HÖGSKOLAN I BORÅS
INSTITUTIONEN INGENJÖRSHÖGSKOLAN

Extraktion av polyfenoler från pressrester av röda vindruvor

Extraction of polyphenols from red grape pomace

Behroz Haidarian

Christina Lidborg

Examensarbetet omfattar 15 högskolepoäng och ingår som ett obligatoriskt moment i Högskoleingenjörsexamen i Kemiteknik, inriktning bioteknik, 180 högskolepoäng
Nr 4/2010

Extraktion av polyfenoler från pressrester av röda vindruvor

Extraction of polyphenols from red grape pomace

Behroz Haidarian, behroz.haidarian@gmail.com

Christina Lidborg, S051534@utb.hb.se

Kandidatuppsats examensarbete

Ämneskategori: Teknik

Högskolan i Borås
Institutionen Ingenjörshögskolan
501 90 BORÅS
Telefon 033-435 4640

Examinator:	Elisabeth Feuk-Lagerstedt
Handledare, namn:	Beatriz Zumalacárregui de Cárdenas, Guido Riera González
Handledare, adress:	Polytechnic University José Antonio Echeverría (CUJAE)
	Calle 127, Marianao, Ciudad de La Habana, Cuba
Uppdragsgivare:	Polytechnic University José Antonio Echeverría (CUJAE),
	Havana, Cuba
Datum:	2010-06-03
Nyckelord:	Antioxidants, polyphenols, grape pomace, winery waste, extraction

Acknowledgements

This bachelor thesis was executed between the period March and June of 2009 at CUJAE (Polytechnic University José Antonio Echeverría), situated in Havana, Cuba, and it was made possible due to a scholarship from the Swedish International Development Cooperation Agency (SIDA). The study was done together with Eric González García, and was a part of his Master degree work in food chemistry. The head of this project was Dr. Guido Riera González. We would like to thank both Eric and Guido for their analytical approach and their deep knowledge in the field of food chemistry, both of which helped us in forming a main theoretical backbone and in performing this study in the most satisfactory way possible. We would also like to thank our tutor, Dr. Beatriz Zumalacárregui de Cárdenas, for her valuable advices and guidance throughout the course of this thesis. Furthermore, we would like to thank our examiner Dr. Elisabeth Feuk-Lagerstedt for her counseling and support from back home. Finally, a big thanks to everyone else, who in one way or another, contributed to this study or was a part of this mission.

Abstract

Antioxidants are compounds that are able to inhibit the oxidation of other molecules. They are widely found in animals and plants and are thought to play a big role in protecting cell tissue from the damaging effects of oxidative reactions. Antioxidants are frequently used as additives in foods and other products to protect against oxidative degradation and to maintain nutritional value. High contents of natural antioxidants are found in grapes, mainly in the form of phenolic compounds. Due to the high output of winery-waste, developing an effective method for the recovery of phenols from the waste is desired.

In this study, a solid-liquid extraction experiment was conducted on winery waste (red grape pomace) of the variety Tempranillo, with the aims of investigating the effects of extraction method, solvent type, extraction temperature and extraction time on the phenol content and the antioxidant activity of the extract. Two types of extraction methods were evaluated; the Soxhlet extractor and direct connect extraction (DCE), and 3 types of solvents; a mixture of ethanol and water (1:1), pure ethanol and ethyl acetate. Total phenolic contents in the extracts were measured using the Folin-Ciocalteu assay and antioxidant activity was measured using the FRAP (ferric reducing ability of plasma) assay.

It was found that a mixture of ethanol and water (1:1) as solvent led to maximum phenolic content and maximum antioxidant activity on all parameters tested, while ethyl acetate gave the lowest values. The Soxhlet extractor was found to be the most effective extraction method in reference to phenolic content and antioxidant activity. For the DCE method it was seen that an extraction time of 2 ½ hours at 55 °C was the most optimum for the ethanolic solvents.

Sammanfattning

Antioxidanter är kemiska ämnen som är kapabla till att förhindra oxidation av andra molekyler. Dessa ämnen återfinns i djur- och växtriket och tycks ha en skyddande effekt på cellvävnad genom att motverka skadliga oxidativa reaktioner. Antioxidanter används bland annat industriellt som tillsater i livsmedel och andra produkter i syfte att förhindra oxidativ degradering och bibehålla näringsvärdet i livsmedel. I vindruvor återfinns höga koncentrationer av naturliga antioxidanter, främst i form av fenoliska ämnen. På grund av att vinindustrin bidrar till stora mänger avfall, främst i form av druvrester, är det önskevärt att hitta en effektiv metod för att återvinna fenoliska antioxidanter från avfallet.

I detta arbete användes lösningsmedel-extraktion för att extrahera fenoliska ämnen från pressrester av röda vindruvor av varianten Tempranillo. Syftet var att uvärdera effekterna av extraktionsmetod, typ av lösningsmedel, extraktionstemperatur samt extraktionstid på fenol-innehåll och antioxidant aktivitet i extrakten. Två typer av extraktionsmetoder jämfördes; Soxhlet-extraktor och direkt-kontakt-extraktion (DCE), samt tre olika lösningsmedel; en blandning av etanol och vatten (1:1), ren etanol samt etyl acetat. Koncentrationen av fenoliskt innehåll i extrakten erhölls med hjälp av Folin-Ciocalteu's metod, och antioxidant aktivetet med FRAP (Ferric reducing ability of plasma) metoden.

Utifrån erhållna data kunde det ses att extraktion med en blandning av etanol och vatten (1:1) som lösningsmedel gav högst fenolisk koncentration och antioxidant aktivitet för alla testade parametrar, medans etyl acetat gav de lägsta värdena. Soxhlet-extraktorn visade sig vara den bästa metoden då den gav högre värden i extrakten jämfört med DCE metoden. För DCE metoden kunde det ses att en extraktionstid på 2½ timmar vid 55 grader var mest optimalt då etanolbaserade lösningsmedel användes.

Table of Contents

1. Introduction

Antioxidants are compounds that are capable of slowing or inhibiting chemical reactions of other molecules with oxygen. They are important additives used mainly in the food industry to prevent the degeneration of foods that are due to oxidative reactions such as the deterioration of fats, vitamins, the loss of color and flavor, and to prevent the formation of potentially harmful metabolites. Antioxidants are also frequently used to prevent degeneration of other oxidaseable goods such as cosmetics, pharmaceuticals and plastics. (Shahidi, 1997; Soong & Barlow, 2003)

Natural antioxidants are primarily derived from plants. These include tocopherols, vitamin C, carotenoids and phenolic compounds. In plants, their functions are to prevent cellular damage by inhibiting the harmful effects of free radicals and high levels of oxygen produced during photosynthesis (Shahidi, 1997). However, a number of antioxidants which are used as food additives are synthetically made. These include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate. A number of reports exist indicating that these compounds may have possible toxic and carcinogenic activities, while on the other hand for natural antioxidants, several health beneficial properties have been reported, including anticarcinogenicity, antimutagenicity, antiallergenicity and anti-inflammatory activity (Spigno & Faveri, 2005).

Despite the favorable profile of natural antioxidants described above, they may also bring about negative effects on the environment. The adverse impacts are due to the presence of high amounts of antioxidants in the form of residual phenols from raw plant material in wastes from many food industries. These compounds significantly increase the chemical oxygen demand (COD) and therefore lead to deteriorating effects on the fauna and flora of the areas where the wastes are discharged (Lafka et al., 2006).

One of the world's largest fruit crops is grapes, which is highly rich on antioxidants, mainly polyphenols. The world-wide harvest of grapes reaches about 60 million tonnes per year with approximately 80% of the harvest being used in the wine industry. The wastes produced in the wine-making process constitutes about 20 % of the weight of the processed grapes which gives a world-wide output of around 5–9 million tonnes wastes per year. A large portion of the wastes are used as animal feed and fertilizers, but due to the high contents of polyphenols and other organic substances in the grape wastes (such as sugars, tannins, polyalcohols, pectins and lipids), the wastes have a considerably negative impact on the environment (Lafka et al., 2006).

On one hand, an increased consumer demand is seen for natural, safe and non-synthetic additives in foods (as many of the currently used additives have possible toxic effects), while on the other hand large quantities of natural antioxidant-rich wastes are being spoiled, causing significant adverse effects on the environment. By developing an effective method for the treatment and recovery of phenolic contents from the wastes, both problems can be addressed.

In this study, solid-liquid extraction experiments were conducted with the aims of investigating the effects of extraction method, solvent type, extraction temperature and extraction time on the phenol content and antioxidant activity of winery waste (red grape pomace) extracts.

Two types of extraction methods were used; Soxhlet extraction and direct connect extraction (DCE). The solvents that were evaluated were ethanol, a mixture of ethanol and water (1:1) and ethyl acetate.

The grape pomace was of the variety Tempranillo and was provided by Bodegas San Cristobal, which is one of Cuba's biggest wineries, with two harvests of a total of 20 tonnes grapes each year.

This is the first study of its kind to be executed in Cuba.

2. Antioxidants

2.1 Oxidants and antioxidants

2.1.1 Reactive oxygen species (ROS)

Molecular oxygen O_2 is vital to most aerobic consuming organisms on earth. Evolutionary, the organisms had to adapt to oxygen in the atmosphere and that has given them a more energetic respiration, but it also brought the danger of reactive oxygen species (ROS). ROS can be both free radicals and non radical oxidants (Chen, 2002). Free radicals are molecules that carry one or more unpaired electrons in their valence shell configurations. When radicals reacts with other compounds a chain reaction will start in the cell which can lead to cell dysfunction, this is because there will always be a new radical as a product in these reactions. The reaction can take different paths as illustrated in the reaction scheme below. The radical can donate its unpaired electron (1A), the radical can take up one electron from a non-radical (1B), or it can bind to a non-radical (1C). The chain reaction will stop first when two radicals pear up (1D & 1E) (SBU, 1997).

1A $X\bullet + Y \longrightarrow X^- + Y\bullet^+$

1B $X\bullet + Y \longrightarrow X^- + Y\bullet^+$

1C $X\bullet + Y \longrightarrow (X^- - Y)\bullet$

1D $X\bullet + X\bullet \longrightarrow X : X$

1E $X\bullet + Y\bullet \longrightarrow X : Y$

The most commonly known reactive oxygen species in biological systems are the free radicals superoxide anions ($O_2\bullet^-$) and hydroxy radicals ($HO\bullet$). The main source of $O_2\bullet^-$ is the aerobic metabolism where up to 1% of the oxygen is converted into $O_2\bullet^-$. The enzyme superoxide dismutase (SOD) can convert $O_2\bullet^-$ into hydrogen peroxide (H_2O_2) (Chen, 2002). The enzyme peroxidase-catalase can then convert it into H_2O as seen in the reaction scheme below (2A) (SBU, 1997). However, if the Hydrogen peroxide comes in contact with iron or copper it will decompose into a hydroxyl radical (2B), which is the most unstable ROS and it will react rapidly and start chain reactions with most biological molecules. One well known chain reaction induced by $HO\bullet$ is lipid peroxidation that damage cell membranes (Chen, 2002).

2A Oxygen → superoxidanion → hydrogen peroxide → water

$$O_2 \xrightarrow{} O_2\cdot^- \xrightarrow{\text{SOD}} H_2O_2 \xrightarrow{\text{peroxidase katalas}} H_2O$$

2B Oxygen → superoxideanion → hydrogen peroxide → hydroxyl radical

$$O_2 \xrightarrow{} O_2\cdot^- \xrightarrow{\text{SOD}} H_2O_2 \xrightarrow{Fe^{2+}\text{-complex}} HO\cdot$$

Other endogenous factors that increase the concentrations of ROS in the body are inflammations. ROS are produced for protection in the immune system. For example the macrophages use them to kill microorganisms that otherwise would hurt us.

Outside factors that can contribute to the formation of ROS is UV light, food that we eat, medicines, gas, smoke and radiation (Chen, 2002)

2.1.2 Properties of antioxidants

The definition of an antioxidant is "*any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate*" (Chen, 2002) Examples of such substrates in biological systems are proteins, lipids, carbohydrates and DNA (SBU, 1997). Our cells produce ROS all the time; oxygen consuming organisms have therefore their own antioxidant system and the anti-oxidative defense works in different ways. The different defense strategies in the antioxidant system are:

- Preventing oxidation to happen.
- Enzymatic neutralization of the radical.
- Reactions with oxidants. For example, the binding of an antioxidant to an oxidant (often referred to chain-breaking antioxidants).
- Reparation of oxidative damages (such as DNA reparation).

(SBU, 1997)

The different types of antioxidants are classified depending of source, mechanisms, function and size. The source of antioxidants consists of different types of endogenous enzymes, for example superoxide dismutase (SOD)-catalase witch prevents oxidation or catalyzes the ROS breakdown. The endogenous antioxidant system also includes small molecules such as glutathione, lipoic acid and urate. Another source for antioxidants is the exogenous, which includes vital vitamins that cannot be produced in the body and therefore must be obtained from the diet, for example vitamin E and beta-carotene. These vitamins are often called the chain-breaking antioxidants because they "trap" the oxidants before they can initiate more reactions that lead to ROS (Chen, 2002).

2.1.3 Mechanisms of antioxidants

Most antioxidants are reducing agents. This means that they donate an electron or proton to "neutralize" the ROS. When a reducing antioxidant reacts with ROS the antioxidant itself will become a radical, but because of the chemical structure of antioxidants they have the ability to reposition the electrons and therefore they will have a much lower reactivity and the chain reaction will inhibit. As can be seen in Table 2-1, less reactive radicals will be produced further down you go. The exception is when catalytic metals are in the presence of oxygen, then more reactive radicals can be the outcome. The antioxidant system often uses antioxidant enzymes to bind these catalyzing ions and thereby prevent these highly reactive radicals to form (Chen, 2002).

Table 2-1. Standard one-electron reduction potential for predicting the course of free radicals. pH 7. NHE, normal hydrogen electrode (Chen, 2002)

Redox couple	E^0/mV vs. NHE
$HO\cdot,H^+/H_2O$	2310
$RO\cdot,H^+$ /ROH (alipathic aloxyl radical)	1600
$ROO\cdot,H^+$ /ROOH (alkyperoxyl radical)	1000
$O_2\cdot^-,2H^+/H_2O_2$	940
$O_3/O_3\cdot^-$	860
O_2(singlet oxygen) $/O_2\cdot^-$	650
PUFA·,H+ /PUFA-H (polyunsaturated fatty acid, bis-allylic-H)	600
HU^-/UH_2^- (URATE)	590
α-tocopheroxyl·,H+ / α-tocopherol (TO,H /TOH) (vitamin E)	500
Trolox /T-O·,H+ /TOH	480
H_2O_2, H^+/H_2O, HO·	320
Ascorbate·$^-$, H^+ /ascorbate monoanion (vitamin C)	282
Fe^{3+}/Fe^{2+}(aqueous)	110
Fe^{3+}/Fe^{2+}(aqueous)	770 (pH 0)
$O_2/O_2\cdot^-$	-330
O_2, $H^+/HO_2\cdot$	-460

2.2 Exogenous vitamins and antioxidants

Vitamins are defined as crucial exogenous substances that can't be produced in the body, they mainly include dietary nutrients. These vitamins are often used as coenzymes in the cells metabolism to enhance some of the cells own enzymes or to make them work properly. Without these vitamins, deficiency diseases like scurvy and beriberi can occur. Deficiency diseases caused by vitamin deficiency are very uncommon in the western world and are often related to medical conditions like low uptake of nutrition in the intestine and other food related diseases. In later years it has been found that some vitamins, specially vitamin E and vitamin C have antioxidant properties (Higdon & Drake, 2008). By eating a healthy diet including a lot of vegetable fruits and berries which contains a lot of antioxidants it's suggested that our antioxidant system could increase and therefore prevent diseases, and it has been proven that some kind of vegetables and fruits protect and lower the risk to get cancer, heart disease and neurological diseases. However, in studies treating consumption of antioxidant supplements such as vitamin E, vitamin C to the ordinary diet, no health benefits has been seen (Thomson, 2000). This is probably because the health benefit comes from the wide and complex mix of substances in the plants. The polyphenolic group is one interesting complex substance found in plants that has been studied a lot in recent years (Buhler & Miranda, 2000).

2.2.1 The function of the dietary antioxidants vitamin E and vitamin C

Tocopherol (vitamin E) is a very important antioxidant in the body. It is a hydrophilic antioxidant and works by preventing lipid oxidation witch otherwise could lead to damages on cell membranes and other lipid containing proteins. The tocopherol becomes a tocopheryl radical with low oxidizing activity by "catching" peroxyl radicals and donate its hydrogen to neutralize them (see reaction 1, A below). The ascorbic acid (vitamin C) could then react

with the tocopherol radical and donate its hydrogen. This way the ascorbic acid enhances the effect of tocopherol (see reaction 1, B below) (SBU, 1997)

(1, A) Peroxyl radicals Tocopherol Peroxyl Tocopheryl radical

$$POO\cdot + TH \longrightarrow POOH + T\cdot$$

(1, B) Tocopheryl radical Ascorbate acid tocopherol ascorbyl radical

$$T\cdot + AH \longrightarrow TH + A\cdot$$

2.2.2 Polyphenols in plants

Aside for the more traditional antioxidants like vitamin E and C, plants also use different kinds of polyphenols for protection against ROS. The polyphenolic molecule is characterized by having two or more phenols bound together. Polyphenols are found mostly in vegetables, fruits and berries and some of them have caught researchers' attention and have been studied a lot in the past 10 years due to their suggested antioxidant properties and their supposed health beneficial effect on the human body. There are several thousand different polyphenolic compounds and the most researched one is the big family of flavonoids. (Buhler & Miranda, 2000).

2.2.3 Flavonoids

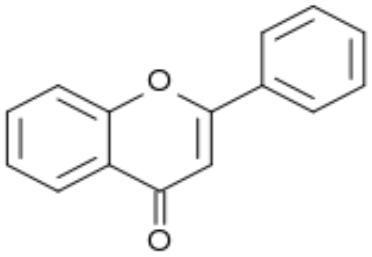


Figure 2-1. Molecular structure of the flavonoid backbone (2-phenyl-1,4-benzopyrone) (Higdon & Drake, 2008)

The flavonoids can be divided in to 6 subgroups.

1. Anthocyanins, found in red/purple grapes and barriers
2. Flavanols, found in teas, cacao beans, grapes, red wine
3. Flavanones, found in yellow and orange fruits
4. Flavonols, found in broccoli, apples, berries and teas
5. Flavones, found in herbs like parsley and thyme
6. Isoflavones, found in soybeans and legumes

(Higdon & Drake, 2008)

There have been a lot of animal studies where flavonoids have been shown to have effect against cancer and cardiovascular diseases, but these studies have not yet been applied on humans. Flavonoids have also shown to have a very strong antioxidants activity *in vitro*, but *in vivo* this effect is thought to be insignificant. In studies with high doses of flavonoids supplement the concentration of flavonoids in the human body was still very low, up to a 1000 times lower than concentrations of the more traditional antioxidants like vitamin C and E. Instead the flavonoids secondary metabolite concentration went up. In experiments with flavonoids in cell cultures, it has been seen that some of the flavonoids secondary metabolites could increase and decrease the availability of different kinds of cell signaling proteins and therefore it is suggested that the biological effects on health from flavonoids is supposed to be due to cell to cell signaling ability, rather than their antioxidant activity (Higdon & Drake, 2008).

2.3 Antioxidants as food preservatives

Oxidation is one of the reasons why food deteriorates. Unsaturated fat can be subjected to peroxidation and therefore turn rancid when it's exposed to oxygen and sunlight. This decreases the nutritional quality and could cause potentially toxic compounds in the food (Moure et al., 2000). Synthetic antioxidants, BHA and BHT have been used as antioxidant preservatives for a long time to prevent fat containing food from oxidation. Both have been studied much, and lots of reports indicate that these compounds could have toxic effect such as allergic reactions and carcinogenic activities. The search for natural antioxidants to replace synthetic antioxidants in food has therefore increased a lot in recent years. Mostly, the studies that involve polyphenols and flavonoids have been done for medical use because of their strong antioxidant effect in vitro. Since there still are uncertainties about their effectiveness as antioxidants in vivo, one very interesting and promising application is the use of polyphenols as natural food preservatives (Spigno & Faveri, 2005)

3. Extraction and testing of phenolic content

3.1 General extraction principles

Extraction is a process where a compound (a solute or solid) is transferred from one phase to another by bringing the two phases into contact, with the objective of separating and isolating the specific compound from a mixture of other compounds or impurities. The two phases can both be liquids, in which case the extraction type is referred to as a "liquid-liquid extraction". The extraction can also be between a liquid and a solid (solid-liquid extraction), or between a gas and a liquid (called absorption) (Gamse, 2002; Harrison et al., 2003).

There are several industrial application areas where extraction is used, such as in the metal industry where extraction is used to separate metals from their ores, or in the pharmaceutical industry where bioproducts and other agents are derived with extraction processes. Other examples include the production of monomers and aromates in the petroleum industry, and wastewater treatment (Gamse, 2002; Harrison et al., 2003).

3.1.1 Distribution equilibrium and the distribution coefficient

The extraction method relies on that the two phases coming in contact with each other are partially or completely immiscible in one another, and the compound to be extracted is soluble in both phases. This is based on the general statement of "like dissolves like", in other words, a compound will dissolve in a solvent that is chemically similar to itself. Upon contact between the two phases, the compound will be transferred from one phase to the other until concentration equilibrium has been achieved. With other words, the compound will be distributed between the two phases until the ratio of concentration of the compound is constant. This ratio is called the *distribution coefficient.* (Shugar & Ballinger, 1996). Mathematically expressed, a compound in a system of two immiscible phases will be distributed according to the following equilibrium:

$$X_{phase1} \Leftrightarrow X_{phase2}$$

It then follows that the distribution coefficient is defined as:

$$K = \frac{\left[X_{phase2}\right]}{\left[X_{phase1}\right]} \tag{3.1}$$

Where $\left[X_{phase1}\right], \left[X_{phase2}\right]$ are concentrations of compound X in each phase. (Shugar & Ballinger, 1996)

The solubility of solutes in liquids varies with temperature and is dependent on the specific solute/solvent; therefore the distribution coefficient is also dependent on temperature. (Zumdahl, 2005)

In solid-liquid systems, the rate of which a solid dissolves in a solvent increases with increasing temperature but the amount that dissolves may decrease or increase depending on the properties of the solute and solvent. The effect of temperature on concentration

equilibrium can be explained by the total enthalpy change that occurs in the system when a solution is formed, and is illustrated in Fig. 3-1. (Zumdahl, 2005)

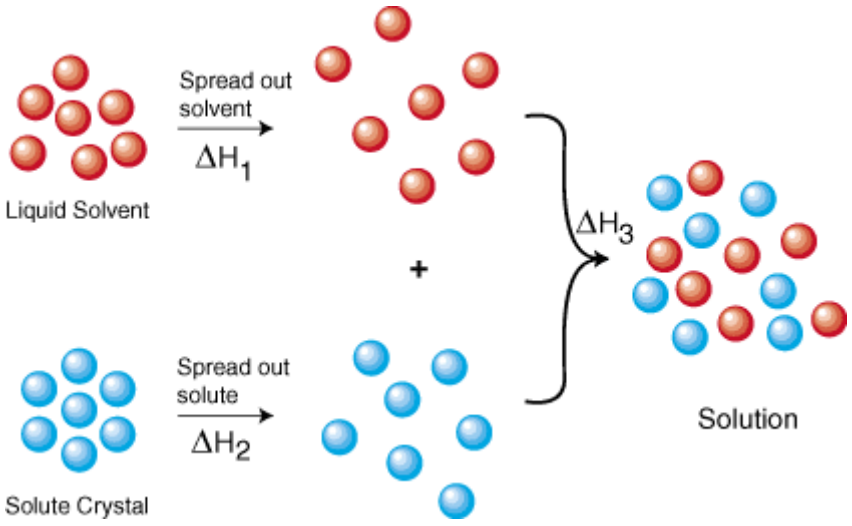


Figure 3-1. Enthalpy change in formation of a liquid solution (SparkNotes Editors, 2010).

The total enthalpy change is called *enthalpy of solution* (denoted ΔH_{soln}), and is the sum of the ΔH values for three distinct steps that are assumed to take place when a solid is dissolved in a solvent, as seen in Fig 3-1. These steps are simplifications of the overall process.

Step 1 (ΔH_1): Separation of solvent-solvent into individual components (endothermic).

Step 2 (ΔH_2): Separation of solute-solute into individual components (endothermic).

Step 3 (ΔH_3): Formation of solute-solvent components (exothermic).

Thus,

$$\Delta H_{soln} = \Delta H_1 + \Delta H_2 + \Delta H_3 \tag{3.2}$$

(Zumdahl, 2005)

If ΔH_{soln} is negative, the overall process is exothermic, and if ΔH_{soln} is positive, the overall process is endothermic. The implication this has on how temperature effects solubility equilibrium is that if the process is endothermic ($\Delta H_{soln} > 0$), then an increase in temperature will shift the position of the equilibrium in such manner that the solubility of the solute is increased, and the opposite will occur if the process is exothermic ($\Delta H_{soln} < 0$). Generally the solubility of solids almost always increase with temperature, however this is not true in all cases. (Zumdahl, 2005; Masterton & Hurley, 2008)

Even though there is a relation between enthalpy of solution and temperature in terms of solubility, there exist important exceptions, and predicting the effect of temperature on solubility equilibrium can be very difficult. The only sure way to thoroughly evaluate this parameter in order to maximize solute solubility in an extraction process is by experimentation. In many cases, finding the optimal temperature and solvent can lead to a

large distribution coefficient, so that nearly all of the solute will dissolve into the solvent. (Zumdahl, 2005; Shi et al., 2002)

Thus, the extraction method relies on the solubility characteristics of both the phases involved and the particular compound involved, with the distribution coefficient being a measure of solubility of the compound between the two immiscible phases. On the basis of this, it follows that the distribution coefficient also describes the maximum amount of a compound X that can be extracted from phase 1 to phase 2, in a single extraction. An implication of the above mentioned, which is crucial to this method, is that several small extractions will often result in a greater recovery than one single extraction with large amounts of solvent. (Shugar & Ballinger, 1996; Rydberg et al., 2004)

3.1.2 Mass transfer and extraction kinetics

The transference of compounds between phases is governed by mass transfer laws, and is a process in which mass is transferred from high concentration to low concentration until concentration equilibrium has been reached and is thus driven by a concentration gradient between the phases. The flow of molecules from the higher concentration to the lower is caused by random molecular motion that ultimately leads to complete mixing (equilibrium). This phenomenon is called *diffusion* and is a time-dependant process, often a very slow one. (Rydberg et al., 2004)

Diffusion is a complex phenomenon. To be able to describe it thoroughly, one must involve theories of molecular interactions and solve complicated differential equations. However, there exist several models that deal with diffusion between phase boundaries. One of the simpler and more useful models is the so-called *two-film model* (also referred to as the Nernst film), and is schematically illustrated in Fig 3-2. (Rydberg et al., 2004)

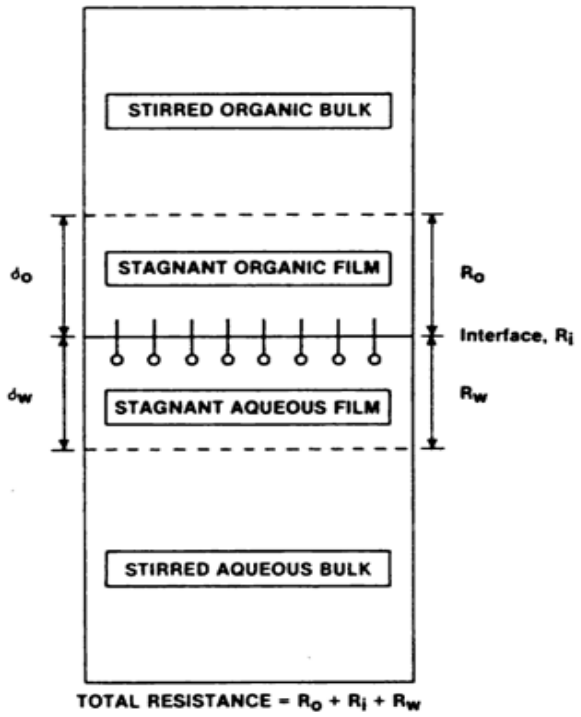


Figure 3-2. Schematic of the two-film model (Rydberg et al., 2004)

In the figure above, the two-film model is applied to a two-phase liquid-liquid system. This model relies on assuming the existence of two hypothetical liquid diffusion films located on each side of the phase boundary (interface), these films are considered to be completely stagnant and of finite thickness. In the Fig. 3-2, the films are represented by R_o and R_w, for the organic and aqueous phases, respectively, and their thickness are denoted δ_o and δ_w. In a stirred system, mass transfer in the bulk will occur by convection (motion of currents in the fluids), and will diminish gradually as the diffusion film is approached. In the diffusion film, convection is absent and thus, no fluid movements exist; there, mass transfer to and from the bulk across the interface will occur by diffusion.

The mass flux across the phases can thus be expressed as: (Mass Flux) = (Convective Mass Flux) + (Diffusional Flux). (Rydberg et al., 2004; Asano, 2006)

The diffusion flow (or diffusional flux) can be described by *Fick's laws of diffusion*, and for a steady-state system assuming linear concentration within the film, it can be expressed as:

$$J = \frac{D}{\delta}(c_2 - c_1) \quad \left[\frac{kg}{cm^2 \times s}\right] \tag{3.3}$$

$c_2 - c_1$ refers to the concentration gradient between the phases, δ refers to the thickness of the diffusion film, and D is a compound-specific constant called the diffusion coefficient. D is independent of concentration but varies with medium and temperature. (Cussler, 2003)

In a stirred system, mass transfer by convection in the bulk occurs very fast and it can even be considered to occur instantaneously, hence it can be neglected. In the film where no fluid movements exist, mass transfer is due to diffusional flux and is a slow process. As can be seen in eq. 3.3, the diffusional flux can be increased by decreasing δ (i.e. the thickness of the diffusion films). The films have thicknesses in the range of 10^{-2} to 10^{-4} cm. The thicknesses are dependent on the geometry of the extraction equipment used as well of the viscosity, velocity and the density of the two liquids. The contact area between the phases is also a significant factor; a large contact area will increase the diffusional flux. For two given liquid phases in a fixed extraction equipment, the thickness of the diffusion films can be decreased with increased stirring rate. However, the thickness can only be decreased to a certain degree and usually settles around 10^{-2} - 10^{-4} cm. Even in a perfectly stirred system, the thickness will never reach zero and thus the compound will always have to diffuse to and from the bulk through the diffusion films. The time required for this is most often longer than the time required for other chemical processes in the extraction procedure. Hence, the overall kinetics of extraction is governed by diffusion through these films. This is also confirmed by experience, as it has been shown that film-diffusion is the primarily rate-limiting factor in many practical extraction processes. (Rydberg et al., 2004; Cussler, 2003)

The fundamentals described in this chapter holds true also for solid-liquid extractions, where a solvent is used to dissolve and extract a soluble compound from an insoluble and permeable solid phase. In case of botanicals, such as grape pomace, the solids are quite porous due to the network of passageways throughout the plant, which are used as routes to distribute nutrients and water while the plant is alive. These same passageways allow the solvent to easily penetrate and recover the desired compound. (Shi et al., 2002)

Mass transfer in a solid-liquid extraction can be modeled according to the two-film model as well. In an agitated system consisting of a solid and liquid phase, convection is occurs in the liquid phase but is obviously absent in the solid phase, since no fluid motion is present in

solids. Mass transfer for a botanical particle is illustrated in Fig. 3-3, and takes place through the following four steps (Shi et al., 2002; Small, 1989).

1. The solvent washes into the solid particle through its internal pores (via convection and diffusion).

2. The soluble compound is dissolved into the solvent, within the solid.

3. The dissolved compound is diffused through the diffusion film across the interface to the surface of the particle.

4. The solute-rich solvent is transferred into the bulk through convection.

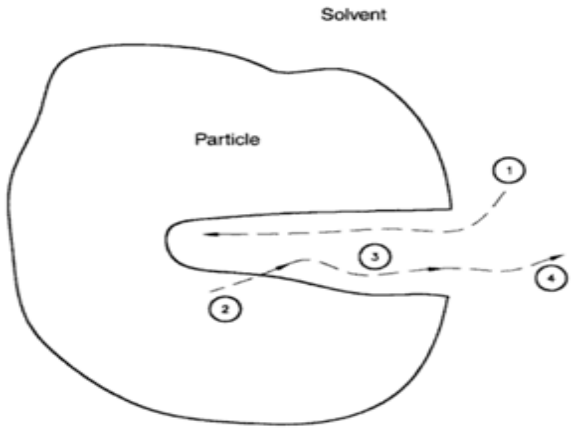


Figure 3-3. Four steps for mass transfer in a solid-liquid extraction (Shi et al., 2002).

As in the case for liquid-liquid extractions, the rate-limiting factor is diffusion through the diffusion film, in solid-liquid extractions as well. That is, step 3, the diffusion of solute from within the particle to the surface. (Shi et al., 2002)

3.1.3 Solvent selection

Choosing the right solvent is essential to having a successful extraction. There are a number of criteria that generally need to be considered when selecting the right solvent.

- Selectivity: This is the most important factor when it comes to choosing a solvent. Selectivity means that the solvent has a preference for the desired compound over other substances that might be present. With other words, the distribution coefficient for the desired compound is large and the distribution coefficients for other, unwanted substances are negligibly small.

- Capacity: A high extraction potency of the solvent means reduced amount of solvent that has to be used and reduced amount of extraction steps.

- Miscibility: In liquid-liquid extraction, a low miscibility between the two phases is desired in order to facilitate separation of the phases after the extraction.

- Density: In liquid-liquid extraction, a high difference in densities between the phases will further facilitate separation. Generally, the density of the lighter phase should be at least 5% less than the heavier phase.

- Surface tension: In liquid-liquid extraction, a low surface tension will result in fine dispersion of one phase in the other and consequently increase the surface area and mass transfer. On the other hand, it will make separation of the phases more difficult. Therefore, the ideal is to choose a solvent with "optimal" surface tension.

- Viscosity: A low viscosity favors mass transfer.

- Other factors to consider: the solvent should be non-corrosive, non-toxic, chemically stable and preferably inexpensive. The solvent should also have low flammability and low volatility.

(Dutta, 2007; Gamse, 2002)

- In extraction of nutraceutical products, even further considerations must be taken into account when choosing the solvent. Ethanol, water and mixtures of these are most often used as solvents in extractions of these types of products. However, even when using ethanol and water, other factors must be evaluated in order to make sure that the compound at hand is stable in the selected solvent. (Shi et al., 2002)

The above mentioned criteria should be viewed as a guideline since finding a solvent that matches all the above mentioned criteria simultaneously would be extremely difficult. The goal should be to find a balance between the solvent (in terms of solvent properties and cost) and the equipment and ease of operation. (Dutta, 2007)

The selectivity of the solute (the first criterion in the above list) can be theoretically evaluated by using the Robbins Chart of Solute/Solvent Group Interactions, shown in Table 3-1.

Table 3-1. The Robbins Chart of Solute/Solvent Group Interactions. (Dutta, 2007)

Solute class	*Group*	*Solvent class* 1	2	3	4	5	6	7	8	9	10	11	12
1.	Phenol	0	0	–	0	0	–	–	–	–	–	–	–
2.	Acid, thiol	0	0	–	0	–	–	0	0	0	0	+	+
3.	Alcohol, water	–	–	0	+	+	0	–	–	+	+	+	+
4.	Active-H on multihalo paraffin	0	0	+	0	–	–	–	–	–	–	0	+
	H-acceptor												
5.	Ketone, amide with no H on N, sulphone, phosphine oxide	–	–	+	–	0	+	+	+	+	+	+	+
6.	Tertiary amine	–	–	0	–	+	0	+	+	0	+	0	0
7.	Secondary amine	–	0	–	–	+	+	0	0	0	0	0	+
8.	Primary amine, ammonia, amide with 2H on N	–	0	–	–	+	+	0	0	+	+	+	+
9.	Ether, oxide, sulphoxide	–	0	+	–	+	0	0	+	0	+	0	+
10.	Ester, aldehyde, carbonate phosphate, nitrate, nitrite nitrile, intramolecular bonding (for example, *o*-nitro phenol)	–	0	+	–	+	+	0	+	+	0	+	+
11.	Aromatic, olefin, halogen aromatic multi-halo-paraffin without active H, mono-halo-paraffin	+	+	+	0	+	0	0	+	0	+	0	0
	Non-H-bonding												
12.	Paraffin, carbon disulphide	+	+	+	+	+	0	+	+	+	+	0	0

The Robbins Chart is based on the activities of the solute in each phase; if the activity of a specific compound is small in the extract phase then the solvent has greater selectivity for that compound, and the distribution coefficient for that compound is large. The chart is primarily based on hydrogen bonding and electron donor-acceptor interactions between solvents and solutes (Dutta, 2007)

In Table 3-1, the most-left column contains the functional groups of solutes along with their respective number. The numbers in the top row under "Solvent class" represents solvents having the corresponding functional groups. A "+" sign at a location in the matrix indicates that the compound belonging to the specific class of solvents in that column raises the activity of the solute belonging to the class in that row. For example, from the chart it can be seen that a solute that has a phenolic group (group 1) has high activity in a solvent that is aromatic (group 11), but low activity in a ketone based solvent (group 5). Thus, a ketone is preferred as solvent in extracting phenol since the activity of phenol in a ketone is low and the distribution coefficient is thereby forecasted to be large for this solute. (Dutta, 2007)

The selectivity can also be evaluated in terms of the polarities of the solute and solvent, based on the fact that more polar solutes dissolve more readily in a more polar solvent, whereas the less polar solutes will dissolve more readily in a less polar solvent. (Schirmer, 1990)

The polarity of a solvent is characterized by a permanent electric dipole in the molecule caused by the atoms having different electronegativities, this leads to the molecule having unequal charge distribution and thus possessing a "positive end" and a "negative end" (Rydberg et al., 2004; Zumdahl, 2005). However, polarity is not a uniquely defined physical property which can be ascribed to a molecule. Thus, the term polarity is often a relative term and somewhat dependent on how it is measured. (Schirmer, 1990)

A widely used polarity index list is the so-called "Snyder's polarity index[55]", which ranks solvent polarities according to a complex theoretical summing of different parameters such as the molecule's proton acceptor/donor properties, dispersion forces and the size of the dipole moment. (Schirmer, 1990) The list is shown in the table below (Table 3-2).

Table 3-2. Snyder's polarity index[55] (Schirmer, 1990)

Solvent	Polarity index	Solvent group	Solvent	Polarity index	Solvent group
n-Decane	−0.3	0	Isopropanol	4.3	2
Isooctane	−0.4	0	Chloroform	4.3	9
n-Hexane	0.0	0	Acetophenone	4.4	6
Cyclohexane	0.0	0	Methyl ethyl ketone	4.5	6
Carbon disulfide	1.0	0	Cyclohexanone	4.5	6
Butyl ether	1.7	1	Nitrobenzene	4.5	7
Carbon tetrachloride	1.7	7	Benzonitrile	4.6	6
Triethylamine	1.8	1	Dioxane	4.8	6
Isopropyl ether	2.2	1	Tetramethyl urea	5.0	3
Toluene	2.3	7	Diethylene glycol	5.0	4
p-Xylene	2.4	7	Ethanol	5.2	2
Chlorobenzene	2.7	8	Pyridine	5.3	3
Phenyl ether	2.8	8	Ethylene glycol	5.4	4
Ethyl ether	2.9	1	Acetone	5.4	6
Ethoxybenzene	2.9	7	Tetramethyl guanidine	5.5	1
Benzene	3.0	7	Methoxyethanol	5.7	4
n-Octanol	3.2	2	Propylene carbonate	6.0	7
Fluorobenzene	3.3	8	Aniline	6.2	6
Benzyl ether	3.3	7	Acetonitrile	6.2	6
Methylene chloride	3.4	5	Methyl formamide	6.2	3
Methoxybenzene	3.5	7	Acetic acid	6.2	4
1-Pentanol	3.6	2	*N*,*N*-Dimethylacetamide	6.3	3
Ethylene chloride	3.7	5	Dimethyl formamide	6.4	3
Bis(-2-ethoxyethyl)ether	3.9	5	Dimethyl sulfoxide	6.5	6
n-Butanol	3.9	2	Methanol	6.6	2
Isobutanol	3.9	2	Nitromethane	6.8	7
n-Propanol	4.1	2	Formamide	7.3	4
Tetrahydrofuran	4.2	3	Water	9.0	9
Ethyl acetate	4.3	6	Tetrafluoropropanol	9.3	9

In Table 3-2, higher polarity index means higher solvent polarity. Linear approximation can be applied to estimate the polarity of a solvent mixture according to:

$$\mathrm{PI(SM)} = \%\,\mathrm{SOL\,(A)} \times \mathrm{PI(A)} + \%\,\mathrm{SOL\,(B)} \times \mathrm{PI(B)} \qquad (3.4)$$

3.1.4 Particle size in solid-liquid extraction

To solve a solid particle, it is required that the solvent has intimate contact with the particle. Therefore, a smaller particle size is preferable in contrast to a larger, as it leads to an increased surface area that needs to be soaked by the solvent. Thus, the ideal is to perform size-reducing treatments of the solids in order to obtain a fine powder (if possible), before an extraction procedure is conducted. Such treatments can be grinding, cutting, chopping, blending, etc. (Kenkel, 2002)

3.2 Types of extraction processes

A large number of different types of extractors are used overall in the chemical industry, but most often certain extractors are preferred over others depending on the type of product that is to be extracted. For example, in the biotechnological field centrifugal extractors and agitated extraction columns are most often used (Harrison et al., 2003).

The extraction processes reviewed in this chapter will be discussed from a solid-liquid extraction point of view.

3.2.1 Single-Stage Batch Processing (Direct-connect-extraction method)

In this process the solids and solvent are simply mixed together in a container and left until equilibrium is reached. The extraction may be facilitated by agitation and heating (using a water bath). After the extraction process, the extract is separated from the solids (e.g. by filtration) (Toledo, 2006). In this study, this method will be referred to as "direct-connect-extraction method" (DCE).

3.2.2 Multistage Cross-Flow Extraction (Soxhlet extraction method)

The principle of this method is that the solids are continuously brought in contact with solute free solvent throughout the extraction process (Toledo, 2006). As discussed in chapter 3.1.1, several small extractions using smaller portions of solvent will often yield a greater solute recovery than a single-stage extraction, which is the reason this process is more efficient than the single-stage batch process (Shugar & Ballinger, 1996). A good example of this process is the Soxhlet extractor (Toledo, 2006) seen in Fig 3-4.

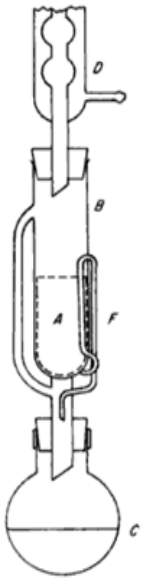


Figure 3-4. The Soxhlet extractor (Shugar & Ballinger, 1996)

In the Soxhlet extractor, the solid substance is placed in a porous container (thimble), which in turn is placed in the Soxhlet tube (A). The thimble functions as a filter, as it retains the insoluble compounds while it lets the dissolved solutes wash through with the solvent. The distilling pot (C) is filled with solvent and serves as the container for the extract. A condenser (D) is assembled just above the Soxhlet tube and is connected to a circulating cooling fluid (cooled water).

When the Soxhlet apparatus is assembled, it is usually placed upon a heating plate. As the solvent is heated up it will vaporize and the vapors will move up along the Soxhlet tube and enter the condensor, where the solvent will condense back into liquid form (reflux) and drop down on the solid substance contained in the thimble where extraction is taken place of soluble compounds. As the level of extract liquid increases in the Soxhlet tube, the liquid will automatically siphon back into the distilling pot through the siphon arm (F).

Thus, fresh solute-free solvent is continuously washing through the solids, and thus each cycle corresponds to one extraction step. The process can be left to continue for as long as needed, and as it is progressed the distilling pot will eventually contain highly concentrated extract. The advantage of the Soxhlet extractor is that small amounts of solvent can be used to obtain high extraction yields. However, large amount of energy is needed in order to vaporize and condense the solvent, therefore the Soxhlet method is usually not used as an industrial extraction method. (Shugar & Ballinger, 1996; Toledo, 2006)

3.2.3 Multistage Countercurrent Extraction

In this process, a number of extractors are interconnected into a single multistage extractor (Toledo, 2006), shown schematically in Fig. 3-5.

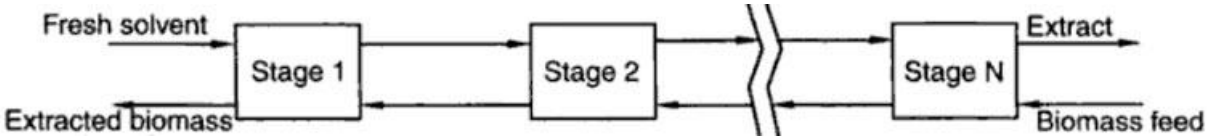


Figure 3-5. Countercurrent extraction (Shi et al., 2002).

In this system, solute-free solvent is fed into the system from one end and fresh solid is fed from the opposite end. The streams leaving each stage are in concentration equilibrium and thus, the solvent will become more and more concentrated with extracted compound after each stage. Countercurrent extractions are usually utilized in the chemical industry and are designed with the number of stages necessary to completely extract the desired compound from the solids. (Toledo, 2006; Shi et al., 2002)

3.3 Assays used in this study

3.3.1 The Folin-Ciocalteu assay for total phenolics

The Folin-Ciocalteu assay is widely used to determine total phenolic contents in foods and beverages. The assay is based on the reduction of the Folin-Ciocalteu reagent in the presence of phenolic compounds. The Folin-Ciocalteu reagent consists of phosphomolybdic-phosphotungstic acid. In an alkaline solution containing phenolic compounds, the reagent will be reduced by the phenolic antioxidants to a blue colored complex. The absorbance change of this reaction can be measured with a spectrophotometer at 765 nm. By constructing a calibration curve with standard solutions of either gallic acid or catechin, samples can be measured for total phenolic content, and the contents expressed as gallic acid or catechin equivalents. (Shahidi & Naczk, 2003)

In this study, the Folin-Ciocalteu assay was done according to the method proposed by Singleton & Rossi, 1965, but with reduced volumes.

3.3.2 The FRAP assay for antioxidant power

The FRAP assay (Ferric Reducing Ability of Plasma) is a simple test that was initially designed to measure “antioxidant power” in plasma. This assay is also frequently applied to dietary substrates such as tea and wine (Pulido et al., 1996). The key components in the FRAP assay are 2,4,6-tripyridyl-s-triazine (TPTZ) and $FeCl_3$ which when mixed together in a solution will form Fe^{III}-TPTZ complexes. At low pH and in the presence of an antioxidant,

the Fe^{III}-TPTZ complex will be reduced to Fe^{II}-TPTZ. This reaction is marked by an intense blue color with absorption maximum at 593 nm which can be measured with a spectrophotometer. This reaction is not compound-specific and any compound that has a lower reduction potential than the Fe^{III}/Fe^{II}-TPTZ half-reaction will reduce the Fe^{III}-TPTZ complex. A calibration curve is constructed by using known concentrations of Fe^{II}-TPTZ. The antioxidant power of samples can then be measured in terms of amount of Fe^{II}-TPTZ generated. (Benzie & Strain, 1996)

In this study, the FRAP assay was done according to the method proposed by Benzie & Strain, 1996. However, the reaction between substrate and reagent was left to undergo for 30 minutes before absorbance measurements were done. This decision was based on a study by Pulido et al., 1996, where it was found that the reaction between polyphenolic antioxidants and FRAP reagent may continue for up to 30 minutes from when the compounds are mixed in a test tube.

4. Material and Methods

4.1 Extraction experiment with the Soxhlet extractor method

4.1.1 Equipment

Soxhlet extractor

Scale: Sartorius, 1700

Measuring cylinders: 200 mL

Kitchen grinder

Laboratory hot plate

Support stands with clamps

1 mm sieve

Falcon tubes

Beakers: 25 mL – 1000 mL

Oven

Refrigerator

4.1.2 Chemicals

Distilled water

Ethanol, C_2H_6O, UNI-CHEM

Ethyl acetate, $CH_3COOCH_2CH_3$, UNI-CHEM

4.1.3 Method

Frozen red grape pomace was prepared in 200 g portions and dried in an oven at 60 °C for 6,5 h . The drying time and temperature were based according to a study by Larrauri et al., 1997, where it was found that drying red grape pomace at 60 °C for 6,5 hours will result in ~10% moisture level and that higher drying temperatures can cause loss of antioxidant activity. The dried grape pomace was then ground using a kitchen grinder and subsequently sieved into particles smaller than 1mm. The choice of particle size was based on a study by Pinelo et al., 2006 on grape pomace, where it was found that a particle size of around 0,5 mm (and up to 5,55 mm) was optimal in terms of extraction kinetics (mass transfer)..

15 g of the dried and sieved grape pomace powder was weighed up and inserted into a Soxhlet thimble. 150 ml of solvent was measured up using a measuring cylinder and then transferred to the Soxhlet distilling pot. The Soxhlet extractor was assembled with the distilling pot, thimble and condenser. The condenser was connected to the tap-water line, using tap-water as cooling fluid. The Soxhlet extractor was fastened to a support-stand above

a heating plate with the distilling pot touching the plate and the solvent was heated until reflux occurred. The extraction was left to run for 6 h. Time of extraction was based on a study by de Campos et al., 2008, where 6 hours of extraction was found to result in a high yield of antioxidants when using the Soxhlet.

When 6 hours had passed, the extract was transferred to a marked falcon tube and stored in a refrigerator.

The extraction was done using the following three types of solvents: ethanol, ethanol-distilled water (1:1), and ethyl acetate.

Samples were tested for total phenol contents according to the Folin-Ciocalteu assay and for antioxidant power according to the FRAP assay. The solids were disposed.

4.2 Determination of extraction time for DCE method

4.2.1 Equipment

Three-neck round-bottom flasks: 500 mL

Condensers

Measuring cylinders: 200 mL

Kitchen grinder

Electric lab stirrers: Eurostar IKA Werke

Cryostat circulator: SCIENTZ DC3006

Heating water bath: SCIENTZ SCI5

Support stands with clamps

Scale: Sartorius, 1700

Volumetric pipettes: 10 mL

Glass funnels

Filter papers: 42 Whatman

Falcon tubes

Beakers: 25 mL – 1000 mL

Oven

Refrigerator

4.2.2 Chemicals

Distilled water

Analytical grade ethanol, C_2H_6O, UNI-CHEM

Analytical grade ethyl acetate, $CH_3COOCH_2CH_3$, UNI-CHEM

4.2.3 Method

For the direct-connect-extraction method, no recommended extraction time was found in previous studies. However, in a study by Spigno & Faveri, 2005, no significant difference was found between 5 hours and 24 hours of extraction in terms of extraction yield of polyphenols from grape stalks. In another study by Yilmaz & Toledo, 2004, a significant correlation was found between total phenol contents and absorbance measurements at 280 nm. Based on these findings, it was decided in this study to determine the best time of extraction for the DCE method by measuring absorbances at 280 nm in extract samples taken over an interval of 6 hours.

Frozen red grape pomace was prepared in 200 g portions and dried in an oven at 60 °C for 6,5 h . The drying time and temperature were based according to a study by Larrauri et al., 1997, where it was found that drying red grape pomace at 60 °C for 6,5 hours will result in ~10% moisture level and that higher drying temperatures can cause loss of antioxidant activity. The dried grape pomace was then ground using a kitchen grinder and subsequently sieved into particles smaller than 1mm. The choice of particle size was based on a study by Pinelo et al., 2006 on grape pomace, where it was found that a particle size of around 0,5 mm (and up to 5,55 mm) was optimal in terms of extraction kinetics (mass transfer).

Two three-neck round-bottom flasks were each filled with 40 g of weighted dried and sieved grape pomace powder along with 400 mL of solvent.

The flasks were immersed into a water bath, so the contents were below the water surface, and fastened in position using support-stands and clamps. The water bath temperature was set to 45 °C.

Two electric lab stirrers were put next to the water bath with the stirrers submerged into each flask through one of the flask-necks. The stirring rate was set to 450 rpm.

A condenser was assembled to the flasks through the second flask-neck. The condensers were connected to a cryostat circulator, using cooled water as cooling fluid. The third flask-neck was capped and used as a route to draw samples.

The extraction was left to run for 6 hours and samples were taken every 30 minutes.

The samples were immediately filtered using glass funnels with Whatman filters (grade no. 42) to separate extract from solids. The filtered extracts were transferred to marked falcon tubes and stored in a refrigerator. The solids were disposed.

The extraction was done with the following solvents: Ethanol-water (1:1), ethanol and ethyl acetate.

Absorbance measurements of the samples were made using a spectrophotometer. The spectrophotometer was set on a reading range of 0A-3.99A, and the samples were diluted with

pure solvent with a dilution factor of 50 prior to measurements. The dilution was done in order to obtain readings within the 0A-3.99A absorbance range. Absorbance readings were measured at 280 nm. Before the measurements, the spectrophotometer was zeroed using a blank solution. A chart was made to correlate absorbance values with time of extraction, for each solvent.

4.3 Extraction experiment with the DCE method

4.3.1 Equipment

Three-neck round-bottom flasks: 500 mL

Condensers

Measuring cylinders: 200 mL

Kitchen grinder

Electric lab stirrers: Eurostar IKA Werke

Cryostat circulator: SCIENTZ DC3006

Heatingkk water bath: SCIENTZ SCI5

Support stands with clamps

Scale: Sartorius, 1700

Volumetric pipettes: 10 mL

Buchner funnel & vacuum pump

Filter papers: 42 Whatman

Falcon tubes

Beakers: 25 mL – 1000 mL

Oven

Refrigerator

4.3.2 Chemicals

Distilled water

Analytical grade ethanol, C_2H_6O, UNI-CHEM

Analytical grade ethyl acetate, $CH_3COOCH_2CH_3$, UNI-CHEM

4.3.3 Method

Frozen red grape pomace was prepared in 200 g portions and dried in an oven at 60 °C for 6,5 h . The drying time and temperature were based according to a study by Larrauri et al., 1997, where it was found that drying red grape pomace at 60 °C for 6,5 hours will result in ~10% moisture level and that higher drying temperatures can cause loss of antioxidant activity. The dried grape pomace was then ground using a kitchen grinder and subsequently sieved into particles smaller than 1mm. The choice of particle size was based on a study by Pinelo et al., 2006 on grape pomace, where it was found that a particle size of around 0,5 mm (and up to 5,55 mm) was optimal in terms of extraction kinetics (mass transfer).

Two three-neck round-bottom flasks were each filled with 40 g of weighted dried and sieved grape pomace powder along with 400 mL of solvent.

The flasks were immersed into a water bath, so the contents were below the water surface, and fastened in position using support-stands and clamps. The water bath temperature was set to 45 °C.

Two electric lab stirrers were put next to the water bath with the stirrers submerged into each flask through one of the flask-necks. The stirring rate was set to 450 rpm.

A condenser was assembled to the flasks through the second flask-neck. The condensers were connected to a cryostat circulator, using cooled water as the cooling fluid. The third flask-neck was capped and used as a route to draw samples.

The extraction was done in two sets using two different times of extraction: 2.5 h and 4.5 h. For each set, the solid-solvent mixtures in the flasks were separated after, using a Buchner funnels and grade no. 42 Whatman filters. 20 mL samples was taken from the filtered extracts and transferred to falcon tubes. The filtered extracts and the solids were mixed back together into the three-neck round-bottom flasks along with 20 mL of pure solvent (to replace the sample volumes taken) and the extractions were resumed for another 1.5 h.

A second batch of extractions was done using the same procedure as above, but this time using a water bath temperature of 55 °C.

Samples were tested for total phenol contents according to the Folin-Ciocalteu assay and for antioxidant power according to the FRAP assay. The solids were disposed.

4.4 Folin-Ciocalteu assay: testing for total phenols

4.4.1 Equipment

Spectrophotometer: Shimadzu UVmini 1240

Volumetric flasks: 25 mL, 50 mL, 100 mL, 1000 mL

Automatic pipettes: Rongtai, 1-5 ml

Automatic pipette: Rongtai: 100 µl

Scale: Sartorius, 1700

Beakers: 25 mL

Test tubes: ~50 mL

Test tube tray

4.4.2 Chemicals
Distilled water

Folin-Ciocalteu reagent, Sigma-Aldrich

Anhydrous Sodium carbonate, Na_2CO_3, Sigma-Aldrich

Gallic acid, $C_6H_2(OH)_3COOH(s)$

4.4.3 Method
10 mL of Folin-Ciocalteu reagent was transferred into a 100 mL volumetric flask using an automatic pipette, and then diluted to a 100 mL solution with distilled water.

A 7.5 % Na_2CO_3 solution was prepared by weighting 75 g of Na_2CO_3 anhydrous and dissolving it to 1 L with distilled water in a volumetric flask.

Preparation of calibration solutions:
A 50 mL solution of 10 mg/mL gallic acid was made by dissolving 500 mg gallic acid in 25 mL ethanol and then diluting to 50 mL in a volumetric flask. From this flask, aliquots of 1 mL to 10 mL were taken and each diluted with distilled water to 100 mL in volumetric flasks. 200 µl from each flask was transferred to test tubes along with 10 mL Folin-Ciocalteu reagent diluted solution and 1.8 mL of distilled water. The tubes were carefully agitated and then left to rest for 5 minutes.

After 5 minutes had passed, 8 mL of 7.5 % Na_2CO_3 solution was added to each test tube. The tubes were agitated and left to rest for 2 h.

After 2 hours, the absorbances in the calibration solutions were measured with a spectrophotometer at 765 nm. Before the measurements, the spectrophotometer was zeroed using a blank solution.

From the obtained absorbance data, a calibration curve was constructed and an equation for the calibration curve was calculated in Excel.

Testing of total phenolics contents in samples:
The same procedure as above was executed with 200 µl sample extracts instead of gallic acid solutions. Before mixing the extract with the reagent solutions, samples were diluted with pure solvents with a dilution factor of 10. This was done in order to obtain absorbance readings within the calibration curve range.

Total phenolics contents in the samples were interpolated from the calibration curve by using the calibration curve equation. All data for the samples were related to the sample masses and expressed as equivalent of gallic acid (mg GAE/g sample) (See Appendix 2: Calculations).

4.5 FRAP assay: testing for total antioxidant power

4.5.1 Equipment

Spectrophotometer: Shimadzu UVmini 1240

Volumetric flasks: 25 mL, 50 mL, 100 mL, 1000 mL

Volumetric pipettes: 1 mL, 5 mL

Automatic pipettes: Rongtai, 1-5 ml

Automatic pipette: Rongtai: 100 µl

Analytical balance: Sartorius

Electric pH meter

Beakers: 25 mL

Test tubes: ~50 mL

Test tube tray

Laboratory incubator

Aluminum foil

4.5.2 Chemicals

Distilled water

Mohr salt, $[NH_4]_2[Fe][SO_4]_2 \cdot 6H_2O(s)$

Hydrochloric acid, HCl, (37%, analytical grade), UNI-CHEM

Acetic acid, CH_3COOH, (analytical grade), UNI-CHEM

Iron(III) chloride, $FeCl_3(s)$

Sodium acetate, $C_2H_3NaO_2(s)$

TPTZ (2,4,6-tripyridyl-s-triazine)(s)

4.5.3 Method

A 40 mM HCl solution was made by measuring 3.3 mL concentrated HCl with a volumetric pipette and diluting up to 1 L with distilled water in a volumetric flask.

A 20 mM solution $FeCl_3$ was prepared by weighting 0.81 g $FeCl_3$ in a 250 mL volumetric flask. 3 drops of the 40 mM HCL solution was added to the salt and then it was diluted with distilled water up to the 250 mL mark.

A 300 mM acetate buffer solution with pH 3.6 was prepared by weighting 1.8688 g of $C_2H_3NaO_2$ in a 1000 mL volumetric flask. 16 mL of analytical grade acetic acid was added to the flask and the solution was diluted up to the 1000 mL mark with distilled water. The pH of the buffer solution was measured with an electric pH meter to make certain a pH of 3.6 was obtained.

A 10 mM TPTZ solution was prepared by weighting 312 mg of TPTZ in a 100 mL volumetric flask and diluting up to the 100 mL mark with the previously prepared 40 mM HCl solution.

Finally, the FRAP reagent solution was prepared by taking 2.5 mL of the 10 mM TPTZ solution, 25 mL of the pH 3.6 buffer solution and 2.5 mL of the 20 mM $FeCl_3$ solution and mixing it all in a beaker. The solution was agitated and the beaker was capped with aluminum foil.

Preparation of calibration solutions:
0,3922 g of Mohr salt was weighted and transferred to a 100 mL volumetric flask. 1 mL of concentrated HCl was added before the salt was diluted to the 100 mL mark with distilled water.

From this solution, calibration solutions were prepared by taking aliquots of 1 mL to 10 mL and diluting each with distilled water up to 100 mL in volumetric flasks. Hence, these solutions corresponded to 100-1000 µM of Fe^{2+}.

500 µl from each calibration solution was transferred to test tubes along with 15 mL of FRAP reagent solution. The tubes were carefully agitated and then incubated at 37 °C for 30 min.

When 30 min had passed the test tubes were taken out of the incubator and absorbance readings were measured for the calibration solutions with a spectrophotometer at 593 nm. Before the measurements the spectrophotometer was zeroed using a blank solution.

From the obtained absorbance data, a calibration curve was constructed and an equation for the calibration curve was calculated in Excel.

Testing of total phenolics contents in samples:
The same procedure as above was executed with 500 µl sample extracts instead of Mohr salt solutions.

Before mixing the extract with the reagent solutions, all extract samples were diluted with pure solvents with a dilution factor of 43.5. This was done in order to obtain absorbance readings within the calibration curve range.

Total antioxidant powers in the extract samples were interpolated from the calibration curve by using the calibration curve equation. The obtained data were related to the sample masses and expressed as µM Fe^{2+}/g sample (See Appendix 2: Calculations).

5. Results and discussion

It should be pointed out that the results for this study are incomplete and some data are missing in order to adequately investigate all the parameters that were intended to be analyzed. A large amount of extraction samples were taken throughout the experiment but not enough samples were accurately tested due to various problems encountered, and also due to changes in the aims of this study at the late stages of the experiment.

5.1 Calibration curve for the Folin-Ciocalteu assay

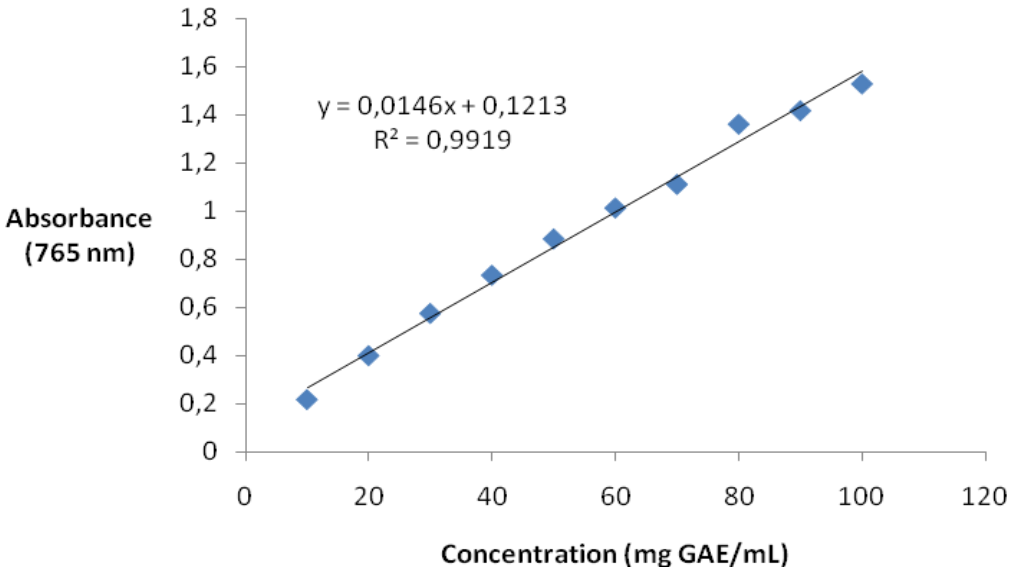


Figure 5-1. Calibration curve for gallic acid.

Above is the calibration curve constructed for gallic acid by applying linear regression to the plotted data (Fig. 5-1). As can be seen, the calibration curve extends linearly from 10 to 100 mg [gallic acid]/mL, which is a wide enough concentration range for sample data interpolation.

The calibration curve equation was calculated in Excel and obtained to be:
y = 0,0146x + 0,1213, with an R-square value of 0,9919 which indicates that the model is very well fit with the data.

5.2 Calibration curve for the FRAP assay

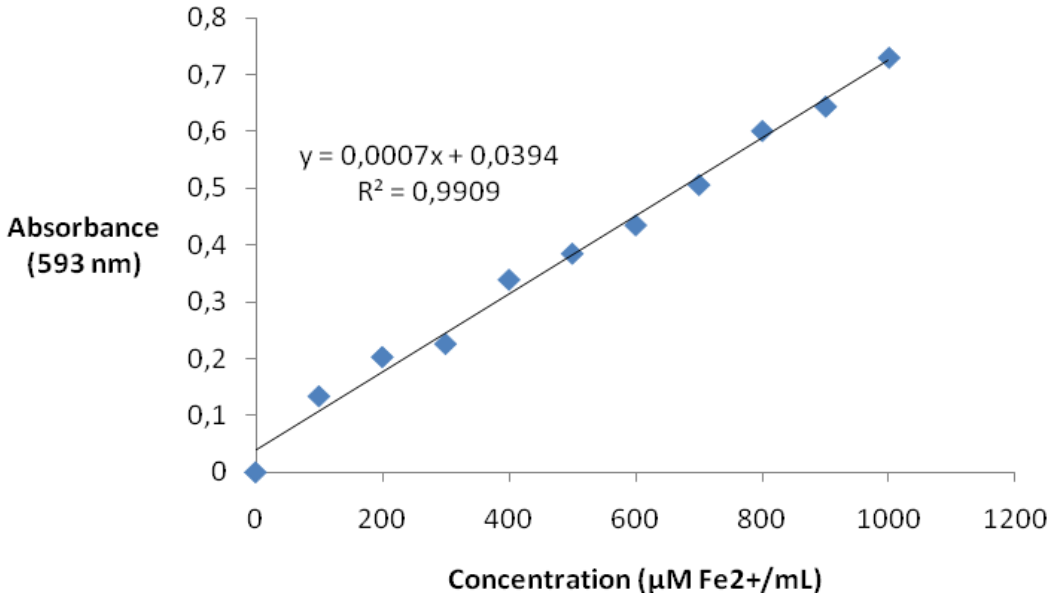


Figure 5-2 Calibration curve for total antioxidant power.

Above is the calibration curve constructed for total antioxidant power by applying linear regression to the plotted data (Fig. 5-2). The concentration is expressed as µM Fe^{2+} and spans between 100 and 1000 µM, which turned out to be an excellent range for interpolation of tested sample values.

The calibration curve equation was calculated in Excel and obtained to be:
y = 0,0007x + 0,0394, with an R-square value of 0,9909 which indicates that the model is very well fit with the data.

5.3 Extraction experiment with the Soxhlet extractor method

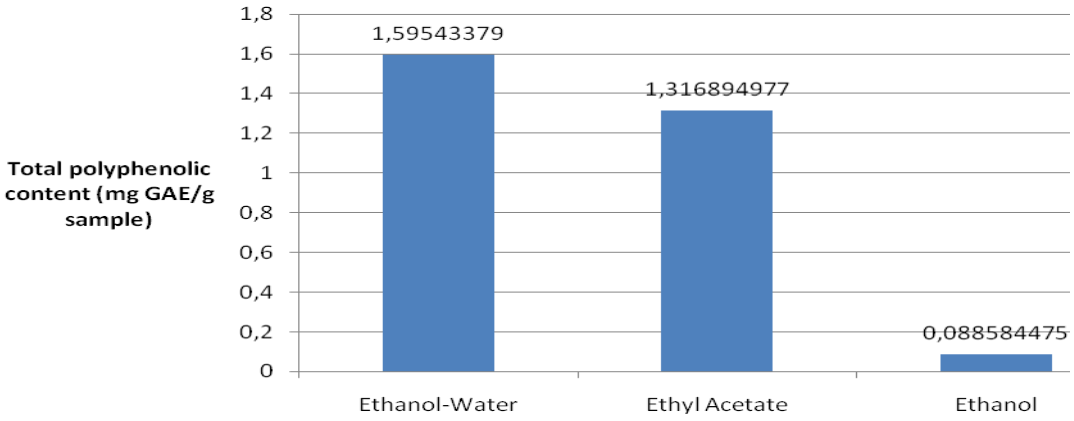


Figure 5-3. Total phenolic contents for Soxhlet extracts.

In the column chart above (Fig. 5-3) it can be seen that the ethanol-water solvent gave the highest extraction of total phenols with a mean value equivalent to ~1.6 mg GAE/g sample. The higher extraction potency of ethanol-water compared to the other solvents was predicted and in accordance to literature since ethanol-water is the solvent that has the highest polarity of the three solvents used (Schirmer, 1990), and therefore has a greater affinity to bind polyphenolic antioxidants (due to their own polar nature) (Tzia & Liadakis, 2003). It can be seen that the value for the ethanol solvent was unnaturally low considering that ethanol is the second most polar solvent of the three. This low value is most likely due to an error in the testing procedure and/or in sample preparation.

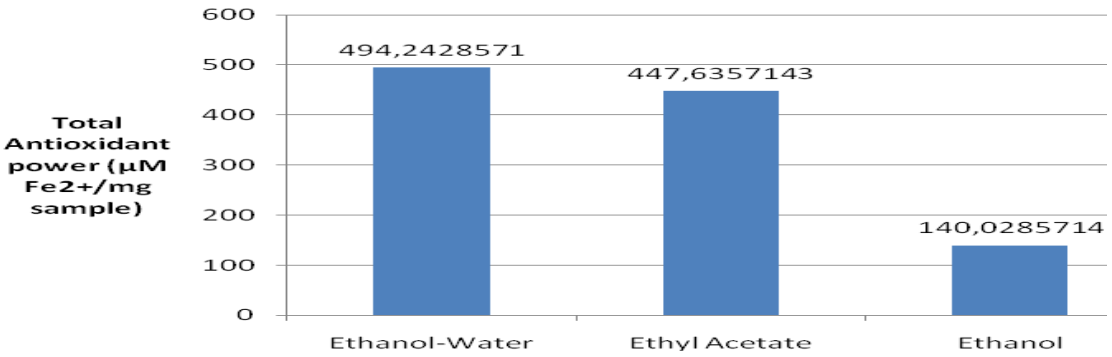


Figure 5-4. Antioxidant powers for Soxhlet extracts.

The chart for total antioxidant power seen above (Fig. 5-4) follows the same pattern as the chart for total phenolic contents, with the ethanol-water solvent having the highest antioxidant power.

5.4 Determination of extraction time for DCE method

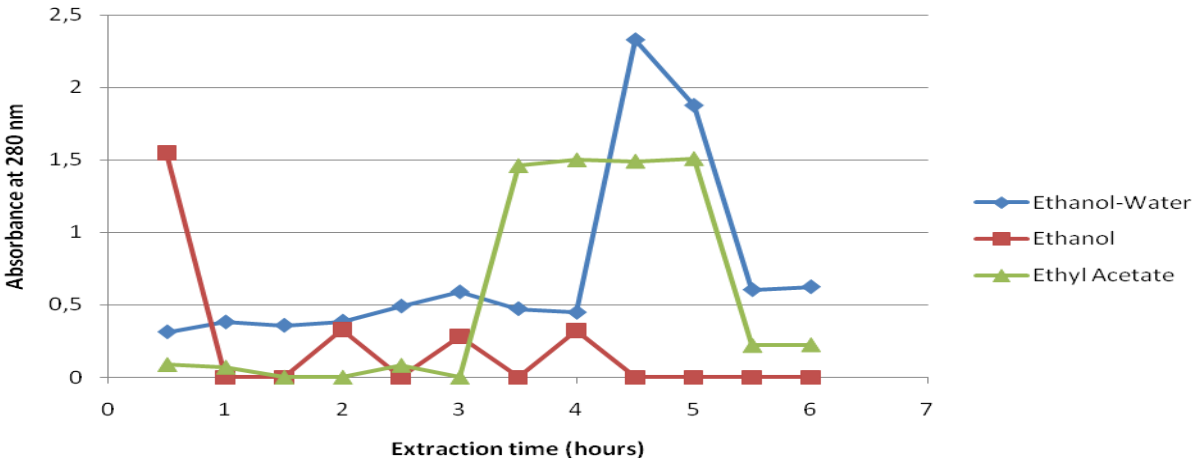


Figure 5-5. Absorbance vs. extraction time for DCE method.

In the absorbance vs. extraction-time graph above (Fig. 5-5), absorbance measurements at 280 nm for each solvent are plotted against the extraction time, which spanned from 0 to 6 hours. The dots in the graph represent tested samples.

An unexpectedly high absorbance at just above 1.5 can be seen for the first ethanol sample at 0.5 hours of extraction. This value can be dismissed as it is most likely due to an error in the dilution process in the preparation of the sample. The absorbance for ethanol followed a zigzag pattern from 0 to around 0.3 from 1.5 h to 4.5 h, with the highest peaks at 2 and 4 hours of extraction.

For ethyl acetate, a small peak at around 0.08 can be seen at 2.5 hours of extraction, which had declined to 0 at 3 hours extraction. Between 3.5 h and 5 hours the absorbance peaked and stayed at around 1.5.

For the ethanol-water solvent a peak at around 0.5 can be seen between 2.5 and 3.5 hours of extraction, and a large peak of around 2.5 after 4.5 hours of extraction.

A general trend for all three solvents can be seen towards higher absorbances after 2-3 hours and after 4-5 hours of extraction and thus indicating higher extraction contents of total phenols are obtained at these times of extraction. Therefore, extraction times of 2.5 h and 4.5 h were used in the experiments with the DCE method.

5.5 Extraction experiment with the DCE method

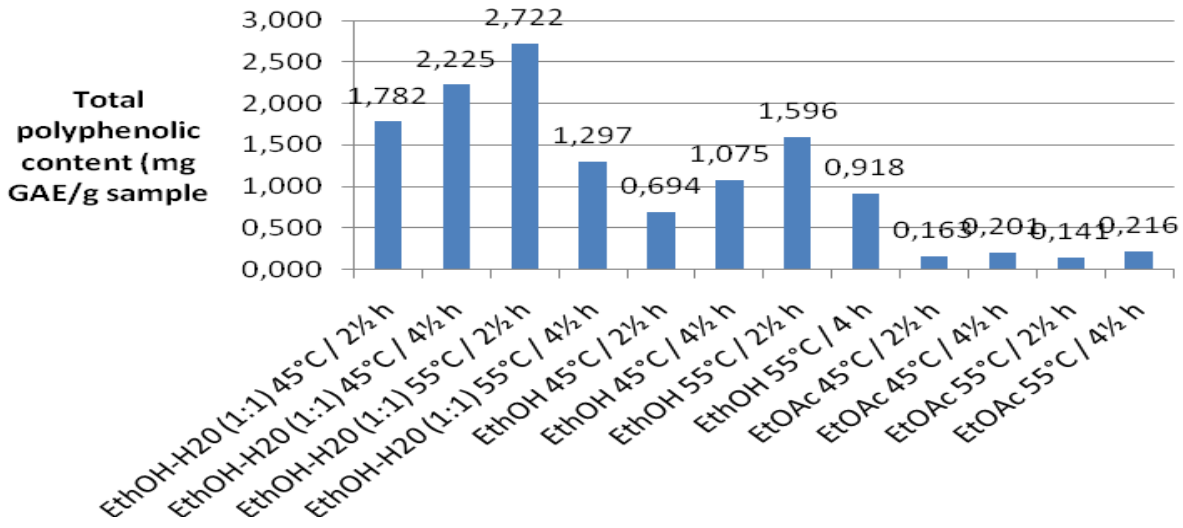


Figure 5-6. Total polyphenolic contents at 45 °C vs. 55 °C for DCE extracts.

In the chart above (Fig. 5-6), mean values from the Folin-Ciocalteu assay are presented. It should be noted that unfortunately no sample for the ethanol solvent at 55 °C and 4 ½ hours of extraction was tested with the Folin-Ciocalteu assay, therefore data for ethanol as solvent at 55 °C and 4 hours of extraction was used in the chart instead.

It can clearly be seen that for the ethanol-water and pure ethanol solvents, the highest extracted concentration of polyphenols is achieved after 2½ hours of extraction when using an extraction temperature of 55 °C. For both solvents, after approximately 4 ½ hours of extraction at 55 °C, the polyphenolic concentration had sharply declined, even below the values obtained after 4½ hours extraction at 45 °C. However, it can be seen that between 2 ½ and 4 ½ hours of extraction at 45 °C, the concentration of polyphenols had increased. This

suggests that a possible degradation of phenolic content may had occurred at the higher extraction temperature when the extraction was left to continue for longer than 2 ½ hours.

For ethyl acetate as solvent a different pattern is seen, and it seems like the extraction temperature does not have a significant impact on the extraction potency. An increase in polyphenolic content is seen from 2 ½ hours to 4 ½ hours for both extraction temperatures. The reason for this different behavior seen for ethyl acetate compared to the other solvents is unclear.

It can also be seen that ethanol-water had the highest extraction potency of the three solvents used, giving a mean extracted concentration of polyphenols equivalent to 2.77 mg GAE/g sample (with the extraction temperature of 55 °C and 2½ hours of extraction), followed by ethanol and lastly ethyl acetate. With other words, the extraction potency increased with ascending polarity of the solvents and this is in accordance to literature, since ethanol-water is the solvent that has the highest polarity of the three solvents used (Schirmer, 1990), it therefore has a greater affinity to bind polyphenolic antioxidants (due to their own polar nature) (Tzia & Liadakis, 2003).

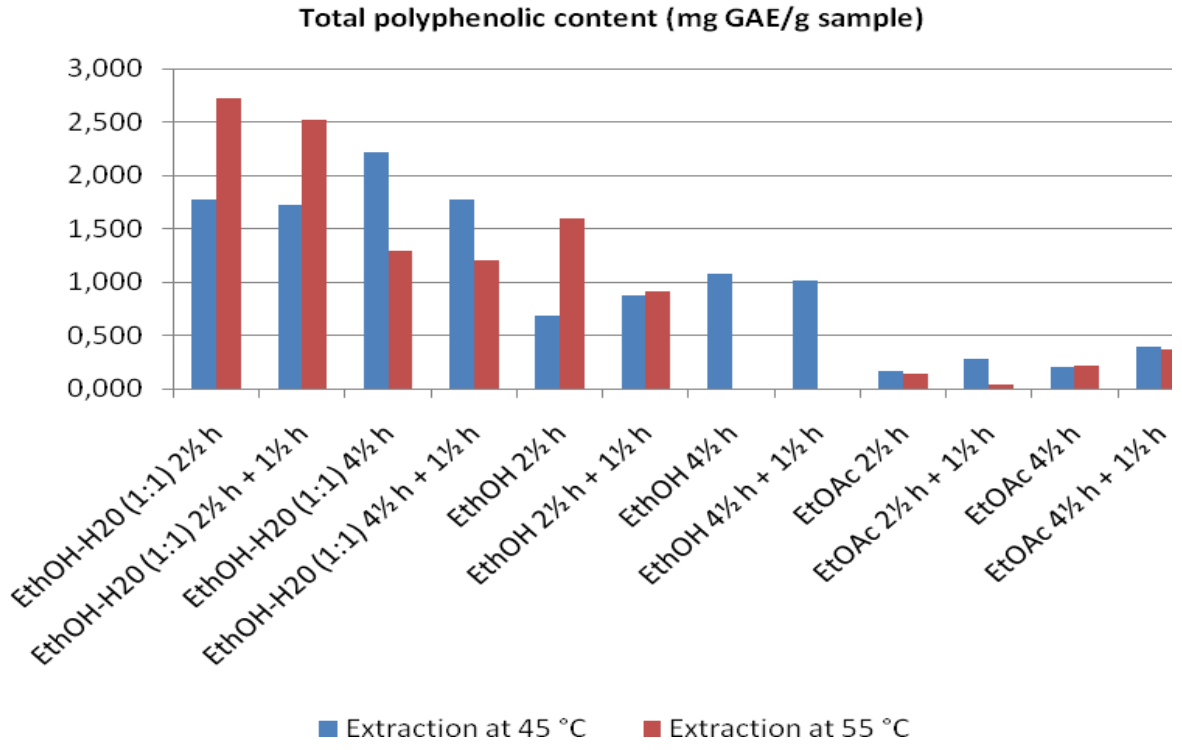


Figure 5-7. Total polyphenolic contents at 45 °C vs. 55 °C and first pass vs. second pass for DCE extracts.

The above discussion is further supported by the chart above (Fig. 5-7), where total polyphenolic content for the first pass (2½ h, 4½ h) versus the second pass (2½ h + 1½ h, 4½ h + 1½ h) have been included. For ethanol at 4½ and 6½ hours of extraction (55 °C), no data was available.

For the ethanol-water solvent at 55 °C extraction temperature, it can again be seen that the polyphenolic concentration decreased steadily after 2½ hours of extraction. At the 6 h mark, the concentration had decreased to almost half of the highest detected value. The same pattern can be seen at 45 °C extraction temperature, where the polyphenolic concentration decreased

after the highest value (after 4½ hours of extraction), again suggesting that a degradation process probably occurs after 2½ hours of extraction at 55 °C and after 4½ hours of extraction at 45 °C extraction temperature.

For the ethyl acetate solvent, a big difference can be noted after 4 hours of extraction, between 45 °C and 55 °C extraction temperatures. This difference may be explained by the fact that only one sample was tested of ethyl acetate at 4 hours of extraction and 55 °C extraction temperature, and this sample had an extraordinary low absorbance, suggesting that the low value obtained for this sample is very likely in error. Putting this value aside, no significant difference can be distinguished for ethyl acetate between 45 °C and 55 °C extraction temperatures.

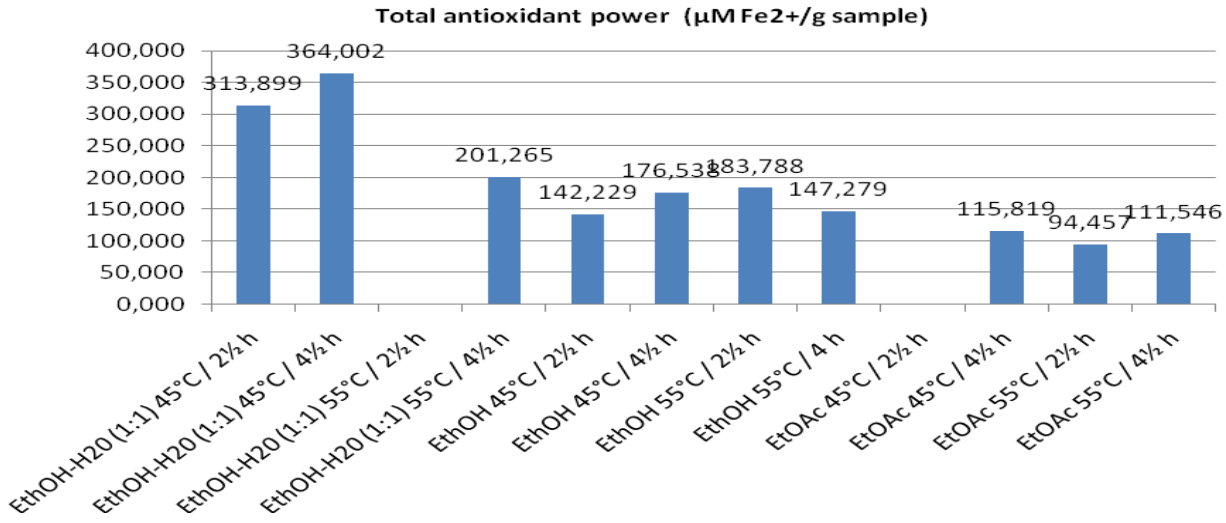


Figure 5-8. Antioxidant powers at 45 °C vs. 55 °C for DCE extracts.

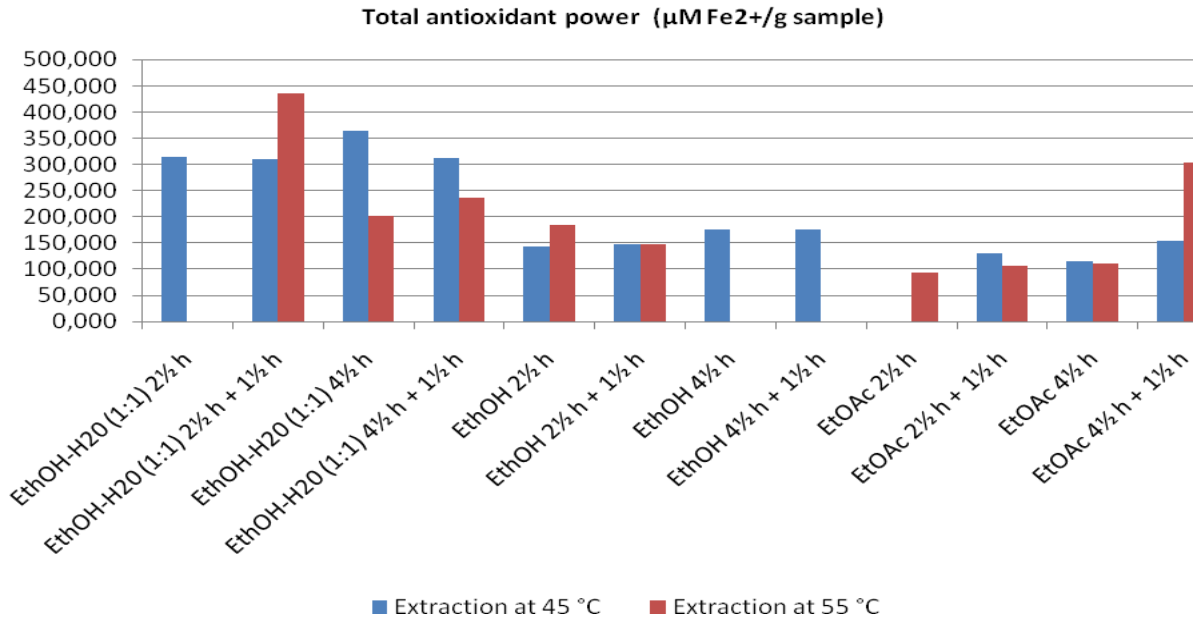
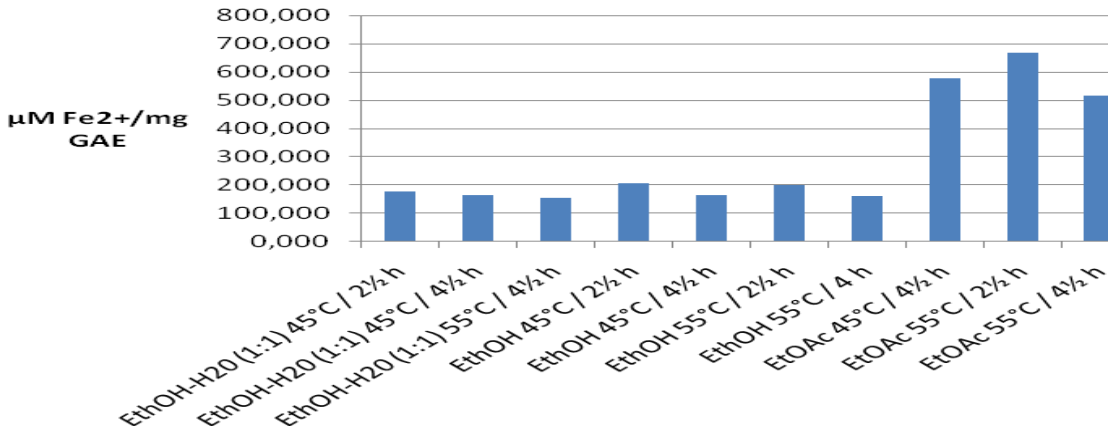


Figure 5-9. Antioxidant powers at 45 °C vs. 55 °C and first pass vs. second pass for DCE extracts.

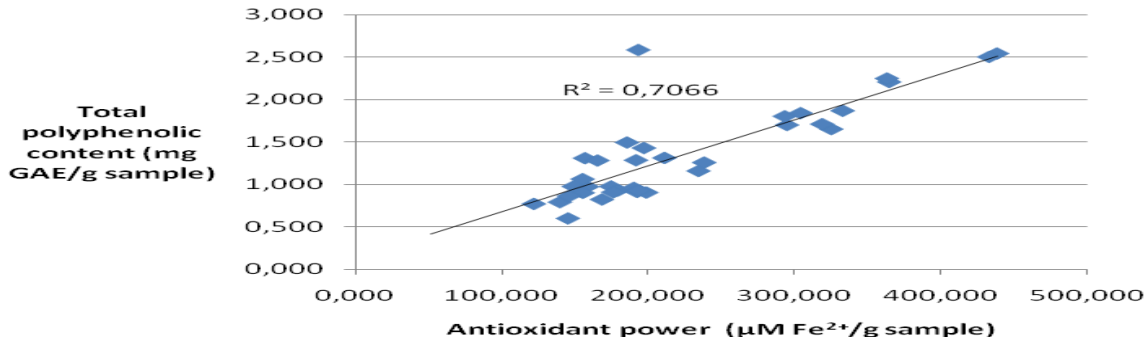
In the above charts (Fig. 5-8 & Fig. 5-9), antioxdant powers according to the FRAP assay are vizualized. Unfortunately, the charts are not complete due to missing data.
As can be seen, ethanol-water as solvent gave the highest antioxidant activity at 45 °C and 4½ hours of extraction, with a mean antioxidant power equivalent to ~ 364 µM Fe^{2+}/g sample. However, taking in account that the above charts follows the same pattern as the data for total polyphenolic content (Fig. 5-6 & Fig. 5-7), it can be guessed that ethanol-water as solvent at 55 °C and 2½ hours of extraction would probably have had the highest antioxidant power. Overall, a clear correlation can be seen between amount of polyphenolic content and antioxidant activity for all solvents.

Relating ethyl acetate to the ethanolic solvents, a remarkably higher antioxidant activity is observed relative to total phenol content for ethyl acetate. This is better visualized in the chart below.



5-10. Antioxidant power/polyphenolic content for DCE extracts.

The antioxidant activities per grams polyphenolic contents in the ethyl acetate extracts were more than 3 times higher than the correspondent values for the ethanolic solvents. One reason for this might be the formation and/or increased solubility of less polar, non-phenolic antioxidants, which were more soluble in ethyl acetate than in the more polar ethanolic solvents.



5-5.6. Relation between total polyphenolic content and antioxidant power.

The correlation between total phenolic content and antioxidant power for the ethanolic solvents is further confirmed by the graph above, where a clearly linear relationship can be observed.

5.6 Soxhlet method versus DCE method

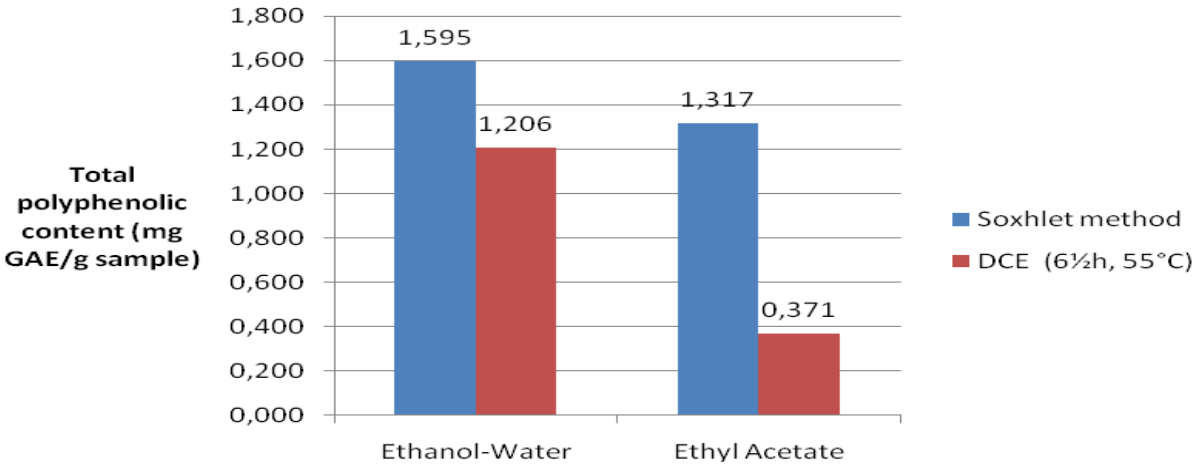


Figure 5-11. Column chart depicting total polyphenolic content for the Soxhlet method vs. DCE method

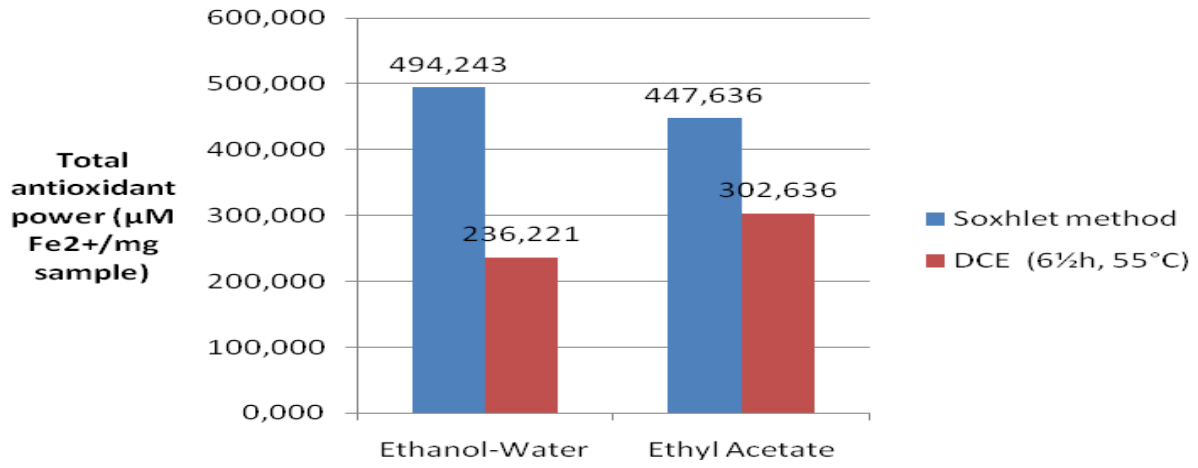


Figure 5-12. Column chart depicting total antioxidant power for the Soxhlet method vs. DCE method

From the charts above (Fig. 5-11 & Fig 5-12), a comparison can be made between the Soxhlet method and the DCE method for ethanol-water and ethyl acetate. Data for ethanol as solvent has not been included in the charts since no reliable data existed for this solvent. Data for 6 hours of extraction at 55 °C was selected to represent the DCE method in the above charts. The reason for this was to make a more accurate comparison between the two methods (based on the fact that the Soxhlet extraction was carried on for 6 hours).

It can clearly be seen that the Soxhlet was the most effective on extracting polyphenols. Both the total phenolic contents and antioxidant powers were significantly higher in the samples extracted with the Soxhlet compared to the DCE method. The better performance of the Soxhlet can most likely be attributed to the recycling (reflux) of the solvent during extraction, this theory is further confirmed by results presented in another study by Soong & Barlow (2003).

6. Conclusions

From the data obtained in this study, it was observed that for both the DCE method and Soxhlet method, a mixture of ethanol-water (1:1) as solvent gave the highest concentrations of extracted phenols (according to the Folin-Ciocalteu assay) and the highest antioxidant activities (according to the FRAP assay), on all parameters tested. For the DCE method, it was seen that the total phenolic concentrations and antioxidant activities in the extracts declined in order with descending polarity of the solvents (i.e. ethyl acetate being less effective than pure ethanol). Unfortunately this could not be confirmed for the Soxhlet extractor since not enough data was available. However, based on the clear correlation seen between extraction potency and solvent polarity in the available data, it can conclusively be said that phenolic extraction potency increases with ascending solvent polarity.

For the DCE method with ethanolic solvents, it was seen that an extraction time of 2½ hours at 55 °C extraction temperature gave the highest extracted concentrations of total phenolic content and highest antioxidant activity. At 45 °C extraction temperature, highest values were observed after 4½ hours of extraction. Continuing extractions longer resulted in declining phenolic concentrations and antioxidant activities, suggesting that a degradation of phenolic content might occur at higher extraction temperatures, which is seemingly accelerated with increasing extraction temperature. For ethyl acetate as solvent no clear relation could be seen between extraction potency and temperature.

On the relationship between total phenols and antioxidant power, a much larger antioxidant capacity per mg extracted phenols was detected for ethyl acetate compared to the ethanolic solvents from data obtained from the DCE method. One possible reason for this could be the formation of less polar, non-phenolic antioxidants from precursors in the extract, which were more soluble in ethyl acetate than in the more polar ethanolic solvents, and/or increased solubility of non-phenolic compounds with antioxidant properties. On the other hand, a clearly linear correlation was observed between total phenolic content and antioxidant power for the ethanolic solvents, further confirming the better efficiency of the more polar solvents on the extraction of phenolic antioxidants.

On the comparison of DCE and the Soxhlet method in terms of extraction potency and antioxidant power, it was found that the Soxhlet was significantly more effective than the DCE method. The comparison was based on extraction data for ethanol-water (1:1) and ethyl acetate as solvents. The better performance of the Soxhlet can probably be attributed to the continuous reflux of the solvent during extraction, resulting in multiple extraction steps.

References

Asano, K. (2006) *Mass Transfer: From Fundamentals to Modern Industrial Applications*. Weinheim: WILEY-VCH.

Benzie, I.F.F. & Strain, J.J. (1996) *The Ferric Reducing Ability of Plasma (FRAP) as a measure of ‘‘Antioxidant Power’’: The FRAP Assay*. Analytical Biochemistry. 239, 70-76.

Buhler, D.R. & Miranda, C. (2000) Antioxidant Activities of Flavonoids <http://lpi.oregonstate.edu/f-w00/flavonoid.html> [2010-05-20].

Chen, J. (2002) *Selenium Compounds and Antioxidant Capacity in Bovine Milk. Studies on Glutathione Peroxidase, Bioavailability and Electrochemical Techniques*. Lund: Pure and Applied Biochemistry (LTH), Lund University.

Cussler, E.L. (2003) Diffusion: Mass Transfer in Fluid Systems. Cambridge: Cambridge University Press.

Dutta, B.K. (2007) *Principles of Mass Transfer and Separation Processes*. New Delhi: Prentice-Hall of India.

Gamse, T. (2002) *Liquid - Liquid Extraction and Solid - Liquid Extraction*, (Electronic). Pdf-document: <http://www.iq.uva.es/separacion/archivos/SkriptumExtraction.pdf>. [2010-05-13].

Harrison, R.G., Todd, P., Rudge, S.R. & Petrides, D.P. (2003) *Bioseparations Science And Engineering*. New York: Oxford University Press.

Higdon, J. & Drake, V.J. (2008) *Flavonoids* <http://lpi.oregonstate.edu/infocenter/phytochemicals/flavonoids/index.html> [2010-05-22].

Kenkel, J. (2002) *Analytical Chemistry for Technicians*. Boca Raton: CRC Press.

Lafka, T.I., Sinanoglou, V. & Lazos, E.S. (2006) *On the extraction and antioxidant activity of phenolic compounds from winery wastes*. Food Chemistry. 104, 1206–1214.

Larrauri, J.A., Ruprez, P. & Saura-Calixto, F. (1997) *Effect of Drying Temperature on the Stability of Polyphenols and Antioxidant Activity of Red Grape Pomace Peels*. Journal of Agricultural and food chemistry. 45, 1390-1393.

Masterton, W.L. & Hurley, C.N. (2008) *Chemistry: Principles and Reactions.* Belmont: Thomson Brooks.

Moure, A., Cruz, J.M., Franco, D., Dominguez, J.M., Sineiro, J., Dominguez, H., Nunez, M.J. & Parajo, J.C. (2000) *Natural antioxidants from residual sources*. Food Chemistry. 72, 145-171.

Pinelo, M., Sineiro, J. & Nunez, M.J. (2006) Mass transfer during continuous solid–liquid extraction of antioxidants from grape byproducts. Journal of Food Engineering. 77, 753-1122.

Pulido, R., Bravo, L. & Saura-Calixto, F. (1996) *Antioxidant Activity of Dietary Polyphenols As Determined by a Modified Ferric Reducing/Antioxidant Power Assay*. 48, 3396-3402.

Rydberg, J., Cox, M., Musikas, C. & Choppin, G.R. (2004) *Solvent extraction principles and practice.* New York: Marcel Dekker.

SBU (Statens beredning för medicinsk utvärdering). (1997) *Att förebygga sjukdom - med antioxidanter*. Stockholm: SB Offset AB.

Schirmer, R.G. (1990) *Modern Methods of Pharmaceutical Analysis: v. 2*. Boca Raton: CRC Press.

Shahidi, F. (1997) *Natural Antioxidants: Chemistry, Health Effects, and Applications*. Urbana: AOCS Press.

Shahidi. F. & Naczk, M. (2003) *Phenolics in Food and Nutraceuticals: Sources, Chemistry, Effects, Applications*. Boca Raton: CRC Press.

Shi, J., Mazza, H. & Maguer, M.L. (2002) *Functional Foods: Vol 2: Biochemical and Processing Aspects*. Boca Raton: CRC press.

Shugar, G.J. & Ballinger, J.T. (1996) *Chemical Technicians' Ready Reference Handbook*. New York: McGraw-Hill.

Small, H. (1989) *Ion Chromatography (Modern Analytical Chemistry).* New York: Plenum Press.

Soong, Y.-Y. & Barlow, P.J. (2003) *Antioxidant activity and phenolic content of selected fruit seeds.* Food Chemistry. 88, 411–417.

SparkNotes Editors. (2010) *SparkNote on Solubility*: SparkNotes LLC. n.d. <http://www.sparknotes.com/chemistry/solutions/solubility/> [2010-05-16].

Spigno, G & Faveri, D.M. (2005) *Antioxidants from grape stalks and marc: Influence of extraction procedure on yield, purity and antioxidant power of the extracts.* Food Engineering. 78, 793–801.

Thomson, P.M. (2000) *Antioxidants: what role do they play in physical activity and health?* American Journal of Clinical Nutrition. 72, 637S-646s.

Toledo, R.T. (2006) *Fundamentals of Food Process Engineering*. New York: Springer

Tzia, C. & Liadakis, G. (2003) *Extraction Optimization in Food Engineering (Food science & technology)*. New York: Marcel Dekker.

Yilmaz, Y. & Toledo, R.T. (2004) *Oxygen radical absorbance capacities of grape/wine industry byproducts and effect of solvent type on extraction of grape seed polyphenols*. Journal of Food Composition and Analysis. 19, 41-48.

Zumdahl, S.S. (2005) *Chemical Principles*. New York: Houghton Mifflin.

Appendix 1: List of Symbols

COD	Chemical Oxygen Demand (mg/L O_2)
K	Distribution coefficient
ΔH_{soln}	Enthalpy change of solution (kJ/mol)
J	Diffusion flux (kg/cm^2·s)
δ	Diffusion film thickness (cm)

Appendix 2: Primary Data

Folin-Ciocalteu calibration curve

Concentration (mg GAE/mL)	Absorbance (765 nm)
10	0,218
20	0,4
30	0,575
40	0,733
50	0,884
60	1,012
70	1,11
80	1,359
90	1,415
100	1,526

FRAP calibration curve

Concentration (µM Fe^{2+}/mL)	Absorbance (593 nm)
100	0,134
200	0,203
300	0,226
400	0,339
500	0,385
600	0,435
700	0,506
800	0,601
900	0,644
1000	0,73

Folin-Ciocalteu assay on Soxhlet samples

Solvent	Absorbance (765 nm)
Ethanol-Water	0,265
Ethanol-Water	0,327
Ethyl Acetate	0,214
Ethyl Acetate	0,317
Ethanol	0,131

FRAP assay on Soxhlet samples

Solvent	Absorbance (593 nm)
Ethanol-Water	0,278
Ethanol-Water	0,278
Ethyl Acetate	0,212
Ethyl Acetate	0,299
Ethanol	0,107

Determination of best time of extraction for DCE

Time (hours)	Absorbance (280 nm) (Ethanol-Water)	Absorbance (280 nm) (Ethanol)	Absorbance (280 nm) (Ethyl Acetate)
0,5	0,311	1,549	0,087
1	0,379	0	0,069
1,5	0,356	0	0
2	0,385	0,328	0
2,5	0,489	0	0,082
3	0,587	0,283	0
3,5	0,471	0	1,459
4	0,446	0,321	1,499
4,5	2,328	0	1,49
5	1,875	0	1,508
5,5	0,602	0	0,219
6	0,622	0	0,224

Folin-Ciocalteu assay on DCE samples

Solvent	Temperature (°C)	Extraction time (hours)	Absorbance (765 nm)
Ethanol	45	2,5	0,296
Ethanol	45	2,5	0,352
Ethanol	45	4	0,406
Ethanol	45	4	0,346
Ethanol	45	4,5	0,392
Ethanol	45	4,5	0,503
Ethanol	45	4,5	0,405
Ethanol	45	4,5	0,538
Ethanol	45	4,5	0,387
Ethanol	45	4,5	0,386
Ethanol	45	6	0,384
Ethanol	45	6	0,431
Ethanol	45	6	0,361
Ethanol	45	6	0,557
Ethanol	45	6	0,385
Ethanol	45	6	0,401
Ethanol	55	2,5	0,495
Ethanol	55	2,5	0,875
Ethanol	55	2,5	0,392
Ethanol	55	4	0,373
Ethanol	55	4	0,406
Ethanol-Water	45	2,5	0,666
Ethanol-Water	45	2,5	0,617
Ethanol-Water	45	4	0,647
Ethanol-Water	45	4	0,603
Ethanol-Water	45	4,5	0,765
Ethanol-Water	45	4,5	0,777
Ethanol-Water	45	6	0,658
Ethanol-Water	45	6	0,62
Ethanol-Water	55	2,5	0,916
Ethanol-Water	55	4	0,863
Ethanol-Water	55	4	0,851
Ethanol-Water	55	4,5	0,496
Ethanol-Water	55	4,5	0,504
Ethanol-Water	55	6	0,459
Ethanol-Water	55	6	0,488
Ethyl Acetate	45	2,5	0,169
Ethyl Acetate	45	4	0,172
Ethyl Acetate	45	4	0,233
Ethyl Acetate	45	4,5	0,183
Ethyl Acetate	45	4,5	0,177
Ethyl Acetate	45	6	0,231

Folin-Ciocalteu assay on DCE samples (continued)

Ethyl Acetate	45	6	0,246
Ethyl Acetate	55	2,5	0,187
Ethyl Acetate	55	2,5	0,138
Ethyl Acetate	55	4	0,133
Ethyl Acetate	55	4,5	0,187
Ethyl Acetate	55	4,5	0,182
Ethyl Acetate	55	6	0,184
Ethyl Acetate	55	6	0,275

FRAP assay on DCE samples

Solvent	Temperature (°C)	Extraction time (hours)	Absorbance (765 nm)
Ethanol	45	2,5	0,226
Ethanol	45	2,5	0,219
Ethanol	45	4	0,264
Ethanol	45	4	0,196
Ethanol	45	4,5	0,27
Ethanol	45	4,5	0,241
Ethanol	45	4,5	0,243
Ethanol	45	4,5	0,293
Ethanol	45	4,5	0,287
Ethanol	45	4,5	0,266
Ethanol	45	6	0,239
Ethanol	45	6	0,239
Ethanol	45	6	0,256
Ethanol	45	6	0,278
Ethanol	45	6	0,295
Ethanol	45	6	0,284
Ethanol	55	2,5	0,252
Ethanol	55	2,5	0,288
Ethanol	55	2,5	0,288
Ethanol	55	4	0,227
Ethanol	55	4	0,231
Ethanol-Water	45	2,5	0,468
Ethanol-Water	45	2,5	0,419
Ethanol-Water	45	4	0,417
Ethanol-Water	45	4	0,458
Ethanol-Water	45	4,5	0,509
Ethanol-Water	45	4,5	0,507
Ethanol-Water	45	6	0,431
Ethanol-Water	45	6	0,45
Ethanol-Water	55	4	0,604
Ethanol-Water	55	4	0,597
Ethanol-Water	55	4,5	0,286

FRAP assay on DCE samples (continued)

Ethanol-Water	55	4,5	0,311
Ethanol-Water	55	6	0,341
Ethanol-Water	55	6	0,346
Ethyl Acetate	45	4	0,181
Ethyl Acetate	45	4	0,234
Ethyl Acetate	45	4,5	0,217
Ethyl Acetate	45	4,5	0,16
Ethyl Acetate	45	6	0,23
Ethyl Acetate	45	6	0,247
Ethyl Acetate	55	2,5	0,161
Ethyl Acetate	55	4	0,175
Ethyl Acetate	55	4,5	0,214
Ethyl Acetate	55	4,5	0,152
Ethyl Acetate	55	6	0,164
Ethyl Acetate	55	6	0,694

Appendix 3: Calculations

Folin-Ciocalteu assay

Equation for the calibration curve:
$A = 0,0147c + 0,1213$, A = absorbance, c = concentration
Interpolating total phenolics contents of samples from calibration curve:
$c = \frac{A - 0,1213}{0,0147}$ [mg GAE/mL sample]
Relating concentration in samples to sample masses [m] (15 g for Soxhlet, 40 g for DCE):
$c_m = \text{concentration} \times \text{sample volume} \times \text{dilution factor}$
$= \frac{c \times 200 \times 10^{-3} \times 10}{m}$ [mg GAE/g sample]

FRAP assay

Equation for the calibration curve:
$A = 0,0007c + 0,0394$, A = absorbance, c = concentration
Interpolating total antioxidant power of samples from calibration curve:
$c = \frac{A - 0,0394}{0,0007}$ [µM Fe^{2+}/mL sample]
Relating concentration in sample to sample mass [m] (15 g for Soxhlet, 40 g for DCE):
$c_m = \text{concentration} \times \text{sample volume} \times \text{dilution factor}$
$= \frac{c \times 500 \times 10^{-3} \times 43,5}{m}$ [µM Fe^{2+}/g sample]