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OPTIMIZATION OF EXTRACTION OF OIL FROM MULBERRY (*MORUS ALBA* L.) SEEDS

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TFM REALIZADO EN PROGRAMA DE INTERCAMBIO

TÍTULO: Optimization of extraction of oil from mulberry (*Morus Alba* L.) seeds

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RESUMEN Y PALABRAS CLAVE

Este trabajo propone la optimización de distintos métodos de extracción para la obtención de aceite de las semillas de moras *Morus Alba* L. El objetivo es estudiar con qué condiciones se obtiene un rendimiento más alto, siendo los métodos empleados los siguientes: extracción Soxhlet (SE), extracción asistida por ultrasonidos (UAE), y extracción con fluidos supercríticos (SFE).

Para la extracción Soxhlet, el máximo rendimiento fue 59.88±3.20% trabajando con 96% de etanol como disolvente y semillas molidas como materia prima.

Para la UAE, las condiciones óptimas son un tiempo de extracción de 34 minutos utilizando semillas molidas y aplicando un 100% de amplitud y pulso, consiguiendo un rendimiento del 39.99%.

Finalmente, para la SFE el mejor resultado fue un rendimiento de 20.22% al usar la mayor presión (450 bar) y temperatura (80 ºC).

El estudio se completa con una evaluación estadística de los resultados.

Palabras clave: *Morus Alba* L., aceite, extracción Soxhlet (SE), extracción asistida por ultrasonidos (UAE), extracción con fluidos supercríticos (SFE).

ABSTRACT AND KEYWORDS

In this Project, an optimization of different extraction methods for obtaining oil from *Morus Alba* L. mulberry seeds is proposed. The aim is to study the conditions with which the highest yield is obtained, being the methods used the followings: Soxhlet extraction (SE), ultrasonic assisted extraction (UAE), and supercritical fluid extraction (SFE).

For the Soxhlet extraction, the maximum yield was 59.88±3.20% working with 96% ethanol as solvent and ground seeds as raw material.

For the UAE, the optimum conditions are an extraction time of 34 minutes using ground seeds and applying a 100% of amplitude and pulse, achieving a yield of 39.99%.

Finally, for the SFE the best results was a yield of 20.22% using the highest pressure (450 bar) and temperature (80 ºC).

The study is completed with a statistical evaluation of the results.

Key words: *Morus Alba* L., oil, Soxhlet extraction (SE), ultrasonic assisted extraction (UAE), supercritical fluid extraction (SFE).

1. INTRODUCTION

In this project, different extraction methods for obtaining oils from a type of mulberry seeds, *Morus Alba L*. seeds, are studied.

This novel raw material is a waste obtained during the mulberry juice manufacturing process. In this process of food industry, seeds and peel are obtained as waste, and they can be sent to a biorefinery in order to take advantage of the high added value products they contain instead of using them as animal feed. Between the different types of mulberry, *Morus Alba* L. contains the highest total fat content. The oil extracted from it contains different fatty acids and other components which gives to this material important properties, like antioxidant properties. These properties are associated with some potential pharmacological health benefits and biological activities. Because of that, mulberry can be used for several applications, being the main one in the medicinal field. For this reason, the main target of this project is to study different methods for extracting the oils contained in mulberry seeds.

The three extraction methods are studied are: ultrasonic assisted extraction (UAE), Soxhlet extraction, and supercritical fluid extraction (SFE). For each one, different operation conditions are going to be tried in some experiments in order to know with which conditions a high yield is obtained and to study the influence of these parameters in the yield. In this way, the process would be optimized to get the maximum amount of extract.

2. OBJECTIVES

The global objective of the project is the **optimization of extraction methods of oils from mulberry seeds** for its application in the field of medicine or pharmaceutical industry.

The partial objectives of the project are the following:

1. **Research the available bibliography** in order to know the raw material to be used as well as its characteristics, composition and applications.

    1.1. In the supercritical fluid extraction case, the behaviour of the process will be estimated with a proposed non-linear model and it will be compared with the experimental results.

2. **Description of the extraction methods** it is going to work with.

3. **Experimental work**:

    3.1. Determine the **moisture content** of the un-ground and ground mulberry seeds.

    3.2. **Ultrasonic assisted extraction (UAE)**. The extraction of un-ground and ground raw material is studied. Firstly, the extraction time is determined. The effects of changeable variables of UAE, which are pulse and amplitude on the overall extraction yield is studied using a design of experiment.

    3.3. **Soxhlet extraction**. Traditional solvent extraction is studied in laboratory scale apparatus. Experiments are carried out with ground and unground raw material and using two different solvents (n-pentane and 96% ethanol).

    3.4. **Supercritical fluid extraction (SFE)**. The effects of extraction parameters (extraction temperature and pressure) on the yield are studied in a pilot plant high pressure apparatus. The overall extraction curves are estimated with a non-linear model and are compared with the experimental data.

3. BACKGROUND

3.1. MULBERRY

The genus *Morus* is the type genus of the family *Moraceae* [1]. It has origin in Asia and it has adapted over time to be able to grow in a wide range of climatic, topographic and soil conditions such as tropical and subtropical areas, and temperate zones of the Northern hemisphere (Asia, Europe, North and South America, and Africa) [2].

Mulberry is a deciduous tree with an average height of 10–13 m [2] that lives 200–300 years [3]. It is variable in form, including drooping and pyramidal shapes and whose leaves are from 10 to 20 cm long. Mulberries can be grown from seed or from large cuttings, being the better way the first one. The plant yields edible and sweet fruits that are 2–3 cm long after they have matured. These fruits present high levels of bioactive compounds, hence it has a very important role in the food industry [2].

There are other parts with important components apart from the fruit, like leaves or seeds. Mulberry is a multiple fruit that each ovary has a single seed inside [4]. These seeds can be obtained as by-products of fruit juice processing and wine production [5, 6] and they are a good source of essential fatty acids and functional foods with important phytochemicals that can reduce the risk of degenerative diseases [5]. In addition, seed residues remaining after oil extraction have a growing interest as valuable sources of dietary antioxidants [5, 7].

3.1.1. Types

There are several species of mulberry plant. The most common are white mulberry (*Morus alba* L.), red mulberry (*Morus rubra* L.) and black mulberry (*Morus nigra* L.) [1, 7, 8]. Their main characteristics are the following [2, 9, 10]:

- *Morus alba* L. (white mulberry)

  This kind of mulberry can produce white fruits with a very sweet taste and low acidity. These fruits are perishable, and they are mostly used for fresh consumption.

  It has the highest total fat content between the three types [7]. This is the reason why it is a good raw material for extracting oils.

- *Morus rubra* L. (red mulberry)

  It is a rapid-growing tree of valleys, flood plains, and low moist hillsides. Their flowers appear in April and May and reach full development from June to August. The valuation of this tree is due to its abundant fruits, which are eaten by people, birds, and small mammals [11].

  Regarding his physical appearance, it is a deciduous tree with a 10-15 m of height. The leaves have a length of 7-14 cm and a width of 6-12 cm, and the fruits have a 2-3 cm of length [12].

  This specie is high in dry matter and as well as *Morus alba L*. has a sweet taste and low acidity. Their fruits use to be eaten fresh and they are good to elaborate marmalades, juices, liquors, natura dyes and in the cosmetic industry [7].

Figure 1. Morus rubra (red mulberry) [12].

- *Morus nigra* L. (black mulberry)

  This plant has a small to medium size reaching nine meters in height. It has juicy fruits with extraordinary dark purple-black colour and a unique and slightly acidic flavour. The fruit can be eaten directly or elaborating jam or juice.

  Their leaves have 10-12 cm of length and 6-10 cm of width and their fruit has 2-3 cm of length [13].

It has the highest antioxidant content [14], having the highest total phenolic and flavonoid contents between the three types [7].

Figure 2. Morus nigra (black mulberry) [13].

Among them, *Morus alba* L. is the dominant [1].

White mulberry has a height of approximately 25 meters with round leaves in which the lower surface only has scattered short hairs on the major veins. Fruits are short with a cylindric or elliptic form and a long/width relation of 1.5-3 [15].

Figure 3. Morus alba L. (white mulberry) [65].

White mulberry has the highest total sugars content compared to black and red ones; therefore, white mulberries can be important as raw materials in processing technology. In addition, *M. alba* L. has the highest total fat content with an 1,1% [15].

3.1.2. Composition

A great diversity of compounds has been found in mulberry fruit depending on the cultivars and maturity stages. Mulberry plant has different components like fatty acids, amino acids, vitamins, minerals, and bioactive compounds. Among the bioactive compounds are anthocyanins, rutin, quercetin, chlorogenic acid, and polysaccharides, according to several studies [8]. Other studies determined that the extract of mulberry contain phenolic compounds as flavonoids and anthocyanins [2]. Depend on the stage of maturity the components appear in different amounts.

Morus alba L. is the first stage of maturity and its composition is going to be explained in the following paragraphs.

Starting with the fruit, mulberry fruits have high water content (71.5 %), therefore they are difficult to preserve, being the solid part (dry material) a 29.5 %. The soluble solids represent a 20.4%. Also, the fruit has a 0.25% of acidity and its pH is 5.6. Due to its high content of soluble solids and soluble solids and dry weight, *Morus alba* L. fruits are recommended for processing [7].

Seeds correspond to the solid part of the fruit. They are formed by nutrients and trace elements.

The amount of the different nutrients is shown in the following table (Table 1):

Table 1. Nutrient content of white mulberry [5, 16, 17].

Content (g)/100 g seeds	*Oil*	*Phenolics*	*Protein*	*Ash*	*Carbohydrates*
	21-29.36	137.1±0.36 mg	21.58±0.13	4.89±0.13	54.76±2.42

Trace elements are minerals like N, P, K, Ca, Mg, Na, Fe, Cu, Mn and Zn which have essential activities in the body. The proportion of the most important minerals is presented below:

Table 2. Trace elements in Morus alba L. seeds [7, 17].

Content (mg)/100 g seeds	*Ca*	*P*	*K*	*Mg*	*Na*	*Fe*
	821.0± 24.58	288.67± 7.51	288.5± 10.61	243.33± 11.59	35.3± 0.46	11.97± 0.61

Finally, the oil contained in the seeds has different fatty acids and tocopherols as well as other components present in minor amount:

Table 3. Fatty acid composition of Morus alba L. seed oil [5, 17].

Acid *Content (%)*	*Linoleic*	*Oleic*	*Palmitic*	*Stearic*
	80.56-81.4	5.75-7.11	7.96-8.4	3.45-3.52

Table 4. Tocopherols in Morus alba L. seed oil [17].

Content (mg)/kg oil	*δ-tocopherol*	*γ-tocopherol*	*β-tocopherol*	*α-tocopherol*
	257.67±4.51	18.23±0.11	6.71±0.13	285.84±4.8

Due to this variety of components, *Morus alba L.* seeds are a good raw material to get important benefits for several applications.

3.1.3. Properties

Mulberry presents important properties due to the compounds that this plant contents. It is a very important resource for its phytochemical composition (as organic acids, monoterpenes and polyphenolic compounds), nutritional value, and antioxidant properties, which gives it quality [2].

As it has been seen in the previous chapter, polyphenols are present in the fruit. They are water-soluble bioactive ingredients that are associated with several potential pharmacological health benefits including anti-cholesterol, anti-obesity, hepatoprotective effects, and anti-diabetic and antioxidative effects [10].
Furthermore, the extracts and active components of mulberry fruit have demonstrated numerous biological activities, including antioxidant, neuroprotective, antiatherosclerosis, immune-modulative, antitumor, antihyperglycemic, hypolipidemic activities, anti-obesity effects, as well as the prevention of cardiovascular diseases [8, 9].

Mulberry fruits are low in calories [1, 8, 10] and have a sour taste, providing a more concentrated flavour for fruit production and for eating fresh [10]. For this reason and for the properties that the mulberry fruit has, it is currently used in various ways, and taking advantage of the different parts of the plant, such as the seeds, to achieve their benefits.

3.1.4. Applications

Mulberry trees have been traditionally cultivated because their leaves serve as food for silkworms or as ruminant livestock feed [18]. However currently and especially due to its nutritive value, mulberry fruits are consumed as both fresh and processed products, such as juices, fruit salads, dried fruits [2, 10], beverages, jellies, jams [5], as well as pulp, cakes, teas and food colorant [18]. Therefore, its production and consumption are increasing because of their aromatic taste, nutritional value, bioactive compound content and biological activities [2, 8].

One of the main applications of mulberry plant is in the medicinal field. Mulberry has been used in traditional Oriental medicine to treat diabetes and premature white hair. Its medical parts are the leaf, twig, root bark, and fruit. As an example, it has potential in treating diabetes mellitus, which is a group of metabolic disorders resulting from defects in insulin secretion and/or reduction of sensitivity of the tissues to insulin action [1]. This disease could cause high blood sugar levels over a prolonged period and predisposes patients to many complications, such as severe microvascular complications [19]. Also, in China, Korea, and Japan, mulberry fruit is also used in folk medicine for its pharmacological effects, including fever reduction, treatment of

sore throat, liver and kidney protection, eyesight improvement, and ability to lower blood pressure [8].

Other uses are bald head, hangover, hypertension and inflammation [5] and in the pharmaceutical industry [18].

Specifically, the biological activities of *Morus alba* L. include antioxidant, neuroprotective, antiatherosclerosis, immune-modulative, antitumor, antihyperglycemic, and hypolipidemic activities [8] as it has been indicated before. These biological activities are present due to its active components which are in the extracts of *Morus alba* L.

Nowadays, there is only a few literature about possible uses of the oil extracted from mulberry seeds because it is a waste, but as it has been seen in the composition section, oil from *Morus alba* L. seeds is mainly composed of linoleic acid, so the oil could be used in cosmetic products due to the fatty amides present in the linoleic acid have beneficial properties for the skin [20].

This diversity of important applications is causing the increase in the research of this plant.

3.2. BIOREFINERIES

Biorefinery is a structure which integrates biomass conversion processes and equipment in order to produce fuels, energy and chemical products.

One kind of biomass is one that comes from agro-food waste. Food industries contribute significantly to problems like climate change and environmental degradation or others like the economic inequality [21]. Also, a very important topic to take into account is the food waste due to a high amount of food is wasted along the food supply chain, what is very inefficient [22]. In addition, food waste is the responsible of the 10% of the emissions of greenhouse gases [23]. For this reason, the United Nations has adopted a specific target in the Sustainable Development Goals aiming at halving per capita global food waste at retail and consumer levels and reducing food losses along production and supply chains by 2030 (Target 12.3) [24].

In order to reduce waste and to take advantage of them due to their high level of homogeneity [25], food waste biorefineries are a solution [23] as they use residues as feedstock to produce value-added products [25] and chemicals [26].

In the case of this project, *Morus alba* L. is used to produce juice in a factory. Of this process, seeds and peel are obtained as waste, so they can be sent to a biorefinery in order to use them for getting valuable products. The complete block diagram of the process would be as follows:

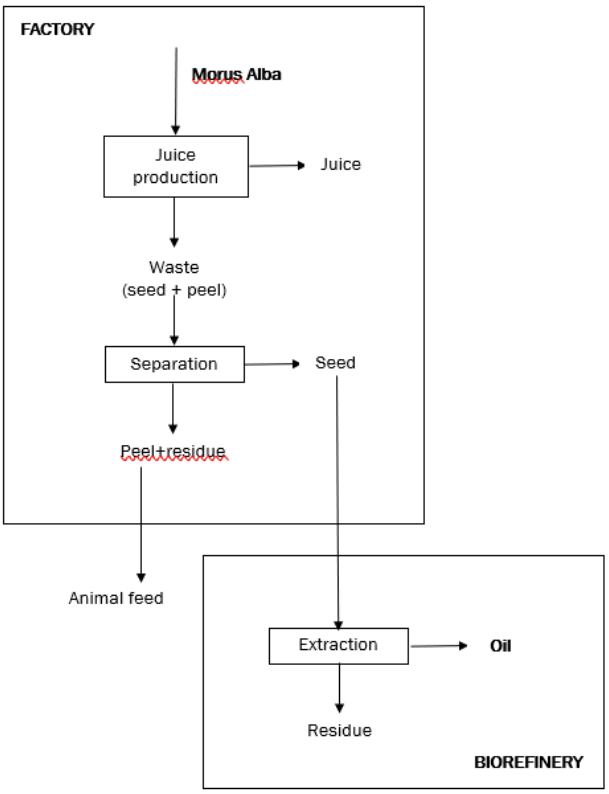

Figure 4. Block diagram of the process to obtain oils from Morus alba L. *(own elaboration).*

Morus alba L. is included in the valorisation of fruit manufacturing. In this project, seeds are used in order to obtain oils rich in bioactive compounds as value-added products [27, 28, 29]. The extraction can be made through different technologies, some of them are conventional oil extractions which involve high temperatures and toxic organic solvents, but others are more environmentally friendly processes such as supercritical fluid extraction with CO_2 [30, 31] or ultrasonic assisted extraction [32, 33] treated in this thesis. There are some investigations using fruit waste like seeds or peels as raw material to obtain oils as product. For example, Ekinci and Guru [30] studied the extraction of oil from peach seeds using supercritical CO_2, Omar and collaborators [32] examined the effect of the supercritical fluid extraction and they focused the ultrasonic method on the extraction of oil and antioxidants using four different citrus peels, Barrales and collaborators [34] examined the extraction of oil from passion fruit seeds and pulp by employing supercritical CO_2 method assisted by ultrasounds, and Cravotto and collaborators [35] investigated different oil extraction techniques on kiwi seeds.

3.3. EXTRACTION METHODS

The extraction is an important step in the recovery of some compounds from the raw material. The extraction process is a phenomenon based on the material transportation of the interest compound from a solid or liquid mixture. This separation is carried out by the different solubilities that the different components of the mixture present in a particular solvent [36].

In this project, the interest compounds are in the *Morus alba* L. seed, so it is a solid matrix. Using a suitable liquid solvent, it is possible to separate these compounds from the matrix for later use. This type of extraction is also called solid-liquid extraction.

There are different methods to carry out this type of extraction. In this thesis three of them are going to be explained. One of them, the Soxhlet extraction, is a more conventional technique, while supercritical fluid extraction and ultrasonic assisted extraction are more advanced techniques.

It is also of interest to name the main parameters that have influence in these processes and in the final yield achieved, like the type of compound to be extracted, type of solvent used, solid/liquid ratio, temperature and pressure, as well as the particle size of the plant material, the moisture content and the residence time.

The pre-treatment step is important because it modifies the structure of the raw material. A high moisture content inhibits the interaction between solute and solvent, reducing the extraction yield. In order to avoid this effect, raw material should have a 10% - 12% moisture content, achieving this result by drying. In addition, high moisture content causes undesirable effects in the final product, like hydrolysis of active compounds or changes in shelf life [37]. Also, the pre-treatment changes the particle size and facilitates the solvent access to the interest compounds in the subsequent extraction stage. When the raw material is broken in smaller particles, the superficial contact area between solvent and solute is increased, so a higher yield should be obtained [38, 39].

Also, a good solvent selection is important. For that, factors like the energy to manufacture the solvent, the cumulative energy demand which is obtained by incinerating or recycling the solvent after use, the impact on health and the environment, and the particular application should be studied [40]. One important point to have into account is the solvent power based on the different polarity between the solvent and the solute. The appropriate solvent is that which can

dissolve the compound of interest but no other compounds. Another point to consider when choosing a solvent is the economic field in which both the price of the solvent and the subsequent stage of its recovery must be taken into account, as well as the availability of the chosen solvent.

Regarding with the flow rate, the solvent flow is important because it affects to the proportion of solute/desired compound between the solvent and matrix. Usually, for continuous processes like the supercritical fluid extraction, a higher flow rate needs shorter extraction time and the extraction yield is higher. In a stirred or static process, like the ultrasonic extraction or Soxhlet extraction, there is no flow rate.

About the extraction part, depending on the technique the parameters are different.

In the solvent extractions, the selection of an appropriate solvent depends on different factors like polarity, melting and boiling point, density, specific gravity, affinity for target component, as well as effect on purity and activity of extracted compound. Another factor to consider is the suitability for the solvent and how the solvent reacts with the desired compounds. In addition, it is important to know the optimum amount of solvent to be used for economic reasons as well as for enhancing extraction outcomes and efficiency.

In the case of ultrasonic assisted extraction, results of some experiments [41] showed that yield increases with an increase in the material porosity, higher solvation, mass transfer due to higher temperatures, and longer extraction times. Added, increased temperature also reduces surface tension and viscosity in extracts, which also enhances extraction yield.

A study for extracting oil from pomegranate seeds [42], a similar raw material to the one used in this project, determined that the optimal conditions are a particle size of 0.2 mm, extraction temperature of 20 ºC, 20/1 solid/liquid ratio, an extraction time between 20 to 30 minutes, and using hexane as solvent. This study shows that increasing the extraction temperature, a decrease in the extraction yield is achieved, because the vapour pressure of the solvent increases when the temperature increases too, and the vapour pressure has influence in the cavitation. Also, with the temperature the surface tension decreases, affecting the bubble formation.

However, in the case of supercritical fluid extraction, there is a study for Argeminean Chia Seed Oil [43] which achieves the highest yield using a mass flow rate of 8 kg/h, 60 ºC, 450 bar, and 138 min. Another study for extracting oil from the mulberry silkworm [44] shows that the optimum conditions are a temperature between 20 and

50 ºC, a pressure between 125 and 225 bar, extraction time from 60 to 180 min and a rate solid/solvent 15-27 g/min, being the optimum conditions a temperature of 45 ºC, pressure of 203 bar, extraction time of 145 min, and a CO_2 flow rate of 24 g/min. With these conditions the total yield achieved is 30.1 g oil / 100 g. This study shows that oil yield increases slowly with an increase in CO_2 flow rate. In the same study the Soxhlet extraction of mulberry silkworm was also studied. The solvent was hexane, and the operation took place at 85 ºC for 90 min. However, the oil yield was 25.82 g/100 g, lower than with the other technique.

3.3.1. Soxhlet extraction

Soxhlet extractor was invented in 1879 by Franz von Soxhlet, a German chemist. The original use of this apparatus was the extraction of a lipid from a solid material. It is an apparatus used in the laboratory for extracting a compound with a limited solubility in a solvent and where the impurity is insoluble in that solvent [45].

This is one of the conventional solid/liquid extraction method which consists in extracting directly from the raw material using a solvent and an extractor.

In this type of extraction, the solid sample is pulverized and placed in a cartridge made of a porous material with cotton on the top. This cartridge is located in the chamber of the Soxhlet extractor. At the same time, the solvent to be used is introduced into a flask and heated, so that the condensing vapours fall on the cartridge containing the sample, extracting the soluble compounds. This technique is easy to perform and does not require any additional filtration step. However, it requires a long extraction time [46, 39] and large amounts of expensive and dangerous solvents, as well as not allowing stirring [46].

In the following figure (Figure 6) a scheme of this equipment is shown:

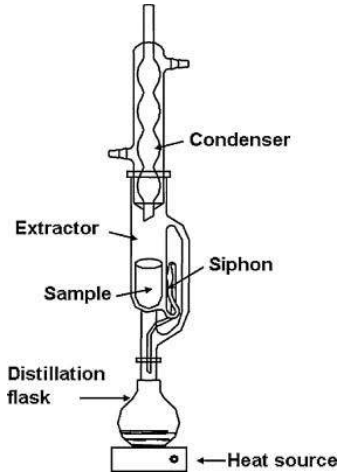

Figure 5. Scheme of a Soxhlet extractor [66]

The operation cycle is the following [47, 39]:

The dry sample is placed inside the cartridge and then paced into the Soxhlet extractor with a siphon. Meanwhile, the solvent located inside the distillation flask is heated until it reaches its boiling point thanks to the heat source. In this moment, the vapour rises up through the neck of the flask, travels along the vapour tube and reaches the condenser. Here, the vapour is condensed and returns to the extractor in liquid form. The solvent drops are collected in the cartridge area and come into contact with the sample. In this moment, the interest compounds are together with the solvent. The extractor collects the solvent with the extract until it reaches a sufficient level to return to the flask through the siphon. The solvent is recycled several times extracting in each cycle a fraction of extract. The desired compound is more concentrated in each cycle and the colour of the solvent in the flask is darker as well due to this reason.

After the extraction, the solvent is removed by evaporation getting only the desired compounds.

3.3.2. Ultrasonic assisted extraction (UAE)

In this technique, mechanical vibrations caused by sound waves with high frequencies are used. The ultrasound waved pass through a medium by creating compression and expansion and forming microbubbles. This effect produces a phenomenon called cavitation, which results in the growth and collapse of bubbles due to the bubbles are unable to keep more energy. The conversion of kinetic energy

of motion causes a temperature and pressure change inside the bubble which produces a large amount of energy useful for chemical reactions. The implosion of cavitation bubbles hits the solid matrix and disintegrates the cells, resulting in the release of the desired compounds. A typical solvent used in this method is ethanol and some of the main advantages of this method is the reduction in the extraction time, a more effective mixing, a faster energy transfer, a reduced extraction temperature, a small equipment, and a high productivity with reduced process steps [48, 46, 49].

This extraction method may be due to several mechanisms or a combination of them [50], which alters the physical and chemical properties of the material [51]:

a) Fragmentation

   Because of the application of ultrasound in the liquid containing the raw material, a rapid fragmentation of the raw material can be observed. This phenomenon is caused by the interparticle collisions and from shockwaves created from collapsing cavitation bubbles in the liquid.

   The consequence of the reduction in particle size is the increase of surface area of the solid, what produces a higher mass transfer and a higher extraction rate and yield.

b) Erosion

   Erosion is caused by the implosion of cavitation bubbles on the particles surface, what induces the erosion of the particle structure releasing the product in the extraction medium.

   Erosion enhances the accessibility of the solvent to the desired compound improving the extraction and solubilization.

c) Capillarity

   This effect causes the increase of depth and velocity of penetration of liquid into canals and pores of the particles under some conditions of sonication.

d) Detexturation

   It is the destruction of plant structures.

e) Sonoporation

   It is useful when a permeability of cell membranes is desired. Ultrasounds form pores in the membrane and it could cause the release of cellular content in the extraction medium.

The scheme of the equipment is the following (Figure 5):

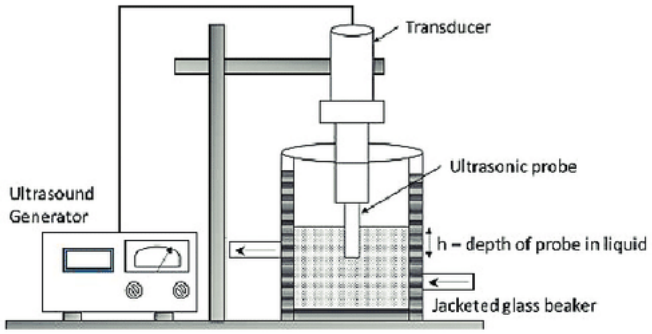

Figure 6. Scheme of ultrasonic assisted extractor [64].

The equipment consists of an ultrasound generator which produces ultrasounds with the amplitude and pulse desired. These ultrasounds are transported to the transducer which causes the ultrasonic probe to vibrate. This causes the movement of the liquid that contains the solid particles with the compounds to be extracted. With the movement the particles collide with each other and disintegrate, extracting the product of interest, which remains dissolved in the solvent. After this operation, a filtration and evaporation are needed on order to obtain the desired products.

The parameters involved in this method are pulse and amplitude. The effect that these parameters have in the yield is the following: the yield increases when the pulse and amplitude are higher because there are more continued collisions in the case of increasing the pulse and the bubbles collapse are more violent in the case of the amplitude since the resonant bubble size is proportional to the amplitude of the ultrasonic wave [41, 50, 51].

3.3.3. Supercritical fluid extraction (SFE)

In this process supercritical fluids (above its critical point [52, 53]) are used to extract the components of interest from a solid matrix, like plants, food by-products and many other feed materials. These supercritical fluids are obtained modifying some conditions like pressure or temperature, and the process is based on the fluid properties, like density, diffusivity, dielectric constant, and viscosity. Under supercritical conditions, the fluid is between gas and liquid because the density of a supercritical fluid is similar to that of liquid while the viscosity is similar to that of gas, having combined properties of gases and liquids. Thus, the supercritical state of a

fluid is the state in which liquid and gas are identical to each other, there is no limit between both phases and there is no surface tension [48, 54]. One of the advantages of supercritical fluids is that these fluids have better transport properties than liquid because the transport properties depend on the density which is adjustable by changing pressure and temperature [48]. Other advantage of supercritical fluid versus organic solvents are that with the first ones a shorter extraction time and a smaller amount of sample is needed, the production of extracts is cleaner, there is a reduction of environmental impact, and they are suitable for extracting thermo-sensitive substances, as well as they have high density, low viscosity, and high diffusivity, so this kind of solvents are effective to dissolve materials and penetrate solid matrices [53].

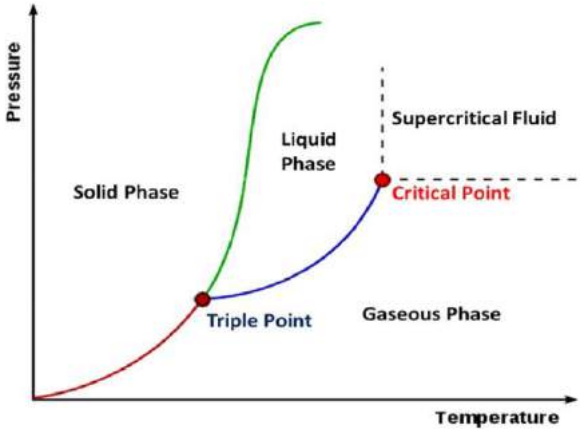

Figure 7. Phase diagram [54].

There are many solvents which can be used in supercritical fluid extraction, like water, ethanol, or methanol. Water is the "greenest solvent", it is available easily and with the purity desired and it can be recycled, it is cheap, non-toxic and non-flammable. Using these solvents higher temperatures are required [55]. Other solvents as ethane and propane have been used because they have a great solvation power which facilitates a higher solubility. The drawback is both solvents are highly flammable and expensive [39]. More possible solvents are propane, n-Butene, n-Pentane, ethane, ethene, nitrous oxide, sulphur hexafluoride, xenon or argon [54, 56]. However, CO_2 is the most commonly one because it is innocuous to human health and to environment and its critical point is at low pressure (7.4 MPa) and at a moderate temperature

(31.1ºC) which is an advantage for the preservation of bioactive compounds in extracts, and the extract does not have contact with air where light oxidation reactions could occur [53, 54, 56]. In addition, CO_2 is inert, non-toxic, non-flammable, non-corrosive, non-explosive and economical [53, 57]. Also, it can be recovered as a by-product of many processes and allows the extraction at low temperature and pressure [57]. It can be easily recycled, it has good solvent characteristics for nonpolar and slightly polar solutes, and it is easily removed from the product by depressurization [53, 54]. Other advantages are that most of the volatile components are present in the extracts, and the ability of supercritical fluid to evaporate non-volatile components, which reduces the energy spent. In terms of solvents, the quality and the purity are superior [54].

The positive point of this method is that the acids are protected by inert atmosphere of CO_2 and the extraction temperature is low, so the quality of the product is superior to that obtained with the use of other techniques [58]. Furthermore, with this method the unique flavour and aroma of the oils extracted are maintained while with other techniques they used to be volatized and lost [39].

This method is used in the food industry for extracting oils among other compounds from several fruits [54]. It is an alternative method for the extraction of low volatile compounds that can be used as nutritional dietary supplements. Specifically, supercritical extraction of oils from seeds has applications in nutraceutical, cosmeceutical, dietary supplement, and animal health products [59]. However, supercritical fluid extraction cannot be widely used due to its high costs [48, 59], the batch-wise extraction, the non-availability of very high pressure plants, and the competing solvent extraction technology [59].

The supercritical fluid extraction operates in two steps [53]: First, the solubilization of the compounds present in the solid matrix takes place when the solvent flows through the packed bed. Then, the separation of the compounds from the solvent by depressurization happens.

During the operation, the plant material is loaded into a tank. This reservoir is pressurized with the fluid using a pump. Subsequently, the fluid with the dissolved compounds passes through some separators where the desired compound can be collected [46]. The extraction takes place when the solid material which contains the desired components contact with the continuous flow of the supercritical solvent. In

that moment, the extract is separated until the solid matrix is empty of this component [60].

A schematic flow diagram of this equipment is represented below (Figure 8):

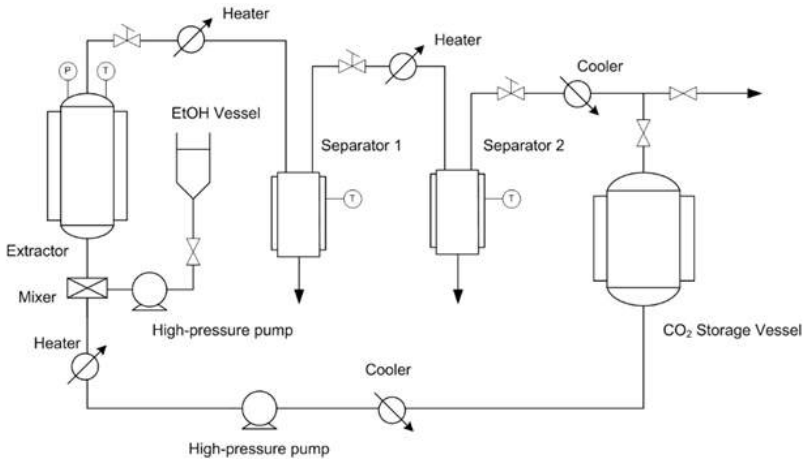

Figure 8.. Scheme of supercritical fluid extraction equipment [62]. 1. CO_2 storage vessel; 2. Cooler; 3. High-pressure pump; 4. Heater; 5. Mixer; 6. Extractor; 7. Separators; 8. Ethyl alcohol vessel.

It is important to know the influence of the pressure and temperature on the yield, due to with small changes in these parameters, large changes in the properties of the extract can be produced. According to some studies [43, 61], the global extraction yield of oil is higher when the pressure increases, while the temperature only has a little influence on it increasinging it slightly.

The explanation of the effect of the temperature on the yield is that when the temperature increases, the solubility of the extract in the supercritical CO_2 also increases because the vapour pressure of the solute increases with the temperature. In addition, a rise in the temperature causes a decrease in the density of CO_2, which leads to an increase in the solvent power to dissolve the solute [39, 44]. So, at higher temperatures, lower pressures and shorter extraction time are required, and vice versa.

The influence of the pressure is because the density of supercritical CO_2 increases proportionally with the pressure, and that controls the power of the solvent and the solubility of extract, since with a higher density, the interaction between molecules and supercritical CO_2 is also higher [39, 44, 61].

There are some mathematical models to describe the extraction of solutes from a solid matrix working with supercritical fluids [62]:

- Empirical models

  The parameters which represent the behaviour of the extractor are obtained by fitting the experimental kinetic curve, so they do not need more information about the material. They are simple models, but they cannot be use for scaling-up since the parameters do not have physical significance.

- Models based on heat transfer analogies

  This kind of models assume that a particle of the raw material is a sphere and for describing the concentration profile inside the particles, the equations of cooling spheres in a uniform medium are used.

- Quasi-steady state shrinking model

  This model considers that the solute is placed inside pores in solid particles and there is a boundary between the oil rich core in the porous particle and the outside region. During the extraction operation, the core shrinks. The model also considers that no adsorption of the oil takes place in the solid matrix. There are two adjustable parameters: the intra particle diffusivity and the external mass transfer coefficient, being the first one used as fitting parameter and the second one calculated.

  It is sometimes used to describe the kinetics of solid and semi-solids extraction with supercritical fluids despite two facts: seed geometries are quite irregular, and the internal walls can affect the diffusion [63].

- Models based on differential mass balances

  These models have physical significance because they consider the characteristics of the plant material, the particle size, and the bed porosity, and they have mass transfer coefficients in fluid and solid phase. They take into account the equilibrium relationships and mass transfer mechanisms. Inside this kind of models, three groups can be differentiated depending on the control step:

  - Models which consider that the external mass transfer controls the extraction.
  - Models which consider that the controlling step is the diffusion inside the particle.

- Models which consider that there is resistance in both the fluid and solid phases, that there is solute-matrix interaction, and a flow like plug flow.

- Mass transfer models [63]

  The supercritical extraction uses to be a semi-batch operation because a fixed bed of solid is mixed with a fluid flow. With this kind of models, the effect of the following variables can be predicted: fluid velocity, temperature, pressure, gravity, particle size, degree of crushing and bed-length. Therefore, they are applied in simulation and design.

  - Desorption with mass transfer

    This model is useful for processes of porous-solid extraction with dense gases.

  - Extraction of solids with high pressure fluid in mixed-flow

    This model works when the solids are stationary in a shallow packed bed and for this reason it can be considered to be in well-mixed conditions. Also, it is considered that the solid is initially saturated by the solute.

It is necessary to know the characteristics of the raw material in order to design or optimize the supercritical fluid extraction process. It is important to know the initial concentration of the solute, the composition of the solid mixture and the humidity and the pre-treatment. But also it is necessary to know the process parameters employed in the operation, like the pressure, temperature and solvent flow rate, and the fluid phase equilibrium data.

In addition, the location of the volatile oil in the particle of the plan material is important for modelling the extraction. The volatile oils can be stored in the internal part, being the intra particle diffusion very important since the supercritical fluid cannot access to the volatile oil, or in the external surface.

As it will be seen in the examples represented in the results section, all the extraction curves have a similar behaviour. The first stage is a straight line, that is because this step is controlled by the equilibrium solubility and shows a quasi-stationary behaviour caused by constant mass transfer resistance [60] depending on the operation parameters. The second stage is an asymptotic curve due to this part of the process is controlled by diffusion in the deeper part of raw material and the extraction velocity decreases quickly because of the difficulties to achieve this part of the plant matrix. Finally, the third stage is a straight line at maximal extraction yield since it is the

slowest step. So, it can be concluded that the extraction velocity is proportional to the concentration of soluble compounds placed in the matrix of the raw material.

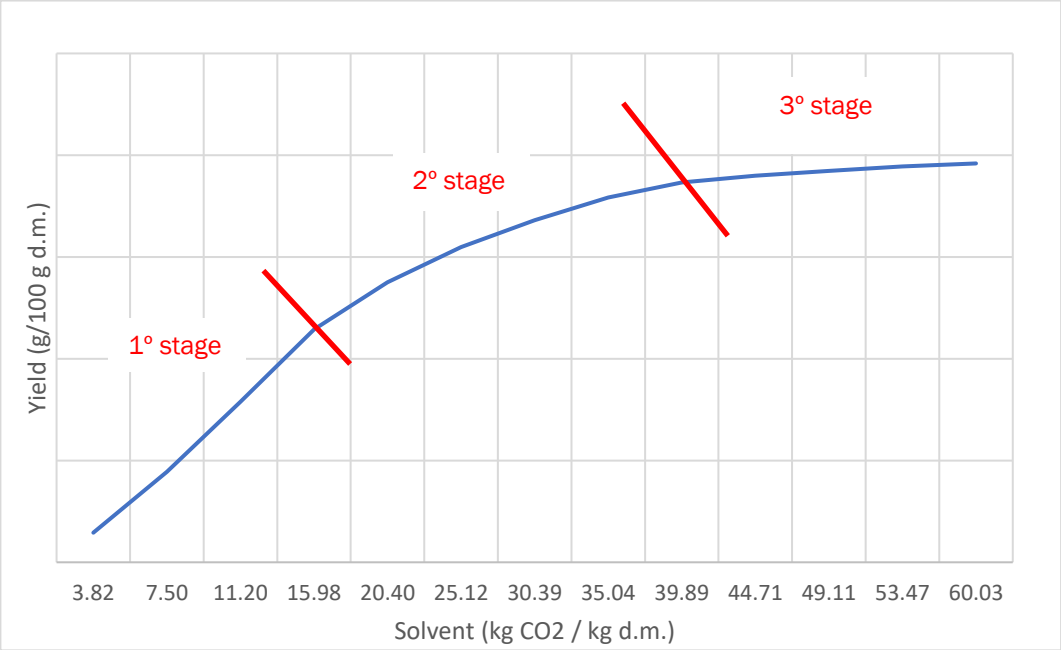

Figure 9. Stages in an extraction curve (own elaboration).

The linear model applied is the following:

$$\frac{dx}{dt} = -kx \quad \textit{Eq. 1}$$

where x is the concentration of soluble compounds in the plant material (kg/kg), t is the extraction time (s), and k is the apparent velocity coefficient (1/s).

Integrating:

$$\frac{x}{x_0} = e^{-kt} \quad \textit{Eq. 2}$$

where x_0 is the concentration of soluble compounds in the plant material at the beginning of the extraction (kg/kg).

In order to obtain the extraction yield, the amount of raw material is used:

$$\frac{m_s \cdot x}{m_s \cdot x_0} = e^{-kt} \quad \textit{Eq. 3}$$

$$\frac{m_s \cdot x_0 - m_s \cdot x}{m_s \cdot x_0} = 1 - e^{-kt} \quad \textit{Eq. 4}$$

The extraction yield is expressed as a fraction of dried raw material. If Eq.4 is expressed in this way:

$$Y = Y_\infty \cdot (1 - e^{-kt}) = x_0 \cdot (1 - e^{-kt}) \quad \textit{Eq. 5}$$

where Y is the extraction yield at t time (kg/kg), Y_∞ is the total possible extraction material (with infinitive extraction time) (kg/kg), k is the velocity coefficient (1/s), and t is the extraction time (s) [63].[54]

Eq.5 will be used in the statistical analysis to compare the experimental results with the theoretical ones obtained applying this equation through a non-linear estimation of the curve and of the parameters Y_∞ and k using the Statistica program.

4. MATERIALS AND METHODS

4.1. RAW MATERIAL

The raw material used in this investigation is white mulberry seeds from a Hungarian food industrial partner (Bio-Drog-Berta Ltd., Kalocsa, Hungary). This industry is a local organic fruit and vegetable farming and processing company. The seeds were washed and dried after fruit juice pressing and separation. The seeds can be treated naturally (unground seeds) or after grinding (ground seeds).

Figure 10. Left: Unground Morus alba L. seeds
Right: Ground Morus alba L. seeds.

The mulberry seeds were ground using a lab scale grinder: Fristcher knife mill with 2 mm sieve plate. After grinding the ground highly scented oily seeds were stored in sealed plastic bag and in fridge (0-7 ºC) until further use. This was needed to avoid the oxidation of oil in the broken seeds. The extraction was studied using un-ground and ground seeds to evaluate the efficiency of extraction.

4.2. CHEMICALS

The chemical solvents and other products that have been used in the different experiments and methods are the following:

- CO_2. supplied by Linde Ltd., Hungary. Purity: 99.5%
- Syringe filter
- Ethanol (C_2H_6O) was supplied by Molar Chemicals Kft, purity: 95.8%.
- n-Pentane (C_5H_{12}) was supplied by Molar Chemicals Kft, purity: 98.7%.
- Distilled water was obtained from a distillation apparatus in the DCS laboratory of BME University.

4.3. METHODS

4.3.1. Moisture content determination

The moisture content of unground seeds was measured as following. Three parallel measurements were carried out in which 7-9 grams of seeds were measured in three Petri dishes (M_b). Then they were dried in the oven at 105 ºC until mass consistency for two days and after that the dishes were weighed again (M_a). The moisture content is the difference between both measurements ($M_c = M_b - M_a$). In order to calculate the moisture percentage, firstly the dry content (M_d) should be known with the following equation:

$$\%M_d = \frac{M_c}{M_b} * 100 \quad \textit{Eq. 6}$$

Then, the moisture content is:

$$\%M_c = 100 - \%M_d \quad \textit{Eq. 7}$$

The moisture content of ground seeds was measured previously in another study in the same way.

4.3.2. Soxhlet extraction

The operation of the Soxhlet extraction has been explained in detail in the previous section. For this operation, an approximate amount of 20 g was measured into the paper thimble placed inside the chamber. Also 240 ml of solvent was introduced in the solvent flask. Two solvents with different polarities were applied: n-pentane and 96% ethanol. The Soxhlet apparatus was connected to a cooler and the round bottom flask containing the solvent was immersed in silicon oil. First the cooling water of condenser was turned on, then the heating element of silicon bath was switched on for the solvent is heated, and the extraction can start. The extraction run until the solvent in the Soxhlet apparatus became transparent again, which showed the end of extraction. It took around 3 days (18 hours more or less) in the case of ground raw material and 4 days (24 hours) for the unground raw material with n-pentane, and 4

days (24 hours) for ground material and 5 days (30 hours) for unground material with 96% ethanol extraction.

Three parallel Soxhlet extraction were carried out for each raw material and solvent and the average yield was calculated.

Figure 11. Soxhlet extraction plant.

After the Soxhlet extraction was done, the evaporation of the liquid is necessary in order to get the extract without solvent. For this, each flask had to be evaporated in the rotary evaporator using a vacuum pump and a control valve.

Figure 12. Rotary evaporator.

After this step only the extract obtained as a product remains in the flask, which is collected in a sample bottle. The extracted material was dried and discarded. The solvent evaporated and condensed is collected in another flask and returned to a bottle for reuse.

Figure 13. Left: Extract after extraction.
Right: Extract inside the sample bottle.

For calculating the yield, the mass of the dry raw material and the mass of the extract must be known. The mass of dry raw material was calculated by using the dry content of raw material, and the weighted mass of seed. The mass of extract was known from the mass of empty flask and the mass of flask with the extract. The yield was calculated with the following equation:

$$Yield(\%) = \frac{m\ ext\ (g)}{m\ dry\ raw\ mat.(g)} * 100 \qquad \textit{Eq. 8}$$

The standard deviation of each measurement was calculated as three parallel measurements were done in the same time.

4.3.3. Ultrasonic assisted extraction

For the ultrasonic assisted extraction, 96% ethanol was going to be used with a proportion of 10 g raw material in 150 ml solvent.

Hielscher UP200St (Germany) apparatus was used, which is composed of an ultrasound generator, a transducer, and an ultrasonic probe. The amplitude and the pulse can be modified in the generator increasing or decreasing it in percentage.

The aim of the experiments was to test the effect of amplitude and pulse on the extraction yield through an experimental design. For that, a 3^2 full factorial experimental design with three repeated measurements in the centre of design was

set in which the variables were the pulse and amplitude of UAE apparatus. The significance level was set at 95%.

During the measurements, some continuous sampling was required by 3-3 minutes in order to know the concentrations at different times of the experiment and thus represent the concentration curve versus time for each experiment and for comparing them for studying the effect of process parameters (amplitude and pulse).

In all experiments the same 400 mL of flask was used to overcome the dimensional changes of liquid and probe position, which might influence the efficiency of extraction.

Firstly, the raw material and the solvent was mixed.

Figure 14. Raw material.

Then the ultrasonic operation can start. Parameters were set on the apparatus, water bath was set at required temperature and extraction was started. The time of extraction was first optimized then that time was used during the experiments. The temperature of water bath was monitored with a thermometer, and when the temperature of extraction solution started to increase above 1-2 ºC of set temperature, the water bath was changed to cold tap water or ice was added to the bath.

Figure 15. Ultrasonic equipment.

Each three or five minutes, the machine was stopped, and the mixture was allowed to stand for two minutes in order that the particles can settle to the bottom. After that, 5 mL of sample was taken with a syringe and deposited into a test tube through a filter.

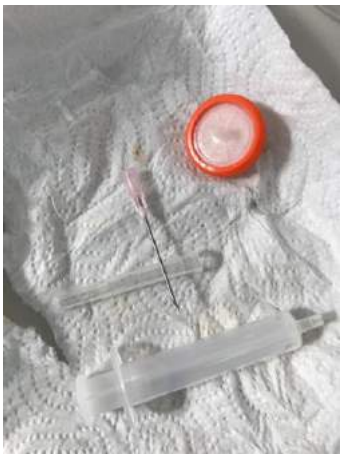

Figure 16. Left: Syringe and filter.
Right: Glass tubes with samples..

When the operation finished, the sample tubes were placed in a stripping apparatus (Figure 15) for two and a half hours with an air flow varying between 1 and 3 L/min and with the temperature of water bath was set to 45-50 ºC. After that time, the solvent evaporated and only the extracted oil remained in the sample tubes.

Figure 17. Stripping

Meanwhile, the rest of the extraction mixture is filtered under vacuum with a Büchner funnel in order to separate the liquid from the solid residue.

Figure 18. Liquid and solid parts.
Right: Filtration

The solid residue was put in the oven at 105 °C for several days to remove the solvent residue it contained.

Figure 19. Residue.

The liquid is placed on a rotary evaporator to evaporate the solvent and to obtain the extract.

Figure 20. Rotary evaporator.

Finally, the oil of the sample tubes and the extract were weighted in order to calculate concentrations at each sampling steps and the extraction yield. Then the extract was collected in a sample bottle.

Figure 21. Left: Oil after evaporitation.
Right: Oil inside the sample bottle.

This experiment was carried out with different durations to determine the optimal extraction time as well as the yields.

In addition, experiments were carried out with the ground seeds and the unground seeds to verify the effectiveness of extraction.

Finally, with the duration and type of grain selected, an experimental design matrix a 3^2 a full factorial experimental design was created to investigate the effects of different amplitudes (%) and pulses (%) for the ultrasound operation on the extraction yield.

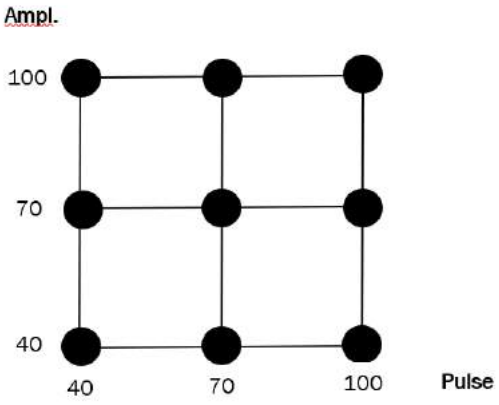

Figure 22. Matrix of experiments (own elaboration).

An experiment is carried out at each of these points, except in the middle point where three experiments were made to estimate the standard deviation of the measurements. The amplitude was set between 40-100% of maximum amplitude which is 175 μm of this apparatus, while the pulses were set between 40-100%. It means that the duty cycle between pause and ultrasonic action will be between 40-100%, which means that 40% the ultrasonic action takes places for 0.4 seconds, while pausing for 0.6 seconds. At 100% setting, there is no pause cycle, only constant ultrasonic action.

Once the operation started, the power and temperature were noted every minute in order to keep a control of them. The samples were taken every 3 minutes in the beginning of the process and every 5 minutes after and they were filtered inside the sample tubes. Then, after the solvent of these tubes were evaporated using the stripping apparatus, they were weight for knowing the exact amount of extract in each tube. With the mass difference between the empty tube and the tube with the extract and the volume of the sample taken, the concentration could be calculated and the extraction curve could be represented.

$$Conc\left(\frac{g}{ml}\right) = \frac{\Delta m}{Vsample} \qquad Eq.\ 9$$

In order to calculate the total yield, the total mass of the extract had to be known. The final amount of extract is form by the mass of extract inside the glass after the evaporation of the liquid and extract inside the sample tubes extracted as samples. Knowing this and the dry raw material used for the experiment, the yield could be calculated with the following equation (Eq. 9):

$$Yield\ (\%) = \frac{\Sigma m\ ext\ (g)}{m\ dry\ raw\ material\ (g)} x100 \qquad Eq.\ 10$$

Finally, the mass balance error could be estimated. The mass balance says that the raw material that enters to the process has to be the same as that obtained at the end. The material at the end is the one obtained as extract plus the one obtained as dry residue:

$$m\ in\ (g) = m\ out\ (g) = m\ ext\ (g) + m\ residue\ (g) \qquad Eq.\ 11$$

And the error in the balance is calculated as follows (Eq. 11):

$$\%\ error = \frac{m\ in\ (g) - m\ out\ (g)}{m\ in\ (g)} x100 \quad \textit{Eq. 12}$$

4.3.4. Supercritical fluid extraction

In this method of operation, the supercritical extraction unit is used, which located at the Department of Environment of BME. It is a pilot scale apparatus with a 5 L extractor vessel and two separators connected in series. It can operate at maximum of 500 bar and 120 ºC. This unit works with CO2 as supercritical fluid in order to extract compounds from the raw material.

Figure 23. SFE plant.

Around 1 kg of raw material was placed in the extractor. Then the pressure and temperature were set up and a bypass is established in the unit until operating conditions are reached. The CO_2 is compressed using a pump until the required pressure, and temperature is adjusted with a heating system since it is necessary to achieve the supercritical state. Also, the refrigerant fluid is cooled in the cooler. It is needed to cool down the CO_2 to became liquid, so the high-pressure liquid pump can carry it as a liquid solvent. After that, the process can start. The supercritical CO_2 passes through the extractor. In this work the supercritical CO_2 extraction of mulberry seeds were studied at three different pressures and temperatures for the extractor:

300, 375 and 450 bar, and 45, 60 and 80 ºC. There the extraction takes place and after it the solvent and the oil leave the vessel and after reducing their pressure go to the first separator. The pressure of first separator set at 40 bar and heated between 30-50 ºC. Here the oil precipitates to the bottom of the vessel and the gaseous CO_2 continues to the 2nd separator. The second separator is used as a buffer and to collect any extract which might have been carried over. The pressure of 2nd separator was set at 20 bar and operated at 30-40 ºC. -From here the CO_2 was not recycled and released to the air. Each more or less 10 minutes all the variables were observed and noted in a table like this (Table 5):

Table 5. Data noted in the SFE experiments.

time	Pressure (bar)					Text	CO_2 flow				m^3	Tª	Tª
	CO_2 bottle	CO_2 tank	Extrac.	Sep.1	Sep.2	(ºC)	kg/h	Tª	Density	Σm	gas	sep.1	sep.2

It is important to control the CO_2 flow rate (7 kg/h approximately) and the pressure of both separators (40 bar for separator 1 and 20 bar for separator 2). The samples are taken after 3 or 4 kg of CO_2 pass along the circuit. These samples are heated, and the oil extracted is weight in order to know the yield in the different points of time. The total time of the process is between 200 and 400 min, depending on the amount of extract achieved in each sample.

The extraction curve represents the yield in the Y axis and the solvent (kg CO_2 / kg d.m.) in the X axis. The kg CO_2 is the cumulative sum of kg of CO_2 (Σm in Table 5) while the dry material (d.m.) is the initial raw material without considering the moisture content.

In order to know the yield, firstly the mass of the extract was calculated. This extract is inside both separators, so the exact amount is the difference between the weight of the cartridge with the extract and the empty cartridge. Then the cumulative mass of extract was calculated. With this information the yield of each separator was obtained, and the total yield is the sum of both of them:

$$Yield\ (\%) = \frac{\Sigma m\ sep.(g)}{m\ d.m.(g)}\ x\ 100 \qquad \textit{Eq. 13}$$

5. RESULTS, DISCUSSION AND STATISTICAL EVALUATION

5.1. RESULTS OF MOISTURE CONTENT

The moisture content of ground seed was previously measured in another study and its results was 11.22 ± 0.48%.

In the case of the unground seed and following the procedure explained previously in Chapter 4.3., the results are showed in the following table (Table 6):

Table 6. Results of moisture content of unground seeds.

	1	2	3
m dish (g)	54.43	50.87	60.96
m mat (g)	8.16	7.59	8.55
m dish+mat (g)	62.59	58.46	69.51
m dish+dry mat (g)	61.83	57.74	68.73
m dry mat (g)	7.4	6.87	7.77
m moisture (g)	0.76	0.72	0.78
% moisture	9.31	9.49	9.12

The average moisture content of un-ground mulberry seed was 9.31 ± 0.18%.

At it can be seen, that the ground seed contained more moisture than that of unground seed. It can be explained by their differences in storage. The ground seed was stored in refrigerator to protect the seed from the oxidation of oil, while the unground seed was stored in a dark, cool storage area.

5.2. RESULTS OF EXTRACTION METHODS

5.2.1. Soxhlet extraction

The Soxhlet extraction was studied in laboratory scale apparatus using two different polarities solvents, the nonpolar n-pentane and polar 96% ethanol. The extractions of ground and non-ground mulberry seeds were also studied. Three-three parallel measurements were carried out. The results of the yields are shown in the following table (Table 11):

Table 7. Average yields achieve with the Soxhlet extraction.

SOLVENT	AV. YIELDS GROUND SEEDS (g/ 100 g d.m. %)	AV. YIELDS UNGROUND SEEDS (g/ 100 g d.m. %)
96% EtOH	59.88 ± 3.20	38.89 ± 0.60
n-Pentane	24.29 ± 0.23	5.00 ± 0.20

Comparing these results in a graph is as follows:

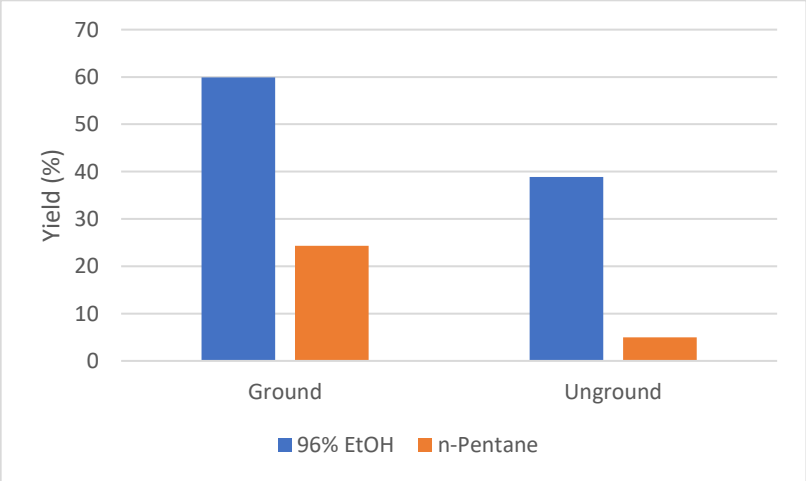

Figure 24. Representation of the yields achieve with the Soxhlet extraction.

It can be concluded that with 96% ethanol the extraction yields were higher using both ground and unground seeds. This polar solvent has stronger solvent power and capable to solve more compounds from the seed. By apolar n-pentane and ground seeds around 60% less extraction yields were achieved, resulting in only clear yellow oil. With ethanol dark brown sticky oil was obtained both with nice, sweet mulberry scent. In the case of un-ground seeds, the yield using n-pentane is around 85% less than with the other solvent. It can be also seen that grinding increased the

effectiveness of extraction: higher yields were obtained from ground seed using both solvents. The physical characters of each extracts were the same whether the extraction was done from ground or unground materials.

5.2.2. Ultrasonic assisted extraction

5.2.2.1. Evaluation of extraction time

Firstly, the optimum extraction time was studied by carrying out several experiments with the ground material at 100% amplitude and 100% pulse in order to observe the behaviour of the concentration curves.

The first experiment (MA-UAE1) had a duration of 24 min and as it can be seen in Figure 24, the concentration of solubles were constantly increasing indicating that the extraction was not yet fully. For this reason, the extraction time of the following experiments was lengthened up to 34 min. The experiments of MA-UAE3, MA-UAE4 and MA-UAE19 were three parallel measurements resulting in similar yield and showing similar extraction curves.

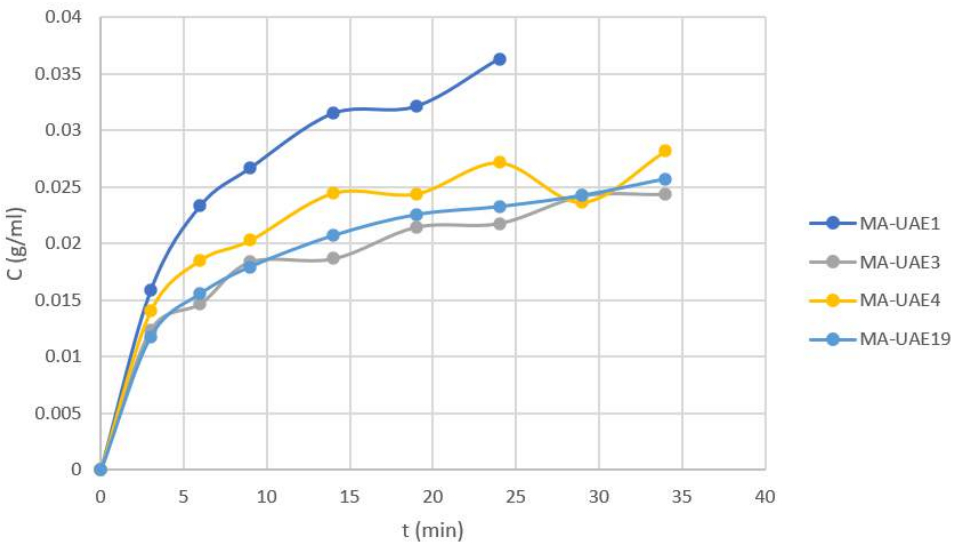

Figure 25. Experiments at 100% amplitude and 100% pulse with ground material.

In Table 7 the overall extraction yields of the three parallel measurement of UAE mulberry seed can be seen.

Table 8. Overall yields of experiments at 100% amplitude and 100% pulse with ground material.

EXPERIMENT	YIELD (%)
MA-UAE3	39.33
MA-UAE4	38.63
MA-UAE19	42.01
Average:	39.99±1.78

The average yield obtained was 39.99% with 1.78% of standard deviation, which seems a bit large, which due to the possible error of sampling and the complexity of ethanolic extract of mulberry seed.

5.2.2.2. Comparison of un-ground and ground seed extraction

Further experiments were carried out using un-ground intact seeds and ground seeds to compare the efficiency of extraction of ultrasound assisted extraction of mulberry seed. The amplitude and pulse were set at 100% in these experiments. They run for 34-44 minutes, using 96% ethanol in 1:15 seed to solvent ratio. In Figure 25, the extraction curves of un-ground seeds can be seen.

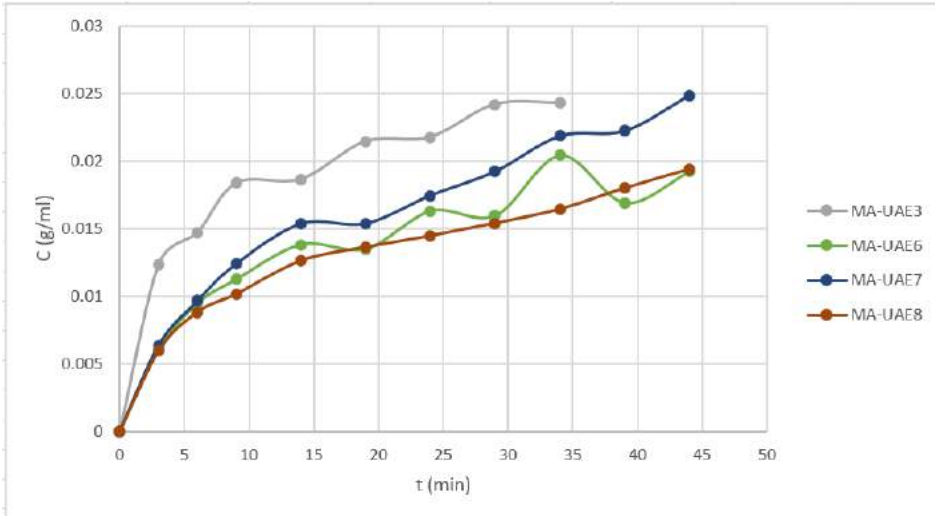

Figure 26. Experiments at 100% amplitude and 100% pulse with unground material.

The first experiment (MA-UAE5) was operated for 34 min, but the overall yield was of 32.65%, lower compared with the ground seed, so the operation time was increased

until 44 min. It can be seen that the slope of extraction curves was constantly increasing even with longer experiments. The extraction is limited as unground seeds were used. Experiments MA-UAE6, 7 and 8 are parallel measurements running at the same conditions (44 min, at 40 °C with 96% ethanol at 1:15 seed to solvent ratio). The overall yield of these three experiments is summarized in Table 8.

Table 9. Yields of experiments at 100% amplitude and 100% pulse with unground material.

EXPERIMENT	YIELD (%)
MA-UAE6	29.72
MA-UAE7	32.71
MA-UAE8	30.58
Average:	31.00±1.54

The average yield obtained was 31.00% with 1.54% of standard deviation, which is quite similar to the one obtained during the extraction of ground seed. The average yield obtained from the unground seeds was almost 10% lower than that obtained from the ground seeds and the extraction time was longer by 10 minutes.

In Figure 26 the average extraction curves of unground (MA-UAE6, MA-UAE7, MA-UAE8) and ground (MA-UAE3, MA-UAE4, MA-UAE19) seeds can be seen. It can be observed that the slope of extraction curve of ground seed is smaller, resulting in a faster extraction in a shorter time.

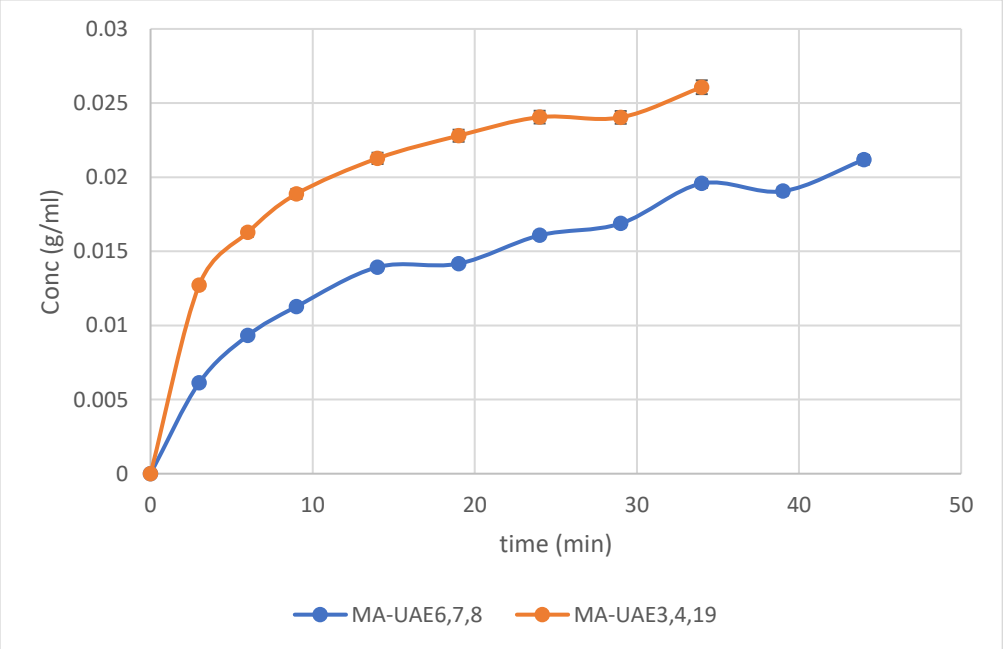

Figure 27. Comparison of extraction curves of ground and unground mulberry seeds.

Comparing the results of ground and unground seeds it can be concluded that the extraction from ground seeds were faster and around 8.99% higher extraction yield could be achieved.

Based on these results, it was decided to use the ground seed in the further optimization study, as using unground seed significantly lower extraction yield can be achieved with longer extraction time.

5.2.2.3. Study the effects of amplitude and pulse of UAE

Finally, the effect of pulsing and amplitude of ultrasound assisted extraction on the extraction yield was investigated using a 3^2 full factorial design with three repeated measurements in the centre of design. The three levels of amplitude were set as the followings: 40, 70, 100 %, while those of the pulse were: 40, 70, m100 %.

During the experiments, the temperature of the extraction was kept constant at 40 ºC and 96% ethanol was used as extraction solvent in 1:15 seed to solvent ratio (w/V). In some cases, where the power output of the sonotrode was rather high (when high amplitudes were combined with high pulse), there beaker was cooled with a water bath.

Regarding to the three repetitions at the central point (70% amplitude and 70% pulse), the measurements are parallel. The extraction curves of these three measurements can be seen in Figure 27.

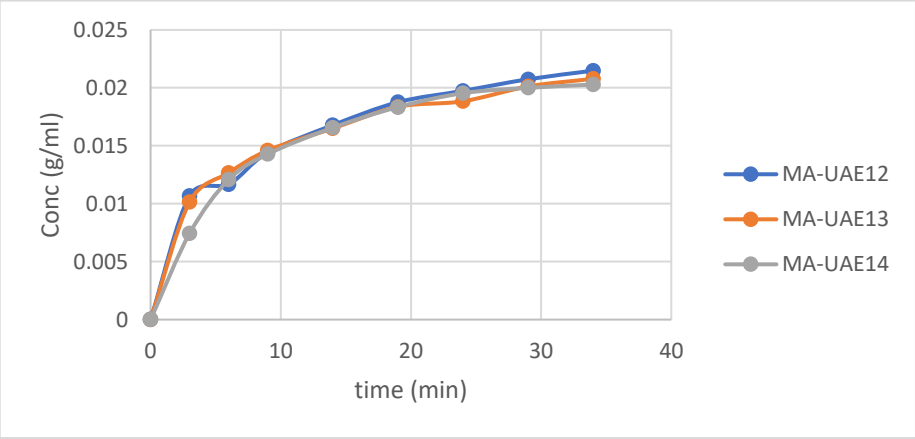

Figure 28. Parallel measurements at 70% amplitude and 70% pulse.

As it can be seen, the extraction curves run very well together, showing only a slight deviation. The overall extraction yields are shown in the following Table 9:

Table 10. Yields of experiments at 70% amplitude and 70% pulse.

EXPERIMENT	YIELD (%)
MA-UAE12	34.51
MA-UAE13	37.79
MA-UAE14	36.95
Average	36.42
SD	1.70

As it can be seen, the standard deviation is very similar to the one of the experiments at 100% amplitude and 100% pulse, which sets this extent of accuracy and repeatability to the measurements.

Keeping the amplitude constant, the effect of pulse can be seen in the following graphs (Figure 28-30).

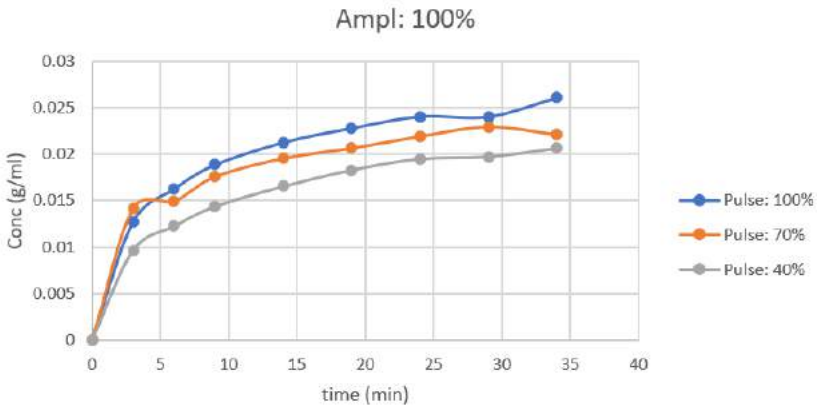

Figure 29. Experiments at 100% of amplitude.

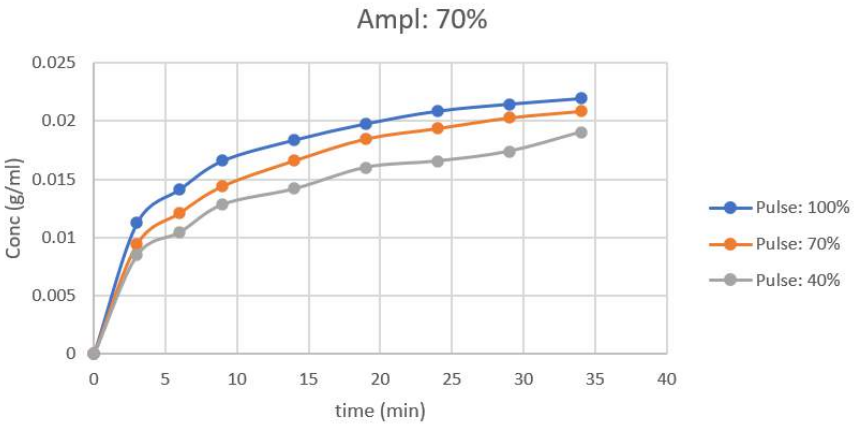

Figure 30. Experiments at 70% of amplitude.

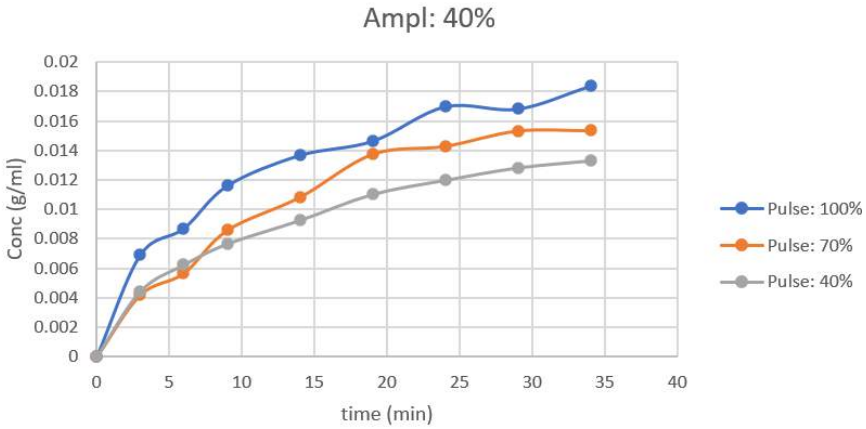

Figure 31. Experiments at 40% of amplitude.

The results obtained at 70 and 100% amplitude show similarities in trends of extraction curves, however at the lowest amplitude setting at 40% the effect of pulse is stronger and much more relevant than in the other two cases. Generally, it can be concluded that in all the cases, the higher the pulse, the higher concentrations are achieved. According to the theory, higher pulses cause more particle collisions and extraction is more effective, realising more desired compound and achieving higher yields.

The extraction curves can be also compared at constant pulse settings in which the effect of amplitude can be observed. The results can be seen in Figure 31-33.

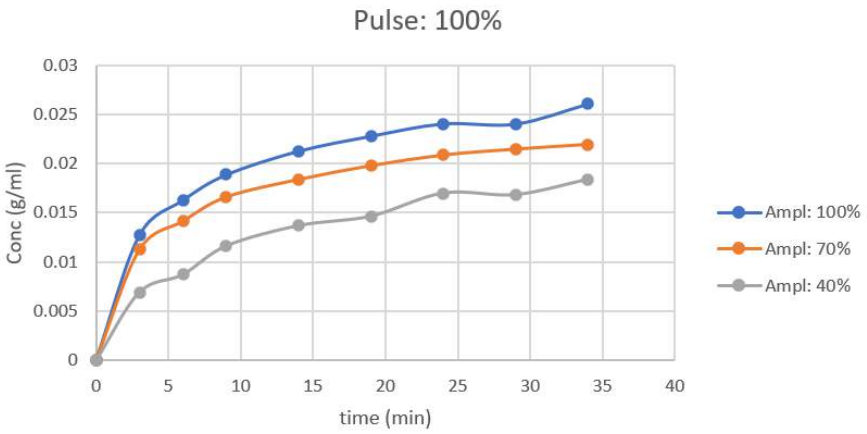

Figure 32. Experiments at 100% of pulsing.

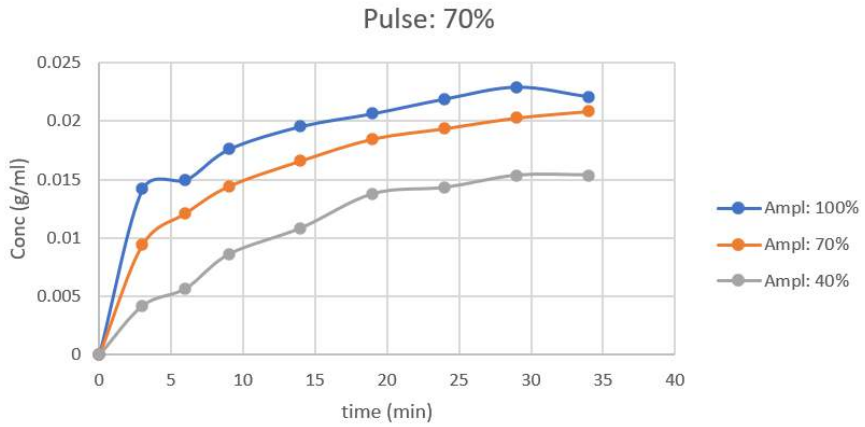

Figure 33. Experiments at 70% of pulsing.

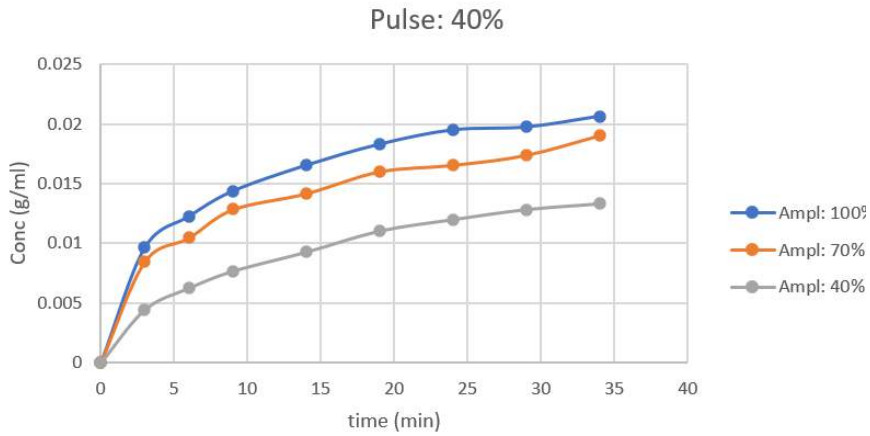

Figure 34. Experiments at 40% of pulsing.

In this case, results obtained with the three different pulses are very similar in trends of extraction curves. The conclusions are the same as in the effect of pulsing, as

higher the amplitude, higher concentrations are achieved. The amplitude is proportional to the size of the resonant bubble, so with higher amplitudes the collapse of the bubbles will be more violent and the extraction is more efficient and better yields are achieved, as it can be checked with the experiments.

Finally, the total overall yields (%) can be compared. The results are summarized in Table 10 and shown in Figure 34:

Table 11. Yields of all the experiments of the design matrix.

AMPLITUDE	40	70	100
PULSE			
40	26.32	32.5	36.38
70	27.62	36.42	38.78
100	31.79	37.77	39.99

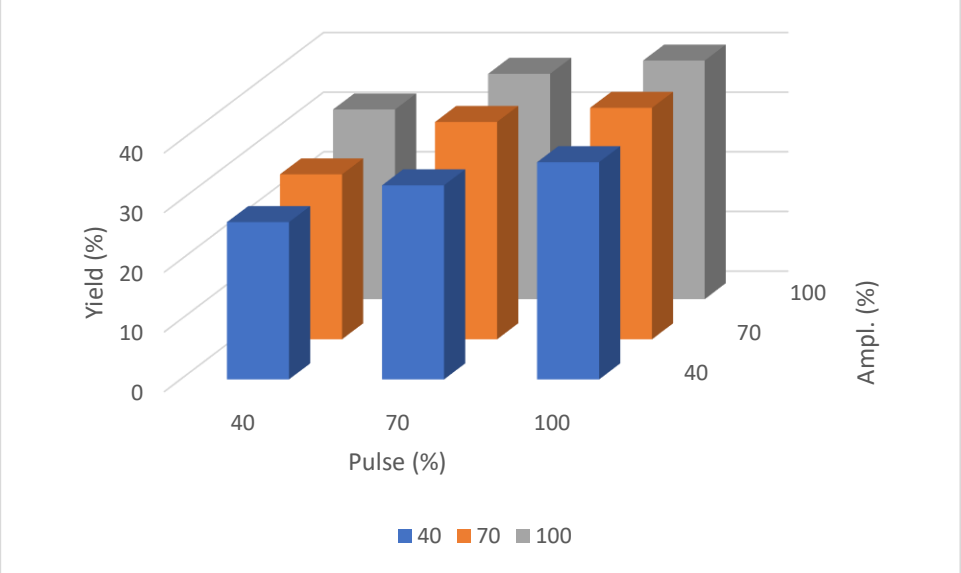

Figure 35. Representation of the yields of the design matrix.

The yields are consistent with conditions. The higher the amplitudes and pulses, the higher the yields. As the amplitudes or pulses decrease, the yields also decrease.

Another appreciation obtained is that both the concentrations and the yields using pulses or amplitudes at 70% or 100% are close numbers. However, when using any of them at 40%, a more notable difference in the results is appreciated.

5.2.2.4. Statistical evaluation of UAE

In order to know which variable is the most influential one in the process, a statistical evaluation of the 3^2 factorial experimental design was carried out. For that, the Statistica program was used with Tibco v13 software. The three variables involved are the yield, the pulse and the amplitude, being the yield the dependent one and the pulse and the amplitude the independent variables. A linear-quadratic model was selected for the evaluation because it is more accurate than others.

The estimated effects of the terms (linear and linear-quadratic terms of pulse and amplitude) can be seen in the Pareto diagram (Figure 45), which is very useful to check the impact that a change in the independent variables has on the process.

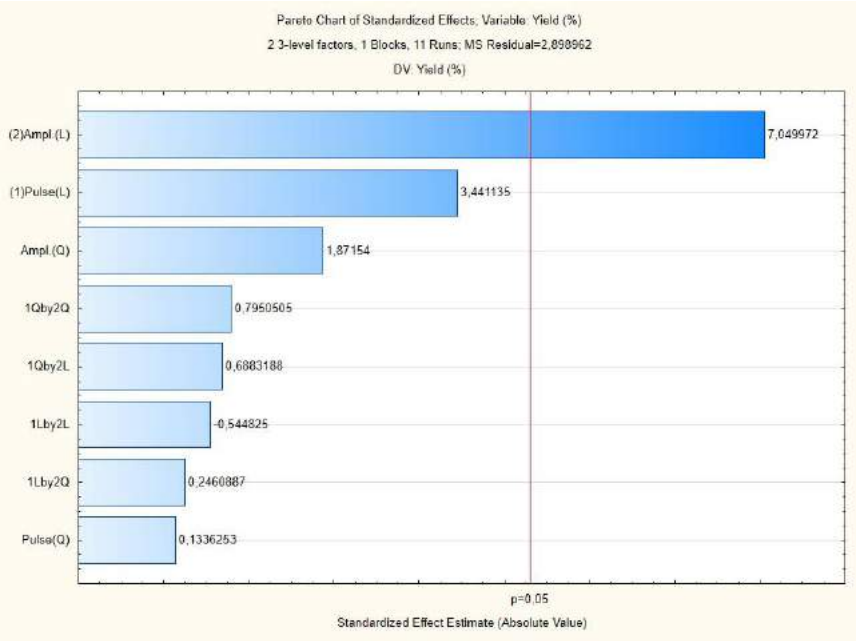

Figure 36. Pareto diagram of the statistical evaluation of the ultrasonic assisted extraction.

This Pareto diagram with 95% significant level shows that a linear term of the amplitude causes the largest change in the yield, because it is the only bar that crosses the critical limit and that means that it is significant for the process at 95% significant level.

The other result obtained is the fitted surface plot (Figure 46), which help to visualize the variation of the yield depending on the independent variables.

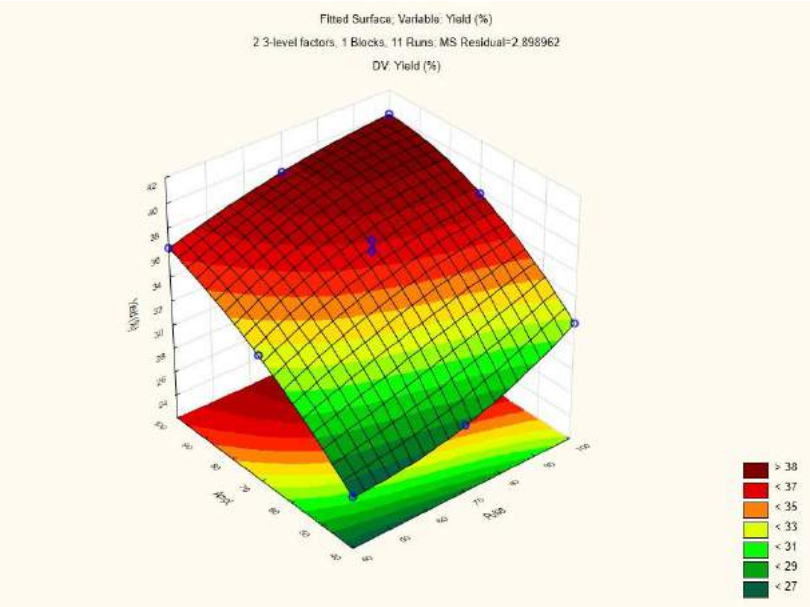

Figure 37. Fitted surface plot of the statistical evaluation of the ultrasonic assisted extraction.

This diagram uses colours to represent the response of the dependent variable to different values of the independent variables. Green colour means a small change in the yield while red colour means a big change and influence of this variable.
In this way, it is seen that changes in the pulse do not lead significant changes in the yield. However, changes in the amplitude cause important variations in the dependent variable. Thus, the surface of the diagram is inclined so that the highest yield is achieves with the highest pulse and amplitude while the lowest is obtained with the lowest values of the independent variables.

5.2.3. Supercritical fluid extraction

A total of 9 experiments with supercritical CO_2 at different temperatures (45, 60 and 80 ºC) and pressures (300, 375 and 400 bar) were carried out. In the operation, two separators were used, but the extract was mainly collected in the first separator. However, in the experiment at 450 bar and 45 ºC (SFE10) there were extracts collected from both separators (as the pressure of 1st separator was set at higher P 90 bar).

The extraction curves can be represented in terms of consumed carbon dioxide (kg CO_2/kg d.m.) and / or in terms of time (min), but the first option was used.

5.2.3.1. Repeatability of experiments

Three parallel measurements were done at 450 bar pressure and 45 °C (Exp No. 2, 3 and 10) and at 375 bar and 60 °C (SFE5, 6 and 7). The extraction curves of those 3-3 parallel runs are shown on Figure 36-39.

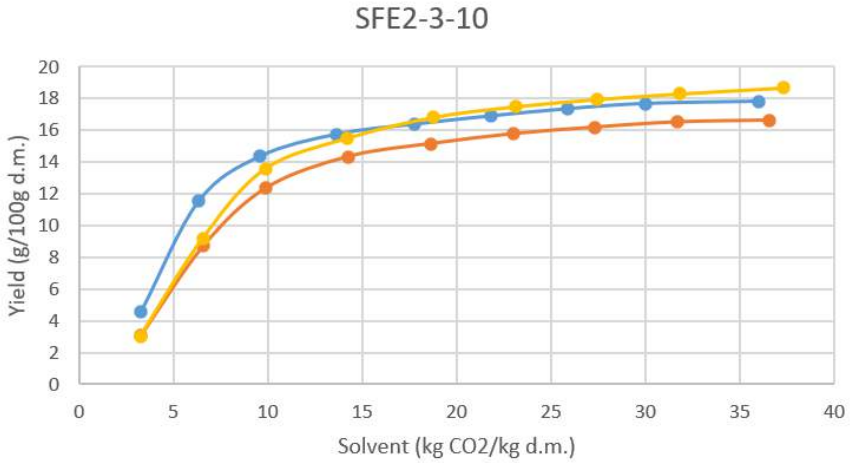

Figure 38. Parallel measurements at 450 bar and 45 °C.

The average overall extraction yield at 450 bar and 45°C was 17.71±1.03%, and the average curve with the standard deviation intervals of each sampling is the following (Figure 37):

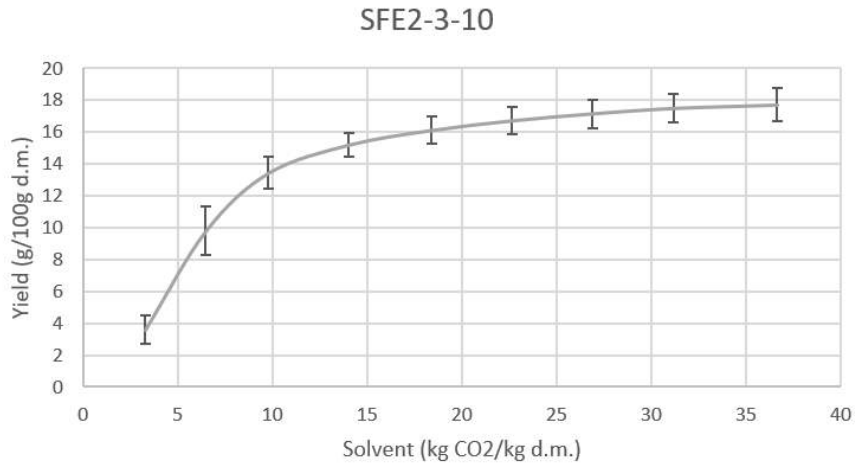

Figure 39. Average curve of the experiments at 450 bar and 45 °C.

The three parallel measurements of SFE at a pressure of 375 bar and 60 ºC of extraction temperature:

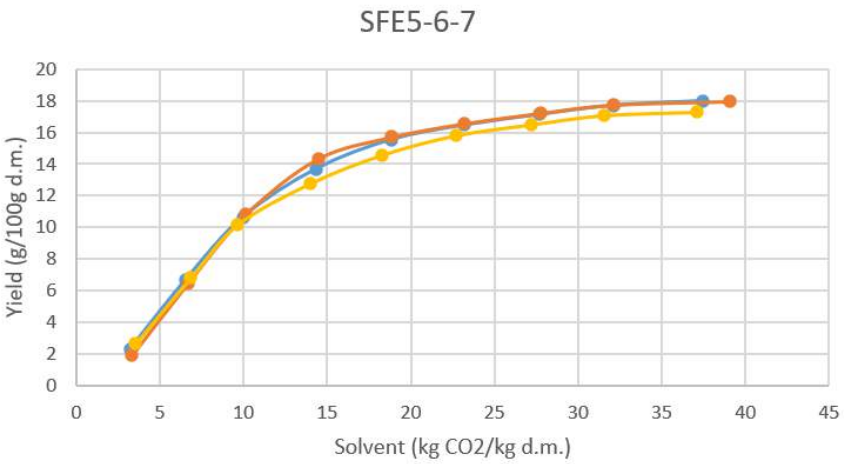

Figure 40. Parallel measurements at 375 bar and 60 ºC.

The average overall extraction yield at 375 bar and 60ºC was 17.74±0.41%. The next graph is the average curve with this data (Figure 39):

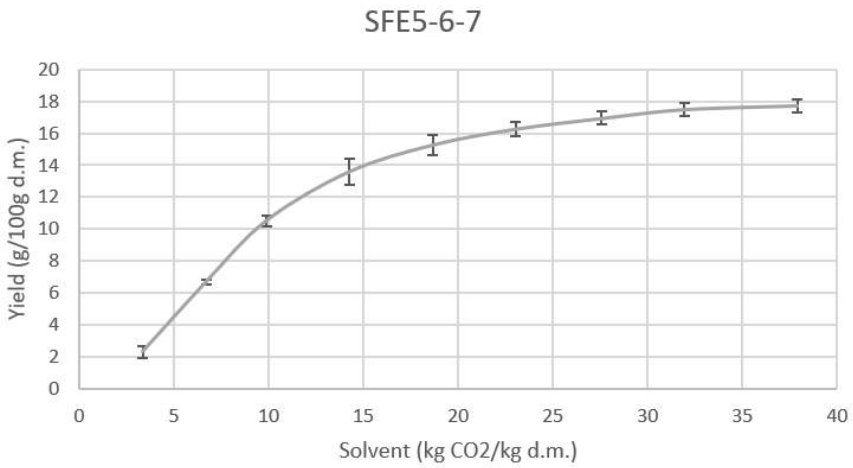

Figure 41. Average curve of the experiments at 375 bar and 60 ºC.

Comparing these graphs, it can be seen that the standard deviation of SFE 5-6-7 is much lower than the experiments run at higher pressure (450 bar). But as these data are from measurement at pilot plant scale, the SD is still rather small, which shows that the measurements can be well repeated and the obtained results are accurate enough.

5.2.3.2. Effect of pressure

The effect of the pressure has been studied at three extraction temperatures: 45, 60 and 80 ºC. The pressure of extraction varied between 300, 375 and 450 bar. The extraction yields are summarized on the following graphs (Figure 40-42).

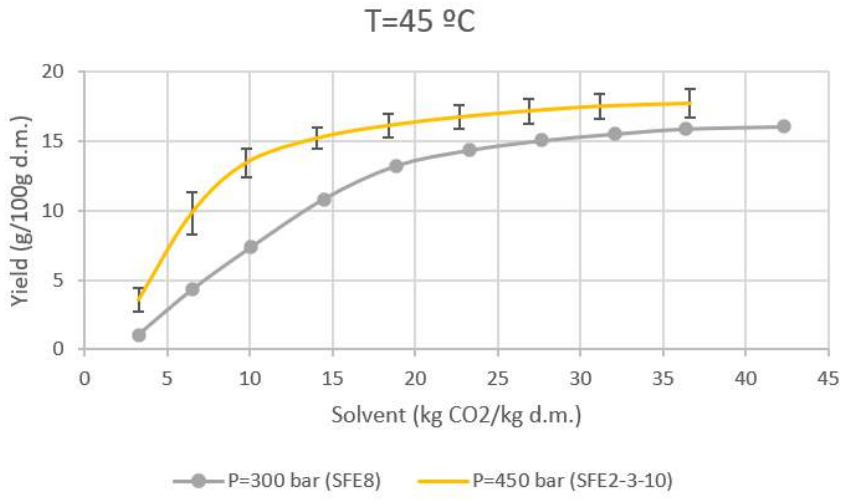

Figure 42. Experiments at 45 ºC.

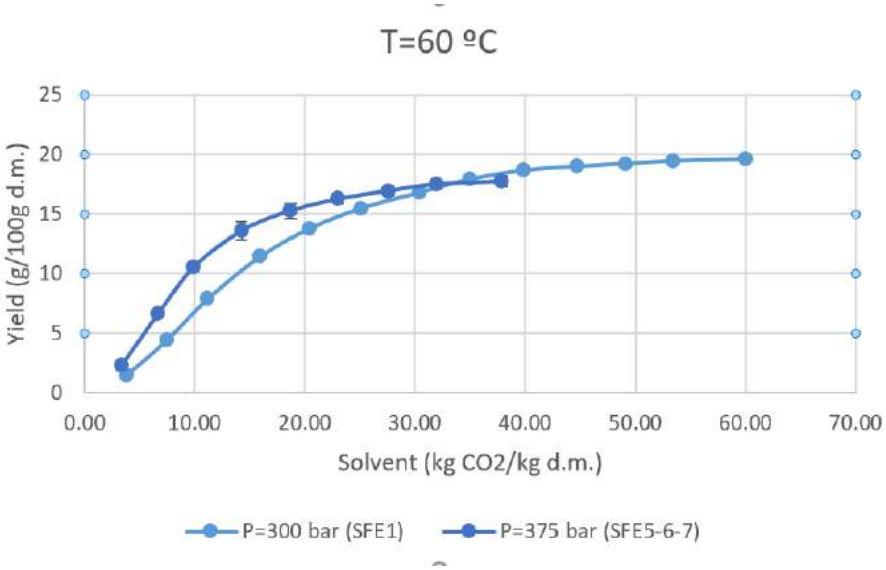

Figure 43. Experiments at 60 ºC.

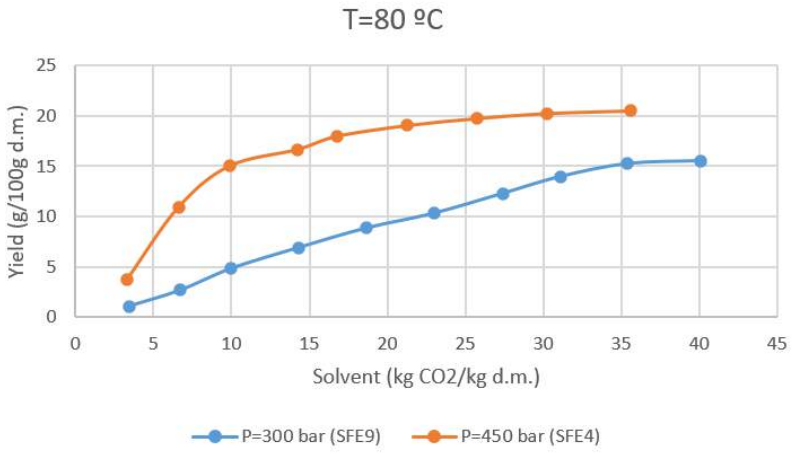

Figure 44. Experiments at 80 ºC.

As it can be seen, with higher pressures the highest yields were obtained. However, at 60 ºC, a slightly higher overall yield was achieved at the lowest pressure applied in these experiments. Also, it can be appreciated that the greater difference between pressures can be observed in the case of a temperature of 80 ºC.

5.2.3.3. Effect of extraction temperature

In the same way, the effect of temperature was studied. Two pressures have been chosen (300 bar and 450 bar) and experiments at different temperatures have been evaluated for each pressure.

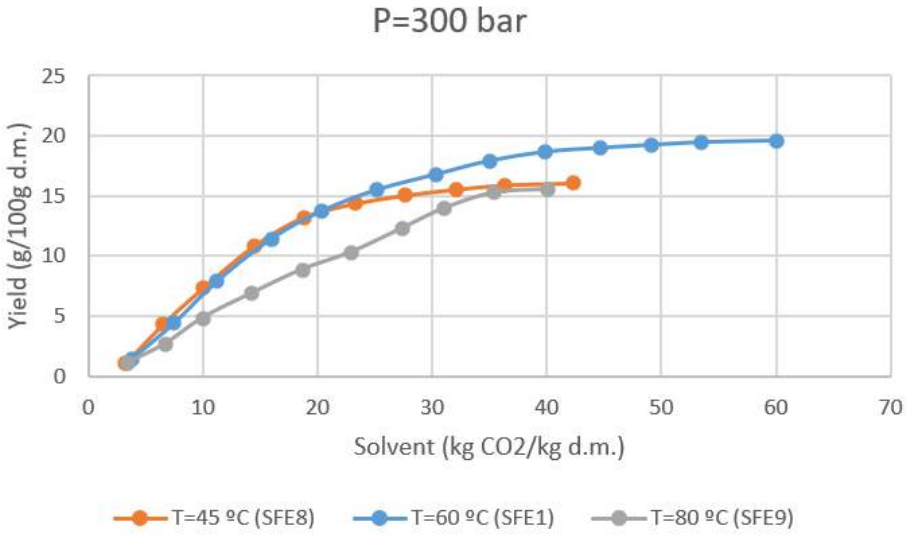

Figure 45. Experiments at 300 bar.

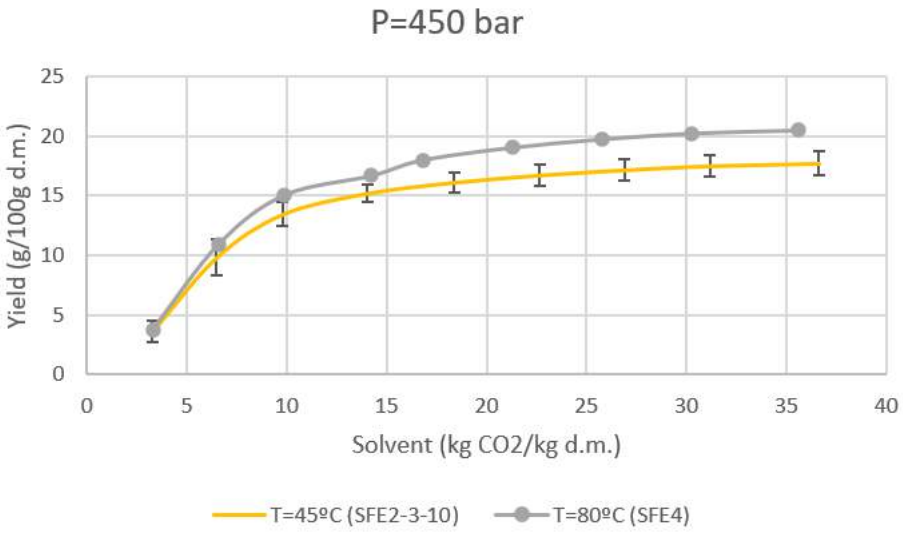

Figure 46. Experiments at 450 bar.

At 300 bar, the extraction curves at 45 and 60 ºC have the same values at the beginning but later the second one achieves higher yield, while the curve at 80 ºC is going lower until the end when it has almost the same value to the one at 60 ºC. In the case of the 450 bar pressure, at all times the curve with the highest temperature is above the one with the lowest temperature, achieving the highest yields at 80 ºC.

The conclusion to this study is that the yield increase when the temperature is higher. The highest yield achieved was at 450 bar and 80 ºC resulting in 20.22% of yield. The product obtained was a clear and yellow oil with mulberry scent.

Therefore, the highest yields were achieved at higher pressures and temperatures in the supercritical CO_2 extraction of mulberry seed.

5.2.3.4. Statistical evaluation of results

For the statistical evaluation, a nonlinear estimation was carried out. The function used to estimate the behaviour of the curves is the one explained in a previous section for a linear model:

$$Y = Y_\infty \cdot (1 - e^{-kt}) = x_0 \cdot (1 - e^{-kt}) \quad \textit{Eq. 14}$$

where Y is the extraction yield at t time (kg/kg), Y∞ infinitive yield which means the total removable material with infinitive extraction time (kg/kg), k is the velocity or mass transfer coefficient (1/s), and t is the extraction time (s) *[63]*.

With this, the effect of pressure and temperature following the same way than in the results section will be shown using this estimation and the respective fitting curves.

5.2.3.3.1. Effect of pressure

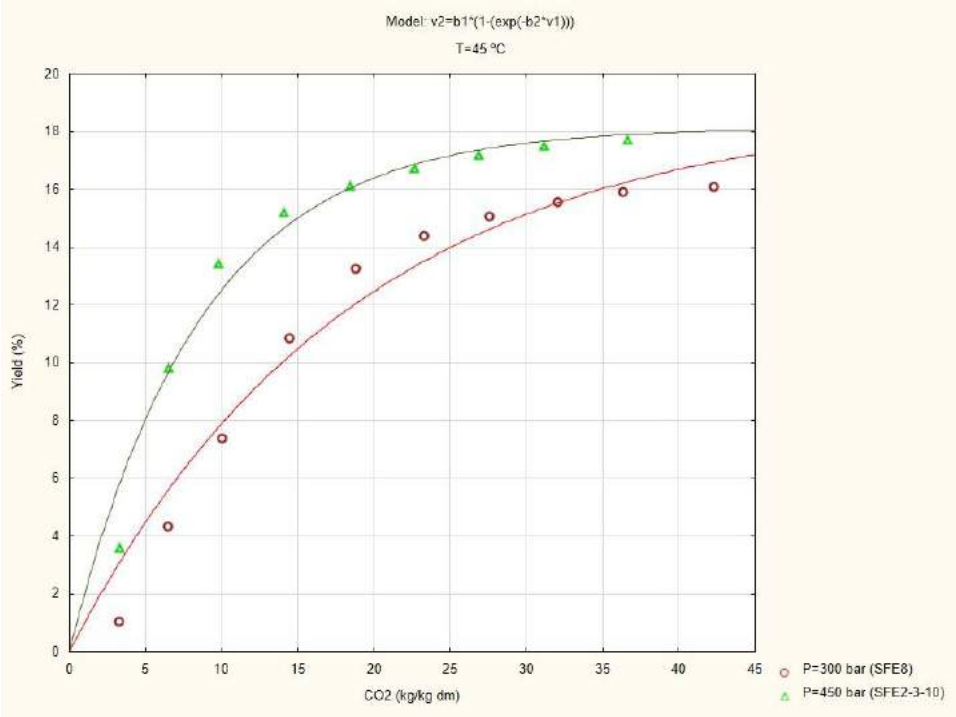

Figure 47. Fitting curve at 45 ºC.

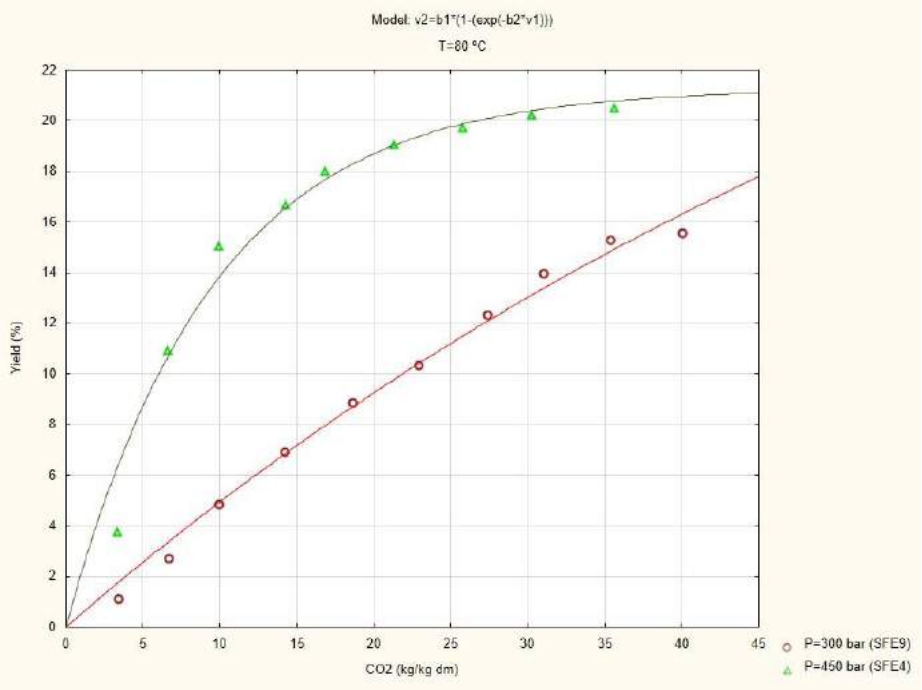

Figure 49. Fitting curve at 80 °C.

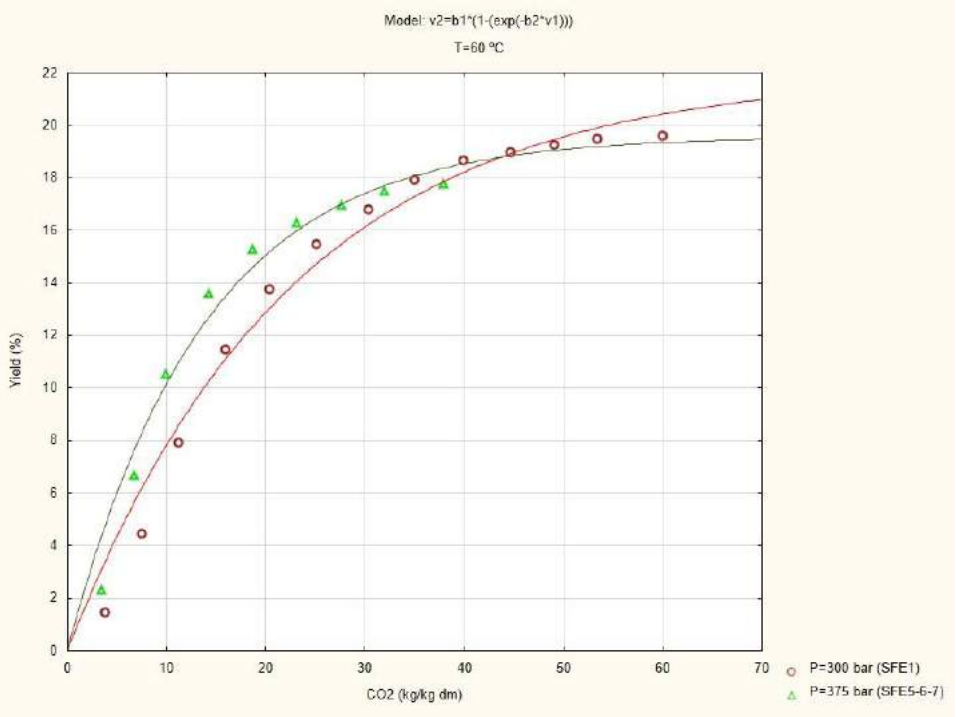

Figure 48. Fitting curve at 60 °C.

The experimental data follow the trend of the estimation model function and fit it with more or less precision.

5.2.3.3.2. Effect of temperature

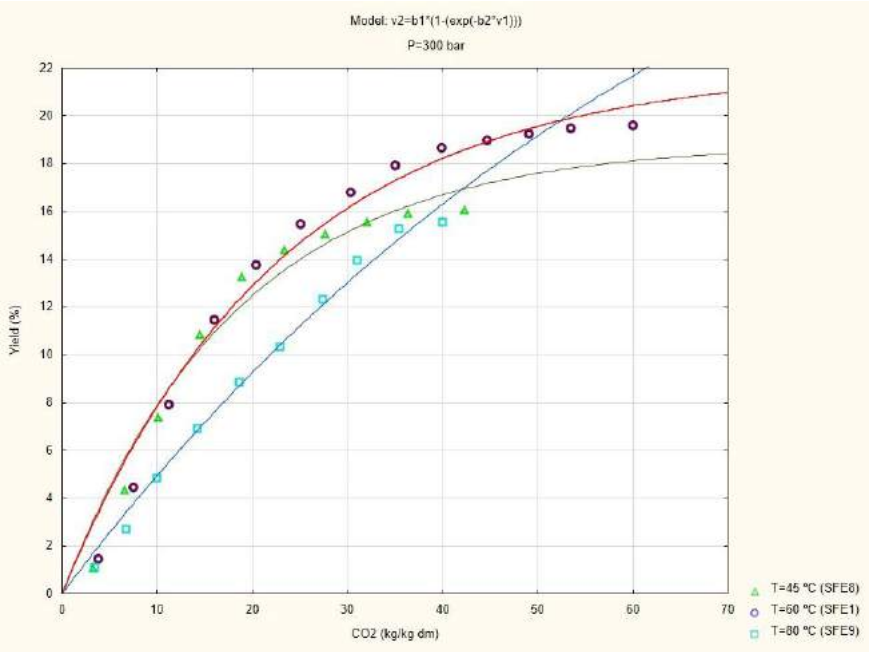

Figure 50. Fitting curve at 300 bar.

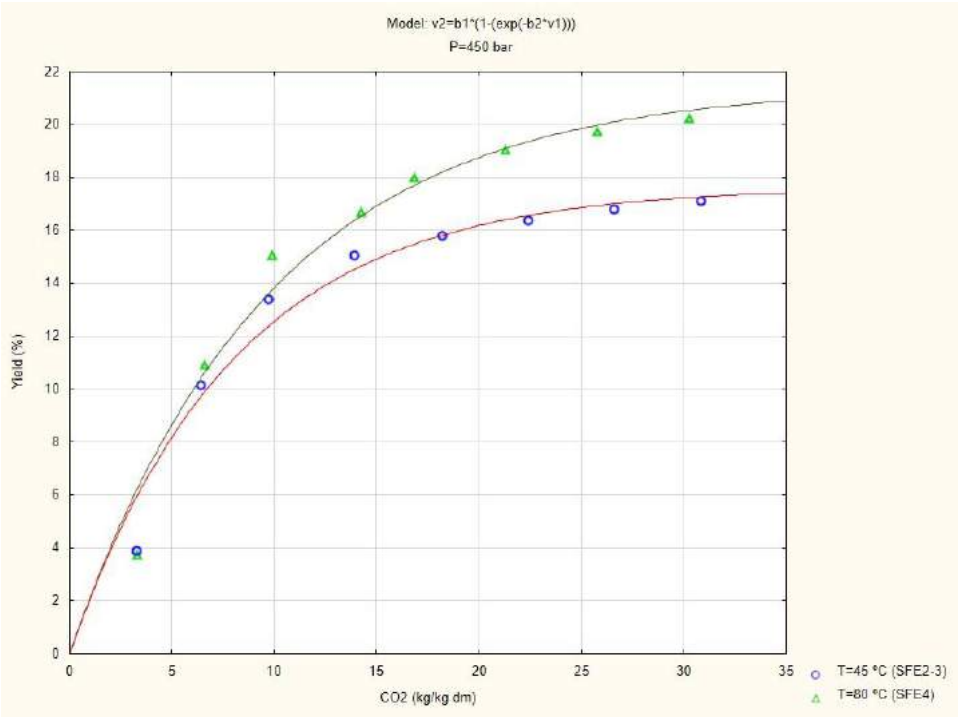

Figure 51. Fitting curve at 450 bar.

The same as before, the fitted model fits well to the experimental data, however some deviations can be observed.

Finally, from this analysis, the parameters of the model Y∞ and k were obtained and collected in Table 12:

Table 12. Parameters of the nonlinear estimation.

	R^2	Y∞ (kg/kg)	K (1/s)
SFE1	0.9884	21.9791	0.0441
SFE2	0.9780	17.9715	0.1435
SFE3	0.9818	17.2295	0.1094
SFE4	0.9827	21.2767	0.1053
SFE5	0.9876	19.7996	0.0732
SFE6	0.9790	19.8517	0.0735
SFE7	0.9891	19.0818	0.0731
SFE8	0.9815	18.8395	0.0542
SFE9	0.9956	38.8298	0.0136
SFE10	0.9805	19.3346	0.1024

This model is used to fit onto the experimental data of SFE extraction of mulberry seed oil. The infinitive yield (Y∞) and the mass transfer coefficient (k) could have been estimated from the fitted equation. As it can be seen, the Y∞ obtained with the model is similar to the yield obtained in the last sample taken. This is the reason why the model can be used for predicting a theoretical yield. However, in one case, at SFE9 (at low extraction pressure) the curve did not fit well onto the experimental data, therefore the predicted values are out of range. The extraction was quite slow at low pressure, perhaps the extraction did not run until similar trend of exhaustion so the model could not have been fitted properly. That k value gives information about the speed of extraction especially at the beginning of the operation when the process is not diffusion controlled yet. k values are close to 0.1 1/s when the extraction run at higher pressure and T, while the k values were lower at lower extraction pressure. Regarding the adjustment, (R^2) high values are obtained but not so close to 1, and in some cases not very high because the experimental data differ from the estimated ones. Due to this non very precise adjustment, the infinitive yield is not exactly the same than the experimental final yield. Therefore, this model is not the best to describe the experimental results it is especially true in the cases of experiments at low pressure (300 bar) and extraction temperature..

6. CONCLUSION

In this work, a study about the optimization of three extraction techniques has been elaborated. The aim of the process is to extract oil from *Morus alba* L. seeds, and the three methods used for that have been Soxhlet extraction, ultrasonic assisted extraction (UAE) and supercritical fluid extraction (SFE).

With the experimental part, different studies were carried out for each technique in order to know the kind of raw material, solvent and optimal conditions which should be used.

- Firstly, the moisture content was determined by weighing and drying seeds. The results showed that the ground seed contains 11.22±0.48% of moisture and the un-ground seeds have a 9.31±0.18% of humidity.
- For the Soxhlet method, experiments with the two solvents (96% ethanol and n-Pentane) were made as well as using both types of seeds (ground and un-ground seeds). After them, it can be concluded that the highest yield has been achieved using ground seeds and 96% ethanol as solvent (Y=59.88±3.20%), given as product a dark brown sticky oil with nice, sweet mulberry scent.
- For the UAE method, a first study to know the optimal extraction time and kind of seeds was made. As conclusion, the optimum operation is extracting the oil for 34 min and with ground seeds as raw material and 96% ethanol as solvent. After that, a 3^2 experimental design matrix was created. Several experiments were carried out at different amplitudes and pulses (40%, 70% and 100% for both parameters) and three repetitions were made in the central point. The results showed that with 100% amplitude and 100% pulse the best yield was achieved (39.99%). In addition, with the statistical evaluation, it could be appreciated in the Pareto diagram and in the fitted surface plot that the variable which makes important variations in the yield is the amplitude instead of the pulse which does not lead significant changes in the dependent variable.
- Finally, for the SFE, different conditions of pressure and temperature were tried in order to know when the highest yield is achieved. The results showed that applying the highest pressure and temperature, the highest yield was achieved (20.22%), so the optimum conditions were 450 bar and 80 ºC for mulberry seed extraction. With the statistical evaluation, the parameters of

one theoretical model proposed were obtained ($Y\infty$ and k), but the adjustment (R^2) was not very good.

As it has been explained, with this work the different extraction methods for obtaining oil from the *Morus Alba* L. seeds have been studied for determining the optimal conditions, kind of raw material and solvent which should be used in order to achieve the best yield.

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Statement of the student

I Leire Nieto Sanz (Neptun code: JR2LWI) as author of the thesis hereby declare that my thesis titled Optimization of extraction of oil from mulberry (*Morus Alba* L.) seeds is my original writing and I have not plagiarised any other work. All third party materials including published and unpublished sources were referenced.

I acknowledge that the intellectual property rights of the methods used and the results of any research or development described in the thesis belong to the participating researchers and institutions/companies, thus their utilization or publication must not be initiated before the approval of all parties.

I also declare that during the preparation and writing the thesis I did not mislead my supervisor(s) and thesis advisor.

03/06/2021, Budapest .....

Leire Nieto Sanz

Statement of the supervisor

I, Erika Vági as supervisor, hereby declare that the thesis written by Leire Nieto Sanz, (Neptune code: JR2LWI) titled Optimization of extraction of oil from mulberry (*Morus Alba* L.) seeds is his/her own writing prepared under my supervision. I also declare that the thesis meets the formal and professional requirements of the Budapest University of Technology and Economics and those of the Faculty of Chemical Technology and Biotechnology, thus I support its submission.

(Date and place) .....

(supervisor)