AN INVESTIGATION INTO THE ANTI-OXIDANT ACTIVITY OF A CIDER YEAST EXTRACT WITH THE AIM OF PROCESS OPTIMISATION.

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Abstract

Cider and/or beer lees has normally either been used as low cost animal feed or been disposed of at great cost. A higher value use for the yeast was therefore sought. This has been developed with the use of environmentally friendly subcritical water extraction. Results have shown that the extract contains anti-oxidant activity using two separate anti-oxidant assays, with a large improvement in activity above a process temperature of 200 °C. This is due in large part to an increase in the concentration of phenolic compounds in the extract. As a result of this, a refined extract was produced using supercritical CO_2 that improved anti-oxidant activity compared to the crude extract. The anti-oxidant activity of the 200 °C and refined extract has also been demonstrated using the comet assay in cells with the performance of the extracts being comparable to that of Trolox. The rheological stability of a number of cosmetic formulations incorporating the extract has also been tested with 2 of the 4 formulations being stable. However, colour change issues have been observed with all four formulations tested. Overall, a novel and biologically effective extract has been produced using data from anti-oxidant assays to improve activity of the extract.

Acknowledgements

I would like to thank my supervisors Dr Regina Santos and Professor Liam Grover, and my industrial supervisors Dr Gary Williams and Dr Judata Wibawa for the help and guidance during my project and during writing my thesis. I would also like to thank my previous industrial supervisor Mr Ed Galley without which I would not being doing this project. Also a big thank you to a family and friends for their endless support during my project.

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CHAPTER 1 : Introduction

1.1 Background to the project

The conversion of waste biomass into more valuable substances has been performed numerous times with a variety of feedstocks such as algae and apple pomace.

The two main waste products from cider production include waste apple pomace and cider lees which is a spent yeast slurry collected after fermentation and collection of alcohol (see figure 1.1-1) (Wijngaard et al., 2012). Apple pomace has traditionally been used as a source of pectin (Schieber et al., 2003) but has also been used as a source of anti-oxidants and other polysaccharides, enzymes and other substances (Vendruscolo et al., 2008). Cider lees however has not found as many disparate uses, the main use for cider lees has been in animal feed which is low in value and is being replaced by other more tailored solutions. Therefore, a more valuable use for the cider lees was sought. Subcritical water was selected as a method of converting the cider lees into a more high value substance, which could also be used as a source of anti-oxidants, polysaccharides and other chemicals.

Previous work has been carried out by Bahari (Bahari, 2010) to develop the production of the extract and to scope potential avenues that could utilise the extract or substances derived from it. In addition previous work by Jumbu, (Jumbu, 2007) appraised the use of the crude cider yeast extract in a cosmetic formulation together with rudimentary characterisation. Both of these studies suggested that the extract contained vitamins and phenolic compounds.

This work builds on and expands on this previous work, by adapting a number of established assays to help further develop and optimise the production of the extract; with the aim of improving the potential activity of the extract (with respect to any anti-oxidant activity

present). While always considering that the ultimate aim of using the extract as an active ingredient in a cosmetic formulation.

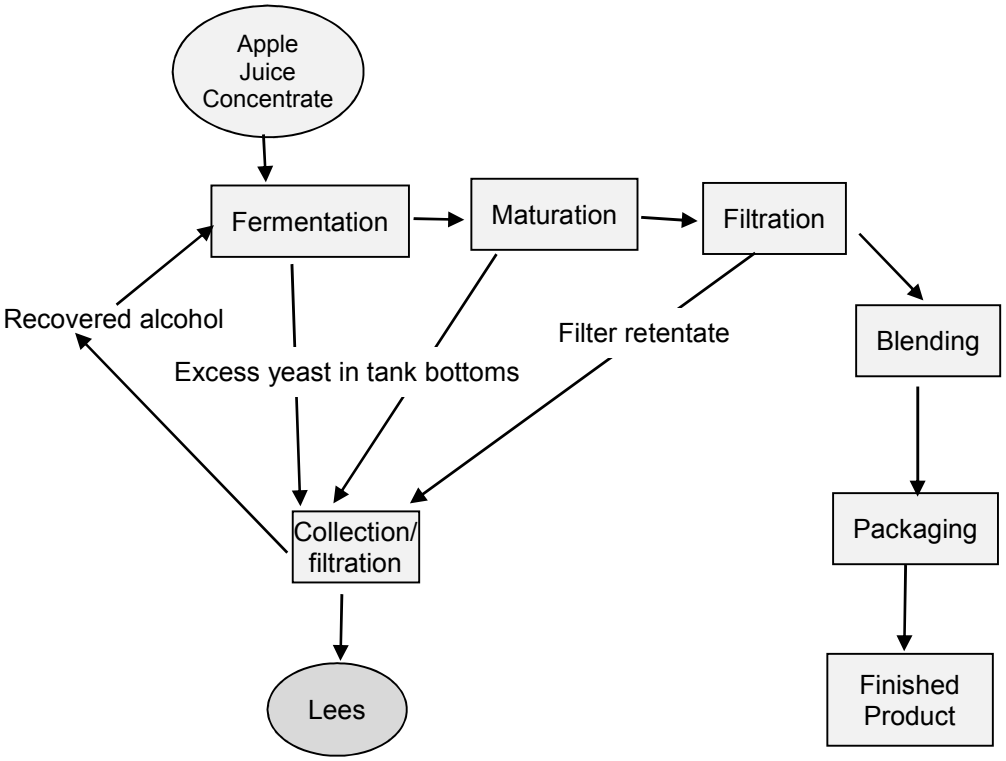

Figure 1.1-1: A diagram illustrating the cider production process (at Bulmers) and demonstrating where the cider lees is taken from during the production of cider .

1.1.1 *The aims of this work*

In summary, the overall aims of this work are as follows:

- To utilise established assays to evaluate any potential anti-oxidant activity of the extract and use these data to further optimise the anti-oxidant activity.
- Further characterise the extract with the aim of evaluating the role of phenolic compounds in the extract with respect to any anti-oxidant activity, and evaluating the composition of the extract and how this relates to any anti-oxidant activity found.
- To evaluate how the extract reacts in a range of prototype cosmetic products including detergents and emulsions.

1.2 Structure of the thesis

The thesis is split up into six separate chapters; chapter 1 will introduce the project and include a review of the literature surrounding the use of subcritical water and subcritical water extraction, together with a brief introduction into yeast extracts and their current uses. This chapter also includes a detailed review of all of the techniques used in the project, with a review of anti-oxidant assays, the comet assay and a background on emulsion structure and stability. This will provide a background to the technique used to generate the extract.

Chapter 2 is composed of all of the methods and materials used in the following results chapters.

Chapter 3 is the first results chapter and focuses on anti-oxidant assays and extract characterisation, with a literature review of all of the various anti-oxidant assays. This chapter also focuses on discussing the anti-oxidant activity of the extract and how this activity relates to the composition of the extract and to the work of other groups using similar extracts.

Chapter 4 aims to corroborate the results of chapter 3 using the comet assay to evaluate the anti-oxidant activity of the extract in cells.

Chapter 5 is a preliminary scoping study focuses on evaluating the extract in a range of cosmetic formulations focusing particularly on emulsion stability and colour change (of formulations with and without extract).

Chapter 6 summarises the work presented and aims to connect all of the work presented together and gives recommendations for future work.

Following this chapter is a list of references cited in the thesis.

1.3 An introduction to subcritical fluids and their uses

The aim of using subcritical fluids and in particular subcritical water is to replace the use of organic and inorganic solvents in extracting valuable compounds from a variety of feedstock. This is motivated by the extensive environmental, toxicological, and removal issues associated with organic or inorganic solvents (Carr et al., 2011).

Subcritical water is defined as water heated to between 100 °C at 0.1 MPa and 374 °C 22.1 MPa (Moller et al., 2011), whereas supercritical water is defined as water which near to or in excess of 374 °C 22.1 MPa (Pavlovic et al., 2013). The locations of both of these water phases is illustrated in figure 1.3-1. Subcritical water has been described as reagent, catalyst and solvent (Simsek Kus, 2012). This is due to the special properties of water between 100 °C at 0.1 MPa and 374 °C 22.1. These properties include a dielectric constant that decreases with increasing temperature from 78.5 (at 25 °C, 0.1 bar) to 32 (at 225 °C 100 bar) (Moller et al., 2011), in addition to a reduced viscosity and an ionic product that also increases with temperature. These properties are mainly a result of the strong hydrogen bonding in water. The strength of these hydrogen bonds reduces with increasing temperature and the water molecules, form water clusters (Simsek Kus, 2012).

The change in the dielectric constant of subcritical water is one the most important aspects of subcritical as is illustrated in figure 1.3-2. As the temperature of water increases water acts more like an organic (or hydrophobic) solvent due to the reduced dielectric constant.

Overall these properties lead to an increase in solubility of phenolic or sparingly soluble organic compounds; together with enhanced hydrolysis of feedstocks such as cellulose and nucleophilic substitutions and eliminations that otherwise would not have occurred in water at ambient temperatures and pressures (Moller et al., 2011).

Due to properties of subcritical water and the inexpensive nature of using water as an alternative solvent and or reactant has led to an explosion in the use of subcritical water by other groups. As of September 2012, there are over 1,500 articles citing the use of subcritical water (Scopus database search, September 2012) with a myriad of uses. Many of which involve extracting compounds of phenolic nature from feedstock such as apple pomace (Wijngaard and Brunton, 2009), algae (Jaime et al., 2010) and whole variety of fruit based feedstock. Subcritical water has also been used to extract environmental contaminants (Wijngaard, 2012) and many other uses. Therefore, subcritical water has become a very useful reactant in a number of scenarios.

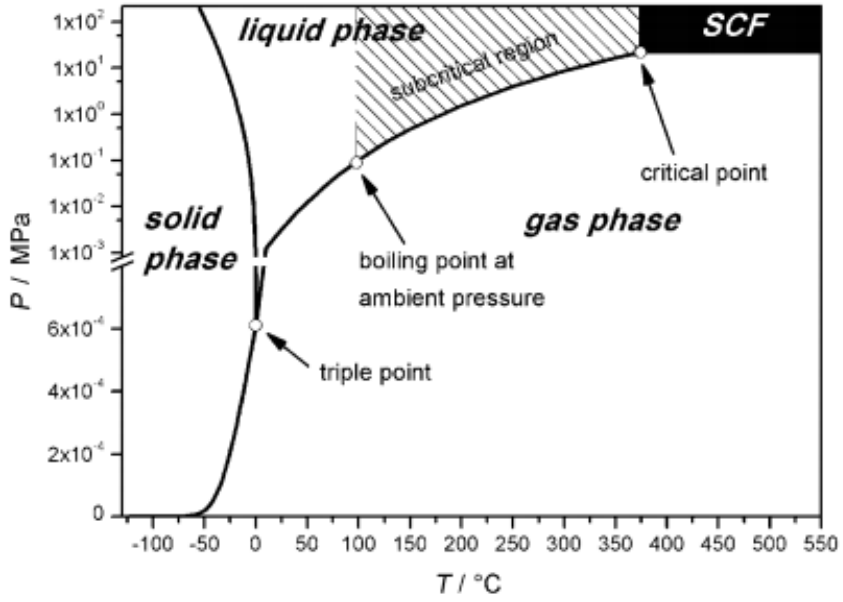

Figure 1.3-1: Water phase diagram (Mok and Antal Jr, 1992) , showing location of the sub and supercritical region with respect to other water phases. SCF refers to the supercritical fluid region.

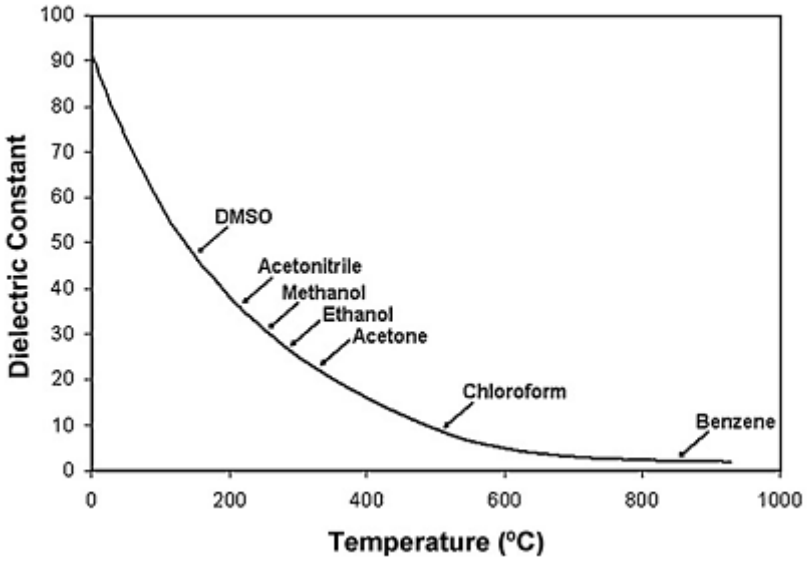

Figure 1.3-2: Comparing the dielectric constant of subcritical water at different temperatures with other organic compounds (Carr et al., 2011).

1.4 Yeast Extracts

Traditionally yeast extracts have been used in a wide variety of fields from animal feed, to food supplements, to cosmetic products (Ferreira et al., 2010). Most of these extracts have used yeast cells as a whole as a source of B vitamins, minerals and polysaccharides.

In the food industry yeast extracts have been used in a whole variety of products with the most popular being autolysed yeast extracts (that is yeast extracts, which have been formed by yeast self-digestion) that have been commonly used for many decades in products such as marmite or vegemite. Autolysis is regarded as a gentle technique as the cells' contents generally remain unmodified. Yeast extracts have also been used as flavour enhancers in a number of food stuffs (Abbas, 2006), such as a source of mono sodium glutamate which is very common food flavouring agent.

Yeast extracts have been used for a sustained period of time in microbiology in the growth of E-coli or variety of other microorganisms such as algae (Ferreira et al., 2010).

Yeast extracts have been used and are continuing to be used in a variety of cosmetic products with a wide range of purported benefits from enhancing skin moisture, skin smoothness and rebuilding skin barrier function (BASF, 2012).

The current and future uses of yeast extracts and their valorisation are illustrated in the figure 1.4-1.

The composition of most yeast extracts is complex, due to the variety of sources from which they are derived. The conditions from which in which the yeast have been cultured also has a large impact on any extract derived from the yeast. For example yeast that have been used in wine fermentation are very different in composition to brewer's yeast and have been known to adsorb polyphenols from wine (Gallardo-Chacón et al., 2010). Yeast extracts in general have

been found to be rich in B vitamins and polysaccharides (such as mannans and β-glucans) and their protein content has been a major reason why they have not been previously used in animal feed (Abbas, 2006). They have also been known to contain chromium.

Some yeast based extracts have been found to contain anti-oxidant activity, the source of which is either due to intracellular enzymes such as superoxide dismutase or catalase (Chassagne et al., 2005) or due to non enzymic products such as glutathione, hydroxyquinone or cell wall polysaccharides (Gallardo-Chacon et al., 2010). Santiago et al (Santiago and Mori, 1993) demonstrated anti-oxidant activity using four different fractions derived from baker's yeast. However only Gallardo and Chacon has shown (Gallardo-Chacon et al., 2010) anti-oxidant activity attributed to polyphenols from yeast used in wine fermentation, illustrating that yeast are not natively known to contain large amount of phenolic compounds.

Investigating the use of subcritical fluids with yeast has revealed a relative dearth of material, with only Lamoolphak et al., (Lamoolphak et al., 2006) reporting use of subcritical fluids with yeast to extract proteins from yeast. Therefore it is of great interest to investigate the effect of treating yeast with subcritical water and to investigate what effect process conditions have on the composition and on any potential anti-oxidant activity of the extract.

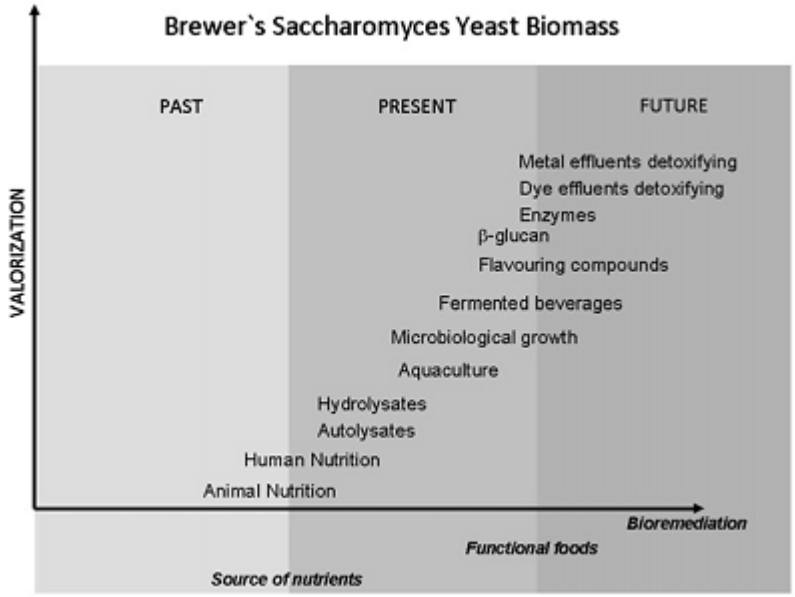

Figure 1.4-1: A diagram showing the past, current and potential future uses of yeast extracts.(Ferreira et al., 2010)

1.5 Anti-oxidants (general)

Anti-oxidants are consumed as a part of human diet and the definition of an anti-oxidant according to the Oxford English Dictionary, is: “An agent which inhibits oxidation” (Oxford English Dictionary, 2012). In other words, it is a substance that helps prevent oxidation and the oxidative reactions caused by free radicals.

This is important as free radicals have been implicated in a wide variety of conditions such as: (a wide variety of) cancers and cellular ageing (Guarente et al., 2008). The free radical theory of ageing, first referred to by Harman; implies that free radicals are the causative agent in ageing in general (Harman, 1956). Free radicals/ radical substances are produced as a natural part of cellular respiration (Johnson et al., 1999). Human cells incorporate anti-oxidant substances and enzymes such as catalase and super oxide dismutase (SOD) (Deisseroth and Dounce, 1970, Kent, 1977). These compounds limit the damage caused by inappropriate oxidation of cellular machinery. Free radicals are also produced in the immune system as an offensive mechanism to kill invading organisms (Knight, 2000).

Free radicals can also be produced by environmental exposure such as through smoking, or exposure to radiation such as UVA light (which is present in sunlight), and other forms of oxidative stress.

Free radicals that are produced as a part of cellular respiration together with environmental free radicals, can overwhelm cellular defences against free radicals (such as SOD and catalase) (Johnson et al., 1999). This can lead to cancer and premature ageing (Apffel, 1976) External anti-oxidants can play a role and help to prevent some of this damage from occurring. These external anti-oxidants can come from fruits and vegetables such as: apples,

strawberries, grapes/grape juice and other foods rich in anti-oxidants (Apffel, 1976). They can also be applied topically to the skin.

Skin in particular is vulnerable to environmental stress such as widespread exposure to UVA light. UVA causes singlet oxygen ($^1O_2$) radicals to be formed and cellular machinery/other cellular proteins or substrates to be oxidised. As UVA light can penetrate to the basal layers of skin; (Agar et al., 2004) it can cause an indirect increase in free radicals leading to a build up of secondary or tertiary oxidation products. These oxidative products often cause damage to other cellular components (such as DNA) and can be the cause of skin cancer (Agar et al., 2004). Mutations caused by UV light impair cellular metabolism leading to a reduction in cell turnover (Liu et al., 2004). Exposure to UV light in general leads to degradation in extra cellular matrix such as collagen leading to a reduction in skin firmness (Marrot and Meunier, 2008).

Anti-oxidants therefore play an important part in keeping cells and the human body as a whole operating normally despite external environmental and internal stresses.

Due to the benefits of anti-oxidants it was of great interest that previous work (Bahari, 2010) demonstrated that the yeast extract contained chlorogenic acid; a well characterised anti-oxidant (Fraga et al., 1987) (Ferrazzano et al 2009). Preliminary work (Jumbu, 2007) also suggested that the extract contained a wide range of phenolic compounds (such as quercetin) which are reported to be rich in anti-oxidants (Wright et al., 2001).

To evaluate and improve the anti-oxidant activity of the extract a number of anti-oxidant assays had to be researched and implemented. This data would be used to assess the effects of processing temperature, pressure and reaction time on the anti-oxidant activity of the extract.

1.5.1 *Anti-oxidant assays*

The aim of an anti-oxidant activity assay is to quantify the ability of particular substance or extract to quench free radicals or their by-products. This is a relatively complex task due to the numerous types of free radical and subsequent oxidation products. There are eight main types of free radical and oxidant sources, which are listed in table 1.5-1, and there are many more secondary oxidation products. Because of this, there is a myriad of different anti-oxidant assays with many variants, some of which are summarised in table 1.5-2. Despite this, most of the anti-oxidant assays fall into two different mechanistic groups, either hydrogen atom transfer (HAT) or (single) electron transfer ((S)ET). One of the main challenges in the area is that there is no standard way of quantifying anti-oxidant activity.

HAT based assays as defined by Prior et al (2005), measure the propensity of an anti-oxidant to quench free radicals by hydrogen donation (Prior et al., 2005). HAT based assays are widely thought to be more biologically relevant as the mechanism is more physiologically relevant (Prior et al., 2005). SET based assays quantify the ability of a potential anti-oxidant to transfer one (or more) electrons to reduce any compound, including metals, carbonyls and radicals (Wright et al., 2001).

Anti-oxidants can follow either one or both mechanism of action. However, their activity in both can vary significantly. (Huang et al., 2005). One class of anti-oxidants that demonstrate this is phenolics which are good at reducing peroxy radicals effectively but are less effective at quenching singlet oxygen (Prior et al., 2005). Bond disassociation and ionisation potential of anti-oxidants play an important part in determining which mechanism is used in each particular case and both of these factors are important in the efficacy of the anti-oxidant (Wright et al., 2001).

Table 1.5.1-1: A list of free radical and oxidant sources together with their chemical notations.(Prior et al., 2005)

Name	Chemical notation
Superoxide	O_2^-
Singlet oxygen	$^1O_2$
Hydroxyl radical	$HO^\bullet$
Nitric oxide	$NO^\bullet$
Peroxynitrile	$ONOO^-$
Hypochlorous acid	HOCl
Alkoxy(peroxy) radical	$RO(O)^\bullet$
Lipid alkoxy(peroxy) radical	$LO(O)^\bullet$

Table 1.5.1-2: A comparison of the major anti-oxidant methods, assessing them with regard to a number of factors. ++[a] (or +++ or +) refers to increased desirability, while --[b] refers to undesirability. ORAC refers to oxygen radical absorbance capacity, TRAP refers to total anti-oxidant trapping parameter, FRAP refers to ferric ion reducing anti-oxidant power, TEAC refers to Trolox equivalent anti-oxidant capacity, and DPPH refers to 2,2-diphenyl-1-picrylhydrazyl based assay. This table was adapted from table 1 in Prior et al. (Prior et al., 2005).

Anti-oxidant assay	Simplicity	Instrument required	Biological relevance	Mechanism	Endpoint	Quantification	Lipophilic and hydrophilic AOC
ORAC	++[a]	+	+++	HAT	Fixed time	AUC	+++
TRAP	---[b]	--specialised	+++	HAT	Lag phase	IC_{50} lag time	--
FRAP	+++	+++	--	SET	Time, varies	ΔOD over a fixed time	---
TEAC	+	+	-	SET	Time	ΔOD over a fixed time	+++
DPPH	+	+	-	SET	IC_{50}	ΔOD over a fixed time	-

To determine which of the several anti-oxidant assays would be used to asses the anti-oxidant activity of the extract, the sections below review each of the major anti-oxidant assays (detailed in table 1.5-2). With a focus on how they were developed, a brief mechanism of action, advantages and disadvantages of each assay (including the ability to screen multiple samples) and finally examples of their use in the literature.

1.5.1.1 ORAC Assay

The oxygen radical absorbance capacity (ORAC) assay is an example of a HAT based assay, where a hydrogen donation is used. The assay was initially developed by Glazer (1990), and further refined by Cao et al., who added the now common Trolox equivalence (Cao et al., 1993, Glazer, 1990), so that the anti-oxidant capacity could be standardised. In these initial ORAC assays β- phycoerythrin was used as a 'probe' and the fluorescence of β-phycoerythrin decreased with incubation time with a free radical generator such as 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH). Anti-oxidant, capacity is measured by using the area under the curve in a plot of fluorescence against time. The assay was then modified by Ou et al., (Ou et al., 2001) who changed the 'probe' to flourescien which addressed issues associated with β- phycoerythrin, such as instability in fluorescence over a period of time with no AAPH and batch to batch variability. The ORAC assay was subsequently automated by Huang et al., (Huang et al., 2002).

The value given by the ORAC assay is a simple way of easily comparing the anti-oxidant activity of a sample against a known standard, in this case; Trolox a vitamin E analogue.

The ORAC value is calculated in accordance with equations 1.5-1 and 1.5-2:

$$AUC = 0.5 + \frac{f_1}{f_0} + \ \ldots \frac{f_i}{f_0} + \ \ldots + \frac{f_{34}}{f_0} + \ 0.5(\frac{f_{35}}{f_0})$$

Equation 1.5.1.1-1

$$relative\ ORAC\ value = \left[\frac{(AUC_{sample} - AUC_{blank})}{(AUC_{Trolox} - AUC_{blank})}\right](molarity\ of\ \frac{Trolox}{molarity}\ of\ sample)$$

Equation 1.5.1.1-2

Equation 1.5-1 and 1.5-2: The ORAC assay equations, where AUC refers to area under the curve, f_1 to fluorescence at time point 1 and f_i to fluorescence at time point i. (Huang et al., 2002)

The ORAC value can then be used to rank samples or extracts in this case. The ORAC value is either expressed in a unitary unit or in µg of Trolox per gram of extract. There are variants of the ORAC assay to measure hydrophilic and hydrophobic anti-oxidants using different solvents.

The advantages of ORAC assay are, that it is easily automated and that many samples can be analysed simultaneously. As the ORAC assay has been found to operate through a traditional HAT mechanism, it is thought to be biologically relevant.(Huang et al., 2005) As the assay is conducted over a period of approximately 45 minutes the assay can take into account anti-oxidants with and without a lag phase and is pH insensitive. One of the disadvantages of the assay is that it is very temperature sensitive due to AAPH generating free radicals thermolytically. In addition, the assay preparation and running time is relatively slow. The ORAC assay has been used extensively in the literature (with over 400 articles using the assay) to screen the anti-oxidant capacity of substances including vegetables (Ninfali et al., 2007), plasma (Prior et al., 2005), algae (Jaime et al., 2010) and many other compounds.

1.5.1.2 TRAP Assay

The total anti-oxidant trapping parameter, commonly referred to as the TRAP assay is another example of a HAT based anti-oxidant assay. It was initially developed by Wayner et al., (Wayner et al., 1985) and subsequently modified by Ghiselli et al., who also documented a more detailed history of the assay in his review article. (Ghiselli et al., 2000).

The assay works by continually subjecting a probe substance (such as R- phycoerythrin) to peroxy free radicals from an initiator such as AAPH; which leads to a reduction of fluorescence or absorbance of the probe with time. The time that an anti-oxidant halts this reduction (referred to as lag time) is measured and is compared to that of Trolox. Using the equation below (equation 1.5-3), a TRAP value is calculated. Trolox is often added and tested after a 50% reduction of the initial fluorescence or absorbance. The assay as a whole has a similar mechanism of action to the ORAC assay.

$$C_{trolox}/T_{trolox} = X/T_{plasma}$$

Equation 1.5.1.2-3 The equation used to calculate the TRAP value, where: C_{trolox} is the concentration of Trolox, T_{trolox} is the lag time of Trolox, T_{plasma} is the lag time of the sample and X is the TRAP value in µmol/L. taken from Ghiselli et al. (Ghiselli et al., 2000)

The advantages of using the TRAP assay are that: it is very flexible as many groups have used a variety of probes, and most groups have used AAPH or ABAP as free radical generators. The other advantage is that it is well used in the literature, which implies that the assay can reliably quantify anti-oxidant capacity. The disadvantages of using the assay are that it requires more effort to automate because the fluorescence or absorbance needs to be monitored closely (to observe a drop in fluorescence). It is also difficult to compare results between studies as many studies have used different probes (Prior et al., 2005). There are conflicting reports into the accuracy of the assay as Howard et al mention that

the assay overestimates weak anti-oxidants (Howard and Ingold, 1964), and Prior et al have concluded that the assay underestimates anti-oxidant activity (Prior et al., 2005). The assay has been used to measure the anti-oxidant activity of a variety of substances particularly bodily fluids such as plasma (Ghiselli et al., 2000), or the consumption of red wine or tea *in vivo* (Serafini et al., 1994, Serafini et al., 1996).

1.5.1.3 Croton Bleaching

The Croton bleaching assay is a HAT based assay that was first developed by Bors et al., (Bors et al., 1984), and relies on the fact that the absorbance of croton (measured at wavelength 443nm) is diminished on incubation with a free radical generator such as AAPH. Anti-oxidant capacity is measured using the amount of anti-oxidant required to limit the reduction in absorbance to fifty percent. The advantages of the assay are that it is relatively rapid to perform and can be readily adapted to microplates so that many samples can be screened. The disadvantages of this assay are: firstly that it is not widely used, due to the difficulty in procuring croton (as it not sold commercially); secondly the difficulty in comparing studies due to variation in expressing the anti-oxidant capacity of a substance.

1.5.1.4 The TEAC assay

The TEAC (Trolox equivalent anti-oxidant capacity) assay is a (S)ET based assay that is based on the formation of the stable radical ABTS· (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), which is the oxidant probe. This is produced by oxidising ABTS to ABTS· with either potassium persulphate (Re et al., 1999) or hydrogen peroxide and horseradish peroxidase (Cano, 1998). The assay was initially developed by Miller et al, (Miller et al., (1993)) who used a combination of metmyaglobin and hydrogen peroxide that went through an intermediate stage to generate the radical ABTS·. This was improved by Re et al., who eliminated this intermediate stage,

followed by Cano et al., who used horseradish peroxidase to shorten the incubation time required to generate the radical from 16 hours to 1-2 hours. ABTS· is an intensely coloured solution with absorption maxima at 415 and 734 nm. Anti-oxidants will rapidly reduce the ABTS· to ABTS, and the reduced ABTS does not have as intense colour and does not absorb at 415 nm. The reaction between the ABTS· and the anti-oxidant is monitored at 415 nm and measured after a period of 6 minutes. It can also be monitored at 734 nm as has been done by many groups (Cano et al., 1998). The anti-oxidant activity is quantified by relating the percentage reduction in absorbance to that achieved with Trolox. The advantages of this assay are that reliable results can be generated rapidly and the assay can be easily automated. The assay is relatively insensitive to pH and due to its simplicity, the assay is widely used. The major disadvantage is that the ABTS is not biologically relevant as the ABTS radical or one like it is not found in mammalian biology (Prior et al., 2005). Van den berg et al., also commented that quantitative evaluation using the TEAC can be difficult but the assay can be used to rank the anti-oxidants (van den Berg et al., 1999).

1.5.1.5 DPPH assay

The DPPH assay is an another (S)ET based assay that uses a rare stable nitrogen radical called 2.2-Diphenyl-1-picrylhydrazyl (DPPH). The assay has a long history and was first reported by Kurechi et al., (Kurechi et al., 1980) and subsequently developed into an assay by Brand Williams et al., (Brand-Williams et al., 1995). The assay involves monitoring the decolouration of the radical DPPH at 515 nm (Brand-Williams et al., 1995). The concentration of anti-oxidant required to achieve 50% reduction in DPPH absorbance is proportional to the anti-oxidant activity of the substance (Sánchez-Moreno et al., 1998). This is referred to as the EC_{50} value. Sanchez-Moreno et al., have introduced an anti radical efficiency value (Sanchez-Moreno, 2002), but this is not widely used in the

literature. The advantages of this assay are that it is very simple, can be easily performed on microplates, is relatively rapid to carry out and the DPPH radical can be easily commercially sourced (as it does not need to be chemically synthesized). However, there are a number of disadvantages to using the DPPH assay. Firstly, DPPH activity bears no relation to anti-oxidant activity either in cells or *in vivo*. Secondly, the assay can underestimate anti-oxidant activity of substances that cannot access the DPPH active site (Prior et al., 2005). Another disadvantage is the inability of the assay to measure carotenoids anti-oxidant activity (Nomura et al., 1997).

1.5.2 ***Conclusion***

In conclusion, all of the assays surveyed could be used to quantify the amount of anti-oxidants in a substance and each techniques has its own advantages and disadvantages. On the basis of this a HAT based assay was selected due to the belief that it more closely represents what physiologically occurs in cells (Prior et al., 2005). Of the HAT based assays assesed, the ORAC assay was chosen instead of other HAT based assays such as the TRAP assay due to fact that it had a great deal of published data (demonstrating reliability) and had already been developed into a 96 well plate based assay (thereby allowing for a large amount of samples to be assessed rapidly). In addition other HAT based assays such as the TRAP assay, have issues with reliability and accuracy (Prior et al., 2005).

To compare the results from the ORAC assay, an assay with an alternative mechanism (such as a SET or ET based assay) was chosen to demonstrate multi-factorial anti-oxidant capacity. Of the SET based assays, the TEAC assay was chosen as it was relatively simple to carry out and had a great deal of published data. However, it needed to be adapted for 96 well plates (Cano et al., 1998 and Erel 2003).

1.6 An introduction to the Comet assay:

The Comet assay (or single cell gel electrophoresis) is a widely used assay that can be used to determine and quantify DNA damage (Collins, 2004). The assay functions by measuring the amount of DNA that has migrated away from a nucleus of a cell during electrophoresis. DNA is used as an indirect method of measuring oxidative stress (and in turn free radicals) in cells, as the amount of oxidative stress is proportional to the amount of DNA damage sustained. The image of the nuclear DNA and migrated DNA resemble a celestial comet, hence the name 'comet assay' (this in illustrated figure 1.6-2). A schematic summarising the comet assay protocol is presented in figure 1.6-1.

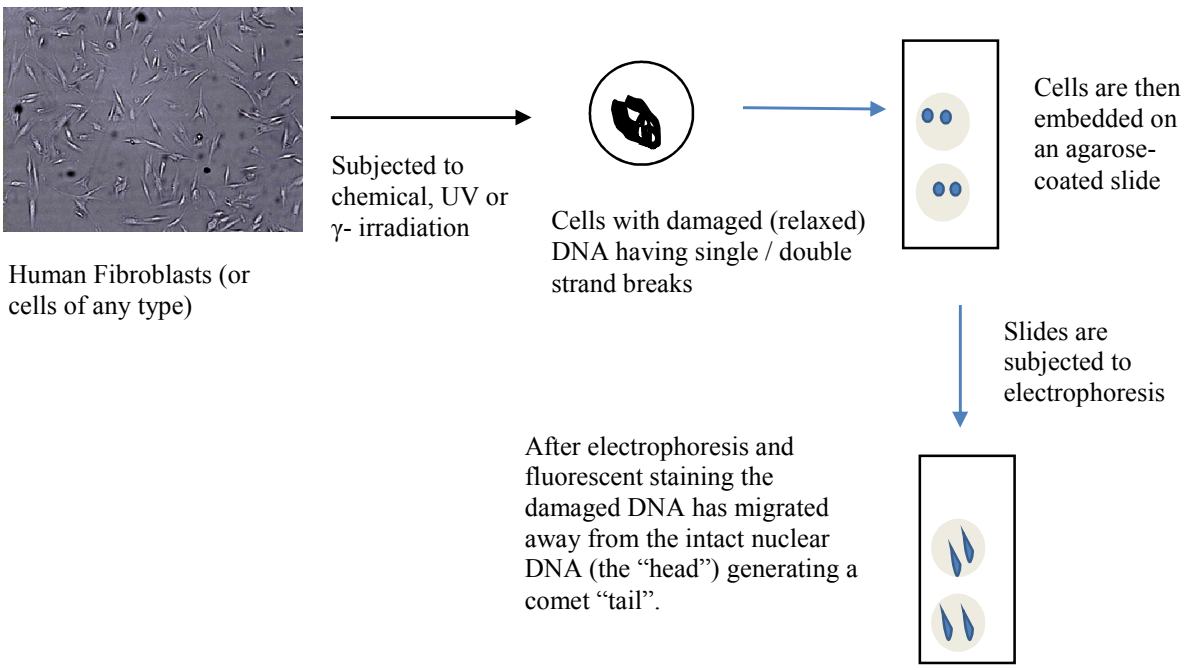

Figure 1.6-1: A schematic summarising the steps in the comet assay. Adapted from a figure from Sigma-Aldrich (Aldrich, 2012).

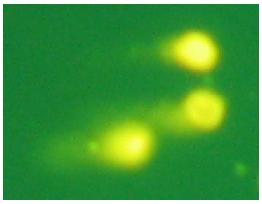

Figure 1.6-2: An image of a 'comet' in the comet assay. The cells in the image were irradiated with UVA light but were not dosed with any extract or Trolox.

The comet assay is over 25 years old and has been modified many times; and there are now many different variations of the comet assay dependant on what the researcher needs. The assay was first developed by Ostling and Johanson in 1984, building on work by Rydberg and Johanson in 1979. The method employed in this paper is not widely used however, due to limited sensitivity of the methods used.

Singh et al., (Singh, 1998) and Olive et al., (Olive et al., 1990) improved on the original Ostling and Johanson method by either using high salt concentration and a detergent (2.5M NaCl and Tritox X-100) or weak alkali to helped denature the DNA and expose alkali labile sites. This improved the sensitivity of the assay by allowing the measurement of single strand breaks and many types of DNA base damage which can be converted into single strand breaks in alkali conditions (Lafleur et al., 1976). Other researchers have subsequently modified the assay but most base their protocol on the work by Singh et al.

The two main comet assay variants in use are the neutral comet assay and the alkaline comet assay. In the neutral comet assay, the electrophoresis stage of the assay is carried out at neutral pH, first demonstrated by Singh et al., and Collins et al., in 1997. The assay is typically carried out at a pH of 7-8 (Singh, 2000) and was first developed to evaluate the amount of double stranded DNA breaks (Singh et al., 1997). The neutral comet assay is also widely characterised as more sensitive to double stranded breaks than single stranded breaks. However, Collins has reported that it can be used to detect single stranded DNA damage although with less sensitivity than the alkaline comet assay (Collins, 2004).

The alkaline comet assay is the more widely used variant of the assay (Collins, 2004). This is mostly likely due to this variant of the assay having a greater sensitivity and range (Collins, 2004). The assay can detect a wide variety of DNA damage including single and double stranded breaks and alkali labile sites (Singh, 2000). It involves the use of strong alkali and a high salt concentration in many stages of the protocol.

The high salt concentration used in both variants of the assay allows for disruption of the cells and solubilisation of the histones. The nuclear DNA then forms a nucleoid which is principally formed of negatively supercoiled DNA. Single stranded breaks caused by oxidative damage or radiation can lead to a relaxation of the DNA supercoiling. This relaxed DNA migrates away from the nucleoid when subjected to electrophoresis (Ostling and Johanson, 1984). The strong alkali is used to enhance this effect and unwinds and denatures the DNA prior to electrophoresis. This all allows for a greater amount of DNA damage to be visualised and detected. Overall, this enhances the range and sensitivity of the assay.

The current incarnation of the assay is an evolution of the method originally developed by Singh et al., This particular method has gone through a number of iterations by Singh from 1988 to 2000 (Singh et al., 1988, 1989, 1990, 1994, 1995, 1997a, 1997b, 1998a, 1998b, 1999, Singh, 2000), Tice (Tice and Vasquez, 1999) and Collins (Collins and Horvathova, 2001). Singh and colleagues have worked for over 10 years on the assay and have added: incubation with a fluorescent stain (Singh et al., 1994), ethanol based DNA precipitation, and DNA neutralisation to the assay. The work of Tice and Collins has principally focused on refining the protocol and developing the assay so that it can be used to monitor site-specific DNA damage and repair. This allows for site-specific damage to be detected.

There are many methods of quantifying the DNA damage measured with the comet assay, including olive moment, % DNA in the comet head, comet tail length and many others. Of these only % DNA in the tail, is directly correlated to amount of DNA damage sustained and is the recommended measurement unit according to Collins (Collins et al., 2008). This unit of measurement is therefore used in this piece of work

The method that is used in this piece of work is principally based on the work of Tice, Collins and the protocol by Trevigen Corp.

1.6.1 ***The documented uses of the comet assay***

The comet assay in all of its variants has been used extensively in the literature (with currently over 6,000 citations), in a wide variety of fields such as carcinogenesis (Bidinotto et al., 2011), human nutrition and oxidative stress *in vivo* and *in vitro* (Hoelzl et al., 2009). However, one of the assay's principal uses has been as an in direct method of measuring the anti-oxidant activity of a particular substance *in vitro* (and *in vivo*) using DNA as a biomarker (Collins, 2005). These substances have covered a very wide spectrum including: plant based extracts such as rosemary and watercress (Casanova and Carballo, 2011, Slameňová et al., 2011), fruits based juices and extracts, such as kiwi juices (Collins et al., 2001); individual substances such as vitamin C, E and Trolox (Anderson et al., 1994, Hseu et al., 2009) and many other extracts and substances. The most commonly used assay variant in all of this work has almost exclusively been the alkaline variant that is also being used in this piece of work.

In contrast to the number of studies using fruit or plant extracts, there is very little literature around using the comet assay with any type of yeast or cider based extract. The work by Trotta et al., (Trotta et al., 2012) and Miorelli et al., (Miorelli et al., 2008) is an exception, that has demonstrated that certain strains of yeast have been shown to have anti-

oxidant activity using the comet assay or have used yeast as the model system of measuring DNA damage. (Rank et al., 2009).

While there is a lack of literature documenting the use of cider or yeast extracts with the comet assay, there is a large amount of literature focusing on the effect of either apple juice extracts or apple extracts (*in vivo* or *in vitro*). Szeto et al., (Szeto et al., 2006) and Schafer et al., (Schaefer et al., 2006b), represented many other studies (such as (McCann et al., 2007)) showing a reduction of DNA damage with cellular DNA damage induced by a chemical oxidiser such as H_2O_2 or menadione. In the studies mentioned, apple homogenate had been used by Szeto et al., while Schafer et al., used a combination of a cider and or conventional apple juice extract. These extracts contained five selected phenolic compounds with the amount of the phenolic compounds related to their concentration in the apple varieties used in the particular extract.

The work by Schafer et al., shows that the cider and table apples both reduced DNA damage induced by menadione, with the cider apple extract more effective at a lower concentration than the table apple extracts used.

In a relatively similar field to cider, a number of groups have investigated the effects of wine components on oxidative stress (Giovannelli et al., 2000) and carcinogenesis (Dolara et al., 2005) using the comet assay as an indirect method of quantifying anti-oxidant activity. Both studies reported significant reductions in basal DNA damage of rats treated with wine polyphenols and Dolara et al.,(Dolara et al., 2005) detailed a reduction in carcinogenesis induced by azoxymethane or dimethylhydrazine. Russo et al.,, (Russo et al., 2003) also investigated the effect of oxidative damage *in vitro* using black grape extracts and reported a reduction in ROS (reactive oxygen species) and subsequently DNA damage caused by amyloid beta.

In contrast to the *in vitro* literature, the *in vivo* literature is much more inconsistent. Work by Castgnini et al., (Castagnini et al., 2009), showed no significant reduction in DNA damage in rats fed a local cider apple variety. This was echoed by Poulsen et al.,(Poulsen et al., 2011), who detailed no significant reduction in DNA damage but did show a reduction in preneoplastic changes in rats which were fed whole apples in conjunction with a carcinogen. Yuan et al., (Yuan et al., 2011) did not report a reduction in DNA damage in a 2 week human intervention study; where the subjects drunk apple or grape juice. In contrast, work by Briviba (Briviba et al., 2007) did show a reduction in DNA damage of lymphocytes treated with $FeCl_2$ and Endo nuclease III taken from subjects who had been on a diet of organic or non organic apples.

1.6.2 ***UVA irradiation and the comet assay***

UVA radiation is well reported in causing DNA strand breaks (Alapetite et al., 1996), and the purported mechanism of action is an indirect increase in amount of ROS (reactive oxygen species), (Pygmalion et al., 2010, Cadet et al., 2009) via the breakdown of photosensitive compounds such as riboflavin or quinines (Marrot and Meunier, 2008, Cadet et al., 2009). It has been demonstrated that UVA irradiation increases intracellular ROS. There are a number of studies that have used the comet assay to quantify the amount of DNA strand breaks (Morley et al., 2003, Marrot et al., 1999) (which are predominately single strand breaks) caused by UVA irradiation. One of the studies by Lyons and O'Brien (Lyons and O'Brien, 2002) details the significant reduction in DNA damage with an algal extract in two different cell types, when compared to the untreated sample. Pygmalion et al., (Pygmalion et al., 2010) and Morley et al., show reductions in DNA damage using a synthetic ion chelator and N-Acetyl cysteine (a precursor to glutathione). Both show significant reduction in DNA damage of cells exposed to UVA radiation. The studies are of particular interest as the work by Pygmalion et al., is targeted for a cosmetic product

(like the yeast extract) and Morley et al., use the same Trevigen comet system that was utilised in this piece of work.

In summary, the comet assay was chosen as the assay to corroborate previous anti-oxidant results. It has been widely used to assess the anti-oxidant activity of a wide range of extracts both *in vitro* and *in vivo*. Of particular interest is the work investigating the effect of apple based extracts and the reduction in DNA damage reported in these studies *in vitro*. Apples are the ultimate feedstock of the cider yeast extract and could ultimately be responsible for the anti-oxidant activity of the extract. The work involving UVA radiation and the comet assay was of particular interest, due to UVA radiation being linked to oxidative DNA damage in skin (Cadet et al., 2009). The comet assay is therefore an ideal method of investigating if the extract could prevent this damage and provide a mechanism of measuring anti-oxidant activity in cells. This would be an important factor of any cosmetic product containing the yeast extract.

1.7 Emulsion formation and rheology

An emulsion is colloidal material where one (or more) liquid phase is dispersed in another liquid phase. For most simple emulsions these two phases are composed of oil and water. Therefore the two main emulsion varieties are the oil in water emulsions (O/W) and water in oil (W/O) emulsions (Binks, 1995), where the dispersed phase is the first phase and the continuous phase the second phase. The dispersed phase is present in the form of droplets suspended in the continuous phase, these are typically 1 μm to 100 μm in size (McClements, 2007). There are more complex double emulsions such as the water in oil in water and oil in water in oil emulsions. However, the emulsions that are dealt with in this chapter are exclusively the oil in water variety.

Most emulsions are inherently thermodynamically unstable and want to separate into their component liquids (due to two immiscible liquids being used) as this reduces the area of contact between the two phases and therefore this reduces the surface tension of the dispersed phase (Friberg and Yang, 1996). Due to the fact that both oil and water phases do not mix spontaneously, energy is required to form a stable emulsion and diffuse the dispersed phase. This is represented by the following equation:

$$\textbf{work done (or } \Delta G) = \gamma A$$

Equation 1.6.21-1 : The equation defining the relationship between work done, surface tension and surface area. Where γ is the surface tension in dynes/cm or mNm^{-1}, A is the increase in area and work done is amount of energy put into the emulsion. (McClements, 2004).

The energy required is provided by the mechanical energy derived from mixing the two phases together. The amount of energy required can be reduced with an emulsifier, which reduces the surface tension (and therefore energy) at the oil/water interface and can therefore stabilise it (Binks, 1995). This mixing is typically carried out with a high shear mixer (referred to as a homogeniser) which can achieve small droplet size due to the shear

stress applied during emulsion formulation. This reduction in particle size due to shear is referred to as comminution.

Rheological properties of emulsions are important, as they define how an emulsion flows and reacts to external forces. Viscosity is defined as a measure of internal friction of a fluid (Schramm, 2006) and defines how much force is required to physically move the fluid (i.e in mixing for example).This can be represented mathematically by the following equation:

$$\eta\ (\mathbf{viscosity}) = \mathbf{shear\ stress/shear\ rate}$$

Equation 1.6.21-2 : The equation used to define viscosity. Where shear stress refers to the amount of force required to move a substance per unit of area and shear rate refers to the rate of movement of layers of the liquid (with respect to each other) (Hulya and Jozef, 2006). Viscosity is either measured in either centipoise (as it is in this chapter) or in milipascal-seconds (mPa•s).

The above equation is only valid if the fluid or emulsion has Newtonian behaviour and most emulsions are not Newtonian. Emulsions are typically pseudo-plastic, which refers to viscosity decreasing as shear rate increases. This is termed shear thinning (Laurier and Elaine, 2005). Due to the complexity of most emulsion formulations, it has been difficult to predict emulsion viscosity (Laurier and Elaine, 2005), and therefore there is not one model which takes into account all of the interactions (and forces) that are present in an emulsion system.

Tracking the viscosity of an emulsion (or other cosmetic formulation) can be used to evaluate its stability as a drop in viscosity leads to an increase in droplet diffusion and a potential increase in coalescence and creaming (with some emulsions it is the first step in emulsion instability). In addition, a drop in viscosity is highly undesirable in a consumer product as it has a negative impact on the product's aesthetics.

1.7.1 *Emulsion stability*

Most emulsions are thermodynamically unstable, however there are emulsions which are kinetically or thermodynamically stable such as micro-emulsions or lyophilic colloids; due to either self emulsifying components or the emulsified state being thermodynamically favourable (Schramm, 2006). However, the emulsions that we shall deal with in this chapter are all thermodynamically unstable. Due to this instability most O/W emulsions will separate given enough time, and 'stable' emulsions are ones that resist change in the period of time measured, and therefore are described to have a degree of kinetic stability (Schramm, 2006).

The DLVO (or Derjaguin and Landau, Verwey and Overbeek) theory is one of the main theories involving emulsion stability; it can be used to predict emulsion stability based on the role of electrostatic interactions and that of London van der Waals forces. Using the theory the energy changes of two particles approaching each other can be calculated; taking into account the attractive van der Waals forces and repulsive electrostatic interactions. The total energy change is a combination of both factors as summed up in the equation below.

$$V_{(T)} = V_R + V_A$$

Equation 1.7.1-3: The relationship between total potential energy (V_T), V_R the energy from electrostatic interactions and V_A, the energy from van der Waals interactions

The van der Waals forces are calculated using the Hamaker constant and the electrostatic interactions forces are calculated using potential of the charged layers around the charged particle (or droplet) (more detail on the mathematical basis for each of the interaction can be found in (Petsev, 2004) and (Binks, 1995)). The overall energy profile is shown in figure 1.7-1. At large film thicknesses attractive (van der Waals) forces dominate but as film thickness (or the distance between the particles or droplets) reduces there is a point

where the interaction energy rises. This is caused by the electrostatic interactions, and providing this rise is greater than the thermodynamic energy in the system, it can prevent droplets getting to the primary minimum and instead settling at the secondary minimum and only reversibly flocculate.

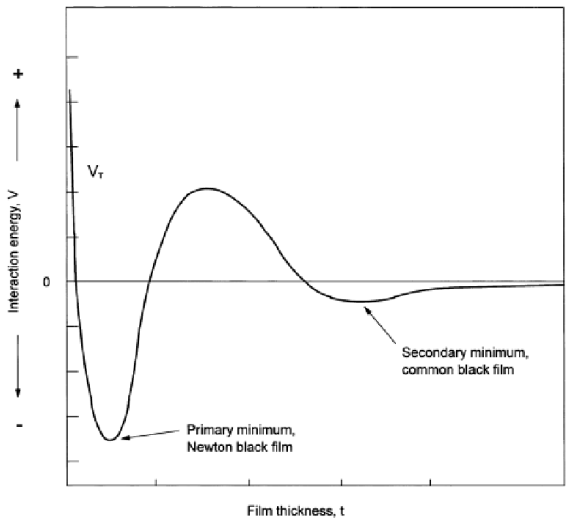

Figure 1.7-1: The Interaction energy V_T as a function of film thickness, based on a figure by Schramm (Schramm, 2006)

Charged droplets are found in most emulsions due to the dispersed phase droplets adsorbing charged substances (such as proteins). The layout of a charged droplet is shown in figure 1.7.2, it shows that there are 2 main layers of particles between the droplet and the bulk solution. The inner layer is termed the Stern layer and is tightly bound to the particle (Hunter, 1996).The outer layer is more diffuse and less tightly bound. The potential difference between this outermost layer and the bulk solution is defined as the zeta potential and can be used as a marker of emulsion stability (Schramm, 2006). The larger the zeta potential the more stable an emulsion is, with a zeta potential of -41 mV or above being defined as good stability with the maximum stability of an emulsion being between -81 and -125 mV (Hunter, 1996). Any emulsion with a zeta potential below -20 mV is predicted to show signs of agglomeration (or flocculation).

There have been criticism of the DLVO theory citing that it does not take into account hydration, hydrophobic and depletion interactions (Grasso* et al., 2002), however Schramm has concluded that that the DLVO theory is a good starting point (Schramm, 2006).

Droplet size is known to have a large impact on emulsion stability, as there is an inverse relationship between droplet size and emulsion stability. Therefore the smaller the droplet size the greater the likelihood that emulsion will be stable (McClements, 2004).

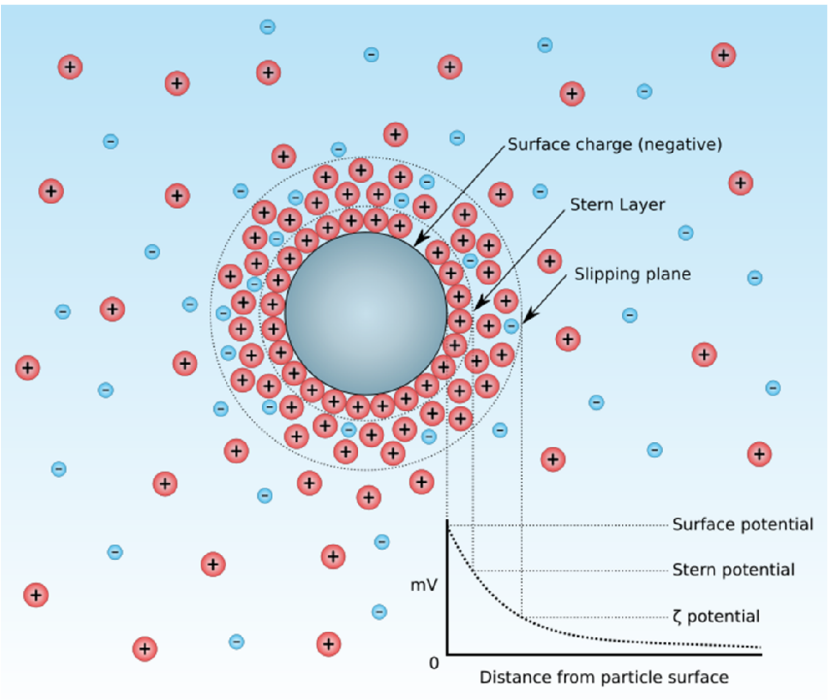

Figure 1.7-2: A Diagram illustrating the layout of zeta potential and slipping plane in a dispersion (such as an emulsion) based on image by (Mjones1984, 2012)

Unstable emulsions are not homogenous systems and go through a gradual process eventually leading to complete phase separation. There are a number of steps in this process including flocculation, coalescence, creaming and sedimentation, Ostwald ripening and phase inversion. They are illustrated schematically in figure 1.7-3.

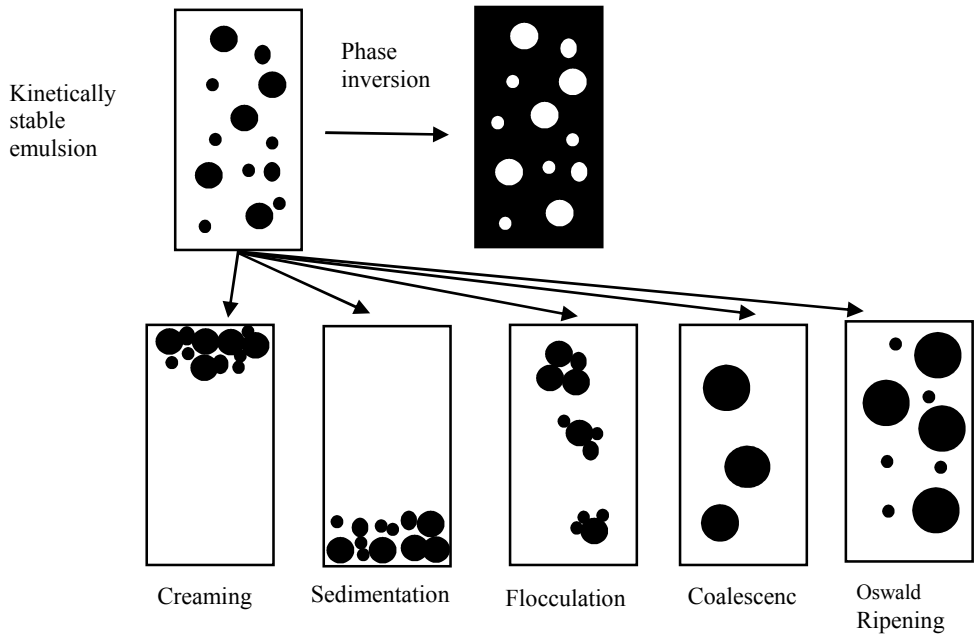

Figure 1.7-3: A schematic diagram of all the common forms of emulsion stability, adapted from a figure by McClements. (McClements, 2007)

Flocculation is one of the first stages in emulsion stability and is where there is no change in the droplet size of the dispersed phase, but the droplets have bound together and are separated by a thin film (Petsev, 2004). The droplets form aggregates and move as a single unit. This occurs without a change of size or shape of the droplets.

Flocculation occurs due to the attraction of neighbouring droplets to each other as a result of van der waals and hydrophobic forces, and lack of any long range repulsive force (such as electrostatic and steric forces) to counter the attractive forces (Freiberg and Yang 1996). This affect can happen due to number of factors including pH and temperature change. A mathematical model of the flocculation, including an analysis of the kinetics involved has been developed by Petsev (Petsev, 2004). However, this is beyond the scope of this chapter.

Flocculation can be studied using images of the emulsion structure from an optical microscope which can be used to determine the type of flocculation occurring

(McClements, 2007). Bridging and depletion flocculation are the names of main types of flocculated structures (referred to as flocs). The organised flocculated structures are referred to as bridging flocculation and are a result of weak forces binding the particles together. The more disorganised and freeform flocculated structures are known as depletion flocculation and are a result of stronger forces acting on the droplets (McClements, 2007). A polydisperse particle or droplet size (in an emulsion) enhances flocculation and can lead to enhanced creaming and coalescence (Binks et al., 1998).

Coalescence is defined as two or more droplets joining together to become one larger droplet (Schramm, 2006). This happens when the van der Waals forces or other forces overcome the repulsive forces and reduce the thickness of thin film separating the two droplets to a point when, the film bursts and the droplets merge (Freiberg and Yang, 1996). Flocculation is a prerequisite for coalescence to occur. Coalescence makes sedimentation and/or creaming occur more rapidly due to an increase in droplet (or particle) size. Coalescence can also lead to a change in colour of the emulsion, due to the larger droplets scattering light differently (McClements, 2004).With regard to coalescence kinetics, larger droplets are known to coalescence faster, than smaller ones, this correlates with the widely held belief that emulsions with smaller droplet size are known to more kinetically stable (Binks, 1995). Coalescence can be monitored using a variety of techniques from optical microscopy to NMR and methods that measure particle (or droplet) size (Schramm, 2006).

The chance of coalescence increases if the viscosity of the continuous phase decreases and if the interfacial viscosity is reduced. This increases the chance that coalescence can occur by droplet collusion.

Creaming is an effect when oil droplets rise to the top of an oil-in water emulsion under gravity, due a difference in density of the oil droplets compared to the continuous phase.

Sedimentation is a similar effect, but with a more dense dispersed phase than continuous phase, with water droplets sedimenting in a bottom of the water-in-oil emulsion. Both sedimentation and creaming do not result in a change in droplet or particle size. It can be monitored using a number techniques such as conductivity, MRI and ultrasound (Binks et al., 1998).

Ostwald ripening a phenomenon where larger drops are formed in favour of smaller droplets, this occurs because the solubility of material in a spherical droplet increases with decreasing droplet size (McClements, 2007).This process only occurs if the droplet sizes are polydisperse and results in an increase in mean droplet size (McClements, 2004). This effect can be measured using any instrument that can measure particle size distribution, such as dynamic light scattering or laser diffraction.

There are a number of steps that take place from an initial emulsion to go to a complete phase separation. The first step is flocculation which causes aggregated droplets, which is followed by the droplets coalescing. This causes the large cream or sediment due to the difference in viscosity of the droplets. This is then followed by further coalescence and complete phase separation (Freiberg and Yang, 1996).

To protect against the various routes of emulsion instability presented a number of steps can be taken, firstly the use of polymers or a non-ionic emulsifier can strengthen the droplet interface. This can lower the interfacial tension and reduce the chance of coalescence when two droplets collide (Laurier and Elaine, 2005). In addition, the use of long chain polymers can stabilise an emulsion due to effects of steric hindrance. Secondly the use of ‘thickeners’ (compounds which make a solution or emulsion more viscous) or polar waxes can increase the viscosity of the continuous phase, limiting the diffusion (and

therefore kinetic energy) of the droplets. This can reduce chance of flocculation or coalescence due to the reduction in collisions and the speed of collisions.

While simple emulsions such as those covered in this chapter are not thermodynamically stable they can be made kinetically stable, with help of an emulsifier.

An emulsifier is a key element in the stability of emulsion; it is a surface active molecule or surfactant which has a lipophilic (tail) and hydrophilic (head) portions. The tail groups generally consist of alkyl or alkylaryl hydrocarbon groups, however oxaalkyl and thiaalkyl are also possible (Kosswig, 2000). Head groups consist of a much wider array of chemical structures and the characteristics of the head group are used to classify emulsifiers (Kosswig, 2000). Emulsifiers are typically found at the oil-water interface, reducing the surface tension and therefore the droplet size. This effect increases the stability of the emulsion (Sjoblom, 1996). They also act as a barrier between phases and prevent droplet coalescence.

The two main groups of emulsifiers are, charged (i.e cationic, anionic and amphoteric) and non-ionic emulsifiers, the most common type is the non-ionic emulsifier which carriers no overall charge; an example of which is glyceryl monostearate (Lauridsen, 1976) .

The group of charged emulsifiers are composed of anionic, cationic and amphoteric surfactants. Anionic surfactants are compounds which contain small counter ions (such as sodium or ammonium) with anionic hydrophobic groups (Kosswig, 2000). Examples include alkyl sulphates and alkyl phosphates. Cationic surfactants are similar but contain a cation instead of an anion, an example is tetraalkyl ammonium chloride (Kosswig, 2000). Amphoteric surfactants have zwitterions which can be positive or negative dependant on the pH of the solution.

Polymeric emulsifiers are another group of non-ionic emulsifiers that have large macromolecules which can act as surfactants. These polymeric structures form large structured films on the outside of the dispersed phase such as an oil droplet. These large structures prevent coalescence and stabilise the emulsion with affects of steric hindrance.

Due the quantity of non-ionic surfactants, it can be difficult selecting the correct surfactant. To solve this issue the HLB or hydrophilic-lipophilic balance system was devised to organise surfactants based on the ratio of the molecular mass of the hydrophilic fraction in the molecule (M_h), to the total molecular mass of the surfactant M multiplied by 20. All non ionic surfactants are classified on a scale from 0 to 20 and surfactants are sorted according to their solubility in water.(Kosswig, 2000)

To demonstrate the role of surfactants and polymers in practice, examples of a number of surfactants and polymers used in formulations later on in this chapter are detailed below.

The surfactants Cithrol GMS and Cetyl alcohol are both used as emulsifiers. Cithrol GMS is used as the main emulsifier; it is a combination of non-ionic emulsifiers polyethylene glycol (PEG) 100 stearate and glycerol monostearate (GMS). The PEG 100 stearate is the more hydrophilic of the two emulsifiers, since it has 100 molecules of PEG, which is hydrophilic. Therefore the glycerol monosterate (GMS) is the more hydrophobic or lipohilic of the two emulsifiers in cithrol GMS. The PEG forms a ‘tail’ on the outskirts of the oil droplet and this results in the steric stabilisation of the emulsion. Using a blend of two non-ionic primary emulsifiers (which is common in industrial applications) enables tighter packing at the oil water interface. This enables a greater reduction in interfacial tension and strengthens the oil/water interface and reduces the chance of coalescence when two oil droplets collide. This is illustrated in figure 1.7-4

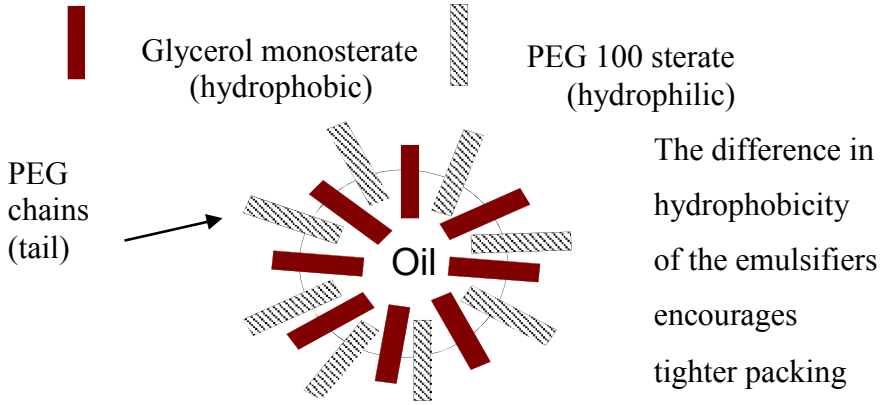

Figure 1.7-4: An illustration showing the effect of the two emulsifiers. The use of two emulsifiers illustrates the effect of tighter packing of the emulsifiers on the oil droplet.

Cetyl alcohol acts as a secondary emulsifier by forming lamellar structures to reduce the chance of droplet collision. It also forms circular structures increasing the viscosity of emulsions and thereby reducing the chance that flocculation or coalescence. At high concentrations cetyl alcohol forms layers surrounding the droplet as illustrated in figure 1.7-4. The entire layered structure is referred to as a liquid crystal structure and traps not just the oil drop lets but limit the movement of the continuous phase around the droplet. All of which stabilises the emulsion.

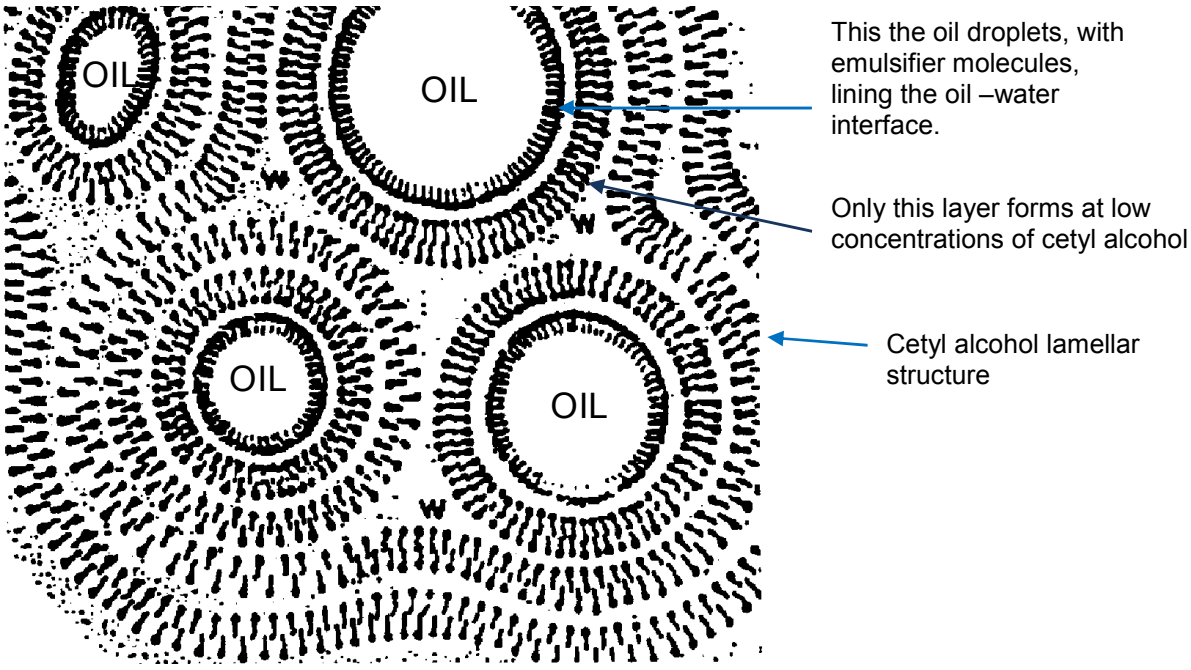

Figure 1.7-5: An illustration of a oil in water (o/w) emulsion with high cetyl alcohol content. Taken from: Emulsions II, Boots training documentation, 2002.

Polyquart 37 is an example of a polycationic polymer (used in one of the formulation in this chapter) which acts as a viscosity modifier (or thickener) which adds structure to an emulsion by increasing the viscosity of the emulsion and thereby enhancing emulsion stability by limiting droplet diffusion.

1.7.2 *Colour change*

Quantification of colour and colour change is important aspect in a wide range of industries from the paint and textile industry to the fast moving consumer goods sector. This is due to colour change being an extremely undesirable characteristic of any product during its shelf life and can be a symptom of much greater problems in the product.

To be able to evaluate colour reproduction and colour change, it is important to first establish the factors affecting colour reproduction and perception. The main factors are the lighting conditions, the observer (as every person can have a different perception of colour) and the object itself. Due to colour being a deeply subjective notion, a standard

way of representing colour was sought and this was carried out by an organisation referred to as the CIE (an abbreviation based on its French name the Commission internationale de l'éclairage or the International Commission on Illumination) (Schanda, 2007). A group at the CIE have derived a number of models over time that aim to standardise colour identification and quantify colour change.

The first of these models was published in 1931 (Schanda, 2007) and was widely adopted. It aimed to mathematically define colour perception into what is termed as colour spaces.

A colour space is a mathematical model defining all of the potential colours that a human can perceive or machine can reproduce. The model represents each colour as a co-ordinate composed of three separate values based initially on the primary colours red (R), green (G) and blue (B). The RGB values are referred to as the tri-stimulus values; in 1931, the CIE defined a colour space where the RGB values were converted into XYZ values by the formulae defined in equation 1.7.2-1. This was done to more closely model human vision and to add a value which accounted for brightness of a colour (the Y value). This was also done to avoid certain colours having negative tri-stimulus values(Hunt and Pointer, 2011).

$$X=0.49R+0.31G+0.20B,\ Y=0.17697R+0.81240G+0.0101063B,\ Z = 0.00R+0.01G+0.99B.$$

Equation 1.7.2-1: The equation required to convert RGB values into XYZ values.(Hunt and Pointer, 2011)

After this model helped standardise colour reproduction, standardisation of the other two major factors affecting colour perception (lighting and the observer) was sought. To do this the CIE defined a range of lighting conditions. The most popular being the D65 standard (which is used in this study), which corresponds to midday sun in northern Europe, therefore is referred to as the standard daylight illuminant. The exact specification of the D65 standard are further detailed by Schanda (Schanda, 2007). The last major factor

in colour measurement is the standard colorimetric observer, which was also defined by the CIE in 1931. It is defined as the response an average human viewing the colour at a 2° angle would give (Hunt and Pointer, 2011).

The CIE1931 colour space was further developed in 1976 into the CIELAB colour space which aimed to be a much closer model of human vision than the 1931 model was and is still used currently. In this colour space the luminescence of a colour was also calculated and XYZ tristimulus values used in the 1931 model were transformed into L^*, a^* and b^* values. The equations used to convert between the XYZ and the L^*, a^* and b^* tristimulus values are defined in equation 1.7.2-2

$$L^* = 116f(Y/Y_n) - 16,\ a^* = 500\,[f(X/X_n) - f(Y/Y_n)],\ b^* = 200\,[f(Y/Y_n) - f(Z/Z_n)]$$

Equation 1.7.2-2: The equation required to convert XYZ values into L^*, a^* and b^* values. X_n, Y_n and Z_n are the X, Y and Z values reference values (Hunt and Pointer, 2011)

All of the three factors used to define a colour (of a material or substance) were used as a basis for defining the colour change formulae released by the CIE in 1976 which helped determine whether a material or substance had changed colour or if two separate substances had imperceptible differences in colour.

The CIE 1976 colour change formula is defined as follows:

$$\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

Equation 1.7.2-3: The CIE 1976 colour change formula (Schanda, 2007). Where ΔL^*, Δa^* and Δb^* represent the change in L^*, a^* and b^* respectively.

The formula uses the quantity of ΔE to quantify the amount of colour change between two samples. A ΔE value of above 1 is commonly referred to as noticeable colour by the standard colour metric observer (Ohta and Robertson, 2005).

The 1976 formula was further refined numerous times (as more data became available) to better model human vision as the 1976 model assumed that the human eye was equally sensitive to all colour hues. However, it was found that human vision is more sensitive to colour change in the green part of the spectrum than it is in the yellow part of the spectrum (Kuehni, 2003). Together with other experimental data, a number of modifications were made to the CIELAB colour change formula including one to take into account interactions between hue and chroma in the blue area of the spectrum. All of these modification resulted in the CIE releasing the CIE2000 formulae in 2001 and the DE2000 scale that takes all of this into account. It is therefore a more accurate model of human perception of colour change particularly small colour changes, than the CIELAB 1976 colour change formula is.

The CIE2000 formula is given in equation 1.7.2-4 alongside a number of supplementary equations. Due to the fact that is the most accurate model of human perception of colour change (particularly small changes in colour) and is currently recommended by the CIE (Hunt and Pointer, 2011), it was chosen as the formula to be used in this piece of work.

$$\Delta E_{00}^{*} = \sqrt{\left(\frac{\Delta L'}{k_L S_L}\right)^2 + \left(\frac{\Delta C'}{k_C S_C}\right)^2 + \left(\frac{\Delta H'}{k_H S_H}\right)^2 + R_T \frac{\Delta C'}{S_C}\frac{\Delta H'}{S_H}}$$

Where: $L' = L^*$

$$\Delta L' = L'_b - L'_s$$

$$\Delta C' = C'_b - C'_s \text{ and } C' = \sqrt{(a'^2 + b'^2)}$$

$$\Delta H' = \sqrt{2(C'_b C'_s)}\sin(\tfrac{\Delta h'}{2})$$

And $\Delta h' = h'_b - h'_s$

Equation 1.7.2-4: the CIDE2000 colour difference formulae with the ΔL', ΔC', ΔH' values defined in separate equations below. The k_L, k_C, k_H, are parametric factors S_L, S_H ,S_C are weighting functions, with R_T the rotational factor (Schanda, 2007). The subscripts b and s denote the values for the test and the reference sample respectively.

1.8 Conclusion and definition of research problem

The literature reviewed has showed that subcritical water is an environmentally benign and versatile substance that can be used to extract a wide range of substances from proteins to phenolic compounds, with the use of temperature as the main variable. In addition a number of other advantages have been detailed including the substance being non-toxic and ease of disposal. This makes it an attractive replacement to organic solvents traditionally used in this instance. Therefore, it makes it the ideal substance to use to optimise the production of the cider yeast extract with a focus on anti-oxidants.

To asses the anti-oxidant activity of the extract, the ORAC and TEAC assays were chosen due the large amount literature citing use of the assay together with the physiological relevance of the ORAC assay. The comet assay was chosen to reinforce any data produced due literature citing the assays use as a route of measuring anti-oxidant capacity in cells via

oxidative stress and DNA damage. Therefore an overall conclusions can be drawn with regard to the anti-oxidant capacity of the extract. This data could be used to refine the extract's production.

Emulsion stability and colour stability is an important factor in any cosmetic product and the background literature surrounding this topic has been summarised, which will be used in scoping study to evaluate the emulsion and colour stability of prototype commercial products containing the extract.

1.8.1 ***Overall research problem***

Overall the research being investigated is:

- Can subcritical water be used to produce an extract from cider lees with anti-oxidant capacity and can changing the process conditions improve this? (which would increase its value and commercial applications).
- If the extract does have anti-oxidant capacity, what in the composition of the extract is responsible?
- How does the extract behave in a prototype commercial formulation with regard to emulsion and colour stability?

CHAPTER 2 : Methods and Materials

2.1 Yeast extract production

2.1.1 *Batch yeast extract sample production*

The 1st set of cider yeast extracts (also referred to as the 1st generation of yeast extracts) were produced in a 300 ml pressurised and stirred reactor (Parr Instrument Company (Illinois, USA), model 5521) by my colleagues Dr Alireza Bahari and Fabio Antas. At any one time 200 ml of cider lees was processed; at various temperatures ranging from 100 °C to 300 °C at either 30 bar or 100 bar (this was used to keep the extract in the liquid phase particularly at 300 °C), with typically a 15 minute reaction time (as summarised in table 2.1-1). All of the extracts were stirred at 500 rpm and the reactor was water cooled and reaction time was only started when the reactor had reached the desired temperature. The reactor control unit controlled both temperature and stir rate, while the reactor was pressurised using a nitrogen gas cylinder and maintained manually using a pressure gauge and release valves.

The processed cider lees was then centrifuged at 4500 rpm for 15 minutes, and the supernatant decanted to remove all of the yeast. Subsequently, this extract was then filter sterilised prior to use with anti-oxidant assays.

The cider lees was sourced from Bulmers (Hereford, UK) and was solution containing waste yeast taken after cider fermentation has been completed and any excess alcohol recovered. The extract source and strains of were commercially sensitive and are unknown.

A diagrammatic representation of the reactor setup is shown in figure 2.1-1

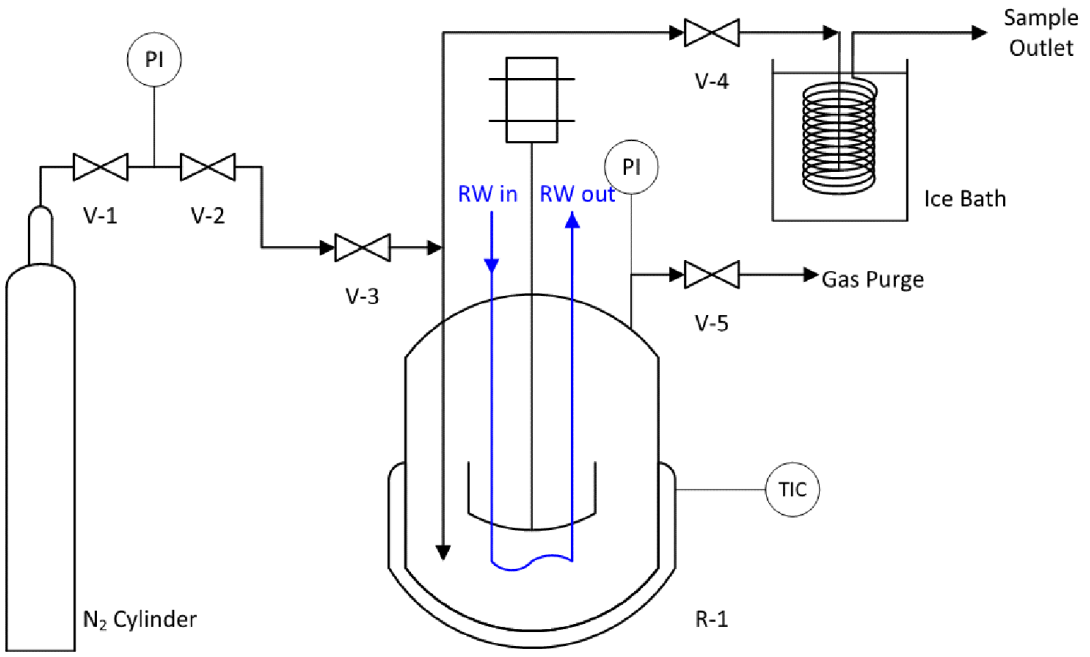

Figure 2.1-1: Showing a representation of the subcritical water reactor setup, where RW is the refrigerated water. (Figure is courtesy of Mr Fabio Antas).

2.1.2 ***Kinetic Extracts***

To study the effect of reaction time on the extract, a much smaller tube reactor (with a total volume of 6 ml and an internal diameter of 4.6 ± 0.1 mm) was used to reduce the heating period from over 20 minutes to just over 5 minutes. This allowed for a reduction in any effects due to the heating up period. This extract was produced by Dr Alireza Bahari.

In the experiment, 3 ml of cider lees was injected into the reactor and heated in an oven to the desired temperature. Independent runs were carried out for each reaction time and temperature shown in figure 3.4-3.

As in section 2.1.1 the cider lees was centrifuged at 4500 rpm for 15 minutes to remove the yeast and the supernatant removed for further analysis.

2.1.3 ***Scale up: 5 L batches***

To scale up the production of the extract, the second generation of extract was produced in a 5 L reactor (Parr instruments, (Maryland, USA) model 4582) arranged in a similar fashion to that shown in Figure 2.1-1. In this instance the cider lees was processed at approximately 200 °C (reactor heating was actually stopped at 180 °C) for 10 minutes at 100 bar. The overall heating up time of the reactor to 200 °C was 40 mins. The cider lees was then centrifuged at 4500 for 15 minutes, to remove all of the yeast and this subsequent extract was then filter sterilised using a 0.2 μm Polyethersulfone (PES) Filter (Milipore , US) prior to use in cell culture (see section 2.4.2).

Table 2.1.3-1: Summarising all of the process conditions of the extracts produced in the 1st and 2nd generation extracts.

Generation	Temperature (°C)	Pressure (bar)	Reaction time (minutes)	Reactor size (ml)
1	100	30	15	300
	125	30	15	300
	150	30	15	300
	175	30	15	300
	200	30	15	300
	225	30	15	300
	300	100	0-10	300
2	180-200	100	10	5000 (5 L)

2.1.4 ***Refined extract production with supercritical CO_2***

The refined extract was produced using the 200 °C hydrolysed yeast extract (1st generation) as the feedstock. The extract was processed using a 500 ml stainless steel reactor (Thar Process, Pittsburgh, USA) with a glass vial containing 5ml of extract with a constant flow of supercritical CO_2 of 50 g/min. The temperature of the reactor was kept at 60 °C, with pressure of 200 bar and an extraction time of approximately 10 minutes. The

extract was collected in a vessel which contained 10ml of ethanol. The pressure in the reactor was maintained using a CO_2 pump and back pressure regulator. This process allowed for an extract rich in phenolics (Antas F, personal communication) to be generated. The extract was produced by my colleague Fabio Antas.

2.2 Anti-oxidant assays

2.2.1 ***ORAC anti-oxidant assay***

The protocol used in this assay was based on the protocol developed by Huang et al., (Huang et al., 2002) for an automated 96 well plate instrument.

Parts of the protocol were adapted for the Promega Glomax Multi (Southampton, UK) that was used in this assay. The parts that were changed involved: preheating the plates to 37 °C, changing the flourescien concentration to 300nM and changing the incubation time to 45 minutes

All of the pure compounds used in this and the TEAC assay were all sourced from Sigma Aldrich (Dorset, UK) and were of analytical grade or better.

For the ORAC or oxygen radical absorbing capacity assay, a stock solution of 6.6 µM fluorescein was prepared in a 100 mM phosphate buffer and kept in the dark in the fridge. This solution is stable for many months if kept in these conditions (Huang et al., 2002).

A 300 nM fluorescein working solution was freshly prepared in 100 mM phosphate buffer, prior to every batch of assays. Also prepared fresh was a 300 mM solution of AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride) in 100 mM phosphate buffer. This solution was pre- warmed to 37 °C, to initiate peroxy free radical generation by AAPH. AAPH is thermolytically unstable and only generates free radicals in a solution above 37 °C.

For this assay 25 µl of sample solutions were added to 150 µl of a 300 nM fluorescein working solution with 25 µl of 300 mM AAPH added as a final step (final concentration of 37.5 mM). The positive control used was 25 µl of 25 µM Trolox.

In every assay each sample had a minimum of 4 replicates and each 96 well microplate was pre-warmed to 37 °C prior to the start of the assay. The assay itself was carried out at room temperature.

The degradation in fluorescence of each sample was tracked by measuring the fluorescence (with an excitation of 490 nm /emittance 520 nm) of each sample every minute, using a Promega glow max multi microplate reader (Promega, Southampton, UK)

2.2.2 *TEAC assay*

This assay protocol was adapted from that reported by Erel et al., (Erel, 2004).

For the TEAC assay, 1.5 mM ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) was dissolved in a citrate buffer at pH 4.5, followed by addition of 15 µM H_2O_2 and 11.3 µM Horse radish peroxidase (HRP) to produce ABTS·. The absorbance of the mixture was measured (at 410 nm in a Promega glow max multi (Promega, Southampton, UK) and the mixture incubated for 30 minutes at room temperature. The absorbance was again measured after the incubation period to see if the absorbance has stabilised. Following stabilisation, 175 µl of ABTS- was added to the 25 µl sample and the reduction in absorbance calculated at 410 nm.

2.2.3 *Extract stability*

To evaluate the stability of the anti-oxidant capacity of the extract, various extracts with different process temperatures were evaluated.

Yeast extracts with hydrolysis (process) temperatures of 150 °C, 175 °C, 200 °C and 225 °C were kept in a 37 °C oven (with no humidity control) in plastic vials for four weeks. Samples were taken every week to evaluate anti-oxidant activity of extracts using the ORAC assay. This was carried out to model long term storage of the extract.

2.2.4 ***Data analysis***

For the ORAC assay, the relative ORAC value (using the performance of Trolox as a benchmark) was calculated as mentioned previously and documented by Huang et al. (please refer to equations 1.5-1 and 1.5-2).

In this study all of the extract concentrations were converted to a dry weight (of extract) /ml of solution and this was also calculated for Trolox. Using equation 1.5-1 and 1.5-2 (with one modification; dry weight is used instead of morality for both Trolox and the substance being tested) to calculate AUC and the ORAC value for all of the samples and including the blank. The units used for the ORAC value are Trolox equivalents due to the units of both the sample and Trolox (of concentration, mg/ml) cancelling out.

For all of the ORAC assay charts presented in chapter 3.4 and 3.5, all the data are represented as Trolox equivalents and results are a mean of $n \geq 3$ samples with error bars represented as standard deviation.

For comparison, the units of the ORAC value are converted to mg/ml of extract per μM of Trolox in section 3.4.

To calculate the TEAC value, the method documented by Cano was adapted (Cano, 1998) (this involved the use of Trolox as the standard compound instead of L- ascorbate) , which is also used by Re et al., (Re et al., 1999). For the TEAC assay, a standard curve was generated using various concentrations of Trolox; plotting the percentage change

(reduction) in absorbance against Trolox concentration in µM. This standard curve was then used to calculate an equivalent concentration of Trolox in µM for all of the samples. To normalise the results and to aid comparison of current and future extracts all of the results are represented in µM of Trolox per milligram of dry extract.

2.3 HPLC

2.3.1 ***HPLC Sample preparation***

All extracts were produced as detailed in section 2.1.1. All extracts were filtered with a 0.2 µM Millipore PES syringe filter (Fisher Scientific, Loughborough, UK), prior to HPLC followed by dilution with water (if necessary) to achieve better separation.

The pure substances such as chlorogenic acid were dissolved in water (at various concentrations) and filtered prior to undertaking HPLC and were used as standards.

The chlorogenic acid and proanthocyanidin breakdown samples were both prepared by processing 2 mg/ml of pure chlorogenic acid (Sigma Aldrich, Dorset, UK) and 18 µg/ml of pure proanthocyanidin B2 (Sigma Aldrich, Dorset UK) using the same vessel and procedure as described for cider lees processed at 200 °C (see section 2.1.1). This allowed the potential role of any break down products to be evaluated.

2.3.2 ***HPLC method***

The HPLC method used was developed by my colleague Dr Ali Reza Bahari (Bahari, 2010).

All of the fine chemicals and solvents were of HPLC grade and were sourced from Sigma Aldrich (Dorset, UK)

The HPLC system used was a Agilent 1100 HPLC (Agilent, Berkshire , UK) with an auto-sampler, a high pressure quaternary pump, an online degasser, a column oven and a diode-array detector (DAD) (with a range of 200-900 nm) (Agilent, Berkshire , UK). Data collection and analysis was carried out initially on the Agilent's ChemStation® software (Version B.01.02).

The HPLC column used throughout the work was a 4 micron Synergi-Fusion (250 × 4.6 mm) column from Phenomenex®, (California, USA) equipped with a column guard (4 × 2.0 mm) using the same stationary phase to protect the column.

The two mobile phases used were an aqueous phase of 0.1% formic acid in water (pH 3.6) (solvent A) and an organic phase of 0.1% formic acid in methanol (solvent B).

The solvents were used as follows: Pure solvent A for the first 5 minutes, a linear gradient of 0-20% of solvent B from 5 to 15 minutes, followed by a gradient of 20-45% of B from 15 to 35 minutes, then a 45-60% of B from 35 to 45 minutes and then back to 0% B / 100% A from 50 to 52 minutes.

The flow rate remained constant at 1 ml/min and either 5 µl or 10µl was injected into the column (in most instances); with the exception of the fractionation samples. The two wavelengths used were 270 nm and 320 nm, along with use of the DAD (diode array detector) to identify specific peaks.

2.3.3 *HPLC fraction collection and fractionation*

To prepare the phenolic and hydroxymethylfurfural (HMF) HPLC extracts, 50 µl of the (1st generation) 200 °C extract was injected into the column using the HPLC protocol that was described in section 2.3.2. The effluent from the column was collected and separated into 3 fractions. The HMF fraction was the 2nd fraction with a retention time of 10-15

minutes. The phenolic fraction was the 3rd fraction with a retention time of approx. 18-26 minutes, which correlates to the retention times of chlorogenic acid, caffeic acid and proanthocyanidin and many other of the phenolic compounds in the extract (Bahari, 2010).

All of these fractions were subsequently dried under vacuum at room temperature for 24 hours. Vials were weighed prior to fractionation and after drying to evaluate the dry weight of the fractions. The fractions were reconstituted with water to the original concentration in the extract, i.e. if 50 µl was injected, all of the fractions would be reconstituted to 50 µl.

2.4 Folin-Ciocalteu method for phenolic content analysis

To measure the phenolic content of the yeast extracts, a method was adapted from a protocol by Waterhouse (Waterhouse, 2001). The assay was carried out on the yeast extracts by my colleague Fabio Antas.

2.4.1 ***Protein precipitation***

Proteins were precipitated prior to analysis of the yeast extract samples, as they are known to interfere with the results of the assay. The proteins were precipitated as follows, 120 µl of 100% (w/v) Trichloroacetic Acid (TCA) (prepared in distilled water) was added to 800 µl of sample. The sample was then centrifuged at 15000g for 15 mins (in a sigma 3K30 centrifuge) with the precipitate removed and the supernatant used for analysis.

2.4.2 ***Assay preparation and protocol***

The gallic acid calibration standard was prepared by using 50, 100, 150, 250 and 500 mg/l of gallic acid (prepared by using a 50 g/l solution of gallic acid dissolved in ethanol and diluting this solution further with distilled water to form the desired concentration).

To analyse the samples, 20 µl of sample or standard was added to 1.58 ml of distilled water and mixed and then incubated at room temperature for 1 min. 300 µl of a 1.89 M

sodium carbonate solution (prepared in distilled water) was then added followed by incubation at room temperature for 2 hours or at 40 °C for 30 mins.

Samples were then measured in a 96 well microplate at 750 nm using a Promega Glowmax Multi (Promega, Southampton, UK).The gallic acid standards were then used to determine the concentration of phenolic compounds in the extract and are expressed in the units of gallic acid equivalents (GAE) mg/L.

2.5 Comet Assay preparation

2.5.1 ***Yeast extract sample preparation***

The yeast extracts used in the comet assay were produced as documented in section 2.1.1 The 200 °C extract was chosen as it was the lowest temperature were high anti-oxidant activity was observed. The three types of extract used in the assay are detailed in table 2.5-1. All extracts were then filter sterilised using a 0.2µm PES (Polyethersulfone) Filter (Milipore, Massachusetts, USA) prior to use in cell culture.

Table 2.5-1: The process conditions of various extracts used in the comet assay.

Generation	Reactor size	Temperature (°C)	Pressure (bar)	Reaction time (minute)
1	300ml	200	30	15
2	5 L	180-200	100	10
2A (a replicate of 2)	5 L	180-200	100	10
4 (referred to as phenolic)	500ml	60	200	10

The phenolic extract was produced as detailed in section 2.1.4 using the 200 °C hydrolysed yeast as the feedstock (also referred to as phenolic in table 2.5-1).

2.5.2 *Cell culture/ cell preparation*

All cell culture and other comet assay reagents were obtained from Sigma Aldrich (Dorset, UK) unless otherwise stated.

Adult Human dermal fibroblasts (aHDF) (ECACC catalogue no.: 06090715) were cultured in supplemented DMEM (Dulbecco's modified eagles medium). The media contained: 10% (w/v) Foetal bovine serum (Mycoplex FBS, PAA labs /GE healthcare, UK), 2% (w/v) L- glutamine (200 mM), 2.2% (w/v) HEPES Buffer, and 1% penicillin-streptomycin. Cells were cultured in T flasks and then detached with 1X trypsin. The cell number and viability of cells was assessed with trypan blue stain. $1.5x10^5$ cells per well were then seeded to 12 well tissue culture plates (Fisher Scientific, Loughborough UK). Prior to seeding, the test substances were aseptically filtered and added to the media containing the cells. Cells were then incubated with test substances for 18-24 hours.

The test compounds included the hydrolysed yeast extract, Trolox and the phenolic extract. The yeast extract was added to achieve a final concentration of 1% w/v, as this was the highest amount of extract permitted without degradation in cell viability due to pH of the extract (for further data please see appendix section A1). The phenolic extract was also added at 1% w/v.

Trolox was used as a positive control, as it is a well characterised anti-oxidant (Cao et al., 1993). To concentration of Trolox used, was calculated using the highest dilution factor of the extract in the ORAC assay. This was approximately 8x, therefore the concentration used was 200 μM (as 25 μM of Trolox is used in the ORAC assay).

2.5.3 *UV Irradiation*

Prior to irradiation, well-plates were checked for cell attachment and cell culture media was removed. Cells were then washed with 1X PBS (phosphate buffered saline) (Fisher

Scientific, Loughborough, UK) and 1 ml of ice cold PBS was added to cells. Cells were then exposed to UVA irradiation from a 150 W xenon arc lamp (l-Oriel, Leicestershire, UK) with a filter centred on 360 nm for 15-20 minutes. The output of the lamp at this wavelength was 38.5 $mWcm^{-2}$ or 150 mW across the whole sample. Cells were then kept at 4 °C on ice for the duration of the exposure.

For a positive control a separate sample was washed with cold PBS (at approx. 4 °C) and then treated with 400 µM hydrogen peroxide for 5 minutes at 4 °C. After this, the cells were again washed with cold PBS to remove any residual hydrogen peroxide.

Cells used as an un-irradiated controls (or blank) were kept at 37 °C in the dark, to limit any UV damage.

2.5.4 ***Preparation of slides for the assay***

All cells regardless of treatment (or in the case of the control no treatment) were washed with cold PBS and all of the cells were gently scrapped off the tissue culture plate with a cell scrapper (Sarstedt, Leicester, UK) in to S-DMEM (supplemented dulbucco's eagles medium). Cells were then pelleted (by centrifugation at 1000 rpm for 3 minutes) and washed with cold (4 °C) PBS (phosphate buffered saline).

Finally, cells were resuspended in cold (4 °C) PBS with a final cell number of approx. $3x10^5$ cells/ml.

25 µl of this solution was added to 500 µl of 0.75% (w/v) low melting agarose and mixed; 50 µl of this mixture was dispensed onto each circular comet slide sample area (Trevigen, Maryland, USA) (a total of three areas per slide).

Each slide was then stored at 4 °C for at least 1 hr to allow the agarose to set and bind to the slide.

2.5.5 ***Comet assay***

After this period, the comet slides were immersed in Trevigen lysis buffer (Trevigen, Maryland, USA) at 4 °C for over 1 hr followed by a treatment with electrophoresis buffer (300 mM NaOH and 1 mM EDTA), for 20 minutes to allow DNA unwinding at room temperature. Slides were then removed from this buffer and put in fresh buffer in an electrophoresis tank (Fisher Scientific, Loughborough, UK) and subjected to electrophoresis (21 V, 300 mA) for 45 minutes at 4 °C in a dark refrigerated room (to prevent further DNA damage during electrophoresis).

After electrophoresis, the comet slides were removed from the buffer and were immersed in a Tris buffer pH 7.5 for 5 minutes. This was repeated at least 3 times to neutralise the slides and to remove any residual electrophoresis buffer.

Following this, the slides were treated with 70% ethanol and dried overnight.

The slides were then treated with 1X Sybr Gold (Invitrogen, UK) in Tris buffer at 4 °C for a minimum incubation of 10 minutes (to stain the slides). Slides were visualised with a fluorescence microscope (Zeiss Axio lab, (Cambridge, UK)) equipped with a zenon arc lamp, with excitation at 490 nm and emittance at 520 nm.

Photos of each slide sample area were then taken with a Canon Powershot G5 (Surrey, UK) and the images analysed using Cometscore image analysis software (version 1.5) (Tritek corp, Virginia, USA). The percentage of DNA in the tail was calculated by using equation 2.5.5-1 and is presented in figures 4.2-1 to 4.2-3.

$$\%\boldsymbol{DNA\ in\ tail} = \mathbf{100}\ \boldsymbol{x}\ (\boldsymbol{I_t}/\boldsymbol{I_C})$$

Equation 2.5.5-1: The equation used to calculate the % DNA in the tail, where I_t denotes the fluorescence intensity in the tail and I_c denotes fluorescence intensity in the comet as a whole.

For all of the comet assay results, the mean was calculated from the % DNA in the tail of at least 50 comets. This was replicated on a minimum of 2 independent comet slides with 3 replicate samples per slide. The mean of all the replicates is presented in the results together with the standard error of the mean. (This error model was chosen as only a representative sample of comets was scored in each slide and therefore the standard error of the mean gives us an estimation of error in the sample had all of the comets in the sample been counted).

To analyse a result's significance a two sample t-test, assuming unequal variances between two samples (e.g. between treated and untreated cells) was carried out. The difference was assumed to be significant if the P value of < 0.05.

2.6 Emulsion formulation

2.6.1 ***Preparation of prototype product formulations:***

To investigate how the extract will behave in a wide range of potential products, four separate formulations were prepared: a face cream, a face wash, a self tan gel and a self tan cream. All of the formulations used are representative of actual commercial formulations (from Boots formulation department). The formulations are summarised in the following tables. In addition, the formulations containing the extract was not established prior to use due to time constraints.

Table 2.6.1-1: A list of ingredients and their purposes used in the face cream formulation (an oil in water emulsion), together with the concentration of each ingredient in the formulation.

Ingredient (Chemical name where appropriate)	Concentration (%)	Purpose
C12-C15 Alcohols benzoate	6.0	Aromatic ester, a moisturiser and a solvent
Parsol MCX (2-ethylhexyl-3-(4-methoxyphenyl)-2-propenoate, or Octinoxate)	6.0	Sunscreen filter
Parsol 1789 (1-(4methoxyphenyl)-3-(4-tert-butylphenyl) propane-1,3-dione or Avobenzone)	3.0	Sunscreen filter
Octocrylene(2-ethylhexyl 2-cyano-3,3-diphenyl-2-propenoate)	1.0	Sunscreen filter
Syncrowax ELRC (C18-C36 Acid Glycol Ester)	1.5	Used for texture, emulsion stability
Shea Butter	0.5	Texture , moisturiser
Cetearyl Glucoside	2.0	Surfactant and emulsifier
Potassium cetyl phosphate	0.5	Surfactant - Emulsifying Agent
Cetyl alcohol (C16 alcohol)	0.5	Secondary emulsifier, builds viscosity
Acrylates/vinyl isodecanoate crosspolymer	0.1	Polymer
Dimethicone/Dimethiconol DC1503 (Dimethicone and Dimethiconol)	1.5	A very large silicone based gum used to get a smooth texture
EDTA (Ethylenediaminetetraacetic acid)	0.05	Ion chelator
Silicone antifoam dc 1500	0.025	Anti foaming agent
Glycerin BP	1.5	Film former, humectant
1,3-Butylene glycol	1.5	Humectant, can act as a solvent
Dry Flo PC (aluminum starch octenylsuccinate)	1.0	Polymer with oil absorbing properties
Caustic potash solution	0.04	pH modifier
Caprylyl glycol and ethylhexylglycerin	0.3	preservative
Phenoxetol nipa (phenoxyethanol)	0.4	Preservative
Sodium benzoate BP	0.1	Preservative
Aristoflex AVC (ammonium acryloyldimethyltaurate/vp copolymer)	1.0	Gelling agent/polymer
Yeast extract processed at 200 °C	5.0	
Purified water	66.5	

The face cream formulation was prepared in the following manner. To 65% of the water phase, EDTA and silicone antifoam was added, followed by acrylates crosspolymer and cetearyl glycoside. Mixing was conducted using a Silverson L4RT homogeniser (Silverson Massachusetts, USA).

To the oil phase: sycrowax, C12-C15 alcohols benzonate, cetyl alcohol, potassium cetyl phosphate, both parsol mcx and 1789 and octocrylene were weighed out into a separate vessel. Both phases were heated to 70-75 °C. Aristoflex was then added to the oil phase, followed by addition of the water phase to form an emulsion using a homogeniser. Following this 1,3 butylene glycol, dryflo, dimethicone, and caustic potash were added with homogenisation to the emulsion. The emulsion was then cooled to 30 °C, and the preservatives, sodium benzoate and the yeast extract were added. The initial viscosity was measured and recorded using a Brookfield DVI prime viscometer (Brookfield, Essex, UK), using spindle D, at 12 rpm, for 30 seconds with a helipath. The pH was measured with a Seven Easy Mettler Toledo pH meter (Mettler-Toledo, Leicester, UK).

Table 2.6.12: A list of ingredients used in the oil phase (and their purposes) of the self tan cream formulation, together with the concentration of each ingredient in the formulation.

Oil Phase	Ingredient (Chemical name where appropriate)	Concentration (%)	Purpose
Oil	Phenyl dimethicone	2	Skin-Conditioning Agent
Oil	Cithrol GMS 1862 (Glyceryl Stearate)	1.5	Emulsifier
Oil	Shea butter	1	For texture and as a moisturiser
Oil	C12-C15 alcohols benzoate	2.5	Aromatic ester, a moisturiser and a solvent
Oil	Cetyl alcohol (C16 alcohol)	2	Secondary emulsifier, builds viscosity
Oil	Dl-a-tocopheryl acetate	0.2	Used to supply vitamin E
Oil	Tween 20 (polyoxyethylene (20) sorbitan monolaurate or polysorbate 20)	0.3	Surfactant

Table 2.6.1-3: A list of ingredients used in (and their purposes) the self tan cream formulation, together with the concentration of each ingredient in the formulation.

Phase	Ingredient (Chemical name where appropriate)	Concentration (%)	Purpose
Water	Ethyl hydroxybenzoate	0.1	Preservative
Water	D-panthenol 75 L (stabilised panthenol)	0.3	Is a stabilised version of vitamin B5, it has a number of cosmetic benefits
Water	Glycerin BP	4.99	Film former
Water	EDTA (Ethylenediaminetetraacetic acid)	0.02	Ion chelator
Water	Methyl hydroxybenzoate bp	0.25	Preservative
Water	Polyquaternium – 37 (Poly(2-methacryloxyethyltrimethylammonium chloride)	1	A stable gelling agent /polymer
Water	Ethyl hydroxybenzoate	0.1	Preservative
Water	D-panthenol 75 L (stabilised panthenol)	0.3	Is a stabilised version of vitamin B5, it has a number of cosmetic benefits
	Dihydroxyacetone	3	Self tan active ingredient
	Erythrulose	1	Self tan active ingredient
	Dimethicone/dimethiconol dc1503	2	A very large silicone based gum used to get a smooth texture
	Phenoxetol nipa (phenoxyethanol)	0.6	preservative
	Yeast extract processed at 200 °C	5	
	Purified water	72.130	

Table 2.6.1-4: A list of ingredients used in (and their purposes) the self tan cream formulation (added after the emulsion had cooled), together with the concentration of each ingredient in the formulation.

Phase	Ingredient (Chemical name where appropriate)	Concentration (%)	Purpose
	Dihydroxyacetone	3	Self tan active ingredient
	Erythrulose	1	Self tan active ingredient
	Dimethicone/dimethiconol dc1503	2	A very large silicone based gum used to get a smooth texture
	Phenoxetol nipa (phenoxyethanol)	0.6	preservative
	Yeast extract processed at 200 °C	5	
	Purified water	72.130	

To prepare the self tan cream formulation the water phase was prepared adding all of the ingredients marked water phase in table 2.5.1-3 to a vessel and heating them to between 70-75 °C. The oil phase was prepared in a similar fashion adding all of the ingredients in Table 2.6.1-3 to a separate vessel and heating them to between 70-75 °C. Polyquarternium-37 was added to the water phase with homogenisation using a Silverson L4RT homogeniser (Silverson Massachusetts, USA), followed by addition of the oil phase to form an emulsion. Dimethicone was then added to the emulsion with homogenisation and it allowed to cool to 30 °C. The preservatives and yeast extract were then added together with dihydroxyactone and erythrulose dissolved in water. The initial viscosity was measured and recorded using a Brookfield DVI prime viscometer (Brookfield, Essex, UK)

using spindle B, at 10 rpm, for 30 seconds with a helipath. The pH was measured with a Seven Easy Mettler Toledo pH meter (Mettler-Toledo, Leicester, UK).

Table 2.6.1-5: A list of ingredients and their purposes used in the self tan gel formulation, together with the concentration of each ingredient in the formulation in percent

Ingredient (Chemical name where appropriate)	Concentration (%)	Purpose
Dihydroxyacetone	4.5	Self tan active ingredient
Erythrulose	1.5	Self tan active ingredient
Glycerin BP	5	Film former
Polyquaternium – 37 (Poly(2-methacryloxyethyltrimethylammonium chloride))	1.3	Polymer/gelling agent
Lactic acid BP	0.05	pH modifier
Denatured ethanol b gde 100%	20	
Yeast extract processed at 200 °C	5	
Purified water bp	67.65	

To formulate the self tan gel, Dihydroxyacetone and erthrulose were dissolved in water and added to glycerine, lactic acid and the ethanol. Polyquarterium-37 was then added and mixed with homogenisation and stirring (using a Silverson L4RT homogeniser (Silverson Massachusetts, USA), until the subsequent gel was lump free and transparent. The extract was then added and mixed into the formulation. The initial viscosity was measured and recorded using a Brookfield LVDI+ viscometer (Brookfield, Essex, UK), using spindle 4, at 12 rpm for seconds. The pH was measured with a Seven Easy Mettler Toledo pH meter (Mettler-Toledo, Leicester, UK).

Table 2.6.1-6: A list of ingredients and their purposes used in the face wash formulation, together with the concentration of each ingredient in the formulation in percent.

Ingredient (Chemical name where appropriate)	Concentration (%)	Purpose
Peg-120 methyl glucose dioleate	0.3	Moisturiser, emulsifying surfactant
Glycerin BP	10	Film former
Dilute sle-2-s (sodium lauryl ether sulphate)	25	Surfactant
Phenoxetol nipa (Phenoxyethanol)	0.6	Preservative
EDTA (Ethylenediaminetetraacetic acid)	0.05	Ion chelator
Sorbistat-k Pfizer (Potassium Sorbate)	0.15	Preservative
Cocamidopropyl betaine 50%	13	Surfactant, thickner
Yeast extract processed at 200 °C	5	
Purified water bp	50.5	

To formulate the face wash, 50% of the total water in the face wash, was added to EDTA, sorbistat-k and PEG 120 methyl glucose dioleate (PEG 120). This mixture was heated to 40 °C to dissolve the PEG 120. The rest of the water was then added, followed by the glycerine and the SLE-2-S. The face wash was cooled to below 30°C and cocomidopropyl betaine and phenoxetol was added as a preservative, followed by addition of the extract into the formulation. The initial viscosity was measured and recorded using a Brookfield LVDI+ viscometer (Brookfield, Essex, UK) using spindle 4, at 10 rpm, for 30 seconds. The pH was measured with a Seven Easy Mettler Toledo pH meter (Mettler-Toledo, Leicester, UK).

For all of the control formulations; yeast extract was removed and the amount of water in the formulations was increased by 5% to compensate.

2.6.2 *Stability testing and microscopy*

Stability testing was carried as described in the standard Boots stability testing protocol. This involved, preparing 5 kg of each formulation, with and without yeast extract, which were prepared and divided up into three separate batches (with three replicates for every temperature). One batch was stored at 4 °C, one at room temperature (approx. 23 °C) and one batch at 40 °C. The samples stored at 4 °C, would be used as a baseline, as no significant changes are expected in these samples. The viscosity and pH of every sample (using a Brookfield LVDI+ and DVI prime viscometer (using the spindle, speed and time settings mentioned in section 2.5.1 for each formulation) and Seven Easy Mettler Toledo pH meter) was measured weekly for the first 6 weeks of the study, followed by measurements taken at 8, 12, and 16 weeks.

For consistency samples that were stored at 4 °C and at 40 °C were allowed to equilibrate at room temperature (approx. 23 °C) prior to viscosity and pH measurement, as all measurement were carried out at room temperature (approx. 23 °C). This allowed for samples to be easily compared and to eliminate any temporary temperature effects.

Due to geometry of the spindles used to measure the viscosity of the self tan cream and face cream a shear rate could not be defined (Brookfield, 2005). These spindles were used as both emulsion were shear thinning and were recommended in the production protocol of both of the formulations. The viscosity of the self tan gel and face wash was however measured at constant shear rate of 2.508 s^{-1} and 2.09 s^{-1} respectively (Brookfield, 2005). This is a departure from standard procedure where a defined shear rate is known due to the instrumentation used.

Digital images of the emulsion formulations (that includes the face cream and the self tan face cream) were taken using a Olympus BX 40 microscope (Olympus, Leicester, UK), and Motic 2300 camera (Motic Europe, Barcelona Spain) at 400x at the same time as the viscosity measurements. Micrographs of the self tan gel and face wash were not taken due to lack of any observable structural details and contrast in the images.

For consistency, all of the samples were equilibrated to room temperature prior to measurement.

2.6.3 *Colour testing*

For the colour testing study, a sample of a formulation was spread using film applicator (Elcometer, Manchester, UK) at a thickness of 200 μm on a Leneta 5DX brush out card. The card was then used to measure the colour of the formulation using a colour i7 spectrophotometer (Xrite, Cheshire, UK) equipped with colour iMatch software. To quantify the colour change the Δ(d)E2000 scale was used as it more closely accounts for human perception of colour (Ohta and Robertson, 2005) (see chapter 5.2) .

The colour of every formulation at the three storage temperatures was measured at the same time (and at the same time intervals) as the pH and viscosity. A minimum of two independent replicate measurements were carried out for every sample measured, in addition to the spectrophotometer reporting a mean of three measurements.

2.6.4 *Yeast extract preparation*

The yeast extract was prepared as documented previously (see section 2.1). The extract processed at 200 °C was chosen for formulation due to it being the lowest temperature where high anti-oxidant activity was observed (see section 3.2).

CHAPTER 3 : Process Optimisation using Anti-oxidant Activity Assays.

3.1 Introduction

Previous characterisation work (Bahari, 2010) (and unpublished data) has shown that cider yeast extract contains a variety of potential and established anti-oxidants such as chlorogenic acid (Fraga et al., 1987). These anti-oxidants could be used to prevent damaged caused to cells by environmental and oxidative stress (Collins, 2005) (as has been previously discussed in section 1.5) and an extract containing a high concentration of these anti-oxidants would be commercially valuable. Other groups have demonstrated that subcritical water can be used to extract anti-oxidant compounds from a variety of feedstocks such as rosemary and pomegranate (Ibanez et al., 2003) (He et al., 2012). Reis et al., have also shown that by optimising the temperature of extraction (from apple pomace) an extract with high anti-oxidant capacity can be produced (Reis et al., 2012). Therefore, the aim of the work presented in this chapter was to investigate this potential anti-oxidant activity and then to understand what affect the process conditions (such as the process temperature) had on this activity of the extract. This information could then be used to help refine the process conditions to produce a more effective extract in terms of anti-oxidant activity.

3.2 Results

3.2.1 *Anti-oxidant results*

The ORAC and TEAC assays have been used extensively by other researchers (Re et al., 1999) to assess the anti-oxidant activity of a range of substances (Huang et al., 2002, Prior et al., 2005).

To investigate the potential anti-oxidant activity of the extract, ORAC assay was chosen. Initial results from the assay were positive and showed that the extract did have anti-oxidant activity with concentrations lower than 1% (v/v) (of the pure extract) diluted in water (preliminary work). To investigate how process temperature could affect anti-oxidant activity, a range of extracts processed from 100 °C to 225 °C were analysed using the ORAC assay and the results are shown in Figure 3.2-1. The results show that ORAC values for extracts processed from 100 °C to 175 °C are relatively consistent and do not show any significant difference ($P<0.05$). This suggests that there is little impact in increasing process temperature from 100 °C to 175 °C. Increasing processing temperature between 200 and 225 °C, resulted in a two fold increase in ORAC value when compared to the 100 °C sample (which is potentially due to an increase in phenolic content seen in figure 3.2-8 and further discussed in section 3.3). This is in contrast to the drop in chlorogenic acid concentration shown in figure 3.2-26 and the breakdown in phenolic content shown in the literature between 175 °C and 200 °C (Wijngaard et al., 2012).

The relative ORAC value for vitamin C (0.64) (which was used as a reference point after consultation with my industrial sponsor), is significantly closer to that of the extracts processed at 200 °C and 225 °C than at lower temperatures, illustrating a considerable improvement in ORAC value.

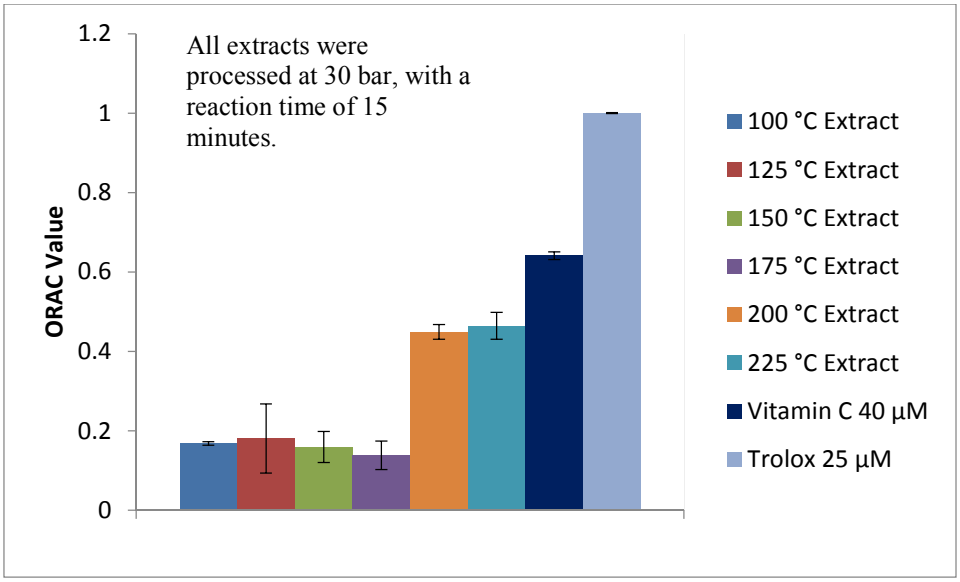

Figure 3.2-1: The relative ORAC values for the all cider yeast extracts, with hydrolysis temperature ranging from 100 °C to 225 °C, with the ORAC value of 40 µM Vitamin C is used as a standard.

Figure 3.2-2 and 3.2-3 shows the effect of reaction time on the anti-oxidant activity of the extract at 225 °C and 300 °C. A longer reaction time particularly after 6 minutes had a detrimental effect on the anti-oxidant activity of the extract. The ORAC value at 8 minutes appears to be anomalous as the ORAC value at 10 minutes is significantly lower and this result is contrary to the overall downward trend. However prior to 6 minutes there appears to be no significant difference in anti-oxidant activity and the ORAC values correspond well with the sample processed at 225 °C. In contrast, the results presented in Figure 3.2-3 show that reaction time at 225 °C does not have any significant effect on extract activity at any reaction time studied. In addition, the extracts produced in tube reactor do not significantly vary with respect to extract anti-oxidant activity. This is also evident for the extract processed at 200 °C.

The results illustrated in Figure 3.2-3 shows that process pressure does not significantly impact ($p<0.05$) the anti-oxidant activity of the 225 °C extract in any measurable way;

despite there being a 70 bar difference between the kinetic extract (see section 2.1.2) and the extract produced in the 300 ml reactor.

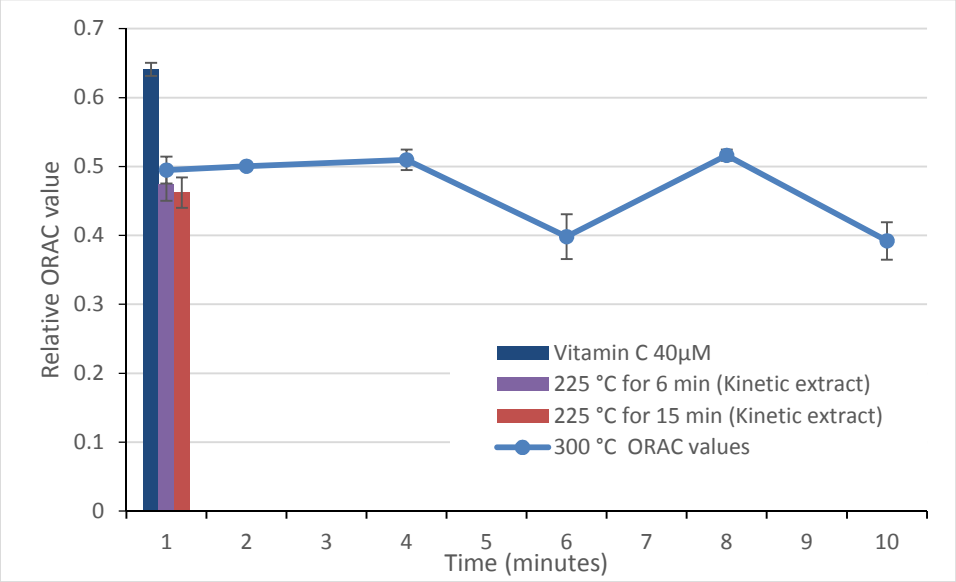

Figure 3.2-2: Effect of reaction time on the anti-oxidant activity of cider yeast extract produced at 300 °C 100 bar, with a sample processed at 225 °C and Vitamin C included for reference.

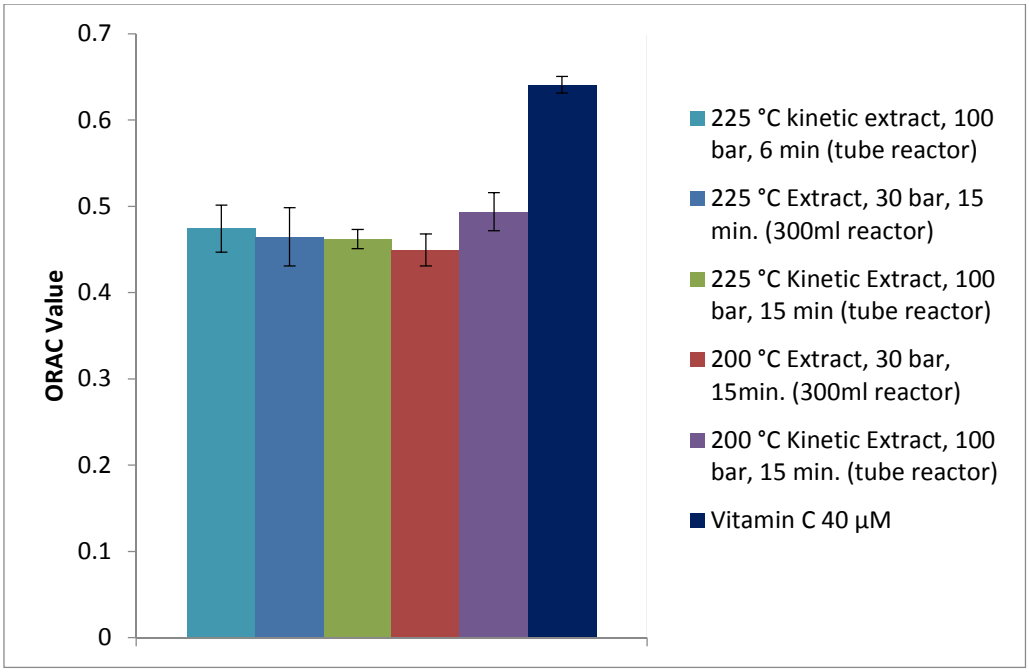

Figure 3.2-3: The influence of reaction time and pressure on the relative ORAC values of the extracts.

To corroborate the results from ORAC assay another anti-oxidant assay was chosen with a different mechanism of action.

The results from extracts analysed with the TEAC assay are shown in Figure 3.2-4, they demonstrate that all of the extracts (at the various process temperatures) have activity via two different mechanisms. In common with the ORAC assay, there is no significant difference in anti-oxidant activity with extracts produced between 125 °C, and 175 °C. In contrast to the results from the ORAC assay, there is only a small increase in anti-oxidant activity at 200 °C, with a much larger increase in activity observed at 225 °C. This probably due to the way that ET (electron transfer) assays (e.g. the TEAC assay) and HAT (hydrogen atom transfer) based assays (e.g. the ORAC assay) measure anti-oxidant capacity.

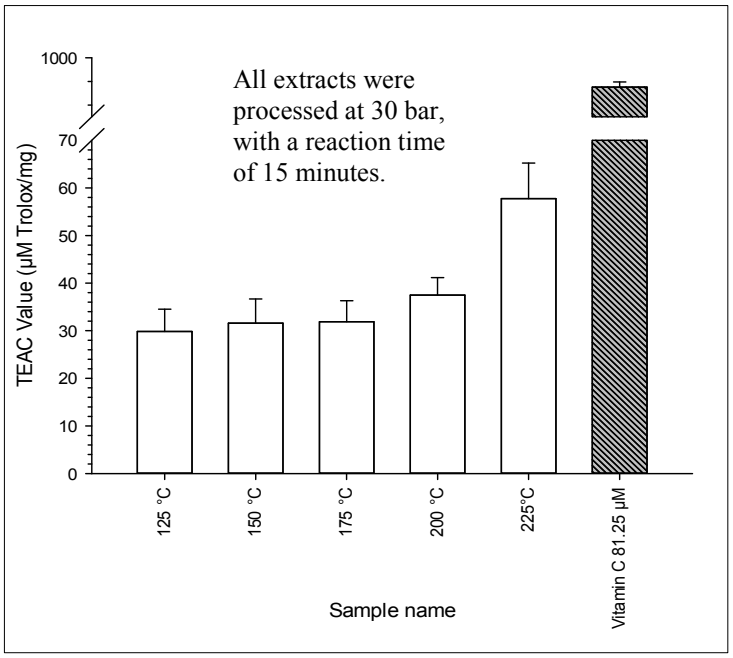

Figure 3.2-4: The effect of temperature on the TEAC value of yeast extracts hydrolysed from 125 °C to 225 °C, are presented in the figure. All results shown are a mean of n≥3 samples with ± standard deviation.

To examine extract stability over a sustained period of time, extracts were kept at 37 °C and the anti-oxidant activity of extracts was assessed every week. The results presented in Figure 3.2-5, illustrate that in all of the samples there is no significant decrease in ORAC value ($P<0.05$) in any of the samples investigated. The ORAC value of extract processed at 200 °C does fluctuate (in ORAC value) during the study, however overall there is no significant difference between the initial (at 0 weeks) and the final (at 4 weeks) ORAC values. With the samples processed at 175 °C and at 225 °C there is a small increase in anti-oxidant activity observed during the period. Only with the sample processed at 150 °C is there a small but insignificant ($P>0.05$) reduction in ORAC value, illustrating that extracts processed between 175 °C and 225 °C are relatively stable for a sustained period of time. This contrasts the results presented in Figure 3.2-6, which shows the degradation of anti-oxidant activity of chlorogenic acid (one of the main anti-oxidants in the extract). The activity of chlorogenic acid over has (at 37 °C) halved over the four week period with

respect to relative ORAC value. The results therefore suggest that the anti-oxidant activity observed in the extract is not heavily dependent on the activity of chlorogenic acid alone.

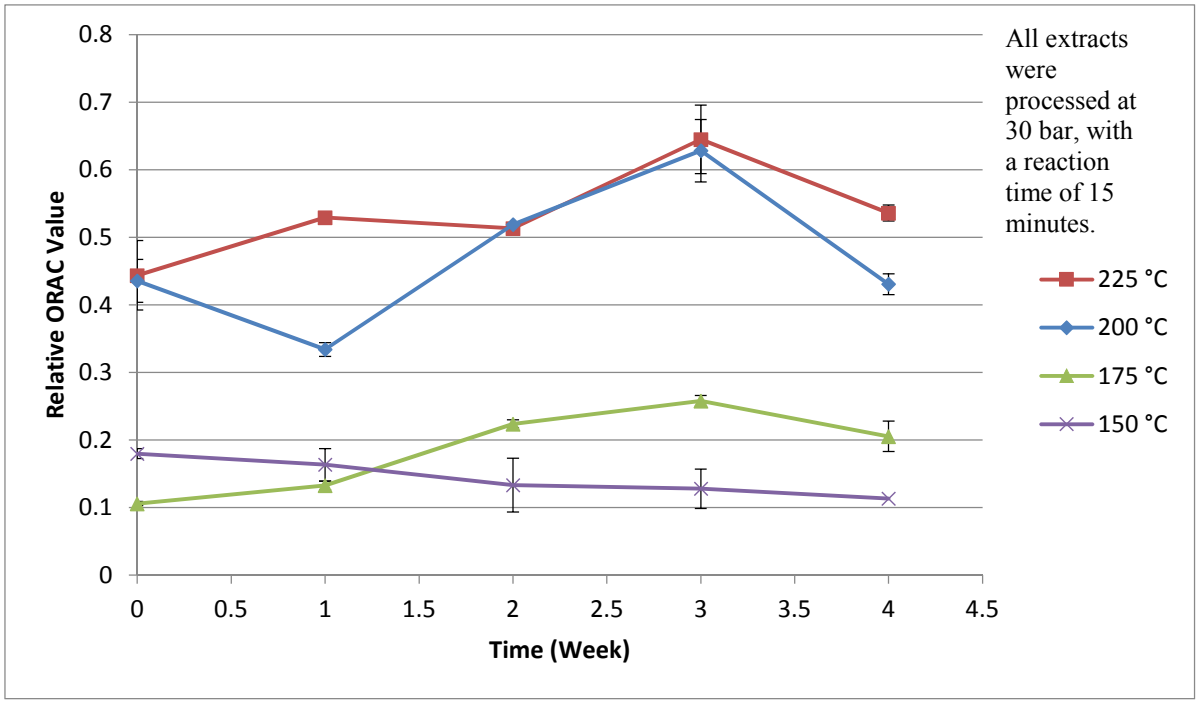

Figure 3.2-5: Results from the extract stability study tracking the extracts relative ORAC value over a period of 4 weeks at 37 °C. With process temperatures ranging from 150 °C to 225 °C.

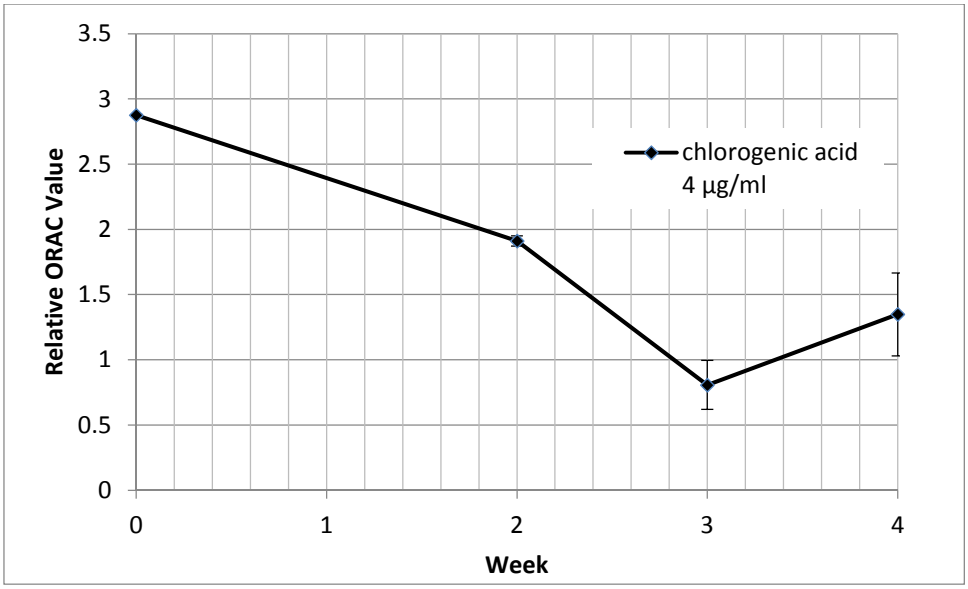

Figure 3.2-6: The change in anti-oxidant activity of Chlorogenic acid with time at 37 °C. All the relative ORAC values are expressed in Trolox equivalents.

The next stage in the development of the cider yeast extract was to show that the anti-oxidant activity could be preserved while the extracts production was scaled up. In this instance, the volume produced was increased from 200 ml (in the initial batch reactor) to 3 L (in the 5 L reactor). To investigate the batch-to-batch variability, 15 independent batches were produced and the anti-oxidant activity quantified with the ORAC assay. The results presented in Figure 3.2-7, show that the majority of the anti-oxidant activity has been retained.

However there is a significant reduction in activity with batches 4 to 8, (excluding batch 6), including batches 14 and 15 when compared to batch I; which was prepared in the 300 ml batch reactor. With batches 4 to 8, this reduction correlates to an increase in extract dry weight. However, there is no significant difference with batches 9-12 and batch I, suggesting that some batches did have equivalent activity to previous extracts. Overall, the figure illustrates that with the majority of samples the scale up process has not harmed the activity of the extract. However the variance in the batches produced in the 5 L reactor is

significant; which is confirmed by single factor ANOVA analysis showing there is a significant difference between all the batches produced in 5 L reactor ($p<0.005$).

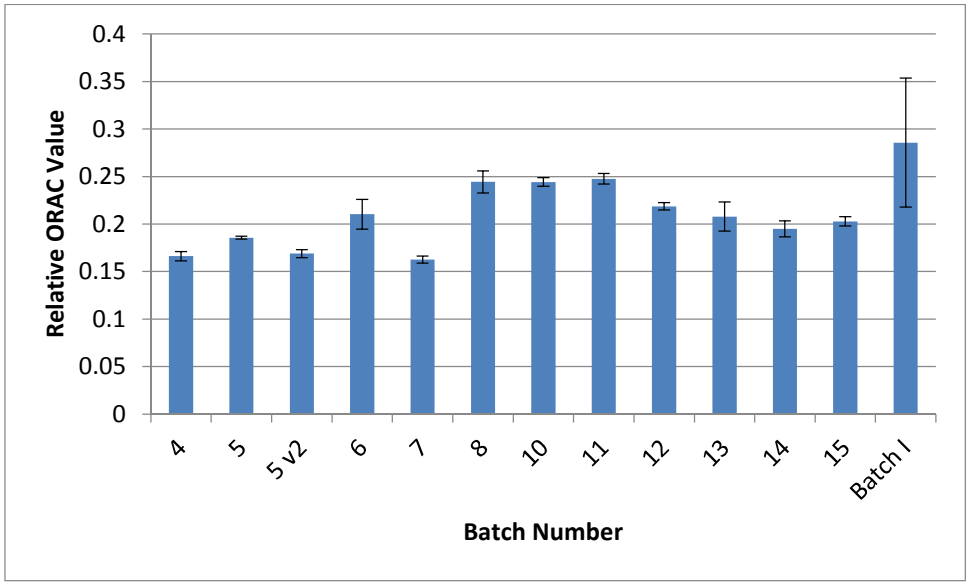

Figure 3.2-7:The relative ORAC values for a number of batches of cider yeast extract produced in the 5 L reactor at 200 °C, 100 bar with reaction time of 10 minutes (the batches were produced by my colleague Fabio Antas). Batch I is an extract in the smaller 300 ml initial reactor produced at the same temperature. The batch labelled 5v2 is a replicate of batch 5 after there were mixing issues with this batch. All results shown are means of n>3 samples.

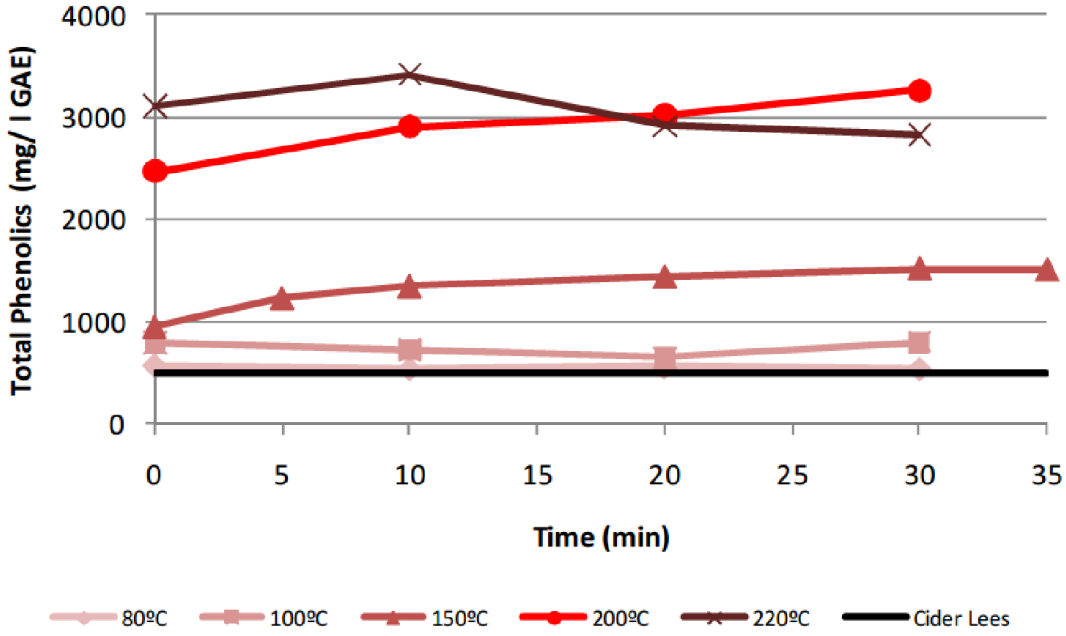

Figure 3.2-8: The change in total phenolic content using the Folin–Ciocalteu assay with process temperature and reaction time; mg/l GAE refers to milligrams per litre of gallic acid equivalents [ref: Antas, 2011 first year report].

3.2.2 *Extract Characterisation*

Results earlier in this chapter (section 3.2.1) have shown that the yeast extract has anti-oxidant activity and that this increases with temperature particularly at 200 °C /225 °C. To investigate this rise in anti-oxidant activity; the extracts were analysed using HPLC.

The HPLC traces for the samples from temperature 150-225 °C; are shown in figures 3.2-9 to 3.2-12.

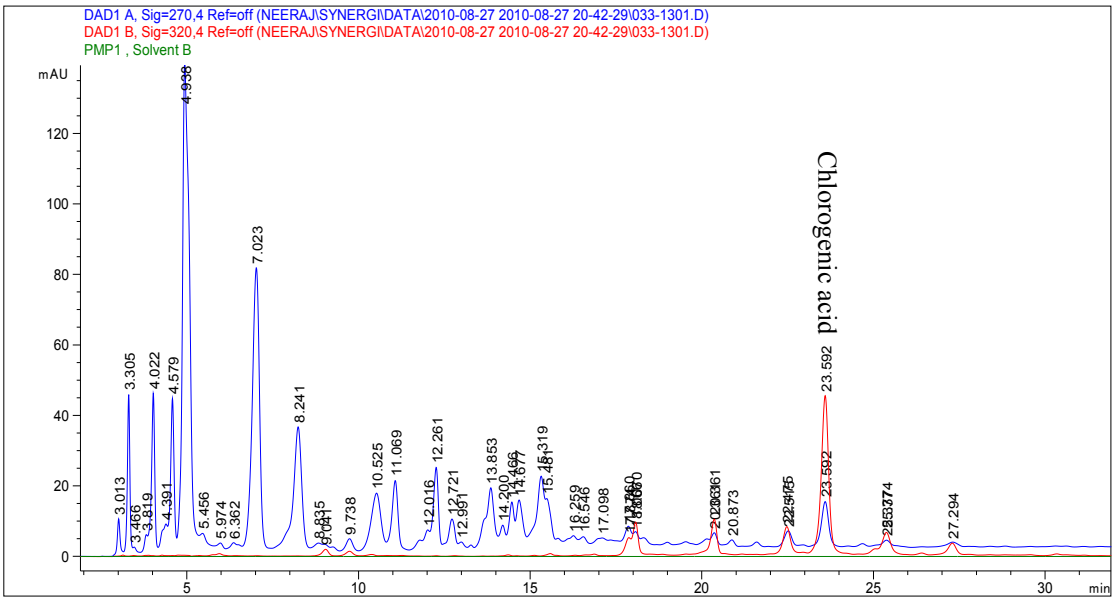

Figure 3.2-9: The HPLC trace of the extract processed at 150 °C, 30 bar with a 15 minute reaction time. The blue trace 270 nm while the red trace is at 320 nm.

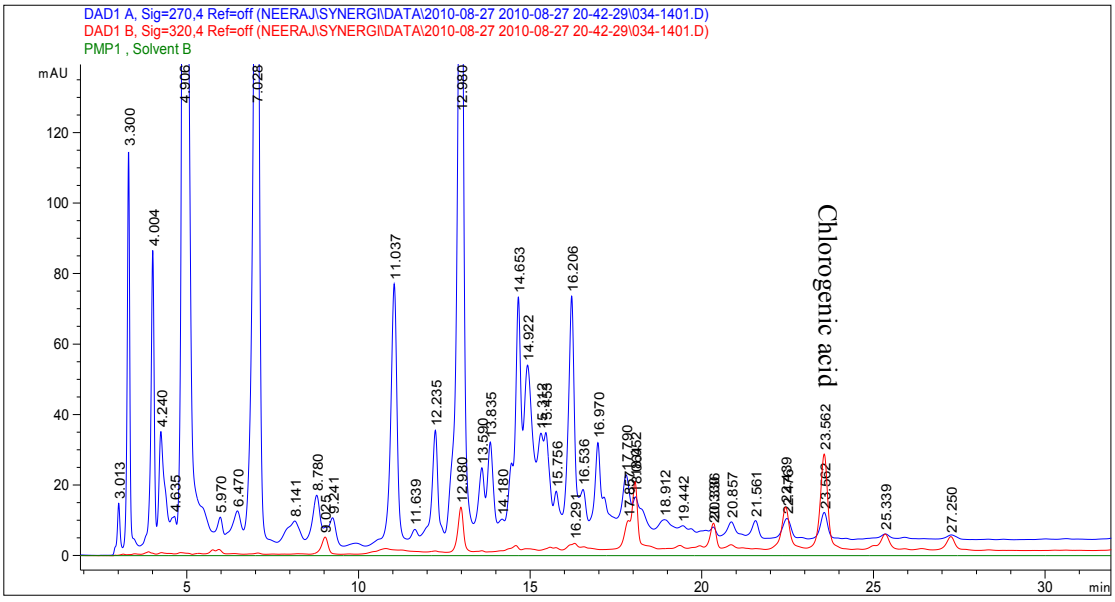

Figure 3.2-10: The HPLC trace of the extract processed at 175 °C, 30 bar with a 15 minute reaction time. The blue trace 270 nm, while the red trace is at 320 nm.

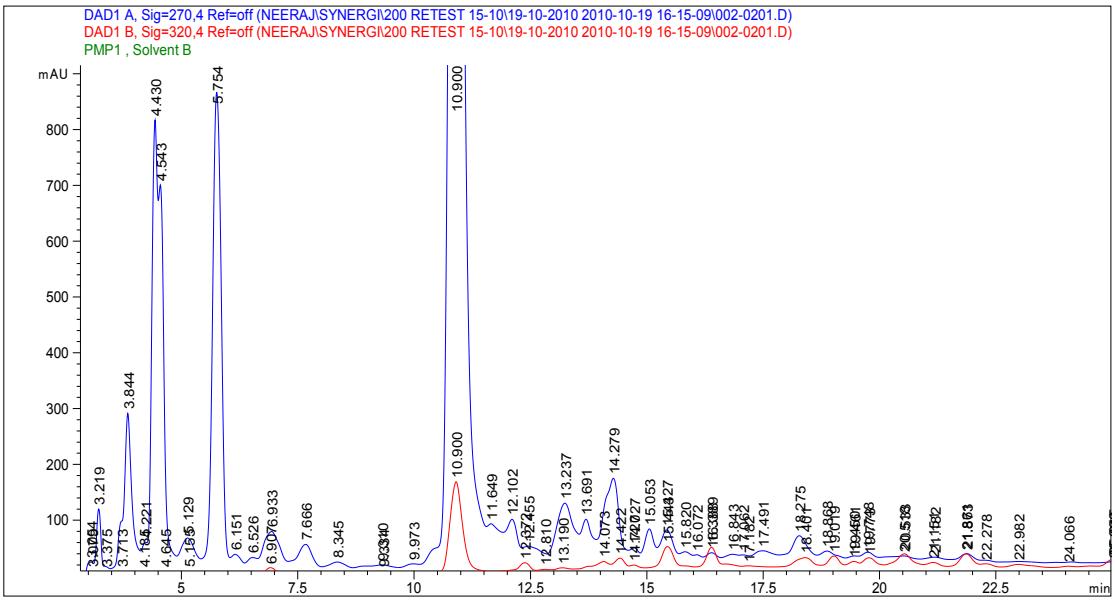

Figure 3.2-11: The HPLC trace of the extract processed at 200 °C, 100 bar with a 15 minute reaction time. The blue trace 270 nm, while the red trace is at 320 nm.

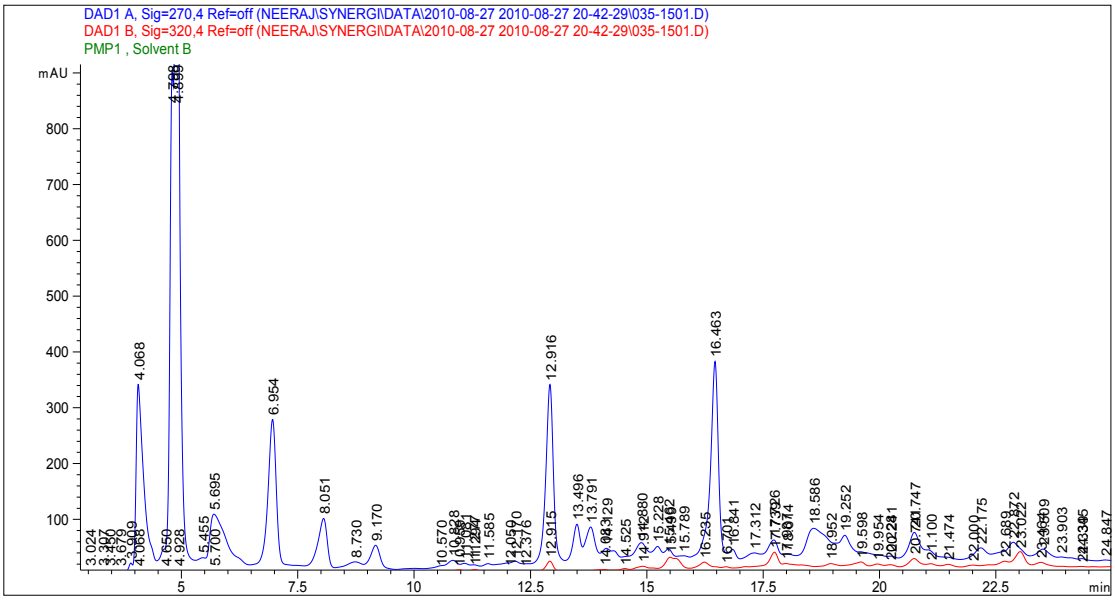

Figure 3.2-12: The HPLC trace of the extract processed at 225 °C, 100 bar with a 15 minute reaction time. The blue trace 270 nm, while the red trace is at 320 nm

The traces for the 150 °C and 175 °C extracts (figure 3.2-9 and figure 3.2-12 respectively) detail that chlorogenic acid appears to be present in the extract as the peak at 23.6 minutes matches the same retention time of pure chlorogenic acid. Further mass spectroscopy data

would be required to confirm this. This peak is not present in the 200 °C and 225 °C extracts (figure 3.2-11 and figure 3.2-12). Comparing the 175 °C and 200 °C extract traces, there are several major difference that become apparent. First of which is a major peak in the 200 °C extract at retention time of 10.9 minutes that is potentially present in the 175 °C extract (at 11.0 minutes), but at much lower absorbance. The second major difference is that overall the peaks in the 200 °C extract have much larger absorbance; twice the absorbance in the 4 to 6 minute and the 13 to 17 minute regions at 270 nm. This indicates that the concentration of substances (in the 200 °C extract) have increased, this correlates with an increase in dry weight from 150 to 200 °C (Bahari, 2010). Therefore, it appears that the efficacy of the extraction from the yeast has improved.

To further investigate the major peak (at a retention time of 10.9 mins) that appeared in the 200 °C extract, a number of compounds were analysed using the HPLC to help identify the peak. The two compounds that had the closest retention time were pyrocatechol and hydroxymethylfurfural (HMF). The spectrums of each compound including the spectrums of the peak (in question) are shown in figure 3.2-13. Comparing the spectra it appears to that the major peak in 200 °C extract could be HMF as it and the 200 °C extract peak have a shoulder at 320 nm which pyrocatechol lacks. This was corroborated by comparing the 200 °C extract spiked with HMF to that without HMF, which is illustrated in figure 3.2-14. However, based on the HPLC data alone a definitive conclusion cannot be made and further mass spectroscopy data of the peak would be required to confirm the existence of HMF.

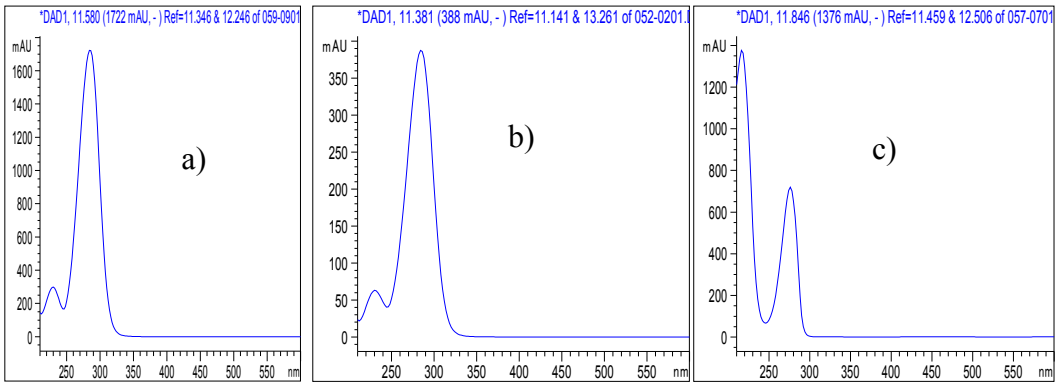

Figure 3.2-13: A) The major peak from the 200 °C extract's HPLC trace taken at a retention time of 10.9 minutes, b) The UV spectrum of HMF with a retention time of 11.1 min, C) is the UV spectrum of pyrocatechol with a retention time of 11.4 minutes.

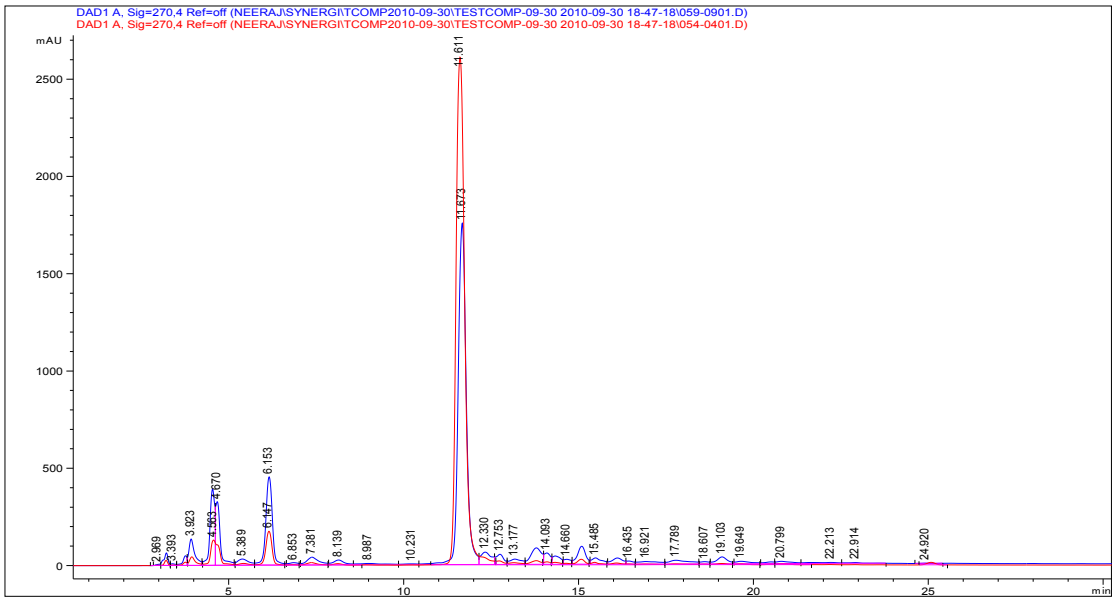

Figure 3.2-14: The HPLC trace of the 200 °C extract spiked with pure HMF dissolved in water.

Comparing the 200 °C extract to the 225 °C extract (Figure 3.2-11 and Figure 3.2-12), the major peak at approx. 11 minutes (the HMF peak) disappears and instead a peak at 4.9 minutes appears, this could a breakdown product of HMF. However, this does not

negatively impact the ORAC or TEAC values and in turn the anti-oxidant activity of the 225 °C extract.

Investigating the role of HMF in the anti-oxidant activity of the extract, results show that it can play a role in the anti-oxidant activity of the extract, despite HMF itself being a weak anti-oxidant (with an ORAC value of < 0.01) (see figure 3.4-16). The mixture of 0.9 μg/ml chlorogenic acid and 0.75 mg/ml HMF has significantly higher AUC (area under the curve) than pure HMF at the same concentration ($p<0.05$) (see figure 3.4-16). In this simple experiment, this mixture outperforms the same concentration (0.9375 μg/ml) of chlorogenic acid. Demonstrating that HMF can act synergistically together with other anti-oxidants present in the mixture to improve the overall ORAC value. Figure 3.2.15 details there is over 1 mg/ml of HMF in the 200 °C extract at a reaction time of 15 minutes. Therefore HMF should contribute some anti-oxidant activity, due to a large amount of HMF in the extract (see Figure 3.2-14 and Figure 3.2-15).

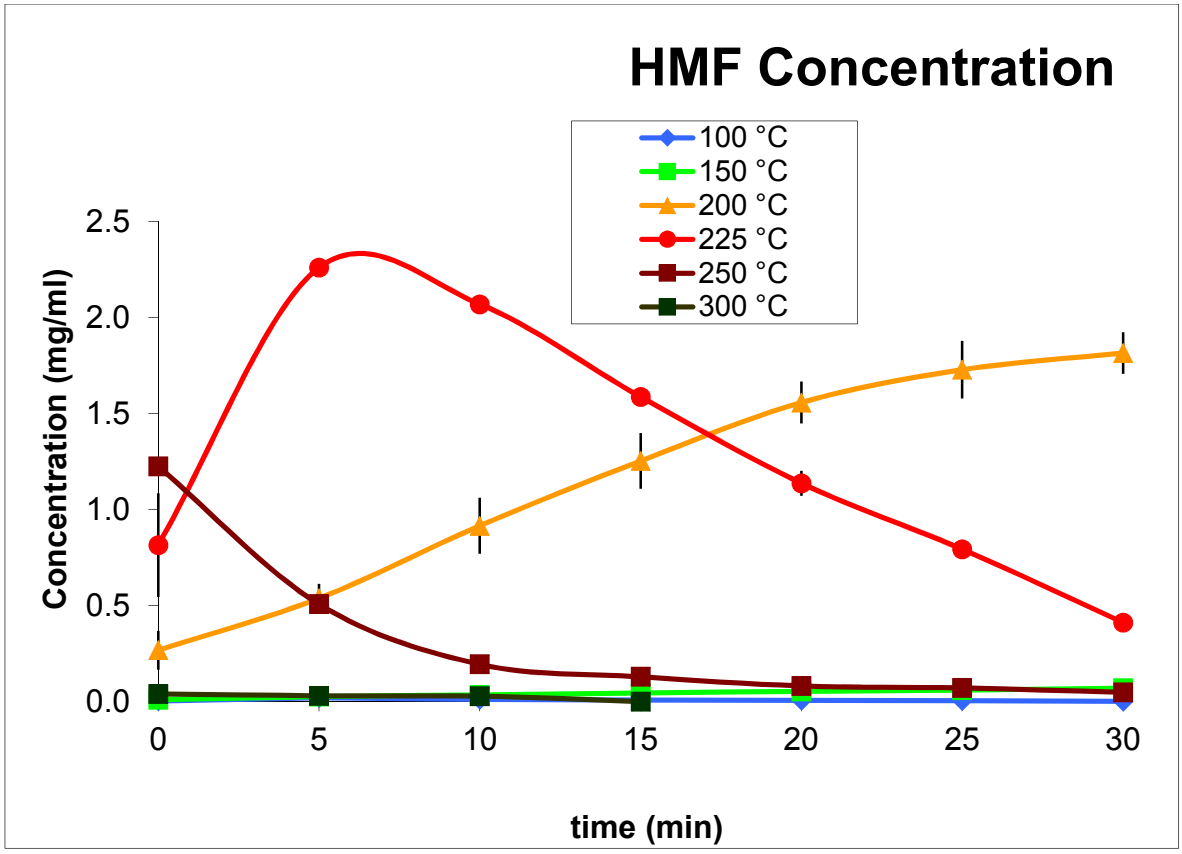

Figure 3.2-15: Showing the change in HMF concentration with process temperature and reaction time. (Figure is courtesy of Dr Alireza Bahari)

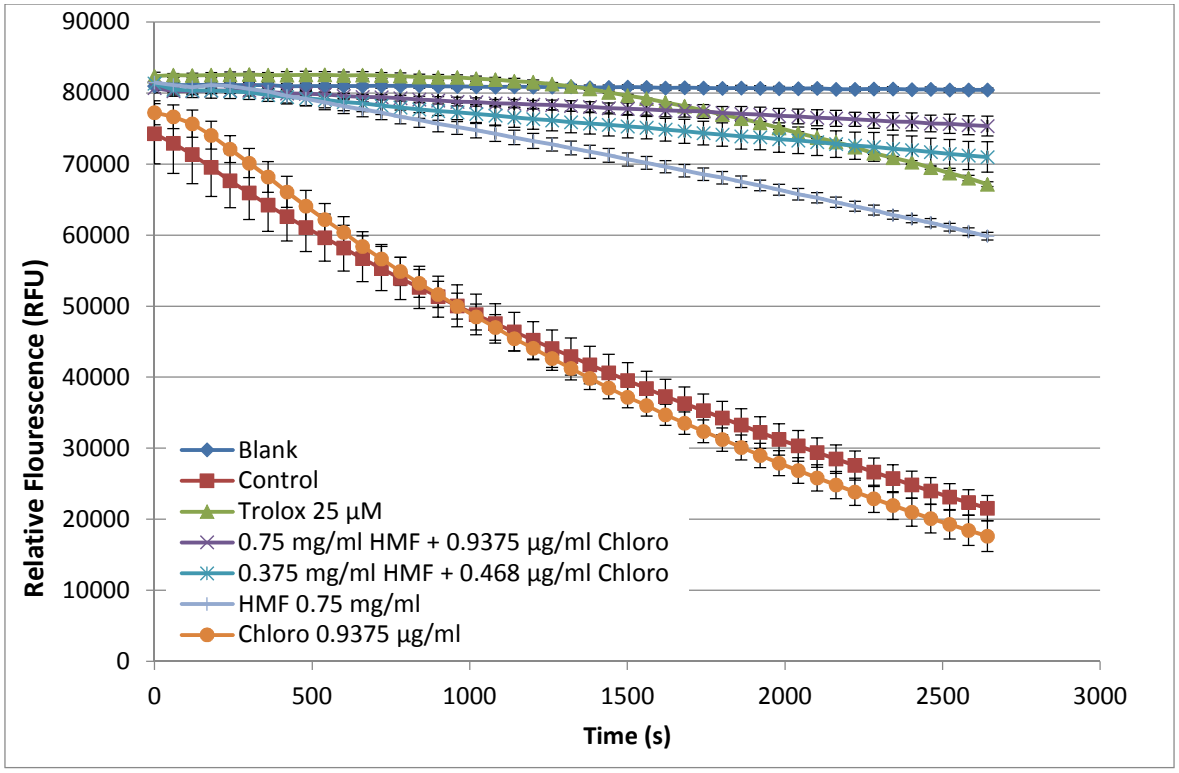

Figure 3.2-16: The ORAC fluorescence decay curve, for samples containing either HMF or a mixture of chlorogenic acid and HMF. For clarity, the blank sample is native fluorescein, while the control sample is fluorescein with AAPH but no anti-oxidant.

To further investigate the role of HMF and that of phenolic compounds in contributing to the anti-oxidant activity of the extract, fractions from the HPLC were collected as described in section 2.3.3, and tested for anti-oxidant activity using the ORAC assay. The results are demonstrated in table 3.2-1. The results illustrate that compounds in fraction 3 (derived from retention time 18-26 mins (see section 2.3.3) where many phenolic compounds have been found to elute); have a much greater role in the anti-oxidant activity of the extract than HMF and other compounds in fraction 2 (thought to contain HMF amongst other compounds) due to the difference in ORAC values. The values indicate that compounds in fraction 3 have a much greater anti-oxidant activity per gram of dry weight than compounds in fraction 2. This is thought to be due to the phenolic compounds that

elute in this region (Bahari, 2010), that could be present in this fraction. The difference in ORAC values between the 200 or 225 °C extracts and fraction 3 show that a focus on the phenolic compounds in a refined extract could dramatically improve the ORAC value and anti-oxidant activity of any refined extract.

Table 3.2.2-1: Comparing the ORAC values of two HPLC fractions taken from the 200 °C extract (see section 2.3.8.1 for the method), alongside native 200 °C and 225 °C extracts.

Sample Name	Relative ORAC Value (± standard deviation)
200 °C Extract	0.449 ± 0.019
225 °C Extract	0.465 ± 0.034
HPLC Fraction 2, retention time: 10-15 min. (fraction thought to contain HMF)	0.375 ± 0.032
HPLC Fraction 3: retention time: 18-26 min. (fraction thought to contain phenolic compounds)	3.351 ± 0.295

To identify which phenolic compounds could be present in the entire extract, the anti-oxidant activity of chlorogenic acid and proanthocyanidin (which has been found in apples and is one of the major anti-oxidants in apples and apple extracts (Guyot et al., 2003)) were assessed, after being processed identically to the 200 °C extract.

The chlorogenic acid results are presented in Figure 3.2-17, they illustrate that despite a large reduction in chlorogenic acid concentration in the 200 °C extract with reaction time, (of almost 70% at 15 minutes) (shown in Figure 3.2-18); the anti-oxidant activity is retained. The results show there is no significant difference between either the processed or unprocessed samples ($P<0.05$). In fact, there is an insignificant increase in anti-oxidant activity.

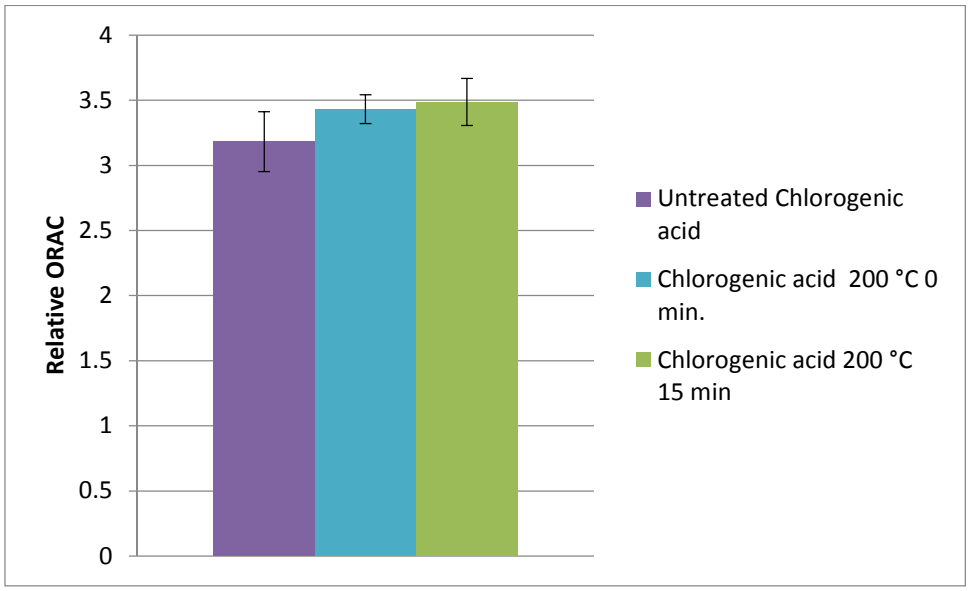

Figure 3.2-17: The change in ORAC value of processed (or untreated) chlorogenic acid with reaction time at 200 °C.

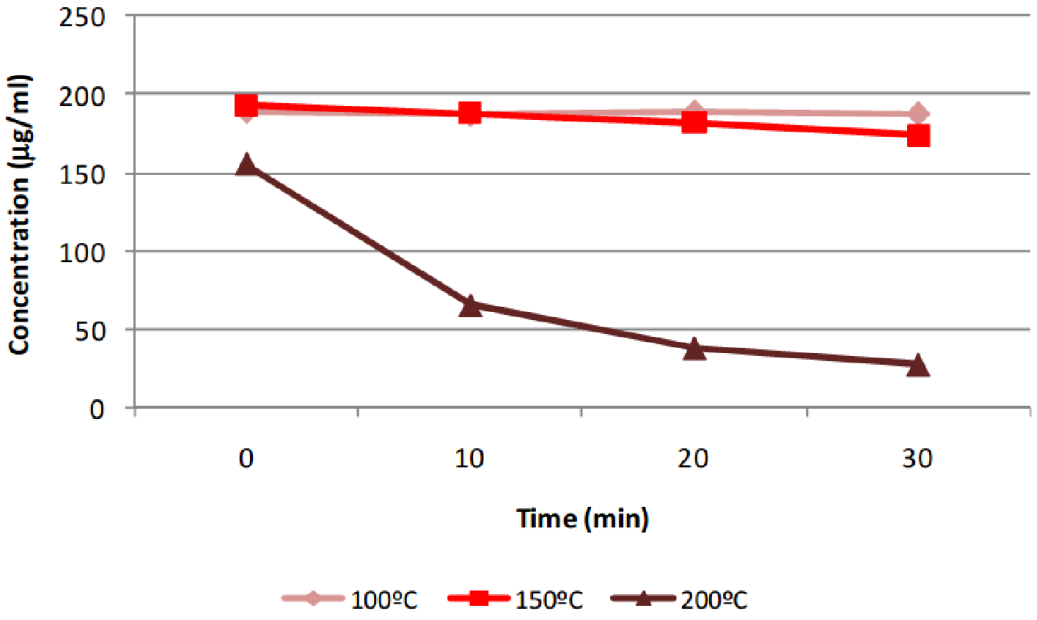

Figure 3.2-18: The change in chlorogenic acid concentration in the extract with temperature and reaction time. (Figure is courtesy of my colleague Mr Fabio Antas).

Therefore, reaction time does not seem to impact the anti-oxidant activity of chlorogenic acid. Analysing this further using the HPLC, reinforces previous work in showing that there is very little native chlorogenic acid at a retention time of 23 minutes (figure 3.2-19 for reference, and figure 3.2-20). Most of the compounds in the processed chlorogenic acid have an earlier retention time, indicating that most of these compounds are more hydrophilic in nature than chlorogenic acid, due to the water and ethanol mix at this retention time. Figure 3.2-20 details that some of these compounds could potentially exist in the 200 °C extract; as peaks from both the processed chlorogenic acid and the 200 °C extract overlap, indicating that they could be the same compound.

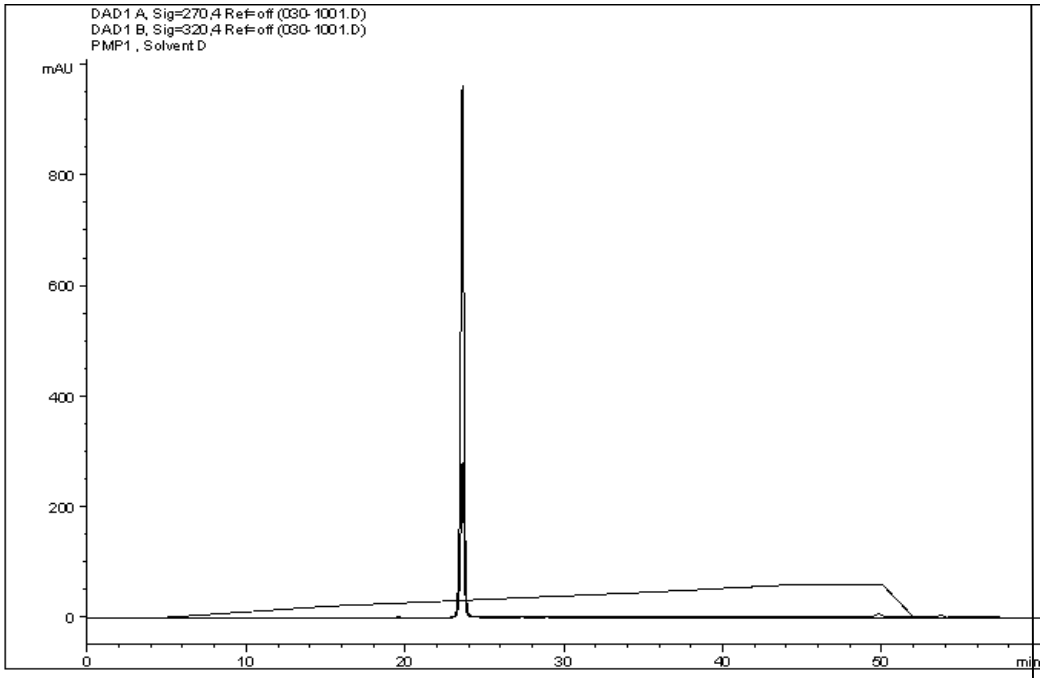

Figure 3.2-19: The HPLC trace of pure, untreated chlorogenic acid at 270 nm and 320 nm.

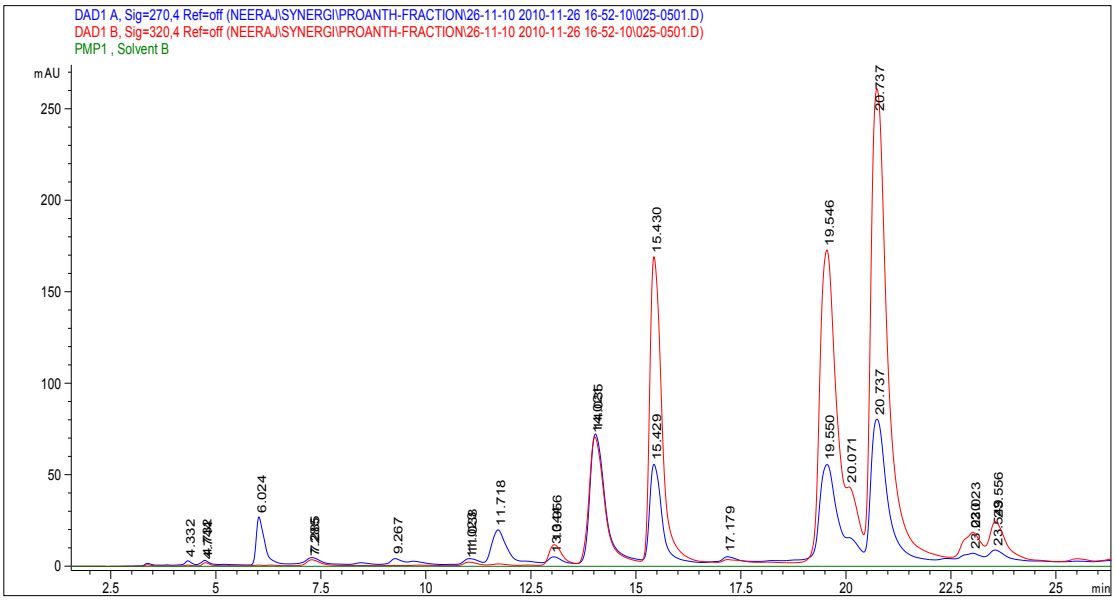

Figure 3.2-20: The HPLC trace of chlorogenic acid processed at 200 °C, 15 min, 100 bar at 270 nm in blue and 320 nm in red.

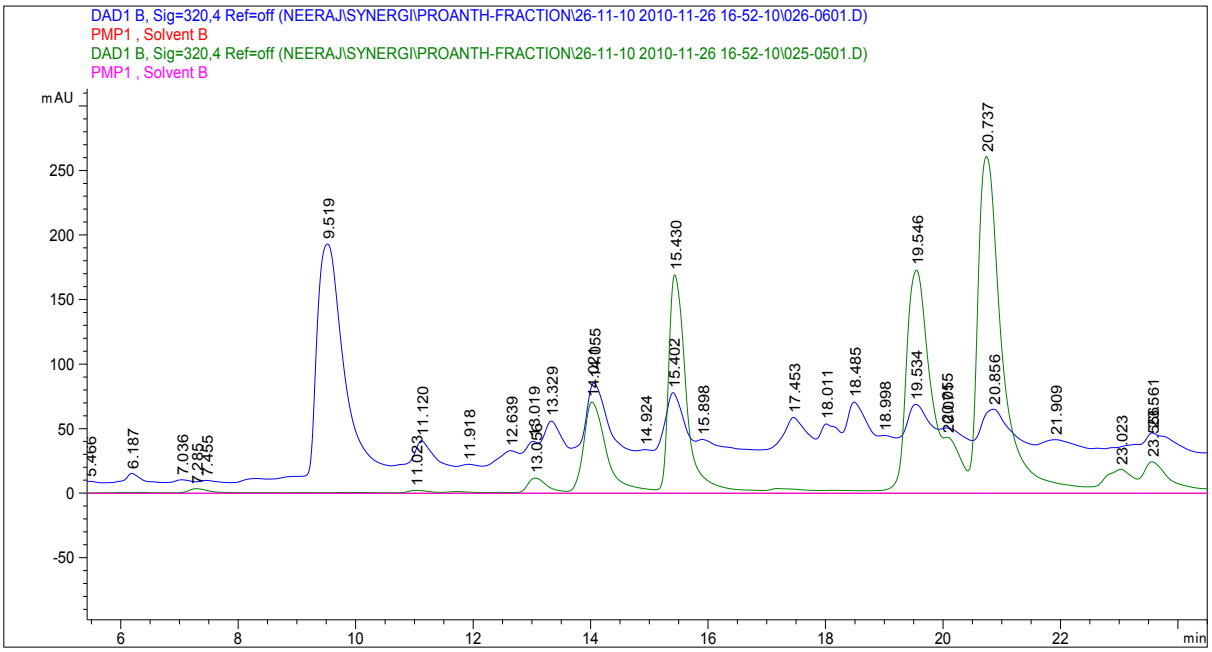

Figure 3.2-21: The HPLC trace of chlorogenic acid processed at 200 °C (in green) at 320 nm overlaying the trace for the extract proceed at 200 °C also at 320 nm.

The processed proanthocyanidin ORAC results are illustrated in Figure 3.2-22, and demonstrate a greater than two fold increase in anti-oxidant activity of processed proanthocyanidin over unprocessed proanthocyanidin. Figure 3.2-23 shows that the UV spectrum and retention time of a peak from the 200 °C extract matches that from the HPLC trace of processed proanthocyanidin. Providing evidence for the existence of proanthocyanidin breakdown products in the extract. Figure 3.2-24 illustrates that phloretin could also be present in the 200 °C extract, as the spectrum of the peak at 17.8 minutes matches that of phloretin.

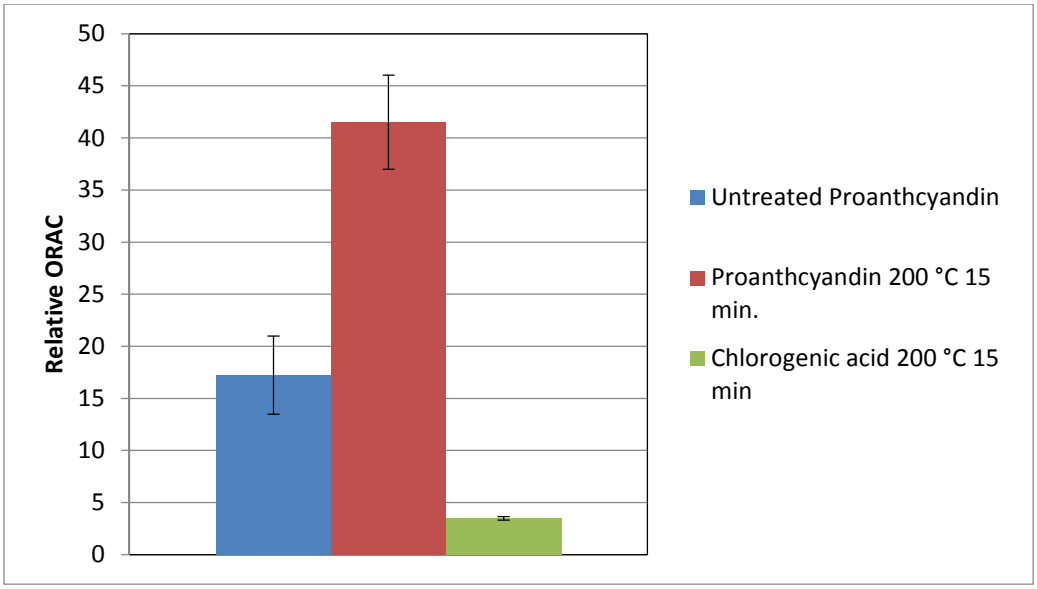

Figure 3.2-22: Comparing the change in ORAC value of unprocessed proanthocyanidin and proanthocyanidin processed at 200 °C, 100 bar 15 minutes.

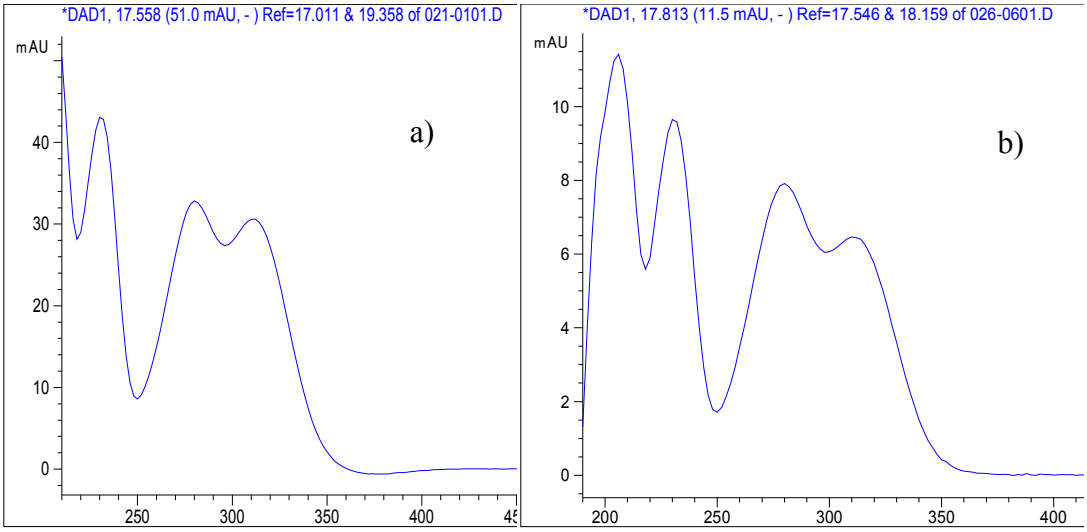

Figure 3.2-23 : a) A peak from the 200 °C extract at retention time of 17.8 minutes b) A peak from Proanthocyanidin 200 °C, 15 min 30 bar at a retention time of 17.558 minutes.

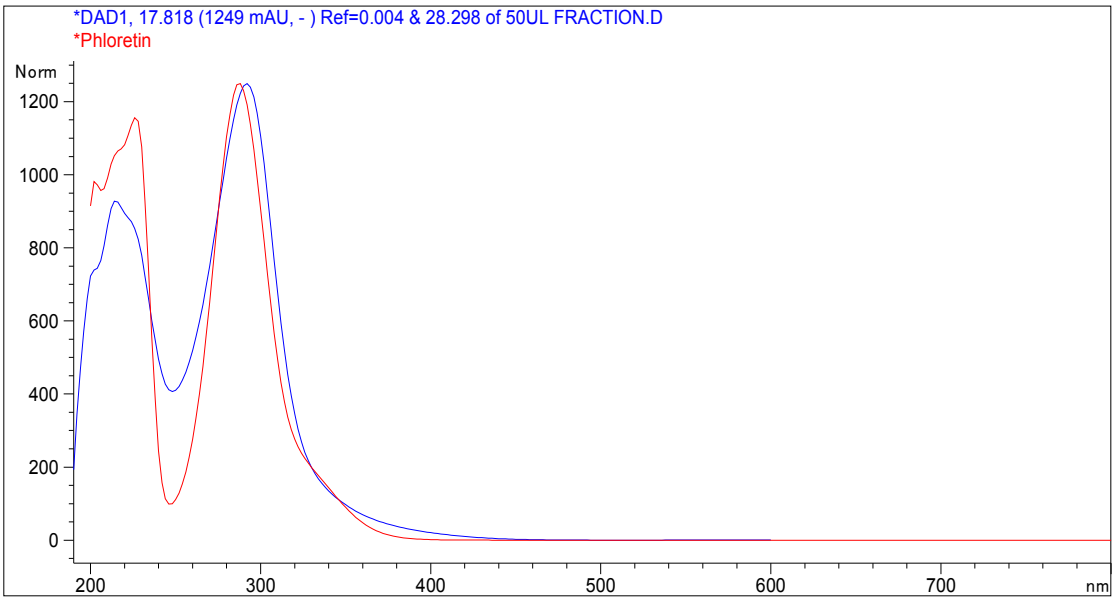

Figure 3.2-24: A UV spectrum from of a peak at 17.818 minutes from the extract processed at 200 °C (in blue) from the DAD (diode array detector), with the spectrum for phloretin overlaid in red (taken from the Agilent Chemstation library).

Based on the potential role of phenolic compounds in the anti-oxidant activity of the extract (see previous results); a refined extract was prepared using supercritical CO_2 extraction and a first generation extract processed at 200 °C used as the feedstock (see section 2.1.4 for the method). Comparing the ORAC value of this refined extract to the previous unrefined extracts, shows that there is a significant increase in anti-oxidant activity (figure 3.2-25), which is greater than the activity of both 40 µM vitamin C and any of the unrefined extracts.

Comparing the second-generation phenolic extract to the HPLC fraction 3 (figure 3.2-26) illustrates that a fourfold increase in ORAC value is possible if the extraction process is improved. This is reinforced by comparing a theoretical ORAC value accounting for impurities in the phenolic extract (as phenolic compounds only account for 22% of the dry weight of the extract (Antas F, personal communication), and the ORAC value of fraction 3, both of which are relatively similar.

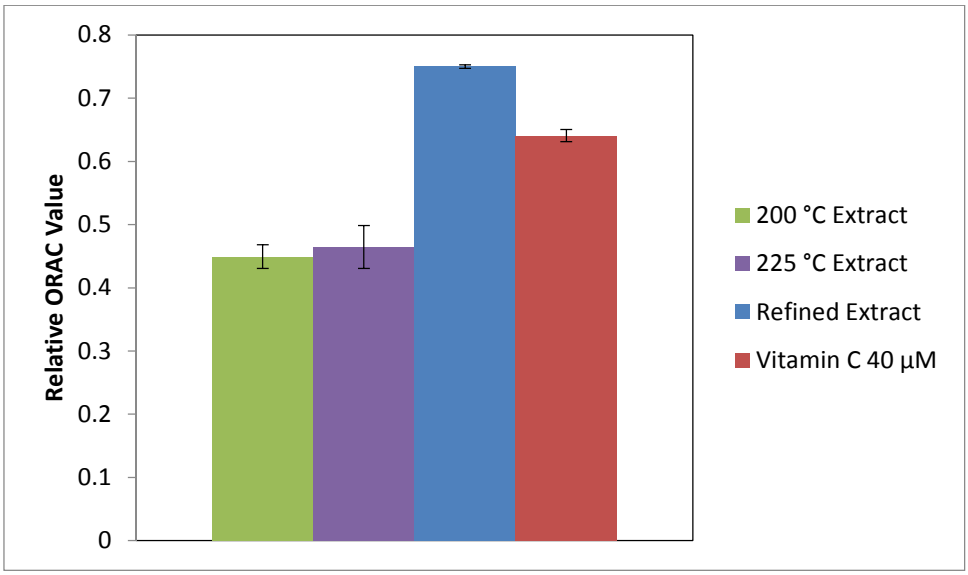

Figure 3.2-25 The ORAC values of the unrefined 200 °C, 225 °C extracts, alongside a refined extract produced using supercritical CO_2 and the 200 °C extract as feedstock.

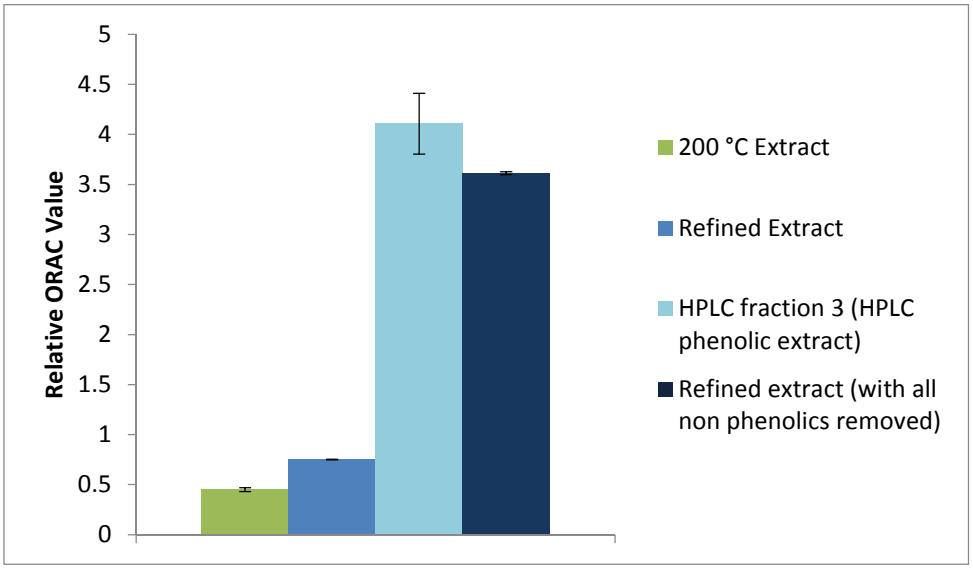

Figure 3.2-26: The ORAC values of: the unrefined 200 °C extract, a refined 200 °C extract (produced using supercritical CO_2 and the 200 °C extract as a feedstock), the HPLC fraction 3 (phenolic extract) and the ORAC value of a hypothetical refined extract with all non phenolic compounds removed .

3.3 Discussion

3.3.1 *Analysis of Anti-oxidant activity*

Yeast extracts of various kinds have been used in the cosmetic and food industry for many years (Gaspar et al., 2008, Abbas, 2006) and with the decline in use of waste yeast in the food industry other high value uses for the yeast were sought. One of the aims of this chapter was to investigate the anti-oxidant activity of the extract and how process conditions could improve the extract.

The results of the anti-oxidant assays presented in section 3.2 illustrate that the extract has definitive anti-oxidant activity, as confirmed by two different assays. The results show that the extracts with process temperatures in the region of 100-150 °C have a slightly higher ORAC value than an extract hydrolysed at 175 °C (figure 3.2-1). This is likely to be due to a reduction in the concentration of free chlorogenic acid above 150 °C (see figure 3.2.-18). This is corroborated by the decrease in absorbance of chlorogenic acid peak observed in 150 °C and 175 °C HPLC traces (Figure 3.2-9 and 3.2-10).

Results show that the 200 and 225 °C extracts have much higher ORAC values than those extracts processed at 175 °C and below. This goes against the perceived wisdom that above a process temperature of 150 °C anti-oxidant activity of any subsequent extract decreases; as is demonstrated by Larrauri. (Larrauri et al., 1997). This has not occurred in this instance due to a large increase in phenolic concentration of the extract processed at 200 °C (see figure 3.2-8) and a lack in of degradation in anti-oxidant activity (with respect to ORAC value) of chlorogenic acid with temperature and residency time (see figure 3.2-17) . Other unidentified factors such as other (as yet) unidentified phenolic compounds could also be responsible.

The ORAC assay results of the HPLC fraction 3 and the phenolic extract further substantiate the role of phenolic compounds in anti-oxidant activity of the extract. This role of phenolic compounds has also been detailed by Betancor–Fernandez et al., (Betancor-Fernandez et al., 2003) , where an increase in phenolic concentrations has lead to an increase in overall anti-oxidant activity.

Results shown in section 3.2.2 demonstrate that as HMF can act synergistically with other anti-oxidants, as has been detailed by Li et al. (Li et al., 2009). Its role in the anti-oxidant activity of the 200 °C extract is limited, due to the ORAC values of HPLC fraction 3 (see Table 3.2-1) being greater than for fraction 2 (containing HMF).

The overlapping peaks in HPLC traces (figures 3.2-22 and 3.2-24) together with the ORAC values of processed proanthocyanidin and chlorogenic acid demonstrate a role for both of these compounds (and there breakdown/polymerisation products) in the anti-oxidant activity of the extract. These compounds could be a part of the phenolic compounds responsible for the anti-oxidant activity of the extract.

The elevation in phenolic concentration with subcritical water or other solvents has been documented by a number of groups, such as Wijngaard and Brunton and Monrad et al., and others (Wijngaard and Brunton, 2009, Monrad et al., 2010, Budrat and Shotipruk, 2009). Fabian et al., and Rodriguez- Meizoso et al., have also reported an increase in phenolic compounds in a microalgae extract together with an increase in anti-oxidant activity (Rodriguez-Meizoso et al., 2008, Fabian et al., 2010). Other groups have used subcritical water (or other solvents) to extract phenolic compounds (García-Marino et al., 2006, Wiboonsirikul and Adachi, 2008) from fruit feedstock.

As most studies use more than one method or assay to validate the anti-oxidant activity of an extract (Zulueta et al., 2009); the results of the TEAC assay confirm that the extract has

anti-oxidant activity. Due to the TEAC and ORAC assays operating via differing mechanisms (Huang et al., 2002), this illustrates that the extract can quench a number of types of free radical. Comparing trends from both the TEAC and ORAC assays (with 225 °C extract having the largest TEAC value) suggests that the composition of the 200 °C and 225 °C differ significantly which is illustrated figures 3.2-11 and 3.2-12. It also suggests that anti-oxidant compounds (particularly in the 200 °C extract) can act differently in both assays as has been documented by Prior (Prior et al., 2005). This is illustrated by comparing the performance of the extract (at 200 °C and 225 °C) against vitamin C in the ORAC and TEAC assay. This demonstrates that the extract performs significantly better in ORAC than in the TEAC assay.

Evaluating the effect of pressure and reaction time illustrated the lack of impact of both factors on the ORAC value of the extract at 200 °C and 225 °C particularly; despite a small change in phenolic content with reaction time (figure 3.2-7) which Carr et al., (Carr et al., 2011) has also been documented.

The drop in ORAC value with reaction time at 300 °C is likely to be due to a breakdown in phenolic content (Bahari, 2010) as there is little literature demonstrating anti-oxidant activity at this temperature.

Work that was carried out independently by my colleague Dr Bahari (Bahari, 2010), while this work was *in situ*; using the DPPH assay, substantiated the trend presented, showing the same increase in anti-oxidant activity at 200 °C with a single batch of extracts and a lack of change with reaction time.

Investigating the stability of the extract over a sustained period of time with various temperatures, shows that the extract was relatively stable (despite some fluctuations on the

extract processed at 200 °C) with no large reductions in ORAC value. This is in contrast to the activity of chlorogenic acid over this period of time, indicating that either other anti-oxidants (possibly formed from chlorogenic acid) were compensating for the reduction in ORAC value of chlorogenic acid or that chlorogenic acid in the extract was being stabilised in some form.

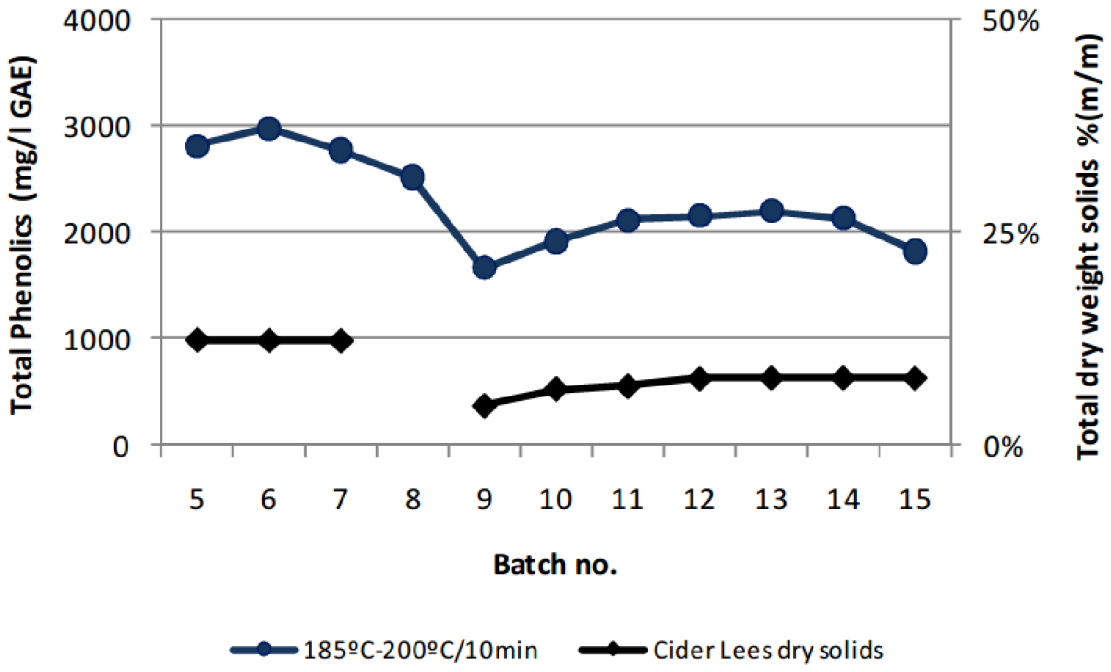

Figure 3.3-1 The change in total phenolic concentration measured using the Folin-Ciocalteu assay (with proteins removed) of large scale (5 L) batches The total dry solid weight of original cider lees (the feedstock in this process) is expressed in percentage with respect to the wet weight (% m/m) (From Antas, 2011)

Table 3.3.1-1 : The total dry weight of extracts produced at 200 °C at 100 bar, with a reaction time of 10 min, in the 5 L reactor. Batch I was produced at the same temperature but in the smaller 300 ml reactor (with data taken from my colleague Mr Fabio Antas).

Batch Number	Dry weight of processed extract (mg/ml)	Mg of phenolic / mg (w.r.t dry weight) of extract	Percentage of dry weight charged into reactor (%/w/w).
5	64.10	0.044	12 %
6	71.90	0.041	12%
7	66.60	0.042	12%
8	61.50	0.041	12%
9	36.30	0.046	5%
10	42.80	0.045	7%
11	46.30	0.046	7%
12	46.00	0.047	8%
13	46.90	0.047	8%
14	46.50	0.046	8%
15	49.20	0.037	8%
Batch I	38.60	0.078	12%

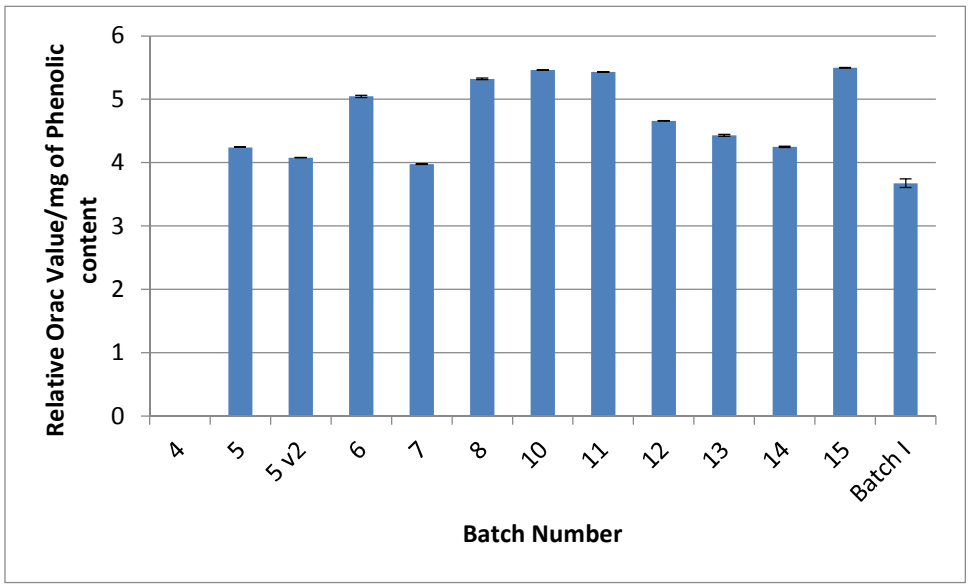

Figure 3.3-2: The relative ORAC values per mg of phenolic in the extract for a number of batches of cider yeast extract produced in the 5 L reactor at 200 °C, 100 bar with reaction time of 10 minutes. Batch I is an extract in the smaller 300 ml initial reactor produced at the same temperature.

Results show that anti-oxidant activity of the first generation extract has been preserved in the second generation extract produced in the 5 L reactor with respect to ORAC value (Figure 3.2-7). However there is significant batch to batch variability that is evident even when dry weight and phenolic content of the extracts are taken into account (Figure 3.3-1, Figure 3.3-2 and table 3.3-1). This could be due to a number of factors; the first is either an error in the processing or analysis of the extract. The second is insufficient mixing prior to production of the extract, which could change the concentration of cider lees prior to production. Finally, but most significantly, the cider lees used to produce the extract is derived from a number of different fermentations; some of which may be richer that others in phenolic content (due to fermentation conditions). This would result in a variation in the quantity and the identity of phenolic compounds either adsorbed or absorbed by the yeast during fermentation. Consequently, the content of this sample of lees could vary sample to sample, as there would be mixture of yeast with differing phenolic content (due to the

mixture / difference in fermentations). Accordingly, the extract produced from the lees would vary batch to batch as the feedstock varies. The change in feedstock dry weight and the change in phenolic content of the extract are illustrated in Figure 3.3-1 as an example. This could explain the batch-to-batch variability observed in extracts produced in the 5 L reactor and this kind of variation would be seen regardless of scale.

Due to the paucity of groups that have used subcritical water with yeast, the anti-oxidant-activity results cannot be directly compared (demonstrating the extract's novelty), as no group has reported generation of a subcritical water processed cider yeast extract. However, there are groups (such as (Rodriguez-Meizoso et al., 2008, Rodriguez-Meizoso et al., 2010, Khuwijitjaru et al., 2012, Herrero et al., 2004)), that have demonstrated an increase in anti-oxidant activity with temperature. Most of these studies have cited 200 °C as an optimal temperature with regard to anti-oxidant activity (Wijngaard and Brunton, 2009). However all of these articles document a variety of different feedstocks including: microalgae (Jaime et al., 2010), waste rice bran (Sereewatthanawut et al., 2008) plants such as oregano (Rodriguez-Meizoso et al., 2006) cinnamon bark (Khuwijitjaru et al., 2012) and rosemary. Of these only Lamoolphak et al., (Lamoolphak et al., 2006) have used yeast with subcritical water. In this study, the extract was not tested for anti-oxidant activity but instead for protein and amino acid content, demonstrating the novelty of the extract.

The rise in phenolic concentration with increasing process temperature as has been documented in this study and in others mentioned, this is probably due to an inverse relationship between the temperature and dielectric constant of water (Carr et al., 2011). Moller et al has shown that as water temperature (and pressure) increases the dielectric constant of water decreases from 78.5 at ambient temperatures to 27.1 at 250 °C (5 MPa,

or 50 bar) (Moller et al., 2011). Therefore, water at this temperature and below starts behaving like an organic solvent. Due to most subcritical water studies showing that 200 °C or above is an ideal temperature, it could be that at this temperature and above the dielectric constant has been reduced to point where phenolic compounds are more readily soluble and therefore can extracted with greater efficiency. This could be further examined by investigating the solubility of phenolic compounds such as chlorogenic or caffeic acid or reservatol in water at 200 °C and above.

Table 3.3.1-2 : Presents the ORAC values of the 200 and 225 °C extracts alongside a number of other compounds taken from the literature. [a] Results taken from Huang et al., 2002. [b] Is the phenolic extract detailed in section 3.2.1

Sample Name	ORAC Value (µmol Trolox/g) (± standard deviation)
200 °C Extract	$(1.77 \pm 0.055) \times 10^3$
225 °C Extract	$(1.78 \pm 0.034) \times 10^3$
Phenolic extract [b]	$(3.00 \pm 0.012) \times 10^3$
Strawberry extract [a]	$(0.54 \pm 0.036) \times 10^3$
Rosemary extract [a]	$(14.3 \pm 0.61) \times 10^3$
Coffee powder [a]	$(11.0 \pm 0.34) \times 10^3$

Table 3.3-2 compares the performance of the extract produced at 200 °C and 225 °C against literature values for other extracts. The ORAC values indicate that the extract performs well, demonstrating a fourfold better activity than the a strawberry extract used by Huang et al.,(Huang et al., 2002) but an order of magnitude worse than the coffee or

rosemary extract. Comparing the ORAC values of the 200 and 225 °C extracts to feedstock from other subcritically processed organisms, such as algae detailed by Plaza et al (Plaza et al., 2010) demonstrate that the extract performs equally as well if not slightly better.

Comparing the performance of the extract against a wide range of vegetable and other plants by Ninfali et al., (Ninfali et al., 2007) illustrates a major issue when comparing anti-oxidant activity results with literature; a lack of standardisation. At first, the extract performs comparably well. However, due to the way that Ninfali et al., data is expressed; direct comparisons cannot be made and this is prevalent throughout the literature and is the reason that more broad performance comparisons with the large amount of literature available could not be made.

3.3.2 *Extract Composition and characterisation*

Figure 3.2-11 showed a what appears to be a dramatic increase in HMF concentration observed in the 200 °C extract, possibly due to a Maillard reaction of a sugar unit from a large polysaccharide such as cellulose (Bahari, 2010). This has been documented by Schroder et al.,, who show that subcritcal water can convert β-D-fructofuranose to HMF (shown in figure 2.5-3) (Moller et al., 2011) Wijngaard and Brunton, have also reported this increase in there apple pomace extract that was processed with subcritical water and ethanol (Wijngaard and Brunton, 2009).

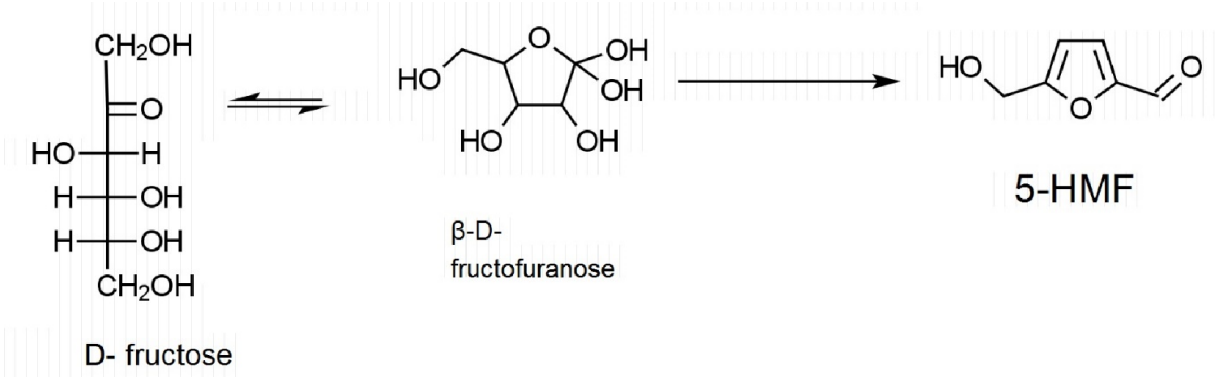

Figure 3.3-3: A schematic of the transformation of fructose to 5-HMF (a Maillard reaction) in subcritical water adapted from Schroder et al., (Moller et al., 2011).

Comparing the compounds found in the extract (with respect to the HPLC data) to work by other groups, they match very closely to apple pomace extract produced by Wijngaard and Brunton (Wijngaard and Brunton, 2009, Reis et al., 2012) which was also produced using subcritical solvents. Due to lack of literature citing yeast extracts natively containing phenolic anti-oxidants such as chlorogenic acid or catechins, it is unlikely that most of the phenolic compounds discovered in this extract are synthesized by the yeast themselves. Indicating that the phenolic compounds observed in the extract were most likely adsorbed or absorbed during cider fermentation; and the literature reports that is occurs in wine fermentation (Morola et al.,, 1999). This theory is confirmed by some work by Bahari (Bahari, 2010), who showed that phenolics could be washed off the yeast cells surface using organic solvents, although this did not account for all of the total phenolic content of the processed extract.

This discrepancy indicates that some synthesis reactions are taking place; and the data presented in figure 3.2-21 to figure 3.2-24 corroborates this view; by showing that the extract contains chlorogenic acid and proanthocyanidin breakdown or polymerisation

products (which would require synthesis). However, the exact mechanism that many of these phenolic compounds are produced remains unclear.

Using the assumption that the phenolic compounds in the extract are from the cider fermentation and in turn the apples, suggests that cinaminic acid, epicatechins and phlordzin could also be involved in the anti-oxidant activity of the extract as they are present in work by Wijngaard and Brunton using apple pomace (Wijngaard and Brunton, 2009).

In general, most of feedstocks used by subcritical solvent literature are either fruit or plant waste based such as apple pomace or rice bran, which indicates that the yeast may have adsorbed phenolic compounds during fermentation

3.4 Conclusions

In summary, it has been demonstrated that the extract has anti-oxidant activity via two different mechanisms. The performance of the unrefined extract produced at 200 and 225 °C, compared to the literature values shows that the extract performs comparably. The rise in anti-oxidant activity above 200 °C is due to an increase in phenolic concentration.

Phenolic compounds have been shown to have a large role in the anti-oxidant activity of the extract, which could be composed of chlorogenic acid breakdown or polymerisation products, and proanthocyanidin breakdown products.

There was a doubling in ORAC value from the refined to unrefined, however results show that the performance of the extract can be improved further, to make it more comparable to other high performance extracts in the literature (Huang et al., 2000) (shown in Table 3.3-1).

In conclusion, by using the 200 °C and/or the 225 °C extracts in all future work the results of the anti-oxidant assays have been used to improve the anti-oxidant activity of the extract as was originally intended. In addition, an effective yeast extract unlike any previously reported has been produced.

The characterisation data together with the lack of documented phenolic compounds found in yeast suggests that the phenolic compounds found in the extract could have been adsorbed from the fruit during the cider fermentation process, further characterisation work would be required to confirm this.

The work further demonstrates the utility of subcritical water in producing an extract rich in phenolic compounds with antioxidant capacity.

CHAPTER 4 : The Comet assay and the quantification of oxidative stress *in vitro*.

4.1 Introduction

The comet assay was developed to measure DNA strand breaks, which are an indirect measure of oxidative stress as discussed in section 1.6. The assay has been used extensively in the literature to investigate the effects anti-oxidants in preventing this oxidative stress both from the environment (such as UV irradiation) and from chemicals (such as hydrogen peroxide).

Therefore, to substantiate previous results shown in chapter 2, which demonstrated the multi-faceted anti-oxidant activity of the extract when processed above 200 °C. The comet assay was chosen to quantify and compare the efficacy of the extract against other benchmark anti-oxidants (such as Trolox) in protection against UVA irradiation which has been shown to cause oxidative stress and DNA damage by free radical generation.(Cadet et al., 2009)

4.1.1 *Background to the work*

Previous characterisation work had shown that the cider yeast extract contains small amounts of vitamin B3 and significant amounts of phenolic compounds, such as chlorogenic acid and hydroxymethylfurfural.

B vitamins are widely used in the cosmetic industry especially vitamin B3 which is used as in cellular metabolism and is used to generate NADH. More significantly vitamin B3 has also been shown by Kang et al; to lengthen the replicative lifespan of human dermal fibroblasts. Vitamin B3 has also been used clinically to treat roseaca (Nirem, N.M. 2006).

Chlorogenic acid especially is well known in the literature as an anti-oxidant, and has been has been proposed as a potential active in a sunscreen (Choquenet et al., 2009). Anti-oxidants in general have been linked to a reduction in cellular DNA damage and an increase in cellular lifespan (Guarente et al., 2008).

To assess the effect of the cider yeast extract on the biological activity of the cells, a number of assays were chosen to match the two key biological areas. They were cellular senescence and metabolism and were chosen due to the and fact that vitamin B3 was is involved in key metabolic pathways and that it seems to have an effect on cellular senescence (Guarente et al., 2008). The criteria for choosing the assays were that they should be relatively simple and rapid to perform and gave us definitive results.

Three assays were chosen to asses the effect of the extract on cells. The first was a MTT (Methylthiazolyldiphenyl-tetrazolium bromide) assay; which is a metabolic assay in which MTT is metabolised by active cells to a formazan (Mosman et al., 1983). The second was a calcein AM (calcein *O*,*O*′-diacetate tetrakis(acetoxymethyl) ester) based assay, which assessed cell viability, as calcein am is a marker that is converted by enzymes in viable cells to calcein (Essodaïgui et al., 1998). The third was a Hoechst assay (from Invitrogen, Paisley UK) which measures total cell number, by measuring the total amount of nuclear DNA present, as Hoechst 33258, fluoresces proportionally to the DNA in the sample. (Daxhelet et al., 1989)

Unfortunately, the results from investigations using these assays proved inconclusive (see Appendix A (which is located at the end of this thesis)) and the focus of the research switched away from cell viability cellular metabolic activity to anti-oxidant activity. The cell culture technique developed within this work package was used as a base for the following Comet assay investigations.

4.2 Results

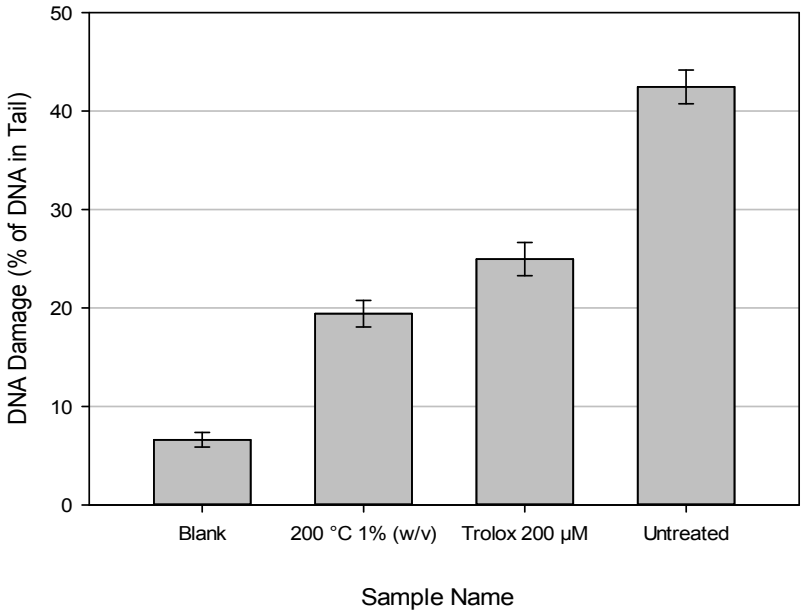

Figure 4.2-1: The results from a comet assay performed with cells treated with the first generation of the 200 °C extract alongside Trolox and a untreated sample.

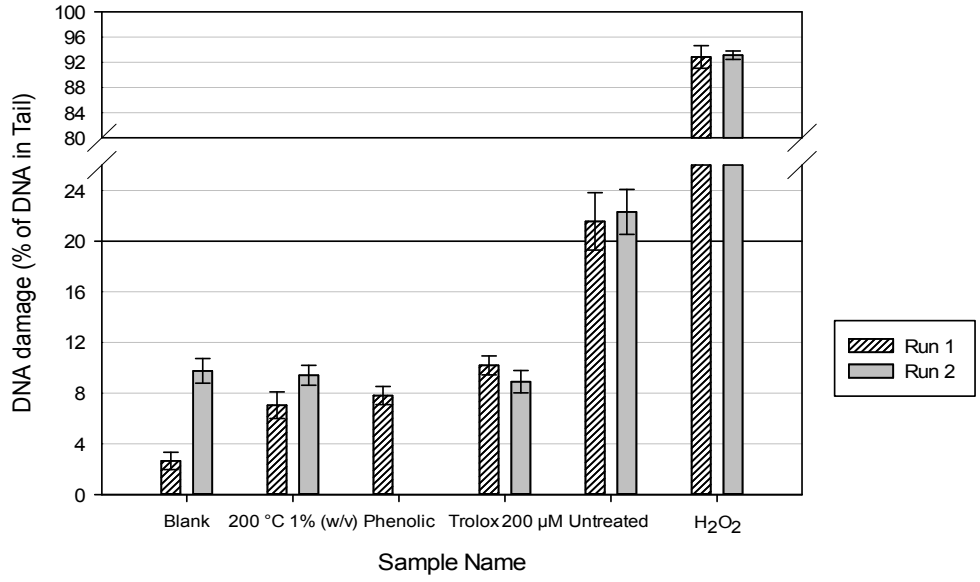

Figure 4.2-2: The comet assay data from two independent runs which were both treated with generation 2 of the 200 °C extract (from the 5 L reactor) alongside a variety of other treatments. The bars labelled blank, were cells that were un-irradiated and untreated.

The comet assay was used as a tool to evaluate the efficacy of cider yeast extract hydrolysed at 200 °C as an anti-oxidant in cells and protect against UV and DNA damage. The comet assay results from the 1st generation of 200 °C extract (produced in the smaller 300ml reactor) are shown in figure 4.2-1. Trolox was used as the positive control, to gauge how well the yeast extract performed in comparison to an established anti-oxidant (Prior et al., 2005). The results show that DNA damage of cells treated with Trolox and extract is significantly lower than with untreated cells ($P<0.05$), with approximately a 50% reduction in damage with both treatments. The irradiated cells did have a significantly higher percentage of DNA damage than un-irradiated cells ($P<0.05$).

Two independent runs of the assay using a single batch produced in the 5 L reactor is presented in figure 4.2-2 (this is referred to as the 2nd generation extract). These data were used to investigate the impact of scaling up production of the extract. The data shows cells treated with a 200 °C extract for 18 hours (prior to UV exposure) have a significant reduction in DNA tail damage of approximately 50%, when compared the untreated cells. Figure 4.2-2 shows that there was a small or no difference between the baseline blank cells (which had not been exposed to any radiation) and those that had been treated with extract and exposed to UVA radiation. This is especially evident in the run 2 where the blank, 200 °C and trolox samples do not have significantly different DNA damage ($P >0.05$) indicating that the extract had negated the effect of the UVA radiation. All of these samples were significantly lower than the untreated sample ($P\leq 0.05$). Cells treated with hydrogen peroxide were used as a positive control.

When comparing to cells treated with extract to those treated with Trolox, both treatments performed similarly with respect to the reduction of DNA damage compared to the untreated sample.

Overall, the variability between both runs is small and is generally within the error bounds of each of the measurements. However there is a significant difference ($P<0.05$) between blank samples. This could be potentially due to the use of a different batch of cells (which were of a different age) used for both runs. However the data for the rest of the samples seems to correspond well with the previous run, so the cells from the run 2 are probably showing unusually high background damage, and this would account for the fact that the cells treated with trolox and UVA radiation show less damage than un irradiated (blank) cells.

Figure 4.2-2 also shows that there is a small amount of variability in the reduction of DNA damage from run to run.

The overall DNA damage observed in irradiated samples treated with the 1st generation extract (figure 4.4-1) is approximately double that observed in figure 4.2-2 and 4.2-3 where a 2nd generation extract was used. This discrepancy is due to the more extensive use of trypsin with the 1st generation extract, which can contribute to increased DNA damage.

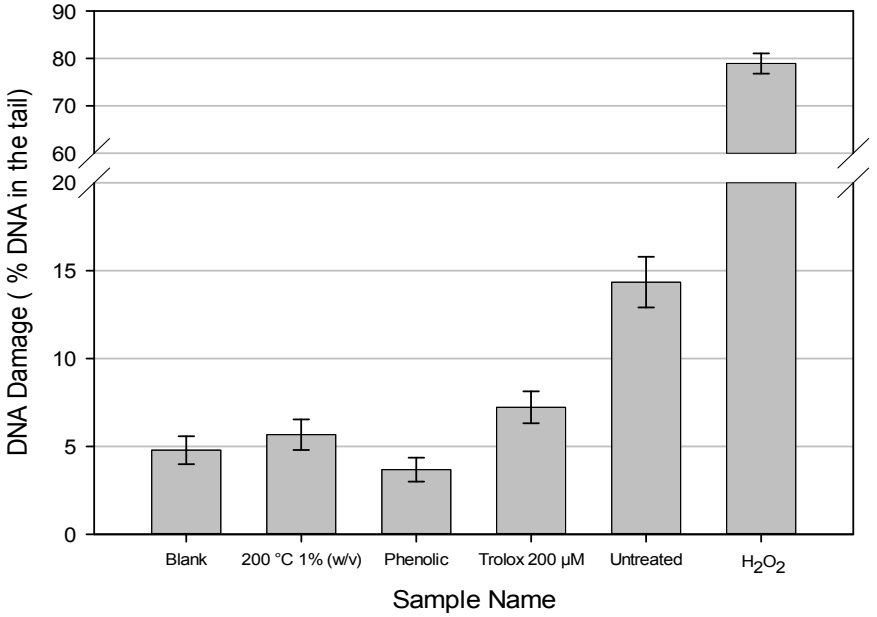

Figure 4.2-3: The DNA damage of cells treated with generation 2A of the 200 °C extract alongside a variety of other treatments with blank cells which were un-irradiated and untreated.

To assess the batch-to-batch variability of the extract batch 2A of 200 °C extract was tested and the comet assay results are shown in figures 4.2-3. The figure shows that cells treated with H_2O_2, have a high degree of damage as expected. Cells treated with extract and irradiated with UVA have half the DNA damage of untreated cells.

When compared to cells treated with Trolox, cells treated with extract had insignificantly less DNA damage. Both of these samples exhibited marginally higher amounts of DNA damage when compared to the blank (un-irradiated) sample. Comparing this to the two previous runs (shown in Figure 4.2-2), both sets of results show a reduction of approximately 50% when compared to the untreated cells which is a significant reduction in DNA damage compared with the untreated cells ($P<0.05$).

The phenolic extract, which is a refined version of the cider yeast extract processed using subcritical CO_2 and contains predominately phenolic compounds. Cells treated with this

extract, exposed to UVA irradiation are shown in figures 4.2-2/3, the results of which illustrate that the phenolic extract performs similarly to the original yeast extract with no significant difference in any of the experiments.

4.3 Discussion

Many groups have demonstrated that apple based extracts or juices have anti-oxidant activity. They have demonstrated that these extracts reduce oxidative stress *in vivo* and *in vitro* measuring the reduction in stress using the comet assay and nuclear DNA as the probe.

While the cider yeast extract is far removed from the apples originally used in the production of cider, the results show that the anti-oxidant activity of the extract is similar to that of the apples and apple based extracts themselves (Bellion et al., 2010, Schaefer et al., 2006a).

Overall the results shown, demonstrate a consistent reduction in DNA damage of approximately 50% in cells exposed to UVA radiation and treated with extract; in comparison to the untreated cells. In almost all of the results shown, there was no significant difference in the performance of the extract in comparison to Trolox, which was encouraging and validates previous anti-oxidant results. As there has been so little literature using yeast extracts with the comet assay previously it is difficult to compare the results to literature. The work involving apples has used (in most cases) hydrogen peroxide as the inducer instead of UVA irradiation used here. Therefore, the results are not directly comparable. However looking more generally; the yeast extract has performed comparably well to the results shown by Schafer et al., and McCann et al (Schaefer et al., 2006a, McCann et al., 2007). In those studies using UVA irradiation, Morley (Morley et al., 2003)

Pygmalion (Pygmalion et al., 2010) and Lyons (Lyons and O'Brien, 2002) have shown a reduction in DNA damage, with Lyons et al using an algal extract instead of yeast based extract.

The refined yeast extract derived from the 200 °C extract, named the 'phenolic extract' performed very similarly to the original 200 °C yeast extract. This corresponds to a large reduction in dry weight of both extracts from 100 mg/ml to 13 mg/ml (unpublished data from Fabio Antas) without a significant loss in activity. This initially seems like a very large increase in activity per gram of extract, but this should be tempered by comparing the phenolic content of both extracts, which is 2.774 mg/ml of the cider yeast extract compared to 13 mg/ml in the phenolic extract (which should contain a much higher concentration of phenolic compounds). This is a fourfold increase in concentration but there isn't a fourfold reduction in DNA damage evident (as both extracts were used at the same concentration w/v). This emphasizes the fact that further work would have to be done to confirm the activity of the phenolic extract. However if this increase in phenolic concentration is taken into account, there is still a significant increase in activity per gram of dry weight of the extract. This substantiates results shown in chapter 2 and reinforces the belief that phenolic compounds play a major role in the overall anti-oxidant activity of the extract. Comparing the activity of the phenolic extract to literature is again difficult as the exact composition of the extract is unknown. However, the nearest match is the work by Schafer et al, who use a tailored phenolic extract to investigate the role phenolic compounds in their apple extract as a whole. The results of the phenolic extract contrast the results documented by Schafer et al; which shows a pro-oxidant effects taking place above 10 μg/ml. The results from the phenolic extract, which was used at a 130 μg/ml (or at 28.6 μg/ml; if pure), show a significant reduction in DNA damage and are contrary to the work shown even if purity issues are taken into account. This is due to a difference in

composition of both extracts, but since Schafer et al used menadione instead of UVA irradiation, only speculative conclusions can be drawn.

The DNA damage of cells treated with all of the extracts compared favourably to cells treated with Trolox as most the results show no significant difference in DNA damage. It is only in the first generation of extract where the yeast extract performs significantly better ($P<0.05$). Trolox has also been used by Hseu et al., to reduce DNA damage *in vitro*, (Hseu et al., 2009), and Trolox is a very widely used anti-oxidant *in vitro*. Therefore the results presented here seem reliable.

In summary, the original aims of this chapter detailed the selection and implementation of an assay that would test the yeast extract in a cell culture model and corroborate the previous anti-oxidant results. The relative consistency of the results and the experimental controls performing as expected shows that this aim has been achieved.

4.4 Conclusion

In summary, the results have demonstrated that the extract has anti-oxidant activity in cells and has limited the damage caused by free radicals generated by UVA irradiation. Results have also shown that cells dosed with extract have performed comparably to those dosed with Trolox.

Due to the complete lack of any comparable yeast extracts which have demonstrated anti-oxidant activity both in cells and in the ORAC assay, it can be concluded that an effective yeast extract with phenolic content has been produced.

Results have also corroborated previous results presented in chapter 2, which was the original aim of the chapter. Therefore, it can be concluded that the aims of the chapter have been achieved.

CHAPTER 5 : PRODUCT FORMULATION

5.1 Introduction

One of the principal destinations of an anti-oxidant (such as the cider yeast extract, as has been demonstrated previously in chapter 2) is as an active ingredient in a cosmetic product (Louli et al., 2004). Therefore, the logical next step was to investigate the effect of the extract in a number of prototype cosmetic formulations. The stability of any such formulation is an important factor and needs to be demonstrated prior to any human testing taking place. The rheological and colour stability of any cosmetic product is also an important factor prior to the commercial release of a product containing the extract. As a change in colour or a breakdown in the rheological properties of a product during its life cycle is extremely undesirable.

Therefore, four commercial base formulations were chosen to form the basis of a scoping study evaluating the effect of the extract on emulsion stability and colour change. This work could then be used as a basis of further work, to address any issues that arise.

5.2 Results

5.2.1 *Emulsion Stability*

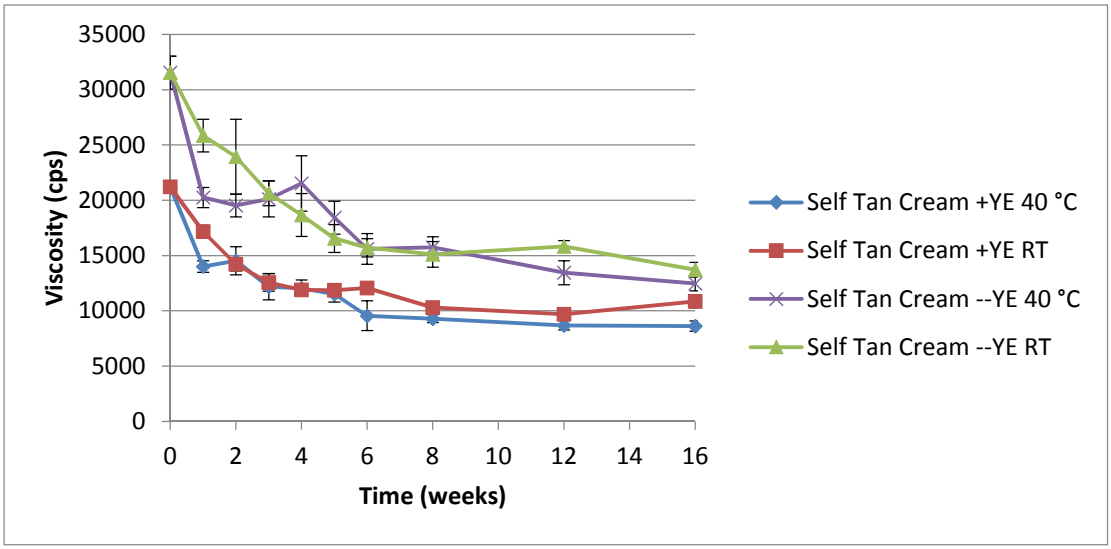

Figure 5.2-1: The viscosity change of the two self tan formulations with time and storage temperature. +YE and –YE refer to either formulations containing extract or without extract respectively. RT refers to formulations stored at room temperature, (which is approximately 23 °C).

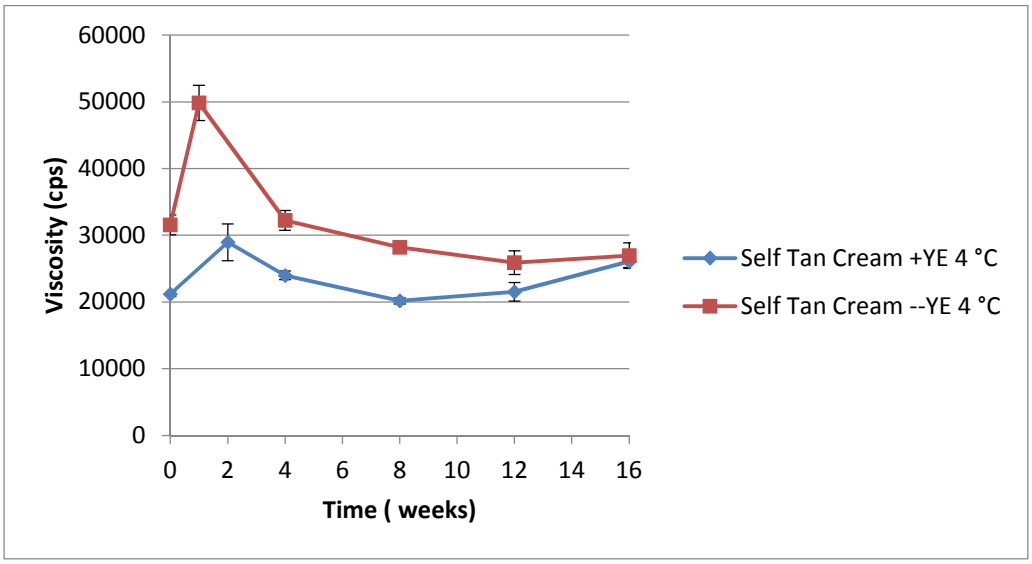

Figure 5.2-2: The viscosity change of the two self tan cream formulations with time at stored 4 °C. +YE and –YE refer to either formulations containing extract or without extract respectively.

Stability testing was used to examine how the yeast extract would interact in a skincare (or cosmetic) formulations over a sustained period of time. The results from the first of these

formulations (the self tan cream) are presented in figure 5.2-1 and 5.2-2. The figures detail a large drop in viscosity of over 50% from the initial viscosity, over the period of 4 months for all samples; with the majority of the drop occurring in the first 4 weeks. The self tan cream without extract has a higher viscosity than the formulation containing yeast extract and this is possibly due to effect of the extracts pH on the formulation, although further work would have to be carried out to confirm this. After the initial 4 week period the viscosity of the formulations stabilises with much less change observed. This occurs in both formulations with and without extract, and at both room temperatures (room temperature and 40 °C) suggesting that the extract itself is unlikely to be a cause of this instability (see section 5.3 for further discussion of this issue). Formulations that were stored at 4 °C do not show this degree of change, while the viscosity of formulations does fluctuate; overall, there is no significant decrease in viscosity of the formulations with or without extract. However there is a significant increase in viscosity after the initial measurement and this is due to the solidification of fats (in the formulation) after the formulation has been stored at 4 °C for 2 weeks, which has been documented previously(Babcock, 1931).

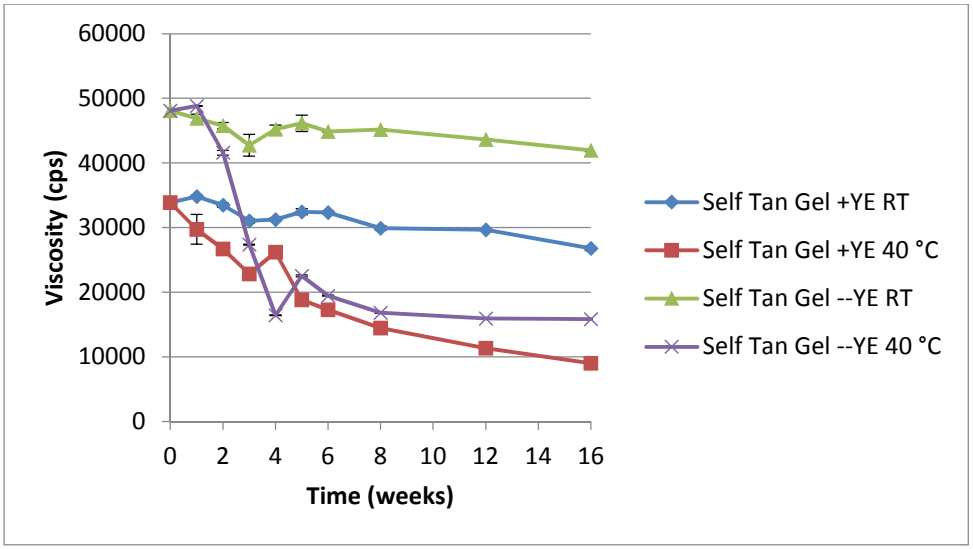

Figure 5.2-3: The viscosity change of two self tan formulations with time and storage temperature measured at a constant shear rate of 2.508 s^{-1}. RT refers to formulations stored at room temperature, (which is approximately 23 °C).

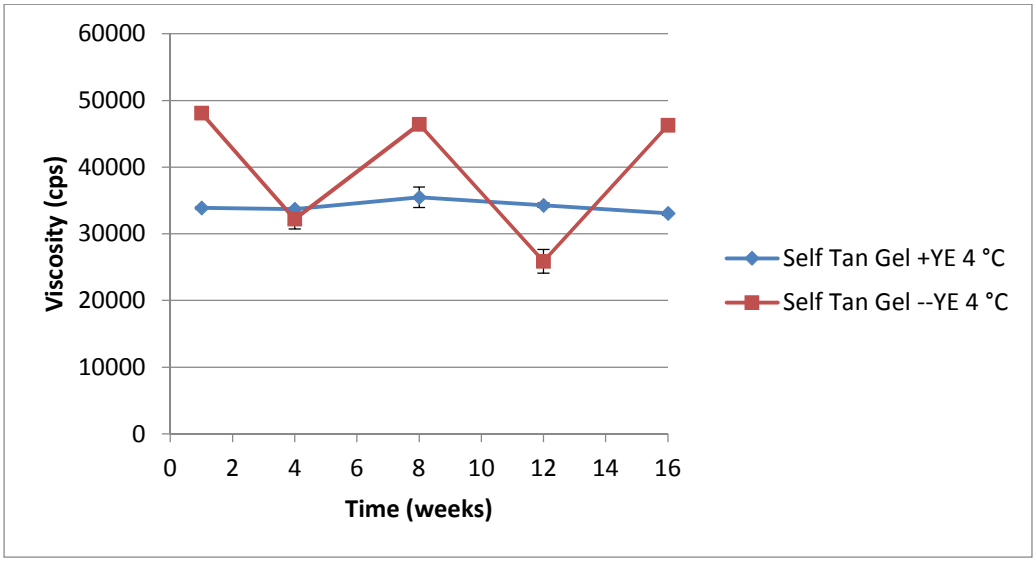

Figure 5.2-4: The viscosity change of two self tan gel formulations with time at 4 °C measured at a constant shear rate of 2.508 s^{-1}.

The results from the second formulation, the self tan gel are shown in figure 5.2-3 and 5.2-4. They illustrate that while formulations stored at room temperature were relatively stable with small reduction of the initial viscosity after 16 weeks. Formulations stored at 40 °C showed large drops in (initial) viscosity in the first 4 weeks which is particularly evident with the

formulation stored at 40 °C without extract. The drop in viscosity is more gradual and is less in overall magnitude with the self tan gel with extract, however by the end of the 16 weeks study there is a drop of over 70% in the initial viscosity of both formulations with and without extract stored at 40 °C. Indicating that the underlying base formulation is unstable at this storage temperature and this is not a result of any interaction with the extract. Self tan gel formulations with yeast extract stored at 4 °C appeared stable with no significant change in viscosity with time, contrasting the formulation without yeast extract whose viscosity fluctuated wildly with time, which is very unusual and no rationale for this fluctuation has been found.

Results from the face cream formulation are presented in figure 5.2-5 and 5.2-6. The results demonstrate that all of the formulations with and without yeast extract are relatively stable with respect to change in initial viscosity, with no significant reduction in initial viscosity observed. This was particularly evident with formulations stored at room temperature, where a ≤10% change in initial viscosity of formulation with and without yeast was observed. While there were fluctuations in the formulation stored at 40 °C at the end of the 16 week period the viscosity of these was not significantly different to samples stored at room temperature.

This contrasts the rise in viscosity of formulations stored at 4 °C over the 16 week period.

Figures 5.2-7 and 5.2-8, show a gradual rise in viscosity in all of the face wash formulations including those with (+YE) and without (--YE) extract. While there was more fluctuations in the viscosity of formulation stored at 40 °C, storage temperature does not seem to have an impact on the viscosity of the formulation (as there is no significant difference ($p<0.05$) in the final viscosity values of those stored at room temperature and those stored at 40 °C). There is however, a significant difference in the viscosity of formulations with or without extracts throughout the 16 week period. Formulation stored at 4 °C showed little change in

viscosity during the 16 week period apart from the viscosity of formulations in week 8, indicating that these formulations were stable.

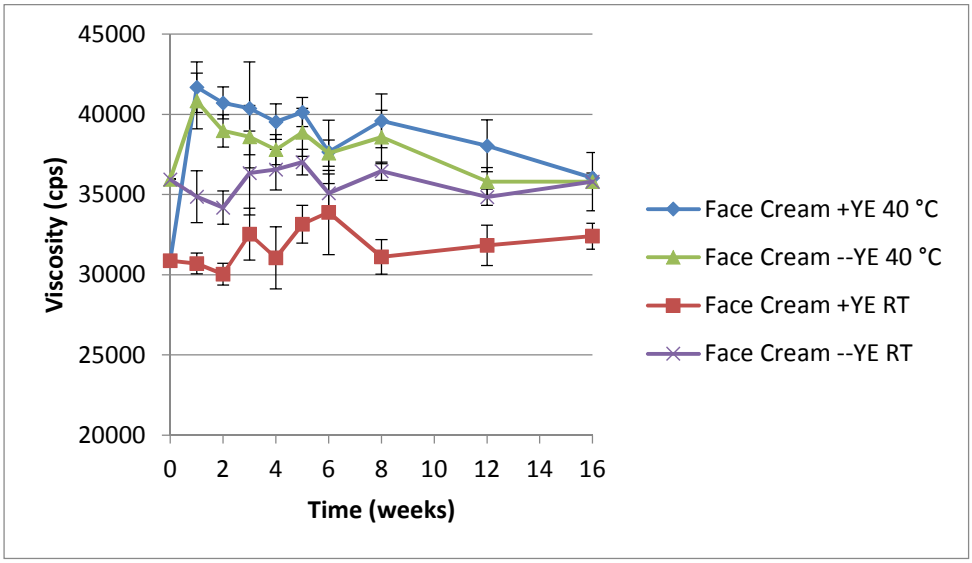

Figure 5.2-5: The viscosity change of two face cream formulations with time at 40 °C and at room temperature (RT).

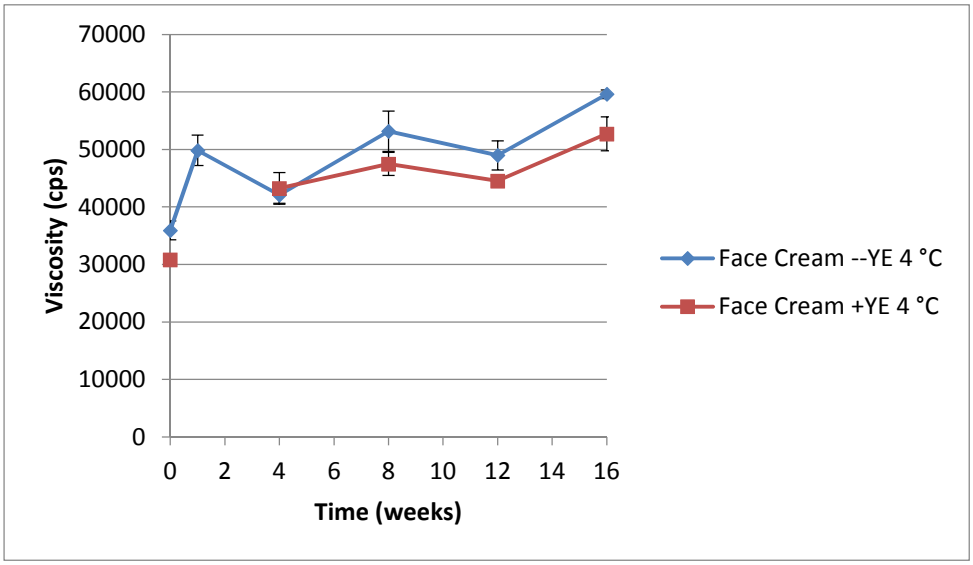

Figure 5.2-6: The viscosity change of two face cream formulations with time at 4 °C and at room temperature (RT) (the face cream +YE is missing data at day 2 due to data loss).

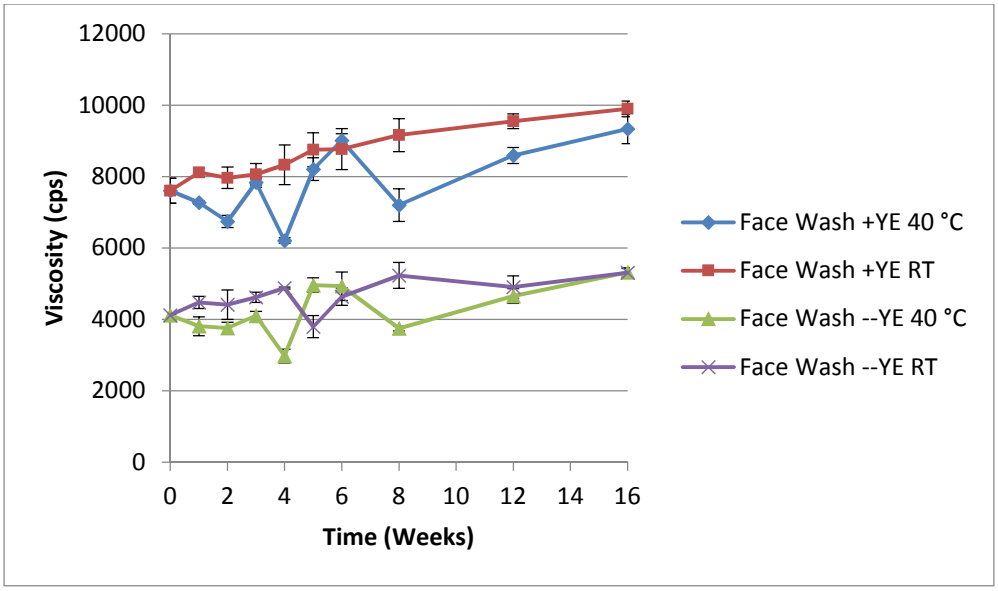

Figure 5.2-7: The viscosity change of two face wash formulations with time at 40 °C and at room temperature (RT) measured at a constant shear rate of 2.09 s^{-1}

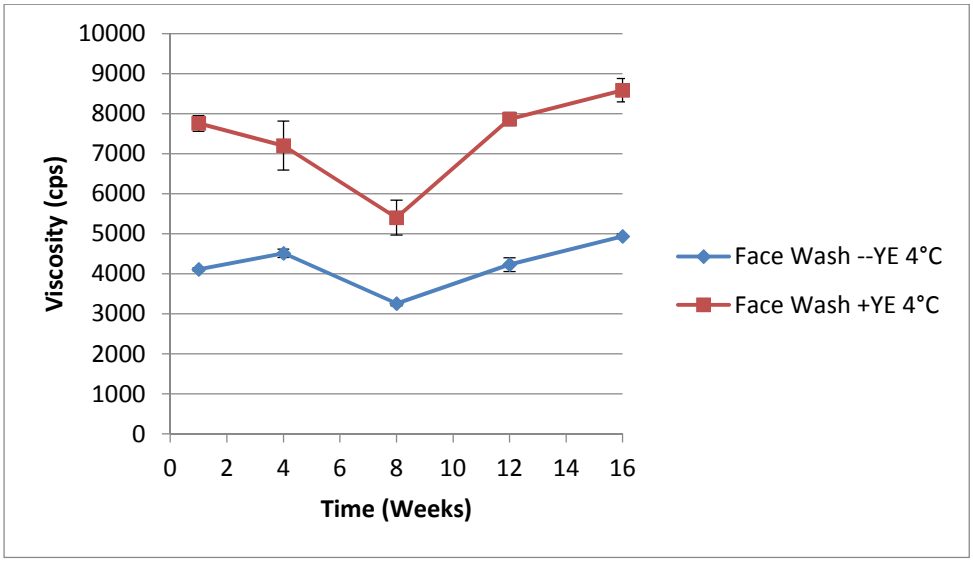

Figure 5.2-8: The viscosity change of two face wash formulations with time at 4 °C measured at a constant shear rate of 2.09 s^{-1}.

Table 5.2.1-1: The change in pH of the various formulations stored at room temperature during the 16 week stability study.

Room temperature	Final pH (at 16 weeks)	Initial pH	Change in pH	± error
Face wash +YE	5.90	6.14	0.25	0.085
Face wash -YE	6.54	6.63	0.09	0.036
Face cream +YE	5.52	5.37	0.15	0.163
Face cream -YE	5.94	5.77	0.17	0.134
Self tan cream +YE	3.28	3.30	0.02	0.135
Self tan cream –YE	3.45	3.47	0.02	0.140
Self tan gel +YE	3.01	3.15	0.14	0.010
Self tan gel –YE	2.60	2.80	0.20	0.022

Table 5.2.1-2: The change in pH of the various formulations stored at 40 °C during the 16 week stability study. +YE and –YE denotes with yeast extract and without extract respectively.

40 °C	Final pH (at 16 weeks)	Initial pH	Change in pH	± error
Face wash +YE	5.895	6.140	0.245	0.085
Face wash -YE	5.630	6.625	0.995	0.029
Face cream +YE	5.515	5.590	0.075	0.092
Face cream -YE	5.835	5.770	0.065	0.177
Self tan cream +YE	2.725	3.295	0.570	0.036
Self tan cream –YE	2.835	3.465	0.630	0.010
Self tan gel +YE	2.450	3.145	0.695	0.043
Self tan gel –YE	2.405	2.795	0.390	0.022

Table 5.2.1-3: The change in pH of the various formulations stored at 4 °C during the 16 week stability study.

4 °C	Final pH (at 16 weeks)	Initial pH	Change in pH	± error
Face wash +YE	5.950	6.140	0.195	0.085
Face wash -YE	6.610	6.625	0.020	0.050
Face cream +YE	5.360	5.580	0.220	0.114
Face cream -YE	5.845	5.770	0.075	0.035
Self tan cream +YE	3.320	3.295	0.025	0.085
Self tan cream –YE	3.730	3.465	0.265	0.142
Self tan gel +YE	3.005	3.145	0.140	0.036
Self tan gel –YE	2.690	2.795	0.105	0.071

The change in pH of the various formulations with storage temperature is presented in tables 5.2-1 to 5.2-3. They illustrate that at room temperature and at 4 °C there is very little change in pH during the 16 week period in majority of the samples. Only the face wash and self tan gel with extract and the face cream formulations have a change in pH of more than 0.1 at room temperature. The change in at pH at 40 °C is much more marked with 4 formulations showing a change in pH of more 0.5. However, the results do not seem to correlate with the changes in viscosity of the formulations. This is illustrated by the large change in pH of the face wash formulation without extract only correlating to a small rise in viscosity; and the much smaller change in pH of the self tan gel formulation (without extract) showing a large drop in viscosity after 16 weeks.

5.2.1.1 Microscopy

To further investigate the stability of the two emulsion formulations the structure of each of was analysed using images of each of emulsion formulation (at the same time intervals as the viscosity measurements). A selection of which is presented in figures 5.2-9 to 5.2-12.

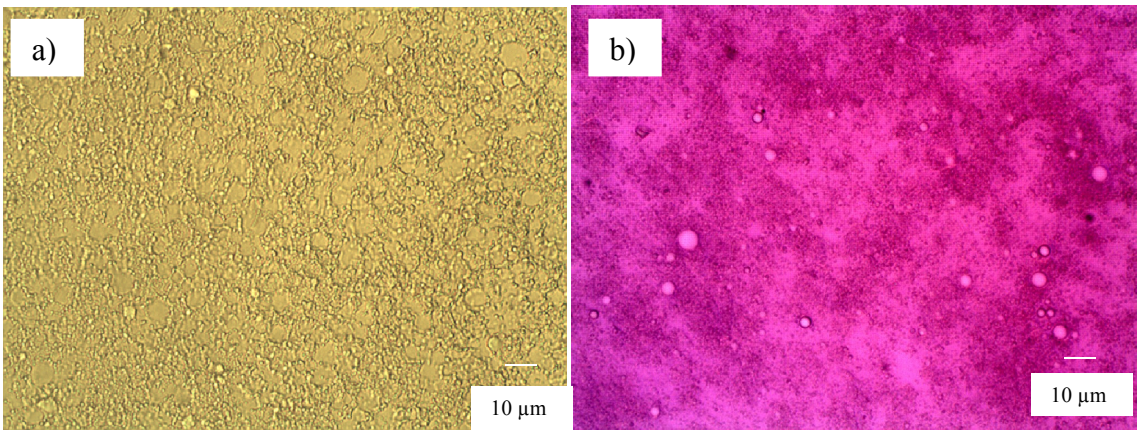

Figure 5.2-9: Micrographs of self tan cream formulation without yeast extract stored at room temperature for either: a) 0 weeks or b) 16 weeks (the initial sample). Both images were captured at a magnification of 400x.

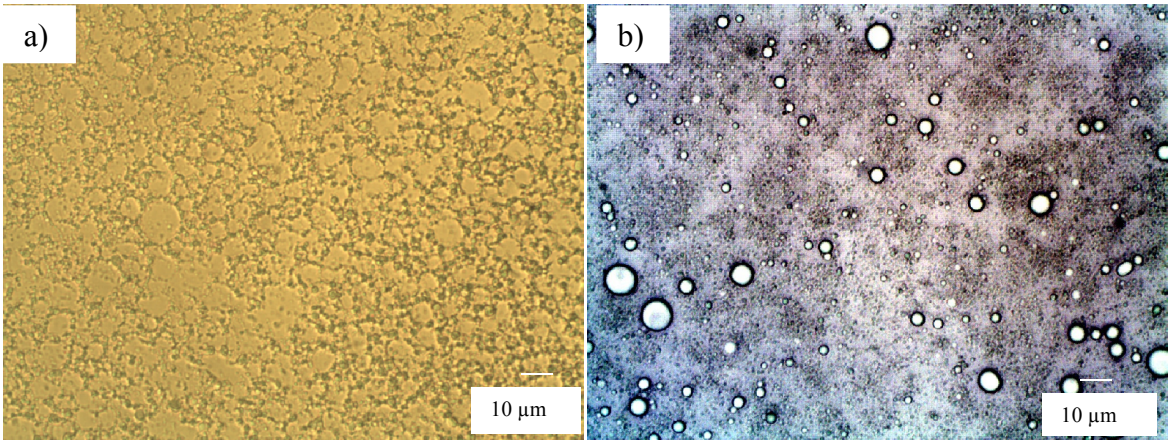

Figure 5.2-10: Micrographs of self tan cream formulation with yeast extract stored at room temperature for either: a) 0 weeks or b) 16 weeks (the initial sample). Both images were captured at a magnification of 400x.

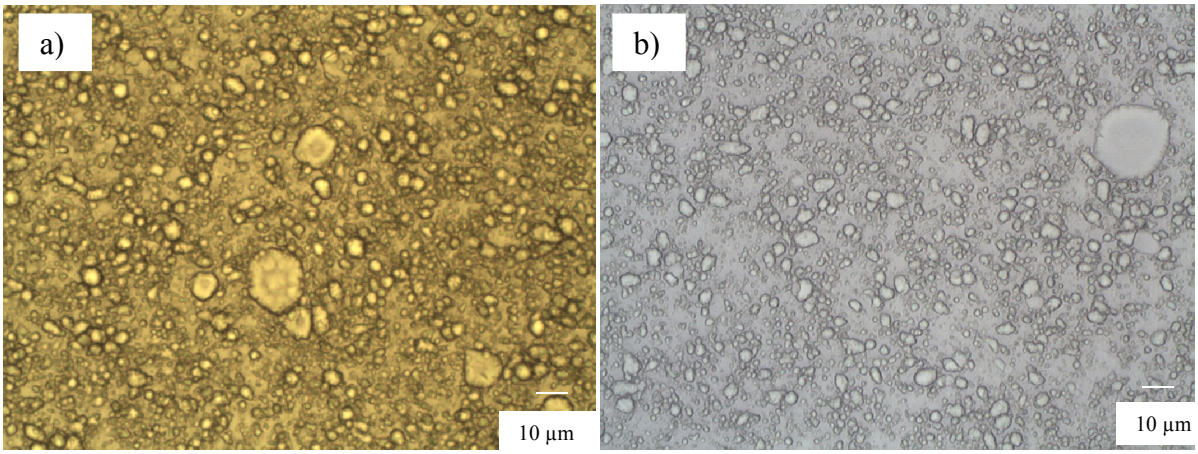

Figure 5.2-11: Micrographs of face cream formulation with yeast extract stored at room temperature for either: a) 0 weeks or b) 16 weeks (the initial sample). Both images were captured at a magnification of 400x.

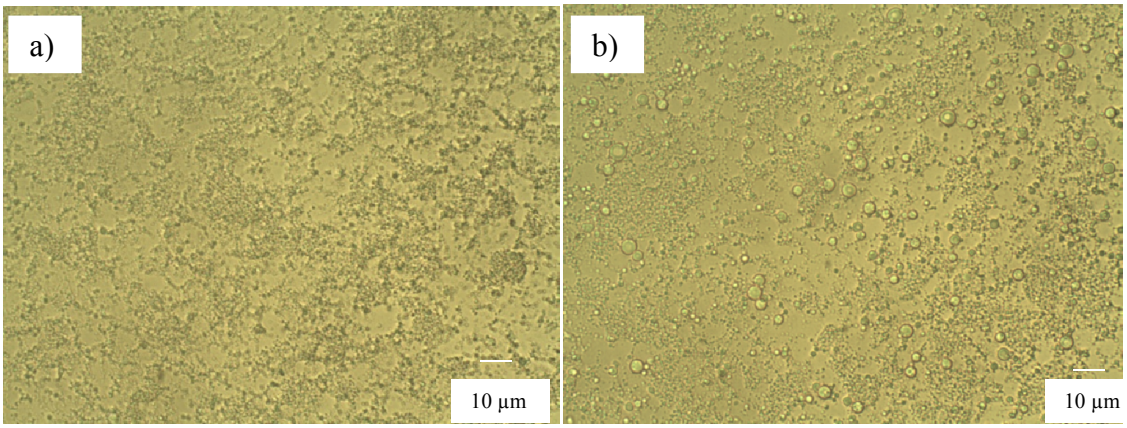

Figure 5.2-12: Micrographs of self tan cream formulation stored at room temperature at 4 weeks: a) without yeast extract or b) with yeast extract. Both images were captured at a magnification of 400x.

Results presented in section 5.2.1 show the viscosity of the self tan cream formulations is unstable, to investigate this in more detail, images of the formulation at 0 (initial) and 16 weeks are presented in figure 5.2-9 to 10 and figure 5.2-12 . The images of the emulsion without yeast extract (figure 5.2-9) illustrate a breakdown in an ordered circular structure of the emulsion from week 0 to week 16. Figure 5.2-9 a) shows a large amount of flocculation and what appears to be aggregates forming (of oil particles), indicating the first stages in emulsion instability. In addition, there appears to be a small quantity of large droplets not observed in figure 5.29 b).

The self tan emulsion with yeast extract (figure 5.2-10), also portrays the same behaviour at week 16, with a decrease in ordered structure, an increase in flocculation and an increase in the quantity of large particles when compared to the emulsion at week 0.

Figure 5.2-12 illustrates that most of changes in emulsion structure observed at week 16 are present at week 4 but to a lesser degree. Both formulations with and without extract demonstrate this, indicating that the emulsion itself is inherently unstable. These images with the signs of emulsion instability correlate well with the drop in viscosity presented in figure 5.2-1 (see section 5.2.1).

Comparing the changes observed with the self tan cream formulation to the face cream formulation (figure 5.2-11). The face cream formulation does not demonstrate the same markers of emulsion instability that the self tan cream presents after 16 weeks. There is no noticeable increase in particle size, flocculation or clumping comparing the images at week 0 and week 16. There is only a small reduction in droplet density, which could be due to the quantity of emulsion on the slide.

5.2.2 ***Colour change analysis***

The change in colour and the rate of change for all of the 4 formulations are presented in figures 5.2-13 to 5.2-17. This is an important factor in a cosmetic product where a stable and consistent colour over a sustained period of time is required.

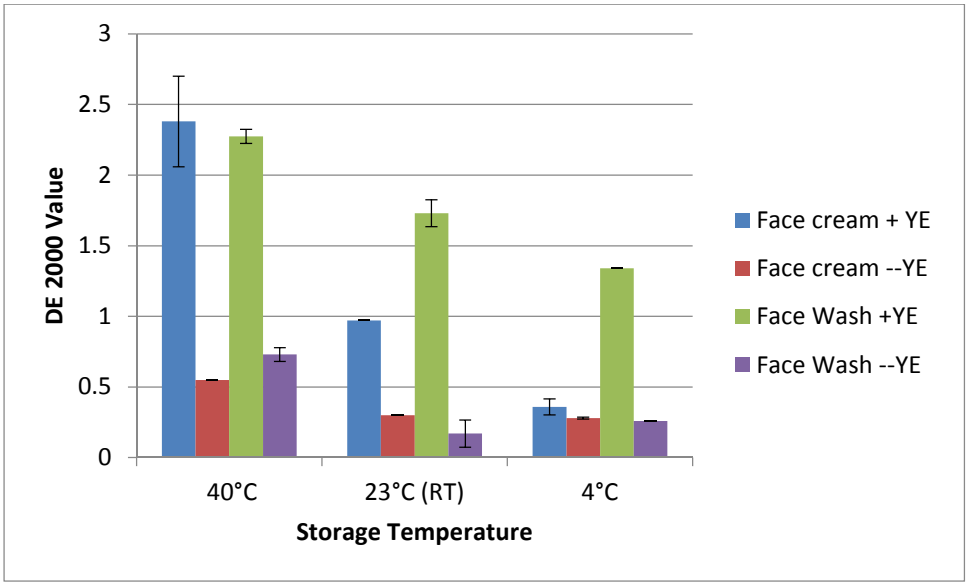

Figure 5.2-13: The colour change of face cream and face wash formulations with storage temperature at week 16. +YE and –YE denotes with and without yeast extract respectively.

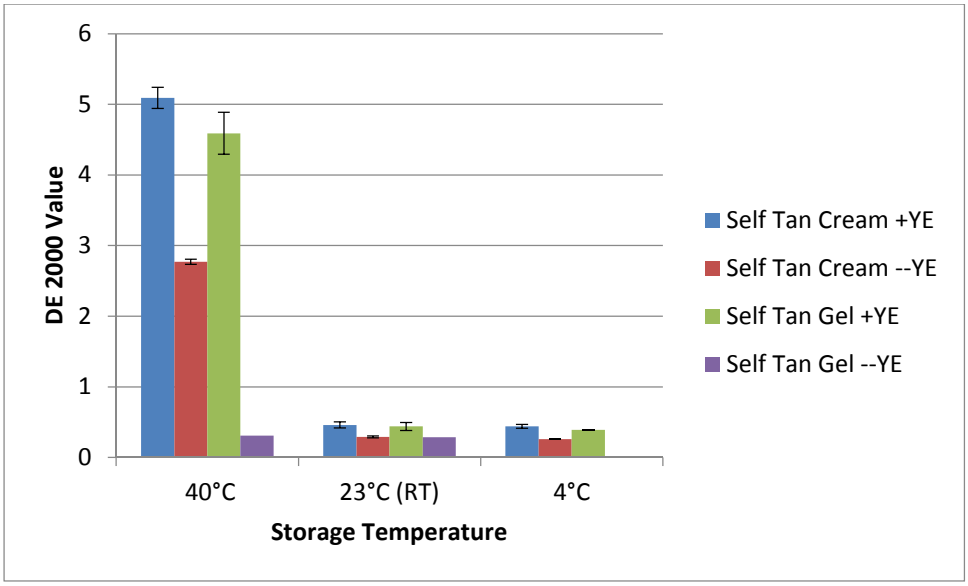

Figure 5.2-14: The colour change of self tan cream and self tan gel formulations with storage temperature at week 16. +YE and --YE denotes with and without yeast extract respectively.

Figure 5.2-13 and 5.2-14 show that formulations with yeast extract stored at 40 °C had significant change in colour after the 16 week period, as the DE2000 values for formulations containing the extract were much larger than 1 (the threshold value). Beyond which value the colour change would be noticeable to normal human vision (Ohta and Robertson, 2005). This contrasts formulations without extract which had significantly smaller values which did not breach the threshold value with exception of the self tan gel formulation without extract stored at 40 °C. All of the formulations showed a significant variation in colour change with storage temperature with respect to DE2000 values, between 40 °C and room temperature (23 °C). This variation is particularly evident with the self tan cream and gel formulations, where a 10 fold increase in DE2000 value is observed. This takes the values from not noticeable (to human vision) to clearly noticeable colour change.

The increase in DE2000 value with storage temperature is more gradual with the face cream and wash formulations; however the DE2000 value of the face cream formulation doubles between room temperature and 40 °C.

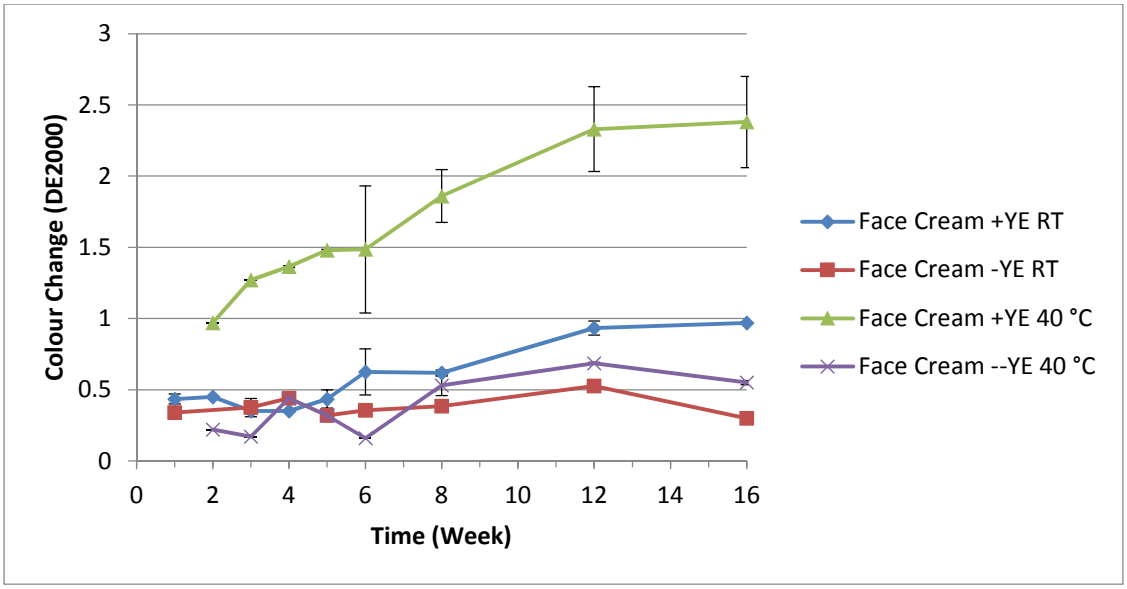

Figure 5.2-15: The colour change of face cream formulations with storage temperature and time. RT denotes room temperature, where formulations were stored at approximately 23 °C.

Figure 5.2-15 to 17 illustrate the rate of colour change with storage temperature and time for all four formulations used. The figures show that for the face cream and self tan formulations with extract, there is a gradual increase in DE2000 value with time at 40 °C, which is also evident in the face cream formulation stored at room temperature. There appears to be a start of a plateau in the rate of colour change with the face cream formulation after week 12 in both samples stored at 40 °C and room temperature, however this does not seem to be the case with the self-tan or the face wash formulations. The colour change of the face wash formulation only seems to increase rapidly after 4 weeks with the formulations stored at room temperature and 40 °C. This is in contrast to the formulations without yeast extract, which show much smaller changes in colour with the exception of the self tan gel. Where the rate of colour change increases after 4 weeks. Of all the formulations without extract only the self tan gel shows any noticeable change in colour (with respect to DE2000 value). The cause of this colour change appears to be an interaction between the extract and the formulations tested, as the majority of formulations without extract do not have any noticeable colour

change and this is further explored in section 5.3.2 and requires future work to elucidate the actual cause of the colour change observed.

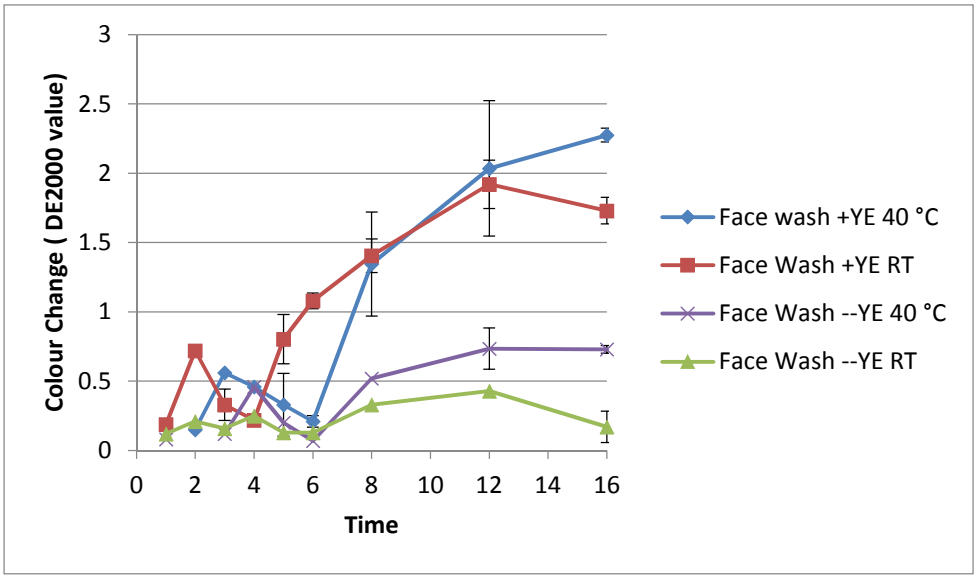

Figure 5.2-16: Depicts the colour change of face wash formulations with storage temperature and time. RT denotes room temperature, where formulations were stored at approximately 23 °C.

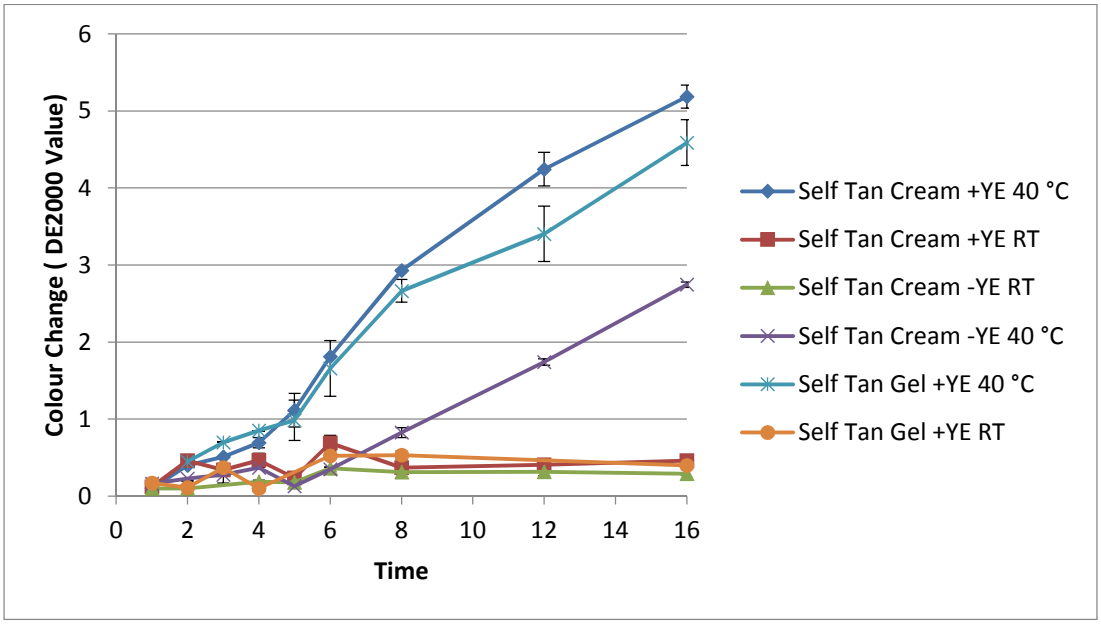

Figure 5.2-17: The colour change of self tan cream and gel formulations with storage temperature and time. RT denotes room temperature, where formulations were stored at approximately 23 °C.

5.3 Discussion

5.3.1 *Formulation stability study*

Emulsion stability and colour change are important factors to investigate in any potential cosmetic product. Therefore, an investigation into the emulsions stability of the yeast extract in a number of prototype formulations (which would be representative of commercial formulations) has been shown in section 5.2.

The results presented demonstrate that out of the four formulations tested: two were stable with no degradation in viscosity or structure (face cream and face wash), two formulations showed signs of instability (the self tan gel and cream formulations) such as a drop in viscosity and flocculation. The large unstructured flocs (or aggregates) formed in the self tan cream formulation suggest that droplets are strongly flocculated together (see section 5.2) and the increase in large droplets suggest either coalescence or Oswald ripening. Further zeta potential and particle size data would be needed to determine whether coalesce or Oswald ripening is occurring and to determine the overall instability of the emulsion. The extract itself is unlikely to be responsible for this as both formulations with and without extract; show signs of instability. Analysing the ingredients in both formulations, results in the conclusion that polyquaternium 37 could be responsible as it is an ingredient found in both formulations and acts as a thickener/stabiliser. Which ideally should maintain the viscosity to the formulation but this does not appear to be the case in this instance. Anecdotal evidence suggests that polyquaternium 37 based gels drop in viscosity at 40 °C and only regain their viscosity when cooled down to room temperature. This could be a factor in the instability (with respect to viscosity) of the emulsion and further work would be required to elucidate the role of polyquarterium 37 (and was not carried out due to a lack of time).

One possible explanation for instability of the self tan formulations could be the repeated heating and cooling cycles that the 40 °C samples undergo. As samples stored at 40 °C were passively allowed to cool to room temperature (approx. 23 °C) during weekly viscosity measurements. This process could have resulted in the structure of the polyquaternium-37 been damaged during the first 4 or 5 weeks of the study, such the compound cannot rebuild the structure of the emulsion at room temperature. This theory is corroborated by the fact that the large drop in viscosity in the formulation stored at 40 °C is not observed with the one stored at room temperature. However further data would have to be carried out to draw any substantive conclusions.

In the self tan cream, cetyl alcohol could also be responsible it acts as a secondary emulsifier, it builds viscosity and forms the circular structure observed in figure 5.2-9 and 5.2-10 (Jumbu, 2007). This circular structure disappears in later images; therefore it could be a result of a breakdown in the cetyl alcohol based structures. As these structure have been seen in previous work (Jumbu, 2007) and they have also been shown to disappear and form aggregates. This occurs when either the pH of the extract added reduces the emulsion pH or the emulsion itself is below pH 5, both of which are true in the self tan cream formulation.

Further zeta potential measurements would have to be carried out to substantiate this theory in a simple formulation where the effect of pH on cetyl alcohol could be investigated. The effect of pH on cetyl alcohol without stabilisation has not been documented previously and the data presented could suggest the need a stabiliser in emulsion where cetyl alcohol is present and the pH of the emulsion is below 5.

The pH change of the overall formulations during the 16 week stability study does not appear to have any correlation to changes in the viscosity or structure of the emulsion, as there does not appear to be any correlation between drops in viscosity and large changes in pH.

In summary cetyl alcohol and polyquaternium 37 could be responsible for the degradation in viscosity and structure of the self tan face cream emulsion; however further formulation work including the measurement of zeta potential and particle size would be required to make any definite conclusions.

5.3.2 ***Colour stability***

The change in colour of a cosmetic product over its shelf life is an important commercial consideration. It can be a symptom of unwanted reactions taking place in the product such as anti-oxidant breakdown.

The results presented in section 5.2 document that the samples do get significant (and noticeable) colour change in all of the prototype formulations with extract stored at 40 °C. This contrasts what occurs at room temperature and at 4 °C, where only the face wash with extract has a noticeable change in colour (with a DE2000 value above 1). The majority of formulations without extract do not have DE 2000 values above 1 (which is noticeable by the human vision), which indicates that either the extract itself is changing colour with storage temperature and /or the extract is reacting with a component of the formulation which is resulting in a change in colour of the formulation. The exact mechanism of action in the formulations can only be speculated at as no definitive analysis was able to be carried out.

If the extract itself is changing colour with storage temperature, this is probably a result of polysaccharide or phenolic breakdown into strongly coloured compounds such as melanoidins. It could also be a result of dihydroxyacetone (DHA) (in the extract), reacting with protein in the extract as Moller et al has shown that subcritical water can convert glucose or fructose into DHA (Moller et al., 2011). This is corroborated by the fact that the colour change occurring in the formulations is in the yellow spectrum (undocumented data), and that melanoidins have been found in apple extracts which have been processed using subcritical water (Wijngaard and Brunton, 2009).

In the face cream and wash formulations the relationship between colour change and storage temperature is more gradual indicating that either one of the two suggested mechanisms could be taking place. Due to lack of data it is impossible to isolate which one (or both) are responsible.

In the self tan formulation it is likely to a result of both of the suggested routes, as DE2000 values of formulations with extract (stored at 40 °C) are double that of the face cream and wash formulation. Dihydroxyacetone (DHA) and the erythlose (the two self tan active ingredients) do react with proteins in skin to form melanoidins (a brown pigment) (Wittgenstein and Berry, 1960). Therefore this could be occurring in the formulation as the yeast extract is known to contain proteins (Bahari, 2010) and the colour change is mostly in the yellow spectrum (undocumented data) . This does not occur with the formulations stored at room temperature probably due to increased viscosity of the formulations at this temperature. Together with the fact that DHA and erythlose are temperature sensitive and it is not advised to store both above room temperature for long periods (Merck (2012)).

5.4 Conclusion

In conclusion, the data from this scoping study suggests that the face cream and wash were stable while the self tan formulations were not. Further work (including zeta potential data) would be required to confirm this and elucidate the role of polyquaternium 37 and cetyl alcohol in the formulations instability. In addition, further zeta potential measurements would be required to confirm the flocculation observed in the micrographs presented in the chapter. The colour change of all the formulations is an issue that needs further work to elucidate the mechanism of action and to reduce the change in colour of formulations.

CHAPTER 6 : CONCLUSIONS AND FUTURE WORK

6.1 Overall conclusions

Overall the work presented has demonstrated that the cider yeast extract has anti-oxidant activity both *in vitro* (in chapter 3) and in cells (in chapter 4) using both the ORAC, TEAC and the comet assays which has not been documented previously.

From the results presented in section 3.2, it can concluded that 200 °C or 225 °C is the optimum processing temperature with regard to anti-oxidant capacity. The data presented have shown that processing the extract below 200 °C results in an extract, which has much less antioxidant capacity, while extracts produced at 300 °C show signs of degradation in anti-oxidant capacity with reaction time. The data presented correlates with data showing a large increase in phenolic concentration and this suggests that the increase in phenolic concentration could be responsible.

This is corroborated by data showing a large increase in anti-oxidant capacity of the HPLC fraction, which is thought to contain a much larger concentration of phenolic compounds as a percentage of its dry weight. The data presented in section 3.2.2 also suggests that phenolic compounds are the major contributor to the anti-oxidant capacity of the extract with regard to the anti-oxidant capacity of the extract.

The data presented has been used to produce a refined extract with a focus on phenolic concentration using supercritical CO_2 and the hydrolysed extract as a feedstock. This extract has shown a two fold increase in anti-oxidant capacity when compared to the hydrolysed extract. Therefore reinforcing the belief that phenolic compounds could be the main contributing factor to the anti-oxidant capacity of the extract.

The results from the TEAC and ORAC assay show that the extract has antioxidant capacity in two independent assays using two different mechanism of action. This difference could be the reason why different trends are observed in both assays with relation to how antioxidant capacity changes with temperature. The differing trends also suggest that composition of the 200 and 225 °C extracts differs significantly which is corroborated by the HPLC traces of both extracts.

The data presented in chapter 3.2 also demonstrates that the scaling up process (from 200 ml to 3 L) has not negatively affected the anti-oxidant capacity of the extract. However, batch-to-batch variability has been observed and could a result of how the cider lees is a mixture of number of fermentations. However further work would have to be carried out to make any definitive conclusions.

The performance of the extract has been shown to be comparable to those produced by Prior et al and appears superficially comparable to the plant and algae based extracts. Further comparisons cannot be drawn due to the way that most studies have expressed their data.

Characterisation data presented in section 3.2.2 has suggested a role for proanthcyandins and chlorogenic acid reaction products in addition to HMF. However further characterisation work is urgently required to establish the compounds that contribute to the anti-oxidant activity of the extract.

The work presented has shown that a combination of anti-oxidant and comet assay can be used to refine process conditions thereby fulfilling one of overall aims of the project.

The results presented in section 4.2 show that the anti-oxidant capacity of the 200 °C extract can be reliably demonstrated across both independent runs and different batches of the extract. In addition, there appeared to be little difference in extracts produced in the 300 ml

reactor and the 5 L reactor. The data also demonstrated that the extract performed comparably to cells dosed with Trolox.

The results from chapter five have shown that of the four prototype formulations investigated, two of which were stable (the face cream and the face wash). The face cream and face wash formulations containing extract appeared to perform comparably to formulations without extract which were representative of standard base formulations used at Boots.

The other two formulations tested (the self tan gel and cream) showed signs of instability; both formulations stored at 40 °C and room temperature had large drops in viscosity of the 16 week period, in addition aggregates/flocculation are visible in the micrographs presented in section 5.2.1.1. Both formulations with and without extract seemed unstable, therefore this suggests that the extract itself is unlikely to be responsible. However further work is required to corroborate this and the conclusion the type of instability observed. Further zeta potential work could be used for this and the data from this work could pinpoint the type of instability present and confirm the suspicion that polyquaterium 37 which is present in both of the formulations is responsible.

The data presented in section 5.3.1 demonstrates that pH change does not seem to have any correlation between emulsion stability and a change of pH over the 16 week period.

The conclusions that can be drawn from the colour stability data presented in section 5.2.2 are that formulations containing extract have colour stability issues. This is particularly evident with samples stored at 40 °C. The self tan formulations appear worst affected by this; literature has suggested that this could be due to an interaction between DHA (dihydroxyacetone) and proteins found in the extract as the colour change is in the yellow spectrum, however further would have to be carried out to corroborate this. For the face

cream and face wash formulations further work would have to carried out to elucidate why the formulation with extract have changed colour while formulations without have not. Currently the rational for the colour change can only be speculated.

Proving these issues can be resolved the extract could be used as an active ingredient in a cosmetic formulation.

6.2 Future work

Further work that could be carried out includes:

Testing the efficacy of the extract in the comet assay in more detail by investigating further dilutions. So that a baseline amount could be established were no further activity could be observed. This would be especially important with the phenolic extract and would help determine purification efficiency; and help determine the role of phenolics in the overall anti-oxidant activity of the extract.

Carrying out an assay evaluating the anti-oxidant capacity of the extract in the prototype formulation using the comet assay, or using a hydroperoxide assay to measure anti-oxidant capacity in an emulsion system (as has been done by Frankel (Frankel et al., 1996)).

Further characterisation of the phenolic extract would be required to define the role of phenolics in the overall extract and would enable easier comparison of results to the literature.

A more detailed investigation into the composition of the extract using a coupled HPLC, mass spectroscopy and NMR (if possible) as this would also help to confirm the presence of HMF (in the 200 °C extract) and chlorogenic acid (in the 150 °C extract). In addition further

characterisation work using samples from various points during the cider production process would help elucidate the source of the phenolics in the extract..

A more detailed investigation into the flocculation and emulsion stability issues seen in two of the formulations with a zeta sizer so that a zeta potential could be established, which would be used to confirm the data already presented.

Further work investigating the role of polyquart-37 and cetyl alcohol in the lack of stability emulsion stability in the two formulations.

Characterisation of the colour change mechanisms seen in formulations would be valuable. This understanding could enable solutions to the colour change to be found which would increase the commercial value of the extracts.

Further investigations to understand the inconclusive results from the cell viability and metabolism results described in appendix A could also be pursued.

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Appendix A1

First year report: An investigation into the biological activity of a cider yeast extract using human fibroblasts

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Sponsors: Alliance Boots

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Abbreviations:

Listed below are abbreviations that are used in the rest of the report.
MTT : Methylthiazolyldiphenyl-tetrazolium bromide (MTT)
Calcein AM : Calcein *O*,*O*′-diacetate tetrakis(acetoxymethyl) ester
Hoechst 33258 or Hoechst: bisBenzimide, 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride hydrate
NADH: Nicotinamide adenine dinucleotide hydrate

1. Statement of Objective/Aim.

The main aim of my project is to investigate how an extract obtained from the hydrolysis of spent cider yeast affects the biological activity of the extract, with a view to improving the processing of the extract. So that a more biologically effective extract can be produced.

The two key biological areas we are focusing on in the PhD are cellular metabolism and cellular senescence.

2. Introduction.

Yeast extracts have been used in a number of different industries including the cosmetic, pharmaceutical, food and a number of other industries. They are used due to high vitamins and minerals content of yeast and, that yeast are readily available and are effective source of these vitamins and minerals (Sweetman, 2007). That is why they have been used in a number of the cosmetic and pharmaceutical products (Zulli et al 1998). Such as yeast supplement tablets and skincare formulations. Yeast extracts are also used as flavour enhancers (Stojanova, 2001).

Characterisation work has shown that the cider yeast extract contains small amounts of vitamin B3 and significant amounts of phenolic compounds, such as chlrogenic acid, levealunic acid and hydroxymethylfurfural. Further characterisation work is ongoing.

B vitamins are widely used in the cosmetic industry especially vitamin B3 which is used as in cellular metabolism and is used to generate NADH. More significantly vitamin B3 has also been shown by Kang et al to lengthen the replicative lifespan of human dermal fibroblasts. Vitamin B3 has also been used clinically to treat roseaca (Nirem, N.M. 2006).

Chlorogenic acid especially is well known in the literature as an anti-oxidant, and has been has been proposed as a potential active in a sunscreen. (Choquenet et al., 2009) Anti-oxidants in general have been linked to a reduction in cellular DNA damage and an increase in cellular lifespan (Guarente et al 2008). Investigation into the effects of the other phenolic compounds is ongoing.

To assess the effect of the cider yeast extract on the biological activity of the cells, a number of assays were chosen to match the two key biological areas. They are cellular senesce and metabolism and were chosen in consultation with my sponsor and that vitamin B3 was is involved in key metabolic pathways and that it seems to have an effect on cellular senescence. The criteria for choosing the assays were that they should be relatively simple and rapid to perform and gave us definitive results.

Initially to assess cellular viability, trypan blue stain in conjunction with a haemocytometer was used. Trypan blue stains dead cells blue, while only stains the outside membrane of viable cells.

The three assays chosen for the plate reader assisted segment of the results included: the MTT (Methylthiazolyldiphenyl-tetrazolium bromide) assay; which is a metabolic assay in which MTT is metabolised by active cells to a formazan (Mosman et al, 1983). Formazan has an absorption maximum at 570nm when re-suspended in isopropanol-HCl. The higher the amount of formazan and absorbance at 570nm indicates either: a) a higher cell number or b) a higher cell metabolic activity.

The second assay is using calcein AM (calcein *O*,*O*′-diacetate tetrakis(acetoxymethyl) ester) which is a cell viability marker that is converted by enzymes in viable cells to calcein (Essodaïgui et al 1998). Calcein is a fluorescene derivative, which fluoresces green and the fluoresce is proportional to the amount of viable cells.

The Hoechst assay (from Invitrogen, paisley UK) measures total cell number, it contains a DNA stain, Hoechst 33258 which fluoresces proportionally to the DNA in the sample. (Daxhelet et al, 1989) This can be used to accurately determine the amount of total cells (alive or dead) as cellular DNA is highly regulated.

3. Achievements

Firstly carried out cell culture without infections, and learnt other cell culture techniques which enabled me to successfully revive and freeze cells that were stored in liquid nitrogen. This was initially done with a preliminary (3T3) cell line.

Establish a new human fibroblast cell bank. This involved growing the cell line successfully and freezing (banking) at specific points or passages and changing the media to a more standard media from a proprietary media. This involved evaluation of a number of different media to find one most suitable for the human fibroblasts.

Carrying out a initial growth curve with the 3T3's, and also looking at the effect of vitamin B3 on the viability of 3T3's over 10 passages (approx 3-4 weeks).

Freezing of fibroblasts at passages 2,3,7,12,14 and 18 which is still ongoing (currently at passage 26). This allows us to build a bank of cells of different ages to use in experiements some of these cells have been used in current work and were used in the first batch of cell viability work and in the initial human fibroblast growth curves.

Viability studies using yeast extract b1-103 (hydrolysed at 200 °C at 15 bar for 30 mins), this initially involved generation of a growth curves at two different seeding densities to establish the ideal seeding density.
Cell viability studies were then carried out with attached and non attached cells at various concentrations.

Set up of the plate reader which would allow many more repeats and many more samples to be screened, this involved finding and purchasing all relevant consumables.

Development of relevant assays; which is still ongoing. Most of the assays were bought in kit form but still needed modification. The assays are based on MTT, Calcein Am and Hoechst 33258 stain.

Running of the first viability and metabolic activity screening, using new samples generated by another PhD student in Chemical Engineering. In this run 17 different conditions were compared with 5 repeats for each. This included 3 different concentrations *(386 µg/ml, 772 µg/ml and 1158 µg/ml)* and 3 different batches which could be compared. Two different conditions (*hydrolysis at 200 °Cand 225°C)* were also compared.
One of the major problems overcome was the set up of the experiment which was done manually. Also feeding all the cells with a single channel was extremely challenging due to time restraints,

Modification of assay protocol for the calcein Am assay (due to initially background issues) which has been changed for all future runs and a new calibration curve has been produced.

Successful running of Hoechst assay which assess the total number of cells. However some of the results from this assay are slightly anomalous and this assay protocol needs to be looked at to see if it can be refined at all.

Running a 14 day screening using the crude, unhydrolysed extract with the three different assays to have a look at the effects of the crude extract.

Sterilising the crude extract as it had a high possibility of containing dormant yeast and as the extract would not filter through a range of filters, (0.22, 0.45, or 1.2um). A solution to this issue was found by diluting the crude extract in cell media and then filter sterilising it.

To summarize all of the runs concluded so far has which have included: (all of the runs have been carried out with Human Dermal Fibroblasts (HDF's) unless otherwise stated.)

Vitamin B3 with 3T3's,

Extract b1-103 with non attached cells at concentration of 2% and 3%

Extract b1-103 with attached cells at concentrations of 2% and 3%

Extract b1-103 with non attached cells at concentrations from 0.1 to 1%.

Viability of cells incubated with extract hydrolysed at 225 °C and 200 °C.

Calibration curves used to trail the assay protocols on the new plate reader.

Runs with extract hydrolysed at 200 °C and 225ºC, with 3 different batches and 3 different concentrations. 200 °C

Run with crude extract (unhydrolysed) at 4 different concentrations.

4. Results and Discussion

4.1 Initial Results

Initially, vitamin B3 was incubated with 3T3 fibroblasts, to investigate how vitamin B3 affected cell viability, as had been done by Kang et al. This involved assessing the overall dead cell count of 3T3's (as a percentage of total cell population) by using a haemocytometer and trypan blue viability stain. 5mM vitamin B3 was added to the cells and the viability of the cells measured at each passage. The results of which are shown in the table 1.

Table 1: The viability results of the vitamin B3 study using 3T3 Fibroblasts.

Passage	Average dead cell % Without B3	Average dead cell % With B3
20	8.9	5.8
21	6.5	6.8
22	7.9	5.5
26	9.1	6.0
27	7.0	5.9
28	6.3	4.9
30	12.7	10.0

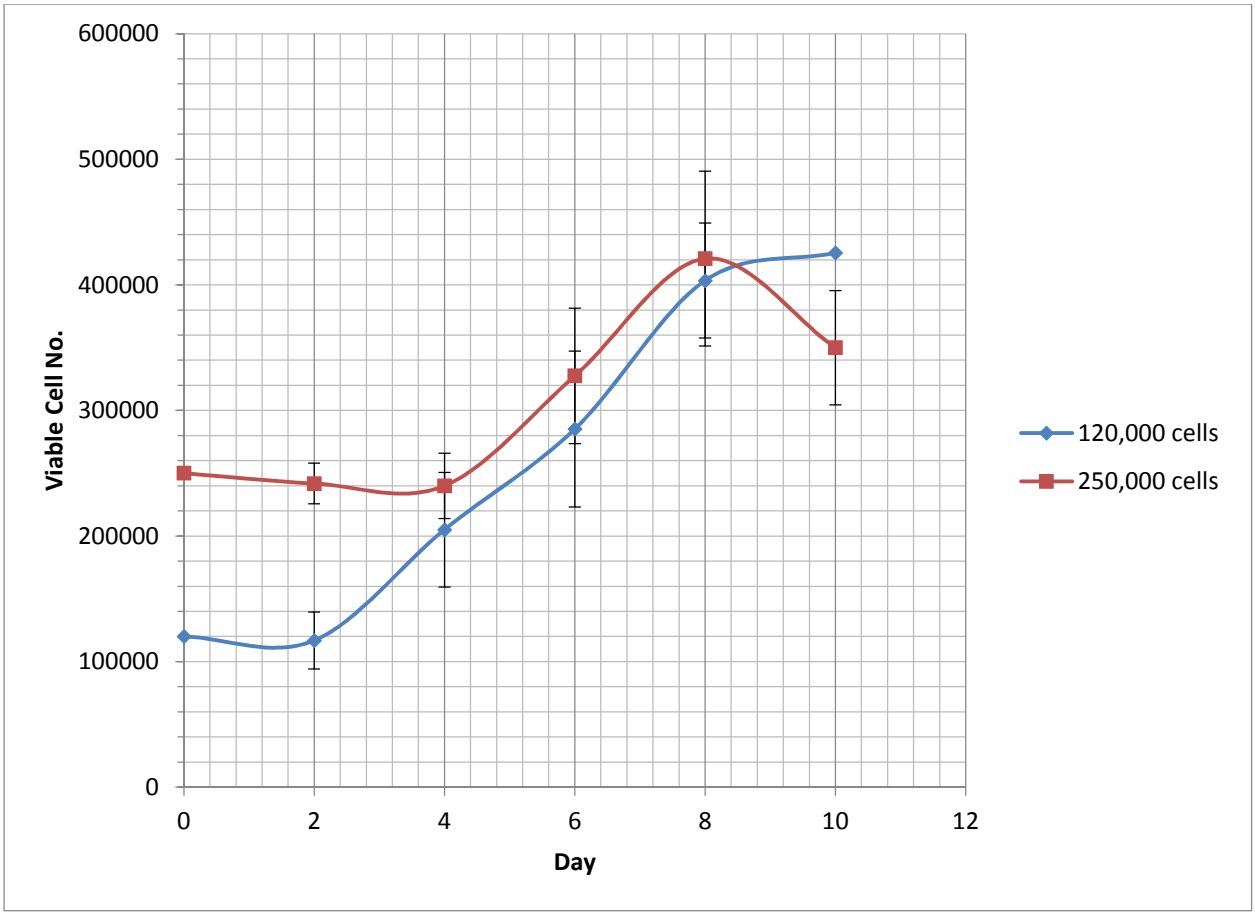
The results shown in table 1 illustrate that vitamin B3 has a small negative impact on the average dead cell percentage when compared to the control sample without B3. On average the difference in the dead cell percentage varies by approximately 2%, between the treated and none treated samples; however this does vary significantly across the passages.

As this assay was done with 3T3's; and with a limited amount of repeats; the experiment would have to be repeated on human fibroblasts with a larger amount of replicates, to get a more reliable result. As vitamin B3 was only found in limited quantities the focus has shifted to investigating the effect of the yeast extract as a whole.

4.1.1 Initial Human Fibroblast Results

A growth curve was generated so that the growth profile of the fibroblasts could be evaluated and the correct seeding density found. This was carried out in a 6 well plate, which has a surface area of 9.6 cm^2/well using the trypan blue viability stain.

The results of the initial human fibroblast growth curve are illustrated in the graph 1 below.



Graph 1: The growth profile of human fibroblasts at two seeding densities.

The results from the growth curve (shown in graph 1) illustrate that the 120 x10^3 seeding density (per 9.6 cm^2) would be the ideal seeding density (for a growth curve experiment) because its shows all of the expected phases of cellular growth with a substantial exponential phase. At this seeding density there is a short lag and extended exponential phases; while the 250 x10^3 growth curve has a long lag phase (till day 4) with short exponential phase.
Also there is a peak of viable cells at day 8 which is earlier than in the 120 x10^3 seeding density, this is to be expected and is due to the fibroblasts running out of surface area (as fibroblast growth is contact inhibited).

4.1.2 Yeast extract b1-103 and subsequent batches

Non attached human fibroblasts were initially incubated with 2% and 3% w/v of extract b1-103 (hydrolysed at 200 °Cat 15 bar for 30 mins) for 3 days. After the 3rd day all of the cells which had been incubated with the yeast extract were found to be dead. One of the reasons for this result could be that the extract could be interfering with the attachment of the fibroblasts.

Therefore pre attached cells were incubated with the extract b1 – 103 at 2% and 3% w/v, with cells initially seeded at 250 $x10^3$ cells per well. The viability of the cells was then assessed with trypan blue and results are shown in table 2.

Table 2: The viability results for pre attached fibroblast incubated with extract at 2% and 3% w/v.

	Well 1 (count)	Well 2 (count)	Well 3 (count)	Average count	Cell No. $X10^6$
2% (w/v)	41	32	32	35	0.356
3% (w/v)	32	33	42	35	0.361
Control	35	32	34	34	0.341

The viability results presented in table 2 show that cells incubated with the extract at 2% and 3% w/v, have no negative effect on cell viability. In fact there seems to be a slight positive effect after the 3 day incubation; when the result is compared to the control.

This indicates that the extract has some negative effect on cell attachment.

As non attached cells failed to survive at 2% and 3% w/v with extract b1-103. Lower concentrations of the extract were incubated with non attached cells. As in previous experiments cells were seeded at 250 $x10^3$ cells per well. The results are shown in the table 3 (below).

Table 3: A Lower concentration of extract b1-103 was incubated with wells seeded with 250 $x10^3$ cells (non attached); the viability of the cells was measured after 3 days.

Concentration (% w/v)	Average count	Average cell No. $x10^6$
1	28	0.283
0.5	33	0.330
0.4	24	0.240
0.3	34	0.339
0.2	45	0.454

The results of this viability experiment (shown in table 3), indicate that as concentration of the extract increases from - 0.2% to 1% w/v there seem to be a small decrease in the number of viable cells. The only exception is the 0.4 % w/v result, which is lower than expected.

To evaluate the effect of a fresh batch of extract at two different processing temperatures, another viability screening was prepared, with a 24 well plate seeded at density of 26 $x10^3$ cells per cm^2 which is equivalent to the previous viability studies.

Three different concentrations were tested to correlate with previous viability experiments; these were: 1%, 2% and 3% w/v, each of the samples were done in triplicate and the average results are shown in the table 4 below.

Table 4: The viability results from incubation with a new batch of samples hydrolysed at 225ºC and 200 °C.

sample processing conditions	225 °C , 30 bar , 15 mins		200 °C , 30 bar , 15 mins		Control	
Concentration (w/v)	Average count	Average cell No. $X10^3$	Average count	Average cell No. $X10^3$	Average count	Average cell No. $X10^3$
1%	17	68	15	58.7	39	59
2%	6	24	14	54.0	n/a	n/a
3%	0	0	5.0	20.3	n/a	n/a

The results shown in table 4 illustrate that as concentration increases the viability of the cells decreases. This is especially stark with cells incubated with the 225ºC extract. The results show that 1% w/v and 2% w/v are the highest concentration of the extract that could be tolerated by the cells with samples hydrolysed at 225ºC and 200 °Crespectively. In addition the results illustrate that cells are more tolerant of the extract hydrolysed at 200 °C.

The results in general, are in contrast to previous viability results that showed that cells only survived with an extract concentration of upto 1% w/v with a similar extract. This could be due to the extract being much older and batch to batch variation.

4.2 Microplate based results

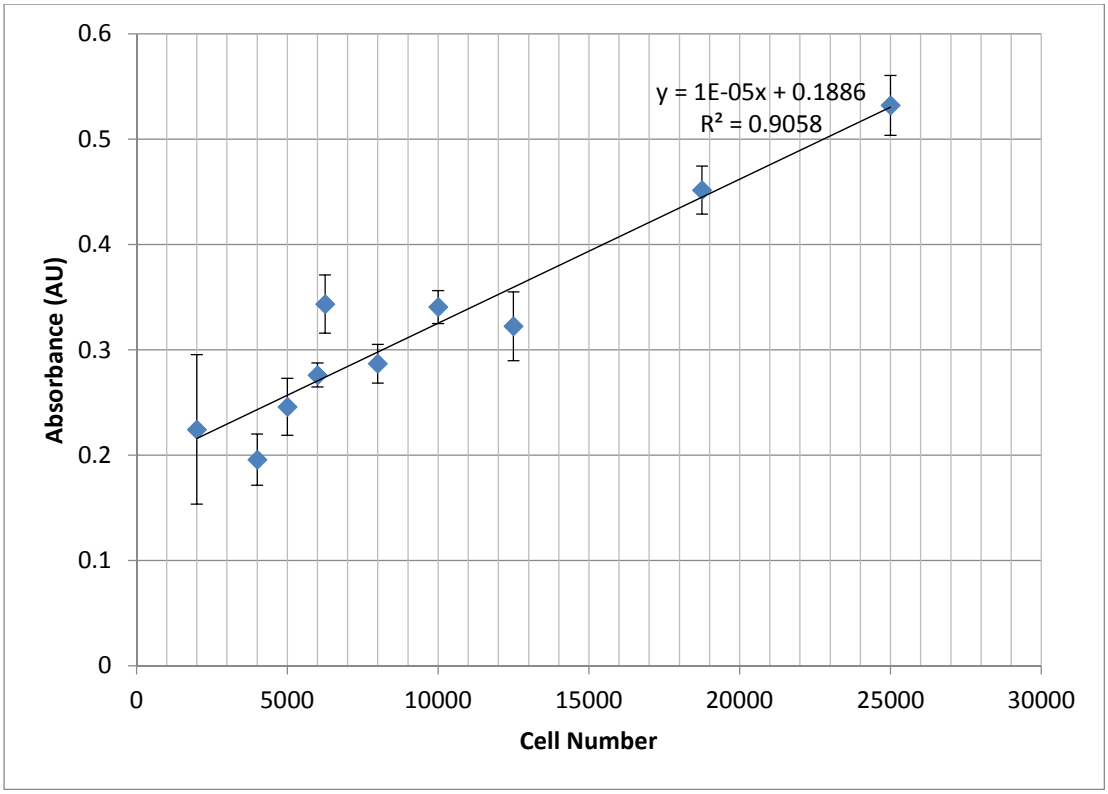
4.2.1 Introduction to microplate based results.

Three microplate based assays were developed to assess the metabolic output and the viability of cells when incubated with hydrolysed yeast extract over a 2 week period. The three assays used were MTT(Methylthiazolyldiphenyl-tetrazolium bromide), Calcein AM (Calcein *O*,*O*′-diacetate tetrakis(acetoxymethyl) ester) and Hoechst stain (bisBenzimide, 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride hydrate), which gave us information on cellular metabolic rate, the number of viable cells and the total cell number respectively.

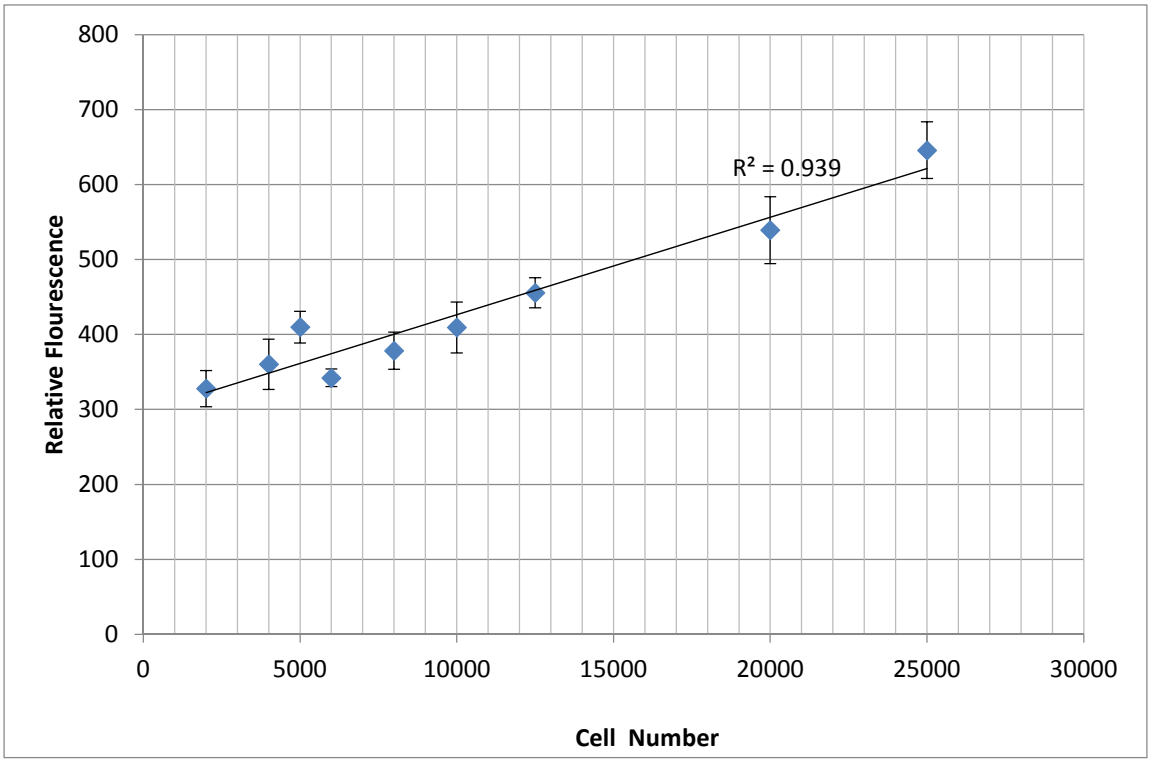
This allowed us to improve throughput; investigate to batch to batch variability and extract concentration.

4.2.2 Calibration Curves, and assay parameters

Prior to commencing the screening calibration charts for MTT and Hoechst assays were produced, these charts are shown in graphs 2 and 3.



Graph 2: Calibration chart for the MTT (metabolic) assay .



Graph 3: Calibration chart for the Hoechst Assay.

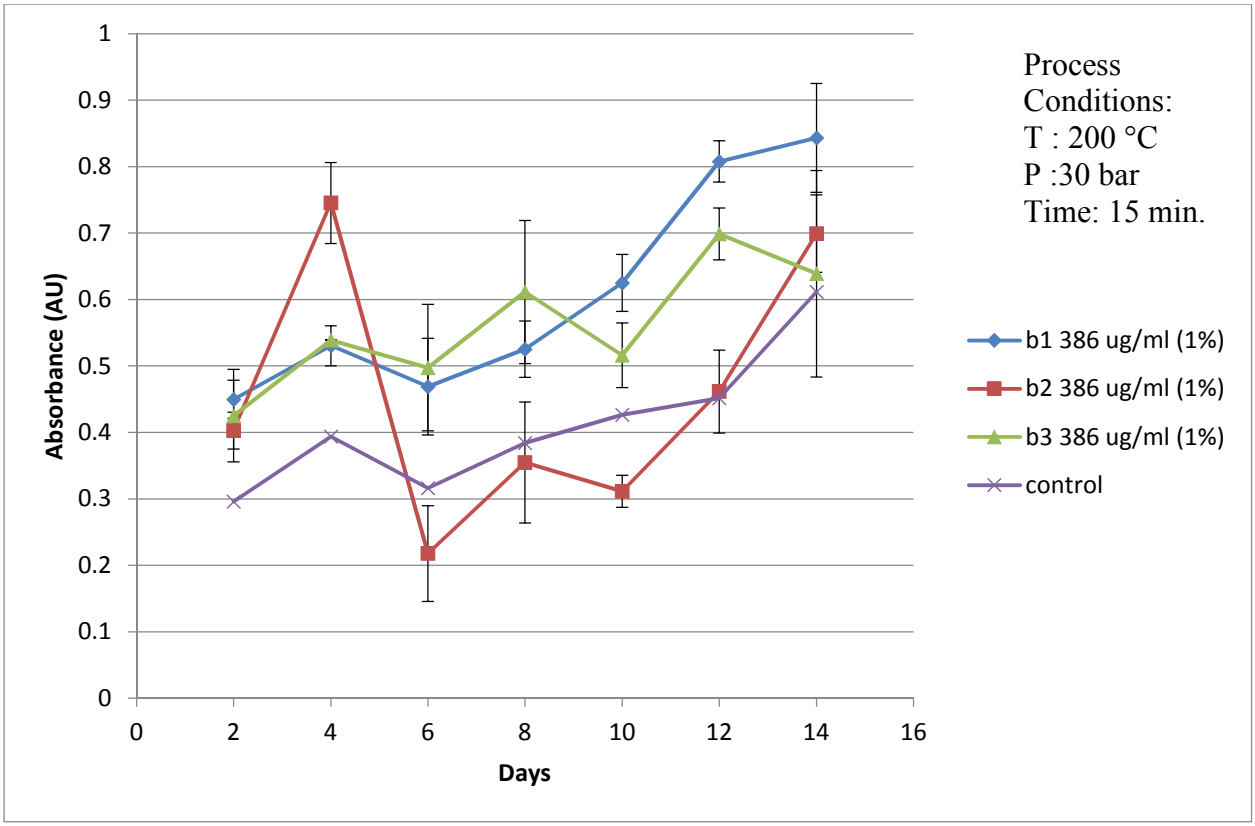
To set up the experiment, 96 well microplates were seeded with 4.1 x10^3 cells / well and incubated with extract that had been hydrolysed at 200 °Cand 225ºC at 3 different concentrations; with 3 different batches for each hydrolysis temperature. Assay results were gathered every 2 days along with a change of media for the cells.

During this first run it became clear that the background readings for the calcein AM based assay were unacceptably high and varied widely between days. This was not picked up when the calibration curve that was prepared for the calcein AM assay. This was due to supplemented media containing non specific esterase's which invalidated the results. Therefore the results for this screening are not included. The protocol for the assay has been modified for all future runs.

4.3 Methylthiazolyldiphenyl-tetrazolium bromide (MTT), Results Part 1

The next series of the results are from the MTT (metabolic) assay, with fibroblasts incubated with a yeast extract hydrolysed at 200 °Cat 30 bar for 15 minutes with various concentrations. All results shown are an average of 4-5 results (with outlying results removed).

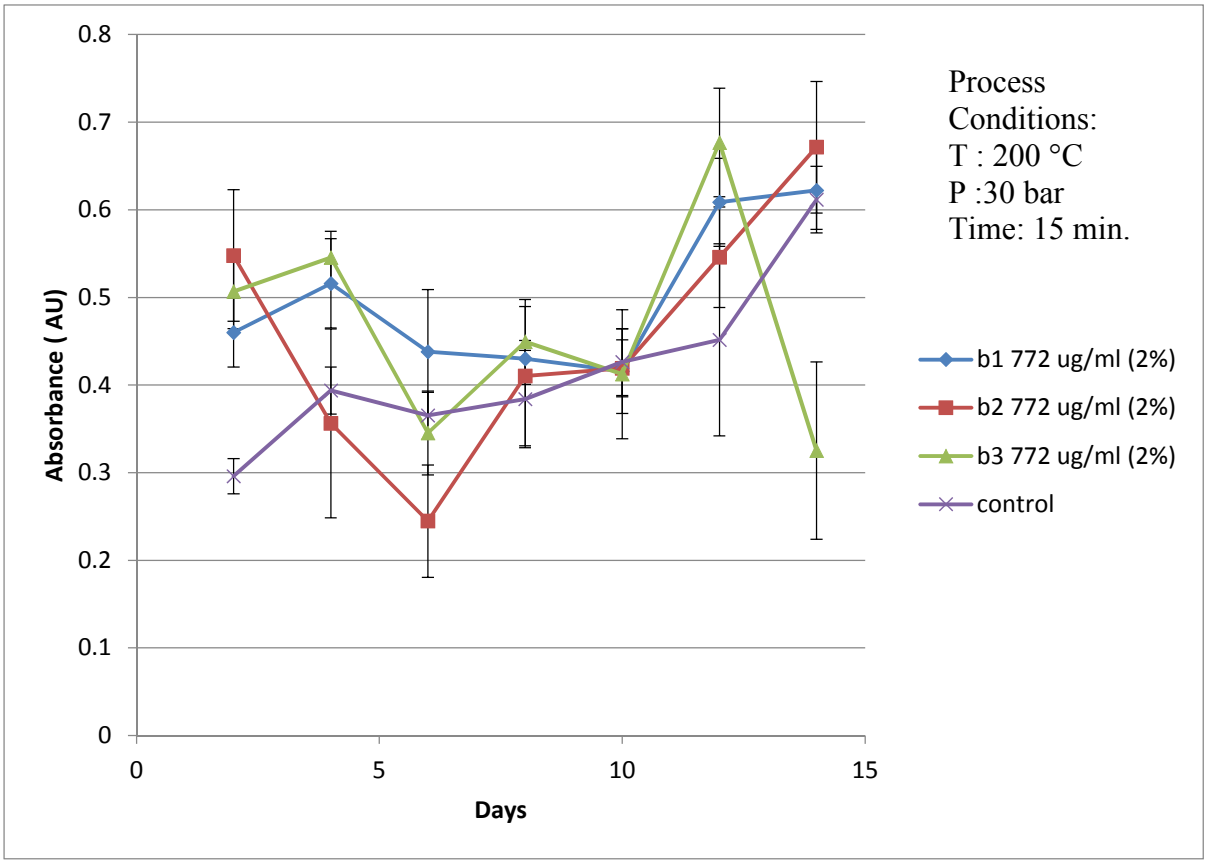
The graph shown in graph 4 shows the change in absorbance (or metabolic activity) of the cells incubated with the yeast extract hydrolysed at 200 °Cat a concentration of 386 ug/ml over a period of 14 days. Abbreviations b1, b2 and b3 refers to batch 1, batch 2 and batch 3 respectively, which are the different batches of the same extract.



Graph 4: showing the change in metabolic activity of cells incubated with extract hydrolysed at 200 °Cat a concentration of 386 µg/ml.

Graph 4 illustrates the batch to batch variability on the absorbance (or metabolic activity) of the cells (especially batch 2). Batch 1 of the extract (b1 in the legend) seems to have an overall positive effect on the metabolic activity over the 14 days when compared to the control sample. The effect of batch 2 from day 2 to 10 overall is negative and from day 6 to 10 there seem to be a small increase in metabolic activity of the cells indicating that the cells are not growing. This is in contrast to the effect of batch 1. After day 10 there is a larger increase in metabolic activity however. In batch 3 there is an overall increase in metabolic activity (which is related to the increase in absorbance) however it not as large as the one

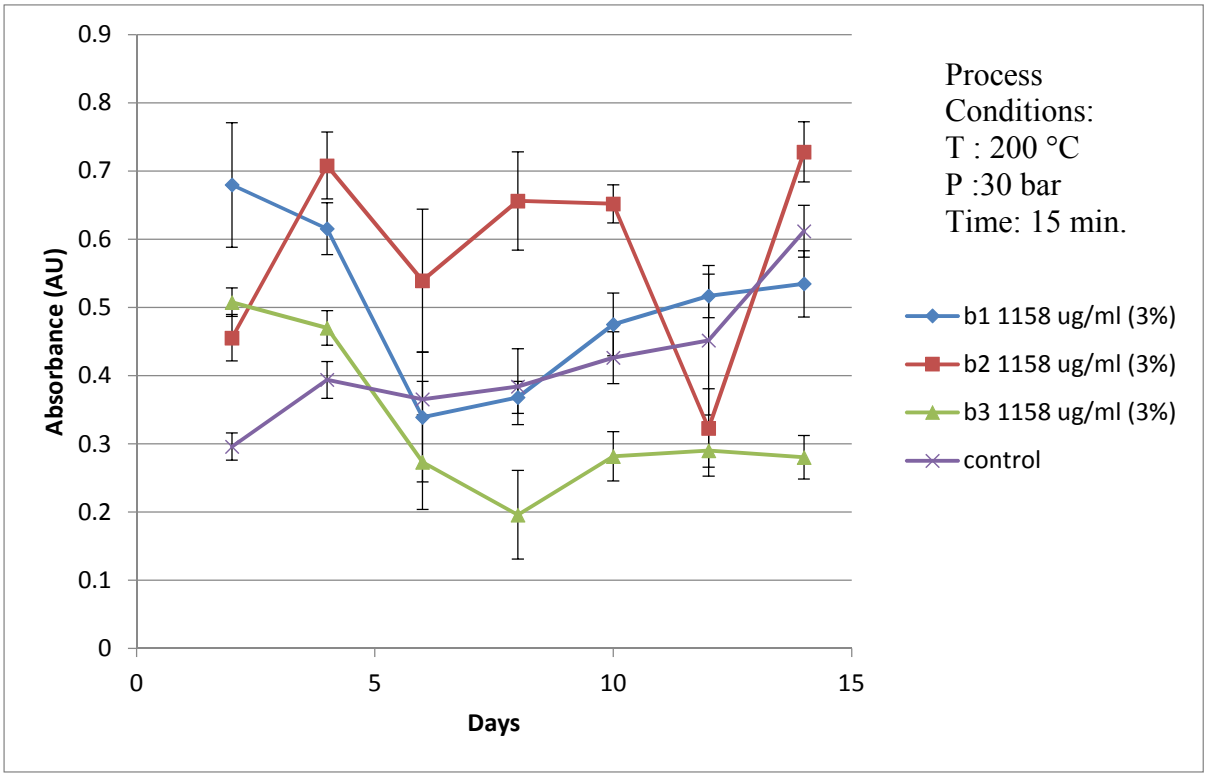
seen in the control or in batch 1 and is variable. However the profile of batch 3 is similar to that seen in batch 1 but the profile of these batches seem quite different to batch 2.



Graph 5: showing the change in metabolic activity of cells incubated with extract hydrolysed at 200 °Cat a concentration of 772µg/ml over a period of 14 days. Abbreviations b1, b2 and b3 refer to batch 1 , 2 and 3 respectively and are different batches of the same extract.

Graph 5 shows that overall; cells incubated with extract show an increase in metabolic activity over the 14 days when compared to the control sample. However the change in metabolic activity from day 2 to 14 is not as great as seen in the control sample. These results seem more reproducible across the batches than the 386µg/ml samples (the last result in batch 3 I would regard as anomalous). The major difference is the graph of batch 2; which

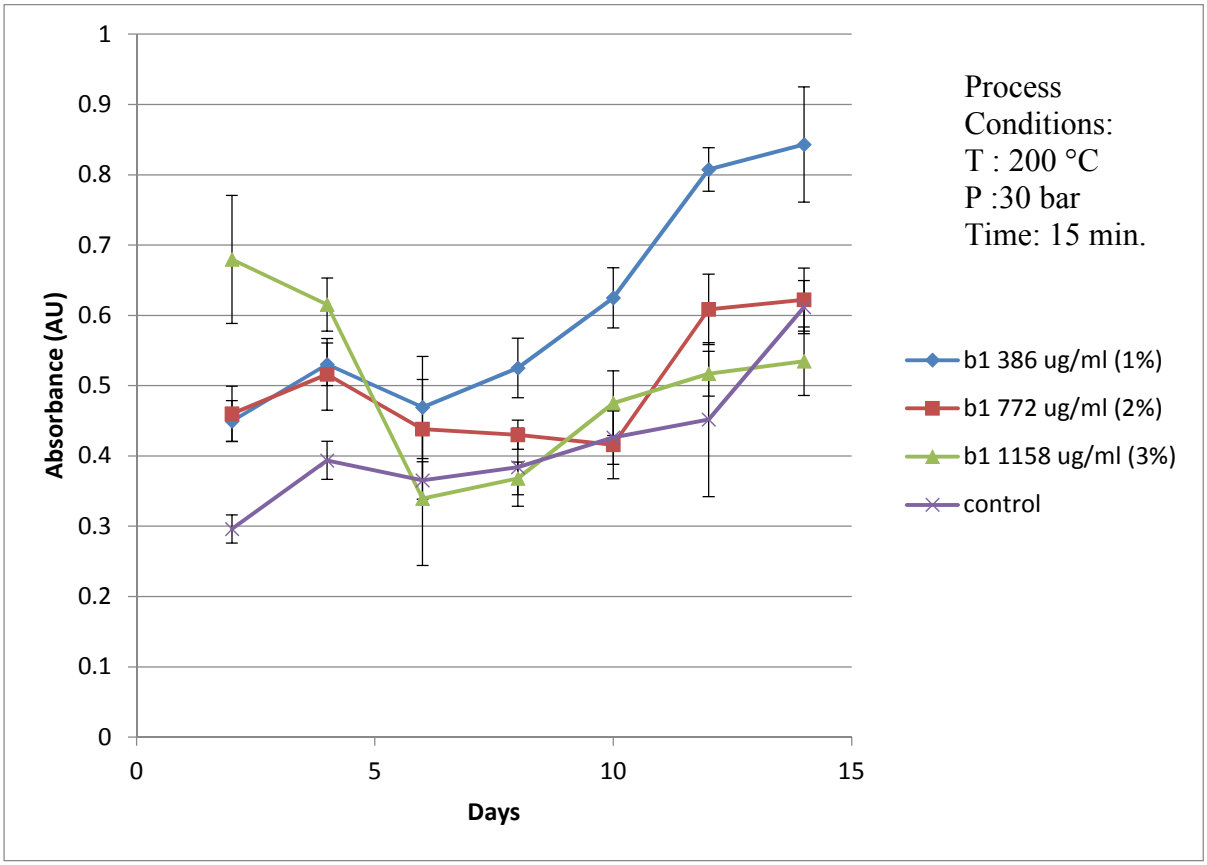
from day 2 to 6 declines rapidly but then recovers from then to day 14. This profile is not mirrored in batches 1 or 3.



Graph 6: showing the change in metabolic activity of cells incubated with extract hydrolysed at 200 °Cat a concentration of 1158µg/ml over a period of 14 days. Abbreviations b1, b2 and b3 refer to batch 1 , 2 and 3 respectively and are different batches of the same extract.

Graph 6 illustrates that at a concentration of 1158µg/ml the 200 °Cextract across batches 1 and 3 has an overall negative effect on the metabolic activity if the cells. Especially when you compare the profile of batch 1 and 3 to the control. In both of these batches from day 2 to 6/8 there is a sharp decline in absorbance (or metabolic activity) of the cells indicating either cell death, or metabolic inhibition. However after day 6 the absorbance (or metabolic activity) increases slightly but does not recover to its initial point. This is in contrast to the results of

batch 2 which show an overall increase in metabolic activity; however after day 4 there is a plateau in metabolic activity of the cells from day 4 to 14.



Graph 7: Shows the change in metabolic activity of cells when incubated with different concentrations of extract hydrolysed at 200 °Cover a period of 14 days within the same batch.

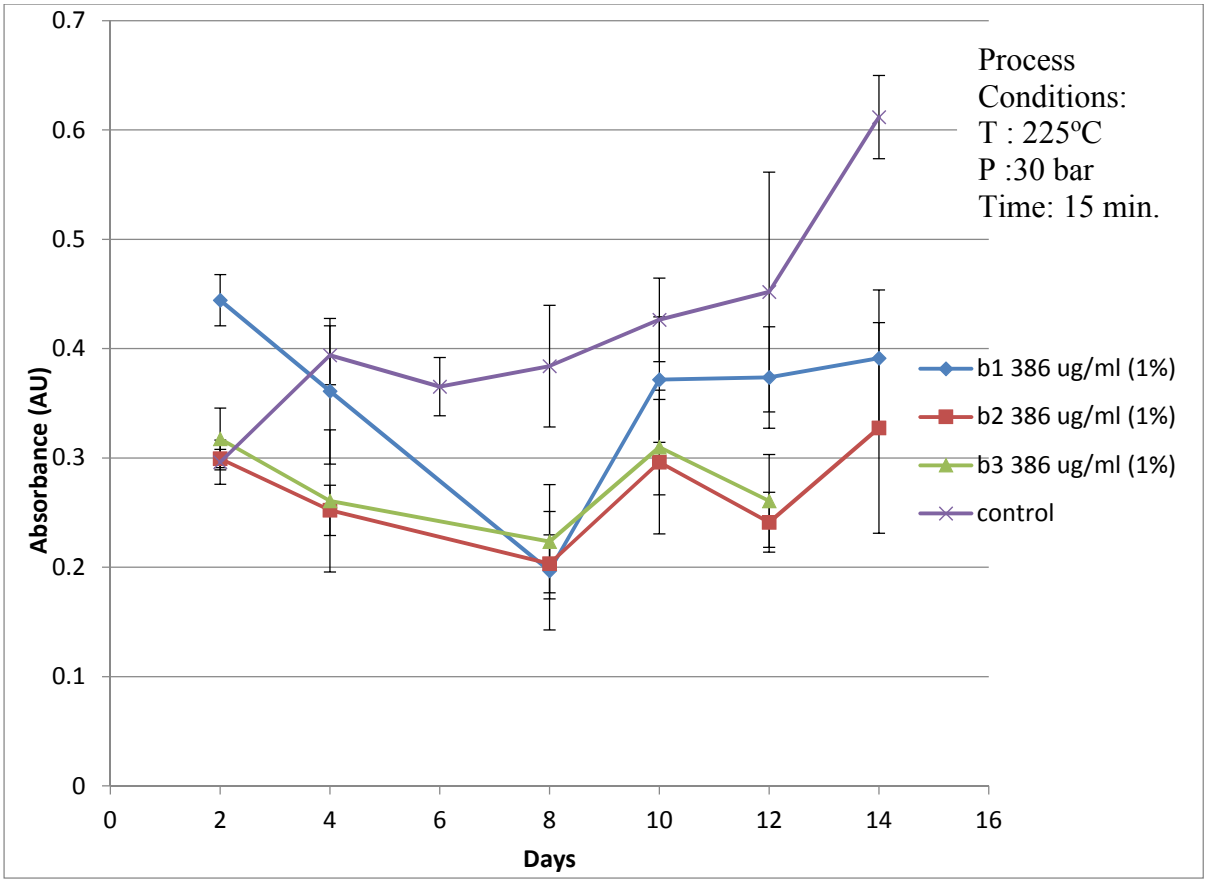
Graph 7 shows that cells incubated with 386µg/ml of extract seem to be more metabolically active than the control sample, as the overall increase in absorbance seems to be higher than the control (even when the error bars are accounted for). This overall increase in metabolic activity seems to decrease with concentration especially at concentration 1158µg/ml which has a large initial dip in absorbance. In addition the 772µg/ml concentration shows only a small overall increase in metabolic activity.

The major conclusion that can be drawn from all three of the concentrations at this hydrolysis temperature is that the results of batch 2 seem to have very different effect on the cells when compared to the other two batches. This is especially noticeable at concentration of 386 and 1158 µg/ml. Also an overall conclusion is that as the concentration of the extract increases is a significant decrease in the absorption or overall metabolic activity of the cells when compared to the control. This could be either due to increase in cellular death or metabolic inhibition. These results seem to mirror the viability results from previous experiments; further reinforcing the theory that concentration of 772µg/ml (2% w/v) would be the highest concentration with this extract.

4.4: Methylthiazolyldiphenyl-tetrazolium bromide (MTT), results Part 2

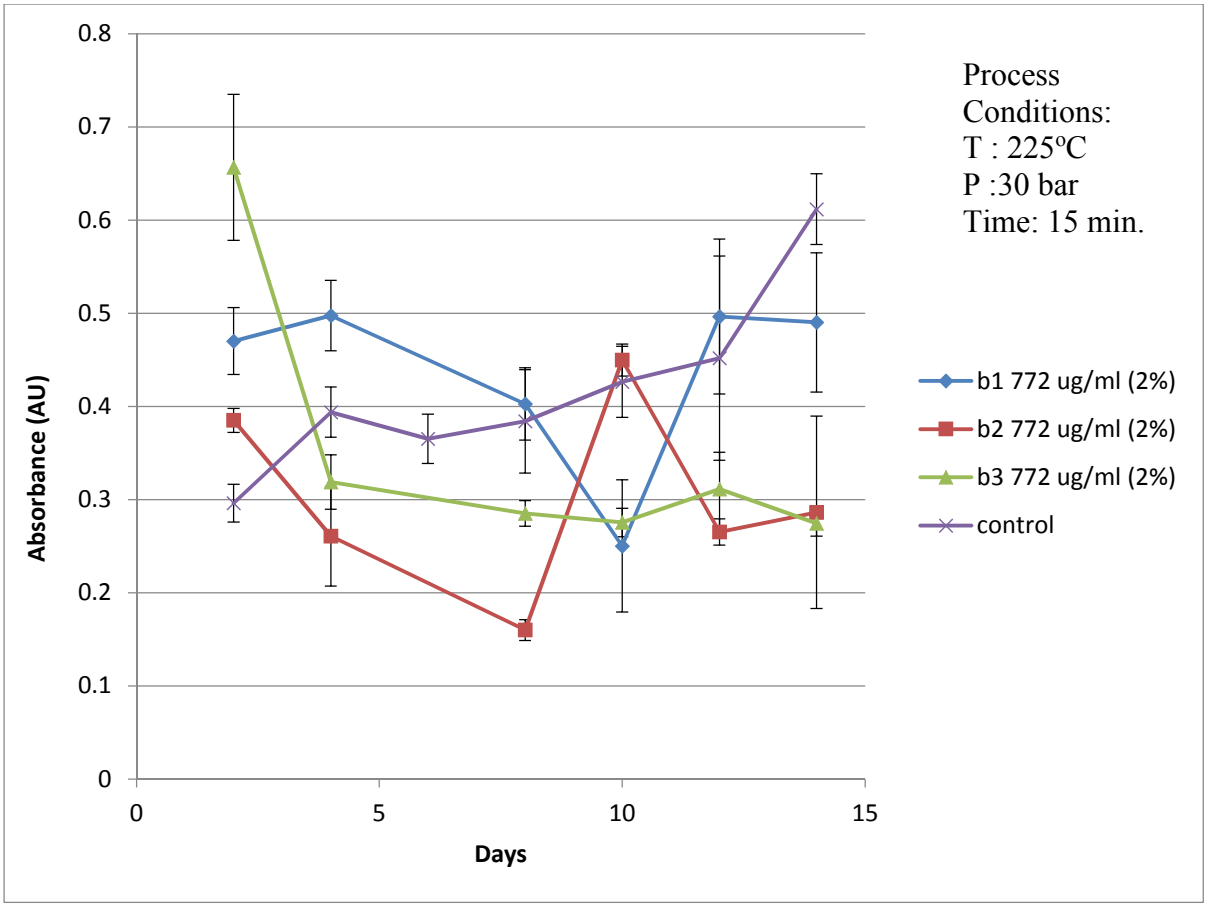
Fibroblasts were incubated with a yeast extract hydrolysed at 225^{o}C at 30 bar for 15 minutes with various concentrations. All of the MTT results shown are an average of 4-5 results (with outlying results removed).

Graph 8 shows the change in metabolic activity of the cells incubated with the yeast extract hydrolysed at 225^{o}C at a concentration of 386 µg/ml over a period of 14 days. Abbreviations b1, b2 and b3 refer to batch 1 , 2 and 3 respectively and are different batches of the same extract.



Graph 8: showing the change in metabolic activity of cells incubated with extract hydrolysed at 225°C at a concentration of 386µg/ml over a period of 14 days.

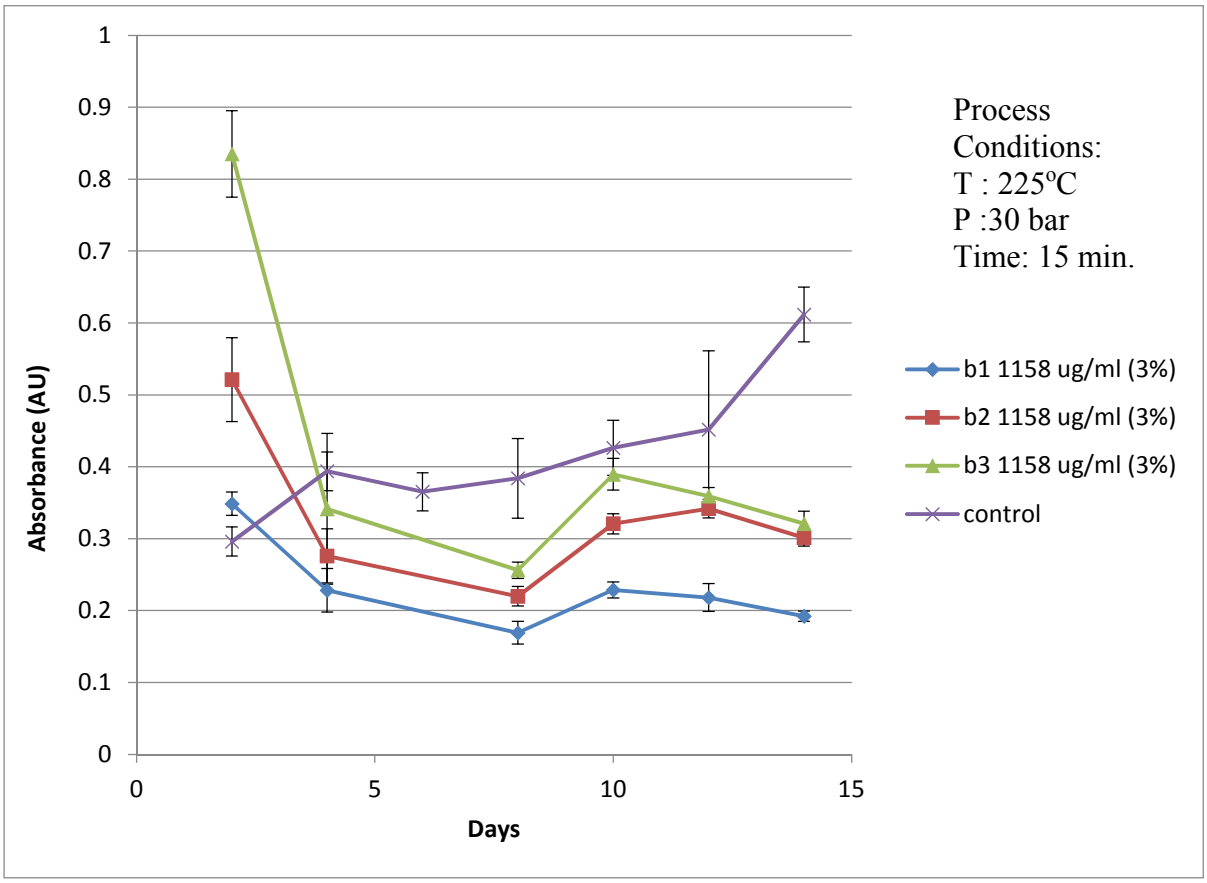
Graph 8 illustrates that overall there seems to be a decline in metabolic activity of the cells from day 2 and that this is shown reproducibly across the batches at concentration of 386ug/ml. This is especially stark when compared to the increase in absorbance and overall metabolic activity seen in the control. This decline is seen from day 2 to 8 however after this there seems to be a small recovery in metabolic activity but the magnitude is not very large. The batch to batch variation observed with the previous extract is not replicated here.



Graph 9: showing the change in metabolic activity of cells incubated with extract hydrolysed at 225°C at a concentration of 772µg/ml over a period of 14 days. Abbreviations b1, b2 and b3 refer to batch 1 , 2 and 3 respectively and are different batches of the same extract.

Graph 9 demonstrates that overall there seems to be a decline or plateau in metabolic activity at 772µg/ml. The plateau is most evident in batches 1 and 3 (if we disregard day 10 from batch 1). In batch 2 there is an initial decline in metabolic activity to day 8 and a small recovery after that

(overall). When comparing the profile of the other graphs to the control it is evident that with batches 2 and 3 there a large initial reduction in absorbance and overall metabolic activity of the cells and after this period the plateau also becomes evident. This is in contrast to the rise in absorbance seen in the control sample.



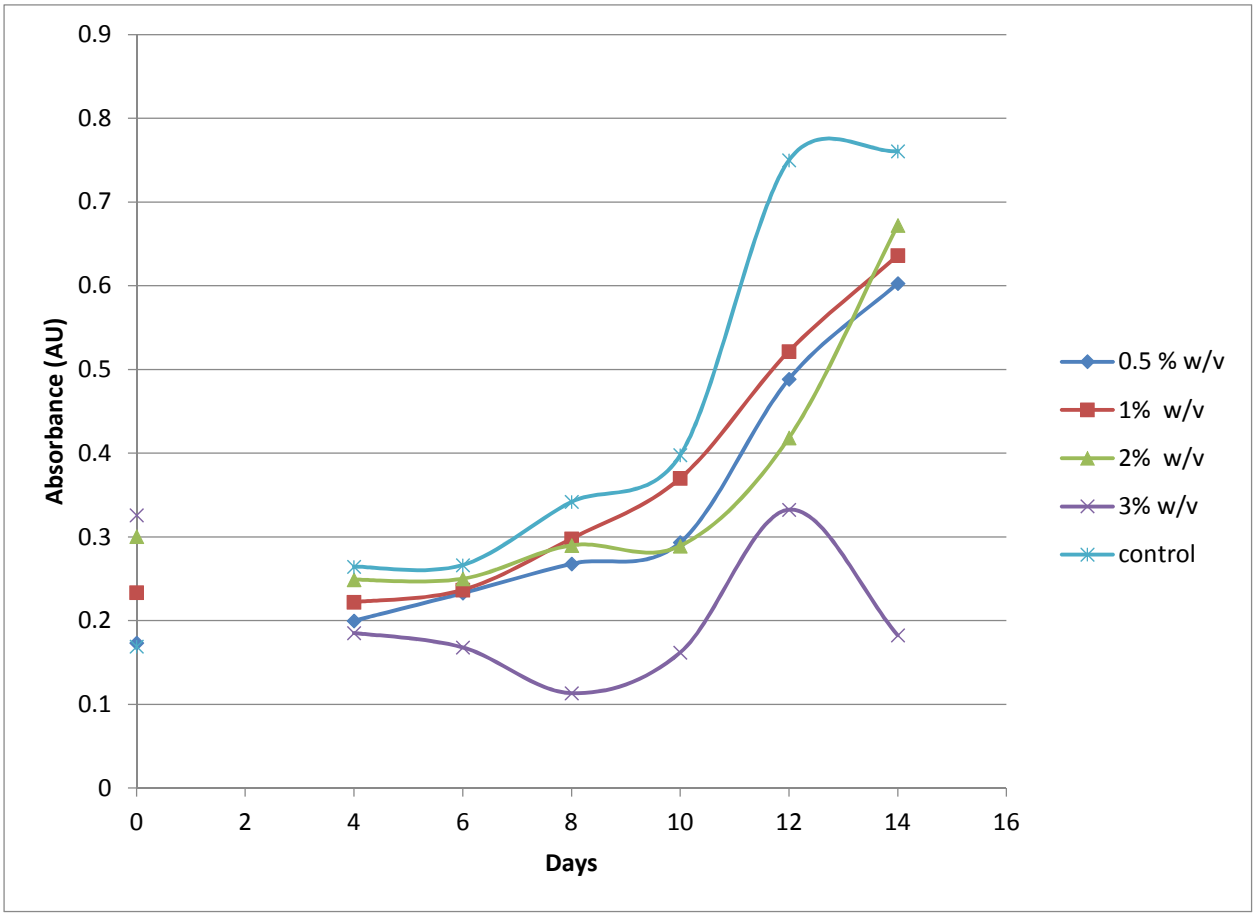
Graph 10: showing the change in metabolic activity of cells incubated with extract hydrolysed at 225°C at a concentration of 1158µg/ml over a period of 14 days. Abbreviations b1, b2 and b3 refer to batch 1, 2 and 3 respectively and are different batches of the same extract.

Graph 10 illustrates that overall (and across the batches); there seems to be a initial steep decline in metabolic activity and then a small recovery absorbance and overall metabolic activity. This indicates that initially the extract reduces the number of metabolically active cells and the surviving cells then recover very slowly. This decrease in overall metabolic activity is in contrast to the profile of the control graph, which gradually increases with time.

Across the three different concentrations with extract hydrolysed 225°C there does not seem to be any positive effect on the metabolic activity of the cells. However the batch to batch variation is not a major issue with this extract and overall the results are reproducible across batches especially at concentration of 386µg/ml and 1158 µg/ml. The results indicate that only at concentration of 386µg/ml do you not get a initial decrease in metabolic activity; indicating that lower concentrations should be tested in future.

4.5 **Crude Methylthiazolyldiphenyl-tetrazolium bromide (MTT) Results**

Graph 11 below shows the change in metabolic activity of the cells incubated with the crude yeast extract at a variety of concentrations over 14 days. As with the previous run, cells were seeded at a density of 4,100 cells/ well or 12,500 cells per cm^2.



Graph 11: showing the change in metabolic activity of cells incubated with an unhydrolysed crude extract at various concentrations.

Graph 11 shows that for most of the crude extract concentrations (except 3% w/v) the profile of the graphs roughly matches that of the control. However by the end of the 14 day growth curve the cells incubated with the crude extract are still in the exponential growth phase while the cells in the control sample have entered the stationary phase. For the cells incubated at 3% w/v the profile of the graph suggests an overall plateau in the metabolic activity of the cells incubated with the result at day 12 could be anomalous. The graph of the 2% w/v suggests that the cells were still in lag phase all the way up to day 10 which is in contrast to the control which is in the lag phase up to day 6. The graphs of 0.5% w/v and 1% w/v are very similar and in fact the profile of both graphs is not far away from that of the 2% w/v graph.

Overall the results suggest is that the crude extract at 3% w/v seems to harm the metabolic activity of the cells and for the other crude concentrations that it either delays or slows down the overall growth of the fibroblasts. This effect could be investigated further by extending the experiment beyond 14 days.

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Appendix B: Poster abstract

This abstract was submitted and accepted for presentation to the conference on: Analysis of free radicals, radical modifications and redox signalling hosted by the Biochemical Society. However this abstract had to be withdrawn due to commercial sensitivity and intellectual property concerns raised by my sponsor Alliance Boots and their attorneys.

P056 An analysis of a yeast extract processed with subcritical water using the TEAC and ORAC anti-oxidant assays.

Neeraj Jumbu, Dr Liam M Grover,
Dr Regina C.D. Santos, Mr Ed Galley
University of Birmingham, and Alliance Boots

The ORAC and TEAC assays are widely used to assess the anti-oxidant capacity of a wide range of substances, using stable free radicals.
As we had characterised chlorogenic acid and other potential anti-oxidant compounds in our processed waste yeast extract, we wanted to use the TEAC and ORAC assays to optimise the processing of the waste yeast extract. This was carried out by relating process temperature to the anti-oxidant activity determined by the TEAC and ORAC assays.
The results of the study revealed that a higher process temperature dramatically improved the ORAC value of the extracts of by over 4x. This is contrary to most of the literature which suggests that anti-oxidant activity of plant and other extracts is reduced with increased temperature due to the breakdown of phenolic and other anti-oxidant compounds processed above temperatures of 150°C.
This has lead to a further investigation into the cause of the increase in the anti-oxidant activity, and to validate the results in cells using the comet assay. Therefore this work demonstrates that the novel use of subcritical water to increase a yeast extracts anti-oxidant activity.