CHALMERS

Supercritical Fluid Extraction of Bilberry Seed Oil

Effects of extraction process conditions on yield and oil quality

Master of Science Thesis in the Master Degree Programme Biotechnology

ELIN HIDEBRING

Department of Biology and Biological Engineering
Division of Food and Nutrition Science
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2017

Supercritical Fluid Extraction of Bilberry Seed Oil

Effects of extraction process conditions on yield and oil quality

ELIN HIDEBRING

Department of Biology and Biological Engineering
Division of Food and Nutrition Science
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2017

Supercritical Fluid Extraction of Bilberry Seed oil
Effects of extraction process conditions on yield and oil quality

ELIN HIDEBRING

Department of Biology and Biological Engineering
Division of Food and Nutrition Science
CHALMERS UNIVERSITY OF TECHNOLOGY
SE-412 96 Göteborg
Sweden
Telephone + 46 (0)31-772 1000

Cover: Bilberry seed oil extracted in pilot scale extractor
Göteborg, Sweden 2017

Abstract

Supercritical fluid extraction is an extraction technique that utilizes solvents in its supercritical state, a state in which the solvent acts as a liquid and a gas simultaneously, resulting in a more efficient extraction process compared to conventional extraction techniques. The most commonly used solvent is carbon dioxide. Carbon dioxide has benefits of being inexpensive, non-toxic and reaches its supercritical state at relatively moderate conditions of 74 bar pressure and 31°C. Extraction of oil from the oil-rich bilberry seed utilizes a by-product from the berry production with the goal to produce a new product with use in the food, pharmaceutical or cosmetic industry. In this thesis, extractions were performed with both laboratory scale and pilot scale equipment to assess how the process conditions affect the oil yield and quality. Optimal yield was determined by the use of a full factorial experimental design investigating the influence of pressure, temperature and particle size. Oil quality from the different extraction experiments was evaluated with respect to thermal and oxidation stability while storing the oil in different environments. The yields from the laboratory scale experiments corresponded well with previous studies using other raw materials, where high pressure combined with high temperature gave the highest yield. Results from the pilot scale extraction were inconclusive due to operational difficulties. Oil from the pilot scale was used to assess stability and quality during storage. Cold storage was most successful in preventing oxidation of the oil while addition of antioxidants unexpectedly accelerated oxidation. The thermal stability of the oil did not change significantly during storage. However, a slight decrease was observed for the oil with added antioxidants. Meanwhile, oil extracted at 60°C showed a significantly improved thermal stability compared to the oil extracted at 35°C, that might be due to a change in the oil composition. This thesis has provided useful data on how to optimize the extraction process and developed promising methods to evaluate the oil's quality for future research.

Acknowledgements

First and foremost, my greatest gratitude goes out to my supervisor Lovisa Eliasson for all of her guidance and support during the long hours of struggling with the extraction equipment, as well as sharing the success of achieving exciting results. I also had PhD student Graziele Gustinelli Carvalho by my side during the extractions, whose continued research on investigating the bioactive compounds in the berry seed oil will be exciting to follow.

I am very grateful to Adj. Prof. Lilia Ahrné, Head of Process and Technology Development at SP Food and Bioscience (previously SIK) who made this master's thesis possible. Many thanks to my examiner Prof. Ulf Svanberg at Chalmers University of Technology for reviewing my work.

I would like to thank all my co-workers at SIK for all the help and support, assistance with equipment and making the time we spent together genuinely enjoyable and memorable. Last but not least I would like to thank Filip, my family and my close friends, who are always there for me and gives their support, even during busy and sometimes frustrating times that is the master's thesis.

Table of Contents

1. Introduction

The Nordic blueberry, more correctly referred as bilberry (*Vaccinium myrtillus*) covers about 17 % of Sweden's surface and 250 million kg of berries are produced annually. Only 4 % of these berries are actually picked and goes to the berry industry or for personal use. Bilberries have high levels of several bioactive compounds, such as flavonoids and tocopherols with antioxidant properties but also lipid soluble vitamins and n-3 fatty acids are found in the bilberry seeds [3]. Seed oil that exhibits these favorable characteristics could therefore be used as an ingredient in functional foods, pharmaceuticals and cosmetics.

Bilberries, among several other species of common Nordic berries, came into focus with the collaborative project "Bärkraft" (2011-2013) where SIK, the Swedish Institute for Food and Biotechnology, was the main project director and included representatives from the berry production industry. Their goal was to increase the knowledge about the potential of the Nordic berries by competence development, product, process and industrial development as well as marketing and counseling to consumers and companies [4]. An area of interest for the berry industry was the possibility to utilize the waste products from the production of juices, jams and jellies etc. since there are initiatives from the European Union and the Swedish government to reduce food waste by 20 %, compared to 2010, by 2020 [5]. The seeds in the bilberries are normally considered as waste and remains after pressing the berries, although most of the nutritional valuable components are in the seeds and skin of the bilberry. A possible way for the berry industry to reduce their waste is to further process the leftover material into a new usable product. The processing relevant for this master's thesis will be to extract the seed oil. However, as seen in Figure 1, the bilberry seeds are small and do not constitute a large part of the total volume, therefore it is essential to process the seeds in an efficient and sustainable way.

Figure 1 – Cross section of a bilberry [1]

Bilberry seed oil is conventionally extracted with the Soxhlet method or cold pressing, by making use of either the bilberries themselves or byproducts from the press- and juicing industry. Soxhlet extraction involves the use of organic solvents such as iso-hexane, and normally results in a high extraction yield. However, organic solvents are not a good option for a sustainable environment and there is a risk of solvent residue in the extract [6]. In this master's thesis, an alternative method for bilberry seed extraction will be evaluated using a green technology; supercritical fluid extraction (SFE) with carbon dioxide. The extraction efficiency will be greatly dependent on the process conditions and pre-treatments of the bilberry, and these parameters need to be optimized to improve both extract quality and oil yield. SFE extraction have shown potential mimic and even transcend the oil yields from conventional extraction methods of other seeds such as kenaf, peach and grape seeds [7-9].

1.2 Objective

The objective of this master's thesis is to optimize the supercritical fluid extraction of bilberry seed oil in terms of yield and oil quality in both pilot and laboratory scale. This will be done by evaluating different extraction process conditions and post-treatments of the bilberry seed oil.

1.3 Specification of the objective

- How does the process conditions influence the extraction yield?
    - Extraction temperature
    - Extraction pressure
- Is it possible to scale up the process?
    - Perform extractions in the pilot scale equipment
    - Optimize process conditions
- How does the pre-treatment of the bilberries affect the final yield and oil quality?
    - Consider drying and particle size reduction
- What physiochemical properties will the extracted oil have?
    - Oxidative stability by non-isothermal differential scanning calorimetry (DSC)
    - Peroxide value by ferrous oxidation in Xylenol orange assay (FOX method)
    - Storage stability

1.4 Delimitations

The project will not include extraction with co-solvent, nor will different pretreatment techniques such as infrared drying or freeze drying be evaluated.

2. Theory

2.1 Bilberry seed oil

Seeds are a common source of vegetable oil for both industrial and food applications. A seed used for vegetable oil production are commonly denoted as oilseeds. An oil seed can contain 10-40 % of lipids inside the seeds. The oil is retained in intracellular organelles called oil bodies, where the oil encapsulated by a membrane of phospholipids. The size of the oil bodies can vary between 0.5-2.0 µm, and smaller the oil bodies are found in oil rich seeds since they have larger surface areas [10]. As mentioned in the introduction, oilseeds are conventionally extracted via solvent extraction using organic solvents where commercial hexane is the most common. Alcohols, carbon disulphide as well as supercritical carbon dioxide are other solvents that has been used since the first development of the solvent extraction process in the middle of the 19th century [11].

Bilberry seeds are an unconventional material to extract oil from and there have been very few studies on the subject. Surprisingly, bilberry seeds have a relatively high oil content of approximately 24 %, according to analysed values (appendix I). In the recent project "Bärkraft" at SIK, the cellular structure of bilberry seeds was examined closely using microscopy. The oil bodies are clearly visible when staining the samples for oil and protein. A bilberry seed in a light microscope at 1 mm and 10 µm zoom is shown in Figure 2 where the dark brown areas in the picture to the right are stained for fat while the green area shows proteins.

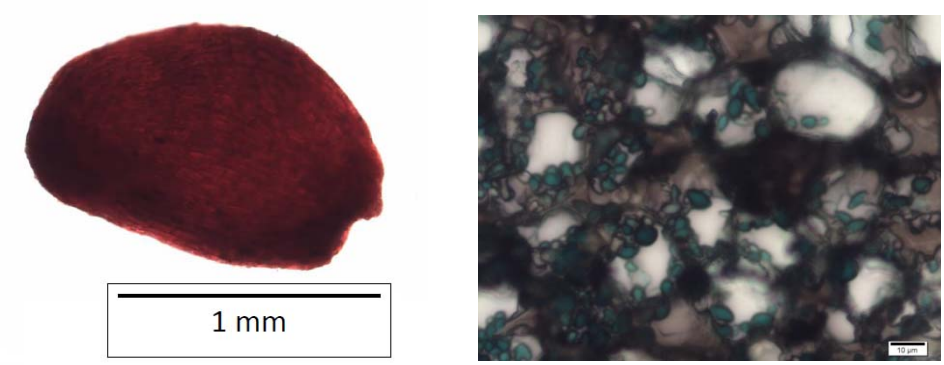

Figure 2 – A whole bilberry seed (to the left) and seed stained for fat (dark brown) and protein (green) (to the right) in a light microscope (Öhgren, C. and Altskär, A. (2013) Determination of the structure of bilberry seeds. The Bärkraft project, SIK –The Swedish Institute for Food and Biotechnology. Unpublished material).

Whole bilberries are famous for their rich content of bioactive compounds which allegedly could improve eyesight and treat gastrointestinal symptoms and diseases [12]. A lot less is known about the specific content of the seeds. The fatty acid profile of the oil as well as the concentration of the bioactive compounds tocopherols, among others has been established in cold pressed bilberry seed oil by Helbig et. al. (2008) [13]. The oil consists primarily of polyunsaturated fatty acids (>60 %) followed by the monounsaturated fatty acids (>20 %) and lastly saturated fatty acids (<10 %). Total tocopherol content expressed as mg/100 oil of the berry press residue (PR) was 24.6 mg/kg PR. However, the oil content of their PR was set at 5.2 %. These properties could offer a balance of mono- and polyunsaturated fatty acids in the human diet, together with bioactive compounds that could add value in many other applications as well.

2.3 Supercritical fluid extraction

Supercritical fluid extraction (SFE) has in the last two decades gained interest as an alternative to conventional solvent extraction. The technique is mostly used in the food industry, for example to decaffeinate coffee beans, but has applications in petrochemical and pharmaceutical industries as well [14]. SFE can be performed by many chemical compounds, nitrogen, ethylene and carbon dioxide among others, as long as they are in a supercritical state and is soluble in the extract of interest. If a compound is in a supercritical state both pressure and temperature must be above the supercritical point, P_C and T_C respectively. Above P_C and T_C there is no clear distinction between the liquid and gaseous phase, the phase diagram in Figure 3 demonstrates different phase regions.

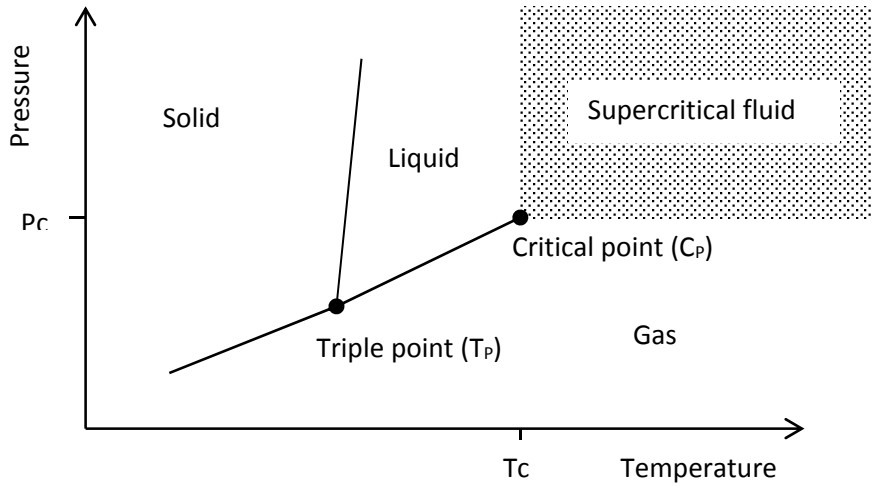

Figure 3 –Phase diagram for a pure component, depicting phase boundaries, including the triple point and the critical point.

In the supercritical region, a compound will adopt properties from both liquid and gaseous states and the phase boundary will be erased. Supercritical fluids are less viscous than liquids and have higher diffusion coefficients, while the density is higher density than for the gaseous state [15]. Due to absence of surface tension in the supercritical liquid it will be less hindered to access the sample matrix compared to a normal liquid.

Carbon dioxide is by far the most commonly used supercritical solvent because it is cheap, non-flammable, easily obtained at a high purity and is allowed to be used in foods because of its GRAS-status (Generally Regarded As Safe). With T_C of 31.1°C and P_C at 73.9 bar, carbon dioxide also provides an accessible critical point that is easily reached and is a suitable choice for heat sensitive components. Supercritical carbon dioxide will transit into gas at room temperature and/or ambient pressure which allows it to leave the extract without further processing and leaves no chemical residue [14]. Carbon dioxide is a nonpolar compound which allows it to extract other nonpolar compounds, such as the oil from various types of seeds, including bilberry seed oil. Supercritical carbon dioxide has generally high affinity for the lipids in the seed oil and the affinity increases for lipids of lower molecular weights. Lipid extraction with carbon dioxide as a solvent is normally carried out in an extraction vessel with a fixed bed of solid extractable material, where the supercritical fluid can flow upwards thru the material. The material will absorb the solvent and the cell membranes of the material swells and facilitates mass transport of lipids out from the cells. In turn, lipids are dissolved by the carbon dioxide and are transported to the material's surface, mainly via diffusion, and follow the supercritical fluid out from the material and accompany the carbon dioxide in the flow

direction [16]. The supercritical carbon dioxide can deliberately be kept in the vessel without any outflow, to take advantage of the increased extraction power when the cells are given time to swell. This part of the extraction process can be referred to as static time. A schematic setup over the extraction vessel is illustrated in Figure 4.

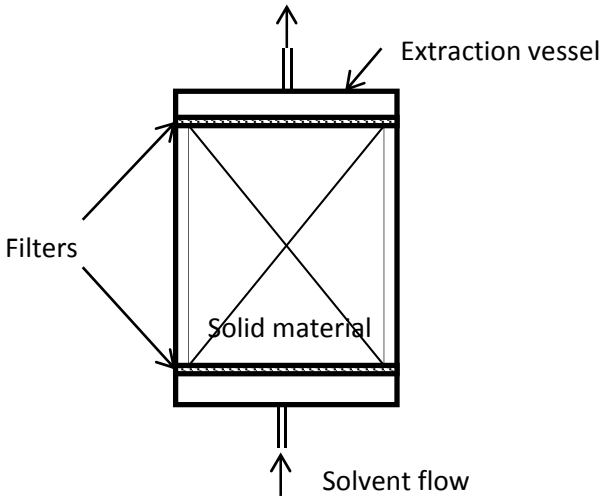

Figure 4 – Extraction vessel for super critical extraction of lipids thru a fixed bed of solid material. The filter distributes the gas and prevents solid particles to follow the solvent flow.

There are several process parameters that influence on the extraction yield where pressure and temperature will have the largest impact. Solubility of the carbon dioxide in the material increases with increased pressures, and the same applies for an increased temperature. Also, mass transport and diffusion is facilitated at higher temperatures. However, at low pressures a higher temperature can decrease the extraction power due to the decrease in density at high temperatures and leads to poor solvent solubility in the material. This behaviour is not observed at high pressures as the increase in vapour-pressure of the solvent will be the main effect.[16]

Besides temperature and pressure, particle size and solvent-to-material ratio are also two important parameters to reach optimal extraction behaviour. Smaller particles, whether natural or thru particle size reduction, offer a shorter length of transport for the extract to travel, as well as an increased surface area and thereby a more efficient mass transfer, leading to higher yield of extract in shorter time. If the particles are too small, they may hinder mass transport of the liquid oil, resulting in a less efficient extraction. Particle size reduction is necessary in the case of lipid extraction of bilberry seeds since the seed coat is too thick to allow mass transport from the intracellular oil bodies. A sufficient solvent-to-material ratio is crucial to achieve an efficient extraction, but it is also a question of economy. High solvent-to-material ratio undoubtedly gives a more efficient extraction but only to a certain point due to limitations in the mass transport and diffusion. Using a high ratio will give higher throughput of oil which could be favourable in an economic sense, especially if the solvent can be recycled in the system. Otherwise, the use of a non-optimal ratio may lead to increased extraction costs for both solvent and equipment without any increase in profit. [16]

2.4. Oil chemistry

Oils derived from plant sources such as seeds, kernels or nuts are all classified as vegetable oils and are the main sources of edible and non-edible oils worldwide. The oils can be classified into more specific areas of applications and production but they have their basic chemical structure in common [17]. A vegetable oil consists of a mixture of organic molecules, where triacylglycerol (TAG) is the largest component [11]. TAGs are esters made up from one glycerol molecule with one molecule of fatty acid (FA) attached with an ester bond to each of the three hydroxyl groups (-OH) on the glycerol via the carboxyl group (-COOH) on the fatty acid, as seen in the simplified figure below, Figure 5.

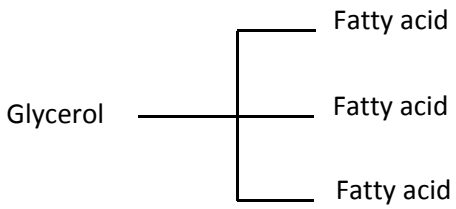

Figure 5 – Basic structure of a TAG

Fatty acids are nonpolar hydrocarbon chains of varying lengths, and fatty acids normally found in vegetable oils are monocarboxylic, i.e. with only one carboxyl group in the end of the molecule. Furthermore, the hydrocarbon chains can have different degrees of saturation in the chains depending on the number of double bonds present [2]. A fatty acid without double bonds is denoted as a saturated fatty acid (SFA) and monounsaturated and polyunsaturated fatty acid (MUFA and PUFA, respectively) for acids with one or multiple double bonds in the hydrocarbon chain, the bond is normally in *cis*-configuration. A *cis*-double bond will also introduce, in the otherwise straight hydrocarbon chain, a kink of about 42°[2]. The number of double bonds and their positions will have a large impact on the chemical and physiological properties of the oil. In general, oil is predominantly composed by MUFAs and PUFAs which contributes to oil's liquid properties, since most MUFA and PUFAs has significantly lower melting points compared to SFA [18, 19]. Double bonds also offer sites for oxidation initiation reactions as the electron rich bonds are more reactive than a single bond.

Although fatty acids make up at least 95 % of unrefined oil, there are other minor components that may have a large impact on oil chemistry. In addition to close relatives of the TAG, as mono-, diacylglycerols and free fatty acids, the oil can also contain phospholipids, phytosterols and tocopherols [11]. Traces of metals, Cu, Fe, Mn and Zn among others, as well as pigments, such as chlorophyll, can also be present in the oil [11, 20]. Most of these components are removed when refining the oil through degumming, neutralization, bleaching and deodorization steps and results in oil with up to 99 % TAG.

2.4.1. Lipid oxidation

The number one process causing rancidity in seed oil is lipid oxidation. Oxidation of lipids is a deteriorative process caused by autoxidation or photosensitized oxidation of unsaturated fatty acids in oil that forms compounds that will ultimately lead to off-flavour and odour [2]. Typically, the autoxidation process can be described in a simplified scheme, consisting of 3 steps, initiation, propagation and termination. The reaction stages are illustrated in Figure 6, and describe the principles of the major reactions in lipid oxidation.

In the initiation step, a fatty acid (RH) is transformed to a free radical ($R^{\bullet}$) via different mechanisms involving catalysts, as it is not a spontaneous reaction. Catalysts can be a metal, photosensitizer, light or heat. The radical can react further with atmospheric oxygen ($^3O_2$, triplet oxygen) and result in peroxy radicals ($ROO^{\bullet}$) in the first propagation step. It is important to note that atmospheric oxygen cannot react directly with double bonds because the spin states of the orbitals are different; the oxygen is in a triplet state, while double bonds are in a singlet state. For the reaction to occur it is necessary for the bonds to be excited into a triplet state which would require a large input of energy. The spin barrier is overcome with the catalysts and thus creating sites on where the atmospheric oxygen can reacts. [21]

Initiation: $RH \rightarrow R^{\bullet}$

Propagation:

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$$

$$2ROOH \rightarrow RO^{\bullet} + ROO^{\bullet} + H_2O$$

Termination: $R^{\bullet},\ RO^{\bullet}, ROO^{\bullet} \rightarrow stable\ compounds$

Figure 6 – Classic reaction scheme of lipid oxidation adapted from Coultate (2009) [2].

Peroxy radicals readily react with other fatty acids and creating hydroperoxides and free radicals. The hydroperoxides are also able to break down into new peroxy radicals and propagate the abovementioned reaction. Oxidation is terminated when radicals react with each other and forms stable compounds, this reaction occur when there is a high concentration of radicals in the lipid matrix. However, because of the autocatalytic nature of the lipid oxidation reactions, it is believed that the reaction is never fully completed. [21]

The photosensitized oxidation involves singlet oxygen ($^1O_2$) which differs from the triplet oxygen on an orbital level by having different electronic configurations in their 2pπ orbital. The singlet oxygen has 1 orbital with paired electrons and one empty, making it electrophilic while triplet oxygen is a radical with 2 unpaired electrons in the orbital[22]. The difference between the two oxygen species' electron configuration in the 2pπ orbitals is illustrated in Figure 7. Due to the electrophilicity of singlet oxygen and since it has the same spin state as the double bonds in the fatty acids, the two can easily react and form hydroperoxides without the formation of fatty acid radicals. The hydroperoxides can follow the same oxidation procedure as the hydroperoxides formed by triplet oxygen in the third propagation reaction in Figure 6.

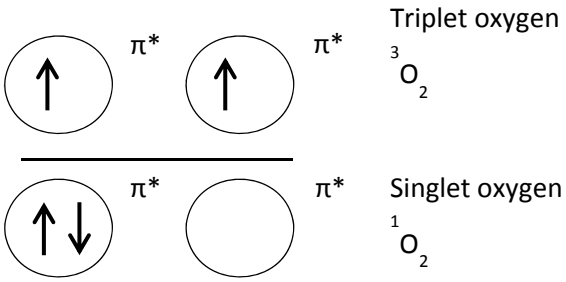

Figure 7 – Electronic configuration of triplet and singlet oxygen in the 2pπ orbital. The rest of the molecular orbitals are not included.

Singlet oxygen is short-lived but acts efficiently on double bonds in the fatty acids, it is also believed that it has a greater influence on the initiation step of the autoxidation than previously thought [23]. Singlet oxygen can be formed by photosensitizers, where chlorophyll is the most relevant for vegetable oils. Chlorophyll absorbs energy from light in picoseconds, excites the electrons and transfers it to triplet oxygen in order to return to its ground state, which forms singlet oxygen. It can also be formed by decomposition of hydroperoxides, enzymatically or chemically [23].

2.4.2. Prevention of lipid oxidation

The oxidation process of oil can be prevented in several ways. The methods most relevant for this thesis are by controlling the temperature, light, headspace atmosphere and addition of radical scavenging antioxidants. Autoxidation is thermodynamically favoured by an increased temperature, a fact that is utilized when assessing lipid oxidation with accelerated oxidation analysis using differential scanning calorimetry (DSC) or the Rancimat method, where an elevated temperature (>100°C) in combination with oxygen flow accelerates the oxidation process [24]. Conversely, a lowered temperature can decelerate the oxidation process to some extent if the oil is stored below room temperature (<10°C) compared to an oil stored at room temperature. However, singlet oxygen oxidation is not affected by temperature as the activation energy is so low [25]. If there are photosensitizers present in the oil, they can still initiate oxidation, despite the low temperature. The effect of light on oxidation has therefore a double influence, both by increasing the temperature of the oil and inducing the formation of singlet oxygen.

The concentration of oxygen, both dissolved in the oil and headspace oxygen, is naturally of great importance for the oxidation process. Oxygen is easily dissolved in vegetable oil and even small amounts can initiate oxidation reactions. The more dissolved oxygen in the oil, the higher level of oxidation is possible [26]. Transport of oxygen can occur via diffusion or thru convection if the oil is stirred or processed. A small sample volume and/or a high surface to volume ratio can facilitate the reaction between oxygen and the lipids by making the oil more accessible for the oxygen [27]. By modifying the headspace atmosphere and replacing the dissolved oxygen with another, non-oxidative, gas the oxidation reactions can be depleted. Commonly used gases are nitrogen and carbon dioxide. Carbon dioxide has shown more potential to supress lipid oxidation simply because it is more soluble in vegetable oil than nitrogen [28].

Addition of antioxidants is a well-known method to limit lipid oxidation. Vegetable oils can naturally contain antioxidants with various modes of operation as scavenge free radicals, quench singlet oxygen and chelate metal ions [22]. Antioxidants can scavenge radicals by relocating hydrogen from the molecule to the radical compound ($ROO^{\bullet}$) and thus incapacitate them into stable compounds. Even though the antioxidant itself becomes a radical when losing hydrogen, its energy will be less than the lipid radical since it has the ability to relocate the radical site by utilizing resonance structures [2]. The most important and biologically available radical scavenging antioxidants are the tocopherols, where α-tocopherol is most effective in moderate concentrations [22]. After donating hydrogen to the peroxy radicals, it has the ability to either form a stable compound with another tocopherol radical or form a complex with a peroxy radical. However, if the concentration of α-tocopherol in the oil is too high it may instead act as a pro-oxidant via tocopherol mediated oxidation.

The tocopherol mediated oxidation is especially likely when the concentration of peroxy radicals (ROO•) is low, as in the case of a freshly extracted oil. A tocopherol radical cannot propagate and react with peroxy radicals, as there are so few, and instead react with the intact PUFA, yielding a lipid radical (R•)[29]. The lipid radical can continue to react with atmospheric oxygen as described in Figure 6.

2.4.3. Assessing lipid oxidation

There are several ways to assess the extent of lipid oxidation in oil. The analysis can for example utilise chromatography, colorimetry, titration or thermal analysis [30]. The methods relevant for this thesis, is a colorimetric determination of peroxide value with ferrous oxidation-xylenol orange (FOX) method and thermal analysis using DSC.

2.4.3.1 Ferrous oxidation-xylenol orange method

The FOX method measures primary products of oxidation, which are the peroxides formed during the propagation step in the lipid oxidation scheme. The extent of oxidation is expressed as peroxide value (PV) which is milliequivalents (meq) of peroxide oxygen per kg oil. As implied by the name, FOX involves ferrous ions and the organic reagent xylenol orange (o-cresolsulfonephthalein-3′-3′-bis-[methyliminodiacetic acid sodium salt]). By taking advantage of the fact that peroxides can selectively oxidize ferrous ions to ferric at an acidic pH, and that ferric ions can complex with the xylenol orange (XO) dye in the presence of sulphuric acid, the concentration of peroxides will be proportional to the amount of ferric-XO complex in the solution. The blue-purple complex will be detectable at 560 nm using a spectrophotometer [31]. The principle of the reactions is displayed in reactions 1 and 2.

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO^{\bullet} + OH^{-} \quad (1)$$

$$Fe^{3+} + XO \rightarrow blue\ purple\ complex \quad (2)$$

To avoid further oxidation reactions by the alkoxyl radicals (RO•) a lipid soluble antioxidant, BHT was added in the solution. BHT has the ability to repair the reactive radical and turn it into a stable compound. By using a standard curve with known amounts of ferrous ions, the peroxide value can be easily calculated [32].The FOX method is reported to be a quick and sensitive (≈0.1 meq/kg oil) alternative to the American Oil Chemists' Society's (AOCS) official standard method involving iodometric titration which is time consuming and requires a large sample volume[30]. However, the FOX method requires careful experimental development depending on the sample as there can be many factors that can influence on the final result [33].

2.4.3.2 Differential scanning calorimetry

The use of differential scanning calorimetry (DSC) is a simple and straight forward method to estimate the oil's tendency to oxidise. With the sensitive equipment of a DSC it is possible to record miniscule changes in thermal release of oxidation reaction when the oil is exposed to heat and oxygen, using sample sizes of only 5-10 mg. The increase in temperature and a forced flow of pure oxygen will accelerate the reactions in a way that it is possible to monitor the changes in real-time. An initiated oxidation reaction will demonstrate itself as a sharp increase in heat flow and form a peak, see Figure 8, and the onset point is determined by extrapolation off the base line and intersecting it with the peak tangent. The analysis can be performed in two ways, either by using isothermal or non-isothermal conditions. Both

conditions can be used to obtain comparable information about the oxidation of the oil but the kinetic information from the data will be different. In isothermal analysis, the sample will be flushed by oxygen (50-100 mL/min) while being heated at constant temperature. The temperature can be set at any level but temperatures from 80 to 180°C are most common [34]. A thermogram from an isothermal DSC analysis will provide information about the onset point, the oxidation induction time (OIT). A long OIT at a high temperature will indicate that the oil is stable and has not undergone extensive oxidation prior to the analysis, while a short OIT at a low temperature would suggest the opposite.

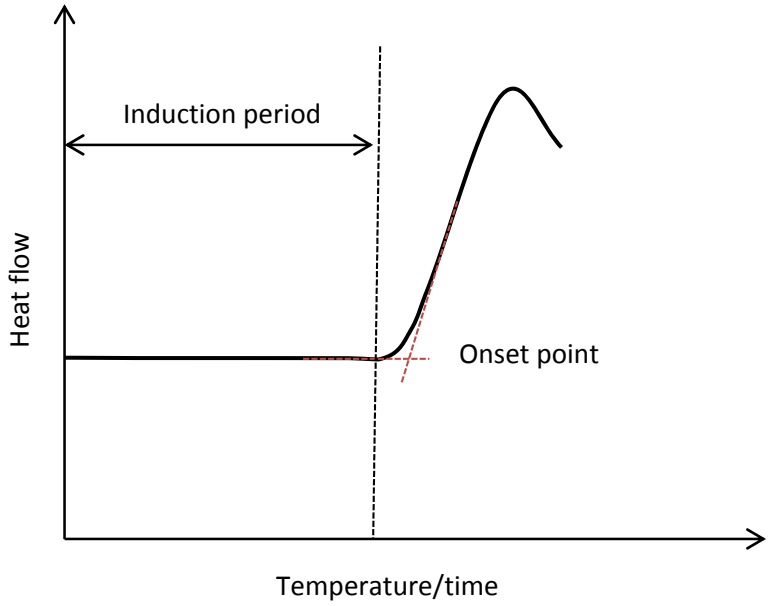

Figure 8 – Model thermogram of a DSC analysis on vegetable oil, analyzed with regard to the onset point (time or temperature) of oxidation.

The non-isothermal DSC can have the same oxygen flow as in the isothermal analysis, while the temperature is continuously increased at a specific rate. Heating rates commonly range from 2-20°C/min [34]. A lower heating rate gives better resolution to the thermogram and if the rate is too high there is a risk of missing the onset point altogether. The heating rate should not be more than 25 °C/min as the heat transfer from the furnace must be allowed time to equilibrate with the oil. Analysis of the non-isothermal thermogram will give the oxidation onset temperature (T_{on}) as onset point. A low T_{on} of a vegetable oil implies that the oil has a higher degree of oxidation than the same oil with a high T_{on}, as less oxidized oil will be more resistant to an elevated temperature and oxygen [35]. A low T_{on} can be correlated to high PV [34]. DSC can also discern between oils with different concentrations of antioxidants and oil of different set of saturated or unsaturated fatty acids.

3. Experimental

3.1. Preparation of bilberry seeds

The bilberry seeds used throughout this thesis work was delivered from Svantes Vilt & Bär AB (Harads, Sweden). The seeds were a by-product from their juice production, using a screw press, and provided seeds clean from both skin and pulp but with a considerable amount of water. Before shipment to SIK, the seeds were frozen in large blocks and cut into manageable pieces. At SIK the seeds were stored at -20°C until use. The preparation of the seeds was divided into thawing, drying, milling and particle size determination.

3.1.1. Thawing and drying

A block of the frozen seeds was placed in room temperature and kept away from light until the thawing was completed (4-6 h). The wet seeds were distributed evenly on trays and dried in a convection oven at 35°C over night, resulting in approximately 60 % weight reduction. The remaining moisture content was determined by heating duplicate fractions (3 g) of the dried seeds in a vacuum oven at 70°C until constant weight. The thawed and dried berry seeds can be seen in Figure 9.

Figure 9 – Thawed and dried bilberry seeds.

3.1.2. Milling and particle size determination

The seeds intended for the laboratory scale extraction was milled in a knife mill (OBH Nordica, 2393) for 10, 20 or 30 sec. Seeds for pilot scale extraction was continuously milled in a flat burr coffee mill (Ascaso i-I) using the same particle size setting for all extractions. The size distribution of the milled seeds was determined with a sieve shaker (Analysette 3 PRO, Fritsch) using sieves with 1.25 mm, 710 µm, 500 µm, 250 µm and 125 µm mesh sizes. The sieving time was 20 min, with an amplitude of 1.5 mm and an interval time of 10 sec, all according to the recommendations from the manufacturer.

3.2. Quantitative peroxide assay

Detection of peroxides in the oil and subsequently the determination of the peroxide value was performed with Pierce Quantitative Peroxide Assay Kit, lipid-compatible formulation from Thermo Scientific (Rockford IL, USA). The kit contained two reagents, A and C and their respective content is displayed in Table 1. For all experiments, the reagents were mixed 1:100 to form the working reagent, WR and at least 1 mL of WR was prepared for each sample. A complete laboratory protocol, developed during this master's thesis, can be found in appendix II.

Table 1 –Overview of components in the Peroxide Assay Kit

Reagent A	25 mM ammonium ferrous (II) sulphate, 2.5 M H_2SO_4
Reagent C	4 mM BHT, 125 µM xylenol orange in methanol

3.2.1. Standard curve

To be able to quantitate the amount of peroxides in the sample a standard curve with $FeCl_3$ was constructed. Fe (III) ions complexes with xylenol orange and yields a purple colour product visible with a spectrophotometer at 560 nm. The procedure was adapted from Shantha and Decker (1994) [32].

The Fe (III) stock solution for the standard curve was made by dissolving 0.5 g $FeCl_3$ in 50mL 10M HCl and 2 mL of 30 % H_2O_2 was added. The excess H_2O_2 was removed by gently heating the solution during continuous stirring. After cooling to room temperature, the solution was diluted with Milli-Q water to 500 mL. Aliquots of 1- 5 mL of the stock solution were transferred to borosilicate tubes and diluted with ACS-grade methanol to a total volume of 5 mL. A tube containing only methanol was also prepared and intended as a sample blank. From each tube, 100 µL of the diluted stock solution was added to 1.5 mL microcentrifuge tubes and 900 µL of WR was added to all tubes, creating a 1:10 mixture. The tubes were vortexed thoroughly and incubated in room temperature for 10 min. Absorbance was measured at 560 nm with a UV/visible spectrophotometer (Ultrospec 1000, Pharmacia Biotech). The standard curve was constructed by plotting absorbance versus µg of Fe (III) and obtaining a straight line where the linear regression equation was calculated according to

$$y = mx + b \tag{3}$$

The inverse of the slope of the curve, m_i, was used to calculate the peroxide value for the oil samples in further experiments, expressed as milliequivalents of active oxygen/kg [36].

$$Peroxide\ value = \frac{A_s - A_b \cdot m_i}{55.84 \cdot m_{oil} \cdot 2} \tag{4}$$

Where A_s and A_b is the absorbance for the sample and the blank respectively, 55.84 is the molecular weight of iron in µg/µmol, m_{oil} is the mass in grams of the oil and the division by a factor of 2 is required to express the peroxide value as meq of active oxygen, i.e. peroxides, instead of meq of oxygen.

3.2.2. Peroxide value of bilberry seed oil

Peroxide value of the oil was established by dissolving the oil in pure ethanol 1:20-1:50, weight by weight proportion, depending on the degree of oxidation of the oil, and 100 µL of the dissolved oil was transferred to 1.5 mL microcentrifuge tubes. In the same procedure as for the standard curve, 900 µL of WR was added, the samples were mixed and incubated for 10 min in room temperature. A blank sample was also prepared with 100 µL of ethanol and 900 µL of WR. Absorbance was measured at 560 nm and the peroxide value was calculated according to equation 4. All samples were prepared in duplicates.

3.3. Oxidative stability with DSC

The oxidative stability of the bilberry seed oil was measured by thermal analysis using a Mettler- Toledo differential scanning calorimeter, DSC-1. The data was analysed with the STARe-software provided from Mettler-Toledo. The method was developed from the manufacturer's booklet with collected applications for thermal analysis on food [35]. Before analysis, a so called "burn out" was performed to remove potential residues on the DSC sensor from former experiments and the instrument was calibrated with indium. Duplicate oil samples of 5 ± 1 mg were weighed into 40 µL aluminium crucibles without a lid and an empty crucible was used as reference. O_2 at 60 ml/min was used as rector gas and N_2 at 200mL/min as the dry gas. The non-isothermal temperature program started at 25°C and heated to 200°C at 15°C/min. The onset temperature, $T_{on,}$ of the oxidation reaction was determined by the intersection of the extrapolated the baseline and the tangent of the oxidation peak.

3.4. Supercritical fluid extraction in laboratory scale

SFE in the laboratory scale was carried out with a multistage automated system from Teledyne Isco (USA). It consisted of the dual chamber SFX 220 supercritical fluid extractor, SFX 200 controller, Restrictor Temperature Controller, 100 DX syringe pump for CO_2, and a cooling bath for the pump (Julabo F3, Sweden). Figure 10 gives an overview of the system and its components.

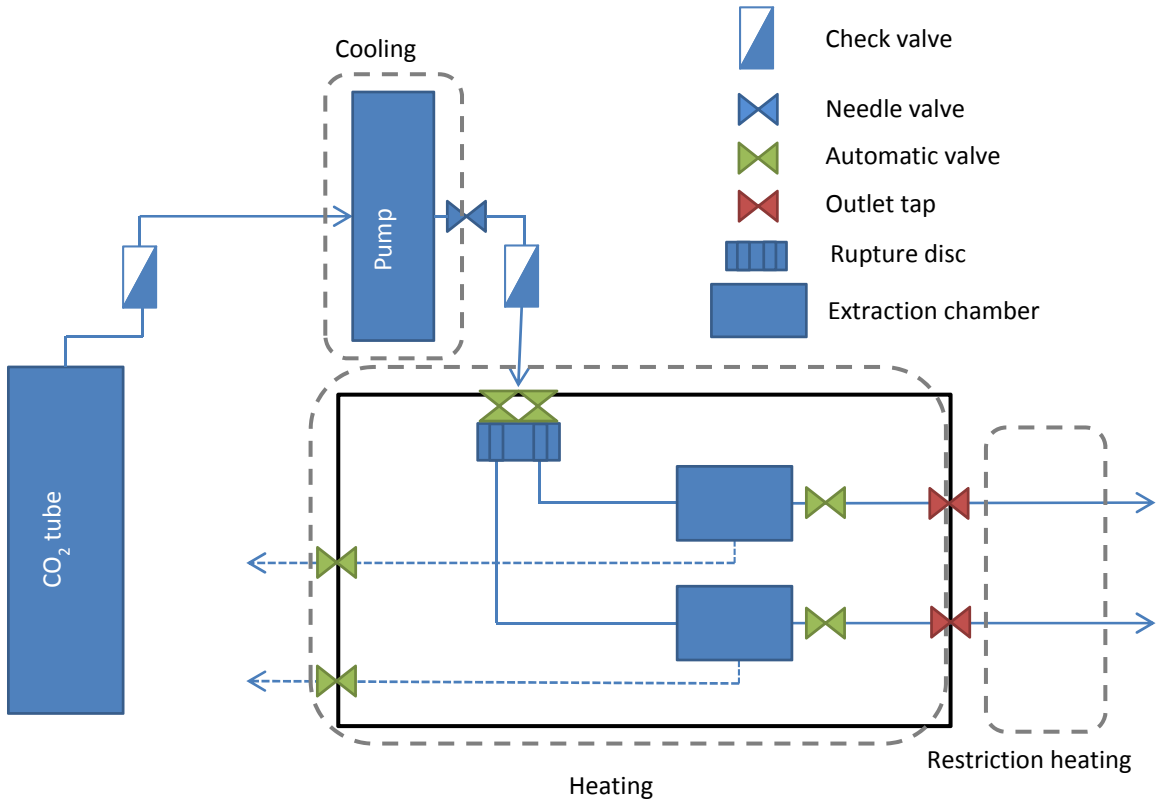

Figure 10 – Schematic image of SFE extraction system used in the laboratory scale experiments. The solid lines indicate the route of CO_2 during extraction, while the dotted lines is the escape route of CO_2 when depressurizing the chambers.

The liquid CO_2 from the tube was continuously cooled to 5°C in the pump by the cooling bath. Pressure and extraction time was controlled by the SFX 200 controller, extraction temperature was set on the SFX 220 supercritical fluid extractor and the same temperature was set on the Restrictor Temperature Controller to maintain the temperature outside the extraction chambers and reduce the cooling effect from the CO_2. Both pressure and temperature settings must be above the critical point for the CO_2.The syringe pump pressurized the CO_2 to the chosen pressure and subsequently pressurized the extraction chamber and the sample. The extract was released through the outlet taps, which also controlled the CO_2 flow rate through the sample. The CO_2 flow rate was shown on the display of the SFX 200 controller and calculated automatically from the volume decrease of CO_2 in the pump.

The samples were prepared according to the methods described in 3.1 and weighed into 10 mL reusable polymer cartridges with caps and filters in both ends, shown in Figure 11, using 2.5 g of bilberry seeds for each extraction. The milled seeds were mixed with 3 mm glass beads (Merck, Germany) to fill up the void volume and helping to avoid agglomeration of the seeds during extraction. Both endcaps were mounted on the cartridge and it was thoroughly shaken to achieve a good mixing. The cartriges was loaded into the extractor and locked in with the extraction chamber cap and the extraction program was started.

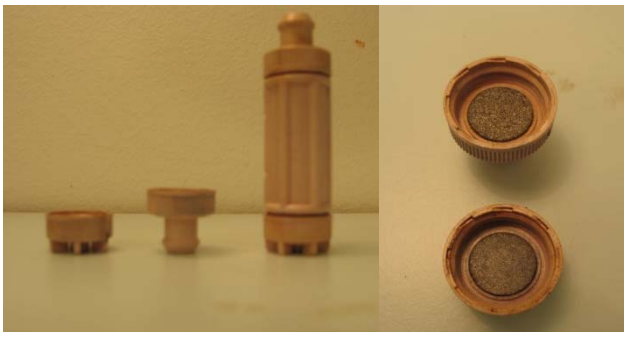

Figure 11 – Polymer cartridge with top and bottom caps with filters.

During the extraction, the oil was collected in two 50 mL falcon tubes that were interchanged with 10 min intervalls and their weights were accounted. The time for extraction was set to 90 min and the flow rate of CO_2 was 2 mL/min for all experiments.

After a finshed extraction run, the yield of extracted bilberry seed oil could be determined in two ways; either by comparing the weight of extracted oil and the sample (5), or compairing weight the weight of the cartrige before and after (6). Differences between the two yields can be due to the loss of volatile compunds that is carried away with the gaseous CO_2 or due to the loss of some extract in tubings of the equipment.

$$Yield_{oil} = \frac{m_{oil}}{m_{sample} \cdot oil\ content} \tag{5}$$

$$Yield_{cartrige} = \frac{m_{weight\ loss}}{m_{oil}} \tag{6}$$

The extractor was cleaned for 15 min using a designated cleaning cartrige filled with ethanol and 3 mm glass beads. The endcap filters were cleaned utrasonically after each extraction using a 1:1 mixture of ethanol and acetone to remove any residual oil traped in the filter.

3.4.1. Experimental design of screening experiments

The experiments in the laboratory scale were designed to optimize extraction yield and investigate which of temperature, pressure or particle size, i.e. milling time, had the most impact on the yield. A full factorial design with two levels, high and low, including a middle point, gave 9 points to evaluate. The high and low levels were chosen with regard to the limitations of the extractor and made sure to be above the critical point of CO_2, the variables and their values are displayed in Table 2.

Table 2 – The variables and the high, low and midpoint values for the factorial design.

Variable	Low level	High level	Middle point
Pressure (bar)	150	300	225
Temperature (°C)	35	60	47.5
Milling time (sec)	10	30	20

The experimental plan was created and evaluated in the statistical software MODDE 9.1 (Umetrics AB), the program provided the individual experimental settings, as seen in Table 3, and randomized the run order.

Table 3 – The experimental settings for the screening experiments in the laboratory extractor.

Experiment name	Pressure (bar)	Temperature (°C)	Milling time (sec)
L1	150	35	10
L2	300	35	10
L3	150	60	10
L4	300	60	10
L5	150	35	30
L6	300	35	30
L7	150	60	30
L8	300	60	30
L9	225	47.5	20

3.5 Supercritical extraction in pilot scale

The equipment to perform SFE in pilot scale was custom built for SIK by SiOx Machines AB (Sollentuna, Sweden). The system was constructed for continuous SFE with liquid CO_2 at a pressure of maximum 300 bar. An overview of the system is shown in Figure 12, where V1-9 are valves, P1-4 are pressure gauges. P1 shows the pressure from the CO_2 bottle, P2 and P3 shows the extraction pressure and P4 should be kept at 15-30 bar to allow CO_2 to transit into

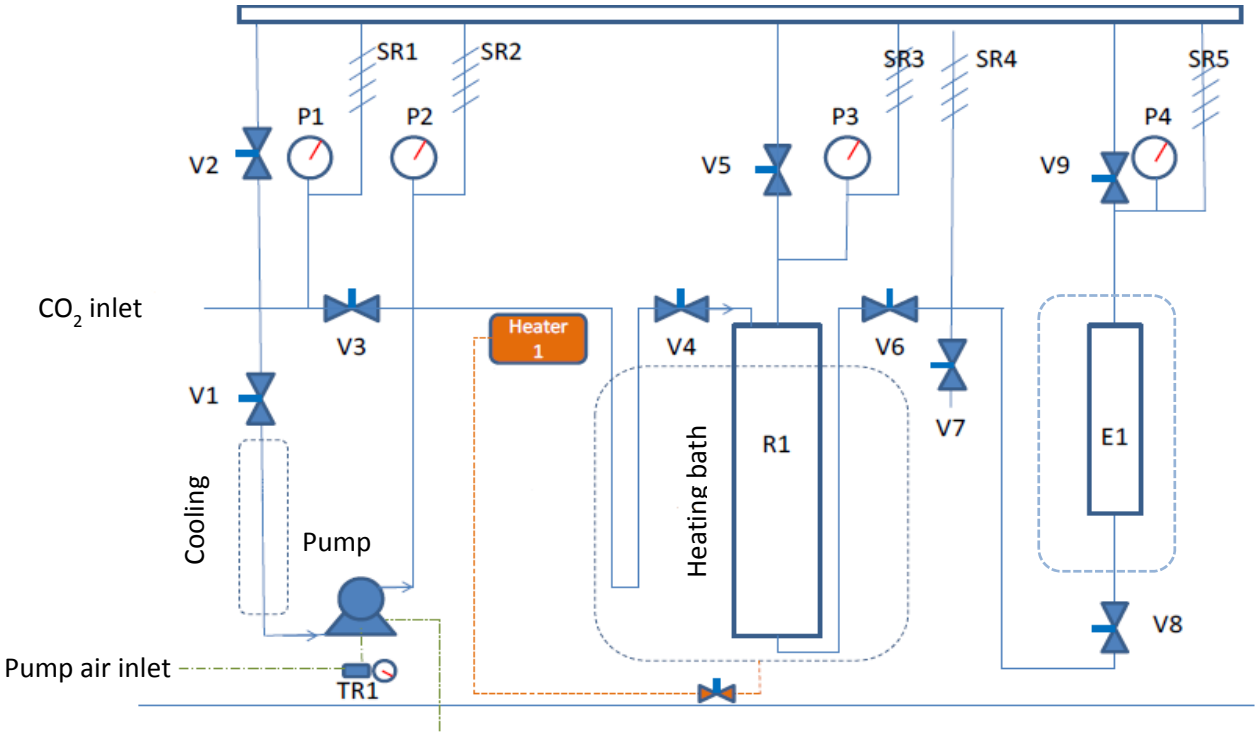

Figure 12 – Overview of the SFE process chart in pilot scale

gaseous phase. SR1-5 air-pressure release systems consisting of an overflow vent and a rupture disc intended to open or break if the pressure in the system is too high. TR1 regulates the air pressure into the pump and thus controls the outlet CO_2 pressure. The pump is cooled to 5°C with the same cooling bath as for the laboratory scale equipment. Heater 1 heats the water bath where the reactor, R1, is placed and set 5°C above the desired extraction temperature to compensate for heat loss into the extraction vessel. Before extraction, R1 is covered with a solid 2” thick stainless-steel lid and sealed with 8 4.5” bolts, cross tightened to 70 Nm with a torque wrench. E1 is the separation vessel where the extracted oil is separated from the gaseous CO_2. E1 is heated by an external heating jacket to compensate for the cooling effect from CO_2 gas.

The bilberry samples were prepared as described in chapter 3.1 and 100g of milled seeds was put into the 1.5 L stainless-steel extraction vessel with filter screw lids in both ends, similar to the cartridges in Figure 11. Glass wool (Merck, Germany) was used to fill up the void volume, to prevent agglomeration of the sample and protect the filters from becoming blocked with small seed particles. The vessel was placed inside R1 before it was sealed with the lid and bolts.

Before initiating the extraction, the system was emptied of air, to reduce the risk of oxidation, by opening up the CO_2 bottle and V1 completely; V4 was opened until P3 showed about 10 bar. V6, V8 and V9 were opened to flush the system with CO_2.

V2 was opened and closed quickly and V5 completely was opened to let the remaining gas escape. This procedure was repeated twice to ensure that no air remained in the system.

To pressurize the system, all valves except V1, V4 and V6 were closed and TR1 was adjusted to increase the air pressure and thereby making it pumping CO_2. TR1 was regulated until desired extraction pressure was reached in P2 and P3. The time (30 min) to reach extraction pressure can be regarded as static time. Extraction began when V8 was opened and the extract could enter E1. CO_2 flow for the majority of pilot scale extractions was 30 g/min and the flow was regulated by TR1 and the opening degree on V8. Flow rate was continuously calculated from the weight decrease of the CO_2 tube over time, with an excel macro (SiOx machines AB, Anders Marcusson) and a scale (VETEK VB2-200-20) with corresponding software (VETEK WeighSoft 1.0) for the tube. Due to the 30 min static time, the dynamic extraction time was set to 60 min to achieve a corresponding time compared to the laboratory scale system. Extraction was terminated by closing the CO_2 tube and TR1 and thus stopping the pump. The remaining CO_2 in the system was escaped through V9 and V5. The extracted oil in E1 had to be thawed before collecting, due to the large cooling effect from the escaping CO_2, when depressurizing the system. Oil yield was calculated according to equation 5 and 6.

Since the extraction system was new, its limitations have not been evaluated fully and difficulties were inevitable. Optimal flow rate, particle size, sample collection and how to avoid formation of dry ice (solid CO_2) were not known beforehand and each extraction was used as a learning opportunity.

3.5.1. Experimental design of pilot scale experiments

The experimental settings in the pilot scale were selected to reflect the extractions in laboratory scale. However, considering the increased sample volume only one particle size was chosen for all experiments and the low temperature was set to 40°C to ensure supercritical CO_2 during the extraction. A pressure of 250 bar instead of 300 was also applied in some cases in order to facilitate the extraction procedure. The experiments performed in pilot scale are compiled in in Table 4.

Table 4 – Extraction settings for experiments in pilot scale. Particle size was kept constant for the majority of extractions.

Experiment name	Pressure (bar)	Temperature (°C)
P1	150	40
P2	200	40
P3	225	47.5
P4	250	40
P5	250	60
P6	300	40

3.6 Storage evaluation of bilberry seed oil

To assess the storage stability of the extracted seed oil from the pilot scale extractor, the oil from experiment P4 (see Table 4) was chosen to be evaluated. The oil was divided equally into 4 glass vials with plastic snap-lids. The vials were stored under different conditions for 4 weeks. Every week, the oils were analyzed with regard to peroxide value and oxidative stability, using the methods described in 3.2 and 3.3. The selected conditions were chosen because of their probability to prevent oxidation compared to a control sample. The conditions included cold storage (4°C), antioxidant addition (500 ppm α-tocopherol) and nitrogen atmosphere, where the oil was flushed with N_2 for 2 min. All samples, besides the cold storage, was stored in room temperature and kept away from light. The different storage conditions are listed in Table 5 . At the day of analysis, enough sample to analyze peroxide value and oxidative stability was removed from the vials and the individual storage conditions was restored as soon as possible.

Table 5 – Overview and specification of the storage conditions used in the storage evaluation

Storage condition	Storage specification
Control	Dark, room temperature
Cold	Dark, 4°C
Antioxidant	Dark, 500 ppm α-tocopherol
Nitrogen atmosphere	Dark, 2 min flush with N_2

4. Results and discussion

This chapter will present the obtained results from the experimental part of this master's thesis, including a discussion of aforementioned results. The results will be presented and discussed in the same order as they were described in chapter 3. Results from the supercritical extraction in the pilot scale will also include a section about the method development associated to the extractions.

4.1 Drying and particle size determination

Replicable results regarding the dry content of the thawed bilberry seed was achieved for all batches of seeds used in both the laboratory and pilot scale. Also, the remaining water content correlated well with the accredited laboratory analysis performed by Eurofins during the "Bärkraft" project, see appendix I. The mean weight loss after drying the wet seeds in the convection oven and remaining water content after drying in the vacuum oven is presented in Table 6.

Table 6 – Percentage weight loss after drying in convection and vacuum oven. The weight loss after drying in vacuum oven represents the total water content of the seeds after drying.

Weight loss after drying in convection oven	59.8 %
Weight loss after drying in vacuum oven	3.7 %

Distribution of particle size after milling seeds for laboratory and pilot scale extractions are presented in Figure 13 a and b.

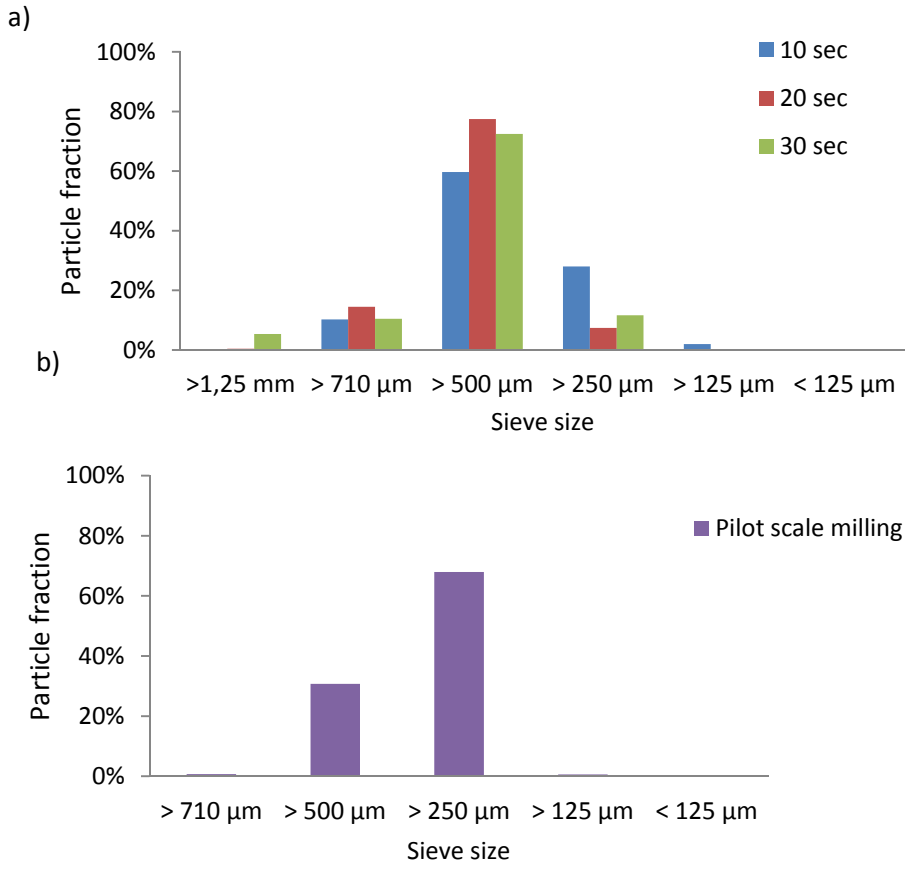

Figure 13 – Distribution of particles after milling for a) laboratory and b) pilot scale respectively.

The distribution of particle sizes of the seeds after different milling times (10, 20, 30 sec) with the knife mill seem to have a similar distribution and a relatively wide range of sizes. The particle size of the seeds after 30 sec was expected to have a high amount of smaller particles (<500 µm) compared to 10 and 20 sec milling, and that was not the case with regard to the seed distribution in the sieves after analysis. However, a visual inspection of the sieves showed that the seeds milled for 30 sec were more prone to agglomeration due to a high amount of released oil. The higher percentage of particles larger than 1.25 mm for 30 sec milling compared to 10 and 20 sec demonstrates this behavior. The particles in that sieve were essentially oil and powdery seeds particles clumped together. These clumps could be observed throughout the sieves and indicate that a higher percentage of the particles actually was smaller than 500 µm. The same behavior was not observed for the other milling times.

Milling at a fixed setting with the flat burr grinder (Figure 13 b) resulted in an even distribution of particles and no agglomeration of particles in the sieves was observed. The particle distribution with the burr grinder is not comparable to the distribution profiles in the knife mill but it gives a better opportunity to control the particle size in extraction experiments.

4.2 Quantitative peroxide assay standard curves

Two standard curves were constructed as described in 3.2.1 as the peroxy kit had to be replaced during the thesis, resulting in the use of two different batches. The curves from the linear regression show slightly different slopes as seen in Figure 14 but are generally quite similar. The slope will have a large impact on PV since the inverse (1/slope) is used for PV calculation. The detection limit of the kit seems to be approximately 10 µg of Fe^{3+} as no linearity was found for standard solutions containing less iron.

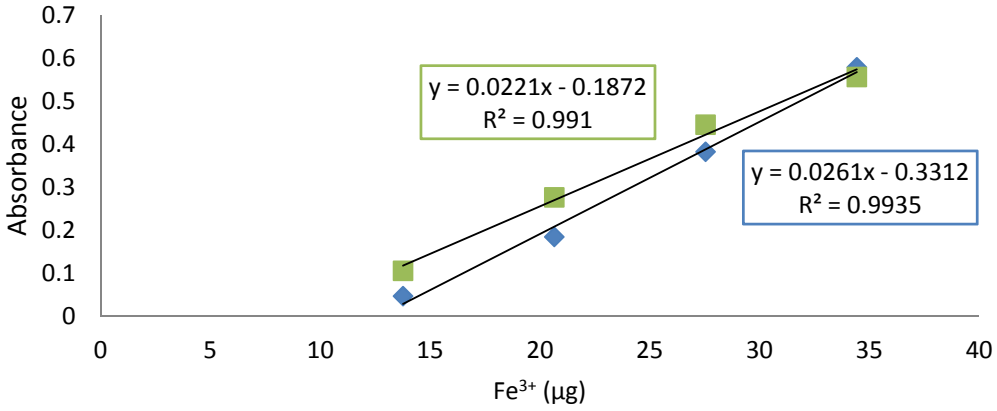

Figure 14 – Standard curves constructed for calculation of PV in bilberry seed oil

4.2. Yield of oil in laboratory scale SFE

The yield from each extraction in the laboratory scale was calculated according to equation 5. Extractions L2, L4 and L8 was performed in duplicates while the middle point L9 was made in triplicates. Though there was every intention to also perform duplicate experiments of L6, it was not possible due to malfunctioning equipment. The duplicate extractions all had large standard deviations (>10 %) while the triplicate extraction had a standard deviation of just 2 %. The large standard deviation could be explained by irregularities with the equipment during extraction, improper cleaning between extractions, small sample volumes or possibly the change of carbon dioxide quality (5.4 to 4.1 purity) between the duplicate extractions. The

duplicate extractions were also performed 3 months apart since the equipment had to undergo reparations, and the yields after the reparations was significantly lower than before. Natural variations in the fat content among the seeds could also have an impact when calculating the oil yield.

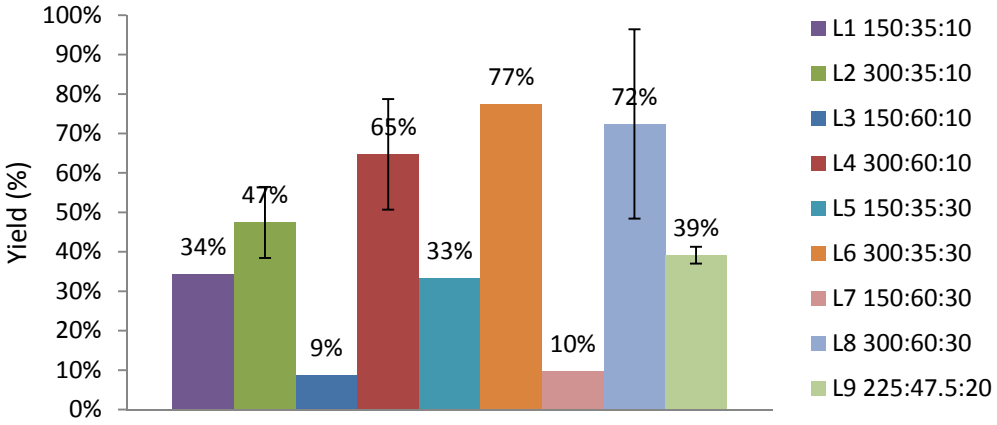

Figure 15 – Yields of all SFE extractions in laboratory scale, presented in the same order as described in Table 3. The yield is calculated as weight extracted oil per weight oil in the seeds. Error bars represent the standard deviation of replicates and triplicates on L2, L4, L8 and L9. Extraction settings are presented according to pressure (bar): temperature (°C): milling time (sec).

The highest extraction yield of 77 % was seemingly obtained in L6 at 300 bar, 35°C and 30 sec milling time as seen in Figure 15. However, considering the large error bars of L2, L4 and L8 could imply that there could be a similar error in L6 if the extraction could have been repeated, and consequently making L8 the best extraction setting with regard to yield. It is difficult to draw conclusions about the yield from the weight loss in the cartridges as the yield with regard to oil content of the seeds could be greater than 100 % even when taking potential loss of water into account as there could be other unknown compounds lost via extraction and evaporation. The oil yield based on the weight loss in the cartridges is presented in appendix III. A notable result is the large difference of the actual extracted oil in the replicate of L8 compared to the cartridge weight loss where the weight loss in the cartridge was twice as large as the actual extracted oil. While the difference in the first extraction of L8 is significantly less. Therefore, the large standard deviation could potentially be explained by an unexpected loss of oil and L8 would theoretically have the highest yield as the conditions were the most promising in a thermodynamic sense.

4.2.1 Impact of process conditions

To determine the impact of the process conditions, the extraction yields were analyzed in the statistical software MODDE 9.1 using multiple linear regression analysis to determine which of the three process conditions had the most impact on yield in laboratory scale. The coefficient of determination (R^2) of the model for predicted versus observed yields was 0.981 and the relative standard deviation (RSD) was 6.9 % which suggests that the model is acceptable but not perfect. If more replicates had been performed for each extraction, the model would likely have a better fit. A plot of the influence for each parameter, pressure, temperature and milling time, is displayed in Figure 16 with a confidence interval of 95 %. The combined effects were also calculated. A parameter has a significant impact if the error bar lies completely on either the positive or negative side of the y-axis. The graph shows the impact of the parameter on extraction yield and how it is influenced when using either the high or low value of the parameter. A large positive effect would therefore imply that yield increases with increased parameter value while a large negative effect would suggest that the yield increases with a decreased value of the parameter. As seen in Figure 16, pressure is the only parameter that has significant effect on extraction yield. All other parameters, whether single or combined, cannot give conclusive information about their influence.

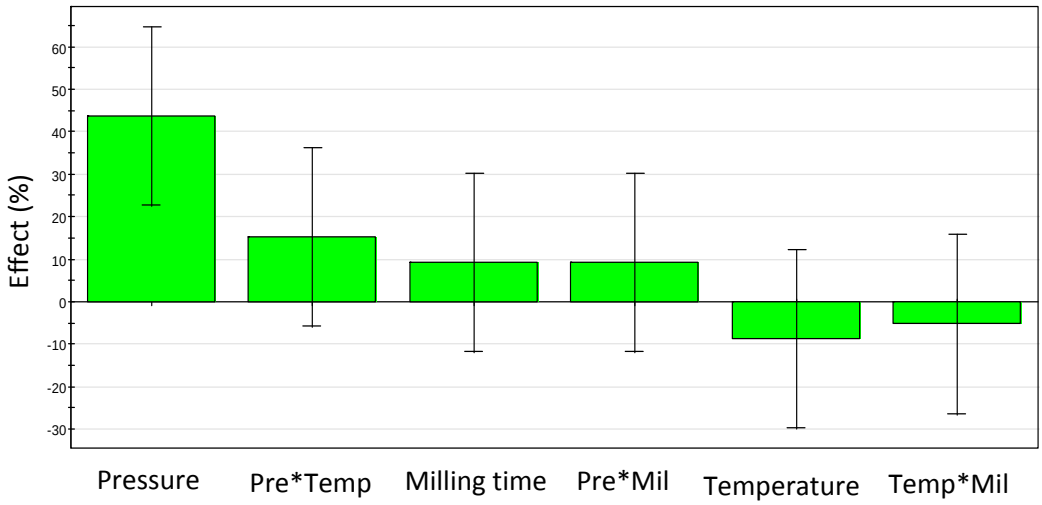

Figure 16 – Effect plot of single and combined parameters pressure, temperature and milling time with regard to extraction yield.

More information about the extraction behavior at different experimental settings could be obtained by studying the extraction curves. By keeping one parameter constant, at either high or low level, it is easier to assess the effect of that particular parameter. The extraction curves are presented in 3 pairs below in Figure 17, Figure 18 and Figure 19.

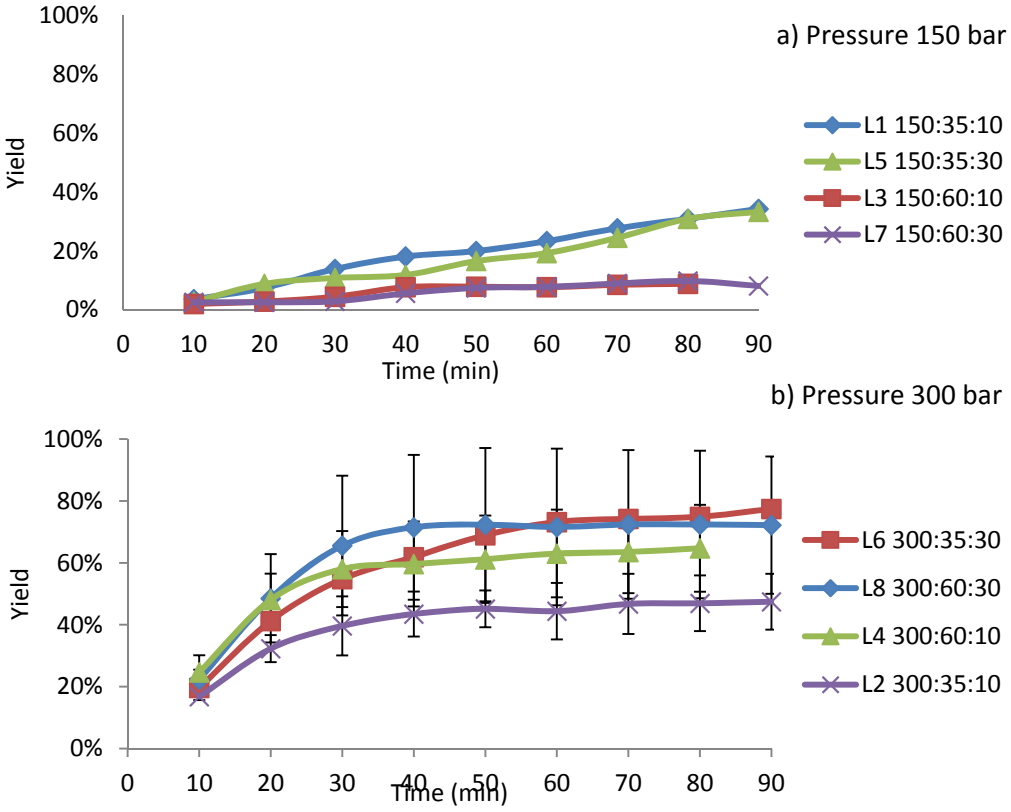

Figure 17 – Compilation of extractions at a) 150 and b) 300bar

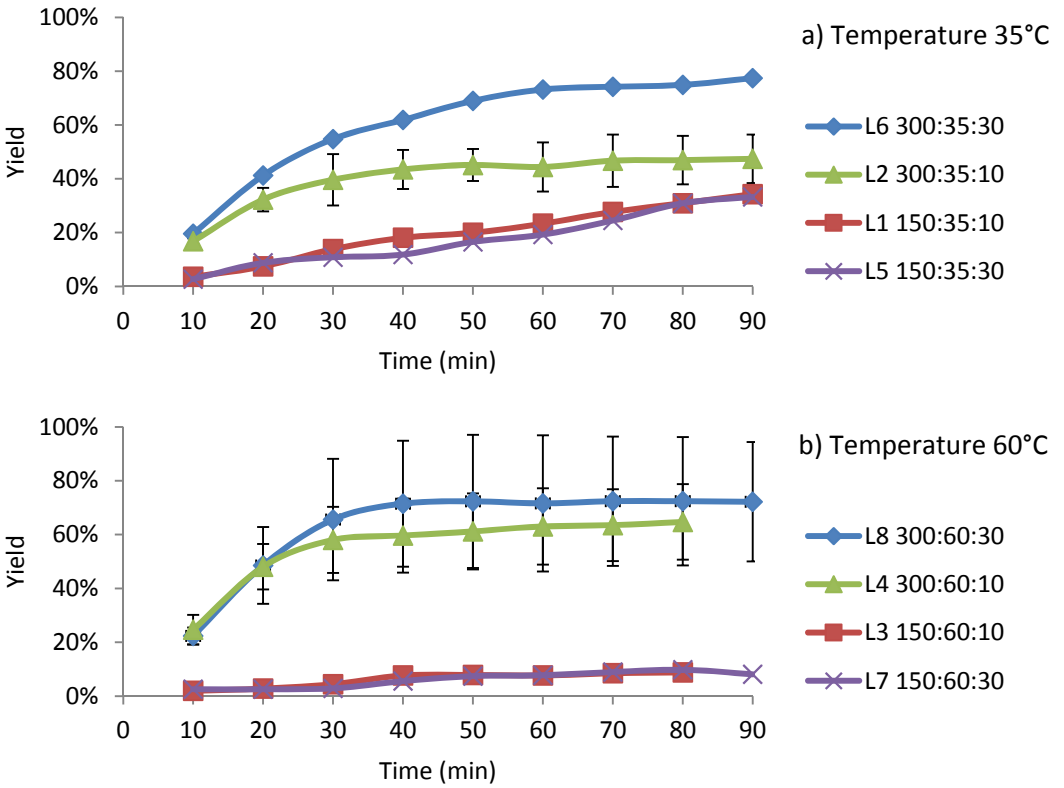

Figure 18 – Compilation of extractions at a) 35°C and b) 60°C

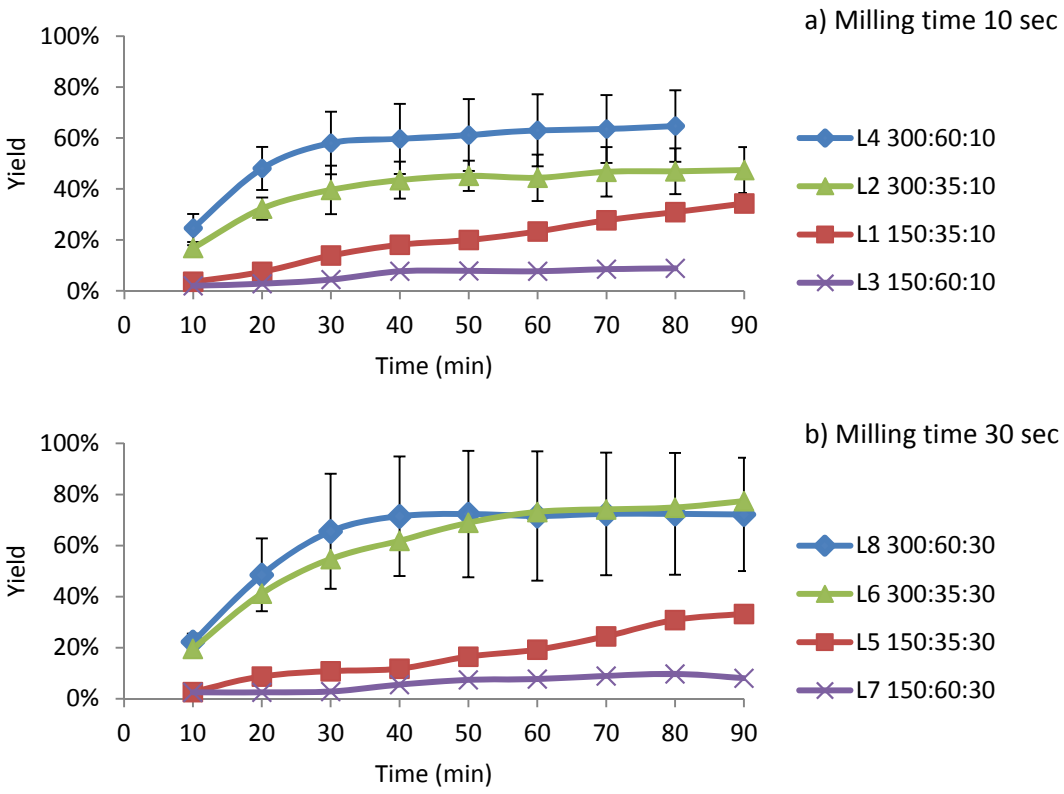

Figure 19 – Compilation of extractions with a) 10 sec milling time and b) 30 sec milling time

It is obvious from Figure 17 that pressure has the largest impact on extraction yield and a high pressure gives a high yield, explained by a better solubility of CO_2 at high pressures. The increase in yield over time for the low pressure is basically linear regardless of the temperature and milling time. It is interesting to note that a lower temperature gives a higher yield compared to the high temperature, a phenomenon only observed at low pressures. The phenomenon could be explained by the decrease of supercritical CO_2 solubility at increased temperatures when extracting at low pressures as explained by Brunner (2005) [15]. A pressure of 300 bar shows a different behavior; where the first 30 min has the same linear slope as for extractions at 150 bar, and from 30- 90 min the slopes planes out. The straight line represents a constant extraction rate, caused by a constant mass transfer resistance from the seeds and the flat part of the curve is where the yield approaches maximum yield for that extraction setting. The total amount of extractable oil is dependent on the availability of oil in the sample and the highest yields were achieved when using a milling time of 30 sec where more oil has been released from the seeds. The effect of temperature is also noticeable in Figure 17 b, where yield decreased with a lower temperature in the first hand and secondly the yield decreased when using the short milling time.

The same behaviors are observed in Figure 18 a and b with constant temperature, where pressure has greatest impact on the yield followed by milling time. Milling time has seemingly less impact at lower pressures regardless of the temperature. Solubility of CO_2 in the seeds at the low pressure is presumably the limiting factor, as described above. Figure 19 a and b also confirms previously stated theories, high pressures gives high yield, and at high pressure a higher temperature gives better yield. A longer milling time unmistakably facilitates extraction at high pressure while the effect is lost at low pressures since solubility of CO_2 is insufficient.

The triplicate extraction of the middle point showed that it is possible to achieve good reproducibility with the laboratory scale extractor. It was observed that a steady flow rate throughout runs and thorough cleaning of the equipment between each run was important. The seeds for the middle point were not from the same drying batch and the seeds were milled fresh for each extraction. However, the extractions were performed in the same week and after the change to the lower CO_2 purity. As seen in Figure 20 the extraction behavior is more similar to the low-pressure extractions but with slightly higher yield.

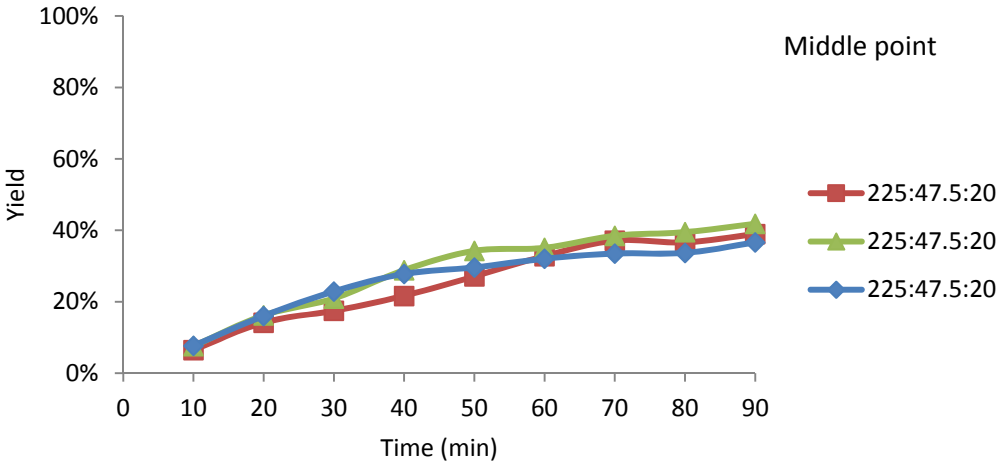

Figure 20 – Triplicate extractions at the same extraction settings to examine reproducibility

4.3. Yield of oil in pilot scale SFE

As shown in Figure 21, it is much more difficult to discern patterns in the oil yields from the pilot scale extractions and it is mostly explained by inexperience of operating the equipment and every step of the process, from pressurizing to collecting the oil, had an element of "learning by doing" as unexpected events often occurred. These events and method development behind the pilot scale extraction are further discussed in chapter 4.3.1

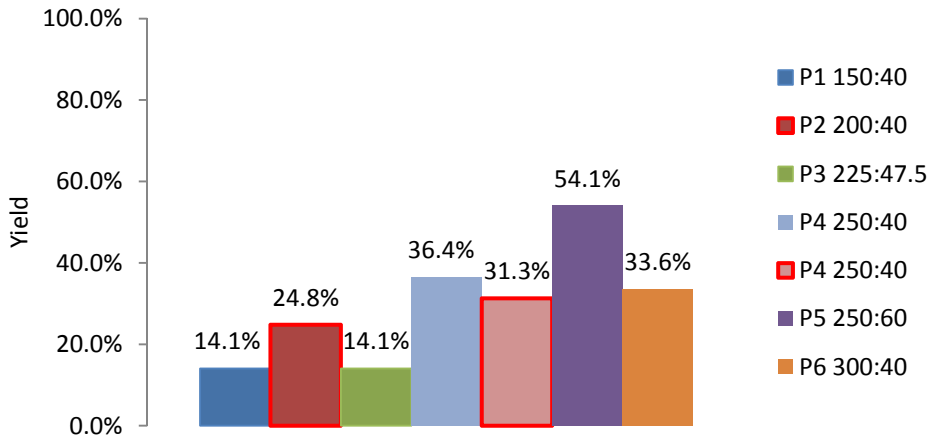

Figure 21 – Yields from all extractions in the pilot scale equipment based on weight of extracted oil per weight available oil in the seeds. Red-marked bars represent possibly misleading yields due to complications during the extraction. Process conditions are presented as pressure (bar): temperature (°C).

The yield was determined by assuming the amount of available oil in the seeds to be 23.7 g/100 g seed, as according to the nutritional analysis of dried bilberry seeds presented in Appendix I. The highest yield from the pilot scale extractions was achieved when the pressure was set to 250 bar and the temperature was 60°C. Unfortunately, this was the only high temperature extraction performed due to time constraints, but by applying the same argumentation as for the laboratory scale extractions a high temperature favors a higher yield for high pressures. The extractions using 300 bar and 40°C was not significantly different from lower pressures at the same temperature which could be explained by difficulties when operating the equipment at high pressures, maintaining a sufficient flow rate was particularly troublesome. The red-marked P2 200:40 had a higher oil yield compared to the yield calculated from the extraction vessel, which was most likely due to residual ethanol in the system from the cleaning process. The other red-marked bar (P4 250:40) represents the yield calculated from the extraction vessel and not the oil yield, as the oil was not weighed before it was divided into individual glass vials for the storage evaluation.

4.3.1 Method development of supercritical extraction in pilot scale

The SiOx SFE equipment was constructed in a logic and straight forward way, where the pump and valves dictated the extraction procedure. As opposed to the laboratory scale equipment, both the pump and valves had to be adjusted manually which influenced both pressure and flow rate considerably so the two systems will never be fully comparable. If the flow rate is too low or unstable, the extraction will be inefficient and it will be difficult to draw conclusions about the other extraction parameters. A lot of experience and knowledge about the system is necessary in order to run a stable extraction. The visual differences of some of the extracted oil are shown in Figure 22 and demonstrate the diversity among the extractions.

There are clear differences in yield among the extractions but also with regard to colour, haziness as well as phase separation.

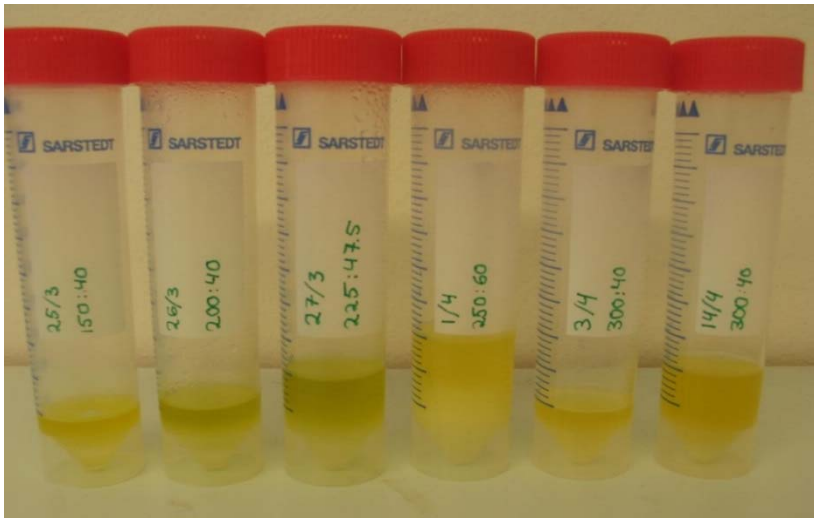

Figure 22 – A selection of extracted oil from the pilot scale equipment.

Particle size is also an important parameter to consider, too large (whole seeds) particles and the supercritical CO_2 will not be able to extract the oil. Small particles increase the risk of channeling inside the extraction vessel and clogging the filters. Channeling is clearly visible when emptying the vessel and extracted material will have lost its initial dark blue-purple colour and transformed into a pale white-purple colour. An example of when substantial channeling occurred is displayed in Figure 23.

Figure 23 – Channeling in the extraction vessel, the darker material has not been exposed to CO_2, while the light material has been properly extracted.

Channeling and clogging of the filters can be avoided by controlling the particle size and incorporating an inert material among the seeds. The filters should also be protected by glass wool to avoid contact with the seed particles.

Another problem that arose during several extractions was the formation of dry ice in the pipes. Dry ice was formed when the flow of supercritical CO_2 was too high and the separation vessel fills up with liquid CO_2. Both liquid and oil could then go up in the pipes.

Expansion of the liquid when it passes the expansion valve V9 causes a sudden phase change into solid CO_2 that could potentially destroy the pipes. If dry ice is formed, the extraction needs to be paused to allow the CO_2 to boil of. Figure 24 shows an example of when dry ice was formed while depressurizing the system and causing the seeds to flow up to the top filter lid. In this case, V5 was opened when the reactor was still above supercritical pressure and the CO_2 filled up the pipes above the reactor.

Figure 24 – Bilberry seeds on the top filter of the extraction vessel after formation of dry ice during depressurization of the reactor.

Dry ice can be avoided by carefully controlling the flow rate (30 ± 10 mL/min) during extraction, depressurize the system slowly and making sure the flow is not restricted at V9.

Depressurizing the extractor causes the separation vessel to cool rapidly when the CO_2 escapes through V8 and V9, temperatures below - 40°C were not unusual despite the heating jacket around the separation vessel. The low temperature made sample collection difficult. The stainless-steel separation vessel was well isolated and often took >3 h to thaw the sample in room temperature. Excessive heating was avoided to speed up the thawing to avoid lipid oxidation as long as possible. However, when thawing in room temperature, the oil will be exposed to oxygen which also can initiate oxidation. An optimal sampling method has yet to be developed.

4.4 Storage evaluation of bilberry seed oil

The different storage conditions were evaluated by determining the peroxide value and the onset temperature of oxidation once a week for 4 weeks. A high peroxide value indicates a higher degree of oxidation while a low T_{on} suggests greater oxidative instability and thus more oxidation. The analysis of peroxide value offered clear indications on how the different storage conditions affected the degree of lipid oxidation; the change in peroxide values over 4 weeks is presented in Figure 25.

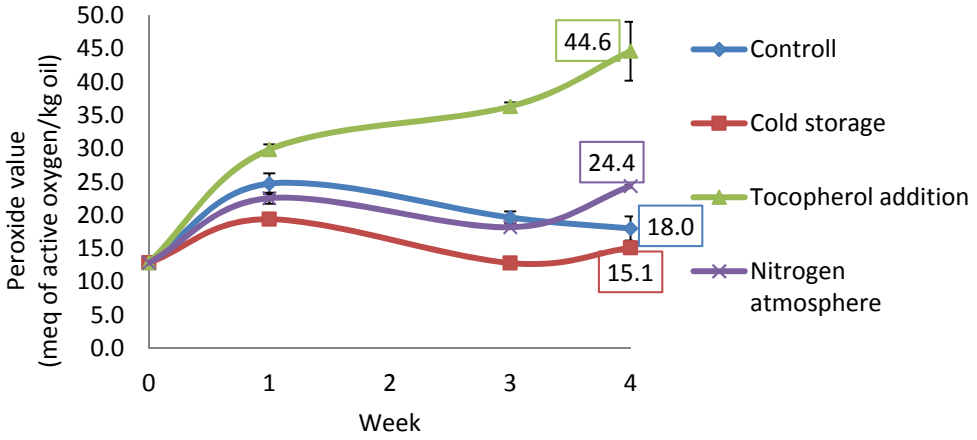

Figure 25 – Evaluation of peroxide values over 4 weeks with bilberry seed oil stored in room temperature, at 4°C, with addition of α-tocopherol and under a nitrogen atmosphere.

The most surprising result is the outstanding peroxide value of the oil with 500 ppm α-tocopherol which exhibited a PV of 44.6 meq of active oxygen/kg oil after 4 weeks. It was originally believed to have the lowest PV since its ability to chemically inhibit the propagation of oxidation if added in correct concentration. However, it seems that a concentration of 500 ppm was too high and initiated tocopherol mediated oxidation instead [29]. Furthermore, the most successful storage condition was obtained in cold storage even though the control sample had similar PV after 4 weeks. Oxidation reactions will progress slower at a lower temperature so therefore this result was expected. The oil treated with nitrogen showed potential of inhibiting lipid oxidation compared to the control but because of the weekly opening and resealing with fresh nitrogen there was probably enough oxygen in the oil to cause oxidation. The upper limit of PV for edible oils for retail sales is 20 of meq active oxygen/ kg oil and these results shows that it is possible to keep even a small volume of unrefined bilberry seed oil below this value for 4 weeks when it is stored dark and cold.

The oil showed changes in solubility after one week of storage, it was considerable more soluble in ethanol compared to the week before. This change indicates changes in the lipid chemistry; long FA chains may have been fragmented thru oxidation reactions into shorter chains that are more soluble. The peak in PV after one week could be a consequence of the fact that the FOX method only can measure primary oxidation products i.e. peroxides formed during the propagation strep of lipid oxidation and after 2 or more weeks the peroxides may have formed stable compounds, undetectable by the method. However, to assess every aspect of the entirety of the numerous oxidation reactions is impossible and the FOX method shows excellent reproducibility, especially for less oxidized oils and serves as a good tool to assess lipid oxidation in bilberry seed oil.

The onset temperatures for oxidation showed less conclusive results for the different storage conditions data presented in Table 7. The highest T_{on} was expected for the oil with added α-tocopherol as it was thought to be the most protected against oxidation. The onset temperature at week 0 gave a T_{on} of 165 °C, while at week 4 the difference between the different storage conditions was too small to be able to draw any practical conclusions about the results (p-value > 0.05 calculated with a two-tailed paired Student's t-test). The small sample size and high surface to volume ratio in the DSC crucible could lead to a decrease in oxidative stability due to oxidation during sample preparation and while the oil is awaiting analysis.

However, studies of fresh olive and sunflower oil with PV< 0.5 had T_{on} of approximately 190°C, which shows that oils of different composition can demonstrate a higher oxidative stability [37]. The only correlation of the peroxide value and onset temperature was seen with the oil treated with α-tocopherol that showed a slight decrease in T_{on} compared to the other oils but this effect was not as pronounced as for the peroxide value.

Table 7 – Onset temperature of oxidation for bilberry seed oil stored 4 weeks under different conditions

Storage condition	T_{on} week 0 (°C)	T_{on} week 1 (°C)	T_{on} week 2 (°C)	T_{on} week 3 (°C)	T_{on} week 4 (°C)
Control	165.4	164.0	165.6	167.1	164.8
Cold		165.7	165.1	164.5	163.7
Nitrogen atmosphere		165.2	166.7	165.3	165.0
α-tocopherol addition		164.4	165.1	161.7	161.7

Differences in onset temperature of oil extracted at a high or low temperature was also evaluated by analyzing freshly extracted oil from L2 and L4 (300:35:10 and 300:60:10 respectively) to further evaluate if DSC could detect differences in T_{on} for different process conditions. A significant difference in T_{on} was observed for the two extraction settings where L4, surprisingly, had the highest $T_{on,}$ see Table 8.

Table 8 – Onset temperature of oil extracted in laboratory scale at low and high temperature

L2 T_{on} (°C)	L4 T_{on} (°C)
163.6	173.8

Hypothetically, oil extracted at a higher temperature could be more prone to oxidation during the extraction process as a high temperature could help initiating oxidation reactions. In this case, a difference in the oil compositions is more likely. A higher amount of lipid soluble antioxidants could have been extracted a 60°C compared to 35°C and thus increasing its oxidative stability [38].

6. Conclusions

Supercritical fluid extraction of bilberry seed oil is a combination of an unconventional technique with a unique material, producing a rare product; a product that could appeal the food industry as well as the cosmetic or pharmaceutical industry, and reinvents a waste product at the same time. The environmentally sustainable and clean extraction technique is timely and gains interest on an international level. The technique must also be economically sustainable and the oil needs to be storable to maintain quality over time.

The laboratory scale extractions showed the influence of the different process parameters on yield and oil quality and gave valuable information on how to optimize the extraction. High pressure and temperature together with a large fraction of available oil in the milled seeds are key points for successful extraction, where pressure has the most influence. There is room for improvement in the preparation process of the bilberry seeds by controlling the particle size more carefully to release a higher amount of oil from the seeds and thus increasing the yield. A significant change was observed in oxidative stability when extracting at 60°C compared to 35°C which was unexpected but could be explained by a higher concentration of lipid soluble antioxidants when extracting at a higher temperature, a phenomenon observed in similar studies.

Pilot scale extraction proved to be a difficult process to operate, so therefore a lot of time and effort was spent on developing the method and not enough time could be spent on optimizing the yield. However, since this equipment is a long-term investment for SIK and has potential to extract oil from numerous other materials and any knowledge on how to operate the equipment more efficiently will be valuable for future projects. A probable limiting factor in the pilot scale extractions is the solvent-to-material ratio which is less than half of the optimal ratio recommended by the literature. A high flow rate increased the risk of dry ice formation in the system which could disrupt the entire extraction. More method development is necessary to reach the same efficient extraction as for the laboratory scale equipment.

Changes in the oil quality during storage as an effect of different storage conditions were easily observed when using the FOX method, but there was not enough variation in the peroxide value to see a significant correlation to the onset temperatures from the DSC. Assessing lipid oxidation by the FOX method and DSC have not been used at SIK before and both show good prospects of rapidly determining the PV while using very small volumes or of investigating differences in onset temperatures of oils extracted at different process conditions. The most successful storage condition was low temperature and darkness. The low temperature slowed down the rate of oxidative reactions. In addition, cold stored samples required least treatment, i.e. no N_2 flush or addition of α-tocopherol, and could be kept in a stable environment for a long time. Addition of α-tocopherol needs to be revised since the treatment lead to the highest degree of oxidation by far. The seed oil is likely to contain tocopherols and is thus already protected from oxidations to some degree.

Supercritical fluid extraction of seed oils is an exciting area of research, providing oils with valuable functional properties with a wide area application while using an environmentally friendly technique. There are good prospects of optimizing the extraction with regard to both efficiency and quality for the extraction of bilberry seed oil.

7. Future work

From the results and conlusions there are several possibilites for future research within the field of supercritical extraction and properties of bilberry seed oil.

- Perform more laboartory scale extraction to validate results regarding yield and oil quality.
- Improving sample preparation in terms of drying time and water content of the seeds to increase avaliability of oil to achive a higher yield. Lyophilisation would be an interesting option due to its ability to break cellwalls.
- Optimize storage conditions by evaluating addition of antioxidants in different concentration and store the oil under a CO_2 atmosphere to further protect the oil during long term storage.
- More extensive method development of the pilot scale extraction equipment and performe more extractions at high temperatures.
- Analyze composition of the bilberry seed oil to confirm the theory about extraction more antioxidants at higher temperatures.
- Develop products and/or specific applications for the bilberry seed oil.

Bibliography

1. Mirtoselect, *Flesh color and crown differences in bilberries and blueberries* 2010: www.mirtoselect.info.
2. Coultate, T.P., *Food - The Chemistry of its Components (5th Edition)*. 2009, Royal Society of Chemistry.
3. SIK. *Bärkraft-Blåbär*. 2013 [cited 2014 January 17]; Available from: http://www.barkraft-sik.se/sv/bar/Sidor/blåbär.aspx.
4. SIK. *Bärkraft-Projekt/aktiviteter*. 2012 [cited 2013 January 21]; Available from: http://www.barkraft-sik.se/sv/projektaktiviteter/Sidor/default.aspx.
5. Naturvårdsverket. *Minskad mängd matavfall – förslag till nytt etappmål i miljömålssystemet*. 2013; Available from: http://www.naturvardsverket.se/Miljoarbete-i-samhallet/Miljoarbete-i-Sverige/Regeringsuppdrag/Redovisade-2013/Fem-nya-etappmal/Minskad-mangd-matavfall/.
6. Yang, B., et al., *Composition and antioxidative activities of supercritical CO2-extracted oils from seeds and soft parts of northern berries.* Food Research International, 2011. **44**: p. 2009-2017.
7. Mariod, A.A., B. Matthäus, and M. Ismail, *Comparison of Supercritical Fluid and Hexane Extraction Methods in Extracting Kenaf (Hibiscus cannabinus) Seed Oil Lipids.* Journal of the American Oil Chemists' Society, 2011. **88**: p. 931-935.
8. Sánchez-Vicente, Y., et al., *Supercritical fluid extraction of peach (Prunus persica) seed oil using carbon dioxide and ethanol.* The Journal of Supercritical Fluids, 2009. **49**: p. 167-173.
9. Beveridge, T.H.J., et al., *Yield and Composition of Grape Seed Oils Extracted by Supercritical Carbon Dioxide and Petroleum Ether: Varietal Effects.* Journal of Agricultural and Food Chemistry, 2005. **53**(5): p. 1799-1804.
10. Huang, A.H.C., *Structure of plant seed oil bodies.* Current Opinion in Structural Biology, 1994. **4**(4): p. 493-498.
11. Hamm, W., R.J. Hamilton, and G. Calliauw, eds. *Edible Oil Processing (2nd Edition)*. 2013, Wiley: Somerset, NJ, USA.
12. Tracy, T.S., *Bilberry*, in *Herbal Products*, T. Tracy and R. Kingston, Editors. 2007, Humana Press. p. 259-268.
13. Helbig, D., et al., *Berry seed press residues and their valuable ingredients with special regard to black currant seed press residues.* Food Chemistry, 2008. **111**(4): p. 1043-1049.
14. Rozzi, N.L. and R.K. Singh, *Supercritical Fluids and the Food Industry.* Comprehensive Reviews in Food Science and Food Safety, 2002. **1**(1): p. 33-44.
15. Brunner, G., *Supercritical fluids: technology and application to food processing.* Journal of Food Engineering, 2005. **67**(1–2): p. 21-33.
16. Brunner, G., *Extraction of Substances with Supercritical Fluids from Solid Substrates*, in *Gas Extraction*. 1994, Steinkopff. p. 179-250.
17. Honary, L., P. Johnston, and E. Richter, *Biobased Lubricants and Greases : Technology and Products*. 2011, Hoboken, NJ, USA: Wiley.
18. Knothe, G. and R.O. Dunn, *A Comprehensive Evaluation of the Melting Points of Fatty Acids and Esters Determined by Differential Scanning Calorimetry.* JAOCS, Journal of the American Oil Chemists' Society, 2009. **86**(9): p. 843-856.
19. SLV, *Livsmedelsverkets livsmedelsdatabas*. 2014.
20. Pehlivan, E., et al., *Determination of some inorganic metals in edible vegetable oils by ICP-AES.* Grasas Y Aceites, 2008. **59**(3): p. 239-244.
21. Karen, S., *Lipid Oxidation in Specialty Oils*, in *Nutraceutical and Specialty Lipids and their Co-Products*. 2006, CRC Press. p. 401-448.

22. Choe, E. and D.B. Min, *Mechanisms and Factors for Edible Oil Oxidation.* Comprehensive Reviews in Food Science and Food Safety, 2006. **5**(4): p. 169-186.
23. David, B.M. and M.B. Jeffrey, *Lipid Oxidation of Edible Oil*, in *Food Lipids*. 2002, CRC Press.
24. Velasco, J.n., M.L. Andersen, and L.H. Skibsted, *Evaluation of oxidative stability of vegetable oils by monitoring the tendency to radical formation. A comparison of electron spin resonance spectroscopy with the Rancimat method and differential scanning calorimetry.* Food Chemistry, 2004. **85**(4): p. 623-632.
25. Yang Wesley, T. and B. Min David, *Chemistry of Singlet Oxygen Oxidation of Foods*, in *Lipids in Food Flavors*. 1994, American Chemical Society. p. 15-29.
26. Min, D.B. and J.Y.E. Wen, *Effects of Dissolved Free Oxygen on the Volatile Compounds of Oil.* Journal of Food Science, 1983. **48**(5): p. 1429-1430.
27. Tan, C.P., et al., *Comparative studies of oxidative stability of edible oils by differential scanning calorimetry and oxidative stability index methods.* Food Chemistry, 2002. **76**(3): p. 385-389.
28. Przybylski, R. and N.A.M. Eskin, *A comparative study on the effectiveness of nitrogen or carbon dioxide flushing in preventing oxidation during the heating of oil.* Journal of the American Oil Chemists' Society, 1988. **65**(4): p. 629-633.
29. Yamamoto, Y., *Role of active oxygen species and antioxidants in photoaging.* Journal of Dermatological Science, 2001. **27, Supplement 1**(0): p. 1-4.
30. Shahidi, F. and Y. Zhong, *Lipid Oxidation: Measurement Methods*, in *Bailey's Industrial Oil and Fat Products*, F. Shahidi, Editor. 2005, Wiley: Hoboken, NJ. p. 357-385.
31. Wolff, S.P., *Ferrous Ion Oxidation in Presence of Ferric Ion Indicator Xylenol Orange for Measurement of Hydroperoxides.* Methods in Enzymology, 1994. **233**(Part C): p. 182-189.
32. Shantha, N.C. and E.A. Decker, *Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids.* Journal of AOAC international, 1994. **77**(2): p. 421-424.
33. Bou, R., et al., *Determination of hydroperoxides in foods and biological samples by the ferrous oxidation–xylenol orange method: A review of the factors that influence the method's performance.* Analytical Biochemistry, 2008. **377**(1): p. 1-15.
34. Saldaña, M.D.A. and S.I. Martínez-Monteagudo, *Oxidative Stability of Fats and Oils Measured by Differential Scanning Calorimetry for Food and Industrial Applications*, in *Applications of Calorimetry in a Wide Context - Differential Scanning Calorimetry, Isothermal Titration Calorimetry and Microcalorimetry*, D.A.A. Elkordy, Editor. 2013, InTech.
35. Mettler-Toledo. *Collected Applications, Thermal Analysis - Food*. 44]. Available from: http://us.mt.com/us/en/home/applications/Application_Browse_Laboratory_Analytics/Application_Browse_thermal_analysis/TA_Appl_Handb/Food/_jcr_content/standardpar/58298/file/file.res/Food_Handbook_%20Example.pdf.
36. Wrolstad, R.E., et al., *Lipid Oxidation/Stability*, in *Handbook of Food Analytical Chemistry*. 2005, John Wiley & Sons, Inc. p. 513-564.
37. Šimon, P., et al., *Analysis of the induction period of oxidation of edible oils by differential scanning calorimetry.* Journal of the American Oil Chemists' Society, 2000. **77**(6): p. 639-642.
38. Shortle, E., et al., *Optimisation of process variables for antioxidant components from Crataegus monogyna by supercritical fluid extraction (CO2) using Box□Behnken experimental design.* The Journal of Supercritical Fluids, 2013. **81**(0): p. 112-118.

Appendix I

Nutritional analysis of dried bilberry seeds

Accredited analysis of the nutritional value and water content of bilberry seeds from Svantes Vilt & Bär AB prepared as described in 3.1.

eurofins

Rapport utfärdad av ackrediterat laboratorium

Report issued by Accredited Laboratory

SWEDAC ACKREDITERING 1977 ISO/IEC 17025

Eurofins Food & Agro Testing Sweden AB
Box 887
Sjöhagsg. 3
SE-53119 Lidköping
www.eurofins.se

Tlf: +46 10 490 8310

SIK AB
Lovisa Eliasson
Box 5401
402 29 GÖTEBORG

AR-13-LW-048409-01

EUSELI-00055243

Kundnummer: LW8400358

Analysrapport

Provnummer: 525-2013-12090132
Provmärkning: 2. Blåbärsfrön
Provet ankom: 2013-12-09
Analysrapport klar: 2013-12-17
Analyserna påbörjades: 2013-12-10 15:09:52

	Analys	Resultat	Enhet	Mäto.	Metod/ref	Lab
LP06U	Vattenhalt	**3.71**	g/100 g	± 10%	NMKL 23	*EUSELI*
LP06V	Aska	**1.66**	g/100 g	± 10%	NMKL 173	*EUSELI*
LP021	Råprotein enl. Kjeldahl (Nx6.25)	**15.7**	g/100 g	± 10%	NMKL 6 mod	*EUSELI*
LP06X	Råfett enl. SBR mod.	**23.7**	g/100 g	± 10%	SLV VF 1980	*EUSELI*
LP06Z	Kolhydrater (beräknade)	**55.2**	g/100 g		SLVFS 1993:21	*EUSELI*
LP072	Energivärde kJ (beräknad)	**2082**	kJ/100 g		SLVFS 1993:21	*EUSELI*
LP072	Energivärde kcal (beräknad)	**498**	kcal/100 g		SLVFS 1993:21	*EUSELI*

Appendix II

Laboratory protocol for FOX assay

Construction of Fe (III) standard curve

1. Prepare Fe (III) standard solution

- Dilute concentrated HCl to 10 M and 50 mL
- Transfer carefully to a 100 mL Pyrex beaker
- Weigh 0.5 g of $FeCl_3$ and note exact weight
- Dissolve the $FeCl_3$ in 50 mL 10 M HCl
- Add 2 mL of 30 % H_2O_2
- Heat gently for 5 min to remove excess H_2O_2
- Cool to room temperature
- Dilute to 500 mL with water (1 mg/mL $FeCl_3$)

2. Create standard curve

- Add aliquots of the Fe(III) standard solution ranging from 0 to 5 mL and dilute with methanol to a final volume of 5 mL

Tube	Fe (III) std solution (mL)	Dilution (MeOH)
1	0	5
2	1	4
3	2	3
4	3	2
5	4	1
6	5	0

- Pipette 100 µL of each calibration tube into microcentrifuge tubes
- Add at least 60 µL of reagent A and 5.940 mL of reagent C in a test tube wide enough for a pipette (1:100 dilution)
- Vortex test tube
- Add 900 µL of WR to each microcentrifuge tube (1:10 dilution)
- Vortex tubes and incubate for 10 min at room temperature
- Transfer the solutions to plastic cuvettes
- Measure absorbance at 560 nm

FOX II assay

1. Prepare working reagent and sample

Prepare at least 1 mL of working reagent for each sample

- Mix 10 µL of reagent A with 990 µL of reagent C (1:100 (v/v)) = WR
- Pipette 100 µL sample into microcentrifuge tube
- To each tube, add 900 µL of WR (1:10 dilution)
- Vortex tubes and incubate for 10 min at room temperature
- Centrifuge at 12 000 × g for 5 min if necessary
- Transfer the supernatant (900 µL) to a plastic cuvette
- Measure absorbance at 560 nm

Appendix III
Laboratory scale data

L1 150:35:10

Table A:III 1 - Data collected during laboratory scale extraction with 150 bar pressure, 35°C extraction temperature and 10 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.044	0.021	0.8	4
20	1.922	0.044	1.8	7
30	1.998	0.082	3.3	14
40	2.026	0.107	4.3	18
50	2.033	0.118	4.7	20
60	1.965	0.138	5.5	23
70	2.034	0.164	6.6	28
80	2.097	0.183	7.3	31
90	1.954	0.203	8.1	34

Cartridge yield: 11 %

Cartridge oil yield: 46 %

L2_1 300:35:10

Table A:III 2 - Data collected during laboratory scale extraction with 300 bar pressure, 35°C extraction temperature and 10 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.078	0.103	4.2	17.9
20	1.870	0.211	8.7	36.7
30	1.803	0.283	11.7	49.2
40	2.317	0.292	12.0	50.7
50	2.067	0.294	12.1	51.1
60	2.019	0.308	12.7	53.5
70	2.017	0.325	13.4	56.5
80	2.117	0.322	13.3	55.9
90	2.179	0.325	13.4	56.5

Cartridge yield: 18 %

Cartridge oil yield: 74 %

L2_2 300:35:10

Table A:III 3 - Data collected during laboratory scale extraction with 300 bar pressure, 35°C extraction temperature and 10 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.097	0.100	3.7	15.7
20	1.975	0.178	6.6	27.9
30	2.288	0.192	7.1	30.1
40	2.045	0.231	8.6	36.2
50	2.273	0.250	9.3	39.2
60	2.071	0.225	8.4	35.3
70	2.101	0.236	8.8	37.0
80	1.928	0.242	9.0	37.9
90	2.149	0.245	9.1	38.4

Cartridge yield: 22 %

Cartridge oil yield: 91%

L3 150:60:10

Table A:III 4 - Data collected during laboratory scale extraction with 150 bar pressure, 60°C extraction temperature and 10 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.098	0.012	0.5	2.0
20	1.978	0.017	0.7	2.8
30	2.037	0.027	1.0	4.4
40	1.760	0.047	1.8	7.7
50	2.164	0.048	1.9	7.8
60	2.145	0.047	1.8	7.7
70	2.355	0.052	2.0	8.5
80	1.978	0.054	2.1	8.8

Cartridge oil yield: 0.2 %

Cartridge yield: 0 %

L4_1 300:60:10

Table A:III 5 - Data collected during laboratory scale extraction with 300 bar pressure, 60°C extraction temperature and 10 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.126	0.175	7.1	30.2
20	2.033	0.328	13.4	56.5
30	2.125	0.408	16.7	70.3
40	1.997	0.426	17.4	73.4
50	2.007	0.437	17.8	75.3
60	2.061	0.448	18.3	77.2
70	2.050	0.446	18.2	76.8
80	1.930	0.457	18.7	78.7

Cartridge oil yield: 82 %

Cartridge yield: 20%

L4_2 300:60:10

Table A:III 6 - Data collected during laboratory scale extraction with 300 bar pressure, 60°C extraction temperature and 10 sec milling time.

Time (min)	Flow rate (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.024	0.116	4.5	19.1
20	2.011	0.240	9.4	39.6
30	1.968	0.277	10.8	45.7
40	1.950	0.278	10.9	45.9
50	1.964	0.285	11.2	47.0
60	1.980	0.296	11.6	48.9
70	1.997	0.304	11.9	50.2
80	2.043	0.307	12.0	50.7
90	2.112	0.312	12.2	51.5

Cartridge yield: 13 %

Cartridge yield: 53 %

L5 150:35:30

Table A:III 7 - Data collected during laboratory scale extraction with 150 bar pressure, 35°C extraction temperature and 30 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.044	0.016	0.6	2.7
20	1.922	0.052	2.1	8.8
30	1.998	0.064	2.6	10.8
40	2.026	0.070	2.8	11.8
50	2.033	0.098	3.9	16.6
60	1.965	0.114	4.6	19.3
70	2.034	0.145	5.8	24.5
80	2.097	0.183	7.3	30.9
90	1.954	0.197	7.9	33.3

Cartridge yield: 8 %

Cartridge oil yield: 36 %

L6 300:35:30

Table A:III 8 - Data collected during laboratory scale extraction with 300 bar pressure, 35°C extraction temperature and 30 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	1.978	0.116	4.6	19.6
20	1.998	0.244	9.8	41.2
30	1.996	0.324	13.0	54.8
40	1.990	0.366	14.7	61.9
50	2.036	0.408	16.3	69.0
60	1.937	0.433	17.3	73.2
70	2.008	0.439	17.6	74.2
80	2.024	0.443	17.7	74.9
90	2.016	0.458	18.3	77.4

Cartridge oil yield: 26 %

Cartridge yield: 110 %

L7 150:60:30

Table A:III 9 - Data collected during laboratory scale extraction with 150 bar pressure, 60°C extraction temperature and 30 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	1.959	0.015	0.6	2.5
20				
30	2.242	0.017	0.7	2.9
40	2.413	0.033	1.3	5.6
50	1.960	0.044	1.8	7.4
60	1.951	0.046	1.8	7.8
70	1.981	0.053	2.1	8.9
80	2.026	0.058	2.3	9.8
90	1.931	0.048	1.9	8.1

Cartridge yield: 3 %

Cartridge yield: 14 %

L8_1 300:60:30

Table A:III 10 - Data collected during laboratory scale extraction with 300 bar pressure, 60°C extraction temperature and 30 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.114	0.151	6.0	25.5
20	2.985	0.372	14.9	62.8
30	2.432	0.522	20.9	88.1
40	2.347	0.562	22.5	94.9
50	1.770	0.575	23.0	97.1
60	1.939	0.574	23.0	96.9
70	2.183	0.571	22.8	96.4
80	2.057	0.570	22.8	96.2
90	1.837	0.559	22.4	94.4

Cartridge yield:28 %

Cartridge oil yield: 117%

L8_2 300:60:30

Table A:III 11 - Data collected during laboratory scale extraction with 300 bar pressure, 60°C extraction temperature and 30 sec milling time.

Time (min)	Flow rate CO_2(mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.091	0.118	4.5	19.2
20	2.043	0.211	8.1	34.3
30	2.071	0.265	10.2	43.0
40	2.092	0.296	11.4	48.1
50	2.069	0.293	11.3	47.6
60	1.967	0.285	11.0	46.3
70	1.844	0.298	11.5	48.4
80	1.971	0.299	11.5	48.6
90	2.013	0.308	11.9	50.0

Cartridge yield: 25 %

Cartridge oil yield: 105 %

L9_1 225:47.5:20

Table A:III 12 - Data collected during laboratory scale extraction with 225 bar pressure, 47.5°C extraction temperature and 20 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.287	0.039	1.5	6.5
20	2.060	0.085	3.4	14.1
30	2.139	0.105	4.1	17.5
40	2.001	0.130	5.1	21.6
50	2.034	0.163	6.4	27.1
60	1.761	0.197	7.8	32.8
70	1.965	0.223	8.8	37.1
80	2.141	0.220	8.7	36.6
90	1.948	0.234	9.2	38.9

Cartridge yield: 25 %

Cartridge oil yield: 107 %

L9_2 225:47.5:20

Table A:III 13 - Data collected during laboratory scale extraction with 225 bar pressure, 47.5°C extraction temperature and 20 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.053	0.045	1.8	7.6
20	2.150	0.096	3.8	16.2
30	2.102	0.124	5.0	20.9
40	2.105	0.171	6.8	28.8
50	2.120	0.203	8.1	34.2
60	2.141	0.208	8.3	35.1
70	1.947	0.228	9.1	38.5
80	1.933	0.234	9.4	39.5
90	2.005	0.248	9.9	41.8

Cartridge yield: 28 %

Cartridge oil yield: 117 %

L9_3 225:47.5:20

Table A:III 14 - Data collected during laboratory scale extraction with 250 bar pressure, 47.5°C extraction temperature and 20 sec milling time.

Time (min)	Flow rate CO_2 (mL/min)	Total weight (g)	Yield (%)	Oil yield (%)
10	2.085	0.047	1.8	7.7
20	2.114	0.098	3.8	16.0
30	1.965	0.140	5.4	22.9
40	1.968	0.170	6.6	27.8
50	2.410	0.181	7.0	29.6
60	2.097	0.196	7.6	32.0
70	2.023	0.205	7.9	33.5
80	2.855	0.206	8.0	33.7
90	2.073	0.224	8.7	36.6

Cartridge yield 23 %

Cartridge oil yield 96 %

Appendix IV
Pilot scale extraction data

P1 150:40

Before extraction	
Glass wool (g)	6.12
Seeds (g)	99.38

CO2	
Consumed CO2 (kg)	3.065

After extraction	
Extractor cell (g)	1464.8
Difference (before-after) (g)	4.3
Oil (g)	3.312

Yield	
Yield extractor cell	4.3%
Yield oil	3.3%

P2 200:40

Before extraction	
Glass wool (g)	3.65
Seeds (g)	92.65
Total weight (g)	1460

CO2	
Consumed CO2 (kg)	4.035

After extraction	
Extractor cell (g)	1456.12
Difference (before-after) (g)	3.88
Oil (g)	5.444

Yield	
Yield extractor cell	4.2%
Yield oil	5.9%

P3 225:47.5

Before extraction	
Glass wool (g)	4.6
Seeds (g)	104.22
Total weight (g)	1472.35

CO2	
Consumed CO2 (kg)	3.665

After extraction	
Extractor cell (g)	1458.14
Difference (before-after) (g)	14.21
Oil (g)	8.577

Yield	
Yield extractor cell	13.6%
Yield oil	8.2%

P4 250:40

Before extraction	
Glass wool (g)	4.97
Seeds (g)	108.08
Total weight (g)	1476.62

CO2	
Consumed CO2 (kg)	3.57

After extraction	
Extractor cell (g)	1459.14
Difference (before-after) (g)	17.48
Oil (g)	13.845

Yield	
Yield extractor cell	16.2%
Yield oil	12.8%

Appendix V

Storage evaluation with regard to peroxide value

Week 2 is excluded due to irregularities with the FOX-II assay

A:V 1 - Compilation of data necessary to calculate the peroxide value according to equation 4, using oil collected directly after extraction

Week 0

Weight oil (g)	0.101
Weight EtOH (g)	1.5195
Oil concentration (g oil/g)	§0.062

	Weight oil solution (g)	Weight oil in tube	Absorbance	Blank	Peroxide value
Tube 1	0.0741	0.0046	0.173	0.151	12.9
Tube 2	0.0757	0.0047	0.171		12.4

A:V 2 - Compilation of data necessary to calculate the peroxide value according to equation 4, using oil stored for 7 days.

Week 1

	1. Control	2. Cold storage	3. α- tocopherol addition	4. Nitrogen atmosphere
Weight oil (g)	0.109	0.101	0.103	0.103
Weight EtOH (g)	1.900	1.907	1.904	1.913
Oil concentration (g oil/g)	0.054	0.050	0.051	0.051

	Weight oil solution (g)	Weight oil in tube	Absorbance	Blank	PV	
1.1	0.080	0.0043	0.293	0.150	23.2	24.7
1.2	0.077	0.0042	0.320		26.3	
2.1	0.078	0.0039	0.231		20.2	19.4
2.2	0.080	0.0040	0.218		18.6	
3.1	0.077	0.0040	0.335		29.1	29.8
3.2	0.076	0.0039	0.348		30.6	
4.1	0.077	0.0039	0.249		21.7	22.5
4.2	0.077	0.0039	0.268		23.4	

A:V 3 - Compilation of data necessary to calculate the peroxide value according to equation 4, using oil stored for 21 days.

Week 3

	1. Control	2. Cold storage	3. α-tocopherol addition	4. Nitrogen atmosphere
Weight oil (g)	0.103	0.101	0.107	0.104
Weight EtOH (g)	3.907	3.907	3.91	3.91
Oil concentration (g oil/g)	0.026	0.025	0.027	0.026

	Weight oil solution (g)	Weight oil in tube	Absorbance	Blank	PV	
1.1	0.076	0.0020	0.099	0.272	20.55	19.6
1.2	0.080	0.0021	0.095		18.73	
2.1	0.075	0.0019	0.059		12.65	12.8
2.2	0.077	0.0019	0.062		12.95	
3.1	0.075	0.0020	0.176		35.69	36.3
3.2	0.075	0.0020	0.182		36.91	
4.1	0.077	0.0020	0.090		18.28	18.2
4.2	0.077	0.0020	0.089		18.07	

A:VI 4- Compilation of data necessary to calculate the peroxide value according to equation 4, using oil stored for 28 days.

Week 4

	1. Control	2. Cold storage	3. α-tocopherol addition	4. Nitrogen atmosphere
Weight oil (g)	0.107	0.105	0.104	0.107
Weight EtOH (g)	3.916	3.905	3.904	3.909
Oil concentration (g oil/g)	0.0266	0.0262	0.0259	0.0266

	Weight oil solution (g)	Weight oil in tube	Absorbance	Blank	PV	
1.1	0.080	0.0021	0.085	0.265	16.19	18.0
1.2	0.080	0.0021	0.104		19.80	
2.1	0.080	0.0021	0.075		14.51	15.1
2.2	0.080	0.0021	0.081		15.67	
3.1	0.075	0.0019	0.193		40.18	44.6
3.2	0.079	0.0020	0.248		49.02	
4.1	0.076	0.0020	0.121		24.21	24.4
4.2	0.077	0.0021	0.124		24.49	