Universidade de Coimbra

Faculdade de Ciências e Tecnologia

Departamento de Botânica

Biodiesel production from extracted
***Cynara cardunculus* L. oil (Soxhlet & SFE)**

Mestrado em Biodiversidade
e Biotecnologia Vegetal

Agostinho Manuel dos Reis Carrondo Alexandre
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Projecto realizado em colaboração com o DEQ da FCTUC

Orientador: Prof. Doutor António Portugal

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O meu sincero bem-haja a todos vós!

Abstract

Suitable and new energy crops are on the day's order to provide a better and sustainable future for planet Earth. The present work aims to use *Cynara cardunculus*, L. seeds' oil to produce biodiesel with the purpose of trying to find a suitable alternative to petroleum based fuels at a regional scale, as it is an endogenous plant of the Mediterranean Flora. The oil was extracted using two different methods, a traditional Soxhlet apparatus using n-hexane as a solvent, and by supercritical carbon dioxide technology, which confers a greener approach to oil extraction using a clean technology. Different temperatures and pressures were used in the SFE's extraction, varying the CO_2 density and consequently oil solubility in CO_2, as well as oil extraction yields and its' properties. It were tested the temperatures of 35ºC and 55ºC at pressures of 150 bar and 300 bar, and a midpoint of pressure and temperature at 45ºC and 225 bar. Transesterification process was performed using alkali based process to transform the oil esters into methyl esters (FAME); methanol as solvent and sodium methoxide as catalyst, in a 6:1 molar ratio. Despite this, biodiesel yields were lower than the expected probably due to saponification reactions (saponins present) occurring in simultaneous with the presence of free fatty acids. These were mainly due to the amount of free fatty acids present in the oil as well as *cynara* saponins. Preliminary tests indicate that acid catalyzed esterification before transesterification are necessary to raise biodiesel yield as well as an increase in FAME content, reaching values stipulated in the norm EN14214. Cardoon proved to be a suitable energy crop as the plants were all randomly selected and had no specific treatment for their development. Taking into account these achievements and the European directive 2003/30 that postulates an increasing insert of biodiesel in all public transport's fuel until 2020, *Cynara* should be considered as an energy crop in alternative to edible plants.

Furthermore it may work as a boost to regional economy of the Portuguese interior areas.

Resumo

Novos e apropriados cultivares energéticos estão na ordem do dia de modo a proporcionar um futuro melhor e mais sustentável para o planeta Terra. Este trabalho tem como objectivo usar o óleo das sementes de *Cynara cardunculus*, L. para produzir biodiesel com a finalidade de tentar encontrar uma alternativa viável aos combustíveis derivados de petróleo a uma escala regional, já que se trata de uma planta endógena da flora mediterrânica. O óleo foi extraído usando dois métodos diferentes, uma extracção com Soxhlet usando n-hexano como solvente, e outra utilizando dióxido de carbono supercrítico, a qual oferece uma alternativa mais ecológica à extracção com solventes orgânicos e uso de uma tecnologia limpa. Diferentes pressões e temperaturas foram usadas na extracção com SFE, de forma a variar a densidade do CO_2 e consequentemente a solubilidade do óleo no mesmo, assim como o seu rendimento. Foram testadas temperaturas de 35ºC e 55ºC a 150 bar e 300 bar. com um ponto intermédio de pressão e temperatura, a 45ºC e 225 bar. O processo de transesterificação foi realizado usando um catalisador alcalino para a conversão dos ésteres do óleo em metil ésteres, já que o solvente usado foi o metanol e o catalisador o metóxido de sódio, num ratio molar de 6:1. No entanto, os rendimentos da reacção foram abaixo do esperado provavelmente reduzidos pela ocorrência de reacções de saponificação (presença de saponinas) em simultâneo com a presença de ácidos gordos livres no óleo. Testes preliminares indicaram que a esterificação ácida do óleo antes da transesterificação para elevar o rendimento de biodiesel e na conversão de ésteres alcançando os valores postulados na norma EN14214. O cardo provou ser um cultivar energético adequado já que as plantas foram todas seleccionadas aleatoriamente e não sofreram qualquer tipo de tratamento para completarem o seu desenvolvimento. Levando em conta este estudo bem como a directiva europeia 2003/30 que prevê uma introdução crescente de biocombustíveis em todo o combustível de transportes públicos até

2020, as plantas de cardo devem ser consideradas como uma alternativa a outras usadas na cadeia alimentar. Além disso pode servir como um impulsionador da economia de áreas do interior português.

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Introduction

Introduction

The demand for new energy solutions, namely by replacing fossil fuels by others from non-edible vegetables, in order to diminish the dependence on petrol, as well as the CO_2 emissions to the atmosphere, is nowadays concerning governments and energetic companies around the world.

Diesel Engine History

By the end of the XIX century a man named Rudolf Diesel invented the diesel engine, a compression-based engine in opposition to the spark ignition engine that ran on petrol. The diesel engine was first postulated in 1892; in 1894 Rudolf Diesel filed in a patent for it but he only achieved to build the first running diesel engine in 1897 being the patent only granted in 1898. Diesel invented an engine that worked with vegetable oils due to their high energetic content. Therefore he believed that vegetable oils could replace fossil fuels and would help to improve agriculture in countries that bet in these fuels and were at the same time petroleum-dependent. To Diesel, "the use of plant oil as fuel may seem insignificant today. But such products can in time become just as important as kerosene and these coal-tar-products of today." However, short after his mysterious death in 1913, the products derived from petroleum get to know an amazing development, and amongst them arose what is nowadays known as "diesel fuel". This sort of fuel gathered the availability and cheapness of petroleum, and as it was a byproduct from raw crude distillation it was almost
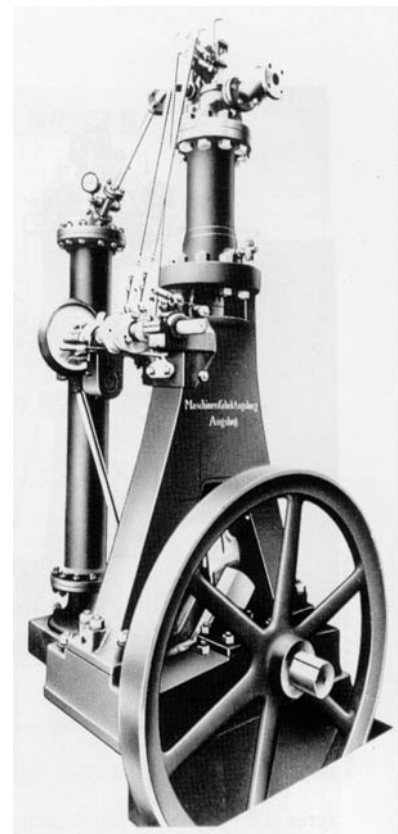
Figure 1 – First Diesel engine (source Ucap, 2010)

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Biodiesel production from *Cynara cardunculus,* L. extracted oil (Soxhlet & SFE)

valueless. By 1920's the engines began to be modified in order to only accept "diesel fuel", the direct injection system upgraded even more Diesel's engine which led to the fall of vegetable origins' fuel to second plan until the 1980's (Grosser, 1978 ; Moon, 1974).

Oil Demand

In spite of the worldwide spread of motor engines as well as World War II, oil demand and CO_2 emissions rose up to unbridled levels. Petroleum had become the "black gold" of the XX century. By the 1970's the world got to know the first oil crisis when the OPEP decided to proclaim an oil embargo in response to the US support to the Israeli military cause. After that two crises came to
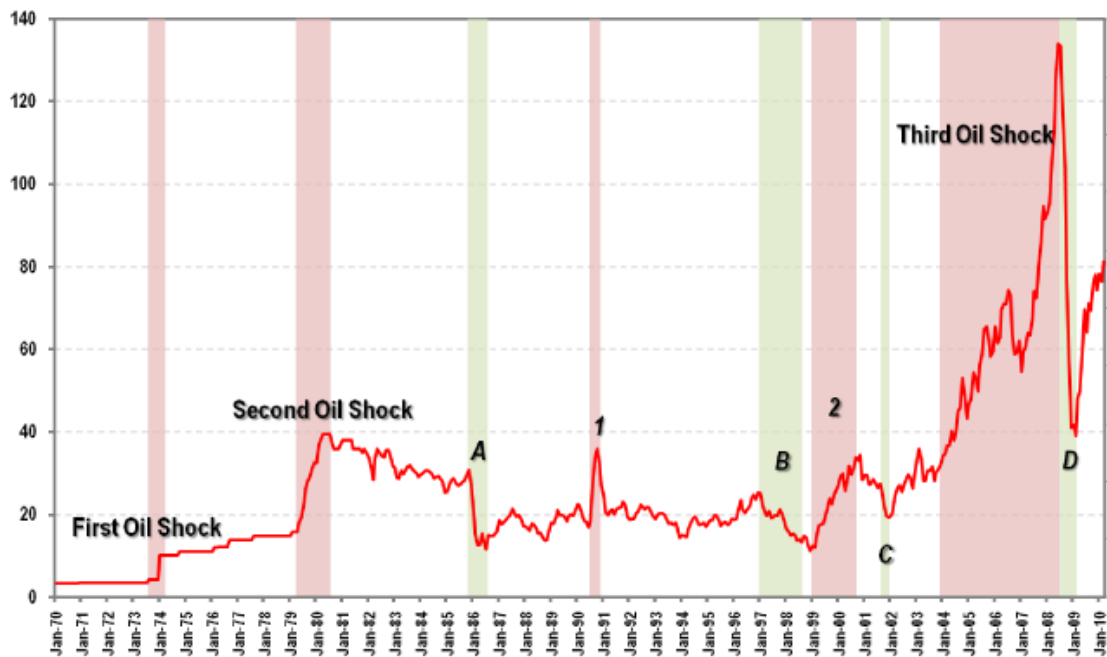
Figure 2 – Oil price changes since 1970, Source: Federal Reserve Bank of St. Louis. (Rodrigue 2010)

raise the oil price, especially in 2008 leading to unseen crude prices' (Barsky and Kilian, 2004). In Figure 2 it is showed that the petrol prices upswing since 1970 and it is possible to confirm the higher climbs during the three oil shocks. Meanwhile, alternative routes of energy consumption came to light in order to suppress the oil dependence and provide cleaner alternatives. Biofuels slowly

started to reenter the market and gaining the public opinion's respect and admiration as they provided a cheaper and greener alternative to fossil fuels.

Biodiesel

Biodiesel is nowadays a common term and a simple search in any internet search engine for scientific articles will easily reveal more than 5000 articles related to the subject. (Science Direct, 2010) Biodiesel is by definition a set of mono alkyl esters of long chain fatty acids derived from vegetable oils or animal fat, in order to be used in a compression ignition engine. A chemical process named transesterification normally produces it, but other process can also be used, such as pyrolysis and emulsification (Meher,et al., 2006 ; Murugesan, et al., 2009).

Transesterification Process

Transesterification is defined by the displacement of alcohol from an ester by another alcohol in a process similar to hydrolysis, except that alcohol is used instead of water. In the majority of cases, methanol is used because of the restrictions to acquire ethanol in large amounts(Knothe, Gerpen e Krahl 2005). In countries like Brazil, where ethanol is cheaper and more available than methanol, this alcohol is preferred for biofuels production, like bioethanol (Pousa et al., 2007 ; Monteiro, et al., 2008).

The transesterification process can be easily described by a general equation, as shown below in Figure 3, producing methyl esters and glycerol in a reaction where the vegetable oils' triglycerides are transformed gradually into diglycerides, monoglycerides and finally into fatty acid alkyl ester of FAME (fatty acid methyl ester), and glycerol as a byproduct of the reaction (Demirbas, 2009). Glycerol was for many years, especially in the XVIII century, the main goal for this reaction, as it can form soap very easily in the presence of water.
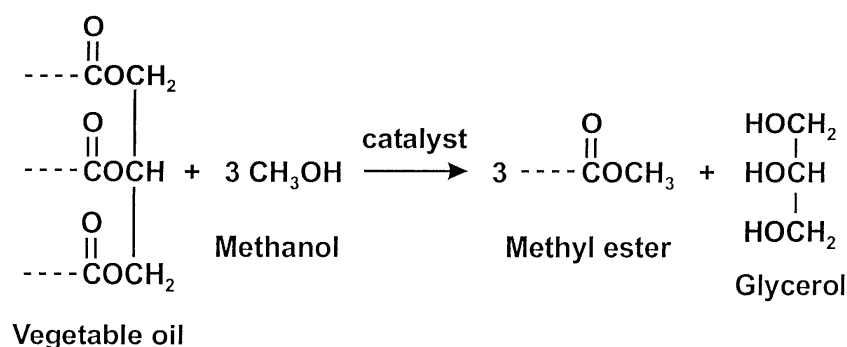
Figure 3 – Transesterification reaction, source Knothe et al., 2005.

According to the origin and characteristics of the oils used to produce biodiesel, slight modifications to the transesterification process may be necessary to achieve better results in terms of yield and quality. These modifications may be related with the catalyst (acid or base) or with different pretreatments made to the oil. These mechanisms are:

- Base catalyzed transesterification
- Acid catalyzed transesterification
- Saponification followed by transesterification
- Acid esterification followed by base catalyzed transesterification

The base catalyzed mechanism is currently the most used method for biodiesel production because of its' higher conversion yields of triglycerides into FAMEs' as well as the velocity of the reaction that is higher (Vyas et al., 2009).

All biodiesel must follow certain norms in order to be sold as a vehicular fuel and to be safe both for storage and for motor safety. Nowadays the accepted are the European standard EN14214 and the USA standard ASTM D6751 (Standardization, 2010 ; ASTM, 2010). Some of the parameters evaluated are listed on Table 1.

Table 1 – EN14214 Standards for Biodiesel

Properties	Limit	Method
FAME content, % (m/m)	Min. 96,5	EN 14103
Density (15ºC), kg/m^3	860 – 900	EN ISO 3675 / EN ISO 12185
Viscosity (40ºC), mm^2/s	3,5 – 5,0	EN ISO 3104
Flash point, ºC	Min.101	ISO CD3679
Sulfur content, mg/kg	Max. 10,0	–
Carbon residue remnant, % (m/m)	Max. 0,30	EN ISO 10370
Cetane number	Min. 51,0	EN ISO 5165
Sulfated ash content, % (m/m)	Max. 0,02	ISO 3987
Water, mg/kg	Max. 500	EN ISO 12937
Total contamination, mg/kg	Max. 24	EN 12662
Copper band corrosion (3h a 50ºC)	Class 1	EN ISO 2160
Oxidation stability (110ºC), Time	Min. 6,0	EN 14112
Acid number, mg (KOH)/g	Max. 0,50	EN 14104
Iodine value	Max. 120	EN 14111
Linolenic Acid Methyl ester, % (m/m)	Max. 12,0	EN 14103
Polyunsaturated methyl ester (≥ 4 double bonds), % (m/m)	Max. 1	EN 14103
Methanol, % (m/m)	Max. 0,20	EN 141101
Monoglyceride, % (m/m)	Max. 0,80	EN 14105
Diglyceride, % (m/m)	Max. 0,20	EN 14105
Triglyceride, % (m/m)	Max. 0,20	EN 14105
Free glycerin, % (m/m)	Max. 0,02	EN 14105 / EN 14106
Total glycerin, % (m/m)	Max. 0,25	EN 14105
Group I metals (Na+K), mg/kg	Max. 5,0	EN 14108 / EN 14109
Phosphorus, mg/kg	Max. 10,0	EN 14107

Most common raw materials used for biodiesel production

Biodiesel can be produced after vegetable oils or animal fats, and the main difference between them is their physical characteristics at

room temperature. These differences are that the oils are liquid while animal fat is solid at the mentioned conditions. The oils are mainly constituted by triglyceride molecules that have three long chain fatty acids that are ester bonded to a single glycerol molecule (Murugesan et al., 2009).

Nowadays the most used crops for biodiesel production are soya-bean, corn and canola due to their availability and high oil content (Patil and Deng, 2009). These are all edible vegetables used commonly in our every day diet. As higher amounts of biofuels are being produced in order to face the challenges of building a greener future for our Planet, sometimes it is forgotten that prior to man's need of vehicular fuel, there are people in the world that depend on these vegetables for their subsistence. Ultimately this unbridled use of these edible crops for biofuels production leads to higher prices for the consumer, along with social and economic instability.

Nevertheless, there are also non-edible vegetables used to produce biodiesel that do not compete with human feeding. Examples of these crops are *Jatropha curcas, Pungamia pinnata* and *Cynara cardunculus*. These crops present major advantages beside non-edible as they may be produced in a massive scale in arid, degrade or waste lands (Murugesan, et al., 2009). Considering that this type of land is normally underutilized and often found in some non-developed countries, this could represent an advantageous opportunity for these crops. The recent climatic changes that have been leading to drier summers and land desertification in countries like Portugal, may also present an alert to the use of suitable energy crops considering each country specifications.

Another problem is related with the extraction of oil from the above-mentioned crops, as it is usually extracted by two methods: a first one using mechanical pressing, which leads to unrefined products and lower yields, and other using organic solvents. A Soxhlet extraction method using n-hexane as solvent is normally

used for this second process. This organic solvent confers higher extraction yields extracting all lipids and higher molecular weight compounds being however a very dangerous solvent to the environment (Chemicals, 2009).

Supercritical fluids extraction

New extraction technologies, such as supercritical fluids that are environmental friendly also provide high extraction yields. If they are used at the appropriate conditions for a specific raw material they will solubilize the compounds of interest as supercritical carbon dioxide presents different selectivity's, and therefore can exhaust the bed. Supercritical fluids have also been used to perform transesterification reaction using only methanol in a supercritical condition. This method has been proved to be faster and economically more viable as it needs less fuel purification (Vyas et al. 2009 ; Kasim et al. 2009 ; Vallea et al. 2010).

A supercritical fluid is a substance that is elevated to a pressure above its' critical pressure and heated up to a temperature superior to its' critical temperature. This confers to the substance properties of liquid and gas in an equilibrium state (Mukhopadhyay, 2000). The carbon dioxide critical temperature is 31ºC and critical pressure is 73,8 bar, so it is possible to manipulate its density by changing values of temperature and pressure above the critical ones and still working at mild conditions (Boutin and Badens, 2009).

Supercritical carbon dioxide presents many advantages when compared to common organic solvent extraction or mechanical extraction. CO_2 is non–toxic, non–flammable and can be easily dissociated from the extract with simple depressurization. This offers advantages at the purity level of the extract as well as the raw material used, which contains no solvent trace and can have other applications at different levels (Boutin and Badens, 2009).
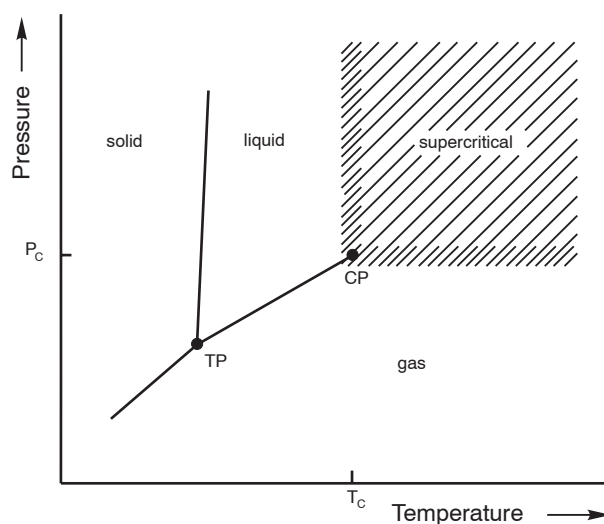
Figure 4 – Supercritical fluid pressure vs. temperature diagram, source Mukhopadhyay, 2000.

Cynara cardunculus

The cardoon (*C. cardunculus*, L.) is a perennial herbaceous plant of the Asteraceae family, native of the Mediterranean ecosystem adapted to dry summers and needing around 500mm of precipitation during winter to perform its' development (Fernández et al., 2006). This rosette shaped thistle is very robust presenting large spiny leaves and branched flowering stems and it has been marginalized along the years amongst the populations of the Mediterranean basin due to its few uses (Raccuia and Melilli, 2007). Nevertheless the cardoon flower was used to make cheese rennet in many traditional productions; in fact it is still used in some regions of Portugal and Spain for this purpose, which led to the remaining of some wild populations of cardoons especially in the mountainous regions where traditional sheep cheese is made (Fernández et al., 2006). Other uses of the cardoon are related with energetic production using their biomass and pellets (Gonzalez et al., 2004), paper pulp (Gominho et al., 2009), green forage for animals (Cajarville et al., 2006), seed oil for human consumption due to the high value of α-tocopherol (Maccarone et al., 1999) and biodiesel production after the seed oil has it presents a high yield of oil in its

seeds (Fernández et al., 2006 ; Curt, et al., 2002 ; Encinar, et al., 2002).

Among others, there are some important arguments that sustain the use of the cardoon. The high yield of extracted seeds' of a randomly chosen crop with low energy input, as they only received water from rainfall, allied to the fact that the cardoon is a species that can easily be planted in many places of the Portuguese landscape. Taking into account the climatic changes leading to drier summers, the cardoon can be a way to deal with this new temperatures as well as it preserves biodiversity. The year of 2009 was one of the years that the average temperature most increased; in fact it was the 5th warmest year ever (WMO, 2010). Also the need of inserting biofuels in the petrol-based diesel sold in the EU can be an interesting way to arid lands of the interior to profit with this low energy input crop and a greener alternative to diesel made out of soya or other edible vegetables used in our country to produce biodiesel. The European Union 2003/30 directive obliges the countries inside the EU to use biofuels in public transportation in proportions of 5,75% by 2010, the present year, and 10% by the year of 2020 (UE, 2003). Public transports are responsible for 84% of the total CO_2 emissions in the EU. Taking into account all of these assumptions *Cynara* may be a possible alternative fuel to help achieve this goals as well as it can be a catalyst to the development of rural areas.

The desire to produce biofuel from a plant that could be at the same time an endogenous species of the Portuguese flora and also a non-edible one, led to the project that is reported in this thesis. *Cynara cardunculus* seeds' oil was extracted with supercritical carbon dioxide and n-hexane in a Soxhlet apparatus with the purpose of producing and characterizing biodiesel.

Materials and Methods

Materials and Methods

Raw material preparation - The material used for this project were the *Cynara cardunculus*, L. seed's. These were obtained from randomly selected plants in Santiago de Cassurrães near Mangualde, Portugal, during August of 2009. Almost 10kg of fresh matter was collected which were processed. This process consisted of drying the matter in a thermo-ventilated oven (Scientific Series 9000, USA) for two days at 35ºC, so the plants could complete their development and expose the seeds for an easier removing.

Figure 5 – Cardoon seed

Afterwards the cardoon "heads" were all manually opened. The obtained seeds were then selected taking into account their development and conservation-state. The final quantity of seeds was almost 3kg that were again dried in the thermo-ventilated oven for 2 hours at 35ºC. The seeds were then milled in a Moulinex knives-mill (Moulinex M-225700, Portugal) for 30 seconds and separated in a mechanical agitator (Retsch, Germany), which was used to determine the average geometric diameter of particle (ASAE, 1993). With the help of 2 different sieves having the following apertures 1mm, 0,250m the matter got retained in this last one used for the whole extraction procedures. The milled seeds were also taken to the light scattering apparatus (Malvern Mastersizer 2000, USA) to determine the medium diameter of the particles.

Density – Measurement at the IPN (Instituto Pedro Nunes) by helium picnometry (Accupyc 1330 Micromeritics, Micromeritics Instrument Corporation, USA), which allows the measurement of the volume and true density of solid objects, without damaging samples. This is accomplished by employing Archimides' principle of fluid displacement and Boyle's law to determine the volume. The displaced fluid is a gas (helium),

which can penetrate the finest pores, thereby assuring maximum accuracy. It was made ten replicates for each condition.

Moisture content – The total moisture content was determined by the xylol distillation method for natural products, using 10g of raw material and 50mL of xylol in each assay. The extraction equipment was set up and turned the heating on for 2 hours. In the end the water content was measured in a graduated beaker. Three replicates were made.

Bed Porosity – The bed porosity was calculated taking into account material's density and the cell volume, as described in the equation below

$$Bed\ Porosity = \frac{Real\ Density}{Apparent\ Density}$$

Where, the real density was measured at the IPN and the apparent density is given by the quotient of the sample mass by the extraction cell's volume.

Extractions – The cardoon oil was obtained from two different methods, extractions using a Soxhlet apparatus (Sox) and with supercritical fluids (SFE).

Soxhlet

Traditional Soxhlet equipment was used to extract the oil of 30g of cardoon seeds using *n*-hexane in a proportion of 1:10(solid-solvent). The extractions lasted for 2,5h and in the end the extract was evaporated using a Rotovapor (Buchi CH-9230, Switzerland), and the oil was weighted and the yield calculated. A total of eighteen replicates were performed.

Figure 6 – Soxhlet apparatus

Supercritical fluids extraction (SFE) – The SFE was performed in an apparatus described by (Braga, et al. 2008). It was equipped with a high-pressure liquid compressor in order to pressurize the liquefied CO_2 into the extraction stainless steel extraction cell that contained ≈14g of raw material. The cell was heated to the operation condition temperatures (35, 45 and 55ºC), and elevated to the desired pressures (150, 225 and 300 bar), which were controlled by a backpressure regulator (26-1762-24-090, Tescom, Selmsdorf, Germany). A static period of 30 min preceded the continuous extraction (300 min) that had a solvent flow rate of 4,7L/min. Two replicates were done for each condition, plus one more providing kinetics data. The use of different pressures and temperatures leads to different CO_2 densities that will be discussed further in the Results and Discussion section.

In the end of every extraction the SFE apparatus was cleaned up with 200mL of EtOH with the help of a high-pressure liquid pump (L-6200A, Hitachi, Merck, Darmstadt, Germany) and a flow of CO_2 to dry the remaining ethanol. The SFE apparatus is described in Figure 7.

Legend:
1. CO_2 cylinder
2. Co-solvent reservoir
3. CO_2 pump
4. Co-solvent pump
5. On-off valve
6. Extraction vessel
7. BPR
8. Micrometric valve
9. Refrigerated recovering flask
10. Heat exchanger
11. Adsorbent packed column
12. Manometer
13. Flow meter
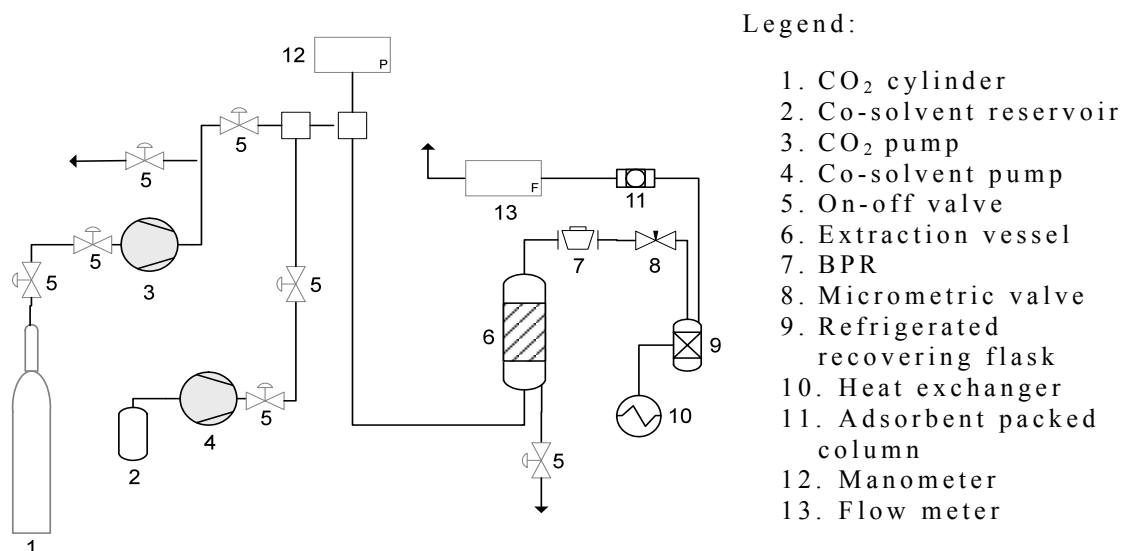
Figure 7 – SFE apparatus scheme, source Seabra, et al., 2010.

Biodiesel production from *Cynara cardunculus,* L. extracted oil (Soxhlet & SFE)

All the extracted oil was stored inside glass flasks in order to prevent any reactions that could damage it and kept inside a refrigerator.

Transesterification – The transesterification process was carried out in an oven (J.P. Selecta, Spain) at the temperature of ≈65°C, where the oil was pre-heated for 30 minutes before the reaction beginning. Then sodium methoxide was used as a catalyst and methanol as reagent in a 6:1 alcohol:oil ratio. The reaction lasted 90 min and was kept with magnetic agitation at 700 rpm. Transesterification was made in duplicate. Acid esterification followed by base catalyzed transesterification was also considered, but only on a late stage of the work in order to improve the reaction yield.

Oil and biodiesel characterization – The biodiesel obtained was then characterized by three different methods, TLC (thin layer chromatography), GC (gas chromatography) and HPLC (high pressure liquid chromatography). TLC analyses were performed using silica gel plates (20cm×20cm, thickness 0.2mm) (Merck, Germany). A hexane-ethyl acetate-acetic acid (80:20:1) mixture was used as the mobile phase, to observe the FAME´s compounds an iron chloride solution was used as the spray reagent and after the plates were heated to 100°C for 3 minutes (Kasim, et al., 2009). To observe the saponins a solvent mixture of toluene-ethyl acetate (65:45) mixture was used as the mobile phase, and an anisaldehyde spray was used as the spray reagent. The plates were then heated to 100°C for 3 minutes (Wagner and Zgainski, 1984).

GC was performed using a GC–MS apparatus (QP-5000, GC–MS, Shimadzu, Tokyo, Japan), it was equipped with a J&W Scientific DB-225 column (30m x 0,25μm x 0,15μm). All the analyses were performed in duplicate. The specifications of the assays are listed on Table 2.

Biodiesel production from *Cynara cardunculus,* L. extracted oil (Soxhlet & SFE)

Table 2 – GC assay conditions

Column	DB-225
Injector	280°C
Detector	300°C (FID)
Split	No split
Injection	1 µL
Oven temperature program	70°C (1 min)
	70–180°C a 10°C/min
	180–220°C a 3°C/min
	220°C (15 min)
Liner	Thiner
Gas	Helium (2,45 mL/min (P=2,5bar))
Total time	45 min
Solvent	Hexane
Internal standard	Methyl myristate (C14:0)
Sample preparation	c = 1 mg/mL in n-hexane

The percentage of esters was determined through the following equation

$$\text{Esters, \% (w/w)} = \frac{(A_t - A_{is})}{A_{is}} \times \frac{C_{is} \times V_{is}}{m_{biodiesel}} \times 100$$

Where, A_t – Ester peak area sum

A_{is}– internal standard peak area (C14:0)

C_{is}– internal standard solution concentration (mg/mL)

V_{is}– volume of the internal standard solution (mL)

$m_{biodiesel}$ – biodiesel mass in sample(mg)

HPLC – In this analysis, the solvent was carrying out using a HPLC system (WellChrom Maxi-Star k-1001, Knauer, Germany). The samples were investigated in a Eurospher column 100-5C18 (250 x4 mm i.d., 5 mm, Germany) equipped with a pre-column, coupled to an UV detector (WellChrom k-2500, Kanuer, Germany). The chromatographic conditions were similar to the conditions described by (Holcapek, et al., 1999), measured at 205 nm, flow-rate 1mL/min. Then samples were filtered through a 0.22 µm teflon filter

prior to injection. The column temperature was 40ºC; mobile phase constitution was: solvent A (water), solvent B (acetonitrile) and solvent C (2-propanol-hexane (5:4, v/v)). A 25-min ternary gradient with two linear gradient steps was employed: 30% A+70% B in 0 min, 100% B in 10 min, 50% B+50% C in 20 min, followed by isocratic elution with 50% B+50% C for the last 5 min. Between two samples was carrying out a run with acetonitrile to clean the column. For each analyze was performed 40 min of equilibration treatment, before the injection (20 µl). All analyses were performed in duplicate.

Kinematic Viscosity – The dynamic viscosity was determined using rheological characterization, tests were carried out in a controlled stress rheometer (Model RS1, Haake, Germany) at 20 ºC using a plate sensor (20 mm diameter) at a gap of 0.250 mm. Flow tests were performed with upward and downward linear shear rate ramps between 0 and 500 s^{-1}. Limit viscosities at high shear rates were reported with triplicate assays. Kinematic viscosity was then calculated according to the following equation

$$\nu = \frac{\mu}{\rho}$$

Where, ν is the Kinematic viscosity, µ is the dynamic viscosity and ρ is the biodiesel's density.

Acid number – The acid value was determined using titration with KOH in according to the EN 14214, where the quantity of KOH in mg used to neutralize the biodiesel was determined by making some changes in the method. As the quantity of biodiesel was very scarce it emerged the need of using smaller quantities in each assay, therefore it was necessary to improve the method using ±0,33g in each assay. This method was optimized using soybean oil and the results are shown in appendix A.

Thermogravimetric Analysis – The thermogravimetric analysis (TGA) curves were obtained in a Thermal Analyzer from TA

Instruments, model Q500, USA, under nitrogen atmosphere, with a flow rate of 40 mL min^{-1}, using platinum crucibles and mass samples of about 8 mg, in a temperature range from 25 to 550°C. A heating rate of 10ºC per minute was employed. All analyses were performed in duplicate.

Fourier Transform Infrared Spectroscopy – FTIR-ATR spectroscopy (Jasco, model 4000, UK) was performed at 128 scans and 4cm^{-1} of resolution between 500 and 4000cm^{-1}. Two replicates were done for each condition.

Results and Discussion

Results and Discussion

Raw Material

The studied *Cynara cardunculus*, L. plants were randomly selected and collected in the small village of Santiago de Cassurrães, near Mangualde, Portugal.
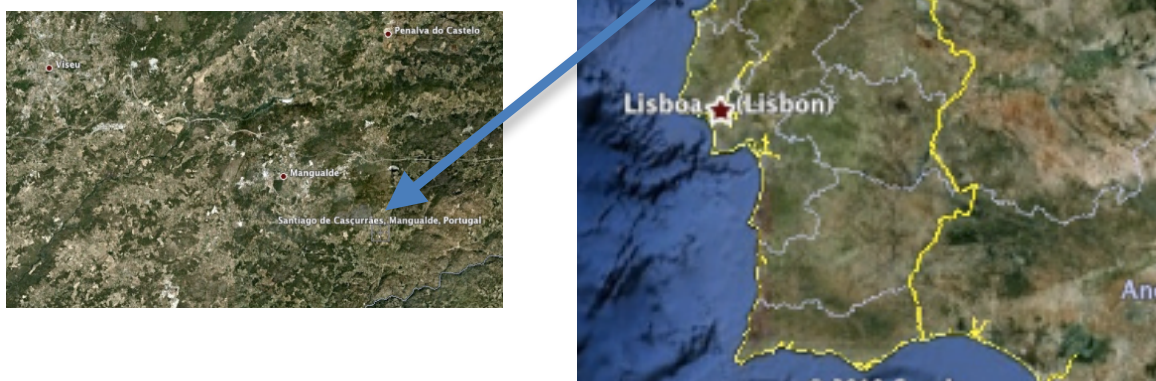
Figure 8 – Map localization of the cardoons' location

The total amount of fresh material collected was of 10kg, which was reduced to 3kg after drying and seeds removal. Seeds were further milled and presented an average particle diameter of 862,15 µm and an average humidity of 8,7%±0,1.

Extraction yields

Considering the extraction techniques, Soxhlet presented a 25,10%±1,70 oil yield being these data consistent with prior extraction of cardoon oil with n-hexane (Maccarone, et al., 1999) while with SFE the yields varied depending on the different conditions used. By comparing these results with the CO_2 density at the different experimental conditions, it was observed that the yield increases with the increment of CO_2' density, as shown in Figure 9.
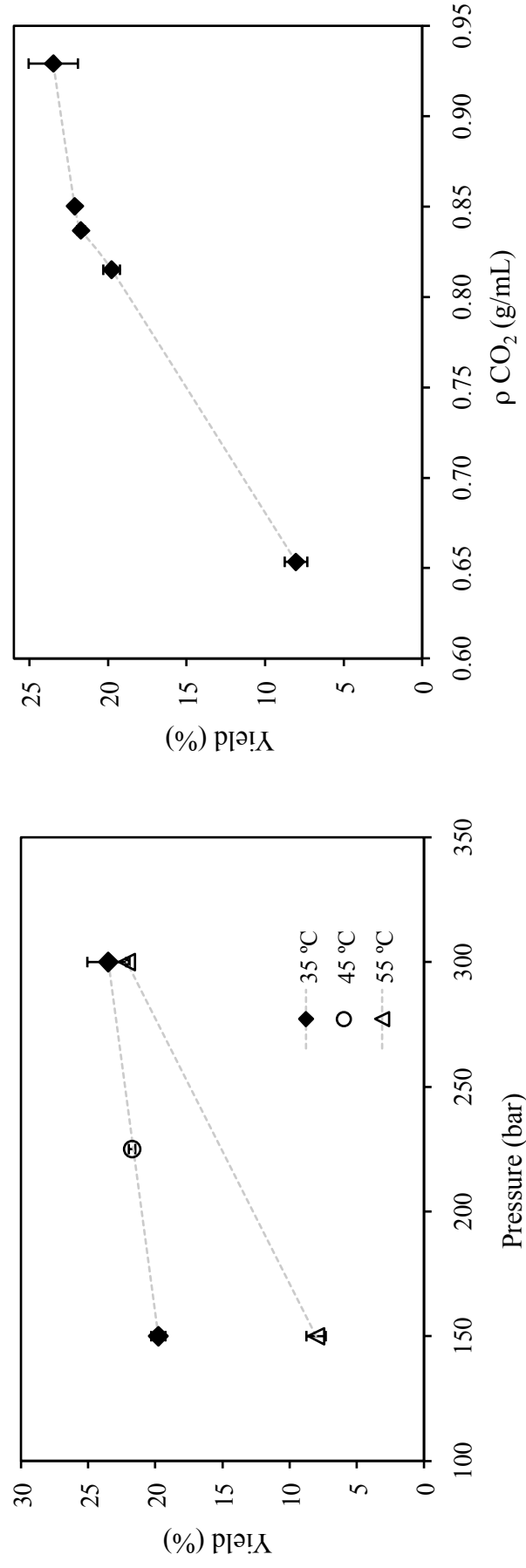
Figure 9 – Cardoon extraction yield vs. pressure (**A**) and carbon dioxide density (**B**)

Biodiesel production from *Cynara cardunculus*, L. extracted oil (Soxhlet & SFE)

Table 3. Extracted oil characterization

Extraction conditions	ρ CO$_2$ (g/mL)	Yield, % (w/w)	Oil density (g/cm^3)	HPLC	
SFE				**FAMEs (%)**	**Di- and triglycerides (%)**
150 bar, 55 °C	0,65350	8,03± 0,72	0,9329 ± 0,0066	20,62±0,85	58,73±0,34
150 bar, 35 °C	0,81506	19,78 ± 0,53	0,9248 ± 0,0045	11,73±0,06	73,79±1,25
225 bar, 45 °C	0,83684	21,73 ± 0,24	0,9212 ± 0,0012	16,84±0,50	64,99±0,67
300 bar, 55 °C	0,85022	22,13 ± 0,04	0,9282 ± 0,0011	14,96±1,24	64,39±1,27
300 bar, 35 °C	0,92911	23,49 ± 1,57	0,9106 ± 0,0018	14,15±0,86	68,55±4,14
Soxhlet	ρ **n-Hexane (g/mL)**	**Yield, % (w/w)**	**Oil density (g/cm^3)**	**FAMEs (%)**	**Di-and triglycerides (%)**
1,013 bar and solvent boiling point (62 -69 °C)	0,61500*	25,10 ± 1,70	0,9256 ± 0,0019	-	-

*data from (Khairulin et al., 2007)

For a more accurate comparison between extraction methods, bed porosity was also calculated for the soxhlet's thimble and the SFE's cell. The calculated bed porosity was 3,99 and 1,99, respectively.

The experimental yield results show a tight relation with CO_2 density. As expected higher density solvents have a larger solvation power and therefore are able to extract higher molecular weight substances such as lipids and fatty acids, originating higher yields.

In Table 3 it is possible to observe the different extraction conditions yields, oil density and content in acylglycerols. The data from the SFE's oil provide evidence that the temperature has a direct relation with oil's density, for the same pressure. For instance, at 150 bar the oil's density at 35°C is lower than at 55°C. Respecting to the extraction yield, the opposite situation occurs as the yields are higher at lower temperatures, for the same pressure. For the soxhlet's oil, the observed density was of 0,9256±0,0019 g/cm^3. This value is similar to the obtained with the SFE condition of 150 bar and 35°C, nevertheless solvents densities and extraction conditions are significantly different.

Regarding the oil composition, the data provided by HPLC showed that beside high values in di and triglycerides, there were also FAME's present in the oil. This fact may be due to the extraction conditions, as CO_2 was used to extract the oil. Carbon dioxide and water could form carbonic acid. This reaction could have led to an *in situ* acidification and therefore some esterification of the oil may have occurred (Seabra, et al., 2010). Nevertheless soxhlet's obtained oil was not analyzed by HPLC due to technical problems concerning the equipment, and therefore this relation cannot be fully proved.

SFE kinetics

The extraction kinetics was also determined in order to identify the point where the maximum extraction yield is achieved, for each process condition assayed. The collect flask was changed every 20 min during the extraction. The results are shown in Figure 10.
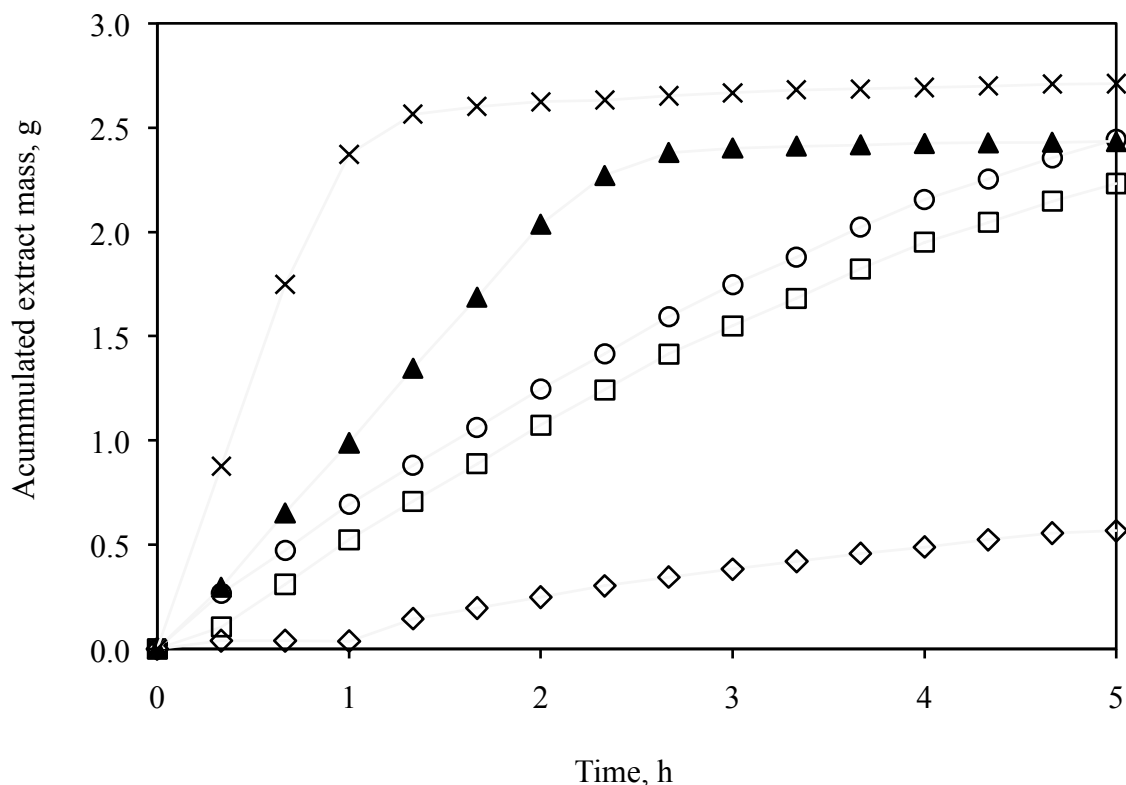
Figure 10 – Kinetics for the SFE extractions: ● 150 bar 35°C; ◇ 150 bar 55°C; □ 225 bar 45°C; ▲ 300 bar 35°C; × 300 bar 55°C

The kinetics clearly show that the maximum extraction yields can be obtained almost after one hour of extraction when working at 300 bar 55°C and two hours if the same pressure is used but at a low temperature (35°C). At these conditions the diffusional period was reached after 2 and 3 hours, respectively. For all the remaining conditions, the extraction yield keeps growing until the end of the extraction time in a more or less continuous way, but without ever exhausting the bed. This information will be useful in further extractions, as diminishing extraction times, when working at higher-pressure conditions, can save energy and CO_2.

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Biodiesel yields

The oil obtained from Soxhlet and SFE at different experimental conditions, were then subjected to alkali-catalyzed transesterification in order to transform the obtained oil into biodiesel. The yields were under the expected, as the conversion reaction for the majority of the literature reported experiments, results in 90% biodiesel and 10% glycerol (Knothe et al., 2005), in this work the highest yield obtained was 61,2% for Soxhlet's oil and 52% for one of the SFE extracts (300 bar 35ºC). Although the results from the transesterification were very low, they were similar for all the SFE's extractions. The results can be observed on Table 4.

The low transesterification yields may be justified by the presence of saponins that exist in *Cynara cardunculus*, L. (Shimizu et al., 1988), which promote saponification reactions that occur during the last minutes of the transesterification reaction. The first transesterification reaction assays were made in goblets that were heated inside a water vessel on a heating plate. Therefore water vapors' could be entering the reaction flask causing the saponification reactions, as all the oil turned into a jelly matter that could only be separated by centrifugation at 6000 rpm. The following assays were performed inside a closed oven to avoid the presence of water in the reaction, but saponification kept occurring.

Table 4 – Biodiesel characterization

Extraction conditions	Yield, % (w/w)	Acid number (mg KOH)	FAMEs (%)		Diglycerides and triglycerides (%)
			GC	HPLC	HPLC
SFE - 150 bar, 55 °C	34,98 ± 3,19	-	23,33±3,16	11,58±0,80	76,43 ± 0,06
SFE - 150 bar, 35 °C	37,68 ± 1,97	0,24 ± 0,0	77,94±10,88	55,96±5,24	38,07 ± 3,35
SFE - 225 bar, 45 °C	38,35 ± 3,57	0,32 ± 0,0	<20	10,78±1,04	80,07 ± 1,21
SFE - 300 bar, 55 °C	37,77 ± 0,16	0,40 ± 0,0	<20	9,85±1,18	81,97 ± 2,42
SFE - 300 bar, 35 °C	44,47 ± 10,87	0,32 ± 0,0	73,25±12,33	60,80±1,74	32,52 ± 0,18
Soxhlet - 1,013 bar and solvent boiling point	52,04 ± 8,15	0,23 ± 0,0065	83,19±12,16	22,57±3,84	75,72 ± 3,79
Acid esterification + Transesterification – 225 bar 45°C	73,90	-	90,05±8,82	-	-

Biodiesel production from *Cynara cardunculus*, L. extracted oil (Soxhlet & SFE)

Thin Layer Chromatography

The presence of saponins in the oil was confirmed by TLC analysis. The TLC plate is shown in Figure 11, confirms the presence of significant amounts of saponins in both soxhlet and SFE obtained oils.

Figure 11 – TLC plate for different oil developed in a mixture of solvent Toluene/ethyl acetate 35/65 v/v

Saponification reactions are undesirable during transesterification as the main goal of this work is the production of biodiesel and saponification is a side reaction (Knothe et al., 2005). Therefore it was necessary to find a way to eliminate saponins from the oil. As there was no literature about this subject for *Cynara* saponins, an approach reported in the literature for the elimination of soybean flour saponins (Tarade et al., 2006) was used. Two methods were considered, where the first consisted in heating the oil to a temperature of 150°C for 60 minutes before the transesterification, and the second in using a higher temperature of SFE extraction, namely 80°C and 300 bar. These two methods proved to be inefficient as the saponification reactions continued to occur.

Although the yields were not satisfactory, some biodiesel was obtained and therefore characterization analyses were performed in order to confirm the production of biodiesel and if it had the desirable properties.

The first test was TLC, which is an easy and fast method of determining if a certain substance is present in a given sample when applying the appropriate solvents' mixture to develop the plate.
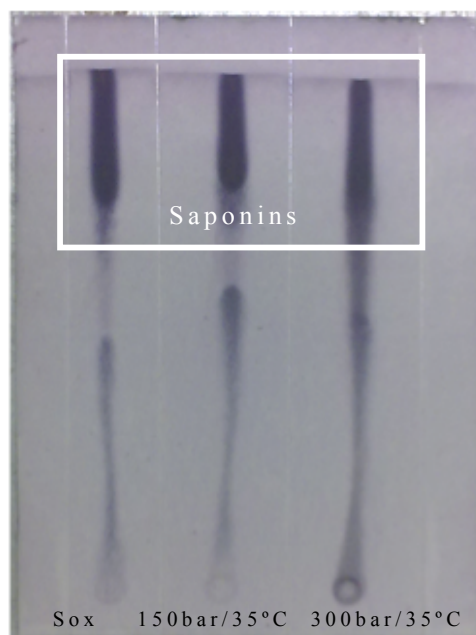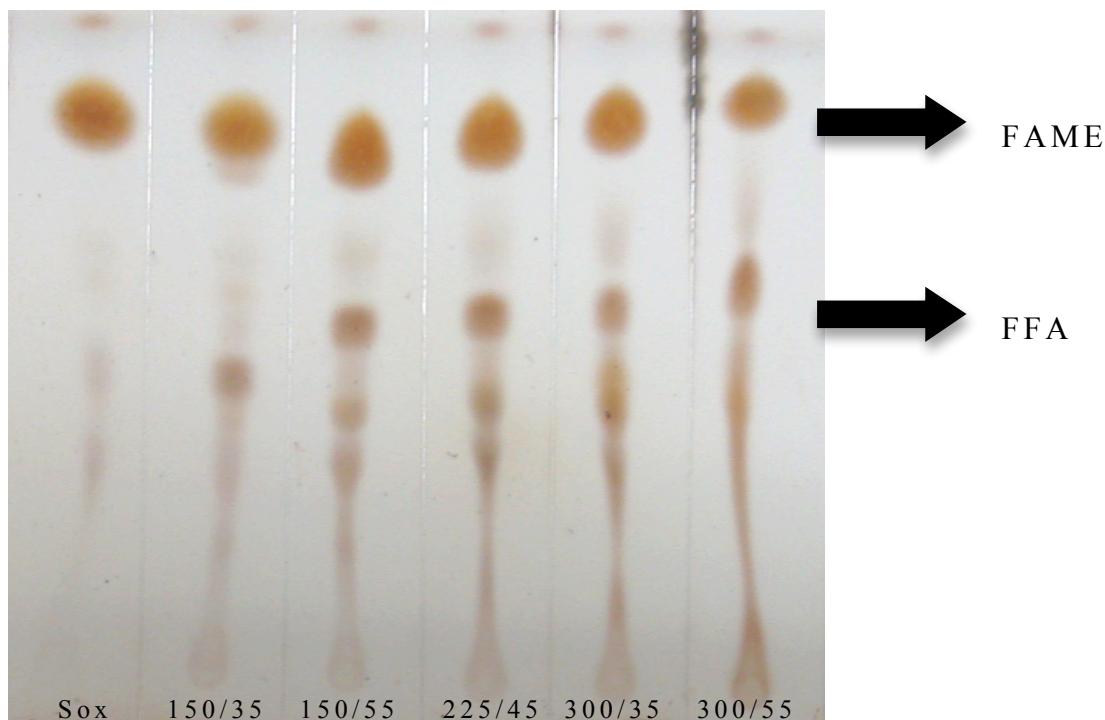
Figure 12 – TLC plate for different biodiesel, developed in a mixture of solvent hexane/ethyl acetate/acetic acid 80/20/1 v/v

The TLC of the biodiesel samples revealed that FAMEs' were present, confirming the production of biodiesel for all samples, but it also revealed that a number of free fatty acids (FFA), responsible for saponification reactions were also present. Therefore, it was necessary to neutralize them by performing an acid esterification before the transesterification reaction (Veljkovic et al., 2006). In an attempt to solve the low yields of biodiesel production, one assay was performed with acid esterification prior to the alkali-based transesterification. The yield of conversion into biodiesel was of 73,90%, and the total ester content was of 90,05%±8,82, as described on the last row of Table 4 (last row). This means that at least one sample had a total FAME content of 98,87%, which is above the required by the standard EN14214. Further characterization for this biodiesel was not possible due to experimental constrains. However, taking into account this promising result, further attention must be paid to this method in a future transesterification process using cardoon's oil.

Acid number

Acid number was determined according to the titration method reported by Rei (Rei, 2007). According to the standard EN14214 its value has to be under 0,50mg KOH/g of biodiesel. A slight modification to use small amounts of biodiesel was performed as described before (see Material and Methods). The values obtained are listed in Table 4. As it is possible to observe all the biodiesel samples are in accordance with the European norm. Although the values were sometimes very close to the upper threshold limit this can be due to the free fatty acids present in the oil that were not completely neutralized (Veljkovic et al., 2006). The acid number of one of the samples (150 bar 55°C) was impossible to measure accurately because of the very low amount of biodiesel left. Further assays for this condition are necessary.

Gas Chromatography

To observe the total ester conversion yield, which according to the standard EN14214 must be over 96,5% in order to be used as a vehicular fuel, GC analyses was performed. Not all the results were satisfactory, but beside all the saponification reactions, there are some conditions that revealed high ester conversion yields.

As can be seen in Table 4 there is no value above the required by the standard EN14214. Nevertheless the biodiesel sample obtained from the soxhlets' oil transesterification almost reached that value (95,35%). In the biodiesel samples obtained from the SFEs' oil, the higher ester conversion was observed in the conditions of 150 bar and 35°C with a conversion yield of 77,94%±10,88 and also in the condition of 300 bar and 35°C with a conversion yield of 73,25%±12,33, being the last one the condition that also presented the highest oil yield. The chromatograms presented in Figures 13 e 14 show the retention times for the different methyl esters present in the biodiesel samples. Those retention times and respective methyl esters are described in Table

Biodiesel production from *Cynara cardunculus,* L. extracted oil (Soxhlet & SFE)

5. In the chromatograms of Figures 13 and 14 it is possible to identify the methyl esters by their retention times. The following methyl esters are present in all samples: methyl palmitate, stearate, oleate and linoleate. This data was also reported by Maccarone (Maccarone et al., 1999). However, for the SFE condition of 150 bar and 55°C, other methyl esters can be observed with higher retention times such as methyl linolenate and others that were not identified.

Table 5 – Retention times of the methyl esters used as standards for GC

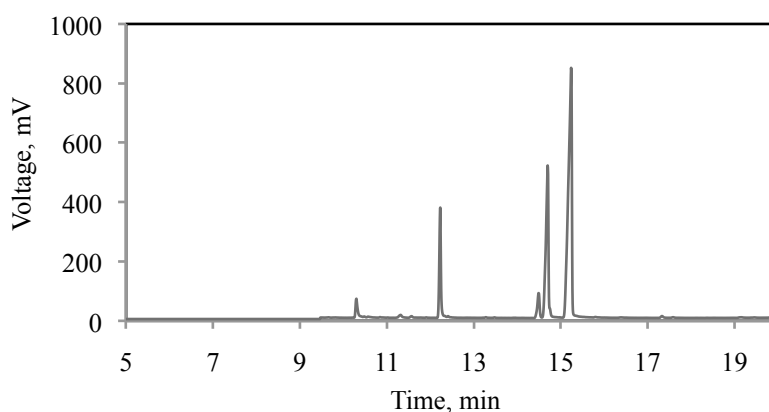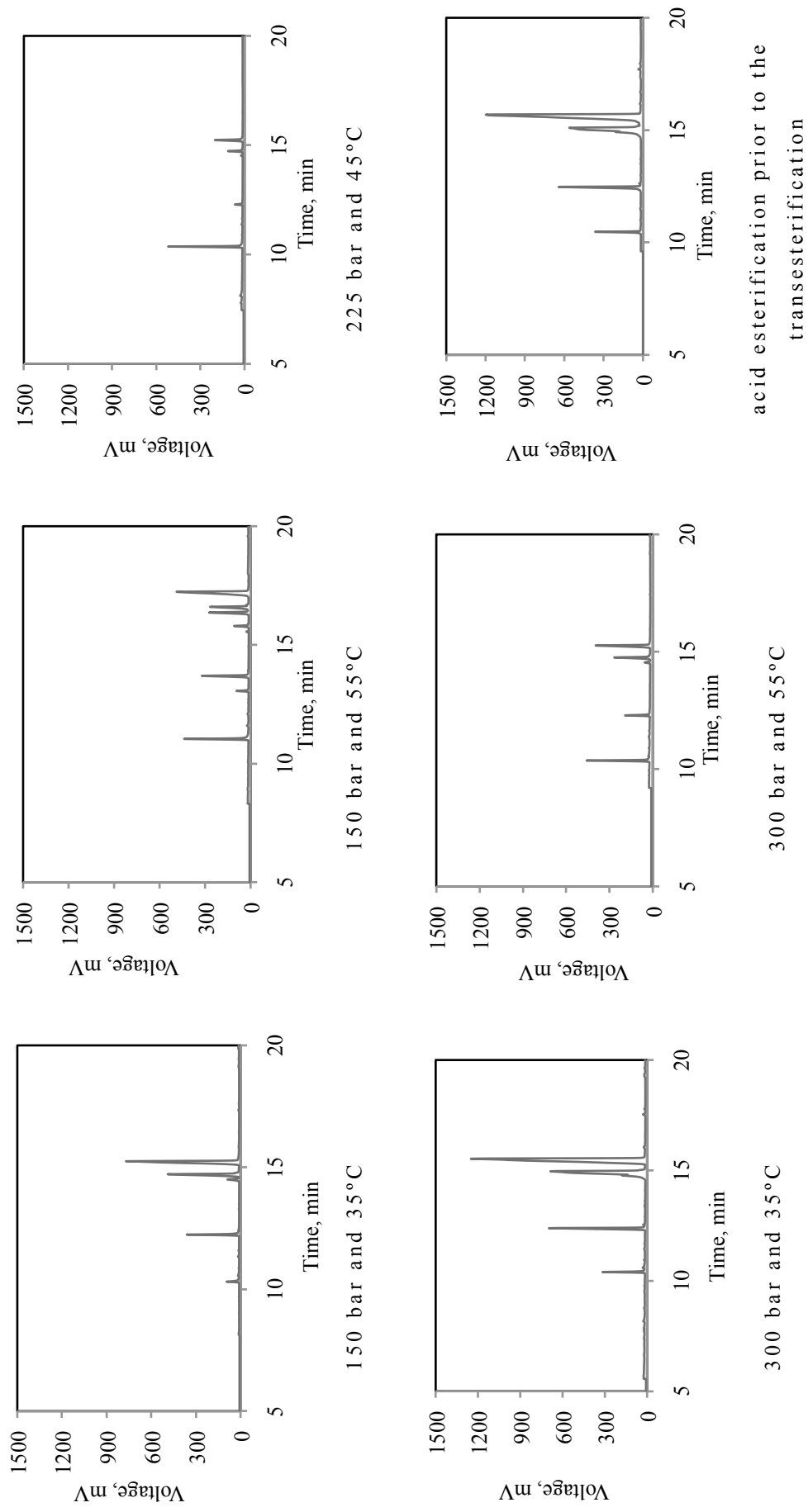| Methyl ester	Time, min
Methyl myristate (internal standard) (C14:0)	10,5
Methyl palmitate (C16:0)	12,5
Methyl stearate (C18:0)	14,9
Methyl oleate (C18:1)	15,0
Methyl linoleate (C18:2)	15,4
Methyl linolenate (C18:3)	16,1

Figure 13 – Chromatogram for the Soxhlets' biodiesel, sample C

Figure 14 – Chromatograms for the biodiesel from SFE's extracted oil

Biodiesel production from *Cynara cardunculus, L.* extracted oil (Soxhlet & SFE)

The low conversion rate of the alkali transesterification may be due to low a ratio of alcohol: oil, the one used was 6:1, and studies demonstrate that higher ratios can provide higher conversion yields (Vyas et al., 2009).

Besides low conversions, below 96,5%, all the oil samples produced biodiesel, as confirmed by the chromatograms from Figures 13 and 14, showing peaks correspondent to the methyl esters standards. The last chromatogram refers to the acid catalyzed esterification prior to the alkali-catalyzed transesterification, and as discussed above the results are very promising.

High Pressure Liquid Chromatography

The HPLC analyses showed complementary data that the GC wasn't able to provide. For instance it shows not only the FAME's in the biodiesel but also diglycerides and triglycerides (Holcapek et al., 1999) which if present indicate that the transesterification reaction was not efficient (Rashid and Anwar, 2008). By comparing the total esters conversion yield provided by GC with the data from the HPLC some answers can be found in order to explain the low conversion yields that were obtained. For the samples where the conversion yields were very low, such as the ones obtained at 225 bar and 45ºC, it is possible to observe that diglycerides and triglycerides are present meaning that the total esters conversion yield was low. On other hand, it is possible to observe that for the 150 bar and 35ºC condition, corresponding to the highest ester conversion yields for the SFE's extracted oils, there are almost no traces of triglycerides, but there are some diglycerides left in the sample. Therefore the conversion yield for this sample was 77,9% (GC), and the diglycerides still present in the sample must be the responsible for the non-total conversion to FAME. The HPLC chromatograms are shown in Figure 15. The acylglycerols present in the cardoon's oil and biodiesel have similar composition as described by Holcapek (Holcapek et al., 1999). The fatty acids

present have retention times between 2 and 8 minutes being FAME's revealed on the sequence at retention times that go from 8 to 12 minutes. The last two groups represented correspond to di- and triglycerides, with retention times between 12 and 19 minutes for the diglycerides and higher than 19 minutes for the triglycerides.
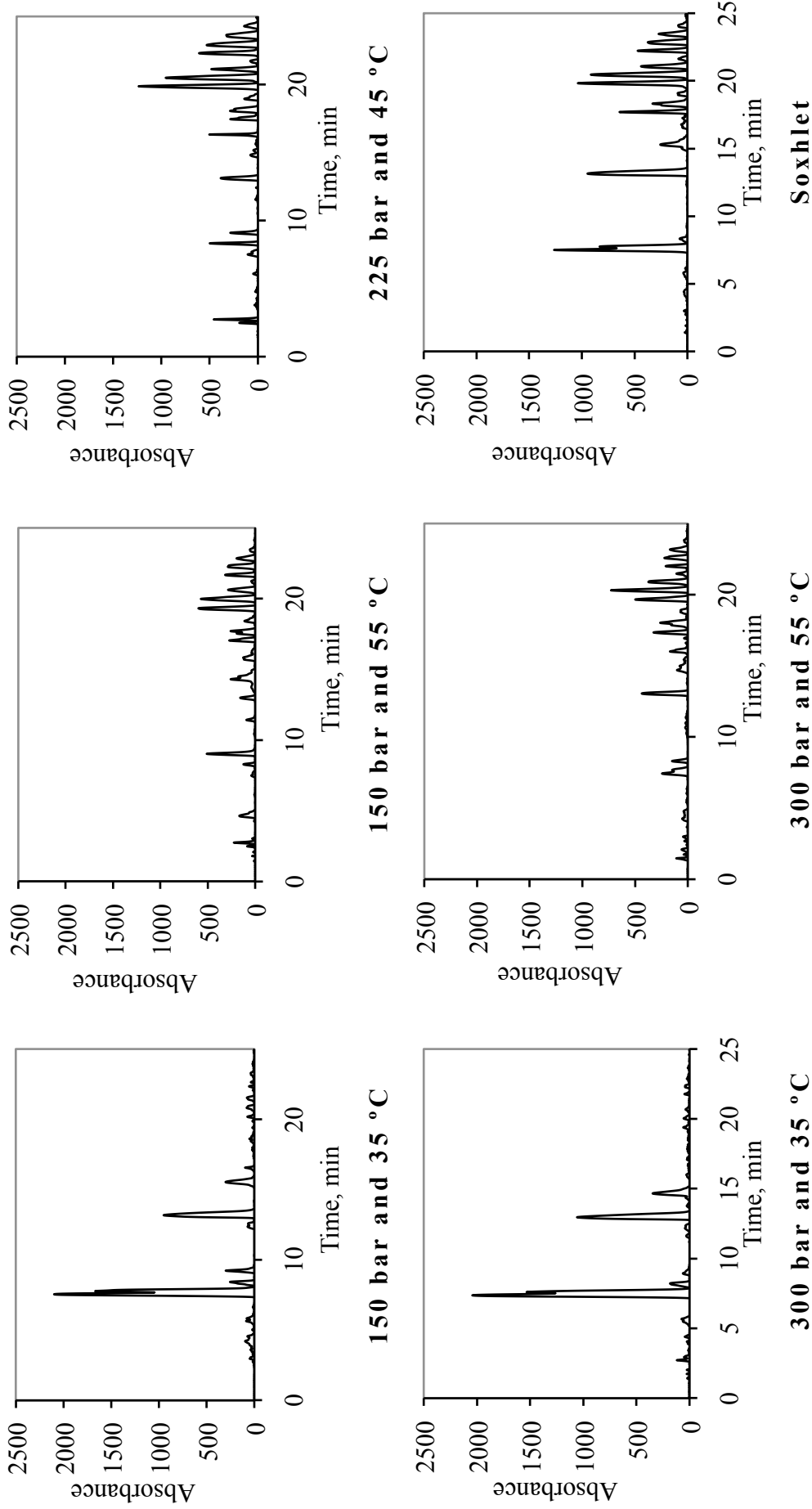
Figure 15 – HPLC chromatograms for biodiesel, SFE & Soxhlet

Biodiesel production from *Cynara cardunculus*, L. extracted oil (Soxhlet & SFE)

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Kinematic Viscosity

Viscosity analyses were also performed on the biodiesel samples at a temperature of 20ºC. Although the standard EN14214 stipulates that the viscosity must be measured at 40ºC, being the obtained values between 3,5-5,0 mm^2.s. These results can provide valid information about the diesel's kinematic viscosity, as it decreases with a temperature increment. Even accounting for the temperature effect the high viscosity observed may be related with the high content of soap produced in the transesterification reactions due to saponification. Despite the non-conforming values for the biodiesel obtained from Soxhlet and two SFE conditions (150 bar 35ºC and 300 bar 35ºC). These samples have already proved to present the higher yields in biodiesel and FAME's, and are once again the most promising values, as presented in Table 6.

Table 6 – Viscosities of different samples of biodiesel and oil

	Average ± S.D.	
Samples	Visc (*10^3 mm^2.s)	Hysteresis
Cardoon oil – Soxhlet	40,27 ± 1,15	426,8 ± 30,8
Biodiesel		
Soxhlet	6,93 ± 0,63	343,8 ± 7,5
SFE (150bar, 35ºC)	8,86 ± 1,09	339,1 ± 36,6
SFE (225bar, 45ºC)	10,25	347,4 ± 25,4
SFE (300bar, 35ºC)	8,48 ± 1,45	329,3 ± 28,3
SFE (150bar, 55ºC)	65,08 ± 32,08	343,3 ± 10,2
SFE (300bar, 55ºC)	73,63 ± 27,46	340,9 ± 76,9

Thermogravimetric Analysis

The biodiesel was also submitted to thermal degradation analysis (TGA), which revealed great differences between the soxhlets' biodiesel and the ones obtained from the SFE's extracts. In the case of the SFE extracts weight loss started to happen at about 200ºC with a slight loss, lower than 15%, while the entire

degradation only happened after 400ºC. The information gathered in the literature only sustains the TGA values for the Soxhlet's biodiesel showing that the results here obtained are in accordance with the ones reported in the literature when using different raw materials (Dantas et al., 2007 ; Santos et al., 2007). Nevertheless the biodiesel obtained from soxhlet's extracted oil presents lower thermal stability when compared with the biodiesel obtained from the SFE extracts.

Therefore the higher degradation temperatures observed for biodiesel obtained from SFE's may be explained by the conditions and method of extraction, and this hypothesis may be sustained by the TLC data from Figure 12, that shows some unidentified compounds present in those biodiesel's.
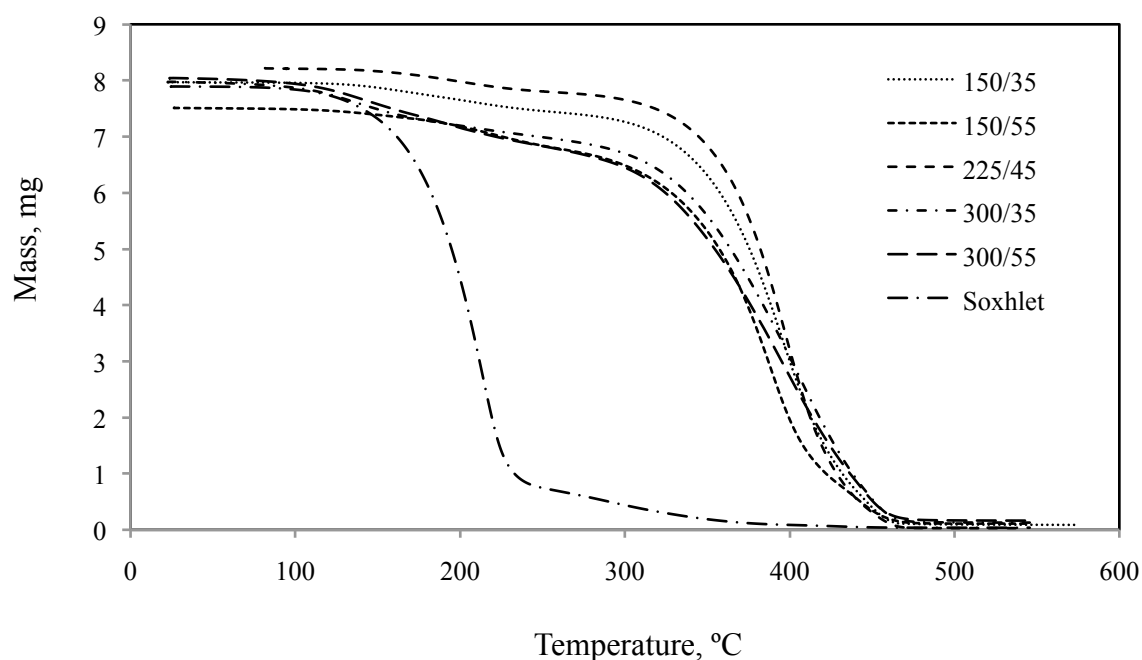

Figure 16 – TGA analyses for biodiesel obtained under different conditions

Fourier Transform Infrared Spectroscopy

The Fourier Transform Infrared Spectroscopy (FTIR) comes to complement some information given before by the GC, as it shows

the conversion of the esters into methyl esters by a slight change in the wavenumber correspondent to the C=O connection that is the characteristic bond for esters. This change is observed in Figures 17 to 19 by observing a change in the peak wavenumber from 1743,33 cm^{-1} in the oil to 1740,44 cm^{-1} in the corresponding biodiesel. The examples shown in the figures present the oil and corresponding biodiesel for Soxhlet, SFE (150 bar, 35°C) and SFE (150 bar, 55°C), representing cases where higher conversion, Figures 17 and 18, and lower conversion, Figure 19, were observed (Zagonel et al., 2004 ; Oliveira et al., 2006).
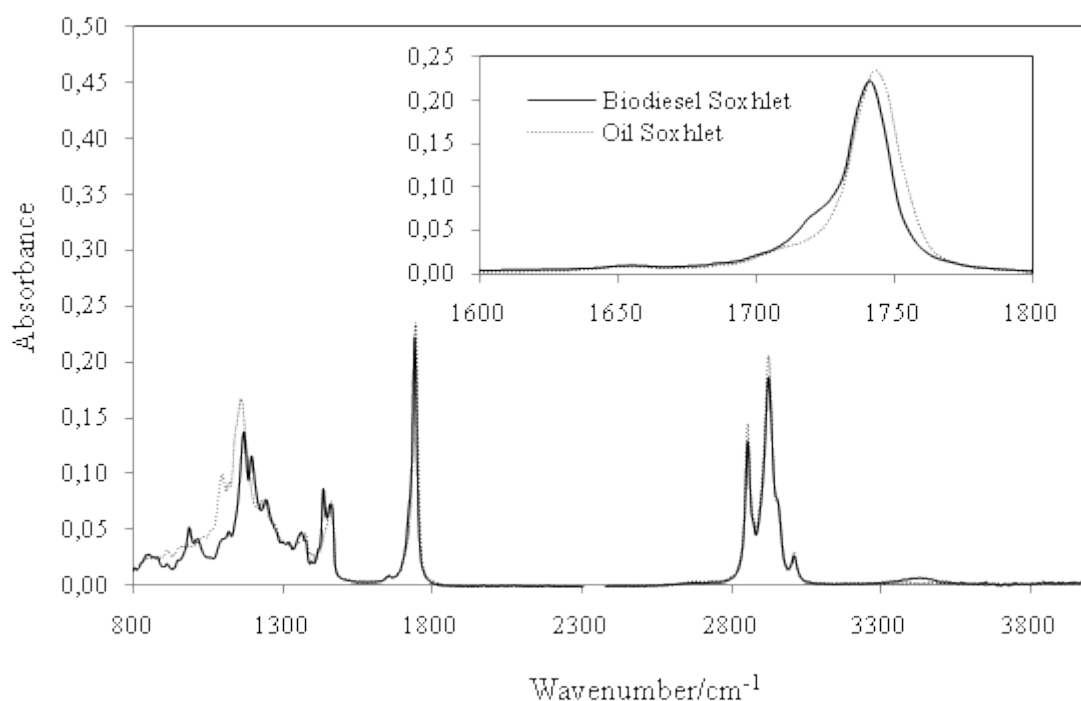

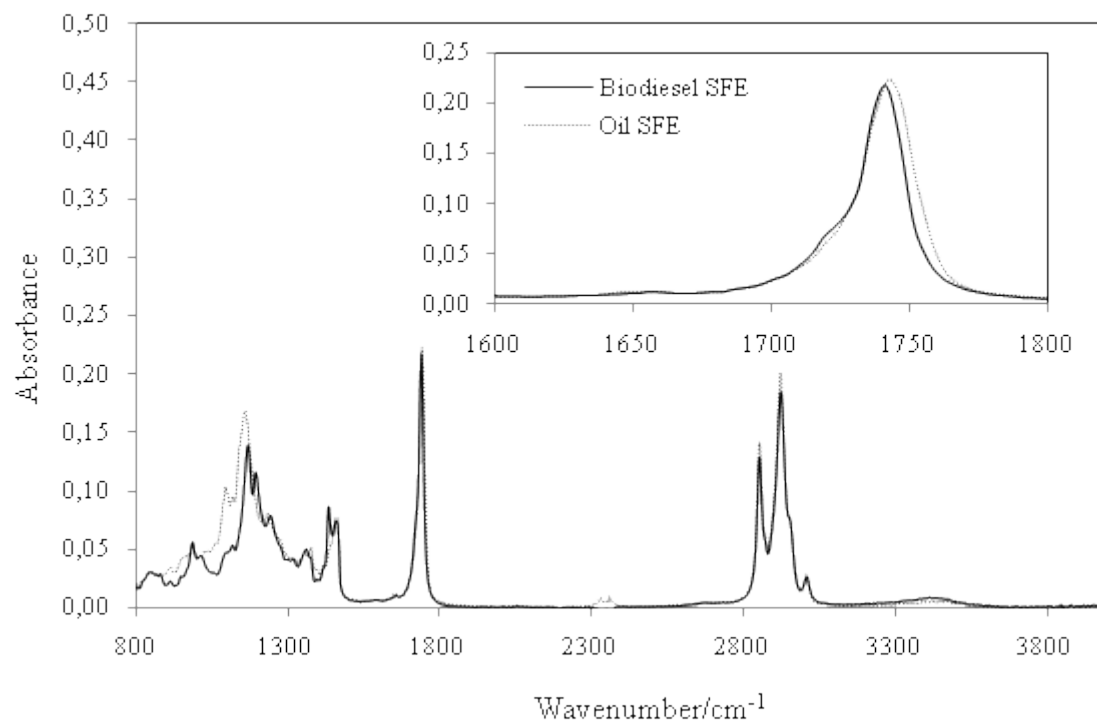Figure 17 – FTIR spectra of biodiesel obtained from Soxhlet's extracted oil

Fig. 18 – FTIR spectra of biodiesel obtained from SFE (150 bar, 35ºC) extracted oil
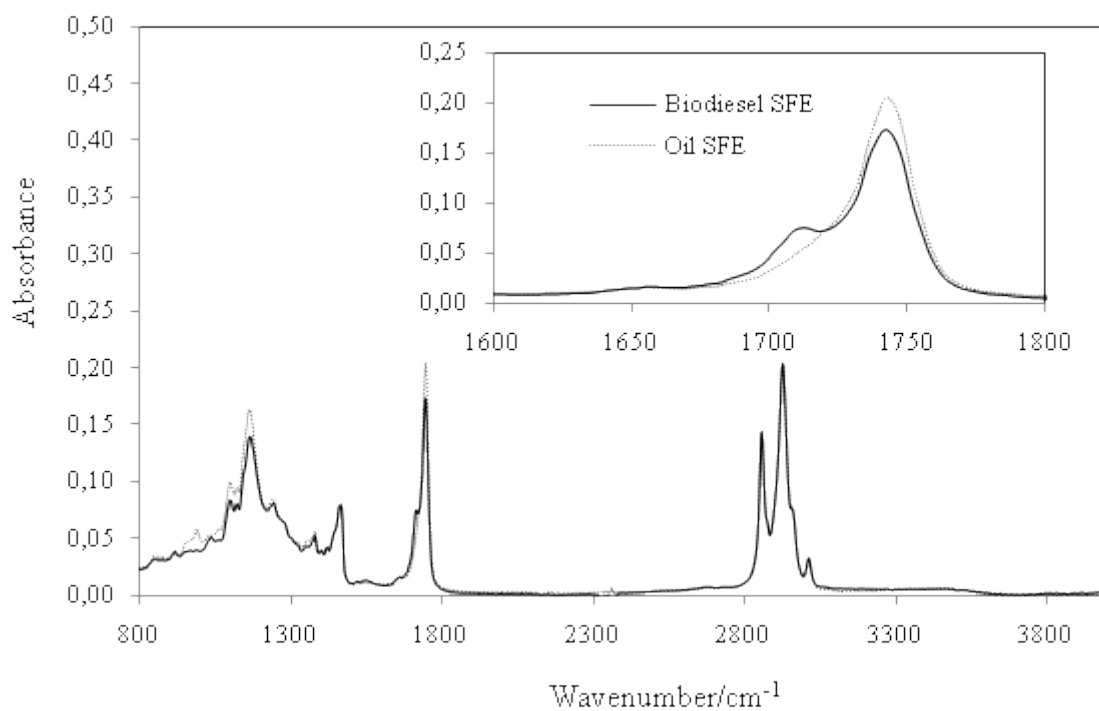

Figure 19 – FTIR spectra of biodiesel obtained from SFE (150 bar, 55ºC) extracted oil

Conclusions and further remarks

Conclusions and further remarks

This work proved that it is possible to go out to the field and randomly select plants from which was possible to remove the seeds and from those extract the oil, and finally produce biodiesel.

The chosen plant for this work, *Cynara cardunculus*, L. had already been used to produce biodiesel, but this work contained a novelty in oil extraction, the use of supercritical carbon dioxide to perform the extractions, leading to more pure oil, but also with more compounds that are still not identified. Therefore further characterization must be done to the extracted oil under different conditions.

The raw material proved to be a satisfactory energy crop, as it was possible to produce biodiesel with it, even though all the plants were randomly selected at harvest time only, and therefore they had no prior treatment, receiving only water when it rained. This kind of plant, that on one hand in an endogenous species of the Mediterranean flora, and on the other hand requires almost no energy input can be a feasible alternative to produce biodiesel, specially in arid areas of the Portuguese interior that due to the desertification need an economic boost.

During the year this project faced some issues as it was a new technology applied to the oil extraction and there is no information about the cardoon oil extracted with SFE. This lead to some delays in the work as every obstacle found need to be solved with the genius of the people involved or with semblances with other works using different raw materials or extractions methods.

Such issues appeared mainly at the transesterification process, and this was the most important step of the work, as it would allow the oil to be converted into biodiesel. In the end of the transesterification, unexpected saponification reactions also occurred that lowered the biodiesel yields and also affected the

conversion yields. As referred before, the extraction techniques with different SFE conditions were first applied to the cardoon seeds in this work. This may have led to the extraction of different compounds in the oil, that may have been the source of the large amount of saponins present in the oil as well as the presence of more free fatty acids and other unknown compounds. These facts could have been responsible for the low yields of biodiesel production. Nevertheless, when acid esterification was applied to the oil prior to the alkali-based transesterification process, the yields of biodiesel and total methyl esters content rose to more acceptable values, especially this last parameter.

In spite of some of these facts, acid esterification has to be considered as well in future transesterification processes using cardoon's oil. Also the cardoon oil extracted with supercritical carbon dioxide must be fully analyzed in further works, as it may have other compounds, that beside of being suitable for biodiesel production may also be used in processes of fine chemistry.

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Appendix

Appendix A

In Table A.1 it is showed the validation for the titration method using smaller amounts of soybean oil, so that it could be used with the cardoon biodiesel. The soybean oil used had an acid number of 0,20 mg/KOH. This method shows that the acid number is possible to determine even with lower amounts of oil, inferior to 1g of oil used by (Rei 2007).

Table A.1 – Titration of soybean oil

EtOH/Iso Solution	Sample (mL)	Acid number Average ± S.D.
A	7,0	0,18±0,02
B	5,0	0,18±0,04
C	3,0	0,20±0
D	1,5	0,20±0

Anova: Two-Factor With Replication

SUMMARY	A	B	C	D	Total
0,1					
Count	3	3	3	3	12
Sum	0,57	0,6	0,73	0,64	2,54
Average	0,19	0,2	0,2433333	0,2133333	0,2116667
Variance	1,15556E-33	1,156E-33	0,0001333	0,0021333	0,0008515
0,05					
Count	3	3	3	3	12
Sum	0,529037143	0,549878	0,5611	0,5611	2,2011151
Average	0,176345714	0,1832927	0,1870333	0,1870333	0,1834263
Variance	0	4,198E-05	1,156E-33	1,156E-33	2,841E-05
Total					
Count	6	6	6	6	
Sum	1,099037143	1,149878	1,2911	1,2011	
Average	0,183172857	0,1916463	0,2151833	0,2001833	
Variance	5,59319E-05	0,0001005	0,0010042	0,0010608	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0,004785123	1	0,0047851	16,581584	0,0008871	4,493998
Columns	0,003356546	3	0,0011188	3,8770759	0,0293488	3,238872
Interaction	0,001705309	3	0,0005684	1,9697666	0,1592004	3,238872
Within	0,004617289	16	0,0002886			
Total	0,014464267	23				

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