Sandra da Fonseca Abrantes
Licenciada em Química Aplicada

Valorization of agroindustrial waste

Dissertação para obtenção do Grau de Mestre em
Biotecnologia

Orientador: Dr. Alexandre Paiva, Investigador Pós-Doc, FCT UNL
Co-orientador: Prof. Pedro Simões, Professor Auxiliar, FCT UNL

Arguente: Dra. Ana Vital Morgado Marques Nunes
Júri: Dr. Alexandre Babo de Almeida Paiva
Presidente: Prof. Dr. Carlos Alberto Gomes Salgueiro

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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"Nothing in life is certain except death, taxes and the second law of the thermodynamics"

Dominic Howard

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"Draw another picture
Of a life you could have had
Follow your instincts
And choose the other path"

"Don't grow up too fast
And don't embrace the past
This life's too good to last
And I'm too young to care"

"My plug in baby
Crucifies my enemies
When I'm tired of giving"

MUSE

Abstract

The main objective of this work is the valorization of residues from agro-industry giving them an added value. The valorization was performed by using a "green" and sustainable solvent - supercritical fluid, in this case carbon dioxide. Two residues and one biomass were used to produce two different final products, thereby emphasizing the versatility of the waste recovery - spent coffee grounds and microalgae *Chlorella protothecoides* to produce biodiesel, and tomato pomace to extract carotenoids.

In the first part of this work it was demonstrated the possibility to obtain a conversion of coffee spent grounds oil into biodiesel, through an enzymatic transesterification reaction, of 98.01% with the following operating conditions: molar ratio oil:methanol 1:24, residence time 0.8 min, pressure 25 MPa, temperature 313,15K. In this first phase, it was also used the microalgae *Chlorella protothecoides,* a biomass, to produce biodiesel and favorable results were obtained with this green process compared with a traditional process - basic catalysis / acid.

In the second part of this work, by an extraction with supercritical CO_2 it was obtained 3.38% oil from tomato pomace under the following conditions: pressure 35.1 MPa, temperature 313,15K. It was found that this oil contains various carotenoids: β-carotene, lutein and lycopene. The latter is present in larger amount.

Keyword: supercritical carbon dioxide, enzymatic transesterification reaction, biodiesel, carotenoids, spent coffee grounds, tomato pomace, microalgae *Chlorella protothecoides*.

Resumo

O principal objectivo desta tese é valorizar os resíduos da industria agro-alimentar dando-lhes um valor acrescentado. Esta valorização foi feita através um processo "verde e sustentável" – fluído supercrítico, neste caso o dióxido de carbono. Foram utilizados dois resíduos e uma biomassa para produzir dois produtos finais diferentes, dando assim enfase a versatibilidade da valorização dos resíduos – borra de café e microalgae *Chlorella protothecoides* para produzir biodiesel, e resíduo de tomate para extrair caratenoides.

Na primeira parte deste trabalho foi demontrado que é possível obter uma converçao de biodiesel, através de uma reação de transesterificação enzimática, de 98,01% com as seguintes condições de operação: rácio óleo:metanol 1:24, tempo de residência 0,8 min, pressão de 25 MPA, temperatura de 313,15K. Nesta primeira fase, utilizou-se uma biomassa também para produzir biodiesel, microalgae *Chlorella protothecoides*, tendo-se obtidos resultados bastantes mais favoráveis através de um processo verde comparado com um processo tradicional – catálise básica/acida.

Na segunda parte desta tese, através de uma extração com CO_2 supercrítico, obteve-se 3,38% de óleo no resíduo de tomate, nas seguintes condições: pressão 35,1 MPa e temperatura 313,15 K. Verificou-se que este óleo contém diversos carotenoides: β-caroteno, luteína e licopeno. Sendo que este último está presente em grande quantidade.

Palavras chave: dióxido de carbono supercrítico, reação transesterificação enzimática, biodiesel, carotenoides, borra de café, resíduo de tomate, microalgae *Chlorella protothecoides.*

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Abbreviations and symbols

μL – microliter
BPR – Back Pressure Regulator
C:18 - linolic acid methyl ester
cm – centimeter
DG - Diglycerides
FAAE – Fatty acid alkyl ester
FAEE – Fatty acid ethyl ester
FAME – Fatty acid methyl ester
FFA – Fatty acid
g – Grame
HPLC – High performance liquid chromatography
IS – Internal Standard
K – Kelvin
Kg – kilograme
MG - Monoglycerides
min – minute
ml – mililiter
MPa – Mega Pascal
PTV – Programmable temperatures vaporizing
s - seconds
SCF – Supercritical fluids
SCG – Spent coffee ground
scCO_2 – Supercritical Carbon Dioxide
scMeOH – Supercritical Methanol
TAG/TG – Triglycerides

CHAPTER 1 – STATE OF ART

Parte I – AN OVERVIEW

I – 1.1 - Worldwide waste

The production of waste appeared during the sedentarization of the human species. Initially this was produced through the practice of agriculture, but little by little its expansion curve was increasing with the modernization of lifestyle. This same curve seen an exponential increase in the last two centuries, such as in the eighteenth century with the industrial revolution in the twentieth century or with the use of oil for the production of materials. Nowadays, the lifestyle of men is based on consumption and materialism, and in Europe it is estimated that each person consumes about 16 tons of material per year where about 6 tons is turned into waste.[1] Aditionally, the energy source most commonly used fossil fuels, has increased.

There are several factors that point to the increased production of waste, such as raising consumption, population growth, the wealth of a country or the development of new technologies. With the combination of these factors, it is expected an increase of five percent of the production of waste by 2025 and an increase in population of around 50% by 2050, whereas 95% of this increase will be in developing countries pathway.[2]

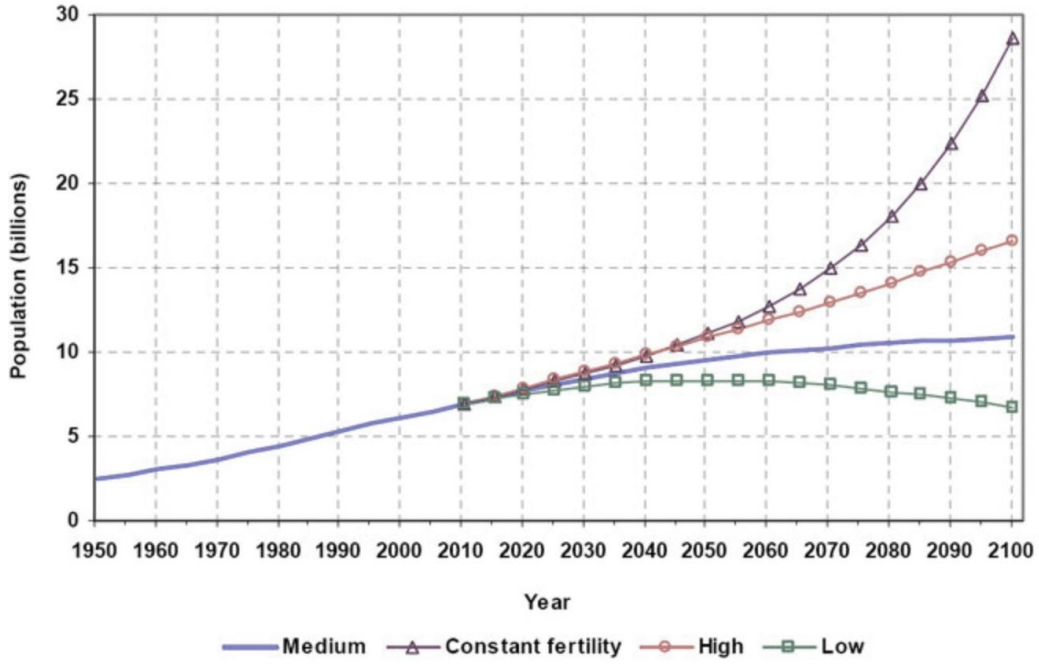


Figure 1.1 – Population of the world, 1950-2100, according to different projections and variants [2]

It is considered a dynamic nature of waste generation process according with three factors: population, technology and affluence, Equation 1.1.

Environmental Impact = Population * Technology * Affluence

Equation 1.1 – Equation to evaluate the environmental impact of the waste.[3]

Waste is the result of processes of different activities according to the source community: industrial, domestic, hospital, commercial, agricultural, services and public cleansing. But Industry is by far one that produces greater volumes of waste. (2)

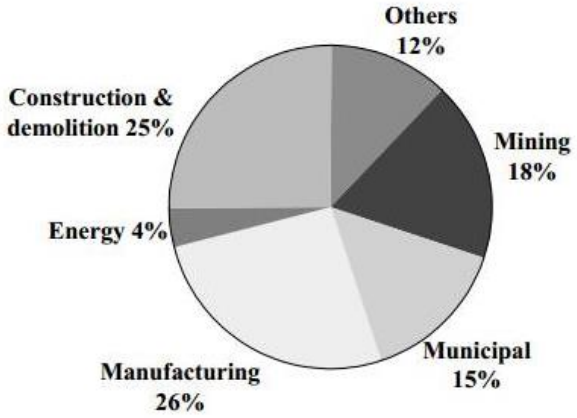


Figure 1.2 - Approximate Generation in the European Union by sector (3)*

The waste can be classified in various ways, according to their source or nature, or can also be classified as wastes, biomedical wastes, construction wastes, industrial wastes solid, sewer, biodegradable waste, non-biodegradable wastes, and hazardous wastes. (4)

Traditionally the waste treatments technologies were based in common activities like landfill or the incineration, which aim at the elimination of the material. Usually solid waste used to be landfilled, liquid waste going to rivers and seas and gas waste was released into the air. (4) But these applications are not effective enough for the treatment of waste due to the high volumes of material to be processed and their different natures. What is importante is that the goal of these techniques is not to bring value to the waste causing environmental problems such as climate change. For these reasons, a more sustainable management and use of waste can reduce the potential impact on the human health and environment.

To reduce the impact of waste on human health and the environment and conserve resources, it is necessary to understand the source of waste and that is made in order to make the best decisions for its treatment. To this end, the concept of hierarchy waste has been implemented and is an important and decisive tool with respect to waste treatment. The aim of the waste hierarchy is to extract the maximum practical benefits from products and to generate the minimum amount of waste, and the hierarchy establishes preferred program priorities based on sustainability. To be sustainable, waste management can not be solved only with technical end-of-pipe solutions and an integrated approach is necessary.

*Foresty and agriculture are not included here, but these two sectors may account for up 30% of overall waste in Europe Union. [2]

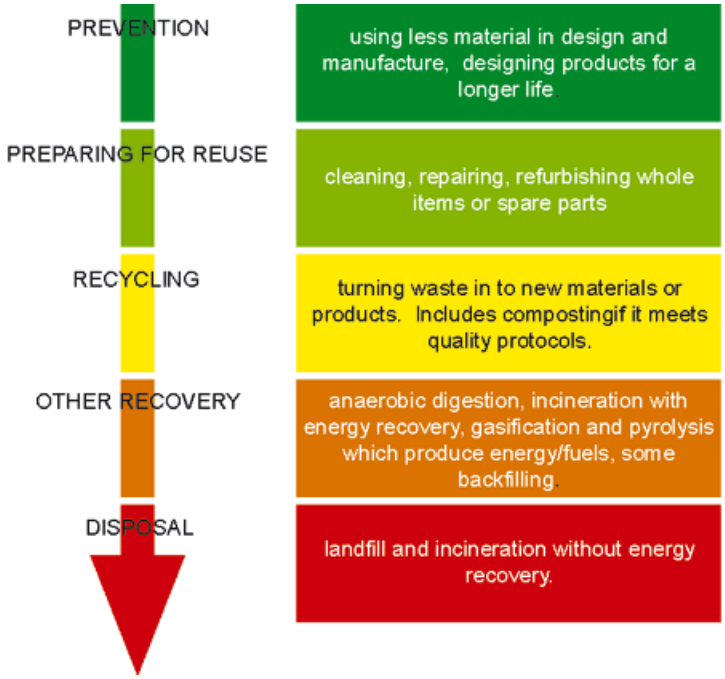


Figure 1.3 – Waste hierarchy diagram [5]

The waste hierarchy captures the progression of a material or product through successive stages of waste management, and represents the latter part of the "life-cycle assessment" for each product. The concept of "life-cycle assessment" is about going beyond the traditional focus and production site and manufacturing processes and the main goals of it are to reduce a products resource use and emissions to the environment as well as improve its socio-economic performance through its life cycle.[4]

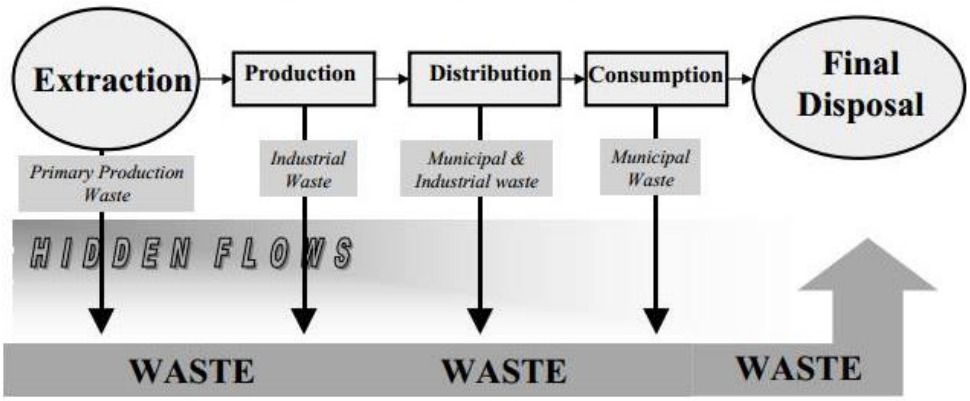


Figure 1.4 – Life cycle of waste[5]

There is a widespread interest in treating the waste material so as to produce energy through it in order to encourage different options to the production of alternative sources of energy facing the actual energy crisis and give an economically value to the viable

constituents that are present in the waste. For that, the forms and techniques are varied and must be adapted in each type of waste, quality, and local conditions.

I – 1.2 - Agro-Food Industry waste

The agro-food industry produces about 26% of the annual waste having a negative environmental impact and consequently economic. This industry tends to grow due to the development and demands of society. The consumer demands are increasingly high that drive to the development of new technologies in the formulation, manufacturing and conservation of food.[6] Currently, by-products of agro-food processing represent a major disposal problem for the industry concerned, due to cost of drying, storage and shipment. Also, these materials are prone to microbial spoilage, thus limiting further exploitation. So, it is necessary to diminish these economically limiting factors, with efficient, inexpensive and environmentally sound utilization of these added-value residues.

The most part of the agro-food industries use traditional methods and consequently a lot of organic solvent to produce their products. So, nowadays, there is increasing public awareness of the health, environment and safety hazards associated with the use of organic solvents in food and ingredient production and possible solvent contamination of final products. The high cost of organic solvents and the increasingly stringent environmental regulations together with the requirements of the food industry for ultrapure and high-added-value products have highlighted the need for the development of new and more environment friendly technologies for the processing of food products.[7]

What is *"food waste"?* In the European Union (Commission Regulations 442/1975/EEC; 689/1991/EEC; 75/442/EEC) food waste was defined as residues of high organic load, which are discarded from the processing of raw material into food product and result in a liquid or solid form. According to the Food and Agriculture Organization, approximately 33% of the edible parts of food produced for human consumption gets lost or wasted globally.[8]

Studies suggest that roughly one-third of food produced for human consumption is lost or wasted globally, which amounts to about 1.3 billion tons per year. This inevitably also means that huge amounts of the resources used in food production are used in vain, and that the greenhouse gas emissions caused by production of food that gets lost or wasted are also emissions in vain. Food is lost or wasted throughout the supply chain, from initial agricultural production down to final household consumption. Five system boundaries were distinguished in the food supply chain:[8]

- Agriculture Production – losses due to mechanical damage and/or spillage during harvest operation.
- Postharvest Handling and Storage – losses due to spillage and degradation during handling, storage and transportation between farm and distribution.
- Processing – losses may occur when crops are sorted out if not suitable to process or during washing, peeling, slicing and boiling or during process interruptions and accidental spillage.
- Distribution – here are included losses and waste from wholesale markets, supermarkets and retailers.
- Consumption – losses and wastes during consumption at the household level.

In medium- and high-income countries food is to a significant extent wasted at the consumption stage, meaning that it is discarded even if it is still suitable for human consumption. In low-income countries food is lost mostly during the early and middle stages of the food supply chain; much less food is wasted at the consumer level. Food waste in industrialized countries can be reduced by raising awareness among food industries, retailers and consumers. There is a need to find good and beneficial use for safe food that is presently thrown away. [8]

Due to increasing of food production, disposal of by-products represents a growing problem. On the other hand, costs of drying, storage and shipment of by-products are economically limiting factors for use in applications with low added value. The problem of disposing by-products is further aggravated by legal restrictions. Thus, efficient, inexpensive and environmentally sound utilization of these materials is becoming more important especially since profitability and jobs may suffer. By-products of plant food processing represent a major disposal problem for the industry concerned, but they are also promising sources of compounds which may be used because of their favorable technological or nutritional properties. For instance, consumers are increasingly aware of diet related health problems, therefore demanding natural ingredients that are expected to be safe and health promoting – in example the use of artificial dyes in the modern food industry concern the consumer about their effect on the human health, so an interest and utilization of natural products, like caronetoids, as alternative food colorants is incrasing.[9]

The large volume of waste produced by the food industry creates an increasing disposal, severe pollution problems and represent a loss of valuable biomass and nutrients. The most past of these waste have been dumped or used as fertilizers or for animal feeds. So, a clean strategies in the manufacturing of new products and compounds must focused on the development of cost effective technology, the optimization of processes including separation steps, alternative processes for the reduction of wastes, optimization of the use of resources and improvement in production efficiency. [10]

The current industrial waste management techniques can be classified into three options:

- Source reduction,
- Waste recovery/recycle or waste treatment by detoxifying, neutralizing
- Destroying the undesirable compounds.

The recycling of residues is important to every manufacturing branch and includes high developing potential. A systematic reduction of product losses and emissions is profitable under both economical and ecological aspects.[10]

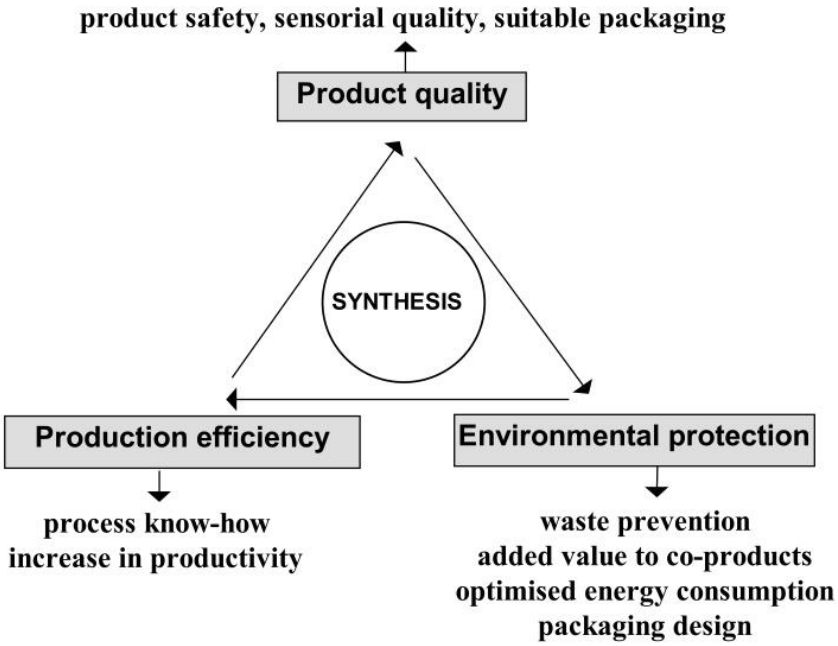


Figure 1.5 – Global concept of food conception.[10]

In this work, it was used spent coffee grounds, microalgae *Chlorella protothecoides* and tomato pomace as the by-product for the production of biodiesel and the extraction of high value-added compounds, respectively.

Parte II – VALORIZATION OF SPENT COFFEE GROUNDS AND MICROALGAE *CHLORELLA PROTOTHECOIDES* AS REBEWABLE SOURCE FOR PRODUCTION OF BIODIESEL

II – 1.1- Biodiesel

"*The use of vegetable oils for engine fuels may seem insignificant today but such oils may in course of time be as important as petroleum and the coal tar products of the present time*" – Rudolf Diesel, 1900, World Fair[11]

Transesterification of a vegetable oil was conducted as early as 1853 by scientists E. Duffy and J. Patrick and the concept of using biofuels in diesel engines originated with the demonstration of the first diesel engine by the inventor Rudolf Diesel, at the World Fair in Paris in 1900, using peanut oil as the fuel.[11] However, due to the abundance and low prices of fossil fuels at that time, the non-conventional fuels never took chance to be developed in order to turn them competitive against fossil fuels. [12]

Nowadays, the scenario for the petroleum fuels is changing drastically. In order to face the global crisis on energy sector introducing in the market an alternative to the conventional fuel which must be technically feasible, economically competitive, environmentally acceptable, readily available and, in the other hand, reduce significantly the global emissions of greenhouse gases, the biofuels arises these days like a feasible bet.

Biofuels are liquid or gaseous fuels for the transport sector that are predominantly produced from biomass. They are renewable, sustainable, biodegradable, carbon neutral for the whole life cycle and environmentally friendly. Several biofuels, bioethanol, biomethanol, biodiesel and biohydrogen, appear to be attractive options for the future of transport sector.[13]

In Europe diesel engine vehicles are the main motors used in the transport sector, and the biodiesel represents an alternative to the convencional fuel for that kind of motors. The use of vegetable oils as alternative renewable fuel competing with petroleum has several advantages of vegetable oils versus diesel fuel but has also some disadvantages [14] that are representanted in the Table 1.1.

Table 1.1 – Advantages and disadvantages of vegetable oils as biodiesel.

Advantages of vegetable oils as biodiesel	Disadvantages of vegetable oils as diesel
• Liquid nature-portability • Ready availability • Renewability • Higher heat content (about 88% of no. 2 diesel fuel) • Lower sulfur content • Lower aromatic content • Biodegradability	• Higher viscosity • Lower volatility • The reactivity of unsaturated hydrocarbon chains

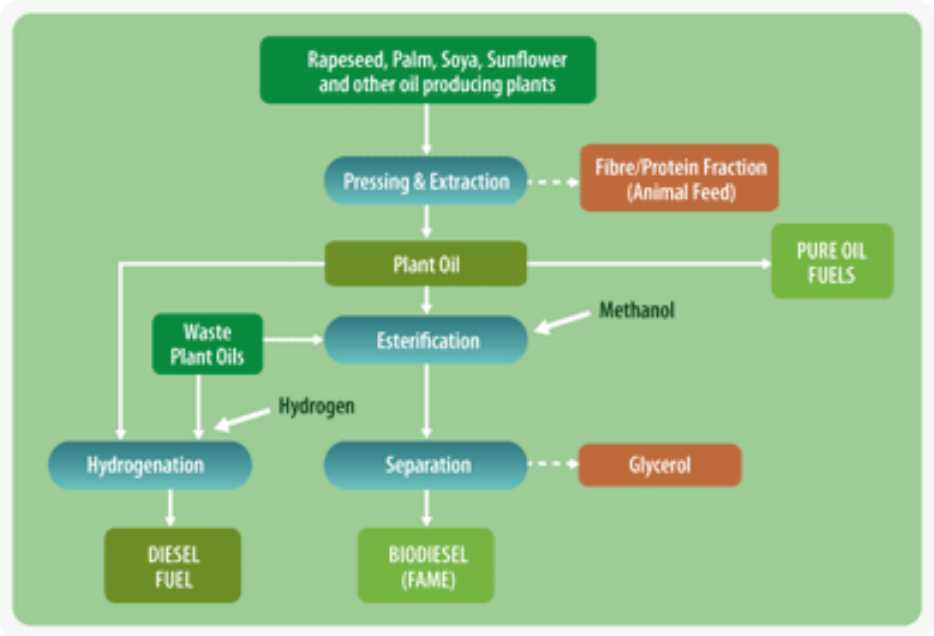


Figure 1.8 – The production of biodiesel from vegetable oils. [15]

Nowadays, biodiesel has attracted great attention, due to the awareness of energy supply and the environmental issues associated to fossil fuels. Biodiesel is presented as a sustainable solution because of its renewability, biodegradability, better gas emissivity[16] and several advantages when considered as the same application of convencional biodiesel, Table 1.2[17, 18].

Table 1.2 – Advantages and disadvantages of biodiesel.

Advantages of biodiesel	Disadvantages of biodiesel
• Biodiegradable; • Renewability; • Lower CO_2 emissions when burned as a fuel; • When compared with convencional diesel: - Higher cetane number (resulting in a larger combustion efficiency); - Lower sulfur and aromatic emissions; - Better lubricant; - Higher oxygen content (improves the combustion process); • The risks of handling, transporting and storing biodiesel are much lower than thos associated with conventional diesel; • Biodiesel is miscible with the convencional diesel and can be employed as a blend.	• The heating value is slightly lower than convencional diesel; • The viscosity, flash point are slightly higher than convencional diesel; • The biodiesel costs are higher than conventional diesel.

The high cost of biodiesel compared to the convencional diesel is the major barrier to its commercialization. It cost approximately 1,5-3 times higer than petroleum-based diesel depending on the sources of feedstock oils.[19] However, Taking into account subjects such as impacts on the environment, employment and climate changes which are not reflected in the price mechanism of biodiesel production, biodiesel turns on the best and competitive alternative to conventional diesel.

In 2012, it was reported that about 85% of the biodiesel production comes from the European Union and the demand for biodiesel in European countries was expected to be up to 10.5 billion liters, in 2010.[20]

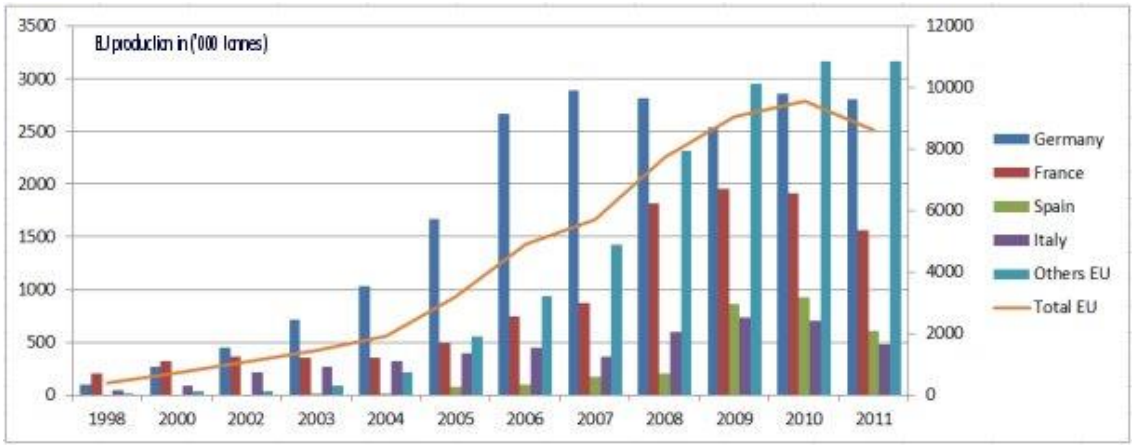


Figure 1.9 – European production of biodiesel. [21]

In order to be used and commercialized in Europe, biodiesel must be produced under some specifications. Those specifications allow biodiesel to become similar to petrodiesel and, because of that, it can be used in the same applications without the need of large changes in engine motors or in the equipment associated. The specifications are described on the Table 1.3.[22]

Table 1.3 – European specifications for the biodiesel commercialization.

Property	Test method	Limits
Ester content	EN14103	96,5 %
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 (min)
Cetane content	EN590 and EN14214	51 mg/kg (max)
Water content	EN14214	500 mg/kg (max)
Total contamination	EN12662	24 % (m/m) (max)
Linolenic acid content	EN14103	12 % (m/m) (max)
Poly-unsaturated FAME	EN14214	1 % (m/m) (max)
Methanol content	EN14214	0,2 % (m/m) (max)
Free glycerol	EN14106:2003	0,02 % (m/m) (max)
Monoglycerides	EN14105	0,80 % (m/m) (max)
Di and tryglycerides	EN14105	0,22 % (m/m) (max)
Total glycerol	EN14105	0,25 % (m/m) (max)
Phosphorous content	EN14107 4	4 mg/kg (max)

II – 1.2- Feedstock

Biodiesel is derived from many different sources, including vegetable oils, animal fats, used frying oils, and even soap stock. There are more than 350 oil-bearing crops identified, among which only sunflower, safflower, soybean, cottonseed, rapeseed, and peanut oils are considered as potential alternative fuels for diesel engines.[(23)] Nowadays, after the controversy over the use of farmland for the production of raw materials for biodiesel versus the use of the land to grow crops for food, actual investigations are more focused on the search of new sources to produce biodiesel, especially sources based in wastes or algae rich on oil components.

Table 1.4 – Conventional and non-conventional feedstock [24]

Conventional feedstock		**Non-conventional feedstock**
Mahua	Soybeans	Lard
Piqui	Rapeseed	Tallow
Palm	Canola	Poultry fat
Karang	Babassu	Fish oil
Tobacco seed	Brassica carinata	Bacteria
Rubber plant	Brassica napus	Algae
Rice bran	Copra	Fungi
Sesame	Groundnut	Micro algae
Safflower	Cynara cardunculus	Tarpenes
Barley	Cotton seed	Latexes
Coconut	Jatropha nana	Microalgae
Laurel	Jojoba oil	
Used cooking oil	Pongamiaglabra	

In Figure 1.10 it is possible to verify that biofuel is characterized on two classifications: primary and secondary. The secondary biofuels are further devided on the basis of raw material and technology used for their production into: first, second and third-generation biofuels. Both the second and third generation biofuels are based on the use of raw materials that do not involve season crops.[25]

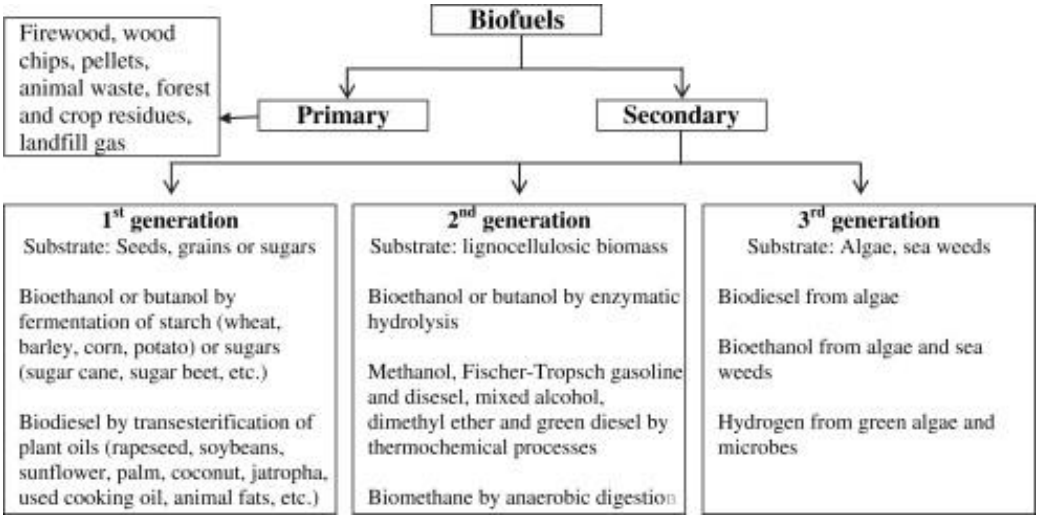


Figure 1.10 - Classification of biofuels.[25]

As said previously, the major barrier to the commercialization of biodiesel it is the high cost of this product which depends of the feedstock used in the process. This work is focused in two different feedstock to the production of biodiesel – Spent coffee ground, that it is incorporate in the second generation and microalgae *Chlorella protothecoides* hat it is incorporate in the third generation of biofuels.

II – 1.2.1 – Spent coffee grounds

Over the years the consumption of coffee and derivatives has increased, making it one of the most consumed beverages worldwide.[26] Coffee is produced in more than 60 countries of which three account for more than half of the world's production: Brazil, Vietnam and Colombia. *Arabica* and *Robusta* are the two species of coffee produced and 75% of all coffee produced is exported, only Brazil and Ethiopia enjoy high domestic consumption.[27] In 2013/2014 about 9 million tones of coffee was produced worldwide.[28]

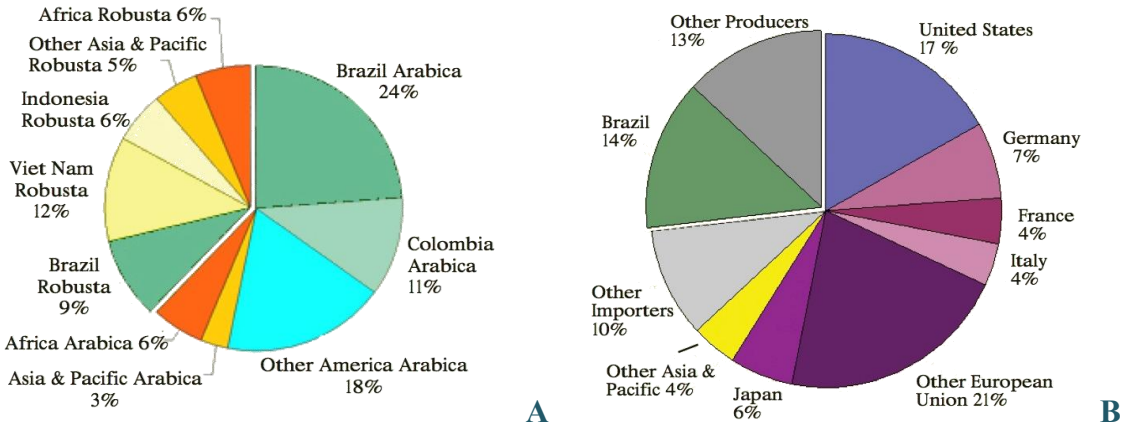


Figure 1.11 – Production (A) and consumption (B) of coffee in 2010. [27]

The solid residues obtained from the treatment of coffee powder with hot water to prepare coffee are the spent coffee grounds (SCG). This organic compound is the main coffee industry residue with a worldwide annual generation of 6 million tons. The SCG can cause contamination and environmental pollution problems due to the presence of tannin,

polyphenols and caffeine. So, considering the huge amount of residue and the environmental impact it's essencial to reuse this material. [29]

Figure 1.12 – Spent coffee grounds. [30]

Nowadays, some attempts for reutilization of SCG have been made, however none of these strategies have yet been routinely implemented and most of these residues remain unutilized:

- As fuel in industrial boilers of the same industry
- As an antioxidant material source
- As fuel pellets
- Used for the production of biodiesel.

There is great political and social pressure to reduce the pollution arising from industrial activities. In this sense, conversion of SCG to value-added compounds is of environmental and economical interest. [29]

The chemical composition of coffee is complex and includes more than two hundred of substances. The composition between green coffee, roasted coffee and SCG are different due the roasted process and the method of preparation of the beverage of the two species of coffee.[31] Coffee is mainly carbohydrates and the amount of this substance increase after the roasted process.[32] In the studies, the compounds that protrude in the SCG composition are hemicellulose (which can be hydrolyzed to produce reduced sugars), phenolic compounds (which can be used in pharmaceutical and food areas[34]) and lipids.[33] The average of lipids is 10%-20 wt%, where 6,5 to 12,5 wt% are diterpene alcohol esters and 87-93% of them are triglycerides.[35] These triglycerides can be converted of biodiedel using the transesterification methods. The fatty acid compostion of the oil extracts are lauric (C12:0), myristic (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linoleic (C18:3) and arachidic acid (C20:0).[33]

In SCG the average of lipid is 10-20 wt%. Assuming an average, the SCG contain 15% oil[36], which can be converted to a similar amount of biodiesel using transesterification methods, the production would be 1 350 000 tones of biodiesel (production of SCG in 2013/2014). Researchers in USA reported that the resulting coffee based biodiesel is much more stable than traditional biodiesel because of coffee's high antioxidant content. The remaining solid waste can be utilized as compost, as a feedstock to produce ethanol or as fuel pellets.[36]

II – 1.2.2 – Microalgae *Chlorella protothecoides*

Microalgae have been discussed as promising solution for biodiesel production. The microalgae consistes in a unicellular microorganism capable to realize photosynthesis (solar energy is converted in organic compounds using CO_2, minerals and water). One of this organic compound is lipids (triglycerides), which represents nutricional reserves for the microalgae. These triglecerides can be converted in biodiesel[37].

Microalgaes have several advantages as[37]:

- Do not compete with food sector;
- Use a small area to grow up, so it is possible to produce a high quantity of triglycerides;
- The culture period is low (generally 15 days).

The content of lipids can be manipulated with the growth condition to increase its production. This increase of lipids can be achieved creating stress condition by depriving microalgae from of the essentials nutrients to its growth: the microalgae will increase its nutritive reserves and consequently the triglycerides.[37]

Figure 1.13 – Microalgae production [38,39]

Chlorella protothecoides is a microalgae that can grow photoautotrophically or heterotrophically under different culture conditions. This microalgae is an important source of many products, such as aquaculture feeds, human food supplements and pharmaceuticals, and it is a very good candite for fuel production. When growth by heterotrophic process and with some manuipulation, the *Chlorella protothecoides* can contain 55,2% of lipids, and

those lipids can be transformed into briodiesel.[40]

II – 1.3 – Transesterification reaction

Biodiesel has been defined as the monoalkyl esters of long-chain fatty acids derived from renewable feedstocks, which content lipids.[41] The physical characteristic of the fatty acid alkyl esters (biodiesel), are very close to those of diesel fuel. Furthemore the esters of fatty acids can be burned directly in unmodified diesel engines, with very low deposit formation.[42] The lipids of the feedstock are mainly consisted of triglycerides molecules (TAG) of three long chain fatty acids (FFA) that are ester bonded to a single glycerol molecule. These fatty acids can differ by the nature, the length, the number and the position of double bonds in the carbon chain.[43]

Several processes can synthesize Biodiesel. Transesterification reaction with small chain alcohols has proved to be the most efficient and promising process.[44] Through catalyzed transesterification reaction, triglyceride reacts with an alcohol molecule producing fatty acid alkyl esters (FAAE) and a glycerol molecule. Transesterification reaction consists of a three consecutive and a reversible reaction where the triglyceride is converted stepwise to diglycerides, monoglycerides (intermediate compounds) and finally glicerol in which 1 mol of FAAE is removed in each one of the three steps[45]. According to the stoichiometric reaction, 1 mol of triglycerides reacts with 3 moles of the alcohol to yield 1 mol of glycerol and 3 moles of Biodiesel. However, in order to favor the direct reaction increasing the yield of the alkyl esters, an excess of alcohol is used.

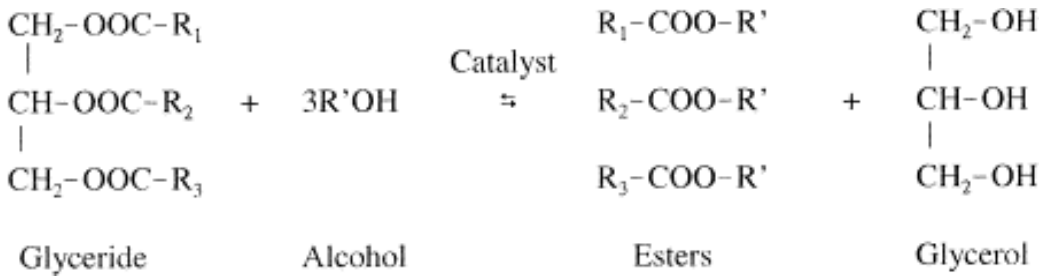


Figure 1.14 – Transesterification reaction.[46]

The selection of one alcohol depends on cost and its performance in the reaction. Two alcohol stands out to be most promising to be used in the transesterification reaction: methanol and ethanol. Even though ethanol represents a renewable source, methanol is preferred over others due to its low cost, simplicity of the process with lower reaction time, spontaneous separation between glycerol and FAAE and high reaction yield.[47] The type of FAAE obtained varies taking into account the type of alcohol used as well as the final properties of the final product since with methanol, fatty acid methyl esters (FAME) are obtained as with ethanol it is obtained fatty acid ethyl esters (FAEE).

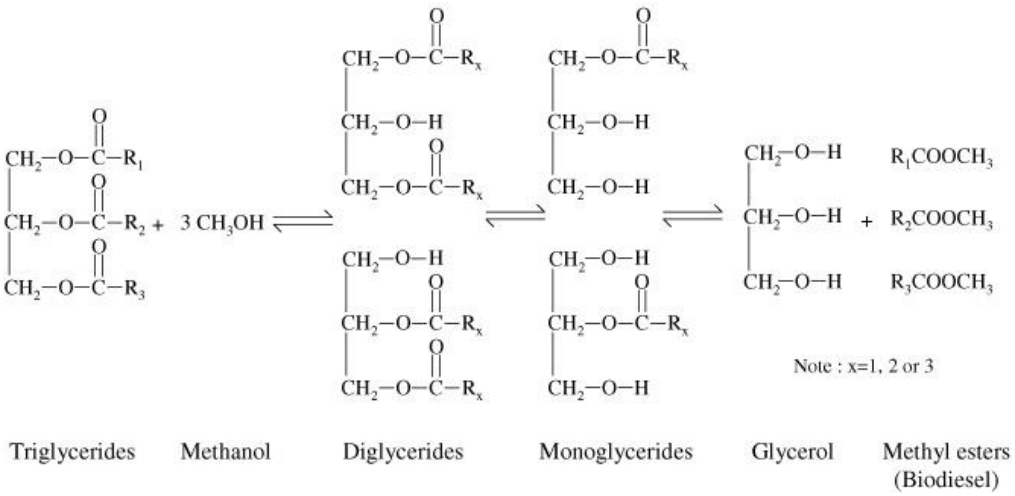


Figure 1.15 – Transesterification reaction with methanol as alcohol. The three steps of the reaction are represented.[48]

Nowadays severeal methods of biodiesel production via transesterification reaction exist and are descrided in the Figure 1.16, some of them are commercially used in today's industrial process and others are still under investigation, like enzymatic method that is the method used in this work. At a high scale production the common process to produce biodiesel is by catalyst transesterification methods, mainly using basic catalyst as sodium hydroxid or potassium hydroxid. However, non-catalytic methods are widely investigated due their advantages in terms of time reaction, low amount of equipment required, no purification process of impurities and high yield achieved.

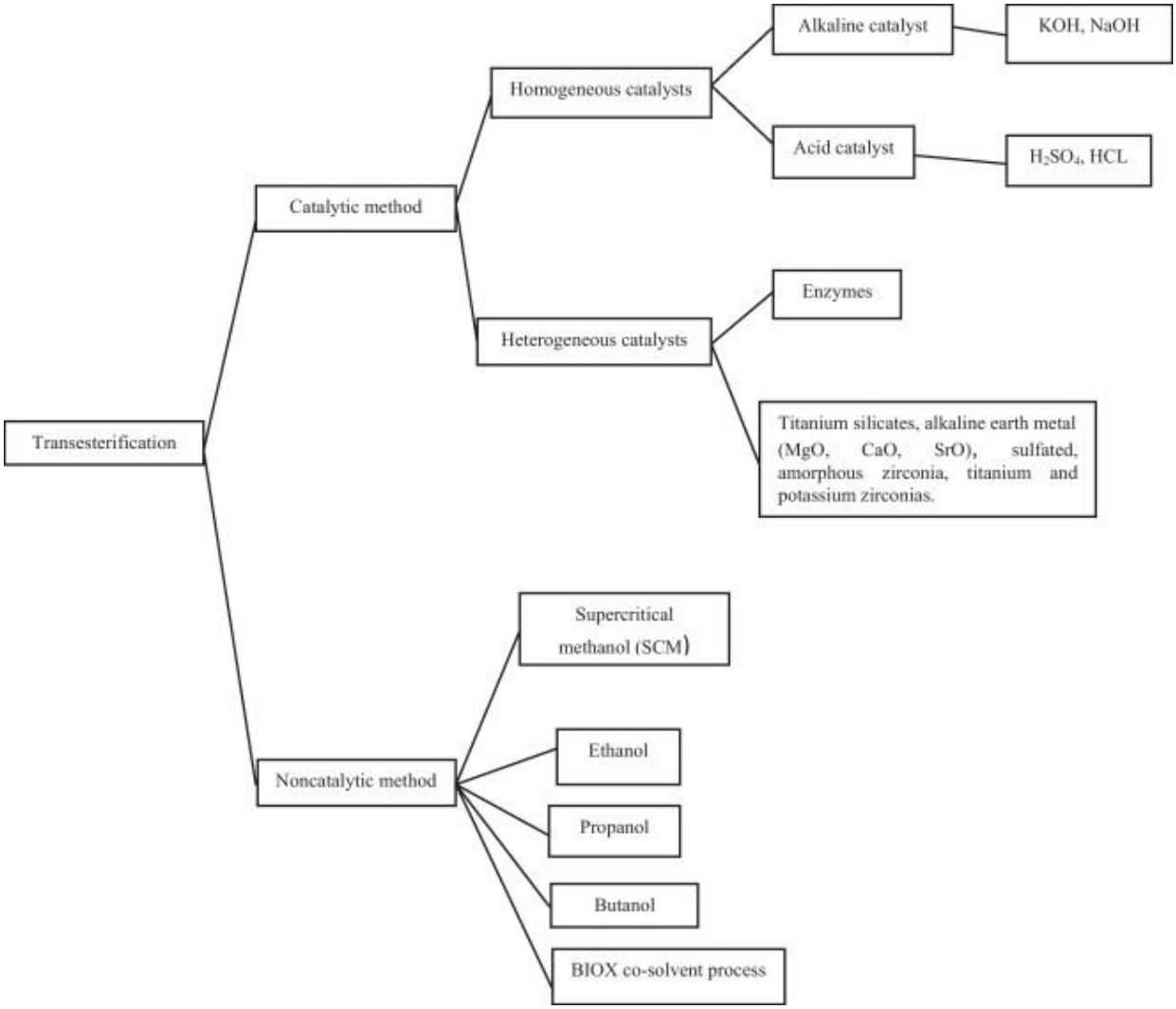


Figure 1.16 - Methods of biodiesel production via transesterification reaction.[20]

The process of transesterification is affected by various factors[49]:

- Molar ratio oil:alcohol;
- Reaction time;
- Type of alcohol used;
- Temperature;
- Catalyst type,
- Water content;
- Pressure;
- Free fatty content.

II – 1.4 – Industrial conventional process

The production process, itself, adds only about 5–30% to the production costs of biodiesel. The best chance to reduce production costs lies in minimizing the cost for the input material. It is reported that approximately 70-95% of the total biodiesel production cost arises from the cost of raw materials.[50]

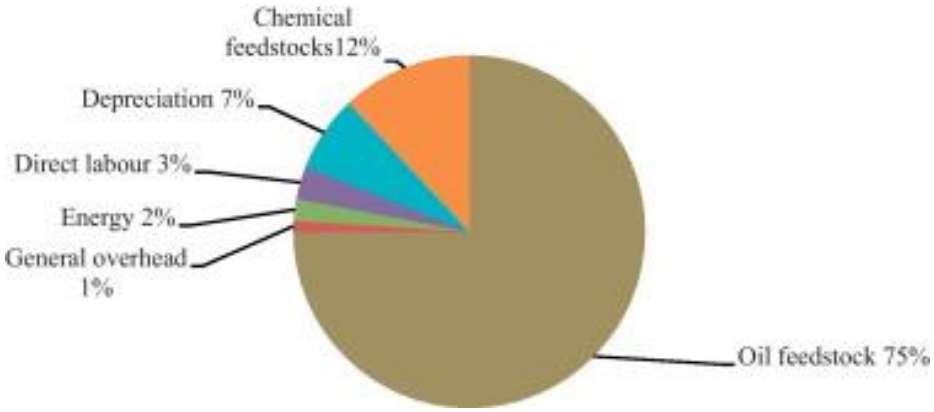


Figure 1.17 - General cost breakdown for production of biodiesel[20]

Most of the biodiesel is currently made from edible oils by using methanol and alkaline catalyst. Frequently, the catalyst is prepared separately from the reaction mixture in order to favor the mass transfer. The catalyst is prepared by potassium hydroxide or sodium hydroxide, this last is often used because it is cheaper and it is more available, and methanol. The convencional process follows these steps[51]:

- The sodium hydroxide (or potassium hydroxide), normally 1% of the total mass of oil, are mixed with methanol in a reactor, yieldind the catalyst;
- The catalyst is tranfered to another reactor, where the transesterification reaction occurs. The molar ratio oil:methanol is 1:6;
- After the rection, the glycerine (by-product) and other contaminants are separated;
- Glycerine and methanol are recuperated by a distillation.
- The biodiesel is watched with distillated water and small amount of hydrochloric acid;
- An evaporation process recovers the water.

Although chemical transesterification using an alkaline catalysis process gives high conversion levels of triglycerides to their corresponding methyl esters, the reaction has several drawbacks: it is energy intensive, recovery of glycerol is difficult, the alkaline catalyst has to be removed from the product, alkaline waste water requires treatment and water interfere with the reaction. Enzymatic catalysts like lipases are able to effectively catalyze the transesterification of triglycerides and can overcome the problems mentioned above. In particular, glycerol can be easily removed without any complex process and also the free fatty acids contained in waste oils and fats can be completely converted to alkyl esters.[52]

II – 1.5 – Enzymatic transterification

An enzyme is a molecule (basically proteins) that catalyses chemical reactions. Like all catalysts, enzymes work by following a reaction way with lower activation energy (Ea) increasing dramatically the rate of the reaction. On the other hand, enzymes are not consumed or do they alter the reactions equilibrium.[53] The convertion of the substrate into the product occur in the active center. Since enzymes are rather flexible structures, the active center is continually reshaped by interactions with the substrates as the substrates interact with the enzyme. The amino acid side chains that make up the active center are molded into the precise positions that enable the enzyme to perform its catalytic function. The active site

continues to change until the substrate is completely bound at which point the final shape and charge is determined.(54)

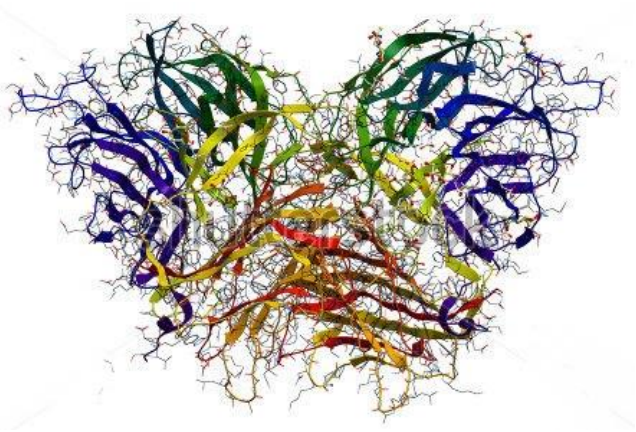


Figure 1.18 – Example of a hydrolase.(55)

The enzymes are classified into six types based on the nature of the catalyzed reaction: oxireductases, transferases, hydrolises, lyases, isomerases and ligases. The enzymes used on the transesterification reaction are lipases that are incorporated on the third type, the hydrolises. So, the lipases catalyze the hydrolise of the lipids. In this work the enzyme used was the Lipozyme *Mucor Miehei*.

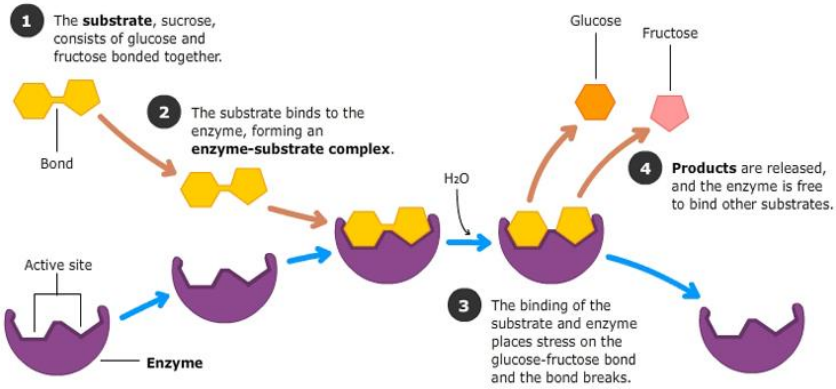


Figure 1.19 – Example of a hydrolysis reaction.(56)

Substrate specificity of lipases consists in the capability of distinguishing structural features of acyl chains such as the length, the number, position or configuration of double bonds or the presence of branched groups, as well as the nature of the acyl source: free acid, alkyl ester, glycerol ester, etc.(57) Differences in catalysis by lipases used for biodiesel synthesis refers to their regiospecificity with respect to the length of hydrocarbon chain of fatty acid. So, the enzymes used for the production of biodiesel display both wide substrate specificity and regiospecificity.

The enzymes can be reutilized a several times and their lifetime can be increased if the enzymes are immobilized. Immobilization is the method most used for maintaining the activity of lipases and has generally been used to obtain a reusable enzyme and increases its stability. This enables easy handling, recovery and recycling of the biocatalyst decreasing the

production cost.[58]

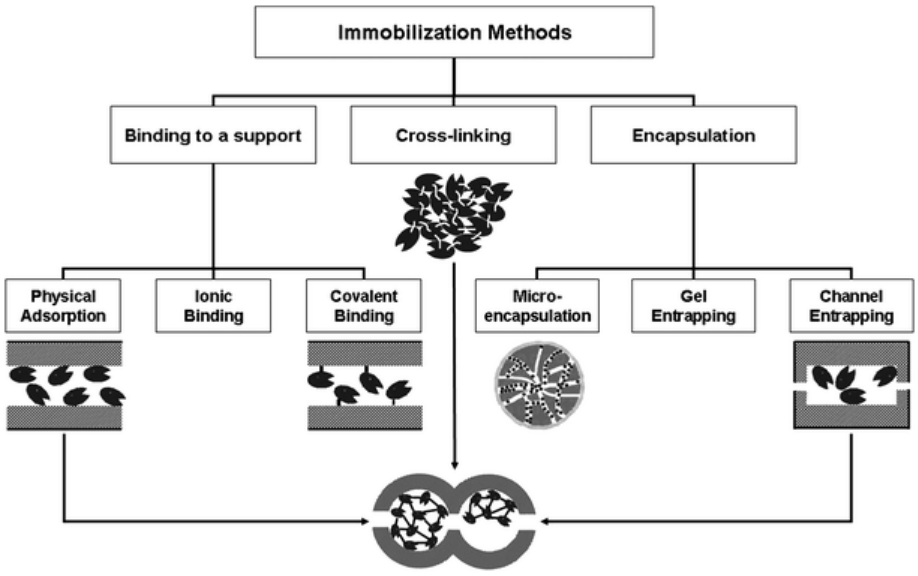


Figure 1.20 – Methods of enzyme immobilization. [59]

Analyzing the Table 1.5 enzymatic catalyst has some advantages and disadvantages comparated with the chemical catalysts used for industrial biodiesel. Taking into account all the parameters, enzymatic catalyst seems to be the best choice for the production of biodiesel.

Table 1.5 – Advantages and disvantages of enzymatic catalysis versus chemical catalyses

Enzymatic catalyst	**Chemical catalysts**
Advantages	
• Synthesis is specific for alkyl esters; • The process need less energy; • Allows the transesterification of glycerides with high free fatty acid contents; • Sustainable; • Environmental friendly; • Easy to recovery of enzymes gives them reusability.	• Excellent productivity.
Disadvantages	
• High cost of the enzyme (but the enzyme can be reutilized, so a long-term the cost is profitable).	• Multi-step purification -> high cost; • Environmental unfriendly; • Chemical catalysts are difficult to recovery.

Severals parameters affect the enzymatic transesterification, affecting the enzyme stability or inibithing it, and consequently decrease the production of biodiesel. It is important to take into account these parameters to optimize the process and obtain the maximum yield of product with a less cost of production. These parameters are[60]:

- Type of alcohol
- Molar ratio oil:alcohol
- Glycerol content
- Temperature
- Residence time;
- Water content;
- Pressure;
- Pressurization-despressurization (when the enzyme is combined with supercritical fluids).

II – 1.6 – Promising alternative biodiesel production process, future trends and outlook

As said previously, nowadays exist a high preoccupation about which feedstock is used for the biodiesel production, giving emphasis to the second and third generation of biofuel, Figure 1.10 because they are based on social, economic and environmental aspects.

Most of the second and third generation biofuels will be derived from perennial plants, wastes sources or microalgae production, but achieving that diversity is likely to depend on greater investment in biofuels R&D and demonstration plants. So, the new generation of biofuels and biodiesel brings a new sustainable business model, figure 1.21.

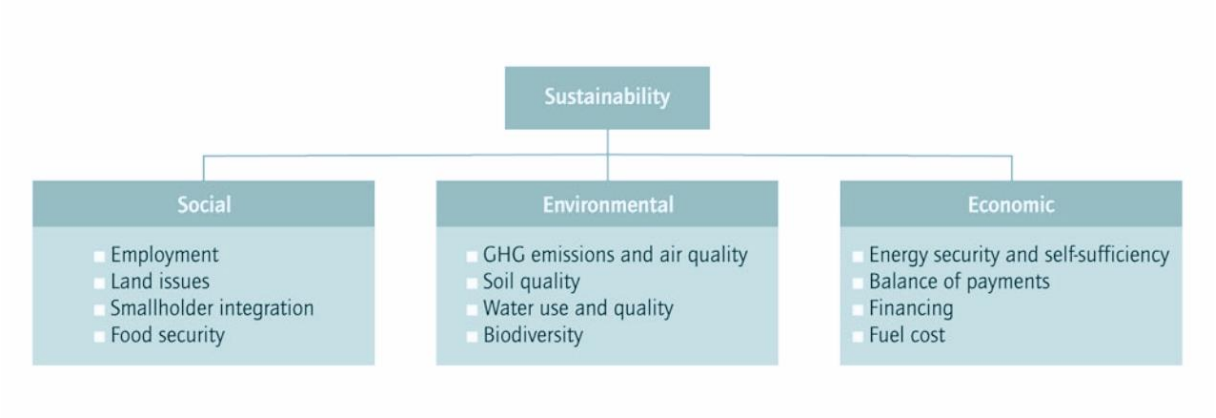


Figure 1.21 - Environmental, social and economic aspects of biofuel and bioenergy production. [52]

The first part of this work is focused in the use of feedstocks that are incorporate in the second and third generation biofuel, the spent coffee grounds, a waste, and the microalgae *Chlorella protothecoides*, a biomass. This work not only uses raw materials that fit with the current scenario but also uses an alternative technology for the production of biodiesel: enzymatic transesterification and supercritical fluids as solvent, more precisely carbon dioxide supercritical, which is detailed in Part IV of this introduction. In this work, the whole process of the biodiesel production is focused on a sustainable method, environmentally friendly and green.

Parte III – VALORIZATION OF TOMATO POMACE BY EXTRACTION OF HIGH VALUE-ADDED COMPOUNDS

III – 1.1 – Value-added compounds

Nowadays it exists an enormous concern about the food and its composition, which drives the search for bio food. So, the Human nutrition science has greatly developed in the past decades, turning from consideration of food and food products as simply energy sources to the recognition of their role in maintaining health and in reducing the risk of diseases.

Lately, there has been a growing interest in functional foods. *So, what is functional food*? It is defined as a food that produces a beneficial effect in one or more physiological functions and additional to nutrional and energetic, increases the welfare and/or decrease the risk of suffering a particular disease. Examples of these functional foods are antihypertensive, anti-inflammatory or antioxidant. Frequently functional food are obtained from tradicional foods enriched and they are preferred by consuers to have a natural origin.(62)

Furthermore, new types of product have been emerged to the market: nutraceutical, cosmeceuticals and nutricosmetics. The nutraceutical are functional foods that are derived from food and they are employed as food supplements and can provide important health benefits. The cosmeceuticals are cosmetics with biologically actives ingredients purporting to have medical benefits. So, they are a combination between cosmestic and pharmaceuticals. The nutricosmetics are nutricional supplements that purport to support the function and the structure of the skin.(63)

The value-added coumpounds can be extracted from waste and residues of the agro-food industry. In this work, the study was focused on extract carotenoids of the tomato pomace.

III – 1.2 – Tomato pomace

Every year are produced 150 million tons of *Lycopersicon esculentum Mill,* tomato, worldwide, being China, India, USA, Spain and Egypt are the bigger producers. The Europe produces approximately 21 million tons.(64) Nearly 20% is sold directly to consumer and the rest is processed into foods, such as ketchup, pasta sauce, soop, canned good, etc.. However, during processing, up to 40% of the raw material ends up as residue, mainly skin and seeds.(65) Those 40% correspond to 48 million tons of potencial added-values compounds.

Figure 1.22 – Tomato pomace.(66)

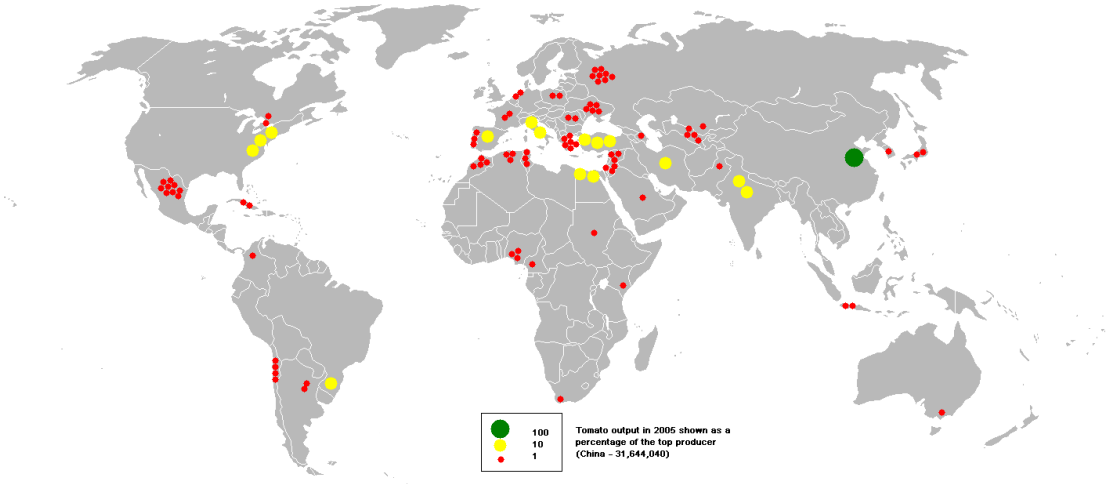


Figure 1.23 – Worldwide tomato production.[67]

Tomato pomace is generally used as pet and livestock food as source of dietary fiber or as biological pesticide.

Tomato pomace consists mainly of peel and seeds and, according some studies, fiber is the major compound of tomato pomace on a dry matter basis, at 25.4–50.0%. Other components ranged between 15.4% and 23.7% for total protein, 5.4% and 20.5% total fat, and 4.4% and 6.8% mineral content.[68] The composition varies with the specie of the tomato. This residue contain a high amount of carotenoids too, approximately 5,1-6,3mg/100g of tomato pomace. The main constituent is lycopene (70–80%) which provides the intensive red colour in tomato fruit. Numerous carotenes can be found above lycopene, like: phytoene (5.3%), phytofluene (2.8%), b-carotene (3.7%) or lutein (2.0%). The tomato contains only small amount of xanthophylls (~2%). The carotenoids are mainly found in skin, the amounts of lycopene and b-carotene are three times higher in skin and at least five times higher in tomato waste than in other tomato products.[69]

In this work, the tomato pomace was used to extract carotenoids, as value-added compounds, by supercritical CO_2.

III – 1.3 – Carotenoids

Carotenoids are a group of phytochemicals that are responsible for different colours of the foods, usually red, orange or yellow in color.[70] They are recognized as playing an important role in the prevention of human diseases and maintaining good health. In addition to being potent antioxidants some carotenoids also contribute to dietary vitamin A.[71] Carotenoids have received much attention because of their various functions and represent a good alternative for the pharmaceutical and food industries (as food colorant) and especially for the human health.[72] They prevent several chronic health disorders including some forms of cancer, heart disease and eye degeneration.[70]

The carotenoids are found in a large number of natural products such as fruit, vegetables and sea products. They are all synthesised by higher plants, algae and bacteria and cannot be manufactured by species in the animal kingdom, so animals adquire them via their diet. Lately, the use of residues from agro-food industry represent a good alternative for

obtains analytes of interest.[73]

All carotenoids are tetraterpenoids, meaning that they are produced from 8 isoprene molecules and contain 40 carbon atoms. Carotenoids in general absorb blue light. They serve two key roles in plants and algae: they absorb light energy for use in photosynthesis, and they protect chlorophyll from photodamage. Carotenoids are split into two classes: xanthophylls, which contain oxygen, and carotenes, which are purely hydrocarbons and contain no oxygen. [70]

Xanthophylls are yellow pigments and are found in highest quantity in the leaves of most green plants. These kinds of carotenoids contain their oxygen either as hydroxyl groups and/or as pairs of hydrogen atoms that are substituted by oxygen atoms acting as a bridge (epoxide).[70] Example of xanthophyills are lutein, zeaxanthin or cryptoxanthin.

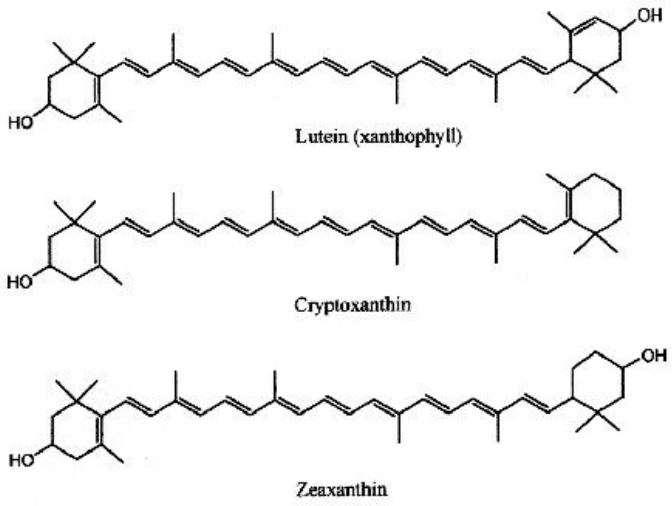


Figure 1.24 – Examples of xanthophylls. [74]

Carotene is an orange photosynthetic pigment importante for photosyntesis and are all synthesised by higher plants, algae and bacteria and are widely distributed in animals. They are composed by unsaturated hydrocarbon having the formula $C_{40}H_x$. The carotene are metabolized by hydroxylation, epoxidation, isomerization, oxidation-reduction and degradation. Some exemples of carotenes are the alfa and beta carotene. [70]

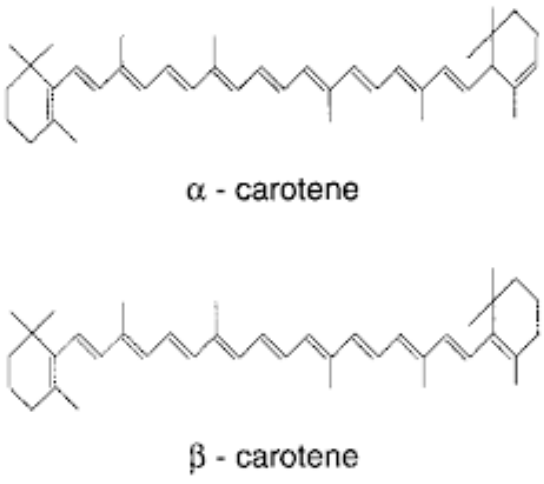


Figure 1.25 – Examples of carotene. [75]

The global market for carotenoids was $766 million in 2007. This is expected to increase to $919 million by 2015. The Beta-carotene has the largest share of the market. Valued at $247 million in 2007, this segment is expected to be worth $285 million by 2015.[76]

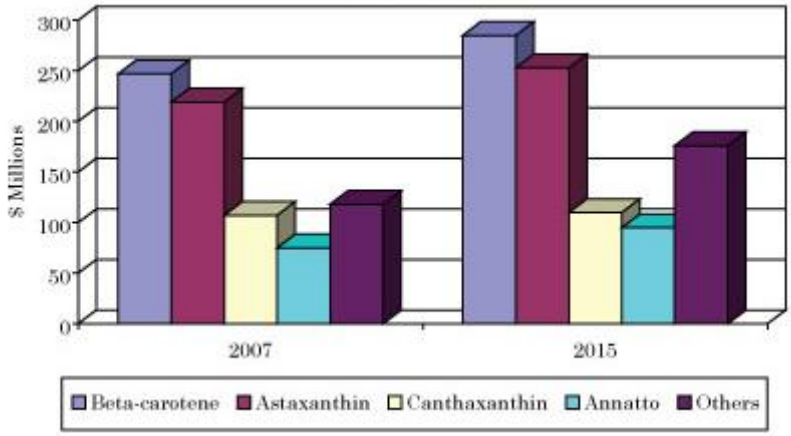


Figure 1.26 - Global carotenoid market value by product 2007 and 2015.[76]

The tomato pomace contains severals carotenoids emphasizing the lycopene.

The lycopene is responsible for the deep red color of ripe tomato. This caronetoid is incorporate in the carotene classe and chemically is an acyclic tetraterpenic hydrocarbon with 13 carbon–carbon double bonds, 11 of which are conjugated. The high degree of conjugation confers strong antioxidant properties to the molecule, making lycopene one of the most potent antioxidants. According to in vitro studies, its ability to quench singlet oxygen is twice as high as that of β-carotene and 10 times higher than that of α-tocopherol. Furthermore, its activity is synergistically enhanced by other tomato components such as phytoene, phytofluene and β-carotene. In addition to being a powerful antioxidant, lycopene is thought to possess other important biological properties, including induction of apoptosis, inhibition of cell proliferation and increase in gap-junctional communication.[77]

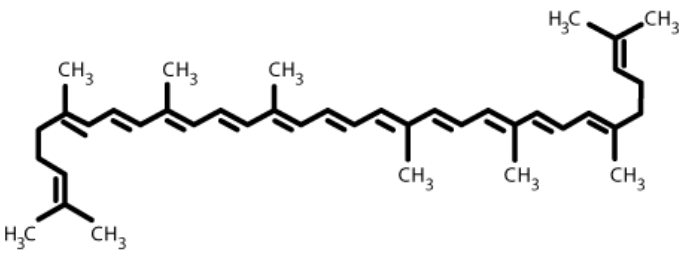


Figure 1.27 - Chemical structure of lycopene.[78]

Natural lycopene is produced by extraction and concentration from whole tomato

fruits that are grown specifically for this purpose. The commercially available product, however, is very expensive. This has prompted the search for alternative sources of lycopene and appropriate technologies for its recovery. In this work, the lycopene was extracted from tomato pomace by supercritical fluids.

III – 1.4 – Carotenoids extraction methods, future trens and outlook

Natural compounds from plants can be extracted by classical techniques as organic solvent extraction, hydrodistillation, steam distillation or low-pressure solvent extraction (LPSE). Carotenoids have a high molecular weight and they can be degraded by the temperature, so it is not possible to extracte them by hydrodistillation or steam distillation. So, the traditional method to extract this coumpond is by LPSE.[79]

LPSE are based on the choice of a solvent coupled with the use of heat and/or agitation. It is based on the principles that the solvent diffuses in the solid matrix and dissolves the soluble compounds. There are several techniques as agitation, homogenization, shaking, centrifugation, ultrasound assisted extraction or soxhlet. LPSE techniques are generally used in industry because they are simple. However, this method as several disadvantages as degradation of biological active compounds because of high temperatures of evaporation step, high consumption of environmentally nonfriendly solvents (for example hexane), high processing time, and, therefore, high energy and operational labor cost. [79]

In most laboratory practices, such as soxhlet extraction, chapter 2.3.2.1.1, Bligh and Dyer method, chapter 2.3.2.1.2, and with acetone extraction, chapter 2.3.2.1.3. The Bligh and Dyer method use a mixture of solvent sufficiently polar to remove the polar lipids but sufficiently non-polar to dissolve the neutral lipids.[80] The soxhlet extraction, like the Bligh and Dyer method can extract both non-polar and polar lipids.

Nowadays and due the government limitation, general concern about the use of organic solvent in the human health and the environmental crisis, it is necessary to invistiguate green technologies to extract the carotenoids. This work is about an alternative extraction by supercritical fluids. Both the method, LPSE and supercritical fluids, have advantages and disvantages, Table 1.6, and taking in account all this factors, the extraction by supercritical fluids could be a good alternative. [79]

Table 1.6 – Advantages and disvantages of supercritical fluids extraction versus LPSE.

Supercritical fluids extraction	**LPSE**
Advantages	
• Reduction, or even the elimination, of the use of organic solvents; • Envirnomental friendly; • Not loss of quality due the temperature.	• Simple technique.
Disadvantages	
• Complex technique and requires several studies to optimize the method for each feedstock; • Expensive equipment.	• Use of organic solvents; • Envioronmental unfriendly; • Several techniques use high temperature that can affect the quality of the product; • Seperation/purification processes are expensive.

Parte IV – SUPERCRITICAL FLUIDS

IV – 1.1 – Supercritical Fluids

Supercritical fluids (SCFs) are substances at pressures and temperatures above their critical values. A pure component is considered to be supercritical if its temperature and pressure are higher than the critical values (Tc and pc, respectively). Above critical conditions for pressure and temperature, there is no sudden change of component properties. The variation of properties with conditions of state is monotonous, when crossing critical conditions. (80)

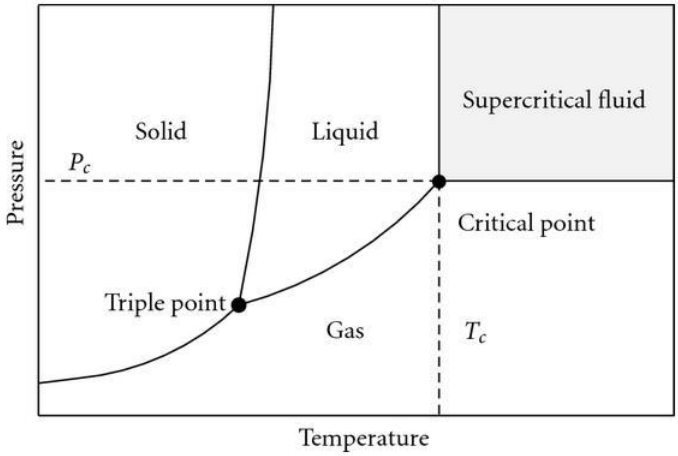


Figure 1.28 – Supercritical fluid diagram.(81)

At the critical point, the densities of the liquid and gas phases become identical; the distinction between the gas and the liquid disappears and it turns a SCF. These fluids display two major properties of having liquid-like density as well as high compressibility (offering large variability of solvency by small changes in temperature and pressure) and gas-like diffusion coefficients as well low as viscosity values. Physical characteristics, including diffusivity, viscosity, and surface tension attribute to the increased solvent capacity of the fluid, which may be exploited for extraction and reaction applications.(82)

Nowadays, the worldwide industries are forced to adopt new sustainable processes that do not require the use of organic solvents because they represent a serious problem to the envirnment. Supercritical fluids (SFC) represent a viable alternative since some of its applications could, partially or totally, substitute organic solvents.

IV – 1.2 – Characteristic of supercritical fluids

As said before, the principal properties of the supercritical fluids are mainly their density, viscosity, diffusivity and solvent strength. In the supercritical region, liquid-like densities are approached, while viscosity is near that of normal gases, and diffusivity is about two orders of magnitude higher than in typical liquids. Properties of the solvents, in the critical area, like temperature and pressure are different from those of a solvent in the liquid or gaseous state far from the critical temperature. In the critical region, properties vary very drastically within narrow ranges of pressures and temperatures, thus producing quite different affects at neighboring conditions of state.(83) Two different effects are present in the SCF properties(84):

- Solvent power of a SCF increases with increasing density;
- Vapor pressure of a solute increases exponentially with the temperature.

So, in order to choose a supercritical fluid as solvent, a number of aspects must be considered, such as[85]:

- Solubility of the solute in the supercritical fluid;
- Viscosity of the fluid in the supercritical region;
- Diffusivity of the supercritical fluid;
- Heat and mass transfer parameters of the solvent;
- All the conditions those are necessary to achieve the supercritical point of the fluid, regarding economical and safety aspects.

To implement SCF in an industry, it is necessary to take in account several characteristics as:

- Inexpensive (< 0.15 €/ Kg ideally);
- No toxic;
- Nonflammable;
- Commercially available;
- Easy purification;
- Pc, Tc near ambient conditions;
- Good solvent properties;
- Co-solvents may be useful.

The more common components used as supercritical fluids are water, dioxide carbon, acetone, alcohols like ethanol and methanol, alkanes such as methane, ethane and butane or unsaturated hydrocarbons like ethylene or propylene, among others.[86]

Table 1.7 – Critical properties of some fluids.

Solvents	Critical temperature (°C)	Critical pressure (bar)
Carbon dioxide	31.1	73.8
Water	374.2	220.5
Propane	96.7	42.5
Ethane	32.2	48.8
Isopropanol	235.2	47.6
Propane	96.7	42.5
Cyclohexane	280.3	40.7

In this work, the SCF used was dioxide carbon (CO_2). This compound is one of the most attractive to apply as supercritical solvent due its several characteristics: its cheap, nonflammable, low toxicity and high availability. Plus, the CO_2 from our own emission can

be explorated to provide a range of environmentally friendly and economically attractive sustainable processes solving a major worldwide problem: global warming.

The solvent properties of sc-CO_2 can be easily modified adjusting temperature and pressure conditions that decrease or increase the solubility of a certain compound. The compound solubility in supercritical CO_2 is related to its density controlled by the pressure and temperature applied. Manipulating these two conditions, a decrease of a certain compound solubility occur resulting in a precipitation. This simplifies the downstream process since carbon dioxide can be easily separated from others components and unreacted compounds.[87]

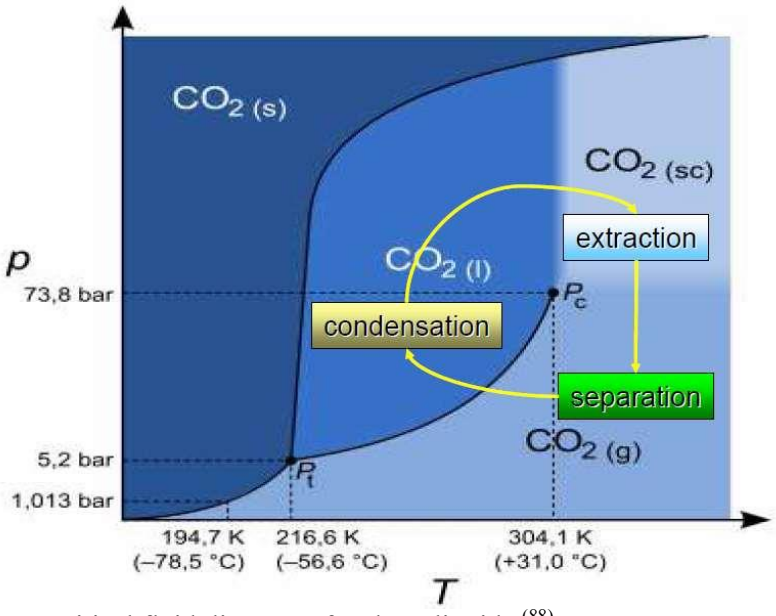


Figure 1.29 – Supercritical fluid diagram of carbon dioxide.[88]

IV – 1.3 – Advantages, disadvantages and applications of the supercritical fluid technology

The SCFs as several advantages one of the principal is the possibility to operate in a large scale of conditions in the same process in order to obtain different product. This can be achieved by applying small change of temperature and pressure that will altere the solvent power of the SCF. This show how versatile is this technology. But, the SCFs have some disadvantages too. The biggest one is the large amount of energy required in order to achieve the operation conditions, as well the high cost of the equipmente required for the high-pressure technology. However, the amount of energy can be reduced with the addition of co-solvent and the cost of the equipment is rentable a long therme.

So, the SCF is a rprofitable a technology and a good choice when the product has a high-added associated, economally interest and when the SCF process gives superior properties than conventional process. The implementation of SCFs technology has already been done for some industrial processes, while the scientific viabilities are being actively explored for others. For example, the Roche Company uses scCO_2 for the production of pharmaceutical products and intermediantes.[89]

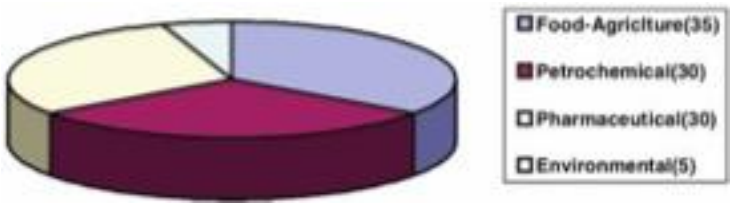


Figure 1.30 – Industry using SCF.[89]

IV – 1.4 – Supercritical fluid – separation process

The most successful process using supercritical fluid is the extraction or reaction and separation process. Intrinsic properties turn $scCO_2$ at an important solvent to these processes since the separation is easily achieved by just adjusting temperature and pressure conditions. After the product(s) recovery, CO_2 is easily recycled.[90]

The most important parameters to extraction/reaction or separation processes are the solubility and phase equilibrium of the systems. Manipulating temperature and pressure conditions, the separation between several components can be achieved by changing compounds solubilities at different conditions of temperature and pressure applied.

Using SCF avoids necessary purification steps with organic solvent or other technologies. With SCF, when a mixture contains several components that have different solubilities at different conditions, it is necessary the implementation of more than one separator. This process is called multi-step separation consists of more than one separator operating at series and each one operates at different conditions in order to recover one or more components. In each separator are applied specific temperature and pressure conditions concerning the solubility of all the compounds. So, this fractionation process is based on the different solubilities of the compounds to be separated in $scCO_2$.

The aim consists in the implementation of the right conditions that allows the precipitation of the components that must be recovered in that separator. The other mixture components remain soluble in scCO2 in order to be recovered in the following separators at different conditions. Joining a few number of separators, is possible to recover several mixture components at the same process just manipulating the operation conditions. In figure 1.31, an example of an ideal multi-step separation process is described assuming a separation between 3 components, A, B e C.

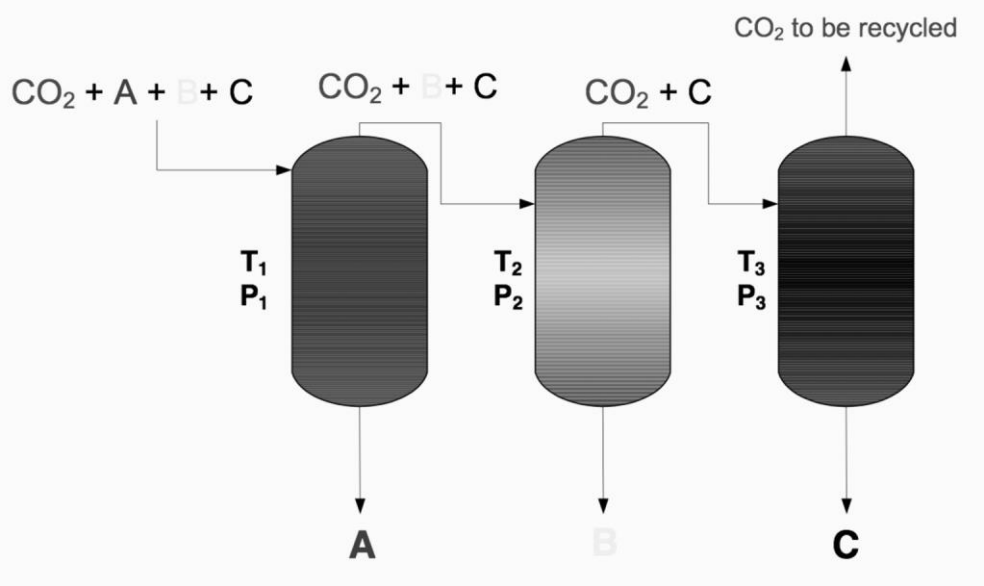


Figure 1.31 – Multi-step separation. [89]

Implementing this kind of process, is possible to obtain a downstream stage that does not mean necessarily high costs production or the application of many utilities due to the high potential of supercritical CO_2 which, in the same process, can be used as solvent for the reaction medium and after, as separator agent.

CHAPTER 2: MATERIALS AND METHODS

2.1 – MATERIALS

2.1.1- Compounds

2.1.1.1 – Spent coffee grounds and microalgae *Chlorella protothecoides* - Biodiesel production

The spent coffee grounds (SCG) used in all the tests was provided by Delta Cafés®.

The microalgae *Chlorella protothecoides* oil used in all the tests was provided by previous experimental works, which used a method of extraction by supercritical CO_2 (sc-CO_2) to obtain this product.

The enzyme used for the enzymatic direct transesterification reaction to produce biodiesel from spent coffee grounds oil and microalgae *Chlorella protothecoides* oil was the Lipozyme RM IM ® (1,3 specific lipase from *Mucor Miehei* fungus, immobilized on ion exchange resin) purchase from Novozymes A/S, Bagsvaerd, Denmark.

All the chemicals compounds used in this experimental work and all there associated information are represented in the Table 2.1.

2.1.1.2 – Tomato pomace – carotenoids production

The tomato pomace without cellulose used in all the tests was provided by Tomaterra Organização de Produtores de Tomate CRL.

All the chemicals compounds used in this experimental work and all there associated information are represented in the Table 2.1.

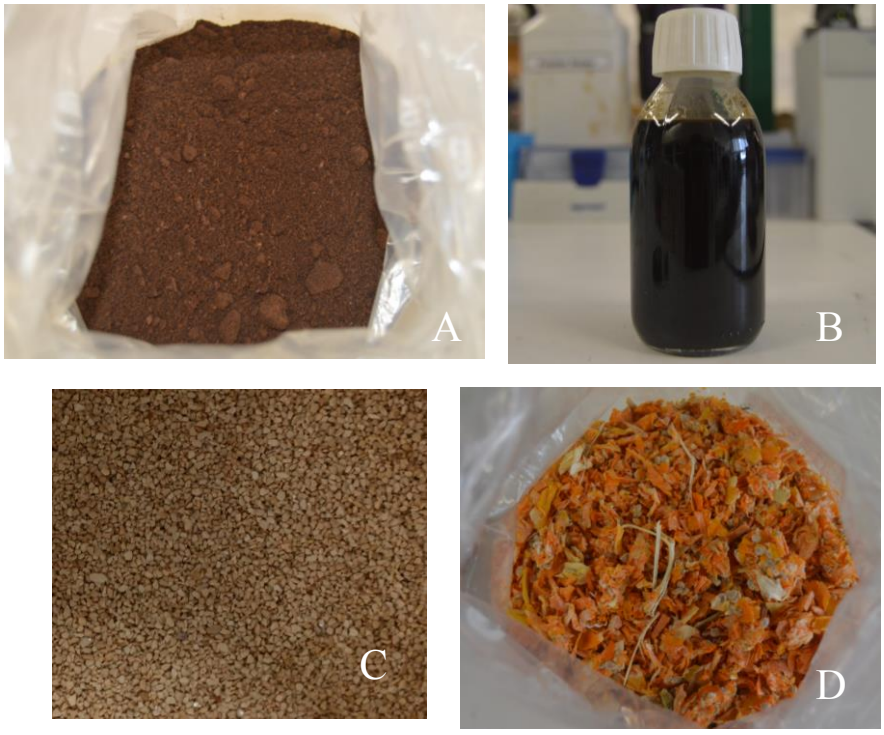


Figure 2.1 – Compounds - **A** - Spent coffee ground; **B** - Microalgae *Chlorella protothecoides* oil; **C**- Lipozyme RM IM ® ; **D** - Tomato Pomace without celullose.

Table 2.1 – Chemicals compounds.

Name	Molecular Formula	Molecular mass (g/mol)	Purity (%)	Brand
Acetone	C_3H_6O	791,0	99	Sigma-Aldrich
Acetonitrile	C_2H_3N	786,0	99.8	Merck
Acetyl Chloride	CH_3COCl	1,1	99	Sigma-Aldrich
Butanotriol	$C_4H_{10}O_3$	106,12	99	Sigma-Aldrich
Carbone Dioxide	CO_2	44,01	95	Air Liquid, Portugal
Chloroform	$CHCl_3$	1,49	99	Pronalab
Ethanol	C_2H_6O	789,0	95	Sigma-Aldrich
Heptadecanoic acid	$C_{17}H_{34}O_2$	270,45	99	Sigma-Aldrich
Hexane	$CH_3(CH_2)_4CH_3$	654,80	96	Carlo-Erba
Methanol	CH_3OH	33,04	99.9	Sigma-Aldrich
Methyl heptadecanoate	$CH_3(CH_2)_{15}COOCH_3$	284,48	99	Sigma-Aldrich
n-Heptane	$CH_3(CH_2)_5CH_3$	100,9	99	Carlo Erba
N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA)	$CF_3CON(CH_3)Si(CH_3)_3$	199,25	-	Sigma-Aldrich
Nitrogen	N_2	1,25	-	Air Liquid, Portugal
Petroleum ether	-	0,64	-	Sigma-Aldrich
Phenolphtalein	$C_{20}H_{14}O_4$	318,32	-	Sigma-Aldrich
Piridine	C_5H_5N	981,90	99	Sigma-Aldrich
Potassium hydroxide	KOH	56,12	94	Sigma-Aldrich
Tricaprin	$C_{33}H_{62}O_6$	54,84	-	Sigma-Aldrich
(DPPH)	$C_{18}H_{12}N_5O_6$	394,32	-	Sigma-Aldrich

2.1.2 – Experiment set-up

2.1.2.1- High-pressure and supercritical CO_2 installations

The extraction of oil from SCG, the production of biodiesel from spent coffee grounds oil and microalgae *Chlorella protothecoides* oil, and the extraction of carotenoids with co-solvent from tomato pomace was performed in the same high-pressure installation (High-pressure Installation 1). The extraction of carotenoids without co-solvent was performed in another a high pressure installation (High-pressure Installation 2, Figure 2.5).

The apparatus used for extraction of oil correspond the Figure 2.2. The apparatus of enzymatic transesterification to produce biodiesel correspond the Figure 2.3. The apparatus of extraction of carotenoids with $scCO_2$ and co-solvent correspond the Figure 2.4.

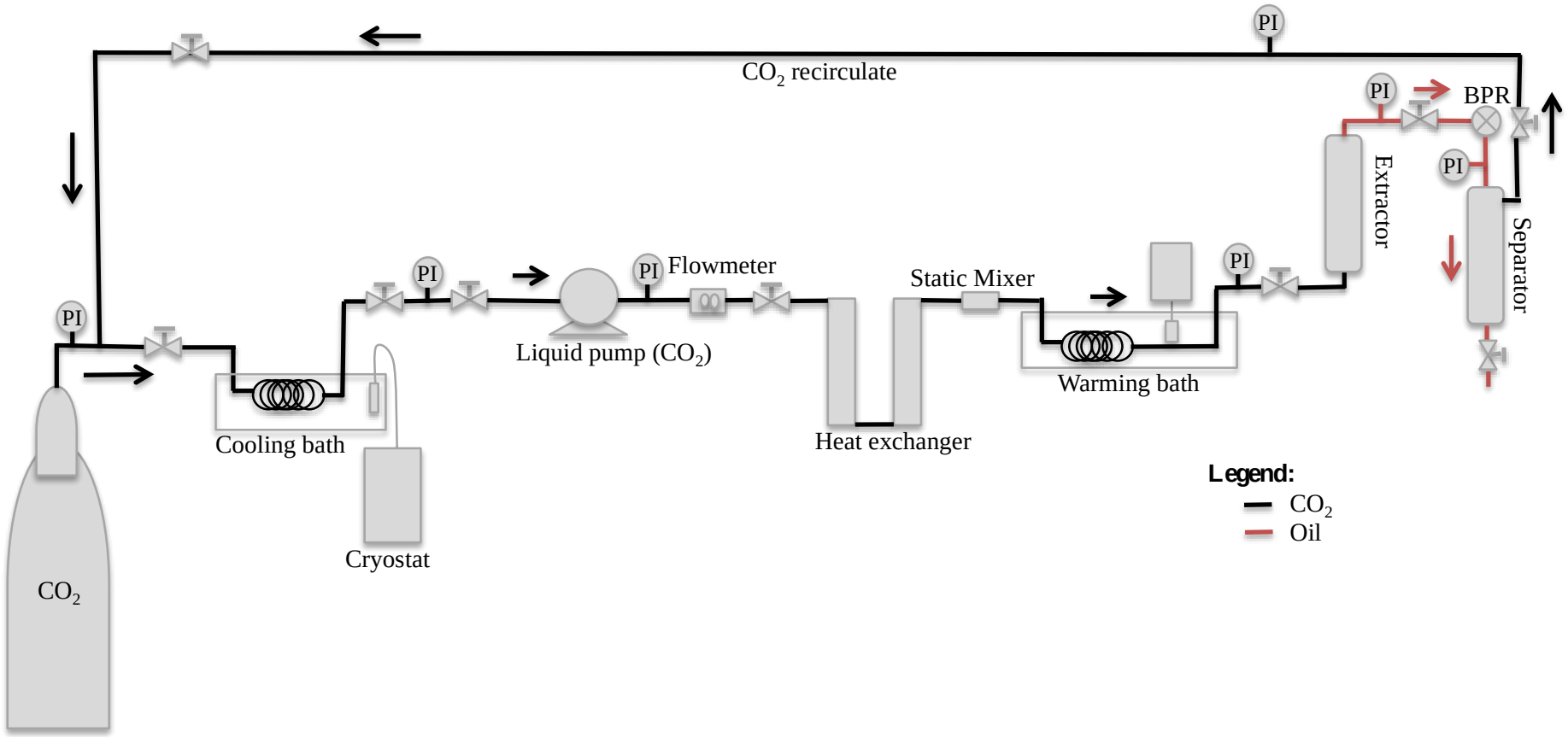


Figure 2.2 – High-pressure installation 1 – Extraction of oil from SCG apparatus.

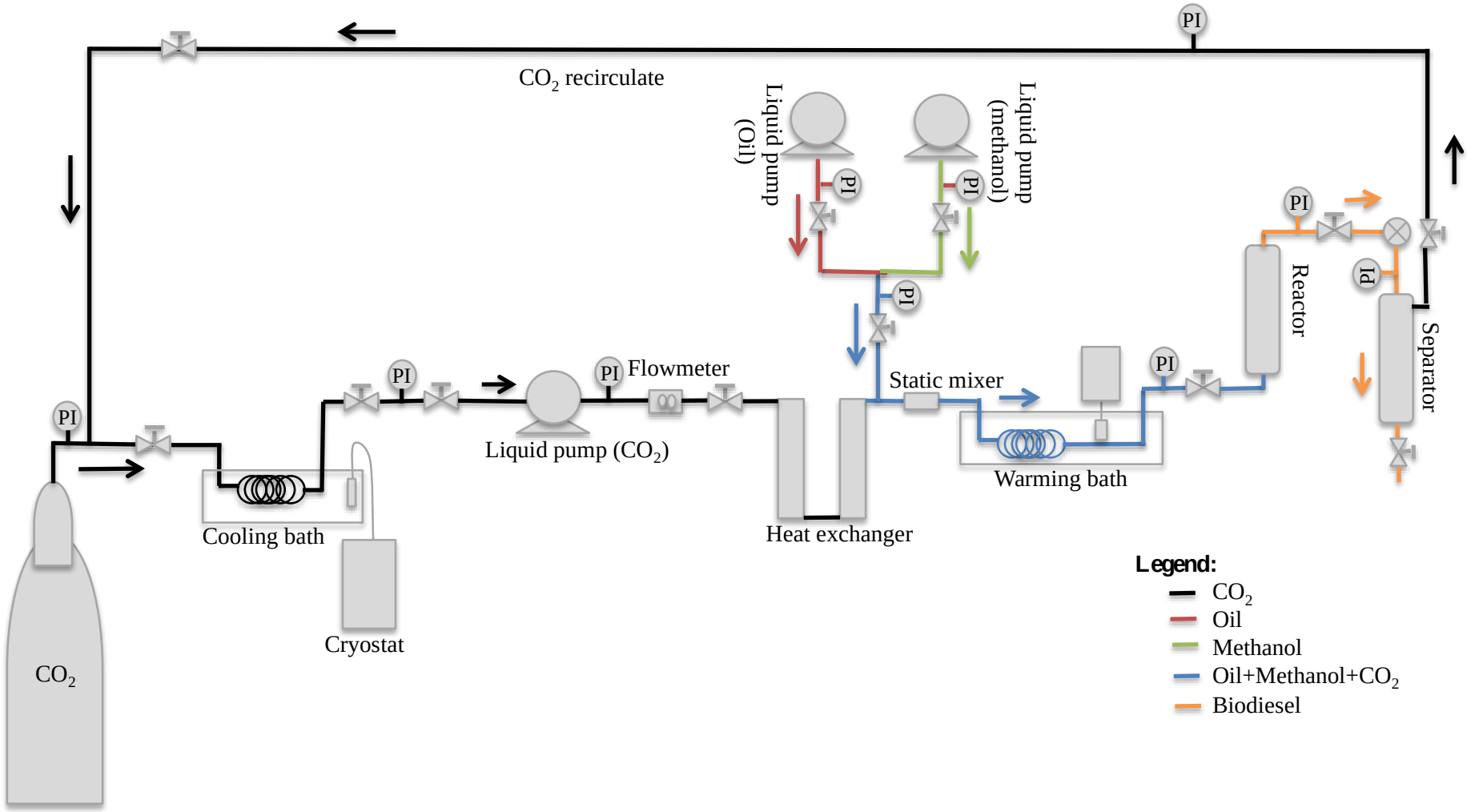


Figure 2.3 – High-pressure installation 1 – Production of biodiesel from SCG oil and microalgae *Chlorella protothecoides* oil apparatus.

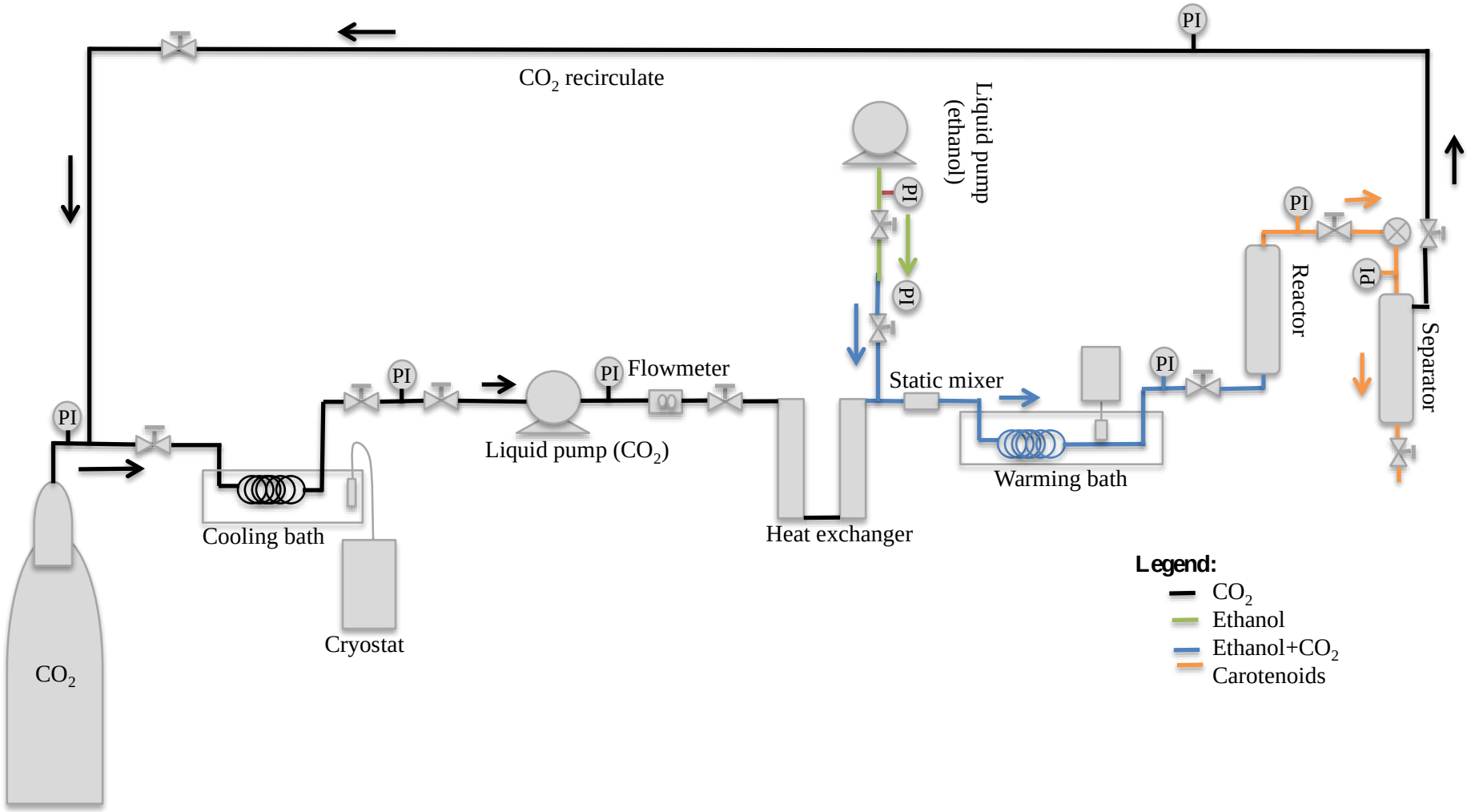


Figure 2.4 – High-pressure installation 1 – Extraction with co-solvent of carotenoids from tomato apparatus.

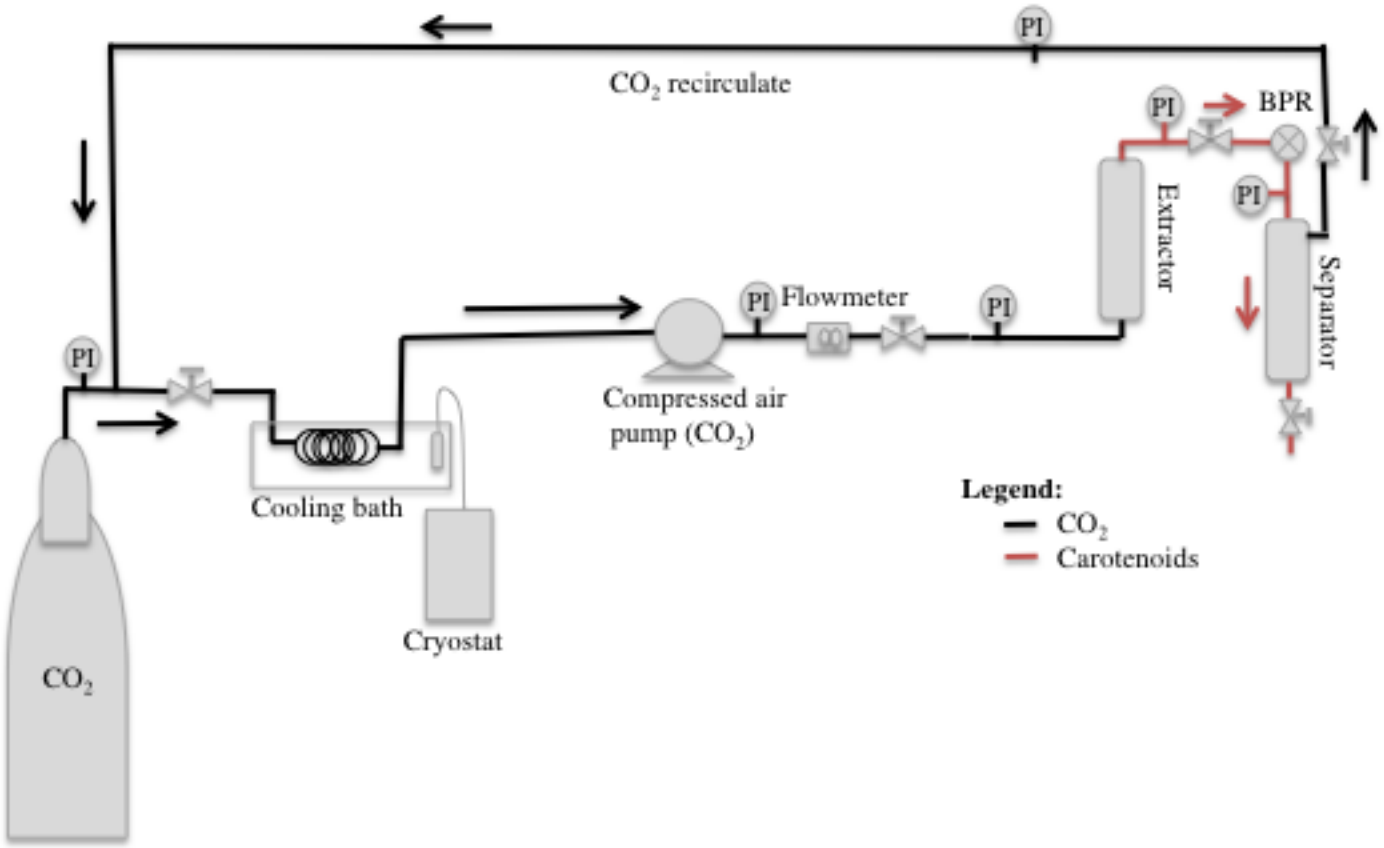


Figure 2.5 – High-pressure installation 2 – Extraction of carotenoids apparatus.

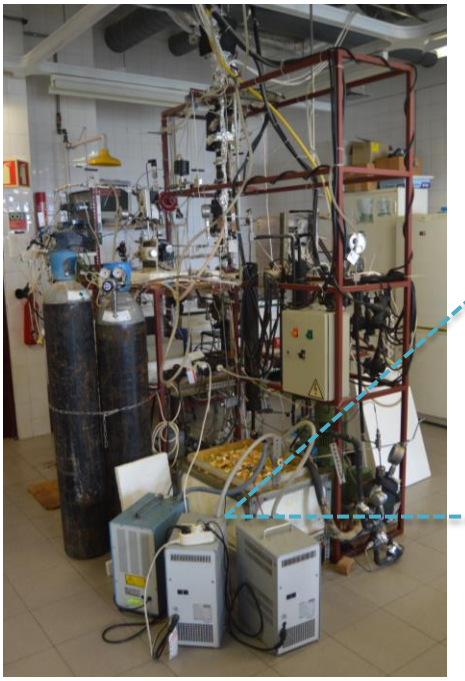


Figure 2.7 – **A** - Cooling bath; **B** – cryostats.

Figure 2.6 – High-pressure installation 1– view from CO_2 bottles and cooling bath.

Figure 2.8 – CO_2 liquid pump of the High-pressure installation 1.

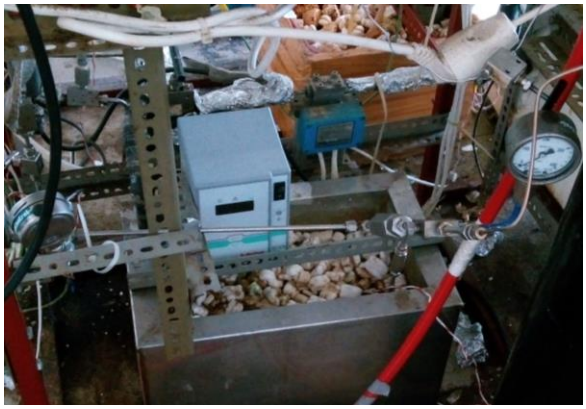


Figure 2.9– High-pressure Installation 1 - **A**- Warming bath, **B** – Static Mixer.

Figure 2.11 – Back pressure regulator (BPR).

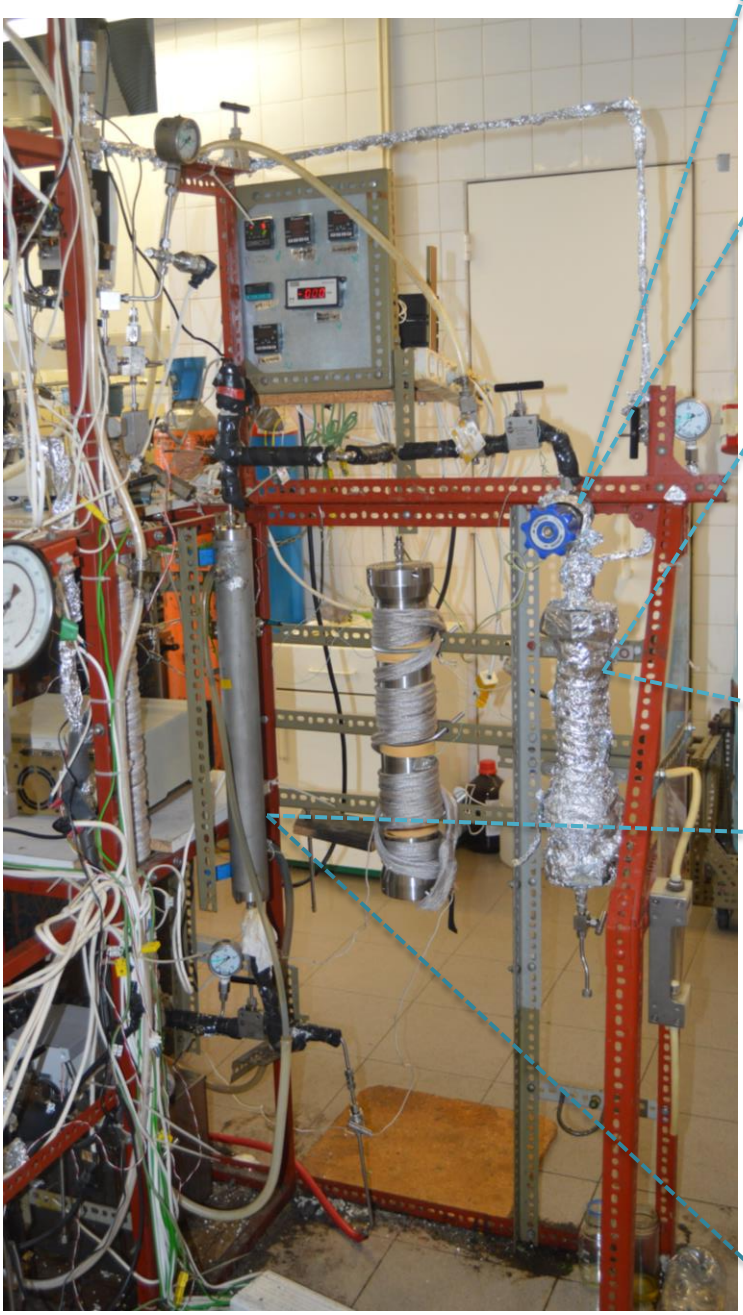


Figure 2.10 – High-pressure instalation 1– view from the separation side.

Figure 2.12 – Extractor.

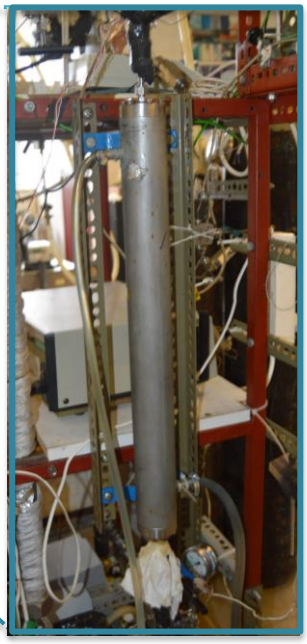


Figure 2.13 – Separator (oil/carotenoids extraction)/ Reactor (enzymatic transesterification).

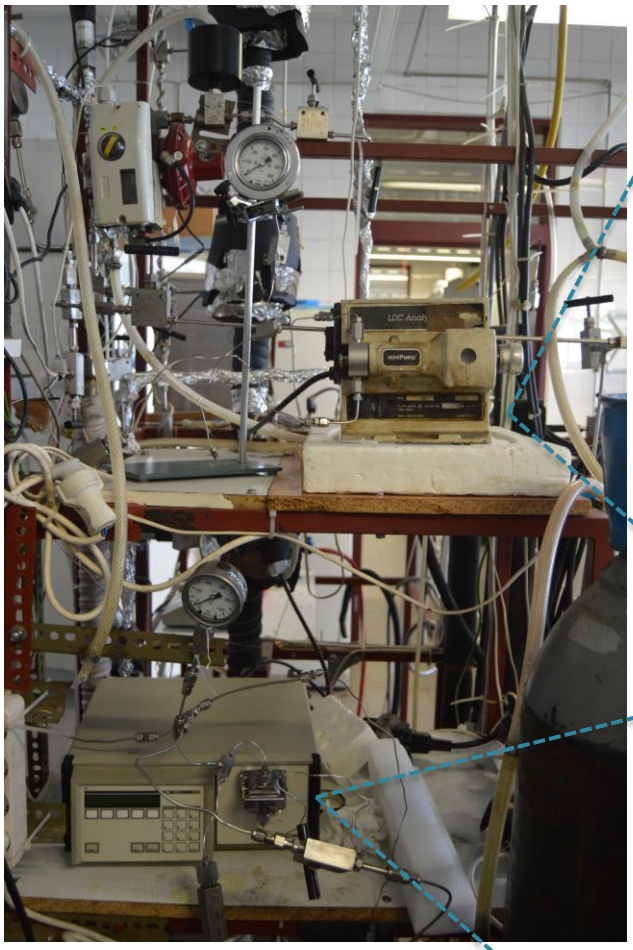


Figure 2.14 – High-pressure instalation 1 – view from the oil and methanol pumps (enzymatic transesterification).

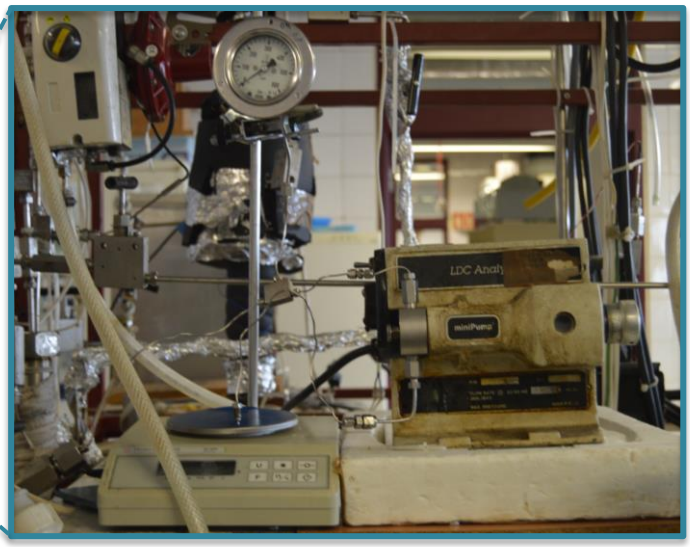


Figure 2.15 – Oil liquid pump.

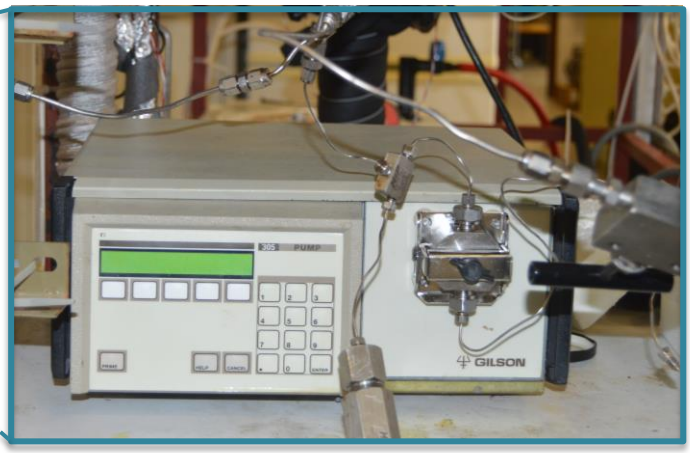


Figure 2.16 – Methanol liquid pump.

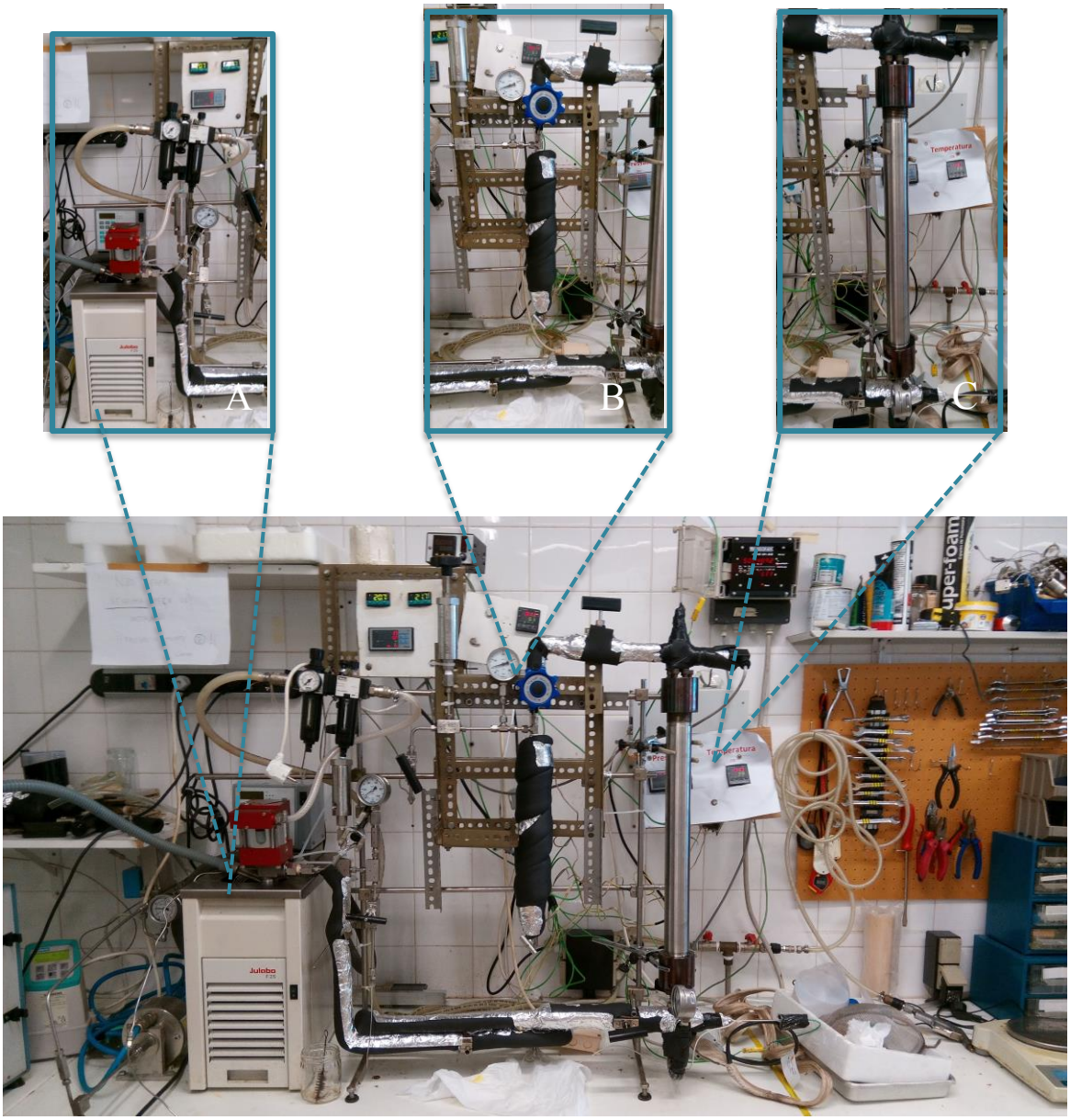


Figure 2.17 – High pressure installation 2 (A – air compressed pump, B – BPR and separator, C – extractor).

2.2 – METHODS

2.2.1 – Biodiesel production

2.2.1.1 – Oil extraction of spent coffee grounds in high-pressure supercritical installation with supercritical CO_2 of spent coffee grounds

At first an amount of 100g of residue (SCG) was weighed and putting inside the extractor (height = 60 cm, inner diameter = 5,5 cm). The assay conditions are on the Table 2.2.

The assays were preceded in the high-pressure installation 1 in a continuous process.

The carbon dioxide (process solvent) in a gas-phase passes through a cold-water bath, cooled by two cryostats (JP Selecta, s.a.), becoming liquid so it can be pumped, with a determinate mass flow (100 g/min), by a liquid pump (Nikkiso) into system. The liquid CO_2 passes through a vessel and is measured with a flow meter (Rheonik, 01.08). The solvent is heated by a heat exchanger heating from a water bath (Julabo ED). Then, the solvent enters into the extractor, also heated. The pressure is controlled by a Back Pressure Regulator (BPR) (Tescom Europe). It's important to refer, that since the liquid pump to the BPR, the solvent is in a supercritical phase due the temperature (323,15 K) and pressure (25 MPa) imposed on the system. After the BPR, the product is collected in a separator, where the pressure is approximately equal to 5,5 MPa and temperature of 328.15 K. In this separator, the CO_2 is separated from the product by a pressure drop returning to a gas-phase (the solvent is recirculate into the system) while the product precipitates and his extraction is possible in a liquid form.

Table 2.2 – Assay condition for the extraction of oil from spent coffee grounds.

Weigh of spent coffee ground (g)	CO_2 flow (g/min)	Assay time (min)	Pressure (MPa)	Temperature (K)
100	100	40	25,1	323,15

2.2.1.2 – Enzymatic direct transesterification in high-pressure installation with supercritical CO_2 of spent coffee grounds oil and microalgae *Chlorella protothecoides*

Both the production of biodiosel from SCG oil and microalgae *Chlorella protothecoides* oil, suffered the same methodology described below and was preceded in the high-pressure installation 1.

At first the enzyme was introduced in the reactor (height = 60 cm, inner diameter = 5,5 cm*), the height of the enzyme varied on each assay according to the Table 2.2.

The carbon dioxide (process solvent) in a gas-phase passes through a cold-water bath, cooled by two cryostats (JP Selecta, s.a.), becoming liquid so it can be pumped, with a determinate mass flow, by a liquid pump (Nikkiso) into system. The liquid CO_2 passes through a vessel and is measured with a flow meter (Rheonik, 01.08).

*A part of the assays were proceeded with a different reactor which the size are height = 33 cm, inner diameter = 2,8 cm.

The solvent is heated by a heat exchanger heating from a water bath (Julabo ED). The methanol and oil (substrates of the process), both in liquid phase, are pumped into the system (LDC analyze - Oil, Gilson - methanol), where they join the solvent in a static mixer. The three compounds then enter into the reactor, also heated, which are the enzyme. The pressure is controlled by a BPR (Tescom Europe). It's important to refer, that since the CO_2, oil and methanol pump to the BPR, the solvent is in a supercritical homogeneous phase due the temperature (313,15 K) and pressure (25 MPa) imposed on the system. After the BPR, the product is collected in a separator, where the pressure is approximately equal to 4,5 MPa and temperature of 318.15 K. In this separator, the CO_2 is separated from the product by a pressure drop returning to a gas-phase (the solvent is recirculate into the system) while the product precipitates and his extraction is possible in liquid form.

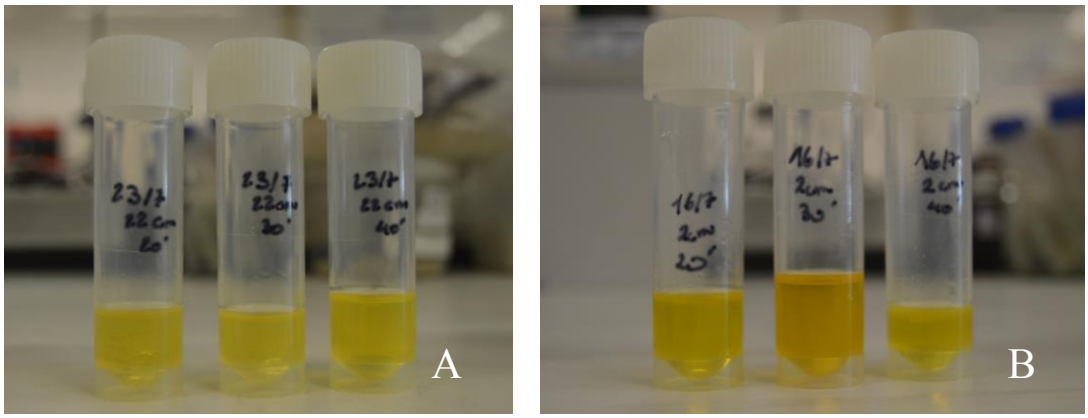


Figure 2.18 – Biodiesel. **A** – Biodiesel from microalgae *Chlorella protothecoides* oil; **B** - Biodiesel from spent coffee grounds oil.

Table 2.3 – Assays conditions for the biodiesel production from spent grounds coffee oil and microalgae *Chlorella protothecoides* oil.

Biodiesel produced from spent coffee grounds oil									
Enzyme bed (cm)	**Weigh of the enzyme (g)**	**Oil flow (mL/min)**	**Methanol flow (mL/min)**	**CO_2 flow (g/min)**	**Assay time (min)**	**Pressure (MPa)**	**Temperature (K)**	**Solubility (g oil/Kg CO_2)**	**Oil:methanol ratio**
5	9,92	0,08	0,470	87	90	25,1	313,15	0,8	1:147
13	20,46								
54,6	88,5								
2	3,46	0,08	0,118	87	90	25,1	313,15	0,8	1:37
7,5	14,39								
13	20,46								
25	42,78								
51,6	86,68								
10	15,20	0,270	0,234	90	50	25,1	313,15	3	1:24
20,8	34,96								
33	52,03								
Biodiesel produced from microalgae *Chlorella protothecoides* oil									
Enzyme bed (cm)	**Weigh of the enzyme (g)**	**Oil flow (mL/min)**	**Methanol flow (mL/min)**	**CO_2 flow (g/min)**	**Assay time (min)**	**Pressure (MPa)**	**Temperature (K)**	**Solubility (g oil/Kg CO_2)**	**Oil:methanol ratio**
2	3,97	0,46	0,1184	127	40	25,1	313,15	3,5	1:6
13,4	21,97								
22	38,46								

2.2.2 – Carotenoids production

2.2.2.1 – Carotenoids extraction from tomato pomace in high-pressure installation with supercritical CO_2 and 5% ethanol as co-solvent

At first an amount of tomato pomace was introduced in the reactor (height = 33 cm, inner diameter = 2,8 cm), the assay condition is on Table 2.4. The process was preceded in the high-pressure installation 1.

The carbon dioxide (process solvent) in a gas-phase passes through a cold-water bath, cooled by two cryostats (JP Selecta, s.a.), becoming liquid so it can be pumped, with a determinate mass flow (65 g/min), by a liquid pump (Nikkiso) in the system. The liquid CO_2 passes through a vessel and is measured with a flow meter (Rheonik, 01.08). The solvent is heated by a heat exchanger heating from a water bath (Julabo ED). The ethanol (co-solvent of the process), in liquid phase, is pumped into the system (Gilson), where it joined the solvent in a static mixer. The two compounds then enter into the extractor, also heated, which are the residue (tomato pomace). The pressure is controlled by a BPR (Tescom Europe). It's important to refer, that since the CO_2 and ethanol pump to the BPR, the solvent is in a supercritical homogeneous phase due the temperature (313,15 K) and pressure (30 MPa) imposed on the system. After the BPR, the product is collected in a separator, where the pressure is approximately equal to 4,5 MPa and temperature of 318.15 K. In this separator, the CO_2 is separated from the product by a pressure drop returning to a gas-phase (the solvent is recirculate into the system) while the product precipitates and his extraction is possible in liquid form.

Table 2.4 – Assay condition for the carotenoids extraction with supercritical CO_2 and ethanol as co-solvent.

Weigh of tomato pomace (g)	Ethanol flow (g/min)	CO_2 flow (g/min)	Assay time (min)	Pressure (MPa)	Temperature (K)
26,9	0,20	65	180	30,1	313,15

2.2.2.2 – Carotenoids extraction from tomato pomace in high-pressure installation with supercritical CO_2 and without co-solvent.

At first an amount of residue (tomato pomace) was weighed and putting inside the extractor (height = 55 cm, inner diameter = 2,5 cm) the assays conditions were on Table 2.5. The process was preceded in the high-pressure installation 2.

The carbon dioxide (process solvent) in a gas-phase passes through a cold-water bath, cooled by a cryostat (JP Selecta, s.a.), becoming liquid so it can be pumped, with a determinate mass flow by a compressed air pump (Maximator) into the system. The solvent was heated by ribbons (Horst GmbH) and entered into the extractor, also heated with ribbons, which are the residue (tomato pomace). The pressure is controlled by a BPR (Tescom Europe). After the BPR, the product is collected in a separator, where the pressure is approximately equal to 4,5 MPa and temperature of 318.15 K. In this separator, the CO_2 is separated from the product by a pressure drop returning to a gas-phase (the solvent is recirculate into the system) while the product precipitates and his extraction is possible in liquid form.

Table 2.5 – Assays conditions for the carotenoids extraction with supercritical CO_2.

Weigh of tomato pomace (g)	CO_2 flow (g/min)	Assay time (min)	Pressure (MPa)	Temperature (K)
18,34	6,0	140	35,1	313,15
16,43	7,7	105	50,1	313,15

2.3 – SAMPLE ANALYSIS

2.3.1 – Analysis of oil and FAME

2.3.1.1 – Determination of unsaponifiable matter of spent coffee ground oil

Unsaponifiable matter includes those substance frequently found dissolved in oils and fats, wish can't be saponified by the usual caustic treatment, but are soluble in ordinary fat and oil solvent. Included in this groud of compounds are higher aliphatic alcohols, sterols, pigments and hydrocarbons. (91)

To analyse unsaponifiable matter existent in the SCG oil, was used the modified AOCS method Ca 6a-40 (2009). Thus, 5 mg of oil were put into Erlenmeyer flask with 30 mL of 95% ethyl alcohol and 5 mL of 50% aqueous potassium hydroxide solution. The round botton flask was boiled under reflux for 1h (until completely saponified). After, the mix of compounds was transferred to the extraction cylinder and washed with 95% ethyl alcohol, cold and warm water (until completed a total volume of 80 mL). Then, added 50 mL of petroleum ether and proceeded to a layer separations with a glass siphon and removed the upper layer (repeated 6 times). The combined extracts (upper layer) were washed with 10% ethyl alcohol until the wash solution no longer gives a pink color after the addition of one drop of phenolphthalein solution. Finally, transferred the petroleum ether extract to a tared beaker and evaporated all the solvent and weighed. The results become the "A", Equation 2.1, in the calculation of percentile unsaponifiable matter.

The "B' of the Equation 2.1, correspond the blank, and it was determinate by repeating the unsaponifiable matter procedure above described but without the 5 mg of oil.

Figure 2.19 – Analysis of unsaponifiable matter. **A-** Apparatus to procede the saponification; **B-** Oil and compounds into the round botton flask ; **C**- Separtion of the upper layer with a extraction cylinder.

$$\text{Unsaponifiable matter, \%} = \frac{A-B}{\text{mass of test portion,g}} \text{ x } 100$$

Equation 2.1 – Equation to calculate the percentile of unsaponifiable matter (Legend: A – mass of residue(g); B – mass of blank (g))

2.3.1.2 - Gas cromatography

Gas chromatography technique is based on the passage of the compounds through a capillary column by the flow of inert gas which consists in the mobile phase. This inert gas can be He, Ar, N etc. The column contains a stationary liquid phase absorbed to the surface in an inert solid where the sample components are retained. The compounds can be separated according to their affinity (polarity) for the stationary phase or its boiling point.

The fatty acid profil of SCG oil and microalgae *Chlorella prothecoides* oil were determinded with a direct transesterification using the Lapage and Roy method (chapter 2.3.1.2.1). The FAME, triglycerides (TG), diglycerides (DG), monoglycerides (MG) were determined by PTV method (chapter 2.3.1.2.2) and on-Column injection (chapter 2.3.1.2.3).

Figure 2.20 – Gas chromatography equipment - THERMO SCIENTIFIC TRACE GC ULTRA.

2.3.1.2.1 – Fatty acid profile of spent coffee ground oil and microalgae *Chlorella protothecoides* oil – Direct transesterification by *Lepage & Roy*

To analyse fatty the acids contents of fat existent in the extracted SCG oil, was used the direct transesterification derived by *Lepage* and *Roy* method, 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 in hexane (5mg/mL) internal standard solution. The mixture, without any contact with light, was heated (at 353,15-358,15 K) for 1 hour. After, the mixture was cooled at room temperature and then was diluted with 1 mL of *n*-heptane and 1 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 (to remove the water) and filtered, collecting the sample. Finally, the sample was analyzed with gas chromatography (GC).

The typical chromatogram obtained is showed in Figure 2.19. Peak identification was carried using known standards and the software Chorm-Card. By the linearization of the peak areas it was possible to correlated the peak with their molar concentrations of fatty acids. The calibration curves are on Appendix A.

2.3.1.2.2 – Determination of total FAME and linolenic acid methyl in Biodiesel from spent coffee ground oil and microalgae *Chlorella protothecoides* oil transesterification – PTV method (EN 14103)

The determination of total FAME and linolenic acid methyl in Biodiesel are essencial to put the product on the market or not. In accordance with the specifications reported in EN 14214:2003 the biodiesel can be put on the market if the esters contente is greater than 96,5% m/m and the linolic acid methyl ester content is lower than 12% m/m. [92]

The EN 14103 is a standard method for determination of esters and linolenic acid methyl ester and can be applied to biodiesel analysis and it requires GC analysis with a programmable temperature vaporizing (PTV) injector and a wax column for a detailed separation of FAMEs. Calculation of the percentage of FAME is achieved with internal standard calibration. This method is suitable for FAMEs which contain methyl esters between C14:0 and C24:0. [92]

Thus, 25 mg of biodiesel extract was weighed in a 10 mL vial, then 500 μL of methyl heptadecanoate internal standard solution was added. The methyl heptadecanoate internal standard solution was made by 100 mg of methyl heptadecanoate and 10 mL of heptane.

The typical chromatogram obtained is showed in Figure 2.21. The percentil of molar concentrations of total FAME and Linolenic Acid Methyl were calculated with the Equation 2.2 and Equation 2.3.

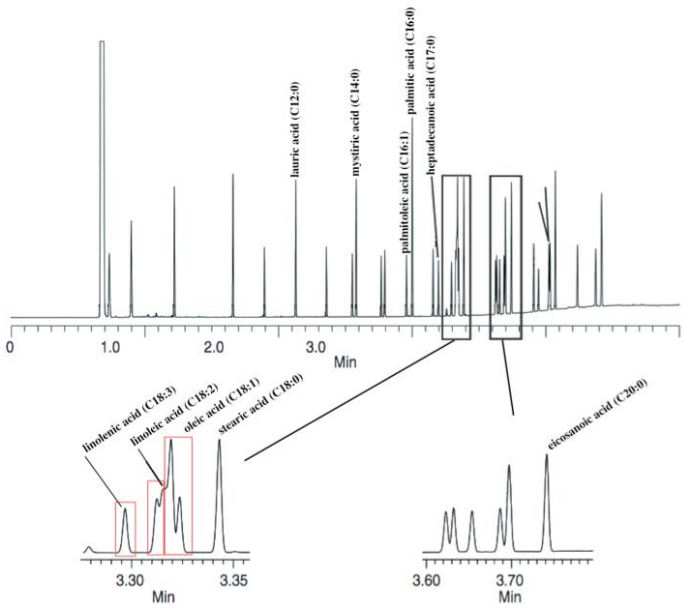


Figure 2.21 – Typical chromatogram obtained by GC for oil analysis by PTV.

$$Total\ FAMEs\ (\%m/m) = \frac{A_{total}}{A_{IS}} * \frac{[Sample]}{[IS]} * 100$$

Equation 2.2 – Equation to calculate the percentil of molar concentrations of total FAMEs. (Legend: A_{total} – Total area ; A_{IS} – Area of internal standard ; $[Sample]$ - Concentrarion of the sample; $[IS]$ – Concentrarion of internal standard)

$$Linolenic\ Acid\ Methyl\ (\%m/m) = \frac{A_{C18:3}}{A_{total}} * 100$$

Equation 2.3 – Equation to calculate the percentil of molar concentrations of Linolic Acid Methyl. (Legend: A_{total} – Total area ; $A_{C18:3}$ – Area Linolenic Acid Methyl)

2.3.1.2.3 – Determination of FAME in Biodiesel from spent coffee ground oil transesterification – On Column method (EN 14105)

The determination of free fatty acids and glycerine levels provides verification that the free glycerin, mono-glycerides (MG), di-glycerides (DG) and tri-glycerides (TG), and total glycerin contentes in pure biodiesel (B100) are lower than the limits shown in the Table 2.6, in accordance with the specification reported in EN 14214:2003. This determination is essencial to know the quality of the product.[93] With this process, the FAME concentration can also be calculated.

Tabela 2.6 – Free fatty acids, glycerine and total glycerin specification according to EN 14214:2003

Compounds	Max % m/m (EN 14214:2003)
Free glycerin (G)	0,02
Mono-glycerides (MG)	0,8
Di- glycerides (DG)	0,2
Tri- glycerides (TG)	0,2
Total glycerin (G+0,255MG+0,146DG+0,103TG)	0,25

The EN 14105 is a standard method for determination of free and total glyceryn can be applied to biodiesel analysis and it requires GC analysis with a non-discrimininative injection system able to transfer both volatile and heavy compounds without discramination or degradation. In this work it was usad A TRACE GC Ultra equipped with a true cold On-column inlet and a flame ionization detector (FID), automated by a TriPlus Autosampler for liquids controlled through the Thermo Scientific Chrom-Card data system. The analytical column used is a non-polar Thermo Scientific TRACE TR-BIODIESEL(G), 10 m, 0.32 mm ID, 0.1 µm f.t. [93]

Thus, two internal standards (IS) are required:

- 1,2,4-butanetriol (IS1) to determinate glycerine;
- Tricaprin (IS2) to determinate MG, DG and TG.

The sample preparation required the following steps:

- 100mg of homogenized sample were weighed and then 80 µL of IS1, 100 µL of IS2 and 100 µL MSTFA* were added;
- The mixture was shaken 1 minute;
- After 15 minutes, 8 ml of n-heptane were added;
- 1 ml of the mixture were transferred to a vial and analyzed. The typical chromatogram obtained is showed in Figure 2.23.

This method also requires four calibration curves – glycerin, mono-olein, dio-olein and trio-olein. These calibration curves are represented on the Appendix B.

* MSTFA (N- methyl-N-trimethylsilyltrifluoroacetamide) – is a compound that derivatives the MG, DG and TC and glycerol into more volatile silytaled, due the low volatility and activity of these compounds.

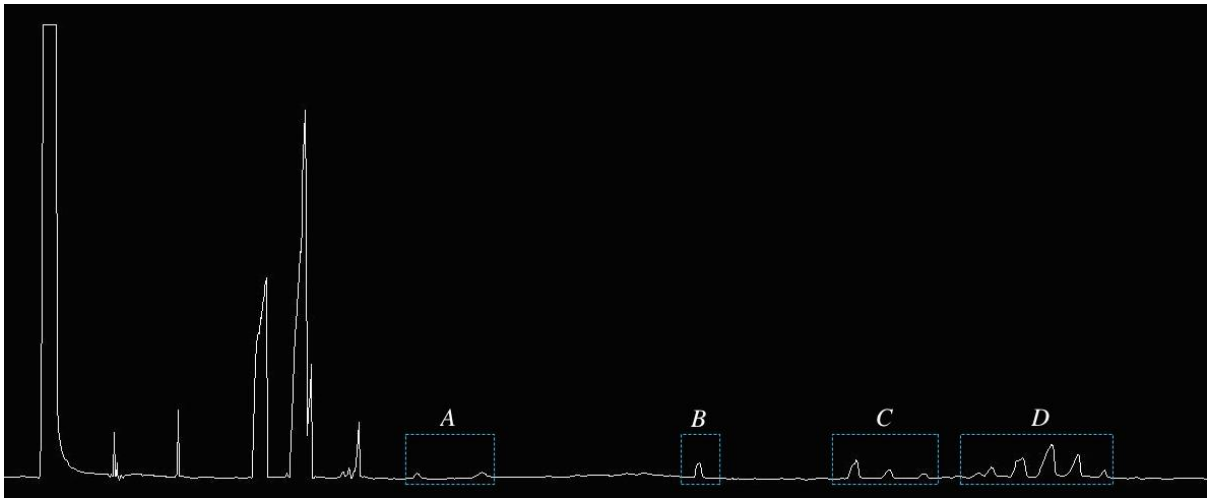


Figure 2.22 – Typical chromatogram obtained by GC for oil analysis by on-column. Legend: A – Monoglycerides; B – IS 2; C- Diglycerides; D – Triglycerides.

2.3.2 – Analysis of carotenoids

2.3.2.1 – Oil extraction of tomato pomace with organic solvents

2.3.2.1.1 - Soxhlet extraction

Tomato pomace was dried in a lyophilizer (Christ alpha 1-4) for 3 days.

The percentil of lipid contained in tomato pomace was obtained through soxhlet extraction that is an extraction method that uses organic solvents. As it can be seen on Figure 2.24, this apparatus has 3 compartments:

- Boiling round-bottom flask - to store the extracting organic solvent
- The extraction chamber (soxhlet extractor) – in which a packet of residue is inside and where the gas (from the solvent) goes through.
- Condenser.

The round-bottom boiling flask is heated by a heating mantle (JP-select) and the organic solvente evaporates and moves up into the condenser where it condensed and trickle backs into the extraction chamber containing the sample. The extraction chamber is designed so that when the organic solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling flask. This cycle is repeated to guarantee that no more lipids can be extracted from the residue.

Extraction was performed during 4h with hexane (1g of residue to 100ml organic solvent) as extraction solvent, and extraction temperature was 343,15-353,15 K (hexane boiling point). [(94)]

At the end of the extraction process, the flask containing hexane and lipid is removed. The hexane is then evaporated from the extracted oil with nitrogen.

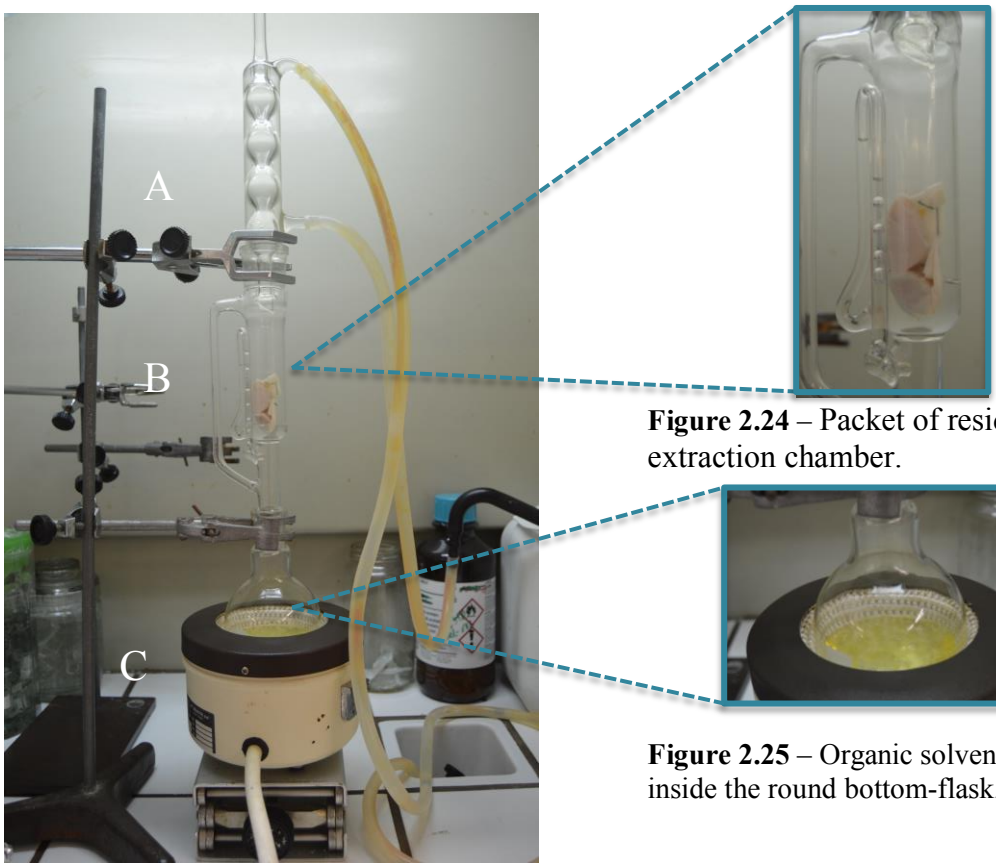


Figure 2.24 – Packet of residue inside the extraction chamber.

Figure 2.25 – Organic solvent and oil extracted inside the round bottom-flask.

Figure 2.23 – Soxhlet extraction apparatus. **A** – Condenser ; **B** – Extraction Chamber (Soxhlet extracor); **C** - Boiling round-bottom flask.

Figure 2.26 – Tomato pomace oil obtained through soxhlet extraction with organic solvent.

2.3.2.1.2 - Bligh and Dyer method

Tomato pomace was dried in a lyophilizer (Christ alpha 1-4) for 3 days.

To perform the lipid extraction from tomato pomace, was used the Bligh and Dyer modified method. This method is characterized to be a cold process and phases separation, where it use a methanol:chloroform:water (10:5:4) solution. The lipids are in the chloroform phase, which can be removed by evaporation.

Firstly, 2g of residue was added to 200ml of methanol:chloroform:water (10:5:4) solution and then was submitted to a magnetic stirring, overnight, or until complete lipid extraction. Subsequently, the solvents mixture with lipids was filtrated and is placed into a separatory funnel. Afterward, the volume is corrected: per 100ml of methanol:chloroform:water (10:5:4) is added 60ml of methanol:chloroform (1:1). Then, the separatory funnel was shaked gently and let stand until two phases are completely formed. Finally, the lower phase (containing lipids) is collected and, subsequently, the organic solvent is evaporated on a rotavapour.[95]

Figure 2.27 – Bligh and Dyer method. **A** – Tomato pomace stirring in methanol:chloroform:water (10:5:4) solution; **B** – Evaporation of the organic solvent on a rotavapour.

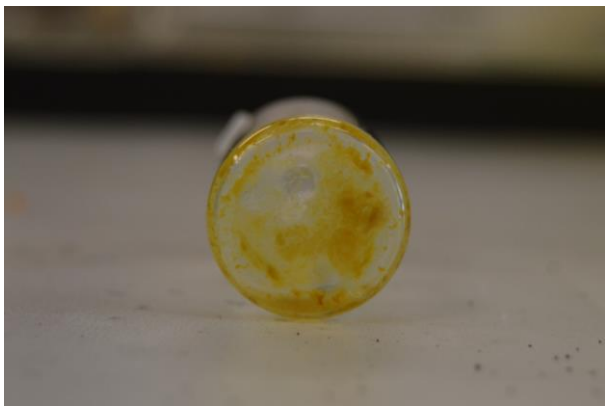


Figure 2.28 – Oil extracted by Bligh and Dyer method from tomato pomace.

2.3.2.1.3 - Acetone method

Tomato pomace was dried in a lyophilizer (Christ alpha 1-4) for 3 days.

To perform the lipid extraction with acetone, were heighed 3g of tomato pomace and put inside an erlenmeyer. Then extracted under agitation for 16 h with 55ml of acetone and the organic solvent was evaporated with gaz nitrogen.

2.3.2.2 – Antioxidant activity – 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical. On accepting hydrogen from a corresponding donor, its solutions lose the characteristic deep purple (λmax 515–517 nm) colour. DPPH is very popular for the study of natural antioxidants. The antiradical activity of tested compounds is expressed as a relative or absolute decrease of concentration of DPPH or as EC_{50} (concentration of a compound decreasing the absorbance of a DPPH solution by 50%). [95]

Dry extracts were dissolvel in 350μl deionized water with 20% of acetonitrilo and mixed with 350μl DPPH solution (in absolutee ethanol with a concentration of 750μM). The mixture was incubated for 30 min in the dark at room temperature and the absorvande was monitored at 517nm with a spectrophotometer. The extracts were analyzed at final concentrations ranging 10-250 μg/ml.[96]

The inhibition of the free radical DPPH, directly related to DPPH bleaching if each sample was calculated with the equation 2.4.

$$\%\ of\ inibition = \frac{A_O - A_s}{A_o}\ x\ 100$$

Equation 2.4 – Equation to calculate the inibition of the free radical DPPH. Legend: A_O – absorbance of the DPPH, A_s – absorbance of the sample.

2.3.2.3- High performance liquid chromatography

A conventional technique for the qualitative and quantitative quantification of carotenoids is high performance liquid chromatography (HPLC). The analysis of β-carotene, lycopene and luteine content in the extracted oil was conducted with Thermo Scientific (Finnigan Surveyor AutoSampler Plus) HPLC, with a reverse-phase analytical 5-μ particule diameter, polymeric C_{18} column equipped with a UV diode array detector (Accela UV/Vis Detector). The mobile phase consisted of metanol and 0,2% H_2O/acetonitrile (75:25 v/v). Total run time was 30 min, with an injection volume of 5 µl. β-carotene and lycopene was monitored at 450 nm at a flow rate of 1 mL/min, and was quantified using a calibration curve (Appendix C) of the corresponding standard compound (trans-β-carotene – 95% type I, Sigma) at the specific absorption maximum.

Figure 2.29 - High Performance Liquid Chromatography equipment

2.3.2.4- UV spectrophotometry

UV Spectrophotometry (DU® 800 Spectrophotometer, Beckman Coulter) analysis was used to determine β-carotene content in the samples, through the beer-lambert law. Spectra was run between 250 and 700 nm, and the concentration was determined at λ_{max} equal to 440, 454 and 474 nm, according to the specific optical coefficient of luteine, β–carotene and lycopene.

β-carotene and lycopene was quantified using a calibration curve (Appendix D) of the corresponding standard compound (trans-β-carotene – 95% type I, Sigma) at the specific absorption maximum.

Figure 2.30 - UV Spectrophotometry equipment

CHAPTER 3: RESULTS AND DISCUSSION

3.1 – Biodiesel production from spent coffee grounds and microalgae *Chlorella protothecoides*

The objective of the first part of this work is the production of biodiesel through a direct enzymatic transesterification reaction in supercritical dioxide carbon ($scCO_2$). The oil used for the production of biodiesel is from SCG.

For these series of experiments the equipment used was the high-pressure installation 1, which is described on the chapter 2.1.2.1 (Figure 2.2/2.3). The conditions used on the assays of oil extraction are exposed on the chapter 2.2.1.1 and for the biodiesel production on the chapter 2.2.1.2.

To complete this work, the SCG oil used on the biodiesel production was extracted with $scCO_2$. The extraction yield is equal to 9,9% and the curve of accumulated oil extracted versus time is represented in the Figure 3.1 (chapter 3.1.1).

So, the main study was focused on the enzymatic direct transesterification reaction in $scCO_2$. For this study we varied two parameters:

- The residence time (amount of the enzyme into the reactor)
- The molar ratio between oil and methanol (the flow rate of methanol on the reaction).

After the experiments proceeded, it was obtained 98,0% of FAME (biodiesel) with a molar ratio oil:methanol equal to 1:24 and a 0,8 min as residence time.

Additional experiments were performed for the production of a third generation biodiesel using biomass – microalgae *Chlorella protothecoides*. The yield of biodiesel achieved was 66,6% for a molar ratio oil:methanol 1:6 and a residence time 4,41 min.

3.1.1 – Extraction with supercritical CO_2 of oil from spent coffee grounds

For the series of extractions, the configuration of the installation used was described in the Figure 2.1 and the conditions used were described in the chapter 2.2.1.1. It is important to refer that the condition for the extractions are based on studies[98] already performed before and was considered as optimal for maximum yield of the global extraction process.

The yield of oil extracted indicates the quantity of triglycerides (TG) extracted in the process from the initial oil in the SCG. It was measured from the amount of oil extracted and the quantity of residue in the reactor, equation 3.1. The curve of accumulated oil extracted versus extraction time is show on the Figure 3.1.

$$Extraction\ yield\ \% = \frac{mass\ total\ of\ residue\ -\ mass\ total\ of\ oil\ extracted\ (g)}{mass\ total\ of\ residue\ (g)}\ x\ 100$$

Equation 3.1 – Equation to calculate yield of extraction.

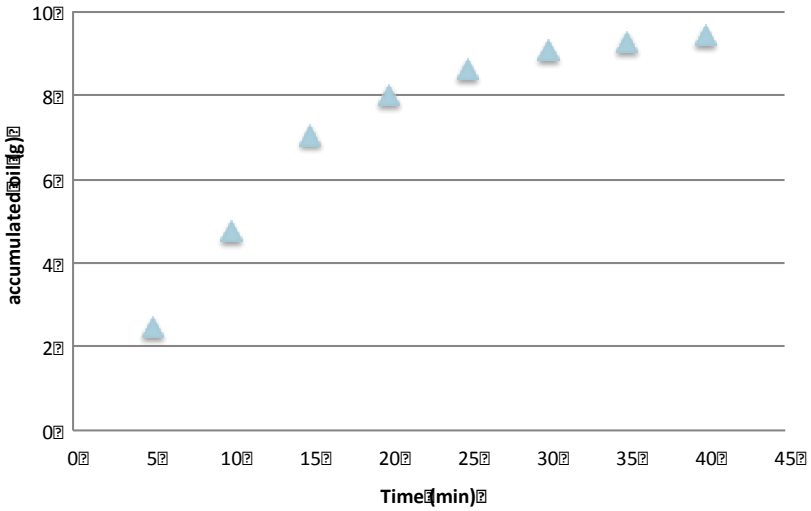


Figure 3.1 - Curve of accumulated oil extracted versus extraction time.

By the Figure 3.1 it is possible to affirm that from 30 minutes, the process reached a stationary level where the yield of extraction is maximum and equal to 9,9%, corresponding to, approximately, 9 g of oil extracted. This yield is in accordance with the literature[98].

Figure 3.2 – SGC oil extracted by extraction with supercritical CO_2.

The unsaponifiable matter and the fatty acid of the SCG oil extracted were analyzed. The unsaponifiable matter, where the method is described on the chapter 2.3.1.1 was 0,68% that is in accordance with the literature.[99] The fatty acid profile was analyzed by the Lepage and Roy method, described on the chapter 2.3.1.2.1, and it containd three satured lipids - lauric acid, palmitic acid and stearic acid; and three unsatured lipids – oleic acid, linelic acid and linolelicacid.

3.1.2 – Biodiesel production from SCG

The main objective of the first part of this work is the production of biodiesel from SCG by enzymatic direct transesterification in $scCO_2$.

As said previously, the study of the biodiesel production was focused on the enzymatic direct transesterification where we varied the residence time and the molar ratio between oil and methanol. The temperature and pressure was constant in all the assays and was chosen by studies realized before. The apparatus of the high-pressure installation used is in the Figure 2.3 and the method and condition are described on the chapter 2.2.1.2.

One of the parameter studied in this work was the residence time. For this study, the amount of enzyme used for the enzymatic transesterification was varied into the reactor. The enzyme used was the lipase Lipozyme RM IM ®. Lipases as very often used as an alternative for the conventional process due to many reasons, as it is described on the chapter II – 1.4. However and despite that are used an immobilized enzyme, it is still be a big investment due the enzyme costs. So it is necessary to optimize the assay conditions to obtain a maximum yield. A lower reaction yield can result from the interference in the enzyme activity. Some examples, the interference can be the exposure to alcohol that can modify the normal enzyme structure or the active center of the enzyme is occupied and they are not able to convert more triglycerides.[100]

The residence time (τ_r) is the average amount of time that the CO_2, and consequently the methanol and oil, spends in the enzyme into the reactor. This measurement varies directly with the amount of enzyme into the reactor and can be calculate with the equation 3.2. This component is important not only to verify the activity of the enzyme to that time of exposure, but also for the design of the reactor with an optimal size (too big represent excessive cost, too small in which the residence time is insufficient for achieving the expect yield). So, taking into account all these parameters, it can be made optimizing the investment and production costs.

$$\tau_r = \frac{\rho_{CO2}}{Q_{CO2}} * V_{enzyme\ into\ the\ reactor}$$

Equation 3.2 – Equation to calculate the residence time. Legend: ρ_{CO2} – Density of the dioxide carbon (0,89 g/cm^3), Q_{CO2} - Flow of the dioxide carbon (g/min), $V_{enzyme\ into\ the\ reactor}$ - Volume of enzyme into the reactor (m^3).

The volume of enzyme into the reactor is calculated with the subtraction of the volume of the reactor, $V_{reactor}$, (Equation 3.3) and the volume of enzyme, V_{enzyme}, (Equation 3.3).

$$V_{reactor} = \pi r^2 h$$

Equation 3.3 – Equation to calculate the volume of the reactor. Legend: r – internal radius of the reactor (cm), h - height of enzyme into the reactor (cm), $V_{reactor}$ - Volume of the reactor (m^3).

$$V_{enzyme} = \frac{m_{enzyme}}{\rho_{enzyme}}$$

Equation 3.4 – Equation to calculate the volume of enzyme. Legend: ρ_{CO2} – Density of the enzyme (0,42 g/cm^3), m_{CO2} – weigh of enzyme into the reactor (g), V_{enzyme} - Volume of enzyme (m^3).

The transesterification reaction needs a molar ration of oil:alcohol 1:3 to be complete, but it is generally used an excess of alcohol to ensure that the reaction equilibrium is shifted in the reaction of the product formation (direct transesterification). The alcohol used in this work was methanol, because is the least expensive alcohol and is widely used for biodiesel production. Nevertheless, it is stated that methanol can have an inhibitory effect to the enzyme activity at high concentration. [101, 102]

The experiments was based on the molar ratio oil:methanol equal to 1:147, and it was tested the following residence times: 0,97; 2,7 and 11,1 minutes, the condition used are written on the Table 2.3, chapter 2.2.1.2. On the table 3.1 are exposed the yield of FAME obtained in each residence time and on the Figure 3.4 it's described the yield of FAME along the assay. The yield of FAME was analyzed by PTV method, chapter 2.3.1.2.2.

Table 3.1 – Yield of FAME and linolic acid methyl ester (C18:3) content obtained in each residence time tested with the molar ratio equal to 1:147.

Molar ratio 1:147				
Weigh of enzyme (g)	**Residence Time (min)**	**Average FAME % m/m**	**Max. FAME % m/m**	**C18:3% m/m (EN 14214:2003)**
9,9	0,97	21,2 +/- 0,4	21,6	0,4 +/- 0,03
20,5	2,7	21,4 +/- 0,5	21,8	0,4 +/- 0,04
88,5	11,1	66,5 +/- 1,2	67,7	0,5 +/- 0,1

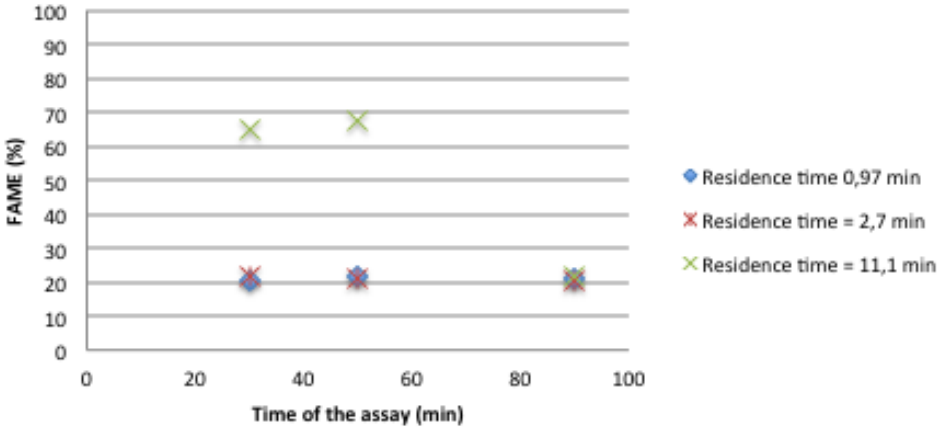


Figure 3.3 – Yield of FAME along the enzymatic transesterification reaction in each assay (molar ratio 1:147)

Analyzing the Table 3.1, the best yield of FAME was achieving when the residence time was 11,1 minutes with 67,7% at 50 min of assay. Despite the content of C18:3 is less than 12%, the percentile of FAME does not reach the lower limit, 96,5%, of ester to be commercialized. About the Figure 3.4, the third point of the resistance time equal to 11,1 min curve (90 min, 21,83 %) can be considered as an experimental error.

The low yield obtained in these experiments can result from the high concentration of methanol. A high concentration of methanol causes a denaturation of the enzyme. So, the loss of the enzyme available reduces the rate of transesterification that it causes an impact on the yield of FAME. So, in this case, it is possible to affirm that the decrease of production of biodiesel is caused by denaturation of the enzyme resulting in a reduction of its activity.[103]

To proceed with the next experimental work, and take in account the results, the molar ratio oil:methanol has decreased to equal to 1:37. It was tested the following residence times: 0,4; 1,5; 2,7; 5,0 and 10,5 minutes and the condition of the assays were the same of the previous experimental work. On the table 3.2 are exposed the yield of FAME obtained in which residence time and on the Figure 3.5 it is described the yield of FAME along the assay. The yield of fame was analyzed by PTV method, chapter 2.3.1.2.2.

Table 3.2 – Yield of FAME and linolic acid methyl ester (C18:3) content obtained in each residence time tested with the molar ratio equal to 1:37.

Molar ratio 1:37				
Weigh of the enzyme (g)	**Residence time (min)**	**FAME % m/m**	**Max FAME % m/m**	**C18:3 % m/m (EN 14214:2003)**
3,5	0,40	27,1 +/- 1,9	29,6	0,55 +/- 0,06
14,4	1,5	33,9 +/- 3,5	37,9	0,42 +/- 0,03
20,5	2,7	32,1 +/- 4,9	37,5	0,50 +/- 0,03
42,8	5,0	86,7 +/- 2,7	90,1	0,34 +/- 0,01
86,8	10,4	95,8 +/- 3,5	99,6	0,29 +/- 0,02

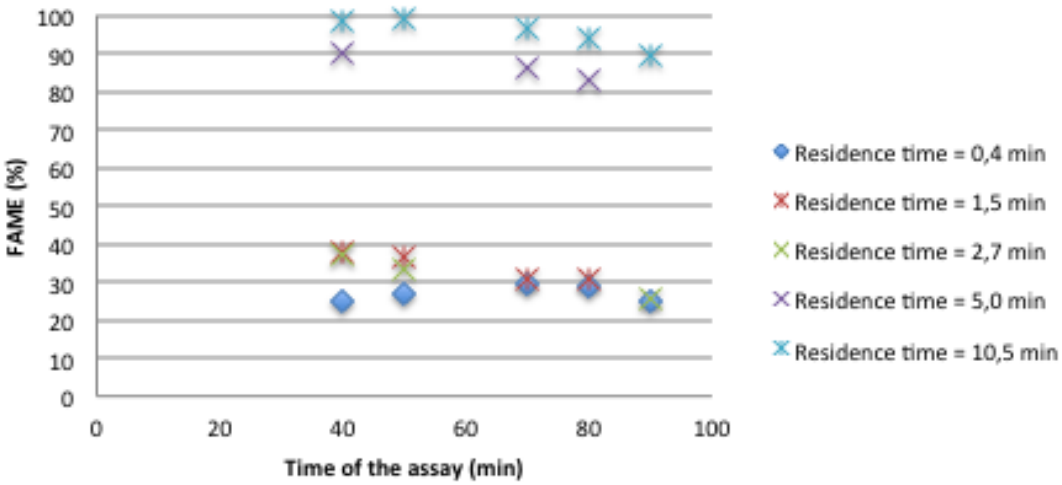


Figure 3.4 – Yield of FAME along the enzymatic transesterification reaction in each assay (molar ratio 1:37).

Analyzing the Table 3.2, the maximum avarege yield of FAME was achieving when the residence time was 10,4 minutes with 95,8%. Despite the content of C18:3 is less than 12%, the percentage of FAME does not reach the lower limit, 96,5%, of ester to be commercialized. However, observing the Figure 3.5, it is possible to see that the maximum yield was obtained at the 50 minutes with 99,6% for the residence time equal to 10,4 minutes, afterwhich the yield decreased.

The decreasing of FAME yield after 50 min can be related with the long exposure of enzyme to glycerol or methanol resulting in the enzyme loss of activity; or the active centers can be all partially occupied reducing the rate of reaction.

An enzyme has a certain number of active centers, where the reaction takes place. When these actives centers of the lipase are occupied, the enzyme is not able to convert more triglycerides into FAME, which results in a drop of the yield.

It was impossible to quantify the quantity of glycerol, but this compounds appeared in few chromatograms and it was possible to see it in some extracts, Figure 3.5. Glycerol molecules can drop the activity of lipases immobilized, because these molecules can be absorbed on the surface of the matrix thereby forming a hydrophilic coating that make enzyme molecules inaccessible to hydrophobic substrates (glycerides).[104]

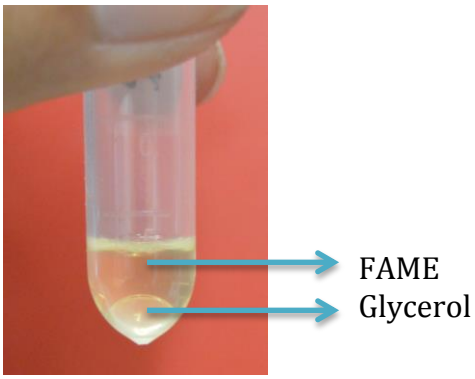


Figure 3.5 – Extract of FAME obtained by $scCO_2$ from SCG.

In this experiments, it is possible to observe that for the residence time equal to 0,4; 1,5; 2,7 min, the convertion of FAME is approxatetly the same. As said previously, this fact can be due from the low quantity of enzymes into the reactor and the flow rate of the solvent did not permit that the enzymes have enough time to converte the oil into biodiesel.

The last experiments were based on previous results. The reaction time was reduced to 50 minutes, to obtain the maximum yield, the molar ratio oil:methanol was reduced to 1:24, the enzyme was renovated and the conditions used are shown in the Table 2.3, chapter 2.2.1.2. The residence times tested were 0,3, 0,4 and 0,8 minutes. The yield of fame was analyzed by PTV method, chapter 2.3.1.2.2, and on-column method, chapter 2.3.1.2.3.

Table 3.3 – Yield of FAME and linolic acid methyl ester (C:18) content obtained in each residence time tested with the molar ratio equal to 1:24.

Molar ratio 1:24				
Weigh of enzyme (g)	**Residence Time (min)**	**Average FAME % m/m**	**Max. FAME % m/m**	**C18:3% m/m (EN 14214:2003)**
15,2	0,3	88,2 +/- 2,1	91,1	0,6 +/- 0,01
34,9	0,4	88,0 +/- 1,0	96,6	0,7 +/- 0,03
52,0	0,8	98,0 +/- 6,3	99,3	0,8 +/- 0,01

Table 3.4 - Free fatty acids and total glycerin contente obtained in each residence time testes with the molar ratio equal to 1:24.

EN 14214:2003				
Weigh of enzyme (g)	**MG % m/m**	**DG % m/m**	**TG % m/m**	**Total Glycerin % m/m**
15,20	0,08 +/- 0,020	0,02 +/- 0,005	0,02 +/- 0,009	0,03 +/- 0,006
34,96	0,01 +/- 0,007	0,02 +/- 0,007	0,05 +/- 0,040	0,01 +/- 0,005
52,03	0,01 +/- 0,001	0,01 +/- 0,006	0,01 +/- 0,013	0,01 +/- 0,002

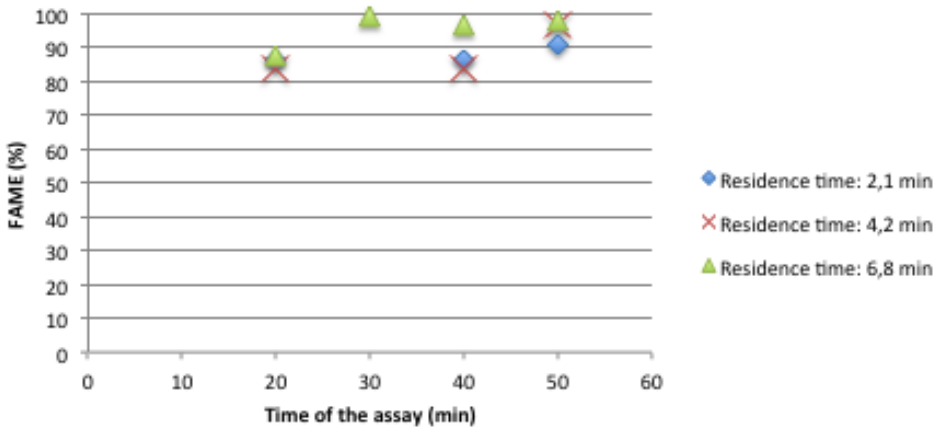


Figure 3.6 – Yield of FAME along the enzymatic transesterification reaction in each assay (molar ratio 1:24)

Analyzing the Table 3.2, the best yield of FAME was achieving when the residence time was 0,8 minutes with 98,0%, so the percentile of FAME reach the lower limit to be commercialized and the specification EN 14214:2003 are met.

Table 3.5 – Comparative results at the same residence time and different assays conditions

Molar ratio oil:methanol	Residence time	Average of FAME % m/m	Max. of FAME %m/m
1:147	2,7	21,4	21,8
1:37	2,7	32,1	37,5
1:24	0,8	98,0	99,3

As it possible to observe on the Table 3.5, the molar ratio oil:methanol is a very important parameter to take in account for the production of biodiesel with enzymes, because he cause an inhibition on the enzyme activity. To conclude this study, it would be interesting to perfom some assays with a molar ratio oil:methanol equal to 1:6.

Comparing all the result obtained and taking into account a high performance, a low cost of production and a product that attempt to all the specification to be commercialized, the better result obtained was 98,01% of FAME with the follow condition: molar ratio oil:methanol equal to 1:24, residence time equal to 0,8 min, 25 MPa, 313,15 K and 3 g oil/ kg CO_2.

3.1.3 – Biodiesel production from SCG microalgae *Chlorella protothecoides*

Nowadays, the biofuels from the third generation can be a good solution for the substitution of the traditional fuels. These biofuels use biomass as feedstock to produce, for example, biodiesel. In this work it was used the microalgae *Chlorella protothecoides*.

As said before, this part of the thesis was focused on a previous work. This study showed that the oil extracted from microalgae *Chlorella protothecoides* is rich on lipids and carotenoids.[105]

The condition of the assays for the production of biodiesel are exposed on the Table 2.3, chapter 2.2.1.2, the molar ratio oil:methanol was equal to 1:6, due to be a common ratio for preliminary assays and the resistance times varied into 0,4 and 4,4 min. The results obtained are exposed on the Table 3.5 and the Figure 3.7.

Table 3.6 – Yield of FAME and linolic acid methyl ester (C:18) content obtained in each residence time tested with the molar ratio equal to 1:6.

Molar ratio 1:6				
Weigh of enzyme (g)	**Residence Time (min)**	**Average FAME % m/m**	**Max. FAME % m/m**	**C18:3% m/m (EN 14214:2003)**
3,97	0,4	21,0 +/- 0,28	21,5	0,41 +/- 0,017
21,97	2,7	24,82+/- 0,67	25,6	0,35 +/- 0,008
38,46	4,4	66,6 +/- 2,48	69,0	0,80 +/- 0,003

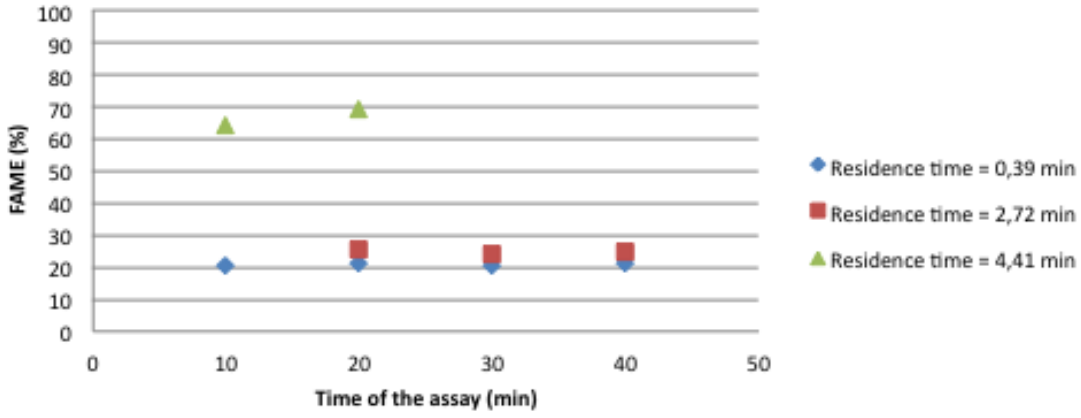


Figure 3.7 – Yield of FAME along the enzymatic transesterification reaction in each assay (molar ratio 1:6)

Analyzing the results obtained, it was not reached the lower limit of FAME to be commercialized, 96,5%, yielding 66.55% after 40 min of reaction time and a molar ratio oil:methanol equal 1:6 and residence time as 4,4 min. The low yield of FAME could be due of the composition of the oil. The oil used has a complex composition and it containt a high

concentration of carotenes, which can interfere with the enzyme. However, it would be interesting to perform assays with a higher residence time and different molar ratio oil:methanol, but that was not possible to effectuate due the lack of oil. The oil was obtained by a partnership with the LNEG.

By acid catalysis, traditional method for biodiesel production, it is obtained 100% of FAME conversion using a molar ratio oil:methanol equal to 1:56.[106] Although the results obtained in this work are not the best, but compared to the traditional process and its disadvantages, chapter II-1.4, biodiesel production from enzymatic transesterification and supercritical CO_2 as solvent is favorable. Plus, with a lowest molar ratio oil:methanol and same resistance time (2,7 min) it was achevied a high conversion of FAME than compared with the yield of FAME obtained with SCG. So, it is expected that with a molar ratio oil:methanol a little higher and an adjustment of operating conditions, it is possible to get results as good as or even better than by traditional production and, added to that, be environmentally friendly and sustainable.

3.2 – Carotenoids from tomato pomace

The second part of this work consists on the extraction of oil that contains carotenoids from tomato pomace. The extraction was realized using $scCO_2$. The study was focused on the modification of two parameters of the extraction $scCO_2$ and basing on articles realized. The parameters varied was:

- Pressure;
- Use of co-solvent.

For these series of experiments the equipment for the extractions with co-solvent was high-pressure installation 1, which is described on the chapter 2.1.2.1 (Figure 2.4) and the conditions used are exposed on the chapter 2.2.2.1; the extraction without co-solvent was the high-pressure installation2, which is described on the chapter 2.1.2.1 (Figure 2.5) and the conditions used are exposed on the chapter 2.2.2.2.

To compare the results obtained by extraction with $scCO_2$, it was proceeded extraction of the carotenoids with traditional methods, which they are described on the chapter 2.3.2.

The quantification of carotenoids was realized by HPLC, chapter 2.3.2.2, and UV spectrophotometry, chapter 2.3.2.3.

After the experiments realized, it was observed that at the condition expressed on the chapter 2.2.2.2 and pressure equal to 35,1 MPa and without co-solvent it was obtained a higher yield of oil extracted, 3,4%. Nevertheless, after the HPLC analysis, the result showed that the concentration of carotenoids was higher on the sample of oil extracted at 50,1 MPa without co-solvent. The analysis demonstrated the presence of lycopen (in higher concentration), β-carotene and lutein.

3.2.1 – Extraction of oil from tomato pomace using traditional methods and supercritical CO_2

Oil extraction was first determined by three different extraction method, using organic solvent: Soxhlet, Bligh and Dyer and acetone. The oil extracted was analyzed by HPLC and UV spectrophotometry.

The yield of oil extracted by soxhlet, Bligh and Dyer and acetone were 5,2%, 7,9% and 2,1% respectively. These values are in accordance with the literature. The HPLC and UV spectrophotometry analyzes show that the oil has several carotenoids, as Lutein, β-carotene and lycopene, and this last has a higher concentration, Figure 3.8.

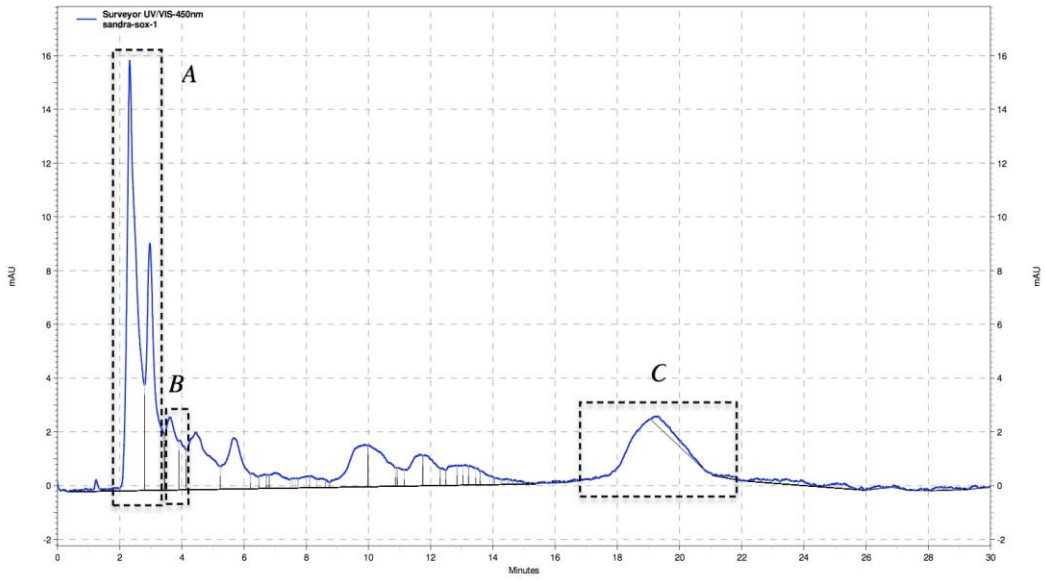


Figure 3.8 – Chromatogram obtained by HPLC – Soxhlet extraction, Legend – A – Lycopene; B – Lutein; C - β-carotene.

The extraction of oil with $scCO_2$ was performed in two different high-pressure installations:

- Extraction with $scCO_2$ and 5% co-solvent (ethanol) (30,1 MPa) was realized in the installation 1, the procedure is described on the chapter 2.2.2.1;
- Extraction with $scCO_2$ without co-solvent was realized in the installation 2 at 35,1 and 50,1 MPa and the procedure is described on the chapter 2.2.2.2.

The pressure and assay condition was chosen after reading several studies.

The yield of oil obtained with the extraction with co-solvent was equal to 2,2% after 180 min of assay. On the Figure 3.9 it is possible to see that after 130 min of assay, the concentration of oil extract start to be constant.

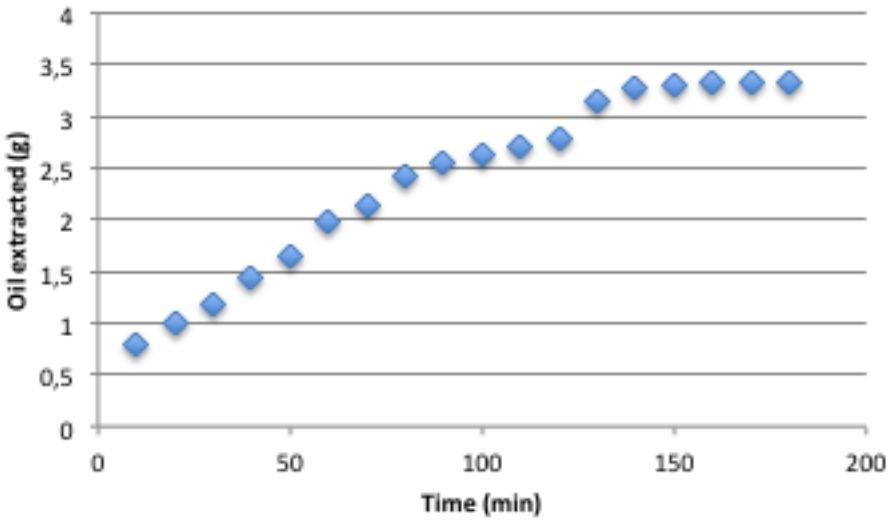


Figure 3.9 - Curve of accumulated oil extracted versus extraction time.

In literature it can be found that with a pressure of 250-450 MPa, with 5% of ethanol as co-solvent and 30 min of assay, the yield of lycopene is 33% (per 100g of dry tomato pomace) and the β-carotene is not extracted.[108] This results are in agreement with the values obtained, as it's possible to see in the Figure 3.10.

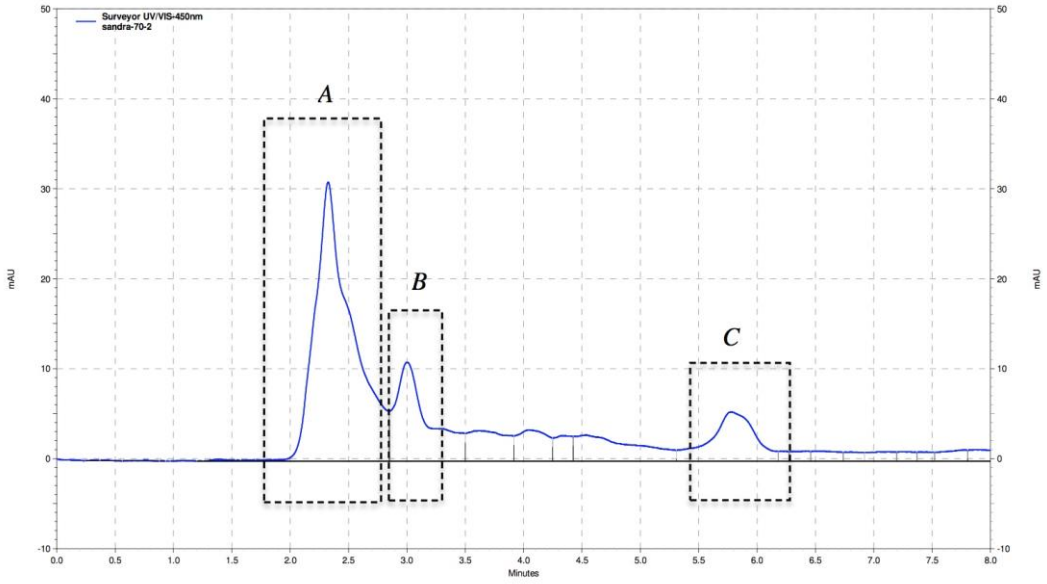


Figure 3.10 - Chromatogram obtained by HPLC that correspond to the assay at 30,1 MPa with co-solvent. Legend – A – Lycopene; B – Lutein; C - β-carotene.

The yields of oil obtained with the extractions without co-solvent were at 3,4% – 35,1 MPa (155 min of assay), and 2,4% at 50,1 MPa (105 min of assay). During the extraction, it is possible to note that the oil extracted is extremely viscous and a high part of the extracted oil stayed in the installation. In all the extract, as it can be seen in the Figures 3.11 and 3.12, it was veriefied the presence of lycopene, lutein and β-carotene. It is possible to verificate that the lycopene is in higher concentration, compareted with the others carotenoids, which is in agreement with the literature.[108] Their quantification was not possible at the time.

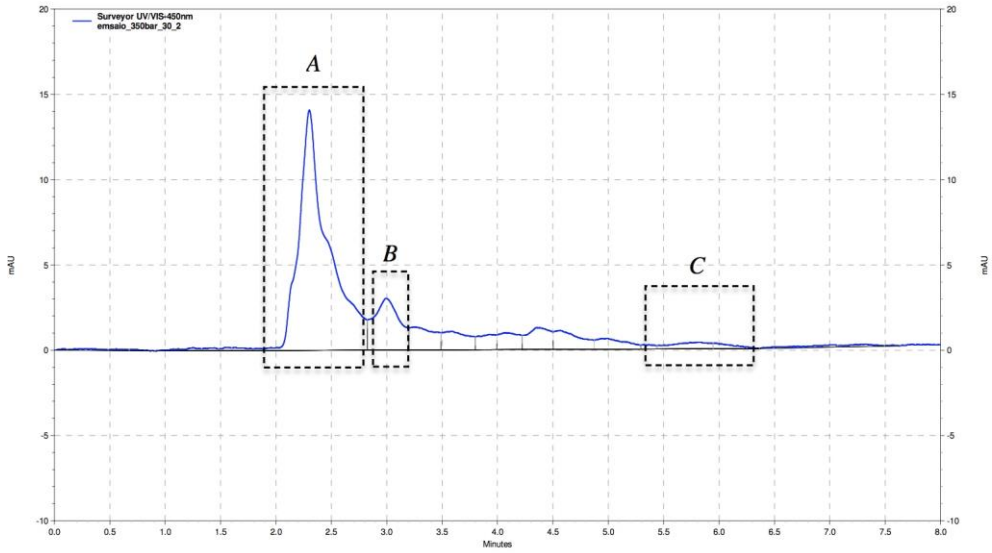


Figure 3.11 - Chromatogram obtained by HPLC that correspond to the assay at 35,1 MPa without co-solvent. Legend – A – Lycopene; B – Lutein; C - β-carotene.

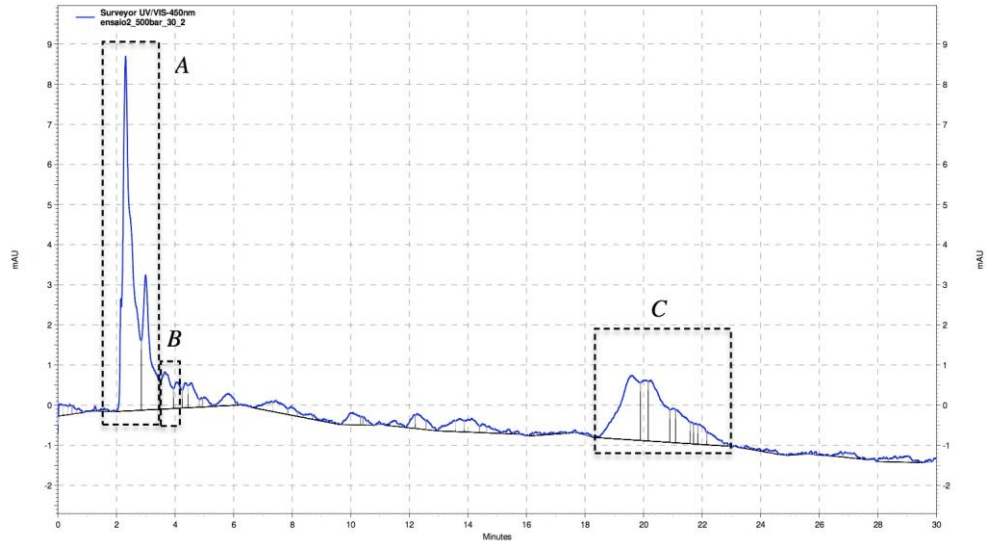


Figure 3.12 - Chromatogram obtained by HPLC that correspond to the assay at 50,1 MPa without co-solvent. Legend – A – Lycopene; B – Lutein; C - β-carotene.

Table 3.7 - Yield of oil extracted with the different methods.

Method		Yield of oil extracted % m/m
Organic solvent	Sohlext	5,2
	Bligh and Dyer	7,9
	Acetone	2,1
Supercritical dioxide carbon	30,1 MPa with 5% ethanol co-solvent	2,2
	35,1 MPa	3,4
	50,1 MPa	2,4

It is important to refer that all these assays are preliminary studies and the result can contain experimentals errors. So, althought the yield of oil extracted by $scCO_2$ is lower than when it is extracted by organic solvent, it is expectable that with an optimization of the assay parameters, the yield can be the same or higher and the oil can contain more carotenoids.

3.2.2 – Antioxidant activity

The antioxidant activity was tested for the samples of oil obtained at 35,1 MPa and 50,1 MPa, without co-solvent. The two samples showed a clear dose-reponse scavenging effect on the free radical DPPH, with specific patterns corresponding to each samples.

The antioxidant capacity, expressed as the half maximal effective concentration (EC_{50}), for the sample obtained at 35,1 MPa is 7,09 μg/ml and it reach the highest scavenging activity at concentration equal to 25 μg/ml, and at 50,1MPa is 6,97 μg/ml, reaching the highest scavenging activity at concentration equal to 100 μg/ml. Comparating with the ascorbic acid, the most used antioxidant, which the EC_{50} is 5 μg/ml, it's possible to affirm that the antioxidant capacity of the carotenoids extracted from the tomato pomace is almost as potent as this acid.

The antioxidante activity is lightly higher on the sample extracted at 50,1 MPa than at 35,1 MPa, it is probably due to the higher amount of carotenoids.

CHAPTER 4: Conclusion and future work

4 - Conclusion and future work

The main objective of this work was the valorization of agroindustrial waste: spent coffee ground, tomato pomace and microalgae *Chlorella protothecoides*, which not being a residue can be a good solution to produce biofuels production. The spent coffee grounds and the microalgae were used to produce biodiesel, and the tomato pomace was used to extract value-added compounds, i.e., carotenoids. Both processes were focused on supercritical fluids, namely supercritical carbon dioxide.

The first part of this work - production of biodiesel with enzymatic transesterification and $scCO_2$ using spent coffee grounds, was successfully achieved after several assays and the optimization of the process parameters. It, was obtained a maximum yield of FAME equal to 99,3% with the follow conditions: molar ratio oil:methanol equal to 1:24, residence time equal to 6,8 min, 25 MPa, 313,15 K. In addition, it was used a biomass, microalgae *Chlorella protothecoides*, to produce biodiesel of the third generation of biofuels. In this work it was not possible to obtain a yield of FAME sufficiently high to be considered a biodiesel with the conditions to be commercialized. Althought, the results show that using this green process - supercritical fluid and enzymes – it is possible to obtain a higher convertion of FAME with less alcohol that the tradicional process. Regarding future work, with the spent coffee ground, it would be interesting realize an integrated process that includes extraction and reaction using the optimized parameters; with the microalgae *Chlorella protothecoides* it would be important to optimize the operating condition, since this study was a preliminary work.

The second part of this work – extraction of value-added compound from tomato pomace, demonstrated that the oil extracted contain severals carotenoids: lycopen (in higher concentration), β-carotene and lutein. The yield of oil rich in carotenoids extracted was approximately 2% and the antioxidant capacity of the carotenoids extracted from the tomato pomace is almost as potent as the ascorbic acid. This work was a preliminary study, so it would be important, as future work, to do more assays and optimize the extraction process, to do an oil characterization, a toxicological evaluation of oil and the incorporation of the carotenoids in cosmetic formulation.

REFERENCES

(1) European Comission – website: www.ec.europa.eu, visited February 2015
(2) United Nations – website: www.un.org, visited on February 2015
(3) Eur-Lex: Acesso a União Europeia – website: www.eur-lex.europa.eu, visited on February 2015
(4) Duty of care waste transfer notes pads – website: www.wastenotes.co.uk, visited on February 2015
(5) OECD, "Strategic Waste Prevention", ed 2000
(6) Senorans, F.J.; Ibanez, E.; Cifuentes, A. "New trends in food processing." Crit. Rev. Food Sci. 2003, 43, 507–526.
(7) Rawsona, A.; Tiwarib, B.K.; Bruntona, N; Brennanc, C.; Cullend P. J.; O'Donnelle C. P., "Application of Supercritical Carbon Dioxide to Fruit and Vegetables: Extraction, Processing, and reservation." Food Reviews International, 2012, Volume 2, 253-276
(8) Gustavsson, J., Cederbeg, C., Sonesson, U., Van Otterdijk, R.; Meybeck A., "Global Food Losses and Food Waste". 2011, 1–38
(9) Alzate, L.M; González, D.; Londoño-Londoño, J, "Recovery of carotenoids from agroindustrial by-products using clean extraction techniques: supercritical fluid extraction and ultrasound assisted extraction.", 2013
(10) Laufenberg, G.; Kunz, B.; Nystroem M., "Transformation of vegetable waste into value added products: (A) the upgrading concept; (B) practical implementations", Bioresour Technol. 2003 Apr; 87(2) :167-98
(11) Progressive Fuels Limited – website: www.progressivefuelslimited.com/, visited February 2015
(12) Meher, L.C., D.V. Sagar, and S.N. Naik, "Technical aspects of Biodiesel production by transesterification- a review. Renewable ans Sustainble Energy.", Reviews 2006, p. 248 - 268
(13) Yusuf N. N. A. N., Kamarudin S. K., Yaakub Z., "Overview on the current trends in biodiesel production," Energy Conversion and Management, vol. 52, 2741-2751, 2011
(14) Vicente, G., Martínez, M., José, A., "Integrated biodiesel production: a comparison of different homogeneous catalyst system". Bioresource Technology 2004, 297 - 305.
(15) European biofuels tecnology platform - website: www.biofuelstp.eu, visited on February 201
(16) Andrade K. S., Goncalvez R. T., Maraschin M., Ribeiro-do-Valle R. M., Martinez J., Ferreira S. R., "Supercritical fluid extraction from spent coffee grounds and coffee husks: Antioxidant activity and effect of operational variables on extract composition," Talanta, vol. 88, pp. 544-52, Jan 15 2012
(17) Demirbas, A., Biodiesel- "A Realistic fuel alternative for Diesel engines. 2008, Springer" Verlag Limited: London
(18) Oliveira L. S., Franca A. S., Camargos R. R. S., Ferraz V. P., "Coffee oil as a potential feedstock for biodiesel production," Bioresour Technol, vol. 99, pp. 3244-3250, May 2008
(19) Zhanga M. A. D. Y, McLeana D.D, Katesb M., "Biodiesel production from waste cooking oil," Bioresour Technol, vol. 89, pp. 1–16, 2003
(20) Atabani A. S. S. A.E., Irfan Anjum Badruddin, Mahlia T.M.I., Masjuki H.H., S.Mekhilef "A comprehensive review on biodiesel as an alternative energy resource and itscharacteristics," Renewable and Sustainable Energy Reviews, vol. 16, pp. 2070-2093, May 2012 2012
(21) EBB - European Biodiesel Board, "EBBQR - European Biodiesel Quality report - Winter 2010/2011 results," Bruxelles, 2011.

(22) Nelson, R.G., Hower, S.A., Potential feedstock supply and costs for biodiesel production, in Bioenergy'94, P.o.t.s.n.b. conference, Editor. 1994.
(23) Giannelos P. N.; Zannikos F.; Stournas S.; Lois E.; Anastopoulos G., "Tobacco seed oil as an alternative diesel fuel: physical and chemical properties", 2011
(24) Shahid E. M.; Jamal Y., "Production of biodiesel: A technical review," Renewable and Sustainable Energy Reviews, vol. 15, pp. 4732-4745, 2011
(25) Nigam, P. S.; Singh A., "Production of liquid biofuels from renewable resources", Progress in Energy and Combustion, 2011, 52-68
(26) USDA Economic Research Service – website: http://webarchives.cdlib.org, visited February 2015
(27) Worldmapper – website: www.worldmapper.org, visited on February 2015
(28) Statista – website: http://www.statista.com, visited on February 2015
(29) Mussatto, S. I.; Carneiro, L.M, Silva, P. A.; Roberto I. C.; Teixeira, J. A., "A study on chemical constituents and sugars extraction from spent coffee grounds", Carbohydrate polymers, 2011, 368-374
(30) www.sossolteiros.bol.uol.com.br, visited on February 2015
(31) Kölling-Speer S. S. I,;Speer K. , "Determination of Free Diterpenes in Green and Roasted Coffees," Journal of High Resolution Chromatography, vol. 22, pp. 43-46, January 1999 1999.
(32) Lercker G. F., Bocci, F., "High resolution gas chromatographic determination of diterpenic alcohols and sterols in coffee lipids," Chromatographia, pp. 29-33, July 01 1995.
(33) R. M. Couto, J. Fernandes, M. D. R. G. da Silva, and P. C. Simoes, "Supercritical fluid extraction of lipids from spent coffee grounds," Journal of Supercritical Fluids, vol. 51, pp. 159- 166, Dec 2009.
(34) Kulkarni MG, D. A., "Waste cooking oil – an economical source for biodiesel: a review.," Ind Eng Chem Res vol. 45, pp. 2901–13, 2006.
(35) Calixto, F.; Fernandes, J.; Couto, R.; Hernandez, E. J.; Najdanovic-Visaka, V.; Simoes, P. C., "Synthesis of fatty acid methyl esters via direct transesterification with methanol/carbon dioxide mixtures from spent coffee grounds feedstock," Green Chemistry, vol. 13, pp. 1196- 1202, 2011.
(36) Kondamudi N.; Mohapatra, S. K.; and Misra, M., "Spent Coffee Grounds as a Versatile Source of Green Energy", J. Agric. Food Chem., 2008, 56 (24), 11757–11760
(37) Poonam Singh Nigama, Anoop Singhb, "Production of liquid biofuels from renewable resources", Progress in Energy and Combustion Science, 2011, 52-68
(38) College of engeneering, Colarado State University, website: www.engr.colostate.edu, visited on February 2015
(39) Energy Region - website: www.energystate.de, visited on February 2015
(40) Han Xu; Xiaoling Miao; Qingyu Wu; "High quality biodiesel production from a microalga Chlorella protothecoides by heterotrophic growth in fermenters", Journal of Biotechnology, 2006, 499-507
(41) Kraeczyk, T.; "Biodiesel-alternative fuel makes inroads but hurdles remain. Inform", 1996, 800 – 815
(42) Demirbas A., "Biodiesel production from vegetable oils via catalytic and non-catalytic supercritical methanol transesterification methods," Progress in Energy and Combustion Science, vol. 31, pp. 466-487, 2005.
(43) Srivastava, A.P.R., "Triglycerides-based diesel fuels. Renewable and Sustainable Energy Reviews", 2000, 111 - 113.
(44) Demirbas A., "Biodiesel fuel from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: a survey Energy conversion and management", 2003, 2093 - 2109.

(45) Freedman, B.; Butterfield; R.O., Pryde, E.H. "Transesterification kinetics of soybean oil." JAOCS, 1986, 1375 - 1380.
(46) Bernardes M. A. S, "Economic Effects of Biofuel Production", 2011
(47) Knothe Gerhard J. K.; Gerpen V.; Jürgen, "The biodiesel handbook", 2005 ed.: AOCS Press, 2005.
(48) Abdullah A. Z.; Razali N.; Mootabadi H.; Salamatinia B., "Critical technical areas for future improvement in biodiesel technologies", 2007
(49) Demirbas A., "Comparison of transesterification methods for production of biodiesel from vegetable oils and fats," Energy Conversion and Management, vol. 49, pp. 125-130, 2008.
(50) Demirbas, A., "Inexpensive oil and fats feedstocks for production of biodiesel," Energy Educ Sci Technol Part A, vol. 23, pp. 1-13, 2009.
(51) Fangrui Maa; Milford; Hannab A., "Biodiesel production: a review", 1999
(52) Fuduka, H.; Kondo, A.,; Noda, H., "Biodiesel fuel production by transesterification of oils." Journal of Bioscience and Bioengineering 2001, 405 - 416.
(53) Smith, A.L., ed. "Oxford dictionary of biochemistry and molecular biology.", ed. Oxfordshire. 1997, Oxford University Press.
(54) Boyer, R., ed. "Concepts in Biochemistry." 2a ed. 2002, John Wiley & Sons, Inc.: Toronto. 137- 138.
(55) Shutter Stock, website: www.shutterstock.com, visited on February 2015
(56) Science Learn, website: www.sciencelearn.org.nz, visited on February 2015
(57) Knez, Z., "Enzymatic reactions in dense gases." Journal of supercritical fluids, 2009, 357 - 372.
(58) Bajaj, A., Lohan, P., Jha, P.N., Mehrotra, R., "Biodiesel production through lipase catalyzed transesterification: An overview". Journal of molecular Catalysis B: Enzymatic, 2009, 1 - 22.
(59) Hartmann, M.; Jung, D, "Biocatalysis with enzymes immobilized on mesoporous hosts: the status quo and future trends", Journal of Materials Chemistry, 2010
(60) Knez, Z., Gamse, T., "Enzymatic reactions. 2009, Department of Chemical Enginnering and Environmental technology", Graz, 1 - 13.
(61) Pringle, D., "The Energy Technology Roadmap.Biofuels: the next generaton.How innovation can brighten Europe's energy future," Science Business Publishing Ltd2011.
(62) Herrero, M.; Cifuentes; Ibanez, E., "Sub and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgaeA review. Food Chem." 98, 2006, 136–148.
(63) Royer, M.; Prado, M.; García-Pérez, M. E.; Diouf, P. N.; Stevanovic, T.; "Study of nutraceutical, nutricosmetics and cosmeceutical potentials of polyphenolic bark extracts from Canadian forest species" PharmaNutrition, 2013, 158–167
(64) Faostat, website: www.faostat.fao.org , visited on February 2015
(65) Food production daily, website: www.foodproductiondaily.com, visited on February 2015
(66) The heart thrills, website: www.theheartthrills.wordpress.com, visited on Februrary 2015
(67) http://upload.wikimedia.org, visited on Februar 2015
(68) Del Valle, M.; Cámara, M.; Torija, M. E., "Chemical characterization of tomato pomace", Journal of the Science of Food and Agriculture, 2006
(69) Vagia,E.; Simandia, B.; Vasarhelyine K.P.; Daoodb, b,H.; Keryc, A.; Doleschalld, F.; Nagya, B., "Supercritical carbon dioxide extraction of carotenoids, tocopherols and sitosterols from industrial tomato by-products", 2006, 218-226.
(70) Caratonoids society, website: www.carotenoidsociety.org, visited on February 2015

(71) Website: http://jocpr.com, visited on February 2015
(72) Héctor Arvayo-Enríquez, Iram Mondaca-Fernández, Pablo Gortárez-Moroyoqui, Jaime López-Cervantes, Roberto Rodríguez-Ramírez, "Carotenoids extraction and quantification: a review" 2006
(73) Taungbodhithama, A. K.; Jonesb, G.P.; Wahlqvistc, M.L.; Briggsb, D.R.; "Evaluation of extraction method for the analysis of carotenoids in fruits and vegetables", Food Chemistry, 1998, 577–584
(74) Cyber Lipid, website: www.cyberlipid.org, visited on February 2015
(75) Website: www.fao.org , visited on February 2015
(76) Website: http://www.bccresearch.com, visited on February 2015
(77) Zuorroa, A.; Fidaleod, M.; Lavecchiaa, "Enzyme-assisted extraction of lycopene from tomato processing waste", Enzyme and Microbial Technology 2011, 567–573
(78) Website: www.lycocard.com, visited on February 2015
(79) Prado, J. M.; Veggi, P. C.; Angela, M; Meireles, A., "Extraction Methods for Obtaining Carotenoids from Vegetables – Review" Current Analytical Chemistry, 2014
(80) Brunner, G., "Supercritical fluids: technology and application to food processing," Journal of Food Engineering, 21-33, 2005
(81) Website: www.hindawi.com, visited on February 2015
(82) Díaz-Reinoso, B., Moure, A., Domínguez, H. & Parajó, J. C. "Supercritical CO2 extraction and purification of compounds with antioxidant activity." J. Agric. Food Chem. 54, 2441– 69 (2006).
(83) Brunner, "Gas Extraction. An introdution to fundamentals of supercritical fluids and the application to separation processes" Steinkopff Darmstadt. Springer New York, 1994.

(84) Williams J. R; Clifford, T., "Supercritical fluid methods and protocols." Totowa, N.J.: Humana Press, 2000
(85) Sovova, H., Stateva, R. P. & Galushko, A. A. "Solubility of i -carotene in supercritical CO 2 and the effect of entrainers." 21, 195–203, 2001
(86) Wen, D., Jiang, H., Zhang, K., "Supercritical fluids techonology for clean biofuel production." Progress in Natural Science 2009, 273 – 284
(87) Jackson, M.A., King, J.W. , "Methanolysis of seed oils in flowing Supercritical Carbon Dioxide." Food quality and Safety Research 1996, 353 – 356
(88) Poonam Singh Nigam, Anoop Singh, "Production of liquid biofuels from renewable resources", Progress in Energy and Combustion Science, 52-68, 2011
(89) Munshi, P. & Bhaduri, S. "Supercritical CO2 : a twenty-first century solvent for the chemical industry."
(90) Reverchon, E., De Marco, I. , "Supercritical fluid extraction and fractionation of natural matter." Journal of Supercritical Fluids 2006, 146 - 166

(91) – Unsaponifiable Matter, AOSC Official Method Ca 6a-40, reapproved 2009
(92) Fausto Munari, Daniela Cavagnino, Andrea Cadoppi, "Determination of Total FAME and Linolenic Acid Methyl Ester in Pure Biodiesel (B100) by GC in Compliance with EN 14103", Thermo Fisher Scientific
(93) Fausto Munari, Daniela Cavagnino, Andrea Cadoppi, "Determination of Total FAME and Linolenic Acid Methyl Ester in Pure Biodiesel (B100) by GC in Compliance with EN 14103", Thermo Fisher Scientific
(94) Maryam Fahimdanesh, Mohammad Erfan Bahrami, "Evaluation of Physicochemical Properties of Iranian Tomato Seed Oil", Nutrition and food science, 2013

(95) Bligh,E.G. and Dyer,W.J. 1959. "A rapid method for total lipid extraction and purification". *Can.J.Biochem.Physiol.* 37:911-917

(96) Gunars Tirzitis, Grzegorz Bartosz, "Determination of antiradical and antioxidant activity: basic principles and new insights", 2010

(97) Nooman A. Khalfa, Ashok K. Shakya, Atif Al-toman, Zaha El-gabar, Husni Farah "Antioxidant Activity of Some Common Plants", 2008

(98) Couto, R. M; Fernandes J.; Gomes da Silva, M. D. R.; Simões, P., "Supercritical fluids extraction lipids from spent coffee grounds", 2009

(99) Shannon D. Sanford, James Matthew White, Parag S. Shah, Claudia Wee, Marlen A. Valverde, Glen R. Meier "Feedstock and Biodiesel Characteristics Report", 2009

(100) Jackson, M.A., King, J.W. , "Methanolysis of seed oils in flowing Supercritical Carbon Dioxide." Food quality and Safety Research 1996, 353 – 356

(101) Wang, Y., Wu, H., Zong, M.H., "Improvement of biodiesel production by Lipozyme TL IM- catalyzed methanolysis using response surface methodology and acyl migration enhancer." Bioresource Technology, 2008, 7232 - 7237.

(102) Dizge, N., Aydiner, C., Imer, D.Y., Bayramoglu, M., Tanriseven, A., Keskinler, B., "Biodiesel production from sunflower, soybean and waste cooking oils by transesterification using lipase immobilized onto a novel microporous polymer." Bioresource Technology, 2008.

(103) Carlo Santambrogio, Francesco Sasso, Antonino Natalello, Stefania Brocca, Rita Grandori, Silvia Maria Doglia, Marina Lotti "Effects of methanol on a methanol-tolerant bacterial lipase" 2013

(104) Miros1awa Szcze ̨sna Antczak, Aneta Kubiak, Tadeusz Antczak, Stanis1aw "Enzymatic biodiesel synthesis – Key factors affecting efficiency of the process", ; 90-92, 2008

(105) Alexandre Paiva, Carmen Montoya, Teresa Lopes, Alberto Reis, Susana Barreiros, Pedro Simões, "Supercritical fluid extraction of added-value compounds from Chlorella Prototecoides"

(106) Han Xu, Xiaoling Miao, Qingyu Wu, "High quality biodiesel production from a microalga Chlorella protothecoides by heterotrophic growth in fermenters", Journal of biotecnology, 499-507, 2006

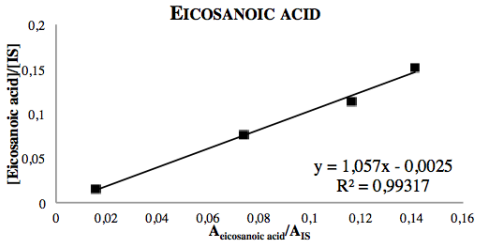
(107) Juliana M. Prado, Priscilla C. Veggi and M. Angela A. Meireles, "Extraction Methods for Obtaining Carotenoids from Vegetables – Review", Current Analytical Chemistry, 2014, 10, 29-66

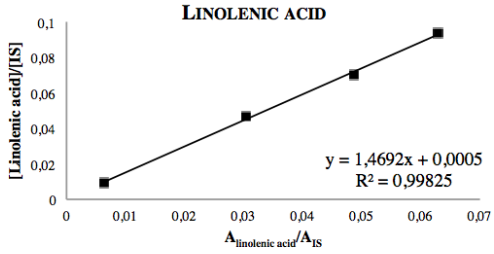
(108) Marleny D.A. Saldaña, Feral Temelli, Selma E. Guigard, Bruno Tomberli, Chris G. Gray "Apparent solubility of lycopene and b-carotene in supercritical CO_2, CO_2 + ethanol and CO_2 + canola oil using dynamic extraction of tomatoes", Journal of Food Engineering 99, 2010, 1–8

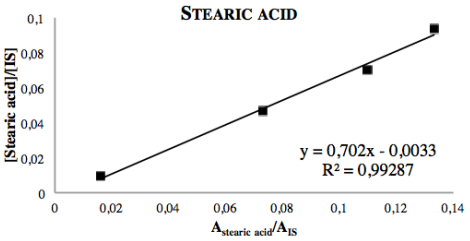
APPENDIX

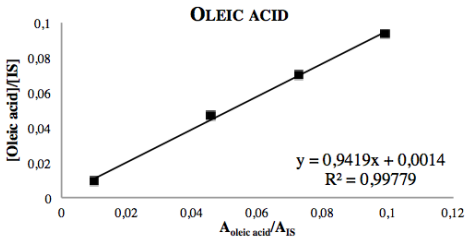
Appendix A

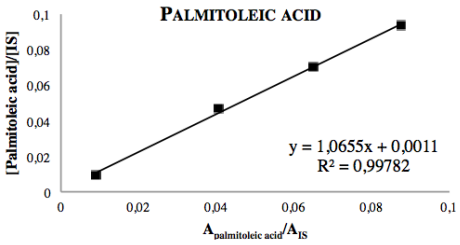
Calibration curves for the Lepage and Roy method

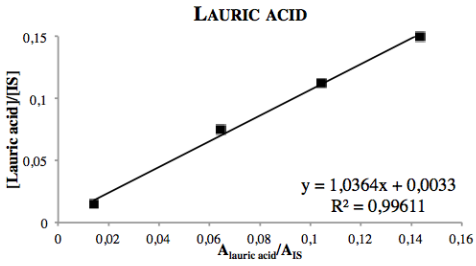


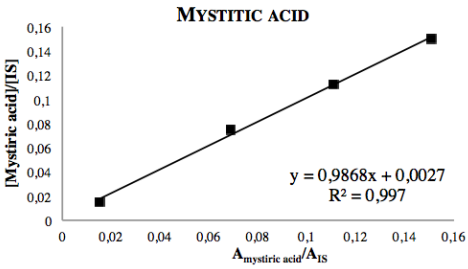






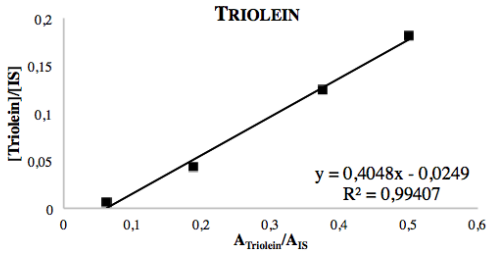

PALMITOLEIC ACID
[Palmitoleic acid]/[IS]
y = 1,0655x + 0,0011
R² = 0,99782
A palmitoleic acid/A IS

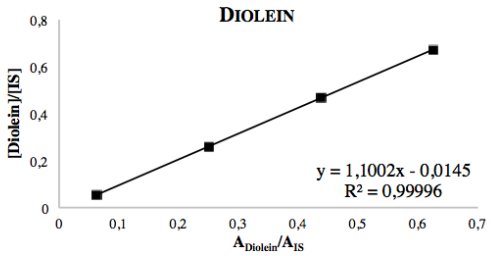

LAURIC ACID
[Lauric acid]/[IS]
y = 1,0364x + 0,0033
R² = 0,99611
A lauric acid/A IS

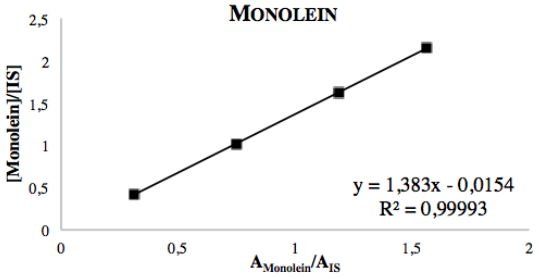

MYSTITIC ACID
[Mystiric acid]/[IS]
y = 0,9868x + 0,0027
R² = 0,997
A mystiric acid/A IS

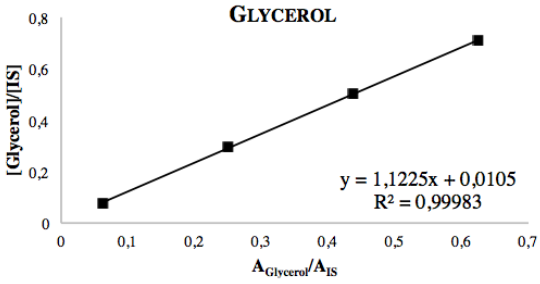
Appendix B

Calibration curves for the On-column method



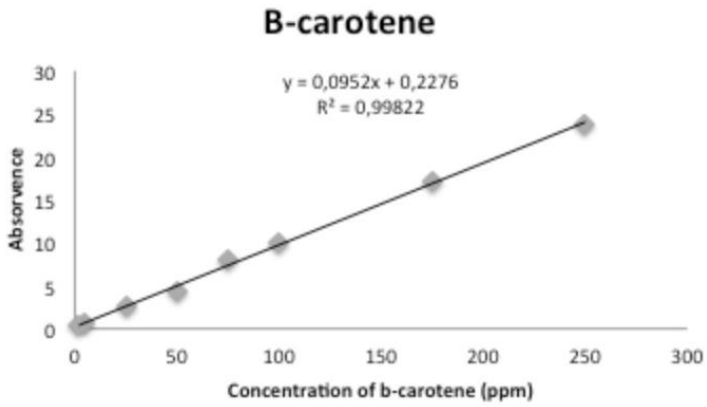


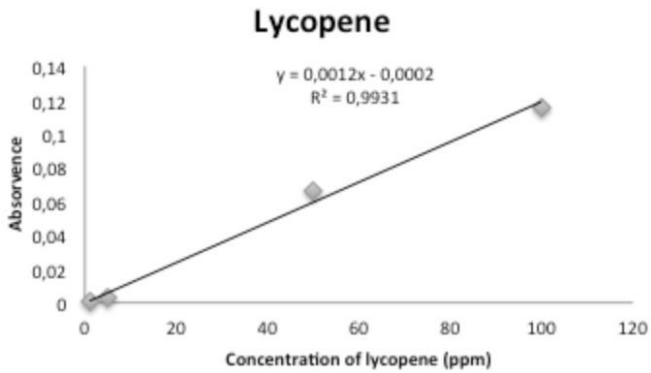




Appendix C

Calibration curves for the b-carotene and lycopene – UV Spectrophotometry





Appendix D

Calibration curves for the b-carotene and lycopene – HPLC