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Supercritical CO_2 extraction of vegetable oils

Determination of fatty acids concentration in sea buckthorn oil

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"In gratitude to my family, who supported me during the time I spent in Leuven; to University Colleges Leuven-Limburg (UCCLL), which gave me the opportunity of completing this research and to Gunther Fleerackers, who guided me during the fulfilment of this project"

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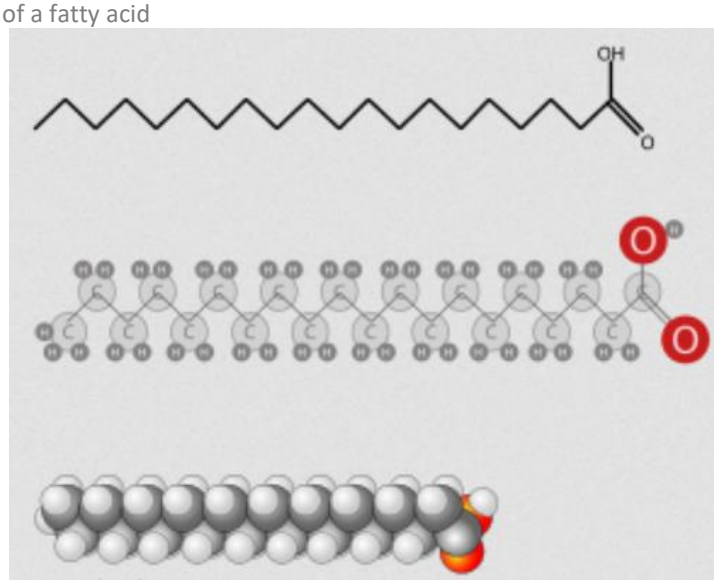
1. INTRODUCTION

Vegetable oils are obtained from plants and are undoubtedly some of the most important ingredients in ambits like food, fuels, chemistry or pharmacology. Depending the source, vegetable oils can be more suitable for one objective or another but they all are known for the important role they play in our everyday life.

The sea buckthorn is the fruit which is obtained from the bush *Hippophae*. Its berries are edible and nutritive, nonetheless, they are very acid, so it is not usual to eat them as a raw fruit. Instead, sea buckthorn berries are used in food industry to make cakes, jams or liquors. Moreover, the oil from the berries has a lot of healing applications against ulcerations and inflammations, so its use is expanded worldwide. Sea buckthorn berry is very rich in essential oils and it is one of the few fruits in the world which contains all four omega fatty acids: omega-3, omega-6, omega-7 and omega-9.

Vegetable oils are compounded mainly of fatty acids. The composition of a fatty acid depends on two parts: the apolar part or the hydrocarbon chain and the polar part or the carboxylic acid group. As the fatty acids have both apolar (hydrophobic) and polar (hydrophilic) parts, they are said to be amphiphilic molecules. This is a very characteristic property of the fatty acids as few natural compounds are capable of being polar/apolar at the same time.

Figure 1: Structure of a fatty acid



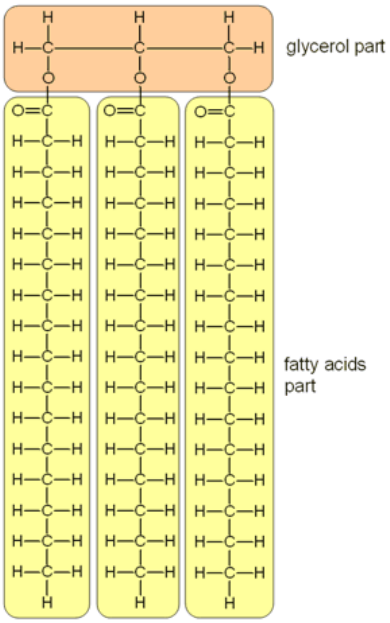
https://upload.wikimedia.org/wikipedia/commons/thumb/0/05/Arachidic_formula_representation.svg/300px-Arachidic_formula_representation.svg.png

Fatty acids differ among each other depending on the number of carbons that compound the hydrocarbon chain and because of the number of unsaturated bonds they have. The higher the number of carbons a fatty acid has, the larger and heavier turns the molecule to be. Then, properties as density, boiling point or retention time for the gas chromatography analysis increase. As we have said, apart from the number of

carbons, fatty acids also depend on the unsaturation level. Fatty acids with the same amount of carbons but with different unsaturation level have very different physical properties between them. Values of those physical properties decrease at the same time the number of unsaturated bonds increase. For instance, fatty acids on food are healthier when the number of double bonds of the molecules is higher, or referring to the gas chromatography analysis, the retention time and boiling time decrease while double bond amount increases.

But fatty acids do not only appear in vegetable oils as free fatty acids (FFA), they can also appear bonded between each other by a glycerol molecule; usually, three fatty acids bond with a glycerol molecule in order to create a triacylglyceride (TAG). When this happens, the carboxylic group of each fatty acid reacts with the hydroxyl group of the glycerol in order to make an esterification happen.

Figure 2: Structure of a triacylglyceride



https://study.com/cimages/multimages/16/triglycerides2.jpg

Triglycerides are bigger compounds, so as they have higher boiling points or density and depending on the number of carbons of the fatty acids composing it they also can be solid at room temperatures. As free fatty acids, triacylglycerides also can be saturated or unsaturated. The three hydrocarbon chains do not even need to be the same size or have the same saturation level. Due to this fact, triacylglycerides have very different physical properties between them.

Physical and chemical properties of vegetable oils depend mainly on the composition of fatty acids composing them. One of the main reason of this research, for instance, is why the sea buckthorn oil solidifies at room temperature. The reason for this resides on the

saturated/unsaturated ratio. This issue would state that if the oil solidifies at room temperature the saturated/unsaturated ratio would be high, while most of the vegetable oil's relation is low. Furthermore, triacylglycerides high concentration also help to raise the melting point, so that could be another reason for the stated problem.

The aim of this research work is to determine, qualitatively and quantitatively, the amount of fatty acids in the oil of sea buckthorn pulp, so as to define the composition of the oil obtained from the pulp. To fulfil this objective supercritical CO_2 extraction will be used instead of organic solvents, as a way of encouragement for the use of green technology, and it will be used for its further study and improvement in the future. Moreover, free fatty acids and triacylglycerides relationship must be defined, as well as saturated/non-saturated fatty acids percentages. As the problem remaining is that at room temperature sea buckthorn extract oil solidifies, this results will help us understand the optimum applications and management of sea buckthorn oil.

So, the main focus of this project is the use supercritical CO_2 extraction. The project is entirely focused on it in order to determine if $scCO_2$ can be a proper replacement for soxhlet extraction. This extraction method has actually been used at industrial level but its scale can be much wider in the future, so it is the spotlight of further researches. As CO_2 is not toxic and its use in supercritical fluid extraction leaves almost no waste, its application in a large range is a total environmental and economic revolution, so it could turn to be the most widespread process in food industry.

For achieving the objective set, a comparison between the compositions of soxhlet extracts and $scCO_2$ extracts has been done. For the determination of free fatty acid composition in each extract FAME (Fatty Acid Methyl Esters) method has been carried out, and the total lipid composition has been analysed by gas chromatography using a flame ionization detector (GC-FID).

2. METHODS AND MATERIALS

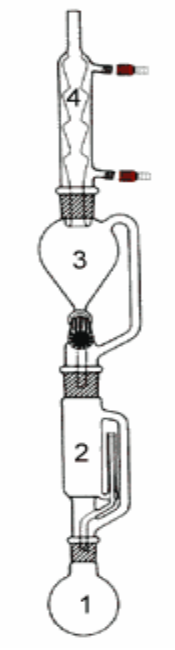
2.1. SOXHLET EXTRACTION

2.1.1. METHOD RESUME

The soxhlet extraction is a typical extraction method in which an organic solvent is continuously extracting a solute from a solid matrix. The reason for using the soxhlet extraction is that the solute of the solid matrix is partially soluble in a solvent. As a solute is not transferred from the sample to the solvent immediately, the principle of this extraction method suits the necessities of the complete extraction from the matrix, as the solvent is continuously passing through the sample matrix, increasing the yield of the extraction.

In this method, the sample is placed in a porous cellulose container inside the extraction chamber *((2) in figure 3)*. This chamber is suspended between a flask *((1) in figure 3)* containing the solvent and a cooler *((4) in figure 3)*. The solvent is heated up until it evaporates; then, it condenses in the cooler and falls to the extraction chamber in which the solid sample is placed. The extraction chamber is designed to fill until a certain point in which the solvent surrounding the solid sample overflows. Reached this filling point, the chamber is totally emptied, and the solvent falls to the flask below again. This process is repeated continuously until no solute can be extracted, which makes the yield of the extraction to be the total amount of extracted solute. [1]

Figure 3: Design of a soxhlet extraction mounting



http://www.cyberlipid.org/images/pict244.gif

The extraction time depends on some different factors. Basically, it is the nature of the sample matrix the one that defines the necessary time for the extraction as the length relies on the ease with which the solute is taken away from the sample. If the solute is extracted easily the necessary time will be shorter than if not. In addition, the more sample is used, the more solute there is to be extracted, so it will take longer to complete it. But more or less, the general time for a soxhlet extraction comes to be between 4 and 8 hours.

The advantage of this method is that the solute is totally extracted from the solid sample. This way, we can determine how much oil there is in a sea buckthorn solid sample. But it has its disadvantages too. It takes a long time to make a total extraction and the solvents used are organic. Large amounts of solvents are required and the vapours could be hazardous, so the soxhlet extraction should not be used as the base method, as one of the aims of this project is to defend and boost the use of green technologies.

2.1.2. REAGENTS AND MATERIAL

The solvent used in this project for the soxhlet extraction is n-hexane (Sigma-Aldrich, C_6H_{14}, technical grade; CAS number: 110-54-3).

The material needed is a soxhlet extractor, a cooler with a water source, a boiling flask, as shown in *Figure 3*. Apart from that stuff, a heat source is also needed to make the extraction happen.

2.1.3. SCOPE

This method is a reference method for solids, sediments, soils and sludges:

- **Method 8270D**, Reference: SW-846, Test Methods for Evaluating Solid Waste: Physical/Chemical Methods, EPA SW-846, Update IV, Feb 2007.

2.2. FAME METHOD (FATTY ACID METHYL ESTERS)

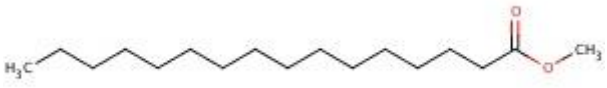
2.2.1. METHOD RESUME

In order to analyse and determinate fatty acids concentration in vegetable oils, both free fatty acids or triacylglycerides, FAME analysis method is used. FAME method is a procedure used for the determination of total lipids expressed as fatty acid methyl esters in a sample of biological source. As the method encompasses all lipids in form of fatty acid methyl esters, both free fatty acids and triacylglycerides can be measured by it. One question it could be done around the FAME method is why fatty acids need an

esterification to be analysed and why they cannot be analysed directly in their acidic form. [2, 3]

Theorically, fatty acids can be measured directly by GC analysis without the need of a trans-esterification, but this leads to two problems when identifying and quantifying the fatty acids. On the one hand, in their free, underivatized form, fatty acids may be difficult to analyze because these highly polar compounds tend to form hydrogen bonds, leading to adsorption issues. When doing the trans-esterification, the polarity of those compounds reduces, making them more amenable for the analysis. On the other hand, to distinguish between the very slight differences exhibited by unsaturated fatty acids, the polar carboxyl functional groups must be first neutralized. This then allows column chemistry to perform separations by boiling point elution, and also by degree of unsaturation, position of unsaturation, and even the cis vs. trans- configuration of unsaturation. [4, 5]

Figure 4: Image of a fatty acid methyl ester



https://media.scbt.com/product/09/23/z/92325.jpg

As it was stated before, this method detects and quantifies the total amount of lipids in a sample. So, the free fatty acids (FFA) cannot be determined apart from the triacylglycerides (TAG). The one thing that can be determined is how much of each fatty acid there is, either if it is free or bonded to a glycerol molecule. Although this analysis method cannot deal with that limitation, it gives exact amount/percentage of each hydrocarbon chain.

So, for the determination of the total fatty acids the trans-esterification is needed. The trans- esterification is done using an acid catalyst, sulphuric acid in this case. It is well known that sulphuric acid is a very dangerous component, especially if it is concentrated, so special care must be taken when proceeding the reaction.

2.2.2. REAGENTS AND MATERIAL

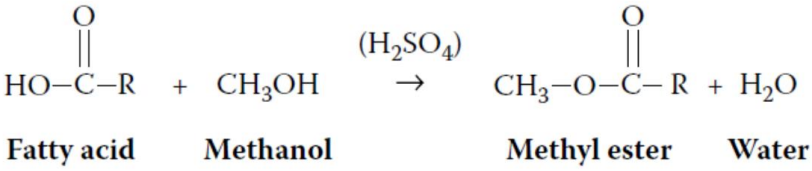
Solvents used in this analysis method are the next ones:

- n-Hexane, technical grade (Sigma-Adrich, C_6H_{14}, CAS number: 110-54-3)
- n-Hexane, analytical standard (Sigma-Aldrich, C_6H_{14}, CAS number: 110-54-3)
- Anhydrous methanol 99,8% (Sigma-Aldrich, CH_3OH, CAS number: 67-56-1)
- Acetone, technical grade (Sigma-Aldrich, C_3H_6O, CAS number: 67-64-1)
- Sulfuric acid, reagent grade 95-98%(Sigma-Aldrich, H_2SO_4, CAS number: 7664-9)

2.2.3. REACTION

This method, is based on a trans-esterification procedure of lipids to FAME, by the use of methanol and sulphuric acid as a catalyst. The fatty acids react with the methanol to give different methyl esters and water molecules as reaction products.

Figure 5: Reaction between a fatty acid and methanol



http://patentimages.storage.googleapis.com/US20130239467A1/US20130239467A1-20130919-C00003.png

The procedure first solubilizes the lipids and then frees the fatty acids by transferring a methyl group from methanol onto the –acyl chain of the lipids. During the reaction, the carboxylic acid group is replaced by an ester bond between the fatty acid and a methyl group, which comes from the methanol, producing this way methyl esters of the fatty acids.

Afterwards, the FAME are extracted using hexane, so the polar unwanted compounds stay in the polar phase. The qualitative and quantitative analysis is done by flame ionization detector gas chromatography, in which only organic compounds are detected. Due to this fact, the hexane is a proper solvent with which to do the GC analysis, as it elution time is known.

2.2.4. APPARATUS

For the FAME analysis of sea buckthorn oil the following apparatus have been used: Thermo Trace 1300 gas chromatograph with Thermo AI 1310 auto sampler and RTX-1 wax column. For detection of the signals Flame Ionization Detector (FID) has been used.

2.2.5. SCOPE

FAME analysis method could be used for any oil, without any dependence on the source of it (vegetable or animal). In this case, FAME analysis is used in sea buckthorn oil, but it can also be used in any other fruits extract.

2.3. SUPERCRITICAL CO_2 EXTRACTION ($scCO_2$)

2.3.1. METHOD RESUME

The supercritical CO_2 extraction part is the main focus of this project. $scCO_2$ is an extraction technique that uses CO_2 in supercritical state to extract solutes from solid matrixes instead of organic solvents. A lot of research has been done around this extraction technique as its advantages are very considerable; yet its full experimental potential has not been discovered and it keeps developing through the years. So this project is in part an intention to keep on developing $scCO_2$ extraction technique for analytical requirements.

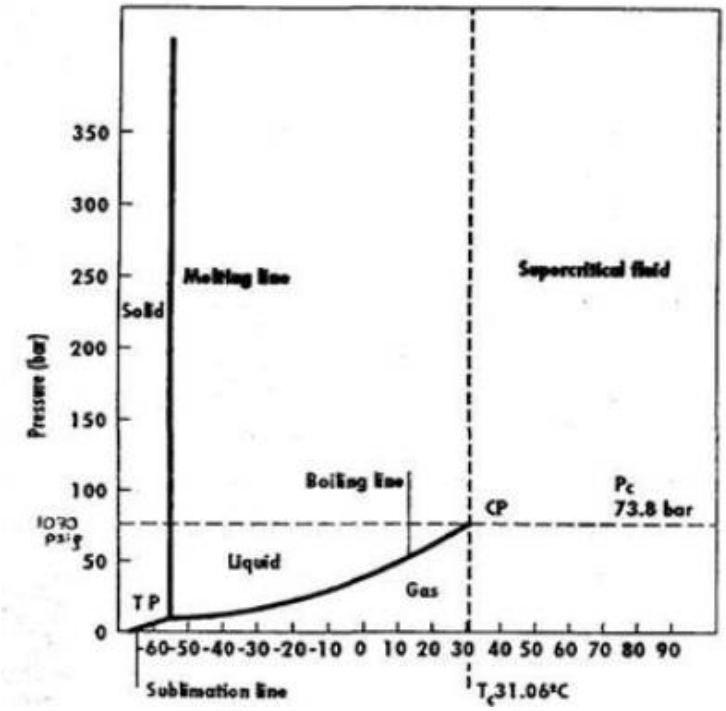
Substances, compounds or matter receive the "supercritical" qualifying when their temperature and pressure are pushed beyond their critical point. This point delimits the subcritical and supercritical states of a compound. When this point is surpassed, there is no clear physical phase of a substance and that is the most remarkable property of supercritical fluids. Supercritical stage is characterized by the inability to distinguish whether a substance is a liquid or a gas, in other words they do not have a definite phase. Supercritical fluids have the low viscosity of a gas and the high density of a liquid, making it impossible to liquefy the substance using any amount of pressure. Nevertheless, it is possible to go from gas to liquid without crossing the boundary between the gas and liquid phase using a supercritical fluid just by lowering the temperature of the liquid. As a consequence of their high density, volatile liquids and solids come to be especially easy to dissolve in supercritical fluids.

Supercritical fluids have no surface tension because they are not subject to the vapour-liquid boundary so no molecules have the attraction to the interior of the liquid. The densities and viscosity of a supercritical fluid change with pressure or temperature, and the supercritical fluid of a substance can have very different properties than the regular fluids. [6]

Each substance of known composition has its own phase diagram, where three lines describe the sublimation, melting and boiling processes, as they define the equilibrium between the phases. These lines also delimit the regions corresponding to the gas, liquid and solid states. The critical region has its origin at the critical point, which is defined by the critical temperature (T_c) and the critical pressure (P_c). Taking a look to its definition, the critical temperature would be the highest temperature at which a gas can be converted into a liquid by increasing the pressure.

The phase diagram of CO_2 can be seen in the picture below *(Figure 6)*. There, the supercritical phase of the gas is shown.

Figure 6: Phase (pressure-temperature) diagram for CO_2



http://eng.ege.edu.tr/~otles/SupercriticalFluidsScienceAndTechnology/image/images/default2_clip_image002.jpg

Taking a look at the phase diagram of CO_2 *(Figure 6)* the critical point can be determined. These are the critical parameters of CO_2:

- T_C: 31,06 ºC
- P_C: 73,8 bar

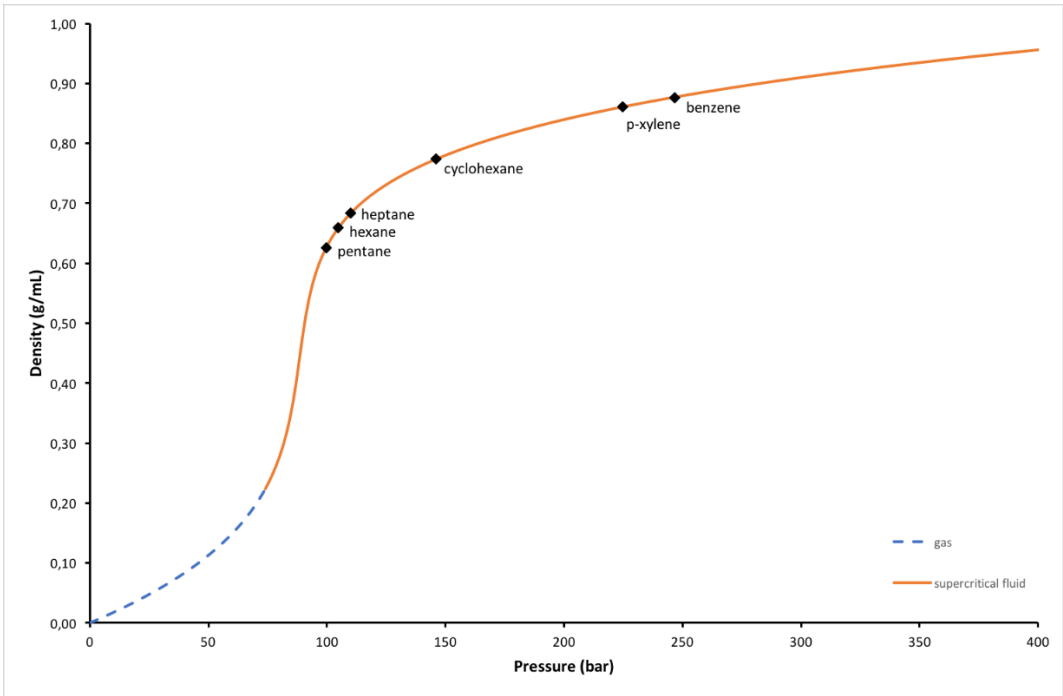
So, noticing its critical parameters, it is easy to achieve the supercritical fluid mode on it, economically and dynamically, as neither the temperature nor the pressure are too high.

It is said that supercritical fluid technology would be better served if experiments were discussed in terms of density instead of pressure. When the parameter pressure is replaced by density, the solubility-temperature relationship becomes much simpler. This anomaly comes about because density decreases dramatically with an increase in temperature at low pressure, whereas at higher pressure, changes in temperature have much less effect on density. Thus density, not pressure, to a first approximation is proportional to the solvent power of the supercritical fluid. [7]

- Solvent power of a supercritical fluid increases with density at a given temperature.
- Solvent power of a supercritical fluid increases with temperature at a given density.

Each compound or substance has its particular density and it is easy to see the change of it depending on the pressure in which is worked. This can be seen in the picture below *(Figure 7)*.

Figure 7: Density of different compounds vs pressure



As this project focuses in $scCO_2$, deeper should be inquired about CO_2 density change, to exactly know about the solvent power of CO_2 working at different parameters. The table below *(Figure 8)* shows density-temperature-pressure relationship in the case of CO_2. There can be seen that each parameter changes a lot with minimum changes in the other ones.

Figure 8: Density-Temperature-Pressure Relationship table for CO_2

Density (g/mL)	40ºC	50 ºC	60 ºC	70 ºC	80 ºC	90 ºC	100 ºC	110 ºC	120 ºC
1,00	526	618							
0,95	383	463	544	644	680				
0,90	281	350	420	489	518				
0,85	211	269	329	401	447				
0,80	164	213	264	314	365	416	467		
0,75	134	175	218	261	305	348	392	436	510
0,70	115	150	187	223	260	297	334	372	425
0,65	104	133	165	196	227	259	290	322	254
0,60	97	122	149	176	203	229	256	284	311
0,55	93	115	138	161	183	206	230	252	276
0,50	91	109	129	148	168	188	207	227	246
0,45	89	104	122	138	155	172	188	205	221
0,40	87	100	115	129	143	157	171	185	197
0,35	84	96	108	120	132	144	155	167	178
0,30	81	90	101	111	121	130	140	149	158
0,25	77	84	93	100	108	116	123	130	137
0,20	70	75	82	88	94	99	105	110	116

Source: Adapted from Hewlett Packard Co. (Wilmington, DE) literature. Pressure is given in bar.

A common practice in supercritical fluid extraction, which has to do with physicochemical properties of the compounds, is the use of modifiers (co-solvents). These are used when the primary fluid in the extraction does not reach the necessary extraction efficiency and are added to it to enhance the yield. In the case of CO_2 the addition of 1 to 10% methanol or ethanol expands its extraction range to include more polar lipids.

Supercritical CO_2 extraction resembles Soxhlet extraction, except that the solvent used is a supercritical fluid. This fluid provides a broad range of useful properties.

The major advantage of using supercritical fluid extraction is the elimination of organic solvents, thus reducing the problems of their storage. Many legislative protocols have focused on supporting a reduction in the use of organic solvents which could be harmful for the environment. Furthermore, CO_2 has low cost, is relatively nontoxic, it does not support combustion and it is environmentally compatible. [8]

Besides ecological benefits, another property of this kind of extraction that draws attention is the high diffusion capacity of lipids in supercritical fluids, far greater than in conventional liquid solvents. According to several studies, supercritical fluid extraction is the replacement method for traditional gravimetric techniques. In addition, carbon dioxide is devoid of oxygen, thus protecting lipid samples against any oxidative degradation.

Currently, the use of supercritical fluids is under study for many applications, such as removal of nicotine from tobacco, synthesis of polymers and organic chemicals or advance in propellants. But right now supercritical fluids are being used for sample

preparation prior to trace analysis and as mobile phases in analytical and preparative scale supercritical fluid chromatography. In summary, the use supercritical fluids in science and engineering in the near future cannot be certainly defined.

2.3.2. REAGENTS AND MATERIAL

In the supercritical CO_2 extraction three compounds have been used: CO_2 as primary fluid and methanol or hexane (both technical) as make-up solvent.

As material stainless steel extraction vessels and borosilicate collectors have been used.

2.3.3. APPARATUS

The apparatus used: Waters supercritical fluid extraction systems, MV-10 ASFE.

2.3.4. SCOPE

Supercritical CO_2 extraction can be used for the extraction of all kind of vegetable oils.

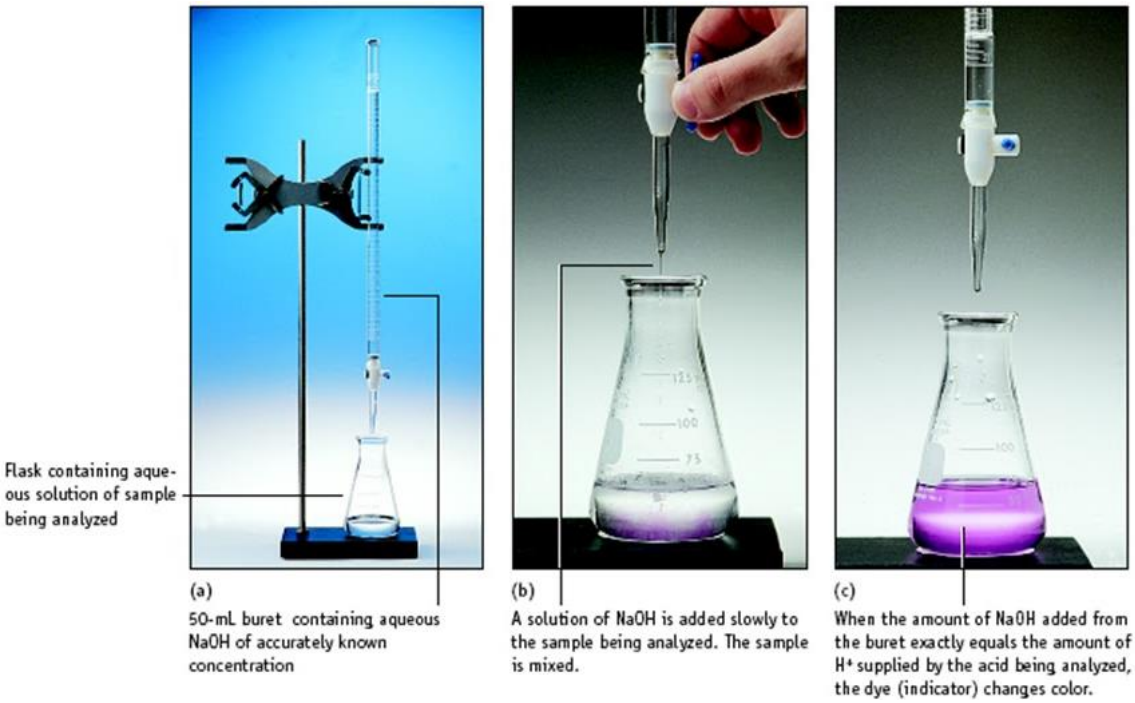
2.4. FFA METHOD

2.4.1. METHOD RESUME

Although the main analysis tool in this project is GC-FID, free fatty acids could not be analysed by it because it quantifies both free fatty acids and triacylglicerides, without the possibility of analysing them separately. So, an alternative analysis method had to be found in order to analyse free fatty acids alone. Taking a look into the literature, some different possible methods appeared and from those the determination by titration using sodium hydroxide (NaOH) was chosen as the most correct one. [9]

This method is based in an acid-basic neutralization reaction, where the analytes are free fatty acids. The analytes, as their name says, are acidic compounds, and when reacting it with a basic compound, sodium hydroxide in this case, the pH of the solution changes. Using phenolphthalein as an indicator, the neutralization point can be seen directly. Phenolphthalein expresses a pink colour in basic solution, and it is colourless in acid solution. Moreover, it changes colour at pH=8. So, knowing which concentration of NaOH is used as titrator, concentration of the analyte can be determined by seeing with our own eyes the point when the solution changes the colour from colourless to pink *(Figure 9)*.

Figure 9: Image of the titration process



https://4.bp.blogspot.com/-RzkjUYDaZ8Q/Vo6GrjgKKNI/AAAAAAAABVE/yYA0rZH1geo/s1600/setup%2Bfor%2Btitration.jpg

Titration by NaOH allows us to determine and quantify free fatty acids alone because the neutralization reaction happening in the analysis can only happen between an acid and a base. So, the carboxyl group from the FFA reacts with sodium hydroxide, producing a neutral salt, sodium palmitate, for instance. Therefore, the reaction only happens with free fatty acids as long as triacylglicerides do not have any carboxyl functional group. As a result, they do not react with NaOH.

As the method relies on the veracity of our eyes and not on that from a flame ionization detector, the reliability of the method is smaller than, for example, gas chromatography. The margin of error is higher when quantification is done with it. But by gas chromatography total lipid amount in sea buckthorn oil only can be quantified, without separating FFA and TAGs, so this method is the only one it could be used.

2.4.2. REAGENTS AND MATERIAL

- Anhydrous methanol 99,8% (Sigma-Aldrich, CH_3OH, CAS number: 67-56-1)
- Sodium Hydroxide 99,9% (Sigma-Aldrich, NaOH, CAS number: 1310-73-2)
- Potassium hydrogen phthalate ≥99,5%(Sigma-Aldrich, KHft, CAS number: 877-24-7)
- Phenolphtalein solution (1%)
- Ethanol 96% (Sigma-Aldrich, C_2H_5OH, CAS number: 64-17-5)
- Diethyl ether, technical grade (Sigma-Aldrich, $(C_2H_5)_2O$, CAS number: 60-29-7)

The material needed for the titration is a burette and an Erlenmeyer flask.

2.4.3. REACTION

The reaction that happens in this analysis method is a neutralization between an acid, free fatty acid, and a base, NaOH. As phenolphthalein is used as an indicator, the neutralization point is detected visually, when a colour change happens. Phenolphthalein expresses an intense pink colour in basic pH, and it is colourless in acid solutions. So, as NaOH is being added, the colour goes from colourless to pink in the neutralization point.

Figure 10: Reaction between FFA and NaOH

$$RCOOH + NaOH \rightarrow RCOONa + H_2O$$

Although that is the main reaction in the method, the titrator, NaOH in this case, needs to be standardised, so another reaction happens here. As a standard, we used potassium hydrogen phthalate, and in this case, as our analyte is NaOH, the colour transition happens the other way, from pink to colourless.

Figure 11: Reaction between KHP and NaOH

$$NaOH + KHFt \rightarrow NaKFt + H_2O$$

2.4.4. SCOPE

The scope of titration analysis by NaOH encompasses any sample that possesses any acid, strong or weak, for the determination of these ones. So, this method can be applied for FFA analysis of any vegetable oil.

2.4.5. METHANOL/STANDARD METHOD

Two different procedure methods were followed when analysing free fatty acids by titration. On the one hand the sample was prepared using methanol as a solvent; on the other hand, the solvent was ethanol-diethyl ether (1:1) solution, as the European Chemistry Association dictates.

At first instance, methanol was used as the solvent to dissolve the extract oil for the analysis by titration, as methanol is a cheaper compound at high purity than ethanol and diethyl ether. Apart from that, diethyl ether is an explosive substance, so there was the intention to avoid it or use it as less as possible.

Then, the solvent was changed to EtOH:Et_2O (1:1), as this one presented better results in the concentration determinations.

2.5. ETHANOL WINTERIZATION

2.5.1. METHOD RESUME

Ethanol winterization, also known as alcohol wash, is an additional procedure technique used to remove from the original $scCO_2$ extract unwanted products as waxes or other type of fats. With the application of this purifying process, the matrix effect that can affect some kind of analysis are erased from the sample, in order to do more accurate and exact measurements.

The cleaning method consists on dissolving the extracted oil into high grade ethanol and freezing it for at least 48 hours. At low temperatures, the waxes and other undesired products separate from the oil and precipitate, resulting in a heterogeneous solid-liquid mixture. Therefore, the solution can be filtrated by vacuum, and the result obtained is a high purity oil. [10]

2.5.2. REAGENTS AND MATERIAL

- Ethanol 96% (Sigma-Aldrich, C_2H_5OH, CAS number: 64-17-5)

The material used for the ethanol winterization is a 500 mL beaker, a Buchner funnel, and a vacuum mounting.

2.5.3. SCOPE

This purification technique can be applied to all kind of vegetable oil extracts. Its most known application is the purification of cannabis oil.

3. MEASUREMENTS AND RESULTS

3.1. PROCEDURE AND MEASUREMENTS

- SOXHLET EXTRACTION

For the procedure of the soxhlet extraction, the dried sea buckthorn fruit peels are first weighed. For a quantitative analysis, more or less 8 g sample is measured and this is transferred to a filter paper thimble, which is made of cellulose. The thimble is placed inside the main chamber of the soxhlet extractor and the mounting for the extraction is prepared. Below the sample, 150 mL hexane are put in a round bottom flask. The solvent is heated up and it is left for between 4-8 hours, depending the exact amount sample weighed.

Note: The extraction finishes when the hexane passes through the solid sample and comes out transparent. When the solvent returns to the round bottom flask colourless it means the total amount of oil that was in the sample has already been extracted, so the extraction has finished. Depending how much sample there is or the temperature of the flask the extraction can last more or less.

When the extraction is finished, the hexane is evaporated and the oil remaining is weighed. Afterwards, the oil will be dissolved in methanol if FFA analysis by titration will be done or the trans-esterification will be made if FAME analysis by GC-FID is required.

- FAME ANALYSIS

For the FAME analysis two different procedures are carried out: one for the commercial sample and one for the extract. The same procedure is followed for all kind of extracts, either soxhlet extracts, scCO_2 extracts or ethanol winterization extracts.

For the commercial sample 0,5 g oil are weighed on the analytical balance and the sample is placed in a round bottom flask. 6 mL methanol-sulphuric acid solution (5%, w/w) are added to the flask; boiling chips are added too. The solution is heated by reflux for 1,5 hours. Once the time passed, the flask is let to cool down. 10 mL hexane and 5 mL water are added to the solution, and the flask is shaken vigorously, in order to do a liquid-liquid extraction. The content is transferred to a clean test tube. The fatty acid methyl esters have been extracted to the upper-apolar phase, so 5 mL of this phase are passed to another test tube and 2 mL water more are added for one more liquid-liquid extraction. The tube is shaken and the upper phase is collected in a vial. This sample is the one to be analysed by GC-FID.

For the extracts, the procedure is a little different. $scCO_2$ extract are already collected in methanol, so 50 mL of it are placed in a round bottom flask and boiling chips and concentrated sulphuric acid are added in order to be 5% (w/w) of the solution. In the case of the soxhlet extract or the ethanol winterization extract, the solvent is first evaporated. Then the oil is dissolved in methanol-sulphuric acid solution (5%, w/w) and boiling chips are added. The solutions are then heated by reflux for 1,5 hours. When the time has passed and the flask has cooled down 20 mL hexane and 10 mL water are added for the liquid-liquid extraction, and the flask is shaken vigorously. The content is then transferred to a test tube and 5 mL of the upper phase are passed to another test tube. Here 2 mL water are added and the test tube is again shaken. The upper phase is taken to a vial and that is the sample to be analysed by GC-FID.

- SUPERCRITICAL CO_2 EXTRACTION

Note: The sea buckthorn berries were first pressed by a juice company. Then, the product remaining was dried and the seed and pulp mass was collected by a responsible from the department. That is the way sea buckthorn solid sample was prepared.

The sea buckthorn peels are weighed in a vessel using analytical balance. More or less 8g sample are weighed and the exact amount is written down. The sample is transferred to the extraction chamber.

Then, the extraction is automatically made, choosing the parameters needed (pressure, temperature, CO_2 flow and make-up solvent flow).

Extract samples are taken each certain time depending how long an extraction lasts. If the extraction lasts less than 2 hours samples are taken each 10 minutes; whether the extraction takes longer than 2 hours samples are taken each 20 minutes.

Extract samples are collected in a round bottom flask and the solvent is evaporated. Afterwards, the flask is weighed through time, and with the data obtained extraction graphs are drawn.

- FREE FATTY ACIDS ANALYSIS BY TITRATION

As explained before, two different procedures were followed to make the FFA analysis: methanol method and standard method.

- Methanol method:

First, the solvent is evaporated. Then, 150 mL methanol are neutralized by titration using NaOH 0,1 M and phenolphthalein as indicator. The extract oil is dissolved into

neutralized methanol and 3 samples of 50 mL each are prepared. Each sample is titrated using NaOH 0,1 M and the volume spent is measured.

- Standard method:

As before, the solvent of the extract is evaporated. Afterwards, 150 mL EtOH:Et_2O (1:1) are prepared and this solution is neutralized with NaOH 0,1 M. The extract oil is dissolved into the neutralized EtOH:Et_2O (1:1) solution. 3 samples of 50 mL each are prepared and they are titrated with NaOH 0,1 M. The volume spent is measured.

With the volume NaOH 0,1 M spent in each case, calculations are made in order to determine free fatty acids percentages (w/w) in the total sample.

- ETHANOL WINTERIZATION

The solvent of the extract obtained from the scCO_2 extraction is evaporated. Then, the remaining oil is dissolved in 150 mL ethanol in a 500 mL beaker and it is covered by paraffin paper. The beaker is left 48 hours in the freezer.

Once the time passed, it can be seen that the waxes have precipitated. The solution is filtered by vacuum and the ethanol is then evaporated. Finally the oil is prepared for FFA analysis by titration or FAME analysis by GC-FID.

3.2. RESULTS AND DISCUSSION

As it was stated before, the main part of this project was the supercritical CO_2 extraction of the sea buckthorn pulp. Several extractions were carried out in order to find out the best instrumental method and parameters to get the major efficiency possible. Apart from that, the free fatty acid amount from the extract was analysed by titration, and the extract were also analysed by GC-FID after the trans-esterification to determine its composition qualitatively and quantitatively.

While doing research on supercritical CO_2 extraction, many parameters were combined in order to get the maximum amount of oil extracted from the dried fruit pulp. The parameters that were changed to achieve that objective were pressure, temperature and CO_2 flow. After many trials with different parameter values we looked up in the literature, we came with some results which defined the path of our project. [11]

Each parameter from those three previously appointed had a different effect on the results and it changed the data obtained particularly:

- Temperature: It was the parameter that had the least effect on the extraction yield and efficiency. Once surpassed the critical temperature of the CO_2, it really did not have such effect a change in the temperature, so we defined the best extraction temperature to be 40ºC, as it is not a very high temperature and it is not difficult to reach it in the oven.

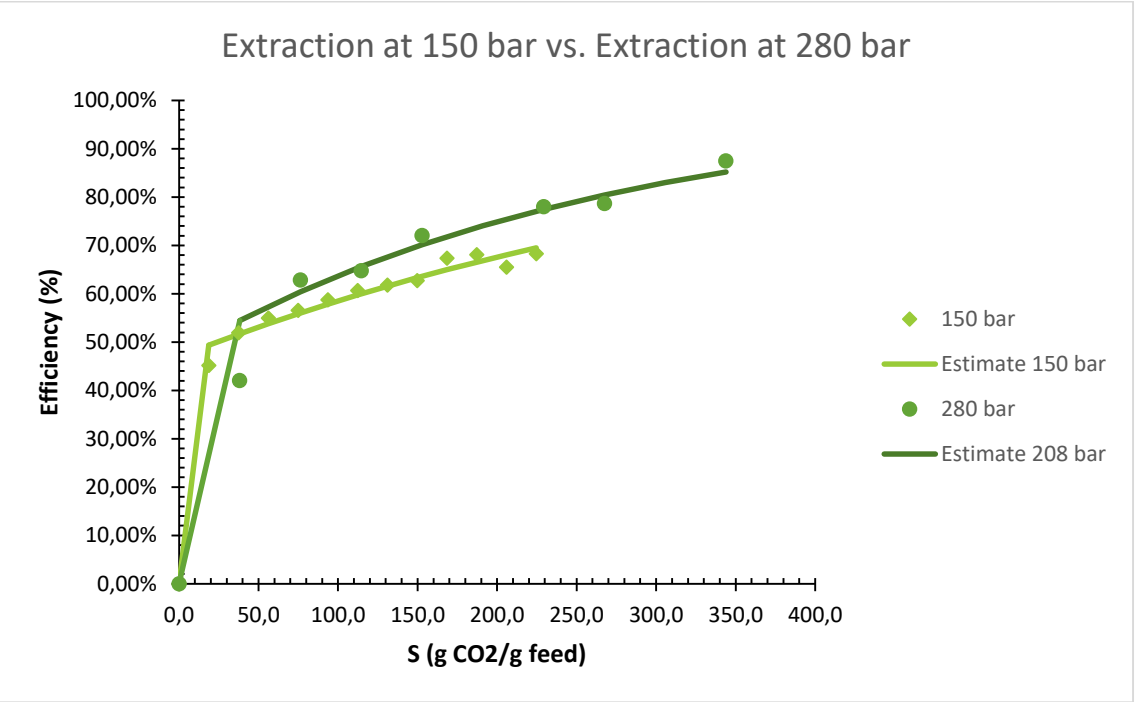
- Pressure: On the contrary to temperature, the pressure did have a crucial role in the extraction efficiency. It could be said that the more pressure it was applied in the extraction vessel, the better extraction efficiencies were obtained. Having viewed that factor, we defined as the best extraction pressure at 280 bar, as it is a pressure relatively easy to reach and not that high so as to be a problem for the extractor.

- CO_2 flow: Possibly this is the parameter which has the most complex explanation. At low flows, the CO_2 moves slowly through the vessel so it has time to saturate. As a result, the efficiency increases slowly and almost constantly through the time. On the contrary, at high flows, the CO_2 moves fast through the vessel, and although it has more extraction capacity as more CO_2 is introduced in the vessel, the gas does not have time to saturate, so its efficiency increase is not as high as in low flows.

The extraction results are given in graphs, were the two parts of an extraction can be clearly seen. In each graph, one of the parameters is changed, as the others are kept constant. This way the effect of each parameter can be appreciated.

First, we drew a graph of two extractions at different pressures in order to see the difference in efficiency that this parameters affects. The extraction were made at different pressures, but the temperature and CO_2 flow were the same in both of them.

Figure 12: Extraction at 150 bar vs. Extraction at 280 bar



- Temperature: 40ºC
- CO_2 flow: 15 mL/min
- Extraction time: 2 hours for 150 bar; 3 hours for 280 bar

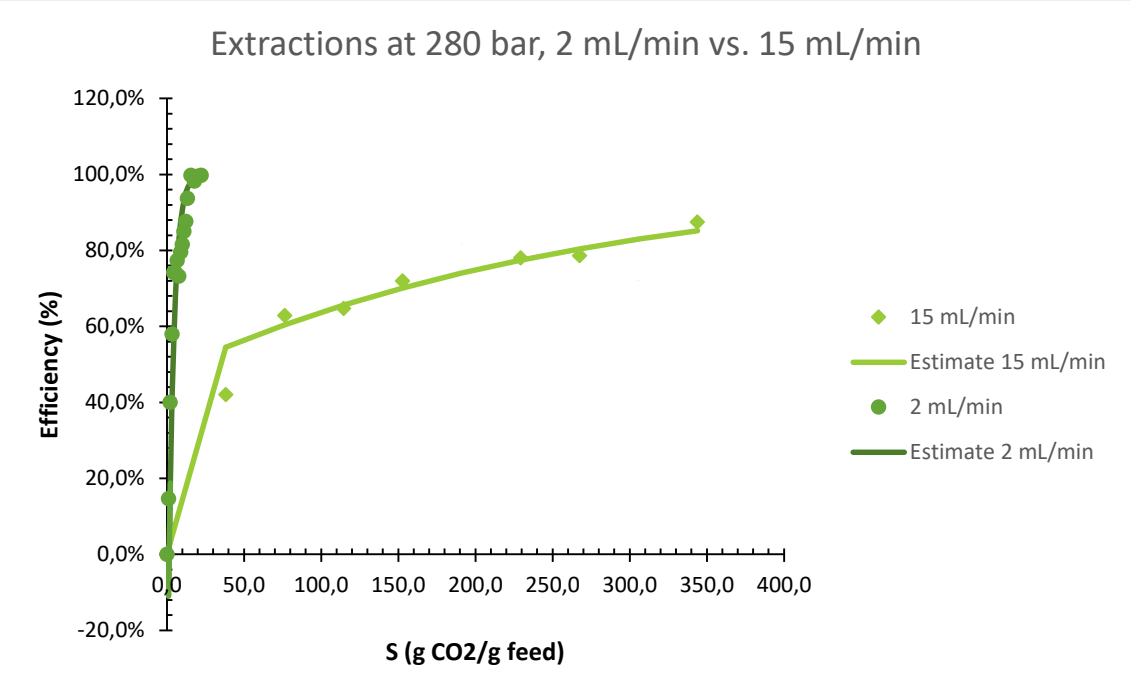
As it can be seen in the graph above *(Figure 12)*, the efficiency of the extraction at 280 bar is higher than at 150, so it can be determined that the higher the pressure applied is, the better efficiency we can get. This is an important factor to take into account as pressure is the most important physical parameter applied in the extractions.

In *Figure 12* it is clearly shown the nature of an extraction process. The extract amount increases through time but it does not do it constantly. The slope changes during the extraction and it can be appreciated that the process can be divided into two parts: the first part, were the slope of the graph increases quickly and lasts a short time, and the second part, which takes a longer time due to a slower increase of the efficiency. In the graph, the first part of the extraction supposes more or less 50% efficiency for both extraction methods; afterwards, in both cases the efficiency increases slowly until reaching around 90% in the case of 280 bar and more or less 70% for 150 bar. This change in efficiency happens because in the first part of the extraction, the oil is extracted from the shells of the dried fruit. The $scCO_2$ does not need to get into the peels to extract oil, so the extraction happens quickly with high yield. But when the oil from the outside of the peels runs out, the $scCO_2$ has to enter inside the peels in order to follow with the oil extraction and this process lasts more time, giving lower efficiency increase.

Knowing this fact, it is logical to think higher pressure leads to higher efficiency, as higher pressures help $scCO_2$ to enter better into the fruit peels.

Having determined the optimal extraction pressure, the effect of different CO_2 flows was analysed with different extractions. In this case, temperature and pressure were kept constant, and the difference in effects of low flows and high flows were compared.

Figure 13: Extraction at 280 bar, 2 mL/min vs. 15 mL/min



- Temperature: 40ºC
- Pressure: 280 bar
- Extraction time: 3 hours

Figure 13 shows the efficiency difference between 2 mL/min flow and 15 mL/min flow. The efficiency increase at 2 mL/min flow is almost constant while at 15 mL/min it is not. At high flows, extraction efficiency increases fast at first, but it keeps increasing slowly after the slope change, without reaching the total extraction efficiency. On the contrary, if we take a look to the extraction at 2 mL/min flow, it can be seen that the extraction reaches almost 100% efficiency in the first stage and then changes its slope. If we would keep doing the extraction at that flow, we would see a totally horizontal line there, because there would not be any more oil to be extracted in the fruit peels. So, from the graph we can conclude that working at high flows the efficiency increases faster than working at low flows but it does not reach its maximum point, as it happens with 2 mL/min flow. This happens because at 15 mL/min the CO_2 does not come out the

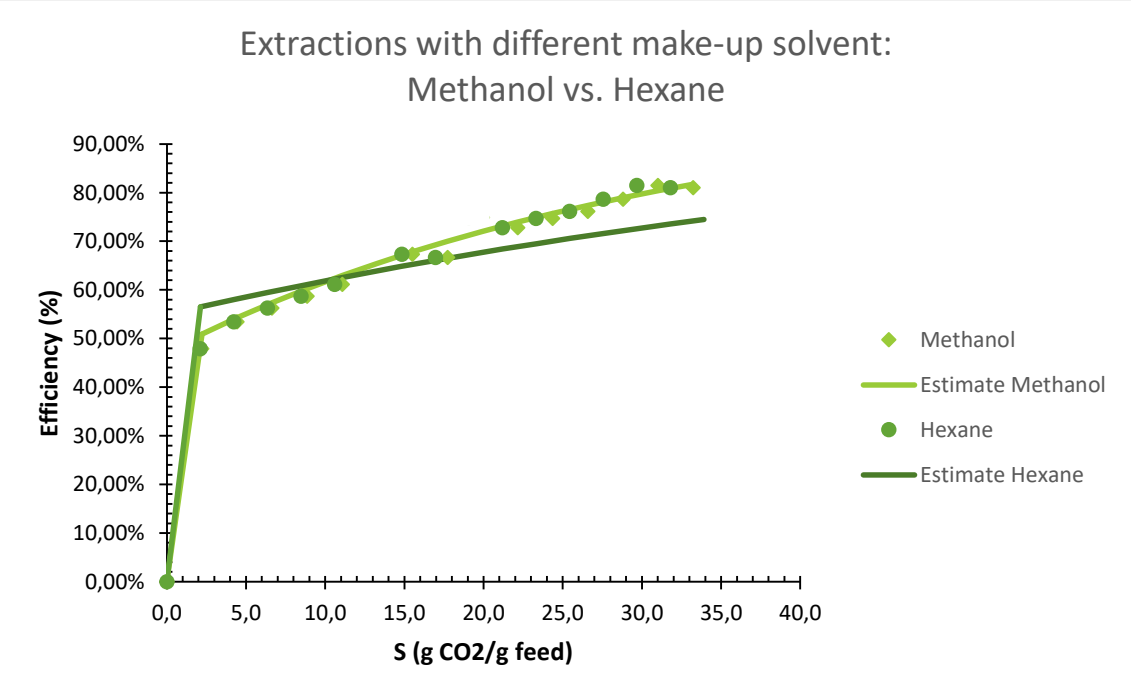
extraction vessel saturated. Conversely, at 2 mL/min the CO_2 passes slowly through the extraction vessel, so it comes out totally saturated of extract oil.

So, the difference between low and high flow rates can clearly be seen in this graph. Nonetheless, that difference does not define which flow rate is better or worse. This can be defined by the objective of an extraction and depending what we want to achieve, the one or the other could be of a better application. For instance, if we want to extract the maximum amount of oil from a sample, low flow rates would be more appropriate, but if our goal is a qualitative analysis, high flow rates would give us the correct results in much less time. What we know is that an extraction at low rates is better if we want to investigate the first stage of the extraction, and high rates are better to define the second stage of it.

These extractions were carried out using methanol as make-up solvent, as it was the standard method, but another question could be made around the extractions: Leaving the physical parameters apart, does the nature of the make-up solvent interfere on the efficiency of the extraction? So, in order to clear out that doubt, more extractions were made, this time comparing methanol with hexane as make-up solvent.

Two long extractions were made with exactly same parameters, but changing the solvent, and this is the graph we obtained.

Figure 14: Extractions using different make-up solvent: Methanol vs. Hexane



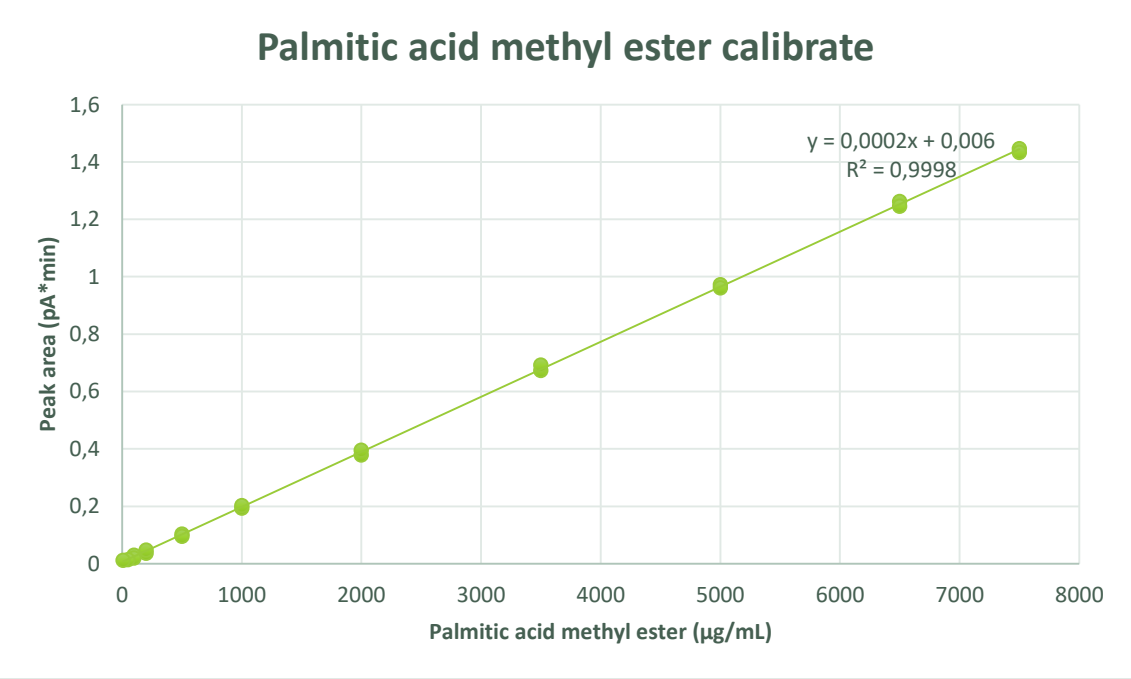
- Temperature: 40ºC
- Pressure: 280 bar
- CO_2 flow: 15 mL/min
- Extraction time: 5 hours

In the *Figure 14* two features can be distinguished: making an analysis of the first stage of the extractions, could be said that the efficiency increase is bigger when using hexane. But then, if we take a look at the second stage, we see that the efficiency grows further when methanol is used. So, as the aim of the research is to obtain the maximum efficiency possible, we could say that methanol is a better make-up solvent than hexane.

These previous results were part of the optimization of the supercritical CO_2 extraction, but apart from it more analysis were also made. Taking scCO_2 extracts as a base, FFA analysis by titration and FAME analysis by GC-FID were made.

In order to determine the concentration of each fatty acid in the extracts, an external calibrate for palmitic acid methyl ester was prepared. The palmitic acid is the most known of the fatty acids from the extract, and as its concentration behaviour in the calibrate changes the same way as the other fatty acid methyl esters that appear in the samples, its calibrate can be used for all of them: Lauric acid methyl ester, Myristic acid methyl ester, Palmitoleic acid methyl ester, Palmitic acid methyl ester, Linoleic acid methyl ester and Stearic acid methyl ester. These fatty acid methyl esters are the ones that appear in the sea buckthorn commercial sample oil *(Figure 16)*.

Figure 15: Palmitic acid methyl ester calibrate



$$Peak\ area\ (pA * min) = 0{,}0002 * Conc.\left(\mu\frac{g}{mL}\right) + 0{,}006$$

So, having prepared a calibrate, concentration of fatty acids from each sample can be calculated using the equation above. These are the GC-FID analysis chromatograms we obtained.

Figure 16: Commercial sample of $scCO_2$ sea buckthorn oil extract chromatogram

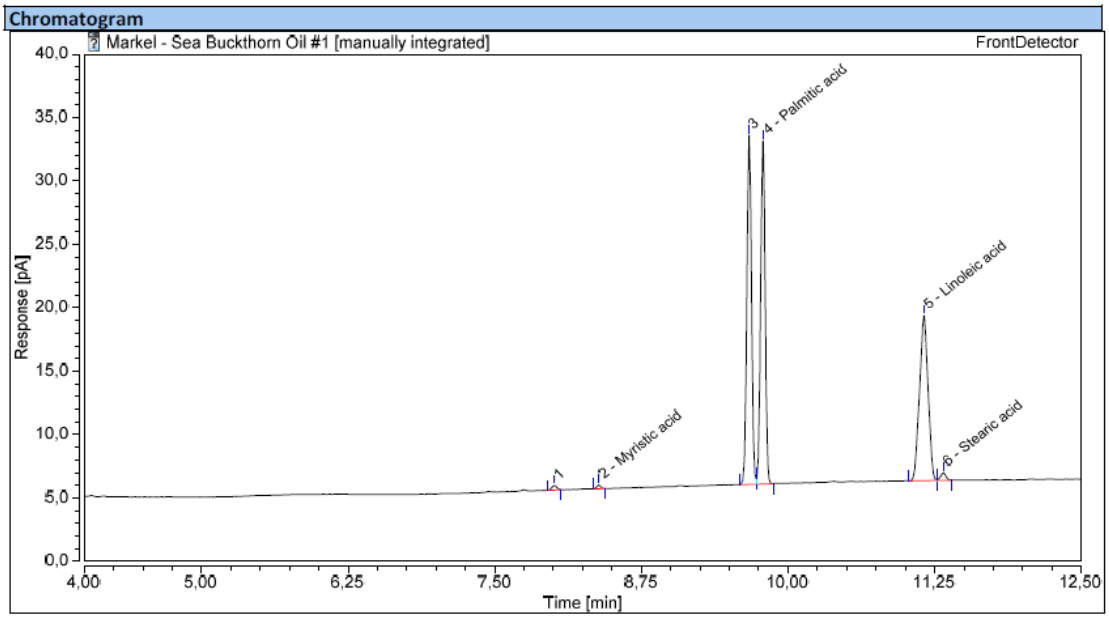


Figure 17: Table for concentration of each fatty acid in the commercial sample

Fatty Acid Methyl Ester	Peak Area (pA*min)	Relative Area (%)	Concentration (µg/mL)
Lauric Acid Methyl Ester	0,019	0,51	65
Myristic Acid Methyl Ester	0,012	0,34	30
Palmitoleic Acid Methyl Ester	1,254	34,63	6240
Palmitic Acid Methyl Ester	1,215	33,57	6045
Linoleic Acid Methyl Ester	1,090	30,10	5420
Stearic Acid Methyl Ester	0,031	0,85	125

These are the 6 fatty acids that appeared in the GC-FID analysis chromatogram of the commercial sample. In the case of palmitoleic acid methyl ester we did not identify it directly, but we qualitatively assumed that the third peak belonged to it because it appeared right next to the one of palmitic acid methyl ester, just a bit earlier. As unsaturated fatty acids appear only a little bit earlier than their saturated homologues, the deduction should be the correct one. Knowing this, it is supposed that in the supercritical CO_2 extract of the sea buckthorn oil there will be the same fatty acids.

Different samples were prepared, using different preparation methods. All of them were analysed in order to see if there was any contrast in the results. These are the other analysis.

Figure 18: Chromatogram of the $scCO_2$ extract

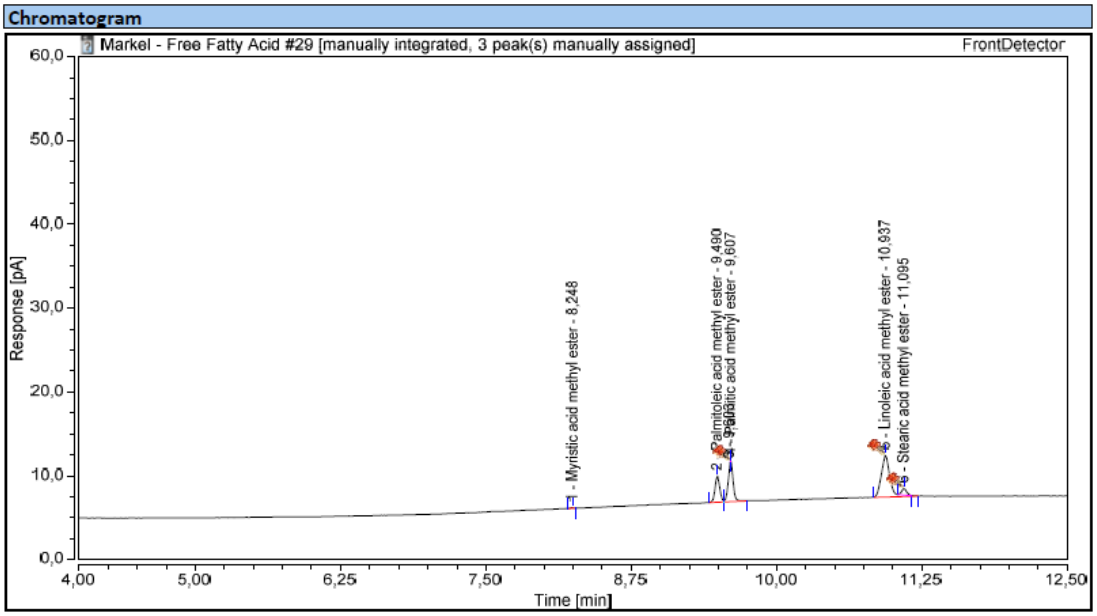


Figure 19: Table for concentration of each fatty acid in the $scCO_2$ extract

Fatty Acid Methyl Ester	Peak Area (pA*min)	Relative Area (%)	Concentration (µg/mL)
Lauric Acid Methyl Ester	-	-	0
Myristic Acid Methyl Ester	0,004	0,35	0
Palmitoleic Acid Methyl Ester	0,148	17,47	710
Palmitic Acid Methyl Ester	0,231	27,24	1125
Linoleic Acid Methyl Ester	0,426	50,31	2100
Stearic Acid Methyl Ester	0,039	4,61	165

In the case of the $scCO_2$ extraction that we made, Lauric Acid Methyl Ester is not even detected or there is not any amount of it in the sample. Apart from that, the concentration of Myristic Acid Methyl Ester cannot be calculated, due to the small signal of it.

Taking a look to the results of the $scCO_2$ extract, one of the questions around this extract could be if $scCO_2$ extraction is effective enough to analyse all the fatty acids. As there was not a sure answer for it, the sample was extracted this time using soxhlet extraction, so this way could be seen if there was any mistake in the analysis.

Figure 20: Soxhlet extraction chromatogram

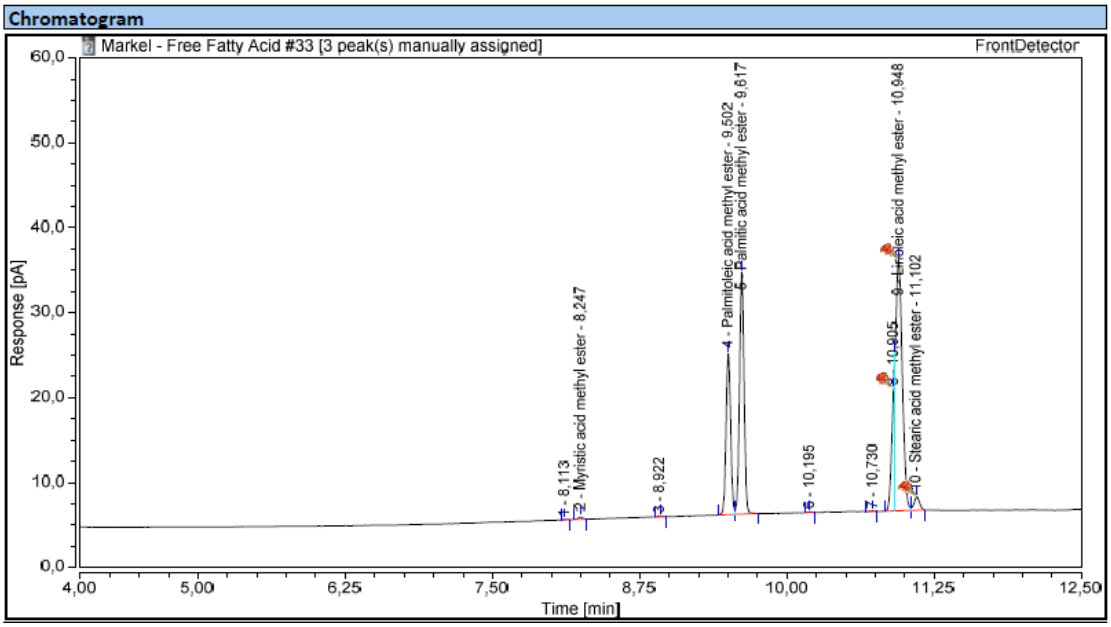


Figure 21: Table for concentration of each fatty acid in the soxhlet extract

Fatty Acid Methyl Ester	Peak Area (pA*min)	Relative Area (%)	Concentration (µg/mL)
Lauric Acid Methyl Ester	-	-	0
Myristic Acid Methyl Ester	0,013	0,32	35
Palmitoleic Acid Methyl Ester	0,847	20,70	4205
Palmitic Acid Methyl Ester	1,260	30,79	6270
Linoleic Acid Methyl Ester	1,883	46,02	9385
Stearic Acid Methyl Ester	0,089	2,17	415

Comparing $scCO_2$ extract and soxhlet extract, it could be said that the concentrations in the second one were much higher. This tells us that soxhlet extraction is more effective than $scCO_2$ extraction. It is a fact that could be expected, as it was known that soxhlet extraction was totally effective, on the contrary to $scCO_2$ extraction. But this was one of the matters from the start and this is important data to keep for future improvements on $scCO_2$ extraction.

Having made this comparison, another question asked was if $scCO_2$ extract could be improved. The answer of lower concentration on the $scCO_2$ extract could be the matrix effect. So, ethanol winterization was applied to the oil obtained in the extractions so as to improve fatty acid methyl esters determination in the sample.

Figure 22: Ethanol winterization extract chromatogram

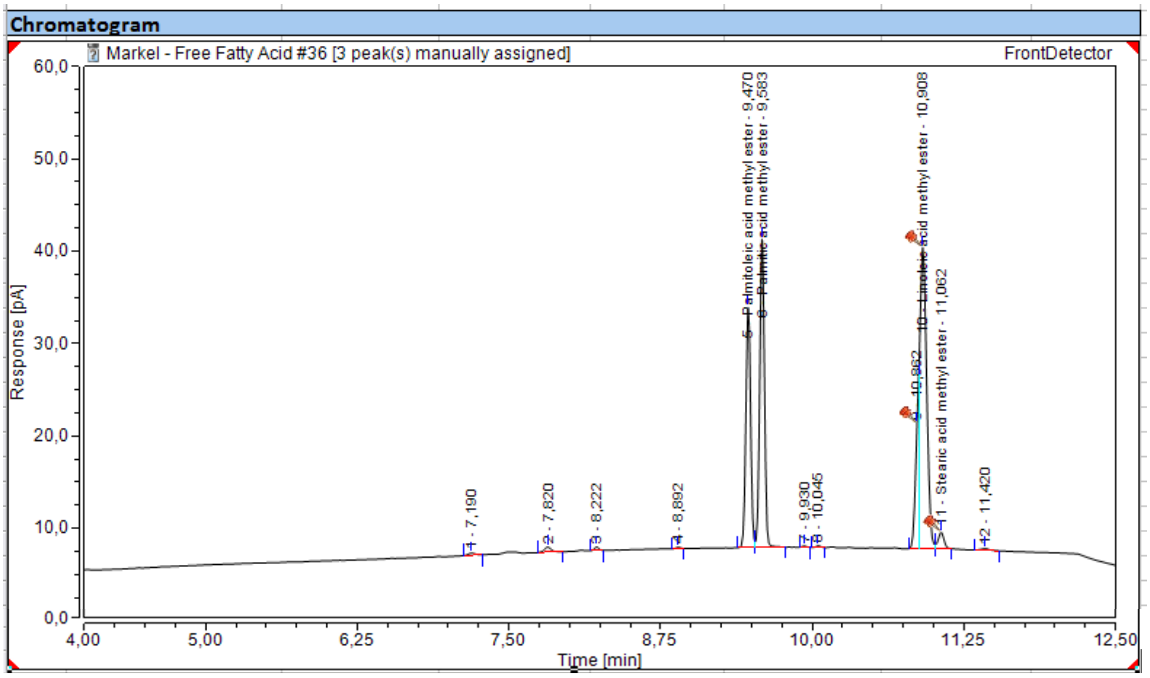


Figure 23: Table for concentration of each fatty acid in the ethanol winterization sample

Fatty Acid Methyl Ester	Peak Area (pA*min)	Relative Area (%)	Concentration (µg/mL)
Lauric Acid Methyl Ester	-	-	0
Myristic Acid Methyl Ester	0,007	0,14	5
Palmitoleic Acid Methyl Ester	1,182	24,09	5880
Palmitic Acid Methyl Ester	1,503	30,64	7485
Linoleic Acid Methyl Ester	2,112	43,05	10530
Stearic Acid Methyl Ester	0,102	2,08	480

As it can be seen in the chromatogram *(Figure 22)* the concentrations obtained are much more comparable and even higher than the soxhlet extract when applying ethanol winterization to the $scCO_2$ extract. According to this information, it can be said that there actually is a negative matrix effect in the $scCO_2$ extract and that this effect erases from there when removing the waxes from the oil.

In fact, $scCO_2$ and soxhlet extracts solidified at room temperature, when judging from their unsaturated fatty acids composition they were supposed not to do it. But when applying ethanol winterization to the $scCO_2$ extract, the oil did not solidify at room temperature. So, we can now ensure that sea buckthorn extract oil is liquid at room temperature.

Apart from each fatty acid methyl ester analysis, which encompasses all fatty acids whether they are free or not, free fatty acid analysis by titration was made so as to

determine the amount of them in the different samples. These are the results of FFA analysis and saturated/unsaturated fatty acids percentages in each sample:

Figure 24: Table for FFA percentages and saturated/unsaturated fatty acid percentages in different samples

	Commercial sample	$scCO_2$ extract in methanol	Soxhlet extraction	Ethanol winterization
FFA (%)	4,27	4,77	4,49	4,24
Saturated fatty acids (%)	35,27	32,17	33,28	32,86
Unsaturated fatty acids (%)	64,73	67,83	66,72	67,14

In the table above *(Figure 24)* it can be seen that the free fatty acid percentage in the sea buckthorn peels is around 4,50 %, in some one more and in some one less. FFA percentage was supposed to be around that number as in all the literature we looked up the amount was more or less that one. Moreover, we confirmed it with the analysis of the commercial sample. So it can be said that FFA results are correct for all the samples. Furthermore, the FFA percentage that most approaches to the commercial oil is the ethanol winterization. When confirming that in the $scCO_2$ extract there was a matrix effect caused by the waxes in it, this was expected to happen, and removing all the waxes from the oil it can be stated that after the application of the ethanol winterization, FFA percentage in the sample gets close to that from the commercial oil. In addition, according to all the literature, sea buckthorn oil is composed by more or less 65% unsaturated and 35% saturated fatty acids. As it can be seen in the table, that is exactly the composition of the commercial oil analysed by us and the rest of the samples have almost the same composition.

So, analysing all the data and results obtained, it seems that applying supercritical CO_2 extraction plus ethanol winterization we can obtain almost an equal sea buckthorn oil as the commercial one. This was the main goal of our project, how to get oil of the same quality as the commercial one.

Applying $scCO_2$ extraction and ethanol winterization, sea buckthorn oil can be obtained at high purity in order to use it in the food industry, so this research is worth it for enterprises dedicating to it.

4. CONCLUSION

Making a slight analysis on the project, we could say that we got the results we expected to obtain. The main objective was to make research and optimize supercritical CO_2 extraction for sea buckthorn in analytical level in order to try to extrapolate these results to an industrial level. Moreover, it was interesting that those results turned to be qualitatively and quantitatively correct, as $scCO_2$ is considered the "green way" when talking about extractions. If the results were to be correct, the major question here would be if the same analytical $scCO_2$ process applied to sea buckthorn pulp could be carried to an industrial level, so as to decrease as much as possible the use of hazardous organic solvents in a large scale.

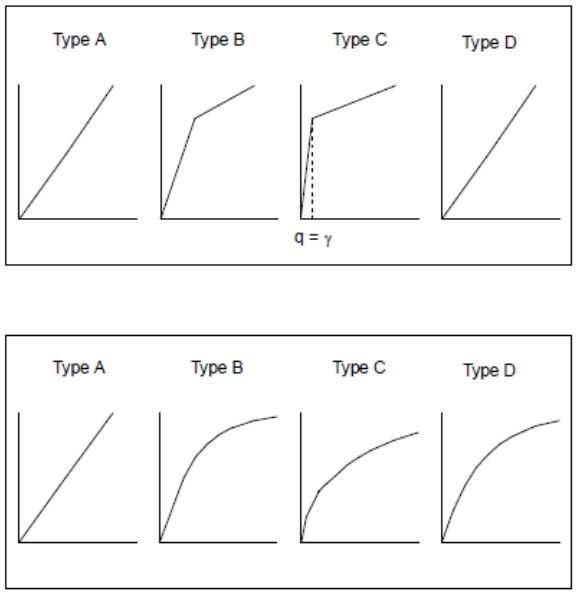
But a comparison between different extraction methods could be done at least in analytical level. This is the reason why we have also made research with soxhlet extraction. When taking a look to *Figure 24*, different fatty acids percentages can be seen, and it can be noticed that $scCO_2$ and soxhlet extraction results are slightly similar. Furthermore, when ethanol winterization is applied to the $scCO_2$ extract, the results obtained are even closer to the commercial sample than the ones from the soxhlet extraction. So, we can assure that at least at analytical level supercritical CO_2 extraction can replace soxhlet extraction, which requires the use of organic solvents.

Following with the results of this project, some talking should be done around the supercritical CO_2 extraction method itself. After many research, the optimum parameters for the extractions were defined. The extraction at these parameters were supposed to give similar extraction graphs as the theoretical models of the literature. Those parameters, previously stated, are 40ºC for temperature, 280 bar for pressure and 2 mL/min and 15 mL/min for CO_2 flow. Those parameters were the ones that most appeared in the literature and after many trials changing them we actually saw that those were the ones that better worked. As previously said, in the case of the CO_2 flow, it cannot be defined which flow is better, high or low, because it is dependent of our objective. If our aim is to get large amount of extract in short time, the working method should be at high flows but if our goal is to get the maximum oil amount from a vegetable feed, the method followed should be carried out at low flow rate.

Several theoretical background has been looked up before and during the project and the researches that mostly have been followed are Helena Sovová and Michel Perrut. In different articles of these researches about $scCO_2$ different extraction types were shown *(Figure 25)*. In those articles it is explained that in the case of vegetable oils, the extraction graphs take a heterogeneous shape, which combines a line in the first part of the extraction and a soft curve in the second part, separated by a spike, more or less as it happens with *Type C (Figure 25)* graph below. At first instance, our graphs also took this shape and we were doubtful about the correctness of them because we thought the curve should be softly drawn, as the graph in *Type D*. But, after many trials and looking up this information in the literature, we noticed that in fact, our graphs must had that

shape, because that one was the model that a $scCO_2$ extract of a vegetable feed followed. This way we knew we were following the correct experimental path. [11, 12, 13]

Figure 25: Different extraction type models



Helena Sovová, Marie Sajfrtova (2005): Experiments, Modelling and Scale-up for Supercritical Extraction from plants (ISASF European meeting on super fluids).

To sum up, the ethanol winterization should be added after $scCO_2$ extraction. The extract without the application of the winterization had an alike fatty acid composition to the one of the commercial sample *(Figure 24)* but the application of the winterization gave even closer results to it. Apart from that, as this washing process removed unwanted compound and waxes from the matrix, the oil remaining looked much more pure and cleaner until the point that looked beautiful to the sight with the characteristic bright orange colour of the sea buckthorn oil. So the ethanol winterization has been an important stage of the $scCO_2$ extract treatment and it should be followed in the future analytical and industrial supercritical CO_2 extraction in sea buckthorn.

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SAFETY CARDS OF THE REAGENTS USED

Substance or compound	Reference
Carbon dioxide	https://www.cdc.gov/niosh/ipcsneng/neng0021.html
Hexane	https://www.cdc.gov/niosh/ipcsneng/neng0279.html
Methanol	*https://www.cdc.gov/niosh/ipcsneng/neng0057.html*
Ethanol	https://www.cdc.gov/niosh/ipcsneng/neng0044.html
Acetone	*https://www.cdc.gov/niosh/ipcsneng/neng0087.html*
Sulphuric acid	*https://www.cdc.gov/niosh/ipcsneng/neng0362.html*
Sodium hydroxide	*https://www.cdc.gov/niosh/ipcsneng/neng0360.html*
Potassium hydrogen phtalate	*https://www.cdc.gov/niosh/ipcsneng/neng1585.html*
Phenolphthalein	*https://www.ch.ntu.edu.tw/~genchem99/msds/exp14/phenolphthalein.pdf*
Diethyl ether	*https://www.cdc.gov/niosh/ipcsneng/neng0355.html*