UNIVERSIDAD DE VALLADOLID

ESCUELA DE INGENIERIAS INDUSTRIALES

GRADO EN INGENIERÍA QUÍMICA

Pressurized liquid extraction of anthocyanins from grape skin

Autor:

de la Cruz Alzaga, Sandra

Responsable de Intercambio en la Uva:

Cocero, María José

BUDAPEST UNIVERSITY OF TECHNOLOGY AND ECONOMICS

FACULTY OF CHEMICAL TECHNOLOGY AND BIOTECHNOLOGY

Valladolid, Junio 2018.

TFG REALIZADO EN PROGRAMA DE INTERCAMBIO

TÍTULO: Pressurized liquid extraction of anthocyanins from grape skin

ALUMNO: Sandra de la Cruz Alzaga

FECHA: 21 de Junio de 2018

CENTRO: Budapest University of Technology and Economics. Faculty of Chemical Technology and Biotechnology

TUTOR: Ildikó Kmecz

RESUMEN

La extracción a alta presión ha demostrado ser un buen método para extraer compuestos de la piel de la uva. La tasa de extracción depende de la temperatura, obteniendo valores más altos a mayores temperaturas. Los máximos extraidos varían también en función del disolvente empleado. Agua y agua-etanol se usan como disolventes. La concentración de antocianinas monoméricas en el extracto se calcula midiendo la absorbancia de las muestras a distintos valores de pH y longitudes de onda. La temperatura es un factor determinante en la cantidad de antocianinas obtenidas. Se estudia la relación entre la temperatura y la tasa de extracción, la concentración de antocianinas y los índices de degradación.

ABSTRACT

The pressurized liquid extraction has proved to be a useful method for extracting compounds from grape skin. The extraction yield is influenced by the temperature, higher rates obtained at a higher temperature. The maximum extraction yield could also variate depending on the composition of the solvent. Water and water-ethanol (50%, v/v) are used as solvents. The concentration of monomeric anthocyanins in the extract has been calculated using absorbance measurements of samples with different pH at different wavelengths. The temperature proves to be a determining factor in the amount of them obtained. The relation between the temperature and the extraction yield, anthocynin concentraction and degradation indexes is studied.

PALABRAS CLAVE

Antocianinas, uva, extracción líquido presión

KEYWORDS

Anthocyanin, grape, pressurized liquid extraction.

INDEX

1. INTRODUCTION 1

2. THEORETICAL BACKGROUND 3

2.1. Grape and grapeskin 3

2.2. Anthocyanins 5

2.3. Anthocyanins in grapeskin 5

2.4. Monomeric anthocyanins 5

2.5. Extraction methods 6

2.5.1. Solvent extraction 6

2.5.2. Microwave-assisted extraction 6

2.5.3. Ultrasound-assisted extraction 6

2.5.4. Supercritical fluid extraction 7

2.5.5. Ionic liquids 7

2.5.6. Enzyme-assisted extraction 7

2.6. Pressurized liquid extraction (PLE) 8

2.6.1. Dielectric constant 9

2.6.2. Effect of temperature 9

2.6.3. Effect of pressure 9

2.6.4. Instrumentation used for pressure hot water extraction 9

2.6.5. Extraction of bioactive compounds by pressure liquid extraction 11

2.7. Spectrophotometric analysis 11

2.7.1. Differential method 12

2.7.2. Subtractive method 12

3. INSTRUMENTATION AND METHOD 13

3.1. Extraction equipment 13

3.2. Extraction method 15

3.2.1. Mass Balance 16

3.3. Vacuum distillation 17

3.4. Spectrophotometer 17

3.5. Measurement of anthocyanines by UV-Visible spectroscopy 18

4 MATERIALS 21

4.1 Grape skin 21

4.2 Solvents 21

4.3 Buffers 21

5 CALCULATIONS 22

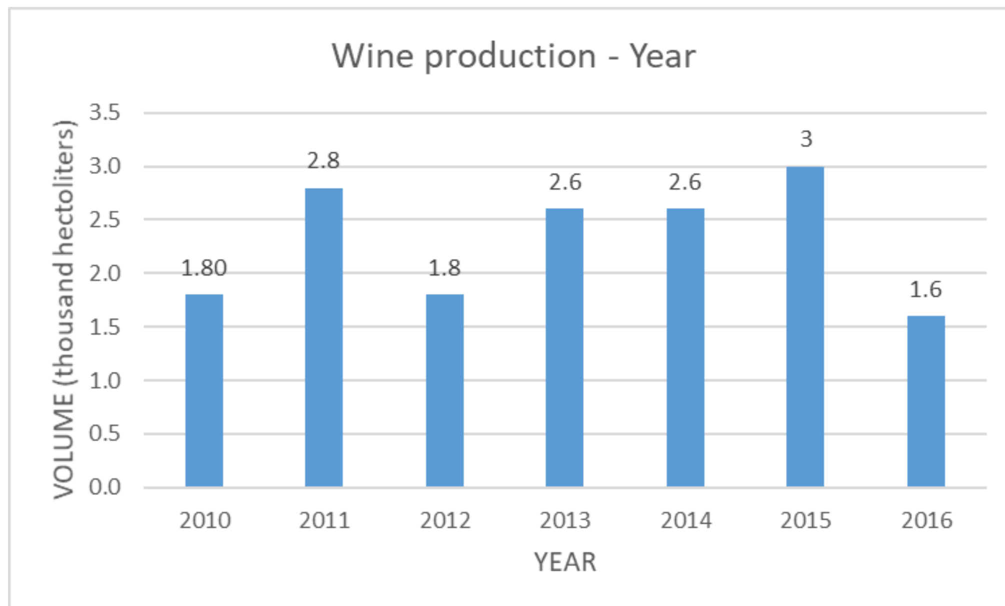
5.1 Dry content 22

5.2 Extraction yield ... 22
5.3 Absorbance and monomeric anthocyanin ... 22
5.4 Colour density, pigment degradation, polymeric colour and browning ... 24
5.5 Percentage of extract and raffinate ... 25
6 RESULTS AND DISCUSSION ... 26
6.1 Dry content ... 26
6.2 Extraction yield ... 26
6.2.1 Pre-experiment for determination of solvent consumption ... 27
6.2.2 Extraction at 90°C with water ... 28
6.2.3 Extraction at 110°C with water ... 29
6.2.4 Extraction at 130°C with water ... 31
6.2.5 Extraction with water-ethanol mixture ... 32
6.2.6 Comparison of the results of PLE with water ... 37
6.3 Percentage of extract and raffinate ... 38
6.4 Anthocyanins content ... 39
6.4.1 Extraction at 90°C with water ... 40
6.4.2 Extraction at 110°C with water ... 41
6.4.3 Extraction with water-ethanol as solvent ... 43
6.4.4 Comparison of the results ... 45
6.5 Colour density, polymeric colour, percent polymeric colour, degradation, and browning ... 47
6.5.1 Extractions at 90°C ... 47
6.5.2 Extraction at 130°C ... 48
7 CONCLUSION ... 52
8 LITERATURE ... 53
9 APPENDIX ... 55
9.1 Extractions ... 55
9.2 Absorbances ... 60

1. INTRODUCTION

The winemaking industry is one of the most important food industries. This industry produces large quantities of heterogeneous solid by-products and residues. The following figure (figure 1.1) is from a wine production study in Hungary. This statistic shows the total volume of wine produced in Hungary from 2010 to 2016. We find the higher volume produced in 2015, approximately three million hectolitres.

Figure 1.1: Wine production in Hungary



The main by-product of the wine industry is the pomace, this is what remains after pressing of the grapes. Pomace consists of skins, seeds and stems. This part represents about 20% of the weight of the processed grapes. Also, pomace contains high levels of polyphenols, substances with antioxidant properties. Polyphenols are not desired for the use as animal feed or for composting. On the other side, pomace is interesting for using it in production of functional foods, dietary supplements, cosmetic industry, and the pharmaceuticals.

Grape skin has component that can be useful, like anthocyanins. Anthocyanins can be found in the cells of all vegetative organs. They are responsible for the colour of many fruits and vegetables. Among them we find the grape, and therefore wine. Anthocyanins extracted from grape skin are the principal compounds involved in the colour of red wines and their interactions with other phenolic compounds (called copigments), normally colourless, allow improving the colour stabilization of aged wines by copigmentation reactions. [1] Anthocyanins are being considered as replacements for the banned synthetic dyes. For this purpose, anthocyanins have the following advantages: [2]

- They have been consumed as part of the human diet many years without suffering any adverse effects to health.
- They are brightly coloured, especially in the red region of the spectrum.
- They are water soluble, this property simplifies their incorporation into aqueous food systems.

Moreover, recent studies show anthocyanins have health benefits:

- Reduction of coronary heart disease. [3]
- Improved visual acuity. [4]
- Antioxidant activities. [5]
- Anticancer activities. [6]

But they also have disadvantages:

- Their tinctorial power and stability in foods are generally low by comparison with coal tar dyes.
- The colour instability is a limitation in the use of anthocyanins as natural food colorants.
- The temperature, the presence of oxygen and light, co-pigmentation, metal ions, the pH value are the major degradation factors of the anthocyanins.

Acylation, glycosylation and condensation with different flavonoids improves the stability of the anthocyanins. [7]

The main goal of this project is to obtain biological compounds, mainly anthocyanins, from grape skin and find out the best extraction method. Among the obtention methods that can be used, the extraction is done in a high-pressure column using different solvents and operating conditions. The operation pressure is set in 50 bars and the temperature varies between 90ºC and 130ºC. The solvents used are water and a mixture of water an ethanol 50:50 (v/v).

Once the extract is collected from the column it is analysed using a spectrophotometer. By using different wavelengths and buffers, it is possible to measure:

- Monomeric anthocyanins.
- Colour density.
- Pigment degradation.
- Polymeric colour.
- Browning.

2. THEORETICAL BACKGROUND

2.1. Grape and grapeskin

One of the most common sources of natural colourants for food use is anthocyanin extracts from grape skins, a by-product of the wine industry. As a result, grape residues have been extensively studied in an attempt to provide an alternative supply of natural colourants. The difference between this and other colorants is that grape is a natural product that doesn´t harm the human body, while other colorants are not so beneficial. [8]

Grapes has not only anthocyanins but many components. We can divide the grape in skin, pulp and seeds. Each part is rich in some kind of components, for example, the skin is rich in pigments, tannins, aromatic substances and minerals (like potassium).

We can find in grapes: [9]

- Sugars
- Aromatic substances
- Organic acids
- Phenolic compounds
    - Anthocyanins
    - Tannins
- Nitrogenous compounds
- Minerals
- Pectic substances
- Antioxidants

- **Sugars**

Sugars are a large amount of the soluble solids, especially glucose and fructose. In the juice of grape there is a sugar concentration between 150 to 250 g/L. The riper the grape is, the more it exceeds the concentration of fructose to the glucose. Glucose and fructose are fermentable sugars.

- **Aromatic substances**

Grapes contain numerous volatile odorous compounds, especially in the skin and the cells beneath it. Their concentration is higher if the grape is ripe.

- **Organic acids**

Organic acids are solids very abundant in grape juice. They are responsible for the wine stability, the pH and the colour. The most common acids are tartaric acid and malic acid, representing over 90% of the total acids present. Grapes are one of the rare fruits that contain tartaric acid. Acid content is related to the pH although the relationship is neither direct nor predictable.

- **Phenolic compounds**

These compounds are also abundant in grape, following sugars and acids. They are structurally diverse and have a great importance in determining the wine's colour and flavour. They are involved in browning reactions in grapes and wines. The two main substances included in this group of compounds are anthocyanins and tannins.

  - Anthocyanins: pigments responsible for the red and purple colour of grapes and wines.
  - Tannins: complex compounds with a molecular weight over 500 that are also astringent and bitter. They are biomolecules that can precipitate proteins and other organic compounds. [10]

- **Nitrogenous compounds**

Grapes contains ammonium cations and organic nitrogenous compounds: such as amino acids, peptides, and proteins.

- **Minerals**

The important mineral compounds include: potassium, sodium, iron, phosphates, sulphate, and chloride. Of these mineral compounds potassium is the most important mineral. During ripening, the potassium content of the grape increases.

- **Pectic substances**

Pectic substances are complex polysaccharides made of polymerised galacturonic acid molecules. When the grapes ripe, pectin is hydrolysed by pectolytic enzymes.

- **Antioxidants**

Antioxidants are molecules that inhibits the oxidation of others. The oxidation is a chemical reaction, it produces free radicals and causes damage to the cells. According to their solubility in water, we can difference two kinds of antioxidants: [11]

  - Lipophilic antioxidants: vitamin A, vitamin D, vitamin E and vitamin K.
  - Hydrophilic antioxidants: vitamin B complex and vitamin C.

All these compounds have interesting properties for their use in many field. An experimental work with determination of all of them in the samples would be very complex. This study is about anthocyanins mainly, so we make no measurements about the other compounds, but it is known that antioxidants and many other compounds are also important in food industry.

2.2. Anthocyanins

Anthocyanins are to be found in the vacuoles of almost every cell type in all vegetative organs. Plants also show tremendous diversity in anthocyanin expression. In leaves, for example, anthocyanins may colour the entire blade, but they can also be only in the margins, stripes, patches, or seemingly random spots on the upper, lower or both lamina surfaces. Given this enormous variation in location, timing, and inducibility of anthocyanins in vegetative tissues, it is not surprising that there is not a unified explanation for the presence of these pigments.

Anthocyanins are responsible for some of the most spectacular natural colours. The term anthocyanin, derived from the Greek words for flower and blue, was used first by Marquart in 1835 to designate the blue pigments of flowers. It was later realized that not only the blue colour, but also the purple, violet, magenta, (in fact many of the colours appearing on flowers, fruits, leaves, stems, and roots) are attributable to pigments chemically similar to the original blue pigment. [12] [13]

2.3. Anthocyanins in grapeskin

The anthocyanin types present in grapes have been reported in numerous papers, with the dominant pigment being peonidin 3-O-glucoside. Other pigments present are cyanidin-, petunidin-, malvidin- and delphinidin-3-O-glucosides. Other studies determined that the principal anthocyanins found corresponded to cyanidin-3-O-glucopyranoside and peonidin-3-O-glucopyranoside. These pigments are also present in the intact plant. These differences show that the anthocyanin type produced from grape can be influenced by environmental conditions and that it is possible to change the anthocyanin composition of grape through careful selection and purification of species. [14]

2.4. Monomeric anthocyanins

Among the anthocyanins we can find monomeric forms. These pigments undergo structural transformations when pH changes.

The two different forms we find are:

- The coloured oxonium form predominates at pH 1.0 (Figure 2.4.1).
- The colourless hemiketal form at pH 4.5 (Figure 2.4.2).

Figure 2.4.1

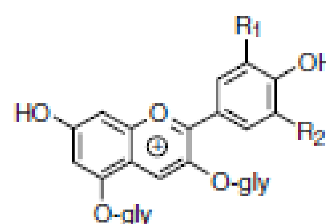
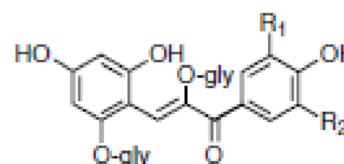


Figure 2.4.2



2.5. Extraction methods [15]

Plants have bioactive metabolites, these components have therapeutic properties but they appear in a very low concentration. This means it is really important to develop an extraction method that obtains elevated yields of extraction and does not damage the component. The damage may be caused by the heat because of the thermolability of the active molecules. [12]

In an extraction, the target compound is removed from the substance due to a diffusion towards the extraction phase to be finally collected. Although the method used was pressurized liquid extraction, there are many other ways to extract the target compounds, the following ones are the most important.

2.5.1. Solvent extraction

There are a large number of inorganic, organic, polar and non-polar solvents that can be used to perform a suitable extraction. They can also be combined to obtain better results. We use polar or apolar solvents depending on the substance of our interest:

- Lipophilic: nonpolar organic solvents, like hexane and n-pentane. They are used, for example, to extract alkaloids, coumarins, fatty acids (FAs), flavonoids and terpenoids.
- Hydrophilic: polar solvents which may be non-protic such as acetone, or protic such as ethanol, methanol or even water. They are used, for example, to extract flavonols, lectins, alkaloids, flavones, polyphenols, tannins and saponins.

The main advantage of this type of extraction is that it is done with simple equipment and it is not expensive.

2.5.2. Microwave-assisted extraction

Microwave-assisted extraction (MAE) is an extractive method based on the utilization of microwave energy that is produced when the perpendicular oscillation between the electric and the magnetic fields generates electromagnetic radiations with a frequency ranging from 0.3 to 300 GHz. If the microwave goes through and interacts with a substance there is a production of heat whose intensity depends on the absorption of the energy by the material and the dissipation of the resulting heat.

The main advantage of this kind of extraction are the high yields. It takes short time, but it is also less eco friendly and not suitable for thermolabile compounds.

2.5.3. Ultrasound-assisted extraction

Ultrasound-assisted extraction, or UAE, is based on the production of ultrasound waves and their transmission throughout the solvent with a resulting cavitation. When the cavitation bubbles collapse, there is a generation of liquid circulation currents and turbulence that improve the mass transfer rate because the permeability of the cell is increased. The solvent must have adequate polarity, viscosity, vapour pressure and surface tension. The most used ones are ethanol, methanol and hexane.

It is interesting because of the great yields that are obtained. It is less expensive than the traditional extractive techniques, it can give high quantities of products in fewer time and with not much solvent.

2.5.4. Supercritical fluid extraction

Supercritical fluid extraction (SFE) is a quite new technique especially used for the recovery of essential oil from plants. SFE is based on the use of carbon dioxide in supercritical phase because CO_2 in this state has a polarity similar to pentane and hexane so is good for lipophilic compounds extraction.

The main advantages of supercritical CO_2 are that it is non-toxic, non-flammable, not expensive, and easy to remove in the end of the process (eco-friendly). It is a good choice with thermolabile compounds because it is possible to obtain a high yield operating at low temperatures.

If the components to extract are polar, a cosolvent like water or ethanol in a little percentage (5-10%) is needed to increase the extraction quantity. When in the plant matrix there are bioactive compounds of different solubility, a method to improve the recovery of all the phytotherapeutics without any loss is the fractionation of the extract. Two strategies could be applied: the multi-step fractionation and the on-line fractionation.

2.5.5. Ionic liquids

The ionic liquid (IL) has many advantages in terms of quality and efficacy of extraction. An IL is a liquid organic that selectively interacts with specific polar and non-polar compounds thanks to ion-exchanges, π-stacking interactions, hydrophobic interactions or hydrogen bonds, improving the selectivity of the extractive method. Ionic liquids enhance the extraction efficiency of other solvents, so they can be used in microwaved-assisted extraction (MAE) and ultrasound-assisted extraction (UAE)

2.5.6. Enzyme-assisted extraction

The advantage of this method is the fact that the enzymes catalyze reactions in a specific way without operating under intense conditions that could lead to the degradation of the desired products. The application of enzymes such as lipases, proteases, phospholipases, allows to reduce the use of the solvent for the extraction. For oil extraction from plants, cellulase, α-amylase and pectinase are the most used enzymes. These proteins can be obtained from fungi, bacteria, animals, and vegetables or from genetic engineering methods. There are some limitations due to the cost, the incomplete disruption of the cell wall and the complicated application in a commercial scale because of the different behaviour of the enzymes depending on the environmental circumstances such as the amount of oxygen, the variety of nutrients and the operating temperature.

2.6. Pressurized liquid extraction (PLE)

Pressurized liquid extraction (PLE or PFE in case of a general fluid) is a novel and eco-friendly technique for the recovery of bioactives from plants. This method often requires water as the solvent and so it can keep away from the environmental and health risks due to the use of organic solvents. It operates at high temperature (until 200°C) and pressure (from 35 to 200 bar). At elevated temperatures there is a reduction of the viscosity of the solvent that can better penetrate the matrix extracting the analytes of interest. The disadvantage is that PLE cannot be used for the thermally unstable compounds and it could lead to a co-extraction of other compounds because of the decreased selectivity of extraction at higher temperatures.

PLE is a technique that involves extraction using liquid solvents at elevated temperature and pressure, which enhance the extraction performance as compared to those techniques done at ambient temperature and atmospheric pressure. The merits of enabling the use of solvents at temperatures above their atmospheric boiling point is the enhanced solubility and mass transfer properties. Dionex Corporation first introduced PLE in 1995 at the Pittcon Conference, where it was introduced as Accelerated Solvent Extraction Technology (ASE). [16]

This technique, which involves extraction with solvents at a high pressure and temperature without their critical point being reached, has received different names, such as: [17]

- Accelerated solvent extraction (ASE).
- Pressurized fluid extraction (PFE).
- Pressurized liquid extraction (PLE).
- Pressurized hot solvent extraction (PHSE).
- High-pressure solvent extraction (HPSE).
- High-pressure, high temperature solvent extraction (HPHTSE).
- Subcritical solvent extraction (SSE).

The use of these different terms may lead to confusion and here we use the term PLE, which is the most widely accepted designation, even though since 1996 the EPA has adopted the term PFE to refer to this technique. When water is employed as the extraction solvent, the authors tend to use a different name to highlight the use of this environmentally friendly solvent. Thus, terms such as:

- Subcritical water extraction (SWE).
- Hot water extraction (HWE).
- Pressurized hot water extraction (PHWE).
- High-temperature water extraction (HTWE).
- Superheated water extraction.
- Hot liquid water extraction.

All these names can be found in literature. Nevertheless, it is important to note that although referring to the same technique, in this case water is employed instead of another organic solvent. The dramatic changes in the physical–chemical properties of water, especially in its dielectric constant (ε), at elevated temperatures and pressures enhance its usefulness as an extraction solvent.

2.6.1. Dielectric constant

The dielectric constant (as a measure of the polarity of the solvent) is a key parameter in determining solute-solvent interactions, and (in the case of water) increasing the temperature under moderate pressure can significantly decrease this constant. At ambient pressure and temperature, water is a polar solvent with a high dielectric constant ($\varepsilon = 78$) but at 300 °C and P = 23MPa this value decreases to 21, which is similar to the value for ethanol (ε=24 at 25°C) or acetone ($\varepsilon = 20.7$ at 25 °C). This means that at elevated temperatures and moderate pressures the polarity of water can be reduced considerably, and the solvent can act as if ethanol or acetone were being used. The main effect of this drop in the dielectric constant when working at elevated temperatures and pressures is that water can be used instead of another organic solvent to extract medium (or low) polarity compounds.

2.6.2. Effect of temperature

Temperature during the extraction is one of the critical factors that affect the efficiency and selectivity in PLE. The use of high temperatures improves the efficiency of the extraction as it helps the disruption interactions caused by van der Waals forces, hydrogen bonding and dipole attraction. The use of thermal energy helps to break molecule–molecule interactions and adhesive interactions between the analyte and the sample matrix, so it is a way to decrease the activation energy required for the desorption process. When increasing the temperature, the diffusivity also increases while the viscosity decreases. Furthermore, elevated temperature decreases the surface tension of the solvent, solutes and matrix and therefore enhances the solvent wetting of the sample. [16]

2.6.3. Effect of pressure

The main advantage of applying pressure during the extraction is that a temperature above the boiling point (at atmospheric pressure) can be used while the solvent maintains its liquid state. The use of elevated pressure at high temperature and reduced solvent surface tension helps to force the solvent within the matrix pore to contact the analyte and extract them. Using pressure during extraction could exert pressure on the matrix resulting in disruption, which could enhance the mass transfer of the analyte from the sample to the solvent. High pressure during the extraction controls problems related to air bubbles found within the matrix that prevent the solvent from reaching the analyte. These conditions boost the analyte solubility and desorption kinetics from the sample matrix.

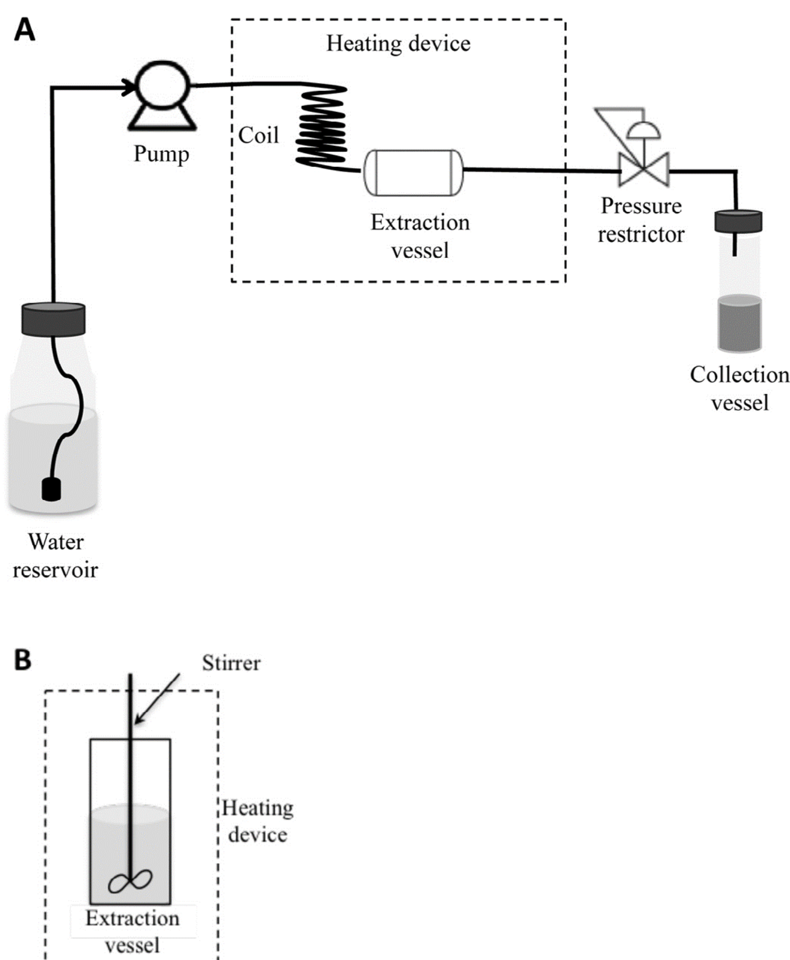
2.6.4. Instrumentation used for pressure hot water extraction [18]

Pressurized hot water extraction (PHWE) is performed with deionized water as solvent. The water should be oxygen-free in order to prevent oxidation of the analytes. The basic equipment to carry out PHWE depends on how the extraction is performed. It can be accomplished in the static mode, the dynamic mode, or a combination of both, so, there are two types of equipment: dynamic (continuous-flow) systems and static (batch) systems, and combinations of the two.

- **Dynamic mode**: the solvent flows through the sample in a continuous manner.

Dynamic PHWE basically needs a pump, an extraction vessel, a heating device, a pressure restrictor and a collection vial. As it is shown in the following figure (Figure 2.6.1 A) the pump delivers water to the extraction vessel, and via the pressure restrictor to the collection vial. The pump should be able to achieve the pressure necessary to keep the water in liquid state during the extraction process (normally 3.5–20 MPa). Heating of the water is by either an oven or a heat exchanger, heating tape or heating jacket. The extraction vessel is usually made of stainless steel, and should have frits at both ends in order to avoid sample losses and clogging the tubing. Pressure restriction is needed to control the pressure inside the extraction vessel and to prevent boiling-off effects of water at the exit of the extraction vessel. The pressure restrictor could be a needle valve, a backpressure regulator, a thin capillary or simply short tubing with a squeezed end providing an exit small enough to maintain an adequate pressure upstream.

Figure 2.6.1: PHWE equipment



- **Static mode**: sample and solvent are maintained for a user-specified time at constant pressure and temperature.

In static PHWE (Figure 2.6.1 B), a pump is unnecessary. However, in the case of a pump is used, pressure can be controlled by adding an increasing amount of water to the vessel with the outlet valve closed. If a pump is not used and water is added manually to the extraction vessel, when the vessel is closed and heated, pressure builds up and extraction is conducted at the

saturation pressure of the system. In static PHWE, convection is accomplished using a stirrer in order to speed up mass transfer. For heating, an oven, a heating jacket or heating tape is appropriate. A pressure restrictor is not needed, unless the speed of removal the extract from the vessel is to be controlled.

2.6.5. Extraction of bioactive compounds by pressure liquid extraction

There is increasing interest in ingredients of bioactive compounds from natural sources to be used in food and pharmaceutical products and in the search for native plants containing interesting bioactive compounds (for example antioxidants and anti-inflammatory compounds). PHWE (pressurized hot water extraction) is one of the most interesting techniques to obtain bioactives from plants and other complex samples, whether for analytical purposes or industry processes. The typical extraction conditions for the extraction of bioactive compounds is 100–200°C and 50–100 bar.

Optimal extraction conditions greatly vary, depending on the kind of phenolic compound to be extracted. For example, extremely labile polyphenols like anthocyanins, whose stability depends on pH and temperature, generally require a lower extraction temperature. For example, static PHWE was optimized for anthocyanins in red cabbage and red onion at 99°C, 50 bar and 7 minutes extraction time. [18]

According to other studies, the higher monomeric anthocyanin yields were achieved at the conditions in which ethanol +water or pure ethanol were used as solvents, at 60 and 80 °C in the extraction from fresh blackberries. The increase of temperature generally decreased the anthocyanin content of the extracts, a negative synergism between high temperatures and acid solvent must have contributed on the degradation of anthocyanins. This is confirmed by the ANOVA analysis, which showed a significant effect of the interaction temperature and solvent on the anthocyanin concentration. Acids at high temperatures possibly resulted in the hydrolysis of acylated components and sugar residues, inactivating the chemical structure of anthocyanins. Temperature itself is well known as a factor affecting the stability of anthocyanins, whose degradation can happen by two mechanisms:

- hydrolysis of the 3-glucoside to form the unstable aglycone.
- hydrolytic opening of the heterocyclic ring, forming chalcone, which is a colourless anthocyanin structure that, at high temperatures, is degraded into an insoluble brown-coloured phenolic compound.

In fact, the extracts obtained in this work at 80 and 100 °C had colour varying from red to brown, indicating some anthocyanin degradation. [19]

2.7. <u>Spectrophotometric analysis</u>

An analysis commonly used to determine anthocyanins concentration is the spectrophotometric analysis. The total anthocyanin content in crude extracts containing other phenolic materials has been determined by measuring absorptivity of the solution at a single wavelength (between 490 and 550 nm in the region of the visible spectra) in their absorption

band. This band is far from the absorption bands of other phenolics (spectral maxima in the UV range).

However, this simple method is inappropriate because of interference from anthocyanin degradation products or melanoidins from browning reactions. In those cases, differential and subtractive methods are used to quantify anthocyanins and their degradation products.

2.7.1. Differential method

The differential method measures the absorbance at two different pH values and relies on the structural transformations of the anthocyanin chromophore as a function of pH. Many pH values have been proposed for this method. Researchers used pH 1.0 and 4.5 buffers to measure anthocyanin content in cranberries, and modifications of this technique have been applied to a wide range of commodities. The pH differential method has been described as fast and easy for the quantitation of monomeric anthocyanins. [2]

2.7.2. Subtractive method

Subtractive methods are based on the use of bleaching agents that will decolour anthocyanins but not affect interfering materials. Polymerized coloured anthocyanin-tannin complexes are resistant to bleaching by bisulfite, whereas the bleaching reaction of monomeric anthocyanins will rapidly go to competition monomeric anthocyanin pigments form a colourless sulfonic acid adduct by react with bisulfite. A measurement of the absorbance at the visible maximum is obtained, followed by bleaching and remeasuring to give a blank reading. The two most used bleaching agents are sodium bisulphite and hydrogen peroxide. [2]

By using both of these spectral procedures, accurate measurement of the total monomeric anthocyanin pigment content can be obtained, along with indices for polymeric colour, colour density, browning, and degradation. To determine total anthocyanin content, the absorbance at pH 1.0 and 4.5 is measured at the λvis-max and at 700 nm, which allows for haze correction. The bisulfite bleaching reaction is utilized to generate the various degradation indices. Absorbance measurements are taken at the λvis-max and at 420 nm on the bisulfite bleached and control samples. Colour density is the sum of the absorbances at the λvis-max and at 420 nm of the control sample, while polymeric colour is the same measurement for the bisulfite treated sample. A measure of percent polymeric colour is obtained as the ratio between these two indexes. The absorbance at 420 nm of the bisulfite-treated sample is an index for browning, as the accumulation of brownish degradation products increases the absorption in the 400 to 440 nm range. The absorption of these compounds is in general not affected by the addition of a bisulfite solution.

3. INSTRUMENTATION AND METHOD

3.1. Extraction equipment

The extraction column is placed in an oven. We can control the temperature on the oven and there are two temperature sensors in it to measure the temperature of the oven and the extract. The following figure (3.1.1) shows the extraction column inside the oven.

Figure 3.1.1. Extraction column

The components of the extraction equipment are:

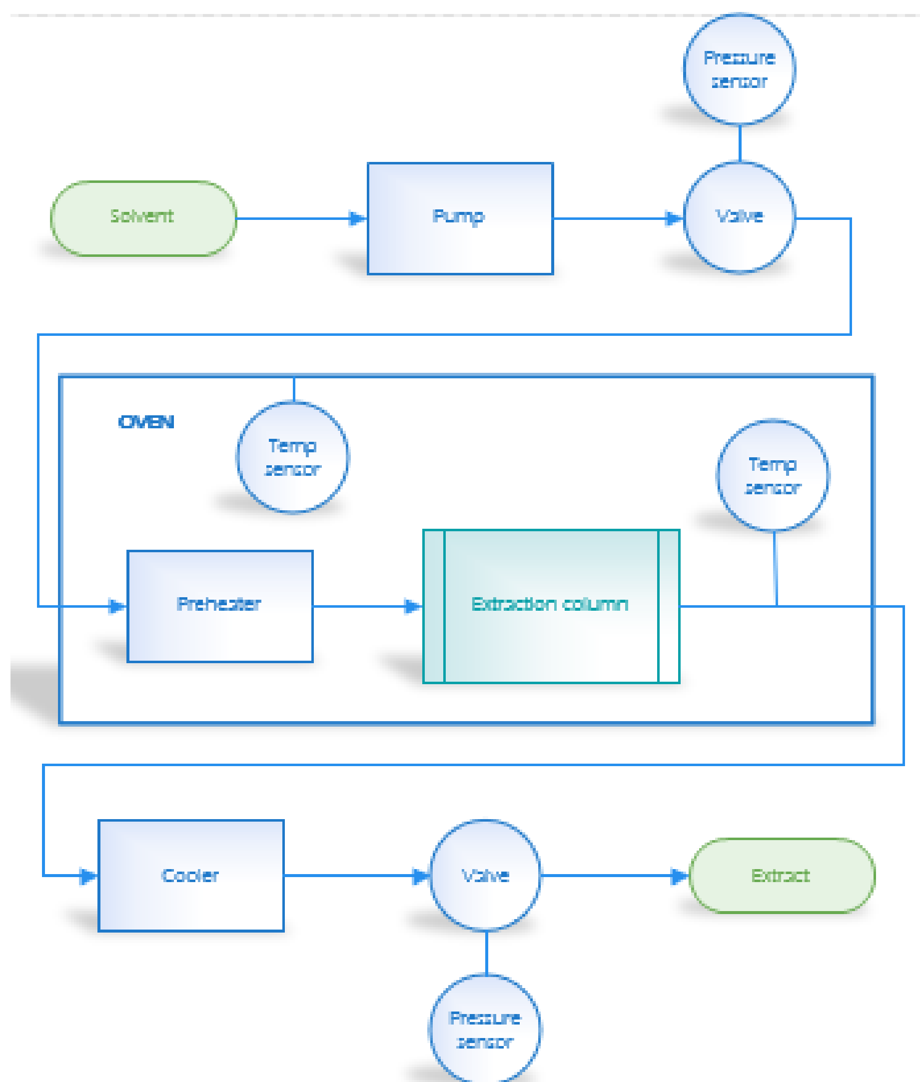
- Outside the oven
    - Solvent: in this case is a liquid. It may be deionized water or a mixture of deionized water and ethanol (50 v/v).
    - Pump (JASCO PU-980): instrument that provides a constant flow of 2ml/minute.
    - Valve: controls the liquid inlet in the oven.
    - Pressure sensor: it is connected to the pump as a safety measure, if the pressure exceeds the maximum value allowed by the pump, the pump turns off.
- Inside the oven
    - Preheater: a spiral where the liquid increases its temperature before feeding the column.
    - Column: cylinder with the grape skin inside. The water can go through the column but the solids like grape skin cannot. This is because of the filters installed at the top and the bottom of the column.
    - Temperature sensor (2): there are two of them inside the oven. One of them works with the temperature controller, while the other measures the temperature of the extract.

- Outside the oven:
  - Cooler (Figure 3.1.2): spiral located into a vessel where there is water flowing to reduce the temperature of the liquid leaving the oven.
  - Valve: controls the extraction pressure.
  - Pressure sensor: it shows the pressure inside the column and makes it possible to control it using the valve.
  - Extract collector: the extract is collected in dark flasks (to prevent degradation because of the light). The samples are more or less 20ml.

Figure 3.1.2 Cooler

The following figure (3.1.3) shows the block diagram of the pressurized liquid extraction.

Figure 3.1.3: Block diagram of the applied extraction arrangement



3.2. Extraction method

The extraction takes place in the oven at different temperatures. Before every extraction the column must be carefully sealed to prevent leaking. A leakage test must be done to be sure the pressure does not drop. During the leakage test the extract leaves the column, but the leakage test is performed at room temperature so the loses should not be high in this case.

The extraction must last enough to obtain as much extract as possible. It means that the yield of extraction in the last sample should be similar to the maximum yield of extraction. The extraction time was determined through trial and error method, and it was finally set on 80 minutes when using only water and 160 minutes when using both water and the mixture of water and ethanol. The data analysis of the samples is easier if the volume of all of them is the same, so, as the pump works at constant flow, all the samples are collected after a certain time. The time was carefully measured with a stopwatch.

The procedure consists of the following steps:

1. About 2g of grapeskin were measured and put into the column.
2. The empty flasks for collecting the samples were weighed.
3. The column had to be connected with the feed and the flasks to gather the extract.
4. A wrench was used to seal the circuit.
5. A leakage test was performed.
6. The oven was turned on and the temperature was set.
7. When the temperature in the second sensor showed the desired temperature, the extract was collected.
8. The flasks were changed every few minutes to obtain different samples.
9. The flasks with the extract were weighed.
10. The raffinate from the column was collected.
11. Three samples of 2ml were taken from every flask to dilute and analyse as is indicated.
12. The remaining solvent was evaporated to obtain the dry extract.
13. The dry extract was weighed and the amount of solid had to be corrected with the part of the sample taken to analyse.
14. The dry solid of every sample was stored separately.
15. The raffinate was placed in a weighted plate and left into the oven at 105°C.
16. The dry raffinate was weighed.

The first extractions are performed using deionized water as solvent, changing only the temperature. But on the last ones the solvent is deionizes water at the beginning but a mixture of water and ethanol later.

When the solvent is changed during an extraction, the procedure is:

1. The pump was turned off.
2. The outlet valve was closed.
3. The feed was changed.
4. The pump was turned on again.
5. The outlet valve was opened as much as it was needed to keep the pressure of the process.

3.2.1. Mass Balance

Weighting the raffinate has nothing to do with the anthocyanin content, but it is desired to compare the percentages of extract and raffinate obtained in each extraction.

All the dry grapeskin introduced in the column should end up being either extract or raffinate but due to some mass loses in the process the addition of extract and raffinate is less than the initial grape skin. The reasons why the raffinate and extract does not have the same mass as the initial dry material are:

- Some extract leaves the column during the leakage text and the heating.
- Some extract remains in the pipes after the extraction.
- Some solid is lost during the collection of the raffinate.

3.3. Vacuum distillation [20]

After the extraction the liquid obtained must be evaporated to get the dry content. A vacuum distillatory was used for that.

Vacuum evaporation is a technique used in the treatment of liquids. Evaporation is a unit operation that consists of concentrating a solution by eliminating the solvent by boiling. In this case, it is performed at a pressure lower than atmospheric pressure. This way, the boiling temperature is much lower than that at atmospheric pressure, thereby resulting in notable energy savings.

After taking some extract for the anthocyanins measure method, the solvent was evaporated with vacuum, so the operating temperature was lower, and the anthocyanins got no damage. The evaporation temperature was controlled with a water bath. The water was at 50°C.

3.4. Spectrophotometer [21]

The spectrophotometer is the instrument used to measure the absorptivity of a sample when the light goes through it.

According to the wavelength, there are two kinds of spectrophotometer:

- Spectrophotometer UV-Vis: uses the UV range (185-400 nm) and the visible range (400-700nm). For the anthocyanin measurement the wavelength needed is the UV-Vis.
- Spectrophotometer of infra-red: uses the range of infra-red (700-15000 nm).

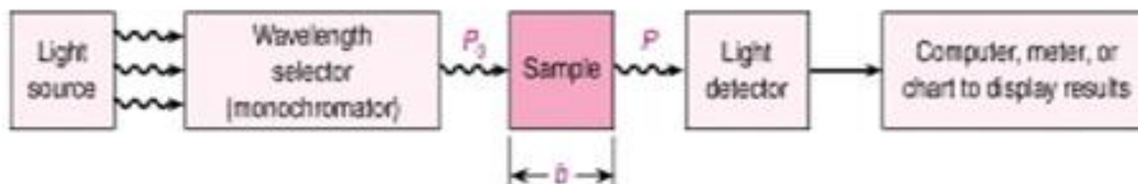
A spectrophotometer has these parts:

- Light source: the light must fulfill the following conditions: stability, directionality, distribution of continuous spectral energy and long life. The light sources used can be xenon arc lamp, tungsten lamp, LED lamp and deuterium lamp.
- Monochromator: it gets the desired wavelength. It consists of: entrance and exit slits, collimator and dispersion element.
- Sample: the subject of the study. The dilution of the sample must be adequate to be on the linear range of the spectrometer.
- Detector: captures the radiation leaving the sample and transform it in information.
- Quartz cell: Cell contains the liquid samples. The material of which they are made varies according to the region of the spectra; glass or plastic if the work is with the visible region, quartz if the work is in the ultraviolet (this case) and NaCl if the work is in the infrared region.

The spectrophotometer used in the analysis was the model JENWAY M6305.

The following picture (Figure 3.4.1) shows the different parts of a spectrophotometer.

Figure 3.4.1: Spectrophotometer block diagram



3.5. Measurement of anthocyanines by UV-Visible spectroscopy

Measuring the absorbance of the sample at different pH, at different wavelengths and with or without bisulfide gives information about the anthocyanins concentration, polymeric colour, browning index, colour density, degradation index and percent polymeric colour. To obtain the information there are some steps that must be carefully followed. The analysis method is explained below.

- **Total monomeric anthocyanin by the pH-differential method [2]**

Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra (Figure 3.5.1). The coloured oxonium form predominates at pH 1.0 and the colourless hemiketal form at pH 4.5 (Figure 3.5.2). The pH-differential method is based on this reaction and permits accurate and rapid measurement of the total monomeric anthocyanins, even in the presence of polymerized, degraded pigments and other interfering compounds.

Figure 3.5.1 Spectral characteristics

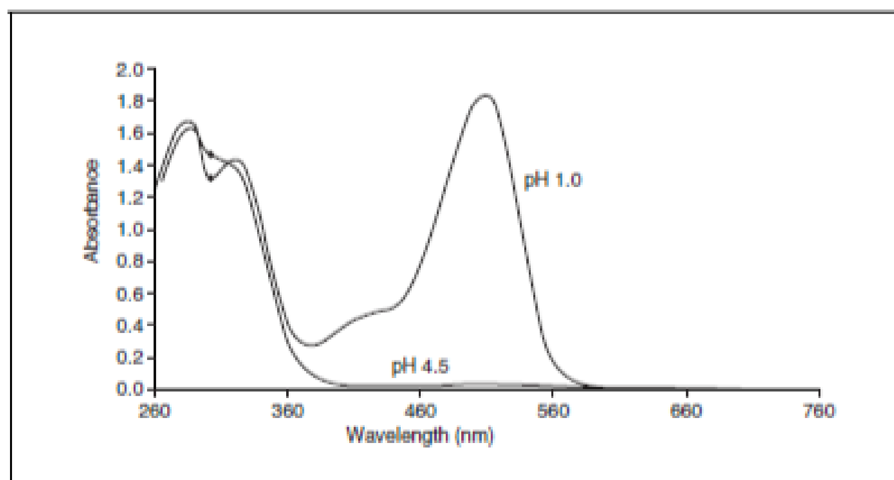
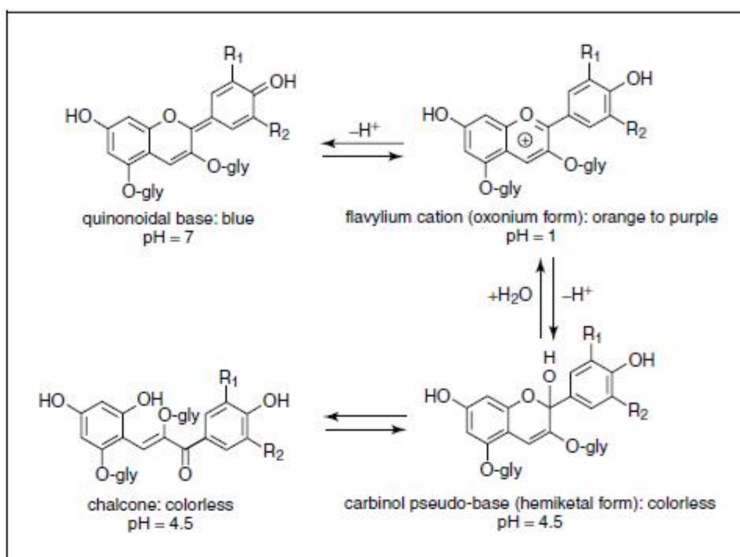


Figure 3.5.2 Structural forms



The steps that were followed to measure the monomeric anthocyanin content are these:

1. The appropriate dilution factor (DF = dilution factor) for the sample had to be determined so the sample at the $\lambda_{vis\text{-}max}$ (for Cyanidin-3-Glucoside, $\lambda_{vis\text{-}max}$ is 520nm) is within the linear range (less than 1.2) of the spectrophotometer. The dilution factor for these samples was generally 5, but in some cases it was 10.

2. Two dilutions of the sample were prepared, one with potassium chloride buffer (pH 1.0), and the other with sodium acetate buffer (pH 4.5), diluting each by the dilution factor. The dilutions needed to equilibrate for 15 minutes.

3. The absorbance of each dilution was measured at the $\lambda_{vis\text{-}max}$ (520nm) and at 700 nm (to correct for haze). The zero point of the spectrophotometer was calibrated before each measurement with distilled water.

- **Indices for pigment degradation, polymeric colour, and browning [2]**

Indices for anthocyanin degradation of an aqueous extract, juice, or wine can be derived from a few absorbance readings of a sample that has been treated with sodium bisulfite. Anthocyanin pigments are combined with bisulfite to form a colourless sulfonic acid adduct. Polymerized coloured anthocyanin-tannin complexes are resistant to bleaching by bisulfite, whereas the bleaching reaction of monomeric anthocyanins will rapidly go to completion. The steps that had to be followed are below:

1. The sample was diluted with distilled water using the dilution factor (DF) from the previous determination. 2.8 ml of the diluted sample was transferred to each of two cuvettes. 0.2 ml of bisulfite solution was added to one and 0.2 ml of distilled water was added to the other. The samples had to be settled 15 minutes before the measurements.

2. The absorbance of both samples was measured at 420 nm, $\lambda_{vis-max}$ (520nm), and 700 nm. The zero point of the spectrophotometer was calibrated before each measurement with distilled water.

3**.** Once the sample absorbance has been measured, the beakers must be washed with acetone and let dry before using again.

4 MATERIALS

4.1 Grape skin

The milled grape skin was obtained from Gere Winery (Villány) after fermentation and drying. The grape used is a red variety.

4.2 Solvents

Water: deionized water.

Water + ethanol (50 v/v): when this solvent mixture was used the extraction begins using only deionized water, by the time there are 8 samples of extract the solvent is changed for the mixture of water and ethanol.

4.3 Buffers [2]

- **Potassium chloride buffer, 0.025 M, pH 1.0**

1.86 g KCl was dissolved in 980 ml of distilled water in a beaker. The pH of the solution was adjusted to 1.0 with concentrated HCl. The solution was transferred to a 1 litre volumetric flask and it was filled to 1 litre with distilled water.

- **Sodium acetate buffer, 0.4 M, pH 4.5**

54.43 g $CH_3CO_2Na \cdot 3\ H_2O$ was dissolved in 960 ml distilled water in a beaker. The pH of the solution was measured and adjusted to 4.5 with concentrated HCl. The solution was transferred to a 1 litre volumetric flask and it was filled to 1 litre with distilled water.

The solutions should be stable at around 20^oC for a few months, but the pH should be checked and adjusted prior to use.

- **Bisulfite solution**

1.1163 g of sodium metabisulfite ($Na_2S_2O_5$) was dissolved in 5 ml of distilled water.

5 CALCULATIONS

5.1 *Dry content*

The grape skin is not dry at ambient conditions. To know the material humidity of a sample must be weighed before and after being in the oven at 105ºC for 72 hours. The humidity was measured with three different samples and the humidity used in the calculations is the average of them. The procedure is:

1. Around 10 g of grape skin were weighted in laboratory conditions.
2. The sample was placed in the oven at 105°C for three days.
3. The grape skin was weighted again when it was cool enough.
4. The dry content (in percentage) is:

Dry content [%] = (final weight (g)/initial weight (g)) x 100

Once the humidity is known, the grape skin mass measured and filled into the column is corrected with this number to obtain the dry grape skin mass.

5.2 *Extraction yield*

The yield of the extract is the percentage of the extract obtained from the dry material. It measures the solvent efficiency in an extraction. The yield can be measured individually for each sample or as an accumulated yield.

- Extraction yield

YE_i (g_{solid}/100$g_{drygrapeskin}$) = Solid obtained (g) / dry grape skin (g) *100

- Accumulated extraction yield

$$YE = \sum YEi$$ **= Accumulate solid obtained (g) / dry grape skin (g) *100**

5.3 *Absorbance and monomeric anthocyanin*

The dilution factor chosen is 5 for most samples (with that dilution the spectrophotometer works in the linear range, generally), but with some samples is necessary to use another dilution factor. The dilution factor 10 was applied for those that exceed the linear range.

The absorbance of the sample is calculated with the data obtained of the sample at pH 1 and pH 4.5 at both wavelengths, vis-max (520nm) and 700nm. Once the absorbance is known it is possible to calculate the monomeric anthocyanins concentration in:

- mg anthocyanin in litre of the sample.
- mg anthocyanin in g of dry extract.
- mg anthocyanin in g of dry material (before extraction).

In these calculations is necessary to know the molecular weight, molar absorptivity, dilution factor and pathlength. According to other studies, if the ε (molar absorptivity) of the major pigment is not available, or if the sample composition is unknown, the calculations are done supposing the pigment is cyanidin-3-glucoside (figure 5.3.1), where: [2]

- MW = 449.2
- ε = 26,900

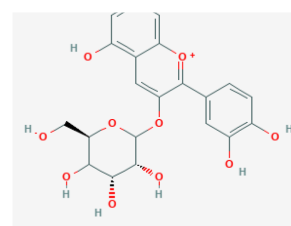
Cyanidin-3-Glucoside [22]

Cyanidin-3-Glucoside is considered the main anthocyanin. It has the following characteristics (table 5.3.1):

Tabla 5.3.1: Characteristics

Cyanidin-3-Glucoside	
Molecular Formula	$C_{21}H_{21}O_{11}$
Molecular Weight	449.388 g/mol

Figure 5.3.1 Cyanidin-3-Glucoside



The absorbance of the diluted sample (A) is calculated as follows, being $(A_{wavelengh})_{pH}$ the absorbance at a determined wavelength and pH:

The monomeric anthocyanin pigment concentration in the original sample is calculated using

$$A = (A_{\lambda\ vis\text{-}max} - A_{700})_{pH\ 1.0} - (A_{\lambda\ vis\text{-}max} - A_{700})_{pH\ 4.5}$$

the following formula. The anthocyanin taken as reference in these calculations is cyanidin-3-glucoside.

$$\text{Monomeric anthocyanin pigment (mg/litre)} = (A \times MW \times DF \times 1000) / (\varepsilon \times l)$$

Where:

- MW is the molecular weight of cyanidin-3-glucoside
- DF is the dilution factor
- ε is the molar absorptivity of cyanidin-3-glucoside
- l is the pathlength (in this case is 1)

The monomeric anthocyanin concentration (MAC) in mg/g extract can also be calculated by dividing the number 50 because of the volume of the samples, they are 20ml each, more or less. The amount in mg we have in the sample (in this case in 20ml of extract) is divided by the amount of dry extract we obtain.

[MAC x 20ml x (1l/1000ml) = MAC/50 = mg anthocyanins in the sample]

$$MAC\ (mg/g\ extract) = (MAC\ (mg/litre)/50)\ /\ extract\ (g)$$

The monomeric anthocyanin concentration in mg/g dry material is calculated according to the following formula:

$$MAC\ (mg/g\ dry\ material) = MAC\ (mg/g\ extract) \times YE\ /100$$

5.4 Colour density, pigment degradation, polymeric colour and browning

From the absorbance measurements we can get some more information about the samples. The absorbance at 420 nm of the bisulfite-treated sample serves as an index for browning. Colour density is defined as the sum of absorbances at the λvis-max (520) and at 420 nm. The ratio between polymerized colour and colour density is used to determine the percentage of the colour that is contributed by polymerized material. The ratio between monomeric and total anthocyanin can be used to determine a degradation index.

Colour density, pigment degradation, polymeric colour and browning are calculated as is indicated below, being DF the dilution factor.

The colour density of the control sample (treated with water) is calculated as follows:

$$\textbf{Colour density} = [(A_{420\ nm} - A_{700nm}) + (A_{\lambda\ vis\text{-}max} - A_{700\ nm})] \times DF$$

The polymeric colour of the bisulfite bleached sample is calculated as follows:

$$\textbf{Polymeric colour} = [(A_{420\ nm} - A_{700\ nm}) + (A_{\lambda\ vis\text{-}max} - A_{700\ nm})] \times DF$$

The ratio between polymerized colour and colour density can be used to determine the percentage of the colour that is contributed by polymerized material. The percent polymeric colour is calculated using the formula:

$$\textbf{Percent polymeric colour (\%)} = (\text{polymeric colour}/\text{colour density}) \times 100$$

The degradation index (DI) is calculated using the formula (treated with water): [7]

$$\text{Degradation index (DI)} = [(A_{420nm} - A_{700nm}) / (A_{\lambda\ vis\text{-}max} - A_{700nm})] \times DF$$

The browning index is calculated using the absorbances of the bisulfite treated samples:

$$\text{Browning index (BI)} = (A_{420nm} - A_{700nm}) \times DF$$

5.5 Percentage of extract and raffinate

- % extract = dry extract accumulate (g) / dry grapeskin (g) x 100
- % raffinate = dry raffinate (g) / dry grapeskin (g) x 100

6 RESULTS AND DISCUSSION

6.1 Dry content

Table 6.1.1 summarizes the results of the determination of the dry content of the milled grape skin. The average dry content in grape skin is 94.19%. It means this percentage of the grapeskin at laboratory conditions is dry material. The grape skin weighted and put into the column must be corrected with this percentage.

Table 6.1.1: Dry content of the milled grape skin

HUMIDITY	Sample	Initial weight (g)		Final weight (g)	Dry content (%)	Average (%)
	S1	9.9474	3 days 105°C	9.3446	93.94	94.19
	S2	10.1560		9.5706	94.24	
	S3	9.9205		9.3628	94.38	

6.2 Extraction yield

The yield of extraction is a relation between the dry grape skin used and the amount of extract obtained. Typically extraction yield as a function of solvent consumption follows a saturation curve.

The data obtained experimentally should fit a determined extraction curve suggested by Brunner [23].

$$YE = YEmax * (1 - \exp(-k * SC))$$

Where:

- YE is the yield of extraction (dependent variable) (g extract/100g dry grape skin)
- SC is the solvent consumption / dry material (independent variable) (g solvent/g dry grape skin)
- YE_{max} is the maximum yield of extraction (g extract/100g dry solvent)
- k is the kinetic parameter (g dry grape skin/g solvent)

The following subchapters give an overview of all the extractions and their yields. Also, there is a graphical representation of the distribution fitting and the values of maximum yield of extraction and the kinetic parameter obtained for each extraction at a determined temperature. The yield values have been obtained from the extraction information detailed on appendix 9.1.

6.2.1 Pre-experiment for determination of solvent consumption

- **EXTRACTION 1**

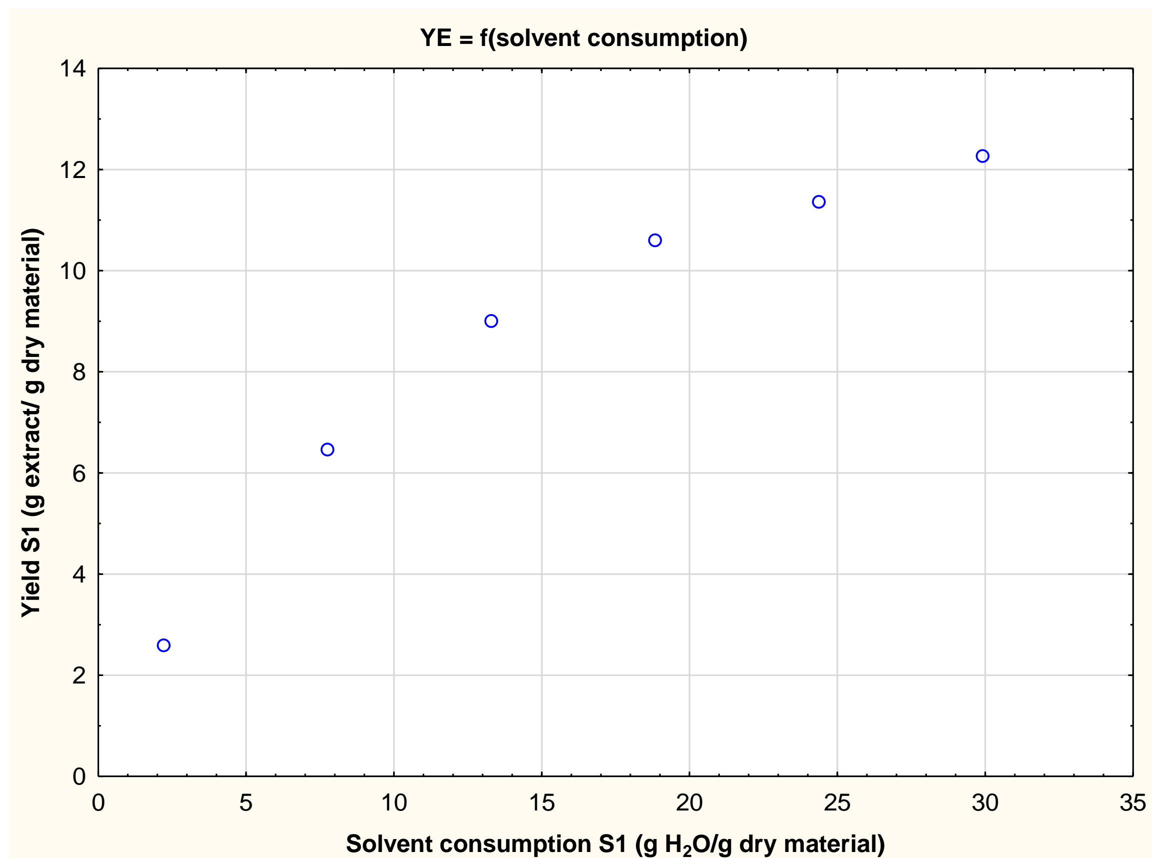
This extraction was intended to determine the parameters of the following ones. It was used to have a general idea of the extraction time, the volume of the samples and the solvent consumption. It was performed at 110ºC and 50 bar. The following table (table 6.2.1) shows the solvent consumption and yield of the extracts obtained for each sample

Table 6.2.1: Extraction yield and solvent consumption of the extraction 1

Sample	Solvent consumption (gH2O/g dry material)	YE dry basis S1 (g extract/100g dry material)
S1/1	2.22	2.59
S1/2	7.76	6.46
S1/3	13.30	9.00
S1/4	18.84	10.60
S1/5	24.38	11.36
S1/6	29.92	12.27

Figure 6.2.1 represents the extraction yield as a function of solvent consumption. It is observed that the maximum yield is not reached because the yield is increasing continuously.

Figure 6.2.1: time course of the extraction 1 yield



6.2.2 Extraction at 90°C with water

These are the results for the extractions performed at 90ºC and 50 bar with deionized water as solvent. These were the extraction number 3 (table 6.2.2) and 7 (table 6.2.3)

- **EXTRACTION 3**

Table 6.2.2: Extraction yield and solvent consumption of the extraction 3

Sample	Solvent consumption (gH2O/g dry material)	YE dry basis S3 (g extract/100g dry material)
S3/1	10.64	10.46
S3/2	21.28	16.63
S3/3	31.92	18.32
S3/4	42.56	19.43
S3/5	53.20	20.40
S3/6	63.85	20.99
S3/7	74.49	21.82
S3/8	85.13	22.52

- **EXTRACTION 7**

Table 6.2.3: Extraction yield and solvent consumption of the extraction 7

Sample	Solvent consumption (gH2O/g dry material)	YE dry basis S7 (g extract/100g dry material)
S7/1	10.75	11.02
S7/2	21.49	16.67
S7/3	32.24	17.83
S7/4	42.99	18.75
S7/5	53.74	19.24
S7/6	64.48	19.72
S7/7	75.23	20.19
S7/8	85.98	20.49

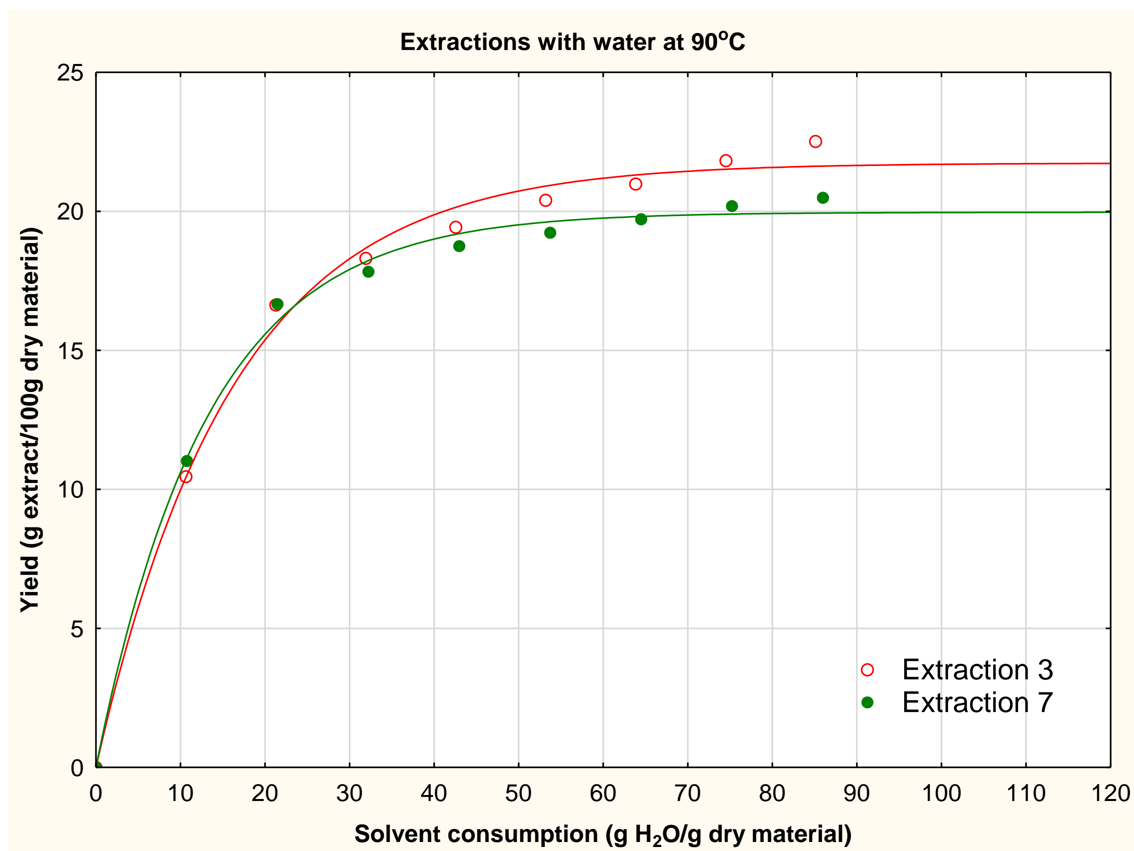
If we fit these data with the extraction curve formula [YE = YEmax*(1-exp(-k*SC))] we obtain a value of maximum yield of the extraction and kinetic parameter for each extraction. They are represented in the following table (table 6.2.4).

Table: 6.2.4: Extraction parameters at 90ºC with water

Extraction	Temperature (ºC)	Pressure (bar)	YE_{max} (g/100g)	k (g dry material/g H_2O)
3	90	50	21.74	0.0614
7			19.97	0.0757

As we can see, the parameters obtained are very similar. The differences between the values are acceptable for an experimental method. Figure 6.2.2 shows the fitted extraction curves.

Figure 6.2.2: Extraction curves at 90°C with water



6.2.3 Extraction at 110°C with water

The following tables summarizes the results of the extractions performed at 110°C and 50 bar with deionized water as solvent. These were the extraction number 2 (table 6.2.5), 5 (table 6.2.6) and 6 (table 6.2.7).

- **EXTRACTION 2**

Table 6.2.5: Extraction yield and solvent consumption of the extraction 2

Sample	Solvent consumption (gH2O/g dry material)	YE dry basis S2 (g extract/100g dry material)
S2/1	5.33	6.41
S2/2	10.66	9.03
S2/3	15.98	11.36
S2/4	21.31	12.85
S2/5	26.64	14.45
S2/6	31.97	15.60
S2/7	37.29	16.96
S2/8	42.62	17.55

- **EXTRACTION 5**

Table 6.2.6: Extraction yield and solvent consumption of the extraction 5

Sample	Solvent consumption (gH_2O/g dry material)	YE dry basis S5 (g extract/100g dry material)
S5/1	10.60	10.42
S5/2	21.19	15.17
S5/3	31.79	17.56
S5/4	42.38	19.42
S5/5	52.98	20.45
S5/6	63.57	21.92
S5/7	74.17	22.97
S5/8	84.76	23.97

- **EXTRACTION 6**

Table 6.2.7: Extraction yield and solvent consumption of the extraction 6

Sample	Solvent consumption (gH_2O/g dry material)	YE dry basis S6 (g extract/100g dry material)
S6/1	10.26	10.53
S6/2	20.52	14.54
S6/3	30.78	16.68
S6/4	41.05	18.01
S6/5	51.31	19.06
S6/6	61.57	19.74
S6/7	71.83	20.33
S6/8	82.09	20.92

When we fit these data with the extraction curve formula used before, we obtain a value of maximum yield extraction and kinetic parameter for each extraction. They are represented in the following table (table 6.2.8):

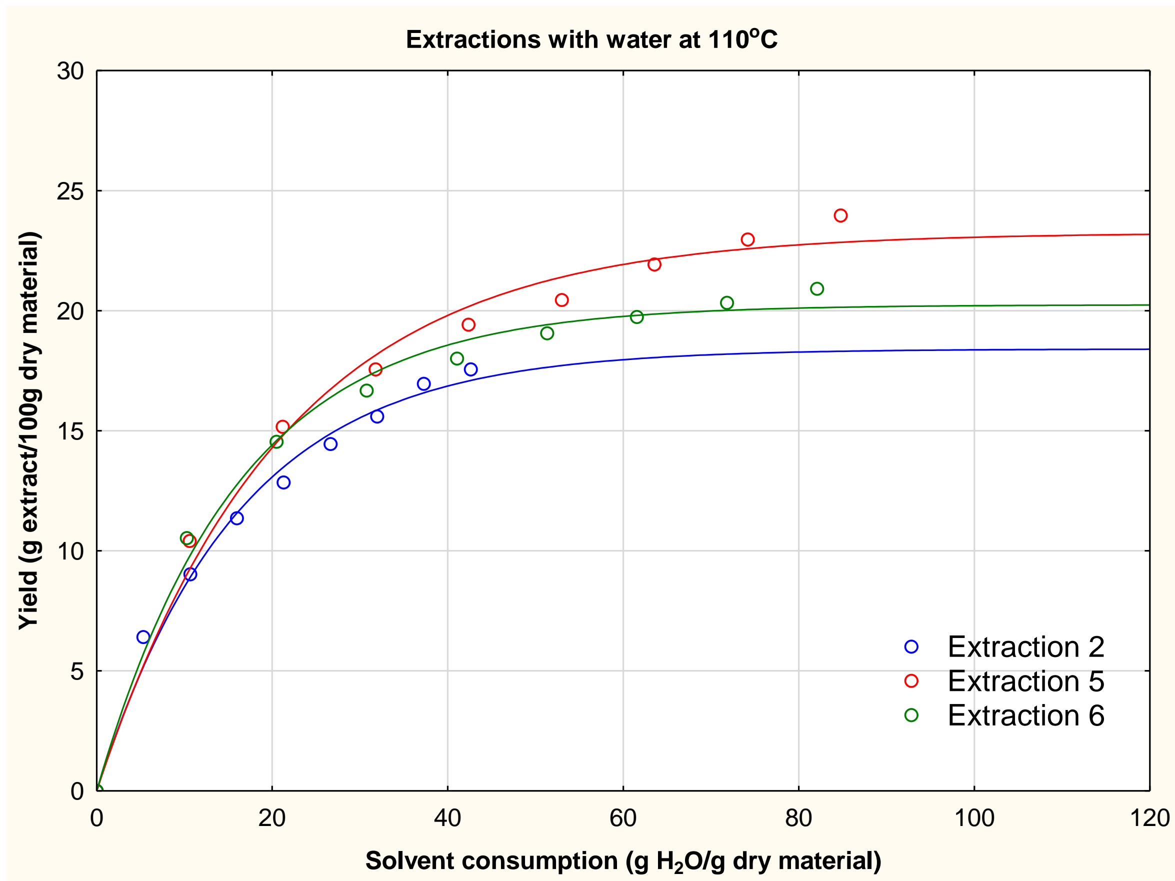
Table: 6.2.8: Extraction parameters at 110ºC with water

Extraction	Temperature (ºC)	Pressure (bar)	YE_{max} (g/100g)	k (g dry material/g H_2O)
2	110	50	18.40	0.0620
5			23.26	0.0477
6			20.25	0.0622

Studying the obtained values, we can see that the constants of the fitted function differ, but they are still reasonable. The kinetic parameter of the extraction 2 and 6 is very similar, while in extraction number 5 it has a lower value. In the following graph (figure 6.2.3) we can observe also how the extraction yield in the extraction 5 take unexpected values from the

sample 6 to the 8. This anomaly means that the function does not describe the time course of the extraction yield precisely.

Figure 6.2.3: Extraction curves at 110ºC with water



6.2.4 Extraction at 130°C with water

These are the results for the extraction performed at 130ºC and 50 bar with deionized water as solvent. That was the extraction number 4 (table 6.2.9).

- **EXTRACTION 4**

Table 6.2.9: Extraction yield and solvent consumption of the extraction 4

Sample	Solvent consumption (gH2O/g dry material)	YE dry basis S4 (g extract/100g dry material)
S4/1	10.40	11.17
S4/2	20.79	16.32
S4/3	31.19	18.83
S4/4	41.59	20.65
S4/5	51.99	21.90
S4/6	62.38	22.94
S4/7	72.78	23.57
S4/8	83.18	24.32

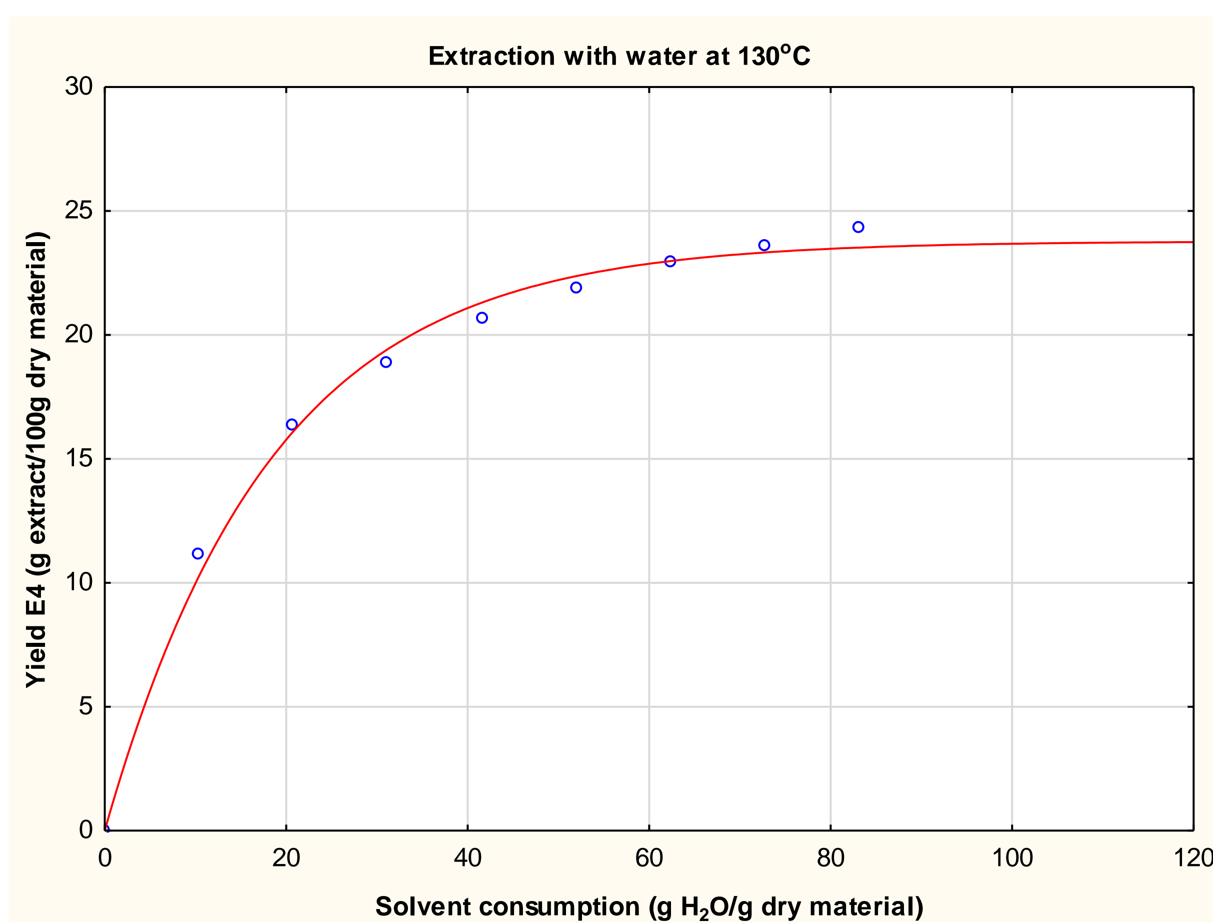
The fitted extraction curve can be seen in figure 6.2.4. The maximum yield of extraction and the kinetic parameter are given in the following table (table 6.2.10).

Table: 6.2.10: Extraction parameters at 130°C with water

Extraction	Temperature (°C)	Pressure (bar)	YE_{max} (g/100g)	k (g dry material/g H_2O)
4	130	50	23.78	0.0544

In this case it is not possible to compare the parameters among extractions at the same temperature. Nevertheless, it is observed that the maximum extraction yiled is slightly higher than at lower temperatures while the kinetic parameter is below the average value for other temperatures.

Figure 6.2.4: Extraction curve at 130°C with water



6.2.5 Extraction with water-ethanol mixture

Since the colour of the raffinate of the PLE with water was nearly the same as the raw material the multi step extraction was performed to obtain more extractable colourants from grape skin. The extraction was executed according to the following extraction the procedure. The extraction was at first like the previous ones, collecting eight samples of the water extract leaving the column for 10 minutes each. But then, with the same grape skin in the column, and

keeping the temperature and the pressure constant, the solvent used was changed. First it was only water, later it is a mixture of water and ethanol at a 50% in volume.

The water – ethanol solvent mixture was already pumped in the 9th sample, but as the column is already filled with water the extract collected is still dissolved in deionized water. In the calculation it is considered that the 10th sample is the first one with the mixture of water and ethanol as solvent. The average density for the mixture of water and ethanol is 0.91g/ml.

In these cases, there are two extraction curves:

- Curve 1: when the solvent is deionized water
- Curve 2: when the solvent is the mixture of water and ethanol

Therefore, there are two YE_{max} and two kinetic parameters for each extraction.

- **<u>WATER-ETHANOL MIXTURE AT 90ºC</u>**

These are the results for the extractions performed at 90ºC and 50 bar with deionized water and a mixture of deionized water and ethanol as solvent. That was the extraction number 10 (table 6.2.11).

- **EXTRACTION 10**

Table 6.2.11: Extraction yield and solvent consumption of the extraction 10

Sample	Solvent consumption (g solvent/g dry material)	YE dry basis S10 (g extract/100g dry material)
S10/1	10.53	8.39
S10/2	21.06	11.07
S10/3	31.59	12.11
S10/4	42.12	13.09
S10/5	52.66	13.62
S10/6	63.19	13.75
S10/7	73.72	14.21
S10/8	84.25	14.98
S10/9	94.78	15.24
S10/10	104.36	17.47
S10/11	113.95	19.85
S10/12	123.53	21.50
S10/13	133.11	22.04
S10/14	142.70	22.21
S10/15	152.28	22.43
S10/16	161.86	22.55

The yield of extraction can not be fitted in an only curve. In the samples 6, 7 and 8 the growing of the yield has almost stopped, but when a new solvent (water-ethanol) is introduced in the column the yield experiences a remarkable increase in the 10, 11 and 12 samples and then the

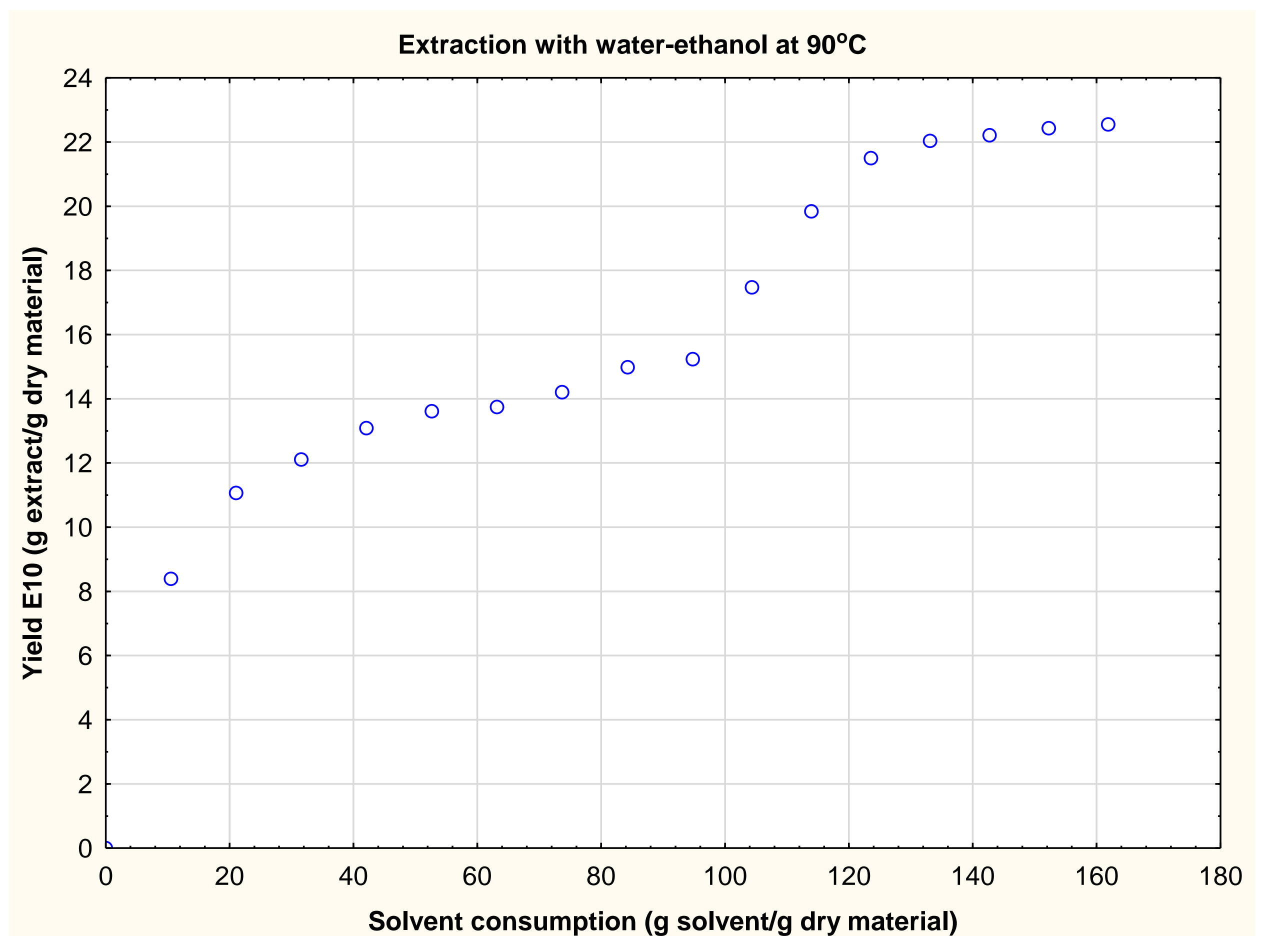
value stabilizes around the value of YE_{max}'. The table below (table 6.2.12) shows the parameters.

Table: 6.2.12: Extraction parameters at 90°C and 50 bar with water-ethanol

Extraction	YE_{max} (g/100g)	k (g dry material/ g solvent)	YE_{max}' (g/100g)	k' (g dry material/ g solvent)
10	14.35	0.0706	23.08	0.0468

The evolution of the extraction yield as a function of the solvent consumption is in the following figure (figure 6.2.5). The fitting in that case was not good enough, so the parameters does not describe the extraction precisely.

Figure 6.2.5: Extraction curve at 90°C with water-ethanol

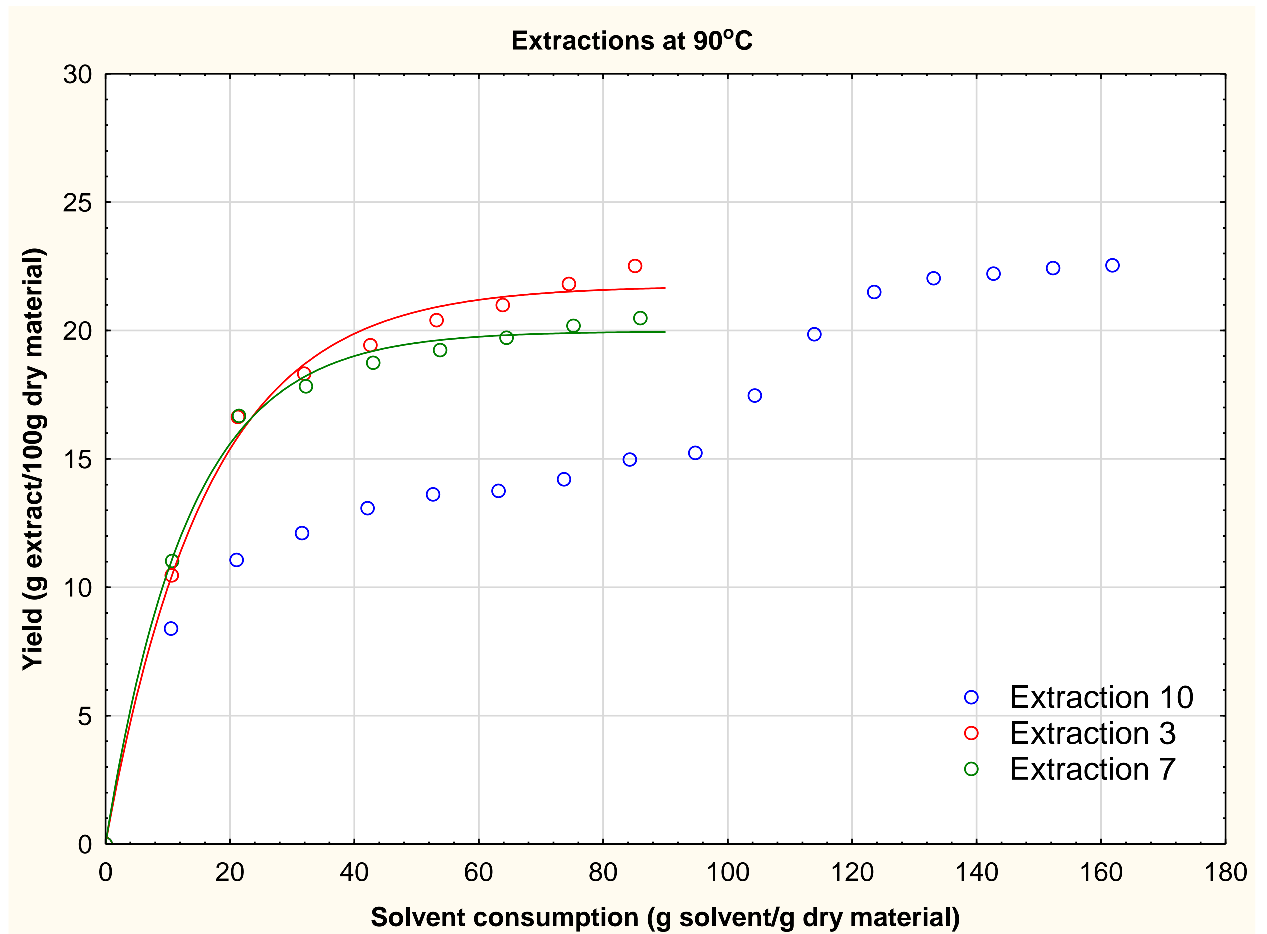


- **COMPARISON OF THE EXTRACTIONS AT 90°C**

As the following figure shows (figure 6.2.6) in case of the two step extraction the extreacton yield significantly increased in the second step. However, in case of the extraction 10, the

extraction yield was lower than the maximum yield was obtained with water (extraction 3 and 7). It might be because of the high mass loses in this extraction or the inhomogeneity of the raw material. Further experiments are necessary to ascertain this discrepancy.

Figure 6.2.6: Extraction curve comparative at 90ºC



- **WATER-ETHANOL MIXTURE AT 130ºC**

Results for the extractions performed at 130ºC and 50 bar with deionized water and a mixture of deionized water and ethanol as solvent are presented in this subchapter. These were the extractions number 8 (table 6.2.13) and 9 (table 6.2.14).

- **EXTRACTION 8**

Table 6.2.13: Extraction yield and solvent consumption of the extraction 8

Sample	Solvent consumption (g solvent/g dry material)	YE dry basis S8 (g extract/100g dry material)
S8/1	10.66	11.51
S8/2	21.33	17.81
S8/3	31.99	20.99
S8/4	42.65	22.88
S8/5	53.31	25.10
S8/6	63.98	26.19
S8/7	74.64	27.14
S8/8	85.30	27.85
S8/9	95.96	28.54
S8/10	105.67	31.44
S8/11	115.37	33.68
S8/12	125.07	34.80
S8/13	134.78	35.54
S8/14	144.48	35.95
S8/15	154.18	36.59
S8/16	163.89	37.26

- **EXTRACTION 9**

Table 6.2.14: Extraction yield and solvent consumption of the extraction 9

Sample	Solvent consumption (g solvent/g dry material)	YE dry basis S9 (g extract/100g dry material)
S9/1	10.53	5.72
S9/2	21.06	12.17
S9/3	31.59	15.08
S9/4	42.12	16.95
S9/5	52.65	19.14
S9/6	63.18	20.10
S9/7	73.71	20.71
S9/8	84.24	21.32
S9/9	94.77	22.06
S9/10	104.36	24.79
S9/11	113.94	26.76
S9/12	123.52	27.59
S9/13	133.11	28.38
S9/14	142.69	28.96
S9/15	152.27	29.40
S9/16	161.85	29.82

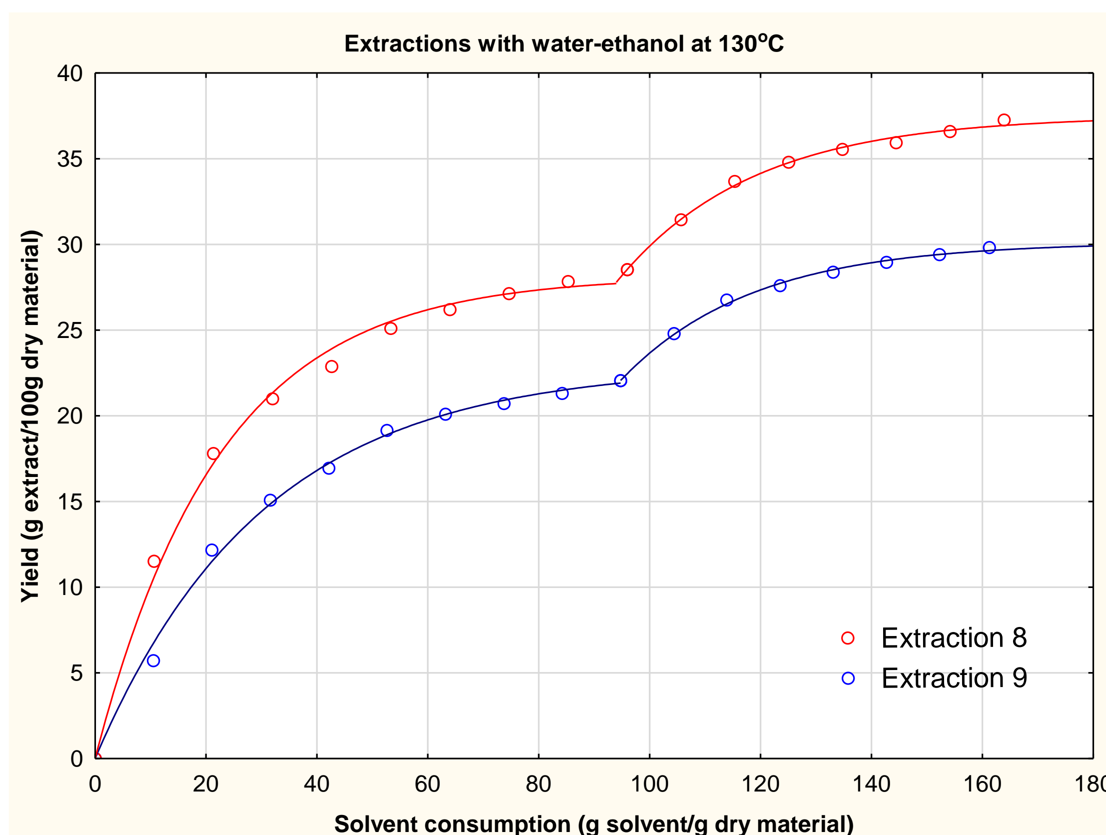
When we fit these data with the extraction curve formula used before, we obtain values for the maximum yield extraction and kinetic parameter. They are represented in the following table (table 6.2.15):

Table: 6.2.15: Extraction parameters at 130ºC and 50 bar with water-ethanol

Extraction	YE_{max} (g/100g)	k (g dry material/ g solvent)	YE_{max}' (g/100g)	k' (g dry material/ g solvent)
8	28.16	0.0444	37.50	0.0408
9	22.91	0.0331	30.12	0.0423

Studying the obtained values, we can see that the extraction yields differs, in the extraction 8 it reaches higher values. However, the kinetic parameters of the extractions 8 and 9 are very similar. In the following graph (figure 6.2.6) we can compare the extraction curves.

Figure 6.2.6: Extraction curves at 130ºC with water-ethanol



6.2.6 Comparison of the results of PLE with water

It is important to compare the information of all the extraction according to the operation temperature. The first extraction is not useful for the calculations because the extraction time was not enough. So, we have:

- 3 extractions at 90°C
- 3 extractions at 110°C

- 3 extractions at 130°C

The results using water as solvent are compared in the following table (table 6.2.16).

Table 6.2.16: Extraction parameters comparative

Temperature	Extraction	YE max (g/100g)	Average YEmax (g/100g)	k (g dry material/ g solvent)	Average k (g dry material/ g solvent)
90°C	3	21.74	18.69	0.0614	0.0692
	7	19.97		0.0757	
	10	14.35		0.0706	
110°C	2	18.40	20.64	0.0620	0.0573
	5	23.26		0.0477	
	6	20.25		0.0622	
130°C	4	23.78	24.95	0.0544	0.0440
	8	28.16		0.0444	
	9	22.91		0.0331	

It can be noticed that the dispersion of the data is remarkable, but it might be due to the mass loses in the process because of the method. The higher the loses are, the lower the YE max value is. Mass balance error of the extractions is explained in subchapter 6.3.

Despite that fact, it is clear that the highest YE values are obtained at 130°C, so there is a direct relation between the temperature and the extraction yield. On the other side, while the temperature increases, the kinetic parameter decreases, so the relation between the temperature and the kinetic parameter is indirect.

6.3 Percentage of extract and raffinate

When the mass balance is calculated, we can appreciate the mass balance error and confirm that this values are related to the extraction yield.

When the loses are lower than 10% (S3, 1.17%; S6, 9.5% or S7, 0.6%) we can observe that the YEmax is bigger than the average YE.

- S3: Loses = 1.17% → YEmax > average YEmax → 21.74 > 18.69
- S6: Loses = 9.55% → YEmax > average YEmax → 20.25 > 20.64
- S7: Loses = 0.60% → YEmax > average YEmax → 19.97 > 18.69

When the loses are higher than 10%, the opposite thing happens.

- S4: Loses = 10.48% → YEmax < average YEmax → 23.78 < 24.95
- S8: Loses = 15.68% → YEmax < average YEmax → 28.16 < 24.95
- S9: Loses = 21.95% → YEmax < average YEmax → 21.74 < 24.95
- S10: Loses = 16.89% → YEmax < average YEmax → 14.35 < 18.69

The mass loss is generally higher at 130°C because it takes more time to reach the extraction temperature, so the extract leaving the column in that period is higher. The following tables (table 6.3.1, 6.3.2 and 6.3.3) represent the percentage of extract, raffinate and loses of the extractions in which the mass balance was done.

Table 6.3.1 Mass balance at 90ºC

EXTRACTION 3 (90 ºC)					
% extract	21.21	% raffinate	77.62	% loses	1.17

EXTRACTION 7 (90 ºC)					
% extract	19.30	% rafinate	80.10	% loses	0.60

EXTRACTION 10 (90 ºC)					
% extract	14.11	% raffinate	69.00	% loses	16.89

Table 6.3.2: Mass balance at 110ºC

EXTRACTION 6 (110ºC)					
% extract	19.71	% raffinate	70.75	% loses	9.54

Table 6.3.3: Mass balance at 130ºC

EXTRACTION 4 (130 ºC)					
% extract	22.91	% raffinate	66.61	% loses	10.48

EXTRACTION 8 (130 ºC)					
% extract	26.23	% raffinate	58.10	% loses	15.67

EXTRACTION 9 (130 ºC)					
% extract	20.08	% raffinate	57.97	% loses	21.95

6.4 Anthocyanins content

It is known that the extract obtained from grape skin contains anthocyanins, but we have to determine the concentration of them. As they are colour pigments, a qualitative method to determine the concentration of them is comparing the colour of the samples.

In the following figure (6.4.1) it can be appreciated how the colour of the samples changes. At the beginning it is similar to wine, but on the 8th sample it is just like water with light colour. The more coloured the sample is, the higher anthocyanins concentration it has.

Figure 6.4.1: Colour variation of the extracts

When a red brown colour appears in the samples, it can be assumed that it is due to the monomeric anthocyanin degradation. Degradation may occur at higher temperature.

The qualitative analysis gives a general idea, but concrete data is required. It is obtained using the UV-VIS spectrophotometric method explained in chapter 3.5. The results obtained were calculated from the absorbance measurements (can be found in the appendix 9.2) and are explained below.

6.4.1 Extraction at 90°C with water

With the extraction at 90ºC samples analysis, we measure the absorbances and then calculate the anthocyanins concentration with the equations from chapter 5.3. The results are summarized in the following table (table 6.4.1).

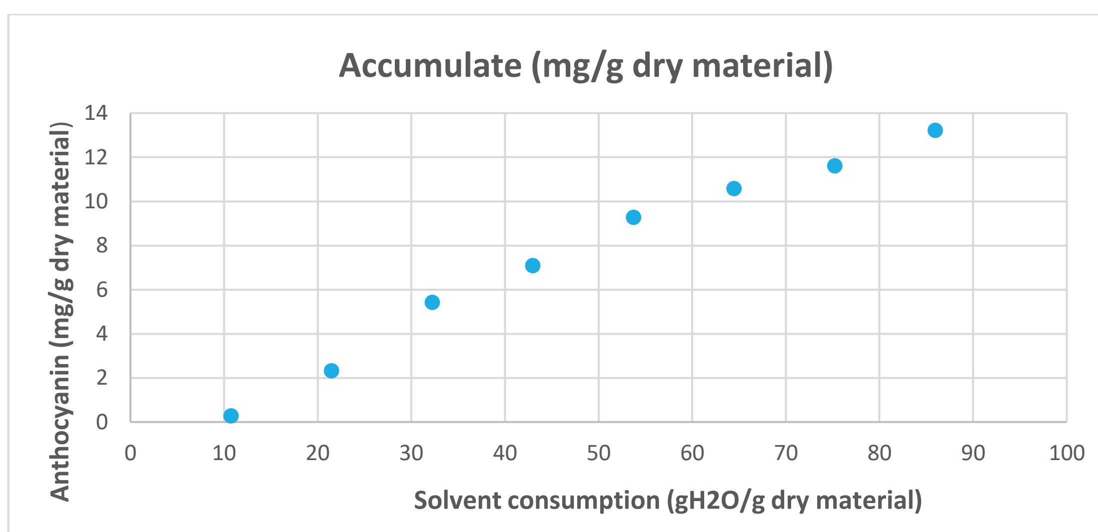
- **EXTRACTION 7**

Table 6.4.1: Anthocyanin concentrations for extraction 7

Sample	A	Anthocyanin (mg/l)	Anthocyanin (mg/g extract)	Anthocyanin (mg/g dry material)	Accumulate (mg/g dry material)	Solvent/dry skin (g/g)
S7/1	0.314	26.2	2.6	0.28	0.28	10.7
S7/2	0.775	64.7	12.3	2.1	2.3	21.5
S7/3	0.226	18.9	17.4	3.1	5.4	32.2
S7/4	0.091	7.6	8.9	1.7	7.1	43.0
S7/5	0.062	5.2	11.4	2.2	9.3	53.7
S7/6	0.035	2.9	6.6	1.3	10.6	64.5
S7/7	0.027	2.3	5.1	1.0	11.6	75.2
S7/8	0.026	2.2	7.8	1.6	13.2	86.0

The figure (figure 6.4.2) shows the mass of anthocyanin obtained.

Figure 6.4.2: Anthocyanin accumulate content in extraction 7



6.4.2 Extraction at 110°C with water

When we use the same equations with the extraction at 110ºC, we obtain the following anthocyanin concentration values (table 6.4.2 and table 6.4.3).

- **EXTRACTION 5**

Table 6.4.2: Anthocyanin concentrations for extraction 5

Sample	A	Anthocyanin (mg/l)	Anthocyanin (mg/g extract)	Anthocyanin (mg/g dry material)	Accumulate (mg/g dry material)	Solvent/dry skin (g/g)
S5/1	0.717	59.9	6.1	0.63	0.63	10.6
S5/2	0.431	36.0	8.0	1.2	1.9	21.2
S5/3	0.180	15.0	6.7	1.2	3.0	31.8
S5/4	0.091	7.6	4.3	0.84	3.9	42.4
S5/5	0.048	4.0	4.1	0.83	4.7	53.0
S5/6	0.027	2.3	1.6	0.36	5.1	63.6
S5/7	0.024	2.0	2.	0.47	5.5	74.2
S5/8	0.009	0.8	0.8	0.19	5.7	84.8

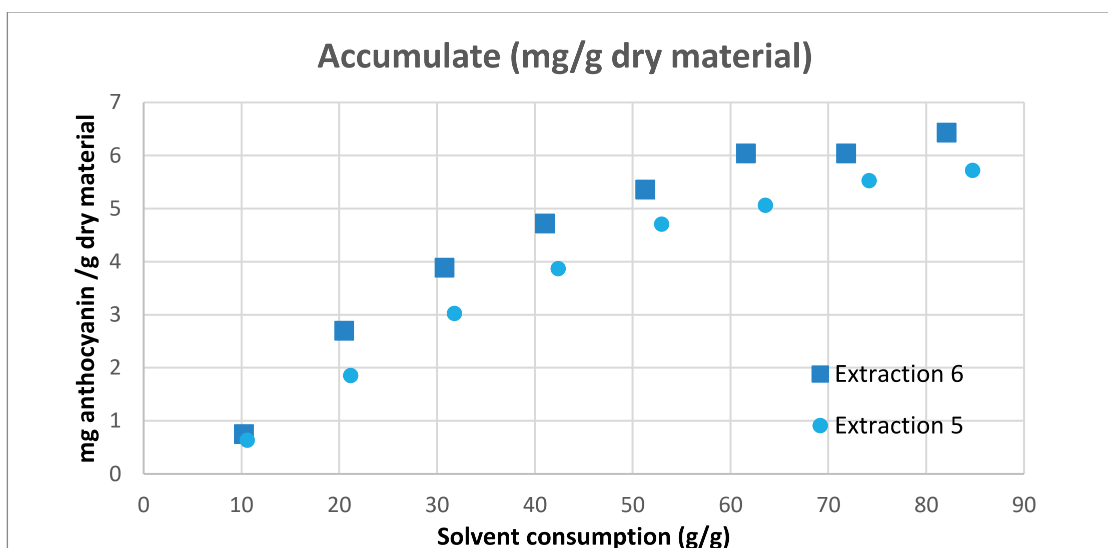
- **EXTRACTION 6**

Table 6.4.3: Anthocyanin concentrations for extraction 6

Sample	A	Anthocyanin (mg/l)	Anthocyanin (mg/g extract)	Anthocyanin (mg/g dry material)	Accumulate (mg/g dry material)	Solvent/dry skin (g/g)
S6/1	0,870	72.6	7.1	0.74	0.74	10.2
S6/2	0,628	52.4	1.4	1.9	2.7	20.5
S6/3	0,178	14.9	7.1	1.2	3.9	30.8
S6/4	0,072	6.0	4.6	0.83	4.7	41.1
S6/5	0,041	3.4	3.3	0.64	5.4	51.3
S6/6	0,027	2.3	3.4	0.68	6.0	61.6
S6/7	0,000	0.0	0.0	0.0	6.0	71.8
S6/8	0,013	1.1	1.9	0.39	6.4	82.1

In these cases, the mass of anthocyanins obtained is quite similar (figure 6.4.3), the variation is acceptable for an experimental method. According to the results, around 6mg of monomeric anthocyanins have been obtained per gram of dry grape skin when the operation temperature was 110ºC.

Figure 6.4.3: Anthocyanin accumulate content in extractions at 110°C



6.4.3 Extraction with water-ethanol as solvent

For the extractions with water-ethanol as solvent the results are showed below (table 6.4.4). The extraction 10 was performed at 90°C while the extractions 8 and 9 were performed at 130°C.

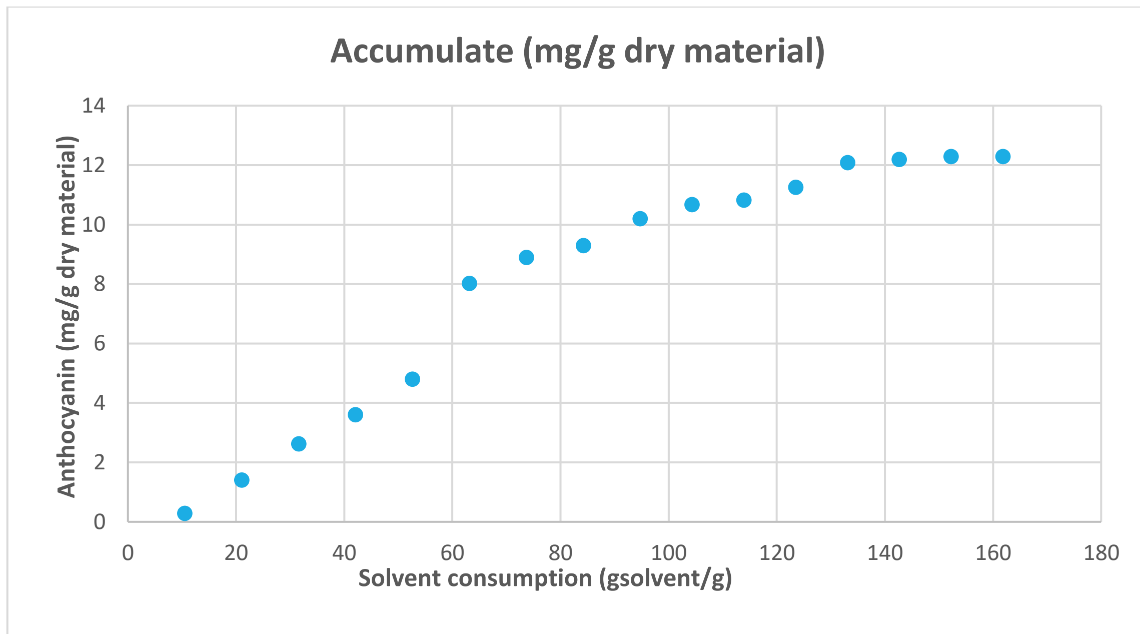
- **EXTRACTION 10**

Table 6.4.4: Anthocyanin concentrations for extraction 10

Sample	A	Anthocyanin (mg/l)	Anthocyanin (mg/g extract)	Anthocyanin (mg/g dry material)	Accumulate (mg/g dry material)	Solvent/dry skin (g/g)
S10/1	0.323	27.0	3.4	0.28	0.28	10.5
S10/2	0.310	25.9	10.2	1.13	1.41	21.1
S10/3	0.120	10.0	10.1	1.22	2.63	31.6
S10/4	0.083	6.9	7.5	0.98	3.61	42.1
S10/5	0.053	4.4	8.7	1.19	4.80	52.7
S10/6	0.035	2.9	23.5	3.23	8.02	63.2
S10/7	0.032	2.7	6.2	0.88	8.90	73.7
S10/8	0.023	1.9	2.6	0.39	9.29	84.3
S10/9	0.018	1.5	6.0	0.91	10.2	94.8
S10/10	0.069	5.8	2.7	0.48	10.7	104.4
S10/11	0.020	1.7	0.74	0.15	10.8	113.9
S10/12	0.038	3.2	2.0	0.44	11.3	123.5
S10/13	0.023	1.9	3.7	0.82	12.1	133.1
S10/14	0.001	0.1	0.51	0.11	12.2	142.7
S10/15	0.001	0.1	0.40	0.09	12.3	152.3
S10/16	0.000	0.0	0.0	0.0	12.3	161.9

The concentration of anthocyanins at 90ºC is two times higher than at 110ºC. It is noticeable that with water, the monomeric anthocyanin content of the grape skin was not extracted completely. The addition of ethanol meant that more monomeric anthocyanins were extracted from the sample. The mg of anthocyanins extracted are represented in the following graph (figure 6.4.4). An unexpected increasing can be seen in the anthocyanin content of the sample 6, moreover, in case of the extraction yield this increasing can also be observed.

Figure 6.4.4: Anthocyanin accumulate content in extraction 10



- **EXTRACTION 8 AND 9**

In these extractions, the degradation of the anthocyanins was significant because of the temperature (130°C), therefore, this temperature should not be used to obtain anthocyanin despite the higher extraction yields obtained at this temperature.

6.4.4 Comparison of the results

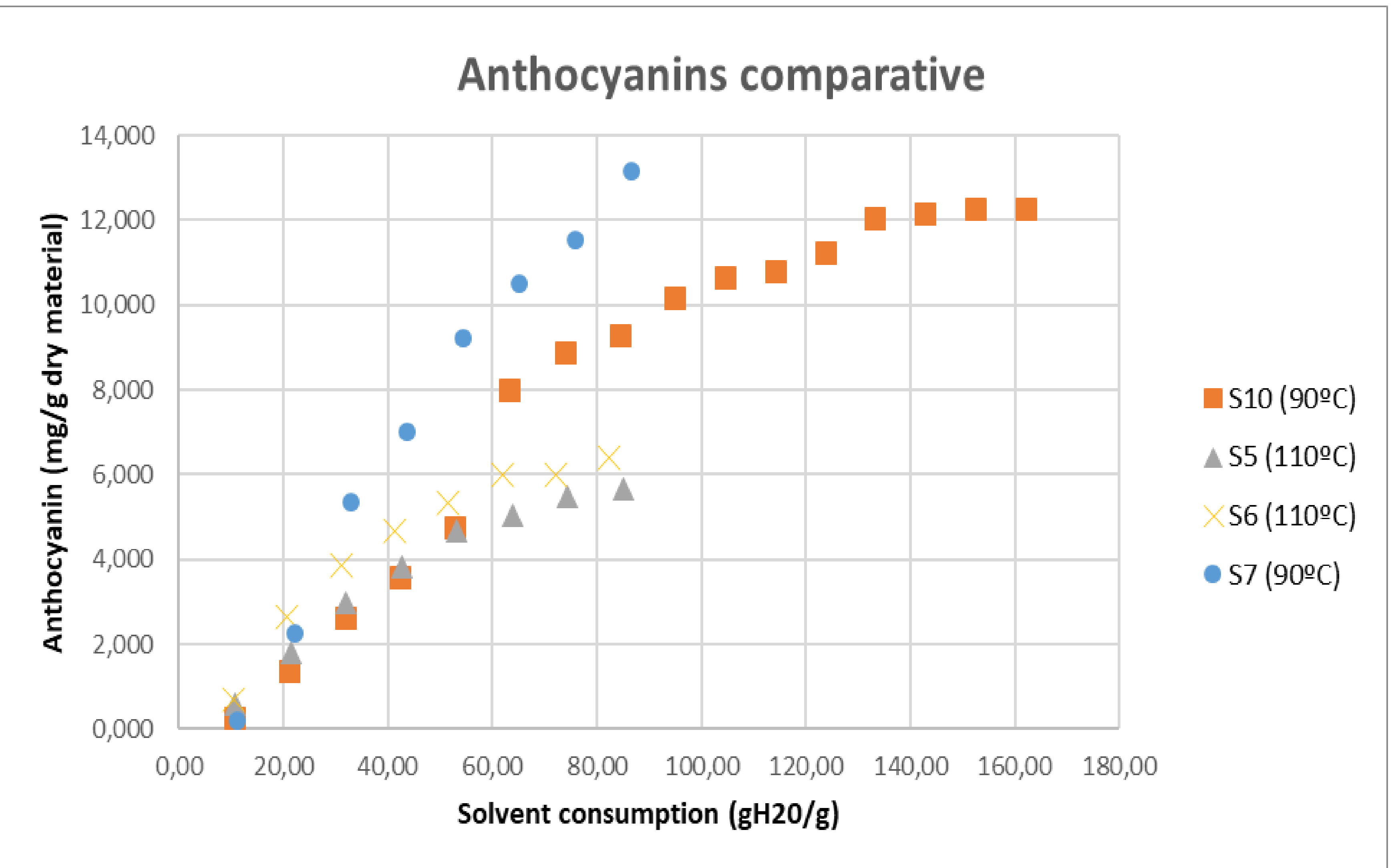
Table 6.4.5 summarizes the anthocyanin yields at the studied extraction conditions. Because there were two different kinds of extraction we can see the amount extracted with only water, so the first 8 samples, but also the amount extracted after the use of water and the mixture of water and ethanol. It is noticeable that the higher anthocyanin concentration is obtained for the lower extraction temperatures (especially extraction 7), in this case 90°C.

Table 6.4.5: Comparative of the results

Temperature (ºC)	Extraction	Anthocyanins (mg/g dry material)		YEmax	
		Water (80 minutes)	Water + ethanol	Water (80 minutes)	Water + ethanol
90	7	13.2		19.97	
	10	9.3	12.3	14.35	23.08
110	5	5.7		23.26	
	6	6.4		20.25	

In the graphic below (figure 6.4.5) it is possible to compare the obtention of anthocyanins for the different temperatures. It is clear that at a lower temperature (90ºC) the anthocyanin concentration is higher. It must be due to the fact that they are thermolabile compounds. So, temperature has influence on the extraction of total monomeric anthocyanin. There is an indirect relation, total monomeric anthocyanin concentration decreases with increasing temperature.

Figure 6.4.5: Anthocyanin accumulate comparative



6.5 *Colour density, polymeric colour, percent polymeric colour, degradation, and browning*

From the absorbance measurements (detailed in the appendix 9.2) it is possible to obtain more information, like:

- **Colour density**
- **Polymeric colour**
- **Percent polymeric colour (%):** the percent polymeric colour is the ratio between polymerized colour and colour density. It is used to determine the percentage of the colour which is contributed by polymerized material.
- **Degradation index:** it gives the accumulation of the browning products. The ratio between monomeric and total anthocyanin can be used for determination of the degradation index.
- **Browning**

With these values we can get a general idea of the amount of monomeric and total anthocyanins, the ratio between them and the degradation of the sample.

6.5.1 Extractions at 90°C

In these cases, the obtained values (table 6.5.1 and 6.5.2) are similar except in certain values that may have been caused by absorbance reading mistakes or contamination of the samples.

As we can see, the sample 2 presents a higher colour density, polymeric colour and browning index than the average of the other samples, while the degradation index is slightly lower than the average.

- **EXTRACTION 7**

Table 6.5.1: Extraction 7 values

Sample	Colour density	Polymeric colour	Percent polymeric color (%)	Degradation index	Browning index
S7/1	0.710	0.48	66.9	7.24	0.34
S7/2	2.155	0.92	42.5	4.84	0.63
S7/3	0.890	0.35	39.3	7.03	0.23
S7/4	0.515	0.13	24.3	7.88	0.10
S7/5	0.355	0.14	39.4	8.65	0.11
S7/6	0.260	0.07	26.9	8.68	0.06
S7/7	0.210	0.03	14.3	8.13	0.04
S7/8	0.175	0.05	28.6	7.50	0.04

- **EXTRACTION 10**

The introduction of the ethanol means a fast increase of the yield of extraction, which means a very elevate colour density, degradation index, polymeric colour, browning index and percent polymeric colour.

Table 6.5.2: Extraction 10 values

Sample	Colour density	Polymeric colour	Percent polymeric color (%)	Degradation index	Browning index
S10/1	0.685	0.41	59.1	7.02	0.26
S10/2	0.725	0.31	42.8	5.51	0.19
S10/3	0.380	0.19	50.0	6.52	0.13
S10/4	0.330	0.13	39.4	7.22	0.09
S10/5	0.240	0.10	41.7	8.33	0.08
S10/6	0.210	0.10	47.6	6.67	0.07
S10/7	0.165	0.04	21.2	8.75	0.04
S10/8	0.135	0.04	25.9	7.27	0.04
S10/9	0.385	0.06	15.6	7.90	0.04
S10/10	4.025	3.79	94.0	8.37	2.40
S10/11	6.040	5.83	96.4	9.80	3.86
S10/12	1.430	1.40	97.5	10.9	0.97
S10/13	0.835	0.90	107.8	10.8	0.62
S10/14	0.380	0.27	71.1	5.27	0.16
S10/15	0.320	0.18	56.3	5.00	0.11
S10/16	0.250	0.22	86.0	5.00	0.13

In the following table (table 6.5.3) there is a summary of the results. There is a remarkable difference between the results using one solvent or other (water or water-ethanol), in all parameters but degradation index. The results are the average of each value with the solvent used.

Table 6.5.3: Comparison of the extraction results at 90ºC

Extraction	Solvent	Colour density	Polymeric colour	Percent polymeric colour (%)	Degradation index	Browning index
7	Water	0.66	0.27	35.2	7.49	0.19
10		0.36	0.15	38.1	7.24	0.10
10	Water + ethanol	1.90	1.80	87.0	7.87	1.18

6.5.2 Extraction at 130°C

- **EXTRACTION 9**

In this case the results are related to the assumed low monomeric anthocyanin concentration in the extract. The monomeric anthocyanins were not measurable in this case because of the high degradation at 130ºC, but the average results indicate the pigment degradation are summarized in the following table (table 6.5.4). It is also remarkable difference between the values obtained with water as solvent and water – ethanol mixture. In case of water – ethanol

mixture used as extraction solvent the polymerized colour content of the extract increased. The accumulation of the brownish degradation products is also determinative.

Table 6.5.4: Extraction 9 at 130°C

Solvent	Colour density	Degradation index	Polymeric colour	Browning index	Percent polymeric colour
Water	1.62	9.28	0.89	0.61	49.5
Water + ethanol	3.52	11.3	3.22	2.14	89.9

The following figures give a comparison between the different averages indicate the polymeric colour content and the degradation (figure 6.5.1, 6.5.2, 6.5.3, 6.5.4 and 6.5.5). The red colour means the extraction is performed at 90°C and the blue at 130°C.

Figure 6.5.1 Comparison of the colour density

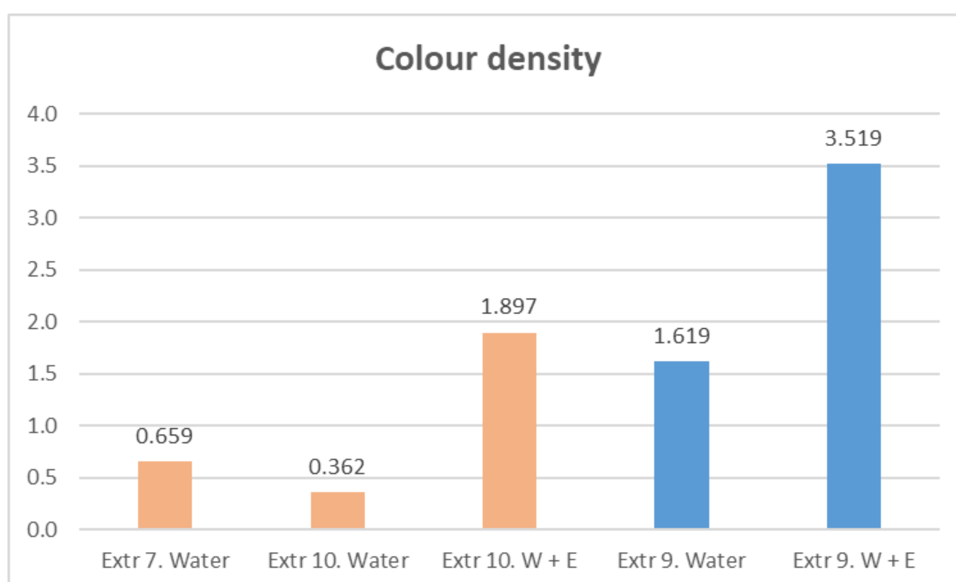


Figure 6.5.2: Comparison of the polymeric colour

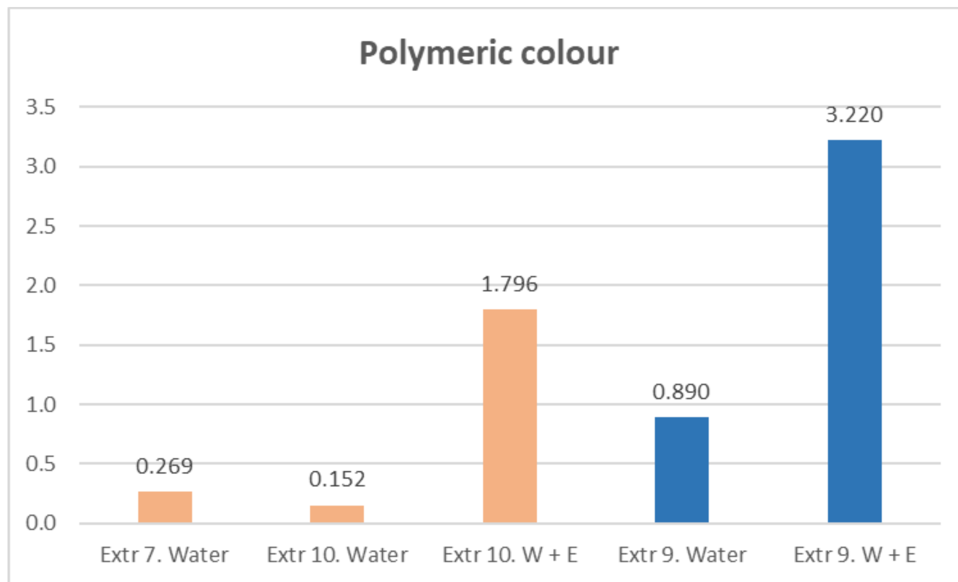


Figure 6.5.3: Comparison of the percent polymeric colour

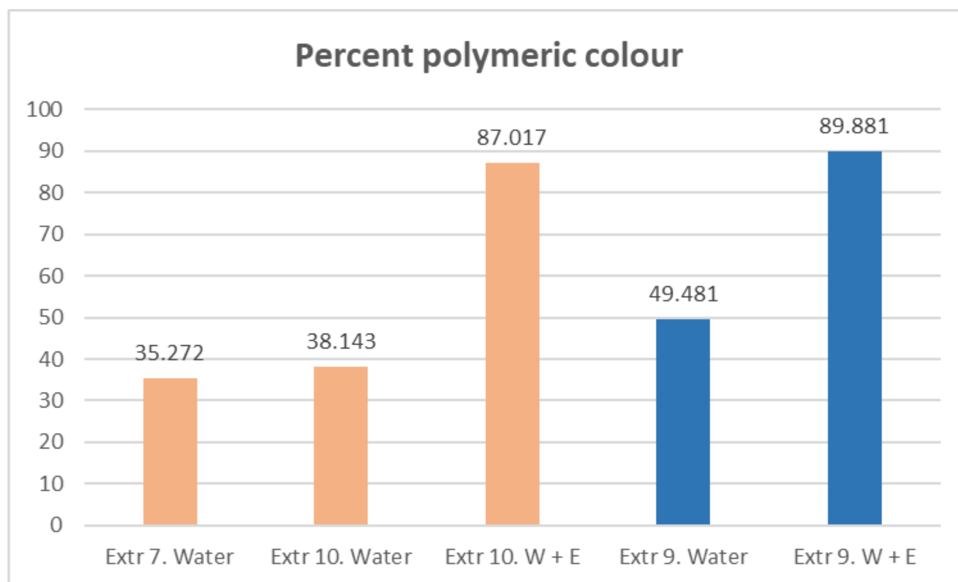


Figure 6.5.2: Comparison of the degradation index

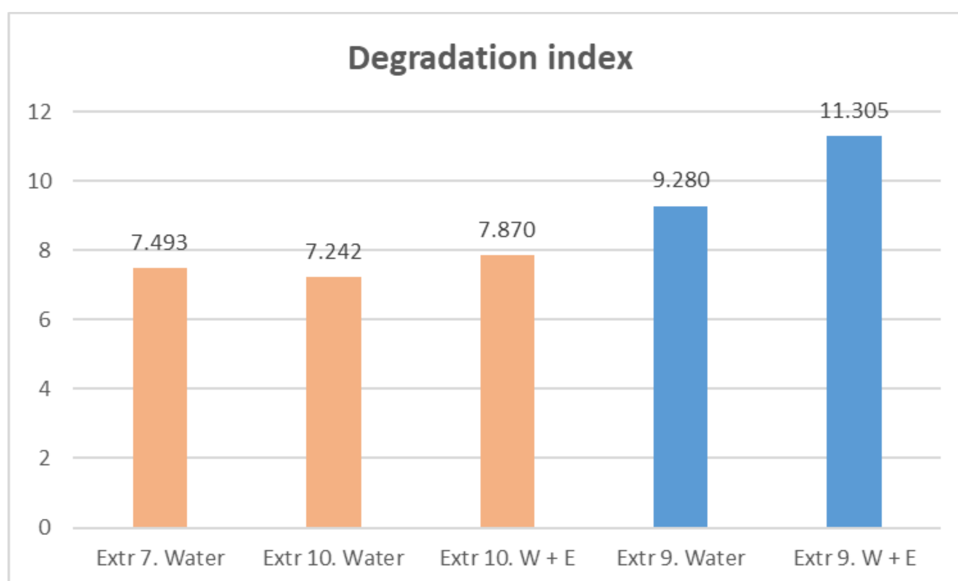
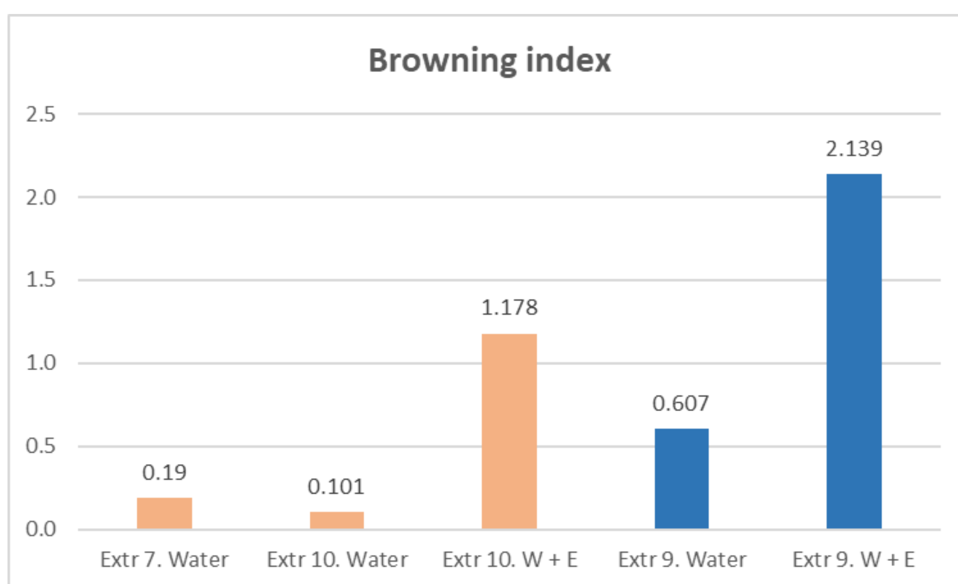


Figure 6.5.4: Comparison of the browning index



In all cases the results with water and ethanol are higher than with only water as solvent. The biggest differences are found in colour density, polymeric colour and browning index.

Moreover, the results at 90°C are lower than at 130°C, but the difference is not so remarkable, and the biggest differences are also found in colour density, polymeric colour and browning index.

7 CONCLUSION

Many components are commonly extracted from natural sources with industrial purposes. Grape skin is an industrial by product (mainly of the wine industry) rich in polyphenols (antioxidants) and anthocyanin (colour pigments) among many other substances.

Anthocyanins are present in grapes, but also in the vacuoles of many other plants. They have some health benefits and can be used to replace banned synthetic dyes. There are many extraction methods to obtain them from their natural sources (solvent extraction, microwave-assisted extraction, ultrasound-assisted extraction). However, as anthocyanins are thermolabile substances, it is important to find an extraction method to get these pigments undamaged.

The method studied in this case was pressurized liquid extraction (PLE), in particular the dynamic mode, with continuous solvent flow. The grape skin was filled in an extraction column and placed into an oven. The experiments were carried at 90ºC, 110ºC and 130ºC, all of them with a pressure of 50 bar, there were temperature and pressure sensors to control the process. Two different solvent were used: deionized water and a mixture of deionized water and ethanol.

The pressurized liquid extraction has proved to be a useful method for extracting compounds from grape skin. The extraction yield is influenced by the temperature, higher rates obtained at a higher temperature. The solvent also is an important variable of the process; when using deionized water, the maximum extraction yield is lower than when using the mixture of 50% in volume of water and ethanol. The maximum extraction yield could also variate depending on the composition of the solvent. The extraction yield data obtained must fit an extraction curve model with two parameters: maximum extraction yield and kinetic parameter. Unlike the maximum extraction yield, the kinetic parameter has higher values at lower temperatures.

The concentration of monomeric anthocyanins in the extract has been calculated using absorbance measurements of samples with different pH at different wavelengths. The temperature proves to be a determining factor in the amount of them obtained. Due to the fact that high temperatures damages anthocyanins, the best extraction temperature is 90ºC despite the higher extraction yields obtained at higher temperatures. The use of ethanol enhances the anthocyanin obtention in almost a 50%, so it is an interesting choice. However, as grape skin is a cheap by product it might not be needed if the process using only water is cheaper and safer.

Other parameters can be calculated from the absorbance measurements, they are: colour density, polymeric colour, percent polymeric colour, degradation index, and browning index. These parameters give information of the ratio between monomeric and total anthocyanins or the degradation of the sample. They all increased when the solvent used was the mixture of ethanol and water. There were differences according to the extraction temperature, but they were not so remarkable. Still, the values of these parameters were slightly higher at higher temperatures, specially the colour density, polymeric colour and browning index.

8 LITERATURE

- [1] B. Baca-Bocanegra, J. Nogales-Bueno, F. J. Heredia, J. M. Hernández-Hierro, *Influence of oak wood chips–grape mix maceration on the extraction of anthocyanins from low-extractable anthocyanin content red grapes,* Food Colour and Quality Laboratory (University of Sevilla), 2017
- [2] M. M. Giusti, R. E. Wrolstad, *Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy*, Current Protocols in Food Analytical Chemistry, 2001, F1.2.1-F1.2.13
- [3] P. Bridle, C. F. Timberlake, *Anthocyanins as natural food colors-selected aspects*, Food Chem, 1996, (58) 103-109
- [4] C. F. Timberlake, B. S. Henry*, Anthocyanins as natural food colorants*, Prog. Clin. Biol. Res., 1988, (280) 107-121
- [5] H. Takamura, A. Yamagami, *Antioxidative activity of mono-acylated anthocyanins isolated from Muscat Bailey A grape*, J. Agric. Food Chem., 1994, (42) 1612-1615
- [6] M. Karaivanova, D. Drenska, R. Ovcharov, *A modification of the toxic effects of platinum complexes with anthocyans,* Eksp. Med. Morfol., 1990, (29) 19-24
- [7] T. Vatai, M. Škerget, Z. Knez, *Extraction of phenolic compounds from elder berry and different grape marc varieties using organic solvents and/or supercritical carbon dioxide*, Journal of Food Engineering, 2009, (90) 246–254
- [8] K. Gould; K. Davies; C. Winefield, *Anthocyanins. Biosynthesis, Functions, and Applications*, Springer, 2009
- [9] M. Dharmadhikari, *Composition of Grapes*, https://www.extension.iastate.edu/wine/files/page/files/compositionofgrapes.pdf (accessed 25-04-2018)
- [10] Bate-Smith, Swain, *Flavonoid compounds*, New York: Academic Press, 1962, 75–809
- [11] K. G. Lee, T. Shibamoto, *Determination of antioxidant potential of volatile extracts isolated from various herbs and spices*, Journal of Agricultural and Food Chemistry, 2002, (50) 4947–4952
- [12] D. Monte, S Carradori et al, *Modern extraction techniques and their impact on the pharmacological profile of Serenoa repens extracts for the treatment of lower urinary tract symptoms*, BMC Urology, 2014
- [13] R. Wrolstad, R. W. Durst, J. Lee, *Tracking color and pigment changes in anthocyanin products*, Trends in Food Science & Technology, 2005, (16) 423–428
- [14] M. L. Blackhall, R. Berry, N. W. Davies, J. T. Walls, *Optimized extraction of anthocyanins from Reid Fruits' Prunus avium 'Lapins' cherries*, Elsevier Ltd, 2018
- [15] H.D. de Faria, C. T. Bueno et al, *Online extraction of antihypertensive drugs and their metabolites from untreated human serum samples using restricted access carbon nanotubes in a column switching liquid chromatography system*, Elsevier B. V, 2017
- [16] A. Mustafa, C. Turner, *Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review*, Elsevier B.V., 2011
- [17] R. Carabias-Martínez, E. Rodríguez-Gonzalo, P. Revilla-Ruiz, J. Hernández-Méndez, *Pressurized liquid extraction in the analysis of food and biological samples*, University of Salamanca, Elsevier B.V., 2005

- [18] M. Plaza, C. Turner, *Pressurized hot water extraction of bioactives*, Trends in Analytical Chemistry, 2015, (71) 39–54
- [19] A. P. Machado, J. L. Pasquel-Reátegui, G. Fernández Barbero, J. Martínez, *Pressurized liquid extraction of bioactive compounds from blackberry (Rubus fruticosus L.) residues: a comparison with conventional methods*, Elsevier Ltd., 2014
- [20] The basis of vacuum evaporation. Condorchem envitech company blog. https://blog-en.condorchem.com/basis-vacuum-evaporation/#.WvWsDYiFPIU (accessed 25-04-2018)
- [21] https://elespectrofotometro.com/que-es/ (accessed 11-05-2018)
- [22] National Center for Biotechnology Information. PubChem Compound Database; CID=92131208 https://pubchem.ncbi.nlm.nih.gov/compound/92131208 (accessed 26-04-2018)
- [23] G. Brunner, *Mass-transfer from solid material in gas extraction*, Berichter der Bunsen-Gesellschaft-Pysical Chem. Chem. Phys., 1984, (88), 887-891

9 APPENDIX

9.1 Extractions

EXPERIMENT S1					
Pressure (bar)		50			
Temperature (ºC)		110			
Flowrate (ml/min)		2			
Grape skin (g)		1.9165			
Dry content (g)		1.8051			
Sample	Ext. Time (min)	Extract (g)	Colour	Solid extract (g)	Accumulate (g)
S1/1	2	4.1508	Wine	0.0468	0.0468
S1/2	5	9.9489	Wine	0.0698	0.1166
S1/3	5	9.9492	Wine	0.0459	0.1625
S1/4	5	9.9943	Wine	0.0289	0.1914
S1/5	5	9.9637	Wine	0.0136	0.2050
S1/6	5	10.0153	Wine	0.0164	0.2214

EXPERIMENT S2					
Pressure (bar)		50			
Temperature (ºC)		110			
Flowrate (ml/min)		2			
Grape skin (g)		1.9929			
Dry content (g)		1.8770			
Sample	Ext. Time (min)	Extract (g)	Colour	Solid extract (g)	Accumulate (g)
S2/1	5	10.0736	Wine	0.1204	0.1204
S2/2	5	9.9433	Wine	0.0491	0.1695
S2/3	5	9.9615	Wine	0.0438	0.2133
S2/4	5	10.0721	Wine	0.0279	0.2412
S2/5	5	9.9751	Lighter wine	0.0300	0.2712
S2/6	5	10.0923	Lighter wine	0.0217	0.2929
S2/7	5	9.9249	Lighter wine	0.0254	0.3183
S2/8	5	9.9621	Lighter wine	0.0111	0.3294

EXPERIMENT S3					
Pressure (bar)		50			
Temperature (ºC)		90			
Flowrate (ml/min)		2			
Grape skin (g)		1.9956			
Dry content (g)		1.8796			
Sample	Ext. Time (min)	Extract (g)	Colour	Solid extract (g)	Accumulate (g)
S3/1	10	20.2569	Wine	0.1966	0.1966
S3/2	10	19.7522	Wine	0.1160	0.3126
S3/3	10	19.8283	Light wine	0.0317	0.3443
S3/4	10	19.7930	Light wine	0.0209	0.3652
S3/5	10	19.7767	Light purple	0.0182	0.3834
S3/6	10	19.8207	Light purple	0.0112	0.3946
S3/7	10	19.8300	Very light	0.0155	0.4101
S3/8	10	19.7490	Almost transp	0.0132	0.4233

EXPERIMENT S4					
Pressure (bar)		50			
Temperature (ºC)		130			
Flowrate (ml/min)		2			
Grape skin (g)		2.0424			
Dry content (g)		1.9236			
Sample	Ext. Time (min)	Extract (g)	Colour	Solid extract (g)	Accumulate (g)
S4/1	10	20.2802	Wine	0.2148	0.2148
S4/2	10	20.0767	Wine	0.0991	0.3139
S4/3	10	19.9982	Purple brown	0.0484	0.3623
S4/4	10	19.8033	Blue brown	0.0349	0.3972
S4/5	10	19.8414	Light brown	0.0241	0.4213
S4/6	10	19.9273	Light brown	0.0200	0.4413
S4/7	10	19.9371	Almost transp	0.0121	0.4534
S4/8	10	19.9382	Almost transp	0.0144	0.4678

EXPERIMENT S5					
Pressure (bar)		50			
Temperature (ºC)		110			
Flowrate (ml/min)		2			
Grape skin (g)		2.0042			
Dry content (g)		1.8877			
Sample	Ext. Time (min)	Extract (g)	Colour	Solid extract (g)	Accumulate (g)
S5/1	10	20.1778	Wine	0.1968	0.1968
S5/2	10	19.7994	Wine	0.0896	0.2864
S5/3	10	19.6992	Wine	0.0451	0.3315
S5/4	10	19.7108	Light wine	0.0351	0.3666
S5/5	10	19.8611	Light wine	0.0196	0.3862
S5/6	10	19.6836	Very light wine	0.0277	0.4139
S5/7	10	19.7534	Very light wine	0.0197	0.4336
S5/8	10	19.7857	Very light wine	0.0189	0.4525

EXPERIMENT S6					
Pressure (bar)		50			
Temperature (ºC)		110			
Flowrate (ml/min)		2			
Grape skin (g)		2.0694			
Dry content (g)		1.9491			
Sample	Ext. Time (min)	Extract (g)	Colour	Solid extract (g)	Accumulate (g)
S6/1	10	20.2552	Wine	0.2052	0.2052
S6/2	10	19.8749	Wine	0.0782	0.2834
S6/3	10	19.8270	Wine	0.0417	0.3251
S6/4	10	19.8280	Wine	0.0260	0.3511
S6/5	10	19.8416	Light wine	0.0205	0.3716
S6/6	10	19.8422	Light wine	0.0131	0.3847
S6/7	10	19.8049	Very light wine	0.0116	0.3962
S6/8	10	19.7682	Very light wine	0.0116	0.4078

EXPERIMENT S7					
Pressure (bar)		50			
Temperature (ºC)		90			
Flowrate (ml/min)		2			
Grape skin (g)		1.9758			
Dry content (g)		1.8609			
Sample	Ext. Time (min)	Extract (g)	Colour	Solid extract (g)	Accumulate (g)
S7/1	10	20.2257	Light wine	0.2050	0.2050
S7/2	10	19.9323	Wine	0.1051	0.3101
S7/3	10	19.7979	Wine	0.0217	0.3319
S7/4	10	19.9334	Wine	0.0171	0.3490
S7/5	10	19.9626	Light wine	0.0091	0.3581
S7/6	10	19.8061	Very light wine	0.0089	0.3670
S7/7	10	19.8467	Very light wine	0.0088	0.3758
S7/8	10	19.8441	Very light wine	0.0055	0.3813

EXPERIMENT S8					
Pressure (bar)		50			
Temperature (ºC)		130			
Flowrate (ml/min)		2			
Grape skin (g)		1.9915			
Dry content (g)		1.8757			
Sample	Ext. Time (min)	Extract (g)	Colour	Solid extract (g)	Accumulate (g)
S8/1	10	20.3392	Dark wine	0.2159	0.2159
S8/2	10	19.7887	Dark wine	0.1182	0.3341
S8/3	10	19.7715	Wine	0.0596	0.3937
S8/4	10	19.7396	Wine	0.0354	0.4291
S8/5	10	19.7519	Light wine	0.0417	0.4708
S8/6	10	19.8495	Light wine	0.0204	0.4912
S8/7	10	19.7970	Very light wine	0.0179	0.5091
S8/8	10	19.8634	Very light wine	0.0132	0.5223
S8/9	10	20.1716	Light brown	0.0131	0.5354
S8/10	10	18.7028	Dirty water	0.0543	0.5897
S8/11	10	18.1927	Dirty water	0.0421	0.6318
S8/12	10	17.9618	light brown	0.0210	0.6528
S8/13	10	18.1511	Light brown	0.0139	0.6667
S8/14	10	18.0583	Very light wine	0.0076	0.6743
S8/15	10	17.9827	Very light wine	0.0120	0.6863
S8/16	10	18.1966	Very light wine	0.0126	0.6989

EXPERIMENT S9					
Pressure (bar)		50			
Temperature (ºC)		130			
Flowrate (ml/min)		2			
Grape skin (g)		2.0165			
Dry content (g)		1.8992			
Sample	Ext. Time (min)	Extract (g)	Colour	Solid extract (g)	Accumulate (g)
S9/1	10	20.0316	Wine	0.1086	0.1086
S9/2	10	19.7808	Dark wine	0.1225	0.2311
S9/3	10	19.7079	Brown wine	0.0553	0.2864
S9/4	10	19.7345	Brown wine	0.0356	0.3220
S9/5	10	19.7149	Dark	0.0415	0.3634
S9/6	10	19.7282	Dark	0.0183	0.3818
S9/7	10	19.7101	Light wine	0.0115	0.3933
S9/8	10	19.8660	Light wine	0.0117	0.4049
S9/9	10	19.9408	Light wine	0.0141	0.4190
S9/10	10	18.4325	Brown green	0.0518	0.4708
S9/11	10	18.3945	Brown green	0.0375	0.5082
S9/12	10	17.6123	Brown orange	0.0158	0.5241
S9/13	10	17.9871	Brown orange	0.0150	0.5391
S9/14	10	17.7651	Brown orange	0.0110	0.5500
S9/15	10	17.8665	Light brown	0.0083	0.5583
S9/16	10	17.8114	Light brown	0.0080	0.5663

EXPERIMENT S10					
Pressure (bar)		50			
Temperature (ºC)		90			
Flowrate (ml/min)		2			
Grape skin (g)		2.0164			
Dry content (g)		1.8991			
Sample	Ext. Time (min)	Extract (g)	Colour	Solid extract (g)	Accumulate (g)
S10/1	10	19.9691	Wine	0.1593	0.1593
S10/2	10	19.7449	Wine	0.0509	0.2101
S10/3	10	19.7017	Wine	0.0199	0.2300
S10/4	10	19.6826	light wine	0.0186	0.2486
S10/5	10	19.7376	light wine	0.0101	0.2587
S10/6	10	19.7722	Very light wine	0.0025	0.2612
S10/7	10	19.7042	Very light wine	0.0087	0.2699
S10/8	10	19.7061	Very light wine	0.0145	0.2844
S10/9	10	20.5761	Very light wine	0.0050	0.2895
S10/10	10	19.4206	Red wine	0.0424	0.3319
S10/11	10	18.6851	Brown	0.0450	0.3769
S10/12	10	18.5884	Light brown	0.0314	0.4083
S10/13	10	18.2759	Light brown	0.0103	0.4185
S10/14	10	18.5774	Very light brown	0.0033	0.4218
S10/15	10	18.6414	Very light brown	0.0042	0.4260
S10/16	10	18.6541	Very light brown	0.0022	0.4282

9.2 Absorbances

- **EXTRACTION 5:**

SAMPLE 1		
Abs	520	700
Water	0.027	0.001
"+KCl	0.905	0.005
"+CH3CO2Na	0.218	0.035

SAMPLE 5		
Abs	520	700
Water	0.001	0.000
"+KCl	0.115	0.011
"+CH3CO2Na	0.096	0.040

SAMPLE 2		
Abs	520	700
Water	0.000	0.001
"+KCl	0.680	0.022
"+CH3CO2Na	0.393	0.166

SAMPLE 6		
Abs	520	700
Water	0.000	0.001
"+KCl	0.068	0.008
"+CH3CO2Na	0.051	0.018

SAMPLE 3		
Abs	520	700
Water	0.001	0.000
"+KCl	0.334	0.032
"+CH3CO2Na	0.194	0.072

SAMPLE 7		
Abs	520	700
Water	0.001	0.001
"+KCl	0.081	0.021
"+CH3CO2Na	0.071	0.035

SAMPLE 4		
Abs	520	700
Water	0.000	0.000
"+KCl	0.173	0.002
"+CH3CO2Na	0.115	0.035

SAMPLE 8		
Abs	520	700
Water	0.001	0.001
"+KCl	0.041	0.002
"+CH3CO2Na	0.062	0.032

- **EXTRACTION 6:**

SAMPLE 1		
Abs	520	700
Water	0.000	0.001
"+KCl	1.105	0.047
"+CH3CO2Na	0.255	0.067

SAMPLE 5		
Abs	520	700
Water	0.000	0.001
"+KCl	0.090	0.018
"+CH3CO2Na	0.057	0.026

SAMPLE 2		
Abs	520	700
Water	0.000	0.001
"+KCl	0.881	0.035
"+CH3CO2Na	0.316	0.098

SAMPLE 6		
Abs	520	700
Water	0.000	0.000
"+KCl	0.073	0.017
"+CH3CO2Na	0.063	0.034

SAMPLE 3		
Abs	520	700
Water	0.000	0.000
"+KCl	0.309	0.023
"+CH3CO2Na	0.173	0.065

SAMPLE 7		
Abs	520	700
Water	0.000	0.000
"+KCl	0.035	0.006
"+CH3CO2Na	0.038	0.009

SAMPLE 4		
Abs	520	700
Water	0.001	0.000
"+KCl	0.132	0.010
"+CH3CO2Na	0.094	0.044

SAMPLE 8		
Abs	520	700
Water	0.000	0.000
"+KCl	0.034	0.005
"+CH3CO2Na	0.034	0.018

- **EXTRACTION 7:**

SAMPLE 1						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.000	0.001	0.001
"+KCl	0.404	0.027	"+water	0.107	0.081	0.023
"+CH3CO2Na	0.087	0.024	"+bisulfide	0.091	0.050	0.023

SAMPLE 2						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.001	0.000	0.000
"+KCl	0.996	0.023	"+water	0.279	0.286	0.067
"+CH3CO2Na	0.273	0.075	"+bisulfide	0.155	0.088	0.030

SAMPLE 3						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.001	0.000	0.000
"+KCl	0.318	0.008	"+water	0.162	0.132	0.058
"+CH3CO2Na	0.131	0.047	"+bisulfide	0.054	0.032	0.008

SAMPLE 4						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.001
"+KCl	0.142	0.005	"+water	0.105	0.082	0.042
"+CH3CO2Na	0.082	0.036	"+bisulfide	0.032	0.019	0.013

SAMPLE 5						
Abs	520	700	Abs	420	520	700
Water	0.001	0.001	Water	0.001	0.000	0.001
"+KCl	0.096	0.004	"+water	0.068	0.049	0.023
"+CH3CO2Na	0.061	0.031	"+bisulfide	0.030	0.016	0.009

SAMPLE 6						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.000	0.000	0.000
"+KCl	0.075	0.011	"+water	0.060	0.046	0.027
"+CH3CO2Na	0.046	0.017	"+bisulfide	0.017	0.009	0.006

SAMPLE 7						
Abs	520	700	Abs	420	520	700
Water	0.001	0.001	Water	0.000	0.000	0.000
"+KCl	0.048	0.005	"+water	0.046	0.036	0.020
"+CH3CO2Na	0.037	0.021	"+bisulfide	0.016	0.008	0.009

SAMPLE 8						
Abs	520	700	Abs	420	520	700
Water	0.001	0.001	Water	0.001	0.001	0.000
"+KCl	0.046	0.003	"+water	0.040	0.033	0.019
"+CH3CO2Na	0.036	0.019	"+bisulfide	0.013	0.009	0.006

- **EXTRACTION 8:**

SAMPLE 1						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.000	0.000	0.000
"+KCl	0.067	0.120	"+water	0.079	0.059	0.082
"+CH3CO2Na	0.065	0.085	"+bisulfide	0.043	0.034	0.043

SAMPLE 2						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.001
"+KCl	0.072	0.106	"+water	0.089	0.063	0.076
"+CH3CO2Na	0.089	0.096	"+bisulfide	0.059	0.042	0.049

SAMPLE 3						
Abs	520	700	Abs	420	520	700
Water	0.001	0.001	Water	0.001	0.001	0.000
"+KCl	0.033	0.047	"+water	0.102	0.079	0.084
"+CH3CO2Na	0.077	0.076	"+bisulfide	0.040	0.034	0.043

SAMPLE 4						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.000
"+KCl	0.124	0.062	"+water	0.211	0.211	0.163
"+CH3CO2Na	0.215	0.166	"+bisulfide	0.101	0.101	0.062

SAMPLE 5						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.001	0.000	0.001
"+KCl	0.072	0.036	"+water	0.088	0.117	0.089
"+CH3CO2Na	0.159	0.120	"+bisulfide	0.031	0.042	0.022

SAMPLE 6						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.000	0.001	0.001
"+KCl	0.071	0.033	"+water	0.094	0.128	0.103
"+CH3CO2Na	0.142	0.109	"+bisulfide	0.037	0.051	0.028

SAMPLE 7						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.001
"+KCl	0.056	0.030	"+water	0.068	0.099	0.079
"+CH3CO2Na	0.110	0.087	"+bisulfide	0.029	0.042	0.023

SAMPLE 8						
Abs	520	700	Abs	420	520	700
Water	0.001	0.001	Water	0.001	0.001	0.000
"+KCl	0.040	0.023	"+water	0.043	0.061	0.044
"+CH3CO2Na	0.079	0.065	"+bisulfide	0.022	0.031	0.020

SAMPLE 9						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.001	0.001	0.001
"+KCl	0.040	0.026	"+water	0.048	0.066	0.052
"+CH3CO2Na	0.064	0.052	"+bisulfide	0.016	0.025	0.014

SAMPLE 10						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.001
"+KCl	1.451	2.000	"+water	1.131	1.531	2.000
"+CH3CO2Na	0.761	2.000	"+bisulfide	1.093	1.492	2.000

SAMPLE 11						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.000	0.001	0.001
"+KCl	0.844	0.666	"+water	1.252	1.046	0.798
"+CH3CO2Na	0.617	0.480	"+bisulfide	1.173	0.989	0.749

SAMPLE 12						
Abs	520	700	Abs	420	520	700
Water	0.001	0.001	Water	0.000	0.001	0.001
"+KCl	0.495	0.321	"+water	0.653	0.493	0.321
"+CH3CO2Na	0.513	0.339	"+bisulfide	0.638	0.473	0.309

SAMPLE 13						
Abs	520	700	Abs	420	520	700
Water	0.001	0.001	Water	0.001	0.001	0.000
"+KCl	0.389	0.236	"+water	0.567	0.408	0.246
"+CH3CO2Na	0.410	0.255	"+bisulfide	0.534	0.377	0.232

SAMPLE 14						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.001	0.000
"+KCl	0.309	0.171	"+water	0.456	0.314	0.186
"+CH3CO2Na	0.321	0.191	"+bisulfide	0.432	0.290	0.168

SAMPLE 15						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.001	0.001	0.000
"+KCl	0.228	0.123	"+water	0.351	0.241	0.141
"+CH3CO2Na	0.263	0.155	"+bisulfide	0.331	0.224	0.129

SAMPLE 16						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.000	0.001	0.001
"+KCl	0.212	0.112	"+water	0.369	0.257	0.147
"+CH3CO2Na	0.238	0.139	"+bisulfide	0.344	0.233	0.126

NOTE: The red colour means the value is above the linear range of the spectrophotometer, so the calculations with this number are not correct. The solution in that case is to analyse the sample using another dilution factor, as it would be done in the following cases.

- **EXTRACTION 9:**

SAMPLE 1						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.000
"+KCl	0.935	0.044	"+water	0.332	0.292	0.069
"+CH3CO2Na	0.310	0.092	"+bisulfide	0.200	0.110	0.029

SAMPLE 2						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.001	0.000	0.001
"+KCl	0.743	0.073	"+water	0.667	0.463	0.155
"+CH3CO2Na	0.548	0.201	"+bisulfide	0.385	0.218	0.072

SAMPLE 3						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.000
"+KCl	0.249	0.042	"+water	0.478	0.303	0.122
"+CH3CO2Na	0.312	0.125	"+bisulfide	0.280	0.163	0.058

SAMPLE 4						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.001	0.000
"+KCl	0.125	0.025	"+water	0.335	0.222	0.099
"+CH3CO2Na	0.210	0.086	"+bisulfide	0.173	0.101	0.031

SAMPLE 5						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.001	0.001	0.000
"+KCl	0.071	0.015	"+water	0.221	0.146	0.068
"+CH3CO2Na	0.150	0.070	"+bisulfide	0.136	0.081	0.028

SAMPLE 6						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.001	0.001	0.000
"+KCl	0.057	0.013	"+water	0.159	0.105	0.047
"+CH3CO2Na	0.107	0.051	"+bisulfide	0.089	0.049	0.021

SAMPLE 7						
Abs	520	700	Abs	420	520	700
Water	0.001	0.001	Water	0.000	0.001	0.001
"+KCl	0.033	0.001	"+water	0.113	0.076	0.037
"+CH3CO2Na	0.077	0.035	"+bisulfide	0.028	0.009	0.001

SAMPLE 8						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.001	0.000	0.000
"+KCl	0.022	0.001	"+water	0.104	0.080	0.045
"+CH3CO2Na	0.051	0.028	"+bisulfide	0.010	0.001	-0.009

SAMPLE 9						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.001	0.000
"+KCl	0.020	0.002	"+water	0.074	0.045	0.025
"+CH3CO2Na	0.057	0.039	"+bisulfide	0.018	0.003	-0.005

SAMPLE 10 x 1/2						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.001
"+KCl	1.038	0.625	"+water	1.385	1.020	0.626
"+CH3CO2Na	1.098	0.678	"+bisulfide	1.319	0.963	0.586

SAMPLE 11						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.001	0.000	0.000
"+KCl	0.809	0.563	"+water	1.284	1.010	0.691
"+CH3CO2Na	0.775	0.570	"+bisulfide	1.134	0.915	0.645

SAMPLE 12						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.001
"+KCl	0.435	0.261	"+water	0.592	0.440	0.274
"+CH3CO2Na	0.465	0.298	"+bisulfide	0.524	0.381	0.245

SAMPLE 13						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.001	0.001	0.000
"+KCl	0.312	0.182	"+water	0.423	0.293	0.167
"+CH3CO2Na	0.345	0.210	"+bisulfide	0.390	0.267	0.155

SAMPLE 14						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.001	0.000	0.000
"+KCl	0.250	0.142	"+water	0.351	0.237	0.127
"+CH3CO2Na	0.297	0.174	"+bisulfide	0.319	0.208	0.112

SAMPLE 15						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.000	0.000	0.000
"+KCl	0.205	0.114	"+water	0.263	0.178	0.096
"+CH3CO2Na	0.252	0.147	"+bisulfide	0.240	0.158	0.084

SAMPLE 16						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.000	0.000	0.000
"+KCl	0.186	0.102	"+water	0.270	0.178	0.094
"+CH3CO2Na	0.234	0.140	"+bisulfide	0.241	0.151	0.079

NOTE: The yellow colour means the absorptivity value is lower than 0. However, it does not mean there is a mistake, only that the sample´s absorbance is lower than distilled water absorbance.

- **EXTRACTION 10:**

SAMPLE 1						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.000	0.000	0.000
"+KCl	0.393	0.018	"+water	0.102	0.079	0.022
"+CH3CO2Na	0.079	0.027	"+bisulfide	0.062	0.039	0.010

SAMPLE 2						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.001	0.000	0.000
"+KCl	0.383	0.011	"+water	0.094	0.087	0.018
"+CH3CO2Na	0.084	0.022	"+bisulfide	0.038	0.026	0.001

SAMPLE 3						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.000
"+KCl	0.167	0.010	"+water	0.063	0.053	0.020
"+CH3CO2Na	0.063	0.026	"+bisulfide	0.029	0.017	0.004

SAMPLE 4						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.001
"+KCl	0.111	0.008	"+water	0.057	0.045	0.018
"+CH3CO2Na	0.040	0.020	"+bisulfide	0.020	0.012	0.003

SAMPLE 5						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.001	0.000	0.000
"+KCl	0.081	0.010	"+water	0.052	0.040	0.022
"+CH3CO2Na	0.049	0.031	"+bisulfide	0.019	0.009	0.004

SAMPLE 6						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.001	0.000	0.000
"+KCl	0.093	0.040	"+water	0.043	0.037	0.019
"+CH3CO2Na	0.042	0.024	"+bisulfide	0.016	0.008	0.002

SAMPLE 7						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.000	0.000	0.001
"+KCl	0.060	0.015	"+water	0.044	0.035	0.023
"+CH3CO2Na	0.038	0.025	"+bisulfide	0.008	0.001	0.001

SAMPLE 8						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.000	0.000	0.000
"+KCl	0.046	0.010	"+water	0.039	0.034	0.023
"+CH3CO2Na	0.032	0.019	"+bisulfide	0.007	0.000	0.000

SAMPLE 9						
Abs	520	700	Abs	420	520	700
Water	0.001	0.001	Water	0.001	0.001	0.001
"+KCl	0.041	0.012	"+water	0.058	0.047	0.028
"+CH3CO2Na	0.032	0.021	"+bisulfide	0.010	0.006	0.002

SAMPLE 10						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.001	0.001	0.000
"+KCl	0.936	0.525	"+water	0.973	0.770	0.469
"+CH3CO2Na	0.868	0.526	"+bisulfide	0.925	0.724	0.446

SAMPLE 11						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.000	0.001	0.000
"+KCl	1.087	0.642	"+water	1.384	0.992	0.584
"+CH3CO2Na	1.079	0.654	"+bisulfide	1.327	0.946	0.554

SAMPLE 12						
Abs	520	700	Abs	420	520	700
Water	0.000	0.000	Water	0.000	0.000	0.000
"+KCl	0.249	0.109	"+water	0.278	0.172	0.082
"+CH3CO2Na	0.195	0.093	"+bisulfide	0.272	0.161	0.077

SAMPLE 13						
Abs	520	700	Abs	420	520	700
Water	0.001	0.001	Water	0.001	0.001	0.000
"+KCl	0.139	0.058	"+water	0.160	0.099	0.046
"+CH3CO2Na	0.106	0.048	"+bisulfide	0.156	0.090	0.033

SAMPLE 14						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.001	0.000	0.000
"+KCl	0.040	0.013	"+water	0.047	0.045	0.008
"+CH3CO2Na	0.029	0.003	"+bisulfide	0.026	0.016	-0.006

SAMPLE 15						
Abs	520	700	Abs	420	520	700
Water	0.000	0.001	Water	0.001	0.000	0.000
"+KCl	0.030	0.004	"+water	0.037	0.037	0.005
"+CH3CO2Na	0.023	-0.002	"+bisulfide	0.020	0.012	-0.002

SAMPLE 16						
Abs	520	700	Abs	420	520	700
Water	0.001	0.000	Water	0.000	0.000	0.001
"+KCl	0.025	0.002	"+water	0.030	0.030	0.005
"+CH3CO2Na	0.023	0.000	"+bisulfide	0.016	0.009	-0.009