Mariana Isabel Correia d'Almeida Mendes Gameiro

Licenciada em Bioquímica

Biodiesel production from chicken feather meal, combining biocatalysis and supercritical technology

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Dr. Alexandre Paiva, Investigador Phos-Doc, FCT UNL

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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AO MEU AVÔ JOAQUIM.

Valeu a pena? Tudo vale a pena
Se a alma não é pequena.

(Fernando Pessoa, in *Mensagem*)

Cada pessoa que passa na nossa vida, passa sozinha, porque cada pessoa é única e ninguém é substituível. Cada pessoa que passa na nossa vida, passa sozinha, e não nos deixa só, porque deixa um pouco de si e leva um pouco de nós. Essa é a mais bela responsabilidade da vida e a prova de que as pessoas não se encontram por acaso.

Se estas páginas servem para escrever o meu eterno agradecimento, que se inicie com um pequeno/grande excerto intemporal. De facto, ao longo deste percurso fui acompanhada pelos normais binómios a ele associados: motivada/desesperada, euforia/fraqueza e é graças ao Dr. Alexandre Paiva, o meu orientador, que posso afirmar que sempre me incentivou e me ofereceu todos os elementos necessários para escolher o lado positivo de cada binómio. Obrigada por toda a tua essência que me motivou ainda mais para enfrentar todo este novo mundo para mim. Foste, e és, sem dúvida, um exemplo a seguir. Mesmo quando estás cheio de trabalho, com mil e uma pessoas para orientar, tens sempre uma palavra para dar, sempre com predisposição para auxiliar mesmo nas dúvidas mais básicas. Sincero, amigo, trabalhador e divertido, são alguns dos adjetivos que te qualificam.

Porque eu sou do tamanho daquilo que sinto, que vejo, e que faço, não do tamanho que as pessoas me enxergam. (Carlos Drummond de Andrade)

Sinto-me bastante orgulhosa por teres sido o meu orientador, aprendi bastante contigo e, sem dúvida, forneceste-me as ferramentas essenciais para continuar a crescer (não, não cresci 10 cm por tua causa... Continuo nos meus míseros metro e meio... Mas, o meu interior já ultrapassa os dois metros!). Por isto tudo, não existem palavras suficientes para demonstrar o meu total agradecimento a ti Alexandre, Dr. B.!

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À Prof. Susana Barreiros, por ter tornado possível este trabalho e pela inspiração na concepção desta ideia na cadeira leccionada por si. Obrigada pela oportunidade que me deu de poder trabalhar no seu laboratório. Toda a sua experiência, sabedoria, respeito, sinceridade, honestidade e simpatia que inspiram e motivam qualquer pessoa. Eu não fui exceção. Espero

poder continuar a aprender consigo, não só a nível académico/profissional, como também a nível pessoal. O meu sincero obrigada por tudo Professora!

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Quero agradecer também às minhas meninas de Castelo Branco (Ana, Caty, Martinha, Tany, Janico, Sil, Loira e Inês Cerdeira). Sem vocês, todo o meu percurso académico não teria sido a mesma coisa. Obrigada pela vossa força, amizade, loucura e por estarem sempre a meu lado quando mais precisava. Quero ainda agradecer à Rute, André, Raqueluxa, Freitinhas, Lavadinho, Ricardo B. (Cadota), Leonor, Mafaldinha e Joana por todo o carinho que me oferecem.

Por último, e não menos importante, quero mencionar o meu especial agradecimento à minha família. Primeiro, aos meus pais. Não existem palavras suficientes para poder agradecer tudo o que me têm prestado. Estou-vos eternamente grata pela educação que me têm dado, pelo carinho que me têm oferecido, pelas palavras amigas que me dão no momento certo, na altura certa. Pelas oportunidade que me têm oferecido ao longo dos anos, pelos pais exemplares que são. Graças a vocês, pelos conhecimentos por vós transmitidos, tornei-me na pessoa que sou.

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Queria também agradecer ao Sr. João Silva, da Lusiaves, que, graças a ele, este trabalho foi possível de realizar, já que nos cedeu o elemento primordial para a realização deste projeto.

A todos vós, e a outros que não referenciei mas que contribuíram igualmente para o meu bem estar, nesta fase da minha vida, bem com o meu crescimento enquanto pessoa, o meu sincero e absoluto Obrigada!

ABSTRACT

The aim of this work is the production of a biofuel - biodiesel, from an innovate and renewable source - chicken feather meal, combining the biocatalysis in supercritical environment - supercritical carbon dioxide.

In a first phase of this project, it's carried out the study of the extraction of oil from chicken feather meal (CFM). The solvent, carbon dioxide in supercritical conditions (sc-CO_2), passes through an extractor filled with raw material, CFM. CO_2, due to its properties, extracts nonpolar compounds, such as triglycerides in which chicken feather meal has in its constitution. It was shown that the optimal extraction conditions are at 300 bar and 313.15 K. Regarding the solvent flow rate, further studies are needed to do in order to establish if the best extraction yield is obtained at 75 or 150 g_{CO2}/min. In fact, the extraction efficiency, in both cases, is very high, yielding 94,2 and 96,2% of extraction efficiency, respectively.

In a second phase of the project, it's carried out the study of transesterification reaction from the oil extracted from CFM. Thus, there was performing a continuous process integrating the oil extraction with the transesterification reaction. Therefore, using also the supercritical carbon dioxide as a solvent, was used the Lipozyme® RM IM to catalyse the transesterification reaction. Thus, initially, the carbon dioxide passes through an extractor filled with CFM inside. When it's saturated in oil (triglycerides), it leaves the extractor and enters in a reactor filled with Lipozyme® RM IM. Here is where the transesterification reaction takes place, converting the oil into biodiesel. The oil, dissolved into sc-CO_2 leaves the reactor. It was demonstrated that the optimum reaction conditions are at 250 bar, 313,15 K with a molar ratio of oil:methanol 1:12 and with a solvent flow rate 75 g_{CO2}/min, yielding 98,54 ± 0,49% of biodiesel.

In the third and last part of the project was performed the fractionation process of the products obtained in the transesterification reaction. Thus, the conditions of pressure and temperature and changing in the two separators/cyclones in order to get the unreacted triglycerides in the first separator and, in the second, the biodiesel with a higher purity, even though in smaller amounts. It was shown that at 100 bar and 333,15 K it is obtained biodiesel with higher quality (93,89 ± 2,89% of yield).

Keywords: Biodiesel; Supercritical carbon dioxide; Residues from food industry; Chicken feather meal; Lipozyme® RM IM.

RESUMO

O objectivo deste trabalho consiste na produção de um biocombustível – biodiesel, a partir de uma fonte inovadora e renovável – farinha de penas de galinha, recorrendo à biocatálise num ambiente supercrítico – dióxido de carbono supercrítico.

Numa primeira fase deste projeto, realizou-se o estudo da extração de óleo da farinha de penas de galinha (CFM). O solvente, dióxido de carbono em condições supercríticas (sc-CO_2), passa por um extractor, contendo no interior a matéria prima. O CO_2, devido às suas propriedades, extrai compostos apolares, como é o caso dos triglicerídos que a farinha de penas de galinha possui. Ficou demonstrado que as condições ótimas de extração são a 300 bar e 313,15K. Relativamente ao caudal mássico do solvente, são necessários mais estudos de modo a comprovar se o melhor rendimento de extração é obtido a 75 ou 150 g_{CO2}/min. Efetivamente, a eficiência de extração em ambos os casos é bastante elevada, tendo-se obtido 94,2 e 96,2% de eficiência de extração, respectivamente.

Numa segunda fase do projeto, realizou-se o estudo da reação de transesterificação, a partir do óleo extraído da farinha de penas de galinha. Assim, realizou-se um processo em contínuo integrando a extração do óleo com a reação de transesterificação. Para isso, além de se usar novamente o dióxido de carbono como solvente, utilizou-se a *Lipozyme® RM IM* para catalisar a reação de transesterificação. Assim, o dióxido de carbono passa inicialmente por um extrator, contendo no interior a matéria prima. Este satura-se em óleo (triglicéridos) saindo do extractor e entra no reator, contendo a *Lipozyme® RM IM* no seu interior. Aqui ocorre a reação de transesterificação, convertendo o óleo em biodiesel. Este (o óleo), dissolvido no sc-CO_2, deixa o reator. Ficou demonstrado que as condições ótimas de reação são a 250 bar, 313,15K com uma proporção de óleo:metanol de 1:12 e com um caudal mássico de solvente a 75 g_{CO2}/min, tendo-se obtido 98,54 ± 0,49% de rendimento de reação.

Numa ultima e terceira fase do projeto realizou-se o fracionamento dos produtos obtidos na reação de transesterificação. Assim, alterou-se as condições de pressão e temperatura nos dois separadores/ciclones com o intuito de obter os triglicerídos que não reagiram na totalidade no primeiro separador e, no segundo, obter o biodiesel com uma pureza mais elevada, mesmo que em menores quantidades. Ficou demonstrado que a 100 bar e 333,15K obtém-se biodiesel com maior grau de pureza (93,89 ± 2,89% de rendimento).

Palavras-chave: Biodiesel; Dióxido de carbono supercrítico; Resíduos da industria alimentar; Farinha de penas de galinhas; Lipozyme® RM IM.

LIST OF FIGURES

AF – animal feed

CFM – chicken feather meal

CHCl$_3$ – chloroform

CO$_2$ – carbon dioxide

CO$_x$ – carbon oxides

DG – diglyceride

F – feed

FA – fatty acid

FAAE – fatty acid alkyl ester

FAME – fatty acid methyl ester

FFA – free fatty acid

GC – gas chromatography

GHG – greenhouse gas effect

Gly – glycerol

K – kelvin

KF – Karl Fisher method

MeOH – methanol

MG – monoglyceride

NGLs – natural gas liquid

min. – minute

NO$_x$ – nitrogen oxides

P$_c$ – critical pressure

Q – flow rate

S – solvent

sc-CO$_2$ – supercritical CO$_2$

SCF – supercritical fluid

SCFOE – supercritical fluid oil extraction

TAG – tryacylglyceride

T$_c$ – critical temperature

TG – triglyceride

INTRODUCTION

1. INTRODUCTION

1.1. MOTIVATION

1.1.1. ENERGY DEMAND

From the 19th century, when Industrial Revolution occurred, until now, the world has been dependent on fossil fuels. Currently, the world produce a total of 12,6 billions tones of oil equivalent for energy use, in which 31,8% is from oil (that includes crude oil, shale oil, oil sands and NGLs – the liquid content of natural gas where this is recovered separately) and 31,5% from coal (referring to commercial solid fuels only, i.e. bituminous coal and anthracite – hard coal, and lignite and brown – sub-bituminous – coal) [1]. The massive consumption and production of fossil fuels have some disadvantages such as: (*i*) concentration in few and problematic regions of the world, which can cause economical and social issues; (*ii*) imminent risk of running out; and (*iii*) cause serious concern over global warming by greenhouse gas emissions (particles, volatile organic compounds, CO_x, NO_x, and SO_x), therefore, affecting directly the public health and the environment [2], [3]. Since 1872, the crude oil has increased to over $100 per barrel and it is causing severe negative impact on the world economy, as it can be seen on Figure 1.1.1.
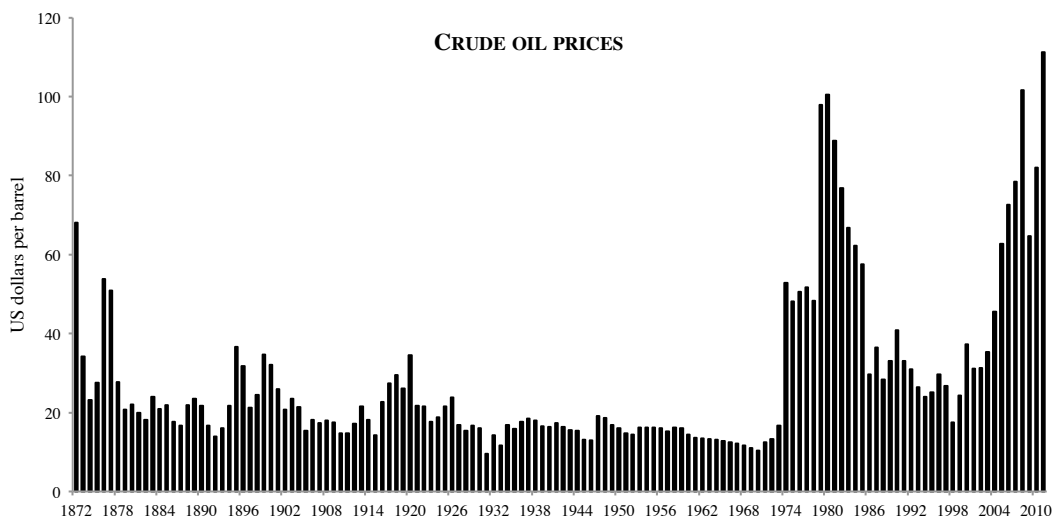
Figure 1.1.1 - Crude oil prices, since 1872 to 2011, in the world [1].

This increase of the crude oil price is related with its massive consumption, as it can be seen on Figure 1.1.2. According to BP statistical review of world energy, published in December 2012, the company estimates that the world's proven reserves of oil are 1,6 billion of barrel

[1] (Figure 1.1.3), which indicates that the world have abundant oil, for now. But taken into account the world crude oil consumption that would account to only 40 years of uninterrupted supply of oil, just based on the volume accessible and proven reserves. The theory of peak oil (the maximum point from which the supply of the planet begin to reduce), Figure 1.1.4, has become a controversial issue in recent years, and it is expected that will occurs on the next year, 2014 [4].
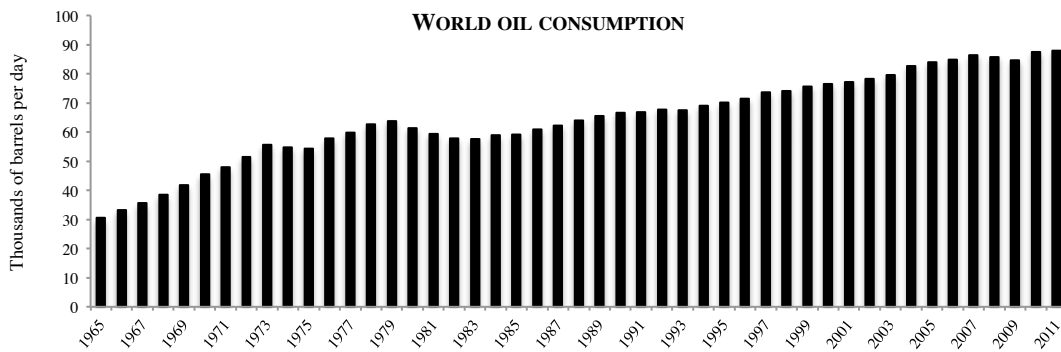
Figure 1.1.2 - World oil consumption since 1965 to 2011 [1].
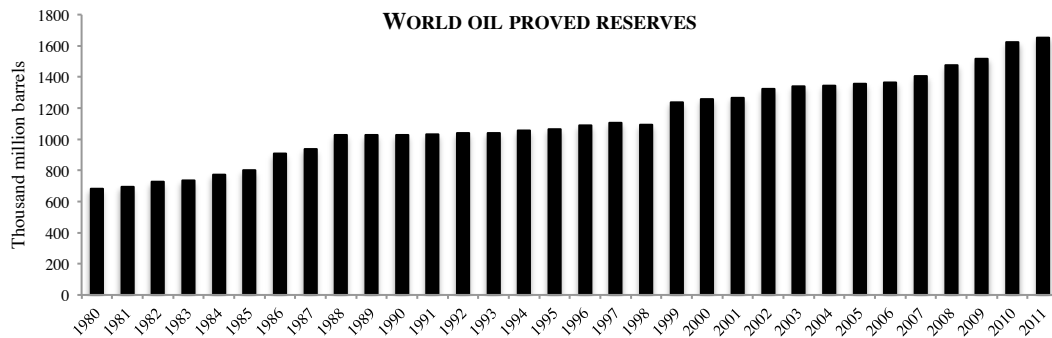
Figure 1.1.3 - World oil proved reserves since 1980 to 2011 [1].

According to William Colton[1], "*Energy is fundamental to our way of life and our future prosperity*" [5]. If demand continues to grow like this, the production of oil begins to decline and the world will enface severe problems.

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[1] Will M. Colton is the vice president (corporate strategic planning) of the ExxonMobil Company.
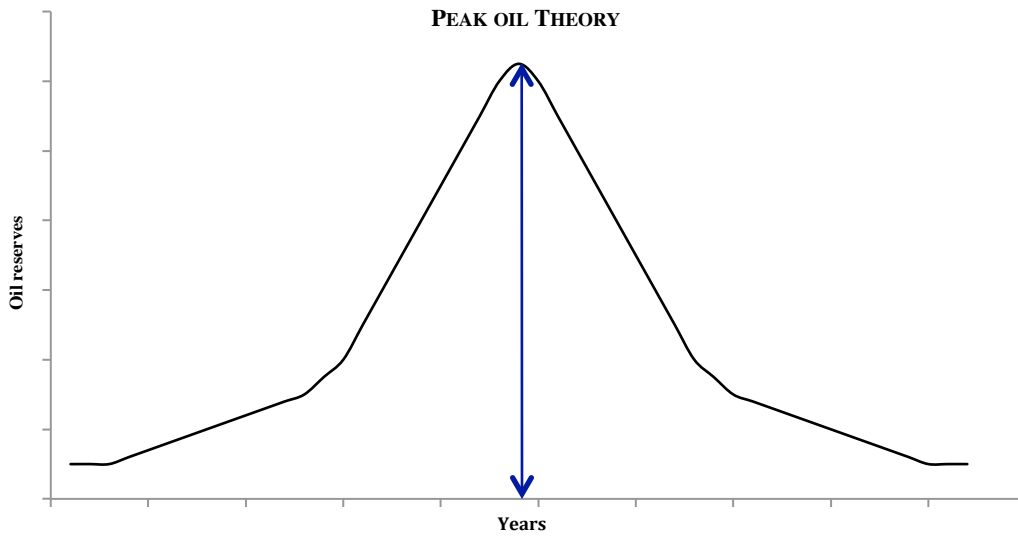
PEAK OIL THEORY

Figure 1.1.4 – Theory of peak oil.

Effectively, the *International Energy Outlook 2013* (IEO2013) projects that world energy consumption will grow more than 50% between 2010 and 2040, and fossil fuels continue to supply almost 80% of world energy use through 2040 [6], Figure 1.1.5.

WORLD TOTAL PRIMARY ENERGY CONSUMPTION, 2009 - 2040
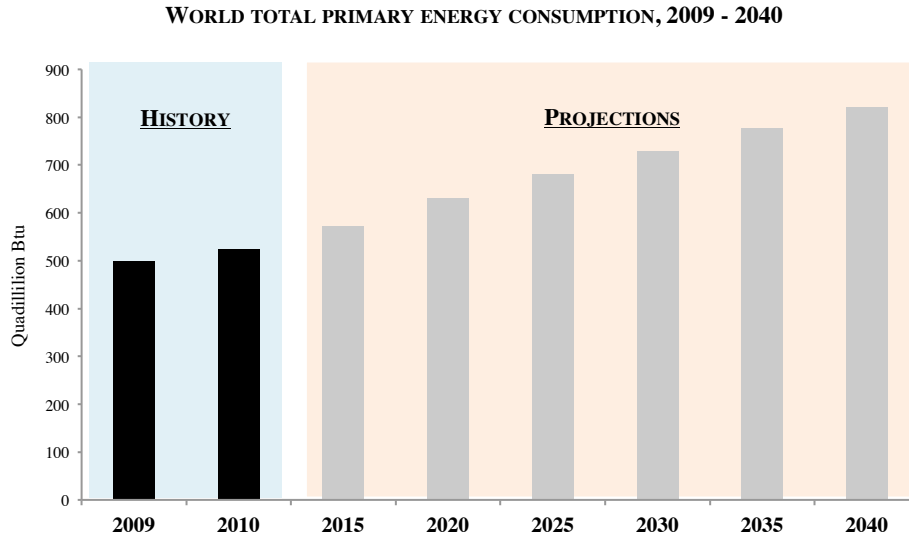
Figure 1.1.5 – World total energy consumption, since 2009 to 2040 projections [6].
(NOTES: 1 quadrillion Btu = 10^{15} Btu = 1,055 J = $22,34 \times 10^{-8}$ metric tonnes).

1.1.2. CLIMATE CHANGE CAUSED BY FOSSIL FUELS

Human activities contribute to climate change by causing changes in Earth's atmosphere in the amounts of greenhouse gases (GHG), aerosols (small particles) and cloudiness. The largest contribution comes from the excessive use of fossil fuels (as mentioned in 1.1.1). The improvement in global average temperature since the mid-20th century is due to the increase of GHG concentrations.

Greenhouse gases absorb and emit radiation at specific wavelengths within the spectrum of thermal infrared radiation emitted by the Earth's surface. The four principal GHG are carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and the halocarbons (group of gases containing fluorine, chlorine and bromine). Although CO_2 is not present in large quantities in the atmosphere (and is release through natural processes such as respiration and volcano eruptions), humans have increased atmospheric CO_2 concentration since the Industrial Revolution (deforestation, land use changes, burning fossil fuels). Carbon dioxide is a GHG that contributes to global warming and climate change. Since 1960, atmospheric CO_2 concentration has increased more than 25%, Figure 1.1.6 and Appendix - Figure 6.2.1 [7], [8].
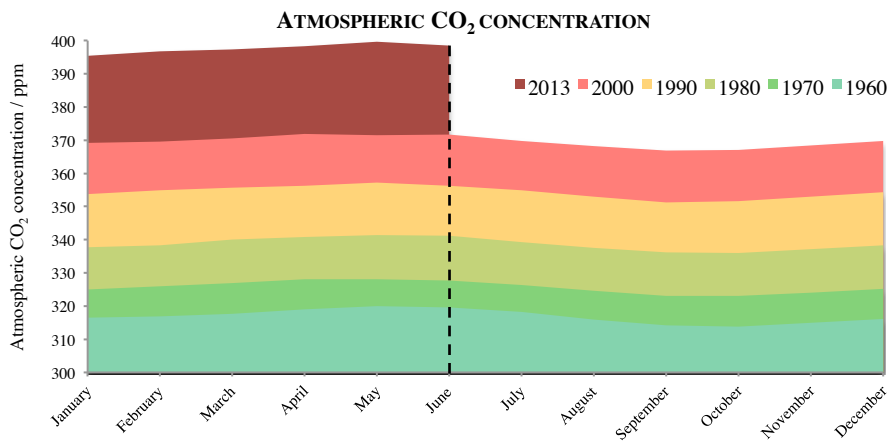
Figure 1.1.6 – World atmospheric CO_2 monthly concentration, since 1960 to 2013 [7].

There are many factors that influence the atmospheric CO_2 concentration, however it can be decompose into four factors:

1. Population;
2. Gross domestic product per capita (GDP);
3. Energy intensity (i.e. total primary energy supply (TPES) per GDP);
4. Carbon intensity (i.e. CO_2 emissions per TPES).

Climate change mitigation is one of the driving forces behind a growing demand of renewable energy technologies. In addition to reducing GHG emissions, these technologies also offer benefits with respect to air pollution and health compared to fossil fuels. However, to evaluate the overall problematic issue on the environment and health, a lifecycle assessment gives a quantitative comparison of 'cradle to grave'[2] emissions across different energy technologies. Figure 1.1.7 illustrates the lifecycle structure for renewable energy, nuclear power and fossil fuels [9].
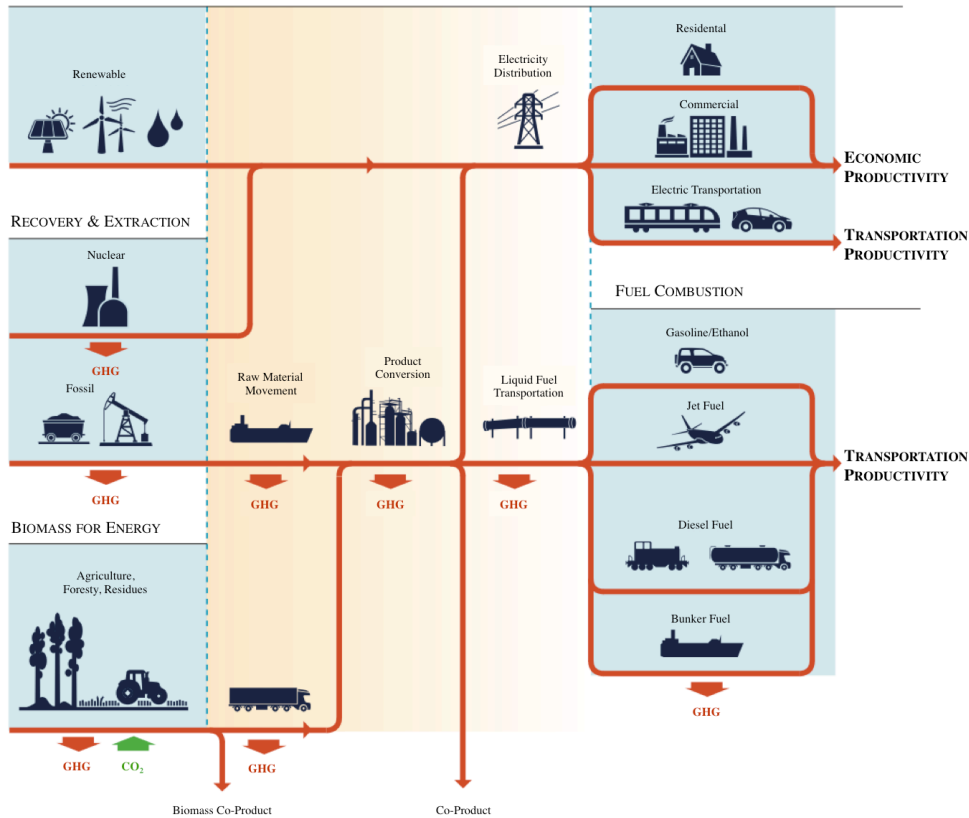
Figure 1.1.7 – Illustrative system for energy production and use illustrating role of renewable energy along with other production options (Adapted from [9]).

Two sectors produced nearly two-thirds of global CO_2 emissions in 2010: electricity and heat generate 41%, while transport produced 22%, Figure 1.1.8 [8].

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[2] 'Cradle-to-grave' is the full life cycle assessment from resource extraction ('cradle') phase to disposal phase ('grave').
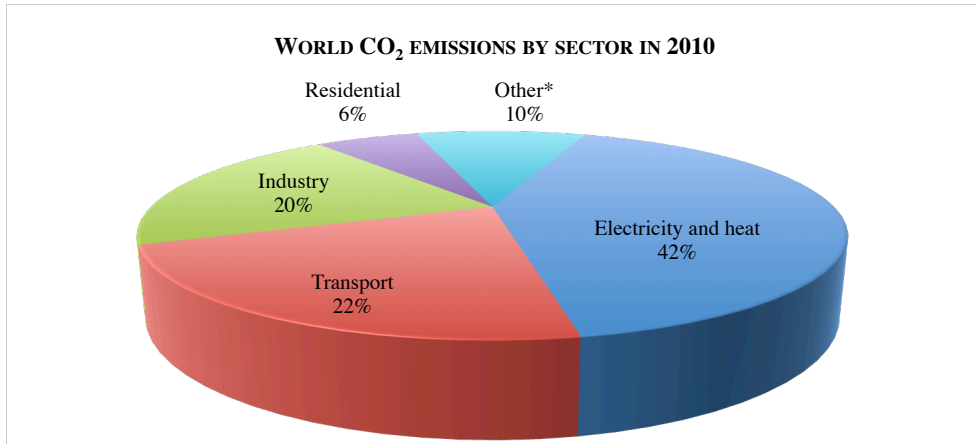
Figure 1.1.8 – World CO_2 emissions by sector, in 2010 [8].
            *Other includes commercial/public services, agriculture/forestry, fishing, energy industries other than electricity and heat generation, and other emissions not specified elsewhere.

According to decreasing of petroleum resources (due to excessive use of fossil fuels), increasing of GHG (and, consequently, amplification of greenhouse effect) and, subsequently, the climate change, the renewable energy technologies offer an alternative of energy from fossil fuels.

1.1.3. BIOFUELS

The production of this renewable energy has been increased over the years, Figure 1.1.9.
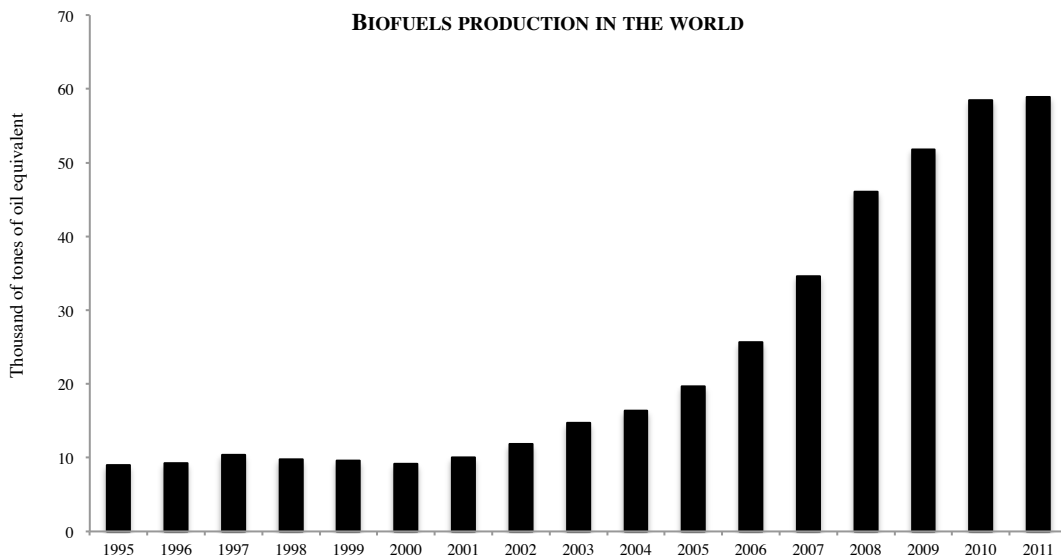
Figure 1.1.9 - Biofuels production in the world since 1995 to 2011 [10], [11].

Effectively, there are many studies of biofuels production from renewable feedstock. The most common biofuels can be produced from sugar or the starch portion of plants like corn [10], [11], sugarcane [12], sunflower [13], soybean [14], lard [15], [16], tallow [16], vegetable oil [17–20], animal fat [21–23], lignocellulosic materials [24] and microalgae [25]. Biofuels provide several benefits such as: alleviation from foreign oil dependence, the security of energy supply, produce lower greenhouse gas emissions, use renewable feedstock resource, provide rural development (which can improve more jobs and income generation through labour-intensive agriculture) and it give a cheaper energy imports [9].

However, biofuels have also many drawbacks that hinder their application, such as the fact that biofuels production aren't enough to fully meet the demands for fuel, but can contribute to sustainable renewable energy resources and reduce the demand for fossil fuels. Another disadvantage of biofuels production is the significant impact on feedstock prices. Naturally, the use of feedstock for biofuels production will, in principle, increase their prices – mainly due to increases in feedstock demands and corresponding higher marginal cost [26].

Furthermore, the land use for biofuels raw material production is another form of environmental impact. Two different impacts: (*i*) direct land use change (dLUC) – occurs when bioenergy feedstock production modifies an existing land use, resulting in a change in above and below-ground carbon stocks; (*ii*) indirect land use change (iLUC) – occurs when a change in production level of an agricultural products (i.e. a reduction in food or feed production induced by agricultural land conversion to produce a bioenergy feedstock) leads to a market-mediated shift in a land management activities outside the region of primary production expansion (iLUC is not directly observable and is complex to model and difficult to attribute to a single cause as multiple actors, industry, countries, policies and markets dynamically interact) [9], [27].

There are a large variety of different biofuels, such as biodiesel, bioethanol, bio-oil, synfuel, biomethanol, hydrogen and biomethane. Their feedstock sources can be divided into animal fat, oil crops, sugar plant, starchy plants, cellulosic biomass and wet biomass. Finally, these biofuels can be classified into liquid or gaseous biofuels (Figure 1.1.10).
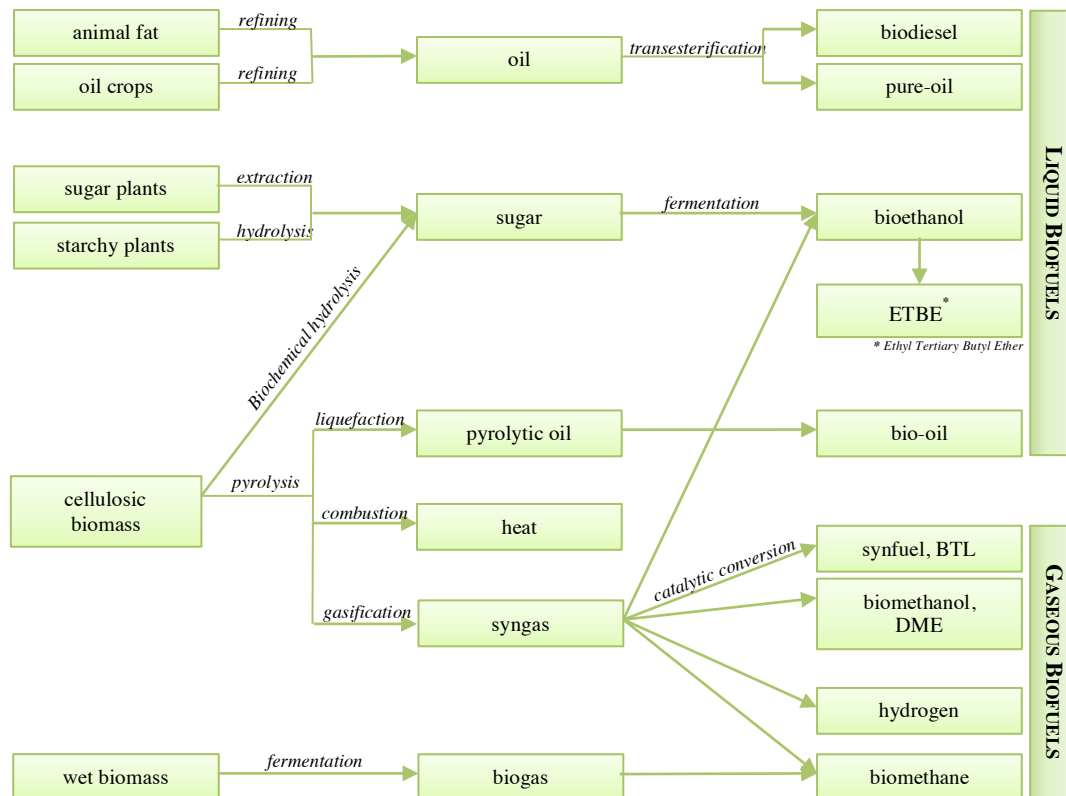
Figure 1.1.10 - Pathways of several biofuels (adapted from [28]).

The best strategic approach to produced biofuels is to avail the wastes from several industries sectors because they (*i*) do not compete directly with food production, (*ii*) can be bred specifically for energy purposes, (*iii*) allow the integration of waste management operations with a variety of other industries offering prospects for industrial symbiosis at the local level. A few studies have been done in wastes recovery, such as the production of biodiesel from coffee ground. In fact, recent decades have seen a significant rise in coffee production and consumption, which increased the coffee waste generation [29], [30]. Thereby, waste management will lead to valorisation strategies, which will contribute for the economy and for the environment.

Over the years, world meat production increased a lot and reached 230 million tons in 2010, from which 42,7%, 33,4% and 23,9% corresponds to pork, poultry and beef, respectively [21] (Figure 1.1.11). In Portugal, the chicken meat production has increased dramatically and so, its predicted that producer price reduces a lot, as it can be seen in Figure 1.3.2. Consequently, in the poultry industry, there are many types of residues that are wasteful. Then, since the non-edible products have a certain amount of fat/oil in its constitution, it's become a good feedstock for biodiesel production.

Therefore, this work will focus in the use of chicken feather meal (non-edible product of poultry industry) for biodiesel production. In chapter 1.3 – Chicken feather meal, the use of chicken feather meal, for biodiesel production, will be further discussed.

WORLD MEAT PRODUCTION
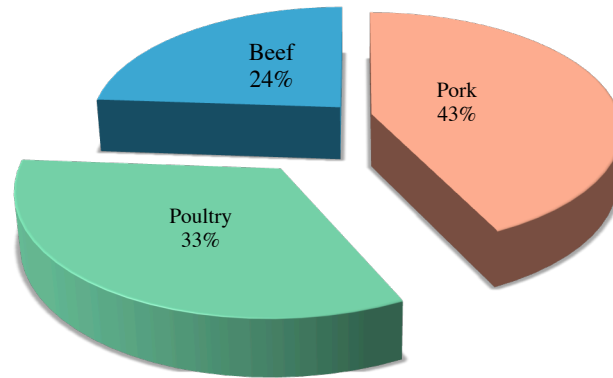
Figure 1.1.11 - World meat Production in 2010.

1.2. BIODIESEL

1.2.1. DEFINITION, ADVANTAGES AND DRAWBACKS

Biodiesel is an alternative fuel that can be produced from any material that contains long-chain of alkyl esters (methyl, propyl or ethyl). Thus, various vegetable fats and oils, animal fats, wastes greases, and edible oil processing wastes can be used as feedstock for biodiesel production. The purity and composition of the feedstock determinates the yield of the products. Generally, feedstock with higher content of free fatty acids (FFA), water, phosphorus, sulphur or other impurities, more difficult the procedure becomes [31].

Biodiesel is a clean source of energy, i.e. it is an environmental friendly fuel, biodegradable, nontoxic (when burned as a fuel, doesn't realise toxic emissions), renewable and it's economical. Biodiesel contains almost no sulphur and does not contribute to green house gas due to their closed carbon cycle. This biofuel is also miscible with usual diesel and it has excellent lubricity, with high flash point. EPA research shows that biodiesel reduces most emissions from usual petroleum diesel, depending on the blend level. A B100 (i.e. 100% of biodiesel), from soybean oil, reduces life cycle CO_2 emissions by 78%; a B5 blend reduces the life cycle CO_2 emission by 3,8%. Oxides of nitrogen (NO_x; which contribute to smog formation) increased slightly with biodiesel: a B50 with 5% and a B≤5 the contamination is almost insignificant (± 0,1% in NO_x emissions) [32].
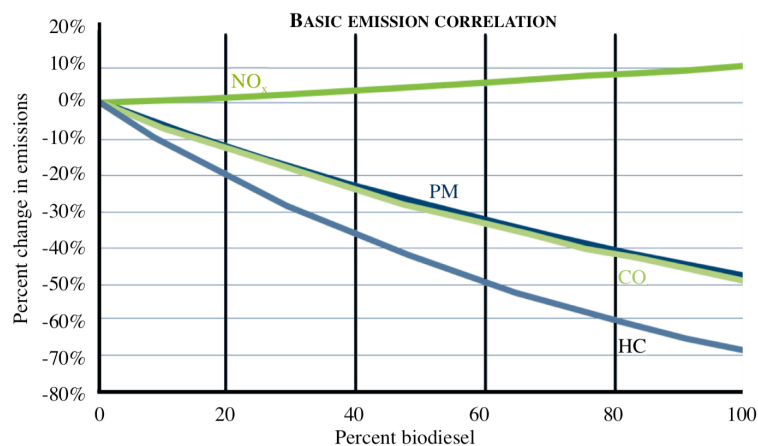
Figure 1.2.1 - Basic emission correlation. Average emission impacts of biodiesel from soybean oil. (Source: U.S. EPA)

Biodiesel has a higher cetane number[3], resulting in a large combustion efficiency, and it also has a higher oxygen content which improves the combustion process too (leading to a

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[3] Cetane number is related with the time between the fuel injection and the start of the combustion. Higher cetane number reflects in a high quality of combustion because the time between the injection and combustion is short. However, if it is lower, the combustion is inadequate which results in smog formation, as well as increase gas emissions.

decreased level of polluting emissions) [3], [17], [33–38]. Another benefit of biodiesel is that it can reduce dependency on crude oil. When this biofuel is used instead of common diesel, it does reduce the amount of crude oil used, which leads to a decreasing demand of petrodiesel [39].

According to European Biodiesel Board [40], the commercial biodiesel must have (Table 1.2.1):

Table 1.2.1 - Properties of biodiesel according to European Biodiesel Board.

PROPERTY	TEST METHOD	LIMITS	UNITS
ESTER CONTENT	EN14103	96,5	% (min)
DENSITY (AT 288,15K)	EN14214	900	kg/m^3 (max)
FLASH POINT	EN14214	374,15	K (min)
SULPHUR CONTENT	EN14214	10	mg/kg (max)
CARBON RESIDUE	CEN	0,3	% (m/m) (max)
CETONE NUMBER	EN590 and EN14214	51	(min)
WATER CONTENT	EN14214	500	mg/kg (max)
TOTAL CONTAMINATION	EN12662[4]	24	mg/kg (max)
LINOLENIC ACID CONTENT[5]	EN14103	12	% (m/m) (max)
POLY-UNSATURATED FAME[6]	EN14214	1	% (m/m) (max)
METHANOL CONTENT	EN14214	0,2	% (m/m) (max)
FREE GLYCEROL	EN14106:2003	0,02	% (m/m) (max)
MONO, DI AND TRIGLYCERIDE	EN14105	0,80	% (m/m) (max)
TOTAL GLYCEROL	EN14105	0,25	% (m/m) (max)
PHOSPHOROUS CONTENT	EN14107	4	mg/kg (max)

Biodiesel besides it advantages, also has disadvantages. This biofuel has a higher viscosity than the conventional diesel, however it is not flammable and explosive, such as the petroleum diesel, because the flash point of the biodiesel is 403 K, and the usual diesel has 337 K [41]. It can be the perfect alternative fuel, when compared to several other one, because biodiesel doesn't require changes to a vehicle to be used[7] [39].

However, the heating value of the biodiesel is lower comparing to petroleum, but it is higher than coal. Yet, the cost of biodiesel is, probably, the main obstacle to commercialization of this product. [42] The biodiesel price varies depending on the feedstock that is used: geographic area, variability in crop production from season to season, the price of the crude petroleum and other factors [43]. Although, the cost of biodiesel can be reduced by using low

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[4] The method is under review so that the measurement accuracy can be improved.
[5] Linolenic acid is a fatty acid, which is considered to have a relatively high oxidation rate.
[6] FAME: fatty acids methyl esters; poly-unsaturated with ≥ 4 bounds.
[7] Ethanol requires specialized changes to the fuel injection system. Natural gas and propane need special tanks to be installed and changes to the fuel injection system must be made as well. The electricity also needs a full different engine. So, because of all this changes, or you either run the alternative fuel, or you do not run the vehicle at all.

cost feedstock such as the waste residues, like animal fat. Actually, it is reported that its price will reduce, approximately, to the half with this kind of feedstock [44].

The biodiesel production in the world has been increasing, as it can be seen on Figure 1.2.2.
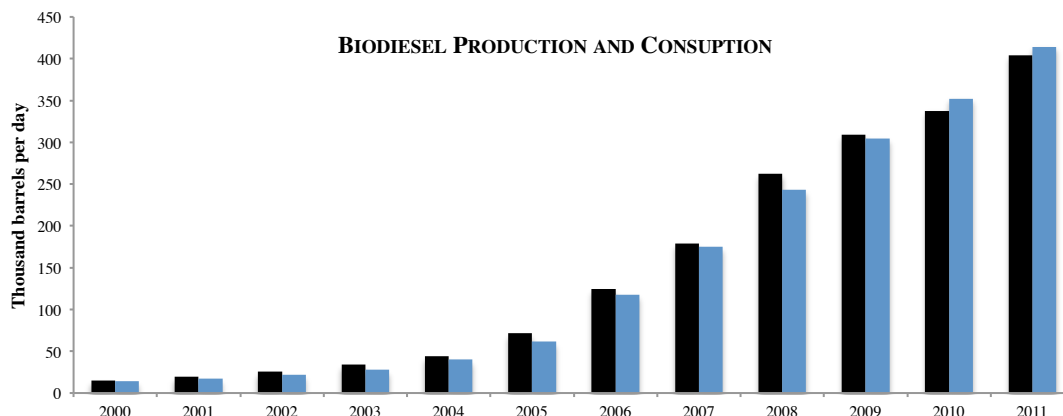
Figure 1.2.2 - Biodiesel world production (black rectangle) and consumption (blue rectangle) since 2000 to 2011 [45].

1.2.2. CONVERSION OF TRIGLYCERIDE TO BIODIESEL: TRANSESTERIFICATION REACTION

The direct use of vegetable oils (large triglyceride molecule content with high molecular mass) isn't adequate in motors devices because of their: (*i*) low stability against oxidation (and the subsequent reactions of polymerization), (*ii*) high viscosity, and (*iii*) low volatility, which influences on the formation of relatively high amount of ashes due to incomplete combustion. Therefore, tryacylglycerides (TAG) has to be cleavage into smaller molecules (pyrolysis). There exists several processes to produce biodiesel from renewable raw material (pyrolysis, or cracking, and microemulsions, which are both an expensive processes and the biodiesel has low quality), however, transesterification reaction seems to be the most viable oil modification process [46], [47], [48].

Biodiesel is made from renewable feedstock such as vegetable oil, animal fat or even microalgae. It is mainly consisted of alkyl esters of fatty acids (FAAE), obtained by transesterification of lipid (acquired from the feedstock used) with an alcohol. The methanol is one of the alcohols that can be used as alkyl donor[8] (alcohol takes the place of the ester linkage to glycerol), due to advantages as easy recovering and it has low cost [49]. The transesterification is a three-step consecutive reaction (Figure 1.2.4), in which diglycerides

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[8] When methanol is used as alkyl donor, the biodiesel is consisted of FAME, fatty acids methyl esters.

and monoglycerides are formed as an intermediate compounds. Three moles of FAME and one mole of glycerol are produced for every mole of triglyceride (TG), or triacylglycerol (TAG), that undergoes completely conversion (Figure 1.2.3) [50]. It is a reversible reaction and accordingly, an excess of alcohol can be used to shift the equilibrium to the products side.

Transesterification reaction can be affected by several factors, such as the nature of TAG, type of alcohol used, molar ratio oil:methanol, type of catalyst, temperature and reaction time.
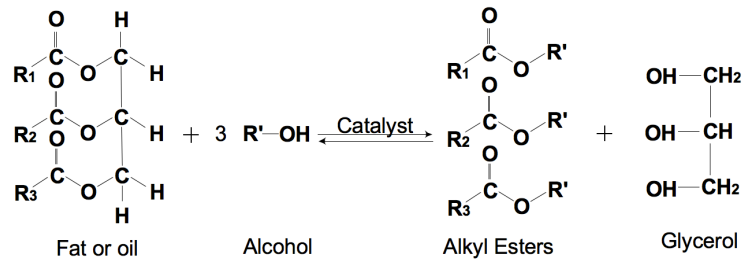
Figure 1.2.3 – General reaction of transesterification of triacilglycerols with alcohol (R' groups = fatty acids)
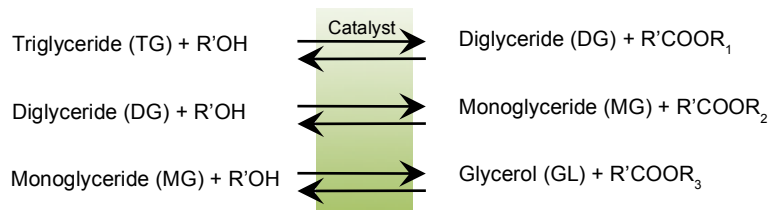
Figure 1.2.4 - Three-step consecutive and reversible reactions in the transesterification process.

Chemically, the lipids from the feedstock are mainly consisted in TAG (Figure 1.2.5). Glycerides are named as the number fatty acids existing in the molecule. Monoglycerides contain only one fatty acid (FA), diglycerides contain two FA and triglycerides contain three FA, bonded to a single glycerol molecule[9]. Consequently, the FA can differ by nature, by the number, position of the double bonds in the carbon chain and can be saturated or unsaturated. The principal saturated acids are lauric acid (C12:0), palmitic acid (C16:0) and stearic acid (C18:0). The more common unsaturated acids are oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) (Appendix - Table 6.3.1).

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[9] When a FA are not bound to some other molecule, are know as free fatty acids (FFA).
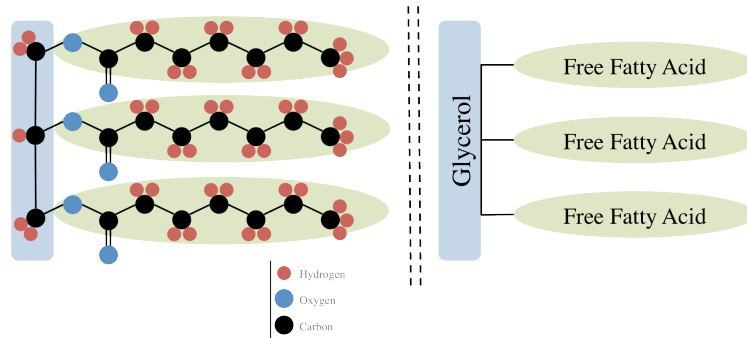
Figure 1.2.5 – Structure of a triglyceride molecule.

Figure 1.2.6 - General reaction of saponification of FA.

Figure 1.2.7 - General reaction of esterification of FA.

1.2.3. INDUSTRIAL CONVENTIONAL PROCESS

Transesterification reactions can be base-catalysed, acid-catalysed or even enzymatic. Currently, in conventional industries is applied transesterification base-catalysed. This reaction takes almost one hour, at room temperature. Despite the high yield, the presence of an alkali (i.e., sodium hydroxide – NaOH, potassium hydroxide – KOH) would lead to the saponification of the FFA and consequent loss of valuable material. Consequently, this saponification reaction (Figure 1.2.6) converts the reagents into 'soap'. This by-product must be removed, resulting in high production cost. Furthermore, the water released during the esterification[10] of FFA (Figure 1.2.7), inhibits the transesterification of the TAG (because instead of the biodiesel production – FAME, there are formation of fatty acid salts). In acid-catalysed transesterification reaction there's no formation of soap, but higher temperature and

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[10] Esterification reaction of FFA:
        FFA + Alcohol ⟶ Biodiesel + Water

higher substrate molar ratio are required (up to 30:1). Besides these facts, it demands three to four days to complete and has lower yield than when hydroxide catalyst [48]. It must have to be divided into two different stages: (*i*) esterification of FFA with presence of an acid (sulphuric acid, sulfonic acid or hydrochloric acid) and heat; (*ii*) transesterification of TAG in a basic medium [51]. In enzymatic-catalysed process, lipase is the enzyme used for biodiesel production. There are two major categories of enzymatic biocatalyst, (in both cases, the enzyme is immobilized): (*i*) extracellular lipases (extracted from several microorganisms, as *Mucor miehei, Rhizopus oryzae, Candida Antarctica* and *Pseudomonas cepacia*); (*ii*) intracellular lipase [48].

Normally, enzymes operate under mild conditions and there are easily recovered from the reaction mixtures. Since the enzymes can be extracted from microorganisms, genetic engineering can be applied in order to improve their catalytic effect (for biodiesel production), thermostability, fatty acid chain length specificity, substrate specificity, alcohol chain length specificity, methanol and ethanol tolerance and pH stability [52]. In Table 1.2.2 is indicate the pros and cons of using lipase as biocatalyst comparing the alkaline process, once is the one that is industrially used.

Table 1.2.2 – Comparison of enzymatic technology vs. conventional alkaline technology, for biodiesel production [48]

	ENZYMATIC PROCESS	ALKALINE PROCESS
PRESENCE OF FFA	FFA are converted into FAME	FFA are converted into soaps
WATER CONTENT ON THE OIL	It does not influence negatively or positively the enzyme	It provides soaps formation. It may also hydrolyse the oil and more soap is formed
BIODIESEL YIELD	High, ± 90%	Very high, ≥ 96%
GLYCEROL RECOVERY	Easy, glycerol recovered with high quality	Complex, glycerol recovered with low quality
CATALYST RECOVERY AND RECYCLE	Easy, is separated from the reaction mixture by filtration. Or it's not necessary when is used PBR (pack bed reactor). The enzyme can be reutilized	Not profitable, because of the successive washing steps or it is lost as soaps.
ENERGY COST	Low, temperature range 293,15-298,15K	Medium, temperature range 333,15-353,15K
CATALYST COST	Very high	Low
ENVIRONMENTAL IMPACT	Low	Medium, because of the alkaline and saline mixtures and there is wastewater treatment required
PROCESS PRODUCTIVITY	Low	High

Methanol and ethanol are the alcohols commonly used for transesterification reaction. As said before, according to the stoichiometric equation, three moles of alcohol would react with one mole of TAG to give three moles of FAME and one mole of glycerol. In other words, an

alcohol to oil molar ration of at least 3:1 is necessary for complete reaction. With both alcohols, emulsions normally form (this emulsions are caused, in part, by the formation of the intermediate MG and DG, which have both polar hydroxyl groups and nonpolar hydrocarbon chains). In methanolysis, the emulsions formed would break down easily to form a lower glycerol rich layer and upper methyl ester rich layer. In the case of ethanolysis, the emulsions formed are much more stable due to the presence of a large non-polar group in ethanol, resulting a separation and purification of biodiesel more difficult [53]. Based on these facts, methanol is the best choice for biodiesel production, resulting in an higher yield [54].

1.3. CHICKEN FEATHER MEAL

1.3.1. POULTRY INDUSTRY AND RENDERING

Generally, a by-product is defined as a secondary product acquired during the manufacture of a principal system. A animal by-product is any part of an animal carcass or any material of animal origin not intended for human consumption. They are a product of food industry, particularly from food processing and dairy plants, which includes animals that die on farm, surplus or waste materials from slaughterhouses and a range of surplus or rejected foodstuffs including catering wastes that contain products of animal origin whether cooked or uncooked.

Meat production as incresead over the years (Figure 1.3.1) and in 2011 was produced almost 230 million tonnes in which 30% corresponds to chicken meat (90 million tonnes).
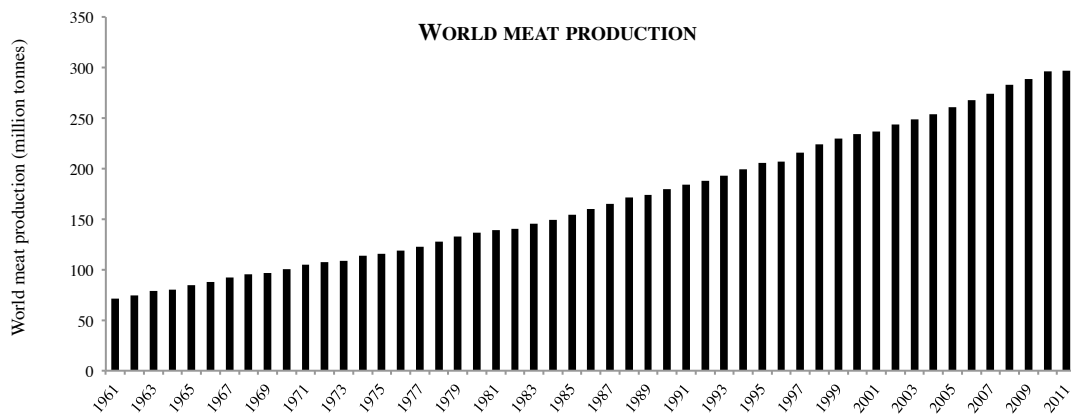
Figure 1.3.1 - World meat production, since 1961 to 2011 [55].

In Portugal, chicken meat production has also increased dramatically as it can be seen on Figure 1.3.2 and consequently, its predicted that produtor price reduces (Figure 1.3.2), which stimulates its consumption. Accordingly to IACA – *Associação Portuguesa dos Industriais de Alimentos Compostos para Animais*, in 2011, Portugal produced 939 tonnes of chicken feather meal (Figure 1.3.3) [56]. Feathers are effectively a waste product generated in large quantities from commercial processing.
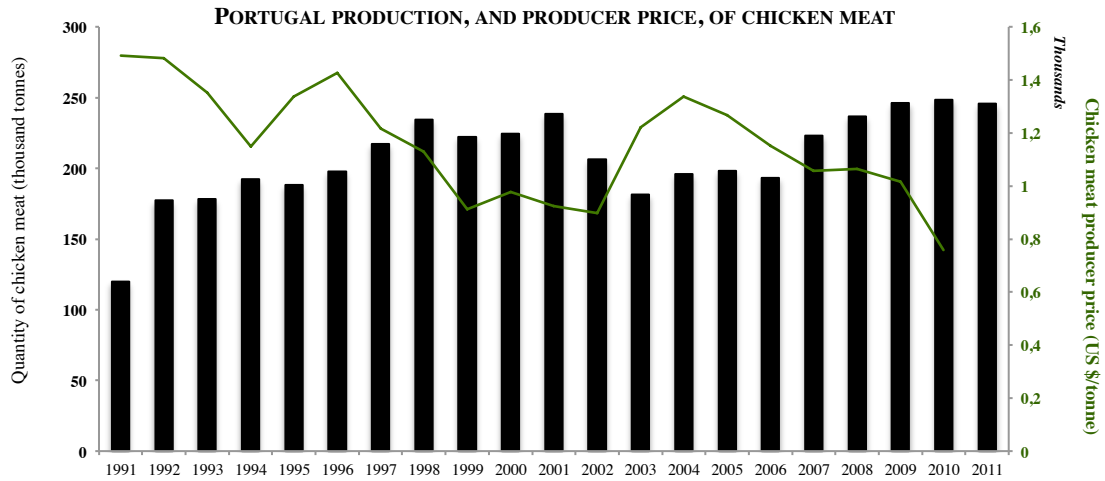
Figure 1.3.2 – Portugal production of chicken meat, in tones, since 1991 to 2011 [57], and Portugal producer price of chicken meat, since 1991 to 2010 [58].
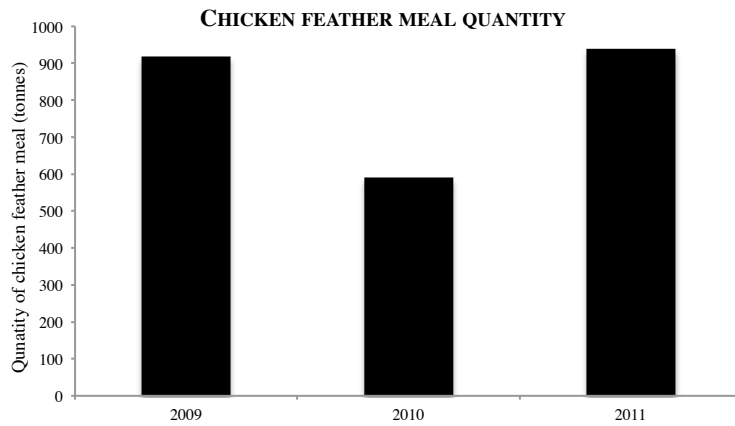
Figure 1.3.3 - Chicken feather meal quantity in Portugal, since 2009 to 2011 [56].

Feather waste has been used as feedstuff for poultry and livestock [59–61]. Figure 1.3.4 shows the main constituent parts of chickens. The non-edible by-products, exhibited in this figure, are the most used in most cases for the manufacture of chicken meal. In 2011, Portugal slaughtered 472.209 chicken per day [62]. Considering the *Paulo Ferroli* study, this number corresponds to 228 tonnes of chicken by-products that are dailly produced, in which 18 tonnes are from chicken feather [61]. Thus, chicken feather meal is consisted in the non-edible by-products of pultry industry.

Products for immediate consuming		Edible sub-products		Non-edible sub-products	
Wings	05,67%	Gizzard	0,92%	Head	2,81%
Thighs and drumsticks	30,30%	Liver	1,02%	Blood	0,83%
Chest	24,80%	Heart	0,50%	Feathers	7,89%
Dorsal, lumbar and cervical	12,68%	Neck	4,40%	Stomach and intestine	2,52%
		Feet	4,60%	Lung	1,06%
TOTAL	**73,45%**	**TOTAL**	**11,44%**	**TOTAL**	**15,11%**
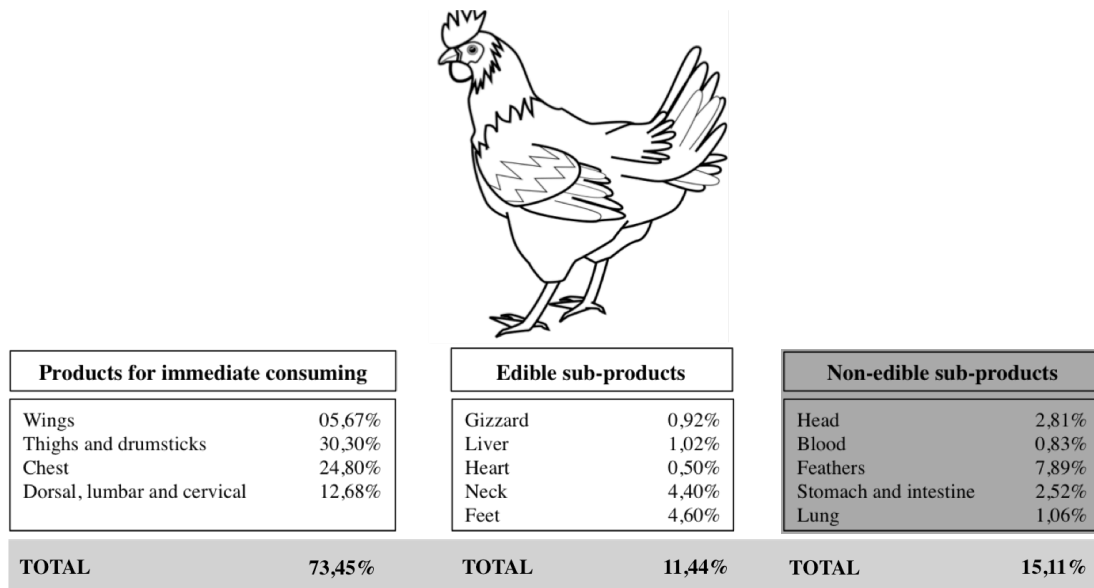
Figure 1.3.4 – Constituent parts of the chicken, in percentage. (Adapted from [61])
The non-edible sub-products are the chicken feather meal (CFM).
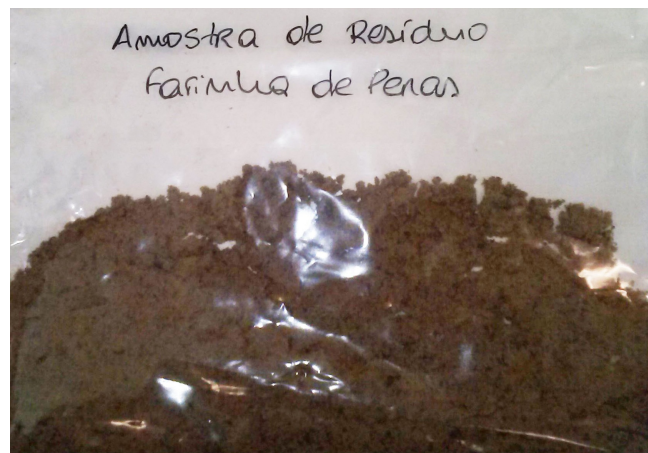
Figure 1.3.5 – Chicken feather meal used in this work.

1.3.2. CHARACTERIZATION OF CHICKEN FEATHER MEAL

According to IACA, chicken feathers meal are produced by hydrolysis, followed by drying and milling [56]. These by-products are cheap and biodegradable and once that feathers are coated by oil/fat, it's become a good feedstock for biofuel production. The nutrient composition of chicken feather meal is shown in Table 1.3.1.

Table 1.3.1 - Nutrient composition of chicken feathers meal (adapted from [63]).

ITEM	CHICKEN FEATHER MEAL	REF.
Crude protein, %	75 - 85	[63], [64], [65]
Fat, %	7 - 12	[22], [63], [66]
Calcium, %	0,3	
Phosphorus, %	0,5	
AMINO ACIDS		
Methionine, %	0,6	
Cystine, %	4,3	
Lysine, %	2,3	
Threonine, %	3,8	
Isoleucine, %	3,9	
Valine, %	5,9	
Tryptophan, %	0,6	
Arginine, %	5,6	
Histidine, %	0,9	
Leucine, %	6,9	
Phenylalanine, %	3,9	
Tyrosine, %	2,5	
Glycine, %	6,1	
Serine, %	8,5	

The production of alternative biofuels should be economically and technically attractive to compete with currently used fossil fuels [67], and chicken feather meal offers another promissing feedstock source for biodiesel production (Figure 1.3.5).

1.4. BIOCATALYSIS

1.4.1. ENZYME – BIOLOGICAL CATALYST

Enzymes are biological catalysts systems and are remarkable molecular devices that determine the profile of chemical transformations. The most impressive feature of enzymes is their catalytic power and specificity. The catalysis occurs at a particular site in enzymes – active site/center (Figure 1.4.1).
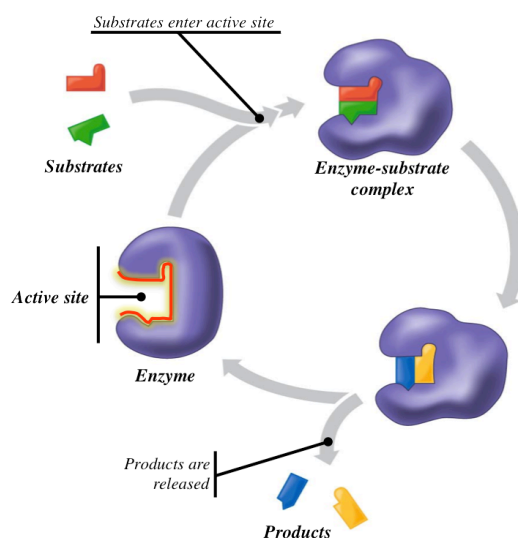
Figure 1.4.1 - Illustration of the enzyme, active site, enzyme-substrate complex and products formation.

Almost all know enzymes are proteins. However, proteins don't have an absolute monopoly on catalysis. They have the ability to catalyse reaction under mild conditions, with a very high degree of substrate specificity, thus decreasing the formation of by-products. Enzymes catalyse reaction by stabilizing transition states, the highest-energy species in reaction pathways. Generally, binding between the substrate and the enzyme is a process that occurs with negative Gibbs energy, and therefore, stabilizes the substrate. This occurs because the affinity of the enzyme for the transition state is greater than the substrate, which explains the decrease in the Gibbs energy, associated with activation of enzyme activity [68]. Enzymes also work by lowering the reaction activation energy and they are not consumed, nor do they alter the reactions equilibrium.

The choice of catalyst for industrial process lies in its energy efficiency. However, the selectivity underlies the increasing demand for enzymes for this purpose, including selectivity to the substrate, enantioselectivity (when one enantiomer is formed in preference to the other), regioselectivity (the preference of one direction of chemical bond making or breaking over all

other possible directions) and chemoselectivity (refers to the selectivity of one function group in the presence of others) [69]. Enzyme-catalysed processes are gradually replacing chemical processes in many areas of industry, such as: starch and fuel, food (including dairy), baking, animal feed, textile, pulp and paper, fats and oils, organic synthesis and also personal care [70].

Enzymes are classified based on reactions that catalyse. They are divided into six major classes, with several subclasses (Table 1.4.1) and lipases are included in hydrolases type.

Table 1.4.1 - The six major classes of enzymes and their function [68].

CLASSIFICATION	REACTION TYPE
OXIDOREDUCTASES	Are involved in oxidation and reduction
TRANSFERASES	Transfer functional group (e.g. amino or phosphate groups)
HYDROLASES • LIPASE	Transfer water, e.g., they catalyse the hydrolysis of a substrate
LYASES	Add (or remove) the elements of water, ammonia, or carbon dioxide (CO_2) to (or from) double bounds.
ISOMERASES	Catalyse rearrangements of atoms within a molecule
LIGASES	Join two molecules

1.4.2. IMMOBILIZED ENZYMES

The high cost of enzymes often makes the enzymatic processes economically unattractive. However, they can be used in an immobilized form, which allows its recovery and reuse [38].

Enzymes can catalyse reactions in different states: as individual molecules in solution, in aggregates with other entities or even as attached/immobilized to surfaces. Immobilized enzymes are currently the object of considerable interest due to the expected benefits over soluble enzymes or alternative technologies [71]. Immobilized enzymes are physically confined, or localized, in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously. The use of immobilized enzyme, in industrial processes, has increased the technical performance and their economy. Thus, the enzyme immobilization has as advantages: (*i*) catalyst reuse (e.g. this technique improves enzyme lifetime, which reduces the cost of the production); (*ii*) easier reactor operation; (*iii*) easier product separation; (*iv*) wider choice of reactor configuration. Therefore, immobilization of enzymes makes them more attractive for industrial processes. Yet, it can promote some disadvantages: (*i*) loss or reduction in activity; (*ii*) diffusional limitation; (*iii*) additional cost [72]. However, the loss or reduction of enzyme activity is compensated by enhanced operational stability. Effectively, there are four main methods of immobilization of

enzymes: *i*) adsorption (*ii*) covalent binding (*iii*) affinity immobilization (*iv*) entrapment [73]. In this work, enzymes are immobilized on a porous matrix.

1.4.3. ENZYMATIC TRANSESTERIFICATION

In catalysed biodiesel production, the enzyme used is a lipase – hydrolase. Lipase is a group of enzymes, water soluble, that catalyses the hydrolysis and synthesis of TAG, e.g. catalyse the hydrolysis of ester bonds. The action of these enzymes on the substrate is a result of a nucleophilic attack on the carbonyl carbon atom from ester groups. In this work it was used the Lipozyme RM (obtained from the *Rhizomucor miehei* fungus).

Substrate specificity of these enzymes consists in the competence of discriminating structural features of acyl chains [length, number of carbons, position or configuration of double bonds, presence of branched groups, nature of acyl source – e.g. FFA, alkyl ester (methyl, ethyl or propyl esters), glycerol]. The differences in catalysis by lipases for biodiesel synthesis are their regiospecificity, regarding the length of hydrocarbon chain of fatty acid. In general, the lipase regioselectivity can be divided into three types: (*i*) 1,3 – specific (hydrolase ester bonds in positions 1 or 3 of TAG), (*ii*) 2 – specific (hydrolase ester bond in position 2 of TAG), (*iii*) nonspecific (which do not distinguish the positions of ester bonds to be cleaved). In biodiesel production, the narrow regioselectivity of overall lipases are not applicable. Therefore, for biodiesel production, the lipases display both wide substrate specificity and regiospecificity [48].

The advantages of enzymatic transesterification over chemical catalysis are notable. With enzymatic transesterification, there's no formation of soaps (during transesterification reaction), which deteriorate the biodiesel quality. Besides that, with this enzymatic process, the glycerol (a by-product of transesterification reaction) is recovery with high quality. Consequently, there's no need of multi-step purification of biodiesel, such as: neutralization steps of the catalyst and water washes, which increase the production cost.

In general, the enzymatic process allows transesterification of glycerides with high FFA contents. Moreover, it is a less energy intensive process. Additionally, the easy recovery of enzymes gives them reusability, reducing production cost [74]. So, the enzymatic transesterification of TAG offers an environmental more attractive option to the conventional process [75].

There are several factors that influence the enzymatic biodiesel synthesis [76]:

- Oil source;

- Reaction temperature;

- Choice of acyl acceptors to oil molar ratio;

- Amount of water in the system;

- Type of lipase.

Reaction temperature may vary from 296,15 to 323,15K. When is used the Lipozyme RM in biodiesel transesterification, the optimum temperature is lower than 313,15K. In general, increasing the temperature leads to an increase of the reaction rate of biodiesel production. However, when the temperature is above the optimum, this leads to decreased catalytic activity of the enzyme, due to denaturation and inactivation of the enzyme. Although, with immobilized enzymes, they provide a more rigid external backbone for lipase molecule, leading to the increase of the optimum temperature and higher reaction rates [76].

1.4.4. ENZYME INACTIVATION TRIGGERED BY ALCOHOLS

As written before (1.2.2. Conversion of triglyceride to Biodiesel: Transesterification reaction), the molar ratio between alcohol to oil, in transesterification reaction, is, at least, 3:1, for complete process. Therefore, an excess of alcohol can be used to shift the equilibrium to the products side [48]. Methanol is the least expensive alcohol and is widely used for biodiesel production. However, methanolysis can provide an enzymatic inhibition because the polar short chain of this alcohol is a major problem for biodiesel production. Actually, the transesterification with longer-chain of fatty alcohols is more efficiently than with methanol (C1), or even ethanol (C2). Fatty alcohols (or long-chain alcohols), with carbon lengths higher than three (> C3), are completely dissolved in the oil, in the stoichiometric amount that is needed. Y. Shimada *et al*. had reported that low methanolysis is due to the inactivation of lipases by contact with insoluble methanol which exists as drops in the oil [77].

1.4.5. ENZYME INACTIVATION TRIGGERED BY GLYCEROL

Once that glycerol possesses high viscosity, it may inhibit the enzyme activity. If occurs an accumulation of this product in reaction mixture, glycerol may cover the enzyme surface resulting in a reduction of reaction yield [48], [78], [79], [80]. However, Watanable *et. al*. has reported that glycerol can increase enzyme stability [79].

1.5. SUPERCRITICAL FLUIDS (SCFS)

The critical point represents the maximum pressure and temperature at which the liquid and gas phase of a fluid are in equilibrium. Therefore, critical temperature (T_c) is obtained above in which liquid-vapour phases can not be formed by increasing temperature at constant pressure. Moreover, the critical pressure (P_c) is obtained above in which liquid-vapour phases can not be formed by increasing temperature at constant pressure [81]. Thus, in the supercritical environment, only one phase exists and consequently, there's no surface tension. An example of supercritical phase diagram is demonstrated on Figure 1.5.1. These fluids have particular physical and chemical properties which offers a suitable variety of applications, e.g. natural products extractions/fractionation (for food and pharmaceutical products) [82], reactions [82], powder technology [82], paints, coatings, polymer processing [83], ceramics and carbons manufacture, foams, aerogels [84], impregnation [85] and also dyeing (process of adding colour to textile products) [86], [87]. In SCFs, physicochemical properties (e.g. density, diffusivity, dielectric constant and viscosity) can be easily controlled by changing the pressure, and/or the temperature. The solvating power of these solvents depends on the density, but in general, SCFs have high solvating power [88]. In fact, supercritical fluids are compressed in liquid-like densities, which promotes the interaction (dispersion, polar hydrogen bonding) with solute molecules. In the supercritical region, the solvent power is similar with the liquids, and the transport properties are common to gases.

Thus, SCFs becomes a good alternative over organic solvents [88]. The overall conditions of supercritical fluids can facilitate the mixing of compounds resulting in a better heat and mass transfer. It can also promote a higher reaction, when comparing with other solvents, which are toxic, expensive and demand extra separation processes. By adding modifiers to a SCF, like methanol, its polarity can be changed. Despite the high investment demanding to apply this technology in industrial processes, disposal costs are much less since those fluids can be easily recovered and recycled.
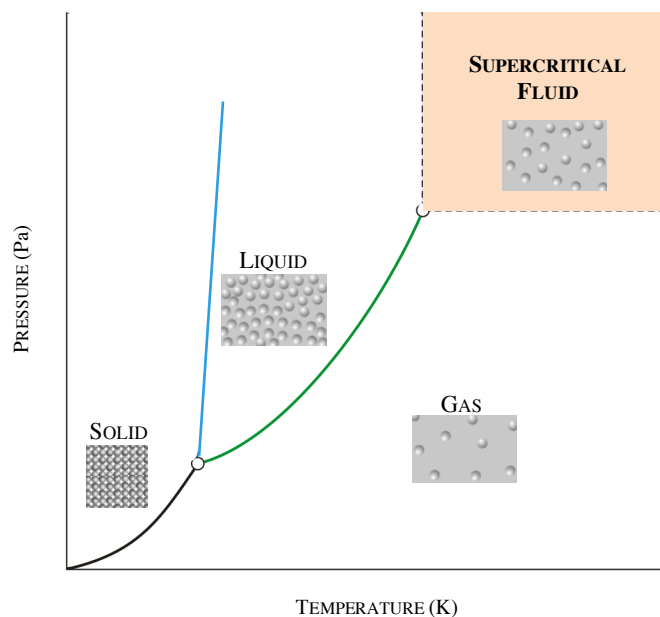
Figure 1.5.1 - Supercritical phase diagram.

Any pure compound can acquired supercritical conditions (Table 1.5.1). In this work its use the supercritical carbon dioxide (CO_2) and its properties is exhibited on Table 1.5.2. Carbon dioxide is the solvent that is normally used because it is considered environmental friendly, it has supercritical conditions relatively easy to work with, when compared with other possible supercritical solvents (without turning it economically unattractive) and is available in high purity at low cost. CO_2 exists on the atmosphere and is a major by-product in several industrial processes (in terms of availability, it's almost as readily abundant as H_2O), therefore, in small amounts is non-toxic and isn't a volatile organic compound (VOC) not contributing to smog formation. Additionally, and as it was written before (1.1.2. Climate change caused by fossil fuels) CO_2 is the major GHG causing global warming and climate change. Therefore, it's important to recycling this by-product gas, reducing emissions into atmosphere. CO_2 is also non-flammable which is an advantage over conventional liquid solvents [89].

Although hydrogen, methane and oxygen have the lowest critical conditions (T_c and P_c), they are compounds highly inflammable (except the oxygen), thus these solvents are considered dangerous to work with.

Table 1.5.1 - Critical point for pure components [81].

COMPONENT	T_c (K)	P_c (MPa)
Hydrogen	33,00	1,29
Oxygen	154,60	5,04
Methane	190,7	4,6
Ethylene	282,40	5,04
Carbon Dioxide	304,15	7,38
Ethane	305,40	4,88
Methanol	512,60	8,09
Ethanol	513,90	6,14
Water	647,30	22,12

Table 1.5.2 - CO_2 properties as liquid, supercritical and vapour [90].

	LIQUID	SUPERCRITICAL (AT 350 BAR AND 335,15K)	VAPOUR
DENSITY ($kg \cdot m^{-3}$)	1170	856	1,87
VISCOSITY ($N \cdot m^{-2} \cdot s$)	$2,5 \times 10^{-4}$	$8,2 \times 10^{-5}$	$1,4 \times 10^{-5}$
THERMAL CONDUCTIVITY ($W \cdot m^{-1} \cdot K^{-1}$)	0,17975	0,1	0,0146

To choose a good supercritical solvent, several aspects must be consider: (*i*) solubility of material in the supercritical fluid, i.e. it's solvent capacity and selecticity (*ii*) viscosity that the fluid adquired in supercritical region, (*iii*) diffusivity of the supercritical fluid, (*vi*) the mass and heat transfer capacity that SCF have and (*v*) conditions required to achivied supercritical region, because of the economical and safety issues.

Sc-CO_2 (supercritical CO_2) is achieved over 7,4 MPa and 304 K conditions (Figure 1.5.2). Carbon dioxide always behaves as an non-polar solvent, therefore it has good solvent properties for extraction of non-polar components such as hydrocarbons (lipids), which are water-insoluble compounds. However, because of this phisical caracteristics, Sc-CO_2 doesn't dissolve hydrophilic compounds such as sugars and proteins, mineral species like salts and metals. The solvent properties of Sc-CO_2 can be easily modified regulating temperature and pressure. Changing this conditons will influence the CO_2 solubility with a certain compound and this fact is related with its density. Therefore, the separation process is easier to obtain. Effectively, manipulating the temperature and pressure conditions, a decrease of a certain compound solubility occur, resulting in a precipitation and there are no costs associated with solvent waste dispostal [91].
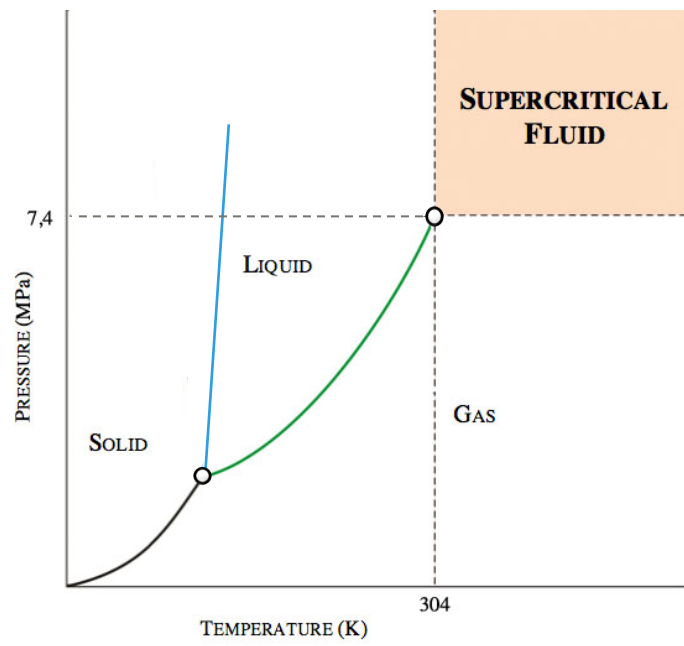
Figure 1.5.2 - Supercritical CO_2 phase diagram.

1.6. SUPERCRITICAL FLUID EXTRACTION

In the recent years, supercritical fluids have emerged substantially, due to a growth in the research and development activities, focused on new approaches, as well as oil extraction, an alternative tecnhology over the tradicional solvent extraction.

Supercritical fluid extraction (SCFE) consists into 2 levels: (*i*) extraction of compounds soluble in SCF solvent, (*ii*) separation/fraccionation of the extracted solutes from the SCF solvent. Effectively, there are 2 kinds of extractions, depending on the experiment purpose [92]:

1. *Carrier material separator*: where the raw material becomes the final product, without the component/s that isn't/aren't desirable. As an exemple, there is the decaffeination process of green tea [93].
2. *Extract material separation*: consists in the removal of desirable compunds, from the initial residue. Exemples of this experiment are the oil (lipids) [94–96] or antioxidants extraction [97].

In this work, oil extraction from chicken feather meal was carried out, using a supercritical fluid. In a first instance, removal of oil with this technology does not appear ecomic, due to the cost of high-pressure batch process. However, there exists areas in which SCFE can be useful in the extraction of high-value oils: food, pharmaceutical and cosmetic applications [98]. Moreover, in this work – biodiesel production using SCF, biodiesel isn't the only reaction product. Glycerol (Gly) is the majour by-product of transesterification reaction, and can be used as a high-value compound, which could be useful for counterpoise the operating cost of biodiesel production.

The most important parameters to perform extraction, or separation, processes are the solubility and phase equilibrium of the systems. The separation of the soluble compounds from the SCF is carried out by modifying the thermodynamic properties of the solvent: the solvation power of it can be modified by manipulating the pressure and temperature. Decreasing the pressure will lead to a reduction of the fluid density and, consequently, a reduction of the solvent power. Operating with temperature can occur 2 differents situations: increasing the temperature, the CO_2 density decrease which leads to a lower solvent power of the solvent and, consequently, decrease the solute solubility into sc-CO_2. However, increasing the temperature, the vapour pressure of the solute increased, which increases the solvation power of the solvent and the solute solubility into sc-CO_2 is higher. Additionally, the

separation can be perform by an external agent, such as an adsorbent, where no significant pressure change occurs. Though, despite a lower operiting cost, the recovery of the product from the adsorbent is difficult. Therefore, to avoid a high losses of the product, the adsorption step may be replaced by and absorption step. The product dissolved in the SCF solvent is aborbed by a wash fluid in a countercurrent flow, using a packed column or spray tower under pressure.

In this work, the separation process was achived by decreasing the pressure. However, and as it was written before, the products obtained in transesterification process are the unreacted methanol, triglycerides, FAME, glycerol, MG and DG. The ideal separation process is the fractionation, based on the different solubilities of the compounds to be separated in sc-CO_2, i.e. the compounds that are desirable to extracted are insoluble on the solvent, while all the other compunds are soluble. Therefore, in these cases, it is possible to perform an extraction in sucessive steps, in order to achieve the maximum difference in solubility among the compounds to be recovered and all the other compounds in the mixture. The scope of this operation is to induce the selective precipitation of different compound families as a function of their different saturation conditions in the SCF. For example, in a first separation process it is possible to operate at high CO_2 density (584,71 kg/m^3, 120 bar, 323,15K) followed by a second extraction step at low CO_2 density (104, 85 kg/m^3, 50 bar, 323,15K). The most soluble compounds are extracted during the first step and the less soluble in the second [99]. The Figure 1.6.1 ilustrates an ideal muti-step separation process, when 3 compounds are needed to separate.
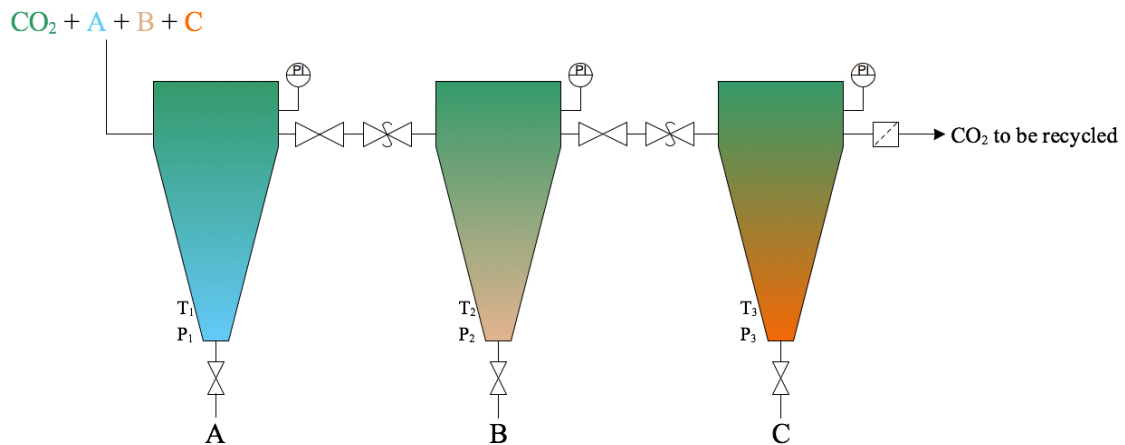
Figure 1.6.1 – Diagram of a separation process in series.

The first separator operates at T_1 and P_1 conditions, in which components B and C are soluble in the sc-CO_2 and component A doesn't, and than precipitats and it's recovered. Components B and C go to the second seperator, dissolved on CO_2. The second separator operated at T_2 and P_2 where component B isn't soluble and is precipitat and recovered, while component C continues soluble in the solvent (CO_2) and flows to the third separator. In this last separator, the conditions applyied are T_3 and P_3 where component C is finally unsoluble on the solvent and precipitates. To not contribute to envrionmental damage, the CO_2 without any component dissolved, is recycled to the process.

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1.7. SUPERCRITICAL TECHNOLOGY COMBINING WITH BIOCATALYSIS IN BIODIESEL PRODUCTION

Economical and financial issues are the major concern in industrial applications. The conventional process of biodiesel production (1.2.3. Industrial conventional process) is the base catalysed. However, it has some drawbacks, such as the hight cost of biodiesel purification and environmental issues. Effectively, to produce biodiesel with this method, more energy is required and it uses hazard compounds to the environment. Moreover, if oil has FFA and water on his constitution, it may produces soaps, due to saponification reaction, and decreased the reaction yield. Besides these, to obtain pure biodiesel with alkali catalysis, additional separation/purification processes must be apply. This separation process is based on a large amount of water and, consequently, effluents are produced. Therefore, there are several negative factors when alkali catalysis is applied for biodiesel production.

To overcome the drawbacks usually associated with the use of conventional process for biodiesel production, supercritical technology has been applied in several studies. Many researchers proposed a non-catalytic process using supercritical methanol (sc-MeOH) [15], [19], [20], [100–102]. The advantages of using this solvent over the conventional process include a faster reaction rate, easy product purification and the ability of using inexpensive feedstock, without any treatment. However, the T_c of methanol is high (512,60 K), which causes high energy required for the process. The sc-CO_2 acquires mild supercritical conditions and it's a good alternative over sc-MeOH.

The biodiesel production using enzymes, as catalyst, is a promising choice to apply in transesterification reaction and is a matter of great scientific and technological interest. The biodiesel usage over diesel from fossil fuels and the production of many chemicals raw materials for food, pharmaceutical and cosmetic industries has inspired researchers in the biotransformation of feedstock with the desired of end result of high-added products, or drastic reduction in environmental investments.

As mentioned before (1.4.2 Immobilized Enzymes), enzymes can be used in the immobilized form, which has advantages over the enzymes in solution and/or in aggregates. In the point of view of an industrial application, the use of immobilized enzymes seems to be a better choice due to its reutilization and improvement of catalyst lifetime, which reflects in a reduction of production cost.

Enzymatic reactions at high pressures, the enzyme behaviour in sc-CO_2 is very important because the loss of activity may lead to undesirable reaction – low reaction rates and low product production [103]. These enzyme modifications can occur because of changes in protein structure, causing denaturation, under extreme conditions and, consequently, lead to a loss of activity. Hence, it's very important to guarantee a good performance of the enzyme under supercritical conditions. However, biocatalysis in sc-CO_2 may offer advantages, since the solubility is greatly influences by fluid temperature and pressure adjustments, the separation process can be easily achieved by a pressure reduction [25]. Lanza *et al.*, investigated the influence of sc-CO_2 on lipase performance and reported that the residual activity of Novozym-435 was approximately 90%.

The biodiesel production under supercritical conditions, and combining biocatalysis, has a high capital cost investment. Despite the advantages that have over the conventional process, in the first instance, seems to be economically unviable. However, a combined continuous process of extracting oil, from chicken feather meal using sc-CO_2 and, consequently, the use of the extracted oil for biodiesel production, using immobilized lipase in the same solvent, in a one integrated process, would economically be feasible. Effectively, the extracted oil is already dissolved on the sc-CO_2 (solvent) and enters directly to the reactor to produce biodiesel, without the need for further expensive pumping.

In Figure 1.2.4, the transesterification is a three-step consecutive reaction, in which diglycerides and monoglycerides are formed as an intermediate compounds and triglycerides are consumed until no more TG exists in the reaction to convert into FAME.
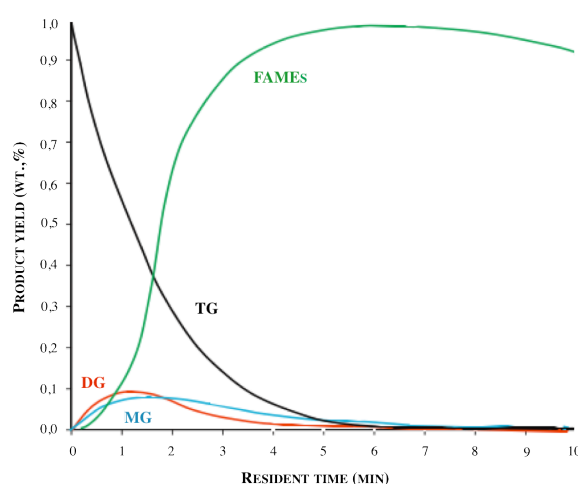
Figure 1.7.1 – Typical temporal profiles of monoglyceride (MG – blue line), diglyceride (DG – red line), triglyceride (TG – black line) and fatty acid methyl ester (FAME – green line) in supercritical transesterification (adapted from [104]).

As written before (1.2.2 Conversion of triglyceride to Biodiesel: Transesterification reaction), the transesterification reaction consists in: 1 mol of triglyceride reacts with 3 mols of alcohol in order to produce 1 mol of glycerol and 3 moles of fatty acid alkyl esters. The alcohol that is used on this work is the methanol. As it can be seen on the Figure 2.2.6, methanol is introduced on the system, by a liquid pump, at a constant flow rate and, additionally, the CO_2 is recycled. It's necessary to guarantee that there aren't accumulation of methanol in the solvent (CO_2) because it can cause problems in the reaction, such as: (***i***) realization of the experiment with determinated conditions and, effectively, the proportion of oil:methanol isn't the expected and, consequently, (***ii***) can lead to a denaturation of the enzyme and decreased the reaction yield. To avoid this situation, after the separators (i.e. before the recycled CO_2 enters again in the system), the amount of methanol must be measured. Therefore, a sample is collected from the system (in this part), the CO_2 is measured by a flow meter and the methanol is recovered in a trap with liquid nitrogen and acetone ($\pm$ 263,15K). The methanol solubility in carbon dioxide at 313,15K and 56,313 atm (57,1 bar) is 0,8%. In the experiments performed, there wasn't significant quantity of methanol in the recycled solvent in which the maximum of methanol achivied was 0,3% dissolved in the CO_2.

1.8. ONE-POT BIODIESEL PRODUCTION: OIL EXTRACTION AND TRANSESTERIFICATION REACTION IN ONE SINGLE STEP PROCESS WITH SUPERCRITICAL TECHNOLOGY

Biodiesel can be also made in one single step process and this is possible by taking advantage of the characteristics of supercritical fluids – the ability to be used as an extractaction agent and reaction solvent.
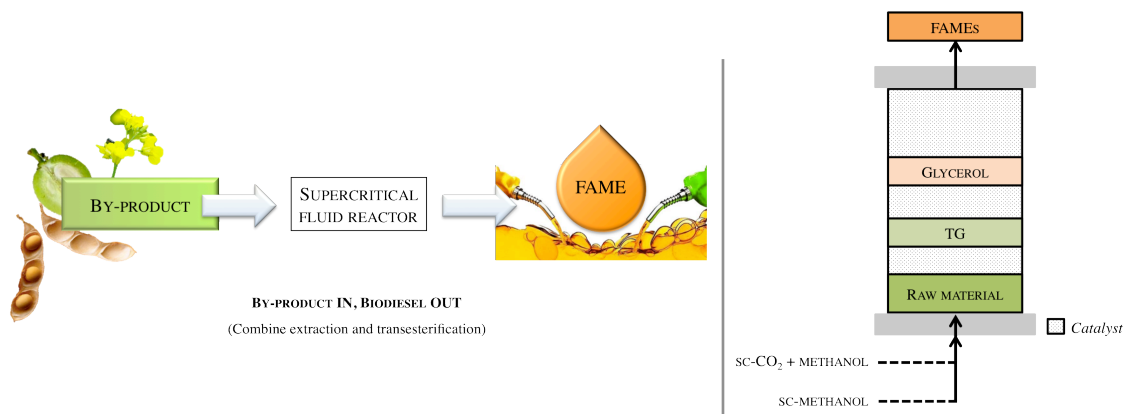
Figure 1.8.1 – One-pot biodiesel production, oil extraction and transesterification reaction in one single step.

In one-pot biodiesel production occurs two different processes in a packed bed reactor, filled with raw material (in which contains oil in it's constitution): oil extraction and the transesterification reaction of that oil into FAME (Figure 1.8.1). Several studies have been done with this technique, using algae [105] or spent cofee ground [106] as raw material, and methanol and carbon dioxide as solvents. To perform this system it is necessary to know the phase behaviour and reactivity of each of the system components. This approach can be done using (*i*) only sc-MeOH, (*ii*) using sc-MeOH and CO_2 as co-solvent [107], or (*iii*) using sc-CO_2/MeOH and a catalyst. sc-MeOH technique has the advantage that could act as an agent of TG extraction and simultaneous direct transesterification reaction solvent and reactant, which means that does not require any addition of catalyst. Non-catalytic process can be superior to catalytic reaction in terms of reaction time and yield [108] and it reduces the total cost of biodiesel production. However, the supercritical conditions of methanol are high (512,60 K and 8,09 MPa) and, consequently, it demands a further energy consuption when compared with sc-CO_2. Moreover, methanol, in supercritical conditions, doesn't required pretreatment of the feedstock since the impurities don't affect the reaction (the same when using sc-CO_2). As mentioned before, the presence of FFA and water influences the transesterification reaction. Effectively, three reactions occurs simultaneously: transesterification, alkyl esterification of FA and hydrolysis of TG (inverted reaction of

esterification). Once that the alkyl esterification is a faster reaction than transesterification, it ensures that all FFAs (present in the feedstock, or formed in TG hydrolysis) are completely transformed into FAME [109]. However, using sc-MeOH, the molar ratio between oil:methanol is higher and, consequently, the methanol must have be removed from the system.

The addition of a co-solvent in a sc-MeOH system can decrease the critical point of methanol, allowing the supercritical reaction to be carried out under milder conditions. Carbon dioxide is a good solvent, increasing the homogeneity of the systems and do not affect the reaction mechanism. Therefore, comparing with the conventional supercritical methanol method, less energy is required for the process because the reaction temperature required is significantly reduced and, consequently, the process is safer and the production costs is lower [100], [110]. However, the reaction conditions are substantively higher when compared with the process used in this work, which reflects in a lower production cost.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. RAW MATERIAL, CHEMICALS AND COMPOUNDS

Chicken feather meal was supplied by Sr. João Silva, from Lusiaves (Industrial Zone of Lavos – Figueira da Foz). Animal feed, from Avenal Rações – Fluffy Manutenção, was purchased and used.

Carbon dioxide (CO_2, MW = 44,01 g/mol) with purity higher then 95% were used and supplied from Air Liquid (Portugal), as well as liquid nitrogen (N_2, MW = 28,01 g/mol).

The enzyme used for transesterification reaction was the Lipozyme® RM IM (1,3 specific lipase from *Rhizomucor miehei* fungus, immobilized on ion exchange resin) purchases from Novozymes A/S, Bagsvaerd, Denmark.

Methanol for transesterification reaction and Bligh and Dyer method (CH_3OH, MW = 33,04 g/mol, PA grade, Sigma-Aldrich), n-heptane for chromatography ($CH_3(CH_2)_5CH_3$, MW = 100,20 g/mol, 99% pure, Carlo Erba Reagents), n-hexane for chromatography and extraction experiments ($CH_3(CH_2)_5CH_3$, MW = 86,18 g/mol, Carlo Erba Reagents), tricaprin ($C_{33}H_{62}O_6$, MW = 554,84 g/mol, $\geq$ 98% pure, TCl), pyridine (C_5H_5N, MW = 79,10 g/mol, $\geq$99% pure, Carlo Erba), N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) ($C_6H_{12}F_3NOSi$, MW= 119,25 g/mol, 97% pure, Alfa Aesar), chloroform ($CHCl_3$, MW = 119,38 g/mol, $\geq$ 99% pure, Carlo Erba Reagents), ethylene glycol ($C_2H_6O_2$, MW = 62,07 g/mol, purity NA, Merck) and acetone (C_3H_6O, MW = 58,08 g/mol, Valente e Ribeiro Lda.®) were purchased and used.

2.1.2. EXPERIMENTAL SETUP

2.1.2.1. OIL EXTRACTION

For oil extraction, carbon dioxide (gaseous CO_2) was cooled by cryostat (JP Selecta, s.a.) and then it's (liquid CO_2) pumped by a liquid pump (Nikkiso) with a determinate mass flow. The liquid CO_2 passes through a vessel and is measured with a flow meter (Rheonik, 01.08). After, the solvent is heated by a heat exchanger (heating from a water bath (Julabo ED) to a desired temperature (supercritical CO_2). Then, Sc-CO_2 enters into the extractor (height = 60 cm, inner diameter = 5,5 cm) that is heated by a heating tape (Horst, HBS 723,15K/623,15K). The

pressure is controlled by a Back Pressure Regulator – BPR (Tescom Europe) and afterward, the products are collected in a cyclone (Separadex 4140/CY01 AS2), also heated by a heating tape (Horst, HBS 723,15K/623,15K). The CO_2 is separated from the product by a pressure drop (returning to a gas-phase), and then the solvent is recovered for another set, and is passage is controlled by an electro-pneumatic positioner (ABB Automation Products GmbH, Process Automation, TZIDC). Before pumping, CO_2 is first cooled.

2.1.2.2. CONTINUOUS PRODUCTION OF BIODIESEL AT PILOT SCALE

For continuous production of biodiesel at pilot scale, CO_2 passes through a flow meter (Rheonik, RHM 01 GNT) and then is cooled by a water spiral-cooling vessel. All the cooling system on the pilot plant is guaranteed by a recirculating cooler (Julabo FL 2503) that chilled the water to a desired value and, by vessels, cooled the CO_2 where is needed to be refrigerated. After, the CO_2 is pump by a liquid pump (Lewa EHM 1) with a desired flow rate, which is measured by a flow meter (Rheonik, 01.08). The liquid CO_2 is then heated by a water bath (Lauda) to a desired temperature. Afterward, the $Sc\text{-}CO_2$ enters into an extractor vessel (316SS with internal diameter of 6,4 cm and 59,6 cm of height) that's is completely packed with raw material (± 500g of chicken feather meal between two metallic porous plates and an amount of cotton to avoid undesired entrainment effect) where the extraction occurred. Before the extraction, it was additionally pumped methanol, for reaction occurs, by a HPLC pump (Gilson 305). In this pilot plant there are 4 extraction vessels that could be used in series, in a continuous extraction, however this experiment wasn't performed. All the extraction vessels were heat by heating tapes (Horst). Besides these 4 extractions vessels, there exist another one with the double of the capacity that is used only for storage the CO_2. It help's the decompression/compression procedures and avoid CO_2 waste. The pressure in the extractors was measured with a pressure transducer (Wika, model 881.14.600).

After the oil extraction, the mixture (CO_2 and extracted oil) passes through a vessel directly to the reactor (with internal diameter of 2,5 cm and 60 cm of height), heated by a heating jacket (Horst), filled with the enzyme (the enzyme was packed between an amount of cotton to avoid undesired entrainment, and in the top of the reactor it was put also a metallic porous plate for the same reason). The pressure in the reactor was measured with a pressure transducer (Wika) and it was controlled by an electro-pneumatic control valve (von Rohr Armaturen AG, VEGP 700 F59). After the reaction, the mixture was separated with 3 cyclones (Separadex 4140/CY01 AS2) connected in series, all heated by water baths (Lauda) through a heating jacket. In the middle of the first and the other two cyclones, there exists a BPR (Tescom Europe) that can control the pressure in the first cyclone, for separations

experiments. The products were collected and CO_2 was recycled for another cycle, but first it is filtered (Stainless steel high pressure filters, Parker) and then, it was cooled before pumped with the liquid pump.

For security advises, the pilot plant was provided with several rupture discs (HIP – High Pressure Equipment Company) to avoid overpressurization or potentially damaging vacuum conditions of the vessels. Moreover, all the high-pressure vessels, valves and fitting are from HIP and Swagelok.

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2.2. EXPERIMENTAL SET-UP

Figure 2.2.1 – Figure 2.2.4 are photography's of the high-pressure installations, used in this work, for oil extraction and continuous production of biodiesel.

2.2.1. HIGH-PRESSURE INSTALLATION FOR OIL EXTRACTION

LEGEND
A) Cryostat and water bath
B) Liquid pump
C) CO_2
D) *i* – Extractor, *ii* – BPR, *iii* – Cyclone
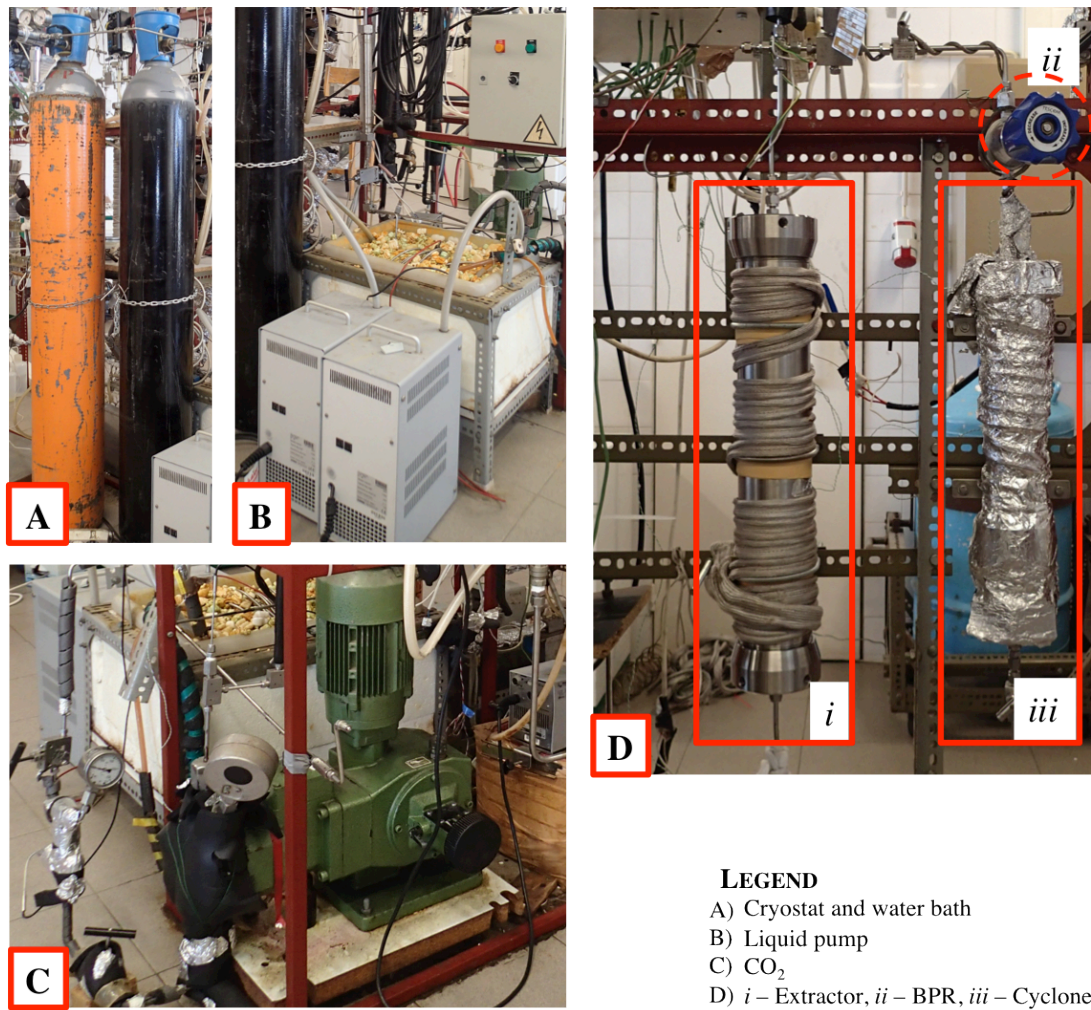
Figure 2.2.1 – High-pressure installation used for oil extraction.

2.2.2. HIGH-PRESSURE INSTALLATION FOR CONTINOUS PRODUCTION OF BIODIESEL

Figure 2.2.2 – High-pressure pilot installation used for continuous biodiesel production.
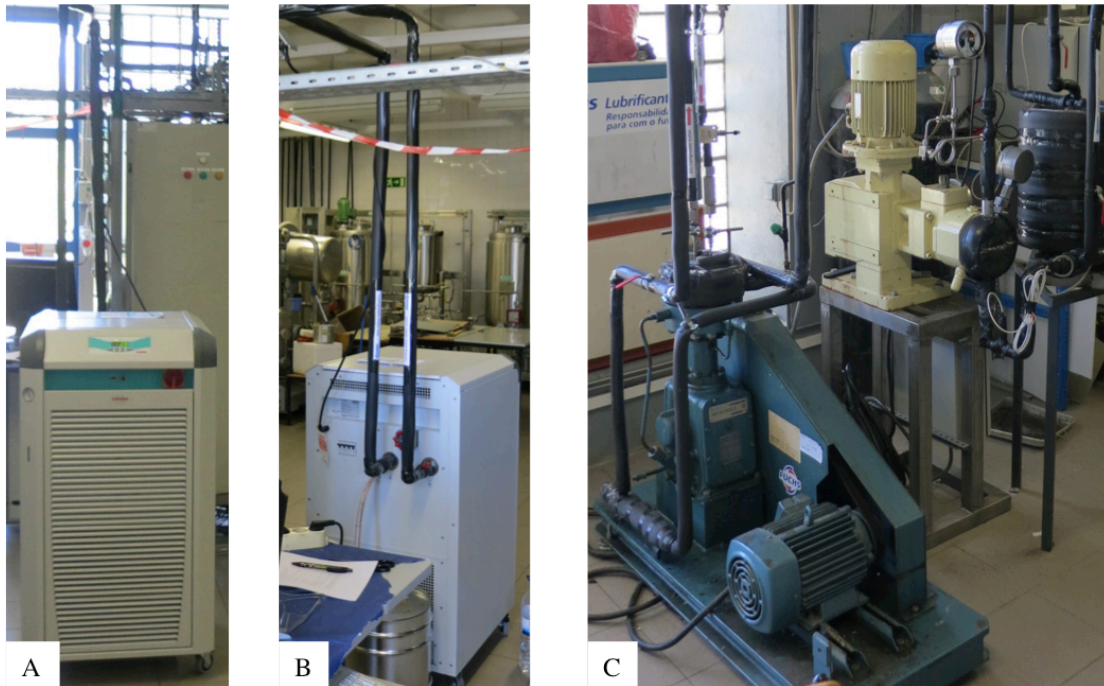
Figure 2.2.3 – Detail image of pilot high-pressure pilot installation. A, B – Recirculating cooler; C – Compressor (green) and liquid pump (yellow).
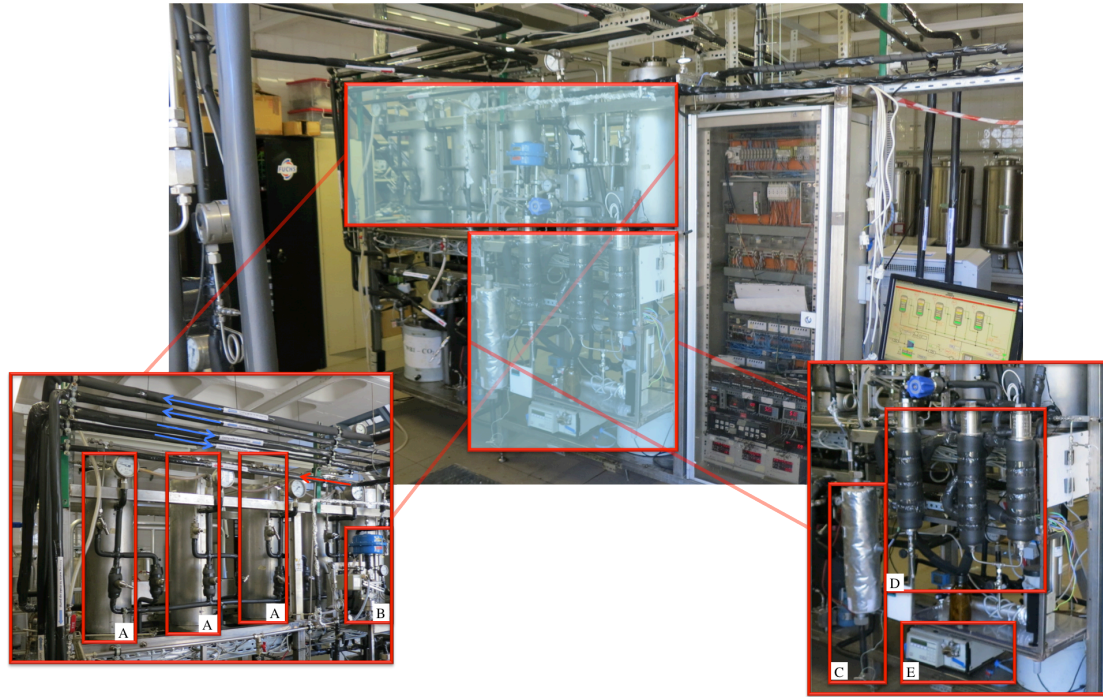
Figure 2.2.4 – Detail image of pilot high-pressure pilot installation: A – Extractors, B – Electro-pneumatic control valve, C – Reactor, D – Cyclones, E – Liquid pump (for methanol).

The flow sheets of the installations are illustrated on Figure 2.2.5 and Figure 2.2.6.
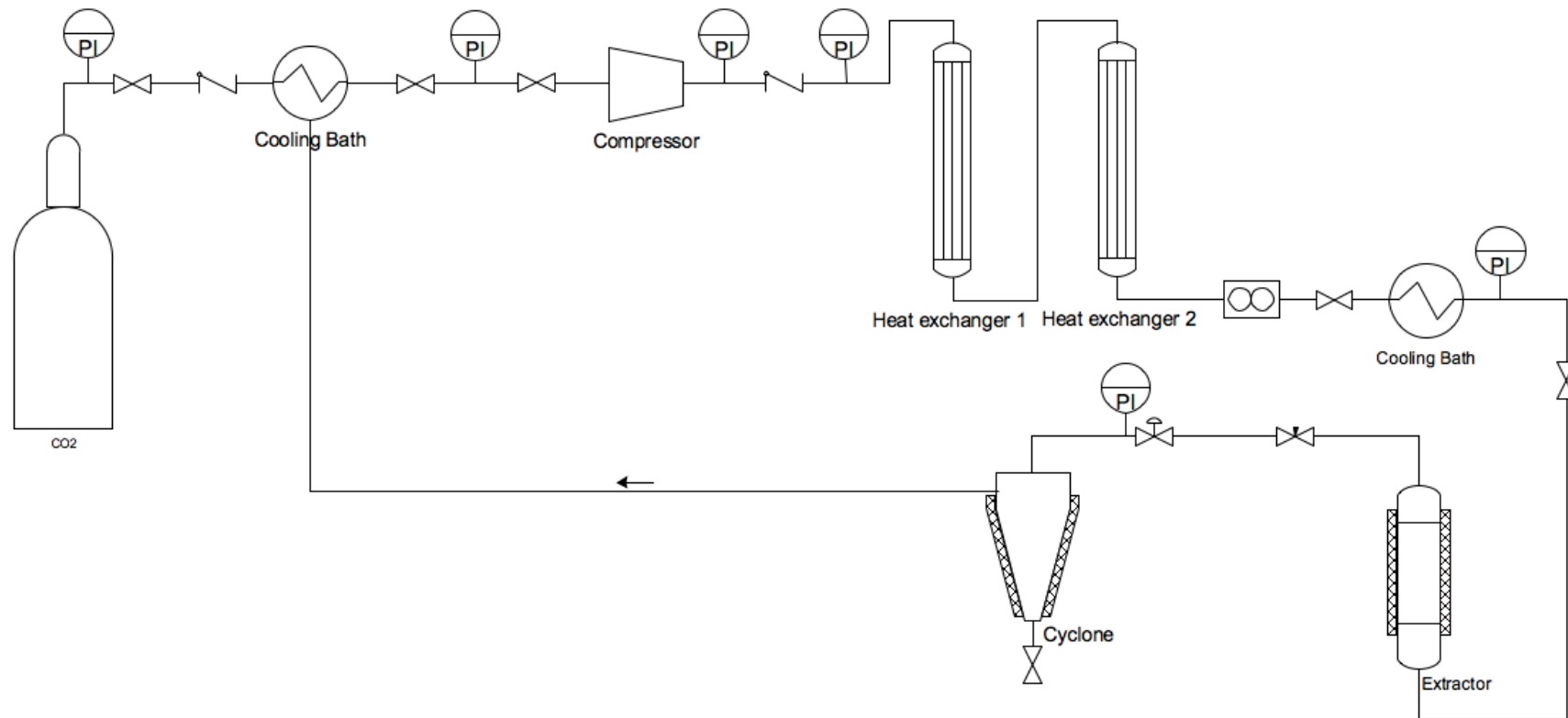
Figure 2.2.5 – Scheme of the high-pressure apparatus for oil extraction with a solvent recycling system.
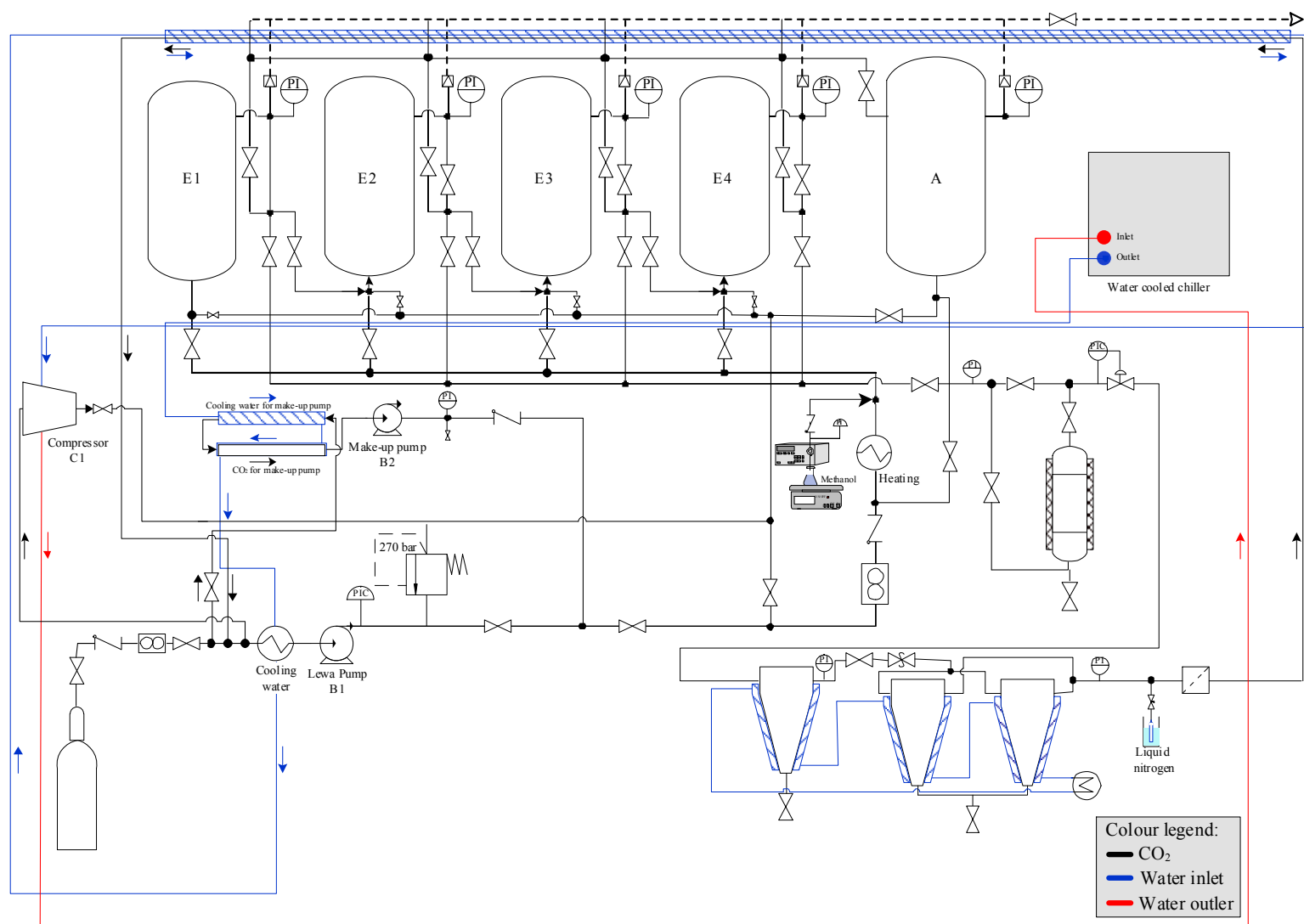
Figure 2.2.6 – Scheme of the high-pressure apparatus for continuous biodiesel production with a solvent recycling system.

2.3. SAMPLE ANALYSIS

2.3.1. SOXHLET EXTRACTION

The practice of solid-liquid extraction is dating from, approximately, 3500 BC, in the preparation of teas and perfumes [111]. The soxhlet extraction is an extraction method that uses organic solvents. Has it can be seen on Figure 2.3.1, this apparatus has 3 compartments: a continuously heat round-bottom flask – to store the extracting organic solvent (*n*-hexane), the Soxhlet extractor – in which a packet of residue is inside and where the steam (from the solvent) goes through and is recover by a cooled reflux condenser. Subsequently, the vapour is condensed and channelled into the Soxhlet extractor again. Once the organic solvent (liquid) in the extractor reaches the overflow level, a siphon unloads the organic solvent-lipids mixture back into the round-bottom flask. This cycle is repeated for 6 hours, to guarantee that no more lipids can be extracted from the residue. Although this apparatus is good for solid-liquid extraction, is requires high energy [94].
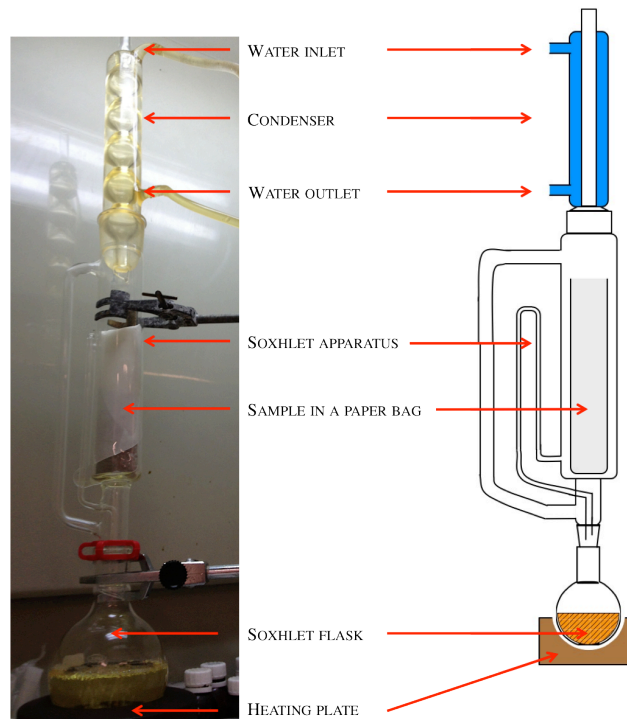
Figure 2.3.1 – Soxhlet apparatus.

2.3.2. BLIGH AND DYER METHOD (OIL EXTRACTION)

The *Bligh and Dyer* modified method (1959), was used to perform lipid extraction from chicken feather meal. In this method are also used organic solvents, methanol and chloroform. Firstly, 1g of residue is added to 100ml of methanol:chloroform:water (10:5:4) solution and then is submitted to a magnetic stirring, for at least 4 hours, or until complete lipid extraction. Subsequently, the solvents mixture with lipids is submitted to centrifugation for ±10 min, and then, the liquid phase is removed, (which corresponds the chloroform phase containing total lipids – fatty acids and sterols) and is placed into a separatory funnel. This step is repeated until the liquid phase has no colour. Afterward, the volume is corrected: per 100ml of MeOH:CHCl$_3$:H$_2$O (10:5:4) is added 60ml of MeOH:CHCl$_3$ (1:1). Then, the separatory funnel is shake gently and let stand for at least 3 hours, or until two phases are completely formed. Finally, the lower phase (containing lipids) is collected and filtered to a soxhlet flask and, subsequently, the organic solvent is evaporated on a rotavapour.

2.3.3. DIRECT TRANSESTERIFICATION – LEPAGE & ROY METHOD

To analyse fatty acids contents of fat existent in the extracted oil (from chicken feather meal), was used the direct transesterification, derived by *Lepage* and *Roy* (1984), resulting the formation of methyl esters. Thus, 10-25 mg of oil extracts were transmethylated with 2 mL of methanol:acetyl chloride (95:5 v/v) and 0,2 mL of heptadecanoic acid (*Sigma-Aldrich*) in hexane (*Carlo Erba*), (5 mg/mL), used as internal standard. The mixture was well sealed, without any contact with light, and heated (at 353,15-358,15 K) for 1 hour. After this step, the vail was cooled at room temperature and then was diluted with 2 mL of *n*-heptane and 2 mL of water, to help the phase separation. Afterward, the heptadecanoic phase (the upper one) was transferred to a cotton filter bed with an anhydrous sodium sulphate (*Fluka*) and filtered, collecting the sample. Before the analysis with gas chromatography (GC), the sample was evaporated and then was added 1 mL of *n*-heptane.

2.3.4. GAS CHROMATOGRAPHY

Triglycerides (TG), diglycerides (DG), monoglycerides (MG), glycerol (Gly) and fatty acid methyl esters (FAME) were quantitatively determined by gas chromatography (GC) with on-Column injection – Thermo Scientific Trace GC Ultra (Figure 2.3.2), equipped with a flame

ionization detector (FID), with a ZB-5HT Inferno column, 10 m x 0,32 mm, 0,10 μm film thickness and polarity of 8 from Zebron. The column is filled with 5%-phenyl and 95%-dimethylpolysiloxane in the stationary phase (non-polar).
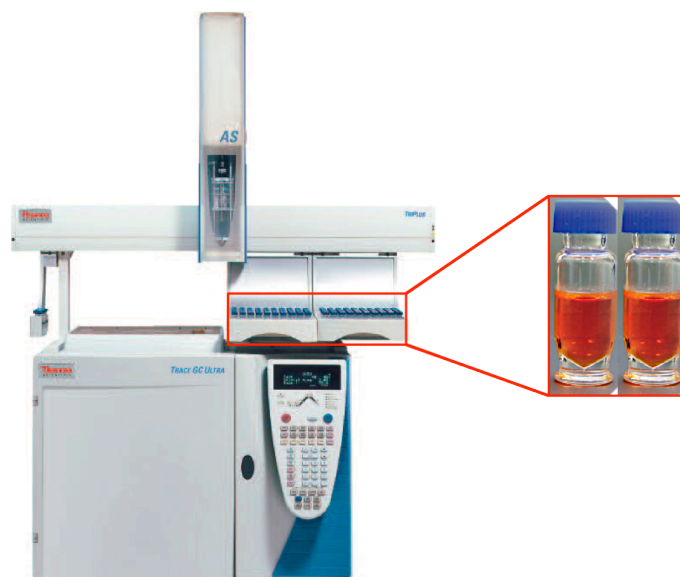
Figure 2.3.2 – Gas chromatograph on-column Thermo Scientific Trace GC Ultra and two vials, used in this analytical method.

Gas chromatography is an analytical method used for separating and analysing compounds passing through a capillary column by the flow of inert gas (mobile phase) – helium (He), Argon (Ar) or Nitrogen (N_2). The column contains a stationary liquid phase absorbed to the surface in an inert solid where the sample to analyse is retained. The compounds can be separated by their affinity (polarity) with the stationary phase or by its boiling points. With an on-column injection the sample is directly introduced in the interior of the column with a syringe, without depressurize it. On this work, all compounds (TG, DG, MG, Gly and FAME) are separated according to the boiling points and unsaturated compounds are eluted before the corresponding saturated acids of the same chain length.

The method used was the EN 14105 modified, exhibited on Table 2.3.1.

Table 2.3.1 – Selected instrument and modified EN 14105 method for the Trace GC Ultra and TriPlus AS.

TRACE GC ULTRA	
INJECTOR	True cold on-column
CARRIER GAS	Helium, 1 ml min⁻¹
FID	653,15 K
OVEN PROGRAM	353,15 K (1 min) to 453,15 K at 288,15 K min⁻¹, then to 503,15 K at 280,15 K min⁻¹, then to 638,15 K (4 min) at 283,15 K min⁻¹
TRIPLUS AUTOSAMPLER	
SYRINGE SIZE	10 μl with 80 mm needle
INJECTED VOLUME	1 μl

Peak identification was carried out using known standards and the Chrom-Card software.

2.3.4.1.   EXTRACTED OIL ANALYSIS

To analyse the fatty acid content in the extracted oil, it was necessary to perform Lepage & Roy method, explained above (2.3.3 Direct transesterification – Lepage & Roy Method). The typical chromatogram obtained is showed in Figure 2.3.3. The molar concentrations of fatty acids were correlated with their corresponding peak areas using linearization parameters of calibration curves previously done. The relationship between concentrations and peak area is showed on Equation 2.3.1, and angular/linear coefficient of each fatty acid is exhibited on Table 2.3.2.

Equation 2.3.1 – Relationship between sample concentration and peak area.
       (A = area; IS = internal standard, heptadecanoic acid)

$$\frac{A_{FA}}{A_{IS}} = \alpha \frac{[FA]}{[IS]} + \beta$$

Table 2.3.2 – Linearization parameters of calibration curves to correlate concentrations and GC peaks areas of fatty acids.

FATTY ACID		ANGULAR COEFFICIENT (α)	LINEAR COEFFICIENT (β)
LAURIC	C12	1,0364	0,0033
MYRISTIC	C14:0	0,9868	0,0027
PALMITIC	C16:0	1,0260	-0,0004
PALMITOLEIC	C16:1	1,0655	0,0011
STEARIC	C18:0	0,7020	-0,0033
OLEIC	C18:1	0,9419	0,0014
LINOLEIC	C18:2	1,1519	0,0010
LINOLENIC	C18:3	1,4692	0,0005
EICOSANOIC	C20:0	1,0570	-0,0025
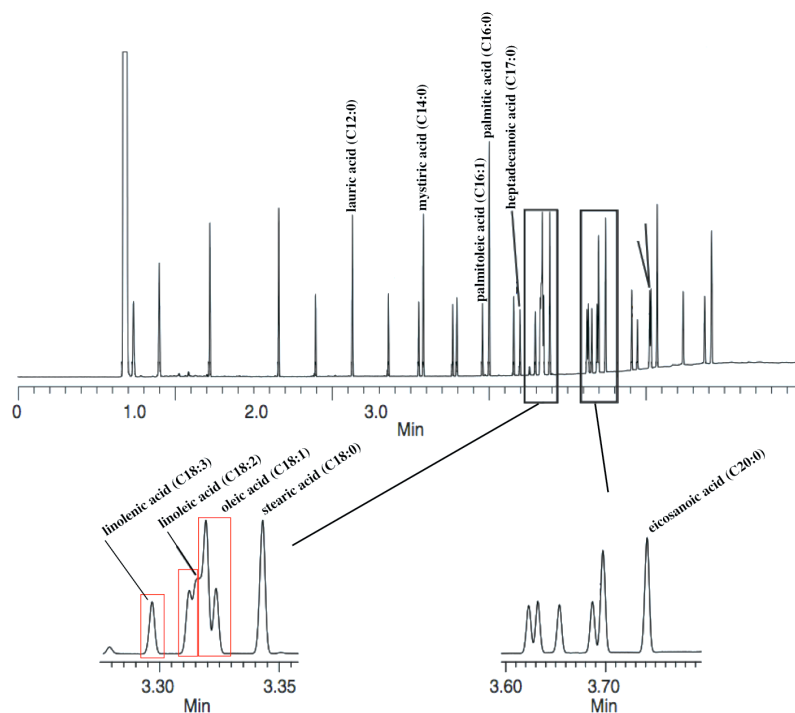
Figure 2.3.3 – Typical chromatogram obtained by GC for oil analysis.

2.3.4.2.   SAMPLES OBTAINED IN TRANSESTERIFICATION REACTION

To determinate the molar concentration of free fatty acids, as well as glycerine and FAME (products of transesterification reaction), calibration curves were previously done. Thus, two internal standards are required: 1,2,4-butanetriol (IS1) for glycerine, and tricaprin (IS2) for mono-, di- and triglycerides. Four reference compounds were also needed – glycerine, mono-olein, dio-olein and trio-olein. Once that glycerine and mono- and diglycerides are polar and high boiling components, they have to be derivatized to increase their volatility and reduce activity before injection into GC. The method used requires derivatization with MSTFA (N-methyl-N-trimethylsilylsilyltrifluoroacetamide) in pyridine, which transforms theses compounds into more volatile silylated derivatives. Therefore, for each 100 mg homogenized sample, 80 μl of IS1 (1 mg/ml), 100 μl of IS2 (8 mg/ml) and 100 μl of MSTFA were added to a 10 ml vial, which was hermetically sealed and shaken vigorously. After 15 minutes, 8 ml of *n*-heptane were added. For analysis, 1 μl of the reaction mixture was automatically injected into the GC, following the instrumental conditions showed on Table 2.3.1. Peak identification and measurements were carried out using Chrom-Card software.

The relationships between concentrations and peak area are showed on Equation 2.3.2 and Equation 2.3.3, and angular/linear coefficient of each compound is exhibited on Table 2.3.3. The typical chromatogram obtained is showed in Figure 2.3.4.

Equation 2.3.2 – Relationship between FAME (mono-, di- and triglycerides) concentration and peak area.
(A = area; IS2 = internal standard 2, tricapin)

$$\frac{A_{TG,DG,or\ MG}}{A_{IS2}} = \alpha \frac{[TG, DG\ or\ MG]}{[IS2]} + \beta$$

Equation 2.3.3 – Relationship between glycerine concentration and peak area.
(A = area; IS1 = internal standard 1, 1,2,4-butanetriol)

$$\frac{A_{Gly}}{A_{IS1}} = \alpha \frac{[Gly]}{[IS1]} + \beta$$

Table 2.3.3 – Linearization parameters of calibration curves to correlate concentrations and GC peaks areas of methyl esters and glycerol.

COMPOUND	ANGULAR COEFFICIENT (α)	LINEAR COEFFICIENT (β)
MONOLEIN	0,4048	0,0249
DIOLEIN	1,1002	-0,0145
MONOLEI	1,3830	-0,0154
GLYCEROL	1,1225	0,0105
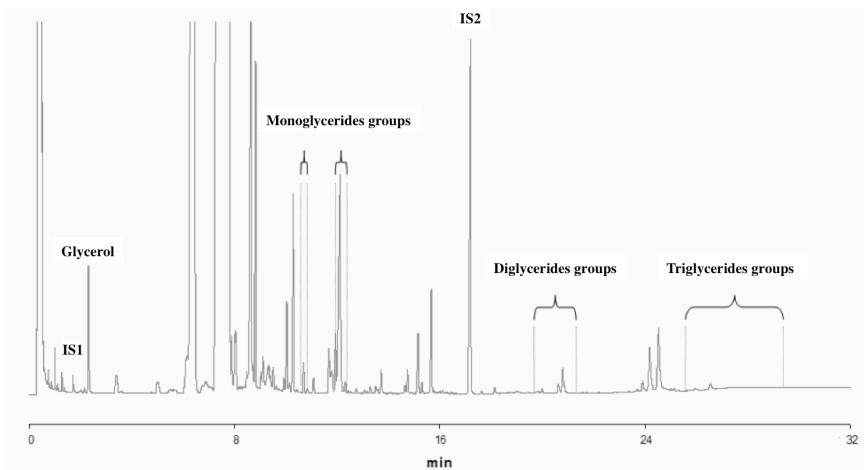
Figure 2.3.4 – Typical chromatogram obtained by GC for FAME analysis.

For calculate FAME yield, using molar concentrations of TG, DG and MG was used the Equation 2.3.4.

Equation 2.3.4 – FAME yield equation ($[TG]_i$ is the triglyceride concentration in the reaction sample if there had been no reaction, i.e. the initial value. [TG], [DG] and [MG] are the final concentrations of tri-, di- and monoglycerides in the reaction sample, respectively).

$$\eta = \frac{[TG]_i \times 3 - ([TG] \times 3 + [DG] \times 2 + [MG] \times 1)}{[TG]_i \times 3}$$

RESULTS AND DISCUSSION

3. RESULTS AND DISCUSSION

3.1. OIL EXTRACTION EXPERIMENTS WITH SC-CO_2

Oil extraction was first determined by two different extractions methods, using organic solvents: Soxhlet extraction and Bligh and Dyer method.

Curves of cumulated oil extraction yield (in %, $g_{oil}/g_{total\ of\ CFM}$) versus extraction time, in the conditions applied (temperature, pressure and flow rate) are shown on Figure 3.1.2 – Figure 3.1.14.

In all these graphics, it is possible to distinguish two different behaviours, which reflect two different extraction mechanics. Effectively, the first linear curve demonstrates a faster extraction due to the removal of oil that is readily available at the solid surface. In this phase the extraction kinetics is governed by the equilibrium solubility of the oil in CO_2 and by the external mass transfer effect. The second region of the graphics shows a decreasing extraction rate due to the oil removal from deeper in the particles of the residue. The effect of internal diffusion – internal mass transfer resistance, controls this phase of extraction kinetics.

3.1.1. OIL EXTRACTION FROM ANIMAL FEED

The main objective of this work is the production of biodiesel from chicken feather meal. However, it was previously extracted oil from animal feed (AF) because chicken feather meal wasn't available yet, and the nutrient compositions are both similar, Table 3.1.1. Once the AF had been purchased in square pieces, it had to be ground with the aid of a coffee grinder (Krups F203 fast-touch coffee grinder).

Supercritical fluid oil extraction (SCFOE) from AF was carried out in a pilot scale apparatus, Figure 2.2.1/Figure 2.2.5. The extraction conditions are illustrated on Table 3.1.2.

Extraction experiments were carry out with one extractor filled with ± 500g of grounded AF and samples were collected every 10 minutes, until complete extraction. The studied parameters were pressure and temperature, at a constant CO_2 flow rate of 150 g/min.

Effectively, it was perform a method to evaluate the quantity of oil present in AF, soxhlet extraction. From this procedure, it was extracted 6,55 ± 0,07% of oil and, in this work, we'll assume this value as the maximum of oil that could be extracted from AF. The extraction

efficiency was determined from the cumulative mass of the extracted fat divided by total amount of fat that present in animal feed (Equation 3.1.1), and range from 49,3% to 158,8% (g_{oil}/g_{feed}), (Table 3.2.1).

Equation 3.1.1 – Extraction efficiency equation.

$$Extraction\ efficiency = \frac{m_{cumulative\ mass\ ext.oil}}{m_{feed} \times m_{max.oil\ (Soxhlet\ ext.)}} \times 100$$

Table 3.1.1 - Nutrient composition of animal feed and chicken feather meal ([112]).

ITEM	ANIMAL FEED	CHICKEN FEATHER MEAL	REF.
Crude protein, %	27 – 30	75 - 85	[63–65]
Fat, %	9 – 15	7 - 12	[21], [22], [60]
Calcium, %	1,3 – 2	0,3	
Phosphorus, %	1,3	0,5	

The water content was measured by Karl Fischer method[11] (KF) (831 and 756 Coulometric Karl Fischer titrator, Metrohm USA Inc. ®). The humidity of dry and normal AF (not dry) is 3,7 and 9,4% of water content, respectively.

Besides the presence of water in AF, unsaponifiable matter in oil is also significant. Unsaponifiable matter includes those substances (higher aliphatic alcohols, sterols, pigments and hydrocarbons) frequently found dissolved in oils, which cannot be saponified by the conventional treatment, but are soluble in ordinary oil solvents. To analysed the unsaponifiable matter present on the extracted oil, the AOCS Official Method Ca 6b-53 (reapproved in 2009) was used, and it was obtained 3,63% of unsaponifiable matter.

Table 3.1.2 – Conditions applied on oil extraction (temperature and pressure) from AF, at 150 g_{CO2}/min, exhibiting the extractions rate (g_{oil}/min) and it efficiency (%).

TEMPERATURE (K)	PRESSURE (bar)	EXTRACTION RATE (g_{oil}/min)	EXTRACTION EFFICIENCY (%)
313,15	200	1,39	124,6
	250	2,19	126,1
	300	1,94	$100,8 \pm 30,5$[12]
323,15	250	1,66	105,8
333,15		1,87	126,2
338,15	200	1,08	135,4

[11] A Karl Fischer (KF) titration determines the water content in a sample, based on an iodine/iodide redox reaction. It's a titration method where water reacts with iodine until the water is consumed and the endpoint is reached. This determination of water content with KF is advantageous over to a determination based on weigh loss, because KF is not affected by volatile compounds.
[12] At 300 bar, 313,15K and 150 g_{CO2}/min was perform a two experiments.

	250	2,00	158,8
	300	0,97	$52,9 \pm 5,1$[13]
343,15	250	1,96	122,4

In Figure 3.1.1 is shown the extraction efficiencies, with increased pressure, at different temperatures.

Firstly, and as it was mentioned before, the maximum oil that could be extracted from the residue is $6,55 \pm 0,07\%$ and, in the experiments – Figure 3.1.2 and Figure 3.1.3, it was obtained higher extraction yields ($\geq 100\%$). Effectively, it was extracted oil in a range of extraction efficiency $56,5\% – 158,8\%$, which indicates that it was extracted more than lipids (oil). In fact, the presence of unsaponifiable matter ($3,63\%$) and the water content ($3,7\%_{dry\ AF}/9,4\%_{normal\ AF}$), do not account for all the extra extractable material. However, the homogeneity of AF square pieces can be reason for these values, i.e. the composition of the squares can be different, which could justify the extraction efficiencies values obtained.
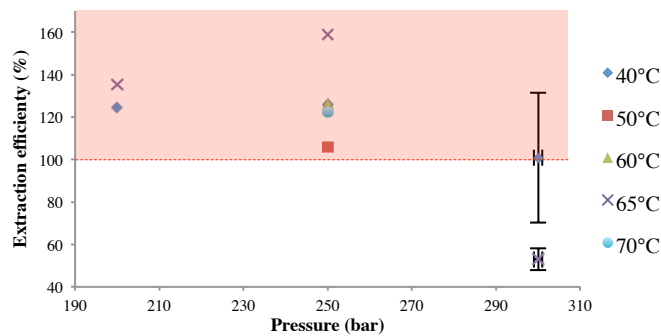
Figure 3.1.1 – Extraction efficiencies, obtained on oil extraction from AF, at 323,15 – 343,15 K.
(The orange rectangle is illustrating the experiments that obtained extraction efficiencies above the 100%.)

3.1.1.1. ISOTHERMAL KINETIC EXPERIMENTS

The pressure is one of the properties that can alter the thermodynamic properties of supercritical fluids. In this chapter (3.1.1. Oil extraction from animal feed) is presenting the experiments made, of oil extraction from AF, investigating the effect of changing the pressure at a constant temperature.

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[13] At 300 bar, 338,15K and 150 g_{CO2}/min was perform a two experiments.

Isothermal kinetic experiments of oil extraction are exhibited on Figure 3.1.2 and Figure 3.1.3, and its extraction rates are illustrated on Table 3.1.2. At constant temperature of 313,15 and 338,15 K, the pressures studied were 200, 250 and 300 bar. The results of pressure effect on oil extraction are not consistent, resulting in yields higher than 100%. As it can be seen on Table 3.1.3, at a constant temperature, the increase of pressure results in an increase of CO_2 density, which means that the solvation power of oil also increases. So, the solvent capacity to solubilize the lipids, from de AF, is higher at higher pressures. This phenomenon reflects in an increasing of oil extraction. However, it wasn't verified in the extractions of oil, from AF.

Table 3.1.3 – Density of Sc-CO_2 (kg/m^3) at 313,15 and 338,15K, changing the pressure.

TEMPERATURE (K)	PRESSURE (bar)	DENSITY (kg/m^3)
313,15	200	839,81
	250	879,49
	300	909,89
338,15	200	691,71
	250	761,94
	300	808,95

In Figure 3.1.2 is shown the kinetic extraction at 313,15 K, at different the pressure. At constant temperature, it is expected to obtain higher extractions rates at higher pressures (300 bar). However, the higher extraction rate obtained was at 250 bar (2,19 g_{oil}/min), which is not coherent and, so, the kinetic curve behaviour was unpredictable.
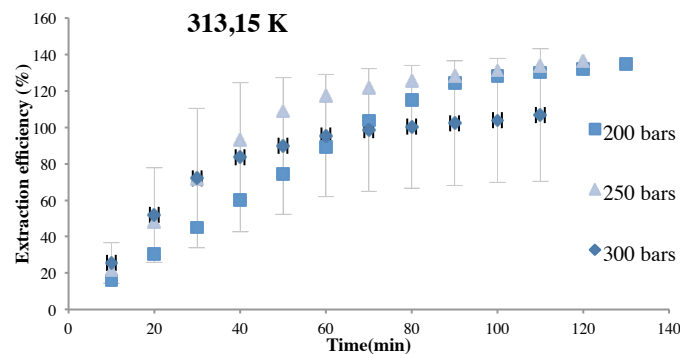
Figure 3.1.2 - Isothermal (313,15K) kinetic curve of oil extraction from AF from a single extractor, using sc-CO_2 as solvent, with a CO_2 flow rate 150 g/min, at 200/250/300 bar.

Increasing temperature to 338,15 K (Figure 3.1.3), the kinetic curves were also unpredictable. The experiment that acquires higher extraction rate was at 250 bar (2,00 g_{oil}/min), followed by 200 (1,08 g_{oil}/min) and then 300 bar (0,973 g_{oil}/min). In accordance with supercritical fluids properties, at a constant temperature, the increase of pressure lead to an increase of solvation

power of the solvent, promoting the oil extraction. However, in the experiments that were made, at 313,15 and 338,15K, is not found this increase, in which should be evident.
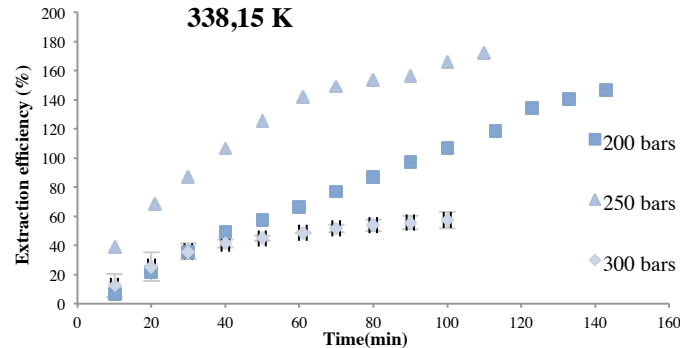
Figure 3.1.3 - Isothermal (338,15K) kinetic curve of oil extraction from AF from a single extractor, using sc-CO$_2$ as solvent, with a CO$_2$ flow rate 150 g/min, at 200/250/300 bar.

Therefore, from the results obtained, it can be concluded that the isothermal kinetic experiments were not coherent at both temperatures.

Although, increasing the temperature in 25K, it was found a decrease in the extraction rates at all the pressure studied. This behaviour can be explained by the possible presence of volatile compounds that evaporate at higher temperatures. In addition, increasing the temperature can also promote degradation of some compounds in the mixture, reflected in the extraction rate.

Consequently, more experiments should be done to understand the kinetic curve behaviour.

3.1.1.2.   ISOBARIC KINETIC EXPERIMENTS

As mentioned before, changing the pressure and temperature can easily alter the thermodynamic properties of supercritical fluids. In the previously subchapter was demonstrated the effect of the pressure in the extraction rate. Now, it will be analysed the effect of the temperature, at a constant pressure.

The kinetics curves of isobaric experiments are showed on Figure 3.1.4, and extraction rates and extraction efficiencies are illustrated on Table 3.1.2. The temperatures studied (at constant pressure – 250 bar) were 313,15/323,15/333,15/338,15 and 343,15 K.

The effect of varying the temperature, at a constant pressure, could reflects into two different effects: (*i*) if on one hand, increasing temperature, the vapour pressure of the solute also increases, which results on an increase of solvation power of oil, (*ii*) on the other hand,

increasing temperature, the solvent density (CO_2) decreases, which leads to a decrease of solvation power.

Analysing Figure 3.1.4, increasing the temperature from 323,15 to 343,15K, it's possible to note an increase in the extraction rate (1,66 to 1,97 g_{oil}/min), which means that the vapour pressure effect prevails at these conditions. Though, at 313,15K the extraction rate is higher (2,19 g_{oil}/min), which indicates that the other effect (CO_2 density) is present at this temperature, i.e. at lower temperatures, the solvent density increase, leading to an increase of solvation power of oil.

However, the extraction rate at 338,15K (2,00 g_{oil}/min) is not coherent with the other conditions, which can be an experimental error.
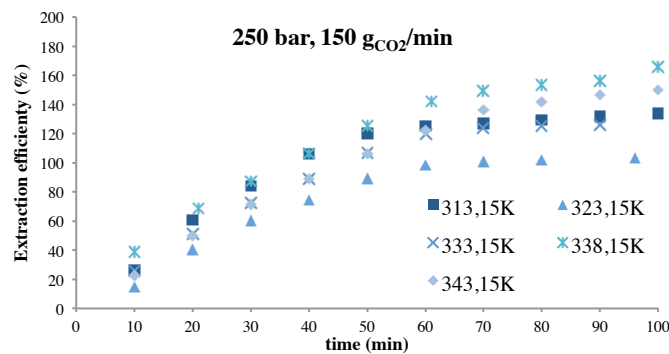
Figure 3.1.4 - Isobaric (250 bar) kinetic curve of oil extraction from AF from a single extractor, using sc-CO_2 as solvent, with a CO_2 flow rate 150 g/min, at 313,15/323,15/333,15/343,15 K.

Considering isothermal and isobaric experiments, more experiments should be done to understand the behaviour of the kinetic curves obtained with the oil extraction from AF.

3.1.1.1.   FATTY ACID COMPOSITION OF EXTRACTED OIL

Analysis of FA content of extracted oil from AF, with hexane and sc-CO_2, was conducted using GC. A total of 7 fatty acids were identified on soxhlet extraction and 9 fatty acids with sc-CO_2, both methods extracted high proportion of unsaturated fats than saturated, as observed in Figure 3.1.5 and Table 3.1.4. The fatty acids were present in similar proportions to the oil extracted with hexane and with sc-CO_2, which indicate that all the fatty acids extracted with hexane are also soluble in sc-CO_2. In general, the extracted oil is mainly constitute by palmitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2).
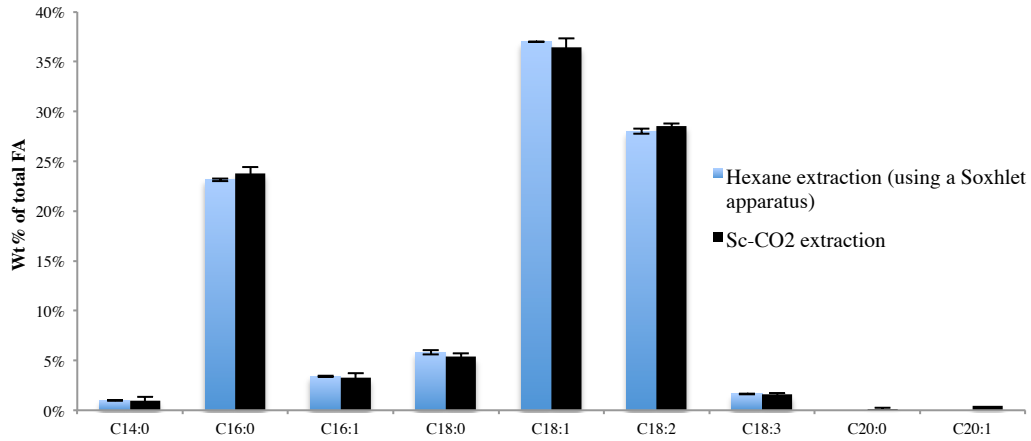
Figure 3.1.5 – Fatty acid content of extracted oil from animal feed, with hexane and sc-CO_2 extraction.

Table 3.1.4 – Fatty acid content on extracted oil, from animal feed.

FATTY ACID	WT.,%	
	SOXHLET EXTRACTION	SC-CO_2 EXTRACTION
$C_{14:0}$	$0,99 \pm 0,04\%$	$0,97 \pm 0,37\%$
$C_{16:0}$	$23,14 \pm 0,13\%$	$23,77 \pm 0,67\%$
$C_{16:1}$	$3,40 \pm 0,06\%$	$3,29 \pm 0,44\%$
$C_{18:0}$	$5,81 \pm 0,21\%$	$5,38 \pm 0,34\%$
$C_{18:1}$	$36,98 \pm 0,01\%$	$36,41 \pm 0,90\%$
$C_{18:2}$	$28,03 \pm 0,26\%$	$28,53 \pm 0,26\%$
$C_{18:3}$	$1,64 \pm 0,04\%$	$1,57 \pm 0,16\%$
$C_{20:0}$	$0 \pm 0,00\%$	$0,11 \pm 0,16\%$
$C_{20:1}$	$0 \pm 0,00\%$	$0,45 \pm 0,00\%$

3.1.2. OIL EXTRACTION FROM CHICKEN FEATHER MEAL

As mentioned before (1.3.1. Poultry industry and rendering), chicken feather meal offers a promising feedstock source for biodiesel production. In the last 10 years, the chicken meat is increasingly embedded in the diet of the consumers. Not just because the preference due to the price/quality ratio, but also because of the awareness of healthy eating has also increased during this period. Thus, due to this high consume of chicken meat, feather meal (non-edible sub-product, Figure 1.3.4) became a by-product of poultry industry. Hence, it is biodegradable and it has oil in its constitution, CFM becomes a good feedstock for biodiesel production.

SCFOE from chicken feather meal (CFM) was carried out in a pilot scale apparatus, Figure 2.2.2/Figure 2.2.6. The extraction conditions are illustrated on Table 3.1.5.

Table 3.1.5 - Conditions applied on oil extraction (temperature, pressure and CO_2 flow rate) from CFM, exhibiting the extractions rate (g_{oil}/min) and it efficiency (%).

TEMPERATURE (K)	PRESSURE (bar)	EXTRACTION RATE (g_{oil}/min)	MAXIMUM EXTRACTION YIELD (%)[14]	EXTRACTION YIELD t = 30 MIN (%)[15]	CO_2 FLOW RATE (g/min)
308,15	200	0,65	91,3	57,29	
313,15	200	0,68	88,7	55,98	
	250	0,96	94,6	79,27	
	300	1,09	96,2	86,90	150
338,15	200	0,31	96,9	34,80	
	250	0,85	98,9	67,02	
	300	0,97	93,8	87,71	
313,15	300	1,21	91,1	81,38	200
		0,89	91,7	78,36	100
		0,83	94,2	64,71	75

Extraction experiments were carry out with one extractor filled with ± 500g of CFM and samples were collected every 10 min, until complete extraction. In Figure 3.1.6 is demonstrated the extracted oil in these experiments.

The objective of this chapter is the optimization of oil extraction from CFM. Therefore, three parameters are considering: temperature, pressure and CO_2 flow rate. Effectively, the quantity of oil present in AF was evaluated by two different methods to, (*i*) soxhlet extraction and (*ii*) Bligh and Dyer method. From these procedures, it was extracted 7,14% and 7,3% of oil, respectively, and, in this work, we will assume these values as the maximum of oil that could be extracted from CFM. The extraction efficiency was determined from the cumulative mass

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[14] Extraction efficiency obtained with complete oil extraction from CFM.
[15] Extraction efficiency obtained at 30 min of oil extraction from CFM.

of the extracted oil divided by total amount lipids fat that present in animal feed (Equation 3.1.1), and range from 89% to 99% (Table 3.1.5).

The water content was also measured by KF (831 and 756 Coulometric Karl Fischer titrator, Metrohm USA Inc.®) and the humidity of dry and normal CFM (not dry) is 2,1 and 7,3%, respectively.

Additionally, the unsaponifiable matter of CFM was also analysed (AOCS Official Method Ca 6b-53 (reapproved in 2009)) and it has 1%, which is in accordance with the literature [21], [63].

Figure 3.1.6 – Extracted oil with supercritical CO_2

In Figure 3.1.7 is shown the extraction efficiencies, obtained on oil extraction from CFM, with increased pressure, at different temperatures.
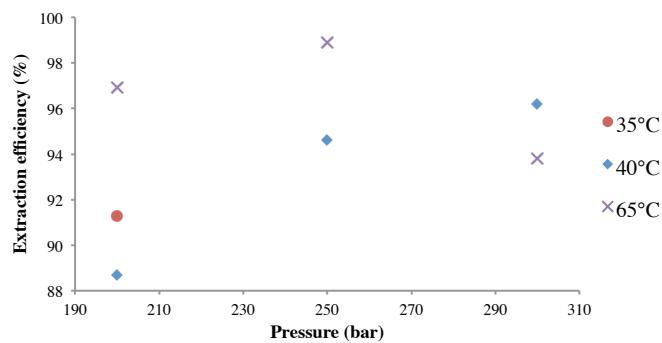
Figure 3.1.7 – Extraction efficiencies, obtained on oil extraction from CFM, at 308,15 – 338,15 K.

The oil extraction from CFM, using sc-CO_2 as an extracting agent, was successfully achieved. Effectively, as it was mentioned before, the maximum of oil that could be extracted from the residue is 7,14% and, in all the extractions performed, the extraction efficiencies were high, ranging from 89 to 99% (g_{oil}/g_{feed}).

3.1.2.1.   ISOTHERMAL KINETIC EXPERIMENTS

Isothermal kinetic experiments of oil extraction are exhibited on Figure 3.1.8 and Figure 3.1.9, and it extraction rates are illustrated on Table 3.1.5.

At constant temperature to 313,15K, Figure 3.1.8, and with CO_2 flow rate of 150 g/min, the pressures studied were 200, 250 and 300 bar. The result of the pressure effect on the oil extraction suggests that the efficiency increased with pressure increasing, which is in accordance with the literature [96], [113], [114]. This is due to the increase of sc-CO_2 density and, consequently, the solvation power of oil increases, which means that the solvent capacity to solubilize the lipids, from the CFM, is higher. Effectively, the CO_2 density at 200 bar and 313,15K, is 839,8 kg/m³ and at 300 bar is 909,9 kg/m³. Therefore, in the first 30 minutes of extraction, the extraction rate at 200 bar is 0,68 g_{oil}/min, which is lower comparing with higher pressure. At 300 bar it can be extracted, in the first 30 minutes of experiment, considerably more oil per minute, 1,09 g_{oil}/min.

Increasing the temperature to 338,15K, (Figure 3.1.9) at constant pressure, the kinetic behaviour was the same. Increasing the pressure, the extraction rate is higher. However, comparing the extraction rates at 313,15 and 338,15K, at the same pressure, at higher temperature, lower extraction rates are obtained. The CO_2 density is showed on Table 3.1.3 and, analysing these values at these temperatures, the density is higher at 313,15K (i.e. at 200 bar, the CO_2 density at 313,15K is 839,8 kg/m³; at 338,15K is 691,7 kg/m³, lower than at 313,15K). Moreover, at higher temperatures is required more time for complete extraction. At 338,15K and 200 bar, it needs a longer time to achieved complete extraction, 160 minutes, while at higher pressures, the extraction is complete at 70 minutes.
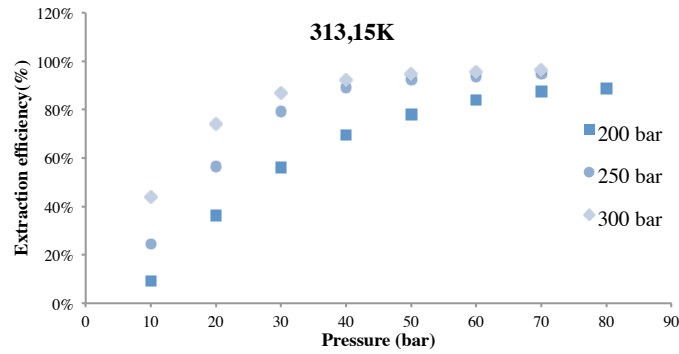
Figure 3.1.8 – Isothermal (313,15K) kinetic curve of oil extraction from CFM from a single extractor, using sc-CO_2 as solvent, with a CO_2 flow rate 150 g/min, at 200/250/300 bar.
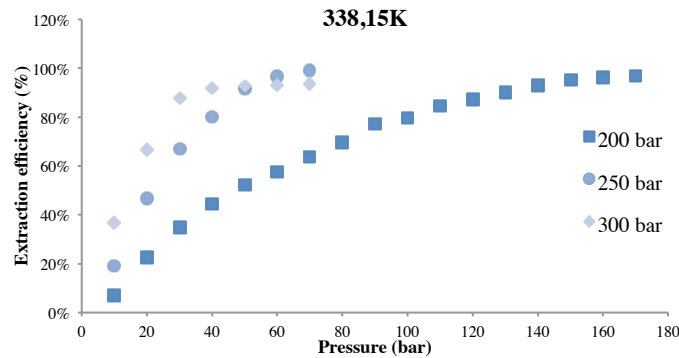
Figure 3.1.9 – Isothermal (338,15K) kinetic curve of oil extraction from CFM from a single extractor, using sc-CO_2 as solvent, with a CO_2 flow rate 150 g/min, at 200/250/300 bar.

3.1.2.2.   ISOBARIC KINETIC EXPERIMENTS

Considering isobaric kinetic experiments of oil extraction, results are exhibited on Figure 3.1.10, Figure 3.1.11 and Figure 3.1.12 , and extraction rates are illustrated on Table 3.1.5. At constant pressure of 200, 250 and 300 bar and with CO_2 flow rate of 150 g/min, the studying temperatures were 313,15 and 338,15K. Besides CO_2 density (which is higher at lower temperatures), increasing temperature the solvation capacity decreases. However, at constant pressure, decreasing the temperature, another effect could be identified, the vapour pressure of solutes decrease, which leads to a lower solubility of oil in supercritical fluids caused by the volatility effect [115]. Therefore, at the pressure conditions studied, the first effect prevails, i.e. the density effect is more pronounced than the volatility, enhancing the extraction efficiency with decreasing temperature. This phenomenon is called retrogradation and is typical in regions of supercritical fluids. This effect does not occur in all extractions experiments. Actually, at a constant pressure, if the extraction efficiency increases with

temperature, means that the volatility effect prevails [114]. It was also studied the extraction of oil from CFM at lower temperature – 308,15K (Figure 3.1.12), and the behaviour was similar to the 313,15K experiment, at 200 bar (150 g_{CO2}/min), because the CO_2 density is alike, 929,11 kg/m^3 (20 kg/m^3 higher than at 313,15K).
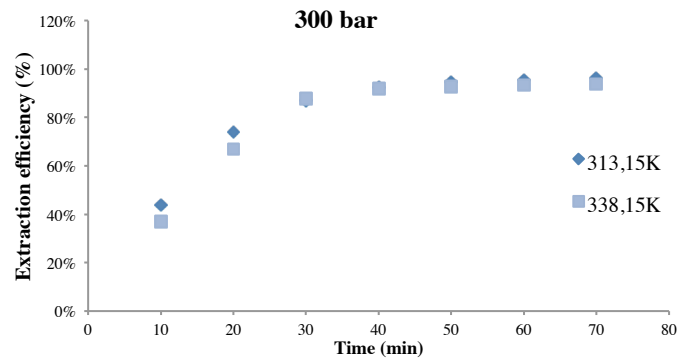
Figure 3.1.10 – Isobaric (300 bar) kinetic curve of oil extraction from CFM from a single extractor, using sc-CO_2 as solvent, with a CO_2 flow rate 150 g/min, at 313,15 and 338,15K.
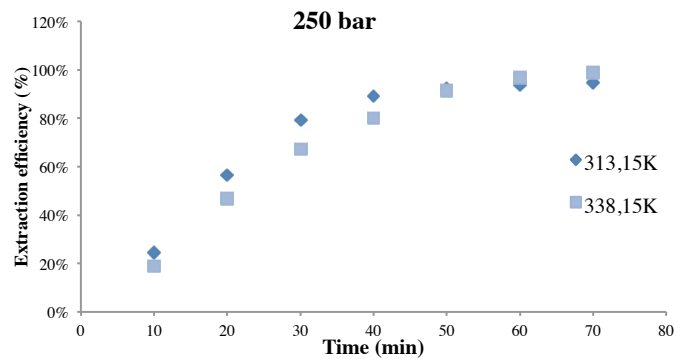
Figure 3.1.11 – Isobaric (250 bar) kinetic curve of oil extraction from CFM from a single extractor, using sc-CO_2 as solvent, with a CO_2 flow rate 150 g/min, at 313,15 and 338,15K.
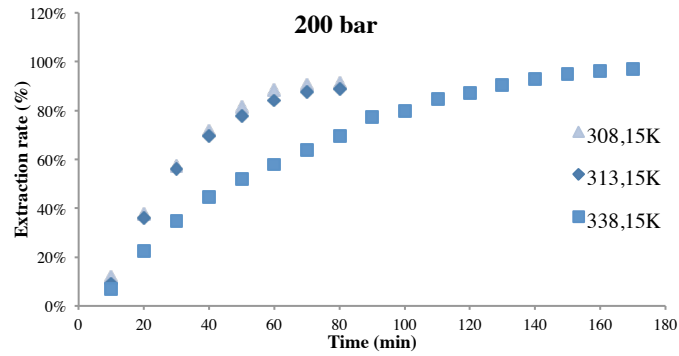
Figure 3.1.12 – Isobaric (200 bar) kinetic curve of oil extraction from CFM from a single extractor, using sc-CO_2 as solvent, with a CO_2 flow rate 150 g/min, at 308,15, 313,15 and 338,15K.

Considering these results, it can be concluded that the best conditions for oil extraction from CFM at a constant CO_2 flow rate of 150 g/min, are at lower temperature and higher pressures, which means: 308,15 or 313,15K and 300 bar.

In Figure 3.1.13 is shown the effect of solubility with an increase of pressure at difference temperatures. As mentioned before, in the experiments made, the density effect prevails. However, at a constant pressure of 300 bar, in both temperatures the extraction rates are similar. This is the crossover point where the density effect no longer prevails, and the volatility effect starts to reveal.
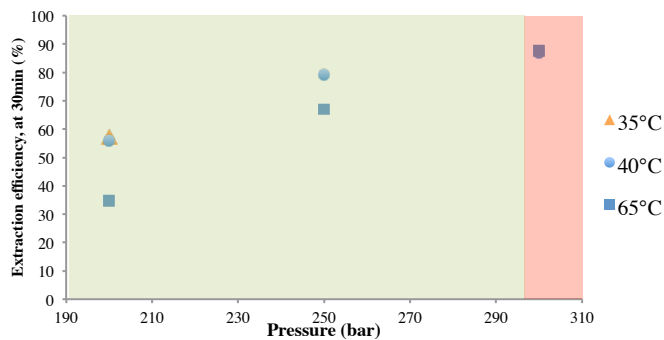
Figure 3.1.13 – Extraction efficiency (%), at 30 minutes of extraction, varying the pressure at different temperatures (308,15 – 338,15K).
        The green rectangle indicates the effect of density effect on the oil extraction. The orange rectangle indicates the crossover point, where the density effect no longer prevails, and the effect of volatility starts to reveal.

3.1.2.3. CO$_2$ FLOW RATE INFLUENCE

The CO$_2$ flow rate was also studied. To maximize the rate of oil extraction, the solvent flow rate must be enough to be completely diffusion limited. Therefore, the optimum flow rate is in the region where both diffusion and solubility are significant factors. Analysing Figure 3.1.14, which refers to extraction conditions at 300 bar and 313,15K, varying the CO$_2$ flow rate – 75, 100, 150 and 200 g$_{CO2}$/min, experiments at higher flow rate seems to have superior extraction efficiency. However, to guarantee this results, it was traced a graphic that represents the oil loading, Figure 3.1.15. At 200 g$_{CO2}$/min flow rate, the residence time is lower than the other CO$_2$ flow rates studied and, consequently, the solvent saturates very quickly. Because of this CO$_2$ effect, the 200 g$_{CO2}$/min curve in Figure 3.1.15 is inferior than 75 g$_{CO2}$/min. At this lower flow rate, the residence time is higher, which leads to an oil loading substantially greater and that's why this curve has a higher slope. However, the important factor in oil extraction is to obtain the highest quantity of oil in a very short time. The loading of oil is an important factor, though, if the extractions at 313,15K/300bar/200-150g$_{CO2}$min^{-1} were comparing with 313,15K/300bar/100-75g$_{CO2}$min^{-1}, the experiments that had the highest extraction rate are at 200 (1,21 g$_{oil}$/min) and 150 g$_{CO2}$/min (1,09 g$_{oil}$/min) of flow rate.
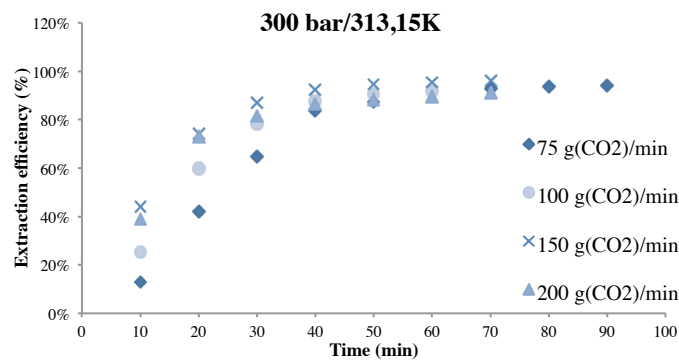
Figure 3.1.14 – Kinetic curve of oil extraction from CFM from a single extractor, using sc-CO$_2$ as solvent, at 300 bar and 313,15K, changing CO$_2$ flow rate: 75/100/200 g$_{CO2}$/min.
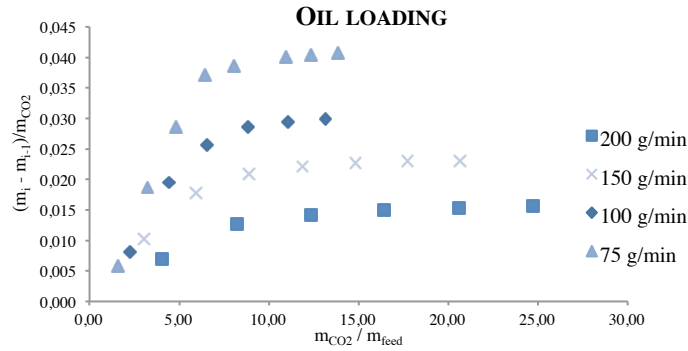
Figure 3.1.15 – Loading of oil in the extraction experiments (m_i = oil mass at time i; m_{i-1} = oil mass at time i-1), examining three different CO_2 flow rate: 75,100 and 200 g/min.

Considering what was mentioned before, Figure 3.1.16 illustrates the extraction efficiency (%), at 30 minutes of extraction, (conditions fixed: 300 bar and 313,15K) with increasing of CO_2 flow rate. It seems, at a first instance, that at 150 g_{CO2}/min is the best flow rate for the extraction because it has higher extraction efficiency at 30 minutes, 86,90%.
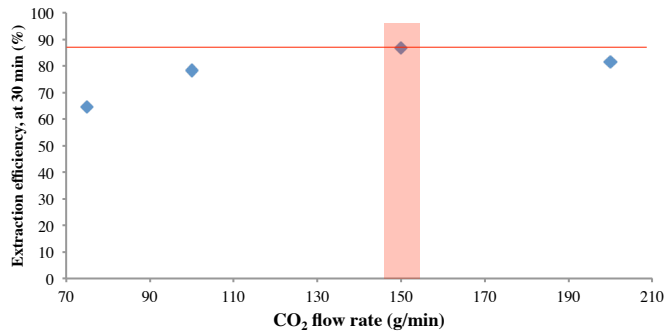
Figure 3.1.16 – Extraction efficiency (%) obtained at 300 bar and 313,15K, varying the CO_2 flow rate (g/min).

However, it's necessary to take into account the amount of CO_2 needed to extract a certain amount of oil. Thus, Table 3.1.6 shows the mass of the extracted oil at 30 and 40 minutes (30' and 40', respectively), as well as the S/F ($m_{solvent}/m_{feed,CFM}$). Considering the quantity of oil extracted at 30' and 40', and taking into account the quantity of CO_2 needed to the extraction, the best solvent flow rates are 75, 100 and 150 g_{CO2}/min. Effectively, at 40', the higher and lower flow rates extracted almost the same quantity of oil. At a S/F (Table 3.1.6 and Figure 3.1.17) of ± 9 at 30', 31,6g of oil extracted is achieved while at 40', only 29,5g of oil are extracted. Therefore, it seems that the extra 10' do not improve the extraction process. Regarding CO_2 flow rate at 30', and although at 150 g_{CO2}/min the extraction efficiency is higher, the energy demand must be taken into account. One must evaluate if the difference in the extracted oil, at higher flow rates, compensates the higher energy required.

Table 3.1.6 – Influence of solvent flow rate on oil extraction from CFM, at a constant extraction pressure (300bar) and at 313,15K.

$S/F = m_{solvent,CO_2}/m_{feed, CFM}$;
$m_{extracted \, oil}$ = mass of oil extracted;
30' = 30 minutes; 40' = 40 minutes.

Q (g_{CO_2}/min)	$m_{extracted \, oil}$ (30')	S/F (30')	$m_{extracted \, oil}$ (40')	S/F (40')
75 g_{CO_2}/min	22,91	4,84	29,70	6,44
100 g_{CO_2}/min	26,34	6,56	29,50	**8,83**
150 g_{CO_2}/min	31,55	**8,86**	33,49	11,88
200 g_{CO_2}/min	28,61	12,33	30,36	16,45
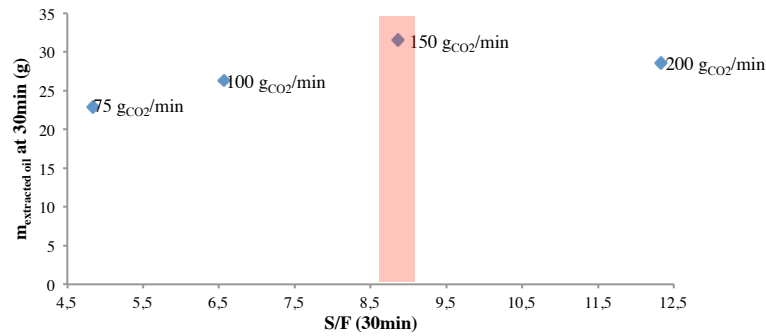
Figure 3.1.17 – Amount of extracted oil, at 30 minutes, varying the S/F.
$S/F = m_{solvent,CO_2}/m_{feed, CFM}$

3.1.2.4. FATTY ACID COMPOSITION OF EXTRACTED OIL

Analysis of the fatty acid fractions of the extracted oil, from CFM, with hexane and sc-CO_2 extraction was conducted using GC (Appendix - Table 6.4.1). A total of 9 fatty acids were identified on hexane extraction, and 8 on sc-CO_2 extraction. Effectively, soxhlet extraction takes, at least, 6 hours to guarantee that no more lipids can be extracted from the residue and, as it can be seen on Figure 3.1.18 and Table 3.1.7, the fatty acid profile of the extracted oil, with both extractions, has similar proportions, which indicate that all FA were extracted with sc-CO_2 (i.e. FA are also similarly soluble on sc-CO_2).
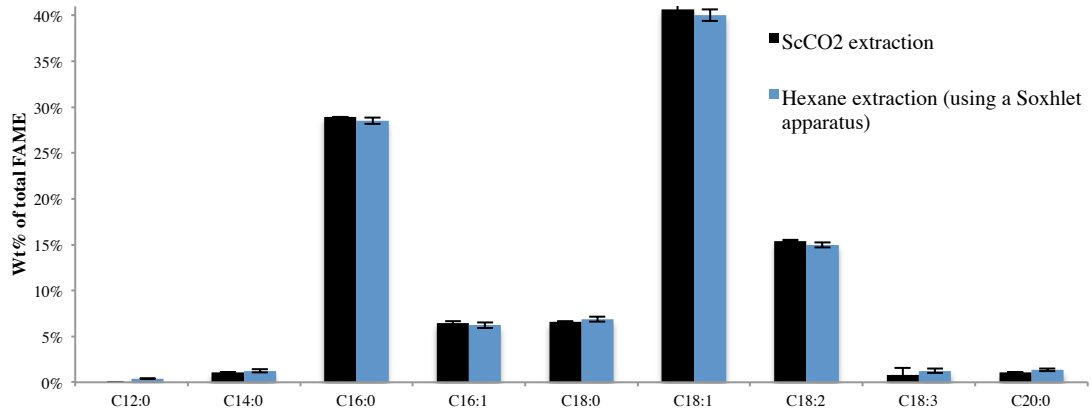
Figure 3.1.18 – Average of fatty acid content of extracted oil from chicken feather meal, with hexane and sc-CO_2 extraction.

Table 3.1.7 – Average of fatty acid content on extracted oil, from chicken feather meal, with soxhlet and sc-CO_2 extraction (the shaded column shows literature values [116]).

FATTY ACID	WT.,%		
	SOXHLET EXTRACTION	SC-CO_2 EXTRACTION	
		$WT_{AVERAGE}$%	$WT_{LITERATURE}$%
$C_{12:0}$	0,40 ± 0,00%	0,00 ± 0,00%	11,40%
$C_{14:0}$	1,23 ± 0,01%	1,06 ± 0,17%	0,00%
$C_{16:0}$	28,51 ± 0,04%	28,92 ± 0,33%	22,98%
$C_{16:1}$	6,22 ± 0,21%	6,47 ± 0,30%	5,24%
$C_{18:0}$	6,90 ± 0,05%	6,60 ± 0,29%	6,56%
$C_{18:1}$	40,03 ± 0,85%	40,65 ± 0,65%	34,71%
$C_{18:2}$	14,98 ± 0,09%	15,41 ± 0,27%	17,31%
$C_{18:3}$	1,25 ± 0,80%	0,77 ± 0,22%	0,00%
$C_{20:0}$	1,37 ± 0,00%	1,08 ± 0,16%	0,00%

3.2. CONTINUOUS PRODUCTION OF BIODIESEL

3.2.1. TRANSESTERIFICATION REACTION STUDY

In the previously section was analysed the best conditions for oil extraction from CFM. The main objective of this work is the production of biodiesel from the oil extracted of CFM. Hence, to perform a continuous production of biodiesel, the system is based into two steps: first it's perform the oil extraction from CFM and then, the same oil is used for the transesterification reaction to produce biodiesel. In this chapter, it will be analysed the best conditions for transesterification reaction.

The continuous production of biodiesel was carried out in a pilot scale apparatus, Figure 2.2.2 - Figure 2.2.4 and Figure 2.2.6. The oil extraction was carry out with one extractor filled with ± 500g of CFM, followed by the reactor filled with 84 g of Lipozyme® RM IM. The oil extracted by sc-CO_2 is reacted in the CO_2 stream as it passes through the enzymatic reactor. Samples were collected every 10 min, until complete extraction (i.e. until no more oil can be converted into biodiesel, which takes 1 hour of each experiment). In Figure 3.2.1 is illustrated one sample obtained from biodiesel production.

Figure 3.2.1 – Biodiesel produced in this work, from the extracted oil.

The transesterification conditions are illustrated on Table 3.2.1. All reaction experiments were executed under 313,15 K and 250 bar. The temperature chosen was 313,15K because the extration of oil offers better results, and because of the enzyme stability [76]. In respect to pressure value, it was mentioned before that the extraction of oil has better results at 300 bar. However, for safety reasons, the experiments were carried out at 250 bar.

Table 3.2.1 – Conditions applied on transesterification reaction (CO_2 flow rate and oil:methanol ratio) and FAME yield(%).

CO₂ FLOW RATE (g/min)	FAME$_{AVERAGE}$ (%)	OIL:MeOH RATIO
30	98,79 ± 0,01%	
50	96,98 ± 0,06%	
75	94,66 ± 1,19%	1:24
100	97,06 ± 0,05%	
150	96,72 ± 0,73%	
75	98,54 ± 0,49%	1:12
	97,68 ± 0,63%	1:6
150	91,69 ± 3,11%	1:12

As mentioned before, there are several factors that influene the enzymatic biodiesel synthesis: oil sorce, reaction temperature, methanol to oil molar ratio, amount of water in the system and the type of lipase.

The water presence is not a problem in this study because the CFM does not have enough water to influence it. Relatively to the choice of the catalyst, the use of Lipozyme® RM IM as been reported to be the best enzyme for transesterification reaction [117], as well has the optimum reaction temperature, 313,15K [76]. For biodiesel production, it's necessary a presence of an alcohol, and in this work its use the methanol since it's the least expensive alcohol. However, the presence of excess alcohol could lead to an enzyme inactivation. Therefore, the molar ratio between methanol:oil will be studied. Moreover, the residence time of the solvent will be take in consideration.

3.2.1.1.   RESIDENCE TIME OPTIMIZATION

The residence time (t_r) consists in the average amount of time that a particle spends in the reactor. It begins from the moment that a particle of a particular substance enters the system, and ends the moment that the same particle of that substance leaves the system. In these experiments, this measurement varies directly with the CO_2 flow rate, i.e. the quantity of solvent that passes through the reactor. Therefore, the reactor was loaded with 84 g of enzyme ($\rho_{enzyme\ particle} = 0,42$ g/cm^3), which fills 60 cm (h) of the reactor, with an internal radius of 1,25 cm (r). At the experiment conditions with 150 g$_{CO2}$/min (Q) at 313,15 K and 250 bar, its density is 0,89 g/cm^3 (ρ). The residence time was calculated using the Equation 3.2.1.

Equation 3.2.1 – Residence time equation.

$$t_r = \frac{\rho_{CO_2}}{Q_{CO_2}} \times \pi \times r^2 \times h$$

As written before (1.4.1 Enzyme – Biological catalyst), an enzyme has a certain number of active centres, where the reaction takes place. When the actives centres of the lipase are occupied, the enzyme is not able to convert more triglycerides into fatty acid methyl esters, which results in a drop of the yield. Besides this fact, the exposure of enzyme to methanol or glycerol could also affect the enzyme activity (1.4.4 and 1.4.5), leading to a lower reaction yield.

The residence time is a very important factor for designing the reactor. Therefore, in a continuous process, the solvent flow rate and residence time are two important factors to take into account in the reactor design – not too big to not increase excessive cost effects, and not too small in which the residence time is insufficient for achieving a high yield.

The molar ratio of oil:MeOH was fixed (1:24) and the residence time used were 4,14, 2,49, 1,09, 0,82 and 0,54 minutes (30, 50, 75, 100 and 150 g_{CO_2}/min respectively). As it can be seen on Figure 3.2.2, all experiments, in general, obtained a high FAME yield. In fact, with a higher residence time (4,14'), it was obtained a higher yield. Decreasing this value, the yield also decreased though, the decrease was not too evident (1,87 ± 0,17% of difference). Effectively, the optimum residence time was not achieved yet. At the lower residence time, the yield remains high which means that it can be reduce in order to determined in which t_r starts to influence the reaction yield.

At 1,09 minutes of residence time, the yield droped 4,13% and, considering the other experiments, it's expected to be an experimental error.
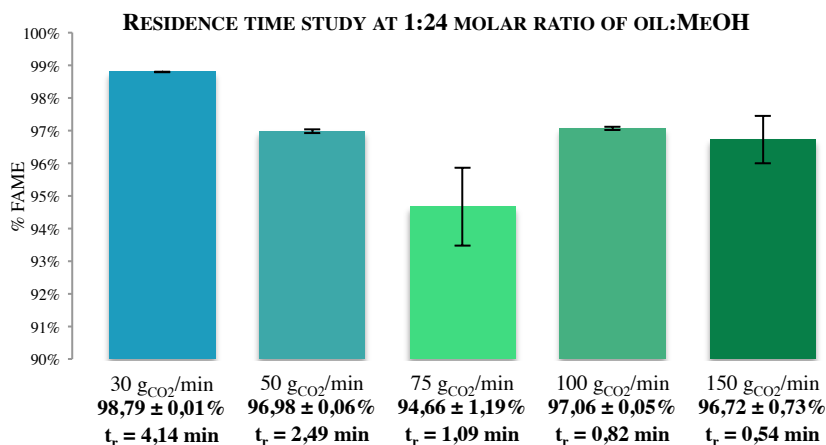
Figure 3.2.2 – Experimental results of CO_2 flow rate study (75, 100 and 150 g_{CO_2}/min) at 1:24 molar ratio of oil:methanol.

Consequently, it's necessary to perform more experiments, in order to achieved the optimum residence time (lower than 0,54 minutes), for the continuous production of biodiesel.

3.2.1.2. OIL:METHANOL RATIO OPTIMIZATION

As mentioned before, according to the stoichiometric of transesterification reaction, three moles of alcohol reacts with one mole of TAG to give three moles of FAME and one mole of glycerol. In other words, an alcohol to oil molar ratio of, at least, 3:1 is necessary for complete reaction. Since it is a reversible reaction, an excess of alcohol can be used to shift the equilibrium to the products formation. However, the exposure of methanol to the enzyme can trigger enzyme denaturation [48].

Therefore, in this section, the molar ratio is analysed, 1:6, 1:12 and 1:24. The residence time is fixed (1,09 minutes, 75 g_{CO_2}/min) and the transesterification reactions were perform at 250 bar and 313,15K.

As it can be seen on Figure 3.2.3, the optimum molar ratio oil:methanol is 1:12, achieving a reaction yield of 98,54 ± 0,49%. Nevertheless, the difference in yield between 1:6 and 1:12 is not significative.

Decreasing the amount of methanol in the system (1:6), can lead to a lower yield because is not quantity enough to promote the direct transesterification reaction. However, at a higher molar ratio oil:methanol (1:24), it's expected to be an experimental error, as it was mentioned before (3.2.1.1. Residence time optimization).
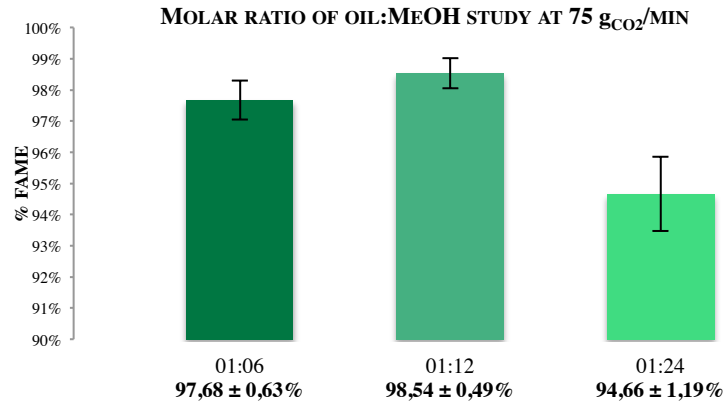
Figure 3.2.3 – Experimental results of molar ratio of oil:methanol study at 75 g_{CO_2}/min flow rate.

Hence, to better understand the influence of the methanol in the transesterification reaction, it's necessary to perform an experiment with a molar ratio oil:methanol 1:24, at the same conditions.

3.2.2. FRACTIONATION PROCESS

After the transesterification reaction, the mixture obtained must be separated in order to recover the biodiesel produced. With the aim of improving the quality of biodiesel, a separation process was integrated after the transesterification reaction.

As it was written before (1.5. Supercritical fluids (SCFs)), in SCFs, physicochemical properties – such as its density – can be easily controlled by changing the pressure or the temperature. The separation process consists in the adjusting of pressure and temperature conditions, inside the cyclone separators, in order to manipulate the CO_2 density, resulting in a change of solubility of the products obtained in this solvent.

In this section, it is study the optimum fractionation conditions in which allow the best product separation/fractionation.

The conditions applied on fractionation process are illustrated on Table 3.2.2. All reaction experiments were executed at 313,15K, 250 bar, 75 g_{CO_2}/min and with a molar ratio of oil:methanol of 1:24. The purpose of chosen these values of solvent flow rate and molar ratio oil:methanol are because of the FAME yield obtained. In fact, and as it mentioned before

(3.2.1. Transesterification reaction study), at these conditions, the FAME yield was the lowest. Therefore, the objective is to improve this FAME yield.

Theoretically, increasing the temperature at a fixed pressure, the CO_2 density decreased, which results in a decreasing of solvation power of the solvent. Therefore, the oil solubility into sc-CO_2 is lower which allows a recover of higher biodiesel purity in the gas phase. Additionally, increasing the temperature another effect could be reveal, the vapour pressure of the solute also increases, which results on an increase of oil in the gas phase. Increasing the pressure at a fixed temperature, the same effect is obtained. The CO_2 density increased, which reflects on an increasing of the solvation power of the solvent. In this fractionation process it's required a low solvation power of the solvent, in order to precipitated the desire product. In other words, it's intended a highly selective solvent.

The conditions applied are shown on Table 3.2.2. At 333,15K and 100/120 bar the solubility of the oil is low, which allows a recover of high quality biodiesel in the gas phase (Separator 2), Figure 3.2.4.

Table 3.2.2 – Conditions applied on fractionation process (pressure and temperature) and FAME content (%).

PRESSURE SEPARATOR 1 (bar)	PRESSURE SEPARATOR 2 (BAR)	TEMPERATURE SEPARATOR 1 AND 2 (K)	FAME$_{AVERAGE}$ (%)
55	55	328,15	94,66 ± 1,19%
100	55	333,15	92,67 ± 2,70%
120	55		88,96 ± 7,45%

In the Figure 3.2.4 is shown the FAME content obtained in the fractionation process. At a constant temperature of 333,15K, increasing the pressure should lead to an increase of oil solubility into sc-CO_2 and consequently, the biodiesel recovered in gaseous phase has lower purity and, in the results obtained, this effect occurs. Effectively, at higher pressure, the solubility of oil in the Separator 1 is higher than with lower pressure. Thus, in this condition of fractionation, at 120 bar, the biodiesel that was recovered in the gas phase has a lower purity when comparing with the fractionation at 100 bar, 93,07 ± 3,52% and 93,89 ± 2,89%, respectively.

However, in respect of the FAME content obtained in both processes, the difference is quite insignificant.
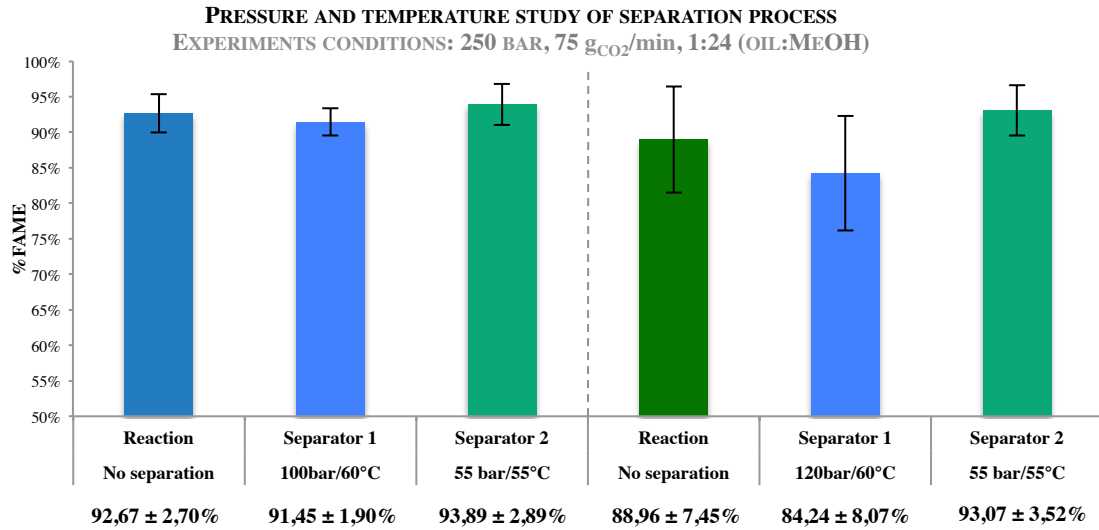
Figure 3.2.4 – Experimental results of fractionation process, revealing the FAME content obtained in the gaseous phase (separator 2), when the pressure is changed.

Accordingly to European Biodiesel Board, the biodiesel, for commercial use, must have 96,5% of ester content. The biodiesel obtained in the fractionation process does not have this ester content. Thus, further experiments must be perform, in order to achieve this biodiesel purity.

CONCLUSION AND FUTURE WORK

4. CONCLUSION AND FUTURE WORK

The main objective of the present work is the biodiesel production using a *green* raw material, chicken feather meal (CFM), combining biocatalysis and supercritical fluids. CFM is a by-product of poultry industry and has 7,1% (g_{oil}/g_{CFM}) of oil, making it a good alternative feedstock for biodiesel production.

Primarily, the oil extraction from CFM using supercritical carbon dioxide (sc-CO_2), as an extracting agent, was optimized. The highest extraction rate for oil extraction was obtained at 300 bar and 313,15K. Considering the CO_2 flow rate, the best extraction efficiency was obtained for a S/F of about ± 9. In the specific case of this work, a S/F of ± 9 corresponded to a flow rate of 150 g_{CO2}/min, with a yield of 96,2%. Nevertheless, further studies are needed to evaluate the energetic cost of this process. So, it was proved that the sc-CO_2 is a good extracting agent of oil from CFM.

The second objective of this work was the integration of oil extraction and transesterification reaction, using methanol, Lipozyme® RM IM as catalyst and sc-CO_2 as solvent. The best results were obtained at 250 bar, 313,15K, 75 g_{CO2}/min and with a molar ratio of oil:methanol 1:12. The fatty acid methyl ester (FAME) yield obtained was 98,54 ± 0,49%. So, it was proved that the extracted oil, from CFM, is a good feedstock for biodiesel production at the conditions applied and using methanol, Lipozyme® RM IM and sc-CO_2 as solvent.

To conclude this work, the fractionation process was performed in order to obtain biodiesel with higher quality, assuming a lower reaction yield. This can be advantageous for reducing reactor volume (and enzyme amount), thus, reducing investment and production cost. The best result was obtained at 100 bar and 333,15K. However, the FAME yield obtained was 93,89 ± 2,89%, which is not in the limits established by the European Biodiesel Board: 96,5% of ester content. Thus, further experiments must have to perform in order to achieve this biodiesel purity.

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APPENDIX

6. APPENDIX

6.1. APPENDIX A

Appendix - Table 6.1.1 – World energy production in million tonnes of oil equivalent [1].

	2009	2010	2011
OIL	3869,3	3945,4	3995,6
NATURAL GAS	2667,4	2866,7	2954,8
COAL	3523,2	3726,7	3955,5
NUCLEAR ENERGY	511,6	521,1	599,3
HYDROELECTRICITY	737,5	778,9	791,5
OTHER RENEWABLES	140,6	165,5	194,8
BIOFUELS	51,8	58,4	58,9
TOTAL	11501,4	12062,7	12550,4

6.2. APPENDIX B
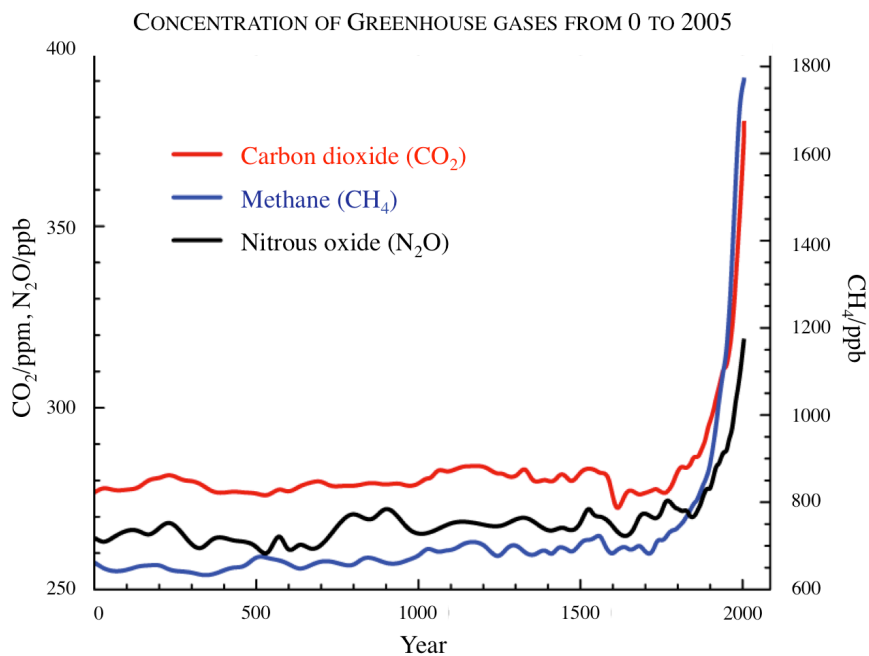
Appendix - Figure 6.2.1 – Atmospheric concentration of important long-lived GHG over the last 2000 years. Increases since about 1750 are attributed to human activities in the industrial era (ppm = parts per million; ppb = parts per billion) (Adapted from [118]).

6.3. APPENDIX C

Appendix - Table 6.3.1 – Structures of fatty acids.

6.4. APPENDIX D

Appendix - Table 6.4.1 – Fatty acid content on extracted oil from chicken feather meal, with sc-CO_2 extraction.
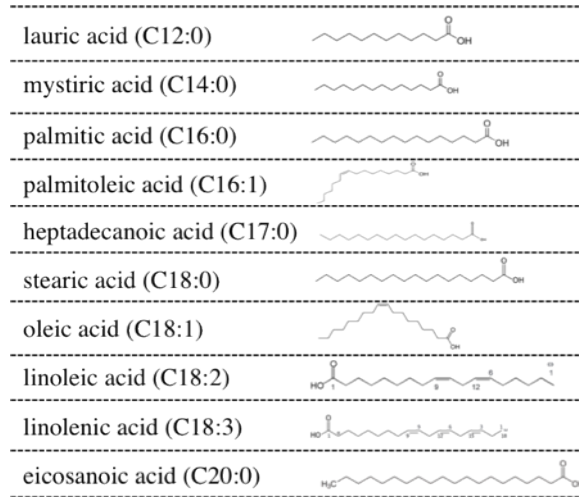
FATTY ACID	WT.,% (SC-CO_2 EXTRACTION)					
	313,15K - 200 BAR	313,15K - 250 BAR	313,15K - 300 BAR	338,15K - 200 BAR	338,15K - 250 BAR	338,15K - 300 BAR
$C_{12:0}$	-	-	-	-	-	-
$C_{14:0}$	1,10 ± 0,08%	1,02 ± 0,18%	1,03 ± 0,20%	0,91 ± 0,16%	1,28 ± 0,00%	1,04 ± 0,27%
$C_{16:0}$	28,98 ± 0,14%	28,77 ± 0,02%	29,28 ± 0,81%	28,93 ± 0,04%	28,84 ± 0,18%	28,71 ± 0,24%
$C_{16:1}$	6,65 ± 0,00%	6,58 ±le 0,28%	5,98 ± 0,46%	6,71 ±0,02%	6,46 ± 0,09%	6,45 ± 0,02%
$C_{18:0}$	6,52 ± 0,07%	6,65 ± 0,18%	6,31 ± 0,71%	6,58 ± 0,06%	6,85 ± 0,05%	6,68 ± 0,18%
$C_{18:1}$	40,20 ± 0,47%	40,91 ± 0,17%	41,37 ± 1,46%	40,58 ± 0,04%	40,14 ± 0,36%	40,72 ± 0,24%
$C_{18:2}$	15,38 ± 0,12%	15,33 ± 0,10%	15,30 ± 0,12%	15,58 ± 0,21%	15,14 ± 0,17%	15,71 ± 0,52%
$C_{18:3}$	0,69 ± 0,04%	0,74 ± 0,01%	1,44 ± 0,00%	0,70 ± 0,03%	0,69 ± 0,01%	0,69 ± 0,05%
$C_{20:0}$	0,97 ± 0,00%	-	-	-	1,19 ± 0,00%	-

6.5. APPENDIX D

6.5.1. CALIBRATION CURVES

LAURIC ACID

$$y = 1,0364x + 0,0033$$
$$R^2 = 0,99611$$

MYSTITIC ACID

$$y = 0,9868x + 0,0027$$
$$R^2 = 0,997$$

PALMITIC ACID

$$y = 1,026x - 0,0004$$
$$R^2 = 0,99905$$

PALMITOLEIC ACID

$y = 1,0655x + 0,0011$
$R^2 = 0,99782$

STEARIC ACID

$y = 0,702x - 0,0033$
$R^2 = 0,99287$

OLEIC ACID

$y = 0,9419x + 0,0014$
$R^2 = 0,99779$

LINOLEIC ACID

$y = 1,1519x + 0,001$
$R^2 = 0,99776$

LINOLENIC ACID

$y = 1,4692x + 0,0005$
$R^2 = 0,99825$

EICOSANOIC ACID

$y = 1,057x - 0,0025$
$R^2 = 0,99317$

TRIOLEIN

$y = 0,4048x - 0,0249$
$R^2 = 0,99407$

DIOLEIN

$y = 1,1002x - 0,0145$
$R^2 = 0,99996$

MONOLEIN

$y = 1{,}383x - 0{,}0154$
$R^2 = 0{,}99993$

GLYCEROL

$y = 1{,}1225x + 0{,}0105$
$R^2 = 0{,}99983$