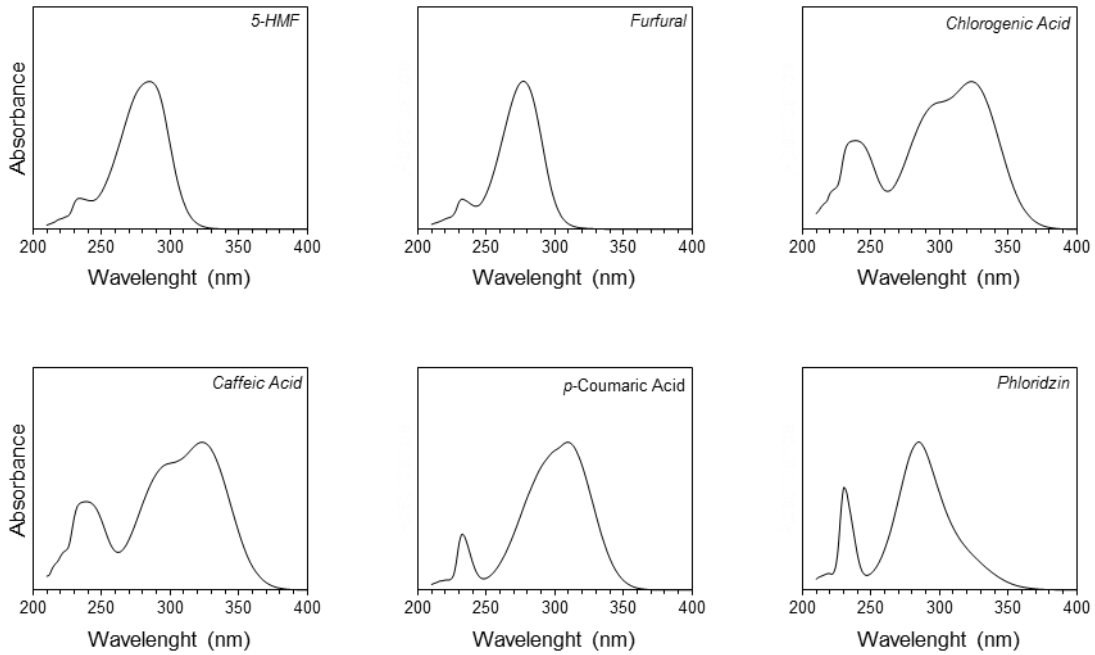

Figure C-1 – UV spectra standards

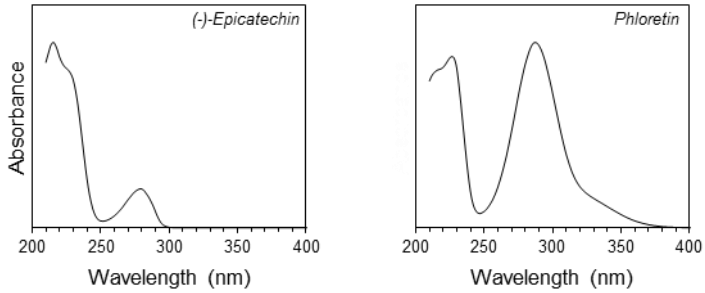

Figure C-2 – UV spectra of Epicatechin[a] and Phloretin[a].

[a]these standards were obtained using a synergi C18 column with water and methanol as mobile phases. As it can be observed in the figure above, absorbance at 200nm is intense, especially in epicatechin spectra. Methanol has maximum absorbance at 205nm while acetonitrile absorbance at 200nm is only 2% of its maximum absorbance so it is expected that UV spectra with methanol in the mobile phase have higher absorbances at lower wavelengths (at 235nm, methanol absorbance is 10% of its maximum).

APPENDIX D Chemical quantification of compounds detected from cider lees using standard subcritical water extraction

The following tables provide the errors associated with the area/concentration of all cmpounds detected in the cider lees extracts using standard subcritical water extraction. Their graphs are illustrated in section 3.3.4.2.2. where to simplify and improve their analysis, the respective error bars were not added.

Appendix D.1 Hydroxycinnamic acids

Chlorogenic acid, caffeic acid and *p*-coumaric acid were quantified using standards, while the other compounds were quantified as equivalents of similar compounds, except compound CL6 which was quantified in terms of area units per cider lees dry weight.

Table D-1 – Chlorogenic acid concentration (*mg/g Cider Lees DW*) in lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-30).

(mg/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	1.247±0.048	1.197±0.011	1.236±0.070	1.248±0.055	1.283±0.010	1.166±0.017
120ºC	1.176±0.048	1.226±0.036	1.210±0.034	1.222±0.034	1.105±0.071	1.062±0.072
150ºC	1.141±0.051	1.073±0.059	1.120±0.078	1.056±0.064	0.983±0.063	0.747±0.039
170ºC	1.132±0.053	0.980±0.057	0.871±0.019	0.729±0.008	0.671±0.048	0.470±0.034
200ºC	0.705±0.038	0.574±0.029	0.464±0.003	0.314±0.004	0.248±0.012	0.109±0.000
220ºC	0.444±0.024	0.248±0.052	0.184±0.015	0.076±0.000	0.045±0.002	nd
250ºC	0.234±0.000	nd	nd	nd	nd	nd

Table D-2 – Caffeic acid concentration (mg/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-30).

(mg/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.051±0.003	0.047±0.001	0.052±0.007	0.052±0.006	0.055±0.002	0.041±0.001
120ºC	0.046±0.003	0.045±0.003	0.043±0.003	0.039±0.004	0.033±0.003	0.023±0.002
150ºC	0.037±0.005	0.023±0.001	nd	nd	nd	nd
170ºC	0.018±0.000	nd	nd	nd	nd	nd
200ºC	nd	nd	nd	nd	nd	nd
220ºC	nd	nd	nd	nd	nd	nd
250ºC	nd	nd	nd	nd	nd	nd

Table D-3 – Compound CL6 concentration (mg CQAE/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-31).

(mg/g DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	nd	nd	nd	nd	nd	nd
120ºC	nd	nd	nd	nd	nd	nd
150ºC	nd	nd	0.135±0.013	0.168±0.019	0.199±0.015	0.236±0.013
170ºC	0.127±0.008	0.173±0.013	0.227±0.021	0.282±0.019	0.322±0.022	0.322±0.024
200ºC	0.270±0.022	0.304±0.007	0.294±0.010	0.241±0.027	0.232±0.013	0.090±0.005
220ºC	0.221±0.019	0.165±0.044	0.140±0.038	nd	nd	nd
250ºC	nd	nd	nd	nd	nd	nd

Table D-4 – Compound LE6 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-33).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	nd	nd	nd	nd	nd	nd
120ºC	nd	nd	nd	nd	0.8±0.0	1.1±0.0
150ºC	1.0±0.3	2.0±0.0	2.8±0.2	4.3±0.3	5.5±0.4	7.3±0.0
170ºC	3.2±0.2	6.3±0.0	8.8±0.0	11.3±0.0	14.3±0.2	18.5±1.7
200ºC	17.5±2.3	20.5±0.4	24.9±1.5	23.3±2.3	21.8±2.3	13.0±0.0
220ºC	24.3±3.0	24.8±1.6	27.1±3.3	23.9±1.5	18.4±3.3	13.3±5.7
250ºC	25.5±1.3	28.1±4.2	25.3±2.2	17.1±5.9	8.0±0.1	nd

Table D-5 – 4-O-p-Coumaroylquinic acid concentration (mg p-CoAE/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-35).

(mg/g Cider LeesDW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.169±0.008	0.161±0.003	0.169±0.009	0.169±0.007	0.162±0.009	0.143±0.003
120ºC	0.151±0.009	0.155±0.014	0.148±0.013	0.144±0.013	0.117±0.008	0.097±0.007
150ºC	0.138±0.007	0.096±0.005	0.105±0.007	0.085±0.000	0.077±0.002	0.047±0.014
170ºC	0.091±0.004	0.068±0.007	0.065±0.008	0.061±0.009	0.055±0.004	0.046±0.003
200ºC	0.054±0.003	0.051±0.000	0.038±0.001	0.028±0.001	0.025±0.000	nd
220ºC	0.037±0.003	0.030±0.000	nd	nd	nd	nd
250ºC	nd	nd	nd	nd	nd	nd

Table D-6 – *p*-Coumaric acid concentration (mg/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-35).

(mg/g DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.020±0.002	0.018±0.001	0.020±0.003	0.020±0.004	0.023±0.000	0.017±0.001
120ºC	0.018±0.001	0.019±0.002	0.019±0.002	0.020±0.001	0.016±0.002	0.015±0.001
150ºC	0.017±0.002	0.016±0.001	0.016±0.002	0.015±0.005	0.010±0.002	0.011±0.001
170ºC	0.017±0.001	0.013±0.001	0.013±0.002	0.008±0.000	0.009±0.002	nd
200ºC	nd	nd	nd	nd	nd	nd
220ºC	nd	nd	nd	nd	nd	nd
250ºC	nd	nd	nd	nd	nd	nd

Table D-7 – Compound CL5 concentration (mg p-CoAE/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-36).

(mg/g DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.019±0.001	0.020±0.002	0.021±0.000	0.022±0.002	0.022±0.001	0.032±0.002
120ºC	0.022±0.002	0.030±0.004	0.035±0.004	0.044±0.004	0.045±0.001	0.060±0.003
150ºC	0.040±0.002	0.060±0.003	0.080±0.003	0.090±0.004	0.088±0.002	0.062±0.002
170ºC	0.073±0.004	0.071±0.005	0.066±0.002	0.060±0.001	0.056±0.000	0.051±0.002
200ºC	0.057±0.002	0.053±0.001	0.049±0.003	0.045±0.004	0.042±0.003	0.022±0.000
220ºC	0.038±0.003	0.033±0.001	0.026±0.010	0.019±0.007	0.017±0.000	nd
250ºC	0.018±0.001	0.017±0.000	nd	nd	nd	nd

Table D-8 – Compound CL8 concentration (mg p-CoAE/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-36).

(mg/g DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.013±0.001	0.014±0.001	0.012±0.002	0.012±0.002	0.011±0.001	0.015±0.002
120ºC	0.013±0.001	0.015±0.000	0.016±0.000	0.016±0.000	0.016±0.000	0.018±0.001
150ºC	0.015±0.001	0.019±0.000	0.024±0.000	0.028±0.001	0.032±0.001	0.030±0.006
170ºC	0.022±0.001	0.029±0.001	0.034±0.002	0.040±0.001	0.043±0.006	0.043±0.005
200ºC	0.036±0.002	0.046±0.002	0.038±0.003	0.031±0.001	0.029±0.003	0.011±0.004
220ºC	0.036±0.003	0.026±0.006	0.022±0.004	nd	nd	nd
250ºC	0.015±0.001	nd	nd	nd	nd	nd

Appendix D.2 Decarboxylation products

Table D-9 – Compound LE7 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-34).

(Area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	nd	nd	nd	nd	nd	nd
120ºC	nd	nd	nd	nd	0.4±0.4	0.6±0.6
150ºC	nd	nd	nd	0.4±0.0	0.6±0.0	2.3±0.0
170ºC	0.3±0.3	1.3±0.0	2.2±0.0	4.4±0.0	8.2±0.2	19.0±1.0
200ºC	9.1±1.9	17.4±0.6	21.0±0.3	19.3±0.6	17.0±3.3	13.3±0.0
220ºC	21.3±2.8	22.9±0.7	25.4±2.8	22.7±2.2	17.0±2.8	12.7±3.2
250ºC	21.6±0.2	19.3±0.9	16.9±1.3	12.4±1.0	8.8±0.5	5.6±0.9

Table D-10 – Compound LE9 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-34).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	nd	nd	nd	nd	nd	nd
120ºC	nd	nd	nd	nd	nd	nd
150ºC	0.5±0.5	0.8±0.8	nd	nd	0.0±0.0	11.4±0.0
170ºC	4.4±2.7	4.8±0.0	12.4±0.3	23.4±1.7	40.8±1.4	89.1±14.0
200ºC	43.3±10.9	84.9±0.4	120.8±12.6	93.5±15.6	96.8±23.0	55.6±4.8
220ºC	89.3±13.2	97.7±12.5	95.3±5.5	74.6±3.2	62.9±17.1	25.6±12.6
250ºC	74.9±15.8	54.1±10.7	29.7±7.2	10.2±3.6	3.7±0.8	1.8±1.8

Appendix D.3 Dihydrochalcones

Table D-11 – Phloridzin concentration (mg/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-37).

(mg/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.215±0.006	0.264±0.015	0.274±0.026	0.317±0.022	0.368±0.000	0.414±0.013
120ºC	0.307±0.014	0.445±0.005	0.497±0.005	0.568±0.008	0.550±0.032	0.583±0.036
150ºC	0.540±0.051	0.587±0.032	0.576±0.075	0.534±0.078	0.481±0.059	0.349±0.024
170ºC	0.572±0.024	0.501±0.028	0.388±0.005	0.245±0.005	0.149±0.013	0.057±0.000
200ºC	0.188±0.015	0.038±0.000	nd	nd	nd	nd
220ºC	0.052±0.000	nd	nd	nd	nd	nd
250ºC	nd	nd	nd	nd	nd	nd

Table D-12 – Compound CL9 concentration (mg PHLE/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-37).

(mg/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.047±0.000	0.054±0.001	0.054±0.000	0.063±0.000	0.080±0.003	0.081±0.001
120ºC	0.064±0.003	0.085±0.000	0.093±0.001	0.106±0.001	0.105±0.009	0.112±0.008
150ºC	0.090±0.010	0.104±0.000	0.092±0.018	0.084±0.016	0.075±0.015	0.072±0.012
170ºC	0.104±0.005	0.097±0.012	0.077±0.011	0.054±0.013	0.030±0.002	nd
200ºC	0.036±0.000	0.000±0.014	nd	nd	nd	nd
220ºC	nd	nd	nd	nd	nd	nd
250ºC	nd	nd	nd	nd	nd	nd

Appendix D.4 Flavanols

Table D-13 – Compound CL4 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-38).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.6±0.0	0.9±0.0	0.7±0.0	1.2±0.0	0.7±0.0	1.3±0.4
120ºC	1.1±0.2	1.8±0.0	2.1±0.0	2.1±0.0	2.3±0.3	3.2±0.2
150ºC	2.7±0.4	5.9±1.0	5.3±0.6	6.0±0.6	4.9±0.8	3.2±0.8
170ºC	6.8±0.3	7.7±0.1	5.4±0.6	2.9±0.0	nd	nd
200ºC	1.9±0.9	nd	nd	nd	nd	nd
220ºC	nd	nd	nd	nd	nd	nd
250ºC	nd	nd	nd	nd	nd	nd

Table D-14 – Compound LE8 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-39).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	1.7±0.1	2.1±0.2	2.5±0.4	3.3±0.1	3.7±0.0	4.1±0.4
120ºC	2.6±0.2	3.9±0.5	4.2±0.5	4.3±0.2	4.4±0.0	4.2±0.2
150ºC	3.7±0.2	3.4±0.1	nd	nd	nd	nd
170ºC	nd	nd	nd	nd	nd	nd
200ºC	nd	nd	nd	nd	nd	nd
220ºC	nd	nd	nd	nd	nd	nd
250ºC	nd	nd	nd	nd	nd	nd

Table D-15 – Compound CL7 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-38).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	1.7±0.3	2.6±0.6	2.8±0.4	3.8±0.4	3.9±0.1	5.5±0.7
120ºC	3.4±0.4	5.0±1.0	5.7±1.0	6.4±0.9	7.8±0.0	8.5±0.1
150ºC	6.2±0.4	8.0±0.4	5.5±0.0	4.4±0.5	2.8±1.2	1.6±0.0
170ºC	6.8±0.4	4.0±0.0	2.1±0.0	0.8±0.0	nd	nd
200ºC	nd	nd	nd	nd	nd	nd
220ºC	nd	nd	nd	nd	nd	nd
250ºC	nd	nd	nd	nd	nd	nd

Appendix D.5 Monosaccharides dehydration products

Table D-16 – 5-HMF concentration (mg/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-40).

(mg/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.020±0.006	0.054±0.015	0.117±0.062	0.107±0.041	0.146±0.000	0.101±0.013
120ºC	0.058±0.012	0.079±0.005	0.110±0.005	0.150±0.003	0.185±0.019	0.242±0.029
150ºC	0.361±0.104	0.381±0.070	0.521±0.050	0.698±0.078	0.946±0.088	1.589±0.058
170ºC	0.566±0.097	0.910±0.005	1.522±0.050	2.705±0.007	3.897±0.166	7.639±0.123
200ºC	2.749±0.295	7.768±0.218	10.902±1.207	12.688±1.392	14.254±0.608	10.390±1.418
220ºC	8.963±0.725	11.686±0.160	11.968±0.690	10.402±0.294	8.006±1.397	2.028±1.175
250ºC	10.677±0.272	5.972±1.043	1.933±0.372	0.428±0.025	0.623±0.106	0.163±0.163

Table D-17 – Furfural concentration (mg/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-40).

(mg/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.094±0.038	0.112±0.005	0.110±0.019	0.116±0.015	0.148±0.000	0.078±0.032
120ºC	0.054±0.033	0.045±0.000	0.064±0.004	0.075±0.013	0.133±0.033	0.208±0.032
150ºC	0.217±0.059	0.354±0.035	0.530±0.109	0.958±0.227	1.170±0.415	4.118±0.147
170ºC	0.543±0.049	1.695±0.085	3.568±0.398	6.541±0.513	10.101±0.498	12.886±1.932
200ºC	5.806±0.646	10.231±0.142	15.440±1.309	11.350±1.847	10.600±2.178	3.841±0.760
220ºC	9.502±0.649	9.649±0.953	8.916±0.887	5.567±0.518	3.835±1.966	0.730±0.542
250ºC	7.934±0.174	2.354±0.756	0.747±0.159	0.122±0.039	0.162±0.046	0.101±0.045

Appendix D.6 Compounds CL1 to CL3 and LE1to LE5.

Table D-18 – Compound LE1and CL1 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (**Error! Reference source not found.**).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	2.5±0.4	2.2±0.2	2.0±0.2	1.4±0.1	1.4±0.2	2.1±0.4
120ºC	3.8±0.8	3.4±0.8	2.4±0.6	2.2±0.5	1.9±0.4	2.9±0.4
150ºC	5.8±0.9	7.4±0.5	11.7±0.7	15.6±1.0	19.4±1.3	25.0±1.6
170ºC	14.7±0.8	23.4±0.7	36.2±2.5	50.3±2.8	87.1±6.1	129.1±8.0
200ºC	90.6±8.9	154.3±0.0	176.7±11.1	203.2±26.3	223.8±15.9	219.3±40.8
220ºC	161.6±16.6	192.8±14.2	213.7±1.2	230.1±0.7	225.1±20.8	236.3±28.0
250ºC	211.6±7.5	219.3±21.8	222.3±21.9	239.1±0.0	237.4±2.0	256.3±10.5

Table D-19 – Compound LE2 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-43).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.6±0.1	0.5±0.1	0.6±0.0	0.7±0.0	0.6±0.0	0.9±0.1
120ºC	0.9±0.1	1.1±0.2	1.3±0.2	1.9±0.2	2.3±0.1	2.9±0.1
150ºC	2.5±0.2	4.2±0.5	7.6±0.8	14.4±1.1	21.4±1.1	28.1±0.0
170ºC	9.0±1.0	22.6±0.8	31.1±0.4	38.0±0.6	34.8±4.3	31.1±2.3
200ºC	41.2±3.3	38.7±0.9	50.5±0.1	34.4±3.6	41.5±0.4	35.0±3.7
220ºC	42.0±0.6	37.8±1.3	41.2±1.7	42.1±1.6	39.8±1.2	38.7±0.4
250ºC	44.8±0.2	48.7±0.0	46.7±0.0	42.4±0.0	38.8±0.4	33.6±0.0

Table D-20 – Compound CL2 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-43).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.7±0.2	nd	0.9±0.0	0.9±0.0	0.9±0.0	0.2±0.0
120ºC	0.4±0.1	0.5±0.3	0.5±0.3	0.5±0.3	0.2±0.0	0.4±0.2
150ºC	0.7±0.2	0.2±0.0	3.5±0.2	5.2±0.3	7.2±0.6	5.4±0.0
170ºC	1.8±0.9	3.0±0.0	3.6±1.5	3.0±0.1	6.3±2.3	14.9±0.6
200ºC	8.9±2.6	19.0±0.9	19.4±1.0	19.8±1.2	21.2±0.7	24.9±0.2
220ºC	16.7±3.2	22.4±0.0	24.6±1.8	28.6±3.0	26.7±5.0	28.1±3.1
250ºC	31.9±0.0	34.7±3.4	34.7±4.3	34.9±4.9	29.5±0.3	30.9±0.8

Table D-21 – Compound CL3 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-44).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	1.0±0.1	0.9±0.0	0.9±0.1	1.0±0.1	1.0±0.0	0.9±0.0
120ºC	0.8±0.1	1.0±0.0	1.0±0.0	1.0±0.1	0.7±0.2	1.1±0.6
150ºC	1.3±0.2	1.6±0.8	2.6±0.9	6.2±0.6	9.7±1.6	17.4±0.4
170ºC	2.3±0.7	6.2±3.0	16.4±0.5	23.2±0.2	26.8±1.0	35.3±5.2
200ºC	24.1±2.6	38.1±1.7	39.7±0.4	42.5±3.0	42.2±6.4	31.3±6.0
220ºC	34.0±2.8	39.0±8.8	36.3±4.9	22.5±5.8	12.2±1.6	16.9±0.9
250ºC	24.6±1.0	11.3±5.1	9.5±3.4	6.8±0.2	6.4±0.4	7.1±2.0

Table D-22 – Compound LE3 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-45).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	0.7±0.0	0.3±0.1	0.3±0.0	0.4±0.0	0.4±0.1	1.0±0.3
120ºC	0.9±0.2	1.5±0.7	2.3±1.1	3.4±1.5	6.2±0.2	11.9±0.8
150ºC	6.8±0.5	19.9±1.1	24.9±0.6	33.0±2.0	35.5±1.8	44.0±0.6
170ºC	24.1±2.6	43.1±2.9	42.3±6.3	36.8±7.2	27.5±2.2	26.4±2.8
200ºC	29.5±3.4	26.9±0.0	26.2±0.3	30.6±1.5	32.2±1.4	15.0±3.2
220ºC	22.2±1.1	21.5±1.5	20.0±4.2	14.5±5.8	4.6±0.3	4.6±1.0
250ºC	10.7±0.6	6.6±0.0	6.1±4.1	7.2±0.4	6.4±0.0	8.1±1.2

Table D-23 – Compound LE4 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-32).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	nd	nd	nd	nd	nd	nd
120ºC	0.8±0.0	1.1±0.0	1.3±0.0	0.5±0.0	0.7±0.7	0.9±0.9
150ºC	1.2±0.1	2.0±0.0	1.5±0.0	1.7±0.0	1.9±0.0	4.1±0.0
170ºC	2.2±0.1	3.6±0.2	4.3±0.0	6.2±0.0	7.4±0.5	16.8±1.8
200ºC	5.8±0.9	13.5±1.0	15.3±0.0	12.4±0.0	8.1±1.3	9.0±0.0
220ºC	12.4±2.1	15.6±3.0	18.8±0.2	11.7±0.0	10.9±0.9	7.0±0.6
250ºC	14.4±1.1	10.9±0.0	4.2±0.0	3.9±0.0	3.8±0.0	3.6±0.0

Table D-24 – Compound LE5 concentration (area/g Cider Lees DW) in cider lees extract from standard SubCW extraction at different temperatures with different extraction times (Figure 3-32).

(area/g Cider Lees DW)	0 min	5 min	10 min	20 min	30 min	60 min
100ºC	nd	nd	nd	nd	nd	nd
120ºC	nd	nd	nd	nd	nd	nd
150ºC	nd	nd	nd	nd	nd	nd
170ºC	nd	nd	nd	nd	1.7±0.0	5.3±0.1
200ºC	3.3±1.7	2.3±0.3	4.9±0.9	6.1±0.5	9.3±0.3	16.7±3.0
220ºC	5.9±1.4	11.2±3.0	16.1±1.5	20.7±4.3	15.5±0.1	11.9±1.7
250ºC	18.9±1.6	19.8±2.4	19.1±0.0	12.4±0.0	1.4±0.0	3.3±0.7